Castanea Recent Genetic Research

Gösta Eriksson
Cover picture. The *C. sativa* plants in this picture were included in an experiment with two sweet chestnut populations from each of Greece, Italy, and Spain. Each population was represented by eight open-pollinated families. Two levels of water availability and temperature were tested. Photograph Alfás Pliura.
Preface

At the end of the 1990-ties Dr. Fiorella Villani invited me to participate in the development of pan-European project on *C. sativa*. It sounded attractive to me, so I accepted the invitation. The project, coined CASCADE, got European Union funding. It was a pleasure for me to work within the CASCADE project and participate in many stimulating discussions with scientists from southern European countries.

This publication tries to cover genetic research since 1990 in all *Castanea* species. I apologize for not treating papers that I may have missed in my search for relevant publications. I was amazed by the number of scientific reports dealing with genetics of *Castanea*, and no less amazed by all scientific achievements. It was a pleasure to compile the results obtained in various projects. It should be noted that several reports have information relevant for more than one of the four chapters. In most cases such papers are treated just once.

As in previous reviews, papers written in languages that are not understood by the scientific community are not treated. As usual, graphic illustrations are in focus in my summary. I am responsible for all illustrations and editing.

The former head of department for Plant Biology at SLU has offered me a working place in the department, which I appreciate much. My sincere thanks to Dr Björn Nicander for his willingness to swiftly solve any computer problems.

I have tried to find a world map with the distributions of the seven *Castanea* species without finding any that illustrates the distribution of all species. Therefore, I present the approximate distributions in Table P below.

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<tr>
<td>C. dentata</td>
<td>Eastern Canada and USA; mainly Appalachian Mountains</td>
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<tr>
<td>C. henryi</td>
<td>Southwestern and south-eastern China</td>
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<tr>
<td>C. mollissima</td>
<td>Large part of China</td>
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<td>C. pumila</td>
<td>Texas to New York, USA</td>
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<td>C. sativa</td>
<td>Portugal to northern Iran</td>
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<tr>
<td>C. segunii</td>
<td>China partly overlapping with <em>C. mollissima</em></td>
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1 Populations

1.1 Metric traits

Pigliucci et al. (1991) studied phenotypic integration in eight populations of *C. sativa* from central Italy. According to Wikipedia: *Phenotypic Integration is the term used to describe when multiple functionally-related traits are correlated with each other*. From a genetic point of view the best method to study phenotypic integration is to estimate genetic correlations among traits. Preferably, this requires large numbers of parents, which were not available at the point of time for this report. Instead 15 leaf traits and 14 nut traits were analyzed in the eight populations. The hypothesis was that nut traits should show more uniformity than leaf traits since nut traits were exposed to strong artificial selection for generations while leaf traits were exposed to natural selection, which probably was less strong. Correlations among populations for traits that showed significant population differentiation were calculated. The geometric mean of the squared correlation coefficients was used as an estimate of the phenotypic integration. A mantel test was used to estimate the similarity of the phenotypic integration between the eight populations.

To illustrate some of the variation among populations I have selected two nut traits and two leaf traits that showed strong population differentiation (Fig. 1-1). It is evident from this figure that population Tss deviated strongly from the other seven populations with respect to the two nut traits. It also contributed strongly to the correlation between the two nut traits, $R^2 = 0.90$. When data from the Tss population was omitted from the estimation of the relationship between nut thickness and nut weight $R^2$ dropped to 0.27. As regards the two leaf traits, the Por population differed most from the other populations. The authors had expected less variation among the populations since selection has taken place over generations for millennia.

Fig. 1-2 reveals that the phenotypic integration within populations was larger for nut traits than for leaf traits as expected by the authors. In five of the eight populations the difference between the two types of trait was pronounced.
The mantel test of the similarity among populations with respect to phenotypic integration revealed more significant relationships for leaf traits than for nut traits (Fig. 1-3). Thus, similarity of leaf traits was greater than for nut traits at the population level, which was somewhat surprising considering the long-term stabilizing selection for nut yield and nut quality in all populations.

Nut traits in five Greek populations from two contrasting localities in northern Greece were studied by Alizoti and Aravanopoulos (2005). From each of the five populations 20 nuts from nine trees per population were sampled. The northern locality had three types of population:
- Old growth natural
- Coppice natural
- Orchard

The southern locality had the first two types of population. Six morphological traits were assessed:
- NL nut length
- NLW length to the widest point
- NTH nut thickness
- NWD nut width
- HL hylum length
- HW hylum width

Besides, nut weight (NWEI), the ratios NWD/NL, NTH/NL, and NTH/NWD were recorded or calculated. A linear model for the ANOVA was used and the coefficient of genetic variance was calculated as $CV_g = (\sigma / x) \times 100$, in which $\sigma$ is the population standard deviation and $x$ is the trait mean value. Repeatability was calculated according to the following: $R = \sigma^2_t / (\sigma^2_t + \sigma^2_e)$, in which $\sigma^2_t$ is the variance due to trees within populations and $\sigma^2_e$ is the error variance.

Except for the two hylum traits and the ratio NTH/NWD all other traits showed significant differences between the two localities. Significant population differences were noted for the first four listed traits above. The repeatability for the ten traits varied in the range 0.10-0.60. The lowest estimate was noted for hylum width and the highest for nut length (Fig. 1-4).

Fernandez-Lopez et al. (2005a) presented terminal bud flushing during three seasons and growth data from two field trials in north-western Spain. The trial at latitude 43.18°N, longitude 7.05°W, and altitude 750 masl contained 16 populations from mainland Spain three from The Canary Islands with 25 single-tree plots per subpopulation. The other trial at latitude 43.08°N, longitude 8.68°W, and altitude 450 masl contained 13 populations with 20 single-tree plots per subpopulation. Frost damage during the first season in field was classified in a five-degree scale based on the percentage of plants damaged. Each population was represented by 2-4 subpopulations selected within each population at a maximum distance of 30 km. $Q_{5\%}$ were reported for the different traits with the assumption of heritabilities 0.20 for flushing and frost damage and 0.40 for plant height. It should be noted that heritabilities were not calculated in these trials. The number of subpopulations in the ANOVAs contained different number of subpopulations and the effects of subpopulation were only presented for the 750 masl trial.

There was a good survival in both trials at age three, 94.5 and 98.3%, respectively. All traits studied showed strongly significant differences among populations. The subpopulation effect was strongly significant for mean date for terminal bud flushing, plant height, and frost damage in the 750 masl trial.

![Figure 1-3](image1.png)

**Figure 1-3.** Number of significant similarities with the other seven populations for leaf traits and nut traits, respectively. Eight Italian *C. sativa* populations from Lazio and Umbria. Pigliucci et al (1991).

The mantel test of the similarity among populations with respect to phenotypic integration revealed more significant relationships for leaf traits than for nut traits (Fig. 1-3). Thus, similarity of leaf traits was greater than for nut traits at the population level, which was somewhat surprising considering the long-term stabilizing selection for nut yield and nut quality in all populations.

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![Figure 1-4](image2.png)

**Figure 1-4.** Repeatability and coefficients for genetic variance x 10^{-2} for nut traits in two combined provenance and progeny *C. sativa* trials in Greece located in two climatically contrasting regions. Below nut traits abbreviations are used:
- NL nut length
- NLW length to widest point
- NTH nut thickness
- NWD nut width
- NWE nut weight
- HL hylum length
- HW hylum width

Alizoti and Aravanopoulos 2005.
Terminal bud flushing. In Fig. 1-5 three estimates of the population variance components are shown; the year with minimum and maximum differentiation as well as a mean for all three years. High percentages of the variance for the year with maximum differentiation were noted for both trials. Unfortunately, it was not reported whether the high estimate originate from the same age in the two trials. It is evident that the site conditions of the populations played a role for the large differentiation among populations. Both the annual precipitation and the mean temperature of the warmest month varied considerably at original localities of the populations; precipitation 600-1,965mm and warmest month 17-25°C, respectively. Such a broad span of climatic variables suggests that there would be a considerable genetic differentiation among populations. The strongly significant population effect in the joint ANOVA suggests that the population x trial effect would be of minor importance. In spite of this, this effect was also strongly significant. In agreement with this, the ranking changes in terminal flushing time between the two trials are considerable for some of the populations (Fig. 1-6). The three populations with the largest rank changes originated from three different parts of Spain. As seen from Fig. 1-7 fairly strong relationships between mean temperature of hottest month and terminal bud flushing were observed in the two trials. Fairly strong relationships between flushing and latitude or summer precipitation were also noted. The strong relationships between flushing date and latitude or temperature of the hottest month indicate that the populations from the north-western and cooler Spain are late-flushing.

This is contrary to the results for tree species from northern Europe with an opposite trend between flushing and latitude. It is likely that there is an adaptive advantage of early flushing in areas with hot and dry summer climate to utilize favorable growth conditions before the summer heat and drought appear. Contrary to this, there was no relationship between tree height and the two climate variables responsible for a large part of the variation for flushing. This means that different climatic factors influence phenology and tree growth.
The QST estimates were very large for the 450 masl trial and the joint analysis of data (Fig. 1-8). The use of such a low heritability as 0.2 for a phenology trait in the calculation of QST may have caused an upward estimation. Plant height. It was stated that there were large differences in plant growth among the populations with estimates of variance in the range 16-24%. No trends as regards relationships with environmental variables were noted for plant height. It is possible that planting chock might have influenced the growth of populations differently during the first year in field. The observation of a much higher QST estimate for subpopulation in the 750 masl trial than for population is surprising considering the geographic closeness of the subpopulations within populations. The QSTs for plant height were based on such a high heritability as 0.40, which might explain part of the difference between the size of QSTs for flushing and plant height.

Frost damage. No percentages of frost damage were reported but there were strongly significant differences both for populations and subpopulations. There was a large QST for this trait at the 450 masl trial (Fig. 1-8). Since no observed percentages were presented it is hard to know if the difference between the trials can be attributed to low percentages of frost damage in all populations in the 750 masl.

General. Some populations had a combined origin in natural regeneration and in development of trees from stumps. Moreover, previous transfers of nuts and scions over wide geographic areas had taken place. These three conditions might have masked the adaptation that had taken place and thus reduce the possibilities to detect strong clinal variation. However, it was stated that the impact of domestication of chestnut orchards or movement of scions was less important than previously believed. It was stated that The correlation between flushing, frost damages and survival indicated that frost is a relevant factor acting in natural selection. The strength of these correlations was not reported.

Diaz et al. (2009) studied spring and autumn frost damage in six Spanish C. sativa populations represented by 41 OP-families from a combined provenance-progeny trial at Rebordero. No human impact on these populations was a criterion for their selection. Twigs were collected in the trial and transported in cool boxes to the laboratory for freeze testing at -7, -10, -12, and -15°C. The temperatures were selected to obtain the best resolution among the entries tested. In the freeze tests the twigs were kept at 5°C for at least 7 hours. After that, temperature was decreased by 2°C per hour. Scoring was carried out 3 or 4 weeks after freezing during spring and autumn, respectively. After freeze testing twigs with buds were cut lengthwise for visual examination of yellowing and browning of them. Six classes (0-5) were used for scoring of frost damage of stem damage while three classes were used for terminal bud damage. Finally, a two-point scale was used for lateral buds, alive or dead. Two phenology traits were assessed in the field trial, bud flushing in 8 classes, and leaf fall in 5 classes. Several climatic variables were used to disclose any relationship with observed data from the present investigation. A prominent climatic variable was drought index which was defined as time span, measured in months, during which the curve of the monthly mean values lies above the precipitation curve.

Both population effects for the field phenology traits were strongly significant. The most interior populations had the earliest bud flushing and leaf fall in the field trial (Fig. 1-9). It was suggested that the early flushing of these populations was attributed to the climatic conditions at their origin. For interior populations it is an advantage...
with an early flushing to utilize the water available before the drought period starts. There was a strong relationship between the drought index and flushing, $R^2 = 0.81$ (Fig. 1-10). The drought index – leaf fall second degree polynomial relationship was still stronger $R^2 = 0.96$. Under Iberian conditions phenology traits are not correlated with geographic variables but with climatic factors, which was strongly emphasized by the authors.

All six traits assessed for frost injuries after freeze testing were strongly significant. However, in the analysis carried out with field bud flushing or leaf fall as covariates the picture changed dramatically. Only terminal bud damage in spring remained strongly significant for populations and stem damage in autumn was non-significant. The four other traits had a weakly significant population effect. The percentage range of frost injuries was approximately 15-65%.

Fig. 1-11 reveals that all three estimates of injuries following freeze testing during spring were significantly related to bud flushing of the six populations in the field trial. Contrary to this, there were no strong relationships between leaf fall in field and the three damage traits (Fig. 1-12). Only after exclusion of population No. 3 there was a strongly significant linear relationship for percentage of damaged lateral buds. It may be concluded that bud flushing in field may be used for prediction of frost damage during spring while phenology data from autumn do not well predict autumn frost damage. As stated in the paper freeze testing might be an option for applied breeding. It was speculated that the early leaf fall of southern populations means that natural selection for autumn frost tolerance had not occurred since leaf fall occurs before presence of any frost exposure at these localities. As corollary of this, southern populations are adapted to drought but not to frost.

**Figure 1-10.** The relationships between drought index at population origin and bud flushing (8 scores), and leaf fall (5 scores) in a North-Western Spanish field trial (blue square) with six Spanish C. sativa populations. The population identification numbers are given. Diaz et al. 2009.

**Figure 1-11.** The relationships between bud flushing in a Spanish field trial with C. sativa and three estimates of freezing test injuries during spring. $DBP_{sp}$ = dead lateral bud percentage in spring; $SD_{sp}$ = Stem damage in spring, scores 0-6; $TB_{sp}$ = Terminal bud damage, scores 0-2. Diaz et al. 2009.

**Figure 1-12.** The relationships between leaf fall in a Spanish field trial with C. sativa and three estimates of freezing test injuries during autumn. The broken line refers to the relationship with exclusion of the encircled population with the earliest leaf fall. $DBP_{au}$ = dead lateral bud percentage, autumn; $SD_{au}$ = Stem damage in autumn, scores 0-6; $TB_{au}$ = Terminal bud damage in autumn, scores 0-2. Diaz et al. 2009.
The strengths of the relationships between field data and damage traits following freeze testing are summarized in Table 1-1. With the exception for two traits, dead lateral bud percentage (DBP<sub>au</sub>) and terminal bud score after autumn freeze testing (TB<sub>au</sub>) there were strong relationships between the majority of the traits. These traits had just one strong relationship with other traits. The absence of any strong relationships between bud damage during autumn and stem damage during autumn was discussed and one explanation might be that different sets of genes were involved in frost tolerance of buds and stems. Alternatively, the gene expression might be different in these two types of tissues. It is somewhat strange that the stem damage after autumn freeze testing (SD<sub>au</sub>) was strongly related to bud flushing in the field trial but with a less strong relationship with leaf fall in the field trial.

As regards relationships with climatic variables at population origins and observed freeze testing results, only two traits from the fall testing were significant, TBD<sub>au</sub> (terminal bud damage score) and DBP<sub>au</sub> (dead bud percentage), with one climatic variable, absolute maximum temperature (Fig. 1-13). Some kind of increase with temperature is expected but a full biological understanding of this relationship is hard to present. Population No. 1 from interior Galicia is the population that disturbs a linear relationship between absolute maximum temperature – stem damage. The linear relationship for this trait was non-significant, $R^2 = 0.44$. Contrary to the results

**Table 1-1.** The strength of the pairwise relationships between bud flushing (BF), leaf fall (LF) in field, and six traits assessed after freeze testing during spring and autumn of six Spanish C. sativa populations. SD<sub>sp</sub> = stem damage score 0-6, TB<sub>sp</sub> = terminal bud damage score 0-2; DBP<sub>sp</sub> = dead bud percentage; sp = spring, au = autumn. Red = non-significant relationships. 06 stands for age 6. Diaz et al. 2009.

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**Figure 1-13.** The relationships between absolute maximum temperature at C. sativa population origin and three estimates of freezing test injuries during autumn. DBP<sub>au</sub> = dead lateral bud percentage during autumn; SD<sub>au</sub> = Stem damage in autumn, scores 0-6; TB<sub>au</sub> = Terminal bud damage in autumn, scores 0-2. sp = spring. Diaz et al.

**Figure 1-14.** The relationships between drought index at C. sativa population origin and three estimates of freezing test injuries during spring. DBP<sub>sp</sub> = dead lateral bud percentage during spring; SD<sub>sp</sub> = Stem damage in spring, scores 0-6; TB<sub>sp</sub> = Terminal bud damage in spring, scores 0-2. sp = spring. Diaz et al. 2009.
from autumn freeze testing, three spring frost damage traits were significantly related to two or three climatic variables; drought index (Fig. 1-14), mean of maximum temperature in month with highest mean, and mean monthly summer precipitation.

The family effect for bud flushing in the field trial was significant at one percent level while it was significant at the five percent level for leaf fall. The individual heritabilities for these two traits were 0.19 and 0.17, respectively. The family effect was significant for two of the six traits assessed after freeze testing; terminal bud damage in spring and percentage of dead lateral buds in spring. The heritabilities were estimated at 0.22 and 0.21 for these traits. The relatively limited within-population differentiation was attributed to strong stabilizing natural selection within populations combined with limited gene flow among populations. The latter might be true for populations 3 and 4, which obviously are growing outside the continuous distribution of *C. sativa*.

In conclusion, a meritorious study, which gives valuable information for breeding *C. sativa* in Spain as well as an attempt to understanding the past evolution of *C. sativa* in Spain.

Phenotypic variation in leaf morphology of three populations from Hyrcanian province in Iran was studied by Zarafshar et al. (2010). The populations are growing in three different valleys at latitudes 37.25, 37.09, and 37.07°N. (There is some confusion as regards longitudes and latitudes in Table 1 of the paper.) Ten leaf characteristics were assessed and four ratios between traits were included in the analysis. Before statistical evaluation was carried out adjustments for allometric differences were done. A principle component analysis was carried out on all 14 traits.

To get some standardization among the traits analyzed I calculated the deviation from the grand means of each of the eight measured and two counted traits (number of teeth and number of veins) for each of the three populations. All relative performance values are found in the range 90-110, which suggests limited differences in performance among the populations. It should be remembered that several of the traits had extremely low mean

values, which means that small deviations among the populations cause large percentage variation among the populations.

The relationships between the northernmost population and the other two populations for the eight measured and the two counted traits are illustrated in Fig. 1-15. If the three populations would be samples of one big population the relationships in Fig. 1-15 should be positive and straight lines. None of the relationships in this figure fit such a pattern. The third relationship between the two southernmost populations (not shown) was negative with an R² estimate of 0.69. Thus, these results indicate real differences among the populations, which agree with the results of the principal component analysis, in which lamina length, lamina width, and length between base of lamina and lamina maximum width contributed most to population differentiation. Lamina width is the trait that contributes strongly to the deviation from a straight line in the 37.25 - 37.07°N relationship (Fig. 1-15).

Even if there are population differences as regards these leaf traits it remains to find out whether they are genetically conditioned.
Míguez-Soto and Fernández-López (2015) published data on growth, phenology, and quality traits in two combined provenance and progeny trials with C. sativa in North-Western Spain. Assessments were taken up to an age of twelve years. One trial contains two populations from each of Greece, Italy, and Spain with strongly varying site conditions (Pan-European trial). The geographic location of the population origins is shown in Fig. 1-16. The other trial contains twelve Spanish populations from six localities; at each locality one high forest and one orchard population are included (Spanish trial). More details about these trials are given in the chapter on progeny testing.

Pan-European trials. From Fig. 1-16 it is seen that the late flushing local populations had the highest stem volume. It also had the latest budset, which with respect to growth more than compensate for the late flushing. The populations from the southern localities with their high xerothermic indices have an early flushing and an early budset. It is believed that there is an evolutionary advantage to have an early bud flushing in areas with pronounced summer drought to capitalize on the good growth conditions during the early part of the growth period. I have illustrated the relationships between xerothermic index and stem volume and the two phenological traits in Fig. 1-17. Except for bud flushing, the 2nd degree polynomial relationships are fairly strong. This suggests that xerothermic index is a fairly good descriptor for evolution of budset. The population effect was strongly significant for all traits studied except for survival at age 3.

Spanish trials. The differences among the Spanish populations were strongly significant for all traits except for survival at ages 7 and 12 in one of the trials as well as apical dominance in the same trial at age 7. Since the orchard populations might not genetically reflect the local conditions I studied separate relationships for high forest and orchard populations (1-18 and 1-19). As seen from these two figures the relationships were strong for all three traits in both types of populations. It suggests that the orchard populations do not differ significantly genetically from their corresponding high forest populations. It should be added that there is a strong relationship between population latitudes and their xerothermic indices in this material.

The relationships with xerothermic index are stronger for the Spanish populations than for the European populations. Other factors than xerothermic index might play a greater role for the European populations with their wider geographic origin.
Tchatchoua and Aravanopoulos (2010a and 2015) reported on growth at ages 4-6 in a Greek trial belonging to the same series of field trials as presented above (Pan-European trials). It is located at latitude 40.75°N, longitude 23.30°E, and altitude 760 masl. Basal stem diameter, stem height, and survival were recorded. Volume index was calculated as $\pi \times \text{height} \times (\text{basal diameter})^2/4$.

Strongly significant differences were noted for survival with the largest survival of the Sicilian population, 76%, and the poorest survival of the Greek Mount Paiko population, 63%. It was suggested that mortality was attributed to drought and weed competition and not to disease. Strongly significant population effects were noted for all growth related traits. The interactions population x year were all non-significant suggesting stability in the trait development over years. This is reflected in Fig. 1-20, which shows that ranking of the populations was more or less constant over the three years of study. This was also the case for volume index but not for basal diameter. I made a comparison of the performance of these populations in this Greek trial and in the Spanish trial presented by Míguez-Soto and Fernández-López (2015). Since I had not access to original data the results could not be presented in exactly the same way for the two trials. Fig. 1-21 reveals that the southern Spanish population from Ronda is characterized by a poor growth at both test localities. The two Italian populations with superior growth in the Greek trial show intermediate growth in the Spanish trial. Not surprisingly, the population originating from the same region as the location of the Spanish trial showed the best growth in that trial. It is somewhat surprising that the two Greek populations had higher ranks in the Spanish trial than in the Greek trial. In conclusion, there seems to be strong population x test locality interaction and coming joint analysis of all field trials belonging to this series will elucidate the previous evolution of C. sativa in Europe.

Thirty healthy nuts from ten trees in each of ten geographically widely separated Croatian populations were analyzed by Idžojtić et al. (2009). The trees in these natural populations were growing at least 50 meters from each other. Nine nut traits and seven derived traits were studied. The nut weights varied in the range 3.8-8.8 grams and had the highest coefficient of variation of the “pure” nut traits, 34.4. Three populations had nut weights above eight grams, one of them originated from central Croatia and clustered together with the coastal cluster. They were all growing on carbonate substrate. Of the 16 traits studied, 13 showed significant differences according to ANOVA. The three non-significant traits were all derived

**Figure 1-19.** The relationship between xerothermic index and least square mean deviations from the mean values of six Spanish C. sativa orchard populations for stem volume at age 12, flushing at age 8, and budset at age 2. Positive values mean above mean stem volume and later flushing or budset than the means of the six populations. Míguez-Soto and Fernández-López 2015.

**Figure 1-20.** Plant height at ages 4-6 in a Greek field trial at Taxiarchis with two C. sativa populations from each of Greece, Italy, and Spain. This trial is located at latitude 44.75°N, longitude 23.30°E, and 760 masl. Tchatchoua and Aravanopoulos 2010a.

**Figure T-21.** Relative stem volume growth in two C. sativa trials, one in Greece and the other in Spain. In Greece it is percentage deviation from the mean value at age 6 and in Spain it is the deviation of least square mean values at age 12. Tchatchoua and Aravanopoulos 2010a and Míguez-Soto and Fernández-López 2015.
Table 1-2. A synthesis of the result from the study of physiological traits hypothesized to be of adaptive significance for drought adaptation in C. sativa. Two populations from each of three climatic regions – wet-dry climate in Turkey - were studied under two different water availability conditions. In some cases only one condition was used and is shown as a merged cell. Population differences, trait relationships and trait relationships with precipitation at population origin are given for the results reported in the paper. Significant differences are indicated. Text in red = negative relationships. Significant differences are indicated. Yellow shading indicates that instructive graphic illustrations occur in the Lauteri et al. (1997) paper.

<table>
<thead>
<tr>
<th>Population differences</th>
<th>Trait relationships</th>
<th>Relationship with precipitation at population origin</th>
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<td></td>
<td>irrigated</td>
<td>drought</td>
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<tr>
<td>Carbon isotope discrimination dry &gt; wet</td>
<td>dry &gt; wet*</td>
<td>dry &gt; wet</td>
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<tr>
<td>Leaf soluble sugar Δ</td>
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<td>Chlorophyll content</td>
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<td>Assimilation rate</td>
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<tr>
<td>Photosynthesis capacity</td>
<td>dry Bursa &gt; wet Hopa; 26.6 vs 11.5 μmol/m²/s estimated as O₂ evolution</td>
<td>Leaf to air vapor pressure</td>
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<td>Stomatal conductance</td>
<td>dry &gt; wet</td>
<td>dry &gt; wet</td>
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<tr>
<td>Mesophyll conductance</td>
<td>dry &gt; wet*</td>
<td>dry &gt; wet</td>
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<tr>
<td>Specific leaf dry weight</td>
<td>dry Bursa &gt; wet Hopa; 86.7 vs 66.8 g/m³</td>
<td></td>
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<tr>
<td>Leaf soluble proteins</td>
<td>dry &gt; wet</td>
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Traits. The dendrogram based on the nut traits consisted of three main clusters with 1, 3, and 6 populations. The nut width, nut height, and nut weight contributed mostly to the discrimination of the populations. Finally, it was stated that human impact on the population structure cannot be excluded.

Physiology traits in each of two populations from three climatic regions in Turkey were studied by Lauteri et al. (1997). Most traits are related to drought adaptation: carbon isotope discrimination Δ photosynthesis chlorophyll content stomatal conductance mesophyll conductance leaf soluble sugar Δ leaf dry matter leaf soluble protein.

Figure 1-22. The relationship between precipitation at population origin and leaf dry matter Δ in Turkish C. sativa populations from three climatic regions. Lauteri et al. 1997.
Measurements took place both in laboratory and in a field trial close to Orvieto, Italy, latitude 42.72°N and 500 masl. Irrigation was carried out in field until July 21. Measurements were done at the end of the watering period and one month after the final irrigation. In addition potted trees from the extreme populations, Bursa and Hopa, were also included in a study of oxygen evolution, maximum RuBP carboxylase activity, as well as specific leaf dry weight. Several relationships between the traits were calculated. The experiments had replications but no information on how the statistical evaluation was carried out. In some cases Pearson correlations were calculated and r-estimates were presented. I calculated the $R^2$ estimates in Figs. 1-22 - 1-24.

I have tried to synthesize the results from this investigation comprising several traits, their relationships, as well as relationships between a few traits and Precipitation at Population Origin (PaPO, Table 1-2). For all the traits included in this investigation there was a difference dependent on the origin of the populations. Significant population differences were found for three of the traits: Carbon isotope discrimination $\Delta$

Photosynthesis rate

Mesophyll conductance

Before any results are presented or discussed it is worthwhile to remember that the field trial used in this study is located in a region with summer drought that might have strengthened the differences between populations originating from different climatic conditions. Even if population differences could not be proven for all traits, it is clear that all results except for $\Delta$ point in the same direction supporting the hypothesis that western Turkish populations are more adapted to summer drought than eastern populations from a more humid climate. Contrary to the present results as regards $\Delta$, this trait was higher in nuts from eastern Turkish populations than in nuts from western Turkey, 25.7 and 22.9%, respectively. The differences among the populations with respect to $\Delta$ were the same in the two treatments. It was stated that low leaf nitrogen content and low photosynthetic capacity characterize wet-adapted populations, a combination that might be detrimental under drought conditions. It was speculated that the higher stomatal conductance in drought-adapted populations might be attributed to enhanced capacity to take up soil water thanks to investment in root development. Mesophyll conductance studied in three populations, two eastern and one western, revealed a relationship of this trait with soluble protein content and specific leaf dry weight. Thus, high mesophyll conductance leads to high photosynthetic capacity and large dry matter production. There was a fairly strong relationship between PaPO and leaf dry matter $\Delta$, $R^2 = 0.82$ (Fig. 1-22). An almost identical relationship for chlorophyll content was observed (Fig. 1-23). These two traits were strongly related, $R^2 = 0.91$. As expected there were moderately strong relationships between chlorophyll content and assimilation rate, $r = 0.97$. The almost parallel relationships for net assimilation rate with PaPO were less strong, $R^2 = 0.60$ (Fig. 1-24). In spite of being parallel it was stated that the interaction population x treatment was significant. The same size of deviations from the lower line as from the upper line is proportionally larger than for the upper line and therefore a population x treatment can be explained.
To illustrate this, I plotted the relative performance in the two treatments in Fig. 1-25. It is clear from this figure that there are rank changes between the two treatments that probably contributed to the significant interaction.

In conclusion several of these physiological traits show considerable population differentiation, which can be attributed to their adaptation to different water availability at population origin. These results call for extended investigations to estimate genetic parameters both between and within populations.

In a later paper by Lauteri et al. (1999) additional data on traits studied in the previous paper are reported together with information on juvenile growth. In this report two eastern and one western Turkish population are included and three populations from the transition zone in Turkey. The plants were growing in a field trial and assessments of carbon isotope discrimination and growth from year two were reported. Gas exchange measurements were carried out on potted plants.

Fig. 1-26 reveals that the growth is not impressive with plant heights in the range 13-26 cm. The relationship between plant height and Δ was weak (Fig. 1-26). In contrast to this, stomatal conductance and transpiration showed a dry-wet gradient with the higher estimates of the populations from dry areas. Much focus of the discussion was centered on evolution of drought tolerance. Since there was no information on the climate, and particularly precipitation, at the origin of the populations it is hard to draw any conclusions on previous adaptations of the studied populations.

1.2 Metric Traits and Markers

Based on earlier results from analysis of variation of isozyme pattern in Turkey (Villani et al. 1991) six Turkish populations from an east-west gradient were for analysis of isoymes, 13 nut traits, and carbon isotope discrimination (Villani et al. 1992). $F_{ST}$s based on 21 isozyme loci were estimated and discriminant analysis of 12 metric nut traits was carried out. One of the nut traits, number of seeds per bur, did not vary among the populations. Seven months after germination plants were divided in two groups; no watering for 40 days and no water stress. After these 40 days carbon isotope discrimination (Δ) in apical parts of the seedlings were determined.

The isozyme analysis confirmed earlier reported results with a strong separation of the two western and drought-adapted populations from the four other populations. A less pronounced difference was noted between the two eastern and two central populations. Nut fresh weight, length of the deepest episperm sinus into the cotyledons and number of sina weighed heaviest in the first function of the discriminant analysis of the nut traits that explained 68.5% of the variance. Length of the style and width of the hylum had the highest weights in the second function, which explained another 14.3% of the variance. It was noted that nut weight also contributed in the two first functions of this analysis. The discriminant analysis clearly separated the western Bursa populations from the other five populations. This was also true for the Istanbul population but to a lesser extent. The second discriminant function separated the two central populations from the two eastern populations.

The two western populations were characterized by the poorest growth and highest Δ of the tested populations. The relationships between Δ and plant height was tested separately for the two watering regimes and were reported as weak but significant, $r = -0.47$ and $r = -0.36$, respectively. Looking at the data in Table 4 of the paper I found these results surprising and tested the relationships using

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**Figure 1-25.** The relative assimilation rate of six Turkish C. sativa populations from three climatic regions. Brown = dry, green = humid, orange = intermediate between the two extremes. Tests were carried out at the end of irrigation treatment at July 21 and one month after that (drought). Lauteri et al. 1997.

**Figure 1-26.** The relationship between plant height and carbon isotope discrimination for six C. sativa populations from Turkey and two full-sib families (encircled). Brown labelled population originates from the drought affected region in western Turkey. The dark green populations originate from mesic climate in eastern Turkey. The rest of the populations originates from the transition zone between the two extremes. Lauteri et al. 1999.
population mean values and found strong degrees of explanation in both treatments (Fig. 1-27). The published r-estimates were based on individual assessments, which explain the difference (Lauteri pers. comm.) The high Δ estimates of the supposedly drought-adapted populations suggest low water use efficiency of these populations. This contradicts the hypothesis that they really are drought-adapted.

In conclusion, this investigation showed, independent of trait studied, similar subdivision of the Turkish C. sativa populations with contrasting characteristics of eastern and western populations and intermediate characteristics of populations in the transition zone in between.

Two natural C. sativa populations in Greece from two geographically different localities, Paikon and Hortiatis, were studied with respect to nine polymorphic isozyme loci, six morphological traits of the nuts, and nut weight (Aravanopoulos et al. 2001). At both test localities a coppice population was studied with respect to isozyme profiles. Only for the Hortiatis coppice population there was enough material for a comparison with the natural population as regards nut traits.

There was no difference in observed heterozygosity between the two types of population at any of the two localities. Contrary to this, observed heterozygosity between the two localities was large; Hortiatis = 0.31 and 0.33, Paikon = 0.23 and 0.22, the first figure in each pair refers to natural population. This difference between the two localities was strongly significant. No estimate of F_{ST} was presented.

All traits related to nut growth had higher values at Paikon than at Hortiatis and the lowest values were noted for the natural population at Hortiatis (Fig. 1-28). For this figure I have selected three traits with differing ratios. The observed difference between the two localities was attributed to better growth conditions at Paikon than at Hortiatis. At the latter locality drought is more pronounced than at Paikon. However, the ratio natural population/coppice population was quite similar for all nut traits except for nut weight (Fig. 1-28), which means that the coppice population at Hortiatis was less affected by the adverse growth conditions than the natural population.

It was concluded that large differences both for isozymes and quantitative traits mean that there is a potential for breeding. As regards the two types of management, markers indicated no human impact while there was a significant difference for the nut traits between the two types of treatment at Hortiatis.
Heterozygosity in five isozyme loci were used by Stilwell et al. (2003) to estimate the relationship with tree growth in one C. dentata population in southern Appalachian Mountains, Virginia, USA. In addition, three other stands were studied to reveal isozyme differentiation among and within populations. All populations originate from high elevations, 1,130-1,210 masl. Trees within a radius of 10 m of a tagged tree were regarded as belonging to a subpopulation. The number of trees per subpopulation varied between 2 and 14. The number of subpopulations was not given. According to the authors the homozygous classification indicates individuals homozygous at all allozyme loci assayed. The heterozygous classification indicates individuals heterozygous at one or two isozyme loci. The size specific growth rate was calculated as \((\text{basal diameter 2001} - \text{basal diameter 1998})/\text{basal diameter 1998}\). Other traits assessed were the health of each tree, the number of sprouts produced at the root collar of each tree, and the number of conspecific stems within a 3-m radius of the focal tree.

There was virtually no differentiation among the four populations, \(F_{ST} = 0.016\). For three of the five loci there was a significantly higher excess of heterozygotes. Contrary to the among-population differentiation, the \(F_{ST}\) for subpopulation differentiation was ten times larger, 0.127. I suspect that the low number of trees per subpopulation is part of the explanation for such large differentiation. There was a disturbing difference among \(F_{ST}\) estimates for individual loci, 0.006-0.204.

As regards the relationship between isozyme heterozygosity and metric traits only the size-specific growth rate showed a relationship with heterozygosity. This is illustrated in Fig. 1-29, which shows an excess of observed heterozygotes in the group of fast growing trees. If there is a disadvantage of heterozygotes the pattern of the medium growth rate group would be intermediate to the two extreme growth rate groups. It would have been useful to know the definitions of slow, medium, and fast growth. (Figure 2 in the paper shows negative values of size-specific growth rate, which indicates shrinkage of the basal diameter.)

A detailed and very laborious study of one cultivar of C. sativa cultivated at different altitudes in Portugal (709-860 masl) was carried out by Dinis et al. (2011). Leaf and nut morphology traits as well as phenological traits were analyzed in 25 trees. Mitotic activity in vegetative buds and time of flowering were recorded. Besides the genotyping of the 25 trees with nine microsatellite loci there was not much genetics in this study.

It was found that there were differences among the trees from different altitudes for morphological and phenological traits, which probably was a response to the ambient conditions at the seven localities. Thus, it showed phenotypic plasticity for the traits studied. The estimated pairwise genetic distances based on the microsatellite analysis confirmed that all 22 trees from six of the seven localities constitute one clone while the mean genetic distance of the three trees from the seventh locality to the other 22 trees was estimated at 0.17. This study was not designed for a comparison of genetic and environmental effects on the development of different traits. In spite of this, it was concluded that the observed differences in morphology and phenology were attributed to environmental conditions and not to small genetic differences.

Leaf and nut traits were recorded together with AFLP analysis of C. mollissima populations from nine localities in Yanshan district and Yangtze valley in China by Lan et al. (2009). It was stated that 82 varieties were tested. It is not clear to me if variety is = cultivar. In all, seven morphological traits, including two ratios, were analyzed. Data were presented for six populations. The cluster UPGMA analysis revealed that one population deviated strongly from the other five populations. This is reflected in the genetic distances illustrated in Fig. 1-30. The mean genetic distances between the group of five populations are low and the means become much larger when the deviating population is included in the
mean values. The low $F_{ST}$s indicate that gene flow among the populations is substantial. There was a weak but strongly significant relationship between genetic distance and geographic distance, $R^2 = 0.24$. (The authors were aware that the degree of explanation is more important than significances.) The relationship was attributed to reduced gene flow with increasing distance. The ratio of within-population variance/among-population variance varied in the range 2.5 – 13.4; leaf width had the lowest ratio and nut length/nut width had the highest ratio.

1.3 Markers

1.3.1 C. sativa

In a series of papers Villani and coworkers (Villani et al. 1991, 1994, 1999, Pigliucci et al. 1990, Fineschi et al. 2000, Mattioni et al. 2010 and 2013) have studied the genetic variation in C. sativa from Mediterranean countries including Turkey. In Villani et al. (1991) the focus was on variation of populations from Turkey and a comparison between Turkish and Italian populations. Sixteen isozyme loci were analyzed in 13 Turkish populations, seven classified as eastern and six as western populations.

In four loci (esterase 1, glucose phosphate isomerase 1, leucine amino phosphatase 1, peroxidase) there was a large difference in allele frequencies between eastern and western populations. The observed heterozygosity was highest in eastern Turkish populations, 0.28, and lowest in Italian populations, 0.23. The mean genetic distances were lowest within each geographic group (Fig. 1-31). This figure shows clearly that the eastern Turkish populations differ much from the western Turkish and the Italian populations. Based on the own data and palynological data a hypothesis for the present differentiation of C. sativa populations was presented:

1. Westward expansion from refugia in eastern Turkey, that took place 40,000 – 1,500 years BC
2. A man-driven expansion into western Turkey and Greece between years 1,500 and 200 years BC
3. A second man-driven expansion to other Mediterranean countries took place up to 200 years AD.

The effect of climate and geographic location of the same 13 Turkish populations was reported by Pigliucci et al. (1990). Focus was put on the type of variation of C. sativa in Turkey, patchy or clinal. Principal component analysis including location, several temperature and precipitation variables as well as solar exposure was carried out. Spatial autocorrelation indices according to Moran (1948) for the alleles in 16 isozyme loci were calculated for four groups of populations with the following upper distance limits of the number of trees: 204, 388, 597, and 1072 kilometers. The limits were selected in such a way that the number of populations should be roughly the same in the four groups.

With the exception of three alleles there was a monotonic drop of Moran’s index with distance class. For the three deviating alleles one depression in the relationship was observed, which suggests that other factors than geographic distance was responsible for the relationships. There was strongest support for clinal variation but the deviating three alleles suggested that differences in precipitation among the populations might have influenced the existing variability.
Villani et al. (1994) used 13 isozyme loci to study the differentiation between 52 *C. sativa* populations from France, Italy, and Turkey. No Wahlund effects were detected in any of the 52 populations. The frequency of polymorphic loci in the four groups increased in the following way: French (58%) < Italian < Western Turkish < Eastern Turkish (83%). The marginal populations had the lowest degree of heterozygosity, which was attributed to successive migration events rather than to natural selection.

The mean genetic distances within and between four geographical groups of populations are shown in Fig. 1-32. The differentiation of populations within each of the four groups increased in the following way: French (58%) < Italian < Western Turkish < Eastern Turkish (83%). The marginal populations had the lowest degree of heterozygosity, which was attributed to successive migration events rather than to natural selection. The mean genetic distances within and between four geographical groups of populations are shown in Fig. 1-32. The differentiation of populations within each of the four groups was limited, all estimates below 0.06. Similarly, the French and Italian populations did not differ much, 0.028. The estimate of 0.228 for the difference between the two Turkish groups is striking and much larger than the differences between the eastern Turkish group and the Italian or French groups. Such a high estimate approaches values typical for differences between two species. The large heterozygosity of the eastern Turkish populations as well as results from the autocorrelation study suggests that the eastern populations are close to the center of origin of *C. sativa*.

In addition to earlier obtained information on genetic differentiation of *C. sativa* in Turkey based on 25 populations, nine more populations from Bithynia region were sampled by Villani et al. (1999). Fifteen isozyme loci were analysed. Number of migrants per population and generation was estimated by $F_{ST} = 1/(1 + 4Nm)$. A hybrid index estimating the number of western alleles in each tree was calculated based on alleles in the eight most diagnostic loci. The mean $F_{ST}$, 0.18, was noted. A still higher estimate, 0.22, was calculated for the comparison of eastern and western populations, which is close to what is expected for species differentiation. The allele frequencies in the eastern and western Turkish populations were relatively uniform but different from each other. The most conspicuous result in this investigation is the steep cline of western alleles within Bithynia for the eight diagnostic loci (Fig. 1-33). An extremely good fit to the curve in this figure was noted, $r = 0.97$. The zone of transition in Bithynia was estimated at 324 km. Two populations constituted bridges, one to the western region and the other to the eastern region.

The focus of this paper was to explain the pattern of allele frequencies from an evolutionary point of view. Two hypotheses for the steep cline in Bithynia were presented: Natural selection

Secondary contact of previously isolated populations

A closer look at the climatic conditions in the three re-
The percentage of fixed loci either absent or 100% in naturalized, coppice, or orchard populations of C. sativa from 29 localities from Spain to Greece. Seventy-three inter-simple sequence repeats, ISSRs were analyzed. Mattioni et al. 2008.

A synthesis of the inter-simple sequence repeats (73 IS-SSRs) variation among C. sativa populations studied in the EU funded project CASCADE was presented by Mattioni et al. (2008). In this project 29 localities from five European countries were included; France (3), Greece (8), Italy (9), Spain (6), and United Kingdom (3). If possible there should be three types of populations selected at each locality; naturalized, coppice, and orchard populations. One objective was to test if management had an impact on the genetic variation, which means that there would be differences among the three categories of population. Several different statistical analyses were carried out. A simple illustration of the differences among the three types of population is shown in Fig. 1-35. The percentages of fixed alleles are much higher in the orchard populations than in the other two types of population. This was attributed to the clonal origin of the trees in the orchard populations. It was noted that the Greek and Spanish orchard populations had higher proportions of fixed alleles than orchard populations from other countries. No differences were noted between coppice and naturalized stands and no specific alleles related to domestication level were observed. It was remarked that many naturalized stands were developed from abandoned coppice. For this reason, differences between these two types of population were not expected. It was hypothesized that the number of trunks in coppice stands were reduced over time owing...
to thinning and in this way approaching conditions similar to high forest stands. If this is the case the difference between coppice and natural stands will be reduced.

The estimates of Nei’s (1978) diversity indices for the populations from individual countries and domestication levels are illustrated in Fig. 1-36. This index is lower for the orchards in all four countries and particularly in Greece (p<0.007). Compared to the situation in the other three countries the total diversity and within country diversity was much less in the Greek orchards. In four of the six Greek orchards the allele frequencies differed significantly from the naturalized and coppice populations at their growing localities. Such differences were also noted for three of the six Spanish and two of the nine Italian orchards populations while no significant difference was found for French orchard populations. This difference between orchard and the two types of populations was attributed to the use of non-local material for grafting in orchards.

The two first components in a principal component analysis explained approximately 35% of the total variance. Three different groups could be distinguished;

- Greek orchards
- Greek naturalized and coppice stands
- All other European populations, although widely spread over the cluster diagram.

It was suspected that the clones in the Greek orchards were selected from a heterogeneous background or that clones were introduced from abroad.

A separate PCA for the 44 coppice and naturalized populations gave a clearer structuring:

- One distinct Greek cluster
- One Italian cluster
- One Spanish cluster

The populations from France and UK were scattered in the Italian and Spanish clusters.

When a cluster analysis based on principal components 1-6 was carried out, the Greek cluster was split into three clusters. Besides, one Galician cluster consisting of two stands and one general European cluster including 27 populations from France, Italy, Spain and UK were distinguished. According to the authors this geographically wide cluster was an effect of human mediated activities.

An AMOVA was carried out to obtain a partitioning of the variance in allele frequencies. This was based on 40 of the 44 populations. Four populations were discarded since their assignment to their clusters was not satisfactorily proven. In Fig. 1-37 one partitioning for all 40 populations is shown as well as one after exclusion of the Greek populations. Most of the variance was attributed to the variation within populations and the variation among populations within clusters was reduced by 50%.

In conclusion this comprehensive study:

1. demonstrated a clear difference in the genetic setup of orchards on one hand and coppice or naturalized forests on the other hand
2. identified five *C. sativa* clusters in Europe, three of them in Greece.

Mattioni et al. (2013) used six microsatellite loci for a study of differentiation and possible refugia during the latest glaciation. In all 779 trees from 31 *C. sativa* popu-
Figure 1-39. Allelic richness (AR) x 0.1 and observed heterozygosity (H_o) in six groups of C. sativa populations based on analysis of six microsatellite loci. Mattioni et al. 2013.

Figure 1-38. The estimated ranges (lowest population mean - highest population mean) for number of alleles per locus (N_a), effective number of alleles per locus (N_e), observed heterozygosity x 10 (H_o), expected heterozygosity x 10 (H_e), allelic richness (AR). Six microsatellite loci were analyzed in 31 C. sativa populations from Turkey, Greece, Italy, and Spain. Mattioni et al. 2013.

lations in Turkey, Greece, Italy, and Spain were included in the analysis. The following estimates were presented:

- Mean effective number of alleles per locus, N_e
- Mean number of alleles per locus, N_a
- Allelic richness, AR
- Private allelic richness, P_Ar
- Observed heterozygosity, H_o
- Expected heterozygosity, H_e
- Inbreeding coefficient over all loci, F_IS
- Inbreeding coefficient for the five loci lacking null alleles, F_IS^5
- Bottle neck events, BE.

Fig. 1-38 reveals that the range for N_a is larger than the range for N_e. Similarly the range for H_e is larger than for H_o. No outliers were observed for any of these measures. Contrary to this, allelic richness for private alleles had a skewed distribution with two extreme values, Galicia in Spain and Hopa in Turkey; 0.52 and 1.12, respectively. None of the other populations exceeded 0.25 for P_Ar and eight populations did not have any private alleles. The Central and Western Turkish populations had larger allelic richness and observed heterozygosity than the rest of the populations, which suggests some geographic differentiation (Fig. 1-39). Estimates of F_IS were calculated for the five loci without any null alleles.
The populations with significant estimates of $F_{IS}$ are shown in Fig. 1-40. All of these estimates were positive, and thus with a deficit of heterozygotes. This figure illustrates that most of the populations with significant estimates of fixation index originate from Turkey. This suggests that the Turkish populations are more isolated than other populations.

The STRUCTURE program analysis suggested a grouping of the populations into three clusters, one Italian-Spanish cluster, one Greek-Western Turkey cluster, and one Central-Eastern Turkey cluster (Fig. 1-40). It was stated that a human impact on the Italian-Spanish cluster could not be totally ruled out. There was some overlap between the two eastern clusters in the central Turkish populations. With a wind-pollinated species with a more or less continuous distribution, sharp boundaries between different clusters are not likely. Rather, an ecoclimatic variation is expected; at least for traits of adaptive significance.

The partitioning of the variance showed that most of the variation was attributed to within-population variation (Fig. 1-41). This result indicates a considerable gene flow among populations. Separate $F_{ST}$ estimates for the three clusters revealed that the lowest differentiation among the populations was noted for the Italian-Spanish cluster, 0.12, and much larger in the other two clusters, 0.19 and 0.21. The results obtained suggest that the present $C. sativa$ populations originate from three glacial refugia, two in Turkey and one in the Iberian-Italian area. Data from several of the Eastern Turkish populations suggested that bottle neck events had taken place. Only one population from Lazio region in the western cluster showed significance for a bottle neck event. The occurrence of bottle neck effects and significant inbreeding in several of the eastern Turkish populations suggest some isolation and limited human impact on high forest populations from this part of Turkey. It was speculated that the ancestors of present day Greek populations migrated from western Turkey to Greece. No chestnut occurred in Greece during the latest glacial maximum, which supports such an interpretation of the origin of the Greek $C. sativa$ populations.

Preliminary results from this investigation were presented by Mattioni et al. (2010).

Chiocchini et al. (2016) used data from the above paper in a combined population genetics, landscape ecology,
and spatial analysis of 31 C. sativa populations. In this report expected heterozygosity, allelic richness, private allelic richness, and membership of each population in the three clusters identified in the above paper were used. Different calculation methods were used to derive maps of genetic diversity.

Inverse distance weighted algorithm were used for $H_e, R$, and $PR_s$; expected heterozygosity, allelic richness, and private allelic richness, respectively.

Monmonier’s maximum difference algorithm implemented in BARRIER 2.2 software was used to find borders between different groups of populations. Genetic discontinuities were based on Delauney triangulation and the resulting Voronoi tessellation. In agreement with the previous paper three main centres for high genetic diversity were identified:

- Easternmost Turkey
- Western Turkey
- Northern and Central Italy

Central Turkey was characterized as a transition zone. Maps with different intensities of the colours of the three clusters dependent on genetic similarity were presented. The study of genetic barriers revealed one barrier separating the Italian-Spanish populations from the other populations, which in turn were separated between the Greek and three south-western Turkish populations and the rest of the Turkish populations. The Dinaric Alps and Adriatic Sea were suggested as barrier for the Italian-Spanish cluster and the Greek-western Turkey cluster. The Taurus Mountains in Turkey was suggested as the barrier between Eastern Turkey and Western Turkey-Greek cluster. In essence the results in this study confirm the results obtained by Mattioni et al. (2013). What is new in this paper? The various maps are good illustrations of genetic variation in C. sativa.

Isozyme and RAPD pattern of variation in two Turkish populations, Bursa (44 trees) and Hopa (38 trees), were studied by Fornari et al. (1999). These populations originate from strongly different site conditions. Fourteen polymorphic isozyme loci and 180 RAPD markers were studied.

Only one isozyme locus in the Hopa population deviated significantly from expectation according to Hardy-Weinberg equilibrium. Owing to the large number of RAPD markers it was estimated that the information content from the RAPD markers is more than five times larger for this marker than isozymes. The isozyme locus Skdh-1 was polymorphic in Bursa only, while 6Pgdh-1 was polymorphic in Hopa only. Fairly high estimates of $F_{IS}$ and $F_{ST}$ were noted, 0.062 and 0.217, respectively. The latter figure suggests a considerable difference between the two populations and that inbreeding had occurred. It would have been valuable to discuss these figures. The genetic identities for the individual populations showed a slightly different pattern with higher estimates for isozymes in Hopa population than in Bursa population (Fig. 1-42). The ratios joint analysis/individual population analysis showed somewhat higher similarity for RAPDs than the identity for isozymes; 0.92 versus 0.79 (Fig. 1-43). The principal component analyses resulted in a finer subdivision of the intra-population dendrogram with RAPD than with isozyme analysis. Both markers showed a clear differentiation between the two populations. It was pointed out that both markers reflect the difference between physiological traits in these two populations as reported by Lauteri et al (1997).

Figure 1-42. Genetic diversities according to Kimura and Crow (1964) estimated by isozymes (16 loci) and RAPDs (180 markers) in two Turkish C. sativa populations from contrasting ambient conditions; Bursa continental and Hopa maritime climate. Fornari et al. 1999.

Figure 1-43. Identities (isozymes) based on 16 isozyme loci and similarities based on 180 markers (RAPDs) of two Turkish populations of C. sativa from contrasting ambient conditions; Bursa continental and Hopa maritime climate. Fornari et al. 1999.
Seabra et al. (2001) used RAPDs to make an inventory of C. sativa genetic resources in Portugal. Sampling of material from northern Portugal was carried out according to the following:

- Fruit orchards: 5 trees in each of eight orchards
- High forest: 5-6 trees in each of three populations
- Coppice: 4-6 trees in each of five stands

It was stated that the three high forest populations were the only ones in Portugal with an age of 50-60 years. Canonical Discriminant Analysis was carried out for each type of population.

Fig. 1-44 reveals that the two first components of the CDA explained different portions of the variation. Three of the eight orchard populations were significantly differentiated from the other five orchard populations while these two components explained all variation among the high forest populations. The coppice populations took an intermediate position with 70% of the variation explained by the two first components. It was stated that the variation within populations was lowest in the orchard populations. The variation was limited in six populations but not in all eight populations. The limited variation was attributed to clonal origin of many of the orchards trees. It was stated that one high forest population and one of the coppice populations originated from the same locality. Both of them showed high homogeneity.

ISSRs (73) and isozymes (16) were used to characterize 82 European C. sativa populations from France, Greece, Italy, Spain, and UK by Aravanopoulos et al. (2005). Each population was represented by 26 trees. Sixteen isozyme loci and 73 ISSR markers were included in this study. To improve the precision of population separation a multiple discriminant analysis (MDA) was carried out. According to the authors, the main purpose of MDA is to classify individuals with minimum probability of misclassification. MDA estimates the true relationship of genetic loci to a specific group. A principle components analysis was also carried out.

The mating pattern was studied in 15 populations from Greece, Italy, and Spain. In each population nuts were collected from 7-9 trees and around 30 other trees were genotyped in each stand for a paternity analysis. For this study 24 isozyme loci and 12 SSR loci were used. Matings with pollen from different distances from the target tree were assessed.

The result of the MDA analysis is illustrated in Fig. 1-45, which shows that the range of the unique gene pool is 25 – 65%. The highest estimate was noted for the southern Greek island population, which means that this population is the most differentiated from the rest of the populations. In Fig. 1-46 it is demonstrated how the “alien” individuals are distributed to the other studied populations for the two extreme populations, the Italian south-central population and the Greek island population. This figure reveals that the Greek island population did not contain any trees that could be assigned to five of the other populations while the corresponding for the Italian population was just one population.

The MDA analysis might be discussed from a gene ecological point of view. Is there ecotypic or ecoclinal variation in C. sativa? If all columns are 100% in Fig. 1-45 we have an extreme ecotypic differentiation. If we have an even size of all columns in Fig. 1-46 it would strongly indicate an ecoclinal variation. The Greek island population gives support for ecotypic variation while the Italian south-central population lends support for ecoclinal variation. It should be reminded that more than one migrant per generation prevents fixation of neutral genes in a recipient population and thus a constraint against ecotypic variation.
differentiation. In the study of the 27 Italian populations the estimated number of migrants per generation was >2 for the high forest and coppice populations, which promotes ecocline variation for these two types of population. It should be remembered that the pattern revealed for neutral markers does not mean that differentiation would be the same for adaptive traits.

The first principle component that explained 17% of the variation separated the Greek populations from the other populations. Thus, lending support to the result from the MDA analysis as regards this population.

It is regrettable that no conventional $F_{ST}$ estimates were presented to allow a comparison with the data from the MDA analysis. In the pan-European project CASCADE $F_{ST}$s for high forest, coppice, and orchard populations from the entire distribution area in Italy were presented with the following estimates based on 16 isozyme loci:

- High forest, nine localities: 0.09
- Coppice, nine localities: 0.10
- Orchard, nine localities: 0.25

The high estimate for the orchard populations might be attributed to different clones in different orchards. As a wind-pollinated species the estimates are fairly high for the high forest and coppice populations too. The considerable climatic differences between the Italian population localities might have contributed to the relatively high $F_{ST}$ estimates. The $F_{ST}$ estimates for the three types of population were -0.09, -0.05, and -0.25, respectively. It may be speculated if the high surplus of heterozygotes in the orchards is a sign of heterozygote advantage of orchard clones.

The assigned matings in the paternity analysis are illustrated in Fig. 1-47. The range was 24 – 46%. It was stressed that too far-reaching conclusions should not be drawn from these figures since all trees in the localities were not genotyped. Moreover, unassigned nuts might be attributed to cryptic or gene flow from other populations. There was a rapid drop of mating events from approximately 8% of assigned matings with close neighbors to 1% at approximately 300 meters from the target tree. This percentage remained up to a distance of 2.2 km. The selfings observed (Fig. 1-48) may also be biased by cryptic gene flow. The high estimate of the southern Spanish population might be attributed to its isolation from other chestnut populations.

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A security distance of two kilometers for in situ conservation populations to other populations was strongly recommended. Such an isolation distance was also recommended for fruit orchards.

**Figure 1-46.** The partitioning of the trees in two contrasting populations with respect to the percentage of trees unique for these two C sativa populations. The own estimates for the two populations are indicated with shading. The capital letters refer to the country identification letters, c = central, is = island, n = northern, s = southern, sc = south-central, se = southeast. The estimates were obtained from multiple discriminant analysis, MDA. Aravanopoulos et al. 2005.

**Figure 1-47.** The percentages of matings with identified males in the own population. Twelve SSR markers and 24 isozyme loci were used in the paternity analysis. The capital letters are the country identification letters, c = central, n = northern, s = southern. Aravanopoulos et al. 2005.

**Figure 1-48.** The percentage of selfing in eight European C. sativa populations. Twelve SSR markers and 24 isozyme loci were used in this analysis. The capital letters are the country identification letters, c = central, n = northern, s = southern. Aravanopoulos et al. 2005.
Akkak et al (2010) tested the cross-species amplification of 17 microsatellite loci from *C. sativa* with 16 other species, among them four other *Castanea* species. Thirteen of the 17 microsatellite loci generated amplification products in the four other species; *C. crenata*, *C. dentata*, *C. mollissima*, and *C. seguinii*. This indicates a high level of conservatism of the DNA within the genus *Castanea*. It was stated that it is important to clone the amplified alleles to confirm identity and avoid misinterpretation of the cross-species amplification.

Thirty-eight *C. sativa* populations from Turkey, Italy, France, Spain and Portugal were analysed with respect to cpDNA and mtDNA by Fineschi et al. (2000). Each population was represented by 3-5 trees. \( G_{ST} \) and \( N_{ST} \) were estimated. Thirteen cpDNA haplotypes were identified, two of them being frequent. The remaining eleven haplotypes occurred in low frequencies 0.02-0.07. Only one mtDNA fragment was found. The most frequent cpDNA haplotype, coined I, occurred in 36 of the populations. It was missing in two south-western Turkish populations. Twenty-five of the populations were monomorphic; twelve of them contained the most common haplotype. Figure 1 in the paper shows in an instructive way the distribution of the haplotypes. This figure illustrates clearly that polymorphism was largest in the Italian populations. \( G_{ST} \) was estimated at 0.43, which is much lower than for other broadleaf tree species. It was even suggested that man had a greater impact on the existing genetic diversity than natural migration. \( N_{ST} \) was significantly higher than \( G_{ST} \) 0.52. This suggests that some geographical structure of the diversity is still retained. It was speculated that the occurrence of different haplotypes in the western and the eastern areas of chestnut distribution lends support to the existence of at least two refugia during the last glaciation. The considerable transfer of chestnut by man complicates the tracing of refugia and migratory routes after the glaciation. The estimated ratio pollen flow/seed flow was close to one, which again singles out *C. sativa* from other wind pollinated broadleaf tree species with ratios of several hundreds. Also in this case it was assumed that the impact of human transfers of nuts and propagation material are responsible for the results.

Three isozyme loci were used to study the genetic differentiation among five geographically, widely separated (approximately five degrees of latitude) pure French *C. sativa* stands by Frascaria et al. (1993b). Each locus had two alleles. Two of the stands were treated as high forest while the remaining three stands were treated as simple coppice system. The mean \( F_{ST} \) for the pairwise comparisons was estimated at 4.1% suggesting a large gene flow among French chestnut populations. Transfer of chestnut by man was also suggested as one explanation for the limited differentiation. There were no signs of inbreeding in the five populations. Even a significant excess of heterozygotes was detected in one locus.

Eleven polymorphic isozyme loci were used to characterize 17 Spanish populations of *C. sativa* by Fernández-López and Monteagudo (2010) (for location of the populations see Fig. 1-49). The focus of the paper was on identifying groups of populations by aid of different calculation techniques. \( F_{ST} \)'s were estimated after exclusion of one population at a time in order to identify populations contributing most to the global \( F_{ST} \). The population with the lowest \( F_{ST} \) after such an exclusion contributes most to \( F_{ST} \).
The mean $F_{ST}$ estimated according to Weir (1990) amounted to 0.15. Such a high estimate is not surprising since the populations sampled covered a wide geographic span of latitudes and altitudes, 7 degrees and 1,200 meters, respectively. The contributions of each population to $F_{ST}$ are illustrated in Fig. 1-49. It should be noted that the differences are not dramatic, 0.14-0.16. The strongest contribution was noted for the north-easternmost population from Ávila province while an Asturian population was the least contributing population to the global $F_{ST}$. This figure also illustrate the clustering of populations into three groups, in which the two southern Galician populations constitute one cluster while another cluster contains nine populations covering the whole range of latitudes in Spain.

The software STRUCTURE was used for another estimation of grouping of the populations. Again three groups were distinguished with five populations in two groups and seven populations in the third group (Fig. 1-50). No geographic grouping could be seen. I made a comparison of the mean $F_{ST}$s obtained among and within two regions with contrasting climatic conditions. One region comprised the five Atlantic populations in Galicia while the other comprised the five populations originating from hot and dry climate. As seen from Fig. 1-51 most of the $F_{ST}$ estimates for populations between climatic regions were larger than for populations within regions. This suggests that a certain geographic differentiation had taken place. The authors claimed that the isozyme data from the 17 populations showed a geographic cline. However, as illustrated in the preceding figures there are some indications of a geographic pattern of the isozyme data but there are signs of patchiness too. The existence of a geographic cline contradicts the assumption that Spanish C. sativa populations originated from different introductions of cultivars. Based on the results presented it is likely that part of the pattern observed might be attributed to introduction of cultivars.

Two of the populations from the hot and dry-climate had a deficit of heterozygotes, $F_{IS}$ 0.15 and 0.23 (1-52). One population originated from Central Spain and the other from Ronda in southern Spain. At least the latter one is growing far from other C. sativa populations, which could explain some isolation and inbreeding. Based on the results it was concluded that the present populations originate from one or two refugia during the latest glaciation. One region in southern Galicia and neighbouring Portugal might have been one refugium since the genetic diversity in this region was large.

Globulin proteins were analyzed in nuts from 146 C. sativa trees collected in 20 populations in Andalucía by Alva-rez et al. (2003). Genetic distances within and among regions were calculated assuming that each band in the chromatograms represented one allele. Thirty-five bands were detected in the chromatograms. There was a geographic grouping of the material with respect to variation of its globulin profiles. The $G_{ST}$ estimates for the variation among the populations within Huelva and Malaga regions were 23 and 22%, respectively. The total $G_{ST}$ from all five regions included in this study was estimated at 39%. These figures emanate from Table 4 in the paper. In the text it was stated that a $G_{ST}$ estimate based on the joint analysis of Huelva and Malaga populations was 10%. This would mean that the variation within each of the two regions is larger than in the joint analysis of the two regions, which is hard to explain.
Fernandez-Cruz and Fernández-López (2016) used nine microsatellite loci for a study of 27 Spanish, two Italian and two Greek populations of *C. sativa*. Genetic diversity within and among geographic groups of populations was presented. The number of alleles in the nine loci varied in the range 3-27. In all, 103 alleles were detected, of which 27 were private and rare. Additional seven alleles were private. The $F_{IS}$ estimates of the individual populations varied in the range -0.15 to +0.15, twenty-one of them were negative. Hybridization with cultivars was suggested as one reason for the surplus of negative $F_{IS}$ estimates. It was speculated that selection of heterozygotes for ink disease tolerance could be an alternative explanation for the mainly negative $F_{IS}$ estimates. Two positive and five negative estimates were significant.

There was a great focus on grouping of the populations by aid of different analyses. Grouping of populations that most likely show clinal variation might be questioned. The observed strong gene flow between populations with a range of 0.9 – 22 migrants per generation must be a great constraint to ecotypic differentiation. The highest estimates of gene flow between populations occurred between populations from adjacent localities. The result of the AMOVA showed that most of the microsatellite variation occurred within the population (78%) but a considerable portion occurred among populations (15%, Fig. 1-53). Four groups of populations were identified:

1. Atlantic cluster containing the Atlantic Galician populations
2. Cantabrian cluster with populations from the Cantabrian and Atlantic coasts
3. Western Mediterranean cluster with populations from mountains in inner Galicia and central Spain
4. Eastern Mediterranean cluster containing the two Greek populations

Besides, several populations of intermediate types were also identified. In the northern and north-western part of Spain many cultivars were selected and probably distributed to many localities. They have probably via pollen spread their genes to adjacent populations. The mean genetic distances ($F_{ST}$) between populations within clusters are more limited than between clusters; (Fig. 1-54). However, the difference between $F_{ST}$s within and between clusters is not pronounced; ranges 0.05-0.07 versus 0.08-0.15. Fig. 1-54 reveals that the Greek populations differed mostly from the other populations. In agreement with this a correlation between geographic and genetic distances was reported. With the large genetic difference between the Greek populations and the other populations, the data from the Greek populations weigh heavily in the relationship. It would have been interesting to estimate the strength of the correlation when the Greek populations are excluded from the relationship.

In Martin et al. (2010b) the genetic differentiation obtained by microsatellites from the entire genome (SSR) and microsatellites from expressed sequence tags (EST-SSRs) in three *C. sativa* populations from each of Greece, Italy and Spain was compared. The populations were selected to represent differing ecological conditions. Six SSR loci and nine EST-SSR loci were studied. $F_{ST}$ was estimated according to Weir and Cockerham (1984), $R_{ST}$ was estimated according to Slatkin (1995), and $Dest$ (= estimator of actual differentiation) was estimated according to Jost (2008). The number of alleles per locus as well as the total number of alleles was higher for the SSRs than for the EST-SSRs; 15.2 versus 3.8 and 81 versus 35. Except for the Greek population Paiko, all populations were polymorphic with respect to SSRs while only three populations were polymorphic with respect to EST-SSRs. The higher

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**Figure 1-53.** The Separation of the variation of 29 *C. sativa* populations from four geographic groups: 3 Spanish and 1 Greek group. Nine microsatellite loci were analyzed. Fernandez-Cruz and Fernández-López 2016.

**Figure 1-54.** The mean $F_{ST}$s within (red columns) and between (blue columns) four geographic groups of *C. sativa* populations. Nine microsatellite loci were analyzed. AT = Atlantic Spain, Cant = Cantabria, W-M = western Mediterranean, GR = Greece. Fernandez-Cruz and Fernández-López 2016.
degree of fixation for EST-SSRs was attributed to the origin of these markers in transcribed regions. Four private alleles were detected for the EST-SSRs while 22 private alleles were found among the SSRs.

The results of population differentiation with three different methods of estimation show that differentiation according to $F_{ST}$ was almost identical for the two markers (Fig. 1-55). $R_{ST}$ and Dest showed different patterns of differentiation based on the two markers with much larger differentiation for SSR markers than for SSR-Est markers when differentiation was estimated according to Dest. The dendrograms for SSR showed a differentiation between the three Greek populations and the other six populations. This result must be attributed to differences in allele frequencies between the populations. Noteworthy from the dendrogram for SSR is the close relationship between the southern Spanish population, Malaga, and the northern Italian Pellice population (The latitudes for mainland Italy populations in Table 1 of the paper are mixed up.). It was stated that the three most northern populations differed from the other six populations with respect to EST-SSR. According to the dendrogram for EST-SSR the Malaga population is the population that deviates most from the other populations. The large discrepancy between the two dendrograms for the Malaga population had deserved a discussion.

All populations had positive $F_{IS}$ estimates for both markers and many populations showed significant deviations from zero (Fig. 1-56). Especially, the $F_{IS}$ estimates for EST-SSR exceeded 0.30 in three populations; Malaga was one of them. Such high $F_{IS}$ estimates suggest that in-breeding had been substantial in several populations. This was not addressed in the paper. Since the populations included in this study originate from high forests and not from vegetatively propagated material the extremely high $F_{IS}$ estimates are puzzling.

One EST-SSR locus discriminated strongly between northern and southern populations and it was suggested that this locus was involved in adaptation.
used seven microsatellite loci for a -
- ST popu-FIS6 4
Fig. FST5 - - - - - ST 5 ST used eleven microsatellite loci for a.
57x93] structure. The Berkovitsa (Ber) population was the geo-
and it cannot be excluded as a contributor to the existing
human impact on the genetic structure was discussed

ing populations had estimates in the range 0.17-0.37. The
estimate of private allelic richness, 0.81, while the remain-
all number allows for larger within-population diver-
genetic impact on the estimates is hard to evaluate. Certainly the
per population. Whether this large number of trees has
by 211 trees while the other populations had 20-42 trees
larger number of unique alleles was found, 30. The
number of private alleles varied between one and four per
population each. The number of populations for the other
graphic groups of populations. MK = Macedonia, XK =
Kosovo, HR = Croatia, BH = Bosnia and Herzegovina, HR C = Coastal Croatia, IT = Italy, RO = Romania, HU
= Hungary. The latter three were represented by one pop-
ulation each. The number of populations for the other
three groups is indicated. Ten microsatellite loci were

There was a high genetic diversity of all populations in
agreement with other investigations of C. sativa popula-
tions in other European countries. The STRUCTURE
analysis resulted in three clusters. The Slavyanka (Sl)
was the most differentiated population followed by the
Brezhani (Br) population. Individuals from the other
four populations were distributed to all three clusters.
The principal component analysis partially supported the
separation into three clusters. I calculated the mean F
ST 58 for each of the six populations. As seen from Fig. 1-57
the Slavyanka population had the largest mean as expected
from the cluster analysis. This population had the highest
estimate of private allelic richness, 1.08. To eliminate the
effect of the Slavyanka population on the mean F
ST values
I calculated a mean for the other five populations (Fig.
1-57). None of these estimates support that the Brezhani
population deviates remarkably from the other four pop-
ulations. The Belasitsa (Bel) population was represented
by 211 trees while the other populations had 20-42 trees
per population. Whether this large number of trees has
impact on the estimates is hard to evaluate. Certainly the
large number allows for larger within-population diver-
sity. The Belasitsa population had the second highest
estimate of private allelic richness, 0.81, while the remain-
ing populations had estimates in the range 0.17-0.37. The
human impact on the genetic structure was discussed and
it cannot be excluded as a contributor to the existing
structure. The Berkovitsa (Ber) population was the geo-

Figure 1-57. Mean FST for six Bulgarian C. sativa populations estimated by seven microsatellite loci. The means of
five populations after exclusion of the most differentiated
distribution are also shown, FST. FIS of the populations
and their significances are illustrated. Lusini et al. 2014.

Lusini et al. (2014) used seven microsatellite loci for a
study of differentiation between six C. sativa populations
from western Bulgaria. The number of trees analyzed in
each population varied between 20 and 211. The traditional
estimates in population genetics such as number of
alleles, observed and expected heterozygosity, FST, FIT
and FIS were estimated. Besides, Jost’s (Jost 2008) unbi-
ased estimator Dest was calculated as an alternative esti-
mate of differentiation. Principal component analysis and
STRUCTURE were used to estimate relationships among
the six populations.

The geographic isolation of the Berkovitsa population is
not reflected in the mean FST estimates for this popula-
tion. As in many other studies of this character there is a
desire to delineate populations into groups in order to
able selection of genetic resource populations. However,
microsatellites are neutral markers, which do not indicate
adaptive variation and as such of less value for pointing
out genetic resource populations.

All FIS estimates were positive and significant in four
populations. This suggests that some inbreeding had taken
place, even in the Belasitsa population with the largest
number of trees examined.

The estimates of Dest were 3-7 times higher than FST estimates of individual loci. This difference between the two
ways of estimating population differentiation was not dis-

Poljak et al. (2017) used eleven microsatellite loci for a
study of differentiation between 15 C. sativa populations
from South-Eastern Europe. The following approximate
latitudinal and longitudinal ranges were included: 41.5-
47.8°N, 13.7-23.5°E, respectively. Twenty trees per pop-
ulation were analyzed except for one population with 15
trees. A sixteenth Croatian cultivated population, Lovran
Marron, was found to be a single clone. The relatedness
to this clone was tested to detect any gene flow from this
plantation to the wild populations.

A large number of unique alleles was found, 30. The
number of private alleles varied between one and four per
cultivated clone. The relatedness
to this clone was tested to detect any gene flow from this
plantation to the wild populations.

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number of private alleles varied between one and four per
cultivated clone. The relatedness
to this clone was tested to detect any gene flow from this
plantation to the wild populations.
The global F$_{ST}$ was estimated at 0.13. In Fig 1-58 I have illustrated the mean F$_{ST}$s for three groups of populations; Hungarian, Italian, and Romanian. It is evident that the three populations from Macedonia and Kosovo are most differentiated from the rest of the populations. The highest F$_{ST}$ was noted for the relationship between the Hungarian and one of the Kosovo populations, 0.30, while the opposite was the case for two of the coastal Croatian populations, F$_{ST}$ = 0.001, which was the only non-significant F$_{ST}$. According to the authors the lowest genetic diversity reported for the coastal Croatian populations suggested that these populations originate from introductions, either from Italy during 14th hundreds or earlier by Romans.

There was a clear clustering of the populations into three groups:
1. Coastal Croatia
2. Continental Croatia, Bosnia-Herzegovina, Italy, and Romania
3. Macedonia, Kosovo, and Hungary

This means that geographic proximity does not always imply close relationship. Especially, the last group contains populations from widely different latitudes. The large difference between the three south-eastern populations and the north-western populations was attributed to migration from separate refugia during the latest glaciation. It was stated that it is likely that these south-eastern populations survived in situ during the latest glaciation. It was noted that the Kosovo population was heterogeneous, which was attributed to anthropogenic effects. It was pointed out that gene flow via pollen from northern populations can be ruled out since there is no C. sativa in intermediate region of central Bosnia-Herzegovina. Similarly, the relationships between the Romanian and Hungarian populations to groups 2 and 3, respectively, were attributed to human impact.

The AMOVA revealed that more than 86% of the variation was attributed to variation within populations (Fig. 1-59). All effects were strongly significant.

Most populations had negative F$_{IS}$ estimates while three populations had high positive estimates with the highest observed for the Italian population, 0.37 (Fig. 1-60). The F$_{IS}$ estimates were not further discussed.

In conclusion, this investigation indicated that the genetic structure of C. sativa in the studied part of Europe is a result of natural differentiation and of human transplantations.
Two chloroplast DNA regions, trnL-F spacer and trnH-psbA spacer were used to estimate variation among three Iranian C. sativa populations from Hyrcania and for phylogenetic relationships among Castanea species (Youzezfazdeh et al. 2014). Ten trees per population were analyzed. Besides, foliar trichomes were examined. It was concluded that low levels of nucleotide diversity and haploid diversity were detected within Hyrcanian samples and the whole species. One possibility for the absence of genetic differentiation among the Iranian populations is that they are panmictic. However, the scattered distribution speaks against such an explanation. It is most likely that a larger number of markers will give more accurate estimation of population differentiation. An opinion supported by the authors. It was also stated that the two markers used could not provide clues as regards species relationships among chestnut species. The Hyrcanian chestnut had fasciculate trichomes on the lower leaf surface, which is a difference between the Hyrcanian chestnut and C. sativa from southern Europe. In spite of all shortcomings it was stated that the Hyrcanian chestnut showed most similarity with C. sativa.

Four small marginal populations of C. sativa in the Hyrcanian region in northern Iran were studied with respect to nuclear microsatellites by Janfaza et al. (2017). Each population was represented by eight trees growing at a minimum distance of 30 meters. Two estimates of population differentiation; according to Nei (1987) and the number of different alleles based on an infinite allele model ($F_{ST}$) were calculated. cpDNA was also analyzed but no polymorphism was noted. I have assumed that the F estimates in Table 3 of the paper stands for $F_{IS}$. The among-population differentiation was estimated at 16%, which must be regarded as fairly high population differentiation. The low number of trees per population might have contributed to this estimate. There was a strong relationship between the two ways of estimation population differentiation (Fig. 1-61). The two geographically most separated populations had the highest estimates of differentiation. In spite of the small size of the populations and their isolation, the $F_{IS}$ estimates were all negative; -0.07 - -0.293. This suggests that inbreeding is not a problem in these populations.

In two papers Pereira et al. (2010 and 2011) analyzed chestnut grafts from the Iberian Peninsula and Canary Islands with methods traditionally used for studies of population diversity. Therefore, they are treated in this chapter. In the first paper ten microsatellite loci were used for studying the genetic set-up of:

- C. sativa 574 accessions from Spain and Portugal
- C. crenata 47 accessions
- C. mollissima 37 accessions
- C. henryi 33 accessions
- Interspecific hybrids 71 accessions from Spain, Portugal, and France

Trees from natural forests 25

Eight chromosomes had one SSR locus each while one chromosome had two loci but located far away from each other resulting in independent segregation. Four loci showed deviations from Hardy-Weinberg equilibrium. These loci were excluded from estimates of factorial correspondence analysis (FCA) as well as AMOVA. It should be remarked that the analysis was carried out after removal of duplicates owing to cloning. The average number of alleles per locus was eleven and a mean observed heterozygosity of 0.50. Fifty-two percent of the alleles were present in all taxa. The proportion of rare alleles was high, which might be attributed to small samples of the exotic species. Eight percent of the alleles were specific to C. sativa while 23% were specific to the exotic species and hybrids. This is a reflection of the observed differentiation among the species. Allelic richness corrected for variations in sample size was largest for C. henryi. The FCA analysis revealed that C. henryi was most differentiated from C. sativa while the other taxa took an intermediate position between the two extremes with some overlap with C. sativa. This is expected since C. sativa was one parent of the interspecific hybrids. It was confirmed that all French and Spain accessions classified as interspecific hybrids were hybrids. Only nine of the 19 assumed hybrids from Portugal were hybrids. This indicates that morphological classification is unsatisfactory for a proper identification of hybrids. Eight of the 25 presumed wild trees from natural populations were found to be interspecific hybrids. The cluster analysis resulted in four clusters:

- C. mollissima and C. henryi
- C. crenata and hybrids
- C. sativa northern Spain
- C. sativa Central Spain

The genetic separation of the two Spanish regions was attributed to the great difference in climatic conditions between these two regions. The accessions from Andalusia and Canary Islands occurred in both Spanish groups. This
was attributed to introduction of cultivars from Northern and Central Spain. The gene diversity varied in the range 0.461-0.722 (Fig. 1-62). The former value was obtained for C. mollissima and the latter for the hybrids. The separation of the variation into within and among populations resulted in several times larger within-population variation (≈92%) than among-population variation (FST=8%). More or less identical estimates were noted for RPP1 (≈8%) and RPP2 (≈92%). In conclusion, the populations of C. sativa in this study behaved as populations in Hardy-Weinberg equilibrium, which justifies their treatment in this chapter.

The objective of the Pereira-Lorenzo et al. (2011) paper was to outline the mechanisms that gave origin to chestnut cultivars, in the Iberian Peninsula, the Canary Islands, and the Azores. As far as I can understand, it is the same material as presented in Pereira-Lorenzo et al. (2010) plus 19 accessions from the Azores Island Terceira. When an accessions shared one allele in each SSR locus this was regarded as hybridization. When the difference between two accessions was just one allele, it was regarded as mutation.

Hybridization was noted for 136 out of 176 different genotypes while 6% of the 375 accessions were characterized as containing a mutation. There was much focus on clonality defined as:

\[ \frac{(\text{No. of accessions} - \text{No. of genotypes}) \times 100}{\text{No of accessions}} \]

Generally, the clonality was higher in south than in north. The 19 accessions from Terceira being extreme consisting of one genotype only. It was concluded that accessions from southern Spain, The Canary Islands, and The Azores were established by offspring or grafts from the two earlier established regions in The Iberian peninsula. Two main groups of cultivars from the Iberian Peninsula were again noted. The northern group contained four subgroups while the southern group consisted of six subgroups. FST's were calculated but no estimate of differentiation of the main groups was presented, nor were there any estimates of differentiation among subgroups. This paper is of more importance for breeding than for genetics.

A data-base of European C. sativa cultivars was developed by Pereira-Lorenzo et al. (2017). One main objective of this investigation was to create a core collection of cultivars that contained all genetic information derived by analysis of 24 SSR loci in 271 accessions. Spain was represented by 96 cultivars out of a total of 118 cultivars, the rest originated from Italy (16), France (4), and Portugal (2). Two of the Spanish cultivars turned out to be interspecific hybrids. Twenty-two of the 24 SSRs used were distributed in eleven of the twelve linkage groups. Traditional population genetic parameters were estimated. The accessions were assigned probabilistically; Reconstructed Panmictic Populations (RPPs).

In all 214 alleles were detected with an approximate average of alleles per locus = 9. Observed and expected heterozygosities were 0.626 and 0.663. Null alleles were found for six loci. The number of unique alleles was 132 and ten groups of synonyms. The STRUCTURE program was used to find a grouping of the cultivars based on SSRs in the 18 loci without null alleles. Seventy-seven cultivars from The Iberian peninsula and Canary Islands were grouped together. Thirty-tree other cultivars constituted a second group while 22 could not be grouped to any of these two groups. Thus 83% of the cultivars could be grouped and related to a geographic region. The subdivision according to STRUCTURE was supported by estimation of Neighbour-Joining tree based on the genetic distance matrix of the 132 genotypes.

A second level of subdivision with five groups was also presented:

- RPP1a 19 mainly Asturian cultivars
- RPP1b 9 related to the Parede cultivar from northern Spain
- RPP1c 24 cultivars from Central and Southern Spain
- RPP2a 14 Italian and 7 Spanish (1 northern and 6 southern) and the 2 French cultivars
- RPP2b 8 interspecific hybrids

The remaining 44 cultivars were not grouped. Three well-known cultivars were grouped into RPP2b together with interspecific hybrids and were characterized by alleles exclusively originating from other chestnut species.
Samples were taken from 11-21 trees in 16 Spanish high forest populations of *C. sativa* for analysis of genetic differentiation by alleles in seven microsatellite loci (Martin et al. 2012). Mantel test, principal coordinate analysis, and program STRUCTURE were used to study any geographic clustering. *F*<sub>ST</sub> was estimated based on all seven loci and six loci with exclusion of a locus with suspected null alleles. Some of the main results are compiled in Table 1-3, which shows that the number of alleles was high in the seven loci studied. The genetic diversity was high and estimated at 0.804. No less than 15 private alleles were detected and 36 occurred in lower frequency than 0.05. The Mantel test run to trace any relationship between geographical distance and genetic differentiation did not result in any significant relationships. Contrary to this, the principal coordinate analysis (PCoA) revealed three groups with close relationship within each group of three pairwise *F*<sub>ST</sub> estimates among the five groups are illustrated in Fig. 1-63, which shows that the estimates between the members in the three RPP1 groups are higher than expected. This can mainly be attributed to the RPP1b group containing northern Spanish cultivars. The mean *F*<sub>ST</sub> for this group was 0.148. Strangely, the *F*<sub>ST</sub> estimated for the difference between RPP1 and RPP2, without subdivision of them, was 0.056. This figure was lower than all estimates shown in Fig. 1-63. These contradictory results were not discussed. The interspecific hybrids, 2b, had also high *F*<sub>ST</sub> estimates, which is logic. The core collection should include a reduced number of cultivars that represents the greatest diversity among the 132 cultivars. To find out this number, the program Power Core (v 1.0, Kim et al. 2007) was used. Hybrids were not included in this process. The analysis resulted in a core collection of 37 cultivars that was composed of 21 Spanish, 12 Italian, and 4 French cultivars. This means that around 14% of the accessions were included in this core collection.

The DARwin 6.0.010 function maximum method (Perrier Jaquemoud-Collet 2006) was also used to estimate the core collection. A comparison of these two methods in case of selection of 37 cultivars resulted in a substantial increase of *F*<sub>IS</sub>, 43%, and a large drop of number of different alleles, 15% (Fig. 1-64). Based on this comparison it is recommended that analysis of the data is done by the program Power Core for selection of genetic resource populations.

**Table 1-3.** Means and ranges for different genetic parameters in seven SSR loci in a study of 16 high forest populations of *C. sativa*. Martin et al. 2012.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alleles per locus</strong></td>
<td>13.1</td>
<td>7-23</td>
</tr>
<tr>
<td><strong>Effective number of alleles</strong></td>
<td>6.5</td>
<td>2.75-9.98</td>
</tr>
<tr>
<td><strong>Observed heterozygosity</strong></td>
<td>0.656</td>
<td>0.321-0.786</td>
</tr>
<tr>
<td><strong>Expected heterozygosity</strong></td>
<td>0.804</td>
<td>0.646-0.898</td>
</tr>
<tr>
<td><strong>Fixation index</strong></td>
<td>0.026</td>
<td>-0.115-0.431</td>
</tr>
</tbody>
</table>

**Figure 1-63.** The genetic distances among the five groups of cultivars identified in the STRUCTURE analysis of 124 cultivars with 24 SSRs. RPP = reconstitution panmictic population:
- **RPP1a** 19 mainly Asturian cultivars
- **RPP1b** 9 related to the Parede cultivar from northern Spain
- **RPP1c** 24 cultivars from Central and Southern Spain
- **RPP2a** 14 Italian and 7 Spanish (1 northern and 6 southern) and the 2 French cultivars
- **RPP2b** 8 interspecific hybrids

Green columns are *F*<sub>ST</sub> within RPP groups one and two, respectively. Pereira et al. 2017.

**Figure 1-64.** The change in some genetic parameters for selection of the 37 cultivars in the core collection of *C. sativa* based on the DARwin 6.0.010 function maximum method instead of the program STRUCTURE. The analysis comprised 24 SSR loci. *N*<sub>a</sub> = number of different alleles, *N*<sub>e</sub> = number of effective alleles, *H*<sub>o</sub> = observed heterozygosity, *H*<sub>e</sub> = expected heterozygosity, *F*<sub>IS</sub> = fixation index. Pereira-Lorenzo et al. 2017.

Samples were taken from 11-21 trees in 16 Spanish high forest populations of *C. sativa* for analysis of genetic differentiation by alleles in seven microsatellite loci (Martin et al. 2012). Mantel test, principal coordinate analysis, and program STRUCTURE were used to study any geographic clustering. *F*<sub>ST</sub> were estimated based on all seven loci and six loci with exclusion of a locus with suspected null alleles.

Some of the main results are compiled in Table 1-3, which shows that the number of alleles was high in the seven loci studied. The genetic diversity was high and estimated at 0.804. No less than 15 private alleles were detected and 36 occurred in lower frequency than 0.05. The Mantel test run to trace any relationship between geographical distance and genetic differentiation did not result in any significant relationships. Contrary to this, the principal coordinate analysis (PCoA) revealed three groups with close relationship within each group of three
populations. One north-western, one north-eastern, and one south-eastern group were identified. The remaining seven populations could not be associated to any of these three groups. The analysis with the program CLUSTER gave a similar result. One striking observation was the strong differentiation between two SE populations growing less than 20 km from each other; \( F_{ST} = 0.102 \). Based on the supplementary Table in the paper, I have calculated the mean \( F_{ST} \)s for each of the 16 populations and the mean \( F_{ST} \)s for geographic groups of populations (Fig. 1-65). I grouped all populations from a certain region in Spain instead of the grouping according to the PCoA analysis. This means that there are two additional populations in the SE group and one in the NW group compared to the results of the PCoA. They are shown with deviating screen. The mean \( F_{ST} \)s within each of the two northern groups were much lower than means based on comparisons with all other populations; around 0.050 versus around 0.095. The three southern populations grouped according to the PCoA had a mean \( F_{ST} = 0.041 \). The global mean for all pairwise comparisons of the 16 populations was estimated at 0.098. This figure is much less than the three estimates of \( F_{ST} \) given in the paper, 0.141-0.152. In the paper the range of \( F_{ST} \) estimates were given as 0.021-0.158. These figures occur in the supplementary table I used for calculation of mean \( F_{IS} \)s for the 16 populations. It is not likely that the range, 0.021-0.158, would lead to a global mean of 0.141, and less likely to a mean of 0.152. There were no significant relationships between \( F_{IS} \)s and geographic variables.

One \( F_{IS} \) estimate differed considerably from all other \( F_{IS} \) estimates; 0.431 in EMCS25 locus. This was attributed to occurrence of null alleles. The two southermost populations had significant positive \( F_{IS} \) estimates, 0.180 and 0.289 (Fig. 1-66). It might be speculated that these two populations were subjected to some inbreeding owing to their position close to the margin for sweet chestnut distribution.

Although there is no straightforward north-south or east-west climatic gradients in Spain it was worthwhile to test relationships between genetic data and geographic variables. This was done for allelic richness, expected heterozygosity, and fixation index, which were related to population longitude, latitude, or altitude. Two of the nine reported relationships were significant:

- allelic richness – latitude, -0.59*
- fixation index – longitude, -0.60*

**Figure 1-65.** Mean \( F_{ST} \) estimates for 16 Spanish C. sativa populations based on microsatellites in seven loci. The mean values (m) for the groups of populations from different geographic regions are shown. The columns with deviating screens stand for populations not identified as belonging to the SE or NW group according to the principal coordinate analysis. The numbers of the populations are those used in the paper. Martin et al. 2012.

**Figure 1-66.** \( F_{IS} \) in 16 Spanish C. sativa populations from the entire distribution area in Spain. Populations with significant deviations from zero are shown in red. Martin et al. 2012.
I plotted the first of these two relationships and found more or less no relationship, $R^2 = 0.09$. A weak positive relationship was noted for allelic richness and longitude, $R^2 = 0.42$ (Fig. 1-67). I also plotted the relationship between $F_{IS}$ and longitude and only found an erratic pattern. Contrary to this, I tested the corresponding relationship between $F_{IS}$ and latitudinal origin and found a weak negative relationship $R^2 = 0.39$ (Fig. 1-68). It is evident that these three genetic parameters do not vary along latitude, longitude or altitude. The great discrepancy between my relationships and those published in the paper is a puzzle to me since I have only used the information published in Tables 1 and 3 in the paper.

It would have been interesting to test relationships between genetic parameters and some climatic variables such as xerothermic index.

Some authors distinguish two chinkapin species $C. pumila$ and $C. ozarkensis$ while others regard the latter as a subspecies and coin the two as $C. pumila$ var. $pumila$ and $C. pumila$ var. $ozarkensis$, respectively. I have followed the taxonomic status used in the individual reports.

The genetic variation among nine $C. pumila$ var. $ozarkensis$ populations from Arkansas, USA, was studied by aid of ten isozyme loci and 18 RAPD loci (Dane et al. 1999). For the RAPD analysis the number of trees that could be analyzed was low. Thus, two populations were represented by five trees and three other populations were represented by eight trees. Additional isozyme loci were later on included in this investigation. $H_o$, $H_e$, $F_{ST}$, $G_{ST}$, $F_{IS}$, and outcrossing rates were estimated. Outcrossing rates, $t$, were estimated using the equation $F_e = (1 – t)/(1 + t)$, for which $F_e$ was estimated by $[(H_e – H_o)/H_e]$ (Berg and Hamrick, 1992). Principal component analysis was carried out on the RAPD phenotypes. Disturbing deviations from Hardy-Weinberg equilibrium expectations were noted for seven of the loci in a varying number of populations, 1-7. Two of the populations with the largest number of deviations had large trees and were growing on a clear-cut area. (Usually this species is a shrub or a small tree.) The genetic diversity in $C. pumila$ var. $ozarkensis$ is half of that in $C. sativa$ and $C. seguinii$. The ratio $H_o/H_e$ (observed heterozygosity/expected heterozygosity) were larger than 1.00 in all populations (Fig. 1-69). As a corollary of this, the outcrossing rate was above 2.0 in seven of the nine populations. The large genetic diversity was attributed to the ancestral character of this species. The Ozark Mountains in Arkansas might have been a refugium during the latest glaciation and for this reason harboring a large genetic diversity. The additional four isozyme loci did not change the results to any
great extent. The $G_{ST}$ estimates varied among the eight loci (0.015 – 0.478; Fig. 1-70); with a large difference between the $Dia6$ locus and the other seven loci. The mean $G_{ST}$ value, 0.147, was fairly high for a wind-pollinated species and unexpected based on the outcrossing rates. When ten additional isozyme loci were included in the analysis the $G_{ST}$ value dropped to 0.122. Owing to the small and isolated populations one would expect genetic drift to cause considerable allele fixation. However, the $F_{IS}$ estimates suggest substantial excess of heterozygotes in four loci. The $F_{IS}$ estimates were exceptionally low for five loci; -0.269 – -0.713, with a mean value of -0.314. Thus, the extremely large excess of heterozygotes and large population differentiation constitute a contradiction. The limited number of trees analyzed probably contributed to these contradictions.

The subdivision of the species based on the RAPD analysis of five populations did not agree with the subdivision based on isozymes. Two of the populations that were closely related according to the isozyme analysis were separated by the second principal component explaining 30% of the variation. The discrepancy between the subdivisions based on the two markers was not discussed.

Fu and Dane (2003) studied the variation in 11 isozyme loci among twelve $C. pumila$ populations from Florida and Mississippi in south to Ohio in north estimated as $G_{ST}$ based on eight polymorphic isozyme loci. The fixation index for these eight loci are presented. Fu and Dane 2003.

Fu and Dane (2003) studied the variation in 11 isozyme loci among twelve $C. pumila$ populations from Florida and Mississippi in south to Ohio in north estimated as $G_{ST}$ based on eight polymorphic isozyme loci. The fixation index for these eight loci are presented. Fu and Dane 2003.
Five microsatellite loci were used by Tanaka et al. (2005) to study genetic differentiation among six Japanese *C. crenata* populations. The northernmost population originates from a locality north of latitude 42°N and the southernmost originates from a locality north of Tokyo, ≈ 35°N. The other four populations originated from localities on both sides of the strait between Hokkaido and Honshu; latitudes 40.8-41.9°N. Besides these populations, 12 cultivars were analyzed.

In all 79 alleles were found in the six populations, two loci had more than 25 alleles. One or two private alleles were detected in each population. Two of the loci showed significant, positive $F_{is}$ estimates. Except for one population, the $F_{is}$ estimates were positive (Fig. 1-72). It was suggested that inbreeding and/or occurrence of null alleles were responsible for the positive $F_{is}$ estimates. The UPGMA dendrogram revealed that the two geographically most distant populations deviated most from the other four populations. This is reflected in Fig. 1-73,

$$
\text{Figure 1-72.} \text{ } F_{is} \text{ estimates for six Japanese C. crenata populations analyzed by five microsatellite loci. \text{O}t = \text{ Otaru, Min = Minamikayabe, Sh = Shimokita, Tsg = Tsugaru, Aa = Aomori, Tsk = Tsukuba. Red column = negative estimate. Tanaka et al. 2005.}
$$

in which the mean of the four central populations is shown together with the individual $F_{is}$ means for the six populations. The results suggest that the Tsugaru Strait between Hokkaido and Honshu does not constitute a great constraint to gene flow between populations on both sides of the strait. Alternatively, chestnut might have been brought to Hokkaido from Honshu by ancient people.

The two types of material did not differ much with respect to genetic diversity within populations (HS) or total genetic diversity of the populations (HT) and the twelve cultivars did not differ much between these two types of material; 0.754 and 0.780 for the populations and 0.727 and 0.779 for the cultivars.

A seventh population from Hokkaido was excluded from the analysis of genetic variability in *C. crenata* in Japan since nine of its trees contained alleles never found in *C. crenata* but found in *C. mollissima*.

$$
\text{Figure 1-73. The mean pairwise } F_{st}\text{s for six Japanese C. crenata populations analyzed by five microsatellite loci. } \text{O}t = \text{ Otaru, Min = Minamikayabe, Sh = Shimokita, Tsg = Tsugaru, Aa = Aomori, Tsk = Tsukuba populations. The mean } F_{st}\text{ for the four populations with purple bars is also shown. Tanaka et al. 2005.}
$$

![Figure 1-74](image-url)
Kubisiak and Roberds (2006) studied the variation in microsatellites and RAPDs in 18 C. dentata populations from the entire distribution area of this species. These results were also reported in an earlier, less detailed report (Kubisiak and Roberds 2003). In the earlier report 22 populations were studied and the conclusions were the same.

To verify that the sampled individuals were truly C. dentata and no other chestnut species or species hybrid, cpDNA haplotypes in the trnT and trnL regions were analyzed in six C. mollissima, seven C. henryi, seven C. sativa, and eight C. pumila individuals. The American chestnut was characterized by unique cpDNA for the two regions studied. This analysis caused a culling of four of the originally 22 populations and 165 of the 1158 sampled individuals. However, the maternal inheritance of chloroplasts means that hybrids with the American chestnut as a female cannot be detected. Since all material was collected in State forests or National forests, in which non-native Castanea species rarely occur, the inclusion of non-American chestnut in this study was minimized. It should be remarked that Dane (2009) later reported that one C. pumila population shared the cpDNA marker that was thought to distinguish C. dentata from C. pumila.

Besides the general statistical evaluations of data in such an investigation, a composite dependent variable (CDV) was computed. The population in the most northeastern position was connected with the population furthest to south west with a straight line. The perpendicular lines starting from this NE-SW line for each population was determined. The positions on the NE-SW line where the perpendicular lines started were determined and used as values for CDV. In this way both latitude and longitude were considered in the geographic evaluation of the results.

The number of alleles in the six microsatellite loci varied in the range 13-31. With one exception, one population from Kentucky, all populations conformed to Hardy-Weinberg expectations. Significant differences of allele frequencies across populations were noted. The $\Phi_{ST}$ estimates according to Michalakis and Excoffier (1996) based on the six microsatellite loci varied in the range 0.030 and 0.097, with a mean value of 0.048. They were significantly different from random expectations. Stepwise regressions were calculated to find if there were any relationships between allele frequency and latitude or longitude. At least one allele showed significance for one or the other geographic variable. The authors reported 23 significant relationships between allele frequency and the composite dependent variable (CDV). Only in four cases $R^2$ was larger than 0.50, which means that the degree of explanation of these relationships was not high. I have illustrated the allele frequencies for two alleles with the highest $R^2$ estimates in Fig. 1-74. The qaCA022-174 allele (green) decreased from south to north while the CcCAT01-187 allele (blue) showed an opposite pattern. Number of rare alleles in three loci showed relationships with CDV too, with two R's larger than 0.50 and largest number of rare alleles in the southern populations. Similarly, diversity was larger in southern populations than in northern populations, $R^2 = 0.46$. Even if there were significant relationships between allele frequencies and geographic data the relationships varied among loci. Thus, neighboring populations did not group together in the principal component analysis. The explanation for the gradients in allele frequencies along a gradient was interpreted as post-glacial migration and founding events. The mean estimate of $G_{ST}$ for RAPD markers was 0.036 and $G_{AT}$ was significantly greater than zero in 14 of the 19 loci analyzed. Allele frequencies in six loci were significantly related to latitude or longitude. As for the microsatellites, a southwest – northeast trend was noted for RAPD loci. Two examples for alleles with a significant relationship between CDV and allele frequency are illustrated in Fig. 1-75. The relationships between CDV and RAPD allele frequencies were significant in four loci. The principal component analysis did not suggest any geographic pattern of the data obtained.
Table 1-4. The number of across-species amplifications between Chinese chestnut species and C. crenata and C. sativa. Fifty-five SSRs were assayed. $H_e$ and $H_o$ for the three Chinese species are shown. The number of polymorphic SSRs of these cross-amplified SSRs is shown in green for each species. Wang et al. 2008.

<table>
<thead>
<tr>
<th>SSR developed in</th>
<th>Cross-species amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. mollissima</td>
</tr>
<tr>
<td>C. crenata 14 SSRs</td>
<td>14,13</td>
</tr>
<tr>
<td>C. sativa 41 SSRs</td>
<td>33,19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Heterozygotes</th>
<th>$H_e$</th>
<th>$H_o$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.440</td>
<td>0.549</td>
</tr>
<tr>
<td></td>
<td>0.506</td>
<td>0.508</td>
</tr>
<tr>
<td></td>
<td>0.615</td>
<td>0.605</td>
</tr>
</tbody>
</table>

In conclusion most of the variation based on microsatellites and RAPDs, >95%, occurred within populations and clinal pattern was observed for alleles in some loci. In spite of substantial gene flow there was significant differentiation among populations for both types of marker. Finally, the authors stressed that their results were based on neutral markers and may not reflect differentiation in adaptive traits.

Across-species amplification of 55 SSRs developed in C. crenata and C. sativa was tested in 30 randomly selected trees from each of C. mollissima, C. seguinii, and C. henry by Wang et al. (2008).

The number of loci deviating from Hardy-Weinberg equilibrium was rather large. In Table 1-4 I have compiled important information from this study. This table reveals that almost all SSRs from C. crenata were cross-amplified in the three Chinese chestnut species while the percentage of cross-amplification of the C. sativa SSRs was considerably lower. This is probably a reflection of a closer relationship between the four Asian chestnut species than with C. sativa. Most of the cross-amplified loci from C. crenata were polymorphic.

Somewhat surprising, the observed heterozygosity was lowest in C. mollissima. The low number of trees per species analyzed might have contributed to these results.

In two papers Cheng and Huang (2009 and 2010) reported on variation among natural populations of C. mollissima in China. In absence of geographic coordinates for the populations it is not clear to me if the same trees or populations were studied in the two papers.

In the first paper (Cheng and Huang 2009) ten microsatellites were used in the analysis of 60 trees from four western and southwestern populations.

The number of alleles per locus varied in the range 4-11. The expected heterozygosity in three of the populations was close to 0.70 while the population from Yunnan had a lower estimate 0.55. It should be noted that only six trees from the Yunnan population were analyzed. The dendrogram based on the cluster analysis did not reveal any geographic pattern in the variation.

In the second paper (Cheng and Huang 2010) four cpSSRs generating eight cpDNA haplotypes were used to study variation among four natural populations of C. mollissima (60 trees) as well as nine cultivar populations from four regions (68 trees). The altitudinal range of the natural populations was 500-1,500 masl. Except for one cultivar at 1,600 masl all other cultivars originated from altitudes below 800 masl.

One haplotype dominated, occurring in almost 60% of all trees analyzed. The diversity was larger in the wild populations than in the cultivars. Only two of the eight haplotypes were found in the cultivars and only one of the cultivars had two haplotypes. The Hanzhong natural population, which had the largest number of analyzed trees, had four haplotypes and 13 of the 28 trees had the dominating haplotype. The Guangde natural population contained five haplotypes in the eight trees analyzed. A geographical pattern of the haplotypes was noted. However, the number of trees analyzed was low in two of the natural populations, 5 and 8 trees, which means that some haplotypes might have escaped from being detected. The cultivars had mainly the dominating haplotype and it was suggested that they might originate from the Tsingling Mountains, in which the Hanzhong population grows.

In a brief report Huang et al. (2014) reported on genetic variation in four C. mollissima populations from China with extreme difference in elevation, 450 – 3,200 masl. They used ten SSR markers in this study comprising 69 trees. The population differentiation estimated as $G_{st}$ was 0.14. The AMOVA run gave essentially the same result, 0.13. With such a variation in altitude of the populations and thereby climate, these figures of population differentiation are not extreme.

A comprehensive molecular genetics investigation of 849 trees from 28 populations covering the entire distribution range of C. mollissima was reported by Liu et al. (2013). The study comprised eight nuclear microsatellites (nSSRs) and six chloroplast microsatellites (cpSSRs). The cpSSRs were studied in 25 populations. A large number of population genetics parameters were estimated. They are shown in Table 1-5, in which results obtained are
Table 1-5. A summary of the result following analysis of 28 Chinese *C. mollissima* populations with eight nuclear and six chloroplast microsatellite markers. Liu et al. 2013.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nuclear DNA 849 trees analyzed</th>
<th>cpDNA 659 trees analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mean number of alleles per locus or haplotypes</td>
<td>16; range 10-22; range for individual populations: 5.0-10.4</td>
<td>17; 39 unique haplotypes; private haplotypes = 24</td>
</tr>
<tr>
<td>2. Mean number of effective alleles per locus</td>
<td>For individual populations; range 2.9-5.1</td>
<td>Effective number of haplotypes 2.0 ($N_e=1/\sum p_i^2$ Table 2 in Liu et al. 2013)</td>
</tr>
<tr>
<td>3. Allelic richness</td>
<td>Mean: 5.4; Range for individual populations: 4.6-6.6</td>
<td>1.5</td>
</tr>
<tr>
<td>4. Heterozygosity observed $H_o$ and mean expected $H_e$</td>
<td>$H_o$ mean: 0.590; range for individual populations: 0.497-0.688 $H_e$ mean: 0.678 range for individual populations: 0.600-0.759</td>
<td>Nei’s haplotype diversity: $H=\frac{n(n-1)}{(1-\sum p_i^2)} 0.359$</td>
</tr>
<tr>
<td>5. Within-population diversity treating alleles as ordered $h_1$</td>
<td></td>
<td>0.433</td>
</tr>
<tr>
<td>6. Total diversity treating alleles as unordered $h_t$</td>
<td></td>
<td>0.895</td>
</tr>
<tr>
<td>7. Mean within-population diversity treating alleles as ordered $v_1$</td>
<td></td>
<td>0.278</td>
</tr>
<tr>
<td>8. Species total genetic diversity treating alleles as unordered $v_t$</td>
<td></td>
<td>0.902</td>
</tr>
<tr>
<td>9. Within-population fixation index $F_{is}$</td>
<td>Mean: 0.148 all estimates positive , range: 0.017-0.256; 18 significant</td>
<td></td>
</tr>
<tr>
<td>10. deviation from mutation-drift equilibrium MDE estimated by Piry et a. (1999)</td>
<td>8 populations showed significant deviation from MDE based on the infinite allele model (IAM) No population showed significant deviation based on the stepwise mutation model (SMM)</td>
<td></td>
</tr>
<tr>
<td>11. Pairwise population differentiation $F_{st}$</td>
<td>0.120 range 0.016-0.278</td>
<td>0.678 range:0.011-0.967</td>
</tr>
<tr>
<td>12. the standardized measure of genetic differentiation averaged over loci $G'_{st}$</td>
<td>0.194 significantly higher than $F_{st}^{***}$</td>
<td>0.629</td>
</tr>
<tr>
<td>13. Bayesian clustering model for population structuring</td>
<td>2 clusters with 5 or 23 populations</td>
<td></td>
</tr>
<tr>
<td>14. spatial analysis of variance SAMOVA</td>
<td>2 clusters with one cluster containing only one population and the other 27</td>
<td>4 clusters 2 clusters with 1 pop. each, the others 6 and 17 populations</td>
</tr>
<tr>
<td>15. Correlations between genetic distances and geographic distances</td>
<td>$r = 0.18^*$</td>
<td>$r = 0.224$</td>
</tr>
<tr>
<td>16. Population geographic boundaries according to BARRIER 2.2</td>
<td></td>
<td>5 boundaries were identified; the most pronounced agreed with Wushan Mountains in central China.</td>
</tr>
</tbody>
</table>

shown. Empty boxes mean that no analysis was carried out. There was a large diversity in the 28/25 populations as revealed by all the data from the first eight rows of Table 1-5. The simplest interpretation of the large diversity is the wide geographic origins of the populations; latitudinal range 23.2-35.1°N, longitudinal range 102.2°-119.7°E.
There was slightly lower diversity, estimated as allelic richness, in the two regions close to the margins of the distribution of *C. mollissima* (Fig. 1-76). The central China population from Shengnongjia in Hubei province with eight different haplotypes showed the highest cpSSR diversity among all 25 populations. Generally the Central China populations exhibited larger diversity than populations from the three other regions. It was suggested that Central China was the origin of *C. mollissima*.

It was striking that all 28 populations had positive estimates of $F_{IS}$. As many as 18 populations had a significant excess of homozygotes (Table 1-5, Fig. 1-77). Seven of the ten central Chinese populations had significant $F_{IS}$ estimates but were also characterized by large genetic diversity. Isolation and limited gene flow or bottlenecks in the past might explain the $F_{IS}$ results. In addition, presence of null alleles might have contributed to the excess of homozygotes. Eight populations showed a significant deviation from mutation-drift equilibrium (MDE) suggesting occurrence of a bottleneck. In the infinite allele model for test of MDE, six of the eight populations had also a significant effect of $F_{IS}$. It was noted that several of the populations showing significant deviations were located in marginal areas. However, five of the eight populations grow in Central China and Southwestern China, including the population showing the largest within-population diversity. When the bottleneck effect was tested with the stepwise mutation model none of the populations had a significant deviation from the mutation-drift equilibrium. Based on the obtained data it is hard to state that bottleneck or isolation is responsible for the positive estimates of $F_{IS}$.

Independent on how the differentiation among populations was estimated cpSSR differentiation was much larger than nSSR differentiation (Fig. 1-78). One reason for this might be the occurrence of fixation of different haplotypes in different populations. This seems to be less pronounced in this material than in other studies.

As seen from Fig. 1-79 just one population was monomorphic. It was noted that most pairwise $F_{ST}$s were significant. The population with highest genetic diversity (GM) is the one with the highest number of haplotypes, 8. The ratio pollen/seed flow based on $G'_{ST}$ data was estimated at 3.0, which is lower than in related species within Fagaceae. This might have contributed to the relatively high differentiation observed in this investigation.

The two methods of estimating grouping of the populations based on nSSRs resulted in two classes. Based on the SAMOVA model one cluster contained just one population and the other cluster contained the other 27 populations while the corresponding figures for the clustering according to the Bayesian model were 5 and 23 populations. It was stated that human impact probably has had a great impact on the population structure owing to movement of cultivars among regions.
Five boundaries were identified based on the cpSSR data. Largely, they agreed with high mountain chains and it was suggested that these chains prevented exchange of pollen over the borders. This is likely true as long as there is no continuous distribution over the highest level of the mountains. One population, Zunyi in Guizhou province, was surrounded by five borders and isolated from the other 27 populations. This population had the lowest expected heterozygosity but also one of the lowest $F_{IS}$ estimates in this study, 0.045, which is an unexpected combination.

It is regrettable that elevations of the individual populations were not given. Nor were there any climatic data for population origins presented. I tested the several relationships between population latitude or longitude with expected heterozygosity for all populations or for part of the populations. None of the relationships were strong. However, they were all stronger than the ones based on genetic distances between populations in Table 1-5. It is obvious that adaptation in C. mollissima has not taken place along geographic variables such as latitude and longitude. It is likely that the feature of the Chinese landscape with several mountain chains does not promote population differentiation along latitudes or longitudes.

1.4 Species comparisons

Genetic pairwise variation between 13 C. mollissima cultivars and the differentiation between this species and C. dentata and C. seguinii was studied by Huang et al. (1994b). The cultivars originated from four localities and should represent the entire range of the diverse germplasm of C. mollissima. C. seguinii was represented by four populations from the Hubei province in China while bulked nuts of C. dentata from various locations in USA represented this species.

Fifteen of the 19 isozyme loci analyzed showed significant differences between the species. Certain alleles could be used to separate species from each other. The mean heterozygosity was highest in C. mollissima and it was claimed that the variability was higher in this species than in any other Castanea species. Fig. 1-80 reveals that the differences among the four C. mollissima populations were limited and that the Changjiang population (CH) differed most from the three other populations. Since these populations were designated as cultivars it might be expected that the differences are limited. Contrary to this, the distances between the three species were of another magnitude, especially the distances between C. dentata and the two other species were pronounced, 51-57% (Fig. 1-81). It should be noted that some loci were monomorphic, which might have contributed to the high estimates if the monomorphism occurs in different loci in the different species. It was concluded that the ancestors of Castanea species most likely were to be found in China.

**Figure 1-79.** Number of C. mollissima populations with different numbers of cpSSR haplotypes out of the 25 populations analyzed. Liu et al. 2013.

**Figure 1-80.** The pairwise genetic distances between four cultivars of C. mollissima from different origins in China. Nineteen isozyme loci were analyzed. Ch = Changjiang river valley, N = northern, SW = south western, and SE = south eastern regional groups. Huang et al. 1994b.

**Figure 1-81.** The pairwise genetic distances between C. dentata, C. mollissima, and C. seguinii based on 19 isozyme loci. Huang et al. 1994b.
Since the within-species variability was higher in *C. mollissima* than in *C. seguinii* the authors suggested the former as a progenitor of the chestnut species of today. This group of authors has revised this hypothesis later on.

An investigation of the variation within and between the three Chinese chestnut species, *C. mollissima*, *C. seguinii*, and *C. henryi* was carried out by Lang and Huang (1999). The number of populations in the three species was 21, 6, and 3, respectively. Twenty isozyme loci were studied. The larger number of *C. mollissima* populations might have contributed to the larger variability in this species than in the other two species. Especially the three populations from Shengnongjia and its surrounding area had a high variability with an observed heterozygosity of 0.381 and an $F_{IS}$ estimate of -0.151. The range of $F_{IS}$ in *C. mollissima* was -0.201-+0.215 with half of the populations showing an excess of heterozygotes. I have illustrated the within- and between species estimates of genetic distances in Fig. 1-82. This figure is based on Table 3 in the paper. It should be noted that the figures given in Tables 3 and 4 of the paper disagree. Even if the figures are inconsistent in the paper, Fig. 1-82 clearly shows less genetic distances within *C. mollissima* and *C. seguinii* than between them. There are other figures that disagree between the text and the tables. As one example the mean number of alleles per locus for *C. mollissima* was 2.9 according to the text but the highest number in Table 1 of the paper was 2.1.

The differentiation between the two American Castanea species (*C. dentata* and two varieties of *C. pumila* var. *pumila* and *ozarkensis*) and three East Asian Castanea species (*C. henryi, C. mollissima*, and *C. seguinii*) was studied by Dane et al. (2003). In all, 33 isozyme loci were examined with a maximum of 14-20 loci occurring in individual taxa. The number of populations per species varied between three for *C. henryi* and 21 for *C. mollissima*. Some of the loci were continent-specific but there was a sharing of most loci and high-frequency alleles. *C. dentata* had lowest genetic variability, which was attributed to its decline caused by *Cryptonectria parasitica*. The differentiation within each taxon was large for a wind pollinated species, with the highest $G_{ST}$ estimate for the three geographically isolated populations of *C. henryi* populations, 0.22 (Fig. 1-83). The lowest estimate was noted for *C. mollissima*, 0.075. Its populations covered a

**Figure 1-82.** The pairwise genetic distance between (blue bars) and within (green bars) the three Chinese chestnut species, *C. mollissima*, *C. seguinii*, and *C. henryi* based on 20 isozyme loci. *moll* = *C. mollissima*, *seg* = *C. seguinii*, *henr* = *C. henryi*. Twenty isozyme loci were studied. Lang and Huang 1999.

**Figure 1-83.** Average population distances within species and subspecies of *Castanea* estimated by 20 isozyme loci. Positive and negative average fixation indices are shown. *moll.* = *C. mollissima*, *seg.* = *C. seguinii*, *henr.* = *C. henryi*, *dent.* = *C. dentata*, *pumO* = *C. pumila* var. *ozarkensis*, *pumP* = *C. pumila* var. *pumila*, *moll.* = *C. mollissima*. The number of populations in each taxon is shown. Dane et al. 2003.
Figure 1-84. The mean species genetic distances estimated by analysis of isozyme loci in six groups of populations from China and USA. \( \text{seg} = \text{C. seguinii}, \text{henr} = \text{C. henryi}, \text{dent} = \text{C. dentata}, \text{puO} = \text{C. pumila var. ozarkensis}, \text{puP} = \text{C. pumila var. pumila}, \text{moll} = \text{C. mollissima}. \) The differentiation between taxa within China and USA, respectively, is shown as green columns. Dane et al. 2003.

A study in Galicia was initiated during the 1990ties with the purpose to identify pure species as well as first and second generation of species hybrids in 356 chestnut trees in stands, plantations, and clone collections (Fernandez-Lopez 2011). Tree morphology and isozyme genotypes based on 13 polymorphic loci were assessed. The statistical evaluation of the obtained data took place more than a decade later than the laboratory work. The computer programs STRUCTURE and HYBRIDS were used in the evaluation. It was admitted that the number of loci and alleles were too low for a precise estimation of hybrids and back crosses. A study with the objectives of detecting pure species and various hybrids including back crosses would today use other markers such as microsatellites to obtain more precise estimates of various kinds of hybrids. It was noted that the introduced species had lower genetic diversity than the domestic species, which was attributed to low diversity in the seed lots introduced to Spain. In this Galician material the \( F_{ST} \) between \( \text{C. sativa} \) and \( \text{C. crenata} \) was lowest while it was highest between \( \text{C. sativa} \) and \( \text{C. mollissima} \) (Fig. 1-85). Of the 356 trees 142 were proven to be pure species (61 \( \text{C. sativa} \), 56 \( \text{C. crenata} \), 26 \( \text{C. mollissima} \)) and 74 \( F_{1} \) hybrids \( \text{C. sativa x C. crenata} \). The remaining 140 trees were \( F_{1} \), \( F_{2} \), or different back crosses.

It was recommended that the existing stands of \( \text{C. crenate} \) and \( \text{C. mollissima} \) should be included in the genetic conservation of chestnuts in Galicia. In case of mortality in these stands new trees should be planted with trees obtained after open pollination in pure stands of these two Asian species. I assume that marker testing would be applied to guarantee the species purity of the seedlings to be planted.

Finally, the results obtained are of limited importance for breeding in general but are of importance for selection of material for breeding and genetic conservation of chestnut resources in Galicia.
The purpose of an investigation by Fernández-Cruz and Fernández-López (2012) on chestnuts was to use morphological traits and nuclear microsatellites for identification of *C. sativa* (228), *C. crenata* (61), and *C. mollissima* (27) and various types of hybrids. The inclusion of genetic material from those two Asiatic species may result in overestimates of genetic differentiation among *C. sativa* populations. Besides, the low drought tolerance and high frost sensitivity of the Asian species are undesired in production populations of chestnut in Galicia. Samples from 316 supposedly pure species trees of these species were collected mainly in Galicia but also in a stand further east along the Northern Atlantic coast; 228 *C. sativa*, 61 *C. crenata*, and 27 *C. mollissima* trees. These trees constitute a reference material for identification of species hybrids. Twenty-one species hybrid trees were sampled in a clone collection in Galicia. Most of the hybrids had *C. sativa* as female partner. The morphology of the three species was examined. *C. crenata* has specific glandular trichomes located on the abaxial surface of the leaves. Besides, *C. crenata* has mucronate leaves. Hairy and light-colored stems are characteristic of *C. mollissima*. The allelic pattern in eleven nuclear microsatellite loci was determined. Different statistical methods for identification were used. Simulations were used based on the characteristics of some of the trees.

Of the 228 “pure” *C. sativa* trees two turned out to be hybrids of some kind. No less than 15 of the *C. crenata* trees were *C. sativa* and one hybrid. Seven *C. crenata* and one hybrid were detected among the 27 *C. mollissima* trees. After exclusion of trees owing to their hybrid nature there remained the below number of trees for analysis by the statistical package CLUSTER:

- 113 *C. sativa*
- 46 *C. crenata*
- 19 *C. mollissima*

The microsatellite data distinguished four groups, the two Asiatic species in separate groups and one southern and on northern group of *C. sativa*. Eleven microsatellite loci were analyzed. The pairwise $F_{ST}$s were largest between the *C. sativa* and the two Asiatic chestnut species ($0.13$).

Based on the nuclear microsatellite genotyping the differentiation between the two *C. sativa* groups was considerable, $F_{ST} = 0.13$. Noteworthy is that three of the western populations contained trees from the southern as well as the northern group of *C. sativa*.

The algorithm HYBRIDLAB (Nielsen et al. 2006) was used in simulations starting with 40 pure trees from each of the four clusters. All possible hybrids between members of the four groups should be generated via simulations leading to 40 hybrid trees. The package CLUSTER was used to assess the ability of the software to assign the simulated pure individuals and simulated hybrids to pure and hybrid groups. The results of this exercise are illustrated in Fig. 1-87, which shows that the pure species *C. crenata* and *C. mollissima* were assigned correctly al-

![Figure 1-86](image1.png)

**Figure 1-86.** The pairwise $F_{ST}$s for all combinations between four groups of European and Asiatic chestnut species. *S* and *N* stand for a northern and a southern group of *C. sativa*. Eleven microsatellite loci were analyzed. Fernández-Cruz and Fernández-López 2012.

![Figure 1-87](image2.png)

**Figure 1-87.** The percentage of correct assignments of pure species ($s$ = sativa, $c$ = crenata, $m$ = mollissima) $F_1$, $F_2$ and back crosses ($B$) following simulations. $sN$ and $sS$ stands for northern and southern groups of *C. sativa*, respectively. Fernández-Cruz and Fernández-López 2012.
most to 100%. The lowest $F_{ST}$ estimate was noted for the two groups of *C. sativa*, which is reflected in the lowest correct assignments in the pure species group. Generally, the back crosses had lower correct assignments than pure species or $F_1$s and $F_2$s. It was stated that the results of the present investigation may be used for assignments of material of unknown genetic origin; exotics or hybrid with exotic species. It was encouraging that the assignment of the pure Asiatic species was almost 100% but correct assignments were less precise with $F_2$ hybrids and various types of back crosses. Besides, morphologically it is difficult to distinguish them from pure species.

**Phylogeny**

An identification of quaternary refugia of *C. sativa* was presented by Krebs et al. (2004), which has bearing for the phylogenetic studies of *C. sativa*. Pollen records from 1,471 sites were examined. An index for refugium probability (IRP) was developed to overcome the irregularity and scarcity of chestnut pollen records. The below factors were included in the calculation of IRP:

- Weighted sum of pre-arboricultural samples with *Castanea*
- Mean pollen % in the pre-arboricultural samples with *Castanea*
- The frequency of pre-arboricultural samples with *Castanea*
- The mean number of pollen grains in the pre-arboricultural samples with *Castanea*
- A correction factor considering the elevation of the sample site
- A correction factor according to the reliability of the chronology

The following five regions had high IRP estimates:
- Transcaucasian region east of The Black Sea
- Anatolia in Turkey
- Tyrrhenian coast from Liguria to Lazio in Italy
- Monte Vulture in southern Italy
- Cantabrian Coast on the Iberian Peninsula

There were other regions with lower IRPs that might have been refugia during the latest glaciation too.

Lang et al. (2006) used three non-coding regions of cpDNA to study the relationship between the seven existing *Castanea* species including two varieties of *C. pumila*. The segments were trnT-L, trnL, and trnL-F. As in many other species of *Fagaceae* the pairwise sequence divergence values for the three cpDNA regions were low, for all loci <1%. Such a low divergence was attributed to slow evolution in *Castanea* or that the divergence occurred recently. The species relationship according to the analysis of the three cpDNA regions is illustrated in the lower part of Fig. 1-88. This figure shows a relationship between the two American chestnut species and *C. sativa*. The three Chinese species form one group and the Japanese *C. crenata* is the only member of a third group. The relationship based on cpDNA is at variance with the morphological classification as illustrated in the upper part of Fig. 1-88.

**Figure 1-88.** The species relationship of Castanea species based on morphological differences and differences revealed by cpDNA, trnT-L, trnL, and trnL-F regions. The same color of the frame indicate relationship. The species furthest apart in each column is least related according to the cpDNA analysis. Lang et al. 2006
A follow-up paper by Lang et al. (2007), five additional cpDNA regions were studied in the same material: rp/16, \textit{ndhF3’} coding region, \textit{ndhF3’} flanking region, \textit{yc/6-psbM} spacer region, \textit{yc/9-trnGM} spacer region. As in the previous study the pairwise sequence divergence values were low but with a little larger range, 0-2.42%. In \textit{C. crenata} many unique substitutions and indels were observed leading to one unique haplotype in this species. In all, seven haplotypes were found in each of the Asian and North American species while two were found in \textit{C. sativa}. There was a clear geographic differentiation among the cpDNA haplotypes. In \textit{C. crenata} and \textit{C. dentata} no intraspecific variability was noted while in \textit{C. pumila} variability was noted for all regions investigated (Table 1-6). As in the previous paper the limited variability was attributed to slow evolution in \textit{Castanea} or that the divergence had occurred recently. In addition, it was speculated that \textit{C. dentata} might have passed a bottle neck, or that \textit{C. dentata} is a young species. Its wide geographic distribution speaks against the latter explanation for the absence of cpDNA variability.

Based on all data from the two reports including the composition of the cpDNA the phylogenetic relationship illustrated in Fig. 1-89 was arrived at. \textit{C. crenata} is mostly related to other genera of \textit{Fagaceae} and most likely with closest relationship to an original \textit{Castanea} species and it was speculated that the most likely ancestral area appears to be located in eastern Asia. There are indications that there was a split in Asia before the split between European and North American chestnuts. It was hypothesized the American species were developed from \textit{C. sativa}. With the geographic split between Europe and North America today such a spreading seems unlikely but it might have taken place when there was a North Atlantic European – North American land bridge around 55 million years before present time. The authors suggested that the shift from three nuts per bur to one nut per bur had occurred independently in the Chinese \textit{C. henryi} and the North American \textit{C. pumila}. Otherwise the phylogeny as outlined in Fig. 1-89 would not be correct. It should be remarked that \textit{C. sativa} was represented by a marginal population from northern Romania, which might have affected the results. The phylogenetic relationships would certainly be strengthened by use of several populations of each species.

\textbf{Figure 1-89}. The phylogenetic relationship of the seven \textit{Castanea} species according to six marker regions in cpDNA. This figure is slightly modified from Fig. 4 in the paper by Lang et al. (2007) with number of nuts per bur indicated. Lang et al. 2007.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|}
\hline
Species & Regions with intraspecific variability \\
\hline
\textit{C. crenata} & none \\
\textit{C. mollissima} & \textit{ndhF3’} flanking, \textit{yc/9-trnGM}, \textit{yc/6-psbM} \\
\textit{C. seguinii} & \textit{yc/9-trnGM} \\
\textit{C. henry} & \textit{ndhF3’} \\
\textit{C. sativa} & \textit{ndhF3’} flanking region, \textit{yc/6-psbM} \\
\textit{C. dentata} & none \\
\textit{C. pumila} & all \\
\hline
\end{tabular}
\caption{Regions showing intraspecific variability in seven \textit{Castanea} species. The following cpDNA regions were tested: rp/16, \textit{ndhF3’} coding region, \textit{ndhF3’} flanking region, \textit{yc/6-psbM} spacer region, \textit{yc/9-trnGM} spacer region. Lang et al. (2007).}
\end{table}
Dane (2009) studied chestnut populations from Florida to Maine with the following chloroplast DNA loci: trnT-trnL, trnL, rpl16 intron, ycf6-psbM and ycf9-trnGM in the large single copy (LSC) and ndhF 3’ coding and 3’ flanking region in the small single copy (SSC) of the cpDNA genome. The number of trees in the C. dentata populations varied between 1-13. Two lineages separated by 6-7 mutations were distinguished in C. dentata. In this species there were ten “dentata haplotypes” and one “pumila haplotype”. There were 15 populations containing just one haplotype, of these 15, six were represented by one tree only and another six populations were represented by two trees (Fig. 1-90). With the low number of trees per population it is obvious that heterogeneity in such small populations might escape detection. Fig. 1-90 shows that populations with larger numbers of trees per population are more heterogeneous than the low-number populations. The two populations with three and four haplotypes/population originated from Kentucky and North Carolina, respectively. It is premature to state that northern populations are genetically less variable than southern populations since the number of trees representing each northern population is too low. What is clear is that haplotype D1 was the most common haplotype and occurred in 28 of the 64 C. dentata trees. Two trees in one of the North Carolina populations had one pumila haplotype suggesting species hybridization. A comparison of Fig. 1-90 and Fig. 1-91 reveals that the pattern as regards population heterogeneity in the eight C. pumila var. pumila populations is different from C. dentata. Seven of the eight populations had more than one haplotype; the eighth from Connecticut could obviously not have more than one haplotype since it was represented by one tree only. The two populations with five haplotypes originated from Virginia and Florida. It was noted that the haplotypes of this species in Virginia differed by more than nine mutations.

Only one of the five C. pumila var. ozarkensis populations represented by five trees had more than one haplotype (3). The other populations were represented by one or two trees. This does not permit a serious comparison with the other two species. There were four different haplotypes in C. pumila var. ozarkensis.

The $F_{ST}$ estimate for within-species differentiation was highest in C. pumila var. pumila, 0.87 and lowest in C. pumila var. ozarkensis, 0.69. High estimates of $F_{ST}$ are obtained when different populations are homozygous for different haplotypes. Therefore, it is somewhat surprising that the differentiation in C. dentata with so many one-haplotype-populations was not the most differentiated of the three species. It was speculated that the high genetic diversity observed in Virginia indicates a refugium during the glaciation. The variation in Virginia was most pronounced for C. pumila. C. ozarkensis was not sampled in Virginia. It was further stated that the southern Appalachian Mountains was another probable refugium based on the great variability in this region.

**Figure 1-90.** The number of C. dentata populations from Florida to Maine having 1-4 cpDNA haplotypes per population. The number of trees in the populations with one cpDNA haplotype are given. The number of trees in the populations with 2-4 haplotypes are indicated. Dane 2009.

**Figure 1-91.** The number of C. pumila var. pumila populations from Connecticut to Florida having 1-5 cpDNA haplotypes per population. The number of trees in the populations with 1-5 haplotypes per population are indicated. Dane 2009.
The species relationships among the two American *Castanea* species were studied by Shaw et al. (2012) using the *trnV-ndhC* region of cpDNA. This region was selected following a screening of nine candidate regions. In addition, another objective of this investigation was to relate the cpDNA profile to morphological traits that differentiate the three American *Castanea* species. Two hundred and thirty-three accessions from the entire distribution of these species were analyzed. Areas where only one of the species occurs as well as areas where two species and putative species hybrids occur were sampled.

Twelve different haplotypes were distinguished. Four primary clades based on the cpDNA analysis were distinguished with some grouping within individual clades (Fig. 1-92). They were coined, D (exclusively *dentata*), M, O (mainly *ozarkensis*), and P (mainly *pumila*). There was some uncertainty about the evolution of the O clade which called for analysis of additional five cpDNA regions in 13 individuals. This analysis largely confirmed the analysis of the first analyzed group of trees.

The morphology and number of individuals belonging to a certain haplotype as well their geographic location are compiled in Table 1-7. It was noted that morphological differences were closely linked to a certain haplotype. Thus, 111 of 119 accessions within clades D, O, and P were closely associated to species *C. dentata*, *C. pumila* var. *ozarkensis*, and *C. pumila* var. *pumila*, respectively. These three clades had to large extent different geographic distributions. The M clade had the largest number of haplotypes and comprised the pure species *C. dentata* and *C. pumila* as well as their intermediate hybrid. This hybrid is characterized by *C. dentata* leaf size, leaf shape and distinct ciliate margins typical for *C. dentata* and one nut per cupule typical for *C. pumila*. Geographically, the M haplotypes were most frequent in southern Appalachian Mountains. Sympatric distribution of the two species

![Figure 1-92. A simplified model of the relationship between twelve chloroplast haplotypes in the three American Castanea species; dentata, ozarkensis, and pumila based on twelve cpDNA haplotypes. The indices indicate the number of different haplotypes within each clade D, M, O, and P. Shaw et al. 2012.](image)

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Type of morphology</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>36 P1</td>
<td>32 <em>C. pumila</em></td>
<td>Virginia – Georgia, Alabama Tennessee</td>
</tr>
<tr>
<td></td>
<td>4 <em>C. dentata</em></td>
<td>1 Alabama, 1 Tennessee, 2 Virginia</td>
</tr>
<tr>
<td>6 P9</td>
<td>6 <em>C. pumila</em></td>
<td>Georgia</td>
</tr>
<tr>
<td>63 D2</td>
<td>63 <em>C. dentata</em></td>
<td>Maine - Georgia</td>
</tr>
<tr>
<td>12 O3</td>
<td>10 <em>C. ozarkensis</em></td>
<td>Arkansas – Missouri</td>
</tr>
<tr>
<td></td>
<td>2 <em>C. pumila</em></td>
<td>Virginia</td>
</tr>
<tr>
<td>1 O11</td>
<td>1 <em>C. pumila</em></td>
<td>Florida</td>
</tr>
<tr>
<td>1 O12</td>
<td>1 <em>C. pumila</em></td>
<td>Arkansas</td>
</tr>
<tr>
<td>63 M4</td>
<td>44 <em>C. dentata</em></td>
<td>Alabama, Georgia, Tennessee</td>
</tr>
<tr>
<td></td>
<td>18 <em>C. pumila</em></td>
<td>Alabama, Georgia, Lousiana, Mississippi</td>
</tr>
<tr>
<td></td>
<td>1 intermediate*</td>
<td>Origin not given</td>
</tr>
<tr>
<td>34 M5</td>
<td>34 intermediate*</td>
<td>Northern Georgia</td>
</tr>
<tr>
<td>8 M6</td>
<td>6 <em>C. pumila</em></td>
<td>Southwest Georgia (5) and northern Alabama (1)</td>
</tr>
<tr>
<td></td>
<td>2 <em>C. dentata</em></td>
<td>Tennessee</td>
</tr>
<tr>
<td>2 M7</td>
<td>2 <em>C. dentata</em></td>
<td>Northeastern Alabama</td>
</tr>
<tr>
<td>6 M8</td>
<td>6 <em>C. pumila</em> (soboliferous)</td>
<td>Northern Florida</td>
</tr>
<tr>
<td>1 M10</td>
<td>1 <em>C. dentata</em></td>
<td>North Carolina</td>
</tr>
</tbody>
</table>

Table 1-7. The number of trees with one of twelve cpDNA haplotypes revealed in 233 Castanea trees in eastern USA. The type of morphology is indicated. Intermediate morphology stands for leaf size and shape and ciliate margins typical for *C. dentata* and one nut per cupule typical for *C. pumila*. Shaw et al. 2012.
and the absence of crossing barriers between the two species were suggested as the explanation for the variety of taxa among the M haplotypes. The great variability in the southern Appalachian Mountains might also be attributed to its role as a refugium during the latest glaciation. One puzzling observation was that haplotype M5, which only occurred in the morphologically intermediate phenotype, did not constitute an intermediary haplotype between D2 and any of the two P1 and P9 haplotypes. It was not closely related to D2 and still less so to P1 or P9. It was concluded that past and recent hybridization are responsible for the complex pattern of cpDNA haplotypes of the two American Castanea species and their subspecies.

1.5 Miscellaneous

The risk for low genetic variability following limited re-introduction in general, and specifically for C. dentata, was addressed by Pierson et al. (2007). In an isolated stand they used 84 minisatellite DNA markers to characterize nine founder trees in a plantation in West Salem, Wisconsin, established in 1880s as well as three stands with offspring from these founders. Trees included in the analysis of the offspring should have a minimum DBH of 20 cm. In each stand 24-25 trees at a minimum distance from each other of ten meters were analyzed. The stands were located approximately 150 meters apart from each other. Besides the stands in Wisconsin, one transect in Ohio with 18 trees and one transect in West Virginia with 14 trees were sampled. All trees, except for one were examined with respect to chestnut blight infection. Bands in the autoradiographies used should have a minimum migration distance of one mm. A similarity index was calculated as $S = \frac{2 \times N_{xy}}{N_x + N_y}$ in which:
- $N_{xy}$ is the number of bands shared between individual X and individual Y
- $N_x$ is the number of bands in individual X
- $N_y$ is the number of bands in individual Y
- $S$ is thus the proportion of bands between two individuals. Heterozygosity and $F_{ST}$ were also estimated.

In the founders 84 distinct bands were found with a mean number of bands per tree varying in the range 31.0-36.0. As seen from Fig. 1-93 the heterozygosity was significantly higher in offspring samples 1 and 3 than in the founder population. This finding was interpreted as a result of higher fitness of heterozygotes. Another contributing factor of the increased heterozygosity was selection against offspring from crosses between related individuals. However, the latter is probably confounded with heterozygote superiority. The heterozygosities of the two transects differed significantly from two of the offspring stands but not from the founder population. The limited difference between the two transect populations was non-significant.

As regards band sharing only one significant difference was noted, offspring 2 versus offspring 3 (Fig. 1-94). The differentiation among the four groups based on bands was limited, $F_{ST} = 0.031$. All pairwise estimates of $F_{ST}$ were lower than the observed for the joint analysis of all four populations. The hypothesis that low number of founders and establishing individuals would lead to genetic drift or localized selection had to be ruled out as explanations for the observed results. None of the 22 low-frequency alleles were fixed or lost, which lends further support to low importance of genetic drift in this study.
The founder population did not differ from the transect populations while significant differences were noted between all three offspring stands and the two transects (Fig. 1-95). The difference between the two transects was non-significant.

Sixty-five of the 82 trees examined with respect to chestnut blight showed signs of infection. The uninfected trees had slightly but significantly higher heterozygosity than the susceptible trees, 0.573 versus 0.532. The infected trees had significantly lower similarity index, 0.525 versus 0.571 for the tolerant trees. Some bands occurred in higher frequency in the tolerant group leading to a higher similarity than in the susceptible group. It was suggested that tolerant trees share some genetic characteristics to a great extent.

The authors summarized their results in the following way: This study demonstrates that populations that develop from a few founders do not always immediately decline in genetic diversity. The absence of genetic deterioration was attributed to:
1. Constant and rapid expansion of the studied population
2. The outcrossing nature of the species
3. Persistence of the founders and their continuous contribution to the developing population.

The outcrossing nature is certainly a key requirement for a successful reintroduction of *C. dentata*.
Table 1-8. Compilation of studies on population differences in *C. sativa* based on various traits. Number of loci for the various markers is indicated in red.

<table>
<thead>
<tr>
<th>Pop./origin</th>
<th>Trait/No. loci</th>
<th>Evaluation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16/Spain 2 trials</td>
<td>Bud flushing</td>
<td>pop. variance ≈15%</td>
<td>Fernández-López et al. 2005a</td>
</tr>
<tr>
<td>6/Spain</td>
<td>Bud flushing freeze testing</td>
<td>Ecoclinal variation</td>
<td>Diaz et al. 2009</td>
</tr>
<tr>
<td>6/Europe</td>
<td>Growth traits</td>
<td>Strongly significant population effects</td>
<td>Aravanopoulos &amp; Tchatchou 2010a</td>
</tr>
<tr>
<td></td>
<td>Phenology; growth traits</td>
<td>Strongly significant population effects</td>
<td>Miguez-Soto-Fernández-López 2015</td>
</tr>
<tr>
<td>6/Turkey</td>
<td>Basic physiology</td>
<td>Strongly significant population effects</td>
<td>Lauteri et al. 1997</td>
</tr>
<tr>
<td>6/Turkey</td>
<td>Isozymes/21; Carbon isotope discrimination (CID)</td>
<td>$F_{ST} = 0.02$-$0.15$ CID significant differences</td>
<td>Villani et al. 1991</td>
</tr>
<tr>
<td>52/France, Italy, Turkey</td>
<td>Isozymes/13</td>
<td>$F_{ST} = 0.071$</td>
<td>Villani et al. 1994</td>
</tr>
<tr>
<td>34/Turkey</td>
<td>Isozymes/15</td>
<td>$F_{ST} = 0.18$; clinal variation</td>
<td>Villani et al. 1999</td>
</tr>
<tr>
<td>28/France, Italy, Greece, Spain</td>
<td>ISSR/73</td>
<td>One Greek, one Italian, one Spanish cluster</td>
<td>Mattioni et al. 2008</td>
</tr>
<tr>
<td>31/Turkey-Spain</td>
<td>SSR/6</td>
<td>One Italian – Spanish cluster</td>
<td>Mattioni et al. 2013</td>
</tr>
<tr>
<td>82/ France, Italy, Greece, Spain</td>
<td>ISSR/73 and</td>
<td>Unique gene pool in individual populations 25 - 65%</td>
<td>Aravanopoulos et al. 2005</td>
</tr>
<tr>
<td>ISSRs 16 multiple discriminant analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38/Turkey - Portugal</td>
<td>cpDNA/13</td>
<td>$G_{ST} = 0.43$</td>
<td>Fineschi et al. 2000</td>
</tr>
<tr>
<td>17/Spain</td>
<td>Isozymes/11</td>
<td>$F_{ST} = 0.15$; 3 clusters without geographic pattern</td>
<td>Fernández-López and Monteagudo 2010</td>
</tr>
<tr>
<td>9/Greece, Italy, Spain</td>
<td>SSR/6 EST-SSR/ 9</td>
<td>$F_{ST}$ for SSR and SSR-EST = 0.17 $R_{ST}$ for SSR: 0.14 $R_{ST}$ for SSR-EST: 0.33</td>
<td>Martin et al. 2010b</td>
</tr>
<tr>
<td>31/Spain, Italy, Greece</td>
<td>SSR/9</td>
<td>$F_{ST} = 0.09$; Atlantic cluster, Cantabrian cl., Western Medit. cl. Eastern Medit. cl</td>
<td>Fernández-Cruz and Fernández-López 2016</td>
</tr>
<tr>
<td>15/ SE Europe</td>
<td>SSR/11</td>
<td>$F_{ST} = 0.13$; 1. Coastal Croatia, 2. Continental Croatia, Bosnia-Herzegovin, Italy, Romania 3. Macedonia, Kosovo, Hungary</td>
<td>Poljak et al. 2017</td>
</tr>
<tr>
<td>16/Spain</td>
<td>SSR/7</td>
<td>$F_{ST} = 0.095$ 3 clusters: NE, NW, ans SE Spain</td>
<td>Martin et al. 2012</td>
</tr>
<tr>
<td>73/entire distribution area</td>
<td>SSR/6</td>
<td>3 main clusters 1. Turkey E - Azerbaijan, 2. Turkey W, Greece, Bulgaria, 3. All western Europe</td>
<td>Mattioni et al. 2017; treated in Chapter 4</td>
</tr>
</tbody>
</table>

One Greek population differed strongly from the other 81 populations. One central Italian population constituted the extreme contrast to the Greek population. One ISSR study demonstrated a clear difference in the genetic setup of orchards on one hand and coppice or naturalized forests on the other hand and identified five *C. sativa* clusters in Europe, three of them in Greece. Several results indicated that there were more than one refugium during the latest glaciation.
One interesting approach to study the genetic differentiation along geographic variables was presented in a study of genetic differentiation in *C. dentata*. A composite dependent variable (CDV) was computed. The population in the most northeastern position was connected with the population furthest to southwest with a straight line. The perpendicular lines starting from this NE-SW line for each population was determined. The positions on the NE-SW line where the perpendicular lines started were determined and used as values for CDV. A clinal variation was noted for some alleles but no strong relationships between CDV and allele frequencies were noted.

A range-wide study of *C. pumila* comprising eight polymorphic isozyme loci revealed an exceptionally large $G_{ST}$ estimate, 0.30, for isozyme differentiation. Several populations were represented by few individuals, which probably contributed to such a high estimate.

The widely distributed *C. mollissima* also displayed large population differentiation based on microsatellites or cpDNA. In China mountain changes constitute a great obstacle for gene flow among populations. Different markers resulted in different structuring of populations, even in studies of the same populations. The phylogeny of *Castanea* was presented with *C. crenata* as the species mostly related to an ancient original *Castanea* species. The three Chinese chestnut species constituted one branch of the chestnut evolutionary tree. Another branch consisted of *C. sativa* and the American chestnut species. The latter were believed to have evolved from *C. sativa*. This requires that the change from three nuts per bur to one nut per bur had taken place independently in the Chinese *C. henryi* and the American *C. pumila*.

The negative consequences of low numbers of founders for reintroduction of *C. dentata* were addressed in one study. The fears were not substantiated in that study.

### Table 1-9. Compilation of studies on population differences in American and Asian Castanea species based on various traits. Number of loci for the various markers is indicated in red.

<table>
<thead>
<tr>
<th>Species</th>
<th>Pop/origin</th>
<th>Trait/No. loci</th>
<th>Evaluation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. dentata</em></td>
<td>USA-wide</td>
<td>SSR/6 RAPD/19</td>
<td>SSR: $F_{ST} = 0.048$ RAPD: $F_{ST} = 0.036$</td>
<td>Kubisiak and Roberds 2006</td>
</tr>
<tr>
<td><em>C. pumila var. ozarkensis</em></td>
<td>9/Arkansas, USA</td>
<td>RAPD/18 Isozymes/10</td>
<td>RAPD: 5 clusters; disagreement with isozyme data</td>
<td>Dane et al. 1999</td>
</tr>
<tr>
<td><em>C. pumila var. pumila</em></td>
<td>12/ Range-wide USA</td>
<td>Isozymes/11</td>
<td>$G_{ST} = 0.30$ 2 distinct clusters</td>
<td>Fu and Dane 2003</td>
</tr>
<tr>
<td><em>C. crenata</em></td>
<td>6/ Japan</td>
<td>SSR/5</td>
<td>3 clusters</td>
<td>Tanaka et al. 2005</td>
</tr>
<tr>
<td><em>C. mollissima</em></td>
<td>4/China W and SW</td>
<td>SSR/10</td>
<td>No geographic pattern</td>
<td>Cheng and Huang 2009</td>
</tr>
<tr>
<td><em>C. mollissima</em></td>
<td>4/China 9 cultivars</td>
<td>cpDNA/8</td>
<td>Geographic pattern</td>
<td>Cheng and Huang 2009</td>
</tr>
<tr>
<td><em>C. mollissima</em></td>
<td>28/nSSR China 25/cpDNA China</td>
<td>nSSR/8 cpDNA/6</td>
<td>$F_{ST} = 0.120$, $F_{ST} = 0.678$, 2 clusters: 1 and 27 pops; 4 clusters: 1, 1, 6 and 17 populations</td>
<td>Liu et al. 2013</td>
</tr>
</tbody>
</table>
2 Progeny testing

2.1 Full-sibs

Míguez-Soto et al. (2016) stated that their investigation of 16 full-sib families from a 6x5 mating was the first one on chestnuts estimating genetic parameters for important traits. The mating was classified as a partial factorial mating but three of the parents were used both as females and males. Partial diallele mating design would be more appropriate. Four females and two males were characterized by good wood quality and two females and three males had good resistance to Phytophthora cinnamomi infection. The latter two females were interspecific hybrids; C. crenata x C. sativa. One of the wood quality males was also such a hybrid. All other parents were C. sativa.

Assessments were carried out at age two and comprised nine traits; growth (height and root collar diameter), form (straightness 5 classes), and phenology (flushing 8 classes) as well as survival and estimated stem volume. Scions were taken from the seedlings of the 16 families. They were dipped into a solution with IBA (indole-3-butyric acid) for three minutes. The rooted cuttings were first planted in 2.5 liter pots and were then transplanted into 4-liter pots. Six cuttings from the individual seedlings were included in the study. Risk for contamination is always great for crosses involving wind pollinated species. Female flowers were bagged and no nuts were obtained from such control bags. Three covariates were tested, nut weight, days of germination of the nuts, and number of roots. Only the latter had a significant effect on the growth traits and was therefore included in the statistical models for these traits.

The below mean values were obtained:

- Height age 2: 133.9 cm
- Root collar diameter: 1.3 cm
- Volume: 236.1 cm³
- Survival: 73%

The variance estimates for GCA (general combining ability) were larger than the corresponding for SCA (specific combining ability). The estimates for GCA were significant for all traits except for branch length. Only flushing was significant for SCA. The ratio $V_D/V_A$ varied in the range 0.11-0.59 according to Table 2 in the paper. The ratio of 0.59 for flushing (TF2) does not agree with the estimates for $V_A$ and $V_D$ in this table. The $V_D$ estimate should probably be 11.7 instead of 17.7 (page 8), which gives a $V_D/V_A$ ratio = 0.529. Anyhow, it is clear that the additive variance is more important for development of the traits studied than non-additive variance. Both narrow sense and broad sense heritabilities were largest for flushing ($h^2$ for the other traits were not impressive; with estimates around 0.10 or less. Besides flushing, $H^2$ for growth traits ($H^2$, $D^2$, and $V^2$) were larger than for the other traits and amounted to $\approx 0.20$. 

![Figure 2-1. Narrow sense heritability, $h^2$, and broad sense heritability (clonal repeatability), $H^2$, in a Spanish chestnut progeny trial with 16 full-sib families. Each seedling was vegetatively propagated. $H2$, $D2$, and $V2$ are height, root collar diameter, and stem volume at age 2, $Bth$ and $Bl$ are branch thickness and branch length at age 2, $Str = straightness$, $Fl = flushing$, $Surv = survival$. Míguez-Soto et al. 2016.](image-url)
In Fig. 2-2 I have illustrated the breeding values for survival and tree height at age 2 for the eight parents included in this study. The survival of the C. sativa parents was positive while the survival of the C. crenata parent was poor. This cannot be attributed to one exceptional full-sib family since this parent occurred in five full-sib families. The authors stated that the estimated genetic correlations suffer from imprecision owing to the limited number of parents in the mating design. Therefore, I have only illustrated the nine correlation coefficients with higher estimates than their standard errors (Fig. 2-3). Owing to autocorrelation the coefficients including the three growth traits were fairly strong 0.65-0.86. The negative relationship between height and branch thickness was unexpected.

In conclusion, this investigation is one important contribution for development of a genetically solid breeding program. As the authors stated more parents have to be included in coming studies and establishment of long-term trials are required for the support of chestnut breeding.

In USA there is a great interest in transferring chestnut blight resistance from C. mollissima to the domestic C. dentata. Via several back crosses it is hoped to obtain a blight resistant hybrid with recovery of C. dentata morphological traits. Several publications by F. V. Hebard and coworkers have treated this issue (Hebard 1991, 1994, Diskin et al. 2006).

Hebard (1991) studied the inheritance of hairiness in 22 trees from a cross C. mollissima x C. dentata and 41 and 183 individuals of its backcrosses with C. dentata and C. mollissima, respectively. C. mollissima has hairy leaves in contrast to C. dentata. Presence of hairs (simple and stellate non-glabrous trichomes) on interveinal portions of the lamina was recorded on field grown individuals. In F1, six of the nine families were hairy, two were hairless, and one had six hairy and two hairless (Fig. 2-4). The occurrence of hairless F1 individuals was attributed to the small size and young age at examination of this trait. The pooled result of segregation in the back cross to the American chestnut was close to the expected 1:1 segregation but with large deviations in two of the families (Fig. 2-4). All 183 back crosses to the Chinese chestnut were hairy. These results suggest that hairiness is dominant.

In a follow-up paper additional progenies and traits (see below) were reported (Hebard 1994a). For several of the traits, assessments were carried out two consecutive

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**Figure 2-2.** Parental breeding values for survival and tree height at age 2 in a Spanish chestnut progeny trial with 16 full-sib families. Each seedling was vegetatively propagated. Cs = C. sativa, HCc = F1 hybrid C. sativa x C. crenata (Cc), Cm = C. mollissima. Míguez-Soto et al. 2016.

**Figure 2-3.** Genetic correlation coefficient between six traits in a Spanish chestnut progeny trial with 16 full-sib families. Each seedling was vegetatively propagated. Only coefficients that were larger than the standard error are shown; red column = negative correlation coefficients. H2, D2, and V2 are height, root collar diameter, and stem volume at age 2, Bth is branch thickness and at age 2, Surv = survival at age 2, Str = straightness, Fl = flushing. Míguez-Soto et al. 2016.
years. In case of agreement between observations from more than one family in a particular type of progeny, data were pooled. The following types of progeny were studied, \( F_1 \), \( F_2 \), \( B_1 \), two different \( F_2 \), and \( B_1 \times B_1 \), as well as the pure species.

In summary, the following results were obtained:

- Interverinal, hairs incompletely dominant trait in \( C. mollissima \)
- Wein hair density, incompletely dominant trait in \( C. mollissima \), three loci
- Twig hairs, incompletely dominant trait in \( C. mollissima \), two loci
- Stipule size, incompletely dominant trait in \( C. dentata \), two loci
- Stem color, dominant trait in \( C. dentata \), two loci with complementary gene action; suggested segregation 9:7
- Bud shape, no simple Mendelian inheritance
- Bud flushing, low heritability trait

I have illustrated the results for interveinal hairs and stem color in Figs. 2-5 and 2-6. The frequency of interveinal hairs in \( F_2 \) deviates from the expected 3:1 segregation, \( p >0.01 \). The segregations in the \( B_1 \) families are both close to the expected 1:1 segregation. Similarly, the segregation in the \( B_1 \times B_1 \) progeny is close to the expected 3:1 segregation.

As regards stem color the four classes do not seem to be well separated from each other, which aggravates interpretation of the observations. The author pooled the observations into two classes; red + greenish red and green + reddish green. This resulted in a fit to the 9:7 segregation in the \( F_2 \) progeny. It is evident from Fig. 2-6 that the red stem color originating from \( C. dentata \) is influencing the color of all hybrid progenies.

Even if the observed segregations in many cases agreed with expected segregations it is evident from the figures reported that no complete dominance existed for any of these morphological traits. I assume that classes for several of the traits studied are not particularly distinct, which means that misclassifications are hard to avoid.

An alternative explanation to regulation of the traits by genes in 1-3 loci would be a continuous variation with polygenic regulation and with some genes with a strong impact on the trait under study.

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**Figure 2-4.** The segregation in hairy:hairless in nine \( C. mollissima \) x \( C. dentata \) \( F_1 \) families and in three back crosses to \( C. dentata \). * recorded during the first growth period. Hebard 1991.

**Figure 2-5.** The percentage of individuals with interveinal hairs in different genetic entries following crosses between \( C. dentata \) (Cd) and \( C. mollissima \) (Cm), their \( F_p \), \( F_z \), \( B_p \), \( B_z \) and \( B_1 \times B_1 \) as well as the pure species. \( B \) stands for back cross with \( C. dentata \). Hebard 1994a.

**Figure 2-6.** The percentage of four stem colors in \( C. dentata \) and \( C. mollissima \), as well as interspecific crosses and backcrosses between these two species; \( F_p \), \( F_z \), \( B_p \), \( B_z \), \( B_1 \times B_1 \) and \( C. dentata \) following interspecific crosses between \( C. dentata \) and \( C. mollissima \). \( B \) stands for back cross. Hebard 1994a.
The study was carried out in a field trial in North Carolina, USA, at the age of four years and at a series of photosynthetic photon flux densities (PPDF), 0 – 2,000 μmol x m⁻² x s⁻¹. The following leaf morphological traits were studied:

- Leaf mass per unit area
- Specific leaf area
- Total leaf mass per leaf
- Total leaf area per leaf
- Nitrogen concentration

It was pointed out that only healthy trees were included in this investigation.

Except for carbon assimilation at high PPDFs (>600) and total leaf area per leaf there were no significant differences among the five materials. The latter trait was significantly lower in C. mollissima than in the other materials. To get information on the relationship between carbon assimilation and the expected C. dentata genome contribution of the three backcross generations Fig. 2-8 is presented. There is a seemingly very good fit to the two straight lines in this figure but it should be remarked that the values for C. mollissima contribute much to this fit. It is evident that the B₁ x B₂ backcross (approximately 94% C. dentata) is close to C. dentata for carbon assimilation.

Tanaka and Kotobuki (1992) studied peeling characteristics in offspring after pollination of C. crenata x C. mollissima hybrids with C. crenata or C. mollissima pollen. Mainly one cultivar of each species was used as male. Four classes of peeling were used:

- 0 = pellicle peeled off together with the shell
- 1 = pellicles removed within less than one minute
- 2 = pellicles removed after 1-3 minutes
- 3 = more than three minutes required for peeling

Fig. 2-9 reveals that C. mollissima as male results in much higher percentage of peeling easiness than after pollination with C. crenata. It should be noted that peeling easiness among the three crosses (F₁ x F₁) x C. crenata varied between 0% and 97%. This suggests that genetic constitution of the female parent has an impact on the easiness of peeling.
gested that the pendula phenotype was regulated by a dominant allele. The authors stated that progenies from additional crosses must be analyzed to reach an understanding of inheritance of pendula habit in *C. crenata*.

Fineschi et al. (1990) analyzed the inheritance of isozymes in six loci of *C. sativa* and observed single-locus codominant inheritance in five of the loci, phosphoglucose-isomerase PGI, isocitrate dehydrogenase IDH, diaphoras DIA, and three aminopeptidase AP loci. One AP locus might have had a null allele but the authors stated that erratic segregation of such an allele made the existence of a null allele uncertain.

The inheritance of isozymes in eleven polymorphic loci was studied by Huang et al. (1994a). Intraspecific and interspecific crosses, F$_1$, and back cross material as well as open-pollinated families of *Castanea* species were included in this study. According to the authors simple Mendelian inheritance was noted for all loci. However, some of the segregations deviated significantly from the expected segregation. Linkage between loci was observed in three cases. Species specificity was noted for shikimate dehydrogenase with difference between the American *C. dentata* and European chestnut *C. sativa* species on one hand and the East Asian *C. mollisima* and *C. crenata* on the other hand. Since *C. dentata* is almost extinct no crosses could be carried out to verify inheritance at this locus in this species.

### 2.2 OP-families

In a short note the inheritance of diaphorases (DIA isozymes) was presented by Dane and Huang (1999). Open-pollinated nuts were collected from individual trees of *C. henryi*, *C. mollisima*, *C. pumila* var. *pumila*, *C. pumila* var. *ozarkensis*, *C. seguinii*. Buds from *C. dentata* were used for the inheritance study. According to the publication *Two alleles at three DIA loci were found to be codominantly inherited*. However, the segregation was not clear-cut in all progenies.

The first results from a combined provenance and progeny testing series with two trials in each of Greece, Italy, and Spain were reported by Fernández-López et al. (2005b). The populations originated from contrasting sites in those three countries. Each population was represented by 26 open-pollinated families. Flushing was recorded in a scale, 1-8. Four stages of budset were assessed. Both phenological traits were recorded once a week during critical periods. Plant height and root collar diameter was measured. ANOVAS were run with population as fixed effect and OP-family as random effect. The results were interpreted as evolutionary responses to ambient conditions. Southern populations benefit from early onset of growth before the dry period appears. The northern populations start their growth later in order to avoid late spring frosts. One of the northern populations had the latest budset in all six trials while others changed ranks between trials leading to strong population x test locality interaction. In Table 2-1 the results from the statistical evaluation are summarized. The interaction OP-family x locality was strongly significant for all traits. With the exception for mortality at age 2, this was also true for population x locality. Only three of the six traits showed significant differences among the open-pollinated progenies while the population effect was significant for all traits. This was expected since the site conditions at the population origins vary considerably.

#### Table 2-1. The significances for different effects in a series of combined population and progeny trials with *C. sativa* in each of Greece, Italy, and Spain. Fernández-López et al. 2005.

<table>
<thead>
<tr>
<th></th>
<th>OP-family</th>
<th>OP x locality</th>
<th>Population</th>
<th>Pop. x locality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flushing</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Budset</td>
<td>ns</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Height 2</td>
<td>**</td>
<td>***</td>
<td>***</td>
<td>**</td>
</tr>
<tr>
<td>Root collar diam.</td>
<td>**</td>
<td>***</td>
<td>***</td>
<td>**</td>
</tr>
<tr>
<td>Mortality age 2</td>
<td>ns</td>
<td>***</td>
<td>***</td>
<td>**</td>
</tr>
<tr>
<td>Mortality age 3</td>
<td>ns</td>
<td>***</td>
<td>***</td>
<td>**</td>
</tr>
</tbody>
</table>
A follow-up of the development in the Fernández-López et al. (2005b) paper was presented by Míguez-Soto and Fernández-López (2015). Besides the populations from the European project, 12 populations from six localities with strongly varying xerothermic indices ($X_i \approx$ biologically dry days during the growth period) in Spain were studied. At each locality one high forest or coppice population and one adjacent orchard population was included. A combined provenance-progeny trial with OP-families was established at Daneiro; latitude 43.15°N, 250 masl, and at Rebordelo; latitude 42.46°N, 505 masl. There is a variable number of trees per family in these single-tree trials with 20 blocks (= replications). Thus, there are two parallel provenance-progeny trials at Daneiro and Rebordelo. They will be referred to as Spanish and European, respectively. For the European trial only data from Rebordelo were presented. The traits studied and their classes are shown in Table 2-2. Since it was assumed that blocks could not take care of all spatial heterogeneity in the trials, corrections were applied before the final genetic evaluation. The corrections were done by iterative spatial analysis. ANOVAs were run separately for each trial as well as jointly on data from both trials. Heritabilities, coefficients of additive variation, genetic correlations, and genetic gains were estimated. The OP-family variance component was multiplied by three rather than by four to get the numerator for the heritability formula. This was done to avoid over-estimation of the heritability and thus compensate for possible inbreeding in the material.

Spanish populations. All traits except for survival showed significant population and family differences with the largest levels of significance for population effects. Population was regarded as a fixed effect, which means that no variance components for population were estimated. This means that the relation between family effects and population effects is not straight-forward. The individual heritabilities for the joint analysis did not exceed 0.20 for any of the traits and was lower than the estimates from the individual trials (Fig. 2-10). The difference between the two heritabilities for straightness was pronounced and must be attributed to strong family x locality interaction.

**Table 2-2.** Traits studied, number of classes used for phenological and quality traits, and other useful information. Míguez-Soto and Fernández-López 2015.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Number of classes</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal flushing</td>
<td>TF</td>
<td>8 Stage 3 = flushed</td>
</tr>
<tr>
<td>Lateral flushing</td>
<td>LF</td>
<td>8 Stage 3 = flushed</td>
</tr>
<tr>
<td>Budset BS</td>
<td>BS</td>
<td>4 Stage 4 budget</td>
</tr>
<tr>
<td>Duration of growth</td>
<td>DUR</td>
<td>BS stage 4 –TF stage 3</td>
</tr>
<tr>
<td>Straightness Str</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Apical dominance AD</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Total height TH</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>DBH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root collar diam RD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume index V</td>
<td></td>
<td>TH x DBH</td>
</tr>
<tr>
<td>Survival S</td>
<td>2</td>
<td>Alive or dead</td>
</tr>
</tbody>
</table>

**Figure 2-10.** Spanish populations. Heritability estimates for different traits in a trial at Rebordelo and for joint analyses of data from this trial and another trial at Daneiro: $ne = $ not estimated. $TF = $ terminal flushing, $BS = $ budset, $AD = $ apical dominance, $Str = $ straightness. The populations originate from six localities in Spain. Míguez-Soto and Fernández-López 2015.

**Figure 2-11.** Spanish populations. Genetic correlation coefficients between five traits in the trial with Spanish populations of C. sativa. $BS = $ budset. The populations originate from six localities in Spain. Ages at assessment are given. Míguez-Soto and Fernández-López 2015.
populations with high xerothermic indices was selected for the so-called Drought Tolerance Breeding Group, DTBG. The same principles for selection were used for the DTBG. The gains for the quality traits are somewhat higher than in the selection for the MBP. Since the growth of the populations from which the selection took place was inferior, the gain in growth is less, but still substantial. The gains in volume from a similar selection from the European populations for the DTBG breeding population are illustrated in Figure 2-13.

As seen from Fig. 2-11, the genetic correlations among the traits were weak except for the relationship between height and stem volume at age 12, which harbors some autocorrelation. The week correlation between heights at ages 3 and 12 is distressing for development of early testing. It was stated that the obtained results confirmed an important relationship between height and form traits. However, according to Table 7 in the paper, apical dominance and straightness were negatively correlated with stem volume and tree height, R² = -0.30 - -0.40. European populations. The heritabilities, which are based on data from one trial (Rebordelo), are generally higher in this material than in the Spanish material (Fig. 2-12). This is especially pronounced for growth traits. The heritability for straightness deviates from the general pattern when this heritability is compared with the corresponding for the Spanish material at Rebordelo. Contrary to the results for the Spanish populations, the genetic correlations between bud set and the growth traits were somewhat stronger for the European populations than for the Spanish populations; R²: 0.45-0.55 versus 0.16-0.19 (Fig. 2-13). As in the Spanish material, correlations between the two quality traits and the three growth traits at age 12 were negative; range -0.31 - -0.48.

Breeding populations. It was stated that multi-trait index selection would be the best for introduction of new material for the main timber breeding population, MBP. The projected gain following selection of one tree in each of the top-3 families based on an index are illustrated in Fig. 2-14. The conspicuous gain for stem volume must be attributed to the strong selection. The gains were less spectacular for the two quality traits. Only material from the populations with high xerothermic indices was selected for the so-called Drought Tolerance Breeding Group, DTBG. The same principles for selection were used for the DTBG. The gains for the quality traits are somewhat higher than in the selection for the MBP. Since the growth of the populations from which the selection took place was inferior, the gain in growth is less, but still substantial. The gains in volume from a similar selection from the European populations for the DTGB breeding population are illustrated in Figure 2-13.

Figure 2-12. European populations. Heritability for various traits in one Spanish progeny trial with C. sativa field trial with 18 open-pollinated families from two localities in each of Greece, Italy, and Spain. AD = apical dominance, BS = bud set, DUR = duration of the growth period, Str = straightness. Míguez-Soto and Fernández-López 2015.

Figure 2-13. European populations. Genetic correlation coefficients between five traits in the trial with European populations of C. sativa. Red = negative coefficient. TF04 = terminal bud flushing at age 4, BS08 = bud set at age 8. Míguez-Soto and Fernández-López 2015.

Figure 2-14. Spanish populations. Mean projected genetic gain after selection of one tree in each of three families with best performance in one progeny trial with C. sativa at Daneiro in North-Western Spain. One type of selection was carried out for the main breeding population (MBP). Another type of selection was carried out for improvement of drought tolerance (DBP). Míguez-Soto and Fernández-López 2015.
tion exceeded 100% with broad margin. The gains in straightness and apical dominance were more in line with the gains in the Spanish populations. It is hard to believe that such extraordinary gains in volume would ever be reached in applied breeding.

In conclusion, an impressive amount of data was presented, which is most useful for timber breeding of *C. sativa* in Spain.

Tchatchoua and Aravanopoulos (2010b and 2015) reported on heritabilities and gains in a Greek trial belonging to the same series of trial. It is located at latitude 40.75°N, longitude 23.30°E, and altitude 760 masl. Essentially, the later paper treats the same data as the old paper. Basal stem diameter, stem height, and survival were recorded at ages 4-6. The number of families per population varied between 19 and 26. Variance components were estimated for the random effects family and family x age as well as for the fixed effects; year, block, and population. Heritability was estimated as 4 x OP-family variance in the numerator.

The development of the heritability over time is illustrated in Fig. 2-15. Especially for stem volume there is a rapid increase of the heritability from year 4 to year 6 and fairly high heritability for a growth trait in a forest tree species. As noted by the authors, heritabilities based on a single trial may be overestimated since no family x site can be estimated. The interactions family x age were non-significant for all traits analyzed. Spectacular genetic gains were estimated following combined selection of 10% of the best individuals in the best performing families.

[Figure 2-15. Heritabilities at ages 4-6 for growth traits in one Greek field trial with 19-26 families in each of six *C. sativa* populations from Greece, Italy, and Spain. Tchatchoua and Aravanopoulos 2010b.]

The survival at Daneiro was 74% and at Rebordele 54%. The higher mortality at Rebordele was attributed to *Phytophthora cinnamomi* damage as well as mechanical damage by machinery. The mean height at the low elevation trial was 5.07 m while it was 3.40 m at the other last es-

Open-pollinated families from 36 *C. sativa* plus trees in the Atlantic area of Galicia were studied in two progeny trials located at Daneiro, latitude 43.15°N, 250 masl and Rebordele, 42.46°N, 505 masl (Míguez-Soto and Fernández-López 2012). Assessments took place at age eight and six. The planting at Rebordele took place two years later than at Daneiro. Thirty-two families were common in the two trials. Twenty-four traits were used in this selection for timber plus trees. Growth including a volume index (= height x DBH²), stem straightness, apical dominance, and survival were assessed. Since it was assumed that blocks could not take care of all spatial heterogeneity in the trial, corrections for heterogeneity were applied before the final genetic evaluation. The corrections were done by iterative spatial analysis. The statistical evaluation comprised individual analyses at the two sites as well as joint analysis of them. Individual heritabilities were estimated separately and jointly for the two trials assuming that the numerator estimated 3 times the family variance that compensates for some inbreeding. A and B genetic correlations between the same trait at the two test locations were calculated. Genetic gains and gene diversity of the retained trees after selection were estimated. The gain in three types of selection for the main breeding population was estimated:

- The best tree in each of 32 families
- Combined family and individual selection of 14 families with 1-5 trees per family (It should be noted that the text referring to this selection is identical with the first selection alternative.)
- Index selection with different values given to the individual traits.

[Figure 2-16. percentage genetic gain in growth traits at ages 4-6 after combined selection of 10% of the best individuals in the best performing families. Data from one *C. sativa* field trial in Greece. Tchatchoua and Aravanopoulos 2015.]

The best tree in each of 32 families

Combined family and individual selection of 14 families with 1-5 trees per family (It should be noted that the text referring to this selection is identical with the first selection alternative.)

Index selection with different values given to the individual traits.

The survival at Daneiro was 74% and at Rebordele 54%. The higher mortality at Rebordele was attributed to *Phytophthora cinnamomi* damage as well as mechanical damage by machinery. The mean height at the low elevation trial was 5.07 m while it was 3.40 m at the other last es-
tablished trial. The stem volume was several times larger at Daneiro (26.4 dm$^3$) than at Rebordelo (7.5 dm$^3$). The apical dominance and straightness did not vary much between the two trials. Except for straightness and apical dominance at Rebordelo, the spatial analysis revealed non-random spatial structures for all other traits. For growth traits the spatial heterogeneity explained approximately 40% of the variation. Fig. 2-17 reveals that estimates of heritability increased for all traits after correction for spatial heterogeneity. This increase was most pronounced for the growth traits. Significant family differences in the separate and the joint analyses of the two trials were noted for all corrected trait values except for apical dominance and straightness at Rebordelo. Generally, the heritabilities for the growth traits were in the same range as for growth traits in other broadleaf forest tree species (Fig. 2-18). Mostly the heritabilities for the joint analysis were lower than the heritabilities based on individual trials, which indicates that family x trial interactions are of some significance. One illustration of the possible impact of this interaction is seen in Fig. 2-19, in which family mean correlation coefficients between the two trials are presented. The between-trait correlation coefficients are shown in Fig. 2-20, which shows a strong relationship between the growth traits. This is due to some autocorrelation. The relationships between the three growth traits and apical dominance (AD) and straightness (Str) were all weak and negative. Finally, the relationship between apical dominance and straightness was positive but weak <0.40.

**Figure 2-17.** Individual heritabilities for 6 traits studied in one C. sativa field trial with 36 open-pollinated families in North-Western Spain at age eight. Corrections for spatial heterogeneity in the trial were carried out. AD = apical dominance. Miguez-Soto and Fernández-López 2012.

**Figure 2-18.** Individual heritabilities for four traits in two North-Western Spanish field trials with 36 open-pollinated families of C. sativa from the same region. Separate estimates as well as estimates based on data from the two trials jointly are shown. Correction for spatial heterogeneity was carried out before estimating heritabilities. Assessments took place at age eight/six. Miguez-Soto and Fernández-López 2012.

**Figure 2-19.** Family mean correlation of the performance between the same traits in two C. sativa field trials with 32 open-pollinated families in North-Western Spain at age eight/six. AD = apical dominance. Miguez-Soto and Fernández-López 2012.

**Figure 2-20.** Genetic correlation coefficients between five traits in two North-Western Spanish field trials with 32 open-pollinated families of C. sativa from the same region. Red columns refer to Daneiro trial and blue columns refer to Rebordelo trial. Two-color columns = negative correlations. Miguez-Soto and Fernández-López 2012.
Considerable gain after selection was found (Fig. 2-21). Especially in the Daneiro trial the gains in volume or straightness dropped conspicuously when the selection was based on the other trait. Survival was not much affected by selection for volume or straightness. The gains were lowest in the Rebordelo trial. Still higher gains might be obtained from the combined selection of 1-5 trees in the 14 best performing families (Fig. 2-22). This number was found to be optimum for selection gain and keeping satisfactory genetic diversity in a breeding population. The loss of gene diversity will be marginal, <3%.

It was pointed out that stem volume was the best trait for selection since there were higher estimated correlations between height and form traits. However, the correlation coefficients ($R^2$) varied in the range -0.35– -0.51, thus these relationships explained at most 51% of the variation.

For the applied breeding population the best performing tree in each of the families was selected with the main focus on growth traits. Gains following selection of the three best trees in three OP-families with different origins were estimated. The percentage gains were dramatic, 89% for stem volume and 50% for straightness. However, a clonal seed orchard with just three clones does not seem to be a viable option. It was not discussed how such gains can be realized in applied breeding. However, it shows the potential for breeding.

In conclusion, this study has generated solid information for applied breeding for timber production of *C. sativa* in Galicia and beyond.

Míguez-Soto et al. (2019) studied growth, phenology, and survival in a Spanish combined provenance and progeny trial located in north-western Spain. Nine natural populations of *C. sativa* originating from varying climatic conditions were included in this trial. According to the map in the paper five populations belong to the Northern Iberian Peninsula gene pool (coined wet here) and four other populations belong to the Mediterranean gene pool (coined xeric here). Each population was represented by 14-28 open-pollinated families. The trial was planned as a single-tree plot trial with 20 replications. Poor quality of some nuts meant that this plan was not totally fulfilled. The following traits were included:

- Terminal bud flushing; eight-degree scale
- Lateral bud flushing was determined as stage 3 in the 8-degree scale, in which stage 3 = green leaves shorter than the brown scales
- Budset assessed weekly during June and August
- Tree height
- Root collar diameter
- Straightness; three classes
- Survival

Assessments took place during two years at ages 3 and 4. The material in this report was genotyped before (Fernández-Cruz and Fernández-López 2016) and reanalyzed for estimates of $F_{st}$, which were compared with $Q_{st}$.

An experiment with periodic drought was carried out in greenhouse, in which 10 open-pollinated families per population were included. When 40% of the weight at field capacity was reached, the pots were watered to full field capacity. The temperature was 27°C in this treatment, T27D, which lasted for ten weeks. The plants in the well-watered treatment were growing in another greenhouse at 21°C. In this experiment focus was on dry weights of different parts of the plants. Defoliation and dry apex were also recorded. Several genetic parameters were estimated and related to environmental variables.

Unfortunately, observed data from this important investigation was more or less totally lacking in this report, which is regrettable. Some recorded data would have fa-
facilitated the interpretation of the results.

Outdoor trial. Except for root collar diameter, all other traits showed significant population differences. There was a tendency to stronger differentiation at age 5 than at age 4. Contrary to this, the family differentiation was lower at age 5 than at age 4. Straightness was the only trait without significant family differences. In Fig. 2-23 I have illustrated the mean $F_{ST}$ within and between climatic groups of populations as well as the mean estimate between the most separated population, Ronda, and the populations from maritime climate. This figure reveals that the within-group estimates are lower than between the two types of origin. Thus lending some support to the classification of populations as done in the paper. The pronounced difference between the Ronda population and the “wet” populations is evident from this figure.

The finding that the differences between $F_{ST}$ and $Q_{ST}$ estimates were significant was interpreted in the following way: neutral processes are insufficient to explain the observed phenotypic trait divergence.

Contrary to the increase of $Q_{ST}$ by age, there was a drop in the narrow-sense heritabilities from age 4 to age 5 (Fig. 2-25). This contradiction is hard to explain.

---

**Figure 2-23.** Mean $F_{ST}$ within and between climatic groups of Spanish natural(ized) C. sativa populations represented by 14-28 open-pollinated families in a combined provenance and progeny trial in Galicia, Spain. The mean $F_{ST}$ estimate for the most differentiated population, Ronda, and the other climate type populations is shown. xer stands for xeric origin. Miguez-Soto et al. 2019.

**Figure 2-24.** $Q_{ST}$ for TF = terminal bud flushing, LF = flushing of lateral buds, BS = budset, H = height, AD = apical dominance, S = survival. The estimates originate from a combined provenance and progeny trial in Galicia, Spain. Nine Spanish natural(ized) C. sativa populations represented by 14-28 open-pollinated families. Miguez-Soto et al. 2019.

**Figure 2-25.** Narrow-sense heritability, $h^2$, for TF = terminal bud flushing, BS = budset, H = height, RCD = root collar diameter, S = survival. The estimates originate from a combined provenance and progeny trial in Galicia, Spain. Nine Spanish natural(ized) C. sativa populations represented by 14-28 open-pollinated families. Miguez-Soto et al. 2019.
The mean genetic correlations within type of traits were strong while two of the three correlations between types of traits were much weaker (Fig. 2-26). Similarly, the correlation between budset at ages 4 and 5 was weak, 0.41. There were strong and positive relationships between latitude at population origin and phenology traits (Fig. 2-27). The relationships between xerothermic index at population origin and various traits were moderately strong, -0.67 - -0.88 (degree of explanation 45-77%). It should be remarked that five of the populations had a xerothermic index of 0, which reduces the possibilities to detect relationships with this variable. The strengths of these relationships point at ecoclinal variation rather than ecotypic variation as stated in the paper. The results mean that flushing and budset take place earlier in the southern and “xeric” populations. This was interpreted as an adaptation to the climatic conditions. Populations growing under severe drought during summer months benefit from an early growth start when water is still available in the ground. The correlations with ancestry of the *C. sativa* gene pool in Spain were significant for flushing and budset. Even if these relationships were significant, none of them explained more than 15% of the variation. Therefore, the statement that *phenology are clearly related to the genetic structure of the Iberian populations that had been identified using microsatellites* is not strongly supported by the results obtained. Significances with low degree of explanation are not particularly informative.

Indoor trial. Larger root systems were noted for the “xeric” populations in the periodic drought treatment (no figures were presented). In Table 2-3 I have compiled the most important results from the joint ANOVAs for the traits studied. For the majority of traits there was a strongly significant population effect while only six of the

<table>
<thead>
<tr>
<th>TRAIT</th>
<th>population</th>
<th>population x treatm.</th>
<th>family</th>
<th>family x treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot dry weight</td>
<td>*</td>
<td>**</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Root dry weight</td>
<td>***</td>
<td>*</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Leaf dry weight</td>
<td>**</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Total dry weight</td>
<td>***</td>
<td>*</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Root collar diameter</td>
<td>**</td>
<td>**</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Defoliation</td>
<td>**</td>
<td>**</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>No. secondary branches</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>**</td>
</tr>
<tr>
<td>Height</td>
<td>***</td>
<td>ns</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Budset</td>
<td>***</td>
<td>*</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Dry apex</td>
<td>***</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Survival</td>
<td>***</td>
<td>**</td>
<td>***</td>
<td>**</td>
</tr>
</tbody>
</table>
In conclusion, this paper shows that there are large genetic differences both among populations and within populations for metric traits of adaptive significance. Drought is a powerful discriminating factor in plant adaptation to ambient conditions. There was a strong relationship between several traits and latitude, which indicates ecolonal variation. It is regrettable that biological data are largely missing in the paper. Instead detailed information on statistical evaluations was presented. Much useful information for breeding of *C. sativa* in Spain can be gained from this investigation.

![Figure 2-28](image)

**Figure 2-28.** The relationship between BLUP estimates for total dry weight in two treatments; periodic drought, and full water field capacity in an experiment lasting for ten weeks. The estimates originate from a combined provenance and progeny trial in greenhouse with nine Spanish naturalized *C. sativa* populations. Míguez-Soto et al. 2019.

Traits analyzed showed a significant family effect. Three of the dry weight traits, budset, and survival showed significances for all four genetic parameters. For three traits, total dry weight, budset, and survival, it was possible to study the relationship in performance between the two treatments. In spite of the 1%-level significance for population x treatment interaction for total dry weight there was a strong relationship between performance in the two treatments ($R^2 = 0.87$, Fig. 2-28). Heritabilities were only presented for the individual treatments. For most of the traits the heritabilities were much higher in the periodic drought treatment than in the other treatment (Fig. 2-29). It is evident that the harsher conditions lead to better resolution among the populations. It would have been useful to have $h^2$ estimates from the joint analysis of this experiment. Relatively high estimates of $CV_A$ were noted in the individual treatments, and except for tree height, they were much larger than in the joint analysis of the two treatments (Fig. 2-30).

There were positive correlations between budset and height, 0.58, and budset and the two dry weight traits, shoot (0.29) and leaf (0.38). Any relationships between budset and total dry weight based on their BLUP estimates in Fig. 5 in the paper were totally absent in both treatments.

The correlations between population means and latitude or xerothermic index were non-significant for all traits in this indoor experiment. It was stated that some of the populations did not perform according to expectation, which was attributed to human impact, such as transfer of material over wide geographic areas.

![Figure 2-29](image)

**Figure 2-29.** Narrow-sense heritability, $h^2$, for SDW = shoot dry weight; RDW = root dry weight; LDW = Leaf dry weight; TDW = total dry weight; $H =$ height; BS = budset; $S =$ survival. The estimates originate from a combined provenance and progeny trial in greenhouse with two treatments: well watered (green columns) and periodic drought (brown). Nine Spanish naturalized *C. sativa* populations, each represented by 10 open-pollinated families. Míguez-Soto et al. 2019.

In conclusion, this paper shows that there are large genetic differences both among populations and within populations for metric traits of adaptive significance. Drought is a powerful discriminating factor in plant adaptation to ambient conditions. There was a strong relationship between several traits and latitude, which indicates ecolonal variation. It is regrettable that biological data are largely missing in the paper. Instead detailed information on statistical evaluations was presented. Much useful information for breeding of *C. sativa* in Spain can be gained from this investigation.

![Figure 2-30](image)

**Figure 2-30.** Coefficient of additive variance, $CV_A$, for SDW = shoot dry weight; RDW = root dry weight; LDW = Leaf dry weight; TDW = total dry weight; $H =$ height. The estimates originate from a combined provenance and progeny trial in greenhouse with two treatments: well watered (green columns) and periodic drought (brown) and joint analysis. Nine Spanish naturalized *C. sativa* populations were each represented by 10 open-pollinated families. Míguez-Soto et al. 2019.
Juvenile height and biomass of open-pollinated families from a 2 x 2 factorial temperature x watering experiment with the same populations as the ones studied above (Fernández-López et al. 2005b) were reported by Pliura and Eriksson (2002). After cultivation for five weeks at 25°C the below four cultivation regimes were used for nine weeks:

- Temperature 25°C well-watered (full field capacity) = T25W;
- Temperature 25°C periodic drought = T25D;
- Temperature 32°C well-watered (full field capacity) = T32W;
- Temperature 32°C periodic drought = T32D.

Periodic drought at 25 and 32°C during the nine-week treatment period was accomplished by watering every 4th and every 3rd day with a balanced nutrient solution. The experimental design was completely randomized single tree plots. Separate and joint analyses of the four treatments were carried out by linear ANOVA models. Ad
ditive genetic variance, additive genetic coefficients of variation, individual tree heritabilities, and genetic correlations were estimated. In addition, ecovariances were estimated for those cases with significant genotype x treatment interaction. As indicated in this figure the interaction family x treatment was significant for three traits, and strongly significant for height and stem dry weight. The highest estimates for heritability of the growth traits were noted for the T25D treatment, which was unexpected since it had the poorest growth. The additive genetic coefficients of variation for biomass traits were large with 12 of the 16 estimates larger than 40% (three traits are illustrated in Fig. 2-32). The biomass ratios between root dry weight and stem or leaf dry weight were in the range 12-28% with one exception root/stem ratio with no genetic variation in the T25D treatment. As seen in Fig. 2-32 the CVAs for height were much less than for biomass traits. Similarly, branchiness had CVAs in the range 10-30%. The ratios of CVAs in the two watering and two temperature treatments were reported (Fig. 2-33). This figure illustrates that temperature treatment influenced the CVAs more than watering regime. Thus, the CVAs in the two watering regimes did not differ much for the five traits illustrated in this figure.

To identify the treatment that contributed most to ecovariance, the performance of the seven families that contributed significantly to the ecovariance is illustrated in Fig. 2-34. The performance in treatment T32D deviated most from the other treatments. In spite of the uniform performance in the four treatments of the Sicily 3 family (S3) it is surprising that this family contributed significantly to the ecovariance.
The uniform growth conditions in the growth chambers resulted in low environmental variances and were interpreted as the reason for the generally high estimates of the genetic parameters. Large estimates of $\text{CV}_A$ may be obtained if the mean value is small. However, it was ruled out that low mean values were the main contributors to the high $\text{CV}_A$s. The large additive variance means that there are good possibilities for the populations to respond by adaptation to changed environmental conditions and good possibilities for the genetic conservation of C. sativa.

At the population level the Hortiatis population from Greece showed the poorest growth while the Coruna population from Spain showed the best growth. This is clearly demonstrated in Fig. 2-35. The most extreme values for biomass were noted for Hortiatis in the T32D treatment, $< 5$ g, and Coruna in the T32W treatment, $> 12$ g. The corresponding for plant height was Hortiatis T25W, $< 30$ cm, and Coruna T32W $> 65$ cm. The population effects were strongly significant for all traits. The expectation for a larger root system of the three populations from the most arid conditions was not confirmed.

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Carbon isotope discrimination (CID) and leaf area size were studied in the same material by Lauteri et al. (2004). It was stated that CID is one out of several components that influence the adaptation to drought. For materials and treatments see above.

There was a limited but significant variation in CID among the four treatments, 0.06‰ (Fig. 2-36). This indicates phenotypic plasticity of this trait. The heritabilities for CID in individual treatments varied in the range 0.15-0.52 while the joint analysis of all treatments resulted in a heritability of 0.16 (Fig. 2-37). It was pointed out that the heritabilities were calculated based on the assumption that the OP-families were true half-sibs. If there were full-sibs in the tested material the heritabilities would be lower. The CVs were all low, 2.6-4.0%, and several times lower than for growth traits in the same material (Pliura and Eriksson 2002 above). The low precision of the separate CID estimates in individual populations in the four treatments was attributed to the low number of families per population. The greatest precision for CID estimates and best growth were noted for the Italian Pellice population while the Greek Paiko population constituted a contrast to Pellice population. The genetic correlations between CID and four growth traits were negative and strongly significant. Two of them are illustrated in Fig. 2-38. At the two temperature levels the relationships were somewhat stronger in the drought treatments than in the well-watered treatments. It was suggested that the strong relationships between limited growth and CID is a sign of adaptation to limited water availability.

In the joint analysis the population differences for CID were strongly significant. Also in the individual treatments there were significant population effects. The population effect could mainly be attributed to the poorly growing Greek Hortiatis population, which had the highest CID. This population was characterized by a low leaf area which under the best growth conditions was more than three times less than the Italian Pellice population with the largest leaf area. The strongest relationship with xerothermic index was noted for the T32W treatment, R² = 0.61, but not significant. Fig. 2-39 reveals that the Spanish population from Coruna was the strongest contributor to the strongly significant population x treatment effect for CID. There was an extraordinary strong relationship between xerothermic index and phenotypic plasticity of
Ecovalences for carbon isotope discrimination $\Delta$ for two C. sativa populations from each of Greece (blue), Italy (green), and Spain (red). Four cultivation regimes were used:

- $T_{25W}$ = well watered at 25°C
- $T_{25D}$ = periodic drought at 25°C
- $T_{32W}$ = well watered at 32°C
- $T_{32D}$ = periodic drought at 32°C. Lauteri et al. 2004.

Contrary to this, there was virtually no such relationship between xerothermic index and phenotypic plasticity for carbon isotope discrimination $\Delta$ for two C. sativa populations from each of Greece, Italy, and Spain. Four cultivation regimes were used:

- $T_{25W}$ = well watered at 25°C
- $T_{25D}$ = periodic drought at 25°C
- $T_{32W}$ = well watered at 32°C
- $T_{32D}$ = periodic drought at 32°C. Lauteri et al. 2004.

It was speculated that the “less xeric” populations respond to favorable growth conditions by increased leaf area and dry matter production in contrast to populations adapted to drought conditions. Such a performance of drought-adapted populations was regarded as a means to avoid damage by later exposure to harsh drought conditions.

### 2.3 Clone trials

López-Villamor et al. (2017) collected scions from the same families as in the paper by Miguez-Soto et al. (2016) for estimation of genetic parameters for rooting ability. The scions were dipped in a solution with IBA with a concentration of 2 g/liter for 3-5 minutes. The scions were placed in so called rooting tunnels in a greenhouse at a temperature of 24°C with a moisture content of 90-100%. The scions were collected during a three-week period starting in mid-May. In a first experiment 16 families were included and in a second experiment 25 families from the partial diallele mating were included. Each seedling was represented by six cuttings. In the 16-family experiment presence of roots and number of roots were assessed. In the 25-family experiment total root length, average root diameter, and root volume were also recorded.

The rooting percentages were high in both experiments with about half of the families showing 100% rooting in the second experiment. In experiment one the range in rooting percentage among families was 72-97%. The great success in rooting was attributed to the use of juvenile material.

The two heritabilities for the five root traits from the second experiment are illustrated in Fig. 2-41. All narrow-sense heritabilities were below 0.10 while the broad-sense heritabilities (clonal repeatabilities) were a few times higher for most of the traits. The two heritabilities in the first experiment did deviate much from the results in the second experiment.
The graphic illustrations of the breeding values in the paper reveal that there was a large variation within families, which explains the low $h^2$ estimates for all traits in the two experiments. Most genetic correlations were weak. Only the correlation between number of roots and presence of roots was strong, $R^2 = 0.86$. However, the limited number of parents means that all genetic correlations suffer from great imprecision. With the assumption that high rootability at young age would remain at mature age it was suggested that early selection for high rootability might be used in applied breeding. However, without any estimates of juvenile – mature correlation such a suggestion is premature. Efforts to keep the juvenility of the ortets would probably be a safer approach.

### 2.4 Genetic maps

Kubisiak et al. (1997) used an $F_2$ progeny for construction of a linkage map for *Castanea*. Eight isozymes, 17 RFLPs, and RAPDs were used for this purpose. This $F_2$ originated from two interspecific half-sib $F_2$ trees with a common female *C. mollissima* tree and two male *C. dentata* trees. In the $F_2$ population of 185 trees 99 with contrasting disease tolerance against *Cryphonectria parasitica* were selected for mapping and detection of markers involved in chestnut blight disease tolerance. At an age of three years inoculations were carried out during spring in cork-bored holes with two isolates of the fungus. Evaluation of the effect of the two isolates used took place in August and September. Markers associated to regulation of five morphological traits that differ between *C. dentata* and *C. mollissima* were searched for.

After exclusion of 45 loci with segregations deviating from the expected Mendelian segregation, 196 loci remained, of them 184 were linked in twelve linkage groups comprising 530.1 Kosami centiMorgan. It was remarked that we cannot be sure that each group represents a unique chestnut chromosome. The number of markers in each linkage group varied in the range 3-30 and twelve markers were unlinked. It was remarked that disturbed meiosis of the interspecific *Castanea* hybrid might be responsible for the deviating segregation in several loci. The deviating loci occurred in five linkage groups and it was speculated that chromosome homology did not exist at these locations and in this way be responsible for the observed aberrant segregation.

As many as 12 markers located on chromosome C affected the presence or absence of interveinal leaf hairs (Table 2-4). There was an indication that this trait was regulated by alleles in two closely linked loci in coupling phase. Modifiers may occur in other loci. Vein hair density was also associated to markers in chromosome C. There was an indication that this trait was regulated by a dominant allele in a single locus in chromosome C. Similarly, one locus seemed to be responsible for twig hair density. As regards stipule size there was a normal distribution of sizes. In spite of this distribution twelve markers were found to be significantly associated to stipule size. Stems of *C. mollissima* have green or tan color while the stems of *C. dentata* are reddish. In the progeny there was a close to 1:1 segregation as regards the two colors. Eight markers in three linkage groups and one unlinked marker were associated to stem color. It is regrettable that there was no information on the five morphological traits in the two $F_1$ trees. This makes the interpretation of the observed segregations for these traits in the progeny problematic. The close to 1:1 segregation for stem color may be the result of a cross cc x Cc but this cannot be verified in the text. Nor was there any information on which of the two traits in a pair that was dominant.

The results from the inoculation with *Cryphonectria parasitica* are summarized in Fig. 2-42, which shows that the four largest expansion classes comprised more than 75% of the $F_2$ seedlings. It was found that 34 markers in seven linkage groups were significantly associated to chestnut blight susceptibility. Of the seven genomic regions it was

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**Table 2-4. Number of markers affecting morphological traits and their linkage groups. Kubisiak et al. 1997.**

<table>
<thead>
<tr>
<th>Trait</th>
<th>Number of markers</th>
<th>Linkage groups involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interverinal leaf hairs</td>
<td>12</td>
<td>12C</td>
</tr>
<tr>
<td>Vein hair density</td>
<td>6</td>
<td>6C</td>
</tr>
<tr>
<td>Twig hair density</td>
<td>2</td>
<td>2C</td>
</tr>
<tr>
<td>Stipule size</td>
<td>12</td>
<td>2B, 3C, 6E, unlinked</td>
</tr>
<tr>
<td>Stem color</td>
<td>9</td>
<td>5A, 1C, 2I, 1 unlinked</td>
</tr>
</tbody>
</table>
found that only three loci had an intermediate to large effect on susceptibility. It was stressed that twelve seedlings had less symptoms than two of C. mollissima check seedlings. The least affected among the check seedlings had an expansion of 1.7 cm; the most affected 3.8 cm. To me the continuous distribution of the susceptibility points at polygenic inheritance with many loci involved and each allele with limited effect on disease tolerance. If this hypothesis turns out to be true, it means that breeding for disease tolerance will be cumbersome. The morphological traits showed all weak correlations with canker susceptibility. The degree of explanation was low, which means that the morphological traits studied do not bring much assistance to breeding for disease tolerance.

The first genetic map of C. sativa was presented by Casasoli et al. (2001). They used 96 trees from one full-sib family with the two parental trees originating from Turkey. The map was based on 311 RAPDs, 65 microsatellites, and five isozyme loci. Of the original ISSR primers tested, 22 were selected for genotyping of the 96 F1 trees to construct a female and male genetic map. The female map contained 92 markers while the male map contained 95 markers. The length of the maps was almost identical 720 and 721 CentiMorgans. The gaps between consecutive markers were wide in both maps, 1 – 34 and 1 – 35 for female and male maps. This suggests that clustering of the markers occurred. Thanks to 37 markers in intercross configuration the homology between the female and male maps could be united. It turned out that eleven of the twelve linkage groups could be joined. It was estimated that the saturation of the two maps amounted to 76 and 68% of the total map size. Casasoli et al. (2004) crossed two Turkish C. sativa trees to generate a large full-sib family for identification of QTL for three adaptive traits; bud flushing, juvenile growth, and carbon isotope discrimination CID. One tree originated from a humid climate while the other originated from a dry climate. In all 152 full-sibs could be analyzed. The traits were assessed during three years at ages 2-4 in a field trial close to Porano in Italy. Budset was recorded in eleven classes at four occasions at age four. Two estimates of bud flushing were observed: The date of the first observed unfolded leaf bud and the date when 70% of the buds showed an unfolded leaf (coined bud70). Basal diameter of the dominating shoot and tree height were measured. Carbon isotope discrimination (D) was analysed on leaf dry matter harvested at the end of the growing season according to Lauteri et al. (1997). In all 21 traits were analyzed. All traits assessed over three years showed a strongly significant effect of year. The map presented by Casasoli et al. (2001) was extended with additional 39 microsatellites to obtain a subset of markers evenly spaced over the genome. The Multiple Interval Mapping according to Kao et al. (1999) was used to detect QTL. The proportion of the phenotypic variation explained by QTL was derived from $\frac{1}{4}(d^2/\sigma^2_{\text{ph}})$, in which $d$ = the substitution effect of the QTL and $\sigma^2_{\text{ph}}$ is the phenotypic variance of the trait. The joint analysis of the trait data over years did not improve the QTL identification. The female and male linkage maps constructed for the QTL analysis were based on 109 and 108 markers respectively. A cluster analysis of the traits was carried out. Three clusters were distinguished.

1. All bud flushing traits
2. Carbon isotope traits + budset age 4 + height age 2
3. All growth traits except for height at age 2 and budset at age 4.

Several of the phenotypic correlations between the traits were significant but only one had a degree of explanation larger than 50%, age 3 diameters at ground level and at 10 cm above soil, $r = 0.83$. It is somewhat surprising that these two traits did not show a still stronger relationship. The total number of QTL detected were 80, of which 50 turned out to be significant. There was a dramatic difference in number of QTL between the female and male linkage groups, 60 and 20 respectively. The impact of the environmental conditions between the two parents was suggested as one explanation for this difference. The female parent originated from Mediterranean type of climate and it was therefore suggested that the growth conditions in the field trial agreed more with the environmental conditions at the origin of the female parent than with the male parent that originated from a humid climate. The small difference in map saturation of the two parents was ruled out as the cause of the difference between female and male QTL detection.
The numbers of significant QTL for the three types of trait were:

- **Phenology**: 23: female 19, male 4
- **Growth**: 16: female 12, male 4
- **CID**: 11: female 9, male 2

In order to get an idea about stability of QTL, mainly over years I listed QTL with identical or close location in a specific linkage group in **Table 2-5**. In the female linkage group 1 one group of QTL for bud flushing and another for height growth occur over time. It should be noted that some autocorrelation occurs between some of the traits, i.e. Hinc01 and H01. It was suggested that different QTL are expressed at different ages or under different environmental conditions as an explanation for lack of QTL agreement over all three years of data collection. In other tree species juvenile growth is characterized by much free growth that disappears with age when most growth is predetermined. If this is the case for chestnut the suggestion of different QTL are expressed different years might be true. The weather conditions during the years of observation varied strongly, which means that differences in expression between years are plausible.

**Table 2-5** also reveals that most QTL had PEVs (phenotypic explanation of variance) between 5-10%. The distribution of PEVs for all QTL agrees with this statement (Fig. 2-43). It was stated that many adaptive traits are regulated by genes in many loci with small effects on the trait in question.

The results were thoroughly discussed from an evolutionary perspective. However, the imprecision in the estimates of QTL is considerable owing to the number of individuals tested. Moreover, one QTL might harbor more than one locus affecting a trait. Therefore, any far-reaching conclusions on evolution of the traits studied in this investigation are hard to draw.

Development of a consensus genetic map of *C. sativa* and *Quercus robur* was one objective in an extension of the previous work (Casasoli et al. 2004). Another objective of this study was to find QTL (quantitative trait loci) common in the two species for three adaptive traits; bud flushing, tree height, and carbon isotope discrimination (CID). Microsatellite markers and newly developed STS (sequence-tagged sites) were used for the two objectives. The estimates of QTL emanate from observations during three years in *C. sativa* and from three different trials in *Q. robur*.

Most of the STS markers amplified in one of the species were also amplified in the other species. Four oak STSs were not amplified in chestnut while one of the chestnut STSs was not amplified in oak. The comparative mapping included 18 microsatellites, 1 isozyme, 1 5srDNA, and 35 STSs. Homeologous linkage groups were found, which contained two to seven anchor markers. Only minor rearrangements between the two species were noted and could be attributed to mapping errors. The observed data

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**Table 2-5.** Significant QTL for traits sharing similar or close positions in a linkage group, their positive or negative substitution effect and their proportion of phenotypic variance explained (PEV) by the QTL; blue = positive PEV, red is negative PEV. Bud00 = terminal bud flushing in year 2000, bud7002 = 70% of all bud have flushed in 2002, bud = joint analysis of bud flushing over years. In analogy with this HXX = height a certain year, HincrXX = height increment a certain year, Dg and Db are diameters at ground level and at 10 cm above ground, Δ is carbon isotope discrimination. Casasoli et al. 2004.

<table>
<thead>
<tr>
<th>Trait</th>
<th>female 1</th>
<th>female 3</th>
<th>female 9</th>
<th>female 10</th>
<th>female 12</th>
<th>male 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phenology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bud01</td>
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<td></td>
<td></td>
<td>Bud02</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>Bud02</td>
<td>12.2</td>
<td></td>
<td></td>
<td>Bud7002</td>
<td>11.6</td>
<td></td>
</tr>
<tr>
<td>BUD7002</td>
<td>6.7</td>
<td></td>
<td></td>
<td>Bud</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>Bud</td>
<td>11.8</td>
<td></td>
<td></td>
<td>Bud00</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bud70</td>
<td>8.0</td>
<td></td>
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<td><strong>Growth</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Hinc01</td>
<td>7.2</td>
<td></td>
<td></td>
<td>Dg01</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td>H01</td>
<td>8.3</td>
<td></td>
<td></td>
<td>H</td>
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</tr>
<tr>
<td>H02</td>
<td>11.5</td>
<td></td>
<td></td>
<td>Dgincr01</td>
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<td></td>
<td></td>
<td>Db01</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td><strong>Carbon isotope discrimina-</strong></td>
<td>7.3</td>
<td></td>
<td></td>
<td>Δ 01</td>
<td></td>
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<td>tion</td>
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<td></td>
<td></td>
<td>Δ</td>
<td>13.2</td>
<td></td>
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</tbody>
</table>

**Figure 2-43.** The number of significant QTL with three percentage levels of PEV (proportion of phenotypic explanation of variance) for three types of traits phenology = bud flushing and budset; growth = height and diameter; carbon = carbon isotope discrimination. Evaluations were carried ot at ages 2-4 in an Italian progeny trial with one full-sib family of *C. sativa*. Casasoli et al. 2004.
suggested that both macrosynteny and macrocolinearity exist between these two species. It should be noted that the numbering of the twelve chromosomes are not the same in oak and chestnut. The *Q. robur* linkage groups corresponding to *C. sativa* linkage groups are shown in Fig. 2-44.

Before the results as regards QTL are presented and discussed it is worthwhile to present some of the shortcomings of such a study according to the authors:

1. only one full-sib family in each species was analyzed
2. relatively low population size, 165 for oak and 147 in chestnut, leading to overestimation of PEV and missing QTL with low PEV
3. large confidence intervals of QTL positions leading to imprecision of QTL positioning
4. sampling strategies differed.

The number of QTL common to both species was much higher for bud flushing than for tree height and CID (Fig. 2-44). Moreover, significant colocation of QTL for phenology was noted while colocation of QTL for height seemed to be more random according to the authors. Colocation for CID was absent. These observations were taken as an indication of strong genetic regulation of bud flushing. It was also suggested that CID and growth are more composite traits than bud flushing and thereby more influenced by environmental conditions than bud flushing. Two linkage groups in *Q. robur* had QTL for bud flushing without any QTL in the corresponding *C. sativa* linkage groups while the opposite situation did not occur. For tree height and CID there were more QTL in *C. sativa* without a counterpart in *Q. robur* than the opposite situation. Dependent on the type of trait, 2-4 linkage groups without QTL were found.

Fig. 2-44 reveals that most mean PEV estimates are below 10%. One CID QTL estimate in *Q. robur* contributed much to the high mean value for this trait, 26%. In the previous paper 12 non-significant QTL had PEVs in the range 3.2-8.0%. With the exception for one QTL for height (10.6%) all PEVs were around 7%. Strong effects of QTL for the three studied traits are evidently rare. There was a considerable difference in number of QTL identified via the female and male parent (Fig. 2-45 and 2-46, next page). It was stated that many QTL regulating bud flushing were heterozygous in the parental generation.
In conclusion an important step in understanding the regulation of one adaptive trait of great significance for survival of *C. sativa* and its future breeding.

The homology of microsatellites of *C. sativa* and *Quercus robur* was determined following cross-species amplification ([Barreneche et al. 2004](#)). Another objective of this study was to include new markers to the genetic maps of these two species. For this study 96 individuals from one full-sib family in each species were analyzed. Cloning of one allele from 17 mapped microsatellite loci was carried out.

Of the 53 oak primer pairs tested 37 gave a positive amplification in *C. sativa*. Seventy percent of the chestnut microsatellites segregated in the chestnut full-sib family while 16 of the oak microsatellites showed segregation in chestnut. Five of the 30 chestnut microsatellites segregated in the oak full-sib family. The mainly tri-nucleotide repeats of chestnut microsatellites was given as reason for the lower percentage of segregation of chestnut microsatellites in oak than vice versa, 5% and 31%, respectively. As many as 14 microsatellites showed corresponding allele size and a high sequence identity at the flanking regions.

Twenty-two new microsatellites from chestnuts and 17 transferred from oak could be introduced into the chestnut genetic map. A comparison of the oak and chestnut maps showed that there were seven homeologous groups. In spite of the observed agreement between the two species from different genera a warning was raised for use in population genetics studies owing to the complexity of the evolution of microsatellite loci.

**Figure 2-46.** Mean proportion of phenotypic explanation of variance, PEV, for QTL in three types of trait; phenology = bud flushing and budset, growth = height and diameter, carbon = carbon isotope discrimination. Only significant QTL are included. Open bars = one QTL only. Evaluations were carried out at ages 2-4 in an Italian progeny trial with one full-sib family of *C. sativa*. Data from the male parent. Casasoli et al. 2004.

In conclusion an important step in understanding the regulation of one adaptive trait of great significance for survival of *C. sativa* and its future breeding.

Bacterial artificial chromosome (BAC) libraries for *C. mollissima* were developed by [Fang et al (2013)](#) to integrate the physical and genetic map of this species. The ultimate goal of this investigation was to facilitate breeding for *Cryphonectria parasitica* resistance in *C. dentata*. In all 691 genetically mapped markers and physically anchored markers were presented in this investigation (Fig. 2-47). Three QTL for chestnut blight resistance were genetically mapped by EST-based genetic markers. The BAC enabled an integrated physical and genetic map for these QTL. It was also disclosed that gene duplication had not taken place to any great extent in *C. mollissima*. The comparison with poplar mapping showed that major macrosynteny between poplars and chestnut does not exist.

Sisco et al. (2005) reported on a comparative study of genetic linkage maps for *C. dentata*, *C. mollissima*, and *C. sativa*, which is a follow-up of two previous papers. Two hundred and seventy-five AFLP markers were added to the *C. dentata/C. mollissima* linkage map. There was a random distribution of the AFLP markers and the map size increased by 21 centiMorgans. Besides the addition of AFLP markers, 24 SSR markers, five S-ribosomal DNA loci, and one isozyme locus was added to the previous map. It was concluded that eleven linkage groups could be correlated between the *C. sativa* and *C. mollissima/C. dentata* linkage groups. Thanks to the large polymorphy in the SSR loci it was possible to eliminate 18 trees from the analysis of chestnut blight tolerance owing to contamination. After this elimination one of the three loci earlier found to be markers for chestnut blight resistance ([Kubisiak et al. 1997](#)) was no longer a marker for this disease. There were no common markers in linkage group B of the Chinese/American map and group 11 of the European chestnut.

It was again pointed out that the distortions in segregation of a fairly high proportion of markers mean that the presented maps suffer from some uncertainties.

**Figure 2-47.** Number of genetically mapped and physically anchored markers in the 12 linkage groups in *C. mollissima*. Fang et al. 2013.
Two full-sib families of *C. crenata* were used by Nishio et al. (2018) for construction of genetic maps of the four parents as well as a joint map for each family. In all, 443 microsatellites and 554 SNPs were used for this construction. These six maps were successfully aligned to the Chinese chestnut consensus map. Eight traits of importance for breeding were analyzed after four and five growth periods:

- Harvesting date
- Number of burs
- Number of nuts
- Nut weight
- Yield per tree
- Pericarp splitting
- Infestation by insects, *Conigethes punctiferalis*
- Trunk diameter (measurement point on the trunk was not given)

Only QTL which showed significances over two years of analysis were regarded as reliable QTL. Owing to the limited insect infestation in one year, QTL for this trait is based on observations during one year only. The most saturated map was based on 409 SSRs and 537 SNPs. Five of the linkage groups had gaps larger than 10cM. The number of loci varied in the range 226-839 among the six maps with map lengths ranging from 453 cM to 668 cM.

I have tried to summarize the most important results as regards QTL location to different linkage groups in Table 2-6 and the agreement with respect to position in the linkage group for those cases with QTL for the same trait in more than one of the six maps (Table 2-7). In all, 21 significant QTL were identified in nine linkage groups. QTL for harvest date, nut weight, pericarp splitting, and insect infestation were detected on more than one map. The positions of the two QTL for nut weight in linkage group A agreed well, which suggest that they may be of the same origin. Fairly good agreement of the QTL for harvesting date in linkage group D and for pericarp splitting in linkage group J was noted.

### Table 2-6. Number of significant QTL in different linkage groups based on individual maps and integrated maps for the two *C. crenata* parents in each cross, K x 709 and T x P. QTL for traits detected in the same linkage group in more than one of the taxa are marked blue. Nishio et al. 2018.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Position agreement in linkage group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harvest</td>
<td></td>
</tr>
<tr>
<td>No. burs</td>
<td></td>
</tr>
<tr>
<td>No. nuts</td>
<td></td>
</tr>
<tr>
<td>Nut weight</td>
<td></td>
</tr>
<tr>
<td>Yield/tree</td>
<td></td>
</tr>
<tr>
<td>Pericarp splitting</td>
<td></td>
</tr>
<tr>
<td>Insect infestation</td>
<td></td>
</tr>
<tr>
<td>Stem diameter</td>
<td></td>
</tr>
<tr>
<td><strong>Individual map</strong></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>D, F</td>
</tr>
<tr>
<td>709</td>
<td>J</td>
</tr>
<tr>
<td>T</td>
<td>D, H</td>
</tr>
<tr>
<td>P</td>
<td></td>
</tr>
<tr>
<td><strong>Integrated map</strong></td>
<td></td>
</tr>
<tr>
<td>K x 709</td>
<td>A, D</td>
</tr>
<tr>
<td>T x P</td>
<td>H</td>
</tr>
</tbody>
</table>

### Table 2-7. The agreement in position on the linkage group in case of QTL identified in more than one genetic map. Nishio et al. 2018.

<table>
<thead>
<tr>
<th>Linkage group with QTL</th>
<th>Trait</th>
<th>Position agreement in linkage group</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Nut weight 2</td>
<td>good</td>
</tr>
<tr>
<td>D</td>
<td>Harvesting date 3</td>
<td>fairly good</td>
</tr>
<tr>
<td>F</td>
<td>Insect infestation 3</td>
<td>poor</td>
</tr>
<tr>
<td>H</td>
<td>Harvesting date 2</td>
<td>poor</td>
</tr>
<tr>
<td>J</td>
<td>Pericarp splitting</td>
<td>fairly good</td>
</tr>
</tbody>
</table>
The variance explained by the various QTL is illustrated in Fig. 2-48, which shows that the range for explanation is approximately 10-25%. The authors mentioned that the variance for harvesting date would amount to around 60% under the assumption that the three QTL in linkage groups A, D, and F act in an additive way. With such a high percentage it is likely that visual inspection of the population would be a less laborious way for breeders to identify superior trees. As in all other cases with QTL, the estimates are valid for the population under study and high precision of the estimates is dependent on large number of individuals tested. No information on the family sizes was given in this report.

Torello-Marinoni et al. (2018) crossed the tolerant *C. sativa* x *C. crenata* hybrid Bouche de Bétizac with *C. sativa* clone Madonna, which is susceptible to *Dryocosmus kuriphilus* to generate a progeny for development of marker-aided selection breeding. One hundred and thirty-two microsatellite loci were used for genotyping of 148 trees at age eight years. Controlled infestation with the insect was assessed visually in two classes susceptible or resistant. The time of bud flushing was assessed during four years and five classes of growth habit were also assessed. Gene mapping of the two parents was carried out by aid of Join mapOR (Van Ooijen 2009). The MapQTLoR 6 software program was used for identification of putative QTL.

A set of 120 microsatellites were used for construction of the female map while 84 were used for the male map. The female map consisted of twelve linkage groups while the male had 13 groups. The resistance against *Dryocosmus kuriphilus* showed a close to 1:1 segregation while bud flushing and growth habit showed normal distributions. The resistance was located to linkage group K in the interspecific hybrid.

Separate QTL for bud flushing were estimated for each year and each parent. Eleven QTL explaining more than 10% of the phenotypic variance (coined major QTL) were identified. Six major QTL were detected on the female map while five were identified on the male map. For the female parent one QTL for bud flushing was found during all four years of observation with a degree of explanation of 28-38%. It was located on linkage group L. Another stable QTL for bud flushing was found for the male parent with a degree of explanation of 10-14%. This QTL was located on linkage group C.

As regards growth habit three and two major QTL were detected on the female and male maps, respectively. One of the female QTL was stable over years. It was concluded that this is a first step in developing tools for breeding. The maps need to be more saturated and this will possibly be achieved by use of SNP markers.

### 2.5 Male sterility

The inheritance of male sterility in *C. sativa* was studied in eight full-sib families by Soylu (1992). Five classes of anthers were identified and their hypothetical genotypes are shown below:

- longistaminate long: XXZZ
- longistaminate short: XxZz
- mesostaminate: Xxzz and xxZZ
- brachystaminate: xxZz
- astaminate: xxzz

In Table 2-8 the segregation in anther classes in three full-sib families is presented together with the expected segregation based on the genotype classes above. As seen from this table only one of the full-sib families supports the hypothesis put forward. The author pooled the data from families 1 and 3 and calculated one $\chi^2$ value for each class and summed the values for all five classes, which is...
a violation of the rules for $\chi^2$ estimation. Doing so, there was no significant deviation from the expected segregation. The absence of intermediate classes in family 2 was attributed to lethal factors. With some exceptions (11 of 100), families with a longistaminate parent had longistaminate offspring. The exceptions were attributed to contamination of nuts. In conclusion, homozygosity for recessive alleles in two loci was suggested as explanation for the results and that the two dominant alleles ($X$ and $Z$) showed almost additive gene expression. It was stated that additional material ought to be studied.

Shi and Hebard (1997) stated that male sterility in hybrids between *C. dentata* and *C. mollissima* only occurred when *C. dentata* was used as female but not in the reciprocal cross. The male sterility has consequences for breeding and therefore its occurrence in backcross generations as well as in $F_1$ was studied.

All offspring in four full-sib families from crosses between *C. dentata* females and two *C. mollissima* males were all male sterile. Eight and five full-sib families from crosses of two *C. mollissima* females and 13 *C. dentata* males did not have any male sterile progeny. One interesting observation was the occurrence of both fertile and male sterile progeny in one of the two backcross families with the same $F_1$ male hybrid. This was interpreted as segregation of a dominant gene for male sterility originating from the $F_1$ male parent. The results of crosses for the BC$_2$ generation revealed that the male sterility in offspring with the interspecific Clapper as male was lower, 9.8%, than in the progenies from the interspecific Graves as male, 41% (Table 2-9). Based on the results Shi and Hebard proposed that the male sterility of *C. dentata*, *C. mollissima*, and their hybrids is controlled by both nuclear and cytoplasmic factors.

### Table 2-8. The observed and expected segregation in three full-sib *C. sativa* families with respect to anther category. Soylu 1992.

<table>
<thead>
<tr>
<th>Supposed genotype and phenotype</th>
<th>family 1</th>
<th>family 2</th>
<th>family 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 XxZZ longistaminate long</td>
<td>6 (6.375)</td>
<td>0 (4.75)</td>
<td>6 (7.625)</td>
</tr>
<tr>
<td>2 XxZz Longistaminate short</td>
<td>7 (12.75)</td>
<td>24 (9.5)</td>
<td>16 (15.35)</td>
</tr>
<tr>
<td>1 XzZ + 1 xxZZ mesostaminate</td>
<td>10 (12.75)</td>
<td>0 (9.50)</td>
<td>18 (15.35)</td>
</tr>
<tr>
<td>2 xxZz brachystaminate</td>
<td>19 (12.75)</td>
<td>0 (9.50)</td>
<td>17 (15.35)</td>
</tr>
<tr>
<td>1 xxzz astaminate</td>
<td>9 (6.375)</td>
<td>14 (4.75)</td>
<td>4 (7.625)</td>
</tr>
</tbody>
</table>

### Table 2-9. Mean percentage of male sterility in second first generations of backcross between *C. dentata* (Cd) and *C. mollissima*. C stands for an interspecific hybrid coined Clapper, G stands for another hybrid, Graves, and N stands for Nanking. Shi and Hebard 1997.

<table>
<thead>
<tr>
<th>Type of cross</th>
<th>No. of families for a certain mating</th>
<th>Number of progeny</th>
<th>Male sterility %/ range</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC2</td>
<td>8 Cd x BC1-C</td>
<td>372</td>
<td><strong>9.8</strong> 0-26</td>
</tr>
<tr>
<td>BC2</td>
<td>7 Cd x BC1-G</td>
<td>422</td>
<td><strong>41.0</strong> 13-67</td>
</tr>
<tr>
<td>BC1</td>
<td>1 Cd-mu x N</td>
<td>70</td>
<td><strong>0</strong></td>
</tr>
<tr>
<td>BC1</td>
<td>1 Cd-mch x N</td>
<td>33</td>
<td><strong>45</strong></td>
</tr>
</tbody>
</table>
Table 2-10. Segregation of male sterility after various types of interspecific Castanea crosses and cpDNA haplotypes. Cc = C. crenata, Cd = C. dentata, Cm = C. mollissima. Sisco et al. 2014.

<table>
<thead>
<tr>
<th>Types of cross and number of families</th>
<th>Mataing</th>
<th>Male fertile</th>
<th>Male sterile</th>
<th>cpDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC1</td>
<td>Cd x (Cm x Cd)</td>
<td>47</td>
<td>0</td>
<td>P</td>
</tr>
<tr>
<td>BC2</td>
<td>Cd x (Cm x Cd)</td>
<td>19</td>
<td>10</td>
<td>D</td>
</tr>
<tr>
<td>6 F1</td>
<td>Cd x Cm</td>
<td>0</td>
<td>41</td>
<td>D</td>
</tr>
<tr>
<td>1F1</td>
<td>Cd x Cc</td>
<td>0</td>
<td>2</td>
<td>D</td>
</tr>
<tr>
<td>3 F1</td>
<td>Cd x Cm</td>
<td>15</td>
<td>0</td>
<td>M</td>
</tr>
<tr>
<td>1 Fpi</td>
<td>Cd x Cm</td>
<td>2</td>
<td>0</td>
<td>P</td>
</tr>
<tr>
<td>3BC1</td>
<td>(Cd x Cm) x Cm</td>
<td>10</td>
<td>0</td>
<td>M</td>
</tr>
</tbody>
</table>

The male sterility issue was followed up by Sisco et al. (2014) and extended to determination of the cpDNA haplotypes of the progeny. It was noted that all materials contained C. dentata cytoplasm. The results in Table 2-10 show that male sterility is developed only in presence of the D haplotype. Nine of the progeny families shared one parent. Only families with the D haplotype caused male sterility. It was stated that cytoplasmic male sterility in chestnut probably results from the interaction between the mitochondrial genes of one species with the nuclear genes of another species. This must be regarded as a bold speculation based on causes of male sterility in other species since no information on mitochondrial genes was presented. The other conclusion that nuclear genes for male sterility are dominant in presence of the D cpDNA haplotypes was supported by the results in this investigation. Finally, it was pointed out that conservation of the different haplotypes should be implemented.

2.6 Miscellaneous

The genome of C. mollissima was compared with genomes of ten other species by Staton et al. (2015). Three QTL regions in C. mollissima related to tolerance against chestnut blight was focused on in the synteny study. The comparison comprised six woody species and four herb species. The integrated genetic and physical C. mollissima map contains 858 shared sequence based markers. The agreement between the chestnut genome and the ten other species varied between 10 and 39%. There was more synteny with the wooden species and chestnut than between chestnut and the four herbal species, even when some of the herbal species were closer related to chestnut than the wooden species. There was some agreement between the QTL regions and the ten other species.

The complete nucleotide sequence of the 1,5-biphosphate carboxylase (rbcL) cpDNA gene was reported by Frascaria et al. (1993a). There were only twelve nucleotide differences for a DNA homology with Quercus rubra. The homology was less with Nicotiana tabacum and Zea mays. The substitution rates in the rbcL locus in sweet chestnut was a few times lower than in annual species such as, Zea mays or Nicotiana tabacum.

Internal kernel breakdown (IKB) was observed in nuts from chestnut orchards in Michigan, USA. Especially one C. sativa x C. crenata cultivar (Colossal) was affected. The impact of pollinator on the development of IKB was studied by Fulbright et al. (2014) in three experimental orchards. Crosses were carried out with Colossal clone as female parent and C. mollissima, C. crenata x C. sativa or C. pumila as pollinators. The C. mollissima male gave rise to 31 and 37% IKB in the two orchards, in which it was used as pollinator. Only two of the 641 nuts obtained after crosses with the three interspecific males were affected by IKB. They were attributed to pollen contamination. Open pollinated nuts from six Colossal trees were collected during two years in one orchard, in which Chinese chestnut trees were the only potential males owing to the isolated location of the experimental seed orchard. In 2010 the mean percentage of IKB nuts from the six Colossal trees was 25% and in 2011 it was 12%. One of the trees had low percentages of IKB both years, 4 and 7%. These findings support the observations in commercial orchards that IKB only occurs when C. mollissima serves as pollinator.

2.7 Summary

Few studies designed to estimate genetic parameters based on full-sib families trials were published. There are many full-sib backcross experiments with C. dentata with the main objective of transferring chestnut blight resistance to this species. Full-sib progeny trials GCA for growth traits and flushing in juvenile material was significant but the heritabilities were low (<0.10) with one exception; flushing 0.30. The clonal repeatability (HF) based on vegetatively propagated plants varied in the range 0.05-0.35. Flushing had also in this case the highest estimate, 0.35.
Inheritance of hairiness in *C. dentata* and its hybrids with Asian chestnuts showed dominant inheritance. Similarly, the reddish stem color of *C. dentata* seems to be a dominant trait. Backcrosses to *C. dentata* following hybridization with *C. mollissima* are treated in the Breeding chapter.

**Open pollinated progeny trials**

Two publications concern two Spanish field trials with 19-26 OP-families from each of six populations from Greece, Italy, and Spain. One Greek trial belongs to the same series of trials. Assessments in the Spanish trials took place up to age twelve and at ages 4-6 in the Greek trial.

In the Spanish trials strong OP-family x locality effects were noted for phenology and growth traits while the family effect was strongly significant for flushing only. The heritabilities at ages 2-12 were in the range 0.25 – 0.40 for flushing and growth traits. The genetic correlations between height at age three and growth at age 12 were weak. In the parallel trials with 36 Spanish populations the heritabilities for growth phenology, and two quality traits were in the range; 0.05 – 0.15. Heritability for basal diameter and stem volume increased rapidly from age 4 to age 6 in the Greek progeny trial. The estimated increase was from 0 to 0.23 and 013 to 0.29, respectively.

High heritabilities for two growth and two quality traits were noted in two Spanish progeny trials with 36 OP-families assessed at age eight and six, 0.20 - 0.40. At a joint analysis of the results from the two trials the heritabilities dropped to around 0.20 for the four traits. The family mean correlation coefficients for the performance of the same trait in the two trials were all 0.80 or stronger. High heritabilities (0.20-0.45) for phenology and growth traits were noted in a combined provenance and progeny trial with nine populations each represented by 14-28 OP-families. The genetic correlations between flushing and bud set or tree height were weak, \( R^2 = 0.40 \).

Based on high heritabilities and different selection schemes, spectacular gains were projected for growth traits in some papers.

Basic gene ecological studies on juvenile open-pollinated families cultivated under controlled conditions in growth chambers revealed high additive genetic coefficients of variance (\( CV_a \)) for growth traits, 10-15%. In this two x two factorial experiment with two temperatures (25 and 32°C) and two water availabilities (temporary drought and full water access) the family x treatment interaction for the growth traits did not exceed 5%. The \( CV_a \) for carbon isotope discrimination (\( \Delta \)) varied in the range 2-4%. There was a strong negative relationship between above ground dry weight and \( \Delta \) in 32°C + temporary drought treatment \( R^2 = -0.95 \).

High narrow-sense heritabilities were noted for flushing and growth traits while budset, apical dominance, and straightness had heritabilities in the range 0.10-0.20 in a Spanish study. In a short-time basic study of effect of water availability on growth traits in young plants from nine Spanish natural populations each represented by ten OP-families, high \( CV_s \) estimates were noted for shoot and root dry weights, 20-30%, in well-watered and periodic drought treatments. In the joint analysis the \( CV_s \) dropped considerably for shoot dry weight but less so for root dry weight. These results suggest that genotype x treatment interaction is substantial.

**Clonal repeatability**

High percentages of rooting were observed in two experiments with 16 and 25 full-sib families. The heritabilities were low, \(<0.10\) for the five rooting traits studied. The low heritabilities were attributed to the large variation within families. Similarly the genetic correlations between rooting traits were in most cases low.

**Maps and QTL**

Genetic maps were constructed for the four important Chestnut species, *C. crenata*, *C. dentata*, *C. mollissima*, and *C. sativa*. One objective in these efforts was to identify QTL for different traits. No less than 34 markers in seven linkage groups were significantly associated with chestnut blight resistance. Only in three regions the effects were intermediate or large.

Significant associations with interveinal leaf hairs in *C. dentata* in two closely linked loci were observed. In *C. sativa* as many as 80 QTL for phenology, growth, and carbon isotope discrimination were detected. A few of the QTL occurred more than one year. Most of the QTL had 5-10% phenotypic explanation of variance (PEV). QTL for nut traits were studied in *C. crenata*. In all, 21 significant QTL were identified. In some cases there was an agreement of position of QTL in more than one of the six maps. The QTL for nut weight showed the closest agreement of the traits tested. Harvesting date and pericarp splitting also showed agreement between maps. The PEVs in *C. crenata* were high in two cases; around 25%. In the study of consensus maps between *Castanea* and *Quercus* minor rearrangements between the two species were noted. Both macrosynteny and macrocolinearity were observed.

It was noted that male sterility in hybrids between *C. dentata* and *C. mollissima* only occurred when *C. mollissima* was used as male parent. A hypothesis that male sterility was regulated by two complementary and dominant alleles in two loci was tested. Some support was found for this hypothesis.

Male sterility in interspecific crosses occurred in presence of one specific haplotype only.
3 Diseases and pest tolerance

3.1 General

In her paper Chestnut breeding in the United States for disease and insect resistance Sandra Anagnostakis gives a historic review of the C. dentata selection and breeding activities starting during the nineteenth century (Anagnostakis 2012). The appearance of chestnut blight and ink disease called for introductions of species tolerant against these diseases and hybridizations and further back crossing. From the 1970ties the introduction of the hypovirulent strain of chestnut blight made it possible to grow C. dentata to fully developed trees and thus facilitate interspecific crossing works.

In another overview paper Steiner et al. (2016) summarizes the results of the backcross breeding program for incorporation of resistance to chestnut blight in American chestnut. One focus was on the negative deviation from expectation of blight resistance in the B₁ x B₁ generation. With a simple Mendelian inheritance full resistance ought to be expressed in such progenies if the hypothesis of resistance as controlled by genes in two loci was true. Initially the back cross breeding program was based on two B₁ hybrid trees, which was regarded as a too limited genetic resource for long-term breeding. Now the number of C. dentata trees in the breeding was extended to 25 trees. The number of backcross lines from the two original hybrids is now 25 and 29. The target is to have 3,000 and 4,000 trees in the B₃ x B₁ generation after selection for blight resistance. It is a laborious task to handle tens of thousands of trees to reach this final target numbers. In 2016 there were 586 planting locations with a total area of 335 hectares. The need for local adaptation was stressed, which means that several breeding populations have to be developed over the whole range of C. dentata in USA; from Alabama in south to Maine in north. The potential of genetic transformation to reach blight resistance was also treated and one example of a successful transformation was given. A recurrent selection program is foreseen for the breeding after reaching the B₃ x B₁ generation. It was noted that the breeding had not addressed the root rot fungus, Phytophthora cinnamomi. Finally, it was emphasized that the program was dependent on philanthropy and it was stated Indeed we have been continually amazed by the talent, enthusiasm, and dedication of TACF volunteers (TACF = The American Chestnut Foundation).

A large number of scientists involved in genetics and breeding of chestnut published an excellent state of the art paper on interspecific hybridization in chestnut in 2016 (Pereira-Lorenzo et al 2016). This publication is not limited to interspecific hybridization but summarizes a broad array of topics such as development of molecular markers, within-species diversity of individual species, and mating system.

3.2 Cryphonectria parasitica

Two strains of Cryphonectri parasitica with rapidly growing mycelia were inoculated into a variety of chestnut species and species hybrids by Anagnostakis (1992). Bark was removed from branches with green bark and plugs of agar containing the fungus were applied in the holes, which had a diameter of 10 mm. The rate of mycelium growth was recorded.

I have summarized most of the observations in Fig. 3-1, which shows that C. dentata is the most susceptible and C. crenata is the least susceptible of the taxa analyzed. The low number of trees in each taxon means that it is hard to draw any far-reaching conclusions. It was suggested that the observed drop of susceptibility from F₁ to the back cross [d x m (mx d) x d] in Fig. 3-1 might be attributed to loss of deleterious genes in the back crossing. Assuming that the susceptibility is a polygenic trait, such an explanation seems unlikely. However, according to Bernatzky and Mulcahy (1992) resistance might be regulated by alleles in two unlinked loci. This was based on published data from a back cross C. mollissima x C. crenata x C. dentata with the segregation 103 resistant and 37 susceptible individuals, which is close to a 3:1 segregation. However, they suggested genotypes of C. mollissima as R, R, R, r, C. crenata as r, r, r, r and C. dentata as r, r, r, r. This would result in resistance of the F₁ genotype as well as in the back cross genotypes and not a 3:1 segregation.

Bernatzky and Mulcahy (1992) did not present any own results but discussed the possibility of using
molecular marker-aided selection in a back cross breeding program to transfer chestnut blight resistance into C. dentata.

Huang et al. (1996) carried out inoculations of seedlings from 13 C. mollissima cultivars and four open-pollinated seedlings of C. dentata with three isolates of Cryphonectria parasitica. All inoculations took place on one-year old seedlings. The spreading of the canker in width and length was assessed. Since these two measurements were strongly correlated only figures for the width of the canker were reported.

Generally, a fast development of the canker took place during the first week after inoculation. Then a gradual decline of the canker was observed in the three of the C. mollissima cultivars (Fig. 3-2). In this figure two of the cultivars that recovered well after inoculation (H and W) and two of the most susceptible cultivars (J and D) are illustrated. In the C. dentata seedlings a linear increment of the canker width took place. As far as I can understand from the paper the results illustrated in this figure does not refer to the isolate with strongest virulence. It was stated that the differences among the American cultivars were insignificant. Three weeks after inoculation 70% of the C. dentata seedlings were girdled and died.

Fig. 3-3 reveals that there was a dramatic difference in susceptibility of the C. dentata cultivars dependent on the isolate used for inoculation. Isolate SLA-155 caused damage of the same magnitude as in C. mollissima. Figure Fig. 3-4 shows that the effect of inoculations was not stable over years. As a corollary of this, the interaction isolate x year was strongly significant. It was suggested that the environmental conditions during the inoculations were responsible for the observed interactions. In conclusion, the findings indicate that the genetic constitution of both host and parasite have pronounced effects on response to inoculations.

Oxalic acid secreted by pathogenic fungi was shown to play a role for cell wall degradation in host plants (references in Zhang et al. 2013). Therefore, several investigations on the possibility to incorporate genes for degradation of oxalic acid such as oxalic acid oxidase were carried out.

Welch et al (2007) studied the effect of oxalic acid in media on cell wall composition in oxalate oxidase transformed and untransformed tissue cultures of C. dentata. The binary vector, pGPOxO with the CaMV 35S promoter, was used for transformation.

Fig. 3-5 shows that the level of lignin dropped to constant level in the range of oxalic acid tested in the untransformed control cultures while a corresponding drop in the transformed material did not occur.
Zhang et al. (2013) carried out experiments aiming at transfer of gene(s) for oxalate oxidase (OxO) from wheat into *C. dentata* to achieve degradation of oxalic acid in *C. dentata*. Somatic embryogenic clumps originating from somatic embryos of two *C. dentata* trees were used in the transformation efforts. Axillary shoots of one *C. dentata* and one *C. mollissima* tree were also used in transformation studies. The vectors *p35S-OxO* and *pTACF3* (*VspB-OxO*) were used for transfer of *OxO* into *C. dentata*. In addition an empty control vector lacking the *OxO* gene was used. *CaMV 35S* and *VspB* were used as promoters. Shoots were regenerated after 28-36 weeks and rooting took place after additional eight weeks. After that plants were grown for three months in growth chamber and then transferred to greenhouse. The *SG2-3* and *EP155* strains of *Cryphonectria parasitica* were used for leaf assays of transfer of *OxO* to the host plants. Lesion length was measured three days after inoculation. The tissue culture *OxO* expression varied considerably among the 16 *35S-OxO* transgenic lines tested; 200 times difference between the extreme lines. The corresponding difference among the four lines obtained from the other vector was 14 times. The expression among the latter lines was 3,000-fold lower than in the former 16 lines. This strong difference was attributed to difference in strength of the two promoters, the tissue specificity of the *VspB*, and positional effect. The mean necrosis length in the leaf assays of the four *C. dentata* transfer control lines did not differ from the necrosis length in untreated *C. dentata* (Fig. 3-6). Similarly, the necrosis length in the four lines obtained after inoculation with the *VspB-OxO* vector was not different from *C. dentata*. Five of the eight lines obtained from the inoculation with the *p35S-OxO* vector showed a strong resistance, which did not differ from the resistance in *C. mollissima*. The relationship between *OxO* expression and length of necrosis in the eight lines inoculated with the *p35S-OxO* vector was plotted (Fig. 3-7) to trace any relationship between these two factors. A fairly good fit to a third degree polynomial curve is evident. As seen from this figure there seems to be a threshold value of *OxO* expression for achieving tolerance against *C. parasitica*. It is likely that this level was not passed in the lines inoculated by the *VspB-OxO* vector.

One take home message from this investigation is the important role of *OxO* to obtain tolerance against *C. parasitica*.

---

**Figure 3-5.** The relationship between media oxalic acid molarity in cultivation media and percentage of lignin in callus tissue in control and transformed tissue. The binary vector, *pGPOxO*, was used for transformation with the *CaMV35S* promoter. Welch et al. 2007.

**Figure 3-6.** Mean necrosis length in leaf assays after inoculations with *Cryphonectria parasitica* strain *SG2-3* in untreated *C. dentata* (*Cd*), *C. dentata* transfer control (*Cd contr*), transfer with *VspB-OxO* vector, *p35S-OxO* vector with two groups of response (3 and 5 lines, respectively), and *C. mollissima* (*Cm*). The leaves were taken from potted plants. Zhang et al. 2013.

**Figure 3-7.** The relationship between oxalic acid oxidase (*OxO*) expression and necrotic length in transformed lines of *C. dentata* following inoculation with *Cryphonectria parasitica* *p35S-OxO* vector. The dotted line indicates the approximate threshold *OxO* expression for development of resistance against *C. parasitica*. Zhang et al. (2013).
One effort to incorporate blight resistance into *C. dentata* via *Agrobacterium*-mediated gene transfer in tissue cultures was reported by Newhouse et al. (2014). The confirmation of successful transfer was evaluated by two marker genes (*GFP* = green fluorescent protein and *OXO* = oxalate oxidase genes) in the plasmid used for transfer of blight resistant genes. Of particular interest is to verify the number of copies of the resistance genes introduced into the host genome. Real-time PCR technique was used for this purpose. Leaves and stems of regenerated plants were inoculated with *Cryphonectria* to identify degree of blight resistance. Transgenic pollen was collected and used for backcrosses with *C. dentata* females. The expression of the markers was confirmed in embryos as well as in leaves formed from the somatic embryos used in the transformation events. Moreover, the *Oxo* expression remained during the study period of four years but with some reduction over the years. Except for the transformed D1, the leaf assays showed a reduction of the necrosis in the transformed lines (Fig. 3-8). Also the two backcrosses to *C. dentata* were less necrotic than the pure *C. dentata*. Stem inoculations with *Cryphonectria* were carried out two years with two strains characterized by different virulence, SG2 and EP155. I have summarized the results from the canker assessments 14 weeks after inoculation in Fig. 3-9. The difference in virulence is particularly pronounced in 2013 with limited cancer development in all three genetic entries. In the other three occasions there was a clear difference between the transgenic line (T0 = D4) and *C. dentata*. Moreover, the T0 line is closer to *C. mollissima* than *C. dentata*. Fig. 3-9 also reveals that the effect of the inoculation is dependent on the ambient conditions and that *C. mollissima* is not resistant to 100%. The strain D5 did not show any increase of the blight resistance following stem inoculations. Non-significant reduction of blight susceptibility was noted for D1, H1, and H2. It was stated that the transgene oxalate oxidase expression T1 individuals from D4 and H1 was fairly similar to the expression in T0 seedlings. Since nuts are harvested for human consumption a comparison of numerous metabolites in transgenic nuts and non-transgenic nuts was carried out. Only two of them were found to differ, pentoside conjugate of ferulic acid and coumaric acid. These differences were attributed to the timing of the experiment and both are cell wall components. One disadvantage with tissue culture plants is a slow growth and reduced apical dominance compared to seedlings originating from nuts. It was concluded that transgenes are inherited and expressed in seedling offspring, which means that blight resistance can be stable across generations. It is also evident from this investigation that there is genetic variation in the ability to transfer blight resistance and a great step is taken to achieve the goal of blight resistant *C. dentata*.

Cipollini et al. (2017) studied the effect of inoculations in three full-sib third backcross families, one F1, two *C. dentata*, and two *C. mollissima* open-pollinated families. Blight inoculation took place during beginning of the fifth growing season. The material was growing in Floyd County, Georgia, USA; latitude 34.32˚N and longitude 85.25˚W, 259 masl. Pesticide and fungicide treatments were used to avoid attacks by insects and root rot. No symptoms of chestnut blight were noted before the inoculation. On each tree two inoculations were applied with each of two strains of *Cryphonectria*; one of them strongly virulent. Trees with small stem diameters were inoculated once. Blight infection was recorded five months and ten months after inoculation. Three classes of chestnut blight cancer were distinguished following visual inspec-
A blight susceptibility index (BSI) was calculated:

\[
\text{(Average score for virulent strain) + (average score for less virulent strain) - 1}
\]

The range of BSI being 1-5, with 1 = low susceptibility and 5 high susceptibility. Twenty morphological traits, known to differ between the parental species were recorded on undamaged branches growing on the south side of the trees at about two meters above ground. The recorded values were standardized according to the following:

\[
\frac{\text{value} - \text{minimum value}}{\text{maximum value} - \text{minimum value}}
\]

The standardized values were arranged such that \(C.\ dentata\) values were low while high values were related to \(C.\ mollissima\). The index of species identity is the mean of thus standardized values for the different families.

In Fig. 3-10 I have summarized the main results from this investigation. The chestnut blight susceptibility is lowest in the \(C.\ mollissima\) progenies and highest in the \(C.\ dentata\) progenies. The agreement between the species identity indices of the backcross and the pure \(C.\ dentata\) is of greatest interest for restoration of \(C.\ dentata\) in USA.

It was noted that three trees in one of the backcross families were morphologically indistinguishable from pure \(C.\ dentata\) and intermediate with respect to blight resistance. These three trees were vigorous 3.5 years after the inoculation and open-pollination nut crop from them will be harvested. To get an impression of the variation in BSI and ISI Fig. 3-11 was constructed. The low number of trees in \(F_1\) and \(C.\ dentata\) makes the percentages rather uncertain. The figure illustrates that progeny \(B_3\) has a fairly large number of trees with low susceptibility against chestnut blight.

The question whether the blight resistance would be fixed in \(B_3\) families as is expected if resistance is regulated by genes in two independent loci was challenged. This challenge was based on own results as well as previous results. It was speculated that the inheritance is less simple than previously believed. The pathogen may pass several generations during one generation cycle in the host species and new mutations overcoming the resistance may occur. Therefore, from an evolutionary perspective it is more likely that resistance to blight is polygenically inherited since the resistance would easily be overcome if the resistance relies on genes in a few loci. It was stated that understanding of the molecular processes leading to resistance might improve the possibilities to incorporate blight resistance into \(C.\ dentata\).

It was pointed out that besides morphology and blight resistance other traits might be of fundamental importance for successful restoration of American chestnut. One such trait is resistance against \textit{Phytophtora cinnamomi}.
The long-term goal of the transcriptome analysis carried out by Barakat et al. (2009) was to identify and isolate genes underlying resistance to Cryphonectria parasitica. Transcriptomes from healthy and canker cambial tissue in C. dentata and C. mollissima were analyzed. Canker tissues were sampled five and twelve days after inoculation with a hypervirulent strain of Cryphonectria parasitica. These points of time correspond to early and late interaction between fungus and host plant. So called 454 technology was used to construct cDNA libraries.

Of primary interest is to compare differences between healthy and canker tissues in the two species as well as species’ differences. This is done in Table 3-1. In both species it was found that several genes related to resistance were differentially expressed between the two types of tissue. As might be expected genes involved in stress responses occurred to a larger extent in C. mollissima than in C. dentata healthy tissues. The differences between the canker tissues in the two species did not show any fundamental differences. Rather, it seems to be a question of degree of difference between the two species.

Besides, the information gained in relation to resistance against Cryphonectria, this investigation presented results from a comprehensive sequencing of chestnut cDNA.

An extension of the above investigation with a larger dataset was presented by Barakat et al (2012). The col-

### Table 3-1. Comparison of observations in stem tissues in healthy and canker tissues in C. dentata and C. mollissima based on transcriptome analyses. Barakat et al. (2009).

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdm healthy vs Cmm healthy</td>
<td>A larger number of genes involved in response to biotic and abiotic stimuli and stresses were identified in Chinese chestnut tissue compared with American chestnut</td>
</tr>
<tr>
<td>Cdm healthy vs Cdm canker</td>
<td>Several resistance genes differentially expressed</td>
</tr>
<tr>
<td>Cmm healthy vs Cmm canker</td>
<td>Several resistance genes differentially expressed</td>
</tr>
<tr>
<td>Cdm canker vs Cmm canker</td>
<td>A small increase in nucleic acid protein binding and transcription factor molecular functions in Chinese chestnut cankers. In the American chestnut there was a small increase in structural molecule activity. American chestnut canker tissue had a larger fraction of genes involved in stress response</td>
</tr>
</tbody>
</table>

Figure 3-12. Genes with differential transcriptome abundance (GDTA) in canker tissue compared to healthy tissue as well as genes responding to biotic and abiotic stress in canker tissue versus healthy tissues of C. dentata and C. mollissima after inoculation with Cryphonectria parasitica. Barakat et al. 2012.

lecion of material for analyses was carried out five and twelve days after inoculation with a hypervirulent strain of Cryphonectria parasitica. Total RNA and cDNA and 454 libraries were constructed.

The focus of this investigation was on identifying genes with differential transcript abundance (GDTA). Fig.3-12 reveals that a large number of genes with differential transcriptome abundance (GDTA) were identified in both species. Of these genes a considerable number were related to response to biotic and abiotic stress; in C. dentata 1,715 and in C. mollissima 720. Most of the defense-related genes were hydrolases, protein binding, transferases, and transporters. There was an overrepresentation of so called house-keeping genes in C. dentata, which was attributed to increase in protein synthesis as a defense against the pathogen.

In the paper 56 defense-related GDTAs from C. mollissima were listed. It is beyond the scope of this genetic summary to bring up all these GDTAs. I have illustrated the gene expression of two with stronger expression in canker tissue in each of the two species in Fig. 3-13. Especially the laccase 15A and laccase17 show much stronger expression in C. mollissima than in C. dentata while laccase15B and disease-responsive-resistance (DRR) are more strongly expressed in C. dentata. It is tempting to believe that the two former laccase genes play a significant role in resistance against Cryphonectria parasitica. However, as stated in the Abstract of the paper, The similar set of GDTAs in American chestnut and Chinese chestnut suggests that the variation in sensitivity to this pathogen between these species may be the result of different timing and amplitude of the response of the two to the pathogen infection. This kind of study shows the complexity of blight resistance but also shows possible ways to understanding of basic processes leading to disease resistance in chestnuts.
from Fig. 3-15. At least in these three experiments, genotype H3 is the most resistant while B4 and H4 are among the most affected in all experiments. It is regrettable that no models for the ANOVAs were presented and that no clear information on the significances of different effects were presented. The design of the experiments and the obtained results would have deserved a clear presentation of the statistical evaluation.

Figure 3-14. The mean necrotic area in branch segments from eight C. sativa x C. crenata genotypes, 13 - H4, following inoculation with three strains of Cryphonectria parasitica. The inoculations took place on June 29 2010, April 12 2011, June 22 2011, and July 13 2012. All genotypes were not studied at every occasion. When there is a gap for one genotype there is a gap in the connecting lines. Bolvanský et al. 2014.

from Fig. 3-15. At least in these three experiments, genotype H3 is the most resistant while B4 and H4 are among the most affected in all experiments. It is regrettable that no models for the ANOVAs were presented and that no clear information on the significances of different effects were presented. The design of the experiments and the obtained results would have deserved a clear presentation of the statistical evaluation.

Figure 3-15. The relative necrotic area in branch segments from six C. sativa x C. crenata genotypes, H4 - H3, following inoculation with three strains of Cryphonectria parasitica. The inoculations took place on April 12, 2011. Bolvanský et al. 2014.
Branches were collected in April and September. The tested trees were previously classified with respect to susceptibility against chestnut blight. The necrotic areas following branch inoculations were used as controls. The least square mean necrotic area following inoculation is April. However, a larger material must be used to obtain reliable relationships with field data.

The necrotic areas in bark and wood following May inoculations showed the same pattern as above, i.e. the strain M6776 was less virulent than the two other C. parasitica strains. There was limited similarity among the three October inoculations but the agreement with field classification was poor in all three treatments.

It was concluded that the most suitable time for artificial inoculation is April. However, a larger material must be studied to obtain reliable relationships with field data.

Crown defoliation caused by Cryphonectria parasitica of interspecific hybrids C. sativa x C. crenata, and reciprocal, was assessed in a Romanian trial by Chira et al. (2018). C. sativa seedlings were used as controls. The most active cankers were inoculated with CHV1 virus in 2014 with the expectation to overcome the blight infections.

The mean crown defoliation was 15% (range 11-26) for the seven C. crenata x C. sativa families and for the two reciprocal OP families it was 19.5% (range 16-23). The five open pollinated families of C. sativa had a mean value of 54%, with a wide range of percentages 22-83. After the inoculation with the CHV1 virus in 2017 the mean crown defoliation for all interspecific families had dropped to 6.3% with seven of the nine families having percentages <6%. The mean percentage of defoliation for the five OP C. sativa families was 9.8%. One C. crenata x C. sativa family deviated much from the other interspecific families with high defoliation percentage both before and after treatment with CHV1 virus, 26 and 17%, respectively. Although this study was not designed for a statistically evaluation of differences in susceptibility against C. parasitica there are signs that genetic differences exist.
Also the pathogen might harbor genetic differences. This was studied by Milgroom and Lipari (1995) who collected canker tissue from *Cryphonectria parasitica* infection in 13 populations in eastern USA; two populations (abandoned orchards) were outside the natural range of *C. dentata*. At two localities in the state of New York, two populations with different history were collected. One at each locality was an understory population, while the other population was growing after the area was clearcut or burned. Six RFLP loci were used for the study of population differentiation of this fungus. The isolation by distance was estimated for nine of the eastern populations. Since most populations from the natural range of distribution were understory populations one estimation of population differentiation was limited to nine populations. Separate estimates were presented for the two populations outside the natural range of chestnut and for all 13 populations. The following $G_{st}$ estimates were obtained:

- 9 populations: 0.20
- 13 populations: 0.31
- 2 orchards: 0.81

It should be noted that there was a large difference of the $G_{st}$ estimates for individual loci. The genetic differentiation of the fairly recently introduced *Cryphonectria parasitica* to USA was expected to be limited. The observed $G_{st}$ of 0.20 was attributed to genetic drift. The high estimate for the abandoned orchard populations was attributed to extreme difference in gene frequencies in two loci, absence in one population and fixation in the other population. Besides, these populations are clonal populations with unknown origin, which might contribute to large differentiation.

There were no indications of isolation by distance in spite of the high $G_{st}$ in the nine populations. Two explanations for this observation were presented:

- Long-distance migration occurs and in this way prevents isolation by distance
- Migration occurs mainly over short distances preventing reaching of equilibrium gene frequencies.

The authors warned for far-reaching interpretations of gene flow since it is not possible to separate historic events from current gene flow.

### 3.3 Phytophthora

The susceptibility of *C. sativa* to *Phytophthora* species raised an interest in hybridization with Japanese chestnut, *C. crenata* and Chinese *C. mollissima*, which are tolerant against Phytophthora-caused diseases. The species hybrids are less drought tolerant and less vigorous than *C. sativa*.

One study of variation in resistance against *Phytophthora cinnamomi*, estimated after inoculation of excised shoots, was presented by Fernandez-Lopez et al. (2001). Thirty-one clones were included in this study, among them 13 *C. crenata* x *C. sativa* and eight pure species clones. Two strains of inoculum were used, one local and one well described strain. Inoculations were carried out in June and September 1996 and 1997 and June 1998. Evaluation took place 14 or 21 days after inoculation when lesion lengths were determined. The local strain caused the largest damage. The interaction clone x isolate increased over the period 1996-1998, which explained the decline of the clonal heritability (repeatability) over time. Based on the joint analysis of all treatments the clonal heritability for inoculations with the local strain was estimated at 0.81, while the corresponding for the other strain just half of that, 0.41. It was pointed out that the most resistant clones did not change rank dependent on the isolate used in the test. The genetic correlations between the evaluations were strong, 0.72-0.85. Finally, the three *C. sativa* clones were all susceptible contrary to the five Asian clones which were resistant.

Inoculation of 27 interspecific hybrids, *C. sativa* x *C. crenata* and *C. sativa* x *C. mollissima* with *Phytophthora cinnamomi* were carried out by Guedes-Lafargue et al. (2005). The evaluation took place ten days after inoculation by measurement of the lesion length. The lesion lengths varied in the range 53-163 mm and the differences were significant. The two most resistant clones will be evaluated with respect to nut and growth traits.
Genetic variation in tolerance against *Phytophtora cambivora* among 23 *C. sativa* populations from nine localities in France, Greece, Italy, Spain, and UK (Fig. 3-18) was studied by Robin et al. (2006). Three types of populations or domestication levels were included; natural forest, coppice, and orchards. All types of populations were not available for testing at all localities. The testing took place in France, Greece, and Italy. Progenies from one hybrid clone and one *C. crenata* clone were included in the testing as resistant materials. This study comprised two types of testing; inoculation of excised shoots and root inoculation. Branches with a diameter of at least 1 cm and up to 100 cm long were collected two weeks before flushing and used for excised shoot testing. Sections 40 cm long were placed in jars with water and placed in a growth chamber at 20°C at 12 hour light. Inoculation took place with *Phytophtora cambivora* isolate P15FC2 after bud flushing. The English and French materials were tested in France, the Italian and Spanish materials were tested in Italy, and the Greek material was tested in Greece. Lesion length was determined seven days after inoculation.

Table 3-2. Number of *C. sativa* populations and trees analyzed for *Phytophtora cambivora* infection estimated as stem lesion lengths. The strength of the significances is shown. Robin et al. 2006.

<table>
<thead>
<tr>
<th>Origin</th>
<th>No. populations</th>
<th>No. trees</th>
</tr>
</thead>
<tbody>
<tr>
<td>France and UK</td>
<td>8 ***</td>
<td>195 ***</td>
</tr>
<tr>
<td>Italy and Spain</td>
<td>8 ***</td>
<td>173 ***</td>
</tr>
<tr>
<td>Greece</td>
<td>5 ***</td>
<td>106 ***</td>
</tr>
<tr>
<td>Coppice France and UK</td>
<td>4 ***</td>
<td>101 ***</td>
</tr>
<tr>
<td>Natural populations Italy and Spain</td>
<td>3 ***</td>
<td>66 ***</td>
</tr>
<tr>
<td>Orchards Italy and Spain</td>
<td>3 ***</td>
<td>66 ***</td>
</tr>
</tbody>
</table>

Nuts were collected for the production of seedlings for the root inoculation experiments. Inoculation of roots took place with isolate P15FC2 at a seedling height of 20 cm and with lignified basal stems. Percentage of infected tap roots was determined on each seedling. Fraction of infected tap roots (FIT) was recorded as well as four growth parameters. FIT was preferred to plant mortality since it was more discriminating than mortality.

The main results as regards excised shoot tolerance against *P. cambivora* are shown in Fig. 3-18. Since the tests were carried out in three laboratories a direct comparison across laboratories is not straightforward. Therefore, the results from each laboratory were analyzed separately. Even if testing took place in different laboratories it is evident that the two coppice populations from UK are most susceptible. It was suggested that the two populations from UK had not been exposed to this pathogen earlier while the French populations had been exposed to *P. cambivora* and had adapted to some extent to the presence of this pathogen. No adaptation to the pathogen had taken place in UK. Contrary to this, *P. cambivora* had not been detected in Sicily and the susceptibility of the three Sicilian populations was lowest in this study. It was speculated that the low susceptibility of the Sicilian hybrid clone and one *C. crenata* clone were included in the testing as resistant materials. This study comprised two types of testing; inoculation of excised shoots and root inoculation. Branches with a diameter of at least 1 cm and up to 100 cm long were collected two weeks before flushing and used for excised shoot testing. Sections 40 cm long were placed in jars with water and placed in a growth chamber at 20°C at 12 hour light. Inoculation took place with *Phytophtora cambivora* isolate P15FC2 after bud flushing. The English and French materials were tested in France, the Italian and Spanish materials were tested in Italy, and the Greek material was tested in Greece. Lesion length was determined seven days after inoculation.

Figure 3-18. Lesion length one week after inoculation of excised shoots with *Phytophtora cambivora* in *C. sativa* populations from France, Greece, Italy, Spain, and UK. NF = natural forest, O = orchard, C = coppice forest. The testing took place in three laboratories. The French and UK materials were tested in France; Italian and Spanish materials were tested in Italy, while Greek populations were tested in Greece. The significances among the populations of different domestication levels are indicated; nt = not tested. Robin et al. 2006.

Table 3-3. Number of *C. sativa* populations and OP families analyzed for *Phytophtora cambivora* infection estimated as percentage of infected taproots. The strength of the significances is shown. Robin et al. 2006.

<table>
<thead>
<tr>
<th>Origin</th>
<th>No. populations</th>
<th>No. OP families</th>
</tr>
</thead>
<tbody>
<tr>
<td>France and UK</td>
<td>8 *</td>
<td>41 *</td>
</tr>
<tr>
<td>Italy and Spain</td>
<td>10 ***</td>
<td>73 ***</td>
</tr>
<tr>
<td>Greece</td>
<td>5 *</td>
<td>28 ***</td>
</tr>
</tbody>
</table>

Table 3-3. Number of *C. sativa* populations and OP families analyzed for *Phytophtora cambivora* infection estimated as percentage of infected taproots. The strength of the significances is shown. Robin et al. 2006.
orchard population could be attributed to a wide origin of the clones in this orchard.

With one exception, the Italian population from Valle Pellice, there were significant differences among the domestication levels at each locality (Fig. 3-18). The coppice populations from Italy were more affected than the other two types of domestication level. In the French material the two orchard populations were more affected than the other two domestication levels. Table 3-2 reveals that all the ANOVAs reported showed strongly significant differences among populations and trees. The three Sicilian populations had lower lesion length than the corresponding populations from Galicia in Spain and Valle Pellice in northern Italy (Fig. 3-18 and Table 3-3). It was noted that ranking of the domestication levels were not consistent among localities. Forty-eight trees from the six French populations were inoculated a second time. The relationship was strongly significant but it explained less than 35% of the variation. (Two figures were given for the Pearson correlation coefficient 0.58 and 0.48; probably a misprint of one of them.)

The progenies from the control clones had FIT of 0.15 or less while only three of the tested families had 0.15 or less infected taproots. Among the families in the French trial FIT varied mainly in the range 0.4-1.0 while the corresponding range for the Italian and Greek laboratory tests was 0.2-0.8. Table 3-3 reveals that there were significant differences for FIT both at population and family levels. The large variation in FIT is encouraging for improvement of tolerance against *P. cambivora*.

The relationships between family means for lesion length of excised shoots and the means for FIT were weak for the materials from all three laboratories. It was significant once for the families tested in Greece but the degree of explanation for this relationship was less than 15%. This means that lesion length of excised shoots does not predict FIT. It was speculated that this lack of agreement between the two ways of estimating tolerance against *P. cambivora* was due to the complexity of the mechanisms involved in susceptibility or resistance and by the fact that different components were assessed in the mother tree and its progeny. The different inoculation techniques for shoot and root inoculation might be one contributing factor for the disagreement between root and shoot inoculations.

The relationships between FIT and five other traits in the seedlings included in the root inoculation study were estimated. The relationships with leaf dry weight and root dry weight were all weak. The strongest relationships were noted for stem lesion length, $r = 0.61-0.62$ (Fig. 3-19). In the French and UK populations there was a moderately strong relationship with stem diameter increment, $r = -0.61$. 

**Figure 3-19. OP family mean correlations between fraction of taproot infection of Phytophtora cambivora in French, Greek, Italian, Spanish, and UK populations of *C. sativa* and three traits assessed in the root inoculated seedlings. SLL = stem lesion length, SDI = stem diameter increment, SHI = shoot height increment. Robin et al. 2006.**
Three experiments with *Phytophthora cinnamomi* inoculations were reported by Miranda-Fontaina et al. (2007). A summary of the three experiments is given in Table 3-4. The large variation for the trait, percentage circumference of collar rot, is illustrated in Fig. 3-20. The continuous variation shown in this figure suggests that there is a polygenic inheritance of resistance against root rot. Interestingly, one of the *C. sativa* clones is one of the least susceptible clones in this experiment. As expected the two Asian clones belong to this category as well. Except for the root rot percentage, the other four traits showed strongly significant differences among clones. This is reflected in the high heritabilities of most traits with small standard errors (0.04 – 0.05) of the estimates (cf. Fig. 3-21). There were significant differences between the isolates for the three traits involving collar rot. Unfortunately, no data on this difference were presented. Except for collar rot length, the interaction clone x isolate was non-significant.

As percentage of circumference collar rot was regarded as the best trait to identify resistance against *Phytophthora cinnamomi* I selected to illustrate the genotypic correlations between this trait as well as growth traits (Fig. 3-22). As seen from this figure the correlations among the root-related traits are all strong and positive while there are negative relationships with the dry weight traits and percentage survival. It is evident that the infection had a negative impact on growth and survival. Experimental shortcomings might be responsible for the coefficients exceeding 1.00. If the estimates of resistance obtained in the experiments with excised and intact stems are strongly correlated with the five resistance traits obtained

![Figure 3-20. Mean clonal value for the percentage circumference of collar rot of 50 clones tested for resistance against *Phytophthora cinnamomi* based on visual examination of roots 14 weeks after inoculation. 44 *C. crenata x C. sativa* clones (red), 4 *C. sativa* clones (blue), 1 *C. mollissima x C. sativa* clone (green), and 1 *C. crenata* clone (yellow). Miranda-Fontaina et al. 2007.](image)

Table 3-4. Number of *C. sativa* clones included in the three experiments, traits, and parameters estimated in the statistical analysis of the results. Miranda-Fontaina et al. 2007.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. clones and isolates of <em>Phytophthora</em></th>
<th>Traits</th>
<th>Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil experiment</td>
<td>50; mainly <em>C. sativa x C. crenata</em> hybrids. 2 isolates of <em>Phytophthora</em></td>
<td>1-2. Symptoms of root and collar rot visually assessed</td>
<td>ANOVA, Broad sense heritability, Genetic and phenotypic correlations</td>
</tr>
<tr>
<td>Inoculation of apex of excised stem</td>
<td>51; 2 isolates of <em>Phytophthora</em>, 33 clones common with soil experiment</td>
<td>3-4. Root rot %, collar rot %</td>
<td></td>
</tr>
<tr>
<td>Inoculation of apex of intact stems</td>
<td>30; 1 isolate of <em>Phytophthora</em>; 20 clones common with soil experiment</td>
<td>5. Level of root rot; scale 0-5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6. % circumference of collar rot</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>7. Collar rot length</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>8. Leaf persistence; scale 0-10</td>
<td></td>
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<td></td>
<td></td>
<td>9. Height growth, collar growth</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>10. Dry weight of plant parts</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>11. % survival</td>
<td></td>
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<td></td>
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<td>Evaluation 14 weeks after inoculation</td>
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</table>
in the soil experiment it would be possible to rely on the simpler and less costly above ground experiments. Fig. 3-23 reveals that there are strong correlations for the experiment including intact stems while the relationships for four of the traits from the experiments with excised shoots were moderately strong. This investigation shows clearly that high tolerance against Phytophtora cinnamomi might be obtained by hybridization of C. sativa with C. crenata but progeny testing is required to identify the tolerant families. The continuous variation in tolerance is striking for the three traits presented in this paper. The high repeatabilities indicate that selection for tolerance might be rewarding.

Interspecific American Chinese chestnut hybrids or backcrosses were used for identification of QTL for resistance against Phytophtora cinnamomi by Zhebentyayeva et al. (2014). Four classes of disease severity of rot were used and three classes for above-ground infection. Two QTL were detected in the first analysis explaining 40 and 34% of the variation in Phytophtora resistance. An additional number of full-sib families were scored for resistance and in eight of them there were a few with total resistance and several more with low susceptibility. DNA typing was under way in 2012. Work was going on to obtain larger progenies than the 48 individuals in the first hybrid family.

Santos et al. (2015a) developed EST-SSR markers for tests of resistance against Phytophtora cinnamomi. Eighteen pure species trees (6 American 3 Chinese, 6 European, and 3 Japanese chestnuts) from breeding programs in Europe and USA were included in this study. Besides, six backcross trees from interspecific Asian x American hybrids were included. Root transcriptomes from inoculated
and non-inoculated European and Japanese chestnut were used as four sources for development of SSRs from EST. Differentially expressed genes (DEGs) in these four materials were identified and two types of candidate genes for resistance against *P. cinnamomi* were selected based on the comparisons:

Inoculated and non-inoculated Japanese chestnut
Inoculated and non-inoculated European chestnut

In the EST sequences for resistance against *P. cinnamomi* 99 SSRs were found, 43 of them in 305 European chestnut ESTs, the corresponding figures for Japanese chestnut was 56 and 283. Of the 58 SSRs selected for testing of PCR reaction, 43 had unique amplification products. The mean number of alleles per locus was 5.3; with three loci being monomorphic. For the 40 polymorphic loci the expected and observed heterozygosities were 0.61 and 0.39, respectively. The difference between expected and observed heterozygosities was not discussed. A dendrogram based on genetic distances resulted in five main clusters. Generally the hybrids were differentiated from the pure species. Twenty-five of the SSRs were amplified in all four species.

The authors concluded that these SSR markers may be used for

- marker-aided selection in breeding programs and for making the marker-aided selection more rapid and accurate,
- development of universal and gene-specific markers for genome synteny studies.

This is one of the first studies, in which functional SSR markers were developed, i.e. SSR markers inside expressed units in the genome.

Resistance to *Phytophthora cinnamomi* of hybrids between *C. sativa* (*Cs*) and *C. crenata* (*Cc*) or *C. mollissima* (*Cm*) was reported by Santos et al. (2015b). Inoculations of roots and excised shoots were carried out with a strongly virulent strain of the pathogen. Four *F₁* Cs x Cm and 16 F₁ Cs x Cc hybrids were included in the root inoculation study. The corresponding numbers for shoot inoculations were 18 *F₁* Cs x Cm and 45 *F₁* Cs x Cc genotypes. *In vitro* clonal propagation was used to get replications in the tests. Five characteristics were used to evaluate the effect of the root inoculations:

- Days of survival
- Root rot level
- Percentage of root color rot
- Percentage of shoot internal lesion
- Percentage of shoot external lesion

The inoculations of shoots took place at two occasions, spring (47 clones) and autumn (60 clones). Some clones were common to the root inoculations. From Table 3-5, in which the significances for different effects are summarized, the strongly significant effect of treatment for all five traits used for evaluation of resistance to *Phytophthora cinnamomi* is evident. No clone or interaction effect was noted for shoot external lesion while both clone and interaction effects were strongly significant for number of days for survival. The latter trait was regarded as most useful for estimation of ink disease resistance. The large variation for this trait is illustrated in Fig. 3-24. The number of clones is too low to evaluate any differences between the two types of interspecific

<table>
<thead>
<tr>
<th>Clone</th>
<th>Days of survival</th>
<th>Level of root rot</th>
<th>Level of root collar rot</th>
<th>Shoot internal lesion</th>
<th>Shoot external lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>***</td>
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<tr>
<td>Clone x treatment</td>
<td>***</td>
<td>***</td>
<td>**</td>
<td>***</td>
<td>ns</td>
</tr>
</tbody>
</table>

Figure 3-24. Number of days of survival after inoculation with *Phytophthora cinnamomi* of clones of *C. sativa* x *C. crenata* and *C. sativa* x *C. mollissima* *F₁* hybrids. Recording ended at day 100 after inoculation. Santos et al. 2015b.
hybrids. As expected the days of survival was negatively correlated with the four other traits; the range being -0.86 - 0.97. There was a moderately strong and positive correlation between days of survival and each of root dry weight and leaf and shoot dry weight. Thus, good growth seems to have a positive impact on resistance against Phytophtora cinnamomi infection.

The clonal differences of lesion growth rate after shoot inoculations were strongly significant for both occasions of inoculation and points of time for assessment. The largest range of estimates was noted for assessments five days after inoculation. As seen from Fig. 3-25 there is a tendency of less susceptibility in the C. sativa x C. mollissima than the other species hybrid. Estimates of coefficients for additive genetic variance and narrow-sense heritabilities were presented. However, clonal variance component and clonal repeatabilities are more appropriate for the results in Table 5 of the paper. Fig. 3-26 reveals high clonal variance components and repeatability for days of survival. An extremely low variance component was noted both for level of root rot and shoot external lesion. In spite of this, the clonal repeatabilities were moderate, which means that the residual variance was exceptionally low (repeatability = [clonal variance/(clonal variance + residual variance)]. These results had deserved a thorough discussion.

Seventeen clones were common to the root and shoot inoculations, which enabled calculations of correlations between the two types of test. There was a strong negative relationship between days of survival in root inoculation test and lesion growth rate in the shoot inoculation test (Fig. 3-27). This is favorable for breeding since the costly root inoculation tests can be substituted for inoculation of shoots. Except for shoot external lesion, the other traits were moderately strongly correlated in the two types of test.

In a follow-up paper Santos et al. (2017b) reported the detection of two QTL for Phytophtora cinnamomi resistance. Two unrelated interspecific C. sativa x C. crenata hybrid families with 52 and 81 seedlings were used for development of maps. In all 452 markers, 180 SSRs and 272 SNPs, were used for mapping.
Table 3-6. Differentially expressed genes (DEG) in inoculated and control tissue of C. crenata and C. sativa as well as up- and down-regulated contigs. Serrazina et al 2015.

<table>
<thead>
<tr>
<th></th>
<th>DEGs</th>
<th>Up-regulated</th>
<th>Down-regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. crenata</td>
<td>283</td>
<td>229</td>
<td>44</td>
</tr>
<tr>
<td>C. sativa</td>
<td>305</td>
<td>117</td>
<td>188</td>
</tr>
</tbody>
</table>

The consensus genetic linkage map consists of 12 linkage groups with 217 markers but there were additional four linkage groups consisting of 25 markers. In spite of the continuous variation in resistance (cf. Fig. 3-24) QTL were detected in linkage groups E and K and they explained 9 and 13% of the variation. One covered a considerable length of the chromosome, 8 cM, while the other was estimated at 1 cM. The E-QTL contained genes involved in:
- resistance via a specific protein hormone signaling,
- transport of phospholipids
- regulation of gene expression
while the K-QTL harbored genes involved in:
- gene expression by alteration of chromatin structure
- cellulose synthesis
- ribosomal protein
- genes with unknown functions.
Thus, a variety of functions, some of which may confer resistance against Phytophthora cinnamomi attacks.

Transcriptome analysis following inoculation with Phytophthora cinnamomi of C. crenata and C. sativa micropropagated plants was carried out by Serrazina et al. (2015). At inoculation the 18 plants of each species were five years old. Half of them were inoculated and the other half was used as control plants. A hypervirulent strain of P. cinnamomi was used. A 5% solution of the inoculum was added to the substrate of the plants to be inoculated. Genes showing higher or lower expression following inoculation compared to the non-inoculated material were regarded as differentially expressed genes (DEG). For each of the species two cDNA libraries were constructed, one for inoculated and the other for control materials. There were large similarities between the Castanea contigs and proteomes of Arabidopsis thaliana (24%), Vitis vinifera (19%), and Populus trichocarpa (15%). A large number of differentially expressed genes in controls and inoculated tissues was noted (Table 3-6). The proportion of up-regulated contigs was larger in C. sativa than in C. crenata. There were numerous differentially expressed genes in this study. In order not to be too detailed about functions of different genes I have preferred to compile what was referred to as Phytophthora cinnamomi resistance-related genes in Table 3-7. The number of genes involved in resistance hints at complex inheritance of Phytophthora resistance.

Three important observations were noted in this investigation:
1. Downregulation of many genes in the susceptible C. sativa
2. A higher number of genes related to biotic stress in C. crenata; most of them upregulated.
3. Both species recognize the pathogen attack

With the large number of transcripts related to resistance it is likely that resistance is polygenically inherited.

Expression profiles of eight genes before and after inoculation of four taxa (1 C. sativa, 1 C. crenata, 3 C. sativa x C. crenata, 1 C. sativa x C. mollissima) with varying susceptibility against Phytophthora cinnamomi were presented by Santos et al. (2017a). Among the 283 differently expressed genes in the above publication eight were selected for a detailed investigation. These genes should represent the three layers of defense against fungal attacks:
1. Means to avoid attacks in form of morphology or antifungal defense around the roots
2. Recognition of the parasite
3. Activation of host resistance genes

The expression of the genes was presented as copy number per microliter, Cn/µL.

All C. sativa plants died one week after treatment while 83% of the C. crenata plants survived. The hybrid with C. mollissima was the most resistant with 46% survival. I
have preferred to illustrate the development of expression for three genes in Figs 3-28 - 3-30. For all three genes there is a clear difference between the pure species before the inoculation with Phytophthora cinnamomi. For *Cast_Gnk-2-like* gene there is a pronounced increase over time in three of the interspecific hybrids while the fourth hybrid and *C. sativa* seedlings did not show any increased gene expression. This gene is believed to have an antifungal activity and it was suggested that it might prevent pathogen growth owing to its antifungal properties or by inducing cell death. *Cast_PE-2* might have a role of enhancement of cell wall quality and in this way obstruct fungal penetration. Most of the taxa increased their expression over the 48 hours (Fig. 3-29). The *C. sativa x C. mollissima* deviated from this pattern but its starting value was highest of all estimates.

It was suggested that the *Cast_LRR-RLK*-gene influences the recognition of the pathogen and an early recognition is part of the resistance against Phytophthora infection. The expression in *C. crenata* did not change during the test period but it was the only taxon with high expression at the start of the experiment (Fig. 3-30). Two of the *C. sativa x C. crenata* hybrids had low expression at the three sampling occasions. They were classified as susceptible or had intermediate susceptibility.

The pattern of development of the expression of the five other genes showed an erratic pattern without any clear relationship to susceptibility or resistance against *Phytophthora*.

Based on the results a hypothetical response mechanism in *Castanea* for attacks by *Phytophthora cinnamomi* was presented, i.e. a crucial role of the *Cast_Gnk-2-like* gene. It was suggested that the first layer of defense was decisive for obtaining resistance. The lower expression in *C. sativa* of the eight genes studied as well as their delayed expressions might be the reason for the susceptibility of *C. sativa*. Since the two Asian chestnuts have coexisted with *Phytophthora cinnamomi* for generations it is likely that selection for resistance against this pathogen has taken place over generations. The task for breeders is to identify these genes and transfer them into the susceptible chestnut species.

**Figure 3-28.** *Cast_Gnk-2-like* gene expression in four types of progeny following inoculation with Phytophthora cinnamomi.

Cs = Castanea sativa, Cc = Castanea crenata  
Cm = Castanea mollissima, CsxCm = interspecific hybrid between C. sativa and C. mollissima  
CsxCc = interspecific hybrid between C. sativa and C. crenata.  
Santos et al. 2017a.

**Figure 3-29.** *Cast_PE-2*-gene expression in four types of progeny following inoculation with Phytophthora cinnamomi.  
Cs = Castanea sativa, Cc = Castanea crenata  
Cm = Castanea mollissima, CsxCm = interspecific hybrid between C. sativa and C. mollissima  
CsxCc = interspecific hybrid between C. sativa and C. crenata.  
Santos et al. 2017a.

**Figure 3-30.** *Cast_LRR-RLK*-gene expression in four types of progeny following inoculation with Phytophthora cinnamomi.  
Cs = Castanea sativa, Cc = Castanea crenata  
Cm = Castanea mollissima, CsxCm = interspecific hybrid between C. sativa and C. mollissima  
CsxCc = interspecific hybrid between C. sativa and C. crenata.  
Santos et al. 2017a.
An investigation with the objective to estimate genetic parameters for *Phytophtora cinnamomi* resistance was carried out by Lopez-Villamor et al. (2018). In a partial diallele mating scheme 25 full-sib families (These families were included in other studies as well) were obtained and multiplied vegetatively to get six cuttings per ortet. The following types of crosses were included:

- **F₁-hybrids backcrossed with** *C. crenata* or *C. mollissima* 4 families
- **F₁-hybrids backcrossed with** *C. sativa* 12 families
- **Hybridization** *C. sativa x C. crenata* or *C. mollissima* 4 families
- **F₂ from the original cross** *C. sativa x C. crenata* 1 family
- **Crosses between** *C. sativa parents* 4 families

The parents in the crosses were selected for different traits. Obviously *Phytophtora* resistance was one criterion; wood quality, nut quality, and vigor were other criteria. Inoculations were carried out with chlamydospore suspensions into the pots, in which the cuttings were growing. Complete randomization was used as experimental design. Evaluations of damage were carried out 15 and 46 days after inoculation. Five classes of foliar damage, root collar damage and root necrosis were used.

In Fig. 3-31 I have illustrated the variance components for general combining ability, GCA, specific combining ability, SCA, and for clone in a Spanish chestnut progeny trial with 25 full-sib families. Each seedling was vegetatively propagated. Fs1 and Fs2 = foliar damage, Rcd1 and Rcd2 = root collar damage, Rn2 = root necrosis. 1 and 2 stand for evaluations 15 and 46 days after inoculation with *Phytophtora cinnamomi*. López-Villamore et al. 2018.

![Figure 3-31](image)

**Figure 3-31.** Variance components for general combining ability, GCA, specific combining ability, SCA, and for clone in a Spanish chestnut progeny trial with 25 full-sib families. Each seedling was vegetatively propagated. Fs1 and Fs2 = foliar damage, Rcd1 and Rcd2 = root collar damage, Rn2 = root necrosis. 1 and 2 stand for evaluations 15 and 46 days after inoculation with *Phytophtora cinnamomi*. López-Villamore et al. 2018.

In a partial diallele mating scheme 25 full-sib families (These families were included in other studies as well) were obtained and multiplied vegetatively to get six cuttings per ortet. The following types of crosses were included:

- **F₁-hybrids backcrossed with** *C. crenata* or *C. mollissima* 4 families
- **F₁-hybrids backcrossed with** *C. sativa* 12 families
- **Hybridization** *C. sativa x C. crenata* or *C. mollissima* 4 families
- **F₂ from the original cross** *C. sativa x C. crenata* 1 family
- **Crosses between** *C. sativa parents* 4 families

The parents in the crosses were selected for different traits. Obviously *Phytophtora* resistance was one criterion; wood quality, nut quality, and vigor were other criteria. Inoculations were carried out with chlamydospore suspensions into the pots, in which the cuttings were growing. Complete randomization was used as experimental design. Evaluations of damage were carried out 15 and 46 days after inoculation. Five classes of foliar damage, root collar damage and root necrosis were used. In Fig. 3-31 I have illustrated the variance components for GCA, SCA, and clones. It should be remembered that these estimates may be exaggerated owing to selection of extreme parents. Another concern is the imbalance in the mating design. The components for clones are much higher than GCA and SCA and the latter are smaller than the GCA components. It was concluded that additive gene action is of significance for resistance to *Phytophtora*. However, the ratio $V_D/V_A$ varied in the range 0.47-0.86, which indicates that dominance has a considerable impact on some traits (foliar damage). To illustrate that the family x disease interaction played a role in this investigation I have illustrated the breeding values for all eight families, in which one *C. crenata x C. sativa* was one parent (tree H1, Fig. 3-32). The breeding values show a broad range -0.47 -+0.83. In addition, I have illustrated the lowest and highest estimates for each type of full-sib family. The high susceptibility of the four purely *C. sativa* families is evident from Fig. 3-33. One half of the *F₁ x C. sativa* had positive breeding values and one family had the highest breeding value of all families. Unfortu-

![Figure 3-32](image)

**Figure 3-32.** Breeding values for one parent in eight full-sib families based on 25 full-sibs in a partial diallele mating. H1 and H2 are interspecific hybrids; C. crenata x C. sativa. Root necrosis was evaluated 46 days after inoculation with *Phytophtora cinnamomi*. Cc = C. crenata, Cm = C. mollissima, Cs = C.sativa. López-Villamore et al. 2018.

![Figure 3-33](image)

**Figure 3-33.** Breeding value for root necrosis 46 days after inoculation with *Phytophtora cinnamomi* based on 25 full-sib families in a partial diallele mating. The lowest (blue) and highest (green) breeding values for each type of progeny are shown. $F₁ = $ interspecific C. sativa x Asian chestnuts, $F₂ = F₁ x F₁$. As = Asian chestnuts C. crenata or C. mollissima, Cs = C.sativa. The number of crosses of each type is given López-Villamore et al. 2018.
Figure 3-34. Genetic correlations between foliar damage and root damage traits in a Spanish chestnut progeny trial with 25 full-sib families. Fd1 and Fd2 = foliar damage, Rcd1 and Rcd2 = root collar damage, Rn2 = root necrosis. 1 and 2 stand for evaluations 15 and 46 days after inoculation with Phytophthora cinnamomi. Note the scale 0.50-1.00. López-Villamore et al. 2018.

nately the *C. sativa* parent in this mating showed a negative breeding value in the other backcross, in which it was included. The genetic correlations among the five traits had all coefficients above 0.50 (Fig. 3-34). Especially, the correlations within traits, foliar damage and root damage had high estimates of the correlation coefficients.

### 3.4 Insects

The interaction between an insect herbivore, *Lymantria dispar*, and chestnut blight susceptibility was studied by Rieske et al. (2003). Blight susceptible seedlings of *C. dentata* and blight resistant *open-pollinated second back cross* seedlings were used. In addition to herbivore feeding traits, seedling growth and foliar content was studied. The caterpillars used in the herbivore feeding assay were starved for 24 hours before individual exposure to fresh leaves in boxes. The following traits were calculated:

- Relative Growth Rate; RGR = caterpillar biomass gained/initial caterpillar weight x time
- Relative consumption rate; RCR = amount of leaf material consumed/initial caterpillar weight x time
- Duration of the fourth larval stadium
- Foliar nonstructural carbohydrates, tannins, and nitrogen in leaves were determined. To promote the uptake of nutrients, inoculation of roots with the mycorrhiza forming fungus *Pisolithus tinctorius* was carried out. One treatment with nitrogen fertilization was included in this investigation as well as an untreated control. Seedling height and basal diameter were measured.

For herbivore performance the multivariate analysis revealed a strongly significant difference (p = 0.001) between the American chestnut and the backcross progenies. Herbivore performance differed between the two progenies as regards soil condition (p=0.01). There was also a strong difference between the two types of progeny in seedling growth. These results are reflected in Figs. 3-35 and 3-36, in which least square means are shown for three traits. Below I have summarized the results of the statistical evaluation of effects on individual traits in the three treatments (mycorrhiza, N-fertilization, and control):

- RGR ns Carbohydrates ***
- RCR ns Nitrogen *
- Development time *** Tannic acid ns
- Height ns Basal diameter **

The least square means of the two traits showing the strongest significances are illustrated in Fig. 3-35, which shows that the backcross has lower estimates in all six comparisons.

No mycorrhiza formation was observed in the visual microscopic inspection. It is somewhat surprising that the nitrogen fertilization had no or limited impacts on percentage of carbohydrates in the back cross family. Contrary to this, N fertilization had a strong impact on basal diameter (Fig. 3-36). *C. dentata* showed better growth in all pairwise comparisons.

Figure 3-35. Least square means for development time of the fourth larval stadium of *Lymantria dispar* and percentage of carbohydrates in *C. dentata* seedlings and *C. dentata x C. mollissima* back cross seedlings with *C. dentata*. I = inoculation with *Pisolithus tinctorius*, N-fertilization (N), C = untreated control. Rieske et al. 2003.

Figure 3-36. Least square means for basal diameter of *C. dentata* seedlings and *C. dentata x C. mollissima* back cross seedlings with *C. dentata*. I = inoculation with *Pisolithus tinctorius*, N-fertilization (N), as well as untreated control (C). Rieske et al. 2003.
To estimate the impact of treatment on insect response a multivariate canonical correlation analysis was performed. In this analysis a linear combination of the three foliar variables (carbohydrate, nitrogen, and tannin) correlated strongly with similar linear combination of the response traits (RGR, RCR, development time). However, the degree of explanation of this relationship was only 10%. It is likely that the large number of plants in this analysis was responsible for the strong significance. The correlation coefficient between the linear combination of the response traits and nitrogen percentage was estimated at 0.55 and for the correlation with carbohydrates the estimate was -0.78. I tested several relationships between the foliar traits and insect response traits based on the least square means presented in Table 2 of the paper. With one exception, the relationship between carbohydrate percentage and development time ($R^2 = 0.50$), none of them had a $R^2 > 0.30$. The second strongest relationship is presented in Fig. 3-37. The largest deviations from the straight line were the $C. dentata$ control (upwards) and the N-fertilized backcross (downwards).

It was concluded that high levels of foliar nitrogen and low levels of foliar carbohydrates are significantly related to rapid caterpillar development. It was further stated that foliage of American chestnut had higher concentrations of carbohydrates and lower concentrations of tannins. At equivalent levels of foliar nitrogen, this means that $C. dentata$ is a higher quality host for generalist herbivores than the hybrid. These conclusions may be true, but they are not fully supported by the data presented. They rely much on significances and not on degree of explanation of relationships, which is most important.

Two backcross families between one $F_1$ tree from a cross between $C. mollissima$ and $C. dentata$ were included in a study of the interaction between chestnut blight and three insect herbivore species ($Lymantria dispar$, $Popillia japonica$, and $Hyphantria cunea$, Kellogg et al. 2005). The families contained 24 and 18 trees respectively and were half-sibs. Chestnut blight was inoculated in June during the fourth growing season of the two progeny families. Also the three parental trees were inoculated. The rating of blight resistance was carried out five months after inoculation with the following result.

- $C. dentata$: 5.0 highest susceptibility
- $F_1$: 2.0
- Back cross 1: 3.0
- Back cross 2: 5.0

Two twigs from each tree were sampled for herbivore assay and foliar properties, respectively. The insects were kept in cages under controlled conditions; 23°C and 15:9 h light and darkness. The $Lymantria dispar$ larvae were reared in small rearing boxes after 24h starvation. The relative growth rate (RGR) was determined and estimated as: \[
\text{final larval weight} - (\text{initial larval weight}) / (\text{initial larval weight/day}).
\]

The $Popillia japonica$ beetles were also starved for 24h before groups of three beetles were exposed to a single leaf in rearing boxes for 48 hours or in groups of nine exposed to three leaves, one from each parent and their progeny. The amount of dry matter consumed was determined.

Caterpillars of $Hyphantria cunea$ were starved for 24 hours and after that placed in groups of nine on chestnut leaves in rearing boxes. Five foliar characteristics were analyzed at three occasions, June 10, July 8, July 29, during the growth period:

- Toughness, mg
- Density, mg/cm²
- Carbohydrates, %
- Nitrogen, %
- Tannins, tannic acid equivalents

Correlations between foliar content/characteristics and the impacts of insect damage were estimated.

The three classes of blight resistance, 3.0 – 5.0, did not affect any of the traits studied in any of the three insect species included in this study. In the three-choice test the $Popillia$ beetles preferred the $F_1$ and the two $C. dentata$ parents, $p <0.001$. There were no significant differences between the five genetic entries with respect to any of the traits studied in the $Lymantria$ assay (cf. Fig. 3-38).

This figure reveals that there were large differences for the $Popillia$ and $Hyphantria$ assays. As regards $Popillia$ the two back crosses and $F_1$ were least affected by this insect. This suggests that there is some dominance for resistance against $Popillia$ contributed by $C. mollissima$ that is conferred from $F_1$ to the two back cross progenies. However, in the three-choice test $F_1$ and the $C. dentata$ parents were the most affected entries and that speaks against such an interpretation. The $F_1$ parent was least affected by $Hyphantria$ insects. It is possible that an increased contribution of the $C. dentata$ genome in the back crosses contributed to an increased susceptibility of them towards $Hyphantria$. 

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Figure 3-37. The relationship between stem basal diameter and development time of the fourth larval stadium of $Lymantria dispar$ in $C. dentata$ seedlings (blue) and $C. dentata$ x $C. mollissima$ back cross seedlings with $C. dentata$ (green). Rieske et al. 2003.
The genetic entries had a strong and significant effect on the foliar traits at the three dates for collection of leaf samples. Except for the sampling date July 29, tannin content was the only foliar trait that differed among the blight resistance classes with its higher content in class 5 than in the two lower classes, 3 – 4. There were several significant correlations between foliar traits and herbivore susceptibility. However, the degree of explanation was mostly low. It was concluded that seasonal changes in foliar characteristics, coupled with differences in insect feeding modes, undoubtedly influenced the results. This conclusion is easy to support.

Another important conclusion from this carefully performed investigation is absence of any correlation between blight resistance and the feeding of the three herbivore species studied.

3.4.1 Dryocosmus kuriphilus

*Dryocosmus kuriphilus* is an invasive insect species introduced to Europe this century. During a 3-year period test of the susceptibility against *Dryocosmus kuriphilus* of 41 Italian cultivars of *C. sativa* was carried out by Sartor et al. (2009). Eight of the cultivars were interspecific hybrids. Observations of infestation in three replicates were carried out over three years in insect-proof screen houses. Number, size and position of galls were recorded the following spring. During years 2 and 3 one susceptible cultivar and one resistant were exposed to a strong attack of the gall wasp to find any difference in DNA between the two cultivars. The observations presented were based on the cultivars that had 100 buds and occurred in all replications. Therefore, only data from 15 cultivars were published. The infestation in one northern Italian provenance field trial with *C. sativa* populations from different localities in Greece, Italy and Spain was recorded during four years.

According to the text, most of the galls appeared on the leaves either at the base of the leaf or all over the leaf surface. This statement does not agree with Fig. 2 of the paper, in which galls on leaf surface has a low incidence. The ratio of galls/No. buds varied in the range 0-2.1 (Fig. 3-39). One *C. sativa X C. crenata* hybrid of French origin did not have any galls while two *C. crenata x C. sativa* were two of the three most affected cultivars. The two extreme cultivars were most differentiated from the rest of the cultivars. There was no information given on the year-to-year variation in infestation.

Figure 3-39. Mean number of galls per bud after infestation with *Dryocosmus kuriphilus* of *C. sativa* and interspecific *C. sativa* and *C. crenata* hybrid cultivars during three consecutive years. The lines indicate non-significant differences among individual cultivars. Sartor et al. 2009.
The infestation percentage increased from a low percentage in 2004 to almost 90% in 2007 (Fig. 3-40). It would have been interesting to know why there was such a dramatic increase of the infestation during this period. Did the increase depend on tree age, weather condition or any other external factor? As seen from Fig. 3-41 there was a difference in infestation with the lowest percentages in the two Greek populations. They showed the poorest growth and there was a significant relationship between growth and percentage infestation suggesting that poorly growing trees are less attractive to the gall wasp.

In the molecular part of the study some sequences were only found in the susceptible cultivar while others were found in both cultivars included in this part of the study. Further studies are required to get an understanding of the mechanism of gall wasp resistance.

In an extended study 62 cultivars of *C. sativa* and its interspecific hybrids with *C. crenata* were infested with *Dryocosmus kuriphilus* (Sartor et al. 2015). Infestation was carried out in June-July in screen houses with one insect per five buds. Three replications were aimed at in the experiment that was run for eight years. The response to infestation was estimated as galls formed per number of buds at infestation. Yield loss in one cultivar (Marsol) was estimated in an Italian orchard at lat. 44.49°N and 500 masl during six consecutive years. At the end of the growing season the tree circumference was measured at 20 cm above the graft union and the nut weight was recorded. The relationship between galls/bud and production data was determined.

Seven cultivars did not develop any galls. This total resistance was confirmed after stronger infestation application of these seven clones. Two of the resistant cultivars were *C. sativa* and one was *C. crenata* the remaining four were interspecific hybrids between these two species. Three of the four hybrids had a common parent a *C. crenata* clone selected in France. Especially for breeding the finding of two resistant *C. sativa* cultivars was welcomed.

The authors preferred to split the cultivars into three groups with different ratios of galls/No. of buds; <0.3, 0.3 – 0.6, and >0.6 (Fig. 3-42). The group with 0-0.3 galls/No buds was the largest group with 35% trees while the smallest was the group without galls, 11%. It was noted that eggs were deposited in all cultivars but with different preference. It was speculated that bud size, bud texture, and volatile substances might be responsible for this difference in preference.

As regards the effect of the infestation there was a strongly significant difference in yield among the three classes. The content of crude fats, crude fibers, carbohydrates, starch, and crude proteins were lower in leaves with galls than in unaffected leaves. Contrary to this, sugar content was higher in the infested leaves.

After reports on a serious attack by *Dryocosmus kuriphilus* in spring of 2008 current generation galls in three cultivars growing in a plantation in Tuscany were recorded by Panzavolta et al. (2012). These cultivars were represented by 21, 29, and 94 grafts. The number of grafts studied in 2009 was 16, 21, and 29. From each of them two current-year shoots were collected and examined with respect to bud traits and shoot length. The results from 2008 indicated that there was a signif-

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**Figure 3-40.** The development of *Dryocosmus kuriphilus* over a 4-year period of six *C. sativa* populations from Greece, Italy, and Spain in a northern Italian provenance trial. Sartor et al. 2009.

**Figure 3-41.** Severe and medium infestation with *Dryocosmus kuriphilus* of six *C. sativa* populations in a northern Italian field trial. ES = Spain, GR = Greece, IT = Italy. Sartor et al. 2009.

**Figure 3-42.** The partitioning of 62 chestnut cultivars into four classes of galls/number of buds after infestation with *Dryocosmus kuriphilus*. Sartor et al. 2015.
significant difference in the mean number of galls per graft; range 1.8-7.4. In the material from 2009 there was a significant difference in percentage of buds attacked by *Dryocosmus kuriphilus* (Fig. 3-43). There was seemingly a discrepancy between the result in 2008 and 2009, which might be attributed to analysis of different traits the two years (Fig. 3-44). In 2008 cultivar Fusca had the highest number of galls per graft while in 2009 Cesurone had the highest mean number of larvae per bud. For the 2009 study it was shown that the bud volume varied. Therefore, bud volume was used as a covariate in the statistical evaluation. It was concluded that the attacks of this insect had passed the injury tolerance level.

Real time PCR and diaminobenzidine (DAB) test were used to possibly reveal difference between two *C. sativa* cultivars in response to *Dryocosmus kuriphilus* attacks; one sensitive and one resistant (Dini et al 2012). Testing took place during different stages of the bud development. The DAB test revealed a difference between the two cultivars. The buds of the resistant cultivar turned brown suggesting the presence of reactive oxygen. The real time PCR revealed a strong expression of the stress-related germine-like gene in the resistant cultivar but not in the susceptible cultivar.

### 3.5 Hypovirulence

Hypovirulence has been regarded as a remedy for chestnut blight. A state of the art report was published 2004 (Milgroom and Cortesi 2004). Cankers on trunks of the trees are treated with the virus causing hypovirulence. The success of such treatment was less in eastern USA than in Europe. One reason might be that attacks by *Cryphonectria parasitica* on *C. dentata* are more severe than the attacks of *C. sativa*. One prerequisite for successful treatment with viruses is that it is spread naturally. It seems as spreading is limited, which means that repeated treatments must be done to combat chestnut blight. Much remains to be studied as regards spreading of the virus as well as its interaction with its host fungus before large scale application with hypovirulence treatment is developed.

Recovery from chestnut blight by aid of *Cryphonectria*-associated hypovirus (CHV-1) was studied by Jezić et al. (2014). In a forest stand in Lovran region in Croatia with a mixture of naturally occurring chestnut trees and grafted Marron trees, bark samples were taken from 26 trees of both types for genotyping and isolation of *Cryphonectria parasitica*. According to the report: Bark samples were taken from the first accessible canker on the tree. All trees analyzed had a DBH of >30 cm. Genotyping of the trees was done by analysis of ten microsatellite loci. The fungus was cultivated on potato dextrose agar and the culture morphology was used to assess presence of hypovirus. Hypovirus-free cultures produce yellow orange mycelium with many conidia while presence of hypovirus results in white mycelium with few conidia. All trees in the region in which this study comprised were infected by *Cryphonectria*. Three types of canker were identified; active/deep, callus, or necrosis. The first and most severe type of damage is described as aggressive and expanding both on bark and cambium. Callus means a wound, which is partially or complete enclosed. Necrosis is a superficial form of damage with slow growth on the bark. The 26 grafted trees constituted one clone with the same genotype while 26 different genotypes were noted for the 26 naturally growing trees. The number of alleles in the ten loci varied in the range 3-9. In spite of individual genotypes of all natural trees it was stated that the study
program was started, which between three Necrosis against this pathogen was regulated by alleles in two or Early results suggested that the inheritance of resistance was followed by repeated backcrossing with dentata to this pathogen. During the eighties hybridization of to be much less susceptible than the American chestnut Asian species, species were carried out in the early nineties. The two efforts to incorporate blight resistance into Much work on trees have low ability to recover from chestnut blight. Marrone genetic constitution. It was speculated that the Marrone difference is the differences in age between the two categories of trees. Nor were there any conditions were ruled out as an explanation for the differ Figure 3-45. Number of trees in three different classes of damage among 26 natural forest trees and 26 Marrone grafts in a mixed forest with naturally growing trees and grafts of C. sativa in Croatia. Active/deep damage is the most severe type of damage. Hypov = presence of hypovirus. Ježić et al. 2014.

showed a homogeneous population. This statement was based on a principle coordinate analysis, in which the first two axes contributed with 26 and 19% of the total variation. The effect of hypovirus on fungal infection is summarized in Fig. 3-45. Of the natural forest trees all ten trees lacking hypovirus had the most severe damage while only four of the trees with hypovirus had this type of damage. The other twelve hypovirus carrying trees had less severe damage. Contrary to this, all Marrone trees except one showed active/deep damage. It is evident that hypovirus does not give the Marrone genotype protection against Cryphonectria attacks. Differences in environmental conditions were ruled out as an explanation for the difference between Marrone trees and natural forest trees since they were growing in a mixed stand. Nor were there any differences in age between the two categories of trees. Therefore, the most likely reason for the difference is the genetic constitution. It was speculated that the Marrone trees have low ability to recover from chestnut blight.

3.6 Summary

Much work on Cryphonectria parasitica was centered on the efforts to incorporate blight resistance into C. dentata. Studies of variation in susceptibility among Castanea species were carried out in the early nineties. The two Asian species, C. crenata and C. mollissima were found to be much less susceptible than the American chestnut to this pathogen. During the eighties hybridization of C. dentata with C. mollissima program was started, which was followed by repeated backcrossing with C. dentata. Early results suggested that the inheritance of resistance against this pathogen was regulated by alleles in two or three loci. If this would be the case total resistance would have been reached in the third backcross generation, which was not obtained. Large variation in virulence among different strains of Cryphonectria parasitica was noted. Strong genetic differentiation in Cryphonectria parasitica was found in a range-wide study in USA. This difference was especially strong in two fruit orchards, $G_{st} = 0.81$.

One reason for transfer of oxalate oxidase, OXO, genes into chestnut is that oxalic acid is believed to be a means for Cryphonectria parasitica to attack cell walls and allow the fungus to enter the host plant and become a pathogen. OXO genes were successfully transferred by Agrobacterium. Dependent on the pathogen strain used for inoculation some improvement of disease tolerance was noted. Since nuts are used as food sources studies were undertaken to study metabolites in transferred nuts. Only two cell wall metabolites differed in the transgenic plants while there was no difference in numerous other cell metabolites in nuts. There was a difference in expression of genes with different transcript abundance (GDTA) between C. dentata and C. mollissima. Whether this is a result of timing and amplitude of the response of the two species remains unresolved.

Significant differences in root lesion length after inoculation with Phytophthora cambivora between three domestication (natural, coppice, and orchard) levels at French, Greek, Italian, and Spanish localities were noted. The coppice populations had largest susceptibility. The Sicilian populations had low susceptibility, which was unexpected since these populations were not previously exposed to this fungus. Thus, this population had no opportunity to respond by adaptation to this parasite. The relationship between stem and root infection with Phytophthora cambivora was weak. Studies with large numbers of cultivars/clones revealed large variation in susceptibility following inoculation with Phytophthora cinnamomi. High clonal repeatabilities for several traits related to pathogen damage were reported. The absence of any indications of distinct classes in response to the inoculations suggests a polygenic inheritance of tolerance against this fungus. In spite of the continuous variation QTL were detected explaining around 10% of the variation in susceptibility. Several genetic parameters were estimated in one Spanish progeny trial with 25 families. Generally, the additive variance was larger than the non-additive variance for susceptibility against Phytophthora. The seedlings were vegetatively propagated and the clonal variance component was several times larger than GCA and SCA variance components. Generally, the interspecific hybrids C. sativa x C. crenata were less susceptible to Phytophthora infection than C. sativa. One of the first studies, in which functional SSR markers were developed, i.e. SSR markers inside expressed units in the genome was published in 2015.
Differences in expression of genes of different taxa before and after inoculation with *Phytophthora cinnamomi* were presented. In one case, *Cast-Gnk-2-like gene*, there was no response in *C. sativa* while there was a strong response in *C. crenata* and *C. mollissima* as well as in their interspecific hybrids.

One hypothesis with three steps in defense against *Phytophthora* attacks was presented:

1. Means to avoid attacks in form of morphology or anti-fungal defense around the roots
2. Recognition of the parasite
3. Activation of host resistance genes

As a consequence of increased problems with insect herbivores, studies were initiated to address problems with insect feeding. The development time for the fourth larval stadium of *Lymantria dispar* was shorter in back cross seedlings (*C. dentata* x *C. mollissima*) x *C. dentata* than in pure *C. dentata*. There was a continuous distribution in susceptibility against *Dryocosmus kuriphilus* in the material studied.

Studies on variation in susceptibility against *Cryphonectria parasitica* after hypovirulence treatment showed that variation exists and that the susceptibility of the famous Marrone cultivar was not reduced.
4 Breeding and conservation

4.1 Breeding programs – applied and outlines

In his paper from 1994 on breeding American chestnut Hebard (1994b) stated that the primary approach was backcross breeding after hybridization with chestnut blight resistant *C. mollissima*. According to this plan backcrossing should take place over three generations. The breeding program will be speeded up by direct seeding at *orchard spacing* in field. As a reference for blight resistance the Chinese Nanking cultivar will be planted. Screening for blight resistance cannot take place with satisfaction until age 4-5 when the stems have reached a DBH of 2.5 cm. In case of higher resistance such as is expected following \( F_1 \times F_1 \), the evaluation may take place earlier at a DBH of 1 cm. Artificial inoculations with *Cryphonectria parasitica* will be carried out. Crosses will take place in field in several states from Georgia to Pennsylvania. Planting of trees for crossing will also take place. Grafting with scions from selected trees onto old flowering trees can be done to obtain hybrids from their grafted parts as well as from non-grafted parts of such trees. Another approach would be planting of Chinese chestnut trees as pollen donors close to solitary American chestnut trees to obtain large number of hybrid nuts.

The needed number of progeny per cross is dependent on the number of loci that regulates blight resistance. For backcrosses it was planned to have 4-5 individuals homozygous for resistance alleles in three loci, which would require 73 individuals to reach this goal to 99%. For intercrosses among \( F_1 \) individuals the corresponding requirement would be 149 individuals. Crosses will be carried out from Georgia to Maine to keep the adaptedness of the local sources.

Therefore, as many as possible hybridizations should take place in the first breeding generation. Hebard discussed the number of lines of American chestnut required, which he defined as the product of one intercross of one Chinese chestnut tree and one American chestnut tree and three backcrosses to American chestnuts. Since different American chestnut trees will be involved at hybridization and the three generations of backcrosses, the number of American chestnut trees within a line would be four. Crosses between trees within a third generation of backcrosses were planned. In reality, more than four American trees might be included in order to obtain the required 73 offspring trees. The author envisaged 20 lines per breeding zone and six zones, which adds up to a total of 120 lines. It was stressed that it is important to keep the obtained adaptedness and a broad base in the breeding population. As regards the number of Chinese chestnut trees it was planned that three highly blight resistant Chinese chestnut trees should be included in the 20 lines. Three Chinese cultivars with high blight resistance were already identified and ready for use in breeding. Finally, a hope was expressed that biotechnology might be a useful tool for identification of genes regulating blight resistance as well as direct gene transfer of blight resistance into the American chestnut genome.

In a paper by Hebard (2005) the back cross breeding program of the American chestnut foundation was presented. Thanks to early flowering of chestnuts the generation turn-over might be five years or less. This makes a back cross breeding program a workable option. Mainly the state of the art of this program was presented by Hebard (2005). Different *C. dentata* individuals are used in the different back cross generations to avoid inbreeding. This means that genes from four different American chestnut trees are included in the \( B_1 \) trees. To keep the variability in the breeding population it was estimated that 20 separate lines should be created but with just one *C. mollissima* parent to increase the probability for homozygosity of blight resistance. For the same purpose crosses between \( B_1 \) lines are carried out coined as \( B_1 \times F_2 \). It seemed as two incompletely dominant genes are responsible for the blight resistance. It was stated that recurrent selection should be used in case further improvement of blight resistance beyond the \( B_1 \times F_2 \) stage would be required. It was noted that all interspecific hybridization attempts were not successful while some interspecific hybrids showed superior growth compared to both parental species. The superiority remained even up to an age of 20 years.

Diskin et al. (2006) presented scientific achievements in the American back cross breeding program aiming at restoring the American chestnut by introduction of chestnut blight resistance into its genome. Thanks to early flowering of chestnuts the generation turn over might be five years or less. This means that a back crossing program is a viable option for restoration of the American chestnut. The authors studied 24 morphological traits in \( F_1 \) and three generations of back crosses together with the parental species *C. dentata* and *C. mollissima*. A principle component analysis was carried and the first principle component captured all of the useful information in the combined data set; thus all traits studied.
Based on this information an Index of Species Identity (ISI) was calculated with a scale 0 - 1, in which 0 stands for *C. mollissima* and 1 stands for *C. dentata*. Such an index gives a general picture of the morphological approach towards *C. dentata*. In case of simple inheritance a straight line is expected between the percentage of *C. dentata* genome in the different materials and the Index of Species Identity. The goal of such a back crossing work is to create a resistant *C. dentata* with its original habitus.

In Fig. 4-1 I have selected to illustrate the relationship between expected percentage of *C. dentata* in the progenies and trait values of *C. mollissima*, *F₁*, *B₁*, *B₂*, *B₃*, and *C. dentata*. Four traits are presented:

- Twig color; 1 = green tan, 4 = reddish brown (red)
- The ratio leaf length/leaf width (blue)
- Interveinal hairs; 1 = present 2 = absent (orange)
- ISI = index of species identity 0 and 1 for *C. mollissima* and *C. dentata*, respectively. (green)

Diskin et al. 2006.

Based on this information an Index of Species Identity (ISI) was calculated with a scale 0 - 1, in which 0 stands for *C. mollissima* and 1 stands for *C. dentata*. Such an index gives a general picture of the morphological approach towards *C. dentata*. In case of simple inheritance a straight line is expected between the percentage of *C. dentata* genome in the different materials and the Index of Species Identity. The goal of such a back crossing work is to create a resistant *C. dentata* with its original habitus. In Fig. 4-1 I have selected to illustrate the relationship between expected *C. dentata* genetic contribution in the two parental species, their hybrid, and three back cross generation and the ISI index as well as some other morphological traits. This figure shows that the fit to the straight line for the relationship with ISI is extremely good. The strong fit suggests that most of the traits analysed showed additive inheritance or alternatively that dominance effects from both parents are cancelled. These results are a most promising step for the restoration of the American chestnut with incorporated tolerance against *Cryphonectria parasitica*. It requires that other important traits also are included in the final back cross generation.

Hebard (2012) stated that the modern breeding for blight resistance of *C. dentata* followed the Multiple Population Breeding System MPBS concept introduced by Namkoong (1984). MPBS means that the main breeding population is split into some 20 subpopulations each with around 50 trees. The subpopulations should be selected to include existing variation of the American chestnut in its distribution area. The American Chestnut Foundation, TACF, has initiated much research in its efforts to restore the American chestnut, *C. dentata*.

Inoculations with two different strains of *Cryphonectria parasitica* and inoculations at different ages were also presented by Hebard (2012). In one experiment the two parental species *C. dentata* and *C. mollissima* and their *F₁* hybrid were included. In this case inoculations at different ages were tested. Another experiment included an array of backcrosses + the parental species, which were inoculated in June at age three and evaluated in December the same year. The *B₃ x B₃* offspring was obtained after open pollination in a seedling seed orchard.

The results as regards canker length in the first experiment are presented in Figs. 4-2 and 4-3. Inoculation with the virulent strain EP 155 caused large cankers in all three families from age two. Only at age one there was a substantial difference between *C. dentata* and the two other families. As expected from the graphic illustrations, the statistical evaluation revealed significant effects for:

![Figure 4-1](https://example.com/image1)

**Figure 4-1.** The relationships between expected percentage of *C. dentata* in the progenies and trait values of *C. mollissima*, *F₁*, *B₁*, *B₂*, *B₃*, and *C. dentata*. Four traits are presented:

- Twig color; 1 = green tan, 4 = reddish brown (red)
- The ratio leaf length/leaf width (blue)
- Interveinal hairs; 1 = present 2 = absent (orange)
- ISI = index of species identity 0 and 1 for *C. mollissima* and *C. dentata*, respectively. (green)

Diskin et al. 2006.

![Figure 4-2](https://example.com/image2)

**Figure 4-2.** The relationship between canker length and age at inoculation for *C. dentata*, *C. mollissima*, and their *F₁*-hybrid following inoculation with *Cryphonectria parasitica* strain SG2-3. Hebard 2012.

Inoculations with two different strains of *Cryphonectria parasitica* and inoculations at different ages were also presented by Hebard (2012). In one experiment the two parental species *C. dentata* and *C. mollissima* and their *F₁* hybrid were included. In this case inoculations at different ages were tested. Another experiment included an array of backcrosses + the parental species, which were inoculated in June at age three and evaluated in December the same year. The *B₃ x B₃* offspring was obtained after open pollination in a seedling seed orchard.

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![Figure 4-3](https://example.com/image3)

**Figure 4-3.** The relationship between canker length and age at inoculation for *C. dentata*, *C. mollissima*, and their *F₁*-hybrid following inoculation with *Cryphonectria parasitica* strain EP 155. Hebard 2012.
It is evident that strain EP 155 has such a high virulence that all materials including *C. mollissima* are strongly affected by this strain. The age effect might be attributed to the change in environment (shading) as trees grow older. Contrary to hybrid and Chinese chestnut, the American chestnut did not vary with age. This explains the significance of the type of cross x age interaction.

**Figure 4-4**. The relationship between canker length and expected part of *C. dentata* genome in different families inoculated with two strains of *Cryphonectria parasitica*, EP 155 and SG2-3. 0 = *C. mollissima*. The inoculation took place in June and the evaluation took place in December the same year at an age of three years in field. Hebard 2012.

Clark et al. (2012) reported on nursery performance of back cross families between *C. dentata* and *C. mollissima* as well as the parental species, one *C. mollissima* family, and three *C. dentata* families in each nursery. I will use the expected percentage of *C. dentata* contribution to the genome in the different back cross families instead of the somewhat confusing terminology in many similar studies. Before sowing in two nurseries, one in Georgia and the other in Tennessee, weights of individual nuts were recorded and a subdivision of nuts into two classes, large and small, was carried out. The following traits were studied at the end of the first growth period:

- Plant height
- Root collar diameter (RCD)
- First order lateral roots (FOLR)
- Missing tap root (MTR)
- Stem Forking (FORK)

At the 94% level of *C. dentata* genome only one family was common to the two nurseries. Of the six *C. dentata* families none was common to the two nurseries. ANOVAs were run to estimate effects of nursery, nut weight, *C. dentata* genome level (0, 75, 88, 94, and 100% American chestnut), family, and interactions. Pairwise Pearson correlations between the above traits were calculated separately for each nursery. Logistic regressions were calculated to estimate if MTR or FORK were influenced by nursery, genetic entry, or nut size class. It should be added that no *C. mollissima* seedlings were included in the Tennessee trial. The limited number of families studied did not allow any estimates of additive variance or potential genetic gain.
The growth of some seedlings was impressive. The range for plant height was 10-262 cm, for RCD 2-31 mm, and for FOLR 0-48. As seen from Fig. 4-5 the plant height was much larger in the Tennessee trial than in the Georgia trial, in which there was a positive relationship between nut weight and plant height. Since only five families were common to the two trials it is hard to know whether the growth differences are due to genetic differences or site conditions. However, the plant heights of four of the common families suggested that the site conditions caused the growth difference between the two trials. There was a significant relationship between nut weight and plant height, R² = 0.57 while the relationships between nut weight and RCD were non-significant. The corresponding relationship with FOLR was significant but with an extremely low R², 0.14.

As regards nut weight strongly significant effects were noted for nursery, genome level, family, and nut class. The significances for different effects on three growth traits are summarized in Table 4-1. Significant effects on plant height were noted for all variables except for the two interactions. No family effect was observed for the two root traits.

For the four genome levels of C. dentata in Georgia nursery plant height at the end of the first growth period showed a slight decrease with increasing level of C. dentata genome (R² = 0.92, Fig. 4-6). Such a trend was not observed in the Tennessee nursery. Whether this can be attributed to different entries studied in the trials was not discussed.

Pearson correlations between all traits were calculated. Only correlation coefficients >0.50 are shown in Fig. 4-7. All correlations with MTR and FORK were weak or even negative. It is expected that the relationship between

<table>
<thead>
<tr>
<th>Trait</th>
<th>C. dentata genome level</th>
<th>Family</th>
<th>Nut size</th>
<th>Nursery</th>
<th>Nut size x Cd genome level</th>
<th>Nut size x family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant height</td>
<td>*</td>
<td>*</td>
<td>**</td>
<td>*</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Root collar diameter</td>
<td>**</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Number of first order lateral roots</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 4-1. The significances for different effects on three growth traits of seedlings grown in two nurseries, Tennessee and Georgia, for one year. Clark et al. 2012.
Plant height and root collar diameter is strong, which was noted $r = 0.79$ and $0.80$ in the two trials. The logistic regression model showed that the *C. dentata* seedlings had lowest probability for stem forks while *C. mollissima* and the first back crosses had the highest probabilities for stem forks and missing tap roots. Based on the difference between the American chestnut and the third back cross generation concern was raised as regards the potential for obtaining a blight resistant chestnut with American chestnut habitus. Finally, it deserves to be added that an applied nursery perspective was frequently discussed in this paper.

Three field trials with the same material were established in North Carolina (Lat. $35.00^\circ$N, 841 masl), Tennessee (Lat. $36.15^\circ$N, 1,036 masl) and Virginia (VA). Only families common to all three trials are included. Before sowing two classes of nuts were identified, large and small. Clark et al. 2016.

Survival. Except for the *C. mollissima* family in the Tennessee trial (<40%), the survival was satisfactory in the other materials, around 80%. The poor survival of Chinese chestnut in TN trial was a strongly contributing factor to the significances of site, genetic type and site x genetic type effects (cf Table 4-2).

Growth. As seen from Table 4-2 the family effect was significant for tree height but not for the other three traits in this table. Site, nut size, and genetic type had strong and significant impact on tree height, which is reflected in Figs 4-8 and 4-9. Noteworthy is the good growth of the two BC$_3$ families in the North Carolina trial. Such a deviating pattern was not revealed in the two other trials, nor was there any deviating growth pattern in the material originating from small nuts (Fig. 4-9).

Table 4-2. The significances of different effects for survival, tree height, Ground Level Diameter (GLD), stem dieback in *C. dentata* and *C. mollissima* as well as different generations of backcross families between these two species (genetic type) at age four in three field trials in North Carolina, Tennessee, and Virginia. Clark et al. 2016.

<table>
<thead>
<tr>
<th>Source</th>
<th>Survival age 4</th>
<th>Height age 4</th>
<th>GLD age 4</th>
<th>Stem die back age 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td>*</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Genetic type</td>
<td>*</td>
<td>***</td>
<td>**</td>
<td>ns</td>
</tr>
<tr>
<td>Size x genetic type</td>
<td>**</td>
<td>ns</td>
<td>ns</td>
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<td>Nut size</td>
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<td>Site x genetic type</td>
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<td>Site x size</td>
<td>ns</td>
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<tr>
<td>Family</td>
<td>ns</td>
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<tr>
<td>Family x size</td>
<td>not presented</td>
<td>ns</td>
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<td>not presented</td>
</tr>
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</table>

Figure 4-8. Four-year mean heights of chestnut backcross families between *C. dentata* and *C. mollissima* as well as families from paternal species growing in three field trials in North Carolina (NC), Tennessee (TN), and Virginia (VA). Only families common to all three trials are included. Before sowing two classes of nuts were identified, large and small. Clark et al. 2016.

Figure 4-9. As Fig. 4-8 but small nuts. Clark et al. 2016.

Chestnut blight were assessed until age four in field. Stem dieback caused by deer browsing was not included in the statistical evaluation. Occurrence of blight was checked three times a year. Seven backcross families, one *C. dentata* OP-family, and one full-sib *C. mollissima* family were common to the three field trials. Four additional families grow in one or two trials only. The focus in this paper was more on finding conditions for successful establishment of advanced backcross generations in commercial plantations. However, genetic information may also be retrieved from this paper.
Fig. 4-10 shows a strong relationship between tree height in trees originating from large and small nuts. This means that there is no family x nut size interaction for tree height. There was not total agreement between the two growth traits studied, tree height and ground level diameter (GLD, Table 4-2). Thus, the family effect was non-significant for GLD while it was significant at one percent level for tree height. Also with respect to site x genotype level there was a difference between these two traits. In Fig. 4-11 I have plotted the relative values for GLD against trial means to illustrate the stability or lack of stability of families common to all trials. Unfortunately, two clusters of trial means occurred, one with two trials and the other with three trials. The *C. dentata* family performs well in most cases while one of the BC₁ families shows poorest growth in most trials. Dramatic rank changes were not observed and as a consequence of this the family x site interaction was non-significant.

**Stem dieback.** This trait was most frequent during the first year in field with the highest percentage in the North Carolina trial. The offspring from large nuts was more affected with stem dieback. It was stated *The only significant breeding effect on first-year dieback was within the large size class, and Chinese chestnut had 33(±6) % dieback compared to the BC₁, F₁ generation, which had 9(±3) % dieback. The family effect was non-significant.*

**Chestnut blight.** The blight was most abundant in the Virginia trial with 14% of trees affected. At this locality there was a significant difference for genetic type. The American chestnut was most affected with 29% blight compared to 7% and 3% for the BC₂ and Chinese chestnut, respectively. The results from the trial in North Carolina are illustrated in Fig. 4-12 for individual families of three generations of backcrosses as well as the parental species. There is considerable variation among the families of *C. dentata*, BC₁, and BC₂. The *C. dentata* family with superior growth of both nut size classes had the highest percentage of blight, 23%. The BC₁ family with the poorest growth in this trial had only 2% of blight affected trees. This called for an analysis of the relationship between growth and blight susceptibility. However, the relationships between tree height and blight percentage for both

![Figure 4-10](attachment://image1.png)

**Figure 4-10.** The relationship for four-year heights between chestnut trees originating from large and small nuts. Backcross families between *C. dentata* and *C. mollissima* as well as paternal species families are included in three field trials in North Carolina, Tennessee, and Virginia. Clark et al. 2016.

![Figure 4-11](attachment://image2.png)

**Figure 4-11.** The relative ground level diameter of *C. dentata* and *C. mollissima* as well as seven families of three backcross generations in three field trials in North Carolina (NC), Tennessee (TN), and Virginia (VA) plotted against the six trial means. Small (S) and large (L) nuts were separated before germination. Clark et al. 2016.
nut size classes were weak. It was pointed out that
development of blight susceptibility must be followed over a
longer time period.
In conclusion, much valuable information as regards the
possibilities for reintroduction of American chestnut in
southeastern USA was obtained.

Dale and Galic (2014) described the breeding for blight
resistance of C. dentata in Canada. Two populations each
with 21 Canadian founders were created. In one popula-
tion the founders were crossed with four backcross trees
from USA. The other population was obtained follow-
ing crosses with Canadian C. dentata trees. Inoculations
of branches were carried out during five years with two
strains of Cryphonectria parasitica and lesion growth
was recorded one and two months after inoculation. The
daily rate of expansion between the two occasions of as-
sessment was calculated in order to enable comparisons
over years. The progenies were planted at two localities.
In Fig. 4-13 I have illustrated the mean expansion rates
for the two types of progeny and test localities for each of
the five years of observation. Significant effects for popu-
lation differentiation were noted in years 2007 and 2008
while the effect of locality was significant the last three
years in spite of the limited difference in year 2011. A
large variation in lesion expansion among trees was not
ed and illustrated graphically. Four trees which had low
expansion rates in 2010 and 2011 were selected for the
breeding population.

Bazzigher and Miller (1991) reported on a 30-year selec-
tion program mainly for resistance against Cryphonectria
parasitica in Tessin Canton in Switzerland. In the initial
selection as many trees as possible free of symptoms
were selected. Later on artificial inoculations took place
and seedlings from C. crenata and C. mollissima were
included in the testing. Artificial inoculations took place
at age 4-5 and evaluations occurred at age 10. Besides
blight resistance, precocity was also selected for. It was
pointed out that more selections took place during years

with limited blight infection than during years with severe
infection. Fig. 4-14 reveals that a substantial number of
selected trees was included in the breeding population in
Canton Tessin. More than 80% of the selected trees were
C. crenata. Experience gained during 30 year of selec-
tion work indicated that there are only limited differences
between resistant and susceptible trees. This suggests that
blight resistance is polygenically inherited.

A summary of the Chinese breeding program of C. mol-
liissima was briefly presented by Qui et al (2005). This
species occupies a vast geographic area from latitude
18.50°N to 43.92°N and with an altitudinal range of
50-2,800 masl. Fifty-one cultivars selected 1970 and
onwards were described with respect to nut yield and
nut traits, precocity of nut production, phenology, and
general resistance. More than 300 cultivars of Chinese
chestnut exist in China. As far as I can understand six
zones for separate breeding were delineated. Nut weights

Figure 4-12. Percentage of chestnut blight at age four
in ten families growing in a field trial in North Carolina
(NC). Results from three backcross generations between
C. dentata and C. mollissima are illustrated together with
results from the two parental species. Clark et al. 2016.

Figure 4-13. The mean daily expansion rate after ino-
culations with Cryphonectria parasitica of branches in
two types of progeny: 21 Canadian C. dentata trees cros-
sed with four backcross males from USA. The same Cana-
dian trees crossed with other Canadian C. dentata trees.
Blue and green refer to the two test localities. Dale and
Galic 2014.

Figure 4-14. The number of selected trees of different
taxa in the chestnut breeding program in Ticino, Switzer-
land. G = generation, Cs = C. sativa, Cc = C. crenata, and
varied widely, 1.7 - 42 g and fruit color varies much too. Two figures on gene flow were presented, 2.2 and 2.7 migrants per generation, which indicates a large exchange of genetic material. Clonal archives are established and continuously new selections are included.

Payne et al. (1994) in their overview of the potential of C. pumila as a crop tree stated that: We believe that after 98 years, the economic potential of this nut crop remains uncertain, although the plant has potential in landscaping and as wildlife food source and shelter. The nut yield per tree at age seven in a closely spaced plantation varied in the range 0-21 kg. The extrapolated nut yields at ages 12 and 14 in 30 trees in a 3 x 6 meter plantation of 30 trees were 1.2 and 3.1 ton/hectare, respectively.

There are some papers treating general aspects of American chestnut breeding from a theoretical point of view without referring to any applied breeding program for American chestnut.

In their paper Restoration of threatened species: A noble cause for transgenic trees Merkle et al. (2007) discussed the general objections against transgenic plants; such as generating weedy plants and transgenic escape. As one example, there is a fear that resistance against pest and diseases are spread by transgenic trees to non-target organisms. In contrast to many agricultural crops, the potential for transgenic escapes from forest trees to undomesticated relatives is higher than to crop plants lacking undomesticated relatives. These concerns have resulted in a demand for sterility of transgenic trees. It was pointed out that any discussion of food products from transgenes had not taken place. In contrast to this, it was argued that the benefit of restoration of the American chestnut would be of value for nut production as a source for human consumption as well as a food resource for wildlife.

It was stated that the main objective for development of transgenic chestnut is for restoration of one once most important tree species of the Appalachian area. Thus, commercial profit is not the focus of transgenic American chestnut, which might be more acceptable for opponents against any transgenic trees. The benefit of chestnut restoration is not limited to the chestnut itself but it is good for restoration of the entire ecosystem.

Since chestnut blight did not play any role in the natural ecology in North America and therefore; one would not expect that blight-resistant chestnuts would be any more weedy than before the introduction of Cryphonectria parasitica.

Finally, objections have been raised against the gene technology approach for development of blight resistant chestnuts since traditional tree breeding is close to the goal of a blight-resistant chestnut. However, it was argued that both traditional and modern gene technology have their role in the work for restoration of the American chestnut.

The role of genomics for restoration of C. dentata was presented by Wheeler and Sederoff (2009). The greatest value of this paper is its role as a policy document that might be followed in the efforts of restoration of the American chestnut. They listed the following topics, in which genomics might contribute to restoration:

1. Identify many of the genes of the organism and their locations
2. Provide for complex traits dissection of blight resistance and other important growth, form and adaptability traits
3. Provide tools for association of genes and traits
4. Identify and clone specific resistance factors, genetic engineering and marker-aided selection
5. Speed backcross breeding and provide pedigree identity capability

It was stated that already approximately 10,000 expressed sequence tags (EST) have been sequenced and hundreds of microsatellites were detected in ESTs. All this information is useful for development of a saturated genomic map of Castanea species.

One detailed treatment of breeding C. dentata for blight resistance was published in 2010 by Worthen et al. (2010). I have tried to summarize their conclusions:

1. Large effective population size of the breeding population,
2. Maintain a large effective population size by incorporation of as many C. dentata genes as possible,
3. Keep equal variance in family size for the final reintroduction generation,
4. Locally adapted individuals should be included in the breeding population i.e. preservation of genetic diversity and local adaptation,
5. Maximize the number of unrelated individuals in small populations and preferably different sources of chestnut blight resistance
6. Prevention of attacks from pests and other diseases
7. Identification of silvicultural factors facilitating reintroduction
8. Control of competition during the establishment might be needed
9. Monitoring of the established populations
10. Acceptance from the general public for reintroduction

As seen many sensible suggestions for a successful reintroduction. Point 5 is related to the MPBS concept of breeding mentioned above.

The role of biotechnology for restoration of American chestnut was addressed in The Forest Health Initiative (Nelson et al. 2014a). Achievements so far and an outline for a successful biological program was presented:

1. Develop and integrate genomic libraries, maps, and sequences for comparison to a model reference species for candidate gene selection;
2. Develop tissue culture and transformation systems for the
species of interest, even if only for functional analysis of candidate genes;
Develop early standardized assays for resistance, measured as directly as possible for the response interest; and
Collaborate with current breeding programs and arboriculturists to make sure the most important and potentially useful materials are included at the earliest and most appropriate opportunity.

Chestnut blight resistance and resistance against ink disease caused by *Phytophthora cinnamomi* are the breeding goals in this initiative. One detailed description of chestnut breeding may be found in the review: Biotechnology of trees: Chestnut by Nelson et al. (2014b).

One example of applied use of molecular genetics in breeding was reported by Georgi et al. (2014). With the purpose of identification of *C. mollissima* genes in American-Chinese hybrids, SNPs specific for the two parental species were successfully identified in a limited material. Thus identified SNPs might be used immediately but for general use the number of trees tested for these SNPs must be extended.

4.2 Description of trees and cultivars

Several papers are just descriptions of cultivars and they are briefly presented in this text. For breeding, identification of cultivars is a key issue. Characterization of cultivars should rely on descriptors that are distinct, uniform and stable. Serdar et al (2018) presented a thorough scrutiny of morphological and phenological descriptors.

4.2.1 Metric traits

Several reports on selection for the breeding population were published without any genetic studies on diversity or discussion of breeding strategies.

To trace the origin of New Zealand chestnut accessions, 49 morphological nut (36) and leaf (13) characters were measured during a 3-year period by Oraguzie et al. (1998a). In all, 23 accessions were included, five of them with known chestnut species. PCA (principle component analysis) and dendrogram based on UPGMA were used to identify species’ belonging of the accessions. The PCA analysis revealed that there was a geographic grouping of the material. The three first factors in this analysis explained 48% of the total variation. The most important traits for separation of the accessions were nut size, hilum length, and pellicle intrusion. The UPGMA grouping gave similar results with four clusters distinguished. The only *C. mollissima* accession included in this study formed an own cluster. Two northern island accessions formed another cluster. Four accessions from the South Island + one from the north island constituted still another cluster together with *C. sativa*. The largest cluster included most accessions from the North Island and a few from the South Island + *C. crenata*.

It was concluded that accessions in the North Island is more *C. crenata* like while the southern island accessions are more *C. sativa* like. As stated in the report, the inclusion of just one accession each of the mentioned two species renders this conclusion somewhat uncertain.

As far as I can understand the same material was used for a comparison with RAPD profiles of this material (Oraguzie et al. 1998b). The RAPD analysis (with ten primers) included three hybrids and *C. dentata*. The latter species was represented by young trees with no nut production.

Two major clusters were obtained in the RAPD dendrogram. The first contained all accessions from the southern island in New Zealand, two accessions from the northern island + the two pure species *C. sativa* and *C. dentata*. The second cluster contained the rest of northern island accessions + *C. crenata* and *C. mollissima*.

A congruence test according to Page (1992) for the agreement between RAPD data and morphology trait data resulted in good agreement for the southern island accessions while for three of the northern island accessions there was no agreement between the two types of traits. Finally, two accessions agreed to 50% with *C. crenata* and *C. mollissima*. Thus, in spite of the clear four clusters for the nut morphology traits there was generally a good agreement between the two types of estimates of relationships. It was suggested that a combined use of RAPD and morphology traits should be used to trace the origin of New Zealand chestnut accessions.

Botu et al. (1999) described 21 selected trees in native stands of *C. sativa* in Romania. Some trees had an impressive sectional area with a maximum of 2.7 m² at age 270. Besides growth, flowering phenology characteristics, nut yield, and disease susceptibility were reported.

Serdar (1999) reported on nut characteristics of 78 selected trees, mainly from the Sinop region in Turkey. The trees were graded with respect to general quality, nut size, earliness, and chestnut paste. Scions will be taken from the selected trees and grafted for inclusion in fruit orchards.

Solar et al (1999) presented a detailed description of seven phenotypically selected trees; four of them originating from the continental part of Slovenia and three from the Mediterranean part. The seven trees belonged to a first plus tree selection of 102 trees from Slovenia that started in 1990.

The same type of report was published by Serdar and Soylu (1999) for 49 selected trees from Samsun region in Turkey.
Mancuso et al. (1999) tested artificial neural networks (ANN) for characterization of chestnut genotypes. According to the authors ANN are powerful computational tools that “learn” with training examples and have the capability for extrapolating their knowledge to new situations in problems of classification. The study of the suitability of ANN for identification of chestnut accessions comprised 17 common cultivars from Italy. Fourteen leaf morphology and color characteristics of leaves from the image analysis were used in the different back-propagation neural networks (BPNN). As a comparison canonical analysis was performed on the same traits.

A comparison of the correct predictions following neural network analysis and canonical analysis of the same traits revealed that percentages were higher for the ANN analysis than for the canonical (Fig. 4-15). For three accessions the canonical analysis gave a more correct prediction of the true accession than the ANN analysis. However, the percentages were all low, 34-36%. It was concluded that the ANN technique was useful for the separation of the Marrone and the chestnut types. Since this technique is inexpensive it can be a useful tool in identification of chestnut cultivars.

Flower morphology and phenology of C. sativa cultivars from southern Switzerland were studied by Rudow and Conedera (2001) with the purpose to identify characteristics useful for certification of cultivars. Five cultivars and trees from natural populations were included in this study. Four north–south transects were used for the pheno-logy part of this study with recording 30 times during the period May 30 – August 4.

The female flowering development in the four trees from natural stands showed limited difference in spite of their origin in different climatic regions. The peak in female flower receptivity is illustrated in Fig. 4-16. Some of the cultivars are protogynous while others are protandrous. Within individual trees flowering started in the basal part of the tree crown and continued upwards. This makes onset of flowering problematic as a characteristic for identification since it is dependent on position of the flowers. As regards morphological characteristics, anthers were grouped in five classes; astaminate = flowering buds without anthers to longistaminate = filaments > 5mm. These classes may be used for identification together with other characteristics. It was concluded that floral characteristics can contribute for identification of cultivars, but for a satisfactory cultivar certification requires more detailed studies of flowering characteristics. Since morphological traits to some extent are dependent upon ambient conditions, highly polymorphic markers seem to be a more fruitful approach for cultivar identification.

In a Slovak progeny trial with C. sativa x C. crenata and its reciprocal cross 114 trees at age 17 were assessed with respect to 30 growth, nut, and phenology traits (Bolvan-ský and Mendel 2001). The parent trees had contrasting phenotypes. The study, which also contained two trees of the parental species, was conducted during 1-4 years. Principle component analysis, PCA, and canonical discriminant analysis, CDA, were applied. ANOVAs were run for ten nut traits, which showed signif-

![Figure 4-15. The number of correct classifications in different classes of percentage following neural network and canonical analysis. This study comprised 17 C. sativa cultivars and 14 leaf morphology and color characteristics. Mancuso et al. 1999.](image1)

![Figure 4-16. Peak of female and male flowering in nine cultivars and one wild tree (W) of C. sativa from southern Switzerland. In four of the cultivars there was no pollen production. Days are counted from May 21. Rudow and Conedera 2001.](image2)
icant effects for female, male, female x male interaction, and trees within families. The first principle component explained 47.2% of the variation. The proportion of nuts with pellicle intrusions, length of filament, number of pellicle penetrations, length of pellicle penetrations, index of pellicle penetration, and nut size, were shown to be the most important. The CDA showed that the beginning of flowering, the beginning of fruit ripening, annual shoot length, the size of hilum, bur size, and the density of bur spines were the most important. Based on these results the following traits were found to be most discriminatory:

- Time of bud flushing
- Time of onset of male flowering
- Time of start of nut ripening
- Proportion of multiple-seed nuts
- Proportion of nuts with pellicle penetration
- Stamen filament length
- Nut weight.

The authors admitted that molecular methods probably will give more reliable discrimination among cultivars and newly selected trees.

The objective of the study carried out by Queijeiro et al. (2005) was to identify morphological traits that well describe 15 cultivars of *C. sativa* from Ourense in Spain. Nine metric traits of leaves and flowers, nine metric traits related to nuts, and six traits related to nut color were studied.

Traits of significance for distinguishing cultivars were:
- height and width of the leaf,
- number of teeth and veins,
- catkin length
- all nut traits except for nut volume and density.

Homonymy and synonymy of 50 Andalusian “cultivars” of *C. sativa* were studied by Alvarez et al. (2005). Eight nut traits were studied, which were used for a preliminary characterization of six important cultivars from Andalusia.

In a brief report Hunt (2005) presented data from 22 cultivars at ages 5-7 of *C. mollissima* and species hybrids in Missouri, USA. In 14 cultivars the nut weight at age five varied in the range 7.4-20.4 g. The corresponding range for age six was 5.5-15.7. One important finding was the large drop in nut size with increased yield. He reported that early results indicate strong differences in form, precocity, nut size and date of nut maturity.

Ten *C. sativa* cultivars were selected among 80 cultivars in Nazilli region in Turkey for a description of 19 morphological, pomological, and biochemical characters (Ertan 2007). This study comprised data collected during three years for most of the traits. ANOVA, multivariate analysis, and cluster analysis were performed. Ertan stated that there was a large variation among the accessions in the region studied, which is regarded as a region with high diversity. One illustration for this is given in Fig. 4-17 for one pomological, one morphological, and one biochemical trait. With the exception of one morphological trait, all others showed significant trait x year interaction. This renders the classification of accessions with these three types of trait problematic. The cluster analysis based on all traits revealed five main groups; one with 4, one with 3, and three with 1 accession.

The objective in two papers by Furones-Pérez and Fernández-López 2009a and 2009b was to identify descriptors, which are distinct, uniform and stable (DUS). Thirteen morphological and phenological traits were tested for characterization of 38 *C. sativa* cultivars. The cultivars were grafted on of *C. crenata x C. sativa* root stocks resistant against *Phytophtora cinnamomi*. The cultivars were growing at two localities in north western Spain with 12 and 35 cultivars, respectively; nine of them were common to the two localities. The assessment of the traits was carried out during 2-3 seasons at the two localities. Phenology recordings took place 23, 24, and 15 times during the three years of observation.

![Figure 4-17. The variation in one pomological, one morphological, and one biochemical trait in ten accessions of C. sativa from Nazilli region in western Turkey. Ertan 2007](image-url)
The objective in the first paper by Furones-Pérez and Fernández-López (2009b) was to identify morphological and phenological traits for distinct, uniform, and stable descriptors (DUS) of 38 cultivars. Separate ANOVAs were carried out for each plantation as well as a joint ANOVA for the cultivars common to the two plantations. (Broad sense heritabilities were calculated for individual plantations and for the joint analysis of the two plantations.) Phenotypic plasticity was estimated as the ratio: environmental variance + the variance of the genotype x environment interaction/ the total variance observed. Broad sense heritabilities were also calculated for phenotypic plasticity. Several traits in one or both plantations did not fulfill the requirement of normality for the ANOVA. For them transformations were carried out. Even so the normality was not always fulfilled. This means that some of the results obtained in the ANOVAs must be regarded as approximations. Variance components were estimated for all effects, which probably is a violation of the requirements for running ANOVAs.

The first step in identifying good descriptors is to find genetically regulated traits, i.e. traits with high estimates of cultivar variance components. Once they are identified their stability over site conditions or weather conditions, i.e. the interactions cultivar x plantation and cultivar x year should be low for good descriptors.

The variation in cultivar variance components among the 13 traits was large (Fig. 4-18). The authors remarked that the lack of genetic differences for some descriptors might be attributed to the restricted geographic origin of the collection. The low number of cultivars common to the two plantations might have a great impact on the results. The cultivar variance percentages did not exceed 10% for nut ripening, nut size, and nut length. Contrary to this, filament, nut split, and hilum size had high percentages in all three ANOVAs. The latter traits would be useful for discrimination among cultivars. The high percentage in the joint analysis for nut shape was unexpected. This must be attributed to the low percentages for the other factors: year, cultivar x year, site, cultivar x site, year x site, cultivar x site, pooled percentage = 10.

To illustrate the stability of the three traits with high genetic regulation I have illustrated the ratios cultivar x year/cultivar and cultivar x locality/cultivar variance components in Fig. 4-19. This figure reveals good agreement over years for filament and nut split while the interaction cultivar x locality is much larger. It should be noted that the variance component for hilum size was zero for hilum. In conclusion none of the traits were ideal for secure identification of cultivars. The estimated plasticity is illustrated in Fig. 4-20, in which low bars indicate good stability.

Figure 4-18. The percentage variance components for 13 traits analyzed separately for the two plantations or analyzed jointly. The plantations are located in north western Spain with C. sativa grafted on Phytophtora cinnamomi resistant root stocks of C. crenata x C. sativa. Furones-Pérez and Fernández-López 2009b.

Figure 4-19. Stability estimated as ratios of variance components for three traits: cultivar x year/cultivar and cultivar x locality/cultivar. For hilum size the cultivar x locality variance component was zero. Furones-Pérez and Fernández-López 2009b.
The author’s recommendations as regards descriptors to be used are summarized in Table 4-3 with filament length and hilum size as best descriptors. The recommendations do not totally agree with the results presented in Fig. 4-18. This might be attributed to the reliance on heritability for recommendations rather than on the cultivar variance components. However, there is full agreement as regards the best descriptors. They also suggested that these two traits together with nut split and nut shape could be used as descriptors thanks to their high heritability and stability. However, the nut shape variance component for cultivar at Sergude plantation was only 3% (Fig 4-18), which means a low heritability. It was remarked that the economically important traits, nut size and nut ripening were poor descriptors, which is evident from Fig. 4-18. In conclusion, the search for universally good descriptors for characterization C. sativa is a demanding task. The use of high-resolution molecular markers for identification of cultivars might be a more advantageous approach.

Table 4-3. Traits recommended as descriptors for various purposes and their characteristics. Furones-Pérez and Fernández-López 2009b.

<table>
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<th>Use</th>
<th>Trait</th>
<th>Characteristics</th>
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<td>Best descriptors</td>
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<td>high heritability, uniformity, and stability</td>
</tr>
<tr>
<td>In situ and collection characterisation</td>
<td>Nut split, Nut shape</td>
<td>High stability and heritability</td>
</tr>
<tr>
<td>Best descriptors for collection characterisation</td>
<td>Onset of bud flushing, onset of female and male flowering</td>
<td>High heritability and moderate uniformity</td>
</tr>
<tr>
<td>Descriptors for collection characterisation</td>
<td>Penetration, Hilum length/hilum width</td>
<td>High heritability and stability; more time consuming and low uniformity</td>
</tr>
</tbody>
</table>

Figure 4-20. The plasticity for 13 traits analyzed separately for the two plantations or analyzed jointly. The impact of weather conditions is included in the estimates from individual trials while the joint analysis estimates the impact of the site conditions at the two plantations, which are located in north western Spain with C. sativa grafted on Phytophthora cinnamomi resistant C. crenata x C. sativa root stocks. Furones-Pérez and Fernández-López 2009b.
Nine of the 13 traits treated in Furones-Pérez and Fernández-López (2009b) were treated in Furones-Pérez and Fernández-López (2009a). The traits and their number of classes are presented in Table 4-4. The excluded traits were nut size, nut length, nut ripening, and embryony. As far as I can see this second paper treats the same cultivars and assessments as the first paper. Photographic illustrations of some of the traits are a merit of this second paper. Detailed description of cultivar performances for individual traits were presented (eg. Table 4 in the paper), which might be useful for chestnut orchard owners but less so for the scientific community. Principal component analyses were carried out on the nine traits as well as on the following five:

- Filament
- Nut shape
- Hilum shape
- Nut split
- Penetration

In both PCAs, filaments and penetration occurred in the first principal component that explained slightly more than 25% of the variation. The PCA with nine traits resulted in a dendrogram with 32 groups while the corresponding for five traits was 23 groups.

In this paper it was stated that the following traits did not vary over years and matched at both sites:

- Filament
- Nut split
- Penetration
- Nut shape
- Hilum shape

It was concluded that all these traits are useful for DUS tests. However, assessment of them is time-consuming. Flushing, hilum size, onset of female and male flowering were regarded as the best descriptors for characterization in the collection because of their high heritability and moderate uniformity.

As far as I understand collection in this paper = the 38 cultivars studied. There are statements about descriptors for the collection, which I interpret as recommendations for just this collection of 38 cultivars. It would be useful to identify descriptors that are valid beyond this collection of 38 cultivars.

Results from two experiments with four and eight cultivars of *C. crenata*, including one hybrid between *C. crenata* and *C. mollissima* were reported by Nishio et al. (2014). The first experiment with four Japanese chestnut cultivars was run to estimate the number of nuts required for obtaining satisfactory precision in the second experiment. Bud flushing, nut weight, pericarp splitting, and insect infestation were reported for ages 4-9 years. ANOVAs were run for each of the four traits. Variance components for clone, clone x year interaction, year, and tree (within clones) were estimated although it is questionable to treat year as a random variable.

![Figure 4-21. The clone and clone x year variance components based on eight clones in a Japanese C. crenata trial with assessments over a six-year period. Flush = flushing date, Nut w = nut weight, Ps = pericarp splitting, Ins = insect infestation. Nishio et al. 2014.](image)

Twenty-eight *C. sativa* trees from different localities in Bursa region, Turkey, were analyzed with respect to five nut and seven leaf traits by Bostan et al. (2018). As far as I understand, Pearson correlations were calculated between all traits. As expected there were strong negative and significant correlations between number of nuts/kg and nut size (-0.83), and number of nuts per bur (-0.97). All other pairwise correlations were non-significant with one exception; petiole length and tooth width (0.62) The three nut traits showing significant correlations explained 25% of the variance in a principal component analysis and constituted the first component in this analysis. In all, four components explained around 69% of the variation.

Table 4-4. The traits analyzed, the number of classes used for evaluation of *C. sativa* cultivars. Traits in red are most useful for classification and those in black least useful. Furones-Pérez and Fernández-López 2009a.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Classes No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bud flushing</td>
<td>5</td>
</tr>
<tr>
<td>Stamens filament length</td>
<td>4</td>
</tr>
<tr>
<td>Onset of male flowering</td>
<td>5</td>
</tr>
<tr>
<td>Onset of female flowering</td>
<td>5</td>
</tr>
<tr>
<td>Seed coat penetration</td>
<td>3</td>
</tr>
<tr>
<td>Nut shape</td>
<td>5</td>
</tr>
<tr>
<td>Size of hilum</td>
<td>3</td>
</tr>
<tr>
<td>Shape of hilum</td>
<td>3</td>
</tr>
<tr>
<td>Presence of split pericarp</td>
<td>3</td>
</tr>
</tbody>
</table>
The objective of the study by Jiang et al. (2017) was to reveal the genetic diversity of 95 cultivars of *C. mollissima* from 10 provinces in China. These cultivars were excellent or traditional varieties cultivated under widely varying site conditions. Forty-one microsatellite loci were analyzed. Another objective was to relate the genetic profiles to 18 agronomic traits; five nut quality traits, four phenotypic traits and nine leaf traits. The association between the markers and phenotypic traits was done by the program TASSEL (Bradbury et al. 2007). A kinship matrix according to Hardy and Vekemans (2002) comparing the identity by descent between all pairs of cultivar was used to detect relatedness among cultivars. The mean observed and expected heterozygosities amounted to 0.32 and 0.45 and the gene flow was estimated at 1.8 migrants per generation. *H*<sub>o</sub> was higher than *H*<sub>e</sub> in 32 of the 41 loci. The mean number of alleles per locus was 2.7 which is several times lower than in natural populations of *C. mollissima*.

According to the STRUCTURE program the 95 cultivars were grouped into three populations with 25 cultivars (from 7 provinces), 40 cultivars (from 8 provinces), and 27 cultivars (from 9 provinces). The remaining three cultivars could not be clustered into any of the three populations. This grouping reveals that the three subgroups of cultivars occurred in several Chinese provinces and thus not a geographic bordering of the three populations. The origin of the cultivars as well as a complex breeding history can explain part of the clustering result. Furthermore, the existence of homonyms and synonyms complicates the tracing of their origin.

Analogous results as regards geographic structuring were obtained in the country-wide study of *C. mollissima* natural populations by Liu et al. (2013). A moderate inbreeding coefficient (0.4783) was reported, which was several times higher than for *C. dentata*, *C. sativa*, and natural populations of *C. mollissima* populations (Liu et al. 2013). It was suggested that common ancestry and domestication could explain the high F<sub>ST</sub> among these 95 cultivars. A F<sub>ST</sub> value of 0.187 was reported and it was stated: indicating significant genetic differentiation among the sites. The meaning of sites was not explained. Linkage disequilibrium was found for six loci. The low number was attributed to low number of markers and samples. In addition, there might be a difference between cultivars and wild populations. All phenotypic traits analyzed showed a large variability. The kinship study of the 18 phenotypic traits revealed that five traits had kinship percentages above 10% with a maximum value of 28%. Another five traits had values between 5 and 10%. The number of traits with no kinship was also five. The kinship for the markers showed that more than half of the 95 populations did not show any kinship with other cultivars while eleven cultivars had kinship estimates of 25%. The highest kinship estimate amounted to 70%. This suggests that total genetic identity did not exist between any of the cultivars.

**Figure 4-22.** The number of associations between 18 economic traits and microsatellite markers in 41 loci. The study comprised 95 *C. mollissima* cultivars. Jiang et al. 2017.

Fourteen of the SSR loci had associations with phenotypic traits explaining 0.05-16.3% of the variation in these traits. One locus, *CrsCAT5*, had the highest number of associations, six. Among the phenotypic traits, starch had the highest number (5) of associations with SSR loci with estimated explanations of 0.06-12% of the variation. Fig. 4-22 reveals that two associations to SSR markers are most common. It was concluded that the results of the association analysis suggest that many of the agronomically important traits are quantitative and thus regulated by genes in many loci.

Offspring from a cross between two interspecific hybrids ([(*C. mollissima* x *C. seguinii*) x (*C. crenata* x *C. sativa* x *C. dentata*), the latter parent is probably one interspecific hybrid which was crossed with still another species] were studied at age nine in Turkey by Macit et al. (2018). Ripening time, growth, tree habitus, and ten nut characteristics of seven offspring trees were studied. The data obtained was compared with a Turkish cultivar. Generally the growth habit, crown width/height was much larger in the new material than in the Turkish cultivar. Stem diameter varied in the range 9.6-13.7 cm. Start of nut yield varied between years 2-7 with cumulated yield per tree varying in the range 0.6-23.5 kg. Six of the hybrid trees outgrew the comparison cultivar in several respects. Three of the seven studied trees were selected for future establishment in fruit orchards. They were characterized by larger polylemethylyoxy than the Turkish cultivar, which might be a drawback.

### 4.2.2 Markers

By aid of six polymorphic isozyme loci Fineschi et al. (1994) reported on genotypic differentiation of 20 varieties of *C. sativa* from three regions in Italy; southern, central, and northern. The central Italian region had two types of varieties, a flour producing and a nut quality variety. All varieties have a long tradition of cultivation and breeding of chestnut fruit varieties.
The vegetative propagation of material for establishment of fruit orchards was evident from their isozyme pattern. Even a single clone occurred in one of the varieties from central Italy aimed for nut production. Least homogeneity was noted for the northern material and the flour varieties in central Italy. There were significant deviations from the Hardy-Weinberg expectation with a surplus of heterozygotes, which speaks for frequent vegetative propagation of materials for fruit orchards.

The genotypic distances between varieties among regions as well as within regions were with one exception large (Fig. 4-23). It was speculated that the low level heterogeneity of most fruit varieties would make them more susceptible against pests and environmental changes. For future it is necessary to establish breeding populations with large genetic variability to guarantee progress in breeding. Such breeding populations may also serve as genetic conservation populations.

RAPDs were developed and used for identification of 16 cultivars from Campania region in Italy by Galderisi et al. (1998). Some of the morphological classifications were incorrect. The RAPD identification of cultivars is a useful technique to support the applied breeding and trade of C. sativa.

Several reports were devoted to locate and describe cultivars used in different provinces by Pereira-Lorenzo and coworkers. The same material occurs repeatedly in several papers and it is not easy to follow what is new information and what was presented earlier. In the two papers by Pereira-Lorenzo et al. 2001a and 2001b 152 cultivars from mainland Spain and 38 from the Canary Islands were described. Of the mainland cultures, 72 were reported as new ones. It remains to determine how many of them are identical genetically but with different names.

The isozyme variation within and among six Spanish cultivars of C. sativa was reported by Pereira-Lorenzo and Fernandez-Lopez (1995). Of the seven enzyme loci studied six were polymorphic. One cultivar produced aesthetically good wood quality. Another cultivar was used for production of beams for building constructions while the rest were cultivated for nut and wood production. Genetic differences between the cultivars were estimated according to Cavalli-Sforza and Edwards’ (1967) chord distances.

The mean genetic distances for the six cultivars based on the figures given in Table 5 of the paper are shown in Fig. 4-24. It should be noted that some of the figures given in the text of the paper do not agree with the figures in Table 5. The Parede and Parese populations showed the smallest difference, 0.159, while the difference between Garrida and Loguesa had the largest chord estimate, 0.339. It might be speculated that there are genetic differences between cultivars depending on the main end use of a cultivar; mainly wood or mainly for nut production. Therefore, I calculated the mean genetic distances within the two groups of end use; wood or wood + nuts, as well as between cultivars with different end use. As is illustrated in Fig. 4-25 there is no indication that this would be the case in the present material; rather the opposite was the case. It should be remarked that the number of entries in the two groups is low. However, if there were clear trends it would call for extension of this study.

The main objective of the study of Galician cultivars of C. sativa was formulated in this way by Pereira-Lorenzo et al. (1996a); to define primary morphological traits to be useful for a simple morphological classification systems of the cultivars. Eighty-two local cultivars with 373 trees were included in this study. Fourteen quantitative traits and three quality traits were studied. It was found that nut size, shape of nuts, male catkin type, and spine length on the burs were the most discriminating characteristics. Based on these traits a discrimination system was suggested. Relationships with ambient factors were determined. All of them had extremely low degrees of explanation of the relationships.
Several studies were devoted to development of molecular markers for *C. sativa*; such as Botta et al. (2001), Marinoni et al. (2003), Gismondi et al. (2015). Although such markers are most useful for a variety of genetic studies on chestnut the technical description of marker development is beyond the scope of this paper. Especially, identification of genotypes for certification and preservation benefit from the possibility to use microsatellite loci for this purpose.

Pafetti et al. (1999) used the RAPD technique to distinguish 13 Italian cultivars of chestnut. Three categories of the cultivars were distinguished, which agreed with the end use of the cultivars; flour, fresh nut and flour, and wood.

A brief report on Portuguese chestnut cultivar synonymy was presented by Pereira et al. (1999). Seven isozyme loci were studied. Thirty-two isozyme genotypes were distinguished, nine of these 32 comprised two or more cultivars.

RAPD genotyping of four *C. sativa*, one *C. crenata*, and two hybrid families between these two species was carried out by Santana et al. (1999). In all, 164 polymorphic bands were obtained with the twelve primers used. There was a clear genotypic separation between the three types of material. Some of the RAPD fragments were specific for each type of material. Three subclusters were identified among the 34 hybrid individuals. Two of the three subclusters contained trees from both hybrid families.

Botta et al. (2001) identified 68 alleles in five microsatellite loci following multiplex analysis, ie simultaneous analysis of several loci mixed together.

English *C. sativa* populations were used for development of a microsatellite library by Buck et al. (2003). In all thirteen microsatellite loci were isolated with the number of alleles varying between two and 14. Eight of them were found to be useful for genetic studies.

Boccacci et al. (2004) tested the possibility of using oak microsatellite loci for studies of polymorphism and fingerprinting in *C. sativa*. Six loci were recommended for use in fingerprinting.

Aravanopoulos and Drouzas (2005) compared the genetic set-up in two Greek orchards of *C. sativa* with the structure in their adjacent natural populations in northern and central Greece. Each population was represented by 26 trees and seven polymorphic loci were studied. Based on the multilocus pairwise comparison of the 52 orchard trees eleven unique genotypes were revealed. Utilizing information from six loci all the clones in the orchard could be identified. Only one of the genotypes (clones) was common to both orchards. The percent-
age of unique genotypes in the orchard was estimated at 21% while the corresponding for the natural populations amounted to 70-86%. Genetic diversity was larger in the natural populations than in the orchards. The expected heterozygosities in the natural populations were more than twice as large as in the corresponding orchards and strongly significant; 0.173 vs 0.073 and 0.265 vs 0.092. The authors argued for additional studies of orchard populations by molecular genetic markers to get still more reliable cultivar identification, which is most useful in chestnut breeding. By advances in breeding it is anticipated that some convergence of characteristics will occur, which with high probability will make morphological cultivar identification difficult.

Six microsatellite loci were used by Martin et al. (2005) to study the variability of 78 C. sativa trees from the main regions for cultivation of this species in Andalusia, Huelva and Malaga. The diversity in Malaga was higher than in Huelva. All pairwise comparisons of the 78 trees were done to estimate similarity coefficient. Two cultivars with three and five trees growing at more than two localities showed limited similarities with the other 75 or 73 trees, Dice’s similarity = 0.11 in both cases.

Costa et al. (2005) studied the differentiation within and among eleven Portuguese cultivars of C. sativa by aid of five microsatellite loci. In all, 92 trees were included in the analysis. In this preliminary report the number of alleles per locus varied in the range 3–6. The differentiation among cultivars was limited but five groups of cultivars were identified.

Microsatellites were used in a series of investigations to characterize C. sativa cultivars from southern Spain and Italy by Martin et al. (2007, 2009, 2010a, 2016). The objective of the paper from 2007 (Martin et al. 2007) was descriptive and formulated in the following way to locate and catalogue the different chestnut groves and to collect samples of fruit and leaves to study the genetic variability existing in Andalusia. In all, nuts and leaves from 156 trees in 14 localities were collected. The variation in altitude among the localities was 554-842 masl in Huelva and 472-995 masl in Malaga district. In addition 29 localities from other parts of Andalucía were included in the collections. No quantitative evaluation of the collected material was given.

In the report from 2009 (Martin et al. 2009), 34 traditional “cultivars” represented by 100 grafted trees from Huelva and Malaga regions in southern Spain were investigated by aid of seven microsatellite loci and ten morphological or phenological traits, three of which turned out to be monomorphic. Precise description of varieties is essential to make a preservation of a broad collection of genetic varieties. This is the more important since plantations were abandoned or were cut down and replaced by new varieties with improved quality. The four stamen types (longistaminate, mesostaminate, brachystaminate, astaminate) of the male catkins were useful in classification of the trees. The number of alleles per locus varied in the range 5-13. The number of possible genotypes in the seven loci varied in the range 9-25. In all, 38 varieties were identified, 12 in Huelva and 26 in Malaga. Approximately one half of them was represented by one tree only. It was concluded that the combination of molecular and morphological classification was useful for distinguishing varieties. It was stressed that genetic preservation of C. sativa by orchard owners is a useful way to pursue.

In one of the papers from 2010 (Martin et al. 2010a), 94 trees supposedly belonging to Italian varieties originating from localities distributed all over Italy were analyzed with the same seven loci as in the previous report. Samples were collected in one southern Italian arboretum while others were obtained from University of Turin. Nine of the varieties were classified as Marrone while 17 others were classified as chestnut. The number of alleles varied between 3 and 11; with a total number of 52 alleles. With one exception rare alleles were present in all loci and four of them had two unique alleles each. The number of genotypes per locus varied in the range 4-16. Twenty genotypes were detected among the 26 accessions in this study. It was noted that all 20 genotypes could be distinguished by the alleles in two loci. The probability for identity could be determined with extremely good precision by use of the genotypes in the seven loci. Thus, these loci are highly informative and would be most useful for certification of accession identity. There was a clear difference between material from southern Italy and other parts of Italy. Similarly, the genotypes of the Marrone accessions differed from other accessions. Three different genotypes were identified for the marrone accessions, one of them was found in 31 trees from the most important Marrone cultivars in Italy.

Medina and Fulbright (2010) carried out a study with the objective to find microsatellite markers for identification of chestnut trees cultivated in orchards in Michigan. One male-sterile C. sativa x C. crenata was used as female in four crosses. Another C. sativa x C. crenata hybrid, one C. crenata x C. pumila, one C. mollissima x (C. crenata x C. dentata) and one C. mollissima were used as males. In all 187 seedlings were analyzed with respect to previously used microsatellite markers. Four primers of the five markers showed polymorphic bands for all parents and progenies. However, only one locus was informative for identification of a genetic entry. The informative locus had six alleles. The probability that two random genotypes could be distinguished by alleles in this locus was 0.72, which is unsatisfactory for identi-
fication purposes. Considering the broad genetic material in these crosses, 0.72 seems to be an unexpectedly low value. The authors concluded that additional microsatellite loci must be tested.

The objective of the investigation by González et al. (2011) was to genetically identify species or interspecific hybrids resistant to Phytophthora species. They used nine microsatellite loci and tested 33 trees from the three Asian chestnut species, *C. crenata* (10), *C. henryi* (13), and *C. mollissima* (10). As regards *C. sativa* 20 accessions from three provenance regions, 20 accessions from 12 *C. sativa* clones, and 8 accessions from three interspecific *C. crenata* x *C. sativa* hybrid clones were analyzed. Finally, 32 accessions from 15 trees selected for resistance against Phytophthora species were studied. Expected and observed heterozygosity, the frequency of null alleles, and polymorphic content were determined. No deviation from Hardy-Weinberg equilibrium was observed. As many as 172 alleles were detected in the four species. In four loci the allele distribution differed between *C. sativa* and the three Asian chestnut species. Four alleles occurred only in the Asian species but not in *C. sativa*. Other alleles occurred only in *C. sativa*. Seventeen percent (= 23 alleles) of the *C. sativa* alleles were shared with the three Asian species; most of them with *C. crenata*. It was concluded that the nine SSR loci were useful for discrimination among the four chestnut species studied.

The AMOVA revealed that that most of the variation occurred among individuals, the percentage variance component being 66%. Two loci explained 86 and 89% of the variance among individuals. Grouping of the species/populations by use of the neighbor joining method showed that *C. henryi* and *C. mollissima* were most separated from the other two species and also separated from each other. *C. crenata* was closer related to *C. sativa* than the two other Asian species. The recently selected trees took an intermediate position between *C. crenata* and *C. sativa*. There was some differentiation among the four groups of *C. sativa* accessions.

As regards the identification of the 15 selected trees for resistance to Phytophthora I have illustrated the occurrence of unique *C. sativa* and Asian alleles in Fig. 4-26. This figure reveals that five of the 14 trees with unique alleles do not contain any unique Asian alleles and most likely are pure *C. sativa* trees while the nine other trees most likely are interspecific hybrids between *C. sativa* and Asian species. Thus, the prime objective of this investigation was fulfilled.

The matings between one clone and four *C. dentata* x *C. crenata* trees in an orchard in Michigan were studied by Medina-Mora et al. (2014). Two trees, one *C. crenata* x *C. pumila* (male 1) and one *C. mollissima* (male 2) served as pollen donors. It was stated that these two trees were the only pollinators in this fruit orchard. In all, 120 female flowers were covered with pollination bags on June 20. Three days later half of them were uncovered and exposed to the general pollen flow. Seventy flowers were successfully pollinated. The matings were analyzed by aid of two microsatellite loci. In case of ambiguity an additional microsatellite locus was used. It was disclosed that male 1 gave rise to 48 of the 70 (≈ 68%) nuts while the corresponding number for male 2 was 21 out of 70 (30%). The pollinator of one nut could not be determined. No discussion of this unbalanced mating pattern was done. Could it be attributed to differences in pollen production or differences in phenology or anything else? Without such a discussion this report just tells that pollinators might be identified.
Fernández-Lopez and Fernández-Cruz (2015) reported on the development of genetic markers for identification of cultivars and clones of *C. sativa*, which today is required for selling of plant material for fruit orchards. The focus was on commercial material for different climatic conditions in Galicia, Spain. Multilocus genotypes based on ten microsatellite loci in 26 varieties were obtained. With the large number of alleles in each microsatellite locus it is easy to separate clones from each other. Such a genotyping offers also opportunities to identify clones that were given different trade names as well as finding molecular genetic relationships among clones and cultivars.

Microsatellite markers from expressed sequence tags, EST-SSR, were used to study the relationship between 50 clones/cultivars from Italy and Spain (Martin et al. 2016). Nine loci with a total number of 46 alleles were used for the genetic separation of cultivars. A tenth locus was excluded from the analysis since it contained null alleles. The program STRUCTURE was used to study whether there was clustering of the clones. Except for one locus, rare alleles with frequencies <0.05, were found in all loci. LOSITAN according to Antao et al. (2008) and BAYESCAN according to Foll and Gaggiotti (2008) programs were used to detect outlier loci that might indicate their role in adaptation. Seven private alleles were detected in five loci. The number of genotypes in each locus varied between 5 and 16. The gene diversity was somewhat higher in the Italian cultivars than in the Spanish ones, 0.66 versus 0.57.

The analysis of F_{is} for the entire material was positive but non-significant for all loci with a mean value of 0.076. Contrary to this, a separate analysis of the Spanish material showed significance. There was an extremely large variation among the loci ranging from -0.197 to +0.681.

Figure 4-27. The percentage attributed to the three clusters obtained from the analysis of nine EST-SSRs in *C. sativa* clones of Marrone type originating from and grown in regions Huelva and Malaga in Spain and in northern Italy. Martin et al. 2016.

Figure 4-28. The percentage attributed to the three clusters obtained from the analysis of nine EST-SSRs in *C. sativa* clones of non-Marrone type coined chestnut originating from and grown in regions Huelva and Malaga in Spain and in southern and northern Italy. Martin et al. 2016.

Both extremes (Italy and Spain) concerned the same locus. The genotypes of the nine loci enabled separation of all cultivars from each other. The genetic differentiation estimated as F_{st} was 0.035 while the corresponding R_{st} was approximately twice as high, 0.078. These estimates are several times lower than reported for the analysis of nine high forest populations from Greece, Italy and Spain (Martin et al. 2010b). The separate dendrograms for Spain that were presented in the paper showed an extremely clear separation of Huelva and Malaga cultivars. Similarly, northern and southern cultivars from Italy differed clearly.

The STRUCTURE program suggested that three clusters had the highest probability for subdivision of the 50 cultivars. Cluster 1 dominated in the Huelva material while cluster 3 dominated in the two Italian groups of clones. The dominance for cluster 2 in the Malaga clones was not as pronounced as was the case for the other two clusters. The conspicuous genetic separation of the Spanish and Italian cultivars was somewhat unexpected since trading of chestnuts between Italy and Spain took place to a great extent already during Roman times. The reason for the presence of a clear difference between Spanish and Italian cultivars was attributed to selection of material by fruit orchard owners resulting in increased adaptedness to local conditions. Thus, a development of land races had taken place.

Since the cultivars included were of two clearly distinguished phenotypes I have illustrated the subdivision separately for Marrone and chestnut clones (4-27 and 4-28). There were no Marrone cultivars from southern Italy. The two phenotypes, Marrone and chestnut, resulted in different clustering for the Malaga cultivars with a fairly low percentage in cluster 2 of the Marrone cultivars but a high estimate for chestnut cultivars in cluster 2. No clear difference for the other two groups of cultivars was ob-
served. Thus Huelva clones occurred mainly in cluster 1 while the majority of northern Italy clones belonged to cluster 3. It was expected that at least some of the loci studied would have an effect on adaptation but it was concluded that: *none of the nine loci showed any significant deviation from neutral expectations.*

One hundred and fifty-four trees from Chestnut germplasm of southern Switzerland were analyzed with respect to eight microsatellite loci by Gobbin et al. (2007). In addition, ten samples were analyzed with alleles in seven loci. Four of the microsatellite loci were developed by the authors. The number of alleles per locus varied in the range 4-21 with a total number of 78 alleles and 98 genotypes. Four main clusters were distinguished, one of them including the hybrids between *C. sativa x C. crenata.* Somewhat surprising was the split of the Marrone type of trees into two groups.

Ten nuclear microsatellites were used for description of 17 *C. sativa* plantations in two Croatian counties by Prgomet et al. (2014). Sixty-two alleles were detected with 3-8 alleles per locus. In all 14 genotypes were detected, of which 13 were represented by single individuals. It was noted that one unique genotype was found in a 900 years old tree.

Abdelhamid et al. (2014) compared the genetic structure following analyses of 73 accessions with RAPD, AFLP, ISSR, and SSR markers. Fifty-two of the *C. sativa* accessions originated from Switzerland and five French and Italian accessions were included in this study. Nine of the Swiss accessions were coppice shoots. *C. crenata* and *C. mollissima* were represented by six and five accessions, respectively. They were regarded as five groups in the estimations of variation among and within groups. Polymorphism information content (PIC), multiplex ratio (MR), effective multiplex ratio (EMR), and marker index (MI) were determined to obtain information on the discriminative potential of the four types of marker.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Clusters and remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAPD</td>
<td><em>C. crenata, C. mollissima,</em> and two <em>C. sativa</em> clusters; no separation of French, Italian and Swiss groups</td>
</tr>
<tr>
<td>AFLP</td>
<td>No separate cluster was noted for <em>C. crenata,</em> a clear separation of the Swiss <em>C. sativa</em> accessions</td>
</tr>
<tr>
<td>ISSR</td>
<td><em>C. crenata, C. mollissima,</em> two <em>C. sativa;</em> no separation of French, Italian and Swiss groups with one exception as regards one <em>C. crenata</em> accession</td>
</tr>
<tr>
<td>SSR</td>
<td>No clear separation of the two Asiatic species</td>
</tr>
<tr>
<td>Joint</td>
<td>Limited differentiation. Asian species were clustered in separate groups</td>
</tr>
</tbody>
</table>

Abdelhamid et al. (2014) showed that AFLP had the highest values for all these variables. Thus for marker index it was 50.77 as compared for 6.52, 5.57, and 1.15 for RAPD, ISSR, and SSR. It was stated that AFLP showed *supremacy* for studies of genetic diversity. The estimated variation among and within populations is illustrated in Fig. 4-29, which shows that the among-population differentiation estimated by aid of AFLP is smallest followed by SSR. The among-groups differentiation estimated by AFLP is unexpected limited considering that three species were included in this study.

The results of the cluster analysis and principle coordinate analysis are summarized in Tables 4-5 and 4-6. It is obvious from these tables that the markers give different results. It was stated that the cluster and PCoA analyses showed unique genetic structure of the Swiss *C. sativa* group. This was true for most of the markers but not for the AFLP cluster analysis.
Correlation coefficients for a distance matrix from a man
tel test of the four markers were calculated. Fig. 4-30 re-
veals that the correlations with SSR data are weakest and
non-significant. It was partly attributed to co-dominance
of this marker. The strong correlation between RAPD
and ISSR, $r = 0.77$ was attributed to dominance of these
markers and that only a small fraction of the genome was
sampled. In three of the five dendrograms published, the
Swiss variety SativalT05 was strongly differentiated from
the other accessions
It was concluded that genetic conservation would benefit
from AFLP studies of genetic diversity. As long as this
and other markers do not reflect the adaptive variation it
is risky to rely solely on markers for genetic differen-
tiation and conservation. It may be of more importance for
genetic preservation. The limited among-population vari-
ation revealed by the AFLP analysis clearly suggests that
this marker does not reflect the adaptive variation among
the three studied *Castanea* species.

![Correlation coefficient](image)

**Figure 4-30.** The pairwise correlation coefficients be-
 tween four types of markers: RAPD, AFLP, ISSR, SSR,
and a combination of these markers. This study com-
pired 52 Swiss *C. sativa*, 5 French and 5 Italian *C. sativa*,
6 *C. crenata*, and 5 *C. mollissima*. Red columns refer to
coefficients $< 0.50$ and all of them non-significant. *Abdel-
hamid et al. 2014.*

Part of the results in the above paper was already pub-
lished by *Abdelhamid et al. 2004.* In the latter paper com-
prising 52 *C. sativa* accessions, only results from the
RAPD and AFLP analyses were presented. Since this pa-
does not contain any Asiatic species it is evident that
the dendrograms of the 52 accessions common to the two
papers are not identical. As an example, accession Verda-
nese 11 takes a dramatically different position in the den-
drograms for RAPD. The correlation coefficient between
RAPD and AFLP from the mantel test was stronger in this
paper than in the 2014 paper, 0.78 versus 0.69.

*Yamamoto et al. (1998)* used AFLP markers for a genetic
description of 24 cultivars used in Japan:
* C. crenata 14
* C. mollissima 7
* C. henryi 1
* Species hybrids 2

Nine sets of primers were used and 18 polymorphic bands
per primer were obtained. The UPGMA dendrogram re-
vealed that nine of the *C. crenata* cultivars were closely
related while two *C. crenata* cultivars from Korea dif-
fered most from the first group of nine. One *C. mollis-
sima* cultivar from USA had one of the lowest similar-
ity indices with the rest of the cultivars. The rest of the
cultivars did not display any clear species groups. It was
noted that the Korean *C. crenata* cultivars resemble the
Japanese *C. crenata* cultivars morphologically but could
be distinguished by the AFLP method.

An investigation with the objective to compare cultivars
of *C. crenata* in Japan and Korea as well as between cul-
tivars and populations in nature (called wild strains in the
paper) was reported by *Yamamoto et al. 2003.* Besides,
a comparison with *C. henryi*, *C. mollissima* *C. sativa*, and
* C. seguinii* was also aimed at.

Fourteen microsatellite loci in *C. crenata* were found to
be polymorphic. Analysis of full-sib families showed no
deviation from Mendelian inheritance. The number of al-

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### Table 4-6. Summary and the author’s interpretation of the results from the principle coordinate analysis, PCoA, as sum-
marized by the authors. *Abdelhamid et al. 2014.*

<table>
<thead>
<tr>
<th>Marker</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAPD</td>
<td>Clear separation of the Asiatic species from <em>C. sativa</em>; clear separation of Swiss <em>C. sativa</em> from the French, Italian, and coppice <em>C. sativa</em>; clear separation of the two Asiatic species</td>
</tr>
<tr>
<td>AFLP</td>
<td>Clear separation of the Swiss cultivars from the other accessions</td>
</tr>
<tr>
<td>ISSR</td>
<td>Clear separation of the Swiss cultivars from the other accessions. My remark: According to the plot shown in Fig. 8 in the paper the separation does not look that clear.</td>
</tr>
<tr>
<td>SSR</td>
<td>Clear separation of the Swiss cultivars from the other accessions</td>
</tr>
<tr>
<td>Joint</td>
<td>Clear separation of the Swiss cultivars from the other accessions. My remark: Three groups with two groups mainly containing Swiss accessions except for coppice shoots, and a third group with the rest of the accessions</td>
</tr>
</tbody>
</table>
leles per locus varied between two and sixteen. A high level of polymorphism was noted for C. crenata; heterozygosity = 0.50, polymorphic information content = 0.54 and a mean number of alleles per locus = 6.5. This high level was attributed to the wind pollination of this species.

In contrast to the previous report, there were no distinctive differences as regards the genetic constitution for these 14 microsatellite loci among the four categories of populations. It was stressed that a larger number of populations must be studied to reach a final conclusion about the origin of C. crenata cultivars. The cultivars and wild populations could be identified by a joint analysis of the alleles in the 14 microsatellite loci. The microsatellites developed in this project might be used to identify the different chestnut species.

Alleles of all 14 loci were found in C. sativa, and C. seguinii. For the two other species, C. henryi and C. mollissima, alleles were missing in two or three loci.

In a brief report Ovesná et al (2005) reported on AFLP characterization of 21 C. mollissima cultivars commercially used in subtropical China. In addition three locally selected rootstocks were included in this study. In all, twelve selective primer pairs were selected for this analysis. The number of detected fragments was 381 and the mean percentage of polymorphism was 52%. According to the authors the UPGMA dendrogram revealed two main groups; the rootstocks and the cultivars. The three rootstocks showed large dissimilarity among themselves. It was stated that the productive cultivars reflected their place of origin.

Nishio et al. (2011) developed twelve SSR markers for a study of 216 chestnut accessions of six Castanea taxa in Japan. One objective was to identify occurrence of synonymy among the accessions. They identified 189 different genotypes and 21 synonym groups. The UPGMA revealed three major groups; 1. C. crenata, 2. C. mollissima + the hybrids between these two species, and 3. C. dentata. The C. sativa and C. seguinii accessions were not included in the UPGMA construction. The expected and observed heterozygosity of the C. crenata accessions were the same, 0.65.

With the aim of finding synonymies and homonymies in 35 cultivars in Missouri, USA, McCleary et al (2013) analyzed eleven EST-SSRs in 214 trees from these cultivars. Their definition of homonymy was cultivars of the same name that appear in different structure groups of cultivars of the same name having more than one mismatch for a pairwise comparison of at least eight loci.

Synonymy = two cultivars of different names that are the same i.e. having no more than one mismatch for a pairwise comparison of at least eight loci. The EST-SSRs were originally developed for C. mollissima and they were found to be useful for the present purpose. The concern that EST-SSRs would be less informative than gSSR (genomic or anonymous microsatellite-containing sequences) was found to be unjustified. Twenty-five of the 36 cultivars with more than one representative in this study were homonymous; 12 had two homonyms and 13 had three or more homonyms. In one case six different cultivars were found to be synonymies. Other cases occurred with four and five synonymies. Clearing up the homonymies and synonymies is useful for breeding and preservation and not the least for trading of cultivars.

4.2.3 Markers and metric traits

In a report from 1997 Pereira-Lorenzo and Fernandez-Lopez (1997a) presented detailed information on chestnut cultivars used in North-Western Spain. The main objective was to bring some order of commercial cultivars such as the occurrence of homonymy, intra-, and inter-cultivar variability. Besides, they selected some promising trees for nut or timber production and grafted them and included these clones into the clonal archive at Centro de Investigaciones Forestales de Lourizan. This article is certainly most valuable for commercial growers of C. sativa in North-Western Spain, in which they can find description by morphological characters as well as their end products of the large analyzed material.

The objective of a Portuguese study of six cultivars was to get clear description of these cultivars by detailed morphological traits and RAPD and ISSR markers (Gouлhлo et al. 2001). In all 17 morphological variables were recorded during five years in four trees per population growing in Alcobaça. The marker study was carried out on one tree per cultivar. ANOVA, multivariate analysis, and regression with climate variables were carried out on the morphological traits. The climatic variables were obtained from an adjacent (7km) meteorological station. An effective thermal index was calculated for each year, which was defined as the accumulated temperature above 7°C between flushing and budset. Besides, precipitation from April to October and from July to October was correlated with the morphological traits. The ANOVA revealed strong tree x year significance for 16 of the 17 morphological traits while only four of the traits showed significant tree effects. As a corollary of the strong interaction the dendrogram for the five years differed.
I have simplified the dendrograms in Fig. 4-31, in which the two main groupings for each year are indicated. The dendrograms for year 1-2 differ from the dendrograms for year 3-5. Both order of the cultivars and their groupings differ. The difference in dendrograms between years is a constraint for the morphological traits as reliable descriptors of *C. sativa* cultivars. Only three of the morphological traits were significantly correlated with some climate variables.

There were suspicions that the pairs of cultivars L5 and L6 as well as M1 and M2 were homonyms. All results in Fig. 4-31 clearly show that this is not the case. The number of polymorphic RAPDs was 125 and the number of polymorphic ISSR fragments was 157. The dendrograms based on the two types of marker were almost identical and different from the morphological dendrograms (Fig. 4-31). The authors concluded characterization based on molecular markers is faster, less expensive and more reliable than the one based on morphological characters. However, it was also stated that morphological characters under strong genetic control might be useful in applied breeding.

Nuts from 171 *C. sativa* trees growing in three geographically isolated populations in Slovenia, eastern interior, central interior and Mediterranean area were examined with respect to eight exterior nut traits and two weight traits (Solar et al. 2001). More or less, the same material and analyses were presented in the paper below and therefore I do not treat this paper further.

Nuts from 244 *C. sativa* trees growing in three geographically isolated populations in Slovenia, eastern interior, central interior and Mediterranean area were examined with respect to eight exterior nut traits and two weight traits (Solar et al. 2005). RAPDs in 46 of these trees were used to estimate genotypic diversity and its relationship with the phenotypic traits.

Generally, the fruits from the central interior region deviated most from the two other regions. Especially the nut weight was lowest from the central interior region. As a corollary of this, the number of nuts per kilogram was highest in this population. The undesirable polyembryony varied in the range 0-23%; with highest percentage in the central interior trees. Cluster assignment of the 46 trees with respect to phenotypic and RAPDs resulted in six clusters for the former. As seen from Fig 4-32, trees from the eastern and central parts of Slovenia occurred in five clusters while the trees from the Mediterranean region occurred in three clusters. Noteworthy is one tree (KOZ1) originating from eastern Slovenia that occurs in the “Mediterranean” cluster IV. Its nut shape and nut weights agree with the Mediterranean trees but its pellicle intrusion was more pronounced. Fig 4-32 shows that no clear geographic differentiation was observed. The result of the comparison of the clusters obtained from phenotypic traits is shown as percentage of agreement between the two estimations of clusters (Fig 4-33). The best agreement was noted for cluster IV, in which the nine trees from the Mediterranean region were very homogeneous. It was speculated that they were grafts possibly belonging to one clone. It is likely that 100% agreement would have been obtained if tree KOZ1 was not assigned to cluster IV.

Cluster assignment of the 46 trees with respect to phenotypic and RAPDs resulted in six clusters for the former. As seen from Fig 4-32, trees from the eastern and central parts of Slovenia occurred in five clusters while the trees from the Mediterranean region occurred in three clusters. Noteworthy is one tree (KOZ1) originating from eastern Slovenia that occurs in the “Mediterranean” cluster IV. Its nut shape and nut weights agree with the Mediterranean trees but its pellicle intrusion was more pronounced. Fig 4-32 shows that no clear geographic differentiation was observed. The result of the comparison of the clusters obtained from phenotypic traits is shown as percentage of agreement between the two estimations of clusters (Fig 4-33). The best agreement was noted for cluster IV, in which the nine trees from the Mediterranean region were very homogeneous. It was speculated that they were grafts possibly belonging to one clone. It is likely that 100% agreement would have been obtained if tree KOZ1 was not assigned to cluster IV. The low number of trees in the phenotypic clusters I-III means that the estimates of percentage agreement between the two types of traits are rather imprecise. Moreover, it is likely that markers like RAPDs are neutral and thus not selected for.

In a brief report by Becarro et al. (2005) leaf and nut morphological as well as phenological characterization of six cultivars from north-western Italy was compared with characterization by aid of seven microsatellite loci. Leaf morphology and phenology were inefficient in classification of these cultivars. It was stated that the following nut traits were useful for distinguishing the cultivars: width, length, thickness and exterior color. Stamen length was also useful for identification of cultivars. The number of alleles per microsatellite locus varied between three and seven. In all, ten genotypes were observed. One cultivar contained three genotypes and two others had two
genotypes. The identification of cultivars based on nut traits and alleles in microsatellite loci gave different results, which calls for further comparative studies of the two methods.

Samples of nuts and leaves from 33 clones belonging to 18 cultivars were examined by Botta et al. (2005). The material was genotyped by aid of microsatellites in ten loci. Different chemical analyses of carbohydrates, lipids and proteins were also carried out.

The clones within each of four cultivars had identical microsatellite genotypes; Marrone being one of the cultivars. Even mislabeling of cultivars was detected. Thus the cultivar Marubia had the same genetic profile as Marrone.

As regards the chemical analysis the range of the substances varied considerably. Moreover, they were strongly affected by the environmental conditions. It was stated that a specific chemical profile for any cultivar could not be obtained.

Ten microsatellite loci, 19 morphological traits, and one phenology trait were analyzed in 68 grafted C. sativa trees from the Piedmont region in north-western part of Italy by Marinoni et al. (2013). Characterization and genetic structure of the material used in commercial plantations were the two main objectives. The genetic structure was estimated by use of the software STRUCTURE. The occurrence of hybridization was investigated by estimation of the paternity exclusion probability according to Tanaka et al. (1999). As a consequence of possible loss of cultivars in the Piedmont region there was a strong applied focus in the paper.

In all, 80 alleles were found with a range of four to 14 alleles per locus. As many as 22 alleles occurred in frequencies below two percent and they were frequently specific to a single genotype. The observed heterozygosity for individual loci varied in the range 0.64-0.89. No deficit of heterozygosity was noted for any of the ten loci. The total number of genotypes was 36, of which 13 were found in two or more trees/cultivars.

Four different clusters were identified. They were designated with different colors (Fig. 4-34); 29 of the 36 genotypes belonged to just one cluster. It was stressed that four homogenous gene pools contributed to the population sampled. However, total homogeneity did not exist as seen from Fig. 4-34. The red cluster contained the famous Marrone cultivar as well as Marrone-like cultivars. The green gene pool contained cultivars from south-eastern Piemonte while the blue cultivars originated from western Piemonte. The cultivars belonging to the yellow cluster did not occur in any specific area in Piemonte. The clusters reflected the geographic origin of the cultivars and the end use of their products. The UPGMA dendrogram showed three clusters: red, green, and blue, while the yellow cultivars were spread into the three mentioned. The discriminant analysis, which was run to identify relationships between morphological traits and microsatellite genotypes revealed that the following morphological traits gave the strongest discrimination:

- nut width/height ratio
- nut hairiness
- foliar blade length/width ratio
- male flower type.

Noteworthy was the observation that almost 40% of the cultivars had astaminate catkins, which do not produce any pollen.

As many as 41 first-degree relationships between the 27 genotypes suggested parentage relationship. The relationships were mainly found between cultivars aimed for specific products such as flour or candy.

The need for saving of germ plasm in form of static preservation was highlighted; especially the risk of loss of cultivars was stressed.
4.3 Vegetative propagation

4.3.1 Grafting

Graft compatibility including 9 C. dentata, 15 C. mollissima, 6 C. crenata, and 2 C. crenata hybrid trees was studied by Huang et al. (1994c). The scion collection, localities, and root stocks used are presented in Table 4-7. The experimental design was three replications each with ten grafts. Isoperoxidase analysis was carried out in cambium of five successful and five unsuccessful grafts. In addition 20 C. seguinii trees were analyzed for isoperoxidase since there is total grafting incompatibility between this species and C. mollissima.

The differences in grafting success for C. dentata were significant with two clones with percentages below 50 while the mean for the seven other clones was 86.6 (Table 4-7). These two clones showed poor union between graft and root stock. The differences were significant among the C. dentata clones even with exclusion of the two clones with poor grafting success. There was no difference in grafting success between the two C. mollissima trees used as root stocks.

The clone without any success in grafting is characterized by vigorous growth, which might be a problem in union between scion and root stock (Table 4-7). The two interspecific hybrids showed low percentages of grafting success, <50, independent on root stock. Poor success in grafting interspecific chestnut hybrids on to C. mollissima was earlier reported. The present observation is another example of this.

It was evident that grafting into phloem bundles should be avoided in grafting of chestnuts (0-4% success, Table 4-7). One C. crenata clone, Kiacheng, deviated strongly from the other five clones with only 6% success as compared to the mean of the other five clones of 84%. It was noted that the Kiacheng scions outgrew the root stock.

No relationship between grafting success and isoperoxidase pattern could be revealed nor did the analysis of C. seguinii give any clues to the grafting incompatibility of chestnut species.

Figure 4-35 Percentage of grafting success at the end of second growth period after grafting of 15 C mollissima trees collected in four different regions in China. Grafting took place on one C. mollissima root stock. Huang et al. 1994c.
Effects of grafting method, time for grafting, and rootstock on grafting success were studied by Pereira-Lorenzo and Fernandez-Lopez (1997b). Rootstocks from nine hybrid clones were used for five types of grafting at twelve occasions. In all, 373 scions from Spanish C. sativa cultivars were grafted. Although the mean success of different rootstocks varied in the range 42-85% there was no significant difference between rootstocks. Moreover, the impact of rootstock on growth up to age ten was non-significant. However, it was stated that there was a strong incompatibility between one French hybrid root stock and the Spanish cultivars.

There was no significant difference on grafting success among grafting methods. The relationship between point of time for grafting and grafting success is illustrated in Fig. 4-36. There was a fairly good fit to a third degree polynomial with highest success during summer. It was reported that growth was good during the second growth period, 123cm, and then dropped the following growth periods to 69 and 14cm, respectively. At age ten in one of the orchards, in which grafting took place the tree height varied between 3.63 and 3.95 meters.

Oraguzie et al. (1998c) reported on three grafting experiments carried out in New Zealand. Experiment 1: Five scions on rootstock originating from the same genotype; 18 clones Experiment 2: Grafting of 75 scions on other rootstock genotypes + precocity of flowering recorded; 8 clones Experiment 3: Grafting of 16 scions on other rootstock genotypes + visual rating of graft failure (1 – 5); 4 clones.

In all experiments number of buds per scion + diameters of scion and rootstock were assessed. Two thirds of the 18 clones in experiment 1 did not show any graft failures while two clones had no successful grafting. All stages between total graft failure and no signs of graft failure were observed. Bark was found inside the wood in several cases of failure. All clones with graft failures originated from the north island while the successful grafts originated from the south island of New Zealand. The selected clones from the north island were of crenata type while sativa types occurred on the south island.

In the second experiment the percentage failure varied in the range 18.7-46.7 (Fig. 4-37). Clonal differences were significant at both assessments, 19 and 38 months. Half of the clones had the highest percentage of failure up to month 19. Between months 19 and 29 there was no increase of graft failures. In clones 1015, LB1, and DW there was a steep increase of graft failures between months 35 and 38, which corresponds to 12 and 15 months after transplanting of the grafts.

In experiment 3 there was no significant difference in graft failures among the four clones studied; the range being 25.0-62.5%. The low number of clones (4) and grafts per clone (16) explained part of the absence of any significant difference. Clone Cr3 had the highest degree of graft failures and had the highest percentages of failed grafts. It was concluded that the rootstock used had an impact on graft failure and that graft failure is more genotypic specific than environmental dependent when scion and rootstock are from the same plant (experiment 1).
Galic et al. (2014) reported on three experiments with vegetative propagation of *C. dentata* from Ontario in Canada. In the first experiment scions were taken from July 12 to August 14 and treated with 1% indole-butyric acid (IBA).

The rooting percentage reached a maximum at July 24 collection but the rooted cuttings failed to leaf out. Somewhat greater success was the propagation of cuttings collected from the 1-2 year old grafts in the second experiment. The scions were collected from potted trees kept indoors. The highest rooting percentage, 60%, was reached for the material collected on February 16 and March 3. Of the rooted cuttings 15% leafed out the following growth season. Softwood cuttings were collected from the 1-2 year old grafts on May 29. The cuttings were treated with 1, 2, and 3% IBA. The rooting percentage was highest for 1 and 2% IBA, 27 and 29%, respectively. Four percent of the control cuttings rooted. A two-stage strategy for cutting propagation was suggested. Step one consists of grafting of the selected material and the second step is collection of scions from these grafts.

Ostere et al. (2004) studied polyphenol in different types of cuttings. Since rooting was less than one per cent and only one clone was studied I will not elaborate on the findings in this paper.

### 4.3.2. Micropropagation and Somatic Embryogenesis

Rooting of *C. dentata* microcuttings was studied by Serres et al. (1990). The original 4-7 cm long cuttings were taken from young shoots. Three factors were investigated:

- IBA concentration, 49, 123, and 369 µM
- Sucrose concentration, 1, 2, and 4% solution
- Basal medium concentration, full and half-strength

The rooting percentage for the lowest IBA concentration was 51% while the two higher concentrations resulted in 93% rooting. The highest sucrose concentration had 80% rooted cuttings after four weeks while 1% sucrose only had 23%. The half basal woody plant growth medium (WPM) had the highest percentage of rooted cuttings, although not significantly different. Based on this investigation it is recommended to have an IBA concentration of 123 µM, the highest sucrose concentration, and half-medium strength of the WPM.

Sánchez and Vieitez (1991) reported on *in vitro* propagation of basal shoots and crown branches from five clones of *C. sativa* and *C. crenata x C. sativa*. The proportion of explants with shoot development, mean number of shoots per explant, length of the tallest shoots, and multiplication coefficient were higher in the basal shoot material than in crown branches material. The subculturing increased the rooting in explants from basal shoots while subculturing did not increase the rooting of the material from crown branches. Clonal differences were noted.

Sánchez et al. (1997a) coined their study reinvigoration treatments for the micropropagation of mature chestnut trees. Five adult trees were included in this study, which comprised spray treatment, pulse treatment, juvenile grafting, *in vitro* culture, and recycling of explants. The grafting took place on two-week old seedlings originating from open-pollination of the five trees. Figure 1 in this paper is an excellent illustration of the design of the experiments.

Spraying (six times with 222 µM benzylaminopurin), grafting, or these two treatments combined showed for three of the trees a greater success in shoot formation than in the untreated control plants (Fig. 4-38). As seen from this figure grafting and especially the combined treatment of grafting and spraying resulted in significantly higher shoot formation than the control. Within trees there was not much variation in number of shoots per rooted explant or length of the longest shoot. Also in this case the highest values were noted for the combined treatment, grafting + spraying. The explants of the HV and 431 trees were exposed to a so called pulse treatment, two hour exposure to 111 µM benzylaminopurin. No significant difference in shoot percentage between this treatment and the control was noted. The results indicate that at least a partial rejuvenation had taken place. It was suggested that the conditions for successful reinvigoration were fulfilled with the vigor of the rootstock and the proximity of the scion to the rootstock.
As regards rooting success the spraying treatment was the most efficient (Fig. 4-39). The rooting percentages for trees HV and 431 were higher in this study than in the report by Sánchez and Vieitez (1991), who studied explants from basal shoots. No significant differences in rooting percentage between the controls and grafting were noted. The test of explant orientation in the medium, horizontal or vertical, resulted in significantly higher rooting percentages for the horizontal cultures. Besides, the turn over of the culture cycles could be reduced to two weeks, which means that this method is an efficient micropropagation technique.

The main contribution of this investigation is that techniques were developed that enable the micropropagation of adult trees.

Sánchez et al. (1997b) carried out experiments to improve micropropagation technique of chestnut. A first experiment concerned initiation of cultures. Cuttings were taken during November-December. After cold storage they were forced to flush. At a size of 10-40 mm five mm long shoot tips or nodes were put into in vitro cultures containing 0.5mg/liter benzyladenine. After six weeks the response was recorded. This material was used for shoot multiplication and rooting experiments. In addition cultures from three months old clones and stump sprouts from three adult clones were studied. The latter being hybrids C. sativa x C. crenata. The shoot multiplication medium contained 0.2mg benzyladenine/liter. Three types of explants were used:

- 7-10 mm shoot tips
- 7-10 mm stem segments with 2-3 axillary buds
- 7-10 mm whole harvested shoots

After each monthly subculture cycle, recordings were taken with regard to number of shoots formed, numbers of new segments.

Shoots 2-3 cm in length were excised from proliferating cultures and exposed to two different treatments: 7 days in medium with 3mg/liter of indole-3-butyric acid (IBA)
Dipping the basal end of the shoots in a solution containing 1g/liter of IBA for one minute.
5 days of darkness + 7 days in medium with 3mg/liter of indole-3-butyric acid (IBA)

The percentage of rooted plants was determined one month after the start of the rooting treatment. Root length and root number were also recorded. Shoot tip explants were more responsive to the treatments than nodal explants. Fig. 4-40 shows that the two interspecific hybrids had higher percentages than the C. sativa trees, which was attributed to the difference in age between the two types of material. The largest number of shoots longer than five mm was noted for clone L2 and the lowest number was obtained in clone L4. The explant type had a significant effect on shoot multiplication with the basal nodal segments showing the largest shoot and segment numbers.
The rooting success was significantly improved by an initial darkness treatment (4-41). Especially, the root number was significantly increased in this treatment. Activated charcoal improved the rooting success and the general condition of the plants produced.

In conclusion this study showed that there are possibilities to mass produce material from adult trees via stump shoots and thus mass produce elite trees.

Micropropagation of one adult and two juvenile American chestnuts were studied by Xing et al. (1997). Stump sprouts of the adult trees were the starting material for micropropagation. Important details about the experiments are given below:

- Shoot-initiation medium MS, 4.4 μM BA, 0.5 μM IBA, 35g/L sucrose
- Shoot multiplication medium
- Woody plant medium with 1 μM BA, 0.5 μM IBA, 35g/L sucrose
- Shoot elongation medium
- Woody plant medium with 0.5 μM IBA, 0.22 or 0.89 μM BA, 30g/L sucrose, 500 mg/L 2-(N-Morpholino) ethansulfonic acid (MES), 500mg/L polyvinylpyrrolidone (PVP)
- Rooting medium MS, 5 or 10 mM IBA a dip for one minute, 2g/L charcoal, 20 g/L sucrose

I have summarized the results as regards rooting and shoot necrosis in Fig. 4-42. This figure reveals that rooting of the adult tree microcuttings (striped green bars) had higher rooting percentages in the low IBA treatment than in the high treatment. The opposite was true for the material from the two juvenile plants. The ANOVA revealed that the genotype effect for rooting was strongly significant in spite of the low number of genotypes. Since there was a difference between the adult microcuttings and those from juvenile material a discussion of a possible effect of the age of the original material would have been justified.

The interaction IBA x genotype was also significant. For the adult material, necrosis was most pronounced at the high IBA treatment. The highest percentage of necrosis was noted for one of the juvenile materials at the low levels of BA and IBA. No significance was noted for genotype as regards necrosis. There were significant effects for BA and genotype x IBA. The latter is reflected in Fig. 4-42.

Harvesting microcuttings during the production phase lead to higher rooting percentages than microcutting harvesting during the stabilization phase. For the juvenile material the percentage of necrosis was much lower after microcuttings harvested during production phase. Thus, again a difference was noted between juvenile and adult origin of the material.

Based on the results obtained, optimum treatment for each of the genotypes was tested. As seen from the Fig. 4-43 the agreement between the results from the original treatment and the optimum treatment for individual clones is close to perfect, which indicates that the experiments were reliable. However, for application it is disturbing that different methods for individual clones are required for optimum rooting and minimum of necrosis.

Estimation of genetic variability in each stage of multiplication via micropropagation, influence of culture media on micropropagation, and correlations between in vitro and in vivo stages were the three objectives of a study by Miranda-Fontana and Fernández López (2001). Based on growth and form a selection of 35 interspecific hybrids between C. sativa and the two Asian species C. crenata and C. mollissima were included in an investigation of vegetative propagation. Multiplication was developed by
axillary shoot production. Stem segments with at least one axillary or apical bud and one centimeter long were excised and inserted vertically into multiplication media. Six propagation media and seven traits were included in this experiment:

<table>
<thead>
<tr>
<th>Media</th>
<th>Traits</th>
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<tbody>
<tr>
<td>GD = Gresshoff and Doy</td>
<td>Number of shoots per explant NSH</td>
</tr>
<tr>
<td>MS = Murashige and Skoog</td>
<td>Length of the tallest shoot LS</td>
</tr>
<tr>
<td>Lp = Lepoivre</td>
<td>Number of segments of one cm per explant NS</td>
</tr>
<tr>
<td>BI = Blaydes</td>
<td>Multiplication coefficient MC</td>
</tr>
<tr>
<td>Hm = Heller modified</td>
<td>% responsive explants RE</td>
</tr>
<tr>
<td>SH = Shenk and Hildebrand</td>
<td>Fraction of apical necrosis RE</td>
</tr>
<tr>
<td></td>
<td>Color, scale 1-10 Color</td>
</tr>
</tbody>
</table>

Clonal repeatabilities were estimated for the traits listed. Fig. 4-44 shows that GD media resulted in the lowest fraction of necrosis and five of the media had fraction of responsive explants in the range 0.92-0.96. Only the GD and MS media had cuttings without apical necrosis, 9 out of 35 cuttings and 3 out of 35 cuttings, respectively. Considering all seven traits, GD medium was the best. Except for color the clonal differences were strongly significant for all traits while the interaction clone x culture medium was strongly significant for all seven traits. In spite of this strong interaction the clonal repeatabilities (Fig. 4-45) were above 0.85 (line shown in this figure) for all traits except for apical necrosis (0.77) and color (0.42). It should be remarked that variance components were estimated for fixed effects, which is a violation of conditions for ANOVA. Two types of apical necrosis were identified. The first coined limited to apex that did not prevent continued growth of lateral branches. The second was coined descending, which expanded downwards and was lethal as far as I can understand. Clonal mean correlations between the two traits apical necrosis and color and all other traits were weak, and for apical necrosis negative. Correlations between the seven traits and several ions such as nitrate, sulphate, phosphate, or metal ions, were with a few exceptions all weak. They never explained more than 35% of the variation.

**Figure 4-43.** C. dentata. The percentage of rooting and necrosis in the original experiment (filled bars) and in the best treatment combination for each of the three genotypes (shaded bars). Xing et al. 1997.

**Figure 4-44.** The fractions of responsive explants and apical necrosis in an experiment with six propagation media and 35 chestnut clones; most clones were hybrids C. crenata x C. sativa. The media were:
- GD = Gresshoff and Doy
- MS = Murashige and Skoog
- Lp = Lepoivre
- BI = Blaydes
- Hm = Heller modified
- SH = Shenk and Hildebrand

Miranda-Fontana and Fernández-López 2001

**Figure 4-45.** The clonal repeatability for seven traits based on an experiment with 35 clones; most of them being interspecific hybrids C. crenata x C. sativa.
- NSH = number of shoots per explant
- LS = Length of the tallest shoots in cm
- NS = number of segments per cm
- RE = percentage of responsive explants
- MC = multiplication coefficient
- AN = apical necrosis; present or absent
- Color = 9 classes from dark green (9) to pale yellow (1)

In a follow-up paper Miranda-Fontaña and Fernández López (2005) reported on further development up to an age of seven years of this material.

At the multiplication stage the separate clonal repeatabilities for the individual media did not differ much and varied in the range 0.60-0.8 and strongly significant. The highest estimate was noted for length of the tallest shoot in the GD medium and lowest estimate was observed for apical necrosis in the same medium. The clonal repeatabilities at the elongation and rooting stages were also high and strongly significant for the traits studied:

- Number of shoots >3 cm: 0.91
- Length of shoots >3 cm: 0.87
- Diameter of shoots >3 cm: 0.86
- Percentage of rooting: 0.81
- Number of roots per rooted shoot: 0.92

The repeatabilities of two traits, height and stem form at age 1 in nursery, were high, 0.89 and 0.96, respectively. Stem form had a high repeatability estimate in field at age 4, 0.96, while the three growth related traits varied in the range 0.65-0.79 with the lowest value for height increment. The correlations between the same trait at different stages: multiplication – establishment and multiplication – elongation were above 0.50 (0.52-0.59) in three of the 28 relationships studied. This means that early stages’ clonal differences are not well reflected at later stages. The relationships of three traits in three culture media were fairly strong for apical necrosis and multiplication capacity while the correlation coefficients for percentage responsive explants were all below 0.50 (Fig. 4-46). The genetic correlations between nursery traits, height at age one and heights at ages 4 and 7, height increment ages 1-7, and stem form at age 4 as well as the corresponding correlations with stem form at age 1. Most ortets were interspecific hybrids C. crenata x C. sativa. Miranda-Fontaña and Fernández-López 2005.

The anatomical and biochemical development during micropropagation of material from the base (Bs material) and crown (Cr crown material) of an 80-year old C. sativa tree was reported by Ballester et al. (1999). The two lines established were subcultured for seven years before this investigation was started. The basal ends of shoots with a length of 2.5-3.0 cm were dipped into a $5 \times 10^{-3}$ IBA solution for one minute. The histological analyses were carried out at eleven times 0.5-14 days after the start of the root induction treatment. Five auxin analyses, 0.5-5 days after IBA treatment, were carried out. Harvesting of whole shoots was sampled three times, 2-6 days after IBA treatment.

The rooting capacity of Bs was maintained at 94% while the corresponding for Cr was <10%. The main anatomical difference at onset of the root induction was to be found in the secondary phloem and xylem, which were more developed in the Cr. At three days after induction root primordia were developed in Bs but not in Cr. The first adventitious roots emerged ten days after induction. The Cr material responded to the root induction treatment by normal cambial derivatives but without any root primordia. More endogenous IAA was found in the Cr material than in the base material. The Cr material had higher concentrations of the polyamines (putrescine, spermine, and spermidine) than the Bs material.

This report contains several instructive micrographs from the histological analysis of the course of development. It was concluded that even after seven years of culture the material remembers its original position in the tree.
San-Jose et al (2001) studied the effect of thidiazuron (TDZ) on shoot induction and plant regeneration from hypocotyl and cotyledonary nodes of C. sativa x C. crenata individuals. Detipped epicotyl segments were also tested. Two preconditioning media were used, 0.1mg/liter of TDZ or 1mg/liter of BA. One control and ten treatments with five concentrations (0-2 mg/liter) of TDZ were tested in the induction medium. Most explants of hypocotyls and epicotyls did not produce any shoot development. As regards the cotyledonary node explants, the two induction media without TDZ, originating from the two preconditioning media 0.1mg/L of TDZ and 1mg/L of BA, had the highest percentages of explant shoots longer than 5 mm, 64 and 80%, respectively. Noteworthy is the high percentage of the control material, 89%. Seven different sets of three clones were used for each of the seven rooting treatments. This means that the comparisons between the seven treatments are not straightforward. Significant clonal differences for rooting percentage were noted for five of the treatments. In most of these cases one of the three clones in a set deviated strongly from the other two. For mean root number significant differences were noted for six of the treatments. One merit of this paper is the microscopic illustration of different developmental stages.

Nodal explants from 2-month old seedlings of C. crenata were used in a micropropagation study by Tetsumura and Yamashita (2004). After subculturing for six months one-node segments were cut and placed on three different basal media supplemented with BA, TDZ, or zeatin. Different levels of zeatin were tested 0.2, 1, 5, and 25 μM. Root induction took place in darkness during five days on three different media (Gellan Gum, Vermiculite + broad-leaved tree medium, and Gellan Gum + vermiculite) containing 15 μM IBA. After 30 days in the cultures for root formation, the surviving shoots were planted. Surviving plants, plant height, and number of leaves were recorded after another 30 days.

The best results as regards in vitro establishment were obtained for zeatin but this treatment suffered from a high percentage hyperhydric shoots, 69%. The percentage of surviving shoots, length of shoots, and shoots showing hyperhydricity increased with increasing concentration of zeatin. The best rooting result was obtained for Gellan Gum + vermiculite. Significant clonal differences were noted both for survival and rooting percentage. Also survival and growth after acclimation reveal significant clonal differences. Unfortunately, no data for individual clones were presented.

Finally it was stated that the same methods were used for explants from adult trees but all attempts failed.

Axillary winter buds from one clone, Sobota, were the starting material for a micropropagation project by Osteric et al (2005). Contrary to other reports it was found that medium with benzyladenine was more efficient than zeatin for the propagation.

4.3.2.1 Somatic embryogenesis

Abbreviations used in this section

2,4-D 0 dichlorophenoxyacetic acid
BA = benzyl adenine
DMSO = dimethyl sulfoxide
LN = liquid nitrogen
MS = Murashige – Skoog growth medium
NAA = naphtaleneacetic acid
PVS2 = 30% glycerol, 15% DMSO, 15% ethylene glycol

There are many papers dealing with somatic embryogenesis in chestnut. The main focus in these papers is on development of methods for production of somatic embryos and successful regeneration of plants from somatic embryos and less on genetic differences in production of plants from somatic embryos. A detailed description of development of methods for production of somatic embryos and their further conversion into plants is beyond the scope of this presentation. An excellent summary of the state of the art of this topic was presented by Correia et al. (2005a). They concluded that the most important factors controlling somatic embryogenesis are:

Developmental stage of the zygotic embryos

Type of growth regulator used such as exogenous auxin

The latter was exemplified by use of 2,4-D (2,4-dichlorophenoxyacetic acid), NNA (Naphtaleneacetic acid) alone or in combination with a cytokinin.

They stated that conversion of somatic embryos to plantlets was the limiting factor for production of plants from somatic embryos. Moreover, that the developmental window of chestnut responsive material being very narrow.

A more recent summary of the state of the art was published by Nelson et al. (2014), in which gene transfer was treated with focus on tolerance against Cryphonectria parasitica. One paper by Maynard et al. (2006) is like a “cookbook” for production of somatic embryo plants.

In a brief report Piagnani and Eccher (1990) reported on successful production of somatic embryos following 2,4-D + BA and NNA treatments of cotyledons from four clones; 2 C. sativa and two C. crenata x C. sativa. Somatic embryos were only obtained in one of the clones.

Merkle et al. (1991) reported on somatic embryogenesis in C. dentata. Ovules were collected 3, 6, and 9 weeks after pollination from one tree. Only the collection at 6 weeks gave rise to embryogenic cultures and in low frequency, six in 178 ovules. Over 300 nuts from two other trees resulted in two somatic embryos each. Thus, a distressing low frequency of success.
Initiation of cultures from ovules and zygotic embryos from 25 *C. dentata* trees was reported by Carraway et al. (1994). Treatments with plant growth regulators were carried out according to the following:

A. Control  
B. Auxin only  
C. Cytokinin only  
D. Auxin + cytokinins  
E. Pulses of B and transfer to A or C  
F. Pulses of C and transfer to A or B  
G. Pulses of D and transfer to A

Somatic embryos from immature zygotic embryos were obtained in 16 of the 25 trees sampled. In five of these cell lines repetitive production of somatic embryos occurred after treatment D. At the time of publication no mature somatic embryos were obtained. Microscopic gold particles with DNA were used for gene transfer via microprojectile bombardment. Sixteen transformed cell lines were obtained.

Xing et al. (1999) collected immature burs of 13 OP- and six full-sib *C. dentata* families 4-7 weeks after anthesis. The ovules were dissected and placed on initiation medium, which contained 18.18 μM 2,4-D, 1.11 μM BA, 1g/L casein. Subculturing of polyembryonic masses (PEM) took place every second week in darkness. Embryos were developed from PEMs on medium with 20g/L of sucrose and 16 growth regulator combinations of 0, 0.05, 0.25, and 0.50 μM of BA and 0, 0.05, 0.25, and 0.50 μM of NAA. Two cell lines from each family were included. The effect of ABA, 2 μM, and sucrose-rich treatments were tested in order to improve embryo development and maturation. The sucrose concentration in the maturation medium was 60 g/L. The variation in embryo yield over time of induction, 4, 8, and 17 months was tested. Embryos were germinated on medium with 500 mg/L 2-[N-morpholino]ethenesulfonic acid (MES), 500 mg/L polyvinylpyrrolidone (PVP40), 0.89 μM BA, and 2g/L of charcoal. The cultures were kept in darkness for embryo initiation and in 16h photoperiod at 23°C for embryo development, maturation, and germination.

The induction rate of somatic embryo formation was 1.6%. Seven of the 19 families did not give rise to any somatic embryos. At most three somatic embryos per family were noted. The yield of cotyledonary stage embryos did not vary among the 16 combinations of BA and NAA. The plant regeneration rate as well as shoot and root regeneration rates were improved by the increase of sucrose from 20 to 60 g/L. Even if the plant regeneration rate increased from 0.7% to 2.6%, the percentages are distressing low. The effect of 2 μM ABA in medium reduced the plantlet regeneration slightly to 0.5% from 0.7%. The regeneration rates of whole plants, shoots and roots were significantly increased in medium containing 500 mg/L MES, 500 g/L PVP40, and 0.89 μM BA (Fig. 4-48). Rooting of the shoots seems to be an option for increase of number of somatic embryo plants. Thus, rooting treatment of such shoots added another 6.3% of rooted plants to the 3.3 directly obtained plantlets.

The results as regards plant regeneration included a study of five families with a variation between zero and 11.8% in one control pollinated family. There was less family variation in shoot regeneration only. 4.8-8.8%.

It was reported that 20 somatic embryo plants were potted and the six tallest were planted in field. At the start of the second growth period four of them were alive. Maintenance of embryogenic competence, maturation and germination of somatic embryos, and the effect of pregermination treatment were studied by Corredoira et al. (2003). Excised leaves from explants of *C. sativa* were used as starting material.

The numbers of secondary embryos and somatic embryos per embryogenic explant are illustrated in Fig. 4-49. In both cases the best result was obtained for the combination of low concentrations of BA and NAA. Sucrose 3 and 6%, activated charcoal + 3% sucrose, maltose 3 and 6%, and sorbitol 6% + 3% sucrose were used in maturation media to study the root and shoot deve-
velopment as well as conversion. Conversion to plants with shoots and roots was only obtained in the two maltose treatments and the 6% sucrose treatment but the percentages were all below 10%. The maltose 3% treatment had a high percentage of shoot development, \( \approx 27\% \). Since the shoots might be stimulated to form roots the percentage of real plants might increase to almost 40%.

Desiccation, cold treatment, and a combination of them were used in pre-germination experiments. Conversion was only obtained in the two treatments including cold storage. In both cases the percentage was below 20%. In conclusion it was stated that a total of 39% of embryos eventually produced plants either through conversion to plantlets or indirectly through rooting of shoots.

Merkle and coworkers have presented a series of papers on somatic embryogenesis of chestnuts (Merkle et al. 1991, Carraway et al. 1994, Carraway and Merkle 1997, Andrade and Merkle 2005). Woody plant medium containing 6mg/liter NAA (naphthaleneacetic acid) and 0.25mg BA (benzyladenine) were used in experiments to obtain somatic embryos from ovules (2 mm or smaller) of five C. dentata trees (Merkle et al. 1991). Another tested plant medium contained the same BA concentration but NAA was substituted for by 4mg/liter of 2,4-D (dichlorophenoxyacetic acid). The material was collected three, six, and nine weeks after anthesis.

Two directly formed somatic embryos and one embryogenic mass were obtained from the 84 ovules collected six weeks after anthesis. Of the 94 ovules collected at the same date and cultivated on the 2,4-D medium only three produced embryogenic masses. Mature somatic embryos were obtained after transfer to hormone-free media but no plantlets were obtained. Collections at three or nine weeks after anthesis did not result in any proembryonic masses.

A continuation of this work was presented by Carraway and Merkle (1997), in which material was collected from 30 trees over several states in eastern USA. Besides BA, NAA, and 2,4-D, treatment with thidiazuron (TDZ) was included in experiments comprising more than 12,000 samples. Thirty and 60g of fructose, maltose, or sucrose were added to the media to stimulate growth of the somatic embryos. For the same purpose activated charcoal was added to the media. Embryogenic cultures were produced from ovules, embryos less than 5 mm in length, and cotyledons <6mm². Auxin without the other hormones generated 2.8% embryogenic response while no response was noted for media containing NAA or TDZ. Only sugar type was significant in the ANOVA run for estimation of the effect of type and concentration of sugar on number of embryos produced. Cell line effects and all interactions were non-significant. As regards morphology of the somatic embryos, cell line effects and sugar type were significant. As far as I can see only three cell lines were included in the experiment. Cold treatments for eight or twelve weeks stimulated root formation. The failure of getting continued growth after transfer to the mixed substrate of peat (55%) and vermiculite (45%) was attributed to inhibiting effects of this substrate.

In well-designed experiments Robichaud et al. (2004) studied the effect of ABA (abscisic acid), PEG (polyethylene glycol), sucrose, and two amino acids, asparagine and L-glutamine, individually or in combination, on maturation and germination of somatic embryos. Different levels of sucrose were also tested. It was anticipated that additions of these substances would improve the germinability of the somatic embryos.

In Table 4-8 I have summarized the results from the statistical evaluation. As regards biomass strongly significant effects for cell lines and treatments were noted for all four variables, amino acids, sucrose, ABA, and PEG. In two cases, amino acids and PEG, the interaction cell line x treatment was strongly significant. This in turn means that the cell lines responded differently upon the treatments. This means that general methods might be hard to develop for germination of somatic embryos. Table 4-8 also shows that the cell line effect was strongly significant for all four variables concerning germination. The amino acid effect was the only significant treatment effect on germination. The germination rate was increased in one cell line after treatment with 25mM L-asparagine.

![Table 4-8](image)

**Table 4-8.** The significances of different treatments on the ratio dry weight/fresh weight and germination frequency. The number of cell lines was three and number of treatments was four for each factor. Robichaud et al. 2004.
During the different steps from the 5,760 somatic embryos to the final six plants in the field there were great losses at several steps (Fig. 4-50). This means that much remains to be done to increase the number of trees in field.

Andrade and Merkle (2005) studied the effect of liquid and semi-solid culture regime on germination and conversion of the somatic embryos. They also studied the effect of charcoal, cold treatment, as well as the somatic embryo morphology on germination and conversion. In Fig. 4-51 I have summarized the routines for production of somatic chestnut embryo plants based on the results in this investigation as well as results from earlier reports.

Axillary shoot and somatic embryogenesis were tested for improvement of micropropagation of C. sativa x C. crenata hybrid cultivars (Ballester et al. 2001). Five different media were tested on five clones to find out the best way of micropropagation via axillary shoots. Somatic embryogenesis was initiated from zygotic tissues during July and August as well as from unfurled expanding leaves. Multiple shoot induction from cotyledonary nodes was also studied. According to the ANOVA clone, treatment, and clone x treatment interaction were all significant in the test comprising axillary shoots. Any general recommendation of media for propagation via axillary shoots could not be presented based on the results in this investigation.

Somatic embryogenesis was also obtained at low frequency (0.5) from leaf explants after cultivation on media with benzyl adenine (BA) and naphthalene acetic acid (NAA). It was reported that five seedlings from somatic embryos were growing in greenhouse.

Clusters of ovules, individual ovules, and immature zygotic embryos from three open-pollinated C. sativa trees growing in Wien were used as starting material for development of somatic embryos (Sauer and Wilhelm 2005). The material was collected 2-10 weeks after anthesis. For induction of somatic embryogenesis 5 μM 2,4-dichlorophenoxyacetic acid plus 0.5 μM 6-benzylaminopurine were used. After three weeks of treatment the material was transferred to medium with 0.89 μM of BA. Subculture interval in this medium was four weeks. Two concentrations of agar, 0.8 and 1.1%, were tested for the maturation medium. For test of germination, medium with indole-3-butyric acid + 0.89 μM of BA were used. After five weeks conversion, shoots formed, as well as the numbers of embryos with developing shoot or root only were recorded. A size index of the zygotic embryos was calculated as length x width.

There was no difference in embryogenic response of the three trees, which allowed a pooling of data from the individual trees. The percentages of embryogenic lines formed from the different starting material were:

- Ovaries: 5.1%
- Ovules: 3.0%
- Zygotic embryos: 27.0%

As expected the latter percentage was significantly different from the two other percentages. The relationship between somatic embryo percentage and collection date is illustrated in Fig. 4-52 for ovaries and zygotic embryos as starting material. The peak frequency occurs at different times following anthesis, which might be attributed to different weather conditions during the two years. There was no relationship between the size index and percentage of somatic embryos induced. It was stressed that the seasonal conditions during individual years must be considered when collection of material for somatic embryogenesis should take place.

For a study of proliferation capacity 21 cell lines were selected and cultured on medium with 0.89 μM BA and three concentrations of glutamine (0, 300, and

Figure 4-50. The steps from somatic embryos to plants in field with the numbers remaining after the different steps. Robichaud et al. 2004.

Figure 4-51. The standard procedure for development of plants from somatic embryogenesis of chestnuts based on the results in Andrade and Merkle 2005.
The effects of glutamine level or cell line did not differ significantly. The agar level of 1.1% in the medium resulted in an increased number of matured embryos compared to agar level 0.8%. At the time of publication five plants derived from somatic embryos were transplanted to a substrate of equal amounts of peat moss and perlite.

Oakes et al (2013) carried out three experiments with the aim of developing good conditions for somatic embryo plants:

1. Light exposure and presence of charcoal
2. Type of light; white, dim, dark, red, blue, and far-red
3. Temperature and time of exposure

In (1) rooting was studied following two minutes dip into 10 mM IBA aqueous solution. The charcoal treatments had 2 g/L of activated charcoal and 3.5 g/L Phytagel. Half of the material was put under 16/8 light/dark conditions while the other half was put in a dark cabinet for eight days. Healthy plants were potted after 21 days in post-rooting medium. Three replications per treatment with ten shoots from each of the two clones were included.

For experiment No. 2 four blocks were used with six different light conditions. Contrary to experiment 1 the shoots were kept in the rooting medium for six days. The red, blue, and far-red light treatments were carried out in one large box in three complete isolated compartments. The three other light treatments were carried out in separate cabinets.

In experiment No. 3 two temperatures, 4 and 25°C, were used with or without activated charcoal.

This paper contains several instructive illustrations of the results for individual traits. I have preferred to bring together the results into unifying illustrations.

**Figure 4-53.** The relative effect of different treatments on percentage of rooted plants, number of roots per rooted plant and percentage necrosis based on micropropagation of two C. dentata trees. The assessment took place three weeks after rooting treatment. Dark = darkness, ch = activated charcoal. Oakes et al. 2013.

compounds and excess plant growth regulators in the substrate. Dark treatment during rooting gave the most positive results with higher rooting percentage and higher number of roots per rooted plant than the two light treatments. The frequency of necrotic shoots was extremely high in the light treatment without charcoal, ≈85%. All treatment effects were significant. As regards the prediction of various traits for survival it was found that leaf number was the best predictor while height was the poorest predictor. Plant height had the largest difference between surviving and non-surviving plants 16 weeks after transplantation.

The light experiment clearly showed the inferiority of the white light treatment with poor survival and low root number accompanied by high necrosis at the assessment 16 weeks after treatment (Fig. 4-54). To avoid shoot top necrosis it is evident that rooting in darkness should be used since this treatment deviates from the other light treatments with an extremely low percentage for this trait, ≈20%.
The survival was strongly affected by temperature and time in rooting induction treatment (Fig. 4-55). The eight-day treatment at 25°C had an extremely low survival, 7%. It is obvious that rooting induction for four days rather than for eight days increases the survival considerably. Moreover, a high temperature at a short rooting induction seems to be beneficial for survival of the explant. Based on the findings in this investigation it was recommended that only shoots >3 cm long and having more than six leaves should be used for potting in applied propagation.

Subculturing of explants from one C. sativa clone was carried out every four weeks to generate a material for studies of growth retardants on somatic embryogenesis by Roussos et al. (2016). Shoot number, shoot length, node number, and nodes per shoot length was recorded after treatments with BA, TDZ, forchlorfenuron (FCF), isopentyl adenine (2iP), and kinetin (KIN) during the proliferation stage. Three concentrations of each substance were tested, 1, 2, and 4mg/L for BA, 2iP, and KIN. The concentrations of FCF and TDZ were 0.025, 0.05, and 0.1 mg/L. For rooting treatments three concentrations of IBA and NAA were used individually or in combination, 1, 2, and 4 mg/L. There was no difference in nodes per shoot length between any of the 15 treatments while BA had significantly higher shoot number and node numbers than any of the other treatments. The three BA treatments did not differ significantly for any of the four traits studied. Once more the efficiency of BA for somatic embryogenesis was confirmed.

The rooting percentage increased with increasing concentration of IBA while an opposite trend was noted for the combined treatment of IBA and NAA (Fig. 4-56). The highest number of roots was noted for the NAA 4 mg/L treatment, but only once significantly higher than NAA 1 mg/L. It is evident that addition of NAA to the highest IBA treatment did not improve the rooting percentage. However, the rooting percentage was already high, 81%, for this treatment (4mg/L IBA).

Cryopreservation of embryogenic axes and somatic embryos were studied by Corredoira et al (2004b). As regards the embryogenic axes desiccation for 0-7 hours was tested before storage in liquid nitrogen. In a second experiment the concentration of BA in the recovery media was studied. Desiccation of somatic embryos were carried out for 0-4 hours and then transferred to liquid nitrogen for 24 hours. After thawing, the material was cultured for three days on proliferation medium followed by six weeks in fresh medium. A vitrification experiment was also conducted, in which somatic embryo clumps were cultured for three days on a medium containing 0.3M sucrose. Then the material was placed on a MS medium containing glycerol, DMSO, and ethylene glycol for 0-120 minutes and after that half of the material was put into liquid nitrogen, the rest served as control. Evaluation took place six weeks later.

**Embryogenic axes.** Plant recovery after LN storage reached the highest value at 5h desiccation time, 63% while the highest value, 100%, for control without LN storage was noted for 2h and 3h desiccation.

**Embryogenic cultures.** The peak in survival was noted for one hour of desiccation, ≈ 50% while the peak in embryo recovery was noted for 2h desiccation, ≈ 30%. The vitrification treatment for 30, 60, 90, and 120 minutes resulted in recovery percentages 60-70%.

It was concluded that cryopreservation of germ plasm may feasibly be carried out.

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**Figure 4-55.** Survival after rooting treatments for four and eight days at two temperatures in a micropropagation experiment with C. dentata. Oakes et al. 2013.

<table>
<thead>
<tr>
<th>8 days</th>
<th>4°C</th>
<th>25°C</th>
</tr>
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<tbody>
<tr>
<td>Survival %</td>
<td>25</td>
<td>100</td>
</tr>
</tbody>
</table>

**Figure 4-56.** The rooting percentage of C. sativa explants following treatments with 1, 2, or 4 mg per liter of indole-3-butyric acid (IBA) and naphtalene acetic acid (NAA) or these two hormones in combination. Roussos et al. 2016.
4.4 Gene transfer

In a brief report by Carraway et al. (1994) ovules and zygotic embryos from 25 C. dentata trees covering a wide range of its distribution area in eastern USA were collected in order to initiate tissue cultures. Four auxins and two cytokinins were tested. Five of the 25 trees produced cell lines having the ability to produce somatic embryos over several generations. Bombardment of suspension culture of cells was carried out with some success but no genetically transferred somatic embryos were obtained.

A report from 1998 by Maynard et al. (1998) summarized the results obtained at this point of time as regards somatic embryogenesis and gene transfer with the ultimate goal of a blight resistant C. dentata. One focus was on development of antimicrobial peptides. Three of the 50 peptides inhibited growth of Cryphonectria parasitica. The authors stressed that it was important to combine at least three different genes that inhibit the growth of the parasite and stated that: It is extremely unlikely that the blight will be able to simultaneously overcome all three mechanisms.

Ballistic gene transfer was tested without much success. Therefore, gene transfer via Agrobacterium tumefaciens was tested with some success. I regard this document as a hybrid between a (popular) scientific report and a grant proposal with admirable clarity.

Gene transfer via Agrobacterium tumefaciens in C. sativa was reported by Seabra and Pais (1998). They used the p35GUSINT plasmid, which has a T-DNA region with the nptII gene that confers kanamycin resistance. It also contains the β-glucuronidase (GUS) gene. Segments of young shoots were cocultivated with the bacterium on medium containing 50 or 150 mg/L kanamycin. Hypocotyl segments were chosen since they were known to have high efficiency of shoot regeneration.

Only two nptII-positive shoots were obtained, one at each kanamycin level. This is the first report on successful transformation by aid of A. tumefaciens in Castanea, even if the percentage was extremely low. A high percentage of so called escapes were noted, 92 and 87%, respectively. Escapes are shoots that survived on the kanamycin media but without the nptII gene. In spite of the meagre results it was recommended that application of 50 mg/L kanamycin directly after coculture with A. tumefaciens and an increase of the kanamycin level gradually to 150 mg/L should be used in future attempts of transformation. At 150 mg/L only transformed shoots will survive.

Corredoira et al. (2004a) reported on successful gene transfer in C. sativa mediated by Agrobacterium tumefaciens. Two strains of Agrobacterium were used. Explant clumps with 2-3 somatic embryos of three lines were isolated from embryogenic cultures for one day on proliferation medium without growth regulators. After that the embryos were immersed in the bacterial suspension for 30 minutes. Washing took place in sterilized water with cefotaxime and thereafter the somatic embryos were transferred to proliferation medium with carbenicillin, cefotaxime, and kanamycin. Different concentrations of them were used.

The transformation efficiency was dependent on the combination of Agrobacterium plasmid and coculture with the bacteria. The highest efficiency, 25%, was noted for four days of coculturing with the EHA 105 Agrobacterium strain containing the plasmid pUbiGUSINT. Only one cell line responded to the germination treatment and with a low frequency, 6.3%. Many of the transformed embryos produced shoots only, which suggests that there was some root inhibitors in the medium.

Acetosyringone was proven to promote transformation in other species but this what not the case for C. sativa, at least not for the two concentrations tested, 100 and 200 μM.

It was concluded that we have defined for the first time a repeatable and efficient genetic transformation protocol in chestnut using somatic embryos as the target material.

Somatic embryos and cotyledonary node explants of C. sativa were cocultivated with strain EHA 105 of Agrobacterium tumefaciens on a medium with kanamycin as selecting agent (Corredoira et al. 2005b). Three different plasmids were tested in this strain of A. tumefaciens pUbiGUSINT or p35SGUSINT. Another strain was also used, C58C1 which contains pBH21 plasmid. The same treatment as in the previous paper was used for transformation of somatic embryos. Three or four weeks of cocultivation was evaluated, as was the optical density of the medium. As regards the explants pre-cultivation medium was supplemented with thiadiazuron or benzyladenine.

Some of the results reported in this paper were already presented in the previous paper. The three levels of optical density of the medium did not influence the selection efficiency much, 57-64% of kanamycin-resistant explants that showed the GUS expression.

Five days cocultivation with A. tumefaciens EHA 105 pUbiGUSINT gave the highest transformation frequency but the percentage was low, 2.3%.

It was concluded that the conversion of the transgenic lines is a hurdle to overcome in the efforts to obtain transformed C. sativa trees.

Polin et al. (2006) reported a successful transformation of five Agrobacterium transformed somatic embryo clones of Castanea dentata. The transformed tissue of one of the clones turned into callus and a sixth clone did not show any transformation. Five of the transformed lines gave rise to morphologically normal shoot development. Two of nine transgenic lines had the marker gene in single copy while seven lines had multiple inserts of the marker gene. The results are encouraging for breeding of traits regulated by genes in a few loci.
4.5 Applied genetic conservation of Castanea

It is important to distinguish between dynamic genetic conservation and static conservation since the methods for these two types of conservation differ. The latter is preferably referred to as preservation and it means as the term says a freezing of the genetic constitution. This can either be carried out with storage of seeds, pollen, or trees in various kinds of collections. Dynamic genetic conservation is an evolutionary way of conservation, in which the genetic resources are exposed to ambient conditions. Most papers treating chestnut species genetic conservation concern preservation.

4.5.1 Dynamic conservation

4.5.1.1 C. sativa

Eriksson (2006) argued that the genetic conservation of C. sativa should be designed such that the methods allow for continued adaptation of the species. This applies both to evolution in nature and improvement in breeding. Adaptation is especially important under rapidly changing environmental conditions. Selection of genetic resource populations both for natural forests and fruit orchards should follow the Multiple Population Breeding System (MPBS) concept developed by Namkoong (1984). In MPBS the genetic resource

Figure 4-57. A suggested model for genetic conservation of C. sativa. For naturalized populations the MPBS concept of genetic conservation is suggested with selection of genetic subpopulations over a broad span of site conditions. For fruit and wood production clonal archives are suggested. In areas, in which interspecific hybrids with C. crenata and C. mollissima are used, clones of these species as well as interspecific hybrids may be included in the clone archives. Slightly modified from Eriksson 2006.
population is split into several subpopulations. The ideal situation for selection is that we have information on variation in adaptive traits to maximize the existing variation of adaptive traits in the genetic resource populations. The observed, large variation among populations as regards adaptive traits suggests that genetic resource populations should be selected over a broad span of site conditions according to the principle outlined in Fig. 4-57. It should be stressed that the number of subpopulations should be much larger than the five illustrated in this figure.

The main advantage of the MPBS is that it combines the capture of the total existing genetic variation with a satisfactory variation within each subpopulation and that it allows the target subpopulations to adapt to the prevailing environmental conditions. Another advantage is that the speed of evolution might be faster in a population of 50 trees than in a large population containing thousands of trees.

There are three types of domestication level in *C. sativa*, orchard, coppice, and naturalized forests. They provide different human utilities and for this reason their genetic constitution may have diverged over the past generations. Especially, the selection for nut quality and yield among orchard clones might have led to loss of Darwinian fitness (= the ability of a genotype to transfer its genes to the next generation) in contrast to domestic fitness, which is the ability to produce human utilities. Introduction of the Asian chestnut species *C. crenata* and *C. mollisima* for hybridization with *C. sativa* has taken place to obtain hybrids tolerant to diseases caused by *Cryphonectria* and *Phytophthora* species. The species hybrids are less drought tolerant and have another growth rhythm. This means that they cannot be used in areas, in which late spring frosts are a constraint for chestnut growth. For regions, in which species hybrids perform well, clones of the exotic species, *C. crenata* and *C. mollissima*, may be included in the clone archives. Also interspecific hybrids might be included in archives. These suggestions were indorsed by Fernández-López (2011). If there is a strong gene flow between orchards and naturalized populations the latter may lose some of their attained Darwinian fitness. To prevent gene flow between orchards or clone archives and naturalized populations, the latter should be isolated as much as possible from orchards and clone archives.

For the subpopulations in the naturalized forest it is important to take measures to guarantee a solid regeneration of these populations.

Within the EU funded project CASCADE Gabriele Bucci developed conservation values for three types of trait; adaptive, disease resistance, and markers. The main results based on Bucci’s derivations were summarized by Eriksson et al. (2005).

The additive conservation value can be based on the evolutionary potential or population divergence. The latter is based on how much a specific population differs from the other populations studied. As seen from Fig. 4-58, the Greek population Paiko was the only population that showed low evolutionary potential. The Spanish population Coruna and the Greek population Hortiatis showed the largest population divergence. The former showed good juvenile growth and the latter showed poor growth. Both populations are important to include in the genetic conservation of *C. sativa*. In this publication many papers report substantial within-population variation of adaptive traits. This indicates that there are good prospects for response of genetic resource populations to changing ambient conditions.
Figure 4-59. Pathogen trait conservation value, PTCV, of *C. sativa* populations. ATCV combines high tolerance to *Phytophthora cambivora* and high potential for improvement of tolerance against this pathogen. The value is given for naturalized, coppice, and orchard populations separately. Eriksson et al. 2005.

The pathogen tolerance conservation value, PTCV, was based on inoculations of the material with one strain of *Phytophthora cambivora* and it was calculated separately for three domestication levels, naturalized, coppice and orchard populations. The PTCV was calculated in such a way that a high PTCV value means good tolerance against *P. cambivora* as well as large evolutionary potential for improvement of tolerance. The two coppice populations from Greece as well as the naturalized Greek population from Hortiatis showed high PTCVs (Fig. 4-59). The French populations and the Spanish orchard populations had low PTCVs.

Three estimates were used for derivation of the marker-based conservation value, MBCV, expected heterozygosity, $H_e$, $F_{ST}$, and $N_e$. For the markers it turned out that $H_e$ had the greatest influence on MBCV. Generally the orchard populations showed the lowest $H_e$ as expected for grafted material (Fig. 4-60). The southern Greek populations had a genetic constitution differing from most other populations and for that reason the Greek populations have a special value for the network of genetic resource populations. Noteworthy is the high $H_e$ in the two English populations, (Glouchestershire and Suffolk), which are both coppice forests.

The calculations were based on too limited sample of populations for a full use of ATCV and PTCV. However, the calculations used in this investigation can be applied in future for chestnut conservation as well as for conservation of other tree species. It was stated that priority should be given to the first two estimates, ATCV and PTCV.

Buffer zones as large as two kilometers around high forest gene resource populations were recommended to avoid pollen contamination. Promotion of flowering in such genetic resource populations should be aimed at.

A comprehensive study of 73 localities with *C. sativa* carried out by Mattioni et al. (2017) is presented in this chapter since the ultimate goal of this investigation was to guide sampling for genetic conservation. They analyzed the allelic variation in six microsatellite loci in these 73 populations originating from almost the entire distribution area of *C. sativa* in Europe. Each population was represented by 9-50 trees. The analysis included data from earlier studies (Martin et al. 2012, Mattioni et al. 2013, Lusini et al. 2014, Chiocchini et al. 2016) as well as analysis of 26 additional populations. The following parameters were estimated:

- Observed and effective number of alleles
- Observed and expected heterozygosity
- Unbiased estimate of expected heterozygosity ($UH_e$)
- Allelic and private allelic richness, $A_r$ and $PA_r$
- Inbreeding coefficient, $F_{IS}$
- UPGMA phylogenetic tree
- Population structure

The priority in selection of genetic resource populations was done by DIVA GIS software (www.diva-gis.org). This software is used to minimize the populations needed for conservation of all genetic diversity. Moreover, it presents the priority in selection and this means that population number 2 to be selected is not necessarily the one with the second highest diversity. Rather, it is the population containing alleles not occurring in the first selected population. Thus, the second population is the best complement to the first selected population.

Figure 4-60. Marker-based conservation value, MBCV, of *C. sativa* populations. From nine regions in Europe. The MBCV is mainly attributed to richness of genetic variability in individual populations. The value is given for naturalized, coppice, and orchard populations separately. Eriksson et al. 2005.
Before a presentation of this report I would like to mention that the report includes many fancy illustrations of significant results. The extension of previous studies did not change the general results of structuring of populations, i.e. three main clusters:

1. One eastern comprising Azerbaijan, Georgia, and eastern Turkey
2. One Bulgaria, Greece, and western Turkey,
3. One with all western European populations

The border between groups 1 and 2 separates southwestern Turkey populations from all eastern populations. A second border separates the western European populations from Greek and Bulgarian populations as well as one of the Romanian populations. The inclusion of populations from Romania and Slovakia in the present study resulted in a slightly different border between groups 2 and 3 compared to previous results (Chiocchini et al. 2016).

Sixteen of the 73 populations had significant positive estimates of $F_{IS}$. Most of them belonged to the eastern population cluster.

The results as regards selection for genetic conservation are illustrated in Fig. 4-61. The authors have kindly supplied me with a list of the ten selected populations as well as the sequence, with which they should be selected. Two populations from the region just east of Black Sea were given high priority (1 and 3). One Central Italian population was given the second rank. The two last selected populations originate from Piemonte in north-western Italy, IT07, and another eastern Black Sea population, GE04.

**Figure 4-61.** The minimum number of geographic units needed to conserve all genetic diversity as revealed by alleles in six microsatellite loci. The analysis comprised 73 natural populations of *C. sativa*. The DIVA-GIS software was used to identify, in sequence of importance, the suggested genetic resource populations. The size of the columns reflects the sequence, in which populations should be selected as well as their importance. This map with *C. sativa* distribution originates from EUFORGEN (www.euforgen.org/distribution_map) Mattioni et al. 2017.
H Provenance research has revealed that much gave a historic survey of preservation of ... genetic conservation rather to present one way for selection of genetic resource populations of *C. sativa*.
clones were included in this preservation repository. An *in vitro* cryo-preservation program was initiated.

Five microsatellite loci were used for classification of 14 cultivars into two clusters. The potential of using molecular markers for identification and breeding was pointed out. Although phenology, flowering, or nut yield traits were recorded during a 6-year period no data were presented. As regards breeding, only breeding goals were mentioned without any outline of a breeding program. Early harvest, high nut yield, excellent nut quality, and disease tolerance were the breeding goals.

### 4.5.2.3 American chestnuts

Dane *et al.* (1999) discussed the need for preservation of American chestnut species via *ex situ* plantations of plants from disease tolerant trees. The selection of the material for conservation or preservation should be populations with a high degree of diversity and populations with unique alleles. *In situ* ecologically managed natural reserves were also suggested. The meaning of ecological management was not explained. An aggressive backcross program designed to transfer the blight resistance of *Chinese chinckapin* into the American chestnuts was initiated.

Alexander *et al.* (2005) reported on the collection of *C. dentata* material from its southeastern range. Populations worthy of conservation were identified. Promotion of flowering by clearing competing vegetation was carried out in the genetic resource populations. Application of hypovirulence to reduce the effects of *Cryphonectria parasitica* on the selected populations was also carried out.

Nuts from crosses of trees in forests were sown and the resulting seedlings were planted in orchards. Collection of scions for grafting onto *C. dentata* and *C. mollissima* rootstocks was included in the conservation efforts. Transplantation of valuable trees was carried out.

### 4.6 Miscellaneous

*In situ* hybridization with ribosomal probes of *C. dentata* root tips was demonstrated by Islam-Faradi *et al.* (2009). They used plasmids with *18S-28S rDNA* from maize and 55 *rDNA* from sugar beet in their experiment. Two *18S-28S rDNA* sites were identified in *C. dentata* while one site was observed for 55 *rDNA*. Similar preliminary results were noted for *C. mollissima*.

Clark *et al.* (2014) reported on early results with field planting of B3 x B3 crosses from the species hybridization program in USA to confer blight resistance into *C. dentata*. No genetic information was given but from a breeding point of view this report deserves being presented in this chapter. Height and survival after one, two or three years in eleven plantations were presented together with remarks on pests and diseases.

The mean survival percentage and final height are presented in Table 4-9. No less than 80% of the plants were browsed by deer in one of the three-year old trials, which affected the plant growth seriously. In spite of this, these trials had the highest survival percentage. One of the two-year old trials had only 49% survival that was attributed to *Phytophthora cinnamomi* infection at this poorly drained trial. Defoliation by the Asiatic oak weevil occurred at relatively high percentage of trees in one of the two-year old trials. Deer repellants reduced deer browsing to one percent only in the youngest trials. In conclusion it was stated that *Success will require a balance among high seedling quality at planting, competition control, disease resistance, and forest management practices to control native and non-native pests and pathogens.* Certainly a demanding task.

<table>
<thead>
<tr>
<th>Age</th>
<th>Number of families</th>
<th>Survival %</th>
<th>Final plant height cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>69</td>
<td>133</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>60</td>
<td>184</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>81</td>
<td>180</td>
</tr>
</tbody>
</table>

The domestication process of *C. mollissima* was studied by LaBonte *et al.* (2018) by resequencing genomes of wild and domesticated Chinese chestnut. Chloroplast haplotypes were also studied in the two types of material. Collection of material for this study was done in six natural populations in China and four orchards; three in China and one in USA. Identification of regions in the genome under selection was carried out by Tajima’s D statistics, which will detect DNA sequences evolving randomly and non-randomly such as directional or balancing selection. Demographic expansion or contraction, genetic hitchhiking, and introgression may also be revealed.

Tajima’s D and II outlier showed that more than 100 intervals were significantly different between the natural population trees and the orchard trees. This analysis led to identification of 25 candidate loci for domestication and 15 of them were attributed to regional differences. Among others these candidate loci contained genes related to flowering time, ethylene synthesis, influence of male fertility, cell wall structure, secondary metabolites, and disease resistance.
Several cpDNA haplotypes not found before were detected. Similar haplotypes were noted for the American Castanea mollissima orchard and southern populations in China suggesting that Chinese chestnuts in the American orchard originate from southern China. It was stated that the differences in diversity between domesticated and non-domesticated populations was not pronounced. Finally this investigation confirmed earlier results suggesting that the Qinling region in Shaanxi province is a center of genetic diversity for C. mollissima.

To overcome the problem with high frequency of empty nuts in commercial Chinese orchards with C. mollissima a study of reproductive biology was carried out by Shi and Stösser (2005). This study did not have a genetic focus but results following mating with five males were reported. Only minute differences in fruit set among the five pollinizers were noted. Slightly higher fruit set was observed in open-pollinated than in artificial cross-pollinated female flowers.

Feng et al. (2011) detected a mutation in a branch of a C. mollissima tree causing a reduction of the male catkin size with 1/6 – 1/8 of the normal size. Anatomical studies revealed that the distal part of the catkin aborted at the stage of staminate differentiation. The branch carrying this mutation produced an increased number of female flowers.

### 4.7 Summary

**Breeding**

The American Chestnut Foundation (TACF) breeding program according to the Multiple Population Breeding System is the only presentation of a long-term breeding program. Some papers could be described as more or less grant proposals in which suggestions for breeding are presented. Several papers treat description of cultivars and thus the possibilities to identify descriptors that are distinct, uniform and stable. One objective was to identify homonymy and synonymy among cultivars. Over time the use of molecular markers for that purpose has increased. Thus, fingerprinting by aid of molecular markers has increased the possibility to distinguish genotypes in breeding populations. These studies are mostly not scientifically path-breaking but still of value for applied breeding.

Matings with one orchard clone surrounded by four other clones and no other pollen sources allowed an estimation of male contribution of the nuts formed. One male gave rise to 48 and another to 21 nuts of the 70 nuts obtained. This illustrates the skewness in spontaneous pollinations. Indications that land races of C. sativa had developed in different parts of Europe were observed. In one study of 73 chestnut trees, classification by aid of five markers was compared. It turned out that SSR markers showed only moderate relationship (r < 0.50) with ISSR, AFLP, and RAPD markers. Another comparison of AFLP, ISSR, RAPD, and SSR markers revealed that AFLPs showed supremacy for studies of genetic diversity.

A search for unique microsatellite alleles in different Castanea species was successful, which means that species and species hybrids in breeding populations can be identified.

A study of 24 morphological traits in F1 and three generations of back crosses together with the parental species C. dentata and C. mollissima was carried out. A principle component analysis revealed that the first principle component captured all of the useful information in the combined data set. Based on this information an Index of Species Identity (ISI) was calculated with a scale 0 - 1, in which 0 stands for C. mollissima and 1 stands for C. dentata. Fig. 4-63 reveals that the phenotype of the B backcross generation is close to the C. dentata phenotype. Strong relationships between backcrosses C. dentata genome and canker length after inoculations with two strains of Cryphonectria parasitica were obtained, R² = 0.81 and 0.84.
Four-year tree height increased with increasing *C. dentata* genome in the backcross progenies from the interspecific *C. dentata x C. mollissima* F₁.

Large variation in grafting success among *C. mollissima* trees from different regions in China was noted. Also in other studies there was large variation in grafting success. In cases of failure bark was found inside the wood.

There are many papers dealing with somatic embryogenesis in chestnut. The main focus in these papers is on development of methods for production of somatic embryos and successful regeneration of plants from somatic embryos. Most papers report results from a low number of genotypes but a few of them contain larger number of genotypes. The state of the art of this topic was presented by Corredoira et al. (2005a). They concluded that the most important factors controlling somatic embryogenesis are:

Developmental stage of the zygotic embryos  
Type of growth regulator and their concentrations.  
Later on effects of sugars, charcoal and light conditions were proven to be significant for somatic embryo production. Charcoal and darkness promoted production. It was stated that conversion of somatic embryos to plantlets was the limiting factor for production of plants from somatic embryos.  
As regards collection time of ovules or zygotes for somatic embryogenesis the results from several studies show that there is a narrow time window for production of somatic embryos. Moreover, this window varies among genotypes. This means that general procedures for somatic embryogenesis production are hard to reach. Even in studies with low number of genotypes, significant differences in production of somatic embryos among trees were proven. High clonal repeatabilities were noted for responsive explants, number of shoots per explant, shoot length, and number of segments per cm in a study of 35 clones. The development of the plants produced was followed up to an age of seven years. The relationship between traits at age one and four or seven were weak. In one study drastic losses occurred at different steps from the 5,760 somatic embryos at the start of the experiment to the final six plants in field. Generally, the percentage of plants obtained from somatic embryos is distressing low. In one study with the starting materials originating from plants obtained from somatic embryos is distressing low. Even in studies with low number of genotypes, significant differences in production of somatic embryos among trees were proven. High clonal repeatabilities were noted for responsive explants, number of shoots per explant, shoot length, and number of segments per cm in a study of 35 clones. The development of the plants produced was followed up to an age of seven years. The relationship between traits at age one and four or seven were weak. In one study drastic losses occurred at different steps from the 5,760 somatic embryos at the start of the experiment to the final six plants in field. Generally, the percentage of plants obtained from somatic embryos is distressing low. In one study with the starting materials originating from crown or base of the tree it was found that the materials “remembered” their original location in the tree even after seven years of cultivation and several cultivation turn-overs.

A cryopreservation technique in liquid nitrogen of somatic embryos was successfully developed.

Reinvigoration by grafting adult material on juvenile rootstocks was tested with some success. Significant improvement of shoot and root formation was noted after combined benzyl adenine spraying and grafting of such a material. Limited success was noted in attempts to transfer genes via ballistic methods. Some success in genetic transformation was obtained by aid of *Agrobacterium tumefaciens* but the percentage of transformation success was extremely low.

### Genetic conservation

Most of the reports on genetic conservation deal with establishment of clonal archives for the economically most important chestnut species. Thus, it mostly concerns preservation of the existing genetic variation. Besides my own contribution, long-term dynamic genetic conservation was not treated. The genetic conservation according to the Multiple Population Breeding System concept was suggested. This means that some 20 subpopulations each with at least 50 flowering trees should be selected to represent the distribution area of the *Castanea* species concerned. The main advantage of the MPBS is that it combines the capture of the total existing genetic variation with a satisfactory variation within each subpopulation and that it allows the target populations to adapt to the prevailing environmental conditions. The selected populations may be used both in breeding and genetic conservation. Clonal archives including material from *C. crenata* and *C. mollissima* were also recommended for the tree breeding of *C. sativa*.

The development of conservation values for different types of trait is an interesting approach for selection of genetic resource populations. Another approach was to use the DIVA GIS software, which was applied on a population genetics study of *C. sativa*, in which alleles in six microsatellite loci were included. This software is used to minimize the populations needed for conservation of all genetic diversity. Moreover, it presents the priority in selection and this means that population number 2 to be selected is not necessarily the one with the second highest diversity. Of the 73 populations studied, ten were selected according to this software. Two eastern populations east of The Black Sea were given priority while no Spanish population was selected by this software.

### Miscellaneous

Tajima’s D and Π outlier analysis of resequenced genomes of cultivars and natural *C. mollissima* trees showed that more than 100 intervals differed between the domesticated and undomesticated *C. mollissima*. Moreover, 25 candidate loci for domestication were identified.
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Gösta Eriksson had his basic university education in botany, genetics, chemistry, and radiobiology at Stockholm University. His thesis *Meiosis and pollen formation in Larix* was publicly defended at the Genetics Department of Stockholm University in January 1969. He was appointed assistant professor the same year. He worked as associate professor in progeny testing at the Royal College of Forestry 1971-1976 and as professor in forest genetics from 1976 to his retirement in 2000.

He was elected member of the Royal Academy for Forestry and Agriculture (KSLA) in 1981 and has served in the board of this academy as well as various committees.

His research areas were cytology, gene ecology, genetic conservation. The phytotron in Stockholm and later on in Uppsala were used for basic studies of population and family responses to environmental conditions such as temperature, photoperiod, water availability, nutrient uptake and nutrient utilization.

Since the nineties genetic conservation is a second focus in his work. Besides the major tree species in northern Europe *Castanea sativa* and *Quercus suber* genetics attracted his interest. He was responsible for and participated in several Nordic and European funded projects.

He was responsible for building up extension service in the forestry faculty at Swedish University for Agricultural Sciences in the early 1980ies. He was the chairperson in the KSLA committee on awards for prominent achievements in extension work from its start in 1984 to 2005. He was the first elected chairman for the Nordic Countries Tree Breeders and Geneticists’ Association. Similarly, he was the first chairperson in EUFORGEN’S network for conservation of noble hardwoods. He has been a member of the network for Mediterranean oaks (earlier cork oak).

He has participated in numerous domestic and international meetings in The Old and the New World.
The author in front of an old *Castanea sativa* tree in Kew Garden London. Photograph Barbro Ekberg.