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# Narrow genetic base in forest restoration with holm oak (*Quercus ilex* L.) in Sicily

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1 **Narrow genetic base in forest restoration with holm oak (*Quercus ilex* L.) in**

2 **Sicily**

3

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21

22 **Running title:** Genetic diversity in holm oak restoration

23 **Abstract**

24

25 In order to empirically assess the effect of actual seed sampling strategy on genetic

26 diversity of holm oak (*Quercus ilex*) forestations in Sicily, we have analysed the

27 genetic composition of two seedling lots (nursery stock and plantation) and their

28 known natural seed origin stand by means of six nuclear microsatellite loci.

29 Significant reduction in genetic diversity and significant difference in genetic

30 composition of the seedling lots compared to the seed origin stand were detected. The

31 female and the total effective number of parents were quantified by means of

32 maternity assignment of seedlings and temporal changes in allele frequencies.

33 Extremely low effective maternity numbers were estimated ( $Nf_e \approx 2-4$ ) and estimates

34 accounting for both seed and pollen donors give also low values ( $N_e \approx 35-50$ ). These

35 values can be explained by an inappropriate forestry seed harvest strategy limited to a

36 small number of spatially close trees.

37

38 ***Quercus ilex* / plantation / genetic diversity / effective population size /**

39 **microsatellite**

40

41

42 **Introduction**

43

44 The maintenance of the natural patterns of genetic diversity of population and species  
45 has been widely recognized as a key factor for the preservation of their evolutionary  
46 potential [30]. The use of autochthonous material is recommended for common forest  
47 practices [24], but there are no guidelines on how much genetic diversity in natural  
48 populations should be represented in an artificially reforested stand to guarantee its  
49 viability in the following generations [30]. The introduction of low diversity material  
50 could result in a reduced long term viability of plantations or in the failure of  
51 demographic rescue of local impoverished populations, due to a decrease in their  
52 effective population size [25].

53

54 Theoretical approaches establish that the genetic drift in the seed collection process is  
55 determined by the number of seed parents before than by the number of seeds per  
56 parent [4, 43]. In wild seed collection the effective size and diversity of pollen donors  
57 is unknown a priori, hence the number of seed trees definitively represents the  
58 operative tool for achieving the conservation of genetic diversity levels [13]. Studies  
59 exploring the genetic diversity of plantations originated from seedlots collected from  
60 natural stands are scarce. In some cases, reduction or biases in genetic composition of  
61 plantations have been linked to a limited or non-random sampling of maternal trees  
62 [13, 26, 35, 40], although this assumption was not verified experimentally.

63

64 On Sicily, natural woods currently occupy only 10% of the area and one third of them  
65 are broadleaf formations. At present, Sicilian woods are scarcely economically  
66 productive, their main interest lying on ecological conservation and landscape values.  
67 Mediterranean vegetation dominates Sicilian ecosystems, where the holm oak,  
68 *Quercus ilex* L., is a key species in many primary and secondary formations from sea  
69 level up to 1 800 m. On the island, this sclerophyllous evergreen tree forms pure and  
70 mixed forests, although it is locally reduced to small relict populations. Over its  
71 western Mediterranean distribution the holm oak shows high levels of nuclear genetic  
72 diversity within populations, and low interpopulation differentiation [31]. Maternally  
73 inherited chloroplast genome suggested glacial refugia in the three Mediterranean  
74 peninsulas and Sicilian populations have shown to represent a reservoir of diversity  
75 [11].

76

77 In order to empirically assess whether forest genetic material from actual seed  
78 sampling strategies suffer changes in genetic diversity relative to natural old-growth  
79 populations, we analysed the genetic composition of seedling lots in comparison with  
80 the known autochthonous seed origin stand. We used *Quercus ilex* as model tree. Its  
81 representative role in Sicilian natural and artificial forest ecosystems makes it one of  
82 the most widely used species for restoring deforested areas and converting introduced  
83 pine plantations. By testing intrapopulation genetic diversity measures and  
84 quantifying genetic drift effects we discuss how actual forestry practices could affect  
85 the long term viability of holm oak plantations in Sicily.

86

87 **Materials and methods**

88

89 *Study site and experimental design.*

90 The study was undertaken in Sicily (Fig. 1): one-year-old seedlings from a plantation  
91 located in the Monte Palmeto and from the nursery of Piano Noce were sampled.

92 These two progeny sets were originated from seeds collected in 2001 on the ground  
93 of a 100 m × 60 m enclosure containing 15 adult holm oaks (hereafter called  
94 candidate mother trees). This group of trees is used as seed source for Piano Noce  
95 nursery, which supplies the holm oak seedling demand for reforestation actions in the  
96 north-western part of Sicily. The 15 individuals and 40 additional adult trees from the  
97 surrounding continuous natural forest of Piano Zucchi were collected (sample  
98 referred as seed origin stand from now on, trees outside the enclosure were chosen  
99 randomly maintaining 50 m distance between them).

100

101 [[[Figure 1]]]

102

103 Sample size for the progeny sets were set to 40 individuals, but only 33 individuals  
104 were available from Monte Palmeto because of the high mortality rate for seedlings  
105 after plantation. Fresh leaves were collected from each individual and stored at -80°C.  
106 DNA was extracted following the method described by Doyle and Doyle [7].

107

108 *Molecular markers*

109 All individuals of the study were genotyped for six microsatellite loci: MSQ4,  
110 developed for *Quercus macrocarpa* [5]; QpZAG15, QpZAG36 and QpZAG46  
111 developed for *Q. petraea* [39]; QrZAG11 and QrZAG20 developed for *Q. robur* [20].  
112 Amplification was performed as described in SOTO *et al.* [37], except for QpZAG36  
113 and QrZAG20. The annealing temperature of 51°C has been used with QpZAG36. A  
114 touchdown procedure has been used for QrZAG20, consisting in 20 cycles starting at  
115 65°C and decreasing 0.5°C each cycle, followed by 20 cycles at 55°C. PCR products  
116 were sized in 6% polyacrylamide gels and electrophoresis was performed on an  
117 automatic sequencer Li-Cor 4200 (Li-Cor Biosciences). Microsatellites were scored  
118 with Gene ImagIR v. 3.56 (Li-Cor Biosciences).

119

120 *Assessment of Hardy-Weinberg model*

121 Preliminary analysis were conducted with MICRO-CHECKER 2.2.0 [42] to assess  
122 the possibility of null alleles or genotyping errors due to stuttering or allelic drop out.  
123 Every pair of loci was tested for linkage disequilibrium by using FSTAT 2.9.3.2 [14],  
124 because the independent transmission of alleles is a required condition for subsequent  
125 analyses (estimation of kinship and genetic differentiation, parentage analysis). Two  
126 of our microsatellites loci belong to the same linkage group in *Quercus robur* [3], but  
127 their linkage in *Q. ilex* has never been studied. According to the results of linkage  
128 disequilibrium test, null allele estimation and exclusion probability computation, we  
129 decided to exclude locus QpZAG36 from analyses requiring unlinked loci (see

130 results). Single locus genotypes were tested for deviations from Hardy–Weinberg  
131 expectations by using FSTAT 2.9.3.2 (1 000 permutations), to assess whether  
132 inbreeding or familiar relationships might produce interferences with the linkage  
133 disequilibrium analysis. The fixation index  $F_{IS}$  was calculated for each locus and  
134 overall loci with the same program. Since the presence of a familiar structure inside  
135 the 15-holm-oak enclosure of Piano Zucchi could produce a reduced genotype  
136 variability in the offspring, as well as some bias in parentage assignment [29], the  
137 relationship among the candidate mother trees has been examined by the estimation  
138 of the kinship coefficient,  $F$  [27], with SPAGEDI 1.2 [16].

139

#### 140 *Genetic diversity and differentiation*

141 The following indices were computed for each locus and for each sample: number of  
142 alleles,  $n_a$ ; allelic richness standardized to the smallest sample,  $A$  [9]; unbiased  
143 effective number of alleles,  $A_e$  [34] and unbiased gene diversity,  $H_e$  [33]. To explore  
144 whether diversity indices in the seedling lots had lower values compared to the  
145 natural seed origin stand, a Monte Carlo resampling approach has been used (10 000  
146 iterations) for each pair of compared samples (pair 1: seed origin stand/nursery stock;  
147 pair 2: seed origin stand/plantation). This approach provided an estimation of the p-  
148 value to reject the null hypothesis of no difference in genetic diversity levels among  
149 samples. Whole multilocus genotypes were permuted to maintain the original  
150 association of allele within the genotypes. The pairwise genetic differentiation  $\theta$  [45]



151 and its significance have been estimated with a permutation procedure (10 000  
152 iterations) with FSTAT 2.9.3.2 software, assuming Hardy-Weinberg equilibrium.

153

#### 154 *Parentage inference and effective number of mothers*

155 The power of our set of loci in parentage assignments was evaluated computing the  
156 value of non-exclusion probabilities [18] for one parent when the genotype of the  
157 other parent is unknown and for parent pair with CERVUS 3.0 [19]. In order to infer  
158 the number of individuals, among the 15 candidate mothers, contributing to the  
159 genetic diversity of the two seedling sets, two types of parentage analysis was  
160 undertaken with CERVUS. The program uses a likelihood-based approach in which  
161 the strength of parentage assignment is evaluated with the log-likelihood ratio  
162 calculated over all loci (LOD-score) for each candidate parent. Using a simulation  
163 procedure CERVUS produces a critical LOD-score value, below which parentage  
164 cannot be attributed at the level of precision chosen (here 80% and 95% were used).  
165 A value of 0.001 has been used for the error rate to take into account the occurrence  
166 of mistyping, null alleles or mutations. Allele frequencies from seed origin stand were  
167 used as reference for CERVUS calculations. We first performed a one parent  
168 analysis, where CERVUS searches for the first parent in absence of genetic  
169 information on the second parent. We assumed the most likely parent assigned being  
170 the mother in consideration of two aspects: i) the knowledge that both seedling lots  
171 proceeded from the 15-trees enclosure and ii) the low probability of finding the  
172 fathers inside the enclosure, taking into account its restricted area and the high level

173 of pollen flow expected in *Quercus*. The sampled percentage of breeding female  
174 population was set to 95%. We considered 95% a conservative estimation as we  
175 cannot exclude the dispersal of seeds from outside the 15-tree enclosure. In any case,  
176 a 100% value was also used to assess the effect of this parameter on the percentage of  
177 unresolved assignments. With the same set of 15 candidate parents a parent pair  
178 analysis was also carried out, in order to quantify, if any, the bias in maternity  
179 assignments due to the identification of male parents among the first-parent  
180 assignments. We set the 15 trees both as candidate male parents and candidate female  
181 parents. As a conservative approach, the proportion of female candidate parents  
182 sampled was set to 0.95, while proportion of male candidate parents sampled was set  
183 to 0.50. An independent control of parentage assignment was made taking advantage  
184 of the putative linkage between loci QpZAG46 and QpZAG36, comparing the match  
185 of association between alleles from each individual of the offspring and the inferred  
186 mother tree or parent pair.

187

188 The reproductive success per mother tree, estimated with maternity analysis with  
189 more than 80% of confidence, allowed to compute the effective maternity number,  
190  $Nf_e$  [34]. Furthermore, to evaluate the potential resistance to random genetic drift of  
191 seedling lots included in the nursery stock of Piano Noce and involved in the  
192 plantation of Monte Palmeto, effective population sizes ( $N_e$ ) for the two seedling  
193 samples have been estimated with a likelihood procedure implemented in MLNE 1.0

194 [44], which takes into account the changes in allelic frequencies between two  
195 generations (considering the seed origin stand, Piano Zucchi, as the first generation).

196

## 197 **Results**

198

### 199 *Assessment of Hardy-Weinberg model*

200 Only one locus (QpZAG36) for one sample (the seed origin stand, Piano Zucchi)  
201 showed a significant excess of homozygotes ( $p = 0.001$ ), which could be due to the  
202 presence of null alleles, however at least one allele was amplified for all individuals  
203 at this locus. No evidence of stuttering or allelic drop out for larger alleles could be  
204 detected. For all samples test for Hardy–Weinberg equilibrium revealed a significant  
205 departure ( $p = 0.002$ ) only for QpZAG36 among the six loci used. No evidence of  
206 inbreeding has been found (data not shown). Close relatedness among the 15  
207 candidate seed mother trees was not detected, as their mean kinship value ( $\overline{F_{ij}} = -$   
208  $0.003$ ) is similar to the mean value relative to all the other individuals sampled in the  
209 whole seed origin stand ( $\overline{F_{ij}} = -0.008$ ). Linkage disequilibrium was significant  
210 between loci QpZAG46 and QpZAG36 in all samples ( $p = 0.008$ ). We will consider  
211 likely that these loci maintain some level of genetic linkage in *Q. ilex* genome.

212

### 213 *Genetic diversity and differentiation*

214 The indices tested show that genetic diversity in both seedling lots has suffered a  
215 significant reduction compared to natural seed origin stand (Table I).  
216 In particular, allelic richness decreases by 22.5% and 33.6%, and the effective  
217 number of alleles by 18.9% and 32.5%, in the nursery and plantation respectively.  
218 The expected heterozygosity ( $H_e$ ) shows a similar pattern of reduction, but less  
219 dramatic. In fact, only the reduction suffered by the planted stand results significant.  
220 The artificial populations showed also a significantly different genetic composition  
221 compared with the seed origin stand, although the significance of the pairwise genetic  
222 differentiation,  $\theta$  (Table I), might be overestimating the differentiation, due to the  
223 particular set of markers used [as shown in 36].

224

225 [[[Table 1]]]

226

#### 227 *Maternity inference and effective number of mothers*

228 One parent assignment was performed by a maternity analysis to infer the number of  
229 trees among the 15 candidate mothers that had contributed to the genetic diversity  
230 observed in the nursery sample and in the plantation. The exclusion of QpZAG36  
231 locus in the maternity analysis because of its putative linkage to QpZAG46 does not  
232 substantially change the parentage inference in the light of the combined non-  
233 exclusion probabilities estimated (Table II).

234

235 [[[Table 2]]]

236

237 At 80% of confidence 98% of the seedlings could be ascribed to one of the candidate  
238 mothers (97% of plantation individuals and 100% individuals from the nursery  
239 sample). Within the assigned offspring over 17% matched at 95% of confidence. For  
240 the 0.001 error rate assumed, only one individual of the plantation are left unassigned,  
241 setting the sampled percentage of the breeding female population either to 95% or to  
242 100%. Among the 15 candidate mothers seven have been identified as source of seeds  
243 for the nursery sample and eight for the plantation (Figure 2). Most of the seedlings  
244 sampled (58%) were identified as offspring of mother trees m1, m9, m12. However,  
245 assigned mothers are not shared between the two offspring groups. In fact, within the  
246 nursery sample 78% individuals come from trees m1 and m9, while within the  
247 plantation 32% come from trees m12 and 43% from trees m14 and m15 (Figure 2).  
248 The parent pair assignment identifies only one parental pair for one seedling (among  
249 66 analyzed) at 80% of confidence. This does not lead to any correction of the  
250 maternity assignment, as the two genotypes correspond to the same candidate parent  
251 (m1).

252

253 [[[Figure 2 ]]]

254

255 Almost every pair offspring-mother genotypes have been confirmed by the control of  
256 the match of allele association for the putative linked loci QpZAG46 and QpZAG36.  
257 For all but six seedlings we observed an allele combination concordant with the

258 assigned mother genotype, and, when more than one seedling was attributed to the  
259 same candidate mother, this was true for the whole group of seedlings (data not  
260 shown). The six mismatches found could be due to either the error rate included in  
261 the mother assignment method or the incorrect identification of alleles during  
262 genotyping. However, among the six mismatches, one individual from the nursery  
263 sample and three from the planted stand match with the second most likely mother  
264 tree. In any case, considering the second most likely mothers as the true ones for  
265 these seedlings would not increase the total number of assigned mothers. The match  
266 of allele association was also compatible for the only trio offspring-parent pair  
267 genotypes.

268

269 Maternity analysis results expressed in terms of the female contribution to parental  
270 population produced very low estimations of effective maternity number ( $Nf_e$ ). For  
271 the nursery sample a value of  $Nf_e = 1.65$  corresponds to the seven putative mother  
272 trees, while for the plantation a value of  $Nf_e = 4.39$  corresponds to the eight putative  
273 mothers. The effective population size accounting for both female and male  
274 contribution gives  $N_e = 52.5$  (95% confidence interval, CI, 32.1–120.0) for the  
275 nursery sample and  $N_e = 35.4$  (95% CI 24.3–61.7) for the plantation.

276

## 277 **Discussion**

278

279 *From natural seed origin stand to plantations*

280 Ecosystem restoration is an issue of major concern to forest management in Sicily  
281 and, regarding *Quercus ilex* genetic resources, priority has to be given to a  
282 conservation forestry to preserve the specific genetic diversity found in Sicilian  
283 populations [11, 28, 31]. Quite the contrary, the results of this study showed an  
284 overall significant reduction of genetic diversity and a significant difference in  
285 genetic composition of seedling lots in comparison with the natural seed origin stand.  
286 The complementary approach of maternal assignment, may be more informative for  
287 the purposes of this study because it allows direct measure of the reduction in  
288 population size, revealed that very few mother trees have finally contributed to the  
289 genetic diversity of artificial populations examined (seven trees for the nursery stock  
290 and eight for the plantation). In terms of effective number of seed donors, the number  
291 of contributing mother trees is further reduced because of the differences in  
292 reproductive success. Estimates accounting for both seed and pollen donors show also  
293 low values. Comparing the genetic behaviour of experimental populations when  
294 founder events were induced [10, 38] with our finding of reduced population  
295 effective size in holm oak seedling lots, we deduce that the latter experienced the  
296 equivalent to a bottleneck process, due primarily to collecting seeds from a limited  
297 number of mother trees.

298

299 Our results conform to previous works which have found that seedlots or planted  
300 stands have a reduced genetic diversity or different genetic composition in  
301 comparison with natural unmanaged populations. In some cases seed harvest from

302 few or non-randomly selected trees has been proposed as one of the most likely  
303 causes [13, 26, 35]. The importance of small population size effect was also shown  
304 by Kitzmiller [21], who compared seedlots of *Pinus ponderosa* and reported the  
305 lowest allelic diversity for the lot collected from the smallest number of trees.

306

### 307 *Factors involved in genetic composition of seedlings*

308 Practical considerations usually determine the selection of seed harvest site in Sicily,  
309 including nursery proximity and road accessibility. Currently, no genetic criteria are  
310 taken into consideration, either because of the general lack of knowledge on the level  
311 and distribution of genetic diversity in Sicilian forest species, or because of the  
312 absence of legal regulations of the genetic composition of seedlots. In theory,  
313 according to *Quercus* species features (allogamy and wind pollination) 15 trees could  
314 be a suitable number to collect the great majority of the population variability [4].  
315 Nevertheless, the strong difference between the number of candidate mother trees and  
316 the actual contributing mothers indicate that other natural and artificial factors, not  
317 taken into consideration for collection planning, could have had a significant  
318 contribution in reducing seedlots diversity.

319

320 In general, reproductive properties as asynchrony in flowering and fruiting phenology  
321 among plants, and individual inter-annual variation in fertility for *Quercus* species [2,  
322 8, 17, 22, 23] determine non-random mating in each reproductive season. In *Quercus*  
323 *ilex*, Lumaret *et al.* [28] recorded that, in a single year, variation in male and female



324 investment involved 15–20% of individuals. In addition, at local scale, differentiation  
325 among the pollen clouds received by different mother trees could significantly  
326 depend on intermate distance, regardless of its dependence on long distance pollen  
327 dispersal [6, 22, 32, 41]. In our study area, the 15-holm-oak enclosure is included in a  
328 wider zone characterized by a low density formation which progressively turns into a  
329 closed wood. Therefore, likely few closer individuals would have the highest  
330 probability of a successful mating in a single reproductive event. Further, the 15 oaks  
331 can be classified into two cohorts, 10 very old trees, and five young trees (Figure 1).  
332 Since fecundity and acorn production are positively correlated with plant or crown  
333 size [1, 15], individuals are expected to differ greatly in their contribution to the next  
334 generation in both male and female fertility. Additionally, the overlapping of crowns  
335 of some old individuals (m3, m4, m5) among them and with an equal-size maple  
336 (*Acer monspessulanum*), may restrict flower and fruit development due to space  
337 competition or light limitation [1, 22]. In fact, it is remarkable that the large tree m5,  
338 the closer to the maple (Figure 1), does not contribute to any of the seedling groups  
339 (Figure 2). All cited factors affecting the individual reproductive performance could  
340 have produced a natural bias in the genetic composition of the 15-trees annual acorn  
341 production and, therefore, in harvested seedlots.

342

343 After collection, genetic variation might be further reduced due to seed and seedling  
344 handling and to plant responses to domestication [21, 24, 40] until successful seedling  
345 in-field establishment is accomplished. More important might be the maladaptation to

346 local conditions of transplanted material [25] (e.g. altitude difference between the  
347 seed origin stand and the plantation is about 700 m). Nevertheless, our data concern  
348 neutral genetic diversity, thus the impact of selection cannot be estimated. We have  
349 no current data on mortality rate for holm oak seedlings in this study, but it is relevant  
350 that Monte Palmeto plantation sample size was constrained to the first-year-survivor  
351 seedlings (33 plants over 1 000 initially planted). The post-nursery selection, whether  
352 human or environment mediated, could have also led to the shift of coincidence in  
353 assigned mother trees between the plantation and the nursery sample.

354

#### 355 *Management implications*

356 The low genetic diversity found for seedlots in this study is likely to concern many  
357 recent forestations on Sicily. In the case of *Q. ilex* acorn harvesting from Piano  
358 Zucchi forest, an increase in the number of seed trees and distance between trees is  
359 recommended. In consideration of the wide extension of Piano Zucchi forest (more  
360 than 1 000 hectares), probably the most effective harvest design includes at least 20–  
361 30 scattered plants, distributed in a few high distance groups (hundred of meters) of  
362 low distance trees (tens of meters). The most efficient model for seed collection and  
363 sampling optimization (i.e. minimal number of tree and seeds per tree for the  
364 maximal yield) could be reached comparing the seedlot genetic diversity from a  
365 number of seed trees progressively higher. In order to achieve this target, setting  
366 minimum species-specific levels of diversity for plantations has been devised as a  
367 difficult key task [12, 21] since it is subjected to the knowledge of the genetic

368 structure of natural stands in an area which is not available in general (except for few  
369 well known temperate species). The genetic diversity of the autochthonous seed  
370 origin stand could be the natural baseline for any plantation, as shown by this study  
371 [but see 40]. It is straightforward that the ideal situation would also be able to ensure  
372 adaptation of genetic material to plantation site [25], but this kind of information  
373 seems to be even more difficult to obtain.

374

375 Our results are based on a single-year seed collection. However, differences among  
376 years are expected as discussed above. In multi-year restoration projects, the annual  
377 addition of seedlings could reduce the loss of variability, increasing progressively the  
378 effective population size and the genetic base (this could be the case of Monte  
379 Palmeto plantation, whose planted area is increased annually). Nevertheless, if  
380 plantation is carried out with only a one-season seed stock, its reduced genetic  
381 diversity could compromise or make ineffective the restoration aim in the long run  
382 (i.e. in isolated condition, in genetic rescuing actions, or under hard environmental  
383 conditions).

384

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392

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519

520

521 **Figure 1**

522 Location of the sampled populations of *Quercus ilex* in Sicily: seed origin stand  
523 Piano Zucchi forest (A), Piano Noce nursery (B) and Monte Palmeto plantation (C).  
524 An enclosed group of 15 holm oaks (D) is the seed-tree source of nursery and  
525 plantation. Relative position of trees and diameter to the breast height (represented as  
526 a circle size) are shown.

527

528 **Figure 2**

529 Distribution of maternity assignments to the 15 candidate mother trees. Individuals  
530 from the nursery and the plantation are assigned with two confidence levels 80% and  
531 95%.

532

533 **Table I** Mean value of diversity indices (number of alleles  $n_a$ , allelic richness  $A$ ,  
 534 effective number of allele  $A_e$  and expected heterozygosity  $H_e$ ) for six microsatellite  
 535 loci and genetic differentiation ( $\theta$ ) per pair of populations.

536

	seed origin	nursery	P-value	seed origin	plantation	P-value
	stand			stand		
$n_a$	10	7	0.0007	10	6	< 0.0001
$A$	8.795	6.978	0.0009	8.795	5.974	0.0001
$A_e$	4.536	3.681	0.0098	4.536	3.062	< 0.0001
$H_e$	0.653	0.641	0.2474	0.653	0.518	< 0.0001
$\theta$	0.023		0.0028	0.036		0.0002

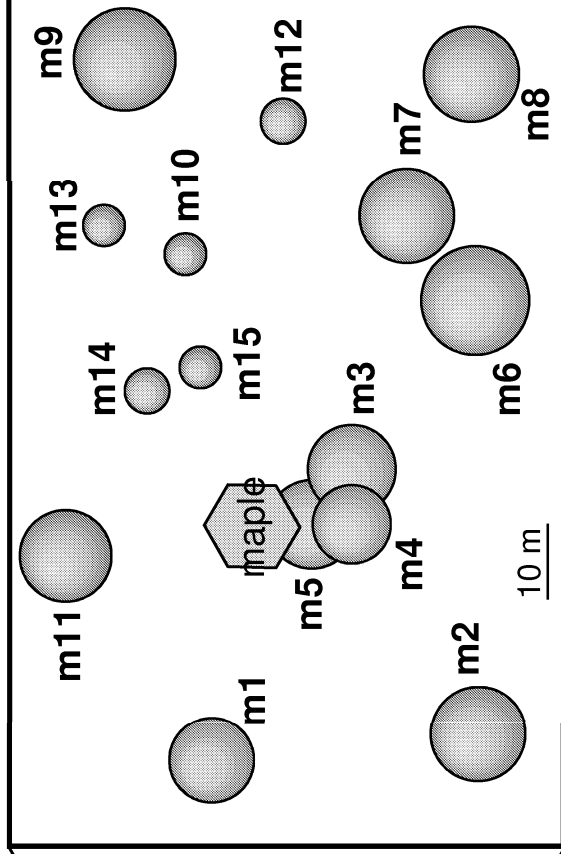
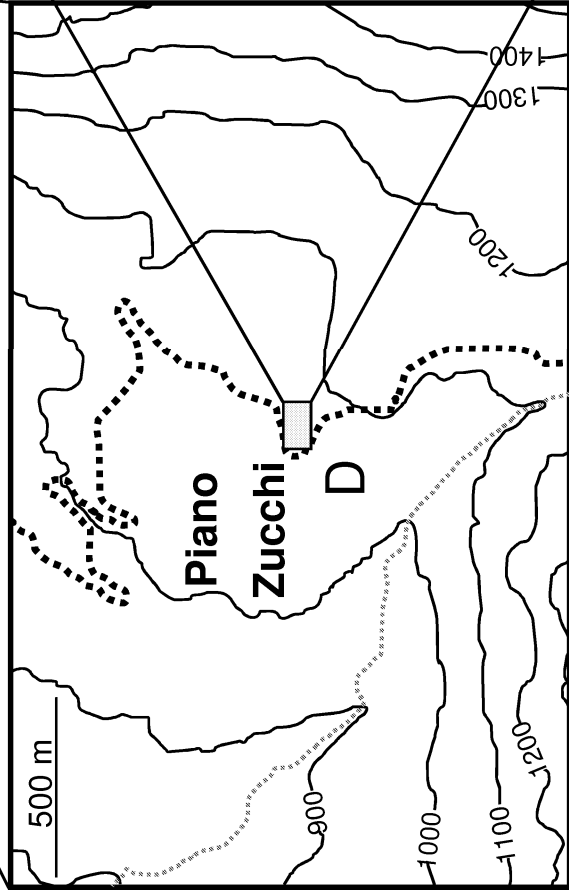
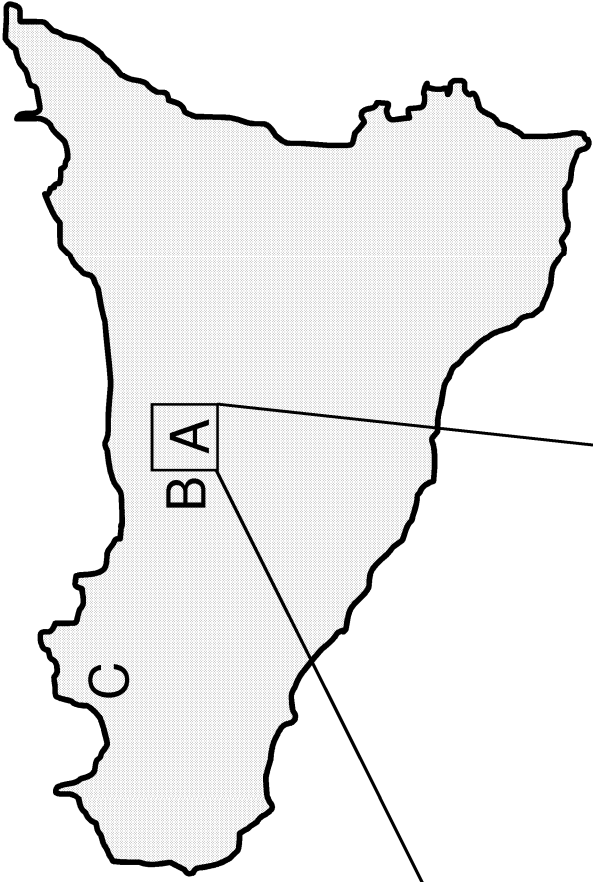
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539 **Table II** Average non-exclusion probabilities in one candidate parent (NE-1P) and  
 540 candidate parent pair (NE-PP) assignments calculated by CERVUS 3.0, separately for  
 541 the six microsatellite loci and combined for two sets of six and five loci (without  
 542 QpZAG36). Loci sorted by increasing values.  
 543

<b>Loci</b>	<b>NE-1P</b>	<b>NE-PP</b>
QrZAG20	0.457	0.124
QrZAG11	0.460	0.125
QpZAG15	0.592	0.232
QpZAG46	0.665	0.277
QpZAG36	0.828	0.516
MSQ4	0.995	0.903
<b>Combined: 6 loci</b>	0.068	<0.001
<b>Combined: 5 loci</b>	0.082	0.001

544



..... Road ..... River

