ALLOZYME VARIATION WITHIN AND BETWEEN NINE ITALIAN POPULATIONS OF ALEPPO PINE (*PINUS HALEPENSIS* MILL.)

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ABSTRACT

The results of a research on the genetic structure of southern Italy populations of *Pinus halepensis* Mill. are presented. For this purpose nine natural populations were sampled and subjected to isozyme analysis. Seven enzyme systems, coded by 13 loci, were utilized: LAP, GOT, PGM, 6PGDH, MDH, GDH, SKDH; only six loci were polymorphic. As observed in former researches, genetic variation in this species is rather low in comparison with variation estimated in most of the studied conifers (mean H_e is 0.112). On the whole, the Apulian and Basilicata populations are more variable than the Calabrian ones. As observed in most conifers, differentiation between populations is low, as revealed by the mean values of parameters G_{st} (3.3 %) and δ (4.2 %), as well as the mean values of genetic distance by NEI (0.011) and GREGORIUS (0.050). Population clusters resulting from the dendrogram based on NEI's genetic distance values are slightly different from those indicated by multivariate analysis. On the basis of the available archaeobotanical data and of the genetic similarity - observed by other authors - between Greek and Apulian populations, which are those having the highest genetic diversity in the whole range of this species, the hypothesis can be raised that Aleppo pine was introduced in Apulia by Greek colonizers in historical time (*Magna Grecia*). Some hypotheses on the evolutionary history of southern Italy populations are discussed.

Key words: *Pinus halepensis*, southern Italy, isozymes, genetic diversity, archaeobotany, evolutionary history.

Introduction

Aleppo pine (*Pinus halepensis* Mill.) is one of the widest-spread forest tree species in the Mediterranean (MIROV 1967) due to its optimum adaptation to the bioclimates of this area, namely to its high tolerance to drought and adverse pedological conditions, as well as to some features of its reproductive cycle and the production of serotinous cones; this latter feature is important as it allows natural regeneration following fire passage (FRANCINI 1958; SARACINO & LEONE 1993a, 1993b; SARACINO *et al.* 1997; LEONE *et al.* 2000a). Its wide spreading is the result of the natural colonization of the Mediterranean basin starting from its center of origin in the Caucasian region (CONKLE *et al.* 1988) and, in all probability, partially also of human activities (SCHILLER & BRUNORI 1992; SCHILLER & MENDEL 1995).

The extent of the surface covered by this species and its naturalistic, ecological and landscape importance have spurred investigations on the provenance variation of its morphological, physiological and biochemical traits (DEBAZAC & TOMASSONE 1965; MIROV *et al.* 1966; PALMBERG 1975; PELIZZO & TOCCI 1978; CALAMASSI *et al.* 1980; PANETSOS 1981; CALAMASSI 1982; FALUSI *et al.* 1983; CALAMASSI *et al.* 1984; MENDEL 1984; SPENCER 1985; CALAMASSI 1986; FISHER *et al.* 1986; ECCHER *et al.* 1987; SCHILLER & GRUNWALD 1987; CALAMASSI *et al.* 1988; GRUNWALD & SCHILLER 1988; BARADAT *et al.* 1989; SCHILLER & WAISEL 1989; WEINSTEIN 1989a, 1989b; BARITEAU 1992; BARADAT *et al.* 1995; KOROL & SCHILLER 1996; CUCCUI *et al.* 1996; GALLIS & PANETSOS 1997; TOGNETTI *et al.* 1997; MENDEL 1998; CALAMASSI *et al.* 2001). Such investigations have been mostly performed on seed samples collected within the FAO-IUFRO 4-bis project "International experiences upon provenances of *Pinus halepensis* and *Pinus brutia*", which was launched in the 70s.

However, probably due to the scarce economic importance attached to Aleppo pine, despite its particular suitability to reforestation of arid zones, relatively few studies have been performed on population genetic structure using isozyme markers (LOUKAS *et al.* 1983; GRUNWALD *et al.* 1986; SCHILLER *et al.* 1986; CONKLE *et al.* 1988; KOROL *et al.* 1995; TEISSEIRE *et al.* 1995; KOROL & SCHILLER 1996; AGÚNDEZ *et al.* 1997; PANETSOS *et al.* 1997; AGÚNDEZ *et al.* 1999) and

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molecular markers (BUCCI *et al.* 1998; MORGANTE *et al.* 1998). SCHILLER *et al.* (1986) and CONKLE *et al.* (1988) obtained important information on the taxonomic and phylogenetic relationships within the *Pinus halepensis* - *Pinus brutia* Ten. species complex from isozyme analysis. They could hypothesize that Aleppo pine is a genetically depauperate derivative from the *P. brutia* subsp. *stankewiczii*-like progenitor of the entire complex (SCHILLER 1994).

SCHILLER *et al.* (1986) carried out what is still today the widest investigation on Aleppo pine population genetic structure by analyzing 26 populations distributed all over the natural range, of which four Italian ones.

In Italy, Aleppo pine high forests cover a total surface of some 20,000 hectares, half of which located in the Apulia region (MAGINI 1955; BERNETTI 1995; PUGLISI *et al.* 1999).

The present research work is the second step in a study still underway aimed to analyzing in detail the genetic structure of Italian populations by isozyme analysis. The first step focused on five southern populations (PUGLISI *et al.* 1999); here the results of these first five populations are presented together with those of four more populations sampled and analyzed later.

Allele frequencies concerning the nine populations under study have been used to calculate, as usual, a set of genetic diversity and differentiation parameters, as well as in multivariate analysis to compare and integrate the results obtained with the two different procedures.

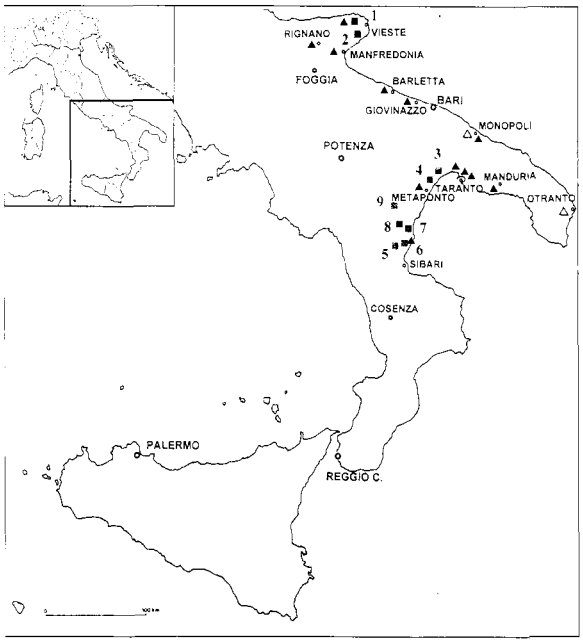

Figure 1. Location of the studied populations (grey squares). 1: Coppa della Nuvola (Peschici, Foggia — Gargano, Apulia); 2: Monte Barone (Mattinata, Foggia — Gargano, Apulia); 3: Perronello (Riva dei Tessali, Taranto — Apulia); 4: Pineta della Regina (Marina di Ginosa, Taranto — Apulia); 5: Albidona 1 (Cosenza — Calabria); 6: Albidona 2 (Cosenza — Calabria); 7: Stràtolo (Valle del Ferro, Cosenza — Calabria); 8: Masseria Sorìa (Valle del Ferro, Cosenza — Calabria); 9: Tursi (Matera — Basilicata). Black and white triangles mark the archaeological sites subjected to archaeobotanical analysis (see text).

MATERIALS AND METHODS

Sampled populations

The nine studied populations (Figure 1) are reputed to be of natural origin and are located in four areas of southern Italy: Gargano (Apulia; two populations), Apulian Ionian coast (two populations), Calabrian Ionian belt (four populations), Basilicata (one population).

For the first eight populations, sampling was carried out by collecting cones from 10 trees per population, located at a minimum distance of 100 meters and uniformly placed on the selected area, in order to have representative enough progenies - especially their pollinic component - on account of the low number of seed parents. Only in population 4 the progenies of nine trees were used for analyses, because all the seeds collected from the tenth tree resulted to be non-vital.

Population 9, on the contrary, due to the proximity of artificial stands and the consequent risk of progeny contamination by external pollen, was sampled by collecting cones from 20 trees in order to obtain a representative sample of seed parent genotypes, rather than of embryo paternal alleles as in the other populations.

The first five populations were sampled in the spring of 1995 and analyzed the following year (PUGLISI *et al.* 1999), the remnant were sampled in the spring of 1997 and analyzed in the same year.

Seeds were dried and stored at 4–5 °C before being analyzed.

Isozyme analysis

For the first eight populations, isozyme analyses were performed on endosperms (which are haploid and identical to the female gametes) and embryos of 12 seeds per mother tree (120 seeds per population, 108 only for population 4) by means of horizontal starch gel electrophoresis in order to distinguish between male and female gamete in each embryo.

As regards population 9, only genotypes of the 20 seed parents were taken into consideration to estimate allele frequencies by analyzing six endosperms from

each individual progeny.

Seven enzyme systems, coded by 13 loci, were utilized: LAP (leucine aminopeptidase, E.C. 3.4.11.1), GOT (glutamate oxaloacetate transaminase, E.C. 2.6.1.1), PGM (phosphoglucomutase, E.C. 2.7.5.1), 6PGDH (6-phosphogluconate dehydrogenase, E.C. 1.1.1.44), MDH (malate dehydrogenase, E.C. 1.1.1.37), GDH (glutamate dehydrogenase, E.C. 1.4.1.2), SKDH (shikimate dehydrogenase, E.C. 1.1.1.25).

Endosperms and embryos were separately homogenized in a buffer 0.08 M tris - 1.00 M HCl, pH 7.2 (MÜLLER-STARCK, pers. comm.).

Electrophoresis was performed using the following buffer systems:

a) electrode buffer: 0.06 M NaOH - 0.30 M boric acid, pH 8.2; gel buffer: 0.08 M tris - 1.00 M HCl, pH 8.7 (POULIK 1957, modified), for LAP and GOT;

b) electrode buffer: 0.135 M tris - 0.047 M citric acid, pH 7.0; gel buffer: 0.034 M tris - 0.012 M citric acid, pH 7.0 (SHAW AND PRASAD 1970, modified), for the remaining enzyme systems.

Starch gel concentration was 11% for buffer system a) and 12% for b). Staining was performed according to MÜLLER-STARCK (1998 and pers. comm.).

The genetic control of the utilized enzyme systems was determined by comparing the endosperm segregation ratio of putative heterozygote seed parents with the expected 1:1 Mendelian ratio (PUGLISI *et al.*, in preparation).

For each enzyme system, loci were designated by capital letters following the enzyme acronym, and the most anodal zone of activity was marked by the first letter. Within each locus, alleles were designated by numbers, starting from the fastest one.

Genetic parameters

Computations were performed with BIOSYS-1 software (SWOFFORD & SELANDER 1989) on embryo genotypes. The contingency table chi-square test (SNEDECOR & COCHRAN 1967) was used in order to estimate the heterogeneity between population distributions of allelic frequencies.

On the basis of the estimated allele frequencies, the following parameters of genetic diversity (variation within populations) were computed: average number of alleles per locus (N); percentage of polymorphic loci (P) computed on the basis of 5% criterion, i.e. the percentage of loci whose more common allele has a frequency lower than 95%; genetic diversity (ν; GREGORIUS 1978; MÜLLER-STARCK & GREGORIUS 1986), also called "effective number of alleles" (CROW & KIMURA 1970), whose average value per population is computed as the harmonic mean of single locus values; expected heterozygosity according to Hardy-Weinberg (H_e; NEI 1978). Observed heterozygosity (H_o) and Wright's fixation index ($F = 1 - H_o/H_e$; WRIGHT 1922) were computed only for population 9 - in order to compare observed heterozygosities with panmittic expectations - since in the remaining populations only pollen allele frequencies were estimated and expected heterozygosities only could be computed.

On the basis of allele frequencies also the following parameters of genetic differentiation (variation between populations) were computed:

- genetic diversity analysis (NEI 1973, 1975), which shows the distribution of genetic diversity: H_t (total diversity), H_s (diversity within populations), D_{st} (diversity among populations, given as the difference between the two former parameters) and G_{st} (relative degree of genetic differentiation, given as the ratio D_{st}/H_t). G_{st} values were computed for each polymorphic locus and then averaged over all loci;
- subpopulation differentiation (δ; GREGORIUS & ROBERDS 1986), which represents GREGORIUS' (1974) genetic distance between each population and the remaining ones, considered as a whole, and is regarded as more sensitive than G_{st};
- genetic distance, computed by means of NEI's (1978) and GREGORIUS' (1974) formulae. NEI's distance is the most used measure, so its usage allows comparisons with other researches. GREGORIUS' distance is a linear measure which can range only between 0 and 1, therefore its values are more directly assessable.

Values of NEI's genetic distance were used for constructing a dendrogram using the UPGMA method (SNEATH & SOKAL 1973).

Multivariate analysis

For multivariate analysis, allele frequencies were transformed into arcsine to reduce the skewness of their distributions (KUNG 1988).

Principal component analysis was performed using the PRINCOMP procedure (ANONYMOUS 1987) of SAS system for Windows release 6.08.

Cluster analysis was performed with the SAS system CLUSTER procedure (ANONYMOUS 1987) using the hierarchical Ward's minimum variance method (WARD 1963).

Both analyses were performed in order to determine independent groupings of populations and to compare them with the clusters resulting from genetic distance values. Principal component analysis was also performed in order to define which alleles explained most of the observed variation.

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RESULTS AND DISCUSSION

First of all, for the first eight populations, allele frequencies estimated on the basis of paternal gametes were compared with allele frequencies drawn from seed parent genotypes. No significant differerences emerged between the two frequency distributions: no heterogeneity chi-square values were significant, and the frequencies which most diverged from average values were almost always very similar in the two sets of samples. Taking account of all the deviations from averages, including the least marked ones, it was possible to observe that they were more numerous in seed parent samples; nevertheless, this could be attributed to sampling error, since these samples were much smaller (Table 1).

Average values of the two most important genetic diversity parameters, reckoned for both sample sets, were very similar (H_s) or equal (v). The same can be said of average values of genetic differentiation parameters δ, G_{st} and GREGORIUS' (1974) genetic distance. As regards NEI's (1978) genetic distance, compared with the average value concerning the paternal allele populations, the mean value relating to the seed parent populations was halved because of reduced sample size, whereas the classic measure (NEI 1972) - which does not take sample size into account - yielded practically equal results.

Although the composition of paternal allele samples could be influenced by many factors (differentiation in pollen production, mating success, genotypes of neighbouring trees, self pollination, etc.), on the basis of the above-mentioned comparisons - for the first eight populations - it was decided to utilize only allele frequencies estimated on the basis of the progeny pollinic component. In this way, a more representative sample could be obtained than using maternal or embryo genotypes, on account of the restricted number of the surveyed seed parents (PUGLISI *et al.* 1999).

For population 9, instead, the genotypes of the 20 sampled seed parents were used, since this is a natural population next to which artificial populations grow which were established with propagation material whose origin is unknown. This would have cast doubts on the representativeness of the paternal embryo alleles.

A distinct analysis was due for the alleles shared only by some - up to four - populations (*6Pgdh-A2*; *6Pgdh-B1* and *4*; *Mdh-A1*; *Skdh-A1*, *2* and *4*), some of which were present only in either the paternal or the maternal set of samples in some populations (Table 1). Exclusively for such alleles, both sample sets were taken into consideration at the same time, in order to compare populations on the basis of their presence or absence and to reduce sampling errors. All the mentions of seed parent samples are hereafter referred to as "s.p.".

Only six out of 13 loci turned out to be polymorphic (Table 1). The frequencies at the locus *Lap-A* show differentiated values in the Calabrian populations 5, 6, 8 and 9; namely, in population 5, allele *2* has a frequency lower than 5% and should thus be considered as rare (Table 1A). Locus *6Pgdh-A* is polymorphic only in the Apulian populations 1 and 2 from Gargano, in the Calabrian population 6 (s.p.) and in the Basilicata population 9 (Table 1, A and B). Alleles *1* and *4* at the locus *6Pgdh-B* are only present in the Apulian Ionian population 4 and in the Calabrian population 6 (s.p.) (Table1, A and B). Locus *Mdh-A* is polymorphic only in populations 1 (Gargano, Apulia; s.p.), 3 and 4 (Apulian Ionian coast) and 5 (Calabria) (Table 1, A and B). Locus *Mdh-D* shows interesting frequency inversions between the two alleles in populations 2 (Gargano, Apulia), 3 and 4 (Apulian Ionian coast), and 9 (Basilicata) (Table 1A). The four alleles at the locus *Skdh-A* are present all together only in the Apulian population 4; they are differently distributed in the other populations, with the exception of the Calabrian populations 6, 7 and 8 which are monomorphic (Table 1A).

Analysing in detail the distribution of the alleles shared only by some populations (Table 1, A and B), the first worth noting fact is the number of their occurences counted over the whole of populations and loci: 19 cases in the paternal samples (Table 1A) and 15 in the maternal samples (Table 1B), which confirms the greater representativeness of the former set. Nevertheless, the alleles *6Pgdh-B1* in population 4, *6Pgdh-B4* in population 6, *Mdh-A1* in population 3, *Skdh-A1* in population 5, *Skdh-A2* in populations 3 and 4 and *Skdh-A4* in populations 2 and 4 are present only in the paternal samples, whereas the alleles *6Pgdh-A2* and *6Pgdh-B1* in population 6, *6Pgdh-B4* in population 4 and *Mdh-A1* in population 1 are present only in the maternal samples (Table 1). Joining the two sample sets, the total of the occurrences amounts to 23.

Both in pollen cloud and in seed parents about 60% of these occurences show frequencies not higher than other tightly linked loci) are hampered by natural 5%, which could suggest these alleles (or any alleles at selection. However, in other populations the frequencies of the same alleles are higher (up to 20 %), except for the allele *6-Pgdh-B1*, present only in populations 4 and 6, which does not exceed the threshold value of 5 % (Table 1). It would not be tenable explaining the even notable differences between allele frequencies on the basis of different selective forces, because the environmental dissimilarity between the sampled stands does not seem marked enough to justify them.

Table 1 — Allele frequencies at the six polymorphic enzyme loci. A): paternal gametes; B): seed parents.

A)

Locus	Allele	Pop. 1	Pop. 2	Pop. 3	Pop. 4	Pop. 5	Pop. 6	Pop. 7	Pop. 8	Pop. 9
Lap - A	1	.583	.567	.558	.546	.958	.776	.588	.795	.525
	2	.417	.433	.442	.454	.042	.224	.412	.205	.475
6Pgdh-A	1	.900	.975	1.000	1.000	1.000	1.000	1.000	1.000	.950
	2	.100	.025	–	–	–	–	–	–	.050
6Pgdh-B	1	–	–	–	.009	–	–	–	–	–
	2	.825	.925	.683	.731	.533	.417	.658	.765	.775
	3	.175	.075	.317	.259	.467	.575	.342	.235	.225
	4	–	–	–	–	–	.008	–	–	–
Mdh-A	1	–	–	.058	.028	.017	–	–	–	–
	2	1.000	1.000	.942	.972	.983	1.000	1.000	1.000	1.000
Mdh-D	1	.558	.325	.400	.361	.575	.546	.617	.550	.375
	2	.442	.675	.600	.639	.425	.454	.383	.450	.625
Skdh-A	1	.192	.025	–	.009	.008	–	–	–	–
	2	–	–	.025	.083	.067	–	–	–	–
	3	.717	.825	.975	.898	.925	1.000	1.000	1.000	.975
	4	.092	.150	–	.009	–	–	–	–	.025

B)

Locus	Allele	Pop. 1	Pop. 2	Pop. 3	Pop. 4	Pop. 5	Pop. 6	Pop. 7	Pop. 8	Pop. 9
Lap-A	1	.700	.650	.650	.556	.950	.700	.550	.800	.525
	2	.300	.350	.350	.444	.050	.300	.450	.200	.475
6Pgdh-A	1	.800	.950	1.000	1.000	1.000	.950	1.000	1.000	.950
	2	.200	.050	–	–	–	.050	–	–	.050
6Pgdh-B	1	–	–	–	–	–	.050	–	–	–
	2	.850	.900	.700	.556	.600	.400	.700	.750	.775
	3	.150	.100	.300	.333	.400	.550	.300	.250	.225
	4	–	–	–	.111	–	–	–	–	–
Mdh-A	1	.050	–	–	.111	.050	–	–	–	–
	2	.950	1.000	1.000	.889	.950	1.000	1.000	1.000	1.000
Mdh-D	1	.400	.450	.400	.556	.600	.450	.750	.650	.375
	2	.600	.550	.600	.444	.400	.550	.250	.350	.625
Skdh-A	1	.200	.050	–	.056	–	–	–	–	–
	2	–	–	–	–	.050	–	–	–	–
	3	.700	.950	1.000	.944	.950	1.000	1.000	1.000	.975
	4	.100	–	–	–	–	–	–	–	.025

In the hypothesis that these alleles are neutral, subjected only to genetic drift and gene flow, their presence or absence in certain populations could give some suggestions about their origin and the migratory movements they can bear witness to. First of all, it is possible to note the total absence of such alleles in the Calabrian populations 7 and 8, located in Valle del Ferro at very close range. Another very interesting remark regards the other Calabrian populations, no. 5 and no. 6 (Albidona), which share none of these uncommon alleles, even though they are located at very close range too. There is also a manifest relationship between the Apulian populations 1 and 2 from Gargano: the uncommon alleles present in no. 1 are always

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present also in no. 2, except for the allele *Mdh-A1*. The two uncommon alleles present in the Basilicata popula tion 9 are present also in populations 1 and 2. The three uncommon alleles present in the Calabrian population 5 are also present in no. 4, and two of them also in no. 3 (populations 3 and 4 are located on the Apulian Ionian coast); these last two alleles are the only uncommon ones carried by population 3, which seems to suggest a relationship between these three populations. The Calabrian population 6 shares two out of its three uncommon alleles with the Apulian population 4, and both populations are the only carrier of these two uncommon alleles (*1* and *4*) at the locus *6-Pgdh-B*; moreover, this is the most evident case of sampling error: the above alleles are shared by these two populations in an alternate way in the two (paternal and maternal) sample sets (Table 1, A and B).

Both populations carrying most occurrences of uncommon alleles are Apulian: no. 4 (six alleles) and no. 1 (four alleles). The Apulian population 3 and the Basilicata population 9 carry the fewest occurrences (two), after populations 7 and 8 (no occurrences). The difference between populations 3 and 4 is remarkable, considering their close range.

Analyzing these data as a whole, no particular groupings seem to emerge. It is also worth noting that, out of the four uncommon alleles present in the paternal sample of population 1, *6-Pgdh-A2* and *Skdh-A1* always show the highest frequencies, and *Skdh-A4* frequency is second only to population 2. This could suggest that populations growing in the whole studied area could have originated from Apulian populations from Gargano, and that the decreasing allele frequencies could be due to founder effect - in all probability, mostly caused by fires, which are very frequent in these zones (LEONE 1999; LEONE *et el.* 2000a, 2000b; SARACINO & LEONE 2001) - in the course of a hypothetical colonization starting from Gargano, and this hypothesis could also explain the total loss of uncommon alleles in Valle del Ferro (Calabria, populations 7 and 8). However, the Apulian Ionian population 4 carries the highest number of uncommon alleles, which would suggest it could be descended from the parent populations, and that differences in allele frequencies could mainly be due to genetic drift.

Table 2 reports the significant values of the contingency table chi-square test, calculated for the polymorphic loci after grouping the alleles having expected absolute frequencies lesser than 4. Four out of the six polymorphic loci show heterogeneous allele frequencies with P<0.005, which assigns statistical significance to some of the above-mentioned differences.

Expected heterozygosity values (Table 3) are rather low compared to those found in most of the studied conifers. These results, together with the high number of monomorphic loci, confirm the relatively low genetic diversity of this species, already reported by SCHILLER *et al.* (1986), GRUNWALD *et al.* (1986), TEISSEIRE *et al.* (1995) and AGÚNDEZ *et al.* 1997. The H_e mean value we obtained (0.112) is lower than the average estimated within gymnosperm populations (0.151) by HAMRICK *et al.* (1992). However, it should be underlined that the values obtained in the present investigation are higher than those obtained in the papers mentioned above. This is also true for the two Apulian populations, from Gargano and the Ionian coast, included in the study by SCHILLER *et al.* (1986). Despite the populations they analized were close to those we sampled, they showed lower H_e values (0.075 and 0.035, respectively; the estimated overall mean on the 26 surveyed populations in the species natural range was 0.040). This is likely due to the higher number of loci analyzed by SCHILLER *et al.* (1986): 30 vs 13. Any influence of the enzyme systems chosen for the present investigation, instead, should be excluded; six out of seven enzyme systems are indeed involved in the primary metabolism (group I) and only LAP belongs to group II, covering secondary metabolism enzymes which could lead to an overestimation of genetic variation (BERGMANN 1991). Nevertheless, LOUKAS *et al.* (1983), KOROL *et al.* (1995) and KOROL & SCHILLER (1996) - in Greek, Israeli and Jordanian populations - recorded H_e values similar or higher than ours.

Even if the direct comparison between variation levels in different researches could be unreliable - because of the use of different loci, sample sizes, methodologies, etc. - such juxtapositions may be helpful, both for stressing the effects of different procedures and for getting some further information, albeit with proper caution.

Also the other genetic diversity parameters (Table 3) show low values. The most informatory among these parameters, v (genetic diversity, or effective number of alleles), has values slightly above 1, which is the minimum value.

Table 2. Heterogeneity chi-square values computed on allelic frequency distributions of the studied subpopulations (D.F.: degrees of freedom; ***: $P < 0.005$).

Locus	χ^2	D.F.	Significance level
Lap-A	180.410	8	***
6Pgdh-B	207.248	8	***
Mdh-D	85.808	8	***
Skdh-A	241.604	8	***
Total	715.070	32	***

Table 3. Parameters of genetic diversity. *N*: mean number of alleles per locus; *P*: percentage of polymorphic loci at the 5% criterion; *v*: genetic diversity; H_e: expected heterozygosity according to Hardy-Weinberg; H_o: observed heterozygosity; *F*: fixation index.

Population	*N*	*P*	*v*	H_e	H_o	*F*
123456789	1.5	38.5	1.170	.146	–	–
	1.5	30.8	1.122	.109	–	–
	1.4	30.8	1.137	.121	–	–
	1.6	30.8	1.140	.123	–	–
	1.5	23.1	1.105	.096	–	–
	1.3	23.1	1.115	.103	–	–
	1.2	23.1	1.121	.109	–	–
	1.2	23.1	1.100	.091	–	–
	1.4	30.8	1.127	.114	.123	– 0.080
Mean	1.4	28.2	1.126	0.112	–	–

The Basilicata population 9, the only one for which H_o and *F* could be calculated, reveals a slight heterozygote excess. In this respect it should be remembered that - only for this population - allele and genotype frequencies refer to the 20 sampled seed parents. In conifers - which, contrary to angiosperms, lack any prezygotic incompatibility mechanisms - an excess of homozygotes is often observed in embryo and seedling populations, which then turns into an excess of heterozygotes under the effect of inbreeding depression and natural selection when individuals reach adult stage (MÜLLER-STARCK & GREGORIUS 1988). Unfortunately, *F* values for the other populations are not available and thus it is not possible to assess whether such a heterozygote excess is normal. It should however be noted that population 9 is the only one growing in clay soils, traditionally considered hard to this species (BERNETTI 1995), and the negative *F* value recorded could be the result of a balancing selection worked by a difficult environment and non homogeneous pedological conditions.

The Gargano (Apulia) population 1 is characterized by the highest diversity parameter values; the lowest values are recorded for the Calabrian population 8. On the whole, the Apulian and Basilicata populations show higher values than the Calabrian ones. Also SCHILLER *et al.* (1986) observed the Gargano population they studied (Vico del Gargano) had a H_e value higher than populations under consideration in the remaining natural range; the Apulian Ionian population they studied (Patemisco), instead, had a value lower than the overall mean. SCHILLER *et al.* (1986) grouped the Gargano population together with the eastern European race, including Greek, Albanian and Libyan populations and characterized by higher H_e values and introgression of alleles from the related species having greater genetic variation, *Pinus brutia*.

Always using isozymes, CONKLE *et al.* (1988) hypothesized *P. halepensis* derived from *P. brutia*-like progenitors, and during the colonization of its present natural range from its geographic center of origin (Caucasian region) it went through several *genetic bottlenecks*. These would have caused the observed loss of genetic diversity (MORGANTE *et al.* 1998).

The greater variability characterizing the eastern European race populations could be due to the migration of Tertiary Aleppo pine populations - characterized by a relatively high heterozygosity - from central Europe into the Balkan peninsula due to climate changes. The Balkan peninsula was only slightly affected by glaciation, unlike West Europe and North Africa where probably *P. halepensis* present races originated from expansion and migration of refugial populations which lost much of their genetic variation (SCHILLER & MENDEL 1995).

BUCCI *et al.* (1998) and MORGANTE *et al.* (1988) studied several populations of *P. halepensis*, among which some Greek and Italian ones, by means of paternally inherited molecular markers (chloroplast microsatellites): the highest values of diversity within populations belonged to the two Greek populations and were similar to the value of the Apulian population from Gargano they surveyed, confirming SCHILLER's *et al.* (1986) results on the similarity between populations from Gargano and from Greece. However, in a Turkish sympatric population, BUCCI *et al.* (1988) detected strong evidence of unidirectional introgression of Aleppo pine haplotypes into seeds of *P. brutia*, but not of a reciprocal gene flow, in contrast with the above-mentioned hypothetical introgression of *P. brutia* alleles into *P. halepensis* seeds (SCHILLER *et al.* 1986).

The trend of Apulian populations to differentiate from the other Italian ones was also observed in some morphological and physiological traits (PALMBERG

1975; CALAMASSI *et al.* 1980; CALAMASSI 1982; CALAMASSI *et al.* 1984; BARITEAU 1992; CUCCUI *et al.* 1996), as well as their similarity to Greek populations (DEBAZAC & TOMASSONE 1965; PALMBERG 1975; Calamassi *et al.* 1980; CALAMASSI 1982; ECCHER *et al.* 1987). BARADAT *et al.* (1989) remarked, based on the terpene composition of four Italian natural populations, a similarity between the two sampled Apulian populations (Gargano and Ionian coast) and their significant differentiation from the others. In a study on the geographical variation of six Italian provenances in several physiological traits and terpene composition, TOGNETTI *et al.* (1997) also observed a noticeable similarity between the Apulian seed sources and their marked differentiation from the remnant.

The parameters listed in Table 4 show the distribution of genetic diversity between and within populations. The mean G_{st} value (3.3%) is similar to the one observed for many other wide-range conifers, characterized by the fact that the majority of the total genetic variation is detected within populations. G_{st} values vary considerably between loci, some of which show greater differentiation between populations having values ranging around 10% (loci *Skdh-A*, *6Pgdh-B* and *Lap-A*; Table 4).

Table 5 shows the values of GREGORIUS' parameter δ ("subpopulation differentiation"), which is regarded to be more sensitive than G_{st}. Indeed, its average value on all loci and populations is 4.2 %, slightly above the mean G_{st}. δ is suitable to graphic representation through the so-called "differentiation snails" (Figure 2), showing clearly those loci which better discriminate between populations: *Lap-A*, *6Pgdh-B*, *Skdh-A* and *Mdh-D*. Generally, the trend of some populations to differentiate from others is immediately highlighted, as for the Calabrian populations 5 and 6 and for the two Gargano (Apulia) populations 1 and 2. However, when observing what happens for single loci, *Lap-A* appears to enhance significantly the features of the Calabrian population 8 and the Basilicata population 9, and *Mdh-D* enhances the features of populations 4 (Apulian Ionian coast) and 7 (Calabria), beside those of population 2 (Gargano, Apulia).

Table 6 reports data from principal component analysis. Eigenvalues, percentages of explained variation and the highest correlation values between transformed allele frequencies and the first three principal components - which account totally for 78% of the standardized variance - are indicated. The loci whose allele frequencies have the highest positive or negative correlation coefficients with the first principal components are the same as above (*Skdh-A*, *6Pgdh-B*, *Mdh-D* and *Lap-A*) plus the other two polymorphic loci, *6Pgdh-A* and *Mdh-A*.

The low overall differentiation level between populations is confirmed by the mean genetic distance values according to NEI (0.011) and GREGORIUS (0.050). However, from Table 7 it can be noted that, for

Table 4. Genetic diversity analysis. H_t: total diversity; H_s: diversity within populations; D_{st}: diversity among populations (H_t–H_s); G_{st}: relative degree of genetic differentiation (D_{st}/H_t). Only values relating to polymorphic loci are reported.

Locus	H_t	H_S	D_{st}	G_{st}
Lap-A	.452	.412	.040	.089
6Pgdh-A	.038	.036	.002	.057
6Pgdh-B	.420	.379	.041	.098
Mdh-A	.023	.022	.001	.032
Mdh-D	.499	.477	.022	.044
Skdh-A	.144	.129	.015	.107
Mean	0.121	0.112	0.01	0.033

Table 5. Genetic differentiation between populations (δ values). D_{ji}: differentiation values of single populations. Only values relating to polymorphic loci are reported.

Locus	Population									δ
	1	2	3	4	5	6	7	8	9	
	D_{j1}	D_{j2}	D_{j3}	D_{j4}	D_{j5}	D_{j6}	D_{j7}	D_{j8}	D_{j9}	
Lap-A	.095	.114	.124	.136	.331	.124	.090	.146	.148	.145
6Pgdh-A	.094	.009	.020	.019	.020	.020	.020	.020	.034	.028
6Pgdh-B	.148	.262	.016	.050	.187	.318	.045	.079	.083	.137
Mdh-A	.014	.014	.052	.018	.005	.014	.014	.014	.013	.018
Mdh-D	.079	.186	.101	.143	.099	.066	.147	.070	.118	.111
Skdh-A	.255	.135	.068	.071	.059	.091	.091	.091	.057	.106
Gene pool	0.053	0.055	0.029	0.034	0.054	0.049	0.031	0.032	0.035	0.042

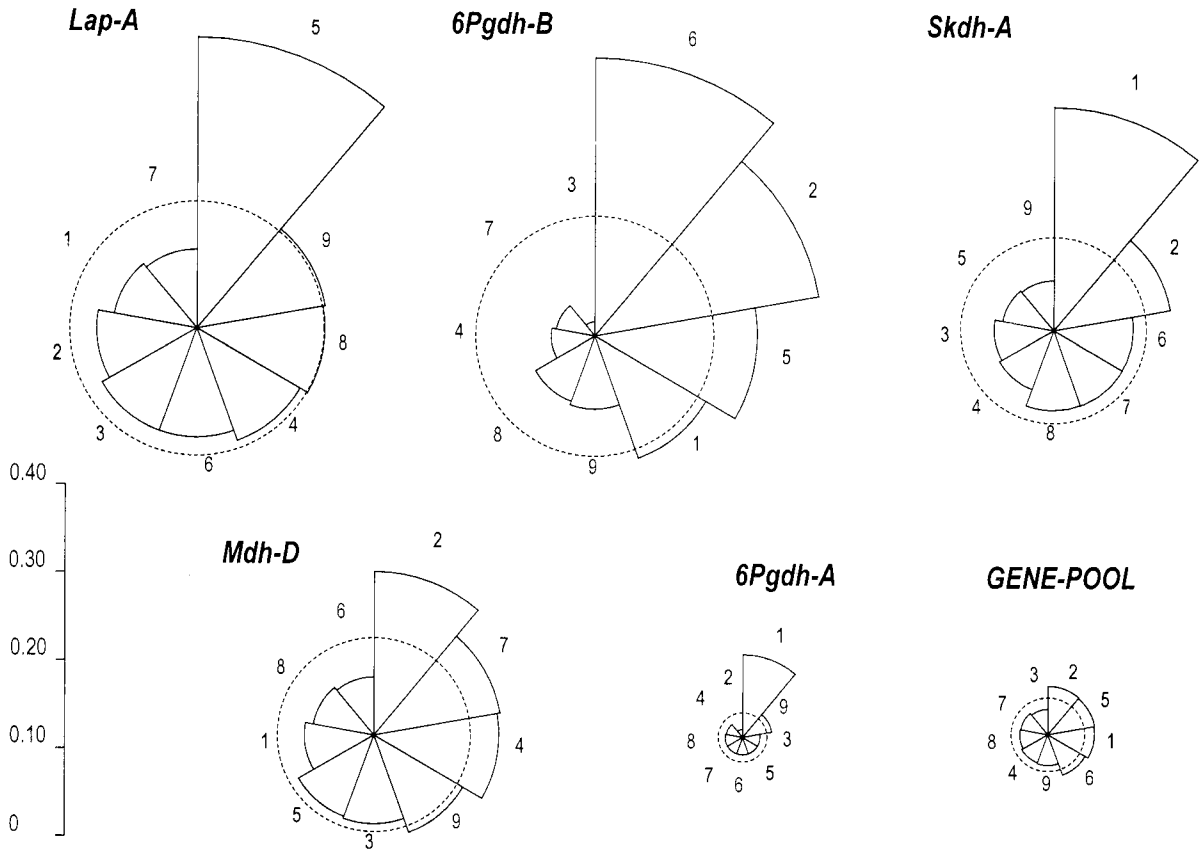

Figure 2. Graphic representation ("differentiation snails") of the values listed in Table 5. Lengths of radii of the dotted circles correspond to the total differentiation level (δ), and lengths of sector radii correspond to the differentiation values of the single populations (D_{Ji}).

both measurements, the Calabrian populations 5 and 6, and, to a lower extent, also the Gargano (Apulia) populations 1 and 2, show on the whole higher genetic distance values that the others.

The trend of populations to differentiate, as well as the similarity between them, appear evident in the dendrogram constructed on the basis of NEI's genetic distance values (Figure 3). The figure shows clearly how populations tend to cluster into three different groups: 1) populations from Apulia (1, 2, 3 and 4) and Basilicata (9); 2) Calabrian populations from Valle del Ferro (7 and 8); 3) Calabrian populations from Albidona (5 and 6).

In particular, the two populations from Albidona (5 and 6) are sharply differentiated from the remnant, including the two other Calabrian populations (Valle del Ferro, 7 and 8), in spite of the short geographic distance which separates them. It is also worthy of note that the population from Basilicata (9) is genetically identical with population 4 and very similar to population 3, both from the Apulian Ionian coast, which enables us to hypothesize a common origin. The trend to differentiation of the Apulian populations from Gargano (1 and 2) is also evident.

Based on principal component analysis (Figure 4), populations arrange in clusters slightly different from the above groups, although the sharp differentiation of some populations is confirmed: populations 1 (Gargano) and 5 (Calabria) are clearly differentiated from the others, which constitute three groups: (1) the Gargano population 2 and the Basilicata population 9; (2) the two Apulian populations from the Ionian coast; (3) the remaining three Calabrian populations.

The dendrogram inferred from cluster analysis (Figure 5) mirrors the plot obtained from principal component analysis (Figure 4). Two clearly distinct clusters can be observed, the one including the Apulian and Basilicata populations and the other for the Calabrian populations. In this latter, population 6 appears to be more similar to populations 7 and 8 than to 5, despite its geographical proximity. It is interesting to observe that with this and the previous analysis, the Basilicata population 9 appears to be more similar to the Apulian populations 1 and 2 from Gargano than to the Ionian populations 3 and 4, in contrast with the results based on genetic distance and in concordance with what was

Table 6. Principal component analysis of allozyme variation: eigenvalues, percentage of explained variation and the first 12 allelic loadings relative to the first three principal components (PRIN1 — PRIN3).

	PRIN 1		PRIN 2		PRIN 3	
Eigenvalue	6.43561		4.09517		1.94410	
Percentage of explained variation	40.2 %		25.6 %		12.2 %	
Cumulative percentage	40.2 %		65.8 %		78.0 %	
Allelic PC-loadings	*6Pgdh-B2*	0.359	*Mdh-A2*	–0.409	*Skdh-A3*	–0.444
	6Pgdh-B3	–0.352	*Mdh-A1*	0.393	*Skdh-A1*	0.411
	6Pgdh-A1	–0.331	*Skdh-A2*	0.344	*Skdh-A2*	0.408
	Skdh-A4	0.327	*6Pgdh-B1*	0.342	*Lap-A1*	0.318
	6Pgdh-A2	0.298	*Mdh-D1*	–0.337	*Lap-A2*	–0.270
	Skdh-A3	–0.285	*Mdh-D2*	0.336	*Mdh-A2*	–0.247
	Lap-A2	0.282	*6Pgdh-A2*	–0.216	*6Pgdh-A2*	0.218
	Lap-A1	–0.272	*Skdh-A1*	–0.210	*Mdh-D2*	–0.213
	Skdh-A1	0.231	*6Pgdh-A1*	0.192	*Mdh-D1*	0.208
	Mdh-D2	0.214	*Lap-A2*	0.167	*6Pgdh-B4*	–0.199
	Mdh-D1	–0.211	*6Pgdh-B4*	–0.157	*Mdh-A1*	0.142
	6Pgdh-B4	–0.187	*Lap-A1*	–0.138	*6Pgdh-B1*	0.126

Table 7. Genetic distance calculated following GREGORIUS (above the diagonal) and NEI (below the diagonal).

Population	1	2	3	4	5	6	7	8	9
1 - Coppa della Nuvola	-	.045	.059	.055	.083	.077	.047	.051	.046
2 - Monte Barone	.007	-	.045	.035	.096	.088	.060	.063	.032
3 - Perronello	.010	.007	-	.017	.063	.055	.027	.042	.022
4 - Pineta della regina	.008	.004	.000	-	.067	.067	.039	.046	.019
5 - Albidona 1	.024	.033	.018	.022	-	.032	.048	.039	.078
6 - Albidona 2	.023	.032	.012	.017	.004	-	.038	.029	.066
7 - Valle del Ferro 1	.008	.016	.004	.007	.013	.008	-	.029	.038
8 - Valle del Ferro 2	.010	.013	.007	.009	.007	.010	.004	-	.041
9 - Tursi	.007	.003	.000	.000	.024	.018	.006	.008	--

observed about the uncommon alleles, as above mentioned: the two uncommon alleles carried by population 9 are also present in populations 1 and 2; only one uncommon allele is shared by populations 4 and 9, and no one between populations 3 and 9, whereas the only two uncommon alleles present in no. 3 are also present in no. 4 (Table 1, A and B). It depends on the fact that multivariate analyses are more sensitive than genetic distances to the presence or absence of alleles, and this divergence could mean either that their occurrence, in this case, is not significant in relation to population origin - since it could mostly depend on casual genetic drift - or alternatively that population 9 has a different origin but its genetic similarity to populations 3 and 4 is due to evolutionary convergence. Their geographic positions (Figure 1) would suggest that the former hypothesis could be more plausible, not least because populations 3 and 4 are very close in both dendrograms even though the former has the lowest number of uncommon alleles and the latter the highest one. Genetic drift and founder effect may play a particularly important role in these zones, characterized by frequent fires (LEONE 1999; LEONE *et al.* 2000a, 2000b; SARACINO & LEONE 2001).

Multivariate analysis applied to allozyme variation of forest tree populations has been used by several authors with interesting results (BONNET-MASIMBERT & BIKAY-BIKAY 1978; KRZAKOWA 1982; YEH *et al.* 1985; KINLOCH *et al.* 1986; MERKLE *et al.* 1988; PIGLIUCCI *et al.* 1990; FURNIER *et al.* 1991; LIU & KNOWLES 1991; RAJORA & DANCIK 1992; TSUMURA *et al.* 1992; CHERNODUBOV 1994; NEET-SARQUEDA 1994; PRAT & ARNAL 1994; KJAER *et al.* 1996; HAZLER *et al.* 1997, and others). The integration of results obtained by means of different kinds of analysis increases the amount of information which can be achieved from the

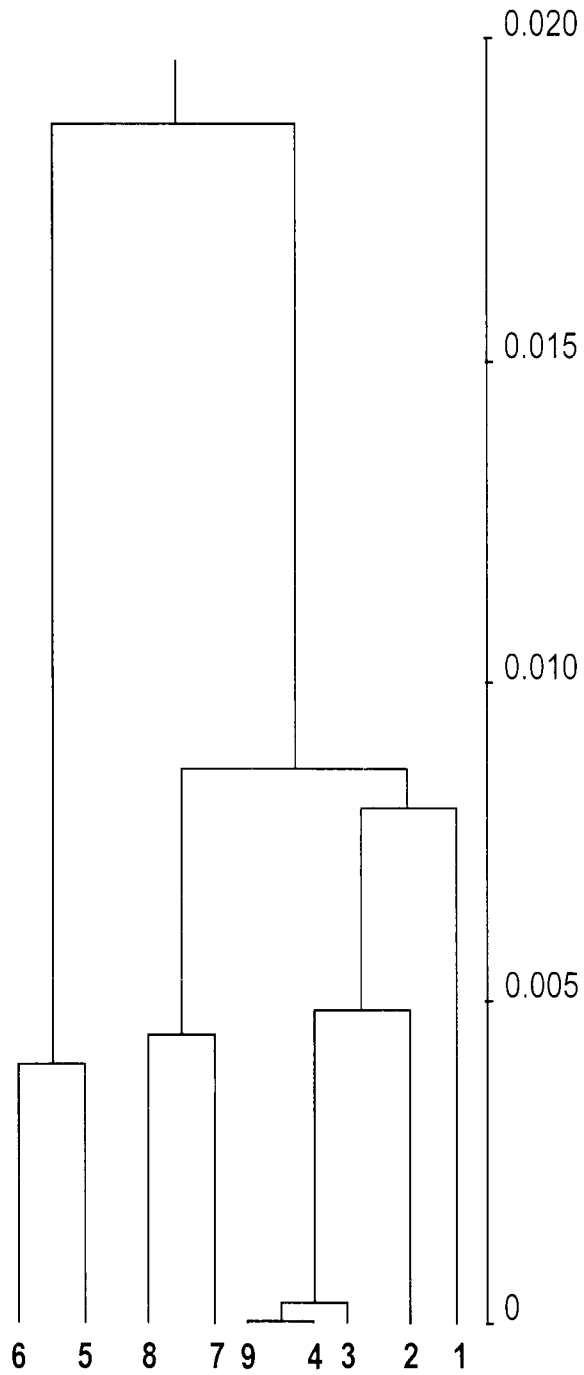

Figure 3. Dendrogram of NEI's genetic distances obtained with UPGMA method.

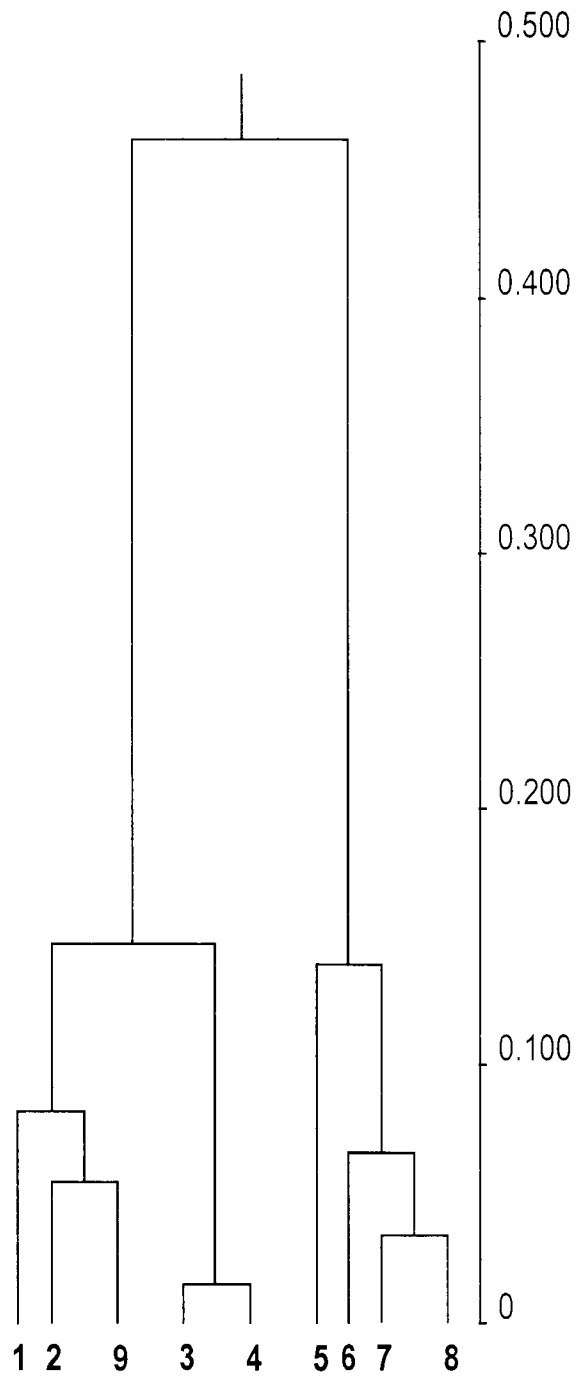

Figure 5. Dendrogram based on cluster analysis.

available data, but a careful evaluation of results is needed, as the above-mentioned comparison shows.

The sharp differentiation of the Calabrian populations is confirmed by their lower genetic variation. These are populations considered to be spontaneous, thus their differentiation could be due to genetic bottle-*necks* causing a loss of variability, probably following fires before trees attained their sexual maturity and maybe in the course of a possible colonization of the Calabrian Ionian belt from the Apulian coast, since Aleppo pine is generally considered autochthonous in Apulia but not in Calabria (FRANCINI 1953; BERNETTI 1995).

Also the Basilicata population 9 could have been originated from a migratory movement which, starting from the Apulian Ionian coast, could have followed the coast of Basilicata and then ascended the valleys of Agri and Sinni rivers; in this case, however, such a

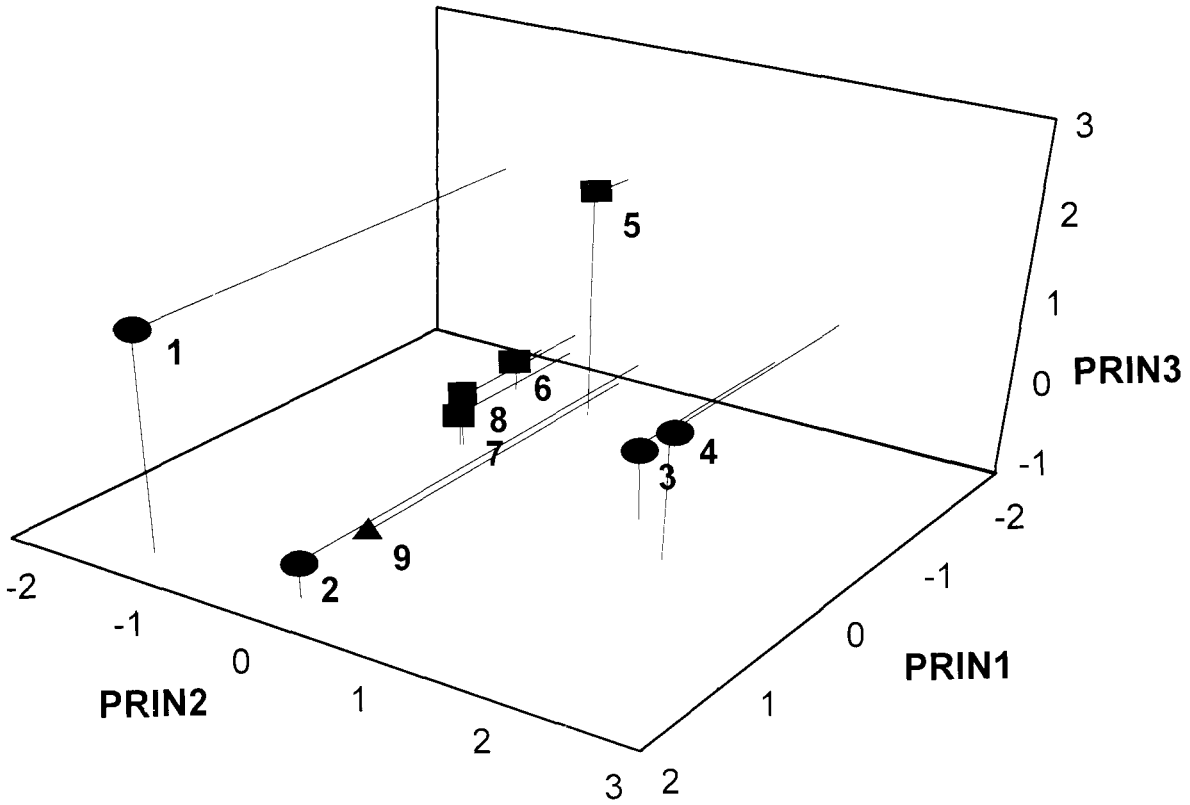

Figure 4. Three-dimensional scatterplot of the first three principal components. Ellipses mark the Apulian populations, rectangles the Calabrian populations and the triangle the Basilicata one.

movement would not have been accompanied by a loss of genetic variation.

The debate on the indigenous nature of Aleppo pine in Apulia has found until now the opposition of few researchers to the dominant hypothesis of the natural origin of the Apulian populations. An important contri bution to the reconstruction of the evolutionary history of this species in southern Italy could be provided by archaeobotany (Figure 1). The studies performed till now in Apulia revealed the presence of carbonized fragments of *P. halepensis* wood dating back to the Late Glacial Period (17.000–10.000 years BP) only in Monopoli, on the Adriatic coast close to Bari (FIORENTINO 1998a) and in the Grotta Romanelli, near Otranto (Salentine Peninsula; FOLLIERI 1968); in Figure 1 these two sites are marked by white triangles. On the contrary, remains of this species are absent in the archaeological sites studied in Vieste (Gargano; FIORENTINO 1995a) and in Manduria (eastern Ionian belt near Taranto; FIORENTINO 1995b), which date back to the Early Neolithic Age (about 7000 years BP) and to the Late Mesolithic/Early-Middle Neolithic Age (about 7200–6500 years BP), respectively. They are also absent in several sites dating back to the Bronze Age (IV–III millennium BP) situated along all the Apulian Adriatic belt (FIORENTINO 1995c, 1998b; FIORENTINO & RADINA 1998) and including a site in Monopoli (FIORENTINO 1995d), where this species was previously present, and in three sites situated along the Ionian belt near Taranto (at present covered by the wide *P. halepensis* stands concerned by our sampling) dating back to the Bronze Age too (FIORENTINO 1999, 2002). Moreover, a Calabrian Bronze Age site, situated near the populations we analyzed (no. 5 and 6), revealed the absence of any Aleppo pine remains (VALLINO & VENTURA 1984). All the sites characterized by the absence of Aleppo pine are marked by black triangles in Figure 1. These data could represent an indirect confirmation of the hypothesis by PIGNATTI (1979) of the anthropic origin of the present populations of Aleppo pine in Apulia; as a matter of fact, he considered this species extra-zonal in Gargano and southern Apulia.

Based on archaeobotanical data, the hypothesis can be raised that Aleppo pine was introduced in Apulia by Greek colonizers in historical time (*Magna Grecia*), and that the genetic diversity of Apulian populations, greater than for the other Italian populations and in the remnant range, could be the consequence of the Greek origin of the propagation material, since Greek popula-

tions are those having the highest variability values in the whole range (LOUKAS *et al.* 1983; SCHILLER *et al.* 1986; SCHILLER & MENDEL 1995; BUCCI *et al.* 1998; MORGANTE *et al.* 1998).

Against this hypothesis the objection can be raised that, as a general rule, when populations originate from individuals imported by humans, a reduction of genetic diversity occurs compared with populations of origin (founder effect), supposing colonizers carry small quantities of propagation material. Nevertheless, considering the magnitude of migratory movements from Greece to southern Italy and the extent of colonizers' native regions, it is not unreasonable to suppose that, on the whole, the introduced samples were rather representative of the diversity contained in Greek populations.

Analyzing in detail the Ionian sector of the studied area, it is possible to note the presence of some important Greek colonies close to the studied populations, often having different origins even if located a short distance away from one another (Figure 1). Taranto was a Spartan city, Metaponto and Sybaris were Achaean. Between Metaponto and Sybaris there were other two colonies, Heraklea and Siris: the former was founded by Taranto and Thourioi (a panhellenic foundation created near the site of the destroyed city of Sybaris at the initiative of Athens, in which many cities in the Peloponnese participated); the latter was Ionic, founded by fugitives from Colophon (western coast of Asia Minor).

Considering such a variety of provenances, it could be hypothesized that within the new populations a large part of the diversity present in the native regions was represented. As regards the possible reasons of such a broad introduction of this species in the colonized areas, an explanation could be found in the very ancient practice of sealing amphorae, vessels and other containers with pine resin, and of using it for overlaying their inner surface, with the twofold effect of an effective preservation of wine and of giving it a distinctive flavour which is still much appreciated at our times (*retsina*; FIORENTINO, pers. comm.). Obviously, other possible uses of pine resin cannot be ruled out.

Therefore, an anthropic origin cannot be excluded also for the Calabrian and Basilicata populations, even if it seems to be less probable on the part of Greeks for the latter, because of its inland location; or the above mentioned hypothetical spontaneous migration from the Apulian Ionian populations took place in more recent times, after the anthropic introduction from Greece. This latter hypothesis could be supported by the highest number of uncommon alleles present in population 4 (Apulian Ionian coast). Anyway, given the absence of Greek cities in Gargano, a progenitor role of Adriatic populations should be excluded, if the hypothesis of an anthropic origin should be valid; these populations could have a more recent origin, like the Basilicata population.

Also for a population in central Italy (Umbria) an anthropic origin was hypothesized on account of its differentiation - detected by morphological and ecophysiological traits as well as with biochemical markers - from other Italian populations and its similarity to Israeli populations; its introduction could have been carried out by a group of monks from Syria in the IV century A. D. (SCHILLER & BRUNORI 1992). Human activity is therefore likely to have played quite a relevant role in the spreading of Aleppo pine in the Mediterranean basin (SCHILLER & MENDEL 1995).

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