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Allozyme, chloroplast DNA and RAPD markers for determining genetic relationships between *Abies alba* and the relic population of *Abies nebrodensis*

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Abstract Allozyme, chloroplast (cpDNA) and random amplified polymorphic DNA (RAPD) markers have been used to estimate genetic and taxonomic relationships among different populations of *Abies alba* and the relic population of *A. nebrodensis*. Twelve isozyme gene loci, as well as restriction fragment length polymorphism (RFLP) at cpDNA spacer regions between t-RNA genes were analysed. Moreover, a set of 60 random sequence 10-mer primers were tested. Over all isozyme loci, evident differences in allele frequencies among *A. nebrodensis* and *A. alba* populations were found, particularly at 2 loci, phosphoglucose isomerase (Pgi-a) and shikimate dehydrogenase) (Skd-a). More than 10% of the total genetic diversity was due to differences among populations. High values of genetic distances among populations were also found. Out of the 60 primers tested, 12 resulted in a polymorphic banding pattern both within and among populations. A total of 84 RAPD fragments were produced by the 12 selected primers. A phenogram of relationships among populations was constructed based on RAPD band sharing: the differentiation of the *A. nebrodensis* population was evident. The analysis of molecular variance (AMOVA) was used to apportion the variation among individuals within populations and among populations. There was considerable variation within each population: even so, genetic divergence was found among populations. This pattern of genetic variation was very different from that reported for inbred species. Identical cpDNA amplification and restriction patterns were observed among all the individuals sampled from the populations. Taken together, the results of allozyme and RAPDs show a clear differentiation among *A. nebrodensis* and *A. alba* populations and provide support for their classification into two different taxonomic groups.

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Introduction

Abies nebrodensis (Lojac.) Mattei is one of the most relevant examples of a relic species in Europe, with a very limited distribution and in an evident state of regression. It's actual natural range has in fact been reduced to a single and small population located in a very restricted area on the Madonie mountains, in Sicily: only 29 trees are considered to belong to this species (Morandini et al. 1994). Several factors could have contributed to the restrictedness of its actual range: among these the most important seems to be strong antropogenic pressure (Raimondo et al. 1990). Knowledge of the structure and pattern of genetic variation of this species is important with respect to the preservation of gene resources: *A. nebrodensis* is considered to be a highly endangered species whose genetic resources need to be analysed and conserved.

Little is known about the origin and the phylogenetic relationships between *A. nebrodensis* and *A. alba*. Some authors (Fenaroli and Giacomini 1958; Morandini 1968) have hypothesised that *A. nebrodensis* originated from the southern populations of silver fir (*A. alba*) as a consequence of isolation during the post-glacial periods. On the other hand, *A. nebrodensis* grows, in its natural range, in a habitat typical of Mediterranean species such as *Crataegus laciniata, Cistus incanus, Genista cupanii, Fraxinus ornus* and *Quercus ilex*. This aspect could support the hypothesis of an independent origin of this species with respect to *A. alba*.

The objective of the research presented here was to use allozyme and DNA markers, namely chloroplast (cp) and random amplified polymorphic DNA (RAPD) markers, for determining genetic relationships among the unique population of *A. nebrodensis* and some Italian *A. alba* populations sampled along a north-south geographic gradient.

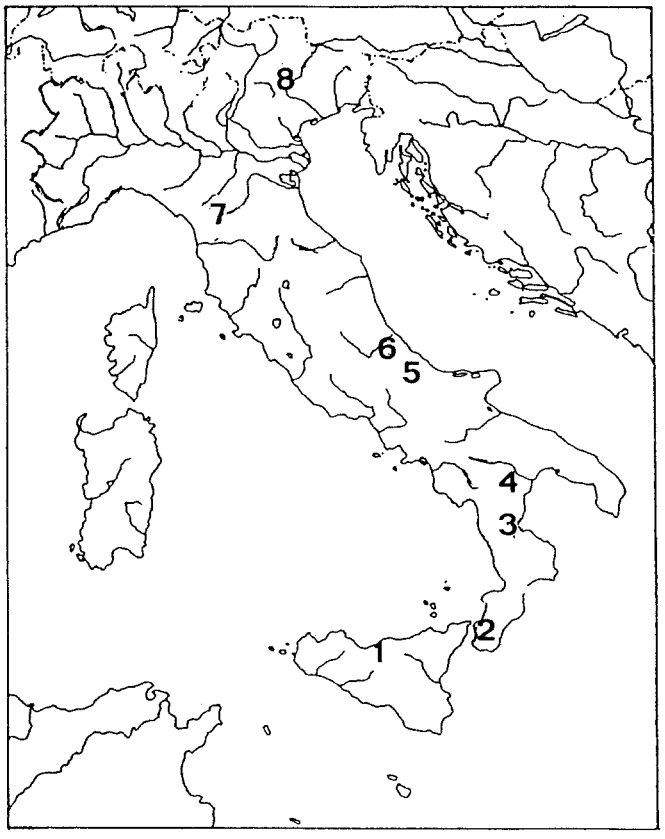

Fig. 1 Geographic location of the *Abies nebrodensis* and *A. alba* populations sampled. See footnote **a** of Table 1 for definition of population numbers

Materials and methods

Plant material

Buds and needles were collected from 14 trees of the natural population of *A. nebrodensis* and from about 30 trees of seven natural populations of *A. alba*, all located in Italy and sampled along a north-south gradient (Fig. 1). The exact number of trees sampled for each population is reported in Table 2. The plant material was frozen at –80°C until DNA extraction.

Allozyme analysis

Bud tissue was used for the isozyme analysis. Extraction and horizontal starch gel electrophoretic procedures were as described in Conkle et al. (1982) and Marty et al. (1984). Eight enzyme systems (Table 1), coded for by 12 gene loci, were analysed by means of starch gel electrophoresis. References on the inheritance of isozyme variants are given in Schroeder (1989), Bergmann et al. (1990) and Villani et al. (personal communication).

Allozyme frequencies, mean number of alleles per locus (n), mean percentage of polymorphic loci ($P_{5\%}$), mean effective number of alleles per locus (n_e) and observed (H_o) and expected (H_e) heterozygosity were calculated. The amount of genetic differentiation among populations at the gene pool level was estimated using the G_{st} measure (Nei 1975). Genetic relationships between populations were calculated by computing the standard genetic distance (Nei 1972). Cluster analysis was performed by the UPGMA method using Nei's genetic distance measure.

Table 1 Allele frequencies

Locus[b]		Population[a]							
		1	2	3	4	5	6	7	8
Gdh-a	1	0.000	0.056	0.033	0.000	0.000	0.000	0.032	0.037
	2	1.000	0.926	0.950	1.000	1.000	1.000	0.968	0.963
	3	0.000	0.018	0.017	0.000	0.000	0.000	0.000	0.000
Got-a	1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Idh-a	1	0.050	0.269	0.293	0.500	0.514	0.667	0.500	0.593
	2	0.950	0.731	0.707	0.500	0.486	0.333	0.500	0.407
Mdh-a	1	0.700	0.889	0.983	0.964	1.000	1.000	0.986	0.981
	2	0.300	0.111	0.017	0.036	0.000	0.000	0.032	0.019
Me-a	1	0.950	0.926	0.933	0.946	0.900	0.778	0.733	1.000
	2	0.050	0.074	0.067	0.054	0.100	0.222	0.267	0.000
Me-b	1	0.950	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	2	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6Pgd-a	1	0.000	0.000	0.000	0.018	0.014	0.000	0.000	0.000
	2	1.000	1.000	1.000	0.982	0.986	1.000	1.000	1.000
6Pgd-b	1	0.200	0.093	0.083	0.018	0.071	0.056	0.065	0.037
	2	0.800	0.907	0.917	0.982	0.929	0.944	0.935	0.963
Pgi-a	1	0.900	0.148	0.067	0.125	0.014	0.111	0.097	0.130
	2	0.100	0.852	0.933	0.875	0.986	0.889	0.903	0.870
Pgi-b	1	0.000	0.074	0.000	0.000	0.014	0.056	0.016	0.037
	2	1.000	0.926	1.000	0.982	0.986	0.944	0.984	0.963
	3	0.000	0.000	0.000	0.018	0.000	0.000	0.000	0.000
Skd-a	1	0.650	0.204	0.138	0.000	0.000	0.000	0.000	0.074
	2	0.350	0.796	0.862	1.000	1.000	1.000	1.000	0.926
Skd-b	1	1.000	0.963	1.000	1.000	1.000	1.000	1.000	1.000
	2	0.000	0.037	0.000	0.000	0.000	0.000	0.000	0.000

[a] 1, Madonie (*Abies nebrodensis*); 2, Aspromonte; 3, Serra San Bruno; 4, Gariglione; 5, Abeti Soprani; 6, Collemeluccio; 7, Abetone; 8, Lavarone

[b] *Gdh*, Glutamate dehydrogenase (1.4.1.3); *Got*, glutamate oxalacetate transaminase (2.6.1.1); *Idh*, isocitrate dehydrogenase (1.1.1.42); *Mdh*, malate dehydrogenase (1.1.1.37); *Me*, malic enzyme (1.1.1.40); *6Pgd*,6-phosphogluconate dehydrogenase (1.1.1.44); *Pgi*, phosphoglucose isomerase (5.3.1.9); *Skd*, shikimate dehydrogenase (1.1.1.25)

Chloroplast DNA analysis

DNA was extracted from needles from the *A. nebrodensis* population and three *A. alba* populations, the latter chosen because they are considered to be representative of the three main parts of its Italian natural range (Lavarone, Northern-Italy; Abeti Soprani, Central-Italy; and Aspromonte, Southern-Italy). Total DNA was prepared following a modification of the method proposed by Guillermaut and Maréchal-Drouard (1992) and Ziegenhagen et al. (1993).

DNA amplification was performed in a Perkin Elmer 9600 thermal cycler. The typical volume of the reaction mixture was 50 µl containing 50 ng DNA, 20 m*M* TRIS-HCl, 100 m*M* KCl, 0.02% gelatine, 3 m*M* $MgCl_2$, 0.2 m*M* of each nucleotide, 0.2 µ*M* of each primer and 1 unit *Taq* polymerase. Six 20-mer primers for the amplification of three non-coding regions of the chloroplast DNA were used. The amplified regions were expected to be intergenic spacers between the *trnT* and *trnL* genes and between the *trnL* and *trnF* genes, and the spacer between *trnL* and *trnF* (Taberlet et al. 1991). DNA amplification was conducted according to the following programme: (1) 95°C step for 5 min × 1 cycle; (2) a 80°C step for enzyme addition; (3) 40 cycles of 1 min at 94°C, 1 min at 54°C and 2 min at 72°C. The last cycle was followed by 8 min at 72°C. Amplification products were separated in a 1.5% agarose gel. The amplification products from each population were cut with 15 restriction endonucleas-

es (*Alu*I, *Cfo*I, *Hae*III, *Hpa*II, *Mbo*I, *Mse*I, *Msp*I, *Rsa*I, *Taq*I, *Tru*9I, *Hin*fI, *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I), with 4, 5 and 6 recognition sites. Every digest was performed twice to avoid problems rising from a misincorporation of nucleotides by *Taq* polymerase. Digestion products were visualised in 3% agarose gel (2% Nu Sieve FMC, 1% agarose Boehringer) after ethidium bromide staining.

RAPD analysis

Genomic DNA was extracted from needles following the procedure described by Ziegenhagen et al. (1993). RAPD amplifications were carried out in 50-µl volumes containing 1% reaction buffer, 2.5 m*M* $MgCl_2$, 0.2 m*M* of each dNTP, 0.2 µ*M* of primer, 1 unit of *Taq* DNA polymerase and 40 ng of genomic DNA. A control polymerase chain reaction (PCR) tube containing all of the components, with the exception of genomic DNA, was run with each primer to check for contamination. Samples were amplified on a DNA thermal cycler Perkin Elmer 9600 using the following cycle parameters: 94°C for 1 min, 36°C for 1 min and 72°C for 2 min, using the maximum ramp time between each temperature. The total number of cycles was 40 with an initial denaturation step at 95°C for 5 min. The last cycle was followed by 8 min at 72°C. Samples were analysed by electrophoresis on 1.5% agarose gels and detected by staining with ethidium bromide. The gels were photographed under UV light with Polaroid film 667. W.M. VI DNA (Boehringer) was used as a molecular size marker. A total of 60 primers (Operon Technologies, Alameda, Calif.) were used. Of the 60 tested primers 12 resulted in good amplification, in terms of reproducibility and clearness of the amplification pattern (Table 5). The reproducibility of the results was evaluated by replicating RAPD analyses on all individuals of the four populations using all of the selected primers.

For each individual of each population a RAPD band profile/absence profile was recorded. Each individual was represented by a vector of 1s and 0s, 1 for the presence of any particular band and 0 for its absence.

The analysis of RAPD band patterns was performed taking into consideration mathematical treatments developed for the analysis of restriction fragment length polymorphism (RFLP) data. We firstly considered a measure of genetic distance among populations (index of genetic distance, IGD) described by Apuya (1988) for RFLP analysis and by Yu and Pauls (1993) for RAPD data:

$$IGD = -\ln\left[\frac{1}{n}\sum_{i=1}^{n} 2\,\frac{f_{i,1}\,f_{1,2}}{f_{i,1}^2 + f_{i,2}^2}\right]$$

where $f_{i,1}$ and $f_{i,2}$ represent the frequencies of the band *i* in population 1 and 2 respectively, and *n* is the number of bands present in each population. The SAS computer program (SAS 1985) was used for cluster analysis of the IGD values (McQuitty procedure of SAS, based on the unweighted pair group method with arithmetic mean, UPGMA).

Furthermore, we considered a measure of which percentage of the total bands is common to the analysed populations, following the approach reported by Yu and Pauls (1993). This parameter, derived from the index of similarity, S_{ab}, introduced by Nei and Li (1979), can be defined in our specific work as follows:

$$1)\ F_3 = \frac{3c_3 + 2c_2}{b_1 + b_2 + b_3}$$

$$2)\ F_4 = \frac{4c_4 + 3c_2 + 2c_2}{b_1 + b_2 + b_3 + b_4}$$

where c_4 is the number of bands common to all four populations; c_3 is the number of bands found in only three of the four populations; c_2 is the number of bands found in only two of the populations; $b_1 \ldots b_4$ represent the total number of bands per population. F_3 and F_4 represent the percentage of common bands among three and four populations, respectively. For a more general definition of F_n see Yu and Pauls (1993).

The average of F_n among all primers attaining a measure of the percentage of population-specific bands (PSB%) was calculated as:

$$PSB\ (\%) = (1\text{-ave}F_n) \times 100$$

where aveF_n is the mean F_n value for all possible combinations of *n* populations.

Many investigations have shown that forest tree natural populations exhibit a very high level of genetic variation, i.e. a large part of the molecular variability is within populations. Therefore, in order to assess relationships among populations it is necessary to estimate, through bands pattern analysis, what fraction of variability is within populations and what fraction reflects variability among populations. To face this question we analysed the RAPD data using the approach of variance partition. We used AMOVA analysis only on the 55 polymorphic RAPD bands. This analysis was introduced by Excoffier et al. (1992) for restriction pattern and was applied successively by Huff et al. (1993) for RAPD patterns.

Results

Allozyme analysis

Of the 12 isozyme loci analysed 11 were polymorphic in at least one population (Table 1). Got-a was the only monomorphic locus. Other GOT and IDH zones of activity were observed but not considered because poor electrophoretic resolution did not allow the identification of other possible gene loci. Evident differences in allele frequencies among *A. nebrodensis* and *A. alba* populations were found particularly at Pgi-a and Skd-a. At the Pgi-a locus the frequency of the *a*1 allele was much higher in *A. nebrodensis* than in all the other *A. alba* populations. Furthermore, at locus

Table 2 Genetic variability measures (*n* mean number of alleles per locus, $P_{5\%}$ percentage of polimorphic loci (5% criterion), n_e effective number of alleles per locus (Crow and Kimura 1970), H_e mean expected heterozygosity, H_o mean observed heterozygosity

Population	Sample size	*n*	$P_{5\%}$	n_e	H_e	H_o
1 Madonie	14	1.6	58.3	1.16	0.138	0.167
2 Aspromonte	27	1.8	66.7	1.18	0.155	0.156
3 Serra San Bruno	30	1.7	50.0	1.11	0.099	0.108
4 Gariglione	28	1.6	25.0	1.09	0.083	0.091
5 Abeti Soprani	35	1.5	25.0	1.08	0.075	0.079
6 Collemeluccio	27	1.4	41.7	1.11	0.100	0.111
7 Abetone	31	1.6	33.3	1.13	0.112	0.087
8 Lavarone	27	1.6	25.0	1.10	0.091	0.080

Table 3 Genetic diversity analyses (Nei 1975) (H_t total gene diversity, H_s gene diversity within populations, D_{st} gene diversity among populations, G_{st} relative degree of genetic differentiation)

Locus	H_t	H_s	D_{st}	G_{st}
Gdh-a	0.047	0.046	0.001	0.026
Idh-a	0.458	0.416	0.042	0.091
Mdh-a	0.120	0.102	0.018	0.150
Me-a	0.187	0.172	0.015	0.079
Me-b	0.012	0.012	0.001	0.001
6Pgd-a	0.008	0.008	0.000	0.012
6Pgd-b	0.144	0.138	0.005	0.037
Pgi-a	0.319	0.175	0.144	0.450
Pgi-b	0.052	0.051	0.001	0.257
Skd-a	0.231	0.144	0.087	0.375
Skd-b	0.009	0.009	0.000	0.033
Mean	0.132	0.106	0.026	0.110

Table 4 Genetic distance between populations calculated according to Nei (1972)

Population	1	2	3	4	5	6	7	8
1 Madonie	****							
2 Aspromonte	0.089	****						
3 Serra San Bruno	0.111	0.002	****					
4 Gariglione	0.133	0.010	0.007	****				
5 Abeti Soprani	0.151	0.011	0.006	0.002	****			
6 Collemeluccio	0.128	0.008	0.005	0.006	0.005	****		
7 Abetone	0.144	0.014	0.010	0.005	0.003	0.003	***	
8 Lavarone	0.116	0.005	0.003	0.002	0.003	0.006	0.008	****

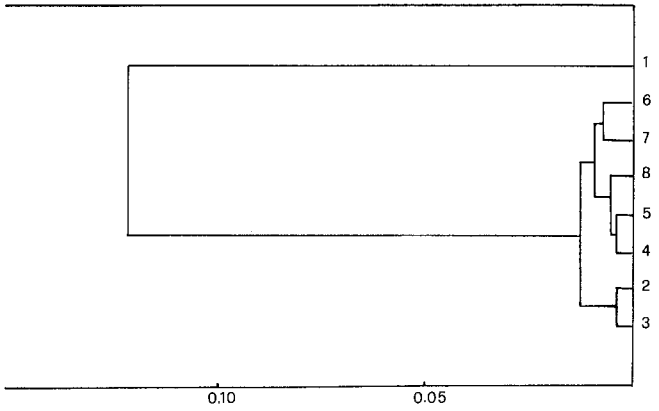

Fig. 2 UPGMA dendrogram based on Nei's genetic distances. Population numbers as described in footnote **a** of Table 1

Skd-a the most frequent allele in *A. nebrodensis* population was *a*1 instead of *a*2. A summary of the measures of genetic variability in populations is given in Table 2. Higher values of the effective number of alleles per locus and of expected and observed heterozygosity were found in the *A. nebrodensis* and Aspromonte populations: Aspromonte represents the most southern *A. alba* population. Mean expected heterozygosity ranged between 0.155 (Aspromonte) and 0.075 (Abeti Soprani). The mean level of genetic differentiation between populations over all loci estimated using G_{st} was 0.11 (Table 3), thus indicating that more than 10% of the total genetic diversity was due to differences among populations. The loci which contributed more than others to the differentiation among populations were Pgi-a and Skd-a (45% and 38% respectively)

The genetic distance between *A. nebrodensis* and the group of *A. alba* populations was 10–70 times greater than that among *A. alba* populations. The lowest genetic distance value was between the Madonie and Aspromonte populations (Table 4). The evident differentiation between the two groups of population is also showed by the UPGMA dendrogram (Fig. 2).

Chloroplast DNA analysis

Three cpDNA fragments were amplified, representing three spacers between t-RNA genes (a-b, e-f and a-f spacers, respectively): no differences in size among the populations were detected with agarose gel electrophoresis. The estimated fragment sizes were 490, 500 and 1440, respectively. Only 8 out of the 15 restriction enzymes succeeded in digesting the a-f spacer, yielding 21 restriction sites. No differences at the 21 restriction sites were observed among the studied populations; the *A. nebrodensis* and the *A. alba* populations showed identical restriction fragment length profiles.

RAPD analysis

Amplification was repeated at least twice for each primer-DNA combination. Out of 60 primers tested, 12 resulted in polymorphic banding patterns both within and among populations (Table 5) and showed a very high reproducibility: using agarose gels and ethidium bromide staining, clear resolution of both major and minor bands with a consistent reproducibility of amplification pattern was obtained. No amplification products were observed in control reactions that did not have the template DNA.

A total of 84 fragments were produced by the 12 selected primers, giving an average frequency of 7 RAPD bands per primer (Table 5). The size of the fragments varied from 480 to 2620 bp. Of these 84 fragments, 29 (35%) were monomorphic, while 55 (65%) were variable in at least one population.

Table 6 shows the pair-wise genetic distance index for four populations. The genetic distance index ranged from 0.221 to 0.076. The results of pair-wise comparisons indicated that the *A. nebrodensis* population was the least similar to all other *A. alba* populations: in fact, the genetic distance value between *A. nebrodensis* and *A. alba* was about three times greater than the values among the *A. alba* populations. Associations among the four populations revealed by UPGMA cluster analysis are presented in Fig. 3. The dendrogram is similar to that obtained with isoenzyme analysis: the differentiation of the *A. nebrodensis* population is again very evident.

The index of genetic distance was also used to derive the percentage of population specific bands (PSB%). The three *A. alba* populations (Aspromonte, Abeti Soprani and Lavarone) present the highest PBS value (91%) with respect to any one of the possible permutations among the four populations.

The AMOVA analysis demonstrated highly significant ($P>0.01$) genetic differences between *A. nebrodensis* and

Table 5 Nucleotide sequences of the 12 selected primers, GC%, number of main fragments for each primer, fragment sizes and number of polymorphic fragments

Primer	Nucleotide sequence	GC%	Number of main fragments	Fragment size range (bp)	Number of polymorphic fragments
C05	GATGACCGCC	70	10	840–1590	7
C07	GTCCCGACGA	70	8	1030–2610	5
E03	CCAGATGCAC	60	5	990–1590	5
E07	AGATGCAGCC	60	9	630–1290	4
E14	TGCGGCTGAG	60	6	730–1300	3
F01	ACGGATCCTG	60	7	690–1790	1
F06	GGGAATTCGG	60	7	810–1680	7
J04	CCGAACACGG	70	7	810–1760	7
J07	CCTCTCGACA	60	5	1380–2620	4
J19	GGACACCACT	60	5	480–1390	5
R02	CACAGCTGCC	70	9	540–1360	3
R12	ACAGGTGCGT	60	6	560–1060	4

Table 6 Values for the index of genetic distance (IGD) calculated from the RAPD pattern

	Madonie	Aspromonte	Abeti Soprani	Lavarone
Madonie	****			
Aspromonte	0.217	****		
Abeti Soprani	0.221	0.076	****	
Lavarone	0.218	0.091	0.080	****

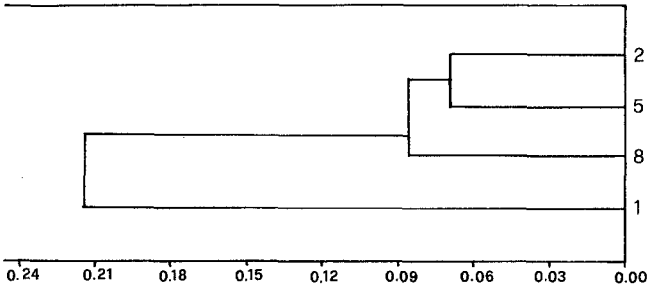

Fig. 3 Clustering of the *Abies nebrodensis* and *A. alba* populations by UPGMA analysis of genetic distance evaluated using single-primer amplification of genomic DNA. Population numbers as described in footnote **a** of Table 1

A. alba populations; nevertheless, there is evident variation within each of the four populations. These results were expected since conifers are outcrossing organisms. Of the total genetic diversity, 16% was attributable to population differences and 84% to individual differences within a population.

Discussion

Differences between *A. nebrodensis* and *A. alba* populations were evident from the allozyme and RAPD analysis. The allozyme analysis revealed that the *A. nebrodensis* population can be distinguished quite clearly from the *A. alba* populations at the gene pool level. The values of genetic differentiation and genetic distance among populations that we found are higher than those generally reported for conifers (Guries 1984; Govindaraju 1988; Hamrick and Godt 1989). The interpopulational variation is about two times greater than the average reported for wind-pollinated conifers (G_{st}=6.8%, Hamrick and Godt 1989). The relatively high level of differentiation among populations could be attributed to the typical scattered distribution of the *Abies* populations in Italy that prevents or limits gene flow. Muller-Starck et al. (1992), in his review of results of studies on genetic variation in various coniferous and angiosperm tree species, observed that species with small and geographically distinct ranges (*e.g. A. alba*) tend to show a great interpopulational differentiation and moderate intrapopulational genetic variation. Our data seems to confirm this trend also taking into consideration that the heterozygosity observed is lower than that generally reported for conifers species (Hamrick et al. 1981; Goncharenko et al. 1993). The highest value of heterozygosity was found in the relic population of *A. nebrodensis*. This result was quite surprising considering the restrictedness and scattered distribution of this population, due probably to a genetic bottleneck experienced in the past. The drastic reduction in the population size, (Raimondo et al. 1990), should have lead to values much lower than those observed as a consequence of genetic drift. Selection favouring heterozygotes in the *A. nebrodensis* population could be acceptable working hypothesis that explain this result.

Among *A. nebrodensis* and *A. alba* populations, genetic variation was easily detectable using single-primer DNA amplifications, and the polymorphisms obtained with the 12 selected primers were easily scorable. The results demonstrated that RAPD technology can be used to identify DNA polymorphisms among the different *Abies* populations. The level of RAPD differentiation among *A. nebrodensis* and *A. alba* populations is quite high and seems to confirm the hypothesis that the two groups of populations

belong to two different taxonomic groups. Unfortunately, no information on RAPD differentiation among conifers species is available and, therefore, a comparison is impossible. Similar and high levels of polymorphisms among different species of *Arachis hypogaea*, *Hordeum* and *Stylosanthes guianensis* were found by Halvard et al. (1992), Gonzales and Ferrer (1993) and Kazan et al. (1993 a, b), respectively. These findings provide strong support for the previous placement of *A. nebrodensis* and *A. alba* into distinct taxonomic groups on the basis of allozyme (in the present study) and morphological characteristics (Raimondo et al. 1990). Moreover, it should be emphasised that the genetic distance between *A. nebrodensis* and Aspromonte *A. alba* populations is much higher than those found among *A. alba* populations located more than 1000 km apart.

The AMOVA approach allowed the apportion of RAPD genetic variation among individuals within populations and among populations. In spite of evident genetic variation among *A. nebrodensis* and *A. alba* populations, within-population differences were very pronounced. This pattern of genetic variation scored in these two conifers is very different from those reported for inbred species and is in accordance with what we might expect of outcrossing organisms. The very low level of RAPD variation within populations and cultivars observed by many authors could be due to the isogenic state or highly inbred nature of the plant materials analysed. The RAPD technique is therefore also a very useful approach to detect genetic variation within populations when it exists. This aspect could be very important for studying conifers which are organisms that generally display a high level of within-population genetic variability. Our result seems to confirm the presence of a correlation between genetic diversity and levels of outcrossing rates, as previously reported by other authors (Gottlieb 1981; Miller and Tanskley 1990; Huff et al. 1993; Kazan et al. 1993a, b). Miller and Tanskley (1990) studied different tomato species and demonstrated that the outcrossing species showed higher RAPD polymorphisms than inbreeders. The high intraspecific variation found in *Stylosanthes guianensis* by Kazan et al. (1993b) was partly explained by the higher outcrossing rate of this species.

No differences in the size and in the restriction patterns of the three non-coding cpDNA regions were found among the *A. nebrodensis* and *A. alba* populations with agarose gel electrophoresis. This approach has already demonstrated its efficiency for the classification of different *Pinus* species: Boscherini et al. (1994) were able to distinguish two closely related species, *Pinus halepensis* and *P. brutia*, digesting the e-f spacer with *MseI* restriction endonuclease. First evidence seems to indicate that the variability obtained upon amplifying and digesting the above mentioned cpDNA regions is much higher within the *Pinus* than within the *Abies* genus (Rossi P, Vendramin GG, Vicario F, unpublished data).

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