Allozyme and chloroplast DNA variation in Italian and Greek populations of *Pinus leucodermis*

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Allozyme and chloroplast (cpDNA) variation was examined in five Italian and two Greek populations of *Pinus leucodermis* Ant. to estimate levels of genetic variability within and among populations and to establish the usefulness of allozyme and cpDNA markers in the taxonomic classification of these populations. Twenty-three isozyme gene loci were analysed, as well as restriction fragment length polymorphisms at two cpDNA spacer regions between tRNA genes. The level of genetic variability tended to be lower in the Greek populations but overall the observed levels of allozyme variation within and among populations were similar to those of other conifers. Identical cpDNA amplification and restriction patterns were observed among all individuals sampled from the seven populations. Taken together, the results of the allozyme and cpDNA analyses indicate that all seven populations belong to the same biological species.

Keywords: allozymes, chloroplast DNA, genetic variability, *Pinus leucodermis*.

Introduction

Pinus leucodermis Ant. is a wind-pollinated conifer species and one of the most narrowly distributed pines in Europe. Its natural range is divided into two main parts, each constituted of small and distinct populations, located either in southern Italy or the Balkan peninsula. Palynological studies show that this species was once a major component of forests in southern Italy (Avolio, 1984). The systematic and phylogenetic relationships between the two groups of populations have tended to remain unclear; according to some authors they represent two different species, *P. leucodermis* and *P. heldreichii*, while other authors (e.g. Mirov, 1967) consider all populations to belong to the same species. Both views are mainly based on morphological and crossability analyses. Recently, isozyme and chloroplast DNA (cpDNA) analyses have been used to reconstruct the phylogenetic relationships among conifer forest tree species (Sigurgeirsson & Szmidt, 1988; Szmidt *et al.*, 1988; Wang & Szmidt, 1990; Shurkhal *et al.*, 1992; Wagner *et al.*, 1992). Most cpDNA analyses conducted to date have examined restriction fragment length polymorphisms (RFLPs) over the entire chloroplast genome or nucleotide sequences of a single gene.

Recently the polymerase chain reaction (PCR) has been used to amplify specific cpDNA sequences which have then been subjected to RFLP analysis using four-base restriction endonucleases (Liston, 1992; Rieseberg *et al.*, 1992).

In the present study allozyme and chloroplast DNA (cpDNA) markers have been used to estimate levels of genetic variability within and among Italian and Greek populations of *Pinus leucodermis*, as well as to establish their usefulness for taxonomic classification. Using cpDNA we amplified and analysed by restriction enzyme digestions two spacer regions located between two pairs of tRNA genes (Taberlet *et al.*, 1991).

Materials and methods

Seeds were collected from seven wild populations of *Pinus leucodermis* (Tables 1 and 2), five of which are located in the south of Italy (in the La Spina and Pollino mountain groups) while two occurred in Greece (in the Olimpo and Pindo mountains). The first Greek population clearly belonged to the group classified as *P. leucodermis*, while the second population was comprised of a type classified by some authors as *P. leucodermis* and by others as *P. heldreichii*. Seeds were collected from between 17 and 29 single trees in the five Italian populations; the exact number of trees sampled for seed in the two Greek populations is unknown but was greater than 30.

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Table 1 Allele frequencies in Italian and Greek populations of *Pinus leucodermis*

Locus		Population						
		1	2	3	4	5	6	7
<i>Aco-a</i>	1	0.879	0.821	1.000	0.806	0.975	0.898	0.923
	2	0.121	0.179	0.000	0.194	0.025	0.102	0.077
<i>G6pd-a</i>	1	0.241	0.483	0.324	0.263	0.250	0.434	0.727
	2	0.759	0.517	0.676	0.737	0.750	0.566	0.273
<i>Got-a</i>	1	0.000	0.017	0.088	0.225	0.175	0.000	0.000
	2	1.000	0.983	0.912	0.775	0.825	1.000	1.000
<i>Got-b</i>	1	0.017	0.052	0.029	0.025	0.000	0.000	0.000
	2	0.983	0.948	0.971	0.975	1.000	1.000	1.000
<i>Got-c</i>	1	0.000	0.000	0.029	0.050	0.050	0.000	0.000
	2	0.879	1.000	0.971	0.925	0.950	1.000	1.000
	3	0.052	0.000	0.000	0.025	0.000	0.000	0.000
	4	0.069	0.000	0.000	0.000	0.000	0.000	0.000
<i>Idh-b</i>	1	0.052	0.000	0.029	0.053	0.000	0.000	0.000
	2	0.983	1.000	0.971	0.947	1.000	1.000	1.000
<i>Lap-a</i>	1	0.948	0.931	0.971	0.925	1.000	0.888	0.981
	2	0.052	0.069	0.029	0.075	0.000	0.112	0.019
<i>Lap-b</i>	1	0.839	0.879	0.906	0.925	1.000	0.423	0.567
	2	0.161	0.121	0.094	0.139	0.000	0.577	0.433
<i>Mdh-b</i>	1	0.000	0.017	0.029	0.000	0.000	0.000	0.000
	2	1.000	0.983	0.971	1.000	1.000	1.000	1.000
<i>Mnr-a</i>	1	0.034	0.071	0.059	0.250	0.200	0.077	0.098
	2	0.880	0.893	0.823	0.750	0.775	0.923	0.902
	3	0.086	0.036	0.118	0.000	0.025	0.000	0.000
<i>Pgm-a</i>	1	0.190	0.018	0.235	0.278	0.175	0.000	0.008
	2	0.810	0.982	0.765	0.722	0.825	1.000	0.992
<i>6Pg-d-a</i>	1	0.017	0.000	0.000	0.000	0.000	0.000	0.000
	2	0.983	1.000	1.000	1.000	1.000	1.000	1.000
<i>6Pg-d-b</i>	1	0.914	0.638	0.824	0.772	0.700	0.728	0.690
	2	0.086	0.362	0.176	0.228	0.300	0.272	0.310
<i>Pgi-a</i>	1	0.017	0.000	0.000	0.000	0.000	0.000	0.000
	2	0.983	1.000	1.000	1.000	1.000	1.000	1.000
<i>Pgi-b</i>	1	0.276	0.304	0.412	0.361	0.200	0.402	0.350
	2	0.724	0.696	0.588	0.639	0.800	0.598	0.462
	3	0.000	0.000	0.000	0.000	0.000	0.000	0.188
<i>Skdh-a</i>	1	0.724	0.804	0.912	0.842	0.725	0.933	0.876
	2	0.276	0.196	0.088	0.158	0.275	0.067	0.124

Aco: aconitate hydratase; *G6pd*: glucose 6-phosphate dehydrogenase;
Got: glutamate oxaloacetate transaminase; *Idh*: isocitrate dehydrogenase; *Lap*:
leucine aminopeptidase; *Mdh*: malate dehydrogenase; *Mnr*: menadione reductase;
Pgm: phosphoglucomutase; *6Pg-d*: 6-phosphogluconate dehydrogenase; *Pgi*:
phosphoglucose isomerase; *Skdh*: shikimate dehydrogenase.

1: La Spina (I); 2: Pollino (I); 3: Pollino A (I); 4: Pollino B (I); 5: Pollino C (I); 6:
Olimpo (GR); 7: Pindo (GR).

Allozyme analysis

For the five Italian populations with seedlots from separate trees, the genotypes of individuals were inferred by examining six endosperms for each tree. Endosperms (140–150) were examined for the two Greek populations for which bulk provenance collections of seeds were available. Thirteen enzyme systems, encoded by 23 gene loci, were analysed by means of starch gel electrophoresis. Details of electrophoretic procedures and the inheritance and linkage relationships of allozyme variants studied are given in Morgante *et al.* (1991, 1993).

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

mean expected heterozygosity (H_e) were calculated. The amount of genetic differentiation between populations at the gene pool level was estimated by calculating G_{st} (Nei, 1975). Genetic relationships between populations were examined by computing standard genetic distances (Nei, 1972) and using these values in a UPGMA cluster analysis.

Chloroplast DNA analysis

DNA was extracted from about 80 germinated embryos from each of the seven populations following a modification of the CTAB method (Bousquet *et al.*, 1989; Doyle & Doyle, 1990).

DNA amplification was performed in a Perkin Elmer 9600 thermal cycler. The typical volume of the reaction mixture was 50 μ L containing 50 ng DNA, 20 mM Tris-HCl, 100 mM KCl, 0.02 per cent gelatine, 3 mM MgCl₂, 0.2 mM of each nucleotide, 0.2 μ M of each primer and 1 unit *Taq* polymerase (commercial preparation). We used two pairs of 20-mer primers for the PCR placed in very conserved regions flanking more variable regions (purchased from Operon Technologies). The amplified regions are expected to be the intergenic spacers between the *trnT* and *trnL* genes and between the *trnL* and *trnF* genes of the chloroplast genome (Taberlet *et al.*, 1991). DNA amplification was conducted according to the following programme: (i) a 5 min, 95°C step for DNA denaturation (hot start), (ii) a 80°C step for enzyme addition and (iii) 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 visualized in a 1.5 per cent agarose gel. Both amplification products were cut with 11 restriction endonucleases (Table 3), all with four base recognition sites.

Table 2 Genetic variability measures in Italian and Greek populations of *Pinus leucodermis*

Population	Sample size	N	$P_{5\%}$	n_e	H_e
(1) La Spina (I)	29	1.7	47.8	1.141	0.124
(2) Pollino (I)	29	1.6	39.1	1.135	0.119
(3) Pollino A (I)	17	1.6	34.8	1.130	0.115
(4) Pollino B (I)	19	1.6	52.2	1.187	0.158
(5) Pollino C (I)	20	1.5	39.1	1.145	0.127
(6) Olimpo (GR)	†	1.3	34.8	1.122	0.109
(7) Pindo (GR)	†	1.4	30.4	1.124	0.110

†Pooled seed samples.

N : mean no. of alleles per locus.

$P_{5\%}$: percentage of polymorphic loci (5% criterion).

n_e : effective no. of alleles per locus (Crow & Kimura, 1970).

H_e : mean expected heterozygosity.

Table 3 Restriction endonucleases used in *Pinus leucodermis* and number of restriction sites found

Endonuclease	Recognition sequence	No. of restriction sites	
		Fragment A	Fragment B
<i>AluI</i>	5'AG/CT ^{3'}	1	2
<i>CfoI</i>	5'GCG/C ^{3'}	1	0
<i>DdeI</i>	5'C/TNAG ^{3'}	0	1
<i>HaeIII</i>	5'GG/CC ^{3'}	0	0
<i>HinfI</i>	5'G/ANTC ^{3'}	3	2
<i>HpaII</i>	5'C/CGG ^{3'}	0	1
<i>MseI</i>	5'T/TAA ^{3'}	0	2
<i>NciI</i>	5'CC/(CG)GG ^{3'}	1	1
<i>RsaI</i>	5'GT/AC ^{3'}	0	1
<i>Sau 3a</i>	5'/GATC ^{3'}	0	2
<i>TaqI</i>	5'T/CGA ^{3'}	3	3

To assess the reliability of this PCR/RFLP method for phylogenetic analysis in the genus *Pinus*, DNA was also extracted and examined from other *Pinus* species: *P. brutia*, *P. halepensis*, *P. nigra* and *P. pinaster*. We chose these *Pinus* species because they all belong to the same subgenus, section and subsection as *P. leucodermis* (Little & Critchfield, 1969). Moreover, *P. brutia* and *P. halepensis* belong to the same complex and are considered to be very closely related, with *P. brutia* formerly treated as a variety of *P. halepensis*. The comparative analysis of *P. brutia* and *P. halepensis* in particular should act as an appropriate test of the reliability of the chosen PCR/RFLP method for separating very closely related pine species. DNA samples from these species were amplified with primers flanking the region between the *trnL* and *trnF* genes, then digested with restriction endonucleases.

As a control, DNA was also extracted and amplified from endosperm tissue, which is of maternal origin. In most conifers, chloroplasts are inherited paternally and amplification of cpDNA is therefore not expected to occur from endosperm tissue. Digestion products were visualized in 3 per cent agarose gel after ethidium bromide staining.

Results

Allozyme analysis

Sixteen of the 23 isozyme loci investigated were polymorphic in at least one population (Table 1). The seven monomorphic loci were *Gdh-a* (glutamate dehydrogenase), *Idh-a*, *Mdh-a*, *Mdh-c*, *Mnr-b*, *Ndh-a* (NADH dehydrogenase) and *Skd-b*. Greek populations were characterized by the absence of rare alleles that were present in Italian populations. Also, Greek populations were often fixed for a particular allele at a locus while Italian populations were polymorphic for two or more alleles. At the *Lap-b* locus the frequency of the *b1* allele was much lower in Greek populations, particularly the Olimpo population. Furthermore, allele *b3* at the *Pgi-b* locus was scored only in the Pindo

population while at locus *G6pd-a* the most frequent allele in the Pindo population was *a1* instead of *a2*.

A summary of measures of genetic variability in populations is given in Table 2. In general, it is evident that the level of genetic variability is lower in the Greek populations than in the Italian populations.

The mean level of genetic differentiation between populations over all loci estimated using G_{st} (Table 4) was 0.054, thus indicating that less than 5.5 per cent of the total genetic diversity is explained by differences between populations. Some loci, e.g. *Lap-b*, *G6pd-a* and *Got-a*, contributed particularly more than others to the observed levels of differentiation among populations (16, 11 and 11 per cent, respectively).

Genetic distance values (Table 5) were in general low, again indicating a low degree of genetic divergence

Table 4 Genetic diversity analyses for *Pinus leucodermis*

Locus	H_t	H_s	D_{st}	G_{st}
<i>Aco-a</i>	0.179	0.171	0.009	0.050
<i>G6pd-a</i>	0.475	0.422	0.053	0.112
<i>Got-a</i>	0.134	0.119	0.015	0.113
<i>Got-b</i>	0.035	0.034	0.001	0.019
<i>Got-c</i>	0.077	0.073	0.003	0.043
<i>Idh-b</i>	0.038	0.037	0.001	0.029
<i>Lap-a</i>	0.097	0.094	0.003	0.026
<i>Lap-b</i>	0.371	0.313	0.058	0.157
<i>Mdh-b</i>	0.013	0.013	0.001	0.018
<i>Mnr-a</i>	0.264	0.253	0.011	0.043
<i>Pgm-a</i>	0.225	0.201	0.024	0.105
<i>6Pgd-a</i>	0.005	0.005	0.000	0.015
<i>6Pgd-b</i>	0.380	0.365	0.015	0.039
<i>Pgi-a</i>	0.005	0.005	0.000	0.015
<i>Pgi-b</i>	0.476	0.457	0.019	0.041
<i>Skdh-a</i>	0.281	0.269	0.012	0.043
Mean	0.191	0.177	0.014	0.054

H_t : total gene diversity; H_s : gene diversity within populations; D_{st} : gene diversity among populations; G_{st} : relative degree of genetic differentiation. See Nei (1973, 1975).

Table 5 Genetic distances between populations of *Pinus leucodermis* calculated following Nei (1972)

Population	1	2	3	4	5	6	7
(1) La Spina (I)	****						
(2) Pollino (I)	0.010	****					
(3) Pollino A (I)	0.005	0.009	****				
(4) Pollino B (I)	0.008	0.010	0.006	****			
(5) Pollino C (I)	0.006	0.009	0.007	0.005	****		
(6) Olimpo (GR)	0.018	0.013	0.017	0.020	0.018	****	
(7) Pindo (GR)	0.025	0.011	0.020	0.018	0.023	0.007	****

among populations. The largest genetic distances were found between the Italian and Greek populations; the average distance between the two groups was 0.016, i.e. about three to four times that within groups (0.004 and 0.005, respectively). The UPGMA dendrogram based on the Nei's genetic distances exhibited two major sub-branches, one containing the Italian populations, the other the Greek ones (Fig. 1). Therefore, the dendrogram shows a clear geographical pattern of genetic variation between these two groups of populations.

Chloroplast DNA analysis

Two cpDNA fragments were amplified representing two intergenic spacers between the *trnT* and *trnL* genes (fragment A) and the *trnL* and *trnF* genes

(fragment B). No differences in the sizes of the two amplified products were found among the populations with agarose gel electrophoresis. The estimated fragment sizes were 520 and 500 bp, respectively. These fragments have been amplified in other conifer species e.g. *Pinus nigra* (Taberlet *et al.*, 1991), *Picea jezoensis*, *P. koraiensis* and *P. pungens* (G. Boscherini & G. G. Vendramin, unpublished data) and estimated to vary between 480 and 600 bp in length.

No differences in size were found between the amplification products of *P. brutia*, *P. halepensis*, *P. leucodermis* and *P. pinaster* (500 bp), while the amplification product of *P. nigra* was smaller (480 bp).

Only five out of the 11 restriction enzymes used succeeded in digesting fragment A, yielding nine restriction sites (Table 3). Fragment B was digested by nine of the 11 enzymes used, corresponding to 15 sites. No differences at the 24 restriction sites were found among the studied populations. Thus the six populations classified as *P. leucodermis* (Pollino, Pollino A, B, C, La Spina, Olimpo) and the one of uncertain classification (Pindo) yielded identical restriction fragment length profiles over all individuals studied (Fig. 2).

Digestion of fragment B with *MseI* and *TaqI* showed different restriction patterns among *P. brutia*, *P. halepensis*, *P. leucodermis*, *P. nigra* and *P. pinaster* (Fig. 3).

DNA purified from endosperm tissue failed to amplify with the above-mentioned four cp-primers.

Fig. 1 UPGMA dendrograms based on Nei's genetic distances. Population numbers refer to Tables 1 and 2.

Discussion

Differences between Greek and Italian populations of *Pinus leucodermis* were evident from the allozyme

Fig. 2 Restriction patterns of fragments A and B obtained using different restriction enzymes (fragment A: 1: *AluI*; 2: *CfoI*; 3: *HinfI*; 4: *NciI*; 5: *TaqI*; fragment B: 1: *AluI*; 2: *DdeI*; 3: *HinfI*; 4: *HpaII* and *NciI*; 5: *MseI*; 6: *RsaI*; 7: *Sau 3a*; 8: *TaqI*); M: pBR322-*HaeIII* digest molecular weight marker (Boehringer). The restriction patterns are identical for all the studied populations of *P. leucodermis*.

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