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Effectiveness of Neutral RAPD Markers to Detect Genetic Divergence between the Subspecies *uncinata* and *mugo* of *Pinus mugo* Turra

Ignazio Monteleone, Diana Ferrazzini and Piero Belletti

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Fifteen populations of *Pinus mugo* subsp. *mugo* (shrub) and *Pinus mugo* subsp. *uncinata* (erect), located in the Alps, were investigated through genetic variation scored at 64 polymorphic RAPD loci. In addition, morphological traits of the female cones were analysed. According to AMOVA most of the genetic variation was found within populations (83.39%), and only 1.25% of it between subspecies. Populations differed in terms of their internal genetic variation, with Nei's gene diversity ranging from 0.227 to 0.397. Morphological data showed differences between subspecies, although none of the populations showed full accordance with expectations. Significant correlation was found between matrices for geographical and morphological distances, while genetic distances were not correlated with any other aspect. The efficacy of morphological and RAPD markers in discriminating between subspecies, and the contribution of the results in relation to the preservation of biodiversity, are discussed.

Keywords *Pinus mugo*, mountain pine, dwarf mountain pine, molecular markers, genetic diversity, population differentiation
Authors' address DI.VA.P.R.A., Plant Genetics and Breeding, University of Turin, via Leonardo da Vinci 44, I-10095 Grugliasco, Italy
E-mail piero.belletti@unito.it
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1 Introduction

Pinus mugo is a highly polymorphic taxon. It is usually divided into two subspecies: *Pinus mugo* subsp. *mugo* Turra (dwarf mountain pine) and *Pinus mugo* subsp. *uncinata* (Ramond) Domin (mountain pine) (Christensen 1987), but there is no general consensus on this classification and different interpretations of the complex have been proposed. Von Tubeuf (1912) suggested a

single species, *Pinus montana* Miller, divided into grex *arborea* and grex *prostrata*. Holubičková (1965) proposed two distinct species (*Pinus mugo* Turra and *Pinus uncinata* Ramond ex De Candolle), further subdivided into subspecies (*mugo* and *pseudopumilio* for *Pinus mugo*; *uncinata* and *rotundata* for *Pinus uncinata*). More recent reviews are still in disagreement, with Farjon (1998) following Christensen (1987), and Richardson (1998) agreeing with Holubičková (1965). The two taxa hybridise extensively, giving the hybrid *P.* × *rotundata* (according to Holubičková) or, alternatively, the subspecies *Pinus mugo* nothossp. *rotundata* (Link) Janchen and Neumayer (according to Christensen) (Businský 1998). Hybridisation can also occur with *Pinus sylvestris* and, less frequently, with *Pinus nigra* and *Pinus heldreichii* (Christensen 1987, Christensen and Dar 2003, Wachowiak et al. 2005).

P. m. mugo is a shrub, 1–2 m tall, with some curved trunks. The branches are long and their base lies on the ground, while the end is erect. Female cones are symmetrical, and have a thin apophysis with a central umbo. *P. m. uncinata* is a tree 12–20 m tall, with a straight and erect trunk. Female cones are strongly asymmetrical and have a thick, hook-shaped apophysis, with the umbo at the apex of the apophysis (Gaussen et al. 1993). However, both morphological and ecological characters used to distinguish subspecies do not occur coherently, so that the systematics of the complex remains uncertain (Sandoz 1987). According to Sandoz, the number and size of hypodermal and endodermal cells are more reliable as discriminating characters, although their analysis is complex and scarcely useful for practical purposes. Other morphometric traits that are known to differ between the two taxa are the length of the needles and the distance between the vascular bundles (Boratyńska and Bobowicz 2001).

The *Pinus mugo* complex is endemic to Southern and Central Europe. In general, the subspecies *mugo* is distributed in the Eastern part of the Alps and in the Carpatian Mountains, while the subspecies *uncinata* is common in the Western part of the Alps and in the Pyrenees. However, the two taxa frequently overlap and give rise to intermediate forms that are difficult to classify. In few locations of Central and Western Alps wide and homogeneous populations, made up entirely of intermediate individuals, grow in the absence of their parental forms, leading to uncertainty about the hybrid origin of such individuals.

The importance of the complex is mainly due to the way it gives protection against soil erosion and retards avalanches. The needles are source of oil (Stevanovic et al. 2005), and spring buds are commonly used in medicine (Hippeli et al. 2004, Graßmann et al. 2005) and in the production of liqueur. Although the wood is hard and heavy it is rarely used due to the small size and slow growth of the trees. The plants are popular as ornamentals, particularly in Central and Northern Europe. Furthermore, *Pinus mugo* is of great ecological and naturalistic importance for mountain regions, where the species is an essential component of their peculiar and vulnerable ecosystems.

Little is known about the genetic variability and the genetic structure of *Pinus mugo* populations, since research has mostly focused on putative hybridisation between *Pinus mugo* and *P. sylvestris* (Filppula et al. 1992, Neet-Sarqueda 1994, Slavov and Zhelev 2004).

The purpose of the present study was to evaluate neutral DNA RAPD (Random Amplified Polymorphic DNA) markers as a tool to distinguish between the *mugo* and *uncinata* subspecies, with particular emphasis on their intermediate forms. The RAPD technique involves the analysis of a large number of loci, thus providing a more complete evaluation of the genome as compared with other biochemical and molecular markers. It also has several other potential advantages, among which is low cost, rapidity, the small amount of DNA required, high potential for detecting polymorphism and the lack of any need for prior knowledge of the genome being studied. Consequently, RAPDs have been intensively used to successfully detect genetic variation between plant populations (Etisham-Ul-Haq et al. 2001), taxa (Roman et al. 2003), and hybrids (Triest et al. 2000, Rajora and Rahman 2003). RAPDs have also some important drawbacks, the most significant being their dominant allelic expression and their low level of reproducibility. The former hampers direct estimates of allele frequencies in diploids and therefore biases the evaluation of genetic variation and differentiation (Lynch and Milligan 1994, Szmidt et al. 1996).

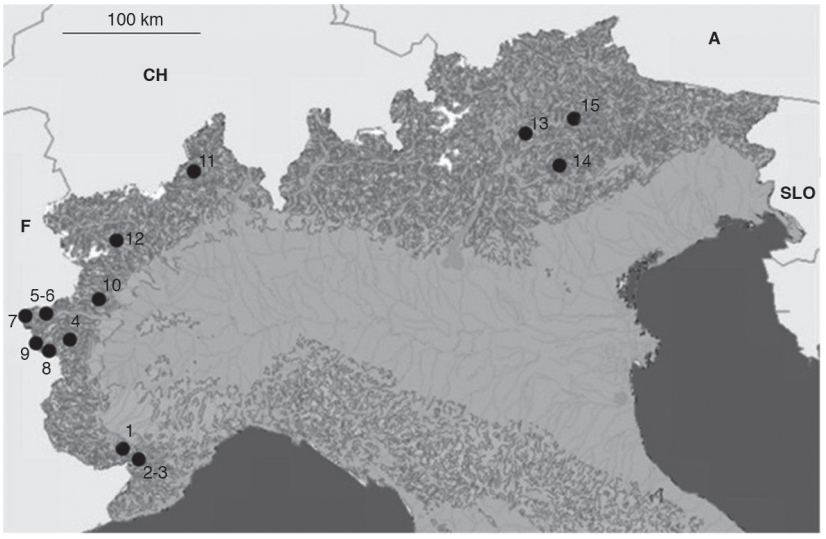

Fig. 1. Geographical distribution of the 15 populations of *Pinus mugo* subsp. *mugo* and *uncinata* analysed in the study.

Neutral genetic markers are currently used in genetic differentiation analysis but their reliability has been questioned, mainly when the objective of the study is to evaluate quantitative variation. Although many assays have been successfully conducted to detect genetic diversity by means of neutral molecular markers (Cheng et al. 2002, Hardy 2003, Shashidhara et al. 2003), it has recently been demonstrated that the correlation between the variation scored with such markers, and the variation for adaptatively important traits, is often poor (Ennos et al. 1997, Reed and Frankham 2001, Van Tienderen et al. 2002, Bekessy et al. 2003, Howe et al. 2003).

A better understanding of the genetic relationship between different forms of *Pinus mugo* could also contribute to the development of *in situ* conservation strategies, identifying populations of high concern. Analysis of the distribution of patterns of genetic variation is also potentially useful for the restoration of degraded populations by designating appropriate seed zones.

2 Material and Methods

2.1 Plant Material

Samples were collected from 15 natural stands, widespread all over the Alps (Fig. 1). The sites were chosen to represent different ecological conditions (Table 1). The populations were divided in two groups that reflect their natural structuring, and which were defined according the growth form of the trees. Erect plants were at least 10 m high, while shrubs reached a height of about 1 m. In two locations (Gola della Chiusetta and Seguret-Pramand) both growth forms (erect and shrub) were present, although at different altitude, and in this case samples were collected separately and considered as distinct stands. In two other stands (Colle Lombardo and Lago di Camplicioli) all of the plants were of intermediate form: they were as high as 3–4 m, although they lacked straight and erect trunks, and their branches originated at ground level (Fig. 2). For this reason, in dealing with the data, intermediate forms were considered as belonging to the shrub group. Intermediate forms were found only in stands nos. 10 and 11, and individuals with such a growth form were not observed elsewhere.

Fig. 2. Erect population of *Pinus mugo uncinata* no. 12 (Monte Avic, above), the shrub population of *Pinus mugo mugo* no. 1 (Passo del Duca, middle) and the intermediate population no.10 (Colle Lombardo, below).

Table 1. Details of site and stand characteristics of the *Pinus mugo mugo* (1, 3, 6, 10, 11, 13, 14, 15) and *Pinus mugo uncinata* (2, 4, 5, 7, 8, 9, 12) populations.

Population	Valley	Province	Latitude (°N)	Longitude (°E)	Altitude range (m a.s.l.)	Growth form	Soil
1. Passo del Duca	Pesio	Cuneo	44.15	7.40	1950–2000	Shrub	Limestone
2. Gola della Chiusetta	Tanaro	Cuneo	44.10	7.45	1550–1650	Tree	Porphyry
3. Gola della Chiusetta	Tanaro	Cuneo	44.10	7.45	1700–1800	Shrub	Porphyry
4. Laval	Troncea	Torino	45.00	6.55	1850–1900	Tree	Schist
5. Seguret-Pramand	Susa	Torino	45.10	6.50	1850–1950	Tree	Dolomite
6. Seguret-Pramand	Susa	Torino	45.10	6.50	2000–2100	Shrub	Dolomite
7. Sette Fontane	Stretta	Torino	45.10	6.40	1550–1600	Tree	Quartzite
8. Ruilles	Thures	Torino	44.50	6.50	1700–1750	Tree	Schist
9. Nevache	Clarèe	Briançon (Fr)	45.00	6.40	1800–1850	Tree	Quartzite
10. Colle Lombardo	Lanzo (Viù)	Torino	45.15	7.20	1650–1700	Intermediate	Gneiss
11. Lago di Camplicciolli	Antrona	Verbania	46.00	8.00	1550–1600	Intermediate	Gneiss
12. Monte Avic	Champdepraz	Aosta	45.40	7.40	1800–1850	Tree	Serpentine
13. Paganella	Non	Trento	46.15	11.00	2000–2100	Shrub	Dolomite
14. Cima Dodici	Valsugana	Vicenza	46.00	11.30	2200–2300	Shrub	Dolomite
15. Val Venegia	Fiemme	Trento	46.20	11.45	1900–2000	Shrub	Dolomite

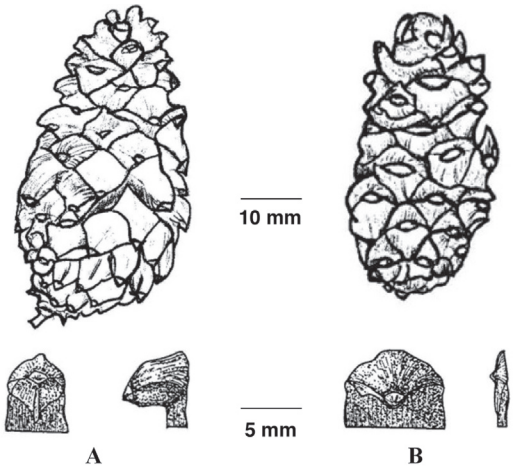

Fig. 3. Cones and prominent apophysis in *Pinus mugo*. Apophysis are drawn in dorsal (left) and lateral (right) view. A, *P. m. uncinata*; B, *P. m. mugo* (modified after Christensen 1987).

Leaves and cones were collected from at least 24 adult trees per population, randomly chosen over a 5 to 10 ha area. About 10 cones per plant were collected and used for morphological analysis, while DNA was extracted from needles.

2.2 Morphological Analysis

Two morphological traits, the symmetry of female cones (presence *vs* absence) and the shape of the cone apophysis (flat *vs* hook-shaped) (Fig. 3), were considered, and were chosen on the basis of a simple evaluation without the need of specialised equipment. The traits were considered as qualitative ones, and only two phenotypes were recorded due to the difficulty of identifying measurements to adequately describe the observed variation. The traits were measured from the same individuals as those used in the molecular genetic study.

Populations were compared by a non-parametric test for nominal data (frequencies), namely one way Chi-Square (SPSS 12.0, 2003). Populations were placed in two groups according to their growth form: shrub and tree. Intermediate forms were combined with shrubs due to their similarity of growth height. Principal Component Analysis (PCA) was used to highlight differences in morphological traits between populations, and also to indicate possible associations between the evaluated characteristics. PCA was performed on the derived trait correlation matrix, and the component values for each individual were projected in two dimensions (programs Eigen, Proj and Mxplot of NTSYSpc2.10j; Rohlf 2001).

Table 2. Characteristics of fragments generated by the 10 primers selected for the genetic analysis.

Primer	Nucleotide sequence	GC basis (%)	Total no of bands	No of polymorphic fragments	Molecular weight range (bp)
OPA01	CAGGCCCTTC	70	8	7	680–2310
OPA02	TGCCGAGCTG	70	7	6	780–2200
OPA03	AGTCAGCCAC	60	5	3	790–2200
OPA04	AATCGGGCTG	60	6	3	470– 850
OPA07	GAAACGGGTG	60	14	14	430–2190
OPA09	GGGTAACGCC	70	12	11	560–1500
OPA10	GTGATCGCAG	60	11	7	380–1420
OPA14	TCTGTGCTGG	60	6	4	900–1600
OPA18	AGGTGACCGT	60	5	5	260–1700
OPA19	CAAACGTCGG	60	7	4	600–1320
Total	–	–	81	64	–

2.3 Molecular Analysis

Total DNA was extracted from young needles, adopting the procedure described by Ziegenhagen et al. (1993). Amplifications were carried out in a Perkin Elmer DNA thermal cycler (PE 9600). The optimal reaction for RAPD analysis was set using the following conditions: 25 µl volume containing 1× reaction buffer, 1 unit of Taq DNA polymerase (Promega), 2.5 mM potassium chloride, 0.2 µM decamer primers, dNTPs mix 0.2 mM and 20 ng DNA. The amplification conditions were as follows: the first step at 95°C for 5 minutes, followed by 40 cycles of 1 minute at 94°C, 1 minute at 36°C and 2 minutes at 72°C. The final step was 8 minutes at 72°C. On the basis of literature reports (Szmidt et al. 1996, Lu et al. 1995, Lu et al. 1997, Hurme and Savolainen 1999), we screened a total of 12 primers from Life Technologies (Table 2). OPA05 and OPA15 were later excluded from the analysis since their amplification products were not reproducible. The high number of bands obtained for the other primers was considered sufficient for a preliminary screening of genetic variation. The amplification products were separated in 1.5% agarose gel with ethidium bromide in 0.5 × TBE buffer. The banding patterns were visualised under UV light and acquired using a fluorimeter linked with a gel documentation system (GelDoc 2000). The bands were scored visually, and those of similar molecular size, scored for the same primer, were assumed to be homologous. Each band was treated as an independent locus with two alleles: presence or absence of the band, respectively indicated as + and –. The reproducibility of the amplification products was tested twice for each sample and each primer.

2.4 Molecular Data Processing

RAPDs were scored as the presence or absence of bands, and a binary matrix of RAPD phenotypes was assembled. Following the commonly accepted advice of Lynch and Milligan (1994), only reliable bands with a frequency of the most common allele not higher than 0.95 were considered for analysis. Each item of data was investigated for non-random association between individual pairs of bands using the correlation test of the Simint program of NTSYS.

Genetic variation was estimated using the PopGen 32 software package (Yeh and Yang 2000) and the following indices were calculated: mean number of alleles per locus, effective number of alleles per locus, percentage of polymorphic loci and Nei's (1973) gene diversity (H_e). The latter was adopted assuming the populations to be in Hardy-Weinberg equilibrium, although we were not able to investigate this since dominant markers were used. Genetic diversity was also measured using the Jaccard similarity coefficient (Jaccard 1908; using SPSS) and Shannon's diversity index ($H_o = -\Sigma p_i \ln p_i$, where p_i is the frequency of a given RAPD fragment; Lewontin 1972). The Pearson correlation analysis was carried out to detect possible relationships between

the three different methods used (SPSS 2003). A non-parametric test (Mann-Whitney) was performed to detect differences in genetic diversity between sub-species.

The Shannon index was used to quantify the degree of within-population diversity (H_o), the average diversity over all populations (H_{pop}) and the diversity of all populations considered together (H_{sp}). Consequently it was possible to estimate the proportion of diversity within (H_{pop}/H_{sp}) and between populations [$(H_{sp} - H_{pop})/H_{sp}$)]. Total diversity, and partitioning of the total variation, were also estimated through Nei's diversity statistics (Nei, 1973; 1987), and analysis of molecular variance (AMOVA) using Arlequin software (Schneider et al. 2000), in order to properly compare our data with that of other studies. AMOVA analysis was computed from a matrix of pairwise distances between individuals using Euclidean distances measured by NTSYS. The genetic differentiation was measured as θ_{st}, according to Weir and Cockerham (1984). The variance components were tested statistically by non-parametric randomisation tests using 1 000 permutations. Genetic differentiation between populations was further estimated using a pairwise F_{ST} values matrix. The null distribution of pairwise F_{ST} values on the hypothesis of no differences between the populations was tested by a permutation test with 10 000 replicates.

Cluster analysis was performed on the values of the Euclidean distances matrix using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) with Sahn program of NTSYS. The cophenetic values matrix (Coph program, NTSYS) was produced from tree matrix obtained from Sahn program. The cophenetic matrix was used to validate the cluster analysis by comparing it to the genetic distances matrix (Mxcomp program, NTSYS). In addition, Principal Coordinates (PCOs) were extracted from the Euclidean distance matrix and projected in three dimensions (programs Dcenter, Eigen and Mxplot of NTSYS).

Mantel tests (Mantel 1967) were performed to correlate the matrix of taxonomic distances (Sneath and Sokal 1973) for all pairs of populations with those based on molecular data and geographical distances. Taxonomic distances were derived from morphological traits (program Simint of NTSYS). The significance of each matrix correlation was evaluated by comparing the observed Mantel test statistic, Z, with its random distribution obtained after 1000 permutations (Program Mxcomp of NTSYS).

3 Results

3.1 Morphological Data Analysis

The morphological results for cones are given in Table 3. In all populations there is variation for at least one of the two traits. The highest level of variation is in population 1, where frequencies are balanced for both traits. High levels of cone asymmetry were scored in all erect populations as well as in shrub populations nos. 3 and 6, and in the intermediate population no. 11. The highest frequencies of flat apophysis were observed in shrub populations, with the exception of nos. 1, 3 and both the intermediate populations. Chi-square analysis showed significant differences for both traits among subspecies ($\chi^2 = 47.51$, $p < 0.001$ for cone symmetry and $\chi^2 = 9.82$, $p < 0.01$ for apophysis shape). PCA analysis confirmed these data (Fig. 4), since the first principal component (cone symmetry) accounted for 69.04% of the total variance and clearly grouped erect populations, while shrub and intermediate populations were plotted as a broad scatter. Furthermore, it was possible to detect a spatial distribution of populations on the basis of their geographical location: all populations from the eastern Alps (nos. 13, 14 and 15) and most of those from the Cottian Alps (nos. 4, 5, 7, 8 and 9) clustered together. The second principal component (apophysis shape) accounted for 30.96% of the total variation and there was no detectable clear clustering of populations according to their growth form.

3.2 Variation of RAPD Loci

Ten primers were used in the analysis, after the exclusion of OPA05 and OPA15. They gave 81 consistent and differential amplification products. Each primer amplified between 5 and 14 fragments, with an average of 8.1, and among

Table 3. Morphological traits scored in populations of *Pinus mugo* ((s) = stands established by shrub plants; (t) = stands with erect individuals; (i) = stands with intermediate individuals).

Population	Female cone		Cone apophysis shape	
	Symmetrical (%)	Asymmetrical (%)	Flat (%)	Hook (%)
1. Passo del Duca (s)	57.1	42.9	31.4	68.6
2. Gola della Chiusetta (t)	21.7	78.3	0.0	100.0
3. Gola della Chiusetta (s)	38.9	61.1	5.6	94.4
4. Laval (t)	0.0	100.0	18.4	81.6
5. Seguret-Pramand (t)	0.0	100.0	33.3	66.7
6. Seguret-Pramand (s)	2.7	97.3	89.2	10.8
7. Sette Fontane (t)	1.9	98.1	7.5	92.5
8. Ruilles (t)	0.0	100.0	16.7	83.3
9. Nevache (t)	2.2	97.8	26.1	73.9
10. Colle Lombardo (i)	96.0	4.0	8.0	92.0
11. Lago di Camplicciolì (i)	0.0	100.0	37.5	62.5
12. Monte Avic (t)	31.0	69.0	0.0	100.0
13. Paganella (s)	93.1	6.9	51.7	48.3
14. Cima Dodici (s)	100.0	0.0	79.3	20.7
15. Val Venegia (s)	94.4	5.6	100.0	0.0

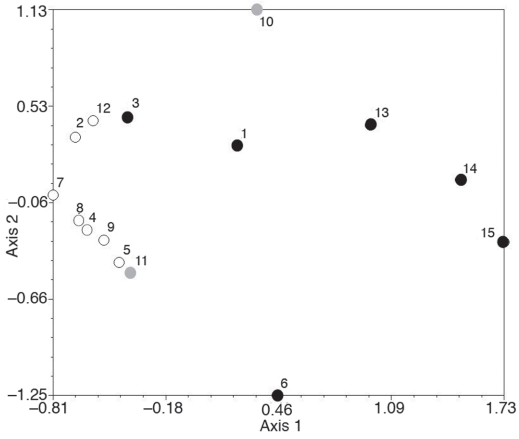

Fig. 4. PCA plot based on morphological characters with the first two principal components showing relationships among the 15 populations of *Pinus mugo*. Clear circles represent stands established by erect individuals, black circles stands with shrub plants and grey circles stands with intermediate individuals.

them 64 bands (about 79%) were polymorphic (Table 2). One of the markers generated by primer OPA07 (about 1550 bp) was fixed in all shrub populations and in two erect populations (Ruilles and Monte Avic): the allele – was therefore only scored in erect and intermediate populations, with a frequency ranging from 0.236 (Lago di Camplicciolì) to 0.711 (Gola della Chiusetta). Apart from this fragment, it was not possible to score any other band unique to a specific growth form, including the intermediate one. No band pair showed any correlation with any other. Therefore, each of the 64 polymorphic bands was considered as an independent marker.

3.3 Genetic Variation within Populations

Within population variation was estimated using different methods (Table 4). Gene diversity (H_e) ranged from 0.227 to 0.397 with a mean of 0.321; Jaccard's similarity coefficient (JSC) ranged from 0.606 to 0.690, with a mean of 0.645; the Shannon index (H_o) ranged from 0.325 to 0.569, with a mean of 0.460. Pearson correlations were calculated between estimations derived by these three parameters. H_e was strongly and significantly correlated with H_o ($r = 0.999$, $P < 0.001$), while JSC showed a significant and negative correlation with both H_e ($r = –0.732$, $P < 0.01$) and H_o ($r = –0.748$, $P < 0.01$). These negative correlations were expected, since JSC measures similarity while H_e and H_o estimate diversity. Looking across all the parameters, the overall lowest diversity was exhibited in populations 1 (Passo del Duca) and 8 (Ruilles), while the highest was found in population 4 (Laval). The mean values of genetic

Table 4. Genetic variation parameters, Nei's genetic diversity, Jaccard's similarity coefficient and the Shannon index scored in the populations analysed. Comparisons of mean values of allelic variation and genetic diversity indices between populations, grouped according to growth form, is also given. N = mean number of alleles per locus, N_e = effective number of alleles per locus, P = percentage of polymorphic loci, H_e = gene diversity, JSC = mean Jaccard's similarity coefficient, H_o = the Shannon index over loci. (s) = shrub; (t) = erect; (i) = intermediate growth.

Population	N	N_e	P	H_e	JSC	H_o
1. Passo del Duca (s)	1.56	1.41	56.3	0.227	0.680	0.328
2. Gola della Chiusetta (t)	1.72	1.51	71.9	0.285	0.615	0.415
3. Gola della Chiusetta (s)	1.66	1.53	65.6	0.287	0.690	0.411
4. Laval (t)	1.91	1.73	90.6	0.397	0.606	0.569
5. Seguret-Pramand (t)	1.78	1.63	78.1	0.341	0.654	0.489
6. Seguret-Pramand (s)	1.75	1.61	75.0	0.333	0.644	0.476
7. Sette Fontane (t)	1.83	1.66	82.8	0.359	0.636	0.514
8. Ruilles (t)	1.53	1.42	53.1	0.227	0.669	0.325
9. Nevache (t)	1.78	1.65	78.1	0.353	0.646	0.503
10. Colle Lombardo (i)	1.63	1.53	62.5	0.284	0.668	0.404
11. Lago di Camplicciolli (i)	1.75	1.55	75.0	0.306	0.654	0.444
12. Monte Avic (t)	1.88	1.65	87.5	0.366	0.618	0.531
13. Paganella (s)	1.72	1.59	71.9	0.317	0.647	0.452
14. Cima Dodici (s)	1.78	1.63	78.1	0.346	0.626	0.495
15. Val Venegia (s)	1.81	1.72	81.3	0.381	0.626	0.538
General mean	1.74	1.59	73.9	0.321	0.645	0.460
Shrub and intermediate	1.71	1.57	70.7	0.310	0.654	0.444
Tree	1.78	1.61	77.4	0.333	0.635	0.478
U of Mann-Whitney	15.5	18.5	15.5	18.5	15.5	18.0
Significance (P)	0.146	0.270	0.146	0.271	0.147	0.247

variation in erect populations were slightly higher than in shrub and intermediate stands. However, according to the Mann-Whitney U-test, these differences were not statistically significant.

3.4 Partitioning of Variation and Genetic Divergence among Populations

The Shannon index calculated from the relation between H_{sp} (0.598) and H_{pop} (0.460) allocated 23.1% of the total variation between populations rather than within them. The RAPD marker generated by primers OPA04 and OPA07 made a relatively high contribution to the total variation, while high between populations variation was indicated by primers OPA01, OPA02, OPA07 and OPA10. Nei's G_{st} was in accordance with the results from the Shannon index, attributing 22.5% of the variation between populations, with the total gene diversity of 0.413, being comprised of 0.321 within populations and 0.092 among populations. Hierarchical analysis of phenotypic diversity was performed to investigate the partitioning of the RAPD variation within and between populations, using the AMOVA procedure. Significant divergence among populations ($\theta_{st} = 16.61$, $P < 0.001$) was obtained, suggesting high phenotypic differentiation. The presence of genetic divergence between populations was also confirmed through calculation of all pairwise F_{ST} values, most of which were significantly different from zero (data not shown). The populations were then joined in two groups for further dissection, according to the growth form of the trees. The results showed that a little and non-significant amount of genetic diversity (1.25%) was attributable to the variance between groups, 15.36% being partitioned between populations within groups while 83.39% of the diversity occurred within populations (Table 5).

Table 5. Analysis of molecular variance (AMOVA) within and among the populations studied.

Source of variation	df	Sum of squares	Variance components	Percentage of variation	p
Among groups	1	71.204	0.132	1.25	0.076
Among populations within group	13	618.673	1.617	15.36	< 0.001
Within populations	345	3029.667	8.782	83.39	< 0.001

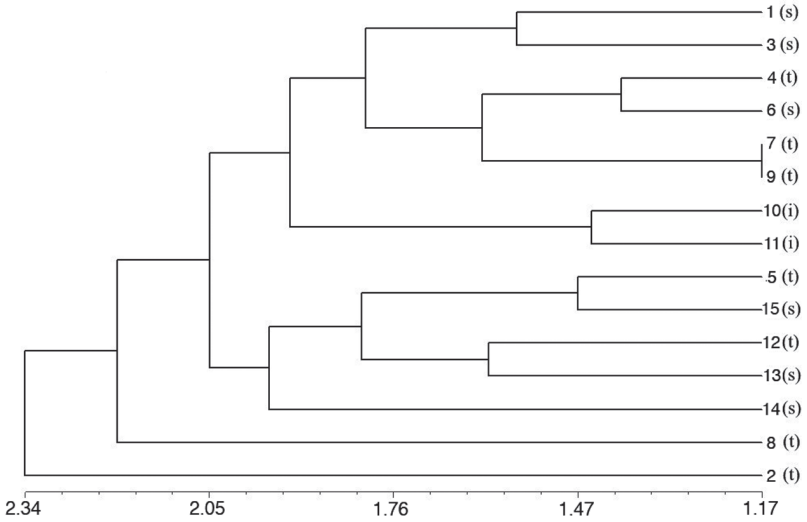

Fig. 5. Dendrogram based on UPGMA of RAPDs profiles, showing relationships among the 15 populations of *Pinus mugo* considered in the study. (s) = stands established by shrub plants; (t) = stands with erect individuals; (i) = stands with intermediate individuals.

3.5 Cluster Analysis

The UPGMA tree, based on Euclidean distances between all pairwise combinations of the populations calculated from the 64 polymorphic markers, did not reveal any clear clustering (Fig. 5) for growth form of the plants or the geographical localisation of the stands. However, it was possible to observe some partial clusterings of populations with the same growth form: i.e. shrub populations nos. 1 and 3, intermediate populations nos. 10 and 11 and erect populations nos. 7 and 9. The locations where both the growth forms were present (Gola della Chiusetta, populations nos. 2 and 3, and Seguret-Pramand, populations nos. 5 and 6) showed a high differentiation between subspecies. On the contrary, in some cases it was possible to score the extent of a correlation between geographical and molecular distances (i.e. populations nos. 1 and 3 as well as 7 and 9). Goodness-of-fit analysis suggested that UPGMA tree is moderately reliable. The Mantel test comparing Euclidean values matrix and cophenetic matrix gave a value of $r = 0.762$ ($P < 0.001$).

3.6 Principal Coordinate Analysis

Calculated pairwise Euclidean distances were used as an input for PCoA. The first three principal coordinate axes accounted for 19.82%, 15.84% and 13.61% of the total variation (cumulative value 49.27%) (Fig. 6). Considering the three principal coordinates, populations plotted as a broad scatter, and clustered independently of each other. However, a low degree of clustering according to the growth form was detected in the case of intermediate populations (nos. 10 and 11). Likewise, some of the populations from Cottian Alps (nos. 4, 6, 7 and 9) showed a certain degree

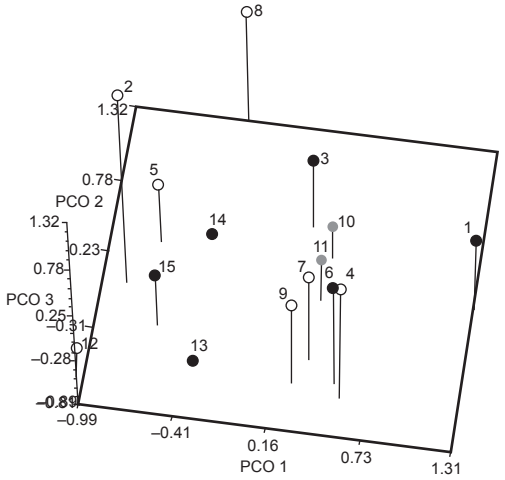

Fig. 6. The plot of principal coordinates analysis obtained from the Euclidean genetic distance matrix among 15 populations of *Pinus mugo*. Clear circles represent stands established by erect individuals, black circles stands with shrub plants and grey circles stands with intermediate individuals.

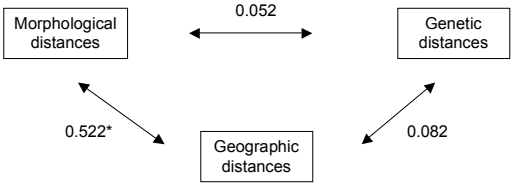

Fig. 7. Mantel tests for populations of *Pinus mugo*. Data show the Pearson coefficient values (r) and tests significance (* = P < 0.01) comparing pairwise matrices of genetic, morphological and spatial distances.

of genetic similarity. Adjacent stands characterised by different growth form (nos. 2 and 3, 5 and 6) were quite distant on all the axes considered.

3.7 Mantel's Z statistic

Matrices of morphological, genetic and geographical distances were compared pairwise to investigate any possible associations. The results of the tests (Fig. 7) showed a moderate but significant correlation (r = 0.522, P = 0.003) only between geographical and morphological distances, while genetic distances showed no correlations with either of the other two.

4 Discussion

We investigated the extent of genetic diversity between the erect *Pinus mugo* subsp. *uncinata* and the shrub *Pinus mugo* subsp. *mugo*, analysing variation for two morphological traits and 64 RAPD markers. Fifteen populations from the Alps were considered, 6 comprising shrub plants, 7 made up of erect plants and two of plants exhibiting an intermediate habit. However, the hybrid origin of these intermediaries is strongly questioned due to the lack of segregant plants. Moreover, intermediate plants were not observed at Gola della Chiusetta and Seguret-Pramand, where both subspecies are present in adjacent although non-overlapping stands.

Morphological traits of the female cones confirmed their partial reliability for distinguishing the subspecies *mugo* and *uncinata*. In fact none of the populations studied exhibited all the traits according to expectations. Erect populations showed a better fit to expectation (on average 95.1% of asymmetrical cones and 84.4% of hook-shaped apophysis) as compared to the shrub ones (60.6% of symmetrical cones and 52.4% of flat apophysis). Intermediate growth populations exhibited peculiar characteristics, with population no. 11 (Lago di Camplicciolì) being similar to subspecies *uncinata* and population no. 10 (Colle Lombardo) having most of its cones with contrasting traits (symmetry and hook-shaped apophysis). Therefore, the morphological analysis of cones proved to be unsuitable for detecting genetic relationship between the intermediate forms and the others. Cone symmetry exhibited a prominent discriminant capacity to distinguish between subspecies, as confirmed by PCA analysis. In conclusion, the two traits analysed proved to be useful to discriminate subspecies, although their efficacy should not be overestimated. Their variability can be high within a population, and sometimes even between cones produced by the same plant (Holubičková 1965). Similar conclusions were reached by other Authors (Cantagrel

1983, Sandoz 1987) analysing different morphological traits, such as cone size. According to Sandoz (1987), morphoanatomical characters that best differentiate subspecies are thickness and size of leaves, and length and number of endodermic cells. However, these features cannot be accurately evaluated directly in a stand, so that growth habit remains the most helpful discriminant character for practical purposes.

Diversity values detected by molecular data for each population, over all loci, indicated a large amount of genetic variation. Pines are considered to be one of the most genetically variable of plant genera, as first revealed by measures of quantitative genetic variation (Cornelius 1994) and by diversity of allozyme loci (e.g. Hamrick et al. 1979, Hamrick and Godt 1990). Lewandowski et al. (2002) confirmed high diversity values, analysing variation at 15 allozyme loci in some native Polish populations of three closely related pine species (*P. uliginosa*, *P. sylvestris* and *P. mugo*). In a study involving 17 populations of *P. mugo* from Bulgaria, gene diversity estimated by allozyme variation was similar to levels reported for several populations of the same species in central and western Europe, but lower than those scored for European pines with large continuous ranges (Slavov and Zhelev 2004). The high level of genetic diversity found in this study can at least be partially explained by the fact that we sampled populations within the main area of diffusion of the species. In fact, it is believed that *Pinus mugo* differentiated during the early Pliocene, when the climate conditions and the snow coverage were much more severe than nowadays (Sandoz 1987). Populations from Bulgaria are located at the south-eastern margin of the species range and a reduced diversity can therefore be expected, as a result of both higher selection pressure and genetic drift due to the smallness of populations and to their fragmentation.

The DNA based markers have revealed high H_e estimates on species belonging to the *Pinus* genus. A level of RAPD variation similar to the one scored in this study was observed in a sample of 210 bristlecone pines (*Pinus longaeva*) from three groves in the White Mountains, California, USA (Lee et al. 2002). There, the mean number of alleles per locus (N) was 1.90, and 84.3% of loci were polymorphic and the expected heterozygosity was 0.321 (in this study the corresponding values are 1.74, 63.9% and 0.321).

In our work, values of within-population diversity were quite different among the 15 populations studied, although no differences were found between mean values of subspecies. All the genetic variation indicators used indicate that populations 4 (Laval, erect), 12 (Monte Avic, erect) and 15 (Val Venegia, shrub) are somewhat more variable, while populations 1 (Passo del Duca, shrub) and 8 (Ruilles, erect) are more homogeneous. We obtained the expected correlations between the indicators used. In particular, estimates of Nei's gene diversity (H_e) and the Shannon index (H_o) exhibited a high and positive correlation ($r = 0.999$, $P < 0.001$).

The Shannon index and Nei's genetic diversity attributed most of the total variation within populations (respectively 76.9% and 77.5%) rather than between them. This pattern of genetic structure is common in conifers with wide distribution ranges. Moreover, moderate genetic variation between populations is expected in a species characterised by early successional status, perennial life form, an outcrossing breeding system and pollen and seeds dispersal by wind or animals, as is the case of *Pinus mugo*. In particular, pollen-mediated gene flow among close populations is highly effective for contrasting population differentiation (Koski 1970, Ledig 1998). The homogenising effect of gene flow can be detected by the distribution of allele frequencies, the high estimate of migration and the low level of population differentiation (Muona and Harju 1989, Beaulieu and Simon 1994, Goncharenko et al. 1994). Between-population differentiation of *P. mugo* estimated in this study is in accordance with the overall RAPD data available in the literature. For instance, Nybom and Bartish (2000) found a mean value of θ_{st} of 0.28 for 46 outcrossing species. Furthermore, the average estimate of genetic differentiation among populations of several pine species based on RAPDs was 0.27 (Lee et al. 2002). The AMOVA procedure, used as an alternative approach to study the relationships between shrub and tree populations, showed a difference in partitioning. In fact, most of genetic variation was still found within populations, but the phenotypic structure of the populations accounted for a slightly lower value ($\theta_{st} = 16.61$, $P < 0.001$).

The phenotypic diversity was mostly distributed between populations (15.36%, $P < 0.001$), with the differentiation between groups being very low and not significant (1.25%, $P = 0.076$). This suggests a substantial gene flow between shrub and tree populations and confirms the close relation between the two *Pinus mugo* subspecies that we investigated. The absence of differentiation among taxa is consistent with the hypothesis of a recent fragmentation of a historically larger population which occurred in the late Tertiary and during the Quaternary interglacial periods (Sandoz 1987). It is known that species are negatively affected by habitat fragmentation (Van Rossum et al. 2004): it increases the isolation of populations and reduces gene flow between them (Slatkin 1995). However, populations of the most widespread forests are characterised by a long life span, and only a relatively few generations have occurred since the end of the last glacial period to enable us to detect changes in their genetic structure.

The lack of separation in the dendrogram reflects a weak genetic differentiation among subspecies, corroborating the results from the analysis of molecular variance. Our cluster analysis did not show a relationship among shrub populations closer than among any other populations. Thus, the different populations of both the *Pinus mugo* subspecies were intermingled in different parts of the UPGMA and PCO analysis. However, it was possible to score relationships between some populations on the basis of the growth form and spatial distribution. In any case, genetic differentiation between populations was independent of geographical and morphological distances, so that the isolation by distance analysis resulted in no significance. The only significant correlation was found among morphological and geographical distances, revealing that spatially closer populations tended to be more morphologically similar. These data appear to be in contrast with the results of Lewandowski et al. (2000), who found a mean genetic distance between *P. uncinata* and *P. mugo* as high as 0.073, much higher than the values estimated within taxa (respectively 0.006 and 0.044). In our study the mean genetic distance between taxa was as low as 0.027 and the value of estimates within subspecies were 0.148 and 0.161 respectively for *P. m. uncinata* and *P. m. mugo*. These data are in accordance with assertions of Crawford (1989) who stated that the mean identity for populations belonging to the same taxon is often above 0.90. However, it must be noted that the study by Lewandowski et al. considered only two populations per taxa, sampled in locations geographically very distant (Spain in case of *P. uncinata* and Ukraine for *P. mugo*). Furthermore, those authors used a different kind of marker system (isozyme), and in a lower number (13 polymorphic loci).

On the basis of our results, it is possible to state that the variation in RAPDs is barely effective in detecting genetic divergence between the subspecies *uncinata* and *mugo* of *Pinus mugo*. The use of RAPD markers to estimate genetic parameters in outcrossing populations has been questioned: RAPDs are dominant markers and therefore the estimation of allele frequencies is less accurate than for codominant markers. In particular, null alleles present only in heterozygous genotypes cannot be detected, thus underestimating gene diversity. These loci, in fact, appear as monomorphic and, according to the Lynch and Milligan (1994) correction, are excluded from data processing. However, the exclusion of such loci could lead to an underestimation of genetic differentiation among populations, in cases where these are characterised by different frequencies of null alleles. On the other hand, RAPDs amplified mostly non-coding DNA sequences, which are subject to weaker selection pressures, so allowing the scoring of a higher amount of genetic variation. It is possible that the use of other molecular markers, such as the codominant microsatellites, could be more efficient in analysing intraspecific genetic variation, and in showing a better correlation with morphological variation.

This research has provided some insight into the genetic structure and variation of *Pinus mugo uncinata* and *P. m. mugo* populations, which could be useful for planning stronger conservation strategies. We found a high level of population differentiation, but a low level of genetic diversity between subspecies, therefore to preserve genetic diversity more reservoirs should be established in order to protect as many stands as possible. However, it must be stressed once again that RAPD variation may not necessarily reflect the true pattern of variation in adaptative genes, so that further studies are needed to improve our

knowledge base on the relationship between genetic and adaptative data.

In conclusion our results seem to be in agreement with the hypothesis of Christensen, concerning the existence of only one species, subdivided into two sub-species on the basis of growth form. In fact, neither morphological traits, nor RAPD markers were able to detect a clear differentiation among taxa, suggesting the sharing of the same gene pool, probably due to a recent fragmentation of the species distribution area. Further studies are needed, however, mainly to better clarify the origin and the position of the intermediate forms as well as to investigate the possible effect of soil type on phenotype.

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