GENETIC VARIATION IN *ABIES NEBRODENSIS*: A CASE STUDY FOR A HIGHLY ENDANGERED SPECIES

Giovanni G. Vendramin, Marco Michelozzi, Lorenzo Lelli & Roberto Tognetti

Istituto Miglioramento Genetico Piante Forestali, CNR, Via Atto Vannucci 13, I–50134 Firenze, Italy

Received March 5, 1996; accepted March 23, 1996

ABSTRACT

Allozyme, DNA markers, sequence data and monoterpenes have been used to estimate genetic and taxonomic relationships among different populations of *Abies alba* Mill. and the unique population of *Abies nebrodensis* (Lojac.) Mattei, one of the most relevant example of relic species in the world. High level of differentiation among populations were found using all the different approaches. The genetic distance between *A. nebrodensis* and the group of *A. alba* populations was much greater than that among *A. alba* populations located more than 1,000 km apart. The results provide support for the classification of *A. nebrodensis* and *A. alba* into distinct taxonomic groups.

Key words: genetic variation, allozymes, DNA markers, monoterpenes, *Abies nebrodensis*

INTRODUCTION

The importance of the conservation of the genetic resources of forest tree species has been recently emphasised during many international congresses (*e.g.*, BARADAT *et al.*, 1995). The conservation of the genetic variability represents a basic strategy for the conservation of high adaptive potential of the populations, which plays an essential role in the stability of the forest ecosystem. The forest tree species are generally characterised by longevity and therefore more subject to the effects of changing environmental conditions. The genetic structure and the genetic systems are important aspects which confer adaptive potential of a population and therefore their analysis is a fundamental prerequisite for establishing appropriate strategies for the conservation of adaptability. This aspect assumes more relevance in the case of relic and highly endangered species, whose genetic resources must be urgently analysed and then conserved. *Abies nebrodensis* (Lojac.) Mattei probably represents the most relevant and evident example of relic and highly endangered species in the world. Its actual natural range is in fact reduced to a single and small population located in the Madonie mountain, in Sicily (Italy); within the population only 29 adult trees are considered to belong to this species (MORANDINI *et al.*, 1994) (Fig. 1). Many factors may have contributed to the restrictedness of the actual range of distribution of this species: among these the most relevant seems to have been the strong antropogenic pressure. *Abies nebrodensis* wood was widely used in this region between the XVII and XVIII century, for the production of roof beams, particularly of churches, and for the domestic use of the local population. Several hypotheses were developed for explaining the origin and the phylogenetic relationships between *A. nebrodensis* and *A. alba* Mill.. While some Authors (FENAROLI & GIACOMINI, 1958; MORANDINI, 1968) considered *A. nebrodensis* to have originated from the southern populations of silver fir (*Abies alba* Mill.), as a consequence of isolation during the past-glacial period, other evidence, such as the presence of typical Mediterranean flora in its natural range, seems to indicate a possible independent origin of this species in respect to *A. alba*.

The main objective of different researches performed in our laboratory was the study of the genetic relationships among the unique population of *A. nebrodensis* and some silver fir populations sampled in the Italian natural range along a north-south geographic gradient. To get as much information as possible about the genetic resources and origin of this highly endangered species, allozyme and DNA markers, namely chloroplast and RAPD markers, as well as terpenes composition were analysed.

MATERIALS AND METHODS

Buds, needles and cortical tissues were collected from 14 trees of the natural population of *A. nebrodensis* and from about 30 trees of seven natural populations of *A. alba*, located in the Italian natural range and sampled along a north-south gradient (Fig.2).

Figure 1 An exemplar of *Abies nebrodensis* in its natural range

Figure 2 Geographic location of the *Abies nebrodensis* and *Abies alba*: 1– Madonie (*A. nebrodensis);* 2 – Aspremonte; 3 – Serra San Bruno; 6 – Gariglione; 5 – Abeti Spoprani; 6 – Collemeluccio; 7 – Abetone; 8 – Lavarone

To assess the entity and distribution of the genetic variation between and among populations, different approaches were performed:

a) *Allozyme analysis.* Eight enzyme systems (Glutamate dehydrogenase, *GDH*, glutamate oxaloacetate transaminase, *GOT*, isocitrate dehydrogenase, *IDH*, malate dehydrogenase, *MDH*, malic enzyme, *ME*, 6-phosphogluconate dehydrogenase, *6-PGD*, phosphoglucose isomerase, *PGI*, shikimate dehydrogenase, *SKD*), encoded by 12 gene loci, were analysed by means of starch gel electrophoresis. Genetic variability (mean number of alleles per locus, *n*, mean effective number of alleles per locus, n_e, and observed (H_o) and expected (H_e) heterozygosity) and differentiation parameters (G_{st} and genetic distances as proposed by NEI (1972, 1975) were estimated using BIOSYS program (SWOFFORD & SELANDER 1981).

b) *RAPD analysis.* 12 decamer oligonucleotide primers, selected, among 60 decamers tested, because they gave good amplification in terms of reproducibility and clearness of the amplification patterns, were used for the amplification of genomic DNA extracted from needles following a modification of the protocols proposed by ZIEGENHAGEN *et al.* (1993). Details on the RAPD amplifications are reported in VICARIO *et al.* (1995). RAPD data was used firstly for the estimation of genetic distances among populations following the approach reported by YU and PAULS (1993) and then for assessing what fraction of variability resides within populations and what fraction among populations following the AMOVA approach proposed by HUFF *et al.* (1993).

c) *PCR/RFLP of chloroplast non coding regions.* Two pairs of 20-mer primers were used for the amplification of two chloroplast intergenic spacers between tRNA genes. The amplification products were then cut with 11 restriction endonucleases. Details on amplification and restriction procedures are given in Vicario *et al.* (1995). The amplified intergenic spacer between the *trnL* and *trnF* genes of *Abies alba*, *A. nebrodensis* and one American fir species, *A. magnifica*, was then cloned into a plasmid vector and then sequenced, using an ALF automated sequencer (Pharmacia), on both strands following the dideoxy chain termination method with T7 polymerase sequencing kit and deaza G sequencing mixes. Alignment of the sequences was performed using MACAW software (SCHULER *et al.*, 1991).

d) *Monoterpene composition.* Monoterpene composition was determined in cortical tissue by means of headspace gas chromatography. Peak area on the chromatograms was expressed as a percentage of the total monoterpenes taken into consideration (α-pinene,

camphene, β-pinene, sabinene, β-myrcene, limonene, cineole). Variance and discriminant analyses were performed on the arcsin transformed monoterpene percentages.

RESULTS AND DISCUSSION

Evident differences between *A. nebrodensis* and *A. alba* were found using allozyme, RAPD and monoterpene composition approaches. The allozyme analysis revealed that *A. nebrodensis* population can be distinguished quite clearly from *A. alba* populations. The level of genetic differentiation, obtained estimating Nei's genetic distances, among *A. nebrodensis* and *A. alba* populations was about 10–70 times greater than among *A. alba* populations. The mean level of genetic differentiation among populations estimated using G_{st} was about 11%, thus indicating a high degree of divergence among the populations. The highest level of G_{st} were found at two enzyme loci, posphogluconate isomerase (*Pgi–A*, 45%) and shikimate dehydrogenase (*Skd–A*, 37.5%), where the differences in the allele frequencies among *A. nebrodensis* and *A. alba* are evident: the main part of the G_{st} is therefore due to the differentiation among the two species. The high level of divergence is also confirmed by the genetic distance values estimated using RAPD data. The genetic distance between *A. nebrodensis* and *A. alba* is much greater than those observed among *A. alba* populations, even in the case of populations located more than 1,000 km apart. The apportion of RAPD genetic variation among individuals within populations and among populations, obtained following the AMOVA approach, indicated that 16% of the total variation was attributable to population differences. Both G_{st} and AMOVA analysis demonstrated therefore high interpopulational genetic variation; the G_{st} value obtained is about two times greater than the average reported for wind-pollinated conifers (6.8%, HAMRICK & GODT 1989). It is surprising to note the high value of heterozygosity, estimated using isozyme data, found in the *A. nebrodensis* population, higher than those observed in the *A. alba* populations (Fig 3). This result was unexpected considering the characteristics of this population, namely its restricted and scattered distribution, probably due to a genetic bottleneck experienced in the past. The drastic reduction in the population size, well documented by RAIMONDO *et al.* (1990), should have lead to lower values of heterozygosity as a consequence of genetic drift. Selection favouring heterozygotes in the *A. nebrodensis* population seems to be an acceptable working hypothesis. Accumulation of genetic variation within individuals could be a very useful strategy of survival.

No differences in size and at the 21 restriction sites of the non-coding chloroplast DNA regions were found among all the individuals of the *A. nebrodensis* and *A. alba* populations. This approach, which showed its efficiency for the classification of *Pinus* species (BOSCHERINI *et al.* 1994), seems not to be highly informative within the genus *Abies*. Analysing the sequence of a portion (about 200 bp) of the intergenic spacer between the *trnL 3' exon* and the *trnF* gene, *A. alba* and *A. nebrodensis* differ by 4 insertions/deletions and only one substitution (transversion), as well as *A. alba* and *A. magnifica*. *A. nebrodensis* and *A. magnifica* differ by 6 insertions/deletions and 3 substitutions (all transversions). Taking into consideration only substitu-

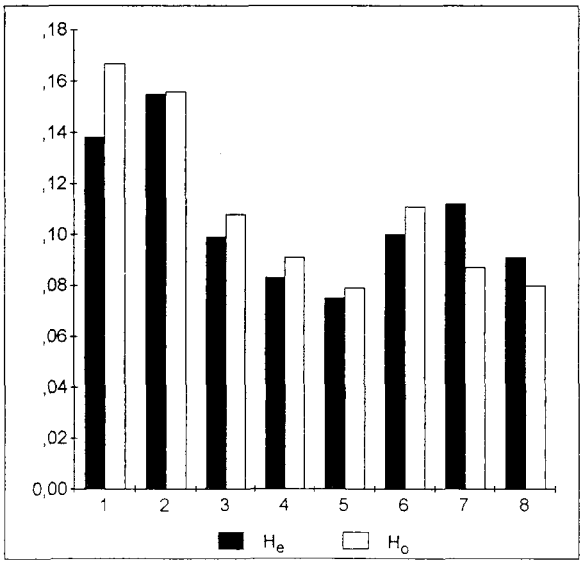

Figure 3 Mean expected (H_e) and observed (H_o) heterozygosity in the 8 sampled populations. Population numbers as described in Fig, 2

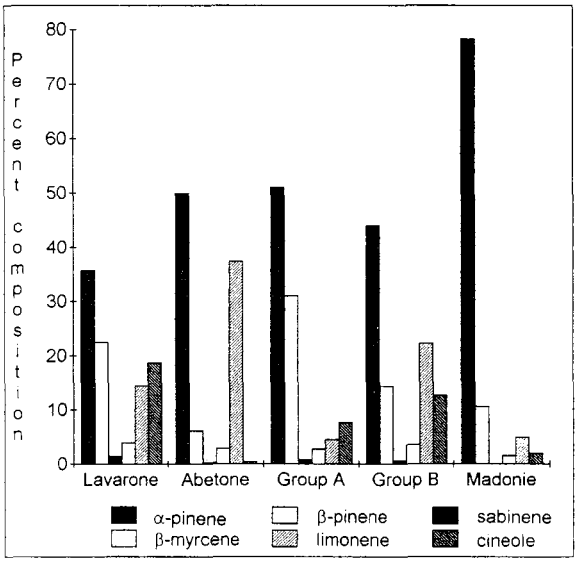

Figure 4 Monoterpene composition in the considered populations

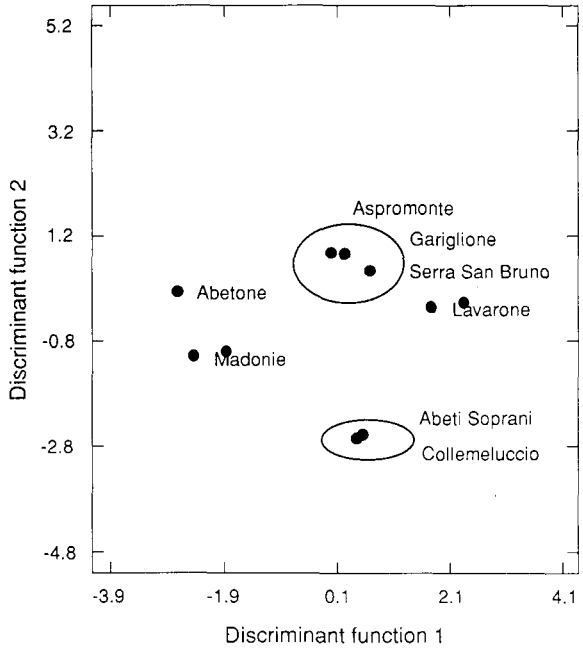

Figure 5 Scatterplot of populations in the plane of the first two discriminant functions based on monoterpene data

tions a sequence divergence of 0.5%, 0.5% and 1.5% was obtained for the three pairs of *Abies* species, respectively. The sequencing of the intergenic spacer of chloroplast DNA appears to be very useful for phylogenetic studies. It allows to distinguish two closely related species like *A. alba* and *A. nebrodensis*, that showed identical restriction patterns (VICARIO *et al.* 1995). These results confirmed the presence of a high level of genetic differentiation among populations of the two species, as already evidenced by isozyme and RAPD data (VICARIO *et al.* 1995).

The pattern of differentiation among populations of *A. alba* and the relic populations of *A. nebrodensis* displayed by terpene profiles is substantially in agreement with the results obtained using allozyme and DNA markers (VICARIO *et al.*, 1995). α-pinene, limonene, β-pinene and cineole were the monoterpenes which appeared in relatively large amount. The results of the analysis of variance showed highly significant differences among populations for all the monoterpenes except canphene. A clear discrimination ($p < 0.05$) among populations resulted applying the Duncan test, except Abeti Soprani and Collemeluccio, and Aspromonte and Serra San Bruno which did not differ significantly from one another in any monoterpene. Gariglione differed significantly from Serra San Bruno only in the amount of β-myrcene. These results are confirmed also by the discriminant analysis: in the plane defined by the projection of the first two discriminant functions (which explain 56% and 26% of the total variation, respectively) Abeti Soprani and Collemeluccio appear very close, resulting in a well defined group (group A), Aspromonte, Gariglione and Serra San Bruno are similar and can be aggregated in group B, while the other populations are well separated from each other (Fig. 4 and 5). These findings are in agreement with LANG's report (1994) which described an extensive study of the geographic variability of *A. alba* based on monoterpene patterns in the cortex resin.

In conclusion data obtained using different approaches clearly indicated a high degree of differentiation between *A. nebrodensis* and *A. alba* and seems to confirm the hypothesis for which the two groups of populations belong to two different taxonomic groups. The need to preserve this highly endangered species has been taken into account by the local administrators. The recent institution of two natural reserves on the Madonie Mountains, where the indigenous station of *A. nebrodensis* lies, aims to preserve this highly endangered species and the great naturalistic richness of this area and should be linked to the development of strategies for the *ex situ* preservation, mainly through the establishment of artificial stands outside the Madonie area and the storage of material as suggested by MAZZOLA *et al.* (1993). The most efficient *in situ* conservation approach could be the creation of new reproduction communities by adding genetically checked material around the solitary trees. In this context the results obtained in our studies may be very useful for determining criteria for genetically adequate strategies of gene conservation.

REFERENCES

BARADAT, PH., ADAMS, W. T. & MÜLLER-STARCK, G. 1995: Population genetics and genetic conservation of forest trees. SPB Academic Publishing, Amsterdam, The Netherlands.

BOSCHERINI, G., MORGANTE, M., ROSSI, P. & VENDRAMIN, G. G. 1994: Allozyme and chloroplast DNA variation in Italian and Greek population of *Pinus leucodermis*. *Heredity* **73**:284–290.

FENAROLI, L. & GIACOMINI, V. 1958: La flora. *In*: Giacomini V. (ed.), Conosci l'Italia. T.C.I. Milano, p. 214

HAMRICK, J. L. & GODT, M. J. W. 1989: Allozyme diversity in plant species. *In*: Brown, A. H. D., Clegg, M. T., Kahler, A. H., Weir, B. S. (eds.). Plant population genetics, breeding, and genetic resources. Sinauer Associates. Inc., Sunderland. MA.

HUFF, D. R., PEAKALL, R. & SMOUSE, P. E. 1993: RAPD variation within and among natural populations of outcrossing buffalograss (*Buchloe dactyloides* (Nutt.) Engelm.). *Theor. Appl. Genet.* **86**:927–934.

MAZZOLA, P., VENTURELLA, G. & SCHICCHI, R. 1993: Piano di gestione e strategie di conservazione *in situ* ed *ex situ* di *Abies nebrodensis* (Lojac.) Mattei. *Giorn. Bot. It.* **3**:584.

MORANDINI, R. 1968: Per la protezione dell'Abete in Sicilia. Sezione Fiorentina del C.A.I., Firenze (1868–1968), 411–414.

MORANDINI R., DUCCI, F. & MENGUZZATO, G. 1994: *Abies nebrodensis* (Lojac.) Mattei. Inventario 1992. *Ann. Ist. Sper. Selv.* **22**:5–51.

LANG, K. J. 1994: *Abies alba* Mill.: differentiation of provenances and provenance groups by the monoterpene patterns in the cortex resin of twigs. *Biochemical Systematics and Ecology* **22**:53–63.

NEI, M. 1972: Genetic distance between populations. *Am. Nature* **106**:282–292.

NEI, M. 1975: Molecular Population Genetics and Evolutions. North Holland Publishing, Amsterdam, Oxford.

RAIMONDO, F. M., VENTURELLA, G. & DI GANGI, F. 1990: Variazioni fenotipiche in *Abies nebrodensis* (Lojac.) Mattei e comportamento vegetativo nella sua discendenza. *Quad. Bot. Ambient. Appl.* **1**:183–210.

SWOFFORD, D. L. & SELANDER, R. B. 1981: BIOSYS –1, a computer program for the analysis of allelic variation in genetics. University of Illinois, Urbana, USA.

SCHULER, G. D., ALTSCHUL, S. F. & LIPMAN, D. J. 1991: A workbench for multiple alignment construction and analysis. *Proteins: Structure, Function and Genetics* **9**:180–190.

VICARIO, F., VENDRAMIN, G. G., ROSSI, P., LIÒ, P. & GIANNINI, R. 1995: Allozyme, chloroplast DNA and RAPD markers for determining genetic relationships between *Abies alba* and the relic population of *Abies nebrodensis*. *Theor. Appl. Genet.* **90**:1012–1018.

YU, K. & PAULS, K. P. 1983: Rapid estimation of genetic relatedness among heterogeneous population of alfaalfa by random amplification of bulked genomic DNA samples. *Theor. Appl. Gen.* **86**:788–794.

ZIEGENHAGEN, B., GUILLERMAUT, P. & SCHOLZ, F. 1993: A procedure for mini-preparations of genomic DNA from needles of silver fir (*Abies alba* Mill). *Plant Mol. Biol. Rep.* **10**:60–65.