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Genetics and Evolution of the Mediterranean *Abies* Species

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Abstract

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The main aim of the studies was to determine the amount and distribution of the genetic variability within and among Mediterranean taxa of *Abies*, at both the nuclear and chloroplast levels, in order to elucidate their origin and evolution and to shed light on the taxonomic position of *A. nebrodensis*.

In studies I, II and V allozyme markers were used to provide information on the level and distribution of genetic variation among and within natural populations of *A. alba, A. cephalonica, A. nebrodensis* and *A. numidica* and to estimate the outcrossing rate within *A. alba*. In studies III and IV, DNA markers from the chloroplast genome were developed and employed at the intra- and inter-specific levels to estimate the degree of cpDNA variation in the genus and to derive inferences concerning species relationships. Two different approaches were used: the first involved a comparative restriction-site analysis of ten different amplified chloroplast DNA fragments and the second involved the analysis of six chloroplast hypervariable repetitive simple-sequence repeats (cpSSRs or microsatellites).

Analyses of both allozyme and cpDNA data indicated that *A. nebrodensis* differs from the neighbouring *Abies* species and justified its classification as a separate species. Based on results from studies III, IV and V we suggested that *A. nebrodensis* originated during the Messinian crises of the latest part of the Miocene, through hybridisation between *A. alba* and *A. numidica*. During the warmer period of the Holocene *A. nebrodensis* became more isolated from both *A. numidica* and *A. alba*, promoting further divergence.

Results from studies III, IV and V agreed well with the hypothesis that the genus *Abies* had an Asiatic origin and then differentiated along an east-west axis (beginning in the Miocene) in the Balkan and Middle East regions. Taxa like *A. alba*, *A. bornmuelleriana*, *A. nordmanniana*, *A. cephalonica* and *A. cilicica* were the first to differentiate from the common *Abies* ancestor. *Abies pinsapo* and *A. numidica* appear to have differentiated much later, and to have remained isolated for a long time after their settlement in Spain and North Africa. The lower levels of polymorphism observed in these two species appear to be a consequence of genetic drift due to isolation and small population size.

Key words: *Abies*, allozyme, chloroplast DNA, mating system, variation, migration and evolution.

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- 6	Genetic structure and mating system of silver fir in Campolino reserve…	Genetic structure and mating system of silver fir in the Campolino reserve…
- 10, 4th par.	grow much large	grow much larger
- 15, 4th par.	…Sorensen & Adam, 1993).	…Sorensen & Adams, 1993).
- 17, 3rd par.	However, chloroplast genes, due to their uniparental mode…	Chloroplast genes, due to their uniparental mode…
- 18, 4th par.	out-crossing rate	outcrossing rate
- 21, 2nd par.	De to all these unique …	Due to all these unique…
- 22, 2nd par.	Unfortunately, due to their high mutation rates, reversion and the possibility of homoplasy…	Unfortunately, due to their high mutation rates, reversion and thus possibility of homoplasy…
- 29, 3rd par.	…and distribution of genetic variation in both animals and plants from…	…and distribution of genetic variation in the plants from…
- 40	Laura wishes to thanks:	Laura wishes to thank:
- Paper IV, p.2, 1st par.	…all European Abies species…	…all European *Abies* species…
- Paper V, p. 9, ref. 8	Genetic variation of *Abies alba* (Mill.) in Italy.	Genetic variation of *Abies alba* in Italy.
- Paper V, Table 2, Table 4	(WEIR & COCKERHAM) 1984) Unbiased genetic distanceS…	(WEIR & COCKERHAM 1984) Unbiased genetic distances…

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Contents

Appendix

This thesis is based on studies reported in the following papers, which will be referred to in the text by the corresponding Roman numerals.

I. Parducci L., Szmidt A. E., Villani F., Wang X-R. & Cherubini M. (1996) Genetic variation of *Abies alba* in Italy. *Hereditas* 125 (1): 11-18.

II. Giannini R., Parducci L., Rossi P. & Villani F. (1994) Genetic structure and mating system of silver fir in Campolino reserve (North Apennines, Italy). *Journal of Genetics and Breeding* 48: 335-338.

III. Parducci L. & Szmidt A. E. (1999) PCR-RFLP analysis of cpDNA in the genus *Abies*. *Theoretical and Applied Genetics* 98: 802-808.

IV. Parducci L., Szmidt A. E., Madaghiele A., Anzidei M. & Vendramin G. G. (2000) Genetic variation at chloroplast microsatellites (cpSSR) in *Abies nebrodensis* (Lojac.) Mattei and three neighbouring *Abies* species. *Theoretical and Applied Genetics*. (In press).

V. Parducci L., Szmidt A. E., Ribeiro M. M. & Drouzas A. D (2000) Allozyme variation in *Abies nebrodensis* (Lojac.) Mattei and three neighbouring *Abies* species. *Forest Genetics*. (Submitted).

Introduction

The genus *Abies* - Taxonomy and biology

The genus *Abies (Pinaceae* family) includes many forest tree species all native of the Northern Hemisphere. All the *Abies* species show a rich variability in their morphological characters. Miller first established the genus in 1754 as a separate entity within the *Pinaceae* family (Miller, 1754) and in 1842 Spach made the first attempt at a generic classification, dividing the genus into five sections. However, only two of Spach's sections contained species of *Abies* as presently circumscribed. Gordon & Glendinning (1858), Egelmann, (1878) and Mayr (1890) made the first classifications that dealt with only true *Abies* species, using morphological characters. Further classifications were later made by Sargent (1898), Kent, (1900), Hickel (1906), Patschke (1913), Franco (1950), Matzenko (1968), Liu (1971), Landry (1984) and Farjon & Rushforth (1989).

Usually, classification of species is a formalized activity in which taxonomists compare new species to other pre-established groups of species and explain how they could be distinguished, based mainly on phenetic (morphological) characters. In biology there are two different methods for classifying species into groups: the *phenetic* and the *phylogenetic* methods. In the *phenetic* method, species are grouped exclusively according to their *phenetic* attributes and nothing is said about their evolutionary and phylogenetic relationships, while the *phylogenetic* method ignores the *phenetic* relationships and classifies species according to how recently they shared a common ancestor. The phenetic and phylogenetic principles are two fundamental types of biological classification, but there are more than two schools of thought about how classification should be carried out. P*henetic classification* ignores evolutionary relationships and classifies species by their similarity in appearance; *cladism* ignores phenetic relationships and classifies species according to their common ancestry; *evolutionary taxonomy* includes both phenetic and phylogenetic relationships (Ridley, 1993).

According to Farjon & Rushforth (1989), who made the most recent classification of the genus *Abies*, the unrestrained use of geographical and ecological characters adopted in many of the previous classifications (*i.e.* Franco, 1950; Liu, 1971), often led to evolutionary assumptions hardly substantiated by the paleobotanical records available for the genus. Similarly, the use of a very limited range of morphological characters, such as those adopted in the classifications of Matzenko (1968) and Landry (1984), often resulted in the grouping of species in an artificial manner. In contrast, the classification made by Farjon & Rushforth (1989) although also based on morphological characters, considered the biology of the whole plant as the primary criterion for grouping species, resulting in a more natural and complete classification than any proposed before. The resulting system puts the *Abies* species with similar ecological

preferences together, and proposes a tentative scheme of relationships among the ten different sections.

The total number of *Abies* species recognized by Farjon & Rushforth (1989) is 49. They are divided into ten different sections further divided into subsections. All the *Abies* species are native to the Northern Hemisphere, where they are widely distributed over both eastern and western regions. The northern limit of distribution lies in the sub-arctic region at about 65° N latitude while the southern limit lies in the high mountains of the tropical regions at 14° N latitude. As a rule, *Abies* species prefer a humid, temperate to boreal climates, however, some species are well adapted to stand drier conditions. Nearly all species are important trees, being major components of the characteristic forests of high altitudes and boreal regions of the Northern Hemisphere. They occur in pure stands at elevations to which they are best adapted, or in mixed forests at lower or higher elevations in their native lands. Most of the species are allopatric, although sympatric zones exist in western North America, on the islands of Japan and to some extent in a few areas in the Mediterranean basin (Liu, 1971).

The Mediterranean species

A large number of *Abies* taxa naturally occur in the Mediterranean area, which is one of the distribution centres of the genus. This region includes not only the coastal areas, islands and peninsulas with high mountains in and around the Mediterranean Sea, but also the Alps, the Carpathians, the mountain ranges in southern Europe and the Caucasus Mountains. For convenience, also, the mountainous regions of Central Europe are included (Liu, 1971).

The total number of *Abies* taxa recognized in this region differs among taxonomists. According to Farjon & Rushforth (1989) there are seven species and one putative hybrid, belonging to two different sections. The section *Abies* includes: *A. alba* (Mill.), *A. cephalonica* (Laud.), *A. cilicica* (Ant. & Kotschy) Carriére, *A. nebrodensis* (Lojac.) Mattei, *A. nordmanniana* (Steven) Spach and the putative hybrid *A. borisii-regis* (Mattf.) Emend. Liu. The section *Piceaster* includes *A. numidica* (De Lann.) and *A. pinsapo* Boiss. Figure 1 shows the geographical distribution of the Mediterranean *Abies* taxa investigated in the present study.

Of the Mediterranean taxa, *A. nebrodensis*, *A. numidica* and *A. pinsapo* show very limited distribution ranges, restricted to the western area of the region, and it is likely that man has had a great influence on them. *Abies nebrodensis* is an extremely rare taxon, represented by just 29 adult individuals growing on the Madonie Range in Sicily (Italy) and very little is known about its origin. Although at first glance it can be easily confused with *A. alba*, it also shares morphological characters with the two neighbouring species, *A. cephalonica* and *A. numidica*.

Figure 1. Distribution of the Mediterranean *Abies* taxa investigated in the present study *A*
◆ *alba* ◆ *A. bornmuelleriana* ◇ *A. cephalonica* ◈ *A. cilicica* ◆ *A. nebrodensis*
◈ *A. nordmanniana* ◆ *A. numidica* ◈ *A. pinsapo* ◈ *A. pinsapo* var. *marocana*

However, several outstanding characteristics, like smaller size, glabrescent branchlets and stiff leaves that are not pectinate in arrangement, clearly differentiate this taxon from the others. *Abies numidica* also has a limited and isolated range, restricted to a small region on Mount Babor in the Kabilye Range in northern Algeria. Finally the range of *A. pinsapo* is limited to the mountains around Ronda and Sierra De Antequera in the province of Granada in southern Spain, as well as to two small mountainous regions in northern Morocco where it occurs in two varieties, var. *marocana* and var. *tazaotana*.

The remaining five taxa show wider distribution ranges. *Abies alba* grows throughout the mountainous regions of central Europe, reaching the southernmost limit of its distribution in the Apennines in Calabria (southern Italy) and in the Balkans. *Abies cilicica* is a native of southern Turkey and northern Syria, occurring in different regions of the Taurus chain, where it is generally accompanied by extensive forests containing *Cedrus* species. *Abies nordmanniana* grows in extensive forests, either pure or mixed, in the central Caucasus and on the high mountains of northern Turkey. Finally *A. cephalonica* grows throughout the mainland of Greece and on the islands of Cephalonia and Evia (Euboea). According to Mattfeld (1930), however, the southern *Abies* populations occurring in southern Greece as far north as latitude 38° 50' N belong to the species *A. cephalonica,* while in central and northern Greece a series of intermediate *Abies* forms occur, belonging to the putative hybrid species *A. borisii-regis*, which at its northern limit most resembles *A. alba* and grows together with individuals of this species while at the southern limit it mostly resembles *A. cephalonica*.

Controlled pollination analyses showed that many of the *Abies* taxa growing in the Mediterranean region can be easily crossed (Klaehn & Winiesky, 1962; Kormutak, 1979; Kormutak, 1981; Kormutak, 1985) although these experiments do not provide direct evidence that hybridisation has taken place in nature. Nevertheless, past hybridisation between *Abies* taxa could be an important factor causing the unstable morphological characters observed today in *A. borisii-regis* in central Greece, *A. equii-trojani* in north-western Turkey, *A. bornmuelleriana* in northern Turkey and *A. nebrodensis* in Sicily. However, reliance upon morphological differences for distinguishing between parental species and their hybrids has often proven to be difficult, even for experts. Molecular taxon-specific markers are required to identify the hybrid individuals and to assess the proportional contribution of parental genomes (Bousquet *et al.*, 1990; Wang & Szmidt, 1994; Bacilieri *et al.*, 1996; Watano *et al.*, 1996; Heinze, 1997).

Genetic variation

Plants differ with respect to the degree and apportionment of genetic variation within and among natural populations (Hamrick *et al.*, 1981). The level and distribution of genetic variation in plant species is affected by both biotic and abiotic factors such as gene flow, mating system, selection, genetic drift, human activities and climatic changes. However, the relative significance of these factors is likely to vary greatly among populations and species. For example, large populations may be immune to drift but may be exposed to highly variable selective pressures. In contrast, small populations may undergo loss of variation due to drift, but experience only weak environmental pressure. Usually, species with wide distributions show higher levels of genetic variability than species with restricted ranges. However, exceptions exist for species with wide ranges (Mosseler *et al.*, 1992; Echt *et al.*, 1998) as well as for isolated and endemic species with restricted ranges (Szmidt, 1982; Hiebert & Hamrick, 1983; Kuittinen *et al.*, 1991; Vicario *et al.*, 1995; Ducci *et al.*, 1999).

In the following paragraphs I will discuss the factors that are likely to have played an important role in shaping the level and distribution of genetic variation of the *Abies* taxa investigated in this thesis.

Climatic changes

Large parts of Europe were directly influenced by the last ice ages of the Pleistocene (1.6-0.01 My BP) and the current distribution of the plant species in the region still reflects colonization into these areas after the retreat of the glaciers. It is now clear that the ice sheets in the Northern Hemisphere began to grow much large around 2.4 My BP and that the last 700 000 years have been dominated by major ice ages with a roughly 100 000-years cycle, interrupted by relatively short warm interglacial periods (Hewitt, 1996). Our knowledge of the last glacial cycle is the most complete, particularly for the progression from the

most recent full ice age condition (20 000 BP) to the warm interglacial of the present day. Extensive palynological, paleobotanical and phylogeographic work has identified several areas around the Mediterranean basin which must have provided glacial refugia for trees (Huntley & Birks, 1983; Taberlet *et al.*, 1998). With the retreat of the ice-sheets, changing climates permitted many plants to expand their ranges again.

According to Hewitt (1996) the process of expansion and contraction occurring during the glacial and interglacial periods may have led to considerable genome reorganization in plant species. After the melting of the glaciers the different genomes mixed to various degrees across the plains of middle and northern Europe, and while the leading populations were expanding northwards from the southern refugia, the warmer climate reduced the ability of the species to survive in the southernmost part of its range, although the mountains of southern Europe provided refugia in the new conditions. It is likely that plant taxa could have survived cold- and warm-stage temperature changes in areas where the change was small relative to climatic zones provided by the available relief, for example in the Alps (up to over 4000 m), the Balkans (over 3000 m) or northwest Greece (2500 m) (Tzedakis, 1993). Therefore, in southern Europe during the warm periods the refugial populations expanded up the mountains. However, the distance dispersed in this manner was relatively low for any given degree of temperature change, and one would expect relatively little genome differentiation to occur among populations and areas and not much bottlenecking or loss of genetic variation. In contrast, extensive and rapid northwards expansions often involved a series of bottlenecks for the colonizing genome, with a loss of alleles and derived genomes spread over large areas. All these range changes would have been, of course, affected by the local and regional topography, with mountains, lakes and plains modifying both expansions and contractions.

Hewitt (1996) also discussed the questions posed by the high levels of genetic diversity found in plant populations occurring in southern parts of Europe, especially around the Mediterranean basin, and the relatively low genetic diversity found in the northern European populations. This partitioning of diversity is explained by the hypothesis that colonization occurred only from the northern fringes of refugial areas (leading edge colonization). Such processes led to only a small fraction of the overall genetic diversity in refugia being dispersed into the areas vacated by the retreat of the glaciers, with the bulk of the genetic variability remaining *in situ* in refugial areas. Evidence from investigations on the genetic variability of pteridophytes (Vogel *et al.*, 1999), *Quercus* species (Dumolin-Lapegue *et al.*, 1997b), *Fagus sylvatica* (Demesure *et al.*, 1996) and comparative phylogeography studies in ten different taxa including mammals, amphibians, arthropods and plants (Taberlet *et al.*, 1998) provide support for this hypothesis.

Fossil remains that are attributable with confidence to the *Abies* species are found in the Northern Hemisphere from periods as early as the Miocene (26-5 My BP) of the Tertiary period, although it is assumed that the genus originated earlier, probably at the beginning of the Palaeocene (65- 54 My BP) (Liu, 1971). In the Miocene an ancient *Abies* progenitor already existed in the Balkan and Middle East regions, and the Aegean basin was a favourable zone for the differentiation of the species, which followed an east-west migration route in their expansion and became widely distributed in the Northern Hemisphere (Liu, 1971) (Figure 2).

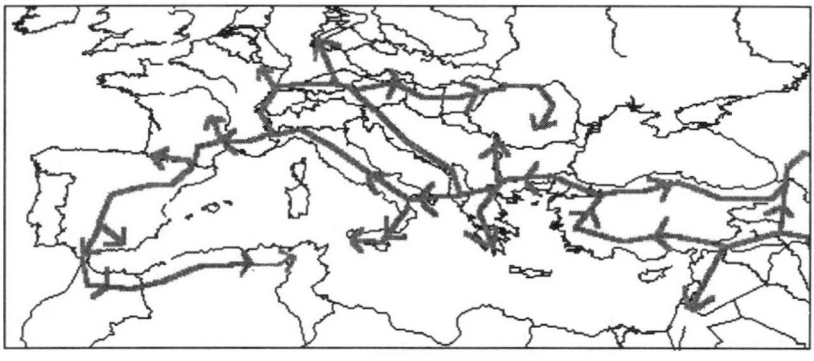

Figure 2. Migration routes followed by the Mediterranean *Abies* species

The *Abies* pollen found in several interglacial and early Pleistocene deposits indicates that extensive *Abies* forests were present in Europe prior to this time (Huntley & Birks, 1983; Huntley, 1990). During the climatic crises of the Miocene, the *Abies* range became fragmented and the individual species became isolated. At this time *A. alba* was confined to the Apennines chain and to central and eastern Europe, where the climate was cooler and the species could benefit from the humid Atlantic streams. It is likely that the other Mediterranean *Abies* species were restricted to the Balkans and to the mountainous regions of Northern Africa and the Middle East. Pollen maps for the last glacial period (90 000-12 000 BP) confirmed that prior to this time *Abies* forests were present in Greece, Italy and south Iberia (Huntley & Birks, 1983; Huntley, 1990) although the lack of *Abies* pollen maps in North Africa and the Middle-East regions does not allow conclusion to be drawn about the presence of *Abies* forests in these areas.

However, according to the hypothesis that the genus had an Asiatic origin, followed by an east-west differentiation, it is reasonable to assume that different *Abies* species were also present in these areas prior to the last glaciations (*i.e. A. numidica* in Northern Africa and *A. cilicica* in Syria and Lebanon).

During the Messinian salinity crisis of the most recent part of the Miocene (12-5 My BP), the Mediterranean became a hyper-saline land-locked sea, substantially below the ocean level so that the Italian Peninsula was connected to the African continent. This situation, which lasted until the early Pliocene (5-1.6 My BP), when the marine flooding took place, offered many possibilities for the different species to come into contact, and this probably happened in various parts of the Mediterranean region. In successive periods when the glaciers grew, during the ice ages of the Pleistocene (1.6-0.01 My BP), the European species withdrew southwards and/or into lower elevations, and in Europe they were restricted to refugial zones.

Information about the location of the refugia might be obtained by comparing pollen diagrams from across a wide area and following the direction of migration of various taxa. Huntley & Birks (1983) and Bennet *et al.* (1991) adopted this approach and identified three different refugia for the *Abies* species in Europe: the southern Balkans, the Apennines (probably Calabria), and the Iberian Peninsula. The detection of the refugia is crucial for understanding the patterns of genetic variation observed in the current Mediterranean *Abies* taxa since the ice ages offered them new possibilities to come in contact.

After the melting of the glaciers (12 000-6000 BP) the rate of spread of plant species was remarkably rapid: between 50 to 1500 m/year (Huntley & Birks, 1983; Bennet *et al.*, 1991). The migration patterns in Europe displayed in the pollen maps available indicate that the *Abies* species moved from the European refugia. We can therefore assume that from the Balkan refugium some species moved north and east to re-colonize central and eastern Europe, while the North African and Middle East species, hardly influenced at all by the effect of the ice ages, probably moved only into lower elevations from the mountain chains of the regions. Lack of pollen evidence from central and southern Greece prior to 6000 BP prohibits any firm conclusion being made about the time at which *A. cephalonica* appeared there; indeed it may have been there since glacial times and remained confined to the mountainous regions of this area (Huntley & Birks, 1983). In addition to the migration from the Balkan refugium, *A. alba* followed two other migration routes, from the southern Italian refugium to northern Italy and from the Iberian Peninsula to central Europe. By 10 000 BP this species was present in the Alps and subsequently expanded steadily in the area, with migration rates of 40-50 m/yr, and by 8000 BP it was present on the Pyrenees. This rapid expansion phase ended by 7000 BP, at which time *A. alba* was well established in the Pyrenees, the western and central Alps, the Apennines and the Balkans. Subsequent expansions into a variety of lower mountain ranges between 6000 and 5000 BP probably reflected some climatic cooling, and

occurred in the mountainous regions of Europe as well as in Northern Africa and the Middle East. The subsequent fragmentation of the *Abies* range resulted either from further climatic changes or, more likely, from anthropogenic influences and forest destruction (Huntley & Birks, 1983 and references therein).

Gene flow

Gene flow is an important factor affecting the distribution of genetic variation in plants. Gene flow among plant populations takes place in two ways. The first involves pollen dispersal to a different population, with fertilization of an ovule and establishment of the resulting seeds. The second involves dispersal of seeds and their successful establishment within the new population (Ennos, 1994). In outcrossing species like *Abies*, the contribution of pollen to gene dispersal is also affected by the frequency of *selfing* (the union of female and male gametes from the same individual) and by the pollen's ability to remain airborne, while the contribution of seeds is mainly dependent on the extent of their physical dispersion from the maternal plant. A complete description of processes that contribute to the distribution of genetic variation in plants requires analysis of separate effects of seeds and pollen on gene flow (Cruzan, 1998).

Attempts to measure gene flow in plants have been essentially based on two approaches. The first makes use of estimates of population subdivision (F_{ST} or G_{ST}) and models of population differentiation with a balance between drift and migration (Slatkin, 1985). However, the obtained estimate of migration rate, *Nm*, provides no direct measure of gene flow, nor does it allow gene flow via pollen to be distinguished from that via seed (Ennos, 1994). In the second approach, pollen migration rates are estimated by calculating the proportion of foreign pollen among all successful pollen that arrives in a population (Friedman & Adams, 1985; Nagasaka & Szmidt, 1985; Ellstrand *et al.*, 1989; Adams & Birkes, 1990; Ellstrand, 1992).

In recent years, due to the advance of molecular genetics, several techniques have been developed for studying population differentiation, not only with respect to nuclear markers but also for chloroplast and mitochondrial markers. In the *Pinaceae*, the inheritance modes of organellar DNAs are unique because chloroplast DNA is paternally inherited but mitochondrial DNA is of maternal origin (Szmidt *et al.*, 1987; Neale *et al.*, 1989; Neale & Sederoff, 1989; Wagner *et al.*, 1989; Wagner *et al.*, 1991a; Mogensen, 1996; Wang *et al.*, 1996). As a consequence of the different modes of inheritance, the extent of gene flow among populations will differ for biparentally and uniparentally inherited markers.

Generally, in the absence of extensive gene flow, uniparentally inherited markers show less variation within populations, and more between populations, than nuclear biparentally inherited markers (Birky *et al.*, 1983). The extent of these differences, however, will also be affected by the biological characteristics of the species concerned. In this respect, differences among species may be substantial.

The greatest difference between pollen and seed migration rates so far recorded was found in the oak species complex, where interpopulation pollen flow was estimated to be 200 times greater than seed flow, while in pine species, corresponding estimates ranges from 20 to 60 (Ennos, 1994 and references therein).

In the last decade, several studies combining information about population genetic structure from both nuclear and chloroplast genomes provided many insights into the relative contribution of seed and pollen movement to the total gene flow (Govindaraju, 1989; Ennos, 1994; Mc Cauley, 1995). New highly polymorphic, paternally inherited DNA markers such as chloroplast microsatellites (cpSSRs) (Powell *et al.*, 1995a; Powell *et al.*, 1995b; Vendramin *et al.*, 1996) are also now available for this purpose.

Mating system

Mating systems affect both the amount and distribution of genetic variation in plants. Mating events in plants are governed by the behaviour of pollen vectors and by processes affecting the post-pollination events. The simplest and most important mating pattern is *random mating*, in which mating takes place at random with respect to the genotypes of a population. However, departures from random mating occur often in natural populations, mainly due to *inbreeding*. Inbreeding refers to mating between relatives and has the effect of increasing the homozygosity of a population. The most extreme form of inbreeding is *selfing*.

In general, conifers have mixed mating systems, and selfing rates at the fertilization stage are intermediate (Sorensen, 1994; Sorensen & Adam, 1993). During the subsequent embryogenesis and stand development, however, selection usually removes selfed progeny so that the mature reproductive populations consist mainly of outcrossed individuals (Shaw & Allard, 1982a; Muona & Szmidt, 1985; Muona, *et al.*, 1988; Bush & Smouse, 1991). Sorensen (1999) demonstrated that the response to inbreeding (inbreeding depression) differed among conifer species. In a comparison of *Abies procera*, *Pseudotsuga menziesii* and *Pinus ponderosa*, the cited author found that over time, at varying stages before reproductive maturity, essentially all the selfed individuals were lost because of inbreeding depression. In accordance with these findings, several studies in conifers have shown an excess of homozygotes over panmictic expectations at the embryo stage, which usually disappears at the adult stage (Shaw & Allard, 1982a; Szmidt & Muona, 1985; Yazdani *et al.*, 1985; Plessas & Strauss, 1986; Muona *et al.*, 1987).

Mating system studies entail estimation of *outcrossing* and *selfing rates* (Muona *et al.*, 1990). An easy and straightforward approach adopted for estimating outcrossing rates in conifers is to use allozymes to identify the paternal parent of seeds collected from the same mother tree by comparing the genotype of the embryo and endosperm (Snow and Lewis, 1993). In the last decades, several

allozyme studies have provided useful information about the mating patterns of several conifer species, as well as the mechanisms that contribute to those patterns (Cheliak *et al.*, 1985; Muona *et al.*, 1990; Beaulieu & Simon, 1995; Changtragoon & Finkeldey, 1995). While allozymes remain the most widely used markers to for evaluating mating systems, the more recently developed AFLP markers are an attractive alternative, due to their high levels of polymorphism (Gaiotto *et al.*, 1997).

In conifers, estimates of outcrossing rates are usually high (>0.85) suggesting that selfing is low in this group of plants (Mitton *et al.*, 1981; Shaw & Allard, 1982b; El-Kassaby *et al.*, 1986; Longauer *et al.*, 1992). Available estimates of outcrossing rates in *Abies* are also high, varying from a maximum of one in a seed orchard of the North American *A. procera* (Siegismund *et al.*, 1996) to 0.89 in nine natural populations of *A. alba* (Schroeder, 1989).

Random genetic drift

Random genetic drift refers to chance fluctuations in allele frequencies, which are particularly pronounced in small and isolated populations, as a result of random sampling among gametes (Ridley, 1993). In a small population, in the absence of mutation, one allele will eventually be fixed at each locus and the population will eventually become homozygous. The main effect of genetic drift is therefore to reduce the amount of genetic variability in small populations.

A particular example of the influence of random sampling is given by the *founder effect.* Mayr (1963) defined the founder effect as the establishment of a new population by a few original founders that carry only a small fraction of the total genetic variation of the parental population: the random genetic drift accompanying a founder event is known as the founder effect. A population may be descended from a small number of ancestral individuals for either of two main reasons. A small number of individuals may colonize a place previously inhabited by their species. Alternatively, a population that is established in an area may fluctuate in size. In the latter case the founder effect occurs when just a few individuals survive but the population later expands again when more favourable times return. Such temporary reductions in population numbers are also called *bottlenecks* and the losses of genetic variation accompanying the reductions in size are called *genetic bottlenecks* (Ridley, 1993).

In practice however, if a small sample of individuals is taken from a highly variable larger population, the founder effect may not be particularly effective at reducing variation at diploid heterozygous loci since the founder population, even if very small (N<10), will usually posses both alleles at each of these loci. On the other hand, the founder effect can have other interesting consequences. Although the individuals forming a founder population are likely to have nearly all the ancestral population's genes, the gene frequencies may be peculiar. Thus, isolated populations tend to have exceptionally high frequencies of rare alleles

and the most likely explanation is that the founder population had a disproportionate number of these alleles (Ridley, 1993).

The situation may be different for uniparentally inherited haploid loci. In such cases the effective population size is half of that of biparentally inherited loci and it will be less likely that the individuals forming the founder population will have all the representative genes of the ancestral population. Thus, genetic bottlenecks at these loci will be more pronounced.

Evolution

Evolution is the result of the progressive changes in the form and behaviour of organisms between generations. While the focus of population genetics is to understand how much genetic variation exists in natural populations, and to explain this variation in terms of its origin, maintenance and evolutionary importance (microevolution), the scope of evolutionary genetic studies is to understand how the genetic composition of specific groups of taxa came to be the way they are, and to infer hypotheses about their origin and evolution using both *phenetic* and *phylogenetic* principles.

A vast array of different characters, including morphological, biochemical and cytological traits, flavonoid patterns, protein variation and DNA markers have been used to study plant relationships and evolution. In the last decade, several studies showed that cpDNA markers were very useful for evaluating phylogenies of a large number of conifer species (Strauss & Doerksen, 1990; Govindaraju *et al.*, 1992; Sigurgeirsson & Szmidt, 1993; Wang *et al.*, 1993; Tsumura *et* al, 1995; Krupkin *et al.*, 1996; Tsumura *et al.*, 1996; Wang *et al.*, 1999; Wang *et al.*, 2000) including several Japanese *Abies* species (Tsumura & Suyama, 1998; Suyama *et al.*, 2000). However, chloroplast genes, due to their uniparental mode of inheritance, behave like haploid loci that do not recombine. The mechanism of recombination, which leads nuclear genes being a mosaic of sequences with different evolutionary histories, is not present in the cpDNA genomes. A consequence of this simplicity of transmission is that the phylogenies derived from cpDNA sequences should be regarded as gene or organellar trees and the relationships inferred will not always tally with the history of the species studied. The approximation involved will, in fact, increase as time goes by, because the biases introduced by intraspecific hybridisation and/or intraspecific polymorphism will diminish as the time scale is extended (Clegg and Zurawski, 1992). Moreover, the usefulness of cpDNA markers for comparisons among species will also depend on the level of polymorphism exhibited by the marker.

At present, phylogenetic information about the Mediterranean *Abies* species is patchy, and the majority of the published phylogenetic studies have been based either on morphological or biochemical traits (Mitsopoulos & Panetsos, 1987; Fady *et al.*, 1992; Fady & Conkle, 1993; Scaltsoyiannes *et al.*, 1999). Similarly, current knowledge of the evolution and migration patterns followed by the *Abies*

species of this region is based on morphological and biochemical comparisons, the degree of cross-compatibility among species and fossil pollen analyses (Liu, 1971; Klaehn & Winiesky, 1962; Huntley & Birks, 1983; Kormutak, 1985; Konnert & Bergmann, 1995; Breitenbach-Dorfer *et al.*, 1997). Finally, controversies and uncertainties among taxonomists and botanists concerning the delimitation of some of these taxa appear, in some cases, to have clouded the biological validity of the available knowledge.

Objectives

The purpose of the project described in this thesis was to study the genetic variability and evolution of the *Abies* taxa occurring in the Mediterranean region. In particular, the investigation focused on the rare species *A. nebrodensis*, which is endemic to the island of Sicily and the three neighbouring species *A. alba*, *A. cephalonica* and *A. numidica*.

The main aim of the five studies included in the thesis was to determine the amount and distribution of the genetic variability within and among taxa, both at the nuclear and chloroplast levels, in order to elucidate their origin and evolution and, especially, to shed light on the taxonomic position of *A. nebrodensis*.

In studies reported in Papers I and II and V, allozyme markers were used to provide information on the level and distribution of genetic variation among and within natural populations of *A. alba*, *A. cephalonica*, *A. nebrodensis* and *A. numidica* and to estimate the out-crossing rate within *A. alba*. In the analyses described in Papers III and IV, DNA markers from the chloroplast genome were developed and employed at the intra- and inter-specific level to estimate the degree of cpDNA variation in the genus and to derive inferences concerning relationships among the species. Two different approaches were used: the first involved a comparative restriction-site analysis of ten different amplified chloroplast DNA fragments (Paper III) and the second involved analysis of chloroplast hypervariable repetitive simple-sequence repeats (cpSSR or microsatellites) (Paper IV).

Methods used

Molecular markers

An important attribute of natural populations of forest tree species in their natural habits is their *phenotypic diversity*. Population genetics deals with that portion of phenotypic diversity that is caused by differences in *genotypes* among individuals in a population. In the past ten years, the advent of molecular techniques has provided a wide array of new methods to study population genetics and evolution

of plant species. First allozyme, then DNA restriction fragment length polymorphisms (RFLPs) and now sequencing of chloroplast, mitochondrial and nuclear DNA fragments, have offered the opportunity to measure, increasingly effectively, the genetic variation across the species' ranges. Molecular markers have been discussed in several recent reviews (Strauss *et al.*, 1992; Geburek, 1997; Karp & Edwards, 1997; Vekemans & Jacquemart 1997; Cruzan, 1998; Haig, 1998; Parker *et al.*, 1998; Szmidt & Wang, 2000)

In the following paragraphs I will describe the molecular markers employed in the studies presented in the thesis.

Allozyme markers

Allozyme markers represent electrophoretically detectable forms of enzymatic proteins. This technique has been the most commonly used in plant population biology over the past several decades, since it is relatively straightforward, easy to carry out and is not expensive, compared to alternative techniques.

Allozyme variation results from changes in protein coding sequences and is associated with biparentally inherited nuclear genes encoding enzymatic proteins. Enzyme loci often show significant amounts of polymorphism, which makes them very useful for investigating various evolutionary factors like gene flow, mating system and genetic drift. They are also useful for measuring the levels of variation within and among populations (Hamrick *et al.*, 1990). Multilocus analysis methods have provided the means for estimating outcrossing rates in many forest tree species and the results of these studies have made important contributions to evolutionary biology, tree breeding, population genetics and the conservation of genetic resources (Muona *et al.*, 1990; Godt & Hamrick, 1991).

A disadvantage of these markers is that they represent a restricted number of structural genes, and extrapolation of the results from a small number of genes to the entire genome may not always be valid. Moreover, the use of these markers in studies comparing individuals belonging to different species is controversial, because two alleles migrating to the same point on a gel and belonging to two different individuals do not necessarily have identical protein sequences. The more unrelated the two individuals, the more likely is this possibility. Finally, the apparent technical simplicity of allozyme markers is not as great as sometimes suggested, due to the lack of standard protocols that are applicable to different species.

DNA markers

DNA markers are potentially the most accurate source of genetic information. Their great advantage is that, unlike allozymes, they provide pure genetic information on the organism studied since they are not the products of transcription or translation. Other great advantages of these markers are the large variety of scales on which evolutionary processes can be studied and their great

potential for detecting variation in all kinds of organisms, in both living and dead tissues.

Genetic information in plants and algae is present in three cellular compartments: the nucleus, the mitochondrion and the chloroplast. Although the use of DNA markers has provided new genetic information about all three genomes, the chloroplast DNA is now the best-known genome in forest trees.

The *chloroplast DNA* (cpDNA) has been extensively studied by genetic and physical mapping not only in forest tree species, but also in a number of other land plants and algae. Probably the most comprehensive and significant contribution to understanding chloroplast gene organization has come from the complete sequencing of chloroplast genomes in several different plants. Besides algae and unicellular eukaryotes, the complete nucleotide sequences of chloroplast genomes are now available for six plants, namely *Marchantia polymorpha* (Ohyama *et al.*, 1986), *Nicotiana tabacum* (Shinozaki *et al.*, 1986), *Oryza sativa* (Hiratsuka *et al.*, 1989), *Epifagus virginiana* (Wolfe *et al.*, 1992), *Pinus thunbergii* (Wakasugi *et al.*, 1994) and *Zea mays* (Maier *et al.*, 1995).

In land plants, the chloroplast genome generally consists of homogeneous circular double-stranded DNA molecules of approximately 110-160 Kilo base pairs (Kbp) that occur in multiple copies per organelle. The gene content of the chloroplast genome is highly conserved and comprises approximately 120 different genes, which are mostly conserved among organisms. In contrast to the nuclear and mitochondrial genomes of plants, which contain vast amounts of DNA of no apparent function, the genetic information contained in the chloroplast genomes is usually very condensed with short intergenic regions. The evolution rate of cpDNA genes is estimated to be several times slower than of the nuclear genes (Wolfe *et al.*, 1987). It is believed that the compact size, structural integrity and genomic content of cpDNA have been maintained by an intensive constraint, either mechanistic or selective (Palmer, 1990).

A prominent feature of the chloroplast genomes found in most angiosperms is the presence of two large inverted repeats ranging in size between 6 to 76 Kbp (Palmer, 1985; Shimada & Sugiura, 1991; Sugiura, 1992). However, this structure is absent from the cpDNA of legumes (Palmer & Thompson, 1981) and most conifers (Lidholm *et al.*, 1988; Strauss *et al.*, 1988; White, 1990a; Raubeson & Jansen, 1992; Karpinska & Karpinski, 1993; Tsumura *et al.*, 1993), including *Abies* (Tsumura *et al.*, 2000). It has been suggested that the absence of the two large repeated regions may confer phylogenetic instability on the chloroplast genome (Palmer & Thompson, 1981; Strauss *et al.*, 1988). Indeed, conifer cpDNA, lacking this structure, has been shown to include several gene rearrangements that have not been observed in angiosperms (Lidholm *et al.*, 1988; Tsumura *et al.*, 2000). However, it is still not clear if this instability is related to the absence of the inverted repeat structure, and there are not enough data to confirm, or refute, this hypothesis.

The cpDNA in conifers also differs from angiosperms with respect to its mode of inheritance. While in most angiosperms cpDNA is transmitted from the female parent, paternal inheritance of cpDNA was found in conifers (Neale *et al.*, 1986; Szmidt *et al.*, 1987; Wagner *et al.*, 1989; Stine & Keathley, 1990) including *Abies* (Ziegenhagen *et al.*, 1995). Due to the uniparental mode of inheritance this molecule behaves like a large, haploid single-locus that does not recombine (Chiu and Sears, 1985). Therefore it is inherited unaltered, except for occasional mutations, across the generations in any line housing it. This implies that a group of associated loci are not separated, so that a great deal of historical information is preserved in their sequences. In addition, its effective population size is half of that of the nuclear DNA and therefore it is more sensitive to reductions in the number of individuals in a population (Birky, 1988). The reduction in effective population size should also lead, theoretically, to lower levels of genetic variation in the chloroplast genome compared to the nuclear genome (Mitton, 1994).

De to all these unique features of the cpDNA, the analysis of its variation has several advantages for evolutionary and systematic studies on plants. The paternal mode of inheritance of this genome implies that cpDNA markers can be very useful for analyses of gene flow *via* pollen in conifers (Ennos, 1994; Mc Cauley, 1995). Furthermore, the non-recombinant nature of cpDNA markers provides unique opportunities for obtaining insights into the origin of hybrid species (Wagner *et al.*, 1987; Govindaraju *et al.*, 1989; Ernst *et al.*, 1990; Wagner *et al.*, 1991b; Wang & Szmidt, 1994; Szmidt *et al.*, 1996). However, in spite of the generally conservative character of cpDNA, in recent years intra-specific variation in it has been found in diverse conifer species (Wagner *et al.*, 1987; Govindaraju *et al.*, 1988; Govindaraju *et al.*, 1989; White, 1990b; Wang & Szmidt, 1994) including *Abies* (Tsumura *et al.*, 1994; Ziegenhagen *et al.*, 1995; Tsumura *et al.*, 2000).

The advent of the *polymerase chain reaction* technique (PCR) (Saiki *et al.*, 1985) has profoundly improved both the speed and efficiency of detecting and analysing all types of chloroplast and other sequences. The technique is based on the enzymatic *in vitro* amplification of a specific or arbitrary DNA sequence. It starts with a tiny amount of DNA and two primers that can bind to the two complementary strands of the DNA sequence, after denaturation by heating. A thermally stable DNA polymerase is then used to extend the primers in the presence of the four nucleotides (dNTPs) in a series of cycles. By repeating the denaturation, annealing and extension steps several times, several million copies of the target sequence DNA determined by the primer sequences are obtained. With the PCR technique specific regions can be amplified if the sequence of the conserved regions flanking the region of interest is known.

Variation in the amplification products can be analysed through digestion with restriction enzymes and separation by gel electrophoresis (PCR-RFLP analysis). This technique is highly reproducible and the only potential disadvantage is that it requires previous sequence information on the species investigated. However,

this problem can be alleviated by designing universal primers based on the cpDNA sequence from organisms related to the one under study (Demesure *et al.*, 1995; Dumolin-Lapegue *et al.*, 1997a). The *PCR-RFLP analysis* of chloroplast genes in forest trees has already proved to be very useful for studying phylogenetic relationships among conifers (Tsumura *et al.*, 1995; Wang *et al.*, 2000), for studying the inheritance of chloroplast and mitochondrial genomes (Dumolin-Lapegue *et al.*, 1995; Ziegenhagen *et al.*, 1995; Wang *et al.*, 1996) and for obtaining evidence for the existence of intraspecific variation in many forest tree species (Demesure *et al.*, 1996; Dumolin-Lapegue *et al.*, 1997b)

More recently, the PCR method has been used to amplify highly variable repetitive DNA sequences such as *microsatellites* or *simple sequence repeats* (*SSRs*). These sequences consist of tandem repeated DNA motifs of six bp or less that occur in high proportion in the nuclear genomes of eukaryotes and have aroused considerable interest due to their ability to generate highly informative DNA markers (Tautz, 1984, 1989). The high degree of variability detected with nuclear SSRs has led to their use for genetic analyses in many plant and animal species (Akkaya *et al.*, 1992; Morgante & Olivieri, 1993; Wu & Tanksley, 1993; Saghai-Maroof *et al.*, 1994). The recent observation that chloroplast genomes contain hypervariable regions (*chloroplast simple sequence repeats* or *cpSSRs*) (Powell *et al.*, 1995a; Powell *et al.*, 1995b) has shown their potential for use in population genetic analyses in many forest tree species. Vendramin *et al.* (1996) designed several primer pairs flanking different monucleotide stretches of DNA present in the chloroplast genome of the pine species *Pinus thunbergii*. The universality of these primers has been confirmed by the results of several recent studies in conifers (Cato & Richardson, 1996; Vendramin *et al.* 1997; Bucci *et al.*, 1998; Echt *et al.*, 1998; Morgante *et al.*, 1998; Vendramin *et al.*, 1998; Echt *et al.*, 1999; Vendramin *et al.*, 1999) and the high level of polymorphism observed suggests that cpSSRs share the same hypervariable nature of nuclear SSRs. Unfortunately, due to their high mutation rates, reversion and the possibility of homoplasy, cpSSRs are not suitable for phylogenetic purposes. This was confirmed by the observation made by (Powell *et al.*, 1995b) that the distribution of cpSSR length variants among different *Pinus* species did not conform to the commonly accepted classification of the genus *Pinus*. Although relatively expensive, SSR analysis is easy to perform when the appropriate equipment is available. The use of automated DNA sequencing apparatus with appropriate software can speed up and automate the genotyping to a considerable extent.

Summary of the results

Genetic variation and evolution of the Mediterranean *Abies* species

Studies presented in Papers I, IV and V provided information on the level and distribution of genetic variation among and within natural populations of *A. alba*, *A. cephalonica*, *A. nebrodensis* and *A. numidica* at chloroplast and nuclear loci.

Variation within populations

The levels of allozyme and cpDNA variation observed in the four *Abies* species were in the range of those found in other conifers and confirmed that the highest polymorphism was concentrated in the *Abies* populations from the eastern Mediterranean regions (northern Greece) while a lower level was found in the isolated species from the western regions (*A. nebrodensis* and *A. numidica)*. Intermediate values were observed in *A. alba.*

The lower level of polymorphism observed in *A. nebrodensis* and *A. numidica* was probably due to genetic drift resulting from isolation and small population size. As expected, the consequences were more pronounced at the chloroplast level than at the nuclear level. This was particularly evident in *A. nebrodensis*, where 41 % of the individuals in the population shared the same cpSSR haplotype (Paper IV). Moreover, results from study V as well from previous allozyme studies (Vicario *et al.*, 1995; Ducci *et al.*, 1999), showed that the extant population, although severely reduced in size, still retained a considerable amount of variation at the nuclear level. Thus, it is likely that just few *A. nebrodensis* pollen-donating parents gave rise to the extant population and the consequently reduced level of variation had a weaker effect at the nuclear level.

The cpSSR analysis presented in study IV revealed that the values of haplotype diversity among individuals (D^2_{sh}) were related with the size and type of location of the population under study. Thus, low levels of diversity were observed not only in *A. nebrodensis* and *A. numidica*, both of which have small and isolated ranges, but also in the *A. cephalonica* population growing on the island of Cephalonia, isolated from the main distribution centre of the species, and in the Calabrian population of *A. alba*, which is also distant from the main distribution centre of the species in central Europe. The increased level of relatedness observed at the chloroplast level among the individuals of these populations was probably caused by the lack of contact with other heterogeneous sources of variation, and in some cases by drift due to small population size. In a similar cpSSR analysis, Morgante *et al.* (1998) attributed the low D^2_{sh} values found in the majority of the populations of *Pinus halepensis* analysed, to a genetic bottleneck experienced by the species at some point in its evolution.

The higher level of polymorphism detected in the *Abies* taxa from Greece was in accordance with previous allozyme studies showing that the *Abies* populations from this region are highly polymorphic compared to the other Mediterranean

taxa (Fady & Conkle, 1993; Scaltsoyiannes *et al.*, 1999). These results can be explained by the presence in central and northern Greece of the hybrid populations of *A. borisii-regis*, although clearly more studies would be necessary to substantiate this hypothesis. It is also possible that this high degree of polymorphism is a consequence of the post-glacial recolonization events of these populations. As already seen, after the last glaciation, the warmer climate drove the northwards expansion of the *Abies* species from the Balkan refugium, while the *A. cephalonica* populations remained confined to the Greek Peninsula. It is likely that the warmer climate reduced the ability of the populations left in the south to survive, which therefore just moved up into the local mountains. Such limited dispersal did not imply much loss of genetic variation or bottlenecking in these populations compared to those that moved northwards, with the bulk of the variation remaining *in situ* (Hewitt, 1996).

Similar hypotheses can be invoked to explain the higher level of variation found in study I in the *A. alba* populations from central and southern Italy, which is supposed to be one of the refugial areas of the species, compared to the northern populations. Reports from previous studies have also shown that the Calabrian populations of *A. alba* are characterized by pronounced genetic variability, high vitality and growth vigour, while provenances from central and north-eastern Europe show little variability and clear signs of dieback (Larsen, 1986; Bergmann & Kownatzki, 1988; Bergmann *et al.*, 1990; Ducci, 1994; Pennacchini & Ducci, 1994; Konnert & Bergmann, 1995; Vicario *et al.*, 1995).

Variation among populations

In *A. alba* the level of population subdivision detected using cpSSR data in study IV accounted for 19 % of the total variation: more than two times greater than the value obtained from the allozyme data (G_{ST}=8.8 %) in study I. A similar level of population differentiation was also found with cpSSRs by Vendramin *et al.* (1999), who analysed multiple populations across the whole range of *A. alba* (G_{ST}=13.3 %). The absence of extensive pollen migration between geographically distant populations and the multiple post-glacial routes followed by *A. alba* may have been major factors influencing this pattern. As already seen, the effect was more pronounced in uniparentally inherited markers (cpSSRs) than with biparentally inherited markers (allozymes).

However, markedly different results were obtained in *A. cephalonica*. For this species, estimates of population differentiation were substantially higher for allozyme markers (F_{ST}=17.8 %) (Paper V) than for cpSSR markers, where as little as 1.2% of the variation was due to differentiation among populations (Paper IV). In Paper V, we suggested that the low level of population differentiation was probably a consequence of the post-glacial migration history of *A. cephalonica*. As already mentioned, this species migrated from the Balkan refugium and subsequently remained confined to the Greek Peninsula. This situation may have promoted intensive gene exchange among the populations, preventing

differentiation. On the other hand, the unexpectedly high level of differentiation observed in the allozyme study presented in Paper V may be due to the choice of material analysed. While in study IV the majority of the populations investigated were collected in Greece below latitude 38° 50' N, where the pure *A. cephalonica* occurs, in study V we also analysed populations growing well above this latitude, where hybrid populations of *Abies borisii-regis* are believed to grow together with populations of *A. alba* (Mattfeld, 1930). Although genetic distance values presented in study V agreed with the hypothesis that hybrid populations were present in this region, additional analyses using species diagnostic markers should be carried out to clarify this issue.

Mating system

As expected, the multilocus estimates of the outcrossing rate obtained for *A. alba* in study I (0.94) did not differ substantially from the values found in other *Abies* species: 0.89 in *A. alba* (Schroeder, 1989), 0.87 in *A. amabilis* (Davidson, 1990) and >1 in *A. procera* (Siegismund *et al.*, 1996). Therefore, it appears that in *Abies* the selfing rate is low, and in this respect the genus does not differ from other conifer species.

Generally conifers show an excess of homozygotes over panmictic expectations at the embryo stage, which often disappears at the adult stage (Shaw & Allard, 1982a; Szmidt & Muona, 1985; Plessas & Strauss, 1986; Muona *et al.*, 1987). In the allozyme analysis presented in Paper V the majority of the loci analysed, in both embryo and adult populations, did not show significant deviations from Hardy-Weinberg equilibrium. Similarly, in *A. alba* Schroeder (1989) found that heterozygote frequencies in nine openly pollinated seed families were all in agreement with panmictic expectations.

However, occasional departures from random mating may occur in outcrossing species even at the adult stage, mainly due to *inbreeding*. In comparison with most pines, the pollen grains of *Abies* are relatively large and heavy, and the bladders relatively small (Liu, 1971). These features of the taxon, combined with the isolated distribution of populations and population subdivision due to restricted gene flow (Wahlund, 1928), are likely to have promoted some form of inbreeding. This, in turn, is likely to have been responsible for the significant heterozygote deficiency observed at the adult stage in three *A. alba* populations from south central Italy in the study presented in Paper I.

The Abies *chloroplast DNA*

Results from the PCR-RFLP analysis presented in Paper III showed that the chloroplast genome is highly variable in *Abies* and that this variation is also present at the intraspecific level. Similarly, diversity estimates based on cpSSR haplotype frequency presented in Paper IV were high, and in some cases even higher than corresponding values recently reported in different *Pinus* species (Powell *et al.*, 1995b; Morgante *et al.*, 1998; Provan *et al.*, 1998; Vendramin *et*

al., 1998). In study III, with only ten pairs of primers we found as many as five different haplotypes in a sample of six *A. alba* individuals, while two haplotypes were found in five individuals of *A. cephalonica*, seven individuals of *A. concolor* and four individuals of *A. numidica*. Similar studies employing larger sample sizes failed to detect intraspecific polymorphism in the chloroplast genome of *Pinus* species (Boscherini *et al.*, 1994; Wang & Szmidt, 1994). The cpDNA variation observed was striking since polymorphism was detected in all the regions investigated and appeared to be distributed throughout the whole genome. Of the 106 variants detected overall, the majority was due to length mutations varying in size between 5 to 165 bp.

In a previous study Powell *et al.* (1995a) examined the number and location of SSR regions (microsatellites) with a minimum of 10 repeats in the chloroplast genomes of six different plant species and found 24 such regions in the cpDNA of *Pinus thunbergii*, clearly demonstrating their abundance and distribution in the cpDNA of this species. In study III, in the absence of sequence information, it was not possible to determine the source of the PCR-RFLP variation observed in the *Abies* cpDNA or whether it was partially associated with microsatellite regions similar to those described by Powell *et al.* (1995a).

Recently, Tsumura *et al.* (2000) detected inter and intraspecific cpDNA variation in five Japanese *Abies* species and demonstrated that the variation was caused by a large 42 Kbp inversion associated with a short inverted repeat. Two different haplotypes were observed in 47 populations analysed and the authors suggested that the polymorphism was maintained within populations and species by the high mutation rate of the inversion. They also suggested that in the *Abies* chloroplast genome rearrangements may have been accelerated by the absence of the large inverted repeat; a structure absent in most conifers, which is thought to give stability to the chloroplast genome (Palmer & Thompson 1982; Strauss *et al.*, 1988). However, in *Pinus*, which also lacks the inverted repeats, the gene arrangements appear to be collinear (Strauss *et al.*, 1988; White, 1990a; Lidholm & Gustafsson, 1991; Karpinska & Karpinski, 1993) and little variation has been found in this genome (Boscherini *et al.*, 1994; Wang & Szmidt, 1994) compared to that found in *Abies* cpDNA. Further studies are required to investigate the source of the high level of variation found in the *Abies* cpDNA.

In study III, a single restriction site polymorphism appeared when the cpDNA region *trn*S-*psb*C was cut with the *Hae*III enzyme and two different variants were observed. A previous analysis of the same region from 12 *A. alba* populations revealed a geographical cline in the distribution of these two variants (Ziegenhagen *et al.*, 1995). One variant was predominant in the eastern populations from Bulgaria, Slovakia and the Carpathians, while the second was predominant in the western populations from the Pyrenees, Vosges and Palatinate Mountains. Accordingly, in our study *A. pinsapo* and *A. nebrodensis* were fixed for the western variant, while both variants were found in *A. alba* and *A. numidica*. The presence of the eastern variant in *A. numidica* strengths the

hypothesis that genetic contact occurred in the past between *A. numidica* and *A. alba*, while the subsequent isolation and bottleneck experienced by *A. nebrodensis* may be the cause of the fixation of the western variant in this species.

Origin and evolution of Abies nebrodensis

In the studies presented in Papers III, IV and V, we studied the genetic variation of *A. nebrodensis* both at the nuclear and chloroplast levels in order to obtain information on the origin of the species and its relationships with the other Mediterranean *Abies* taxa. In particular, in studies IV and V we focused on its relationships with the neighbouring *A. alba*, *A. cephalonica* and *A. numidica*.

Results from both allozyme and cpDNA analyses indicated that *A. nebrodensis* differs from the other *Abies* species studied, justifying its classification as a separate species. However, some affinities to *A. alba* and *A. numidica* were observed in both analyses. In study IV, with the allozyme markers, *A. nebrodensis* appeared to be genetically closest to the population of *A. alba* from southern Italy (Calabria), while in the PCR-RFLP analysis presented in study III, it shared one haplotype with *A. numidica*. Finally, in study IV, we found one cpSSR haplotype in common between the *A. alba* population from Calabria and *A. numidica*.

Although firm conclusions about the relationships of *A. nebrodensis* with any of the *Abies* species investigated requires additional studies employing cladistic methods and larger sample sizes per taxon, results obtained in Papers III, IV and V provided sufficient information to construct the following tentative explanation for the origin and the evolution of this taxon.

The origin of *A. nebrodensis* can be dated back to the end of the Tertiary period, probably in one of the climatic crises of the latest parts of the Miocene (26-5 BP). At that time the *Abies* species were already differentiated and well distributed in the Northern Hemisphere. During the climatic crises the *Abies* range became fragmented and the species more isolated: *A. alba* was probably confined to the Apennines chain and to central and northern Europe, while the other *Abies* species were restricted to the mountainous regions of the Balkans, Northern Africa and the Middle East. The most striking event of this period took place at the end of the Miocene, in the Messinian. As indicated by the presence of vast quantities of evaporates, it is evident that the Mediterranean became a hyper-saline land-locked sea, located substantially below ocean level. The European and African continents were then connected and this marked the time of mammalian migrations to several Mediterranean islands (Hallam, 1994). It is likely that the connection, which lasted until the early Pliocene (5-1.6 My BP), when the marine flooding took place, offered a possibility for the North African *A. numidica* to come in contact with *A. alba*, probably in the region today occupied by Sicily, resulting in the appearance of *A. nebrodensis* through

hybridisation. During the successive ice ages of the Pleistocene (1.6-0.01 My BP), the land was subjected to considerable climatic fluctuations as the polar icecap successively expanded and retreated. One major consequence of this was a series of sea-level changes in the Mediterranean area, with the establishment of new land-links (Hallam, 1994), which may have facilitated additional contacts between *A. nebrodensis* and *A. numidica*. In the warmer period of the Holocene, *A. nebrodensis* became isolated from both *A. numidica* and *A. alba*, promoting further divergence. The affinities described in Papers III, IV and V among the *A. alba* from Calabria, *A. nebrodensis* and *A. numidica* seem to agree with this hypothesis.

The decline of *A. nebrodensis* occurred in recent times, mainly due to human activities. Indeed, several authors attested to the existence of extensive *Abies* forests on the Madonie Range until approximately 200 years ago (Morandini *et al.*, 1994 and references therein). By the beginning of the 19th century *A. nebrodensis* was considered lost by the scientific community, although later investigations led to the discovery of a few individuals in a restricted area in Sicily. These individuals, together with those discovered in the following years, constitute the extant *A. nebrodensis* population, all of which is at least 70-80 years old. It is likely that due to the severe reduction in *A. nebrodensis* size that occurred in the last two centuries, many alleles were lost in this species. Consequently, only a fraction of the original genetic variation was present in the small number of founders that gave rise to the extant population (founder effect). At the same time, the few available mates and the lack of contact with other heterogeneous sources of variation increased the level of relatedness among the individuals. This is in accordance with the findings in *A. nebrodensis* of a decreased level of cpDNA variation and a high percentage of individuals sharing an identical cpSSR haplotype (study IV).

However, it appears that despite the extremely small population size of *A. nebrodensis* and the reduced level of cpDNA variation, the few individuals left in this population still retain a considerable amount of the original genetic variation at the nuclear level (Vicario *et al.*, 1995; Ducci *et al.*, 1999; Paper V). Therefore, special attention should be given to their preservation as well as in the propagation of material for *ex situ* preservation.

Origin and evolution of the Mediterranean species

The complexity of the genus *Abies* and the variability in morphological traits of its species has often led to uncertainties in the classification of the Mediterranean taxa. Results from studies presented in Papers III and IV contributed to elucidate the relationships among these taxa and their migration history using cpDNA markers.

It was evident from the PCR-RFLP analysis presented in Paper III that the chloroplast genome of the ten *Abies* taxa studied shows pronounced intra-specific

diversity. Therefore we could not use our data for phylogenetic analysis. Usually, in phylogenetic studies, inter-specific comparisons of cpDNA variation have involved from one to few individuals per species analysed (Strauss & Doerksen, 1990; Wang & Szmidt, 1994; Tsumura *et al.*, 1995; Krupkin *et al.*, 1996). It was clear that, at least in the genus *Abies*, phylogenetic studies would require analysis of much larger numbers of individuals. However, the distribution of cpDNA haplotypes over the ten taxa investigated in study III, and the detection of haplotypes specific to the most differentiated taxa (*A. concolor*, *A. numidica*, *A. pinsapo* var. *marocana* and *A. pinsapo*) suggested that, at least at the haplotype level, the PCR-RFLP analysis offer considerable potential for the identification of species-specific cpDNA markers in *Abies*.

The pattern of variation observed among the *Abies* taxa studied in Paper III and IV suggested some preliminary hypotheses about their genetic relationships. *Abies alba*, *A. bornmuelleriana*, *A. nordmanniana*, *A. cephalonica* and *A. cilicica*, growing in relatively close regions in the eastern part of the Mediterranean area, harboured the same group of haplotypes. In contrast, the taxa growing in scattered and isolated areas in the southwestern Mediterranean regions, namely Sicily (*A. nebrodensis*), North Africa (*A. pinsapo* var. *marocana* and *A. numidica*), the Iberian Peninsula (*A. pinsapo*) and North America (*A. concolor*), were characterized by a different and more heterogeneous group of haplotypes. These results, together with the finding of different levels of genetic variation in the two groups of taxa, indicate that the two groups are genetically distinct and agree well with the hypothesis that the Mediterranean *Abies* species originated from a common *Abies* ancestor growing in the Balkan and Middle East regions and differentiated along an east-west axis in Europe.

Closing remarks

The studies presented in this thesis, illustrating different patterns of spatial population structure among the Mediterranean *Abies* species investigated, provided insights into the evolutionary processes resulting from courses of post-glacial colonization and subsequent isolation of the taxa, in conjunction with biotic factors like gene flow and mating system. The results provide a good example of how heavily cold periods of the Quaternary in Europe have influenced the amount and distribution of genetic variation in both animals and plants from the Mediterranean region.

On the whole the results agree with the hypothesis that species of the genus *Abies* in Europe had an Asiatic origin and differentiated along an east-west axis. According to this hypothesis, the differentiation started at the beginning of the Miocene period (26-5 My BP), or maybe earlier, from a common *Abies* ancestor growing in the Balkan and Middle East regions then followed an east-west

migration route (Figure 2). Taxa like *A. alba*, *A. bornmuelleriana*, *A. nordmanniana*, *A. cephalonica* and *A. cilicica* were the first to differentiate from the ancestor, and moved northward and eastward, expanding their ranges across large areas of Central Europe and the Middle East. In contrast *A. pinsapo* and *A. numidica* differentiated much later, when they reached the mountainous regions of southern Spain and North Africa, and have remained isolated there for a long time. It is likely that events like the Messinian salinity crisis of the last part of the Miocene, and the subsequent retreat of the species to their various refugia during the glaciations offered many opportunities for the differentiated *Abies* taxa to come into contact in several parts of the Mediterranean basin, resulting in the appearance of new species like *A. nebrodensis* in Sicily. It is also possible that other taxa like *A. borisii-regis* from central and northern Greece, *A. equii-trojani* from northwestern Turkey, and *A. bornmuelleriana* from northern Turkey originated at such times. The investigation of the origin of these taxa would provide important additional information on the genetics and evolution of the Mediterranean species of *Abies*.

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