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THE DISTRIBUTION OF THE GENETIC DIVERSITY IN *ARAUCARIA ANGUSTIFOLIA* (BERT.) O. KUNTZE POPULATIONS AND ITS IMPLICATIONS FOR THE CONSERVATION OF THE SPECIES' GENETIC RESOURCES

Institute of Forest Genetics and Forest Tree Breeding
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The distribution of the genetic diversity in *Araucaria angustifolia* (Bert.) O. Kuntze populations and its implications for the conservation of the species' genetic resources

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To my beloved Family

Gisele, Victor and Sofia

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1. GENERAL INTRODUCTION

1.1. CONSERVATION OF FOREST GENETIC RESOURCES

The need to conserve forest genetic resources has been widely recognized in recent times because of the risk associated with global changes in environment, including climate changes (Geburek and Turok, 2005). The longevity of trees makes a rapid adjustment to changing conditions relatively difficult. Therefore, conservation strategies of forest trees should take into consideration the subject of environmental changes in an evolutionary perspective (Mátyás, 2005).

A gene resource is defined as the biological material either knows to or expected to contain either specific or extensively variable genetic information (Ziehe et al., 1998). Genetic conservation is understood as the preservation of genetic resources in a condition allowing for their regeneration (Finkeldey and Hattemer, 2007). Based on the work of Ziehe et al. (1989), Finkeldey and Hattemer (2007) suggest the following sequence of procedures for conserving genetic resources of forest tree species:

- Defining priorities - due to their rarity or importance, certain sorts of genetic information are in more urgent requirement of conservation than others. Thus, the choice of priority targets for conservation enterprises has to be based on the ecological and/or economic importance of populations, species, or species groups and the potential risks to their gene pools and their hierarchical, historically formed structure (see section 7).

- Identifying clear objectives - in general, one can consider three main goals: (i) preservation of the potential for particular trait expressions; (ii) preservation of maximum variation; and (iii) preservation of adaptability (see section 9).

- Selecting genetic resources - it is fundamental to find populations most worthy of conservation, as well as to identify how the diversity wished to conserve is distributed in space (see section 7).

- Choosing the method of physical preservation of the genetic information - genetic resources can be preserved, through *in situ* (Figure 1.1) or *ex situ* approaches. Some strategies for *in situ* and *ex situ* conservation of forest genetic resources are reviewed by Rotach (2005), Skrøppa (2005), Klumpp (2005) and Wilhelm (2005).

- Regenerating the resource - the main goal of conservation enterprises for forest genetic resources is the establishment of a population that is both adapted to the environmental conditions existing during the regeneration of the resource and adaptable to future environmental changes. Thus, the regeneration of the resource has to be considered as an integral part of the conservation program.

1.2. CHARACTERIZATION OF GENETIC DIVERSITY IN NATURAL POPULATIONS

Diversity is one of the characteristics of living organisms and has primary biological implications. In most organisms, diversity is observed at morphological and molecular levels, and is the main factor allowing species, populations and ecosystems existence. Many traits observed in living organisms are effects of strict genetic control, while the expression of others may be shaped by the environment or may have no genetic basis at all (Hattemer, 2005). Genetic diversity is a fundamental feature of species, populations and ecosystems, because it represents the evolutionary potential to survive in a changing environment. Such a capability allowed the continued existence of many extant species during the glacial times, while several others went extinct.

Characterization of diversity has long been based primarily on morphological traits, which may be significantly affected by the environmental conditions. Molecular markers are expected to avoid many complications of environmental effects and have been widely applied as a complementary strategy to traditional approaches for characterizing genetic resources for conservation (Vendramin and Hansen, 2005). With the advances of molecular biology, a variety of different molecular genetic markers are available, allowing a relatively easy way to characterize the genetic diversity of natural populations. However, each molecular marker has its benefits and drawbacks. Therefore, choosing the most appropriate marker will depend on many factors as the precise purpose, the desired levels of polymorphism, the availability of technical facilities and the efficiency in terms of costs and time (Vendramin and Hansen, 2005).

In 1966, J. L Hubby and R. C. Lewontin introduced the analysis of isozymes in population genetics, a method expected to “detect a large proportion, if not all, of the isoallelic variation at a locus” (Hubby and Lewontin, 1966). With the progress of the molecular techniques, new markers emerged allowing the analysis of variation directly at the DNA level. These techniques are based on the use of restriction enzymes (Restriction Fragment Length Polymorphism, RFLP; Botstein et al., 1980), the polymerase chain reaction (PCR; Mullis and Faloona, 1987) using thermostable enzymes (e.g. microsatellites; Litt and Luty, 1989; Random Amplified Polymorphic DNA, RAPD; Williams et al., 1990) or a combination of both approaches (Amplified Fragment Length Polymorphism, AFLP; Vos et al., 1995). Modern sequencing strategies permit the assessment of variation at single nucleotide level (Single Nucleotide Polymorphism, SNPs), as well as the analysis of entire genomes. In addition to these methods, numerous variants are available (for a review, see Weising et al., 2005).

1.3. THE MIXED OMBROPHILOUS FOREST OR ARAUCARIA FOREST

The Araucaria forest is a particular ecosystem in southern and southeastern Brazil. It originated by an admixture of two distinct vegetations: the tropical afro-Brazilian and the temperate austro-Brazilian floras (Guerra et al., 2002). The dominant tree species in this forest is *Araucaria angustifolia* (Bert.) O. Kuntze, the unique representative of the family Araucariaceae in Brazil. The distribution of *A. angustifolia* is predominant in altitudes between 500 and 1800 m, from 19°15' to 31° southern latitude (Reitz and Klein, 1966). This forest is usually associated with grassland, mainly in the Santa Catarina state, southern Brazil.

During the later Holocene, the subtropical highlands of Brazil lacked forest formations and were covered by grassland, because of the cold and dry climate (Ledru et al., 1998). About 3,000 years ago, the climate changed and species of Araucaria forest started to migrate into the highlands of southeastern Brazil, in São Paulo state (Behling, 1997, 1998). About 1500 to 1000 years ago, the post-glacial migration followed in the states of Paraná, Santa Catarina and Rio Grande do Sul, in southern Brazil. Pollen records from southern Brazil suggest that grassland patches in highlands are natural remnants of a large Glacial and Early-Mid Holocene area (Behling and Pillar, 2007). With the expansion of Araucaria forests mainly during the last 1500 years, the grassland areas became markedly reduced. A current expansion of Araucaria forest over the grassland does not occur, mainly due to human activities such as forest logging and conversion of forest into pasture and agricultural lands. Figure 1.2 illustrates a classical formation of grassland and small patches of Araucaria forest in southern Brazil.

1.4. *ARAUCARIA ANGUSTIFOLIA*: TAXONOMICAL AND BOTANICAL ASPECTS

Araucaria angustifolia is the unique representative of family Araucariaceae in Brazil and together with the closely related species *A. araucana* (Setoguchi et al., 1998; Stefenon et al., 2006), the unique extant representative of the family in the American continent. The genus *Araucaria* (de Jussieu) includes 19 species, with current geographic distribution restricted to the Southern hemisphere (Golte, 1993). Based on ripening time and seed colour nine botanical varieties of *A. angustifolia* are described: 1) *elegans*; 2) *sancti josephi*; 3) *angustifolia*; 4) *caiova*; 5) *indehiscens*; 6) *nigra*; 7) *striata*; 8) *semi-alba*; and 9) *alba* (Reitz and Klein, 1966). Matos (1994) proposed an additional variety, *catarinensis*, which has seeds with an uncovered ventral face.

A. angustifolia is a long-lived dioecious conifer species with seeds dispersed mainly by gravity (barochory) and with wind dispersed pollen (anemophily). Alternatively, seeds may be dispersed by vertebrates. However, the transported seeds are often damaged by these animals and not able to germinate (Müller and Macedo, 1980; Mello Filho et al., 1981). The pollination occurs between September and October, and seed ripening occurs from March to June (Mantovani et al., 2004).

The stem is cylindrical and straight (20-50 meters height and 1-2 meters diameter; Figure 1.3). The young trees exhibit a pyramidal form with many branches (Figure 1.4). Adult individuals lack branches up to two thirds of their height, presenting an umbrella-shaped crown (Reitz and Klein, 1966).

1.5. *ARAUCARIA ANGUSTIFOLIA*: ECOLOGICAL, ECONOMICAL AND CULTURAL ASPECTS

Araucaria angustifolia is a dominant tree in the mixed ombrophilous forest. This species generates a particular micro-environment within the forest which allows the growth and survival of many shade-tolerant plant species (Figure 1.5). Many small vertebrates and invertebrates take advantage of the trees trunk and branches as housing and reproductive points. Additionally, seeds feed the wild fauna, supplying the most important source of food during the winter for mammalians and birds.

Because of its high quality wood, *A. angustifolia* was the most important Brazilian forest resource during the 1960's, corresponding to about 90% of the country's wood exportation (see Figure 1.6) at the end of this decade (Hueck 1972). Although covering around 200,000 km^2 of the Southern states of Brazil at the beginning of the 20th century, the intensive exploitation process reduced its area to about 3% (Guerra et al. 2002), leading this species to the vulnerable category of the IUCN Red List of Threatened Species. Despite the vulnerable status of the species, the exploitation continuously advances over the remnants of Araucaria forest, which is replaced by exotic fast-growing tree species (mainly *Pinus* spp. and *Eucalyptus* spp.) or agricultural lands.

Before the Brazilian colonization by European people, the native folks used to live near and inside Araucaria forests, which had high cultural importance. Indians of the Taquara/Itararé Tradition used to build pit houses inside the forests, throughout the southern Brazilian highlands until about 200 years ago (Bitencourt and Krauspenhar, 2006). One important feature of the current *A. angustifolia* distribution may be the seed dispersion by harvesting seeds, and possible management and planting of this species by the pottery-producing hunter-gatherers of the Taquara/Itararé folk (Bitencourt and Krauspenhar, 2006). Another proof of the cultural importance of the species is its close relationship with city names. Important cities in southern Brazil carry Indian names related to Araucaria forest. For instance, the name Curitiba, the capital of Paraná states, derivates from two Indian words, *Kurit* and *Yban* and means "huge amount of pine".

A

B

Figure 1.1: Natural regeneration into areas of *in situ* conservation of *A. angustifolia*. (A) Seedling growing in a forest gap. (B) Young individuals growing in a grassland area. (Photos: V.M. Stefenon)

Figure 1.2: Grassland landscape with small patches of *A. angustifolia* in the Acauan Farm, Bom Jesus municipality, Rio Grande do Sul state, Brazil. (Photo: V.M. Stefenon)

Figure 1.3: Measurement of the DBH (diameter at breast height) of an adult tree of *A. angustifolia* in Santa Catarina state, Brazil. (Photo: V.M. Stefenon)

Figure 1.4: A young individual of *A. angustifolia* (in first plane) and a group of adult trees in Santa Catarina state, Brazil. (Photo: V.M. Stefenon)

Figure 1.5: Shade-tolerant species (Bromeliaceae) growing over the stem of *A. angustifolia*. (Photo: V.M. Stefenon)

Figure 1.6: A truck ready to transport *A. angustifolia* timber at beginning of the 1970’s, in Santa Catarina state, Brazil. (Photo: personal archiv)

1.6. RESEARCH STRATEGIES AND OBJECTIVES OF THE STUDY

Genetic markers have been widely used as a tool to assess levels of genetic diversity, to determine species conservation status and to point out conservation and management strategies for different species. In this study, seven species of genus *Araucaria* were investigated using AFLP markers in order to explore phylogenetic relationships and evolutionary patterns of *A. angustifolia.* Patterns of among and within population diversity of this species were further investigated in six natural populations (n = 384) and five plantations (n = 192) using nuclear microsatellite and AFLP markers. Details about molecular and statistical methods are described throughout sections 6 to 10.

The central objective of this study was to characterize the distribution of genetic diversity of *A. angustifolia* at phylogenetic and population levels. The main hypotheses tested were:

- Morphological and molecular phylogenetic classification of *Araucaria* species are congruent, revealing high relationship among species growing in the same geographic region (section 6).

- Populations of *A. angustifolia* display high levels of differentiation, following an isolation-by-distance model, mainly as result of limited gene dispersal (sections 7 and 8).

- Different glacial refugia partly explain high differentiation between distant populations (sections 7 and 10).

- Migration through seed and pollen within and between populations are central factors in determining population structure of *A. angustifolia* (section 8).

- Production of reproductive material for plantation establishment will result in reduction of gene diversity and alteration of its original genetic structure (section 9).

- As effect of post-glacial colonization of highlands with small effective population sizes, *A. angustifolia* populations have undergone genetic bottlenecks (section 10).

2. General Discussion and Conclusions

2.1. Evolutionary history of *A. angustifolia*

Both macro- and microfossil data have much information to yield regarding species' evolutionary history, mainly when used in conjunction with ecological and morphological knowledge (Hill and Brodribb, 1999). According to Kershaw and Wagstaff (2001), all extant sections of the genus *Araucaria* (*Eutacta, Bunya, Araucaria* and *Intermedia*) likely evolved before the final break-up of the Gondwana continent. At the present time, this genus is restricted to the Southern Hemisphere, with 17 species in Australia and South Asia (15 species from section *Eutacta*; one species from section *Bunya* and one species from section *Intermedia*) and two species in South America (*A. angustifolia* and *A. araucana* from section *Araucaria*). Macrofossils belonging to section *Araucaria* have been recorded from the Cenozoic and Early Cretaceous of Australia and Argentina (Hill and Brodribb, 1999). *Araucaria nathorstii*, the earliest unequivocal fossil of leaves belonging to a species similar to extant representatives of section *Araucaria* was discovered in Argentina and stems from the Tertiary (about 65 million years ago; Stockey, 1994). The well supported monophyletic origin of the section *Araucaria* (100% bootstrap in the AFLP analysis, section 6; 88% bootstrap in the *rbcL* analysis, Setoguchi et al., 1998) supports its origin from a common ancestor. This ancestor should have migrated northward in the Gondwana continent (southern South America at the present time). Due to different environmental conditions, the process of speciation led the species *A. araucana* to the arid Andean areas and *A. angustifolia* to the moist regions of the southern Brazilian highlands. The phylogenetic relationship between these two species is clearly resolved at morphological and molecular level (see section 6). High similarity between these species is also suggested by caryologycal similarities (Bandel, 1970), the possibility of controlled hybridization (Barret, 1974; Vidaković, 1991) and the high transferability of microsatellite markers in these species (Salgueiro *et al.,* 2005). Likely the occurrence of natural hybridization between *A. angustifolia* and *A. araucana* is just prohibited by their geographical isolation.

Concerning microfossils, pollen of *A. angustifolia* dating from more than 40,000 years ago has been recorded in southern Brazil (Behling, 2002). Although the highlands in southern and southeastern Brazil have never been covered by ice sheets, the cold and dry conditions during the Last Glacial Maximum and late Holocene did not allow the survival of forest formations in these regions. *A. angustifolia* was found just in protected valleys and/or slopes where the moisture was elevated. The increase of the rainfall after the early Holocene allowed the expansion of forest species from refugia and *A. angustifolia* migrated onto the highlands, substituting the grassland. The different times of migration from refugia onto highlands (see sections 7 and 10) may have played a very important role in shaping the current genetic structure of *A. angustifolia* populations. The fitting of climatic dynamic reconstruction with the molecular signatures of population demography discussed in section 10 gives strong evidence of the importance of the post-glacial expansion for the current populations' genetic structure of this species.

2.2. THE GENETIC STRUCTURE OF *A. ANGUSTIFOLIA* POPULATIONS

The genetic structure observed in *A. angustifolia* populations is likely the effect of an isolation by distance process caused by limited gene flow through both seed and pollen (at least in some populations; see section 8). Effects of fragmentation due to recent forest exploitation on genetic diversity of *A. angustifolia* populations were suggested by Auler et al. (2002) in the analysis of nine natural populations with different levels of disturbance using isozyme markers. In general, the most preserved populations revealed a higher level of genetic diversity (percentage of polymorphic loci, H_e, H_o and number of alleles) in comparison to more degraded stands. The authors suggested that the fragmentation of the forest followed by the exploitation of the remnant fragments contributed to the genetic differentiation of the studied populations. However, they agree that "the time after fragmentation has so far been insufficient to allow a more substantial differentiation among populations" (Auler et al., 2002). Even though the exploitation of the forest during the last 200 years may have influenced the current genetic structure of the *A. angustifolia* populations, likely it is more strongly affected by long term evolutionary events, given the long generation span of this conifer species.

The results of the present study show that natural populations display a clear pattern of geographic differentiation, justifying the use of the species' geographic distribution as criterion for *in situ / ex situ* conservation strategies. Both marker systems (microsatellites and AFLPs) suggested a clear differentiation between southeastern and southern populations in Brazil. A particular differentiation related with geographic distribution of populations was revealed by microsatellites. These markers suggested the presence of three main groups of populations, with significant correlation between geographical distance and genetic differentiation over all populations (see section 7). These patterns corroborate previous studies that suggested the presence of geographical ecotypes in *A. angustifolia*. Studies based on provenance/progeny tests (Shimizu and Higa, 1980; Monteiro and Spelz, 1980; Kageyama and Jacob, 1980; Sebben et al., 2003) revealed evidences of geographical races among natural populations based on quantitative traits. In general, provenances from the southeastern region grow more in height while provenances from southern region revealed superior growth rates. Analogous conclusions concerning geographical differentiation were obtained from isozyme analyses, which revealed markedly differentiation among populations from southeastern and southern Brazil (Sousa et al., 2004). Similarly, natural populations from Santa Catarina state analysed by Auler et al. (2002) were differentiated in a northernmost and in a southernmost group based on isozyme variation. Hampp et al. (2000) found a south-to-north gradient in the presence of a sequenced DNA fragment of unknown origin. These author suggested that this fragment may be linked to adaptation to frost, which is less intense in southeastern Brazil. RAPD analyses performed by Mazza (2002) also revealed an accentuated differentiation of a population from southeastern Brazil from southern populations.

The presence of significant within population spatial genetic structure (SGS) revealed by both markers systems in this study suggests the occurrence of biparental mating. The patterns of fine-scale spatial genetic structure revealed by AFLPs suggested limited seed and pollen dispersion at the intra-population level. Data from microsatellite makers revealed somewhat weaker spatial genetic structure (see section 8). Significant spatial genetic structure up to 70 meters was also revealed in one natural population analyzed with isozyme markers by Mantovani et al. (2006). Given that *A. angustifolia* is a dioecious species, biparental inbreeding is the only source of family structure. Evidences of biparental inbreeding were obtained from isozyme analyses by means of outcrossing rates estimations. Although dioecious species are obligatory outcrossing, the difference between the multilocus and single-locus outcrossing rates is used as an inference of the biparental inbreeding within a population (Sousa et al., 2005). Mantovani et al. (2006) found a value of 0.058 for one population, while Sousa et al. (2005) reported values ranging from 0.018 to 0.061 in their estimations of biparental endogamy in four populations.

According to these patterns of within population spatial genetic structure, gene flow is comparatively limited. However, the extent of gene dispersal differs among populations. While population NG revealed the highest level of spatial genetic structure for microsatellite and AFLP data, population CJ revealed patterns congruent with comparatively larger gene dispersal (section 8). Congruent with the patterns of the spatial genetic structure analyses, the estimation of the effective number of migrants between two neighboring populations obtained from microsatellite data was around one individual per generation, revealing a dominance of short-distance gene dispersal (section 8). Contrasting with this result, relatively low genetic differentiation was revealed among populations from Santa Catarina and Rio Grande do Sul states (section 7), suggesting more effective gene flow, likely by means of migration through a stepping-stone model.

2.3. CONSERVATION OF THE *A. ANGUSTIFOLIA* GENETIC RESOURCES

Considering the results from this work and from previous studies, some basic recommendations may be discussed towards the conservation of the remainder fragments of *Araucaria* forest. The present work (section 7) and previous studies based on isozyme markers (Shimizu et al., 2000; Sousa et al., 2004; Mantovani et al., 2006) suggest that *A. angustifolia* remnants maintain relatively high levels of genetic diversity. Besides to focus on the geographical pattern of the species' distribution, this high diversity must be considered when selecting areas for *in situ* protection, collecting seeds for *ex situ* conservation and for reforestation enterprises. However, the maintenance of this high genetic diversity depends on the promotion of connectivity among remnants and the support of natural regeneration. In section 8 it was shown that although usually restricted, gene dispersal by means of both pollen and seed may occur at comparatively large distances. However, gene flow among remnants is completely dependent on their connectivity, allowing secondary seed dispersal by animals and stepping-stone pollen dispersal by wind.

Moreover, it was demonstrated in section 10 that isolation of small stands tends to impede the recovery of effective population size. On the other hand, even limited gene flow among small populations at equilibrium has the tendency to reduce negative effects of small population sizes.

Programs of reforestation may be a very important tool towards conserving *Araucaria* forest (section 9), through reforestation of degraded areas and recovery of impoverished stands lacking natural regeneration. All five plantations analysed in this study revealed high levels of genetic diversity and no remarkable changes in the original genetic structure (in terms of Hardy-Weinberg equilibrium and allelic frequencies), when compared to natural populations of the same geographical region where seeds were collected for the forest establishment. These results suggest that planted forests of *A. angustifolia* may be useful as source of species' genetic resource conservation. Ultimately, programs of reforestation should essentially incorporate local knowledge and skills, as well as the rational exploitation of secondary forest products and agroforestry by local people in order to be successful and sustainable.

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4. SUMMARY

Genetic markers have been widely used for the assessment of levels of genetic diversity in various species, for the determination of their conservation status and for the derivation of conservation and management strategies. In the present study, the pattern of phylogenetic relationships within the genus *Araucaria,* the genetic structure of natural populations and levels of genetic diversity in plantations of *A. angustifolia* were assessed using AFLP and nuclear microsatellite markers. Phylogenetic relationships were investigated for seven species of the genus *Araucaria* using four AFLP primer combinations (678 loci). Genetic diversity and structure of natural and planted populations of *A. angustifolia* were investigated using five microsatellite and 166 AFLP loci.

Section 6 deals with the utility of AFLP fingerprints to provide informative phylogenetic data on the genus *Araucaria*. The results of the ordination (PCO), cladistic (neighbour-joining cladogram) and phenetic (maximum parsimony) analyses revealed three distinct groups: [1] *A. angustifolia* and *A. araucana* (= section *Araucaria*), [2] *A. bidwillii* (= section *Bunya*) and [3] *A. cunninghamii, A. heterophylla, A. rulei,* and *A. scopulorum* (= section *Eutacta*). In the cladistic and phenetic analyses, phylogenetic trees were subdivided into two sister clades. One clade comprised all samples from section *Eutacta*. The other clade was divided again into two sister clades corresponding to sections *Araucaria* and *Bunya*. These results are congruent with the classification of the genus *Araucaria* based on chloroplast DNA sequences (*rbcL* region) and morphological traits. Whereas the fossil record points out that section *Bunya* is one of the oldest within the genus, the AFLP phylogenetic analysis does not support this hypothesis.

In section 7 results on the distribution of the genetic variation within and among natural populations of *A. angustifolia* growing in different regions in Brazil are reported. Both AFLP and microsatellite markers revealed high gene diversity, moderate overall differentiation but pronounced divergence of the northernmost, geographically isolated population. In a model-based Bayesian analysis of microsatellite data, this population was clearly differentiated from the southern populations. This result was confirmed by a cluster analysis of microsatellite data (bootstrap support > 95%). Non-hierarchical analysis of molecular variance revealed high variation among populations from different *a posteriori* defined geographical groups for both markers. The genetic distance between sample locations increased with geographical distance for microsatellites and AFLPs. These patterns of population differentiation agree with the geographical distribution of populations and are likely to be related to population history such as geographical isolation and postglacial colonization of highlands.

In section 8 the role of gene flow in determining genetic structures of *A. angustifolia* at intra- and inter-population levels is addressed. Due to morphological features of pollen and seed, limited gene dispersal has been assumed for this species. According to the estimation of both fine-scale

spatial genetic structure (SGS) and migration rate, the analysis of both nuclear microsatellite and AFLP markers suggested relatively short-distance gene dispersal. However, the intensity of gene dispersal differed among populations, and effects of more efficient dispersal were observed in at least one population. In addition, the results suggest that, even if some proportion of seed is aggregated, reasonable secondary seed dispersal within populations is presumably facilitated by overlaps of seed shadows and by vertebrates seed transport. In general, no correlation was observed between SGS and levels of inbreeding, population density or age structure, except that a higher level of SGS was detected in the population with a greater proportion of juvenile trees. Also, AFLPs revealed more SGS than microsatellites, which is probably due to broader genome coverage of the former. A low estimate of the number of migrants per generation between neighbouring populations was obtained, which explains the significant increase of genetic differentiation with increasing geographical distance (isolation-by-distance) as described in section 7. The comparatively low genetic differentiation described among southern populations may be explained through stepping-stone pollen flow.

Results on genetic diversity of planted populations are presented in section 9. Levels of genetic diversity were assessed in natural and planted populations of *A. angustifolia* in order to test the value of planted forests in genetic resources conservation programs. The results suggest that, in general, the original genetic structure of populations was not strongly altered in the plantations. For microsatellites, gene diversity (*H*) and allelic richness were significantly higher in plantations, while the degree of inbreeding did not differ between natural populations and planted stands. For AFLPs, no significant difference between groups in the measures of genetic diversity was found. The cluster analysis of natural populations based on microsatellite data mainly reflected a geographical pattern of grouping as shown in section 7. However, in cluster analysis based on AFLP data plantations were differentiated from natural populations. This pattern may be result of genetic hitchhiking of AFLP fragments with genes under selective pressure due to plantation establishment and management.

Further investigations on the evolutionary history of *A. angustifolia* populations are presented in section 10. Analyses were performed using the microsatellite data scored in the six natural populations and published isozyme allelic frequencies scored in 11 natural populations. The analysed populations covered the main area of the distribution range of the species in the states of São Paulo (4 populations), Paraná (2), Santa Catarina (10), and Rio Grande do Sul (1). The aim of this section was to investigate the relationship between historical demography and the current genetic structure of *A. angustifolia*. Traces of genetic bottlenecks were detected in four populations of southeastern Brazil. However, small past effective population size was indicated in only three out of the 13 southern populations. Based on the results on the current patterns of genetic diversity, it is suggested that southern populations experienced faster recovery of their effective size after migration onto highlands, while populations from southeastern Brazil recovered comparatively slower. In general, demographic history of *A. angustifolia* matches the climatic dynamics of

southern and southeastern Brazil during the Pleistocene and Holocene. The hypothesis of differential speed of the recovery of populations from southeastern and southern Brazil is also supported by palynological records and paleobotanical data of these regions.

On the basis of these results conservation efforts for *A. angustifolia* genetic resources are suggested: [1] *In situ* conservation of the relic populations and the promotion of their natural regeneration are crucial for the maintenance of the current patterns of genetic variation; [2] the geographic distribution of the species can be used as a rough and simple criterion to select *in situ* conservation areas, for planning seed collection and for the delineation of seed zones; [3] connectivity among fragments should be promoted in order to permit gene flow among relic populations; [4] sustainable management of the extant forest remnants and forestation and/or reforestation efforts should comply with the observed trends concerning population structure and gene flow; [5] the conservation of the genetic resources of the species may be strongly supported by planting seed stands and/or by enrichment plantings in degraded populations.

5. ZUSAMMENFASSUNG

Genetische Marker dienen häufig zur Messung genetischer Diversität, zur Feststellung des Erhaltungsstatus von Arten und zur Herleitung von Strategien der Erhaltung und Behandlung von Arten. In der vorliegenden Untersuchung wurden Muster der phylogenetischen Verhältnisse innerhalb der Gattung *Araucaria*, die genetische Struktur natürlicher Populationen und der Grad der genetischen Diversität in gepflanzten Beständen von *A. angustifolia* mit AFLPs und Kernmikrosatelliten ermittelt. Phylogenetische Verhältnisse wurden für sieben Arten der Gattung *Araucaria* mit vier AFLP-Primerkombinationen (678 Genloci) untersucht. Genetische Diversität und die Struktur natürlicher und gepflanzter Populationen von *A. angustifolia* wurden mit fünf Mikrosatelliten und 166 AFLP-Genloci analysiert.

Abschnitt 6 behandelt die Verwendbarkeit von AFLP-Fingerabdrücken zur Gewinnung informativer phylogenetischer Daten innerhalb der Gattung *Araucaria*. Die Ergebnisse der Ordination(PCO), phänetischer (neighbour-joining cladogram) und kladistischer (maximum parsimony) Analysen erbrachten drei eindeutige Gruppen: [1] *A.angustifolia* und *A. araucana* (= Sektion *Araucaria*), [2] *A. bidwillii* (= Sektion *Bunya*) und [3] *A. cunninghamii, A. heterophylla, A. rulei* und *A. scopulorum* (= Sektion *Eutacta*). In den kladistischen und phänetischen Analysen wurden die phylogenetischen Bäume in drei Gruppen (Kladen) unterteilt. Ein Klade enthielt alle Proben der Sektion *Eutacta*. Die andere Klade wurde abermals in Schwestergruppen unterteilt, welche den Sektionen *Araucaria* und *Bunya* entsprechen. Diese Resultate befinden sich in Übereinstimmung mit der Klassifikation der Gattung *Araucaria* anhand der Sequenzierung der Chloroplasten-DNA (*rbcL* Region) und anhand morphologischer Merkmale. Während Fossilfunde für die Sektion *Bunya* als eine der ältesten Sektionen der Gattung *Araucaria* sprechen, bestätigt die phylogenetische Analyse anhand von AFLPs diese Hypothese nicht.

In der Abschnitt 7 wird die Verteilung der genetischen Diversität in und zwischen natürlichen Populationen von *A. angustifolia* in verschiedenen Regionen Brasiliens analysiert. AFLP- und Mikrosatellitenmarker zeigten hohe Gendiversität, mäßige allgemeine Differenzierung, aber deutliche Abweichung der nördlichsten, geographisch isolierten Population. In einer modell-gestützten Bayes'schen Analyse der Mikrosatellitendaten erwies sich die nördlichste Population als von den südlichen klar differenziert. Dieses Resultat wurde durch eine Gruppierungsanalyse der Mikrosatellitendaten bestätigt (Bootstrap-Absicherung von > 95%). Die nicht-hierarchische Analyse der molekularen Variation beider Marker ließ hohe Variation zwischen Populationen verschiedener *a posteriori* definierter geographischer Gruppen erkennen. Der genetische Abstand zwischen Populationen erhöhte sich mit deren geographischem Abstand bei Mikrosatelliten als auch AFLPs. Diese Muster der Populationsdifferenzierung stimmen mit der geographischen Verbreitung der Populationen überein und stehen mit ihrer Geschichte wie ihrer geographischen Isolierung und der postglazialen Rückwanderung in die Hochlandgebiete in Einklang.

Im Abschnitt 9 wird die Rolle des Genflusses bei der Ausbildung genetischer Strukturen von *A. angustifolia* in und zwischen Populationen angesprochen. Aufgrund der morphologischen Eigenschaften von Pollen und Samen wird für diese Baumart begrenzter Genfluss angenommen. Schätzungen sowohl der kleinräumlichen genetischen Struktur (SGS) als auch der Migrationsrate anhand der Analyse von Kernmikrosatelliten und von AFLP-Markern legen verhältnismäßig kurze Abstände des Genflusses nahe. Jedoch unterschieden sich die Populationen in der Intensität des Gentransports, und in mindestens einem Bestand wurden Effekte effizienteren Genflusses beobachtet. Die Resultate legen ferner nahe, dass, selbst wenn ein Anteil von Samen nur über kurze Distanz verbreitet wird, z. B. Wirbeltiere für eine Sekundärverbreitung der Samen sorgen, so dass es zu einer Überlappung der Samenverbreitung („seed shadows") einzelner Bäume kommt. Insgesamt wurde eine Wechselbeziehung zwischen der Intensität der SGS, dem Inzuchtniveau, der Populationsdichte oder Altersstruktur nicht beobachtet, außer dass SGS in der Population mit größerem Anteil junger Bäume eine höhere Intensität aufwies. Auch ließen die AFLPs deutlichere SGS erkennen als die Mikrosatelliten, was vermutlich auf deren höherer Genomabdeckung beruht. Eine geringe geschätzte Anzahl von Migranten pro Generation zwischen benachbarten Populationen erklärt die bedeutende Zunahme der genetischen Differenzierung mit der Zunahme ihres geographischen Abstandes (isolation-by-distance), über die im Abschnitt 7 berichtet wurde. Die vergleichsweise geringe genetische Differenzierung unter den südlichen Populationen lässt sich durch Pollentransport nach dem stepping-stone-Modell erklären.

Die genetische Diversität künstlich begründeter Populationen wird im Abschnitt 9 dargestellt. Um die Verwendbarkeit von gepflanzten Beständen im Rahmen von Erhaltungsprogrammen der genetischen Ressourcen der Art zu prüfen, wurde der Grad genetischer Diversität sowohl in natürlichen Populationen als auch Pflanzbeständen von *A. angustifolia* ermittelt. Die Resultate wiesen darauf hin, dass die ursprüngliche genetische Struktur der Populationen sich im allgemeinen nicht stark veränderte. Für Mikrosatelliten waren Gendiversität (H) und allelischer Reichtum (allelic richness) in den Kunstbeständen erheblich höher, der Inzuchtgrad künstlicher und natürlicher Populationen aber nicht unterschiedlich. Für AFLPs wurde in den Maßen der genetischen Diversität kein bedeutsamer Unterschied zwischen Gruppen gefunden. Die auf Mikrosatellitendaten basierende Gruppierungsanalyse reflektierte im wesentlichen ein geographisches Muster der Populationen, wie es für die natürlichen Populationen in Abschnitt 7 beschrieben wurde. Nach der auf AFLP-Daten basierenden Gruppierungsanalyse der Populationen unterschieden sich die künstlichen von den natürlichen Populationen. Dieses Ergebnis kann auf Kopplung der AFLP-Fragmente mit Genen (oder andere hitch-hiking-Effekte) beruhen, die einem von Begründung und Behandlung der Pflanzbestände ausgehenden Selektionsdruck ausgesetzt sind.

Weitere Untersuchungen über die Entwicklungsgeschichte der Populationen von *A. angustifolia* werden im Abschnitt 10 dargestellt. Die Analysen wurden mit Daten über Mikrosatelliten in den sechs natürlichen Populationen bzw. mit solchen Daten angestellt, die andere Autoren über

Allelfrequenzen von Enzymgenloci in 11 Populationen veröffentlicht hatten. Die untersuchten Populationen umfassten den Hauptbereich des Verbreitungsgebiets in den Staaten São Paulo (4 Populationen), Paraná (2), Santa Catarina (10) und Rio Grande do Sul (1). Ziel der Analyse war die Untersuchung des Zusammenhangs zwischen historischer Demographie und gegenwärtiger genetischer Struktur von *A. angustifolia*. Anzeichen für Flaschenhalseffekte wurden in allen vier Populationen im Südosten Brasiliens beobachtet. Spuren geringer effektiver Populationsgröße waren jedoch nur in drei der 13 südlichen Populationen festzustellen. Die Ergebnisse über die gegenwärtigen Muster der genetischen Diversität legen nahe, dass sich nach Besiedlung höherer Lagen die effektive Größe südlicher Populationen rascher erholte als die der südöstlichen. Ganz allgemein steht die demographische Geschichte von *A. angustifolia* mit der Dynamik des Klimas im südlichen und südöstlichen Brasilien während des Pleistozäns und Holozäns in Einklang. Die Hypothese unterschiedlich rascher Erholung südöstlicher und südlicher Populationen wird auch durch palynologische Funde und paläobotanische Daten dieser Regionen gestützt.
Auf der Grundlage dieser Ergebnisse werden Verfahrensweisen für die Erhaltung genetischer Ressourcen von *A. angustifolia* vorgeschlagen: [1] Die *in-situ* Erhaltung der Restvorkommen und die Förderung ihrer natürlichen Verjüngung sind für die Aufrechterhaltung der gegenwärtigen Muster der genetischen Diversität entscheidend; [2] bei der Vorauswahl der Bereiche für die Erhaltung *in situ*, bei der Planung der Samengewinnung und der Ausweisung von Saatgutzonen (Herkunftsgebieten) kann die geographische Verteilung der Art als näherungsweises und einfaches Kriterium dienen; [3] die Konnektivität der Populationsfragmente sollte gefördert werden, indem Genfluss ermöglicht wird; [4] die beobachteten Tendenzen von Populationsstrukturen und Genfluss sollten bei der nachhaltigen Behandlung der vorhandenen Restvorkommen und der Aufforstung bzw. Wiederaufforstung Beachtung finden; [5] die Begründung von Saatguterntebeständen und/oder die Anreicherung degradierter Populationen können die Erhaltung der Art wirksam unterstützen.

6. PHYLOGENETIC RELATIONSHIP WITHIN GENUS *ARAUCARIA* (ARAUCARIACEAE) ASSESSED BY MEANS OF AFLP FINGERPRINTS [1] [2]

Abstract

Highly polymorphic AFLP markers were applied to analyse the phylogenetic relationships of seven species from three sections within genus *Araucaria* (Araucariaceae) with cladistic and phenetic approaches. The objectives of the study were to compare the intrageneric relationships within *Araucaria* assessed by AFLP markers with the classification according to chloroplast DNA sequences and morphological characters. The AMOVA revealed 48% of the variation among species. The results of the principal coordinate analysis revealed three distinct groups: (1) *A. angustifolia* and *A. araucana* (= section *Araucaria*), (2) *A. bidwillii* (= section *Bunya*) and (3) *A. cunninghamii, A, heterophylla, A. rulei* and *A. scopulorum* (= section *Eutacta*). In the cladistic and phenetic analyses, phylogenetic trees were subdivided into two sister clades, one comprising the samples from section *Eutacta*, the other one was divided again into two sister clades corresponding to sections *Araucaria* and *Bunya*. These results are congruent with a previous phylogenetic study of the family Araucariaceae based on *rbcL* sequences and with the classification of genus *Araucaria* based on morphological characters. Both *rbcL* sequence data and AFLP analyses do not support section *Bunya* as one of the oldest sections within genus *Araucaria,* as suggested by the fossil record. The utility of AFLP markers for phylogenetic analyses is discussed.

Key words: Araucaria, AFLP, phylogeny, phylogenetic relationships

[1] Stefenon, V.M., Gailing, O. and Finkeldey, R. (2006) **Silvae Genetica** 55(2): 45-52.

[2] VMS conceived, designed and performed the experiments, analyzed the data and wrote the paper. All authors improved the final manuscript.

Introduction

The genus *Araucaria* de Jussieu (Family Araucariaceae*,* Order *Coniferales*) includes 19 species. Its current geographic distribution is restricted to the Southern hemisphere (Golte, 1993). Despite their important ecological and economical role, some species like the South American *A. angustifolia* (Bert.) O. Ktze and *A. araucana* (Mol.) K. Koch are nowadays classified as vulnerable due to intense human pressures (Bekessy *et al.,* 2002; Stefenon and Nodari, 2003).

From an origin in the Triassic, the family Araucariaceae expanded and diversified in both hemispheres in the Jurassic and Early Cretaceous (Kershaw and Wagstaff, 2001). Within genus *Araucaria,* the fossil records suggest a basal position of section *Bunya* as one of the oldest recorded sections (Stockey and Taylor, 1978).

A phylogenetic study of the *rbcL* gene for the family Araucariaceae (Setoguchi *et al.,* 1998) revealed a clear structure within the genus *Araucaria*. This structure is in accordance with the taxonomic classification based on morphological characters in sections *Araucaria*, *Eutacta, Intermedia* and *Bunya*. However, molecular data of the *rbcL* gene did not support the early divergence of the monotypic section *Bunya* (Setoguchi *et al.,* 1998). These authors suggested that further molecular data should be added to enhance the statistical probability concerning the position of *A. bidwillii* Hook. (the only extant species in section *Bunya*) in the phylogenetic tree. The *rbcL* sequence is very commonly used for phylogenetic analyses. However, some studies have shown that its sequence is much conserved and sometimes not able to clarify relationships between closely related taxa (Wang *et al.,* 1999; Rydin and Wiström, 2002). According to Wang *et al.* (1999), *rbcL* tends to be conservative among some genera of the gymnosperm family Pinaceae.

AFLPs are highly polymorphic dominant markers that cover a larger proportion of the whole genome (Mueller and Wolfenbarger, 1999), randomly accessing both coding (rather conservative) and non-coding (not necessarily conservative) regions. Thus, they may provide many informative markers to complement the single gene *rbcL* information within genus *Araucaria*. The AFLP technique has been used to reveal evolutionary relationships at the species or genus level (Koopman *et al.,* 2001; Beardsley *et al.*, 2003; Brouat *et al.,* 2004) and is considered to be able to resolve phylogenetic relationships congruent with analyses based on morphological characters and on nuclear markers as internal transcribed spacers (ITS) or restriction fragment length polymorphisms (RFLPs) (Brouat, *et al.* 2004). Here, we report a phylogenetic analysis of the genus *Araucaria* generated by means of AFLP markers and discuss the capacity of these markers to produce informative phylogenetic data to an ‘ancient’ genus of gymnosperms.

Material and Methods

Plant material

Plant material was collected from botanical and private gardens (see Table 6.1). Seven species of genus *Araucaria* (*A. angustifolia* (Bert.) O. Ktze., *A. araucana* K. Koch, *A. bidwillii* Hook., *A. cunninghamii* Aiton ex D. Don., *A. heterophylla* (Salisb.) Franco, *A. rulei* F. Muell. and *A. scopulorum* de Laub.) corresponding to three sections (*Araucaria, Bunya* and *Eutacta*) were investigated. *Agathis robusta* (F. Muell.) F. M. Bailey (Araucariaceae) was used as an outgroup (see Table 6.1). Species identification of the samples was performed in the respective botanical gardens, with exception of sample 'ang5' (cultivated in a private garden in Brazil) that was identified by V. M. Stefenon. Identification of the samples was confirmed in our laboratory and doubtful samples were excluded from the analysis. Voucher specimens were deposited in the Institute of Forest Genetics and Forest Tree Breeding of the Georg-August-University Göttingen. The natural distribution of the species is shown in Figure 6.1.

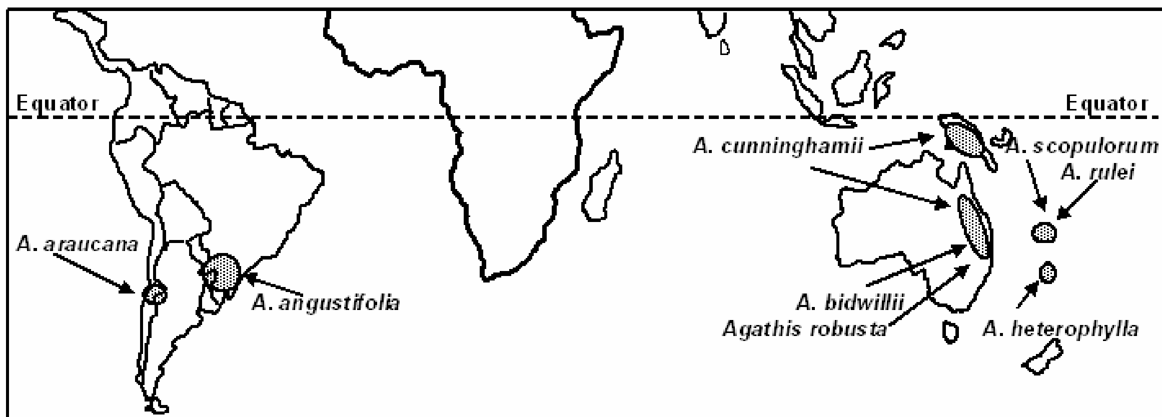

Figure 6.1: Natural geographical distribution of *Araucaria* species analysed and outgroup species *Agathis robusta. A. angustifolia* (Brazil, Argentina and Paraguay), *A. araucana* (Chile and Argentina), *A. bidwillii* (Australia), *A. cunninghamii* (Australia and New Guinea), *A. heterophylla* (Norfolk Island), *A. rulei* (New Caledonia), *A. scopulorum* (New Caledonia) and *Agathis robusta* (Australia). After SETOGUCHI *et al.* (1998) and GOTE (1993).

Table 6.1: Plant material sampled for the phylogenetic analysis and names applied.

Species (Section)	Sample name	Source
A. angustifolia (Araucaria)	ang1	University of Freiburg – Germany
	ang2	University of Tübingen – Germany
	ang3	University of Gießen – Germany
	ang4	University of Oldenburg – Germany
	ang5	Cultivated in Private Garden – Lages - Brazil
A. araucana (Araucaria)	ara1	University of Gießen – Germany
	ara2	University of Tübingen – Germany
	ara3	University of Oldenburg – Germany
	ara4	Free University of Berlin – Germany
	ara5	University of Göttingen – Germany
A. bidwillii (Bunya)	bid1	University of Freiburg – Germany
	bid2	University of Gießen – Germany
	bid3	University of Tübingen – Germany
A. heterophylla (Eutacta)	het1	University of Freiburg – Germany
	het2	University of Gießen – Germany
	het3	University of Oldenburg – Germany
A. cunninghamii (Eutacta)	cun1	University of Tübingen – Germany
	cun3	University of Göttingen – Germany
A. scopulorum (Eutacta)	sco	University of Tübingen – Germany
A. rulei (Eutacta)	rul	University of Tübingen – Germany
Agathis robusta (outgroup)	Agathis	University of Göttingen – Germany

DNA isolation and AFLP analysis

About fifty milligrams of plant material were disrupted in a 96-well block and the total DNA was extracted using the DNEasy 96 Plant Kit (Qiagen), following the instructions of the manufacturer. The AFLP reactions were performed as described by Vos *et al.* (1995), with slight modifications as described by Gailing and von Wuehlisch (2004). About 150 ng of genomic DNA was incubated at

room temperature for about 16 hours for the digestion with the restriction enzymes *Eco*RI and *Mse*I and the ligation of the corresponding *Eco*RI- and *Mse*I-adapters to the ends of the restriction fragments. A pre-selective amplification was performed with the primer pairs displaying one selective nucleotide, namely *Eco*-primer + A (E-A) and *Mse*-primer + G (M-G). The PCR protocol for the pre-selective amplification consisted of an initial step at 72 °C for 2 min followed by 20 cycles at 94°C for 10s, at 56 °C for 30s, at 72°C for 2 min and of a final extension step at 60°C for 30 min. Four microliters of the diluted (1:10) pre-selective reaction were used as template for the selective amplification with the following primer combinations: E-AGA/M-GGA, E-AGA/M-GGG, E-AGC/M-GCC and E-AGC/M-GGA. The PCR protocol for the selective reaction was: a 2 min denaturation at 94 °C, 9 cycles at 94°C for 10s, an annealing step at 65°C for 30s (which was decreased by 1 °C every cycle until 56 °C was reached) and an extension step at 72°C for 2 min. The reaction was continued with an annealing temperature of 56 °C for the last 24 cycles ending with a final extension step at 60 °C for 30 min. All PCR reactions were carried out in a Peltier Thermal Cycler PTC-200 (MJ Research). The *Eco*RI selective primers were labelled with the fluorescent dyes NED or HEX. The fragments were separated on an ABI Genetic Analyser 3100 with the internal size standard GS 500 ROX (Applied Biosystems). The data were analysed using GeneScan 3.7® and Genotyper 3.7® software (Applied Biosystems). Bands between 50 and 350 bp (>50 rescaled peak height) were analysed. Absence (0) and presence (1) of fragments was scored and transformed into a binary matrix for data analysis. After confirming that the analysed species were monophyletic, the pre-amplified DNA of up to five samples of each species (see Table 6.1) was bulked and this bulked DNA served as template for a selective AFLP amplification using the same selective primer pairs and analysis parameters (see above).

Data analysis

Initially, genetic relatedness of species and sections were assessed for the data set of all individuals using an analysis of molecular variance (procedure AMOVA from Arlequin 2.0; Schneider *et al.,* 2000) and a principal coordinate analysis (PCO) based on Dice's coefficient of similarity (Dice, 1945) using the procedures SIMQUAL, DCENTER and EIGEN from NTSYSpc 2.0 (Rohlf, 1998). In addition, the data set of all individuals and the data set of bulked DNA were analysed with phenetic (Neighbor-Joining; NJ) and cladistic (Maximum Parsimony; MP) approaches using the software PAUP* version 4.0b10 (Swofford, 1998). The NJ analysis was performed using the genetic distance of Nei and Li (Nei and Li, 1979), which is the complement of Dice's coefficient of similarity, equalling 1 – "Dice". The parsimony heuristic tree searches were carried out under equal weight criterion, the tree bisection-reconnection (TBR) branch swapping algorithm and the option to collapse branches at zero length. A bootstrap analysis (Felsenstein, 1985) with 1000 replicates was conducted to assess the internal support for taxa in NJ and MP analyses.

The NJ and MP trees generated with the data set of all individuals were visually compared to assess the congruence between both analyses (see Fig. 6.3). Additionally, the topology of the MP

tree derived from bulked DNA samples was compared with the MP tree calculated from *rbcL* sequences after Setoguchi *et al*. (1998), in order to assess the congruence between AFLP and cpDNA analyses (see Fig. 6.4).

Results and Discussion

Relationship among species

Following the parameters applied for markers selection, the four primer combinations generated a total of 678 polymorphic markers. From 136 to 210 markers could be analysed per primer combination (mean number = 169.5 markers).

The partitioning of the molecular variance (AMOVA) among species was calculated for the data set of all individuals and revealed that 48% of the variation reside among species (Φ_{ST}=0.48; $p<0.001$).

The PCO analysis generated three groups that were clearly differentiated and corresponded to sections *Araucaria, Bunya* and *Eutacta.* The first principal coordinate explained 23% and the second principal coordinate explained 13% of the total variation. The three represented sections were clearly differentiated. Samples of *A. angustifolia* and *A. araucana* (Section *Araucaria*) group together, while the monotypic section *Bunya*, represented by *A. bidwillii*, is also clearly separated in this analysis. The structure among species within section *Eutacta* was not clarified, but the four species analysed of this section form a distinct group (Fig. 6.2).

For the data set with all individuals, ninety percent (608 out of 678) of the AFLP markers were parsimony-informative, while the NJ tree was generated with all 678 markers. Pairwise genetic distances (Nei and Li, 1979) between samples are shown in Table 6.2. In the MP analysis heuristic search yielded two shortest trees of 2003 steps, a consistency index (CI) of 0.31 and a retention index (RI) of 0.53. The consistency index in the parsimony analysis suggests a high level of homoplasy. Nevertheless all sections and species were supported by high bootstrap values (Fig. 6.3). *Araucaria rulei* and *A. scopulorum* (section *Eutacta*) that are represented only by unique samples cluster together in clade *Eutacta* and show the same position in *rbcL* and AFLP trees (Fig. 6.4) suggesting that also unique samples are informative to represent the respective species.

Comparing cladistic and phenetic analyses no difference was observed in the general topology of the generated trees (see Fig. 6.3). The monotypic section *Bunya* revealed to be sister group to section *Araucaria* and the *Eutacta* clade is sister to the *Araucaria*/*Bunya* clade. Thus, the basal position of *Bunya* as one of the oldest recorded sections, as indicated by the fossil record (Stockey and Taylor, 1978), is not supported by our analysis. Congruence with *rbcL* data (Setoguchi *et al.,* 1998) and more recent paleobotanical evidence (Stockey, 1994) suggest that many fossils formerly named as *Bunya* should be re-evaluated.

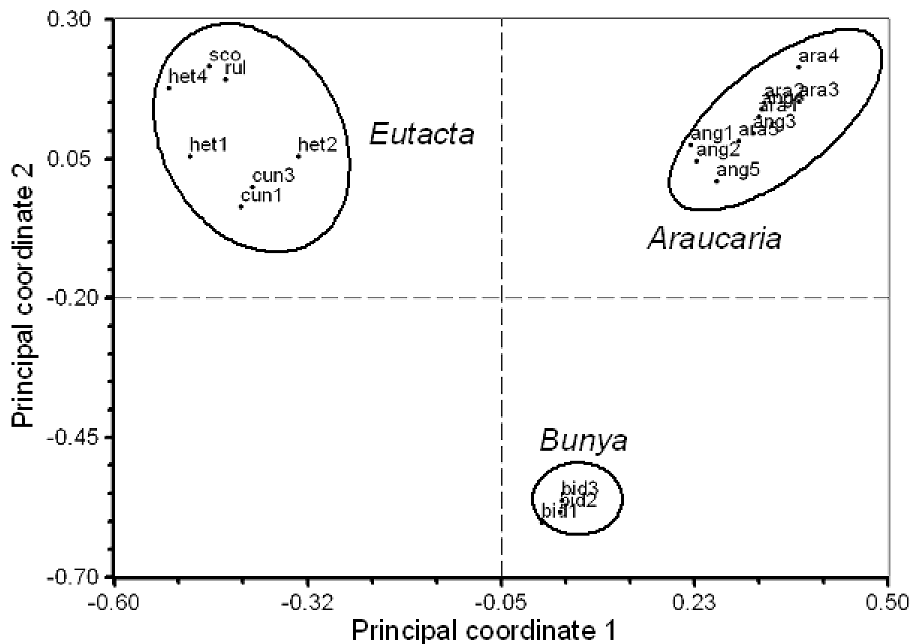

Figure 6.2: Principal coordinate analyses (PCO) based in Dice genetic distance showing differentiation among sections of genus *Araucaria*. The first coordinate describes 23% and the second coordinate 13% of the total variation. For samples codes see Table 6.1.

According to the NJ tree *A. cunninghamii* is sister to the other species of section *Eutacta*. *A. heterophylla* forms a well supported sister clade to *A. rulei* and *A. scopulorum*. In the MP tree these species form a polytomy. The clade comprising *A. heterophylla* and *A. cunninghamii* as sister species has only 50% bootstrap support.

Phylogenetic trees calculated for individual samples (Fig. 6.3) and from bulked DNA (Fig. 6.4) show the same topology and are congruent with the *rbcL* Maximum Parsimony analysis from Setoguchi *et al.* (1998). Figure 6.4 shows a comparison between the AFLP phylogram generated from bulked DNA (678 AFLP markers, 430 parsimony-informative markers, tree length=1152 steps, CI=0.48, RI=0.36) and the *rbcL* phylogeny.

Section *Araucaria* (*A. angustifolia* and *A. araucana*) and the monotypic section *Bunya* (*A. bidwillii*) that group together in the AFLP tree also share important taxonomic characters. Both sections are characterized by large and flat leaves, hypogeal germination, fleshy seedlings and two cotyledons that are long-stalked during germination and retained in seed coats (Stockey, 1982; Golte, 1993). Species from section *Eutacta* display smaller leaves, epigeal germination, four sub-sessile cotyledons that are freed from seed walls at germination and no fleshy seedlings (Stockey, 1982; Golte, 1993).

Table 6.2: Pairwise genetic distances (Nei and Li) between samples of *Araucaria*. For samples codes see Table 6.1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 ang1	-																				
2 ang2	0.099	-																			
3 ang3	0.085	0.073	-																		
4 ang4	0.055	0.069	0.048	-																	
5 ang5	0.080	0.035	0.070	0.058	-																
6 ara1	0.128	0.081	0.102	0.115	0.077	-															
7 ara2	0.130	0.095	0.101	0.111	0.099	0.055	-														
8 ara3	0.125	0.093	0.082	0.097	0.091	0.058	0.052	-													
9 ara4	0.123	0.123	0.086	0.089	0.123	0.097	0.073	0.080	-												
10 ara5	0.122	0.060	0.095	0.101	0.073	0.039	0.061	0.060	0.105	-											
11 het1	0.174	0.139	0.158	0.163	0.149	0.157	0.167	0.174	0.190	0.143	-										
12 het2	0.142	0.124	0.149	0.150	0.131	0.130	0.156	0.153	0.177	0.129	0.039	-									
13 het4	0.177	0.143	0.158	0.165	0.152	0.153	0.172	0.164	0.197	0.137	0.019	0.042	-								
14 cun1	0.164	0.162	0.186	0.189	0.159	0.190	0.197	0.195	0.229	0.172	0.117	0.110	0.117	-							
15 cun3	0.165	0.150	0.175	0.178	0.152	0.163	0.174	0.187	0.220	0.154	0.095	0.092	0.102	0.053	-						
16 rul	0.218	0.158	0.178	0.185	0.167	0.194	0.190	0.196	0.212	0.181	0.094	0.121	0.095	0.155	0.141	-					
17 bid1	0.142	0.119	0.129	0.146	0.112	0.149	0.145	0.144	0.172	0.122	0.145	0.142	0.158	0.150	0.152	0.195	-				
18 bid2	0.158	0.116	0.139	0.145	0.113	0.127	0.132	0.126	0.171	0.119	0.148	0.142	0.153	0.160	0.161	0.186	0.048	-			
19 bid3	0.162	0.120	0.130	0.152	0.116	0.126	0.138	0.126	0.159	0.116	0.153	0.145	0.164	0.177	0.168	0.190	0.054	0.050	-		
20 sco	0.203	0.164	0.181	0.193	0.174	0.184	0.171	0.177	0.195	0.167	0.089	0.106	0.086	0.132	0.129	0.081	0.192	0.194	0.179	-	
21 Agathis	0.228	0.180	0.219	0.223	0.169	0.191	0.210	0.224	0.326	0.168	0.216	0.195	0.218	0.204	0.197	0.261	0.192	0.194	0.200	0.234	-

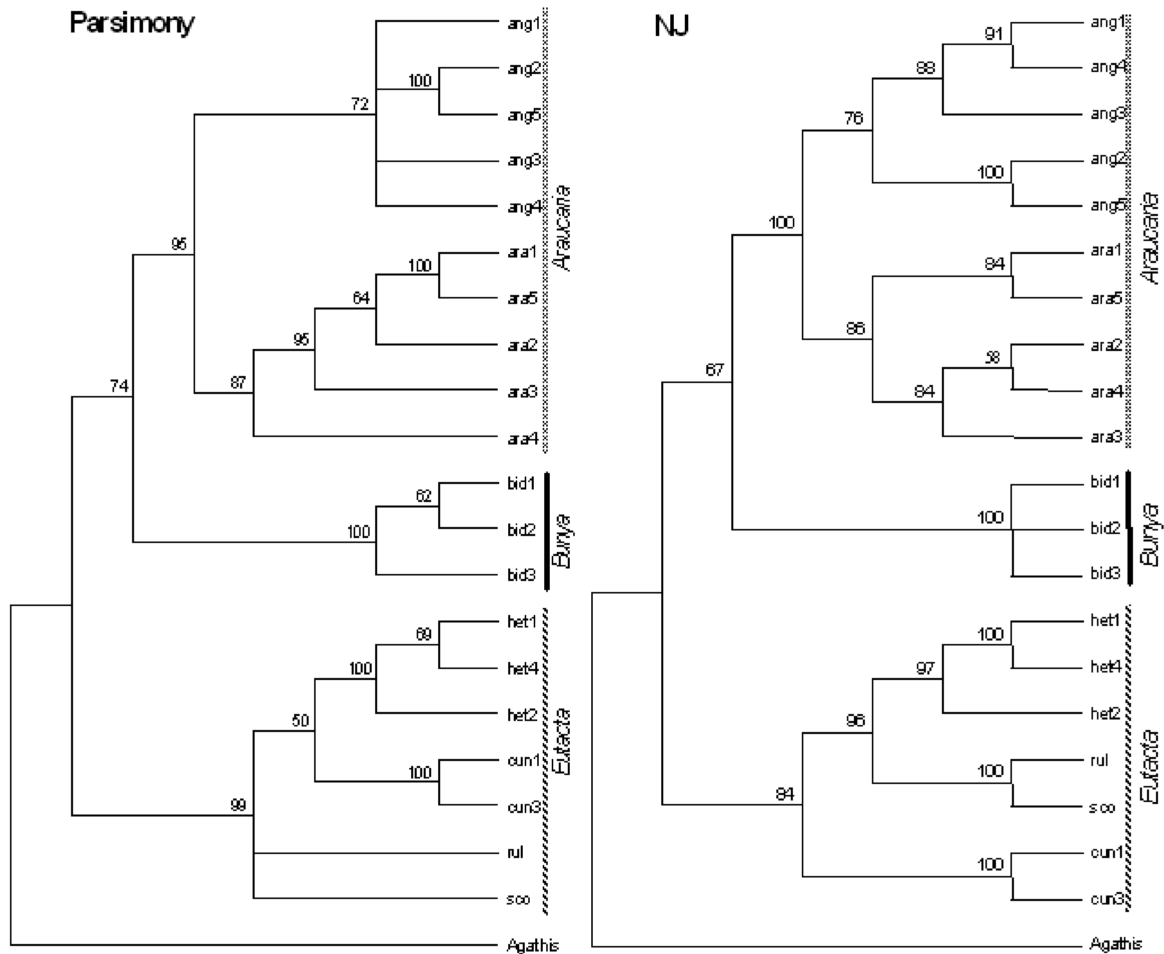

Figure 6.3: Parsimony and neighborjoining (genetic distance of Nei and Li) phylogenetic trees generated with 678 AFLP markers for individuals of seven species of *Araucaria* from South America (*A. angustifolia* and *A. araucana*) and Australasia (*A. cunninghamii, A. heterophylla, A. rulei, A. scopulorum* and *A. bidwillii*). Bootstrap values for 1000 replicates are shown for each node. Sections are indicated below branches. For samples codes see Table 6.1.

Within section *Araucaria* a considerable differentiation supported by high bootstrap values was observed among individuals within species *A. angustifolia* and *A. araucana* suggesting that the individuals sampled in Botanical Gardens originated from different geographic locations.

In conclusion, the relationship among species of *Araucaria* revealed by AFLPs are in accordance with prior classifications based on molecular (*rbcL* sequences; Setoguchi *et al.*, 1998) and morphological (Stockey, 1982) studies.

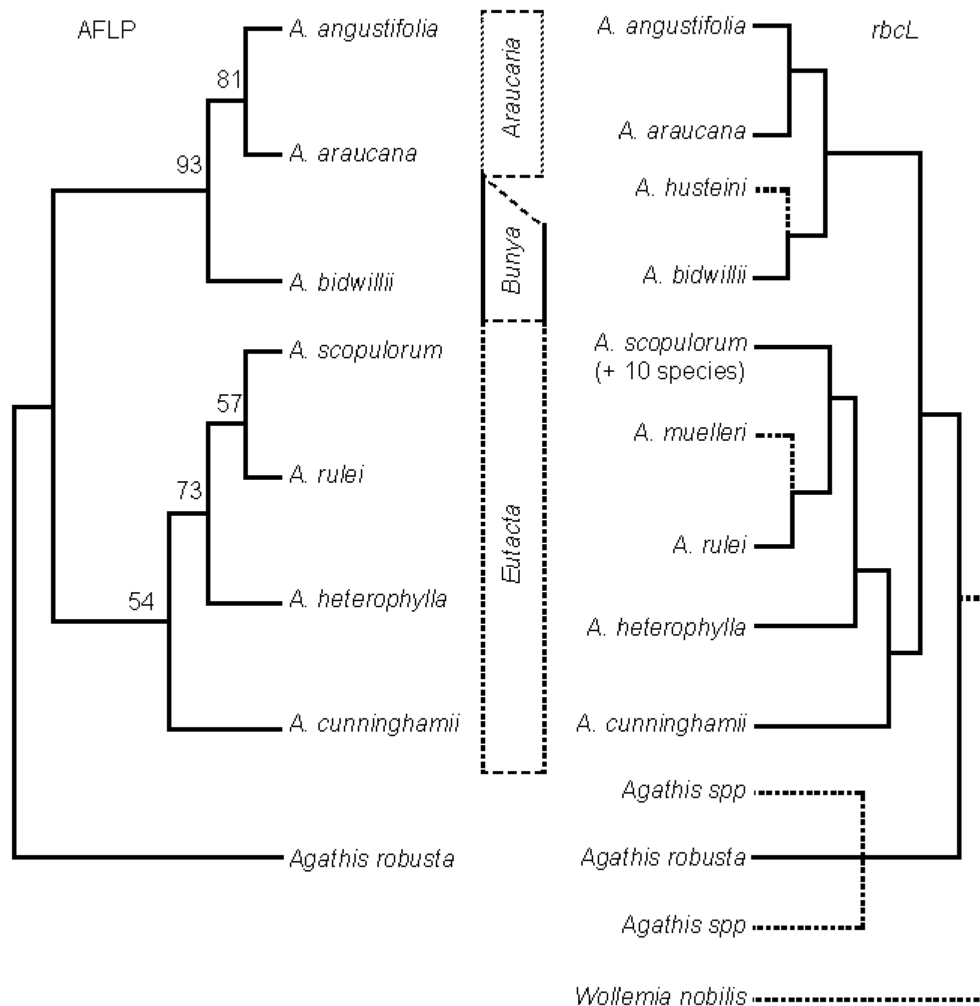

Figure 6.4: Comparison between AFLP (bulked DNA) and *rbcL* gene sequences maximum-parsimony phylogenies of *Araucaria.* Numbers at each node in the AFLP tree represent bootstrap values (1000 replicates). Doted branches in the *rbcL* tree show species not included in the present AFLP study. *rbcL* phylogenetic tree are adapted from SETOGUCHI *et al*. (1998).

Usefulness of AFLP technique

Despite the wide use of AFLP markers for genetic studies, there are doubts of using this technique to determine phylogenetic relationships. In order to establish phylogenetic relationships among taxa, the character analysed must show homologous similarities (modification by descent). One of the weaknesses of AFLP markers to assess phylogenetic relationships is the fact that fragments of related taxa may have the same length, but a different sequence and are therefore not orthologous. With increasing genetic differentiation among taxa fragments of the same size are more likely to be not orthologous (Mechanda et al. 2004).

Furthermore, it is known that there are many duplication events during species evolution resulting in paralogs that constitute a general problem in deducing phylogenies. This problem is even more acute,

if single genes from a multigene family (and not single copy genes) are analysed.

However, the strongest advantage of AFLP markers to infer phylogenetic relationships is that they sample from many regions of the genome, generating a large number of markers (Mueller and Wolfenbarger, 1999; Weising *et al*., 2005). These genome-wide data sets may provide high power in testing specific phylogenetic relationships (Rokas *et al.,* 2003).

If a large number of AFLP markers are investigated, many of them are likely to be orthologous. Indeed, Rouppe van der Voort *et al.* (1997) found 19 identical sequences out of 20 putatively homologous AFLP markers sequenced in potato. Parsons and Shaw (2001) sequenced ten AFLP fragments co-migrating in cricket species (genus *Laupala*) and found a degree of sequence similarity of the same-sized bands between 97 and 100%. They suggested that same-sized AFLP fragments can be confidently considered as homologous. Thus due to the large number of markers analysed any bias in phylogenetic inference are likely to be small and the results will accurately reflect the genetic relationships among taxa (Parsons and Shaw, 2001).

Since the relative amount of homoplasic AFLP fragments and their effect on reconstructing phylogenetic relationships is difficult to assess, the application of either phenetic or cladistic approaches when using AFLP markers has been discussed (Koopman *et al.,* 2001; Lara-Cabrera and Spooner, 2004).

Koopman *et al.* (2001) suggested that, if topologies of the phenogram and the cladogram generated by AFLP fingerprints are identical, homoplasies do not influence the cladistic analysis and will not affect conclusions of species relationships. Besides, in bootstrap or jackknife branch support analyses, the presence of internal conflict caused by homoplasies will lead to an exclusion of these branches as uninformative and they will not affect the conclusions on species relationships (Koopman *et al.,* 2001).

Despite the potential limitations for the use of the AFLP technique in phylogenetic analyses, in particular false fragment homology, congruence has been reported between AFLP and single gene sequence phylogenetic analyses (present study; Spooner *et al.*, 2005), between AFLP and ITS/ETS phylogenetic analyses (Koopman *et al.*, 2001; Beardsley *et al.*, 2003; Spooner *et al.*, 2005) and between AFLP and morphological characters analyses (present study; Spooner *et al.*, 2005). Furthermore, analyses of just one or few sequences, as well as analyses of a large number of “biased” genes are likely to produce incorrect phylogenetic trees with even high bootstrap support (Rokas *et al*., 2003). Thus, AFLP fingerprints can be a useful technique to complement the information about phylogenetic relationships among related taxa.

Since the present AFLP phylogenetic analysis of genus *Araucaria* showed high congruence with morphological and cpDNA sequence classifications, AFLP markers can be used to confirm or complement the information about phylogenetic relationships among ancient taxa, especially if DNA sequence variation is limited or sequence information of only few loci is available.

Conclusions

In the present study, species within genus *Araucaria* proved to be well separated from each other with strongly supported monophyletic sections. The relationship between the South American species *A. angustifolia* and *A. araucana* (section *Araucaria*) is also clearly resolved. Within section *Eutacta* from Australasia the species relationships are only resolved in the NJ tree. In addition, our data and previous reports (Stefenon *et al.,* 2003; Stefenon and Nodari, 2003) suggest that AFLPs provide suitable molecular markers to study the relationships among species within genus *Araucaria* and also within *Araucaria* species. In an ongoing project the usefulness of AFLP markers to distinguish *A. angustifolia* populations from different geographic origins in Brazil will be tested.

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7. Genetic structure of *Araucaria angustifolia* (Araucariaceae) populations in Brazil: implications for the *in situ* conservation of genetic resources [3] [4]

Abstract

The distribution of the genetic variation within and among natural populations of *A. angustifolia* growing in different regions in Brazil was assessed at microsatellite and AFLP markers. Both markers revealed high gene diversity (H = 0.65; A_R = 9.1 for microsatellites and H = 0.27; P = 77.8% for AFLPs), moderate overall differentiation (R_{ST} = 0.13 for microsatellites and F_{ST} = 0.10 for AFLPs), but high divergence of the northernmost, geographically isolated population. In a Bayesian analysis, microsatellite data suggested population structure at two levels: at K = 2 and at K = 3 in agreement to the geographical distribution of populations. This result was confirmed by the UPGMA dendrogram based on microsatellite data (bootstrap support > 95%). Non-hierarchical AMOVA revealed high variation among populations from different *a posteriori* defined geographical groups. The genetic distance between sample locations increased with geographical distance for microsatellites (r = 0.62; p = 0.003) and AFLPs (r = 0.32; p = 0.09). This pattern of population differentiation may be correlated with population history such as geographical isolation and postglacial colonization of highlands. Implications of the population genetic structure for the conservation of genetic resources are discussed.

Key words: AFLPs, *Araucaria angustifolia*, genetic diversity, genetic resources, microsatellites, population structure, population history

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[4] VMS and RF conceived and designed the study. VMS performed the experiments, analyzed the data and wrote the paper. All authors improved the final manuscript

Introduction

The organisation of genetic variation within and among species is an outcome of the evolutionary history of ecosystems and a significant aspect of biodiversity. Therefore, it should be considered whenever conservation strategies are developed or implemented. Conservation of genetic diversity will remain empty talk until we begin to understand how the diversity we wish to conserve is distributed in space (Bawa and Krugman, 1990). Without information on genetic variation patterns, the securest conservation guidelines involve conserving virtually everything (National Research Council, 1991). However, *in situ* conservation relies on the incorporation of large areas of land in order to adequately represent the gene pool of a species. The knowledge on spatial patterns of intraspecific variation greatly enhances the efficiency of conservation strategies by guiding the selection of genetic resources (Finkeldey and Hattemer, 2007).

The loss of genetic diversity often precedes total extinction. According to IUCN estimations, the geographical distribution of about 31000 vascular plant species is limited to a single country. Many of these species are under varying degrees of threat, with more or less high potential for extinction (Phartyal et al., 2002). Thus, the development and implementation of conservation strategies is essential both at the species and the genetic level. In this sense, the assessment of intraspecific biodiversity within and among regions is crucial to recognize and prioritise areas for monitoring, protection and sustainable management. The integration of information on historical population processes is important for the selection of priority areas for conservation (Moritz and Faith, 1998). The observation of variation at a limited set of putatively neutral gene markers often allows concluding on the evolutionary history of populations. This information provides a suitable basis for the selection and the design of plant genetic resources (Finkeldey and Mátyás, 1999).

Araucaria angustifolia (Bert.) O. Ktze. is the unique representative of family Araucariaceae in Brazil and together with the closely related species *A. araucana* (Setoguchi et al., 1998; Stefenon et al., 2006), the unique extant representative of the family in the American continent. The distribution of *A. angustifolia* is predominant in altitudes between 500 and 1800 m, from 19°15' to 31° southern latitude (Reitz and Klein, 1966).

A. angustifolia is a long-lived dioecious conifer species, endemic to the sub-tropical Brazilian highlands and to small patches in Argentina and Paraguay (Reitz and Klein, 1966). Although covering about 200,000 km^2 of the Southern states of Brazil at the beginning of the 20th century (Auler et al., 2002; Guerra et al., 2002), the intensive exploitation process reduced its area to about 3%. Today, the species is regarded as vulnerable according to the IUCN Red List of Threatened Species. The conservation of the species is safeguarded by the creation of nature reserves. However, few efforts have been directed towards the conservation of its genetic resources. In this study, population genetic structure was assessed in Brazilian *A. angustifolia* populations using nuclear microsatellite and AFLP (amplified fragment length polymorphism; Vos et al., 1995) markers. The central aim of this survey was to evaluate the distribution of the genetic variation within and among natural populations of *A.*

angustifolia growing in different regions in Brazil, in order to enhance the knowledge about genetic characteristics useful for the conservation of the genetic resources of the species. Considering results of previous studies pointing towards limited gene flow and population differentiation in *A. angustifolia* (Auler et al., 2002; Sousa and Hattemer, 2003), we intended to test four main hypotheses: (i) populations of *A. angustifolia* display high levels of differentiation, following an isolation-by-distance model; (ii) limited gene dispersion generated sub-structured populations (iii) forest fragmentation resulted in a reduction of within-population diversity; and (iv) different glacial refugia partly explain high differentiation between distant populations.

Materials and Methods

Plant material and DNA extraction

The area of distribution of *A. angustifolia* belongs to the Southern Brazilian highlands, showing quite variable soils, topography and climatic conditions (Reitz and Klein, 1966). Provenance-progeny tests performed with samples from different regions in Brazil revealed evidence of geographical ecotypes (Kageyama and Jacob, 1980; Shimizu and Higa, 1980). Assuming the existence of genetic differentiation due to putatively different histories of populations as well as geographic and climatic variation, six populations (n = 384) were sampled in order to cover different environmental zones and putative ecotypes in Brazil (Fig. 7.1). Population Campos do Jordão (CJ) in the Mantiqueira Hills (São Paulo state, 1507 m above sea level, 22°41'S and 45°29'W) is the current northernmost limit of *Araucaria* forest occurrence in Brazil. This forest is isolated from the southern *Araucaria* formations, mainly due to soil and topographic conditions found in the central region of São Paulo state. The Fazenda Velha (FV; 970 m altitude, 24°15'S and 50°25'W) and Restingão (RG; 729 m altitude, 24°20'S and 50°34'W) populations are old stands located in the West-central region of Paraná state. Populations Paredão (PD; 1034 m asl, 27°12'S and 50°23'W) and Negrinha (NG; 885 m asl, 27°45'S and 49°39'W) located in Santa Catarina state and population Bom Jesus (BJ; 967 m asl, 28°32'S and 50°39'W) in Rio Grande do Sul state represent a region where *A. angustifolia* occurs in small groups of trees as well as dense formations surrounded by grassland. The minimum linear distance between populations is 17.8 km (FV and RG) and the maximum distance is 653.2 km (BJ and CJ). At least sixty putatively mature trees were selected at each location for analyses.

Healthy leaves were selected for each sample and dried in silica gel. About 50 mg of plant material was washed with 70% ethanol, disrupted into the collection microtubes using a Mixer Mill MM 300 (Qiagen) and total DNA was extracted using the DNeasy 96 Plant Kit (Qiagen), following the instructions of the manufacturer. Isolated DNA was eluted in 100 mL TE buffer and deposited at -20°C until use.

Microsatellite analysis

For the microsatellite analysis, genomic DNA was diluted to a concentration of about 10 ng/µL. Genotypes of all samples were scored at five microsatellite loci, namely CRCAc2 (Scott et al., 2003), AA01 (Andrea Schmidt, personal communication), Ag20, Ag45 and Ag94 (Salgueiro et al., 2005). For loci CRCAc2 and AA01, PCR amplifications were performed as described by Scott et al. (2003), with the reverse primer fluorescently labelled (4 µL template DNA, 1X PCR buffer, 0.05 U/µL *Taq* polymerase (Qiagen), 100 µM of each dNTP and 250 µM of each primer in 15 µL reaction). The amplification for loci Ag20, Ag45 and Ag94 was performed with a tailed-primer technique, as described by Schuelke (2000), comprising 4 µL template DNA, 1X PCR buffer, 1.0 U *Taq* polymerase, 100 µM of each dNTP and 100 µM of the reverse primer with a M-13 tail (5'-TTTCCCAGTCACGACGTT-3') at its 5' end, 100 µM of the forward primer and 100 µM of the M-13 labelled primer (5'-AGGTTTTCCCAGTCACGACGTT-3') in a 20 µL reaction. Primer sequences and PCR conditions of all loci are described in Table 7.1. PCR reactions were carried out in a Peltier Thermal Cycler PTC-200 (MJ Research). All fragments were separated on an ABI Genetic Analyser 3100 with the internal size standard GS 500 ROX (Applied Biosystems) and the data were scored using GENESCAN 3.7® and GENOTYPER 3.7® software (Applied Biosystems).

AFLP analysis

The AFLP reactions were performed as described by Vos et al. (1995) with slight modifications. For the restriction/ligation reaction, about 150 ng of genomic DNA was incubated with the restriction enzymes *Pst*I and *Mse*I and the corresponding *Pst*I- and *Mse*I-adapters at room temperature for about 16 hours in a single reaction (1X *T4* DNA-ligase reaction buffer, 0.25 pMol of each adapter, 0.07 U *Mse*I, 0.4 U *Pst*I, 0.08 U *T4* DNA-ligase, 0.05 µg/µL BSA and 0.05 M NaCl). The reaction mixture was diluted 4-fold and used as template for the PCR pre-selective amplification (5 µL template DNA, 1X PCR buffer, 0.08 U *Taq* polymerase (Qiagen), 0.25 mM of each dNTP and 0.05 pMol of each primer). The pre-selective amplification was performed with the primer pairs displaying one selective nucleotide, namely *Pst*+A and *Mse*+G. The PCR conditions for the pre-selective amplification consisted of an initial step at 72°C for 2 min followed by 20 cycles at 94°C for 10s, at 56°C for 30s, at 72°C for 2 min and of a final extension step at 60°C for 30 min. The pre-selective amplified DNA was diluted 10-fold and used as template for the selective amplification with the primer combination *P*-AT/*M*-GCC (4 µL pre-amplified DNA, 1X PCR buffer, 0.07 U *Taq* polymerase (Qiagen), 0.25 mM of each dNTP, 0.08 pMol fluorescently labelled *Pst*-primer and 0.16 pMol *Mse*-primer). The PCR conditions for the selective reaction were an initial denaturation step at 94°C for 2 min, 9 cycles at 94°C for 10s, an annealing step at 65°C for 30s (decreasing 1°C every cycle) and an extension step at 72°C for 2 min. The reaction was continued with an annealing temperature of 56°C for 24 cycles ending with a final extension step at 60°C for 30 min. All PCR reactions were carried out in a Peltier Thermal Cycler PTC-200 (MJ Research). AFLP fragments were electrophoretically separated as described above for microsatellites. All steps of the AFLP reactions from DNA restriction to selective PCR-amplification were repeated twice separately using seven samples and one negative control to

test the reproducibility of the amplified fragments. Fragments between 75 and 400 bp (> 50 rescaled peak height) that were consistent through the two runs were selected for the population analysis. Fragments were automatically scored and transformed into a binary matrix for data analysis (0 for absence and 1 for presence of the fragment). An additional visual check of the raw data was made to correct possibly mislabelled peaks.

Analysis of genetic diversity within populations

For the analysis of microsatellite data, allelic frequencies, allelic richness per locus (A_R; Petit et al., 1998) with a standard sample size of 114 gene copies, unbiased gene diversity (*H;* Nei, 1973) and the Weir & Cockerham's (1984) estimator of inbreeding (*f*) were estimated using the software FSTAT version 2.9.3 (Goudet, 2001). Statistical significance of *f* was based on Bonferroni-corrected *p*-values after 10000 permutations. Observed heterozygosity (H_o), was assessed by direct count using the software ARLEQUIN 3.01 (Excoffier et al., 2005).

Genetic diversity of AFLP data was assessed assuming an inbreeding coefficient equivalent to the mean *f* from the microsatellite analyses (f = 0.1) using the software AFLP-SURV (Vekemans, 2002). Allelic frequencies of AFLPs were estimated using a Bayesian approach with non-uniform prior distribution of allele frequencies (Zhivotovsky, 1999), which gives an unbiased estimate of allele frequencies (Zhivotovsky, 1999; Kraus, 2000). Genetic diversity (*H*) and percentage of polymorphic loci at the 5% level (*P*) were computed following the approach of Lynch and Milligan (1994), i.e. pruning loci with null-allele frequency higher than 1 – 3/*n*, where *n* is the population sample size.

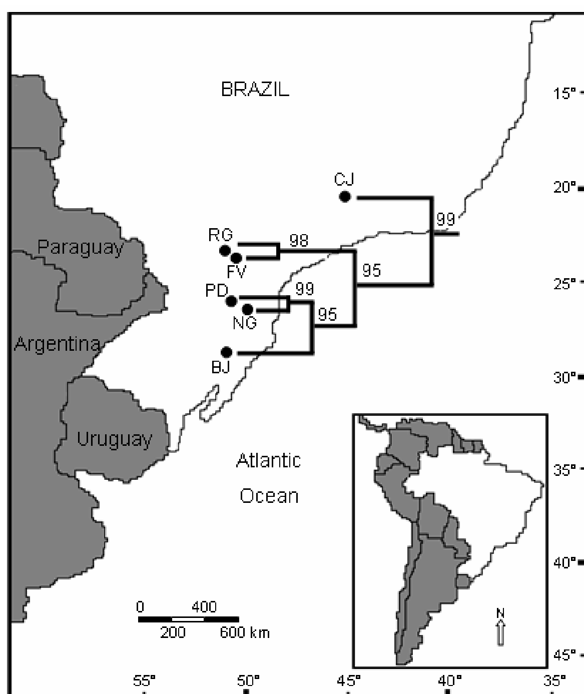

Figure 7.1: Geographic distribution of the six analyzed populations in Brazil. The UPGMA dendrogram based on microsatellite data was superimposed upon the geographic distribution of populations.

Table 7.1: Primer sequences and PCR conditions for the five microsatellite loci analyzed in this study.

Locus	Primers Sequences (5'-3')	Initial denaturation	Denaturation	Annealing	Extension	Cycles	Final extension
AA01 [a]	R: TGACGGGTTCACTCCTACCT F: TAGGAACCCCCATTCATTTG	94°C / 4 min	94°C/45 s	56°C / 45 s	72°C/1 min	31	72°C/4 min
CRCAc2 [b]	R: ATGCATGACTAGGATGAACA F: ATAGTTCTGCTTATCACATCT	92°C / 1 min	92°C/10 s	50°C / 25 s	72°C/1 min	40	72°C/2 min
Ag20 [c]	R: AAGGTATGGCATGTCTC§ F: ACTAGGAATGGATGTTGGTG	95°C / 2 min	95°C/45 s 95°C/45 s 95°C/45 s	68°C/4 min(-2°C/cycle) 58°C/1 min(-2°C/cycle) 47°C / 30 s	72°C/1 min 72°C/1 min 72°C/1 min	5 5 27	no no 72°C/10 min
Ag45 [c]	R: TCCCTCCCTATGTCCCAAAG§ F: CCATCCTCCATCATTCATCC						
Ag94 [c]	R: AGTAAAATCCGCTAACAAATGC§ F: CCCCACAATAACCCAAGATG						

[a] Andrea Schmidt, personal communication; [b] Scott et al., 2003; [c] Salgueiro et al., 2005. § For these primers, the PCR amplification was performed with an M-13 tail at its 5' end (see text for details).

Analysis of population structure

In order to closely investigate the population structure, a Bayesian model-based clustering analysis (Pritchard et al., 2000) was implemented for the microsatellite data set. In this analysis, individual multilocus genotypes are assigned probabilistically to a defined number K of clusters, according to a particular membership coefficient, or into multiple groups with membership coefficients summing up to one across groups. Bayesian analysis of population structure was performed using the non-admixture and the frequency independent alleles models with 50000 Markov chain Monte Carlo (MCMC) steps and 10000 burn-in periods using the software STRUCTURE version 2.1 (Pritchard et al., 2000). Number of K was set from two to twenty and ten replicates were run for each K. The optimum number of clusters K was selected using the approach suggested by Evanno et al. (2005). This method is based on the computation of ΔK, the second order rate of change of the likelihood function with respect to K and is assumed to be reliable when values of $\ln(X|K)$ increase continually with the number of clusters.

Additionally, the relationship among populations was analyzed by means of a cluster analysis using the UPGMA algorithm based on the chord genetic distance (Cavalli-Sforza and Edwards, 1967) estimated for microsatellite data with the software POPULATIONS 1.2.28 (Langella, 2000). Bootstrap values were obtained after 1000 permutations over loci.

The overall and pairwise population differentiation was calculated for microsatellites following R_{ST} (Slatkin, 1995) and F_{ST} (according to Weir & Cockerham, 1984) approaches, using the software RST-CALC 2.2 (Goodman, 1997) and FSTAT 2.9.3 (Goudet, 2001), respectively. For AFLPs, F_{ST} was calculated according to Lynch and Milligan (1994) using the software AFLP-SURV (Vekemans, 2002). Significance of genetic differentiation was determined by permutation tests (1000 permutations).

Additionally, a non-hierarchical analysis of molecular variance (AMOVA; Excoffier et al., 1992) as implemented in ARLEQUIN 3.01 (Excoffier et al., 2005) was applied to estimate among-population differentiation (significance test by 10000 permutations of microsatellite genotypes or AFLP haplotypes among populations) and within population differentiation. Based on the pattern of geographical subdivision identified in the Bayesian and UPGMA analyses (see results), an additional analysis was performed for *a posteriori* defined geographical subsets (population CJ, Paraná group and Santa Catarina/Rio Grande group), as well as between population CJ and the southern group. Groups were created by assigning individuals from different populations to larger units as follows: Paraná group: individuals from populations FV and RG; Santa Catarina/Rio Grande group: individuals from populations BJ, NG and PD; southern group: individuals from all populations except CJ. The correlation between genetic differentiation and geographical distance among populations was evaluated by regressing the population pairwise genetic differentiation matrix (R_{ST} or F_{ST}) against the pairwise geographical distance matrix (in km), using a Mantel test with 10000 permutations performed in the software NTSYS-pc 2.0 (Rohlf, 1998).

Results

Genetic diversity assessed by microsatellites

Genetic diversity parameters estimated for microsatellite data are summarized in Table 7.2. A total of 73 alleles with an average of 14.6 alleles per locus were observed across the five analyzed microsatellite loci, ranging from 7 (Ag45) to 21 alleles (AA01). The multilocus analysis revealed a total gene diversity H = 0.71 for all six populations combined and a mean observed heterozygosity H_o = 0.58. With the exception of population NG, all populations showed private alleles ranging from two to five, with relatively low frequencies. In the multilocus analysis, population CJ displayed the lowest mean values for all genetic parameters, while the highest mean values were observed in population RG (Table 7.2). The mean inbreeding coefficient over all loci indicated a heterozygote deficit with significant statistical support for all populations. However, in the analysis of each individual locus per population, just 43% of inbreeding values were significant and none of the populations revealed a significant deficit of heterozygotes at all loci, suggesting the presence of null alleles. Estimations of overall and pairwise population differentiation calculated for the SMM (R_{ST}) or IAM (F_{ST}) models revealed very similar trends (data not show). Here, just values of R_{ST} estimates will be presented.

Genetic diversity assessed by AFLP markers

The AFLP primer combination applied generated a total of 166 reliable polymorphic markers. Following the approach of Lynch and Milligan (1994), the percentage of polymorphic fragments ranged from 61.4% (FV) to 91.0% (NG) with a mean value of 77.8%. Gene diversity H ranged from 0.21 (FV) to 0.31 (NG) with a mean value of H = 0.27. For the total set of six populations, the estimated gene diversity was H = 0.30 (Table 7.2).

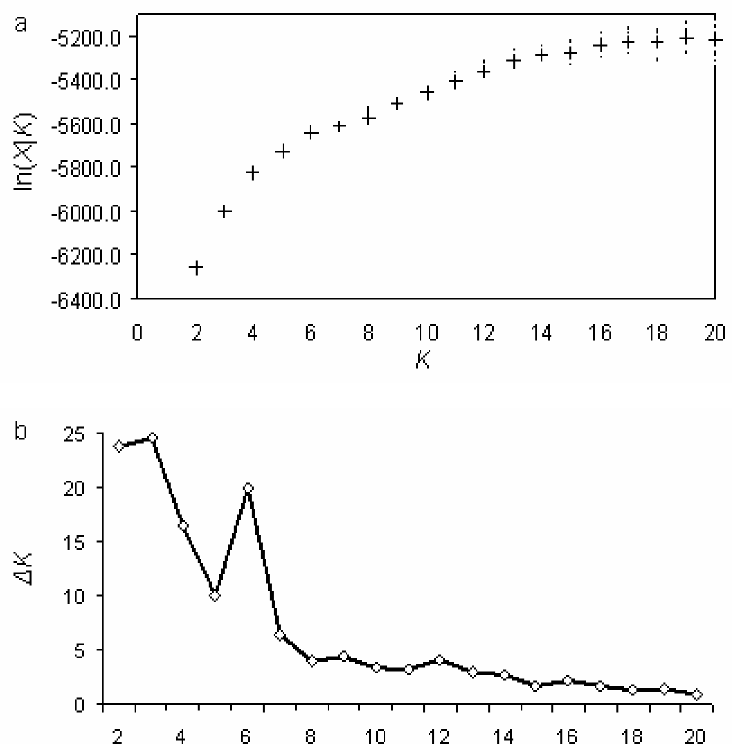

Figure 7.2: Determination of the population structure based on Bayesian clustering analysis applied to microsatellite data. (**a**) Values of log likelihood ln(X|K) as function of the number of clusters (K). (**b**) Values of the second order rate of change of ln(X|K) as function of K. The modal value of ΔK represents the true number of populations or the uppermost level of structure.

Table 7.2: Summary of genetic variation parameters at five microsatellite loci and at 166 AFLP fragments for each population and for the total (six populations).

	BJ	NG	PD	FV	RG	CJ	Mean	Total
AA01 (201 – 245 bp)								
n	62	63	63	64	64	64	63.3	380
A	13	15	16	15	16	12	14.5	21
A_R	12.6	14.8	15.8	14.8	15.9	11.9	14.3	20.9
H	0.84	0.84	0.86	0.90	0.92	0.87	0.87	0.91
H_o	0.77	0.62	0.75	0.81	0.89	0.73	0.76	0.76
f	0.075^{ns}	0.259^{***}	0.137^{**}	0.101^{**}	0.034^{ns}	0.158^{**}	0.127	-
Ag20 (238 – 255 bp)								
n	63	63	64	63	62	57	62	372
A	11	12	11	8	12	9	10.5	18
A_R	10.8	11.8	10.8	7.9	11.7	9.0	10.3	17.9
H	0.81	0.83	0.83	0.69	0.83	0.71	0.78	0.88
H_o	0.59	0.56	0.59	0.63	0.65	0.56	0.60	0.60
f	0.278^{***}	0.330^{***}	0.281^{***}	0.077^{ns}	0.226^{***}	0.206^{**}	0.233	-
Ag45 (154 – 172 bp)								
n	63	64	60	62	63	62	62.3	374
A	3	2	3	5	5	5	3.8	7
A_R	3.0	2.0	2.9	4.8	4.8	4.8	3.7	6.9
H	0.45	0.22	0.32	0.51	0.48	0.41	0.40	0.41
H_o	0.49	0.25	0.38	0.50	0.57	0.32	0.42	0.42
f	-0.100^{ns}	-0.135^{ns}	-0.206^{ns}	0.023^{ns}	-0.186^{ns}	0.215^{*}	-0.045	-
Ag94 (138 – 182 bp)								
n	64	63	63	57	60	61	61.3	368
A	7	8	8	10	7	5	7.5	13
A_R	6.9	7.9	7.9	10.0	6.9	4.9	7.4	13.0
H	0.31	0.54	0.46	0.49	0.69	0.34	0.47	0.61
H_o	0.34	0.52	0.52	0.30	0.53	0.31	0.42	0.42
f	-0.109^{ns}	0.025^{ns}	-0.149^{ns}	0.387^{***}	0.227^{**}	0.092^{ns}	0.079	-
CRCAc2 (183 – 211 bp)								
n	64	63	62	64	64	64	63.5	381
A	8	10	9	10	14	7	9.7	14
A_R	7.9	9.9	8.9	9.8	13.5	6.9	9.5	13.9
H	0.73	0.78	0.75	0.75	0.82	0.60	0.74	0.80
H_o	0.70	0.71	0.68	0.83	0.72	0.59	0.71	0.71
f	0.037^{ns}	0.088^{ns}	0.097^{ns}	-0.099^{ns}	0.123^{*}	0.006^{ns}	0.043	-
Mean values for Microsatellites								
n	63.2	63.2	62.4	62	62.6	61.6	62.5	384
A	8.4	9.4	9.4	9.6	13	7.6	9.6	14.6
A_R	8.2	9.3	9.3	9.5	10.6	7.5	9.1	14.5
H	0.63	0.64	0.64	0.67	0.75	0.59	0.65	0.71
H_o	0.58	0.53	0.58	0.61	0.67	0.50	0.58	0.58
f	0.076^{*}	0.169^{***}	0.090^{***}	0.081^{**}	0.103^{***}	0.139^{***}	0.11	-
AFLPs								
n	62	63	63	64	63	62	62.8	377
P	78.3	91.0	81.3	61.4	78.9	74.7	77.8	100.0
H	0.28	0.31	0.28	0.21	0.28	0.27	0.27	0.30

n: sample size; *A*: number of alleles; A_R: allelic richness; *H*: gene diversity; H_o: observed heterozygosity; *f*: inbreeding coefficient; *P*: percentage of polymorphic fragments. Significance level: $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$; ns: not significant.

Analysis of population structure and isolation by distance

In the Bayesian analysis of population structure, the values of ln(X|K) increased progressively with the number of clusters (K) and it was not possible to determine an appropriate number of K that represents the population structure based solely on this estimate (Fig. 7.2a). Using the analysis of ΔK, microsatellite data suggested population structure at two levels (Fig. 7.2b). At K = 2 (Fig. 7.3a) population CJ was differentiated from the other five populations and at K = 3 (Fig. 7.3b), one cluster was formed by population CJ (membership > 98%), a second cluster by populations FV and RG (membership > 67%) and a third cluster comprised populations PD, NG and BJ (membership > 71%). These patterns are in conformity with pairwise R_{ST} and F_{ST} values (Table 7.3), with lower values among populations PD, NG and BJ (R_{ST} < 0.034 and F_{ST} < 0.102) and between FV and RG (R_{ST} = 0.012 and F_{ST} = 0.059), and higher values between CJ and the other populations (R_{ST} > 0.182 and F_{ST} > 0.123). In accordance with the Bayesian analysis, the UPGMA dendrogram (superimposed upon the geographic map in Figure 7.1) resolved the populations in complete congruence with their geographic distribution. Bootstrap support was higher than 95% for all clusters. CJ was plotted as the most divergent population (bootstrap = 99%), while populations FV and RG formed one group (hereafter designated as Paraná group) with bootstrap support of 98%, sister to a cluster formed by populations PD, NG and BJ (hereafter designated as Santa Catarina/Rio Grande group) with bootstrap support of 95%. The five southern populations clustered with a bootstrap support of 95% and populations PD and NG grouped with a bootstrap support of 99%.

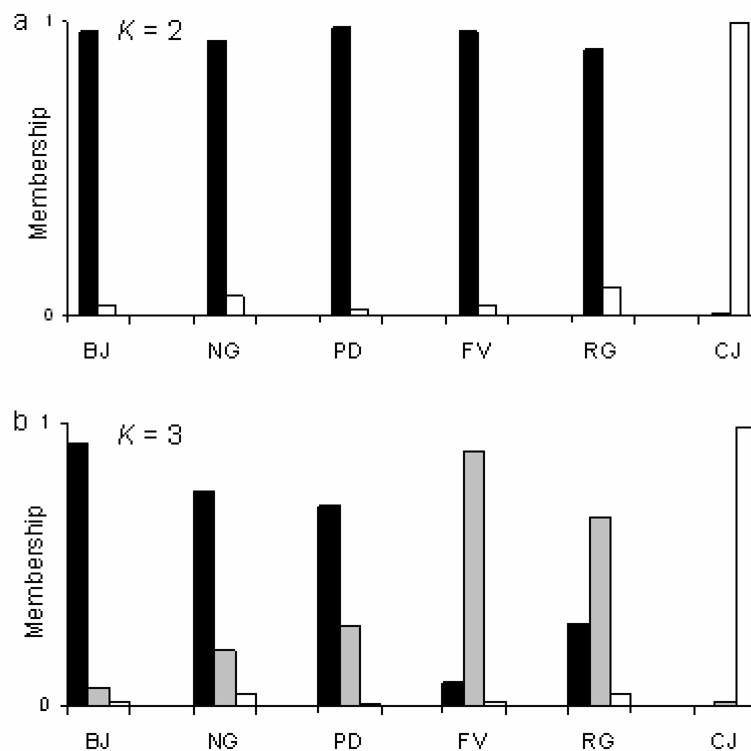

Figure 7.3: Membership of each population as measured by means of the Bayesian clustering analysis of microsatellite data for (**a**) two clusters and (**b**) three clusters.

For both markers, total population differentiation was highly significant ($P < 0.001$), with $R_{ST} = 0.13$ for microsatellites and $F_{ST} = 0.10$ for AFLPs. In the non-hierarchical AMOVA, microsatellites and AFLPs revealed very similar values concerning the apportionment of the genetic diversity in all analyses (Table 7.4). Besides, for the *a posteriori* defined groups, AMOVA revealed patterns congruent with the results of the Bayesian analysis as well as with the UPGMA clustering: low differentiation between Paraná and Santa Catarina/Rio Grande groups (< 5%) and a comparatively high differentiation between CJ and the other groups (> 11%). The differentiation between CJ and the Southern populations was higher than 19% for both markers (Table 7.4). In terms of isolation by distance among populations, the Mantel test showed that the degree of genetic differentiation between sample locations increased with geographical distance for microsatellites ($r = 0.62$; $p = 0.003$) and AFLP data ($r = 0.32$; $p = 0.09$), although not statistically significant for the later marker (Figure 7.4). If isolated population CJ is excluded from the analysis, the correlation increases for microsatellites ($r = 0.93$; $p = 0.014$) and decreases for AFLPs ($r = 0.15$; $p = 0.26$).

Table 7.3: Pairwise population differentiation calculated for microsatellite (R_{ST}; below diagonal) and for AFLP (F_{ST}; above diagonal) markers. Correlation between estimations: $r = 0.79$; $p = 0.03$.

	BJ	NG	PD	FV	RG	CJ
BJ		0.063	0.102	0.065	0.077	0.213
NG	0.034		0.029	0.081	0.038	0.123
PD	0.031	0.014		0.103	0.042	0.129
FV	0.085	0.088	0.052		0.059	0.235
RG	0.101	0.082	0.055	0.012		0.125
CJ	0.237	0.182	0.249	0.237	0.186	

Discussion

Comparison between marker systems

Microsatellites and AFLPs revealed very similar trends when measuring population differentiation by means of AMOVA, R_{ST} and F_{ST} estimations. Similarly, pairwise R_{ST} and F_{ST} revealed analogous trends. Concerning population ranking based on gene diversity estimates, the two markers revealed a non-significant negative correlation (Spearman's $r_s = -0.020$; $p = 0.67$). Even if there is some difference in diversity estimates, it can be generated just by random variation if populations have not reached equilibrium between drift, migration and mutation (Mariette et al., 2002; Gaudeul et al., 2004).

Genetic diversity within A. angustifolia *populations*

The gene diversity assessed by microsatellites and AFLPs in the present survey suggest a relatively high level of genetic variation within *A. angustifolia* populations. Considering the species life history traits, the estimated values are within the range summarized for species with endemic distribution, long-lived perennial life forms, outcrossing breeding system and attached seed dispersal, and are higher than the mean values reported for microsatellites and AFLP markers in plant species (Nybon, 2004). In comparison to other species of the genus, *A. angustifolia* revealed an amount of AFLP polymorphism similar to values estimated using RAPDs in *A. araucana* (Bekessy et al., 2002) and *A. bidwillii* (Pye and Gadec, 2004), and higher than estimated for *A. cunninghamii* using microsatellites and AFLPs (Peakall et al., 2003).

Table 7.4: Summary of the non-hierarchical analysis of molecular variance (AMOVA) for all populations and *a posteriori* defined groups.

Source of variation	d.f.	Variance components [a]	Variation
Microsatellites			
Among all populations	5	0.196	11.16%
Among individuals within populations	762	1.562	88.84%
Among *a posteriori* defined groups	2	0.232	12.80%
Among individuals within populations	765	1.583	87.20%
Between CJ and Southern populations	1	0.389	19.39%
Among individuals within populations	766	1.617	80.61%
Between CJ and Paraná group	1	0.133	11.33%
Among individuals within populations	382	1.039	88.67%
Between CJ and Santa Catarina/Rio Grande group	1	0.425	21.97%
Among individuals within populations	510	1.509	78.03%
Between Santa Catarina/Rio Grande and Paraná groups	1	0.085	4.94%
Among individuals within populations	638	1.627	95.06%
AFLPs			
Among all populations	5	3.509	14.40%
Among individuals within populations	371	20.864	85.60%
Among *a posteriori* defined groups	2	3.144	12.57%
Among individuals within populations	374	21.871	87.43%
Between CJ and Southern populations	1	5.527	19.88%
Among individuals within populations	375	22.273	80.12%
Between CJ and Paraná group	1	6.278	24.22%
Among individuals within populations	187	19.638	75.78%
Between CJ and Santa Catarina/Rio Grande group	1	5.433	19.05%
Among individuals within populations	248	23.080	80.95%
Between Santa Catarina/Rio Grande and Paraná groups	1	0.993	4.27%
Among individuals within populations	313	22.246	95.73%

[a] Significance level after 10000 permutations: $p < 0.001$ for all analyses.

A significant deficit of heterozygotes was revealed in 13 out of 30 tests, but no population showed significant deviation from Hardy-Weinberg proportions (HWP) in more than 3 out of 5 loci. Besides, loci Ag45 and CRCAc2 showed significant deviation from HWP in just one of the six populations. Although deviations from random mating have been noticed in *A. angustifolia* populations (Sousa et al., 2005), strong inbreeding is not expected in adults of most conifer species (e.g. Perry and Knowles, 1990). Furthermore, selfing is obviously excluded as a cause of inbreeding in this dioecious species. Numerous factors apart from particularities of the mating system can account for an excess of homozygotes relatively to Hardy-Weinberg proportions. The presence of null alleles is an important reason for a heterozygote deficit at microsatellite loci (Nascimento de Souza et al., 2005). The occurrence of null alleles cannot be ruled out in the present study.

Genetic differentiation and population history

The Bayesian structure analysis revealed a clear relationship between population differentiation and their geographical distribution, suggesting a northernmost group (population CJ), a Paraná group (FV and RG) and a Santa Catarina/Rio Grande group (BJ, NG and PD). An analogous result was revealed in the UPGMA dendrogram. A high divergence of the southeastern populations from southern stands was also reported for isozyme loci (Sousa et al., 2004). The AMOVA revealed a high contribution to the variation among populations by the inclusion of population CJ, since about 20% of the variation was found between CJ and the group of southern populations. In contrast, less than 5% of the variation was found between Santa Catarina/Rio Grande and Paraná groups, while at least 11.2% of the total variation was apportioned among all populations and 12.6% among the three geographical groups. This pattern of population differentiation may be caused by different population histories such as geographical isolation (population CJ is geographically isolated in the Matiqueira's Hills, at least 100 km far from southern formations with *A. angustifolia*) and postglacial colonization of highlands by *A. angustifolia* populations.

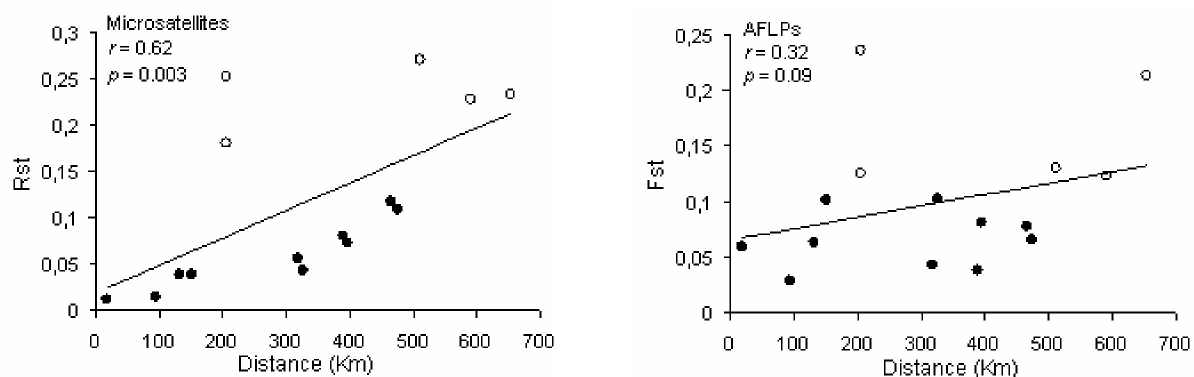

Figure 7.4: Plots of the Mantel test for the correlation between genetic differentiation (R_{ST} and F_{ST}) and geographic distance. White points represents the pairs containing population CJ and filled points all other pairs.

Throughout the Last Glacial Maximum (about 18000 to 15000 years before present; Brewer et al., 2002), the subtropical region of Brazil displayed a cold climate, with relatively long dry periods that did not permit the establishment of *A. angustifolia* in this region (Behling, 1997, 1998; Ledru et al., 1998). The existence of refugia in protected valleys within the highlands and/or coastal slopes in southern and central Brazil, where suitable microclimates existed, is suggested by palynological data (Behling, 1997, 1998, 2004; Ledru et al., 1998). Approximately 4000 years ago, polar fronts did not proceed further than to the southern region of Brazil (Ledru et al., 1998) and it is likely that *Araucaria* forest expanded firstly in its northernmost region of its occurrence, about 3000 years ago (Behling, 2004), followed by a progressively southern expansion from the refugia into the highlands. According to palynological data (Behling, 2004), migration from refugia started 1500 years ago in Paraná state (location of populations FV and RG), 1000 years ago in Santa Catarina state (populations NG and PD) and 800 years ago in Rio Grande do Sul state (population BJ). Thus, besides to the geographical isolation, population CJ may be “isolated in time” from the southern populations due to an earlier expansion and may have experienced evolutionary forces different from the southern populations.

Implications for genetic resource conservation

Most genetic effects of population decline and fragmentation are expected to be manifested only after several generations. Since *A. angustifolia* is a long-living tree species with long reproduction intervals, the extensive exploitation during the last century did not strongly affect the genetic structures of existing populations. However, the maintenance of the current patterns of genetic variation depends on the *in situ* conservation of the remnants and the promotion of natural regeneration, which is often scarce or even lacking. Among the six studied populations, only population NG exhibited abundant natural regeneration in the forest margin and in the neighbouring grassland, while the other five populations comprise formations with old trees and rare regeneration. Absence of young trees has been reported also for other populations of *A. angustifolia* in Brazil (Soares, 1979; Sousa et al., 2005). Natural regeneration of *A. angustifolia* is aided by partial shading during early development, but the species is light demanding in later juvenile and adult stages (Inoue et al., 1979; Inoue and Torres, 1980). Thus, regeneration is mainly confined to forest margins and neighbouring grassland. Periodic natural disturbance of ecosystems due to fire or other catastrophic events is assumed to promote regeneration of *A. angustifolia*. The scarcity or even absence of regeneration in many *Araucaria* forests has partially been attributed to the lack of an appropriate disturbance regime in today’s forests (Soares, 1979).

From a conservation perspective, the present results suggest that the maintenance of genetic variation in *A. angustifolia* populations requires special attention. Our data confirm previous studies (Auler et al., 2002; Sousa et al., 2004) that the remaining *A. angustifolia* populations contain ample genetic diversity although they represent only a small fraction of previously existing forests. Most genetic variation in *A. angustifolia*, like in almost all other long-living, outcrossing woody plants, is found within populations.

However, several lines of evidence suggest that it is not sufficient to conserve a single, large population in order to maintain the genetic resources of the species. In particular, the northernmost population CJ revealed a high differentiation from the southern stands. This differentiation is likely to result from a unique evolutionary history of the isolated CJ population. Thus, the differentiation is likely to be reflected not only at the investigated, mostly neutral gene markers, but also at other loci, presumably including genes of adaptive significance. Thus, this population deserves particular attention concerning the conservation of its genetic resources due to its distinctive genetic attributes.

The large-scale geographic distribution of the populations of a tree species is frequently regarded as a basis for the conservation of its genetic resources and for the delineation of seed zones for commercial seed harvesting. It is recommended to conserve at least one population in each of the main distribution areas of a species (FAO, FLD, IPGRI, 2004). This general recommendation is practical and intuitively appealing, but it is rarely based on sound genetic data (Finkeldey and Hattemer, 2007). Here, we describe genetic structures confirming the appropriateness of an approach for the selection of genetic resources based on the large-scale geographic distribution of *A. angustifolia* in Brazil. The positive correlation between spatial and genetic distances and the identification of three geographic groups by Bayesian analysis indicates that it is justified to use the geographic distribution of the species as a rough and simple criterion to select *in situ* conservation areas, for planning seed collection for *ex situ* conservation, and for the delineation of seed zones.

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8. The role of gene flow in shaping genetic structures of the sub-tropical conifer species *Araucaria angustifolia* [5] [6]

Abstract

Due to morphological features of pollen and seed, limited gene dispersal has been proposed for *A. angustifolia.* We applied nuclear microsatellite and AFLP markers in order to assess patterns of genetic variation at the intra- and inter-population level, and to relate our findings to gene dispersal in this species. Six natural populations were analysed using nuclear microsatellite and AFLP markers. Estimations of both fine-scale spatial genetic structure and migration rate suggest relatively short-distance gene dispersal. However, gene dispersal differed among populations, and effects of more efficient dispersal within population were observed in at least one stand. In addition, even though some proportion of seed dispersal may be aggregated in this principally barochorous species, reasonable secondary seed dispersal presumably facilitated by animals and overlap of seed shadows within populations is suggested. Overall, no correlation was observed between levels of SGS and inbreeding, density or age structure, except that the higher level of SGS was revealed for the population with higher level of juvenile individuals. A low estimate of the number of migrants per generation between two neighbouring populations was estimated, implying in limited gene flow. We expect that stepping-stone pollen flow may have contributed for low genetic differentiation among populations observed in a previous survey. Thus, strategies for maintenance of gene flow among remnants should be considered in order to avoid degrading effects of population fragmentation in the evolution of *A. angustifolia.*

Key words: AFLPs; Brazilian pine; gene dispersion; microsatellites; spatial genetic structure

[5] Stefenon, V.M., Gailing, O. and Finkeldey, R. (in press) **Plant Biology**

[6] VMS conceived, designed and performed the experiments, analyzed the data and wrote the paper. All authors improved the final manuscript.

Introduction

The spatial distribution of genotypes is not only the result of evolutionary forces acting on populations during their past, but also has considerable evolutionary consequences for plant populations, since multiplicity of local gene and genotype frequencies generated over space and time may increase considerably the potential for adaptive evolution (Heywood, 1991). Gene flow is a critical factor for this distribution of genetic variation, because high gene flow tends to homogenize genetic structures while low gene flow allows a non-random distribution of alleles and genotypes. Understanding connectivity of individuals within and among populations (intra-population and inter-population gene flow, respectively) is a primary aspect of populations' ecology and evolution. Furthermore, the understanding of existing gene flow should be complemented by comparisons to historically pertinent reference values, which are reflected in the genetic structures of the extant populations (Dutech et al., 2005).

Fine-scale spatial genetic structure (SGS) of forest trees has been assessed mainly by means of isozyme and microsatellite analyses (Epperson, 1992; Vekemans and Hardy, 2004). The same marker types are frequently employed to assess population differentiation by the computation of F_{ST} or similar measures. These results form the basis for the estimation of inter-population gene flow. The development of statistical approaches allowing the analysis of SGS using dominant markers (e.g. Hardy, 2003) and methods based on likelihoods calculated by coalescent processes (e.g. Beerli and Felsenstein, 1999) allow to get a more accurate understanding of gene flow within and among natural populations. The advantage of using universal dominant markers like AFLPs resides in the possibility of generating hundreds of markers without previous knowledge about the species' genomic constitution (Mueller and Wolfenbarger, 1999). Likelihood methods are often superior to estimations of gene flow among populations based on the "classical" F_{ST} approach ($F_{ST} = 1/4Nm + 1$) and can be applied under circumstances where the use of F_{ST} has limitations (Neigel, 2002).

In this study, we focus on the analysis of the role of gene flow in determining genetic structure in the sub-tropical species *Araucaria angustifolia* (Bert.) O. Ktze. (Araucariaceae). *A. angustifolia* is a long-lived dioecious conifer species endemic to Southern and South-eastern Brazil and to small areas in Argentina and Paraguay at the Brazilian border, in altitudes between 500 and 1800 m (Reitz and Klein, 1966). Although it is an anemophilous species, the pollen is expected to be transported over only relatively short distances due to its morphological characteristics (Sousa and Hattemer, 2003). Besides, the seeds are dispersed essentially through gravity, mainly near the mother-tree because of their size and weight. In a previous survey, we identified significant differentiation among *A. angustifolia* populations and deviations from Hardy-Weinberg proportions with a deficit of heterozygotes found in four out of five microsatellite loci (Stefenon et al., 2007). Furthermore, isozyme analysis revealed deviation from random mating and significant spatial autocorrelation up to 70 metres in natural populations of this species (Sousa et al., 2005; Mantovani et al., 2006). Since *A. angustifolia* is a dioecious species, the results of these studies point towards the existence of biparental

inbreeding, likely due to limited gene flow. Similarly, limited gene flow may be responsible for the significant differentiation among populations. Here, we expand the analysis of gene flow within and among natural populations by using indirect, model-based estimations originated from spatially explicit microsatellite and AFLP marker data. The main goal of this study was to estimate patterns of SGS within populations and migration rates between neighbour populations and relate our findings to gene flow.

Material and Methods

Sampling strategy and DNA extraction

Samples of *A. angustifolia* were collected in six natural populations covering the main range of species distribution in Brazil (Figure 8.1). Details about location and ecological constitution of each population are summarized in Table 8.1. At least sixty neighbouring mature trees were sampled in the investigated areas. For each sampled tree, diameter at breast height (DBH) and spatial position (altitude, latitude and longitude recorded using a GPS equipment Garmin® e-trex) were determined. For the analysis, latitude and longitude were transformed into the Universal Transverse Mercator projection (UTM coordinates). Healthy leaves of 64 individuals were selected in each population, deposited in plastic bags with silica gel and maintained at room temperature. For DNA isolation, leaves were washed with 70% ethanol and about 50 mg of plant material were disrupted in a Mixer Mill MM 300 (Qiagen). Total DNA was extracted using the DNeasy 96 Plant Kit (Qiagen), following the instructions of the manufacturer. Isolated DNA was eluted in 100 µL TE buffer and deposited at -20°C until use.

Molecular analyses

Genotypes of all samples were scored at five nuclear microsatellite loci [CRCAc2 (Scott *et al.,* 2003) , Ag20, Ag45, Ag94 (Salgueiro *et al.,* 2005) and AA01 (Schmidt *et al.,* 2007)], and at one AFLP primer combination (*Pst*I-AT/*Mse*I-GCC). Number of microsatellite alleles and AFLP fragments considered for each population are listed in Table 8.2. Primer sequences, details of PCR amplification and scoring criteria of both marker systems were described by Stefenon *et al.* (2007). All PCR reactions were carried out in a Peltier Thermal Cycler PTC-200 (MJ Research). PCR products were combined with the internal size standard GS ROX 500 (Applied Biosystems) and the electrophoresis was performed on a capillary sequencer ABI Genetic Analyser 3100 (Applied Biosystems). Data were collected and aligned with the internal size standard using GENESCAN 3.7® (Applied Biosystems) and fragments were scored with GENOTYPER 3.7® (Applied Biosystems).

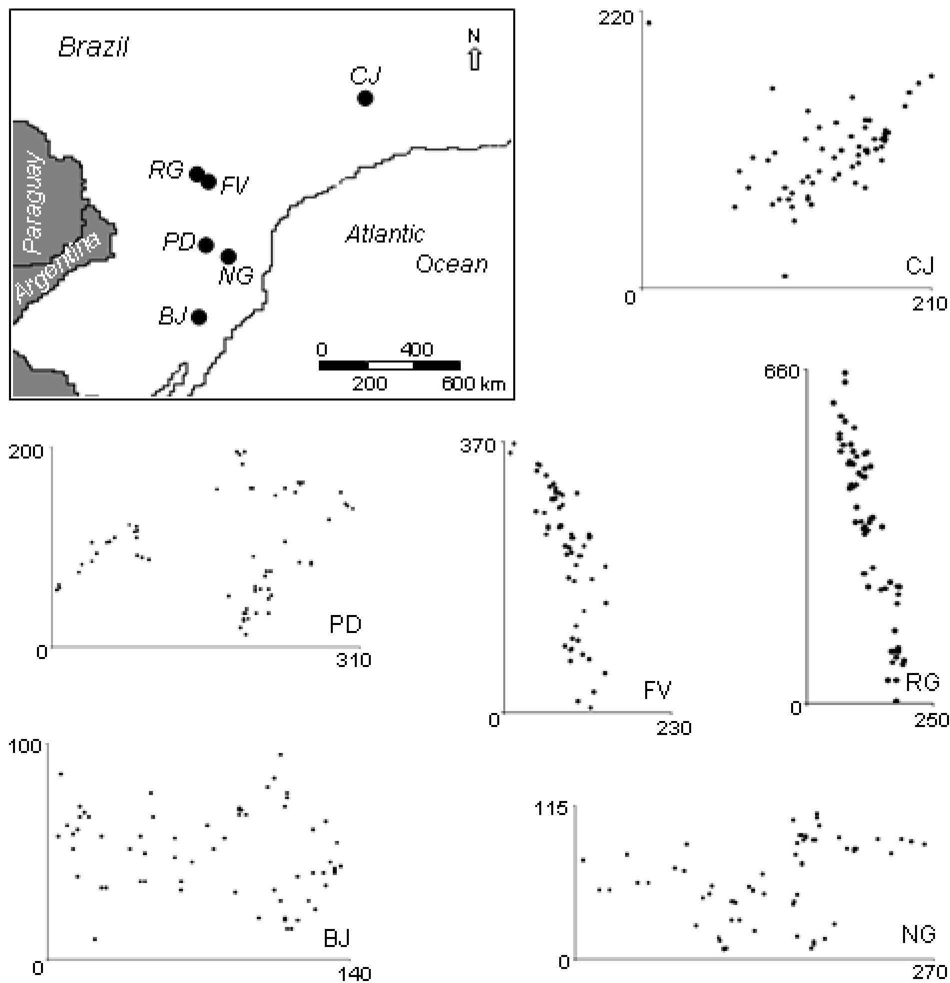

Figure 8.1: Location of the studied populations in Brazil and the distribution of individuals within each population. Distances among individuals are given in metres.

Table 8.1: Location and description of the studied populations.

	Sample (n)	Latitude (S)	Longitude (W)	Altitude (m)	Sampled area (m^2) [a]	Census density (ind/ha) [b]	Mean DBH (cm) [c]	Juvenile trees (%) [d]
BJ	64	28°32’	50°39’	967	9157.0	120.2	25.8	18.6
NG	64	27°45’	49°39’	885	16075.0	68.2	15.1	40.0
PD	64	27°12’	50°23’	1034	30941.5	32.2	38.5	7.1
FV	64	24°15’	50°25’	970	22622.5	44.2	58.9	2.9
RG	64	24°20’	50°34’	729	30411.0	32.9	70.1	2.9
CJ	64	22°41’	45°29’	1507	20274.0	49.3	33.9	11.4

[a] Area corresponding to the polygon formed by the sampled trees within the population.
[b] Actual density of adult trees estimated in the total population.
[c] Mean values computed for the sampled trees.
[d] Percentage of juveniles individuals within the entire population based on DBH and presence of reproductive structures.

Characterization of the intra-population gene flow

Initially, inbreeding coefficient (*f*) was estimated from microsatellites for each population according to Weir and Cockerham (1984), with statistical significance estimated by means of 10000 permutations among individuals. Fine-scale spatial genetic structure (SGS) was analysed in each population using kinship coefficients (F_{ij}). For microsatellites, SGS was assessed according to Loiselle et al. (1995) and for AFLPs using the approach described by Hardy (2003). For each population, the average inbreeding coefficients estimated from five microsatellites were used in the SGS analysis of AFLP data. The number of distance classes (distance intervals within which all pairs of sampling points are considered) was determined for each population in order to display at least 50 pairs of individuals and an increment of about 10 metres per distance class. Within each population, an even number of pairs of individuals was analysed across distance classes. The relationship of the genetic similarity and geographic distance between individuals was computed for each population as the regression slope of kinship coefficients on log-transformed distances (b_F). The standard errors were estimated using the jackknife method. Additionally, the *Sp*-statistic (Vekemans and Hardy, 2004) was computed for each population, based on the regression slope of kinship coefficients as $Sp = -b_F/(1-F_1)$, where F_1 is the mean kinship coefficient between individuals belonging to the first distance class. This measure is expected to quantify SGS, permitting a quantitative comparison among species or populations (Vekemans and Hardy, 2004). The statistical significance of F_1 and b_F was determined through the upper and lower bounds of the 95% confidence interval of F_{ij} defined after 10000 permutations of locations among individuals.

An indirect estimation of gene dispersal from SGS was performed assuming equilibrium of isolation-by-distance in the fine-scale genetic structure. In such a case, the extent of gene dispersal can be expressed in terms of Wright's neighbourhood size as $\hat{N}_b \equiv 4\pi D\sigma^2$, where D is the effective population density and σ^2 represents the physical distance between parent and offspring (Fenster et al., 2003). Given that values of neither D nor σ^2 are known, indirect estimates of $D\sigma^2$ were obtained from the regression of the pairwise F_{ij} values on geographical distance as $\hat{N}_b = (F_1 - 1)/b_F$ and gene dispersal was estimated through the iterative procedure suggested by Vekemans and Hardy (2004). When successive estimates did not converge and the procedure cycled periodically around a set of values, a mean of the cycling estimations was considered as the actual estimation. In cases where b_F became null or positive at one step or became larger than d_{ij} for all *ij* pairs, the estimations did not converge and no results were obtained. Estimations of SGS and gene dispersal were performed using the software SPAGEDI 1.2 (Hardy and Vekemans, 2002).

In order to test the informative content of our AFLPs concerning their genomic distribution, sub-sets of 50 and 100 loci were randomly sampled among the full AFLP data-set and the SGS was re-analysed in population BJ with both sub-sets. Each sub-set was sampled and analysed 10 times independently. If AFLPs are informative due to wide genome coverage, it is expected that sub-sets will be significantly correlated with the full AFLP data-set.

Characterization of the inter-population gene flow

Most of the studied populations are separated by distances that preclude direct gene flow among them, through both pollen and seed dispersion. Therefore, migration rates were estimated just between the neighbouring populations FV and RG, using sub-sets of 50 samples scored randomly within each population. These populations are around 18.0 km apart and were part of an uninterrupted forest before fragmentation started about 100 years ago, allowing gene flow between them. Rates of migration were assessed from microsatellites using a maximum-likelihood framework (Beerli and Felsenstein, 1999) based on coalescent theory (Kingman, 1982). Unlike traditional F_{ST}-based methods, coalescent-based techniques take into account the differences in population sizes, allowing the computation of directional gene flow. The amount of gene flow (*Nm*) was estimated for microsatellite data as $Nm = M\theta_i/4$, with $M = m/\mu$ (the scaled immigration rate) and $\theta_i = 4N_e\mu$ (theta parameter of the recipient population conditional on the underlying genealogy) scaled by the mutation rate per generation per locus (μ), where m is the migration rate per generation and N_e is the effective population size (Beerli and Felsenstein, 1999; Beerli, 2004). It is important to note that the unknown mutation rate (μ) is absorbed into the parameters θ and M, which were initially generated from F_{ST}-calculations. Computations were performed assuming constant mutation rates for all loci. The Markov Chain Monte Carlo simulations were run using 30 short chains (10000 genealogies sampled, 500 genealogies recorded per chain) and five long chains (100000 genealogies sampled, 5000 genealogies recorded per chain). An adaptive “heating scheme” was used to search for additional compatible genealogies, using four chains with start temperatures 1.0, 1.2, 1.5 and 3.0. The analysis was run three times and the mean value over the runs is reported. Data were analysed using a Brownian motion in the software MIGRATE 2.1.2 (Beerli, 2004).

Results

Marker polymorphism and levels of inbreeding

Estimations of the numbers of alleles for microsatellites, numbers of polymorphic AFLP loci and levels of inbreeding are summarized in Table 8.2. Across the five microsatellite loci a total of 73 alleles were characterized. From 38 to 54 alleles were observed per population. Concerning AFLP markers, the number of polymorphic loci ranged from 138 to 164 per population. The mean inbreeding coefficient over all microsatellite loci indicated a heterozygote deficit with significant statistical support for all populations, ranging from 0.07 ($p < 0.05$) to 0.17 ($p < 0.001$).

Table 8.2: Estimation of the fine scale genetic structure and gene dispersion in *A. angustifolia.*

	BJ	NG	PD	FV	RG	CJ
D_e (x10^{-2})	0.30	0.16	0.08	0.11	0.08	0.13
F	0.07 **	0.17 ***	0.09 ***	0.08 **	0.10 ***	0.13 ***
Microsatellites						
A	42	47	47	48	54	38
F_1 (SE)	0.004 ^{ns} (0.013)	0.017 ^{ns} (0.011)	0.017 ^{ns} (0.013)	0.004 ^{ns} (0.016)	0.038 * (0.019)	-0.009 ^{ns} (0.019)
b_F (SE)	-0.003 ^{ns} (0.005)	-0.016*** (0.004)	-0.015*** (0.006)	-0.009** (0.006)	-0.012 *** (0.006)	0.0001 ^{ns} (0.003)
Sp	0.003	0. 016	0.015	0.009	0.012	-0.0001
σ_g	nc	42 m	64 m	65 m [a]	101 m	nc
AFLP						
NP	146	164	145	138	156	149
F_1 (SE)	0.044 *** (0.008)	0.116 *** (0.017)	0.054 *** (0.009)	-0.009 ^{ns} (0.009)	0.097 *** (0.012)	0.018 ^{ns} (0.008)
b_F (SE)	-0.022*** (0.004)	-0.053 *** (0.008)	-0.021*** (0.003)	-0.0008 ^{ns} (0.002)	-0.039*** (0.004)	-0.006** (0.002)
Sp	0.023	0.060	0.022	0.0008	0.043	0.006
σ_g	28 m	25 m	67 m [a]	nc	51 m	nc

D_e: assumed effective population density per m^2 (¼ of the actual census density); *f*: inbreeding coefficient; *A*: number of alleles at five loci: *nP*: number of polymorphic AFLP loci; F_1: multilocus kinship coefficient between individuals of the first distance class; SE: standard error; b_F: regression slope of *F* on log distance; *Sp*: quantification of the SGS; σ_g: gene dispersal; nc: not computed; Significance level after 10000 permutations: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: not significant.

[a] Mean of the cycling estimations (see text for details)

Estimations of SGS and gene dispersal within populations

Among populations, the number of analysed pairs ranged from 50 to 149 across distance classes. Negative values were reported for the regression slope of F_{ij} (b_F; Table 8.2) in five populations for microsatellites (statistically not significant in BJ) and in all populations for AFLPs (statistically not significant in FV). The negative values of b_F indicate that on average, individuals spatially close are genetically more similar to each other than individuals separated by larger distances. Indeed, a pattern of positive F_{ij} at short distance classes (< 30 m) and negative F_{ij} at long distance classes (> 80 m) is evident for AFLPs in most populations, where a near monotonic decrease of the mean kinship coefficient with the increase of distance is observed (Figure 8.2). The average of F_{ij} between individuals at the first distance class (F_1) ranged from -0.009 to 0.039 for microsatellites and from -0.009 to 0.116 for AFLPs (Table 8.2). Significant SGS was detected in the first distance class in population RG for microsatellites (Figure 8.3) and in populations BJ, NG, PD and RG for AFLPs (Figure 8.3). For all populations but one (FV), microsatellites revealed lower values than AFLPs for *Sp*-statistic. Comparing the populations with significant SGS according to *Sp*-statistic, the strongest SGS was revealed by population NG for both markers (Table 8.2). With exception of population CJ, in which the inference failed for both marker systems, estimations of gene dispersal were obtained at

least for one data set in each population. The estimations of gene dispersal (Table 8.2) obtained from microsatellite data ranged from 42 (population NG) to 101 (population RG) metres, while the estimations from AFLP data ranged from 25 (population NG) to 67 (population PD) metres. The re-analysis of SGS in population BJ using sub-sets of 50 and 100 AFLP loci suggested high genomic coverage of these markers. A correlation higher than 95% was revealed for all sub-sets with 100 loci and higher than 80% for eight out of ten sub-sets with 50 loci (Figure 8.4).

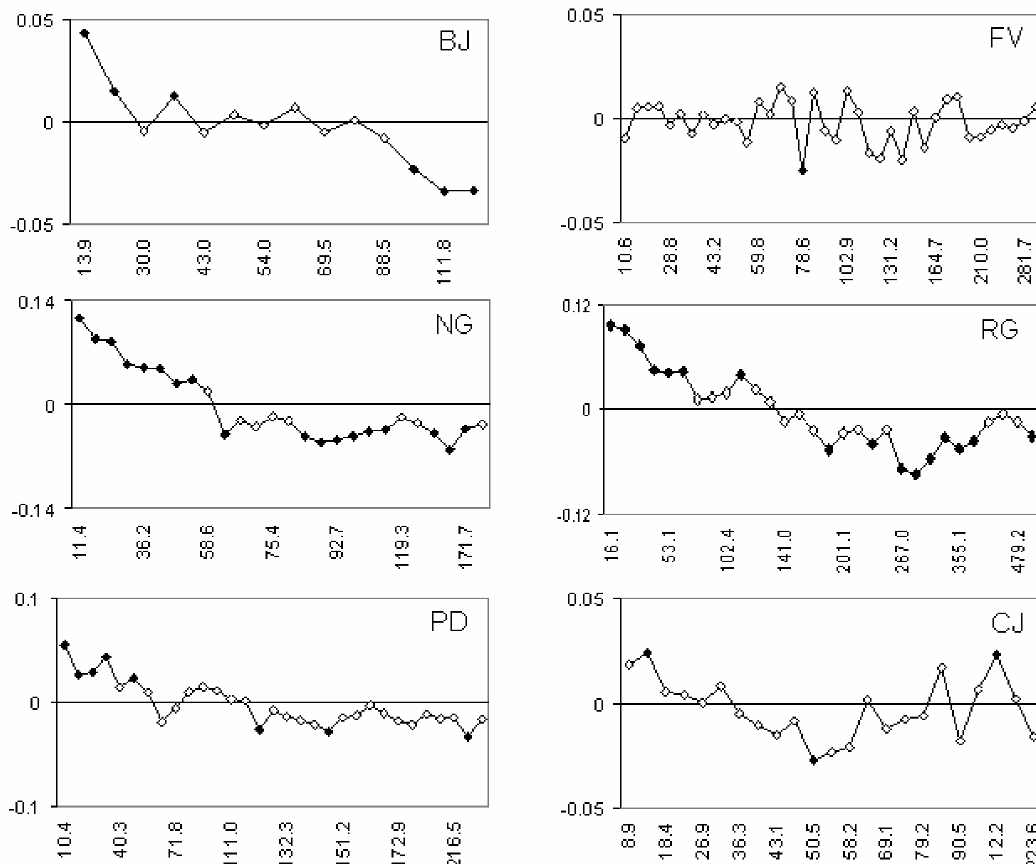

Figure 8.2: Correlograms of kinship coefficient measures (F_{ij}) plotted against the distance (given in metres) based on AFLP data. Filled symbols are significant at the 5% level.

Estimations of inter-population gene flow

According to coalescent analysis of microsatellite data, the effective number of migrates (*Nm*) between FV and RG was near unity. The number of immigrants from FV into RG was 1.24 individuals per generation, while in the opposite direction it was 0.93 individuals per generation. Assuming μ = 10^{-4}, the effective population size ($N_e = \theta / 4\mu$) was estimated as N_e = 2228 for FV and N_e = 1743 for RG.

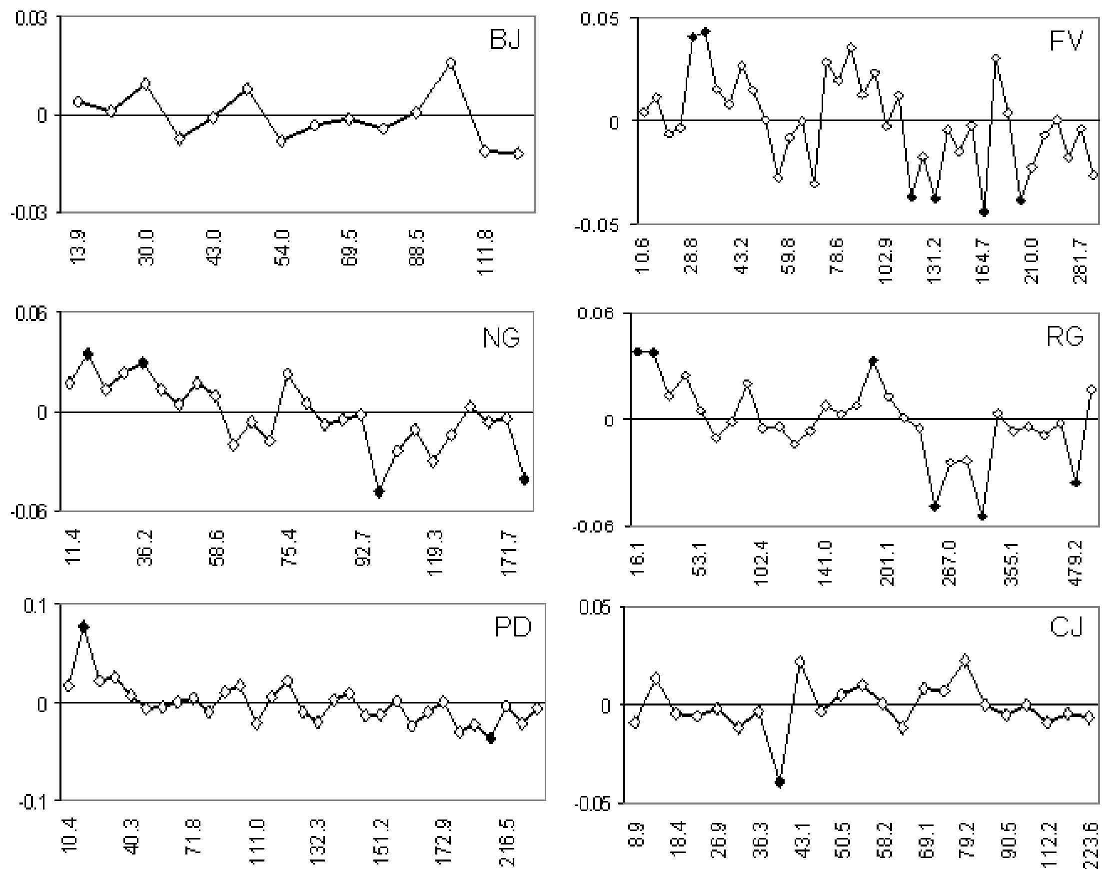

Figure 8.3: Correlograms of kinship coefficient measures (F_{ij}) plotted against the distance (given in metres) based on microsatellite data. Filled symbols are significant at the 5% level.

Discussion

In the present study, microsatellite and AFLP markers were applied to analyze the role of gene flow in shaping genetic structure of *A. angustifolia* populations. Besides revealing limited gene dispersal, the results show considerable variation in the internal structure of the analysed populations. For instance, while population NG revealed strong SGS, our results suggest extended gene dispersal in population CJ. In general, AFLPs were more effective to detect SGS than microsatellites. Similar results were observed by Jump and Peñuelas (2007) in a population of *Fagus sylvatica* using six microsatellite and 250 AFLP loci. Conversely, Hardesty et al. (2005) detected significant SGS in *Simarouba amara* with microsatellites (five loci) but not with AFLPs (155 loci). The higher capacity of AFLPs in detecting SGS observed in our study is likely to result from the large number of unlinked loci, which allowed wider genome coverage in comparison to the five microsatellite loci. This hypothesis is reinforced by the high correlation between results obtained with the full data set (166 loci) and sub-sets of 100 and 50

loci applied to re-analyse SGS in population BJ. While the variation observed at a large number of AFLP loci was high informative for the intra-population analysis of gene flow, the large number of alleles observed at microsatellites was crucial for inter-population analysis. In good agreement to the SGS analysis, a dominance of short-distance gene dispersal was suggested by the estimations of migration between neighbour populations based on microsatellite data.

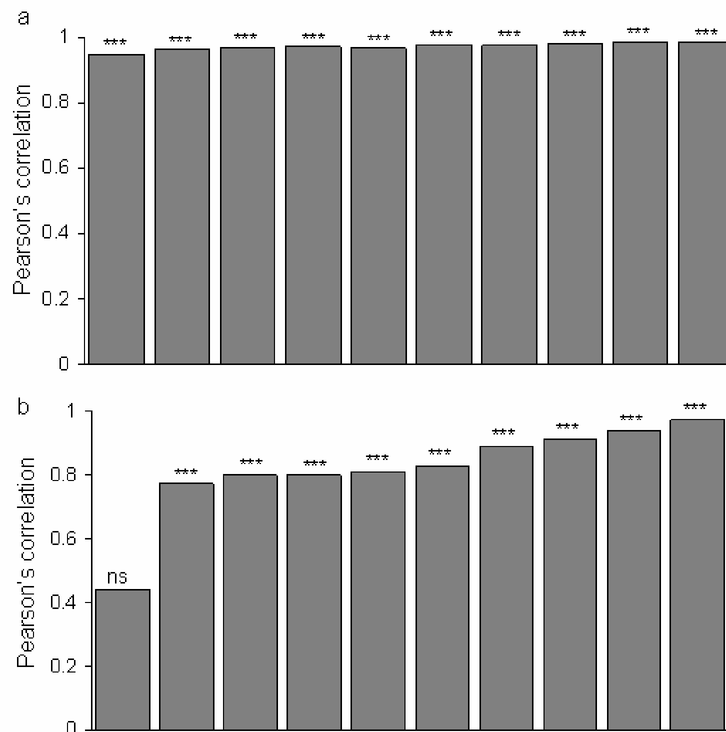

Figure 8.4 Correlation between the full AFLP data set (166 loci) and sub-sets of (a) 100 and (b) 50 randomly scored AFLP loci for SGS analyses in population BJ. The ten different sets were randomly scored and analyzed independently. Significance of the t-test for correlation: ***: $P < 0.001$, ns: not significant.

Gene flow at intra-population level

Due to morphological features of the pollen grain (Sousa and Hattemer, 2003), pollen dispersal is expected to be comparatively limited for *A. angustifolia*. Besides, a mean of only three to four male individuals contributing to the effective pollen clouds of single seed trees were detected by Sousa et al. (2005), increasing the chance of correlated mating. Seed dispersal is also expected to be relatively limited in *A. angustifolia*. The gravity-dispersed seeds of *A. angustifolia* weigh about 8.0 g (Mantovani et al., 2004) and lack structures that aid the dispersion. These features result in dispersal at relatively short distances. Alternatively, long distance seed dispersal is facilitated by rodents (e.g. *Agouti paca* and *Sciurus ingrami*), parrots (e.g. *Amazona petrei*) and crows (e.g. *Cyanocorax coeruleus*). However,

the transported seeds are often damaged by these animals and not able to germinate (Müller and Macedo, 1980; Mello Filho et al., 1981).

Indirect inferences concerning the relative efficiency of seed and pollen dispersal, respectively, can be obtained from an analysis of the linear regression of the kinship coefficients against the geographical distance. Simulation studies (Heuertz et al., 2003) showed that deviations from the linear relationship at distances shorter than the total gene dispersal distance (σ_g) are related to the different contributions of pollen (σ_p) and seed dispersal (σ_s). For *A. angustifolia*, a pattern analogous to $\sigma_p \approx 10\sigma_s$ in the simulations is visualized in populations BJ, NG, PD and RG for the AFLP data (Fig. **2**). These results suggest that despite limited pollen dispersal, the narrow seed distribution is by far the most important factor in generating SGS in *A. angustifolia*. Assuming that the estimations of gene dispersal $\hat{\sigma}_g$ correspond to the largest distance reached by pollen, the maximum distance of seed dispersal ($\sigma_s = \hat{\sigma}_g / 10$) based on AFLP estimations ranges from 2.8 to 6.7 metres. Estimations based on microsatellite data are somewhat larger, from 4.2 to 10.1 metres. In general, our field observations of seed fall in *A. angustifolia* revealed distances shorter than eight metres in relation to the mother-tree for seed dispersal through gravity.

If restricted seed dispersal is important in generating SGS, it is expected that juveniles reveal larger estimates of coancestry than adults (Epperson, 1992). Given that *A. angustifolia* seeds usually aggregate around the mother tree, the juvenile individuals within a given area around it are likely to be at least half-sibs and display a kinship value of 0.125 or larger. Field observations based on diameter (DBH) and reproductive status (presence of cones) of individuals revealed only few young trees in five populations, while population NG showed a relatively high number of juveniles. This population revealed the highest level of SGS in this study and the kinship value estimated for neighbour individuals with AFLP markers in this population was F_1 = 0.116 (±0.017), suggesting high relatedness among trees in a neighbourhood area of up to 10 metres. Even though some proportion of seed dispersal may be aggregated, the level of coancestry estimated for putative neighbour individuals in the other populations is lower than expected for half-sibs ($F_1 \leq 0.038$ for microsatellites and $F_1 \leq 0.097$ for AFLPs), suggesting that there is reasonable secondary seed dispersal and overlap of seed shadows within populations. Besides, much of the SGS caused by seedling clumping may be later removed by severe competition (Epperson, 1992).

Gene flow among populations

In a previous study, we observed significant correlation between genetic differentiation (R_{ST}) and geographic distance among these six populations (*r* = 0.62; *p* = 0.003) suggesting a pattern of isolation-by-distance among them (Stefenon et al., 2007). Our estimations of effective migration rates between two neighbouring populations revealed about one migrant per generation, a scenario congruent with limited gene flow. Using the traditional F_{ST}-approach, Schuster et al. (1989) estimated a level of 11.1 migrants per generation in *Pinus flexilis*. Such amount of gene flow was explained as likely stepping-stone pollen transfer between intermediate populations and high levels of seed

dispersal. High levels and long distance inter-population gene flow were suggested in populations of the same species, in which migration rate was estimated as 6.9 migrants per generation (Schuster and Mitton, 2000). In *P. coulteri*, a species with comparatively larger seeds, just 1.27 migrants per generation were reported by Ledig (2000). Considering that *A. angustifolia* formed a continuous forest about 100 years ago, the hypothesis of migration through a stepping-stone model can not be discarded. Such a model could explain the low genetic differentiation among the southernmost populations BJ, NG and PD (R_{ST} < 0.034; Stefenon et al. 2007), despite relatively short distance gene flow.

Remarks on the species evolutionary history

In general, the levels of SGS based on *Sp*-statistic revealed for *A. angustifolia* are higher than values estimated for other outcrossing tree species with wind-dispersed pollen and gravity-dispersed seeds, such as *Quercus robur, Q. petraea, Q. lobata* and *Larix laricina* (Vekemans and Hardy, 2004; Dutech et al., 2005). In *A. angustifolia*, limited gene dispersal and a relatively low number of pollen donors may be considered as main courses of family structures. However, some features may extend seed and pollen dispersal in this species. For instance, no SGS was evident from microsatellite analyses, weak SGS was revealed by AFLPs and no estimation of gene dispersal was obtained in population CJ, suggesting more extended gene dispersal in this stand compared to other populations with similar age structure. This conclusion is further supported by two lines of evidence. First, the correlograms of population CJ for AFLP data revealed a shape analogous to cases where simulated dispersal of pollen and seed exhibit similar magnitude (see Heuertz et al., 2003). This pattern suggests more extensive seed dispersal in comparison to the other populations, where pollen is expected to be dispersed about 10-fold more widely than seeds (see above). Second, the failure of gene dispersal estimation occurred because the slope b_r became positive at one step (meaning no pattern of isolation-by-distance) or because $\hat{\sigma}_g$ became larger than the greater distance between individuals in the sampled area (Hardy et al., 2006). Although the physical attributes of the pollen grains suggest limited gene dispersion, *A. angustifolia* occupies the upper canopy of the forest, with strobili located primarily at the end of the branches (Mantovani et al., 2004). These features may facilitate the pollination of the female strobili and partially compensate the poor flight ability of pollen. Concerning secondary seed dispersal by animals, more studies are needed to highlight the behaviour of potential seed dispersers and its importance for the gene flow in natural populations.

Considering the distance estimated for gene dispersal in *A. angustifolia*, current gene flow among the remaining forest fragments is expected to be restricted. For instance, our estimation of migrants between two neighbouring populations approximates one migrant per generation. While the exchange of one migrant per generation prevents the fixation of neutral loci in the recipient population at equilibrium (Erikson, 2005), absence of gene flow tends to enhance the effect of genetic drift. Present day *A. angustifolia* forests are mostly composed of fragments isolated by several kilometres. Therefore, exchange of migrants among these remnants is virtually absent, likely increasing genetic differentiation among them in future generations. Small reproduction-effective population sizes

enhance the effect of genetic drift in fixing alleles and promote mating among relatives as a type of inbreeding. Fixation of alleles and the consequent reduction in heterozygosity, genetic drift and inbreeding erode quantitative variation and diminish population fitness (van Buskirk and Willi, 2006). Because of the environmental heterogeneity in the range of *A. angustifolia* distribution, high adaptability is vital for the survival of natural populations. Hence, strategies for maintenance of gene flow among remnants (e.g. providing connectivity among them) should be considered in order to diminish the negative consequences of population fragmentation in the future generations of *A. angustifolia.*

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9. Genetic structure of plantations and the conservation of genetic resources of Brazilian pine (*Araucaria angustifolia*) [7] [8]

Abstract

With growing concern about maintenance of genetic variation and conservation of gene resources, the question arise on the extent to which a planted population should be considered a resource able to preserve the gene pool of a species. In this study, levels of genetic diversity were assessed in natural and planted populations of *A. angustifolia* using AFLPs and nuclear microsatellites, in order to test the usefulness of planted forests in programs of species' genetic resource conservation. In general, the original genetic structure of the plantations was not strongly altered. For microsatellites, gene diversity (*H*) and allelic richness was significantly higher in plantations, while inbreeding was not different between planted and natural populations. For AFLPs, no significant difference was found between groups in the measures of genetic diversity. In the cluster analysis based on microsatellite data, plantations and natural populations from Santa Catarina state grouped together, suggesting that plantations preserved genetic information very similar to natural populations. The cluster analysis of populations based on AFLP data differentiated plantations from natural populations. This pattern may be result of genetic hitchhiking of AFLP fragments with genes under selective pressure due to plantations establishment and management. We suggest that the moderate to high level of genetic diversity retained in *A. angustifolia* populations after the intense fragmentation of the natural forest has the potential to supply plant material with sufficient genetic diversity for the species conservation through the establishment of planted forests. A sustainable management of the extant forest remnants and forestation/reforestation enterprises should additionally attend to trends revealed in previous studies concerning population structure and gene flow.

Key words: Brazilian pine, microsatellites, AFLPs, genetic diversity, genetic resource conservation

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[8] VMS conceived and designed the study, performed the experiments and wrote the paper. VMS and OG analyzed the data. All authors improved the final manuscript.

Introduction

With growing concern about maintenance of genetic variation and preservation of gene resources, the question arise on the extent to which a planted population should be considered a gene resource able to preserve the gene pool of the original populations (Bergmann and Ruetz, 1991). Planted forests are the result of afforestation of previously non-forested lands, reforestation of degraded areas or conversion of primary and secondary forests. The maintenance of genetic diversity and of the evolutionary adaptive potential of planted forests is an important issue if it is planned to use reproductive material from these plantation, for example by its conversion to a seed production area (Finkeldey and Hattemer, 2007) or by using natural regeneration for the establishment of the second generation. Monitoring genetic diversity parameters of planted forests may help to evaluate whether forest establishment may contribute to the conservation of the species' gene pool. In addition, several selection factors may affect a set of the propagation materials used in afforestation programs. For example, the rejection of seedlings considered as unsuitable might result in remarkable changes to the original genetic structure of the propagated material.

In this study we focused on the analysis of the genetic structure in plantations of *Araucaria angustifolia* (Bert.) O. Kuntze established in southern Brazil, in comparison with natural populations of the same region. *A. angustifolia* is a long-lived perennial outcrossing conifer species endemic to southern Brazil and small areas in Argentina and Paraguay at the Brazilian border (Reitz and Klein, 1966). Because of its high quality wood, *A. angustifolia* was the most important Brazilian forest resource during the 1950's to 1970's (Guerra et al., 2002). Covering around 200,000 km^2 of the Southern states of Brazil at the beginning of the 20th century, the intensive exploitation process reduced its area to about 3% (Guerra et al. 2002), leading this species to the vulnerable category of the IUCN Red List of Threatened Species (IUCN, 2006). Despite the vulnerable status of the species and recommendations of sustainable use and recovery management (IUCN, 2006), the exploitation continuously advances over the remnants of *Araucaria* forests, which are replaced by exotic fast-growing tree species (mainly *Pinus* spp. and *Eucalyptus* spp.) or agricultural lands.

Given the long reproduction intervals of *A. angustifolia*, it is expected that the forest exploitation occurred during the last century did not strongly affect the genetic structures of remnant populations. Instead of that, genetic effects of population reduction and fragmentation are expected to be manifested in future generations. In a previous study (Stefenon et al., 2007) we suggested that the maintenance of the current patterns of genetic variation depends on the *in situ* conservation of the remnants and the promotion of natural regeneration. The central goal of the present study was to assess the levels of genetic diversity in plantations of *A. angustifolia* using AFLPs (Vos et al., 1995) and nuclear microsatellites, in order to test the usefulness of planted forests in programs of species' genetic resource conservation. With this intent, we focus on the question whether population establishment and management change genetic structure of planted populations in comparison to natural stands.

Material and Methods

Sampling strategy

For the present study, plant material was collected in five plantations (n = 192) and five natural populations (n = 320) of *A. angustifolia* in southern Brazil. These natural populations were not necessarily the source of the seeds used in the plantations establishment, but likely represent the gene pool of each geographic region, considering the patterns of genetic differentiation revealed for this species in a previous study (Stefenon et al., 2007). Plantations were established between 1961 and 1992 by the paper manufacturing company Klabin S/A. Plantations CEU, GUA and PAI were established in the state of Santa Catarina with seeds obtained in the same state. Seeds were purchased from local farmers and were likely collected from different stands and mixed before plantation establishment. Plantations TEL1 and TEL2 were established with seeds collected from single populations in the states of Santa Catarina (TEL1) and Paraná (TEL2) and established in the municipality of Telêmaco Borba, in Paraná state. Details about location of natural populations and plantations, as well as about plantations establishment are given in Figure 9.1 and Table 9.1. Leaves of all 512 samples were collected, dried in silica gel and maintained at room temperature until DNA extraction.

Table 9.1: Number of individuals sampled and location of natural and planted populations. Origin of the seeds and year of establishment is given for the planted stands.

Samples		Location				Establishment	
Name	N	Alt. (m)	Lat.	Long.	State[1]	Origin of seeds[1]	year
Natural populations							
BJ	64	967	28°32'S	50°39'W	RS	-	-
NG	64	885	27°45'S	49°39'W	SC	-	-
PD	64	1034	27°12'S	50°23'W	SC	-	-
FV	64	970	24°15'S	50°25'W	PR	-	-
RG	64	729	24°20'S	50°34'W	PR	-	-
Plantations							
CEU	48	773	27°41'S	49°18'W	SC	SC	1975
GUA	48	896	27°41'S	50°00'W	SC	SC	1992
PAI	48	884	27°23'S	50°32'W	SC	SC	1976
TEL1	24	790	24°15'S	50°39'W	PR	SC	1961
TEL2	24	758	24°15'S	50°38'W	PR	PR	1963

[1] RS = Rio Grande do Sul State; SC = Santa Catarina State; PR = Paraná State

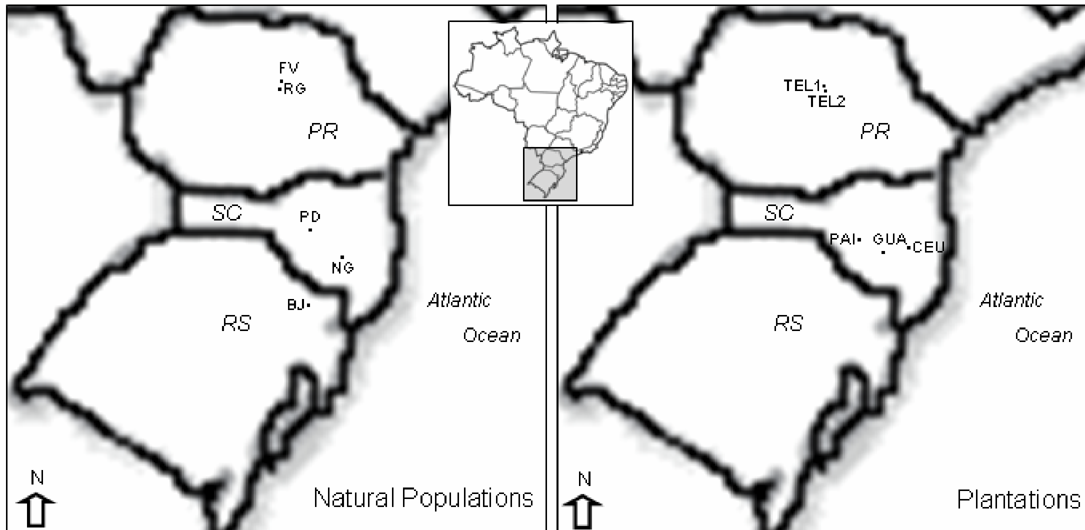

Figure 9.1: Distribution of the five natural populations (BJ, NG, PD, FV and RG) and the five plantations (CEU, GUA, PAI, TEL1 and TEL2) in southern Brazil.

DNA extraction and molecular analyses

Silica dried leaves were washed with 70% ethanol and disrupted into the collection microtubes using a Mixer Mill MM 300 (Qiagen). Total DNA was extracted from about 50 mg of leaves using the DNeasy 96 Plant Kit (Qiagen), following the instructions of the manufacturer. Isolated DNA was eluted in 100 µL TE buffer and deposited at -20°C until use. Genotypes of all samples were scored at five nuclear microsatellite loci [CRCAc2 (Scott et al. 2003), Ag20, Ag45, Ag94 (Salgueiro et al. 2005) and AA01 (Schmidt et al., 2007)] and at one AFLP primer combination (*Pst*I-AT/*Mse*I-GCC). Primer sequences, details of PCR amplification and scoring criteria of both marker systems were described elsewhere (Stefenon et al., 2007). All PCR reactions were carried out in a Peltier Thermal Cycler PTC-200 (MJ Research). PCR products were combined with the internal size standard GS-500 ROX (Applied Biosystems). The electrophoresis was performed on a capillary sequencer ABI Genetic Analyser 3100 (Applied Biosystems). Data were collected and aligned with the internal size standard using GENESCAN 3.7® (Applied Biosystems) and fragments scored with GENOTYPER 3.7® (Applied Biosystems). An additional visual check of the raw data was made to correct mislabelled peaks.

Analysis of population diversity

For microsatellite loci, gene diversity (*H*; Nei 1973), allelic richness per locus ($A_{r[g]}$; El Mousadik and Petit, 1996) and inbreeding coefficient (*f*; Weir & Cockerham's 1984) were estimated. Statistical significance of *f* was based on Bonferroni-corrected *P*-values after 1000 permutations of alleles among individuals within populations. Plantations and natural populations were tested for differentiation in *H*, $A_{r[g]}$ and *f* using 1000 permutations (two sided test). All analyses were performed using FSTAT 2.9.3 (Goudet, 2001). Linkage disequilibrium (LD) between pairs of microsatellite loci was tested in each

population by means of Fisher's exact test using a Markov chain (1000 dememorization steps, 1000 batches with 10000 iterations per batch) performed in GENEPOP web version (Raymond and Rousset, 1995).
For AFLP markers, genetic diversity was measured through the percentage of polymorphic loci (*PPL*) and gene diversity (H_j) estimated with AFLP-SURV (Vekemans 2002) according to Lynch and Milligan (1994), i.e. restricted to loci with band presence frequencies below 1-(3/*N*), where *N* is the sample size. Allelic frequencies were computed using a Bayesian approach with non-uniform prior distribution of allele frequencies (Zhivotovsky, 1999) assuming an inbreeding value f = 0.1 (mean value computed from microsatellites). Fragment richness per locus ($F_{r[n]}$; Coart et al., 2005) was computed based on a sample size of 24 individuals using AFLPDIV 1.0 (R. J. Petit; available at http://www.pierroton.inra.fr/genetics/labo/Software/). Plantations and natural populations were tested for differentiation in *PPL*, $F_{r[n]}$ and H_j using an analysis of variance (ANOVA).

Correlation between estimations of gene diversity obtained with the different markers was assessed using the Spearman's rank correlation coefficient and an analysis of variance (ANOVA).

Genetic differentiation over all populations, between groups (i.e. plantations and natural populations), among natural populations and among plantations was assessed for both marker systems. For microsatellites, the proportion of total genetic variation distributed among populations was computed based on genetic diversity using the F_{ST} estimator of Weir and Cockerham (1984) and on allelic richness according to Petit et al. (1998) as $A_{ST} = 1 - \left(A_{s[g]} - 1\right) / A_{T[g]} - 1$, where $A_{s[g]}$ is the mean allelic richness within populations and $A_{T[g]}$ is the total allelic richness. For AFLPs, differentiation was assessed based on the genetic diversity as $F_{ST} = \left(\overline{H}_T - \overline{H}_S\right) / \overline{H}_T$ (Finkeldey, 1994).

Additionally, a clustering analysis was performed based on the UPGMA algorithm derived from the chord genetic distance (D_C; Cavalli-Sforza and Edwards, 1967) for microsatellites and from Nei's genetic distance (D_N; Nei, 1973) for AFLPs. Genetic distance matrix and UPGMA dendrogram were computed for microsatellites using the software POPULATIONS 2.0 (Langela, 2002). For AFLPs, genetic distance was calculated for each pair of populations using AFLP-SURV (Vekemans 2002) and the dendrogram was constructed with the programs NEIGHBOUR (UPGMA algorithm) and CONSENSE (expanded majority rule approach) of the package PHYLIP (Felsenstein, 1989) release 3.66. Statistical support of the clusters was assessed by means of 1000 bootstrap replicates over loci for both marker systems.

Results

Population diversity

For microsatellites, 92 alleles were characterized over all populations, the allelic richness equalled 18.36, the gene diversity was H = 0.721 and the inbreeding coefficient was 0.154 (Table 9.2).

Considering two groups (i.e. plantations and natural populations) the measures of genetic diversity and inbreeding coefficient computed for plantations (A = 85, $A_{r[48]}$ = 8.81, H = 0.741 and f = 0.141) were higher than for natural populations (A = 69, $A_{r[48]}$ = 7.49, H = 0.693 and f = 0.102). Allelic richness and gene diversity revealed significant differences between groups (P = 0.004 and P = 0.05, respectively), while the inbreeding coefficient was not significantly different between plantations and natural populations (P = 0.18). Among ten pairs of loci, significant linkage disequilibrium between loci (LD; Table 9.3) was observed in 4 natural populations (1 pair in BJ, PD and RG; 2 pairs in NG) and two plantations (1 pair in CEU and TEL1). For plantation TEL2, the test of four pairs failed because locus Ag45 was fixed. Thus, out of 96 tests for LD conducted, seven showed significant LD at the 5% level.

For AFLPs, natural populations revealed higher levels of diversity in comparison to plantations ($F_{r[24]}$ = 1.862 versus $F_{r[24]}$ = 1.738; PPL = 100.0% versus PPL = 97.6 and H_j = 0.291 versus H_j = 0.240; Table 9.2). However, according to the ANOVA, the difference between plantations and natural populations was statistically not significant (P > 0.33) for all indices of genetic diversity.

Estimations of gene diversity obtained from microsatellites and AFLPs were negatively correlated (Spearman's rank correlation R^2 = -0.30) but not statistically significant (P = 0.30). Similarly, according to the analysis of variance, these measures were not statistically different between marker systems (P = 0.27).

Table 9.2: Measures of gene diversity based on five microsatellite loci and on 166 AFLP loci.

	Microsatellites				AFLPs		
	A	$A_{r[48]}$	H	f	PPL	$F_{r[24]}$	H_j
Natural populations							
BJ	42	6.71	0.622	0.076 **	78.3	1.897	0.283
NG	47	7.54	0.635	0.169 **	91.0	1.936	0.305
PD	47	7.50	0.637	0.090 **	81.3	1.917	0.281
FV	48	7.54	0.663	0.081 **	61.4	1.713	0.214
RG	54	8.15	0.743	0.103 **	78.9	1.849	0.275
Overall	69	7.49 (a)	0.693§ (a)	0.102 (a)	100 (a)	1.862# (a)	0.291§ (a)
Plantations							
CEU	56	9.13	0.766	0.176 **	65.7	1.754	0.237
GUA	54	8.65	0.743	0.066 *	64.5	1.739	0.233
PAI	56	9.27	0.718	0.178 **	63.3	1.769	0.222
TEL1	43	8.43	0.677	0.153 **	60.2	1.723	0.221
TEL2	44	8.55	0.657	0.142 **	59.0	1.705	0.217
Overall	85	8.81 (b)	0.741§ (b)	0.141 (a)	97.6 (a)	1.738# (a)	0.240§ (a)
Total	92	18.36	0.721	0.154 **	-	-	0.298

A: total number of alleles; $A_{r[48]}$: mean allelic richness over loci based on 48 gene copies; H: mean gene diversity over loci; f: mean inbreeding coefficient over loci; PPL: percentage of polymorphic loci; $F_{r[24]}$: Fragment richness over loci based on 24 individuals; H_j: mean gene diversity over loci. Values followed by the same letter in a column are not significantly different at 5% probability level. §: Total gene diversity H_T according to Nei (1973); #: mean value over populations.

Population differentiation

At all hierarchical levels, estimations of F_{ST} based on AFLPs revealed higher values than estimations from microsatellites (Table 9.4). Estimations of A_{ST} among plantations and among natural populations were higher than values of F_{ST} estimated from AFLPs, while overall and between groups A_{ST} values were lower than AFLPs' F_{ST} estimations. As expected, the proportion of total genetic variation distributed among populations estimated as F_{ST} (based on gene diversity) and A_{ST} (based on allelic richness) for microsatellites revealed a congruent ranking. However, estimations obtained from microsatellites and AFLPs were not congruent with respect to the ranking of F_{ST} (or A_{ST}) values. For microsatellite markers, the highest values of F_{ST} and A_{ST} were observed among natural populations, while the lowest values were found between groups. For AFLPs, the highest value of F_{ST} was found overall populations, while the lowest value was observed among plantations (table 9.4).

The UPGMA dendrogram generated from microsatellite data (Figure 9.2) separated the natural populations according to geographical distribution with relatively high bootstrap support (77% for populations NG and PD and 90% for populations FV and RG). The low bootstrap support for the other clusters reflects the high variation at the intra-population level, mainly in the plantations, since bootstrap values higher than 95% are obtained for all groups when just natural populations are considered (Stefenon et al., 2007). Plantations which were established with seeds collected in Santa Catarina state formed a cluster sister to natural populations from SC/RS. Plantation TEL2, established with seeds from Paraná state was the most divergent population. In the UPGMA dendrogram generated from AFLP markers (Figure 9.3) natural populations formed a cluster with high bootstrap support (99%), clearly separated from plantations. All clusters revealed high bootstrap support (> 72%), but no clear geographical pattern is observed.

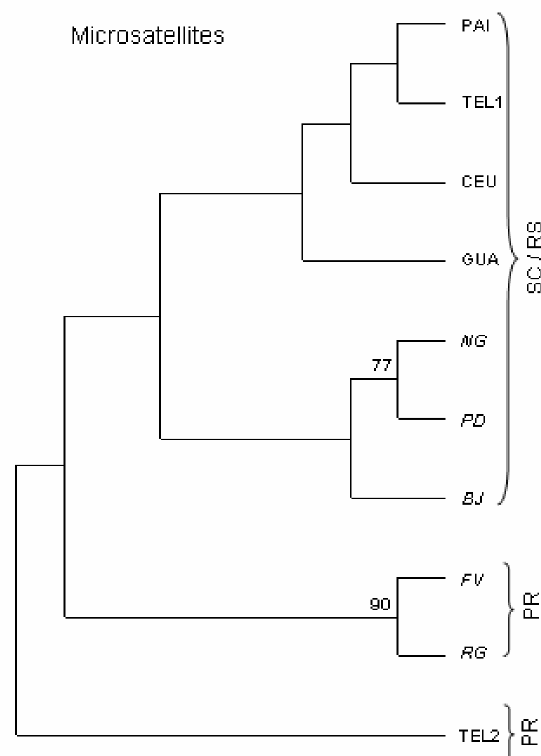

Figure 9.2: UPGMA dendrogram based on D_C genetic distance (Cavalli-Sforza and Edwards, 1967) computed for microsatellite data. Numbers at nodes are percentage over 1000 bootstrap replicates. See Table 9.1 for legends.

Discussion

Considering the emergent climatic instability, adaptedness (the degree to which an organism is capable to survive and reproduce in a particular environment; Eriksson, 2005) and adaptability emerge as fundamental problems for forestry (Mátyás, 2005). Such capacity of populations to react to changing environmental conditions by evolutionary adaptations critically depends on the maintenance of high levels of genetic diversity, both in natural populations and plantations. Several studies have been performed in order to assess the levels of genetic variation within planted forests (e.g. Medri et al. 2003; Korshikov et al., 2004; Li et al. 2005; Íçgen et al., 2006) and reported different results. The present study adds further information to this subject through the comparison of results obtained using two contrasting marker systems.

As frequently found in the literature (e.g. Mariette et al., 2002a, b; Gaudeaul et al., 2004; Woodhead et al., 2005; Garoia et al., 2007), different patterns of population diversity assessed with microsatellites and AFLPs were observed in this study. However, this divergence can be generated just by random variation if populations have not reached equilibrium between drift, migration and mutation (Mariette et al., 2002b). Our estimations of gene diversity obtained from microsatellites and AFLPs were negatively correlated according to populations ranking, but this correlation was not statistically significant. In the same way, these measures were not statistically different between markers. Thus, the observed ranking of populations was likely generated just by the random variation of diversity and may not bias our general conclusions.

Table 9.3: Summary of linkage disequilibrium (LD) analyses. The plus signal means significant LD at 5% level. Minus signal means non-significant LD.

	AA01/ Ag20	AA01/ Ag45	Ag20/ Ag45	AA01/ Ag94	Ag20/ Ag94	Ag45/ Ag94	AA01/ CRCAc2	Ag20/ CRCAc2	Ag45/ CRCAc2	Ag94/ CRCAc2
Natural populations										
BJ	+	-	-	-	-	-	-	-	-	-
NG	-	-	-	-	-	-	+	-	+	-
PD	+	-	-	-	-	-	-	-	-	-
FV	-	-	-	-	-	-	-	-	-	-
RG	-	-	-	-	-	-	+	-	-	-
Mean	2/5	0/5	0/5	0/5	0/5	0/5	2/5	0/5	1/5	0/5
Plantations										
CEU	-	+	-	-	-	-	-	-	-	-
GUA	-	-	-	-	-	-	-	-	-	-
PAI	-	-	-	-	-	-	-	-	-	-
TEL1	-	-	-	-	-	+	-	-	-	-
TEL2	-	nc	nc	-	-	nc	-	-	nc	-
Mean	0/5	1/4	0/4	0/5	0/5	1/4	0/5	0/5	0/4	0/5

nc: not computed because locus Ag45 was fixed in population TEL2.

Genetic diversity of plantations

Remarkable changes in the genetic structure of propagated material in comparison to its source populations may occur due to screening and rejection of unsuitable seedlings before plantation establishment (Korshikov et al., 2004). In general, the results of this study suggest that the analysed plantations were not strongly altered in comparison to the genetic structure of the natural populations. This pattern is mainly revealed by the microsatellite data through the inbreeding estimations, the linkage disequilibrium (LD) analysis and UPGMA cluster analysis. The estimations of inbreeding coefficients were slightly higher in the plantations than in natural populations, but not significantly different between groups. This suggests that seeds were widely collected within natural stands for the plantation establishment, maintaining a level of individual relatedness similar to natural populations. Congruently, low estimations of LD for pairs of microsatellite loci in the plantations suggest that seeds applied in plantations' establishment were sampled from many different mother trees. In the cluster analysis based on microsatellite data, all plantations from Santa Catarina state grouped with natural stands from the same geographic region where seeds were collected for their establishment. This result suggests that plantations preserved genetic information very similar to natural populations from the same geographic region. This is in agreement with the pattern revealed by Stefenon et al. (2007) which showed that the analysed natural populations can be differentiated in a 'Paraná group' and a 'Santa Catarina/Rio Grande group' based on their genetic structures.

Despite of the smaller sample sizes (mean n = 38.4) plantations revealed significantly higher allelic richness and gene diversity at microsatellite loci than natural populations (mean n = 64). Medri et al. (2003) reported a reduction of RAPD polymorphism of 11.58% in a managed population of *A. angustifolia* (*PPL* = 72.5%) in comparison to a natural stand (*PPL* = 82.0%). In a progeny test, the reduction of polymorphism was 27.43% (*PPL* = 59.7%) in relation to the natural population. Analysing six isozyme systems, Gömöry (1992) reported lower gene diversity in plantations than in natural populations of *Picea abies.* In contrast, Thomas et al. (1999) found no difference in gene diversity between natural and planted populations of *Pinus contorta* using microsatellite and RAPD markers. It has been suggested that the admixture of organisms from different source populations can result in an increase of diversity (Comps et al., 2001; Petit et al., 2003). This increase tends to upset the effects of transitory reduction in the effective population size, a liable fact in establishing new populations. The high levels of genetic diversity in the plantations may be related with high allelic diversity of the natural stands where seeds were collected, or with the admixture of seeds that originated from different natural stands. This pattern can be clarified by the inspection of A_{ST} values. Inference of A_{ST} is mostly dependent on the distribution of rare alleles, mainly whether they tend to be clustered in some populations (case where A_{ST} is high) or are distributed more evenly (Comps et al., 2001). The low differentiation among plantations based on allelic richness (A_{ST} = 0.089) suggests that seeds were mixed (at least for some plantations) from different natural populations before plantations establishment, generating a more homogeneous distribution of alleles among them. Indeed, plantations CEU, GUA and PAI were established with seeds acquired from local farmers, which likely collected seeds from different natural stands. These plantations revealed higher allelic richness than

natural stands and than plantations TEL1 and TEL2, which were established with seeds from single natural populations. On the other hand, A_{ST} among natural stands is 1.5-fold higher than among plantations. The clustering of rare alleles in natural populations is expected as effect of limited gene flow in *A. angustifolia* (Stefenon et al., in press) and consequent isolation-by-distance (Stefenon et al., 2007).

Table 9.4: Partition of genetic variation based on gene diversity (F_{ST}) and on allelic richness (A_{ST}).

	Overall	Between groups	Among natural populations	Among plantations
A_{ST} (Microsatellites)	0.116	0.049	0.136	0.089
F_{ST} (Microsatellites)	0.045	0.020	0.048	0.027
F_{ST} (AFLPs)	0.133	0.113	0.066	0.059

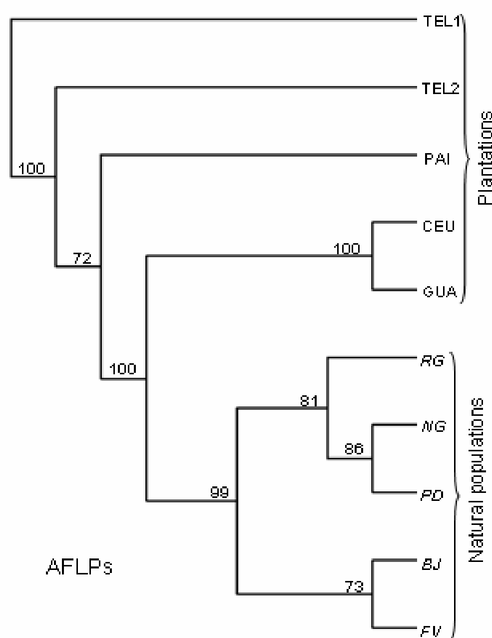

Figure 9.3: Consensus UPGMA dendrogram based on Nei's genetic distance (Nei, 1973) computed for AFLP data. Numbers at nodes are percentage over 1000 bootstrap replicates.

Remarks on conservation and management of Brazilian pine

Although the requirement of genetic conservation of araucaria forests has been recognized in Brazil since early in the last century, limited interest has been shown in establishing Brazilian pine plantations (Bittencourt et al., 2004). Our results suggest that planted forests may have abundant genetic diversity for species conservation, even after the strong fragmentation of the natural populations (e.g. plantation GUA, established in 1992). In general, the levels of gene diversity revealed by microsatellites and AFLPs in the plantations indicate good representation of the overall genetic variation. Concerning species sharing the same life history with *A. angustifolia* (i.e. long-lived perennial, outcrossing with gravity or attached seed dispersion, mid successional status), the level of

genetic diversity assessed for both marker systems in plantations and natural populations is higher than mean values reported in the literature (H = 0.42 to 0.68 for microsatellites and H_j = 0.16 to 0.27 for AFLPs; Nybom, 2004). Consequently, the moderate to high level of genetic diversity retained in *A. angustifolia* populations after the intense fragmentation of the natural forest (Shimizu et al., 2000; Sousa et al., 2004; Mantovani et al., 2006; Stefenon et al., 2007) has the potential to supply plant material with sufficient genetic diversity for the species conservation through the establishment of planted forests.

Despite the high genetic diversity observed, the analysed markers are expected to be primarily neutral and therefore will likely fail in detecting signatures of selection induced by seedling assortment, forest management and/or different environmental pressure in natural populations. As result of seedling assortment and forest management, differences in frequency of potentially adaptive genes between plantations and natural populations may exist. Despite the assumed neutrality of the analysed markers, some of them may be linked to genes under selective pressure although they do not behave as outlier loci (see Le Corre and Kremer, 2003). In fact, adaptive variation matters for conservation and must be considered. Therefore, genetic variation based on morphological adaptive traits can not be ignored. The clear differentiation between plantations and natural populations observed in the cluster analysis of populations based on AFLP data may occur as effect of genetic hitchhiking of some AFLP fragments. This issue should be focus of future studies, for instance assessing population differentiation for quantitative traits and for neutral gene markers with similar measures (e.g. F_{ST} estimations based on variance components; Yang et al., 1996).

A sustainable management of the extant forest remnants and forestation/reforestation enterprises should additionally attend to trends revealed in previous studies concerning population structure and gene flow. For instance, the significant geographic structure among populations validates the use of the species geographic distribution as a criterion for planning *in situ* conservation, seed collection and for the delineation of seed zones (Stefenon et al., 2007). In addition, trends of fine-scale spatial genetic structure demonstrate that, in some populations, individuals within a neighbourhood of up to 70 meters are genetically more related than expected, suggesting the existence of family structures (Mantovani et al., 2006; Stefenon et al., in press). Planning seed collection should focus also on this feature, in order to increase the sampled genetic diversity. Similarly, thinning enterprises should concentrate on this aspect, in order to ensure the maintenance of high diversity in managed stands.

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10. Evidences of delayed size recovery in *Araucaria angustifolia* populations after post-glacial colonization of highlands in Southeastern Brazil [9] [10]

Abstract

Up to date, little is known about the relationship between historical demography and the current genetic structure of *A. angustifolia*. As a first effort towards overcoming this lack, microsatellite data scored in six populations and isozyme allele frequencies published for 11 natural stands of this species were analyzed in order to assess molecular signatures of populations' demographic history. Signatures of genetic bottlenecks were captured in all analysed populations of southeastern Brazil. Among southern populations, signatures of small effective population size were observed in only three out of 13 populations. Likely southern populations experienced faster recovery of population size after migration onto highlands. Accordingly, current genetic diversity of the southern populations gives evidence of fast population size recovery. In general, demographic history of *A. angustifolia* matches climatic dynamics of southern and southeastern Brazil during the Pleistocene and Holocene. Palynological records and paleobotanical data of the past climatic dynamics of southeastern and southern Brazil support the hypothesis of different population size recovery dynamics for populations from these regions.

Key words: Araucaria angustifolia – genetic bottleneck – population size recovery – demographic history

[9] Stefenon, V.M., Gailing, O., Behling, H. and Finkeldey, R.

[10] VMS conceived, designed and performed the experiments, analyzed the data and wrote the paper. All authors improved the final manuscript.

Introduction

Although the lands in Brazil have never been covered by ice sheets, the remarkable climatic changes during the Pleistocene and Holocene have influenced distribution and evolution of different species. For instance, due to the cold climate with long dry periods during the last glaciation (Ledru et al. 1998), the subtropical highlands of Brazil lacked forest formations and were covered by grassland throughout the Late Pleistocene, about 48000 to 18000 ^{14}C yr BP (uncalibrated radiocarbon years before present). During this adverse period, dispersed stands of *Araucaria angustifolia* (Bert.) O. Kuntze were likely found in refugia where the moisture allowed species' survival (Behling 2002). The transition to wetter climatic conditions started about 6000 to 5000 ^{14}C yr BP in southeastern and around 3000 ^{14}C yr BP in southern Brazil (Behling 2002). Based on palynological data (see Behling 2002 and references therein), it is presumed that a remarkable post-glacial recolonization of the southeastern and southern Brazilian highlands by *A. angustifolia* populations occurred from 3500 ^{14}C yr BP in form of larger gallery forests. From about 1000 ^{14}C yr BP Araucaria forest expanded markedly into the Campos (grassland) vegetation, when climatic conditions became wetter with no marked dry season.

To date, different techniques have been applied to assess patterns of genetic structure in *A. angustifolia* (e.g. Shimizu and Higa, 1980; Auler *et al.,* 2002; Sousa *et al.,* 2004; Mantovani *et al.*, 2006; Stefenon *et al.,* 2007; Schögl *et al.,* 2007). However, little is known about the relationship between historical demography and the current genetic structure of this species. The coalescent approach (Kingman, 1982) provides a powerful way to investigate the spatiotemporal process of genetic changes. The coalescent approach can be interpreted as a large-population approximation to gene genealogies in a number of neutral models with finite population size (Nordborg and Krone, 2001). It is a stochastic model, which follows gene genealogies backwards in time and, based on robust statistical approaches, allows making inferences about the past demography of populations. Each segment of the genome is a replicate of the coalescent process, and the comparisons across multiple loci are more likely to accurately reflect population history. Multilocus microsatellite and isozyme data have been considered as very useful for inferring recent reductions in census and/or effective population size (Cornuet and Luikart, 1996; Beaumont, 1999). Here, two coalescent-based methods (the heterozygosity excess and the *M*-ratio analyses), a graphical analysis of allele frequency distribution and classical clustering analysis were applied to investigate molecular signature of demographic events based on individual microsatellite genotypes scored in six natural populations of *A. angustifolia* sampled in four states: São Paulo, Paraná, Santa Catarina and Rio Grande do Sul (see Table 10.1). Additionally, allelic frequencies at isozyme loci scored in three populations from São Paulo state (Sousa *et al.,* 2004; Mantovani *et al.,* 2006; see Table 10.2) and in eight populations from Santa Catarina state (Auler *et al.,* 2002; see Table 10.2) were analyzed using the heterozygosity excess and the allele frequency distribution approaches. The main goal of this study was to investigate molecular signatures of genetic bottleneck due to post-glacial establishment of *A. angustifolia* populations in the highlands of southeastern and southern Brazil. It is believed that when a population has colonized a region through migration, it is much harder for following populations to advance. Therefore, it is likely that during a range change the last surviving population should be

severely bottlenecked (Hewitt, 2000). Given that migration of *A. angustifolia* into highlands occurred relatively recently, signatures of bottleneck events may be retained in its genome and are likely to be assessed through genetic analyses. Additionally, if such a reduction in effective population size occurred as effect of post-glacial migration from refugia onto highlands, it is expected that climate dynamics match the genetic demographic history of populations.

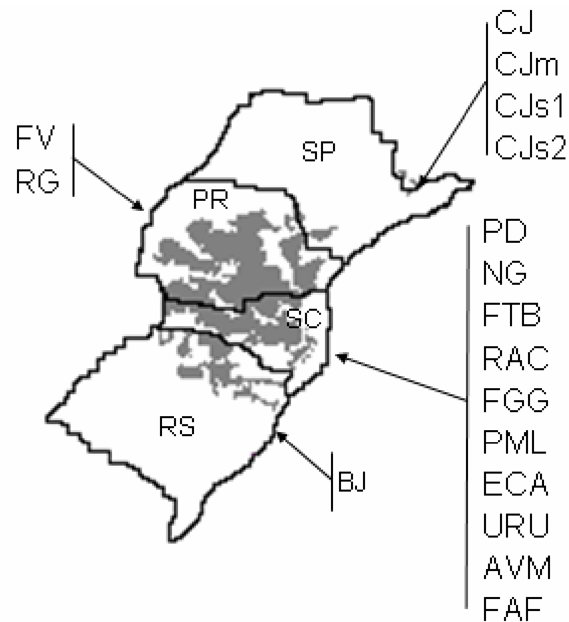

Figure 10.1: Map showing the natural distribution range of *A. angustifolia* in southern and southeastern Brazil (grey areas). The state to which each population belongs is indicated by the arrows. Geographic coordinates of individual populations are given in Tables 10.1 and 10.2.

Material and Methods

Multilocus microsatellite genotypes were scored at five loci as described by Stefenon *et al.* (2007). Isozyme allelic frequencies were obtained from their original publications (Auler *et al.,* 2002; Sousa *et al.,* 2004; Mantovani *et al.,* 2006). Founded on the principle that rare alleles are lost in populations which experienced a strong reduction in effective size (Nei *et al.,* 1975), three methods were employed to search for signature of population bottlenecks. The first method consists in testing for heterozygosity excess in comparison to predictable heterozygosity under mutation-drift equilibrium considering the observed number of alleles (Cornuet and Luikart, 1996). Heterozygosity excess should not be confused with the excess of heterozygotes (comparison of observed and expected heterozygoties). Bottlenecked populations are expected to reveal a significant excess of heterozygosity than estimated under equilibrium, because rare alleles are lost faster than the heterozygosity. Heterozygosity excess was tested by comparing the expected heterozygosity under

mutation-drift equilibrium (H_{EQ}) with the Hardy-Weinberg heterozygosity (H_e). Estimates of H_{EQ} were obtained by simulating the coalescent process (10000 iterations) for each population following the two-phase mutation model (TPM; Di Rienzo *et al.*, 1994), which assumes that most mutational changes result in an increase or decrease of one repeat unit but also incorporates mutations of larger size. The isozyme data were investigated using the infinite allele model (IAM; Kimura and Crow, 1964), which assumes that each arising allele is unique. Statistical significance of the analyses was assessed using the Wilcoxon test (Luikart *et al.*, 1998a).

The second approach is based on the assumption that populations which experienced a recent reduction in effective size tend to show a distortion of allele frequency distribution (Luikart *et al.,* 1998b). For this mode-shift analysis, alleles were grouped into 10 allele frequency classes and plotted in a frequency histogram. Bottlenecked populations have a propensity to display a shifted distribution, with the incidence of alleles at frequency lower than 0.1 becoming lower than the incidence of alleles in an intermediate allele frequency class.

The third method consists in analysing the ratio of the total number of alleles (k) to the overall range in allele size (r) as $M = k/r$ (Garza and Williamson, 2001). For this analysis, alleles of the microsatellite locus Ag20 were binned into classes to fit the step-wise model. To test whether *M*-values are lower than expected (cases in which a population experienced a bottleneck), 10000 replicates of the coalescent process were simulated assuming a proportion of one-step mutations (p_s) of 0.2, a mean size of non one-step mutations (Δ_g) of 3.5 and two different bottleneck population sizes, assuming a mutation rate (μ) of 10^{-4} mutations per locus per generation: N_e = 50 (θ = 0.02) and N_e = 500 (θ = 0.2), where $\theta = 4N_e\mu$. Analyses were performed using the programs BOTTLENECK 1.2.02 (Piry *et al.,* 1999) and M_P_VAL (Garza and Williamson, 2001).

Further evidences of bottleneck events were investigated assuming that distance values increase rapidly when a bottleneck occurs and, therefore bottlenecked populations tend to present elongated branches in cluster analyses (Takezaki and Nei, 1996). Phylograms were constructed for microsatellite data with the program POPULATIONS 2.0 (Langella, 2002) using the $(\delta\mu)^2$ genetic distance (Goldstein *et al.,* 1995) and the neighbor-joining clustering algorithm.

Table 10.1 - Summary of the coalescent-based analyses of microsatellite data. Statistically significant values are highlighted in bold.

Population (*n*)	Location of Populations				Polymorphic loci / alleles (mean H_e)	Loci with heterozygosity excess §	*P*-value Multilocus ‡	*M*-ratio analysis		
	Latitude (S)	Longitude (W)	Altitude (m)	State †				*M*-ratio	*P* (θ =0.02)	*P* (θ = 0.2)
BJ (64)	28°32’	50°39’	967	RS	5 / 42 (0.63)	2 (0)	0.68	0.72	**0.05** *	0.07 ns
NG (64)	27°45’	49°39’	885	SC	5 / 47 (0.64)	1 (0)	0.97	0.86	0.27 ns	0.36 ns
PD (64)	27°12’	50°23’	1034	SC	5 / 47 (0.64)	1 (0)	0.95	0.79	0.13 ns	0.19 ns
FV (64)	24°15’	50°25’	970	PR	5 / 48 (0.67)	1 (0)	0.92	0.78	0.12 ns	0.17 ns
RG (64)	24°20’	50°34’	729	PR	5 / 54 (0.75)	1 (1)	0.59	0.73	**0.05** *	0.08 ns
CJ (64)	22°41’	45°29’	1507	SP	5 / 38 (0.59)	1 (0)	0.97	0.69	**0.03** *	**0.04** *

† RS: Rio Grande do Sul; SC: Santa Catarina; PR: Paraná; SP: São Paulo.

§ Number of loci revealing heterozygosity excess. Number of loci with significant excess is given within parenthesis.

‡ Statistical significance of the Wilcoxon test for multilocus analysis of heterozygosity excess.

Results

The accentuated genetic differentiation reported among populations from southeastern and southern regions (e.g. Sousa *et al.,* 2004; Stefenon *et al.,* 2007) has been explained as effect of post-glacial migration from different refugia, which in addition to the geographical isolation, resulted in temporal isolation of the southeastern population (Stefenon *et al.,* 2007). Four populations analyzed in this study belong to the southeastern region (CJ, CJm, CJs1 and CJs2 from São Paulo state; Figure 10.1) and revealed signatures of bottleneck in at least one of the analyses. Among the southern populations (from Paraná, Santa Catarina and Rio Grande do Sul states), molecular signature of low population size was detected just in three out of 13 stands.

In the analysis of heterozygosity excess (Tables 10.1 and 10.2) signature of bottleneck was detected only for population CJm, with significance excess of heterozygosity observed in six out of seven loci (Table 10.2). Three out of these six loci revealed significant excess of heterozygosity at 5% level. The Wilcoxon test for the multilocus analysis was highly significant (*P* = 0.008).

In the mode-shift analysis populations CJm, CJs1, CJs2 and FTB revealed a shifted distribution of alleles (Figure 10.2A), characteristic of bottlenecked populations. All populations analysed with microsatellite markers revealed a normal distribution of alleles in this analysis (Figure 10.2B), suggesting absence of bottleneck signature.

In the *M*-ratio analysis, populations BJ, RG and CJ revealed signatures of bottleneck (Table 10.1) when considering a effective population size of 50 individuals for the post-migration population (θ = 0.02). When θ was set to 0.2 in the *M*-ratio analysis (N_e = 500; Table 10.1), signature of low effective population size was retained just in population CJ, emphasizing the occurrence of a bottleneck in this stand. Additional evidence of a bottleneck event in population CJ is given by the elongated branch revealed by this population in the neighbor-joining phylogram (Figure 10.2C). Although displaying a relatively low bootstrap support (55%), such elongated branches are usually observed for bottlenecked populations (Takezaki and Nei, 1996).

Discussion

In this study, the occurrence of genetic bottlenecks in *A. angustifolia* populations was tested with complementary methods, providing a more comprehensive view of the demographic events related with historical low effective population size. The graphical method and the heterozygosity excess analysis are more powerful in detecting molecular signature when pre-bottleneck θ is small, the bottleneck was severe and the population recovered sample size quickly, while the *M*-ratio analysis is more efficient in opposite circumstances (Busch et al., 2007). Moreover, the heterozygosity excess and the shift-mode analyses are more powerful in capturing signatures of bottleneck from loci evolving under the IAM model (Cornuet and Luikart, 1996), while the *M*-ratio was specifically designed to the analysis of allele distributions of loci evolving under the stepwise mutation model (SMM) and TPM model (Garza and Williamson, 2001). Concerning the timeframe since the occurrence of the bottleneck, the methods cover different and complementary time windows: $0.2N_e$ to $4N_e$ generations for the heterozygosity excess method (Cornuet and Luikart, 1996), $2N_e$ to $4N_e$ generations for the model shift method (Luikart *et al.,* 1998b) and a comparative larger timeframe for the *M*-ratio analysis (Garza and Williamson, 2001). Assuming a very low population size in the colonization of highlands by *A. angustifolia* may be unrealistic, given the high amount of pollen and seeds produced (one single tree can produce more than 1000 seeds per year; Mantovani *et al.,* 2004). Thus, N_e = 50 individuals should be a reasonable approximation of the effective population size of the post-glacial founder populations of *A. angustifolia*. Assuming this post-bottleneck effective population size, the timeframe ranges from 10 to 200 generations for the heterozygosity excess analysis and from 100 to 200 generations for the shift-mode analysis. For the *M*-ratio analysis, this timeframe goes up to more than 200 generations. We intended to detect bottleneck events occurred as effect of the post-glacial migration of the species. Assuming a generation interval ranging from 15 to 30 years for *A. angustifolia*, the methods applied will detect signatures of bottleneck events occurred between 150 and more than 6000 years ago, covering the period of the migration onto highlands (1000 to 3500 ^{14}C yr BP).

Table 10.2 - Summary of the analysis of heterozygosity excess based on coalescent simulations of isozyme data. Statistically significant values are highlighted in bold.

Population (*n*)	Location of Populations				Polymorphic loci / alleles (mean H_e)	Loci with heterozygosity excess §	*P*-value Multilocus ‡
	Latitude (S)	Longitude (W)	Altitude (m)	State †			
FTB (33) [a]	26°06'	59°19'	802	SC	6 / 14 (0.10)	3 (0)	0.66
RAC (34) [a]	27°25'	48°50'	600-910	SC	7 / 17 (0.08)	1 (0)	0.99
FGG (41) [a]	27°53'	50°43'	960	SC	10 / 23 (0.12)	2 (0)	0.99
PML (23) [a]	27°47'	50°22'	918	SC	5 / 11 (0.07)	2 (0)	0.92
ECA (45) [a]	26°46'	51°00'	920	SC	10 / 22 (0.11)	2 (0)	0.99
URU (37) [a]	27°57'	49°53'	980	SC	9 / 20 (0.10)	1 (0)	0.97
AVM (43) [a]	26°40'	49°49'	400-800	SC	7 / 15 (0.06)	1 (0)	0.98
FAF (42) [a]	27°48'	50°19'	918	SC	4 / 10 (0.06)	2 (0)	0.91
CJs1 (35) [b]	22°44'	43°44'	~1800	SP	6 / 12 (0.26)	5 (1)	0.08
CJs2 (35) [b]	22°44'	43°44'	~1800	SP	6 / 13 (0.25)	3 (0)	0.42
CJm (334) [c]	22°45'	45°30'	1450	SP	7 / 17 (0.17)	6 (3)	**0.008**

† SC: Santa Catarina; SP: São Paulo.

§ Number of loci revealing heterozygosity excess. Number of loci with significant excess is given within parenthesis.

‡ Statistical significance of the Wilcoxon test for multilocus analysis of heterozygosity excess.

Original data from: [a] Auler *et al.*, 2002; [b] Sousa *et al.*, 2004; [c] Mantovani *et al.*, 2006.

Genetic dynamics of bottlenecked populations

After a bottleneck, populations enter a recovery phase and new alleles are created by mutation. This population expansion and arise of new alleles generates a heterozygosity deficiency which can erase the signature of population decline (Cornuet and Luikart 1996). Consequently, signature of population reduction will be lost if population size recovery is fast. Recent forest exploitation should not be a source of bias in comparing *A. angustifolia* populations from southeastern and southern Brazilian regions, given that both share the same recent events (comprising the last 100 years). Assuming that the southeastern region was colonized earlier than the southern region, the preserved signature of a bottleneck suggests that southeastern stands recovered slowly after post-glacial migration onto highlands. On the other hand, southern populations may have displayed somewhat quicker effective size recovery, even if founded with low population size. Additionally, migration events among the southern stands might have overturned the effect of a bottleneck event on the allelic diversity of these populations. In small populations, the movement of just a few migrants can erase the signature of bottleneck in two to three generations (Busch et al. 2007).

Based on analyses of allelic structure of microsatellite loci, molecular signatures of genetic bottleneck were detected in an isolated stand but not in a large population of *Pinus taeda* in central Texas (Al-Rabab'ah and Williams 2004). The occurrence of long dry periods during the Holocene was the reason postulated for the persistent low population size of the isolated population. Similar to *P. taeda*,

prolonged drought is a likely factor to have intensified the effect of reduced effective size of *A. angustifolia* populations in southeastern Brazil. Ledru et al. (1998) propose that the modern climate in central Brazil was reached just about 2500 ^{14}C yr BP. If *A. angustifolia* colonized the southeastern Brazil about 3000 ^{14}C yr BP as suggested by the reconstruction of vegetation and polar advections trajectory (Ledru et al. 1998) and by the palynological record (Behling 1998, 2002), relatively dry periods followed the post-glacial migration for at least 500 years (these dating should be considered with caution due to imprecision of the radiocarbon method) and may have delayed population recovery in this region. At present day, the region of Campos do Jordão, southeastern Brazil, displays a low rainfall period of four months between May and August (Behling 1997b).

A shifted frequency distribution of alleles at five isozyme loci in the endemic *P. maximartinezii* was also interpreted as effect of an extreme bottleneck (Ledig et al. 1999). The authors evoked a rapid post-bottleneck expansion as the reasoning for the diminished effects of genetic drift observed. Similarly, rapid population expansion from a bottleneck event was suggested to explain the unimodal distribution of pairwise differences among individuals (chloroplast microsatellites analysis; Echt et al. 1998), the small estimated effective population size and the high selfing rate (nuclear microsatellites analysis; Boys et al. 2005) assessed in the widely distributed *P. resinosa*.

In the present study the mean number of alleles per locus observed was similar among bottlenecked and non-bottlenecked populations for microsatellites (44.7 and 44.3 respectively; $p > 0.05$; t-test = 0.61; d.f. = 4). Among the populations analysed with microsatellite markers, population CJ revealed the lowest number of alleles and gene diversity (H_e). On the other hand, population RG revealed the highest number of alleles and gene diversity among the six investigated populations, although signature of bottleneck was captured in this stand in the *M*-ratio analysis with $N_e = 50$ ($\theta = 0.02$). The high diversity observed in this population supports a quick effective size recovery in the southern region. As for microsatellites, the mean number of alleles per locus at isozyme markers was statistically not different between bottlenecked and non-bottlenecked populations (2.2 and 2.3 respectively; $p > 0.05$; t-test = 0.59; d.f. = 9). Comparing the eight populations analysed by Auler et al. (2002), population FTB revealed the third highest gene diversity (H_e) and mean number of alleles per locus. Considering the signature of bottleneck captured in this population in the mode-shift analysis, this comparatively high diversity is also evidence of a quick effective size recovery of the southern populations.

Matching of past climatic dynamics and signature of bottleneck events

If the observed signatures of past population demography is an effect of the post-glacial migration of *A. angustifolia* from refugia onto highlands, it is expected that reconstructed climate dynamics match the genetic inferences. The presence of small population of *A. angustifolia* in low elevated areas along rivers in southeastern Brazil and in deep protected valleys and/or on the Atlantic facing slops at lower elevations in southern Brazil during the glacial period is indicated by pollen analytical studies (Behling and Lichte 1997, Behling et al. 2002, 2004, 2007). Expansion of *A. angustifolia* from refugia as gallery

forest at low elevations onto the higher mountains in southeastern Brazil (e.g. Campos do Jordão) started already during the late glacial period (Behling 1997b), while in southern Brazil a significant expansion onto highlands occurred when climate conditions were more suitable, about 3500 ^{14}C yr BP by the expansion of gallery forests and somewhat latter since about 1000 ^{14}C yr BP into the grassland (Behling 1998, 2002, Behling and Pillar 2007).

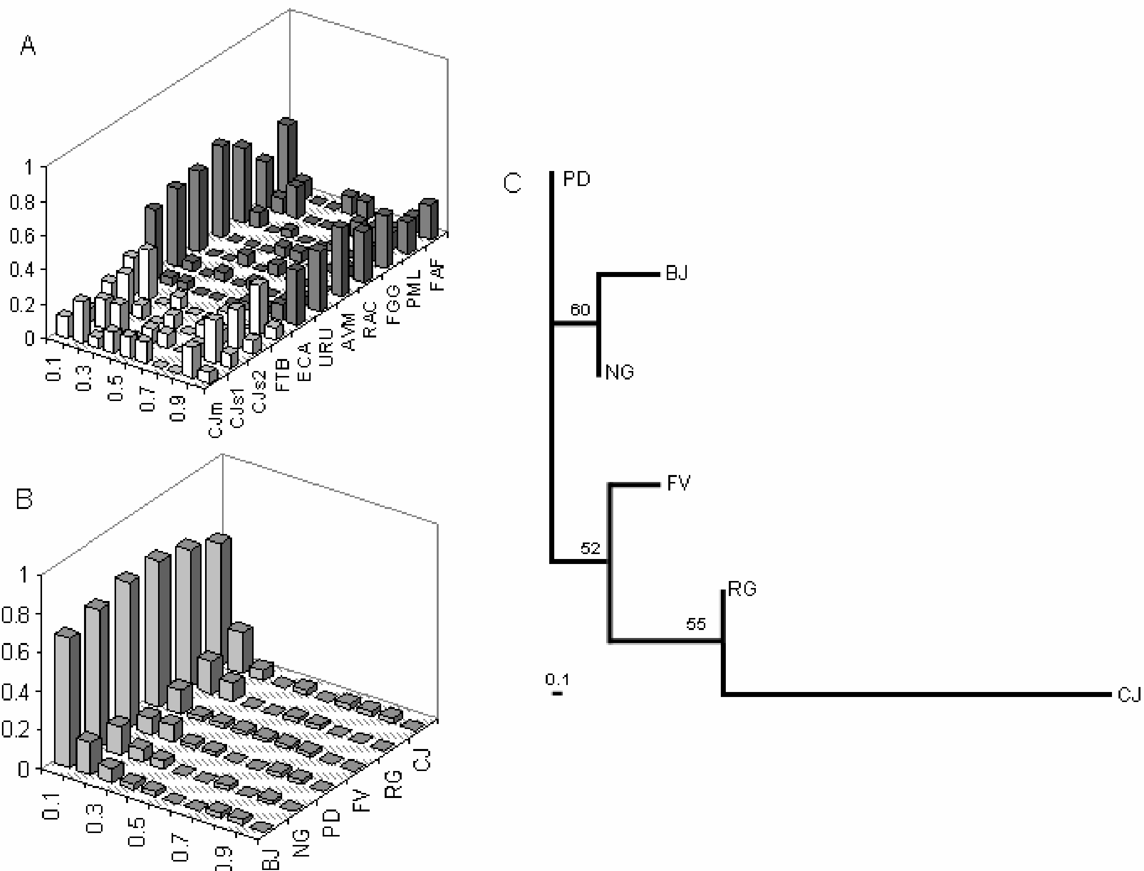

Figure 10.2: Molecular signatures of bottleneck events in *A. angustifolia* populations. (**A**) Plotting of the frequency distribution of allele classes for isozymes. Populations CJm, CJs1, CJs2 and FTB revealed a shifted distribution, indicating historical reduction of the effective population size. (**B**) Plotting of the frequency distribution of allele classes for microsatellites. All populations revealed a non-shifted alleles distribution, meaning absence of bottleneck signatures. (**C**) Neighbor-joining phylogram computed for microsatellite data based on $(\delta\mu)^2$ genetic distance. Evidence of a bottleneck event in population CJ is given by the elongated branch length. Numbers at nodes in the phylogram are bootstrap values after 1000 replicates.

Based on the pollen record from four peat bogs in Santa Catarina, Behling (1995) suggested that *A. angustifolia* might have persisted in protected highland valleys during the Late Pleistocene (> 10000 years ago). Just minor expansion as gallery forest along rivers occurred at the end of this period. The first major expansion of *A. angustifolia* onto highlands in Santa Catarina occurred as a result of a very moist climate, around 1000 ^{14}C yr BP (Behling 1995). Palynological data from Serra dos Campos

Gerais in Paraná State (Behling 1997a) revealed a Late Quaternary vegetation and climate history very similar to that of Santa Catarina with a long dry season until the beginning of the Holocene. The expansion of *A. angustifolia* onto highlands in the Late Holocene is evidence of a shorter annual dry period around 2850 ^{14}C yr BP. The first broad expansion of *A. angustifolia* onto highlands in Paraná State occurred about 1500 ^{14}C yr BP (Behling 1997a). Past environmental changes reconstructed on basis of the pollen record from Cambará do Sul, in the Rio Grande do Sul State (Behling et al. 2004) corroborated the results from Santa Catarina and Paraná. *A. angustifolia* was likely found just in deep protected valleys and/or wetter coastal slopes. Replacement of grassland vegetation by *A. angustifolia* started around 1100 ^{14}C yr BP, reflecting the arrival of a wetter period without marked annual dry season. The pollen record from the southeastern highlands is more restricted, but a rather comprehensive reconstruction of the past climatic dynamics is available by Behling (1997b). The late Quaternary period (from about 35000 to 17000 ^{14}C yr BP) was represented by a cold and dry climate in this region. A comparatively warmer but still dry climate followed until around 2600 ^{14}C yr BP, forcing *A. angustifolia* to continue in moist refugia. After this period, a cool and moist climate allowed the expansion of *A. angustifolia* from refugia onto São Paulo highlands.

A comparative analysis of palynological records from southeastern and southern Brazilian highlands (Figure 10.3) corroborates the occurrence of an expressive expansion of *A. angustifolia* populations in the southern states starting about 1500 to 1000 ^{14}C yr BP (Cambará do Sul, Rio do Rastro and Campos Gerais in Figure 10.3), but a lack of such a major expansion in the southeastern region (São Paulo State; Itapeva in Figure 10.3). This fact matches the molecular evidences of a post-glacial bottleneck in Campos do Jordão region followed by a slow recovery of effective population size, while southern populations experienced a fast size recovery after the post-glacial migration.

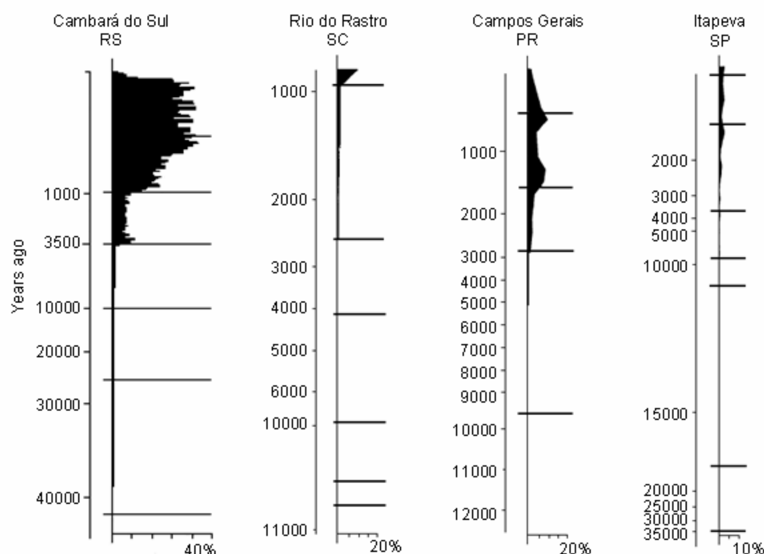

Figure 10.3: Pollen diagrams of *A. angustifolia* from Cambará do Sul (RS), Serra do Rio do Rastro (SC), Serra dos Campos Gerais (PR) and Morro do Itapeva (SP). Percentages were calculated from the total pollen sum (arboreal and non-arboreal), excluding aquatic taxa. Reproduced from Behling (2002) and Behling et al. (2004).

Conclusions and outlook

Despite the evidences of genetic bottlenecks revealed in this study, Busch et al. (2007) demonstrated that some biological features may lead to violations of the assumptions made for each of the methods employed here, obscuring the molecular signature of genetic bottlenecks. In addition, a low number of markers (both isozymes and microsatellites) were analysed. Therefore, corroboration of the demographic patterns observed should be obtained through genome sequencing which allows the use of more powerful tests for departure from mutation-drift equilibrium.

Here, signature of low effective size during populations' establishment and subsequent generations was assessed. However, consequences of recent population reduction and fragmentation in patterns of reproduction and species adaptedness will be observed just in future generations. Considering the increasing concern in conservation of *A. angustifolia* genetic resources, highlighting the species demographic history may aid to foresee and minimize unwanted events related to decreasing demographic and genetic population size. For instance, since isolation likely delays genetic recovery from a small population size (as suggested by the molecular signature of the southeastern populations), promoting connectivity among fragments may be a fundamental issue in scheduling genetic conservation of the extant remnants of *A. angustifolia*.

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11. APPENDICES

Appendix 1: Allele frequencies for the microsatellite locus AA01 in six natural populations and five plantations of *A. angustifolia* in Brazil.

Appendix 2: Allele frequencies for the microsatellite locus Ag20 in six natural populations and five plantations of *A. angustifolia* in Brazil.

Appendix 3: Allele frequencies for the microsatellite locus Ag45 in six natural populations and five plantations of *A. angustifolia* in Brazil.

Appendix 4: Allele frequencies for the microsatellite locus Ag94 in six natural populations and five plantations of *A. angustifolia* in Brazil.

Appendix 5: Allele frequencies for the microsatellite locus CRCAc2 in six natural populations and five plantations of *A. angustifolia* in Brazil.

Appendix 6: Frequency of the fragment presence (1) for the 166 AFLP loci in six natural populations and five plantations of *A. angustifolia* in Brazil.

Appendix 1: Allele frequencies for the microsatellite locus AA01 in six natural populations (BJ, NG, PD, FV, RG and CJ) and five plantations (CEU, PAI, GUA, TEL1 and TEL2) of *A. angustifolia* in Brazil.

Allele	BJ	NG	PD	FV	RG	CJ	CEU	PAI	GUA	TEL1	TEL2
201	0.040	0.024	0.032	0.109	0.016	0.000	0.000	0.000	0.000	0.022	0.023
203	0.008	0.056	0.008	0.008	0.016	0.000	0.000	0.011	0.067	0.000	0.023
205	0.008	0.000	0.000	0.094	0.023	0.000	0.000	0.000	0.000	0.000	0.000
207	0.032	0.056	0.016	0.023	0.039	0.000	0.023	0.011	0.044	0.065	0.068
209	0.000	0.032	0.024	0.008	0.008	0.000	0.070	0.033	0.056	0.022	0.000
211	0.226	0.135	0.214	0.070	0.094	0.039	0.186	0.167	0.122	0.130	0.227
213	0.242	0.357	0.238	0.086	0.102	0.203	0.174	0.133	0.144	0.196	0.136
215	0.194	0.048	0.159	0.070	0.117	0.039	0.233	0.111	0.144	0.196	0.159
217	0.121	0.016	0.048	0.055	0.055	0.000	0.058	0.100	0.022	0.043	0.114
219	0.008	0.063	0.071	0.211	0.094	0.000	0.093	0.111	0.089	0.065	0.068
221	0.008	0.040	0.048	0.086	0.094	0.063	0.070	0.133	0.067	0.065	0.045
223	0.065	0.008	0.040	0.023	0.070	0.000	0.035	0.056	0.078	0.087	0.000
225	0.000	0.087	0.024	0.023	0.133	0.117	0.000	0.078	0.067	0.022	0.023
227	0.008	0.016	0.016	0.078	0.055	0.141	0.012	0.000	0.022	0.043	0.000
229	0.040	0.056	0.016	0.055	0.047	0.180	0.000	0.011	0.056	0.022	0.023
231	0.000	0.000	0.040	0.000	0.000	0.016	0.000	0.033	0.000	0.000	0.068
233	0.000	0.000	0.000	0.000	0.000	0.141	0.035	0.011	0.022	0.022	0.000
235	0.000	0.008	0.000	0.000	0.000	0.000	0.012	0.000	0.000	0.000	0.000
237	0.000	0.000	0.008	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000
239	0.000	0.000	0.000	0.000	0.039	0.031	0.000	0.000	0.000	0.000	0.023
245	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000	0.000	0.000	0.000

Appendix 2: Allele frequencies for the microsatellite locus Ag20 in six natural populations and populations (BJ, NG, PD, FV, RG and CJ) and five plantations (CEU, PAI, GUA, TEL1 and TEL2) of *A. angustifolia* in Brazil.

Allele	BJ	NG	PD	FV	RG	CJ	CEU	PAI	GUA	TEL1	TEL2
237	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021
238	0.000	0.040	0.000	0.056	0.137	0.184	0.000	0.000	0.011	0.000	0.000
239	0.000	0.000	0.016	0.175	0.048	0.000	0.074	0.021	0.033	0.000	0.000
240	0.048	0.032	0.078	0.000	0.153	0.000	0.149	0.083	0.056	0.045	0.125
241	0.008	0.032	0.086	0.516	0.210	0.105	0.000	0.021	0.089	0.068	0.042
242	0.127	0.286	0.305	0.071	0.266	0.009	0.277	0.083	0.167	0.432	0.396
243	0.349	0.238	0.227	0.103	0.000	0.053	0.223	0.354	0.300	0.114	0.208
244	0.190	0.127	0.133	0.048	0.121	0.000	0.106	0.073	0.089	0.091	0.063
245	0.063	0.040	0.055	0.000	0.008	0.491	0.074	0.198	0.078	0.068	0.083
246	0.000	0.135	0.063	0.016	0.008	0.000	0.064	0.021	0.022	0.068	0.000
247	0.008	0.008	0.000	0.000	0.016	0.096	0.000	0.000	0.011	0.000	0.021
248	0.095	0.040	0.008	0.000	0.000	0.009	0.011	0.146	0.000	0.023	0.021
249	0.024	0.000	0.000	0.016	0.016	0.044	0.011	0.000	0.011	0.023	0.021
250	0.000	0.000	0.000	0.000	0.000	0.009	0.011	0.000	0.022	0.045	0.000
251	0.024	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.044	0.000	0.000
252	0.000	0.016	0.000	0.000	0.008	0.000	0.000	0.000	0.022	0.000	0.000
253	0.000	0.008	0.016	0.000	0.008	0.000	0.000	0.000	0.044	0.023	0.000
254	0.000	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
255	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Appendix 3: Allele frequencies for the microsatellite locus Ag45 in six natural populations (BJ, NG, PD, FV, RG and CJ) and five plantations (CEU, PAI, GUA, TEL1 and TEL2) of *A. angustifolia* in Brazil.

Allele	BJ	NG	PD	FV	RG	CJ	CEU	PAI	GUA	TEL1	TEL2
154	0.063	0.000	0.017	0.024	0.032	0.040	0.074	0.010	0.000	0.000	0.000
158	0.000	0.000	0.000	0.000	0.008	0.000	0.021	0.031	0.011	0.045	0.000
160	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.000	0.000
162	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.000	0.000	0.000	0.000
164	0.000	0.000	0.000	0.000	0.000	0.008	0.032	0.010	0.000	0.000	0.000
166	0.230	0.125	0.175	0.355	0.294	0.202	0.266	0.323	0.149	0.068	0.000
168	0.706	0.875	0.808	0.605	0.659	0.742	0.574	0.604	0.830	0.886	1.000
170	0.000	0.000	0.000	0.008	0.008	0.008	0.021	0.000	0.000	0.000	0.000
172	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000
176	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.000	0.000

Appendix 4: Allele frequencies for the microsatellite locus Ag94 in six natural populations (BJ, NG, PD, FV, RG and CJ) and five plantations (CEU, PAI, GUA, TEL1 and TEL2) of *A. angustifolia* in Brazil.

Allele	BJ	NG	PD	FV	RG	CJ	CEU	PAI	GUA	TEL1	TEL2
117	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000	0.000
127	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.000	0.000	0.000
129	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000	0.000
131	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000	0.000	0.000
133	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.011	0.000	0.000	0.000
135	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000
137	0.000	0.000	0.000	0.026	0.000	0.000	0.000	0.011	0.000	0.000	0.000
139	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.034	0.012	0.000	0.000
141	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.000	0.000	0.022	0.000
143	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.011	0.023	0.000	0.000
145	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.000	0.000
147	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.023	0.000	0.000
149	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000
151	0.023	0.024	0.032	0.000	0.000	0.000	0.047	0.045	0.035	0.022	0.045
153	0.063	0.048	0.063	0.079	0.167	0.025	0.058	0.148	0.128	0.087	0.068
155	0.828	0.667	0.730	0.711	0.500	0.098	0.384	0.466	0.349	0.326	0.227
157	0.000	0.032	0.024	0.061	0.167	0.066	0.186	0.034	0.163	0.174	0.136
159	0.016	0.063	0.040	0.044	0.083	0.803	0.070	0.057	0.081	0.152	0.068
161	0.031	0.111	0.079	0.035	0.067	0.000	0.081	0.045	0.023	0.152	0.159
163	0.000	0.000	0.000	0.009	0.000	0.000	0.023	0.023	0.023	0.000	0.023
165	0.023	0.048	0.024	0.009	0.008	0.000	0.023	0.000	0.058	0.065	0.250
167	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000
169	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000
171	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.000	0.000	0.000	0.023
173	0.016	0.008	0.000	0.009	0.000	0.000	0.000	0.011	0.000	0.000	0.000
175	0.000	0.000	0.000	0.018	0.000	0.008	0.035	0.000	0.000	0.000	0.000
181	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000
185	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000	0.000	0.000

Appendix 5: Allele frequencies for the microsatellite locus CRCAc2 in six natural populations (BJ, NG, PD, FV, RG and CJ) and five plantations (CEU, PAI, GUA, TEL1 and TEL2) of *A. angustifolia* in Brazil.

Allele	BJ	NG	PD	FV	RG	CJ	CEU	PAI	GUA	TEL1	TEL2
183	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000
185	0.000	0.008	0.016	0.023	0.008	0.008	0.035	0.011	0.000	0.043	0.042
187	0.469	0.373	0.419	0.414	0.258	0.070	0.442	0.311	0.422	0.457	0.333
189	0.000	0.016	0.032	0.000	0.086	0.000	0.058	0.044	0.011	0.022	0.042
191	0.109	0.214	0.226	0.188	0.266	0.109	0.105	0.344	0.256	0.196	0.188
193	0.133	0.095	0.129	0.180	0.195	0.125	0.105	0.067	0.133	0.152	0.063
195	0.039	0.016	0.016	0.031	0.016	0.000	0.012	0.122	0.056	0.000	0.063
197	0.141	0.119	0.097	0.008	0.047	0.609	0.116	0.089	0.100	0.043	0.167
199	0.016	0.024	0.032	0.016	0.031	0.023	0.012	0.011	0.011	0.087	0.000
201	0.031	0.119	0.000	0.102	0.023	0.055	0.058	0.000	0.000	0.000	0.021
203	0.063	0.000	0.000	0.016	0.016	0.000	0.000	0.000	0.000	0.000	0.042
205	0.000	0.016	0.032	0.023	0.031	0.000	0.012	0.000	0.000	0.000	0.000
207	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.021
209	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.000	0.000	0.000	0.000
211	0.000	0.000	0.000	0.000	0.008	0.000	0.035	0.000	0.011	0.000	0.021

Appendix 6: Frequency of the fragment presence for the 166 AFLP loci in six natural populations (BJ, NG, PD, FV, RG and CJ) and five plantations (CEU, PAI, GUA, TEL1 and TEL2) of *A. angustifolia* in Brazil.

Locus	BJ	NG	PD	FV	RG	CJ	CEU	GUA	PAI	TEL1	TEL2
G075	0.919	0.921	0.937	1.000	0.968	0.968	0.938	0.957	0.979	1.000	1.000
G076	0.339	0.095	0.048	0.094	0.016	0.081	0.063	0.043	0.085	0.083	0.000
G078	1.000	0.905	0.841	0.891	0.905	0.984	0.521	0.500	0.489	0.417	0.583
G080	0.129	0.079	0.143	0.078	0.000	0.129	0.042	0.043	0.064	0.042	0.083
G081	0.226	0.444	0.159	0.391	0.524	0.597	0.021	0.174	0.000	0.000	0.083
G084	0.613	0.651	0.524	0.516	0.667	0.823	0.188	0.196	0.021	0.000	0.000
G086	1.000	0.984	0.873	1.000	0.984	0.919	0.958	0.978	1.000	0.917	0.958
G087	0.145	0.111	0.095	0.047	0.032	0.000	0.021	0.000	0.000	0.000	0.042
G088	0.661	0.365	0.698	0.375	0.270	0.145	0.083	0.065	0.106	0.000	0.000
G090	0.065	0.063	0.127	0.016	0.143	0.113	0.000	0.043	0.064	0.000	0.000
G092	0.097	0.159	0.048	0.125	0.032	0.000	0.000	0.000	0.000	0.000	0.000
G093	0.645	0.365	0.238	0.797	0.635	0.113	0.813	0.500	0.404	0.375	0.250
G094	0.048	0.254	0.222	0.094	0.159	0.145	0.042	0.239	0.213	0.292	0.250
G098	0.048	0.159	0.079	0.031	0.000	0.258	0.083	0.022	0.021	0.000	0.000
G099	0.048	0.063	0.063	0.031	0.048	0.032	0.042	0.022	0.000	0.042	0.000
G100	0.435	0.143	0.143	0.031	0.000	0.032	0.021	0.000	0.043	0.000	0.000
G101	0.500	0.317	0.190	0.500	0.302	0.177	0.417	0.196	0.362	0.125	0.333
G102	0.306	0.286	0.571	0.406	0.429	0.258	0.292	0.565	0.149	0.250	0.167
G105	0.355	0.302	0.127	0.031	0.048	0.161	0.000	0.022	0.021	0.000	0.042
G107	0.855	0.857	0.952	0.984	0.921	0.887	0.958	0.804	0.468	0.542	0.500
G108	0.548	0.460	0.365	0.297	0.286	0.081	0.125	0.130	0.426	0.375	0.375
G109	0.258	0.587	0.270	0.188	0.460	0.629	0.021	0.000	0.021	0.000	0.000
G111	0.742	0.365	0.317	0.125	0.143	0.048	0.271	0.022	0.085	0.042	0.042
G112	0.839	0.429	0.714	0.734	0.556	0.355	0.313	0.543	0.128	0.125	0.083
G113	0.629	0.857	0.714	0.359	0.603	0.919	0.250	0.130	0.085	0.083	0.125
G115	0.435	0.540	0.302	0.609	0.778	0.597	0.563	0.478	0.766	0.875	0.750
G118	0.952	0.841	0.794	0.953	0.921	0.629	0.688	0.457	0.681	0.250	0.375
G120	1.000	0.968	0.937	0.984	0.968	0.565	0.896	0.826	0.915	0.917	0.792
G122	0.532	0.206	0.095	0.313	0.063	0.387	0.104	0.196	0.319	0.083	0.000
G123	0.952	0.714	0.683	0.688	0.651	0.774	0.875	0.522	0.574	0.458	0.375

Appendix 6: Continued

Locus	BJ	NG	PD	FV	RG	CJ	CEU	GUA	PAI	TEL1	TEL2
G124	0.032	0.206	0.127	0.234	0.302	0.532	0.000	0.022	0.064	0.167	0.125
G125	0.935	0.698	0.460	1.000	0.937	1.000	0.917	0.935	0.787	0.625	0.958
G126	0.387	0.254	0.063	0.453	0.381	0.065	0.021	0.043	0.021	0.000	0.000
G128	0.790	0.857	0.810	0.906	0.810	0.968	0.771	0.652	0.723	0.833	0.667
G129	0.323	0.413	0.381	0.250	0.540	0.581	0.646	0.783	0.979	1.000	0.958
G131	0.290	0.508	0.365	0.109	0.190	0.581	0.083	0.065	0.085	0.167	0.167
G134	0.952	0.984	0.825	0.984	0.968	0.968	0.708	0.848	0.957	0.958	0.958
G138	0.581	0.810	0.524	0.766	0.683	0.177	0.458	0.261	0.426	0.375	0.333
G139	0.806	0.825	0.603	0.516	0.714	0.161	0.625	0.543	0.681	0.542	0.625
G142	0.952	0.794	0.921	1.000	0.889	0.500	0.938	0.652	0.213	0.292	0.125
G143	0.032	0.222	0.016	0.000	0.048	0.242	0.042	0.196	0.468	0.542	0.583
G144	0.210	0.032	0.016	0.063	0.016	0.097	0.000	0.000	0.021	0.000	0.000
G146	0.871	0.937	0.714	0.938	0.984	0.968	0.604	0.652	0.574	0.875	0.833
G149	0.516	0.175	0.206	0.250	0.270	0.226	0.313	0.152	0.234	0.000	0.042
G150	0.758	0.413	0.556	0.422	0.413	0.677	0.083	0.000	0.021	0.000	0.000
G151	0.903	0.810	0.762	1.000	0.968	0.952	0.729	0.609	0.191	0.333	0.167
G152	0.984	0.952	0.921	1.000	0.937	0.887	0.979	0.913	0.957	0.958	0.958
G154	0.984	0.889	0.889	0.984	0.984	0.952	0.813	0.587	0.766	0.667	0.667
G157	0.984	0.968	0.841	1.000	0.952	0.887	0.979	0.935	0.787	0.958	0.833
G158	1.000	0.937	0.873	0.984	0.952	0.903	0.917	0.826	0.936	0.958	0.875
G163	0.194	0.206	0.143	0.000	0.000	0.065	0.000	0.000	0.043	0.000	0.000
G164	0.097	0.111	0.127	0.016	0.016	0.000	0.063	0.022	0.000	0.000	0.000
G166	0.355	0.635	0.365	0.094	0.222	0.806	0.021	0.000	0.064	0.000	0.000
G168	0.742	0.746	0.571	0.844	0.238	0.226	0.417	0.283	0.064	0.042	0.083
G169	0.113	0.254	0.286	0.078	0.571	0.661	0.375	0.609	0.915	1.000	0.875
G170	1.000	0.952	0.952	1.000	0.968	0.968	0.979	0.935	1.000	0.958	0.958
G174	0.097	0.127	0.111	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042
G175	0.871	0.667	0.651	0.922	0.937	0.968	0.667	0.457	0.383	0.583	0.375
G177	0.952	0.905	0.857	0.984	0.968	0.968	0.938	0.891	0.894	0.958	0.917
G180	0.855	0.746	0.746	0.719	0.873	0.742	0.813	0.543	0.894	0.833	0.917
G181	0.210	0.444	0.397	0.156	0.254	0.565	0.021	0.022	0.021	0.042	0.125

Appendix 6: Continued

Locus	BJ	NG	PD	FV	RG	CJ	CEU	GUA	PAI	TEL1	TEL2
G185	0.516	0.127	0.063	0.094	0.111	0.000	0.021	0.022	0.043	0.000	0.042
G186	0.968	0.952	0.921	1.000	0.984	0.952	0.458	0.413	0.851	0.958	0.958
G187	1.000	0.921	1.000	0.984	0.952	0.952	0.958	0.957	0.979	0.958	0.958
G190	0.145	0.349	0.111	0.000	0.095	0.613	0.042	0.087	0.128	0.083	0.042
G192	0.081	0.063	0.048	0.000	0.000	0.258	0.021	0.000	0.021	0.000	0.042
G193	0.919	0.952	0.905	0.734	0.778	0.919	0.875	0.826	0.936	0.750	0.833
G194	0.210	0.111	0.159	0.078	0.063	0.048	0.104	0.043	0.064	0.000	0.000
G195	0.065	0.159	0.048	0.016	0.000	0.226	0.000	0.000	0.000	0.000	0.000
G196	0.710	0.540	0.381	0.688	0.667	0.613	0.458	0.391	0.234	0.292	0.250
G197	0.242	0.175	0.159	0.094	0.095	0.387	0.208	0.152	0.447	0.292	0.458
G200	0.452	0.460	0.413	0.266	0.476	0.306	0.125	0.152	0.426	0.292	0.208
G201	0.113	0.190	0.302	0.266	0.413	0.403	0.125	0.348	0.511	0.583	0.667
G203	0.323	0.143	0.238	0.078	0.159	0.000	0.542	0.370	0.617	0.583	0.375
G204	0.065	0.048	0.032	0.094	0.016	0.661	0.000	0.022	0.000	0.000	0.042
G205	0.145	0.095	0.063	0.156	0.063	0.742	0.125	0.000	0.085	0.042	0.000
G207	0.758	0.730	0.508	0.422	0.540	0.935	0.188	0.087	0.191	0.083	0.250
G210	0.710	0.190	0.095	0.344	0.444	0.339	0.521	0.196	0.511	0.292	0.208
G211	0.597	0.556	0.317	0.016	0.302	0.806	0.000	0.000	0.021	0.000	0.000
G214	0.919	0.714	0.413	0.594	0.635	0.129	0.729	0.543	0.468	0.375	0.208
G215	0.968	0.937	0.873	1.000	0.905	0.919	0.813	0.696	0.851	0.833	0.875
G217	0.242	0.476	0.397	0.141	0.175	0.516	0.021	0.022	0.021	0.000	0.000
G220	0.016	0.032	0.032	0.094	0.048	0.419	0.000	0.000	0.021	0.042	0.000
G221	0.177	0.222	0.095	0.078	0.095	0.532	0.042	0.043	0.021	0.042	0.042
G222	0.048	0.032	0.063	0.063	0.016	0.823	0.000	0.000	0.021	0.000	0.000
G223	0.597	0.746	0.302	0.125	0.381	0.919	0.021	0.000	0.064	0.000	0.000
G224	1.000	0.984	0.952	1.000	0.952	0.242	0.958	0.935	1.000	0.958	1.000
G225	0.887	0.635	0.603	0.938	0.794	0.677	0.813	0.543	0.319	0.375	0.292
G226	0.032	0.111	0.095	0.063	0.079	0.113	0.063	0.261	0.277	0.458	0.250
G228	0.903	0.397	0.190	0.344	0.381	0.000	0.521	0.239	0.426	0.167	0.208
G229	0.903	0.746	0.746	0.891	0.714	0.645	0.792	0.522	0.638	0.375	0.375
G230	0.468	0.667	0.524	0.438	0.397	0.726	0.229	0.391	0.468	0.542	0.542

Appendix 6: Continued

Locus	BJ	NG	PD	FV	RG	CJ	CEU	GUA	PAI	TEL1	TEL2
G232	0.823	0.810	0.841	0.906	0.698	0.597	0.521	0.435	0.149	0.125	0.125
G233	0.129	0.254	0.095	0.063	0.175	0.290	0.333	0.522	0.894	0.875	0.833
G242	0.355	0.302	0.254	0.297	0.222	0.371	0.292	0.196	0.277	0.208	0.208
G244	0.452	0.206	0.127	0.078	0.048	0.113	0.000	0.000	0.085	0.042	0.042
G245	0.242	0.079	0.032	0.016	0.016	0.000	0.000	0.000	0.000	0.000	0.042
G246	0.081	0.190	0.032	0.000	0.016	0.048	0.000	0.000	0.000	0.000	0.000
G247	0.323	0.222	0.079	0.047	0.063	0.145	0.125	0.348	0.213	0.167	0.167
G248	0.177	0.111	0.063	0.094	0.032	0.048	0.083	0.000	0.043	0.000	0.000
G249	0.984	0.937	0.921	0.922	0.968	0.887	0.500	0.304	0.681	0.667	0.667
G253	0.548	0.524	0.317	0.516	0.254	0.129	0.146	0.109	0.021	0.042	0.000
G254	0.403	0.365	0.476	0.375	0.587	0.677	0.708	0.739	0.915	0.917	0.958
G256	1.000	1.000	0.937	1.000	0.921	0.903	0.979	0.935	1.000	0.958	0.958
G259	0.226	0.063	0.063	0.313	0.095	0.065	0.042	0.043	0.021	0.042	0.042
G260	0.903	0.587	0.508	0.766	0.651	0.387	0.063	0.000	0.106	0.042	0.000
G261	0.887	0.794	0.857	0.969	0.794	0.806	0.646	0.326	0.128	0.292	0.042
G262	0.097	0.286	0.079	0.031	0.270	0.371	0.271	0.500	0.809	0.750	0.833
G263	0.177	0.111	0.048	0.016	0.095	0.048	0.021	0.022	0.000	0.042	0.000
G264	1.000	0.921	0.921	0.922	0.921	0.935	0.042	0.000	0.043	0.042	0.083
G267	0.790	0.635	0.175	0.531	0.556	0.016	0.521	0.217	0.404	0.250	0.250
G268	0.210	0.095	0.032	0.031	0.143	0.016	0.063	0.000	0.064	0.000	0.000
G271	0.823	0.857	0.857	0.906	0.937	0.952	0.625	0.457	0.851	0.958	0.875
G272	1.000	0.889	0.968	0.953	0.984	0.984	0.979	1.000	1.000	0.958	1.000
G273	0.177	0.190	0.190	0.078	0.143	0.290	0.000	0.022	0.021	0.000	0.000
G274	0.968	0.635	0.714	0.953	0.873	0.758	0.771	0.761	0.553	0.417	0.417
G279	0.355	0.048	0.016	0.078	0.000	0.000	0.000	0.000	0.000	0.000	0.000
G280	0.355	0.127	0.032	0.047	0.286	0.000	0.063	0.000	0.128	0.042	0.000
G281	0.871	0.762	0.683	0.813	0.889	0.919	0.104	0.043	0.213	0.375	0.375
G284	0.000	0.175	0.016	0.047	0.111	0.226	0.000	0.000	0.021	0.000	0.000
G285	0.758	0.746	0.540	0.734	0.651	0.290	0.563	0.326	0.426	0.583	0.500
G292	1.000	0.857	0.952	0.984	0.952	0.968	0.979	0.957	0.936	0.917	0.875
G293	0.952	0.968	0.889	1.000	0.857	0.887	0.604	0.913	0.787	0.875	0.792

Appendix 6: Continued

Locus	BJ	NG	PD	FV	RG	CJ	CEU	GUA	PAI	TEL1	TEL2
G296	0.113	0.016	0.143	0.078	0.063	0.145	0.042	0.000	0.000	0.000	0.000
G300	1.000	0.857	0.937	1.000	0.937	0.871	0.979	0.957	0.596	0.583	0.458
G301	0.032	0.175	0.079	0.000	0.048	0.065	0.000	0.043	0.319	0.250	0.500
G302	0.597	0.429	0.222	0.375	0.413	0.161	0.000	0.022	0.000	0.000	0.000
G303	1.000	0.984	0.937	1.000	0.937	0.935	0.979	0.935	1.000	0.958	1.000
G304	0.677	0.556	0.238	0.828	0.254	0.032	0.125	0.283	0.000	0.000	0.042
G305	0.274	0.429	0.603	0.125	0.635	0.919	0.792	0.696	1.000	0.958	0.917
G307	0.742	0.778	0.746	0.469	0.413	0.919	0.688	0.826	0.851	0.792	0.792
G308	1.000	0.952	0.952	0.969	0.952	0.984	0.979	1.000	1.000	0.958	1.000
G314	0.613	0.302	0.333	0.281	0.238	0.048	0.188	0.217	0.021	0.000	0.042
G315	0.032	0.222	0.190	0.094	0.190	0.210	0.375	0.283	0.596	0.375	0.833
G330	0.871	0.635	0.714	0.719	0.698	0.000	0.583	0.761	0.596	0.667	0.542
G331	0.274	0.063	0.127	0.063	0.159	0.000	0.063	0.043	0.043	0.042	0.083
G332	0.161	0.127	0.016	0.094	0.032	0.000	0.000	0.000	0.021	0.000	0.000
G333	0.952	0.905	0.778	1.000	0.873	0.516	0.833	0.565	0.787	0.625	0.625
G334	0.984	0.905	0.905	1.000	0.905	0.355	0.979	0.957	0.872	0.917	0.792
G337	0.355	0.254	0.286	0.359	0.603	0.339	0.583	0.391	0.745	0.708	0.583
G340	0.532	0.333	0.016	0.109	0.000	0.016	0.000	0.000	0.064	0.000	0.000
G341	0.919	0.746	0.508	0.984	0.603	0.371	0.146	0.283	0.000	0.042	0.000
G342	0.726	0.651	0.667	0.391	0.540	0.903	0.813	0.630	0.979	1.000	0.958
G344	0.532	0.413	0.238	0.391	0.556	0.000	0.417	0.217	0.000	0.083	0.000
G346	0.645	0.349	0.381	0.719	0.270	0.000	0.229	0.174	0.064	0.042	0.000
G347	0.258	0.143	0.095	0.031	0.238	0.371	0.104	0.174	0.128	0.250	0.125
G348	1.000	1.000	0.968	1.000	0.937	0.242	0.958	0.935	1.000	0.958	0.958
G350	0.258	0.079	0.016	0.094	0.238	0.032	0.104	0.000	0.213	0.167	0.042
G351	0.000	0.254	0.190	0.031	0.016	0.210	0.000	0.022	0.000	0.000	0.042
G352	0.919	0.889	0.778	0.750	0.698	0.855	0.500	0.370	0.532	0.458	0.500
G353	0.484	0.302	0.333	0.422	0.587	0.532	0.667	0.630	0.766	0.500	0.792
G366	0.984	0.984	0.952	1.000	0.937	0.952	0.979	0.913	0.979	1.000	0.958
G367	0.371	0.016	0.095	0.047	0.048	0.000	0.167	0.000	0.128	0.333	0.000
G368	0.274	0.111	0.095	0.250	0.190	0.032	0.104	0.000	0.170	0.208	0.042

Appendix 6: Continued

Locus	BJ	NG	PD	FV	RG	CJ	CEU	GUA	PAI	TEL1	TEL2
G369	1.000	0.952	0.857	1.000	0.889	0.871	0.917	0.870	0.957	0.958	0.875
G388	0.629	0.127	0.016	0.406	0.286	0.065	0.458	0.413	0.574	0.792	0.708
G392	1.000	0.952	0.921	0.969	0.889	0.403	0.938	0.891	0.957	0.958	0.917
G398	0.210	0.143	0.159	0.078	0.190	0.032	0.271	0.261	0.298	0.250	0.292
G399	0.581	0.349	0.190	0.406	0.317	0.032	0.208	0.283	0.170	0.208	0.125
G407	0.032	0.206	0.302	0.031	0.175	0.081	0.458	0.261	0.574	0.375	0.375
G408	0.919	0.730	0.540	0.922	0.683	0.806	0.458	0.587	0.319	0.583	0.500
G416	0.806	0.476	0.413	0.797	0.667	0.452	0.792	0.522	0.511	0.667	0.458
G418	0.145	0.127	0.063	0.078	0.000	0.032	0.063	0.043	0.085	0.042	0.000
G419	0.968	0.905	0.873	0.891	0.921	0.919	0.917	0.826	0.979	0.958	1.000
G426	0.984	0.984	0.937	0.969	0.905	0.952	0.979	0.957	1.000	0.958	0.958
G435	1.000	0.952	0.921	1.000	0.937	0.903	0.958	0.935	0.915	0.875	0.875

CURICULUM VITAE

Personal data

Name:	Valdir Marcos Stefenon
Date of birth:	01 July, 1973
Place of birth:	Lages, Santa Catarina state, Brazil
Marital status:	Married
Nationality:	Brazilian

Education

1980 – 1990	High School at C.E. Gal. J. P. Sombra, Lages, Brazil.
1990 – 1993	Licence in Sciences and Mathematics at the University of the Highlands of Santa Catarina (UNIPLAC), Lages, Brazil.
1997 – 1999	Licence in Biology at the University of Ijuí (UNIJUI), Ijuí, Brazil.
1999 – 2000	Post-graduation course in Morphophysiological Sciences at the University for the Development of the State of Santa Catarina (UDESC), Lages, Brazil.
2001 – 2003	MSc Biotechnology at the Federal University of Santa Catarina (UFSC) Florianópolis, Brazil.
2004 – 2007	Doctoral study at the Institute of Forest Genetics and Forest Tree Breeding at the Faculty of Forest Science and Forest Ecology, Georg-August University, Göttingen, Germany.

Work experience

1994 – 2001	Teacher at the High School level.
2001 – 2003	Lecturer at the Department of Biological Sciences, University of the Highlands of Santa Catarina (UNIPLAC), Lages, Brazil.

Language skills

English, German, Portuguese (native).

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