Theor Appl Genet (2001) 102:97–103

M.M. Ribeiro · C. Plomion · R. Petit · G.G. Vendramin
A.E. Szmidt

Variation in chloroplast single-sequence repeats in Portuguese maritime pine (*Pinus pinaster* Ait.)

Received: 15 February 2000 / Accepted: 14 April 2000

Abstract Genetic variation in 12 *Pinus pinaster* (maritime pine) populations spanning most of the distribution range of the species in Portugal was evaluated using six polymorphic chloroplast microsatellite (cpSSR) loci. Thirty-two haplotypes were found. There were indications of very weak differentiation among populations (Weir's θ coefficient, 0.023), and the R_{ST} value, derived from the stepwise mutation model (SMM), was not significantly different from zero. The pattern, in which similarities in allele size, in base pairs, do not contribute to the genetic structure, may be due to the recent mixing of genetic material from different stands through plantations. Overall, a high level of haplotypic variation within populations was detected. Using the SMM estimator (mean genetic distance of individuals within populations, $\overline{D^2_{sh}}$) we divided the populations into two groups, with above and below average values. The first group contained 5 populations, mainly from the central part of the country, which possess, in general, high levels of haplotypic diversity. Among them, 2 populations were divergent from the others based on the pair-wise Nei's distance. The results indicate that there is no discernible geographic genetic pattern for the Portuguese populations of *P. pinaster* investigated. The history of expansion of the species range in Portugal during the twentieth century (mainly due to human activity) and extensive gene flow among populations associated with the expansion could explain this finding.

Communicated by P.M.A. Tigerstedt

M.M. Ribeiro · A.E. Szmidt (✉)
Department of Forest Genetics and Plant Physiology,
Swedish University of Agricultural Sciences, S-901 83, Umeå, Sweden
e-mail: Alfred.Szmidt@genfys.slu.se
Fax: +46 90 786 90 92

C. Plomion · R. Petit
INRA, Laboratoire de Génétique et
Amélioration des Arbres Forestiers, BP45, F-33610 Cestas, France

G.G. Vendramin
Istituto Miglioramento Genetico Piante Forestali, CNR,
Via Atto Vannucci 13, 50134, Florence, Italy

Keywords Chloroplast microsatellites · Haplotypes · Pines · Human impact · *Pinus pinaster* Aiton

Introduction

Pinus pinaster covers more than 4 million hectares in southwestern Europe (Portugal, Spain, France and Italy). In Portugal, 31% of the total area covered with forest is occupied by this species, i.e. 1 million hectares (see http://pinus.dgf.min-agricultura.pt/abre.htm). The forested area occupied by *P. pinaster* has increased by 30% over a 60-year period during the twentieth century due to reforestation by humans, in most cases with seeds and seedlings of unknown origin (Devy-Vareta 1993). Natural regeneration has become important in the past 25 years because of the high frequencies of fire. On average, 51000 ha of forest were burned down every year in the period between 1990 and 1997 (see http://pinus.dgf.min-agricultura.pt/abre.htm), and sometimes the fire-free intervals were too short for successful pine re-establishment (Buting and Rego 1988).

Very little is known about the genetic variability of this species despite intensive use and management. Baradat and Marpeau-Bezard (1988) carried out a chemosystematic study throughout the distribution area of *P. pinaster* based on terpene composition of the cortical tissues. The authors identified three different geographic groups, one of them being the Atlantic group, which includes populations in the western part of the Iberian Peninsula and the Aquitaine region in France. In another study, 6 Portuguese stands were analysed using isozymes to evaluate genetic diversity. Strong within-population variability was found, but genetic differentiation among populations was low (Castro 1989). Petit et al. (1995) also estimated the genetic differentiation using isozymes, total protein and terpenic loci in 6 populations of *P. pinaster* widely spaced across the range of the species. A high degree of differentiation in this pine was found with all types of markers (G_{ST}=0.17). Salvador et al. (2000) studied 12 populations of *P. pinaster* from the Iberian

Peninsula with isozyme markers, and they found three groups of populations with different levels of diversity. The only Portuguese population they included in their study clustered with the Atlantic group. Vendramin et al. (1998) screened 10 populations distributed throughout the range of *P. pinaster* with chloroplast simple-sequence repeats (cpSSR). These authors detected a higher degree of genetic differentiation among populations of *P. pinaster* (R_{ST}=0.23) than anything previously reported by authors using other markers (Castro 1989; Petit et al. 1995). They found that 2 of the Portuguese populations included in their study showed the lowest levels of genetic diversity

The method used by Vendramin et al. (1998) is a polymerase chain reaction (PCR)-based analysis of microsatellites specific to the chloroplast genome. This methodology has allowed the investigation of population genetic parameters at the intraspecific level (e.g. Powell et al. 1995a; b). Primers flanking a mononucleotide repeat located in the intergenic region between the *trnK* and *pbsA* genes that was retrieved from the completely sequenced chloroplast genome of *P. thunbergii* (Wakasugi et al. 1994) were used to detect variation in different pine species (Powell et al. 1995b). Sequencing of the fragments confirmed that the size differences were due to variation in the number of repeat units forming the microsatellite region (Tautz 1984).

In the study presented here, six cpSSR loci were used to screen 12 *P. pinaster* populations from Portugal to quantify the variation within and among populations. The main objectives of this study were to evaluate the effect of domestication of the species on the pattern and degree of genetic variation, to analyse the geographic genetic pattern of the populations studied and to verify the usefulness of chloroplast microsatellites for population identification.

Materials and methods

Plant material and DNA extraction

The plant material analysed in this study is listed in Table 1. Needles were collected from individual trees growing in a provenance trial established in 1993 in the Central Eastern part of the country (Serra da Malcata). Eleven provenances were used, and the trees were randomly sampled from each of them. The provenances were initially selected, following phenotypic evaluation, to represent the entire distribution range of the species in Portugal (Aguiar et al. 1995). Needles were also collected from the randomly selected trees of a 12th population, designated Oleiros. Total DNA was extracted from the needles according to the protocol described by Lerceteau and Szmidt (1999). The number of individuals used per population is shown in Table 1.

CpSSR, PCR conditions and sizing of PCR products

Six of the twenty primers flanking pine chloroplast microsatellites, designed on the basis of the chloroplast genome of *P. thunbergii* (Vendramin et al. 1996), were used in this study: Pt1254, Pt15169, Pt30204, Pt36480, Pt71936 and Pt87268. These primers were chosen since they detected a relatively high level of polymorphism in an analysis of a sub-sample of individuals. PCR reactions were performed in a final volume of 10 µl that contained 2.5 m*M* $MgCl_2$, 0.05 m*M* of each dNTP, 0.2 µm*M* of forward primer (5′-fluoresceine-labelled), 0.2 µ*M* of reverse primer, 0.4 U *Taq* DNA polymerase (Gibco BRL), 10 ng of DNA and 1× reaction buffer (Gibco BRL). Amplifications were carried out using a Perkin Elmer 9600 thermal cycler. The following cycling parameters were used: a preliminary denaturation step (4 min, 94°C), followed by 25 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C, with a final extension step of 60 min at 72°C. After the amplification, cpSSR fragments were denatured by adding an equal volume of loading-buffer (95% formamide, 10 m*M* EDTA pH 7.6, 0.1% bromophenol blue and 0.1% xylene cyanol) to each sample and subsequently heating for 5 min at 75°C. Diluted template was loaded into a 25-cm-long 8% acrylamide/bisacrylamide denaturating gel.

Fragments were separated using Li-Cor automatic DNA sequencers (models 4000 and 4000L) at 50 W constant power for approximately 80 min. For those amplified fragments whose size in base pairs varied so as to enable sufficient discrimination, the amplified products from different loci were loaded simultaneously in the same lane. The presence of the bands was visually scored,

Table 1 Geographical and genetic parameters of 235 individuals from 12 Portuguese *P. pinaster* populations

Code	n[a]	Region	Altitude (m)	Latitude	Longitude	n_h[b]	H_e (SD)[c]	$\overline{D^2_{sh}}$[d]
A	20	Aveiro	30	40°39′N	8°36′W	6	0.76 (0.061)	0.702
B	20	Oleiros	750	39°55′N	7°50′W	10	0.90 (0.027)	2.319
C	19	Alcácer do Sal	20	37°52′N	8°30′W	8	0.84 (0.044)	1.478
D	20	Bragança	800	41°52′N	6°32′W	9	0.85 (0.058)	1.453
E	20	Figueira da Foz	30	40°18′N	8°44′W	9	0.82 (0.073)	4.635
F	19	Lousã	250	40°09′N	8°11′W	11	0.91 (0.039)	4.519
G	19	Monção	310	42°04′N	8°23′W	7	0.85 (0.040)	0.780
H	20	Mondim de Basto	480	41°25′N	7°55′W	10	0.88 (0.035)	1.529
J	20	Leiria	50	39°46′N	8°57′W	11	0.93 (0.020)	6.104
L	20	Manteigas	625	40°24′N	7°26′W	9	0.87 (0.036)	6.028
M	20	Montalegre	690	41°49′N	7°56′W	8	0.87 (0.024)	2.400
N	18	Sintra	250	38°46′N	9°22′W	11	0.91 (0.045)	4.227
Mean						9.1	0.87	3.014

[a] Number of individuals sampled
[b] Number of haplotypes
[c] Unbiased haplotypic diversity (H_e), with standard error between brackets
[d] Mean genetic distance of individuals within populations

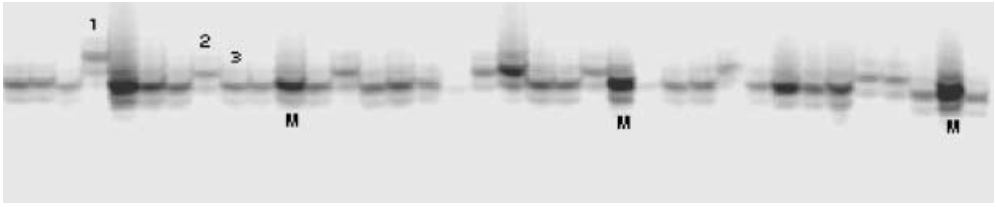

Fig. 1 Example of allelic variation detected at locus *Pt 15169*. Three different alleles are shown: *1* 115 bp, *2* 114 bp and *3* 113 bp. *M* Fragment size standards (113 bp). *Lower-sized bands* are 'stutter' bands commonly found in PCR amplification of SSR regions

with the help of fragment size standards (Fig. 1). To confirm the accuracy of the visual reading and to evaluate the size of the alleles (bp), we used a sample of 21 individuals containing all the alleles found with the 6 primers. The sizing of the amplified fragments from this sample was done with FRAGMENT MANAGER version 1.2 conversion software (Pharmacia) using both external and internal standards (50, 100, 150 and 200 bp) as reference molecular weights.

Data analysis

Size scores for the six fragments analysed were combined in order to derive the chloroplast haplotype of each individual. Unbiased haplotypic diversity based on haplotype frequencies, H_e, was computed for each population (Nei 1987). The degree of genetic differentiation among populations was estimated using the parameter θ (Weir and Cockerham 1984). Based on haplotypes, overall θ and pair-wise θ coefficients between populations were calculated. Nei's unbiased distances, *D*s, were obtained for pair-wise populations using the haplotype frequencies (Nei 1978).

Genetic distances among individuals within populations were computed according to the stepwise mutation model (SMM) estimator $\widehat{D^2_{sh}}$ (Goldstein et al. 1995), modified and adapted to fully linked haplotype markers by Morgante et al. (1998) and Bucci and Vendramin (unpublished results):

$$\widehat{D^2_{sh}} = \frac{1}{[n\cdot(n-1)]/2}\cdot\frac{1}{L}\cdot\sum_{i=1}^{n}\sum_{i'=(i+1)}^{n}\left[\sum_{k=1}^{L}\left|a_{ik}-a_{i'k}\right|\right]^2$$

where n is the total number of individuals analysed within each population, L is the number of loci, a_{ik} and $\frac{a_{i'k}}{i'\text{-th}}$ are the allele sizes (bp) of the i-th and $\frac{a_{i'k}}{i'\text{-th}}$ individual at the k-th locus, respectively, and $|a_{ik}-a_{i'k}|$ is the absolute difference between the allele size (bp) of the individuals considered. Genetic differentiation among populations was assessed by R_{ST} (Slatkin 1995), which is the ratio of the between-population variance and the total variance of allele size (bp), adapted to fully linked haplotype markers. Within-population haplotypic diversity was estimated using a modified version of the parameter S_w (Slatkin 1995). The formula has been modified and also adapted to fully linked haplotype markers (Bucci and Vendramin, unpublished results). Symbols previously described have the same meanings as above, and d_s is the number of populations:

$$S_W = \frac{1}{d_s}\cdot\sum_{j=1}^{d_s}\left\{\frac{1}{n\cdot(n-1)}\cdot\frac{1}{L}\cdot\sum_{i=1}^{n}\sum_{i'=(i+1)}^{n}\left[\sum_{k=1}^{L}\left|a_{ijk}-a_{ij'k}\right|\right]^2\right\}.$$

The exact tests for population differentiation based on absolute frequencies of haplotypes were carried out using the contingency table approach (Fisher's exact test) (Sokal and Rohlf 1981). The number of dememorisation steps was 1000, with 60 batches and 2000 permutations per batch.

The physical distance matrix, between every two stands, was compared using the genetic distance matrices, D_s and pair-wise θ coefficients. The strength of the relationships between the matrices was measured using the Mantel matrix-correspondence test (Sokal 1979). The null hypothesis refers to the absence of association between the elements of the pairs of matrices. A normalised *Z* test was performed in which the observed value after 999 permutations should be significantly larger than that expected by chance in order for an association to be accepted as valid. Grouping of the populations was carried out by multivariate analysis. Principal component analysis (PCA) was performed on the Nei's distance genetic matrix (D_s). The programmes used to compute the required parameters were TFPGA version 1.3 (Mark P. Miller, Northern Arizona University, USA), FSTAT version 1.2 (Jérôme Goudet, University of Lausanne, Switzerland) and SIMCA-P version 3.01 (Umetri AB & Ericsson Erisoft, Sweden).

Results

Characterisation of cpSSR loci

A total of 21 alleles (2–7 per locus) were detected in the analysed populations. A description of the alleles at each locus and definition of haplotypes can be obtained upon request from the corresponding author. The alleles were combined in 32 different haplotypes, all found in the set of 235 individuals from the 12 different populations. The frequency distribution in each population is given in Table 2. All of the populations had 1 haplotype in common (the most frequent, no. 2). In accepting cpSSR marker haplotypes for identifying a given population, the haplotype had to be found at a threshold level of at least 2 individuals (10%) of the population sample (Table 2). Only 3 populations have "population-specific haplotypes": Monção (haplotype 21), Figueira da Foz (haplotype 18) and Lousã (haplotype 10). Manteigas differs from the other populations because it does not have haplotype 3, which is otherwise common to all of them.

Within-population diversity

A very high within-population diversity (H_S=0.866) was found. The most and the least diverse populations for all the computed diversity parameters were Leira and Aveiro, respectively (Table 1). Some populations occurring in the north of Portugal (Aveiro, Bragança and Monção) displayed diversity values below average, as did the most southerly stand, Alcacér do Sal.

With respect to the $\widehat{D^2_{sh}}$ estimates, populations could be split into two different groups: Figueira da Foz, Lousã, Leiria, Manteigas and Sintra, which had above average values, and the others, which had below average values (Table 1). The first group contains populations from the central region of the country (Fig. 2) and they possess, in general, a high effective number of haplotypes.

Table 2 Haplotypes and their relative frequencies (percentage) in the tested Portuguese populations (A to N) of *P. pinaster*, a total of 235 sampled individuals

Code[a]	A	B	C	D	E	F	G	H	J	L	M	N
1	10[c]	0	5	10[c]	0	5	0	5	5	0	5	0
2	40[c]	5	32[c]	35[c]	5	26[c]	16[c]	25[c]	20[c]	15[c]	20[c]	28[c]
3	30[c]	20[c]	21[c]	20[c]	15[c]	16[c]	32[c]	20[c]	5	0	25[c]	11[c]
4	0	0	0	0	0	0	0	0	0	5	0	0
5	10[c]	20[c]	0	10[c]	0	11[c]	21[c]	5	10[c]	0	20[c]	0
6	0	5	5	0	40[c]	5	11[c]	20[c]	15[c]	25[c]	0	17[c]
7	0	0	0	0	0	0	0	0	0	5	0	0
8	0	5	0	0	0	5	0	0	0	0	0	0
9	0	0	0	0	5	5	0	0	5	5	0	0
10	0	5	5	0	0	11[c]	0	5	0	0	5	0
11	0	0	5	0	0	0	0	0	0	0	0	0
12	5	20[c]	21[c]	5	5	0	5	5	15[c]	25[c]	15[c]	6
13	0	0	0	0	0	0	0	0	0	0	0	6
14	0	0	0	0	0	0	5	0	0	0	0	0
15	0	0	0	0	5	5	0	0	10[c]	10[c]	5	6
16	0	0	0	0	5	0	0	0	5	0	0	0
17	0	0	0	0	0	0	0	0	0	0	0	6
18	0	5	0	0	15[c]	0	0	0	0	0	0	6
19	0	0	0	0	0	0	0	0	0	0	0	6
20	0	0	0	5	0	5	0	0	5	5	5	0
21	0	0	0	0	0	0	11[c]	0	0	0	0	6
22	0	5	0	0	0	0	0	0	0	0	0	0
23	0	10	0	5	0	0	0	0	0	0	0	6
24	0	0	0	5	0	0	0	0	0	0	0	0
25	0	0	5	0	0	0	0	5	0	0	0	0
26	0	0	0	5	0	0	0	0	0	0	0	0
27	5	0	0	0	0	0	0	5	0	0	0	0
28	0	0	0	0	5	0	0	0	0	0	0	0
29	0	0	0	0	0	5	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	5	0	0
31	0	0	0	0	0	0	0	0	5	0	0	0
32	0	0	0	0	0	0	0	5	0	0	0	0
Count[b]	6	10	8	9	9	11	7	10	11	9	8	11

[a] Code numbers of the haplotypes
[b] Number of haplotypes found in each population
[c] Haplotypes with at least a 10% occurrence in the population

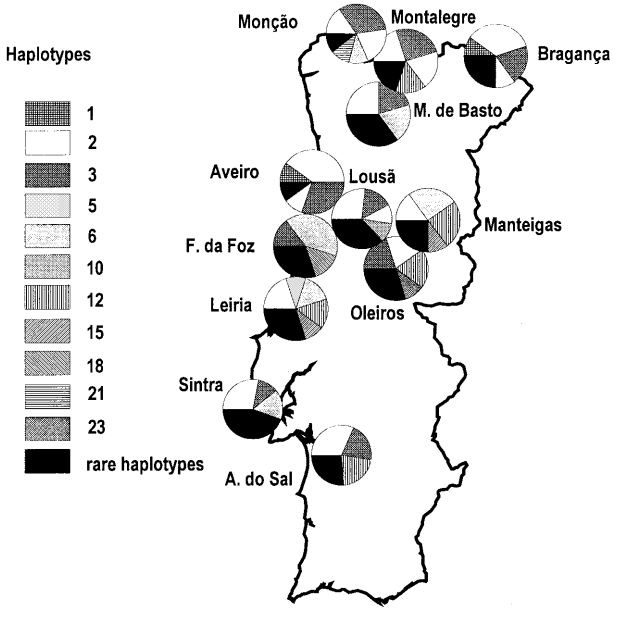

Fig. 2 Distribution of haplotypes in each population. The *number* indicates the code of the haplotype. Rare haplotypes (one individual per haplotype) were pooled together

Among-population diversity

There was a high level of total diversity (H_T=0.885) based on the haplotype chloroplast microsatellite data, and the diversity among populations (computed for haplotypes) was very low,θ=0.023, although θ was significantly different from zero [P (θ not>0)<0.002]. However, the R_{ST} value found in this study was not significantly different from zero.

Using PCA analysis, based on pair-wise Nei's genetic distances, we were able to differentiate three main groups (Fig. 3): group 1, with populations above the X-axis; group 2 with below-X-axis populations, group 3, comprising the Figueira da Foz and Manteigas populations. The percentage of the total variation explained was about 82%. The exact test for differentiation of populations, based on haplotype absolute frequency, confirmed that the Figueira da Foz and Manteigas populations were significantly divergent from the others with *P*=0.0008±0.0007 (±SD). A contrast analysis was also performed between groups 1 and 2, which showed there was a significant difference (*P*=0.042±0.0049).

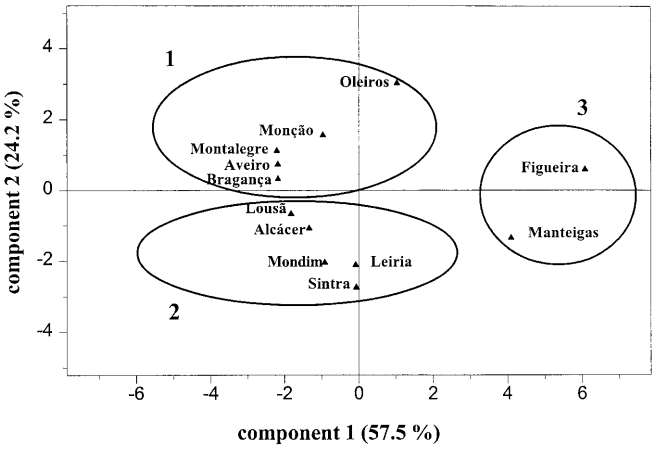

Fig. 3 Principal component analysis of the 12 populations based on pair-wise Nei's genetic distances (D_s). The proportion of the variance explained is about 82%

Geographical pattern

The physical distance matrix between every 2 stands was compared using the genetic distance matrices *D*s and θ by performing Mantel tests. The correlation values (*r*) were very low and not significant. In both cases, the Z values for the majority of the 999 random permutations were larger than the observed Z values. Also, the one-tail probability, *P* coefficient [random Z≤observed Z] indicated that the null hypothesis can not be rejected. This result indicates that there is no discernible geographical genetic pattern in the investigated populations**.**

The geographic proximity of the stands does not have any clear correlation with the haplotype profiles (Fig. 2). Some relatively close stands do not share common haplotypes. The Leiria and Figueira da Foz populations, for instance, share only 1 frequent haplotype (no. 6), although the distance between them is only about 60 km. In contrast, the Sintra and Mondim de Basto populations share the same frequent haplotypes (nos. 2, 3 and 6), although they are separated by 320 km.

Discussion

The high within-population haplotypic diversity observed in some Portuguese populations of *P. pinaster* of this study is probably due to a relatively recent transfer of genetic material by humans. However, when allozyme markers were used Salvador et al. (2000) found higher values of within-population diversity of *P. pinaster* in the eastern region of the Iberian Peninsula than in the Atlantic region (that includes Portugal). It would be necessary to compare our results with similar data obtained using our methodology in the populations of that region in order to verify if the two types of markers follow the same trend of diversity in both regions (Portugal and eastern Spain).

Fossil, charcoal and palynological records indicate that *P. pinaster* was present in Portugal during the Middle Würm (55000–25000 BP), the late Pleniglacial (25000–15000 BP) and terminal Pleistocene (12000–11000 BP) (Teixeira 1945; Mateus and Queiroz 1993; Figueiral 1995). In addition, pollen analyses have also indicated the presence of *P. pinaster* forests in the coastal area south of Lisbon during the Atlantic period (7580–6550 BP). This species was probably able to survive the latest glaciation in Portugal in sheltered areas at low altitudes close to the Atlantic Ocean (Figueiral 1995), a suggestion in contrast to that of Salvador et al. (2000) who propose that *P. pinaster* disappeared from Portugal during the last glaciation.

The scattered distribution of *P. pinaster* in Portugal in ancient times with different populations evolving separately could have been a source of haplotypic diversity, together with subsequent human activity. An additional hypothesis to be taken in consideration is the possible migration pathway coming from putative refugia in the southeastern region of Spain, as defended by Salvador et al. (2000). A combination of these factors could explain why some of the populations investigated show high levels of diversity and effective number of haplotypes.

In this study, the average diversity within populations was found to be similar to the value obtained by Vendramin et al. (1998). The data from both studies were made comparable by using the same loci and estimating the genetic diversity parameters, H_e and $\overline{D^2_{sh}}$ (recomputed, data not shown). These diversity values are difficult to compare with those previously reported for other conifers, however, due to differences in the number and types of loci analysed (e.g. Echt et al. 1998; Morgante et al. 1998; Provan et al. 1998).

Our data show that the genetic differentiation among populations (θ=0.023) is similar to that reported by Castro (1989) using allozyme markers (G_{ST}=0.020). Genetic divergence values are expected to be different when nuclear and cytoplasmic markers are used due to differences in seed and pollen migration (Petit et al. 1993). In our case the mix of genetic material (plants and seeds) due to human activity could explain the similarity of those values. Vendramin et al. (1998) also studied the distribution of genetic diversity in *P. pinaster* with cpSSR. After finding that 23% of the haplotypic variation was due to differences among populations, they postulated that parcelling of the natural range of *P. pinaster* after the last glaciation may have caused the strong differentiation observed for this species at the range level. In contrast, in our study, the Portuguese populations of *P. pinaster* showed a low level of among-population genetic divergence. Genomic divergence is expected to arise among geographically isolated populations due to the accumulation of new mutations through time and the absence of homogenisation by gene flow (Orr 1995). Our results showing that the θ, but not the R_{ST}, value differed from zero are in agreement with the results obtained by Bucci et al. (1998). These authors in their study of hybridisation in *halepensis*-complex pine species using cpSSR markers inferred that the G_{ST} was more effective than the R_{ST} for evaluating low levels of inter-population divergence.

According to Devy-Vareta (personal communication), by the end of the eighteenth century and the beginning of the nineteenth century, *P. pinaster* was clearly expanding and supplanting other species or invading non-cultivated areas in Portugal. *Pinus pinaster* has several characteristics that have played a role in it being species of preference; in particular, it is fast growing and grows well on mineral soils (Farjon 1984). In Portugal, the area covered by this species increased from about 900000 ha at the beginning of the twentieth to 1300000 ha in the 1960s, mainly due to human activity, but the origin of the seed and/or seedlings is unknown (Devy-Vareta 1993). The absence of a geographical pattern, as demonstrated in this study by the Mantel tests and the PCA analysis, support these observations, and there are historical records of a strong anthropogenic influence (Devy-Vareta 1993). Our results are in agreement with those obtained by Salvador et al. (2000) and Burban et al. (1999). In the first study, in which isozyme markers were used to study a set of populations from the Iberian Peninsula, the authors observed that a linear relationship between geographic and genetic distances could only be found if the populations of *P. pinaster* from the Atlantic group – which includes 1 Portuguese population – were removed. In the second study, the authors found a blurred geographic pattern for a specific pest of maritime pine, the bast scale *Matsucocus feytaudi*, in Portugal.

In our study, the genetic divergence among populations is very low but different from zero, and the Manteigas and the Figueira da Foz populations are divergent from the others. The Manteigas population is located in a relatively isolated part of the country surrounded by high mountains. Moreover, the probably old origin of these 2 stands and the absence of genetic transfer could explain their divergence from the other populations.

The high level of diversity found in the Leiria population should be particularly emphasised. As early as the thirteenth century, an order of monks was responsible for reforesting the sandy soils of the Leiria region (Mattoso and Sousa 1993). This area might have been the origin of much of the transferred genetic material (Castro 1989 and references therein). In contrast, the Aveiro, Bragança, Monção and Alcácer do Sal populations display below average values of diversity. They may have experienced a severe reduction in population size, with consequent founder effect, with the Aveiro population being an extreme case. The haploid cpDNA is more sensitive than nuclear DNA to severe reductions in the number of individuals in a population because of its effective population size being half that of the nuclear DNA (Birky 1988; Mitton 1993).

Another factor that should be taken into consideration is the influence of fire. Since the mid-1970s an average of 51000 hectares has burned down every year in Portugal. Young *P. pinaster* trees can start to produce cones at the top shoot at approximately 6 or 7 years of age (Farjon 1984). If the fire-free interval is not shorter than the time needed for producing seeds, fire could alter the reproduction pattern. The Oleiros and Sintra populations have high levels of genetic diversity, and they are both examples of stands with a post-fire regeneration origin. However, detailed information was not available for all the populations, and more data would be needed to fully evaluate the impact of fire on genetic diversity.

Vendramin et al. (1998) detected relatively low values of diversity in 2 of the populations from Portugal in their range-wide study of cpDNA variation in *P. pinaster*. Further comparisons, reported here, based on a subset of common loci, proved that Leiria is the most polymorphic population among all the populations used in both studies, and conclusions about the migration history of the species should be based on a larger number of populations for each region. However, it should be recognised that, due to the strong human impact, some *P. pinaster* Portuguese populations might represent genetic 'melting pots' (being derived from mixtures of seeds of varied origin) rather than 'hot spots' of diversity.

On the basis of our study the populations can not be identified by "population-specific" haplotypes because only a few populations possess them. The data also suggest that there is no discernible geographical genetic pattern in *P. pinaster* in Portugal. Man's influence over the expansion of this species in Portugal during the last century and extensive associated gene flow among populations seem to be largely responsible for this. This study confirms the influence of man in shaping the genetic structure of a species and how the mixing of genetic material of unknown origin can affect its genetic resources. As a consequence of the blurred pattern found in the Portuguese populations of *P. pinaster* the interpretation of the history of this species in this area may turn out to be difficult if not impossible.

Acknowledgements Special thanks are extended to Prof. N. Devy-Vareta, for exchanging stimulating ideas and for her help in interpreting historical, geological and ecological data concerning *P. pinaster* in Portugal. We are very grateful for the technical help provided by D. Pot. This work could not have been done without the assistance of the full-time researcher at the Estação Florestal Nacional of the INIA, A. Aguiar, who was responsible for establishing the *P. pinaster* provenance trials in Portugal. He kindly provided all the plant material used in this study. Thanks are also due to M.A. Antunes, C. Gracio, A. Serra and S. Elvas for helping us during the needle collection. M.M. Ribeiro is supported by a PRODEP II – Acção 5.2 fellowship, and her stay in France was funded by the KSLA/Carl Fredrik von Horns fund, H-381. We acknowledge funding from the INRA in France and from GIS (Programme Cooperatif Pin maritime du futur). A. Szmidt acknowledges support from the Swedish Council for Forestry and Agricultural Research (SJFR) and the Swedish International Development Cooperation Agency (SIDA). C. Plomion and G.G. Vendramin are supported by a grant from the European Union (contract ERBIC 18 CT 970 200). We hereby declare that the experiments comply with the current laws of France and Sweden.

References

Aguiar A, Alpuim M, Roldão MI (1995) Ensaio de proveniência de *Pinus pinaster* Ait. (resultados preliminares). Silva Lusitana 3:53–63

Baradat PH, Marpeau-Bezard A (1988) Le pin maritime, *Pinus pinaster* Ait.. Biologie et génétique des terpènes pour la conn-

aissance et l'amélioration de l'espèce. PhD thesis, University of Bordeaux I, Bordeaux, France
Birky CW (1988) Evolution and variation in plant chloroplast and mitochondrial genomes. In: Gottlieb L, Jain S (eds) Plant evolutionary biology. Chapman & Hall, London, pp 23–53
Bucci G, Anzidei M, Madaghiele A, Vendramin GG (1998) Detection of haplotypic variation and natural hybridization in *halepensis*-complex pine species using chloroplast simple sequence repeat (SSR) markers. Mol Ecol 7:1633–1643
Burban C, Petit R, Carcreff E, Jactel H (1999) Rangewide variation of the maritime pine bast scale *Matsucoccus feytaudi* Duc. (Homoptera: *Matsucoccidae*) in relation to genetic structure of its host. Mol Ecol 8:1593–1602
Buting SC, Rego FC (1988) Human impact on Portugal's vegetation. Rangelands 10:251–255
Castro LFT (1989) Isoenzimas do *Pinus pinaster* Ait. numa prespectiva de aplicação ao melhoramento genético da espécie. PhD thesis, Universidade de Trás-os-Montes e Alto Douro
Devy-Vareta NL (1993) A floresta no espaço e no tempo em Portugal. A arborização da Serra da Cabreira (1919–1975). PhD thesis, Faculdade de Letras da Universidade do Porto
Echt CS, DeVerno LL, Anzidei M, Vendramin GG (1998) Chloroplast microsatellites reveal population genetic diversity in red pine, *Pinus resinosa* Ait. Mol Ecol 7:307–316
Farjon A (1984) Pines: drawings and descriptions of the genus. E. Brill/DR. Backhuys, Leiden, The Netherlands
Figueiral I (1995) Charcoal analysis and the history of *Pinus pinaster* (cluster pine) in Portugal. Rev Palaeobot Palynol 89:441–454
Goldstein DB, Linares AR, Cavallisforza LL, Feldman MW (1995) An evaluation of genetic distances for use with microsatellite loci. Genetics 139:463–471
Lerceteau E, Szmidt AE (1999) Properties of AFLP markers in inheritance and genetic diversity studies of *Pinus sylvestris* L. Heredity 82:252–260
Mateus JE, Queiroz PF (1993) Os estudos da vegetação quaternária em Portugal: Contextos, balanço de resultados, prespectivas. O quaternário em Portugal, balanço e prespectivas. Colibri, pp 105–131
Mattoso J, Sousa A (1993) História de Portugal. Círculo de Leitores, Lisbon
Mitton JB (1993) Molecular approaches to population biology. Annu Rev Ecol Syst 25:45–69
Morgante M, Felice N, Vendramin GG (1998) Analysis of hypervariable chloroplast microsatellites in *Pinus halepensis* reveals a dramatic bottleneck. In: Karp A, Isaac PG, Ingram DS (eds) Molecular tools for screening biodiversity: plants and animals. Chapman and Hall, London, pp 407–412
Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583–590
Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York
Orr HA (1995) The population genetics of speciation: the evolution of hybrid incompatibilities. Genetics 139:1805–1813
Petit RJ, Kremer A, Wagner DB (1993) Finite island model for organelle and nuclear genes in plants. Heredity 71:630–641
Petit RJ, Bahrman N, Baradat P (1995) Comparison of genetic differentiation in maritime pine (*Pinus pinaster* Ait.) estimated using isozyme, total protein and terpenic loci. Heredity 75:382–389
Powell W, Morgante M, McDevitt R (1995a) Hypervariable microsatellites provide a general source of polymorphic DNA markers for the chloroplast genome. Curr Biol 5:1023–1029
Powell W, Morgante M, McDevitt R, Vendramin G, Rafalski J (1995b) Polymorphic simple sequence regions in chloroplast genomes: applications to the population genetics of pines. Proc Natl Acad Sci USA 99:7759–7763
Provan J, Soranzo N, Wilson NJ, McNicol JW, Forrest GI, Cottrell J, Powell W (1998) Gene-pool variation in Caledonian and European Scots pine (*Pinus sylvestris* L.) revealed by chloroplast simple-sequence repeats. Proc R Soc London Ser B 265:1697–1705
Salvador L, Alía R, Agúndez D, Gil L (2000) Genetic variation and migration pathways of maritime pine (*Pinus pinaster* Ait) in the Iberian peninsula**.** Theor Appl Genet 100:89–95
Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. Genetics 139:457–462
Sokal R (1979) Testing statistical significance of geographic variation patterns. Syst Zool 28:227–232
Sokal RR, Rohlf FJ (1981) Biometry. W.H. Freeman and Co, San Francisco
Tautz D (1984) Simple sequences are ubiquitous repetitive components of eukariotic genomes. Nucleic Acid Res 12:4127–4138
Teixeira C (1945) Subsídios para a história evolutiva do pinheiro dentro da flora portuguesa. Bol Soc Broteriana 19:209–221
Vendramin GG, Lelli L, Rossi P, Morgante M (1996) A set of primers for the amplification of 20 chloroplast microsatellites in *Pinaceae*. Mol Ecol 5:595–598
Vendramin GG, Anzidei M, Madaghiele A, Bucci G (1998) Distribution of genetic diversity in *Pinus pinaster* Ait. as revealed by chloroplast microsatellites. Theor Appl Genet 97:456–463
Wakasugi T, Tsudzuki SI, Shibata M, Sugiura M (1994) A physical map and clone bank of the black pine (*Pinus thunbergii*) chloroplast genome. Plant Mol Biol Rep 12:227–241
Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution 38:1358–1370