Robledo-Arnuncio JJ, Collada C, Alía R, Gil L. Genetic structure of montane isolates of Pinus sylvestris L. in a Mediterranean refugial area. J Biogeogr 32: 595-605

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ORIGINAL ARTICLE

Genetic structure of montane isolates of *Pinus sylvestris* L. in a Mediterranean refugial area

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ABSTRACT

Aim This work investigates the population genetic effects of periodic altitudinal migrations and interstadial fragmentation episodes in long-term Scots pine (*Pinus sylvestris* L.) populations at a regional scale.

Location The study focuses on Scots pine populations in the northern Meseta and peripheral mountain chains, central and north-western Iberian Peninsula. The ample macrofossil record in the area shows that this 60,000-km^2 region represent a glacial refugium for Scots pine. The species occupied large areas on the Meseta plains during glacial cold stages, but it has periodically sheltered at high elevation in the surrounding mountain chains during warm episodes, conforming to a fragmented pattern similar to its present-day distribution.

Methods We perform a fine-scale chloroplast microsatellite (cpSSR) survey to assess the genetic structure of 13 montane Scots pine isolates in the northern Meseta (total $N = 322$ individuals). Using a hierarchical analysis of molecular variance (AMOVA), we test the hypothesis of genetic isolation among disjunct mountain areas. We use a standard coalescence model to estimate genealogical relationship among populations, investigating the potential role of the regional relief as a factor influencing historic gene exchange among Scots pine populations.

Results Population haplotypic diversity was high among Scots pine populations ($H_e = 0.978$), greater than values reported for other more thermophilic pine species in the Iberian Peninsula. The AMOVA revealed low (but significant) differentiation among populations ($\Phi_{ST} = 0.031$, $P = 0.010$), showed that the disjoint montane distribution could not account for the genetic divergence among areas ($\Phi_{CT} = 0.012$, $P = 0.253$), and that there was non-trivial subdivision among populations within the same mountain region ($\Phi_{SC} = 0.021$, $P = 0.012$). The genealogical relationships among populations showed that Scots pine isolates growing on disjoint mountain blocks, but on slopes flowing to the same basin, were genetically closer than populations growing on different slopes of the same mountain chain, flowing to different basins.

Main conclusions The observed genetic structure for Scots pine is consistent with its population history, inferred from the palaeobotanical record, with vertical migrations throughout climatic pulses and with the drainage basins and large long-term population sizes connecting different mountain blocks during the cooler glacial periods. Overall, the results suggest that, despite periodic interstadial fragmentation episodes, Scots pine biology provides for the long-term maintenance of high within-population and low among-population genetic diversity at neutral genetic markers.

Keywords

Chloroplast microsatellites, genetic structure, Holocene fragmentation, Iberian Peninsula, mountain isolation, *Pinus sylvestris*, vertical migration.

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INTRODUCTION

The present native distribution of European trees has been shaped mainly by expansion following Holocene climatic warming, notably northward expansion from southern refugia (Bennett *et al.*, 1991). Populations of widespread species at different latitudes may have undergone contrasting demographic and genetic processes. In northernmost regions, expansion and founding events into large areas, subsequent to ice sheet retreat, are expected to erode allelic richness and heterozygosity of colonizing populations (Wade & McCauley, 1988; Hewitt, 2000; but see Austerlitz *et al.*, 2000 and Petit *et al.*, 2003). By contrast, southern regions, free from ice sheets and permafrost soils, would have allowed more stable population dynamics for many species, resulting in higher genetic diversity (Hewitt, 1996, 2000). The importance of southern areas of temperate regions for the long-term survival of plant species is supported both by the fossil record and molecular data (Comes & Kadereit, 1998). The palynological record suggests that the populations of several tree genera, such as *Quercus*, *Tilia* and *Ulmus*, that recolonized northern Europe from southern refugia during interstadials, became extinct in the North during subsequent cold periods, not being able to migrate back rapidly enough to southern milder regions (Bennett *et al.*, 1991). The persistence of these species in southern refugia during both interstadials and cold stages could explain their long-term survival, which would have required suitable local habitats under both climatic extremes.

Mountainous areas of southern temperate regions provide a varied topography that results in very different ecological and microecological conditions. Steep gradients of temperature and rainfall may permit a rapid response of tree species to climatic shifts via short altitudinal migrations (Hewitt, 1996), allowing persistence of large, stable populations through several glacial cycles (Tzedakis, 1993; Hewitt, 2000). For those species adapted to cold and wet conditions, however, uphill retreat following a climatic warming may lead to fragmentation and population size reduction, with populations confined to disjunct high-elevation zones, separated by unsuitable habitat at lower elevations (Oline *et al.*, 2000). There is limited and inconclusive empirical evidence of the population genetic consequences of this process. Some isozyme studies of montane conifer isolates have revealed a strong genetic structure among populations, indicating rapid genetic isolation and drift, subsequent to Holocene warming (Ledig *et al.*, 1997; Oline *et al.*, 2000), while others have failed to find significant differences from the weak genetic structure typical of more continuous distributions, suggesting the maintenance of genetic connectivity or absence of substantial drift (Hiebert & Hamrick, 1983; Hamrick *et al.*, 1989; Ledig *et al.*, 2000, 2002). A detailed knowledge of the demographic history of the populations is crucial, but seldom available, to interpret and compare observed patterns of genetic variation. The combination of molecular and fossil record data from well-documented regions may provide an unusually good opportunity for hypothesis development (Cruzan & Templeton, 2000; Pastorino & Gallo, 2002).

The southernmost populations of Scots pine (*Pinus sylvestris* L.), located in the Iberian Peninsula, constitute an interesting system to investigate this evolutionary aspect of tree species further. Scots pine is a wind-pollinated, wind-dispersed, predominantly outcrossing conifer (Kärkkäinen *et al.*, 1996) and the most widely spread among pine species, extending from arctic latitudes in Norway (70° N) to warm southern areas of Spain (38° N). Both palaeobotanical information and broad-scale molecular surveys suggest that Iberian Scots pine populations are Tertiary relicts that have persisted in the region through several Quaternary glacial cycles (Bennett *et al.*, 1991; Willis *et al.*, 1998; Sinclair *et al.*, 1999; Soranzo *et al.*, 2000). The present-day Iberian distribution of the species conforms to a warm-stage pattern, with fragmented populations sheltering at high elevation on different mountain chains. During the extended glacial cold stages, the macrofossil record suggests that Scots pine occupied large areas on the lower-elevation mesetas (plateaus) between mountain ranges (Costa-Tenorio *et al.*, 1990). The Holocene demographic dynamics of the species has been especially well documented in the northern Meseta and surrounding ranges, with macrofossil and subfossil records suggesting the permanence of widespread Scots pine woodlands in the plateau at least until the mid-Holocene (*c.* 7000 BP; Franco-Múgica *et al.*, 2001).

In this study, we perform a fine-scale chloroplast microsatellite (cpSSR) survey across Scots pine populations in the northern Meseta region, focusing on the following questions: (1) What is the level of neutral genetic diversity within this region for this usually more northern species, relative to that of the more Mediterranean pine species in the Iberian Peninsula? (2) Has the present interstadial distribution led to genetic differentiation among populations on different mountain blocks? (3) What are the imprints of altitudinal migrations, known from the fossil record, on present-day genetic diversity distribution?

MATERIALS AND METHODS

Study area and population sampling

The northern Meseta region of Spain (central and north-western Iberian Peninsula, Duero river basin) is a 60,000-km^2 plateau, ranging in elevation from 600 to 1000 m, surrounded by mountain chains, and with an extreme continental climate. Scots pine occupies high-elevation areas of these peripheral mountain chains, growing mainly at elevations between 1000 and 1800 m, and usually forming the timberline (Fig. 1). Several wood macrofossils from the species, dated between 12,000 and 7700 ^{14}C years BP, have been found in different inner locations of the Meseta (Fig. 1), establishing the low elevation distribution of Scots pine during early and mid-Holocene (Muñoz *et al.*, 1996; Sánchez *et al.*, 1999; Alcalde *et al.*, 2001). A high-resolution and continuous pollen-sediment analysis at a valley mire at Espinosa de

Figure 1 Orographic map of the northern Meseta region, showing *Pinus sylvestris* L. distribution and the sites sampled in this study (circles). The stars indicate known locations of Scots pine macrofossils or sub-fossil wood (12,000–7700 yr BP). Underlined population codes stand for sites where Pt71936 141-bp mutation was found.
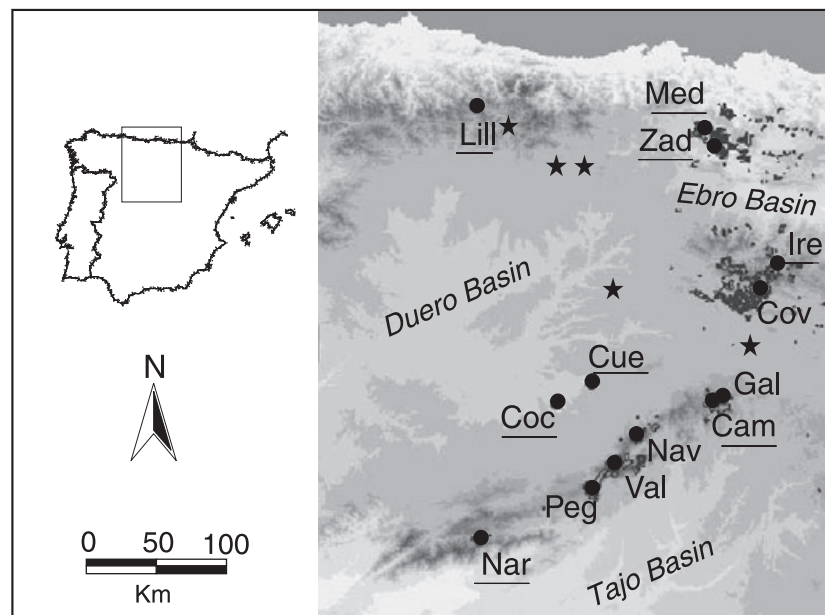
Cerrato (north-eastern Northern Meseta), suggests that pine woodlands dominated this area of the plateau throughout almost the entire Holocene, and it has been pointed out that *P. sylvestris* was probably one of the species best represented in these ancient forests during the early Holocene (10,000–9000 yr BP; Franco-Múgica *et al.*, 2001). Some relictual isolates still survive in the dry plains, thanks to microecologically suitable conditions, such as those near Cuéllar and Coca (Fig. 1), further outlining the extent of Scots pine's early to mid-Holocene distribution.

We collected needle samples from 25 randomly selected adults from each of 13 sampling sites (Fig. 1 and Table 1). The aim was to cover the full range of Scots pine in the Northern Meseta, including the Cuéllar and Coca relict populations in the inner plateau, as well as two other small, geographically marginal populations (Lillo and Navarredonda). At least two different sampling sites were selected on the same mountain range (and on different slopes, whenever possible) in those areas where Scots pine woodlands extended over several thousand hectares.

Genetic analysis

We isolated total genomic DNA, following the protocol by Dellaporta *et al.* (1983), and assessed the chloroplast haplotype of each tree at six polymorphic, mononucleotide-repeat, microsatellite regions (Pt15169, Pt26081, Pt30204, Pt36480, Pt71936, Pt87268; Vendramin *et al.*, 1996). Chloroplast microsatellites (cpSSR) provide useful information about the recent evolutionary history of pine species, due to their high polymorphism and non-recombinant, paternally inherited nature (Powell *et al.*, 1995; Provan *et al.*, 2001), which allows

Table 1 Geographical and genetic diversity parameters of 13 sampling sites of *Pinus sylvestris* L. in the northern Meseta region. Sample size was 25 trees in all cases, except for the Ire population (22 trees)

Code	Population	Elevation (m)	Latitude	Longitude	H_e (SD)*	$\bar{D}_{sh}^2$†
Lill	Puebla de Lillo	1550	43°04′ N	5°15′ W	0.973 (0.018)	4.5
Zad	San Zadornil	1000	42°50′ N	3°11′ W	0.980 (0.017)	4.7
Med	Medina de Pomar	860	42°57′ N	3°16′ W	0.970 (0.019)	4.4
Cov	Covaleda	1550	41°56′ N	2°48′ W	0.980 (0.016)	3.8
Ire	Hoyos del Iregua	1350	42°05′ N	2°39′ W	0.987 (0.017)	4.5
Gal	Galve de Sorbe	1400	41°15′ N	3°07′ W	0.960 (0.023)	3.2
Cam	Campisábalos	1400	41°13′ N	3°12′ W	0.987 (0.015)	4.5
Nav	Navafría	1700	41°00′ N	3°50′ W	0.993 (0.013)	4.5
Val	Valsaín	1500	40°49′ N	4°01′ W	0.993 (0.013)	4.8
Peg	Peguerinos	1500	40°39′ N	4°12′ W	0.987 (0.015)	4.5
Nar	Navarredonda	1550	40°21′ N	5°07′ W	0.973 (0.022)	5.2
Cue	Cuéllar	800	41°16′ N	4°13′ W	0.943 (0.026)	3.7
Coc	Coca	790	41°12′ N	4°30′ W	0.983 (0.015)	4.3
Average					0.978	4.3

*Unbiased haplotypic diversity. Standard deviation is shown between brackets.
†Mean genetic distance among individuals within populations.

a genealogical approach to investigation of haplotype relationships (Wilson & Balding, 1998). We carried out the PCR in a total volume of 10 µL, containing 2.5 mM MgCl$_2$, 1x reaction buffer (Ecogen, Barcelona, Spain), 275 µM of each dNTP, 1.5 pmol IRD-800 labelled forward primer, 1.5 pmol reverse primer, 0.16 U *Taq* polymerase (Ecogen) and 10 ng of genomic DNA. We used a Perkin Elmer model 9700 thermal cycler, Applied Biosystems Norwalk, CT, USA, with the following profile: initial denaturation at 95 °C for 5 min, followed by 15 cycles of 1 min at 94 °C, 45 s at 55 °C and 1 min at 72 °C, and a final extension step of 8 min at 72 °C. We then resolved amplification products on 6%, 25-cm-long, 0.25-mm-thick, denaturing polyacrylamide gels, containing 7 M urea and 1x TBE buffer. We ran gels at 45 W, constant power, for about 1 h, using a Li-Cor 4200 Series automatic sequencer, Li-Cor Bioscience Lincon, NE USA. Sizing of the amplified fragments was carried out by Gene ImagIR ver. 3.56 software (Scanalytics, Fairfax, VA USA), using external standards, followed by a visual check of all the size scores. We cloned and sequenced the amplification products of all samples carrying a rare cpSSR size variant (141 bp at Pt71936). To do so, we precipitated PCR products with ethanol, and then cloned using the pGEM-TEasy vector (Promega, Madison, WI USA). We carried out DNA sequencing using dye terminator sequencing reagents (Perkin-Elmer, Wellesley, MA USA) in an automatic ABI 377 sequencer. We aligned sequences with the CLUSTAL-W method, included in MegAlign software (DNA-STAR, Inc., Madison, WI USA), following with manual alignment adjustments.

Data analysis

We defined different haplotypes as a unique combination of size variants across the six microsatellite regions. We then characterized the genetic diversity of each population by computing the unbiased haplotypic diversity, $H_e = n(n-1)^{-1}(1 - \Sigma p_i^2)$, where p_i is the population frequency of the *i*th haplotype (Nei, 1987), and the average genetic distance among individuals within populations, $\bar{D}_{sh}^2$, according to a microsatellite stepwise mutation model (SMM). This distance is based on the Goldstein *et al.*'s (1995) distance, but treating the non-recombinant chloroplast genome as a single locus (Echt *et al.*, 1998; Vendramin *et al.*, 1998). The distance between the *i*th and *j*th individuals is defined as:

$$D_{sh}^2(i,j) = K^{-1}\left[\sum_{k=1}^{K} |a_{ik} - a_{jk}|\right]^2$$

where a_{ik} and a_{jk} are the allele size of the *i*th and *j*th individuals at the *k*th microsatellite region, and $K = 6$ is the number of microsatellite regions assayed. Genetic distance metrics that account for specific microsatellite mutation mechanisms (such as the microsatellite SMM) define appropriate haplotype relationships (rather than relying solely on haplotypic identity) that allow more efficient recovery of evolutionary information (Goldstein *et al.*, 1995). We tested

genetic differentiation among groups of populations from different mountain regions by performing a hierarchical analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992), partitioning total cpSSR variation into (1) within-population, (2) among-population-within-isolated-mountain-area, and (3) among-isolated-mountain-area components (seven groups: Lill; Med–Zad; Ire–Cov; Gal–Cam; Nav–Val–Peg; Nar; Cue–Coc; see Fig. 1). We calculated distance among haplotype pairs using the D_{sh}^2 measure. This yields an analogue of Slatkin's (1995) R_{ST} for population differentiation. We computed the significance of the Φ-statistics using standard permutation procedures (10,000 random permutations in all cases), implemented and described in Arlequin ver. 2000 software (Schneider *et al.*, 2000).

From the genetic data, we inferred demographic parameters for Scots pine in the northern Meseta with the Bayesian method proposed by Wilson & Balding (1998). This method, designed for fully linked loci, is based on a standard coalescent model, combined with a stepwise microsatellite mutation model. Assuming prior distributions for mutation rate (µ) and effective population size (*N*), we obtained posterior distributions of $\theta = 2N\mu$, *N*, and TMRCA (time since most-recent common ancestor, in generations), via a Markov chain Monte Carlo (MCMC) algorithm. We performed computations with MICSAT software (by Wilson & Balding, 1998; available from http://www.maths.abdn.ac.uk/~ijw/), using uniform priors on mutation rate and effective population size. We attempted 40 changes to the genealogical tree between every attempt to change the hyperparameters, and did 100 of these attempts between samples (Wilson & Balding, 1998). After 2000 burn-in iterations, we retained 10,000 samples for parameter estimation. We repeated the process three times using different seed numbers, so as to test whether the MCMC scheme had been run for long enough (Stephens, 2001). We examined the genealogical relationships among haplotypes obtained in the final coalescence tree, after the last iteration of the MCMC. Based on this tree, we executed a principal components analysis (PCA) to group populations on the basis of their genealogical relationships, considering as variables the proportions of individuals from each population carrying haplotypes contained within each of the main clades of the coalescence tree. In doing so, we hypothesized that lineage sorting might have occurred in the formation of separate populations, and we performed the PCA under the *a priori* assumption that this sorting would have been reflected in the distribution of haplotypes from each population across the coalescent tree topology.

RESULTS

All six chloroplast microsatellites regions were polymorphic, yielding a total of 29 size variants (3–7 per region). The distribution of size variants for each region was unimodal (Fig. 2), with variants differing by 1-bp from each other, consistent with a microsatellite SMM. An exception was found for the Pt71936 region, in which a 2-bp gap was detected
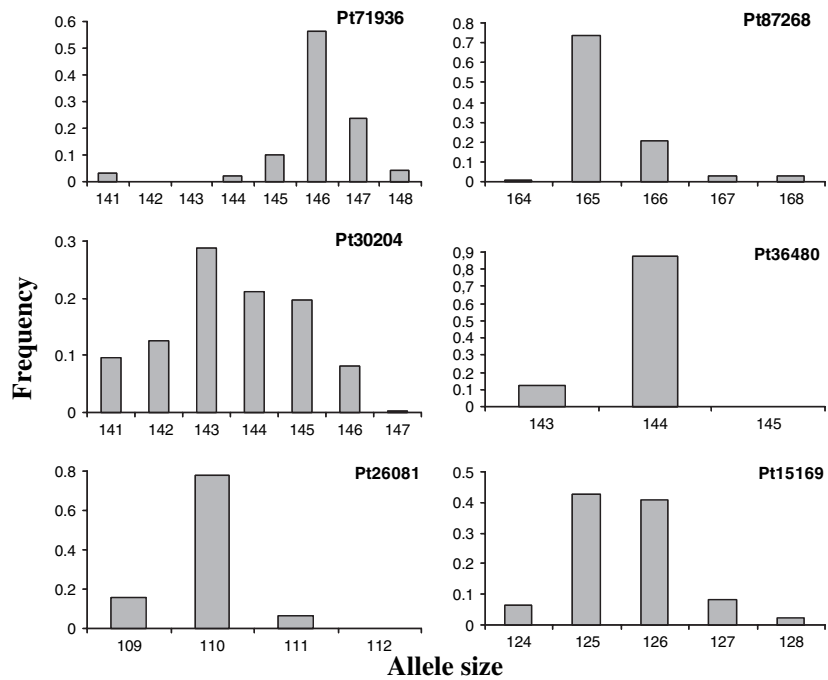
Figure 2 Frequency distribution of size variants at six *Pinus sylvestris* L. chloroplast microsatellite loci. Allele sizes are given in base pairs.

between the 141-bp size variant and the rest (Fig. 2). All 11 samples carrying the 141-bp variant, as well as four other samples (carrying the 145-, 146- and 147-bp variants), were sequenced. We found that the presence of the rare 141-bp variant was due to a 5-bp deletion in the microsatellite flanking region, rather than a 5-bp decrease of the repeat region itself, which turned out to have, in all the analysed samples, the same 14 repeats as the most frequent 146-bp fragment (Fig. 3). The 11 trees carrying this mutation were found in eight of the 13 sampling sites, spread over different mountainous areas of the region (Fig. 1). Five different haplotypes carried this rare variant, with the most abundant found in seven individuals from five populations, and the other four, differing by a single mutation step from the most abundant haplotype (data not shown), found in single individuals from each of other four populations. This pattern suggests that the five haplotypes carrying the rare 141-bp variant constitute a clade, geographically widespread across the Meseta.

The 29 size variants defined 139 different haplotypes among the 322 individuals from 13 populations. All haplotypes had a frequency below 0.05, averaged over the total set of 322 trees, and most of them (83%) had frequencies below 0.01. The most abundant haplotype was found in 15 trees from eight different populations (overall frequency of 0.046), while 77 haplotypes (55.3%) were found in a single individual, and 85 (61.2%) in a single population each. Haplotype population frequencies were also very low, with most (average 84%) of the haplotypes in

each population showing a frequency below 0.05. On average, 33.2% of the haplotypes found in each population were unique to it, and population pairs shared only 6.3% of their combined set of haplotypes. This narrow intersection among the haplotypic arrays of different populations could indicate some degree of lineage sorting, although the very low haplotypic frequencies renders this inference risky, as population comparisons based on an identity criterion may be highly subject to the stochastic process of sampling.

As a result of the low haplotype population frequencies, very high within-population diversity values were found (Table 1), with an average haplotypic diversity (H_e) of 0.978. The average mean distance among individuals within populations, according to an SMM ($\bar{D}_{sh}^2$), was 4.3. Populations located in the Guadarrama Chain (Valsaín, Navafría and Peguerinos; Fig. 1) had the highest haplotype diversity values, with a maximum $H_e = 0.993$, while one of the relictual populations in the Meseta plains (Cuéllar) showed the lowest haplotypic diversity ($H_e = 0.943$; Table 1), significantly smaller than for the rest of the populations (Fisher's LSD multiple comparison of means, $P < 0.001$).

The analysis of molecular variance (Table 2), based on D_{sh}^2 distance among haplotypes, and excluding the 11 samples that carried the 141-bp rare variant, which violated the SMM, showed that genetic variation among populations was low ($\Phi_{ST} = 0.031$) but significant ($P = 0.010$), with most of the total variation attributable to within-population differentiation. To

Figure 3 DNA sequence alignment of different size variants at locus Pt71936. The repeat region is shown in bold. The rectangle shows the 5-bp deletion at 141-bp variant.

P. thunbergii	AGAATC**TTTT TTTTTTTTTT TT**CAATTCCA CGGATTACTC
P. sylvestris (147 bp)	AGAAC**TTTT TTTTTTTTTT T**-AAAACCCA GGGTTTCCCC
P. sylvestris (146 bp)	AGAAC**TTTT TTTTTTTTTT** --AAAATCCA CGGTTTACCC
P. sylvestris (145 bp)	AGAAC**TTTT TTTTTTTTT**- --AAATCCCA CGGTTTACTC
P. sylvestris (141 bp)	AGAATC**TTTT TTTTTTTTTT** --AAATCCCA CGG-----TC

Table 2 Analysis of molecular variance (AMOVA), based on D_{sh}^2 distance among *Pinus sylvestris* L. haplotypes. (a) Assuming no population structuring. (b) Assuming population structuring based on isolation in disjoint mountain areas. (c) Assuming population structuring as defined by the PCA, based on genealogical relationships among populations (see Fig. 5 and Materials and methods)

Source of variation	d.f.	Variance component	% Variation	Φ-statistic	P
(a)					
Among populations	12	0.0417	3.08	0.031	0.010
Within populations	298	1.3121	96.92		
(b)					
Among groups	6	0.0162	1.20	0.012	0.253
Among populations within groups	6	0.0270	2.06	0.021	0.012
Within populations	298	1.3121	96.74	0.032	0.010
(c)					
Among groups	6	0.0390	2.87	0.029	0.050
Among populations within groups	6	0.0088	0.65	0.007	0.070
Within populations	298	1.3121	96.48	0.035	0.010

test genetic differentiation among disjunct mountain areas, we partitioned the among-population variance into separate components for mountain ranges and populations within mountain ranges. Results indicated that a small and non-significant portion of the total variation ($\Phi_{CT} = 0.012$, $P = 0.253$) separated mountains, but that a larger and significant ($\Phi_{SC} = 0.021$, $P = 0.012$) portion of variation separated populations occupying the same isolated mountain block.

Three different runs of the MCMC algorithm, performed to estimate demographic parameters of Scots pine in the Northern Meseta, yielded very similar posterior probability distributions for all variables, suggesting adequate convergence. They were all combined, yielding a total of 30,000 samples. The median (and 95% probability interval) for μ, N, θ and TMRCA were 0.00096 (0.00001–0.09554), 12,744 (44–364,132), 24.5 (19.2–36.6) and 4210 (77–224,857), respectively. This lack of precision is reflected in the posterior probability distributions of the parameters (Fig. 4), which are sharply peaked, but with long tails, indicating limited support for a wide range of high values. The estimate for θ is more precise, as expected from the fact that, under the standard coalescent, information about N and μ can only be obtained from the data through their product (Nμ = θ/2; Wilson & Balding, 1998). These results very broadly suggest a mutation rate of about 10^{-3} for Scots pine chloroplast microsatellite loci, a long-term effective population size above 10,000 individuals, and more than 4000 generations since the most recent common ancestor of Scots pine cpSSR haplotypes in the Northern Meseta, although the minimal precision implies little confidence in the point estimates.

A first examination of the coalescence tree showed no clear relation between the geographical location of haplotypes and the tree topology. Both recent and deep clades contained haplotypes from several populations, spread over the different mountain areas. Conversely, haplotypes from any single population were drawn from most of the main branches of the tree. The geographically based groupings seemed not to be coherent haplotypic clades, suggesting shared ancestry or extensive chloroplast exchange among populations. A more detailed analysis, however, revealed a noticeable geographical pattern of haplotypes across the four main clades of the coalescent tree (Fig. 5). The PCA ordination, based on the
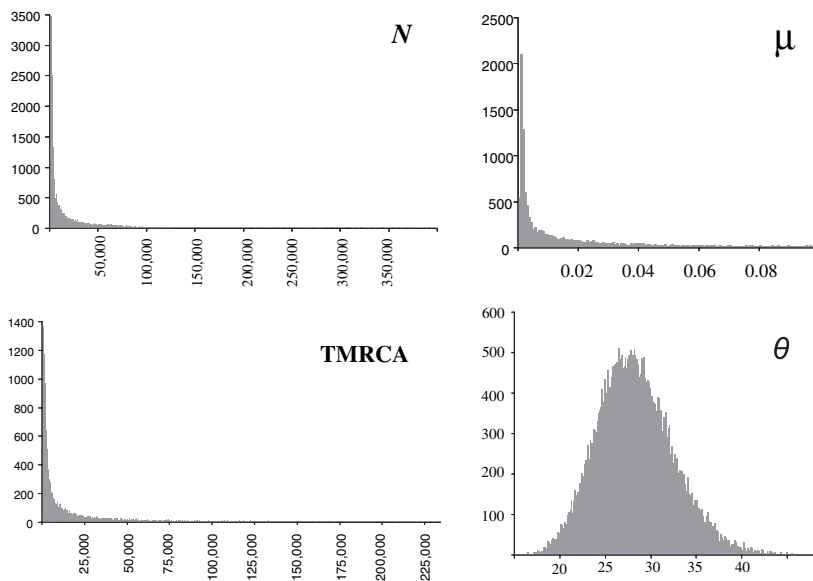

Figure 4 Probability distributions of the mutation rate (μ), 2Nμ(θ), effective population size (N), and time since most recent common ancestor (TMRCA) for *Pinus sylvestris* L. cpSSR haplotypes, obtained using the Bayesian method of Wilson & Balding (1998).

proportion of individuals from each population carrying haplotypes contained within each of the four main branches of the tree (Fig. 5), showed: (1) most of the small, geographically marginal populations (Lill, Cue, Nar) appearing as outliers, (2) populations located on different north-eastern mountains, but growing on opposite sides of the Ebro river basin (Med, Zad, Ire), clustering together, (3) another cluster including populations located on disjunct southern and eastern chains (Val, Nav, Peg, Cov), but all on slopes flowing into the Duero basin and (4) populations on the south face of the Central Chain (Cam and Gal, within the Tajo basin) appearing very differentiated from populations on the northern face of the Central Chain (Val, Nav, Peg, within Duero basin), and very divergent from each other. The Coca relict population, despite being geographically located in the

inner planes of the Duero basin, lay within the cluster of populations from the Ebro basin. An AMOVA, based on D_{sh}^2 distance among haplotypes, showed that genetic differentiation among the groups defined by the PCA was significant ($\Phi_{CT} = 0.029$, $P = 0.05$, Table 2), and accounted for most of the total among-population variation.

DISCUSSION

High haplotypic diversity and low among-population differentiation observed in 13 Iberian mountain isolates of Scots pine are consistent with the hypothesis, suggested by the palaeobotanical information, that there has been recent fragmentation of a historically larger population. The average within-population genetic diversity of Scots pine in this study
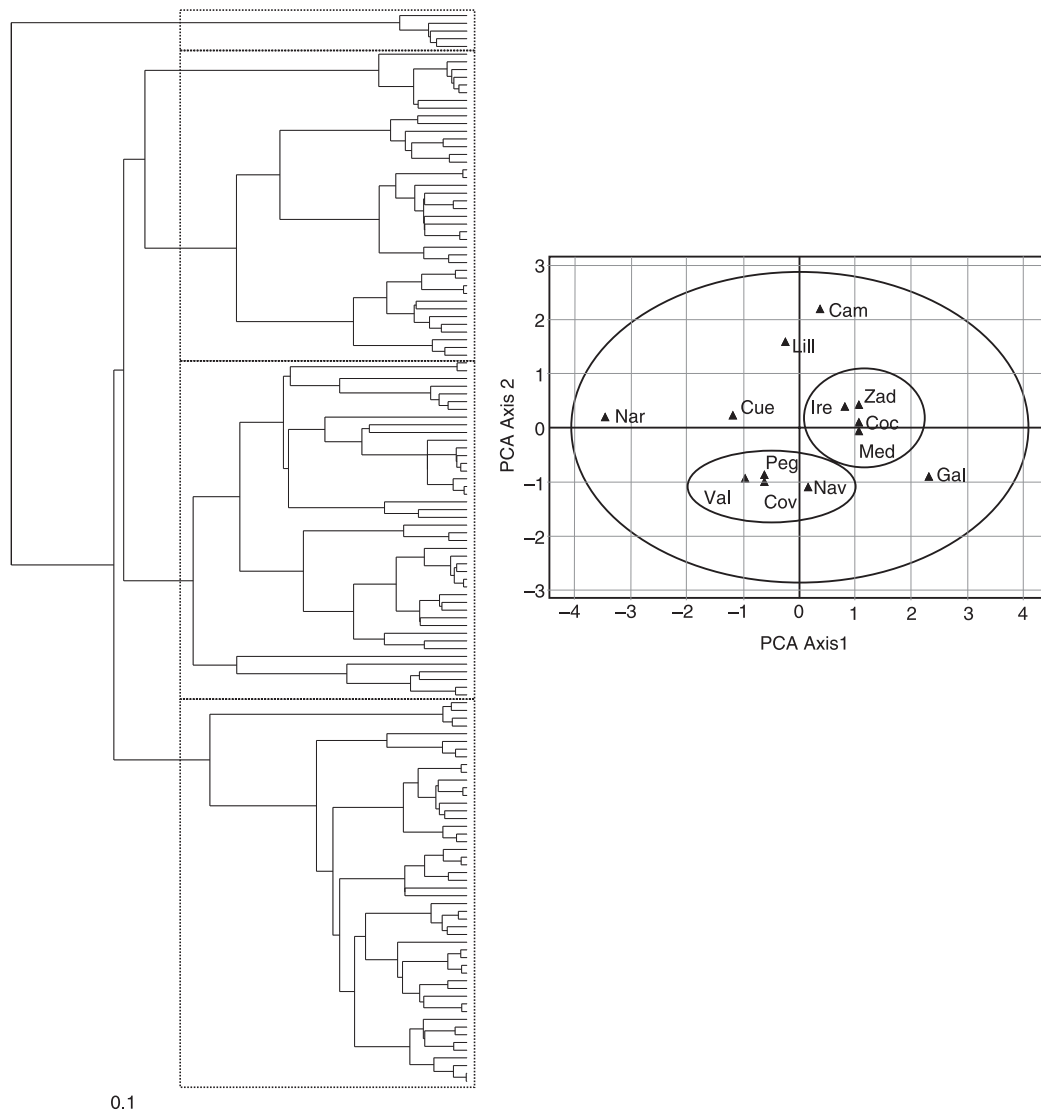

Figure 5 Genealogical relationships among *Pinus sylvestris* L. haplotypes (left), based on a standard coalescent model and a stepwise microsatellite mutation model (Wilson & Balding, 1998). The dotted-line squares in the tree indicate the four main clades considered for the principal components analysis (PCA) of Scots pine population relationships (right). PCA was based on the proportion of individuals from each population carrying haplotypes contained within the four main branches of the coalescence tree. The proportion of the variance explained by the first two principal components from the PCA is 77%.

($H_e = 0.978$, $D_{sh}^2 = 4.3$; Table 1) is higher than values, based on the same set of cpSSR loci, reported for the Iberian populations of three Mediterranean pine species, *P. pinaster* Ait. ($H_e = 0.938$, $D_{sh}^2 = 4.2$; G.G. Vendramin, pers. comm.), *P. halepensis* Mill. ($H_e = 0.626$, $D_{sh}^2 = 0.6$; Gómez, 1998), and *P. pinea* L. ($H_e = 0.522$, $D_{sh}^2 = 0.5$; Gómez *et al.*, 2000), although not significantly different from the *P. pinaster* result (Fisher's LSD, $P > 0.05$).

The level of cpSSR diversity is markedly higher in *P. sylvestris* than in the more thermophilic taxa, *P. halepensis* and *P. pinea*. All three species currently show a similar pattern of fragmentation on the Iberian Peninsula. There is strong palaeobotanical and genetic evidence, however, suggesting that while *P. sylvestris* established widespread woodlands on the inner plateaus during full glacial stages (Franco-Múgica *et al.*, 2001), *P. halepensis* and *P. pinea* were unable to endure the harsh continental climate of inner regions, suffering further fragmentation and dramatic range contraction into the milder climates of small coastal refugia, which may have led to repeated bottlenecks and subsequent gene diversity loss (Morgante *et al.*, 1998). From a long-term perspective, Scots pine adaptation to low temperatures, coupled with the Iberian orographic and climatic profiles, could have been a crucial factor allowing its demographic stability in the region throughout consecutive climatic pulses. The fact that the two other Iberian mountain pines (*P. uncinata* Ram. and *P. nigra* Arn.), adapted to a similar temperature range as Scots pine, show comparably high levels of cpSSR diversity (authors, unpubl. data), argues in favour of this hypothesis.

Such high haplotypic variation as that found in this study, on the contrary, may have undesirable methodological implications. Inferences on population relationships may be vulnerable to stochastic sampling, especially if based solely on haplotype frequencies (76–92% of haplotypes had an observed population frequency below 0.05). This factor reinforces the advisability of using genetic distance metrics or genealogical models that do not rely solely on haplotypic identity, such as the microsatellite SMM, which, apart from recovering evolutionary information more efficiently, are somewhat less sensitive to the hypervariability of the markers (Goldstein *et al.*, 1995; Hedrick, 1999). Microsatellite size homoplasy, however, is an inevitable and serious consequence of a stepwise mutation mechanism, especially when size constraints exist and the mutation rate is high. Its effects on the inferred genetic and phylogeographical structure, mimicking shared ancestry or gene exchange, remain difficult to circumvent (Goldstein & Pollock, 1997), although phylogenetic reconstructions among closely related populations would be somewhat less affected by the mutation model and thus by size homplasy (Estoup *et al.*, 2002).

The low among-population variation ($\Phi_{ST} = 0.031$) found for Scots pine relictual populations in the Iberian northern Meseta region is very similar to the value among northern populations of this same species in Scotland ($\Phi_{ST} = 0.032$; Provan *et al.*, 1998) and in Finland ($\Phi_{ST} = 0.030$; M.R. García-Gil, pers. comm.), all based on the same cpSSR markers

and the D_{sh}^2 haplotypic distance measure. Comparably low differentiation has been observed at nuclear isozyme markers in 14 populations across the entire Iberian range of the species ($F_{ST} = 0.038$; Prus-Glowacki *et al.*, 2003). These results strongly contrast with values reported for maternally inherited mtDNA markers for Scots pine, showing very substantial genetic structure in Spain ($F_{ST} = 0.595$–0.817), but somewhat lower differentiation among northern European populations ($F_{ST} = 0$–0.370) (Sinclair *et al.*, 1999; Soranzo *et al.*, 2000). This contrasting pattern suggests the effective role of pollen flow in homogenizing the genetic structure of Scots pine, eroding the genetic imprints of population history that maternally inherited mtDNA markers seem to retain (Hu & Ennos, 1999). The widespread presence of the haplotypic clade containing the 141-bp rare variant across the northern Meseta (Fig. 1) is consistent with the hypothesis of extensive pollen exchange. Burban & Petit (2003) present similar results from a range-wide genetic survey of *P. pinaster* populations across the Mediterranean region, revealing three non-overlapping regions, fixed for different mitotypes ($F_{CT} = 1$ and $F_{SC} = 0$), but much lower genetic structure for chloroplast markers ($F_{ST} = 0.19$), suggesting extensive pollen flow within and among areas colonized from different refugia. Analogous findings have been reported for European populations of *Abies alba* Mill. (Liepelt *et al.*, 2002).

The low differentiation among Scots pine montane populations in the northern Meseta region contrasts with some previous studies, showing a strong 'montane island effect' for conifers, i.e. effective genetic isolation and strong differentiation among mountain isolates. Ledig *et al.* (1997) found high levels of isozyme differentiation ($F_{ST} = 0.25$) for the endemic *Picea chihuahuana* Martínez in Mexico, subsequent to Holocene fragmentation. Virtually the same value of isozyme multilocus F_{ST} (0.24) was found in northern populations of foxtail pine (*Pinus balfouriana* Grev. and Balf.), scattered on the few peaks that rise above 2000 m in the Klamath Mountains of California (Oline *et al.*, 2000). The contrast of these results with those for *P. sylvestris* probably arises from different long-term effective population sizes. In the cited examples, populations were in the low hundreds, or even smaller for *P. chihuahuana*. Moreover, *P. balfouriana* shelters at the very crests of the mountains, under extremely marginal ecological conditions, and with no migratory path to higher elevation areas under past scenarios of increased temperature, which could have led to repeated bottlenecks and subsequent episodes of genetic drift (Oline *et al.*, 2000). By contrast, Scots pine populations in the northern Meseta are relatively larger, with mountain isolates of several thousand (or hundred thousand) trees growing in suitable habitats, and seldom reaching the crests of the chains, despite being the tree species that usually forms the timberline. Further uphill migration would have been possible under a warmer climate, avoiding severe population size reductions. This fact, in addition to the recency of fragmentation (less than 6000 years BP) and the potential for long-distance pollen dispersal (Koski, 1970), has probably minimized the impact of genetic drift and divergence.

An alternative argument that could explain the low differentiation among Scots pine montane isolates found in this study might be the high variability of the microsatellite markers, which may result in small differentiation measures even when the populations have non-overlapping sets of alleles (Hedrick, 1999). However, the fact that Scots pine populations across the whole Iberian Peninsula have shown a similarly small value of among-population genetic variation at lowly polymorphic isozyme loci ($F_{ST} = 0.038$; Prus-Glowacki *et al.*, 2003) makes this hypothesis unlikely.

Genealogical inferences about population parameters are consistent with the absence of long-term effective-size constraints for Scots pine in the Northern Meseta. Our estimate of N c. 13,000 can be considered large enough to avoid serious inbreeding and genetic drift (Savolainen & Kuittinen, 2000). This value is greater (although not significantly so, as precision was minimal) than the estimate (N, c. 3000) reported for lodgepole pine (*Pinus contorta* Dougl.), based also on cpSSR haplotypes and on the same Bayesian inference method (Marshall *et al.*, 2002). Unlike the small-scale sampling of the present study, *P. contorta* trees were surveyed from the very wide (encompassing 25° of latitude) and continuous range of the species in western North America, which highlights the relatively large effective population size observed for Scots pine across the much smaller northern Meseta region.

Our fine-scale genetic survey failed to detect significant genetic differentiation among populations from different mountain blocks ($\Phi_{CT} = 0.012$, $P = 0.253$), but did reveal variation among populations within the same chain ($\Phi_{SC} = 0.021$, $P = 0.012$). The phylogeographical analysis of haplotypes was consistent with these results, with wide physical spread of single lineages and mixing among them, but suggesting an important role for watersheds in shaping the genetic structure of Scots pine in the Northern Meseta. Populations located on different mountain chains, but growing on slopes flowing to the same basin, were genetically closer than populations on opposite slopes of the same mountain chain but contained within different drainages (Fig. 5). This fact suggests that valleys have served as corridors for historical gene exchange among presently distant populations, consistent with cold-stage expansions of Scots pine into the lowlands of the region (Franco-Múgica *et al.*, 2001). A similar role of valleys as genetic corridors has been pointed out for a *Pinus pinaster* refugial area in south-eastern Spain (González-Martínez *et al.*, 2004).

Finally, together with the general pattern of low genetic divergence among populations, it is noteworthy that the smallest and most isolated Scots pine populations in the northern Meseta region showed relatively high genetic differentiation (e.g. Lill, Cue, Nar; Figs 1 & 5). A possible explanation for this genetic divergence from other populations in the region is that their remote geographical location may reflect lower levels of long-term gene exchange. On the contrary, local episodes of genetic drift can lead to high genetic differentiation estimates among recently split populations (Gaggiotti & Excoffier, 2000), as could be the case for the Cuéllar relict, located on the inner plateau, only 60 km North (and downhill) from the widespread woodlands of the Guadarrama Chain. Besides its small size, its singular linear-shaped structure along the Cega River could be reducing its effective neighbourhood size by favouring mating among near neighbours (Loveless & Hamrick, 1984), enhancing local inbreeding and the action of genetic drift, thus increasing the chances of genetic differentiation. It is more difficult to find a logical explanation for the observed genetic similarity between the other relict population in the plateau, Coca, and north-eastern populations lying within the Ebro basin (Fig. 5). In the absence of stronger genetic evidence, we hypothesize that it could be due to a stochastic effect.

Overall, the results of this study suggest that, despite periodic interstadial fragmentation episodes, Scots pine biology provides for the long-term maintenance of high within-population and low among-population diversity at neutral genetic markers. Comparative studies, including different Iberian forest species, and both molecular and quantitative information, would be of further utility in providing deeper insights into the evolutionary history of tree species in this Mediterranean refugial area.

ACKNOWLEDGEMENTS

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BIOSKETCHES

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Editor: Philip Stott