

Phylogeography of maritime pine inferred with organelle markers having contrasted inheritance

C. BURBAN* and R. J. PETIT†

*INRA, Equipe d'Entomologie Forestière, †INRA, Equipe de Génétique des Arbres Forestiers, 69 Route d'Arcachon, 33612 Cestas Cedex, France

Abstract

Range-wide variation of maritime pine was studied at maternally inherited and paternally inherited markers (mitochondrial DNA and chloroplast DNA). While chloroplast DNA exhibits the highest diversity, phylogeographic inferences from this marker are blurred by homoplasmy and extensive pollen flow. In contrast, the only three mitochondrial haplotypes found provide a clear picture of nonoverlapping areas colonized from different refugia, with no single population having a mixed composition ($G_{ST} = 1$). Comparison of the genetic structure inferred from both organelle genomes allows the investigation of differential seed and pollen dispersal, pointing to pollen, but not seed, dispersal across the Strait of Gibraltar (from Morocco into Iberia). A comparison with already available genetic information, especially that of one of the maritime pine's most threatening insect pests, the bast scale *Matsucoccus feytaudi*, further completes the picture.

Keywords: chloroplast DNA, *Matsucoccus*, mitochondrial DNA, *Pinus*, RFLP

Received 18 October 2002; revision received 20 January 2003; accepted 20 January 2003

Introduction

In any phylogeographic survey, the accuracy of the spatio-temporal reconstruction will be conditioned by the resolution that can be achieved in terms of geographical structure and phylogeny. Geographic resolution will be greater if the genomes targeted are subject to limited gene flow, whereas phylogenetic reconstructions will be facilitated if they are uniparentally inherited. While mitochondrial DNA (mtDNA) has been extensively used in animal studies (Avice 1998), plant mtDNA is known to exhibit a lower rate of nucleotide substitution and to be prone to extensive intramolecular recombination (Newton 1988; Palmer 1992). Chloroplast DNA (cpDNA) has therefore been used preferentially in plants, especially for species where it is maternally inherited (Schaal *et al.* 1998; but see Desplanque *et al.* 2000). In hermaphrodite plants, both organelles are transmitted many together, through gametes of the same sex, the female in most angiosperms (e.g. Dumolin-Lapègue *et al.* 1999), the male in some gymnosperms (Taxodiaceae, Cupressaceae and Cephalotaxaceae, Chesnoy 1987). Cases of opposite inheritance of the two organelles also exist. In

Pinaceae or in the genus *Actinidia* (kiwifruit), chloroplasts are paternally inherited and mitochondria are maternally inherited (Wagner *et al.* 1987; Neale & Sederoff 1989; Cipriani *et al.* 1995; Chat *et al.* 1999; but see Wagner *et al.* 1991 for a case of paternal leakage of mtDNA in *Pinus*). The reverse situation also exists: in banana (*Musa acuminata*) and in cucumber and melon (genus *Cucumis*), chloroplasts are maternally inherited and mitochondria are paternally inherited (Fauré *et al.* 1994; Havey *et al.* 1998). Such cases of discordant uniparental inheritance offer the opportunity to study the effects of differential levels of gene flow through seeds and pollen on levels of geographical structure, reminiscent of, but not strictly comparable with, studies of animals when genetic information is available from both mitochondria and Y-chromosomes. Moreover, because plant mtDNA and cpDNA are known to differ in their rates of evolution (generally very slow in both cases, but more so in mtDNA; Wolfe *et al.* 1987), the levels of diversity that can be expected from surveys of each organelle genome are likely to differ, and hence the precision of the phylogenetic reconstruction. Here we compare the potential of both cytoplasmic genomes for phylogeographic investigations in a pine species.

Geographic variation at cpDNA and mtDNA were studied comparatively early in conifers, especially in pines

Correspondence: C. Burban. Fax: + 33 556680546; E-mail: chris@pierroton.inra.fr

(Wagner *et al.* 1987, 1989, 1991). As a result of their opposite mode of inheritance, mtDNA is expected to have a greater genetic differentiation than cpDNA in Pinaceae (Petit *et al.* 1993). This has been verified for *Pinus banksiana* and *P. contorta* (Dong & Wagner 1994), for *P. flexilis* (Latta & Mitton 1997) and for *P. albicaulis* (Richardson *et al.* 2002), but the disjunct populations of *P. muricata* display high genetic differentiation for both organelle genomes (Strauss *et al.* 1993). In studies based on cpDNA, phylogeographic insights have been reduced because of the low levels of geographical structure and the limited phylogenetic resolution [cf. the nature of these polymorphisms: variable number of repeats (VNTRs) at mini-or microsatellites; Doyle *et al.* 1998; Liepelt *et al.* 2001]. On the other hand, despite its low level of polymorphism, mtDNA has proved to be very useful in several studies of conifers, even when VNTRs were investigated (e.g. Soranzo *et al.* 2000; Gugerli *et al.* 2001; Sperisen *et al.* 2001). The development of universal plant cytoplasmic primers (Demesure *et al.* 1995; Dumolin-Lapègue *et al.* 1997; Grivet *et al.* 2001; Dumnil *et al.* 2002) should allow a better exploration of several non-coding regions from both plant organelle genomes. We have made an attempt to characterize the phylogeographic structure of a pine species using polymorphisms from non-coding regions of both cpDNA and mtDNA.

The species selected, maritime pine (*Pinus pinaster* Aiton), occurs naturally in fragmented populations in the western part of the Mediterranean basin, limited notably by limestone outcrops. This distribution has been reshaped during the last two centuries by heavy afforestation, particularly in southwest France and in the Iberian Peninsula, and by invasive behaviour in disturbed areas (Devý-Vareta 1988; Carrion *et al.* 2000).

The genetic structure of the species has been described using several sets of markers, including terpenes (Baradat & Marpeau-Bezard 1988), total proteins (Bahrman *et al.* 1994), isoenzymes (Petit *et al.* 1995; Salvador *et al.* 2000; González-Mártinez *et al.* 2001), chloroplast microsatellites (Vendramin *et al.* 1998; Ribeiro *et al.* 2001), nuclear microsatellites, and amplified fragment length polymorphisms (Mariette *et al.* 2001). Most of these studies focused on a reduced part of the natural range or sampled only a few populations. Therefore, our understanding of the history of the species refers mainly to the large survey of Baradat & Marpeau-Bezard (1988). These authors analysed terpene polymorphism in 105 *P. pinaster* populations and reviewed the available fossil data; they inferred a complex scenario of differentiation from Portugal and Andalusia, involving different routes of migration before and after the last ice age. They also proposed the existence of three major groups of maritime pine: an Atlantic group comprising populations from western France and the larger part of Spain and Portugal, a Mediterranean group consisting of all eastern European populations, and including eastern

Spanish populations up to Andalusia and the small stand of Punta Cires in Morocco, and a North African group comprising all the other African populations, the island of Pantelleria and one Andalusian stand. They suggested that the present range of these groups is partly the result of human influences, as the introduction of nonautochthonous plant material may have accompanied human migrations and commercial exchanges that occurred all around the Mediterranean basin.

Because of the fragmentation of its natural range, the maritime pine exhibits a relatively high genetic differentiation among populations at nuclear markers in comparison to other conifer species ($G_{ST} \approx 0.15-0.20$, Petit *et al.* 1995). Genetic differentiation of a specific pest of maritime pine, the bast scale *Matsucoccus feytaudi*, estimated using mtDNA markers, is again particularly high, probably because of the fragmented distribution of its host ($N_{ST} = 0.87$, Burban *et al.* 1999). The phylogeographic pattern of *P. pinaster* derived from both organelle markers will be discussed in comparison with previously derived nuclear data and with mtDNA data from its pathogen *M. feytaudi*.

Materials and methods

Sampling

Fifty-seven populations were used for genetic analysis, covering the natural range of the species. In each population, 10 individuals, distant from each other by at least 50 m, were sampled (one cone per tree). Seedlings were grown in a greenhouse and conserved frozen until DNA isolation. The material for the Corsican and Aquitaine populations and for nine of the Spanish populations had been obtained separately but using similar sampling approaches (Agúndez *et al.* 2001; Mariette *et al.* 2001).

Polymerase chain reaction (PCR), restriction fragment length polymorphism–single-strand conformation polymorphism analysis (RFLP–SSCP) and sequencing

DNA was isolated according to the protocol of Doyle & Doyle (1990), modified following Dumolin *et al.* (1995) and Belahbib *et al.* (2001). PCR were performed in a thermocycler allowing a gradient of annealing temperatures. The reaction mixture (25 μ L) contained 20 ng DNA template, 0.2 U Silverstar *Taq* polymerase (Eurogentec), 1 \times Buffer (Eurogentec 10 \times PCR Buffer), 1.8–2.5 mM $MgCl_2$, 0.1 mM of each dNTP, 5 μ g bovine serum albumin. The PCR conditions for four mtDNA and five cpDNA fragments that were successfully amplified are given in Table 1.

RFLP–SSCP techniques are described in Bodénès *et al.* (1996) and Burban *et al.* (1999). Two individuals from 48 populations were used for the screening of polymorphic fragments, by combining RFLP and SSCP techniques

Table 1 PCR and RFLP conditions for screening polymorphism of *Pinus pinaster* mtDNA and cpDNA

Primer pair	Fragment amplified	Reference	Annealing temperature	Extension time	Restriction enzyme
Mitochondrial DNA					
<i>nad1</i> /2–3	<i>nad1</i> intron 2	Demesure <i>et al.</i> (1995)	57 °C	2' 15"	<i>HinfI</i>
<i>nad4</i> /3–4	<i>nad4</i> intron 3	Dumolin-Lapègue <i>et al.</i> (1997)	49 °C	2'	<i>HinfI</i>
<i>nad5</i> /1–2	<i>nad5</i> intron 1	Dumolin-Lapègue <i>et al.</i> (1997)	47 °C	2'15"	<i>HinfI</i>
<i>cox1</i> *	<i>cox1</i>	Soranzo <i>et al.</i> (2000)	47 °C	45'	<i>HinfI</i>
Chloroplast DNA					
AS	<i>psbA-trnS</i>	Demesure <i>et al.</i> (1995)	53 °C	4'	<i>HinfI</i>
AS1	intern AS	this study	59 °C	45'	<i>DraI</i>
AS2	intern AS	this study	58 °C	45'	<i>HinfI</i>
CD	<i>psbC-trnD</i>	Demesure <i>et al.</i> (1995)	50 °C	4'	<i>HinfI</i>
CS	<i>trnC-trnS</i>	Demesure <i>et al.</i> (1995)	57 °C	1'20"	<i>HinfI</i>
K1K2	<i>trnK</i> intron	Demesure <i>et al.</i> (1995)	61 °C	3'	<i>DraI</i>
SfM	<i>trnS-trnFM</i>	Demesure <i>et al.</i> (1995)	60 °C	1'10"	<i>TaqI</i>

SSCP was tested after digestion for most fragments, but directly after PCR for the smaller one: *cox1*. It was not applied to subfragments AS1 and AS2.

*MgCl₂ concentration was 2.5 mM for *cox1* and 1.8 mM for others fragments.

(Sunnucks *et al.* 2000). RFLP was applied first and then combined with SSCP to enhance the detection of polymorphisms for the digested fragments. One fragment from each genome, *nad1* intron 2 (mtDNA) and *psaA-trnS* (cpDNA) exhibited variation using restriction enzymes alone. SSCP did not allow the detection of additional variation in these and in six other PCR fragments among the 48 populations (Table 1), nor when it was applied to the whole sample set in the case of *nad1*.

Sequencing conditions for *nad1* intron 2 and *psaA-trnS* were as in Burbank *et al.* (1999). We obtained the entire sequence for *nad1* intron2 and a partial one for the intergenic cpDNA spacer *psaA-trnS*, for 12 individuals. This allowed full characterization of the polymorphisms detected by RFLP, but no additional mutation was detected elsewhere in the sequence. As a consequence, we performed simple RFLP analysis for all the samples. This was done directly from the *nad1* PCR product. For the longer *psaA-trnS* fragment, we designed two internal primer pairs: AS1 (71360F: 5'-GGAGCCATCGGGATTATTC-3' and 70668R: 5'-CGGAAAGACGAAAGGACATT-3') and AS2 (73395F: 5'-ATATAAAAGGCCATCCATTA-3' and 72660R: 5'-GCGGTGTAGCATCAGAT-3') and the two amplified fragments (respectively 674–709 bp and 791–811 bp) were analysed by RFLP. Numbers refer to 5' nucleotide position in the *Pinus thunbergii* cpDNA complete sequence (GenBank accession number D17510).

Differentiation indices G_{ST} and N_{ST} were compared with an analytical test (Pons & Petit 1996) and a permutation test (Burbank *et al.* 1999), using the programs HAPLONST and permut, available at <http://www.pierroton.inra.fr/genetics/labo/Software/>. These parameters were first applied to the whole data set for each marker. The differentiation para-

eters were also calculated for paired populations, and analysed in relation to their geographical distances.

The sequences obtained were compared to GenBank using BLAST (Altschul *et al.* 1990) and aligned with CLUSTAL W (Thompson *et al.* 1994). Percentages of identity with other species were determined with ALIGN (Myers & Miller 1988). The cpDNA network was built with the help of TCS (Clement *et al.* 2000); no *a priori* model of evolution for the cpDNA repeats was considered (unordered states).

Results

Three mitotypes were detected from RFLP analysis of *nad1* intron 2 (Table 2). Their geographical distribution and their phylogenetic relationship are provided in Fig. 1. No single population was polymorphic and the subdivision is therefore maximal ($G_{ST} = N_{ST} = 1$). The distribution of these mitotypes clearly divides the range of maritime pine into three regions: the first mitotype ('Moroccan') is found in all populations from Morocco except Punta Cires; the

Table 2 Sequence data (polymorphism in bold) of *nad1* intron 2 for maritime pine and *Pinus ponderosa**

Mitotype	Sequence
Moroccan	... ACTA AAAGCGG TGGT ...
Western	... ACTA - GCTTAT GGT ...
Eastern	... ACTA - GCTTAT GGT ...
<i>Pinus ponderosa</i>	... ACTA - GCTTAT GGT ...

*The 34 bp repeat 'ACCATATGAATAGTGTGCTTACGCACCCCTC', characterizing the eastern haplotype, cannot be aligned unambiguously with *P. ponderosa* sequences (see the text).

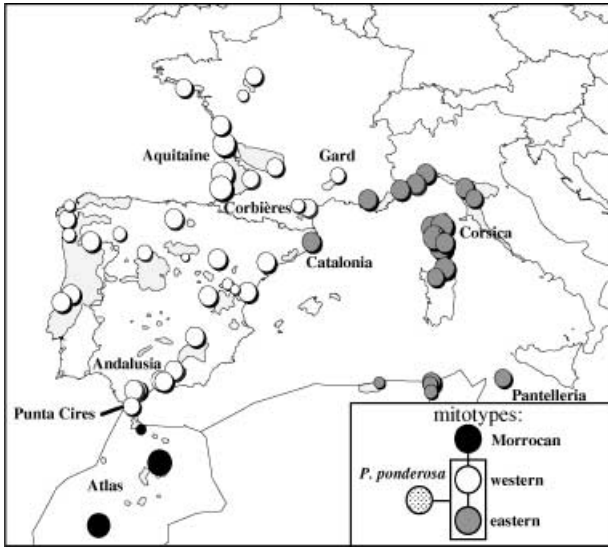


Fig. 1 Distribution of maritime pine mitotypes and phylogenetic relationships based on variation in *nad1* intron 2.

second (western) is present in all populations from the Iberian peninsula, except for that of Catalonia in north-eastern Spain, and all populations from continental France except the two easternmost; the last ('eastern') mitotype is found in all populations from southeastern France, Corsica, Italy, Pantelleria island, Tunisia and Algeria. Sequencing allowed characterization of two mutations differentiating the three mitotypes (Table 2). The Moroccan mitotype is characterized by the replacement of a 5-base-pair (bp)

sequence by an unrelated 8-bp sequence, whereas the eastern mitotype had a 34-bp duplication in a region of complex rearrangements (EMBL, accession no. AJ509804 to AJ509806).

Among the *nad1* sequences available from other conifers, that of *Pinus ponderosa* had a 91% identity and that of *Picea abies* a 76% identity with that of *P. pinaster*; intermediate values were observed for *Pinus* from other sections (*Sylvestris* and *Strobus*). Comparison with the *P. ponderosa* sequence demonstrated that the Moroccan haplotype is likely to be derived, because its 5-bp insertion is unique. The complex rearrangements observed for both species in the region of the 34-bp repeat prevent the direct use of the result of alignments. They also preclude establishing preferential relationship of the *P. ponderosa* sequence with either the eastern or western haplotype.

RFLP analysis of the two cpDNA regions studied in *psaA-trnS* (AS1 and AS2) differentiated 15 chlorotypes (Fig. 2). The analysis of these sequences (EMBL accession nos AJ509858 and AJ509859) indicates that the mutations correspond to a variable number of tandem repeats at four loci of 5–10 bp each (Table 3). The resulting network is particularly complicated (Fig. 3). Applying a more restrictive (i.e. stepwise mutation) model for the evolution of these repeated sequences did not help to resolve the phylogenetic relationships (results not shown). Despite the difficulty of reconstructing a clear phylogeny, a phylogeographic structure was detected, because the fixation index N_{ST} (0.26) is significantly higher than the corresponding G_{ST} (0.19), using both the permutation approach of Burban *et al.* (1999) and the analytic method of Pons &

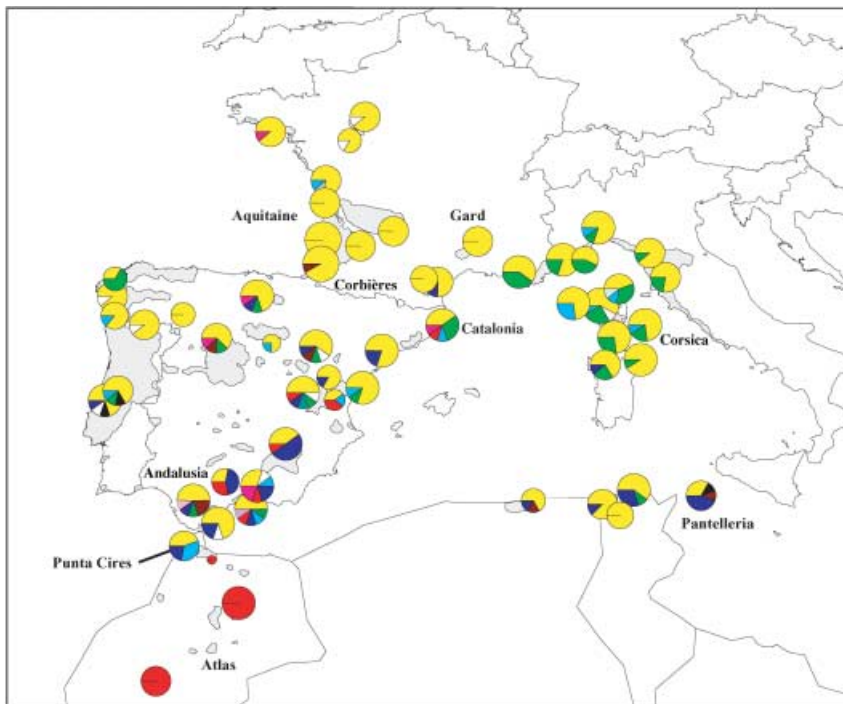


Fig. 2 Distribution of maritime pine chlorotypes (*psaA-trnS* subfragments). Colour codes for the chlorotypes are as in Fig. 3.

Table 3 Description of *Pinus pinaster* chlorotypes (*psaA-trnS* subfragments) and comparison with *P. thunbergii* (*t)

Chlorotype number	Number of repeat (repeated bases)			
	AS1R1 (CAATCTAATTC)	AS2R1 (ATGGA)	AS2R1 (AATCA)	AS2R2 (ATATT)
a	3	2	2	3
b	3	2	2	4
c	3	1	2	3
d	3	1	2	2
e	3	2	1	3
f	4	2	2	3
g	3	2	1	2
h	3	2	ND	3
i	5	2	1	3
j	5	2	2	3
k	5	2	2	2
l	5	2	1	2
m	6	2	2	3
n	6	2	2	2
o	3	2	ND	3
*t	2	2	1	1

ND, not determined.

Chlorotypes h and o exhibit particular ASR1 RFLP pattern and were not sequenced.

Petit (1996). Moreover, N_{ST} increases more than G_{ST} as a function of geographical distances between paired populations (Fig. 4).

The distribution of some of the chlorotypes can be compared with that of the three mitotypes (Fig. 5). Chlorotype A is widely distributed and associated with both the western and the eastern mitotypes. Chlorotype J, which is less frequent, is also associated with these two mitotypes, but is more frequent in the south. Other chlorotypes are mainly associated with one particular mitotype, except that they are generally more widely distributed. This is the case for chlorotype K, fixed in the Moroccan populations (except Punta Cires) but also found at lower frequency up to northern Iberia, and chlorotype E, particularly abundant in association with the eastern mitotype.

Discussion

mtDNA data

Only two polymorphisms have been found in *Pinus pinaster* mtDNA. Available universal plant mitochondrial primers were designed from angiosperm sequences, because of the absence of a completely sequenced mtDNA genome in conifers, and only a fraction of them can amplify conifer sequences, which limits the chances of detecting variation. The polymorphisms detected are not substitutions but occur in a region which is prone to complex rearrange-

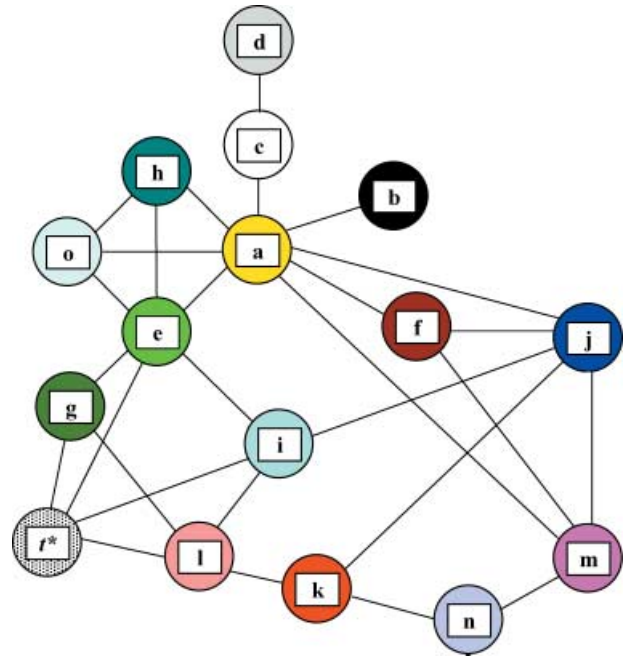


Fig. 3 Relationships between maritime pine chlorotypes determined from the sequences of *psaA-trnS* subfragments (*t: *P. thunbergii*). The tree was built on the basis of the nature of the length variants found at each of the four loci (described in Table 3) considered as unordered states (no *a priori* model of evolution).

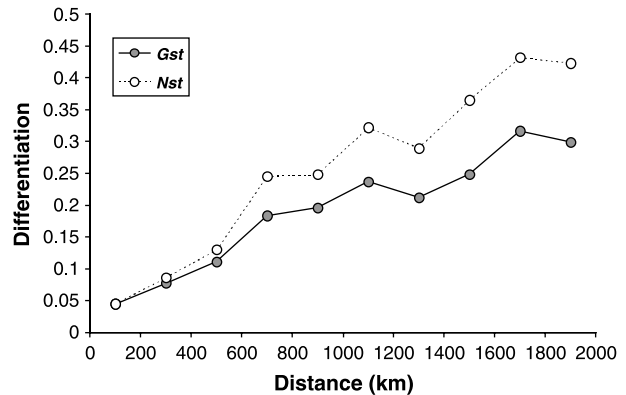


Fig. 4 Chloroplast differentiation in *Pinus pinaster* as related to geographical distances between population pairs.

ments. In fact, in this particular intron (*nad1* intron2), a minisatellite had already been identified, which is common to several conifers (Sperisen *et al.* 2001). It is noticeable that the repeat of 34 bases that differentiates the western and eastern maritime pine haplotypes matched exactly the R1 repeat described in the same intron for *P. ponderosa* (Mitton *et al.* 2000). Furthermore, the absence of polymorphic populations in *P. pinaster* matches results obtained in *P. ponderosa* with the same marker: in the latter species, the only polymorphic population was located at the junction of the range of two different varieties, *P. p. ponderosa* and

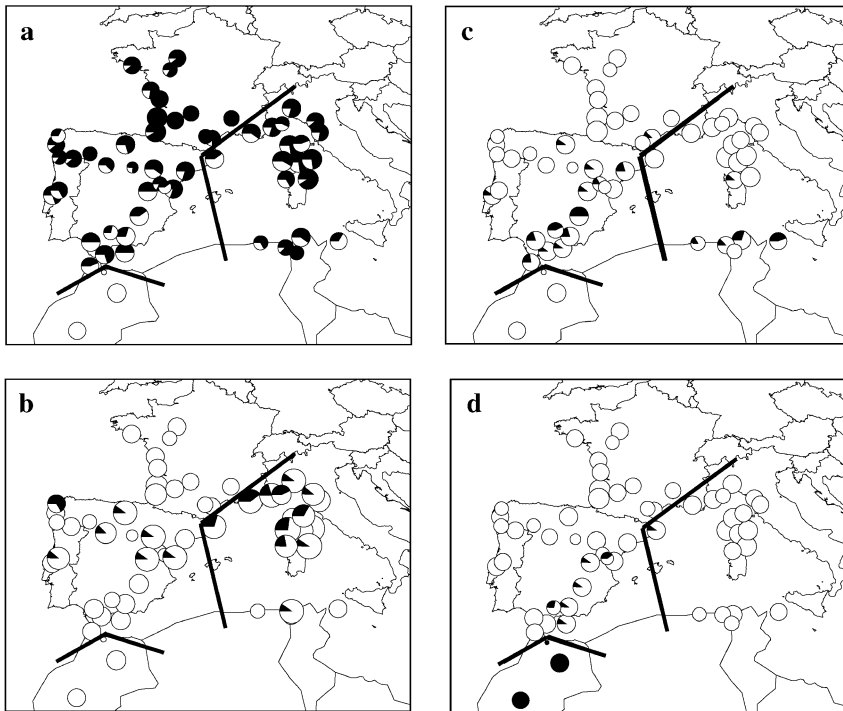


Fig. 5 Comparative distribution of maritime pine mitotypes (solid lines) and chlorotypes (circles). (a) Widespread distribution of chlorotype A; (b) possible introgression of eastern chlorotype E into Iberia; (c) distribution of chlorotype J in both eastern Spain and in Tunisia/Algeria; and (d) possible introgression of Moroccan chlorotype K into Iberia.

P. p. scopulorum (Latta & Mitton 1999). In *P. pinaster*, as in *P. ponderosa*, genetic differentiation at mtDNA is therefore exceptionally large (i.e. close to one), even by comparison with other conifers (mean $G_{ST} = 0.76$, averaged over 10 species; Petit *et al.* 2003). Particularly low seed flow in these two species may account for this observation.

cpDNA data

With a screening effort comparable to that used for mtDNA, up to 15 different chlorotypes were detected. The level of differentiation measured is much lower than that found with mtDNA but of the same magnitude as that found in a previous study based on chloroplast microsatellites ($G_{ST} = 0.15$; G.G. Vendramin, personal communication). Paternal inheritance, leading to gene flow through both pollen and seeds, instead of through seeds only, is the simplest explanation to account for the contrasted levels of differentiation observed for cpDNA vs. mtDNA markers (Petit *et al.* 1993). Despite the nature of the polymorphisms identified (tandem repeats prone to recurrent or parallel mutations), a phylogeographic structure was present (as demonstrated by the fact that N_{ST} is significantly higher than G_{ST}). The faster increase of N_{ST} with geographical distances, compared to G_{ST} , further confirms this finding. This situation differs from that observed with chloroplast microsatellites, where R_{ST} (a measure comparable to N_{ST} but adapted to the stepwise mutation process of microsatellites; Slatkin 1995) was not higher than G_{ST} (G.G. Vendramin, personal communication).

Phylogeographic inferences

Despite the low level of mtDNA polymorphism, the pattern detected is of great significance for our understanding of the history of the species. The existence of at least three separate refugia during the last ice age can explain the present distribution of the mitotypes. This subdivision matched some previous taxonomic subdivisions of the species (e.g. Del Villar 1933).

Although we had attempted to avoid available chloroplast microsatellites in an effort to recover more reliable markers for phylogenetic purposes, the only cpDNA polymorphisms detected proved to be tandem repeats, which are prone to recurrent or parallel mutations. The distribution of most chlorotypes should therefore be interpreted with caution, and pollen dispersion may not be the only factor involved. Nevertheless, the differential dispersion of mtDNA and cpDNA haplotypes is most likely a consequence of differential gene flow through pollen and seeds. The pattern found for the chlorotype K, fixed in Morocco but extending into Iberia, could reflect pollen flow across the straight of Gibraltar, all the way to northern Iberia. No evidence for pollen flow in the other direction is present. It will be particularly interesting to check if such pollen flow has also played a role in the introgression of adaptive traits, such as resistance to the bark scale *Matsucoccus feytaudi*, which is particularly high in Moroccan provenances (Harfouche *et al.* 1995). Extensive pollen flow (in the absence of seed flow) across hundreds of kilometres has been inferred for other conifers as well: for *Pinus albicaulis* in

western USA (Richardson *et al.* 2002), or for *Abies alba* throughout Europe, resulting in the extensive introgression of populations originating from different ice age refugia (Liepelt *et al.* 2002).

The distribution of mtDNA haplotypes based on *nad1* intron 1 variation does not match the three geographical groups previously defined with terpene analysis (Baradat & Marpeau-Bezard 1988). Analysis of both mtDNA and cpDNA did not confirm the existence of a North African group; exchanges by pollen rather than by seeds between the disconnected populations from Morocco and Algeria/Tunisia could in principle account for the similarity of terpene composition, but the distribution of chlorotypes suggests otherwise.

The claim for extensive seed transfer that would have taken place during historical time, and which would be responsible for the present distribution of terpene groups (Baradat & Marpeau-Bezard 1988), is not supported by the findings based on organelle DNA markers, especially the maternally inherited mtDNA. Exceptions are the populations in Gard and Corbières (southern France) characterized by the western type; Aquitaine provenances are known to have been introduced into Gard for timber production during the last century (Baradat & Marpeau-Bezard 1988). The presence of the eastern mitotype in Catalonia may be related either to a non-native status or to the western geographical extent of the eastern mitotype. Comparisons with other tree species suggest that this region indeed had a separate postglacial dynamic compared with the rest of the Iberian Peninsula (Agúndez *et al.* 2001; Petit *et al.* 2002).

The resolution of the phylogeography of *P. pinaster* based on organelle DNA is limited, given the low level of polymorphism in mtDNA, but can be supplemented with data from animal mtDNA. The insect *Matsucoccus feytaudi* (maritime pine bast scale) is a specific pest of this tree, characterized by a strong mtDNA geographical structure (Burban *et al.* 1999). Trees characterized by the eastern mitotype all belong to maritime pine provenances known to be sensitive to the pest (Harfouche *et al.* 1995). When (naturally or artificially) introduced in those stands, the insect causes severe decay (Schvester 1967; Covassi & Binazzi 1992; Jactel *et al.* 1996, 1998). The stands from Middle Atlas in Morocco are colonized by a particular lineage of the scale insect, endemic to Morocco, in agreement with an ancient differentiation of the pest and its host. Except for Andalusia, all the stands exhibiting the western mitotype are infested by *M. feytaudi* whose mtDNA belongs to an Atlantic lineage. Furthermore, the insect genetic diversity is higher in the Iberian Peninsula than in France, suggesting a northward colonization from Iberian refugia. The stand of Punta Cires in northern Morocco (the only *P. pinaster* stand in Morocco which has neither mtDNA nor cpDNA Moroccan haplotypes) is generally assumed to have been

introduced (Baradat & Marpeau-Bezard 1988). It hosts an endemic population of the pest belonging to the Atlantic lineage but characterized by private haplotypes. This would argue in favour of a native status of both the tree and the insect in this marginal stand, a finding which should have important consequences for conservation.

Andalusian populations of *M. feytaudi* exhibit a particular mtDNA lineage, whereas no difference was found between maritime pines from this area and from the rest of Spain. However a detailed survey of Iberian maritime pine based on isoenzyme data allowed the distinction of Andalusian stands from other Iberian stands (Salvador *et al.* 2000). Palynological and palaeoecological data also suggest that there were several refugia in the Iberian peninsula during the last ice age (Carrion *et al.* 2000), in line with findings for other tree species (such as the deciduous oaks, Petit *et al.* 2002). This illustrates the limits of the resolution with mtDNA polymorphism found at present in maritime pine. Analysis of mtDNA from Spanish populations of another Mediterranean pine, *P. halepensis*, has revealed no polymorphism at all (Agúndez *et al.* 2001), while isoenzymes (Agúndez *et al.* 1999) and chloroplast microsatellites (Gomez 1998) were useful at this scale. Nevertheless, mtDNA remains a promising source of markers for studying the phylogeography of conifers, being the only genome that is maternally inherited; this genome is comparatively large in plants, and future sequencing studies should eventually allow the appropriate markers to be targeted more easily.

Acknowledgements

This work was supported by grant from the EU as part of the FOSILVA project (no. EVK2-1999-00015P). We thank particularly D. Agúndez and S. Mariette for providing the already extracted DNA from adequate maritime pine sampling from, respectively, Spanish and French Corsican and Aquitaine stands. D. Agúndez has also participated in the mitochondrial RFLP analysis of some Spanish populations. E. Bertocchi was efficiently responsible for seedling production at INRA. This study would not have been possible without the precious collaboration of M. Bertin, R. Bigel, J.-M. Corti, N. Delaire, R. Delpont, V. Didier, M.-R. Fleisch, J.-F. Gueguen, R. Icher, G. Leroy, A. Lévy, J.-M. Linder, S. Normand, J. Regad (France); P. Luciano (Italy); D. Ghaioule (Morocco); J.C. Franco Santos Silva (Portugal); F.J.F. Ana Magán, P. Cabezuolo, J.S. Gutierrez, R. Hernández Alonzo, M.J. Lombardero Díaz, P.M. López, M. Mozos Pascual, M.R. Ocón, J.M. Sierra Vigil (Spain); M.L. Ben Jamaa, K. Hamdi and M. Othmani (Tunisia) who collected maritime pine cones. Sequence analyses were performed using the server INFOBIOGEN (Villejuif, France).

References

- Agúndez D, Degen B, von Wuehlisch G, Alía R (1999) Multilocus analysis of *Pinus halepensis* Mill. from Spain: genetic diversity and clinal variation. *Silvae Genetica*, **48**, 173–178.

- Agúndez D, Burban C, Robledo JJ, González-Martínez SC, Petit RJ, Alía R (2001) Estudio de poblaciones naturales españolas de *Pinus pinaster* Ait. y *Pinus halepensis* Mill. mediante ADN mitocondrial. In: Montes para la sociedad del Nuevo milenio. *Proceedings of the II Congreso Forestal Español, 25–28 septiembre, Granada* (ed. Junta de Andalucía), pp. 189–194. Coria Gráfica, Sevilla.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology*, **215**, 403–410.
- Avice JC (1998) The history and purview of phylogeography: a personal reflection. *Molecular Ecology*, **7**, 371–379.
- Bahrman N, Zivy M, Damerval C, Baradat P (1994) Organisation of the variability of abundant proteins in seven geographical origins of maritime pine (*Pinus pinaster* Ait.). *Theoretical and Applied Genetics*, **88**, 407–411.
- Baradat P, Marpeau-Bezard A (1988) Le pin maritime *Pinus pinaster* Ait: biologie et génétique des terpènes pour la connaissance et l'amélioration de l'espèce. Thèse, University of Bordeaux-I.
- Belahbib N, Pemonge M-H, Ouassou A, Sbay H, Kremer A, Petit RJ (2001) Frequent cytoplasmic exchanges between oak species that are not closely related: *Quercus suber* and *Q. ilex* in Morocco. *Molecular Ecology*, **10**, 2003–2012.
- Bodénès C, Laigret F, Kremer A (1996) Inheritance and molecular variations of PCR-SSCP fragments in pedunculate oak (*Quercus robur* L.). *Theoretical and Applied Genetics*, **93**, 348–354.
- Burban C, Petit RJ, Carcreff E, Jactel H (1999) Rangewide variation of the maritime pine bast scale *Matsucoccus feytaudii* Duc. (Homoptera: Matsucoccidae) in relation to the genetic structure of its host. *Molecular Ecology*, **8**, 1593–1602.
- Carrion JS, Navarro C, Munuera M (2000) The distribution of cluster pine (*Pinus pinaster*) in Spain as derived from paleoecological data: relationships with phytosociological classification. *Holocene*, **10**, 243–252.
- Chat J, Chalak L, Petit RJ (1999) Strict paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in interspecific crosses of kiwifruit. *Theoretical and Applied Genetics*, **99**, 314–322.
- Chesnoy L (1987) L'origine des organites du cytoplasme embryonnaire chez les Gymnospermes. *Bulletin de la Société Botanique de France*, **134**, 51–56.
- Cipriani G, Testolin R, Morgante M (1995) Paternal inheritance of plastids in interspecific hybrids of the genus *Actinidia* revealed by PCR-amplified chloroplast DNA fragments. *Molecular and General Genetics*, **247**, 693–697.
- Clement MD, Posada MD, Crandall KA (2000) TCS: a computer program to estimate gene genealogy. *Molecular Ecology*, **9**, 1657–1660.
- Covassi M, Binazzi A (1992) Primi focolai di *Matsucoccus feytaudii* Duc. nella Liguria orientale (Homoptera: Margarodidae). *Estratto Da Redia*, **75**, 453–466.
- Del Villar EH (1933) Sobre el habitat calizo de pinus pinaster. *Boletín de la Sociedad española de Historia Natural*, **33**, 133–138.
- Demesure B, Sodzi N, Petit RJ (1995) A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Molecular Ecology*, **4**, 129–131.
- Desplanque B, Viard F, Bernard J, Forcioli D, Saumitou-Laprade P, Cuguen J, Van Dijk H (2000) The linkage disequilibrium between chloroplast DNA and mitochondrial DNA haplotypes in *Beta vulgaris* ssp. *maritima* (L.): the usefulness of both genomes for population genetic studies. *Molecular Ecology*, **9**, 141–154.
- Devy-Vareta N (1988) La question du reboisement au Portugal, un processus de longue durée. *Revue Géographique Des Pyrénées et Du Sud-Ouest*, **59**, 159–186.
- Dong J, Wagner DB (1994) Paternally inherited chloroplast polymorphism in *Pinus*: estimation of diversity and population subdivision, and tests of disequilibrium with a maternally inherited mitochondrial polymorphism. *Genetics*, **136**, 1187–1194.
- Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. *Focus*, **12**, 13–15.
- Doyle JJ, Morgante M, Tingey SV, Powell W (1998) Size homoplasy in chloroplast microsatellites of wild perennial relatives of soybean (*Glycine* subgenus *Glycine*). *Molecular Biology and Evolution*, **15**, 215–218.
- Duminil J, Pemonge M-H, Petit RJ (2002) A set of 35 consensus primer pairs amplifying genes and introns of plant mitochondrial DNA. *Molecular Ecology Notes*, **2**, 425–427.
- Dumolin S, Demesure B, Petit RJ (1995) Inheritance of chloroplast and mitochondrial genomes in pedunculate oak investigated with an efficient PCR method. *Theoretical and Applied Genetics*, **91**, 1253–1256.
- Dumolin-Lapègue S, Demesure B, Fineschi S, Le Corre V, Petit RJ (1997) Phylogeographic structure of white oaks throughout the European continent. *Genetics*, **146**, 1475–1487.
- Dumolin-Lapègue S, Kremer A, Petit RJ (1999) Are chloroplast and mitochondrial DNA species-independent in oaks? *Evolution*, **53**, 1406–1413.
- Fauré S, Noyer J-L, Carreel F, Horry J-P, Bakry F, Lanaud C (1994) Maternal inheritance of chloroplast genome and paternal inheritance of mitochondrial genome in bananas (*Musa acuminata*). *Current Genetics*, **25**, 265–269.
- Gomez A (1998) Analisis de la variabilidad genética mediante marcadores moleculares de ADN en poblaciones españolas de *Pinus halepensis* Mill. Tesis Doctoral, Universidad Politécnica de Madrid.
- González-Martínez SC, Agúndez D, Alía R, Salvador L, Gil L (2001) Geographical variation of gene diversity of *Pinus pinaster* Ait. in the Iberian Peninsula. In: *Genetic Response of Forest Ecosystems to Changing Environmental Condition* (ed. Müller-Starck G), pp. 161–171. Kluwer Academic Press, Dordrecht.
- Grivet D, Heinze B, Vendramin GG, Petit RJ (2001) Genome walking with consensus primers: application to the large single copy region of chloroplast DNA. *Molecular Ecology Notes*, **1**, 345–349.
- Gugerli F, Sperisen C, Büchler U, Magni F, Geburek T, Jeandroz S, Senn J (2001) Haplotype variation in a mitochondrial tandem repeat of Norway spruce (*Picea abies*) populations suggests a serious founder effect during postglacial re-colonization of the western Alps. *Molecular Ecology*, **10**, 1255–1263.
- Harfouche A, Baradat P, Kremer A (1995) Variabilité intraspécifique chez le pin maritime (*Pinus pinaster* Ait) dans le sud-est de la France. I. Variabilité des populations autochtones et de l'ensemble de l'aire de l'espèce. *Annales Des Sciences Forestières*, **52**, 307–328.
- Havey MJ, McCreight JD, Rhodes B, Taurick G (1998) Differential transmission of the *Cucumis* organellar genomes. *Theoretical and Applied Genetics*, **97**, 122–128.
- Jactel H, Ménassieu P, Burban C (1996) Découverte en Corse de *Matsucoccus feytaudii* Duc. (Homoptera: Margarodidae), cochenille du Pin maritime. *Annales Des Sciences Forestières*, **53**, 145–152.
- Jactel H, Ménassieu P, Ceria A, Burban C, Regad J, Normand S, Carcreff E (1998) Une pullulation de la cochenille *Matsucoccus feytaudii* provoque un début de dépérissement du pin maritime en corse. *Revue Forestière Française*, **50**, 33–45.

- Latta RG, Mitton JB (1997) A comparison of population structure across four classes of gene markers in limber pine. *Genetics*, **146**, 1153–1163.
- Latta RG, Mitton JB (1999) Historical separation and present gene flow through a zone of secondary contact in ponderosa pine. *Evolution*, **53**, 769–776.
- Liepert S, Kuhlenkamp V, Anzidei M, Vendramin GG, Ziegenhagen B (2001) Pitfalls in determining size homoplasy of microsatellite loci. *Molecular Ecology Notes*, **1**, 332–335.
- Liepert S, Bialozyt R, Ziegenhagen B (2002) Wind-dispersed pollen mediates postglacial gene flow among refugia. *Proceedings of the National Academy of Sciences of the USA*, **99**, 14590–14594.
- Mariette S, Chagné D, Lézier C, Pastuszka P, Raffin A, Plomion C, Kremer A (2001) Genetic diversity within and among *Pinus pinaster* populations: comparison between AFLP and microsatellites markers. *Heredity*, **86**, 469–479.
- Mitton JB, Kreiser BR, Rehfeldt GE (2000) Primers designed to amplified a mitochondrial nad1 intron in ponderosa pine, *Pinus ponderosa*, limber pine, *P. flexilis* and Scots pine, *P. sylvestris*. *Theoretical and Applied Genetics*, **101**, 1269–1272.
- Myers EW, Miller W (1988) Optimal alignments in linear space. *Computational Applied Biosciences*, **4**, 11–17.
- Neale DB, Sederoff RR (1989) Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. *Theoretical and Applied Genetics*, **77**, 212–216.
- Newton KJ (1988) Plant mitochondrial genomes: organization, expression and variation. *Annual Review of Plant Physiology and Plant Molecular Biology*, **39**, 503–532.
- Palmer JD (1992) Comparison of chloroplast and mitochondrial genome evolution in plants. In: *Cell Organelles* (ed. Hermann R), pp. 99–133. Springer-Verlag, Vienna, Austria.
- 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 Ph (1995) Comparison of genetic differentiation in maritime pine (*Pinus pinaster* Ait) estimated using isozymes, total proteins and terpenic loci. *Heredity*, **75**, 382–389.
- Petit RJ, Brewer S, Bordács S *et al.* (2002) Identification of refugia and post-glacial colonisation routes of European white oaks based on chloroplast DNA and fossil pollen evidence. *Forest Ecology and Management*, **156**, 49–74.
- Petit RJ, Porter K, Vendramin GG (2003) Plant phylogeography based on organelle genes: an introduction. In: *Phylogeography of Southern European Refugia* (ed. Wiess S, Ferrand N), in press. Kluwer Academic Press, Dordrecht.
- Pons O, Petit RJ (1996) Measuring and testing genetic differentiation with ordered vs. unordered alleles. *Genetics*, **144**, 1237–1245.
- Ribeiro M, Plomion C, Petit R, Vendramin GG, Szmidi AE (2001) Variation of chloroplast single-sequence repeats in Portuguese maritime pine (*Pinus pinaster* Ait.). *Theoretical and Applied Genetics*, **102**, 97–103.
- Richardson BA, Brunfeldt SJ, Klopfenstein NB (2002) DNA from bird-dispersed seed and wind-disseminated pollen provides insights into postglacial colonization and population genetic structure of whitebark pine (*Pinus albicaulis*). *Molecular Ecology*, **11**, 215–227.
- Salvador L, Alía R, Agúndez D, Gil L (2000) Genetic variation and migration pathways of maritime pine (*Pinus pinaster* Ait) in the Iberian peninsula. *Theoretical and Applied Genetics*, **100**, 89–95.
- Schaal BA, Hayworth DA, Olsen KM, Rauscher JT, Smith WA (1998) Phylogeographic studies in plants: problems and prospects. *Molecular Ecology*, **7**, 465–474.
- Schvester D (1967) Observations générales sur le dépérissement du pin maritime dans les Maures. *Revue Forestière Française*, **6**, 374–384.
- Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, **139**, 457–462.
- Soranzo N, Alía R, Provan J, Powell W (2000) Patterns of variation at a mitochondrial sequence-tagged-site locus provides new insights into the postglacial history of European *Pinus sylvestris* populations. *Molecular Ecology*, **9**, 1205–1211.
- Sperisen C, Büchler U, Gugerli F, Matyas G, Geburek T, Vendramin GG (2001) Tandem repeats in plant mitochondrial genomes: application to the analysis of population differentiation in the conifer Norway spruce. *Molecular Ecology*, **10**, 257–263.
- Strauss SH, Hong Y-P, Hipkins VD (1993) High levels of population differentiation for mitochondrial DNA haplotypes in *Pinus radiata*, *muricata* and *attenuata*. *Theoretical and Applied Genetics*, **86**, 605–611.
- Sunnucks P, Wilson ACC, Beheregaray LB, Zenger K, French J, Taylor AC (2000) SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. *Molecular Ecology*, **9**, 1699–1711.
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673–4680.
- Vendramin GG, Anzidei M, Madaghiale A, Bucci G (1998) Distribution of genetic diversity in *Pinus pinaster* Ait as revealed by chloroplast microsatellites. *Theoretical and Applied Genetics*, **97**, 450–463.
- Wagner DB, Furnier GR, Saghai-Marouf MA, Williams SM, Dancik BP (1987) Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. *Proceedings of the National Academy of Sciences of the USA*, **84**, 2097–2100.
- Wagner DB, Govindaraju DR, Yeatman CW, Pitel JA (1989) Paternal chloroplast DNA inheritance in a diallel cross of jack pine (*Pinus banksiana* Lamb.). *Journal of Heredity*, **80**, 483–485.
- Wagner DB, Dong J, Carlson MR, Yanchuk AD (1991) Paternal leakage of mitochondrial DNA in *Pinus*. *Theoretical and Applied Genetics*, **82**, 510–514.
- Wolfe KH, Li W-H, Sharp PM (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proceedings of the National Academy of Sciences of the USA*, **84**, 9054–9058.

This study is part of a research programme dealing with an evolutionary and functional approach to bast scale–pine relationships at the Laboratoire d'Entomologie Forestière, INRA Bordeaux. The work of Christian Burban focuses on the molecular evolution of both insect and host populations and benefits from a close collaboration with Rémy Petit, a population geneticist interested in the phylogeography of forest trees at the Laboratoire de Génétique et Amélioration des Arbres Forestier, where the molecular investigations were carried out.
