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Population genetic structure of *Tomicus piniperda* L. (Curculionidae: Scolytinae) on different pine species and validation of *T. destruens* (Woll.)

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Abstract

Genetic diversity and population structure of *Tomicus piniperda* was assessed using mitochondrial sequences on 16 populations sampled on 6 pine species in France. Amplifications of Internal transcribed space 1 (ITS1) were also performed. Our goals were to determine the taxonomic status of the Mediterranean ecotype *T. piniperda destruens*, and to test for host plant or geographical isolation effect on population genetic structure. We showed that *T. piniperda* clusters in two mtDNA haplotypic groups. Clade A corresponds to insects sampled in continental France on *Pinus sylvestris*, *P. pinaster* and *P. uncinata*, whereas clade B gathers the individuals sampled in Corsica on *P. pinaster* and *P. radiata* and in continental France on *P. pinea* and *P. halepensis*. Insects belonging to clade A and clade B also consistently differ in the length of ITS1. Individuals belonging to both clades were found once in sympatry on *P. pinaster*. Genetic distances between clades are similar to those measured between distinct species of *Tomicus*. We concluded that clade B actually corresponds to the *destruens* ecotype and forms a good species, *T. destruens*. Analyses of molecular variance (AMOVA) were conducted separately on *T. destruens* and *T. piniperda* to test for an effect of either geographical isolation or host species. Interestingly, the effect of host plant was significant for *T. piniperda* only, while the effect of geographical isolation was not. Pine species therefore seems to act as a significant barrier to gene flow, even if host race formation is not observed. These results still need to be confirmed by nuclear markers.

Keywords: AMOVA, host specialization, ITS1, mtDNA, *Pinus*, *Tomicus*

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Introduction

Natural population structure is determined by genetic isolation, which depends on gene flow, selection and drift. Among the isolating factors responsible for the genetic differentiation, low dispersal ability, geographical barriers, habitat distribution (including host plant availability), and host plant longevity (Mopper 1996) play a central role. For oligo- or polyphagous insects, it is now clear that the host plant can play a major role in isolating specialized populations via unique selection pressures, leading to the formation of host-races, and eventually to sympatric speciation (Bush 1975; Kondrashov & Mina 1986; Tauber & Tauber 1989; Bush & Smith 1997). These selection pressures are expected to be even greater for endophagous vs. exophagous insects, as they are confined to the same plant throughout larval development (Mopper *et al*. 1995).

A number of recent studies have been developed, leading to contrasting conclusions. In *Rhagolettis pomonella* for instance, the existence of two host races is clear, and this structure is attributable to differential host plant usage and fidelity (Feder *et al*. 1988; McPheron *et al*. 1988; Feder *et al*. 1994). On the other hand, the host races found in pea aphids are due to specialized feeding behaviour and direct selection against migrants and hybrids rather than to effective host location (Via 1999; Caillaud & Via 2000; Via *et al*. 2000). On the contrary, other insects seem to easily shift hosts and do not exhibit any genetic pattern linked to host

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plant use (e.g. Radtkey & Singer 1995; Brown *et al*. 1997). In forest systems, very few studies already exist concerning the role of the host plant in genetic structure of the associated insects. For the European larch budmoth *Zeiraphera diniana*, the larch and pine-forms are strongly genetically differentiated (Emelianov *et al*. 1995). On the other hand, the structure of *Dendroctonus brevicomis* (Scolytinae) in the United States is mainly due to geographical isolation, with very weak host effect (Kelley *et al*. 1999) while two studies found some evidence of host effect on the genetic structure of *D. ponderosae* using allozyme data (Sturgeon & Mitton 1986; Kelley *et al*. 2000).

Among bark beetles (Coleoptera: Curculionidae: Scolytinae), the genus *Tomicus* comprises five described species, but only two are present in Europe, namely *T. piniperda* and *T. minor*. *T. piniperda* (L), the pine shoot beetle, has a Palearctic distribution from Europe to Japan, and has been repeatedly introduced in North America in the XXth century (Balachowsky 1949; Alosi Carter *et al*. 1996). Typically, in late winter or at the very beginning of spring, adults of *T. piniperda* disperse and attack a host trunk where mating takes place. Most attacks occur on recently fallen trees, but living host can also be chosen in epidemic conditions. In the Mediterranean region at low altitude however, trunk attacks occur in late fall. Females bore a longitudinal gallery in the inner bark where they lay eggs in lateral niches. The larvae feed on the inner bark, and the complete larval development takes place on the same host. Young adults emerge in late spring or early summer and fly to surrounding shoots where their maturation feedings take place until fall. Adults overwinter either in the shoots or in the thick bark at the base of the trunk depending on climatic conditions (Chararas 1962). At least for some host species, it is now clear that individual trees strongly differ in their ability to resist to attacks (Paine *et al*. 1997; Bois & Lieutier 2000). *T. piniperda* causes damages on various pine species throughout Europe, mainly due to its shoot feeding behaviour (Langström & Hellqvist 1990; Lieutier 1991). However, trees can sometimes be killed following stem attacks, essentially in the Mediterranean area (Ghaioule 1994). Heavy pine mortality caused by this beetle has been reported in southwestern China (Ye & Dang 1986).

In Europe, the Mediterranean populations differ from the populations of other areas. In addition to the above-mentioned details, few larval characters separate the two groups, but adults have been morphologically indistinguishable so far. Depending on the authors, the peculiar Mediterranean populations are considered either as a separate species, namely *T. destruens* (Wollaston 1865; Lekander 1971; Pfeffer 1994), or as an ecotypic form of *T. piniperda*, namely *T. piniperda* var *destruens* (Eggers 1929; Balachowsky 1949; Carle 1975). Whether *T. destruens* is a valid species is still a matter of debate. Given that no objective morphological diagnose is currently available to distinguish the two forms, we will hereafter use the term *T. piniperda sensu lato* to include both the typical populations and the *destruens* ecotype.

Moreover, several characteristics of the biology of the polyphagous species *T. piniperda s.l.* make it an ideal candidate for local adaptive structure, or even host race formation to occur (Mopper 1996; Bush & Smith 1997): (i) mate location takes place on the host plant; (ii) larval development is completed on one individual host; (iii) selection pressures due to the host (e.g. resistance capacity) are probably highly variable between hosts and are magnified by the intimacy of the insect—plant relationship; (iv) host-tree longevity compared to that of the insect can act as an additional isolating factor (Mopper 1996).

The objectives of the present work are (i) to determine on a molecular basis whether or not the Mediterranean populations of *T. piniperda* can be considered as a distinct, valid species; and (ii) to study population genetic structure in order to determine if the host-plant acts as an effective isolating barrier between populations within species. To meet this goal, we conducted an analysis of COI-COII mitochondrial sequences on a large set of *T. piniperda s.l.* populations in France. As a consequence of our results, we also conducted morphological observations to separate the adults of *T. piniperda* from those of *T. destruens*.

Materials and methods

Beetle sampling

In December 1997, October and December 1999, trap trees were cut in large pine stands of either *Pinus sylvestris*, *P. pinaster*, *P. halepensis*, *P. uncinata*, *P. nigra laricio* or *P. pinea*, in order to attract beetles during the trunk attack period throughout France. Collecting attacking *Tomicus* rather than emerging adults prevents sampling siblings and thus underestimating intra–population genetic diversity; it also allows to better separate the effect of differential host preference from effects of differential survival of genotypes (Langor & Spence 1991). The sites were chosen in stands where only one pine species was present, in the natural range of the host (except in Orléans where Scots pines are at the edge of their natural distribution). To avoid confounding the effect of geographical isolation and the effect of the host plant, we sampled beetles in 2 or 3 different locations per host plant whenever possible. However, we collected beetles in only one site for *P. pinea*, which is restricted to southeastern France and for *Pinus nigra laricio*, which is naturally present only in Corsica. Additionally, we sampled *Tomicus* from one Corsican stand of *P. radiata* (although obviously out of the host natural range) as it was heavily infested. The sampling sites are summarised in Table 1, and the locations are shown in Fig. 1. In each site where *Tomicus* attacks occurred, about

Table 1 Sampling sites and date of capture of *Tomicus piniperda s.l*

Date of capture	Locality	Host species	Code	No of individuals sequenced
February 1999	Mazaugues	*P. pinaster*	D	5
February 1999	Mont Ventoux	*P. sylvestris*	S1	4
March 1999	Comps-sur-Artuby	*P. sylvestris*	S2	2
April 1998	Dax	*P. pinaster*	B1	3
April 1998	Vendrays	*P. pinaster*	B2	3
March 1998/March 2000	Orléans	*P. sylvestris*	O/OS	9
February 1999/November 99	Lubéron Trou du Rat	*P. halepensis*	A/LA	7
April 2000	Mont-Louis	*P. uncinata*	LC	5
March 2000	Quillan	*P. sylvestris*	QS	5
March 2000	Mulhouse	*P. sylvestris*	MS	3
December 1999	Toulon	*P. halepensis*	TA	5
November 1999	St Chinian	*P. halepensis*	SA	5
March 2000	Les Arcs	*P. pinea*	AP	5
February 2000	Calvi	*P. pinaster*	CM	4
February 2000	Aléria	*P. pinaster*	AM	2
February 2000	Pietrosella	*P. radiata*	PR	5

NB: Trap trees were set in 3 additional localities in 2000 (*P. pinaster* in Mazaugues, *P. uncinata* in the Alps and *P. nigra laricio* in Niello, Corsica), but were not attacked by any *Tomicus* during the course of this study.

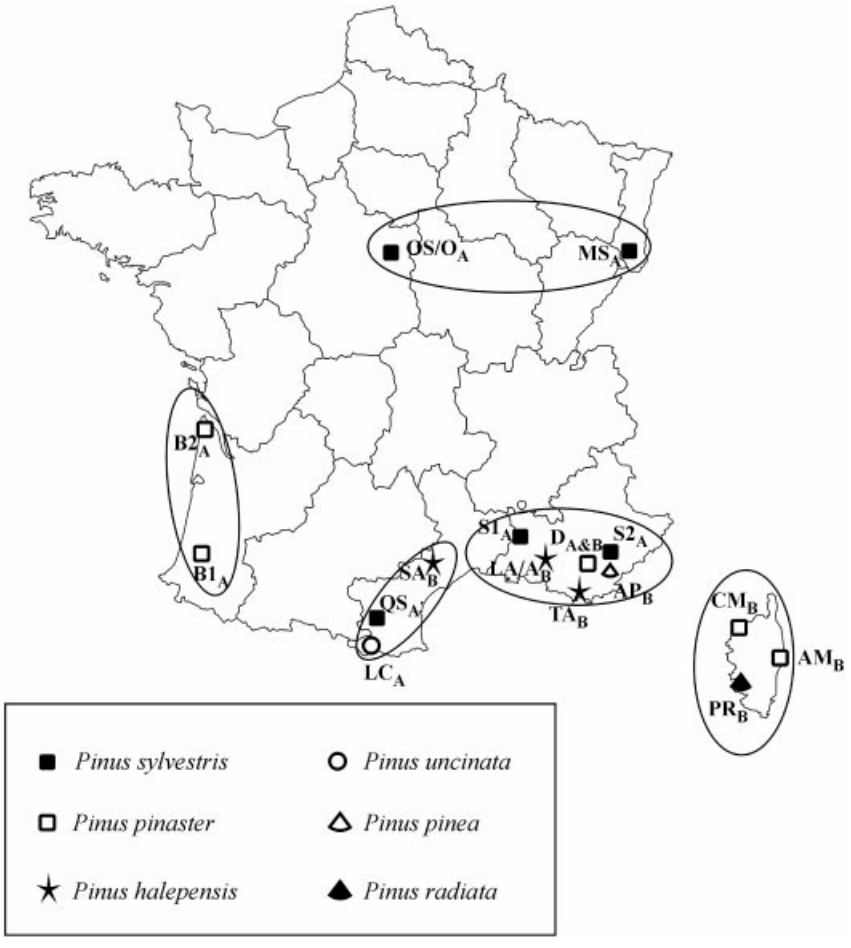

Fig. 1 Sampling sites of *T. piniperda s.l.* in France. The codes for the localities are given in Table 1. The indices A and B refer to the clade the insects were proved to belong to (see text). The ellipses show the regional groupings used for the AMOVA analysis. Southern populations were grouped on each side of the Rhône valley.

50 insects were collected in the parent galleries and immediately killed and stored in absolute ethanol. Additionally, 30 individuals of *Tomicus minor* were sampled on *P. sylvestris* in St André les Alpes (code TMAS) and Comps-sur-Artuby (code TMCS) to be compared to the populations of *T. piniperda sensu lato*. These two locations are situated near the population S2 (see Fig. 1). The tubes were kept at –20 °C until DNA extraction.

DNA protocols

DNA extractions. DNA was extracted from the head and thorax of five individual *Tomicus* per population, except where fewer than five insects were caught. The abdomen, elytras and antennas were kept apart to avoid contamination by fungi and nematodes and to permit subsequent morphological observations. Genomic DNA was isolated and purified using procedures from the DNeasy Tissue Kit (Qiagen) and eluted in 200 µL of pure water.

mtDNA polymerase chain reaction (PCR) and sequencing. We amplified a 950-bp fragment of the mitochondrial genes COI and COII by PCR. The primers were designed using published sequences of *T. minor* and *Ips typographus* (Accession numbers U82583 and AF036108): 5′-CCTCATCATTATGAGCTATTGG-3′ and 5′-TCATAGGATCAATATCATTG-3′ (primer pair #1). Using the Promega *Taq* package, 30 cycles of amplification were performed as follows in 50 µL reaction volumes: denaturation step at 92 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. For the populations sampled on *P. halepensis* and on *P. pinea* as well as for the populations from Corsica (*P. pinaster* and *P. radiata*), we had very little success amplifying using these PCR primers. We used the few obtained sequences from these populations to design new primers (5′-TCAATAGGAGCAGTATTTGCTA-3′ and 5′-AAGTAATCGTAAAGACGGAAGA-3′, primer pair #2) using the Primer3 software (Rozen & Skaletsky 1998). We could then successfully amplify a 719-bp fragment (same conditions as above, except that the annealing temperature was set to 55 °C). All PCR products were then purified with QIAquick PCR purification kit (QIAgen).

Purified PCR products were directly sequenced with the amplification primers. Sequencing was performed using the big-dye terminator sequencing kit (PE Applied Biosystem) and carried out with a ABI 373 automatic sequencer. All sequences were carefully checked by hand before analysis.

Nuclear DNA amplification. We amplified the nuclear domain ITS1 using the primer pair ITS1F (GCGTTCGAARTGCGATGATCAA) and ITS1R (GTAGGTGAACCTGCAGAAGG) developed by Vogler & DeSalle (1994). PCR conditions were similar to those used for the mitochondrial domain, except that the elongation step was increased to 1 min 30 s. The PCR products were subsequently deposited on a 2% agarose gel and migrated during 2 h to compare the length of the amplified products obtained for all individuals. The PCR product was cloned using the TOPO TA Cloning kit (Invitrogen) for two individuals of each clade (see Results) and subsequently sequenced to check that the amplified product was beetle DNA.

Data analysis

The obtained sequences were aligned using Clustal W (Thompson *et al*. 1994) as implemented in BioEdit. The genus *Dendroctonus*, which belongs to the tribe Tomicini (Pfeffer 1994), can be considered as the sister group of the genus *Tomicus* and was used as outgroup in our study. We thus aligned our sequences together with a published sequence of *D. micans* (accession number AF 296556). Kimura 2-parameter genetic distances between the haplotypes were calculated. Phylogenetic trees were reconstructed with PAUP 4*B8 (Swofford 2000) using the maximum parsimony method (MP trees). We conducted a heuristic search with a simple stepwise addition of sequences and tree bisection-reconnection (TBR) branch-swapping option. In addition, analyses were conducted using the distance-matrix method with the Neighbour-Joining (NJ) algorithm (Saitou & Nei 1987) on Kimura 2-parameter distances with MEGA 2.0 (Kumar *et al*. 2001). Both for MP and NJ methods, a bootstrap procedure of 500 iterations was completed.

The genetic structure was examined by Analysis of Molecular Variance (AMOVA) using the ARLEQUIN 2.001 software package (Excoffier *et al*. 1992; Schneider *et al*. 1997). This method was used to partition the genetic variance within populations, among populations within groups and among groups. The populations were grouped either by geographical location (regions) as shown in Fig. 1 or by host species (see Table 1). Due to the strong differentiation found among populations of *Tomicus* in France (see below), we conducted separate AMOVAS on the two haplotypic groups (clade A and clade B, see results). Levels of significance were determined through 1000 random permutation replicates.

Morphological characters

In order to determine the taxonomic status of the *destruens* ecotype, we also conducted morphological observations. Wollaston (1865), cited in Lekander (1971), proposed a few interesting characters on adults such as the length of the antennae, the width of the tibia, or the number of spines on the tibia. He also stated that *T. destruens* has 'more coarsely rugulose' elytra than *T. piniperda*. We thus performed a

careful observation of 20 individuals of each sampled population.

Results

Depending on the sites, beetle attacks took place between November and April (see Table 1). In a few sites however, the trap trees did not attract any insects. This is the case in 2000 for *P. uncinata* in the Alps, *P. pinaster* in Mazaugues and *P. nigra laricio* in Niello. For this last pine species however, the sampling failure was mainly due to extremely bad weather conditions.

Mitochondrial DNA sequences and nuclear DNA PCR products

Mitochondrial DNA. For the 38 *Tomicus piniperda s.l.* and the six *T. minor* individuals that we successfully amplified and sequenced using primer pair #1, we obtained 800 bp sequences, including 458 bp in COI, 69 in tRNA Leu and 273 bp in COII. We obtained 21 different haplotypes for *T. piniperda*, due to 23 polymorphic sites; two of these corresponded to seven and eight individuals, three haplotypes were shared by two to three insects, and the remaining 16 haplotypes were unique. Two haplotypes were found for *T. minor*, corresponding to two and four of the sequenced individuals, respectively. All sampled populations contained one to three private haplotypes (i.e. found only in that population).

For the 34 individuals amplified with primer pair #2, the resulting sequences were 657 bp long including 391 bp in COI, 68 in tRNA Leu and 198 bp in COII. They showed 9 different haplotypes due to 9 polymorphic sites. One of these corresponded to a large majority of the sampled individuals (22), two haplotypes were shared by three individuals and six haplotypes were unique. Five out of eight populations had one to three private haplotypes. One haplotype was found for three individuals sampled from different populations in Corsica.

The two sets of sequences together with the published *Dendroctonus micans* sequence could be unambiguously aligned as only one insertion occurred in tRNA Leu between the *Tomicus* sequences. The complete data set thus contains 79 individuals and is 658 bp long.

Nuclear DNA amplifications: The PCR products obtained for the ITS1 domain were 1400–1450 pb long for all individuals successfully amplified with mtDNA primer pair #1 and 1300 bp long for all insects amplified with primer pair #2. No intermediate length was observed. We obtained partial sequences (435–610 bp on each strand), from three of the four cloned PCR products. A blast search confirmed that we did amplify insect DNA.

All sequences have been deposited in GenBank under accession numbers AF457785–AF457873.

Distance matrix and phylogenetic trees

Among the *T. piniperda s.l.*, the genetic distances measured on the total alignment of 658 pb ranged between zero and 0.124, but clearly fall in two classes (class 1 from zero to 0.015 and class 2 from 0.107 to 0.124: Fig. 2A), separating *T. piniperda s.l.* in two major haplotypic groups. All pairwise comparisons within groups fall into distance class 1, whereas the distances between groups fall into distance class 2. One group (hereafter clade A) comprises the haplotypes found on *P. sylvestris*, *P. uncinata* and *P. pinaster* in continental France [except for one haplotype (D1) found on *P. pinaster* in Mazaugues]. The second group (clade B) gathers the haplotypes sampled in Corsica on *P. pinaster* and *P. radiata*, in continental France on *P. halepensis* and *P. pinea*, and the haplotype D1 collected on *P. pinaster* in southern France. This latter group also corresponds to the insects we amplified and sequenced with the second primer pair. Interestingly, the distances between the haplotypes of *T. minor* and any haplotype of *T. piniperda s.l.* are comprised between 0.121 and 0.13 (i.e. are similar to the class 2 distances presented above), whereas the distances between *Dendroctonus* and *Tomicus* ranged from 0.229 to 0.246 (Class 3, see Fig. 2B). Within haplotypic groups, the number of transitions ranges from zero to 8 and the number of transversions from zero to 2. Between haplotypic groups (i.e. between clades A and B), these numbers reach 41–53 for transitions and 18–22 for transversions. These results can be compared to those obtained between *T. minor* and *T. piniperda s.l.*, i.e. 39–48 transitions and 28–34 transversions. Between any *Tomicus* and the outgroup *D. micans*, the number of transitions ranges from 50 to 66 and the number of transversions from 67 to 74.

Phylogenetic trees were reconstructed using a subset of sequences. We retained only one sequence for each haplotype in *T. piniperda s.l.*, and excluded the sequences that differed from all others by unique substitutions. With the maximum parsimony method, six equally parsimonious trees of 244 steps were obtained. MP and NJ trees both show three strongly supported clades among the *Tomicus* haplotypes, bootstrap values reaching 100 (Fig. 3). One of the monophyletic groups corresponds to the *T. minor* individuals, whereas the two groups previously identified in *T. piniperda s.l.* form the two other clusters. Within group, the phylogenetic structure of the different haplotypes is not resolved, as shown by the low bootstrap values.

Genetic structure

The results of the AMOVA analyses are summarized in Table 2. For both analyses conducted on clade A (i.e. populations grouped by region and populations grouped by host) most of the haplotype diversity (81.86–86.08%) is found within each population, this result being significant.

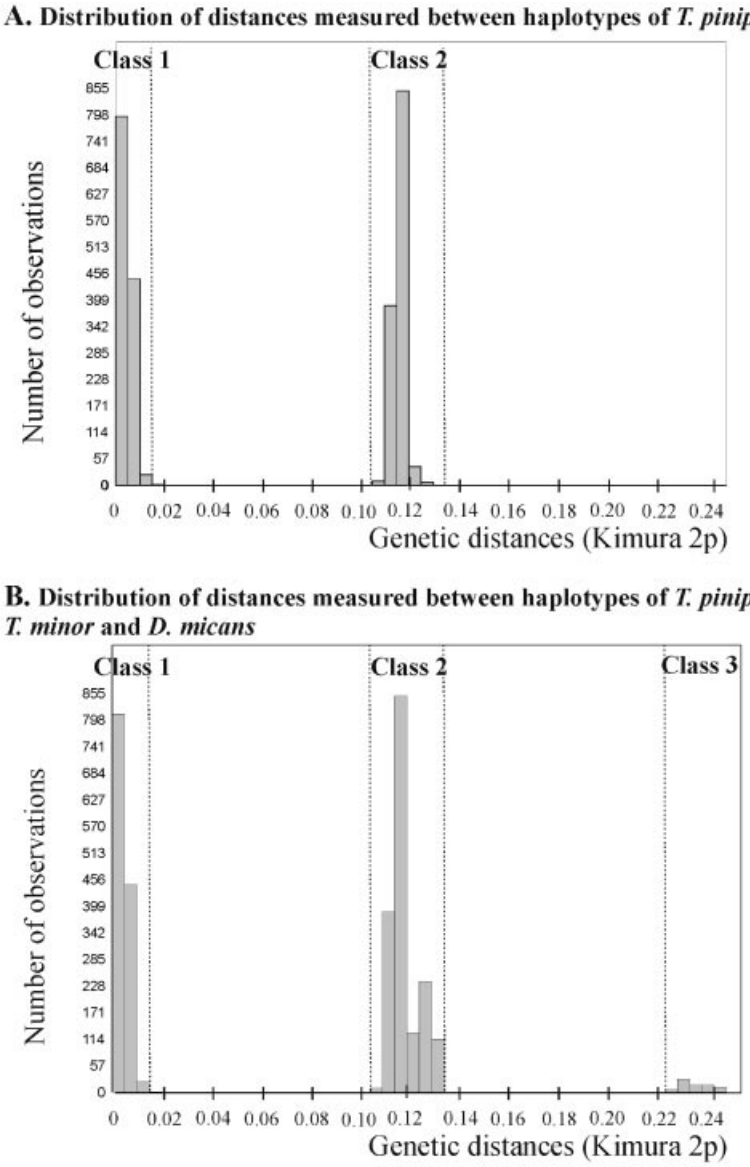

Fig. 2 Histograms of Kimura 2-parameter distance frequencies based on nucleotide sequences. A. Including all *T. piniperda s.l.* B. Including *T. piniperda s.l.*, *T. minor* and *D. micans*.

Table 2 AMOVA results

	Clade A (*T. piniperda*)				Clade B (*T. destruens*)			
Variance component	Variance	% total	*P*-value	Φ-stats	Variance	% total	*P*-value	Φ-stats
Between regions	0.22848	12.69%	0.09	$\Phi_{CT} = 0.127$	0	0	0.57	$\Phi_{CT} = 0$
Between populations within regions	0.02205	1.22%	0.32	$\Phi_{SC} = 0.014$	0.09273	17.68%	0.07	$\Phi_{SC} = 0.177$
Within populations	1.54992	86.08%	0.021	$\Phi_{ST} = 0.139$	0.43190	82.32%	0.06	$\Phi_{ST} = 0.103$
Between hosts	0.34357	18.14%	0.028	$\Phi_{CT} = 0.181$	0	0	0.86	0
Between populations within hosts	0	0	0.036	$\Phi_{SC} = 0$	0.14281	24.85%	0.20	$\Phi_{SC} = 0.248$
Within populations	1.54992	81.86%	0.028	$\Phi_{ST} = 0.181$	0.43190	75.15%	0.07	$\Phi_{ST} = 0.087$

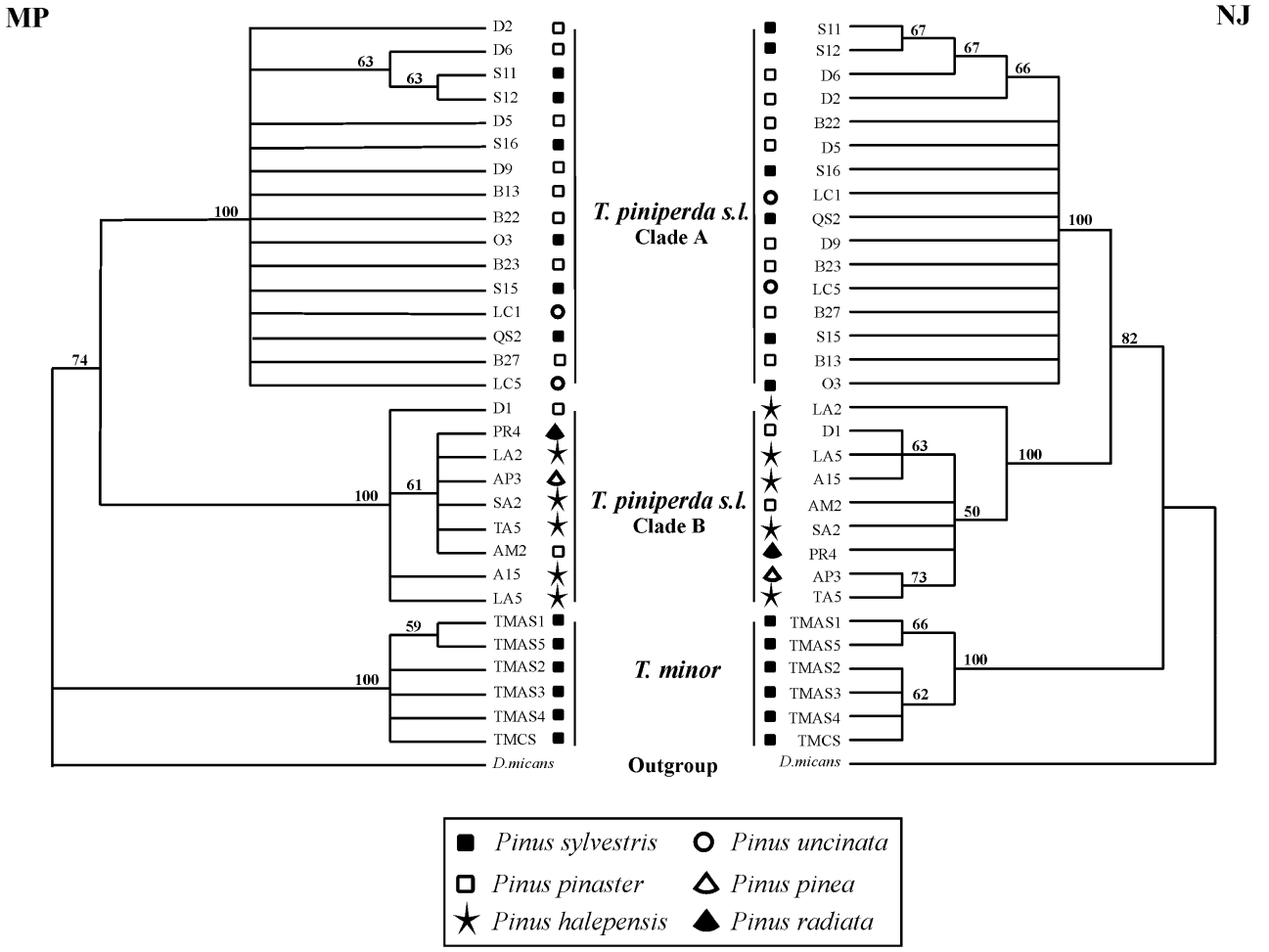

Fig. 3 Phylogenetic trees reconstructed from the 658 bp nucleotide sequences obtained. Right: Maximum-parsimony tree (MP), 50% majority rule consensus. Left: Neighbour-Joining tree (NJ) reconstructed with Kimura 2-parameter distances. Bootstrap values over 50 are given for both MP and NJ.

On the contrary, the diversity between populations within groups (regions or hosts) is negligible. When populations are grouped by region (see Fig. 1), an appreciable but non-significant amount of the variation is found among regions (12.69%). Interestingly, when populations are grouped by host plant, a greater amount of the diversity is found among groups (18.14%), and the partition is significant.

In clade B, most of the diversity is also found within populations (75.15–82.32%), but the partition of the residual variance differs drastically from that of clade A. In that case, the variation between groups is negligible, whereas the variation between populations within groups reaches 17.68–24.85% (see Table 2). However, this partition is not significant.

Morphological characters

Observations concerning the length of the antennae, the width of the tibia, or the number of spines on the tibia showed that these characters exhibit intra-populational variability or between sexes differences. On the other hand, the rough aspect of the elytra was proved to be due to the presence of additional micropunctuations on all individuals sampled from the clade B populations compared to clade A (see Fig. 4). All individuals of clade A exhibit only one row of micropunctuations between the main punctuations, whereas two to three micropunctuations can be observed in clade B individuals. This is easily seen on the elytral declivity where setae are absent and can be used as a diagnostic character to separate the two clades.

Discussion

The molecular marker we chose proved to be useful for the study of *Tomicus piniperda* populations in France. Mitochondrial DNA is widely used for understanding animal population genetic processes (see Hillis *et al*. 1996). DNA sequencing further enables obtaining detailed

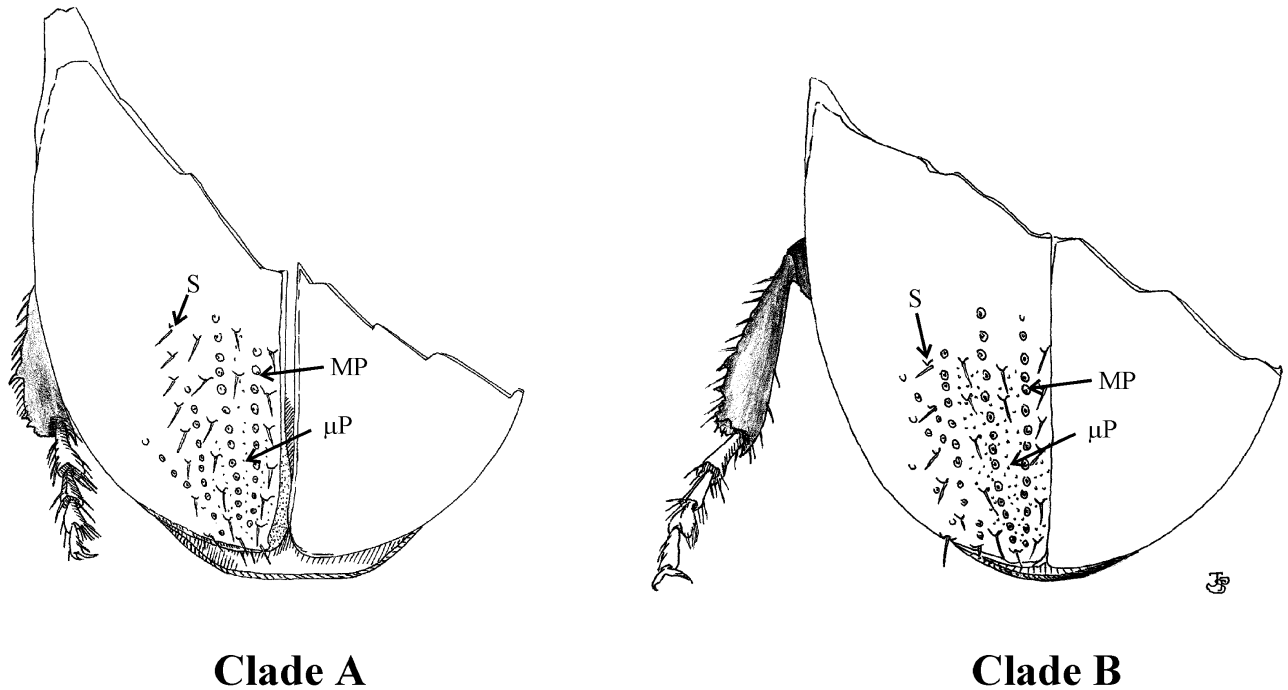

Fig. 4 Elytral declivity of clades A and B (Drawing by J. Sainsard). S: setae; MP: main punctuations; μP: micropunctuations. Note that only one rank of micropunctuations is present between 2 rows of main punctuations in clade A, whereas 2–3 micropunctuations are irregularly arranged between the rows of main punctuations in clade B.

information that can be used to characterize the haplotypes and reconstruct their relationships in a wide range of genetic distances (intra as well as interspecific levels). Although this technique cannot be applied to a great number of samples, the resulting data permitted us to obtain conclusions about the genetic pattern of the studied populations due to the haplotypic diversity that we could find. The following discussion will be structured in two main points, that is: (i) the taxonomic status of the Mediterranean populations and; (ii) the respective roles of the host plant and geographical isolation on intra-specific genetic structuring.

Taxonomic status of the Mediterranean T. piniperda s.l. T. destruens is a valid species

As shown in Figs 2 and 3, the sequenced individuals are strongly structured in two haplotypic groups. One of this group (clade A) corresponds to most insects collected in continental France on *Pinus sylvestris*, *P. uncinata* and *P. pinaster* whereas the second group (clade B) clusters all Corsican and Mediterranean populations on *P. halepensis*, *P. pinea*, *P. radiata* and *P. pinaster*. The distribution of this Mediterranean clade thus follows the expected range of *T. piniperda* var *destruens* (Carle 1973). The distances measured within group do not overlap those observed between groups. Moreover, the distances we found between clade A and clade B (0.107–0.125) are very close to the distances between any *T. piniperda s.l.* and *T. minor* (0.121–0.136, see Fig. 2). Moreover, the distances between clade A and clade B are greater than interspecific distances calculated in the genus *Dendroctonus* (Kelley & Farrell 1998) on the overlapping region of COI between their sequences and ours (311 bp).

On the other hand, the intra-group distances are fully compatible with intra-specific variation commonly observed in insects (see for instance Kelley & Farrell 1998; Kerdelhué *et al*. 1999). We can therefore confidently conclude that the clade B populations studied here form a valid species rather than an ecotype, and that these populations correspond to *T. destruens* (Woll.) while the populations in the clade A correspond to the species *T. piniperda sensu stricto*. Moreover, the results obtained on the nuclear domain ITS1 are consistent with those obtained on the mt DNA genes, as the PCR products obtained for the individuals identified as *T. piniperda* were 100–150 bp longer than the PCR products obtained for *T. destruens*.

T. destruens was found on *P. pinea* and *P. halepensis* in Southern continental France and in Corsica while *T. piniperda* was never found in these localities. Interestingly, both *T. piniperda* and *T. destruens* were found on the same trap trees on *P. pinaster* in Mazaugues, which shows that the two species can be found in sympatry contrarily to previous observations (Lekander 1971; Carle 1973; Carle 1975).

Even though more precise ecological studies will now be necessary, a differential host use seems to appear between the two species. Whether this apparent specialisation is due to an actual host preference or is due to differential climatic and habitat preferences (*P. halepensis* and *P. pinea* being restricted to the Mediterranean region) remains unknown. The distribution of *T. destruens* is most likely limited by climatic conditions, as it has been found so far neither over 600 m in altitude, nor on its potential hosts at higher latitudes (in particular on more northern plantations of *P. pinaster*). This feature could be related to the fact that dispersion flight and trunk attack occur in autumn rather than in spring, and that cold winter temperatures could be lethal for its larvae. However, we cannot rule out the hypothesis that *T. destruens* actually shows a host preference behaviour. On the other hand, *T. piniperda* is occasionally found in the Mediterranean regions, but has never been trapped on *P. halepensis* or on *P. pinea*. Whether this is due to environmental conditions or to host suitability still needs to be tested. It could be hypothesised that these pine species are not suitable hosts for *T. piniperda*. In an unpublished experiment, we put trap logs of *P. halepensis*, *P. sylvestris* and *P. pinaster* in northern forests where *T. destruens* seems to be absent. The logs were all attacked by *T. piniperda* females (no significant differences appeared in the number of entrance holes), but the galleries were consistently and significantly shorter on *P. halepensis* than on *P. sylvestris* and *P. pinaster* logs (Forichon 1999). Such results will need confirmation by further experiments, but would show that *P. halepensis* could be somewhat toxic to *T. piniperda*. No data is currently available concerning larval differential mortality.

These results bring up the question of the reason for the split between the two sister species. *T. piniperda* and *T. destruens* share at least one host species, namely *P. pinaster* and can occur in sympatry. *T. destruens* was found on *P. radiata* and *T. piniperda* can attack American host species in the United States where it was accidentally introduced (Alosi Carter *et al*. 1996), which shows that both species can develop on nonnative hosts. These observations suggest that sympatric effects like host specialisation alone cannot lead to complete divergence of the two taxa all by themselves. On the other hand, the two species show geographical differences in their distribution ranges, *T. destruens* being restricted to the Mediterranean area whereas *T. piniperda* occurs in northern Europe. A scenario could be that the speciation event between the two species was primarily due to geographical or climatic barriers. The places where the two species are now found in sympatry would then result from a secondary contact after the split. A consequence of the geographical separation of the species is that they later evolved on different pine species, and developed adaptations to their local hosts. The situation would then be partly similar to that of *Dendroctonus brevicomis* that was proved to be composed of two cryptic sister species (Kelley *et al*. 1999). Even if host effect does not seem to be the main reason for the speciation of *T. piniperda* and *T. destruens*, it can still be of importance in the intra-specific genetic structure of either of the two species (see below 'genetic diversity and population structure within species').

Carle (1973) obtained fertile hybrids between *T. piniperda* and *T. destruens*, and concluded that the differences between them were ecotypic rather than specific. Unfortunately, no data are given about larval mortality or offspring fitness, which prevents any conclusion about hybrid selection. If hybridization in the lab could be confirmed, then it would mean that endogenous selection (due to incompatibilities between parental genomes, see Arnold 1997) does not occur. The possibility would remain that exogenous (i.e. ecologically based) selection acts against hybrids in the parental environment. Indeed, temporal differences in adult emergence and mating, and differential host choice could also act as premating barriers in natural conditions (Feder *et al*. 1988) wherever *T. piniperda* and *T. destruens* occur in sympatry. Whether natural hybrids can be found in the field remains unknown. Geographic isolation is probably also an important factor to explain the maintenance of the two species. *T. piniperda* occurs only with *T. minor* in most of its geographical range. The extent of sympatry of *T. piniperda* and *T. destruens* in the Mediterranean region would need to be more precisely determined by further sampling effort.

Following the genotypic cluster species concept (Mallet 1995), our results definitely show that *T. piniperda* and *T. destruens* are two distinct species. This finding is of importance for the management of this forest insect pest in Europe, as it means that most Mediterranean 'populations' of the pine shoot beetle found on *P. halepensis* and *P. pinea* are genetically isolated from the northern 'populations'. Our study has drastic applied consequences on the understanding of epidemics. In the case of local eruptive development of a population and if segregation by host species really exists, one can expect the damages to spread to neighbouring forest patches suitable for this particular species, rather than to pines infested by the other *Tomicus* species. The same expectation can be drawn regarding possible climatic changes.

A practical consequence of our work is that we are now able to propose a molecular diagnostic to separate the species. *T. piniperda* and *T. destruens* are differentially amplified with the two mtDNA primer pairs we used, which can be used as a first clue. The identification can then be easily confirmed after digestion of the PCR product by diagnostic restriction enzyme. For instance, *Bcl*I has a restriction site for *T. destruens* and *T. minor*, and none for *T. piniperda*, whereas *Hin*dIII cuts the sequence of *T. piniperda* and *T. minor* but not *T. destruens*. Additional diagnostic enzymes can easily be found on our published

sequences. The length of the nuclear ITS1 domain could also simply be used as a key character to separate both species. Such interspecific differences in ITS length were observed in various taxa (Schlötterer *et al*. 1994; Tang *et al*. 1996; Fenton *et al*. 1997; Krüger *et al*. 2000). Using both mitochondrial and nuclear diagnoses could further allow to identify hybrids.

However, since this method is destructive it can only be used to sort the beetles a *posteriori*. Fortunately, we believe that identification of the species can be based on the elytral micropunctuations that seem to separate *T. piniperda* and *T. destruens* although this assumption still needs to be tested on additional populations. Moreover, the elytra of *T. destruens* are more or less ferruginous whereas they are black for *T. piniperda*. However, this character is only valid on mature adults, as all young adults are reddish when exiting from the galleries where they developed.

Genetic diversity and population structure within species

There were marked differences between *T. piniperda* and *T. destruens* in mtDNA diversity, as 21 haplotypes were found for 38 individuals in *T. piniperda* whereas only nine haplotypes were uncovered for 34 individuals in *T. destruens*. Among the 658 bp sequenced for both species, we found 18 polymorphic sites for *T. piniperda* and only nine for *T. destruens*. The situation is similar to that recently found in the sister species *Dendroctonus ponderosae* vs. *D. jeffreyi* (Kelley *et al*. 2000). In that latter study, the authors concluded that diet breadth could play a role in the disparity of genetic diversity and structuring between species. In our case, both species were sampled on three or four host pine species. However, diet breadth could be an important parameter in the observed genetic patterns, as the major hosts of *T. destruens* are either rare (*P. pinea*, *P. radiata*), or of restricted distribution (*P. halepensis*). Mitochondrial markers are known to be more sensitive than nuclear ones to factors restricting effective population sizes and shortening coalescence times (Moore 1995), such as dispersal ability, mating system, bottlenecks or smaller overall population sizes. Mating behaviour and sex ratios seem to be similar in *T. piniperda* and *T. destruens*. Whether the two species differ in dispersal patterns remain unclear. The observed disparity in genetic diversity could thus result from either a historical bottleneck undergone by *T. destruens*, or from smaller population sizes in that species that make it more prone to genetic drift (Whitlock & Barton 1997). Another explanation could be that *T. destruens* experiences more episodes of flushes and crashes than *T. piniperda*. In France, the populations of *T. piniperda* are endemic, while those of *T. destruens* are more often epidemic, as can be seen by the highest damages observed in the Mediterranean area compared to other places.

Concerning the distribution of genetic diversity within species, most of the variability is found within population for both *T. piniperda* and *T. destruens* as shown by the AMOVA results, which shows that their populations are not strongly structured. However, the distribution of the residual molecular variance is drastically different between the two species. When populations are grouped either by region or by host, the residual variance is mostly found between groups in *T. piniperda* whereas it is distributed within groups for *T. destruens*. For that latter species, it thus means that the grouping of populations we tested has no biological reality. The results obtained for *T. destruens* would rather show that the populations are differentiated at a very fine scale, and that no isolation can be detected at a greater scale. This could indicate that the species has very low dispersing abilities (Peterson & Denno 1998). However, the corresponding AMOVA parameters are not significant, and this pattern could also be due to the relatively low genetic diversity measured in *T. destruens*.

Concerning *T. piniperda*, a significant structure is observed when the populations are grouped by host species rather than by region. Even if most of the variance is found within populations, our results show that the host plant plays a significant role in the insect genetic structure even if the species does not appear to be differentiated in host races. No differentiation by population appears within host group. *T. piniperda* is thus not locally structured, which shows that its dispersal ability does not significantly limit gene flow at a fine scale. On the contrary, the host plant seems to act as a relative barrier to genetic exchange between insect populations at least when the beetles are sampled during the host colonisation phase. The host plant effect detected here therefore reflects differential host preference, which can be due to effective host choice behaviours, or to selection against migrants. In future works, collecting beetles prior to emergence would also determine whether there is larval differential survival due to host selection pressures. Even if the present study was conducted on a single mitochondrial locus, the results show the significant role plaid by the host plant in population structuring of an oligophagous forest insect without host race formation.

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