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ORIGINAL PAPER

Lack of genetic differentiation after host range extension argues for the generalist nature of *Pityogenes chalcographus* (Curculionidae: Scolytinae)

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Abstract
• ***Context*** The bark beetle, *Pityogenes chalcographus*, is one of the main pests in *Picea abies* stands, and it has also been found on other European Pinaceae species. With massive introductions of North American conifer species into European forests, this insect has extended its host range to exotic Pinaceae species.
• ***Aim*** This study assessed whether a wider host range has influenced the genetic structure of *P. chalcographus* and has led to host specialization.

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Contribution of the co-authors Coralie Bertheau: was a PhD student in the University of Orléans (France) when the experiments took place. Now she is a post-doctoral research associate at the Institute of Forest Entomology, Forest Pathology and Forest Protection, Boku, University of Natural Resources and Life Sciences in Vienna (Austria). She coordinated the project from its elaboration until the submission of the paper. She designed the experiments, sampled beetles, did technical experiments and phylogenetic data analyses, and wrote the paper.
Stéphanie Bankhead-Dronnet: is Assistant Professor at the University of Orléans. She contributed in phylogenetic data analyses and in writing of the paper.
Carine Martin: is a technical assistant at the University of Orléans and participated to the sampling and the experimental work.
François Lieutier: is a Professor at the University of Orléans. He led this project, supervised the work, helped in beetles sampling and read, corrected, discussed and approved the final version of the paper.
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• ***Methods*** Insects were collected from two different regions of France, where eight native and exotic conifer species coexist and were analyzed using mitochondrial and nuclear genetic markers.
• ***Results*** Considerable haplotypic diversity was observed within the regions and within host species from where *P. chalcographus* populations were collected. No genetic differentiation, especially with respect to host species associations, could be detected. Moreover, no relationship could be established between closely related *P. chalcographus* haplotypes and taxonomically related conifer species.
• ***Conclusion*** The capacity of *P. chalcographus* for host shifting and dispersal may have played a key role in the rapid extension of its host range. These findings are important for pest management in forests and health and phytosanitary measures in the timber trade, especially for risk assessment in mixed coniferous forests including tree species of major economic importance.

Keywords Bark beetle · Conifers · Native · Exotic · COII-ITS2 markers

1 Introduction

Many phytophagous insects exploit a limited number of host plants, mostly belonging to the same genus (monophagous species) or the same family (oligophagous species) (Schoonhoven et al. 2005). They have a well-defined host range corresponding to the major physiological and behavioral adaptive traits of the insects (Vanbergen et al. 2003). According to Diegisser et al. (2009), the incorporation of a new host into the host range of phytophagous insects could result in two types of consequences differing significantly in their evolutionary implications. The first scenario, *host-range expansion*, suggests that insect populations are able to use the new host without losing any of their ability to exploit their natural hosts (Hare 1990). This outcome may be observed if no new morphological or physiological adaptations are required or if adaptations evolve without decreasing their fitness to the natural host. In both cases, the populations with a wide host range are not expected to be genetically differentiated (Diegisser et al. 2009). On the other hand, the second scenario, *host shift*, proposes that insects may not be able to adapt to a new host while remaining adapted to their natural hosts. Adaptations to one host are often maladaptions for other hosts, resulting in host plant associated fitness trade-offs (Via 1990), which may lead to specialized populations via selection pressure, sympatric speciation, and possibly to the formation of host races (Drès and Mallet 2002; Jaenike 1990).The apple maggot, *Rhagoletis pomonella* Walsh (Diptera: Tephritidae) is a well-known example of phytophagous insect speciation by adaptation to new host plants. This species shifted from its ancestral host, hawthorn, *Crataegus* spp., to the newly introduced Palearctic apple, *Malus pumila* Walsh, resulting in two host races (Feder et al. 1994, 1988; McPheron et al. 1988).

Shared chemical, physical, and ecological characteristics in the natural and new hosts are important for increasing the probability of an insect adapting to a new host plant (Becerra 1997; Lopez-Vaamonde et al. 2003; Strong et al. 1984); among others). Such similarities can arise between related plants, belonging to the same genus or family. Host taxonomic proximity is, therefore, one of the main controlling factors involved in successful host shift in phytophagous insects. Although it is not uncommon to find taxonomically distant plant species sharing similar secondary compounds (Becerra 1997; Strong et al. 1984), host shifts in phytophagous insects tend to occur between closely related host plants (Degomez and Wagner 2001; Lopez-Vaamonde et al. 2003; Strong et al. 1984; among others).

The European six-toothed bark beetle, *Pityogenes chalcographus* L. (Curculionidae, Scolytinae) is one of the main pests of the Norway spruce, *Picea abies* L. KARST., forests. Although *P. abies* is its preferred host and the most suitable for its development (Bertheau et al. 2009a), it is not strictly a monophagous species. This beetle has been frequently reported using other European Pinaceae species as alternative hosts, such as Scots pine, *Pinus sylvestris* L., European Silver-fir, *Abies alba* MILL. and European Larch, *Larix decidua* MILL. (Pfeffer 1994). Moreover, with the intensive reforestation of European forests (Zobel et al. 1987), *P. chalcographus* has extended its host range to various exotic North American Pinaceae species belonging to the genus *Picea*, *Pinus*, *Abies*, and *Pseudotsuga* (Pfeffer 1994). *P. chalcographus* thus constitutes a useful model for testing whether a population's genetic structure can be modified by the introduction of a new host species into the existing host range. Behavioral assays on both native and exotic Pinaceae species within the *P. chalcographus* host range showed that the two exotic species *Picea sitchensis* (Bong.) Carrière and *Pseudotsuga menziesii* (Mirb.) Franco were the second and the third choices of *P. chalcographus* after its main host *P. abies* and that the fitness was similar or only slightly lower than on *P. abies* (Bertheau et al. 2009a). The ability of *P. chalcographus* to colonize and develop on exotic Pinaceae species reveals a rapid adaptation to new hosts over a period of nearly 200 years

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in extensive plantations of North American conifers in Europe (Agnoletti and Anderson 2000). A combination of a wide range of suitable hosts and good fitness to exotic hosts might be expected to lead to a lack of genetic differentiation between *P. chalcographus* populations. However, bark beetles have an endophytic life cycle and spend most of their life under the bark. This high degree of intimacy with their hosts may reinforce local adaptation (Kerdelhué et al. 2002; Mopper 1996) and favor host specialization, leading to genetic differentiation between populations. So far, the potential effect on the population genetics of the insect of introducing a new tree species into the insect's host range has not been studied for bark beetles, despite their significant impact on the economics of European forestry.

Mitochondrial (mtDNA) and nuclear DNA (nuDNA) markers were used to analyze *P. chalcographus* populations within two regions in France (Limousin and Jura) that have undergone intensive reforestation with exotic North American conifers. The first objective was to evaluate the genetic variability among populations on native and exotic host species. The second objective was to test whether any genetic differentiation among *P. chalcographus* samples grouped by host species was correlated with taxonomic proximity between the associated host species. Despite a rather low number of analyzed insects due to the necessity of sampling from several different tree species within each region, the results should improve our understanding of how a host range expands with new conifer species after recent extensive reforestation and how this may influence the genetic structure of this bark beetle.

2 Materials and methods

2.1 Beetle sampling

During 2006 and 2007, 61 *P. chalcographus* adults were obtained from trap trees in 17 sites in two regions of France (Limousin and Jura), which were chosen for the abundance of exotic North American and native conifers. In each region, the samples came from single species stands of native conifers (*P. abies*, *P. sylvestris*, and *A. alba*) and exotic conifers [*P. sitchensis*, *Pinus strobus* L., *Abies grandis* Douglas ex (D. Don) Lindl., *P. menziesii* (Mirb.) Franco, and *Thuja plicata* Donn ex D. Don.]. The minimum and maximum distances between each stand in each region were 0.06–45 km in Limousin and 0.05–11 km in the Jura. This study considered a *site* to be a given conifer species stand from which bark beetles samples were taken. Only one individual per mother gallery was collected to prevent the sampling of siblings. Samples were stored in absolute ethanol immediately after collection. Six additional samples of *P. chalcographus*, whose DNA extracts were given by C. Stauffer (Vienna, Boku University), were included in the analyses. These samples, chosen in different European countries to represent the major clades identified by Avtzis et al. (2008a) in a previous phylogeography study, may help in haplotype assignation to particular *P. chalcographus* reference lineage previously designed. All the sampling sites and host conifer species are given in Table 1.

2.2 DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA was extracted from the head of the insects using the GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich, France) as described by the manufacturer. DNA was eluted in 100 μl of the elution solution provided in the kit and stored at −20°C. The remaining parts of the body were stored at −80°C and kept as vouchers at Orleans University.

A 676-bp fragment of the mitochondrial gene of the cytochrome oxidase subunit 2 (COII) was amplified by polymerase chain reaction (PCR), using the Sigma Red Taq package (Sigma-Aldrich, France) for up to five individuals for each population (Table 1). The first experiment produced only a few sequences using the non-specific primers C2-J-3138 (Simon et al. 1994) and TK-N-3782 (Bogdanowicz et al. 1993). Two microliters of DNA extraction in 50-μl reaction volumes containing 1.5 mM $MgCl_2$, 100 μM dNTPs, and 0.4 μM of the primers were used as templates for 35 cycles of amplification. The reaction was performed under the following conditions: denaturation step at 95°C for 1 min, annealing at 46°C for 1 min, and extension at 72°C for 1 min 30 s. The initial cycle was 3 min denaturation at 95°C, and the final cycle had an extension step of 72°C for 10 min. New *P. chalcographus*-specific primers C2PcF 5′-TAGAACAAC-TAAATTTCTTCCATG-3′ and C2PcR 5′-GTCATCTAAT-GAGGTTT TATCTGTGG-3′ were designed using OLIGO© (Rychlik 2007) to amplify a 670-pb sequence in the same conditions as above but with the annealing temperature set to 61°C.

Amplification of the full ITS2 region, including the end of the 5.8S and the beginning of the 28S ribosomal gene, was carried out for 50-μl reaction volumes containing 1.5 mM $MgCl_2$, 100 μM dNTPs, 0.2 μM of the primers ITS2F and ITS2R (Campbell et al. 1993), and 1 U of Red Taq Polymerase (Sigma-Aldrich, France). The reaction was performed under the following conditions: denaturation step at 95°C for 1 min, annealing at 51°C for 1 min, and extension at 72°C for 1 min for 30 cycles. The initial cycle had 3 min denaturation at 95°C, and the final cycle had an extension step of 72°C for 5 min.

All PCR products were purified using the GenElute PCR Clean-Up kit (Sigma-Aldrich, France) and directly sequenced

Table 1 Characteristics of *P. chalcographus* sampling sites listed by host conifer species and host origin (native or exotic)

	Tree species	Tree origin	Locality	Country	Latitude	Longitude	Code	Haplotype (COII)	Clade (COII)	Allele (ITS2)
French sites	*Picea abies*	Native	Limousin-Chamboux	France	45°40	2°00	ChaNS	Pc1(3), Pc21(1)	A (3), C (1)	I(1), II(1)
	Picea abies	Native	Limousin-Larfeuil	France	45°30	1°57	LarNS	Pc1(3), Pc18(1), Pc24(1)	A (4), C (1)	II(2)
	Picea abies	Native	Limousin-St Pierre Bellevue	France	45°54	1°53	SpbNS	Pc1(1), Pc7(1), Pc8(1), Pc22(1), Pc23(1)	A (3), C (2)	I(1), II(1)
	Picea abies	Native	Jura-Chaux	France	47°04	5°31	ChxNS	Pc1(1), Pc2(1), Pc5(1), Pc13(1), Pc25(1)	A (4), C (1)	Ia(1), IIa(1)
	Picea abies	Native	Jura-Montbarrey	France	47°01	5°37	MonNS	Pc1(2), Pc14(1), Pc17(1), Pc19(1)	A (5)	II(2)
	Pinus sylvestris	Native	Jura-Chaux	France	47°02	5°33	ChxSP	Pc9(1), Pc10(1)	A (2)	I(1), II(1)
	Abies alba	Native	Jura-Chaux	France	47°04	5°32	ChxSF	Pc1(1)	A (1)	I(1)
	Picea sitchensis	Exotic	Limousin-Chamboux	France	45°41	2°01	ChaSS	Pc1(5)	A (5)	I(1), II(1)
	Picea sitchensis	Exotic	Limousin-Larfeuil	France	45°30	1°57	LarSS	Pc1(2), Pc12(2)	A (4)	I(1), II(1)
	Pinus strobus	Exotic	Jura-Chaux	France	47°02	5°35	ChxWP	Pc1(3), Pc3(1), Pc11(1)	A (5)	I(2)
	Abies grandis	Exotic	Limousin-Larfeuil	France	45°32	1°58	LarGF	Pc1(1), Pc15(1)	A (2)	I(1)
	Abies grandis	Exotic	Jura-Chaux	France	47°02	5°38	ChxGF1	Pc1(1), Pc17(1)	A (1)	II(1)
	Abies grandis	Exotic	Jura-Chaux	France	47°04	5°33	ChxGF2	Pc16(1)	A (2)	I(1)
	Pseudotsuga menziesii	Exotic	Limousin-Chamboux	France	45°40	2°01	ChaDF	Pc1(2), Pc4(1), Pc6(1)	A (4)	II(1), Ib(1)
	Pseudotsuga menziesii	Exotic	Jura-Chaux	France	47°02	5°33	ChxDF	Pc1(2), Pc4(1), Pc10(1), Pc18(1)	A (5)	I(1), II(1)
	Pseudotsuga menziesii	Exotic	Jura-Montbarrey	France	47°00	5°37	MonDF	Pc1(2), Pc9(1), Pc10(1), Pc20(1)	A (4), B (1)	I1), II(1)
	Thuja plicata	Exotic	Jura-Chaux	France	47°04	5°30	ChxWR	Pc18(1)	A (1)	–
European sites	*Picea abies*	Native	Heubronn	Germany	47°45	7°45	Ge-IIIa	–	A(1)	–
	Picea abies	Native	Asiago	Italy	45°52	11°30	It1-II	–	B(1)	–
	Picea abies	Native	Tolmezzo	Italy	46°24	13°01	It2-IIIa			
	Picea abies	Native	Vilnius	Lithuania	54°41	25°19	Li-IIIa	–	A(1)	–
	Picea abies	Native	Eina	Norway	60°38	10°36	No-I	–	C(1)	–
	Picea abies	Native	Hajnowka	Poland	52°45	23°36	Pl-IIIa	–	A(1)	–

For each site, mtDNA haplotypes and nuDNA alleles are followed by the number of individuals (in *brackets*). For mtDNA, haplotypes are grouped within three clades A, B, and C, according to the phylogenetic reconstruction (see Section 3). European individuals, previously analyzed using COI sequences (Avtzis et al. 2008a), are assigned to the same clade according to their COII sequences

with the corresponding amplification primers. Sequencing was performed using the BigDye Terminator sequencing kit (Applied Biosystems, France) and carried out with an ABI 3100 automatic sequencer. All sequences were carefully checked by hand and then aligned using ClustalW (Thompson et al. 1994) as implemented in BIOEDIT version 7.0.9 (Hall 1999). The alignments were straightforward because no gaps occurred. Due to the inability to have a COII sequence of another *Pityogenes* species, no outgroup species could be used in this study and take another Scolytinae species as outgroup would not be enough close to allow inference from sequence or trait data.

2.3 Data analyses

2.3.1 Phylogenetic reconstructions and haplotype network estimation

Maximum parsimony (MP) and maximum likelihood (ML) tree reconstructions were produced using PAUP* 4.0 beta 10 (Swofford 2002). The ML trees were generated using the nucleotide substitution model that had the best fit for the data, using the hierarchical likelihood ratio test (hLRT) criterion, determined using MODELTEST v3.7 (Posada and Crandall 1998). MP tree reconstruction used a heuristic

search method with 1,000 random-addition sequence replicates and explored tree space by tree bisection and reconnection branch swapping. The robustness of the trees was assessed by 1,000 bootstrap replicates. To test for constancy in rates of COII evolution between lineages, maximum likelihood trees were constructed, with and without a molecular clock enforced, using PAUP. A likelihood ratio test (Felsenstein 1988) was used with a homogeneous rate of evolution as the null hypothesis. The likelihood ratio was defined as twice the difference of log-likelihood scores from constrained and unconstrained trees and compared to a χ^2 distribution with $N-2$ degrees of freedom (N=number of sequences in the tree). Uncorrected "*p*" genetic distances were calculated using PAUP* 4.0 beta 10.

A statistical parsimony network was calculated using TCS version 1.21 (Clement et al. 2000), and topological, geographical, and frequency criteria (Crandall and Templeton 1993) were used to solve the few cladogram ambiguities that occurred.

All these analyses were intended for both markers, but were mostly done for the mtDNA fragment due to the lack of polymorphism of the ITS2.

2.3.2 Population genetic parameters and analyses of population structure

The haplotype diversity H_d (mean±SD), nucleotide diversity π (mean±SD), and mean number of pairwise differences (mean±SD) were calculated using ARLEQUIN 3.11 (Excoffier et al. 2005) for both COII and ITS2 sequences. They were calculated first for each region and secondly for each host tree species. When samples were grouped by tree host species, the corresponding allelic richness r was calculated from the haplotype frequencies using the rarefaction method proposed by (Petit et al. 1998) using CONTRIB (http://www.pierroton.inra.fr/genetics/labo/Software/Contrib/). The rarefaction size r was set to 5 among the groups for the mtDNA marker and to 4 for the nuclear marker. The occurrence of a significant phylogeographic structure was assessed by testing whether *Gst* (coefficient of genetic variation over all populations) was significantly smaller than *Nst* (equivalent coefficient taking into account the similarities between haplotypes) using 1,000 permutations (see Pons and Petit 1996) in the program PERMUT (http://www.pierroton.inra.fr/genetics/labo/Software/Permut/).

Analysis of molecular variance (AMOVA; Excoffier et al. 1992) was used to partition the molecular variance into different hierarchical levels using ARLEQUIN 3.11. Samples were grouped either according to the region (Jura and Limousin), conifer species, or host origin (native vs. exotic). The significance level of F_{ST}-statistics was determined using a non-parametric permutation procedure with 1,000 randomizations, also implemented in ARLEQUIN.

An exact test of population differentiation based on haplotype frequencies (Raymond and Rousset 1995) was performed to test the null hypothesis of random distribution of individuals between pairs of sites.

2.3.3 Correlation matrix

The study tested whether the genetic divergence between the samples from different host conifer species was influenced by the taxonomic proximity between tree species. To determine whether there was any specialization of *P. chalcographus* onto its various host species, it was assumed that, for more genetically distant conifer species, there would be greater genetic differences between *P. chalcographus* samples developing on those conifer species. First, pairwise values of Kimura-2 parameter genetic distances (Kimura 1980) between the eight conifer species were taken from Bertheau et al. (2009b), who had performed such analyses using the DNA sequences from GenBank. The dissimilarity matrix including all possible tree species pairs was constructed from these genetic distances. Secondly, pairwise Φ_{ST} values were calculated for *P. chalcographus* samples grouped by host species using ARLEQUIN 3.11. In addition to using the variation in haplotype frequencies, as in conventional F_{ST} statistics, the index Φ_{ST} takes into account pairwise differences between haplotypes, which makes it the molecular analogy of Wright's fixation index (Excoffier et al. 1992). Finally, a permutation test strategy (10,000 permutations; (Smouse et al. 1986)) was used to carry out Mantel tests to determine whether the matrix of genetic distances between host species was correlated with each of the independent matrices of genetic divergence between *P. chalcographus* samples, using XLSTAT 7.1.

3 Results

3.1 Mitochondrial DNA

3.1.1 Sequence alignment and haplotypes reconstruction

The final alignment of the COII sequences comprised 638-bp, with a total of 34 (5.3%) polymorphic nucleotides of which 23 were parsimony informative (21 transitions and two transversions). Twenty-five different haplotypes within *P. chalcographus* were identified and named Pc1 to Pc25 (Table 1). They are available from GenBank under accession numbers JQ066282 to JQ066306. One main haplotype Pc1 was shared by 29 individuals; six were shared by two to three individuals (Pc4, Pc9, Pc10, Pc12, Pc17, and Pc18); and 18 haplotypes were found only once

(Table 1). All the haplotypes gave clear, unambiguous sequence chromatograms, and no indicator of pseudogenes was observed (see Zhang and Hewitt 1996). There are non-synonymous mutations but with no major changes of the COII amino acid structure. Our findings are in agreement with those found by Arthofer et al. (2010) where no pseudogene was detected in the COI sequences of *P. chalcographus*. The geographic distribution of the 25 haplotypes is shown Fig. 1a. The widespread haplotype Pc1 was observed for both native and exotic conifer species, in all sites studied except in three populations of Jura, on *P. sylvestris* (native), *A. grandis*, and *T. plicata* (exotic). Overall, the haplotype distribution of *P. chalcographus* did not appear to be correlated with geographic origin or host species (Fig. 1a, Table 1).

3.1.2 Phylogenetic trees and haplotype network

For the hierarchical likelihood ratio tests, MODELTEST analyses revealed that the most appropriate sequence evolution model was the K81uf+I model, including invariable sites (I=0.90) with unequal base frequencies (freqA=0.3524; freqC=0.1870; freqT=0.3489; freqG=0.1116). The likelihood ratio test supported a molecular clock model for *P. chalcographus* (χ^2=32.29, df=23, P<0.05). Both ML and MP phylogenetic analyses gave congruent trees, which differed only in the placement of haplotypes within clades (Supplementary Information, Fig. S1). Three clades were identified and labeled from A to C. Clade A comprised 23 haplotypes, corresponding to 59 individuals from the eight native and exotic host conifer

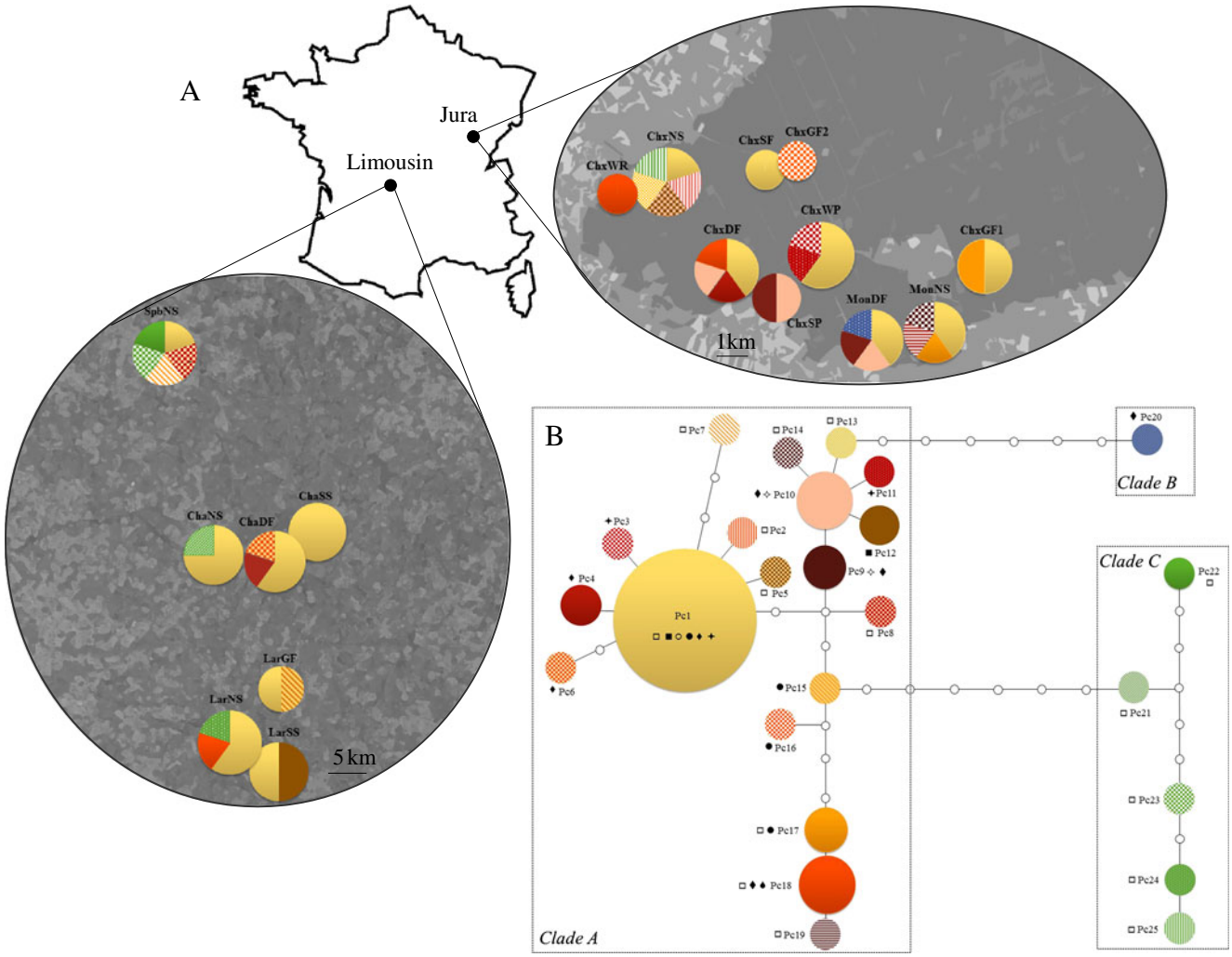

Fig. 1 Haplotype distribution and haplotype network of the 61 *Pityogenes chalcographus* cytochrome oxidase II (COII) sequences. **a** Geographical distribution of the haplotypes among the 17 sampled populations. Population frequencies are approximated by the area of the circle. Each population and haplotype is defined as in Table 1 and color codes are the same as for the haplotype network. **b** Haplotype network of the 25 haplotypes detected in *P. chalcographus* with different symbol codes for each host conifer species, *Picea abies* (*empty square*), *Picea sitchensis* (*filled square*), *Pinus sylvestris* (*empty star*), *Pinus strobus* (*filled star*), *Abies alba* (*empty circle*), *Abies grandis* (*filled circle*), *Pseudotsuga menziesii* (*filled diamond*), and *Thuja plicata* (*filled drop*). Haplotype frequencies are represented by the area of the circle. Each *line* corresponds to a mutational step and each *empty circle* to a missing intermediate

species. Nineteen haplotypes belonged to French individuals and the four others to Germany, Italy, Lithuania, and Poland. Clade B regrouped two haplotypes, a unique one Pc20 belonging to a French specimen sampled on the exotic tree species *P. menziesii* and the haplotype It1-II from Italy collected on *P. abies*. Clade C had six haplotypes with five unique (Pc21, Pc22, Pc23, Pc24, and Pc25) from French individuals sampled both in Limousin and Jura, and one corresponding to a Norwich specimen (No-I), all collected on the native *P. abies*. The distribution of haplotypes within each group was not restricted to one particular locality since they were found in both Limousin and the Jura. However, although clade A appeared to be a generalist clade (with all native and exotic conifers represented) and clade C appeared to be a specialist clade (with only *P. abies*), this structure was not correlated with the ability to use host trees. Instead, they rather matched with the structure obtained in the European phylogeography of *P. chalcographus*, sampled on *P. abies* only and analyzed with the COI gene (Avtzis et al. 2008a). Indeed, by adding to our analyses, these individuals from Norway, Lithuania, Poland, Germany, and Italy, and analyzing them with COII, it became possible to associate our clades A, B, and C to the clades IIIa, II, and I, respectively, published in the European phylogeography study (Avtzis et al. 2008a, Fig. S1, Supporting Information). Bootstrap values of the ML and MP phylogenetic trees were similar and supported the phylogenetic structure between the three clades. Genetic *p*-distances between all haplotypes, between and within the three clades, were calculated and are shown in Table 2.

The 25 haplotypes were joined into a single haplotype network with 95% probability, which showed the host plant association and phylogenetic relationships of all haplotypes (Fig. 1b). This network revealed three haplotype groups corresponding to the three previously defined clades (A, B, and C). Clades B and C diverged by six mutation steps from clade A. As mentioned above, the structure of haplotypes corresponded to the three clades of the European phylogeographic study (Avtzis et al. 2008a) and not associated with the host plants.

Table 2 Comparison of average (min–max) genetic *p*-distance, within each clade, between clades, and between all haplotypes

		p-distance
Within clades	A	0.008 (0.002-0.016)
	B	–
	C	0.008 (0.002–0.014)
Between clades	A–B	0.009 (0.002–0.019)
	A–C	0.013 (0.002–0.030)
	B–C	0.012 (0.002–0.025)
Total (between all haplotypes)		0.013 (0.002–0.030)

3.1.3 Population genetic parameters and analyses of population structure

The proportion of the various haplotypes yielded a high haplotype diversity of 0.77±0.06. However, nucleotide diversity (π=0.008±0.001) and mean number of pairwise differences (4.87±2.41) were generally low. The indices of population structure *Gst* and *Nst* were 0 and 0.042, respectively, and did not differ significantly from each other, indicating a weak phylogeographical structure. The within-population diversity indices were summarized per region and per host in Table 3. The haplotype diversity was higher in the Jura than in Limousin, while the nucleotide diversity and the mean number of pairwise nucleotide differences between haplotypes showed a similar pattern in the two regions. The haplotype diversity (H_d) within host species groups ranged from 0.39 to 0.90 with an average of 0.73. The lowest H_d was found in the exotic species *P. sitchensis*, with two different haplotypes out of nine sampled individuals. H_d was high for the native *P. abies* and the three exotic species *A. grandis*, *P. menziesii*, and *P. strobus*. According to the values of the allelic richness after rarefaction, individuals from *P. abies* did not have more haplotypes when the number of samples was similar for each host species. Nevertheless, allelic richness was still high for most host species, since more than two haplotypes were found on average for only five individuals.

AMOVA results for all three grouping options are presented in Table 4. When populations were grouped "by region," "by host," or "by host origin," the main part of the observed variability was due to the genetic difference within populations (97%, 98%, and 98%, respectively), but the results were not significant. Moreover, the exact test of differentiation among sites was not significant (*P*=0.35149).

3.1.4 Correlation matrix

The genetic distances between the six host tree species were not correlated with the Φ_{ST} genetic differentiation indices between *P. chalcographus* samples grouped by host species (Mantel test, r_S=0.022, *P*=0.99), indicating that tree species taxonomically close to each other did not tend to be exploited by genetically close insect populations.

3.2 Nuclear DNA

3.2.1 Sequence alignment and alleles reconstruction

Twenty-nine ITS2 sequences were aligned over 512-bp. No insertion and deletion of sequence was observed between the individuals analyzed. Only five distinct alleles were

Table 3 Within-population diversity indices of *P. chalcographus* samples for COII and for ITS2: because of unequal sampling size for different host species, allelic richness was calculated using rarefaction size as indicated between square brackets

		Number of individuals	Number of haplotypes	Haplotypic diversity (H_d)	Mean number of pairwise difference	Nucleotide diversity (π)	Allelic richness after rarefaction (r)
COII							
Region	Limousin	29	12	0.66	4.51	0.007	
	Jura	32	16	0.85	4.99	0.008	
Host species	*Picea abies*	24	15	0.84	6.87	0.01	2.87 [5]
	Pinus sylvestris	2	2	–	–	–	–
	Abies alba	1	1	–	–	–	–
	Picea sitchensis	9	2	0.39	1.17	0.002	0.83 [5]
	Pinus strobus	5	3	0.7	2.40	0.004	2.00 [5]
	Abies grandis	5	4	0.9	4.20	0.006	3.00 [5]
	Pseudotsuga menziesii	14	7	0.81	3.91	0.006	2.61 [5]
	Thuja plicata	1	1	–	–	–	–
ITS2							
Region	Limousin	14	3	0.6	0.68	0.0013	
	Jura	15	4	0.66	0.8	0.0015	
Host species	*Picea abies*	10	4	0.58	0.87	0.0016	1.46 [4]
	Pinus sylvestris	2	2	–	–	–	–
	Abies alba	1	1	–	–	–	–
	Picea sitchensis	4	2	0.67	0.67	0.0013	1.00 [4]
	Pinus strobus	2	1	–	–	–	–
	Abies grandis	4	2	0.5	0.5	0.0009	1.00 [4]
	Pseudotsuga menziesii	6	3	0.73	0.93	0.0018	1.60 [4]

obtained (GenBank accession numbers JQ066307-JQ066311) with four polymorphic sites (one of which was parsimony informative) and all substitutions were transitions. No heterozygous individual was observed in *P. chalcographus*. This phenomenon was not rare due to concerted evolution which usually results in the presence of a single dominant ITS allele per individual, with little intra-specific variation (Hillis and Dixon 1991). Three alleles were found only once, two on *P. abies* coded Ia and IIa and one on *P. menziesii* coded Ib, whereas both alleles I and II were found in 13 individuals and were observed in the two regions and on both native and exotic conifer species (Table 1).

The proportion of different alleles in the samples yielded a genetic diversity of 0.615±0.052, and the nucleotide diversity was very low with a value of 0.0014±0.0012. The genetic distances between all alleles calculated with the *p*-distance model ranged from 0.002 to 0.006, with an average of 0.004. All phylogenetic analyses as well as the network described no obvious pattern of host-associated group (data not shown).

Table 4 Analysis of molecular variance (AMOVA) between French populations of *Pityogenes chalcographus*, with grouping by region, host species, and host origin

	Source of variation	Variance components	Percentage of variation
Grouping by region	Between groups	0.00868 Va	2.22% NS
	Between pops within groups	0.00253 Vb	0.65% NS
	Within populations	0.37955 Vc	97.13% NS
Grouping by host	Between groups	0.00713 Va	0.84% NS
	Between populations within groups	0.00140 Vb	0.36% NS
	Within populations	0.37955 Vc	97.80% NS
Grouping by host origin	Between groups	−0.00273 Va	−0.71% NS
	Between populations within groups	0.00864 Vb	2.24% NS
	Within populations	0.37955 Vc	98.47% NS

3.2.2 Population genetic parameters

The diversities within population are shown by region and by host in Table 3. The diversities within regions were approximately similar, and the genetic and nucleotide diversities within regions corresponded to those of the total genetic and nucleotide diversities over all regions. *P. menziesii* and *P. sitchensis* showed higher genetic diversity per host species than *A. grandis* and *P. abies*. Nevertheless, values of the allelic richness after rarefaction ranged from 1 to 1.6 among the host species tested. Given the low polymorphism level in ITS2 sequences, no further analyses of genetic structure were performed.

4 Discussion

The diversity of European and North American conifer species in France made it possible to estimate whether the expansion of the insect host range following extensive reforestation with exotic conifers may have contributed to the differentiation of populations of *P. chalcographus*. The COII gene was useful because of its high variability and its ability to explain recent historical events. Conversely, the nuclear marker ITS2, mainly used to highlight cryptic species, did not provide any additional information in this study. The sampling size was limited by the following criteria: (1) individuals were sampled in sympatric areas on a total of eight native and exotic host species; (2) the colonization rate on the different host tree species studied varied because the beetles preferred and better performed their development and fecundity with some tree species, which led to different sample sizes (Bertheau et al. 2009a, b). Despite this limitation, our results tended to show that the host tree species may not constitute an effective isolating barrier between *P. chalcographus* populations.

One finding that emerged from this study was the low nucleotide diversity with few mutations observed in the COII gene sequences as opposed to the high haplotypic diversity in the *P. chalcographus* samples. This significant haplotype diversity expressed as the number of haplotypes (25 haplotypes for 61 individuals) is in agreement with the diversity observed with COI gene sequences for the same species (58 haplotypes for 262 individuals, in Avtzis et al. 2008a); more than 140 haplotypes for 523 individuals, in Bertheau, personal communication). Based on COI and/or COII gene diversity, *P. chalcographus* had the highest haplotype richness compared to other European bark beetles species studied, such as *Ips typographus* (Stauffer et al. 1999), *Tomicus destruens* Woll. (Horn et al. 2006) or *Tomicus piniperda* L. (Horn et al. 2009). The wider diet breadth of the bark beetle may explain this genetic diversity. Gaete-Eastman et al. (2004) highlighted that local aphids populations with broadest diet breadth showed higher genetic diversity compared to a strict specialist aphid species. This could be the case of *P. chalcographus* since it has broader host range than the other bark beetles mentioned above (Bertheau et al. 2009b; Pfeffer 1994).

Despite a rather low sampling size per host tree species, a general tendency to an absence of global genetic differentiation among insect populations appears through the genetic analyses. First, AMOVA showed that the greatest genetic diversity was found within populations, whatever the grouping factor tested (i.e., region, host species, or host origin). A similar pattern has been found in *T. destruens* (75–82% variation within populations, Kerdelhué et al. 2002) and in *Orthotomicus erosus* Woll. (87–93% variation within populations, Pointeau et al., personal communication). Secondly, the phylogenetic analyses as well as the haplotype network also reflected the lack of differentiation since the main clade (A) grouped individuals from different localities (Limousin and Jura) and from different host species (native and exotic) (Fig. 1). Nuclear DNA sequencing confirmed these results since the main two alleles I and II were observed whatever the sampling site (region) and host species (Table 1). Although the diet breadth is known to play a significant role in structuring bark beetle populations (Cognato and Sperling 2000; Sturgeon and Mitton 1986), the lack of differentiation by host is not rare, especially for beetle species living in regions under strong human pressure from major reforestation and wood transport. For example, the lineage diversification of *Ips confusus* Leconte was not greatly influenced by the host specificity (Cognato et al. 2003); no difference was found between populations of *Dendroctonus jeffreyi* Hopkins or *D. ponderosae* Hopkins collected from sympatric host species (Kelley et al. 2000), and no host effect was observed in *Polygraphus grandiclava* Thomson populations (Avtzis et al. 2008b). Finally, the Mantel test did not provide any correlation between genetic distances between host tree species and genetic differentiation among *P. chalcographus* samples grouped by host tree species. Even if the sampling needs to be completed to assess this assumption, it seems plausible to say that taxonomically closely related tree species did not tend to be exploited by genetically closely related insect populations.

Subject to confirmation using a larger sampling size, the high genetic diversity associated with the lack of genetic differentiation of *P. chalcographus* populations might result from certain gene flow between the populations on the various host species exploited by the beetle. There may be several explanations for this pattern. The dispersal ability of phytophagous insects appears to be an important ecological factor determining population structure (Peterson and Denno 1998). These authors showed that species with moderate mobility may undergo a significant decline in gene flow with distance, while more vagile species display

a complete lack of isolation by distance. Comparably high effective dispersal abilities with lack of genetic differentiation have generally been reported for bark beetle species such as *I. typographus* (Sallé et al. 2007), *T. piniperda* (Kerdelhue et al. 2006) and *O. erosus* (Pointeau et al., personal communication). *P. chalcographus* is known to have considerable dispersal ability since it is able to fly as far as 86 km (Nilssen 1984). Like most bark beetle species confined to weakened or felled trees, especially on broken tops and branches and on logging waste for *P. chalcographus* (Hedgren et al. 2003), this high dispersal ability may be the result of an adaptation to a patchy, ephemeral breeding resource. Selection pressure and evolution may increase dispersal abilities in insects for which availability of suitable hosts is unpredictable in space and time (Gandon et al. 1998). In addition to natural dispersal capacities, the transportation of cut trees via human activities such as wood trade could play a significant role in increasing the movement of the beetles (Kerdelhue et al. 2006). The short distances between studied plots in each region compared to the *P. chalcographus* flight distance could also facilitate displacement between the various available tree species. All these factors would have homogenized *P. chalcographus* populations.

In a previous behavioral study of *P. chalcographus*, Bertheau et al. (2009a) showed that, although *P. abies* was the preferred and most suitable host of *P. chalcographus*, all conifer species tested were colonized. Furthermore, little or no difference was found in beetle fitness on *P. sitchensis* and *P. menziesii*. The similarities between its natural and alternative host species, such as morphological characteristics (i.e., thin bark thickness) and probably chemical compounds, provided good conditions for adaptation to new hosts (Bertheau et al. 2009a). All these considerations, together with its biological and behavioral traits and its tendency to a lack of population genetic differentiation, suggest that *P. chalcographus* may use alternative hosts without losing any ability to exploit its natural host *P. abies*. A more complete sampling, combined with more polymorphic markers (as microsatellites) would permit to draw more general conclusions. Nevertheless, these findings are important to consider for forest pest management, health, and phytosanitary measures for the timber trade, especially for risk assessment in mixed coniferous forests.

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