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Phylogeography of the Eurasian pine shoot beetle *Tomicus piniperda* (Coleoptera: Scolytidae)

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Abstract. *Tomicus piniperda* is a pest in pine stands in Eurasia and is also found in the USA, where it has caused a decline in the abundance of pine since 1992. Knowledge of the genetic structure of pine shoot beetle populations is important for understanding their phylogeographic history and for quarantine control. In this study, European, Asian and American *T. piniperda* populations were analyzed by sequencing a region of the mitochondrial COI gene. Twenty-five haplotypes (HT) were detected and over 70% of these HT were found in individual areas, e.g. 5 HT in China, 5 HT in France and 3 HT in Spain. Nested clade analysis revealed that most European and the American population was in a clade containing 9 HT connected by one to two mutational steps. A second clade contained HT from France (2 HT), Spain (2 HT), Sweden (1 HT), Russia (1 HT) and China (5 HT). In this clade, one to 13 mutational steps and 13 missing or theoretical HT were detected. The third clade had 5 HT from France, Russia, Poland, Finland and Switzerland; 1 to 7 mutational steps and 5 missing or theoretical HT were detected. Although only a few significant relationships were found in the nested clade analysis, the following conclusions can be drawn: (1) *T. piniperda* is a polymorphic species with numerous HT throughout Europe, and HT are likely to exist regarding the missing or theoretical HT; (2) It is likely there were refugial areas in Southern Europe and Western Russia; (3) The Pyrenees formed a barrier to migration after the last ice age; (4) Chinese and European populations have been separated for at least 0.6 MYA.

INTRODUCTION

The bark beetle genus *Tomicus* Latreille 1802 contains six species in Europe and Asia, all of which infest coniferous trees of the genus *Pinus*. *Tomicus piniperda* L. and *T. minor* are Eurasian species, *T. destruens* occurs in the Mediterranean area, and *T. brevipilosus*, *T. puellus* and *T. pilifer* are found only in Asia. The pine shoot beetle *T. piniperda* and the Mediterranean sibling species *T. destruens* are often synonymized (e.g. Schedl, 1932). Recent phylogenetic analyses based on mitochondrial and nuclear DNA, however, reveal that they are two distinct species (Gallego & Galian, 2001; Kerdelhué et al., 2002; Kohlmayr et al., 2002), thereby confirming the classification of Pfeffer (1994) and Wood & Bright (1992).

Maturation of callow *T. piniperda* beetles involves feeding within young shoots of *Pinus sylvestris*, which damages vigorous trees. Adults attack standing pine trees and establish breeding galleries in the bark of weakened trees. The beetles are vectors of blue stain fungi, which help to overcome the resistance of the trees (Solheim et al., 1993). During mass outbreaks, the beetles successfully attack living trees (Postner, 1974). In China, *T. piniperda* is reported to cause substantial mortality of *Pinus yunnanensis* (Långström et al., 2002).

Besides *P. sylvestris*, Pfeffer (1994) listed 16 other species in the genus *Pinus* as hosts; Wood & Bright (1992) also mention various *Picea* species as host trees of *T. piniperda*. Host association is an important factor in the speciation of herbivorous insects such as scolytids (Kelley et al., 2000). Analysis of mitochondrial and nuclear DNA, however, gave no indication of host race formation in *T. piniperda* (Kerdelhué et al., 2002). The genetic structure of scolytids might be influenced by their postglacial history. In *Ips typographus* (Stauffer et al., 1999) and *Ips pini* (Cognato et al., 1999), for instance, the genetic structure based on mitochondrial DNA sequences is a consequence of their migration history since the last ice age.

The aim of this study was to reconstruct the post-glacial history of European *T. piniperda* populations. A gap in the geographical distribution of the main host *P. sylvestris* in Europe, between France and Spain – the Pyrenean Range – suggests that gene flow in the associated bark beetle might also be interrupted. Furthermore, divergence between the Asian and European populations may be expected due to the geographic distance. Specimens of the morphologically and ecologically related *T. minor* from European and Asian populations were analyzed for comparison. We used the sequence data from the mitochondrial cytochrome oxidase I (COI) gene and performed a nested clade analysis.

MATERIAL AND METHODS

DNA sequencing

Beetles were collected from the bark layer of infested pine trees and kept in absolute ethanol. DNA of single specimens was extracted using the Sigma Genelute Extraction (St. Louis, MO) kit. PCR reactions were carried out in reaction volumes of 50µl. A touchdown program was used with an initial annealing temperature of 55°C, finishing with 47°C. A 629 bp long fragment, including the partial 3' end of the COI gene and a non-

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transcribed region between COI and the tRNA-leu gene, was amplified with the primers C1-J-2441 5'cctacaggaattaaaatttttagatgattagc3' and TL2-N-3014 5'tccattgcactaatctgccatatta3' described by Simon et al. (1994). PCR products were purified using the Qiaquick® PCR purification kit (Hilden, Germany) and subsequently sequenced using primer C1-J-2441 and the Big Dye termination reaction kit (Applied Biosystems, Foster City, CA). Sequences were analyzed on an ABI 377 automatic sequencer (Applied Biosystems, Foster City, CA). Each haplotype was sequenced at least twice from independent PCR amplifications in order to avoid PCR artefacts.

Phylogenetic reconstruction

Sequence alignment was done with Clustal X (Thompson et al., 1997). To infer phylogenetic relationships, maximum parsimony analysis was performed with the software package PAUP* 4.0b3 (Swofford, 1998). Support for the nodes was calculated with bootstrap replicates using maximum parsimony (1000 steps) and neighbour joining (1000 steps) search methodologies.

Nested clade analysis

We evaluated the data with an unrooted network using a statistical parsimony (SP) criterion (Templeton et al., 1992). The SP network was constructed with the computer program TCS version 1.13 (Clement et al., 2000). To infer population histories, nested clade analysis (Templeton et al., 1995) was carried out. The nesting design was constructed on the SP network following the rules in Crandall (1996) and Templeton (1998). After transforming the network into a nested series of clades (Templeton et al., 1987; Templeton & Sing, 1993), association between these clades and geographic distances were tested using the permutational contingency analysis in the program GeoDis 2.0 (Posada et al., 2000). Appendix I of Templeton (1998) was used to interpret the results.

RESULTS AND DISCUSSIONS

For the phylogeographic analysis, 74 *T. piniperda* specimens were sequenced. All sequences collected for this study were deposited in Genbank and accession numbers are listed in Table 1. Twenty-five haplotypes (HT) were detected; sequence divergence was between 0.18% and 2.48%. All substitutions were transitions and on the 1st or 3rd codon positions. Eighteen HT (72%) were found only in individual areas, such as China (5 HT), France (5 HT), Spain (3 HT), Russia (2 HT), Austria (2 HT) and Sweden (1 HT).

Because maximum parsimony methods (MP) do not necessarily reflect population history and may indeed be misleading for shallow genealogies (Posada & Crandall, 2001), a nested clade analysis was performed. The MP analysis is not presented because it showed too little resolution. The nested clade design included the 25 HT across three nesting levels (Fig. 1). Most Central European and the American population were in clade 3-1 containing 9 HT, which were separated by one or two mutations. Only one missing or theoretical HT was detected. Clade 3-2 contained HT found in individual countries: France (2), Spain (2), Sweden (1), Russia (1) and China (5). One to 13 mutational steps were detected and the calculations indicated 13 missing or theoretical HT. The Russian HT XX was 4 steps apart from the Chinese HT and 6 steps apart from the European HT XVIII. The Spanish HT XVII was 7 steps apart from the Swedish HT X. Clade 3-3 had 5 HT from France, Spain, Russia, Poland, Finland and Switzerland. Here, 1 to 7 mutational steps and 5 missing or theoretical HT were detected. Four clades (1-1, 3-1, 3-2 and total cladogram) revealed significant associations between geographic and genetic variation (Table 2). No inference on population history could be made on clade 1-1 because values for clade distance (Dc) and nested clade distance (Dn) were not significant (Templeton, 1998). The values for clade 3-2 containing populations from Europe and Asia could not distinguish between fragmentation and isolation by distance. This is congruent with the hypothesis (see below) of fragmentation of the European population during the glacial period and the obvious geographical isolation of the Asian populations. As a result of the ambiguities in the cladogram there were no tip clades in the total cladogram. The nested clade analysis revealed that more sampling will be needed to fully resolve the population history of *T. piniperda*. It is likely that many more HT will be detected. This is also indicated by the 18 missing or theoretical HT detected in the nested clade analysis.

T. piniperda can be considered as a highly polymorphic species because other European scolytid species have fewer HT (Stauffer, 2003). This polymorphism may be due to the existence of several distinct refugial areas during the last ice age. A prolonged genetic isolation in the refugial areas could have led to the selection of different HT. The data presented here provide little information about the origins of most HT and their migration routes when the temperature increased after the last ice age. Our data suggest the area of Southern France, the Iberian Peninsula and the area south of St. Petersburg, Russia, served as possible refugial areas. The HT found there were not detected elsewhere. Moreover, they showed a high sequence divergence. Four main refugial areas were proposed for the main host, *P. sylvestris*, according to pollen findings: (1) Iberian Peninsula, (2) South France, (3) Balkan Mountains, (4) the area north of Moscow (Huntley & Birks, 1983). We did not incorporate data from the Balkan Mountains, but for other areas it is suggested there is a parallel evolution of *T. piniperda* and its host *P. sylvestris*.

The Pyrenees apparently formed a migratory barrier after the temperature increased. Assuming a constant substitution rate of 2% per MYA (DeSalle et al., 1987), the Spanish HT supposedly diverged about 0.3 MYA. Genetic studies based on chloroplast DNA of *Pinus sylvestris* revealed a potential parallel evolution of the main host of *T. piniperda*. In *P. sylvestris*, haplotypes detected on the Iberian Peninsula were not found in other parts of Europe (Soranzo et al., 2000). The Pyrenean Range forms a gap in the geographic distribution of *P. sylvestris* between Northern Spain and Southern France (Fig. 1 - inset). According to Willis et al. (1998), no other pine species in that gap could have served as "bridge" species for *T. piniperda*.

The Chinese populations revealed four closely related HT. The average sequence divergence between European

TABLE 1. Countries and abbreviations, locations, geographic coordinates and host trees from which *T. piniperda* and *T. minor* populations were collected. Accession numbers and collector's names are listed.

Country	Location	n	lat.	long.	Host tree	Haplotype (HT)	Accession No.	Collected by
T. piniperda								
Austria AUT	Mattersburg	4	47°45'N	16°24'E	*P. sylvestris*	V, VII, IX	AF367041 AF367043 AY239113	Gallego & Galliàn, 2001 B. Kohlmayr
	Kaindorf	1	47°22'N	15°90'E	*P. sylvestris*	VI	AY239114	authors
China CHN	Jinlin	6	43°53'N	126°35'E	*P. thunbergii*	XXIV, XXV	AY234048 AY239112	Chen Guofa
	Liaoning	4	41°00'N	123°00´´E	*P. tabliformis*	XXI, XXII, XXIII, XXIV	AY234046 AY234047 AY234048 AY239111	Chen Guofa
Croatia HRV	Gaj	4	45°29'N	17°02'E	*P. sylvestris*	I, II	AY234039 AY234041	B. Hrasovec
Czech R. CZE	St. Boleslav	5	50°12'N	14°44'E	*P. sylvestris*	I, II	AY234039 AY234040	M. Knizek
Finland FIN	Joensuu	3	62°36'N	29°46'E	*P. sylvestris*	I, XII	AY234039 AY234044	M. Robbo
France FRA	Bordeaux	3	44°50'N	0°34'W	*P. sylvestris*	I, III, XI	AY234039 AY234042 AY234043	C. Kerdlhué
	Mulhouse	4	47°44'N	7°21'E	*P. sylvestris*	I, VIII	AY234039 AY239115	C. Kerdlhué
	Quillant	3	42°80'N	2°15'E	*P. sylvestris*	XIII, XIV	AY234050 AY239109	C. Kerdlhué
	Pierronton	2			*P. pinaster*	VIII, XV	AY239110 AY239115	C. Kerdlhué
Italy ITA	P. Bocco	4	44°30'N	9°04'E	*P. nigra*	I, IV	AY234039 AY234040	M. Faccoli
Poland POL	Sekocin	5	52°06'N	20°52'E	*P. sylvestris*	I, II, XII	AY234039 AY234041 AY234044	J. Hilszczanski
Russia RUS	Rybachii	3	54°78'N	20°51'E	*P. sylvestris*	IV, XX	AY234040 AY234049	M. Mandelshtam
	Yashchera	5	59°94'N	30°29'E	*P. sylvestris*	I, XII, XIII, XVI	AY234039 AY234044 AY234045 AY234050	M. Mandelshtam
Spain ESP	Teruel	3	40°21'N	1°06'W	*P. pinaster*	XVII, XVIII, XIX	AF367047 AF367048 AF367049	Gallego & Galliàn, 2001
Sweden SWE	Uppsala	3	59°52'N	17°38'E	*P. sylvestris*	VII, X	AF367041 AF367046	Gallego & Galliàn, 2001
	Tormestorp	3	56°07'N	13°44'E	*P. sylvestris*	I	AY234039	Schlyter
	Lund	2	55°42'N	13°11'E	*P. sylvestris*	IV	AY234040	Schlyter
Switzerland CHE	Liesberg	4	47°38'N	7°42'E	*P. sylvestris*	I, XII	AY234044 AY234039	M. Kenis
USA	Snow Cap	3	42°41'N	84°30'W	*P. sylvestris*	I, IV	AY234039 AY234040	B. Haack
T. minor								
Austria	Mattersburg	2	47°45'N	16°24'E	*P. sylvestris*		U82583	B. Kohlmayr
China	Liaoning	2	41°00'N	123°00"E	*P. tabliformis*		AY237724	Chen Guofa

and Chinese populations was 1.24%. The closest relationship between the Chinese HT XXII and the Russian HT XX was 0.77%. Little information exists about the genetic divergence of Asian and European populations in the subfamily Hylesininae. In the subfamily Ipinae, *Ips cembrae* collected from Asia and Europe showed a 4%

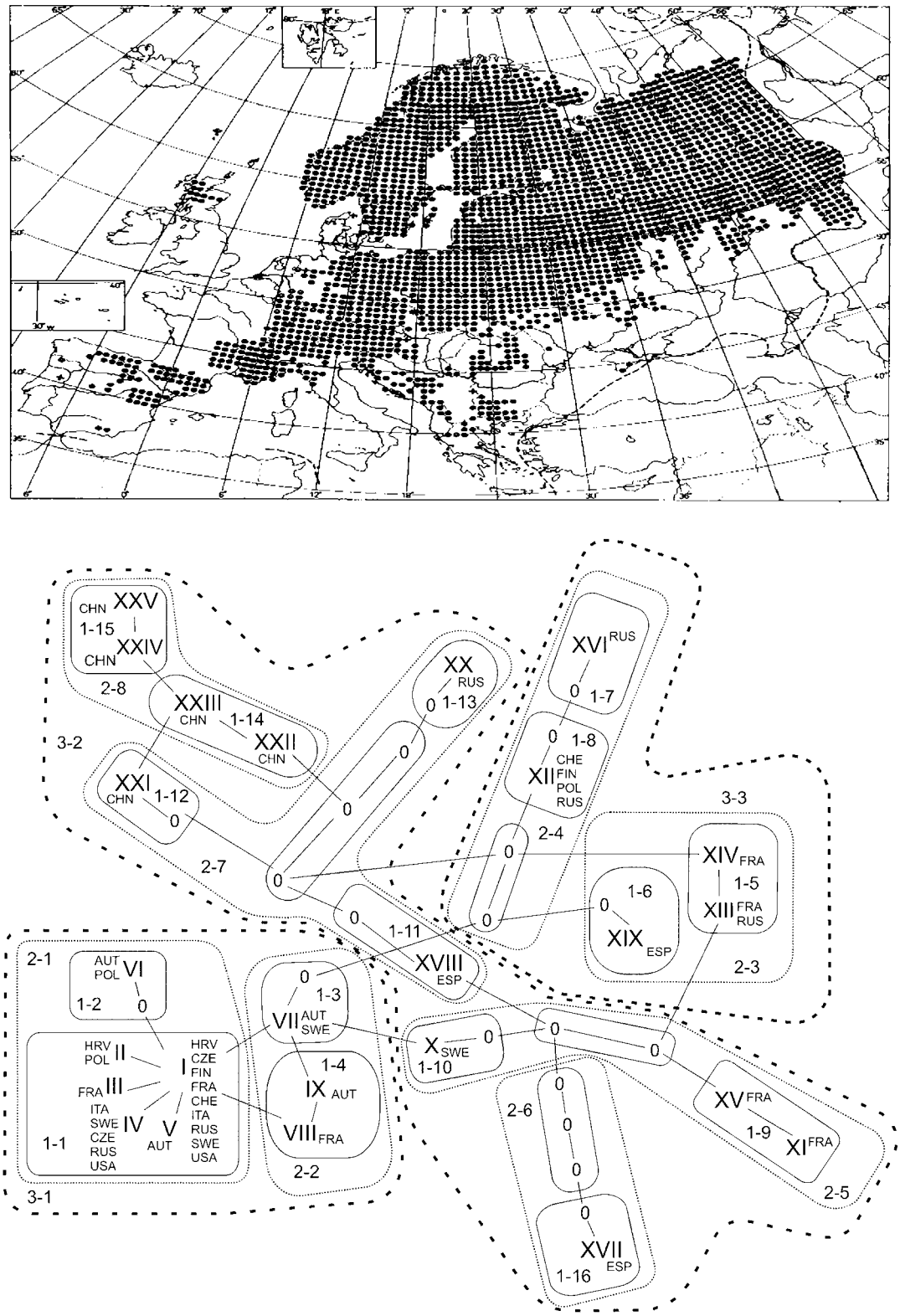

Fig. 1. Haplotype network based on mutational differences in the COI mtDNA sequences of pine shoot beetle *T. piniperda* populations. Haplotypes are presented as Roman numerals and each bar represents a mutational step, and "0" refers to missing or theoretical haplotypes. The origins of the haplotypes are listed in Table 1. Inset shows the current distribution of *Pinus sylvestris* in Europe (from Willis et al., 1998). For abbreviations of the countries see Table 1.

genetic divergence (Stauffer et al., 2001), whereas the genetic differentiation in *Ips typographus* from European and Asian populations was similar (< 1.5%) to the one found in *T. piniperda* (Stauffer, 2003). To obtain more information about the genetic structure of European and Asian *Tomicus* species, specimens of the closely related

TABLE 2. Result from the nested geographic analysis. *Dc* is the clade distance and *Dn* is the nested clade distance, *I-T* is the average difference between interior vs. tip clades for both distance measures (no *I-T* value was computed when no interior or tip clade was present) (see Templeton (1988) for details). The superscript *S* means that the measure was significantly small and *L* that the distance was significantly large (both at the 5% level). Interior clades are shaded.

Haplotypes			1-step clades			2-step clades			3-step clades		
Clade	*Dc*	*Dn*	Clade	*Dc*	*Dn*	Clade	*Dc*	*Dn*	Clade	*Dc*	*Dn*
I	1463	147									
II	372	1105									
III	0	725									
IV	1878	1656									
V	0	891									
I-T	221	72	1-1	1450	1409						
VI	—	—	1-2	171	860						
			I-T	1279	549	2-1	1352	1321			
VII	—	—	1-3	660	802						
VIII	255	354									
IX	0	768	1-4	458	764	2-2	785	844	3-1	1246^S	1224^S
XIII	986	886									
XIV	0	489	1-5	775	508						
XIX	—	—	1-6	0	507						
			I-T	776	0.87	2-3	508	1127			
XII	—	—	1-7	0	552						
XVI	—	—	1-8	861	851						
			I-T	861	299	2-4	800	1382			
						I-T	–292	–254	3-3	1216	1424
X	—	—	1-10	0	1421						
XI	0	75									
XV	0	50	1-9	60	604						
I-T	0	–25	*I-T*	–60	817	2-5	838	3252^S			
XVII	—	—	1-16	0	0	2-6	0	3823			
XVIII	—	—	1-11	0	2014						
XX	—	—	1-13	0	1081						
XXI	—	—	1-12	0	7552						
			I-T	0	3702	2-7	2411	3527			
XXII	—	—									
XXIII	—	—	1-14	0	249						
XXIV	193	174									
XXV	0	86									
I-T	192	87	1-15	139	198^S						
			I-T	–138	51	2-8	213^S	6109^L			
						I-T	1545^L	-2471^S	3-2	4298^L	3571^L

T. minor were sequenced (Table 1). A divergence of 6.4% between the European and the Chinese populations was detected. Thus, the evolutionary histories of *T. piniperda* and *T. minor* in Europe and Asia are different. According to the molecular clock of arthropod species (DeSalle et al., 1987), the Asian *T. minor* diverged from the European populations about 3 MYA whereas the Asian *T. piniperda* diverged from the European populations about 0.6 MYA. Although these two species occur sympatrically it seems that their history is diverse.

Two HT of *T. piniperda* were found in North America that were identical to HT found in Europe. This indicates that Europe was the source of the American populations of *T. piniperda*. The beetle was found for the first time on

the American continent in 1992 (Haack & Kucera, 1993). In 2000, *T. piniperda* was already present in 12 US states and in two provinces of Canada (Haack et al., 2000). Carter et al. (1996) suggested that the populations in the US were established separately in Illinois near Lake Michigan and in Ohio along Lake Erie, according to population analysis by RAPD. Knowing the exact origin(s) of North American *T. piniperda* populations could help in predicting important life history traits of this pest species, such as their hibernation behaviour and in using natural enemies as biocontrol agents (Metcalf & Luckmann, 1994). More individuals and finer tuned markers, like the recently developed microsatellites (Kerdelhué et al., 2003), are needed for a clearer picture.

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