Phylogeographic structure and past history of the circum-Mediterranean species *Tomicus destruens* Woll. (Coleoptera: Scolytinae)

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Abstract

Phylogeographic studies are often focused on temperate European species with relict footholds in the Mediterranean region. Past climatic oscillations usually induced range contractions and expansions from refugial areas located in southern Europe, and spatial distribution of genetic diversity show that northward expansions were usually pioneer-like. Actually, few studies have focused on circum-Mediterranean species, which probably were not influenced in the same way by climatic oscillations. We present the phylogeography of the bark beetle *Tomicus destruens*, which is restricted to the whole Mediterranean basin and the Atlantic coasts of North Africa and Portugal. We systematically sequenced 617 bp of the mitochondrial genes COI and COII for 42 populations (*N* = 219). Analysis revealed 53 haplotypes geographically structured in two clades, namely eastern and western clades, that diverged during the Pleistocene. A contact zone was identified along the Adriatic coast of Italy. Interestingly, we found contrasting levels of genetic structure within each clade. The eastern group was characterized by a significant phylogeographic pattern and low levels of gene flow, whereas the western group barely showed a spatial structure in haplotype distribution. Moreover, the main pine hosts were different between groups, with the Aleppo-brutia complex in the east and the maritime pine in the west. Potential roles of host species, climatic parameters and geographical barriers are discussed and the phylogeographic patterns are compared to classical models of postglacial recolonization in Europe.

Keywords: glacial refugia, Mediterranean basin, mitochondrial DNA, phylogeography, *Pinus*, *Tomicus destruens*

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Introduction

During the Quaternary period, climatic oscillations shaped the distribution of most species all over the world, leading to genetic consequences and particular phylogeographic patterns (Hewitt 2000). In Europe, temperate species responded to ice ages by local extinctions in northern regions and survival in warmer, southern ones. On the contrary, interglacial periods were characterized by retreat of the ice core and northward range expansions to newly suitable habitats, and by eventual extinctions in the southern rear edge due to extreme conditions (Hewitt 1996). Some of the southern refugia did not act as effective sources for the recolonization of northern Europe and rather appeared as relict populations (Petit *et al*. 2005). Two main, extreme models of expansion were previously described (Nichols & Hewitt 1994; Hewitt 1996; Ibrahim *et al*. 1996). The 'pioneer expansion' due to leptokurtic dispersal with occasional long-range movements of individuals often leads to reduced genetic diversity, and is classically hypothesized for north European populations exhibiting low levels of diversity. On the contrary, the 'phalanx-like expansion' is produced by normal or stepping-stone models and tends to preserve most of the allelic diversity. It is supposed to have occurred in the southern limits of species ranges, where populations could survive by limited movements between suitable locations.

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Taberlet *et al.* (1998) and Hewitt (1996, 1999, 2001) have reviewed documented cases of postglacial colonization routes in Europe. Even if no clear congruence between phylogeographies were found, common glacial refugia (i.e. southern Iberia, Italy, Balkans and Greece) and common hybrid or suture zones of secondary contacts between formerly isolated clades were found in most species. However, a great majority of the European taxa for which phylogeographic data are available are temperate species exhibiting patterns of 'southern richness and northern purity' (Hewitt 1999) typical for pioneer-like expansions. Few data are yet available concerning exclusive Mediterranean taxa that also experienced climatic oscillations. Even if little is known about the impact of the glaciations in the southern shore of the Mediterranean, there is clear evidence that southwestern Europe was subject to dramatic cooler and dryer episodes during full ice ages (Sànchez-Goñi *et al.* 2002; van Andel 2002). Hewitt (2001) suggested that 'unravelling the spatial genetic history of species in such regions is more complicated than for expansion further north'. The quaternary ice ages probably did not affect southern species in the same way, as they could benefit from more and larger refugial areas. Moreover, they are supposed to have experienced phalanx-like interglacial expansions that are more difficult to trace back using molecular markers.

Here we present the range-wide phylogeographic study of an oligophagous insect restricted to the Mediterranean basin, to infer how the periodic Quaternary ice ages influenced the population history of a strictly southern species, including North Africa, southwestern Europe and part of the Middle East. The pine shoot beetle *Tomicus destruens* (Woll.) (Coleoptera: Scolytinae) has long been confounded with its Palaearctic sibling species *Tomicus piniperda* (L.). It is found all around the Mediterranean, where it develops on local pine species (Kerdelhué *et al.* 2002; Kohlmayr *et al.* 2002; Gallego *et al.* 2004; Faccoli *et al.* 2005). Even if little is known about its specific biology, it probably has high dispersal capacity due to the occurrence of two dispersal phases per generation; its life cycle consists of a phase of trunk attack followed by oviposition and larval development in the inner bark, and a phase of shoot attack during warm season for maturation feeding (Chararas 1962). A study of its distribution in Spain (Gallego *et al.* 2004) revealed that it preferentially occurs in warm and dry places. Indeed its biological cycle suggests that it probably cannot survive cold winter conditions, as larval development occurs in fall and winter. As *T. destruens* strictly depends on pines for reproduction, it was necessarily restricted to the same or to some of the refugial area of its hosts, and the interglacial expansions of its populations were limited by the occurrence of suitable trees. Thus, we can hypothesize that the phylogeography of the bark beetle was not independent of those of its main hosts *Pinus*

halepensis and *Pinus pinaster* that both exhibit significant phylogeographic patterns. *P. halepensis* is present all around the Mediterranean basin and is divided in three main lineages (Korol *et al.* 2002), i.e. eastern Mediterranean (Israel, Jordan, Turkey), east European (Greece and Italy) and western Mediterranean (Morocco, Spain and France). On the other hand, *P. pinaster* is found only in the western Mediterranean region and shows three divergent groups (Burban & Petit 2003), i.e. a Moroccan, a western (Portugal, Spain and most France) and an eastern group (Tunisia, Italy, southeastern France). For both pine species, Tunisian populations were related to clades from across the sea, showing events of Mediterranean crossing.

Hence, the recent history of *T. destruens* must have been influenced both by the climate oscillations and by the occurrence and postglacial history of its hosts. Preliminary genetic data in Italy suggested an east–west differentiation of the species (Faccoli *et al.* 2005), but a range-wide sampling is necessary to confirm this hypothesis. We sampled *T. destruens* all around the Mediterranean basin from all occurring pine species, and systematically sequenced a part of the mitochondrial genes COI and COII to reconstruct its recent history. Our goals were to determine the phylogeographic patterns and to infer the respective roles of climate oscillations, habitat fragmentation and hosts pine history on the genetic structure of the associated insects.

Materials and methods

Beetle sampling

Beetles were collected throughout the Mediterranean basin and on the Atlantic coast of the Iberian Peninsula and France from 1999 to 2004. Twenty-one populations were collected in 10 countries and on four *Pinus* species to complement existing samples from France, Spain and Portugal (Kerdelhué *et al.* 2002; Vasconcelos *et al.* 2006) and obtain a representative sampling of the species across its distribution range. Samples were immediately stored in absolute ethanol. The sampling sites, pine host and date of capture are summarized in Table 1 and the localities are shown in Fig. 1.

DNA extraction and amplification

DNA was extracted for all samples from head, thorax and legs. The abdomen, elytra and antennae were kept as vouchers in ethanol to allow further morphological analyses. Genomic DNA was extracted and isolated either with the DNeasy Tissue Kit (QIAGEN) or the GenElute Mammalian Genomic DNA miniprep kit (Sigma).

The amplification of a part of the cytochrome oxydase I and II genes was performed using the Sigma Red *Taq*

Table 1 Sampling sites, date of capture, host trees, geographical coordinates and abbreviations of *Tomicus destruens'* populations. Collectors' names are listed

Code	Country	Location	Host species	Date	Collected by	Latitude	Longitude
SEN-AL	Algeria	Senalba	*P. halepensis*	09/2002	N. Brague	34°31′ N	2°44′ E
PU-CR	Croatia	Pula	*P. halepensis*	03/2004	B. Hrasovec	44°54′ N	13°51′ E
UB-ESP	Spain	Uriz-Begonte	*P. radiata*	12/2002	M. Lombardero	43°08′ N	7°39′ W
ERO-ESP	Spain	Roquez	*P. halepensis*	11/2000	Vasconcelos *et al.* (2006)	37°57′ N	02°26′ W
BIL-ESP	Spain	Bilbao	*P. radiata*	02/2001	Vasconcelos *et al.* (2006)	43°22′ N	03°01′ W
MAD-ESP	Spain	Madrid	*P. radiata*	01/2002	Vasconcelos *et al.* (2006)	40°25′ N	04°44′ W
ELA-ESP	Spain	Valence	*P. pinaster*	03/2002	Vasconcelos *et al.* (2006)	39°48′ N	01°16′ W
BAL-ESP	Spain	Baleares	*P. halepensis*	04/2002	Vasconcelos *et al.* (2006)	39°08′ N	02°55′ E
SEV-ESP	Spain	Seville	*P. pinea*	06/2002	Vasconcelos *et al.* (2006)	37°12′ N	06°23′ W
LA-FRA	France	Lubéron	*P. halepensis*	11/1999	Kerdelhué *et al.* (2002)	43°48′ N	5°07′ E
AP-FRA	France	Les Arcs	*P. pinea*	03/2000	Kerdelhué *et al.* (2002)	43°25′ N	6°24′ E
PR-FRA	France	Pietrosella	*P. radiata*	02/2000	Kerdelhué *et al.* (2002)	41°50′ N	8°50′ E
CM-FRA	France	Calvi	*P. pinaster*	02/2000	Kerdelhué *et al.* (2002)	42°34′ N	8°45′ E
TA-FRA	France	Toulon	*P. halepensis*	12/1999	Kerdelhué *et al.* (2002)	43°09′ N	5°55′ E
PAM-FRA	France	Pautilles	*P. pinaster*	04/2000	Vasconcelos *et al.* (2006)	42°30′ N	03°07′ E
COA-FRA	France	Collioures	*P. halepensis*	04/2000	Vasconcelos *et al.* (2006)	42°32′ N	03°06′ E
SA-FRA	France	St Chinian	*P. halepensis*	11/1999	Kerdelhué *et al.* (2002)	43°22′ N	3°02′ E
OLM-FRA	France	Oléron	*P. pinaster*	09/2002	J. Garcia	45°55′ N	1°18′ E
PIM-FRA	France	Pierroton	*P. pinaster*	12/2004	P. Ménassieu	44°44′ N	0°46′ W
COM-FRA	France	Forêt de la Coubre	*P. pinaster*	12/2004	D. Piou	45°46′ N	1°13′ W
POL-GR	Greece	Polygyros	*P. brutia*	11/2002	M. Kalapanidas	40°22′ N	23°26′ E
DOM-GR	Greece	Domokos	*P. brutia*	09/2002	M. Kalapanidas	39°07′ N	22°18′ E
AGI-GR	Greece	Agia-Anastasia	*P. brutia*	08/2002	M. Kalapanidas	40°28′ N	23°07′ E
JER-IS	Israel	Jérusalem	*P. halepensis*	03/2004	Z. Mendel	31°47′ N	35°07′ E
GIO-IT	Italy	Gioiosa — Taranto	*P. halepensis*	03/2002	M. Faccoli	40°32′ N	17°06′ E
ALB-IT	Italy	Alberese — Grossetto	*P. pinea*	03/2002	M. Faccoli	42°40′ N	11°06′ E
VVC-IT	Italy	Vallevechia — Venezia	*P. pinaster*	06/2002	M. Faccoli	45°54′ N	12°36′ E
EM-IT	Italy	Eraclea Mare — Venezia	*P. pinea*	03/2001	M. Faccoli	45°32′ N	12°43′ E
TAR-IT	Italy	Ginosa Marina — Taranto	*P. pinaster*	11/2003	E. Tarasco	40°34′ N	16°45′ E
ALE-LIB	Lebanon	Aley	*P. pinea*	06/2004	N. Nemer	33°48′ N	35°35′ E
BRO-LIB	Lebanon	Broumana	*P. pinea*	06/2004	N. Nemer	33°50′ N	35°34′ E
TAM-MA	Morocco	Tamrabta	*P. pinaster*	11/2002	D. Ghaioule	33°35′ N	05°00′ W
				03/2003	D. Ghaioule		
LAR-MA	Morocco	Larache-Leghdira	*P. pinaster*	11/2002	D. Ghaioule	35°08′ N	06°06′ W
				03/2003	D. Ghaioule		
MAM-MA	Morocco	La Mamora	*P. pinaster*	12/2002	D. Ghaioule	34°07′ N	06°34′ W
				04/2003	D. Ghaioule		
TORA-POR	Portugal	Ota	*P. halepensis*	09/2002	Vasconcelos *et al.* (2006)	39°06′ N	8°59′ W
TOT-POR	Portugal	Ota	*P. pinaster*	10/2002	Vasconcelos *et al.* (2006)	39°06′ N	8°59′ W
TAR-POR	Portugal	Aveiro	*P. pinaster*	09/2002	Vasconcelos *et al.* (2006)	40°36′ N	8°41′ W
TPLV-POR	Portugal	Ponte Lima	*P. pinaster*	02/2003	Vasconcelos *et al.* (2006)	41°48′ N	8°34′ W
TALR-POR	Portugal	Alcacer do Sal	*P. pinea*	09/2002	Vasconcelos *et al.* (2006)	38°19′ N	8°31′ W
SAK-TUN	Tunisia	Sakia	*P. halepensis*	10/2002	M. Ben Jamâa	36°11′ N	8°42′ E
				03/2004	M. Ben Jamâa		
TAB-TUN	Tunisia	Tabarka	*P. pinaster*	11/2003	M. Ben Jamâa	36°56′ N	8°43′ E
				03/2004	M. Ben Jamâa		
TEK-TUR	Turkey	Teknepinar	*P. brutia*	10/2004	M. Doganlar	36°09′ N	36°01′ E

package. We used a *Tomicus destruens*-specific primer pair described in Kerdelhué *et al.* (2002). The annealing temperature was 55 °C, and a total of 30 cycles of amplification were performed in 50-μL reaction volume. Polymerase chain reaction (PCR) products were purified using the GenElute PCR Clean-Up kit (Sigma) and directly sequenced. Sequencing was performed systematically on both strands with the PCR primers using the BigDye Terminator
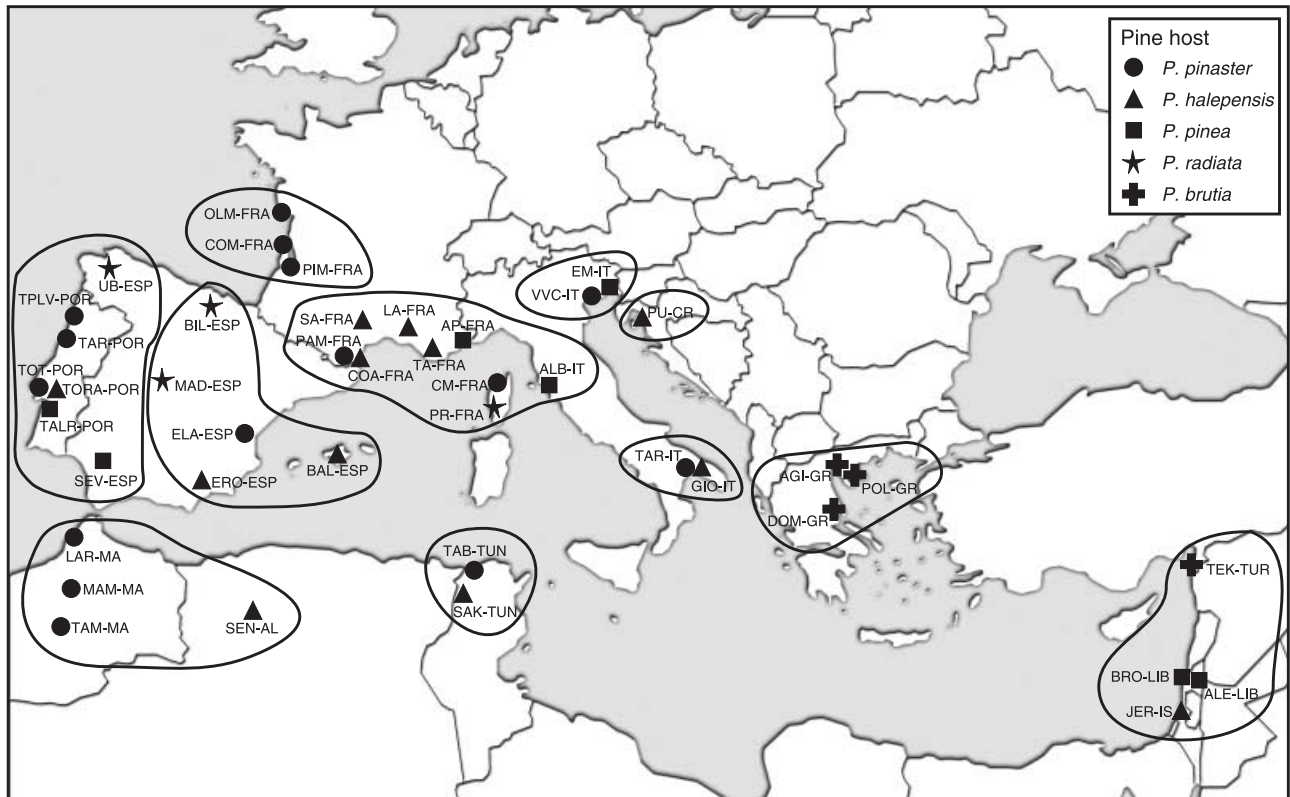
Fig. 1 Sampling sites of *Tomicus destruens* populations in the Mediterranean basin. Codes of the localities are given in Table 1. The ellipses show the 11 regional groups used for AMOVA.

sequencing kit (PE Applied Biosystems) and carried out with an ABI 3100 automatic sequencer.

Data analysis

All the obtained sequences as well as sequences from France and Iberian Peninsula (Kerdelhué *et al.* 2002; Vasconcelos *et al.* 2006; GenBank Accession nos AF457827, AF457831–AF457854, AF457858–AF457861, DQ182709–DQ182712, DQ182714, DQ182716–DQ182731, DQ182733, DQ182734) were aligned using CLUSTAL W version 1.4 (Thompson *et al.* 1994) as implemented in BIOEDIT version 7.0.5.

The best-fit model of sequence evolution was estimated using hierarchical likelihood-ratio tests with MODELTEST version 3.7 (Posada & Crandall 1998). The chosen model of sequence evolution was applied to calculate genetic distances between haplotypes with PAUP*4b10 (Swofford 2000). To test for constancy in rates of COI/COII evolution among lineages we constructed maximum-likelihood phylogenetic trees with and without a molecular clock enforced using PAUP, with the closely related species *Tomicus minor* as outgroup (accession number AF457866). We used a likelihood-ratio test (LRT; Felsenstein 1988) with a homogeneous rate of evolution as the null hypothesis. The LRT statistic 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). A statistical parsimony network was computed using TCS version 1.21 (Clement *et al.* 2000), which estimates gene genealogies from DNA sequences following the method described in Templeton *et al.* (1992). We used topological and frequency criteria (Crandall & Templeton 1993; see also Pfenninger & Posada 2002) to solve cladogram ambiguities. As the results showed a clear pattern of genetic divergence between two clades respectively distributed in the western and the eastern parts of the Mediterranean basin (see below), all the subsequent data analyses were performed first on the whole data set, and then within each clade (hereafter called 'western group' and 'eastern group'). Two Italian populations, namely EM-IT and TAR-IT, appeared to contain both western and eastern haplotypes. To avoid overestimation of the genetic diversity, these two populations were excluded from the within-clade analyses.

The genetic structure was examined by analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) as implemented in ARLEQUIN version 3.0 (Excoffier *et al.* 2005). AMOVA conducted on the whole data set was performed using three grouping options. In the first option ('by clade'), two groups corresponding to the western and eastern clades

were used. The second option ('by region') was to cluster the populations in 11 geographical groups shown on Fig. 1. In the third option ('by host'), the populations were grouped according to the pine species from which they were sampled (see Table 1). AMOVAS were then performed separately within clade, grouping populations either by host species or using the regional clusters shown on Fig. 1.

Allelic richness *R* was computed after rarefaction to four individuals (Petit *et al*. 1998) using CONTRIB, for all populations including at least four individuals. Gene diversity *H* and within-population mean number of pairwise differences π were calculated using ARLEQUIN. Correlations between the intrapopulation parameters (*H* and π) and the latitude were assessed by means of linear regressions using SYSTAT version 10 (SPSS Inc., 2000).

Occurrence of a significant phylogeographic structure was inferred by testing if G_{ST} (coefficient of genetic variation over all populations) and N_{ST} (equivalent coefficient taking into account the similarities between haplotypes) were significantly different by use of 1000 permutations (see Pons & Petit 1996) using PERMUT. Pairwise G_{ST} and N_{ST} were calculated using DISTON, and Nei's average number of differences between populations (corrected Nei's *D*, Nei 1975) was calculated using ARLEQUIN. The geographical distances were computed between all sampling locations using the geographical coordinates (see http://jan.ucc.nau.edu/~cvm/latlongdist.html). Matrices of genetic distances (pairwise G_{ST} and N_{ST}, and corrected Nei's *D*) were constructed separately for the western and the eastern clade of *T. destruens*. Genetic distances matrices were compared to the matrix of geographical distances by means of a simple Mantel test (Legendre & Legendre 1998) to detect isolation by distance. We used 999 random permutations to test for Mantel statistic significance. CONTRIB, PERMUT and DISTON are available at www.pierroton.inra.fr/genetics/labo/Software/.

To infer between historical and contemporary processes, a nested clade phylogeographic analysis (NCPA) was performed. Nesting design was constructed on the haplotype network, following the rules given in Templeton *et al*. (1987) and Templeton & Sing (1993). The NCPA was performed using GEODIS version 2.4 (Posada *et al*. 2000). Phylogeographic interpretation for each clade with significant geographical association were inferred using the latest revised inference key (Templeton 2004), updated at http://darwin.uvigo.es/software/geodis.html.

Results

Sequence alignment

Tomicus destruens sequences were added to the previously published sequences from France, Spain and Portugal (see Table 1) to obtain a final alignment of 219 individuals from 12 countries around the Mediterranean basin. Sequences were 617 bp long, including 351 bp in COI, 68 bp in tRNALeu and 198 bp in COII. A total of 53 haplotypes were identified (Table 2) with 48 polymorphic sites. Two major haplotypes AC and AA were shared by 59 and 50 individuals, respectively, all from the western part of the Mediterranean basin. Another main haplotype (ZZ) grouped 17 individuals from the eastern part of the Mediterranean basin. Thirty-five unique haplotypes were identified. The geographical distribution of the 53 haplotypes is shown in Fig. 2(a). Haplotype sequences are available in GenBank under accession nos DQ295748–DQ295777.

Haplotype parsimony network and genetic distances

All 53 mitochondrial haplotypes were joined in a single network with 95% probability. One ambiguous loop (connecting AC, AP and AA) could not be broken as the haplotype AP had a similar probability to be attached to haplotype AC or haplotype AA. Two main clades were observed, with a distance of five mutational steps (Fig. 2b). One clade contained only individuals caught from the western part of the Mediterranean basin (from Portugal to Italy as well as from North Africa), while the other group clustered individuals caught from the eastern part of the Mediterranean basin (from Italy to Israel). For convenience, the 41 western haplotypes were named AA, AB, AC, … , AZ and BA, BB, … , -BR, and the 12 eastern haplotypes were named ZZ, ZY, ZX, … , -ZO. Two Italian populations, namely EM-IT and TAR-IT, were composed of individuals bearing either eastern or western haplotypes (Fig. 2a, b).

The most appropriate model of sequence evolution was HKY + I + G model (Hasegawa–Kishino–Yano 85; Hasegawa *et al*. 1985) including invariable sites (I = 0.7411) and rate variation among sites (G = 1.6450) with unequal base frequencies (freqA = 0.3522; freqC = 0.1563; freqG = 0.1118; freqT = 0.3797). For the whole data set, genetic distances between haplotypes calculated with the HKY + I + G model ranged from 0.002 to 0.028. Within the western clade, the average distance value was 0.005 (range 0.002–0.011). The average distance value within the eastern clade was 0.006 (range 0.002–0.011). Between clade distances ranged from 0.008 to 0.028, with an average of 0.010. The likelihood-ratio test supported a molecular clock model for *T. destruens* (χ^2 = 46.48, d.f. = 52, *P* = 0.68). The maximum-likelihood tree of haplotypes is available as Supplementary material (Fig. S1). We therefore estimated divergence time between eastern and western clades using the general molecular clock estimate for arthropods mitochondrial DNA (2.3% per million years; Brower 1994), and obtained an average divergence time of 430 000 years (range 350 000–1 200 000 years). Despite the lack of precision of such estimates, we can confidently conclude that the divergence between the eastern and the western clade is of recent Pleistocene origin.

Table 2 Haplotypes found in each population and population parameters

Code	N	# HT.	Haplotypes	H	R	π
SEN-AL	5	4	AC(2), AS, BC, BJ	0.90	2.400	2.00
PU-CR	5	1	ZZ(5)	0.00	0.000	0.00
UB-ESP	5	2	AA(4), AC	0.40	0.800	0.40
ERO-ESP	5	5	AC, AD, AN, AQ, AR	1.00	3.000	1.80
BIL-ESP	5	3	AA, AC(2), AI(2)	0.80	1.800	1.00
MAD-ESP	5	3	AC(3), AO, AP	0.70	1.600	0.80
ELA-ESP	3	3	AA, AC, AL	—	—	—
BAL-ESP	5	3	AA(3), AJ, AK	0.70	1.600	1.80
SEV-ESP	5	3	AB, AC(3), AM	0.70	1.600	1.20
LA-FRA	5	2	AA, AC(3), AJ	0.70	1.600	0.80
AP-FRA	5	3	AA, AC(3), AW	0.70	1.600	0.80
PR-FRA	4	2	AC(3), AD	0.50	1.000	0.50
CM-FRA	4	3	AC(2), AD, AY	0.83	2.000	1.17
TA-FRA	5	3	AC(2), AS(2), AV	0.80	1.800	1.80
PAM-FRA	4	3	AA(2), AT, AU	0.83	2.000	2.67
COA-FRA	5	3	AA(3), AT, AZ	0.70	1.600	1.60
SA-FRA	5	2	AC(4), AS	0.40	0.800	0.40
OLM-FRA	1	1	AC	—	—	—
PIM-FRA	5	2	AA, AC(4)	0.40	0.800	0.40
COM-FRA	5	1	AC(5)	0.00	0.000	0.00
POL-GR	1	1	ZW	—	—	—
DOM-GR	5	1	ZZ(5)	0.00	0.000	0.00
AGI-GR	5	3	ZZ(3), ZY, ZX	0.70	1.600	0.80
JER-IS	4	2	ZU(2), ZS(2)	0.67	1.000	2.00
GIO-IT	5	3	AS(3), BA, BB	0.70	1.600	1.20
ALB-IT	5	4	AA, AC(2), AD, AQ	0.90	2.400	1.20
VVC-IT	5	1	AA(5)	0.00	0.000	0.00
EM-IT	5	2	AC(2), ZZ(3)	0.60	1.000	4.80
TAR-IT	5	3	AS(2), BA(2), ZZ	0.80	1.800	3.80
ALE-LIB	4	3	ZV(2), ZU, ZT	0.83	2.000	1.67
BRO-LIB	5	5	ZU, ZT, ZQ, ZP, ZO	1.00	3.000	2.80
LAR-MA	5	3	AA(3), AD, BO	0.53	1.200	0.80
	5	2	AA(4), BN			
MAM-MA	5	4	AD, AI, BK(2), BQ	0.91	2.500	2.29
	5	4	AA, AD(2), BC, BR			
TAM-MA	5	3	AC(3), BL, BM	0.76	1.843	1.16
	5	4	AC(2), AQ, BL, BP			
TORA-POR	5	2	AA(3), AB(2)	0.60	1.000	0.60
TOT-POR	5	4	AA(2), AB, AD, AH	0.90	2.400	1.80
TAR-POR	5	2	AA(4), AB	0.40	0.800	0.40
TPLV-POR	5	1	AA(5)	0.00	0.000	0.00
TALR-POR	5	3	AA, AC(3), AF	0.70	1.600	1.40
SAK-TUN	5	4	AA(2), AJ, BC, BH	0.96	2.733	2.18
	5	4	AC(2), BD, BE, BG			
TAB-TUN	5	2	AC(4), BI	0.69	1.603	1.17
	4	3	AA(2), AC, BF			
TEK-TUR	5	3	ZV(3), ZT, ZR	0.70	1.600	2.20

N, number of sequenced individuals; *H*, gene diversity; *R*, allelic richness after rarefaction to four; π, nucleotide diversity. Codes for populations are in Table 1. Numbers in bracket after haplotype name is the number of individuals with that haplotype. Haplotypes which first letter is A and B belong to the western clade, and haplotypes which first letter is Z belong to the eastern clade. *H*, *R*, and π were not computed for populations ELA-ESP, OLM-FRA and POL-GR because the number of individuals was too low.
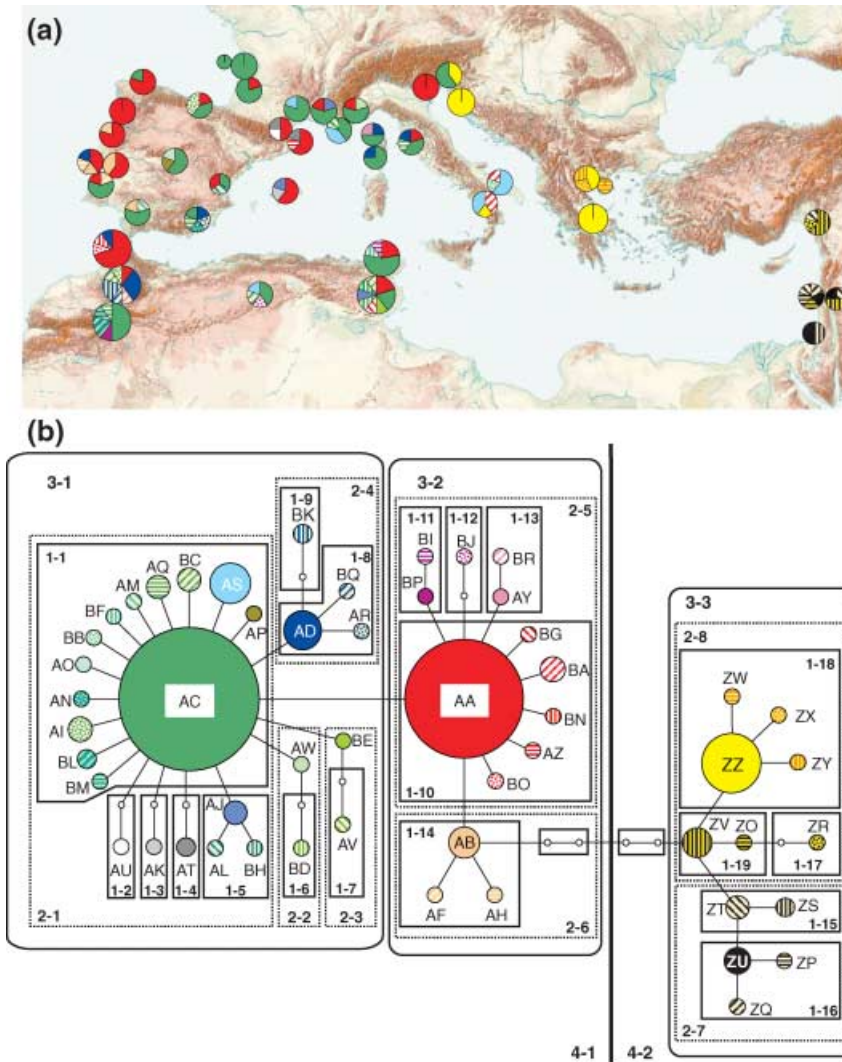
Fig. 2 Haplotype distribution and haplotype network of 219 *Tomicus destruens* cytochrome oxydase I and II sequences. a. Geographic distribution of the haplotypes among the 42 sampled populations. b. Haplotype network of the 53 haplotypes with corresponding colour codes and nested design for the NCPA. 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. Ambiguous haplotype AP is presented here connected to haplotype AC (option APAC, see text for details). Boxes represent the n-step clades. The thick line separates the 4-step clades (clades 4-1 and 4-2).

Population genetic parameters and phylogeographic structure

Total gene diversity H_T was 0.87, while the average within-population diversity H_S was 0.64. The indices of population structure G_{ST} and N_{ST} were 0.269 and 0.632, respectively. The permutation test showed that these two values were significantly different from each other. Within the western clade, G_{ST} and N_{ST} values were 0.202 and 0.215, respectively, and did not differ significantly. Within the eastern group, G_{ST} and N_{ST} were 0.353 and 0.457, respectively, and proved to be significantly different ($P < 0.05$).

For each population, gene diversity H, allelic richness R and mean number of pairwise differences π are given in Table 2. H and π were found to be negatively and significantly correlated with latitude in the whole data set (H: $R^2 = 0.25$, $P = 0.001$ and π: $R^2 = 0.24$, $P = 0.002$) as well as within the eastern clade (H: $R^2 = 0.59$, $P = 0.045$ and π:

$R^2 = 0.69$, $P = 0.021$) and within the western clade (H: $R^2 = 0.26$, $P = 0.004$ and π: $R^2 = 0.26$, $P = 0.004$).

For all three grouping options, AMOVA showed that all components of variance partitioning (among groups, among populations within groups and within populations) were significant (Table 3). When populations were grouped by clade or by region, most of the genetic variation was found among groups (77.65%, $P < 0.001$ and 57.87%, $P < 0.001$, respectively). On the contrary, when populations were grouped by host, most of the variation was found among populations within groups (36.46%, $P < 0.001$) and within populations (34.98%, $P < 0.001$), and a slightly lower amount of the variability was found between hosts (28.56%, $P < 0.001$).

In the western clade, a vast majority of the variance was found within population whatever the grouping option (81.47% when populations were grouped by host and 80.40% when populations were grouped by region) while the among-group component was always negligible. On

Table 3 Analysis of molecular variance (AMOVA) among populations of *Tomicus destruens*. Results are shown for the whole data set as well as for within-clade analyses. *$P < 0.05$ **$P < 0.01$ ***$P < 0.001$ NS: nonsignificant

		Whole data set		Western clade		Eastern clade	
	Source of variation	Variance components	Percentage of variation	Variance components	Percentage of variation	Variance components	Percentage of variation
Grouping by clade	Among groups	3.30903 Va	77.65***	—	—	—	—
	Among pops within groups	0.26852 Vb	6.30***	—	—	—	—
	Within populations	0.68385 Vc	16.05***	—	—	—	—
Grouping by region	Among groups	1.13187 Va	57.87***	0.04687 Va	7.87*	0.53852 Va	35.45*
	Among pops within groups	0.14025 Vb	7.17***	0.09682 Vb	11.73***	0.31360 Vb	20.64*
	Within populations	0.68385 Vc	34.96***	0.60013 Vc	80.40***	0.66689 Vc	43.90***
Grouping by host	Among groups	0.54968 Va	28.56***	−0.00455 Va	−0.62NS	0.08182 Va	5.96NS
	Among pops within groups	0.70160 Vb	36.46***	0.14108 Vb	19.15***	0.62326 Vb	45.43***
	Within populations	0.67326 Vc	34.98***	0.60013 Vc	81.47***	0.66689 Vc	48.61***

Table 4 Clades with significant geographical structure ($P < 0.005$) with their biological interpretation according to the inference key (Templeton 2004)

Clades	χ^2-statistic	P	Chain of inference	Inference
Clade 2-1	202.4936	0.0049	1, 2, 3, 4 — NO	Restricted gene flow with isolation by distance
Clade 2-5	148.5643	0.0044	No significant clade distance	—
Clade 3-3	25.3589	0.0004	1, 2, 3, 4 — NO	Restricted gene flow with isolation by distance
Clade 4-1	84.8395	0	1, 2, 3, 4 — NO	Restricted gene flow with isolation by distance
Total cladogram	205.0538	0	4-2 as interior: 1, 2, 3, 4, 9 — NO	Allopatric fragmentation
			4-1 as interior: 1, 2, 3, 5, 15 — NO	Past fragmentation

the contrary, in the eastern clade, a significant part of the genetic variation was found between regions (35.45%) even if a larger proportion of the variation was due to population effect (43.90%). On the other hand, no host effect could be detected in the eastern clade (only 5.96%, $P > 0.05$), the genetic variance being found almost exclusively within populations (48.61%) and among populations within hosts (45.43%).

The Mantel test showed a significant effect of isolation by distance in the western group as the matrix of geographical distances was significantly correlated to either G_{ST} (standardized Mantel statistics $r_M = 0.1505$, $P = 0.026$), N_{ST} ($r_M = 0.2119$, $P = 0.002$) or corrected Nei's D ($r_M = 0.2361$, $P = 0.001$). A significant effect of isolation by distance was also found in the eastern group as the matrix of geographical distances was significantly correlated to either G_{ST} ($r_M = 0.7714$, $P = 0.01$), N_{ST} ($r_M = 0.8292$, $P = 0.002$) or corrected Nei's D ($r_M = 0.6703$, $P = 0.003$).

Geographical nested clade analysis

The entire network was composed of two four-step clades. Two options were analysed due to the ambiguous loop AA-AP-AC (option 'APAC' when AP was attached to AC,

and option 'APAA' when AP was attached to AA). There was no difference in the nesting design and the inference of the population history was identical for these two options. The network was alternatively rooted at haplotype groups 4-1 or 4-2. The nesting design is shown in Fig. 2(b). Results of the NCPA are shown in Table 4. Significant association at the 0.05 level between haplotypes and geographical distribution was found at different clades levels. Within clade 2-1, restricted gene flow with isolation by distance was inferred. The same inference was found within the clade 4-1 (corresponding to the western clade of Mediterranean basin). Within clade 3-3 (the eastern part of the Mediterranean basin), restricted gene flow with isolation by distance was also found. The total network was inferred with both rooting options, and it showed a past fragmentation when rooting at 4-1 and an allopatric fragmentation when rooting at 4-2.

Discussion

We conducted a range-wide phylogeographic analysis of the circum-Mediterranean species *Tomicus destruens*. The number of haplotypes (53 for 219 individuals) was high when compared to other Ipinae species (Stauffer *et al.* 1999;

Cognato *et al.* 2003) but still lower than its sibling *Tomicus piniperda* (Ritzerow *et al.* 2004) or the red turpentine beetle *Dendroctonus valens* (131 mitochondrial haplotypes for 218 individuals, Cognato *et al.* 2005). This variability is in accordance with Kelley *et al.* (2000) who showed that specialists had a lower genetic diversity than generalists. Both indices of genetic differentiation over all populations (namely G_{ST} and N_{ST}) were quite low when compared to other Mediterranean species like the maritime pine bast scale *Matsucoccus feytaudi* (Burban *et al.* 1999). However, such differences could be due to the poor dispersal capacities of *M. feytaudi* compared to *T. destruens*. In the same way, the bark beetle populations appeared to be less differentiated than the main hosts *Pinus pinaster* and *Pinus halepensis*, which could also be related to the comparatively lower dispersal of seeds. On the contrary, total gene diversity was high (0.87), when compared to other beetle species such as the white pine weevil *Pissodes strobi* (0.258; Lewis *et al.* 2001), or to the main host *P. halepensis* (0.304; Gomez *et al.* 2001). High gene diversity and low differentiation is often found in widely distributed species as the result of gene flow among interconnected populations (Hamrick *et al.* 1992; Fady 2005).

Phylogeographic analysis demonstrated that the circum-Mediterranean species *T. destruens* was clearly separated in two groups, namely the western and eastern clades. This spatial pattern was supported by the haplotype network that showed that both groups had their own haplotypes and were separated by five mutational steps. This clear geographical structuring was also supported by the AMOVA results. Moreover, all distance values clearly fell into intraspecific distances typically found in the genus *Tomicus* (Kerdelhué *et al.* 2002; Kohlmayr *et al.* 2002; Duan *et al.* 2004), and show that the split between both groups did not date back to the Tertiary, but rather occurred during the Pleistocene (Hewitt 1996). Nested clade phylogeographic analysis indicated that the split between western and eastern group (i.e. clades 4-1 and 4-2) might be due to past or allopatric fragmentation. The occurrence of desert and subsequent absence of pines in Egypt and Libya (see distribution maps in Barbéro *et al.* 1998) could have acted as a natural barrier between the sister groups, maintaining this fragmentation in the southern range of the species.

On the other hand, analyses of molecular variance showed a significant and high effect of host species on the distribution of the genetic variance. The geographical distribution of the eastern and western clades in *T. destruens* partially match those of its main hosts, namely *P. pinaster* in the western side of the Mediterranean basin (Burban & Petit 2003) and the *P. halepensis/Pinus brutia* complex in the eastern range (Gomez *et al.* 2001). The apparent host effect we found in the distribution of genetic diversity of the beetle could, however, be due to a similar split in the distribution of its main hosts (due for instance to historical and environ-

mental constraints) rather than to a direct effect of host association and local adaptation on the evolutionary history of *T. destruens*.

A contact zone between the western and the eastern group was identified along the Adriatic coast of Italy, as two populations (respectively EM-IT near Venice on *Pinus pinea* and TAR-IT in southern Italy on *P. pinaster*) shared haplotypes from both clades. This was in accordance with Faccoli *et al.* (2005) who found a highly divergent haplotype in a northern Italian population of *T. destruens*, and suggested that Italy could be a contact zone between genetically divergent groups. Suture zones are expected when two postglacial colonization routes originating from two distinct refugia make secondary contact, i.e. when different genomes expanding from their refugia meet (Hewitt 1999). So far, four 'classical' hybrid zones have been described in European biotas usually found near natural barriers or where lineages expanding from distinct refugia merge (Pyrenees, Alps, Scandinavia, and between France and Germany; Taberlet *et al.* 1998), but the contact zone we found along the eastern Italian coast appears to be new. This result was probably due to the strict Mediterranean distribution of *T. destruens* that did not follow the classical routes of European postglacial history. It will now be necessary to characterize this area with a systematic sampling along the Adriatic coasts both in Italy and from Croatia to western Greece, to determine the width of the hybrid zone as well as the degree of interpenetration of both clades. As the divergence between the eastern and western clades is of recent origin, the most plausible hypothesis would be that there is no reproductive isolation between groups. Yet, the possibility still remains that local adaptations or genetic drift have led to assortative mating or selection against hybrids in mixed populations. Using both mitochondrial and nuclear markers would help to unravel the origin of individuals along this contact zone and to examine the existence of any kind of reproductive isolation between individuals of the western and the eastern clades.

A phylogeographically structured eastern group

Among the eastern clade, a significant phylogeographic structure was found as shown by the significant difference in the differentiation indices G_{ST} and N_{ST}. Moreover, the AMOVA showed that 35.45% of the genetic variation was found among the geographical groups while no host effect could be detected. The eastern clade was clearly structured in two geographical subgroups, one restricted to the easternmost part of the species' range (Israel–Lebanon–Turkey) and the other occurring in Croatia, Greece and Italy. Interestingly, these subclades strictly mirror the phylogeographic groups found for the main host *P. halepensis* (Korol *et al.* 2002) which would suggest a parallel evolution of the hosts and associated insects at the intraspecific level.

As no common haplotype was found between these subclades, we could hypothesize that maternal gene flow was strongly limited between these regions, as also suggested by the NCPA inference. The eastern regions of the Mediterranean basin are highly mountainous, and environments suitable for *T. destruens* are most probably fragmented and isolated in the landscape, which could explain the low levels of gene flow. As we failed to sample *T. destruens* in western Turkey, it is unclear if it is actually absent from this region and hence if the subclades are geographically disjunct, or if a contact zone may occur there between Greek and Turkish populations. Our results could be biased by the limited number of sampled populations, and thus the possibility remains that our data underestimated gene flow between clades and consequently overestimated the phylogeographic patterns found. The genetic parameters *H* and π were shown to decrease with latitude, showing a loss of diversity in northern populations within the eastern clade. This result suggested that a recent expansion occurred northwards, leading to founder effects (see below).

Within the first subclade, corresponding to the populations from Israel, Lebanon and Turkey, allelic richness and genetic diversity among populations were quite high. No evidence of rapid expansion and loss of genetic diversity was observed, which shows that population size did not fall under a threshold where alleles could have been lost. This part of the Mediterranean basin may not have been affected by Pleistocene climatic oscillations. Consequently the beetles inhabiting such regions certainly experienced either no cycles of populations contractions/expansions, or limited range reduction followed by slow movements to newly suitable habitats during interglacial periods in a phalanx-like expansion, as expected for southern species or subspecies (Hewitt 2001). This eastern-most subclade apparently did not engage in postglacial colonization further north and should rather be seen as an isolated relict zone (Petit *et al.* 2005). Interestingly, after a case review, Hewitt (1999) pointed out that refugial populations from the eastern part of the Mediterranean basin were often blocked in their regions and did not effectively contribute to the recolonization of northern biotas. The same was apparently true for the eastern populations of the Mediterranean *T. destruens*, probably because of the absence of suitable hosts in northern Turkey and the fragmentation of host distribution further west (Barbéro *et al.* 1998).

On the other hand, the Balkan subclade, that occurred in Greece, Croatia and Italy, had a reduced haplotype diversity and a different pattern of spatial distribution of genetic diversity. Populations from southern Greece, Croatia and Italy were fixed for the main haplotype ZZ, while genetic variability was found in other Greek populations although only five individuals were sequenced in each case. This peculiar pattern suggests the existence of a glacial active refugium in Greece, from which beetles colonized northwards to Croatia during re-warming periods via a classical pioneer-like expansion (Ibrahim *et al.* 1996). The beetles that reached southern Italy may have crossed the Adriatic Sea or progressively colonized the whole eastern Italian coast along a north–south route. Whether beetles bearing eastern haplotypes are present in intermediate populations between EM-IT and TAR-IT still needs to be tested. In the context of global warming, the hypothesis of an ongoing northward range expansion cannot be ruled out, but the Alps may well have acted as a natural impediment to the expansion of the Balkan genomes. The apparent lack of diversity in southern Greece could be due to local bottlenecks at the southern rear edge due to extreme climatic conditions during interglacials. However, this hypothesis should be tested by sampling both additional populations in the southern Balkans and more individuals per population.

The western group: a complex pattern of recolonization

The western clade was characterized by a significant pattern of isolation by distance, but unlike the eastern group, no clear phylogeographic structure was detected. The low value of N_{ST} showed that extensive gene flow occurred within this clade. Yet, the geographical distributions of haplotypes and of diversity indices allow the inference of the existence of past southern refugia and to describe the main northward colonization processes. The genetic diversity was highest in the southern rear edge of the beetle's distribution (North Africa and the extreme south of both the Iberian and Italian peninsulas). Moreover, all common haplotypes (i.e. those shared by at least three individuals) are found today in these three places. The results suggest that these particular populations were probably not significantly affected by glaciations and that they did not experience drastic population reductions leading to significant loss of alleles. A similar hypothesis was proposed by Fady (2005) to explain the retention of high gene diversity in Mediterranean trees. The distribution of several haplotypes show the existence of relict populations in southern Italy — with the occurrence of the private haplotype BA — and in the Iberian Peninsula where the related haplotypes AB, AF and AH were exclusively found. The absence of these haplotypes elsewhere shows that their lineages did not contribute to subsequent northward colonization during interglacial periods. Some other haplotypes were also preferentially found in Morocco, Spain and Portugal (lineage AD, BK, BQ, AR) or in Italy (AS) but eventually experienced long-range dispersal through the crossing of the Mediterranean Sea, as haplotype AD was also found in Corsica and Italy, and haplotype AS was found in Algeria and southern France. Both southern Italy and the Iberian Peninsula are classical refugia for temperate

European species (Hewitt 1996; Taberlet *et al.* 1998), and our data show that they also were active refugia or relict populations for the Mediterranean *T. destruens*.

Within the western clade, both indices of population diversity *H* and π were negatively correlated with latitude, indicating a loss of genetic variability in northern populations. The haplotype network was characterized by the existence of two main haplotypes AA and AC, and a star-shape pattern that is usually interpreted as the consequence of founder effect(s) followed by rapid population expansion (Slatkin & Hudson 1991; Avise 2000). This particular pattern is close to the phylogeographic results expected for species recolonizing northwards following the 'pioneer-like' expansion model (Ibrahim *et al.* 1996). Yet, a certain degree of genetic variability (with many single haplotypes) was retained during the expansion process. This variability can be explained either by (i) the existence of several long-range colonization episodes through the sea, bringing together a limited number of genomes from the refugial areas and preventing fixation in northern populations or by (ii) an increased substitution rate in the clade due to the rapid expansion of the populations as suggested by Petit *et al.* (2005). Only the northern-most populations (COM-FR and VVC-IT) were found to be monomorphic which could be explained by drastic founder effects at the leading edge of the species distribution. As *T. destruens* seems dependent on warm and dry climatic conditions (Gallego *et al.* 2004), one can hypothesize that its populations are still expanding northwards due to the present global warming and the occurrence of its main host *P. pinaster* outside of *T. destruens*'s distribution range (Burban & Petit 2003).

The absence of clear phylogeographic pattern within the western group and the evidence of repeated long-range movements of individuals contrast with the results found within the eastern clade. Even if the natural barriers of the Pyrenees, the Alps and the Mediterranean Sea limited the expansion further north of many southern haplotypes, they did not strictly prevent gene flow between regions. Interestingly, host fragmentation and mountain ranges apparently prevented any long-range dispersal in the eastern part of the Mediterranean basin. Occurrence of multiple host species in the west and thus of a more continuous distribution of suitable hosts could partially explain the extensive gene flows. We did not detect any host effect on the distribution of genetic variability, which shows that host species probably does not limit dispersal in the western *T. destruens*. Moreover, the Gibraltar Strait and Mediterranean islands could have allowed stepping-stone dispersal from one side of the sea to the other. For example, individuals from Morocco could have reached the Iberian Peninsula via Gibraltar Strait as shown for other beetles (Palmer & Cambefort 2000) or the shrew *Crocidura russula* (Cosson *et al.* 2005), and beetles from Tunisia could

have migrated to Italy as already suggested for the host trees *P. pinaster* (Baradat & Marpeau-Bezard 1988; Vendramin *et al.* 1998) and *P. halepensis* (Korol *et al.* 2002). Yet, as *T. destruens* probably cannot reproduce in cold places (Gallego *et al.* 2004), the physical barriers of the Alps and the Pyrenees were expected to be more effective in preventing dispersal. We cannot rule out the hypothesis that different selection pressures between the western and the eastern clade could have resulted in higher dispersal abilities of the beetles in the former region. The success of bark beetle development is largely linked to its host's inherent resistance capacity, and *Tomicus* are often found on fallen or recently dead trees rather than on vigorous living hosts. Different host species are found between the western and the eastern clade, with *P. halepensis* and *P. brutia* in the eastern region and *P. pinaster* mostly present in the western zone (see distribution maps in Barbéro *et al.* 1998). Higher individual tree resistance and/or general health in the western region could have acted as a force selecting higher dispersal abilities of the beetles to enhance successful host localization. Finally, recent long-range movements of insects could also be due to human activities, as individuals can be transported with rough timber, or within the shoots of transplanted young trees.

Supplementary material

The supplementary material is available from
http://www.blackwellpublishing.com/products/journals/
suppmat/MEC/MEC2872/MEC2872sm.htm

Fig. S1 Maximum-likelihood phylogenetic tree of *Tomicus destruens* haplotypes generated under the model of sequence evolution HKY + I + G. No branch length information is provided as the resolution was too small. Bootstrap values = 50% for the major nodes are shown above branches. The tree was rooted using *Tomicus minor*.

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