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ORIGINAL ARTICLE

Incongruent evolutionary histories of two parasitoids in the Mediterranean Basin: influence of host specialization and ecological characteristics

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ABSTRACT

Aim The pine processionary moth (PPM) is a species complex containing two congeneric taxa, namely *Thaumetopoea pityocampa* and *Thaumetopoea wilkinsoni*, with a circum-Mediterranean distribution and a strong geographical structure. We developed comparative phylogeographies for two of the main natural enemies of the PPM, the egg parasitoids *Baryscapus servadeii* and *Ooencyrtus pityocampae*, to determine to what extent their Quaternary histories were parallel and mirrored that of their host.

Location Pine and cedar forests around the Mediterranean Basin.

Methods Egg masses of the PPM were sampled from its whole range and parasitoids allowed to emerge in the laboratory. We sequenced one mitochondrial fragment from 303 individuals of *B. servadeii* and 239 of *O. pityocampae*, and the nuclear ITS2 for a subset of the samples. The distribution of genetic diversity, network of alleles and AMOVAs were analysed to decipher the phylogeographical structures, and PPM and host plant effects.

Results Results differed drastically between the two parasitoid species. We found 87 haplotypes and five ITS alleles for *B. servadeii*, which showed a strong phylogeographical structure over its distribution range. We identified four divergent clades, one of them further forming four haplogroups. Refugial areas were similar to those of the host. AMOVA showed that over 40% of the variance could be explained by the insect host structure. In contrast, *O. pityocampae* showed 16 closely related haplotypes, one corresponding to 60% of the individuals. PPM structure explained only 15% of the variance. The effects of the pine host were limited in both parasitoid species.

Main conclusions *Baryscapus servadeii* probably survived Quaternary climatic oscillations in long-term refugia where the PPM host was also continuously present. Although not strictly parallel, its history showed high degrees of similarity with that of the host. Conversely, results suggest that *O. pityocampae* had different climatic requirements and experienced severe bottleneck(s) during the Quaternary. Yet, it efficiently recolonized its main host range, probably helped by its parthenogenetic reproduction and possibly also by local host shifts.

Keywords

Dispersal, egg parasitoids, genetic structure, glacial refugia, Mediterranean Basin, mitochondrial marker, phylogeographical patterns, pine processionary moth, Quaternary history, *Thaumetopoea*.

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INTRODUCTION

During the Quaternary, long-term climatic cycling has occurred over the last 700 kyr. These cycles have consisted of a series of 100 kyr glaciations followed by 10–15 kyr interglacial periods. They affected the distribution and genetic diversity of animal and plant species and greatly influenced their present-day characteristics (Avise, 2000; Hewitt, 2004). In European temperate taxa, glacial maxima caused local extinctions in northern regions, while populations survived in several southern refugia usually located in the European peninsulas (Hewitt, 1996). In contrast, when the ice retreated during the warmer interglacial periods, northern territories became suitable and could be recolonized through geographical expansion from the glacial refugia (Taberlet *et al.*, 1998; Schmitt, 2007). In recent decades, phylogeographical studies have improved our knowledge of refugial areas and the patterns of recolonization routes, demonstrating individualistic responses and the importance of species' ecological characteristics in relation to their response to past climate changes (Stewart *et al.*, 2010). The roles of biotic interactions and species interdependence have received much less attention (Espíndola & Alvarez, 2011).

Comparative phylogeographical methods can be used to test for concordant geographical distributions of lineages in co-distributed species (Hickerson *et al.*, 2010). The approach allows common historical patterns of population differentiation and the influence of species-specific characteristics to be identified (Bertheau *et al.*, 2013). When studying interacting species (plant and pollinators, phytophagous or parasite species associated with the same host), comparative phylogeography allows for the testing of the congruence of phylogeographical patterns across trophic levels or between species belonging to the same community. Congruence can be expected when a dependent species is strongly constrained in its migration pathways by its partner and must follow its colonization routes (Nicholls *et al.*, 2010; Borer *et al.*, 2012), while incongruent histories can occur if the species have different dispersal propensities or if the dependent species can switch hosts (Espíndola & Alvarez, 2011; Kohnen *et al.*, 2012). The level of similarity in phylogeographical patterns is thus expected to be lower in generalist species, which can survive on alternative hosts, than in specialists.

In the present study we analysed the Quaternary history of a multi-trophic system that involves two egg parasitoids (Hymenoptera: Chalcidoidea) associated with the pine processionary moth species complex (PPM; Lepidoptera: Notodontidae) and its pine host species around the Mediterranean Basin. The PPM is known to exhibit a very strong biogeographical genetic structure, with differentiated lineages occurring in allo- or parapatry (Kerdelhué *et al.*, 2009; Rousselet *et al.*, 2010). This circum-Mediterranean species complex is rapidly expanding towards higher elevations and latitudes in Europe as a consequence of global warming (Battisti *et al.*, 2005; Robinet *et al.*, 2014). The PPM species complex is currently known to be composed of two congeneric taxa, namely *Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775) and *T. wilkinsoni* Tams, 1925 (Salvato *et al.*, 2002; Simonato *et al.*, 2007). Nevertheless, Kerdelhué *et al.* (2009) showed that the species complex is composed of three (rather than two) parapatric mitochondrial clades without geographical overlap (Fig. 1). The *pityocampa* clade occurs throughout Europe as well as in Morocco and Algeria. The second clade of the complex, named the eastern North African (ENA) clade, is distributed in northern Algeria, Tunisia and Libya. The third clade corresponds to *T. wilkinsoni* and is present in the eastern part of the Mediterranean Basin. The differentiation of these three clades corresponds to major biogeographical events such as the opening of the Aegean trench and the rise of the Tellian Atlas combined with late Tertiary climate change (Kerdelhué *et al.*, 2009). Within-clade spatial structure was further identified (Fig. 1). Genetic diversity was retained in the PPM in all southern populations (Anatolia, North Africa and Iberian Peninsula).

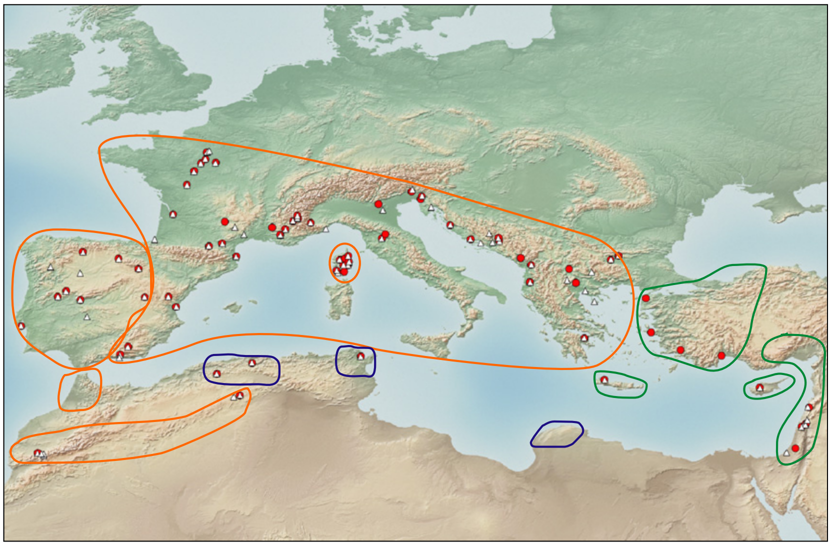

Figure 1 Map showing the sites in which *Baryscapus servadeii* (white triangles) and *Ooencyrtus pityocampae* (red circles) were sampled and successfully sequenced. The curves correspond to the subclades of the pine processionary moth *Thaumetopoea pityocampa*/*T. wilkinsoni* complex that were used in the analyses of molecular variance (orange: subclades of the *pityocampa* clade; dark blue: ENA clade; green: *wilkinsoni* clade).

European glacial refugia were identified and mostly corresponded to southern regions with moderate elevations (Kerdelhué *et al.*, 2009; Rousselet *et al.*, 2010).

We studied the phylogeographical patterns of two parasitoids, namely *Baryscapus servadeii* (Domenichini, 1965), which is specifically associated with the PPM, and *Ooencyrtus pityocampae* (Mercet, 1921), which can also parasitize other species of Lepidoptera and Hemiptera (Battisti *et al.*, 1988; Binazzi *et al.*, 2013). They oviposit in PPM egg masses and their larvae develop at the expense of the insect host's embryos. They occur across the Mediterranean Basin (i.e. wherever their main host is present), reproduce through thelytokous parthenogenesis (i.e. without male production) (Battisti *et al.*, 1990) and have two generations per year. As *B. servadeii* is supposed to depend strictly on its host for reproduction, we expect it to share common phylogeographical patterns with the PPM. Conversely, the generalist *O. pityocampae* could switch to other hosts in regions where the PPM became rare or went extinct in the past, and its phylogeographical structure could thus differ because different hosts could represent a continuity of bridges among refugial populations (Porretta *et al.*, 2013). Additionally, these species could be directly or indirectly dependent on the distribution and history of host pines. Arnaldo & Torres (2006) showed that the frequency of *B. servadeii* and *O. pityocampae* changed according to PPM host tree species in north-eastern Portugal, while Simonato *et al.* (2012) reported an absence of correlation between population genetic variability and host plant species in northern Italy.

Using mitochondrial and nuclear markers, we analysed the phylogeographical patterns of these specialist and generalist parasitoid species over their whole range in order to assess their level of congruence with the known evolutionary history of their shared PPM host. We also took into account the potential structuring impact of the pine tree species. Our aims were (1) to describe the phylogeographical structure of each parasitoid species, as the intraspecific diversity of these natural enemies was so far unknown, and (2) to identify similarities and discrepancies between the Quaternary histories of both parasitoid species and their PPM host. We specifically asked whether the history of the specialist species would mirror that of its host (localization of refugial areas, colonization routes, main phylogenetic differentiations), and tested the hypothesis that plasticity in host choice has led to a divergent phylogeographical history for the generalist parasitoid.

MATERIALS AND METHODS

Sampling

Egg masses of the PPM *T. pityocampa/T. wilkinsoni* complex were collected from 99 different locations in 19 countries around the Mediterranean Basin on various pine species (*Pinus nigra, P. sylvestris, P. halepensis, P. pinea, P. pinaster, P. brutia, P. canariensis* and *P. radiata*) or on *Cedrus atlantica* (in Algeria). Approximately 30 egg masses were sampled per site, stored at room temperature and monitored on a daily basis until emergence and morphological identification of adult parasitoids. We obtained *B. servadeii* from 83 localities in 17 countries and *O. pityocampae* from 71 localities in 19 countries. Moreover, two individuals of *B. servadeii* and four of *O. pityocampae* were obtained from eggs of the cedar processionary moth, *Thaumetopoea bonjeani*, collected in two cedar forests in Algeria, and four individuals of *O. pityocampae* were sampled from eggs of the caper bug *Stenozygum coloratum* found on the host plant *Capparis spinosa* in Biriya, Israel (Fig. 1, and see Appendix S1 in Supporting Information). All samples were transferred to ethanol 96% and stored at −20°C until DNA extraction.

DNA extraction, amplification and sequencing

Genomic DNA was extracted using the DNeasy Tissue kit (Qiagen, Hilden, Germany). For both parasitoid species, a fragment of the mitochondrial gene cytochrome *c* oxidase subunit I (*COI*) was amplified and sequenced for 1 to 10 individuals from each sampling locality. Each individual was chosen from a different egg mass to avoid the risk of sampling siblings. The PCR primers are given in Table 1. We used the pairs Clyde3 – Bas-R1271 for *B. servadeii* and Clyde2 – Opi-R1275 for *O. pityocampae*. When amplification failed, we amplified two overlapping fragments using

Table 1 PCR and sequence primers used in this study. Op: *Ooencyrtus pityocampae*; Bs: *Baryscapus servadeii*. The primers shown in italics were specifically designed for this study. Jerry was from Simon *et al.* (1994) and the ITS2 primers from Ji *et al.* (2003).

Primer name	Forward (F) or Reverse (R)	Primer sequence	Marker	Species
Clyde2	*F*	*5′-CGAATAAATAATATAAGTTTTTG-3′*	*COI*	Op
Clyde3	*F*	*5′-CTCGGATAAATAATATAAGTTTTTGGCT-3′*	*COI*	Bs
BasR1271	*R*	*5′-AAATAATATTTCAACATGTATATGAATCTGG-3′*	*COI*	Bs
Opi-R1275	*R*	*5′-CAACATAAATAAGAATCTGGA-3′*	*COI*	Op
Jerry	F	5′-CAACATTTATTTTGATTTTTTGG-3′	*COI*	Bs and Op
Lou	*R*	*5′-AGTATCAACATCTATGCCCACTGTAA-3′*	*COI*	Bs
Tess	*R*	*5′-AACTTCTTTTTTTATCCTTTCATTTC-3′*	*COI*	Op
CAS5p8sFc	F	5′-ATGAACATCGACATTTCGAACGCACAT-3′	ITS2	Bs and Op
CAS28sB1d	R	5′-TTCTTTTCCTCCGCTTAGTAATATGCTTAA-3′	ITS2	Bs and Op

Clyde3 – Lou and Jerry – Bas-R1271 for *B. servadeii* and Clyde2 – Tess and Jerry – Opi-R1275 for *O. pityocampae*. We also amplified a fragment of the nuclear ribosomal internal transcribed spacer 2 (ITS2) for a subset of the samples, including all sampled countries and the mitochondrial clades identified for *B. servadeii* (see Results and Appendix S1).

PCR products were purified using Nucleospin gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany). Both DNA strands were sequenced on an ABI Prism 3500 automatic sequencer using the Big-Dye Terminator sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the primers used for PCR amplification. All sequences were checked and aligned using Codon Code Aligner 4.2.4 (CodonCode Corporation, Dedham, MA, USA).

ITS2 data analyses

In *O. pityocampae*, the ITS2 fragment contained a microsatellite locus $(AC)_n$ that led to ambiguous sequences and difficulties in determining the exact number of tandem repeats. We thus used only the sequence data flanking the microsatellite locus in this species. The complete ITS2 sequence could be analysed in *B. servadeii*. A network of alleles was built for both species using tcs 1.21 (Clement *et al.*, 2000), and their geographical distribution was determined. Because of the limited number of alleles found in both genera, no further statistical analyses were developed.

COI data analyses

Diversity and phylogenetic analyses

All sequences were translated into amino acids using mega 6 to check that no stop codons occurred (Tamura *et al.*, 2013). To quantify the divergence between haplotypes, a pairwise genetic distance matrix was calculated using the Kimura 2-parameter distance (K2P). Mean, minimum and maximum genetic distances within and between the clades identified in *B. servadeii* were also calculated using mega. Indices of genetic diversity in the studied species (gene diversity, mean number of pairwise differences π and nucleotide diversity per site) were estimated using Arlequin 3.11 (Excoffier *et al.*, 2005). Because a network approach is well adapted to infer intraspecific genealogical relationships (Posada & Crandall, 2001), a haplotype network based on statistical parsimony was built for each species using tcs allowing 20 connection steps. To infer the demographic histories of each species and detect signs of recent population expansions, we used the frequency-based indicators Tajima's D (Tajima, 1989), Fu's F_S (Fu, 1997) and R_2 (Ramos-Onsins & Rozas, 2002) statistics available in DnaSP 5.10 (Librado & Rozas, 2009). The significance of each index was evaluated by performing 10,000 coalescent simulations. Significantly negative Tajima's D and Fu's F_S values and significantly positive R_2 values were taken as evidence of a population expansion.

Hierarchical analyses of molecular variance

To infer the possible impact of the PPM phylogeographical patterns on the parasitoid genetic structures, we used analyses of molecular variance (AMOVAs; Excoffier *et al.*, 1992). These analyses evaluate the variation in genetic diversity at different hierarchical levels by testing the proportions of molecular variance explainable by *a priori* defined grouping factors. AMOVAs were performed with Arlequin using K2P genetic distances between haplotypes and excluding the few individuals sampled from *T. bonjeani* or from *S. coloratum*. We tested several groupings corresponding to the main genetic structures identified in the PPM host (Kerdelhué *et al.*, 2009). First, we considered the regions associated with the three main mitochondrial clades (*pityocampa*, eastern North African and *wilkinsoni* clades). Second, we used the eight subclades of the PPM [(western Iberian Peninsula), (south Algeria and south Morocco), (Corsica), (rest of Europe), (Tunisia), (northern and western Turkey), (eastern Turkey, Lebanon and Israel), (Crete)]. Finally, the subclade (rest of Europe) was split into the three mitochondrial groups defined by Rousselet *et al.* (2010) [(from western Massif Central to Italy, the Balkans and eastern regions), (from eastern Iberia to western France) and (both sides of the Pyrenean range)].

AMOVAs were also used to explore the possible effect of host tree species on parasitoid structures. The samples were first grouped by pine host species. Next we grouped the hosts into three ecological groups, namely the mountain pines (*P. nigra* and *P. sylvestris*), the Mediterranean pines (*P. halepensis, P. brutia, P. pinea* and *P. pinaster*) and introduced species (*P. canariensis* and *P. radiata*).

RESULTS

***Baryscapus servadeii*, the specialist species**

Genetic diversity, haplotype distribution and network for both markers

We sequenced the *COI* marker for 303 individuals and the ITS2 fragment for a subset of 159 samples in this species. The alignment of ITS2 sequences was 484 bp long, including gaps, and contained five alleles. All-B3 (10 individuals) had a central position in the network, with All-B1 (77 individuals), All-B2 (60) and All-B4 (5) linked to it by a single mutational step. All-B5 (7 individuals) was separated by 12 mutation steps from All-B4. All-B1 was found in the northern rim of the Mediterranean Basin (from Portugal to the Balkans); All-B2 was distributed around the Mediterranean, mostly in sites close to the sea [from the Balkans to Israel, Cyprus, Italy, France (including Corsica), Spain and North Africa]; All-B3 was found in northern Algeria (both in individuals sampled from *T. pityocampa* and from *T. bonjeani*) and in Corsica. All-B4 was restricted to Crete and All-B5 was found only in the Balkans (Albania, Bulgaria and Macedonia).

Concerning the mitochondrial marker, we obtained 898 bp long sequences. We found 87 haplotypes and observed high levels of haplotype and nucleotide diversity. Gene diversity reached 0.96 and the mean number of pairwise differences (π) was 14.18 (i.e. 0.016 per nucleotide). Sixty-three haplotypes were endemic from single sampling sites, and most of the others were shared only between neighbouring localities. Nevertheless, two haplotypes had large geographical distributions (H40 from Spain to Israel and H50 from Croatia to Israel), while two others were present over large regions (H1 in western France and northern Spain and H42 in Italy and most of the Balkans; see Appendix S1).

The haplotype network showed four divergent clades that did not branch together (Fig. 2a); the haplotypic genetic distances, which estimate the proportion of substitutions between haplotypes, ranged from 3.1% to 6.2% between clades and from 0.1% to 1.6% within clades, except for Clade IV where it reached 2.4% (Table 2). The geographical distributions of the four clades and of the haplogroups identified within Clade I (see below) are shown in Fig. 2b. Clade I (CI) was the most diverse, with 67 haplotypes found in most localities in France, Italy and in all sites from the Iberian Peninsula, the Balkans, the Near East and Tunisia. Clade II (CII) included 14 haplotypes (nine in North Africa and five in south-eastern France, Italy and Corsica); the two individuals sampled from *T. bonjeani* belonged to that clade. Clade III was restricted to individuals from Crete (and having the ITS allele All-B4) and contained two closely related haplotypes. Clade IV contained four divergent haplotypes that corresponded to seven individuals sampled in the Balkans, all having the ITS2 divergent allele All-B5.

CI was further structured into three shallowly divergent haplogroups, HG1 to HG3, and a more divergent haplogroup, HG4. HG1 contained seven haplotypes, occurring mostly in western and southern France. H1 was shared by 48 individuals (47 from France and 1 from northern Spain). This haplotype was the only one found at the northern edge of the PPM's distribution range in France. HG2 corresponded to 16 haplotypes found in the Southern Alps, northern Italy and the western Balkans. HG3 grouped 16 haplotypes. Twelve were found in the Balkans, one was restricted to Cyprus, two were found only in Israel, and one (H50) was present both in the Near East and the Balkans. Finally, the most divergent haplogroup, HG4, gathered 24 haplotypes. Nineteen were found only in the Iberian Peninsula, two in the French Pyrenees and two in the Southern Alps. The last haplotype (H40) was remarkable, as it presented the largest distribution around the Mediterranean Basin.

The haplotype network obtained for *B. servadeii* thus showed a geographical structure. In some cases, however, divergent haplotypes belonging to different clades co-occurred in the same region. This was observed in Corsica, the Southern Alps and Tunisia (co-occurrence of CII and CI), and in the Balkans (CI and CIV). Moreover, haplotypes from different haplogroups also co-occurred in the Southern Alps (HG2 and HG4), in the Balkans (HG2, HG3 and HG4) and in the Near East (HG3 and HG4). Haplotype diversity was retained in most of the sampling sites, in particular in the Iberian Peninsula, in the Southern Alps and in the Balkans. Endemism was high, as most haplotypes were not shared between sites.

Demographic history

To detect recent population expansions, Tajima's D, Fu's F_S and R_2 were estimated for the species as a whole and ignoring Clade IV, which probably corresponded to a distinct species (see below), as well as for Clade I and Clade II separately. When studying only Clade II, none of the three indices was significant, suggesting long-term demographic stability in this group. In all other cases, only Fu's F_S was significant (Table 3), suggesting a slight population expansion that was only detected by the most sensitive test.

Hierarchical analyses of molecular variance

AMOVAs were first performed using all the individuals sampled from the *T. pityocampa*/*T. wilkinsoni* complex. Most of the variation was found among populations within groups in all cases (44.0% to 67.1%, $P < 0.001$). Nonetheless, the among-group percentage of variation was always significant, and reached 39.3% when populations were grouped according to the PPM subclades. It was 33.0% when using the PPM haplogroups, and decreased to 17.2% when using the PPM clades. When populations were grouped following the host tree species or ecological groups, the among-group variation was *ca*. 24%.

The same analyses were further implemented using only individuals belonging to Clade I. Most of the variation was again found among populations within groups in all cases (44.9% to 56.4%, $P < 0.001$). Here again, the among-group percentage of variation was always significant. It reached 28.5% (26.0%) when grouping the populations depending on the PPM subclades (haplogroups) and 26.8% when grouping the populations according to the host plant species. This proportion was lower when grouping the populations following the PPM clades (17.0%) or the host pine ecological groups (15.4%).

***Ooencyrtus pityocampae:* the generalist species**

Genetic diversity, haplotype distribution and network for the two markers

In this species, we obtained *COI* sequences for 239 individuals and the ITS2 fragment for a subset of 93 samples. The ITS2 fragment was 536 bp long. When ignoring the $(AC)_n$ region, it exhibited three alleles (All-O1, All-O2 and All-O3) connected by a single mutational step due to two polymorphic sites. Two of the four individuals sampled from eggs of *S. coloratum* had a highly divergent allele that could not be aligned to the rest of the data set. The main allele, All-O1,

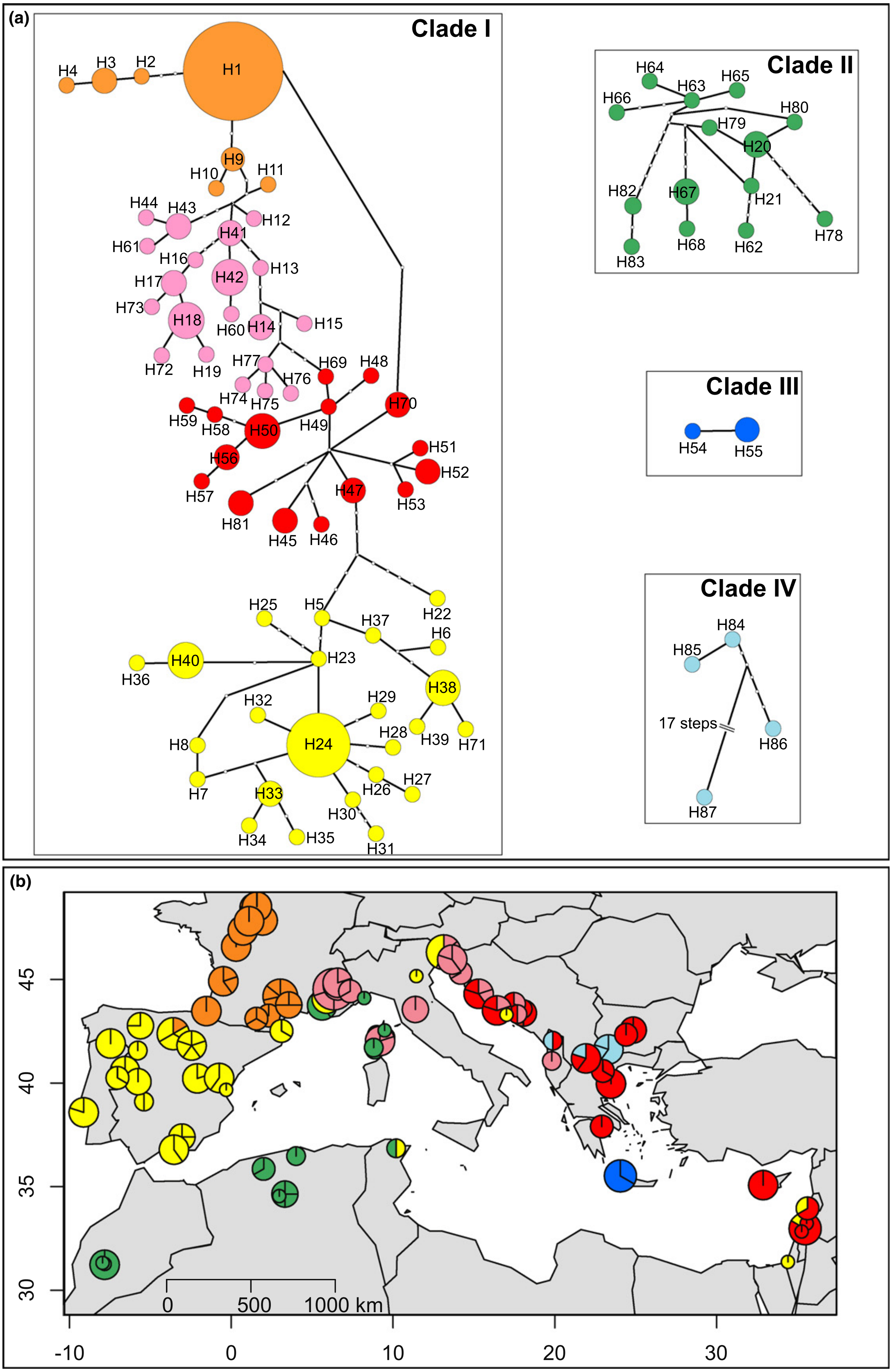

Figure 2 (a) Haplotype networks obtained for the four mitochondrial clades observed for *Baryscapus servadeii*, and (b) geographical distribution of the corresponding clades and haplogroups. In the networks, the size of each circle is proportional to the haplotype's frequency, and the small white dots correspond to missing haplotypes.

Table 2 Mean (min–max) intra- and inter-clade K2P genetic distances measured between haplotypes in *Baryscapus servadeii*.

	CI HG1	CI HG2	CI HG3	CI HG4	CII	CIII	CIV
CI HG1	0.005 (0.001–0.010)						
CI HG2	0.008 (0.003–0.014)	0.007 (0.001–0.012)					
CI HG3	0.008 (0.002–0.012)	0.009 (0.004–0.016)	0.005 (0.001–0.010)				
CI HG4	0.009 (0.004–0.015)	0.010 (0.004–0.015)	0.010 (0.004–0.016)	0.006 (0.001–0.016)			
CII	0.037 (0.033–0.040)	0.038 (0.033–0.043)	0.036 (0.031–0.039)	0.038 (0.032–0.046)	0.008 (0.001–0.016)		
CIII	0.038 (0.036–0.041)	0.039 (0.034–0.043)	0.035 (0.031–0.038)	0.038 (0.033–0.044)	0.036 (0.033–0.038)	0.001 (0.001–0.001)	
CIV	0.050 (0.042–0.056)	0.052 (0.044–0.058)	0.052 (0.046–0.058)	0.053 (0.046–0.062)	0.053 (0.050–0.057)	0.047 (0.046–0.050)	0.014 (0.001–0.024)

Table 3 Tajima's *D*, Fu's F_S and R_2 values estimated for *Baryscapus servadeii* (whole data set, whole data set except Clade IV, Clade I and Clade II) and *Ooencyrtus pityocampae* (whole data set). *n*, number of individuals. Values in brackets correspond to the *P*-value. Asterisks (*) indicate significant values ($P < 0.05$).

	n	Tajima's *D*	Fu's F_S	R^2
B. servadeii, whole data set	303	−0.4780 (0.3684)	−41.1028* (0.0002)	0.0664 (0.3779)
B. servadeii except Clade IV	296	−0.1119 (0.5500)	−27.3709* (0.0039)	0.0763 (0.5413)
B. servadeii Clade I	254	−1.2558 (0.0786)	−41.1028* (0.0000)	0.0483 (0.0906)
B. servadeii Clade II	36	0.1269 (0.6250)	−0.7305 (0.4284)	0.1206 (0.6242)
O. pityocampae, whole data set	239	−1.6136* (0.0213)	−13.0102* (0.0019)	0.0285* (0.0253)

was distributed in the western part of the Mediterranean Basin and was found in approximately two-thirds of the individuals. It was not found in Corsica, Albany, or in the eastern Mediterranean. All-O2 (one-third of the individuals) also had an extensive geographical distribution. However, it did not occur in North Africa or in most of the Balkans. All-O3 (12 individuals) was observed mostly in North Africa (including the individuals collected on *T. bonjeani*) and was present in Israel and Turkey. The two individuals sampled from egg masses of *S. coloratum* in Israel had alleles All-O2 and All-O3. The distribution of the three alleles can be found in Appendix S1.

Concerning the *COI* mitochondrial marker, owing to technical difficulties we obtained a final alignment of 752 bp long sequences. The two individuals sampled from eggs of *S. coloratum* that had a divergent ITS2 allele exhibited a single *COI* haplotype that was well differentiated from all other observed haplotypes [pairwise K2P distance of 8% whereas all other pairwise distances were 0.5% at most, consistent with interspecific divergence; see Hernández-López *et al.* (2012)]; these two individuals were thus excluded from the data set for all further analyses, as they probably corresponded to a different species. Finally, we found 16 haplotypes, the gene diversity was 0.57, the mean number of pairwise differences was 0.72 and the nucleotide diversity per site was 0.0009. The haplotype network was star-shaped and did not reveal any phylogenetic structure. It was characterized by one main haplotype, HA, shared by 147 individuals sampled all over the Mediterranean Basin from the PPM, 4 from *T. bonjeani* in Algeria and 1 from *S. coloratum* in Israel. All other haplotypes were separated from HA by two mutation steps at most (Fig. 3a). The geographical distribution of haplotypes is shown in Fig. 3b. The second most frequent haplotype (HB, 28 individuals) occurred in the Iberian Peninsula, the Pyrenees and Tunisia. Haplotype HN was found in nine individuals from Greece, Crete, Cyprus and Turkey. We could also identify three groups of linked haplotypes. HK–HL–HM occurred in the Balkans, in Tunisia and in Israel (one individual from *S. coloratum*). HI–HJ was restricted to the northern Balkans while HD–HE–HF–HG was mostly found in the western Mediterranean Basin.

Demographic history

Given the absence of phylogenetic structure in *O. pityocampae*, the tests were only performed at the species level. Tajima's *D*, Fu's F_S and R_2 were all significant and unambiguously suggested a recent demographic expansion in this species (Table 3).

Hierarchical analyses of molecular variance

We first explored the effect of PPM genetic differentiation. Whatever the genetic pattern tested (PPM clades, subclades or haplogroups), most of the variance was found among populations within groups (41.6% to 50.7%, $P < 0.001$). The PPM genetic clusters explained 10.7% ($P > 0.05$) of the total variance when considering the main clades, 15.2% ($P < 0.05$) when considering the subclades and 17.0% when considering the haplogroups ($P < 0.001$).

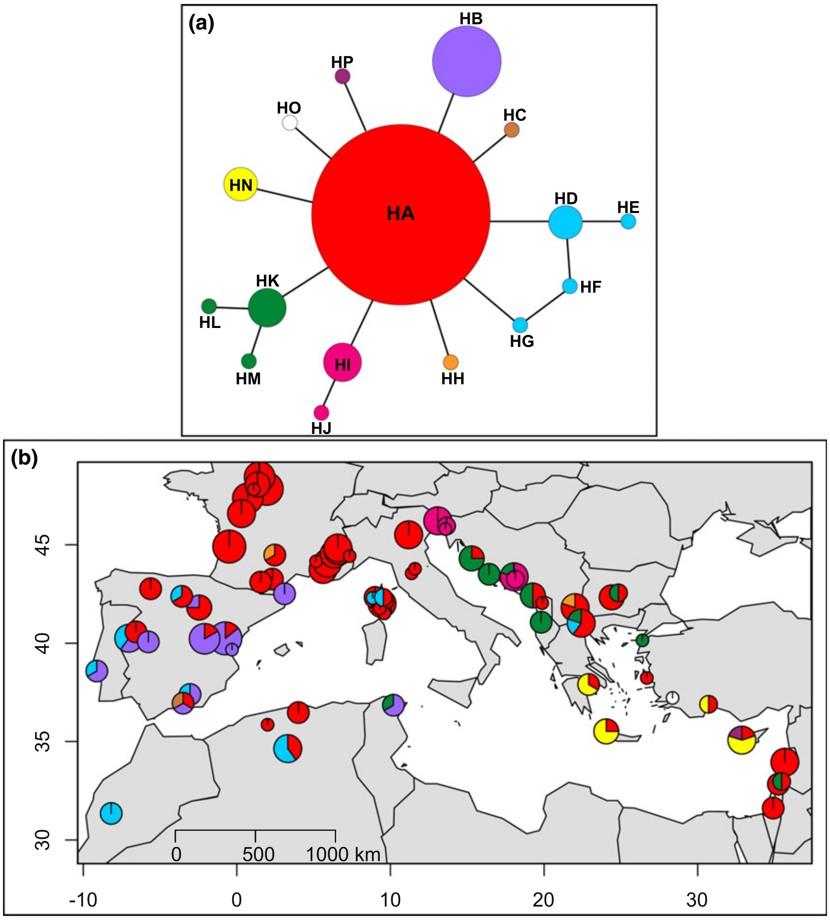

Figure 3 (a) Haplotype network obtained for *Ooencyrtus pityocampae*, and (b) geographical distribution of the corresponding haplotypes and haplotype groups. In the network, the size of each circle is proportional to the haplotype's frequency, and the small white dots correspond to missing haplotypes.

We then performed AMOVAs taking into account the identity of pine host species from which egg batches were sampled. We obtained similar results when considering each species of pine as a separate group, or when considering ecological groups. The total variance was in both cases mostly found within populations (45.0% and 44.7%, respectively) and among populations within groups (51.9% and 52.5%, respectively). Less than 3% of the variance occurred among groups.

DISCUSSION

Various footprints of Quaternary climatic cycles can be identified in present-day patterns of genetic diversity, depending on species' climatic requirements and demographic responses to climate change. Refugial areas in which a species could survive several glacial periods ('long-term refugia'; Stewart *et al.*, 2010) will be characterized by a high local genetic diversity and a relatively stable demographic history, while newly recolonized areas will usually be genetically depauperate and show signs of recent population expansion (Hewitt, 2004). Close phylogenetic relationships between the haplotypes found in refugial areas and those found in recently invaded regions will give insights into the most plausible recolonization routes used by the species during the current interglacial period. A strong divergence between lineages within a species, indicated by large haplotypic distances between clades, is expected if the populations continuously survived the glacial periods in isolated refugia.

Our study aimed to examine the range-wide phylogeographical structures of two parasitoid species possessing similar reproductive systems (thelytokous parthenogenesis) and associated with the same insect host across the Mediterranean Basin. We hoped to compare their histories relative to the strong geographical patterns documented for the PPM at the same geographical scale, and expected the specialist species to show a greater similarity with the host than the generalist. Interestingly, we found very contrasting results in the two studied species concerning not only the observed phylogeographical structures but also the levels of genetic diversity and differentiation. As detailed below, we suggest that the discrepancies found between species can originate from their degree of host specialization as well as from drastically different demographic histories during the Quaternary glacial cycles. The effect of pine host was low for both species.

Baryscapus servadeii, a diverse and strongly structured specialist parasitoid

This species was genetically diverse, and structured into four divergent lineages. One of these phylogenetic clades (CIV) diverged for both *COI* and ITS2 markers. This clade was represented by relatively few individuals in the central Balkans, where they were found in sympatry with individuals from CI, from which they were morphologically indistinguishable. Even though a dedicated taxonomic study would be necessary to reach a definitive conclusion, we suggest that they could correspond to a sister species of the 'typical' *B. servadeii*, possibly the cryptic hyperparasitoid *Baryscapus transversalis* Graham, 1991 (Tsankov *et al.*, 1998).

The other three identified mitochondrial clades were found in parapatric geographical regions (Crete, North Africa, and the rest of the Mediterranean Basin). They probably corresponded to distinct glacial refugia where populations occurred independently throughout the Quaternary: this inference is based on the large genetic distance between the clades, and an assumption of rate evolution for insect mitochondrial DNA of between 1.5% and 2.5% per Myr (Borer *et al.*, 2010). The observed levels of divergence between the clades are consistent with long-term spatial isolation.

Most of the sampled individuals belonged to Clade I, which had a large geographical distribution over the northern rim of the Mediterranean Basin and in the Near East. This clade was genetically diverse and showed a clear geographical substructure from which identification of refugial areas and colonization routes was possible. The most divergent and diverse haplogroup was mainly restricted to Spain and Portugal. As observed in many European taxa, *B. servadeii* probably occurred in this region over a long period of time, in a multitude of allopatric refugia within the peninsula that allowed genetic diversity to be maintained (Gómez & Lunt, 2006). Unlike its PPM host, however, for which we have evidence that southern Spanish refugial populations recolonized northwards to western France (Rousselet *et al.*, 2010), the Iberian lineage of *B. servadeii* is inferred to have expanded eastwards to the Alps and the northern Balkans, as indicated by the occurrence of some Iberian haplotypes in these regions. Its contribution to the post-glacial recolonization of suitable habitats elsewhere in Europe was limited. One haplotype (H40) had a surprisingly large and discontinuous geographical range, which could suggest repeated long-distance dispersal or recent connections due to human-mediated dispersal. The three other haplogroups showed a shallow structure, but clearly corresponded to distinct refugial areas in which diversity was maintained. The relatively limited haplotypic distances observed between haplogroups suggest a fairly recent isolation. HG1 was found in southern France and the Pyrenees, and expanded northwards in western France up to the current PPM expansion front in the Paris Basin, where only H1 occurs. HG2 corresponded to a centre of genetic diversity located in the Alps and in the northern Balkans. Whether this lineage contributed to northward expansions cannot be determined without samples located further north. Finally, the last haplogroup (HG3) was distributed from the Balkans to the Near East, including the island of Cyprus. The observed diversity was maximal in the Balkans, but we cannot rule out that refugia were also located in Turkey, from which we did not obtain any specimens despite a strong sampling effort. A similar geographical lineage was found in the Mediterranean bark beetle *Tomicus destruens*, but genetic diversity was located in the Near East in this case (Horn *et al.*, 2006).

The other identified clades (CII and CIII) corresponded to North Africa and Crete. They probably remained isolated over long periods of time and therefore diverged genetically. We infer that these clades did not contribute to northern recolonization of suitable habitat during the interglacials, as demographic analyses suggest stability over time and their haplotypes were found only in these restricted regions. The Cretan Clade III corresponded to the most differentiated PPM subclade in the Near East. These individuals also had a specific ITS2 allele (All-B4). Clade II was well diversified and the corresponding individuals had two ITS2 alleles, namely the geographically restricted All-B3 and the common All-B2. These results are consistent with a long-lasting presence of the species in North Africa, which remained suitable even during glaciations and so could serve as a 'long-term' refugial area (Stewart *et al.*, 2010), as is already known for the PPM. This clade is also present in Corsica, southern France and Liguria (Italy), where a contact zone between Clades I and II was observed. Major migration routes thus crossed in these regions that could be reached from North Africa either through long distance, potentially wind-assisted dispersal over the Mediterranean, as documented for certain other arthropods (Horn *et al.*, 2006; Kerdelhué *et al.*, 2014), or through Sicily and southern Italy, which were not included in the present study. Mutations accumulated locally over time, resulting in the existence of many endemic haplotypes in Corsica. Interestingly, the classical recolonization route from North Africa to Europe through the Strait of Gibraltar and Spain could be ruled out.

Overall, diversity was retained in most regions except north-western France, where the PPM and its specific parasitoid expanded in distribution only recently. This result is consistent with the expectations for a Mediterranean species that occurred in regions where the intensity of glaciation events was relatively limited, and where populations could survive locally via limited movements (phalanx-like expansion: Hewitt, 1996; Ibrahim *et al.*, 1996). The results hence suggest that *B. servadeii* survived the series of Quaternary climatic oscillations throughout its present range in various refugial areas. The existence of extensive diversity shows that its populations remained relatively stable over time. The AMOVA results clearly show that the distribution of genetic diversity in the parasitoid was significantly influenced by that of its main host, and the data reveal extant shared refugia in the European peninsulas and in all southern regions of moderate elevation, as well as in North Africa and Crete (Kerdelhué *et al.*, 2009; Rousselet *et al.*, 2010). The main discrepancies concern the Near East, where a highly differentiated clade exists for the PPM while local populations of *B. servadeii* are closely related to the populations that diversified in the southern Balkans.

Ooencyrtus pityocampae, a generalist parasitoid with low genetic diversity and structure

In spite of a shared insect host and a similar reproductive system, *O. pityocampae* exhibited drastically different genetic features when compared to *B. servadeii*. We obtained only 16 haplotypes over the whole Mediterranean Basin, with very low gene diversity and π values. *O. pityocampae* was characterized by a major haplotype that was found in all regions, and a series of closely related haplotypes with restricted geographical distributions. The star-shaped network with one over-represented haplotype and an excess of rare ones, as well as the demographic indices, suggest a recent expansion history following a drastic bottleneck (Avise, 2000), and local occurrence of new, recent haplotypes that emerged through mutation during the rapid expansion process (Petit *et al.*, 2005). These features are consistent with the hypothesis that *O. pityocampae* was strongly affected by the Quaternary glaciations, and that the genetic structure observed nowadays mostly corresponds to a fairly recent history that could date back from the last (or one of the very last) glacial maxima. Genetic diversity was mostly found in the Iberian Peninsula and in the Balkans. Populations located north of a line from the Pyrenees to Croatia were all monomorphic, which further contrasts with *B. servadeii* and the PPM host. Moreover, the colonization route from North Africa occurred preferentially through the Straits of Gibraltar and the Iberian Peninsula, as shown by the distribution of haplotypes HB and HD (Fig. 3, Appendix S1). The occurrence of haplotype HH in southern France and in Macedonia could be a sign of allopatric survival of this haplotype in disjunct refugia during the LGM, but could also be due to homoplasy. It is interesting to note that Corsica contained both the main haplotype HA and an endemic haplotype closely related to the North African ones, which recalls the results obtained in *B. servadeii* and suggests that the position of this island in the Mediterranean, where the PPM has a long-lasting history (Kerdelhué *et al.*, 2009), favours the 'interception' of accidentally migrating individuals. Overall the results thus suggest that *O. pityocampae* had different climatic requirements compared to the PPM host and *B. servadeii*, and that it survived only in restricted regions during the Quaternary. Its current pattern of genetic diversity shows that it probably recolonized from a single refugium, as has been suggested for other species (Bertheau *et al.*, 2013). Thelytokous parthenogenesis probably favoured the survival of populations in spite of strong bottlenecks and rapid expansions, as parthenogenesis strongly limits Allee effects. The capacity of this species to exploit various hosts further assisted the local maintenance of populations even after drastic reductions in size. These bottlenecks, together with rapid dispersal dynamics, led to some stochasticity, which might also explain why the mitochondrial and ITS2 markers did not convey consistent information.

Comparative phylogeographical studies aim at testing whether species belonging to the same community responded as a single unit to Quaternary environmental perturbations ('community phylogeography', commonly expected in closed communities; Nicholls *et al.*, 2010) or whether the genetic structure of interacting species results from idiosyncratic biogeographical histories (Kohnen *et al.*, 2012). Incongruent patterns are expected when host switches occur in dependent species, or when their dispersal characteristics and ecological requirements are different. In that case, interaction may still persist despite differences in recolonization routes as long as the host species occurs frequently enough for the dependent species to survive in locations where suitable abiotic and biotic factors overlap (Espíndola & Alvarez, 2011). Comparative studies over a large spatial scale are still relatively rare in the literature, and usually reveal similar patterns for the various studied species (either congruence or incongruence with the host species for all the studied taxa). The results obtained in the present paper show that two parasitoid species associated with the same main host throughout their range with similar reproductive characteristics can still exhibit contrasting histories. We showed that the evolutionary history of the specialist species was partially congruent with that of its host, yet with regional discrepancies, which was not the case for the generalist species. Interestingly, when studying two bark beetle species associated with the same coniferous host, Bertheau *et al.* (2013) also found contrasting patterns between the specialist and the oligophagous species but with opposite conclusions. Vulnerability to extreme climatic events and low survival during glacial maxima, rather than the generalist feature *per se*, probably explain the observed discrepancies between *O. pityocampae* and its main host in our case.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Sampling details and sequence results for each studied locality.

DATA ACCESSIBILITY

The sequences generated for this study are available in GenBank, with accession numbers KP420032–KP420118 for the *COI* haplotypes of *B. servadeii*, KP420119–KP420134 for the *COI* haplotypes of *O. pityocampae*, and KP420135–KP420142 for the ITS2 alleles.

BIOSKETCH

The authors belong to a research consortium dealing with insect evolutionary biology and natural genetic structure at different scales. They have been involved in various projects studying the range-wide phylogeography of several species of forest insects (seed chalcids, scolytids, processionary moths) in Europe and the Mediterranean Basin, and recently started to collaborate on multi-trophic systems.

Author contributions: M.-A.A.-R. and C.K. designed the study and organized the sampling; C.C. performed the molecular laboratory analyses; M.-A.A.-R., C.K., J.-P.R. and M.T.-L. analysed the data and drew the figures; M.T.-L., M.-A.A.-R. and C.K. wrote the manuscript, which was revised and approved by all co-authors.

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