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ORIGINAL PAPER

Did the parasitoid *Pnigalio mediterraneus* (Hymenoptera: Eulophidae) track the invasion of the horse chestnut leafminer?

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Abstract How communities of natural enemies, such as parasitoids, adapt to the range expansion of their hosts or the arrival of a novel invasive host is an important question in invasion biology. Do parasitoids track the expansion of their hosts from their shared native range? Do they locally adapt both behaviorally and physiologically to the arrival of a novel species by shifting hosts? Few studies have addressed those questions, yet they are important to develop efficient methods to manage invasive species. Here we focus on *Pnigalio mediterraneus* Ferriére and Delucchi (Hymenoptera: Eulophidae), an important parasitoid of two major agricultural and ornamental pests, the olive fruit fly *Bactrocera oleae* Rossi (Diptera: Tephritidae) and the horse chestnut leafminer *Cameraria ohridella* Deschka & Dimic (Lepidoptera: Gracillariidae). *C. ohridella* recently invaded Europe starting from the Southern Balkans, whereas *B. oleae* has been associated since the Quaternary with wild olives in the Mediterranean, where it largely spread after the domestication of cultivated olives. We used two markers, the ribosomal spacer ITS2 and the mitochondrial gene COI. Although the ITS2 dataset provided little variation and no phylogeographic signal, analysis of mtDNA of 188 individuals of *P. mediterraneus* from 54 European localities allowed us to identify 53 haplotypes. Both nucleotide and haplotype diversity were higher for Mediterranean samples, and from samples reared from *B. oleae*. The statistical parsimony network identified one haplotype as the most frequent, ancestral and mainly associated with *C. ohridella*. Our findings suggest that *P. mediterraneus* locally host switched to *C. ohridella* from other hosts in the Balkans and later tracked the horse chestnut leafminer invasion over Europe. Therefore both host-tracking and ecological sorting could explain the current distribution of *P. mediterraneus* haplotypes.

Keywords *Bactrocera oleae* · *Cameraria ohridella* · Invasive pest · Leafminer · mtDNA diversity · Phylogeography

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Introduction

Although the rate of biological invasions of terrestrial invertebrates has dramatically increased during the second half of the 20th century (Roques et al. 2008), invasive invertebrates are not a new phenomenon. Ever since humans started shaping nature to their

needs, insects and other invertebrates have quickly adapted and exploited the new available ecological niches.

A recent example of range expansion is the horse chestnut leafminer *Cameraria ohridella* Deschka & Dimic (Lepidoptera: Gracillariidae). First collected in Greece in 1879 (Lees et al. 2011), it was described in 1986 from a Macedonian outbreak from 1984 (Deschka and Dimić, 1986), spread via Vienna in 1989 and colonised most European countries in the next 20 years (Augustin et al. 2009; Valade et al. 2009). By using both mitochondrial and microsatellite DNA markers, Valade et al. (2009) showed that *C. ohridella* originated in the southern Balkans, confirming the hypothesis that it is a relict species that survived the glacial cooling of the tertiary period together with its main host tree, *Aesculus hippocastanum* L. (Hippocastanaceae). Additional data gathered from historical herbarium collections from Albania and Greece by Lees et al. (2011) gave further support to a Balkan origin of *C. ohridella*. The high invasive potential of this micromoth is mainly attributed to its high population growth, which results, among other factors, from low rates of predation and parasitism (Ferracini and Alma 2007; Freise et al. 2002; Girardoz et al. 2007; Grabenweger et al. 2007; Volter and Kenis 2006), although approximately 30 native generalist parasitoid species have been recorded attacking this leafminer (Grabenweger et al. 2010).

The *enemy release-hypothesis* (Keane and Crawley 2002), that is, the scarcity and poor adaptation of native natural enemies at the beginning of the invasion, is commonly advocated as one of the best explanations for the invasion success. Indeed, release from parasites and pathogens is thought to be one of the primary reasons for the success of both alien invasive species (Phillips et al. 2010) and species expanding their range under climate change (Menendez et al. 2008).

Several studies have focused on the parasitoid complex of invasive insect species in their native range (Grabenweger et al. 2005; Lupi 2005; Lozier et al. 2008, 2009; Zappalà et al. 2012; Quacchia et al. 2013). In contrast, few studies have focused on whether and how parasitoids adapt behaviourally and physiologically to the arrival of a new potential host at the invasion front (Klug et al. 2008). The mechanisms involved in such adaptive processes are poorly understood. For instance, what are the (pre-)adaptations that allow a parasitoid to switch to a new host and what are the (post-)adaptations that take place after the host switch? Knowledge of the processes that take place before and after the host switch is of crucial importance for the development of efficient pest management programmes (Grabenweger et al. 2010).

Two main hypotheses may explain how parasitoid communities assemble during a process of invasion or range expansion by an insect host: (1) the *Host-Pursuit Hypothesis* predicts that parasitoids will track the expansion range of their hosts from a shared area of origin (Menendez et al. 2008; Nicholls et al. 2010; Hernandez-Lopez et al. 2012; Stone et al. 2012); (2) the *Ecological Sorting Hypothesis* predicts that local parasitoids will adapt to the arrival of the novel host by host shifting (Klug et al. 2008; Hernandez-Lopez et al. 2012). Here we address those hypotheses using phylogeographic data for *Pnigalio mediterraneus* Ferriére and Delucchi (Hymenoptera: Eulophidae), one of the main parasitoids of the invasive *C. ohridella*. *P. mediterraneus* is a primary ectoparasitoid of larvae of leafminers and/or gall-making insects belonging to three orders: Diptera (Tephritidae and Agromyzidae), Hymenoptera (Tenthredinidae) and Lepidoptera (Gracillariidae) (Freise et al. 2002; Grabenweger and Lethmayer 1999; Grabenweger 2003; Gebiola et al. 2009; Grabenweger et al. 2010; Volter and Kenis 2006). *P. mediterraneus* was described as a parasitoid of the olive fruit fly *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) in the Mediterranean basin (Ferrière and Delucchi 1957), was later synonymized with *Pnigalio agraules* Walker (Askew 1984) and has been recently validated as a distinct species (Gebiola et al. 2009).

In a survey of the *C. ohridella* parasitoid complex in Europe, Grabenweger and Lethmayer (1999) found *P. mediterraneus*, then known as *P. agraules*, in every sample, leading authors to consider it as one of the best candidates for the biological control of *C. ohridella*, although acknowledging that climatic barriers could restrict its use due to a distribution limited to southern parts of Central Europe (Mey 1993). More recently, Grabenweger et al. (2010) reported that *P. mediterraneus* dominates *C. ohridella*'s parasitoid complex in northern, western and central Europe along with *Minotetrastichus frontalis* (Nees) (Hymenoptera: Eulophidae), while it is less frequent, sometimes almost lacking, in the Balkans (see also Freise et al. 2002). The absence of *P. mediterraneus* from Central-Northern Europe is argued indirectly. In fact, both *P.*

mediterraneus and *P. agraules* have been reported from this area, but due to the former synonymy, it is difficult to assess the reliability of many records. Nevertheless, host records prior to the synonymy suggest that *P. mediterraneus* is the species with Southern distribution (Noyes 2012). Furthermore, although an important natural enemy of *C. ohridella*, prior to *C. ohridella*'s invasion, the preferred host of *P. mediterraneus* was *B. oleae*, a fly of Mediterranean origin. Following the Quaternary differentiation between African and Asian lineages of its native host plant *Olea europaea* subsp. *cuspidata* and the Mediterranean *O. e.* subsp. *europaea* var. *sylvestris* (also known as oleaster or wild olive), Mediterranean populations of olive fly present on wild olives started to diverge from African populations and remained associated with their host in the process of retreat to separate Pleistocene refugia. Later, as the cultivated olive was introduced in historical and recent times from the Levant to cover all the Mediterranean area, olive flies were already established and transferred from the original wild olive host to cultivated olive, *O. e.* subsp. *europaea* (Nardi et al. 2010). Therefore, as a parasitoid of two invaders that colonized Europe thousands of years apart, *P. mediterraneus* represents an interesting case study. Recent studies that integrate different types of data show that parasitoids believed to be generalists are instead complexes of often host-specific cryptic species (Chesters et al. 2012; Gebiola et al. 2012). Among parasitoids of *C. ohridella*, Hernandez-Lopez et al. (2012) found evidence for a complex of at least five haplogroups within *Pediobius saulius* (Walker) (Hymenoptera: Eulophidae), but no evidence of host-tracking and the existence of a *C. ohridella*-specialized Balkan haplogroup of *P. saulius* that could be potentially used for the biological control of the invasive leafminer. Finally, several studies have focused on the phylogeography of alien invasive species or species that are experiencing an expansion of their distribution range (Valade et al. 2009; Rousselet et al. 2010). However, only very few studies have focused on the phylogeography of the parasitoids of invasive species (Baer et al. 2004; Hayward and Stone 2006; Nicholls et al. 2010; Hernandez-Lopez et al. 2012; Stone et al. 2012).

Here, we report the phylogeography and population genetic structure of *P. mediterraneus* based on analysis of mitochondrial and ribosomal sequences of wasps collected from the native (Balkans) and introduced (rest of Europe) range of *C. ohridella*, and from the native range of *B. oleae* (the Mediterranean region). Elucidating the phylogeography of *P. mediterraneus* in native and introduced ranges allows us to address several questions related to host-parasitoid evolution. The main aim of the present study is to infer the ecological mechanism by which *P. mediterraneus*, a species previously restricted to southern Europe, has become a frequent parasitoid of *C. ohridella* in Central and Northern Europe: did it track the host from the Balkans (*Host-Tracking Hypothesis*)? Or did it locally adapt to the new host (*Ecological Sorting Hypothesis*)?

Materials and methods

Taxa sampling

One hundred and seven adults identified as *P. mediterraneus* based on both morphology and COI sequence data were reared from *C. ohridella* mines, while 81 specimens were reared from nine other hosts, including 54 specimens reared from *B. oleae*. Overall, *P. mediterraneus* samples were obtained from 54 localities in 14 different countries (Fig. 1, Supplementary Table 1).

Molecular procedures

We used two markers. The first is a fragment of the mitochondrial COI gene, a widely used marker suitable for investigating processes at the species level due to its high mutation rate, haploid inheritance and absence of recombination. Owing to its reduced coalescence time, the possibility to observe groups of haplotypes associated with structured populations that reflects their evolutionary history is maximized, making it an excellent tool for phylogeographic investigations (Pauls et al. 2006; Valade et al. 2009). The second is the ribosomal internal transcribed spacer 2 (ITS2), a widely used nuclear marker that has been successfully used to discriminate between closely related Hymenoptera species (Rugman-Jones et al. 2009; Li et al. 2010; Chesters et al. 2012), and also in the genus *Pnigalio* (Gebiola et al. 2012).

DNA from whole specimens preserved in alcohol was extracted using a Chelex–proteinase K-based non-

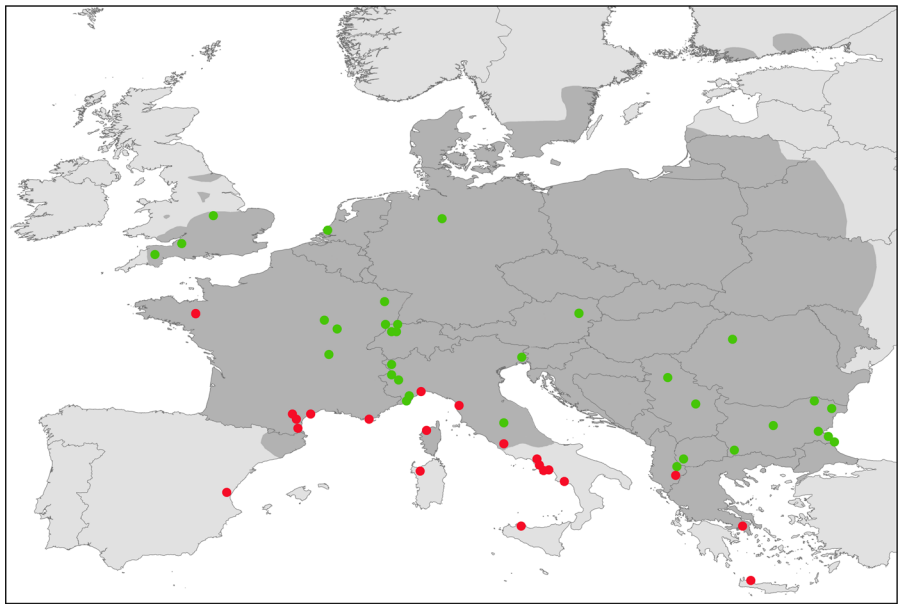

Fig. 1 Map showing the localities sampled in our study (*green dots* indicate presence of H8, *red dots* indicate absence of H8). The *shaded area* indicates the current distribution of *C. ohridella* modified from Valade et al. (2009) and Augustin et al. (2009) by personal observations. (Color figure online)

destructive protocol as in Gebiola et al. (2009). The same DNA extraction protocol was used for dried specimens but specimens were kept in Chelex-proteinase K at 55 °C for 5 h instead of 1 h. After DNA extraction, specimens were card mounted and stored as vouchers at the Museo di Entomologia "F. Silvestri", Portici, Italy. 126 fresh specimens were used to amplify the 3′ half of COI (a 820 bp fragment) using either of the forward primers C1-J-2195 or C1-J-2183 (Simon et al. 1994) with the reverse primer TL2-N-3014 (Simon et al. 1994). For 39 dried samples, two overlapping shorter fragments of 400 bp and 570 bp were amplified by using primer pairs C1-J-2195/CO1-Hco-extB (Schulmeister 2003) and C1-J-2441/TL-N-3014 (Simon et al. 1994), respectively. ITS2 was amplified using primers ITS2F (Campbell et al. 1993) and ITS2RevTrich (Stouthamer et al. 1999). PCR protocols for COI and ITS2 are reported in Gebiola et al. (2009, 2010). Amplicons were checked on 1.2 % agarose gel, purified and sequenced at XiLin sequencing in Beijing, China. Chromatograms were unambiguously edited by eye using BioEdit (Hall 1999). ITS2 sequences were aligned by MAFFT 6 (Katoh and Toh 2008) using the G- INS-i algorithm. Sequences were submitted to GenBank with accession numbers KC171455–KC171619 for COI and KF484913–KF485079 for ITS2. Accessions EF507488–EF507493 from Bernardo et al. (2008), and FJ812249–FJ812267 from Gebiola et al. (2009) were retrieved for analyses.

Phylogeographic analyses

To compare haplotype diversity across sites and different hosts, we used a rarefaction method to take into account differences in sample sizes (Kalinowski 2004). The Chao-1 estimator was calculated ($\mathbf{S}_1^*$) (Chao 1984; Colwell and Coddington 1994) by using Rarefaction Calculator (www2.biology.ualberta.ca/jbrzusto/rarefact.php#ColCod1994). To test whether genetic diversity is structured by geography and/or host associations, samples were grouped according to origin (Balkans/Mediterranean/rest of Europe) and to host (*C. ohridella*/*B. oleae*/other hosts). Polymorphism analyses (haplotype and nucleotide diversity) were performed by locality, and by region and host groupings using DnaSP 5 (Librado and Rozas 2009). Genetic differences among haplotypes were represented by a statistical parsimony network (Templeton et al. 1992) using TCS 1.21 (Clement et al. 2000).

Regional genetic diversity was visualized by plotting genetic data as pie charts on a map using PhyloGeoViz (Tsai 2010). We compared the partition of genetic variability at different hierarchical levels by an analysis of molecular variance (AMOVA), estimated by computing conventional F-statistics for COI haplotypes using Arlequin 3.5 (Excoffier and Lischer 2010) with 10,000 permutations.

A Mantel test was performed in Arlequin 3.5 to check if the genetic structure of the populations fits an isolation by-distance model (Slatkin 1993), using pairwise F_{ST} values and straight-line geographical distances in km estimated by using Google Maps Distance Calculator (http://www.daftlogic.com/projects-google-maps-distance-calculator.htm).

In order to assess whether *P. mediterraneus* followed a range expansion similar to the one experienced by its hosts, two methods were used. First, nested clade phylogeographic analysis (NCPA) (Templeton et al. 1995) was performed by using the software ANeCA (Panchal 2007). This is a widely used method that combines information on the spatial distribution of sequences (in particular mitochondrial haplotypes) and the phylogenetic relationships among them (but see Beaumont and Panchal 2008). Where significant geographical association among sequences is detected, an inference key allows discrimination among patterns generated by contiguous range expansion, long-distance colonization and isolation by distance. Second, mismatch distribution (MMD) analysis was also performed in Arlequin to infer recent demographic changes according to the model of sudden expansion (Rogers 1995). Raggedness (R) indices (Harpending 1994) were used to assess the significance of the fit of the MMD distribution to that of an expanding population, where a non-significant, low R *P*-value infers population expansion. Additionally, differences between expected and observed mismatch patterns were tested by calculating the sum of the squared deviations (SSD) (Rogers and Harpending 1992) and its significance based on 10,000 replications. A significant SSD *P*-value is interpreted as departure from the estimated demographic model of population expansion. Furthermore, the number of pairwise differences observed was plotted against those expected under a sudden expansion model. In such a plot, an unimodal distribution is taken as support for a population expansion (Rogers and Harpending 1992).

Results

Mitochondrial and nuclear DNA sequences

COI was successfully sequenced for all specimens; trimmed sequences were 729 bp. A total of 53 COI haplotypes were found among the 188 individuals analysed in all localities sampled, with 35 unique haplotypes. Polymorphism data and haplotype distribution by locality and by groups as defined above are reported in Tables 1 and 2.

Internal transcribed spacer 2 (ITS2) sequences were obtained for 167 specimens. Sequence length ranged from 405 to 410 bp, and the final alignment consisted of 412 nucleotides. Eleven ribotypes were recovered, one of which, H1, was predominant, being represented 87 times. However, variation for this marker was very limited, and statistical parsimony analysis showed no geographic or host-associated structure (Supplementary Figure 1). Therefore, ITS2 sequences were not subjected to further analyses.

Spatial distribution of genetic diversity: mediterranean origin

Both nucleotide and haplotype diversity were higher for Mediterranean samples, and from samples reared on *B. oleae* (Table 2). Haplotype diversity was significantly correlated to sampling effort (number of individuals sampled per population) (Spearman correlation: $r_S = 0.72$, $N = 54$, $P < 0.001$). Rarefaction analysis showed that haplotype richness was higher in the Mediterranean area (24.0 ± 2.18 SD; $S_1^* = 147.1 \pm 40.25$) than Balkan (11 ± 0; $S_1^* = 29 \pm 15.17$) and Rest of Europe (8.3 ± 1.05 SD; $S_1^* = 28.0 \pm 15.17$). Haplotype richness was higher for *P. mediterraneus* reared from *B. oleae* (32 ± 0; $S_1^* = 120.2 \pm 37.90$) than for specimens reared from *C. ohridella* (14.19 ± 1.86; $S_1^* = 54.7 \pm 18.08$). The highest genetic diversity was found in the islands of Corsica (Lumio) and Sicily (Palermo), with seven haplotypes out of seven and eight individuals analysed, respectively (Table 1). It is also worth noting that from a single plant of *Celtis australis* L. (Ulmaceae) in Portici (Italy) four different haplotypes were sampled (Table 2). The statistical parsimony network showed the haplotype H8 as the most frequent, and, given coalescent theory, also the ancestral one, with 11 haplotypes connected to it by 1-step mutations, most

Table 1 Number of individuals, haplotype designation, host associations and genetic diversity for sampled populations grouped according to geographical origin

Country	Locality	Host	Individuals sampled	Number of Haplotypes	Hd (SD)	π (SD)	Haplotype distribution
Albania	Korcae	*C. ohridella*	3	2	0.667 (0.314)	0.00640 (0.00312)	H28(2), H29(1)
	Pogradec	*C. ohridella*	4	4	1 (0.500)	0.00640 (0.00156)	H4(1), H8(1), H19(1), H30(1)
Austria	Wien	*C. ohridella*	5	2	0.400 (0.237)	0.00055 (0.00033)	H8(4), H35(1)
Bulgaria	Burgas	*C. ohridella*	3	1	0	0	H8(3)
	Dobrich	*C. ohridella*	4	3	0.833 (0.222)	0.00206 (0.00075)	H8(2), H26(1), H27(1)
	Kazanlak	*C. ohridella*	3	2	0.667 (0.314)	0.00549 (0.00259)	H8(2), H10(1)
	Primorsko	*C. ohridella*	3	2	0.667 (0.314)	0.00091 (0.00043)	H8(2), H25(1)
	Sandanski	*C. ohridella*	3	3	1 (0.272)	0.00183 (0.00061)	H8(1), H13(1), H19(1)
	Silistra	*C. ohridella*	3	2	0.667 (0.314)	0.00091 (0.00043)	H8(2), H13(1)
	Tsarevo	*C. ohridella*	2	1	0	0	H8(2)
England	Bristol	*C. ohridella*	2	2	1 (0.500)	0.00823 (0.00412)	H8(1), H10(1)
	Devon	*C. ohridella*	2	2	1 (0.500)	0.00823 (0.00412)	H8(1), H10(1)
	Harpenden	*C. ohridella*	5	2	0.400 (0.237)	0.00055 (0.00033)	H8(4), H13(1)
		Phyllonorycter sp.					
France	Argèles sur mer	*B. oleae*	4	4	1 (0.177)	0.00754 (0.00164)	H2(1), H10(1), H47(1), H48(1)
	Ferrals les Corbières	*B. oleae*	2	2	1 (0.500)	0.00960 (0.00480)	H20(1), H47(1)
	Lumio	*B. oleae*	7	7	1 (0.076)	0.00928 (0.00136)	H29(1), H33(1), H38(1), H39(1), H43(1), H45(1), H49(1)
	Mèze	*B. oleae*	2	2	1 (0.500)	0.00823 (0.00412)	H2(1), H41(1)
	Montfort	*B. oleae*	2	2	1 (0.500)	0.00549 (0.00274)	H2(1), H50(1)
	Porquerolles	*B. oleae*	2	2	1 (0.500)	0.00549 (0.00274)	H2(1), H14(1)
	Rieux Minervois	*B. oleae*	3	3	1 (0.272)	0.00823 (0.00224)	H42(1), H44(1), H47(1),
	Bettborn	*C. ohridella*	3	3	1 (0.272)	0.00183 (0.00061)	H8(1), H13(1), H19(1)
	Chartre	*C. ohridella*	6	5	0.933 (0.122)	0.00412 (0.00166)	H8(2), H10(1), H13(1), H31(1), H46(1)
	Dijon	*C. ohridella*	2	2	1 (0.500)	0.00823 (0.00412)	H8(1), H10(1)
	Fontenay	*C. ohridella*	4	2	0.500 (0.265)	0.00069 (0.00036)	H8(3), H25(1)
	Lucelle	*C. ohridella*	2	2	1 (0.500)	0.00137 (0.00069)	H8(1), H19(1)
Germany	Hannover	*C. ohridella*	5	3	0.700 (0.218)	0.00439 (0.00139)	H8(3), H10(1), H51(1)

Table 1 continued

Country	Locality	Host	Individuals sampled	Number of Haplotypes	Hd (SD)	π (SD)	Haplotype distribution
Greece	Khania	*B. oleae*	4	2	0.500 (0.265)	0.00480 (0.00225)	H23(1), H24(3)
	Kifissia	*B. oleae*	4	3	0.833 (0.222)	0.00572 (0.00223)	H2(1), H4(2), H22(1)
Italy	Caprie	*A. torquatella*	4	3	0.833 (0.222)	0.00480 (0.00215)	H8(2), H10(1), H21(1)
	Albisola	*B. oleae*	2	2	1 (0.500)	0.00686 (0.00343)	H2(1), H10(1)
	Licusati	*B. oleae*	2	2	1 (0.500)	0.00823 (0.00412)	H2(1), H17(1)
	Palermo	*B. oleae*	8	7	0.964 (0.077)	0.00852 (0.00146)	H4(1), H5(1), H6(2), H7(1), H20(1), H37(1), H52(1)
	Peagna	*M. latifoliella*	3	2	0.667 (0.314)	0.00549 (0.00259)	H8(1), H10(2)
	Portici	*P. millierella*	8	4	0.643 (0.184)	0.00392 (0.00156)	H1(1), H2(5), H10(1), H12(1)
	S. Giovanni a Teduccio	*B. oleae*	4	3	0.833 (0.222)	0.00732 (0.00217)	H2(2), H9(1), H10(1)
	San Giuliano Terme	*B. oleae*	2	2	1 (0.500)	0.00549 (0.00274)	H2(1), H14(1)
	Sassari	*B. oleae*	4	3	0.833 (0.222)	0.00914 (0.00250)	H3(1), H18(1), H36(2)
	Varcaturo	*B. oleae*	2	2	1 (0.500)	0.00686 (0.00343)	H11(1), H14(1)
	Capua	*H. ochropodus* *P. pygmaea*	2	2	1 (0.500)	0.00823 (0.00412)	H2(1), H20(1)
	Albenga	*C. ohridella*	6	5	0.933 (0.122)	0.00613 (0.00110)	H8(1), H12(1), H13(1), H14(1), H33(2)
	Assisi	*C. ohridella*	2	1	0	0	H8(2)
	Cividale del Friuli	*C. ohridella*	2	2	1 (0.500)	0.00686 (0.00343)	H8(1), H34(1)
	Grugliasco	*C. ohridella*	6	2	0.333 (0.215)	0.00091 (0.00059)	H8(5), H40(1)
	Saint Nicolas	*C. ohridella*	3	2	0.667 (0.314)	0.00091 (0.00043)	H8(2), H13(1)
	Roma	*C. ohridella*	2	1	0	0	H53(2)
Macedonia	Ohrid	*C. ohridella*	6	4	0.800 (0.172)	0.00631 (0.00213)	H8(3), H10(1), H19(1), H28(1)
Netherlands	Den Haag	*C. ohridella*	5	3	0.800 (0.164)	0.00549 (0.00144)	H8(2), H10(2), H13(1)
Romania	Cluj Napoca	*C. ohridella*	4	3	0.833 (0.222)	0.00434 (0.00189)	H8(2), H10(1), H32(1)
Serbia	Padinska Skela	*P. platani*	3	2	0.667 (0.314)	0.00091 (0.00043)	H8(2), H19(1)
	Paracin	*P. platani*	4	2	0.500 (0.265)	0.00069 (0.00036)	H8(3), H13(1)
Spain	Valencia	*P. citrella*	2	2	1 (0.500)	0.00274 (0.00137)	H15(1), H16(1)
Switzerland	Bellach	*C. ohridella*	2	1	0	0	H8(2)
	Choindez	*C. ohridella*	2	2	1 (0.500)	0.00137 (0.00069)	H8(1), H13(1)
	Delemont	*C. ohridella*	4	3	0.833 (0.222)	0.00503 (0.00225)	H8(1), H10(1), H13(2)

Table 2 Polymorphism data and haplotype distribution by region and by host

Group	Samples	Localities	Haplotypes	HD ± SD	π ± SD	Tajima's D (ns)	Haplotype distribution
Balkans	41	12	11	0.676 (0.079)	0.00372 (0.00079)	−0.87769	**H4(1), H8(23)**, **H10(2)**, **H13(3), H19(4)**, **H25(1)**, H26(1), H27(1), H28(3), **H29(1)**, H30(1)
Mediterranean	91	26	42	0.943 (0.014)	0.00719 (0.00034)	−1.53474	H1(1), H2(16), H3(1), **H4(3)**, H5(1), H6(2), H7(1), **H8(12)**, H9(1), **H10(7)**, H11(1), H12(2), **H13(1)**, H14(4), H15(1), H16(1), H17(1), H18(1), H20(3), H21(1), H22(1), H23(1), H24(3), **H29(1)**, H33(3), H34(1), H36(2), H37(1), H38(1), H39(1), H40(1), H41(1), H42(1), H43(1), H44(1), H45(1), H47(3), H48(1), H49(1), H50(1), H52(1), H53(2)
Rest of Europe	56	16	10	0.656 (0.061)	0.00304 (0.00052)	−0.43914	**H8(31), H10(9), H13(8), H19(2), H25(1),** H31(1), H32(1), H35(1), H46(1), H51(1)
C. ohridella	107	31	22	0.696 (0.046)	0.00387 (0.00046)	−1.20473	**H4(1)**, **H8(57)**, **H10(11)**, **H12(1)**, **H13(11)**, **H14(1)**, **H19(5)**, H25(2), H26(1), H27(1), H28(3), **H29(1)**, H30(1), H31(1), H32(1), **H33(2),** H34(1), H35(1), H40(1), H46(1), H51(1), H53(2)
B. oleae	54	16	32	0.956 (0.018)	0.00746 (0.00051)	−1.52617	**H2(10)**, H3(1), **H4(3)**, H5(1), H6(2), H7(1), H9(1), **H10(3)**, H11(1), **H14(3)**, H17(1), H18(1), **H20(2),** H22(1), H23(1), H24(3), **H29(1)**, **H33(1),** H36(2), H37(1), **H38(1),** H39(1), H41(1), H42(1), H43(1), H44(1), H45(1), H47(3), H48(1), H49(1), H50(1), H52(1)
Other hosts	27	9	11	0.838 (0.050)	0.00545 (0.00048)	−0.34795	H1(1), **H2(6), H8(9), H10(4)**, **H12(1), H13(1),** H15(1), H16(1), **H19(1)**, **H20(1),** H21(1)

Shared haplotypes are indicated in bold

of them present exclusively on *C. ohridella* (Fig. 2). H8 was present in all countries and in 28 out of 31 localities sampled for *C. ohridella* (90 %), was recovered 66 times out of 188 samples (35 %) and in 57 % of parasitoids reared on *C. ohridella* (Table 1). H8 and its derived 1-step haplotypes were found in 75.5 % of individuals reared on *C. ohridella*. Other frequent haplotypes were H10 (n = 18), H2 (n = 16), H13 (n = 12,) and H19 (n = 6). H8, H10, H13 and H19 were mostly restricted to *Cameraria* and four other gracillarids, *Phyllonorycter platani* Staudinger, *Phyllonorycter millierella* Staudinger, *Phyllonorycter* sp. and *Metriochroa latifoliella* (Millière), the only exception being the yponomeutid *Atemelia*

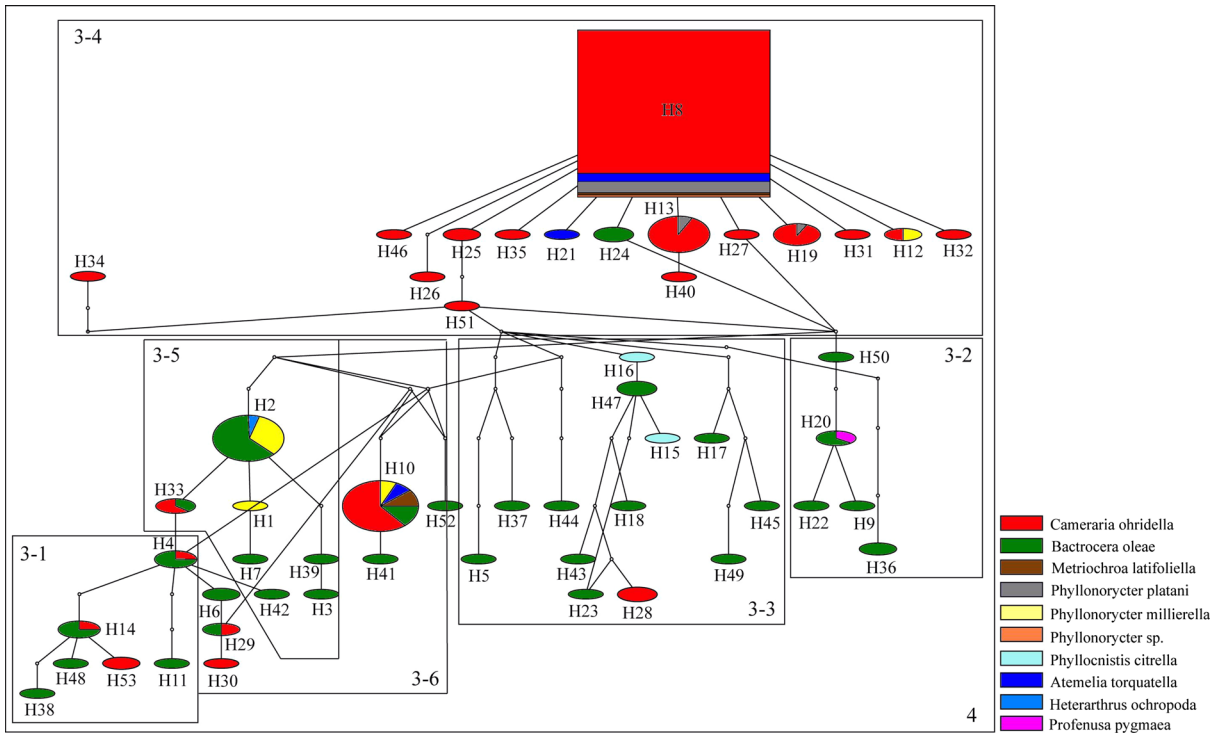

Fig. 2 COI statistical parsimony network and nesting levels identified by NCPA

torquatella (Lienig & Zeller). H8, H13 and H19 were never recorded on *B. oleae*. Furthermore, H8 was the most frequent haplotype both in the Balkans (54.8 %) and in central and northern Europe (56.4 %), whereas in the Mediterranean area it was the second most frequent (13.2 %, although never recorded on samples ex *B. oleae*) after H2 (17.6 %), which was exclusively found here. Haplotypes were genetically similar, with a maximum of 11 substitutions between pairs of haplotypes, and maximum uncorrected *p*-distance of 1.5 %. There was a clear divide between Balkans, Mediterranean and central and northern European haplotypes, as only three haplotypes (H8, H10, H13) were present in all geographic regions, whereas when samples were grouped by host as described above, only H10 was recovered in the three groups (Table 2). Geographical distribution of haplotype diversity in Fig. 3 showed a decrease in genetic diversity away from southern Europe, highlighting the Mediterranean as the most genetically diverse regions.

There is a split in the network, roughly corresponding to host distribution, with most haplotypes recovered on individuals attacking *C. ohridella* on the upper part, and most haplotypes of parasitoids of *B. oleae* in the lower part. Furthermore, the upper part of the network has a star-like pattern, while the lower part is more reticulated (Fig. 2). This suggests that there is genetic structure associated with the host. For both geographic and host groupings, the AMOVA revealed that most of the variation is explained by differences between individuals within populations, with a much smaller, but significant, amount of variation found among groups and among populations within groups (Table 3). The Mantel tests showed no significant correlations between geographic distance and pairwise F_{ST} ($r = 0.097$, $P = 0.104$) or corrected Kimura 2-parameter distance of pairwise differences between populations ($r = 0.075$, $P = 0.071$).

Nested clade phylogeographic analysis (NCPA) identified a 4-level nesting design, and the null hypothesis of no geographic association among sequences could be rejected only for clades 3-1, 3-3 (the inference key detected isolation by distance) and 3-2, for which long-distance colonization possibly coupled with subsequent fragmentation or past fragmentation followed by range expansion was inferred. Except for PM_CO2, PM_CO18, PM_CO58, PM_CO72, PM_PC2 and PM_PY1, the remaining

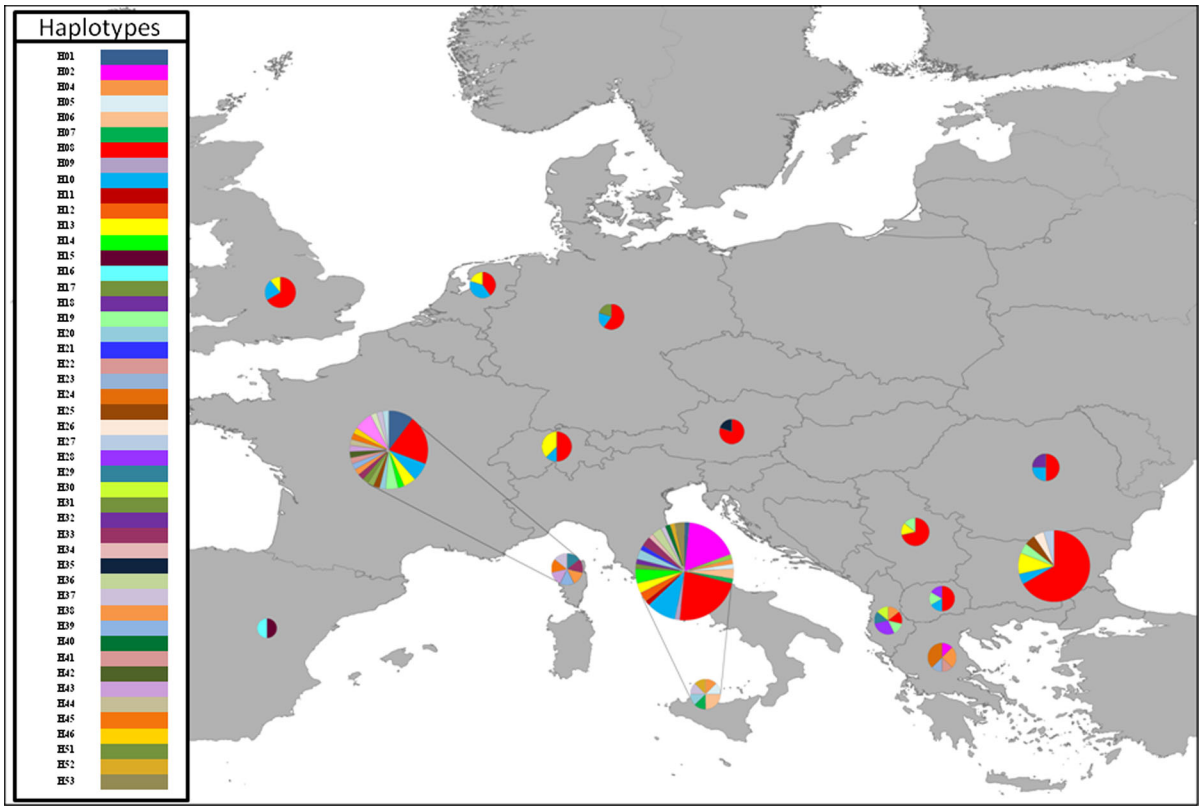

Fig. 3 Geographic distribution of the 53 haplotypes among the 14 sampled countries. Each pie represents a country. The size of the pie charts is correlated to the number of individuals sampled per country

Table 3 Partitioning of genetic variation at different hierarchical levels

Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation indices
Grouping by region					
Among groups	2	53.209	0.41630	17.66	$F_{CT} = 0.17658$*
Among population within groups	68	162.389	0.27823	11.80	$F_{SC} = 0.14333$*
Within populations	117	194.165	1.66296	70.54	$F_{ST} = 0.29460$*
Grouping by host					
Among groups	2	65.606	0.56644	23.22	$F_{CT} = 0.23219$*
Among population within groups	51	119.689	0.19495	7.99	$F_{SC} = 0.10408$*
Within populations	134	224.869	1.67813	68.79	$F_{ST} = 0.31211$*

* $P < 0.01$

22 samples for which there was a significant association between geography and haplotypes were reared on *B. oleae* from this host's entire Mediterranean range (Fig. 2).

Mismatch distribution (MMD) analysis could not reject the model of sudden demographic and spatial expansion because of a high concordance of COI data with the predicted distribution. Indeed, R p-values and SSD p-values were all not significant for the Mediterranean, the Balkans and central-western Europe (for demographic expansion: SSD *P*-value = 0.100, 0.591 and 0.402, and R *P*-value = 0.270, 0.780 and 0.540, respectively; for spatial expansion: SSD *P*-value = 0.404, 0.681 and 0.220, and R *P*-

value = 0.712, 0.884 and 0.693, respectively) (5,000 bootstraps). However, the distribution was unimodal only for the Mediterranean group.

Discussion

Genetic variability and choice of molecular markers

Phylogeography is a powerful tool to infer evolutionary processes at the intraspecific level and among closely related species (Avise 1998, 2009). Finding nuclear markers that are variable at intraspecific level in parasitoids can be a difficult task (Hernandez-Lopez et al. 2012; Gebiola et al. 2012). ITS2 showed low levels of intraspecific variability. The low intraspecific variation and the wide sharing of ribotypes among specimens throughout the geographic range and hosts suggest high levels of gene flow among populations of *P. mediterraneus*. Therefore, we used mitochondrial data to determine the demographic history of a parasitoid associated with invasive pests by direct interpretations from geographically contextualized gene genealogies, by reporting hierarchically partitioned summary statistics, and by permutation tests on various summary statistics to test for demographic expansion and/or geographic structuring of genetic variation.

Recent demographic range expansion

Data presented here are somewhat contrasting. On one hand, Tajima's D is always not significantly different from zero, which should indicate lack of geographic structure and rejection of the sudden expansion model and no founder effect. Similarly, NCPA did not detect range expansion for *Cameraria*-associated haplotypes. On the other hand, the star-like pattern of the haplotypes associated with *C. ohridella*, along with their geographic distribution and frequency, is typical of a recent expansion indicating that *P. mediterraneus* could have experienced a demographic expansion similar to *C. ohridella*. This finding seems to be confirmed by the fact that, based on MMD analysis (non-significant R and SSD *P*-values), the model of sudden demographic and spatial expansion cannot be rejected, although only the Mediterranean grouping has a unimodal distribution. Furthermore, AMOVA results show that variation is somewhat related to geographic origin and host association, with a small but significant percentage of variation explained by difference among groups. However, the mantel test rejected the hypothesis of isolation by distance. This might be due to the fact that the *Cameraria* invasion is very recent and to the ongoing movement of haplotypes, in particular of H8, throughout Europe. Such movements might have obscured the pre-existing geographic structure before the spread of *Cameraria* in Europe.

Recent host switch to *Cameraria ohridella*

The lower genetic diversity of *P. mediterraneus* on *C. ohridella* than on *B. oleae* could be explained by a recent shift to *C. ohridella* or by the fact that *C. ohridella* feeds on a toxic plant, which could serve as a barrier to the adaptation of many haplotypes to this host. The reduction of haplotype diversity in *P. mediterraneus* when moving away from the Mediterranean area might be explained by a climatic barrier that could prevent many Mediterranean haplotypes from establishing in central and Western Europe. Indeed, mild winter conditions are likely to allow larger effective population sizes and a larger amount of within-population genetic variation as recorded also for other insects (Ferreira and Ferguson 2010). However, this seems not to be our case, because in Southern Balkans, for example, the mean temperatures are very similar to those recorded in Italy and because parasitism rate by *P. mediterraneus* seems not to be influenced by environmental conditions or by the residence time of *C. ohridella* (Grabenweger et al. 2010), which would indicate a co-introduction of both pest and parasitoid. However, a historical series of *P. mediterraneus* collected on *C. ohridella* should be analysed to confirm this hypothesis.

Our results indirectly confirm data recorded by Nardi et al. (2010) on the associations between olive fly and wild olive, as we found a high haplotype diversity of *P. mediterraneus* in Corsica and Sardinia, but also in Sicily and in part in Southern Italy where oleasters are common (Bronzini de Caraffa et al. 2002). This should be a further evidence of a long coevolution with *B. oleae* that is congruent with the hypothesis that the shift of *B. oleae* on wild olives happened during the Quaternary and post-glacial periods (Nardi et al. 2010), differently from what

previously hypothesized (Nardi et al. 2005). Therefore it is interesting to note that, notwithstanding this long coevolution, H8 has not yet been found in these islands and in general in the area where wild olives are common and *C. ohridella* is absent, and not even in areas of sympatry of olives and horse chestnuts. Therefore, H8 seems to be mainly specialized on *C. ohridella* and was found only where this host is present.

A parasitoid haplotype specialized on *C. ohridella*

Considering the pattern of *C. ohridella*'s haplotype distribution, in particular haplotype A (Valade et al. 2009), it is possible that there has been a specialization of *P. mediterraneus*'s H8 on *C. ohridella*'s invasive haplotype A, a hypothesis deserving further testing. In a recent study on another eulophid parasitoid, *Pediobius saulius* (Walker) a haplogroup associated with *C. ohridella* and highly prevalent in southern Balkans was found (Hernandez-Lopez et al. 2012). The adaptation of a particular haplotype or haplogroup to *C. ohridella* is challenging since leaves of *A. hippocastanum* are known to contain toxic substances that are believed to play a role in protecting the leafminer against natural enemies (Ferracini and Alma 2007). It would be interesting to test whether specimens with the ancestral haplotype H8 have a higher capacity to detoxify those toxins. Ancestral haplotypes are indeed known to have a higher adaptive capability in other systems (Diamantidis and Carey 2011). The high specificity of metabolic differences due to the quantitative expression of existing or novel enzymes, especially detoxifying ones, may allow shifts onto novel hosts which may ultimately become adaptive to the point that a new sub-specific forms or ultimately full species arise (Janz 2011; Loxdale 2010). If trade-offs in fitness on alternate hosts are common, adaptation to one host can prevent adaptation to another (Baer et al. 2004), and this could explain why we did not find H8 on *B. oleae*. Therefore, it is not to be excluded that H8 individuals may at some point become specialized on *C. ohridella* and reproductively isolate themselves from other populations. However, another possible explanation for the prevalence of H8 could be 'gene surfing', a phenomenon that is due to strong genetic drift taking place in populations located at the front of the expansion. Low-frequency alleles can thus surf on the wave of advance of a population range expansion, reaching high frequencies and spreading over large areas, leading to potentially large allele frequency differences between the source and the edge of the spatial expansion (Lawson Handley et al. 2011).

Parasitoid host switch in the Balkans and host-tracking of *C. ohridella* invasion

We argue that our data support the host-tracking hypothesis, that is, a few haplotypes that appear to be more specialized on *C. ohridella* (H8, H13, H19) followed the leafminer from the Balkans and spread into central and northern Europe. Some questions arise: had such haplotypes always been associated with *Cameraria* in the Balkans? Did they come from the Mediterranean and/or Europe? Or did they locally adapt from other hosts in the Balkans and did they later track the horse chestnut leafminer over Europe? Multiple evidences converge toward the latter scenario. First, parasitism of *C. ohridella* by *P. mediterraneus* in the Balkans, and especially in the Southern Balkans, is very low or null (Freise et al. 2002; Tomov, personal communication) and the parasitoid phenology is not well synchronized with that of *C. ohridella* (Freise et al. 2002; Grabenweger et al. 2010). If *P. mediterraneus* had always been associated to *C. ohridella*, we would expect higher percentages of parasitism, a well-adapted life cycle, and likely the existence of a host-specific and genetically distinct lineage. Second, although lower than that observed in the Mediterranean, haplotype diversity is slightly higher in the Balkans than in the rest of Europe. If the *C. ohridella*-specialized haplotypes had come from Europe, we would expect lower haplotype diversity in the Balkans due to founder effect related to the inaccessibility of several natural and artificial horse chestnut stands in the Southern Balkans (Lees et al. 2011). Third, the mountain regions of the Iberian, Italian and Balkan Peninsulas have been identified as the three main ice-free refugia from the Quaternary glaciations for many plants and animals, and the populations in them eventually recolonized Europe (Hewitt 2001). The hosts that can be surely associated with *P. mediterraneus* are indeed *B. oleae* and those feeding on *Celtis*, *Ulmus*, *Populus* and *Aesculus* (Gebiola et al. 2009). *Olea*, *Celtis*, *Ulmus*, *Populus* have a Balkan-Mediterranean distribution, while *Aesculus* is a Tertiary relict only present on the Balkan

Peninsula as *A. hippocastanum* (Xiang et al. 1998). Fourth, considering a maximum distance among haplotypes of 1.5 %, and a mutation rate of 2.3 %/million years (Brower 1994, but see Welch and Bromham 2005 when there is no constant rate of molecular evolution), the variability we observe (especially on samples ex *B. oleae*, that are the most diverse) could have been generated on this host. Therefore, we argue that the natural hosts of *P. mediterraneus* are *B. oleae* and Gracillariidae, Tenthredinidae and Yponomeutidae feeding on plants with Mediterranean-Balkan distribution. Then, over the past 25 years, some haplotypes switched to *C. ohridella*. When *C. ohridella* expanded its range from the southern Balkans, some haplotypes of *P. mediterraneus* commonly present in the Balkans on the above-mentioned native hosts might have locally adapted first, then tracked the host all over Europe. Hence, both host-tracking and ecological sorting could be at play.

Conclusions

Although several recent studies have highlighted that adaptation to invaders by parasitoids often requires a time lag (Roy et al. 2011), few studies (Nicholls et al. 2010; Hernandez-Lopez et al. 2012) have shown what type of adaptation occurs in different populations of a parasitoid wasp. In the present paper, we highlight the importance of studying the intraspecific variability of parasitoids to understand how different haplotypes adapt to different hosts across their distribution range. Our data suggests that *P. mediterraneus* may have switched from native hosts in the Balkans to *C. ohridella* and then tracked the recent invasion of this host from the Balkans to central and Western Europe. The hypotheses here put forward, based on mtDNA dataset, need further testing by expanding the sampling effort on native hosts, especially from the Southern Balkans. If confirmed, this would be the first case of a single or few haplotypes of a parasitoid that successfully host-tracked an invasive host.

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