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Geographic structure with no evidence for host-associated lineages in European populations of *Lysiphlebus testaceipes*, an introduced biological control agent

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Abstract

Lysiphlebus testaceipes (Cress.) is an aphidiine parasitoid originally introduced to Europe as a biological control agent of citrus aphids in the Mediterranean. It has rapidly become widespread in coastal areas continuing gradually to expand inland. *Lysiphlebus testaceipes* exploited a large number of aphids in Europe, including new hosts and significantly changed the relative abundance of the native parasitoids. This behavior may reflect a broad oligophagy of the introduced parasitoid or it may require the evolution of host specialization that results in genetically differentiated subpopulations on different hosts. To address this issue we used the mitochondrial cytochrome oxidase subunit I and seven microsatellite loci to analyze the structure of genetic variation for *L. testaceipes* samples collected from 12 different aphid hosts across seven European countries, as well as some samples from Benin, Costa Rica, USA, Algeria and Libya for comparison. Only five COI haplotypes with moderate divergence were identified overall. There was no evidence for the association of haplotypes with different aphid hosts in the European samples, but there was geographic structuring in this variation. Haplotype diversity was highest in France, where *L. testaceipes* was introduced, but only a single haplotype was detected in areas of south-eastern Europe that were invaded subsequently. The analysis of microsatellite variation confirmed the lack of host-associated genetic structure, as well as differentiation between populations from south-western and south-eastern Europe. The parasitoid *Lysiphlebus testaceipes* in Europe is thus an opportunistic oligophagous species with a population structure shaped by the processes of introduction and expansion rather than by host exploitation.

Key words: *Lysiphlebus testaceipes*; microsatellite; cytochrome oxidase I; biological control; parasitoids

1. Introduction

Genetic variability and behavioral plasticity are important traits of parasitoids to be used as potential biological control agents (Rehman and Powell, 2010). Parasitoids may vary in terms of their capacity to include the target species in their host range, how quickly they establish and spread in the introduced area, but also in their competitive effects on native parasitoids and the potential of invading non-target habitats. Many cases of classical biological control failed because the introduced parasitoid populations were not adapted to the local environment, whereas others have had undesirable impacts on non-target species (Boivin et al., 2012).

The evolution of host specialization is an important consideration when employing parasitoids for biological control of pest aphids. This is particularly true for parasitoids of the subfamily Aphidiinae (Hymenoptera: Braconidae), as different species show different degrees of host-specificity, ranging from strict specialization in only one species to parasitization of more than a hundred aphid hosts in different types of habitats and geographical areas (Starý, 1981). This diversity in the host range of aphid parasitoids has been explained by different authors using various ecological and biological factors affecting the parasitoid-host interactions over the evolutionary time scale e.g. invasion status, host plant associations and seasonal host plant alternations of the aphid hosts, chemical responses of the plants to aphid infestation and the ability of the parasitoid to recognize these chemical cues during host search, interactions with other parasitoids etc. (Porter and Hawkins 1998; Vinson, 1998; Storeck et al., 2000; Tentelier et al., 2005). The host use patterns of aphidiine parasitoids are not only determined by the aphids that physiologically support the development of parasitoids, but also by the host acceptance that may be constrained by different behavioral processes (Strand and Obrycki, 1996; Poppy et al., 1997; Vinson, 1998; Tentelier et al., 2005). In search for a suitable aphid host, parasitoids are faced with a complex environment and their success depends on

several actions including the host habitat location, host location, host recognition, host acceptance, host suitability and host regulation (Vinson, 1998; Rehman and Powell, 2010).

Different aphid hosts in the introduced area of an aphid parasitoid used for biological control may represent different selective environments that require different adaptations (Antolin et al., 2006), which in turn may affect their potential as biocontrol agents. Specialization in a specific aphid host along with physiological and morphological adaptations can lead to genetic isolation by adaptive divergence (Dres and Mallet, 2002; Lajeunesse and Forbes, 2002). For this reason, the impact of host specialization on the genetic structure of aphid parasitoids is an important question for both evolutionary and applied entomology (Tremblay and Pennacchio, 1988, Lozier et al., 2008,b).

Studying the patterns of molecular variation in parasitoid populations could provide an answer to the question of whether geographic or ecological factors prevail in promoting the population differentiation. The increased use of genetic markers in population studies of biological control agents provides an opportunity to study the evolutionary processes underlying the establishment after their introduction. Additionally, it contributes to increasing the precision of the pre-release risk assessment of potential agents and also provides an opportunity for controlled mass production of specific parasitoids (Rehman and Powell, 2010).

Lysiphlebus testaceipes (Cress.) (Aphidiinae) is a solitary parasitoid with a host range exceeding 100 aphid species in association with diverse plants (Pike et al., 2000). This parasitoid has been introduced from Cuba to Southern France in 1973 to control the aphids *Toxoptera aurantii* (Boyer de Fonscolombe) and *Aphis spiraecola* Patch on *Citrus* trees (Stary et al., 1988a). Post-colonization studies in the introduced area determined that within a short period of time *L. testaceipes* had established over the whole of Mediterranean, including the coastal areas of southeastern Europe, North Africa and Turkey (Starý et al., 1988b; Cecilio, 1994; Suay and Michelena, 1997; Kavallieratos et al., 2004; Laamari and Coeur d' Acier, 2010; Havelka et al., 2011; Satar et al., 2012). Moreover, it continued to gradually expand towards the interior of the Iberian Peninsula (Starý et al., 2004), in

109 accordance with its potential to establish in cooler climates of northern Europe as well (Hughes et al.,
110 2011).

111 In the introduced area *L. testaceipes* exhibited an opportunistic pattern of acquiring new hosts.
112 Besides the citrus groves with their target aphids, it also established in other ecosystems acquiring
113 over 20 other aphid species as hosts, some of them new for its world host range (Starý et al., 2004;
114 Kavallieratos et al., 2005; Tomanović et al., 2009; Kavallieratos et al., 2010). Eventually, the
115 numerous non-target effects led to its exclusion from the positive list of recommended biological
116 control agents by EPPO in 2008 (EPPO, 2008-03-26/28).

117 It was unknown whether the introduced species' broad host range reflected extreme generalism
118 or the co-occurrence of multiple, host-associated lineages with narrower host ranges. This lack of
119 genetic information about the initial release and postcolonization changes of *L. testaceipes* was
120 classified as a lost unique chance in aphid parasitoid research (Stary et al.,1988a). The present study
121 aimed to obtain some of the missing data about the underlying processes of adaptation and gene flow
122 in the parasitoid populations. We presumed that the adoption of new aphid hosts might have required
123 some specialization that would be reflected by genetic divergence among parasitoids attacking
124 different hosts. To test this hypothesis, we analyzed variation at the mitochondrial cytochrome c
125 oxidase subunit I and seven microsatellite loci in *L. testaceipes* populations collected from different
126 aphid hosts across seven European countries.

127

128 **2. Material and methods**

129 *2.1. Field sampling*

130 Parasitoids were collected between 2006 and 2011 at localities in Spain, Italy, France,
131 Slovenia, Montenegro, Switzerland and Greece (Table 1). In addition to the European material, *L.*
132 *testaceipes* samples from the USA (Florida) and Costa Rica (close to the area of founder populations),
133 as well as Libya, Algeria and Benin were also included in molecular analyses. Lacking the samples of

the founder populations from Cuba, we have included in these non-European specimens to potentially gain insights into additional accidental or undocumented introductions that may have occurred. The material was collected from 12 different aphid hosts, including *Aphis nerii* Boyer de Fonscolombe, *A. gossypii* Glover, *A. parietariae* Theobald, *A. craccivora* Koch, *A. fabae* Scopoli, *A. ruborum* (Börner and Schilder), *A. fabae cirsiiacanthoidis* Scopoli, *A. hederae* Kaltenbach, *A. punicae* Shinji, *Toxoptera aurantii*, *Dysaphis plantaginea* (Passerini) and *Brachyunguis tamaricis* (Lichtenstein) (Table 1). Leaves with mummified aphid hosts were collected and placed into plastic boxes with gauze lids for parasitoid rearing. Adults of *L. testaceipes* emerging from the mummies were captured, placed in tubes with 96% ethanol and stored at 4 °C until molecular analyses.

2.2. DNA extraction, amplification and sequencing

Two genetic markers were chosen for molecular analyses of *L. testaceipes* populations in association with different aphid hosts: COI mtDNA sequences and microsatellites. Total nucleic acids from single wasps were extracted using a non-destructive TES method (Mahuku, 2004) in order to save the specimens for possible re-examination.

We genotyped part of the specimens at seven microsatellite loci developed by Fauvergue et al. (2005) for *L. testaceipes* (Lysi5a12, Lysi6f4, Lysi1b6, Lysi5c4, Lysi5e1, Lysi6b12, Lysi H02) (Table 1). Microsatellites were amplified in a single PCR reaction using the QIAGEN Multiplex PCR Kit in 10 µl volumes. Each reaction contained 1xQIAGEN Multiplex PCR MasterMix, including PCR-buffer (3mM MgCl2), a dNTP Mix and HotStarTaq DNA polymerase, 1µl of genomic DNA and 0.1mM of every locus-specific primer, each with specifically adjusted proportions of labeled/unlabelled forward-primers. The PCR cycling conditions were as follows: denaturation for 15min at 95 ^{o}C, followed by 30 cycles consisting of 30s at 94 ^{o}C, 90s at 52 ^{o}C and 60s at 72 ^{o}C. The final extension step was performed at 60 ^{o}C for 30min. Products were diluted 5 times and submitted to a fragment analysis on an ABI3130xl 16-capillary automated sequencer. The GeneMapper® Software v 4.1 (Applied Biosystems) was used to score the alleles.

The mitochondrial COI gene was amplified using the LCO1490 and HCO2198 primers (Folmer et al., 1994). Each PCR reaction was carried out in a volume of 20 µl, containing 1µl of extracted DNA, 11.8µl of H_20, 2µl of High Yield Reaction Buffer A (with 1xMg), 1.8µl of MgCl2 (2.25mM), 1.2µl of dNTP (0.6mM), 1µl of each primer (0.5µM) and 0.2µl of KAPA*Taq* DNA polymerase (0.1U/µl) (Kapabiosystems). The PCR protocol included an initial denaturation at 95 °C for 5 min, 35 cycles consisting of 1 min at 95 °C, 1 min at 54 °C, 2 min at 72 °C, and a final extension at 72 °C for 10 min. Amplified products were run on 1% agarose gel, stained with ethidium bromide and visualized under a UV transilluminator. All amplified COI products were purified using QIAquick PCR purification Kit (QIAGEN) according to the manufacturer's instructions and sequenced using automated equipment (BMR Service, Padova, Italy).

Table 1.

2.3. Phylogenetic analyses

Sequences of COI were manually edited in FinchTV v.1.4.0 (www.geospiza.com) and aligned using the Clustal*W* program integrated in MEGA5 (Tamura et al., 2011). Estimates of evolutionary divergence between sequences were conducted using the Kimura-2-parameter model (Kimura, 1980). Mitochondrial COI was amplified and sequenced for two other parasitoids of the same subfamily, *Areopraon chaitophori* Tomanović and Petrović and *Ephedrus plagiator* (Nees), which were used as outgroups to root the trees. A maximum parsimony tree was constructed using PAUP*4.0b10 (Swoford 2002). A Bayesian phylogenetic tree was constructed using the program MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). The best-fitting model of sequence evolution based on the Akaike Information Criterion was the general time reversible model, as determined with Modeltest 3.7 (Posada and Crandall, 1998). The Bayesian Inference analysis was conducted running two Markov Chain Monte Carlo searches each with one cold and three heated chains, for 5 million generations,

sampling every 100 generations. The first 12500 trees were discarded as a burn-in. The average standard deviation of split frequencies was below 0.01. Potential scale reduction factors (PSRF) were all approximately equal to one. To confirm the convergence of the parameters we used the program Tracer v1.5.0 (Rambaut and Drummond, 2003) and the program FigTree 1.3.1. to view the consensus tree with posterior probabilities (Rambaut, 2006-2009). A haplotype network using statistical parsimony with a confidence limit of 95% was created using the program TCS ver. 1.21 (Clement et al., 2000).

2.4. Population genetic analyses

Standard population genetic analyses were restricted to microsatellite genotypes of *L. testaceipes* from southern France, because this was the only large sample from a restricted region that had multiple host aphids represented in meaningful numbers. We used the FSTAT 2.9.3 software (Goudet, 2001) to test for deviations from Hardy-Weinberg and linkage equilibrium and to test for genetic differentiation among subsamples collected from different aphid hosts. We used the option of the test for genetic differentiation in FSTAT that does not assume Hardy-Weinberg equilibrium.

All microsatellite genotypes were included in a Bayesian clustering analysis using the software STRUCTURE 2.3.3 (Pritchard et al., 2000; Falush et al., 2003) to infer population structure without prior knowledge of the genotypes' host- and geographic associations. For all simulations we used the admixture model and uninformative priors. The number of genetic clusters (K) was varied from 1 to 7, and we ran 5 independent simulations for each value of K with a burn-in period of 20'000 iterations, followed by 50'000 iterations. To infer the most probable number of genetic clusters based on the log probability of the data, we used the method of Evanno et al. (2005), as implemented in the software STRUCTURE HARVESTER (Earl and vonHoldt, 2012).

3. Results

3.1. Mitochondrial COI variation

Amplification of COI mtDNA sequences was successful for all 116 samples of *L. testaceipes* submitted to the analysis (Table 1). Aligned sequences were indel-free with 10 variable sites, all of which were parsimony informative. Only five different haplotypes were identified. Their sequences were deposited in GenBank (http://www.ncbi.nim.nih.gov) under accession numbers: haplotype H1 - JX470529, H2 - JX470530, H3 - JX470531, H4 - JX470532, H5 - JX470533.

The analysis involved mitochondrial sequences from all 5 haplotypes, with a total of 609 positions in the final dataset. Overall mean divergence between haplotypes of *L. testaceipes* was 0.8% (range 0.2–1.3 %).

The most numerous and widely distributed haplotype was H1 (67 sequences) which was found in samples collected from Montenegro, Slovenia, Libya, Switzerland, Greece and France, in association with eight different aphid hosts (Table 2). The haplotype designated as H2 was not determined in populations from Europe and included samples from Benin, Costa Rica and United States collected from *A. gossypii, T. aurantii* and *A. fabae*, respectively. Haplotype H3 was detected only in two samples from Spain parasitizing *A. nerii* and H5 only in France in association with *A. fabae, A. nerii, A. hederae, A. ruborum* and *A. fabae cirsiiacanthoidis* (Table 2). Haplotype H4 is represented by 32 individuals from Spain, Italy, France and Algeria, in association with 5 different aphid hosts.

Table 2.

Estimation of a haplotype network using TCS ver. 1.21 produced a single network with no ambiguities (Fig. 1). There was no consistent pattern of haplotype association with hosts or the

sampled region. Different aphid hosts within the same region yielded parasitoids with the same haplotype and parasitiods from the same aphid in different regions often possessed different haplotypes, suggesting a lack of clear genetic differentiation among *L. testaceipes* populations associated with different host taxa.

Fig. 1

Depicting the haplotype frequencies on a map of Europe (Fig. 2) shows the highest diversity of haplotypes in southern France (H1, H2, H4, H5), whereas further east and south east, from Slovenia to Greece, just one haplotype occurs (H1). Haplotypes detected in Spain were H3 and H4, with the latter also being present in Italy. The Bayesian and maximum parsimony phylogenetic trees inferred from the COI fragments of *L. testaceipes* from 12 different aphid hosts and 12 countries are also presented in Fig. 2. Grouping of haplotypes within the same taxon has maximal bootstrap support of 100% under maximum parsimony and of 100 posterior probability under Bayesian inference. Within the *L. testaceipes* group, tree topology obtained poor statistical support for individual haplotypes which corresponds to the low overall divergence of the COI sequences.

Fig. 2

3.2. Microsatellite variation

We observed a moderate degree of variation at the microsatellite loci in our sample of *L. testaceipes* specimens. One locus was monomorphic (Lysi5c4), at the others we observed between three and seven alleles (mean number of alleles: 4.14). There was no evidence for significant linkage disequilibrium between any pair of loci in the sample from France, but two loci exhibited significant homozygote excess: Lysi1b6 ($P = 0.014$) and Lysi5a12 ($P < 0.001$). Based on tests not assuming

Hardy-Weinberg equilibrium, there was no evidence for genetic differentiation between wasps collected from different aphid hosts in the French *L. testaceipes* (global $P = 0.546$), with an estimate of F_{ST} according to Weir and Cockerham (1984) of -0.013, that is effectively zero.

The Bayesian clustering analysis with STRUCTURE including all genotypes confirmed the lack of host-associated genetic differentiation. The distribution of log-likelihoods for the number of genetic clusters (K) increased rapidly with K and plateaued already at $K \geq 3$. Accordingly, the method of Evanno et al. (2005) identified $K = 2$ as the most likely number of genetic clusters and $K = 3$ as the second most likely number. Higher values of K were very unlikely. There was a strong geographic signal in the distribution of individuals assigned to the different clusters, but no evidence for host-associated genetic structure (Fig. 3). Under $K = 2$, all European individuals from France, Spain, Italy and Switzerland were assigned with high probabilities to cluster 2, independent of what aphid species they emerged from (Fig. 3A). All individuals from Montenegro and Greece were assigned with high probabilities to cluster 1, again independent of aphid host. Only the sample from Slovenia, which is also geographically in-between, consisted of intermediate genotypes that could not be assigned to either cluster with confidence. As a *post hoc* analysis following from this observation, we split all European samples into two groups, those from south-eastern Europe (Slovenia, Montenegro and Greece) versus all others (mostly France), and estimated their genetic differentiation at the microsatellite loci. The groups were strongly and significantly differentiated ($F_{ST} = 0.267$, $P < 0.001$).

The few non-European samples we had obtained also exhibited some interesting patterns in the STRUCTURE analysis. Individuals from Florida, Costa Rica, Benin and Libya fell into the same cluster as those from south-eastern Europe (Greece and Montenegro), whereas the two individuals from Algeria as well as the only individual from the North of the USA (Washington State) fell into the same cluster as all the French samples (Fig. 3A). Under $K = 3$, the genotypes from France and neighboring areas remained a well-defined group, but the genotypes belonging to cluster 1 under $K = 2$

were split into two distinct groups (Fig. 3B), one comprising individuals from Florida, Costa Rica and Benin, the other comprising the individuals from south-eastern Europe and Libya.

Fig. 3.

4. Discussion

After its introduction in Europe to control pest aphids on citrus trees, *L. testaceipes* has shown a rapid spread beyond the target habitats and a substantial expansion of its host range (Starý et al., 1988b). Here we show that the acquisition of new hosts in the invaded range is unlikely to be driven by the evolution of host-specialized lineages. Neither the mitochondrial COI sequences nor the nuclear microsatellite loci provided any evidence of host-associated genetic differentiation in European populations of *L. testaceipes*. On the other hand, the genetic variation shows a clear geographic structuring in Europe, apparently reflecting the population history of this biocontrol agent in its introduced range.

The highest diversity of haplotypes was determined in France, the area of introduction from where the populations of the parasitoid expanded along the Mediterranean coast and subsequently into central and south-eastern Europe (Starý et al., 1985; Costa and Starý, 1988; Lumbierres et al., 2003; Kavallieratos et al., 2005; Havelka et al. 2012). With a total of only five haplotypes across all specimens, the level of genetic variation was moderate for mitochondrial COI sequences. Only haplotype H1, the rarest of the three haplotypes found in French samples, was detected in south-eastern Europe between Slovenia and Greece, suggesting a narrow genetic basis of the parasitoids that colonized the Balkan peninsula. The genetic differentiation between *L. testaceipes* populations in south-eastern and south-western Europe was also obvious in the analysis of the nuclear microsatellite data. Individuals from France and the Balkans were assigned to different genetic clusters with high confidence, whereas individuals from Slovenia were intermediate and exhibited genetic admixture

306 between these clusters. Note that this structure would also be consistent with the scenario of a second,
307 undocumented introduction of *L. testaceipes* somewhere on the Balkan peninsula, followed by a
308 northward spread. This is purely speculative, however, since we have no independent evidence for
309 such an event.

310 Samples from outside of Europe were too few to allow any firm conclusions, but they did
311 exhibit some patterns worth mentioning. The presence of a COI haplotype in American samples that
312 was not found in Europe as well as some nuclear genetic differences (at least under $K = 3$) is not
313 surprising for a species native to the New World. The parasitoids introduced to Europe could only
314 have comprised a small subset of the genetic variation present in the native range. The few individuals
315 we obtained from African countries were genetically very different. When we assumed $K = 2$ genetic
316 clusters in the STRUCTURE analysis, the two individuals from Algeria clustered with the French
317 samples, whereas the individual from Libya clustered with the samples from the Balkans. The
318 individuals from Benin were also closer to parasitoids from the Balkans, but in the analysis assuming
319 $K = 3$ clusters, they clearly grouped with New World samples from Florida and Costa Rica. This was
320 further supported by parasitoids from Benin, Costa Rica and Florida sharing haplotype H2, which was
321 not present in any European samples. Thus, the *L. testaceipes* populations currently present in Africa
322 appear to have very diverse origins.

323 While our results suggest that different host use is not a driving agent for genetic
324 differentiation within introduced *L. testaceipes* populations in Europe, this question remains to be
325 investigated for the native range of *L. testaceipes*. In this context it is worth pointing out that a
326 congener of *L. testaceipes* native to Europe, *L. fabarum*, has a broad host range as well, but exhibits
327 significant genetic differentiation among populations collected from different hosts (Sandrock et al.
328 2011).

329 Situations similar to that of *L. testaceipes* in Europe have been reported for other aphidiine
330 parasitoids in biological control programs as well, e.g. for *Diaeretiella rapae*, which was reported to

exhibit fitness trade-offs between alternative hosts indicative of host specialization in the introduced area of North America (Baer et al., 2004). However, mtDNA sequence analyses revealed some geographical structuring, but no association between mitochondrial haplotypes and host species in either the ancestral or the introduced range (Baer et al., 2004). Another post-introduction study conducted by Baker et al. (2003) on the same parasitoid species in Australia (using microsatellites) also found no evidence of host-associated genetic structure after introduction. A similar case was reported by Lozier et al. (2009) who have analyzed mitochondrial DNA and seven microsatellite loci of the parasitoid *Aphidius transcaspicus*, an important natural enemy of *Hyalopterus* spp. in the Mediterranean. Also in this parasitoid, there was significant geographic structuring but no evidence for host-associated diversification.

Overall, these data suggest that there is sufficient gene flow among parasitoids using different host aphids in their introduced range as to disrupt any associations between particular genotypes and aphid host species. These introduced species appear to have already possessed the ability to exploit new ecological ranges before they were introduced, and there is little or no evidence at present that genetic specialization of the introduced parasitoids occurs and is important for their success in biological control (Louda et al., 2003; Hufbauer and Roderick, 2005). Yet it should be considered that the period over which the effects of biological control are typically monitored might be insufficient to observe the evolution of host-associatied differentiation (Roderick and Navajas, 2003).

The absence of evident genetic diversification in the European populations of *L. testaceipes* could be accounted for by a high behavioral plasticity that is not depending on the initial genetic variability. Tentelier et al. (2005) indicated that *L. testaceipes* uses information from both, plants and hosts to adapt the patch use behavior. Among the major factors influencing a host selection behavior in parasitoids are experience and learning (Vinson, 1998). Parasitoids such as *L. testaceipes* that attack hosts on different plant species, learn to respond to specific plant volatile cues through associative learning during foraging (Lopez Perez et al., 2007). Associative learning redirects and broadens a

parasitoid's response to changing environments, including new aphid host/plant associations (Vinson, 1998), thus reducing the potential for genetic differentiation while at the same time increasing the probability of acquiring non-target hosts.

In contrast to biological control of weeds by herbivores, biological control programs of herbivorous arthropods with parasitoids have involved much less extensive host range testing to enhance the safety of introductions (Van Driesche and Hoddle, 1997). The case of *L. testaceipes*, and other aphidiine parasitoids exhibiting similar patterns in the invaded areas implies that a more cautious approach would be warranted. Louda et al. (2003) recommended that biological control programs with natural enemies of herbivores should be improved by primarily avoiding the use of exotic generalist parasitoids, by expanding the host-specificity tests, by incorporating population-level measurements of ecological risk and by defining the ecological risk criteria to target selection and consequently prioritize host-specific agents according to their effectiveness.

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602 **Table 1** List of *Lysiphlebus testaceipes* samples submitted to molecular analysis with designated
603 geographic origin and aphid host / plant associations
604

605 **Table 2.** Association of *Lysiphlebus testaceipes* COI haplotypes with aphid hosts
606

607 **Fig. 1.** Haplotype network obtained from 116 *Lysiphlebus testaceipes* mtDNA COI nucelotide
608 sequences using TCS. Numbered circles represent specific haplotypes, size of circle reflects the
609 number of individuals with that haplotype (not to scale). Smaller filled circles represent missing
610 haplotypes; lines between circles are mutational steps; colors represent the aphid host haplotypes are
611 associated with.
612

613 **Fig. 2.** A map of Mediterranean Europe is presented on the right, with the pie charts with haplotypes
614 frequencies. On the left is a phylogram obtained by Bayesian inference and maximum parsimony
615 analysis from the *L. testaceipes* COI sequences. Haplotypes are presented as H1, H2, H3, H4 and H5;
616 Ar ch – *Areopraon chaitophori* as the first outgroup; Ep pl – *Ephedrus plagiator* as the second
617 outgroup; Bayesian posterior probabilities ≥70% colored in black are shown above branches; Maxium
618 parsimony bootstrap support values are colored in red below branches with values above 50%
619 presented; scale bar indicates substitutions per site (0.03).
620

621 **Fig. 3.** Results from the Bayesian clustering analysis in STRUCTURE, using (A) $K = 2$ clusters or (B)
622 $K = 3$ clusters. Each vertical bar represents the genotype of an individual with different shadings
623 indicating the assignment probabilities to each of the clusters. Their geographic origins and the aphid
624 hosts from which parasitoids emerged are indicated at the bottom and the top of the Fig., respectively.

625 *Ac = Aphis craccivora, Afc = A. fabae cirsiiacanthoidis, Aff = A. fabae fabae, Ag = A. gossypii, Ah =*

626 *A. hederae, An = A. nerii, Ar = A. ruborum, Bt = Brachyunduis tamaricis, Dp = Dysaphis*

627 *plantaginea*.

628

629
630

Table 1

Aphid host	sampling date	Country	Locality	Plant	No of samples collected	No of COI sequences	No of microsatellite genotypes
Aphis nerii	5/26/2006	France	Antibes	*Nerium oleander*	2	2	0
Aphis ruborum	5/16/2006	France	Lunel, Camargue	*Rubus fruticosus*	9	7	9
Aphis fabae cirsiiacanthoidis	5/16/2006	France	Lunel, Camargue	*Carduus tenuiflorus*	4	4	3
Aphis hederae	5/16/2006	France	Lunel, Camargue	*Hedera helix*	3	2	3
Aphis ruborum	5/16/2006	France	Cote d'Azur, Grimaud	*Rubus fruticosus*	7	7	7
Aphis fabae cirsiiacanthoidis	5/17/2006	France	Cote d'Azur, Grimaud	*Carduus tenuiflorus*	3	2	3
Aphis hederae	5/17/2006	France	Cote d'Azur, Grimaud	*Hedera helix*	1	1	1
Aphis hederae	5/20/2009	France	Montélimar	*Hedera helix*	1	1	1
Aphis fabae cirsiiacanthoidis	5/20/2006	France	Montélimar	*Cirsium arvense*	1	0	1
Aphis hederae	5/21/2006	France	Remoulins	*Hedera helix*	1	0	1
Aphis fabae	5/21/2006	France	Remoulins	*Chenopodium album*	1	0	1
Aphis ruborum	5/21/2009	France	Remoulins	*Rubus fruticosus*	1	1	0
Aphis nerii	5/17/2006	France	Cote d'Azur, Grimaud	*Nerium oleander*	1	1	1
Aphis fabae cirsiiacanthoidis	5/22/2009	France	Romans	*Cirsium arvense*	3	1	1
Aphis fabae cirsiiacanthoidis	5/18/2006	France	Cote d'Azur, Le Muy	*Carduus tenuiflorus*	2	1	2
Aphis fabae	5/18/2006	France	Cote d'Azur	*Vicia faba*	4	4	3
Aphis fabae	5/17/2006	France	Cote d'Azur	*Chenopodium album*	2	0	1
Aphis fabae	5/2/2010	Greece	Kyparissia	*Galium aparinae*	1	1	1
Aphis gossypii	5/1/2010	Greece	Kyparissia	*Citrus aurantium*	3	3	1
Aphis fabae	5/2/2010	Greece	Kyparissia	*Papaver rhoeas*	1	1	0
Aphis parietariae	5/1/2010	Greece	Kyparissia	*Parietaria diffusa*	1	1	0
Aphis nerii	5/2/2010	Greece	Kalamata	*Nerium oleander*	2	2	1
Aphis nerii	5/4/2010	Greece	Kifissia	*Nerium oleander*	1	1	1
Aphis gossypii	5/1/2010	Greece	Kyparissia	*Hibiscus rosa sinensis*	1	1	0
Aphis fabae	5/2/2010	Greece	Kalamata	*Galium aparinae*	2	2	1
Aphis fabae	5/5/2010	Greece	Kalamata	*Pinpinella anisum*	2	0	1
Aphis hederae	5/9/2006	Italy	Romagna, Cesena	*Hedera helix*	1	1	1
Aphis nerii	8/7/2010	Libya	Derna	*Nerium oleander*	1	1	1
Aphis nerii	5/11/2008	Algeria		*Nerium oleander*	1	1	1
Dysaphis plantaginea	5/14/2008	Algeria		*Malus communis*	4	1	1
Aphis gossypii	5/29/2010	Benin	Hla Avame	*Capsicum annuum*	4	4	3
Aphis gossypii	5/12/2011	Benin	Benin	*Phaseolus sp.*	2	2	2
Toxoptera aurantii	1/10/2007	Costa Rica	San Hoze	*Eugenia wilsonii*	1	1	1

Aphis nerii	5/17/2010	Montenegro	Budva	*Nerium oleander*	1	1	1
Aphis nerii	5/24/2011	Montenegro	Bar	*Nerium oleander*	6	6	3
Aphis gossypii	5/24/2011	Montenegro	Bar	*Citrus deliciosa*	2	2	2
Aphis gossypii	5/25/2011	Montenegro	Tivat	*Citrus aurantifolia*	2	2	0
Aphis gossypii	5/23/2011	Montenegro	Ada bojana	*Citrus deliciosa*	2	0	1
Aphis fabae	5/24/2011	Montenegro	Petrovac	*Pittosporum tobira*	2	2	1
Aphis fabeae	5/24/2011	Montenegro	Bar	*Cirsium* sp.	1	1	0
Aphis fabae	5/24/2011	Montenegro	Bar	*Galium aparine*	1	1	0
Aphis fabae	5/24/2011	Montenegro	Bar	*Magnolia grandiflora*	1	1	0
Aphis fabae	5/24/2011	Montenegro	Bar	*Hedera helix*	1	1	0
Aphis gossypii	5/24/2011	Montenegro	Bar	*Tecoma radicans*	1	1	0
Aphis punicae	5/24/2011	Montenegro	Bar	*Punica grandiflora*	2	2	0
Aphis gossypii	5/24/2011	Montenegro	Bar	*Hibiscus rosa sinensis*	1	1	0
Aphis gossypii	5/25/2011	Montenegro	Tivat	*Citrus aurantifolia*	2	2	2
Aphis gossypii	5/24/2011	Montenegro	Bar	*Hybiscus syriacus*	2	2	0
Aphis fabae	5/24/2011	Montenegro	Bar	*Chamomilla recutita*	2	2	0
Aphis punicae	5/24/2011	Montenegro	Bar	*Punica granatum*	4	4	0
Aphis gossypii	5/25/2011	Montenegro	Tivat	*Citrus aurantium*	1	1	0
Aphis gossypii	5/24/2011	Montenegro	Bar	*Citrus japonica*	2	2	1
Aphis fabae	5/24/2011	Montenegro	Bar	*Abutilon sp.*	1	1	0
Aphis parietariae	5/24/2011	Montenegro	Bar	*Parietaria sp.*	1	1	0
Aphis gossypii	5/24/2011	Montenegro	Bar	*Chaenomeles japonica*	1	1	0
Branchyunguis tamaricis	5/24/2011	Montenegro	Bar	*Tamarix sp.*	6	6	4
Aphis nerii	6/17/2009	Slovenia	Portorož	*Nerium oleander*	6	6	6
Aphis craccivora	6/17/2010	Slovenia	Strujan	*Robinia pseudoacacia*	1	1	1
Aphis nerii	6/17/2010	Slovenia	Izola	*Nerium oleander*	3	2	3
Aphis fabae	11/27/2006	Spain	La Grania - Madrid	*Chenopodium album*	2	2	1
Aphis nerii	6/7/2010	Spain	Lleida	*Nerium oleander*	2	2	0
Aphis hederae	7/1/2006	Switzerland	St. Margrethen	*Hedera helix*	1	1	0
Aphis fabae	6/25/2009	Switzerland	Genève	*Chenopodium album*	1	0	1
Aphis fabae	7/20/2010	USA	Florida	*Solanum nigrum*	3	2	3
Aphis ruborum	12/30/2009	USA	WA, Yakima Co. Buena A9K	*Rubus* sp.	1	0	1

Table 2

aphid host	H1	H2	H3	H4	H5
Aphis fabae	13	2	0	5	1
Aphis nerii	20	0	2	2	1
Aphis gossypii	18	6	0	0	0
Aphis parietariae	2	0	0	0	0
Aphis craccivora	1	0	0	0	0
Aphis hederae	1	0	0	4	1
Aphis ruborum	0	0	0	14	1
Aphis fabae cirsiiacanthoidis	0	0	0	6	2
Aphis punicae	6	0	0	0	0
Toxoptera aurantii	0	1	0	0	0
Brachyunguis tamaricis	6	0	0	0	0
Dysaphis plantaginea	0	0	0	1	0
total	67	9	2	32	6

Figure 1

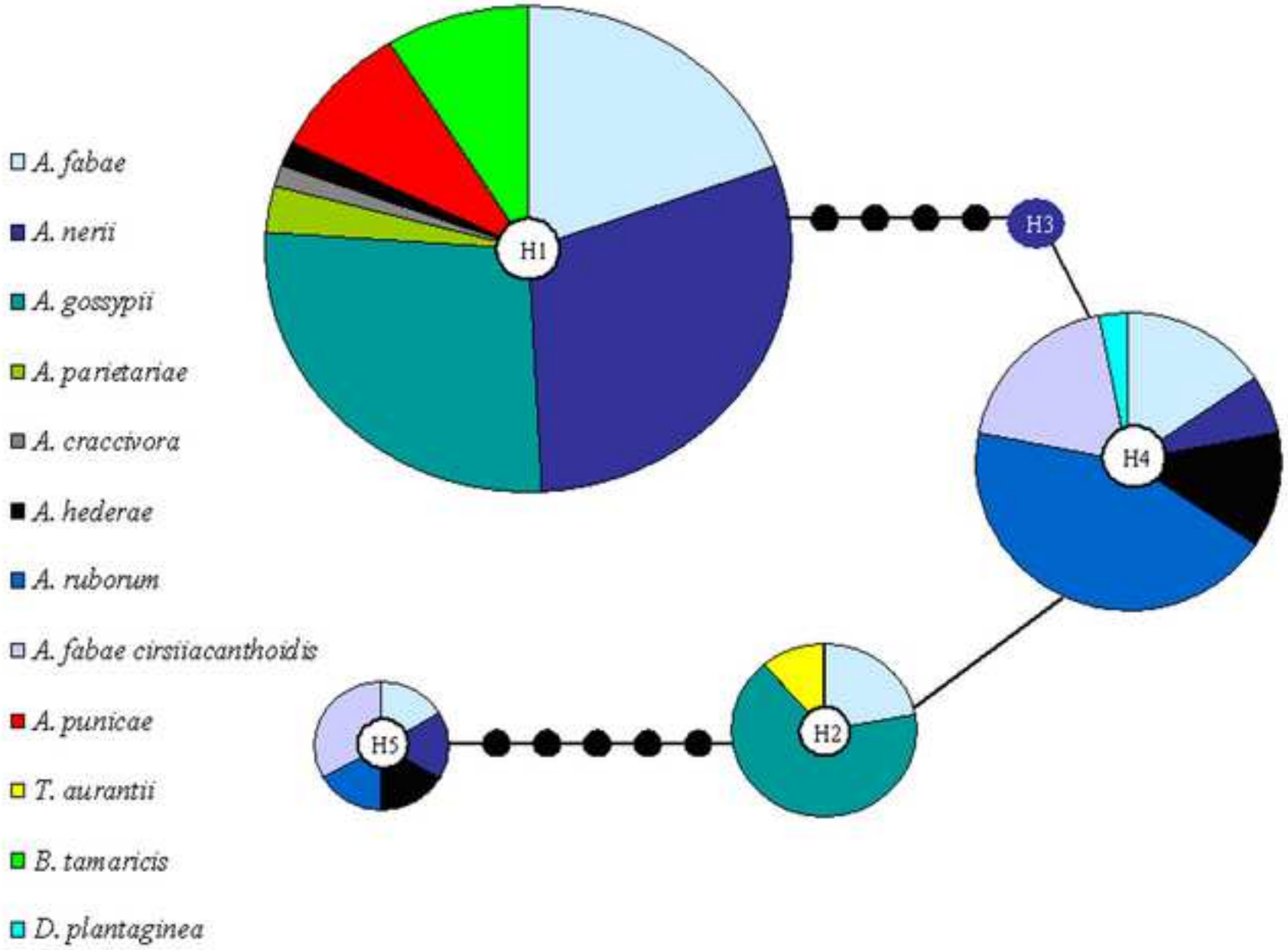
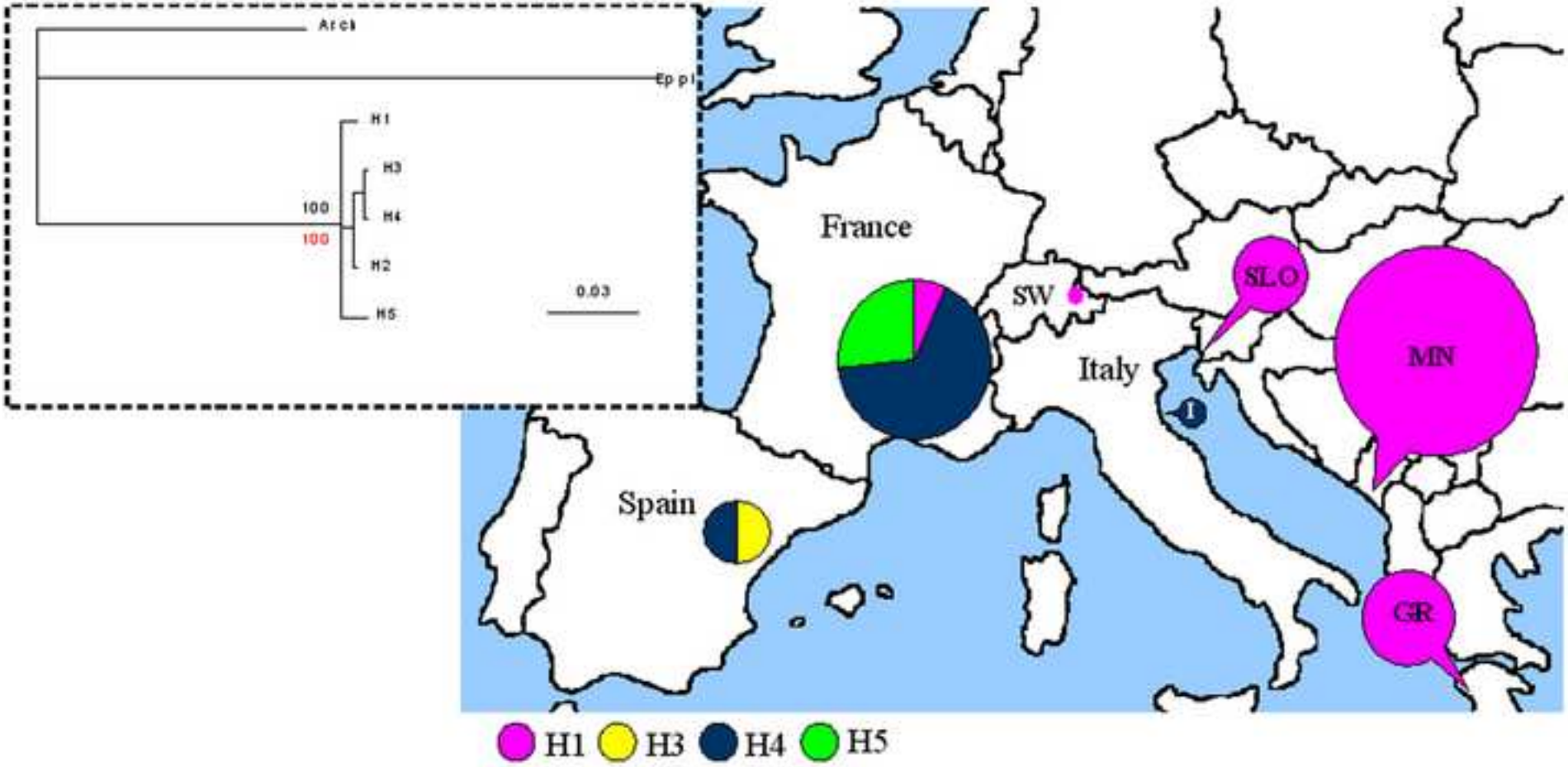
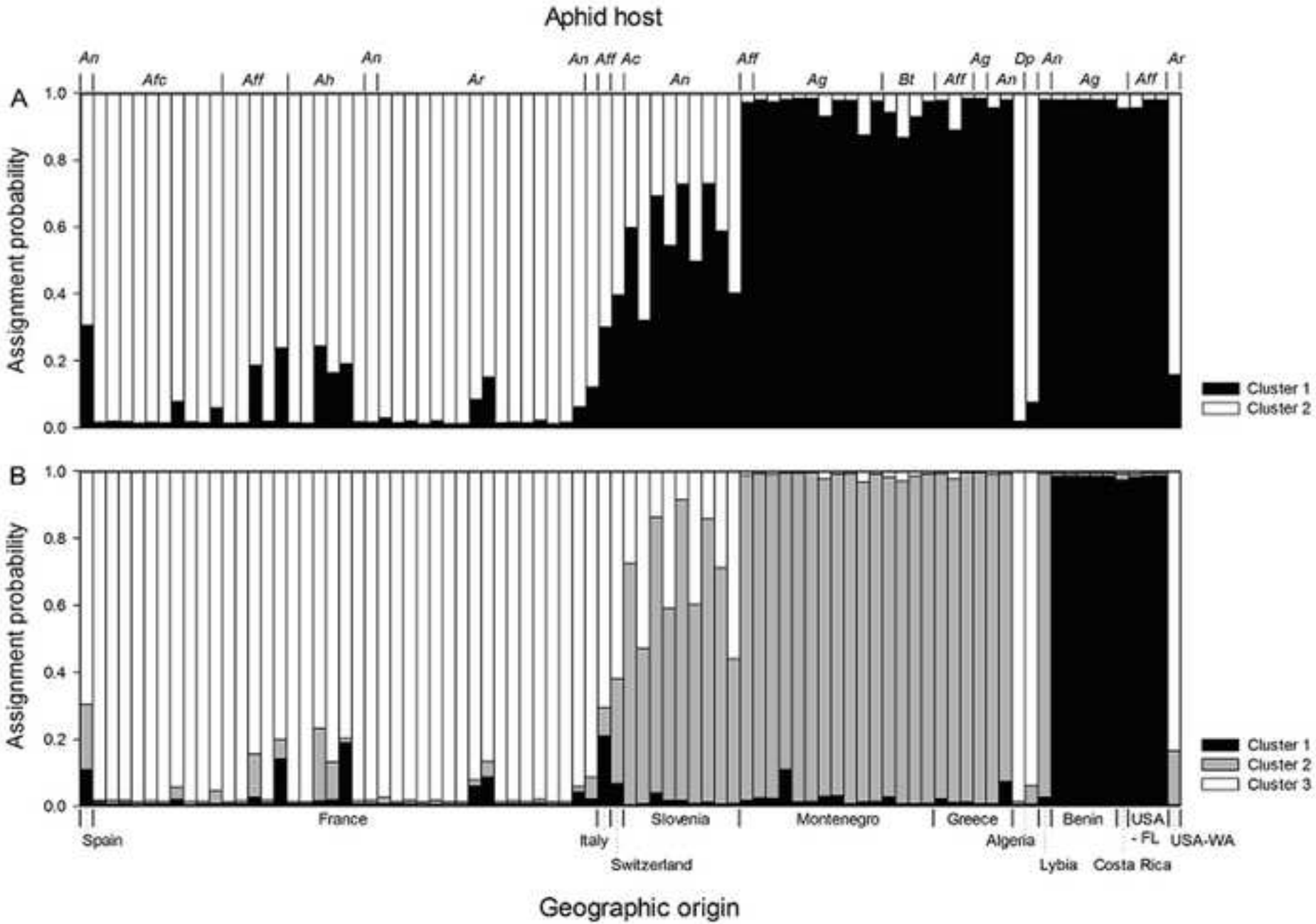


Figure 2



MN-Montenegro; SLO-Slovenia; GR-Greece; SW-Switzerland; I-Italy

Figure 3



637 Highlights

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639 *Lysiphlebus testaceipes* is an aphid parasitoid with opportunistic oligophagous behavior. ▯ Five mitochondrial
640 COI haplotypes identified with moderate divergence in European populations. ▯ No evidence of host-
641 associated genetic differentiation of COI gene or microsatellite loci. ▯ Geography substantially affects variation
642 of mitochondrial and nuclear loci in European samples. ▯ Genetic structure of populations is shaped by the
643 history of introductions and range expansion.

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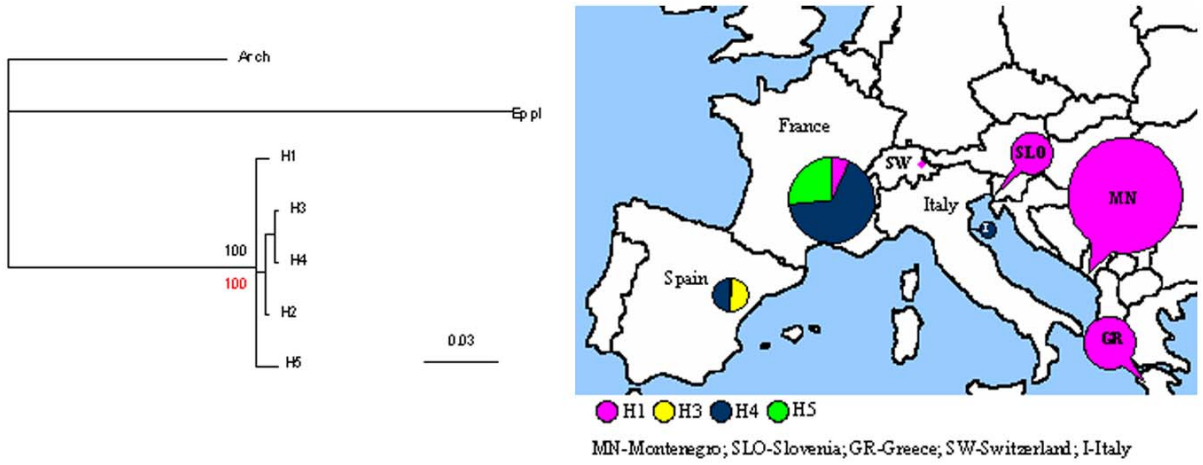

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