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RESEARCH

Interference of Field Evidence, Morphology, and DNA Analyses of Three Related *Lysiphlebus* Aphid Parasitoids (Hymenoptera: Braconidae: Aphidiinae)

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ABSTRACT. This study provides evidence on integrating the morphological, field, and laboratory data, and application of the cytochrome oxidase subunit I (COI) barcoding gene to the three asexual or sexual *Lysiphlebus* spp., i.e., *Lysiphlebus cardui* (Marshall), *Lysiphlebus confusus* Tremblay and Eady and *Lysiphlebus fabarum* (Marshall) (Hymenoptera: Braconidae: Aphidiinae). New aphid–invasive plant association, *Aphis fabae* Scopoli (Hemipreta: Aphididae) on *Impatiens glandulifera* Royle, has been used in the same model area in the Czech Republic under the same sampling and rearing method for several consecutive years and throughout the season. For molecular identification of these three species, we used DNA sequences of the barcoding region of the mitochondrial COI gene. Although our results confirmed ecological and morphological differences among *L. cardui, L. confusus,* and *L. fabarum*, genetic analysis on the basis of COI mitochondrial barcoding gene does not support species status of the mentioned *Lysiphlebus* taxa. The level of morphological differentiation in these *Lysiphlebus* Förster species is in accordance with the usual species variability within subfamily Aphidiinae. However, it should be examined how appearance of asexual lineages affects the morphological or genetical variability.

Key Words: *Aphis fabae*, *Impatiens glandulifera*, *Lysiphlebus*, Europe, taxonomy

Aphidiine parasitoids are one of the few groups of endoparasitoid insects whose fauna can be studied relatively easily by region. The updated approach to faunistical research around the world has been based mainly on the tritrophic (parasitoid–aphid–plant) associations and subsequent derivable interactions of the individual food webs in an ecosystem (Pike et al. 2000, Kavallieratos et al. 2004, Starý 2006). The results can be utilized in a set of approaches starting from the fauna, ecology, biodiversity, and ecosystem interactions, among others, up to the ecologically friendly management of pest aphids.

Lysiphlebus spp. belong to the most common aphidiine parasitoids of aphids. Their host range is predominantly associated with the Aphidinae aphids who are phylogenetically the youngest; in addition, most of the species are numerous and abundant in ecosystems (Starý 1981, Starý and Rejmánek 1981, Kavallieratos et al. 2004, Ortiz-Rivas and Martinez-Torres 2010). Species composition of the genus *Lysiphlebus* is not very rich. Three centers of origin tend to be distinguishable: West Palaearctic, Far East (Starý et al. 2002), and North American (Pike et al. 2000). Some of these species have invaded other areas either as naturally expansive (Starý 1995) or as purposely introduced species (i.e., Australia; Carver and Franzmann 2001).

The taxonomy of the genus and the species identification has been examined in this research. Because of the disorder of the molecular, ecological, and morphological data, the status of at least three European species is questionable: *Lysiphlebus cardui* (Marshall), *Lysiphlebus confusus* Tremblay and Eady, and *Lysiphlebus fabarum* (Marshall). Moreover, there are both sexual and asexual populations distinguishable in their distribution range (Němec and Starý 1985, Belshaw et al. 1999, Sandrock et al. 2011).

On the basis of their long-term evidence concerning the host range of these three sexual or asexual *Lysiphlebus* species, their host preferences, and their sympatric occurrence, we have attempted to demonstrate their taxonomical differentiation in a definable model situation: the same model site in Czech Republic, the same sampling and rearing method in several subsequent years, and throughout the season and the same plant–aphid association. This study brings evidence of integrating the morphological, field, and laboratory data, and application of the COI barcoding gene to the three asexual or sexual *L. cardui*, *L. confusus,* and *L. fabarum*.

Materials and Methods

This study provides the background to long-term research on the associations of the exotic invasive *Impatiens glandulifera* Royle and the local parasitoid guilds from 1996 to 2011. A new, partially exotic aphid–plant association, *Aphis fabae* Scopoli (Hemiptera: Aphididae) on *I. glandulifera*, is now quite common all over the country (Slavík 1996, 1997). It has become widely distributed in wetland habitats, prevailing along the rivers, ponds and brooks, humid lowland forests, and even the edges of wet meadows and bare grounds all over the Czech Republic. It is a progressively expanding species, extending rather quickly even in 1 yr (Slavík 1996) to nearby habitats through the natural dissemination of seeds by birds in flood waters.

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The respective field research was realized in the model locality: Czech Republic, southern Bohemia, between Třeboň and Jindřichův Hradec, close to the city Stráž nad Nežárkou, and the village Pístina (faunistic grid 6955), a mixed lowland forest (*Picea abies* (L.), *Betula pendula* Roth, *Fraxinus betulus* (L.), *Alnus glutinosa* L., and *Salix caprea* L.). The length of the model site is about 140 m and contains a fish pondside and a birch avenue along the road, with 5 m wide groves of *I. glandulifera* on the sides.

Sources of potential target host aphid–plant associations were occasionally found on the site edges or in a close neighboring area*: Aphis farinosa* J.F. Gmelin (Hemiptera: Aphididae) on *S. caprea* and *Aphis fabae cirsiiacanthoidis* Scopoli (Hemiptera: Aphididae) on *Cirsium arvense* (L.) Scop.

Samples were collected at two weekly intervals. At each sampling date, only one-third of an aphid infested plants were sampled to prevent an adverse effect on the aphid population. Parts of aphid-infested plants were gently cut with scissors and transferred into plastic translucent containers with nylon mesh at their tops. The containers were transported in field cool boxes up to the laboratory, where they were maintained under a 22°C temperature, 18 h of photoperiod (fluorescent light), and 70% relative humidity (RH) and were lightly sprinkled with water twice a day. The containers were inspected daily for the emergence of parasitoids. The aphid species transfer trials were realized in the same environment, and *A. fabae* on *Vicia faba* L. association was used. The bean plants were grown from seeds in wet conifer sawdust, in a rearing container of 35- by 35- by 35-cm size, covered with nylon mesh. Interactions of three parasitoid species associated with *A. fabae* on *I. glandulifera* and the attending ants were investigated from 2000 to 2006.

The number of *A. fabae* on *I. glandulifera* infested plants varied depending on the overall degree of aphid infestation. In most cases, there was a single aphid colony found per plant, but if two or more were found, these were sampled together.

The overwintering of parasitoid mummies sampled in the fall (October) and kept in the open until the next February was examined in air-conditioned cabinets under the conditions mentioned above. All the examined material is deposited in coll. P. Starý (České Budějovice). New records of aphidiines on aphid–host plant associations are indicated with an asterisk in front of each host plant name.

Molecular Analyses

For the molecular identification of these three species, we used DNA sequences of the barcoding region of the mitochondrial cytochrome oxidase subunit I (COI) gene. In total, 15 specimens were sequenced (Table 1). DNA was extracted using the KAPA Express Extract kit (Kapa Biosystems, Inc. Boston, USA) following the manufacturer's instructions. A barcoding region of COI gene was amplified using primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′- TAAACTTCAGGCTGACCAAAAAATCA-3′); (Folmer et al. 1994). We used polymerase chain reaction protocols and cycling conditions, which were previously published by Petrović et al. (2013).

Table 1. Sampling data for specimens used for molecular analyses of three *Lysiphlebus* species collected in Pístina, Czech Republic

Code	Date	Parasitoid	Aphid	Plant
Lys4	5 Sept. 2000	*L. fabarum*	*A. fabae*	*I. glandulifera*
Lys6	3 July 2003	*L. fabarum*	*A. fabae*	*I. glandulifera*
Lys7	3 July 2003	*L. fabarum*	*A. fabae*	*I. glandulifera*
Lys9	9 July 2003	*L. fabarum*	*A. fabae*	*C. arvense*
Lys11	24 July 2003	*L. cardui*	*A. fabae*	*I. glandulifera*
Lys12	24 July 2003	*L. cardui*	*A. fabae*	*I. glandulifera*
Lys13	24 July 2003	*L. cardui*	*A. fabae*	*I. glandulifera*
Lys14	24 July 2003	*L. cardui*	*A. fabae*	*C. arvense*
Lys15	24 July 2003	*L. cardui*	*A. fabae*	*C. arvense*
Lys16	24 July 2003	*L. cardui*	*A. fabae*	*C. arvense*
Lys19	24 July 2003	*L. confusus*	*A. fabae*	*I. glandulifera*
Lys20	24 July 2004	*L. confusus*	*A. farinosa*	*Salix* sp.
Lys21	24 July 2004	*L. confusus*	*A. farinosa*	*Salix* sp.
Lys22	24 July 2004	*L. confusus*	*A. farinosa*	*Salix* sp.

Sequences were edited using FinchTV (www.geospiza.com). After alignments, conducted using CLUSTAL W integrated in the MEGA5 software (Tamura et al. 2011), sequences showed no indels (insertion or deletion) and were trimmed to a length of 627 bp. All sequences are deposited under accession numbers KM408522-KM408535 in GenBank. For the calculation of genetic distances, we used Kimura's two-parameter method (K2P) of base substitution. Maximum parsimony and neighbor-joining trees were also obtained using the MEGA5 software. The robustness of the trees was assessed using a bootstrap analysis with 1,000 replicates. A COI sequence of *Lysiphlebus testaceipes* (Cresson), from GenBank (accession no. KC237764.1) *Aphidius avenae* Haliday from GenBank (accession no. JN164785.1), and *Ephedrus blattnyi* Starý also from GenBank (accession no. JN164786.1) were used as outgroups for molecular analyses.

Results

Morphology. The three target species can easily be distinguished morphologically using the characters on the marginal side of the forewing in combination with the pubescence of the hind femur as follows, key (Figs. 1–6):

1 Forewing marginal side with short setae, equal to these on the wing surface (Figs. 1 and 5); hind femur with erected (Fig. 2) or adpressed setae (Fig. 6)……2
Forewing marginal side with setae distinctly longer than the surface setae (Fig. 3); hind femur with semi-erected setae (Fig. 4)……*L. confusus*

2(1) Hind femur with erected setae (Fig. 2)……*L. cardui*
Hind femur with adpressed setae (Fig. 6)……*L. fabarum*

Review of Parasitoid–Aphid–Plant Associations in Czech Republic. The list of the hosts is briefly summarized below using original information by Starý (2006). For all species, only the *Aphis* spp. hosts are listed. Populations of all three *Lysiphlebus* species in the *A. fabae* on *I. glandulifera* and *C. arvense* associations dealt with in this article were asexual.

L. cardui (Marshall). ***Aphis brohmeri*** Börner on *Anthriscus sylvestris* Hoffmann, ***A. fabae*** Scopoli on *Chenopodium* sp., *C. arvense*, *Arctium lappa* L., *Matricaria perforata* Merat, **I. glandulifera, V. faba, Rumex* sp., *Calendula officinalis* L., *Philadelphus coronarius* L., *Helianthus annuus* L., *Sylibun marianum* L., *Solanum nigrum* L., ***A. farinosa*** J.F. Gmelin on *S. caprea* L., *Salix* sp., ***Aphis grossulariae*** Kaltenbach on *Epilobium parviflorum* Schreber, ***Aphis intybi*** Koch on *Cichorium intybus* L., ***Aphis nasturtii*** Kaltenbach on *I. glandulifera*, ***Aphis sambuci*** L. on *Sambucus nigra* L., ***Aphis spiraephaga*** F.P. Müller on *Spiraea vanhouttei* (Briot), ***Aphis urticata*** J.F. Gmelin on *Urtica dioica* L.

A. fabae was the dominant host species for *L. cardui*, especially in association with *C. arvense.*

L. confusus Tremblay and Eady. ***A. fabae*** Scopoli on **I. glandulifera, C. arvense,* ***A. farinosa*** J.F. Gmelin on *S. caprea, Salix viminalis* L.

L. confusus attacking *A. farinosa* feeding on *Salix* spp. and *A. fabae* on *I. glandulifera* have become common associations in the Czech Republic.

L. fabarum (Marshall). ***A. brohmeri*** Börner on *An. sylvestris*, ***Aphis chloris*** Koch on *Hypericum perforatum* L., ***Aphis confusa*** Walker on *Knautia arvensis* L., ***Aphis coronillae*** Ferrari on *Trifolium repens* L., ***Aphis craccivora*** Koch on *Medicago sativa* L., *Onobrychis sativa* Lamarck, *Lathyrus pratensis* L., *Vicia* sp., ***Aphis cytisorum*** Hartig on *Laburnum anagyroides* Medik, ***A. fabae*** Scopoli on *Beta vulgaris* L., *S. nigrum, Chenopodium album* L., *Arctium lappa, C. arvense*, **I. glandulifera, Euonymus europaeus* L., *C. officinalis, Carduus acanthoides* L., *Dahlia variabilis* (Willd), *P. coronarius, Spiraea* sp., *Fagopyrum convolvulus* (L.), ***A. grossulariae*** Kaltenbach on *E. parviflorum*, ***Aphis hederae*** Kaltenbach on *Hedera helix* L., ***Aphis hieracii***

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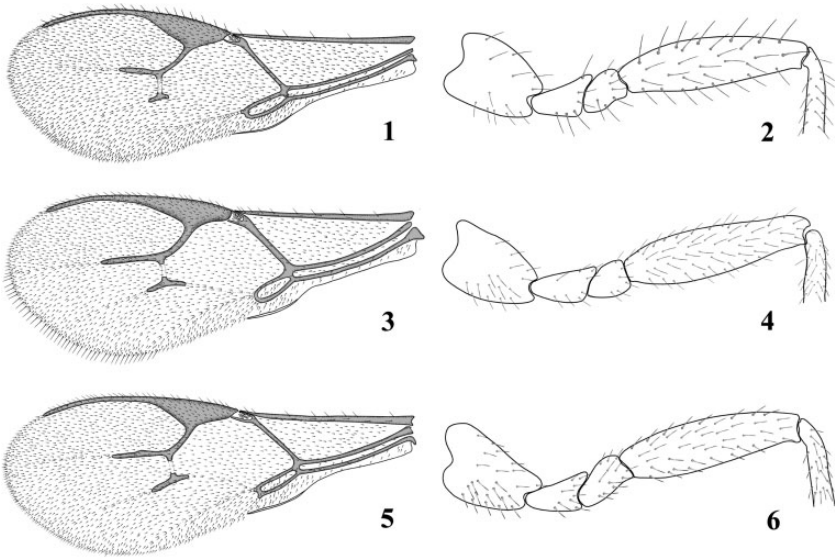


Fig. 1–6. Forewing and hind femur (females). 1. Forewing of *L. cardui* (Marshall); 2. Hind femur of *L. cardui* (Marshall); 3. Forewing of *L. confusus* Tremblay and Eady; 4. Hind femur of *L. confusus* Tremblay and Eady; 5. Forewing of *L. fabarum* (Marshall); 6. Hind femur of *L. fabarum* (Marshall).

Schrank on *Hieracium* sp., ***Aphis idaei*** van der Goot on *Rubus idaeus* L., ***A. intybi*** Koch on *C. intybus*, ***Aphis janischi*** (Börner) on *Cirsium oleraceum* L., ***Aphis lambersi*** (Börner) on *Daucus carota* L., ***A. nasturtii*** Kaltenbach on *Camelina alyssum* (Miller), *Capsicum* sp., ***Aphis newtoni*** Theobald on *Iris germanica* L., ***Aphis origani*** Passerini on *Origanum* sp., ***Aphis plantaginis*** Goetze on *Plantago major* L., *P. media* L., ***Aphis polygonata*** (Nevsky) on *Polygonum* sp., ***Aphis pomi*** de Geer on *Malus domestica* Borkh, ***Aphis poterii*** Börner on *Sanguisorba minor Scopoli*, ***Aphis roepkei*** (Hille Ris Lambers) on *Potentilla reptans* L., ***Aphis rumicis*** L. on *Rumex* sp., ***Aphis salviae*** Walker on *Salvia pratensis* L., ***A. sambuci*** L. on *Helichrysum* sp., ***Aphis schneideri*** (Börner) on *Ribes aureum* Pursh, ***A. spiraephaga*** F.P. Müller on *S. vanhouttei*, ***Aphis stachydis*** Mordwilko on *Stachys recta* L., ***Aphis symphyti*** Schrank on *Symphytum officinale* L., ***Aphis taraxacicola*** (Börner) on *Taraxacum officinale* Weber, ***Aphis thomasi*** (Börner) on *Scabiosa columbaria* L., ***A. urticata*** J.F. Gmelin on *U. dioica*, ***Aphis vandergooti*** (Börner) on *Achillea millefolium* L., ***Aphis verbasci*** Schrank on *Verbascum austriacum* Schott, ***Aphis carlinae*** (Börner) on *Carlina vulgaris* L.

L. fabarum was a dominant species in association with *A. craccivora, A. fabae* on *B. vulgaris*, *Chenopodium* L., and thistles in farmland habitats. Its association with *A. fabae* on *I. glandulifera* seems less typical for this species because of its prevailing occurrence in open field type of habitats.

***I. glandulifera–A. fabae*–Parasitoid Associations at the Model Site and Ant Attendance Association.** *I. glandulifera* was infested by the aphid populations somewhat later in the season because of its phenology. The aphid occurrence lasts almost up to the first freeze in the autumn. *A. fabae* was observed to start populations from a few individuals occurring on upper parts of the stem and terminal leaves, soon increasing to more or less higher populations, terminating in dense colonies covering even the terminal parts and leaves of the plants, and decreasing at the end of the season (increase of alatae still before the freeze). Aphid numbers varied in the course of the season and through the years.

Parasitoids appeared somewhat later than aphids. Parasitoid numbers varied significantly in the course of the season, specifically representing a combination of *L. cardui* and *L. confusus* and less frequently even *L. fabarum* (Fig. 7). It is also necessary to emphasize the highly important role of *I. glandulifera* in the oversummering of the aphid as its colonies can be found even outbreaking in the course of the whole summer during the period when most of the local alternative herbal hosts become dried-up and free of aphids.

Altogether (2000–2006), 17.151 *Lysiphlebus* individuals were identified to species, with respective numbers (in brackets) for these years as follows: 2000 (3,014), 2001 (1,634), 2002 (3,752), 2003 (5,663), 2004 (34), 2005 (1,920), and 2006 (1,134) specimens.

Ant attendance started almost immediately with the appearance of *A. fabae* colonies and lasted until the end of the season. Several ant species (Hymenoptera: Formicidae) were observed: *Formica fusca* L., *Lasius emarginatus* (Olivier), *Lasius platythorax* Seifert, *Myrmica rubra* L., and *Myrmica ruginodis* Nylander (Hymenoptera: Formicidae). All the five identified ant species manifested rigorous defensive responses to mechanical stimuli. However, whereas the ants mostly disregarded the parasitoid adults, if present, in an aphid colony the *Lasius* species manifested a different behavior: they defended unparasitized colonies, colonies with some mummies, colonies consisting of merely mummies with no live aphids and empty mummies with emergence holes and without any live aphids.

Interactions of Aphids and Parasitoids Within and With Nearby Ecosystems. An environmental analysis of all the available host species of three *Lysiphlebus* species in the wetland ecosystem and nearby agroecosystems has determined specific differences. *L. confusus* is almost uniquely associated with *A. farinosa* on *Salix* spp. (Table 2). It apparently switches to *A. fabae* on *I. glandulifera* subsequent to a decrease in *A. farinosa*. Its association with *A. fabae* on *I. glandulifera* is significant and common in the studied ecosystem, whereas it rearly parasitizes *A. fabae* on *C. arvense*.

L. cardui manifests a clearly dominant position of *A. fabae* on *C. arvense* and switches from there to *A. fabae* on *I. glandulifera*. This can be easily derived from species and from the abundance of data on this association both in the wetland ecosystem and in a nearby agroecosystem. Rarely, it also attacks *A. farinosa* on *Salix* spp.

L. fabarum is a less common parasitoid of *A. fabae* on *Cirsium* spp. and less commonly parasitizes *A. fabae* on *I. glandulifera.*

Laboratory Transfer Trials. The trials targeted the verification of the host alternation of parasitoids of *A. fabae* on *I. glandulifera* to conspecific or other aphids in the ecosystem.

L. cardui. A. fabae on *I. glandulifera* to *A. fabae* on *V. faba* (positive: 1998, 2000, 2003, 2004, 2005).

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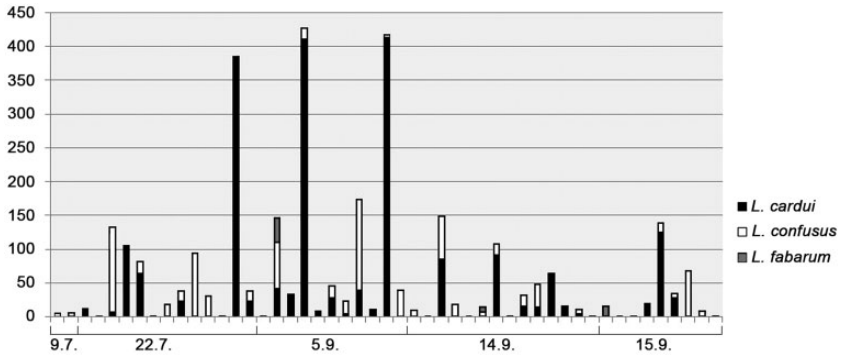


Fig. 7. Seasonal occurrence and abundance of *L. cardui, L. confusus,* and *L. fabarum* parasitizing *A. fabae* on *I. glandulifera* association. Model site: Pístina, 2000. Numbers of parasitoid adults counted from field sampled aphid colonies reared in the laboratory.

Table 2. Occurrence and abundance of *Lysiphlebus* species on representative samples in wetland and nearby habitats

Date	*L. cardui*	*L. confusus*	*L. fabarum*	Total
Wetland: *A. farinose* on *Salix* spp.				
10 May 2000		87 (100%)		87
5 June 2000		310 (100%)		310
Pondside: *A. fabae* on *C. arvense*				
9 July 2003	18 (54%)		15 (46%)	33
Field: *A. fabae* on *C. arvense*				
17 July 2005	9 (100%)			9
24 July 2005	94 (96%)	4 (4%)		98

Numbers of parasitoid adults obtained from field-sampled aphid colonies reared in the laboratory. Model site: Pístina.

Note: Overwintering *A. fabae* on *I. glandulifera* in the open 2003–2004 accepted *A. fabae* on *V. faba* when transferred in the laboratory conditions in February 2004.

A. farinosa on *Salix* sp. to *A. fabae* on *V. faba* (positive: 2004; adverse 1995).

L. confusus. A. fabae on *I. glandulifera* to *A. fabae* on *V. faba* (positive: 1991, 1998, 1999, 2001).

A. farinosa on *Salix* sp. to *A. fabae* on *V. faba* (positive: 1985, 1995, 1996, 1998, adverse 1998).

L. fabarum. A. fabae on *Chenopodium* sp., *C. arvense, B. vulgaris* to *A. fabae* on *V. faba* (positive: 1983, 1985, 1987, 1997, 1996, 2005).

DNA Identification. Calculated K2P distances show that all analyzed specimens of *L. fabarum* belong to a single haplotype. The same is recorded in *L. cardui* regardless of the aphid-plant association from which it is collected. Specimens of *L. confusus* occur in two haplotypes with a K2P distance of 0.6%.

The topology of both the maximum parsimony and neighbor-joining trees showed weak separation of specimens into four clades (Fig. 8). Three clades correspond to analyzed morphospecies, while the fourth represented with one specimen of *L. confusus*. Although these three species did not share any haplotype, the calculated K2P distances between them were very low (0.2–0.8% between *L. fabarum* and *L. confusus*; 0.5% between *L. fabarum* and *L. cardui*; and 0.3–0.8% between *L. cardui* and *L. confusus*).

Discussion

Although commonly distinguished in the respective morphological keys, the validity and separation of *L. cardui*, *L. confusus,* and *L. fabarum* have been targeted in several articles, reporting more or less different approaches and results (Carver and Franzmann 2001, Kavallieratos et al. 2004). Moreover, a mixture of all three of these species may occur even together per sample, and such phenomena may contribute to opinions on their species conspecificity. However, morphological, biological, and DNA analyses may sometimes contradict the integration of respective results. Several updated studies bring focus to the species, or species-complex, occurrence of individual species up to specifically identical morphospecies in *Lysiphlebus*. Belshaw et al. (1999) raised doubts about the *L. cardui* separation from *L. fabarum* but also found genetical isolation of *L. fabarum* on *Brachycaudus cardui* (L.) (Hemiptera: Aphididae) biotype from other *L. fabarum* biotypes. Barahoei et al. (2011) carried out morphometric analyses of wing venation, petiole, and femur of five *L. fabarum* biotypes in Iran. Although they used a small number of biotypes, this result demonstrated morphometric analysis as an efficient tool to distinguish host associated lineages. Sandrock et al. (2011) on the basis of COI and ATP6 data sets found that among sexual and asexual *L. cardui*, *L. confusus,* and *L. fabarum*, there is no isolation between reproductive modes, and thus, these three definable morphospecies represent a single species. Derocles et al. (2012) suggested a combination using two or more genes for accurate identification of the Aphidiinae. *L. confusus* and *L. fabarum,* were also considered, noting a sole nuclear gene (long wavelength rhodopsin) and mitochondrial COI gene are not variable enough to discriminate both related species.

The invasion and spread of *I. glandulifera* into local ecosystems has also been connected with the development of new food webs: *I. glandulifera*, *A. fabae, L. cardui, L. confusus, L. fabarum*. Correspondingly, its invasion has been secondarily followed by increasing the biodiversity of the target ecosystem represented by two species of aphids, *A. fabae* and *Impatientinum balsamines* (Kaltenbach) (Hemiptera: Aphididae) (Starý 1970), including several predators (Starý and Láska 1999) and pollinators (Starý and Tkalců 1998).

The local associations in the wetland ecosystem interacted with *I. glandulifera* through the *L. confusus*, attacking *A. farinosa* on *Salix* spp. and *L. cardui* parasitoids and less frequently with *L. fabarum*. Both *L. fabarum* and *L. cardui* are derivable from the *A. fabae* on *C. arvense* association. For all three parasitoid species, the new association and respective refugium on *A. fabae* feeding on *I. glandulifera* in wetland habitats is important as a refugium for oversummering and overwintering, at rather high population levels. The field-derived interactions between the associations on *A. farinosa* feeding on *Salix* spp. and *A. fabae* feeding on *C. arvense* though the parasitoids were also verified on grounds of the laboratory transfer trials. Ant attendance of *A. fabae* on *I. glandulifera* realized by five ant species did not affect adversely the presence of *Lysiphlebus* adults in the colonies, irrespective of (morpho) species identity.

The relatively new and common association for all three *Lysiphlebus* (morpho)species studied in an identical environment through the season for several subsequent years has supported the demonstration of their specific ecological peculiarities manifested in the host range and host alternation illustrated also by their relative

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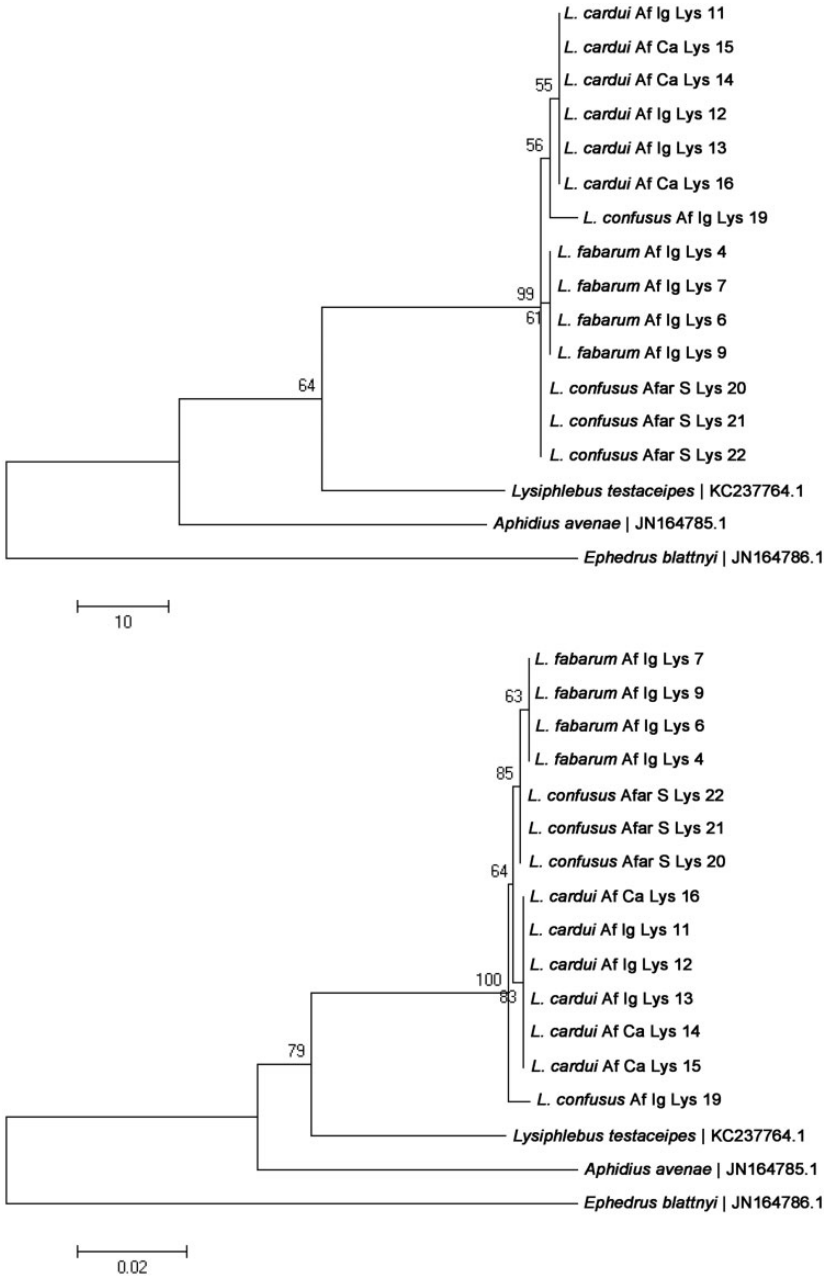


Fig. 8. Top: The first among the 96 most parsimonious trees for the cytochrome oxidase I (COI) gene of a priori determined specimens of *L. cardui*, *L. confusus,* and *L. fabarum*. Bottom: neighbor-joining tree based on Kimura-2-Parameter (K2P) distances. *L. testaceipes*, *Aphidius avenae,* and *Ephedrus blattnyi* were used for the outgroup species. Numbers above or below the branches represent the bootstrap values (%).

abundance during the season. However, using the COI mitochondrial barcoding gene, we could not clearly separate these three species because of small genetic distances. Although these three species are separately clustered, with small overlapping of *L. confusus*, genetical distances are rather low (0.2–0.8%) and with relatively small bootstrap values.

In conclusion, although our results confirmed ecological and morphological differences between *L. cardui, L. confusus,* and *L. fabarum*, genetical analysis on the basis of the COI mitochondrial barcoding gene does not support species status of the studied *Lysiphlebus* taxa. Although we designated experiments to the same locality and association following these three species, our results are in agreement with Belshaw et al. (1999) and Sandrock et al. (2011) as far as the use of molecular markers is concerned. The level of morphological differentiation in these *Lysiphlebus* species is in accordance with the usual species variability within subfamily Aphidiinae, but it should be examined how appearance of asexual lineages affects the morphological or genetical variability.

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