

# *Lysiphlebus orientalis* (Hymenoptera, Braconidae), a new invasive aphid parasitoid in Europe – evidence from molecular markers

Andjeljko Petrović<sup>1\*</sup>, Milana Mitrović<sup>2</sup>, Petr Starý<sup>3</sup>,  
Olivera Petrović-Obradović<sup>4</sup>, Vladimir Žikić<sup>5</sup>,  
Željko Tomanović<sup>1</sup> and Christoph Vorburger<sup>6</sup>

<sup>1</sup>Institute of Zoology, Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia: <sup>2</sup>Department of Plant Pests, Institute for Plant Protection and Environment, Banatska 33, Zemun, Serbia: <sup>3</sup>Laboratory of Aphidology, Institute of Entomology, Biology Centre, Academy of Sciences of the Czech Republic, Branišovská 31, 37005 České Budějovice, Czech Republic: <sup>4</sup>Department of Plant Protection, Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11081 Zemun, Serbia: <sup>5</sup>Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Višegradska 33, 18000 Niš, Serbia: <sup>6</sup>Institute of Integrative Biology, ETH Zürich, Switzerland, and EAWAG, Swiss Federal Institute of Aquatic Science and Technology, Überlandstrasse 133, 8600 Dübendorf, Switzerland

## Abstract

We report the occurrence of *Lysiphlebus orientalis* in Serbia, an aphid parasitoid from the Far East that is new to Europe and has the potential to become invasive. Our finding based on morphological characters is confirmed by analyses of mitochondrial cytochrome oxidase subunit I sequences. An increase in number and an expansion of the host range were observed during field studies over the past two years, and it is determined that the current host range encompasses nine aphid hosts on 12 different host plants, forming 13 tri-trophic associations. A host range determined for European populations of *L. orientalis* appears wider compared with that in its Far Eastern native habitats where *Aphis glycines* Mats. is the sole known host. Moreover, it overlaps considerably with the host ranges of European parasitoids that play an important role in the natural control of pest aphids.

**Keywords:** new invasive species, Serbia, DNA barcoding, biological control, parasitoid

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## Introduction

Non-native arthropods abound worldwide and many cause substantial ecological and economic damage (Hallman

& Schwalbe, 2002). While invasions of herbivorous pest arthropods are typically the consequence of unintentional introductions, predators and parasitoids of pest arthropods are often introduced deliberately for biological control. Nevertheless, there are numerous cases of alien arthropod predators and parasitoids that became invasive and have undesirable effects on the invaded ecosystems (Roy *et al.*, 2011). Here we report the case of an accidentally introduced parasitoid of aphids in Europe that appears to be expanding its host range.

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\*Author for correspondence  
Phone: +381 11 2638 890  
Fax: +381 11 2638 500  
E-mail: andjeljko@bio.bg.ac.rs

The subfamily Aphidiinae (Hymenoptera, Braconidae) consists of solitary endoparasitoids of aphids, several of which are of great value in pest aphid control (Hagvar & Hofsvang, 1991; Brewer & Elliott, 2004) and are commercially available as biocontrol agents (Boivin *et al.*, 2012). More than 200 species have been recorded in Europe (van Achterberg, 2011), but there is variation in how comprehensively the fauna of Aphidiinae is documented in different areas/countries. With 100 recorded species, Serbia is one of the most extensively surveyed European countries (Petrović *et al.*, 2009; Petrović *et al.*, 2011). The genus *Lysiphlebus* Förster, with about 30 known species, belongs to a taxonomically and biologically less studied genera (Mackauer, 1961; Kambhampati *et al.*, 2000). It comprises highly specialized species such as *L. hirticornis* Mackauer which parasitize *Metopeurum fuscoviride* exclusively (Nyabuga *et al.*, 2009), as well as relative generalists such as a *L. testaceipes* (Cr.) with a host range of more than 50 species in Europe (Starý *et al.*, 2004) and even more in its native home, North America (Pike *et al.*, 2000). An important characteristic of the genus *Lysiphlebus* is the occurrence of species that have sexual and asexual reproduction such as *L. fabarum*, *L. confusus* and *L. cardui* (summarized in Sandrock *et al.*, 2011).

Aphidiinae parasitoids have been used in biological control programs against pest aphids on various crops worldwide (Hagvar & Hofsvang, 1991). Although new biocontrol agents are typically subject to a risk assessment prior to their field release, post-release studies often focus on establishment success and target effects, paying less attention to the effects of introduced parasitoids on non-target hosts. However, the best example on how important the latter studies are is *L. testaceipes*, a biocontrol agent introduced to Mediterranean Europe from Cuba to control *Aphis citricola* and *Toxoptera aurantii* in citrus crops. Within less than three decades *L. testaceipes* expanded its host range to 50 different aphid species (Cecilio, 1994; Starý *et al.*, 1988, 2004), replacing native parasitoid species. In 2008, *L. testaceipes* was removed from the positive list of biocontrol agents in Europe by the European Plant Protection Organization (EPPO 2008-03-26/28).

*Lysiphlebus orientalis* Starý & Rakhshani was recently described from northeast China as a specialized parasitoid of the soybean aphid (*Aphis glycines* Matsumura) on *Glycine max* (L.). On the basis of field-collected material and laboratory rearing, Starý *et al.* (2010) determined that this parasitoid occurs in all-female populations that reproduce by thelytokous parthenogenesis. Since the soybean aphid has become a major source of economic loss for soybean production in North America (Heimpel *et al.*, 2010; Ragsdale *et al.*, 2011), *L. orientalis* is currently under investigation as a potential biocontrol agent (Starý *et al.*, 2010; Ragsdale *et al.*, 2011). In their description of *L. orientalis*, Starý *et al.* (2010) determined on the basis of morphological characters that some *Lysiphlebus* specimens found recently in Europe may be related to *L. orientalis*. Here we present molecular evidence that *L. orientalis* is indeed present in Europe, presumably due to an accidental introduction. It was collected repeatedly in Serbia and it appears to be spreading geographically as well as expanding its host range.

## Materials and methods

### Collecting

For the past two decades, members of *Lysiphlebus* were intensively collected throughout Serbia as a part of ongoing

research on Aphidiinae fauna. Plant samples infested with live and mummified aphids were collected in the field and transferred into plastic containers covered with nylon mesh. Voucher specimens of live aphids from each plant sample were preserved in 70% ethanol for later identification. The remaining aphids were maintained in the laboratory until parasitoid emergence.

The external morphology of emerged parasitoids was studied using a ZEISS Discovery V8 stereomicroscope. All materials used in this study are deposited in the collection of the Institute of Zoology, Faculty of Biology, University of Belgrade.

### DNA extraction, polymerase chain reaction (PCR) amplification and sequencing

Thirty-five *Lysiphlebus* specimens were used for the molecular confirmation of species status and for the analysis of genetic variability (table 1). *L. orientalis* was represented with 22 specimens originating from Serbia and five from China. We also obtained sequences from two specimens of *L. testaceipes* and three specimens of *L. balcanicus*. Both species are members of the 'testaceipes' group which is closely related to *L. orientalis*. In addition to members of the 'testaceipes' group, we sequenced three specimens of *L. fabarum* which shares aphid hosts with *L. orientalis*. Prior to DNA extraction, all specimens were stored in 96% ethanol at  $-20^{\circ}\text{C}$ . DNA was extracted from each individual adult wasp using the KAPA Express Extract kit (Kapa Biosystems) following the manufacturer's instructions. A region of approximately 710bp of the barcoding region of the mitochondrial cytochrome oxidase subunit I (COI) gene was amplified using the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGCTGACCAAAAAATCA-3') (Folmer *et al.*, 1994).

DNA amplification was performed in a final volume of 25  $\mu\text{l}$  containing 1  $\mu\text{l}$  of the extracted DNA, 1  $\times$  KAPA2G Robust HotStart ReadyMix (contains 2 mM  $\text{MgCl}_2$  at 1X) and 0.5  $\mu\text{M}$  of each primer. All PCRs were conducted in an Eppendorf Mastercycler<sup>®</sup> using the following thermal profile: initial denaturation at  $95^{\circ}\text{C}$  for 5 min, followed by 35 cycles of  $94^{\circ}\text{C}$  for 60 s,  $54^{\circ}\text{C}$  for 60 s,  $72^{\circ}\text{C}$  for 90 s and a final extension step at  $72^{\circ}\text{C}$  for 7 min. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions, while DNA sequencing in both directions was performed by Macrogen Inc. (Seoul, Korea).

### Genetic analysis

Sequences were edited using FinchTV ([www.geospiza.com](http://www.geospiza.com)). After multiple alignments, conducted using CLUSTAL W integrated in MEGA5 software (Tamura *et al.*, 2011), sequences showed no indels (insertion/deletion) and were trimmed to a length of 630 bp. All sequences were deposited under accession numbers KC237734-KC237768 in GenBank. For calculation of average genetic distances between sequences, within each group and between groups of species, we used Kimura's two-parameter method (K2P) of base substitution. Maximum parsimony (MP) and neighbor joining (NJ) trees were also obtained using MEGA5 software. The robustness of the trees was assessed using a bootstrap analysis with 1000 replicates. A COI sequence of *Aphidius rhopalosiphii* De Stefani Perez from GenBank (Accession No. JN164775.1)

Table 1. Sampling data for specimens used for molecular confirmation of species status and genetic variability study of *L. orientalis*.

Parasitoid_Code	Geographic origin	Aphid host	Plant	Accession no.
<i>L. orientalis</i> _SER1	Serbia	<i>Aphis hederæ</i>	<i>Hedera helix</i>	KC237734
<i>L. orientalis</i> _SER2	Serbia	<i>Aphis fabæ cirsiiacanthoidis</i>	<i>Cirsium arvense</i>	KC237735
<i>L. orientalis</i> _SER3	Serbia	<i>A. f. cirsiiacanthoidis</i>	<i>C. arvense</i>	KC237736
<i>L. orientalis</i> _SER4	Serbia	<i>Aphis fabæ fabæ</i>	<i>Chenopodium album</i>	KC237737
<i>L. orientalis</i> _SER5	Serbia	<i>Aphis craccivora</i>	<i>Medicago sativa</i>	KC237738
<i>L. orientalis</i> _SER6	Serbia	<i>A. f. cirsiiacanthoidis</i>	<i>C. arvense</i>	KC237739
<i>L. orientalis</i> _SER7	Serbia	<i>A. f. cirsiiacanthoidis</i>	<i>C. arvense</i>	KC237740
<i>L. orientalis</i> _SER8	Serbia	<i>A. f. cirsiiacanthoidis</i>	<i>C. arvense</i>	KC237741
<i>L. orientalis</i> _SER9	Serbia	<i>A. f. cirsiiacanthoidis</i>	<i>C. arvense</i>	KC237742
<i>L. orientalis</i> _SER10	Serbia	<i>A. f. fabæ</i>	<i>C. album</i>	KC237743
<i>L. orientalis</i> _SER11	Serbia	<i>A. f. fabæ</i>	<i>C. album</i>	KC237744
<i>L. orientalis</i> _SER12	Serbia	<i>Aphis schneideri</i>	<i>Ribes</i> sp.	KC237745
<i>L. orientalis</i> _SER13	Serbia	<i>A. schneideri</i>	<i>Ribes</i> sp.	KC237746
<i>L. orientalis</i> _SER14	Serbia	<i>A. hederæ</i>	<i>H. helix</i>	KC237747
<i>L. orientalis</i> _SER15	Serbia	<i>A. hederæ</i>	<i>H. helix</i>	KC237748
<i>L. orientalis</i> _SER16	Serbia	<i>Aphis</i> sp.	<i>Rubus</i> sp.	KC237749
<i>L. orientalis</i> _SER17	Serbia	<i>Aphis gossypii</i>	<i>Althea officinalis</i>	KC237750
<i>L. orientalis</i> _SER18	Serbia	<i>Aphis</i> sp.	<i>Geranium</i> sp.	KC237751
<i>L. orientalis</i> _SER19	Serbia	<i>A. fabæ</i>	<i>C. album</i>	KC237752
<i>L. orientalis</i> _SER20	Serbia	<i>Aphis</i> sp.	<i>Laserpitium halleri</i>	KC237753
<i>L. orientalis</i> _SER21	Serbia	<i>Aphis</i> sp.	<i>L. halleri</i>	KC237754
<i>L. orientalis</i> _SER22	Serbia	<i>A. craccivora</i>	<i>Robinia pseudoacacia</i>	KC237755
<i>L. orientalis</i> _CN1 <sup>1</sup>	China	<i>Aphis glycines</i>	<i>Glycine max</i>	KC237756
<i>L. orientalis</i> _CN2 <sup>2</sup>	China	<i>A. glycines</i>	<i>G. max</i>	KC237757
<i>L. orientalis</i> _CN3 <sup>2</sup>	China	<i>A. glycines</i>	<i>G. max</i>	KC237758
<i>L. orientalis</i> _CN4 <sup>2</sup>	China	<i>A. glycines</i>	<i>G. max</i>	KC237759
<i>L. orientalis</i> _CN5 <sup>3</sup>	China	<i>A. glycines</i>	<i>G. max</i>	KC237760
<i>L. balcanicus</i> _1	Serbia	<i>Aphis psammophila</i>	<i>Jasione heldreichii</i>	KC237761
<i>L. balcanicus</i> _2	Serbia	<i>A. psammophila</i>	<i>J. heldreichii</i>	KC237762
<i>L. balcanicus</i> _3	Serbia	<i>A. psammophila</i>	<i>J. heldreichii</i>	KC237763
<i>L. testaceipes</i> _1	Spain	<i>A. hederæ</i>	<i>H. helix</i>	KC237764
<i>L. testaceipes</i> _2	Slovenia	<i>Aphis nerii</i>	<i>Nerium oleander</i>	KC237765
<i>L. fabarum</i> _1	Serbia	<i>A. gossypii</i>	<i>A. officinalis</i>	KC237766
<i>L. fabarum</i> _2	Serbia	<i>A. f. cirsiiacanthoidis</i>	<i>C. arvense</i>	KC237767
<i>L. fabarum</i> _3	Serbia	<i>A. craccivora</i>	<i>M. sativa</i>	KC237768
<i>Aphidius rhopalosiphii</i>	Serbia	<i>Sitobion avenae</i>	<i>Triticum aestivum</i>	JN164775.1

<sup>1</sup> lab culture1 Newark USA.

<sup>2</sup> lab culture2 Newark USA.

<sup>3</sup> lab culture3 Newark USA.

Table 2. Number of collected samples with *Lysiphlebus* specimens and proportion of samples with *L. orientalis* in Serbia for period 1995–2011. Only years in which *L. orientalis* was found are shown.

	1995	2002	2008	2010	2011
No. of <i>Lysiphlebus</i> samples	18	5	32	31	27
No. of <i>L. orientalis</i> samples	1 (5.55%)	1 (20%)	2 (6.25%)	9 (29.03%)	9 (33.33%)

was used as an outgroup taxon for molecular phylogenetic analyses.

## Results

During a nearly two-decade-long research of the Aphidinae fauna of Serbia, specimens that are morphologically identical to *L. orientalis* were encountered very sporadically. The first record is from 1995, but finding these parasitoids only became a regular occurrence in 2010 and 2011 (table 2). In total, 214 females, but no males, were collected in two distinct regions of Serbia, the Belgrade region and northern Serbia (fig. 1). These parasitoids emerged from

nine aphid hosts on 12 different host plants, forming 13 tri-trophic associations, which are summarized in the next paragraph. The lack of males is consistent with asexual reproduction as reported for *L. orientalis* in its native range. Molecular identification of the collected material as *L. orientalis* (see below) makes these the first records of this Far East species in Europe. Based on the number of records and diversity of aphid hosts, it appears that the Belgrade region is the center of introduction where *L. orientalis* gained a foothold and from where it could expand its geographic range as well as its host range. This opinion is supported by the results of a long-term research on aphid parasitoids (including *Lysiphlebus*) realized since 1956 in the Czech Republic (Starý, 2006) and nearby

Table 3. Mean K2P genetic distances at COI between and within (gray fields) groups of *Lysiphlebus* parasitoids included in the present study.

	<i>L. orientalis</i> _SERBIA	<i>L. orientalis</i> _CHINA	<i>L. balcanicus</i>	<i>L. testaceipes</i>	<i>L. fabarum</i>
<i>L. orientalis</i> _SERBIA	<b>0.001 (0.000–0.005)</b>				
<i>L. orientalis</i> _CHINA	0.008	<b>0.000</b>			
<i>L. balcanicus</i>	0.040	0.043	<b>0.000</b>		
<i>L. testaceipes</i>	0.031	0.031	0.026	<b>0.01</b>	
<i>L. fabarum</i>	0.068	0.065	0.074	0.067	<b>0.004 (0.002–0.006)</b>

Fig. 1. Map of Serbia with marked regions where *L. orientalis* was detected.

Slovakia (Starý & Lukáš, 2009). In spite of a high number of samples and reared individuals, no *L. orientalis* have been found in these areas.

#### Tritrophic associations

##### *L. orientalis* Starý & Rakhshani

*Aphis affinis* Del Guercio on *Mentha aquatica*, Radmilovac, 12.6.1995. (10f); *Aphis craccivora* Koch. on *Medicago sativa*, New Belgrade, 13.7.2010. (5f); on *Robinia pseudoacacia*, Slankamen, 24.6.2011 (1f); *Aphis fabae* Scop. on *Chenopodium album*, Zemun, 7.6.2011. (28f), Slankamen, 24.6.2011. (4f); on *Vicia* sp., Zemun, 18.6.2010. (6f); *Aphis fabae cirsiacanthoidis* Scop. on *Cirsium arvense*, Besni fok, 27.5.2010. (3f), Jabuka, 18.5.2008. (35f), New Belgrade, 26.5.2002. (11f), Surčin 1, 15.6.2010. (8f), Surčin 2, 15.6.2010. (1f), Surčin 3, 15.6.2010. (1f), Vrbovski, 3.6.2008. (1f); *Aphis fabae fabae* Scop. on *C. album*, Slankamen, 20.7.2010. (1f), Zemun 1, 18.6.2010. (1f), Zemun 2, 18.6.2010. (6f), Zemun 3, 18.6.2010. (21f); *Aphis gossypii* Glover on *Althea officinalis*, Zemun, 7.6.2011. (2f);

*Aphis hederae* Kaltenbach on *Hedera helix*, Zemun, 2.9.2010. (1f), Zemun, 14.6.2011. (2f); *Aphis schneideri* (Borner) on *Ribes* sp., Konjarnik, 6.6.2011. (13f); *Aphis* sp. on *Geranium* sp., Zemun, 7.6.2011. (12f); on *Laserpitium halleri*, Dobanovci, 17.6.2011. (2f), Zemun, 9.6.2011. (5f); on *Picris hieracioides*, Palić, 6.9.1995. (34f).

#### Genetic relationships

The identical topology of both, the MP (fig. 2) and NJ (not shown) trees, shows that specimens of *L. orientalis* from Serbia and China are each other's closest relatives and, with bootstrap values of 99% and 100%, respectively, form one phylogenetic clade.

The calculated genetic distances (K2P) between specimens of *L. orientalis* from Serbia and China are very low with a mean distance of 0.008 (table 3).

For the COI barcoding region used here, this is within the range of intraspecific divergence observed in other species of Aphidiinae and well below the level typically found for between-species divergence (Derocles *et al.*, 2011; Sandrock *et al.*, 2011). Both trees further show that *L. orientalis* is close to other members of the 'testaceipes' group, *L. testaceipes* and *L. balcanicus*, and more distantly related to *L. fabarum*.

#### Discussion

Although Hymenoptera constitute the largest proportion (63.3%) of alien arthropod predators and parasitoids in Europe, there are presently only five alien Aphidiinae species reported (Roy *et al.*, 2011). All of them (*Aphidius colemani* Viereck, *Aphidius smithi* Shama & Subba Rao, *Lysiphlebus testaceipes*, *Pauesia cedrobii* Starý & Leclant, and *Pauesia unilachni* (Gahan)) were introduced as biocontrol agents (Starý, 2006). Here we report the occurrence of *L. orientalis* in Serbia, an aphid parasitoid that is new to Europe and which has the potential to become invasive. Its arrival in Europe appears to predate the recognition of its invasion substantially and it predates its formal species description as a parasitoid of soybean aphid in China (Starý *et al.*, 2010). The first records in Serbia are from 1995, but previous to 2010 there are only five records. These specimens were mistakenly treated as rare female morphotypes of *L. fabarum*. Only when the number of records of these 'morphotypes' increased at about the same time as *L. orientalis* was described in China did we suspect that they belong to *L. orientalis*. This suspicion is now confirmed by our analyses of COI sequences. The introduction of a parasitoid attacking agricultural pests such as aphids may not appear undesirable at first glance. However, new aphid parasitoids can become invasive by replacing native parasitoid species, as documented for *L. testaceipes* (EPPO 2008-03-26/28). Important determinants of their potential impact are host range and reproductive mode. *L. orientalis* is thought to

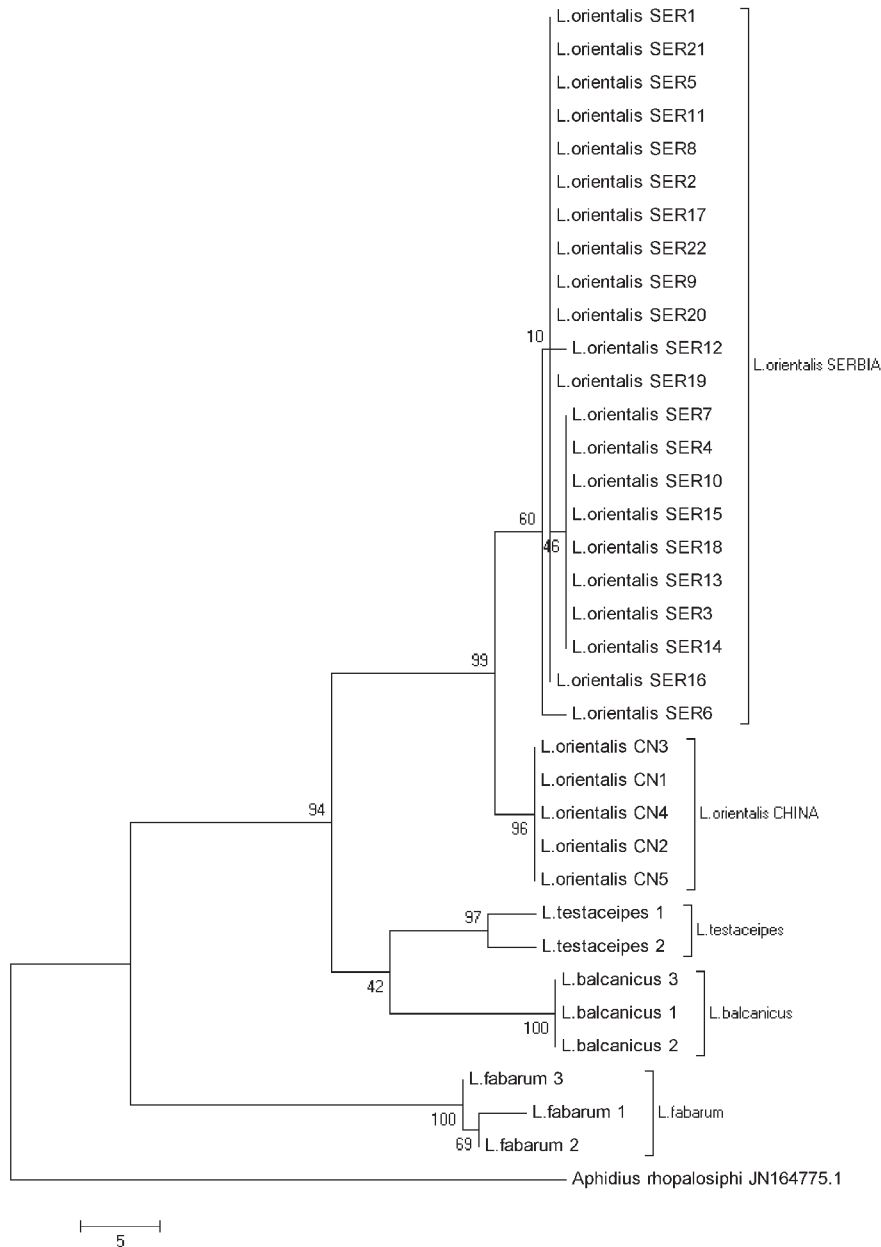


Fig. 2. The first out of the 438 most parsimonious trees (length=126) for COI of *Lysiphlebus* specimens. *A. rhopalosiphi* was used as the outgroup species. Numbers above/below the branches represent the bootstrap values (%).

be monophagous on *A. glycines* in China (Starý *et al.*, 2010), but in Serbia it parasitizes several species of the genus *Aphis*. Based on the limited data available so far, its host range overlaps strongly with that of *L. fabarum*, which is the most abundant native parasitoid on hosts of the genus *Aphis*, providing an important ecosystem service through its impact on pest aphid populations (Starý, 2006). *L. orientalis* also shares its mode of reproduction with *L. fabarum*. Based on the complete absence of males we concluded that *L. orientalis* in Europe reproduces by thelytokous parthenogenesis, as in its place of origin (Starý *et al.*, 2010). This mode of reproduction clearly facilitates invasion, because a single female is sufficient to form a new

population, and it can make exotic species more competitive because they forego the cost of producing males (Maynard Smith, 1978), resulting in a higher rate of population increase.

Kaiser & Heimpel (2011) described another phenomenon that makes *L. orientalis* a potentially problematic invader. *L. orientalis* preferentially attacks older aphids (*A. glycines*) that are mature enough to reproduce before being killed by the parasitoid larva. Furthermore, offspring of parasitized *A. glycines* reproduce earlier and faster than offspring of unparasitized *A. glycines*. These are undesirable attributes from a pest control perspective as they allow the host aphids to maintain high numbers despite high rates of parasitism

(Kaiser & Heimpel, 2011). As yet there are no investigations of such phenomena in Serbian populations of *L. orientalis*. If present, they could have significant implications for the structure of plant-aphid-parasitoid food webs in agroecosystems. For example, *L. orientalis* may become a serious competitor of *L. fabarum*, but not have equivalent effects on aphid population densities.

*L. orientalis* is indubitably established in Serbia since it has been present in the same area for at least 17 years, but it appears to still be restricted to ruderal vegetation in urban areas. This is consistent with the community maturity concept of Shea & Chesson (2002), which states that man-made environments have high levels of niche opportunities for alien species, because such communities have had less time to assemble, and less time for their constituent species to adapt to the local conditions. Their species might also have lower competitive abilities than those in mature communities (Shea & Chesson, 2002). The occurrence of *L. testaceipes* in the Mediterranean also manifests similar occurrence patterns (Starý et al., 1988, 2004). The expansion of host range observed during the past two years and the increase in population density suggest that *L. orientalis* is moving past the lag phase of population growth in Serbia. The reasons mentioned above suggest that *L. orientalis* has a great potential to become a problematic invasive parasitoid in Europe. Constant monitoring is therefore necessary, as well as further research on its distribution, biology and ecology.

The pathways of introduction of *L. orientalis* are still unknown, but some conjectures can be made. Over the past two decades, a very intensive official and unofficial trade of goods has developed between China and Serbia. New exotic species could thus be introduced together with imported soybean, for example. Belgrade as the capital of Serbia is under the biggest pressure of immigration and trade, and is therefore the most likely center of exotic species introductions. To date Serbia is the only European country in which *L. orientalis* has been detected, despite the fact that the Aphidiine fauna is extensively investigated also in other parts of Europe (Kavallieratos et al., 2004; Starý, 2006; Starý & Lukáš, 2009). Considering the level of trade and transfers between western European countries and China, it is possible that *L. orientalis* is already present but still undetected elsewhere in Europe.

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