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# Morphological and molecular characterization of common European species of *Adialytus* (Hymenoptera: Braconidae: Aphidiinae) based on the mtCOI barcoding gene and geometric morphometrics of forewings

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**Key words.** Hymenoptera, Braconidae, *Adialytus*, parasitoids, morphology, genetic diversity, key

**Abstract.** In this study three common European species of the genus *Adialytus* Förster, 1862 were examined: *Adialytus ambiguus* (Haliday, 1834), *Adialytus salicaphis* (Fitch, 1855) and *Adialytus thelaxis* (Starý, 1961). Molecular analysis involved the DNA barcoding of a region of the mitochondrial gene cytochrome oxidase I (COI). The genetic difference based on Kimura's two-parameter model for computing pairwise distances showed that *A. thelaxis* differs from both *A. ambiguus* and *A. salicaphis* by 4.9 and 6% on average, respectively. The genetic distance between *A. ambiguus* and *A. salicaphis* was 1.5% on average, suggesting that barcodes based on the COI gene are insufficiently informative for separating these two species. Geometric morphometrics analysis of forewing size and shape revealed statistically significant differences. The R1 vein on the forewing of *A. ambiguus* is more elongated than on the wings of *A. salicaphis* and *A. thelaxis*. The geometric morphometrics analysis of the forewings also revealed that *A. salicaphis* and *A. thelaxis* have much broader forewings, suggesting strong flight ability associated with their parasitizing arboricolous aphids. The distal part of the forewing of *Adialytus ambiguus* is narrower, which in this case suggests poor flight ability associated with parasitizing *Sipha* aphids on grasses. An illustrated key for identifying the European species of *Adialytus* is provided.

## INTRODUCTION

The genus *Adialytus* Förster, 1862 belongs to the koinobiont endoparasitic wasps of the subfamily Aphidiinae (Hymenoptera: Braconidae). *Adialytus* resembles the genus *Lysiphlebus* Förster, 1862, but can be easily distinguished by its reduced wing venation. The forewings of the genus *Adialytus* lack the m-cu and r-m veins, present in most species of the genus *Lysiphlebus* (Starý, 1975) Fig. 1. The two genera can also be separated by the shape of the petiole, which is slender and elongated in *Adialytus* and triangular and short in *Lysiphlebus* (Starý, 1975). The genus *Adialytus* was established by Förster (1862) with the type species *Adialytus tenuis* Förster, 1862. However, it was later joined by Gahan (1910) to the genus *Diaeretus* Förster, 1862 on the basis of forewing venation. After redescription by Starý & Schlinger (1967), *Adialytus* was recognized as a subgenus of *Lysiphlebus*, from which it differs in the previously mentioned characters. Finally, its generic rank was restored by Mackauer & Starý (1967) and Mackauer (1968). It is usually considered as a genus of the subtribe Lysiphlebina Mackauer, along with the genera *Lysiphlebus* and *Lysiphlebia* Starý & Schlinger, 1967. In later studies (Starý, 1970, 1975), *Adialytus* was still considered as a subgenus of *Lysiphlebus*. Furthermore, when the species *Adialytus veronicaecola* Starý, 1978, [originally *Lysiphlebus* (*Adialytus*) *veronicaecola*] was described, the subgeneric status was preserved (Starý & Juchnevic, 1978). One year later, Starý & Rakauskas (1979) described *Adialytus balticus* and confirmed the generic status of *Adialytus*. Currently, seven valid *Adialytus* species are recognized: *A. ambiguus* (Haliday, 1834), *A. balticus* Starý & Rakauskas, 1979, *Adialytus fuscicornis* (Ashmead, 1891), *Adialytus kaszabi* Takada, 1979, *A. salicaphis* (Fitch, 1855), *A. thelaxis* (Starý, 1961) and *A. veronicaecola* (Starý, 1978). *Adialytus ambiguus* and *A. salicaphis* have the widest distribution, inhabiting the entire Holarctic (Mackauer & Starý, 1967). The most common hosts of *A. ambiguus* are species of the genera *Sipha* Passerini, 1860 and *Atheroides* Haliday, 1838, both of which feed on Poaceae (Starý & Rakauskas, 1979). There are also a few records of successful parasitization of *Rhopalosiphum padi* (Linnaeus, 1758) by *A. ambiguus* (Michelena et al., 2004; Stanković et al., 2013). *Adialytus salicaphis* parasitizes many species of the genus *Chaitophorus* Koch, 1854, which feed on poplars and willows (Takada, 1968; Shujauddin, 1978; Pike et al., 2000; Tomanović et al., 2006; Žikić et al., 2012). *Adialytus thelaxis* has been recorded so far from Asia and Europe,

parasitizing species of the genus *Thelaxes* Westwood, 1840 (Starý, 1979; Kavallieratos et al., 2004; Žikić et al., 2012). The Nearctic species *A. fuscicornis* is recorded in Canada and the USA as a parasitoid of aphids of the genus *Aphis* (Linnaeus, 1758) (Smith, 1944; Pike et al., 2000). The remaining three species have much narrower distributions, especially *A. balticus*, which is recorded in Lithuania as a parasitoid of *Dysaphis anthrisci* Börner, 1950 on the root collar of *Anthriscus* sp. (Starý & Rakauskas, 1979). *Adialytus kaszabi* is recorded only in Mongolia (Takada, 1979), and *A. veronicaecola* is restricted to Central Asia. The species *A. veronicaecola* parasitizes leaf-curling aphids of the genus *Aphis* (Starý & Juchnevic, 1978; Rakhshani et al., 2012).

Here we investigate all the European species except *A. balticus*, for which we do not have any specimens (there is only type material in P. Starý's collection). All three species analyzed (*A. ambiguus*, *A. salicaphis* and *A. thelaxis*) are morphologically similar. According to the existing identification keys, these three species differ in the shape and length ratio of the flagellar segments and in their petioles and ovipositor sheaths (Starý & Rakauskas, 1979; Mescheloff & Rosen, 1990). However, the differences between species are unclear because the morphological traits are often subtle and inconspicuous. Two new phenotypes of *A. ambiguus* are reported from Iran (Rakhshani et al., 2012). These phenotypes are denoted as "*A. arvicola*" and "*A.* cf. *ambiguus*" and both are parasitoids of the genus *Sipha*, like the common *A. ambiguus*. However, they differ primarily in the length of the R1 vein, which is much shorter in these two phenotypes, especially in "*A. arvicola*". These phenotypes of *A. ambiguus* further complicate the already difficult identification of *Adialytus* spp. In the past three decades, several identification keys for *Adialytus* species were published (Starý & Rakauskas, 1979; Mescheloff & Rosen, 1990; Rakhshani et al., 2012). However, the description in these keys of their coloration is vague and the measurements inconclusive.

*Adialytus* species are currently not considered to be economically important parasitoids. However, *A. ambiguus* parasitizes *Sipha* aphids, which are common on maize and wheat (Kavallieratos et al., 2004; Starý, 2006). *Adialytus ambiguus* was successfully introduced and established in Hawaii as a biocontrol agent of the sugarcane aphid *Sipha flava* (Forbes, 1884) (Culliney et al., 2003). Furthermore, *A. salicaphis* is an important parasitoid of *Chaitophorus* spp. in poplar nurseries (Tomanović et al., 2006; Žikić et al., 2012). An attempt was made in Chile to utilize *A. salicaphis* as a biocontrol agent against the poplar-feeding *Chaitophorus leucomelas* Koch, 1854, which is a nuisance because it produces large quantities of honeydew that contaminates roads and parked cars, but it failed, probably due to climatic conditions (Rodriguez et al., 2001). Living in or near agroecosystems, *Adialytus* spp. constitute an additional food source for predators and hyperparasitoids and thus has an important role in maintaining ecological stability in these habitats (Hågvar & Hofsvang, 1991; Starý & Pike, 1999).

The aim of the present research was to explore the genetic structure and morphological variability of European species of *Adialytus* (*A. ambiguus*, *A. salicaphis* and *A. thelaxis*) using partial sequences of the mitochondrial cytochrome oxidase subunit I (mtCOI) gene and geometric morphometrics analysis of forewing shape. We also provide a new identification key for the European species of *Adialytus*, based on the results of the geometric analysis and other morphological traits.

## MATERIAL AND METHODS

All specimens of *Adialytus* were collected during 1970–2013 from several European countries, i.e., the Czech Republic, France, Greece, Lithuania, Montenegro, Serbia, Slovenia, Spain and Iran. The nomenclature of parasitoid morphology follows Sharkey & Wharton (1997). The wasps analyzed along with their host associations are presented in Table 1. Aphid hosts were collected along with the plant material on which they fed and put into plastic containers covered with muslin cloth. The containers were placed in a growth cabinet kept at 22.5°C, 65% RH and 16L : 8D (Kavallieratos et al., 2010) for 2–3 weeks until the emergence of the wasps. Specimens subjected to DNA analysis were kept in 96% ethanol. For the molecular analysis of the species we chose the mtCOI gene, which is widely used as a tool for discriminating closely related species. This barcoding gene is very informative for aphidiine parasitoids (Derocles et al., 2012; Mitrovski-Bogdanović et al., 2013; Petrović et al., 2013). This molecular marker is not only an efficient tool for use in the phylogenetic analysis of insects, but can also be used for rapid species identification (Hebert et al., 2003; Packer et al., 2009). Furthermore, molecular identification does not have to be invasive. The inspected specimens remain intact and can be used for further morphological study, in contrast to morphological analysis requiring dissection of the specimens.

Prior to molecular analysis, all genomic DNA material was extracted from the wasps using the KAPA Express Extract Kit according to the manufacturer's instructions. The barcoding region of the mtCOI gene was amplified using the primers LCO1490 and HCO2198 (Folmer et al., 1994). The PCR amplification of DNA was done in a 25 µl volume containing 1 µl of extracted DNA, 12.5 µl of 1 × KAPA2G Robust HotStart ReadyMix (which contains 2 mM $MgCl_2$ in a concentration of 1X), 1.25 µl (0.5 µm) of each primer and 9 µl of molecular grade water. All PCR reactions took place in an Eppendorf Mastercycler® according to the following protocol: initial denaturation at 95°C for 5 min; 35 cycles consisting of 1 min at 94°C, 1 min at 54°C and 1.5 min at 72°C; and a final extension at 72°C for 7 min. Purification of the amplified samples was done using a QIAGEN QIAquick® PCR Purification Kit according to the manufacturer's instructions. Sequencing of the amplified DNA was performed by Macrogen Inc. (Seoul, South Korea).

Acquired sequences were edited using FinchTV (Geospiza, Inc., Seattle, WA) and prepared for alignment using Clustal *W* incorporated in the MEGA5.2 software package (Tamura et al., 2011). To calculate the average genetic distance between obtained sequences, we used Kimura's two-parameter procedure of base substitution using the bootstrap method with 1000 replicates. Phylogenetic relationships were reconstructed using the maximum likelihood (ML) and maximum parsimony (MP) methods, also incorporated in MEGA5.2. One thousand bootstrap replicates were performed to assess branch support in the given trees. We obtained sequences from a total of 65 specimens of *Adialytus* (species, hosts and sampling data are given in Table 1). Specimens of *Lysiphlebia mirzai* Shujauddin, 1975, *Lysiphlebus faba-*

Table 1. The list of specimens used in analysis. N-haplotype – number of different mitochondrial haplotypes per population. N-morph. – number of specimens used in geometric morphometrics analysis. GeneBank (NCBI) accession numbers for each haplotype are given.

| Species | Host | Plant | Locality | N-haplotype | N-morph. | GeneBank accession no. |
|---|---|---|---|---|---|---|
| *A. ambiguus* | *Sipha maydis* | *Lolium perenne* | GRC, Kyparissia | 1 (A8) | 15 | KJ719612 |
| *A. ambiguus* | *Sipha* sp. | *Zea mays* | SRB, Niš, Popovac | 2 (A2, A8) | 10 | KJ719606<br>KJ719612 |
| *A. ambiguus* | *Sipha maydis* | *Hordeum murinum* | GRC, Kalamata | 1 (A6) | 13 | KJ719610 |
| *A. ambiguus* | *Sipha maydis* | *Bromus tectorum* | IRN, Mane | 1 (A8) | – | KJ719612 |
| *A. ambiguus* | *Sipha* sp. | *Hordeum murinum* | MNE, Bar | 1 (A8) | 10 | KJ719612 |
| *A. ambiguus* | *Sipha* sp. | *Digitaria sanguinalis* | SRB, Lebane | – | 10 | – |
| *A. ambiguus* | *Sipha maydis* | *Hordeum murinum* | FRA, Le Luc | – | 10 | – |
| *A. ambiguus* | *Sipha maydis* | *Plantago* sp. | SRB, Tošin bunar | – | 12 | – |
| *A. ambiguus* | *Sipha* sp. | *Dactylis glomerata* | SRB, Vlasina lake | 1 (A1) | – | KJ719605 |
| *A. ambiguus* | *Sipha* sp. | *Arrenantherum elatior* | SRB, Tara mt. | 1 (A7) | 10 | KJ719611 |
| *A. ambiguus* | *Sipha maydis* | *Agropyrum repens* | SRB, Kula | 1 (A2) | 8 | KJ719606 |
| *"A. arvicola"* | Malaise Trap | | IRN, Qazvin, Reveskk | 1 (A4) | – | KJ719608 |
| *"A. arvicola"* | *Sipha maydis* | *Poa pratensis* | LTU, Punciai | 2 (A6, A8) | – | KJ719610<br>KJ719612 |
| *"A. arvicola"* | Malaise Trap | | IRN, Qazvin, Koohin | 1 (A3) | – | KJ719607 |
| *"A. arvicola"* | *Sipha flava* | *Agropyrum repens* | IRN, Kermanshah | 1 (A4) | – | KJ719608 |
| *"A. cf. ambiguus"* | *Sipha elegans* | *Triticum aestivum* | IRN, Mashhad | 1 (A5) | – | KJ719609 |
| *"A. cf. ambiguus"* | *Sipha* sp. | *Hordeum* sp. | IRN, Isfehan | 1 (A9) | – | KJ719613 |
| *"A. cf. ambiguus"* | *Sipha elegans* | *Gastridium phleoides* | IRN, Isfehan | 1 (A9) | – | KJ719613 |
| *A. salicaphis* | *Chaitophorus populeti* | *Populus alba* | ESP, Leida | 1 (S2) | – | KJ719615 |
| *A. salicaphis* | *Chaitophorus vitellinae* | *Salix alba* | SVN, Zbilje | 2 (S3, S5) | – | KJ719616<br>KJ719618 |
| *A. salicaphis* | *Chaitophorus leucomelas* | *Populus* sp. | SVN, Zbilje | 1 (S7) | – | KJ719620 |
| *A. salicaphis* | *Chaitophorus leucomelas* | *Populus* sp. | SVN, Zbilje | 1 (S8) | – | KJ719621 |
| *A. salicaphis* | *Chaitophorus* sp. | *Salix* sp. | CZE, N. Dvur, Silesia | 1 (S8) | – | KJ719621 |
| *A. salicaphis* | *Chaitophorus* sp. | *Salix* sp. | CZE, Ceske Budejovice | – | 10 | – |
| *A. salicaphis* | *Chaitophorus niger* | *Salix* sp. | FRA, Antibes | – | 10 | – |
| *A. salicaphis* | *Chaitophorus salijaponicus* | *Salix alba* | IRN, Shirvan | 1 (S6) | 14 | KJ719619 |
| *A. salicaphis* | *Chaitophorus populeti* | *Populus alba* | IRN, Tehran | 1 (S8) | – | KJ719621 |
| *A. salicaphis* | *Chaitophorus populeti* | *Populus alba* | SRB, Niš, Popovac | 1 (S4) | 15 | KJ719617 |
| *A. salicaphis* | *Chaitophorus* sp. | *Salix alba* | IRAN, Isfehan | 2 (S6, S8) | – | KJ719619<br>KJ719621 |
| *A. salicaphis* | *Chaitophorus* sp. | *Populus alba* | SRB, Niš, Popovac | 1 (S4) | 11 | KJ719617 |
| *A. salicaphis* | *Chaitophorus niger* | *Salix fragilis* | SRB, Niš, Popovac | 1 (S1) | 9 | KJ719614 |
| *A. salicaphis* | *Chaitophorus salicti* | *Salix caprea* | SRB, Vlasina lake | 1 (S1) | 10 | KJ719614 |
| *A. salicaphis* | *Chaitophorus* sp. | *Salix caprea* | SRB, Dukat mt. | 1 (S4) | 10 | KJ719617 |
| *A. salicaphis* | *Chaitophorus salicti* | *Salix caprea* | SRB, Stara mt. B. zub | 2 (S1, S3) | 10 | KJ719614<br>KJ719616 |
| *A. salicaphis* | *Chaitophorus populeti* | *Populus alba* | IRN, Karadj | 1 (S6) | – | KJ719619 |
| *A. thelaxis* | *Thelaxes* sp. | *Quercus cerris* | SRB, Lebane | 2 (T1, T2) | 15 | KJ719622<br>KJ719623 |
| *A. thelaxis* | *Thelaxes* sp. | *Quercus ilex* | MNE, Bečići | – | 9 | – |
| *A. thelaxis* | *Thelaxes* sp. | *Quercus cerris* | SRB, Niš | 1 (T2) | 9 | KJ719623 |
| *A. thelaxis* | *Thelaxes* sp. | *Quercus castanifolia* | IRN | – | 7 | – |

*rum* (Marshall, 1896), *Areopraon lepelleyi* (Waterston, 1926) and *Cotesia* sp. Cameron, 1891 (Braconidae: Microgastrinae) were used as out group taxa. To calculate the genetic distance between the *Adialytus* species, the sequences of 29 specimens of *A. ambiguus*, 29 of *A. salicaphis* and seven of *A. thelaxis* were used in this analysis (Table 1). Of the 29 specimens of *A. ambiguus*, six belong to the "*A. arvicola*" phenotype and six to the "*A.* cf. *ambiguus*" phenotype (Rakhshani et al., 2012). To obtain phylogenetic trees with a clearer topology, we used one specimen per haplotype. The species *A. ambiguus* encompassed nine haplotypes (A1–A9), each consisting of 1 to 10 specimens, while *A. salicaphis* encompassed eight haplotypes (S1–S8), with 1 to 7 specimens per haplotype. *Adialytus thelaxis* was represented by only two haplotypes (T1–T2), one with one specimen and the other with six specimens (Table 1). All sequences were indel-free trimmed to a length of 619 bp and in this form used for the phylogenetic analysis.

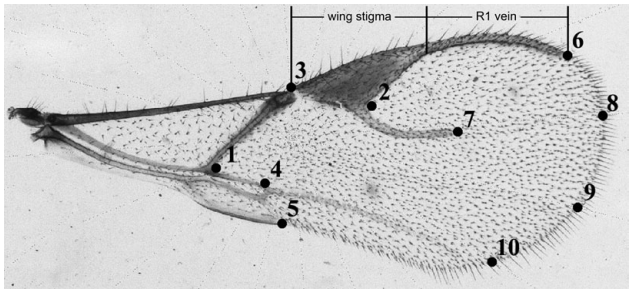


Fig. 1. Positions of the landmarks (1–7) and semi-landmarks (8–10) on the forewing of a species of *Adialytus*. The stigma and R1 vein are also marked.

The right forewings of female specimens were used to determine the variation in wing size and shape using a geometric morphometrics approach (Zelditch et al., 2004). In total, we used 237 specimens from 22 *Adialytus* populations; nine populations of *A. ambiguus*, nine of *A. salicaphis* and four of *A. thelaxis* (Table 1). In this study we defined a population as a set of specimens sampled at the same location on the same date. Most of the chosen phenotypes embodied 7 to 15 specimens per population. The wasps were dissected and mounted on microscope slides in Berlese solution. All wings were photographed using a Leica system DM2500 microscope with a Leica DFC490 digital camera (Leica Microsystems©, Wetzlar, Germany) under the same magnification and at the same microscope settings. After digitalization, the wings were analyzed using a series of computer software packages.

Due to the reduced wing venation in the genus *Adialytus*, we used a combination of seven landmarks and three semi-landmarks (positions and definitions are given in Fig. 1) in order to analyze forewing size and shape.

Both landmarks and semi-landmarks were digitalized using Tps Dig2 software (Rohlf, 2005) by the same person (S. Stanković). Before placing the semi-landmarks, all wings were processed in the MakeFan6 (Sheets, 2003) program by adding star-shaped lines, which serve for consistent placement of semi-landmarks at equal angular displacements along the curves superimposed by generalized Procrustes analysis. Thus, all variations due to scale, position and orientation of landmark configurations were eliminated (Rohlf & Slice, 1990; Bookstein, 1991). Superimposition of the semi-landmarks was done by allowing them to slide along curves bounded by landmarks, thus minimizing the Procrustes distances among the specimens analyzed (Bookstein, 1997).

Forewing size was computed as centroid size (CS), which represents the measure of size in geometric morphometrics and indicates the dispersion from the centroid of the landmark configuration. To explore the variation in size among forewings, we used analysis of variance (ANOVA) performed on centroid size, while for the shape variables we used multivariate analysis of variance (MANOVA) performed on eigenvalues of principal components, i.e., PC scores. The MorphoJ program was used to analyze and visualize shape changes described by canonical axes (Klingenberg, 2011). Principal component analysis (PCA) was used to analyze variability in wing shape among the specimens investigated. The differences in wing shape of the three *Adialytus* species was visualized using canonical variate analysis (CVA). The PC scores and centroid size (CS) were obtained using MorphoJ software. All statistical analyses were performed in the Statistica program package (Stat Soft Inc. 7.0).

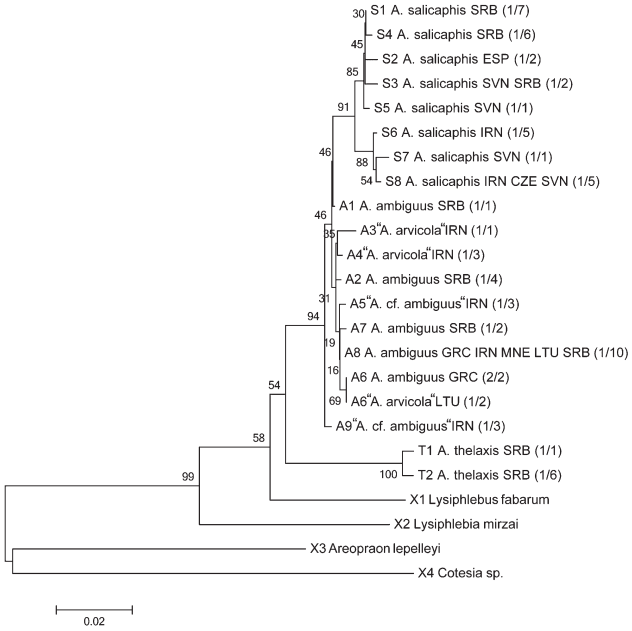


Fig. 2. Phylogenetic tree inferred using the maximum likelihood method. A discrete Gamma distribution was used to model the differences in evolutionary rate; Branches corresponding to partitions that were reproduced in less than 50% of the bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Numbers in parentheses represent the number of specimens per given haplotype.

## RESULTS

### Phylogenetic analysis

The topology of the phylogenetic trees inferred using ML and MP methods reveals that the clade that includes *A. thelaxis* is clustered apart from the mutual clade of *A. ambiguus* and *A. salicaphis*. However, relationships between *A. ambiguus* and *A. salicaphis* are not clear. Both of the trees presented have almost the same topology (Figs 2, 3). The A1 and A9 haplotypes of *A. ambiguus* are separated from the clade, whereas all other haplotypes of *A. ambiguus* are clustered together and all those of *A. salicaphis* are grouped in the same clade (Figs 2, 3). The consistency of the *A. salicaphis* clade is well supported by both phylogenetic trees, in which more than 90 percent of the cases this branch was separated from the *A. ambiguus* branch. However, the topology of *A. ambiguus* on the phylogenetic trees is not so clear (Figs 2, 3). Haplotype A9 (*A.* cf *ambiguus*) is positioned as a basal taxon to the mutual clade formed by *A. ambiguus* and *A. salicaphis*. Furthermore, the *A. salicaphis* clade has one sister taxon represented by haplotype A1, which is actually *A. ambiguus*. The analysis of genetic distances revealed that *A. thelaxis* differs from *A. ambiguus* on average by 4.9% and from *A. salicaphis* by 6%. The average genetic distance between *A. ambiguus* and *A. salicaphis* is 1.5%, but it ranges from 0.8 to 2.2%. All specimens of *A. ambiguus*, which are denoted as "*A. arvicola*" phenotypes, are clustered together with other specimens of *A. ambiguus* and showed no particular grouping within the *A. ambiguus* clade. The genetic dis-

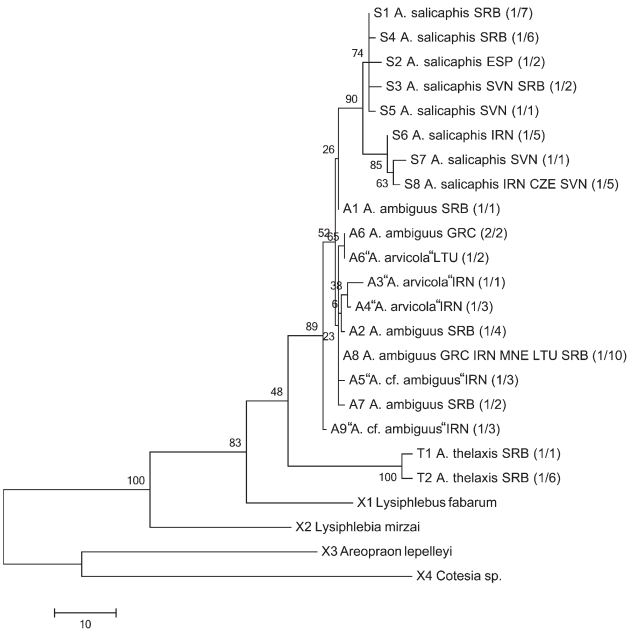


Fig. 3. The first of the 55 most parsimonious trees (length = 249) with a consistency index of 0.691358. Branches corresponding to partitions that were reproduced in less than 50% of the bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Numbers in parentheses represent the number of specimens per given haplotype.

tance between phenotype "*A. arvicola*" and other *A. ambiguus* specimens ranges from 0 to 1% (0.3% on average). The situation is almost the same for phenotype "*A.* cf *ambiguus*": average genetic distance is 0.4% (range 0.2–1%), but one of the haplotypes (A9) is not included in the main *A. ambiguus* clade (Figs 2, 3). Although the topologies of the phylogenetic trees indicate that the *A. salicaphis* clade is closest to the *A. ambiguus* specimens mentioned above, the genetic distance between the A1 haplotype of *A. ambiguus* and all other specimens of *A. ambiguus* is 0.3% on average (range 0.2–0.8%). However, the distance between the same haplotype (A1) and all specimens of *A. salicaphis* is 1.2% (0.8–1.7%). A similar situation exists with the A9 haplotype. The average genetic distance between this haplotype and all other specimens of *A. ambiguus* is 0.4% (0.3–1%), while the distance from *A. salicaphis* is on average 1.6% (range 1.2–2.2%). The average genetic distances within each particular group are: *A. ambiguus*: 0.4%, *A. salicaphis*: 0.8% and *A. thelaxis*: 0.5%.

**Geometric morphometrics**

The variation in the shape of the forewing of our material was determined using principal component analysis (PCA). Ordination of the specimens in morphospace defined by the first two principal axes is shown in Fig. 4. From the scatterplot it is evident that all specimens of *A. ambiguus* are grouped together on the right side of the PC1 axis, while all those of *A. salicaphis* and *A. thelaxis* are grouped on the left side and overlap. Overlapping is due to the similar length of the R1 vein.

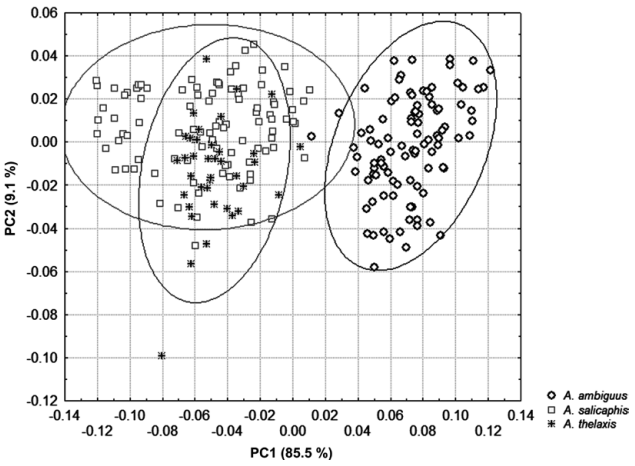


Fig. 4. Principal component analysis scatterplot of the results for the shape of the forewing: the PC1 axis accounts for 85.5% and PC2 axis for 9.1% of total variability. The confidence interval of the ellipses is 95%.

Analysis of variance of the forewing size of *Adialytus* spp. showed marginally significant differences among the species (ANOVA: $F_{(2, 234)} = 5.99$; $P = 0.002899$). Apart from forewing size, multivariate analysis of shape using PC scores also showed significant differences (MANOVA: Wilks' $\lambda = 0.026928$; $F_{(32, 438)} = 69.72$; $P < 0.000001$). As all differences were statistically significant, we performed a canonical variate analysis (CVA) in order to obtain a clearer distinction in terms of wing shape. In the morphospace defined by canonical axes, all three *Adialytus* spp. occupied discrete positions, with a clear distinction between all the species analyzed (Fig. 5).

Along the first canonical axis (CV1), which accounts for 82.91% of the total differences, *A. ambiguus* is separated from both *A. salicaphis* and *A. thelaxis* primarily by the length of the stigma and R1 vein on the forewing, which were described by landmarks 3 and 6. It is also noticeable that the forewings of this species are narrower distally than in the other two species. This is evident from the shift in semi-landmarks 8, 9 and 10 towards the center of the wing. The second canonical axis (CV2) explains the remaining variability and separates *A. thelaxis* from the other two species, especially from *A. salicaphis*, which it resembles in length of the R1 vein. *Adialytus thelaxis* has a slightly wider forewing proximally than *A. salicaphis*, which is evident from landmarks 1 and 2, as well as in the upper distal part, described by landmark 6. However, the most conspicuous feature is the shape of the R1 vein. In *A. salicaphis* the upper right portion of the wing is more rounded than in *A. thelaxis* (landmark 6).

**Key to European species of *Adialytus* (based on females)**

1 Flagellomeres F3 and F4 about 1.5 times as long as wide. Three preapical flagellar segments square (Figs 6f, 7c) ..... 2
– Flagellomeres F3 and F4 ≥ 2 times as long as wide. Three preapical flagellar segments not square (Figs 6d, 6e)..... 4
2 Ovipositor sheath sharply pointed (Fig. 6i) .....*A. thelaxis*
– Ovipositor sheath broad at tip (Figs 6h, 7b) ..... 3

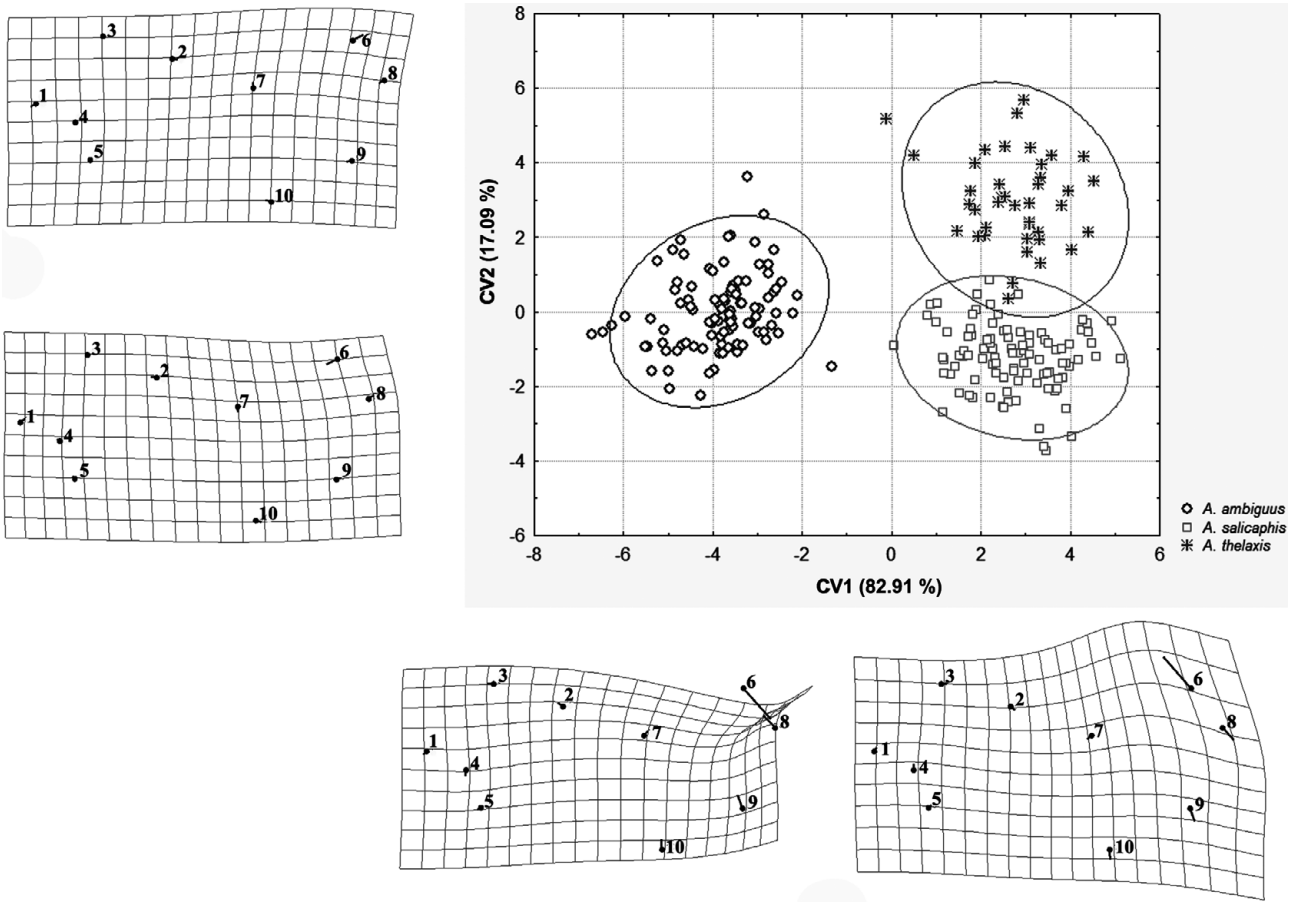


Fig. 5. Position of specimens of *Adialytus* in morphospace based on the shape of their forewings and defined by the first (CV1 = 82.91%) and second (CV2 = 17.09%) CV axis. The changes in wing shape are presented as thin-plate spline deformation grids. The scale factors for the transformation grid for both axes are –6 and 6. The confidence interval of the ellipses is 95%.

3 Dorsal outline of ovipositor sheath almost linear (Fig. 7b).... ..... *A. balticus*
– Dorsal outline of ovipositor sheath strongly convex (Fig. 6h) ..... 4
4 R1 vein longer than stigma (Fig. 6a). Ovipositor sheath pointed at apex (Fig. 6g). Two divergent carinae present at base of propodeum (Fig. 6j)..... *A. ambiguus*
– R1 vein as long as stigma (Fig. 6b). Ovipositor sheath broad at tip (Fig. 6h). Carinae absent or not clearly defined (Fig. 6k).....*A. salicaphis*

## DISCUSSION AND CONCLUSIONS

In this study we explored and identified variation in the morphology of the forewings of *A. ambiguus*, *A. salicaphis* and *A. thelaxis*. It is well known that wings of parasitoids are important in host location and mating behaviour (Godfray, 1994) and also a reliable taxonomical character (Starý, 1970). Geometric morphometrics of wing size and shape revealed some characters that are useful for identification at the species level. This method has proved to be very useful in resolving taxonomical problems of parasitoid species complexes (Baylac et al., 2003; Žikić et al., 2009; Mitrovski Bogdanović et al., 2013; Tomanović et al., 2013). The PCA analysis revealed that the length of the R1 vein varies between the species analyzed. Thus, this character can be used to distinguish *A. ambiguus* from the other two species analyzed. Although all specimens of *A. ambiguus* analyzed had a long R1 vein, which reached the upper right margin of the forewing, it is likely that the two phenotypes "*A. arvicola*" and "*A.* cf. *ambiguus*" described by Rakhshani et al. (2012) will not only be confused with other *Adialytus ambiguus* phenotypes, but also with *A. salicaphis*, if the identification is solely based on the length of the R1 vein. However, reliable identification of *A. ambiguus* is possible if a large number of specimens are available. The results of geometric morphometrics of the forewing also revealed that *A. salicaphis* and *A. thelaxis* have much broader forewings, suggesting they are strong fliers and this trait might be associated with their being parasitoids of arboricolous aphids. The distal part of the forewing of *Adialytus ambiguus* is narrower, which in this case suggests poor flight ability associated with parasitizing *Sipha* aphids on grasses. According to geometric morphometrics, *A. salicaphis* and *A. thelaxis* are morphologically more closely related to each other than either is to *A. ambiguus*.

In addition to the morphological differences between species of *Adialytus*, which are not conspicuous, we felt that it would be useful if they could be correctly identified using molecular analysis. However, the molecular analysis of these three species revealed a different picture of their phylogenetic relations. According to Kimura's two-pa-

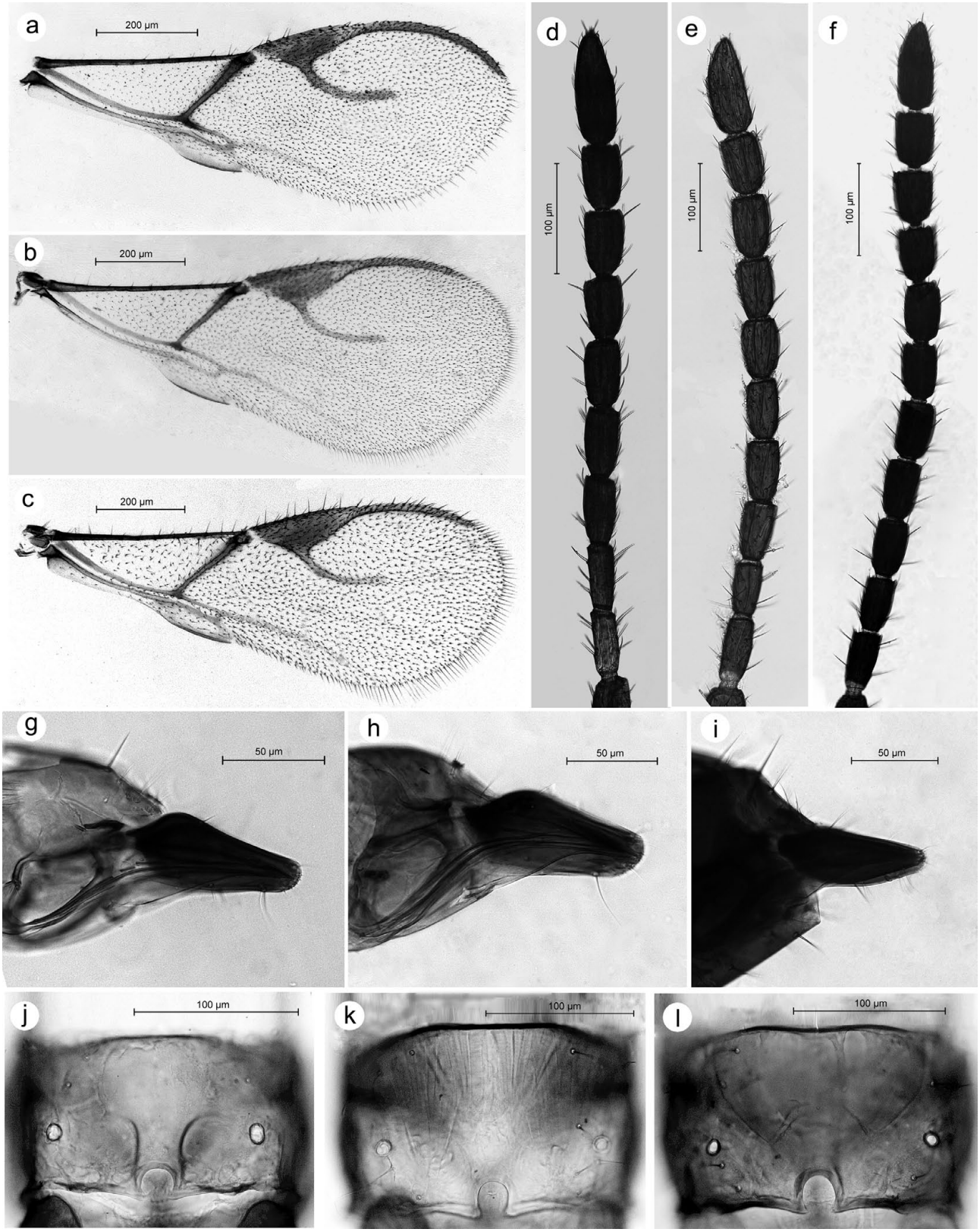


Fig. 6. Forewings, antennae (flagellomeres), ovipositor sheath and propodeum of the three species of *Adialytus*. *A. ambiguus* – a, d, g, j; *A. salicaphis* – b, e, h, k; *A. thelaxis* – c, f, i, l.

rameter model of pairwise distances among insects, 2% is enough to consider two entities as separate species (Packer et al., 2009; Kim et al., 2012). However, this percentage should not be used alone for the delimitation of species. We strongly advocate the use of morphological traits and ecological data along with genetic distances, because the combination of these three methods can give more accurate information on species separation. The present study con-

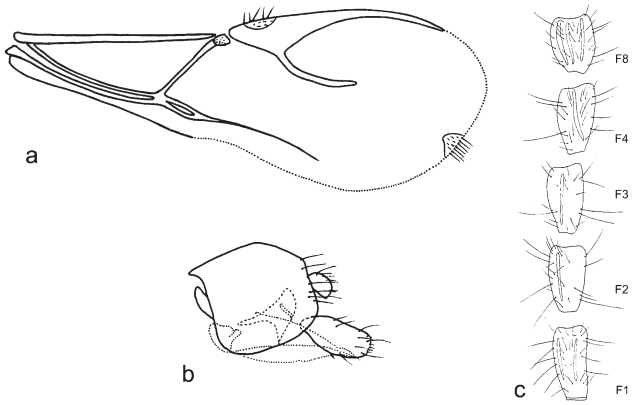


Fig. 7. *Adialytus balticus*: a – forewing; b – ovipositor; c – flagellar segments (after Starý & Rakauskas, 1979).

firms that *A. thelaxis* is a "bona species" based on its genetic distance from both *A. ambiguus* and *A. salicaphis*, which is on average 4.9 and 6%, respectively. This is clearly evident in both of the phylogenetic trees (Figs 2, 3) in which the separation of the *A. thelaxis* branch has the greatest "bootstrap" support. Although the phylogenetic reconstruction showed that *A. salicaphis* is a monophyletic group, the genetic distance based on the mtCOI gene of 1.5% (range from 0.8 to 2.2%) between *A. ambiguus* and *A. salicaphis* indicates that in this case the mtCOI gene is not informative enough. The "bootstrap" support for separating the *A. salicaphis* branch is about 90%, showing that this species is very consistently identified as monophyletic. However, *A. ambiguus* is a much more complex species, both genetically and morphologically. According to the topology of the phylogenetic trees there is relatively little support for the monophyly of this species. Haplotypes such as A1 seem to be close to *A. salicaphis*, whereas A9 is not included in the mutual clade consisting of these two species with a relatively high "bootstrap" support of about 50 percent. It seems that there is no congruence between the molecular and morphological variation within *A. ambiguus*. Haplotypes denoted as "*A.* cf *ambiguus*" and "*A. arvicola*" are not clustered in the same clade but rather scattered between other *A. ambiguus* haplotypes. A similar example is that of the *Lysiphlebus fabarum* aphidiine wasp group, where the mtCOI gene is not informative enough for the separation of three morphological and ecological different sexual/asexual taxa – *L. fabarum*, *L. cardui* and *L. confusus* (Derocles et al., 2012; Sandrock et al., 2011). According to Žurovcová et al. (2010) the mtCOI gene is not informative enough for separating species complexes of Adelgidae, but is informative enough to identify the genera. Both *A. ambiguus* and *A. salicaphis* parasitize aphids of the same subfamily (Chaitophorinae). However, *A. thelaxis* parasitizes aphids of the subfamily Thelaxinae indicating that this could be an evolutionary young complex. *Adialytus ambiguus* parasitizes aphids on herbaceous plants (Kavallieratos et al., 2004), while *A. salicaphis* attacks only aphids on trees, i.e., poplars and willows (Tomanović et al., 2006), which means that they have separate ecological niches.

Morphological variability of *A. ambiguus* together with the phenotypes "*A. arvicola*" and "*A.* cf *ambiguus*" is not reflected in genetic distance measured using the mtCOI gene. Both of these phenotypes are genetically very close or even identical to other specimens of *A. ambiguus* and are not grouped on the phylogenetic tree. Thus, we cannot state that these phenotypes are indeed different species, even though they are morphologically different (Rakhshani et al., 2012). The occurrence of these phenotypes also confirm that *A. ambiguus* is morphologically and genetically highly variable, suggesting that this particular species has great evolutionary plasticity.

This study reveals an apparent disparity between genetic traits on the one hand and morphological and ecological traits on the other. Incongruence between morphological and genetic traits is frequently recorded for parasitic wasps (Quicke & Belshaw, 1999; Tomanović et al., 2013). For example, Tomanović et al. (2013) report that there is no relationship between variation in the barcoding area of the mtCOI gene and the level of morphological variation in wing shape in closely related species of the genus *Aphidius* Nees, 1818. Similarly, there is no relationship between morphology and genetic diversity in the *Lysiphlebus fabarum* species group (Sandrock et al., 2011). On the other hand, incongruence between ecological traits and genetic diversity has not been well studied within the subfamily Aphidiinae. An interesting example is the case of *Aphidius microlophii* Pennacchio & Tremblay, 1987 and *Aphidius ervi* Haliday, 1834. Although, these species are morphologically similar, they are completely separated from an ecological point of view: *A. microlophii* parasitizes only *Microlophium carnosum* (Buckton, 1876) (Pennacchio & Tremblay, 1986) and *Wahlgreniella ossiannilssoni* Hille Ris Lambers, 1949 (Petrović et al., 2006), while *A. ervi* parasitizes a large number of aphid hosts, but not the previous two species. Analyses of mtCOI sequences showed no differences between these two parasitoid species (Derocles et al., 2012), although it is known that there is significant reproductive isolation between them (Tremblay & Pennacchio, 1988).

The results of this study revealed new relationships with respect to morphological and genetic variation in the genus *Adialytus*. Morphological differences in the shape of the forewings and patterns in wing venation, as well as other characters used in the key, had little support at the molecular level based on the mtCOI gene analyzed. However, each of the three species has a distinct ecology and a strong preference for different aphid hosts, which could be the main factor determining the morphological differences and consequent further separation of the species. Analysis of mating behaviour and reproductive isolation could provide additional information on the mechanism of speciation among species of the genus *Adialytus*.

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