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**Evolutionary relationships of wing venation and wing size and shape in Aphidiinae
(Hymenoptera: Braconidae)**

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Abstract

We explored evolutionary changes in wing venation and wing size and shape in
Aphidiinae, one of the well-known groups of parasitic wasps from the family Braconidae.

Forewings of 53 species from 12 genera were examined, for which a molecular phylogeny was constructed on the basis of the mitochondrial barcoding gene COI. By covering all types of wing venation within the subfamily Aphidiinae and by using landmark-based geometric morphometrics and phylogenetic comparative methods, we tested whether evolutionary changes in wing shape correlate to the changes in wing venation and if both changes relate to wing size. The relationship between wing morphology and host specificity has been also investigated. We found that six types of wing venation, with different degree of vein reduction, could be recognised. Wing venation type is largely genus specific, except in the case of maximal reduction of wing venation which could be found across examined Aphidiinae taxa. The reconstruction of evolutionary changes in wing venation indicates that evolutionary changes in wing shape are related to the changes in wing size, indicating that miniaturization play a role in evolution of wing morphology while host specialization does not affect the wing shape within the subfamily Aphidiinae.

Key words: parasitoids, geometric morphometrics, host specialization, miniaturization, comparative analyses.

Introduction

The wing venation pattern in braconids is a conspicuous and an important taxonomic trait (e.g., Riegel 1948; van Achterberg 1991) which confers important information when inferring phylogeny (Sharkey and Roy 2002). The reduction in wing venation within braconid wasps is a general evolutionary trend within parasitoid insects and could be associated with miniaturization of body size (Quicke and van Achterberg 1990). Relatively larger Braconidae species usually

have many longitudinal and cross veins, e.g., most species of the cyclostome groups, but also Sigalphinae, Betylobraconinae, Helconinae, Opiinae, Macrocentrinae or Homolobinae (van Achterberg 1991). Such wing venation is considered to be an ancestral trait. For example in the subfamily Euphorinae, and more specifically in the more basal tribe Meteorini, all existing forewing veins are present, contrary to the modern group Perilitini where many veins are absent (Stigenberg et al. 2015). The maximum reduction of wing venation in Braconidae in the distal part of the forewing occurs in *Aneurobracon annulipes* van Achterberg, 1990, from the subfamily Agathidinae. Wharton (1980) mentions that the reduction of wing venation in Braconidae is achieved through different paths, following elongation of forewing cells and reduction of various groups of veins in separate phylogenetic lineages.

Parasitic wasps from the subfamily Aphidiinae represent a unique group of aphid-specific parasitoids within the family Braconidae. To date, between 420-505 species of Aphidiinae have been described worldwide with a great majority inhabiting the Holarctic region (Yu et al. 2013; Žikić et al. 2017). In the subfamily Aphidiinae, wing venation pattern varies considerably among taxa. The fully developed wing venation such as in the species from the genus *Ephedrus* or *Toxares* is considered a plesiomorphic trait (Mackauer 1961; Gärdenfors 1986). An illustrative example is a variation in wing venation within the Aphidiini lineage where different paths of vein reduction occurred; through partial reduction in *Aphidius* and *Lysiphlebus* species where two medial cross veins and a longitudinal medial vein disappear to the reduction of all medial and distal veins retaining only a part of the radial vein in various degree such as in the genera *Adialytus* and *Diaeretiella* (Wharton et al. 1997). Further, the reduction and narrowing of the stigma occurs in some species of the tribe Alysiini of the subfamily Alysiinae (e.g., *Aphaereta* and *Asobara*), and in *Pseudephedrus* species in Aphidiinae (Starý 1972).

Many factors, such as evolutionary changes in parasitoids' life history traits, aphid exploitations, e.g., shifting hosts, could have contributed to determine wing morphology, including wing venation pattern (e.g., Henry et al. 2006). Active flying, through oviposition, foraging and mating behaviour are connected with adaptive advantages of wing morphology in aphidiine parasitoids (Starý 1970; Rehman and Powell 2010). All Aphidiinae species are endoparasitic koinobionts, laying a single egg in the aphid host body. The entire larval stage of Aphidiinae takes place inside a living host body. The majority of aphidiine wasps are oligophagous, according to their host range (Starý 1981), attacking several to more than a hundred of aphid hosts (Žikić et al. 2017). Often, the hosts come from different genera or even different subfamilies (e.g. Kavallieratos et al. 2004; Žikić et al. 2012), frequently in different types of habitats, usually not affecting on the morphology of the parasitoid altered by the host in which it develops (Žikić et al. 2009). Gagić et al. (2016) found that for efficient aphid host exploitation by a specialist or generalist parasitoid, host aphid traits appear to be more important than the habitat or host-plant characteristics. As adults, Aphidiinae feed on nectar, and also aphid honeydew (Starý 1970).

Traditionally, Aphidiinae were considered as a separate family, Aphidiidae, within the superfamily Ichneumonoidea, mostly because of specific morphological characteristics and their specialization to parasitize only aphids (Starý 1966). However, according to subsequent molecular studies, they were given the subfamily status within the family Braconidae (Quicke and van Achterberg 1990; Wharton et al. 1992). Although there is no absolute consensus about phylogenetic relationships within the Aphidiinae subfamily, one of the widely accepted classification proposed by Mackauer (1961), recognise four tribes: Aclitini, Ephedrini, Praini and Aphidiini. Additionally, the Trioxini tribe is usually separated from Aphidiini (Tremblay and

Calvert 1971; O'Donnell 1989; Finlayson 1990; Belshaw and Quicke 1997). For a review of proposed Aphidiinae classification in the past see Smith et al. (1999).

Biogeographically, Aphidiinae follow their aphid hosts, having a Northern Hemisphere distribution. Exceptions are three Southern Hemisphere genera: *Pseudephedrus* inhabiting South and Central America, *Parephedrus* in Australia (Mackauer 1968) and *Choreopraon* in New Zealand (Mackauer and Finlayson 2012). Those "southern" genera are considered to be the oldest within the Aphidiinae having a Gondwanan origin which was supported by molecular evidences (Schlinger 1974) and Belshaw et al. (2000). More recently, Ortega-Blanco et al. (2009) discovered the earliest known Aphidiinae fossil from Spain (dated more than 100 Mya). They questioned Gondwanan origin of Aphidiinae and proposed a Northern hemisphere origin with a possibility of significant extinction of basal Aphidiinae. It became apparent that the great diversity of aphids and their parasitoids on the Northern Hemisphere strongly connect them with angiosperms. The particular interest in our study is a wide range in parasitoid body length that usually varies from 1 to 4 millimetres, but never exceeds 4 mm (Starý 1970).

By exploring wing venation, wing size and shape in 53 species from 12 genera covering all general types of wing venation within the subfamily, we explored the morphological variation in wing venation and wing shape of parasitoid wasps from the subfamily Aphidiinae. By mapping the wing venation and the wing shape onto an independently derived molecular phylogeny, we inferred the main evolutionary changes in wing structure. We tested if there is a phylogenetic signal concerning the change or reduction of wing venation and whether the evolutionary change in wing venation affects the shape of the wing. We also explored whether the evolutionary change in body size or host specificity affect wing morphology.

Material and methods

Sample overview

For this investigation we sampled 53 species from 12 genera. The list of analysed species is given in the Supplement 1. The material studied covers the largest part of the Holarctic including the endemic genus *Pseudephedrus* from the Neotropical region. The analysed species were classified based on their host specificity according to the number of aphid hosts they parasitize, taking into account their phylogeny and also the phylogenetic relations among plants (Starý 1981; Žikić et al. 2017). We accepted five host specificity groups; from monophagous (species that parasitize a single host) to polyphagous (that parasitize a large number of aphid hosts, often from different genera, even subfamilies). The following data are provided in Starý (1981), Yu et al. (2012) and Žikić et al. (2017), and the host specificity categories for this analysis were given in Supplement 1.

DNA analysis and molecular data

In order to test the phylogenetic signal in the evolution of wing venation, we constructed a phylogenetic tree based on the sequences of the mitochondrial gene of cytochrome oxidase 1 (COI) from 53 Aphidiinae species. This molecular marker has been widely used, especially for delimiting closely related species (Kos et al. 2011; Derocles et al. 2012; Mitrovski-Bogdanović et al. 2013; Petrović et al. 2013). For this purpose the sequences were acquired from the gene bank (NCBI) for 41 species while for 12 of them we performed DNA extraction. The sample overview and the GenBank codes are provided in Supplementary Table 1. The detailed

description of the PCR amplification, sequencing and phylogenetic reconstruction are provided in Supplement 1.

Wing venation and landmark data

In total, 748 right wings of female specimens were dissected to make microscopic slides for geometric morphometrics analysis. The wings were mounted on microscope slides using Berlese medium, dried at room temperature for seven days and photographed using a Leica DM2500 microscope with a Leica DFC490 digital camera. Wing venation was examined and the selected species were classified based on their wing venation type into groups (Fig. 1). To each of these groups, categorical state of characters was assigned according to wing venation presence and distribution and possible way of the wing veins reduction taking into account Aphidiinae phylogeny (Belshaw and Quicke 1997; Kambhampati et al. 2000). Hypothetically the way of reduction of veins started from the wing architected as typical braconid venation (Ephedrini), by losing veins in different modes, firstly cross veins, than longitudinal such as in many Trioxini and some Aphidiini. For the description of wing venation pattern we followed the nomenclature of wing venation after Wharton et al. (1997) (Fig. 2).

Landmark selection and size/shape variables

In comparative studies that include evolutionary novelties and losses, application of landmark based geometric morphometrics analysis can be a challenging task (Polly 2008; Klingenberg 2008; Gómez-Robles et al. 2011). To capture wing shape in all 53 species with disparate wing venation and extensive reduction of wing veins in some species, we selected 11

anatomical points (Fig. 3; Table 1). As medial veins (2M and 3M), including RS+M, 2RS and r+m were entirely reduced in some species the anatomical points related to these veins could not be considered (Fig. 2). Landmarks were limited to stigma, metacarpus, radial vein, intersections of veins on the proximal part of the wing, and the projections of radius, cubitus and anal vein to the very edge of the wing. A different degree of reduction of metacarpus and radial veins was captured with landmarks 8, 9 and 10. If a sclerotized radial vein reaches the edge of the wing, landmarks 9 and 10 fall at the same point. There are species with complete wing venation and others where, similarly, the metacarpus reaches the distal end of the radial vein or even projects over and beyond the end of the radial vein. In these species, the landmarks 8, 9 and 10 either fall at the same point, or even switch positions between landmarks 8 and 10 (see Fig. 3). The description of the landmarks is given in the Table 1. The set of eleven landmarks was positioned on forewings using the TPSdig2 software package (Rohlf 2005). Wing size was computed as the centroid size (CS), calculated as the square root of the sum of squared distances from a set of landmarks. Because of wide range in CS, all analyses were performed on log transformed CS. We applied a generalized Procrustes analysis (Rohlf and Slice 1990; Dryden and Mardia 1998) to obtain a matrix of shape coordinates – Procrustes coordinates. The software used to obtain size and shape variables and their statistical analyses was MorphoJ (Klingenberg 2011).

Statistical analyses

To trace changes in wing venation and reconstruct evolutionary changes in wing venation pattern, we applied maximum-parsimony criteria under two assumptions: 1) that evolutionary change between each wing type has equal "cost" (unordered characters) and 2) that the evolution of a particular character exhibits directionality, i.e., based on step-matrix derived from

assumptions on gain and loss of wing veins (see Fig. 4). A step-matrix explicitly specifies the number of steps from state to state by a matrix. The modular system Mesquite (Maddison and Maddison 2016) was used for ancestral state reconstruction.

The landmark-based, geometric morphometric analyses of wing shape were done using MorphoJ software (Klingenberg 2011). Principal Component Analysis (PCA) based on the covariance matrix of the shape variables (Procrustes coordinates) was used to explore shape variation within and between species. To reconstruct and visualize evolutionary shape changes, we mapped the PC scores onto the phylogeny. The shapes corresponding to the internal nodes were reconstructed using the weighted squared-change parsimony (Maddison 1991; Klingenberg and Gidaszewski 2010). To test for phylogenetic signal in wing size and shape, we used the permutation approach to simulate the null hypothesis of the absence of phylogenetic structure by randomly reassigning shape configurations to the terminal nodes of the phylogeny (Laurin 2004; Klingenberg and Gidaszewski 2010). Phylogenetic independent contrasts were calculated as weighted differences of values for sister nodes (Felsenstein 1985; Rohlf 2001).

As change in wing shape could be related to evolutionary change in wing size (Gould 1966; Pélabon et al. 2014; Klingenberg 2016), we applied a multivariate regression, with independent contrasts of shape as the dependent variable, and independent contrasts of size as the independent variable to test for evolutionary allometry (for detailed description see Klingenberg and Marugán-Lobón 2013). To test further for patterns of wing shape evolution, including an association of changes in wing size, wing shape and wing venation, we did a multivariate regression of the independent contrast of wing size and wing shape onto the independent contrast of the wing venation types. We also explored the association between changes in wing size and shape and host specificity, by regressing independent contrasts of size

and shape on independent contrast of host specificity. For the regression analyses, statistical significance was assessed by a permutation test (see Klingenberg 2016 and references therein).

Results

The phylogenetic tree based on COI barcoding region of 53 species from 12 genera of the subfamily Aphidiinae was constructed (Supplementary Fig. 1) and used in a subsequent comparative analyses. The phylogeny shows that the four tribes Ephedrini, Praini, Trioxini and Aphidiini are monophyletic clades. In addition, *Pseudephedrus* (unranked) stands out as a completely separate clade at the very base of the phylogenetic tree. The tribes Ephedrini and Praini are clustered at the base of the tree. Trioxini assembles the species of the genera *Trioxys*, *Binodoxys* (Trioxina) and *Monoctonus* (Monoctonina) leaving out *Lipolexis gracilis* Foerster 1862 on a separate clade. Aphidiini, a diverse tribe, is further subdivided into three subtribes: Protaphidina, Lysiphlebina and Aphidiina.

The wing venation pattern falls to six recognisable types (Figs. 1 and 4): A (*four cells type*) – fully developed venation of the forewing, forming four cells (1st discal, 1st submarginal, 2nd submarginal and marginal), in *Ephedrus*. Taking the complete wing venation as plesiomorphic trait, the reduction of r+m and 2RS veins leads to C (*axe stigma type*); the stigma is in the shape resembling an axe (narrow in its proximal part, triangular in the distal part, in *Praon* species. Further reduction of the C type by losing m-cu vein leads to wing type which is marked as B (*fork type*) which is present in *Pseudephedrus*. Two main veins, the radial sector (3RSa+3RSb) and the medial vein (RS+M, 2M and 3M) together with the metacarpus (R1) form a fork-like pattern. This type of wing has a stigma almost the same width as the metacarpus.

225 However, transition from the A to the B type could have happened in the direction to lose all tree
226 cross veins (r+m, 2RS and m-cu) (Fig. 4).

227 The third way of reduction of wing venation in the type A (*four cells type*) leads to the
228 type E (*horse head type*) by losing 2RS and RS+M veins (Figs 2 and 4). This wing venation type
229 has the unique discocubital cell, formed by the fusion of the 1st discal (VI), 1st submarginal (II)
230 and 2nd submarginal (III) (Fig. 2). This discocubital cell is in the form of horse head, and it is
231 present in the genera *Aphidius*, *Pauesia*, and almost all *Monoctonus*. Further reduction of m-cu
232 and 2M veins leads to type D (*H letter type*) – venation is reduced, the rest of the vein fragment
233 2M together with 3M, r+m, 3RSa and 3RSb form the uppercase letter H, as it can be seen in
234 *Lysiphlebia* and *Lysiphlebus*. The reduction of the above mentioned fragment of 2M vein and
235 3M leads to the most reduced wing venation F (*hook type*). This wing type has only a part of the
236 radius vein left (RS), present in *Adialytus*, *Binodoxys*, *Diaeretiella*, *Lipolexis* and *Trioxys*.

237 Mapping of scored character states over phylogeny revealed that wing venation types are
238 largely clade-specific, except E and F wing types (Fig. 5). However, ancestral states were largely
239 unresolved for the reconstructions based on step-matrix inferred character state changes (Fig. 5
240 right tree).

241 The distribution of the species in the wing shape morphospace is presented in Fig. 6. The
242 first three PC axes describe 84.3% of the total variance in wing shape (PC1 = 52.1%, PC2 =
243 25.1%, PC3 = 7.1%). These three PCs clearly separate tribes in the morphospace (Fig. 6A).
244 Wing venation pattern mapped over phylomorphospace (Fig. 6B) showed that change in wing
245 shape does partially coincide with the change in wing venation. Closely related species that
246 differ in wing venation pattern grouped together with their conspecifics (Fig. 6B).

The shape changes associated to the given PC axes are shown in the Fig. 7. The PC1 separated relatively shorter and narrower wings with the radial vein reaching the distal edge of the wing and elongated metacarpus (*Ephedrus*, *Pseudephedrus*, *Lipolexis*) from broader and relatively elongated wings, with partially reduced radial vein and short metacarpus, such as in *Monoctonus*, *Diaeretiella* and also in *Aphidius* (*Lysaphidus*) *viaticus* Sedlag 1968, and *Praon exsoletum* (Nees 1811). The PC2 axis separates the wings with elongated metacarpus and shorter radial vein (*Binodoxys* and *Trioxys*) from those with shorter metacarpus and relatively elongated radial vein (*Adialytus*, *Lysiphlebia*, *Pauesia*). The changes in the stigma shape (described by landmarks 5, 6 and 7, see Fig. 3) and the shape of the distal part of the wing below the radial vein are described by PC3 (Fig. 7). The PC3 separated the species with a relatively elongated wings with a narrower stigma or almost completely reduced stigma such as *Pseudephedrus*, from the wings with a wider distal part of the wing and a robust stigma as in *Monoctonus mali* van Achterberg 1989. Most *Aphidius* species have an intermediate wing shape relative to the species at the extremes of PC axes. In general, congeneric species are clustered together. The largest within-group variation in the wing shape characterises analysed members of the genus *Lysiphlebus*. Species with the short metacarpus and reduced radial vein, *L. testaceipes* (Cresson 1880), *L. orientalis* Stary & Rakhshani 2010 and *L. balcanicus* Starý 1998, group together and separate from the congeneric species with elongated metacarpal vein and longer radial vein (*L. fabarum* (Marshall 1896), *L. cardui* (Marshall 1896), *L. confusus* Tremblay & Eady 1978 and *L. hirticornis* Mackauer 1960).

The distribution of wing venation types relative to the forewing size indicates that D and F wing types occurred in the species with smaller log centroid size values, while the wing type E covers a wider range of the wing size (Fig. 8). The significant phylogenetic signal was found for

wing venation pattern ($P < 0.0001$) and for both wing size and wing shape (in both cases $P < 0.0001$), but no phylogenetic signal was found for host specificity ($P = 0.1958$). The multivariate regression of independent contrasts of wing shape on the independent contrast of wing size was marginally significant (6.02% variance explained, $P = 0.042$). No significant relationship was found between independent contrasts of wing size and shape and independent contrasts of host specificity (0.75 % variance explained, $P = 0.537$ for independent contrast of log centroid size and independent contrast of the host specificity; 2.67 % variance explained, $P = 0.217$ for independent contrast of shape and independent contrast of host specificity).

Discussion

We explored evolutionary changes in wing venation and wing size and shape of 53 species of parasitic wasps from the subfamily Aphidiinae with the aim of uncovering the major patterns of diversification in wing morphology and revealing possible trends and directions of evolutionary change in wing. To do this, we used landmark-based geometric morphometrics along a well resolved molecular phylogeny. In the subfamily Aphidiinae, the wing venation patterns fall into six recognizable types. Although fully developed wing venation with four closed cells in the distal part of the wing (type A) is regarded as plesiomorphic state in Aphidiinae (Mackauer 1961; Gärdenfors 1986; Wharton et al. 1997), the reconstruction of an ancestral character state at the basal branches of the phylogenetic tree are largely unresolved. Such patterns could be the result of a long and independent evolution of analysed lineages of aphid parasitoids. The first braconid fossils come from the Upper Cretaceous found in ambers from Canada and North Siberia, later in the Tertiary in Baltic amber, and also from Miocene to the middle Oligocene in North America (Florissant) (Willemstein 1987). Larsson (1978)

suggests that the main lineages of Braconidae, about 18 subfamilies, including Aphidiinae, were already present in the middle Tertiary, or even from the Lower Cretaceous (Ortega-Blanco et al. 2009). Such early separation of main phylogenetic clades, could lead to independent changes of wing venation within lineages, and explain largely “unresolved” evolutionary changes in wing venation among taxa in this study.

Aphidiinae tribes are clearly separated in the wing shape morphospace, while closely related species groups are clustered tight in the wing shape morphospace (Fig. 6). The most basal taxa, *Pseudephedrus* and *Ephedrus* are clearly separated from the other lineages. *Pseudephedrus* are specialized parasitoids of Spicaphidinae (*Neuquenaphis*) aphid hosts associated with the endemic South American plants of *Nothofagus* (Starý 1976). On the other hand, *Ephedrus* is a cosmopolitan genus whose species parasitize over hundred species from different aphid subfamilies (Žikić et al. 2012, 2017; Yu et al. 2012). However, there are some exceptions with marked divergence in the morphospace between related species. For example, within Aphidini, genus *Lysiphlebus*, *L. testaceipes*, *L. orientalis* and *L. balcanicus* with short metacarpus clearly separated from the other members of *Lysiphlebus* such as *L. fabarum*, *L. confusus*, *L. cardui* and *L. hirticornis* with a long metacarpus almost reaching the apex of the wing (Petrović et al. 2015). Our phylogenetic analysis supports the polyphyletic origin of the genus *Lysiphlebus*, and is further supported by the close clustering of phylogenetically related taxa in the wing shape morphospace.

In general, homoplasies in Aphidiinae are relatively rare. Similar comparative studies that apply a phylomorphospace approach on various taxa, have found species to be more evenly dispersed in shape space, with overlapping clades (Stayton 2005; Monteiro and Nogueira 2011; Prevosti et al. 2012; Klingenberg and Marugán-Lobón 2013; Hipsley et al. 2014). Such,

homogenous dispersion in the shape space, with substantial intersecting of branches among closely-related taxa characterizes ants (Pie and Tschá 2013) and phytophagous chafers (Scarabaeidae: Pleurosticti) (Eberle et al. 2014) among insect taxa. The strong phylogenetic structure in Aphidiinae taxa, with distinct clustering of clades and relatively few crisscrossing of branches produce discontinuous morphospace (Fig. 6). Such a discontinuous or starburst distribution has been observed in caecilians, (Sherratt et al. 2014). According to Starý (1970) there are two main groups of early phylogenetic differentiation within Aphidiinae related to ancestral aphids group: 1) ancestors of recent parasitoid genera (e.g. *Pauesia*, *Diaeretus*) which are related with lachnid aphid hosts in coniferous forest habitats, and 2) ancestors of remaining parasitoids genera (e.g. *Aphidius*, *Lysiphlebus*, *Ephedrus*) which are connected with aphid hosts in deciduous forest habitats and subsequent steppe habitats. *Pauesia* wasps are specialized parasitoids of ancient lachnid aphid hosts and Starý (1970) considered their origin from early parasitoid group associated with conifer habitats. Although, there are no simple relationships among recent aphidiine tribes and their aphid hosts, Aphidiini and Trioxini predominantly attack Macrosiphini and Aphidini aphid hosts, respectively (Starý 1981). However, we found that wing morphology (size, shape or venation pattern) is not related to host specificity in Aphidiinae. Host specialization in parasitoids is related to the efficiency of host use and especially with local host characteristics (Gagić et al. 2016) but not necessarily linked with change in morphology.

Indication of miniaturization

Brachyptery or complete wing loss is not a trait that is typical for aphidiines (Starý 1970). However, *Diaeretellus svalbardicum* Chaubet & Tomanović, 2012, a parasitoid species from Arctic area, was found in both forms, as macropterous and micropterous (Chaubet et al. 2012).

This state was also recorded in *Trioxys apterus* Gärdenfors, 1990 collected in a high mountain area in Ecuador (Gärdenfors 1990). In both these cases the wing reduction is driven by selection pressure induced by extreme climate conditions and short seasonal activity rather than direct influence of aphid hosts. Our results showed that Aphidiines with the smallest wings have the most reduced wing venation, such as the species in group F (hook type) and also in the group D (H letter type) (Fig. 5). Based on the pattern of the reduction of wing venation in the analysed taxa we assumed that 3RS vein is important in the basic armature of the distal part of the wing. Therefore the radial vein (3RS) is a single longitudinal vein left in the most reduced hook type wing venation (F type). This is obvious from *Trioxys pannonicus* Starý, 1960 (not included in this study), where the R1 vein is much shortened and only a small part is projecting from the stigma, while the radial sector remains as is in other species of the same wing type. This reduction was scored in the subtribes Lysiphlebina and Aphidiina of the tribe Aphidiini, and Trioxina and Monoctonina of the tribe Trioxini including *Lipolexis gracilis* (see Fig. 5). Such type of wing venation also occurs in other genera which belongs to Aphidiini tribe but have not been analysed here (due to a small number of available specimens) – *Diaeretellus* Starý and *Diaeretus* Förster (Mackauer 1958).

Our results support the notion that the evolution of the most reduced wing venation occurred several times independently, and a possible biological explanation would be economization of developmental resources as well as miniaturization, as suggested by Dudley (2002) when exploring the biomechanics of insect flight. An example of this is *Diaeretiella* which has the hook type wing among the subtribe Aphidiina, where all other members have the horse head wing type. Also, *Adialytus* with the hook type wing venation is nested among species with the H letter type of wing venation. In all analysed taxa, 2RS vein is the first vein that is

reduced from complete wing venation (four cells type) whichever, way the reduction goes. On the other hand, the radial sector (3RS) is the vein in wing architecture being present in all aphidiines. However, the high ambiguity of character changes, especially in the reconstruction based on step-matrix (Ree and Donoghue 1998), and absence of information on any functional and adaptive significance of wing venation patterns, prevents us from any further interpretation of homoplasy in wing venation pattern. Considering all above, it is evident that miniaturization but also, complex natural history, including early expansion and coevolution with hosts results, in the diversity in Aphidiinae wing venation patterns we observe today. In a further perspective including more genetic markers, such as sequence of the gene for 28S ribosomal subunit, could contribute in resolving the relationships of Aphidiinae joined with morphological analysis. The gene for 28S ribosomal subunit is conservative and it is already used in resolving phylogenetic relations of Aphidiinae (Belshaw and Quicke 1997; Dowton et al. 2002).

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585

586

587 **Table 1** The description of 11 landmarks identified on the forewings of Aphidiinae species. See
588 also Fig. 3.

Landmark	Description
LM1	The corner formed by the medial vein (1M) and the cubitus (1CU)
LM2	The corner formed by (1CU) and transverse medio-cubital vein (m-cu)
LM3	The projection of the vein analis to the edge of the wing, following its curvature
LM4	The corner formed by (1M) and radial sector + media (RS+M)
LM5	The beginning of the stigma
LM6	The junction of the transverse radial vein (r) and the stigma
LM7	The endpoint of the stigma
LM8	The endpoint of the radius (R1)
LM9	The endpoint of the radial sector (3RS)
LM10	The projection of the radial sector (3RS) following the curvature
LM11	The projection of the cubitus (3CU) following the curvature

589

590

591

Figures

Fig. 1 Diversity of wing types characterised by the presence/absence of wing veins and cells. A) *Ephedrus plagiator*, B) *Pseudephedrus* sp., C) *Praon barbatum*, D) *Lysiphlebus fabarum*, E) *Aphidius ervi*, F) *Binodoxys angelicae*. Detailed wing type definition is given in the text.

Fig. 2 Nomenclature of Aphidiinae wing venation following Wharton et al. (1997). CU – cubitus, M – media, R – radius, RS – radial sector, m-cu – transverse medio-cubital vein, r – transverse radial vein, r+m – transverse radio-medial vein; cells: I – marginal, II – 1st

601 submarginal, III – 2nd submarginal, IV – 3rd submarginal, V – basal, VI – 1st discal, VII – 2nd
 602 discal, VIII – subbasal, IX – 1st subdiscal, X – 2nd subdiscal, XI – anal.

603
 604 **Fig. 3** Set of landmarks positioned on the forewing of (A) *Ephedrus plagiator*, species with fully
 605 developed venation, and forewing of (B) *Binodoxys angelicae*, species with a reduced wing
 606 venation. First five landmarks (landmarks 1 to 5) describe the proximal part of the wing, the
 607 landmarks 5, 6 and 7 describe the stigma, 7 and 8 mark the length of metacarpus, 6 and 9 mark
 608 the radial vein, and together from 6-11 represent the distal area of the wing.

609

610 **Fig. 4** Wing venation types in Aphidiinae and number of character state changes. Distribution of
 611 veins and cells in the medial and distal part of the wing were considered, as proximal part of the
 612 wing has the same structure in all Aphidiinae. A – *four cells type*; B – *fork type*; C – *axe stigma*
 613 *type*; D – *H letter type*; E – *horse head type*; F – *hook type*. The changes in venation are
 614 highlighted in red and marked by smaller arrows.

Fig. 5 Phylogenetic hypothesis for the subfamily Aphidiinae based on the mtCOI gene and character states of wing venation allocated based on Parsimony ancestral state reconstruction method: left – unordered; right – step-matrix reconstruction as shown on Fig. 4. Wing venation character states were shown in colour as explained in legend. For the explanation of wing types see Fig. 4; empty branches = ambiguous.

Fig. 6 Distribution of species in the morphospace defined by three PC axes. Phylogeny is mapped over graph. A) Mapped distribution of the species means in phylomorphospace. Ellipses of the symbols represent genus affiliation; the genera affiliations are symbol-coded. B) Distribution of the six main types of the wing venation. The character states are colour-coded as in Fig. 5.

Fig. 7 Shape changes associated with the first three PCs are shown as extreme wing shapes (black shape) representing the shape of species with maximal positive and negative score of each axis comparing to the mean shape of the sample (grey shape).

Fig. 8 The distribution of the wing venation types relative to the species mean sizes (log centroid size). The phylogeny is superimposed according to the reconstructed ancestral values. The character states are colour-coded same as in the Fig. 5.

Supplement captions

Supplement 1 The data and parameters used for the analysis of selected parasitoids. Country's abbreviations: BEL=Belgium, CHE=Switzerland, CHL=Chile, CHN=China, CZE=Czech Republic, FRA=France, GRC=Greece, IND=India, IRN=Iran, JPN=Japan, MNE=Montenegro, NLD=Netherlands, SLO=Slovenia, SRB=Serbia. Legator's abbreviations: AM=A. Mitrovski-Bogdanović, AP=A. Petrović, BL=B. Lavandero, CV=C. Vorburger, ER=E. Rakhshani, HT=H. Takada, KK=K. Kos, MB=M. Brajković, MD=M. Djordjević, MI=M. Ilić Milošević, MJ=M. Janković, NK=N. Kavalliaratos, PS=P. Starý, SS=S. Stanković, VŽ=V. Žikić, ZK=Z. Kojičić, ŽT=Ž. Tomanović

Supplement 2 DNA extraction, amplification, sequencing and phylogenetic reconstruction.

Supplementary figure

Suppl. Fig. 1 Phylogenetic relationships of Aphidiinae obtained from sequences of cytochrome *c* oxidase I using Maximum likelihood method. Bootstrap values are indicated above/below branches. Scale bar indicates the number of substituted bases per site. Information is presented with parasitoid species name following with specimen GenBank accession number or voucher

654 code (Appendix 1). A phylogenetic tree supports the traditional phylogenetic relations of
655 Aphidiinae species based on morphological traits (Mackauer 1961; Gärdenfors 1986) and
656 molecular data (Belshaw and Quicke 1997; Sanchis et al. 2000). Also in accordance with
657 literature, *Pseudephedrus* stands out as a completely separate clade at the very base of the
658 phylogenetic tree like it is earlier shown based on other genes (Belshaw et al. 2000). The position
659 of *Lipolexis gracilis* out of the clade that comprise *Trioxys* and *Monoctonus* (subtribe Trioxina)
660 is unexpected as estimations of phylogenetic relationships based on ribosomal DNA indicated
661 that *Lipolexis* was coherent within the subtribe Trioxina (Sanchis et al. 2000).