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Morphology versus DNA – what will bring clarity to the relationships of phylogenetically unclear genera of Anthomyzidae (Diptera)?

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Abstract

A hypothesis of phylogenetic relationships of mainly Holarctic Anthomyzidae based on multigene analysis of combined mitochondrial + nuclear gene markers is compared with those of previously published cladistic analyses of morphological characters with the aim to elucidate affinities of phylogenetically unsettled taxa. The placement of *Fungomyza* Roháček, 1999, *Amygdalops* Lamb, 1914 + *Typhamyza* Roháček, 1992 and *Quametopia* Roháček & Barber, 2011 + *Paranthomyza* Czerny, 1902 proved to be well supported by molecular data but are inconsistent with those suggested by morphological data analyses, therefore demanding further phylogenetic study. In other groups, the relationships recognized by multigene molecular analysis are in agreement with previous or subsequent morphological examination: the *Mumetopia nigrimana* group is postulated as the closest ally of *Stiphrosoma* Czerny, 1928 and hence needs to be excluded from *Mumetopia* Melander, 1913; intrageneric relationships of *Arganthomyza* Roháček, 2009 and allies revealed that *Ischnomyia spinosa* Hendel, 1918 is, in fact, a species of *Arganthomyza* and that the concept of the genus *Ischnomyia* Loew, 1863 has to be redefined; *Epischnomyia* Roháček, 2006 proved to be closest to *Anthomyza* Fallén, 1810. The placement of the genera *Anagnota* Becker, 1902, *Carexomyza* Roháček, 2009, *Cercagnota* Roháček & Freidberg, 1993 and *Santhomyza* Roháček, 1984 have not been resolved either by molecular or morphological analyses and their sister groups remain unknown. The new molecular evidence provides new insight into the phylogeny of Anthomyzidae, but like morphology, fails to resolve some key nodes, suggesting that new studies using both sources of information will be necessary to fully reconstruct the history of the family.

Key words

Diptera, Anthomyzidae, phylogeny, mitochondrial + nuclear markers, inter- and intra-generic relationships, impact on systematics.

1. Introduction

Anthomyzidae are delicate slender flies with elongate wings and relatively short legs (Figs. 2–4, 6, 7, 9, 10). Most of them are inhabitants of wetland and grassland plant communities. Their larvae are micro(phyto)saprophagous, feeding usually in damaged tissues between the sheathing leaves of the tillers or terminal shoots of graminoid plants. There are also some species developing in soft and often partly rotten tissues of dicotyledonous and non-graminoid monocotyledonous plants (ROHÁČEK 2009, 2013a; ROHÁČEK & BARBER 2011) and even in horsetails (Equisetopsida) and possibly also ferns (Polypodiopsida) (K.N. Barber, pers. comm. 2010). Exceptional larval feeding habits are only known in species of the genus *Fungomyza* Roháček, 1999, whose larvae develop in rotting fungi (ROHÁČEK 2009).

Taxonomically the family has been best studied in the Palaearctic Region, in Europe in particular (ROHÁČEK 2006, 2009); more recently the revision of Nearctic species has also started (ROHÁČEK & BARBER 2004, 2005, 2011, 2013). The knowledge of the anthomyzid fauna in

other major biogeographical areas remains rather fragmentary despite a number of recently described species from the Afrotropical (Roháček 1993, 2004; Roháček & Barraclough 2003) and Oriental (Roháček 2008) Regions. A total of 130 (including 11 fossil) named species of Anthomyzidae belonging to 27 (three fossil) genera are currently recognized but at least three times this number of unnamed species (chiefly from tropical areas) are known to await description in various insect collections (Barber & Roháček 2010). All ancient members of Anthomyzidae were found in Tertiary amber inclusions (see Roháček 2013b), including an exclusively fossil subfamily, Protanthomyzidae, with nine species from Baltic (including Bitterfeld) amber (Middle Eocene, 38–50 MYA), and two exclusively fossil genera of Anthomyzinae, viz. *Lacrimyza* Roháček, 2013 with two species from Baltic amber and *Grimalantha* Roháček, 1998 with the single species *G. vulnerata* Roháček from Dominican amber (Middle Miocene, 17–20 MYA).

The family Anthomyzidae has usually been classified in the superfamily Opomyzoidea (Hennig 1958; J.F. McAlpine 1989; Roháček 1998, 2006, 2013b) as the sister group of the family Opomyzidae. However, in the past there were also different opinions about its relationships (cf. Hennig 1971, 1973; Griffiths 1972; Colless & D.K. McAlpine 1970, 1991). Recently, based on reconstructions using analyses of molecular data, Anthomyzidae has even been linked to quite different clades of Acalyptrates, e.g. to Carnidae or to Milichiidae + Chloropidae (Winkler et al. 2010) or to Heleomyzidae (Wiegmann et al. 2011), but these sister-group relationships are poorly supported and they conflict with the morphological data; therefore we currently consider them unlikely (see also Roháček 2013b). Based on six synapomorphic characters (Roháček 1998, 2006) the Opomyzidae thus remains the most probable sister group of Anthomyzidae. The monophyly of Anthomyzidae (particularly of its subfamily Anthomyzinae, which contains all extant species) is well supported both by morphological (Roháček 1998, 2006) and molecular data (Roháček et al. 2009; present results, see Fig. 1).

The relationships within Anthomyzidae have hitherto been discussed mainly on the basis of cladistic analyses of morphological characters. Roháček (1998) studied the phylogenetic relationships of the family and its two subfamilies Protanthomyzinae (fossil only) and Anthomyzinae, defined their taxonomic limits and confirmed their monophyly. Roháček & Barber (2009) analysed the affiliation and phylogeny of the genera *Mumetopia* Melander, 1913, *Chamaebosca* Speiser, 1903, *Stiphrosoma* Czerny, 1928 and *Cercagnota* Roháček & Freidberg, 1993, and considered them derivatives of the same clade (probably of Neotropical origin) of Anthomyzidae. The relationships of the Palaearctic genera and species were discussed by Roháček (2009) including a comparison with results of the first phylogenetic reconstruction based on molecular data by Roháček et al. (2009; see below). Other cladistic analyses hitherto performed in Anthomyzidae were focused on the relationships of species within selected genera: Roháček & Barraclough (2003) treated the Afrotropical genus *Margdalops* Roháček & Barraclough, 2003; Roháček (2004) the Afrotropical species of *Amygdalops* Lamb, 1914; Roháček & Barber (2005) the world species of *Stiphrosoma*; Roháček (2008) the Oriental, Oceanian and Australasian species of *Amygdalops*; and Roháček & Barber (2013) the world species of *Arganthomyza* Roháček, 2009. Despite relatively good progress achieved by the above studies, the phylogenetic relationships of a number of extant genera of Anthomyzidae remain uncertain, mainly because of analytical problems caused by an abundance of homoplasies and difficulties in recognizing the polarity of some morphological characters.

To address these problems, Roháček et al. (2009) performed the first study of the phylogenetic relationships of European genera of Anthomyzidae using molecular analysis of two mitochondrial DNA markers, viz. 12S and 16S rRNA. Results of this study uncovered affinities of several species of previously unclear relationship, e.g. *Anthomyza socculata* (Zetterstedt, 1847) and *Paranthomyza caricis* Roháček, 1999, taxa that proved to be incorrectly affiliated and, therefore, were subsequently placed in newly established genera, viz. *Arganthomyza* and *Carexomyza* by Roháček (2009). Nevertheless, the relationships of some analysed genus-level taxa remain uncertain, not to mention those rare ones which could not be included because material suitable for molecular study was not yet available. The phylogenetic hypothesis posited by Roháček et al. (2009) was in considerable agreement (including unclear affinities of some genera) with the morphology-based phylogenetic results presented by Roháček (2009).

In order to understand better the relationships within Anthomyzidae, we (1) expand the number of analysed taxa, particularly to include rare ones representing genera of poorly known affinities, and (2) use more gene markers with the aim to increase the power of the molecular analysis. It was supposed that accomplishing these two conditions could bring more clarity to the relationships of the problematic taxa, particularly those where cladistic analyses of morphological characters provided ambiguous results. During the past three years a number of additional species have been obtained and analysed, including some of those from the East Palaearctic, Nearctic and even Neotropical Regions and even the very rare *Cercagnota collini* (Czerny, 1928) representing the only European genus not covered by Roháček et al. (2009). Altogether 40 species of Anthomyzidae plus 3 outgroup taxa were analysed. The number of gene markers used was enlarged to include a total of seven genes, viz. 12S, 16S, COI (two fragments), COII, CytB, 28S, ITS2 (for details see below). This combination of markers achieved reliable results in previous studies on families Sciomyzidae (Tóthová et al. 2013) and Mycetophilidae (Ševčík et al. 2013).

The main goal of this study is to clarify the relationships of some of the most phylogenetically problematic

genera or species groups of Anthomyzidae by comparing the results of a new phylogenetic reconstruction based on analysis of molecular data with the results previously obtained by cladistic analyses of morphological characters and of 12S & 16S sequence data.

2. Materials and methods

2.1. Analysed specimens

The list of analysed species with full names and authors is given in Table 1. The selected species cover **(1)** the apparent main lineages of Holarctic Anthomyzidae with some Neotropical taxa, **(2)** the monotypic or species-poor and homogeneous genera by a single (usually type) species, **(3)** the more speciose and/or heterogeneous genera (*Anthomyza* Fallén, 1810, *Arganthomyza*, *Mumetopia* and *Stiphrosoma*) by species belonging to various species groups to cover better the morphological diversity of these taxa. We used one specimen per species; only for *Mumetopia nigrimana* group and *Anthomyza trifurca* Sueyoshi & Roháček, 2003 did we process two specimens to test the reliability of the method. The sequences of *Geomyza tripunctata* Fallén, 1823 and *Opomyza florum* (Fabricius, 1794) from Opomyzidae, the sister family of the Anthomyzidae, and *Clusia flava* (Meigen, 1830) from Clusiidae, the most generalized family of the Opomyzoidea, represented the outgroup and were used to root the phylogenetic trees.

2.2. DNA extraction, PCR and sequencing

All the insect material used for DNA analysis was air-dried. The DNA was extracted using DNeasy Blood & Tissue Kit (QIAGEN) following the manufacturer's protocol. Individual flies or tissue portions were rinsed in PBS, placed in sterile Eppendorf tubes and pulverised in liquid nitrogen. After adding proteinase K, samples were incubated at 56°C for 3 hours. PCRs (total volume = 20 µl) were performed using primers published in Cook et al. (2004) (ribosomal 12S), Roháček et al. (2009) (ribosomal 16S), Folmer et al. (1994) (protein-encoding COIa; COI was amplified in two fragments, a and b), Simon et al. (1994) (protein-encoding COIb) and Su et al. (2008) (protein-encoding CytB and COII). Two nuclear genes, ribosomal 28S and ITS2, were amplified using primers according to Belshaw et al. (2001) and Beebe & Saul (1995), respectively. Amplified products were purified using the QIAquick PCR Purification Kit (QIAGEN). Sequencing was carried out with BigDye Terminator ver.3.1 (Applied Biosystems, Foster, CA) on an ABI 3100 genetic analysis sequencer (Perkin Elmer Applied Biosystems, Norwalk, CT). All sequences were assembled and edited in SEQUENCHER 4.8 (Gene Codes Corporation, Ann Arbor, MI). GenBank accession numbers for the sequences are listed in Table 1.

2.3. Alignment and phylogenetic analyses

The ribosomal genes 12S, 16S, 28S and ITS2 and protein-encoding genes CytB, COI and COII were aligned using MEGA 5 (Tamura et al. 2011) with default settings and manually inspected. The protein-encoding CytB, COI and COII sequences were checked based on amino-acid translations and yielded indel-free nucleotide alignments. The final dataset consisted of 45 specimens as terminal "taxa" (43 species) and 4478 characters: 12S – 348 bp, 16S – 356 bp, 28S – 622 bp, COI – 1286 bp, COII – 633 bp, CytB – 650 bp, ITS2 – 583 bp.

The dataset was analysed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) in order to explore the strength of the phylogenetic signal under different optimality criteria.

The MP analyses of the dataset were performed using TNT v.2.0 (Goloboff et al. 2008) with the following parameters: New technology search, level 50, initial addseqs = 9, find minimum tree length 5 times. Analyses were carried out both with gaps coded as 5th character states and as missing data. Nodal support was assessed by jackknife resampling (JK, 250 replicates with 36.8% character deletion). Trees were rooted by the outgroup taxon *Clusia flava*.

To evaluate the best fit model for the BI and ML analyses, the concatenated dataset was partitioned into eight sets: 12S, 16S, 28S, CytB, COIa, COIb, COII and ITS2. Each of the partitions was processed in PAUP v4b10 (Swofford 2002) and evaluated with MrModeltest v.2.2 (Nylander 2004) using both hierarchical likelihood ratio tests (hLRTs) and Akaike Information Criterion (AIC). The model GTR + Γ + I (Rodriguez et al. 1990) was favoured for each of the individual gene regions.

The partitioned BI of 10 million generations on the concatenated dataset was implemented in MrBayes (Huelsenbeck & Ronquist 2001) and carried out on the CIPRES computer cluster with a burn in of 30% (Cyberinfrastructure for Phylogenetic Research, San Diego Supercomputing Center; Miller et al. 2010).

The ML analyses were conducted in Garli v.2.0 (Zwickl 2006). Two independent runs of 5 million generations using the default automated stopping criterion were carried out. Nodal support was assessed using a nonparametric bootstrap with 250 replicates.

The resultant tree was edited in TreeView (Page 1996) and the layout was prepared using Adobe Photoshop CS 8.0.

Table 1. List of analysed species of Anthomyzidae + outgroup taxa (the last 3 taxa), authors, localities and GenBank sequence accession numbers.

Species	Author	Locality	12S	16S	COIa	COIb	COII	CytB	28S	ITS2
Amygdalops thomasseti	Lamb, 1914	Crete	EU268496	EU268522	KJ418529	KJ418574	n/a	n/a	n/a	KJ418664
Anagnota bicolor	(Meigen, 1838)	Czech Rep.	EU268497	EU268523	KJ418530	KJ418575	KJ418619	KJ418651	KJ418484	KJ418665
Anthomyza anderssoni	Roháček, 1984	Czech Rep.	EU268498	EU268524	KJ418531	KJ418576	n/a	n/a	KJ418501	KJ418666
Anthomyza baezi	Roháček, 1999	Madeira	EU268499	EU268525	KJ418532	KJ418577	n/a	n/a	KJ418485	n/a
Anthomyza collini	Andersson, 1976	Austria	EU268516	EU268542	KJ418549	KJ418593	n/a	n/a	KJ418502	KJ418683
Anthomyza dissors	Collin, 1944	Austria	EU268519	EU268545	n/a	KJ418596	KJ418628	n/a	KJ418503	KJ418686
Anthomyza elbergi	Andersson, 1976	Poland	FJ372994	FJ372993	KJ418554	KJ418599	KJ418629	KJ418648	KJ418500	KJ418688
Anthomyza gracilis	Fallén, 1823	Czech Rep.	EU268515	EU268541	KJ418548	KJ418592	n/a	n/a	KJ418498	KJ418682
Anthomyza macra	Czerny, 1928	Austria	EU268500	EU268526	KJ418533	n/a	KJ418620	KJ418650	KJ418506	KJ418667
Anthomyza neglecta	Collin, 1944	Poland	EU268501	EU268527	KJ418534	KJ418578	n/a	n/a	KJ418486	KJ418668
Anthomyza pallida	(Zetterstedt, 1838)	Czech Rep.	EU268502	EU268528	KJ418535	KJ418579	n/a	n/a	KJ418487	KJ418669
Anthomyza paraneglecta	Elberg, 1968	Slovakia	EU268518	EU268544	KJ418551	KJ418595	KJ418626	KJ418649	KJ418505	KJ418685
Anthomyza pleuralis	Czerny, 1928	Austria	EU268517	EU268543	KJ418536	KJ418580	KJ418621	KJ418645	KJ418504	KJ418670
Anthomyza socculata	(Zetterstedt, 1847)	Slovakia	EU268520	EU268546	KJ418552	KJ418597	KJ418627	n/a	n/a	n/a
Anthomyza trifurca 1	Sueyoshi & Roháček, 2003	Korea	EU268503	EU268529	KJ418537	KJ418581	n/a	n/a	KJ418489	KJ418671
Anthomyza trifurca 2	Sueyoshi & Roháček, 2003	Korea	KJ418457	KJ418478	KJ418570	n/a	n/a	KJ418661	KJ418523	KJ418704
Anthomyza umbrosa	Roháček, 2006	Madeira	EU268504	EU268530	KJ418538	KJ418582	n/a		KJ418490	KJ418672
Arganthomyza bivittata	Roháček & Barber, 2013	Canada (Ontario)	KJ418452	n/a	KJ418566	KJ418612	KJ418640	KJ418659	KJ418518	KJ418699
Arganthomyza carbo	Roháček & Barber, 2013	Canada (Ontario)	KJ418447	KJ418469	KJ418561	KJ418607	n/a	KJ418654	KJ418513	KJ418694
Arganthomyza duplex	Roháček & Barber, 2013	Canada (Ontario)	KJ418448	KJ418470	KJ418562	KJ418608	KJ418636	KJ418655	KJ418514	KJ418695
Arganthomyza versitheca	Roháček, 2009	Korea	KJ418451	KJ418473	KJ418565	KJ418611	KJ418639	KJ418658	KJ418517	KJ418698
Carexomyza caricis	(Roháček, 1999)	GB, England	EU268521	EU268547	KJ418553	KJ418598	n/a	n/a	KJ418499	KJ418687
Cercagnota collini	(Czerny, 1928)	GB, England	KJ418454	KJ418475	n/a	n/a	n/a	n/a	KJ418520	KJ418701
Epischnomyia merzi	Roháček, 2009	Korea	KJ418453	KJ418474	KJ418567	KJ418613	KJ418641	n/a	KJ418519	KJ418700
Fungomyza albimana	(Meigen, 1830)	Czech Rep.	EU268514	EU268540	KJ418547	KJ418591	KJ418624	n/a	KJ418497	KJ418681
Ischnomyia spinosa	Hendel, 1911	Canada (Ontario)	KJ418446	KJ418468	KJ418560	KJ418606	KJ418635	n/a	KJ418512	KJ418693
Mumetopia nigrimana group sp. 1	unnamed	Peru	KJ418461	KJ418482	n/a	n/a	n/a	n/a	KJ418527	KJ418708
Mumetopia nigrimana group sp. 2	unnamed	Peru	KJ418462	KJ418483	n/a	n/a	n/a	n/a	KJ418528	KJ418709
Mumetopia occipitalis	Melander, 1913	Canada (Ontario)	KJ418443	KJ418465	KJ418557	KJ418603	KJ418632	n/a	KJ418509	KJ418690
Paranthomyza nitida	(Meigen, 1838)	Poland	EU268505	EU268531	KJ418539	KJ418583	n/a	n/a	n/a	KJ418673
Quametopia clintonia	Roháček & Barber, 2011	Canada (Ontario)	KJ418442	KJ418464	KJ418556	KJ418602	KJ418631	KJ418652	KJ418508	KJ418689
Quametopia terminalis	(Loew, 1863)	Canada (Ontario)	KJ418441	KJ418463	KJ418555	KJ418601	KJ418630	n/a	KJ418507	n/a
Santhomyza inermis	Roháček, 1984	Malta	EU268513	EU268539	n/a	n/a	n/a	n/a	KJ418496	KJ418680
Stiphrosoma balteatum	Roháček & Barber, 2005	Canada (Ontario)	KJ418445	KJ418467	KJ418559	KJ418605	KJ418634	n/a	KJ418511	KJ418692
Stiphrosoma cingulatum	(Haliday, 1855)	Czech Rep.	EU268506	EU268532	KJ418540	KJ418584	n/a	n/a	KJ418491	KJ418674
Stiphrosoma fissum	Roháček, 1996	Korea	EU268507	EU268533	KJ418541	KJ418585	n/a	KJ418646	KJ418492	KJ418675
Stiphrosoma hirtum	Roháček & Barber, 2005	Canada (Ontario)	KJ418455	KJ418476	KJ418568	KJ418614	KJ418642	KJ418660	KJ418521	KJ418702
Stiphrosoma humerale	Roháček & Barber, 2005	Canada (Ontario)	KJ418456	KJ418477	KJ418569	KJ418615	KJ418643	n/a	KJ418522	KJ418703
Stiphrosoma laetum	(Meigen, 1830)	Slovakia	EU268508	EU268534	KJ418542	KJ418586	n/a	n/a	n/a	KJ418676
Stiphrosoma sabulosum	(Haliday, 1837)	Czech Rep.	EU268509	EU268535	KJ418543	KJ418587	KJ418622	n/a	n/a	KJ418677
Stiphrosoma setipleurum	Roháček & Barber, 2005	Canada (Ontario)	KJ418444	KJ418466	KJ418558	KJ418604	KJ418633	KJ418653	KJ418510	KJ418691
Typhamyza bifasciata	(Wood, 1911)	Austria	EU268510	EU268536	KJ418544	KJ418588	KJ418623	n/a	KJ418493	KJ418678
Clusia flava	(Meigen, 1830)	Slovakia	KJ418458	KJ418479	KJ418571	KJ418616	n/a	n/a	KJ418524	KJ418705
Geomyza tripunctata	Fallén, 1823	Madeira	EU268511	EU268537	KJ418545	KJ418589	n/a	n/a	KJ418494	n/a
Opomyza florum	(Fabricius, 1794)	Czech Rep.	KJ418459	KJ418480	KJ418572	KJ418617	KJ418644	KJ418662	KJ418525	KJ418706

3. Results and discussion

3.1. Overall phylogenetic results

The results based on the BI, ML and MP analyses of the dataset are summarized in Fig. 1. The tree displays Bayesian topologies with nodal support values from the BI, ML, and MP (indels treated as 5th character state) analyses. For the Bayesian analysis the standard deviation of split frequencies was in all cases < 0.004. The log likelihood value for the best tree of the dataset was −35817.48. Both MP analyses (tree length = 8732) of the dataset (with gaps coded as a 5th character state and as missing data, respectively) resulted in two most parsimonious trees. Overall in all analyses, the gap treating had no significant influence on the tree topology.

The topologies of ML and BI trees are very similar conserving the main branches and species clusters. The MP topology is rather different mostly due to several polytomies. The clustering of analysed representatives of *Anthomyza*, *Stiphrosoma*, *Arganthomyza* and *Mumetopia* was consistent across all analyses. Incongruence between MP and model-based methods was observed with regards to the positions of *Amygdalops*, *Typhamyza* Roháček, 1992 and their relationships to the rest of the family and the position of *Mumetopia occipitalis* Melander, 1913; the position of *Santhomyza*, *Cercagnota* and *Mumetopia nigrimana* group was poorly supported overall. All performed analyses (BI, ML, MP) strongly support the monophyly of the family (though with only three outgroup taxa this was tested to a very limited extent).

The analysed ingroup dataset is split into two main clades, one with the single species *Fungomyza albimana* (see 3.2. below), the other with all remaining species (PP = 1.0, ML = 100, MP = 99). Further branching is dichotomous, with a clade comprising all analysed species of *Anthomyza* plus the single representative of the genus *Epischnomyia* (PP = 0.88, ML = 62, MP < 50) and a branch with representatives of all remaining genera. Surprisingly, the latter clade seems to be relatively well supported (PP = 0.88, ML = 100, MP < 50), this being contrary to expectation because it also includes all members of the genus *Arganthomyza* forming a well-supported branch also containing *Ischnomyia spinosa*. The sister group to the *Arganthomyza* clade is formed by another surprisingly rather well-supported cluster (PP = 1.0, ML = 76, MP < 50) formed by representatives of the remaining anthomyzid genera in the dataset. However, further splitting of the latter clade results in a quadrichotomy having branches with *Anagnota* (one species), *Carexomyza* (one species), *Santhomyza* + *Cercagnota* (each with a single species; this one poorly supported) and a large cluster of species in the remaining six genera.

The monophyly of the latter cluster also appears to be rather well supported (PP = 1.0, ML = 76, MP < 50) and its further branching is dichotomous with both clades strongly supported by model-based methods (PP = 1.0, ML = 100), one clade with the pair *Paranthomyza* (one species) + *Quametopia* (two species), the other with the remaining four genera. Members of these four genera were split into two distinct clades (both with PP = 1.0, ML = 98), viz. that with the *Amygdalops* + *Typhamyza* pair, and that with the members of the *Chamaebosca* genera group (*Mumetopia*, *Stiphrosoma*, *Mumetopia nigrimana* group). The phylogenetic relationships of most of the above genera are discussed in more detail below (see 3.2.–3.8.) comparing the above results with hypotheses proposed by previous analyses based on morphological (Roháček 2009; Roháček & Barber 2009, 2013) and molecular data (Roháček et al. 2009). Generally, the main differences of the present phylogenetic hypothesis based on multigene analysis (Fig. 1) against those previously published (Roháček 2009; Roháček et al. 2009) can be seen in the topology of the basal clades of the tree, particularly those with the genera *Fungomyza*, *Anthomyza* and *Arganthomyza*, which were previously thought to constitute a monophyletic clade derived from the common ancestor of the *Anthomyza* clade (see more in 3.2.).

3.2. Phylogenetic relationships of *Fungomyza*

The genus *Fungomyza* Roháček, 1999 is exceptional within the world Anthomyzidae because of the trophic biology of its members which are (supposedly all three species, see Roháček 2009) mycophagous in contrast to all other anthomyzids being phytosaprophagous. Hitherto, *Fungomyza* has been considered to belong to the *Anthomyza* clade together with *Arganthomyza* (closest), *Anthomyza*, *Ischnomyia*, *Epischnomyia* and possibly *Receptrixa* Roháček, 2006 based on morphological analysis (Roháček 2009); it was likewise clustered in the previous hypothesis based on molecular data (Roháček et al. 2009). Very surprisingly, its only European representative, *Fungomyza albimana* (Fig. 2), is postulated as the sister group to all other analysed anthomyzid species in the present hypothesis (see Fig. 1) and this position in the tree seems to be very well supported by high values in all analyses (PP = 1.00, ML = 100, JK = 99). This is a striking discrepancy considering the fact that morphologically *Fungomyza* was previously linked (as sister group) to *Arganthomyza* on the basis of two synapomorphies (Roháček 2009), a relationship which also had molecular (though not very strong) support (Roháček et al. 2009); thus, it does not differ significantly from other genera of the *Anthomyza* clade (as defined by Roháček 2009), except perhaps for retaining more apparent plesiomorphies than other groups of this clade. Consequently, mycophagy seems to be the only obvious difference distinguishing *Fungomyza* from all other Anthomyzidae. However, could this distant separation of *Fungomyza* in the resulting tree (Fig. 1) be caused by its

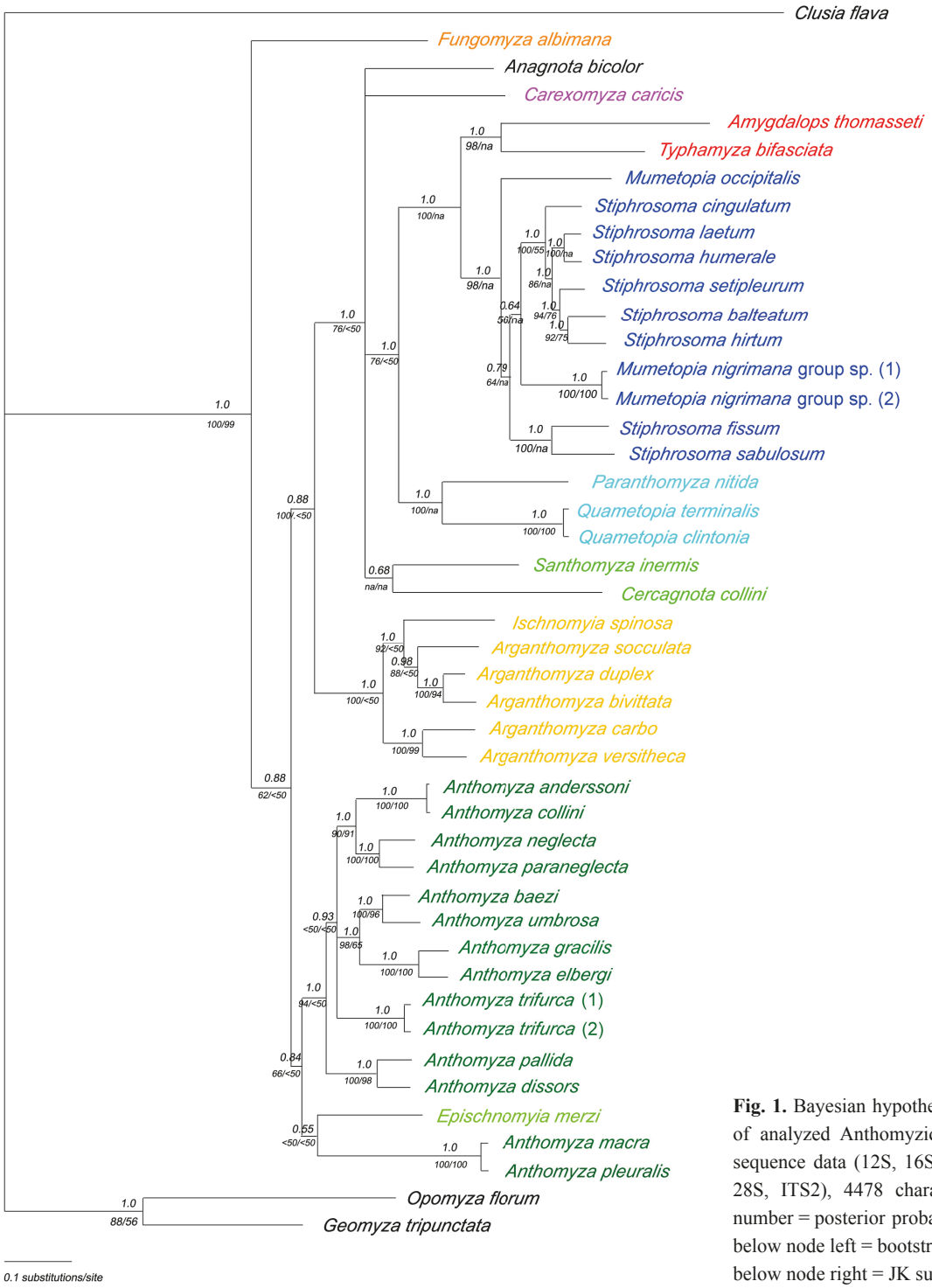

Fig. 1. Bayesian hypothesis for relationships of analyzed Anthomyzidae based on DNA sequence data (12S, 16S, COI, COII, CytB, 28S, ITS2), 4478 characters. Above node number = posterior probability (PP) if > 0.5; below node left = bootstrap support for Garli; below node right = JK support for MP.

different larval diet and, consequently, digestive physiology? Physiological characters ought not to be reflected at all when using only 12S and 16S mitochondrial gene markers in the analysis by Roháček et al. (2009) so that their results are closer to the morphology-based ones in Roháček (2009). If the present hypothesis (Fig. 1) is correct could this genus really branch off so early from other extant Anthomyzidae owing to a change of feeding strategy? *Reliquantha variipes* Roháček, 2013, a peculiar new genus and species of Anthomyzidae which has recently been described from Great Britain, could also be associated with (probably tree) fungi as is indicated by the label data of the female paratype (see Roháček 2013c). This very rare species is, however, considered not to be related to any extant Anthomyzidae but most probably to the Eocene genus *Lacrimyza*. Because it is a potential fungus-feeder it would be very important to test its affinity to other Anthomyzidae (particularly to *Fungomyza*) with molecular data analysis, particularly inasmuch as the genus *Reliquantha* supposedly belongs to a clade branching off very early from the common stem of the subfamily Anthomyzinae (see Roháček 2013c).

Fig. 2. *Fungomyza albimana* (Meigen, 1830), mating pair on fungus (Czech Republic), body length ca. 2.5 mm. Photo by J. Roháček.

Fig. 3. *Amygdalops thomasseti* Lamb, 1914, female on leaf of *Arundo donax* (S. Turkey), body length ca. 2.6 mm. Photo by J. Roháček.

3.3. Phylogenetic relationships of *Amygdalops* and *Typhamyza*

The relationships of these distinctive genera also proved to be dissimilar in the phylogenetic hypotheses based on previous (both morphological – Roháček 2009 – and molecular – Roháček et al. 2009) and current analyses. *Typhamyza* Roháček, 1992 is a monotypic genus represented by a W. Palaearctic species *T. bifasciata* (Wood, 1911) while *Amygdalops* Lamb, 1914 is a speciose group widespread in the tropical and subtropical belts of the Old World (cf. Roháček 2004, 2008). Both Roháček (2009) and Roháček et al. (2009) hypothesize *Typhamyza* as the probable sister group of the genus *Paranthomyza* Czerny, 1902 but the support of this cluster was rather poor (PP only 0.70; a single synapomorphic feature – female sternite 10 with anterior strip-like part). The genus *Amygdalops* was not distinctly clustered with any other Palaearctic (European in Roháček et al. 2009) genera in either previous analysis. However, the new hypothesis based on molecular data postulates *Typhamyza* and *Amygdalops* as a sister pair whose relationship is strongly supported in model-based methods (PP=1.0, ML=98). This clustering is surprising because there seem to be no distinct shared apomorphic characters in the structures of the male and female terminalia (cf. Roháček 2006); the marked differences in genitalic structures can be thought to reflect considerable phylogenetic distance between these genera. And yet, species of both genera appear to be strikingly similar in some external features, such as the convex ellipsoid eyes, the dorsally flattened head, the long orbital setae distant from each other, the narrow elongate thorax or the narrow and often patterned wings (cf. Figs. 3 and 4). It is notable that *Amygdalops* has its closer relatives in the tropics, one being the Afrotropical genus *Margdalops* Roháček & Barraclough, 2003, the other (probably the closest ally of *Amygdalops*) an unnamed genus from the Neotropical Region (cf. Roháček 2008, 2009). Based on the above result it is not excluded

Fig. 4. *Typhamyza bifasciata* (Wood, 1911), female on dry stem of *Typha latifolia* (S. Turkey), body length ca. 3.3 mm. Photo by J. Roháček.

that *Typhamyza* actually originates from this Pantropical clade. However, to demonstrate this supposition definitely it is necessary not only to re-evaluate the use of external body characters in the morphological analyses but also to include the tropical species of *Amygdalops*, *Margdalops* and the undescribed Neotropical genus (hitherto unavailable) in future molecular studies.

3.4. Phylogenetic relationships of the *Mumetopia nigrimana* group

Roháček & Barber (2009) examined the phylogeny of the largely New World *Chamaebosca* clade including the genera *Mumetopia* Melander, 1913, *Chamaebosca* Speiser, 1903, *Stiphrosoma* Czerny, 1928 and *Cercagnota* Roháček & Freidberg, 1993 on the basis of morphological data. Results of that study (cf. Fig. 5) suggested that the genus *Mumetopia* is not monophyletic because its

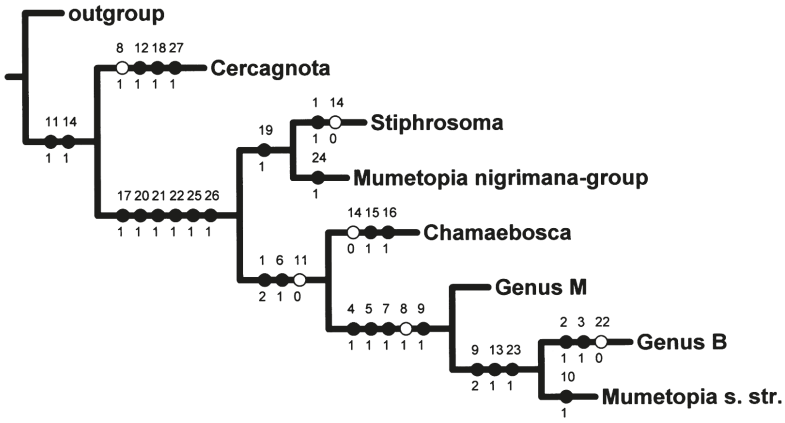

Fig. 5. The most parsimonious tree (L = 34 steps, CI = 0.85, RI = 0.82) resulting from cladistic analysis of the *Chamaebosca* clade. ACCTRAN optimization. Full circles = non-homoplasious character transformations; empty circles = homoplasious character transformations. Numbers below branches indicate change to apomorphic (1) or plesiomorphic (0) states of characters (reversals in case of (0)). Adapted from Roháček & Barber (2009: fig. 38).

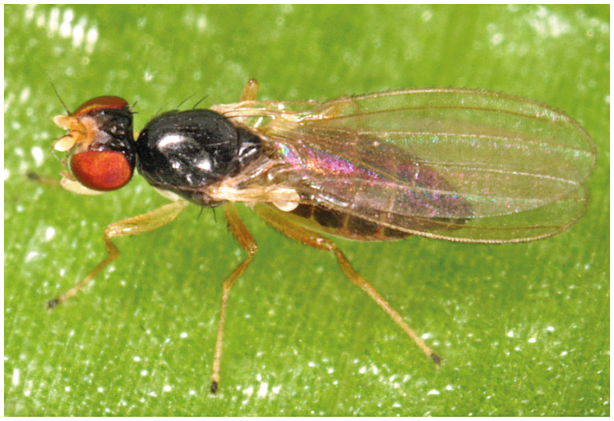

Fig. 6. *Quametopia clintonia* Roháček & Barber, 2011, female on leaf of *Clintonia borealis* (Canada: Ontario), body length ca. 2.3 mm. Photo by J. Roháček.

Fig. 7. *Paranthomyza nitida* (Meigen, 1838), male on leaf of grass (S. Sweden), body length ca. 2.4 mm. Photo by J. Roháček.

representatives belong to three different lineages: (1) *Mumetopia* s.str. being closely related to two unnamed Neotropical groups and (less closely) to *Chamaebosca*, (2) the *Mumetopia nigrimana* group being closely allied with *Stiphrosoma* and (3) the fairly isolated *Mumetopia terminalis* (Loew, 1863). Consequently, to render *Mumetopia* monophyletic it is necessary to exclude representatives of the two latter clades from the genus. To solve this taxonomically, Roháček & Barber (2011) first removed *M. terminalis* and placed it (plus two other formerly unnamed relatives) in a new genus *Quametopia* whose affinities are discussed below. Although Roháček & Barber (2009) suggested treating the *M. nigrimana* group as another separate genus, this systematic solution has been postponed in order to clarify first its relationships to *Stiphrosoma*. Because we managed to obtain fresh specimens of an unnamed species of this group from Peru (see Material and methods) it was included in our molecular analysis together with several Nearctic species of *Stiphrosoma*. The resulting tree (Fig. 1) demonstrated very close affinity of this unnamed species to *Stiphrosoma* (thus confirming the conclusion of Roháček & Barber 2009) but clustering it even among clades of the latter genus. Although its topology is not well supported (PP = 0.64, ML = 56), this result raises the question as to whether it would not be better to include species of the *M. nigrimana* group in *Stiphrosoma* instead of erecting a new genus for them. However, this will depend upon the revision of the *M. nigrimana* group because it contains numerous unnamed Neotropical species in addition to the single described one, *M. nigrimana* (Coquillett, 1900).

3.5. Phylogenetic relationships of *Quametopia*

The genus *Quametopia* Roháček & Barber, 2011 was established recently for three closely allied Nearctic species including the one formerly known as *Mumetopia terminalis*, see also above. Although previously considered unrelated to the *Chamaebosca* clade (see Roháček & Barber 2009), it was later postulated by Roháček & Barber (2011) as a probable sister group of this clade (excluding *Cercagnota*). The close relationship of *Quametopia* to the genera of the *Chamaebosca* clade was considered to be supported by the following shared morphological apomorphies: female synsclerite 7 dorsomedially depigmented or divided, female sternite 8 short and posterodorsomedially (though not deep-

ly) incised, posterior internal sclerites of female genital chamber well developed and spermathecae with surface spines (although of different structure in *Quametopia* than in the *Chamaebosca* group of genera). It is significant that all these characters are of the female terminalia. In contrast, the male genitalia hardly indicate this relationship because genitalic synapomorphies of the *Chamaebosca* group of genera are lacking in *Quametopia* species. The phylogenetic hypothesis based on analysis of molecular data has not confirmed the above relationship (see Fig. 1). Instead, representatives of *Quametopia* were unexpectedly clustered (with strong support PP=1.0, ML=100) with the monotypic Palaearctic genus *Paranthomyza* Czerny, 1902, a group with quite different morphological structures of the male and female terminalia. On the other hand, *Quametopia* and *Paranthomyza* species resemble each other in external appearance (cf. Figs. 6 and 7), particularly regarding their rounded head, large and more or less shining frontal triangle, only 1 long ors seta, relatively to strongly shining body (due to reduction of microtomentum) and well-developed ctenidial spine. Although some of these characters are obviously apomorphic, they all occur scattered as homoplasies in other groups of Anthomyzidae and, hence, cannot be used to demonstrate directly a close relationship between the two genera. Thus, it is a further example (see above *Amygdalops* and *Typhamyza*) where externally similar taxa were linked by analysis of molecular data. This can hardly be considered coincidental and again indicates that external characters should be included in morphological analyses despite their often homoplastic transformation. Roháček & Barber (2011) also described all preimaginal stages of two *Quametopia* species and compared them with (a few of) those hitherto known in Anthomyzidae. Interestingly, the puparium of *Quametopia* was found to be most similar to that of *Paranthomyza*, while the mouthhooks, intermediate sclerite and posterior spiracles of the 3rd-instar larva were also similar in these two taxa. The resemblance of the larval structures may be a reflection of their similar feeding strategies as larvae of both genera develop in damaged tissues of soft plants in undergrowth of moist woodland – this could also be further reflected in shared physiological characters.

3.6. Phylogenetic relationships of *Cercagnota*

The enigmatic genus *Cercagnota* Roháček & Freidberg, 1993 includes the single rare species, *C. collini* (Czerny, 1928). Because of difficulties in obtaining material for molecular study, the affinity of this taxon has previously been discussed only on the basis of morphological data analyses. Roháček (2009, treating only Palaearctic genera) considered it to be most probably related to *Stiphrosoma*; Roháček & Barber (2009) placed it as the probable sister group of the *Chamaebosca* clade of Anthomyzidae, which includes *Stiphrosoma*. Because this relationship was rather poorly supported (cf. Roháček & Barber 2009: figs. 36–38, and Fig. 5 herein), it is significant that *Cercagnota collini* could be included in our current molecular analysis. However, this has not confirmed the affiliation of *Cercagnota* with the *Chamaebosca* clade (represented by *Stiphrosoma* and *Mumetopia* species in Fig. 1). Its current clustering with *Santhomyza inermis* Roháček, 1984 is not considered reliable because this clade is poorly supported (PP=0.68) and there is no morphological synapomorphy to support this relationship. *Cercagnota* is therefore to be considered a distinct genus whose nearest relative (sister group) remains unknown as are those of the genera *Anagnota* Becker, 1902, *Carexomyza* Roháček, 2009 and *Santhomyza* Roháček, 1984 (as given in the tree in Fig. 1). The topological separation of these taxa seems to be concordant with results found with morphological data analysis (Roháček 2009).

3.7. Phylogenetic relationships of *Ischnomyia spinosa* and *Arganthomyza*

The world species of the genus *Arganthomyza* Roháček, 2009 (nine species) were recently reviewed with revision of the Nearctic species and with a hypothesis of their phylogenetic relationships based on cladistic analysis of morphological characters (Roháček & Barber 2013). On the other hand, the species of the genus *Ischnomyia* Loew, 1863 (two currently known) have not been revised so that the relationships of this genus have remained inadequately understood – only Roháček (2006, 2009) discussed its probable affinity to *Epischnomyia*. To test the results achieved by Roháček & Barber (2013), we have included as many *Arganthomyza* species as available in our molecular analysis, plus one species of *Ischnomyia*, viz. *I. spinosa* Hendel, 1911. The resulting hypothesis of relationships among *Arganthomyza* species (Fig. 1) agrees perfectly with that based on morphological data by Roháček & Barber (2013) (see their tree in Fig. 8) with one addition: *I. spinosa* was placed within the *Arganthomyza* clade (PP=1.0, ML=92). Subsequent revision of the types and comparative material of both *Ischnomyia* species, viz. the type species *I. albicosta* (Walker, 1849) and *I. spinosa*, revealed that the latter is not closely allied to the former and may actually belong to *Arganthomyza* as molecular data suggest, although it differs strikingly from other *Arganthomyza* species (Fig. 10) in having wings with brown and white longitudinal ornamentation (Fig. 9) strikingly similar to those of *I. albicosta*. Consequently, this distinctive wing pattern seems to have evolved independently in three different lineages (i.e. genera) of Anthomyzidae, viz. in *Ischnomyia* (one species) and *Arganthomyza* (one species, the former *I. spinosa*), which are probably derived from the same clade, but also in the

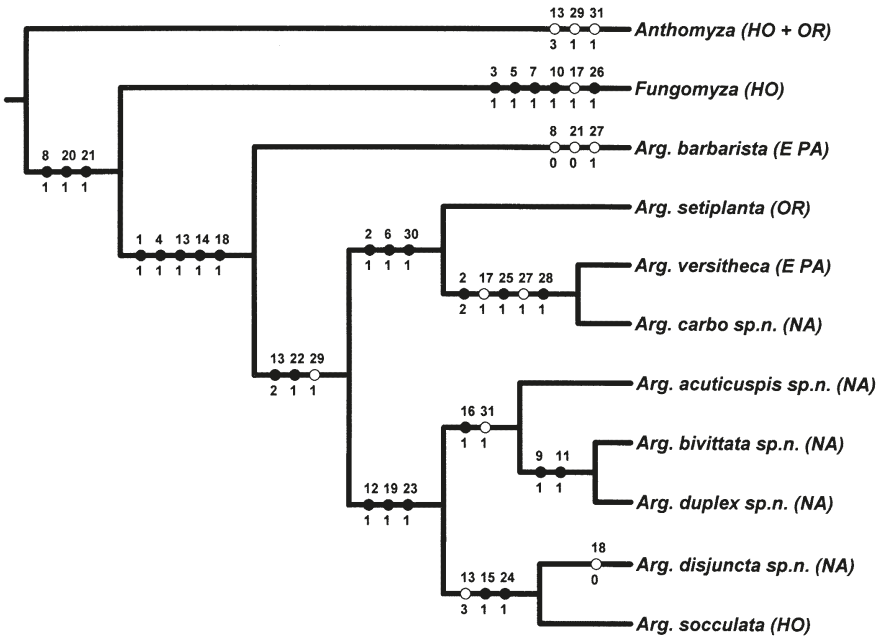

Fig. 8. One of two most parsimonious trees (L = 42 steps, CI = 0.80, RI = 0.81) chosen to represent the phylogeny of *Arganthomyza* species. Characters with ambiguous evolution (8, 13, 14, 18, 21, 29) are optimized assuming fast transformation (ACCTRAN). Full circles = non-homoplasious character transformations, empty circles = homoplasious character transformations (both referring to the selection of taxa included in the tree). Numbers below branches indicate change to apomorphic (1, 2, 3) or plesiomorphic (0) states of characters (reversals in case of (0)). Faunal regions: E PA = East Palaearctic, HO = Holarctic, NA = Nearctic, OR = Oriental, PA = Palaearctic. Adapted from Roháček & Barber (2013: fig. 173).

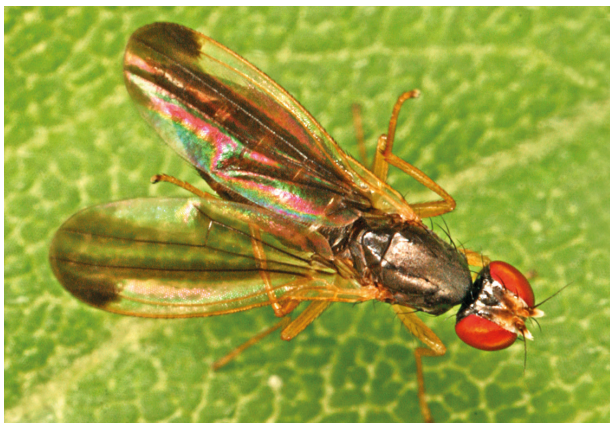

Fig. 9. *Ischnomyia spinosa* Hendel, 1911, female on leaf (Canada: Ontario), body length ca. 3.3 mm. Photo by J. Roháček.

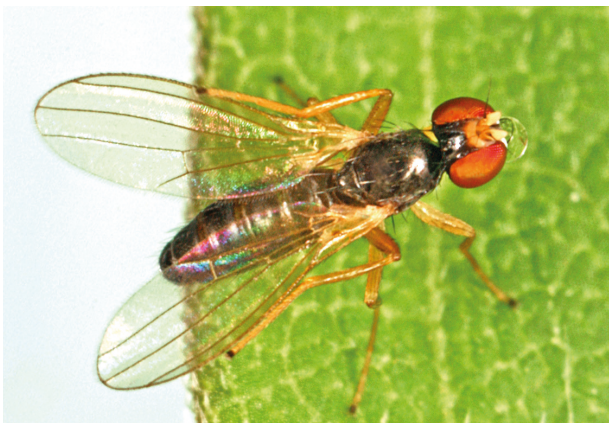

Fig. 10. *Arganthomyza carbo* Roháček & Barber, 2013, male on leaf (Canada: Ontario), body length ca. 2.6 mm. Photo by J. Roháček.

much more distantly related *Epischnomyia* (both known species, see section 3.8.). Inasmuch as there is also a nomenclatural problem with *I. spinosa*, the species will be redescribed and nomenclaturally clarified as a member of *Arganthomyza* in a subsequent study (Roháček & Barber in prep.). Thus, the present molecular study revealed a false generic affiliation of one of the *Ischnomyia* species and, in turn, a misleading taxonomic concept of the genus (which will be redefined by Roháček & Barber in prep.).

3.8. Phylogenetic relationships of *Epischnomyia*

Epischnomyia Roháček, 2006 comprises two closely related East Palaearctic species, viz. *E. triarmigera* (Sueyoshi & Roháček, 2003) (type species) and *E. merzi* Roháček, 2009. *Epischnomyia triarmigera* was originally described by Sueyoshi & Roháček (2003) as an aberrant species of *Ischnomyia* because its wing ornamentation closely resembles that of *Ischnomyia albicosta*. Subsequently, *Epischnomyia* was established to accommodate this species (Roháček 2006) when essential differences in the male and female genitalia compared to those known in *Ischnomyia* were recognized. In spite of this, *Epischnomyia* has continued to be considered a close relative of *Ischnomyia* (although as its highly derived ally) and both these genera were clustered within the *Anthomyza* clade in the phylogenetic hypothesis of Palaearctic genera of Anhomyzidae (Roháček 2009). However, our molecular data place *Epischnomyia merzi* within *Anthomyza* (Fig. 1), but of uncertain placement within this lineage (the sister-group relationship with the *A. macra* group is very weakly supported). The subsequent comparison of postabdominal structures revealed that *Epischnomyia* shares a number of features of the male genitalia (e.g. the compact filum of the distiphallus, the robust spine-like armature of the saccus) and female

postabdomen (e.g. well-developed synsclerite 7, sclerotization of the female genital chamber, elongate ventral receptacle with twisted but secondarily sclerotized apex) with *Anthomyza* that would link these genera if assigned (as synapomorphies) to the groundplan of these taxa.

4. Conclusions

(1) The new phylogenetic hypothesis based on multigene analysis of 40 species of Anthomyzidae belonging to the majority of Holarctic genera provided useful information about previously unclear relationships of a number of genera. Comparison of its results with previously suggested hypotheses (largely based on analyses of morphological data) revealed distinct discrepancies in the clustering of certain genera but also agreement in the relationships among other taxa.
(2) It is significant that the largest discrepancies or even unclear relationships were found in groups whose relationships proved to be difficult or impossible to resolve by means of cladistic analyses of morphological characters (mainly due to frequent homoplasies). In contrast, the morphologically well-founded relationships (particularly of species within genera) have been strongly supported by the molecular data analysis.
(3) The finding of sister-group relationships that are strongly supported by the current multigene data analysis, viz those of *Fungomyza* + remaining genera, *Amygdalops* + *Typhamyza* and *Quametopia* + *Paranthomyza*, but not by previous hypotheses based on morphological data, is very encouraging for future phylogenetic studies, particularly in the search for additional morphological and other characters which could help to test their validity.
(4) It is suggested that the relationships of taxa which are supported by both molecular and morphological data should be reflected in their re-classification in future taxonomic studies.

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