

Combining Nuclear and Mitochondrial Loci Provides Phylogenetic Information in the Philopterus Complex of Lice (Psocodea: Ischnocera: Philopteridae)

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Abstract

The Philopterus Complex includes several lineages of lice that occur on birds. The complex includes the genera *Philopterus* (Nitzsch, 1818; Psocodea: Philopteridae), *Philopteroides* (Mey, 2004; Psocodea: Philopteridae), and many other lineages that have sometimes been regarded as separate genera. Only a few studies have investigated the phylogeny of this complex, all of which are based on morphological data. Here we evaluate the utility of nuclear and mitochondrial loci for recovering the phylogeny within this group. We obtained phylogenetic trees from 39 samples of the Philopterus Complex (Psocodea: Philopteridae), using sequences of two nuclear (*hyp* and *TMEDE6*) and one mitochondrial (*COI*) marker. We evaluated trees derived from these genes individually as well as from concatenated sequences. All trees show 20 clearly demarcated taxa (i.e., putative species) divided into five well-supported clades. Percent sequence divergence between putative species (~5–30%) for the *COI* gene tended to be much higher than those for the nuclear genes (~1–15%), as expected. In cases where species are described, the lineages identified based on molecular divergence correspond to morphologically defined species. In some cases, species that are host generalists exhibit additional underlying genetic variation and such cases need to be explored by further future taxonomic revisions of the Philopterus Complex.

Key words: Passeriformes, Phthiraptera, genetic divergence, molecular data

The Philopterus Complex of feather lice (Ischnocera: Philopteridae, Table 1) contains about 225 species parasitizing a wide variation of songbirds and a few other avian groups (Price *et al.* 2003; Mey 2004; Valim 2006; Cicchino 2007; Sychra *et al.* 2010; Najer *et al.* 2012a, b, 2016, 2020; Valim and Palma 2013; Gustafsson and Bush 2014, 2017). Mey (2004) splits up the previously single genus *Philopterus* Nitzsch, 1818 into 11 separate genera recognized as the Philopterus Complex: *Corcorides* (Mey, 2004; Psocodea: Philopteridae); *Philopterus* (Nitzsch, 1818; Psocodea: Philopteridae); *Mayriphilopterus* (Mey, 2004; Psocodea: Philopteridae); *Philopteroides* (Mey, 2004; Psocodea: Philopteridae); *Tyranniphilopterus* (Mey, 2004; Psocodea: Philopteridae); *Australophilopterus* (Mey, 2004; Psocodea: Philopteridae); *Cinclosomicola* (Mey, 2004; Psocodea: Philopteridae); *Paraphilopterus* (Mey, 2004; Psocodea: Philopteridae); *Trirabeculus* (Uchida, 1948; Psocodea: Philopteridae); *Cincloecus* (Eichler,

1951; Psocodea: Philopteridae); *Clayiella* (Eichler, 1940; Psocodea: Philopteridae).

In addition to these genera, Gustafsson *et al.* (2019a) described *Vinceopterus* (Gustafsson, Lei, Chu, Zou, and Bush, 2019; Psocodea: Philopteridae), increasing the number of genera in this complex to 12. Members of this group possess a triangular head, rounded body shape, and specialize on feathers of the host's head (Gustafsson *et al.* 2019b). Despite this taxonomic work, several phylogenetic issues regarding this complex remain. No thorough taxonomic revision of the entire complex has ever been published. The key characters for determination of species, and even genera, are often tentative, and can overlap between species, making species delimitation only possible when large numbers of specimens from the same host are available. Most studies to date have been taxonomic, focusing solely on morphology based on a limited number of slide-mounted specimens (e.g., Mey 2004, Najer *et al.* 2016).

Table 1. Taxonomic classification of the Philopterus Complex

Taxonomic rank	Taxa in manuscript	Other taxa in the same higher taxon
Order	Psocodea—lice	Thysanoptera—thrips, Hemiptera—hemipterans
Suborder	Ischnocera—feather lice, ischnocerans	Anoplura—sucking lice, Amblycera—amblycerans
Family	Philopteridae	Trichodectidae
Genera complex	Philopterus Complex	Brueelia Complex, Oxylipeurus Complex
Genus	<i>Philopterus</i> , <i>Philopteroides</i> , <i>Tyranniphilopterus</i>	<i>Columbicola</i> (Ewing, 1929; Psocodea: Philopteridae), <i>Physconelloides</i> (Ewing, 1927; Psocodea: Philopteridae)

Other taxa include those mentioned in the text, as well as other examples following Price *et al.* (2003).

DNA sequencing provides the opportunity to further evaluate the phylogenetic relationships of the Philopterus Complex. Most prior molecular phylogenetic studies of lice have used only short fragments of the mitochondrial cytochrome-oxidase subunit I (*COI*) and nuclear elongation factor-1 alpha (*EF1α*). Some of these studies (Cruickshank *et al.* 2001, Johnson *et al.* 2003) included Philopterus Complex samples, but both of these studies were carried out prior to Mey's (2004) morphological review, so it is not clear whether they belong either to *Philopterus* or some other genus of the complex. More recently, Sweet *et al.* (2014) provided new primers for four additional nuclear loci that may provide resolution for species-level relationships in lice.

Here, we test the utility of six DNA sequence markers previously used in Ischnocera for phylogenetic analysis of the Philopterus Complex. We use these sequences to reconstruct phylogenetic relationships between members of the complex for which fresh material was available for DNA sequencing.

Material and Methods

We obtained 39 specimens of the Philopterus Complex originating from material (Table 2) collected by the research team of the Department of Biology and Wildlife Diseases, University of Veterinary and Pharmaceutical Sciences, Brno, Czechia, during field trips in the Azores, Borneo, Czechia, Greece, Honduras, Slovakia, Sweden, and Vietnam between 2008 and 2016; in addition, 19 samples from Australia, China, and New Guinea collected between 2002 and 2007 were provided by the Price Institute of Parasitological Research, University of Utah, Salt Lake City, United States (PIPeR). All ethical and permitting documents are listed in the acknowledgments. We extracted lice using a Qiagen Dneasy Blood and Tissue kit (Qiagen, Venlo, The Netherlands) according to the standard manufacturer's protocol. After the extraction, the vouchers were slide-mounted in Canada balsam. The lice on the slides were morphologically identified to genus and species, respectively, using a CX21FS1 light wide-field upright microscope as described by Palma (1978) (Olympus, Tokyo, Japan), based on Gustafsson *et al.* (2019b), Mey (2004), Najer *et al.* (2012), Najer *et al.* (2020), Palma and Price (2006), and Price and Hellenthal

(1998). All the vouchers are deposited at the Department of Biology and Wildlife Diseases, University of Veterinary and Pharmaceutical Sciences, Brno, Czechia.

We tested whether these 39 specimens could be amplified for mitochondrial *COI* gene and five nuclear protein coding genes. In addition to *COI* (379 bp fragment, primers COI-L6625 and COI-H7005; Hafner *et al.* 1994) and *EF1α* (343 bp fragment, primers EF1α-Cho10 and EF1α-EF1; Danforth and Ji 1998) genes, we chose four nuclear loci which Sweet *et al.* (2014) published as consistently amplifying in lice (VATP21, 278 bp; *hyp*, 386 bp; DIPP, 133 bp; TMEDE6, 220 bp), using the same primers as in that study (BR12-223L, BR12-578R; BR50-181L, BR50-621R; BR62-295L, BR62-429R; BR69-190F, and BR69-432R, respectively). PCR reactions were set up in total volume of 25 µl, including 22 µl of master mix (5 µl of Taq polymerase, 0.5 µl of each primer, 16 µl of dH₂O) and 3 µl of DNA extract. For *EF1α*, we used the following PCR protocol: 94°C for 2 min, 94°C for 30 s, annealing temperature 50°C for 30 s, 72°C for 1 min, repeated 34 times, and a final extension step 72°C for 7 min. For the other loci, we used the same protocol, with exception of an annealing temperature of 46°C. In some cases, we doubled the volume while changing the proportions of some reagents (50 µl; 25 µl of master-mix, 2 µl of each primer, 18 µl of dH₂O, and 3 µl of DNA extract). In poorly performing samples, we also tried increasing the amount of DNA template to 5 µl and with 50 and 53°C annealing temperatures. Reaction products were run on 1% agarose gels at 120 V for ca. 20 min. We evaluated the success of each reaction according to the brightness and clarity of bands from the gels, and based on these results, we used only three well-amplifying loci (*COI*, *hyp*, *TMEDE6*) for downstream analyses. The final products were purified using a Gel/PCR DNA Fragments Kit (Geneaid, New Taipei City, Taiwan) according to the standard protocol. The purified fragments were then sequenced either with an ABI Prism BigDye Terminator Kit (Applied Biosystems, Foster City, CA) on an AB 3730x capillary sequencer (Applied Biosystems) at the Roy K. Carver Biotechnology Center (University of Illinois, Champaign, IL), or through the commercial services of Macrogen Europe (Amsterdam, The Netherlands). All newly generated DNA sequences are deposited in GenBank (Table 2).

We aligned sequences of *COI*, *hyp*, and *TMEDE6* genes in Geneious 9.1.8 (Kearse *et al.* 2012), utilizing the built-in alignment algorithm with a 65% similarity matrix. In order to assess genetic divergences among the samples, we computed both net average interspecific p-distances and average intraspecific p-distances in MEGA 7.0.14 (Kumar *et al.* 2016) on four data sets—*COI* (379 bp), *hyp* (386 bp), *TMEDE6* (220 bp), and the concatenated sequences of all three fragments combined (985 bp). Unknown taxa were defined according to their distance from other taxa and clustering within the trees.

We used the same four data sets for phylogenetic analyses (i.e., each gene individually and all genes combined), including published sequences of *Paragoniocotes* sp. (Cummings, 1916; Psocodea: Philopteridae) (GenBank accession numbers: AF348870 (*COI*), KF841398 (*hyp*), KF841433 (*TMEDE6*)) as an outgroup. We used the Akaike information criterion (AIC) computed in MEGA 7.0.14 (Kumar *et al.* 2016) to identify the most appropriate models of nucleotide substitution. We then used two phylogenetic methods: 1) Bayesian inference analysis (BI) and 2) Maximum Likelihood (ML). For BI, we used the Mr.Bayes 3.2.6 plugin in Geneious 9.1.8 (Ronquist and Huelsenbeck 2003, Kearse *et al.* 2012) with a GTR+G+I model for 10⁷ generations for each partition, with trees sampled every 1,000 generations. A majority rule consensus tree was summarized after discarding 1,000 trees as a burn-in. We ran ML analysis with the PhyML 2.2.3 plugin in Geneious 9.1.8 (Guindon and Gascuel 2003, Kearse *et al.* 2012) with a GTR+G+I model and

Table 2. List of specimens used for molecular analyses of relationships between groups of Philopterus Complex lice

Voucher number	Louse species	Host species	Host family	State	Location	Date of collection	GenBank numbers		
							COI	hsp	TMEDE6
PM58	<i>Philopteroides cucphuongensis</i>	<i>Calliope calliope—straggler</i>	Muscicapidae	Vietnam	Pù Mát	25.10.2015	MT468926	MT468947	MT468967
PM54	<i>Philopteroides cucphuongensis</i>	<i>Pycnonotus finalysoni</i>	Pycnonotidae	Vietnam	Pù Mát	25.10.2015	MT468925	MT468946	MT468966
108	<i>Philopteroides cucphuongensis</i>	<i>Pycnonotus melanoleucos</i>	Pycnonotidae	Borneo	YSEMA [†]	12.8.2015	MT468927	MT468948	MT468968
2483	<i>Philopteroides cucphuongensis</i>	<i>Pycnonotus xanthorrhous</i>	Pycnonotidae	China	Guizhou	7.4.2007	MT468928	MT468949	MT468969
2370	<i>Philopteroides flavala</i>	<i>Hemixos castanonotus</i>	Pycnonotidae	China	Guizhou		MT468919	MT468940	MT468960
2365	<i>Philopteroides flavala</i>	<i>Hemixos castanonotus</i>	Pycnonotidae	China	Guizhou		MT468920	MT468941	MT468961
2375	<i>Philopteroides flavala</i>	<i>Hemixos castanonotus</i>	Pycnonotidae	China	Guizhou		MT468921	MT468942	MT468962
CP142	<i>Philopteroides flavala</i>	<i>Hemixos flavala</i>	Pycnonotidae	Vietnam	Cúc Phương	7.2.2010	MT468922	MT468943	MT468963
CP56	<i>Philopteroides flavala</i>	<i>Iole propinqua</i>	Pycnonotidae	Vietnam	Cúc Phương	3.2.2010	MT468923	MT468944	MT468964
2750	<i>Philopteroides flavala</i>	<i>Ixos mcllellandii</i>	Pycnonotidae	China	Guizhou	3.5.2006	MT468924	MT468945	MT468965
43	<i>Philopteroides sp. 1</i>	<i>Alphoixox bres</i>	Pycnonotidae	Borneo	YSEMA [†]	23.7.2015	MT468929	MT468950	MT468970
PM176	<i>Philopteroides sp. 2</i>	<i>Pycnonotus flaviventris</i>	Pycnonotidae	Vietnam	Khe Kèm	4.11.2015	MT468930	MT468951	MT468971
2508	<i>Philopteroides sp. 3</i>	<i>Spizixos semitorques</i>	Pycnonotidae	China	Guizhou	5.4.2007	MT468931	MT468952	MT468972
57	<i>Philopteroides sp. 4</i>	<i>Pycnonotus erythrophthalmos</i>	Pycnonotidae	Borneo	YSEMA [†]	24.7.2015	MT468932	MT468953	MT468973
S221674	<i>Philopterusacrocephalus</i>	<i>Acrocephalus melanopogon</i>	Acrocephalidae	Slovakia	Parížske močiare	18.4.2016	MG565983	MG566002	MG566021
S221707	<i>Philopterusacrocephalus</i>	<i>Acrocephalus melanopogon</i>	Acrocephalidae	Slovakia	Parížske močiare	20.4.2016	MG565984	MG566003	MG566022
CU18816	<i>Philopterus citrinellae</i>	<i>Acanthis flamma</i>	Fringillidae	Sweden	Umeå	12.9.2013	MG565987	MG566004	MG566023
2KS44708	<i>Philopterus citrinellae</i>	<i>Emberiza citrinella</i>	Emberizidae	Sweden	Umeå	17.9.2013	MG565992	MG566011	MG566030
1EV21759	<i>Philopterus citrinellae</i>	<i>Emberiza schoeniclus</i>	Emberizidae	Sweden	Umeå	17.9.2013	MG565989	MG566008	MG566027
1EV20889	<i>Philopterus citrinellae</i>	<i>Fringilla coelebs</i>	Fringillidae	Sweden	Umeå	12.9.2013	MG565990	MG566009	MG566028
1EV21218	<i>Philopterus citrinellae</i>	<i>Fringilla montifringilla</i>	Fringillidae	Sweden	Umeå	15.9.2013	MG565991	MG566010	MG566029
CT98119	<i>Philopterus citrinellae</i>	<i>Spinus spinus</i>	Fringillidae	Sweden	Stora Fjädräggs	22.9.2013	MG565988	MG566007	MG566026
LV066	<i>Philopterus fringillae</i>	<i>Passer domesticus</i>	Passeridae	Greece	Loutra Volvis	6.6.2013	MG565985	MG566004	MG566023
LV62	<i>Philopterus fringillae</i>	<i>Passer domesticus</i>	Passeridae	Greece	Loutra Volvis	6.6.2013	MG565986	MG566005	MG566024
F48388	<i>Philopterus gustafssonii</i>	<i>Regulus ignicapillus</i>	Regulidae	Czechia	Klec	16.5.2015	MG565996	MG566015	MG566034
AZ14	<i>Philopterus gustafssonii</i>	<i>Regulus regulus</i>	Regulidae	Azores	Sete Cidades	14.4.2013	MG565993	MG566012	MG566031
F49358	<i>Philopterus gustafssonii</i>	<i>Regulus regulus</i>	Regulidae	Czechia	Lubno	18.3.2015	MG565994	MG566013	MG566032
F49362	<i>Philopterus gustafssonii</i>	<i>Regulus regulus</i>	Regulidae	Czechia	Janovice	19.3.2015	MG565995	MG566014	MG566033
1621/1*	<i>Philopterus Complex sp. 1</i>	<i>Dicrurus bracteatus</i>	Dicruridae	Australia	Northern Territory	24.10.2002	MT468913	MT468934	MT468954
1621/2*	<i>Philopterus Complex sp. 1</i>	<i>Dicrurus bracteatus</i>	Dicruridae	Australia	Northern Territory	24.10.2002	MG565999	MG566018	MG566037
1467	<i>Philopterus Complex sp. 2</i>	<i>Dicrurus bracteatus</i>	Dicruridae	New Guinea	Oro	14.10.2002	MG566001	MG566020	MG566039
2450	<i>Philopterus Complex sp. 3</i>	<i>Pterococcus solaris</i>	Campephagidae	China	Guizhou		MG566000	MG566019	MG566038
LA50	<i>Philopterus Complex sp. 4</i>	<i>Euphonia hirundinacea</i>	Fringillidae	Honduras	Atlántida	12.8.2014	MG565997	MG566016	MG566035
1EV21209	<i>Philopterus Complex sp. 5</i>	<i>Poecile montanus</i>	Paridae	Sweden	Umeå	15.9.2013	MG565998	MG566017	MG566036
1491/1*	<i>Philopterus Complex sp. 6</i>	<i>Chaetorhynchus papuensis</i>	Rhipiduridae	New Guinea	Oro	3.11.2002	MT468916	MT468937	MT468957
1491/2*	<i>Philopterus Complex sp. 6</i>	<i>Chaetorhynchus papuensis</i>	Rhipiduridae	New Guinea	Oro	3.11.2002	MT468917	MT468938	MT468958
LB028	<i>Philopterus Complex sp. 7</i>	<i>Rhipidura albicollis</i>	Rhipiduridae	Vietnam	Lang Biang	12.9.2012	MT468918	MT468939	MT468959
2549	<i>Philopterus urocisae</i>	<i>Urocissa erythrorhyncha</i>	Corvidae	China	Guizhou	4.5.2006	MT468933		MT468974
LA65/1*	<i>Tyranniphilopterus sp.</i>	<i>Pitangus sulphuratus</i>	Tyrannidae	Honduras	Atlántida	13.8.2014	MT468914	MT468935	MT468955
LA65/2*	<i>Tyranniphilopterus sp.</i>	<i>Pitangus sulphuratus</i>	Tyrannidae	Honduras	Atlántida	13.8.2014	MT468915	MT468936	MT468956

Voucher numbers represent ring or identification field numbers of hosts from which the lice were collected. All the vouchers were deposited in the personal collection of Oldrich Sychra.

*Specimens were collected from the same host individual.

[†]Yayasan Sabah Forest Management Area, Sabah, North East Borneo, Malaysia.

parameters estimated from the data. Branch supports were generated with 1,000 bootstrap replicates.

Results

COI, *hyp*, and *TMEDE6* successfully amplified in all samples apart from *Philopterus urocissae* (Price and Hellenthal, 1998; Psocodea: Philopteridae) from blue magpie (*Urocissa erythroryncha*) (Boddaert, 1783; Passeriformes: Corvidae), from which we obtained only sequences of the *COI* and *TMEDE6* genes. For most taxa only one or two individuals were sequenced (Table 2). More than two individuals were sequenced from *Philopterus citrinellae* (Schrank, 1776; Psocodea: Philopteridae) (6 individuals ex 6 host species), *Philopterus gustafssoni* (Najer et al., 2020; Psocodea: Philopteridae) (4 individuals ex 2 host species), *Philopteroides flavala* (Najer and Sychra, 2012; Psocodea: Philopteridae) (6 individuals ex 4 host species), and *Philopteroides cucphuongensis* (Mey, 2004; Psocodea: Philopteridae) (4 individuals ex 4 host species, but one is considered to be a straggler). Genetic divergence between species was much higher for the mitochondrial (*COI*) than for the nuclear (*hyp*, *TMEDE6*) markers (Table 3). The mitochondrial gene also provided a tree with more resolution and support (Fig. 1 and Supp Fig. S1 [online only]) than trees based on the nuclear markers (Supp Figs. S2 and S3 [online only]). The most resolved and well-supported tree was based on the concatenated sequences of all three markers (Fig. 2). The results of genetic analyses (Figs. 1 and 2, and Supp Figs. S1–S3 [online only]) showed 19 putative taxa, 12 of which belong to morphologically undescribed species (Philopterus Complex spp. 1–7, *Tyranniphilopterus* sp., and *Philopteroides* spp. 1–4).

The net p-distances between putative taxa are shown in Table 3 for individual markers and in Supp Table S1 (online only) for concatenated sequences. The net p-distances within putative taxa are shown in Table 4. These distances were computed only for taxa including more than one specimen. The net interspecific p-distances lay within a range of 0.044–0.301 for *COI* (average 0.230, $n = 171$ pairwise distance comparisons, SD = 0.048; Table 3), 0.006–0.148 for *hyp* (average 0.094, $n = 153$ pairwise distance comparisons, SD = 0.042; Table 3), 0.004–0.115 for *TMEDE6* (average 0.071, $n = 171$ pairwise distance comparisons, SD = 0.036; Table 3), and 0.021–0.193 for concatenated sequences (average 0.141, $n = 153$ pairwise distance comparisons, SD = 0.041; Supp Table S1 [online only]). In most of the cases, except four *Philopteroides* species (*Philopteroides flavala*, *Philopteroides* spp. 1, 3, and 4), the interspecific divergences for *COI* exceed 12%. Although interspecific divergences were generally high, especially for *COI* (Table 3), divergences within named species (Table 4) were much lower (0–0.06 for *COI*; 0–0.01 for *hyp*, 0–0.005 for *TMEDE6*; and 0–0.027 for concatenated sequences). The highest intraspecific divergence (0.024–0.027 for concatenated sequences, Table 4) was in species in which the examined samples originated from more than two host species (six host species in *Philopterus citrinellae* and four host species in each *Philopteroides flavala* and *Philopteroides cucphuongensis*, Table 2).

Phylogenetic trees for each gene analyzed separately were generally well resolved, although there were some polytomies and weakly supported relationships (Figs. 1 and 2). These trees had generally high support for monophyly of most named species. Bayesian trees had identical topologies to the consensus ML trees for the nuclear genes (not shown) but had slight differences in the case of *COI* (Fig. 1 and Supp Fig. S1 [online only]). However, many terminal branches were collapsed in the ML tree, so it provides considerably less resolution regarding the relationships among the groups. The concatenated data set of all three genes provided the most resolution and support (Fig. 2).

Discussion

Combining DNA sequences from two nuclear (*hyp* and *TMEDE6*; Supp Figs. S2 and S3 [online only]) and one mitochondrial (*COI*; Fig. 1 and Supp Fig. S1 [online only]) gene provided phylogenetic information within the Philopterus Complex of feather lice (Psocodea: Ischnocera). When all three loci were compared (Fig. 2), *COI* exhibited the highest interspecific pairwise divergences (Table 3), as has been consistently found for lice and most other insects (e.g., Lozano-Sardaneta et al. 2020). Although variation in nuclear loci was lower (Table 3), they provided similar phylogenetic information to *COI*, and the combination of all three genes (Supp Table S1 [online only] and Fig. 2) appeared to provide the most resolution and support. This suggests that in our case, increasing the number of base pairs improves phylogenetic resolution, and is consistent with previous work (Rokas and Carroll 2005) that implies including additional informative genes (e.g., Sweet et al. 2014) can improve the accuracy of phylogenetic estimation.

Bush et al. (2016) suggest 5% *COI* divergence as a relevant threshold for species differentiation in the Brueelia Complex of ischnoceran lice. The interspecific divergences between almost all our inferred species (except *Philopteroides cucphuongensis* and *Philopteroides* sp. 4; Table 3) are higher than this number, so they can be well-defined species if this limit is applied. Another study considering genetic divergences of avian lice (Kolencik et al. 2017) proposed that a 12% *COI* divergence is an appropriate species-level threshold for the amblyceran louse genus *Myrsidea* (Waterston, 1915; Psocodea: Menoponidae). Other than a few comparisons within the *Philopteroides* genus, the interspecific distances in our data set are almost all above 12%, suggesting that nearly all our species are well defined even using this higher threshold. Some *Philopteroides* species have a distance between the 5 and 12%, but this might relate to the fact that we only analyzed species from one host family and one geographical area in this case. On the other hand, Sychra et al. (2014) kept lice in the genus *Penenirmus* (Clay and Meinertzhagen, 1938; Psocodea: Philopteridae) conspecific despite their relatively large intraspecific variability (evaluating Johnson et al. 2001 and Sychra et al. 2014). The studies of Sychra et al. (2014) and Kolencik et al. (2017) differ from those of Bush et al. (2016) mainly in the number of specimens examined. Although the former studies include rather modest numbers of samples, Bush et al. (2016) refer to hundreds of specimens, often not identified to species level. Our study includes rather low numbers of specimens, which has several implications: 1) it is more similar to the studies of Sychra et al. (2014) and Kolencik et al. (2017), so the suggested relevant genetic distance threshold should be higher, as it is in those studies; 2) the interspecific divergences have limited values, as the number of putative taxa may change if more specimens are included; 3) the values represent only a limited part of the Philopterus Complex (and the distances among genera may differ, see, e.g., *Philopterus* vs *Philopteroides*, Table 3), they do not say anything about other genera or Philopterus Complex as a whole; 4) it is still necessary to confirm the species-level taxonomy of Philopterus Complex lice with additional information, such as traditional morphology-based approaches. In cases where the analyzed specimens belong to morphologically described species, the molecular data are consistent with this taxonomy. At the generic level, the molecular data imply that *Philopterus* might be further split into several groups. In the case of *Philopterus citrinellae*, the molecular data support recent taxonomic changes of this species (Palma and Price 2006). The data also support morphological similarity of *Philopterus gustafssoni* and *Philopterus* species described from hosts of the avian family Paridae (Najer et al. 2020).

Table 3. Net interspecific p-distances of *Philopterus* Complex lice computed from *COI*, *hyp* and *TMEDE6* gene sequences

Taxon	1	2	3	4	5	6	7	8
<i>Philopterus acrocephalus</i>		0,198	0,245	0,231	0,269	0,253	0,245	0,227
<i>Philopterus citrinellae</i>	0,018/0,012		0,189	0,148	0,214	0,197	0,189	0,165
<i>Philopterus fringillae</i>	0,018/0,014	0,016/0,009		0,18	0,222	0,224	0,243	0,219
<i>Philopterus gustafssoni</i>	0,016/0,008	0,016/0,004	0,016/0,004		0,193	0,2	0,221	0,216
<i>Philopterus</i> Complex sp. 1	0,084/0,046	0,08/0,041	0,085/0,041	0,085/0,037		0,206	0,206	0,256
<i>Philopterus</i> Complex sp. 2	0,091/0,041	0,088/0,037	0,093/0,027	0,093/0,033	0,023/0,023		0,201	0,227
<i>Philopterus</i> Complex sp. 3	0,078/0,032	0,075/0,028	0,08/0,027	0,08/0,024	0,018/0,032	0,026/0,027		0,24
<i>Philopterus</i> Complex sp. 4	0,021/0,014	0,023/0,009	0,023/0,009	0,023/0,005	0,078/0,041	0,085/0,036	0,073/0,027	
<i>Philopterus</i> Complex sp. 5	0,026/0,014	0,026/0,009	0,026/0,009	0,016/0,005	0,078/0,041	0,085/0,036	0,073/0,027	0,028/0,009
<i>Philopterus</i> Complex sp. 6	0,124/0,101	0,126/0,096	0,121/0,096	0,126/0,092	0,11/0,091	0,115/0,086	0,115/0,077	0,123/0,095
<i>Philopterus</i> Complex sp. 7	0,138/0,11	0,145/0,105	0,145/0,105	0,145/0,101	0,137/0,105	0,145/0,1	0,124/0,1	0,137/0,105
<i>Philopterus urocissae</i>	-/0,06	-/0,055	-/0,055	-/0,051	-/0,059	-/0,064	-/0,055	-/0,055
<i>Philopteroides cucphuongensis</i>	0,114/0,109	0,118/0,104	0,118/0,095	0,118/0,1	0,113/0,09	0,118/0,085	0,113/0,085	0,115/0,103
<i>Philopteroides flavala</i>	0,116/0,115	0,123/0,109	0,123/0,1	0,123/0,106	0,118/0,096	0,123/0,091	0,114/0,091	0,12/0,109
<i>Philopteroides</i> sp. 1	0,117/0,115	0,124/0,109	0,124/0,1	0,124/0,106	0,114/0,095	0,119/0,091	0,114/0,091	0,122/0,109
<i>Philopteroides</i> sp. 2	0,128/0,106	0,13/0,1	0,135/0,091	0,135/0,096	0,119/0,086	0,124/0,082	0,119/0,082	0,132/0,1
<i>Philopteroides</i> sp. 3	0,117/0,11	0,124/0,105	0,124/0,096	0,124/0,101	0,119/0,091	0,124/0,086	0,119/0,086	0,122/0,105
<i>Philopteroides</i> sp. 4	0,112/0,115	0,119/0,109	0,119/0,1	0,119/0,106	0,109/0,095	0,119/0,091	0,114/0,091	0,117/0,109
<i>Tyranniphilopterus</i> sp.	0,136/0,087	0,132/0,082	0,132/0,082	0,132/0,078	0,124/0,068	0,127/0,073	0,119/0,073	0,132/0,082

Distances for the mitochondrial gene (*COI*) above the diagonal and distances for the nuclear genes (*hyp*/*TMEDE6*) below the diagonal.

In particular, for species that were found on only a single host species, intraspecific divergences were generally very low (often 0%). In contrast, for louse species found on more than one host species, intraspecific divergences are relatively higher (e.g., *Philopterus citrinellae*—0.063 for *COI*, 0.025 for concatenated sequences; *Philopterus gustafssoni*—0.03 for *COI*, 0.014 for concatenated sequences; and both *Philopteroides* species—0.056 and 0.061 for *COI*, 0.024 and 0.027 for concatenated sequences). This phenomenon might have two potential explanations. The host generalists could be more genetically variable than host specialists simply because we examined more specimens of these species. Here we define host specialist as those parasitizing a single host genus (e.g., *Philopterus fringillae* (Denny, 1842; Psocoda: Philopteridae), *Philopterus gustafssoni*), whereas species parasitizing multiple host genera are host generalists (e.g., *Philopterus citrinellae*, *Philopteroides cucphuongensis*, *Philopteroides flavala*). On the other hand, it might be that species with higher host specificity indeed have lower genetic variability, as was shown by Sweet and Johnson (2018) in the dove louse genus *Physconelloides* (Ewing, 1927; Psocoda: Philopteridae). However, the same study showed that more host-specific species of the genus *Columbicola* (Ewing, 1929; Psocoda: Philopteridae) are more variable compared with generalists in the same genus, suggesting that the differences between *Physconelloides* and *Columbicola* are actually driven by factors other than host specialist versus generalist. Thus, it would be interesting to broaden this analysis to include a larger set of samples of generalist lice from across their host species and compare intraspecific variability to a comparable number of specimens of highly host-specific louse species. We also cannot exclude the possibility that when more molecular data become available, the morphologically different species will become a single species, as suggested by phylogenetic work on, e.g., fig-wasps (Anstett *et al.* 1997 vs Haime *et al.* 2006) or avian malaria parasites (Bennet *et al.* 1994 vs Ricklefs and Fallon 2002).

In terms of geographic distribution, the vast majority of the specimens that we were able to obtain originate either from Europe or Southeast Asia (Table 2; Fig. 1). In the case of host generalists, the intraspecific distances were similar in both European and

Southeast Asian species (*Philopterus citrinellae* vs *Philopteroides cucphuongensis* and *Philopteroides flavala*). Concerning morphologically described host specialists from Asia, this study includes only one specimen of *Philopterus urocissae*. Therefore, comparison to the European species is not possible, and more analyses of further specimens and species are needed. In the European species, although the specimens of *Philopterus gustafssoni* were collected in two distant locations (Azores and Czechia), they were not dramatically divergent, particularly for *COI* (0.03 vs 0.063). In this case, the divergence was smaller than that within *Philopterus citrinellae*, specimens of which were collected from a broader spectrum of hosts from two locations that are close to one another.

Species-Level Taxonomy Within the *Philopterus* Complex

Although not recovered in the analyses of individual genes, the concatenated analysis supports monophyly of the *Philopterus* Complex. Unfortunately, the position of *Philopterus urocissae* could not be assessed in the context of this analysis because of missing sequences. However, it may be that further subdivision of *Philopterus* is needed to include the separate major clades found in the trees (Fig. 1). These groups were constructed solely on the basis of the trees and are not currently officially recognized taxa; therefore, they are not included in the classification scheme (Table 1):

- 1) *Philopterus* from the avian family Corvidae (here represented by the only one specimen of *Philopterus urocissae*). Compared with the rest of our data set, the genetic divergences of this specimen and the rest of the samples are relatively high for both *COI* (0.196–0.264) and *TMEDE6* (0.055–0.109). These divergences support the morphological results, showing that this group may represent a separate genus. For clear genetic-based conclusion, however, the molecular analyses of more specimens are sorely needed. Concerning other characteristics of this group (assumed from the results of morphological work, Price and Hellenenthal 1998), it is strictly host specific and geographically widespread all around the world. Because only a single specimen was

9	10	11	12	13	14	15	16	17	18	19
0,23	0,28	0,282	0,261	0,25	0,258	0,29	0,293	0,28	0,272	0,301
0,156	0,231	0,239	0,234	0,201	0,205	0,234	0,236	0,246	0,224	0,242
0,185	0,272	0,259	0,245	0,224	0,212	0,256	0,23	0,235	0,243	0,274
0,144	0,271	0,24	0,237	0,205	0,211	0,241	0,231	0,232	0,239	0,255
0,237	0,256	0,251	0,243	0,196	0,203	0,232	0,219	0,227	0,232	0,259
0,224	0,282	0,272	0,24	0,21	0,219	0,253	0,24	0,248	0,23	0,264
0,235	0,261	0,253	0,235	0,22	0,225	0,264	0,264	0,261	0,24	0,272
0,208	0,272	0,301	0,248	0,25	0,25	0,282	0,285	0,282	0,274	0,274
	0,272	0,269	0,272	0,257	0,247	0,277	0,28	0,259	0,29	0,285
0,128/0,095		0,216	0,248	0,242	0,25	0,288	0,269	0,272	0,277	0,264
0,148/0,105	0,073/0,082		0,222	0,232	0,235	0,266	0,259	0,256	0,282	0,264
-0,055	-0,1	-0,109		0,201	0,209	0,24	0,264	0,23	0,243	0,256
0,126/0,103	0,083/0,09	0,11/0,103	-0,103		0,044	0,093	0,124	0,11	0,047	0,225
0,131/0,109	0,089/0,096	0,115/0,108	-0,108	0,007/0,007		0,1	0,117	0,098	0,074	0,246
0,132/0,109	0,084/0,095	0,114/0,109	-0,109	0,012/0,005	0,016/0,014		0,15	0,148	0,127	0,266
0,142/0,1	0,1/0,086	0,124/0,1	-0,1	0,027/0,005	0,031/0,014	0,031/0,009		0,124	0,156	0,251
0,132/0,105	0,089/0,1	0,114/0,109	-0,105	0,012/0,013	0,016/0,018	0,016/0,018	0,021/0,018		0,15	0,259
0,127/0,109	0,089/0,095	0,119/0,109	-0,109	0,006/0,007	0,011/0,009	0,016/0,014	0,031/0,014	0,016/0,018		0,266
0,132/0,082	0,115/0,059	0,14/0,082	-0,073	0,102/0,063	0,1/0,064	0,104/0,068	0,114/0,064	0,101/0,073	0,109/0,068	

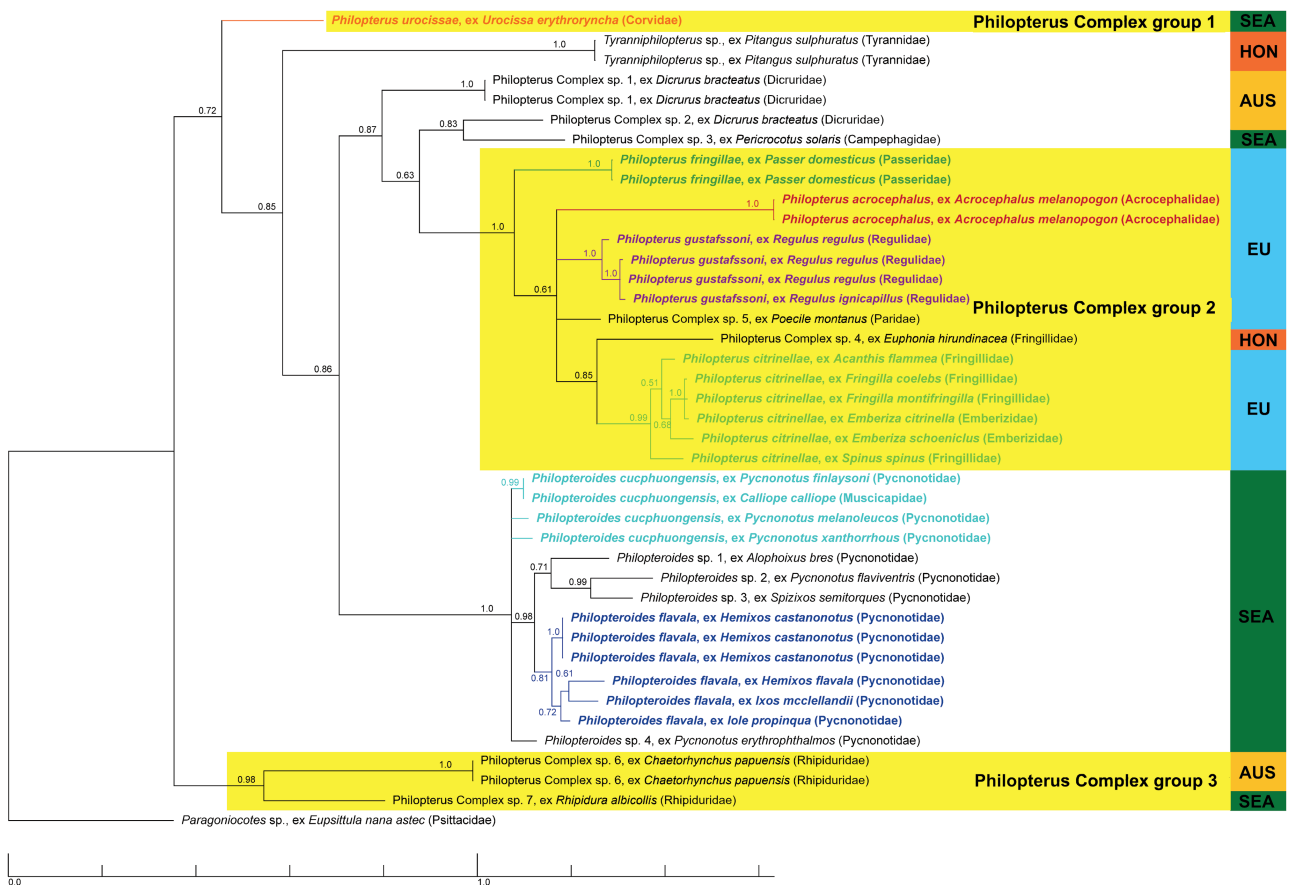


Fig. 1. Phylogenetic tree of Philopterus Complex estimated with Bayesian analysis based on a 379 bp alignment of a *COI* gene fragment. Morphologically described species indicated in other colors than black. Branch lengths indicate substitutions per nucleotide site. Numbers above the branches indicate Bayesian posterior probabilities. Branches with posterior probabilities < 0.5 were collapsed. Separate major clades of Philopterus Complex indicated with yellow. Geographic distribution of the examined specimens indicated on the right side: SAE – Southeast Asia (Borneo, Vietnam, southern China); HON – Honduras; AUS – Australian realm (Australia, New Guinea); EU – Europe (including Azores).

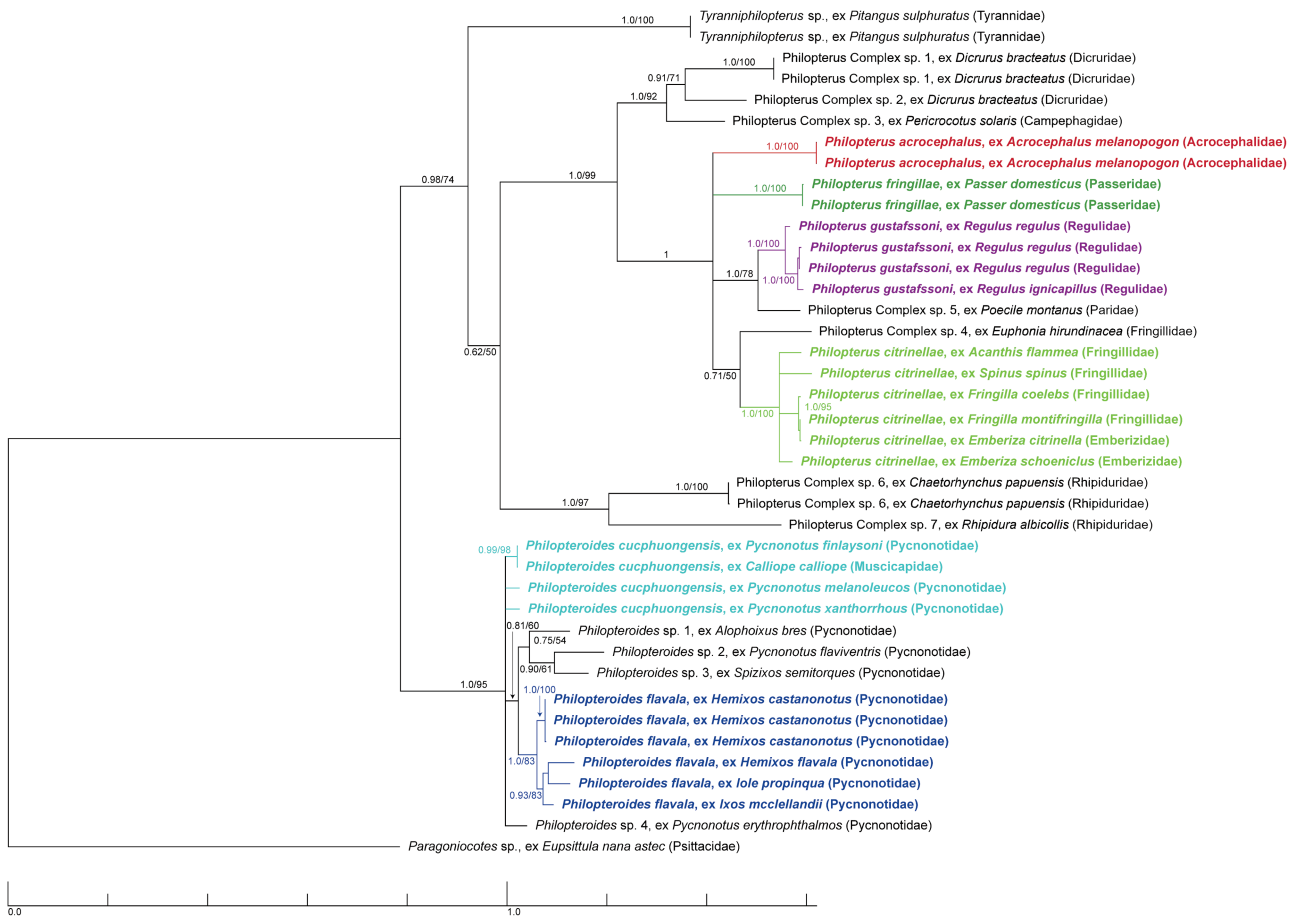


Fig. 2. Phylogenetic tree of *Philopterus* Complex as revealed by Bayesian analysis based on 985 bp long alignment of *COI*, *hyp*, and *TMEDE6* gene fragments. Morphologically described species indicated in other colors than black. Branch lengths indicate expected numbers of substitutions per nucleotide site. ML tree provided identical topology. Numbers along branches indicate Bayesian posterior probabilities/percent bootstrap support values as obtained by ML analysis. Branches with posterior probabilities < 0.5 were collapsed.

Table 4. Average intraspecific p-distances of *Philopterus* Complex lice with two or more available sequences

Taxon	<i>COI</i>	<i>hyp</i>	<i>TMEDE6</i>	Concatenated
<i>Philopterus acrocephalus</i>	0	0	0	0
<i>Philopterus citrinellae</i>	0.06	0	0.004	0.025
<i>Philopterus fringillae</i>	0	0	0	0.001
<i>Philopterus gustafssoni</i>	0.03	0	0.003	0.014
<i>Philopterus</i> Complex sp. 1	0	0	0	0
<i>Philopterus</i> Complex sp. 6	0	0	0.005	0.001
<i>Philopterooides cucphuongensis</i>	0.06	0.01	0.002	0.024
<i>Philopterooides flavala</i>	0.06	0.01	0.002	0.027
<i>Tyranniphilopterus</i> sp.	0	0	0	0

analyzed in this study, we are unable to comment further on the status of this group.

- 2) *Philopterus* Complex from European small passerine birds (including both host specialists and generalists). Genetic divergences within this group are variable. More data about the other groups are needed to show if the cause of this variability is the higher number of individuals, or if the group is more variable itself.

Morphologically, this group is well described (e.g., [Gustafsson et al. 2019b](#), [Najer et al. 2020](#)), and results of this study confirm the morphological species delimitation. However, all the analyzed specimens were collected in Europe, so more extensive sampling could help us to confirm these results.

- 3) *Philopterus* Complex from fantails (avian family Rhipiduridae, i.e., *Philopterus* Complex spp. 6 and 7). This group contains two species. The genetic divergences between them are slightly lower than average in *COI* (0.216) and *hyp* (0.073) and slightly higher than average in *TMEDE6* (0.082) and concatenated sequences (0.13). Both species are morphologically undescribed, so we cannot compare the species delimitation to the morphological studies. It is the only group represented in two geographical regions, Vietnam and New Guinea; however, more specimens would be needed to show whether there is any geographical pattern in the genetic diversity.

Should the delimitation of these clusters be confirmed in the future (i.e., when sufficient numbers of representatives from each particular group will be analyzed), it may be possible that each of these groups should be designated as a separate louse genus, with the generic name *Philopterus* applied to the clade parasitizing corvids, since the type species of the genus belongs there. Morphologically, there were

confirmed the groups 1 and 2, nothing is known about morphology of the group 3.

From the second group (host generalists), *Philopterus acrocephalus* and *Philopterus gustafssoni* (placed in one of the most terminal branches of the trees, Figs. 1 and 2, and Supp Figs. S1–S3) are the most recently revised species based on morphology. Until recently, they were placed in the so called ‘reguli’ species group (Złotorzycka and Lucińska 1976, Mey 1983), assuming their morphological similarity. A thorough morphological revision (Najer *et al.* 2020) showed that this categorization was based on incomplete descriptions and the two species do not share any similarities justifying existence of the group. Our analyses thus support the morphological results showing that interspecific divergence between these species (0.231 for COI, 0.016 for *hyp*, 0.008 for *TMEDE6*, and 0.097 for concatenated sequences) is not substantially smaller than divergences between each of them and either *Philopterus citrinellae* or *Philopterus fringillae*, two species that are even more morphologically distant. Furthermore, our results show that the interspecific distances between *Philopterus gustafssoni* and both *Philopterus citrinellae* and *Philopterus fringillae* are much lower than those between *Philopterus gustafssoni* and *Philopterus acrocephalus* (Carriker, 1949; Psocodea: Philopteridae). This study itself, however, does not explicitly rule out the existence of the species group, since molecular data from its many potential members are still missing.

Phylogenetic relationships within the host generalist group (group 2 among the abovementioned, Fig. 1) could be further clarified by additional examination of more *Philopterus acrocephalus* and *Philopterus fringillae* specimens. Although both of these species are host generalists, they seem to occur more rarely (i.e., with much lower prevalence) than *Philopterus citrinellae* and *Philopterus gustafssoni* (e.g., Gustafsson *et al.* 2019b, Najer *et al.* 2020). In each of these species, we examined only two specimens from one host species, which is too few to make any firm conclusion regarding their systematics.

Based on the sampling in our analyses, the genus *Philopteroides* appears to be phylogenetically well defined. Both *Philopteroides cucphuongensis* and *Philopteroides flavala* show a relatively high intraspecific variability, which leads to some uncertainty in their relationships to other *Philopteroides* species. *Philopteroides* is a very variable genus consisting of two morphologically well-defined species groups (Valim and Palma 2013). It is known from a large range of host families across all the Old World tropics, with many species still expected to be described. This study includes only two morphologically described species known from the same host family and geographical area. Therefore, we avoid concluding its monophyly from our limited sampling.

Overall, this study confirms the delimitation of morphologically described species in the *Philopterus* Complex based on sequence divergence values. The phylogeny based on these sequences shows potential support for existing genera, based on our limited sampling. It also appears that the less host-specific groups within the *Philopterus* Complex (*Philopterus* group 2, *Philopteroides*) are associated with more recently derived species. Further studies are needed, dramatically increasing taxon sampling, to provide a comprehensive phylogenetic picture for this extremely diverse and widespread group of lice. That goes along with trend in amblyceran genus *Menacanthus* (Neumann, 1912; Psocodea: Menoponidae), as showed by Martínů *et al.* (2015).

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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