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DNA barcoding of Palaearctic Ulidiidae (Diptera: Tephritoidea): morphology, DNA evolution, and Markov codon models

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Abstract

Background: Here, for the first time, we report a barcoding survey of the dipterian family Ulidiidae (with two subfamilies Ulidiinae and Otitinae) coupled with morphology. To date, this is the first comprehensive analysis of the family that relies on molecular data. To reconstruct probable higher-level phylogenetic relationships between the genera of Ulidiidae, we exploited maximum likelihood and Bayesian inference, and additionally, we utilized a modern Markov model of codon substitutions for protein-coding genes coupled with the maximum likelihood approach to establish more realistic evolutionary scenarios connecting Ulidiinae and Otitinae.

Results: Though we found morphological synapomorphic characters that clearly distinguish two groups of genera, formerly relating to two subfamilies, the monophyly of the clade Otitinae was not firmly supported by molecular phylogenetic reconstructions. The subfamily Ulidiinae was recovered as the basal likely paraphyletic group with high reliability. Overall, our results suggest the inclusion of the genera *Homalocephala* and *Seioptera* into the tribe Seiopterini (Otitinae). Three genera of Ulidiinae (*Physiphora*, *Timia*, *Ulidia*), eight genera of Otitinae (*Ceroxys*, *Herina*, *Melieria*, *Myennis*, *Otites*, *Pseudotephritis*, *Seioptera*, *Tetanops*), and the genus of *Homalocephala* with still unconfirmed systematic status were included in the analysis.

Conclusions: On all phylogenetic trees obtained in our survey, there is a clear tendency of clustering at the genus level with separation of (*Physiphora* + (*Timia* + *Ulidia*)) (Ulidiinae) and (Otitinae + *Homalocephala*). The genus *Homalocephala* takes basal or subbasal position relatively to Otitinae. Phylogenetic reconstruction based on Markov models of codon evolution provided a good resolution for our limited dataset.

Keywords: Barcoding; Diptera; DNA evolution; Markov codon models; Palaearctic; Ulidiidae

Background

The true fruit fly family Ulidiidae Macquart, 1835 consists of subfamilies Ulidiinae Macquart, 1835 and Otitinae Westwood, 1840 and belongs to the superfamily Tephritoidea of the acalyptrate Diptera. Inability to separate Ulidiidae from other Tephritoidea families using morphological characters alone introduced great uncertainty about its taxonomic status including multiple divisions versus unifications into a single family (Figure 1). The subfamily Ulidiinae includes approximately 430 species assigned to about 60 genera. There have been 91 species of Ulidiinae described from 3 genera in the Palaearctic. These flies are small- to medium-sized (2 to 13 mm). Ulidiins consist of diversely colored flies (black or reddish-yellow, often dull gray, sometimes with a metallic green or blue shine) with predominantly hyaline wings, often with sub-basal and apical dark spots. This subfamily occurs nearly worldwide, with more than half of the species and 75% of the genera in the Neotropical region (Kameneva 2008). About 100 species of Ulidiinae belonging to 5 genera inhabit the Palaearctic territory. The subfamily Otitinae includes about 260 species assigned to 31 genera, distributed mainly in the Palaearctic region and the Americas. There have been 128 species of Otitinae described from 12 genera in the Palaearctic. Otitins are generally small- to medium-sized

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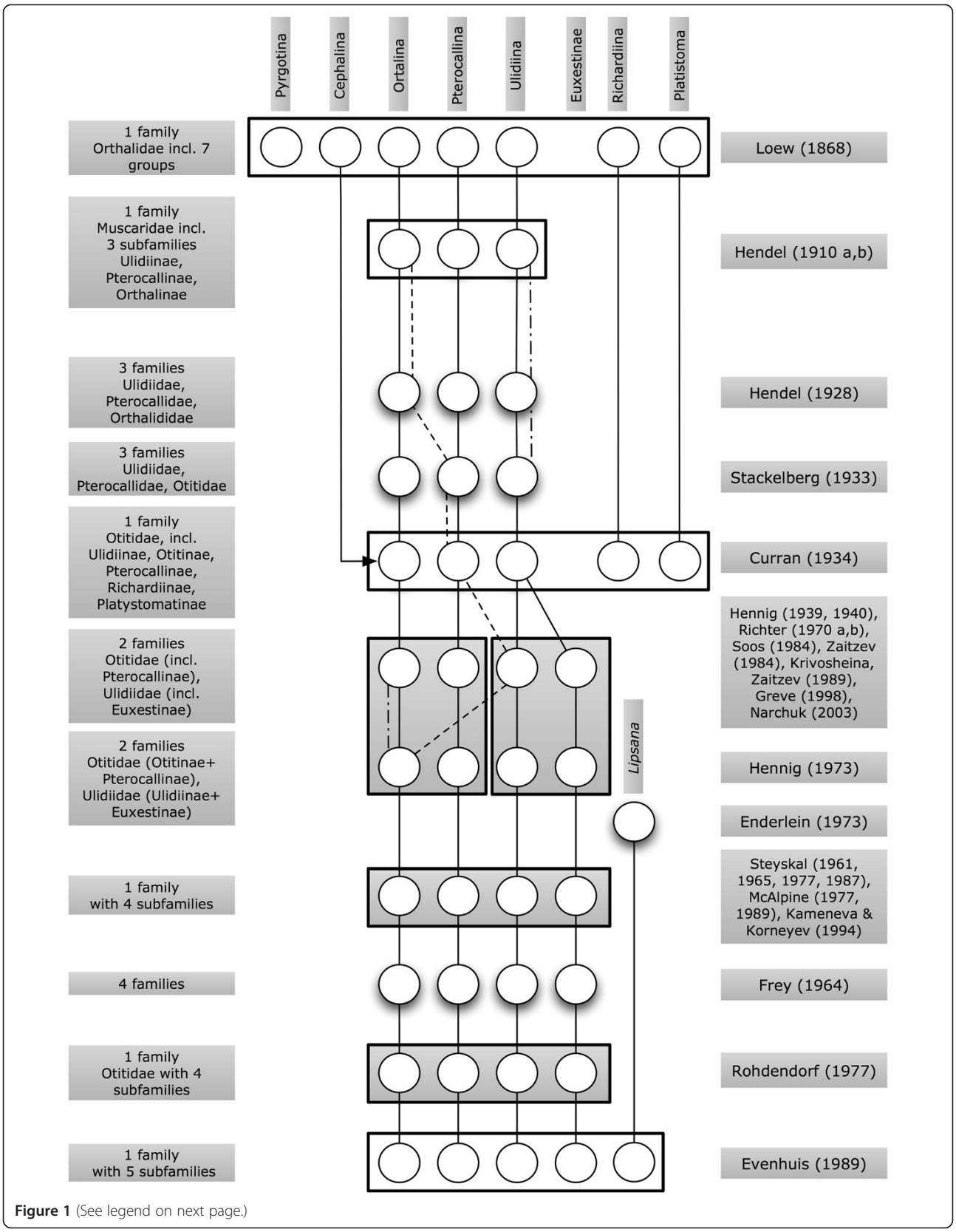

Figure 1 (See legend on next page.)

(See figure on previous page.)
Figure 1 Historically most significant taxonomic changes and modifications of Ulidiidae at the family level. Circles represent families or subfamilies. Dashed line corresponds to the position of *Homalocephala* throughout the development of the system. Dot-dashed line corresponds to the position of *Seioptera* throughout the development of the system. Rectangles combine subfamilies or groups into families, where gray color denotes major classification trends. The arrow indicates inclusion of American species of Otitidae by Curran (1934a, b) into Otitinae of the Ulidiinae + Otitinae group.

(3 to 12 mm) flies with diverse coloration; their wings are either predominantly hyaline or with a number of dark spots. Both subfamilies are characterized by a high level of species diversification and by a varying degree of morphological genus differentiation. Within the superfamily Tephritoidea, the family Ulidiidae occupy a proximal position to the root of the phylogenetic tree (Figure 2) and thus play a crucial role in understanding the major evolutionary trajectories of tephritoidous flies. To date, researches have not reached any strict consensus regarding the taxonomic status of the group Ulidiinae + Otitinae.

Loew (1868) was the first to propose a detailed classification of the family named Ortalidae (Figure 1). Within the family boundaries, he discerned seven groups: Pyrgotina, Ortalina, Cephalina, Platistoma, Pterocallina, Ulidiina, and Richardiina. Later, Ulidiinae, Pterocallinae, and Ortalinae have often been considered as the subfamilies in Muscaridae (Hendel 1910a, b) or as the separate families Ulidiidae, Pterocallidae (syn. Ulidiidae), and Ortalidae (syn. Otitidae) (Hendel 1928; Stackelberg 1933). Hennig (1939, 1940, 1973a, b) discriminated Otitidae (including true Pterocallinae) and Ulidiidae (including Euxestinae) as independent families. Hennig (1939, 1940) showed that these two families might be only differentiated by the distiphallus structure and by the presence/absence of setulae on vein R_1. Solid synapomorphic characters uniting Ulidiidae and Otitidae into a single monophyletic group were not found. In addition, Hennig (1939, 1940) included the genus *Homalocephala* Zetterstedt, 1838 into the family Ulidiidae. Nonetheless, *Homalocephala* was later moved to the family Otitidae (Hennig 1973a, b), whereas *Homalocephala* had been previously included either in Otitidae (Hendel 1910a, b) or in Pterocallidae (Stackelberg 1933). Most European dipterists (Richter 1970a, b; Soós 1984; Zaitzev 1984; Krivosheina and Zaitzev 1989; Greve 1998; Nartshuk 2003) use two separate family names, Ulidiidae and Otitidae, following Hennig's earlier opinion (Hennig 1939, 1940, 1973a, b).

Based on American fly collections, Curran (1934a, b, 1944) proposed an alternative classification combining Ulidiinae with Otitinae into a single family called Otitidae. Besides Ulidiidae and Otitidae, he also included Pterocallinae, Richardiinae, and Platystomatinae in this family. At this time, the concept of synapomorphic and symplesiomorphic similarities was not yet developed; therefore, observed symplesiomorphic characters had been viewed as the evidence of systematic relationships. Thus, Ulidiinae + Otitinae + Pterocallinae + Richardiinae + Platystomatinae was thought to be a natural group, and the problem of acceptance of either five families or one family with five subfamilies had mostly classification agreement reasons. Steyskal (1961, 1965, 1968, 1977, 1987) modified existing classification by the exclusion of Richardiidae and Platystomatidae, treating them as separate families, that was also supported by McAlpine (1977, 1989). Rohdendorf (1974, 1977), in his system of Diptera, considered the single family Otitidae as including the following four subfamilies: Otitinae, Ulidiinae, Euxestinae, and Pterocallinae. In addition, Evenhuis (1989) determined a single subfamily Lipsaniinae for genus *Lipsana* Enderlein, 1940 (current syn. *Physiphora* of the family Otitidae) from the Australian region. Kameneva and Korneyev (1994) showed that the genera *Seioptera* Kirby, 1817 and *Pseudoseioptera* Stackelberg, 1955 (Otitinae) are phylogenetically close to *Homalocephala* (Ulidiinae) and combined them into the tribe Seiopterini (Ulidiidae: Ulidiinae). The monophyly of the tribe is supported by several morphological characters: the absence of anepisternal bristles and the presence of two pairs of katepisternal bristles, the epandrium expanded in the anterocaudal direction, the distiphallus pubescent or short spinulose in its medial part, no sclerotized preglans or long bristles, and two pairs of spermathecae in females. Therefore, Kameneva and Korneyev (Kameneva and Korneyev, 1994, 2006; Korneyev, 1999) included the rest of the Palaearctic Otitidae into the tribes Cephaliini, Otitini, and Myennidini, whereas the remaining Ulidiidae (without genus *Homalocephala*) were included into the tribes Ulidiini and Pterocallini, while considering all of the tribes within the scope of Ulidiidae. For uniting Ulidiinae and Otitinae into a single family, Kameneva and Korneyev used the following characters: non-developed fronto-orbital plates without strong setae (except in *Chaetopsis* Loew and close genera); absence of presutural supra-alar (except in *Dyscrasis* Hendel and some species of *Otites* Latreille) and katepimeral setae (presutural supra-alar present in the ground plan of most other families; katepimeral setae or setulae present in Platystomatidae, Ctenostylidae, Pyrgotidae, and Tephritidae); slightly bowed continuous Sc vein, neither bent anteriorly at right angle nor constricted before apex (bent anteriorly at right angle in Pyrgotidae Toxurini and most Tephritidae; constricted or broken before apex in Ctenostylidae, most Tephritidae, and some Pyrgotidae); R_1 vein

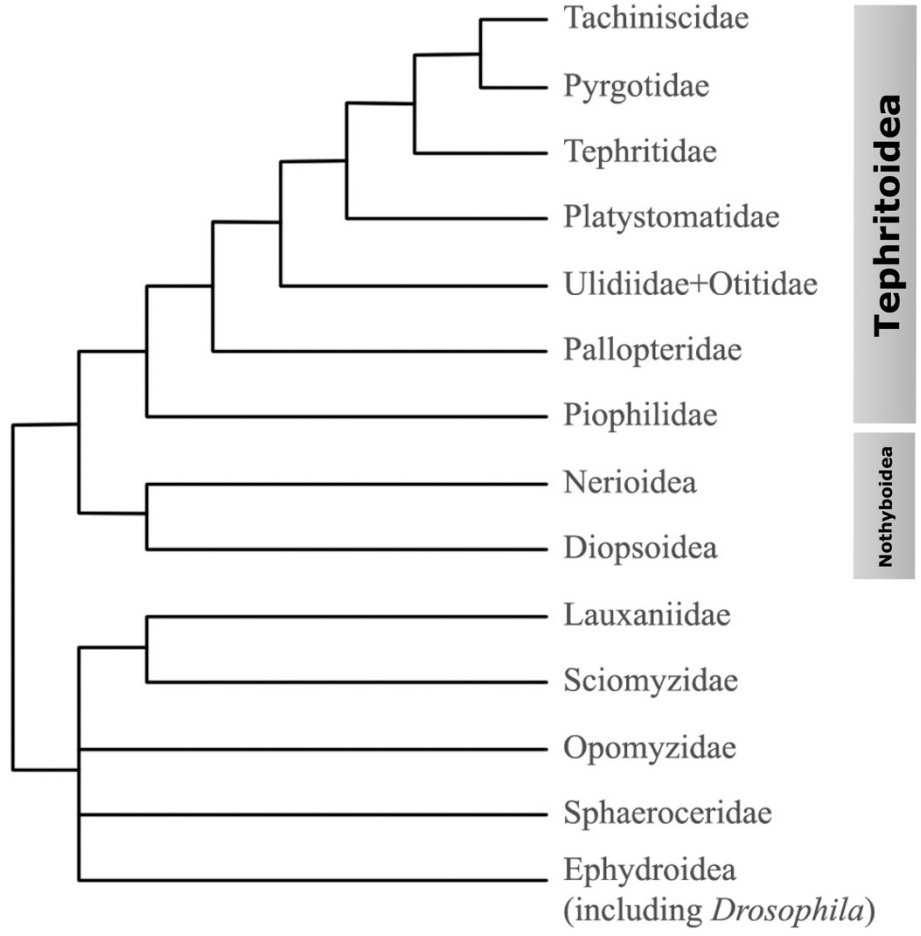

Figure 2 Phylogenetic relationships of Tephritoidea, Nothyboidea, and Ephydroidea flies (based on McAlpine 1981; Gibson et al. 2010; Wiegmann et al. 2011). Outgroups were selected based on current hypotheses of phylogeny: the superfamily Nothyboidea (including Psilidae) form a sister group to the superfamily Tephritoidea (including Ulidiidae). Drosophilidae (superfamily Nothyboidea) is a sister group to Nothyboidea + Tephritoidea.

usually setulose only on apical portion (bare in all Ulidiini and most Lipsanini, and certain species of other tribes sometimes either bare or entirely setulose; bare in most or all Piophilidae, Pallopteridae, Lonchaeidae, and Richardiidae, and entirely setulose in Platystomatidae, Ctenostylidae, Pyrgotidae, and Tephritidae); a basal cubital (bcu) cell with triangular posterodistal lobe, except in *Homalocephala* Zetterstedt and few Pterocallini (absent in lower Tephritoidea, all Platystomatidae and Ctenostylidae, and in few genera of Pyrgotidae and Tephritidae); absence of male tergite 6 (rudimentary in few Lonchaeidae and Pallopteridae); bare male sternite 6 (setulose in lower Tephritoidea); absence of abdominal spiracles 6 and 7 in males (present in many lower Tephritoidea); phallus, when at rest, stored in pouch on ventral aspect of abdomen; apex of the phallus without separated glans (stored on dorsal side underneath tergite 6 with glans in other higher Tephritoidea); bare taeniae of eversible membrane without trichoid sensilla (with trichoid sensilla along whole length in most Piophilidae, Pallopteridae, and Lonchaeidae) and with two ducts of spermathecae; apically bifurcated single duct with two spermathecae, except in Lipsanini (two spermathecae on two ducts) (three spermathecal ducts in ground plan of Pyrgotidae and Tephritidae; two of them bifurcated close to vagina) (Kameneva and Korneyev 2006).

Since this is currently the most thorough and comprehensive taxonomic review of the group, we followed their hypothesis to consider the single family Ulidiidae with two subfamilies Ulidiinae and Otitinae.

Molecular phylogenetic studies of the superfamily Tephritoidea were conducted at the genus, subfamily, and family levels with the use of various molecular markers: 16S rDNA and COI (Zhang et al. 2010), COII (Smith and Bush 1997), 16S rDNA (Han et al. 2006), and 12S, 16S rDNA and COII (Han and Ro 2005, 2009). Often phylogenetic inferences reached agreement with morphological data. Nevertheless, in most cases, a reconstruction of

phylogenetic relationships among species/genera was based on relatively small sample surveys, which do not represent the full taxonomic diversity of the superfamily Tephritoidea. In addition, the phylogenetic schemes derived by different authors, with the implementation of various species samples and sets of molecular markers, make it difficult to compare these results. Importantly, the number of species from the family Ulidiidae included in the studies mentioned above was very small (two to three species, one individual).

In our study, we compared the external morphology and genital structures of Ulidiidae coupled with rigorous molecular phylogenetic analyses, in order to clarify its interspecific relationships. For species identification, we used external and genital morphology; for phylogenetic reconstruction, we performed stable morphological characters and sequence analysis based on a commonly used COI mitochondrial molecular marker. We found justification for the uniting of Ulidiinae and Otitinae into a single family.

Methods

Taxon sampling and morphological analysis

Sampling for subsequent phylogenetic analysis was focused on Ulidiidae flies of the Palaearctic region (Additional file 1). The specimens were identified, labeled, and stored in the fly collection of Zoological Museum at Lomonosov Moscow State University, Moscow, Russia (ZMUM).

For the investigation of external morphology and the anatomy of terminal male and female structures, in total, approximately 6,800 fly specimens belonging to 82 species of 3 genera of the subfamily Ulidiinae and 2,700 fly specimens from 107 species of 12 genera of the subfamily Otitinae were analyzed from museum collections. Morphological characters were examined and visualized under a stereomicroscope Stemi SV11 Apo Carl Zeiss (Oberkochen, Germany) and microscope Olympus CX41 (Shinjuku, Tokyo, Japan). The male and female terminalia were dissected out, treated with 10% KOH solution at approximately 80°C for several minutes, and observed in a droplet of glycerol under a compound light microscope. Photographic series were created with the use of CombineZM software (Hadley 2007).

This study is based mainly on the material deposited in the Zoological Institute of the Russian Academy of Sciences, Saint-Petersburg, Russia (ZISP), the Zoological Museum at Lomonosov Moscow State University, Moscow, Russia (ZMUM), the Hungarian Natural History Museum, Budapest, Hungary (HNHM), the Museum of Natural History at Humboldt University in Berlin, Germany (ZMHB), and Naturhistorisches Museum Wien, Austria (NHMW).

Morphological terminology generally follows McAlpine (1981) and Nartshuk (2003) (Additional file 2). Abbreviations of veins and setae follow White et al. (1999).

DNA analysis - extraction, amplification, and sequencing

The mitochondrial cytochrome *c* oxidase (COI) gene barcodes were successfully obtained for 69 specimens. Three genera of Ulidiinae (*Physiphora* (two species), *Timia* (four species), *Ulidia* (two species)), eight genera of Otitinae (*Ceroxys* (six species), *Herina* (two species), *Melieria* (four species), *Myennis* (one species), *Otites* (three species), *Pseudotephritis* (one species), *Seioptera* (one species), *Tetanops* (one species)), the genus *Homalocephala* (four species), and *Psila fimetaria* (L., 1761) (Psilidae) as an outgroup were included in a barcode analysis. Polymerase chain reaction (PCR) amplification and DNA sequencing were performed at the Canadian Centre for DNA Barcoding following standard throughput methods. The specimen information, vouchers, sample distribution, photographs. and GenBank accession numbers can be assessed through the Barcode of Life Data System (BOLD; http://www.barcodinglife.com) (Ratnasingham and Hebert 2007) in the publically available project 'TGSPA-Schizophora of the Palaearctic.' In order to broaden the sample space of the group Ulidiidae, we additionally obtained five COI barcodes of *Physiphora clausa* Macquart, 1843, *Timia monticola* Becker, 1906, *Timia* (*Empyelocera*) *libani* Gregor, 1970, *Ulidia megacephala* Loew, 1845, and *Ulidia ruficeps* Becker 1913 [GenBank:KC663633-KC663637]. DNA was extracted from whole specimens following the manufacturer's protocol of the DIAtomTM DNA Prep 100 kit (Isogene, Russia). PCR amplification was performed using LCO1490 (forward, 5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (reverse, 5′-TAAACTTCAGGGTGACC AAAAAATCA-3′) primers (Folmer et al. 1994). The following are the conditions: PCR conditions - hot start 94°C for 5 min; pre-PCR 5 cycles - denaturation at 94°C for 1 min, annealing at 45°C for 1 min 30 s, elongation at 72°C for 1 min 30 s; and 35 cycles - denaturation at 94°C for 1 min, annealing at 50°C for 1 min 30 s, elongation at 72°C for 1 min. Amplification products were run on 1% TBE agarose gel (Sigma-Aldrich, St. Louis, MO, USA). PCR products were extracted from an agarose gel via the manufacturer's protocol of the JETQUICK Gel Extraction Spin Kit 250 (GENOMED GmbH, Löhne, Germany). Sequencing was performed using a sequenator ABI PRISM 310 with reagents from an Applera kit (Norwalk, CT, USA). Contigs were made using CodonCode Aligner Ver3.7.1.1 (CodonCode Co.).

Sequence data analysis

The COI sequence alignment was produced using MAFFT v. 6.864b (Katoh et al. 2002) with the 'auto'

option, which enables the user to automatically select an appropriate strategy among speed-oriented and accuracy-oriented methods. We included *P. fimetaria* (Psilidae) and four species of *Drosophila* Fallen, 1823 (derived from [GenBank:HQ979116.1, HQ979110.1, EU493633.1, JN019869.1]) as outgroups. They were selected based on current hypotheses of phylogeny: the superfamily Nothyboidea (including Psilidae) form a sister group to the superfamily Tephritoidea (including Ulidiidae) (McAlpine 1981; Gibson et al. 2010). Nothyboidea (Hennig 1958, 1971, 1973a, b) (=Diopsoidea (McAlpine 1989) = Tanypezoidea (Pape et al. 2011)) includes families Tanypezidae, Strongylophthalmyiidae, Psilidae, Nothybidae, Megamerinidae, Syringogastridae, and Diopsidae, implying that the family Psilidae (superfamily Nothyboidea) has sister relationships with Tephritoidea. Belonging to the superfamily Ephydroidea, Drosophilidae represents a phylogenetically more progressive lineage relative to the basal Nothyboidea and Tephritoidea. Thus, Drosophilidae (superfamily Nothyboidea) is a sister group to Nothyboidea + Tephritoidea (Figure 2) (Rohdendorf 1974; McAlpine 1981; Hennig 1958; Wiegmann et al. 2011).

The substitution model of DNA sequence evolution was chosen among 88 competing models using a model test implemented in the IQ-Tree v. 0.9.4 software (http://www.cibiv.at/software/iqtree) (Minh et al. 2013) according to the model log-likelihood values. The TVM model, with a rate variation among sites following a gamma distribution (G), was selected according to the highest log-likelihood of −7706.4766. This model was used to construct a maximum likelihood (ML) tree. To do this, we used the IQ-Tree v. 0.9.4 software. In order to obtain branch support values, 1,000 bootstrap replicates were performed in the same software utilizing the chosen evolutionary model. In addition, a replicated Bayesian analysis coupled with Markov chain Monte Carlo (MCMC) simulations starting from an initial random tree was performed in MrBayes v 3.2.1. (http://mrbayes.sourceforge.net/) (Ronquist and Huelsenbeck 2003) for four million generations in two independent runs with a sampling frequency every 500 generations. All trees that were sampled before the two runs reached a value of standard deviation less than 0.015 were discarded as burn-in. The resulting trees were summarized to a consensus, and posterior probabilities (PP) as branch statistical support were then calculated. Since the ML and Bayesian trees agreed on one topology, we drew the bootstrap support values (in %) from 1,000 bootstrap trees onto the Bayesian tree using the IQ-Tree software, thus combining bootstrap support (BS) with posterior probability values.

In addition to a typical phylogenetic analysis based on common models of nucleotide substitutions in DNA sequence, we reconstructed phylogenetic relationships utilizing a model of codon evolution. Markov models of codon substitution have emerged as a powerful method modeling biologically more realistic scenarios for protein-coding sequences (Gil et al. 2013). The most important advantages of codon models over DNA models are being achieved by naturally incorporating selection regimes acting on protein and the structure of the genetic code as relevant model parameters. For further phylogenetic inference, the YAP parametric codon model was *a priori* selected, reasoning that estimation of the non-synonymous to synonymous substitution ratio (ω) is not affected by base composition at each codon compared to other codon models, namely GY and MG (for details, see Yap et al. (2010)). The selective variability (distribution of ω ratios) among codon sites was modeled using the model M3 with three discrete site classes (Yang et al. 2000). Empirical codon frequencies were estimated under CFx4 option, which enables correction for absence of stop codons (Kosakovsky Pond et al. 2010). Then, ten initial trees generated by the BioNJ method corresponding to ten random starts were estimated. Optimization of each tree topology was carried out using simultaneously two ML heuristic tree search algorithms - nearest neighbor interchange (NNI) and subtree pruning and regrafting (SPR). The most likely tree was obtained with SPR moves (log-likelihood = −6781.1666). The branch support was assessed by the Bayesian-like modification of the approximate likelihood ratio test (aBayes) (Anisimova et al. 2011). aBayes was defined to be the most powerful among other branch support methods based on an approximate likelihood ratio test. Although aBayes supports tend to be very similar to PP supports, the aBayes method exhibits more conservative behavior and robustness to model violations (Anisimova et al. 2011). All of the above procedures and codon ML tree construction were implemented in CodonPhyML Ver1.0 (http://sourceforge.net/projects/codonphyml/) (Gil et al. 2013).

For the barcoding data, the distance matrix was produced under the Kimura two-parameter (K2P) (Kimura 1980) model of base substitution with uniform rates using MEGA 5 (Tamura et al. 2011). In addition, we calculated uncorrected *p*-distances as an alternative distance metric that may be preferable to K2P for short, closely related sequences. The intraspecific variation and interspecific divergence levels were investigated by comparing pairwise K2P distances within and between fly species.

Saturation testing was carried out in DAMBE5 (http://dambe.bio.uottawa.ca/dambe.asp) (Xia 2013). All statistical analyses were implemented in R.

Results

Sequence details and nucleotide composition

The alignment of 78 COI sequences of unequal lengths (min = 246 bp, max = 701 bp), including 4 *P. fimetaria*

and 4 *Drosophila* outgroup sequences, contained 704 positions without gaps; 269 sites were variable, 251 of which were identified as parsimony-informative. Although short COI sequences (<600 bp) may not provide sufficient information for phylogenetic reconstruction, short barcode sequences (<150 bp, mini-barcodes) have been reported to provide an efficient taxonomic signal for species discrimination (Meusnier et al. 2008). In their survey, a correct species identification rate of 95% was achieved with 250-bp barcodes. Estimated average base frequencies for the barcode region (all sites) in our dataset were A 28.53%, C 19.84%, G 17.69%, T 33.94% ($\chi^2 = 38.82$, $df = 3$, $P = 1.896e-08$; Additional file 3). As expected, the A/T bias (79.35%) was significant for the third codon positions of the sequence ($\chi^2 = 223.03$, $df = 1$, $P < 2.2e-16$) and considerably greater than at first (50.05%) and second (58.03%) positions ($\chi^2 = 51.69$, $df = 2$, $P = 5.972e-12$).

Barcoding gap

The K2P model has been recently criticized as being inappropriate in some cases for divergence estimation and having no evident advantages over uncorrected *p*-distance (Srivathsan and Meier 2012). Indeed, a comparison of K2P with *p*-distances in our dataset showed high correlation between both distance metrics (Pearson correlation = 0.9980, $P < 2.2e-16$; Additional file 4). However, for barcoding gap assessment, we used the K2P distance, which is widely accepted in DNA barcode literature. The interspecific genetic K2P distances averaged at 0.1483 (SD = 0.0262, $n = 2356$), while conspecific distances averaged at 0.0078 (SD = 0.0154, $n = 59$). An apparent non-significant (Wilcoxon rank-sum test, $W = 100.50$, $P < 2.2e-16$) overlap was found between intraspecific and interspecific distributions of K2P distances. Interspecific distance values between *Homalocephala albitarsis* Zetterstedt, 1838 and *Homalocephala biumbrata* (Wahlberg, 1839) created the overlap with intraspecific K2P distribution (Figure 3, first asterisk). These two species are morphologically close based on the following characters: face, parafacials, and genae are white; katepisternum is silvery pubescent and with long bright setae on its inner margin. However, they are currently considered as independent species (Andersson 1991) based on median wing spot size, apical wing spot color intensity, and a surface structure of scutellum. Therefore, the low value of K2P distance (0.0109) occurs most likely due to a recent split between these two species. Due to the high intraspecific sequence variation between *Melieria cana* (Loew, 1858), two K2P distances (Figure 3, second asterisk) formed an overlap with the overall K2P divergence distribution. Interestingly, left-tail distances located on the 0.03 to 0.08 interval of the interspecific K2P distribution were generated mostly by pairwise comparisons between species within the *Melieria* R.-D., 1830 genus. This observation may indicate that species within the genus *Melieria* are currently undergoing speciation events. To rule out the possibility that K2P distances may be affected by sequence length parameter, we compared the distribution of K2P within short (<400 bp) and long (>400 bp) sequences. No significant differences were observed (Wilcoxon rank-sum test, $W = 713{,}950.50$, $P = 0.06$; Additional file 5).

Phylogenetic analysis

To test whether our sequence dataset possesses the phylogenetic signal necessary for tree reconstruction, we performed Xia's substitution saturation method based on the concept of Shannon entropy (Xia et al. 2003) implemented in DAMBE5 (Xia 2013). The empirical proportion of invariant sites ($P_{inv} = 0.2$) was estimated from the data. Xia's test for fully resolved sites, with the replicate number of 1,000 computed the index of substitution saturation ($I_{ss} = 0.205$) for 32 operational taxonomic units, was substantially smaller than the critical index of substitution saturation ($I_{ss.c} = 0.684$ assuming a symmetrical topology and $I_{ss.c} = 0.362$ assuming an asymmetrical topology). In addition, the rates of transitions and transversions increased approximately linearly with the K2P genetic distance (Figure 4, Additional file 6), with transitions being higher than transversions (mean $\kappa = 1.37$, Wilcoxon one-tailed rank-sum test, $W = 7{,}602{,}731$, $P < 2.2e-16$), which is expected for closely related species (Ong et al. 2011). Overall, saturation tests revealed no significant substitution saturation in our data, implying validity for phylogenetic reconstruction.

Both ML and Bayesian trees showed identical topologies for each clade (Figure 5A,B). The monophyly of the group Ulidiinae + Otitinae was strongly supported (PP = 1.00, BS = 95), and most of the individual species branches within it were also well supported (PP > 0.95, BS > 75). The clade *Physiphora* Fallén, 1810 was sufficiently supported by ML (BS = 88) but not by Bayesian analysis (PP = 0.88). The monophyly of the group *Timia* Wiedemann 1824 + *Ulidia* Meigen, 1826 was not well supported by Bayesian analysis (PP = 0.90), and not by ML (BS = 34). The clade Otitinae was not recovered as monophyletic by Bayesian analysis (PP = 0.45) nor by ML (BS = 8).

The codon ML analysis resulted in an altered topology (Figure 6A,B). Interestingly, however, the codon ML tree showed clustering patterns that reach an agreement with phylogeny corroborated by morphological observations. Overall, major clades on the codon ML tree generally received higher branch supports than on ML and Bayesian trees. We put a threshold value of 0.90 for aBayes support to be sufficient for recovering significant branches, as it is a more conservative metric than PP (Anisimova et al. 2011). The monophyly of the group Ulidiinae + Otitinae was strongly supported (aBayes = 1.00). The

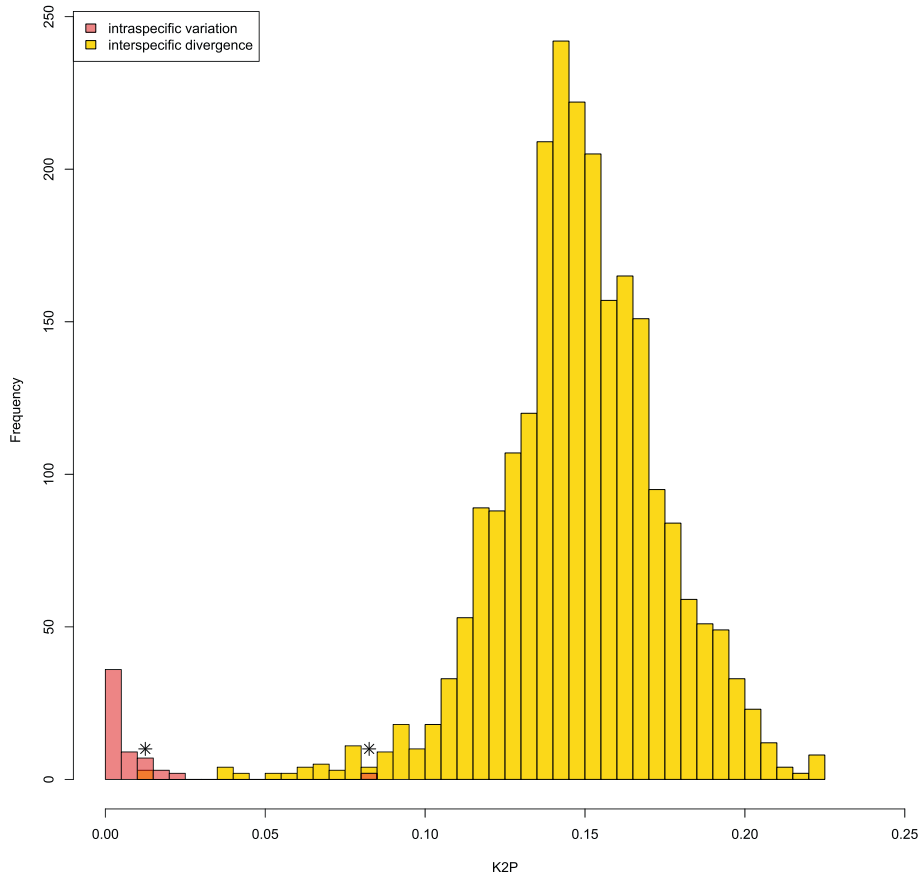

Figure 3 Pairwise intra- and interspecific (congeneric) comparisons of genetic K2P distances between COI sequences of different fly species. The first asterisk represents an overlap with intraspecific distribution generated by pairwise comparisons between *Homalocephala albitarsis* and *Homalocephala biumbrata*. The second asterisk represents overlap with interspecific distribution generated by pairwise comparisons within *Melieria cana*.

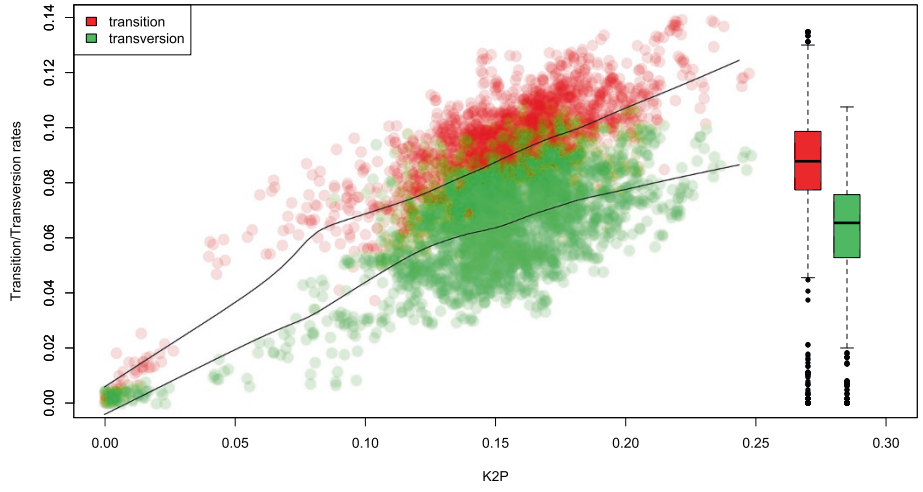

Figure 4 Transition/transversion rate distributions with statistical jittering (random noise ~ Unif ($a = 0$, $b = 0.005$)). Transition/transversion rates are approximately linearly increasing with divergence (K2P). Black lines represent a LOWESS fit.

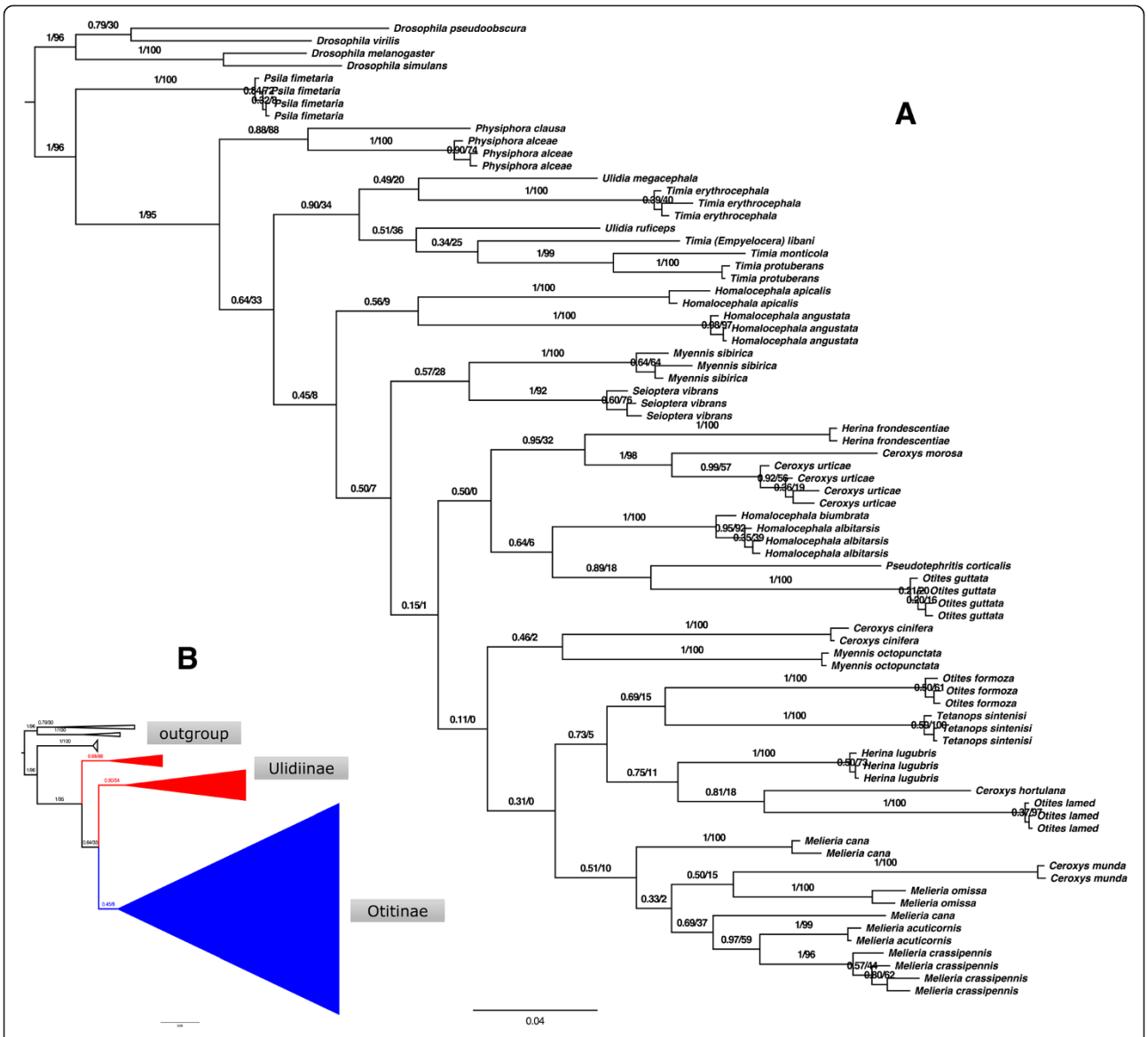

Figure 5 ML and Bayesian analyses. (A) Combined tree of ML and Bayesian analyses. Branches are labeled with Bayesian posterior probabilities (PP) and bootstrap support (BS). Significant support values range from PP = 1 and BS = 100 to PP ≤ 0.95 and BS ≤ 75 (all supports are shown). **(B)** The cartoon Bayesian/ML tree represents high-level relationships between Ulidiinae (red) and Otitinae (blue).

paraphyletic relationship of the clade *Physiphora* to the group Ulidiinae (excluding *Physiphora*) + Otitinae was weakly supported (aBayes = 0.60). Within significant, the monophyletic (aBayes = 0.91) group *Ulidia* + *Timia*, *Ulidia* and some species of *Timia* formed a highly supported cluster (aBayes = 0.98). Although the branch Otitinae was also not significant on the codon ML tree, it received higher aBayes support (0.58) than original Bayesian PP (0.45). It is important to note that within Otitinae, *Homalocephala* species combined into a single cluster, but with low support (aBayes = 0.57). The genus *Melieria* received a very high aBayes support (0.98) implying its monophyly. Deeper branches mostly recovered with high supports (aBayes > 0.9) as on ML and Bayesian trees.

Morphological analysis

We examined external morphology of approximately 6,800 fly specimens belonging to 82 species of 3 genera in the subfamily Ulidiinae and 2,700 fly specimens from 107 species of 12 genera in the subfamily Otitinae. Based on this analysis, we confirmed morphological characters that are able to separate all studied genera. In particular, the genital structures (surstyles, phallus, and phallus apodeme) were shown to be the most useful

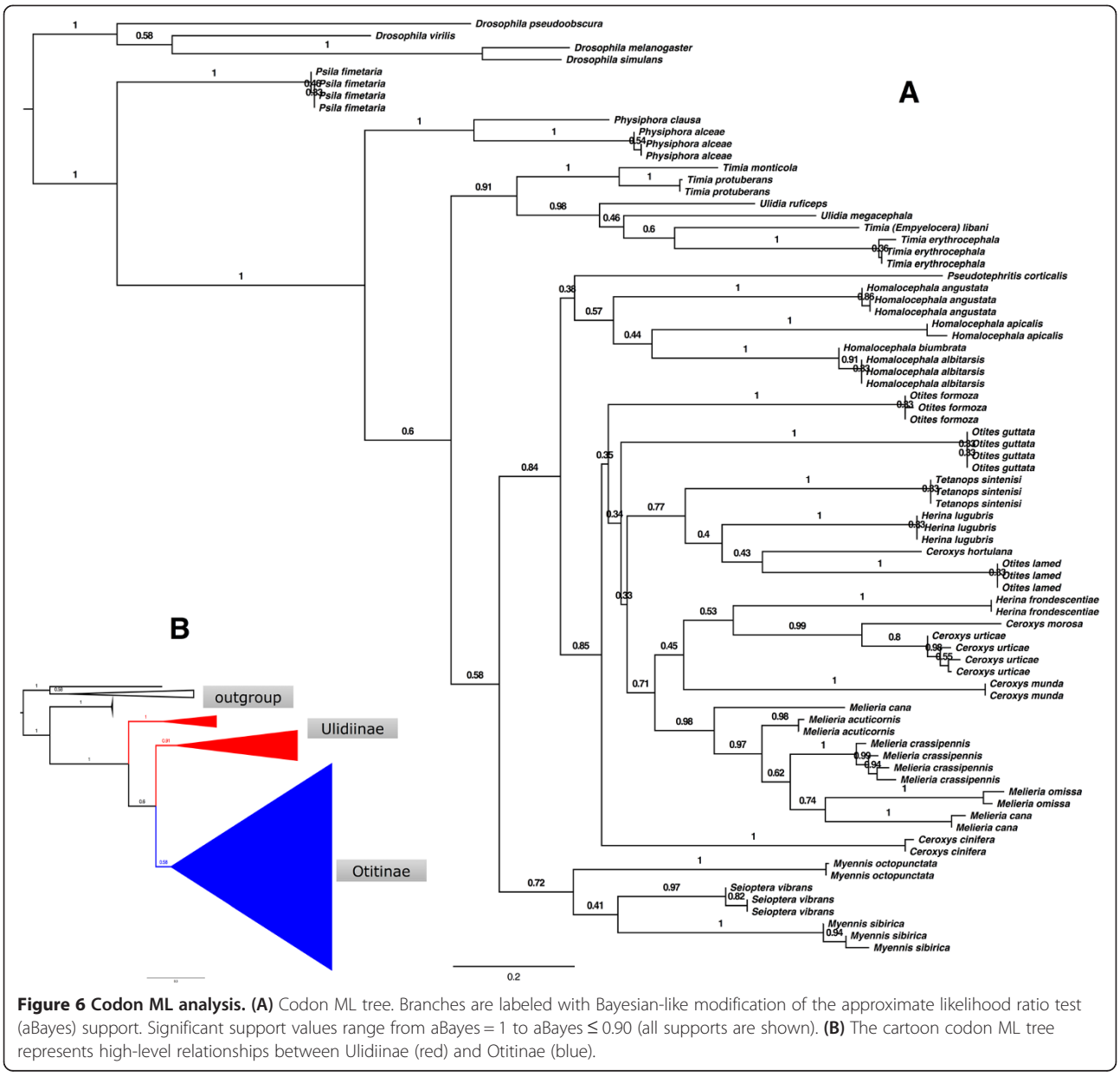

Figure 6 Codon ML analysis. (A) Codon ML tree. Branches are labeled with Bayesian-like modification of the approximate likelihood ratio test (aBayes) support. Significant support values range from aBayes = 1 to aBayes ≤ 0.90 (all supports are shown). **(B)** The cartoon codon ML tree represents high-level relationships between Ulidiinae (red) and Otitinae (blue).

for distinguishing species. We moved the results of the morphological analysis to the 'Discussion' section for easier perception.

Discussion

Our phylogenetic analysis suggests the existence of three major clusters - *Physiphora* (Ulidiinae), *Timia* + *Ulidia* (Ulidiinae), and Otitinae + *Homalocephala* (Figures 4 and 5B). Inclusion of *Homalocephala* into Otitinae coincides with Hennig's classification [1], whom previously considered it within Ulidiidae. The subfamily Ulidiinae (*Physiphora* + (*Timia* + *Ulidia*)) forms a basal paraphyletic group (BS = 33, PP = 0.64, aBayes = 0.60) nested between *P. fimetaria* and Otitinae. Nevertheless, morphologically these genera are similar in having a bare vein R_1, as well as in genital structures (non-rupted closed hypandrium, well-developed parameres, a bare distiphallus (rarely with a group of setae in the center of distifallus) with membranous lobes, and a phallus apodeme with lateral lobes). Thus, there might be symplesiomorphic similarity. The subfamily Otitinae occupies the apical position on all of the phylogenetic trees (Figures 5 and 6). Despite this, the lineage was not statistically recovered as a monophyletic group, with low support values (BS = 8, PP = 0.45, aBayes = 0.58). Morphologically, the monophyly of Otitinae (without *Homalocephala*) was supported by a spinulose phallus, absence of the glans, absence of membranous lobes on the distiphallus and by the presence of

the female sternal apodemes. We assume these characters to be an apomorphic similarity. Interestingly, the *Homalocephala* genus takes intermediate position between Ulidiinae Otitinae, which can be corroborated by previous studies (including Hennig 1973a, b). Recovery of basal and subbasal positions of the *Seioptera* species within Otitinae is partially supporting the hypothesis of Kameneva and Korneyev (1994), which suggests inclusion of the *Homalocephala* and *Seioptera* genera into the Seiopterini tribe, however, within Ulidiinae. Our results confirm the uniting of Ulidiinae and Otitinae into a single family.

Physiphora (mostly the Afrotropical genus, 24 species) branches out as a monophyletic clade with relatively high support (BS = 88, PP = 0.88, aBayes = 1.00). This observation is in agreement with morphological data about the genus. All *Physiphora* species possess a very similar structure of exoskeleton and genital characters: *Physiphora* always have transparent wings (Figure 7X,Y); spermathecae covered by small papillae, the shape of the phallus tip (glans) in combination with a bare distiphallus with membranous lobes; and surstyles without prensisetae (Figure 8W,X). The genus *Physiphora* is distributed mostly in the Old World, but includes several cosmopolitan species with the worldwide area. Thus, early divergence might have allowed *Physiphora* species to spread across such a vast range.

Ulidia (Palearctic genus, 21 species) and *Timia* (Palearctic genus, 59 species) most likely form a monophyletic (BS = 34, PP = 0.90, aBayes = 0.91) cluster, although these two genera do not create particular clusters within it. The morphological data that closely relates these two genera, the overall external characters and genital structures and having little or no hiatus, firmly supports the phylogenetic inference. Group specialists often encounter issues in separation between *Ulidia* and *Timia*, so the possible merging of these genera has been suggested. The differences used for separating *Ulidia* and *Timia* so far are mainly the following: the frons is smooth (in *Timia*) versus dimpled (in *Ulidia*) (with some exceptions); the head and thorax are microtrichose (in *Timia*) versus bare (in *Ulidia*, but some assigned to *Timia* have a shiny head and thorax, and the frons of *Ulidia metope* Kameneva, 2010 is widely microtrichose) (Chen and Kameneva 2009; Kameneva 2010). The monophyletic status of the group *Timia* + *Ulidia* is also confirmed by the distribution of the species with a shared ecological niche - the majority of *Ulidia* and *Timia* occur in arid and semiarid areas of the Palaearctic. Based on these data, it is more likely that the formal characters defined *Ulidia* and *Timia*, unambiguously identifying just a fraction of species in each genus. Likewise, the subgenera *Timia* and *Empyelocera* in the genus *Timia* seem to be unnatural taxa, which is only differentiated by width of parafacial area (Zaitzev 1982). This assumption is congruent with our hypothesis that the genera *Ulidia* and *Timia* have recently diverged and are currently on a course of differentiation. Within these groups, typical representatives as well as transitional forms can be found. The molecular phylogenetic reconstruction does not exclude a more complicated scenario such as *Ulidia* and *Timia* not being natural groups but forming a cluster of several subgroups connected with multiple transitions. *Timia* have transparent wings, some species have dark basal costal (bc), costal (c), and subcostal (sc) cells and with apical wing spot. A basal bcu cell has a well-developed posteroapical projection, whose length has an identification value (Figure 7Z, A1,A2,A3). Male surstyles do not possess strong prensisetae, occasionally with setae on the inner lobe (Figure 8Y,Z, A1,A2). The genus *Ulidia* possesses a poorly developed wing pattern, the species *Ulidia megacephala*, *Ulidia melampodia* Loew, 1873, *Ulidia salonikiensis* Hennig, 1940, *Ulidia kandybinae* Zaitzev, 1982, *Ulidia splendida* Zaitzev, 1982, *Ulidia nigricubitalis* Zaitzev, 1982, and *Ulidia transcaspica* Galinskaya, 2011 exhibit the greatest development of the character: dark costal, subcostal, and (sometimes) bcu cells and an apical wing spot (Figure 7A4,A5). *Ulidia* possess three smooth spermathecae with two of them having a collective sperm duct. Male genitalia has the shape of the phallus tip (glans) in combination with a bare distiphallus (rarely with a group of setae in the center of the distiphallus) and distiphallus with membranous lobes. Male surstyles do not possess strong prensisetae, occasionally with setae on the inner lobe (Figure 8A3,A4). It is important to note that within *Timia*, we found three species (*Timia albifacies* Gorodkov and Zaitzev, 1986, *Timia gussakovskyi* Gorodkov and Zaitzev, 1986, and *Timia komarowi* Mik, 1889) that have the distiphallus in the middle part with an area densely covered by relatively strong setae. We hypothesize that setae on the distiphallus of *Timia* and Otitinae emerged in parallel, possibly having unique origins.

Homalocephala species (nine species total: four Holarctic, four Palaearctic, and one Nearctic) have always been included into the clade Otitinae with different, lowly supported clustering patterns on the Bayesian/ML tree (Figure 5A) and codon ML tree (Figure 6A). On the codon ML tree, the *Homalocephala* cluster likely exhibits sister relationships (aBayes = 0.84) with the *Otites* + *Tetanops* + *Herina* + *Ceroxys* + *Melieria* group (aBayes = 0.85). *Homalocephala* species possess the following relevant morphological characters: no glans on the distiphallus, two katepisternal setae, and four spherical spermathecae. This relates these species to the *Seioptera* and *Pseudoseioptera* genera, whereas having vein R_1 covered with setulae along its full length relates *Homalocephala* to *Pseudoseioptera*. We notice that *Homalocephala* and *Seioptera* genera were placed basally or subbasally relatively to Otitinae (Figures 5A and 6A). Species of *Homalocephala* possess

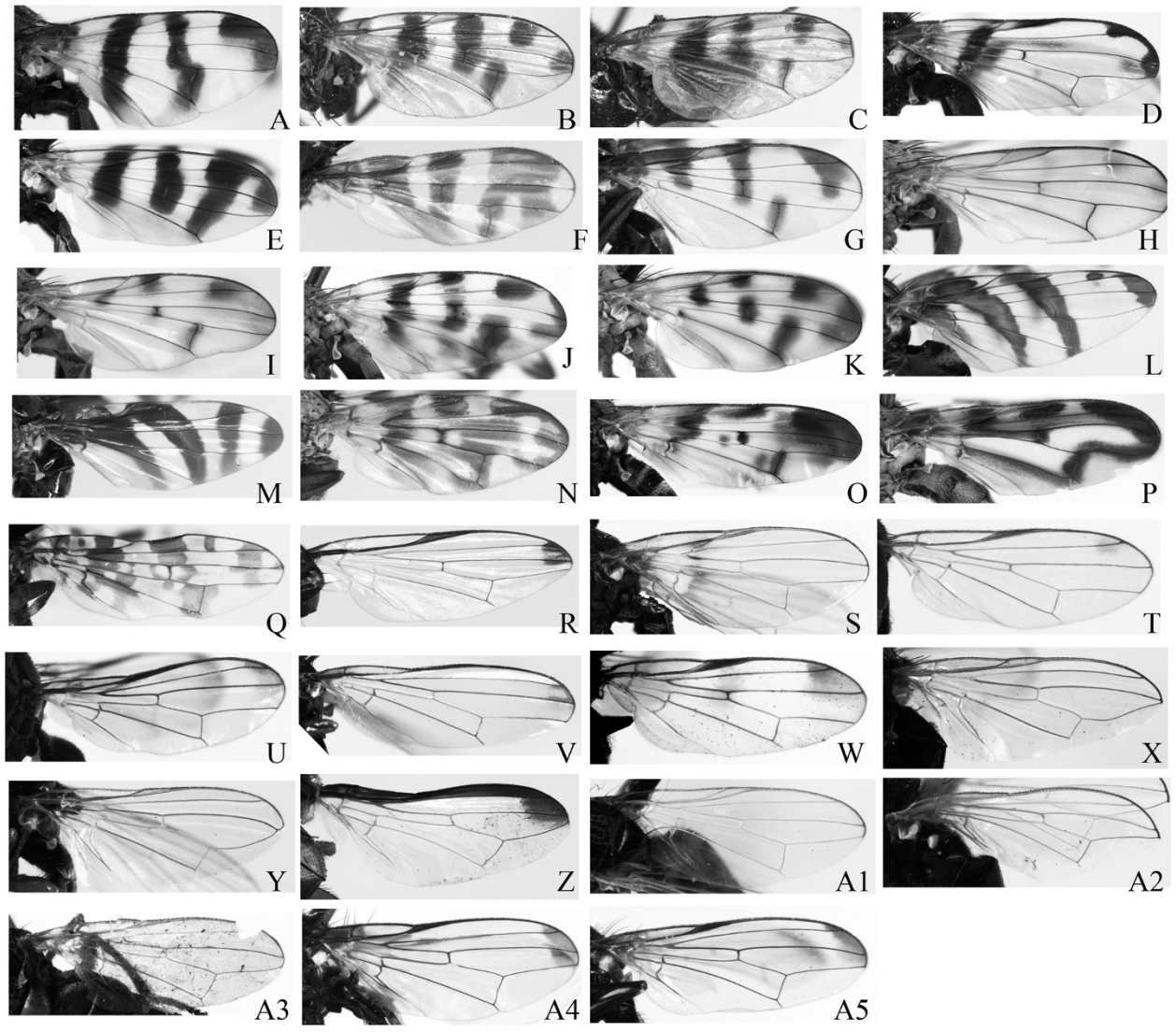

Figure 7 Wings of Ulidiidae species. Otitinae: **(A)** *Ceroxys cinifera*, **(B)** *Ceroxys hortulana*, **(C)** *Ceroxys morosa*, **(D)** *Ceroxys munda*, **(E)** *Ceroxys urticae*, **(F)** *Herina frondescentiae*, **(G)** *Herina lugubris*, **(H)** *Melieria acuticornis*, **(I)** *Melieria cana*, **(J)** *Melieria crassipennis*, **(K)** *Melieria omissa*, **(L)** *Myennis octopunctata*, **(M)** *Myennis sibirica*, **(N)** *Otites centralis*, **(O)** *Otites formosa*, **(P)** *Otites lamed*, **(Q)** *Pseudotephritis corticalis*, **(R)** *Seioptera vibrans*, and **(S)** *Tetanops sintenisi. Homalocephala*: **(T)** *Homalocephala albitarsis*, **(U)** *Homalocephala angustata*, **(V)** *Homalocephala apicalis*, and **(W)** *Homalocephala biumbrata*. Ulidiinae: **(X)** *Physiphora alceae*, **(Y)** *Physiphora clausa*, **(Z)** *Timia erythrocephala*, **(A1)** *Timia* (*Empyelocera*) *libani*, **(A2)** *Timia monticola*, **(A3)** *Timia protuberans*, **(A4)** *Ulidia megacephala*, and **(A5)** *Ulidia ruficeps*.

transparent wings, occasionally with apical spots (on apexes of veins R_{2+3} and R_{4+5}) or with medial spots occupying pterostigma and sometimes extending downward. The bcu cell is enclosed with arcuate vein without breaking (Figure 7T,U,V,W). Male surstyles possess figured prensisetae (Figure 8S,T,U,V) that are typical only of genera *Homalocephala*, *Pseudoseioptera*, and *Ceroxys* and are probably the result of parallel origin or convergent evolution. Interestingly, the areal of *Homalocephala* partially coincide with the areal of *Pseudoseioptera* inhabiting Holarctic temperate forests; moreover, both of their larvae live under the bark of falling trees. The position of this genus is disputed, with differerent scientists including it in Otitidae, Ulidiidae, or Pterocallidae (Kameneva and Korneyev 1994; Hendel 1910a, b, 1928; Stackelberg 1933; Hennig 1939, 1940; Richter 1970a, b; Soós 1984; Zaitzev 1984; Krivosheina and Zaitzev 1989; Greve 1998; Nartshuk 2003; Curran 1934a, b; Steyskal 1965, 1977; McAlpine 1977, 1989; Rohdendorf 1977; Evenhuis 1989; Frey 1964) (Figure 1). Based on phylogenetic inferences about the genus, *Homalocephala* (Figures 5A and 6A) tend to be placed within the Otitinae clade, though the taxonomic status of the genus needs to be extensively revised. Morphologically, the *Homolocephala* lineage

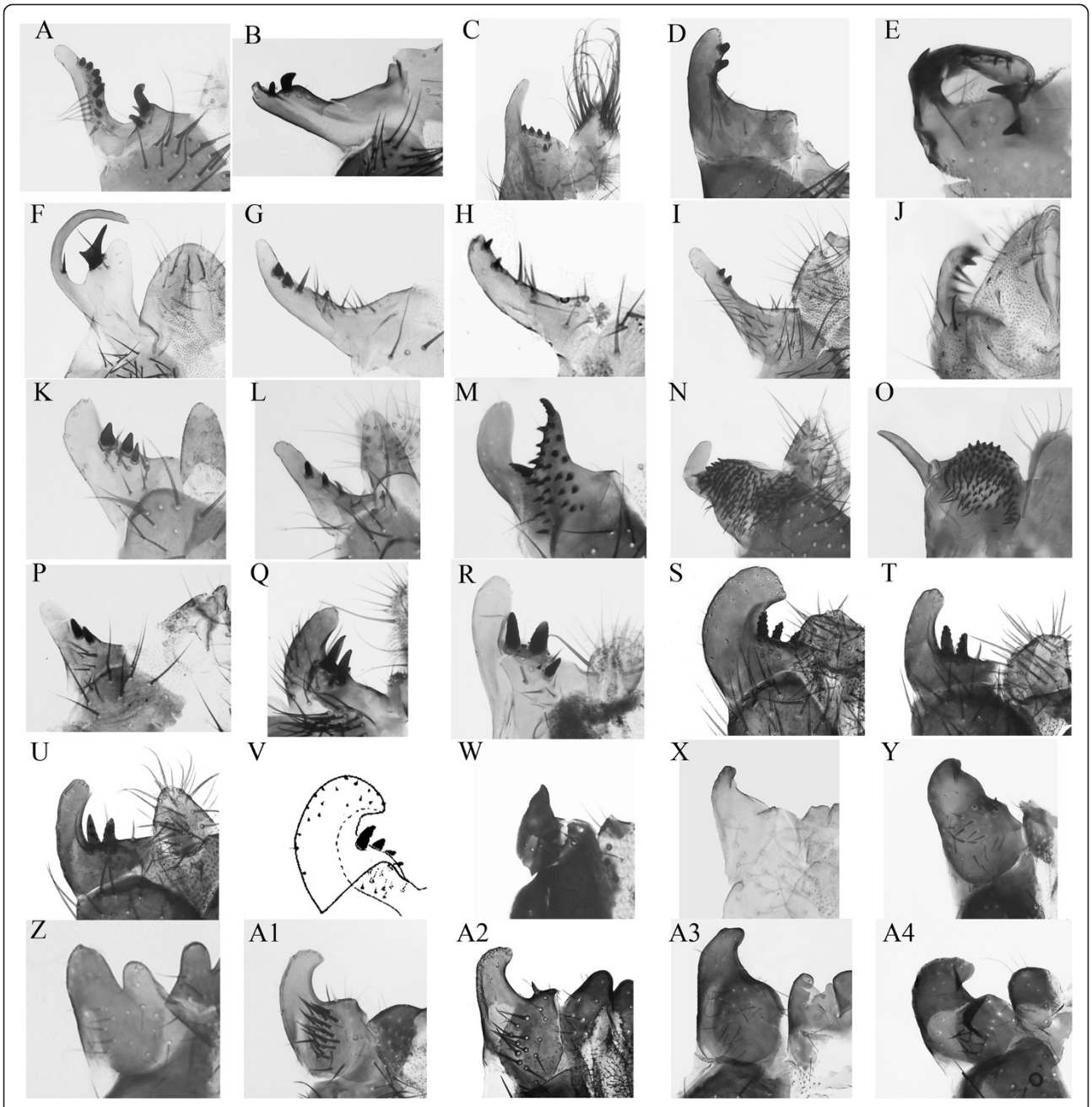

Figure 8 Surstyles in male genitalia of Ulidiidae species. Otitinae: **(A)** *Ceroxys cinifera*, **(B)** *Ceroxys hortulana*, **(C)** *Ceroxys munda*, **(D)** *Ceroxys urticae*, **(E)** *Herina frondescentiae*, **(F)** *Herina lugubris*, **(G)** *Melieria acuticornis*, **(H)** *Melieria cana*, **(I)** *Melieria crassipennis*, **(J)** *Melieria omissa*, **(K)** *Myennis octopunctata*, **(L)** *Myennis sibirica*, **(M)** *Otites centralis*, **(N)** *Otites formosa*, **(O)** *Otites lamed*, **(P)** *Pseudotephritis corticalis*, **(Q)** *Seioptera vibrans*, and **(R)** *Tetanops sintenisi. Homalocephala*: **(S)** *Homalocephala albitarsis*, **(T)** *Homalocephala angustata*, **(U)** *Homalocephala apicalis*, and **(V)** *Homalocephala biumbrata*. Ulidiinae: **(W)** *Physiphora alceae*, **(X)** *Physiphora clausa*, **(Y)** *Timia erythrocephala*, **(Z)** *Timia* (*Empyelocera*) *libani*, **(A1)** *Timia monticola*, **(A2)** *Timia protuberans*, **(A3)** *Ulidia megacephala*, and **(A4)** *Ulidia ruficeps*.

remains unresolved too, as most of the characters examined are plesiomorphic and cannot reliably relate it to either Otitinae or Ulidiinae.

Melieria contains 38 species: 2 Holarctic, 4 Nearctic, and 29 Palaearctic. In our study, *Melieria omissa* (Meigen, 1826), *Melieria cana*, *Melieria acuticornis* (Loew, 1854), and *Melieria crassispennis* (Fabricius, 1794) exhibited a tendency to form a single cluster with an apical topology (Figures 5A and 6A). While the codon ML tree gave the genus high branch support (aBayes = 0.98), the Bayesian/ML tree ambiguously clustered it with *Ceroxys munda* (BS = 10, PP = 0.51). Interestingly, *Melieria* shows maximal

levels of intraspecific diversity, whereas interspecific comparisons within the genus attain the minimal distances. Perhaps, these results suggest deep conspecific diversity of *M. cana*, which in turn may indicate a current process of speciation. Species of *Melieria* possess an R_1 vein with setulae in its apical portion and a bcu cell with very short posteroapical extension (Figure 7H,I,J,K). The male distiphallus is covered by long bristles, lacks membranous lobes, and lacks a glans; it also has surstyles with two to five prensisetae (Figure 8G,H,I,J).

The single representative of *Tetanops* Fallén, 1820 (Holarctic genus with eight Nearctic and six Palaearctic species) - *Tetanops sintenisi* Becker, 1909 - was placed with *Otites formosa* on the Bayesian/ML tree with weak support (BS = 15, PP = 0.69), but on the codon ML tree, it was placed within the lowly supported clade containing *Herina lugubris*, *Ceroxys hortulana*, and *Otites lamed* (aBayes = 0.77). *T. sintenisi* possesses an R_1 vein with setulae and a bcu cell with short posteroapical extension (Figure 7S). The male distiphallus is covered by long strong bristles and lacks membranous lobes and a glans; it has surstyles with two to eight prensisetae on the medial lobe (Figure 8R).

Herina R.-D., 1830 has 31 species: 19 Palaearctic species, 4 Nearctic, 6 Oriental, and 2 Neotropic. *Herina lugubris* (Meigen, 1826) and *Herina frondescentiae* (L., 1758) were differently clustered with low branch support values (Figures 4A and 5A). Species of *Herina* possess an R_1 vein with setulae and a bcu cell with short, often indistinct posteroapical extension (Figure 7F,G). The male distiphallus is covered by short strong bristles and lacks membranous lobes and a glans; it has surstyles with prensisetae and its lobes are often elongated and modified (Figure 8E,F). *Herina* are generally widespread in the majority of zoogeographic regions, excluding the Afrotropic (Kameneva and Korneyev 2006).

Representatives of *Otites* Latr., 1804 (31 species: 5 Nearctic and 26 Palaearctic species) - *Otites guttata* (Meigen, 1830), *Otites lamed* (Schrank, 1781), and *Otites formosa* were scattered across all the trees showing no particular clustering patterns (Figures 5A and 6A). Species of *Otites* possess an R_1 vein with setulae and a bcu cell with short posteroapical extension (Figure 7N,O,P). The male distiphallus is covered by strong bristles and lacks membranous lobes and a glans. The medial lobes of the surstyles are covered by an abundance of prensisetae (15 to 40), which clearly represent the genera's character (Figure 8M,N,O). *Otites* is the only Holarctic genus, with the majority of the other species distributed in Southern Europe.

The single representative of *Seioptera* (two species: one Holarctic and one Nearctic) - *S. vibrans* (Linnaeus, 1758) - was nested within the lineage *Myennis* on the codon ML tree (aBayes = 0.72; Figure 6A). However, on the Bayesian/ML tree, it showed different topology clustering with *Myennis sibirica* (BS = 28, PP = 0.57; Figure 5A). Morphologically, this genus is close to *Pseudoseioptera* and *Homalocephala* based on the following characters: no glans on the distiphallus, two katepisternal setae, and four spherical spermathecae. Species of *Seioptera* possess a bare R_1 vein, a cup cell with short posteroapical extension, and an sc cell darkened and an apical spot (Figure 7R). The male distiphallus lacks a glans and membranous lobes and is covered by relatively strong bristles along its full length; it has surstyles with two big prensisetae on the medial lobe (Figure 8Q).

Ceroxys (Macquart, 1835) contains 16 species: 15 Holarctic and 1 Nearctic. *Ceroxys hortulana* (Rossi, 1790), *Ceroxys urticae* (L., 1758), *Ceroxys morosa* (Loew, 1873), *Ceroxys cinifera* (Loew, 1846), and *Ceroxys munda* (Loew, 1868) exhibit highly inconsistent clustering patterns on all of the trees (Figures 5A and 6A). Only *C. urticae* and *C. morosa* jointly cluster with moderate branch support (BS = 18, PP = 0.81, aBayes = 0.99). Species of *Ceroxys* possess an R_1 vein with setulae and a bcu cell with short posteroapical extension (Figure 7A,B,C,D,E). The male distiphallus is covered with strong bristles and lacks membranous lobes and a glans; it has surstyles with prensisetae (Figure 8A,B,C,D).

Representatives of *Myennis* R.-D., 1830 (four Palaearctic species) - *Myennis octopunctata* (Coquebert, 1798) and *Myennis sibirica* Portschinsky, 1892 - formed the single cluster with non-significant branch support on the codon ML tree (aBayes = 0.72; Figure 6A). No particular clustering was observed on the Bayesian/ML tree. Species of *Myennis* possess an R_1 vein with setulae and a bcu cell with short posteroapical extension (Figure 7L,M). The male distiphallus is covered by short strong bristles and lacks membranous lobes and a glans; the lateral lobes of the surstyles have two to three prensisetae (Figure 8K,L). The genus *Myennis* is mostly restricted to forest zones. *Myennis* and *Seioptera* genera form a singe branch on all the phylogenetic hypotheses.

The single representative of *Pseudotephritis* Johnson, 1802 (five species: two Palaearctic and three Nearctic) - *Pseudotephritis corticalis* (Loew, 1873) - is not consistently placed on all of the trees. Species of *Pseudotephritis* possess an R_1 vein with setulae and a bcu cell with long posteroapical extension (Figure 7Q). The male distiphallus is covered by strong bristles and lacks membranous lobes and a glans; it has surstyles with prensisetae (Figure 8P).

In summary, based on phylogenetic inference, the subfamily Otitinae did not form a statistically supported monophyletic group. However, we found morphological synapomorphic characters that may unambiguously distinguish Otitinae from Ulidiinae, namely a spinulose phallus, the absence of the glans, the absence of

membranous lobes on the distiphallus, and the presence of the female sternal apodemes. Presumably, the COI region has no sufficient phylogenetic signal to resolve genera divergence.

Conclusions

The phylogenetic analysis of COI showed satisfactory results in recovering some major lineages of the Ulidiidae family. On all phylogenetic trees obtained in our survey, there is a clear tendency of clustering at higher levels with separation of (*Physiphora* + (*Timia* + *Ulidia*)) (Ulidiinae) and (Otitinae + *Homalocephala*). Otitinae take apical position within the Ulidiidae family. However, monophyly of the Ulidiinae subfamily was not supported, forming basal paraphyletic group. Our tree inferences provide evidence that the *Homalocephala* genus has ambiguous evolutionary relationships with other Ulidiinae and Otitinae that was also shown by morphological analyses. In addition, our results support the inclusion of the *Homalocephala* and *Seioptera* genera into the Seiopterini tribe that was initially proposed by Kameneva and Korneyev (1994).

Additional material from the subfamilies Ulidiinae and Otitinae would aid to further investigation to confirm the phylogenetic status of Ulidiinae, Otitinae, and the genus *Homalocephala*.

We also conclude that analysis of other molecular markers may help to support the hypothesis of an evolutionary split having occurred between Ulidiinae and Otitinae. Nevertheless, phylogenetic reconstruction based on Markov models of codon evolution provides a good resolution for our limited dataset. Most likely, the codon models coupled with the ML approach are capable of capturing more general phylogenetic patterns, making them very promising and attractive tools for modern systematic entomology.

Additional files

Additional file 1: Collection sites. Locations are marked with the circles.

Additional file 2: General morphology and abbreviations. (A) Head lateral, *Ulidia salonikiensis*; (B) thorax, *Ulidia salonikiensis*; (C) head frontal, *Timia* (*E.*) *paramoena*; (D) wing, *Ulidia salonikiensis*; (E) surstyles, *Homalocephala albitarsis*; (F) male genitalia, *Euxesta pechumani*.

Additional file 3: Nucleotide composition at each site. The A/T bias (79.35%) is significant for the third codon positions of the sequence and considerably greater than at first (50.05%) and second (58.03%) positions.

Additional file 4: Correlation between K2P and *p*-distance. The red line is a LOWESS fit.

Additional file 5: The distribution of K2P within subsets of sequences of lengths <400 and >400 bp. No significant differences are observed.

Additional file 6: Species pairwise comparisons (transition/transversion values, minimal sequence length, K2P, and *p*-distance). The summary table.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
TVG conceived, designed, and performed the experiments. AS and MVO analyzed the data. AS, MVO, and AIS contributed the reagents, materials, and analysis tools. TVG and AS wrote the paper. All authors read and approved the final manuscript.

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