Molecular phylogeny and biogeography of the aquatic dance fly subfamily Clinocerinae (Diptera: Empididae)

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Abstract

This study presents the first molecular phylogenetic analysis of the Clinocerinae, challenging the traditionally accepted monophyly of this subfamily. DNA was extracted from fresh and museum specimens representing all biogeographical regions. Maximum likelihood (ML) and Bayesian inference (BI) phylogenetic analyses were performed based on sequences from two mitochondrial genes, cytochrome c oxidase subunit I (COI) and cytochrome β (Cytβ), and three nuclear genes, carbomoylphosphate synthase domain of rudimentary (CAD), elongation factor-1α (EF-1α) and isocitrate dehydrogenase (IDH). Through molecular data and morphological examination, our results reveal a division within Clinocerinae, distinguishing "typical" or Clinocerinae (s.s.) from several genera, specifically *Afroclinocera* Sinclair, *Asymphyloptera* Collin and *Proagomyia* Collin, possibly lending support for a reclassification of these genera outside Clinocerinae. *Bergenstammia* Mik is proposed as a junior synonym of *Phaeobalia* Mik, **syn. n.**, and the following new combinations are recognized: *Phaeobalia albanica* (Wagner) **comb. n.**, *Phaeobalia aurinae* (Pusch & Wagner) **comb. n.**, *Phaeobalia carniolica* (Horvat) **comb. n.**, *Phaeobalia frigida* (Vaillant) **comb. n.**, *Phaeobalia glacialis* (Palaczyk & Słowińska) **comb. n.**, *Phaeobalia multiseta* (Strobl) **comb. n.**, *Phaeobalia nudimana* (Vaillant) **comb. n.**, *Phaeobalia nudipes* (Loew) **comb. n.**, *Phaeobalia pulla* (Vaillant & Wagner) **comb. n.**, *Phaeobalia pyrenaica* (Vaillant & Vinçon) **comb. n.**, *Phaeobalia slovaca* (Wagner) **comb. n.** and *Phaeobalia thomasi* (Vaillant & Vinçon) **comb. n.** Re-evaluation of the genus *Roederiodes* resulted in the following new combinations: *Clinocerella macedonicus* (Wagner & Horvat) **comb. n.** and *Clinocerella montenegrinus* (Wagner & Horvat) **comb. n.** The origins of Clinocerinae (s.s.) are traced back to the Holarctic region, Laurasian origin, with a likely complex history of dispersal events into the Southern Hemisphere. Based on current knowledge, the greatest generic and species richness is confined to the Palaearctic Region. These findings provide valuable insights into the evolutionary relationships and distribution patterns of

Clinocerinae (s.s.), challenging existing taxonomic classifications and shedding light on their historical biogeography.

KEYWORDS

Bayesian inference, biogeography, Clinocerinae, maximum-likelihood, new combinations, new synonymy, phylogeny

INTRODUCTION

More than 3,000 described species of Empididae are recorded worldwide (Pape *et al*. 2009), but the numbers are presumed to be higher (Bickel, 2009). The Empididae are important as pollinators, especially for early spring flowers and in colder ecological zones (Kevan, 1972; Lefebvre *et al*. 2014), as biological indicators in various biodiversity research (Ivković & Plant, 2015; Plant *et al*., 2017), as well as in studies of behaviour and evolution (Cumming, 1994; Funk & Tallamy, 2000), and as models in sexual selection theory (Gwynne, 2008; Wheeler *et al*., 2012).

The classification of the superfamily Empidoidea has been revised and analysed over the last few decades, based on morphological characters (Chvála, 1983; Wiegmann *et al*., 1993; Cumming *et al*., 1995; Yang, 2004, Sinclair & Cumming, 2006, Badano *et al*., 2023) and molecular data (Collins & Wiegmann, 2002; Moulton & Wiegmann, 2004, 2007; Wahlberg & Johanson, 2018). These studies have resulted in recognition of as many as nine families within the Empidoidea, although not all have been accepted by specialists. The traditional family Empididae (Empididae *sensu lato*) has been restricted in concept (i.e., Empididae *sensu stricto*) either to include the subfamilies Empidinae, Clinocerinae and Brachystomatinae sensu Wahlberg & Johanson (2018), or Empidinae, Clinocerinae and Hemerodromiinae sensu

Sinclair & Cumming (2006), with several genera still unplaced (Sinclair & Cumming, 2006; Wahlberg & Johanson, 2018).

The Clinocerinae mostly occur in temperate regions with all known larvae occurring in aquatic habitats, and adults of most genera collected from emergent rocks in fast flowing streams and rivers, or from moss mats and madicolous habitats, and for some genera from vegetation near the water (Fig. 1). They are relatively good and active fliers that are predators and scavengers both as larvae and adults (Sinclair, 1995; Ivković *et al.*, 2019). Adults are most sufficiently obtained by hand collecting and netting along water courses and mossy surfaces. Malaise traps stretched across streams can also produce modest catches, but yellow pan traps are not as efficient as for other aquatic empidoids (e.g., Ceratomerinae).

Sinclair (1995) provided a detailed morphology-based cladistic analysis of the subfamily Clinocerinae, at the time recognizing 15 genera (*Dipsomyia* Bezzi was removed in a "note added in proof" and later transferred to the *Ragas*-group in Empidoidea (Sinclair, 1999a)) (Fig. 2): *Asymphyloptera* Collin, *Bergenstammia* Mik, *Clinocera* Meigen, *Clinocerella* Engel, *Dolichocephala* Macquart, *Hypenella* Collin, *Kowarzia* Mik, *Oreothalia* Melander, *Phaeobalia* Mik, *Proagomyia* Collin, *Proclinopyga* Melander, *Rhyacodromia* Saigusa, *Roederiodes* Coquillett, *Trichoclinocera* Collin, and *Wiedemannia* Zetterstedt. Two additional genera were subsequently described, *Afroclinocera* Sinclair and *Asioclinocera* Saigusa & Sinclair (Sinclair 1999b; Saigusa & Sinclair, 2022), so currently 17 genera are assigned to the subfamily.

The subfamily Clinocerinae is distinguished from other Empididae subfamilies and unplaced genera on the basis of the following synapomorphies: pubescent eyes, narrow wings (anal lobe not developed, and forming an obtuse angle), lacinia appressed or fused to labial paraphysis, sucker-like labellum without pseudotracheae (except *Proclinopyga*), pulvilliform empodium, and stout, erect costal setae (Sinclair, 1995; Sinclair & Cumming, 2006). In all, more than 464 species are presently described from various biogeographical regions (Yang *et* *al*., 2007; Sinclair, 2007a, b, 2008, 2015, 2023a, b, c; Grootaert & Yang, 2008; Raffone, 2011; Sinclair & MacDonald, 2012; Ivković *et al*., 2012, 2014, 2017, 2019, 2022; Kustov & Zherebilo, 2014, 2015; Sinclair & Shamshev, 2014; Palaczyk *et al*., 2015; Saigusa & Sinclair, 2016, 2021, 2022; Sinclair & Plant, 2017, 2022; Sinclair *et al*., 2020; Wagner *et al*., 2022). The genera *Clinocera* and *Wiedemannia* are the richest in number of species. The phylogeny and biogeography of *Wiedemannia* was analysed by Ivković *et al*. (2019), and previous subgeneric concepts were found not to be supported and their continued recognition rejected.

In the hypothesis by Chvála (1983), the subfamily Clinocerinae was well established by at least the Early Paleocene, based on the numerous fossils identified from other lineages of the Empidoidea. The first confirmed fossil of the subfamily Clinocerinae, †*Proclinopyga ulrichi* Sinclair, was described from Baltic amber (Eocene) (Sinclair, 2010). Clinocerinae are underrepresented in amber compared to other groups of empidoids, possibly due to their very close relationship with flowing water habitats and infrequent association with sources of amber (Sinclair, 2010). Waters (1989) suggested that Empidinae lineages were well established by the early Late Cretaceous or 93 million years ago (Mya), and Grimaldi & Cumming (1999) suggested that they were established even earlier in Lower Cretaceous, much earlier than suggested by Chvála (1983). With present distribution patterns and fossil evidence, it is probable that Clinocerinae were also established as early as the Cenomanian period (97-91 Mya) of the Late Cretaceous (Sinclair, 1995).

Species richness of Clinocerinae in the Palaearctic and Nearctic regions is much higher than in other biogeographic regions and does not appear to be a collecting artefact. The dominance of other groups (e.g., Ceratomerinae) in the Southern Hemisphere appear to have replaced certain clinocerine genera. The Palaearctic species of Clinocerinae have had a long history of taxonomic study in Europe, beginning with Mik (1880, 1881) and Engel (1918, 1939-1940), followed by the important works of Collin (1961) and Vaillant (1965), as well as recent revisions by Ivković *et al*. (2019, 2022). In addition to these major syntheses, there have been numerous papers describing new species in various clinocerine genera. The Nearctic also has a long history of clinocerine research, beginning with Loew (1860, 1862, 1876), with later major works of Melander (1928), Vaillant (1960), Chillcott (1961), and Wilder (1981). Since then, the New World clinocerine genera have been revised in a series of revisions published over the past 30 years (Sinclair, 1994, 1998, 2007, 2008, 2015, 2023c; Sinclair & MacDonald, 2012). Additional revisions of certain genera have been published from other biogeographical regions, such as Australasia (Sinclair, 2000), Afrotropics (Sinclair, 1999b, c, 2003a, 2023b), or East Asia (Sinclair & Saigusa, 2005; Saigusa & Sinclair, 2016, 2022). Still most of these works were done solely using morphological data, and only in Ivković *et al*. (2019, 2022) and Sinclair (2023c) have molecular data been included.

Phylogenetic relationships suggested by Sinclair (1995), based on morphological characters and shown in Figure 2, places *Proagomyia*, and then *Asymphyloptera* as sister groups to the remaining genera of Clinocerinae, but the placement of the former genera within Clinocerinae was questioned even then. *Proagomyia* includes a single described species, with many undescribed species recognized from southern South America and Australia. *Asymphyloptera* can be divided into two groups, the New World species group revised by Sinclair (2015), and Australasian species group of which not much is known (Sinclair, 1995). *Asymphyloptera* is Gondwanan in origin, and has probably dispersed northwards along the New World Cordillera as far as southwestern North America (Sinclair, 2015) (Table 1, Fig. 3).

Proclinopyga is one of the “primitive” clinocerine genera, where the cercus is not fully divided into the lower cercal plate and upper clasping cercus as found in all other “higher” clinocerines. The genus was hypothesized as being most closely related to *Rhyacodromia* and *Trichoclinocera*, which together is sister group to the remaining clinocerines, based on the configuration of the female terminalia (Sinclair, 1995) (Fig. 2). Only two species of

Rhyacodromia are described, with one species from Japan (East Palaearctic), and the other from Nepal (Oriental) (along with several undescribed species; T. Saigusa pers. comm.). *Trichoclinocera* is distributed through northern Scandinavia, central and eastern Palaearctic, and the Nearctic and Oriental regions (Table 1, Fig. 3).

Clinocera was considered most closely related to *Oreothalia* on the basis of a large, wide surstylus, and today this genus has nearly the greatest number of described species, which are sorted into several species groups (Sinclair, 2008). The genus is present in all biogeographical regions, except Antarctica, with most species situated in the Nearctic Region. On the other hand, *Oreothalia* is strictly confined to the Nearctic Region.

Roederiodes was hypothesized to be most closely related to *Hypenella* and *Clinocerella* (Sinclair, 1995), forming a well-supported monophyletic group based on morphology (Fig. 2). Detailed morphological and COI based species delimitation of New World *Roederiodes* species has been provided by Sinclair (2023c).

Dolichocephala is closely related to seven clinocerine genera (*Bergenstammia*, *Clinocerella*, *Hypenella*, *Kowarzia*, *Phaeobalia*, *Roederiodes*, and *Wiedemannia*) in which the macrosetae of the cercal plate are confined to a small apical region (Sinclair, 1995) (Fig. 2).

Mik (1881) first introduced *Kowarzia* as a genus, but that was mostly disregarded and was classified as a subgenus of *Clinocera* until the detailed morphological revision of all clinocerine genera by Sinclair (1995). Since then it has been recognized as a valid genus most closely related to *Wiedemannia* and *Dolichocephala*, and not to *Clinocera* (Fig. 2). Additionally, Mik (1881) also erected both *Phaeobalia* and *Bergenstammia* to genus level, but as with *Kowarzia*, this was not accepted until Sinclair (1995) established them as valid genera and not as subgenera of *Clinocera*. The two former genera are closely related, and together related to *Wiedemannia* (Sinclair, 1995) (Fig. 2).

Wiedemannia was formerly known to have several subgenera that were ill defined (Sinclair, 1995). The subgeneric concepts have not been used following the conclusions of the molecular phylogeny of the genus by Ivković *et al*. (2019). It is hypothesized that *Wiedemannia* is most closely related to *Phaeobalia* and *Bergenstammia* (Sinclair, 1995) (Fig. 2). Its wide biogeographical distribution was discussed in detail by Ivković *et al*. (2019).

Afroclinocera was established by Sinclair (1999) for two Southern African species (Table 1, Fig. 3). Sinclair (1999) hypothesized that *Afroclinocera* is sister genus to *Proagomyia* based on several morphological characters.

Asioclinocera is the newest genus in the subfamily Clinocerinae described by Saigusa & Sinclair (2022), and it includes two species. This genus appears to be most similar to *Rhyacodromia* on the basis of the convex-shaped labrum and spine-like setae on the fore femur (Saigusa & Sinclair, 2022). Both species are confined to the Oriental Region (Table 1, Fig. 3).

To date there is only one published phylogeny of the Clinocerinae based on morphological data (Sinclair, 1995). Due to questions arising in some of the genera, we decided to examine the clinocerines further based on molecular data in order to compare the results to the morphological phylogeny. In this paper, we test support for the monophyly of the subfamily Clinocerinae and its genera. This will also aid in building upon the knowledge of the biogeographical history of the subfamily, and may provide better insight into its fossil history and biogeographical hypotheses.

MATERIALS AND METHODS

The analysis included 66 taxa from the subfamily Clinocerinae (Empididae) and 76 representative taxa from the following Empidoidea families: Atelestidae, Brachystomatidae, Dolichopodidae, Empididae, Hybotidae, Iteaphilidae (sensu Sinclair & Shamshev, 2021), and other Diptera families: Asilidae, Micropezidae, Phoridae, Rhagionidae, Syrphidae and Tabanidae. The samples from the subfamily Clinocerinae from which DNA was extracted, were made available from the Canadian National Collection of Insects, Ottawa, Canada, the private collection of Rüdiger Wagner, and collection of Marija Ivković, University of Zagreb, Croatia.

All genera of the subfamily Clinocerinae were included in the phylogenetic analysis, except *Asioclinocera* (not available) and *Rhyacodromia*, where gene amplification failed for any of the tested primer pairs. The origin of all samples is listed in Table S1. Some COI gene sequences from the Clinocerinae specimens were obtained from the Barcode of Life Data (BOLD database). Sequences not belonging to the subfamily Clinocerinae were taken from previous works (Wahlberg & Johanson, 2018; Ivković *et al.*, 2019). DNA fragments of five genes were targeted for the present study, the mitochondrial genes for cytochrome β (Cytβ) and cytochrome c oxidase subunit I (COI), and the nuclear genes for carbomoyl phosphate synthase domain of rudimentary (CAD), elongation factor-1α (EF-1α), and isocitrate dehydrogenase (IDH). The primers used are listed in Table S2.

DNA extraction, PCR amplification and Sequencing

DNA was extracted from specimens stored in ethanol and museum dried specimens using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) or the QIAamp® DNA Micro Kit (Qiagen, Hilden, Germany), which is designed for small samples. The entire body or specific body parts (legs) were utilized for extraction, following the manufacturer's protocol. Following DNA extraction (without squashing), specimens were briefly rinsed in Milli-Q water, subsequently transferred to 96% ethanol, and preserved as vouchers (Table S1).

Amplification mixtures consisted of 1 x GoTaq® Flexi reaction buffer (Promega) containing 2.5 mM MgCl2 (Promega), 0.2 mM dNTP mix (Promega), 0.3 µM each primer, 0.125 units of GoTaq® Flexi DNA Polymerase (Promega), 3 µL of DNA and ultrapure Milli-Q water to make up the final 20 µL reaction volume. PCR reaction protocols were optimized regarding temperature and number of cycles for each primer pair according to previous work (Wahlberg & Johanson, 2018) as listed in Table S3. Enzymatic purification of PCR products was conducted utilizing the ExoI-rSAP system (NEB), following the protocol prescribed by the manufacturer. Sequencing was performed in both directions in Macrogen Inc. (Amsterdam, The Netherlands), using amplification primers. Sequence chromatograms of each sample were inspected and edited using Geneious v9.1.8 (https://www.geneious.com), and potential contamination was checked using BLASTn against the NCBI database.

Phylogenetic analyses

Multiple sequence alignments of genes were generated with MAFFT v7.308 (Katoh & Standley, 2013) using the algorithm AUTO, implemented in Geneious v9.1.8. (Biomatters Ltd). Alignments were manually inspected to ensure the accurate selection of the reading frame for all genes. For the nuclear gene Ef1-α, only the first part (~300 bps) of the sequence was used since the rest of the sequence could not be properly aligned. Concatenated datasets consisting of five genes (COI, Cyt*β*, CAD, EF-1α, and IDH) were generated in Geneious v9.1.8. Maximum-likelihood (ML) analyses were performed on the concatenated nucleotide sequence data matrices. We partitioned the concatenated dataset consisting of the five genes into distinct subsets based on codon regions. The best-fitting models were estimated using ModelFinder in IQ-TREE v2.0 (Kalyaanamoorthy *et al.*, 2017; Minh *et al.*, 2020) to find the optimal model for each partition. Maximum likelihood tree inferences were subsequently conducted with 1,000 ultrafast bootstraps (Minh *et al.*, 2013) and with SH-aLRT (Guindon *et al.*, 2010) support values to examine their robustness. Ultrafast bootstrap (UFBoot) support values were considered well-supported at a threshold of 95% (Minh *et al.*, 2013), and SH-aLRT support values were significant at 80% (Guindon *et al*., 2010). The command included the option --runs set to 10. The input file was partitioned into 14 subsets based on codons using the "charset" commands.

The Bayesian inference (BI) analysis was carried out using MrBayes v3.2.7 (Ronquist *et al*., 2012). The best partition scheme and corresponding model were determined using program Partition Finder v2.1.1 (Lanfear *et al*., 2017) using the Bayesian information criterion with linked branch lengths. The analysis involved running the program for 150 million generations, sampling every 10000 generations. Four simultaneous Markov chain Monte Carlo (MCMC) chains were used with a 'heating temperature' of 0.15, and a relative initial burn-in of 25%. Support values were calculated as posterior probabilities (PP) and were considered well-supported at a threshold of 0.95 (Erixon *et al*., 2003). Convergence of the chains, correct mixing and the number of burn-in generations, including the assessment of the effective sample size (ESS) were monitored with Tracer v. 1.7 (Rambaut *et al*., 2018). The resulting trees were edited for aesthetic purposes using FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and Paint v.22H2.

To determine statistical support for alternative tree topologies, we constructed five constrained trees (Table S4). The likelihood scores of five constrained and unconstrained tree topologies were compared using the approximately unbiased (AU) test (Shimodaira, 2002) implemented in IQ-TREE v2.0. Moreover, marginal likelihood (in natural log units) values were estimated using stepping-stone sampling (Xie *et al*. 2011; Fan *et al*., 2011) implemented in MrBayes, based on 50 steps with 75000000 generations in total. The obtained marginal likelihood estimates were compared using Bayes factors.

Biogeographical data and distribution

Based solely on described species, a species richness map per biogeographical region was created (Fig. 3). Editing and finalization was done with Paint.

RESULTS

Phylogenetic analysis

Among the 142 taxa analysed in this study, sequencing data was successfully obtained for 162 specimens for cytochrome c oxidase subunit I (COI) gene, 126 specimens for the Cytβ gene, and 84, 65, and 41 specimens for the nuclear CAD, EF-1α, and IDH genes, respectively. GenBank accession codes for successfully sequenced genes for each individual taxon are available in Table S1.

Aligned nucleotide lengths of gene fragments were 658 for COI (291 parsimony-informative, 27 singleton sites), 433 for Cytβ (222 parsimony-informative, 37 singleton sites), 846 for CAD (446 parsimony-informative, 55 singleton sites), 300 for EF-1α (123 parsimony-informative, 10 singleton sites) and 724 for IDH (359 parsimony-informative, 53 singleton sites). The total dataset included 2961 nucleotide sites. Resulting tree files are deposited in Zenodo.

Both PartitionFinder and ModelFinder identified GTR models with additional parameters (F, I, and G) for all partitions with ModelFinder applying a gamma distribution with four rate categories, whereas PartitionFinder six. Phylogenetic relationships are similar between the trees obtained in the ML and BI analyses (Fig. 4, Fig. S1). The monophyly of Clinocerinae is rejected based on the tree inference. In our study, the maximum likelihood (ML) and Bayesian inference (BI) methods consistently positioned the genera *Afroclinocera*, *Asymphloptera*, *Hypenella*, and *Proagomyia* outside the clade of eleven Clinocerinae genera, from now on referred to as Clinocerinae (s.s.). These four genera are nested within the subfamilies Empidinae and Brachystomatinae sensu Wahlberg & Johanson (2018), with strong support from ML (SH-aLRT of 97%, uBV of 100%) and lower support in BI analysis (PP of 0.91). We subsequently explored alternative phylogenetic topologies to assess the potential integration of these four genera within Clinocerinae (s.s.) clade. This involved the construction of five distinct topologies: each topology individually incorporated one of the four genera into Clinocerinae (s.s), alongside a topology that placed all four genera simultaneously within Clinocerinae (s.s). Based on the calculated Bayes factors (Table S4), all constrained topologies were strongly rejected (Bayes factor values > 5). Conversely, the approximately unbiased (AU) test results did not reject the hypotheses for the individual inclusion of *Afroclinocera* and *Proagomyia* within Clinocerinae (s.s.), as evidenced by p-values of 0.193 and 0.173, respectively. Nevertheless, the unconstrained topology where the four genera are outside the Clinocerinae (s.s.), had the highest p-value = 0.913. In conclusion, both the Bayes factor assessments and the AU test analysis strongly suggest that the integration of all four genera, *Afroclinocera*, *Asymphloptera*, *Hypenella*, and *Proagomyia*, into Clinocerinae (s.s.) is highly improbable based on the current molecular data.

Of the four genera placed outside Clinocerinae (s.s.), only *Proagomyia* is monophyletic (SH-aLRT of 98.9%, uBV of 97%, PP of 0.91). *Hypenella* and *Asymphyloptera* were recovered as closely related in ML analysis (SH-aLRT of 98.9%, uBV of 97%). *Afroclinocera* and *Hypenella* are each represented by a single species; with *Afroclinocera obesa* Sinclair placed as the sister taxon to *Chelifera* sp. and *Hemerodromia* sp. In general, internal topology of this whole clade is poorly resolved, probably due to undersampling, so no reliable conclusions on generic relationships can be drawn.

Within Clinocerinae (s.s.), whose monophyly is supported in ML analysis with SH-aLRT and uBV values of 100, and in BI analysis with PP 0.92, four genera were retrieved as monophyletic with various levels of support, namely *Wiedemannia* (SH-aLRT of 100%, uBV of 99%, PP of 0.93), *Trichoclinocera* (SH-aLRT of 100%, uBV of 100%, PP of 1), *Proclinopyga* (SH-aLRT of 100%, uBV of 100%, PP of 1), and *Dolichocephala*, (SH-aLRT of 36.9%, uBV of 91%, PP of 0.41). As supported by the ML analysis (SH-aLRT of 100%, uBV of 100%), *Proclinopyga* is sister genus to the remaining genera of Clinocerinae (s.s.). *Trichoclinocera* is divided into two well supported groups (SH-aLRT of 100%, uBV of 100%, PP of 1), the *T. stackelbergi* group (*T. falcata* Sinclair, *T. pectinifemur* Sinclair, *T. longipes* (Walker), *T. minor* (Melander), and *T. dasycoxa* Sinclair) and the *T. comata* group. The genus *Bergenstammia* is nested with high support in both analyses (SH-aLRT of 90%, uBV of 98%, PP of 0.97) within *Phaeobalia*. The genus *Oreothalia* is represented by two species, and in ML analysis *O. rupestris* Vaillant, characterized by three genes (COI, Cyt*β*, CAD), is positioned as a sister taxon to *Clinocera* clade C (SH-aLRT of 63.7%, uBV of 88%) (see explanation below), whereas *O. spinitarsis* Wilder, represented solely by the COI gene, is nested within *Kowarzia* (SH-aLRT of 99.5%, uBV of 63%), thereby potentially making the genus polyphyletic.

The genus *Clinocera* was recovered as paraphyletic and is divided into three well-supported clades: *(i) Clinocera* clade A (*Clinocera nigra* group: *C. appendiculata* (Zetterstedt), *C. aucta* (Zetterstedt); *Clinocera lineata* group: *C. lineata* Loew; *Clinocera stagnalis* group: *C. stagnalis* (Haliday), *C. binotata* Loew, *C. maculata* Loew, *C. nivalis* (Zetterstedt), *C. wesmaeli* (Macquart), *C. fontinalis* (Haliday)), *(ii) Clinocera* clade B (*Clinocera fuscipennis* group: *C. fuscipennis* Loew, *C. disjuncta* Sinclair; *Clinocera tripunctata* group: *C. smithi* Sinclair), and *(iii) Clinocera* clade C (*Clinocera conjuncta* group: *C. conjuncta* Loew, *C. olivacea* Melander, *C. subtrunca* Sinclair). Clade A is sister to the remaining Clinocerinae (s.s.), exclusive of *Proclinopyga*, and this relationship is supported by both analyses. The position of the other two clades in not resolved, but based on ML analysis, clade C is sister to *O. rupestris* (SH-aLRT of 63.7%, uBV of 88%), and together with *Trichoclinocera* is sister to clade B (Fig. 4, Fig. S1).

Biogeography of Clinocerinae

An analysis of literature data has provided insights into the distribution and abundance of 464 described species of the subfamily Clinocerinae. Key genera include *Wiedemannia* and *Clinocera*, which together account for approximately 50% of all described species (119 and 115 species, respectively). While *Clinocera* is present in all biogeographic regions (except Antarctica), *Wiedemannia* does not have described representatives in the Neotropical and Australasian regions (Fig. 3). *Dolichocephala* and *Trichoclinocera* contribute a lesser, but still substantial proportion of the Clinocerinae (12% and 10%, respectively). Both of these genera exhibit a geographical presence spanning from the Palaearctic, Nearctic and Oriental regions, with *Dolichocephala* having an additional distribution in the Afrotropical and Australasian regions. Some genera exhibit region-specific distribution, such as *Afroclinocera* (restricted to Southern Africa of the Afrotropical Region), *Asioclinocera* (Oriental Region), *Oreothalia* (Nearctic Region), *Proagomya* (Neotropical Region; but many undescribed species in Australia), and *Bergenstammia*, *Clinocerella*, and *Phaeobalia* (exclusive to the Western Palaearctic region).

In terms of regional abundance, the Palaearctic Region is the most species-rich with 223 species of Clinocerinae. This region predominantly features *Wiedemannia* (103 species), *Kowarzia* (30), *Dolichocephala* (19), *Trichoclinocera* (17) and *Phaeobalia* (16). The western Palaearctic stands out as a region with 195 species of Clinocerinae. In this region, *Wiedemannia* and *Kowarzia* are the most speciose genera, with 97 and 30 species, respectively. The Nearctic Region is populated mostly by species from the genera *Clinocera* (44 species) and *Trichoclinocera* (16). In the Oriental Region, 68 species of Clinocerinae have been described, primarily in the genera *Clinocera* (24 species), *Dolichocephala* (19) and *Trichoclinocera* (12). In contrast, the Neotropical (Central and South America) and Australasian regions (Australia, New Zealand, Oceanic Islands, e.g. Fiji) are predominantly inhabited by species from the genus *Clinocera*, with 27 and eight species, respectively but three species of *Dolichocephala* occur in

Fiji Islands of the Australasian region. In the Neotropical Region, besides *Clinocera* there are *Roederoides* (6) in Central America and *Asymphyloptera* (8) in Central and South America.

DISCUSSION

Phylogenetic relationships in Clinocerinae

The phylogeny based on molecular data is not congruent with the historical and current morphological classification of the subfamily (Chvála & Wagner, 1989; Sinclair, 1995). The monophyly of Clinocerinae as suggested by Sinclair (1995) was not confirmed by either the maximum likelihood (ML) or the Bayesian inference analyses (BI). Four genera, *Afroclinocera*, *Asymphyloptera*, *Hypenella* and *Proagomyia* in both analyses were placed outside of Clinocerinae (s.s.) (SH-aLRT of 97%, uBV of 100%, PP of 0.91). Sinclair (1995) already suggested doubt that *Proagomyia* belonged to the Clinocerinae, and his cladistic analysis placed it together with *Asymphyloptera* as sister groups to the remaining clinocerine genera. Although these two genera share the synapomorphies defining the Clinocerinae (costa spines, sucker-like labellum, empodium pulvilliform), the latter feature is certainly an adaptation to aquatic habitats (see the discussion in Sinclair & Cumming, 2006: 62), and the male terminalia of these two genera distinctly differs from the remaining clinocerine genera. When *Afroclinocera* was described as a new genus, Sinclair (1999b) stated that it is most closely related to *Proagomyia* based on several morphological characters. Placement of *Proagomyia* and *Afroclinocera* appears to be outside of Clinocerinae (s.s), but their relationship within the remaining Empididae was not resolved with our dataset. Moulton & Wiegmann (2007) in their ML tree retrieved *Proagomyia* in the clade with the rest of the Hemerodromiinae which partially corresponds to our ML analysis. In both analyses, *Proagomyia* was not included within

Clinocerinae (s.s.). The robust rejection of the constrained topology which integrates these four genera within Clinocerinae (s.s.), showed by Bayes factor values and corroborated by approximately unbiased test results, supports their placement outside of Clinocerinae (s.s.). Based on the results of our analyses we herein view the genera *Afroclinocera*, *Asymphyloptera* and *Proagomyia* as doubtful clinocerines and removed from Clinocerinae (s.s.). Future phylogenetic studies into other subfamilies of the Empididae should include these three genera, combined with better gene, species and generic coverage in order to test and assess our results.

Hypenella is also placed outside of Clinocerinae (s.s.) based just on a COI sequence of a single species. There is strong morphologically based support that this genus belongs to Clinocerinae (s.s.), specifically in the form of the clasping cercus, position of setae on the cercal plate and phallus at apex of hypandrium (Sinclair, 1995). Consequently, the placement of *Hypenella* needs to be further analysed to confirm its classification.

In agreement with Sinclair (1995) who previously characterized *Proclinopyga* as one of the most basal clinocerine genera with plesiomorphic male terminalia and thoracic chaetotaxy, both of our analyses confirm this assessment, showing high support for *Proclinopyga* as the sister group to the remaining Clinocerinae (s.s.). Unfortunately, the status of *Rhyacodromia* and *Asioclinocera* could not be tested as the DNA extraction was unsuccessful (*Rhyacodromia*), or the sample was unavailable at the time the analysis was conducted (*Asioclinocera*). The statement by Sinclair (1995) that *Rhyacodromia* is a sister genus to *Trichoclinocera* based on morphological characteristics cannot be rejected or confirmed at this point. *Asioclinocera* was only recently described (Saigusa & Sinclair, 2022) and based on morphology it is possibly most closely related to *Rhyacodromia*.

Both ML and BI analyses recovered *Bergenstammia* nested within *Phaeobalia*. The lack of substantial phylogenetic or morphological evidence to justify their separation as genitalia of both genera are similar, with clasping cercus consisting of two lobes. The absence of empodium and pulvilli that define *Bergenstammia* occur in a species of *Trichoclinocera*, so it is not a good generic character (Sinclair, 1995), resulted in their distinct classification as genera primarily based on historical tradition (Mik, 1881). Consequently, we propose that *Bergenstammia* Mik, 1881, with type species *Clinocera nudipes* Loew, is a junior synonym of *Phaeobalia* Mik, 1881, **syn. n.**, with type species *Clinocera trinotata* Mik. This generic synonymy also results in the following new combinations: *Phaeobalia albanica* (Wagner) **comb. n.**, *Phaeobalia aurinae* (Pusch & Wagner) **comb. n.**, *Phaeobalia carniolica* (Horvat) **comb. n.**, *Phaeobalia frigida* (Vaillant) **comb. n.**, *Phaeobalia glacialis* (Palaczyk & Słowińska) **comb. n.**, *Phaeobalia multiseta* (Strobl) **comb. n.**, *Phaeobalia nudimana* (Vaillant) **comb. n.**, *Phaeobalia nudipes* (Loew) **comb. n.**, *Phaeobalia pulla* (Vaillant & Wagner) **comb. n.**, *Phaeobalia pyrenaica* (Vaillant & Vinçon) **comb. n.**, *Phaeobalia slovaca* (Wagner) **comb. n.** and *Phaeobalia thomasi* (Vaillant & Vinçon) **comb. n.**

Kowarzia is resolved as polyphyletic with *Oreothalia spinitarsis* grouped within it. Because of the morphology and distribution patterns of the two genera this is highly unlikely and the observed result could be due to the well-recognized problem in Diptera where COI of distant species can be similar or identical (Meier *et al*., 2006, 2022). Since only COI from *O. spinitarsis* was available to us for the analysis, the observed topology should be taken with caution and tested further.

The monophyly of *Wiedemannia* was already previously resolved in Ivković *et al.* (2019), and it is monophyletic in our ML and BI trees as well, although highly supported only in the ML tree.

Clinocerella and *Roederiodes macedonicus* Wagner & Horvat form a monophyletic clade with high support. Sinclair & Harkrider (2004) had transferred *R. macedonicus* to *Clinocerella*, but Sinclair (2023c) returned it to *Roederiodes*, along with *R. montenegrinus* Wagner & Horvat. The resulting placement with *Clinocerella* in this study warranted generic reassessment, that is supported by not only gene coverage and phylogenetic analysis, but by detail morphological analysis of the specimens. The phallus of *Clinocerella macedonicus* and *Clinocerella montenegrinus* is bi-articulated, a feature not present in *Roederiodes*, which instead has membranous lobes on the apex of the phallus, but often not well drawn if the specimen was cleared in very strong acid. The transfer of these two species to *Roederiodes* was an error in Sinclair (2023c) and this molecular result encouraged a re-evaluation and correction. Both species should now be classified in *Clinocerella* Engel, with type species *Atalanta sorex* Engel, as *C. macedonicus* **comb. n.** and *C. montenegrinus* **comb. n**.

The genera *Dolichocephala*, *Roederiodes* (excluding *R. macedonicus*), and *Trichoclinocera* are monophyletic, with *Roederiodes* and *Trichoclinocera* having support for their monophyly, while *Dolichocephala* lacks support. The *Dolichocephala irrorata* group, defined by stout setae near the apex of the hypandrium (Sinclair, 1995), includes many species throughout the world, and in our analysis they are resolved as a clade (*D. irrorata* (Fallén), *D. vockerothi* Sinclair & MacDonald, *D. quadrispina* Smith, and *D. chillcotti* Sinclair & MacDonald). The groups of *Trichoclinocera* that were established by Sinclair (1994) and somewhat revised by Saigusa & Sinclair (2016), correspond to the groups retrieved in the ML tree.

Clinocera is the only genus that was resolved as paraphyletic with three distinct clades, A, B, and C. Clade A is distantly related to all others, while clade B and C more closely related and group together in a clade with *Trichoclinocera* and *Oreothalia rupestris*. Several *Clinocera* species groups within each of the clades, established on the basis of morphological characters by Sinclair (2008), were confirmed and supported in the ML tree. Morphological characters used to distinguish species groups seem to have phylogenetic signal, but have poor resolution on deeper nodes. Since our taxon coverage of *Clinocera* diversity is very low, further study is required to test the internal relationships within this paraphyletic genus.

Oreothalia was viewed as closely related to *Clinocera* by Sinclair (1995), and this is confirmed with the assignment of *O. rupestris* (with genes COI, Cyt*β* and CAD) as sister to the *Clinocera* clade C (Sinclair, 1995, 2008), whereas *O. spinitarsis* was assigned within *Kowarzia*, although with poor gene coverage (COI only). Given this conflicting result, the classification of *Oreothalia* remains unchanged at this time.

Biogeography and current distribution of Clinocerinae (s.s.)

The geographic distribution of Clinocerinae (s.s.) is of complex origin, with the highest number of species and genera in the Palaearctic Region (Fig. 3). Despite intensified sampling efforts in this area, the observed geographic distribution pattern suggests factors beyond simple collection bias. There are still undescribed species in various regions, and the same is plausible for the Palearctic as well, since some areas of Palearctic have not been explored at all (huge areas of Central Asia). Collection in the Nearctic, Australia, and New Zealand has been considerable as well, suggesting that the absence of certain genera in these biogeographical regions is most probably correct. Moreover, it is observed that some other empidoids (e.g. Ceratomerinae) have replaced certain clinocerine genera in some biogeographical regions (Sinclair, 2003b). In the Palaearctic Region *Wiedemannnia* and *Clinocera* are having the highest number of described species (Ivković *et al*., 2019; Sinclair 2008; Sinclair & Plant, 2022). Six genera are exclusively confined to the Holarctic (*Clinocerella*, *Hypenella*, *Oreothalia*, *Phaeobalia* (including *Bergenstammia*), *Proclinopyga* and *Rhyacodromia*), with four of them only found in the Palaearctic (*Clinocerella*, *Hypenella*, *Phaeobalia* (including *Bergenstammia*) and *Rhyacodromia*). Furthermore, *Phaeobalia* (including *Bergenstammia*) and *Clinocerella* are confined to European Mountains, and *Rhyacodromia* is only found in the Eastern Palaearctic (Sinclair, 1995; Yang *et al*., 2007). There is strong evidence in some other genera of

Empidoidea (*Acarterus* Loew (Hybotidae), *Empis* Linnaeus (Empididae)) of an Afrotropical and Oriental relationship among several genera (Sinclair, 1996; Daugeron & Grootaert, 2003), but this is not found in the Clinocerinae. On the other hand, a potential link between Afrotropical and Neotropical regions is present in some *Clinocera* and *Dolichocephala* present in Southern Africa . Plant (2011) hypothesized that divergence of Hemerodromiinae and Empidinae was by the early Cretaceous based on sister group relationship of Southern Africa *Afrodromia* Smith to the rest of the Hemerodromiinae, suggesting an early origin, predating Gondwanaland fragmentation, and we hypothesize that this could also explain patterns for Clinocerinae.

Hypenella is present only in the Eastern Palaearctic and Oriental regions, with several undescribed species from Japan, China and Thailand (Smith, 1965; Horvat, 1998; Grootaert & Yang, 2008).

Proclinopyga is sister genus to the remaining genera of Clinocerinae (s.s.), and is confined to the Nearctic and Eastern Palaearctic with a single species described from Nepal (Sinclair, 1995; Yang *et al.*, 2007). The discovery of the Baltic amber species †*Proclinopyga ulrichi* suggests that the genus had a wider distribution range in the past, but still confined to the Palaearctic (Sinclair, 2010). We are hypothesizing that the major radiation and diversification of Clinocerinae (s.s.) is of Laurasian origin with several instances of some genera dispersing into the Southern Hemisphere (Sinclair, 2008). The *Clinocera* clade A, which is sister to all other Clinocerinae (s.s.) excluding *Proclinopyga*, probably dispersed south from the Nearctic Region via an intercontinental land bridge, likely to have formed during a late Campanian/early Maastrichtian uplift (78 Mya), and which possibly existed into the early Paleocene (Hoernle *et al.*, 2002; Iturralde-Vinent and MacPhee, 1999; Sinclair, 2008). There are many interconnected groups between various biogeographical regions, suggesting many sister lineages between the regions, which are known in other groups of Empidoidea (Sinclair *et al*., 2019; Cumming *et al*., 2014).

Clinocera possibly has a Laurasian origin with speciation occurring in various geographical regions (Sinclair, 2000, 2008; Sinclair & Plant, 2022). *Clinocera* is one of the most numerous and most widely distributed genera of Clinocerinae, with records from all biogeographical regions, except Antarctica (Sinclair, 2008; Sinclair & Plant, 2022). Greater species richness of *Clinocera* in the Western Nearctic has been connected to higher number of mountain ranges facilitating geographic isolation and speciation (Sinclair, 2008), similar to *Wiedemannia* in the Western Palaearctic (Ivković *et al*., 2019). Speciation of Nearctic *Clinocera* lineages was probably during Late Eocene (38–33.9 Mya), and they diversified through vicariance created by Miocene orogenic events (Sinclair, 2008). *Clinocera* clade A is sister to the other *Clinocera* clades (B and C) and the remaining Clinocerinae, and radiation and diversification of clade A presumably started earlier. Possibly for that reason, the representatives of *Clinocera* clade A are found in all biogeographical regions (Sinclair, 2000, 2003a, 2008; Sinclair & Plant, 2022). In South America only representatives of *Clinocera* clade A (*Clinocera stagnalis* group) can be found, and surprisingly, one species occurs on Campbell Island in the subantarctic region of New Zealand. A late Cretaceous Nearctic dispersal through South America and to Australia and New Zealand via Antarctica (see the review of Sanmartín & Ronquist, 2004), could explain how the *C. stagnalis* group reached Campbell Island (Sinclair, 2008). On the other hand, only representatives of the *Clinocera lineata* group from clade A are present in Australia, occurring from northern Queensland along the Great Dividing Range in the southeast to Tasmania (Sinclair, 2000). Hypothesized older age of *Clinocera* clade A compared to the rest of the Clinocerinae diversity (without *Proclinopyga*), could explain their wider distribution in the world. *Clinocera* clade B (*C. fuscipennis* and *C. tripunctata* groups) is a well-supported clade, with all representatives present in Nearctic Region or Southern Africa

, and with several undescribed species from Eastern Palaearctic (Japan) in the *C. fuscipennis* group (Sinclair, 2008). The direct connection between South America and Africa lasted until Late Cretaceous (~100 Mya Reguero & Goin, 2021; or 90 Mya, White, 1994), but it could have been connected longer via islands that were used as stepping-stones (Sinclair, 2003a). All this is indicating that *Clinocera* clade A and possibly clade B were well established prior to the separation within Gondwanaland. *Clinocera* species present in East Africa belong to the *C. stagnalis* group of the clade A, and they arrived in Africa from dispersal from the north, but the *Clinocera* species in Southern Africa that belong to *Clinocera* clade B did not (Sinclair 1999b, 2003a). The most probable routes for colonization by *Clinocera* clade A of southeast Asia are up-elevation dispersal of “old Oriental” elements in response to climate change, and lateral immigration of “new” Palaearctic elements along the orogenic eastern arc of the Himalayas (Sinclair & Plant, 2022). Additionally, dispersal went from Western Nearctic, through Eastern Palaearctic, to southeast Asia and further down to Australia (Sinclair 2000, 2008). East Asia – Eastern Nearctic distribution patterns are well documented among Empidoidea (Saigusa, 2012; Saigusa & Sinclair, 2016).

The *Trichoclinocera stackelbergi* group is distributed through the Eastern Nearctic and in the Palaearctic. The other group retrieved by ML tree is the *T. comata* group that is restricted to the Western Nearctic. All *Trichoclinocera* species are restricted to the Palaearctic and Nearctic, and only a handful of species are distributed in the Oriental Region where they possibly arrived via the Himalayas from the Palaearctic, or through the Beringian passage from the Nearctic (Yang *et al*., 2007; Sinclair, 1994; Saigusa & Sinclair, 2016).

The biogeography of *Wiedemannia* was previously discussed by Ivković *et al*. (2019), where it is hypothesized that *Wiedemannia* diversified from Early and Middle Eocene to the Miocene, commencing around 50 Mya. The speciation of the group was probably triggered by several sudden changes in global sea-levels, which promoted dispersal, and subsequent isolation and speciation mostly through vicariance (Haq *et al*., 1987; Ivković *et al*., 2019). Intercontinental dispersal between Eurasia and the Nearctic may have occurred via the Bering and North Atlantic land bridges (Sanmartín *et al*. 2001; Mao *et al*., 2010; Ye *et al*., 2018) not just for *Wiedemannia*, but for other Clinocerinae genera, such as *Clinocera* clade A, *Dolichocephala*, *Proclinopyga*, *Roederiodes* and *Trichoclinocera*. *Wiedemannia* in the Afrotropical Region is distributed in the Eastern and Southern areas of the continent, probably reaching Africa from the north (Ivković *et al*., 2019). Although there is only one described species of *Wiedemannia* from the Oriental region bordering the Palaearctic, there are still no described species of *Wiedemannia* from the Australasian and Neotropical regions. Undescribed species are known from Indonesia, India, Myanmar (Burma), and Papua New Guinea (Sinclair, 1995, 1998). The highest species richness of *Wiedemannia* is in the mountains of Europe (Ivković *et al*., 2019).

Kowarzia is present in the Western Palaearctic and the Afrotropical Region. It is present in Eastern and Southern Africa, and an undescribed species is known from Madagascar. *Kowarzia* probably arrived in Africa from Europe before the increased aridity of the Oligocene (Willassen & Cranston, 1986), with dispersal to Southern Africa of a recent origin, possibly during the last Glacial Maximum (Sinclair, 2003a).

Roederiodes has the highest species richness in the Nearctic Region and extending into Central America, but it is also present in Palaearctic (Canary Islands, southern Europe, and Japan), Oriental Region bordering the Palaearctic and Afrotropical Region (Sinclair, 2023b). The two Afrotropical species share affinities to European and Asian taxa (Sinclair, 2003a). The genus is not known from the Australasian Region and South America (Sinclair, 2023b).

Dolichocephala is present in all biogeographical regions except in Australia and New Zealand, although it does occur in New Guinea and Fiji (Sinclair, 1995; Sinclair & Evenhuis, 2005). The greatest number of species is present in the Palaearctic Region (Yang *et al*., 2007).

Phaeobalia (including *Bergenstammia*) is exclusively European and usually confined to higher mountains, while *Clinocerella* can be found at a wider range of altitudes (Sinclair, 1995; Ivković *et al*., 2013).

These findings provide a comprehensive overview of the distribution and abundance of Clinocerinae species across various biogeographic regions, aiding in further understanding of their ecology and evolution.

CONCLUSIONS

The phylogenetic hypothesis presented above represents a valuable insight into the internal relationships of the Clinocerinae. Results imply that the Clinocerinae are not a monophyletic group. The genera can be divided into Clinocerinae (s.s.), and three genera are questionable members of the subfamily (*Afroclinocera*, *Asymphyloptera*, *Proagomyia*). Morphological characters used to define genera seem to be valid for most genera except for *Clinocera*. This paraphyletic genus is composed of three independent clades, and additional work, including better sampling, is needed to resolve this matter. Additionally, on the basis of morphology and now DNA phylogeny, we conclude that there is no necessity for *Bergenstammia* and *Phaeobalia* to be separate genera, and therefore propose the former as a junior synonym of *Phaeobalia*, **syn. n.** The proposed origin of Clinocerinae (s.s) is the Holarctic Region, of Laurasian origin, while the origin of *Afroclinocera*, *Asymphyloptera* and *Proagomyia* is hypothesized to be Gondwanan.

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Data Sharing and Data Availability

The data that support the findings of this study are openly available in Zenodo at https://zenodo.org/records/10539136.

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Figures

Figure 1. Live images of Clinocerinae. A. *Dolichocephala irrorata* (Fallén), photo by R. Usami, Galerie-insecte; B. *Trichoclinocera pectinifemur* Sinclair, photo by Z. Dankowicz; C. *Wiedemannia* sp., photo by N. Vikhrev; D. *Clinocera lineata* Loew, photo by J. van der Linden.

Figure 2. Hypothesized morphological cladistic relationships of genera of the subfamily Clinocerinae modified from Sinclair (1995).

Figure 3. World distribution of the genera of Clinocerinae in biogeographical regions. Numbers in the centre of the pie charts indicate the total number of described species, and the charts represent the proportional species richness for each genus. Two genera, marked with asterisks, are not included in the phylogenetic analyses.

Figure 4. Maximum-likelihood tree inferred from the concatenated matrix of two mitochondrial and three nuclear genes including 142 taxa from the subfamily Clinocerinae (Empididae) and other representative taxa from different Diptera families. Branch support is expressed as SH-aLRT support values (significant at 80% (Guindon et al., 2010)) and as ultrafast bootstrap (UFBoot) values (supported at a threshold of 95% (Minh *et al.*, 2013)) presented as SH-aLRT/UFBoot. The tree is rooted with *Rhagio tringarius* De Geer (Rhagionidae) and *Chrysops viduatus* (Fabricius) (Tabanidae). Each of the 15 genera of the Clinocerinae is highlighted with a different colour. The genus *Clinocera* is divided into three clades (A, B and C).

Table

Table 1. World distribution of the described species in genera of Clinocerinae according to biogeographical regions.

Supplementary Files

Supplementary Figures

Figure S1. Majority-rule consensus topology from the Bayesian inference (BI) analysis of the partitioned dataset, with a burn-in of 25%. Numbers above branches represent posterior probability values (PP) which are considered well-supported at a threshold of 0.95 (Erixon et al., 2003). The tree is rooted with *Rhagio tringarius* De Geer (Rhagionidae) and *Chrysops viduatus* (Fabricius) (Tabanidae). Each of the 15 genera of the Clinocerinae is highlighted with a different colour. The genus *Clinocera* is divided into three clades (A, B and C).

Supplementary Tables

Table S1. Included taxa and country of origin. The GenBank accession numbers are provided for the newly submitted sequences.

Table S2. Primer names and their respective sequences used for gene amplification in this study.

Table S3. Individual PCR reaction protocols were designed for amplifying each gene fragment following the methodology established by Whalberg and Johanson in their previous work (2018).

Table S4. Topology testing results. Taxonomic constraints range from an unconstrained topology to topologies constrained by specific genera within the Clinocerinae clade. Marginal likelihood (in natural log units) were estimated using stepping-stone sampling based on 50 steps with 1470500 generations (2941 samples) within each step. Log Bayes factor (ln (BF)) was obtained by comparing unconstrained against the constrained tree topology. Values greater than

3 are typically considered as strong evidence in favour of the better model, and values greater than 5 indicate very strong evidence (Kass and Raftery, 1995). The four genera* refers to *Afroclinocera*, *Hypenella*, *Asymphyloptera* and *Proagomyia*.