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Phylogenomics reveals the timescale of diversification in Amblycera

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Abstract

Recently, genomic approaches have helped to resolve phylogenetic questions in many groups of parasitic organisms, including lice (Phthiraptera). However, these approaches have still not been applied to one of the most diverse groups of lice, Amblycera. To fill this gap, we applied phylogenomic methods based on genome-level exon sequence data to resolve the relationships within and among the families of Amblycera. Our phylogenomic trees support the monophyly of the families Ricinidae and Laemobothriidae. However, the families Trimenoponidae and Gyropidae are not monophyletic, indicating that they should be merged into a single family. The placement of *Trinoton* is unstable with respect to Boopiidae and Menoponidae, and we suggest recognizing Trinotonidae as a separate family. At the genus level, the genera *Colpocephalum*, *Hohorstiella*, *Menacanthus* and *Ricinus* were recovered as paraphyletic. Regarding generic complexes, the tree revealed the *Menacanthus* complex to be monophyletic, but the *Colpocephalum* complex paraphyletic, including genera not traditionally placed in this group. Dating analysis suggests that the divergence among families of Amblycera occurred shortly after the Cretaceous–Paleogene boundary 66 Mya. Cophylogenetic analyses revealed many host-switching events during the diversification of Amblycera, indicating that the evolutionary history of Amblycera does not tightly mirror that of its hosts. Ancestral host reconstructions revealed that the ancestral host of Amblycera was most likely a bird, with two host switching events to mammals. By combining phylogenomics, molecular dating and cophylogenetic analyses, we provide the first large-scale picture of amblyceran evolution, which will serve as a basis for future studies of this group.

KEYWORDS
coevolution, lice, next-generation sequencing, parasites, Phthiraptera, Psocodea

INTRODUCTION

In the past decade, methodological advancements in genomic sequencing technologies have revealed unprecedented details about the evolutionary history of many organisms. High-throughput sequencing has made it possible to work with thousands of genes in many groups, ranging from birds (Jarvis et al., 2014; Prum et al., 2015), mammals (Álvarez-Carretero et al., 2022), non-avian reptiles (Card et al., 2023), molluscs (Smith et al., 2011), to insects (Misof et al., 2014). At the same time, advances in phylogenomic methods have allowed for phylogenetic reconstruction from large data sets of hundreds or thousands of genes for many taxa (Chernomor

et al., 2016; Hoang et al., 2017; Kalyaanamoorthy et al., 2017; Minh et al., 2020; Mirarab et al., 2021). In addition, advances in Bayesian molecular-clock dating (Dos Reis, 2022) approaches have allowed for reconstruction of the timing of diversification events in these large data sets.

One group in which these phylogenomic approaches have been widely applied is parasitic lice (Insecta: Phthiraptera), which are permanent ectoparasites of birds and mammals. Members of this group are characterized by secondary losses of organs traditionally used for morphological identification in other insects, such as wings or eyes (Johnson, 2022). Recent phylogenomic studies have confirmed that parasitic lice originated from free-living bark lice, being the sister taxon of the family Liposcelididae (De Moya et al., 2021). Liposcelids are small, dorso-ventrally flattened insects that occasionally feed on organic debris in the nests of birds and mammals and are sometimes found on the animals themselves (Johnson, 2022; Mockford, 1993). As the life cycles of ancestral parasitic lice became closely associated with their hosts, structures typically used for mobility and orientation were reduced or eliminated (Johnson, 2022; Lyal, 1985). Across parasitic lice, phylogenomic approaches have also been applied to relationships within and among major groups, such as within the parvorder Ischnocera (De Moya et al., 2019) and among the three parvorders parasitizing mammals (Johnson et al., 2022). Phylogenomic techniques have also proved useful at lower taxonomic levels, such as in primate lice (Boyd et al., 2017), seal lice (Leonardi et al., 2019) and within the genus *Columbicola* (Boyd et al., 2022).

One major group of lice in which these genomic approaches have not yet been widely applied is the parvorder Amblycera. This group is sister to all other parasitic lice (De Moya et al., 2021; Johnson et al., 2022; Najer et al., 2024), but a comprehensive phylogeny of this group has only been investigated using morphological characters (Marshall, 2003). Amblycera is a diverse group of parasitic lice containing over 1500 species and some of the most speciose louse genera (Kolencik et al., 2024; Marshall, 2003; Martinů et al., 2015). Members of Amblycera parasitize a broad diversity of birds and mammals (Johnson et al., 2022; Price et al., 2003). Compared with the dietary specialists in the other parvorders of lice (e.g. Ischnocera feeding exclusively on feathers or Anoplura exclusively on blood), different species of Amblycera encompass notable variability in feeding strategies, including blood- and feather-feeding lice within a single group (*Colpocephalum* complex; Kumar et al., 2018). Typically, most Amblycera are dietary generalists, feeding on tissue debris (Whiteman & Parker, 2004). Relatively long legs enable them to move around the host body and even off of the host (Grossi & Galloway, 2022). Perhaps most notably, species of *Trinoton* can move across the surface of the water to move between waterfowl or their nests (Eichler & Vasjukova, 1981). Members of Amblycera vary widely in host specificity, with some species being restricted to a single host species, while others parasitize dozens of hosts (Kolencik et al., 2024; Martinů et al., 2015; Price et al., 2003). Some genera have unusual lifestyles with specific adaptations. For example, members of the genus *Piagetiella* exclusively parasitize the throat pouches of pelicans (Rékási & Kiss, 2006), while members of other genera (e.g. Gustafsson, Lei, et al., 2019) are adapted to living inside the feather quills of their avian hosts.

From the standpoint of classification, Amblycera is traditionally divided into six families: Ricinidae, Laemobothriidae, Menoponidae, Gyropidae, Trimenoponidae and Boopiidae (Clay, 1970; Price et al., 2003). Ricinidae, Laemobothriidae and Menoponidae exclusively parasitize birds, whereas Gyropidae and Trimenoponidae exclusively parasitize mammals. Members of Boopiidae are found almost exclusively on marsupial mammals; however, one species has been described from a cassowary in New Guinea. The family Abrocomophagidae, parasitizing mammals, was synonymized with Gyropidae (Price et al., 2003; Price & Timm, 2000). The phylogenetic relationships among all families have not been investigated in detail. The morphological data set of Marshall (2003) focuses on relationships among the avian Amblycera and Boopiidae and did not explicitly examine the relationships among other mammalian Amblycera. While molecular phylogenetic studies of lice have included some representation of Amblycera (e.g. De Moya et al., 2021; Johnson et al., 2022), no comprehensive molecular study has investigated relationships among all amblyceran families across a diversity of genera. At lower levels, some molecular phylogenetic data sets have examined relationships within or among closely related genera (Catanach et al., 2017; Kolencik et al., 2024; Martinů et al., 2015). However, these studies have inferred trees from only a few genes and failed to resolve most relationships with high support.

A recent study of mitochondrial genome fragmentation analysed genomic reads from 90 samples of Amblycera representing 53 genera and all currently recognized families (Najer et al., 2024). While the study was primarily focused on mitochondrial genomics, it also produced phylogenomic trees based on nuclear loci for Amblycera. The concatenated and coalescent trees from this study (Figures S1 and S2 in Najer et al., 2024) differed in positions of some groups, most notably the family Boopiidae and the genus *Trinoton*. However, this study did not exhaustively analyse these phylogenomic data sets. To more clearly understand instability in the positions of Boopiidae and *Trinoton*, here we conduct additional phylogenomic analyses, including exclusion of third codon positions, comparisons of partitioned and non-partitioned analyses of the concatenated tree and exclusion of the genus *Trinoton*. We also conduct a Bayesian dating analysis of this data set to compare with similar studies in the literature (De Moya et al., 2021; Johnson et al., 2018). Using the information from the dating analysis as constraints, we also compare the tree for Amblycera with those for birds and mammals to shed more light on the history of host switching and co-divergence. Altogether, this work represents a substantial step in understanding the evolution of Amblycera, a major diversification of parasitic lice.

MATERIALS AND METHODS

Phylogenomic analysis

We used existing genomic data of 90 amblyceran lice (89 species, 53 genera) from a study of mitochondrial genome organization in this

group (Najer et al., 2024). This sample represents all families, the majority of host groups and most biogeographic regions across which Amblycera occur. For analysis, we used annotated and trimmed exon alignments of 2375 single-copy nuclear orthologs between 156 and 14,466 bases long from Najer et al. (2024). From this prior study, we compare the concatenated and coalescent trees (Figures S1 and S2 in Najer et al., 2024), to those generated from a new partitioned analysis of the concatenated matrix in IQ-TREE 2 v.2.1.2 (Minh et al., 2020). We used the -p (Chernomor et al., 2016), -m TESTNEWMERGE (Kalyaanamoorthy et al., 2017) and -rclusterf 10 (Lanfear et al., 2017) parameters to search for the optimal number of partitions, yielding a total of 396 partitions and a model selection for each of them (IQ-TREE identifies the optimal partition scheme by default). Support was estimated using ultrafast bootstrapping (UFBoot2; Hoang et al., 2017) with 1000 replicates.

In addition to these comparisons, we evaluated the impact of removing the more rapidly evolving third codon positions from the data set. To estimate a tree based on only first and second codon positions, we used the splitting and concatenating functions of AMAS (Borowiec, 2016) to remove every third base from the individual gene alignments. The resulting sequences were concatenated, and the entire alignment was used for phylogenetic analyses in IQ-TREE 2 v.2.1.2 (Minh et al., 2020). We ran partitioned and non-partitioned analyses, including the -p (Chernomor et al., 2016) parameter and removing it, respectively. The -m TESTNEWMERGE (Kalyaanamoorthy et al., 2017) and -rclusterf 10 (Lanfear et al., 2017) parameters were used in the same way as in the partitioned analysis of the full genomic sequences. Support was estimated using ultrafast bootstrapping (UFBoot2; Hoang et al., 2017) with 1000 replicates. From the individual gene alignments with third codon positions removed, separate gene trees were inferred using IQ-TREE 2 based on the optimal models for each gene. These gene trees were used in a coalescent analysis in ASTRAL-III v5.7.4 (Zhang et al., 2018) to account for gene-tree/species-tree discordance.

To explore how the unstable position of the genus *Trinoton* (see below) affects the topology of other tree branches, we removed three sequences of the genus *Trinoton* from the alignments of all orthologs. We repeated the partitioned concatenated and coalescent phylogenomic analyses of full genomic sequences using the same methods as with the *Trinoton* sequences.

Dating analysis

We estimated the timing of amblyceran evolution using Bayesian dating techniques with MCMCtree implemented in PAML v4.9 (Yang, 2007) over the concatenated amblyceran tree. For calibration, we used fossil and codivergence dates from previous studies (Johnson et al., 2021, 2022; Najer et al., 2024; split between human and chimpanzee lice 5–7 Mya, split between the lice from Old World primates and Great Apes 20–25 Mya, the minimum age for Menoponidae of 44 Mya based on a fossil) and a root age of 127.1 Mya (De Moya et al., 2021). We estimated branch lengths and substitution rate using baseml; then, we used the substitution rate to calculate priors for the MCMCtree run. For the Markov chain Monte Carlo (MCMC) simulations, we used the independent rates clock model (clock = 2) and GTR + G model of sequence evolution. Node age priors for the nodes without calibrations were uniformly distributed between present time and root age (BDparas = 1 1 0). The gamma-Dirichlet prior calculated from the substitution rate (0.453053 ± 0.000368) was set for rgene_gamma = 1 2.22, and the rate variance prior was set for sigma2_gamma = 1 10. We ran all the simulations with 100 million generations, burning 50,000 generations and sampling every 100 generations. As the first step of the MCMCtree run, we estimated the gradient and Hessian of the loglikelihood (usedata = 3), which we later used for MCMC sampling of posterior distribution using normal approximation (usedata = 2). We ran the entire dating analysis two times to ensure the consistency of the results.

Cophylogenetic analysis

For cophylogenetic analyses, we used the concatenated and coalescent trees for the lice (Figures S1 and S2 in Najer et al., 2024). To construct the host tree, we compiled phylogenetic information from four sources. As a backbone avian tree to the level of families, we used the higher-level tree from Prum et al. (2015). Within avian families, we downloaded information about relationships between genera and species, respectively, from BirdTree (Jetz et al., 2012, 2014) and grafted it to the backbone. For the time-constrained analysis in Jane (below), we obtained the 95% time confidence interval of divergence between birds and mammals (316–322.4 Mya) from TimeTree 5 (Kumar et al., 2022). For mammals, we used the tree and dating analysis from Álvarez-Carretero et al. (2022).

To determine the overall congruence between the phylogenies of lice and their hosts, we ran a distance-based cophylogenetic analysis in PACo (Balbuena et al., 2013) with default parameters and 100,000 permutations. As an additional cophylogenetic analysis, we used eMPRess v1.0 (Santichaivekin et al., 2020) to compare host trees with concatenated and coalescent parasite trees. This software summarizes events across equally parsimonious solutions. While it allows exploration of adjacent cost spaces, it does not necessarily provide time-consistent cophylogenetic reconstructions, and it does not provide an option for using information from dated trees. To facilitate comparison with previous cophylogenetic studies of lice (Johnson et al., 2021, 2022), we initially used a cost scheme of cospeciation, 0; duplication, 1; loss, 1; and host-switching, 2. The first iteration did not produce any a time-consistent reconciliation, in which all host-switching events are between contemporaneous lineages. Therefore, we increased clustering as described by Johnson et al. (2021), and if the increased clustering did not provide at least one time-consistent solution, we explored adjacent cost spaces.

Even after exploring this variation, the solutions reconstructed by eMPRess were only weakly time-consistent (e.g. Figure S13). Therefore, we repeated the cophylogenetic analysis in Jane v4 (Conow et al., 2010) with the dated concatenated parasite tree, applying time constraints to both host and parasite trees, cost scheme as above with cost of failure to diverge, 1, and genetic algorithm parameters set as recommended on the Jane website (number of generations, 30;

population size, 1300; https://www.cs.hmc.edu/~hadas/jane/). As time constraints, we used the 95% confidence intervals assigned to time zones 20 million years wide (i.e. 0–20, 20–40, 40–60, 60–80, 80–100, 100–120 Mya) and assigned nodes in the dated louse tree to these corresponding intervals. Confidence intervals for the host tree were obtained from the source relevant for each node (above), and confidence intervals for the parasite tree were obtained from the MCMCtree dating analysis. While Jane produces results that are strongly time-consistent reconciliations (Libeskind-Hadas, 2022), a large number of solutions are typically produced without general overview. To facilitate evaluation of these solutions, we clustered the solutions into isomorphs and manually inspected one solution from each isomorph that contained more than 200 solutions. To test whether the total costs of the solutions suggested by Jane were lower than those obtained by chance, we randomized the tip mapping (host-associations) in Jane with default parameters (initial population size, 30; number of generations, 30) and 1000 samples. Due to the absence of terminal branch lengths, we did not apply the time constraints to the parasite coalescent tree. To test whether our concatenated parasite tree provides lower solution costs than a random parasite tree, we ran the cophylogenetic analysis in Jane without time constraints, using the same parameters as for the time-constrained analysis. Then, we randomized the parasite tree with the initial population size (30), number of generations (30), sample size (100) and Yule beta parameter (−1.0), and compared costs obtained by both unconstrained analyses.

Ancestral host reconstruction

We reconstructed ancestral host (bird or mammal) over the dated tree using the *ace* function of the APE v5.4 R package (Paradis et al., 2004) under equal-rates (ER) and all-rates-different (ARD) model. The model fit was assessed with the *fitDiscrete* function in the geiger v2.0.7 R package (Pennell et al., 2014); the best model was selected using the corrected Akaike information criterion (AICc) and Aikake information criterion (AIC) weight. With the best model (ER, AIC = 21.48181, AICc = 21.52727, AIC weight = 0.6788871), we performed stochastic mapping with 1000 simulations using the phytools v0.7 R package (Revell, 2012). The coalescent tree (Najer et al., 2024) differed from the concatenated tree in the position of the family Boopiidae, which occurs predominantly on mammals. Although the coalescent tree produced by ASTRAL does not have terminal branch lengths, we repeated the ancestral host reconstruction with the coalescent tree only to see whether the ancestral host changes between analyses. To enable this analysis, R used a constant value 1 instead of terminal branch lengths.

RESULTS

Phylogenomics

All our phylogenomic analyses recover monophyly of Amblycera with 100% support (Figures 1 and 2, Figures S1–S8). Across all trees (Figures 1 and 2, Figures S1–S8), Amblycera is sister to all other parasitic lice. Of the 118 internal nodes recovered in the phylogenomic trees including the genus *Trinoton*, 93 nodes are highly supported (100%) across all these trees (Figure 1). According to our dating analysis (Figure 2 and Figure S6), Amblycera diverged from other parasitic lice in the Late Cretaceous (103–75 Mya), and the deepest divergences within Amblycera occurred around the time of Cretaceous–Paleogene (K-Pg) boundary (66 Mya). The 95% confidence intervals for the earliest divergences within Amblycera overlap the K-Pg boundary (83–58 Mya for Amblycera; Figure S8).

Consistent with a morphological phylogeny (Marshall, 2003), all our analyses suggest that three amblyceran families (Ricinidae, Laemobothriidae and Boopiidae) are monophyletic with 100% support (Figures 1 and 2, Figures S1–S8). For Boopiidae, our study contains only two species from one genus (*Heterodoxus*), so the monophyly of this family needs to be verified with additional taxon sampling from other genera in the future. Gyropidae and Trimenoponidae, two families parasitizing neotropical mammals, are mutually intertwined and paraphyletic in all trees. Together, they form a monophyletic group strongly supported across all our phylogenomic trees (Figures 1 and 2, Figures S1–S8). Thus, we propose merging the families Gyropidae and Trimenoponidae, and subsequently, we now refer to the entire clade containing both families as Gyropidae (see Discussion).

The positions of different families of Amblycera vary among phylogenomic trees, providing four different topologies. From the concatenated analyses, the trees inferred from the full gene sequences (Figures 1–3, Figure S1) indicate that the families diverged from the rest of parasitic lice in the order (1) Ricinidae, (2) Laemobothriidae, (3) Gyropidae, (4) Boopidae, (5) *Trinoton* and (6) Menoponidae, i.e. Ricinidae is sister to all other Amblycera. In the concatenated trees inferred from the first and second positions only (Figures S3 and S4), the families diverged from the rest of Amblycera in the order (1) Boopidae, (2) *Trinoton*, (3) Ricinidae+Laemobothriidae and (4) Gyropidae +Menoponidae, i.e. Boopidae is sister to all other Amblycera. The coalescent analysis from the full gene sequences (Figure S2) suggests that the families diverged from the rest of Amblycera in the order (1) Boopidae, (2) Ricinidae+Laemobothriidae, (3) Gyropidae and (4) Trinotonidae+Menoponidae, i.e. Boopidae is sister to all other Amblycera. Finally, in the coalescent tree from the first and second positions only (Figure S5), the families diverged in the order (1) Boopidae, (2) Ricinidae+Laemobothriidae, (3) *Trinoton* and (4) Gyropidae+Menoponidae, i.e. Boopidae is sister to all other Amblycera. In the trees with the genus *Trinoton* excluded from the analyses, both concatenated (Figure S6) and coalescent (Figure S7) trees strongly support Boopidae as sister to all other Amblycera. In all of these trees (Figures 1–3, Figures S1–S7), some nodes involving the relationships among families have low support, but the details of which nodes are strongly versus weakly supported differ among the trees (Figure 1).

The position of the enigmatic genus *Trinoton*, currently placed in Menoponidae, varies among different phylogenies and in some cases renders the family Menoponidae paraphyletic. While morphological analysis places *Trinoton* in the crown group of Menoponidae (Marshall, 2003), our various phylogenomic analyses place this genus in different positions depending on analysis, lacking strong support in

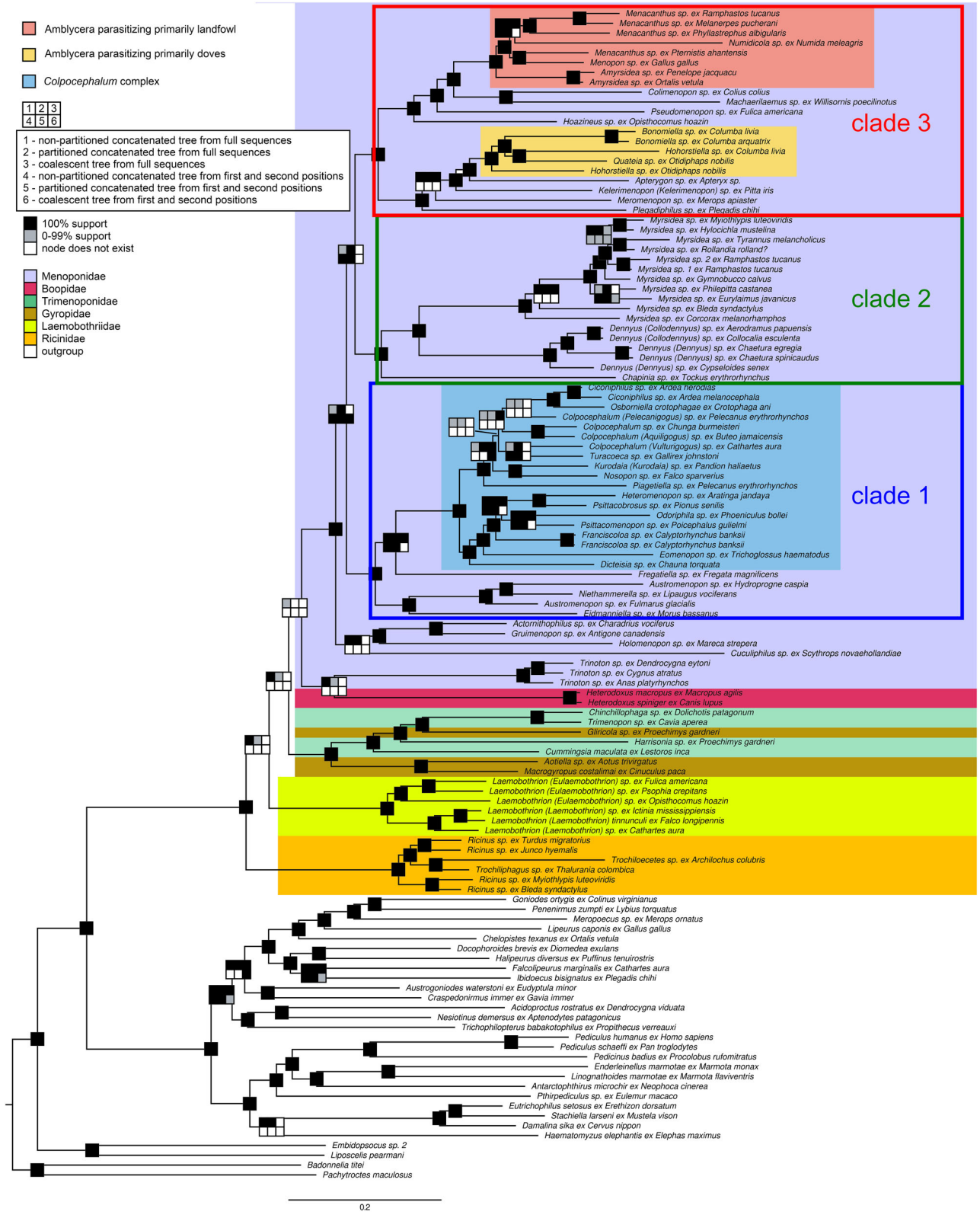

FIGURE 1 Non-partitioned concatenated maximum likelihood phylogenomic tree constructed by Najer et al. (2024) from the full genomic sequences of Amblycera. Based on a set of 2375 protein-coding genes. Colour-coded matrices show support and congruence among six phylogenomic trees analysed in this study as indicated in legend. A single black square on an internal node indicates high (100%) support across all six trees. Amblyceran families and outgroups are marked in colour as indicated in legend. Three main clades within the family Menoponidae are marked with blue (clade 1), green (clade 2) and red (clade 3), respectively. Groups of Amblycera parasitizing primarily landfowl are marked with red background, groups of Amblycera parasitizing primarily doves are marked with yellow background, and the *Colpocephalum* complex is marked with blue background.

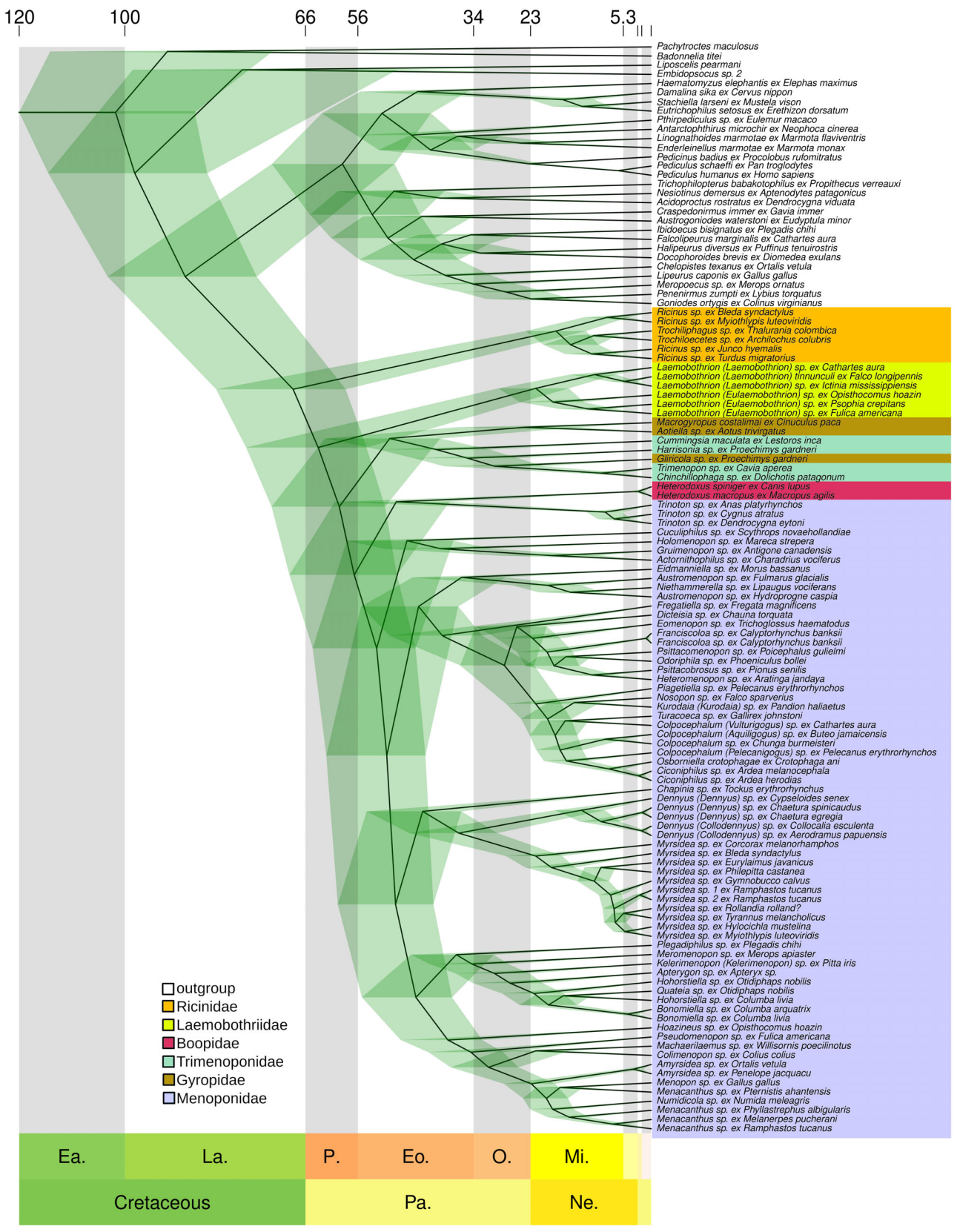

FIGURE 2 Dated phylogenomic tree of Amblycera. Based on the concatenated tree from Najer et al. (2024). The 95% dating confidence intervals are marked in green, and amblyceran families and outgroups are marked in colour as indicated in legend. Geological timescale is at the bottom, and absolute ages are at the top. Abbreviations of geological units: Ea., Early Cretaceous; La., Late Cretaceous; Pa., Paleogene; P., Paleocene; Eo., Eocene; O., Oligocene; Ne., Neogene; Mi., Miocene.

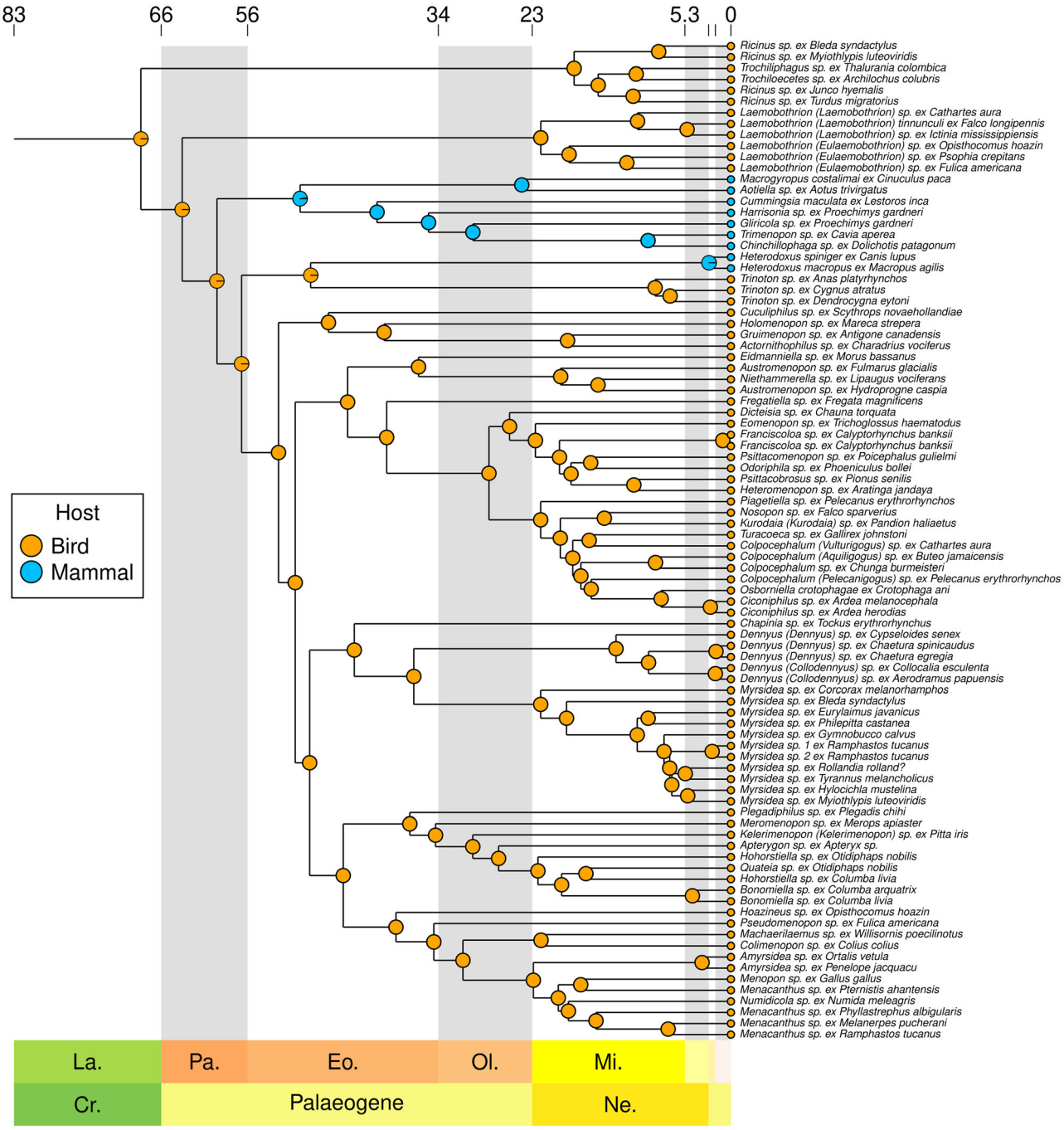

FIGURE 3 Dated phylogenomic tree with reconstruction of ancestral hosts under the equal rates (ER) model. Circles at the tips indicate the host (bird vs. mammal). Pie charts at the nodes show the frequency distribution of the reconstructed host after 1000 simulations of stochastic character mapping using an ER model. Geological timescale is at the bottom, and absolute ages are at the top. Abbreviations of geological units: Ea., Early Cretaceous; La., Late Cretaceous; Pa., Paleogene; P., Paleocene; Eo., Eocene; O., Oligocene; Ne., Neogene; Mi., Miocene.

any of them. Concatenated maximum likelihood analyses (Figures 1 and 2, Figures S1 and S6) place *Trinoton* as sister to the family Boopiidae, rendering Menoponidae paraphyletic. Concatenated analyses excluding third positions (Figures S3 and S4) place *Trinoton* as sister to all other Amblycera except Boopiidae. Coalescent analysis with third positions excluded (Figure S5) places *Trinoton* as sister to all other Amblycera except Boopiidae, Laemobothriidae and Ricinidae. Removing the genus *Trinoton* from the phylogenomic analyses enabled reaching highly supported and consistent topology across our phylogenies. Then, the concatenated and coalescent trees both suggest that the families diverged from the rest of Amblycera in the order (1) Boopidae; (2) Ricinidae+Laemobothriidae; (3) Gyropidae+Menoponidae (Figures S6 and S7).

Our analyses recover the genera *Colpocephalum*, *Menacanthus*, and *Hohorstiella*, and *Ricinus* as paraphyletic. Our study includes several genera of the *Colpocephalum* complex, and relationships among

them have low support across most phylogenomic trees (Figures 1 and 2, Figures S1–S6). The genera *Menacanthus* and *Hohorstiella* both appear to have other morphologically distinct genera embedded within them. The genera *Menopon* and *Numidicola*, which both parasitize landfowl (Galliformes) (Price et al., 2003), render the genus *Menacanthus* paraphyletic consistently across all trees and with 100% support (Figures 1 and 2, Figures S1–S7). The species of *Menacanthus* that also parasitizes landfowl is more closely related to *Menopon* and *Numidicola* than to other *Menacanthus*. Furthermore, also with consistent support across phylogenomic trees (Figures 1 and 2, Figures S1–-S7), this expanded *Menacanthus* clade is sister to *Amyrsidea*, another amblyceran genus parasitizing landfowl (Price et al., 2003). With similarly broad and robust support (Figures 1 and 2, Figures S1–S7), the genera *Bonomiella* and *Quateia* render the genus *Hohorstiella* paraphyletic. Although all of these genera are morphologically distinct (Marshall, 2003; Price et al., 2003), they all parasitize pigeons and doves (Aves: Columbiformes) (Price et al., 2003). Within the family Ricinidae, the genera *Trochiloecetes* and *Trochiliphagus* are embedded within *Ricinus*, rendering *Ricinus* paraphyletic across all our trees and with 100% support (Figures 1 and 2, Figures S1–S7).

We could not fully verify the monophyly of Boopiidae because we only had two specimens of one genus (*Heterodoxus*; Figures 1 and 2, Figures S1–S8) available. The position of this family differs among different analyses, although all phylogenomic analyses resolve monophyly of *Heterodoxus* with 100% support. In the trees inferred from the full concatenated sequences including *Trinoton* (Figures 1 and 2, Figures S1 and S8), Boopiidae is sister to *Trinoton*, while in other genomic analyses (Figures S2–S7), it is sister to all other Amblycera. This latter position appears to be more likely, given it is the only one supported by multiple independent analyses.

Cophylogenetics

Our distance-based cophylogenetic analysis indicates significant congruence between parasite and host trees in Amblycera ($m^2 = 10833.77$, p value < 0.001). Forty-two squared residuals were higher than the median squared residual value, and in 71 host–louse associations, the 95% confidence interval was higher than the median value (Figure S9). Using the concatenated tree without dated nodes, we did not find strongly time-consistent reconciliations in eMPRess. Despite increased clustering as described by Johnson et al. (2021), weakly time-consistent reconciliations provided by eMPRess contained biologically improbable host switches back in time, indicating that information from dated trees is required for further analyses. In this case, a cophylogenetic analysis in Jane with time constraints (Figures S10–S12) recovered 32,125 equally parsimonious solutions with a total cost of 149 each (Table S1). These solutions were clustered in 326 isomorphs (Table S1), from which we manually inspected representatives of 46 isomorphs, i.e. representatives of 21,958 solutions (more than two thirds of all solutions, Table S2). Randomizing tip mapping (host-associations) 1000 times produced costs ranging from 310 to 348 (Figure S11), thus the cost of actual trees (149) was much less than this randomized distribution (p < 0.001). The unconstrained analysis in Jane suggested 11,770 possible solutions with a total cost of 124 each, while the parasite tree randomization produced costs ranging from 155 to 167. Thus, the cost of actual trees was significantly lower than all costs obtained with random parasite tree (p < 0.001). The isomorphs of time-constrained reconstructions that were examined in detail contained 14–16 cospeciations, 4–5 duplications, 69–70 host switches and 4–7 losses (Table S2). Thus, host switching is a major component of amblyceran diversification. The events revealed by the most frequent solution (Figure S12) generally agree with those in other solutions, and differ only in the position of some events on different branches. All manually inspected solutions reveal an ancestor of hoatzin (*Opisthocomus hoazin*) as the ancestral host of Amblycera, with a subsequent early switch to placental mammals.

Using the coalescent tree, the cophylogenetic analysis in eMPRess provided only weakly time-consistent reconciliations, again with a few improbable switches back in time (Figure S13). It suggested 12,551,454,720 possible solutions containing 31 cospeciations, 2 duplications, 56 host transfers and 8 losses each (total cost 122; Figure S13). The eMPRess software automatically summarizes the results and tests them by randomization with 100 replicates. This randomization provided costs 155–165 (p value < 0.01). With the coalescent tree, host switches again represented the majority of inferred cophylogenetic events. The coalescent analysis reveals the common ancestor of placental mammals as the ancestral host of Amblycera.

In general, both concatenated and coalescent cophylogenetic analyses (Figures S12 and S13) indicate that host switching played a substantial role in the evolution of Amblycera. Despite its higher cost, it was more common than cospeciation. Using the dated concatenated tree helps constrain the reconstructions to be time consistent and potentially more plausible. Both analyses reveal that Amblycera originated on neotropical hosts. Together with the predominantly Southern Hemisphere (specifically Australia and South America) distribution of Boopiidae and Gyropidae, the congruence between cophylogenetic analyses further supports Southern Hemisphere origin of Amblycera.

Ancestral host reconstruction

In our ancestral host reconstructions, the ER model was optimal when using the concatenated tree (AIC = 21.48181, AICc = 21.52727, AIC weight = 0.6788871; vs. AIC = 22.84213, AICc = 22.98006 and AIC weight = 0.3211129 for the all-rates-different model). MCMCtree relies on the concatenated data matrix for the dating analysis; therefore, we did not perform the dating analysis with the coalescent tree, because it does not have a concatenated matrix underlying it. However, we did perform the reconstruction with the coalescent tree to test whether the positions of Boopiidae and *Trinoton* affect results obtained from the concatenated tree. Both reconstructions reveal that the ancestral host of Amblycera was a bird, and Amblycera switched to mammals within two events (Figure 3): (1) A common ancestor of the family Gyropidae switched from birds to a

common ancestor of neotropical mammals between the late Cretaceous and early Eocene (72–40 Mya; Figure 3); (2) A member of the family Boopiidae or its common ancestor switched to mammals in Australia (Figure 3). However, only two samples analysed from Boopiidae obscure details of the second switch event, and we do not know whether the switch between birds and mammals in Australia happened once or twice (see below).

DISCUSSION

Phylogenomic analyses of 2395 target nuclear orthologs for 90 samples of Amblycera, plus outgroups, produced generally well resolved and well supported trees, although some branches were unstable across analyses. We found that Amblycera is sister to all other parasitic lice, which is consistent with previous studies (De Moya et al., 2021; Johnson et al., 2018; Najer et al., 2024). Furthermore, this result is stable across a number of phylogenetic methodologies, including concatenated and coalescent analyses, and analyses with third codon positions removed. Within Amblycera, multiple phylogenomic analyses produced a higher-level molecular phylogeny of Amblycera broadly consistent with published morphological studies (Clay, 1969, 1970; Marshall, 2003). All our analyses strongly support the monophyly of the amblyceran families Ricinidae, Laemobothriidae and Boopiidae. The families Gyropidae and Trimenoponidae are both paraphyletic. These families were not included in Marshall's morphological phylogeny (Marshall, 2003), but the close relationship among them has been suggested by Clay (1970) and Price et al. (2003). Thus, we propose merging Gyropidae and Trimenoponidae into one family, Gyropidae Kellogg, 1896 (urn:lsid:zoobank.org:act:95931689-1121-45E4-9AAB-BBEBBDF412EC).

The most diverse amblyceran family, Menoponidae, as currently defined, is paraphyletic in most trees, because the genus *Trinoton* is separated from the rest of Menoponidae. The exception is the coalescent tree from all codon positions (Figure S2) in which the genus *Trinoton* is sister to the remainder of Menoponidae. In a phylogeny based on the 18S nuclear ribosomal gene, Barker et al. (2003) recovered *Trinoton* as the sister to all other Amblycera. From a morphological perspective, the separation of *Trinoton* and Menoponidae has been discussed by Price et al. (2003). However, Marshall (2003) placed this genus within the crown group of Menoponidae, based on its morphological resemblances to *Meromenopon* and *Menacanthus*. While the exact placement of *Trinoton* varies among different trees in our analyses, it always remains separated from the rest of Menoponidae, suggesting the morphological similarity to genera within Menoponidae is a result of homoplasy. To express the distinctiveness of the genus *Trinoton* and to ensure the monophyly of Menoponidae, we suggest recognition of the family Trinotonidae (urn:lsid:zoobank.org:act:0F17E73F-97B9-47CE-987E-A37DDB813ECC). This family was established without a clear definition (Eichler, 1941). However, since the establishment of the family in 1941, its name has been used by several authors (e.g. Eichler, 1963; Złotorzycka, 1976), including The International Commission on Zoological Nomenclature (Steyskal, 1972). According to Article 13.1 of the International Code of Zoological Nomenclature, this makes the name Trinotonidae Eichler, 1941 valid. Also, it was used as a valid name by Marshall (2003), even though she ultimately placed *Trinoton* within Menoponidae. The morphology of *Trinoton* was thoroughly explored by Marshall (2003), and keys for the identification of the genus were provided by Clay (1969) and Gustafsson, DiBlasi, et al. (2019). At the moment, *Trinoton* would be the only genus belonging to Trinotonidae. Therefore, the diagnostic combination of characters for the genus *Trinoton* becomes the diagnosis for Trinotonidae. Thus, the family Trinotonidae differs from Menoponidae by (1) division of sternal plates on pterothorax (Clay, 1969; Gustafsson, DiBlasi, et al., 2019), (2) large numbers of setae on pterothorax (Clay, 1969; Gustafsson, DiBlasi, et al., 2019) and (3) generally large size of adult individuals (over 4 mm long; Clay, 1969).

The unclear position of Trinotonidae also seems to be the main cause of the differences among our phylogenomic analyses. Once we remove *Trinoton* from the data set (Figures S6 and S7), the topology becomes stable between concatenated and coalescent trees, and several core nodes of the concatenated tree obtain high support (Figures S1 vs. S6). Identifying Trinotonidae as the cause of instability across phylogenomic trees increases the robustness of the rest of these trees, providing more convincing evidence that the relationships among other amblyceran families revealed by our analyses are correct. Causes of the phylogenetic instability observed in Trinotonidae could include gene introgression, incomplete lineage sorting or other data biases. These causes can be relatively hard to differentiate (Meyer et al., 2016). In this case, introgression could be possible, given that members of Trinotonidae are highly mobile (Eichler & Vasjukova, 1981), and an increased rate of introgression has been observed in lice with higher dispersal capabilities (Doña et al., 2020).

Within Menoponidae, several insights into phylogenetic relationships emerge. Three large clades within the family are highly supported and consistent among topologies. The first, clade 1 (Figure 1), comprises members of the *Colpocephalum* complex and related genera. Three studies subsequently redefined the complex over time (Catanach et al., 2017; Clay, 1969; Price et al., 2003). In each of them, the complex includes different genera. Our results show that for it to be monophyletic, the *Colpocephalum* complex needs to take something from each of these definitions plus a few additional genera. Among the genera we sampled, this complex should contain the genera *Ciconiphilus*, *Colpocephalum*, *Dicteisia*, *Eomenopon*, *Franciscoloa*, *Heteromenopon*, *Kurodaia*, *Nosopon*, *Piagetiella*, *Psittacobrosus*, *Psittacomenopon*, *Odoriphila*, *Osborniella* and *Turacoeca*. Genera related to the *Colpocephalum* complex include *Austromenopon*, *Eidmaniella*, *Fregatiella* and *Niethammerella*. Our trees revealed the genus *Colpocephalum* itself is paraphyletic, with several other genera of the complex embedded within it. The paraphyly of the genus *Colpocephalum* was also previously suggested in a phylogenetic study of the *Colpocephalum* complex based on Sanger sequencing of a few genes (Catanach et al., 2017). Further taxon sampling is needed to fully understand the extent of paraphyly of *Colpocephalum*.

Another major clade comprises the genera *Chapinia*, *Dennyus* and *Myrsidea* (clade 2, Figure 1). This clade is sister to a larger clade (clade

3, Figure 1) containing lice of landfowl (Galliformes) and doves (Columbiformes), among others. We find that *Menacanthus* is paraphyletic, with *Menacanthus* from landfowl being separated from *Menacanthus* from Piciformes and Passeriformes, the latter of which forms a clade within various amblyceran genera from landfowl. The division of *Menacanthus* into two distinct clades is consistent with Martinů et al. (2015) who divided *Menacanthus* into two clades but did not explore the relationships of these clades to other Amblycera from landfowl. Within clade 3, we also find the genus *Hohorstiella*, which occurs on doves, to be paraphyletic with other lice from doves being embedded within it. Thus, the lice of landfowl and doves each form major distinctive groups, with various genera in each of them, indicating a radiation of genera of lice within these host groups.

Generally, the relationships revealed by our phylogenomic analyses broadly agree with published morphological data, although individual cases of disagreement occur. For instance, Marshall (2003) inferred a close relationship between the genera *Chapinia*, *Dennyus* and *Myrsidea* as was found in our trees (clade 2; Figure 1). However, she also placed the genera *Ancistrona*, *Bonomiella* and *Pseudomenopon* in this clade, but we found these three genera to be outside of this group. Marshall (2003) also finds close relationship between *Numidicola*, *Menopon*, *Amysidea*, *Menacanthus* and *Colimenopon*, a result broadly consistent with our analyses. Some authors have placed the genus *Cuculiphilus* within the *Colpocephalum* complex (Marshall, 2003; Scharf & Price, 1965). However, Clay (1969) suggested that *Cuculiphilus* should not be included in the *Colpocephalum* complex, a finding consistent with our results. While the exact position of *Cuculiphilus* is unstable across some of our analyses, it is always outside of the three major clades discussed above. When comparing genomic and morphological data, our findings suggest that morphological analysis, when it is based on a sufficient number of characters, may reveal relationships which broadly correspond to those inferred from phylogenomics.

Patterns of diversification

Our dating analysis showed that amblyceran families diverged around the time of, or shortly after, the Cretaceous–Paleogene (K-Pg) boundary (Figure 2 and Figure S8). These divergence events correspond to the time of diversification of birds and mammals after the K-Pg mass extinction (O'Leary et al., 2013; Prum et al., 2015). Subsequent diversification of modern lineages within the families Ricinidae and Laemobothriidae did not occur until much later, in the early Miocene. These families contain only a few genera, they are both morphologically homogeneous (Marshall, 2003; Price et al., 2003), and exclusively blood-feeding (Eichler, 1963; Kumar et al., 2018). In contrast, the family Menoponidae, which also occurs on birds, began diversifying extensively in the Eocene with continual radiation until the present.

One important question concerns the ancestral host of Amblycera, whether it was a bird or mammal. Prior phylogenomic studies using ancestral character reconstruction have suggested that the ancestral host of all parasitic lice was a bird and that mammalian lice originated as a result of four host-switching events, two of which occurred in Amblycera (Johnson et al., 2022). On the basis of morphological evidence, Clay (1970) had also suggested two host switches to mammals within Amblycera. We reconstructed the ancestral host considering only the phylogeny of Amblycera (Figure 3). This reconstruction also indicated that the ancestral host was a bird. However, the uncertainty regarding the early divergences within Amblycera could affect this result. For example, the position of the family Boopiidae changes markedly between different analyses, and these different positions possibly affect the results of the ancestral host reconstruction. However, ancestral reconstruction over the coalescent tree, even though this is not an ultrametric tree, also indicated that the ancestral host of Amblycera was a bird, with two host switches to mammals.

One possible consideration, however, is that a member of Boopiidae may also occur on birds: *Therodoxus* parasitizing cassowaries. Although not documented by Clay (1970), specimens of *Therodoxus* have been obtained from cassowaries on at least three different occasions (slides NHMUK010648801, NHMUK010648803 and NHMUK010648806 in the Natural History Museum, London, UK), confirming the likely validity of this host association. No fresh material of *Therodoxus* exists for sequencing, so we were not able to include this important genus in our study. This raises the question of whether the ancestor of Boopiidae originated on cassowaries and then switched to marsupials, or whether it originated on marsupials and then switched to cassowaries (Clay, 1970). On the basis of morphology, Marshall (2003) placed *Therodoxus* as sister to all other Boopiidae, so either scenario seems possible.

Another way to reconstruct ancestral hosts is through cophylogenetic analyses. Overall, our cophylogenetic analyses revealed that phylogenetic tree of Amblycera is significantly congruent with that of its hosts. However, host-switching has been frequent and ongoing during the diversification of Amblycera. In our concatenated cophylogenetic analysis, the ancestral host is a bird (i.e. the ancestor of the hoatzin lineage). In contrast, cophylogenetic analyses of the coalescent tree reconstructs the ancestral host as a mammal (i.e. the common ancestor of placental mammals). This difference likely reflects the changing position of Boopiidae. One other caveat is that we only had two samples of Boopiidae, one from a marsupial and one from the domestic dog. In fact, one of the few places of agreement between the concatenated and coalescent cophylogenetic reconstructions is the ancestor of Boopiidae on carnivores. However, it has long been suggested that occurrence of Boopiidae on dogs and other canids is a result of host switching of lice from marsupials to canids, likely originally to dingos in Australia in prehistoric times (Clay, 1976; Murray & Calaby, 1971). Assuming this was the case, then the ancestral host of mammalian Boopiidae would be a marsupial, which would likely impact the interpretation of the cophylogenetic reconstructions. Both analyses also agree that the ancestral host of the family Gyropidae is a common ancestor of Neotropical placental mammals, and then later switched to marsupials. However, this result may also be a result of undersampling of this clade, particularly from Neotropical marsupials. Another point of agreement between the analyses with concatenated and coalescent trees is that passerine birds were colonized multiple times throughout the diversification of Amblycera. However, in many

other respects the details of cophylogenetic reconstructions using the concatenated versus coalescent trees are different. These differences likely arise because of differences in arrangement of the earliest diverging branches, which in turn impact the inferred ancestral host and nearly all other inferred cophylogenetic events.

One limitation of this study with respect to cophylogenetics is that it is not densely sampled across all the lineages of hosts on which Amblycera occurs. Rather the goal was to gain a broad understanding of amblyceran phylogeny. Thus, our data set typically contains representatives from each larger group of Amblycera, but not from each important host group of Amblycera. This uneven host sampling becomes apparent in the analysis with time constraints. For example, the ancestral host of Amblycera is reconstructed as the hoatzin likely because our data set contains two distantly related lice from hoatzin (*Laemobothrion* and *Hoazineus*). In addition, the hoatzin represents an early diverging long branch in the host tree. Thus, the ancestral host reconstructed by cophylogenetics could well change with additional, broader sampling. Although our data set represents unprecedented dense sampling for Amblycera, more is needed, especially in mammalian lice. Most notably, future sampling of placental versus marsupial mammals needs to include more lice from marsupials. From the family Boopiidae, our data set includes only two samples, one from a marsupial and one from a placental host. As mentioned above, this family parasitize predominantly marsupials, with one species known from placentals and one species described from birds (Clay, 1970; Price et al., 2003). In Neotropical mammals, our data set contains only one sample from a marsupial, and several more representatives are possible. This undersampling of marsupials potentially distorts the cophylogenetic results so that both analyses suggest the origin of mammalian Amblycera on placental mammals. In general, for cophylogenetic analyses without dense taxon sampling, minor differences in sampling or tree structure can have a major impact on the reconstruction, which is done by parsimony, minimizing overall cost. In contrast, maximum likelihood ancestral host reconstruction using broadly defined character states (such as bird versus mammal in this case) incorporate uncertainty in reconstruction and may be more reliable when taxon sampling incorporates major lineages, rather than all species, as in this study.

In conclusion, this study is the first to evaluate the higher phylogeny of Amblycera based on molecular data. We use dense sampling across the entire parvorder and suggest taxonomic changes strongly supported by our data but also discussed in earlier literature. We show that the history of amblyceran host-louse associations is complex. Thus, this study establishes a framework for future taxonomic work in Amblycera.

AUTHOR CONTRIBUTIONS

Tomáš Najer: Funding acquisition; writing – original draft; writing – review and editing; formal analysis; visualization; resources; data curation; validation. **Jorge Doña:** Formal analysis; writing – review and editing. **Aleš Buček:** Conceptualization; writing – review and editing. **Andrew D. Sweet:** Conceptualization; writing – review and editing. **Oldřich Sychra:** Formal analysis; writing – review and editing. **Kevin P. Johnson:** Conceptualization; writing – review and editing; funding acquisition; data curation; supervision; resources; methodology; validation.

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CONFLICT OF INTEREST STATEMENT

We declare we have no competing interests.

DATA AVAILABILITY STATEMENT

Data associated with this study are available in the Supplementary material and Figshare. The code used for phylogenomic, cophylogenetic and dating analyses, including mcmctree control files, is available on GitHub (https://github.com/tomas-najer/Amblycera-systematics). Additional data, including manually inspected isomorphs of cophylogenetic reconciliations, are available on Figshare (https://doi.org/10.6084/m9.figshare.26367217, https://doi.org/10.6084/m9.figshare.26367229, https://doi.org/10.6084/m9.figshare.26367235).

ETHICS STATEMENT

Research on animals was conducted according to University of Illinois Institutional Animal Care and Use Committee protocols 10119, 13121, 15212.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Partitioned concatenated maximum likelihood phylogenomic tree of Amblycera. Based on a set of 2375 protein-coding genes. Numbers associated with branches indicate ultrafast bootstrap support.

Figure S2. Coalescent tree of Amblycera constructed by Najer et al. (2024) from the full genomic sequences of Amblycera. Based on ASTRAL analysis of a set of 2375 protein-coding genes, combining individual gene trees into a species tree. Numbers associated with branches indicate local posterior probability.

Figure S3. Non-partitioned concatenated tree of Amblycera with third codon positions of the sequences removed. Based on a set of 2375 protein-coding genes. Numbers associated with branches indicate ultrafast bootstrap support.

Figure S4. Partitioned concatenated tree of Amblycera with third codon positions of the sequences removed. Based on a set of 2375 protein-coding genes. Numbers associated with branches indicate ultrafast bootstrap support.

Figure S5. Coalescent tree of Amblycera with third codon positions of the sequences removed. Based on ASTRAL analysis of a set of 2375

protein-coding genes, combining individual gene trees into a species tree. Numbers associated with branches indicate local posterior probability.

Figure S6. Partitioned concatenated tree of Amblycera without the genus *Trinoton*. Based on a set of 2375 protein-coding genes. Numbers associated with branches indicate ultrafast bootstrap support.

Figure S7. Coalescent tree of Amblycera without the genus *Trinoton*. Based on ASTRAL analysis of a set of 2375 protein-coding genes, combining individual gene trees into a species tree. Numbers associated with branches indicate local posterior probability.

Figure S8. Dated phylogenomic tree of Amblycera. Based on the concatenated data set and a set of 2375 protein-coding genes. Numbers at nodes indicate 95% confidence intervals. Absolute ages are indicated at the bottom.

Figure S9. Squared residuals (bars) associated with each host-louse association. Error bars indicate upper 95% confidence intervals, and the dashed line indicates the overall median squared residual value (n = biologically independent samples).

Figure S10. Host tree used for cophylogenetic analysis in Jane. Numbers at nodes indicate time zones assigned to these nodes.

Figure S11. Distribution of total costs obtained from Jane tip mapping randomization with initial population size 30, 30 generations and 1000 samples. The dashed line indicates the total cost of 149, the lowest total cost obtained from the actual solution.

Figure S12. The most frequent isomorph of cophylogenetic reconstruction of optimal MPRs from Jane using the dated concatenated tree with the host tree in Figure S8 represents 1512 solutions. Cost scheme cospeciation, 0; duplication, 1; loss, 1; host-switching, 2; and failure to diverge, 1. Hollow circles indicate cospeciations, solid circles indicate duplications, dashed line indicates loss, and jagged line indicates failure to diverge. Arrows indicate the direction of host-switching. Colours of the circles indicate relative costs of reconstructed events compared with other possible solutions: Green events may be mapped at lower costs in other solutions, yellow events have equal costs in other solutions, red events have higher costs in other solutions.

Figure S13. Summary of cophylogenetic reconstruction of optimal MPRs using coalescent tree. Cost scheme duplication, 1; loss, 1; host-switching, 2. Arrows indicate the direction of host-switches. Numbers associated with events are the percentage of MPRs with that event.

Table S1. Solutions and their isomorphs obtained from cophylogenetic analysis using the dated concatenated tree and time constraints.

Table S2. List of manually inspected cophylogenetic solutions as representatives of isomorphs.

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