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Evolution and Biogeographic History of Rubyspot Damselflies (Hetaerininae: Calopterygidae: Odonata)

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Abstract: The damselflies Hetaerininae, a subfamily of Calopterygidae, comprise four genera distributed from North to South America: *Hetaerina*, *Mnesarete*, *Ormenophlebia* and *Bryoplathanon*. While several studies have focused on the intriguing behavioral and morphological modifications within *Hetaerina*, little of the evolutionary history of the group is well understood. Understanding the biogeographical history of Hetaerininae is further complicated by uncertainty in important geological events, such as the closure of the Central American Seaway (CAS). We generated a phylogenetic hypothesis to test the relationships and divergence times within Hetaerininae using IQtree and BEAST2 and found that *Mnesarete* and *Ormenophlebia* render *Hetaerina* paraphyletic. Reclassification of the genera within Hetaerininae is necessary based on our results. We also tested the fit to our dataset of two different hypotheses for the closure of CAS. Our results supported a gradual closure, starting in the Oligocene and ending in the Pliocene. Using Ancestral Character State Reconstruction, we found that the rubyspot, which is associated with higher fecundity in several species, was ancestral for Hetaerininae and subsequently lost four times. Estimates of diversification in association with the rubyspot are needed to understand the plasticity of this important character. Forest habitat was the ancestral state for Hetaerininae, with transitions to generalist species of *Hetaerina* found primarily in the Mesoamerican region. These results add to our understanding of the relationship between morphology, biogeography and habitat in a charismatic group of damselflies.

Keywords: biogeography; Zygoptera; wing coloration; mating behavior

1. Introduction

Extant Odonata (damselflies and dragonflies) represent some of the earliest branching lineages of winged insects [1]. While some species are long distance migrants, others do not stray far from their natal nymphal water source; indeed, dispersal capabilities are heterogeneous among this diverse clade of ~6300 species [2,3]. The Zygoptera (damselflies) are an extant suborder of Odonata and comprise over 3000 species distributed globally [4], with a hotspot in tropical Central and South America. Within Zygoptera, the Calopterygidae comprises over 150 species, including many species with metallic bodies and conspicuous wing coloration. The family is divided among three subfamilies: the clearwings or Caliphaeinae Tillaryd and Fraser, 1939, the demoiselles or Calopteryginae Selys, 1859 and the Hetaerininae Tillyard and Fraser, 1939.

Hetaerininae damselflies comprise four genera: *Hetaerina* Hagen, 1853 (rubyspot damselflies), *Mnesarete* Cowley, 1934, *Ormenophlebia* Garrison, 2006 and *Bryoplathanon* Garrison, 2006 (see Figure 1). Hetaerinines are easy to recognize due to their combination of dense wing venation, metallic green or reddish body coloration and unique male caudal appendage morphology [5]. We will use Hetaerininae and the common name rubyspot interchangeably, as the greatest diversity within hetaerinines is found within *Hetaerina*, the genus traditionally referred to as rubyspot damselflies.

Figure 1. Habitus images showing diversity between the four genera of Hetaerininae. (**A**) *Ormenophlebia imperatrix*, photo by Jim Johnson; (**B**) *Hetaerina amazonica*, photo by R. Guillermo-Ferreira; (**C**) *Mnesarete guttifera*, photo by R. Guillermo-Ferreira; (**D**) *Bryoplathanon globifer*, photo by Tom Kompier.

The nearly 70 species of Hetaerininae are distributed from North to South America, with some species occupying only forested habitats and others occupying both forest and grassland. Throughout their range, habitat and climate vary from arid desert to tropical rainforest. Variations in habitat over geological time may have caused barriers following dispersal, leading to speciation. Another likely barrier preventing dispersal was the Central American Seaway (CAS) [6], a body of water that separated North and South America until approximately 20–4 mya. As damselflies, in general, are relatively weak fliers compared to dragonflies and migratory insects [3,7,8], the likelihood that Hetaerininae could travel between Central and South America over open ocean, before the CAS was closed, seems low. However, a phylogenetic hypothesis does not exist for this subfamily, limiting our ability to test how these barriers may have influenced Hetaerininae speciation. If the Caribbean Sea was a barrier to lineage dispersal, then we expect that dispersal events would take place only after the closure of the CAS by continental landmasses or stepping stone islands.

Timing for the closure of the CAS may also have greatly influenced Hetaerininae speciation. Two main hypotheses for the timing of the closure of the CAS exist: (1) a gradual closure starting during the Oligocene and ending in the Pliocene (5.3–2.6 mya), with islands connecting landmasses [9]; (2) an abrupt closure during the Middle Miocene (16.0–11.6 mya) [10].

While studies have tested divergence times for Hetaerininae, they were limited in taxa inclusion and data, making them challenging to use in biogeographic studies. The earliest example is Dumont [11], who estimated the absolute rates of divergence times using the program r8s and suggested the age of Hetaerininae to be approximately 60 mya. The nodes were calibrated with enforcement of a minimum and maximum age constraint based on the fossil evidence. However, r8s assumes autocorrelated rates based on a user-inputted phylogeny, and some note that r8s-estimated node ages may be older than the estimates generated with Bayesian methods and may be less consistent among analyses (e.g., [12]), thus, the age of Hetaerininae may be far younger. Suvorov et al. [13] and Kohli et al. [14] found the age of Hetaerininae to be 43 million and 40 million years in age, respectively, using transcriptomes.

A younger age, as found by Suvorov et al. [13] and Kohli et al. [14], would mean an increased likelihood that dispersal between Central America and South America occurred early on in Hetaerininae evolution; while, with an older age of 60 million years, well before the CAS was closed, dispersal between these two landmasses would have been less likely early on.

For several species of rubyspot damselflies (e.g., *Hetaerina americana*, *H. titia* and *H. cruentata*), reproductive behavior, territoriality and overall fitness displays are well documented [15–21]. Males are territorial, and experimental manipulation of the size of their rubyspot has been shown to affect their fitness by increasing male fecundity [7,20,22] and reducing male survival and successful prey capture [23]. The rubyspot has been used to distinguish between two highly similar genera within Hetaerininae: *Hetaerina* and *Mnesarete* [5,24]. This taxonomic use of the rubyspot to separate the two groups assumes that the fitness benefits of red wing spots are present in all environments of Hetaerininae. That would suggest that once wing spots appear they are unlikely to disappear. However, populations of *H. aurora* have been found both with and without the rubyspot, and *H. titia* varies seasonally in wing color and size, supporting an alternative hypothesis that they might be advantageous as signals in some environments, but disadvantageous in other environments. In this scenario, we might expect to see repeated gains and/or losses of red wing pigmentation, making use of the basal rubyspot as an identifying character misleading. Additionally, we would expect to see an association between the basal rubyspot and habitat type. However, despite the behavioral and taxonomic importance of the rubyspot, evolution and habitat association of both the basal and apical rubyspots are not well understood and have never been studied across the entire subfamily.

Herein, we generate a phylogenetic hypothesis to: (1) test whether the CAS created a barrier to Hetaerininae dispersal using two different closure hypotheses; (2) explore the evolution of basal and apical wing coloration and preferred habitat (forest vs. generalist species) of Hetaerininae; (3) test the monophyly of the species groups, genera (*Hetaerina*, *Mnesarete* and *Ormenophlebia)* and the relationships between the genera.

2. Materials and Methods

2.1. Taxon Sampling

We sampled extensively across *Hetaerina* and *Mnesarete*, including 214 ingroup and outgroup taxa. The taxon sampling consisted of 13 of the 24 species of *Mnesarete* (40 specimens), 31 of the 39 species of *Hetaerina* (152 specimens) and two of the four species of the rare *Ormenophlebia* (three specimens), for a total of 46 ingroup species (192 specimens). We were not able to obtain *Bryoplathanon* spp. for the analysis. Multiple individuals from each species at different geographic locations were included to evaluate the monophyly for the proposed taxonomic genera and species within the family. Geographic origin, collector and Genbank Accession Numbers are summarized in Table S2. We identified specimens using the taxonomic keys provided in the revisions of this group [5,24]. Rosser Garrison confirmed the IDs.

2.2. DNA Extraction, Amplification and Sequencing

DNA extraction was performed primarily on the legs of dried specimens provided from both museums and personal collections. These included the Florida State Collection of Arthropods, as well as co-authors and collaborators at Rutgers University, Newark, USA, UFSCAR and GCEUMSNH (Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Mexico). For some species, a small portion of flight muscle tissue was also included. Additional species were collected in the field. All field-collected specimens were preserved in 95% ETOH following collection and deposited at the Monte Bean Museum Insect Cryo Collection.

For the majority of the taxa, DNA extraction, amplification and sequencing was performed at Brigham Young University (Provo, UT, USA). Three mitochondrial loci (COI 5′ end, COI 3′ end, 12S) and two nuclear loci (ITS1/ITS2, ef1a) were targeted for amplification using primers specifically designed for *Hetaerina* (Table S3; [25,26]). Gene fragments were amplified using standard polymerase chain reaction (PCR) techniques. Yield and potential contamination were monitored by gel electrophoresis. Sequencing was performed at the BYU DNA sequencing center. Collaborators also provided sequence data and additional sequence data was gathered from GenBank for 21 specimens (Table S2).

2.3. Phylogenetic Analyses

Sequence data was uploaded to Geneious 11.1.5 (https://www.geneious.com) (accessed on 12 October 2020), where it was assembled, edited, aligned and concatenated. All genes were aligned using MAFFT [27]. Phylogenetic relationships were reconstructed using maximum likelihood partitioned analyses in IQ-tree v1.6.12 [28]. Best-fit partitioning schemes were estimated using ModelFinder [29] in IQ-tree, allowing partitions to be merged to reduce over-parameterization and increase the model fit. One thousand replicates of Ultrafast bootstrap (BS) and SH-like approximate likelihood ratio test (SH-aLRT) were performed to estimate the node support [30,31]. The ggplot2, ggtree and ape packages in R v4.0.5 were used to view and analyze the phylogeny, and devtools, harrietr and phytools were used to summarize support values [32–35].

2.4. Divergence Dating Analysis

We ran a relaxed log-normal clock divergence dating analysis in BEAST v2.6.2 [36] using the birth death tree model to account for the possibility that rates changed continuously along the branches. We reduced our taxon sampling in the molecular matrix to include one sample per species, choosing the sample with the largest number of loci to minimize missing data among taxa, including a total of 62 individuals in the analysis. We used a fixed starting tree, generated in IQ-tree and made ultrametric in R using chronos in ape [33] to reduce computation time. We used ModelFinder [29] to estimate site models and merge similar partitions. Based on our results, COI 5′ end and COI 3′ end were merged for the BEAST2 analysis. As the default uniform clock prior is improper (i.e., the substitution rate cannot be less than 0), we used a lognormal prior for ucldMean and increased the speed of convergence by providing a mean value of 0.000001 [37]. Analysis of the log file with Tracer v1.7.1 [38] confirmed the rejection of the strict clock model with the observation that the 95% credible interval of ucld.stdev excluded zero.

Chosen fossils, their accession numbers, publication date and justifications for node calibrations are shown in Table 1 below. For fossil priors, we chose the oldest fossil crown member of a clade when multiple were available; all fossils were chosen with Parham et al. [39] best practices in mind. A lognormal distribution was used for three fossil priors; for this distribution, we used the minimum possible age in the fossils range as the zero offset and chose parameters such that the median was the maximum age range for the fossil. We used *Sinocalopteryx shangyongensis*, the oldest known Calopterygidae fossil, to set the maximum age for a uniform prior of our ingroup [40].

Table 1. Age of selected nodes and fossils included in analysis. See Figure S6 for numbered nodes.

Fossil	Accession Number	Publication	Fossil Placement	Age and Justification	Prior	Shape	Mean Age	CI
Calopteryx andancensis	PaleoDB collection 113893	Nel and Brisac [41]	Node 68	9.0–5.3 mya; Steininger et al. [42]	Lognormal	Offset = 5.3 Mean = 13.4	25.4	19.1, 32.0
Sapho legrandi	PaleoDB collection 194946	Nel and Petrulevičius [43]	Node 73	27.82–24.8 mya; Steininger et al. [42]	Lognormal	Offset = 24.8 Mean = 31.4	25.7	24.8, 27.9
Chlorocypha cordasevae	PaleoDB collection 105962	Nel et al. [44]	Node 119	11.1–9.4 mya; Steininger et al. [42]	Lognormal	Offset = 9.4 Mean = 13.2	42.5	31.2, 53.3
Sinocalopteryx shangyongensis	PaleoDB collection 194570	Lin et al. [40]	Node 65	56–5.3 mya	Uniform	Min = 5.3 Max = 56	53.5	47.6, 56.0
MRCA of Hetaerininae			Node 74				36.2	30.1, 42.1
North & Central American clade			Node 76				29.3	24.1, 34.8
South American clade			Node 87				28.4	23.1, 33.6
Ormenophlebia split			Node 82				23.2	18.5, 28.1
Ormenophlebia diverged			Node 86				4.7	2.6, 7.1

Three identical but separate BEAST2 analyses were run for 100,000,000 generations to ensure the mixing of the data. These results were combined, and the log files were evaluated in Tracer v1.7.1 to confirm the mixing of the data and to check ESS values before combining the tree file results. Ten percent burnin was an appropriate cut off for this dataset after observing the distribution, and so we removed 10% burnin from the tree files following the evaluation of log files in Tracer.

2.5. Ancestral Character State Reconstruction

Wing characters were coded based on voucher specimens. Maximum likelihood Ancestral Character State Reconstruction (ACSR) was run using the equal rates model in ace:ape [33]. Characters were mapped onto the ultrametric dated phylogeny generated in BEAST2 (see *Divergence dating analysis*).

Habitat data were divided into two categories: generalist (species is present in non-forested areas) and forest specialist (species only found in forested areas). Habitat type was determined based on a literature review [5,24,45–48] and personal observation of adult reproductive behavior.

Characters were as follows:

1. Male, Hindwing, color: hyaline wings (0), color only basally on wing (1), entire wing colored (2)
2. Male, Hindwing, apical color: absent (0), apical melanization only (1), multiple cells colored black or red (2)
3. Male, Forewing, apical color: absent (0), apical melanization only (1), multiple cells colored black or red (2)
4. Male, apical color: absent from forewings and hindwings (0), present in hindwing (1), present in hindwings and forewings (2). Presence was indicated by any color present, melanization or multiple cells.
5. Habitat: generalist (0), forest specialist (1)

2.6. Biogeography Analysis

We tested the fit of two different closure hypotheses of the CAS on the evolution of Hetaerininae using time stratified analyses in BioGeoBEARSv1.1.2 [49]. The analysis was limited to the ingroup only and run using standard options with a maximum range size of six. Areas were delimited based on Morrone et al. [50] and determined for each species based on Garrison [5,24] and collection records. We tested three different scenarios: two-time stratified analyses for the CAS closure, and a control with no distance constraints. The two-time stratified analyses tested the following hypotheses for the CAS closure timing: (1) a gradual closure starting during the Oligocene and ending in the Pliocene 4 mya, with islands connecting land masses [9]; (2) an abrupt closure during the Middle Miocene 14 mya [10]. Minimum geographic distance between landmasses was determined using paleogeography literature [9,10,51]. Time multiplication matrices for all the scenarios were coded as follows: separation between landmasses greater than 200 km was coded as 0, no separation was coded as 1 and variation between the two was coded between 0 and 1, depending on the distance between landmasses. A distance of 200 km and greater was treated as a barrier to dispersal, as that distance prevents dispersal of Hetaerininae to Cuba in the present day.

We also tested the fit of three different models in BioGeoBEARS; likelihood-based Dispersal-Extinction Cladogenesis (DEC), likelihood version of the Dispersal-Vicariance Analysis (DIVALIKE) and a likelihood range evolution model BAYAREALIKE [49] (Table S5). Each of the three models were tested with and without founder-event speciation (+J). We picked the best fitting biogeographical model for within and among scenarios using the lowest AICc values. Recent work has shown that +J models can be included in AICc comparisons [52]. The best selected reconstructed areas models for each scenario were mapped over the best time calibrated phylogeny. See File S1 for BioGeoBEARS scripts and results.

3. Results

3.1. Phylogenetic Analysis

ModelFinder merged the models for four partitions, resulting in seven partitions being included in the analysis, with protein coding loci coded for each position (see Supplementary Table S1). The total alignment length was 4979 sites, with 36.8% missing data.

The monophyly of both Calopterygidae and Hetaerininae was recovered with high support (91.9% SH-aLRT and 99% BS and 100% SH-aLRT and BS, respectively). The *Hetaerina pilula* clade was a sister to all other Hetaerininae. *Hetaerina* was not recovered as monophyletic, with both *Mnesarete* and *Ormenophlebia* nested within the genus. *Mnesarete* was also recovered as polyphyletic. Within the Hetaerininae clade, our results suggest that most of the species are highly supported monophyletic groups (Figure 2). There are two major clades within the phylogeny, one largely South American and one largely Mesoamerican (Figure 3).

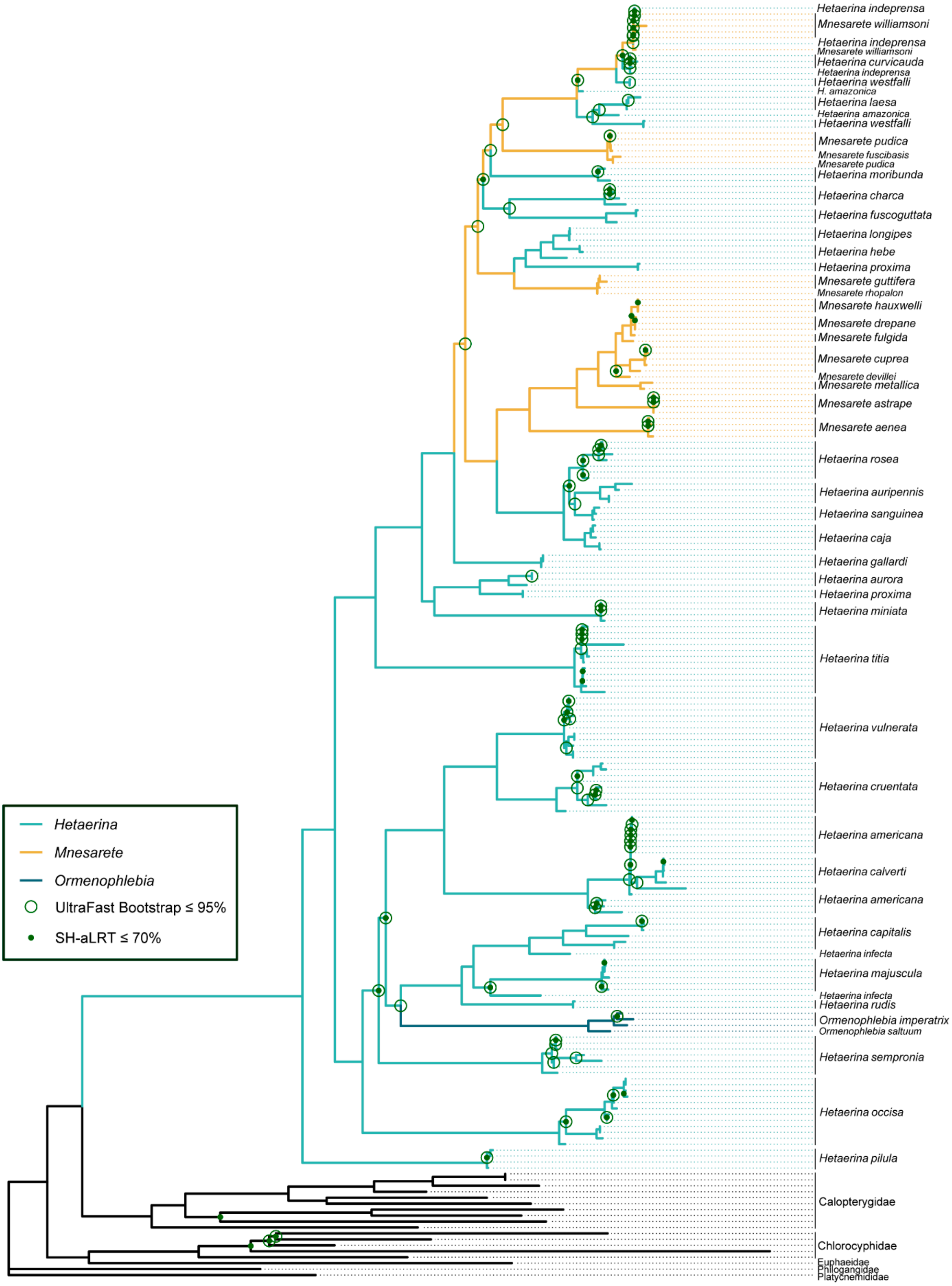

Figure 2. Maximum likelihood phylogenetic reconstruction of Hetaerininae estimated in IQtree. Relationships within and between species shown. Light blue branches: *Hetaerina* spp. Yellow branches: *Mnesarete* spp. Dark blue branches: *Ormenophlebia* spp. Large empty green circle: UltraFast bootstrap support less than or equal to 95%. Small filled green circle: SH-aLRT support less than or equal to 70%.

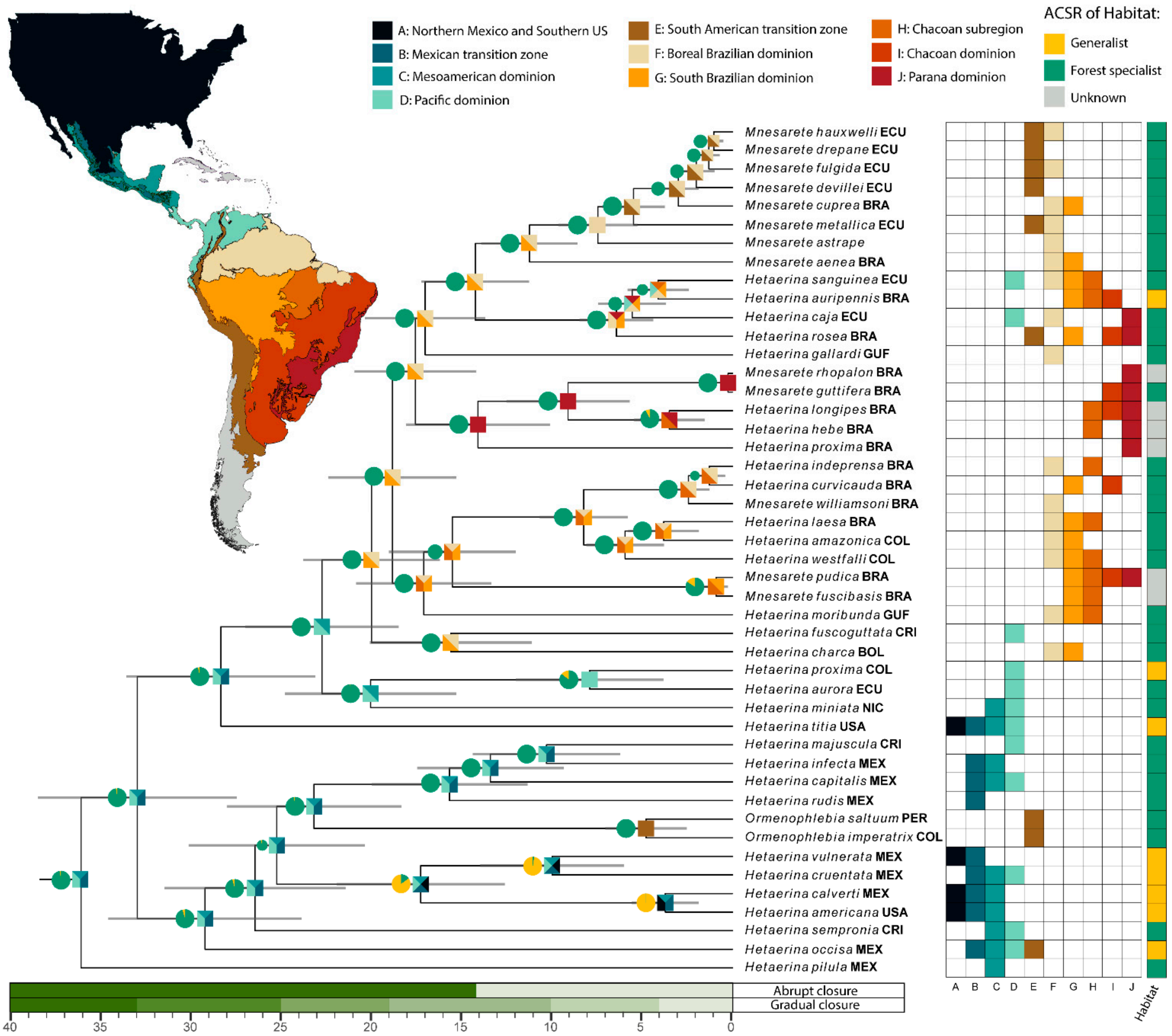

Figure 3. Hetaerininae biogeography analysis on time-calibrated phylogenetic tree. Biogeographic analysis conducted in BioGeoBEARS v1.1.2 with a maximum range size of six. Bayesian time-calibrated tree estimated in BEAST2 v2.6.6. Color-coded matrix corresponds with current species distribution. Squares at nodes represent the ancestral range with the highest probability from the Pliocene BAYAREALIKE +J analysis. Likelihood of dispersal between Central and South America for two hypotheses (abrupt Middle Miocene closure and gradual closure) shown. Dark green represents low likelihood of dispersal, light green easy dispersal. Map based on Morrone et al., 2022 area map [50]. Pie charts represent ACSR of habitat, generalist or forest specialist. Far right column on matrix corresponds to character states coded for ACSR of habitat.

3.2. Ancestral Character State Reconstruction: Wing Coloration

Male coloration, or the "rubyspot", at the base of the hindwing was ancestral to Hetaerininae (Figure 4). The rubyspot was lost at least four times within Hetaerininae. Independent losses were reconstructed in *Ormenophlebia* (6 mya), *Mnesarete williamsoni* (1 mya), the clade of *M. rhopalon* and *M. gutiffera* (1 mya) and the clade with *M. drepane*, *M. cuprea*, *M. metallica*, *M. astrape*, *M. aenea*, *M. fulgida*, *M. devillei* and *M. hauxwelli* (12 mya).

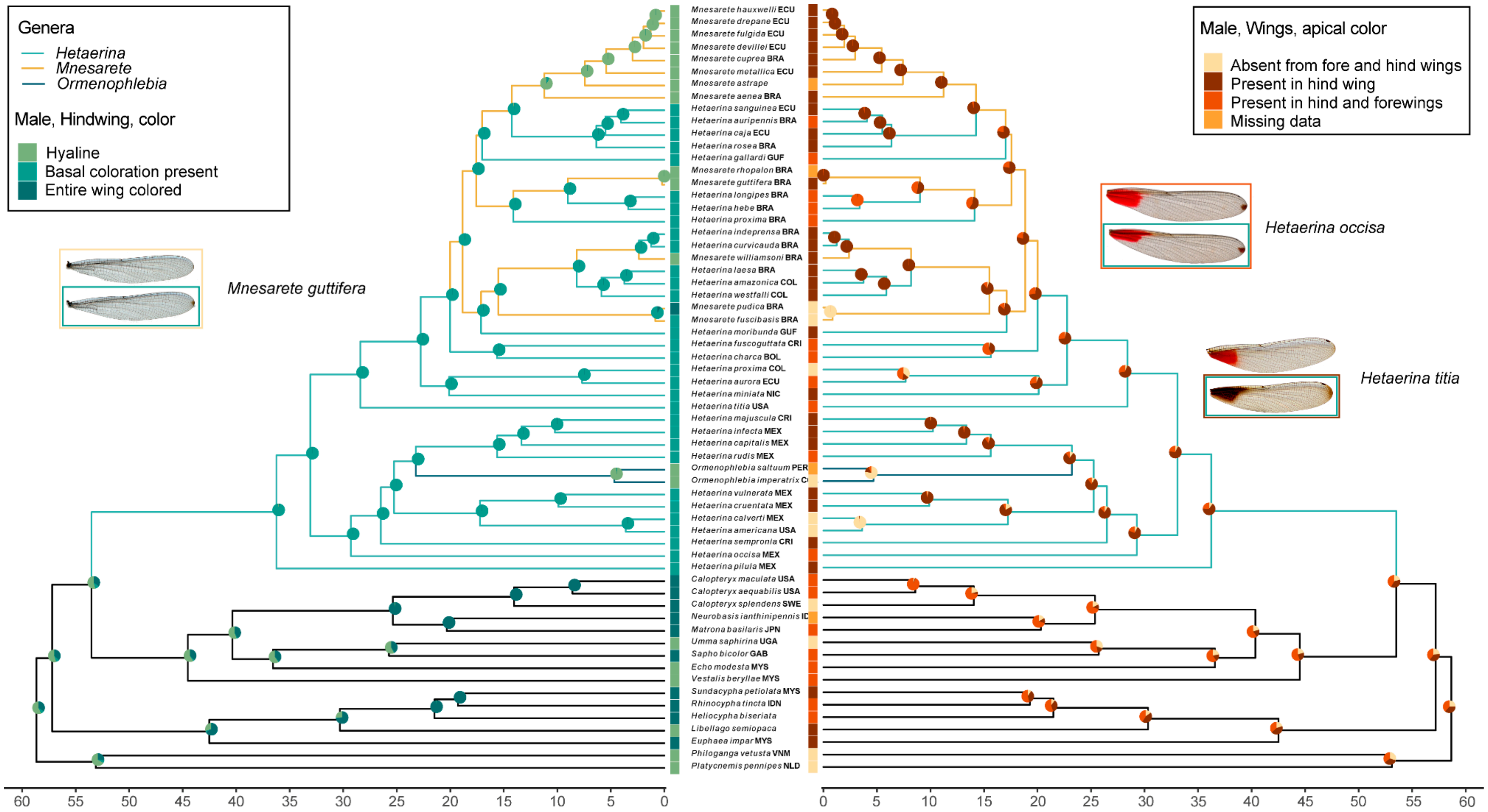

Figure 4. Likelihood ancestral character state reconstruction of coloration in male wings. Characters mapped onto divergence dated phylogeny generated in BEAST2. Pie charts show probability of presence or absence of state at each node. **Left:** ACSR of male hindwing color mapped onto phylogeny. Green: Hyaline wings. Teal: Basal coloration present. Dark blue: Entire wing colored. Basal coloration has the highest likelihood for the ancestral node of Hetaerininae. **Right:** ACSR of apical color in male wings mapped onto phylogeny. Tan: Apical color absent from forewings and hindwings. Dark red: Apical color present in hindwing. Red: Apical color present in hindwings and forewings. Orange: Missing data. Wings for three species shown, forewing on top, hindwing on bottom: *Mnesarete guttifera*, *Hetaerina occisa* and *Hetaerina titia*. Colored boxes show character state.

Male coloration apically in the hindwing was ancestral to Hetaerininae, and completely lost three times (in the past 2–6 my), with transitions to melanization only occurring five times (Figure S3). In contrast, male coloration apically in the forewing was probably absent ancestrally and gained five times (1–16 mya) (Figure S4). Apical coloration in both the hindwings and forewings has been gained eleven times, and the complete loss of coloration apically in both the forewings and hindwings occurred three times (2–6 mya).

3.3. Ancestral Character State Reconstruction: Habitat

The ancestral habitat for Hetaerininae was as a forest specialist, with five transitions to generalist habitat behavior, occupying either grassland or grassland and forest (Figure 3). One of the transitions occurred ~17 mya in the basal clade and included *H. vulnerata*, *H. cruentata*, *H. calverti* and *H. americana*. Within this clade, species were widespread, occurring from the Nearctic to the Neotropics. Other transitions between habitat preference were at the species level.

3.4. Divergence Dating and Biogeography

Four partitions, with a total of 3488 sites, were included in the analysis, with 20.5% missing data. Log files showed ESS values above 6000 for all parameters and mixing across all analyses. The most recent common ancestor (MRCA) for all the Calopterygidae originated 53.5 mya [CI 47.6, 56.0] during the early Eocene epoch (Figure 5 and Table 1). The MRCA of *H. pilula* and all other members of Hetaerininae originated 36.2 mya [CI 30.1, 42.1] (Figure 5) during the late Eocene. The two well-supported geographical clades, the primarily North and Central American clade (10 of 12 spp) and the primarily South American clade (31 of 33 spp), originated concurrently during the Oligocene epoch 29.3 mya [CI 24.1, 34.8] and 28.4 mya [CI 23.1, 33.6], respectively (Figure 5). Both of these clades diversified mostly during the Miocene; however, the South American clade also showed diversification during the Pliocene and Pleistocene epochs (Figure 5). Within the North/Central American clade, *Ormenophlebia* split off from the *Hetaerina* relatives 23.2 mya [CI 18.5, 28.1] and diverged approximately 4.7 mya [CI 2.6, 7.1]. On the other hand, within the South American clade, despite the polyphyletic recovery of *Mnesarete* species, the MRCA of all these lineages originated ~21 mya. All the lineages containing *Mnesarete* diversified in the Miocene, Pliocene, Pleistocene and Holocene epochs (Figure 5).

For each of the two tested scenarios for the CAS closure (i.e., an abrupt Middle Miocene closure and a gradual closure) and the control, the BAYAREALIKE +J model had the best fit AICc values (Table S5). In the time stratified analyses, closure starting during the Oligocene and ending in the Pliocene had the lowest AICc value. The ancestral areas reconstructed were consistent for all of the CAS closure scenarios. The most likely areas for the MRCA for all Hetaerininae and the other two geographical clades are the Mexican transition zone, Mesoamerican dominion and the Pacific dominion (Figure 3).

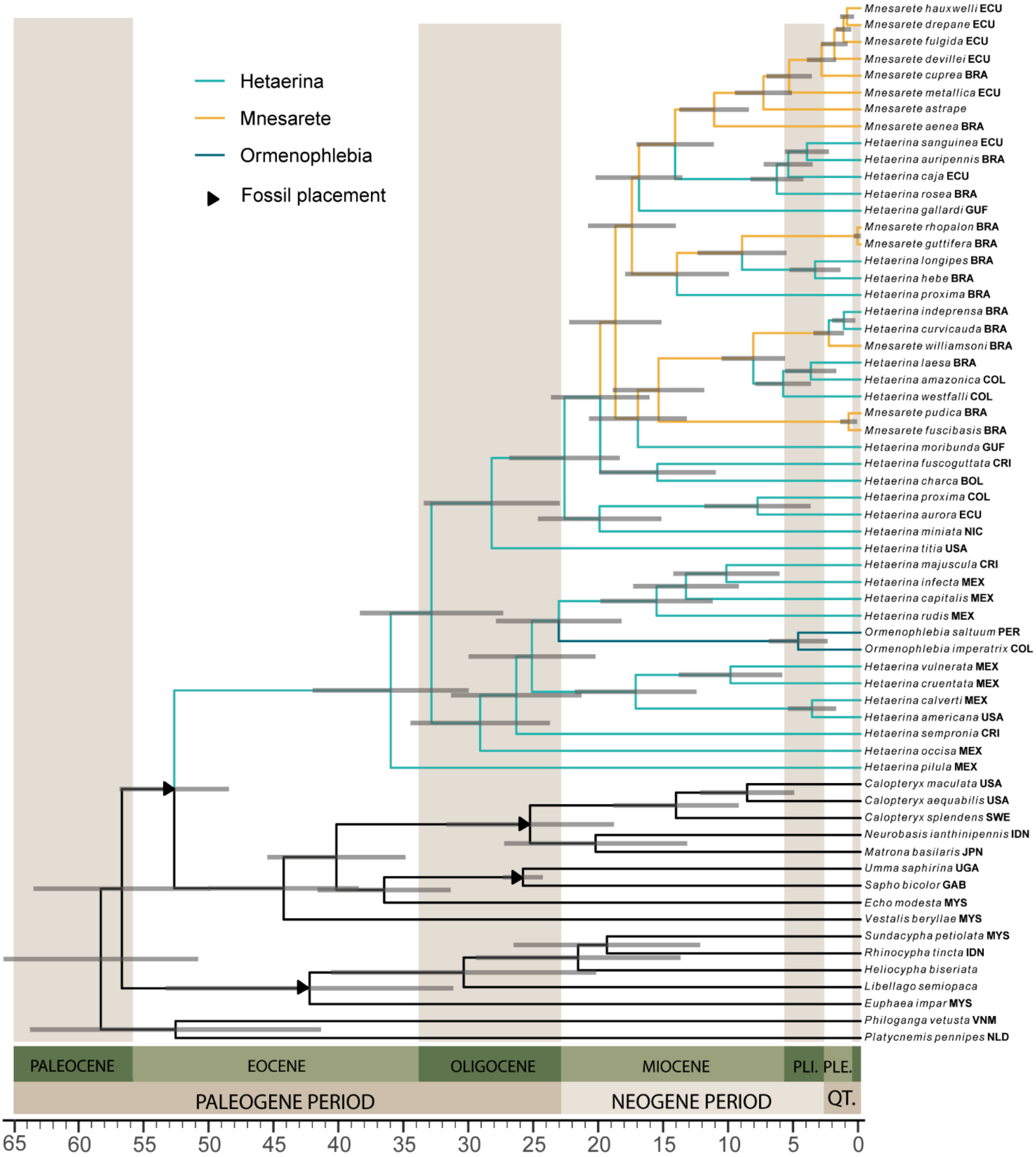

Figure 5. Phylogenetic relationships and time calibration of Hetaerininae. Bayesian time-calibrated tree estimated in BEAST2 v2.6.6 with median node ages, 95% high probability density (HPD) intervals for each node displayed in gray bars. Fossil-calibrated nodes represented with black triangles.

4. Discussion

Biogeography: Hetaerininae split off from the rest of Calopterygidae ~36 mya. Our reconstructions do not definitely place the pre-CAS ancestor in North or South America, with Hetaerininae having an ancestral area that comprised the Mexican transition zone, Mesoamerican dominion and the Pacific region. However, they are consistent in having a later dispersal into southern South America and northern North America and a gradual connection between the regions. Support for the gradual closure model suggests that

Hetaerininae dispersal was not restricted exclusively to overland dispersal and must have included some transoceanic dispersal. Surprisingly, this indicates that the Caribbean was not a complete barrier to dispersal.

The MRCA of *Ormenophlebia* dispersed to the southern range of the Pacific dominion ~5 mya. Starting during the Miocene, the Pebas System encompassed numerous wetlands and lakes covering >1 million km^2 [53] and may have facilitated further dispersal south of *Ormenophlebia* to the South American transition zone. As the Pebas wetland transitioned to a fluvial-dominated system ~10 mya, this would have promoted further dispersal. As dispersal was possible but unlikely ~20 mya, two separate clades formed within Hetaerininae: one primarily northern and one primarily southern (Figure 3). Shortly after dispersal to South America, the Andean uplift would have been another barrier to dispersal back to North and Central America. The Pebas System likely influenced dispersal in the South American clade as well, facilitating dispersal throughout the Boreal Brazilian dominion and the South Brazilian dominion [54].

Habitat and distribution: Interestingly, the generalist species of *Hetaerina* appear to be found primarily in the Mesoamerican region and are more widespread than most of the forest specialists. Towards the end of the Miocene, the temperature began dropping in North America, coinciding with a decrease in swamp forest and an increase in evergreen mixed forest [55]. This change in climate may have selected for species in North America with more diverse habitat preferences, while still retaining the ability to occupy tropical forests. This increased flexibility would then have allowed them to disperse more easily and occupy a wider range. In contrast, the increasingly tropical climate in South America may have supported the high diversification seen following dispersal to South America (Figure 3).

Phylogeny and taxonomy: Overall, the phylogenetic reconstruction shows unique patterns for the neotropical subfamily Hetaerininae. Our results suggest that *Mneserate* and *Ormenophlebia* are nested within *Hetaerina* (Figure 2); *Mnesarete* is paraphyletic and closely related to the mostly South American *Hetaerina* clade, while *Ormenophlebia* is monophyletic but nested within the mostly Central American clade (Figure 2). The general morphology of both *Mnesarete* and *Hetaerina* is similar, and they both possess cross-veins in the mid-basal space of their wings [5,24]; however, most of what is currently known as *Hetaerina* have a distinctive red basal spot on their wings [5]. Our results support Garrison's [5,24] view that the definition of *Mnesarete* is problematic and that this genus is paraphyletic. Further, while the majority of species form monophyletic clades, a few do not. The newly described *Hetaerina calverti* [56] is nested within *H. americana* in our analyses. *Mnesarete fulgida, M. drepane* and *M. hauxwelli* form a clade, with *M. fulgida* nested within *M. drepane. Mnesarete fuscibasis* is nested within *M. pudica* and renders it paraphyletic. *Hetaerina indeprensa, M. williamsoni* and *H. curvicauda* form a clade, with *H. indeprensa* nested within *M. williamsoni* and *H. curvicauda*. These results may be due to missing data. This is especially an issue when all members of a species do not have overlapping loci sequenced (e.g., *H. indeprensa*).

Geographic isolation has clearly played a large role in speciation in this cryptic group. For example, there are two separate clades of *M. westfalli*, one from Brazil and the other from Colombia. Similarly, *H. cruentata* is monophyletic but has two distinct clades, one from Central America (Costa Rica and Panama) and the other from Mexico. *Hetaerina occisa* is monophyletic but also forms two clades, one from Mexico and the other from Ecuador, Colombia and Peru. *H. infecta* is recovered as polyphyletic, and *H. hebe* is recovered as paraphyletic, but again this may be due to missing data as the taxa only had one locus for phylogenetic reconstruction.

What is clear is that further molecular and morphological studies that investigate the taxonomy of the group are needed. Our data calls for a new revision for all the neotropical calopterygid damselflies to establish valid monophyletic groupings. The task is not small and if our data are supported with further study, will likely require doing away with several genera, as *Hetaerina* Hagen in Selys 1853 has priority over *Mnesarete* Cowley 1934, *Ormenophlebia* Garrison 2006 and *Bryoplathanon* Garrison 2006.

Wing "ruby" spot evolution: Our results suggest that wing spot gain and loss is not correlated with habitat preference or phylogenetic position. In several species of *Hetaerina*, male red spots are sexually selected traits that mediate territorial interactions between males [16,57–62]. Wing spot traits (e.g., color and size) also play a role in mediating aggressive interactions between members of different species in the genus, and, in some instances, have been targeted by selection acting to reduce energetically costly interspecific fighting [7,61,62], but see [63,64] for instances where interspecific territoriality is maintained by selection. It is intriguing that one of the few *Mnesarete* species with extensively colored male wings also exhibits male-male territoriality and elaborate courtship displays [65]. Our phylogenetic analyses demonstrate that wing spots have been lost four times in this clade (Figure 4). Given the importance of wing spots in territorial interactions, further research into the reproductive strategies of species without wing spots is imperative to understanding the selective pressures that led to the loss of wing spots.

5. Conclusions

A revised taxonomical classification is needed, and color is not a diagnostic character for classification purposes in Hetaerininae. Morphological traits, such as male cerci and female intersternites, may provide morphological evidence for a stronger classification scheme. Further research on the behavior and ecology of species with hyaline wings is needed to better understand why wing coloration was lost in some lineages. Our results support a gradual dispersal of Hetaerininae from North America to South America that began in the Oligocene and that the Caribbean was not a barrier to dispersal. We suggest that extension of the Isthmus of Panama during the Oligocene, shortening the distance between Central America and South America, assisted in their dispersal. However, the lack of Hetaerininae on Caribbean islands suggests transoceanic dispersal to be challenging for this group. Further work is needed to fully understand their complicated dispersal history. An interesting avenue for research that our study only superficially examined is the evolution of Hetaerininae coloration (both body and wing) and habitat, specifically the light environment of forest vs. grassland habitat. This combined with estimates of diversification should allow for greater insight of the ecological shifts in their evolutionary history.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14090757/s1, Figure S1: Maximum likelihood IQtree results with full node support; Figure S2: Male, Hindwing color, Ancestral Character State Reconstruction; Figure S3: Male, Hindwing, apical color, Ancestral Character State Reconstruction; Figure S4: Male, Forewing, apical color, Ancestral Character State Reconstruction; Figure S5: Male, apical color in fore and hind wings, Ancestral Character State Reconstruction; Figure S6: Dated phylogenetic results with numbered nodes; Table S1: Partitions for IQTree analysis; Table S2: Taxa included in analysis; Table S3: Primers used in analysis; Table S4: Taxa included in BEAST2 and BioGeoBEARS analyses; Table S5: BioGeoBEARS model testing results; Table S6: Ancestral Character State Reconstruction matrix. Sequence data submitted to GenBank. File S1: BioGeoBEARS scripts and results.

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Data Availability Statement: The data presented in this study are openly available in GenBank, see Table S2 for accession numbers.

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