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Diversification patterns of Haeterini butterflies (Nymphalidae: Satyrinae)

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The understanding of evolutionary relationships among insect lineages has received special attention in recent years, leading to an increasing number of studies aiming to shed light on their diversity, biogeography, and species boundaries. Despite the growing use of integrative taxonomic approaches, the positioning and relationships of some groups remain controversial, mainly due to limited sampling and low-resolution genetic markers. This is the case of the Neotropical butterfly tribe Haeterini (Nymphalidae: Satyrinae), characterized by unique coloration patterns in which species display either transparent or dark wings. To date, species boundaries within Haeterini remain uncertain due to limited knowledge of their natural history and reliance on traditional morphology. Our study investigated species delimitation patterns and phylogenetic relationships within Haeterini using molecular and morphological data. Our sampling included most of the tribe's distribution range, including previously unsampled regions. We applied quantitative methods to analyze wing coloration patterns and explored the tribe's biogeographic history and diversification. Our findings contrast with previous studies and reveal weak support for some currently recognized species and subspecies, challenging Haeterini taxonomy. Additionally, the biogeographical analysis failed to identify a region of origin for the tribe but suggested dispersal-driven diversification with 8 vicariance events during the Miocene, consistent with major physiographic events such as the Pebas and Acre wetlands and the Andes orogeny. The tribe's diversification likely occurred at a relatively low, constant rate, with few speciation events, aligning with the museum model of diversification in the Neotropics.

Keywords: phylogenetics, diversification, morphology, butterflies, Haeterini

Introduction

Accurate species delimitation is essential for reliable biodiversity assessments and for properly estimating species extinction rates, especially in biodiversity hotspots such as the Neotropics (Brown 2014, Liu et al. 2022). Despite the description of ~18,000 new species every year (Zamani et al. 2021), many taxa remain undescribed, limiting our understanding of evolutionary relationships in taxa with high morphological variability or cryptic diversity (Gaston et al. 1995, Vodă et al. 2015). Also, traditional species descriptions based solely on morphology and geographic distribution often fail to account for intraspecific variation or cryptic diversity, leading to misclassifications (Padial et al. 2010). To address these limitations, integrative taxonomy has emerged as a powerful approach that combines morphological, molecular, ecological, and behavioral data to improve species delimitation (Padial et al. 2010). This approach has been successfully applied to multiple taxa, from crustaceans to butterflies, revealing hidden diversity and clarifying evolutionary relationships (eg Zimmermann et al. 2018; Shimizu et al. 2020), including those at the intrageneric level, such as in rotifers of the genus *Brachionus* (Hwang et al. 2013).

Butterflies are among the most studied insects, but species delimitation and phylogenetic relationships in certain groups,

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such as the subfamily Satyrinae, remain unsolved. The reasons may be 2-fold. First, the large number of species in the subfamily (~2,400 worldwide; Ackery et al.1998) makes it difficult to sample all lineages for comprehensive phylogenetic analyses. Second, the rapid radiation of the subfamily leads to inconsistencies among phylogenetic hypotheses generated by different methods, thereby obscuring its evolutionary history (Peña et al. 2011). However, several studies have attempted to clarify the phylogenetic relationships between groups in the subfamily (Price et al. 2011, Verovnik and Wiemers 2016), while others have gone further to study their biogeographical history, diversification patterns, and the factors influencing their distribution (Peña et al. 2011, Kodandaramaiah et al. 2018).

Within Satyrinae, the tribe Haeterini is a small but fascinating group of Neotropical butterflies known for their transparent (hereafter "clearwing") and opaque wing coloration (Penz et al. 2014). These wing phenotypes could serve either as a camouflage strategy, reducing their detectability by predators (as in other butterflies), or as a sexual strategy (as suggested by some species that exhibit sexual dimorphism; McClure et al. 2019). This tribe comprises 5 genera that exclusively occur in the Neotropical rainforests, from southern Mexico to the Atlantic Forest of Brazil. To date, species boundaries within Haeterini remain poorly explored and understood, largely because their taxonomy relies on traditional morphological traits and limited sampling across their distribution (Matos-Maraví et al. 2019). The latter may be due to their efficient camouflage and low population density, which make them difficult to find in the wild.

Traditional species descriptions in the tribe based on wing phenotype, genitalia morphology, and geographical distribution (Constantino 1995, Lamas 2004, Penz et al. 2014) have led to the identification of 29 species and 39 subspecies (Lamas 2004, Penz et al. 2014, Willmott 2015, Zacca et al. 2016). However, these numbers may be an over- or underestimation. In consequence, a recent coalescent multispecies approach using molecular data tested species boundaries in Haeterini and included 18 species and 18 subspecies (Matos-Maraví et al. 2019). The results supported the recognition of 22 species and raised questions about the status of some described lineages, such as *Pierella keithbrowni*. However, this study did not include samples from northern South America, a region with complex topography where the Andes mountain range and the inter-Andean valleys provide diverse niches that have likely contributed to the diversification of groups like spiders and birds (Cadena et al. 2020, Salgado-Roa et al. 2021). In this study, we expanded the geographical and molecular sampling of Haeterini to test evolutionary relationships within the tribe. We also evaluated delimitation of species by combining new and previous samples and implementing an integrative taxonomic approach. Additionally, we assessed whether a quantitative method for analyzing wing color patterns could distinguish between species and subspecies. Finally, we explored biogeographical patterns associated with major physiographic events (eg the uplift of the Andes) and described the diversification patterns observed within the tribe.

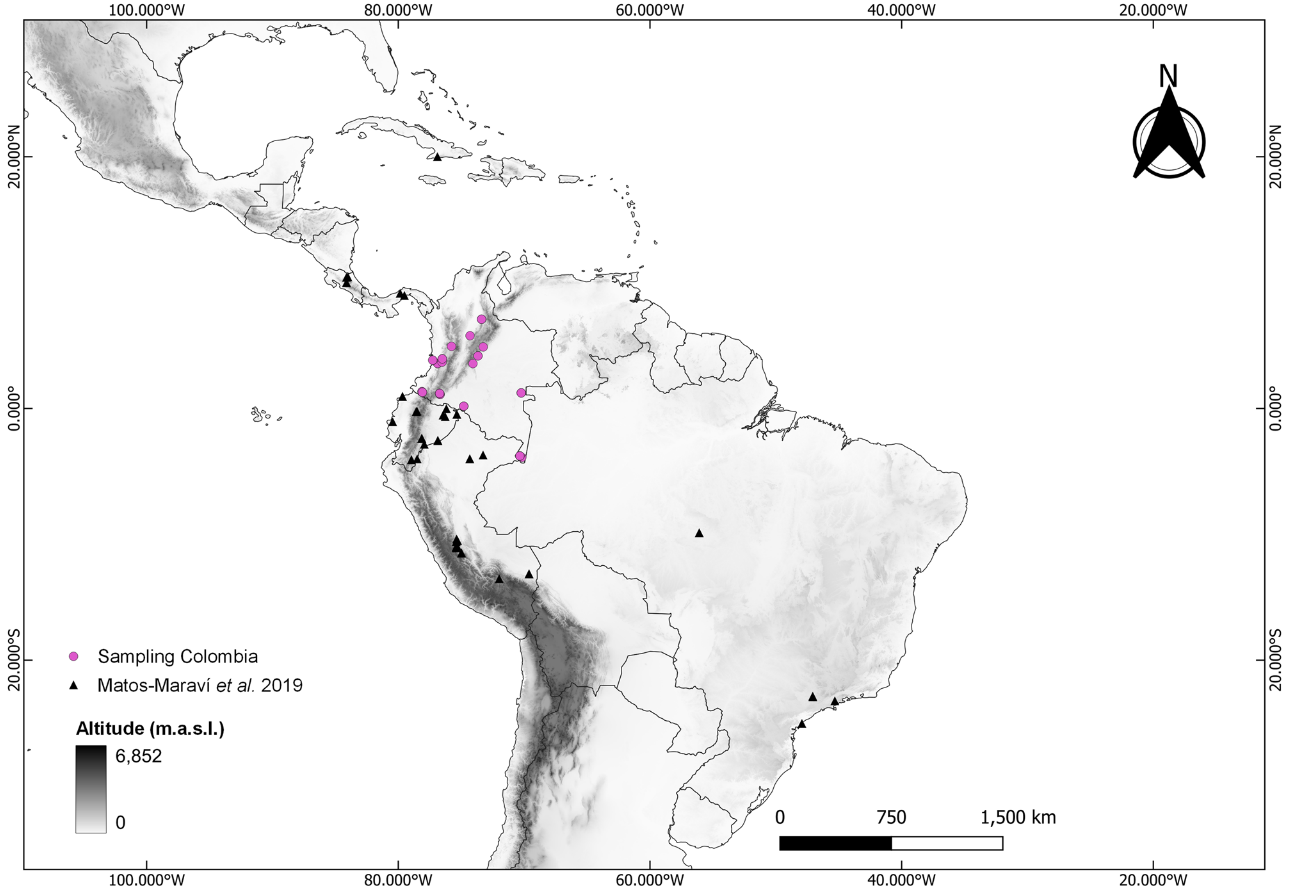

Fig. 1. Geographical distribution of Haeterini specimens collected in Colombia, including previously sampled individuals from Matos-Maraví et al. (2019).

Materials and Methods

Taxon Sampling

We collected Haeterini butterflies across most of their distributions in Colombia (Fig. 1). Specimens were identified at the species and subspecies level using wing morphological traits and following the taxonomic revisions (Constantino 1995, Lamas 1997, Salazar and Constantino 2007, Penz et al. 2014, Paluch et al. 2015, Willmott 2015, Zacca et al. 2016, Penz 2021) and photographs of type specimens available at https://www.butterfliesofamerica.com (Warren et al. 2023). The assigned identification was then verified by an expert on the tribe. In this way, our sampling consisted of 5 genera, 16 species, and 12 subspecies.

DNA Extraction, PCR, and Sequencing

We extracted DNA from the thorax of 68 individuals (Supplementary Table S1) using either the DNeasy Tissue Kit (QIAGEN) or SeraPure magnetic beads (Rohland and Reich 2012, Arias-Cárdenas et al. 2024), incubating with Proteinase K at 56 °C for 24 h. The quality and quantity of DNA were verified using spectrophotometry and electrophoresis. Then, we amplified 2 gene fragments, *Cytochrome Oxidase I* (*COI*; ~700 bp) with the primers LCO1490 and HCO2198 (Folmer et al. 1994) and *Elongation Factor 1-alpha* (*EF1α;* ~700 bp) with the primers EF1-F2361 and EF1-R3093 (Jordan et al. 2003). Amplicons were Sanger sequenced at Macrogen (South Korea), and a quality check was performed using Geneious Prime (v.2023.0.4; Biomatters Ltd.). Sequences were aligned with MAFFT (Katoh and Standley 2013) and manually adjusted with AliView (Larsson 2014). For the nuclear gene *EF1α*, we performed haplotype reconstruction using PHASE on DNAsp v.6 (Rozas et al. 2017) to retrieve the 2 alleles. We also downloaded *COI* and *EF1α* sequences from GenBank for 18 Haeterini and 16 Satyrinae species. Accession numbers for sequences generated in this study and those already available are in Supplementary Table S1.

Phylogenetic Analyses

We estimated phylogenetic relationships within the Haeterini tribe using Maximum Likelihood (ML) and Bayesian Inference (BI) with both individual-gene and concatenated alignments. The sequences of the nuclear genes (*EF1α*, *CAD*, *GAPDH*, *rps5*, and *wingless*) were left with heterozygous sites for the concatenated alignment. We then inferred ML phylogenies with IQ-TREE v1.6.12 (Nguyen et al. 2015). For individual gene fragments, we identified the best nucleotide substitution model with ModelFinder (Kalyaanamoorthy et al. 2017) based on the Bayesian Information Criterion (BIC; Schwarz 1978), whereas for the concatenated alignment, we identified the best partition scheme using PartitionFinder (Lanfear et al. 2012) and the best nucleotide substitution model for each partition using IQ-TREE v.2 (Minh et al. 2020). Node supports were calculated with 1,000 ultrafast bootstrap pseudo-replicates (Hoang et al. 2018), SH-alrt (Guindon and Gascuel 2003), local bootstrap test (Adachi and Hasegawa 1996), and aBayes (Anisimova et al. 2011). For the BI phylogenies, we used JmodelTest2 (Darriba et al. 2012) to estimate the best nucleotide substitution model for each gene using the BIC criterion. All BI analyses followed (Matos-Maraví et al. 2019) and were performed in MrBayes v3.2.7a (Ronquist et al. 2012). We executed 2 independent runs with 4 Markov Monte Carlo Chains (MCMC) for 20 million generations, sampling every 1,000 generations and applying a 25% burn-in. For the concatenated alignment, we used a mixed model (I + G) with 20 million generations.

Estimation of Divergence Times

The divergence times of the Haeterini tribe were estimated using the concatenated alignment using BEAST 2.7.4 (Bouckaert et al. 2019) on the CIPRES server (Miller et al. 2010). We set and optimized a relaxed clock with the ORC package, applying a GTR + F + I + G4 substitution model for *COI*, a GTR + I model for *EF1α*, a GTR + F + I + G4 for *CAD-GAPDH-RpS5*, and a GTR + F + I + G4 for *wingless*, with a Yule Model. The tree was secondarily calibrated using the 95% high posterior density (HPD) from Chazot et al. (2019) and followed a conservative approach with uniform distributions for the nodes: (i) Brassolini and Morphini (32 to 58 Ma), (ii) Melaniti and Dirini (23 to 47 Ma), (iii) Lethina, Paragina, and Mycalesina (28 to 65 Ma based on the fossil of *Lethe corbieri*, which is part of Lethina), (iv) crown age of Satyrini (32 to 53 Ma), and (v) crown age of the Satyrini's sunclade, which includes Pronophilina, Euptychiina, Satyrinae, Erebiina, and others (32 to 53 Ma). We used a lognormal distribution with the following parameters ($M = 47.5$, $S = 1.0$, and Offset = 38) for the crown age of Satyrinae. MCMCs were run for 50 million generations, which were sampled every 1,000 generations. The effective sample size was checked in Tracer v.1.6 (Rambaut et al. 2018), and the maximum clade credibility tree was produced in TreeAnnotator v.2.6.6 (Drummond and Rambaut 2007) with a burn-in of 25%. Tree editing and visualization were performed in FigTree v1.4.4 (Rambaut 2018).

Color Analysis

To explore the variation in hindwing color patterns across the Haeterini tribe, we performed a color pattern analysis using the *Patternize* and *Recolorize* packages in R (Van Belleghem et al. 2018, Weller et al. 2024). For this purpose, we scanned the wings of 76 individuals using an EPSON Perfection V500 scanner at 12,000 dpi resolution; wings that were broken or bent were excluded. The resulting images were aligned with the landmark scheme depicted in Supplementary Fig. S1 using Fiji (Schindelin et al. 2012). We used 3 bins per color channel for *Pierella* and *Pseudohaetera* (Brown 1942), 4 bins per color channel for *Haetera*, and 5 bins per color channel in *Cithaerias*, with a cutoff of 35 in all genera. The resulting color maps were transformed into a pattern object using the "*recolorize_to_patternize*" function, and this object was used to plot a Principal Components Analysis (PCA) and visualize the relationship between the hindwing phenotype of all species in each genus. Because *Haetera* and *Cithaerias* exhibit sex dimorphism in wing coloration, we analyzed these genera by combining data from both sexes, as well as independently for each sex.

Biogeography

To infer the biogeographic history of Haeterini, we collected information on the current species distributions available from primary literature and taxonomic sources (Lamas 1997, 2004, Salazar and Constantino 2007, Constantino and Rodríguez 2009, Penz et al. 2014, Paluch et al. 2015, Zacca et al. 2016, Vila-Verde and Paluch 2020, Penz 2021) and distribution information provided by Patrick Botto (personal communication). We defined 10 regions based on those proposed by Sánchez-Herrera et al. (2020), which were coded as follows: (A) Amazon Basin, (B) Brazilian Shield, (C) Central America, (D) Central Andes, (E) Guiana Shield, (F) Northeastern Andes, (G) Northwestern Andes, (H) Tumbes-Chocó-Magdalena, (I) Venezuelan Highlands, and (J) Atlantic Forest, the latter noted for its high endemism. We used the BioGeoBEARS package (Matzke 2013) implemented in RASP v.4.0 (Yu et al. 2020) to test 6 biogeographical models for 3 different scenarios: (i) a control with free dispersion—S0, (ii) a scenario that considers the Pebas and Acre system—S1, and (iii) a scenario based on the uplift of the Andes—S2. Because no extant species occupies more than 5 areas, ranges were limited to a maximum of 5 areas. Impossible adjacency combinations were manually excluded from the BioGeoBEARS range list used for the inferred ancestral biogeographical reconstructions. We favored the BAYAREASLIKE model in the control scenario and DEC in the remaining scenarios based on the AICc_wt score in BioGeoBEARS (Supplementary Table S2).

Species Delimitation Analysis (iBPP)

We used Bayesian species inference analysis (iBPP; Solís-Lemus et al. 2015) to delimit species boundaries in the Haeterini tribe using 3 datasets: (i) by genus, (ii) in clearwing species, and (iii) in opaque species. The first dataset included both molecular and morphological data. The second and third datasets comprised only molecular data. We specified 9 parameter combinations of the prior distribution for the ancestral population size (θ) and the root age of the tree (τ), ranging from scenarios that represent large population sizes and a deep divergence time ($\theta = G(1, 10)$ and $\tau = G(1, 10)$) to those representing small population sizes and a shallow divergence time ($\theta = G(2, 2000)$ and $\tau = G(2, 2000)$) as previously described (Eberle et al. 2016, Olave et al. 2017, Pardo-Diaz et al. 2019, Arias-Cárdenas et al. 2024). The MCMC analysis was run for 50,000 generations, sampling every 1,000 steps, and using a 10% burn-in. The locus-specific evolution rate was fine-tuned with the auto-option.

Diversification Time

We used RevBayes (Höhna et al. 2016) to identify the model that best explains diversification within Haeterini. Bayes factors (Kass and Raftery 1995) were calculated using both stepping stone sampling (Xie et al. 2011) and path-sampling (Lartillot and Philippe 2006) in 3 models: (i) Yule Model, (ii) Birth-Death Model, and (iii) Episodic Birth-Death Models with 4, 5, and 10 intervals (Supplementary Table S3). For the last model, we used the function "*setMRFGlobalScaleHyperpriorNShifts*" from the R package RevGadgets (Tribble et al. 2022) with the HSMRF model to estimate the hyperpriors. We used an incomplete sampling fraction of 35/67 (0.52) lineages across all models and ran 50,000 MCMC generations with a burn-in of 5,000. The parameter outputs were examined using Tracer V.1.6. We estimated the mlnL of each model by sampling the power posterior for 1,000 MCMC generations with a burn-in of 25%, and we visualized the results using the RevGadgets R package (Tribble et al. 2022, R Core Team 2023).

Results

Phylogenetic Analyses

Our phylogenetic analysis in the Haeterini tribe is the most taxonomically complete to date, expanding the sampling of Matos-Maraví et al. (2019) with the inclusion of species and subspecies not previously analyzed, such as *Pierella hyalinus*, *Pierella lamia*, *Pierella amalia*, *Pierella astyoche*, *Pseudohaetera mimica*, *Cithaerias pireta magdalenensis*, and *Pierella helvina pacifica*, from multiple regions in Colombia. The ML and BI phylogenetic reconstructions for the concatenated dataset recovered 2 major groups that were consistent with the wing phenotype: (i) opaque species (*Pierella*) and (ii) clearwing species (*Dulcedo*, *Pseudohaetera*, *Haetera*, and *Cithaerias*). Both groups were monophyletic and well-supported sister clades (ultrafast bootstrap = 100%, PP = 1; Fig. 2).

Most species within the opaque clade were recovered as well-supported clades (ultrafast bootstrap >90%, PP >0.9). We observed a clade formed by *P. lamia*, *Pierella chalybaea*, and *P. keithbrowni* as sister to *Pierella hyceta ceryce*. This larger clade was the sister of *Pierella hyceta hyceta*. Although, *P. h. ceryce* and *P. h. hyceta* are currently recognized as subspecies of *P. hyceta* and were recovered as monophyletic, they were not grouped together, thus suggesting the paraphyly of this species. In addition to these 4 species, *Pierella luna*, *Pierella nereis*, and *Pierella hortona* formed a larger clade as shown in Fig. 2. All of the species above cluster in a group closely related to the strongly supported clade of *Pierella lucia* + *P. astyoche* (ultrafast bootstrap = 100%, PP = 1), within which the 2 species are not reciprocally monophyletic. The latter clade was sister to *P. helvina*, and together, they formed a clade closely related to *P. hyalinus*. Finally, all of these species were sister to *Pierella lena*, which formed a clade that also contained the only sample of *P. amalia*. None of the individuals of the newly included species (*P. lamia*, *P. astyoche*, and *P. amalia*) were monophyletic except for *P. hyalinus*. The subspecies showed mixed patterns. For example, we did not observe clusters consistent with the subspecies described for *P. luna*, *P. lena*, and *P. hortona*. In contrast, within *P. helvina*, we recovered 3 clades consistent with 3 subspecies, although *Pierella helvina pacifica* and *Pierella helvina ocreata* showed little differentiation based on the branch length (Fig. 2).

In the clearwing clade, we observed 4 monophyletic groups consistent with the described genera (ultrafast bootstrap = 100%, PP > 1). The first, *Dulcedo*, appeared as a monotypic clade sister to the other clearwing species. The second, *Pseudohaetera*, was sister to the clade formed by the genera *Haetera* and *Cithaerias* and showed that *Pseudohaetera hypaesia* and *P. mimica* are differentiated and monophyletic (ultrafast bootstrap = 100%, PP = 1). The third and fourth clades, corresponding to *Haetera* and *Cithaerias*, respectively, were sister. The species in *Haetera* were not monophyletic, and the subspecies (*Haetera piera negra*, *Haetera piera piera*, and *Haetera piera pakitza*) were also genetically indistinguishable (Fig. 2; Supplementary Table S4).

In the case of *Cithaerias*, we observed 2 distinct clusters (ultrafast bootstrap = 100, PP = 1). The first cluster consists of *C. pireta*, *Cithaerias aurora*, and *C. pireta magdalenensis*, with the latter being the only monophyletic (ultrafast bootstrap = 100%, PP = 1). The second cluster is a mixture of *Cithaerias aurorina*, *Cithaerias cliftoni*, *C. aurora*, *Cithaerias pyropina*, *Cithaerias bandusia*, and *C. pireta*. Within this

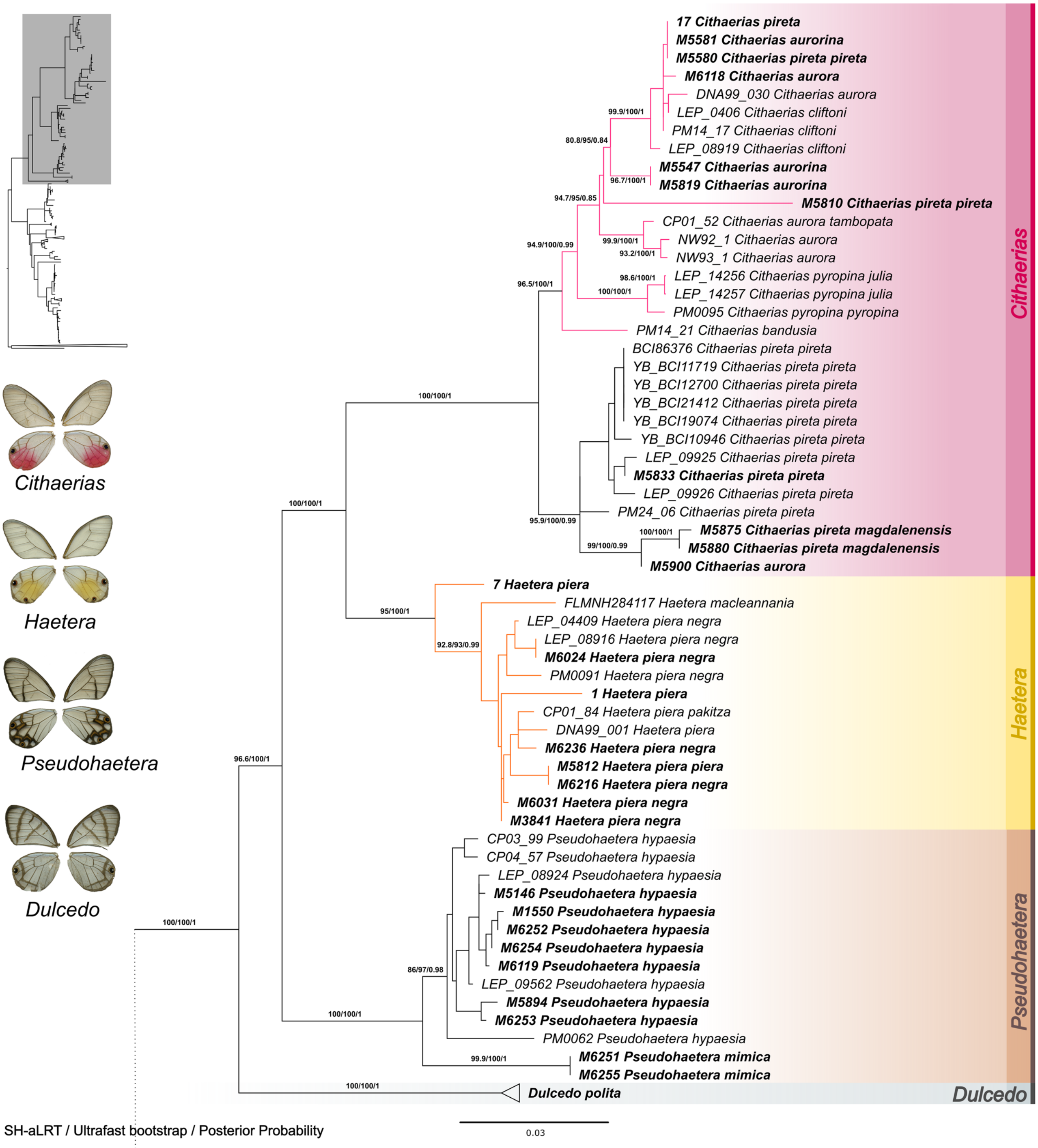

Fig. 2. ML consensus phylogenetic tree based on the concatenated dataset (COI, EF1α, CAD, GAPDH, rps5, and wingless). Nodes show the SH-aLRT support on the left, ultrafast bootstrap support in the middle, and the posterior probability on the right. An asterisk (*) indicates lack of support. Bold text indicates newly included samples. The inset tree in the top left highlights the position within the larger tree.

cluster, we recovered the species *C. pyropina* and its subspecies as differentiated and monophyletic (ultrafast bootstrap > 90%, PP = 1). Furthermore, 1 sample of *C. aurora* (M5900) and *C. pireta magdalenensis* were grouped together (ultrafast bootstrap = 99%, PP = 0.99).

When we inferred gene topologies for *COI* and *EF1α* (ie the genes for which we obtained sequence data), we also recovered 2 major sister clades that matched the wing phenotype (ie clearwing and opaque species; *EF1α*: PP = 0.98, ultrafast bootstrap = 99%; *COI*: PP = 0.86, ultrafast bootstrap = 79%). We also recovered 5 clades corresponding to the genera in the tribe (color coded, Supplementary Fig. S2). The relationships between genera revealed by the 2 genes were highly consistent, except for the sister group of *Cithaerias* (*Haetera* in the mitochondrial gene and *Pseudohaetera* in the nuclear gene).

At the species level, within *Pierella*, the nuclear gene was unable to resolve the relationships among some species (clade

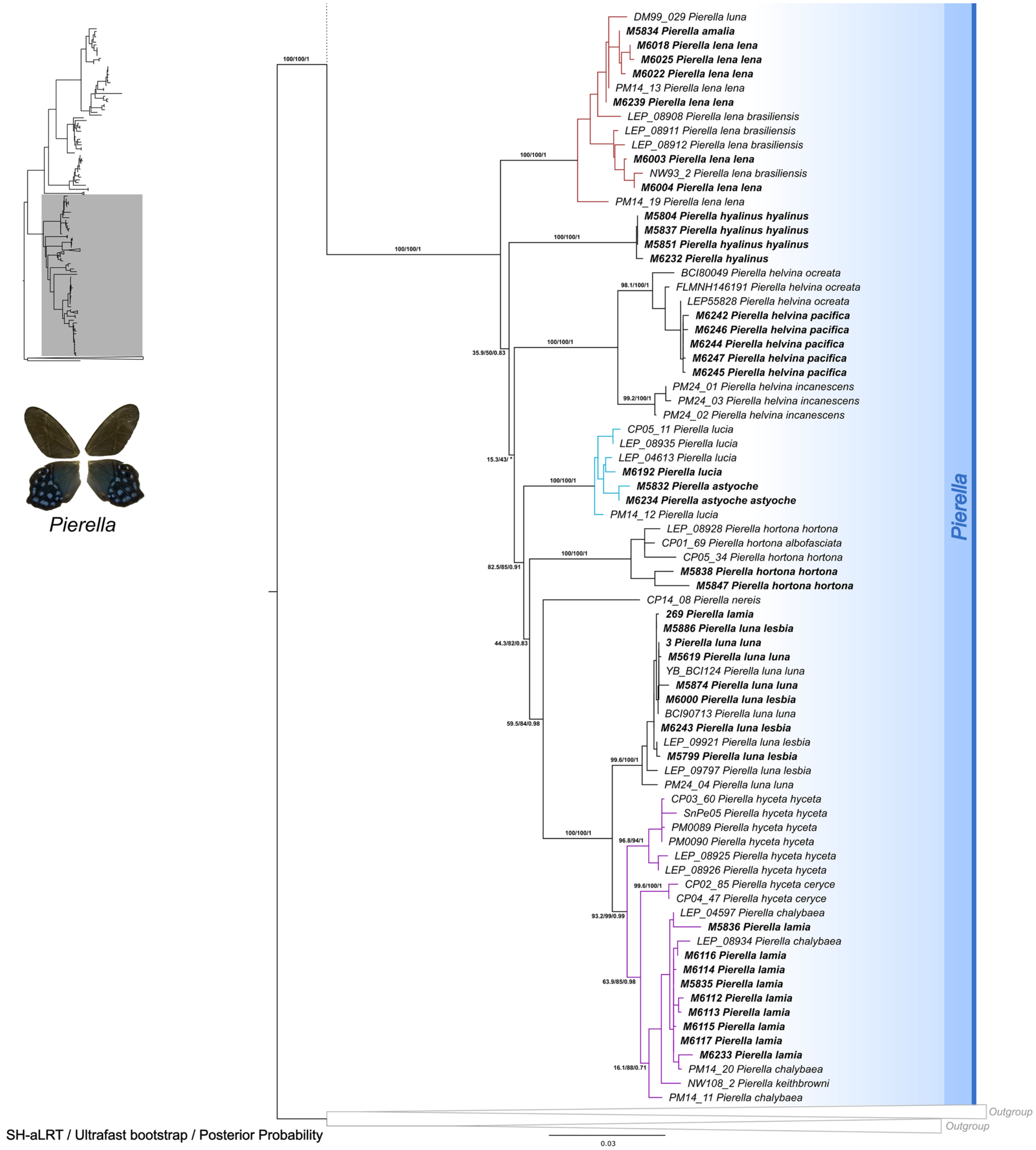

Fig. 2. Continued.

FGH, Supplementary Fig. S2), whereas the remaining species clustered similarly to the mitochondrial topology but with discrepancies in their relationships (clades A, B, C, D, and E). Likewise, species of *Pseudohaetera* were recovered in both topologies, but *EF1α* failed to establish them as sister groups. Finally, the lineages of *Haetera* were indistinguishable in both cases, and in *Cithaerias*, the relationship of the clades (A, B, and C) was discordant between the nuclear and mitochondrial genes.

Species Delimitation Analysis

The species delimitation test based on both molecular and morphological data (Fig. 3) and molecular data only (Supplementary Fig. S3) failed to recover most of the predefined subspecies and even some species. In most of the scenarios tested, the posterior probability for the subspecies was lower than 50% for both clearwing and opaque butterflies. In the case of *Pierella*, the pairs *P. amalia*/*P. lena*, *P. astyoche*/*P. lucia*, and *P. lamia*/*P. chalybaea* were not recovered as different lineages under any

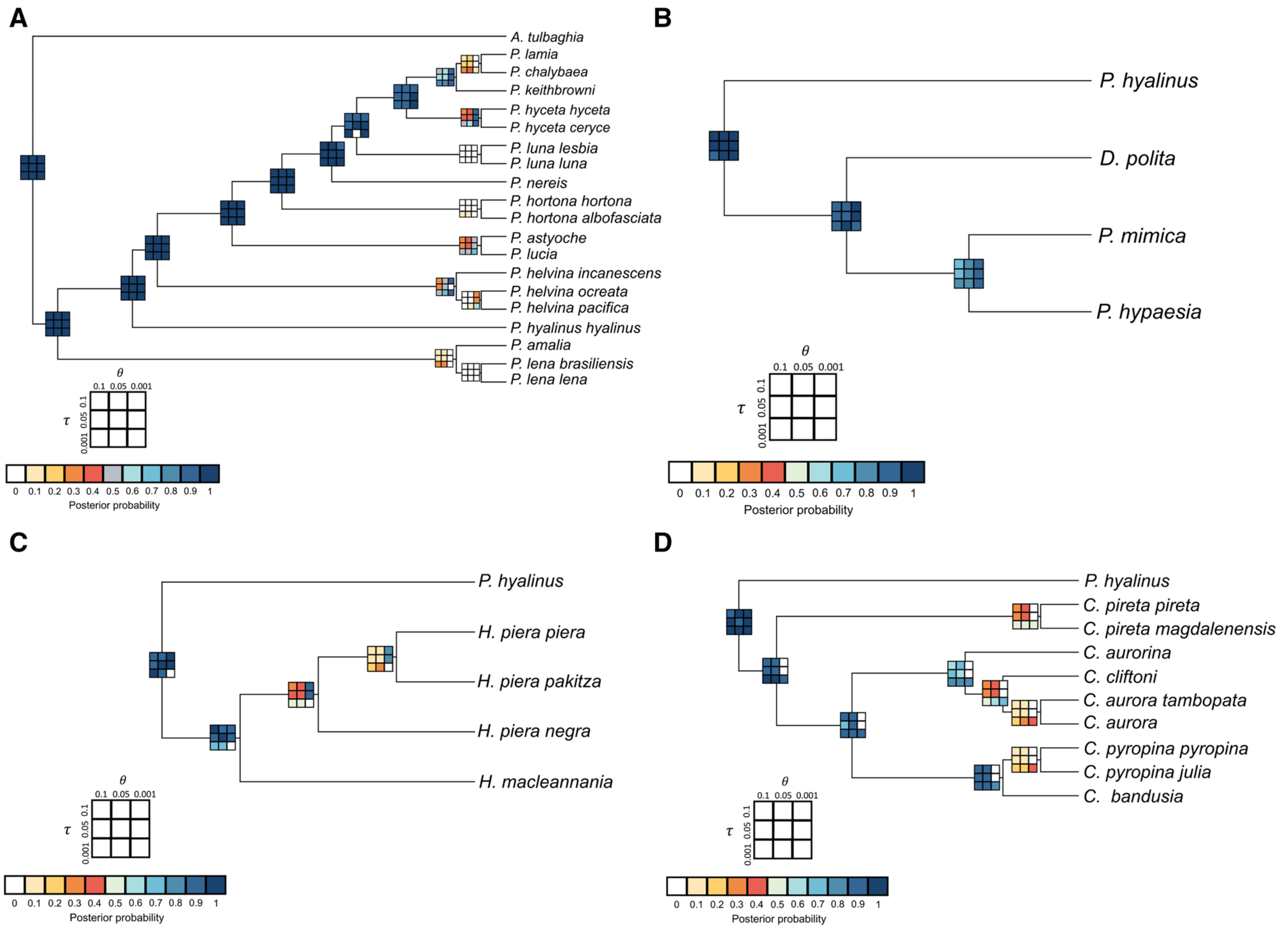

Fig. 3. iBPP species delimitation based on the molecular + morphological data for A) opaque butterflies *Pierella*, B) *Dulcedo* and *Pseudohaetera*, C) *Haetera*, and D) *Cithaerias*. Color scales indicate the support for each node based on 9 different models.

scenario, consistent with the phylogenetic results. Interestingly, *P. keithbrowni* showed higher support (PP = 90%) in 3 scenarios, whereas in the remaining scenarios, it showed lower posterior probabilities (PP = 0.50 to 0.60).

Dulcedo polita and the 2 species of *Pseudohaetera* (*P. hypaesia* and *P. mimica*) showed high support in all prior combinations. In the case of *Haetera*, we found strong support for the species (*Haetera macleannania* and *H. piera*), but this decreased when we incorporated morphological data (Fig. 3; Supplementary Fig. S3). In *Cithaerias*, we observed low support for most species and subspecies in all scenarios. However, the inclusion of morphological data increased the posterior probability at some nodes and in some scenarios (Fig. 3; Supplementary Fig. S3).

Wing Pattern Analysis

Overall, no correlation was observed between color patterns and phylogenetic clades. In *Pierella* (Fig. 4A), the first principal component (PC1) separated individuals by wing coloration (from orange to blue), while the second principal component (PC2) differentiated by color pattern (from a solid color patch to a speckled pattern). In this genus, we observed phylogenetically distinct species like *P. hyalinus* and *P. lena* clustering together based on the color pattern, which is speckled at the distal section of the wing in both species. In addition, the subspecies of *P. luna* (*P. l. luna* and *P. l. lesbia*), which differ in their color patterns, were discriminated in the PCA despite not being valid subspecies according to the phylogenetic and species delimitation analyses (Figs 2 and 3). We observed the same for *P. amalia*, where the wing color pattern separates it from *P. lena*, but the phylogeny and species delimitation analyses suggest otherwise. Unexpectedly, *P. h. pacifica* grouped with *P. hyalinus* and *P. lena* despite their differences in the color pattern. This could be due to the similarity in their wing patterns.

In *Cithaerias* (Fig. 4B), PC1 clustered individuals based on the amount of pink across the wings, whereas PC2 discriminated based on color pattern (ie by sex due to dimorphism). Consequently, we observed 2 major clusters separating males from females. In males, *C. aurorina* (wing almost fully pink) was detected as different from *C. aurora* (half of the wing pink), but females were not differentiated. Despite marked differences in their wing patterns, the molecular phylogeny and species delimitation analyses did not indicate that they are separate species. Wing pattern analysis also detected differences between the 2 species of *Haetera* due to sexual dimorphism, and the females of these species were separated. Unfortunately, we were unable to obtain molecular data for *Haetera piera sanguinolenta*, and we do not have information on its phylogenetic relationships, thus preventing its comparison with the wing pattern.

Pseudohaetera is the only genus in which the wing phenotype was concordant with the phylogenetic signal. PCA revealed the distinction between the species based on the brown patterns in the distal portion of the wing (Supplementary Fig. S4). Because

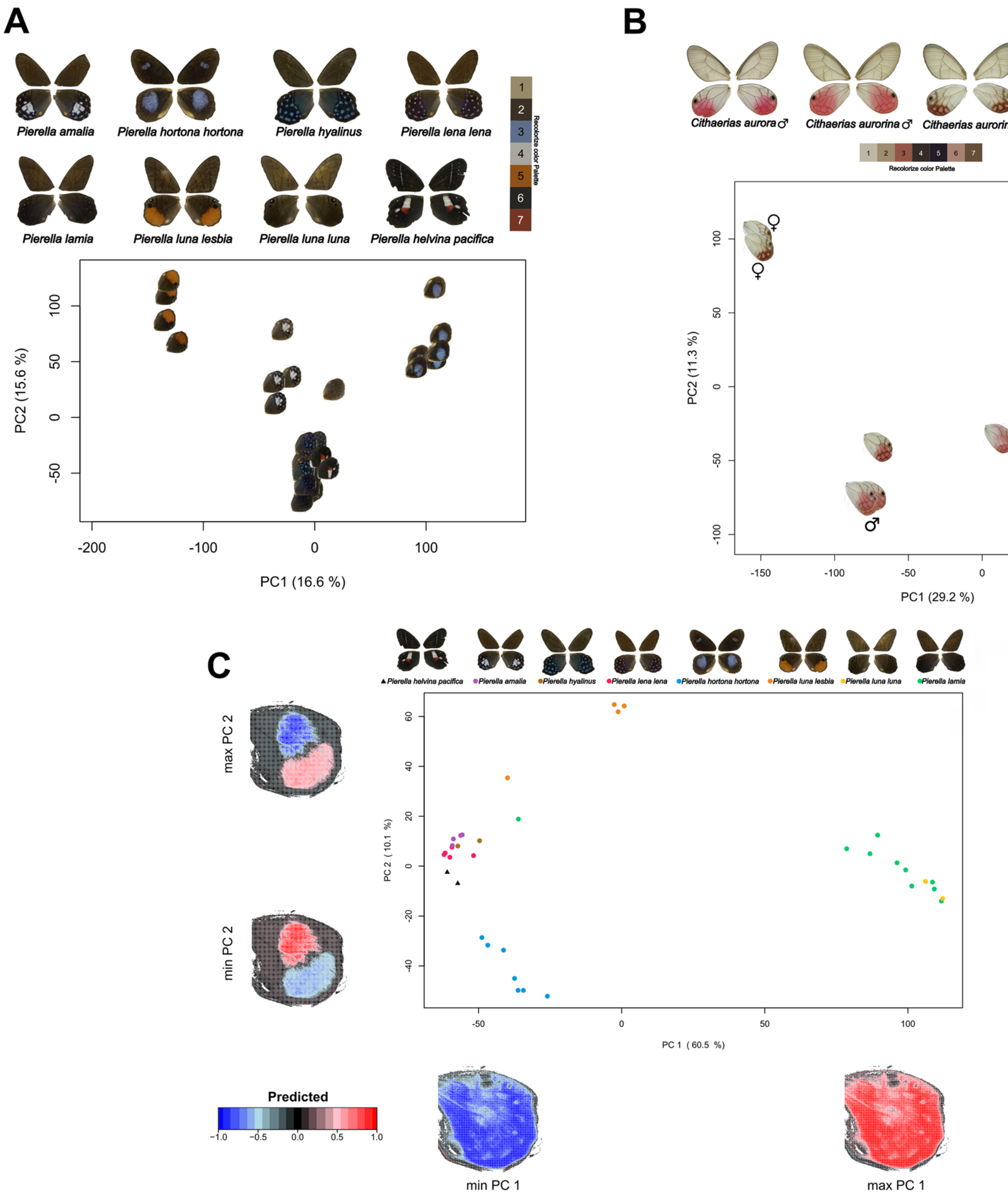

Fig. 4. Principal Components Analysis (PCA) for the wing phenotype of A) *Pierella* and B) *Cithaerias*. C) Comparison of color patterns between species of *Pierella*. Scale of predicted values indicates full presence (positive/red) and full absence (negative/blue).

Dulcedo is a monotypic genus, the results for its analysis only showed variation in the wing pattern between individuals of the same species (Supplementary Fig. S4).

Divergence Times Estimation, Biogeography, and Lineages Diversification

Our BEAST time-calibrated tree was consistent with the main clades identified using ML and BI (Fig. 5). However, the credibility intervals for node age estimations are wide, especially for nodes closest to the root. The divergence between clearwing and dark-winged species was estimated at ~33 Ma (95% HPD = 22 to 44 Ma [HPD = high probability density intervals]). Our divergence-time reconstruction suggests that the Most Recent Common Ancestor (MRCA) of *Pierella* diverged around 16 Ma (95% HPD = 12 to 22 Ma) during the Early Miocene. The first split within the clearwing clade is estimated to have occurred during the late Oligocene, with *Dulcedo* diverging from the other species around 25 Ma (95% HPD = 17 to 34 Ma), followed by *Pseudohaetera* at ~21 Ma (95% HPD = 13 to 29 Ma). The 2 species within the *Pseudohaetera* clade (*P. hypaesia* and *P. mimica*) diverged around 6 Ma (95% HPD = 2 to 12 Ma) in the Late Miocene. Finally, the sister clades *Haetera* and *Cithaerias* diverged at approximately 16 Ma (95% HPD = 9 to 23 Ma).

Due to the low resolution of species within the clades of *Cithaerias* and *Haetera*, we were cautious with the estimated divergence times between these taxa. However, the split between *C. pireta pireta*/*C. pireta magdalenensis* and other species of *Cithaerias* appears to have occurred ~5 Ma. This date coincides with a geographic division that separated individuals from the western side of the eastern Cordillera of Colombia from those on the eastern side (Supplementary Fig. S5). In *Pierella*, most divergences occurred during the Miocene.

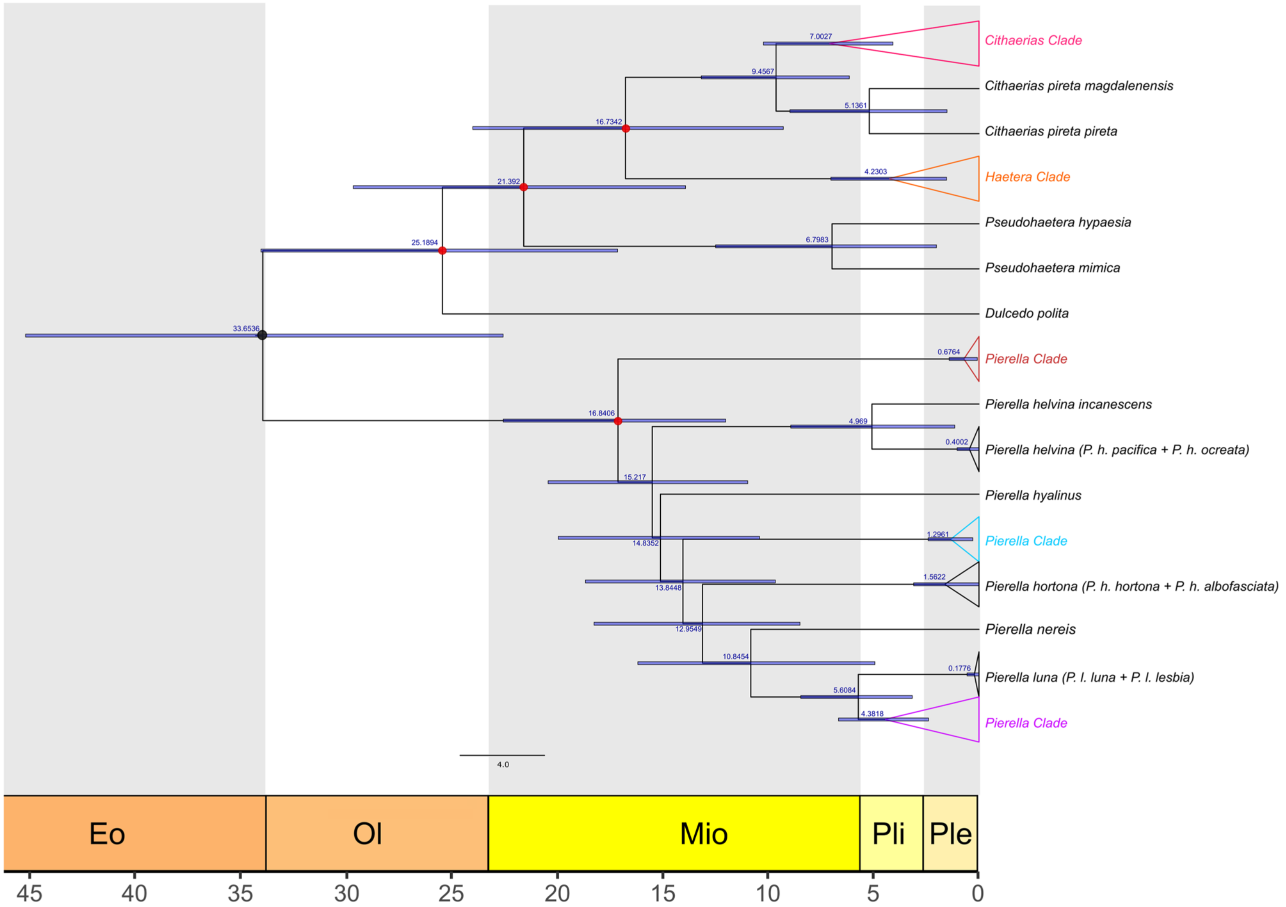

Fig. 5. Bayesian time-calibrated best tree obtained in BEAST 2.7.7, showing median node ages. Blue bars represent the 95% High Probability Density (HPD) intervals for each node. Nodes marked with red circles indicate genera within the Haeterini tribe, and the black circle indicates the separation of the clearwings and dark-winged groups. The time axis is scaled in millions of years ago (Ma), and the grey and white shaded regions mark different geological epochs. Clades are collapsed according to Supplementary Table S4 due to the lack of resolution for some lineages.

For example, *P. lena* and *P. amalia* were the first to diverge at approximately 16 Ma (95% HPD = 12 to 22 Ma), followed by the clade of *P. helvina* which diverged ~15 Ma (95% HPD = 10 to 20 Ma). *Pierella hyalinus* diverged ~14 Ma (95% HPD = 10 to 19 Ma). Similarly, *P. astyoche* and *P. lucia* diverged at around 13 Ma (95% HPD = 9 to 18 Ma), followed by *P. hortona* at around 12 Ma (95% HPD = 8 to 18 Ma). Next, *P. nereis* diverged around 10 Ma (95% HPD = 4 to 16 Ma), followed by the divergence of *P. luna* from the sister groups *P. lamia* and *P. hyceta* at ~5 Ma (95% HPD = 3 to 8 Ma). Finally, the clade composed by *P. hyceta*, *P. lamia*, *P. keithbrowni*, and *P. chalybea* diverged around 4 Ma (95% HPD = 2 to 6 Ma).

To reconstruct the ancestral areas, we tested 3 different scenarios: (i) Control (S0), (ii) Pebas and Acre systems (S1), and (iii) Andes Uplift (S2). We tested whether phylogenetic relationships could reconstruct ancestral areas in the absence of major geographical barriers (S0), such as marine and freshwater incursions into the continent (S1) or the formation and uplift of the Andes (S2). The tested scenarios did not produce ancestral areas that differed significantly from the control, which suggests a simple scenario with free dispersal between areas. However, we were unable to estimate the ancestral area of origin for Haeterini because of the resulting wide distribution, which includes areas like the Amazon basin, Central America, Tumbes–Choco–Magdalena, Guiana Shield, Venezuelan Highlands, and Atlantic Forest (Fig. 6). Although the ancestral areas identified have a low likelihood, they allow us to infer possible ancestral states for the nodes. For example, the *Pierella* node favors an Amazon origin because its distribution includes the Amazon basin, whereas scenario S2 also includes the Guiana Shield, Atlantic Forest, and Venezuelan Highlands. In contrast, in the node of the clearwing butterflies, the MRCA was distributed in the Tumbes–Choco–Magdalena. The MRCA of *Pseudohaetera* likely occurred in the Tumbes–Choco–Magdalena region, although the Northeastern and Northwestern Andes are also identified as potential ancestral distributions. The origin of *Haetera* is suggested to have a broader distribution, including the Amazon basin, the Guiana Shield, the Venezuelan Highlands, and the Tumbes–Choco–Magdalena region. Finally, the MRCA of *Cithaerias* is inferred to have originated in the Amazon basin (S0) and Tumbes–Choco–Magdalena region (S1 to S2).

The best model in each scenario involved several dispersal events (~30 events) and 8 vicariance events, including dispersals into regions such as the Guiana Shield, Venezuelan highlands, Atlantic Forest, Central America, and Andes mountains. One of the vicariance events involves the MRCA of *Pseudohaetera*, which likely restricted *P. mimica* to the valleys, while *P. hypaesia* dispersed toward the Northwestern and Northeastern Andes. Another involves the MRCA of *Cithaerias* and explains the presence of *C. pireta* in the

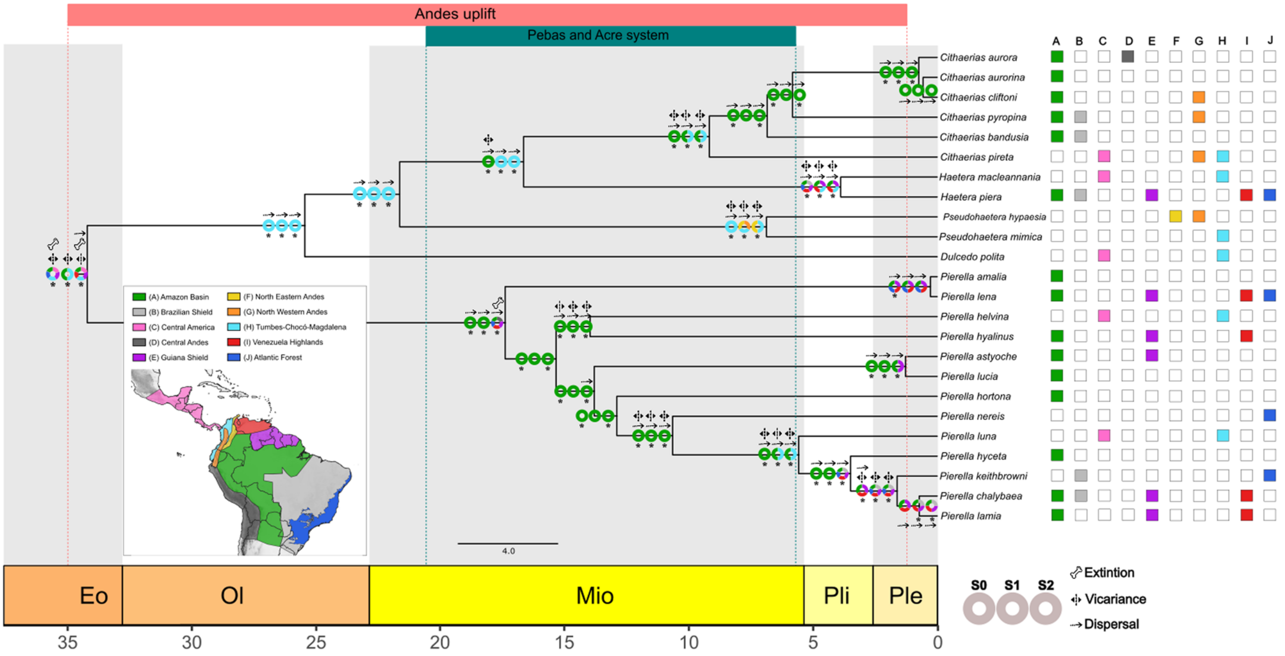

Fig. 6. Areas of ancestral distribution for each node of the reconstructed phylogeny for Haeterini. Colors on the map correspond to biogeographical regions, and circles represent the most likely ancestral distribution of the nodes. Multiple colors within the same circle indicate a combination of ranges for that node. The asterisk (*) indicates that the likelihood supporting the areas is <70%, but the MLAS is still displayed. The time axis is scaled in millions of years ago (Ma), and the grey and white shaded regions mark different geological epochs.

Tumbes–Choco–Magdalena region, whereas the other species are distributed in the Amazon. The remaining events occurred within the *Pierella* and Clearwing clades.

Finally, the Episodic Birth–Death with multiple periods was the best model for explaining diversification patterns within the Haeterini (Supplementary Table S3). The speciation rate (λ) showed a constant and stable trend at an approximate rate of 0.01 Myr^{-1} (lineages per million years), whereas the extinction rate (μ) was consistently low at 0.02 Myr^{-1}. These rates resulted in a steady net diversification rate ($\lambda - \mu$) ranging from 0.09 to 0.1 Myr^{-1} and a relative extinction rate (λ/μ) of 0.02 Myr^{-1} over the analyzed time periods (Fig. 7).

Discussion

The application of integrative taxonomy offers a powerful framework for properly assessing biodiversity (Struck et al. 2018). This approach is particularly effective when dealing with taxa where species have mostly been described based on single-line evidence, such as morphology alone, leading to multiple cases of species synonymization (Dayrat 2005, Alevi et al. 2021, Lewis and Anderson 2023). Additionally, most taxonomic studies suffer from limited sampling, precluding accurate phylogenetic reconstruction and species delimitation (Ahrens et al. 2016).

Our findings show that species and subspecies in the tribe Haeterini, particularly those in the genera *Pierella*, *Cithaerias*, and *Haetera*, require major, integrative taxonomic revisions due to the lack of support in our phylogenetic and species delimitation analyses. We found support for only 9 of the 24 species analyzed, while the remaining 15 clustered into 5 groups and revealed 13 lineages in total. This is also true for subspecies, where only a few cases show clear distinctions, such as *C. pireta pireta* and *C. pireta magdalenensis* or *Pierella helvina incanescens*. Although we recovered the same phylogenetic relationships among the genera as in recent studies (Matos-Maraví et al. 2019, Kawahara et al. 2023), our findings do not support recent suggestions to elevate some lineages to the status of species (Matos-Maraví et al. 2019). For instance, our results do not support the previously reported differentiation between *Pierella lena lena* and *Pierella lena brasiliensis*, as they were never resolved as distinct lineages in our analyses. In addition, our delimitation test recovered fewer lineages than those currently recognized taxonomically at the tribe level. This is evident in groups like *Cithaerias*, where the distinction between *C. aurora* and *C. cliftoni* as different species is poorly supported, and in *Haetera*, where our analyses failed to differentiate between *H. piera* and *H. macleannania*.

Our analysis of wing color highlights the complexity of wing morphology variation within the Haeterini tribe. While we observed distinct groups, they do not consistently match the recognized species or subspecies (with some exceptions, such as *P. hortona*). We consistently observed a lack of correspondence between wing color patterns and phylogenetic signals, suggesting that morphological differences do not reflect genetic divergence. In fact, intraspecific variation in wing coloration has been reported in *Cithaerias*, with instances of color pattern overlap between species (Penz et al. 2014). Furthermore, rapid transitions in wing coloration in Preponini butterflies are not necessarily linked to diversification shifts (Ortiz-Acevedo et al. 2020). These examples further highlight that wing color patterns do not always correspond to evolutionary relationships, suggesting that wing color patterns alone, as morphological characters, are not reliable for species delimitation and do not necessarily reflect phylogenetic relationships. Interestingly, sexual dimorphism in *Cithaerias* and *Haeterini* resulted in sex-specific groupings in our wing-phenotype analysis. Because of the role of visual cues in mate choice in butterflies (Costanzo and Monteiro 2007,

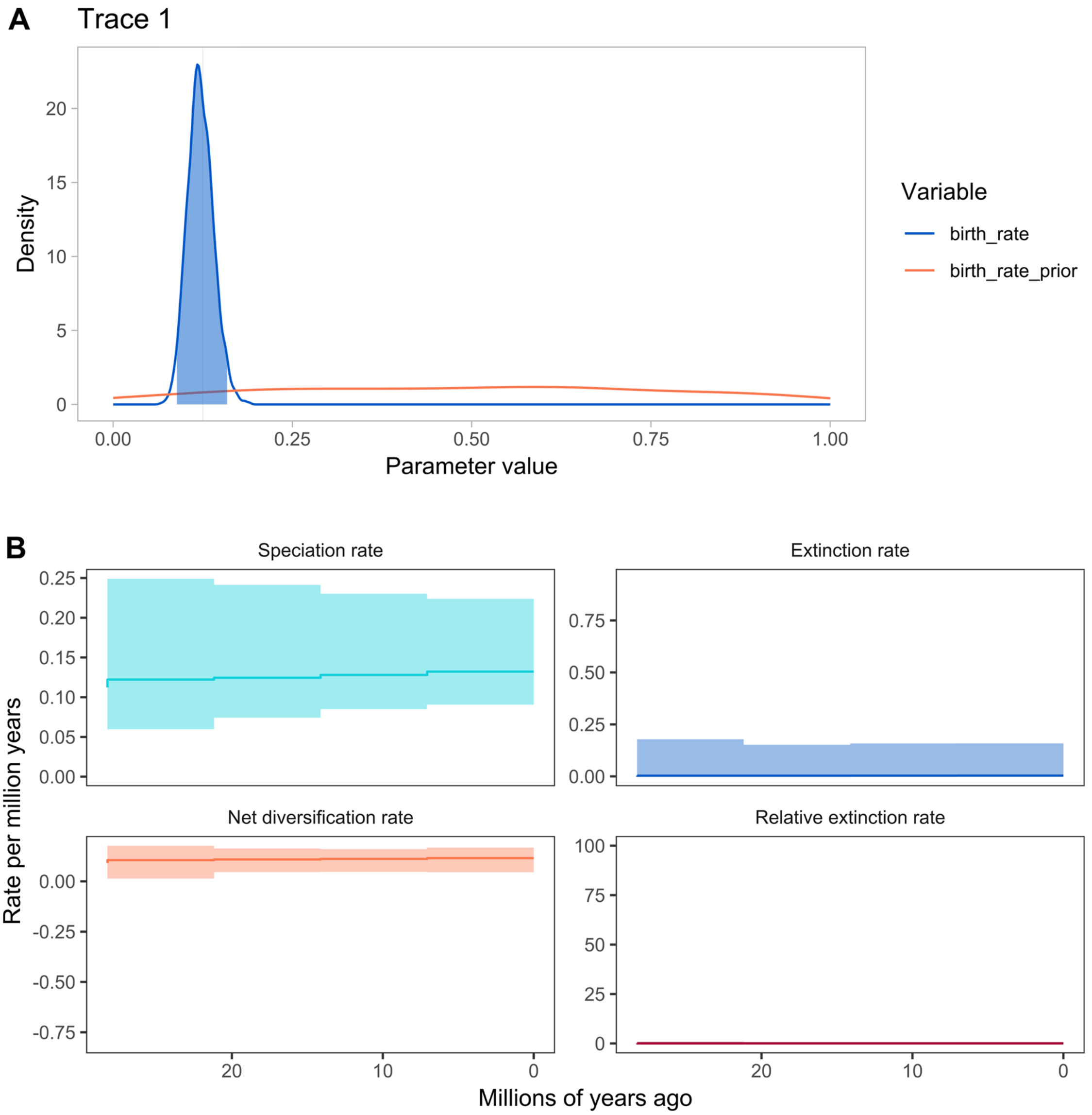

Fig. 7. Episodic Birth–Death model with 5-time intervals (EBD-5N) for lineages in Haeterini. A) Estimates of the posterior and prior distribution for the Yule Model. The red curve shows the selected lognormal prior distribution. B) Posterior distributions of speciation rate (λ), extinction rate (μ), net-diversification rate (λ – μ), and the relative extinction rate (λ/μ) for the Episodic Birth–Death model with 5 intervals. Dark lines correspond to the posterior-mean estimate of each parameter for each time interval, and shaded regions correspond to the 95% credible interval.

Kemp 2007), sexual selection may contribute to diversification in these groups. However, experimental evidence on mate choice in this tribe is needed to support this claim.

Our divergence-time estimates suggest that Haeterini originated around the Eocene–Oligocene boundary (~33 Ma), with most of its diversification occurring in the Miocene. Although our estimates are older than those previously reported (Matos-Maraví et al. 2019; Kawahara et al. 2023), they are still consistent with these findings. The estimations we obtained coincide with major geographical events in the Neotropics, including the formation of the Pebas and Acre floodplains and the uplift of the Andes (Hoorn et al. 2010). While these events greatly shaped the diversification of lineages such as *Taygetis* butterflies (Matos-Maraví et al. 2013), spiders (Salgado-Roa et al. 2021, Salgado-Roa et al. 2024), and *Polythore* damselflies (Sánchez-Herrera et al. 2020), they seem to have had a lesser impact on Haeterini, resulting in fewer opportunities for geographic isolation and speciation. This is similar to Anaeini butterflies, in which the Andean orogeny was not crucial for their diversification and did not constitute a strong barrier to dispersal (Toussaint et al. 2019). In fact, our findings indicate that most diversification in Haeterini is due to dispersal rather than vicariance, even during the marine incursions of the Pebas and Acre systems. This aligns with the growing understanding that the Pebas and Acre systems had mixed consequences for Neotropical taxa, restricting some groups while creating ecological opportunities for others, as seen in the tribe Brassolini, where these systems promoted their diversification by facilitating the dispersal of host plants (Matos-Maraví et al. 2021), However, in Haeterini, we lack ecological and biogeographical information on their host plants to establish the role of host plants in their diversification. This pattern of dispersal-driven diversification is particularly evident in *C. pireta magdalenensis*, whose divergence coincides with an uplifting phase of the

Northern Andes (12 to 4 Ma; Hoorn et al. 2010, Zapata et al. 2023), as well as the establishment of forested habitats after the demise of the Acre and Pebas systems (Hoorn et al. 2010), likely creating the ecological and topographic conditions that facilitated the divergence and isolation from *C. p. pireta*.

The low and relatively constant speciation and diversification rates observed for the Haeterini tribe ($\lambda = 0.01$) are similar to those observed for *Morpho* butterflies ($\lambda = 0.081$; Chazot et al. 2021a), but they sharply contrast with those reported in other butterfly groups. For instance, the genus *Erebia* shows an initial burst of diversification ($\lambda = 0.5$), followed by a decline to a stable rate of $\lambda = 0.266$ (Peña et al. 2015), whereas *Heliconius* has an estimated speciation rate of $\lambda = 0.318$ (Kozak et al. 2015). Similarly, genera in the tribe Ithomiini, such as *Mechanitis* and *Melinaea*, exhibit much higher rates (Van Der Heijden et al. 2025). Interestingly, the butterfly subtribe Dircennina (Nymphalidae: Ithomiini) has region-specific speciation rate estimates: $\lambda = 0.230$ for the Andean region and a lower $\lambda = 0.118$ in non-Andean regions (Chazot et al. 2018). Despite these examples of high diversification rates in multiple butterfly lineages, Chazot et al. (2021b) did not detect an elevated average net diversification rate for Neotropical nymphalids, except during the Eocene—a period likely pivotal for the build-up of Neotropical diversity. This aligns with findings from Meseguer et al. (2022), who suggested that most of the diversity in Neotropical clades stems from the gradual accumulation of lineages, with only a small proportion resulting from exponential diversification. Overall, the low speciation rate observed in Haeterini aligns with the results of the species delimitation test and phylogenetic reconstructions, and together they support the conclusion that Haeterini has more described lineages than it actually does. The mismatch between morphological and genetic divergence further supports this.

While we were unable to identify the most probable region of origin for the tribe, our findings indicate that the evolution of Haeterini was somewhat shaped by abiotic factors in the Neotropics, beginning in the Miocene. This aligns with a possible museum model of diversification (Stebbins 1974, Chown and Gaston 2000, Jablonski et al. 2006), in which species accumulation is slow and relatively stable rather than characterized by rapid bursts of speciation.

Although we have highlighted discrepancies in species delimitation within Haeterini, further studies with more species are necessary to accurately test species boundaries. These should integrate multiple data sources, including morphology (eg wing and genitalia morphology), genomics, and extensive georeferenced sampling from underrepresented regions. Expanding sampling efforts could also help address current over-splitting tendencies by providing an accurate picture of the diversity of this tribe across its distribution. This will also allow future studies to explore other evolutionary processes that shape the speciation and diversification patterns of this group of butterflies.

Specimen Collection Statement

Insect Systematics and Diversity supports compliance with the Nagoya Protocol. The authors attest that all legal and regulatory requirements, including export and import collection permits, have been followed for the collection of specimens from source populations at any international, national, regional, or other geographic level for all relevant field specimens collected as part of this study.

Nomenclature

This paper and the nomenclatural act it contains have been registered in Zoobank (www.zoobank.org), the official register of the International Commission on Zoological Nomenclature. The LSID (Life Science Identifier) number of the publication is: urn: lsid:zoobank.org:pub:0533B2D3-A677-45A0-8ED8-330C6079C9B2

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Author Contributions

Frasella De Martino (Conceptualization [equal], Formal analysis [equal], Investigation [equal], Methodology [equal], Visualization [equal], Writing—original draft [equal], Writing—review & editing [equal]), Nicol Rueda-M (Data curation [equal], Supervision [equal], Validation [equal], Writing—review & editing [equal]), Lucas S. Barrientos (Data curation [equal], Investigation [equal], Writing—review & editing [equal]), Paula Vela (Investigation [equal], Writing—review & editing [equal]), Melissa Sanchez-Herrera (Formal analysis [equal], Writing—review & editing [equal]), Carolina Pardo-Díaz (Funding acquisition [equal], Project administration [equal], Resources [lead], Validation [equal], Writing—review & editing [equal]), and Camilo Salazar (Conceptualization [equal], Funding acquisition [equal], Methodology [equal], Resources [equal], Supervision [equal], Validation [equal], Writing—original draft [equal], Writing—review & editing [equal])

Supplementary Material

Supplementary material is available at *Insect Systematics and Diversity* online.

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Conflicts of Interest

None declared.

Data Availability

The data underlying the findings of this study are available on Zenodo (DOI: 10.5281/zenodo.17737701).

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