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Title: Species limits in butterflies (Lepidoptera: Nymphalidae): Reconciling classical taxonomy with the multispecies coalescent

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Abstract.—Species delimitation is at the core of biological sciences. During the last decade, molecular-based approaches have advanced the field by providing additional sources of evidence to classical, morphology-based taxonomy. However, taxonomy has not yet fully embraced molecular species delimitation beyond threshold-based, single-gene approaches, and taxonomic knowledge is not commonly integrated to multi-locus species delimitation models. Here we aim to bridge empirical data (taxonomic and genetic) with the latest coalescent-based species delimitation approaches. We use the multispecies coalescent model as implemented in two recently developed Bayesian methods (DISSECT/STACEY and BP&P) to infer species hypotheses. In both cases, we account for phylogenetic uncertainty (by not using any guide tree) and taxonomic uncertainty (by measuring the impact of using or not a priori taxonomic assignment to specimens). We focus on an entire Neotropical tribe of butterflies, the Haeterini (Nymphalidae: Satyrinae). We contrast divergent taxonomic opinion—splitting, lumping and misclassifying species—in the light of different phenotypic classifications proposed to date. Our results provide a solid background for the recognition of 22 species. The synergistic approach presented here overcomes limitations in both traditional taxonomy (e.g., by recognizing cryptic species) and molecular-based methods (e.g., by recognizing structured populations, and not raise them to species). Our framework provides a step forward towards standardization and increasing reproducibility of species delimitations.

KEYWORDS: Bayes factor; clearwing Satyrinae; Haeterini; Neotropics; speciation; systematics

INTRODUCTION

Traditionally, taxonomic delimitations have relied on diagnostic phenotypic characters to classify distinct populations into species and subspecies (hereafter, the 'traditional taxonomic approach'). More recently, coalescent-based methods that quantify reproductive isolation using genetic data have been proposed as a means to calculate the probability of speciation (hereafter, the 'coalescent approach'; e.g., Knowles and Carstens 2007; Yang and Rannala 2010; Fujita et al. 2012). Both approaches do not necessarily agree in their species hypotheses because their scopes are centered on different sections of the speciation continuum; while traditional taxonomy depends on the evolution of informative and consistent morphological characters, the coalescent approach is guided by any gene flow reduction that could be associated with the onset of reproductive isolation. As a consequence, phenotype-based delimitation may not identify cryptic and incipient species (i.e., genetically divergent lineages embarked in the process of speciation; Rosindell et al. 2010) whereas coalescent-based delimitation may simply reveal population structure (i.e., subpopulations with a long non-breeding history) (Sukumaran and Knowles 2017). Despite the paramount importance of delimiting species for multiple disciplines and practices in science and society (e.g., ecology, evolution, conservation biology, among others), it still remains unclear how to reconcile conflicts between traditional taxonomy and the coalescent approach while taking into account their respective benefits and limitations.

Taxonomists have not fully embraced the recent developments in molecular species delimitation beyond threshold-based, single-gene approaches (e.g., DNA barcoding based on COI; Hebert et al. 2003) (Pons et al. 2006; Puillandre et al. 2012). End-users of the coalescent approach, on the other hand, do not usually incorporate taxonomic knowledge to inform their models (e.g., Leaché

and Fujita 2010; Olave et al. 2014; but see Aydin et al. 2014; Jones et al. 2015). In a Bayesian framework, taxonomic information could be explicitly acknowledged in the form of prior distributions, and thus alternative species hypotheses can be statistically weighed. Indeed, the latest Bayesian implementations, which include the multispecies coalescent model (MSC; Degnan and Rosenberg 2009), can accommodate parameters that control the number of species in a dataset, their divergence times and ancestral population sizes, all in a single probabilistic framework. These properties among others, such as the recognition of genealogical incongruence and incomplete lineage sorting, arguably make MSC-based methods more biologically realistic than threshold-based molecular species delimitation (Knowles and Carstens 2007; Fujita et al. 2012). However, it remains unexplored how divergent taxonomic opinions affect species delimitations, when these opinions (such as "splitters" *vs.* "lumpers") are translated into prior distributions for molecular species delimitation analyses.

Here we aim to reconcile genetic and taxonomic data using species delimitation models that estimate coalescence and species divergence in a fully Bayesian framework. We study the butterflies classified in the tribe Haeterini (Nymphalidae: Satyrinae), insects that exclusively inhabit tropical rainforests in southern Mexico, Central and South America. Transparent wings are the most obvious characteristic of this group, an attribute shared by four out of five genera within the tribe—*Pierella* being the exception by having full scale-cover (Fig. 1).

Taxonomic work on this charismatic group involves numerous researchers over the last 150 years (e.g., Herrich-Schäffer 1864; Weymer 1910; Miller 1968; Constantino 1995; Lamas 1997; Penz et al. 2014; Paluch et al. 2015; Willmott 2015; Zacca et al. 2016). Haeterini is a

88 monophyletic tribe (Wahlberg et al. 2009; Chazot et al. 2018), and current taxonomic
89 understanding (Lamas 2004; Penz et al. 2014; Willmott 2015; Zacca et al. 2016) is that Haeterini
90 consists of 29 described species and 39 subspecies. However, these estimates remain contentious
91 because published taxonomic studies have mostly focused on smaller groups within Haeterini
92 and have relied on different morphological character systems (wing coloration, genitalia shape or
93 male androconial organs), and have not taken into account variation in DNA sequence data. The
94 taxonomic opinions among these authors reflect the well-recognized subjectivity of species-level
95 taxonomic work, even though Haeterini has a comprehensive taxonomic knowledge as compared
96 to most other tropical insects. This offers the opportunity to quantify taxonomic opinion and
97 definitions among authors, as a preliminary step for standardization.
98
99 Specifically, we aim to test alternative species delimitation hypotheses and to evaluate the
100 following two interconnected expectations:
101
102 1. Lineage delimitation using the multispecies coalescent model (MSC) will recover the
103 taxonomic subspecies in Haeterini. Butterfly subspecies are commonly described based on
104 parapatric or allopatric geographical distributions, and reduced gene flow could facilitate
105 phenotypic divergence. If phenotype divergence resulted from genetic differentiation, then any
106 reproductive isolation among subspecies will be recovered by the MSC.
107
108 2. Taxonomic knowledge modeled as prior distributions will group specimens into MSC Clusters
109 (MSCC). However, regardless of prior distribution, the MSC will standardize species
110 delimitation because divergent taxonomic opinion among authors, if any, will be evident in the

phylogeny of Haeterini. Therefore, the MSC will inform on whether different morphological characters (wing coloration, genitalia shape or male androconial organs) result in narrower or broader species definitions.

MATERIALS AND METHODS

Taxon sampling and molecular dataset

Haeterini butterflies were collected by the authors and collaborators throughout most of the geographical range of the tribe, including localities from northern Costa Rica to southeastern Brazil. Specimens were identified to the species and subspecies level following published taxonomic revisions (Constantino 1995; Lamas 1997; Penz et al. 2014; Paluch et al. 2015; Willmott 2015; Zacca et al. 2016) and comparing wing morphology to type specimen photographs at https://www.butterfliesofamerica.com (last accessed August 2018). We sampled all five Haeterini genera, including 18 of 29 currently valid species, and 18 of 39 subspecies (Lamas 2004; Penz et al. 2014; Paluch et al. 2015; Willmott 2015; Zacca et al. 2016). Note that several subspecies were not represented in our sample because they are only known from type collections (hampering their access for genetic studies) or have rarely been collected in recent years.

We followed standard lab protocols (Wahlberg and Wheat 2008) to sequence 6 gene fragments from 63 specimens: the mitochondrial locus COI (1,475 bp) and the nuclear loci CAD (850 bp), EF1α (1,240 bp), GAPDH (691 bp), RpS5 (617 bp), and *wingless* (400 bp). Sanger sequencing was conducted by the company Macrogen (South Korea), and sequence quality control and DNA alignments were carried out using the program Geneious R7. We retrieved from GenBank the

DNA sequences of sixteen species classified in the subfamily Satyrinae in order to root the phylogeny of Haeterini. All DNA sequences were deposited in GenBank (Table 1) and the DNA alignments used in this study were archived in TreeBASE (ID: 23439). Detailed specimen voucher information can be found in Table 1 and voucher photographs can be found at http://www.nymphalidae.net/db.php.

Phylogenetic analyses

We inferred phylogenies using single-gene datasets partitioned by codon position to rule out any tree topology conflict due to contamination. In addition, in order to test the monophyly of each genus, we inferred a phylogeny using the concatenated multi-locus dataset consisting of 6 genetic markers that proved to be phylogenetically informative (Table 2), and 63 Haeterini specimens and 16 outgroup taxa. We used PartitionFinder v2.1.1 (Lanfear et al. 2017) to estimate the best-fit partitioning strategy for the concatenated dataset using 18 data blocks (each codon position separately for each gene region) and the following settings: branchlengths = linked (higher likelihood than the unlinked option), models = mrbayes, model_selection = bic and search = greedy. All phylogenetic analyses using single-gene and concatenated datasets were carried out using MrBayes v3.2.3 (Ronquist et al. 2012) through the CIPRES portal (Miller et al. 2010). We used the reversible-jump Markov chain Monte Carlo approach (rjMCMC) to allow moving across nucleotide substitution schemes (nst = mixed) with different rate variation across sites (+I and +Γ). Two independent analyses and four chains, one cold and three heated, were run for 10 million cycles and sampled every 1,000 cycles, discarding the first 25% sampled parameters as burn-in. We evaluated convergence using the average standard deviation of split frequencies (< 0.005), potential scale reduction factor (~ 1.000), estimated sample sizes (ESS >

200), and by inspection of stationary distribution of log-probabilities in both independent runs. We summarized the 7,500 sampled trees using the 50% majority-rule consensus method.

Molecular species delimitation

Once we had confidence on the monophyly of each genus and a notion of the phylogenetic relationships among the 63 specimens, we carried out molecular species delimitation under the MSC framework. Although the inference of phylogenetic relationships is not a pre-requisite for subsequent delimitation analyses, it becomes informative when large phylogenies need to be divided into smaller well-supported subclades, and analyzed separately due to computational limitation (see BP&P analyses below). We used a comprehensive taxon sampling of Haeterini, a multi-locus dataset, and two popular Bayesian implementations of the MSC, namely STACEY (Jones 2017) and BP&P (Yang 2015):

STACEY.—We used DISSECT (Jones et al. 2015) which is a taxonomic assignment-free Bayesian method for grouping individuals into multispecies coalescent clusters (MSCC). The method is implemented in the STACEY v1.2.4 package (Jones 2017) available in BEAST v2.4.7 (Bouckaert et al. 2014). All gene markers sequenced for this study are likely unlinked in the genome of the butterflies and thus gene trees, substitution and clock models were all treated as unlinked in the analysis. We assigned to the mitochondrial COI locus a gene ploidy of 0.5 and to the remaining nuclear loci a gene ploidy of 2.0 (diploid). Uncorrelated relaxed-clock models were chosen for all loci, and we estimated nuclear clock rates relative to the COI mean clock rate fixed to 1.0. The relative clock mean priors were all log normal (M = 0, S = 1). We used the birth-death-collapse model following Jones et al. (2015) with GrowthRate prior as log normal (M

= 5, S = 2) and relative DeathRate as uniform in [0, 1], while the popMean prior was set to log normal (M = −7, S = 2).

In STACEY, the discovery of MSCCs relies on two parameters that control node collapsing in the phylogeny, the collapseHeight (ε) and collapseWeight (ω). The parameter ε distinguishes very shallow species divergences (node heights) and should be assigned a small value (Jones et al. 2015), thus, we set ε to 1*e*−4. The parameter ω controls the number of MSCCs and can be used as a proxy for prior taxonomic knowledge. The 1/X distribution of the prior for the number of MSCCs has a mean of $1 + (n - 1) \times (1 - \omega)$, where n is the number of individuals in the dataset. We set ω to 0.73 or 0.59, corresponding to 18 described species and 26 taxa (the 18 sampled subspecies elevated to species), respectively. In addition, we carried out a third analysis that did not take into account prior taxonomic information on the number of species by using a Beta distribution (α = 2, β= 2) as a prior for the parameter ω. The analyses were run four independent times for 200 million cycles each, with parameters sampled every 20,000 cycles. Sampled trees were combined after discarding the first 25% samples as burn-in and checking that ESS values were > 200. MSCCs, their posterior probabilities and pairwise similarity probabilities were obtained using SpeciesDelimitationAnalyser v1.8.0 (Jones et al. 2015) acknowledging ε = 1*e*−4. We chose the clustering with the highest posterior probability (counts) as the working species hypothesis.

BP&P.—We used the multispecies coalescent model as implemented in BP&P v3.4 (Yang 2015) to jointly infer species trees and delimit MSCCs, without using any guide tree (Yang and Rannala 2014; Rannala and Yang 2017) nor taxonomic assignment to specimens (Yang and

Rannala 2010; Rannala and Yang 2013). The heredity scalar was set to 1 for all nuclear loci and to 0.25 for the mitochondrial locus. The prior for ancestral population sizes controlled by the parameter θ was assigned the inverse gamma distribution (IG[α, β]), and we evaluated three different scenarios: i) large ancestral population size (IG[3, 0.2]), ii) medium ancestral population size IG[3, 0.1], and iii) small ancestral population size IG[3, 0.02]. These three different scenarios are expected to impact the inferred number of MSCCs, so that a larger ancestral population prior would favor fewer species in the model. We separately analyzed two Haeterini subclades, namely i) *Pierella* and ii) the remaining genera, to overcome computational limitation in BP&P. We used the inverse gamma distribution for the divergence time of the root in the species trees, which is controlled by the parameter τ_0. The prior distribution of τ_0 was diffuse ($\alpha = 3$) and we specified $\beta = 0.042$ for the *Pierella* species tree and $\beta = 0.098$ for the remaining Haeterini genera. These values enforced a sequence divergence mean of 2.1% for *Pierella* and 4.9% for the remaining genera, which translate into absolute times of ~7 Ma for *Pierella*'s crown age and ~17 Ma for the remaining genera, assuming a butterfly mutation rate of 2.9×10^{-9} (Keightley et al. 2015). The analyses were run two independent times using the rjMCMC algorithm 1 with gamma variable fine-tuning shape $\alpha = 2$ and mean $m = 1$, each for 500,000 cycles with sampling frequency of 50 and burn-in of the first 10,000 cycles. We chose the species delimitation model with the highest posterior probability as the working species hypothesis.

Species hypothesis testing and divergence time estimation

We compared the statistical support (model adequacy) for eight species hypotheses in a fully Bayesian framework:

i) Taxonomic species (18 lineages);

ii) Taxonomic subspecies elevated to species (26 lineages);

iii) STACEY's clusters under $\omega = 0.73$ (accounting for number of taxonomic species; 22 lineages);

iv) STACEY's clusters under $\omega = 0.59$ (accounting for number of subspecies elevated to species; 24 lineages);

v) STACEY's clusters under ω = Beta ($\alpha = 2$, $\beta = 2$) (non-informative prior on the number of MSCCs; 63 lineages);

vi) BP&P's clusters under θ = IG[3, 0.2] (large ancestral population size prior; 21 lineages);

vii) BP&P's clusters under θ = IG[3, 0.1] (medium ancestral population size prior; 28 lineages);

viii) BP&P's clusters under θ = IG[3, 0.02] (small ancestral population size prior; 55 lineages).

In order to account for incomplete lineage sorting and to avoid any of the pitfalls of using concatenated datasets (Edwards et al. 2016; Bravo et al. 2018), we inferred species tree topology and divergence times using the Bayesian multispecies coalescent method implemented in StarBEAST2 (Ogilvie et al. 2017). We used jModelTest v2.1.7 (Darriba et al. 2012) to evaluate the substitution models available in starBEAST2, including or not rate variation among sites (+I and +Γ). Nucleotide substitution models for each locus were chosen on the basis of the Bayesian Information Criterion (BIC). We preliminarily evaluated the fit of two tree models, namely Yule and birth-death, using 50 path-sampling steps under thermodynamic integration (Lartillot and Philippe 2006), each running for 60 million cycles to ensure final ESS values > 200. The Yule tree model had the highest marginal likelihood estimate in all cases and it was preferred over the birth-death model. We therefore report here the path-sampling analyses based on the Yule tree

model for the eight species delimitation hypotheses. Other parameters, including gene ploidy, clock models and popMean prior, were set as in the STACEY analyses. The support for each of the eight species delimitation hypotheses was assessed via Bayes factors (*ln*Bf) (Kass and Raftery 1995) calculated from the posterior tree samples, and we considered *ln*Bf = 2–10 to represent positive but not conclusive support and *ln*Bf > 10 as decisive support for the species hypothesis with the highest marginal likelihood estimated through path sampling.

We must rely on secondary calibrations to date the phylogeny of Haeterini because there are no described fossils assigned to the tribe. Based on a densely sampled, fossil-calibrated butterfly phylogeny (Chazot et al. 2018), we constrained the ages of six well-supported nodes that do not belong to Haeterini. We followed a conservative approach by using uniform priors encompassing the 95% highest posterior density (HPD) intervals from Chazot et al. (2018). The constrained nodes included the divergences of:

i) Brassolini and Morphini to 32–58 Ma;

ii) Melaniti and Dirini to 23–47 Ma;

iii) Lethina, Parargina and Mycalesina to 25–44 Ma;

iv) The crown age of Satyrini to 32–53 Ma;

v) The crown age of the Satyrini's subclade encompassing the tribes Pronophilina, Euptychiina, Satyrina, Erebiina, and other closely related subtribes, to 25–43 Ma;

vi) The crown age of Satyrinae to 41–67 Ma.

Time-calibrated species trees were inferred using BEAST v2.4.7 and the analyses were run four independent times for 200 million cycles each, with parameters sampled every 20,000 cycles.

Trees were summarized in TreeAnnotator (part of BEAST v2.4.7) using the maximum clade credibility method after discarding the first 25% samples as burn-in and merging the four independent runs in LogCombiner (part of BEAST v2.4.7). Convergence among runs was evaluated on the basis of ESS values > 200. For qualitative evaluation we present "cloudograms" (Figs 2 & 3), which are phylogenetic diagrams that reflect topological uncertainty of species trees. Cloudograms were recovered using DensiTree v2.2.5 (Bouckaert 2010) based on 500 trees from the posterior distribution.

RESULTS

Data compilation and potential phylogenetic biases

The single-gene and concatenated multi-locus tree topologies were congruent and showed no evident signature of cross-contamination (Fig. S1). The inferred inter-generic relationships were robust as indicated by high posterior probabilities (PP = 1.0 for the concatenated multi-locus tree) and the posterior MSC trees (Figs 2 & 3). All clearwing Haeterini form a monophyletic group sister to the Haeterini butterflies having full scale-cover on wings. Therefore, in terms of phylogenetic branching pattern, the genus *Pierella* diverged early in the evolution of Haeterini, followed by the monotypic genus *Dulcedo*, the genus *Pseudohaetera*, and the divergence between the genera *Haetera* and *Cithaerias*. Mixed node support for inter-specific relationships were recovered in single-gene and in the concatenated multi-locus datasets, with PP ranging from ~0.6 to 1.0. The six loci chosen for this study have been previously utilized in butterfly species-level systematics, thus, we expected these loci to be phylogenetically informative (Table 2). Instead, we recovered low node support among certain Haeterini species (Fig. S1), such as

294 those that rapidly diverged early in the evolution of *Pierella*, or in the recent radiation (< 2.5 Ma)
295 of the genera *Pierella* and *Cithaerias*.
296
297 *Species delimitation*
298 Regardless of which prior was used to take into account taxonomic knowledge for the
299 collapseWeight parameter (ω), STACEY converged in similar MSCCs suggesting that either 22
300 (under parameter ω = 0.73) or 24 (under parameter ω = 0.59) lineages are the most adequate
301 representation of species in our dataset. Indeed the pairwise similarity matrices generated for the
302 two analyses are highly congruent, though the pairwise posterior probabilities seem to decrease
303 in the STACEY analysis using ω = 0.59 (Figure S2). On average, the posterior probability that
304 two or more specimens inferred by STACEY under parameter ω = 0.73 belong to a single MSCC
305 ranged from 0.42 to 0.99, with a median of 0.85 and mode of 0.93. The lowest posterior
306 probability (0.42) was for the MSCC encompassing *Pierella lena lena*. In contrast, the STACEY
307 analysis that did not take into account any taxonomy information (parameter ω with Beta
308 distribution [α = 2, β = 2]) did not group any specimen in the dataset into MSCCs.
309
310 The MSCCs inferred by BP&P showed high sensitivity to prior distributions for the parameter θ.
311 The scenario with small ancestral population size, i.e., θ = IG[3, 0.02], suggested that nearly
312 every specimen in the dataset represent a single divergent lineage. This extreme scenario
313 suggesting 55 MSCCs was considered in the hypothesis testing exercise using Bayes factors,
314 even though it significantly departs from current taxonomic understanding of Haeterini. The
315 most likely numbers of MSCCs recovered by the other two BP&P analyses were 21 (when θ =
316 IG[3, 0.2]; prior mean of 0.1) or 28 (when θ = IG[3, 0.1]; prior mean of 0.05). The two

317 independent runs for each analysis converged in the same MSCCs, and the posterior probabilities
318 of the delimited species for the analysis under θ = IG[3, 0.2] ranged from 0.44 to 1.00, with a
319 median of 0.96 and mode of 0.99. The two lowest posterior probabilities (0.44) were for the
320 MSCCs encompassing *Pierella lena lena* and for *Pierella hyceta hyceta*. Overall, the large
321 impact of the prior distribution of the parameter θ in BP&P, resulting in 21, 28 or 55 MSCCs,
322 suggests that more data, both genetic and taxon sampling, is needed to strongly inform the
323 molecular species delimitation. However, the resulting most-probable number of MSCCs favored
324 under the two scenarios for large and medium ancestral population sizes are congruent with the
325 taxonomic knowledge of the group, as well as with the species delimitation exercises carried out
326 in STACEY (Figure 2).
327
328 The eight species delimitation hypotheses ranged from 18 to 63 species (or MSC clusters)
329 (Figure 2). The species hypotheses recovered by STACEY using ω = 0.73 (22 MSCCs) and
330 BP&P using θ = IG[3, 0.2] (21 MSCCs) were strongly supported compared to the remaining six
331 species delimitation hypotheses (*ln*Bf >> 10). The STACEY analysis recovering 22 MSCCs is
332 supported, but not conclusively (*ln*Bf = 9.69), over the BP&P analysis recovering 21 MSCCs
333 (Table 3). The differences between these two species hypotheses are: 1) the delimitation of two
334 lineages of *Pseudohaetera hypaesia*, one from central Peru (Chanchamayo Valley) and the other
335 from southeastern Ecuador (Morona Santiago and Zamora Chinchipe Provinces); BP&P
336 suggested a single MSCC for these two lineages whereas STACEY suggested two separate
337 MSCCs, 2) split of the subspecies *Pierella helvina ocreata* and *P. helvina incanescens* into two
338 separate species only by STACEY, and 3) split of *Pierella chalybaea* from Ecuador (Sucumbios)
339 from remaining *P. chalybaea* only by BP&P. On the other hand, the BP&P analyses under the

340 medium ancestral population size (θ = IG[3, 0.1]) recovered most of the described subspecies in
341 Haeterini, thus, we used this delimitation as a proxy for recognizing taxonomic subspecies in the
342 framework of the MSC despite it not being supported by the Bayes factor model comparison
343 (Figure 2).
344
345 *Absolute divergence times*
346 We show in Figure 3 two time-calibrated species trees, one approximating species in Haeterini
347 (i.e., STACEY's clusters under ω = 0.73; which had the highest marginal likelihood estimate
348 among the eight species delimitation hypotheses) and the second approximating subspecies in
349 Haeterini (BP&P's clusters under θ = IG[3, 0.1]; which recovered most of the described
350 taxonomic subspecies). The remaining six species trees can be found in the Supplementary
351 Information (Fig S3). Species tree topologies and branch lengths in absolute time remained
352 virtually the same regardless of species delimitation hypothesis. The median crown age of extant
353 Haeterini was estimated at 27 Ma (95% HPD: 22 to 33 Ma). The rapid early radiation of *Pierella*
354 happened in the late Miocene, at about 5 to 7 Ma (95% HPD: 3 to 9 Ma), whereas the recent
355 radiation of *Pierella* and *Cithaerias* occurred in the Pleistocene, at about 1 to 2 Ma (95% HPD:
356 0.5 to 2.5 Ma). We estimate that these two diversification events gave rise to about 70% of extant
357 Haeterini species.
358
359 DISCUSSION
360 Regardless of the species concept advocated by different researchers, traditional taxonomy and
361 coalescent-based approaches can act in synergy to infer statistically robust species hypotheses.
362 Importantly, the multispecies coalescent (MSC) model and the Bayesian framework to delimit

363 MSC clusters (MSCCs) allow the quantification and testing of species boundaries informed by
364 taxonomic knowledge. Furthermore, divergent taxonomic opinion among authors working on
365 particular species groups has been statistically weighed by the approach followed in this study.
366 This is important as a first step towards reliable standardization of taxonomy and higher-level
367 systematics based on models that take into account the process of speciation (e.g., incomplete
368 lineage sorting) and not just arbitrary genetic distances as in threshold-based approaches (e.g.,
369 based on COI barcoding). More realistic models of speciation that include, for example, inter-
370 specific gene flow might be more accurate at estimating species histories (Müller et al. 2018).
371 For example, in Europe alone, around 16% of butterfly species are known to hybridize
372 (Descimon and Mallet 2009), which is in line with the idea of speciation as a continuum (De
373 Queiroz 2007) and that inter-specific gene flow in animals might not be uncommon (Mallet et al.
374 2007). Another source of information for taxonomic conclusions is the development of new
375 methods that jointly model phenotypic traits and genetic data (Solís-Lemus et al. 2015). Taken
376 together, these methodological advances and the generation of biological data from various
377 sources suggest that in the near future coalescent-based approaches based on multi-locus data
378 may be able to overcome many current shortcomings in delimiting species.
379
380 *Limitations and strengths*
381 The dataset and approach that we have followed here are not exempt of limitations. First, the
382 amount of missing information, including taxonomic sampling and gapped molecular dataset,
383 may have reduced the power of our analyses. However, it has been noted previously that the
384 MSC might still be accurate with sampling schemes including fewer than five individuals per
385 lineage, as long as multi-locus datasets (in our case 6 unlinked loci) are utilized (Zhang et al.

386 2011). Although the impact of missing data in the MSC framework needs to be further
387 examined, a recent simulation study suggested that coalescent-based species tree inference might
388 be highly accurate even with severely gapped multi-locus datasets (Nute et al. 2018). Second,
389 inter-specific gene flow may impact current implementations of the MSC by obscuring lineages
390 divergence (Luo et al. 2018). Nevertheless, unless there is a high level of gene flow and
391 hybridization, the MSC-based delimitation and species tree inference should be robust (Eckert
392 and Carstens 2008; Zhang et al. 2011). However, we note that this issue needs to be further
393 studied in the light of more data and using recently-developed approaches, such as the isolation-
394 with-migration model (Müller et al. 2018). Third, the impact of priors in MSC-based species
395 delimitation might be high when the molecular dataset does not hold sufficient signal to
396 converge on the same MSCCs (Leaché and Fujita 2010; Jones et al. 2015). Our results showed
397 that the choice of priors heavily influenced the number of MSCCs estimated by MSC-based
398 methods. However, our priors rely on existing taxonomic knowledge and thus we have reduced
399 the clustering space of 63 specimens based on actual evidence coming from previous
400 morphology-based studies. The number of most likely MSCCs inferred here (from 21 to 28
401 clusters) remains highly congruent with the morphological diversity encountered in this group of
402 butterflies.
403
404 The strengths of our study rely on three key aspects. First, we have not used any a priori
405 taxonomic assignment of individuals to species, nor any guide species tree to delimit species.
406 This allowed us to include both tree topology and taxonomic assignment uncertainties explicitly
407 into the models, avoiding potential biases that may have precluded accurate estimation of
408 speciation probabilities (Leaché and Fujita 2010). However, we note that the posterior

409 probabilities of certain species delimitations, such as *Pierella lena lena*, remain low, which may
410 be explained by non-sufficient signal of our molecular dataset. We expect that adding more
411 genetic data might increase the posterior probabilities while holding the same delimitations,
412 given that different approaches converged in the same species hypotheses regardless of the low
413 posterior probabilities (e.g., *Pierella lena lena* was recovered as most probable by both STACEY
414 and BP&P, Fig. 2). Second, taxonomic knowledge has been formally taken into account in our
415 probabilistic scenarios. Previous studies have put forward the statistical comparison (e.g., via
416 Bayes factors) of alternate species delimitation models, but these mostly evaluated different
417 individual reassignments based only on node collapsing of sister lineages in a phylogeny
418 (Grummer et al. 2014; Yu et al. 2017). Our pipeline, on the contrary, has aided the exploration of
419 alternate MSCCs that are congruent as well with other sources of information in butterfly
420 taxonomy, such as morphology and geography. Third, the MSCCs found in this study are not
421 nested, meaning that specimens could be re-assigned to any combination of clusterings. The use
422 of Bayes factors as a selection tool is appropriate because of its flexibility in testing non-nested
423 models (Leaché et al. 2014). We used path sampling under thermodynamic integration which has
424 been shown to be highly accurate in testing non-nested species delimitation models (Grummer et
425 al. 2014).
426
427 *Performance of methods*
428 Our approach focused on using the multispecies coalescent model, informed by taxonomic
429 knowledge, to assign individuals to MSCCs based on different priors and methods implemented
430 in STACEY and BP&P. This is arguably a less arbitrary approach to reduce the space of all
431 possible clusterings for model testing using Bayes factors, compared to other approaches based

solely in taxonomic expertise (Leaché et al. 2014) or in multi-locus networks (Grummer et al. 2014) and population assignments (Olave et al. 2014). Furthermore, the approach outlined here is flexible, simple and fast because it avoids preliminary estimation of guide trees and *a priori* taxonomic assignments of individuals. Taxonomic knowledge modeled as prior distribution has the potential to ameliorate potential biases (e.g., population structure raised to species), which have been observed when relying solely on genetic information and guide trees (Olave et al. 2014; Sukumaran and Knowles 2017). We showed that the number of inferred MSCCs heavily depends on priors, and indeed, under the scenario of medium ancestral population sizes (θ = IG[3, 0.1]) BP&P recovered traditionally viewed infra-specific diversity, and perhaps structured populations. Note that subspecies in butterfly taxonomy are seen as populations with limited gene flow due to allopatric distributions (Lamas 2008). Overall, we highlight the importance of taxonomically-informed molecular species delimitation and the use of Bayes factor model comparison.

Both MSC-based methods used in this study, STACEY and BP&P, were fast and simple in their implementation thanks to available guidelines and manuals (e.g., Jones et al. 2015; Yang 2015). The most supported BP&P's delimitation followed a conservative prior distribution for the size of ancestral population size, that is θ = IG[3, 0.2] with a prior mean = 0.1, which was pointed out as the most appropriate prior mean (Leaché and Fujita 2010). This species delimitation model is highly similar to the most supported STACEY model, except for three species hypotheses. In line with morphological differences, STACEY favored the split of *Pierella helvina incanescens* and *P. helvina ocreata*, two subspecies that were originally considered separate species but synonymized due to allopatric geographical distribution by Constantino (1995). On the other

455 hand, BP&P favored the split of one *Pierella chalybaea* specimen (Ecuador, Sucumbíos) from
456 other conspecific individuals, including others from Ecuador. STACEY did not favor such a
457 split, which again is in line with the absence of any clear morphological difference in *P.*
458 *chalybaea* from western Amazonia (Zacca et al. 2016). Finally, STACEY delimited three
459 separate species within *Pseudohatera hypaesia* while BP&P delimited only two. However, there
460 are no clear wing coloration differences among populations of *P. hypaesia* throughout its range
461 from Colombia to Bolivia (Gerardo Lamas, *pers. comm.*). The supported STACEY's
462 delimitation here thus points out that large genetic divergences exist in the otherwise similar-
463 looking *P. hypaesia*, warranting a taxonomic revision of these montane butterflies from the
464 tropical Andes.
465
466 *Taxonomic implications*
467 The probabilistic framework applied in this study allows the statistical test of alternative species
468 hypotheses in a taxonomic group that has likely evolved for nearly 27 million years. The most
469 likely scenario among those tested here suggests that at least four divergent lineages should be
470 elevated to species by current taxonomic standards. It is clear that a more comprehensive
471 sampling and datasets, including morphological and molecular characters, are needed to robustly
472 delineate species boundaries in Haeterini. Nonetheless, the four divergent lineages found in this
473 study corroborate morphological differences that have been previously acknowledged in these
474 groups but that taxonomists considered conspecific variations (subspecies) mainly because of
475 allopatric distributions, a historical practice in butterfly taxonomy (Lamas 2008). In almost all
476 MSC-based analyses, the following taxa were considered independent evolutionary lineages: i)
477 *Pierella hyceta hyceta* and *Pierella hyceta ceryce*, ii) *Pierella lena lena* and *Pierella lena*

478 *brasiliensis*, iii) *Pierella helvina incanescens* and *Pierella helvina ocreata*, iv) two divergent
479 sympatric populations in central Peru (Chanchamayo Valley) of *Pseudohaetera hypaesia*.
480 According to our results, these eight lineages are likely full species (a taxonomic revision in
481 preparation is addressing this).
482
483 Zacca et al. (2016) split *Pierella lamia* into seven species mainly based on allopatric
484 geographical distributions, and also on genitalia and androconial patches on male wings (despite
485 their similar wing coloration). We did not find support for the species status of *Pierella*
486 *keithbrowni* from southeastern Brazil proposed by these authors. In the case of *P. keithbrowni*,
487 the main diagnostic characters were the androconial patch shape, and ductus bursae in female
488 genitalia longer than in *P. nice*, albeit male genitalia in *Pierella lamia* complex are not
489 differentiated. In all MSC-based analyses, *P. keithbrowni* is not genetically different from other
490 populations in central Brazil and Ecuador. Our results therefore suggest that differences in the
491 aforementioned morphological characters may represent variation within a single evolutionary
492 lineage, and thus their usage needs to be complemented with other lines of evidence. Note that
493 androconial patch morphology has been widely used to diagnose butterfly species boundaries in
494 other satyrine groups (e.g., Núñez Aguila et al. 2013; Penz et al. 2017), and our results suggest
495 that the reliability of this character system in different lineages should be further investigated. As
496 noted previously, granting species status to butterfly populations primarily based on geographical
497 distribution (allopatric populations) might be unjustified if other evidence fails to recognize clear
498 divergence (Descimon and Mallet 2009). Therefore, we suggest that *P. keithbrowni* should be
499 synonymized with *P. chalybaea* (work in preparation will address this).
500

CONCLUSIONS

Here we show that for the butterfly tribe Haeterini, the multispecies coalescent model generally recognizes traditionally viewed butterfly subspecies and species, with some exceptions linked to the use of different morphological character systems. By using a probabilistic framework, we have shown that divergent taxonomic opinion (concepts) were used by different authors, including butterfly species that were over-split (*lamia* complex), lumped (at least 6 subspecies raised to species here), or misclassified (e.g. *Pierella lena browni* was previously synonymized with *P. lena brasiliensis* (Lamas 2004), despite *browni* being evolutionarily more closely related to *P. lena lena* than to *brasiliensis*). Androconial patch morphology is commonly used as informative character to diagnose species, but we showed that at least in *Pierella* it alone may not be well-suited to distinguish infra- and inter-specific diversity. Furthermore, taxonomic knowledge informed as priors in MSC-based species delimitation using genetic data is a robust approach to reduce the clustering space in model testing.

The low node support recovered among certain Haeterini species may be attributed to incomplete lineage sorting due, for example, to ancient rapid radiation as in the crown node of *Pierella* or to recent speciation as in *Pierella* and *Cithaerias*. Haeterini butterflies evolved for nearly 27 million years but most extant species (*ca*. 80%) likely diverged rather recently—within the past 2 million years. Future macroevolutionary studies using the revised species boundaries of Haeterini might address this puzzling diversification history, whether it was the result of high tropical species turnover over millions of years (with constant and high extinction and speciation rates, in line with Antonelli et al. 2015) or a Pleistocene major burst in diversification (in line with the Quaternary diversification model, as characterized via simulated data; Matos-Maraví 2016).

SUPPLEMENTARY MATERIAL

Figure S1: Consensus trees based on single-gene and concatenated loci datasets (pages 2–9).

Figure S2: Pairwise similarity matrices based on delimitation analyses with STACEY (pages 10–14).

Figure S3: Time-calibrated Maximum Clade Credibility species trees of the eight species delimitation hypotheses (pages 15–24).

DNA alignments and phylogenies of the single-gene and concatenated datasets were archived in TreeBASE (ID: 23439). Accession URL: http://purl.org/phylo/treebase/phylows/study/TB2:S23439

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549

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REFERENCES

Antonelli A., Zizka A., Silvestro D., Scharn R., Cascales-Miñana B., Bacon C.D. 2015. An engine for global plant diversity: Highest evolutionary turnover and emigration in the American tropics. Front. Genet. 6:130.

Aydin Z., Marcussen T., Ertekin A.S., Oxelman B. 2014. Marginal likelihood estimate comparisons to obtain optimal species delimitations in *Silene* sect. *Cryptoneurae* (Caryophyllaceae). PLoS One. 9:e106990.

Bouckaert R., Heled J., Kühnert D., Vaughan T., Wu C.-H., Xie D., Suchard M.A., Rambaut A., Drummond A.J. 2014. BEAST 2: a software platform for Bayesian evolutionary analysis. PLoS Comput. Biol. 10:e1003537.

Bouckaert R.R. 2010. DensiTree: making sense of sets of phylogenetic trees. Bioinformatics. 26:1372–1373.

Bravo G.A., Antonelli A., Bacon C.D., Bartoszek K., Huynh S., Jones G., Knowles L.L., Lamichhaney S., Marcussen T., Nakhleh L.K., Oxelman B., Pfeil B., Schliep A., Werneck F.P., Wiedenhoeft J., Willows-Munro S., Edwards S. V. 2018. Embracing heterogeneity: building the Tree of Life and the future of phylogenomics. PeerJ Prepr. 6:e26449v3.

Chazot N., Wahlberg N., Freitas A.V.L., Mitter C., Labandeira C., Sohn J.-C., Sahoo R.K., Seraphim N., de Jong R., Heikkilä M. 2018. The trials and tribulations of priors and posteriors in Bayesian timing of divergence analyses: the age of butterflies revisited. bioRxiv.:259184.

Constantino L.M. 1995. Revisión de la tribu Haeterini Herrich-Schäffer, 1864 en Colombia (Lepidoptera: Nymphalidae, Satyrinae). Shil. Rev. Lepidopterol. 23:49–76.

Darriba D., Taboada G.L., Doallo R., Posada D. 2012. jModelTest 2: more models, new

574 heuristics and parallel computing. Nat. Methods. 9:772.

575 Degnan J.H., Rosenberg N.A. 2009. Gene tree discordance, phylogenetic inference and the

576 multispecies coalescent. Trends Ecol. Evol. 24:332–340.

577 Descimon H., Mallet J. 2009. Bad species. In: Settele J., Shreeve T., Konvička M., Van Dyck H.,

578 editors. Ecology of Butterflies in Europe. Cambridge, UK: Cambridge University Press. p.

579 219–249.

580 Eckert A.J., Carstens B.C. 2008. Does gene flow destroy phylogenetic signal? The performance

581 of three methods for estimating species phylogenies in the presence of gene flow. Mol.

582 Phylogenet. Evol. 49:832–842.

583 Edwards S. V., Xi Z., Janke A., Faircloth B.C., McCormack J.E., Glenn T.C., Zhong B., Wu S.,

584 Lemmon E.M., Lemmon A.R., Leaché A.D., Liu L., Davis C.C. 2016. Implementing and

585 testing the multispecies coalescent model: a valuable paradigm for phylogenomics. Mol.

586 Phylogenet. Evol. 94:447–462.

587 Fujita M.K., Leaché A.D., Burbrink F.T., McGuire J.A., Moritz C. 2012. Coalescent-based

588 species delimitation in an integrative taxonomy. Trends Ecol. Evol. 27:480–488.

589 Grummer J.A., Bryson R.W., Reeder T.W. 2014. Species delimitation using Bayes factors:

590 simulations and application to the *Sceloporus scalaris* species group (Squamata:

591 Phrynosomatidae). Syst. Biol. 63:119–133.

592 Hebert P.D.N., Cywinska A., Ball S.L., DeWaard J.R. 2003. Biological identifications through

593 DNA barcodes. Proc. R. Soc. B Biol. Sci. 270:313–321.

594 Herrich-Schäffer G.A.W. 1864. Prodromus systematis Lepidopterorum: versuch einer

595 systematischen Anordnung der Schmetterlinge. Corresp. des Zool. Vereines zu Regensbg.

596 18(7/8):89112.

597 Jones G. 2017. Algorithmic improvements to species delimitation and phylogeny estimation
598 under the multispecies coalescent. J. Math. Biol. 74:447–467.
599 Jones G., Aydin Z., Oxelman B. 2015. DISSECT: an assignment-free Bayesian discovery
600 method for species delimitation under the multispecies coalescent. Bioinformatics. 31:991–
601 998.
602 Kass R.E., Raftery A.E. 1995. Bayes Factors. J. Am. Stat. Assoc. 90:773–795.
603 Keightley P.D., Pinharanda A., Ness R.W., Simpson F., Dasmahapatra K.K., Mallet J., Davey
604 J.W., Jiggins C.D. 2015. Estimation of the spontaneous mutation rate in *Heliconius*
605 *melpomene*. Mol. Biol. Evol. 32:239–243.
606 Knowles L.L., Carstens B.C. 2007. Delimiting species without monophyletic gene trees. Syst.
607 Biol. 56:887–895.
608 Lamas G. 1997. Lista sinonímica de los géneros *Cithaerias* Hübner y *Haetera* Fabricius
609 (Lepidoptera: Nymphalidae: Satyrinae), con la descripción de dos subespecies nuevas del
610 Perú. Rev. Peru. Entomol. 40:133–138.
611 Lamas G. 2004. Atlas of Neotropical Lepidoptera: Checklist: Part 4A. Hesperioidea-
612 Papilionoidea. Gainesville, Florida: Association for Tropical Lepidoptera.
613 Lamas G. 2008. La sistemática sobre mariposas (Lepidoptera: Hesperioidea y Papilionoidea) en
614 el mundo: estado actual y perspetivas futuras. In: Llorente Bousquets J., Lanteri A., editors.
615 Contribuciones Taxonómicas en Órdenes de Insectos Hiperdiversos. México D.F.: Las
616 Prensas de Ciencias, UNAM. p. 57–70.
617 Lanfear R., Frandsen P.B., Wright A.M., Senfeld T., Calcott B. 2017. Partitionfinder 2: new
618 methods for selecting partitioned models of evolution for molecular and morphological
619 phylogenetic analyses. Mol. Biol. Evol. 34:772–773.

620 Lartillot N., Philippe H. 2006. Computing Bayes factors using thermodynamic integration. Syst.
621 Biol. 55:195–207.
622 Leaché A.D., Fujita M.K. 2010. Bayesian species delimitation in West African forest geckos
623 (*Hemidactylus fasciatus*). Proc. R. Soc. B Biol. Sci. 277:3071–3077.
624 Leaché A.D., Fujita M.K., Minin V.N., Bouckaert R.R. 2014. Species delimitation using
625 genome-wide SNP Data. Syst. Biol. 63:534–542.
626 Luo A., Ling C., Ho S.Y.W., Zhu C.-D. 2018. Comparison of methods for molecular species
627 delimitation across a range of speciation scenarios. Syst. Biol. 67:830–846.
628 Mallet J., Beltrán M., Neukirchen W., Linares M. 2007. Natural hybridization in heliconiine
629 butterflies: the species boundary as a continuum. BMC Evol. Biol. 7:28.
630 Matos-Maraví P. 2016. Investigating the timing of origin and evolutionary processes shaping
631 regional species diversity: Insights from simulated data and Neotropical butterfly
632 diversification rates. Evolution 70:1638–1650.
633 Miller L.D. 1968. The higher classification, phylogeny and zoogeography of the Satyridae
634 (Lepidoptera). Mem. Am. Entomol. Soc. 24:1–174.
635 Miller M.A., Pfeiffer W., Schwartz T. 2010. Creating the CIPRES Science Gateway for
636 inference of large phylogenetic trees. Proc. Gatew. Comput. Environ. Work. (GCE), 14
637 Nov.:1–8.
638 Müller N.F., Ogilvie H.A., Zhang C., Drummond A., Stadler T. 2018. Inference of species
639 histories in the presence of gene flow. bioRxiv.:348391.
640 Núñez Aguila R., Matos-Maraví P., Wahlberg N. 2013. New *Calisto* species from Cuba, with
641 insights on the relationships of Cuban and Bahamian taxa (Lepidoptera, Nymphalidae,
642 Satyrinae). Zootaxa. 3669:503–521.

643 Nute M., Chou J., Molloy E.K., Warnow T. 2018. The performance of coalescent-based species
644 tree estimation methods under models of missing data. BMC Genomics. 19:286.
645 Ogilvie H.A., Bouckaert R.R., Drummond A.J. 2017. StarBEAST2 brings faster species tree
646 inference and accurate estimates of substitution rates. Mol. Biol. Evol. 34:2101–2114.
647 Olave M., Solà E., Knowles L.L. 2014. Upstream analyses create problems with DNA-based
648 species delimitation. Syst. Biol. 63:263–271.
649 Paluch M., Zacca T., Siewert R.R., Mielke O.H.H., Casagrande M.M. 2015. A new subspecies of
650 *Pierella lena* (Lepidoptera: Nymphalidae: Satyrinae) in northeastern Brazil. Florida
651 Entomol. 98:916–920.
652 Penz C.M., Alexander L.G., DeVries P.J. 2014. Revised species definitions and nomenclature of
653 the rose colored *Cithaerias* butterflies (Lepidoptera, Nymphalidae, Satyrinae). Zootaxa.
654 3873:541–559.
655 Penz C.M., Casagrande M.M., Devries P., Simonsen T.J. 2017. Documenting diversity in the
656 Amazonian butterfly genus *Bia* (Lepidoptera, Nymphalidae). Zootaxa. 4258:201–237.
657 Pons J., Barraclough T.G., Gomez-Zurita J., Cardoso A., Duran D.P., Hazell S., Kamoun S.,
658 Sumlin W.D., Vogler A.P. 2006. Sequence-based species delimitation for the DNA
659 taxonomy of undescribed insects. Syst. Biol. 55:595–609.
660 Puillandre N., Lambert A., Brouillet S., Achaz G. 2012. ABGD, Automatic Barcode Gap
661 Discovery for primary species delimitation. Mol. Ecol. 21:1864–1877.
662 De Queiroz K. 2007. Species concepts and species delimitation. Syst. Biol. 56:879–886.
663 Rannala B., Yang Z. 2013. Improved reversible jump algorithms for Bayesian species
664 delimitation. Genetics. 194:245–253.
665 Rannala B., Yang Z. 2017. Efficient Bayesian species tree inference under the multispecies

666 coalescent. Syst. Biol. 66:823–842.

667 Ronquist F., Teslenko M., van der Mark P., Ayres D.L., Darling A., Höhna S., Larget B., Liu L.,
668 Suchard M.A., Huelsenbeck J.P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic
669 inference and model choice across a large model space. Syst. Biol. 61:539–542.

670 Rosindell J., Cornell S.J., Hubbell S.P., Etienne R.S. 2010. Protracted speciation revitalizes the
671 neutral theory of biodiversity. Ecol. Lett. 13:716–727.

672 Solís-Lemus C., Knowles L.L., Ané C. 2015. Bayesian species delimitation combining multiple
673 genes and traits in a unified framework. Evolution 69:492–507.

674 Sukumaran J., Knowles L.L. 2017. Multispecies coalescent delimits structure, not species. Proc.
675 Natl. Acad. Sci. 114:1607–1612.

676 Wahlberg N., Leneveu J., Kodandaramaiah U., Peña C., Nylin S., Freitas A.V.L., Brower A.V.Z.
677 2009. Nymphalid butterflies diversify following near demise at the Cretaceous/Tertiary
678 boundary. Proc. R. Soc. B Biol. Sci. 276:4295–4302.

679 Wahlberg N., Wheat C.W. 2008. Genomic outposts serve the phylogenomic pioneers: designing
680 novel nuclear markers for genomic DNA extractions of Lepidoptera. Syst. Biol. 57:231–
681 242.

682 Weymer G. 1910. Familie: Satyridae. In: Seitz A., editor. Die Gross-Schmetterlinge der Erde.
683 Vol. 5. Stuttgart, Alfred Kernen Verlag. p. 173–283.

684 Willmott K.R. 2015. A new subspecies of *Cithaerias pyropina* (Salvin & Godman, 1868) from
685 the Cordillera de Cutucú in southeastern Ecuador (Nymphalidae: Satyrinae). Trop. Lepid.
686 Res. 25:1–4.

687 Yang Z. 2015. The BPP program for species tree estimation and species delimitation. Curr. Zool.
688 61:854–865.

689 Yang Z., Rannala B. 2010. Bayesian species delimitation using multilocus sequence data. Proc.
690 Natl. Acad. Sci. U. S. A. 107:9264–9269.
691 Yang Z., Rannala B. 2014. Unguided species delimitation using DNA sequence data from
692 multiple loci. Mol. Biol. Evol. 31:3125–3135.
693 Yu G., Rao D., Matsui M., Yang J. 2017. Coalescent-based delimitation outperforms distance-
694 based methods for delineating less divergent species: the case of *Kurixalus odontotarsus*
695 species group. Sci. Rep. 7:16124.
696 Zacca T., Siewert R.R., Casagrande M.M., Mielke O.H.H., Paluch M. 2016. Taxonomic revision
697 of the "*Pierella lamia* species group" (Lepidoptera: Nymphalidae: Satyrinae) with
698 descriptions of four new species from Brazil. Zootaxa. 4078:366–386.
699 Zhang C., Zhang D.X., Zhu T., Yang Z. 2011. Evaluation of a Bayesian coalescent method of
700 species delimitation. Syst. Biol. 60:747–761.
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702

703 FIGURES AND TABLES

704 Figure 1: Representatives of the tribe Haeterini. a) *Pierella nereis* (Brazil, Minas Gerais, Santa
705 Barbara; Milwaukee Public Museum), b) *Dulcedo polita* (Costa Rica, Sarapiqui, Tirimbina
706 Biological Station; Phil DeVries Collection), c) *Pseudohaetera mimica* (Peru, Junin, Satipo;
707 Natural History Museum of Los Angeles County), d) *Haetera piera* (Ecuador, Napo, Garza
708 Cocha; Phil DeVries Collection), e) *Cithaerias cliftoni* (Ecuador, Oriente; Natural History
709 Museum of Los Angeles County), f) *Cithaerias aurora tambopata* (Peru, Madre de Dios,
710 Pakitza, Manu National Reserve; Smithsonian Institution).

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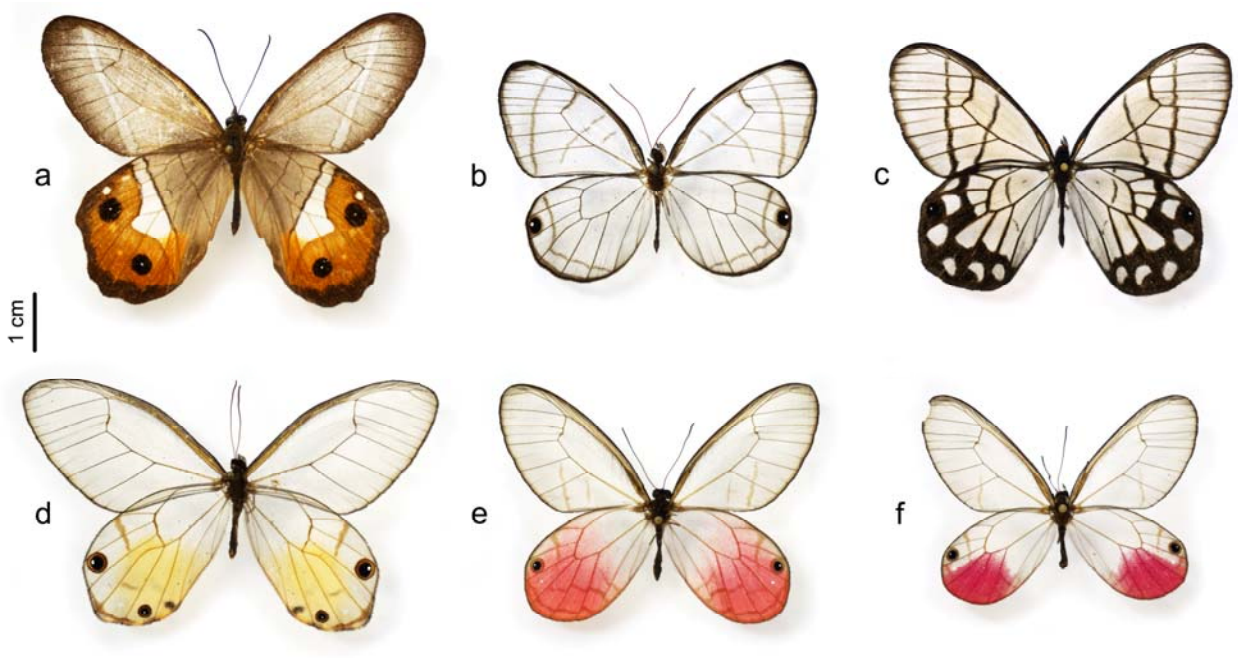

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Figure 2: Evaluated species delimitation hypotheses using Bayes factors. The eight scenarios were: Taxonomic species (*spp*, 18 lineages) or subspecies raised to species (*sspp*, 26 lineages), STACEY's delimited species under prior accounting for number of taxonomic species (*spp prior*, 22 lineages) or number of subspecies raised to species (*sspp prior*, 24 lineages), as well as with prior not informed by taxonomy (*no prior*, 63 lineages), and BP&P's delimited species under prior for ancestral population size as large (21 lineages), medium (28 lineages), or small (53 lineages). The "cloudogram", which is a diagram representing phylogenetic uncertainty of the 63 Haeterini specimens, was generated based on 500 posterior trees from STACEY analysis (thicker blue line represents the consensus phylogeny). The delimitation model STACEY under prior accounting for taxonomic species (outlined by a surrounding box) received significant support based on Bayes factors over all other models, and thus is the classification that we propose here.

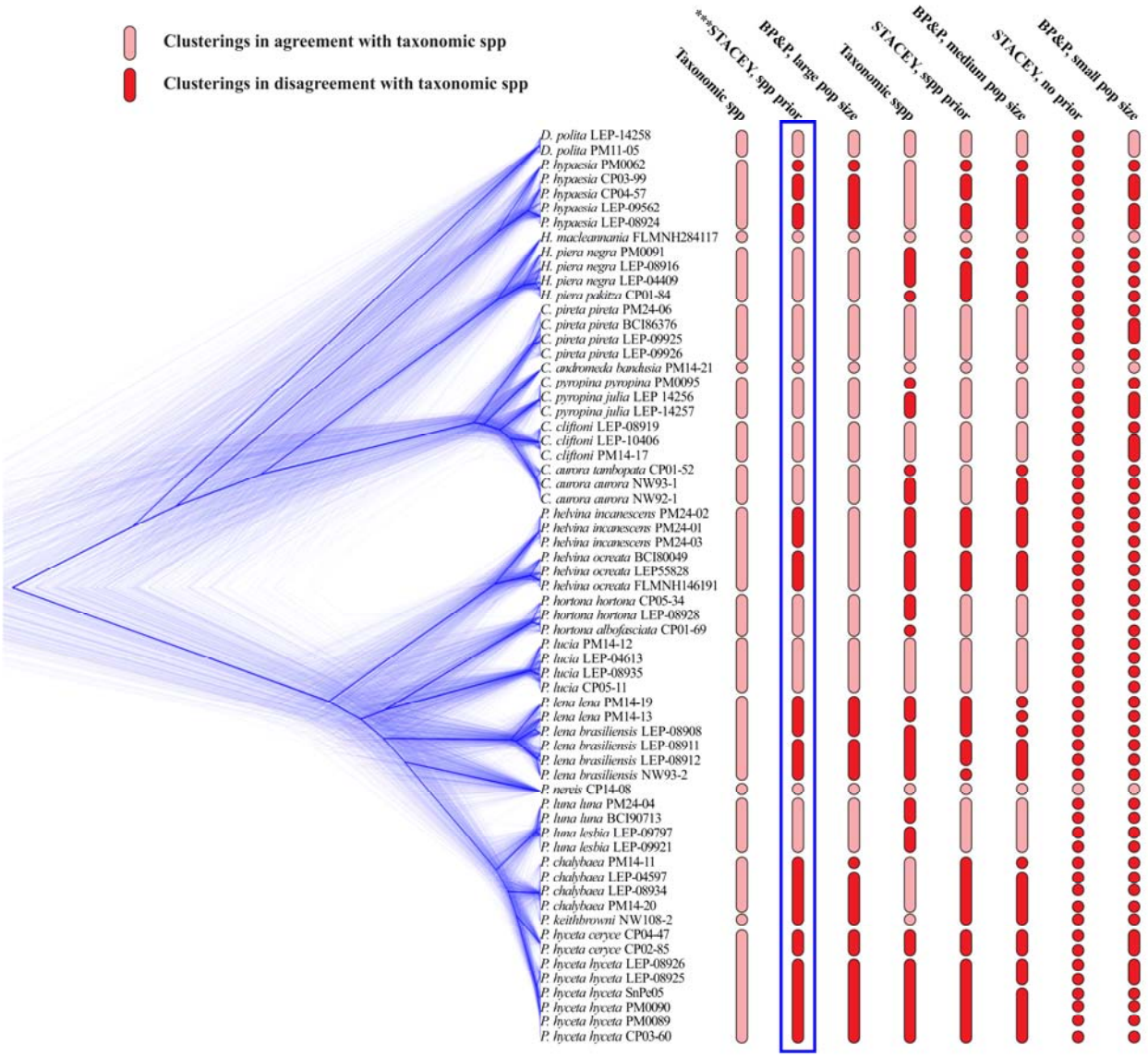

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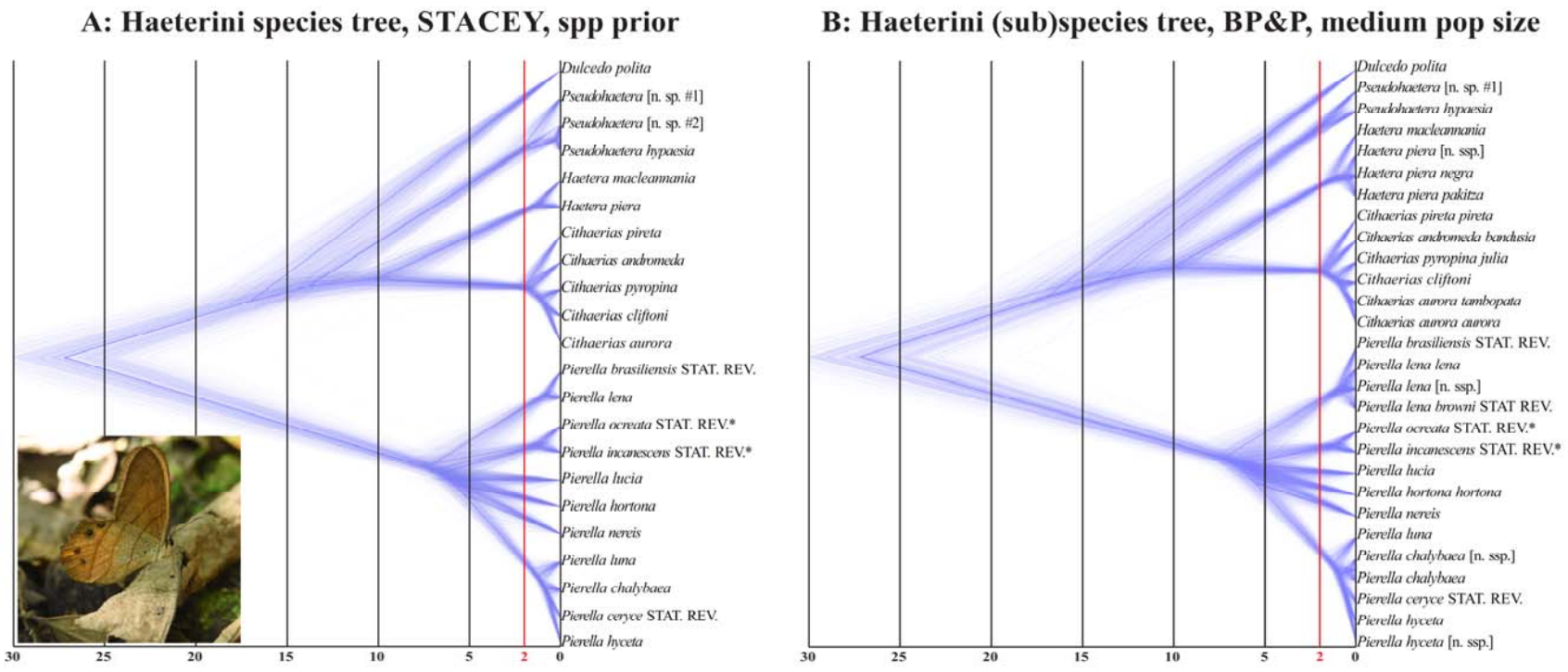
Figure 3: Time-calibrated phylogenetic hypotheses of models that best approximate species and subspecies in Haeterini. A) “Cloudogram” of the best-fit species delimitation model based on Bayes factors, STACEY under prior accounting for taxonomic species. B) “Cloudogram” of the delimitation model that best approximated described subspecies, BP&P under prior for medium ancestral population size. Time axis in both panels is scaled to million years. *The species status of *Pierella helvina ocreata* and *P. helvina incanescens* may change with the inclusion of *P. helvina helvina*, but it is likely that *P. helvina ocreata* and *P. helvina incanescens* are separate species. Inset butterfly: *Pierella hyceta hyceta*; Peru, Pasco, Cañón de Huancabamba, 1200 masl, 29.vii.2017. Photo: Markéta Aubrechtová.



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740 Table 1: Voucher locality information and associated genetic data. All Haeterini specimens were
741 identified to the species and subspecies level based on the most recent taxonomic revisions
742 (Constantino 1995; Lamas 1997; Penz et al. 2014; Paluch et al. 2015; Willmott 2015; Zacca et al.
743 2016). GenBank accession numbers for each of the sequenced locus are presented for every
744 specimen.

Code	Genus	Species	Subspecies	Country	Locality	CAD	COI	EF1a	GAPDH	RpS5	*wingless*
EW1-1	Pararge	aegeria		FRANCE	Carcassonne, 2 km S Trassonel	EU141293	DQ176379	DQ338913	EU141476	EU141372	DQ338620
NW121-17	Lethe	minerva		INDONESIA	Bali	EU141309	DQ338768	DQ338909	EU141492	EU141387	DQ338616
EW10-5	Bicyclus	anynana		ZIMBABWE	Harare	EU141295	AY218238	AY218258	EU141478	EU141374	AY218276
CP06-89	Oressinoma	sorata		PERU	Pasco, Oxapampa	MH802140	GQ357209	GQ357278	GQ357440	GQ357570	GQ357342
EW25-17	Orsotriaena	medus		BANGLADESH	Sylhet Div., Lowacherra Forest	-	DQ338766	DQ338906	EU528405	EU528453	DQ338633
NW108-3	Taygetis	virgilia		BRAZIL	SP, Campinas, Santa Genebra	EU141305	DQ338812	DQ338958	EU141487	EU141383	DQ338683
PM07-05	Calisto	smintheus		CUBA	Alrededores de La Platica	JN881807	JN881904	JN881778	JN881827	JN881845	JN881870
CP03-63	Manerebia	cyclopina		PERU	Junín, Quebrada Siete Jeringas	-	DQ338785	DQ338928	EU528397	EU528443	GQ864477
NW162-21	Satyrus	actaea		FRANCE	Aude, Villegly	GQ864709	GQ864807	GQ864901	GQ865030	GQ865494	GQ864495
EW24-7	Erebia	oeme		FRANCE	Languedoc, Ariege 09, Ustou	EU141296	DQ338780	DQ338923	EU141479	EU141375	DQ338640
CP13-01	Aeropetes	tulbaghia		S. AFRICA	Mpumalanga Verloren Valei	-	DQ338579	DQ338907	EU528381	EU528419	DQ338634
NW66-6	Melanitis	leda		AUSTRALIA	Queensland, Cairns	EU141330	AY090207	AY090173	EU141508	EU141408	AY090140
NW122-21	Brassolis	sophorae		BRAZIL	SP, Campinas	-	EU528314	EU528291	GQ357384	EU528425	EU528270
NW66-5	Morpho	helenor		-	London Pupae Supplies	EU141329	AY090210	AY090176	EU141507	EU141407	AY090143
NW121-20	Elymnias	casiphone		INDONESIA	Bali	EU141310	DQ338760	DQ338900	-	EU141388	DQ338627
NW101-2	Zeuxidia	dorhni		INDONESIA	Java, Bandung	-	DQ338752	DQ338892	EU528417	EU528471	DQ338609
PM14-21	Cithaerias	andromeda	bandusia	BRAZIL	MT, Alta Floresta	KJ145993	KJ145988	KJ145996	KJ146001	KJ146004	KJ146008
NW92-1	Cithaerias	aurora	aurora	PERU	Loreto, Río Paiwa	-	MH802187	-	MH802251	MH802286	MH802332
NW93-1	Cithaerias	aurora	aurora	PERU	Loreto, Río Paiwa	-	DQ338756	DQ338896	MH802252	MH802287	DQ338613
CP01-52	Cithaerias	aurora	tambopata	PERU	Tambopata Research Center	MH802134	MH802157	MH802205	MH802239	MH802258	MH802304
LEP-08919	Cithaerias	cliftoni		ECUADOR	Pastaza, Kapawi lodge	MH802148	MH802174	MH802218	MH802246	MH802276	MH802320
LEP-10406	Cithaerias	cliftoni		ECUADOR	Sucumbios, Cuyabeno lodge	KM013123	KM012939	KM012990	KM013275	KM013174	KM013055
PM14-17	Cithaerias	cliftoni		ECUADOR	Sucumbios, Garza Cocha	-	MH802196	-	-	MH802295	MH802341
PM24-06	Cithaerias	pireta	pireta	COSTA RICA	Sarapiqui, Agrícola Sofía, nr. Tirimbina	-	-	MH802238	-	MH802299	MH802345
LEP-09925	Cithaerias	pireta	pireta	ECUADOR	Carchi, Finca San Francisco	MH802154	MH802182	MH802225	MH802249	MH802282	MH802327
LEP-09926	Cithaerias	pireta	pireta	ECUADOR	Pichincha, km. 20 Pacto-Guayabillas Rd.	KM013135	KM012940	KM013007	KM013282	KM013181	KM013069
BCI86376	Cithaerias	pireta	pireta	PANAMA	BCI	-	HM406591	MH802203	-	MH802256	MH802302
LEP-14256	Cithaerias	pyropina	julia	ECUADOR	Morona Santiago	-	MH802183	MH802226	-	MH802283	MH802328
LEP-14257	Cithaerias	pyropina	julia	ECUADOR	Morona Santiago	-	MH802184	MH802227	-	MH802284	MH802329
PM0095	Cithaerias	pyropina	pyropina	PERU	Pasco, Cañón de Huancabamba	-	MH802192	MH802232	-	MH802291	MH802337

PM11-05	Dulcedo	polita		COSTA RICA	Heredia, Tirimbina	-	KJ145990	KJ145998	KJ146002	KJ146006	KJ146010
LEP-14258	Dulcedo	polita		ECUADOR	Esmeraldas, Río Chuchubi	-	MH802185	MH802228	MH802250	-	MH802330
FLMNH284117	Haetera	macleannania		ECUADOR	Esmeraldas, Finca Cypris	-	MH802165	MH802209	MH802242	MH802267	MH802311
LEP-04409	Haetera	piera	negra	ECUADOR	Morona Santiago, nr. Yaupi	MH802142	MH802167	MH802211	MH802244	MH802269	MH802313
LEP-08916	Haetera	piera	negra	ECUADOR	Pastaza, Kapawi lodge	MH802147	MH802173	MH802217	MH802245	MH802275	MH802319
PM0091	Haetera	piera	negra	PERU	Pasco, Cañón de Huancabamba	-	MH802191	MH802231	MH802253	MH802290	MH802336
CP01-84	Haetera	piera	pakitza	PERU	Tambopata Research Center	EU141292	DQ018959	DQ018926	EU141475	EU141371	DQ018897
PM14-20	Pierella	chalybaea		BRAZIL	MT, Alta Floresta	KM013148	KM012978	KM013025	-	KM013197	KM013087
LEP-04597	Pierella	chalybaea		ECUADOR	Orellana, Napo Wildlife Center	-	MH802168	MH802212	-	MH802270	MH802314
LEP-08934	Pierella	chalybaea		ECUADOR	Pastaza, Kapawi lodge	MH802151	MH802178	MH802221	-	MH802279	MH802323
PM14-11	Pierella	chalybaea		ECUADOR	Sucumbios, Garza Cocha	MH802155	MH802193	MH802233	-	MH802292	MH802338
PM24-01	Pierella	helvina	incanescens	COSTA RICA	Sarapiqui, Finca Starke	-	MH802197	-	-	-	-
PM24-02	Pierella	helvina	incanescens	COSTA RICA	Heredia, Tirimbina	-	MH802198	MH802235	-	MH802296	MH802342
PM24-03	Pierella	helvina	incanescens	COSTA RICA	Heredia, Tirimbina	-	MH802199	MH802236	MH802254	MH802297	MH802343
FLMNH146191	Pierella	helvina	ocreata	ECUADOR	Esmeraldas, Río Chuchubi	-	MH802164	MH802208	-	-	MH802310
LEP55828	Pierella	helvina	ocreata	ECUADOR	Esmeraldas, San Francisco Ridge	-	MH802166	MH802210	MH802243	MH802268	MH802312
BCI80049	Pierella	helvina	ocreata	PANAMA	BCI	-	MH802156	MH802202	-	MH802255	MH802301
CP01-69	Pierella	hortona	albofasciata	PERU	Tambopata Research Center	MH802135	-	MH802206	-	MH802259	-
LEP-08928	Pierella	hortona	hortona	ECUADOR	Pastaza, Kapawi lodge	MH802150	MH802177	MH802220	-	MH802278	MH802322
CP05-34	Pierella	hortona	hortona	PERU	Paraíso, Río Momón	MH802139	-	-	-	MH802265	MH802308
CP02-85	Pierella	hyceta	ceryce	PERU	Junín, Aldea	MH802136	MH802158	-	-	MH802260	MH802305
CP04-47	Pierella	hyceta	ceryce	PERU	Junín, Río Colorado, Quebrada Perla	MH802138	MH802160	-	-	MH802263	MH802307
LEP-08925	Pierella	hyceta	hyceta	ECUADOR	Zamora-Chinchipe, San Roque, Ridge E	KJ145994	KJ145989	KJ145997	-	KJ146005	KJ146009
LEP-08926	Pierella	hyceta	hyceta	ECUADOR		-	MH802176	-	-	-	-
CP03-60	Pierella	hyceta	hyceta	PERU	Yanachaga-Chemillén	MH802137	MH802159	-	-	MH802261	MH802306
PM0089	Pierella	hyceta	hyceta	PERU	Pasco, Cañón de Huancabamba	-	MH802189	MH802230	-	MH802289	MH802334
PM0090	Pierella	hyceta	hyceta	PERU	Pasco, Cañón de Huancabamba	-	MH802190	-	-	-	MH802335
SnPe05	Pierella	hyceta	hyceta	PERU	Cuzco, San Pedro	-	MH802201	-	-	MH802300	MH802346
NW108-2	Pierella	keithbrowni		BRAZIL	SP, Ilha do Cardoso, Cananéia	-	MH802186	MH802229	-	MH802285	MH802331
LEP-08908	Pierella	lena	brasiliensis (browni)	ECUADOR	Pastaza, Kapawi lodge	MH802144	MH802170	MH802214	-	MH802272	MH802316
LEP-08911	Pierella	lena	brasiliensis	ECUADOR	Morona-Santiago, Wachirpas airfield	MH802145	MH802171	MH802215	-	MH802273	MH802317
LEP-08912	Pierella	lena	brasiliensis	ECUADOR	Pastaza, Kapawi lodge	MH802146	MH802172	MH802216	-	MH802274	MH802318

NW93-2	Pierella	lena	brasiliensis	PERU	Loreto, Río Paiwa	-	DQ338757	DQ338897	-	MH802288	DQ338614
PM14-19	Pierella	lena	lena	BRAZIL	MT, Alta Floresta	KM013139	KM012979	KM013011	-	KM013185	KM013073
PM14-13	Pierella	lena	lena	ECUADOR	Sucumbios, Garza Cocha	-	MH802195	-	-	MH802294	MH802340
LEP-04613	Pierella	lucia		ECUADOR	Orellana, Napo Wildlife Center	MH802143	MH802169	MH802213	-	MH802271	MH802315
LEP-08935	Pierella	lucia		ECUADOR	Pastaza, Kapawi lodge	MH802152	MH802179	MH802222	-	MH802280	MH802324
PM14-12	Pierella	lucia		ECUADOR	Sucumbios, Garza Cocha	-	MH802194	MH802234	-	MH802293	MH802339
CP05-11	Pierella	lucia		PERU	Cordillera del Cóndor	-	MH802162	-	-	-	-
LEP-09797	Pierella	luna	lesbia	ECUADOR	Manabi, above Camarones, Pedernales-Jama Rd.	MH802153	MH802181	MH802224	-	MH802281	MH802326
LEP-09921	Pierella	luna	lesbia	ECUADOR	Esmeraldas, Tundaloma lodge	KM013149	KM012980	KM013026	-	KM013198	KM013088
PM24-04	Pierella	luna	luna	COSTA RICA	Heredia, Tirimbina	-	MH802200	MH802237	-	MH802298	MH802344
BCI90713	Pierella	luna	luna	PANAMA	Argos Plantations	-	HM406585	MH802204	-	MH802257	MH802303
CP14-08	Pierella	nereis		BRAZIL	SP, São Luiz do Paraitingo	MH802141	MH802163	MH802207	-	MH802266	MH802309
LEP-08924	Pseudohaetera	hypaesia		ECUADOR	Zamora-Chinchipe, Quebrada Guayzimi (close to La Merced)	MH802149	MH802175	MH802219	MH802247	MH802277	MH802321
LEP-09562	Pseudohaetera	hypaesia		ECUADOR	Morona Santiago, El Boliche	-	MH802180	MH802223	MH802248	-	MH802325
CP03-99	Pseudohaetera	hypaesia		PERU	Junín, Quebrada Siete Jeringas	-	DQ338758	DQ338898	MH802240	MH802262	DQ338625
CP04-57	Pseudohaetera	hypaesia		PERU	Junín, Mina Pichita	-	MH802161	-	MH802241	MH802264	-
PM0062	Pseudohaetera	hypaesia		PERU	Junín, San Ramón, Nueva Italia	-	MH802188	-	-	-	MH802333

745 Table 2: Characteristics of the molecular dataset used in this study, including gene and specimen
746 coverage, GC content and the number of variable sites.

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Genes	Specimens	Length (bp)	Variable sites	Missing data (%)	GC content (%)
CAD	29 (46%)	850	157	25.8	33.5
COI	60 (95%)	1475	474	26.5	29.5
EF1α	49 (78%)	1240	230	17.5	48.6
GAPDH	21 (33%)	691	129	2.6	45.4
RpS5	55 (87%)	617	124	1.9	45.2
wingless	58 (92%)	412	96	9.3	58.6
TOTAL	63	5285	1210	39.2	41.4

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Table 3: Marginal-likelihood calculations using path sampling and Bayes factor model testing. Eight competing, non-nested species delimitation models were compared, and the STACEY analysis under prior accounting for the number of taxonomic species (*spp prior*) had the highest marginal likelihood estimate. Bayes factors (*ln*BF) = 2–10 were considered to represent positive support, while *ln*Bf > 10 were considered as decisive support.

Delimitation model	**Marginal L (Path Sampling)**	**Delimited species**	***ln*BF STACEY, spp prior**
STACEY, spp prior	-43522.54	22	0
BP&P, large pop size	-43532.23	21	9.69
Taxonomic spp	-43537.35	18	14.81
STACEY, sspp prior	-43674.24	24	151.70
BP&P, medium pop size	-43689.95	28	167.41
Taxonomic sspp	-43701.81	26	179.27
STACEY, no prior	-43719.44	63	196.90
BP&P, small pop size	-43737.40	55	214.86