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Title: Mesoamerica is a cradle and the Brazilian Atlantic Forest is a museum of Neotropical butterfly diversity (Lepidoptera: Nymphalidae: Brassolini)

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Abstract

Regional species diversity is ultimately explained by speciation, extinction, and dispersal. Here we estimate dispersal and speciation rates in Neotropical rainforest biomes to propose an explanation for the distribution and diversity of extant butterfly species. We focus on the tribe Brassolini (owl butterflies and allies): a Neotropical group that comprises 17 genera and 108 species, most of them endemic to rainforest biomes. We infer a total-evidence species tree using the multispecies coalescent framework. By applying biogeographical stochastic mapping, we infer ancestral ranges and estimate rates of dispersal and cladogenesis at the scale of millions of years. We suggest that speciation in Mesoamerica and the northwestern flank of the Andes have only increased within the past 2 million years. In contrast, speciation in the Brazilian Atlantic Forest has been constant throughout the past 10 million years. The disparate species diversification dynamics may be partly explained by the geological and environmental history of each bioregion. Importantly, the dispersal rates into the Atlantic Forest and Mesoamerica plus NW Andes increased simultaneously in the middle-Miocene, suggesting that lineages from such regions have had comparable times for speciation despite their decoupled diversification dynamics. Diversification of extant Amazonian lineages, on the other hand, has episodically increased since the late Miocene, including a rise in speciation rate during the Pleistocene. Altogether, our results reveal a mosaic of biome-specific evolutionary histories within the Neotropics, where species have diversified rapidly (cradles: e.g., Mesoamerica), have accumulated gradually (museums: e.g., Atlantic Forest), or have alternately diversified and accumulated (e.g., Amazonia).

Keywords: Speciation; dispersal; multispecies coalescent; total-evidence analyses; ancestral state inference; biogeographical stochastic mapping.

Introduction

Terrestrial biomes are heterogeneous, and some areas accumulate greater diversity and endemic species than others. An explanation for this biogeographical pattern includes three evolutionary mechanisms: species origination, extinction, and dispersal (Goldberg et al. 2005, Jablonski et al. 2006). For example, a species-rich region can attain its present diversity by exceptionally high rates of speciation, becoming a "cradle of diversity", or by the persistence of old lineages, e.g., via low species extinction rates, becoming a "museum of diversity" (Stebbins 1974). Furthermore, a species-rich region might be a "source of diversity" when dispersal out of the region is high, or a "sink of diversity" when regional species diversity accumulates from external sources by dispersal into the region. Characterizing the interplay among speciation, extinction, and dispersal is thus highly relevant for understanding the origin and evolution of total regional diversity and endemism (Wiens and Donoghue 2004, Roy and Goldberg 2007).

The Neotropical region contains a great deal of the world's biodiversity and endemism. Most of such diversity is concentrated in tropical rainforests in Central America, the Amazon basin, and the Atlantic Forest in southeastern Brazil. However, there is no consensus on whether the currently scattered rainforests were once connected, nor for how long (Jaramillo and Cárdenas 2013). It is also unclear whether rainforests represent centers of "ecological stability" that share a common evolutionary history (Moritz et al. 2000). Mammals and birds in Neotropical biodiversity hotspots, including the Brazilian Atlantic Forest, and rainforests in Mesoamerica and the neighboring area Chocó (Myers et al. 2000) apparently originated at faster rates and exported more lineages compared to surrounding areas, suggesting that such hotspots are both cradles and sources of diversity (Igea and Tanentzap 2019). Some non-hotspots regions are, in

contrast, museums of diversity given the old and gradual accumulation of species through time, such as Amazonia for Troidini butterflies (Papilionidae) (Condamine et al. 2012). It thus seems that the checkered spatial assemblage of rainforests acting as cradle and museum areas might explain why the Neotropics is considered both museum and cradle of diversity (McKenna and Farrell 2006, Moreau and Bell 2013, Antonelli et al. 2018a). However, the impacts of geological and paleoenvironmental changes in the Neotropics together with the roles of speciation and dispersal have seldom been jointly elucidated in individual rainforest hotspots.

Here we test whether there was significant biotic exchange among Neotropical rainforests during the Neogene and Quaternary periods (i.e., the past 23 Ma), which might indicate physical connectivity of biomes. If connection among biomes existed, we aim to quantify its timing and magnitude in terms of dispersal and speciation of rainforest taxa. To do this, we estimate dispersal rates and speciation of Neotropical butterflies that occur mainly in rainforest hotspots, such as Mesoamerica, the northwestern slope (NW) of the Andes (including e.g., Chocó), the Atlantic Forest, Amazonia, and the South American “dry diagonal” (Fig. 1). Butterflies in the tribe Brassolini (Nymphalidae: Satyrinae) are particularly suited to address our question because they are a monophyletic group (Freitas and Brown 2004, Wahlberg et al. 2009, Espeland et al. 2018, Chazot et al. 2019a), exclusively Neotropical, and have about one third of their species diversity endemic to rainforest hotspots (see Penz 2007 for an overview).

Studies that focused on individual brassoline genera used adult morphology (Penz 2008, 2009a, b, Garzón-Orduña and Penz 2009) and DNA data (Penz et al. 2011a, Shirai et al. 2017) to infer species-level phylogenies, but several deep nodes had weak or even conflicting phylogenetic

support in tribal-level analyses (Penz et al. 2013). We infer a time-calibrated species phylogeny of Brassolini by merging all previous datasets and generating new DNA and morphological data. Specifically, we use this phylogeny to revisit and test the following predictions on diversification and dispersal among three Neotropical rainforest biomes:

1. Amazonia is the main source of Brassolini diversity by means of dispersal, reflecting the patterns from Neotropical vertebrates and plants during the past 60 million years (Antonelli et al. 2018b). Rapid species diversification of some butterfly groups occurred there since at least the Oligocene (Matos-Maraví 2016), and old lineages might have gradually accumulated in Amazonia since that time (Condamine et al. 2012). More recent diversification episodes supporting Amazonia as alternating museum and cradle of diversity could have been triggered by paleoenvironmental changes, such as the retreat of the Pebas wetland system (Antonelli et al. 2009, Antonelli and Sanmartín 2011, Chazot et al. 2019b).

2. Mesoamerica and the NW Andes are together a sink of Brassolini diversity due to dispersal events into this region during the Miocene, and cradle of diversity due to more recent, rapid speciation in the Pleistocene. This scenario conforms with the likely emergence of landmass in Central America and Chocó after the collision of the Panama Block and northwestern South America by the late Oligocene–early Miocene (Coates and Stallard 2013, Jaramillo 2018), and with Uranium-lead dating confirming that segments of the Panama arc had already emerged by 13–15 Ma (Montes et al. 2015). Shallow seawaters probably existed in the region until ~3 to 4 Ma (O'Dea et al. 2016, Jaramillo et al. 2017a), and while dispersal might have significantly increased since the early Miocene about 20 Ma, facilitated by land availability (Bacon et al.

2015), regional species diversification might have been hindered by lowland area water inundations until the Plio-Pleistocene (Mullen et al. 2011, Hosner et al. 2016).

3. The Atlantic Forest might have been a sink of Brassolini diversity for old Amazonian lineages (>10–15 Ma) and/or a recent source of diversity for present dry diagonal region (<10 Ma). The onset of a period of global cooling at ~10 to 15 Ma likely drove the expansion of open environments between Amazonia and the Atlantic Forest (Simon et al. 2009). Apparently, intermittent forest expansions into the dry diagonal occurred throughout the Neogene and Pleistocene (Werneck 2011, Trujillo-Arias et al. 2017), and Amazonia and the Atlantic Forest might have repeatedly been in contact (Ledo and Colli 2017, Capurucho et al. 2018). Biotic interchange from the Atlantic Forest toward the dry diagonal might have been a geologically more recent event, since at least 5 Ma (Costa 2003, Batalha-Filho et al. 2013, Thomé et al. 2016).

Material and methods

Taxon sampling

Specimens were collected in Mexico, Costa Rica, Ecuador, Peru, and Brazil (Supplementary material Appendix 1 Table S1), and identified to species based on various sources (Casagrande 2002, 2004, Austin et al. 2007, Penz 2008, 2009a, b, Garzón-Orduña and Penz 2009, Penz et al. 2017, 2011a, b, Chacón et al. 2012). To evaluate taxonomic bias in relation to geography, we compiled the year of description of each Brassolini species and the year of the latest revision of their current taxonomic status (e.g., when former subspecies were raised to species).

Molecular dataset

Based on protocols in Wahlberg and Wheat (2008), we Sanger-sequenced fragments of the mitochondrial COI gene (1,475 bp) and the nuclear genes CAD (850 bp), EF1α (1,240 bp), GAPDH (691 bp), RpS5 (617 bp), and *wingless* (400 bp) (outsourced to Macrogen, Seoul). We obtained genetic data from 81 individuals classified in 57 out of 108 valid species in 15 out of 17 Brassolini genera (Penz 2007). The two missing genera (*Aponarope* and *Mielkella*) are monotypic. We retrieved COI sequences available from the BOLD database (http://boldsystems.org) for seven additional species; thus, our molecular dataset consisted of 15 genera and 64 species. To root the phylogeny, we included genetic data of 19 outgroup taxa in the subfamily Satyrinae from public data deposited in GenBank. All DNA sequences from this study are in GenBank (accession numbers MK551348–MK551551).

Morphological and total-evidence dataset

Our morphological dataset included 255 characters (201 from adults and 54 from early stages) for 64 species from all 17 Brassolini genera. The morphological matrix was concatenated from previous studies (Penz 2007, 2008, 2009a, b, Garzón-Orduña and Penz 2009, Penz et al. 2013) by eliminating duplicate characters and scoring data for species missing entries. Specimens used were the same as listed in the original publications. The combined molecular and morphological dataset comprised all genera and 84 out of 108 Brassolini species. We call this the total-evidence dataset, which we used to infer a time-calibrated phylogeny.

Distribution dataset

We delineated bioregions (*sensu* Vilhena and Antonelli 2015) using geo-referenced occurrence points of Brassolini identified at the species level, of which 6,378 points came from GBIF (https://gbif.org; retrieved on 21st January 2019) and 881 points from the ATLANTIC BUTTERFLIES dataset (Santos et al. 2018). We used the R v3.5.3 (R Core Team 2019) package CoordinateCleaner v2.0-11 (Zizka et al. 2019) for automated cleaning of species occurrences. The software flagged 81 potential errors, such as occurrence records in the ocean, geographic coordinates identical to country centroids, and occurrences in close proximity to biodiversity institutions. We uploaded the 7,174 vetted geo-referenced occurrences to the web application Infomap Bioregions (Edler et al. 2017) at http://bioregions.mapequation.org/. We set the maximum and minimum values for cell sizes to 4 and 2, and for cell capacities to 40 and 4. We used different values of cluster cost, from 0.1 to 3.0, in steps of 0.1. The number of inferred areas remained stable between values of 1.8 and 2.4 and, thus, received the strongest support. The bioregions identified correspond to previously identified biomes or combinations of these: 1) Mesoamerica plus NW Andes, 2) Brazilian Atlantic Forest, and 3) the remaining tropical South America, including the Amazon drainage basin and the seasonally dry diagonal biomes Cerrado and Caatinga (Fig. 1).

To inform the biogeographical and within-area speciation rate analyses, we generated a species-level distributional dataset. We surveyed the literature to score species occurrences in the defined bioregions because the geo-referenced dataset did not include all Brassolini species. To investigate dispersal patterns across the South American dry diagonal, we kept this area separate. We used distributional maps and observations, not always with primary geographic coordinates, from taxonomic revisions (Bristow 1981, 1982, 1991, Casagrande 2002, Furtado and Campos-

Neto 2004, Penz 2008, 2009a, b, Garzón-Orduña and Penz 2009, Penz et al. 2017) and published butterfly inventories in Mesoamerica (DeVries 1983, 1994, Janzen and Hallwachs 2009, Basset et al. 2015), Amazonia (Pereira Martins et al. 2017), Cerrado (Pinheiro and Emery 2006, Emery et al. 2006, Silva et al. 2012, Pereira Martins et al. 2017, Dickens et al. 2019), Caatinga (Zacca and Bravo 2012), and Atlantic Forest (Santos et al. 2011, 2018, Pérez et al. 2017, Melo et al. 2019, Soldati et al. 2019). We call this the presence/absence biogeographical dataset.

Total-evidence phylogenetic inference and divergence time calibration

We inferred time-calibrated species trees using the StarBEAST2 package (Ogilvie et al. 2017) available in BEAST v2.5.2 (Bouckaert et al. 2014). We removed the species *Opsiphanes camena* from the dataset because it had only 325 bp of the COI locus, and its phylogenetic position was not stable in a preliminary analysis (Supplementary material Appendix 1). We used a gene-based partitioning strategy, each having unlinked tree models in the multispecies coalescent framework. We performed model averaging over 31 substitution models available in the bModelTest v1.1.2 package (Bouckaert and Drummond 2017). We applied uncorrelated relaxed-clock models for all partitions because strict clock models were rejected based on the stepping-stone method (Supplementary material Appendix 1). We estimated clock rates relative to the mitochondrial mean clock rate fixed to 1.0, and we used log normal distribution for the relative clock mean priors (M = 0, S = 1). We used the Yule tree model because it received decisive support over the birth-death tree model based on path-sampling steps (Supplementary material Appendix 1). We added the morphological dataset onto the species tree and we conditioned the characters to the Markov model (Lewis 2001).

Inasmuch as there are no described fossils of Brassolini, we relied on secondary calibrations to time-calibrate the species tree. We conservatively used uniform distributions encompassing the 95% highest posterior density (HPD) from a large fossil-calibrated, genus-level butterfly phylogeny (Chazot et al. 2019a) to constrain six outgroup nodes:

1) The crown age of the subfamily Satyrinae (46–65 Ma),
2) The divergence of the tribes Melaniti and Dirini (27–41 Ma),
3) The crown age of the tribe Satyrini (38–55 Ma),
4) The crown node of the subtribes Lethina, Parargina and Mycalesina to (31–46 Ma),
5) The crown node of the subtribes Pronophilina, Euptychiina, Satyrina and Erebiina (31–44 Ma),
6) The divergence of the tribes Brassolini and Morphini (34–50 Ma).

The analyses were run four independent times for 300 million generations, via the CIPRES Science Gateway v3.3 (Miller et al. 2010). We sampled 20,000 trees from the posterior distribution and discarded the first 25% as burnin. We checked that the estimated sample sizes (ESS) were above 200 using Tracer v1.7.1 (Rambaut et al. 2018). We merged the independent runs using LogCombiner (part of the BEAST v2.5.2 package) and we summarized the post-burnin species trees as a maximum clade credibility (MCC) tree in TreeAnnotator (part of the BEAST v2.5.2 package).

Within-area speciation and dispersal among bioregions through time

We estimated ancestral geographic ranges and colonization rates among bioregions using the dispersal-extinction-cladogenesis (DEC) model (Ree et al. 2005), as implemented in the R package BioGeoBEARS v1.1.2 (Matzke 2013). We used the presence/absence biogeographical dataset and the four defined bioregions: 1) Mesoamerica plus NW Andes, 2) Atlantic Forest, 3) Amazonia, and 4) Dry diagonal. We did not constrain any *a priori* dispersal multiplier, nor did we stratify dispersal rates across the phylogeny. To account for ancestral state uncertainty, we carried out 100 biogeographical stochastic mappings (BSM; Dupin et al. 2017). To account for divergence time uncertainty, we used 100 random topologies from the BEAST2 posterior distribution. To account for phylogenetic uncertainty of 25 missing species, we randomly added such lineages to their currently assigned genera in the 100 posterior phylogenies. In this case, we assumed that phylogenetic diversity per genus was maximized and we placed missing taxa in the crown genera given our comprehensive taxonomic sampling. All genera are monophyletic, but we conservatively joined *Selenophanes* and *Catoblepia* (posterior probability, PP = 1.0; Fig. 2) when assigning missing species because the monophyly of *Catoblepia* was only moderately supported (PP = 0.75).

We estimated the number of dispersal events and within-area-cladogenesis events by simulating areas on nodes based on the 10,000 pseudoreplicated biogeographic histories (100 BSM × 100 posterior species trees). We calculated colonization rates through time as $c_{\mathrm{XtoY}}(t_1) = d_{\mathrm{XtoY}}(t_1) / \mathrm{L}(t_1)$, where $d_{\mathrm{XtoY}}(t_1)$ is the number of inferred dispersal events from area X to Y in a 1-million year interval, and $\mathrm{L}(t_1)$ is the total length of all branches in the 1-million-year interval (Antonelli et al. 2018b). In addition, we calculated the relative number of *in situ* speciation events through time as $\lambda(t_1) = \mathrm{s}(t_1) / \mathrm{s}(t_0 / t_{0+1})$, where $\mathrm{s}(t_1)$ is the number of within-area cladogenesis in a 1-

million-year interval, and s(t_0 / t_{0+1}) is the number of within-area cladogenesis in the previous 1-million-year interval (t_0) relative to the number of within-area cladogenesis in time intervals t_0 and t_1 (modified from Xing and Ree 2017).

Speciation within bioregions through time

For a second, independent estimate of within-area speciation, we calculated speciation rates for all tips and branches in the MCC species tree using BAMM v2.5.0 (Rabosky 2014). To account for the 25 missing species, we generated clade-specific sampling fractions at the genus level. We used the R package BAMMtools v2.1.6 (Rabosky et al. 2014) to estimate prior values for the evolutionary rate parameters, and we assigned the expected number of shifts to 1.0 as recommended for phylogenies including ~100 species (Rabosky 2014). We ran the analysis for 10 million generations, sampling event data every 1,000 generations. After discarding the first 20% samples, we checked that the ESS value was higher than 200 and the likelihood estimates reached a stationary distribution.

First, we estimated speciation rates within bioregions at present using the function “getTipRates” in BAMMtools. We computed tip-specific mean speciation rates for those species occurring only in one bioregion, i.e., the endemic lineages. Second, we used the function “dtRates” in BAMMtools to compute mean speciation rates along branches in ~1-million year intervals (tau parameter set to 0.05). We assigned the most probable inferred area from the 10,000 BSMs to the points where branches cross time intervals (Igea and Tanentzap 2019). When the most probable area for a node and its parental one were the same, we assumed that the branch connecting both nodes occurred in such an area. When inferred areas for a node and its parental one were

different, we assumed that the dispersal event occurred stochastically along the branch. We computed mean speciation rates only for nodes that most probably occurred in a single bioregion.

All the datasets from this study can be found in TreeBASE (ID 25040) and Dryad (DOI: XXXXX).

Results

Phylogenetic and divergence time uncertainty

We found no disparate trend among Neotropical bioregions in taxon discovery, description and taxonomic opinion (i.e., oversplitting species *vs*. multiple infraspecific taxa) (Supplementary material Appendix 1). The multispecies coalescent model recovered low posterior probabilities for deeper nodes (0.39–0.90; Fig. 2). In contrast, a concatenation-based analysis using only the molecular dataset (Supplementary material Appendix 1) produced higher posterior probabilities for the deep nodes (0.97–1.00). This suggests that the molecular dataset held strong phylogenetic signal, but the multispecies coalescent model might have better reflected the phylogenetic uncertainty in the morphological dataset. We estimated these nodes had ages between 15 and 17 Ma (entire highest posterior density HPD, 10.87–20.89 Ma). The total-evidence dataset supported posterior probability of 1.0 for the crown node of almost all non-monotypic genera, regardless of phylogenetic method.

Ancestral bioregions and colonization rates through time

The inferred ranges coming from the 10,000 pseudoreplicated biogeographic histories and plotted against the MCC species tree in Fig. 2 suggested that Brassolini most probably (*Pr* = 0.36) originated in Amazonia at 21 Ma (entire HPD, 16.67–27.16 Ma). The early rapid radiation of Brassolini between 15 and 17 Ma also probably took place in Amazonia (*Pr* = 0.30–0.52).

Dispersal out of Amazonia had the highest rates compared to other bioregions (Fig. 3). Colonization rate from Amazonia to Mesoamerica showed a sharp increase by ~5 Ma and is currently the highest rate among other dispersal types (Fig. 3A). Dispersal into Mesoamerica from other bioregions occurred throughout most of the Miocene, and peaked at ~10 Ma (Supplementary material Appendix 1). Dispersal out of Mesoamerica + NW Andes, however, was very low through time, and only since the past ~2 Ma did it exponentially increase toward Amazonia (Fig. 3A). Dispersal from Amazonia to the dry diagonal increased throughout the Plio-Pleistocene and has since then surpassed dispersal to Atlantic Forest (Fig. 3B & 3C). Dispersal from the Atlantic Forest into Amazonia was higher at ~10 Ma than from any other region (Fig. 3C). Nonetheless, dispersal into or out of the Atlantic Forest from/to any other bioregion declined from ~10 to 2 Ma, rendering the Atlantic Forest isolated for most of the late Miocene and Pliocene; only since the Pleistocene ~2 Ma dispersal into and from the dry diagonal has increased until the present (Fig. 3D).

Regional speciation through time

The relative rates of within-area cladogenesis estimated in BioGeoBEARS and speciation estimated in BAMM were congruent (Fig. 4). Speciation rates increased through time in all areas except in the dry diagonal, which has had near zero endemic cladogenesis. Diversification began

earlier in the Atlantic Forest, at ~12 Ma when the cladogenesis rate was slightly above zero (Fig. 4A) and speciation rate increased (Fig. 4B), specifically in the clade comprising *Penetes*, *Orobrassolis* and *Blepolenis*. However, diversification in the Atlantic Forest decreased throughout most of the Miocene until ~ 5 Ma, when it steadily rose again towards the present. In contrast, speciation in Mesoamerica remained low during most of the Miocene and Pliocene, and sharply increased only at ~2 to 3 Ma. Speciation in Amazonia increased since the late Miocene at higher rate than in the Atlantic Forest and Mesoamerica, but it also dramatically increased during the Pleistocene (<2 Ma). At present, the speciation rate in Mesoamerica + NW Andes is similar to Amazonian lineages, both being much higher than in the Atlantic Forest (Fig. 4C).

Discussion

Here we investigated the role of regional speciation and dispersal to understand the origin of species in the butterfly tribe Brassolini. One third of those species are endemic to rainforests in Mesoamerica, NW Andes and the Brazilian Atlantic Forest. Recent and rapid butterfly speciation occurred in Mesoamerica and NW Andes, suggesting that it acted as a cradle of diversity. In contrast, low and constant diversification since at least the late Miocene has shaped Brassolini endemic diversity in the Atlantic Forest. This finding, coupled with the survival of old lineages found mainly in montane habitats (e.g., *Penetes*, *Dasyophthalma*) suggests that the Atlantic Forest acts as a museum of diversity.

A longer time for speciation in the Atlantic Forest potentially explaining the older events of cladogenesis is not supported because dispersal into Mesoamerica and NW Andes occurred near-simultaneously. We argue that the uneven origin of endemic species among the Neotropical

rainforest biomes reflect the geological and environmental changes that took place throughout the Neogene and Pleistocene, including the availability of substantial landmass in Mesoamerica and Chocó by ~3 to 4 Ma and the spread of wet forest into what is now the South American Dry Diagonal during the past 10 Ma.

Strengths and weaknesses of the approach

We are aware of two main limitations and took them into account in the interpretation of our results. First, by using the DEC model, we assumed no species extinction in the phylogeny of extant Brassolini. Simulation analyses comparing the DEC and state-dependent diversification models suggested that the estimation of ancestral areas can be robust when extinction rates were low, or not associated to particular geographic areas (Matzke 2014). The lack of Brassolini fossils precludes assessing this directly, but a better fit of the Yule process over the birth-death model suggests that extinction was probably low to moderate during the timeframe of their diversification, and might not have substantially biased our inferences.

Second, BAMM's prior model used for estimating diversification rate shifts were contested by Moore et al. (2016), but further evaluation via simulated data suggested that BAMM can infer speciation rates accurately from extant-taxon phylogenies (Rabosky et al. 2017). Although low extinction rates might explain the museum of diversity pattern, we have conservatively focused only on regional species origination rates to explain lineage accumulation within bioregions. We additionally used a fundamentally-distinct measure of within-area cladogenesis (Xing and Ree 2017) that corroborated the pattern of recent and rapid speciation in Mesoamerica + NW Andes, and an older lineage accumulation and lower diversification in the Atlantic Forest.

Our approach to disentangle regional speciation from dispersal to understand the origin of within-area species diversity has two strengths. First, we defined bioregions that fit the distribution and composition of Brassolini species by using geo-referenced occurrences, rather than expert opinion or another subjective criterion (Vilhena and Antonelli 2015). This approach revealed a clustering of butterfly communities from southern North America to Mesoamerica and the NW Andes, which are regions that otherwise belong to different biogeographic dominions (Morrone 2014). Our inference of large bioregions for Brassolini agrees with its apparent poor spatial structuring and high dispersal capabilities. For instance, given a suitable environment, ancestral *Bia* had the capability to disperse across pan-Amazonia in only 1,463–3,115 years (Penz et al. 2015). The delimitation of Mesoamerica + NW Andes and the Atlantic Forest as separate bioregions has likely been determined by ecological and habitat suitability rather than geographical barriers to Brassolini dispersal; this being a similar biogeographical pattern found in other Neotropical rainforest taxa (Dexter et al. 2017, Pérez-Escobar et al. 2019).

Second, we used a multispecies coalescent framework which outperforms the concatenation-based approach (Edwards et al. 2016), in the face of incomplete taxon sampling and incomplete lineage sorting (Liu et al. 2015, Ogilvie et al. 2017). For additional gene-tree discordance tests, see Supplementary material, Appendix 1. Our comprehensive dataset including molecular and morphological characters allowed us to simultaneously use several lines of evidence for recognizing and dating speciation events. The Bayesian probabilistic framework applied here also considered three types of uncertainties: phylogenetic, temporal and taxonomic sampling.

This approach contributed toward a better assessment of confidence intervals when estimating dispersal and diversification rates.

Species diversification and dispersal in bioregions

The calculated probability densities of dispersal and speciation rates supported three distinct evolutionary processes: recent and fast speciation in Mesoamerica + NW Andes, old diversification dynamics with low speciation rates in Atlantic Forest, and episodic increases in speciation rate of Amazonian lineages. Our results suggest that Brassolini diversified mainly in Amazonia and the Atlantic Forest from about 23 to 5 Ma (speciation rate remained above 0.4 in Amazonia, and below 0.4 in the Atlantic Forest, Fig. 4B), and early Brassolini diversification might have been accompanied by increasing dispersal out of these two bioregions.

In the case of Amazonia, the Pebas wetland system (Wesselingh and Salo 2006) may not have drastically limited dispersal and diversification of Brassolini as it likely did for other butterfly groups (Chazot et al. 2019b). These wetland system was occasionally affected by marine flooding events, which were probably short-lived, and at least two dated events at ~17.8 Ma and ~13.7 (Jaramillo et al. 2017b) were concurrent with the early and rapid cladogenesis events of Brassolini in Amazonia. The Pebas wetland might in fact have increased opportunities for dispersal and diversification of some typical coastal plants (Bernal et al. 2019), including many of the butterflies' hostplants in the region: Arecaceae (palms), Poaceae (bamboo) and Zingiberales such as Marantaceae (Beccaloni et al. 2008, Janzen et al. 2009). Therefore, the evolutionary consequences of the Miocene marine incursions in western Amazonia might have

been mixed, restricting dispersal and diversification in some lineages while providing ecological opportunities for others, such as Brassolini butterflies.

The recent increased diversification in Amazonian lineages (< 3 Ma) might be related to younger paleoenvironmental changes, such as fluctuating river dynamics mediated by Quaternary glacial cycles (Ribas et al. 2012). For example, species in the genus *Bia* seem to have strong biogeographical structure driven by major Amazon-basin rivers (Penz et al. 2017), suggesting that riverine barriers might have been involved in allopatric differentiation. Indeed, in areas of the Amazon River, the distance between river banks is larger than the estimated daily dispersal of species such as *Bia actorion* (Tufto et al. 2012, Penz et al. 2015). However, a phylogeographic evaluation including more samples per species would be necessary to estimate the influence of river barriers in differentiating Brassolini populations, and potentially to explain the major increase in diversification during the Pleistocene.

In the case of the Atlantic Forest, Miocene expansions of wet forests into Amazonia through the dry diagonal might have been determinants of ancient butterfly diversity. Forest connections have been documented mainly for the Quaternary based on palynological data and phylogeographic studies (e.g., Werneck 2011, Prates et al. 2016, Trujillo-Arias et al. 2017). Although evidence of Neogene connection between the Atlantic Forest and Amazonia is still scarce, the origin of the modern transcontinental Amazon river by ~10 Ma might have facilitated dispersal of rainforest taxa (Albert et al. 2018). Indeed, we found that late Miocene dispersal rates between these wet forest biomes were higher than in the Plio-Pleistocene. We suggest that episodic Miocene wet forest expansions might have represented ecological opportunities for

Brassolini to disperse across Central Brazil, either by increased sizes of wet forest galleries in the dry diagonal biome, or by fully connecting Amazonia and Atlantic Forest.

By the Plio-Pleistocene, speciation in Mesoamerica and NW Andes seems to have increased and surpassed the speciation rate in the Atlantic Forest. Biotic dispersal into Mesoamerica from South America likely occurred throughout most of the Miocene (e.g., Mullen et al. 2011, Bacon et al. 2015). However, only at the end of the Pliocene did shallow water fully recede from the region (Coates et al. 2004, Montes et al. 2012), thus increasing opportunities for terrestrial species diversification. In contrast, speciation in the Atlantic Forest remained low and dispersal out of this region decreased until the mid-Pleistocene. These events coincided with the expansion of open environments in the American continent and between Amazonia and Atlantic Forest since the late Miocene (Simon et al. 2009, Edwards et al. 2010, Strömberg 2011, Werneck 2011). The contraction of Atlantic Forest may have restricted speciation to montane habitats in southeastern Brazil where most extant species occur, and old lineages having low speciation rates through time survived in remnants of rainforest.

Consequences for Brassolini classification

Brassolini has been subdivided in three subtribes, the monotypic Biina and Naropina, and polytypic Brassolina (Casagrande 1995, Penz 2007), but here *Narope* falls within Brassolina. At the genus level, our analyses support sister taxa relationships between *Dynastor* and *Dasyophthalma*, *Opoptera* and *Narope*, *Selenophanes* and *Catoblepia*, *Orobrassolis* and *Blepolenis*, and *Brassolis* appears as sister to the *Opsiphanes*-group (sensu Penz 2007). We propose the following changes to the Brassolini classification at the subtribe and species levels:

1) Naropina is subsumed within Brassolina. Brassolini is thus redefined to include the subtribes Biina (monotypic) and Brassolina.

2) The monotypic *Aponarope* is subsumed within *Narope*. Stichel (1916) originally described *Narope sutor*, but this species was later segregated in the monotypic *Aponarope* (Casagrande 1982). We revise the genus assignment for *Narope sutor*, REV. COMB., and synonymize *Aponarope*, NEW SYN.

3) *Selenophanes orgetorix*, NEW COMB. This species grouped with members of *Selenophanes* with high posterior probability (see also Shirai et al. 2017).

Conclusions

This study suggests that the Neotropics consists of a regional network of museums and cradles of butterfly diversity. We found that endemic species to Mesoamerica plus NW Andes have originated by old dispersal events into the region, and that speciation rates increased only at about 2 Ma (cradle of diversity). In contrast, endemic species to the Brazilian Atlantic Forest likely arose under low and continuous speciation rates since about 12 Ma (museum of diversity), and was likely intermittently connected to Amazonia since the Miocene. This dynamic evolutionary history of Neotropical rainforests appear to reflect paleoenvironmental changes, including landscape reconfigurations in Central America and paleoclimate fluctuations during the Neogene and Quaternary.

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Author contributions

PMM, NW, and CMP conceived the project, PMM, AVLF, PJD, and CMP conducted fieldwork, PMM carried out the lab experiments and led the data analyses, PMM wrote the first draft of the manuscript, and all authors discussed and contributed to the final product.

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Supplementary material (available online as Appendix 1 at website). Appendix 1.

Figures

Fig. 1: Map of the Neotropics and the defined bioregions based on occurrence data of Brassolini. Inset map shows the most stable bioregions under different cluster cost values by the software Infomap Bioregions using validated geo-coordinates from GBIF and ATLANTIC BUTTERFLIES databases. Bioregions are delineated based on Morrone's (2014) Neotropical provinces, encompassing the Mesoamerican plus Pacific dominions, except for three provinces east of the northern Andes; the Paraná dominion (Brazilian Atlantic Forest); the remaining dominions and provinces where Brassolini occurs. Shapefile from Löwenberg-Neto (2014).

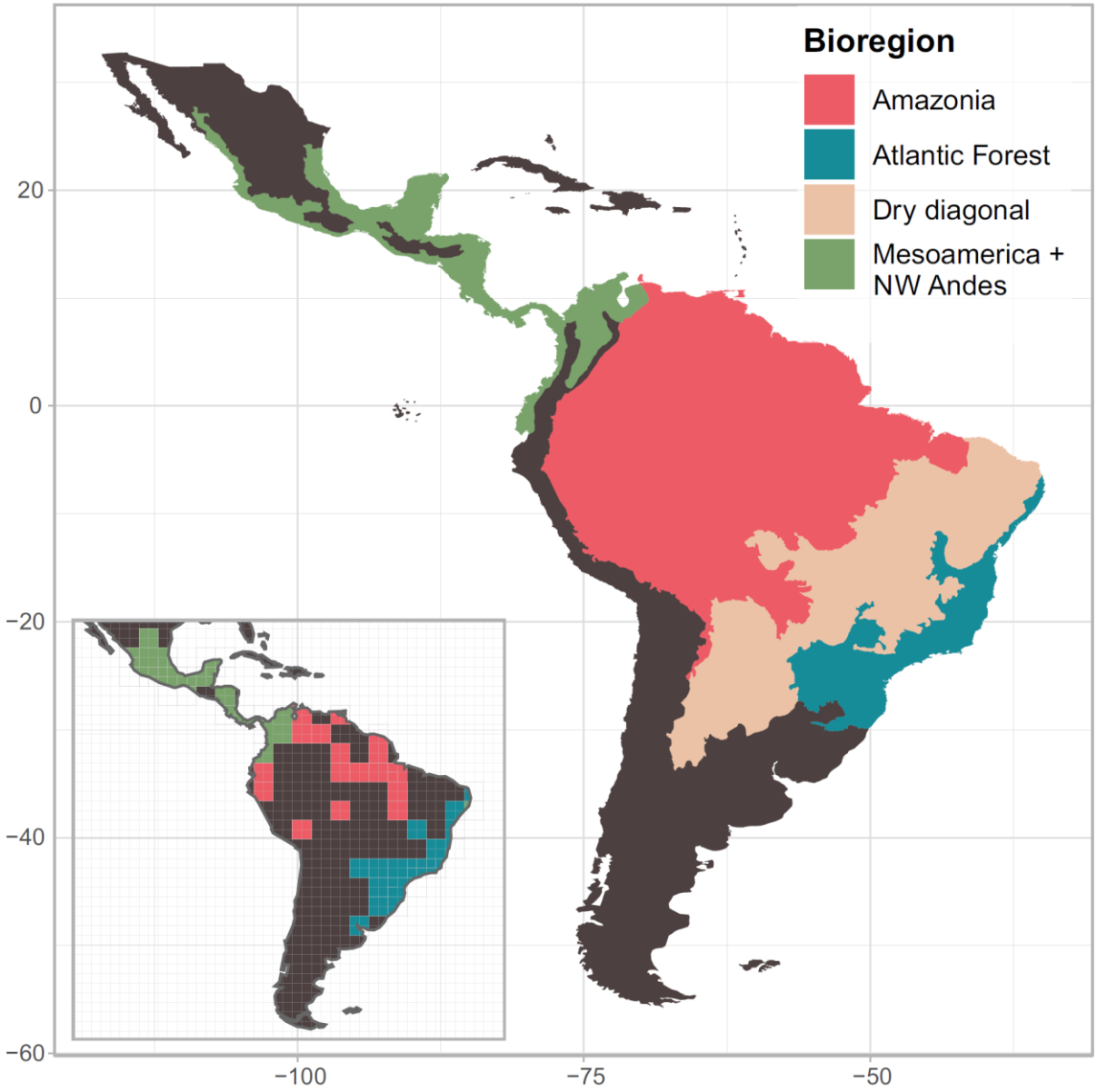
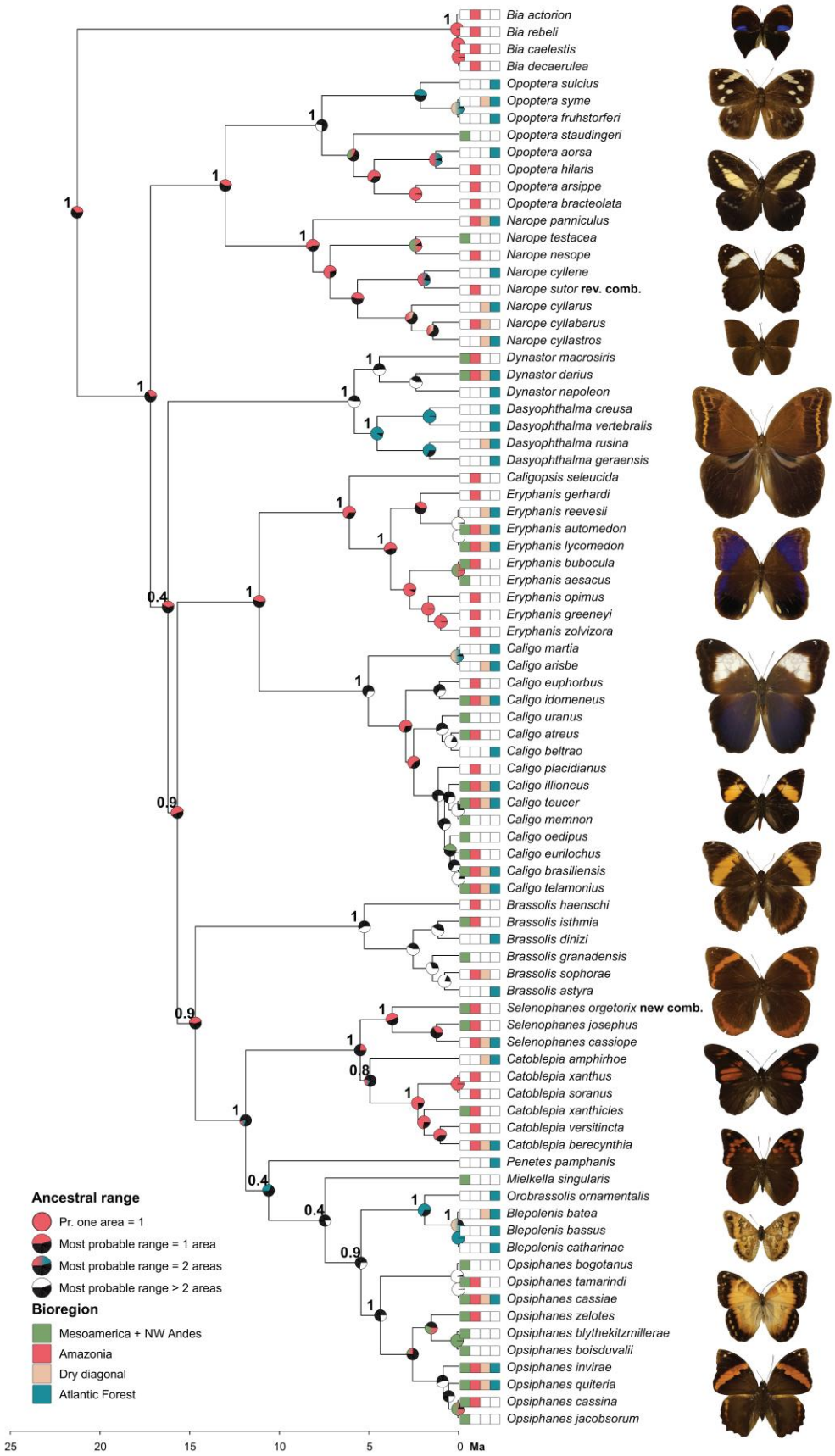
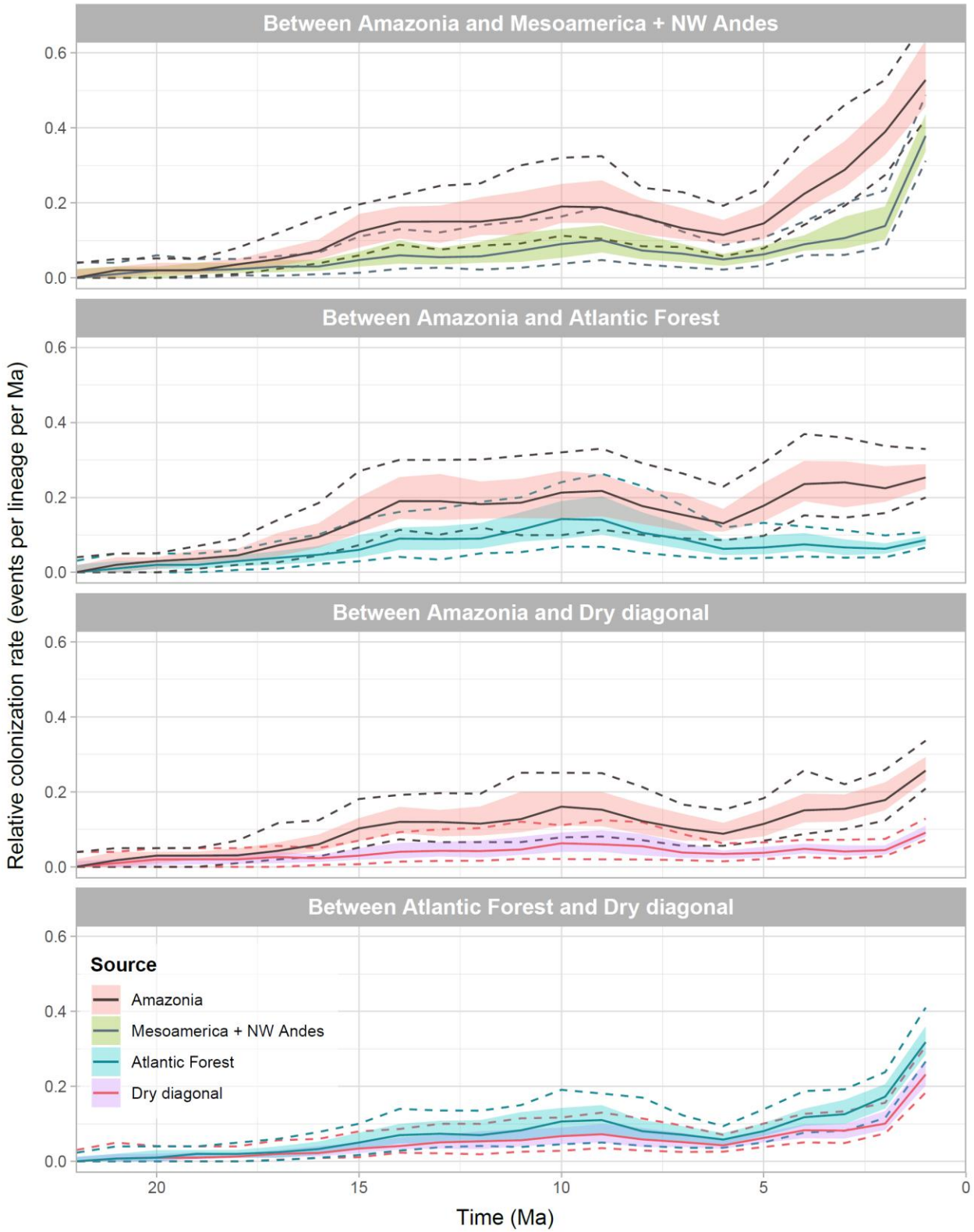

Bioregion
Amazonia
Atlantic Forest
Dry diagonal
Mesoamerica + NW Andes
20
0
−20
−40
−60
−100
−75
−50

Fig. 2: Time-calibrated species tree of the butterfly tribe Brassolini. The posterior probabilities for all genera and among genera are displayed next to the crown node. Ancestral range probability based on the DEC model and 10,000 biogeographical stochastic mappings is depicted as a pie chart following colors in legend. Images of representative butterflies of all Brassolini genera, all at the same scale: From top to down, *Bia actorion* (DeVries collection, PJD), *Dynastor darius* (Milwaukee Public Museum, MPM), *Dasyophthalma creusa* (MPM), *Opoptera fruhstorferi* (American Museum of the Natural History, AMNH), *Narope panniculus* (AMNH), *Caligopsis seleucida* (PJD), *Eryphanis aesacus* (MPM), *Caligo martia* (MPM), *Brassolis astyra* (MPM), *Selenophanes cassiope* (PJD), *Catoblepia berecynthia* (MPM), *Penetes pamphanis* (MPM), *Mielkella singularis* (AMNH), *Orobrassolis ornamentalis* (MPM), *Blepolenis bassus* (Museu de Zoologia - USP), *Opsiphanes quiteria* (PJD).


Bia actorion
Bia rebeli
Bia caelestis
Bia decaerulea
Opoptera sulcius
Opoptera syme
Opoptera fruhstorferi
Opoptera staudingeri
Opoptera aorsa
Opoptera hilaris
Opoptera arsippe
Opoptera bracteolata
Narope panniculus
Narope testacea
Narope nesope
Narope cyllene
Narope sutor rev. comb.
Narope cyllarus
Narope cyllabarus
Narope cyllastros
Dynastor macrosiris
Dynastor darius
Dynastor napoleon
Dasyophthalma creusa
Dasyophthalma vertebralis
Dasyophthalma rusina
Dasyophthalma geraensis
Caligopsis seleucida
Eryphanis gerhardi
Eryphanis reevesii
Eryphanis automedon
Eryphanis lycomedon
Eryphanis bubocula
Eryphanis aesacus
Eryphanis opimus
Eryphanis greeneyi
Eryphanis zolvizora
Caligo martia
Caligo arisbe
Caligo euphorbus
Caligo idomeneus
Caligo uranus
Caligo atreus
Caligo beltrao
Caligo placidianus
Caligo illioneus
Caligo teucer
Caligo memnon
Caligo oedipus
Caligo eurilochus
Caligo brasiliensis
Caligo telamonius
Brassolis haenschi
Brassolis isthmia
Brassolis dinizi
Brassolis granadensis
Brassolis sophorae
Brassolis astyra
Selenophanes orgetorix new comb.
Selenophanes josephus
Selenophanes cassiope
Catoblepia amphirhoe
Catoblepia xanthus
Catoblepia soranus
Catoblepia xanthicles
Catoblepia versitincta
Catoblepia berecynthia
Penetes pamphanis
Mielkella singularis
Orobrassolis ornamentalis
Blepolenis batea
Blepolenis bassus
Blepolenis catharinae
Opsiphanes bogotanus
Opsiphanes tamarindi
Opsiphanes cassiae
Opsiphanes zelotes
Opsiphanes blythekitzmillerae
Opsiphanes boisduvalii
Opsiphanes invirae
Opsiphanes quiteria
Opsiphanes cassina
Opsiphanes jacobsorum
Ancestral range
Pr. one area = 1
Most probable range = 1 area
Most probable range = 2 areas
Most probable range > 2 areas
Bioregion
Mesoamerica + NW Andes
Amazonia
Dry diagonal
Atlantic Forest
25
20
15
10
5
0 Ma

786

Fig. 3: Colonization rates through-time based on 10,000 biogeographical stochastic mappings in BioGeoBEARS. Rates are displayed for a select pair of areas. Source areas (dispersal from) follow the color in the legend. Solid lines are the median values, colored ribbons are the lower and upper quartiles (0.25 and 0.75 quantiles), and dashed lines are the 0.1 and 0.9 quantiles.


Between Amazonia and Mesoamerica + NW Andes
Between Amazonia and Atlantic Forest
Between Amazonia and Dry diagonal
Between Atlantic Forest and Dry diagonal
Relative colonization rate (events per lineage per Ma)
0.0
0.2
0.4
0.6
Source
Amazonia
Mesoamerica + NW Andes
Atlantic Forest
Dry diagonal
20
15
10
5
0
Time (Ma)

792

Fig. 4: Within-area speciation through-time based on 100 posterior phylogenies and 100 biogeographical stochastic mappings (BSMs) in BioGeoBEARS. A) The relative number of within-area cladogenesis through time was calculated using a formula modified from Xing and Ree (2017) based on cladogenesis events occurring only in one area. Solid lines represent median values, colored ribbons represent lower and upper quartiles (0.25 and 0.75 quantiles) and dashed lines represent 0.1 and 0.9 quantiles. The x-axis is truncated at the middle Miocene, as most Brassolini cladogenesis events have occurred since then. B) Per-lineage speciation rates through time estimated by BAMM were assigned to a given bioregion based on the BSMs. Solid lines represent mean speciation rates per lineage and the colored ribbons the 5% and 95% confidence intervals. The x-axis is truncated at the middle Miocene. C) Species (tips) specific speciation rates as estimated by BAMM. Box plots per area show estimates from the posterior distribution, and the cross within each box plot represents mean speciation rates per area.

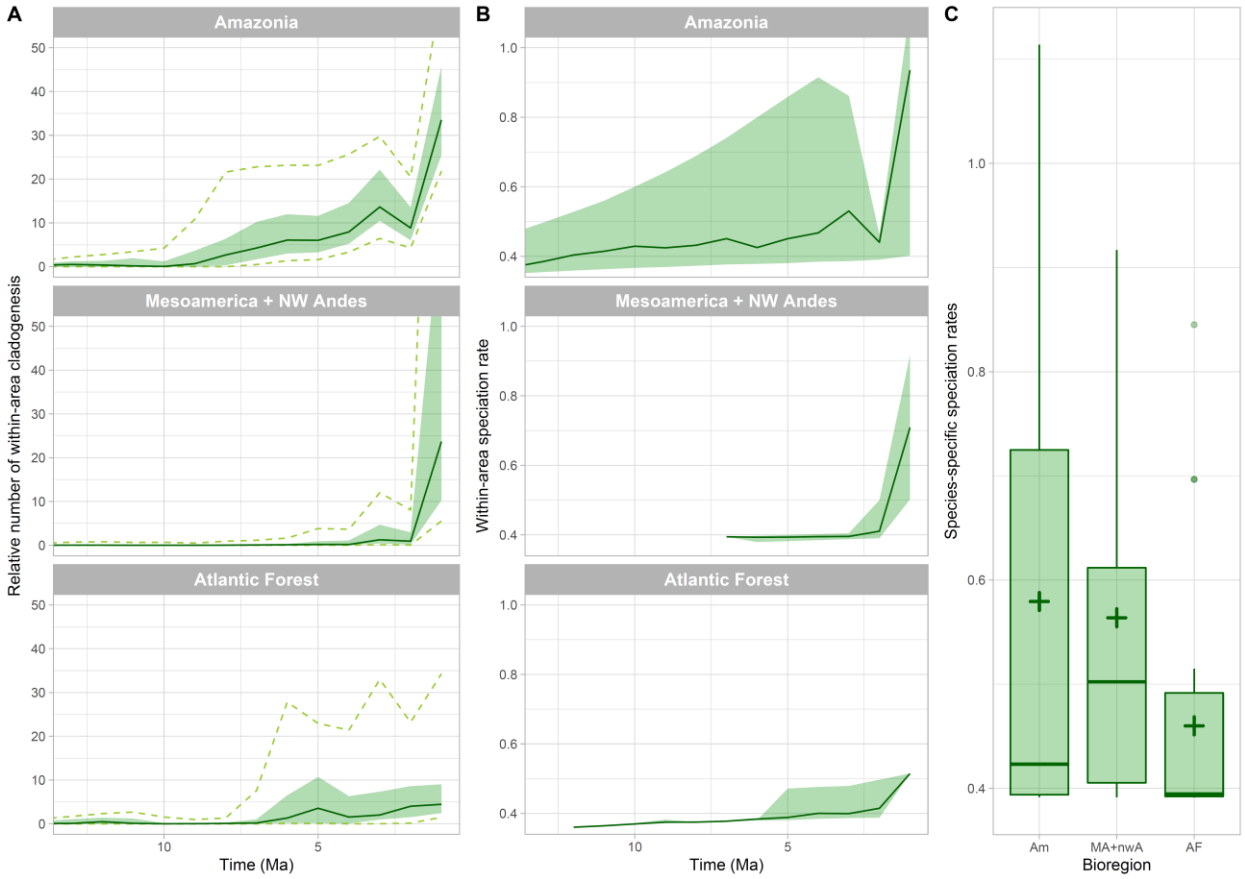
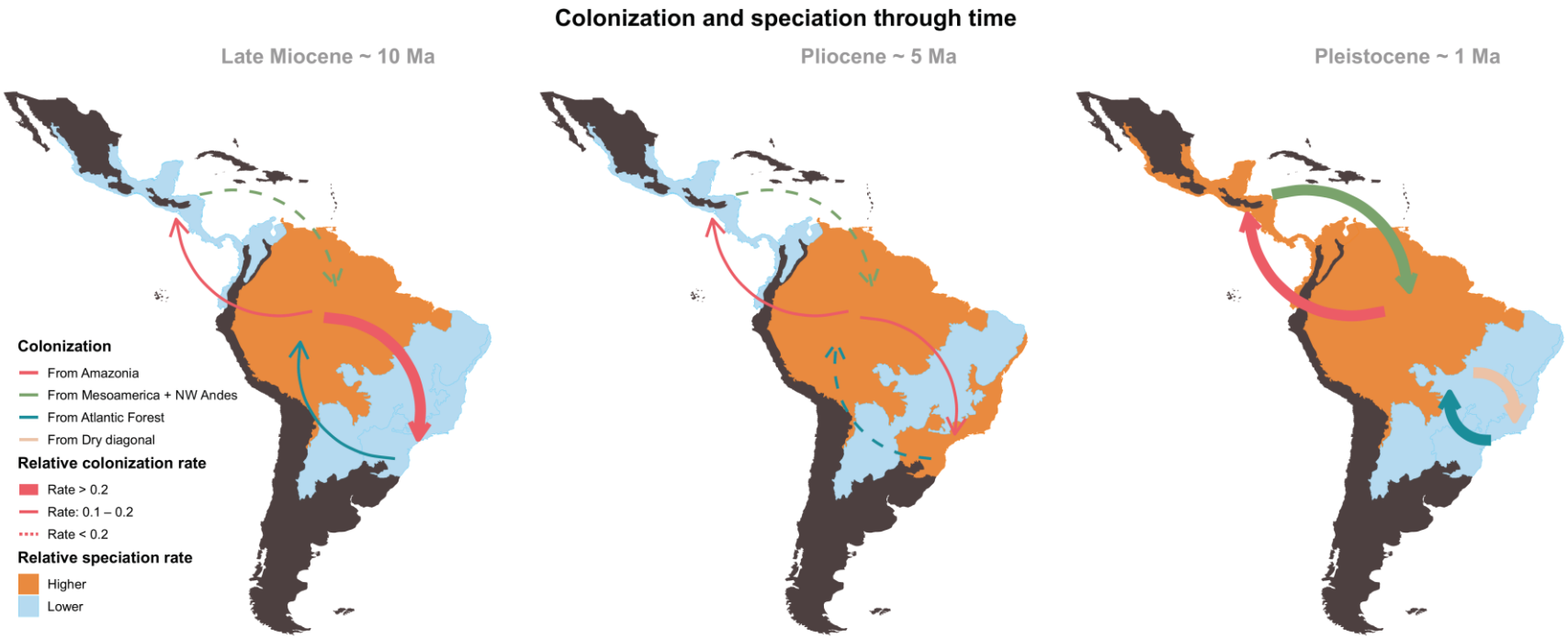


Fig. 5: Summary of Brassolini colonization and speciation estimates in three time-windows, at 10, 5 and 1 Ma. Colored directional arrows represent dispersal from source areas, following colors in legend. The width and shape of arrows represent the estimated relative colonization rate between two areas in BioGeoBEARS, as depicted in Fig. 3. Bioregions were colored to qualitatively represent the estimated relative cladogenesis/speciation rates, as either center of speciation or area with comparatively lower speciation rates per time window.


Colonization and speciation through time
Late Miocene ~ 10 Ma
Pliocene ~ 5 Ma
Pleistocene ~ 1 Ma
Colonization
From Amazonia
From Mesoamerica + NW Andes
From Atlantic Forest
From Dry diagonal
Relative colonization rate
Rate > 0.2
Rate: 0.1 – 0.2
Rate < 0.2
Relative speciation rate
Higher
Lower

814