Title page

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 butterfly 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 of Brassolini using the multispecies coalescent framework. By applying biogeographical stochastic mapping, we infer ancestral ranges and estimate rates of butterfly dispersal and cladogenesis at the scale of millions of years. We suggest that speciation in Mesoamerica and the northwestern flank of the Andes has 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 explained by the geological and environmental history of each bioregion: the Central American Arc was likely intermittently inundated by water for most of the Miocene (15 to 3 Ma), thus, reduced landmass area until the Plio-Pleistocene may have hindered species diversification; the Atlantic Forest, in turn, likely went through episodes of expansion and contraction and has probably been biologically connected with the Amazonian forest since at least the early Miocene until the Pliocene, about 5 Ma. Importantly, a longer time for speciation in the Atlantic Forest than in Mesoamerica plus NW Andes is ruled out because the dispersal rates into both regions increased simultaneously in the middle-Miocene. Our results reveal a mosaic of bioregion-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).

47

48 **Keywords:** Speciation; dispersal; multispecies coalescent; total-evidence analyses; ancestral

49 state inference; biogeographical stochastic mapping

50

Introduction

Terrestrial bioregions are heterogeneous, and some areas accumulate greater diversity and endemic species than others. An explanation for this biogeographical pattern includes three evolutionary mechanisms: *in situ* 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 low rates of species extinction, becoming a "museum of diversity". 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 *in situ* 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, and for how long (Jaramillo and Cárdenas 2013), and whether they are 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

74 the old and gradual accumulation of species through time, such as Amazonia for butterflies
75 (Condamine et al. 2012). It thus seems that the checkered spatial assemblage of Neotropical
76 rainforests acting as cradle and museum areas might explain why the Neotropics as a whole can
77 be now considered both museum and cradle of diversity (McKenna and Farrell 2006, Moreau
78 and Bell 2013, Antonelli et al. 2018a). However, the impacts of geological and
79 paleoenvironmental changes in the Neotropics together with the roles of *in situ* speciation and
80 dispersal have seldom been jointly elucidated in individual rainforest hotspots.
81
82 Here we test whether there was significant biotic exchange among Neotropical rainforests during
83 the Neogene and Quaternary periods (i.e., the past 23 Ma), which might indicate physical
84 connectivity of biomes. If connection among biomes existed, we aim to quantify its timing and
85 magnitude in terms of dispersal and speciation of taxa associated to rainforests. To do this, we
86 estimate colonization rates (dispersal and establishment in a new area) and *in situ* speciation of
87 butterflies that occur in tropical rainforest hotspots. We focus on Mesoamerica, the northwestern
88 slope (NW) of the Andes (including e.g., Chocó), and the Brazilian Atlantic Forest, compared to
89 neighboring areas such as the South American “dry diagonal” (Fig. 1). Butterflies in the tribe
90 Brassolini (Nymphalidae: Satyrinae) are particularly suited to address our question because they
91 are exclusively Neotropical and have about one third of their species diversity endemic to
92 rainforest hotspots (see Penz 2007 for an overview).
93
94 Brassolini constitutes a monophyletic group (Freitas and Brown 2004, Wahlberg et al. 2009,
95 Espeland et al. 2018, Chazot et al. 2019a). Studies that focused on individual genera used adult
96 morphology (Penz 2008, 2009a, b, Garzón-Orduña and Penz 2009) and DNA data (Penz et al.

2011a, Shirai et al. 2017) to infer robust 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 by merging all previous datasets and generating new DNA and morphological data to clarify the phylogeny of Brassolini under the multispecies coalescent framework. Specifically, we use this phylogeny to test the following hypotheses on diversification and dispersal among Neotropical rainforest biomes:

1. Amazonia is the primary source of butterfly species diversity in the Neotropics due to its large size, high number of standing lineages, long-term accumulation of species, and episodic increases of diversification rate (alternating cradle and museum of diversity), in association with high dispersal toward other Neotropical regions (source of diversity).

Rapid species diversification of some butterfly groups occurred since at least the Oligocene (Matos-Maraví 2016), and species might have gradually accumulated in Amazonia since that time (Condamine et al. 2012). More recent diversification episodes supporting Amazonia as a cradle of diversity could have been triggered by paleoenvironmental changes, such as the retreat of the Pebas wetland (Antonelli et al. 2009, Antonelli and Sanmartín 2011, Chazot et al. 2019b). For vertebrate and plant lineages, Amazonia has also been the main source of species diversity by means of dispersal during the past 60 million years (Antonelli et al. 2018b).

2. Mesoamerica and the NW Andes, considered together, became more diverse due to dispersal events into this region during the Miocene (sink of diversity), and more recent, rapid *in situ* speciation in the Pleistocene (cradle of diversity).

Landmass likely emerged 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); with Uranium-lead dating confirming that segments of the Panama arc had already emerged by 13-15 Ma (Montes et al. 2015). However, shallow water likely 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), *in situ* species diversification might have been restricted by lowland area water inundations until the Plio-Pleistocene (Mullen et al. 2011, Hosner et al. 2016).

3. The Brazilian Atlantic Forest is characterized by old biotic interchange with Amazonia until ~10 to 15 Ma (sink of diversity), and younger <10 Ma biotic interchange with the dry diagonal (source of diversity). However, it is not clear whether endemic diversity to the Atlantic Forest arose by recent, fast diversification in the Pleistocene (cradle of diversity) or by gradual accumulation of species with old and constant *in situ* speciation (museum of diversity).

The onset of the 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 Brazilian 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).

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 at 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

166 previous studies (Penz 2007, 2008, 2009a, b, Garzón-Orduña and Penz 2009, Penz et al. 2013)
167 by eliminating duplicate characters and scoring data for species missing entries. Specimens used
168 were the same as listed in the original publications. The combined molecular and morphological
169 dataset comprised all genera and 84 out of 108 Brassolini species. We call this the total-evidence
170 dataset, which we used to infer a time-calibrated phylogeny.
171
172 *Distribution dataset*
173 We delineated bioregions using geo-referenced occurrences of extant Brassolini, rather than
174 defining areas based on geological or other sources of data. We retrieved 7,255 geo-referenced
175 occurrence points identified at the species level, of which 6,378 points came from GBIF
176 (https://gbif.org; retrieved on 21st January 2019) and 881 points from the ATLANTIC
177 BUTTERFLIES dataset (Santos et al. 2018). We uploaded the geo-referenced occurrences to the
178 web application Infomap Bioregions (Edler et al. 2017) at http://bioregions.mapequation.org/.
179 We set the maximum and minimum values for cell sizes to 4 and 2, and for cell capacities to 80
180 and 4. We used different values of cluster cost, from 0.1 to 3.0, in steps of 0.1. The number of
181 inferred areas remained stable between values of 1.9 and 2.5 and, thus, received the strongest
182 support: 1) Mesoamerica plus NW Andes, 2) Brazilian Atlantic Forest, and 3) the remaining
183 tropical South America, including the Amazon drainage basin and the seasonally dry diagonal
184 biomes Cerrado and Caatinga (Fig. 1).
185
186 To inform the biogeographical and *in situ* speciation rate analyses, we generated a species-level
187 distributional dataset. We surveyed the literature to score species occurrences in the defined
188 bioregions because the geo-referenced dataset did not include all Brassolini species. To

189 investigate dispersal patterns across the South American dry diagonal, we kept this area separate.
190 We used distributional maps and observations, not always with primary geographic coordinates,
191 from taxonomic revisions (Bristow 1981, 1982, 1991, Casagrande 2002, Furtado and Campos-
192 Neto 2004, Penz 2008, 2009a, b, Garzón-Orduña and Penz 2009, Penz et al. 2017), and also
193 published butterfly inventories in Mesoamerica (DeVries 1983, 1994, Janzen and Hallwachs
194 2009, Basset et al. 2015), Amazonia (Pereira Martins et al. 2017), Cerrado (Pinheiro and Emery
195 2006, Emery et al. 2006, Silva et al. 2012, Pereira Martins et al. 2017, Dickens et al. 2019),
196 Caatinga (Zacca and Bravo 2012), and the Atlantic Forest (Santos et al. 2011, 2018, Pérez et al.
197 2017, Melo et al. 2019, Soldati et al. 2019). We call this the presence/absence biogeographical
198 dataset.
199
200 *Total-evidence phylogenetic inference and divergence time calibration*
201 We inferred time-calibrated species trees using the StarBEAST2 package (Ogilvie et al. 2017)
202 available in BEAST v2.5.2 (Bouckaert et al. 2014). We removed the species *Opsiphanes camena*
203 from the dataset because it had only 325 bp of the COI locus, and its phylogenetic position was
204 not stable in a preliminary analysis (Supplementary material Appendix 1). We used a gene-based
205 partitioning strategy, each having unlinked tree models in the multispecies coalescent
206 framework. We performed model averaging over 31 substitution models available in the
207 bModelTest v1.1.2 package (Bouckaert and Drummond 2017). We applied uncorrelated relaxed-
208 clock models for all partitions because strict clock models were rejected based on the stepping-
209 stone method (Supplementary material Appendix 1). We estimated clock rates relative to the
210 mitochondrial mean clock rate fixed to 1.0, and we used log normal distribution for the relative
211 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 but we discarded the first 25% as burnin. We checked convergence among runs by inspecting that the estimated sample sizes (ESS) were above 200 using Tracer v1.7.1 (Rambaut et al. 2018). We merged the four independent runs using LogCombiner (part of the BEAST

v2.5.2 package) and we summarized the post-burnin species trees using the maximum clade credibility (MCC tree) method in TreeAnnotator (part of the BEAST v2.5.2 package).

In situ 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 v3.5.3 (R Core Team 2019) package BioGeoBEARS v1.1.2 (Matzke 2013). We used the species-level 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 (*in situ* speciation) 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 $c_{XtoY}(t_1) = d_{XtoY}(t_1) / L(t_1)$, where $d_{XtoY}(t_1)$ is the number of inferred dispersal events from area X to Y in a 1-million year interval, and $L(t_1)$ 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 $\lambda(t_1) = s(t_1) / s(t_0 / t_{0+1})$, where $s(t_1)$ 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

To have a second independent estimate of *in situ* speciation through time, 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 and we sampled the event data every 1,000 generations. After discarding the first 20% samples as burnin, we checked convergence by inspecting 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

The multispecies coalescent model recovered low posterior probabilities for deeper nodes (0.39–0.90 in the MCC tree; 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. The high uncertainty in such nodes coincided with short branches typical of a very fast diversification process. We estimated that 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.

303

304 *Ancestral bioregions and colonization rates through time*

305 The inferred ranges coming from the 10,000 pseudoreplicated biogeographic histories and
306 plotted against the MCC species tree in Fig. 2 suggested that Brassolini most probably (*Pr* =
307 0.36) originated in Amazonia at 21 Ma (entire HPD, 16.67–27.16 Ma). The early rapid radiation
308 of Brassolini between 15 and 17 Ma also probably took place in Amazonia (*Pr* = 0.30–0.52).
309 Although we allowed any lineage to occur in an unlimited number and combination of areas, the
310 DEC model mostly inferred ancestral ranges that were single areas.

311

312 Dispersal out of Amazonia had the highest rates compared to other bioregions (Fig. 3). It
313 increased since the early Miocene and peaked at ~10 Ma, followed by decreasing dispersal to
314 other bioregions until ~6 Ma. Dispersal from Amazonia to Mesoamerica had a sharp increase by
315 ~5 Ma and is currently the highest rate among other dispersal types (Fig. 3A). Dispersal into
316 Mesoamerica from other bioregions occurred throughout most of the Miocene, and peaked at
317 ~10 Ma (Supplementary material Appendix 1). Dispersal out of Mesoamerica + NW Andes,
318 however, was very low through time, and only since the past ~2 Ma did it exponentially increase
319 from this region toward Amazonia (Fig. 3A). Dispersal from Amazonia to the dry diagonal
320 increased throughout the Plio-Pleistocene and has since then surpassed dispersal from Amazonia
321 to Atlantic Forest (Fig. 3B & 3C). Dispersal from the Atlantic Forest into Amazonia was higher
322 at ~10 Ma than from any other region into Amazonia (Fig. 3C). Nonetheless, dispersal into or
323 out of the Atlantic Forest from/to any other bioregion declined from ~10 to 2 Ma, rendering the
324 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. The Atlantic Forest is currently linked by dispersal mostly with the dry diagonal biome (Fig. 3D).

In situ speciation through time

The relative rates of *in situ* cladogenesis estimated in BioGeoBEARS and *in situ* speciation rates estimated in BAMM were congruent (Fig. 4). *In situ* speciation rates slightly increased through time in all areas except in the dry diagonal, which has had near zero *in situ* cladogenesis. Diversification began earlier in the Atlantic Forest at ~10 Ma, specifically in the clade comprising *Penetes*, *Orobrassolis* and *Blepolenis*, among others. In contrast, speciation sharply increased in Mesoamerica only at ~2 to 3 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 *in situ* 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 ~10 Ma has shaped Brassolini endemic diversity in the Atlantic Forest. This finding, coupled with the survival of old lineages found mainly in montane habitats in the Atlantic Forest suggests that this region acts as a museum of diversity.

348 A longer time for speciation in the Atlantic Forest is not supported because dispersal into
349 Mesoamerica and NW Andes occurred near-simultaneously as dispersal into the Atlantic Forest.
350 Therefore, we argue that the uneven origin of endemic species among the Neotropical rainforest
351 bioregions reflect the geological and environmental changes that took place throughout the
352 Neogene and Pleistocene, including the availability of substantial landmass in Mesoamerica and
353 Chocó by ~3 to 4 Ma and the spread of wet forest into what is now the South American Dry
354 Diagonal during the past 10 Ma.
355
356 *Strengths and weaknesses of the approach*
357 There have been great improvements in tools that help quantifying geographic range evolution
358 and diversification rates, but their assumptions might not be biologically realistic. We are aware
359 of these limitations and took them into account in the interpretation of our results. First, by using
360 the DEC model, we assumed no species extinction in the phylogeny of extant Brassolini.
361 Simulation analyses comparing the DEC and state-dependent diversification models suggested
362 that the estimation of ancestral areas can be robust when extinction rates were low, or not
363 associated to particular geographic areas and clades (Matzke 2014). The lack of Brassolini fossils
364 precludes assessing this directly, but a better fit of the Yule process over the birth-death model
365 suggests that extinction was probably low to moderate during the timeframe of their
366 diversification, and might not have substantially biased our inferences.
367
368 Second, BAMM's likelihood function and the prior model used for estimating diversification
369 rate shifts were contested by Moore et al. (2016), although further evaluation of BAMM via
370 simulated data suggested that it can infer speciation rates accurately from phylogenies of extant

371 species (Rabosky et al. 2017). We have conservatively focused on *in situ* species origination rate
372 to explain lineage accumulation within bioregions, and have not used the extinction rate
373 estimates of Brassolini. We also used a second, fundamentally-distinct measure of *in situ*
374 cladogenesis (Xing and Ree 2017) that corroborated the pattern of recent and rapid speciation in
375 Mesoamerica + NW Andes, and an older and lower speciation rate in the Atlantic Forest.
376
377 Our approach to disentangle *in situ* speciation from dispersal to understand the origin of within-
378 area species diversity has two strengths. First, we defined bioregions that fit the distribution and
379 composition of Brassolini species by using geo-referenced occurrences. This approach revealed a
380 clustering of butterfly communities in southern North America, Mesoamerica, and the NW
381 Andes, which are regions that belong to different biogeographic dominions (Morrone 2014). Our
382 inference of large bioregions for Brassolini agrees with its apparent poor spatial structuring and
383 high dispersal capabilities. For instance, given a suitable environment, ancestral *Bia* had the
384 capability to disperse across pan-Amazonia in only 1,463–3,115 years (Penz et al. 2015). The
385 delimitation of Mesoamerica + NW Andes and the Atlantic Forest as separate bioregions has
386 likely been determined by ecological and habitat suitability rather than geographical barriers to
387 Brassolini dispersal; this being a similar biogeographical pattern found in other species restricted
388 to Neotropical rainforests (Dexter et al. 2017, Pérez-Escobar et al. 2019).
389
390 Second, we used a multispecies coalescent framework which outperforms the concatenation-
391 based approach when facing incomplete taxon sampling and incomplete lineage sorting (Liu et
392 al. 2015, Ogilvie et al. 2017). Our comprehensive dataset including molecular and morphological
393 characters allowed us to simultaneously use several lines of evidence for recognizing and dating

394 speciation events. It is important to note that the Bayesian probabilistic framework applied here
395 considered three types of uncertainties: phylogenetic, temporal and taxonomic sampling. This
396 Bayesian approach contributed toward a better assessment of confidence intervals when
397 estimating dispersal and diversification rates.
398
399 *Species diversification and dispersal in bioregions*
400 We calculated probability densities of dispersal and speciation rates that supported three distinct
401 evolutionary processes: recent and fast speciation in Mesoamerica + NW Andes, old
402 diversification dynamics with low speciation rates in Atlantic Forest, and episodic increases in
403 speciation rate of Amazonian lineages. Our results suggest that Brassolini diversified mainly in
404 Amazonia and the Atlantic Forest from about 23 to 5 Ma. Early diversification might have been
405 accompanied by increasing dispersal out of these two bioregions. In the case of Amazonia, the
406 Miocene marine incursions known as the Pebas wetland system (Wesselingh and Salo 2006) may
407 have not drastically limited dispersal and diversification of Brassolini as it likely did for other
408 butterfly groups (Chazot et al. 2019b). These marine flooding events were probably short-lived,
409 and at least two dated events at ~17.8 Ma and ~13.7 (Jaramillo et al. 2017b) were concurrent
410 with the early and rapid cladogenesis events of Brassolini in Amazonia (Fig. 2). The Pebas
411 wetland might in fact have increased opportunities for dispersal and diversification of some
412 typical coastal plants (Bernal et al. 2019), including many of the butterflies' hostplants in the
413 region: mainly Aracaceae (palms), Poaceae (bamboo) and Zingiberales such as Marantaceae
414 (Beccalconi et al. 2008, Janzen et al. 2009). Therefore, the evolutionary consequences of the
415 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.

In the case of the Brazilian 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 Period based on palynological data and phylogeographic studies at the population level (e.g., Werneck 2011, Prates et al. 2016, Trujillo-Arias et al. 2017, and references therein). Although evidence of Neogene connection between the Atlantic Forest and Amazonia is still scarce, this study suggests that Miocene dispersal rates between these wet forest biomes were higher than in the Plio-Pleistocene. We suggest that 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. Two key events might explain this shift in diversification dynamics: the emergence of landmass in Panama, and the expansion of open environments during the late Miocene and Pliocene. 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, which explains why speciation rates in this region might have apparently remained low through time, with old lineages surviving 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) — some of which are novel. 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.

462

463 3) *Selenophanes orgetorix*, NEW COMB. This species grouped with members of *Selenophanes*
464 with high posterior probability (see also Shirai et al. 2017), justifying the proposed new
465 combination.

466

467 *Conclusions*

468 This study suggests that the Neotropics as a whole consists of a regional network of museums
469 and cradles of diversity. We tested this hypothesis using a total-evidence phylogeny of the
470 butterfly tribe Brassolini. We found that endemic species to Mesoamerica plus NW Andes have
471 originated by old dispersal events into the region, and that speciation rates increased only at
472 about 2 Ma (cradle of diversity). In contrast, endemic species to the Brazilian Atlantic Forest
473 likely arose by low and continuous speciation rates since about 10 Ma (museum of diversity),
474 and this region was likely intermittently connected to Amazonia since the Miocene. This
475 dynamic evolutionary history appears to reflect paleoenvironmental changes, including
476 landscape reconfigurations in Central America and paleoclimate fluctuations during the Neogene
477 and Quaternary periods. Our findings reinforce the interpretation of Neotropical rainforests
478 expanding during Miocene, which likely facilitated dispersal and speciation of taxa associated to
479 such biomes.

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

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Figures

Fig. 1: Map of the Neotropics and the defined bioregions based on occurrence data of Brassolini. Inset map shows the strongest clustering 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).

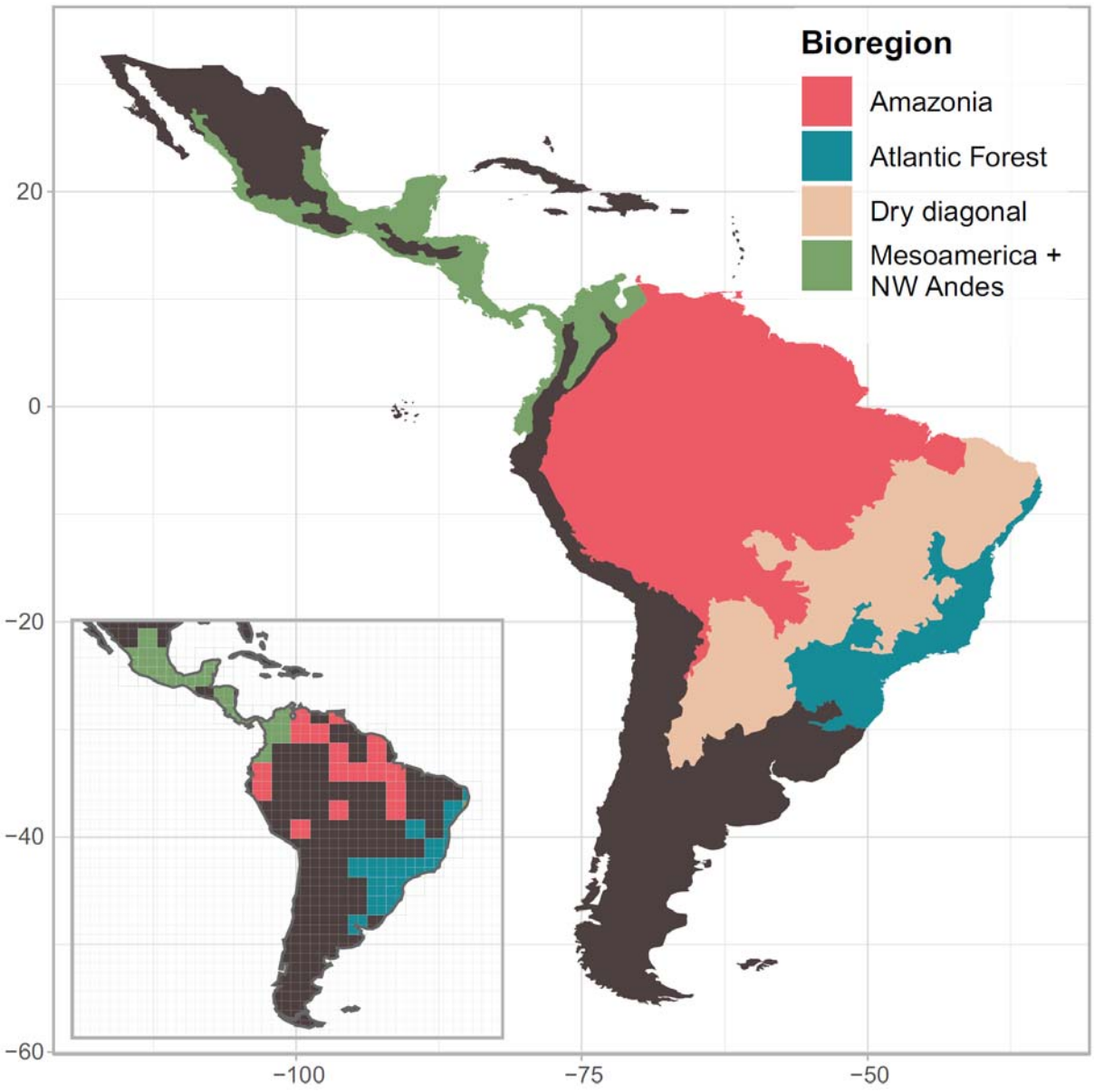

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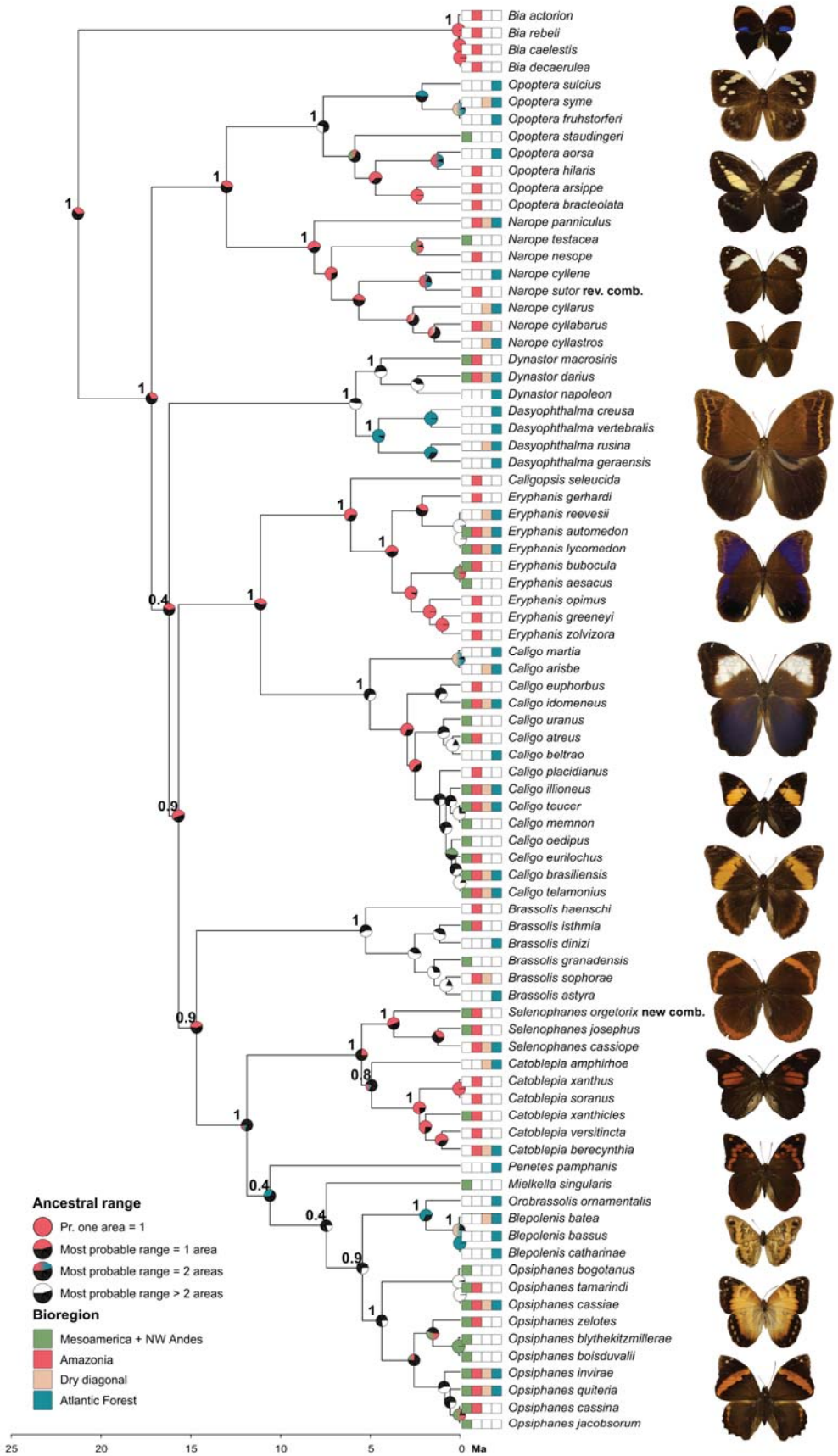
Fig. 2: Time-calibrated species tree of the butterfly tribe Brassolini. The posterior probabilities for all genera and among genera are displayed next to the crown node. Ancestral range probability based on the DEC model and 10,000 biogeographical stochastic mappings is depicted as a pie chart following 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).



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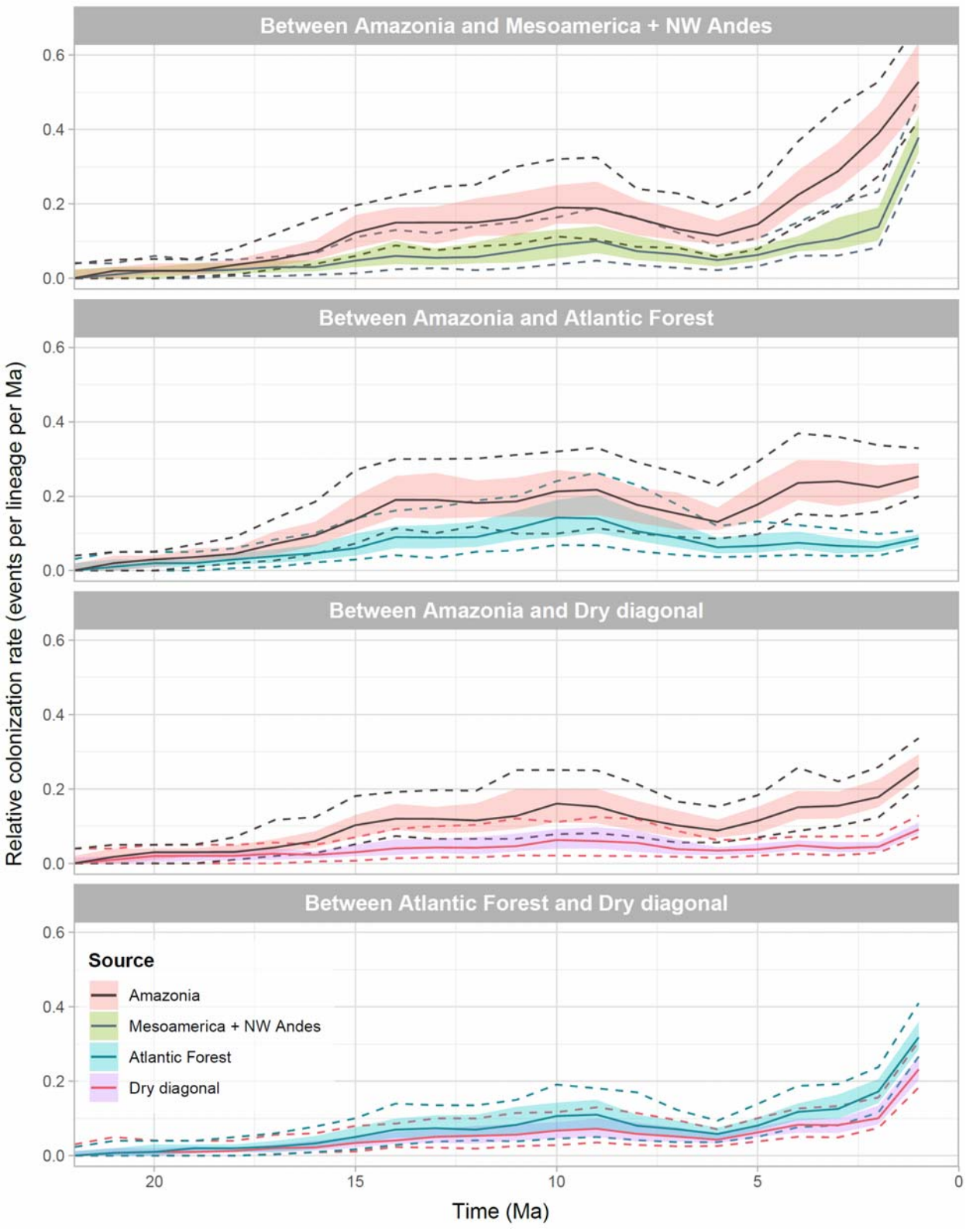

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778 Fig. 4: Within-area speciation through-time based on 10,000 biogeographical stochastic
779 mappings in BioGeoBEARS and on per-lineage speciation rates by BAMM. A) The relative
780 number of *in situ* cladogenesis calculated using a formula modified from Xing and Ree (2017).
781 Solid lines represent median values, colored ribbons represent lower and upper quartiles (0.25
782 and 0.75 quantiles) and dashed lines represent 0.1 and 0.9 quantiles. The x-axis is truncated at 8
783 Ma. B) Solid lines represent mean speciation rates per lineage and the colored ribbons the 5%
784 and 95% confidence intervals. The x-axis is truncated at 8 Ma. C) Species (tips) specific
785 speciation rates as estimated by BAMM. Box plots per area show estimates from the posterior
786 distribution, and the cross within each box plot represents mean speciation rates per area.
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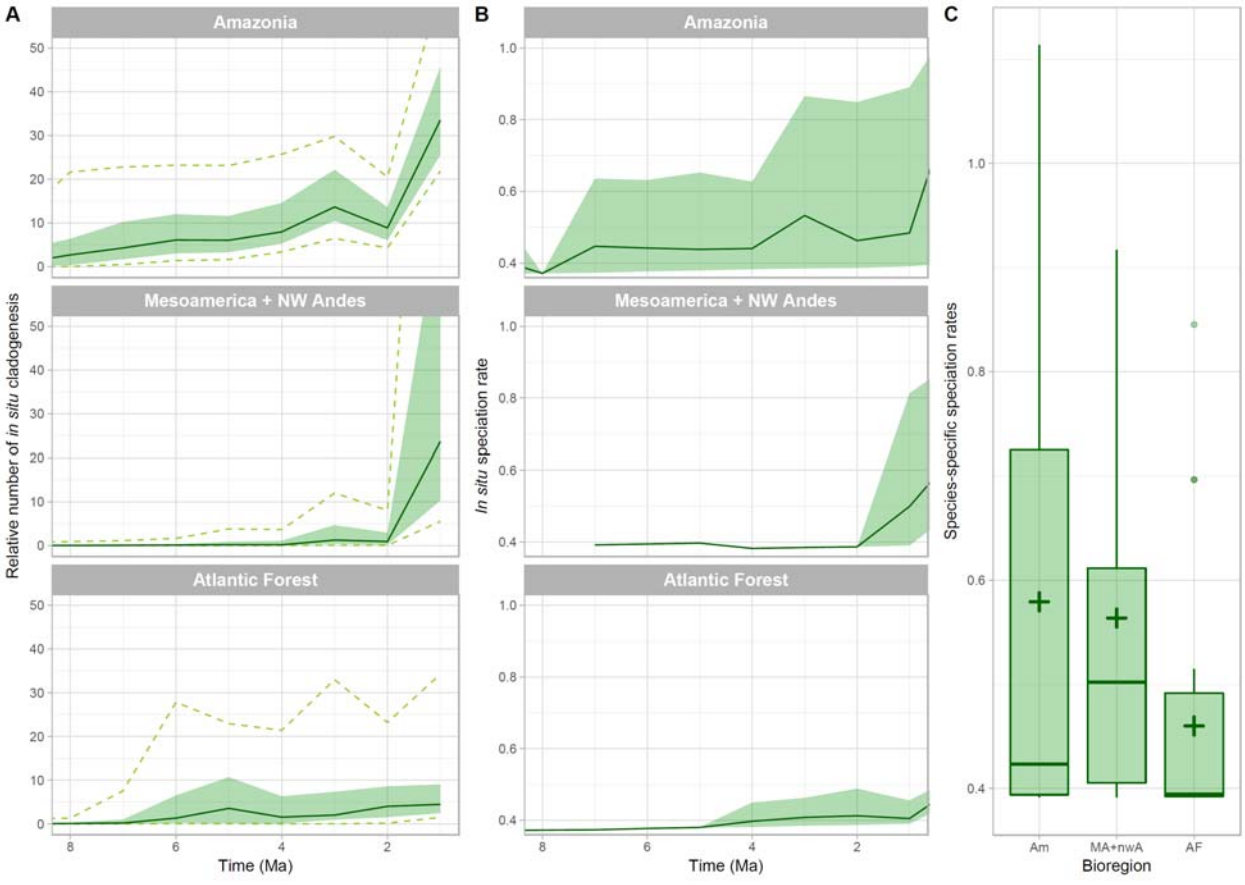

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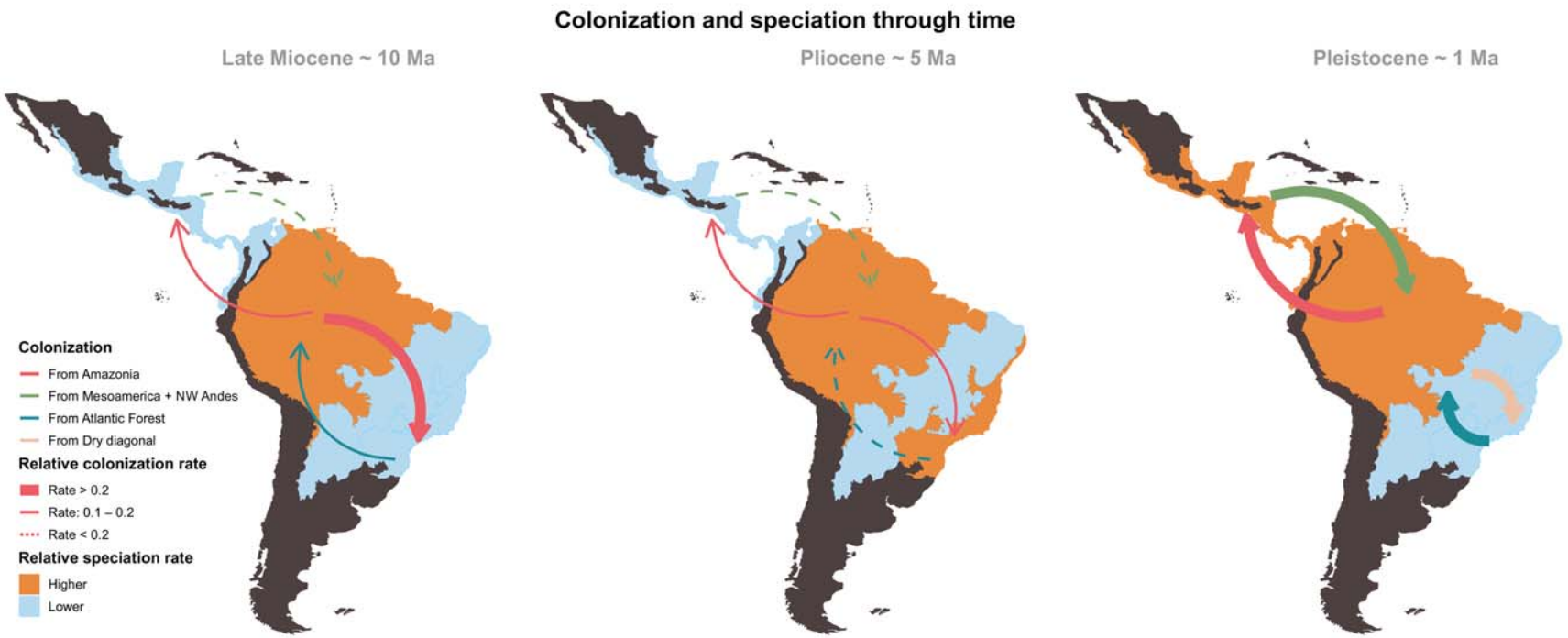
Fig. 5: Summary of Brassolini colonization and speciation estimates in three time-windows, at 10, 5 and 1 Ma. Colored directional arrows represent dispersal from source areas, following colors in legend. The width and shape of arrows represent the estimated relative colonization rate between two areas 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.



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