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# Biogeographic evidence supports the Old Amazon hypothesis for the formation of the Amazon fluvial system

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The Amazon has high biodiversity, which has been attributed to different geological events such as the formation of rivers. The Old and Young Amazon hypotheses have been proposed regarding the date of the formation of the Amazon basin. Different studies of historical biogeography support the Young Amazon model, however, most studies used secondary calibrations or are performed at the population level preventing evaluation of a possible older formation of the Amazon basin. Here, we evaluate the fit of molecular phylogenetic and biogeographic data to previous models regarding the age of formation of the Amazon fluvial system. We reconstructed time-calibrated molecular phylogenies through Bayesian inference for six taxa belonging to Amphibia, Aves, Insecta and Mammalia. We used both nuclear and mitochondrial DNA sequence data and fossils as calibration points, and explored priors for both data sources. We detected the most plausible vicariant barriers for each phylogeny and performed an ancestral reconstruction analysis using areas bounded by major Amazonian rivers. Furthermore, we evaluated the effect of different dispersal rates over time based on geological and biogeographical information. The majority of the genes analysed fit a relaxed clock model. The lognormal distribution fits better and leads to more precise age estimation than the exponential distribution. The data suggested that the first dispersals to the Amazon basin occurred to Western Amazonia from 16.2–10.4 Ma, and the taxa covered most of the areas of the Amazon basin between 12.2–6.2 Ma. Additionally, we obtained evidence for two rivers, Tocantins and Madeira, acting as vicariant barriers. Given the molecular analyses, we suggest that the temporal range for the beginning and complete formation of the Amazon fluvial system might be older than previously proposed.

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## Abstract

The Amazon has high biodiversity, which has been attributed to different geological events such as the formation of rivers. The Old and Young Amazon hypotheses have been proposed regarding the date of the formation of the Amazon basin. Different studies of historical biogeography support the Young Amazon model, however, most studies used secondary calibrations or are performed at the population level preventing evaluation of a possible older formation of the Amazon basin. Here, we evaluate the fit of molecular phylogenetic and biogeographic data to previous models regarding the age of formation of the Amazon fluvial system. We reconstructed time-calibrated molecular phylogenies through Bayesian inference for six taxa belonging to Amphibia, Aves, Insecta and Mammalia. We used both nuclear and mitochondrial DNA sequence data and fossils as calibration points, and explored priors for both data sources. We detected the most plausible vicariant barriers for each phylogeny and performed an ancestral reconstruction analysis using areas bounded by major Amazonian rivers. Furthermore, we evaluated the effect of different dispersal rates over time based on geological and biogeographical information. The majority of the genes analysed fit a relaxed clock model. The lognormal distribution fits better and leads to more precise age estimation than the exponential distribution. The data suggested that the first dispersals to the Amazon basin occurred to Western Amazonia from 16.2–10.4 Ma, and the taxa covered most of the areas of the Amazon basin between 12.2–6.2 Ma. Additionally, we obtained evidence for two rivers, Tocantins and Madeira, acting as vicariant barriers. Given the molecular analyses, we suggest that the temporal range for the beginning and complete formation of the Amazon fluvial system might be older than previously proposed.

## Introduction

The Amazon basin harbors a high biodiversity, which has been attributed to different geological events such as the formation of rivers in a scenario that was proposed by Wallace (1852) known as the "riverine barrier hypothesis". This hypothesis has been supported by different studies (Maldonado-Coelho et al., 2013; d'Horta et al., 2013). Likewise, two geological models (Old and Young Amazon) supporting this hypothesis have been proposed regarding the date of the formation of the Amazon basin. The "Old amazon" model suggest that the fluvial system was established during the late Miocene (~10–7 Ma, Hoorn et al., 1995; Figueiredo et al., 2009; Hoorn et al., 2010, 2017, Bernal et al., 2019), while some authors consider an upper limit of 10.5 Ma (Figueiredo et al., 2010). The "Young Amazon" model covers a wider temporal range, with studies agreeing that the basin was completely formed during the Pilo-Pleistocene (~2.5 Ma, Campbell, Frailey & Romero-Pittman, 2006; Horbe et al., 2013; Rossetti et al., 2015). Both models have been evaluated using biogeographical approaches (e.g. Ribas et al., 2011; Fernandes, Wink & Aleixo, 2012), which suggested that the Amazon basin was formed in the Plio-Pleistocene, and therefore support the "Young amazon" hypothesis. Alfaro et al., (2015) also proposed plausible dates for the formation of some main Amazonian rivers, providing a deeper understanding of how the basin reached its current configuration.

The temporal range of the formation of the basin remains controversial. The changes in the Amazonian landscape that led to the formation of the current Amazon basin have been attributed to a series of geological events starting with the gradual uplift of the Eastern Cordillera in the Central and Northern Andes, which caused the closure of the Western Andean Portal and created a large watershed in the middle Miocene (Hoorn et al., 1993, 2010; Parra et al., 2009; Horton, 2018) known as the Pebas mega-wetland system (hereafter, PMWS). Located in the Western Amazonia, the PMWS was connected to the Caribbean Sea and bounded by the Purus Arch on the east (Figueiredo et al., 2009, 2010), but some details are still unresolved (Jaramillo et al., 2017). In the late Miocene, the PMWS disappeared (Wesselingh et al., 2002) due to the uplift of the Eastern Colombian Andes (~15–3 Ma, Mora et al., 2008), and as consequence, the Vaupes Arch was drawn close to the Andes, causing the Orinoco-Amazonas separation event (Olivares et al., 2013) and gradually reducing the water flux towards the Caribbean (Lundberg et al., 1998). Finally the Amazon River extended eastward either by the subsidence of the Purus Arch (Dobson, Dickens & Rea, 2001; Figueiredo et al., 2009, 2010; Nogueira, Silveira & Guimarães, 2013; Hoorn et al., 2017; van Soelen et al., 2017). The role of these events in the formation of the modern Amazon basin has been highlighted in different studies (e.g. Matos-Maravi et al., 2013; Horbe et al., 2013). In general. Most studies support a "Young Amazon" model (e.g. Horbe et al., 2013; Rossetti et al., 2015), but others disagree (e.g. Figueiredo et al., 2009, 2010; Hoorn et al., 2010).

The main idea in historical biogeography analyses is that geological events have influenced the diversification of the biota, and therefore this diversification pattern is seen in the phylogeny as biogeographical cladogenetic patterns, and from a Panbiogeographic view, those patterns will agree for different taxonomic groups, following Croizat's idea that the earth and biota have evolved as a whole. To date, there are no biogeographical studies that incorporate different taxa. Most of the studies that have addressed the Amazon basin formation used secondary calibrations (e.g. Buckner et al., 2015) —which increase uncertainty in age estimates (Schenk, 2016)— or are performed at the population level (e.g. Ribas et al., 2011), preventing evaluation of a possible older formation of the Amazon basin. Furthermore, the number of species used has been low in some studies (10–68% less than the number of species used in the present study) (e.g. Pramuk et al., 2007; Maciel et al., 2010; Ramírez et al., 2010). Therefore, the goal of the present study is to evaluate the fit of molecular phylogenetic and biogeographic data to the previously described models regarding the age of formation of the Amazon fluvial system.

## Materials & Methods

**Selection of taxa.** We selected taxa using the following criteria: (1) We considered only monophyletic taxa with clades currently distributed in the Amazon basin; (2) The phylogeny must include clades with divergence times covering the temporal ranges proposes by the two models (1 – 11.8 Ma, Hoorn et al., 2010; Figueiredo et al., 2009, 2010; Ribas et al., 2011); (3) At least 60% of the described species in the phylogeny must have both mitochondrial and nuclear genes, and a minimum of three genes available at GenBank; (4) The taxa must have fossil records available in the literature for the ingroup and/or outgroup (see Table S2), and the fossils must not have uncertain phylogenetic positions (Kay & Maldrum, 1997; Hosner, Braun & Kimball, 2016; Kay & Cozzuol, 2006) or poor stratigraphic information.

**Molecular and Distributional data.** We downloaded all nucleotide genes sequences available at GenBank for each taxon (see Data S1). Each gene was aligned separately using MUSCLE v3.8.31 (Edgar, 2004) with default settings. The nucleotide substitution models were selected with the Akaike Information Criterion (AIC, Akaike, 1974) with the modelTest function in the R package ‘phangorn’ (Schliep, 2011). For the distributional data, we used the available literature and occurrences from the Global Biodiversity Information Facility (GBIF, see Table S1). The dataset was checked removing points out of the known IUCN distributional range and distributional range cited in literature.

**Phylogenetic reconstruction.** We carried out a partitioned phylogenetic analysis under Bayesian Inference as implemented in MrBayes 3.2.6 (Ronquist et al., 2012) via the CIPRES Science Gateway (Miller, Pfeiffer & Schwartz, 2010). For each partition, we applied the nucleotide model previously selected, and we tested whether the evolutionary rate was constant across the phylogeny (strict molecular clock) or it varies on each branch (relaxed molecular clock). Then, we chose the model that best fit our data through Bayes Factors (Kass & Raftery, 1995). Once the evolutionary rate of the model was settled, we modeled the relaxed clock using the independent gamma rate model (Lepage et al., 2007; Bakiu, Korro & Santovito, 2015), which is a continuous uncorrelated model of rate variation across lineages that assumes an independent rate on each branch following a gamma distribution. We used a fossilized birth-death process (Heath, Huelsenbeck & Stadler, 2014; Zhang et al., 2016) to model the cladogenetic process, taking into account speciation, extinction and fossilization.

**Priors and clock dating.** For each calibration point, we tested two informative prior distributions (exponential and log-normal) and chose the model that best fit the data through Bayes Factors (Kass & Raftery, 1995). This step is crucial for the estimation of divergence times of the species. It has been proposed that the reason for over-estimation of branch lengths is the poor choice of the prior distribution (Rannala, Zhu & Yang 2011). Both prior distributions differ in the location of the highest likelihood for the age of a node (Jones et al., 2013) and the parameters established for each one (Ho & Phillips, 2009; Arcila et al., 2015).

We conducted two independent runs with 30 million generations using the selected parameters, sampling every 2000 generations and discarding the first 25% samples (burn-in). Each run consisted of four Metropolis-coupled Markov Monte Carlo chains with default temperatures ($\Delta$=0.09 between heated chains). Each analysis was carried out until the average standard deviation of split frequencies was below 0.05 (Ronquist et al., 2012). The convergence between runs was assessed using the effective sample size as reported in TRACER v1.7 (Rambaut et al., 2018) and the potential scale reduction factor (Ronquist et al., 2012). We also used the command 'mcmcp data=no' to perform the analyses without data and sample from the priors only (Ronquist et al., 2012) to evaluate the impact of including only the priors on ages and monophyly.

**Infinite sites.** We plotted the posterior means of each clade against the 95% posterior confidence interval values (CIs, Yang & Rannala, 2006) for each phylogeny to evaluate the uncertainty source in posterior estimates of the divergence times, either by molecular or fossil sampling. The plot is based on the fact that even with infinitely long sequences, uncertainties will remain in the posterior time estimates because the posterior converges to a one-dimensional distribution (Yang & Rannala, 2006).

**Isolation barriers and ancestral area inference.** We determined the potential isolation barriers for each clade following Hovenkamp (1997, 2001), who suggested that the only evidence of a speciation process in a geographical context is the allopatric distributions. We used the Vicariance Inference Program (VIP, Arias, Szumik & Goloboff, 2011) for this analysis. This method does not require predefined areas, so the percentage of vicariant events can be higher compared with other methods. Grid size was selected to minimize the under-sampling and low resolution, and we used a strict non-overlapping rule.

We reconstructed the ancestral distribution using areas bounded by major Amazonian rivers (Alfaro et al., 2015, Fig. 1, Fig. S1) and under the dispersal-extinction-cladogenesis model (DEC, Ree et al., 2005; Ree & Smith, 2008) implemented in the R package 'BioGeoBEARS' (Matzke, 2013). We assessed two different biogeographic models, the base model with a constant dispersal rate in which all areas have the same probability, and a stratified model with different dispersal rates over time (Ree & Smith 2008), abruptly changing the dispersal rates along the time span. Therefore, we can evaluate whether there are changes related to the time and distribution of the ancestral areas in a given temporal range and if those changes are related to the ranges proposed for the formation of the Amazon basin. We generated four models, with three time slices for each one: (1) The "Young Amazon" model, in which the dispersal rates were low before 11.8 Ma (proposed lower limit, code as 0.25), then started to increase (code as 0.50) during the temporal range in which the Amazon system began forming (11.8–2.5 Ma), and the rates became maximal when the Amazon basin reached its current form and size (after 2.5 Ma, code as 1.0). (2) The "Old Amazon" model, where rates were intermediate (0.50), between 10–7 Ma, suggesting that the system was mostly formed during this time, and the Amazon basin was completely established after 7 Ma (1.0). (3) The Lineage Through Time (LTT) model (Fig. S2), based on a lineage-through-time plot for all taxa together, and assumed the rates were low until 17 Ma (0.25) and became intermediate (0.50) where the PMWS existed between 17 and 10 Ma, then, for the maximal rates (1.0) in the last 10 Ma to the present, considering the end of the PMWS and that the Amazon basin reached its present form. (4) The “River Model”, where the rates were minimum until 10 Ma (0.25), intermediate from 10 to 7 Ma (0.50) when the Amazon River flowed eastward, and the rates were minimum after 7 Ma (0.25) when the Amazon basin was completed. We used a log-likelihood difference of two units to compare the models (Edwards, 1992).

The methodology work flow can be found in the supplemental file Figure S3.

**Congruence between events.** To quantify the congruence, we relied on the approach presented by Cunningham & Collins (1994), who proposed biogeographic congruence, namely, synchronous vicariance or dispersal events that reflect shared historical processes. The use of different taxa reduces the possible bias that a single taxon could exhibit. We obtained the vicariant events associated with the formation of Amazonian rivers for each clade based on the position of the rivers and through the reconstruction of the ancestral distribution and the evaluation of the vicariant barriers based on grids, and therefore, we did not require prior assumptions of areas. We checked whether the same river could be assigned as a vicariant barrier for two or more taxa and if they shared similar divergence times for that event. The congruence was evaluated for each method separately. Finally, we verified whether the two aforementioned methodological approaches showed congruent vicariant events associated with the formation of rivers.

Data and R-scripts implemented in this research are available at:

https://github.com/karen9/Amazonia

## Results

**Taxa and calibration points.** The dataset was composed of six phylogenies representing four different taxonomic groups (Table 1). For most of the taxa analyzed in this study, there are not biogeographic studies evaluating the role of rivers as speciation barriers. However, for Cebidae, there are analyses regarding the Madeira (Buckner et al., 2015), Negro, Branco, Tapajos, Xingu and Tocantins rivers (Alfaro et al., 2015; Boubli et al., 2015). For mammals, we found fossil information for the ingroup, while for the remaining groups, we used the fossils that were available for the outgroup (Table S2).

**Molecular clock.** The strict molecular clock model was rejected for all genes (Table S3), except for the ATP7A and COI for Stenodermatinae (log-difference of three to five, Kass & Raftery, 1995). We verified the individual topologies of each of the two genes to assess the overall impact of these genes, which fit a strict model based on the total evidence topology. We found that they produced unresolved topologies, therefore COI did not present common nodes and ATP7A presented 2% common nodes with the total evidence topology. Nonetheless, removing the ATP7A and COI genes was not useful, as the removal resulted in a less resolved topology (50% of common nodes) with lower Posterior Probabilities (hereafter, PP).

**Prior and posterior estimates.** The exponential and log-normal distributions generated the same topology, but differed in branch lengths. All calibration points for each phylogeny fit best to a log-normal distribution, showing the narrowest 95% CIs without divergence date estimates (Fig. 2). Furthermore, for the majority of the calibration points were overlap between the prior and the posterior distributions. For 16 calibration points, the prior overlapped with the posterior about 80–100%, and for nine of these 16 calibration points, the posterior was narrower (Fig. 3).

The only exception in which the prior and the posterior did not overlap was *Pampamy emmonsae* Verzi, Vucetich & Montalvo, 1995 (Echimyidae), where the posterior distribution was older with a difference of 3 Ma (media: 17.58, CI-95%: 21.58 – 14.09 Ma. Fig. 3). The absence of this fossil in the analysis led to a wider 95% CI (median: 18.84, CI-95%:23.35 – 14.68 Ma) than the analysis including it, with a difference of ~1 Ma. While, the PP did not change between both analyses (100%). On the other hand, the incorporation of the new fossil record in the *Cebuella* Gray, 1870 lineage (Cebidae) led to a narrower 95% CI (10 – 10.9 Ma) and a higher PP (100%) than the analyses without this calibration point (CI-95%: 8.7 – 4.5 Ma, and PP: 73%).

Both, the posterior age means and the CIs fit best to a straight line under the exponential fossil prior distribution (gray, Fig. 2), and led to older ages and a larger 95% CIs than the log-normal distribution (red, Fig. 2). The taxa with the oldest ages and the widest CIs (lower precision) in decreasing order were *Melipona*, Cracidae and *Rhinella*. While the taxon with most recent ages and highest precision was Cebidae (Fig. 3).

Finally, the analyses using only priors resulted in unresolved topologies (results not shown), although monophyly was enforced on the calibrated nodes (nodes constrained by fossil ages).

**Isolation barriers and ancestral reconstruction.** Dispersal is the main driver of most cases of speciation (Fig. 4), and these events are concordant with dates proposed by another analysis (Ribas et al., 2011). We obtained 55 isolation barriers for all analysed clades (264), which were mostly concentrated in Echimyidae and *Melipona*. Seven of these 55 isolation barriers were associated with some of the Amazonian rivers (Tocantins, Madeira, Amazonas, Marañón and Ucayali, Fig. 5). However, only two of these nine barriers were congruent among different taxa and matched the Tocantins (10.85–9.75 Ma) and Madeira (10.40–8.27 Ma) rivers. On the other hand, the reconstruction of the ancestral distribution only reported one vicariant event associated with the Negro river (Table S4). But, this vicariant barrier was not congruent between the taxa or methodological approaches (VIP and BioGeoBEARS).

Although all taxa fit stratified models, four were better fitted to more than one stratified model with a log-likelihood difference of 0.1–0.77 (Table S5). Otherwise, Cracidae and Stenodermatinae only fit the “Young Amazon” model. For *Rhinella*, *Melipona*, Echimyidae and Cebidae, the first dispersal to the Amazon basin occurred from the Atlantic Forest to some areas of Western Amazonia (Napo, Marañon and Ucayali) in the range of 16.2–10.4 Ma, and all taxa covered most of the areas of the Amazon basin from 12.2–6.2 Ma (Fig. 1D; Fig. 4). Nevertheless, for Cebidae and Echimyidae, the first dispersal involved all areas of the Amazon basin (Fig. 4), then experienced a contraction of the ancestral range, but there was no congruence in the temporal range in which these events took place (Fig. 4). Both taxa experienced expansion to some areas of Western Amazonia (Ucayali and Napo) following a contraction of the ancestral range to the Atlantic Forest and the Andes, respectively. For the remaining two taxa, Cracidae and Stenodermatinae, the first dispersal to the Amazon basin occurred from Central America and the Andes, respectively(Fig. 4), in different temporal ranges for each taxon and out of the suggested ranges for the taxa mentioned above (CI-95%: 3.3 – 0.99 Ma, and 7 – 3 Ma, respectively).

## Discussion

**Historical Amazon.** We found that species dispersal agrees with a progressively developing Amazon drainage system from 16.2–10.4 Ma, the first dispersal to Western Amazonia, similar to other studied groups (Sánchez-Herrera et al., 2020). This pattern recreates a new scenario that suggests that the PMWS disappeared or was reduced for that time, allowing colonization of these areas. We agree with Shephard et al., (2010), who suggested that the PMWS was greatly reduced in the middle-late Miocene. Hence, we propose that the biota follow the development of the Amazon drainage basin, which, given the data, likely reached its shape and size from 12.2–6.2 Ma (Fig. 1), when the taxa extended their distributions to Eastern Amazonia. In general, the temporal range proposed here agrees with the “Old Amazon” hypothesis, but we might consider a wider temporal range than those proposed by Hoorn et al., (2010) and Hoorn et al., (2017).

Although both Cracidae and Stenodermatinae fit a stratified model of the “Young Amazon” hypothesis where the dispersal rates are maximum after 2.5 Ma, the reconstruction of the ancestral range indicates a different scenario in which the first dispersal to the Amazon basin occurred before 2.5 Ma, and occurred at different ranges for each taxon. However, we cannot discard the possibility that after the first dispersal to the basin, the number of dispersal events could have increased in response to the complete establishment of this fluvial system.

The temporal range proposed here for the formation of the Amazon fluvial system agrees with the geological process sequence that occurred during that time. Hoorn et al., (1995, 2010) suggested that the formation of the Amazon River can be attributed to the Andean uplift, and our findings are consistent with stratigraphic studies regarding the emergence of the Eastern Cordillera of the Colombian Andes (Mora et al., 2008), and with paleobotanic observations, which suggest that elevations of the Eastern Colombian Andes were homogeneous between the early and middle Miocene and Pliocene (Gregory-Wodzicki, 2000), as well as with studies regarding the sedimentation rates of the Foz do Amazonas, which highly increased since the mid-Miocene (Figueiredo et al., 2009, 2010; Hoorn et al., 2017). On the other hand, our results are also incongruent with other geological events, such as the date of activation of the Vaupes arch in the Miocene (Olivares et al., 2013) and the subsidence of the Purus arch that allowed the Amazon to flow to the east. Although in the present study we cannot establish the exact range of its subsidence, we can say that it might be assigned to the mid-Miocene, which contrasts with other proposals (e.g. Nogueira, Silveira & Guimarães, 2013). The geological events together have been broadly accepted as the main causes of the disappearance of the PMWS. Notwithstanding, our data and analyses do not allow us to establish the complete temporal range in which the system existed.

Our results are not affected by the number of time slices and different probabilities of dispersal in the range-inheritance scenarios (Ree & Smith, 2008), and consequently, we cannot establish the temporal ranges in which there is a change in the dispersal rates according to the geological ranges proposed for the two aforementioned hypothesis. Moreover, using the biogeographical approach we demonstrated that the large Amazonian rivers can limit species distribution ranges (Moraes et al., 2016; Oliveira, Vasconcelos & Santos, 2017; Godinho & Da Silva, 2018), and the dates proposed here differ from Ribas et al., (2011) and Alfaro et al., (2015) —which suggested that the Tocantins river was formed between 0.8–0.3— and Buckner et al., (2015) —which suggested that the Madeira river was formed around 5 Ma—. It is worth noting that the methods used in this study to identify rivers as vicariant barriers are based on different algorithms, and therefore, different results could be expected, also, that the rivers dates proposed here are just estimations and many rivers changed during time. The main differences between our findings and those of other authors who proposed recent dates for rivers, are the type of analyses and the implemented data. Some studies have incorporated populations and species (Ribas et al., 2011; Buckner et al., 2015), while some only took into account mitochondrial genes (Alfaro et al., 2015) or secondary calibrations (Alfaro et al., 2015; Buckner et al., 2015) and did not carry out and exploration of priors.

**Ages estimates.** The chosen priors are reasonable for modeling our data as there is a considerable overlap between the prior and the posterior density functions, with the posterior more concentrated that the prior (Fig. 3, Nascimento et al., 2017), but priors only do not determine the results. The infinite-sites plot (Fig. 2) suggests that the uncertainty in the posterior age estimates is mainly due to limited molecular data (Yang & Rannala, 2006) and is not due to the fossil points used, as is reflected in the regression value, which is low in most cases (with the exception of Cracidae).

We cannot rule out the possibility that both an increased density of taxon sampling at genome scale and fossil sampling could improve age estimates (Yang & Rannala, 2006). We noted that incorporation of fossils for the clade in which posterior is older and out of the prior (e.g. *Pampamys emmonsae*) led to more precise age estimation (Foote et al., 1999; Smith & Peterson, 2002; Sytsma, Spalink & Berger, 2014).

The molecular clock model used here does not incorporate uneven fossil sampling (Drummond et al., 2012; Zhang et al., 2016), which could have impacted the posterior age estimates. Thus, the ancient and less precise ages produced in the present study could be due to a lack of internal node constraints, which leads to ancient ages (Bibi, 2013; Arcila et al., 2015). This could be highlighted by the fact that the taxon with most fossils (Cebidae) presents more precise date estimates for the entire dataset, and the amount of uncertainty added in the posterior CI is lower (Fig. 2). On the other hand, in contrast with Heads (2012), our results show that the use of an exponential distribution for the calibration points generates older and less precise posterior estimates (Fig. 2), as was previously suggested (Heath, 2012; Sauquet et al., 2012; Arcila et al., 2015).

## Conclusions

Our results provide support for the “Old Amazon” hypothesis as well as for a middle-late Miocene time origin for the Amazon drainage system. We only obtained evidence for the date of formation of two rivers (Tocantins and Madeira), although there might be other rivers acting as biogeographical barriers. For the temporal range studied here, the rivers did not structure the Amazonian biota. It is likely that there are other physical factors involved in Amazonian biota evolution, emphasizing the complexity and dynamics of the Amazonian system. It is, therefore, necessary to consider this issue with different tools with multiple sources of data. Furthermore, our analyses highlight the importance of including numerous fossil calibration points distributed throughout the phylogeny and an exploration of priors, resulting in more precise age estimates.

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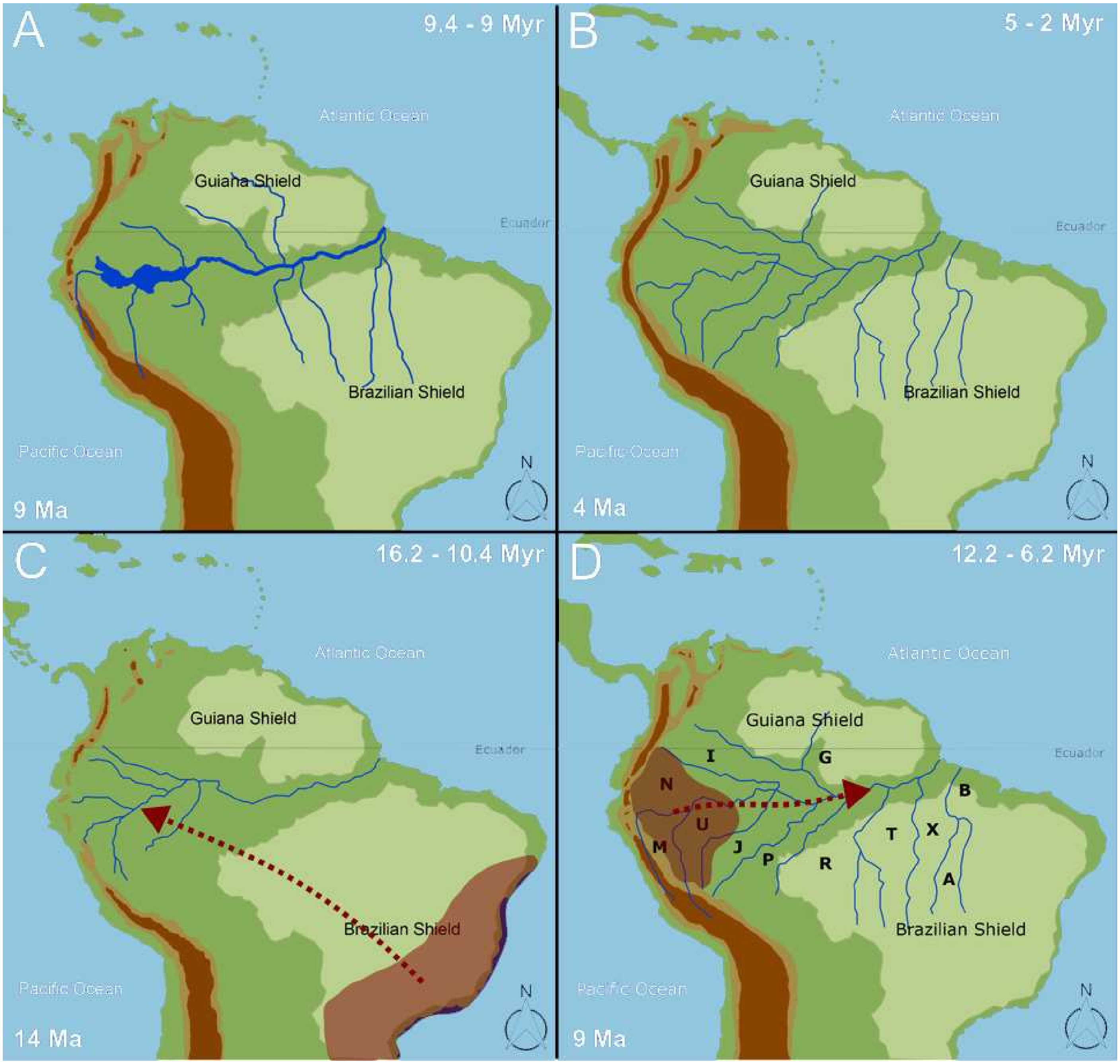
# Figure 1

Figure 1. The biogeographical pattern for formation of the Amazon basin.

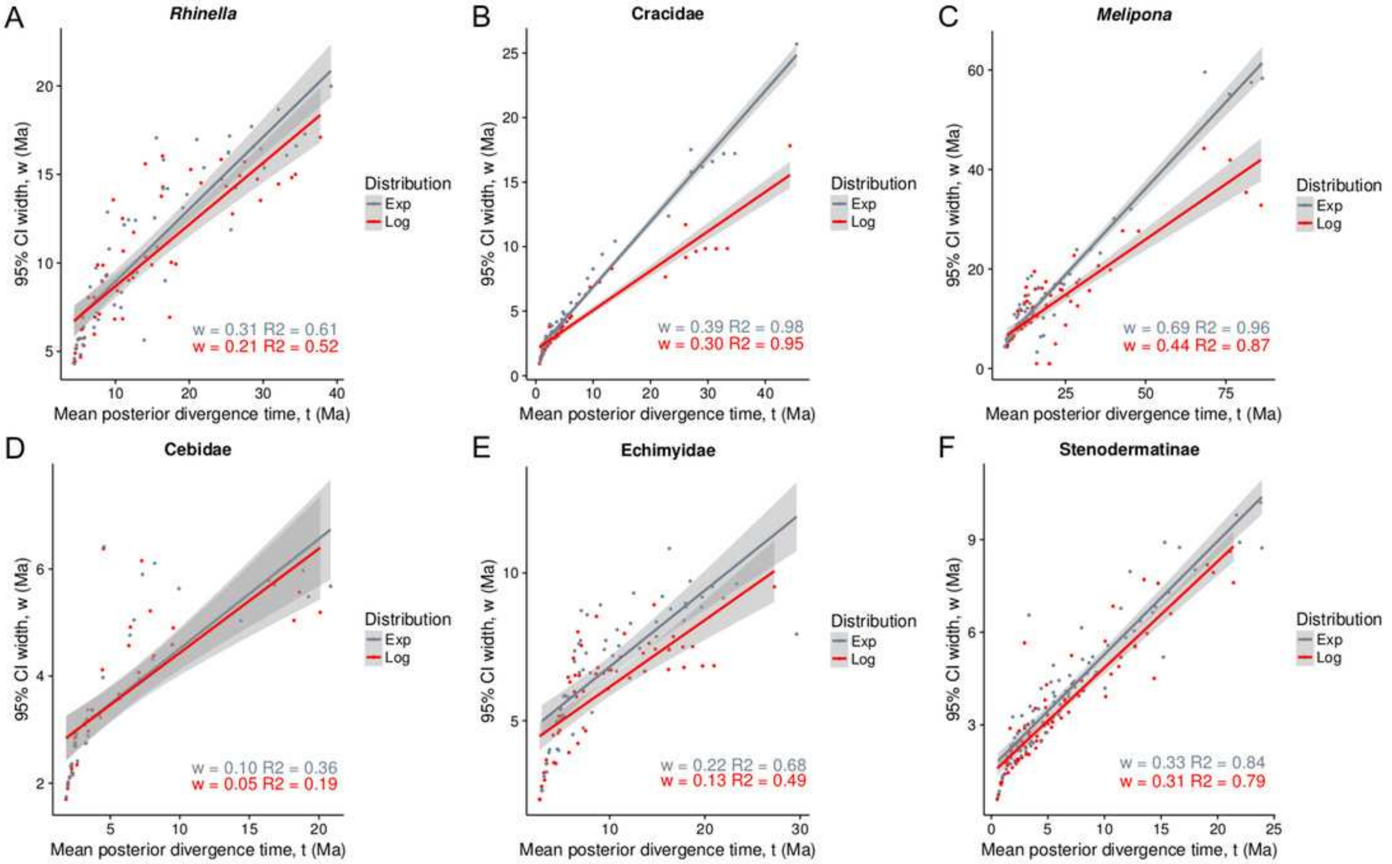
Maps are modified from Hoorn et al. (2010). Dates for the closure of the Panama isthmus according to Bacon et al. (2015), Caribbean islands according to Ali (2012). The numbers in the bottom right correspond to Andean uplift according to Hoorn et al. (2010). The panels (A) and (B) correspond to the temporal events proposed by Hoorn et al. (2017) for the transcontinental rivers and the establishment of the Amazon fluvial system respectively. The panels (C) and (D) present the temporal events proposed in the present study, in which the first dispersals occur to Western Amazonia (Napo, Marañón, and Ucayali) and the expansion of the distributional range from Western Amazonia to Eastern Amazonia. Area labels as follows: Guiana (G), Imeri (I), Rondonia (R), Tapajos (T), Belem (B), Mata Atlantica (F), Xingu (X), Napo (N), Marañon (M), Ucayali (U), Jurua (J), Purus (P), Araguaia (A), Andes (D), Y area = Araguaia+Xingu+Tocantins+Rondonia.


A
9.4 - 9 Myr
Atlantic Ocean
Guiana Shield
Ecuador
Brazilian Shield
Pacific Ocean
N
9 Ma
B
5 - 2 Myr
Atlantic Ocean
Guiana Shield
Ecuador
Brazilian Shield
Pacific Ocean
N
4 Ma
C
16.2 - 10.4 Myr
Atlantic Ocean
Guiana Shield
Ecuador
Brazilian Shield
Pacific Ocean
N
14 Ma
D
12.2 - 6.2 Myr
Atlantic Ocean
Guiana Shield
Ecuador
I
G
N
B
U
T
X
M
J
P
R
A
Brazilian Shield
Pacific Ocean
N
9 Ma

# Figure 2

Figure 2. Infinite-sites plot for all taxa under lognormal and exponential distributions.

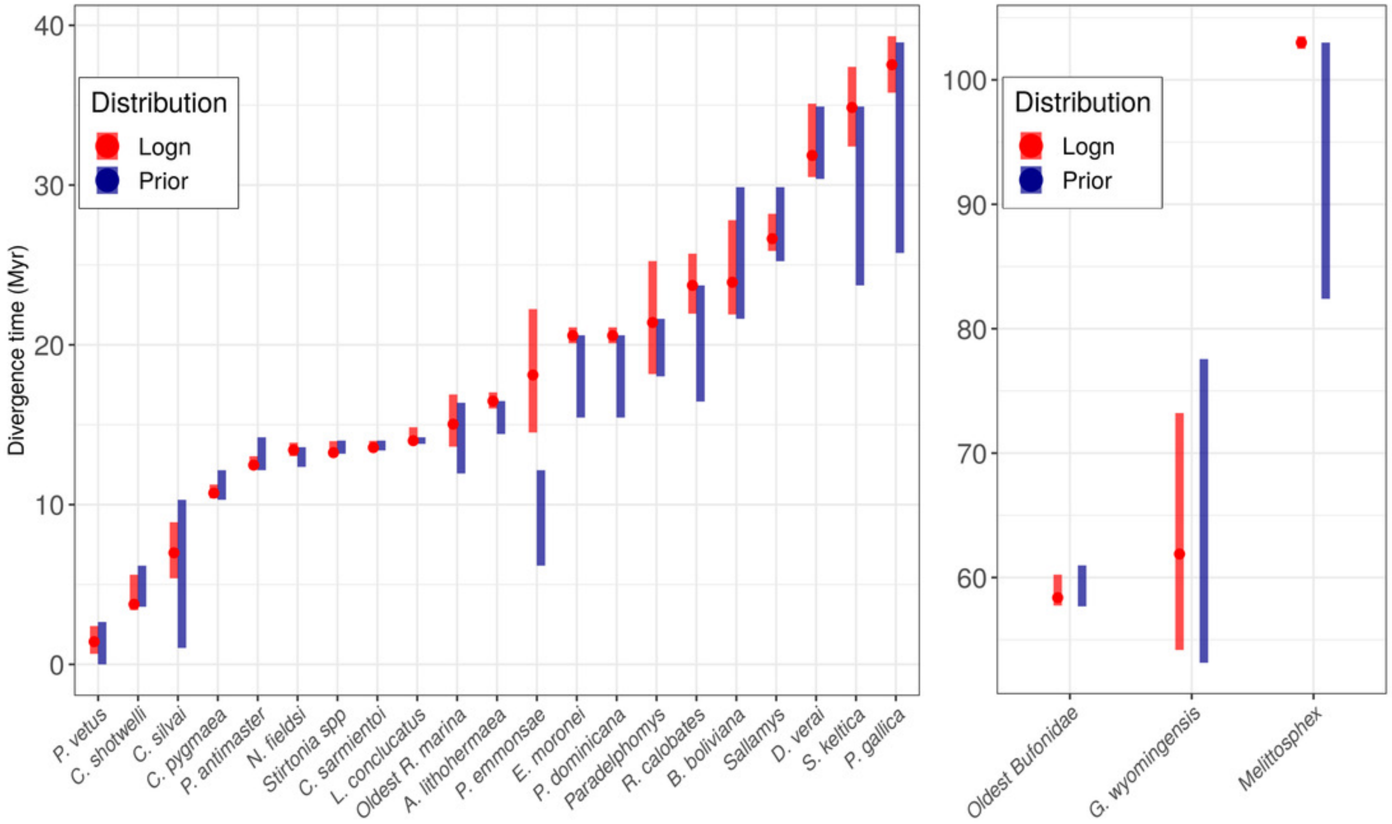
The x-axis is the posterior means of each node age, and the y-axis is the 95% posterior confidence interval (CI) width values. The slope (w) is a measure of fossil precision and represents the direct relationship between divergence time and uncertainty in the posterior CI (Yang & Rannala 2006).



# Figure 3

Figure 3. Prior age distributions and posterior age estimation for all the fossils used.

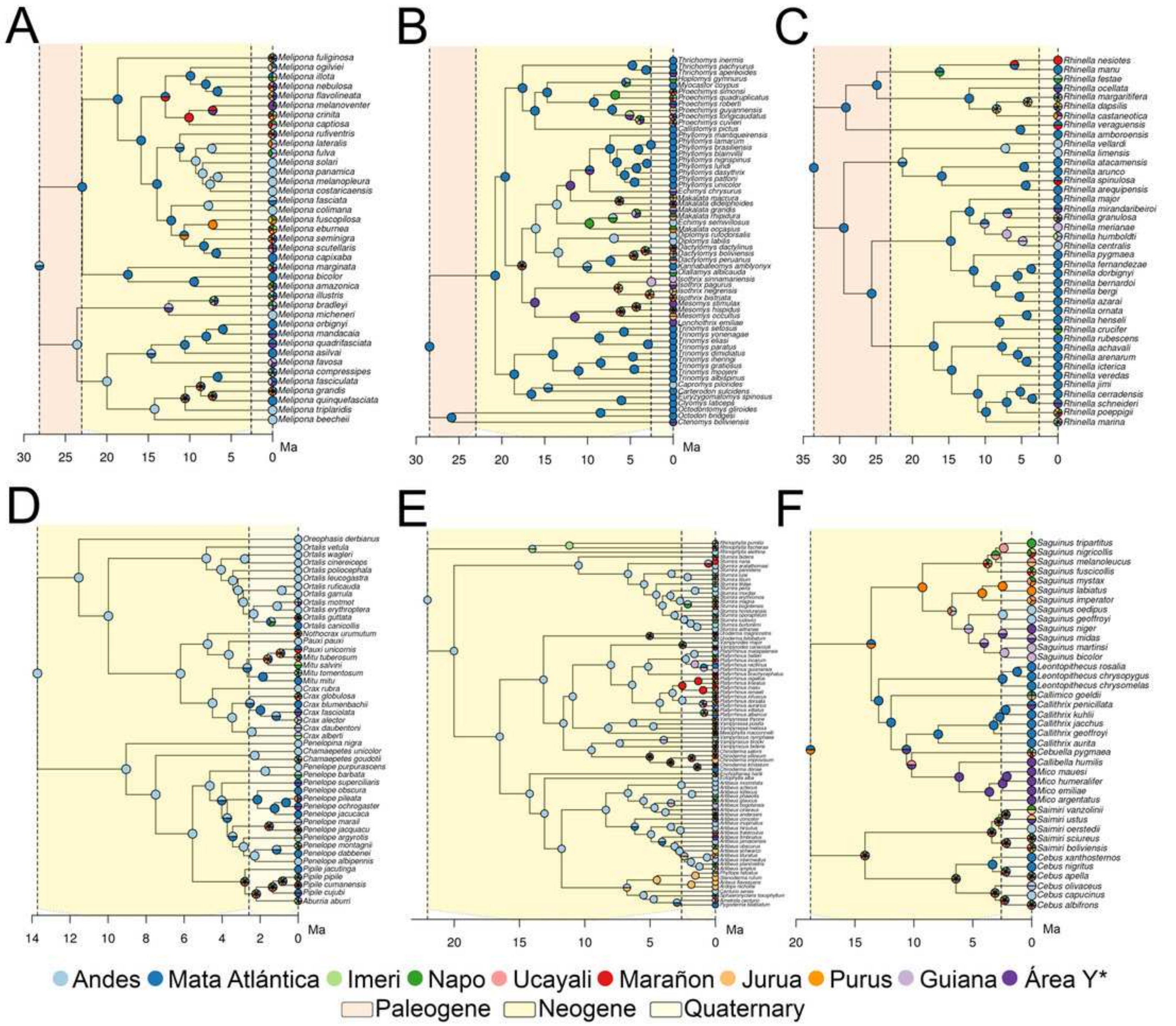
Red bars indicate the 95% posterior Confidence Interval (CI) under a lognormal distribution, and the points represent the posterior median values. Blue bars correspond to the temporal range for each prior.



# Figure 4

Figure 4.Ancestral Range Reconstruction under the model with the smallest likelihood value.

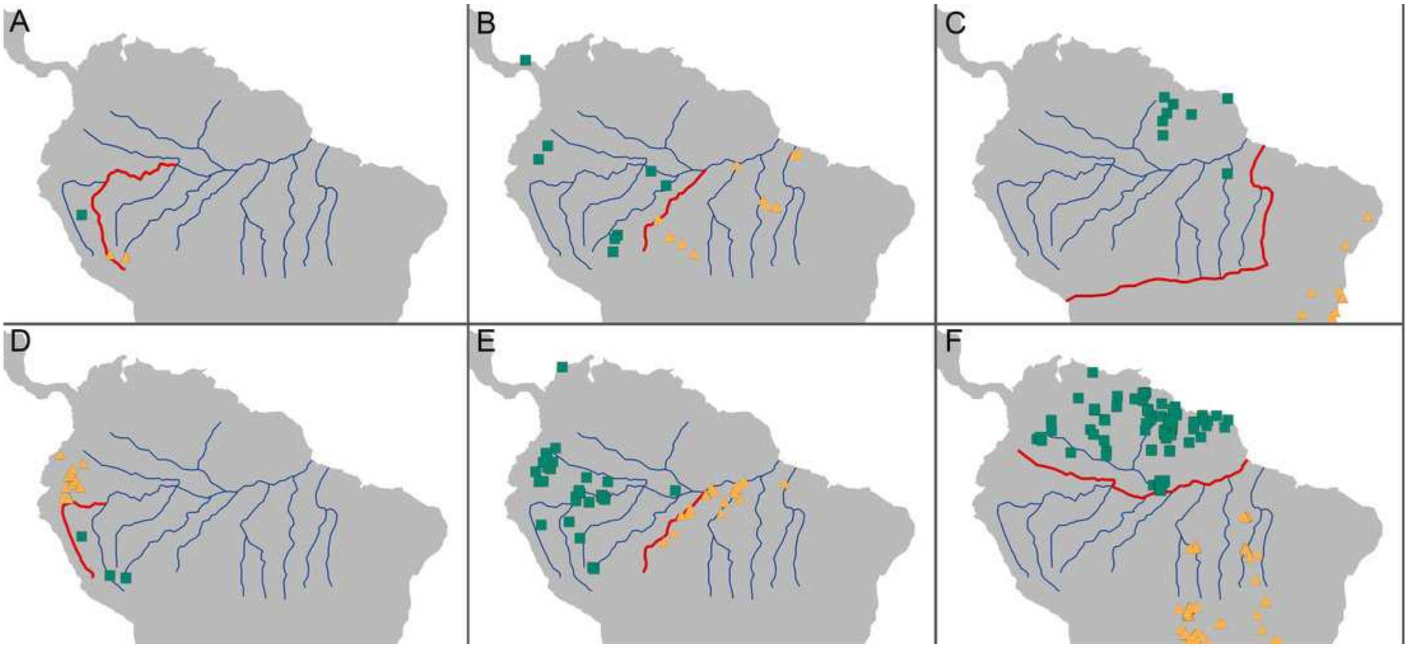
*Melipona* (A), Echimyidae (B), *Rhinella* (C), Cracidae (D), Stenodermatinae (E), and Cebidae (F). For Cracidae, Stenodermatinae, and Echimyidae the model with intermediate dispersal rates between 11.8–2.5 Ma (Million years ago) and maximal after 2.5 Ma, and for the rest of the taxa the non-stratified model. Area Y* = Araguaia+Xingu+Tocantins+Rondonia.


A
B
C
D
E
F
30 25 20 15 10 5 0 Ma
35 30 25 20 15 10 5 0 Ma
14 12 10 8 6 4 2 0 Ma
20 15 10 5 0 Ma
Andes
Mata Atlántica
Imeri
Napo
Ucayali
Marañon
Jurua
Purus
Guiana
Área Y*
Paleogene
Neogene
Quaternary

# Figure 5

Figure 5. Rivers found as biogeographical barriers for each node, as determined under Hovenkamp's context (1997, 2001).

*Rhinella*-Ucayali (A), *Melipona*-Madeira (B), Echimyidae-Tocantins (C), *Rhinella*-Marañon (D), Cebidae-Madeira (E), and Cracidae-Amazonas (F).



## Table 1(on next page)

Table 1. Groups used in the phylogenetic reconstructions.

The number of species used for each group, the percentage of those species that ocurrs in the Amazon basin and the calibration points used.

**Table 1. Groups used in the phylogenetic reconstructions.**

The number of species used for each group, the percentage of those species that ocurrs in the Amazon basin and the calibration points used.

| | Species in the phylogeny | Distributed in the Amazon basin | Calibration points |
|---|---|---|---|
| *Rhinella*<br>Fitzinger, 1826 | 41 | 37% | 2 |
| *Melipona*<br>Illiger, 1806 | 39 | 61% | 4 |
| Cracidae<br>Vigors, 1825 | 47 | 53% | 5 |
| Cebidae<br>Bonaparte, 1831 | 44 | 59% | 6 |
| Echimyidae<br>Gray, 1825 | 58 | 43% | 4 |
| Stenodermatinae<br>Gervais, 1856 | 78 | 49% | 3 |