RESEARCH PAPER

Historical biogeography identifies a possible role of Miocene wetlands in the diversification of the Amazonian rocket frogs (Aromobatidae: *Allobates*)

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Abstract

Aim: We investigate the spatiotemporal context of the diversification of *Allobates*, a widespread genus of Amazonian frogs with high species diversity particularly in western Amazonia. We tested if that diversity originated in situ or through repeated dispersals from other Amazonian areas and if this diversification took place during or after the Pebas system, a vast lacustrine system occupying most western Amazonia between 23 and 10 million years ago (Mya).

Location: Amazonia.

Taxon: *Allobates* (Anura: Aromobatidae).

Methods: We gathered a spatially and taxonomically extensive sampling of mitochondrial DNA sequences from 932 *Allobates* specimens to delimit Operational Taxonomic Units (OTUs). Complete mitogenomes of these OTUs were assembled to reconstruct a time-calibrated phylogeny used to infer the historical and spatial origin of the Amazonian *Allobates* lineages.

Results: Phylogenetic analyses and ancestral area reconstruction suggested that most of the western Amazonian lineages resulted from in situ diversification and that these events occurred between the inferred origin of the genus (25 Mya) and 10 Mya, with a possible peak between 14 and 10 Mya. Dispersal among areas mostly occurred from western Amazonia towards the Brazilian and the Guiana Shields. Closely related *Allobates* OTUs display an allopatric pattern of distribution, matching interfluves delimited by modern Amazonian rivers.

Main Conclusions: In western Amazonia, diversification of *Allobates* appears to have been simultaneous with the last stages of the Pebas system (14–10 Mya). Subsequently (within the last 10 Mya), modern Amazonian river courses shaped the distribution pattern of *Allobates* species and possibly promoted allopatric speciation.

K E Y W O R D S

anura, DNA-based species delimitation, historical biogeography, mitogenomics, Neogene, Neotropics, riverine barrier

1 | INTRODUCTION

The number of species found in the Neotropics exceeds that of the other tropical regions (Qian & Ricklefs, 2008) and the origins of this astonishing diversity have puzzled biologists for over two centuries (Wallace, 1854). Within the Neotropics, lineages of Amazonian origin frequently dispersed into other Neotropical regions, positioning Amazonia as a major source of diversity for the entire continent (Antonelli et al., 2018). However, processes responsible for diversification within Amazonia remain poorly understood. Speciation within Amazonia has been hypothesized to be linked to climatic fluctuations during the Quaternary with successive stages of forest fragmentation promoting allopatric speciation (Haffer, 1969; Hooghiemstra & van der Hammen, 1998), although recent empirical evidences tend to refute this hypothesis (Wang et al., 2017). Andean orogeny during the Neogene may also have created the conditions for in situ diversification (Hoorn et al., 2010; Santos et al., 2009) notably by modifying the hydrological system of Amazonia (Shephard, Müller, Liu, & Gurnis, 2010). Phylogenetic analyses suggest that both Neogene and Quaternary events have contributed to Neotropical

diversification, but they vary in importance across Amazonian regions and taxa (Hoorn et al., 2010; Turchetto-Zolet, Pinheiro, Salgueiro, & Palma-Silva, 2013).

During the Neogene, the Brazilian and the Guiana Shields remained geologically stable. However, Quaternary climatic fluctuations appear to have had an important influence on the biota in these regions (Fouquet, Noonan, et al., 2012; Wang et al., 2004). Western Amazonia, in contrast, underwent profound landscape changes during the Neogene (Hoorn et al., 2010; Shephard et al., 2010). One of these major transformations is related to the formation of the Pebas system, a freshwater system the size of Western Europe initially connected to the Caribbean Sea. The Pebas system supposedly occupied most of western Amazonia during a period ranging from early Miocene (23 Mya) to around 10–9 Mya, when this system started to be drained eastward into the Atlantic Ocean (Albert, Val, & Hoorn, 2018). Subsequent hydrological changes have frequently taken place during the Pliocene and the Pleistocene as well, particularly in western Amazonia (Pupim et al., 2019; Rossetti et al., 2015), with various consequences on Amazonian diversification, such as promoting allopatric speciation or favoring dispersals (Albert et al., 2018). These different temporal and spatial contexts may have

had distinctive contributions to the diversification of Amazonian flora and fauna. Moreover, different taxonomic groups with distinct life history traits are likely to have responded differently to the dramatic landscape changes (Paz, Ibáñez, Lips, & Crawford, 2015).

Continued scientific exploration of Amazonia and large international collaborative efforts have made samples available throughout Amazonia and shed light on the highly taxon-specific patterns of diversification (e.g. Fouquet, Loebmann, et al., 2012). For example, many biogeographical studies within Amazonia have focused on birds, arguably the best-known group of vertebrates in terms of Amazonian biogeography. The consensus emerging from these studies is that Amazonian bird diversification patterns throughout Amazonia during the last 6 Mya have resulted from a complex interplay of vicariance and dispersals events, promoted by Amazonian rivers (Smith et al., 2014) and ecological gradients (Silvia et al., 2019). Bird lineages diversified rapidly throughout Amazonia, while similarly distributed frog lineages display much older crown ages, suggesting that drivers promoting these diversifications might be different (Antonelli et al., 2010; Rull, 2011).

Several studies that have sought to unravel the phylogenetic relationships among Neotropical anurans have identified certain clades that started to diversify within Amazonia during early Neogene (Fouquet, Cassini, Haddad, Pech, & Rodrigues, 2014; Fouquet, Loebmann, et al., 2012; Fouquet, Recoder, et al., 2012; Santos et al., 2009). Here, we focus on one of these clades, the genus *Allobates* (Anura: Aromobatidae), a group of small-bodied, diurnal and territorial frogs that deposit their clutches in the forest leaf litter. This genus contains 55 recognized species distributed mostly in Amazonia but occurring also in the Atlantic Forest, Cerrado, Northern Andes and trans-Andean lowland forests. With 25 described species occurring in Western Amazonia, this region seems to harbour more species (25 species) than the Brazilian (11 species) and the Guiana Shields (six species; Table S1; Frost, 2019; IUCN, 2019). These numbers are probably underestimated because recent molecular phylogenetic studies suggest that many species still await formal description (e.g. Melo-Sampaio et al., 2020). Given their habitat and their reproductive biology, *Allobates* species have limited dispersal abilities, which is consistent with marked range boundaries and the profound intraspecific genetic structure documented for several species at regional scale, notably throughout large rivers (Kaefer, Tsuji-Nishikido, Mota, Farias, & Lima, 2013; Maia, Lima, & Kaefer, 2017).

Previous phylogenetic studies proposed a crown age of approximately 26 Million years (My) (CI 95%: 18–35 My) for *Allobates* (excluding the *A. olfersioides* clade; Santos et al., 2009). Subsequent studies improved taxonomic coverage but left virtually unexplored the temporal and spatial context of *Allobates* diversification within Amazonia (e.g. Melo-Sampaio et al., 2020). We speculate that *Allobates* diversification was likely influenced by the important hydrological and geomorphological changes that took place especially in western Amazonia. These changes were related to the existence of the Pebas system until 10 Mya and subsequently to the dynamic river courses (e.g. Albert et al., 2018; Wesselingh et al., 2001). Before 10 Mya, we hypothesize that the Pebas system could have either fostered diversification by providing a mosaic of fragmented habitats or prevented it by creating unsuitable habitats. The subsequent transformation of

western Amazonia may have not only provided opportunity for in situ diversification but also for dispersals from the Brazilian Shield and Guiana Shield towards western Amazonia. In this complex biogeographical context, we aim to test whether the higher *Allobates* diversity in western Amazonia results predominantly from: (1) dispersals from neighbouring provinces; (2) in situ diversification or (3) a combination of both processes. We also investigate whether most of *Allobates* Amazonian diversification preceded the transition from the Pebas to the modern Amazonian hydrological system (14–10 Mya), post-dated this event or was continuous throughout the Neogene. To answer these questions, we evaluated boundaries among *Allobates* species, reconstructed a time-calibrated phylogeny of the genus and estimated patterns of diversification through time and space.

2 | MATERIALS AND METHODS

2.1 | Species delimitation

Our first objective was to delimit all major mitochondrial DNA (mtDNA) lineages. Our sampling included 73 *Allobates* tissue samples, obtained through fieldwork and loans from collaborators. We sequenced a fragment of the 16S rDNA region, a classical marker for Neotropical amphibian taxonomy and systematics (Vences, Thomas, van der Meijden, Chiari, & Vieites, 2005). We also retrieved homologous sequences from GenBank (859 accessions). In total, we obtained 932 16S mitochondrial sequences for this study (sequencing protocols can be found in Appendix S1 and database details are provided in Table S2). These samples span the entire range of the genus *Allobates*. DNA sequence alignment was conducted on the MAFFT online server under the E-INS-i option with default parameters, which is designed for sequences with multiple conserved domains and long gaps (Katoh, Rozewicki, & Yamada, 2017).

We first clustered DNA sequences into taxonomic units, which may represent valid species. To this end, we applied three DNA-based single-locus species delimitation approaches: (a) a distance-based method, the Automated Barcode Gap Discovery (ABGD; Puillandre, Lambert, Brouillet, & Achaz, 2012); (b) a single-threshold coalescent-based method, the Generalized Mixed Yule Coalescent approach (single-threshold GMYC; Monaghan et al., 2009; Pons et al., 2006) and (c) a multi-rate coalescent-based method, the multi-rate Poisson Tree Processes model approach (mPTP; Kapli et al., 2017). Operational Taxonomic Units (OTUs) were defined using a majority rule consensus from the results of the three methods, i.e. a lineage is considered as being an OTU if supported by at least two of the three methods. Details of the species delimitation analyses are provided in Appendix S1.

2.2 | Time-calibrated species phylogeny

We selected one representative for each OTU ($n = 50$, see Results) for complete mitogenome sequencing in order to infer the topology and obtain time-calibrated relationships. These mitogenomic

sequences were obtained through low-coverage shotgun sequencing. They were combined with available mtDNA sequences in GenBank for OTU for which we could not obtain tissue samples (see database details in Table S2).

To reconstruct a phylogenetic tree, we recovered mitochondrial genome assemblies for 32 OTU representatives (see Appendix S2 for details regarding mitogenomes sequencing, assembling and annotation). We then extracted 12S, 16S and all coding DNA sequences (CDS), thus removing D-loop and tRNAs from complete mitogenomes. For the remaining 18 OTUs, we gathered all the available mitochondrial DNA loci (12S, 16S, ND1, COI and Cytb) from GenBank; six of these OTUs were represented by the 16S region only (12%). We also selected 10 species as outgroups (three with complete mitogenomes), including one for each genus of Aromobatidae and six genera within Dendrobatidae, the sister clade of Aromobatidae (Table S3).

We then aligned each locus independently using the MAFFT7 online server. For rRNA genes we chose the E-INS-i strategy, which is designed for sequences with multiple conserved domain and long gaps, while for the protein-coding genes, we chose the G-INS-i strategy, recommended for sequences with global homology (Katoh et al., 2017). Coding regions were realigned considering reading frame and concatenated in Geneious V9.1.8.

We selected the best-fit partition scheme and model of evolution for each partition using PartitionFinder V2.1.1 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2016), according to the Bayesian Information Criterion (BIC). We predefined four data blocks, one for rRNA genes (12S and 16S) and one for each codon position of concatenated CDS regions.

We reconstructed a time-calibrated gene tree using a birth–death tree prior in BEAST 2.5 that accounts for extinction processes and incomplete sampling. We parametrized unlinked substitution models according to the estimates obtained in the PartitionFinder V2.1.1 analysis (Lanfear et al., 2016). Time calibration was implemented using an uncorrelated relaxed lognormal clock model of the distribution of rates among branches for each partition (Drummond, Ho, Phillips, & Rambaut, 2006). In the absence of a Dendrobatoidea fossil record, we relied on a secondary calibration point. The alternative, to expand the matrix to distantly related lineages with fossil records, may lead to an overestimation of calibration dates (Molak & Ho, 2015; Papadopoulou, Anastasiou, & Vogler, 2010). We based our secondary calibration on the results of Feng et al. (2017), whom relied on an extensive nuclear genomic dataset (88 kb) of anurans including all major frog lineages and inferring estimates for the crown age of Dendrobatoidea. We consequently constrained our analysis based on the Feng et al. (2017) estimates. Specifically, we assumed a normal prior distribution (Mean = 34.6 Mya, SD = 3 Mya), a time range consistent with several other studies (i.e. Hedges, Marin, Suleski, Paymer, & Kumar, 2015; Roelants, Haas, & Bossuyt, 2011). We acknowledge that our phylogenetic reconstruction is based only on mtDNA sequences only and can lead to an overestimated speciation rate (e.g. McCormack, Heled, Delaney, Peterson, & Knowles, 2011; Near et al., 2012). We emphasize that future work, e.g. using nuclear genomic approaches, may lead to slightly different time estimates. As the availability of material remains challenging, gathering nuDNA data for all the terminals included herein was out of reach. However, we could prioritize spatial and taxonomic completeness over genomic coverage.

In the BEAST2 tree reconstruction, the Markov chain Monte Carlo (MCMC) parameters were set to four independent runs of 100 million iterations each, recording every 10,000th iteration and discarding the first 10% of iterations as burn-in. We combined the log files of the independent runs using LogCombiner 2.5 (Bouckaert et al., 2014) and checked the convergence of our parameters via time-series plots and confirming that all parameters achieved an effective sample size above 200. We extracted the maximum clade credibility tree using Tree annotator 2.5 (Bouckaert et al., 2014).

2.3 | Biogeographical analysis

Biogeographical inferences assumed the resulting time-calibrated phylogeny and were conducted using the BioGeoBEARS R package (Matzke, 2013). BioGeoBEARS infers the geographical distributions of ancestral species and explores for each node the relative probabilities of potential biogeographical events based on a maximum likelihood algorithm. We compared three models: (i) a likelihood version of the Dispersal-Vicariance model (DIVALIKE; Ronquist, 1997) which would be selected in case of a prevalence of vicariance events, such as newly formed riverine barriers; (ii) a likelihood version of the BayArea (BBM) model (Landis, Matzke, Moore, & Huelsenbeck, 2013) that would be favoured in case of prevalence of in situ diversification within biogeographical areas, such as a radiation in newly formed environments; and (iii) the Dispersal Extinction Cladogenesis model (DEC; Ree & Smith, 2008) which would be favoured if both vicariance and in situ diversification occurred. We also compared versions of these models allowing jump dispersal as described by the J parameter (Matzke, 2013), although there is an ongoing debate around the use of this parameter (Klaus & Matzke, 2020; Ree & Sanmartín, 2018). We modified the list of geographical ranges in order to forbid state transitions between non-adjacent areas. Models were compared with the Akaike Information Criterion (AIC). We ran the BioGeoBEARS biogeographical stochastic mapping 50 times to determine biogeographical event counts for the best-fit model (Dupin et al., 2017).

To identify processes at different spatiotemporal scales, we adopted two contrasting biogeographical partitioning schemes that differed within Amazonia but similarly included five non-Amazonian Neotropical areas: trans-Andean forests (Tf), northern Andes (nA), dry diagonal (DD), Atlantic forest (Af) and Lesser Antilles (lA).

The first Amazonian partitioning differentiates major geological features within Amazonia: western Amazonia, the Brazilian Shield and the Guiana Shield; for a total of eight Neotropical areas (referred hereafter as the 8A analysis). The western Amazonia underwent dynamic hydrological and geological changes throughout Neogene, while the Brazilian and Guiana Shields remained more stable (Albert et al., 2018; Hoorn et al., 2010). Boundaries for these three Amazonian areas are roughly delimited by modern riverine

barriers: the Madeira River, the Negro River and the lower course of the Amazon River and also correspond to the large biogeographical regions known as Wallace's districts (Hoorn et al., 2010; Wallace, 1854). These districts were recently confirmed as major breaks of species composition in bird (Oliveira, Vasconcelos, & Santos, 2017) and amphibians (Godinho & da Silva, 2018), strengthening their status of biogeographical region. This spatial partitioning of Amazonia will be used to investigate the East/West and North/South divergences expected during early Neogene diversification of *Allobates* species according to their distributions and phylogenetic relationships previously inferred (Melo-Sampaio et al., 2020).

The second Amazonian partitioning is based on the major Amazonian interfluves, recognized as the nine Amazonian areas of endemism (AAE) by Cracraft (1985). They have been used in many other studies investigating the historical biogeography of taxa that diversified during late Neogene notably because of riverine barriers (e.g. Smith et al., 2014). We used this partitioning to identify patterns of diversification within the previously described larger Amazonian areas (8A analysis). In this second scheme, following Smith et al. (2014), western Amazonia is subdivided into four areas (Napo, Inambari, Huallaga and Imeri) as well as the Brazilian Shield (Rondônia, Tapajos, Xingu and Belém), for a total of 14 Neotropical areas (referred hereafter as the 14A analysis; see Appendix S3 for additional details on area boundaries).

2.4 | Diversification through time

To investigate diversification through time in *Allobates*, we used two complementary approaches. First, we tested whether diversification of *Allobates* varied through time and was significantly different from a Yule pure-birth model accounting for randomly distributed missing taxa, using a Monte Carlo Constant Rate (MCCR) test implemented in the package LASER V2.4.1 (Pybus & Harvey, 2000; Rabosky, 2006).

Once we assured that diversification of *Allobates* varied through time, we explored this variation by fitting seven models of diversification through time using the same package. They include two constant-rate models (pure-birth and birth-death), two density-dependent models (DDX and DDL) and three variants of the Yule-n-rate model (respectively, with two, three and four speciation rates categories across the tree). Adjustments of these models were compared with the Akaike Information Criterion (AIC).

Finally, we computed a lineage through time (LTT) plot using APE V5.3 package and compared it to a Yule pure-birth model with a 95% CI (Paradis, Claude, & Strimmer, 2004), to confirm whether and when *Allobates* diversification varied through time.

3 | RESULTS

3.1 | Species delimitation

Of the three tested methods of species delimitation, the mPTP method was found to be the most conservative, delimiting 45 candidate species while GMYC and ABGD delimited 51 and 66 candidate species respectively. Results were largely congruent among methods, as expected from previous studies (Paz & Crawford, 2012). The majority rule consensus of the three methods led to the delimitation of 50 OTUs in the genus *Allobates*. Closely related OTUs are rarely spatially overlapping (Fig. S1) and these allopatric ranges are generally separated by major Amazonian rivers. Following OTU delimitation we confirmed that the diversity in Amazonian *Allobates* species is heterogeneously distributed, having a higher diversity in western Amazonia (22 OTUs) than in the Brazilian Shield (15 OTUs) and the Guiana Shield (9 OTUs; Table S1). Results of species assignation for each method are available in Table S2 and plotted in Fig. S2.

3.2 | Time-calibrated species phylogeny

Our final matrix totalized 60 taxa and 14,167 aligned nucleotide sites. Among these taxa, 33 were complete and 49 comprised at least 3,000 nucleotides. The inferred mitogenome-based phylogenetic tree had clade posterior probabilities above 0.95 for 53 of the 59 nodes. We estimated the crown age of *Allobates* to be 28.8 My (CI 95%: 23.3–34.2 Mya). The genus comprises one Atlantic forest clade (*A. olfersioides*) with an onset of diversification at 16.1 Mya (CI 95%: 10.8–24.5 Mya) and its sister clade encompassing all other Amazonian *Allobates* species, which started to diversify 25.6 Mya (CI 95%: 20.8–30.5 Mya) (Table S4; Figure S3). The topology was consistent with the supported relationships recovered by Melo-Sampaio et al. (2020), except for the placement of *A. granti*. In our analysis, *A. tinae* is the supported sister species of a supported clade formed by *A. granti* + *A.* aff. *granti* (Figure 1a–c).

3.3 | Ancestral area reconstructions

Model comparisons identified DEC + J as the best model for each geographical partitioning (Tables S5 & S6), suggesting that vicariance, sympatry (within our areas) and jump dispersal played a role in the history of diversification in *Allobates*. The ancestral range of the most recent common ancestor (MRCA) of all extant *Allobates* was ambiguous (Figure 1a), as all options included two or more areas, but most included the Guiana Shield. Initial diversification of Amazonian *Allobates* was inferred to have taken place in the Guiana Shield, around 25 Mya (Figure 1a). Around 22 Mya, Amazonian and trans-Amazonian sister clades (Chocó + Central America) diverged. This Amazonian clade encompasses four large clades, as follows. The *A. masniger* clade likely initiated its diversification in the Brazilian Shield around 17 Mya (Figure 1a). A major *Allobates* group, the *A. trilineatus* clade, comprising 21 OTUs, formed around 14 Mya in western Amazonia (Figure 1a). The origin of the *A. caerulodactylus* clade was uncertain in western Amazonia, the Brazilian Shield or both (Figure 1a). Finally, we recovered a
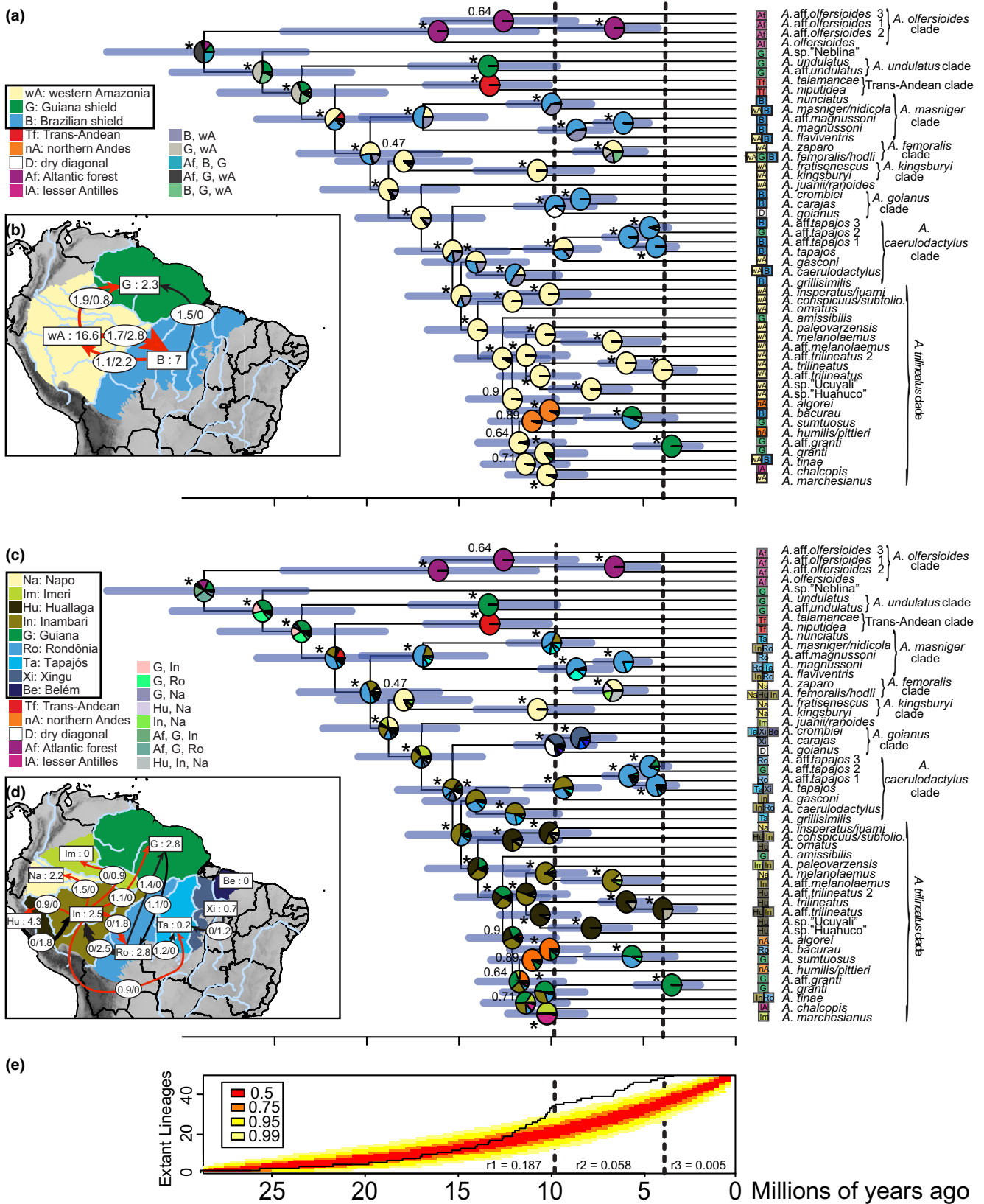
poorly supported node comprising the *A. kingsburyi* and *A. femoralis* clades, with a crown age of 18 Mya and possibly originating from western Amazonia (Figure 1a).

From the 8A analysis we inferred a majority of in situ speciation events, western Amazonia had an average of 16.6 speciation events; versus in average only 7 and 2.3 of such events within the

FIGURE 1 Ancestral area reconstruction of the genus *Allobates* using BioGeoBears assuming a DEC + J model, with: a) and c) being most likely biogeographical scenarios plotted on the Maximum clade credibility chronogram obtained with BEAST 2.5, for the 8 Neotropical biogeographical areas and the 14 Neotropical biogeographical areas respectively; with b) and d) being summaries of the 50 biogeographical stochastic mappings (BSMs) for the 8 Neotropical biogeographical areas and the 14 Neotropical biogeographical areas respectively; and e) lineage through time (LTT) plot performed on the same tree. Numbers on branches are posterior clade probabilities, those ≥ 0.95 are indicated with an asterisk. Node bars indicate the 95% highest posterior distributions of node dates. Squares on the tips of the trees indicate the geographical distribution of extant species sampled in the phylogeny. Pie charts on nodes show the most likely reconstructions of ancestral areas, the size of each slice being proportional to the maximum likelihood. Colours corresponding to the different geographical distributions are depicted on the left. Black dotted lines represent changes in diversification rate through time according to the Yule three-rate model. In the summaries of the BSMs results, we present values of the within-area speciation events for each region (values within rectangles) as well as the anagenetic dispersal events and founder speciation events between each region (values within ellipses). Values correspond to the mean number of inferred events of a specific type across the 50 independent BSMs. Anagenetic dispersals events and founder events are represented only for intra-Amazonian events with a mean of > 0.8. Red arrows highlight dispersals emitted by major dispersal hubs. The different colour ranges on the LTT plot indicates different confidence intervals of lineage accumulation through time predicted by a Yule pure-birth diversification model where the black line represents empirical data. Dotted vertical black lines indicate the dates of mean diversification rate changes according to the Yule three-rate model, and associated diversification rates are displayed on top of the LTT plot

Brazilian Shield and the Guiana Shield respectively (Figure 1b). At a finer scale it appears that in situ diversification occurred notably along the Andean foothills within western Amazonia as well as in the Rondônia area within the Brazilian Shield (Figure 1d). In the 8A analysis, we recovered few dispersal events towards western Amazonia from the Brazilian Shield (1.1 founder events, or FE, and 2.2 anagenetic dispersals, or AD, on average) and none from the Guiana Shield (Figure 1b). Similarly, few dispersal events occurred from western Amazonia towards the Guiana Shield (FE = 1.9; AD = 0.8) and Brazilian Shield (FE = 1.7; AD = 2.8), as well as from the Brazilian Shield towards the Guiana Shield (FE = 1.5; Figure 1b). At a finer scale, it appears that the Inambari and Rondônia areas played a major role as hubs of Amazonian dispersal with, respectively, a total of 17.6 and 13.2 dispersal events emitted/received on average, twice as much as other areas (Table S7).

3.4 | Diversification through time

The MCCR test suggested an overall decrease in diversification rate across time ($\gamma = -5.429$, *p*-value $= 2.831 \times 10^{-8}$). The diversification through time model that best fit *Allobates* phylogenetic reconstruction is the Yule pure-birth three-rate model, with a high diversification rate until 10 Mya, followed by a decrease (Table S8, Figure 1c). The second-best model was a Yule pure-birth four-rate model that further detected a diversification peak around 10 Mya (Table S8). The LTT plot illustrates this rapid accumulation of lineages at approximatively 10 Mya (Figure 1c).

4 | DISCUSSION

As discussed below, the results presented here are consistent with the role of landscape changes in western Amazonia in the rapid diversification of the genus *Allobates* before 10 Mya. After 10 Mya, diversification rate decreased and a complex combination of dispersal and isolation throughout rivers as well as ecological opportunities

seem to have played a major role in *Allobates* diversification, paralleling the situation found in birds (Silva et al., 2019; Smith et al., 2014). Although the lack of fossils of aromobatids and the use of mitogenomic data alone (see Material and Methods) limit the accuracy of time estimates, our study still represents one of the most thorough in terms of spatial and taxonomic sampling of a vertebrate clade in lowland Amazonia. As such, our results contribute to the understanding of diversification in the Amazonian lowlands.

4.1 | Species delimitation and distribution pattern

We used 16S DNA sequences of 932 specimens to delimit major extant lineages within *Allobates* (Table S2). We identified 50 OTUs and confirmed that *Allobates* species richness is higher in western Amazonia than in the rest of the region (Table S1; Frost, 2019; IUCN, 2019).

Although our delimitation clarified some species boundaries, *Allobates* diversity remains undersampled. Considering that we included 41 of the 55 currently recognized taxa, we estimate that the genus may approach 70 species, which represents 21% of missing diversity. This figure still likely represents an underestimation, as most *Allobates* species have small ranges and large parts of Amazonia remain poorly sampled (Mayer, da Fonte, & Lötters, 2019). Additional information on reproductive isolation, phenotypic and genomic variation is needed to evaluate if our proposed OTUs correspond to species on independent evolutionary trajectories (Hickerson, Meyer, & Moritz, 2006). Nevertheless, we are confident that our study will contribute to foster such extended studies. We briefly refer to the taxonomic implications of our results in Appendix S1.

4.2 | In situ western Amazonian diversification during the Pebas system era

The early diverging lineages of *Allobates* are distributed in the Brazilian Shield, with the *A. masniger* clade, and along the Andean foothills with the *A. femoralis* and *A. kingsbury* clades. Geographical

origins of lineages arising between 20 and 15 Mya remain largely ambiguous as well as the potential number of vicariance or dispersal events. Nevertheless, the current spatial distribution of these early diverging lineages suggests that during early Miocene the genus had a disjunct distribution with a gap in the western Amazonian lowlands between the Andean foothills and the Brazilian Shield. Moreover, biogeographical inferences suggest a large amount of in situ diversification in western Amazonia (8A; Figure 1b). In contrast, we identified only few dispersal events from the Brazilian Shield towards western Amazonia and none from the Guiana Shield during the same period (8A; Figure 1b). Complementary biogeographical inferences considering 14 Neotropical areas (14A) suggest that central Andean foothills (particularly Huallaga) acted as a centre of diversification for *Allobates* (similar findings were obtained in different frog taxa; e.g. Guayasamin et al., 2017; Santos et al., 2009). Furthermore, the Andean foothills remained largely unflooded during the Neogene (Shephard et al., 2010), while a large part of western Amazonia was likely unsuitable for most terrestrial organisms and likely endured high extinction rates and species turnover following the setup of the Pebas system around 23 Mya (Antoine, Salas-Gismondi, Pujos, Ganerød, & Marivaux, 2017). Consequently, the central Andean foothills might have acted as a refuge during the Pebas system era for *Allobates* (~23–10 Mya). These findings also suggest that the Pebas system prevented *Allobates* diversification in western Amazonia by constituting an unsuitable habitat.

Most of the in situ diversification in western Amazonia occurred between 14 and 10 Mya and is highlighted by a rapid accumulation of lineages in the *A. trilineatus* clade, which was followed by a decrease in diversification rate (Figure 1a–c). In the *A. trilineatus* clade, this 14–10 Mya period encompasses 14 of 20 nodes, among which 12 correspond to western Amazonian in situ speciation events (Figure 1a). This represents approximately 72% of the total western Amazonian in situ speciation events inferred by our analysis (Figure 1a,b). This 14–10 Mya period coincides with the last phase of the Pebas system that led to the formation of new *terra firme* habitats, as well as the setup of the modern Amazon watershed (Albert et al., 2018; Boonstra, Ramos, Lammertsma, Antoine, & Hoorn, 2015). We speculate that this period might have promoted speciation through an increased availability and diversity of habitats and niches (see below) and reconfiguration of riverine barriers (Hoorn et al., 2010; Rull, 2011). Previous studies suggested that the Pebas system might also have fostered the diversification in other amphibians through similar processes (e.g. Waddell, Crotti, Lougheed, Cannatella, & Elmer, 2018).

4.3 | Allopatric speciation as a driver of diversification

Given the drastic hydrological reconfiguration of western Amazonia during the 14–10 Mya period, i.e. the transition from the Pebas system to the modern Amazon drainage (Hoorn et al., 2010), the role of rivers as dispersal barriers in the context of the more ancient history

of the genus *Allobates* remains unclear. Many *Allobates* species ranges are currently circumscribed to single major interfluves, suggesting that rivers may act as barriers, limiting the distribution ranges of species pointing to the role of rivers in the recent diversification of the genus (Kaefer et al., 2013; Moraes, Pavan, Barros, & Ribas, 2016). The lower course of the Amazon River is among these major disjunctions, with related species facing each other across the river within the *A. caerulodactylus* clade (*A.* aff. *tapajos* 3 vs. *A.* aff. *tapajos* 2) and the *A. trilineatus* clade (*A. granti* + *A.* aff. *granti* vs. *A. tinae*; *A. bacurau* vs. *A. sumtuosus*) (Figure 1c). Conversely, we observe that western Amazonian rivers appear to play a modest role as barriers, a pattern already suggested by the classic phylogeographical study on *Allobates femoralis* (Lougheed, Gascon, Jones, Bogart, & Boag, 1999). Many species are found on both sides of the Madeira River, for example, which marks the transition between the Brazilian Shield and western Amazonia (*A. caerulodactylus*, *A. flaviventris*, *A. masniger/nidicola*, *A. paleovarzensis*, *A. conspicuus/subfolionidificans* and *A. femoralis/hodli*; Figure 1a). In addition, we recovered numerous dispersal events from and towards the two interfluves bordered by the Madeira River (Table S8). Courses of western Amazonian rivers, such as the Juruá and Purús Rivers, have high sediment loads that increase river meandering rates, making them highly dynamic compared to the more channelled rivers with lower sediment loads running on the cratons (Tapajos, Xingu, Tocantins and Negro) (Constantine, Dunne, Ahmed, Legleiter, & Lazarus, 2014). These characteristics might partly explain why these western Amazonian rivers have been more permeable than cratonic rivers, whose courses have remained much more stable over time (Albert et al., 2018). Quaternary events of river avulsions and captures in the Amazon drainage may help explain part of the dispersal and species accumulation in particular interfluves (Pupim et al., 2019; Rosseti et al., 2015). Overall, the major Amazonian rivers seem to have acted as semi-permeable barriers for *Allobates*, allowing dispersal and isolation among neighbouring interfluves, as reported for other taxa (Naka & Brumfield, 2018; Pirani et al., 2019).

5 | CONCLUSION

In *Allobates*, western Amazonia acted as a source of diversity for the rest of Amazonia, and did so mostly before the end of the Pebas system. Subsequently (last 10 My), riverine semi-permeable barriers may have been the main cause of speciation. This study is among only a few to document a history of diversification spanning the last 23 My of the Neogene within Amazonia. The prevalence of the roles of geological and hydrological changes throughout Neogene in driving the diversification of many other organisms within Amazonia remains to be investigated.

DATA AVAILABILITY STATEMENT

Newly generated DNA sequences are available on GenBank under the accessions MT524105-MT524178 for 16S and MT627173-MT627207 for mitogenomes. Input and output files, as well as scripts used for the phylogenetic reconstruction and the biogeographical inferences are available on Dryad https://doi.org/10.5061/dryad.79cnp5hsg.

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BIOSKETCH

Alexandre Réjaud is a PhD student broadly interested in diversification, biogeography and evolution. The research team consists of herpetologists who are interested in amphibian diversity and the evolutionary processes generating Amazonian diversification.

Author contributions: A.R., J.C. and A.F conceived the study. A.R., M.T.R., A.J.C., S.C.F., A.F.J., J.C.C., F.G., G.L.A.G–U., J.M., I.J.D.R., P.P., A.P.L., F.P.W., T.H., S.R.R., R.E., P.J.R.K. and A.F. conducted sampling. A.R, A.J.C, S.C.F, A.D and A.F. generated DNA sequence data. A.R. analysed the data and wrote the article; and all authors commented on the final draft.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.