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A complex biogeographic history of diversification in Neotropical lancehead pitvipers (Serpentes, Viperidae)

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Abstract

Based on the literature, we had predicted that the diversification within the Neotropical snake genus *Bothrops* occurred along a latitudinal gradient from north to south, with diversification into unoccupied niches through ecological opportunity, not correlated with geoclimatic events. Using a dated phylogeny and estimating likelihoods of ancestral states at cladogenesis events, we reconstructed ancestral areas and assessed major events of the diversification of *Bothrops* clades, and we also discuss systematic implications for this group. Based on the phylogeny we produced, *B. lojanus* was not considered as part of the genus *Bothrops* since the results recovered this species nested within the *Bothrocophias* clade. We infer that the diversification of the Miocene *Bothrops pictus* and *Bothrops alternatus* clades may be related to the uplift of the western slopes of the Andes and the Argentinian Patagonian Andes, respectively. The Pliocene *Bothrops taeniatus* and *Bothrops osbornei* clades may be related to the uplift of the eastern and northern Andes, respectively. The Plio-Pleistocene *Bothrops neuwiedi* clade may be related to the habitat transitions from a warmer and forested environment to a cooler and open landscape; the *Bothrops jararaca* (i.e. island endemic species) and *Bothrops lanceolatus* clades to over-water dispersal with island speciation; and *Bothrops atrox* clade to the appearance of the Panamanian land bridge. We found that a multitemporal and multidirectional history of diversification may be correlated with geoclimatic and dispersalist events. We argue that the vacant niche hypothesis by itself does not explain *Bothrops* diversification.

KEYWORDS
distribution, diversification, ecological opportunities, molecular phylogeny, Neotropical region, patterns of speciation

1 | INTRODUCTION

The Neotropical region, which corresponds to most of South America, Central America, southern and central Mexico, and the Antilles (Morrone, 2014), comprises the largest biodiversity on Earth (Antonelli & Sanmartín, 2011; Myers, Mittermeier, Mittermeier, Fonseca, & Kent, 2000). Some of the hypotheses on diversification in the Neotropics assert it was due to allopatric speciation and ecological displacement due to the uplift of mountain ranges (Antonelli, Nylander, Persson, & Sanmartín, 2009), to rivers that have acted as prezygotic barrier (Wallace, 1852), to habitats fragmented through Pleistocenic climatic changes (Haffer, 1969), or to a continuous diversification trend, without significant differences between Paleogene, Neogene and Quaternary speciation rates (Bacon, Molnar, Antonelli, Crawford, Montes & Vallejo-Pareja, 2016; Hoorn et al., 2010; Rull, 2011). Finally, some hypotheses argue that intrinsic ecophysiological barriers such as temperature for ectothermic vertebrates (e.g. Hamdan, Pereira, Loss-Oliveira, Rödder, & Schrago, 2017), and rapid diversification due to radiation into niches that were largely unoccupied (Wüster, Salomão, Quijada-Mascareñas, Thorpe, & BBBSP, 2002) contributed to this diversification. The last one implies that when colonizing a habitat with vacant niches, lineages may respond to this "ecological opportunity" by diversifying into several daughter species, each occupying different parts of the ecological space (Schluter, 2000).

Despite numerous attempts to determine the role of historical and ecological factors in the diversification, evolutionary studies of Neotropical groups are still needed to provide a comprehensive picture of the origin of the biodiversity (Lohmann, Bell, Calió, & Winkworth, 2013). Many of these studies focus on groups that are geographically or ecologically restricted (Guarnizo et al., 2016; Smith et al., 2014; Werneck, Leite, Geurgas, & Rodrigues, 2015). Study systems spanning the Neotropics across numerous ecological settings provide a greater ability to test these broad models of diversification.

Venomous snakes of the family Viperidae are a well-known component of the faunal community of the Neotropics, with several species co-occurring in most areas. *Bothrops* (sensu Carrasco, Mattoni, Leynaud, & Scrocchi, 2012), commonly referred to as "lanceheads," is one of the most speciose genera within the subfamily Crotalinae, with 46 known species widespread in the Neotropical region from Mexico to Argentina (Carrasco et al., 2019; Guedes et al., 2018; Timms et al., 2019; Uetz, Freed, & Hošek, 2018; Wallach, Williams, & Boundy, 2014). Based on phylogenetic data (Fenwick, Gutberlet, Evans, & Parkinson, 2009; Wüster, Salomão, et al., 2002), *Bothrops* can be grouped into six evolutionary clades: *B. alternatus* clade, *B. atrox* clade, *B. jararaca* clade, *B. jararacussu* clade, *B. pictus* clade, *B. neuwiedi* clade, and

B. taeniatus clade. First estimates for divergence time of *Bothrops* were around 13 million years ago (Ma) (Hedges, 1996; Savage, 1966). The study of Wüster, Salomão, et al. (2002), who were the first to amass a significant volume of mitochondrial DNA data of the genus, suggested that the ancestor of *Bothrops* might have colonized South America during the Miocene, *c*. 23–10 Ma. Among latest studies, some have estimated similar divergence time for bothropoids (*Bothrops* + *Bothrocophias*) (Alencar et al., 2016), while others estimated a more recent period of diversification for the group (around 13 Ma or less) (Fenwick, Greene & Parkinson, 2012; Wüster, Peppin, Pook, & Walker, 2008).

Bothrops distribution patterns, high richness and the wide variety of niches they occupy provide an ideal study system for assessing general diversification patterns, testing and refining biogeographic hypotheses in the Neotropics. At first, a biogeographic study suggested that the ancestor of modern *Bothrops* dispersed into South America from the north (Parkinson, Campbell, & Chippindale, 2002). Then, Wüster, Salomão, et al. (2002) reported that the adaptive radiation of *Bothrops* into niches devoid of other viperids might have led to their rapid and great diversification through ecological opportunities. However, this study used 26 of 43 described species at that time and did not perform biogeographic analysis.

We used a data set of mitochondrial DNA, incorporating four gene regions (total 2,058 bp), with sequences from 34 species of *Bothrops* to produce a calibrated phylogeny, and reconstruct the diversification of *Bothrops* clades across the Neotropics. Based on the vacant niches hypothesis, we predict that this geographic pattern of speciation was mostly unidirectional, which means that *Bothrops* had a chronologically southward movement and that the diversification history was not correlated with geoclimatic events. We then use this new information to assess the implications for the systematics of this group.

2 | MATERIAL AND METHODS

2.1 | Study system: genus *Bothrops* (Viperidae: Crotalinae)

The *Bothrops* genus is distributed across the Neotropics. The genus comprises abundant and widely distributed species (e.g. *B. asper* and *B. atrox*) as well as rare and/or restricted species (e.g. *B. pirajai* and *B. muriciensis*). Species occur from sea level to 3,500 m (*B. jonathani, B. ammodytoides*) (Campbell & Lamar, 2004; Carrasco, Harvey, & Muñoz Saravia, 2009; Carrasco, Leynaud, & Scrocchi, 2010; Harvey, 1994; Lira-da-Silva et al., 2009; Wallach et al., 2014). Most species are continental, terrestrial, and inhabited forested habitats (e.g. *B. atrox, B. jararaca* and *B. leucurus*). However, some are arboreal in rainforests (e.g. *B. bilineatus*), and some inhabit

open landscapes (e.g. *B. erythromelas*) (Barbo et al., 2016; Barbo, Grazziotin, Sazima, Martins, & Sawaya, 2012; Campbell & Lamar, 2004; Guedes, Nogueira, & Marques, 2014; Guedes et al., 2018; Turci, Albuquerque, Bernarde, & Miranda, 2009).

Some evolutionary relationships remain controversial, including the status of the Andean *B. lojanus*, reported as incertae sedis by Carrasco et al. (2012) and found to be nested within the genus *Bothrocophias* by Arteaga et al. (2016) and Alencar et al. (2016). Fenwick et al. (2009) proposed a rearrangement in the classification of *Bothrops* recognizing the genera *Bothriopsis*, *Bothropoides* and *Rhinocerophis*, subsequently considered synonyms of *Bothrops* by Carrasco et al. (2012). This synonymy was supported by Alencar et al. (2016) and has also been adopted in the current study.

2.2 | Taxon sampling and data acquisition

We sampled 34 species of *Bothrops* representative of the major clades within the genus, as well as 51 species from 23 other genera than *Bothrops* and the lizard *Iguana iguana* for rooting and allowing a greater number of calibration points. DNA sequences, incorporating four mtDNA genes (12S, 16S, ND4, CYTB), were downloaded from GenBank for each species, except for *B. leucurus,* which was sequenced as part of the current study. Alcohol-preserved tissue samples of *B. leucurus* were extracted from liver using the Qiagen DNeasy kit. Sequence amplification via polymerase chain reaction (PCR) was performed using gene-specific primers as follows: 12S, L12 "5-CGCCAAAYAACTACGAG-3" (Vidal, Lecointre, Vié, & Gasc, 1997) and H1557 "5-GTACACTTACCTTGTTACGACTT-3" (Knight & Mindell, 1994); 16S, 16Sar "5-CGCCTGTTTATCAAAAA CAT-3" and 16Sbr "5-CTCCGGTCTGAACTCAGATCACG TAGG-3" (Palumbi et al., 1991); ND4 ND4f, "5-CACCTA TGACTACCAAAAGCTCATGTAGAAGC-3" and ND4fr, "5-TTCTATCACTTGGATTTGCACCA-3" (Arévalo, Davis, & Sites, 1994); and CYTB LI4724, "5-TGACTTGAARAAC CAYCGTTG-3" and H15915, "5-TGAGAAGTTTTCYGGG TCRTT-3" (Irwin, Kocher, & Wilson, 1991; Kocher et al., 1989). The GenBank accession number for sequences are provided in Appendix S1.

Ribosomal 12S and 16S markers were aligned with MAFFT software (Katoh & Standley, 2013) using the Q-INS-i algorithm, which considers the secondary structure of RNA. Sequences of CYTB and ND4 were aligned using the default settings of MAFFT. Minor adjustments were done manually. The final data set consisted of 2,058 bp from the following four genes: two mitochondrial protein-coding genes, cytochrome b (CYTB, 635 bp; 86 taxa) and NADH subunit 4 (ND4, 624 bp; 86 taxa); and two non-coding ribosomal genes, 12S (333 bp; 74 taxa) and 16S (478 bp; 76 taxa).

2.3 | Supermatrix tree analyses and time of diversification

The GTR+G+I model of sequence evolution was chosen for each partition independently by jModelTest using both AIC and BIC criteria (Darriba, Taboada, Doallo, & Posada, 2012; Guindon & Gascuel, 2003). We inferred maximum likelihood topologies using RAxML 8.2.0 on the CIPRES science gateway (Miller, Pfeiffer, & Schwartz, 2010) and assessed the statistical confidence of branches by 1,000 bootstrap replicates (Felsenstein, 1985). We performed Bayesian Inference in MrBayes 3.1.2 software and ran the Markov chain Monte Carlo (MCMC) algorithm for 80,000,000 generations using three independent runs with four chains each. Chains were sampled every 1,000th generation. We applied a burn-in of 25% and evaluated effective sample size values using TRACER v.1.5.

A time tree was inferred using the RelTime method in MEGA 7 (Tamura et al., 2012) and the GTR model. The timetree was computed using eight calibration constraints. A discrete Gamma distribution was used to model evolutionary rate differences among sites (four categories (+G, parameter = 0.6745)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 33.21% sites).

The calibration points used to convert relative times into absolute time estimates of divergence times were based on snake fossils (Buffetaut, Marandat, & Sigé, 1986; George & Vincent, 1977; Head, 2015; Head, Mahlow, & Müller, 2016; Parmley & Holman, 2007; Parmley & Hunter, 2010; White, 1942) and were defined as follow: (a) the split between "*Eryx*–Boinae" between 58.0 and 64.0 (hard minimum–hard maximum ages) million years ago (Ma), which corresponds to the divergence between "Erycinae–Boinae"; (b) the split between "*Boa*–(*Epicrates* + *Eunectes*)" was constrained to be older than 20.43, which corresponds to the minimum age of the oldest *Boa* fossil (stem group age of Boinae); (c) the split between "*Eunectes*–*Epicrates*" was constrained to be older than 12.3 Ma, which corresponds to the minimum age of the oldest *Eunectes* fossil; (d) the split between "*Oxyuranus*–*Laticauda*" was constrained to be older than 10.0 Ma, which corresponds to the minimum age of the oldest Oxyuraninae fossil (minimum divergence between *Laticauda* and Oxyuraninae); (e) the split between "Elapoidea–Colubridae" was constrained to be older than 30.8 Ma, which corresponds to the minimum age of divergence of crown colubroid lineages; (f) the split between "Booidea–Caenophidia" was constrained to be older than 72.1, which corresponds to the oldest fossil assigned to Caenophidia crown group; (g) the split between "Viperidae–(Elapoidea + Colubridae)" was constrained based on the oldest fossil of this clade, an Elapidae of 48.6 Ma; and viii) the split between "*Crotalus*–*Sistrurus*" was constrained to be older than 10.3 Ma, which corresponds to the oldest *Sistrurus* fossil.

There has been much critical attention towards dated phylogeny, especially on the use of calibration, which is the

rate-determining step in every molecular clock analysis. To minimize the issue on distinguishing good from bad calibrations, we run a complementary timetree analysis using Viperidae species and only *Sistrurus* fossil as the closest constraint. The results are placed in Appendix S5.

2.4 | Historical biogeographic analysis

We sought to identify factors or processes related to shaping the composition and distribution of *Bothrops* major groups over time. Thus, the posterior distribution of divergence times of the *Bothrops* clades was then compared with past geoclimatic events reported in the specific literature.

Another goal was to infer the ancestral areas of clades by using the retrieved timetree and test whether *Bothrops* had a chronologically southward movement and that the diversification history was not correlated with geoclimatic events. We inferred the historical biogeography of *Bothrops* based on RAxML topology due to the high resolution and bootstrap values and, when appropriate, the age nodes from Mega 7, RelTime method. We assigned the distribution of the species to the following pre-defined Neotropical areas (according to Morrone, 2014): Antillean subregion: Antilles or West Indies (Greater and Lesser Antilles) and the Bahamas Islands, including the Bahamas, Cuban, Cayman Islands, Jamaica, Hispaniola, Puerto Rico and Lesser Antilles provinces; Brazilian subregion: Southern and central Mexico, Central America and north-western South America including the Mesoamerican, Pacific, Boreal Brazilian and Southwestern Amazonian dominions; Chacoan subregion: south-eastern South America including the Southeastern Amazonian, Chacoan and Parana dominions; and South American Transition Zone: highlands of the Andes between western Venezuela and northern Chile and central western Argentina, including the Paramo, Desert, Puna, Atacama, Prepuna and Monte provinces. The geographic distribution of each species of *Bothrops* was obtained from scientific literature (Campbell & Lamar, 1989, 2004; Carrasco et al., 2012; Fenker, Tedeschi, Pyron, & Nogueira, 2014; Silva & Rodrigues, 2008; Uetz et al., 2018). Detailed information on the assignment of each species of *Bothrops* in the Neotropical areas adopted is provided in Appendix S2.

We reconstructed the ancestral areas for each node in the phylogeny of *Bothrops* using the Reconstruct Ancestral States in Phylogenies software (RASP; Yu, Harris, Blair, & He, 2015). We chose the Dispersal–Extinction–Cladogenesis model for geographic range evolution (DEC; Ree, Moore, Webb, & Donoghue, 2005), for biogeographic analysis. DEC specifies instantaneous transition rates between ranges along phylogenetic branches and applies these rates to estimate likelihoods of ancestral states (range inheritance scenarios) at cladogenesis events. We limited the maximum number of

regions in ancestral areas to two. Based on results from our phylogeny, *Bothrops lojanus* was not considered as part of *Bothrops* in our biogeographical analyses given that the results recovered the species nested within the *Bothrocophias* clade. This grouping is consistent with the results reported by Arteaga et al. (2016) and Alencar et al. (2016).

3 | RESULTS

3.1 | Phylogenetic relationships

The concatenated tree topologies inferred using ML and BI were highly congruent and generally well resolved. The tree estimated in RaxML showed the best topological resolution. Clades recovered in both ML and Bayesian (Appendix S3) analyses are indicated in Figure 1 with their respective statistical support.

Bothrops lojanus is nested within the genus *Bothrocophias*, recovering *Bothrops* as paraphyletic. *Bothrops pictus* was recovered as the sister group of all remaining representatives of the genus (Figure 1 and Appendix S3). Except for *B. lojanus*, all species of *Bothrops* formed a well-supported monophyletic clade. Thus, based on these results, we herein define the following *Bothrops* clades to be used in the diversification and biogeographic analyses: (a) *B. pictus* clade (contains only *B. pictus*); (b) *B. taeniatus* clade (*B. taeniatus*, *B. pulchra*, *B. bilineatus* and *B. chloromelas*); (c) *B. jararacussu* clade (*B. jararacussu* and *B. brazili*); (d) *B. osbornei* clade (*B. osbornei* and *B. punctatus*); (e) *B. lanceolatus* clade (*B. lanceolatus* and *B. caribbaeus*); (f) *B. atrox* clade (*B. atrox*, *B. asper*, *B. moojeni*, *B. colombiensis*, *B. isabellae*, *B. marajoensis* and *B. leucurus*); (g) *B. jararaca* clade (*B. jararaca* and *B. insularis,*); (h) *B. neuwiedi* clade (*B. neuwiedi*, *B. erythromelas*, *B. lutzi*, *B. mattogrossensis*, *B. pubescens*, *B. diporus*, *B. marmoratus* and *B. pauloensis*); and (i) *B. alternatus* clade (*B. alternatus*, *B. ammodytoides*, *B. fonsecai*, *B. cotiara* and *B. itapetiningae*).

3.2 | Diversification and patterns of speciation of *Bothrops*

Overall, most *Bothrops* clades are widely distributed in the Neotropical areas adopted here. Fourteen species are restricted to the Chacoan subregion (highest species richness), eight in the Brazilian subregion, two in the Antilles subregion and five to the South American Transition Zone. Five species were assigned to occur in both Chacoan + Brazilian subregions, one in the Chacoan subregion + South American Transition Zone and one in the Brazilian subregion + South American Transition Zone. The *B. jararaca* clade is restricted to the Chacoan subregion, while *B. lanceolatus* clade is exclusive to the Antilles subregion and *B. pictus* to the South American Transition Zone (see Appendix S2).
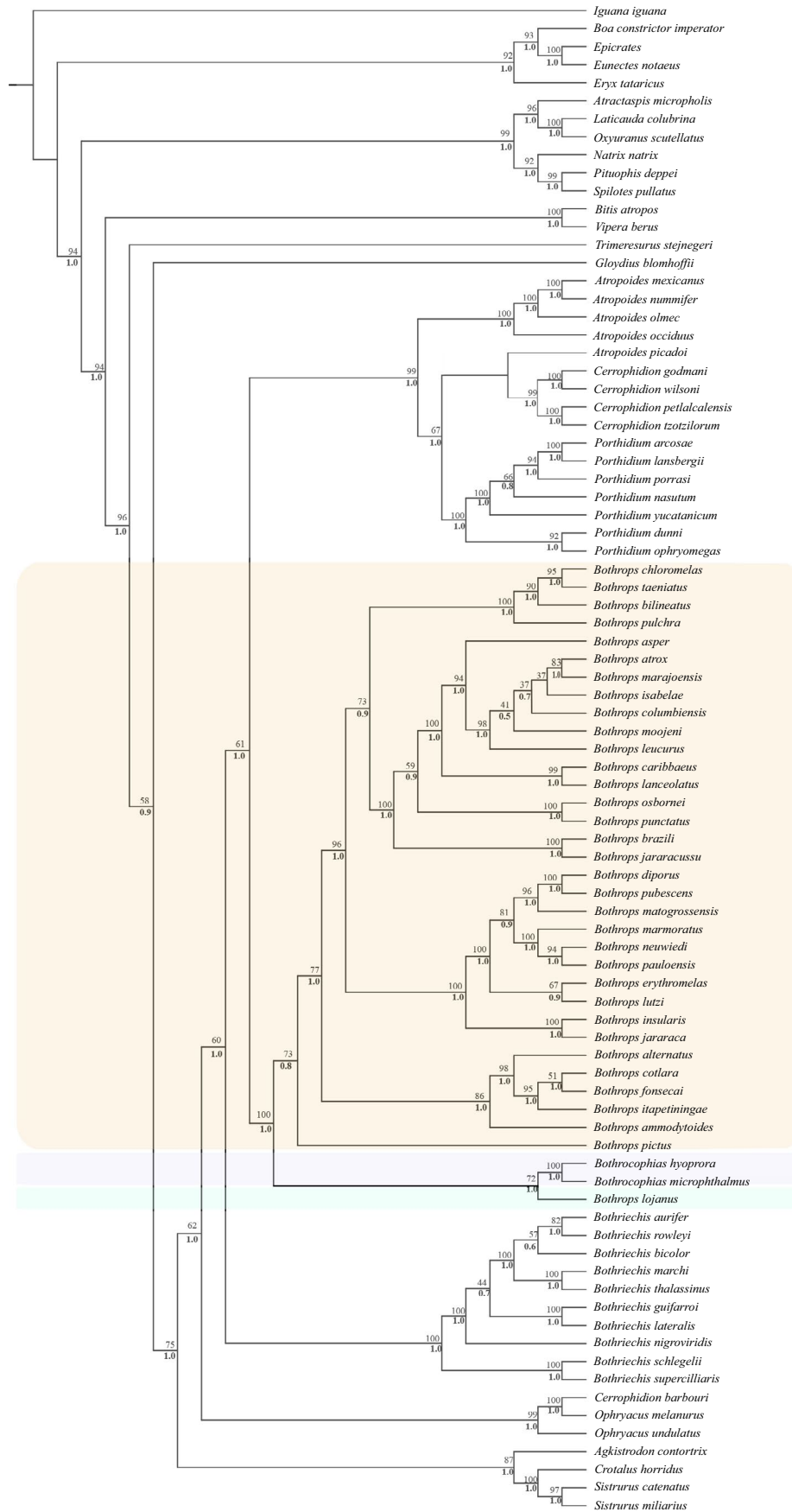
FIGURE 1 Phylogenetic relationships of the species of the genus *Bothrops* based on maximum likelihood inferred in RaxML 8.2.0. Numbers on branches indicate the bootstrap support (not bold) and posterior probability (bold)

Our results show that the most likely ancestral scenario for the diversification of the genus *Bothrops* (nodes 68–71; Figure 2) was within mainland of the South American Transition Zone + Chacoan subregion during the Miocene *c*. 12.03–11.08 Ma (95% divtime: 0.14–32.03). The *B. pictus* clade is supported as having diversified in the South American Transition Zone, splitting from the common ancestor of all other *Bothrops* species (node 67) during the Miocene *c*. 11.08 Ma (95% divtime: 1.91–30.95 Ma).

Results suggest that the *B. alternatus* clade (node 66) has evolved within the Chacoan subregion during the Miocene *c*. 8.81 Ma (95% divtime: 0.29–26.17 Ma). *Bothrops ammodytoides*, the sister group to the other representatives of this clade, is the only taxon distributed in both Chacoan and South American Transition Zone areas.

The *B. neuwiedi* clade (node 60) is inferred to have diversified from a common ancestor (node 61) shared with the *B. jararaca* clade, both restricted to the Chacoan subregion. The diversification of the *B. neuwiedi* clade was found to take place during the Pliocene *c*. 3.04 Ma (95% divtime: 0.2–9.25 Ma), while the diversification of the *B. jararaca* clade (node 53) was reconstructed to the Chacoan subregion from an ancestor (node 61) restricted to this region during the Plio/Pleistocene transition *c*. 2.37 Ma (95% divtime: 0.3–7.66 Ma).

The best-supported scenario for the diversification of the *B. taeniatus* clade (node 51) involves the Brazilian subregion + South American Transition Zone during the Pliocene *c*. 5.47 Ma (95% divtime: 0.10–16.77 Ma). The diversification of the *B. jararacussu* clade (node 47) occurred at the Chacoan + Brazilian subregions in the Pleistocene *c*. 2.68 Ma (95% divtime: 0.2–8.66 Ma). The diversification of the *B. osbornei* clade (node 45) involved the Brazilian + South American Transition Zone areas during the Pliocene and posterior speciation from the ancestor node 46 *c*. 3.64 Ma (95% divtime: 0.1–11.9 Ma). The *B. lanceolatus* clade (node 43) is inferred to have diversified in the Antillean subregion from an ancestor that inhabited the Brazilian subregion during the Plio/Pleistocene transition *c*. 1.91 Ma (95% divtime: 0.2–6.43 Ma).

Finally, our results support the diversification of the *B. atrox* clade (node 42) in the Chacoan + Brazilian subregions during the Plio-Pleistocene *c*. 3.02–2.32 Ma (95% divtime: 0.5–7.59 Ma).

4 | DISCUSSION

4.1 | Early diversification—Pliocene/Miocene orogenic events

The crown nodes in *Bothrops* range from the middle Pleistocene *c*. 0.54 Ma, Quaternary (split *B. diporus* + *B. pubescens*) to 11.08 Ma between Middle and Late Miocene, Neogene (*Bothrops* root). A Miocene occurrence of viperids

at South America is supported by the oldest fossil record of the family in the southern continent, a viperid vertebra reported for the late Miocene Cerro Azul Formation at Caleufú, in La Pampa Province, central Argentina (Albino & Montalvo, 2006). The oldest fossil record for *Bothrops* corresponds to a compound bone exhumed from sediments attributed to the Ensenada Formation (La Plata, Buenos Aires Province, Argentina) from the Early to Middle Pleistocene (0.915–1.01 Ma), which indicate that the cladogenetic events that led to the extant *Bothrops* occurred before 1 Ma (Scanferla & Nenda, 2005).

The uplift of the Andes, which began in the late Oligocene to early Miocene (*c*. 23 Ma) with the formation of the highest peaks during the late Middle Miocene (*c*. 11–12 Ma) (Folguera et al., 2011; Hoorn et al., 2010), coincides with the age estimates for the diversification of *Bothrops*. This continuous strip of mountain ranges that separates the rest of the continent from the narrow Pacific coastal region potentially acted as an effective moisture barrier (Aragon et al., 2011), leading to vicariance speciation, while also presenting new opportunities for ecological adaptation and dispersal (Antonelli et al., 2009; Elias et al., 2009). In the late middle Miocene (*c*. 12.9–11.8 Ma), the eastern Cordillera started developing, extending for almost 9,000 km along the western coast of South America. Thus, it is possible that the ancestor of *Bothrops* was widely distributed and was then splitted by the Andean uplift, which probably also played an important role in the diversification events within *Bothrops* during the Miocene. According to Martins, Araujo, Sawaya, and Nunes (2001), it is possible that the ancestor of *Bothrops* was a small snake, with stout body, and had terrestrial habits.

According to our results, *B. pictus* is the sister group to all *Bothrops* species. The species distribution is restricted to Peru, from the western slopes of the Andes to the Pacific coast, from La Libertad to Arequipa Departments, inhabiting arid to semiarid coastal foothill environments, river valleys and dry western Andean slopes (Campbell & Lamar, 2004; P. A. Carrasco & P. J. Venegas, unpublished data). There is evidence that in the late Miocene the Andean uplift continued to play a role in the diversification and speciation within *Bothrops*. The divergence of *B. ammodytoides* from all *B. alternatus* clade *c*. 8.81 Ma, supports the hypothesis of its occurrence during Pliocene/Miocene orogenic events, potentially due to the uplift of the Patagonian Andes. The current distribution of *B. ammodytoides*, endemic to Argentina, includes areas of the Puna, Prepuna and Monte (part of the South American Transition Zone) and extends to areas in the dry Chaco, Espinal and Pampas, from sea level to altitudes of more than 3,000 m, reaching the highest austral latitudes among snakes. This distribution pattern suggests a south-east expansion from an Andean ancestor, as has been proposed previously (Carrasco et al., 2010; Werman, 2005).
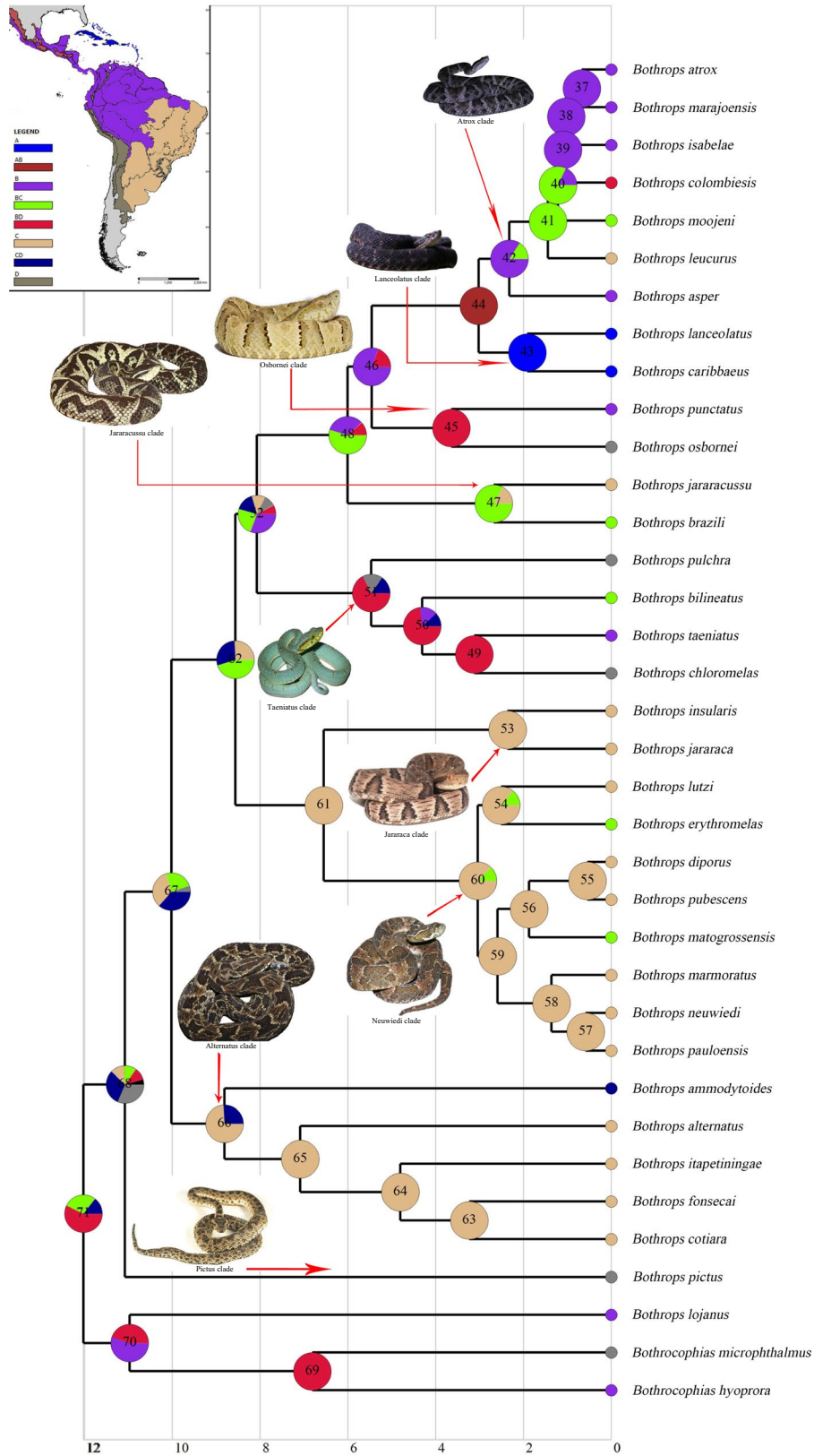
FIGURE 2 Biogeographic history of the snakes genus *Bothrops* through temporal and geographical aspects of diversification from DEC analysis. The bar at the bottom side gives the absolute time scale in millions of years before the present. The modern range for each taxon is colour-coded and is drawn on the terminal lineages before each taxon's name. Pie charts at internal nodes represent the marginal probabilities for each alternative ancestral area. Only ancestral areas for basal nodes are shown. Codes. Chacoan subregion: C; Brazilian subregion: B; South American Transition Zone: D; Antillean subregion: A. Photograph credits: Rodrigo Tinoco (Jararaca clade), Marco Freitas (Taeniatus clade and Jararacussu clade), Omar Torres-Carvajal (Osbornei clade), Germán Chávez (Pictus clade), Catherine Godefroid (Lanceolatus clade)

More recent diversification during the Pliocene led to the formation of the *Bothrops taeniatus*, *B. osbornei* and *B. jararacussu* clades, a period in which Andean elevations rapidly increased by 60% between 2 and 5 Ma, reaching modern elevations by around 2.7 Ma (Gregory-Wodzicki, 2000). This may have led to heterogeneity at local and regional scales, potentially enhancing environmental opportunity for ecological divergence and speciation (Werneck, 2011). The Pliocene rapid uplift of the Eastern Cordillera of the Andes at rates of 0.6–3 mm/year (Gregory-Wodzicki, 2000) may have played a role in the diversification of the *B. taeniatus* clade (node 51), with the speciation of *B. pulchra* c. 5.47 Ma along the South America Transition Zone. *Bothrops pulchra* currently inhabits montane temperate forests in the eastern slopes of the Andes, from southern Colombia to northern Peru (Campbell & Lamar, 2004). The diversification of *B. osbornei* clade (node 45), with the speciation of *B. osbornei* at the South American Transition Zone and *B. punctatus* at the Brazilian subregion, seems to be coupled to the uplifts of the northern Andes c. 5.46 Ma (node 46) (Hoorn et al., 2010). Currently, *B. osbornei* inhabits montane forests in the Pacific slopes of the Andes in Ecuador, and *B. punctatus* inhabits coastal plains and montane forests from eastern Panama to the Pacific coast and Pacific slopes of the Andes in Colombia and Ecuador (Campbell & Lamar, 2004; Cisneros-Heredia, Borja, Proaño, & Touzet, 2006; Ospina-L, 2017). The evolution of the *B. jararacussu* clade is the subject of an ongoing research, and it will not be discussed in details here.

4.2 | Plio-Pleistocene scenario of climatic oscillation, Panamanian land bridge uplift and dispersal over water

The Plio-Pleistocene was a period of transition from humid and warmer forest-like habitats to cooler and drier savannah-like habitats (Hooghiemstra & Cleef, 1995). This period was characterized by pronounced dry seasons (Jacobs, 2004) associated with drastic lowering of global temperatures and increasing aridity, resulting in the replacement of lowland rainforests by savannah woodlands (Plana, 2004). From that time, environmental shifts occurred during the entire Quaternary (2.588 Ma to present) and were linked to climatic changes (temperature and aridity oscillations). It is possible that these dramatic changes in climate and habitats shaped the diversification and speciation patterns of younger clades in *Bothrops*. The influence of Plio-Pleistocene events related to extreme environmental heterogeneity have been reported in a range of Neotropical fauna, including snakes of the genus *Pituophis* (Bryson, García-Vázquez, & Riddle, 2011), mosquitos (Conn & Mirabello, 2007) and birds (Grau, Pereira, Silveira, Wajntal, & Höfling, 2005).

The role of such climate shifts in the diversification of *Bothrops* is denoted in the common ancestor of *B. neuwiedi* and *B. jararaca* clades (node 61), which seem to have diversified in the late Miocene c. 8.56–6.55 Ma, predominantly in the Chacoan subregion with few subsequent dispersal events towards the Brazilian subregion (nodes 54 and 56) during the Pleistocene (c. 2.6–1.95 Ma). The diversification of the *B. neuwiedi* clade (node 60) took place with the split of two major clades (*B. erythromelas* + *B. lutzi*) and a clade that comprises the rest of the species of the group. Machado, Silva, and Silva (2014) highlighted the importance of Neogene events in the diversification of the *B. neuwiedi* clade and proposed that Quaternary climate fluctuations were responsible for the diversification within the clade. During Pleistocene climatic shifts associated with glaciation, areas of moister vegetation types (such as rainforest) and their populations contracted and became isolated, surrounded by drier vegetation such as savannah. During warmer, wetter periods, these vegetation islands (refugia) and their populations expanded. The contractions and expansions occurred cyclically and are thought to have resulted in diversification (Haffer, 1969).

The *Bothrops jararaca* clade (node 53) diversified within the Chacoan subregion with the split of mainland *B. jararaca* and the insular species *B. insularis* c. 2.37 Ma, values that are much older than the c. 10,000 years previously reported (Grazziotin, Monzel, Echeverrigaray, & Bonatto, 2006), but in agreement with the recent literature (e.g. Alencar et al., 2016). Geologic estimates of maximum Pliocene sea level ranged from +5 to +40 m relative to present, with +25 m typically used by the modelling community (Raymo et al., 2009). Thus, considering that there is no strong evidence of sea-level fluctuation by that time, we infer that the diversification of the *B. jararaca* clade may be due to a dispersal event, from mainland populations to islands through dispersal over water. Another existing island endemic species of the *B. jararaca* clade such as *B. alcatraz*, *B. otavioi* and *B. sazimai* may support this hypothesis. Nevertheless, the shared mitochondrial haplotypes between the continental and island forms (Grazziotin et al., 2006) as well as the other terrestrial species found on the Queimada Grande island may suggest secondary contact by connection via land, perhaps mediated by more recent cycles of sea-level transgressions.

In addition to climate and vegetation transition during the Plio-Pleistocene, the uplift of the Isthmus of Panama linking North and South America during the late Pliocene, led to unprecedented ecological and evolutionary consequences for previously isolated biotas (Simpson, 1980). Among the many evolutionary inferences gleaned from the mammalian fossil record are the following: (a) immigrant taxon appeared on each continent soon after the formation of the Panamanian Isthmus, (b) an early wave of xeric-adapted species was followed by a second wave of mesic-adapted species; and (c)

most exchanges had ceased by the onset of the mid-Pleistocene (Webb, 1976).

The *B. atrox* clade (node 42) started its diversification *c*. 3.02–2.32 Ma. Our results support the hypothesis that *B. asper* was the first lineage to diversify, which matches with the appearance of the Panamanian land bridge, allowing this taxon to be the only *Bothrops* species to have reached Central America. *Bothrops asper* currently occurs in the forests of Central and northern South America (see Wüster, Salomão, Duckett, Thorpe, & BBBSP, 1999; Wüster, Salomão, et al., 2002). Speciation between North, Central and South America *c*. 3 Ma has been reported in many faunal clades (Marshall, Webb, Sepkoski, & Raup, 1982), suggesting the formation of the Isthmus of Panama may explain the early diversification of the *B. atrox* clade (see Parkinson et al., 2002). Our estimates of the diversification of the *B. atrox* clade during the Plio-Pleistocene, together with the enormous variety of habitats occupied by its species —from rain forests (e.g. *B. leucurus* and *B. atrox*) to savannas (e.g. *B. moojeni*)—are suggestive of the Refuge Hypothesis (Haffer, 1969, 2008) as an explanation for subclade diversification. Finally, the ancestor of the *B. atrox*–*B. lanceolatus* clade may have dispersed over water to the islands of the Antillean subregion during the Pleistocene and diversified into the species of the *B. lanceolatus* clade, as previously reported by Wüster, Thorpe, et al. (2002). Currently, *B. lanceolatus* and *B. caribbaeus* inhabit the Martinique and Santa Lucía Islands, respectively, in the Lesser Antilles (Wüster, Thorpe, et al., 2002). Saldarriaga-Córdoba, Parkinson, Daza, Wüster, and Sasa (2017) consider an alternative hypothesis to explain the allopatric distribution of *B. atrox* group and *B. asper* lineages on each side of the Eastern Andes Cordillera, suggesting that the final uplift of this mountain range played a significant role in the cladogenesis of these lanceheads. They report that *B. asper* stock would diverge to the west of the Eastern Andes, possible within the foothills along the Pacific coast of northern South America and that the uplift of the Panamanian land bridge would have played a role in the diversification within *asper* lineages.

Overall, our data are in congruence with the history of diversification found for snakes of the genus *Lachesis* (Zamudio & Greene, 1997) and *Corallus* (Colston et al., 2013), frogs (Weigt, Crawford, Rand, & Ryan, 2005), birds (Ribas, Aleixo, Nogueira, Miyaki, & Cracraft, 2012), bees (Ramírez, Roubik, Skov, & Pierce, 2010), butterflies (Brower, 1994) and plants (Antonelli et al., 2009; Dick, Abdul-Salim, & Bermingham, 2003), which would have involved geoclimatic events during the Neogene, dispersals from South America to Central America via the Panamanian land bridge and over-water dispersals to the Antilles during the late Neogene. This congruent biogeographic patterns provide empirically robust diversification hypotheses and support the history of speciation for the clades of *Bothrops* presented herein.

The choice of calibration points is a critical part of diversification studies (Inoue, Donoghue, & Yang, 2010). The complementary timetree analysis using Viperidae species and only *Sistrurus* fossil as the family closest constraint presented younger ages overall, different age values from those found in the literature (e.g. Alencar et al., 2016) and wider confidence intervals (Appendix S5). This is consistent with literature observations on mean substitution rate and coefficient of variation in rates. Sauquet et al. (2012) suggest that, even when suitable internal age constraints for the in-group are available, more accurate age estimates can be obtained by combining both in-group and out-group calibrations. Thus, we have decided to discuss the results for the tree calibrated with all the fossil constrains (Appendix S4, Table 1).

4.3 | Systematic implications

The phylogenetic relationships among the major clades within *Bothrops* shown by our results are in agreement with previous studies (Alencar et al., 2016; Carrasco et al., 2012); these clades are well supported by both molecular and morphological evidence. Our results also support the monophyly of *Bothrops* + *Bothrocophias*; *Bothrocophias* being a genus composed of six species whose distribution is mainly Andean. However, some phylogenetic relationships at the point of divergence between both lineages are not yet sufficiently clear.

Our analyses recovered *Bothrops lojanus* nested within *Bothrocophias*; this relationship was well supported by bootstrap values and is in agreement with previous results (e.g. Alencar et al., 2016). The systematic position of *B. lojanus* was enigmatic until it was included in phylogenetic analyses, showing different results. *Bothrops lojanus* is an Andean species known from a restricted area in southern Ecuador and northern Peru (Campbell & Lamar, 2004; Carrasco, Venegas, Chaparro, & Scrocchi, 2016. Based on morphological evidence, the species has been recovered as the sister taxon of the remaining *Bothrops* (Fenwick et al., 2009) or closely related to *B. pictus* and *B. ammodytoides* (Carrasco et al., 2012). Phylogenies based on mtDNA (Alencar et al., 2016; Arteaga et al., 2016; the present study) recovered the species basal to or rooted within *Bothrocophias*, regarding *Bothrops* paraphyletic. A recent phylogenetic study combining molecular and morphological evidence recovered *B. lojanus* in a position that led *Bothrocophias* to be paraphyletic (Carrasco et al., 2019). Hence, although undoubtedly *B. lojanus* is a basal member of the *Bothrops* + *Bothrocophias* radiation, it may still be considered an "unstable" taxon whose different positions regarding both genera will likely lead to some taxonomic rearrangement. To clarify the phylogenetic position of the Andean *B. lojanus* is crucial for a better understanding of the geographical and historical patterns of divergence of the sister lineages *Bothrops* and *Bothrocophias*, one of the aims of ongoing research projects.

Bothrops clades	Ancestral area	Divergence times mean [95% CI]	Putative events (references)
B. pictus	SATZ	11.08 [1.91–30.95]	Uplift of the Andes (Folguera et al., 2011; Hoorn et al., 2010)
B. alternatus	CS	8.81 [0.29–26.17]	Uplift of the Patagonian Andes (Carrasco et al., 2010; Werman, 2005)
B. taeniatus	BS + SATZ	5.47 [0.10–16.77]	Rapid uplift of the Eastern Cordillera of Andes (Gregory-Wodzicki, 2000)
B. osbornei	BS + SATZ	3.64 [0.1–11.9]	Uplifts of the Northern Andes (Hoorn et al., 2010)
B. neuwiedi	CS	3.04 [0.2–9.25 Ma]	Climate and vegetation changes (e.g. Machado et al., 2014)
B. jararaca	CS	2.37 [0.3–7.66]	Sea-level fluctuations (e.g. Grazziotin et al., 2006) and dispersal over water
B. atrox	CS + BS	2.32 [0.5–7.59]	Appearance of the Panamanian land bridge (Parkinson et al., 2002; Simpson, 1980; Webb, 1976; Wüster, Salomão, et al., 2002)
B. lanceolatus	AS	1.91 [0.2–6.43]	Water dispersal with island speciation (Wüster, Thorpe, et al., 2002)

TABLE 1 Estimated divergence times (in millions of years), ancestral area and putative events for evolution of *Bothrops* clades

Note: The *B. jararacussu* clade is object of study of an ongoing research, and it will not be presented herein.

Abbreviations: AS, Antillean subregion; BS, Brazilian subregion; CS, Chacoan subregion; SATZ, South American Transition.

5 | CONCLUSION

We had predicted a priori that (a) the diversification in *Bothrops* is ancient and (b) the geographic pattern of speciation was most likely unidirectional, having a north to south movement and diversification pattern that was not correlated with geoclimatic events. Our results showed a complex pattern of diversification for *Bothrops* in the Neotropics, with the uplift of the Andes likely playing an important role in the early diversification during the Pliocene-Miocene, and multifaceted past geoclimatic events helping to explain the diversification of younger clades of the genus along forested and open landscapes in the Neotropics during the Pleistocene-Pliocene. It is probable that some of the simpler biogeographic models proposed for the Neotropics are applicable at a more local scale but have limited applicability at such a vast regional scale. Our findings warrant further investigation of diversification and historical biogeographic studies across speciose, widely distributed clades.

CONFLICT OF INTEREST

We have no competing interests.

AUTHOR CONTRIBUTIONS

BH conceived the paper main goals; BH and TBG collected the data, BH and TBG performed the analyses; and BH led the writing with contribution from TBG, PC and JM in the interpretation and discussion of the results.

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SUPPORTING INFORMATION

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