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Vastly underestimated species richness of Amazonian salamanders (Plethodontidae: *Bolitoglossa*) and implications about plethodontid diversification

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ABSTRACT

We present data showing that the number of salamander species in Amazonia is vastly underestimated. We used DNA sequences of up to five genes (3 mitochondrial and 2 nuclear) of 366 specimens, 189 corresponding to 89 non-Amazonian nominal species and 177 Amazonian specimens, including types or topotypes, of eight of the nine recognized species in the region. By including representatives of all known species of Amazonian *Bolitoglossa*, except for one, and 73% of the currently 132 recognized species of the genus, our dataset represents the broadest sample of *Bolitoglossa* species, specimens, and geographic localities studied to date. We performed phylogenetic analyses using parsimony with tree-alignment and maximum likelihood (ML) with similarity alignment, with indels as binary characters. Our optimal topologies were used to delimit lineages that we assigned to nominal species and candidate new species following criteria that maximize the consilience of the current species taxonomy, monophyly, gaps in branch lengths, genetic distances, and geographic distribution. We contrasted the results of our species-delimitation protocol with those of Automated Barcode Gap Discovery (ABGD) and multi-rate Poisson Tree Processes (mPTP). Finally, we inferred the historical biogeography of South American salamanders by dating the trees and using dispersal-vicariance analysis (DIVA). Our results revealed a clade including almost all Amazonian salamanders, with a topology incompatible with just the currently recognized nine species. Following our species-delimitation criteria, we identified 44 putative species in Amazonia. Both ABGD and mPTP inferred more species than currently recognized, but their numbers (23–49) and limits vary. Our biogeographic analysis suggested a stepping-stone colonization of the Amazonian lowlands from Central America through the Chocó and the Andes, with several late dispersals from Amazonia back into the Andes. These biogeographic events are temporally concordant with an early land bridge between Central and South America (~10–15 MYA) and major landscape changes in Amazonia during the late Miocene and Pliocene, such as the drainage of the Pebas system, the establishment of the Amazon River, and the major orogeny of the northern Andes.

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1. Introduction

The study of species richness patterns is a highly active research line in biology (e.g., Mittelbach et al., 2007; Weir and Schluter, 2007; Jetz et al., 2012; Mannion et al., 2014; Hutter et al., 2017; Wiens, 2018). A key assumption in these studies is that species richness is sufficiently well known within each unit of comparison so that the observed pattern reflects the true proportion of species differences (Hortal et al., 2015; Gill et al., 2016; Fišer et al., 2018). However, biologists working in tropical regions may look at this premise as containing more wishful thinking than corroborated knowledge. The task at hand is gargantuan, since Earth's diversity is estimated in more than one billion species (Locey and Lennon, 2016; Larsen et al., 2017), but only about 1.5 million have been formally described (Roskov et al., 2018). Even in birds, arguably the best-known group of animals, our understanding of their species richness seems to be so limited as to compromise the results of detailed inferences (Tobias et al., 2008; Barrowclough et al., 2016).

Within amphibians, plethodontid salamanders have been the focus of multiple studies addressing the causes of differences in species richness in relation to latitude and elevation (Kozak, 2017) and are considered a model in evolutionary biology and ecology (Wake, 2009). Plethodontidae (478 spp.; Frost, 2019) harbors about 66% of the currently recognized species of Caudata. Most species occur in Central America and the Mesoamerican Highlands of Mexico (hereafter collectively called Central America), with a second center of diversity in the southern Appalachian Highlands of eastern North America.

According to current data (Frost, 2019), South America is particularly poor in plethodontid salamanders when compared to Central America (280 species distributed in 17 genera), with only 35 species in the genus *Bolitoglossa* (all of them in the subgenus *Eladinea*) and two more in the genus *Oedipina*. This pattern of species richness is explained by a relatively late dispersal from the Nearctic into the Neotropics, followed by an increase in diversification in Central America (Vieites et al., 2007, 2011; Kozak and Wiens, 2010; Rovito et al., 2015; Shen et al., 2016) and colonization of South America 8.4–30.3 MYA (Elmer et al., 2013; Rovito et al., 2015). This comparatively low number of South American species seems at odds with other variables that usually are good predictors of species richness, such as available area and environmental heterogeneity that favor isolation and speciation (Stein et al., 2014). Amazonia encompasses more than 7 million km^2 and has a geological history that produced an important topographical complexity, including the longest and second highest mountain chain and the largest rivers in the world. This seems to offer no shortage of opportunities for groups to diversify into many species (Hoorn and Wesselingh, 2010; Gehara et al., 2014). Not surprisingly, many groups reach their peak of species richness in Amazonia (Myers et al., 2000; Jenkins et al., 2013), even in taxa that originated elsewhere (Hughes and Eastwood, 2006), but this is not the case for salamanders. A time-dependent diversification model (Stephens and Wiens, 2003) seems like a good explanation for the limited number of salamander species in South America. Relatively small amphibians such as *Bolitoglossa* are poor dispersers, with little ability to cross oceanic barriers—such as the land gap postulated to exist between Central and South America until relatively recently (~3.2 MYA), thus limiting the time for diversification.

However, a series of geological, paleontological, and biogeographic breakthroughs open the possibility of an older colonization for South American salamanders. These include the existence of an older land bridge between Central and South America (Montes et al., 2015), possible amphibian oceanic dispersals (Fonte et al., 2019), considerably older estimated dates for the colonization of South America by salamanders than the ~3 MYA land-bridge (Elmer et al., 2013; Rovito et al., 2015), and the first fossil of a Caribbean salamander, apparently a Bolitoglossini of at least 15 MYA (Poinar and Wake, 2015). On the other hand, the number of species of *Bolitoglossa* may be more underestimated in South America than in other regions. Different studies indicate that, in Plethodontidae, cladogenesis—as inferred from phylogenetic analyses of DNA sequences—is often not accompanied by detectable morphological changes, and phenotypic homoplasy is very common (Larson and Chippindale, 1993; Tilley and Bernardo, 1993; Parra-Olea and Wake, 2001; Adams et al., 2009; Wake, 2009; Elmer et al., 2013). Independently of the causes of this morphological stasis, the implications for the systematics of these salamanders are obvious: species delimitation solely based on the variation of a handful of morphological characters traditionally used in the group is likely biased towards an under-estimation of species richness. To date, no study has addressed the systematics of South American salamanders using DNA sequences of a collection of samples that truly reflects their distribution. The most extensive study, based on Ecuadorian samples, indicates high levels of cryptic species richness even at a moderate geographic scale (Elmer et al., 2013).

In summary, there may be many more species of plethodontid salamanders in South America than currently known, because the group may have arrived earlier from Central America than previously thought (~23 MYA; Elmer et al., 2013) and our understanding of the diversity of the group is superficial.

Amazonian salamanders are relatively small (snout-vent length = 25.2–58.9 mm; Brame and Wake, 1963; Crump, 1977; Brcko et al., 2013) and have hands and feet modified as pads, apparently to increase adherence, which may facilitate their arboreal and epiphyllous life. Like other plethodontids, they are lungless, with the hyoid system modified to dart their tongue to capture prey. Females deposit terrestrial eggs and embryos undergo direct development so that a miniature version of the adult hatches from the egg (Brame and Wake, 1963; Wake, 1966). Currently, nine species of *Bolitoglossa* are recognized in Amazonia, from the lowlands of the Amazon River to around 2000 m a.s.l. in the eastern Andean slopes. Four of them—*B. caldwellae* Brcko, Hoogmoed and Neckel-Oliveira, 2013, *B. madeira* Brcko, Hoogmoed and Neckel-Oliveira, 2013, *B. paraensis* (Unterstein, 1930), and *B. tapajonica* Brcko, Hoogmoed and Neckel-Oliveira, 2013—are relatively well characterized morphologically as the result of a recent taxonomic revision (Brcko et al., 2013). The other five—*B. altamazonica* (Cope, 1874), *B. digitigrada* Wake, Brame and Thomas, 1982, *B. equatoriana* Brame and Wake, 1972, *B. palmata* (Werner, 1897), and *B. peruviana* (Boulenger, 1883)—represent more challenging situations (Wake et al., 1982; Acosta-Galvis and Gutiérrez-Lamus, 2012; Elmer et al., 2013).

The phylogenetic relationships among Amazonian salamanders are also poorly studied, and the taxonomic identification of specimens is problematic. Until 2004, they were grouped in different phenetic clusters. Parra-Olea et al. (2004) studied the phylogeny of 61 species of *Bolitoglossa* analyzing DNA sequences of two mitochondrial genes and proposed the recognition of seven subgenera (*Bolitoglossa*, *Eladinea*, *Magnadigita*, *Mayamandra*, *Nanotriton*, *Oaxakia*, and *Pachymandra*). All South American species analyzed were part of the subgenus *Eladinea*. Parra-Olea et al. (2004) divided *Eladinea* into four species groups (*B. adspersa*, *B. epimela*, *B. schizodactyla*, and *B. subpalmata*), with all South American species placed in the *B. adspersa* group. Following the work of these authors, several studies published phylogenetic hypotheses including DNA sequences of South American salamanders (García-Gutiérrez et al., 2013; Pyron and Wiens, 2011; Acevedo et al., 2013; Elmer et al., 2013; Batista et al., 2014), although with limited taxon sampling, as they were designed to study either particular species-level systematic issues or very broad phylogenetic questions across amphibians. Most of these studies agree that the Amazonian species included in their respective analyses are paraphyletic with respect to other species of the *B. adspersa* group. However, they all differ about the details of the relationships. Given than these studies vary in their combinations of characters, terminals, and optimality criteria, their results cannot be easily compared.

Considering the situation outlined above, the main objectives of this study are to assess the evolutionary relationships among specimens of

Bolitoglossa from Amazonia, evaluate their species diversity, as well as their diversification and biogeography.

2. Material and methods

2.1. Taxon sampling

We aimed to include as many specimens as possible of *Bolitoglossa* salamanders from Amazonia, including representatives of all currently recognized subgenera and all species groups within *Eladinea*. Considering the current difficulty in assigning species names to specimens, we made an effort to include data from type material and/or topotypes so that binomials could be assigned to clades. Representatives of other genera of Plethodontidae (*Aquiloeurycea*, *Chiropterotriton*, *Ixalotriton*, *Parvimolge*, and *Pseudoeurycea*) were used as outgroups, and *Thorius* was set as the root in all analyses (Rovito et al., 2015). Our final dataset included 366 terminals, 189 corresponding to 89 non-Amazonian nominal species and 177 to Amazonian specimens, including types or topotypes of eight of the nine recognized species in the region (Table S1). By including representatives of all the known species of Amazonian *Bolitoglossa*, except for *B. digitigrada*, and 73% of the currently 132 recognized species of the genus, our dataset represents the broadest sample of species, specimens, and geographic localities studied to date.

2.2. DNA sequences collection

We used the molecular markers most frequently sequenced in previous studies of *Eladinea* to be able to incorporate as much published data as possible. After a review of the relevant literature (Parra-Olea et al., 2004; Rovito et al., 2012; Batista et al., 2014; Elmer et al., 2013), we selected three mitochondrial—16S rRNA (16S), cytochrome *c* oxidase subunit I (*COI*), cytochrome *b* (*cytb*)—and two nuclear markers—proopiomelanocortin (*POMC*) and recombination activating gene 1 (*RAG1*). Laboratory protocols for newly generated sequences followed standard procedures described by Palumbi et al. (1991), Moritz et al. (1992), Ivanova et al. (2006), Vieites et al. (2007), and Elmer et al. (2013). The primers used are listed in Table S2.

Sequences were obtained from samples listed in Table S1. PCR amplification products were sequenced in both directions. The resulting chromatograms were visualized in Sequencher 4.1.4 to trim low-quality sequences and to correct errors or ambiguous nucleotides. Additionally, we downloaded homologous sequences from GenBank of ingroup and outgroup taxa (up to 27 November 2017). We filtered all terminals from non-South American salamanders (i.e., García-París et al., 2000; Boza-Oviedo et al., 2012; Rovito et al., 2012), incorporating only those that had genetic distances greater than 1% in 16S and *cytb* (these genes were sequenced in more than 85% of the terminals), to reduce search space during phylogenetic analyses (Wilkinson, 1995; Kearney, 2002; Brower, 2018). In order to reduce wildcard terminals, incomplete sequences from outgroup samples (i.e., *B. colonnea*, *B. engelhardti*, *B. helmrichi*, *B. occidentalis*, *B. orestes*, *B. rufescens*; Table S1) were merged with sequences from other individuals of the same species to construct a single complete composite sequence. This was done only after checking that the genetic distances in 16S and/or *cytb* fragments were < 1.0%.

In total, 353 sequences were generated, including the first sequences of six South American species: *B. altamazonica*, *B. hypacra*, *B. madeira*, *B. peruviana*, *B. tapajonica* and *B. walkeri*. Nine terminals from GenBank were re-identified (Table S3) based on two criteria: (i) secondary literature, for recently described species with sequences submitted to GenBank as belonging to undescribed taxa (i.e., sp.); and (ii) discordance in the species name between the GenBank database and the original publication.

2.3. Phylogenetic analyses

2.3.1. Theoretical considerations

Under the parsimony criterion, the best explanation of the observed variation is the one that requires fewer transformations (Kluge and Grant, 2006; Grant and Kluge, 2009). In other words, minimizing the number of necessary evolutionary changes maximizes the explanatory power of the hypothesis.

A different and currently more popular approach considers that the best phylogenetic hypothesis is the one that maximizes the likelihood of observing the data. This requires a probabilistic model of character change, together with several further assumptions.

We performed two types of phylogenetic analyses that reflect the two views outlined above, an equally weighted parsimony analysis, which is consistent with the first view, and a maximum likelihood (ML) analysis, compatible with the second perspective. The purpose of these analyses is twofold. First, we want to evaluate the sensitivity (sensu Giribet and Wheeler, 2007) of our results to the different optimality criteria. Second, we want to foment collegiality among colleagues (including the authors of this study), which include conflicting preferences regarding the analytical approaches outlined above.

Regardless of optimality criterion, a phylogenetic hypothesis that is the optimal solution according to a criterion (parsimony or ML in this study) was considered supported if not contradicted by other, equally optimal hypotheses (i.e., evidence is ambiguous, such as when multiple most-parsimonious cladograms are obtained). Frequency of clades based on resampling measures (i.e., Jackknife and Bootstrap) are interpreted as a proxy of the relative amount of favorable and contradictory evidence for each clade present in the optimal topology inferred from a specific dataset when frequency ≥ 50% (Goloboff et al., 2003; Ramírez, 2005).

2.3.2. Parsimony

Analyses were performed under direct optimization in POY 5.1.1 (Varón et al., 2010; Wheeler et al., 2014), which evaluates hypotheses of nucleotide homology dynamically by optimizing unaligned DNA sequences directly onto alternative topologies (Wheeler et al., 2006). First, sequences of each marker were individually aligned using the MUSCLE algorithm in AliView 1.17.1 (Larsson, 2014) under default parameters. Each aligned gene fragment was partitioned into smaller blocks so that within each block, length variation among DNA sequences was attributed only to insertions and/or deletions of nucleotides and never to missing data (Wheeler et al., 2006). Each block was flanked by conserved regions with no gaps and few or no nucleotide substitutions. Before tree searches in POY, all gaps were removed from each block. Tree searches were conducted using the cluster Amazonia, from the *Laboratório de Alto Desempenho* (LAD)-PUCRS high performance computing. The Amazonia cluster consists of an enclosure HP Blade System C3000 with 4 blades L620cG7 and a dedicated storage with access through Fiber Channel Protocol (8 Gib/s). It is composed by two Intel Xeon E7-2850 2.0 GHz Hyper-Threading processors with 160 GB and 512 GB of memory, and 20 cores (40 threads) for each processor (160 threads in total for the cluster). Three searches of 50 h each on 40 CPUs (giving a total of 6024 CPU-hours) were run using the command “search”, which implements an algorithm based on random addition sequence (RAS) Wagner builds, tree bisection and reconnection (TBR) branch swapping (Goloboff, 1996, 1999), parsimony ratcheting (Nixon, 1999), and tree fusing (Goloboff, 1999), storing the shortest trees from each independent run and performing a final round of Tree Fusing on the pooled trees. Next, 3000 rounds of Tree Fusing of the optimal trees from driven searches were performed, using the standard direct optimization algorithm. Then, we used the exact iterative pass algorithm (Wheeler, 2003a) to improve the cost of the optimal trees identified in the previous analyses. Finally, tree-alignment matrices of all the optimal trees were generated (i.e., the implied alignment; Wheeler, 2003b). To search for additional optimal trees for

the tree-alignment, we performed searches using the New Technologies algorithms (Sectorial Search, Ratchet, Drift, Tree Fusing) in their default modes in TNT 1.5 (Goloboff et al., 2008; Goloboff and Catalano, 2016). Searches were set for all taxa, at level 70, with minimum tree length set to be found 100 times and random seed = 1. Finally, we visually compared the resulting consensus trees from each tree-alignment. Jackknife frequencies (JK) were calculated in TNT from the implied alignment for 1000 pseudoreplicate searches with the Traditional Search option with 50 replicates and 50 trees saved per replication, gaps as a fifth state, and removal probability of 0.36 ($\sim e^{-1}$) to render bootstrap (BS) and JK values comparable (Farris et al., 1996).

Given the heterogeneity of gene coverage (1–5 loci per terminal) in our data set, we analyzed the potential wildcard behavior of terminals (Simmons, 2012; Simmons and Norton, 2013; Simmons and Goloboff, 2013; Padial et al., 2014) for all terminals with YBYRÁ (Machado, 2015) using all optimal topologies from the parsimony analyses and the program "ybyra_sa.py". Briefly, this analysis ranks all terminals according to their average matching split distance (MSD; Bogdanowicz and Giaro, 2012) calculated from all optimal topologies when the given terminal is pruned. Terminals that, when removed, resulted in the smallest average MSD are considered potential wildcard taxa and will cause the greater resolution decrease in the strict consensus. In other words, the exclusion of the terminal from the optimal trees will cause the trees to be more similar. The objective of this analysis was to detect samples that may collapse important or large sections of the optimal trees but not to exclude such samples from our analyses.

2.3.3. Maximum likelihood

We combined the similarity alignments mentioned above into a single matrix using SequenceMatrix 1.8 (Vaidya et al., 2011). We used the greedy algorithm of PARTITIONFINDER v.1.1.1 (Lanfear et al., 2016) and the corrected Akaike information criterion to select the optimal combination of partition schemes and DNA substitution models for the concatenated matrix. We followed Simmons and Ochoterena (2000) and coded continuous indels as the largest possible single events as implemented in the option "simple coding" of SeqState (Müller, 2005, 2006), which was included as an independent data partition. The best-fitting partition scheme and DNA substitution models were applied to search the ML tree. As indel characters were coded as binary (0 or 1), we used Mkv model of evolution for discrete morphological data (Lewis, 2001), which assumes that the data collected contain only variable characters. Tree searches of the final matrix (the similarity alignment of DNA sequences with gaps recoded as unknown nucleotides plus the additional partition of indels as binary characters) were performed in Garli (Zwickl, 2006) on XSEDE (CIPRES Science Gateway; Miller et al., 2010). We conducted 500 independent searches using a random tree ("streefname = random"), 100,000 generations without topology improvement required for termination (genthreshfortopoterm), tree rejection threshold at 50 (treerejectionthreshold), and the maximum number of branches away from original location that a branch may be reattached during a limited SPR move was 10 (limsprrange). The best tree from these independent searches was selected according to the highest value of log likelihood score. Bootstrap frequencies were calculated with 1000 pseudoreplicates under the same tree search parameters outlined above. The replicates were compiled in a single tree file using the R package Ape 4.1 (Paradis et al., 2004), and BS frequencies were assigned to the corresponding clades of the optimal tree using SumTrees 4.3.0 (Sukumaran and Holder, 2010a) of the DendroPy 4.3.0 package (Sukumaran and Holder, 2010b).

2.3.4. Genetic distances

We calculated genetic distances for the mitochondrial markers 16S and *cytb* because they are the best represented in our dataset (sequenced for 130 and 115 Amazonian samples, respectively). Uncorrected p-distances were estimated in Mega 7 (Kumar et al., 2018) for each marker independently using a similarity alignment (453 and 528 bp for 16S and *cytb*, respectively). We used the option "Site Coverage Cutoff (%) = 5%", which means that only sites present in at least 5% of the sequences/taxa will be used in each pairwise comparison (Mello, 2018).

2.4. Species: Conceptual and operational considerations

We consider a species as the single lineage segment of ancestor–descendant populations or metapopulations delimited by a splitting event (Simpson, 1951; Wiley, 1978; de Queiroz, 1998; Wiley and Lieberman, 2011). Under this theoretical perspective, species exist (i.e., they are ontological historical individuals, regardless of our ability to discover them), evolve, and are discoverable to the degree that footprints of their evolutionary history—characters observed on organisms—allow us to infer their existence (Ghiselin, 1975; Hull, 1976; Wiley, 1978; Frost and Kluge, 1994).

Operationally, we first used the optimal trees, inferred as explained above, as guides to identify putative species. On these trees, we identified topotypic or type samples of nominal Amazonian species and checked that they were morphologically concordant with the most updated descriptions. After assigning these specimens to nominal species, we navigated the trees from those tips towards the root to identify the most inclusive concordant clades with BS and JK $\geq$ 75 and that did not include clear gaps in branch lengths, genetic distances, and geographic distribution. These clades were assigned to nominal species. Samples not assigned to nominal species were evaluated in a similar fashion—although the criterion of monophyly took precedence over values of BS or JK—and assigned to putative new species. Our protocol attempts to maximize the consilience of current availability of names, evolutionary history (in the form of phylogenetic trees and clades), and gaps in the amount of divergence—as indicated by branch lengths and genetic distances, and geographic distribution—, at the same time that considers congruence between analyses (parsimony and ML) and among characters within a specific analysis (BS and JK). For this reason, we call it *congruence* approach and we opted to use the species delimitation resulting from it. We used the adjective *unconfirmed* for candidate species when dealing with singletons and ambiguous monophyly (Padial et al., 2009; Vieites et al., 2009; Padial et al., 2010).

We compared our *congruence* approach to two currently used automatized species delimitation methods but solely based on analyses of DNA sequences: Automated Barcode Gap Discovery (ABGD; Puillandre et al., 2012) and multi-rate Poisson Tree Processes (mPTP; Zhang et al., 2013; Kapli et al., 2017). The ABGD analyses were performed using the online server http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html. We used the simple distance method, with a relative gap width of 0.01, and intraspecific p-distance of 0.001 to 0.029 (16S) or 0.057 (*cytb*). The upper value used for both genes corresponds to the maximum intraspecific distance found between the two terminals of *Bolitoglossa tapajonica*. We used this species because it has the highest intraspecific distance among the currently recognized and well characterized Amazonian species. The other parameters were set according to the default configuration (Steps = 20, Nb bins = 20). For the reasons exposed by Padial et al. (2009) and Padial and De la Riva (2010), we do not endorse using global thresholds of genetic divergences alone to propose putative species and we used them in this work solely for comparative purposes. We used the software mPTP 0.2.4 v. (Kapli et al., 2017) on the ML tree based on nuclear and mitochondrial data, inasmuch as it contains information about the nucleotide substitution rate that is used by the algorithm to identify speciation events (Kapli et al., 2017). The analyses were conducted with the MCMC method and multi-rate command with 50,000,000 generations, sampling every 10,000 generations and with a burn-in phase of 1000 generations. Although we considered applying other methods for species delimitations such as BPP (Yang, 2015) our sequence coverage of nuclear loci was too incomplete due to diverse reasons. For example, many specimens were represented by sequences exclusively taken from

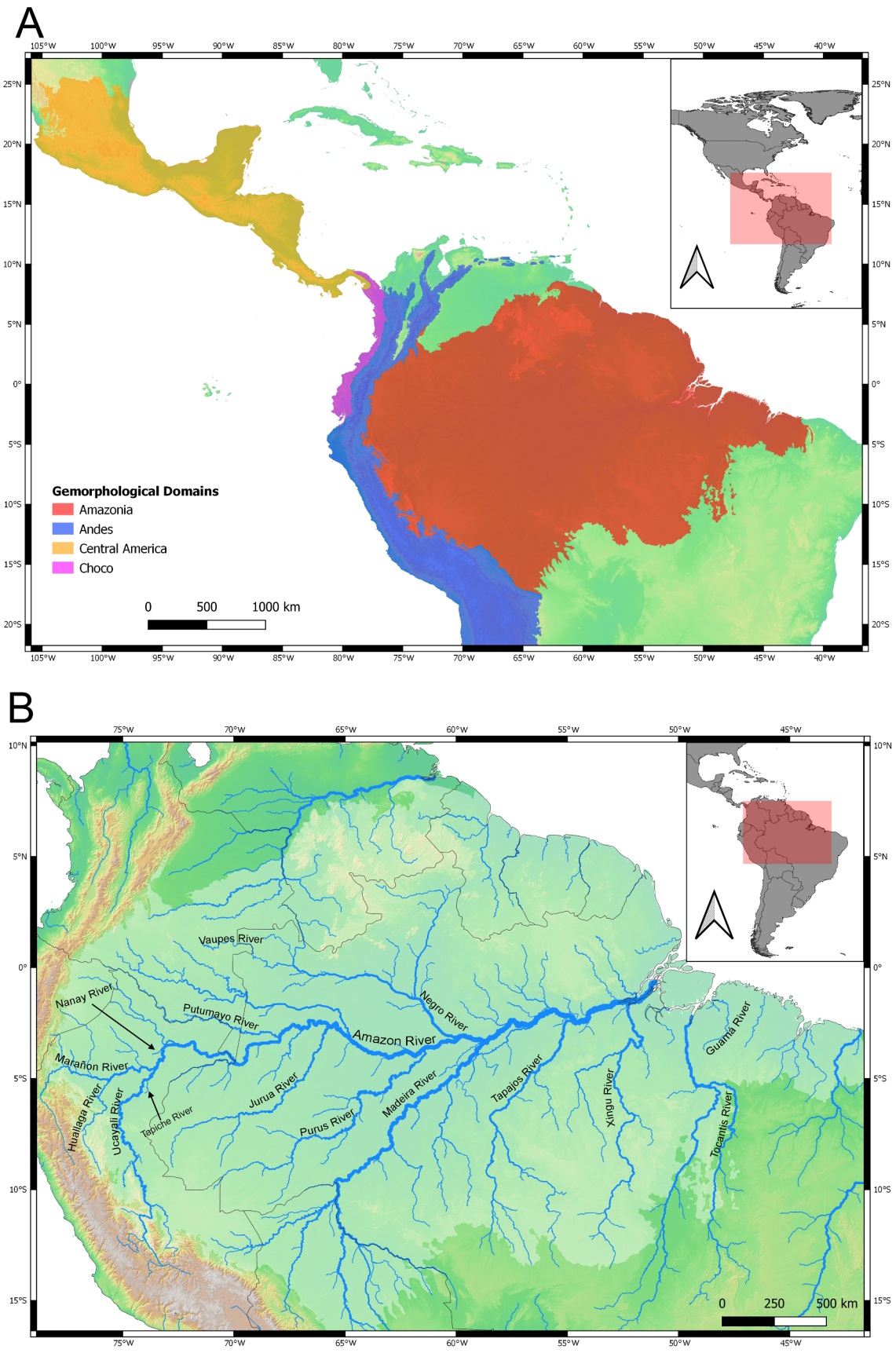

Fig. 1. (A) Map of Central and northern South America with biogeographic areas used in the study marked with colors. (B) Map of Amazonia (light green) highlighting the major rivers discussed in the text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

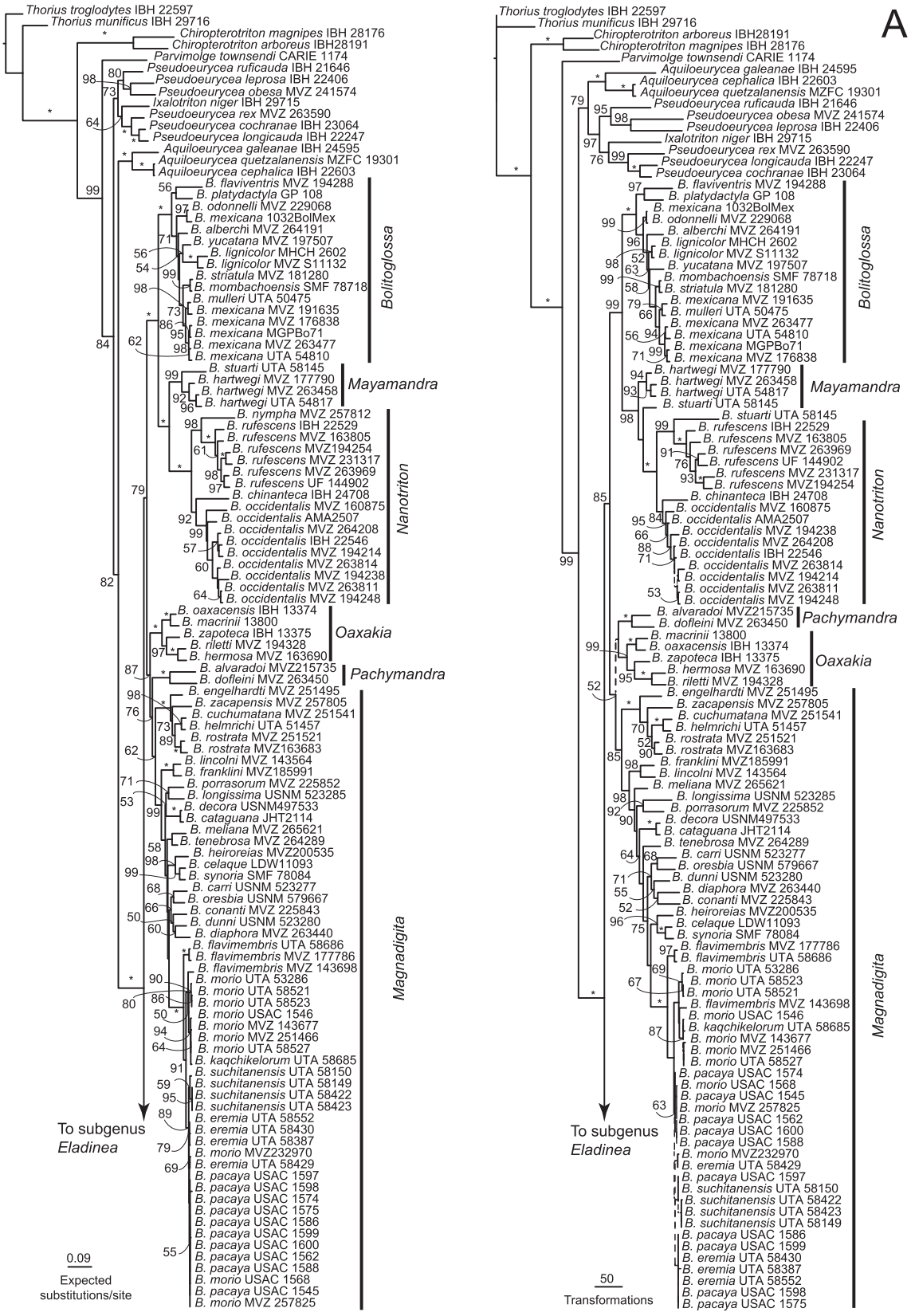

Fig. 2. Phylogenetic relationships of *Bolitoglossa* and outgroups inferred from up to three mitochondrial (16S, *COI*, and *cytb*) and two nuclear (*POMC* and *Rag1*) partial gene sequences. On the left, maximum likelihood tree (log likelihood = − 5,9863.1) from a similarity alignment and considering indels as the longest possible binary characters. On the right, one of the 1752 shortest trees (12,597 transformations) from a tree-alignment parsimony analysis, coding indels as fifth character, with dashed lines indicating collapsed clades in the strict consensus. Numbers on branches are bootstrap (left) and jackknife (right) frequencies (percent) of 1000 searches. Subgenus and species groups are labeled. Specimens in shaded boxes correspond to Amazonian samples in the parsimony tree (in the maximum likelihood tree, all Amazonian samples form a clade). A, outgroups and subgenera of *Bolitoglossa* other than *Eladinea*; B, non-Amazonian *Eladinea*. The relationships among Amazonian *Eladinea* are shown in Figs. 3 and 4.

GenBank and only mitochondrial genes were available. Also, several of our samples were of low quality, limiting amplification and sequencing to mitochondrial genes. In summary, only 29% of Amazonian terminals had sequences of both nuclear markers.

To quantify the differences among the delimitation methods, we used the Relative Taxonomic Resolving Power Index (R_{tax}) and the Taxonomic Index of Congruence (C_{tax}) following Miralles and Vences (2013). The R_{tax} quantifies the relative power of a method to infer all estimated speciation events present in a data set (large R_{tax} means small type II error), but does not necessarily imply correct delimitations (i.e.,

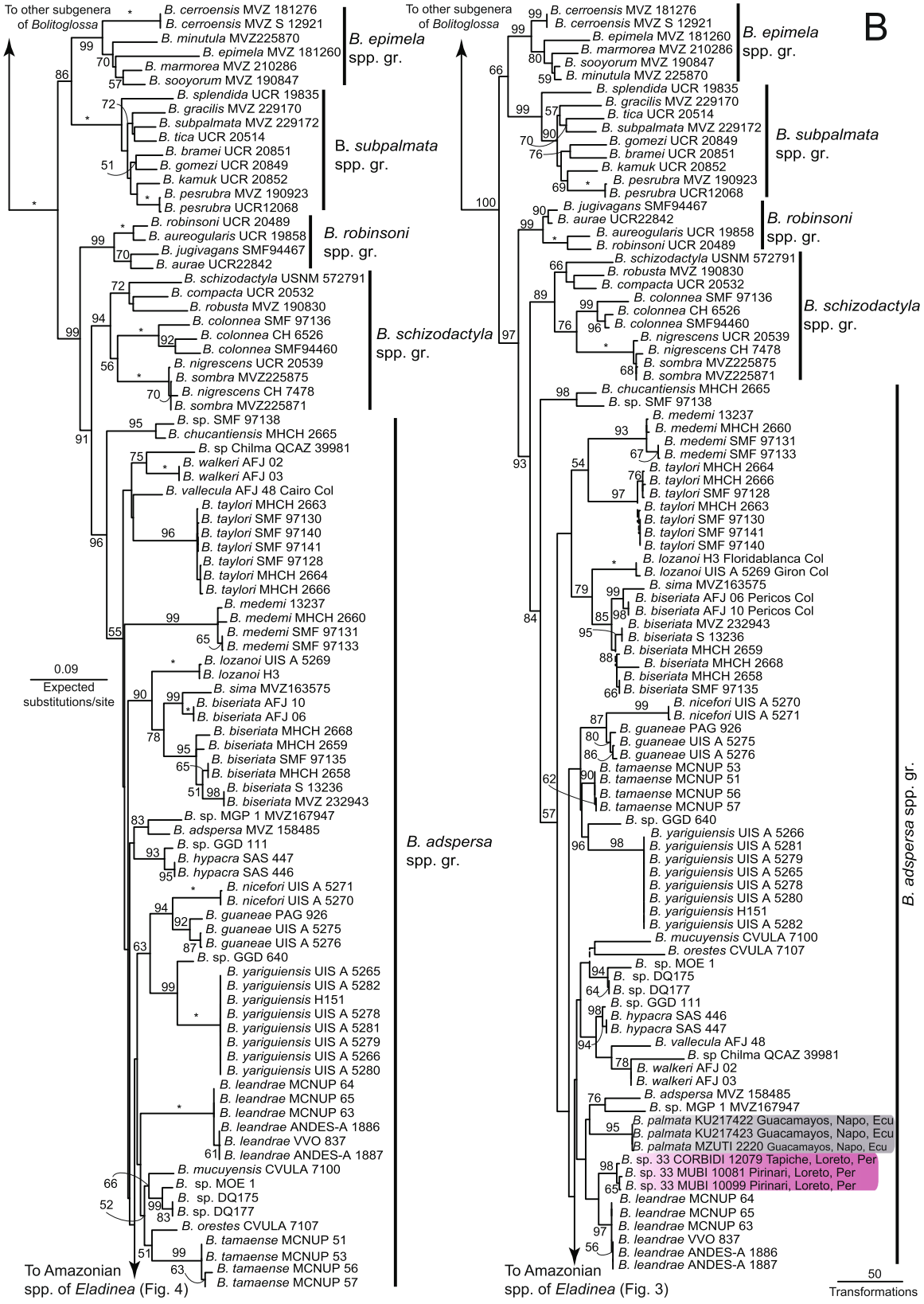

Fig. 2. (*continued*)

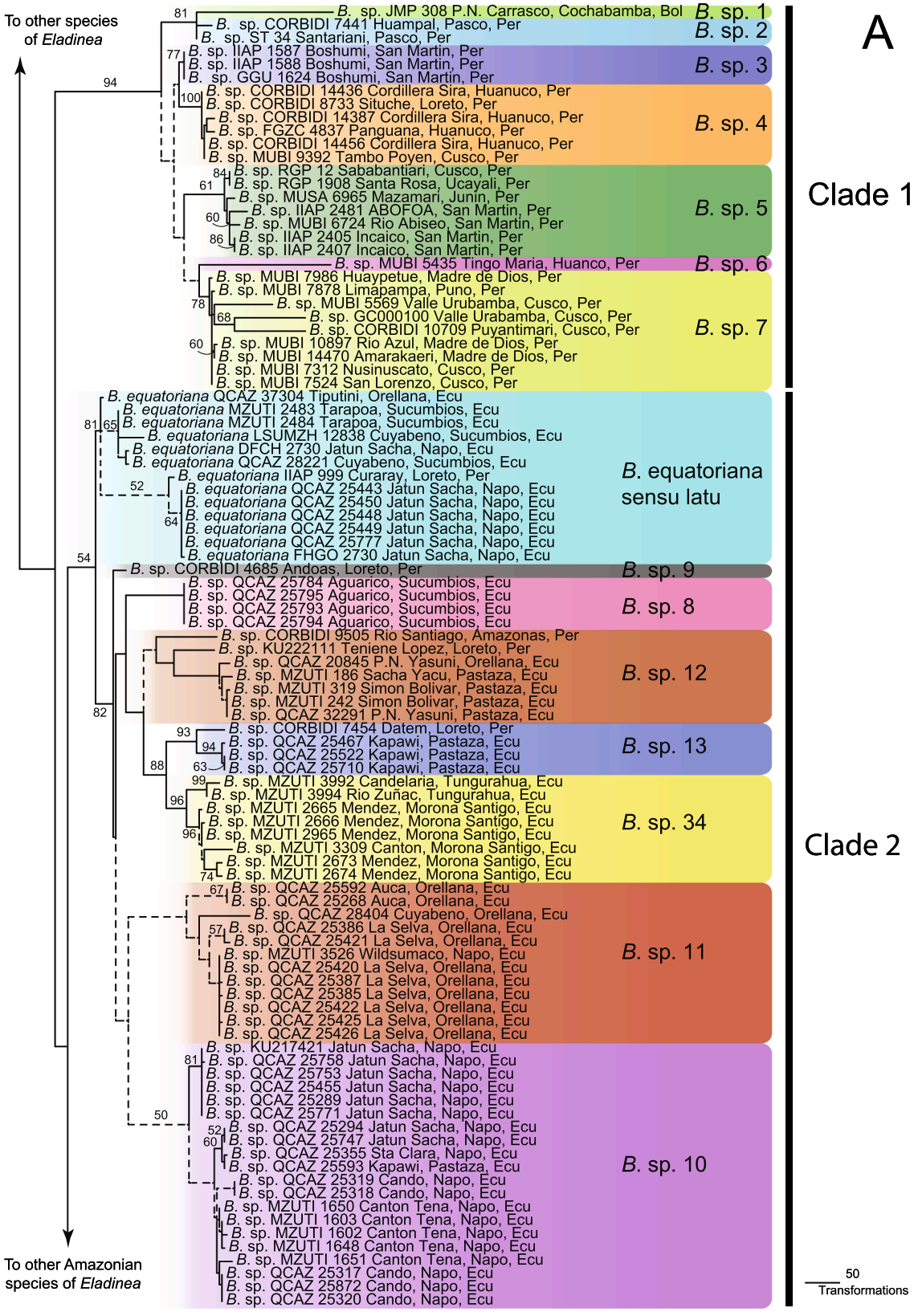

Fig. 3. One of the 1752 shortest trees (12,597 transformations) illustrating the relationships among Amazonian *Eladinea* and inferred from up to three mitochondrial (16S, *COI*, and *cytb*) and two nuclear (*POMC* and *Rag1*) partial gene sequences from a tree-alignment parsimony analysis coding indels as a fifth character. Dashed lines indicate collapsed clades in the strict consensus. Numbers on branches are jackknife frequencies (percent) of 1000 searches. Nominal and candidate species according to this work are indicated with color shading. Clades 1 to 7 indicate groups with identical content in the maximum likelihood analysis (except for *Bolitoglossa palmata*); see main text for discussion. This tree is a continuation of Fig. 2 (right). A, Clades 1 to 2; B, Clades 3 to 7.

it can lead to oversplitting). On the other hand, the C_{tax} measures the congruence in delimitation assignments between two methods, with a value of 1 indicating complete congruence. For details of calculation of both indexes, see Miralles and Vences (2013).

We want to highlight that our proposal merely flags evolutionary lineages that may represent species. We acknowledge that further evidence needs to be gathered and analyzed before formalizing these species hypotheses with names that follow the rules of the ICZN (Anonymous, 1999).

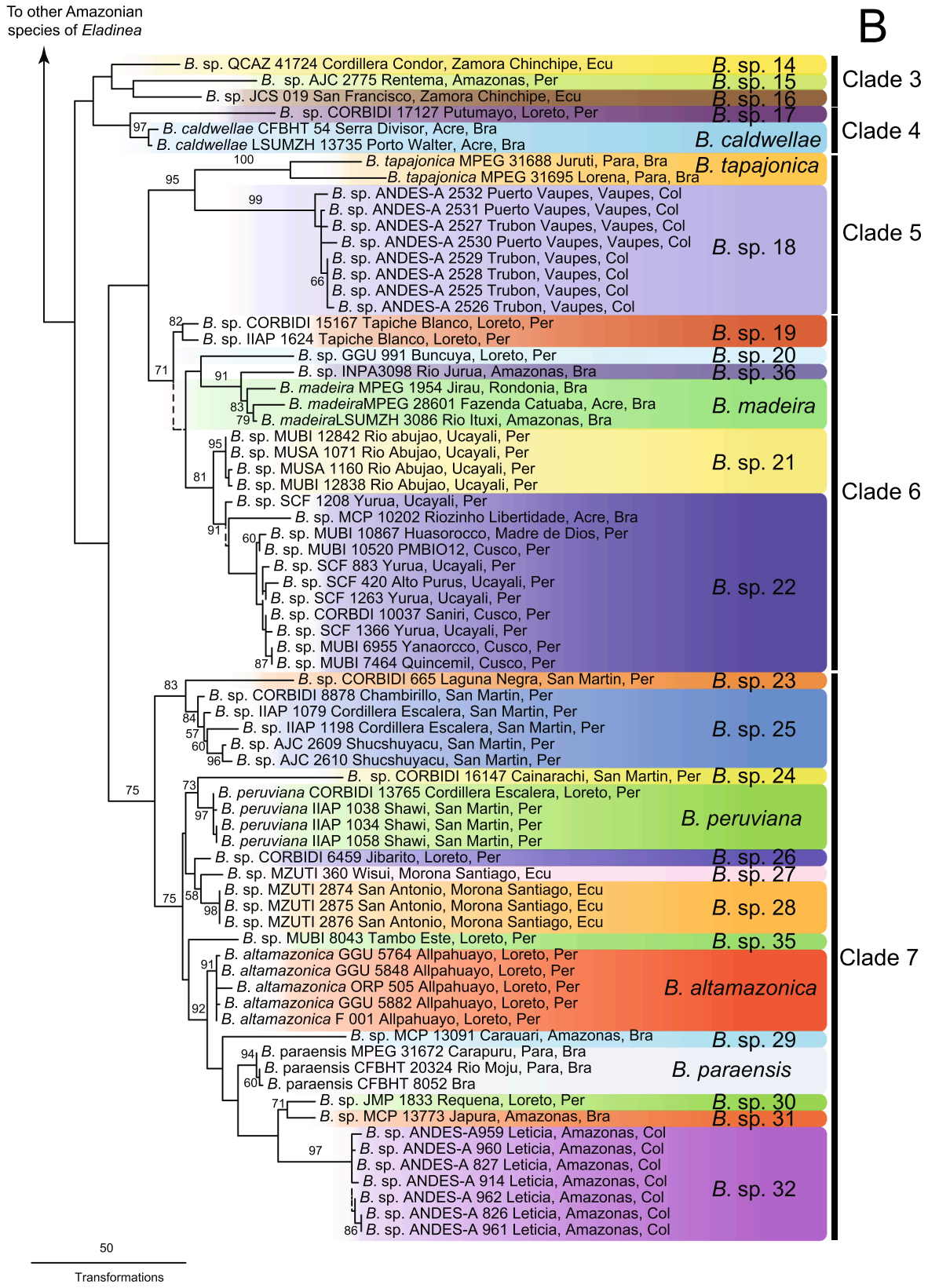

Fig. 3. (*continued*)

2.5. Biogeographic analysis

2.5.1. Time-calibrate phylogeny

To infer a temporal framework for the diversification of Amazonian salamanders, we created a new dataset by sampling a single terminal per putative species—the one with the most complete representation of the five loci—of *Eladinea* and one species from each of the other *Bolitoglossa* subgenera as outgroup. We used *Pseudoeurycea rex* to root all trees. For the new reduced datasets, we used the alignments generated with all the data to avoid artifacts described by Simmons and Freudenstein (2003). We selected the best-fit partition scheme and model of nucleotide evolution as explained above. We used the optimal topologies resulting from the ML and parsimony analysis explained above but pruned to match the taxon sampling of the dataset. We randomly resolved the unique polytomy of the ML pruned topology using the command *multi2di()* in the APE package (Paradis et al., 2004), while we randomly selected one of the most parsimonious trees.

Because there are not described fossils of *Bolitoglossa*, we used a secondary calibration point taken from Shen et al. (2016). We used the mean (23.2 MYA), youngest (16.8 MYA) and oldest (33.2 MYA) age of the most recent common ancestor (MRCA) of *Bolitoglossa* and *Pseudoeurycea* considering the results of all dating analyses of Shen et al. (2016) (MultiDivTime, MCMCTREE, and BEAST).

We used BEAST v. 10.4 (Suchard et al., 2018) to date both topologies. Prior to running BEAST, we performed a pseudo-calibration using the command *chronos()* of APE, constrained with the youngest and oldest ages of our calibration point and using a relaxed clock model. We performed this analysis to generate a start time-calibrated topology with the ages matching the calibration point prior and obtaining the tree height prior for BEAST analysis. We used a Yule process tree prior and each topology was fixed omitting the tree operators "*subtreeSlide*", "*narrowExchange*", "*wideExchange*" and "*wilsonBalding*". We selected an uncorrelated relaxed log-normal clock model with rate variation among the branches for each partition (Drummond et al., 2006). We constrained the root and calibration point assuming a normal prior distribution (mean = 23.2 MYA, SD = 1 MYA, upper = 33.2 MYA, lower = 16.8 MYA). We ran two independent MCMC chains of 50 million generations each, sampling every 10.000 generations. We combined each Log file using LogCombiner v. 10.4 (Suchard et al., 2018) and checked the convergence of each MCMC parameter in Tracer v. 1.7 (Rambaut et al., 2018). We used TreeAnnotator v. 10.4 (Suchard et al., 2018) with a burn-in of 10% to calculate the maximum clade credibility tree.

2.5.2. Ancestral area reconstruction

To identify dispersal, vicariance, and extinction events between geographic areas, we used a dispersal-vicariance analysis (DIVA; Ronquist, 1997), as implemented in RASP v.4.0 (Yu et al., 2015), on the time-calibrate phylogenies.

Based on the known species distribution and the South American geomorphological domains proposed by Ab'Saber (1977), we selected three biogeographic units: Chocó, Andes, and Amazonia (Fig. 1). We combined Central America and Mesoamerica into a single region, hereafter Central America, as we were not investigating biogeographic events between those two regions. To separate South American species into Amazonian, Andean, and Chocoan, we compiled data of the distribution and elevation ranges for all South American species from the IUCN's web page, new data published here, and recent taxonomic and species descriptions (Brcko et al., 2013; Acevedo et al., 2013; Meza-Joya et al., 2017). Based on the elevation ranges plot (Fig. S1), we considered 1200 m a.s.l. as the elevational range limit. The break between highland (i.e., Andean) and lowland (i.e., Amazonian or Chocoan) taxa roughly coincides with the lower limit of mountain rain forest belts (c. 800–1200 m a.s.l. depending on specific local conditions; Hooghiemstra et al., 2006 and references therein) and only two of the evaluated species cross this elevational "barrier".

3. Results

3.1. Parsimony

Tree searches of the complete dataset in POY yielded six most parsimonious trees (12,610 steps). A final round of swapping using iterative pass optimization on these trees further reduced the cost (12,597 steps). The implied alignment contained 3,441 molecular transformation series (Supplementary data 1). Tree searches of the implied alignment in TNT yielded 1,751 most parsimonious trees. The strict consensus (Figs. 2 and 3) is well resolved with 34% polytomies of 381 possible nodes of a fully bifurcating tree. Most polytomies correspond to shallow clades involving specimens of the same or closely related species. Jackknife values are ≥ 75 in 59 clades and ≤ 50 in 31 clades. The implied alignment is available at https://datadryad.org/stash/share/EasgCOWXAduHRDJ5cx8Lck4kKWghDj4GrWywggaq8mg

The results recovered the monophyly of all sampled genera (Fig. 2) except *Pseudoeurycea*, which is paraphyletic in relation to *Ixalotriton niger*—this is the sister taxon of a clade formed by *P. cochranae*, *P. longicauda*, and *P. rex*. Within the genus *Bolitoglossa* (JK = 100), all currently recognized subgenera are monophyletic except *Mayamandra*, which is paraphyletic in relation to *Nanotriton* (JK = 100), because *B. stuarti* is more closely related to *Nanotriton* than to *B. hartwegi* (JK ≤ 50). The first split within *Bolitoglossa* separates a clade (JK = 85) comprising the subgenera *Bolitoglossa*, *Magnadigita*, *Mayamandra*, *Nanotriton*, *Oaxakia*, and *Pachymandra* from the subgenus *Eladinea* (JK = 100). The subgenus *Bolitoglossa* (JK = 100) is the sister taxon of *Mayamandra* and *Nanotriton* (JK = 98). Within the subgenus *Bolitoglossa*, *B. mexicana* is non-monophyletic because the sample *B. mulleri* UTA 50,475 is embedded within five samples of *B. mexicana* and because *B. mexicana* 1032 is more closely related to *B. odonnelli* MVZ 229,068 than to the other samples of *B. mexicana*. The sister clade of *Bolitoglossa*, *Mayamandra*, and *Nanotriton* contains the subgenera *Oaxakia* (JK = 99), *Pachymandra* (JK = 100), and *Magnadigita* (JK = 85) forming a polytomy. Within the latter subgenus, samples of *B. morio* are not monophyletic. The species *B. eremia*, *B. flavimembris*, and *B. pacaya* are also non-monophyletic.

Within *Eladinea*, all species groups currently recognized are monophyletic with the exception of *B. schizodactyla* and *B. adspersa* groups due to the position of *B. compacta*. This species is currently considered part of the *B. adspersa* group based on similarity to other species (Parra-Olea et al., 2004); however, our results indicate that it is nested within the *B. schizodactyla* group. Although Boza-Oviedo et al. (2012) included *B. compacta* in their analysis, their dataset did not include representatives of the *B. adspersa* group. The *B. epimela* (JK = 99) and *B. subpalmata* (JK = 99) groups are sister taxa (JK = 66). This clade is the sister taxon of the *B. robinsoni* (JK = 99), *B. schizodactyla* (JK = 89), and *B. adspersa* (JK = 84) groups. *Bolitoglossa nigrescens*, of the *B. schizodactyla* group, is non-monophyletic with respect to *B. sombra*.

The *Bolitoglossa adspersa* group includes all South American species of the genus plus a few species from the Chocó and Darién of Panama, such as *B. biseriata*, *B. chucantiensis*, *B. medemi*, and *B. taylori*. Our results indicate that samples identified as *B. biseriata* are non-monophyletic because the two samples from Pericos, Colombia (AFJ 06 and 10) are more closely related to *B. sima* than to the other samples of *B. biseriata*. *Bolitoglossa walkeri* is also non-monophyletic because one of our samples is more closely related to a sample of a putative new species from Chilma, Ecuador. All of our 177 samples of *Bolitoglossa* from Amazonia, but six, form an exclusive monophyletic group. The exception includes specimens of *B. palmata* (highlands of Ecuador) and three samples of a putative new species (*B.* sp. 33) from the Amazonian lowlands of Loreto, Peru; both taxa are more closely related to Andean species from outside Amazonia such as *B. adspersa* (from the western flank of the Cordillera Oriental of Colombia) and *B. leandrae* (from the Andes of the Orinoco basin).

To facilitate comparisons and discussions among results of

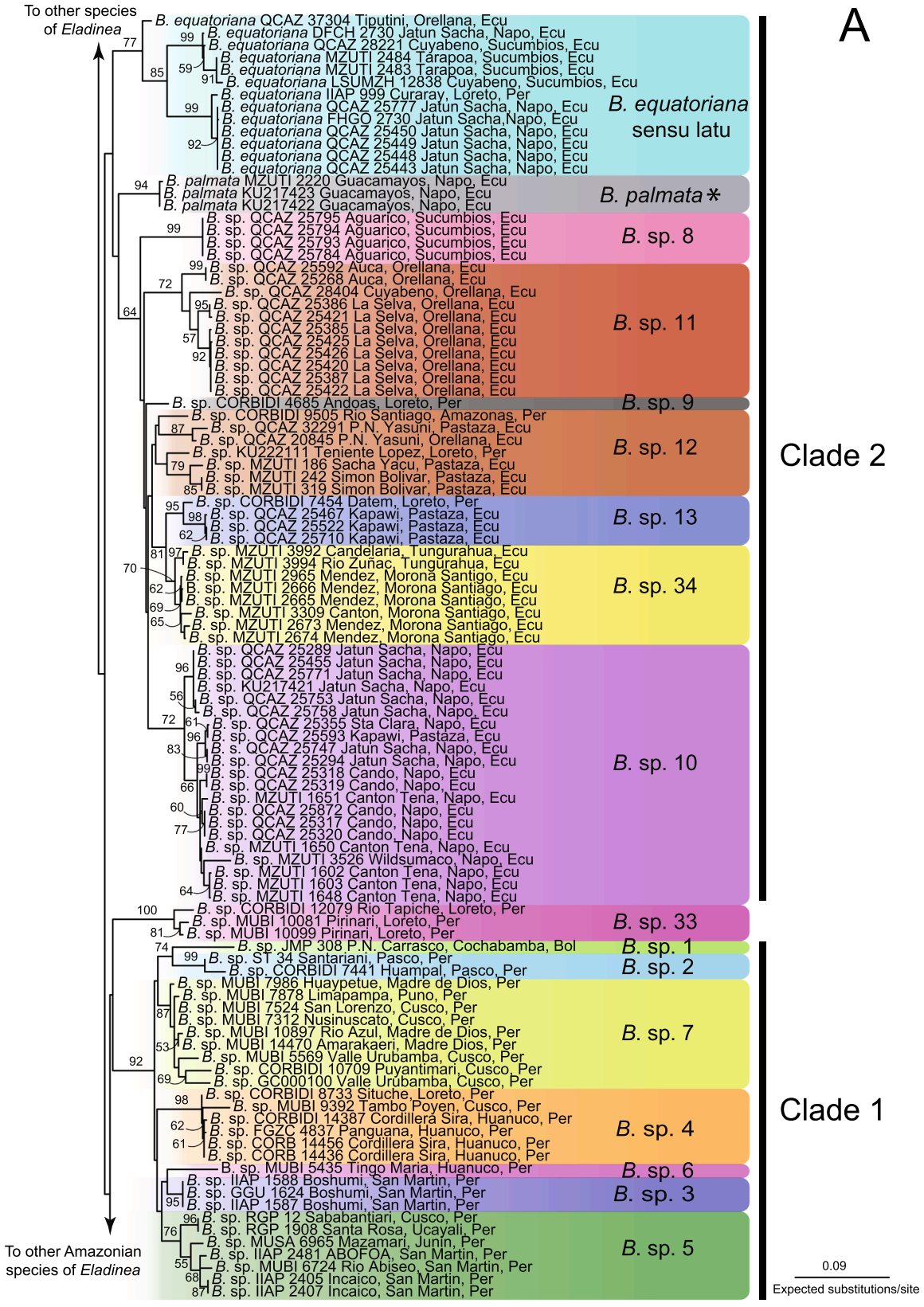

Fig. 4. Maximum likelihood tree (log likelihood = − 59863.1) of Amazonian *Bolitoglossa*, subgenus *Eladinea*, inferred from up to three mitochondrial (16S, *COI*, and *cytb*) and two nuclear (*POMC* and *Rag1*) partial gene sequences from a similarity alignment and considering indels as the longest possible binary characters. Numbers on branches are bootstrap frequencies (percent) of 1000 pseudoreplicates. Nominal and candidate species according to this work are indicated with color rectangles. Clades 1 to 7 indicate groups with equal content in the parsimony analysis (except for *Bolitoglossa palmata* marked with an asterisk); see main text for discussion. This tree is a continuation of Fig. 2 (left). A, Clades 1 to 2; B, Clades 3 to 7.

parsimony and ML for Amazonian *Eladinea*, we labeled seven clades (numbers 1 to 7, Figs. 3–5) that are identical in content in both analyses except for Clade 2 because the parsimony optimal trees do not include *B. palmata*. Nevertheless, the optimal evolutionary relationships among these clades have in general JK and BS ≤ 50, indicating that there is conflicting evidence (i.e., transformations that are against the optimal clades) in both alignments, few transformations supporting the clades (regardless of conflict) or a high proportion of missing data. Clade 1 (JK = 94) is the sister taxon of a clade formed by all other six clades (JK ≤ 50) (Fig. 3). None of the currently recognized species of

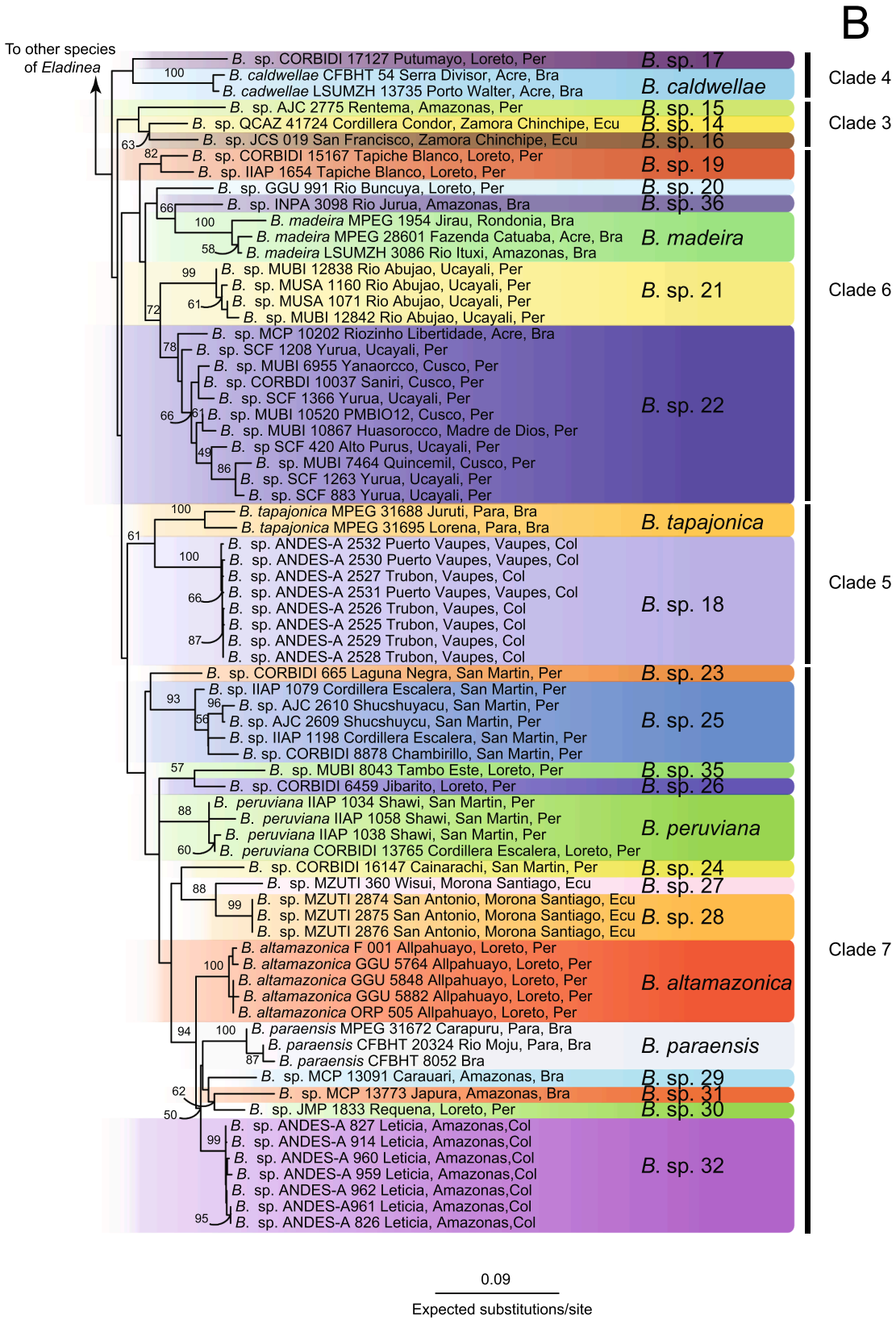

Fig. 4. (*continued*)

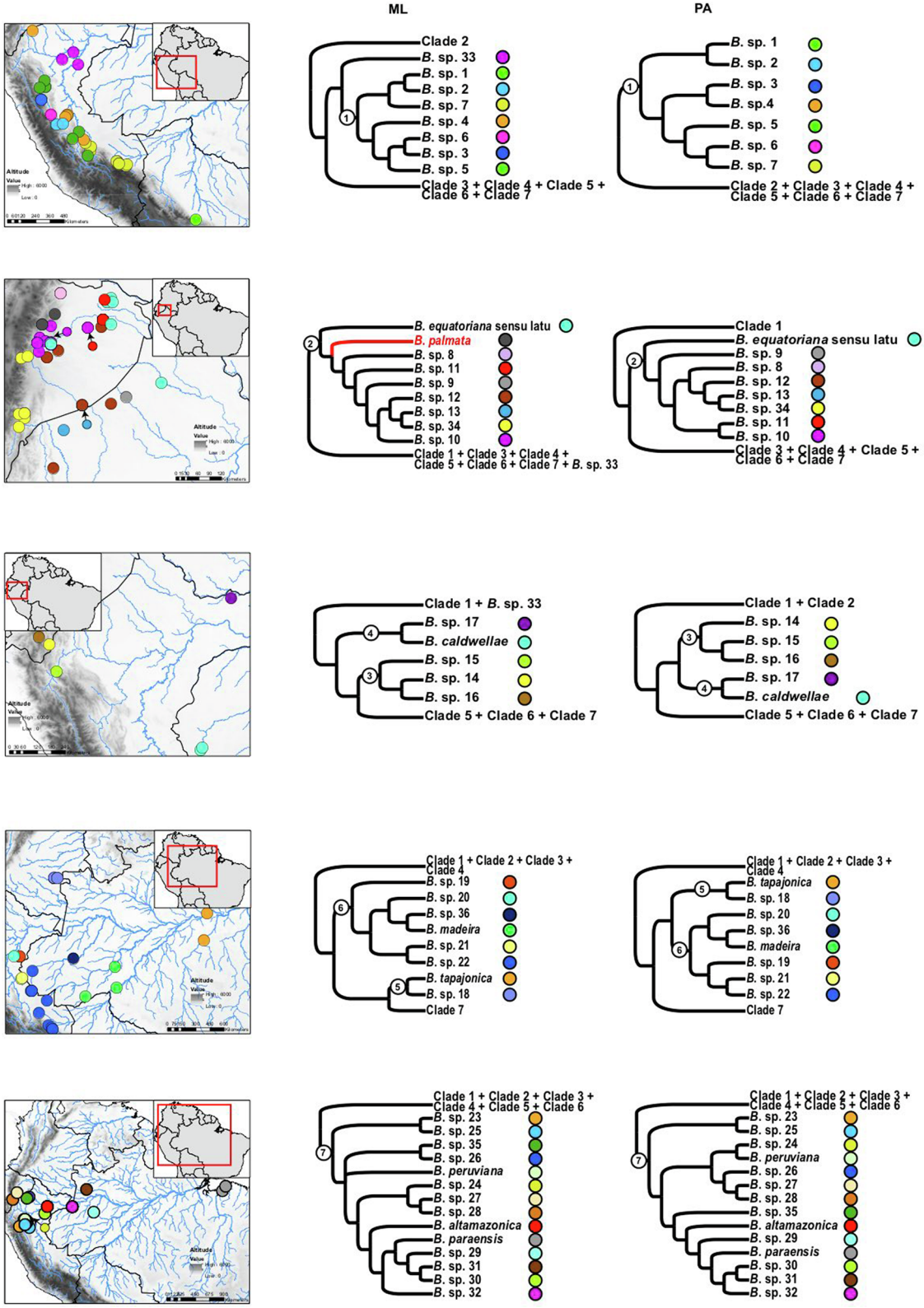

Fig. 5. Maps illustrating the known localities (dots) of nominal and candidate species of *Bolitoglossa* from Amazonia according to the results of this study. Trees represent schematic relationships according to maximum likelihood (ML) and parsimony analyses (PA) for the different clades—marked as circles in ancestral nodes—and follow those illustrated in Figs. 3 and 4. Arrows in maps indicate overlapping localities.

Bolitoglossa from Amazonia is part of this clade. All specimens that are part of Clade 1 were found in western Amazonia between 236 and 1050 m a.s.l., following the arc described by the eastern slopes of the Andes from central Bolivia in the south to the Peruvian border with Ecuador in the north (Figs. 1 and 5). Relationships among clades within Clade 1 are not resolved (i.e., polytomy in the strict consensus). Clade 2 (JK = 54) comprises samples that are restricted to Ecuador and northern Peru between 187 and 1920 m a.s.l. (Figs. 1 and 5), including *B. equatoriana sensu lato*. Clade 3 (JK ≤ 50) comprises specimens from different localities on the eastern slopes of the Andes (664–1953 m a.s.l.) of southern Ecuador and northern Peru (Figs. 1 and 5). Clade 4 (JK ≤ 50) is restricted to the northwestern Amazonian lowlands and includes *B. caldwellae* from the Jurua River in Brazil, and a sample from the Putumayo River in Peru (Figs. 1 and 5). Clade 5 (JK = 95) is exclusively represented by lowland taxa (30–195 m a.s.l.) and it includes specimens from the Vaupés River in Colombia, and *B. tapajonica* from the Tapajós River in eastern Amazonia (Figs. 5 and 1B). Clade 6 (JK = 71) also is exclusively represented by lowland taxa (64–777 m a.s.l.) and it contains *B. madeira* and specimens from Rio Tapiche in Loreto to the foothills of the Andes in Madre de Dios, both in Peru, following a north–south axis, and from there to the Rivers Juruá, Purús, and Madeira in Brazil (*B. madeira*; Figs. 1 and 5). Clade 7 (JK = 75) includes *B. altamazonica*, *B. paraensis*, *B. peruviana*, and several lineages not assigned to these nominal species. Samples originated from 12 to 1788 m a.s.l (Figs. 1 and 5) including western localities associated with the eastern slopes of the Andes of northern Peru and southern Ecuador (e.g., *B. peruviana*) and others distributed along the axis of the Amazon River from Requena, Loreto, Peru in the west to the right bank of Guamá River, Santa Isabel do Pará, Pará, Brazil (*B. paraensis*).

Among outgroup taxa, the YBYRÁ analysis identified as the top wildcards (Table S4) the terminals of *Bolitoglossa pacaya* (all USAC series) and *B. morio* (USAC 1568 and MVZ 257825). These terminals are responsible for most of the incongruence among optimal topologies, resulting in a large polytomy that also includes the terminals of *B. eremia* and *B. suchitanensis* (Fig. 2). Most of these terminals are represented in our dataset only by 16S and *cytb*, indicating that, at this level of universality, either there is not enough information in these markers or that the information is contradictory. Other terminals represented in our dataset by these markers alone (i.e., *B. adspersa* MVZ 158485, *B. aurae* UCR 22842, *B. palmata* KU 217422, *B. robusta* MVZ 190830, *B. tica* UCR 20514, and *B. zapoteca* IBH 13375) were not recovered as wildcards. Within the ingroup, the terminal *B.* sp. MZUTI 3526 was the top potential wildcard. This terminal is represented by sequences of 16S and *Rag1* and causes the polytomy of *B.* sp. 10 and *B.* sp. 11 (Fig. 3, see below for the reasoning to identify some clades as putative new species). The terminal *B. equatoriana* QCAZ 37304, only represented by *Rag1* in the dataset, was recovered as the 9th top wildcard terminal (Table S4) and causes the collapse of the *B. equatoriana* complex. Other wildcard terminals seem to collapse conspecific relationships, such as *B.* sp. MZUTI 1603 and 1650 within *B.* sp. 11 or the terminals belonging to *B. yariguiensis*.

3.2. Maximum likelihood

The similarity alignment of DNA sequences includes 3,252 transformation series and the binary block codifying indels (Supplementary data 2). The selected models and partition scheme are indicated in Table 1. Tree searches of the complete dataset in Garli revealed a single most likely tree (log likelihood = − 5,9863.1). The optimal tree is shown in Figs. 2 and 4. As in the parsimony strict consensus, several shallow clades corresponding to intraspecific relationships are collapsed. Nonetheless, the optimal tree is well resolved with 17% polytomies of 381 possible nodes of a fully bifurcating tree. Bootstrap values are ≥ 75% in 50 clades and ≤ 50% in 23 clades. In general, the topology is consistent with the topology obtained with parsimony, and we only report relevant differences below.

Relationships among the subgenera of *Bolitoglossa* (BS = 100) are similar to those of parsimony except that all currently recognized subgenera are monophyletic in ML and that *Pachymandra* (BS = 100) is the sister taxon of *Magnadigita* (BS = 76). Within the *Bolitoglossa adspersa* species group, *B. walkeri* is monophyletic.

Regarding Amazonian salamanders, ML recovers all of them as a monophyletic group (BS ≤ 50) separated from salamanders from other regions. In this regard, it differs from the parsimony trees, where *B. palmata* and *B.* sp. 33 are more closely related to species outside Amazonia. There are important differences regarding how Clades 1 to 7 relate to each other. The best ML tree recovers Clade 2 (BS ≤ 50) as the sister taxon of a group that includes Clades 3 to 7.

3.3. Species diversity of Amazonian salamanders

Assigning clades to nominal species was straightforward except for *Bolitoglossa equatoriana* due to the conflicting results between parsimony and ML (Figs. 2–4). For this species, the problem seems to be the sample *B. equatoriana* QCAZ 37304 from Tiputini, Napo, which is the closest (about 43 km) to the type locality in Limón Cocha, Napo, Ecuador, and causes a polytomy on the strict consensus of the parsimony optimal trees. This sample is one of the top 10 wildcard terminals of the ingroup, probably because it is represented just by *Rag1*—a nuclear-protein coding gene with low nucleotide variation at this level of comparison. The ML optimal tree places this sample as the sister taxon of two clades, one (BS = 99) including samples from Cuyabeno, Jatun Sacha and Tarapoa, Ecuador, and the other one (BS = 99) with samples just from Jatun Sacha, Ecuador. Given the current situation and until more data are gathered for sample QCAZ 37304 or from new samples from the type locality, we prefer to be conservative and consider all the

Table 1
Partition scheme, models of nucleotide substitution, and number of sites per partition selected by the PARTITIONFINDER analysis.

Partition	MUSCLE (complete)	# sites	Implied alignment (reduced)	# sites	MUSCLE (reduced)	# sites
16S	GTR + I + G	560	GTR + I + G	656	GTR + I + G	560
COI, first position	TRN + I	196	TRN + I + G	201	TRN + I	196
COI, second position	TRN + G	196	K80 + G	201	TRN + G	196
COI, third position	SYM + G	195	TRN + G	200	SYM + I	195
cytb, first position	TRN + I + G	269	TRN + I + G	289	TRN + I + G	269
cytb, second position	GTR + G	269	HKY + I + G	289	GTR + G	269
cytb, third position	SYM + I + G	269	HKY + I + G	288	SYM + I + G	269
POMC, first position	TRN + G	161	HKY + G	162	TRN + I	144
POMC, second position	GTR + I + G	160	TRN + G	162	TRN + G	144
POMC, third position	TRN + I	160	K80 + G	161	GTR + I	143
Rag1, first position	GTR + G	273	K80 + I + G	278	GTR + G	273
Rag1, second position	SYM + I + G	272	SYM + I + G	277	GTR + I + G	272
Rag1, third position	GTR + I + G	272	HKY + G	277	GTR + I + G	272
AICc	117854.64		70850.02		63296.63	

aforementioned samples as *B. equatoriana* sensu lato, although it is obvious that at least two independent lineages are currently included under this name.

Considering the tree topologies and the identification of nominal species, the phylogenetic position of the other samples from the Amazonia clearly indicates that the results are not compatible with the recognition of just nine independently evolving lineages—the current number of recognized species in the region—unless one is ready to consider rampantly non-monophyletic species with very large ranges across important geographic barriers (e.g., the Amazon River and its main tributaries) and encompassing levels of interspecific morphological variation unknown in other plethodontids. We prefer to explain the observed historical (i.e., topologies) and phenetic (i.e., genetic distances) patterns of nucleotide variation, in consilience with the geographic distribution of the samples and the known morphological variation (e.g., Brcko et al., 2013), as compatible with the existence of 36 candidate new species of *Bolitoglossa* in the Amazonia (color boxes in Figs. 2–5). Within these 36 candidate new species, the pairs *B*. sp. 1 and *B*. sp. 2 and *B*. sp. 10 and *B*. sp. 11 are not reciprocally monophyletic in the parsimony analysis, although they are in the ML tree, and deserve further evaluation. In the first case, the only sample available from Bolivia (*B*. sp. 1 JMP 308) forms a polytomy with two samples from Pasco, in central Peru in the parsimony consensus tree (JK = 81). On the other hand, the ML optimal tree recovers the two samples from Pasco as monophyletic (BS = 99) and as the sister taxon of the sample from Bolivia (BS = 74). The branch corresponding to the Bolivian sample is much longer than those of the Pasco samples (in both parsimony and ML), and the genetic distance between the Pasco samples is 1.1% for 16S (the only shared marker between them), while it is 11.0% between the Bolivian and the Pasco sample for *cytb* (the only shared marker between them). Taking into account the reciprocal monophyly in ML, the longer branch length and larger genetic distance of the Bolivian sample, and the large geographic gap between Carrasco, Bolivia and Pasco, Peru, we consider these specimens as part of two unconfirmed candidate species rather than of a single biological entity. In the second case, the strict consensus of the most parsimonious trees collapses samples labeled *B*. sp. 10 and *B*. sp. 11 into a large polytomy. However, the ML optimal tree recovers them not only as reciprocally monophyletic but also as non-sister groups, although the branches separating these clades in ML are short and with BS ≤ 50. It is also relevant that the samples forming clade *B*. sp. 10 are all from the Andean foothills (277–705 m a.s.l.) of Napo and Pastaza, Ecuador, while those within *B*. sp. 11 are all from the lowlands (≤277 m a.s.l.) of Orellana, Ecuador. Genetic distances for *cytb* (the only shared mitochondrial marker) within *B*. sp. 10 = 0.0–3.6% and within *B*. sp. 11 = 0.0–5.5%, while distances between samples of *B*. sp. 10 and *B*. sp. 11 = 8.7–12.1%. The wildcard analysis recovered the terminal *B*. sp. MZUTI 3526 as the top potential wildcard within the ingroup. This terminal changes position between different places within *B*. sp. 10 and *B*. sp. 11 in the different most parsimonious trees, apparently causing the polytomy in the strict consensus. The sample *B*. sp. MZUTI 3526 contains information just for 16S and *Rag1*, and this may be the cause of its wildcard behavior because samples of *B*. sp. 11 only share *Rag1*. Considering all the aforementioned factors, we preferred to maintain *B*. sp. 10 and *B*. sp. 11 as two different unconfirmed candidate species.

Genetic intra and interspecific uncorrected genetic *p*-distances for 16S and *cytb* among the delimited units are summarized in Table S5. The minimal distance between nominal species (excluding *B. equatoriana* sensu lato for the reasons outlined above) and other Amazonian salamanders (including putative species proposed here) ranges from 1.6 to 3.2% and 5.1–10.4% in 16S and *cytb* respectively. Interestingly, the topotype samples of *B. altamazonica* and the sample *B. sp*. MCP 13,091 from Japurá, Brazil (*B*. sp. 29) show the smallest genetic distance, but both parsimony and ML recovered this specimen as more closely related to specimens of *B. paraensis* and candidate species from Requena (Peru) and Leticia (Colombia) (Figs. 3 and 4).

The ABGD analyses did not reveal a well-defined barcoding gap for 16S or *cytb*. Based on the distribution of genetic distances, the program calculated the number of potential species using four threshold values of maximum divergence of intraspecific diversity (P) (Table 2). However, according to Puillandre et al. (2012) and Pardo et al. (2014) the lowest and highest thresholds of an analysis can lead to trivial delimitations, where every terminal is considered a species or all terminals are included into a single one. Thus, we focused on two intermediate values for each marker that we labeled 16S1 (P = 0.006), 16S2 (P = 0.014), *cytb*1 (P = 0.009), and *cytb*2 (P = 0.036). It is worth noting that (i) not all samples are represented by the same markers so that some candidate species cannot be evaluated by the ABGD analyses and (ii) several of the putative species proposed by the ABGD analyses of 16S1 and 16S2 are incompatible with the inferred evolutionary history represented by the optimal phylogenetic trees (Fig. 6). The mPTP analyses recognized 42 species, including eight singletons, with the best score of multi coalescent rate of 1039.7 (Fig. 6).

When we compared the results of the number and limits of species suggested by our *congruence* approach with the results of the ABGD and mPTP analyses, they all agree in recognizing many more (23–49 species) than the currently nine recognized species in the Amazonia (Fig. 6; Table 3). Nonetheless, the total number and limits of these units vary among analyses, which is expected considering their different assumptions and type of information used to make inferences. Regarding nominal species and using the results of the *congruence* approach as a measuring stick, *Bolitoglossa altamazonica*, *B. palmata*, and *B. paraensis* have coinciding limits in all analyses; *Bolitoglossa caldwellae* is delimited differently by ABGD 16S2 and mPTP; both ABGD (except for 16S2) and mPTP recognized the existence of more than one species within *B. equatoriana* sensu lato; *Bolitoglossa madeira* is delimited differently by all analyses but ABGD *cytb*; *Bolitoglossa peruviana* differs only in the delimitation of ABGD 16S2; *Bolitoglossa tapajonica* is subdivided into two species by ABGD 16S1, 2 and *cytb*1. Among the 36 putative new species identified through our *congruence* approach, six are delimited identically among all the ABGD and mPTP analyses, while 13 are incongruent with only one of the objective species-delimitation approaches (Fig. 6). The R_{tax} values for our data set were lowest for ABGD 16S 2 (R_{tax} = 0.37) and highest for ABGD *cytb*1 (R_{tax} = 0.79) and *congruence* approach (R_{tax} = 0.73), consistent with the total number of species suggested by the different approach (Table 3). Congruence

Table 2
Number of sample clusters resulting from the ABGD analyses of a similarity alignment of 16S and *cytb* according to different values of maximum divergence of intraspecific diversity (P) assigned by the program. Number of clusters can be used as a proxy to number of species, notwithstanding important assumptions. Values in italics represent intermediate values that are further discussed in the main text.

Maximum divergence of intraspecific diversity	**16S** P = 1.0×10^{-3}–4.5×10^{-3}	P = *6.5×10^{-3}*	P = 9.4×10^{-3}	P = *1.4×10^{-2}*	P = 2.0×10^{-2}
# clusters	53	*41*	25	*23*	2
	cytb				
Maximum divergence of intraspecific diversity	P = 1.0×10^{-3}–6.0×10^{-3}	P = *9.4×10^{-3}–1.5×10^{-2}*	P = 2.3×10^{-2}	P = *3.6×10^{-2}*	P = 5.7×10^{-2}
# clusters	52–55	*49*	41	*29*	1

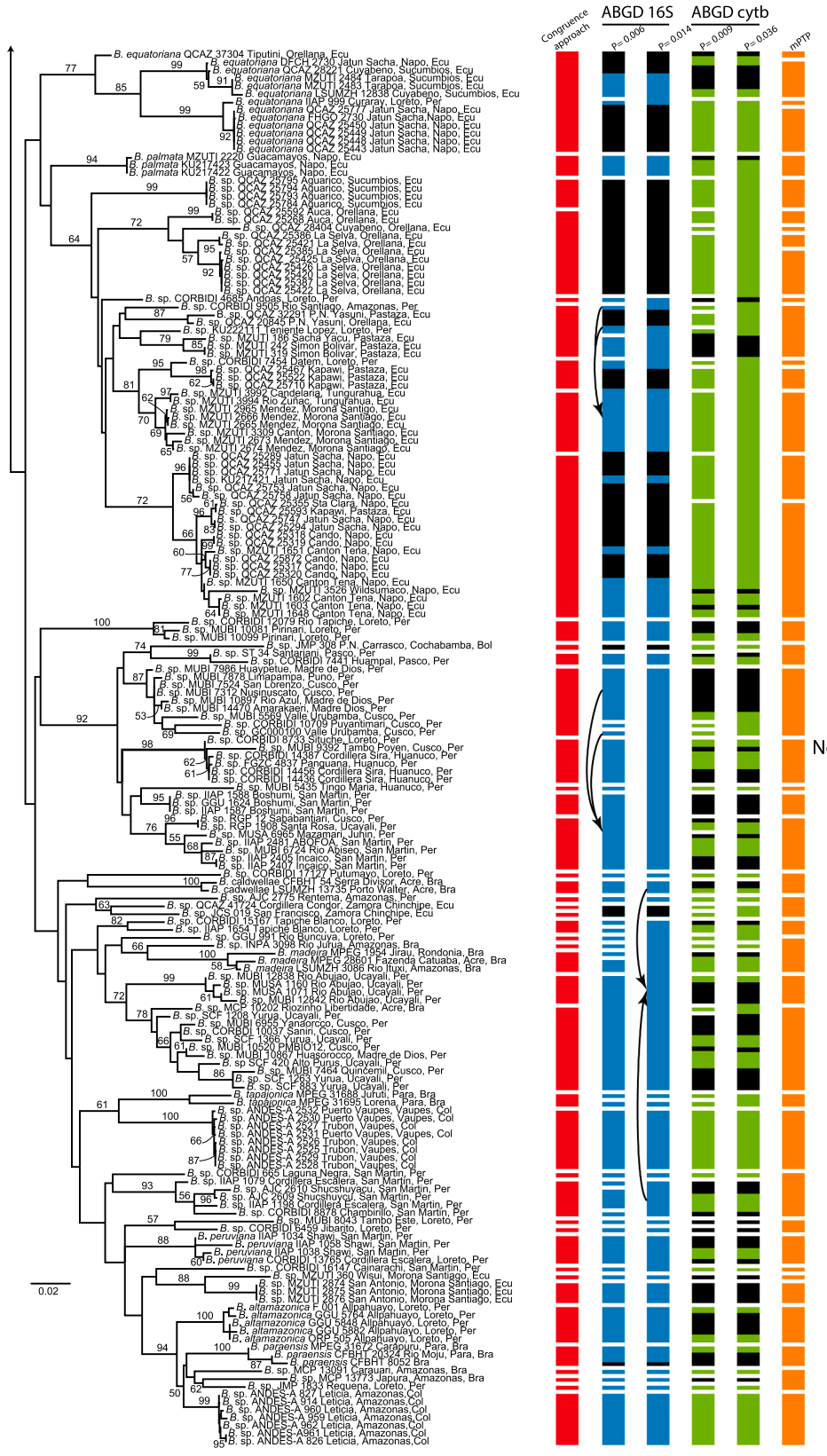

Fig. 6. Putative species as inferred by our *congruence* approach or by mPTP or ABGD using different genes and distance thresholds. On the left, maximum likelihood phylogenetic relationships of Amazonian *Bolitoglossa* with numbers on branches indicating bootstrap values. Bars on the right indicate inferred species according to different approaches. Arrows indicate terminals or clades that were clustered inside other clades for a given analysis.

Table 3
Summary of performance of methods using the Relative Taxonomic Resolving Power Index (R_{tax}) and the Taxonomic Index of Congruence (C_{tax}). R_{tax} quantifies the relative power of a method to infer all estimated speciation events (large Rtax means small type II error). C_{tax} measures the congruence in delimitation between two methods, with a value of 1 indicating complete congruence.

Delimitation method	N°. species	Rtax	Mean Ctax	Ctax				
				ABGD 16S 1	ABGD 16S 2	ABGD *cytb* 1	ABGD *cytb* 2	mPTP
ABGD 16S 1	41	0.66	0.74					
ABGD 16S 2	23	0.37	0.56	0.63				
ABGD *cytb* 1	49	0.79	0.65	0.78	0.48			
ABGD *cytb* 2	29	0.47	0.65	0.74	0.63	0.59		
mPTP	42	0.69	0.64	0.64	0.50	0.65	0.55	
Congruence	44	0.73	0.72	0.74	0.56	0.80	0.72	0.71
All speciation events	62							

among methods (C_{tax}), the similarity among the results obtained, was highest between our *congruence* approaches and ABGD *cytb*1 (C_{tax} = 0.80) and lowest between ABGD 16S2 and ABGD *cytb*1 (C_{tax} = 0.48) (Table 3).

3.4. Biogeography

The selected models and partition scheme for the reduced alignments are indicated in Table 1. The inferred ages of nodes are overall similar between both datasets, with the difference that implied alignment + parsimony topology (IA + P) resulted in slightly older ages and narrow range in the 95% highest posterior density than MUSCLE alignment + Maximum Likelihood topology (M + ML) (Fig. S3 and Table S6). The split between *Eladinea* with the other *Bolitoglossa* subgenera occurred about 21.3 MYA (HPD 95%: 19.0 – 23.6 MYA, IA + P) or 20.4 MYA (HPD 95%: 17.5 – 23.4 MYA, M + ML), and started to diversify 14.3 MYA (12.4–16.2 MYA, IA + P) or 14.5 MYA (11.9–17.1 MYA, M + ML). Within *Eladinea*, the South American clade diverged from Central American species 10.1 MYA (8.8–11.5 MYA, IA + P) or 10.2 MYA (8.4–12.0, M + ML). The main Amazonian clade split from Andean species 8.3 MYA (7.2–9.3 MYA, IA + P) or 8.1 MYA (6.8–9.5, M + ML), and clades 1, 2 and the large clade that contains the

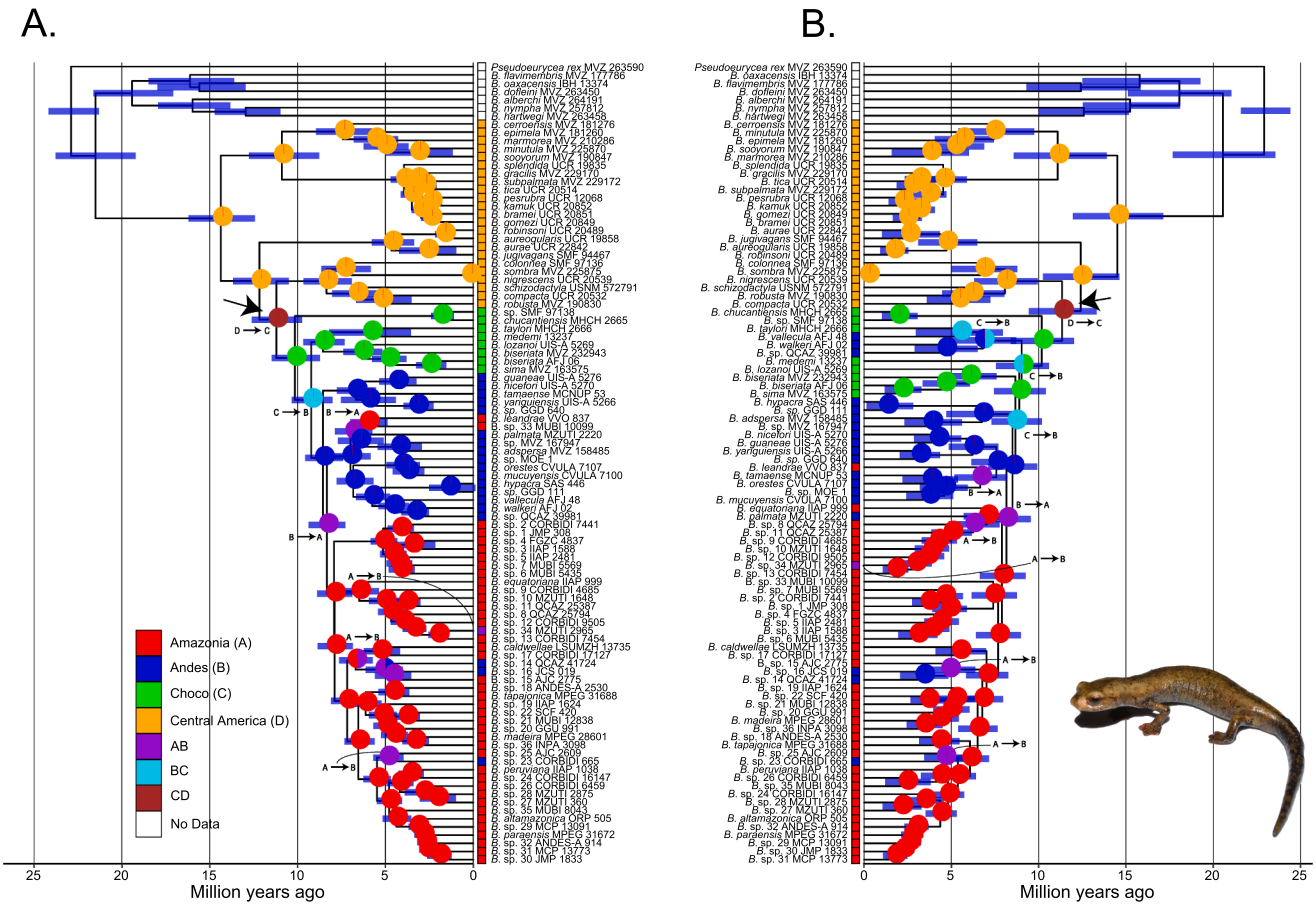

Fig. 7. Ancestral area reconstruction and time-calibrated phylogeny of the subgenus *Eladinea* using one of the most parsimonious trees (A) and the most likely tree (B). In both cases, the tree was pruned to keep one terminal per species. Polytomies were solved randomly, but the alternatives do not affect the results. Blue bars indicate the lower and upper values of 95% highest posterior density for divergence times. Colors indicate presence of a taxon in an area, with squares indicating distribution of terminals and circles inferred distribution of ancestors. Dispersals are marked with letters on the corresponding branches. The arrows indicate the inferred colonization of South America from Central America. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Amazonian clades 3 to 7 diverged from each the other in a short time period 7.85–7.88 MYA (IA + P) or 7.66–7.88 MYA (M + ML) (Fig. 7).

The species relationships inferred by both phylogenetic methods included some differences in the ancestral area reconstructions of parsimony and ML (Fig. 7). However, the incongruences are minor, and the most important biogeographic events are shared between reconstructions. Both biogeographic histories show a unique dispersal event from Central America to Chocó, explaining the presence of salamanders in South America. This was followed by one (parsimony) or two (ML) dispersals into the Andes from the Chocó and two dispersals from the Andes to Amazonia (Fig. 7). One contributed with just one (ML) or two species (parsimony), while the second dispersal event was followed by an impressive diversification of Amazonian salamanders (37–38 spp. according to our results) that went back into the highlands of the Andes in three (parsimony) or four occasions (ML).

4. Discussion

4.1. Phylogenetic relationships of Bolitoglossa within Bolitoglossini

Different topologies resulted from each optimization method; *Bolitoglossa* is the sister taxon of *Aquiloeurycea* (ML) or of a clade including *Aquiloeurycea*, *Pseudoeurycea*, and *Ixalotriton* (parsimony). Both alternatives have been suggested by previous studies using different analytical premises and datasets. For example, Rovito et al. (2015: Figs. 4 and 5) found *Bolitoglossa* as the sister taxon of *Aquiloeurycea* + *Isthmura* (the latter not represented in our dataset), whereas Wiens et al. (2007), Pyron and Wiens (2011) and Rovito et al. (2015: Figs. 2, 3, 6) found *Bolitoglossa* as the sister taxon of more inclusive clades, in terms of supraspecific taxa, that included *Aquiloeurycea*, *Pseudoeurycea*, and *Ixalotriton*. Several non-mutually exclusive factors could be behind these differences—such as taxon and character sampling, optimality criteria, exhaustiveness of tree searches, treatment of indels, data partition schemes, model selection, and alignment parameters—and, without detailed sensitivity analysis, it is impossible to assess which factor or combination of them is causing the incongruence among different studies. Our study was designed with different objectives and our only germane contribution is that relationships among Bolitoglossini should be revisited in light of our new data.

4.2. Phylogenetic relationships within Bolitoglossa

After Parra-Olea et al. (2004), only a few studies have a comparable taxon sampling of *Bolitoglossa* (Wiens et al., 2007; Pyron and Wiens, 2011; Elmer et al., 2013). Nonetheless, all of them differ in important aspects of the relationships among subgenera and species within them, and even the monophyly of some subgenera has been questioned. For example, some *Magnadigita* species were nested within *Pachymandra* (Wiens et al., 2007; Elmer et al., 2013) or *Oaxakia* (Pyron and Wiens, 2011). Our study, with 73% of the currently described species of the genus and sequences from up to five genes, constitutes the largest effort to address the evolutionary relationships of *Bolitoglossa*. Despite important differences in our analytical assumptions regarding nucleotide homology, indel coding, and optimization criterion, the results of parsimony and ML analyses are very much congruent (although not identical) regarding the relationships among subgenera of *Bolitoglossa*. Both analyses agree in placing a monophyletic *Eladinea* as the sister taxon of a clade with the other six subgenera. Within the latter clade, the subgenus *Bolitoglossa* is the sister taxon of *Mayamandra* + *Nanotriton*, although in parsimony, *B.* (*Mayamandra*) *stuarti* is more closely related to *Nanotriton* than to other species of *Mayamandra*. Regarding the position of *B.* (*Mayamandra*) *stuarti*, it is relevant to note that this is the first time that this species is included in a large-scale phylogenetic study of *Bolitoglossa*, and that in our dataset is represented only by a single marker (609 nucleotides of *cytb*). We consider evidence still too limited to suggest nomenclatural changes, although future studies should revisit the subgeneric placement of this taxon.

The relationships among the three remaining subgenera are also different between the two analyses, with *Oaxakia* as the sister taxon of *Pachymandra* + *Magnadigita* in ML while in parsimony we retrieved a polytomy among the three subgenera. Similar cases in which parsimony retrieved a polytomy while ML found a bifurcating tree involve the basal relationships of *B. equatoriana* or those between *B.* sp. 11 and *B.* sp. 12. Two non-exclusive explanations could be behind the observed pattern of more polytomies in the strict consensus of the parsimony optimal trees. On the one hand, current implementations of ML and Bayesian posterior probability perform a limited number of less intense heuristic searches than thorough search strategies in programs such as TNT (Goloboff and Pol, 2005; Goloboff, 2014), holding only one tree for every tree search and pseudoreplicate, at least in ML. As a result, unsupported clades may be resolved and a high BS value or clade posterior probability assigned to them (Goloboff and Pol, 2005; Simmons and Goloboff, 2013; Simmons and Randle, 2014; Sanderson et al., 2015; Dobrin et al., 2018). These tree undersampling artifacts are more likely when analyzing supermatrices, consisting mostly or entirely of locally sampled characters, but can also affect smaller and more complete matrices (Simmons and Goloboff, 2013). Thus, clades recovered as a polytomy by parsimony analyses and completely resolved by ML or Bayesian analyses must be interpreted cautiously. For example, Padial et al. (2014) provided a clear empirical case of such artifact with *Eleutherodactylus* frogs. At least for some ML implementations, new approaches are being developed to evaluate some of these cases (Biczok et al., 2018), although they need a root with fully sampled characters, which our dataset lacks. On the other hand, the increase in resolution observed in our ML results, when compared to parsimony, could be related to the expectations of homogeneity incorporated in the models used in our ML analysis. These expectations could count as evidence nucleotides that would be rendered uninformative under parsimony.

4.3. Species richness of Amazonian salamanders

With more than 7 million km^2 (more than twice the area of India), the Pan-Amazonian lowlands constitute the largest stretch of tropical rainforest in the world. It also seems to be the most species-diverse region, with amphibians as a clear example of this pattern. With reports of more than 100 species in less than 6 km^2 (Bass et al., 2010), these amphibian communities have no rival among tropical ecosystems (Jenkins et al., 2013). However, this already outstanding amphibian species richness is dramatically underestimated. Several studies with anurans document an unexpected high diversity of new species, representing an increase of 22–350% over the known diversity (e.g., Fouquet et al., 2007a,b; Funk et al., 2012; Jungfer et al., 2013; Gehara et al., 2014; Rojas et al., 2018). Elmer et al. (2013) showed that the diversity of Amazonian salamanders in Ecuador was higher than previously thought. Our results not only corroborate the findings of Elmer et al. (2013), but also show very high levels of species richness of Amazonian salamanders elsewhere. If we considered all candidate species from our *congruence* approach, in Amazonia alone there would be 36 new species, an increase of 400% over those previously known. This result surpasses any previous estimation of amphibian cryptic diversity (Fouquet et al., 2007a,b; Padial and De la Riva, 2009; Angulo and Icochea, 2010; Funk et al., 2012; Jungfer et al., 2013; Caminer and Ron, 2014; Fouquet et al., 2014; Gehara et al., 2014; Lourenço et al., 2015), and confirms that South American *Bolitoglossa* is one of the most poorly studied amphibian groups. Even if the number of new species is smaller than our current inferences, large portions of the Andes and the Amazonian lowlands remain to be explored (Mayer et al., 2019) and more new species are likely to be discovered.

Our results have important implications for the currently recognized Amazonian species of *Bolitoglossa*. The type locality of *B. altamazonica* is Nauta, Loreto, Peru, and our samples assigned to this species are from just around 50 km from the type locality on a continuous stretch of

forest without barriers. The only sample in the literature with DNA sequences identified as *B. altamazonica* (KU 222111 from Loreto, Peru; Parra-Olea et al., 2004; Elmer et al., 2013) is distantly related to our samples of *B. altamazonica* (4.3–5.4% in 16S and 10.3–13.6% in *cytb*) and is herein considered part of *B.* sp. 12. With the evidence at hand, *B. altamazonica* has changed from a catchall name used for specimens from Venezuela to Bolivia and from Ecuador to Brazil into a micro-endemic species restricted to *terra-firme* forests in northern Peru, between the rivers Nanay in the north, Tigre and Marañón in the south, and Amazon in the west (Fig. 1). Considering that the type material of this species is lost, the designation of a neotype and a careful re-description is most needed.

The type locality of *B. peruviana* is Moyobamba, San Martín, Peru. Our samples of *B. peruviana* come from Shawi, San Martín, Peru, located at about 41 km from the type locality. The Ecuadorian samples identified as *B.* cf. *peruviana* by Elmer et al. (2013) are distantly related to our samples of *B. peruviana* and with considerably large genetic distances (5.4–6.5% in 16S and 10.6–14.0% in *cytb*). Herein, we considered these samples as part of three candidate new species (*B.* sp. 8, *B.* sp. 10, and *B.* sp. 11). As in the case of *B. altamazonica*, our data indicate that *B. peruviana* has a much more restricted distribution than previously thought. We currently consider this lineage restricted to the northeastern flank of the Cordillera Escalera in Peru.

4.4. Biogeography and diversification of South American salamanders

Our results agree with previous studies showing that *Bolitoglossa* colonized South America from Central America (Dunn, 1926; Brame and Wake, 1963; Wake and Lynch, 1976; Parra-Olea et al., 2004; Elmer et al., 2013; Rovito et al., 2015), although we estimate that this dispersal may have occurred 14.7–9.4 MYA (Fig. 7; Table S6). This time interval is coincident with a recently proposed land-bridge connecting Central and South America (Montes et al., 2015). Following the results of our DIVA analysis, the MRCA of *B. colonnea* and *B. altamazonica* could have used this land connection to move into South America, and this led to a speciation by vicariance between Central and South America once the sea isolated both land-masses (Fig. 7).

Within South America, we infer a clear pattern of dispersal between adjacent areas from Central America to the Chocó, from the Chocó into the Andes, and from the Andes into Amazonia (Fig. 7). According to our results, these dispersals happened in rapid succession (Fig. 7; Table S6). The presence of *Bolitoglossa* in the Amazonian lowlands is due to two independent dispersal events from the Andes. One is rather anecdotal in terms of diversification because it explains the presence of only one (*B. leandrae*, ML) or two species (*B. leandrae* and *B.* sp. 33, parsimony) in Amazonia. The other dispersal event originated a large diversification of Amazonian species (39 or 40 species, as indicated by parsimony and ML respectively) and it dated to at least 6.8 MYA (Table S6). Contrary to other studies of amphibians (Castroviejo-Fisher et al., 2014; Mendoza et al., 2015; Santos et al., 2009), Amazonia was the source of more dispersals into the Andes (3 or 4) than the opposite. Similarly, Faivovich et al. (2005) identified at least three hylid frog clades that may have radiated into the Andes after a dispersal event from lowland regions. The contribution of Amazonian lineages to other Neotropical regions is emerging as a general biogeographic pattern (Antonelli et al., 2018).

The diversification of Amazonian salamanders coincides with major modifications of this region. The drainage of the Pebas system and the formation of the modern Amazon drainage system is dated at 10.0–4.5 MYA (Albert et al., 2018). We speculate that the MRCA of Amazonian species was restricted to the Andes foothills in western Amazonia. Following the drainage of the Pebas system, salamanders expanded into eastern Amazonia and diversified into several species. Later dispersals into the Andes from the Amazonian lowlands are coincident in time with the major uplifts of the Andes ~ 8–2 MYA (Hooghiemstra et al., 2006; Hoorn and Wesselingh, 2010).

The discovery of this large diversification of lowland salamanders in the Amazonian rainforest bears important implications with regard to the study of the mechanisms behind observed differences in species richness between regions and among clades. Plethodontids have been used as a model group to test hypotheses regarding differences in species richness over space and time (see review by Kozak, 2017).

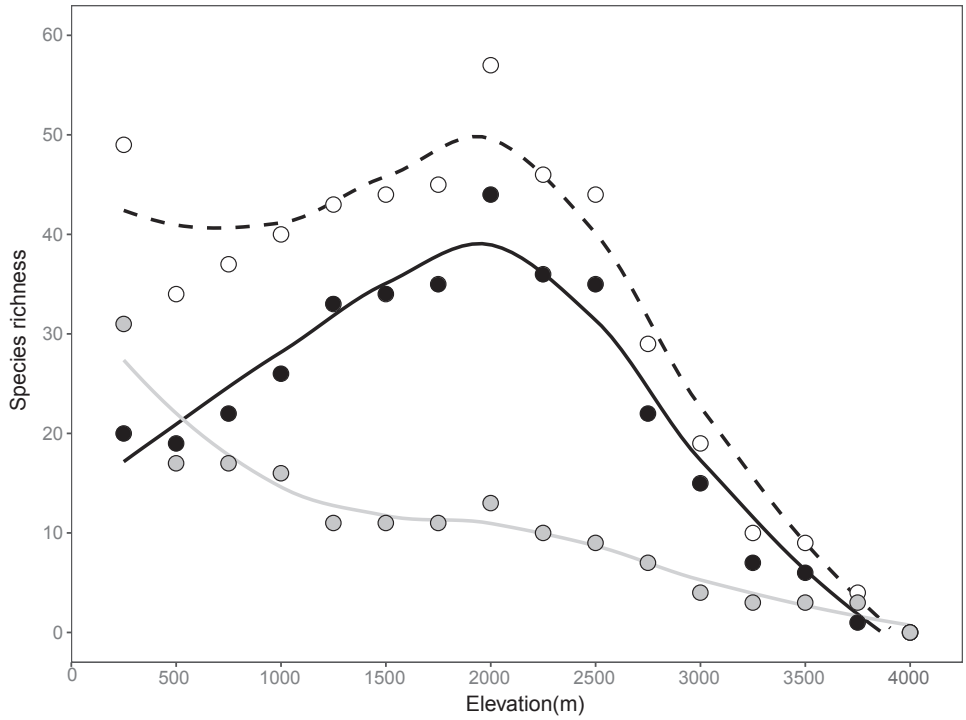

Fig. 8. Elevational pattern of species richness of Plethodontidae globally (white), in Central America (black), and in South America (grey) following the results of our study.

Generally, these studies rest upon two key assumptions related to the geographical pattern of species of plethodontids: (i) Species diversity is concentrated in two hotspots, in the Appalachian and Central American highlands, and (ii) most species are found in midelevation habitats. Our results question these premises. First, the number of South American species currently recognized is vastly underestimated. From 35 nominal species of *Bolitoglossa* currently recognized in South America (Frost, 2019), we report up to 42 new candidate species (36 from the Amazonian rainforest and six from the Andes). If confirmed by future studies, South America would move from harboring 37 species of plethodontids (7.7% of the current 478 species) to 79 species (15% of 520 species) so it should be considered an important center of plethodontid diversification. Second, the greatest species richness within South America is found in lowland rainforests below 1000 m a.s.l. (Fig. 8).

The great task ahead is to continue the study of the species-level systematics of *Bolitoglossa* and to find additional information, to refine all these species hypotheses. Furthermore, although our study has greatly increased the sampling of salamanders in the Amazonian lowlands and midlands, the Andes of Colombia remain poorly sampled. Even our meager sampling of Andean salamanders indicates the presence of eight potential new species from Colombia, Ecuador, and Venezuela. All these facts taken together clearly point out that species richness of salamanders in the Neotropics is not sufficiently well known, which means that the observed patterns have great potential to reflect our ignorance rather than our knowledge.

Supplementary data 1

Implied alignment obtained by POY. https://datadryad.org/stash/share/EasgCOWXAduHRDJ5cx8Lck4kKWghDj4GrWywggaq8mg.

Supplementary data 2

Similarity alignment obtained by MUSCLE. https://datadryad.org/stash/share/EasgCOWXAduHRDJ5cx8Lck4kKWghDj4GrWywggaq8mg.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2020.106841.

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