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## Research Article



# Systematics and biogeography of the *Boana albopunctata* species group (Anura, Hylidae), with the description of two new species from Amazonia

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The outstanding species richness of Amazonia has fascinated biologists for centuries. However, the records of actual numbers and distribution of species forming its ecosystems are so incomplete that the understanding of the historical causes and regional determinants of this diversity remain speculative. Anuran clades have repeatedly been documented to harbour many unnamed species in this region, notably the *Boana albopunctata* species group. Considering the documented distribution and the ecology of the species of that group, we hypothesized that it diversified via successive trans-riverine dispersals during the late Miocene and Pliocene, after the formation of the modern Amazon watershed. To test this hypothesis, we gathered an extensive dataset of 16S rDNA sequences sampled throughout Amazonia and a mitogenomic dataset representative of the diversity of the clade to (1) re-evaluate species boundaries and distributions, and (2) infer the spatio-temporal history of diversification within Amazonia. We delimited 14 Operational Taxonomic Units (OTUs) in an Amazonian clade, i.e., 75% higher than currently recognized (14 OTUs for eight described species). Combining molecular data with morphological and acoustic data, two new species, *Boana courtoisae* sp. nov. from the eastern Guiana Shield and *Boana eucharis* sp. nov. from Southern Amazonia, are described herein. These species belong to a clade that diversified throughout Amazonia during the last 10 Ma, thus more recently than co-distributed small terrestrial anurans but concomitantly with other more vagile vertebrates. Our time-scaled phylogeny and biogeographic analyses suggest an initial east-west divergence and confirm reciprocal trans-riverine dispersals during the last 5 Ma. The geomorphological evolution of the region and species-specific dispersal ability largely explain these distinct spatio-temporal patterns across anurans.

<http://www.zoobank.org/zoobank.org:act:4F8ACA9F-F6F1-4605-BD6C-6D4650AACCBE>

<http://www.zoobank.org/zoobank.org:act:51CC7B40-2D6B-4A9E-AF50-AB34D4CE1042>

**Key words:** Amphibia, biodiversity, Guiana Shield, mitogenomics, neotropics, phylogenetics

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## Introduction

The Neotropics harbour the most diverse ecosystems on the planet (Jenkins et al., 2013). Within the Neotropics,

Amazonia stands as a major biogeographic region covering more than 6.5 million km<sup>2</sup>, including the largest continuous tract of tropical forest (40% of the world's tropical rainforests), encompassing the most important drainage (Amazon River basin) and probably hosting the highest continental biodiversity on earth (Myers *et al.*, 2000). The outstanding species richness of this region has raised many questions concerning its origins and diversification mechanisms. While all agree on the astounding diversity of Amazonia, our understanding of the actual number and distribution of species forming its ecosystems is in fact so incomplete (Ficetola *et al.*, 2014; Meyer *et al.*, 2015; Vacher *et al.*, 2020) that it has so far hampered investigations at the regional scale. Consequently, the understanding of the historical causes and regional determinants of this outstanding diversity remain speculative (Antonelli *et al.*, 2018). Another consequence is that the collision between the Linnean shortfall and the biodiversity crisis is particularly evident in Amazonia (Guerra *et al.*, 2020). With almost the entire eastern and southern parts (25%) of its extent being already deforested for agriculture, an unknown proportion of endemic species, and thus of the testimony of Amazonian history, has already vanished (Lovejoy & Nobre, 2019).

Anuran clades have repeatedly been documented to harbour many unnamed species in Amazonia (Vacher *et al.*, 2020) and to display strikingly allopatric distribution patterns that could provide crucial insights into Amazonia's past (Fouquet, Cassini, Haddad, Pech, & Rodrigues, 2014; Réjaud *et al.*, 2020). This is notably the case of the *Boana albopunctata* species group (Caminer & Ron, 2014; Funk *et al.*, 2012). This group is only defined on the basis of molecular data (Faivovich *et al.*, 2005), and currently comprises 16 valid nominal species distributed in the Caribbean and South America: *Boana albopunctata* (Spix, 1824), *Boana alfaroi* (Caminer & Ron, 2014), *Boana almen-darizae* (Caminer & Ron 2014), *Boana caiapo* Pinheiro *et al.*, 2018, *Boana calcarata* (Troschel, 1848), *Boana dentei* (Bokermann, 1967), *Boana fasciata* (Günther, 1858), *Boana heilprini* (Noble, 1923), *Boana lanciformis* (Cope, 1871), *Boana leucocheila* (Caramaschi & de Niemeyer, 2003), *Boana maculateralis* (Caminer & Ron, 2014), *Boana multifasciata* (Günther, 1859), *Boana paranaiba* (Carvalho, Giaretta & Facure, 2010), *Boana steinbachi* (Boulenger, 1905), *Boana tetete* (Caminer & Ron, 2014) and *Boana raniceps* (Cope, 1862). Within this clade, *B. heilprini* is the most distinct since it is confined to Hispaniola and is phylogenetically distant from a clade formed by all the other species of the group (Duellman, Marion, & Hedges, 2016). We can also distinguish *B. raniceps* and the *B. albopunctata*

clade (*B. lanciformis*, *B. albopunctata*, *B. multifasciata*, *B. paranaiba*, *B. caiapo*, *B. leucocheila*), which occur widely in the open habitats of the Cerrado and Amazonia (Camurugi *et al.*, 2021), from the Amazonian clade. According to Caminer and Ron (2014), this Amazonian clade is itself subdivided in two main groups, hereafter called the *B. calcarata* (*B. fasciata*, *B. calcarata*, *B. almen-darizae*, *B. maculateralis*) and the *B. steinbachi* (*B. tetete*, *B. alfaroi*, *B. steinbachi*) clades.

Within this Amazonian clade, Funk, Caminer, and Ron (2012) identified no less than seven putative unnamed species based on mitochondrial divergence and acoustic data. These species add up to the 10 taxa that were valid at that time. Caminer and Ron (2014) completed this picture by documenting two additional lineages, describing four of the previously discovered species, and removing *B. steinbachi* from its synonymy with *B. fasciata*. However, sampling of both studies was mostly circumscribed to western Amazonia (mostly Ecuador) while the species complex occurs throughout Amazonia. Populations from the eastern Guiana Shield lowlands are already assumed to belong to a yet unnamed species (Caminer & Ron, 2014), but the status of numerous other populations throughout Amazonia, generally identified as *B. fasciata*, remain virtually unknown. More recently, Vacher *et al.* (2020) suggested, based on 16S mitochondrial DNA (mtDNA) sequences, that up to 22 species could exist in that group and that the distribution of most species could be circumscribed to small ranges within Amazonia.

These aforementioned studies improved our understanding of the actual diversity in the *B. calcarata/steinbachi* clade, but left virtually unexplored the temporal and spatial context of the diversification within Amazonia. The crown age of this Amazonian clade was estimated between 16–11 million years ago (Ma) by Funk *et al.* (2012) and between 14–9 Ma by Duellman *et al.* (2016). Therefore, the initial diversification of that clade throughout Amazonia may have taken place during the final stage of the Pebas system, a freshwater lake occupying most of Western Amazonia from the early Miocene (23 Ma) until 10–9 Ma (Hoorn *et al.*, 2017). Meanwhile, most of the diversification within the *B. calcarata/steinbachi* clade took place during the late Neogene (last 10 Ma). From 9 Ma onward, this system has drained eastward into the Atlantic Ocean, but enormous flooded ecosystems (Acre system) still occupied a large portion of what we currently consider as Western Amazonia until 7 Ma (Hoorn *et al.*, 2010; Albert, *et al.*, 2018a). Subsequent river captures were common (Ruokolainen, Moulatlet, Zuquim, Hoorn, & Tuomisto, 2018), as were climatic fluctuations that may have modified vegetation possibly to the point of forest

fragmentation, at least in some peripheral parts of Amazonia (Cheng et al., 2013; Kirschner & Hoorn, 2019). The relative roles of hydrological evolution (Albert et al., 2018b; Ribas, Aleixo, Nogueira, Miyaki, & Cracraft, 2012) and rainforest expansion and contraction due to climate oscillations (Haffer, 1969) have been the focus of intense debate (Leite & Rogers, 2013). Since the species of the *B. calcarata* and *B. steinbachi* complexes are associated with riparian forests, and these species mostly breed in pools formed on the banks of the beds of small to medium-sized rivers, very large rivers may act as barriers to dispersal, thus reducing or completely impeding gene flow and inhibiting homogenization of differentiating populations (Naka & Brumfield, 2018). However, smaller and highly dynamic meandering rivers probably represent more permeable barriers. Therefore, we assume that successive dispersals across major Amazonian rivers (parapatric speciation) (Pirani et al., 2019), as well as hydrological changes (vicariant speciation) (Naka & Brumfield, 2018), could have been major processes of diversification in these frogs.

We gathered a large mtDNA dataset for the *B. albopunctata* group sampled throughout Amazonia combined with morphological and acoustic data for a subset of this group (the *B. steinbachi* clade), to address two main goals: (1) reevaluate species boundaries (in or among members) of the *B. albopunctata* group and their respective distributions, and (2) investigate its diversification history within Amazonia using mitogenomic data for one terminal of each delimited species. Furthermore, two species in the *B. steinbachi* clade that were unnamed are described herein.

## Materials and methods

### Species delimitation

Our first objective was to delimit all major Operational Taxonomic Units (OTUs) based on mtDNA main lineages. Our sampling included 55 new *Boana* tissue samples, obtained through fieldwork throughout Amazonia and adjacent Dry Diagonal (DD; Chaco, Cerrado, Caatinga; Werneck, 2011) and loans from collaborators (from collections QCAZ, MPEG, INPA, CORBIDI, AAGUFU, and from personal loans JMP, SCF, FTA; Supplemental Appendix 1). Samples unambiguously identified (near type locality and/or phenotypically corresponding to type material) as belonging to the currently recognized taxa were included except *B. paranaiba* and *B. caiapo*. We sequenced a ~400 bp portion of the end of the 16S rDNA gene, a locus commonly used for Neotropical amphibian taxonomy and systematics

(Vences, Thomas, Bonett, & Vieites, 2005). We also retrieved homologous sequences from GenBank (389 accessions). In total, we gathered 444 16S sequences for this study (dataset details are provided in Supplemental Appendix 1 and DNA extraction and sequencing protocols can be found in Supplemental Appendix 2). These samples cover the whole distribution of the *B. albopunctata* species group (14 included taxa). DNA sequence alignment (alignment available from figshare.com at <https://doi.org/10.6084/m9.figshare.13536302.v1>) was conducted on the MAFFT7 online server under the E-INS-i option with default parameters, an algorithm designed for sequences with multiple conserved domains and long gaps (Kato et al., 2019).

We applied three DNA-based single-locus species delimitation approaches: (a) a distance-based method, the Automated Barcode Gap Discovery (ABGD; Puillandre, Lambert, Brouillet, & Achaz, 2012); (b) a multi-rate coalescent based method, the multi-rate Poisson Tree Processes model approach (mPTP; Kapli et al., 2017); and (c) a single-threshold coalescent-based method, the Generalized Mixed Yule Coalescent approach (single threshold GMYC; Monaghan et al., 2009; Pons et al., 2006).

The ABGD delimitation was performed with a prior of intraspecific divergence  $P$  between 0.001 and 0.1, a proxy for minimum relative gap width,  $X$ , of 1, and a number of steps  $n$  equal to 30. We kept the partition such that  $P=0.016$ , as it corresponds to the end of a plateau for group number and it matches thresholds of intraspecific divergence proposed in other vertebrate delimitation studies using 16S barcodes (Puillandre et al., 2012). For mPTP delimitation, we first reconstructed a Maximum Likelihood (ML) tree with RAxML v.8.2.4 (Stamatakis, 2014) using the GTR + G + I substitution model and estimated nodal support via 1000 parametric bootstraps. We used nine outgroups representing most other *Boana* species groups (Supplemental Appendix 3). The mPTP delimitation was undertaken on the rooted ML tree, with 5 million Markov chain Monte Carlo (MCMC) iterations, sampling every 10,000th iteration, and a 10% burn-in. For the GMYC delimitation, we reconstructed a time-calibrated phylogeny using BEAST 2.5 (Bouckaert et al., 2014). We used a birth-death population model to account for extinction processes and incomplete sampling. We included the same nine *Boana* outgroups used in RAxML reconstruction and five additional representatives of other Cophomantini genera (*Nesorohyla*, *Myersiophyla*, *Hyloscirtus*, *Aplastodiscus*, *Bokermannohyla*) in order to include relationships that can be time-calibrated. We used a single partition with a GTR + G + I substitution model, with an uncorrelated relaxed lognormal clock model of rate variation among branches (Drummond, Ho, Phillips, & Rambaut, 2006).

We used two calibration points, the ages of the most recent common ancestor (MRCA) of *Aplastodiscus* and *Boana*, as well as the MRCA of a clade formed by *Hyloscirtus*, *Boana*, *Aplastodiscus*, and *Bokermannohyla*, which were estimated by Feng *et al.* (2017) and consequently constrained here assuming normal prior distributions of 25.2 Ma (standard deviation [SD]=2.8 Ma) and 32.3 Ma (SD = 3 Ma) respectively. We considered these calibrations to represent the best prior because Feng *et al.* (2017) included a comprehensive dataset of nuclear loci for an extensive sampling of anurans and used many reliable fossils as calibration points. Moreover, Hime *et al.* (2021), analysing an even larger genomic dataset, found a very similar time estimate for the TMRCA of *Boana*+*Hyloscirtus*. Instead, using fossil and/or biogeographic calibrations would have implied to expand the matrix to lineages distantly related to *Boana* and would likely lead to an overestimation of calibration dates with our mitogenomic dataset (Molak & Ho, 2015; Papadopoulou *et al.*, 2010). For the MCMC parameters, we used four independent chains of 100 million iterations, recording every 10,000th iteration, and a 10% burn-in. We combined the log files of the independent runs using LogCombiner 2.5 (Drummond & Rambaut, 2007) and checked the convergence of our parameters, confirmed by all Effective Sample Size (ESS) being above 200. Then, we extracted the maximum clade credibility tree using Tree annotator 2.5 (Drummond & Rambaut, 2007) with a burn-in of 10%. After removing outgroups, we performed a GMYC delimitation on the ultrametric tree using the GMYC function of the splits R package v.1.0-11 (Ezard, Fujisawa, & Barraclough, 2009), with a threshold interval between 0 and 10 Ma and by using the single threshold method. Operational Taxonomic Units (OTUs) were defined using a majority-rule consensus from the results of the three methods, i.e., a lineage is considered as being an OTU if supported by at least two of the three methods.

All but five occurrence records were georeferenced and used to create distribution maps with convex polygons under QGIS 2.14 (QGIS Geographic Information System). OTUs were assigned to taxa based on the field/museum identification and sometimes corrected in accordance with type localities and known distribution (Frost, 2019; IUCN, 2020).

### **Morphological and acoustic variation in the *Boana steinbachi* clade**

The following morphometric measurements were taken on 38 males and 9 females: snout-vent length (SVL), head length (HL), head width (HW), eye diameter (ED), tympanum diameter (TD), tibia length (TL), foot length (FL), thigh length (THL) and calcaneal appendage length (CL). In addition to these, we also measured

hand length (HAL), forearm length (FLL), and eye–nostril distance (EN) for the morphometric characterization of holotypes. All measurements followed the definitions and terminology of Watters, Cummings, Flanagan, and Siler (2016). We classified the calcaneal appendage into three-character states: (i) a calcar (skin appendage with length > 1 mm, flattened or conical); a (ii) skin flap (skin appendage with length < 1 mm, flattened; or a (iii) tubercle, a conical structure with reduced size and, as such, not forming an appendage coming out of the heel. The specimens were measured by P. Marinho (Alta Floresta, Mato Grosso State and Assis Brazil, Acre State) using a Mitutoyo digital calliper (to the nearest 0.05 mm), by A. Mollo Neto (Jirau and Pacaás Novos, Rondônia State) using a Digimess digital calliper (to the nearest 0.01 mm), by A. Fouquet (French Guiana and Suriname) using a DigiMax digital calliper (nearest 0.01 mm) and by M. Caminer (Ecuador, Peru and Bolivia) using a Mannesmann digital calliper (nearest 0.01 mm). Snout shape was assessed according to Heyer *et al.* (1990). Digital webbing formulae followed the notation system of Savage and Heyer (1997).

Acoustic analysis of 27 recorded calling males (Supplemental Appendix 4) was conducted in Raven Pro 1.5, 64-bit version (Center for Conservation Bioacoustics, 2014); sound figures were produced using Seewave version 2.1.0 (Sueur, Aubin, & Simonis, 2008) and tuneR version 1.3.2 (Ligges, Krey, Mersmann, & Schnackenberg, 2014), in R version 3.5.0 (R Core Team 2018). Raven Pro settings: window size = 512 samples; window type = Hann; 3 dB filter bandwidth = 124 Hz; window overlap = 85%; hop size = 77 ms; discrete Fourier transform (DFT) size = 1024 samples; grid spacing = 43.1 Hz; seewave settings: window type = Hanning; fast Fourier transform (FFT) size = 256 samples; FFT overlap = 90%. The analysed files are deposited in the sound collections of the ‘Museu de Biodiversidade do Cerrado’ (AAG-UFU), ‘Fonoteca Neotropical Jacques Vielardi’ (FNJV), ‘La Sonothèque du Muséum National d’Histoire Naturelle’ (MNHN) and the Macaulay library (MC). Detailed information about the analysed sound files and accession numbers are provided in Supplemental Appendix 4.

Call types were distinguished based on the number of notes per call and pulsing. We defined as type 1 calls the multinote calls formed by nonpulsed notes, which consist of the main call type emitted by males of all studied species. Type 2 calls are emitted less often than type 1 and consist of a one-note call formed by poorly defined pulses. We only included type 1 calls in the acoustic diagnoses and the interspecific comparisons. The acoustic traits were generally analysed manually (if not stated otherwise), as follows: temporal traits (call duration, note duration, interval between notes, number of notes, and call rise time; the latter using the ‘Peak

Time' function); frequency traits (dominant frequency, using the 'Peak Frequency' function; minimum and maximum frequency using the 'Frequency 5%' and 'Frequency 95%' functions, respectively). Call traits used in the description followed the definitions and terminology of Köhler et al. (2017), using a note-centred approach.

### Time-calibrated species phylogeny

We selected a representative for each of the 25 OTUs identified by the species delimitation (see Results), for complete mitogenome sequencing to investigate interrelationships and divergence times. Mitogenomic sequences were obtained using low-coverage shotgun sequencing. We recovered high-quality mitochondrial genome assemblies for 18 OTU representatives (see Supplemental Appendix 2 for details regarding mitogenome sequencing, assembling and annotation). For the remaining seven OTUs for which we could not obtain tissue samples (Supplemental Appendix 3), we gathered all the available mitochondrial loci (12S, 16S, ND1, COI, and Cytb) from GenBank; four of these OTUs were represented by 16S only. We also selected 14 species as outgroups (nine with complete mitogenomes), including representatives for other *Boana* species groups and most other Cophomantini genera (Supplemental Appendix 3).

We extracted 12S, 16S and all protein-coding sequence regions (CDS, thus removing D-Loop and tRNAs) from complete mitogenomes as well as GenBank accessions and aligned each locus independently using the MAFFT7 online server (alignment available from figshare.com at <https://doi.org/10.6084/m9.figshare.13560371.v1>). For rDNA genes, we chose the E-INS-i strategy, recommended for sequences with multiple conserved domains and long gaps. For the protein-coding genes, we chose the G-INS-i strategy, designed for sequences with global homology (Kato et al., 2019). Realignment of CDS considering the reading frame were done and concatenated in Geneious v.9.1.8 (<https://www.geneious.com>). Our final matrix totalled 39 terminals and 13,620 aligned nucleotide sites. Among these terminals, 27 were complete and nine had fewer than 3,000 nucleotides (20%) but all were putatively closely related to terminals with complete data.

We selected the best-fit partition scheme and model of evolution for each partition using PartitionFinder V2.1.1 (Lanfear et al., 2016), based on the Bayesian Information Criterion (BIC). We predefined four data blocks, one for rRNA genes (12S and 16S) and one for each codon position of all CDS regions and found the GTR+I+G model to best-fit all partitions. We

reconstructed a time-calibrated tree using a birth-death tree prior using BEAST 2.5, to account for extinction processes and incomplete sampling. We parameterized unlinked substitution models according to the estimates obtained in the PartitionFinder V2.1.1 analysis (Lanfear et al., 2016). We used the same two calibration points as in the previous BEAST analysis. Analyses were undertaken using uncorrelated relaxed lognormal clock model of distribution of rate variation among branches for each partition (Drummond et al., 2006). The Markov chain Monte Carlo (MCMC) parameters were set with four independent chains of 100 million iterations, storing every 10,000<sup>th</sup> iteration and a 10% burn-in. We combined the log files of the independent runs using LogCombiner 2.5 and confirmed the convergence of our parameters as all ESS were above 200. Finally, we extracted the maximum clade credibility tree using Tree annotator 2.5. We acknowledge that our phylogenetic reconstruction solely based on mtDNA sequences prioritizes spatial and taxonomic completeness over genomic coverage and can lead to overestimated divergence times (e.g., McCormack, Heled, Delaney, Peterson, & Knowles, 2011; Near et al., 2012).

### Biogeographic analysis

Biogeographic inferences were undertaken on the time-calibrated phylogeny using the BioGeoBEARS R package (Matzke, 2013). This package reconstructs ancestral geographic distributions and investigates the role of each biogeographic event with a maximum likelihood algorithm. We compared three different models: (i) a likelihood version of the Dispersal-Vicariance (DIVALIKE) model (Ronquist, 1997); (ii) a likelihood version of the BayArea (BBM) model (Landis et al., 2013); and (iii) the Dispersal Extinction Cladogenesis (DEC) model (Ree & Smith, 2008). We also compared versions of these models allowing jump dispersal as described by the J parameter (Matzke, 2013). Models were compared with the Akaike Information Criterion (AIC). We ran 50 independent BioGeoBEARS biogeographic stochastic mapping, to determine biogeographic event counts for the best-fit model (Dupin et al., 2017). Ree and Sanmartín (2018) voiced several criticisms against the use of J parameter arguing that it inflates the contribution of cladogenetic events to the likelihood, and minimizes the contribution of anagenetic, time-dependent range evolution. We thus also compared the results from the best model without jump dispersal.

Since this work focuses on the historical biogeography of the Amazonian clade, we discarded the two early diverging lineages *B. heilprini* and *B. raniceps* from the biogeographic analyses since their distribution

ranges lie mostly outside of Amazonia. We considered three areas within Amazonia: Western Amazonia, the Brazilian Shield, and the Guiana Shield. These areas correspond to major geological features roughly delimited by modern riverine barriers: the Madeira River, the Negro River, and the lower course of the Amazon River and to three large biogeographic regions known as Wallace's districts (Hoorn *et al.*, 2010; Wallace, 1854). These districts were recently confirmed as major breaks in birds' species composition (Oliveira, Vasconcelos, & Santos, 2017) and amphibians (Godinho & da Silva, 2018; Vacher *et al.*, 2020), strengthening their status of biogeographic regions. Because the distribution range of the *B. albopunctata* group extends outside Amazonia, we included in the ancestral range reconstruction one additional non-Amazonian Neotropical area, the Dry Diagonal.

## Results

### Species delimitation

Of the three tested methods of species delimitation, the ABGD method was found to be the most conservative, delimiting 21 OTUs while mPTP and GMYC delimited 24 and 47 OTUs, respectively (Supplemental Appendix 1). In one case, the consensual partitioning was overly conservative given that *B. leucocheila* and *B. multifasciata* were considered as a single OTU which is contradicted by the fact that these two taxa are phenotypically distinct. Therefore, we kept the different lineages delimited by GMYC in this group as distinct OTUs. The final delimitation led to 25 OTUs (including *B. heilprini*) in the *B. albopunctata* species group (Fig. 1, Supplemental Appendix 1). Thirteen of these OTUs could be linked to nominal species (except *B. caiapo* and *B. paranaiba* that were not included in the analysis because material was not available). Conversely, 11 could not be linked to any nominal species, representing a possible 44% increase in the species richness of the group. The Amazonian clade itself was represented by 14 OTUs including eight nominal species and six putative new species (Fig. 1). These results also imply important changes to the geographic distribution of the species of the group. In Supplemental Appendix 5, we detail and justify the identification of these OTUs and their respective geographic ranges.

### Taxonomic accounts

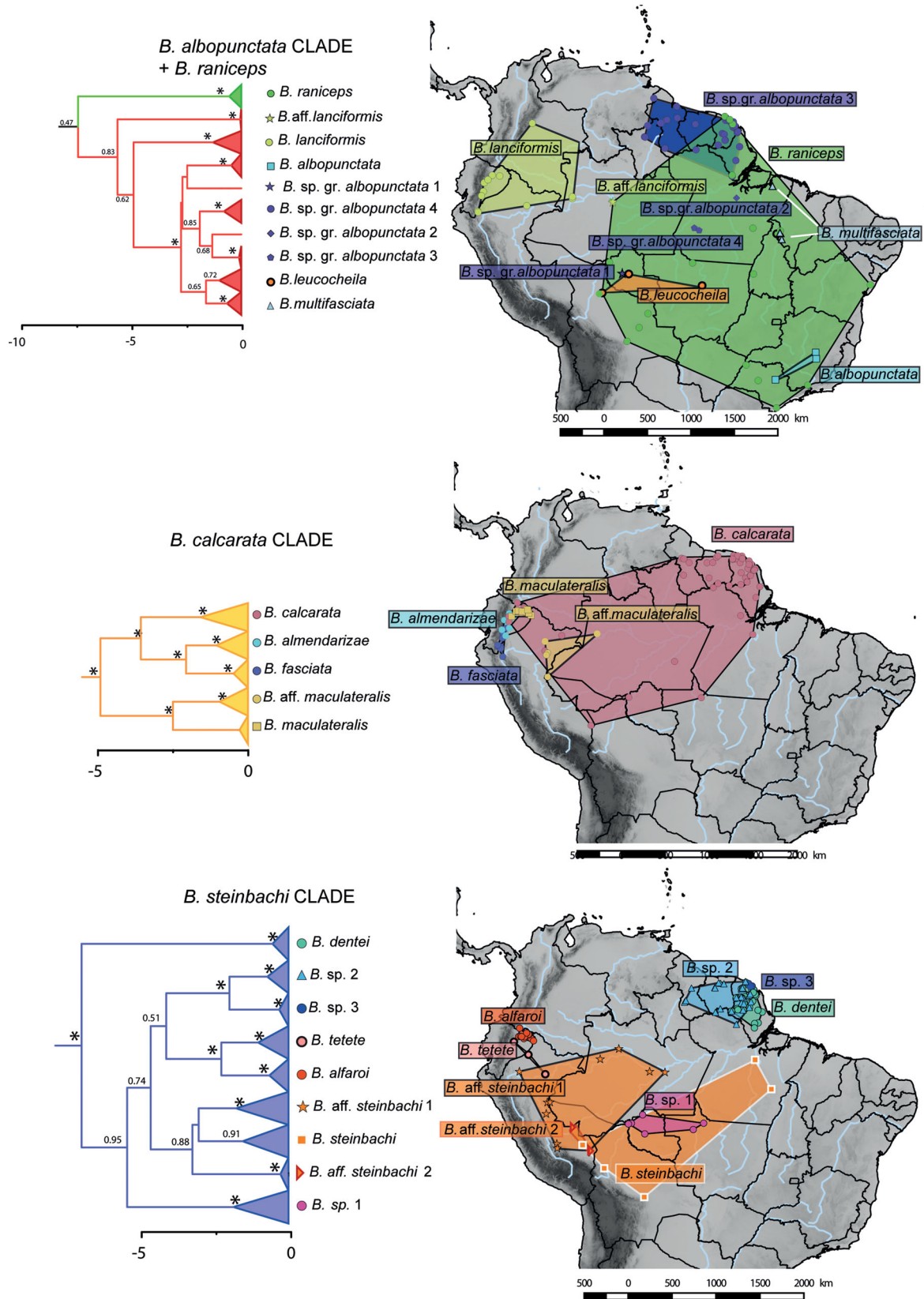
The Amazonian clade of the *B. albopunctata* group, previously reported as the *B. calcarata*–*B. fasciata* species complex by Caminer and Ron (2014), is composed of

two major clades (the *B. calcarata* and the *B. steinbachi* clades), defined by the following combination of character states: (1) truncate snout in dorsal view; (2) rounded head shape; and (3) presence of calcaneal appendage on heel.

The *B. calcarata* clade (Fig. 1) is composed of four species possessing a calcar and having advertisement calls formed by a single type of call (Caminer & Ron, 2014). The *B. steinbachi* clade, on the other hand, includes four described and two confirmed undescribed species (Fig. 1). Members of this clade lack a calcar (skin flap or tubercle present, or the complete absence of calcaneal appendages) and have vocal repertoires composed of more than one type of call, except for *B. alfaroi* for which a single type of call has been documented so far (Caminer & Ron, 2014).

*Hyla steinbachi* Boulenger, 1905, currently valid as *Boana steinbachi* (Boulenger, 1905), was described from the Bolivian Province Sara, Departament Santa Cruz de la Sierra, Bolivia. We assume that Buenavista is the type locality of *B. steinbachi*. Between 1910 and 1950 the Steinbach family collected many amphibians and reptiles in Bolivia, mainly at Buenavista in the Department of Santa Cruz (see Parker, 1927), which is the type locality of several anurans (*Hamptophryne boliviana* (Parker, 1927); *Pseudopaludicola boliviana* Parker, 1927; *Scinax parkeri* (Gauge, 1929)), and the snake *Apostolepis tenuis* Ruthven, 1927. The material collected by the Steinbach family is on display in various museums, such as the University of Michigan Museum of Zoology (UMMZ, see Gauge, 1929), the Natural History Museum, London (BMNH, see Parker, 1927), the Zoologisches Museum Berlin (ZMB, see Müller, 1924), and the Naturhistorisches Museum Basel (NBM, pers. comm. Wüest). Many decades later, De la Riva (1990) synonymized *H. steinbachi* with *H. fasciata*. Subsequently, Jansen, Bloch, Schulze, and Pfenninger (2011) found differences in call and mtDNA data between populations of *B. fasciata* from Bolivia and Ecuador and suggested that their results imply a resurrection of *B. steinbachi*. In the following years, Caminer and Ron (2014) tentatively assigned *B. steinbachi* to a genetic lineage (clade J) inhabiting Bolivian Amazonia, near its type locality. By assessing its phylogenetic relationships in the group, Caminer and Ron (2014) formally revalidated *B. steinbachi* as a distinct species from nominal *B. fasciata*. The revalidation of *B. steinbachi* was also supported by phenotypic data obtained from syntypes but did not include a reexamination of all diagnostic characters of the species.

Next, we present an amended diagnosis of *B. steinbachi* based on novel data on life colours and morphology, and describe its vocal repertoire based on topotypes



**Fig. 1.** Subtrees of the three main clades from the chronogram obtained from the analysis of 16S sequences using BEAST2. Terminals are collapsed according to the OTU recovered from the species delineation analysis. The distribution of each of these OTU is depicted on the maps.



from Bolivia and distinct OTUs from Peru and Brazil also assigned to this species in this study. Specimens and calls from Acre (“*B. aff. steinbachi* 1”) and calls from the lower Madre de Dios River (“*B. aff. steinbachi* 2”) were included in the variation of *B. steinbachi*.

*Boana steinbachi* (Boulenger, 1905)  
*Hyla steinbachi* — Boulenger, 1905  
*Hyla fasciata* De la Riva, 1990  
*Hypsiboas fasciatus* Jansen *et al.*, 2011  
*Hypsiboas* sp. (Clade G) Funk *et al.*, 2012  
*Hypsiboas steinbachi* Caminer & Ron, 2014  
*Boana steinbachi* Dubois, 2017.  
*Boana* sp. (Clade J) Meza-Joya *et al.*, 2019  
*Boana fasciata* Vacher *et al.* 2020

**Syntypes.** BMNH 1947.2.13.61–63, two adults of unknown sex and one juvenile, respectively, from Sara province, Department of Santa Cruz de La Sierra, Bolivia. Collected by Hf. J. Steinbach.

**Diagnosis.** *Boana steinbachi* is characterized by the following combination of character states: (1) skin flap on heel; (2) vocal repertoire composed of more than one call type; (3) multinote call; and (4) regular internote intervals (Figs 2, 3 & 4).

**Comparisons with congeners of the Amazonian clade.**

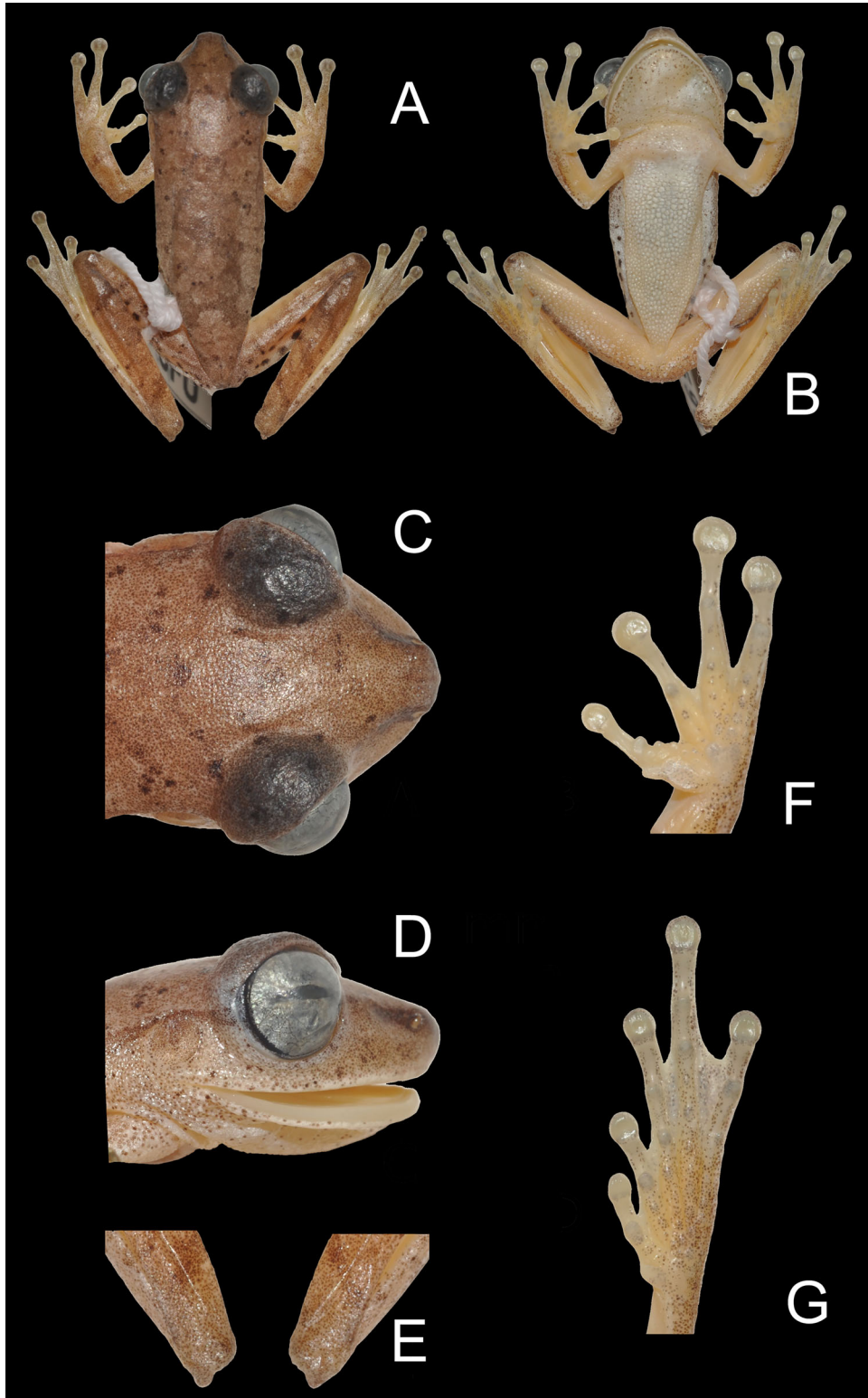
*Boana steinbachi* can be distinguished from the members of the *B. calcarata* clade (*B. almendarizae*, *B. fasciata*, *B. calcarata*, and *B. maculateralis*) by the absence of a calcar and by the vocal repertoire composed of more than one type of call. Among members of the *B. steinbachi* clade, it can be distinguished from *B. dentei*, *B. alfaroi*, and *B. tetete* by the presence of a skin flap on heel (tubercle in *B. alfaroi* and *B. tetete*; tubercle on one side or completely absent in *B. dentei*), by its multinote call (one-note call in *B. dentei* and *B. tetete*), by having regular internote intervals between call notes (notes with irregular intervals, sometimes partly fused one with the next in *B. alfaroi*), and by the vocal repertoire composed of two distinct types of calls (one call type in *B. alfaroi*) (Caminer & Ron, 2014; Marinho *et al.*, 2020).

**Variation.** Body size varies between 30.4 and 37.4 mm in males and between 42.5 and 48.8 mm in females (Supplemental Appendix 7). Individual AAG-UFU 5918 lacks the heel skin flap on the left side, topotypes SMF 88394–95 and SMF 88397 lack the skin flap on both sides. In life (Fig. 3a, b), dorsal colouration is beige, light brown, bright yellow or orange brown in calling males, with faint brown transversal bands and dark

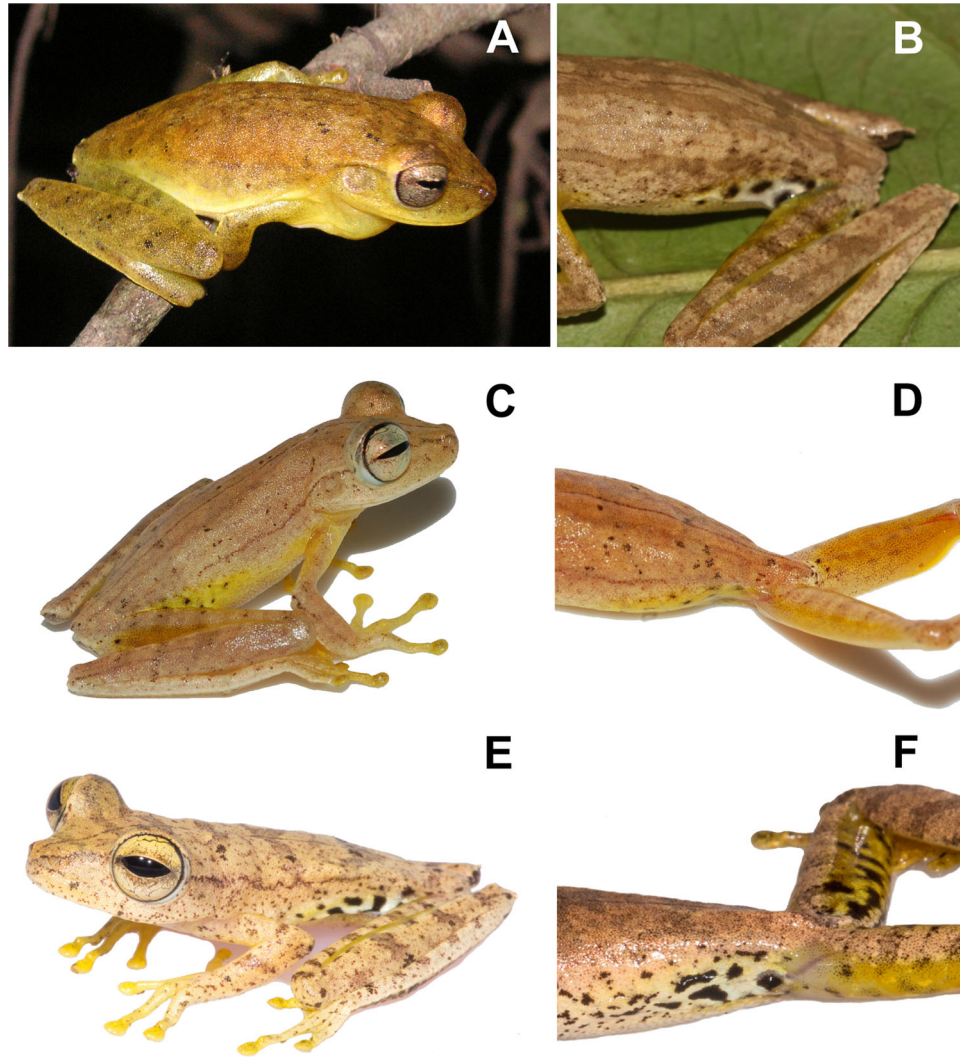
purplish-brown, faint brown or grey longitudinal lines extending from the snout to vent, a second line extending from behind the eye to pelvic region, and a third line as a canthal stripe extending from the posterior corner of the nostril to anterior corner of the eye, and from the posterior corner of the eye to midbody length on flank (the topotypes SMF 88394–97 lack transversal bands on dorsum; individuals SMF 88394 and CORBIDI 13414 lack longitudinal lines on dorsum and the middorsal line is extending from snout to interocular region; AAG-UFU 5921 lacks longitudinal lines on dorsum). Iris cream or grey, sometimes with yellow pigmentation on the upper part of the iris. Throat varying from white to yellow, chest and anterior belly varying from white to cream. Ventral surface of hand and hind limb beige to bright yellow. Posterior surface of thigh, groin and posterior half of flank spotted in black, with a bright yellow or white background colour. Brown dots scattered on dorsum, dorsal surface of limbs, bordering the lower lip, mental region, and chest. In preservative, the colours fade: the bright yellow colour sometimes present on dorsum, ventral surfaces of limbs, groin, and flank are beige or pale cream. Iris grey. Flank and groin maculation are denser and more extensive in females.

**Vocal repertoire.** (Fig. 4) We analysed calls of 13 males (see Supplemental Appendix 4 for information about sound recordings and Supplemental Appendix 6 for the complete descriptive statistics). The vocal repertoire of *Boana steinbachi* is composed of two distinct types of calls (type 1:  $n = 149$  calls of 13 males; type 2:  $n = 43$  calls of five males) that are emitted sporadically at irregular intervals. The type 1 call of *B. steinbachi* lasts 130–430 ms and is composed of 3–8 nonpulsed notes lasting 4–52 ms, separated by intervals of 1–67 ms. The rise time is at 2–98% of call duration. The minimum frequency ranges from 1335–1981 Hz, the maximum frequency from 2713–4479 Hz, and the dominant frequency from 1688–3402 Hz. The type 2 call is composed of one note with poorly defined pulses. Notes last 47–87 ms. The rise time is at 18–60% of note duration. The minimum frequency ranges from 1453–2016 Hz, the maximum frequency from 2713–4522 Hz, and the dominant frequency from 1688–2813 Hz. The limited sample size for each population with recorded calls prevented us from evaluating the geographic variation of acoustic traits across populations of *B. steinbachi*.

**Distribution and ecology.** In addition to the type locality in central Bolivia, occurrence records of *B. steinbachi*, based on molecular and phenotypic data, encompass the south-western, central, and eastern Brazilian Amazonia (Acre, Amazonas, and Pará; Fig. 1), and south-western Peruvian Amazonia (Tambopata and the



**Fig. 2.** Adult male of *Boana steinbachi* (AAG-UFU 5921) from the Assis Brasil population (Acre, Brazil): (A) dorsal and (B) ventral views of the body (SVL = 33.7 mm), (C) dorsal and (D) lateral views of the head (HL = 12.3 mm; HW = 10.2 mm), (E) detail in dorsal view of the skin flap on heels, (F) palm of the hand (HAL = 10.6 mm), and (G) sole of the foot (FL = 13.9 mm).



**Fig. 3.** Life colours in dorsolateral view and detail of the patterns on flank and groin of males. (A, B) *Boana steinbachi* (topotype SMF88394 and non-topotype AAG-UFU 5918, respectively), (C, D) *B. eucharis* sp. nov. (AAG-UFU 6503; holotype), and (E, F) *B. courtoisae* sp. nov. (holotype, MNHN-RA-2020.0001, and an unvouchered specimen, respectively).

lower Madre de Dios River). Collected males were calling perched on shrubs in the periphery of flooded areas and in forest clearings of secondary Amazonian lowland forests. Its range is extensive and encompasses numerous protected areas. Moreover, the species seems to tolerate habitat disturbance. Therefore, its status should be considered of Least Concern.

*Boana eucharis* sp. nov.

*Hypsiboas fasciatus* Ávila & Kawashita-Ribeiro, 2011

*Hypsiboas fasciatus* Rodrigues et al., 2015

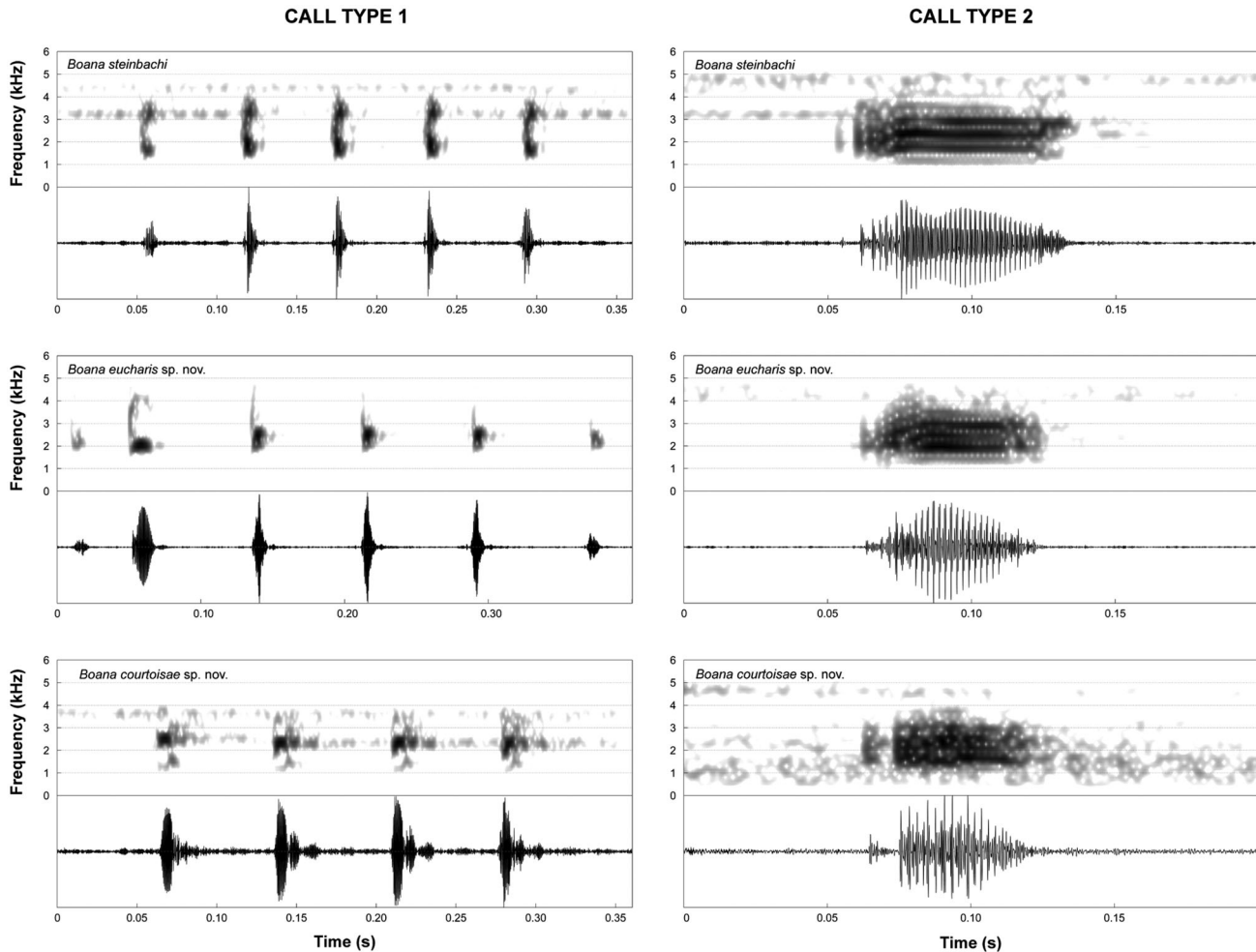
*Boana fasciata* Vacher et al. 2020

**Holotype.** AAG-UFU 6503, adult male collected from the municipality of Alta Floresta, Mato Grosso state,

Brazil (−9.642839°, −56.271408°) by Davi L. Bang, André G. Lopes, and Pedro Marinho on 11 January 2019 (Fig. 3 & 5).

**Paratopotypes.** Five adult males (AAG-UFU 6504–6508) collected with the holotype. AAG-UFU 6904, adult male collected on 20 January 2020 by Ariovaldo A. Giaretta, Pedro Marinho, and André G. Lopes.

**Paratypes.** (1 male, 4 females). MZUSP 143323, adult male and MZUSP 143324, adult female, collected at UHE Jirau, Abunã, state of Rondônia, Brazil (−9.699410°, −65.358875°); MZUSP 143238, adult female collected at UHE Jirau, Porto Velho, state of Rondônia, Brazil (−8.748483°, −63.903465°); MZUSP 159228 (field No. MTR25896), adult female collected at



**Fig. 4.** Type 1 (left) and type 2 (right) calls (spectrograms and corresponding oscillograms) of *B. steinbachi* (Top; accession number MNHN-SO-2020-2934); *B. eucharis* sp. nov. (Middle; file: B\_eucharisAltaFlorestaMT5hPM\_AAGm671; MNHN-SO-2020-2969); and *B. courtoisae* sp. nov. (Bottom; MNHN-SO-2020-2947). See [Supplemental Appendix 4](#) for additional information on sound recordings.

PARNA Pacaás Novos, state of Rondônia, Brazil ( $-10.786979^{\circ}$ ,  $-63.627305^{\circ}$ ); MZUSP 159227 (field No. MTR25798), adult female collected at PARNA Pacaás Novos, state of Rondônia, Brazil ( $-10.786979^{\circ}$ ,  $-63.627305^{\circ}$ ).

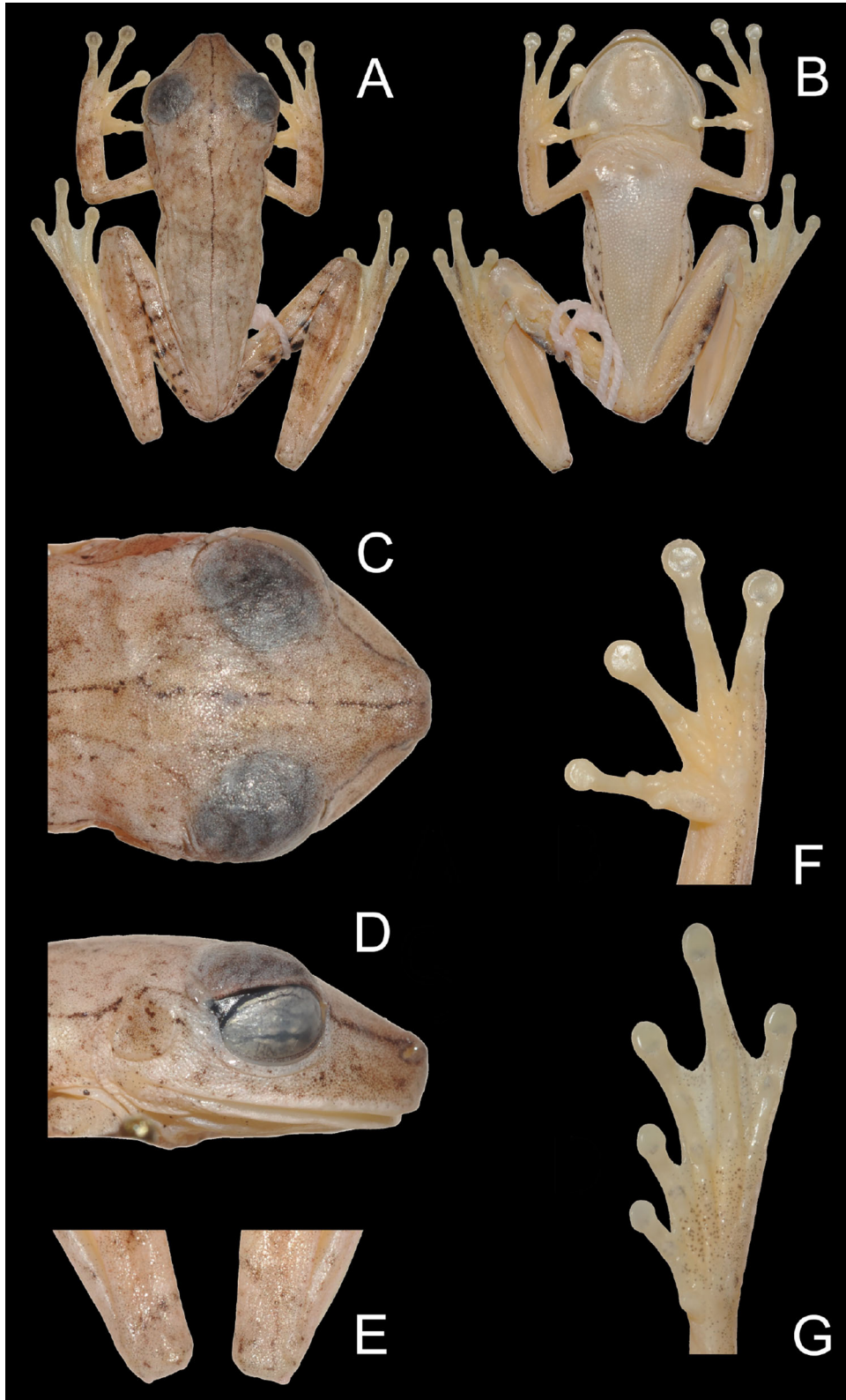
**Diagnosis.** *Boana eucharis* sp. nov. is characterized by the following combination of character states: (1) tubercle on heel; (2) vocal repertoire composed of more than one call type; (3) multinote call; and (4) regular internote intervals (Figs 3, 4 & 5).

**Comparisons with congeners of the Amazonian clade.**

*Boana eucharis* sp. nov. is distinguished from the members of the *B. calcarata* clade (*B. almendarizae*, *B. fasciata*, *B. calcarata*, and *B. maculateralis*) by the absence of a calcar and by the vocal repertoire composed of more than one type of call. Within the

*B. steinbachi* clade, *B. eucharis* can be distinguished from *B. dentei*, and *B. tetete* by its multinote call (one-note calls in *B. dentei* and *B. tetete*), and from *B. alfaroi* by having regular internote intervals and vocal repertoire composed of two distinctive types of calls (in *B. alfaroi*, call notes having irregular intervals, sometimes partly fused one with the next, and repertoire formed by one call type; Caminer & Ron, 2014; Marinho et al., 2020). *Boana eucharis* can be distinguished in almost all cases from *B. steinbachi* by the presence of tubercle on heel (skin flap in *B. steinbachi*; but see Variation). In addition, the phylogenetic relationships within this clade and spatial distribution strongly support the distinct specific status of *B. eucharis* relative to its closest relatives (Fig. 7).

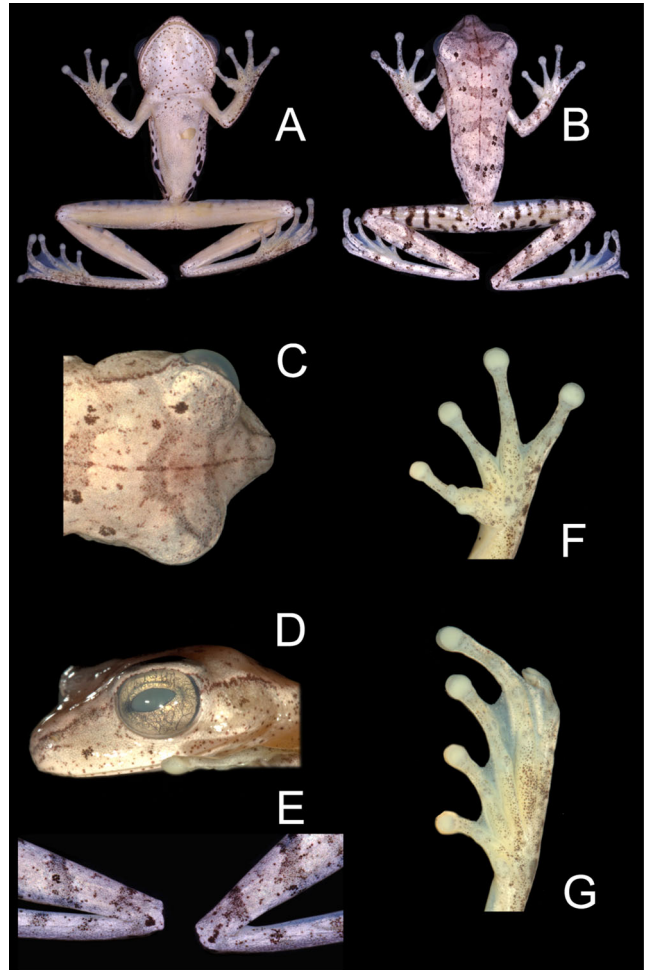
**Description of holotype.** (Figs 3 & 5) Adult male, SVL 31.8 mm, FL 13.1 mm, ED 3.8 mm, TD 2.0 mm, TL



**Fig. 5.** Holotype of *Boana eucharis* sp. nov. (AAG-UFU 6503) from Alta Floresta (Mato Grosso, Brazil): (A) dorsal and (B) views of the body (SVL = 31.8 mm), (C) dorsal and (D) lateral views of the head (HL = 11.9 mm; HW = 10.3 mm), (E) detail in dorsal view of the tubercle on heels, (F) palm of the hand (HAL = 8.7 mm), and (G) and sole of the foot (FL = 13.1 mm).

17.8 mm, THL 15.8 mm, CL 0.2 mm, HAL 10.4 mm, FLL 5.9 mm, EN 3.3 mm, head slightly longer (HL 11.9 mm) than wide (HW 10.3 mm), and wider than body; snout rounded in lateral view, truncate in dorsal view; EN shorter than ED; canthus rostralis indistinct, rounded; loreal region concave; internarial area convex; nostril slightly protuberant, directed laterally; interorbital area slightly convex; eye large, strongly protuberant; ED 1.9 times TD; tympanic membrane undifferentiated; tympanic annulus evident, rounded, concealed posteriorly by the supratympanic fold, running from the posterior corner of the eye to arm insertion. Tongue ovoid, widely attached to mouth floor; six vomerine teeth on each vomer, vomers barely separated, posteromedial to choanae; choanae ovoid. Arm slender, axillary membrane absent; ill-defined, low tubercles present along ventrolateral edge of forearm; relative length of fingers  $I < II < IV < III$ ; fingers bearing large, oval discs; subarticular tubercles prominent, ovoid to conical, single; supernumerary tubercles present; palmar tubercle small, elongated; prepollical tubercle large, flat, elliptical; prepollex enlarged, covered by skin; nuptial excrescences absent; webbing absent between fingers. Tubercle on tibiotarsal articulation; scattered tubercles along the external edge of tarsus and foot; toes bearing discs slightly wider than long, smaller than those of fingers; relative length of toes  $I < II < V < III < IV$ ; outer metatarsal tubercle ill-defined, small, rounded; inner metatarsal tubercle large, ovoid; subarticular tubercles single, low, rounded; supernumerary tubercles restricted to the sole of foot; webbing formula of toes  $I 2-2^{1/2} II 1^+-2^{1/2} III 1^{1/2}-2^{1/2} IV 3-1^{1/2} V$ . Skin on dorsum, head, and dorsal surfaces of limbs and flank mostly smooth; skin on belly and thigh coarsely granular; skin on throat and chest finely granular, arm, forearm, and shank smooth. Cloacal opening directed posteriorly at upper level of thigh; short simple cloacal sheath covering cloacal opening; round tubercles below and on the sides of the opening.

**Colours of holotype.** In preservative, dorsum greyish brown with scattered minute black dots; faint brown middorsal line extending from the tip of the snout to pelvic region, fragmented in interorbital region, a second line extending from behind the eye to pelvic region, fragmented and faint at midbody length, and a third line as a canthal stripe extending from the posterior corner of the nostril to anterior corner of the eye, and from the posterior corner of the eye to midbody length on flank; dorsal surface of limbs greyish brown with transversal faint brown bars; flank beige with dark irregular spots; posterior surface of thigh beige with dark irregular spots; venter cream white with brown spots on the mental region and chest; ventral surface of limbs cream with



**Fig. 6.** Holotype of *Boana courtoisae* sp. nov. (MNHN-RA-2020.0001) from Alikéné (French Guiana): (A) dorsal and (B) ventral views of the body (SVL = 31.0 mm, adult male), (C) dorsal and (D) lateral views of the head (HL = 11.5 mm; HW = 10.8 mm), (E) detail in dorsal view of the skin flap on heels, (F) palm of the hand (HAL = 9.3 mm), and (G) sole of the foot (FL = 12.0 mm).

a narrow brown stripe on the outer edge of the hand, forearm, thigh, tarsal fold, and foot; limb bones partially visible through skin, white. In life (Fig. 3c, d), dorsum beige with a purplish brown middorsal line extending from the tip of snout to pelvic region, a second line extending from behind the eye to pelvic region, fragmented and faint at midbody length, and a third as canthal stripe from the posterior border of nostril to anterior corner of the eye, and from the posterior corner of the eye to midbody length on flank; dorsal surface of hindlimbs with faint brown transversal bands; minute dark brown dots scattered on the dorsal surface of limbs and dorsum; flank bright yellow with dark brown irregular blotches on groin; posterior surface of thigh pale yellowish with dark brown blotches.

**Variation.** Body size varies between 31.8 and 34.7 mm in males (Supplemental Appendix 7). The paratopotype AAG-UFU 6504 lacks the heel tubercle on the right side, and MZUSP 80790 (from Rondônia) does not possess the tubercle on either side. In life (Fig. 3c, d), dorsal colouration varies from beige to brown, orange brown in calling males, with dark purplish-brown longitudinal lines and faint brown transversal bands (individuals MZUSP 159227 and MZUSP 159228 do not have longitudinal lines extending from snout to pelvic region or a canthal stripe). Throat varies from white to yellow, chest and anterior belly varying from white to cream. Iris cream or grey, sometimes with yellow pigmentation on the upper iris. Ventral surface of hand and hind limb mostly bright yellow. Posterior surface of thigh, groin and posterior half of flank spotted in black, with a bright yellow or white background colour. Brown dots scattered on dorsum, dorsal surface of limbs, bordering the lower lip, mental region, and chest. We did not observe dichromatic patterns between male and female specimens. In preservative, the colours become paler: the bright yellow tone occasionally on ventral surfaces of limbs, groin and flank is beige or pale cream. Iris grey.

**Vocal repertoire.** Calls of seven males were recorded at the type locality, in southern Brazilian Amazonia (Supplemental Appendix 6). The vocal repertoire of *Boana eucharis* is composed of two distinct calls (type 1: n = 133 calls from seven males; type 2: n = 62 calls from seven males) that are emitted sporadically at irregular intervals. The type 1 call (Fig. 4) lasts 90–420 ms and consists of 3–7 nonpulsed notes that last 3–60 ms, separated by intervals of 27–82 ms. The rise time is at 2–97% of call duration. The minimum frequency ranges from 1723–2250 Hz, the maximum frequency from 2196–4220 Hz, and the dominant frequency from 1981–2972 Hz. The type 2 call consists of one note with poorly defined pulses. Notes last 29–64 ms. The rise time is at 24–78% of note duration. The minimum frequency ranges from 1594–2067 Hz, the maximum frequency from 2712–3422 Hz, and the dominant frequency from 1938–3144 Hz.

**Distribution and ecology.** *Boana eucharis* is known from southern Amazonia in the Brazilian states of Mato Grosso and Rondônia (Fig. 1). Males call perched on shrubs in flooded areas associated with the border of secondary-growth or disturbed forests. Sympatric anuran species at the type locality of *B. eucharis* are *Boana leucocheila*, *B. albopunctata*, *Dendropsophus cruzi*, *Engystomops freibergi*, *Leptodactylus vastus*, *L. petersii*, *Pithecopus hypochondrialis*, *Scinax garbei*, and *S. nebulosus*. The species is not abundant. It is possible

that the species range is more extensive than the five populations reported in this study, which suggests a Data Deficient conservation category for *B. eucharis*. However, it is important to highlight that the southern limits of Amazonia are overall highly impacted by habitat conversion and this species could be classified at least as Vulnerable. Nevertheless, the known occurrence recordings comprise at least two protected conservation units: Pacaás Novos National Park and possibly the Cristalino State Park, and the species seems to tolerate a certain extent of human disturbance to forest habitats, since calling males were sampled at forest borders and clearings.

**Etymology.** The specific epithet is derived from the Greek word *eúkharis*, which means gracious or charismatic, as a reference to the delicate and gracious aspect of the species.

***Boana courtoisae* sp. nov.**

*Hyla fasciata* Lescure & Marty, 2000

*Hyla fasciata* Faivovich *et al.*, 2005

*Hypsiboas fasciatus* Fouquet *et al.*, 2007

*Hyla fasciata* Ávila-Pires *et al.*, 2010

*Hypsiboas* sp. (Clade H) Funk *et al.*, 2012

*Hypsiboas fasciatus* Ouboter & Jairam, 2012

*Hypsiboas fasciatus* Cole *et al.*, 2013

*Hypsiboas* sp. (Clade H) Caminer & Ron, 2014

*Boana* cf. *fasciata* Fouquet *et al.*, 2019

*Boana fasciata* Vacher *et al.*, 2020

**Holotype.** MNHN-RA-2020.0001 adult male, collected at Alikéné, French Guiana (3.20906°, –52.402000°) by J.P. Vacher and S. Cally on 12 February 2015 (Figs 3 & 6).

**Paratopotypes.** An adult female (MNHN-RA-2020.0002) collected with the holotype.

**Paratypes.** (12 males, 3 females). MNHN-RA-2020.0004 adult male, collected at Saut Taconet, French Guiana (4.03249°, –52.526188°); MNHN-RA-2020.0005 adult female, collected at Saut Grand Machicou, French Guiana (3.897416°, –52.583565°); MNHN-RA-2020.0006–7 two adult males, collected at Saul, French Guiana (3.615576°, –53.227093°); MNHN-RA-2020.0008 adult male, collected at Flat de la Waki, French Guiana (3.089500°, –0°, 398460°); MNHN-RA-2020.0009–12 four adult males, collected at Sipaliwini, Suriname (2.097530°, –56.147200°); MNHN-RA-2020.0013 an adult male, collected at Ekini, French Guiana (4.050000°, –52.466700°); MNHN-RA-2020.0014–16 three adult males, collected at Mitaraka, French Guiana (2.235770°, –54.449280°); MNHN-RA-

2020.0003 an adult female collected at Inini Tolenga, French Guiana (3.663159°, -53.928308°).

**Other material.** Twelve additional males and three females (Supplemental Appendix 8) from French Guiana and Suriname were also assigned to *B. courtoisae*. They were examined and included in the analysis of the variation but were not deposited and not included in the type series.

**Diagnosis.** *Boana courtoisae* sp. nov. is characterized by the following combination of character states: (1) skin flap on heel; (2) vocal repertoire composed of more than one call type; (3) multinote call; and (4) regular internote intervals; (5) multi-blotched pattern on groin and flank of males (Figs 3, 4 & 6).

**Comparisons with congeners of the Amazonian clade.**

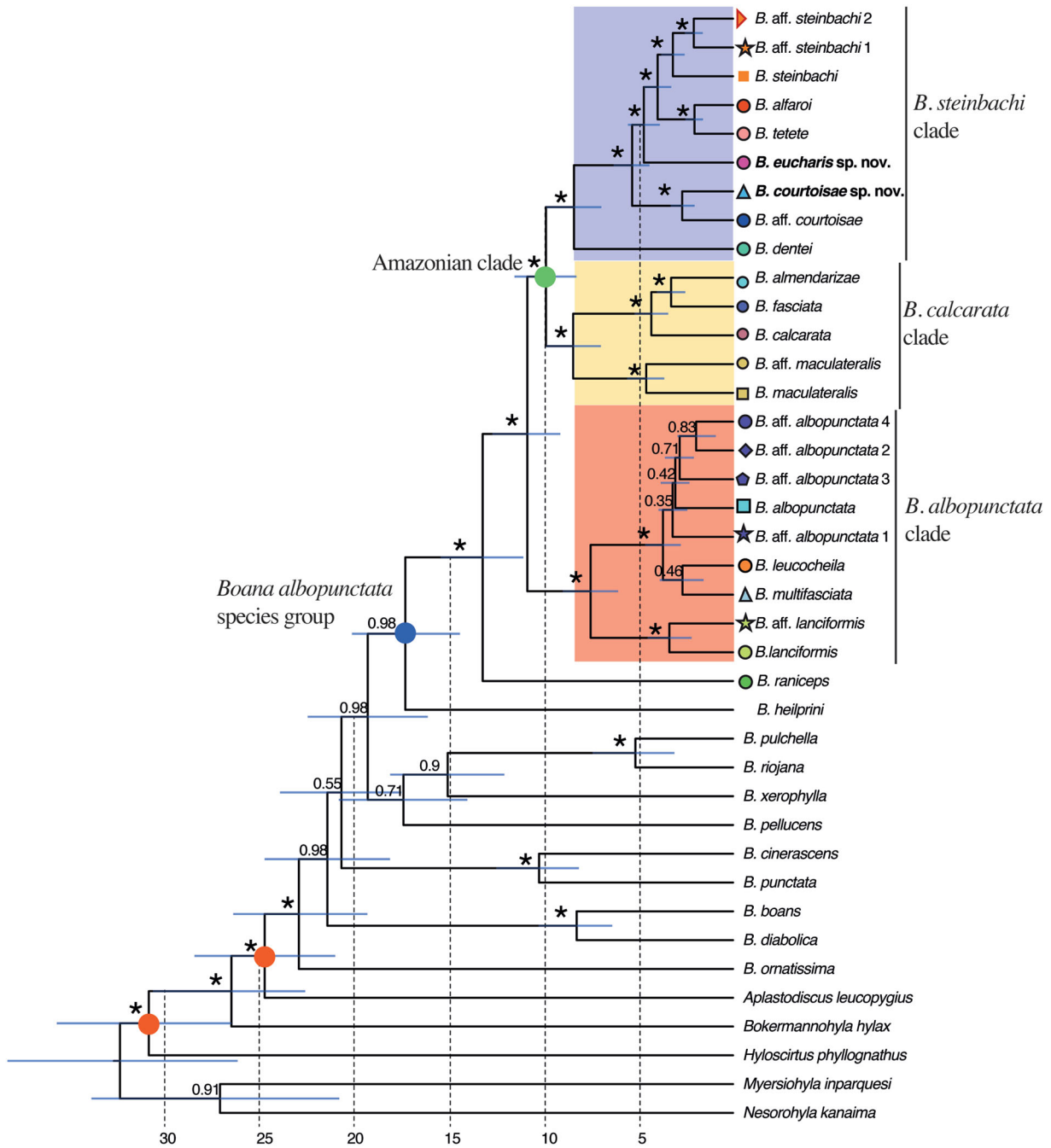
*Boana courtoisae* sp. nov. is distinguished from members of the *B. calcarata* clade (*B. almendarizae*, *B. fasciata*, *B. calcarata*, and *B. maculateralis*) by the absence of a calcar and by the vocal repertoire composed of more than one type of call. Within the *B. steinbachi* clade, *B. courtoisae* can be distinguished from *B. dentei*, and *B. tetete* by its multinote call (one-note calls in *B. dentei* and *B. tetete*), and from *B. alfaroi* by its regular internote intervals and vocal repertoire composed of two distinctive types of calls (in *B. alfaroi*, call notes having irregular intervals, sometimes partly fused one with the next, and repertoire formed by one call type) (Caminer & Ron, 2014; Marinho et al., 2020). *Boana courtoisae* can be distinguished in almost all cases from *B. eucharis* by the presence of skin flap on heel (tubercle in *B. eucharis*; but see Variation). In addition, *B. courtoisae* can be distinguished from *B. eucharis* and *B. steinbachi* by a multi-blotched pattern on flank and groin of males (fewer blotches, or transversal bands and spots in males of the other two species; Fig. 3). Although *B. courtoisae* and *B. eucharis* are distinguished by subtle differences in morphology and colouration, the two species are not directly phylogenetically related to each other since *B. courtoisae* is sister of the remaining species that form the *B. steinbachi* clade (*B. steinbachi*, *B. alfaroi*, *B. tetete*, and *B. eucharis*) except *B. dentei*, which strongly supports the distinct specific status relative to its closest relatives (Fig. 7).

**Description of holotype.** Adult male, SVL 31.0 mm, FL 12.0 mm, ED 4.4 mm, TD 1.8 mm, TL 18.9 mm, THL 15.8 mm, CL 0.4 mm, HAL 9.3 mm, FLL 6.2 mm, EN 4.2 mm, head slightly longer (HL 11.5 mm) than wide (HW 10.9 mm), and wider than body; snout rounded in lateral view, truncate in dorsal view (Fig. 6); EN shorter

than ED; canthus rostralis indistinct, rounded; loreal region concave; internarial area convex; nostrils slightly protuberant, directed laterally; interorbital area slightly convex; eye large, strongly protuberant; ED 2.5 times TD; tympanum membrane undifferentiated; tympanic annulus evident, rounded, concealed posteriorly by supratympanic fold, running from the posterior corner of the eye to arm insertion. Tongue ovoid, widely attached to mouth floor; vomerine odontophores triangular with arched base, barely separated, posteromedial to choanae, bearing eight vomerine teeth on each side; choanae ovoid. Arm slender, axillary membrane absent; indistinct low tubercles present along ventrolateral edge of forearm; relative length of fingers I < II < IV < III; fingers bearing large, oval discs, subarticular tubercles prominent, ovoid to conical, single; supernumerary tubercles present; palmar tubercle small, elongated; prepollical tubercle large, flat, elliptical; prepollex enlarged, claw shaped; nuptial excrescences absent; webbing absent between fingers. Skin flap on tibiotarsal articulation; scattered tubercles on tarsus and along ventrolateral edge of foot; toes bearing discs slightly wider than long, smaller than those of fingers; relative length of toes I < II < V < III < IV; outer metatarsal tubercle ill defined, small, round; inner metatarsal tubercle large, elongated and elliptical; subarticular tubercles single, low, rounded; supernumerary tubercles restricted to the sole of foot; webbing formula of toes I2<sup>-</sup>2<sup>1/2</sup>III<sup>+</sup>-2<sup>1/2</sup>III1<sup>1/2</sup>-2<sup>1/2</sup>IV3-1<sup>1/2</sup>V. Skin on dorsum, head, and dorsal surfaces of limbs smooth; skin on flanks smooth with weak longitudinal wrinkles posterior to the arm; skin on venter coarsely granular; skin on ventral surfaces of head and thighs granular, those of shanks smooth. Cloacal opening directed posteriorly at upper level of thighs; short simple cloacal sheath covering cloacal opening; round tubercles below and on the sides of the opening.

**Colour of holotype.** In preservative, dorsum beige with scattered minute black dots and spots (Fig. 6); faint brown narrow middorsal line extends from the tip of the snout to the vent; faint brown transversal bands on dorsum; dorsal surface of limbs beige with transversal faint brown bars; flank white with dark irregular spots; posterior surface of thigh white with dark irregular blotches; venter creamy white with brown spots on the throat and chest; ventral surface of limbs whitish cream with scattered dots on the forearm; ventral surface of shank cream; a discontinuous brown stripe, varying in width along its length, on the outer edge of the hand and forearm, limb bones (visible through skin) white. In life, dorsum beige with a faint brown narrow longitudinal line from snout to vent; dorsal surface of limbs beige with faint brown transversal bands; scattered



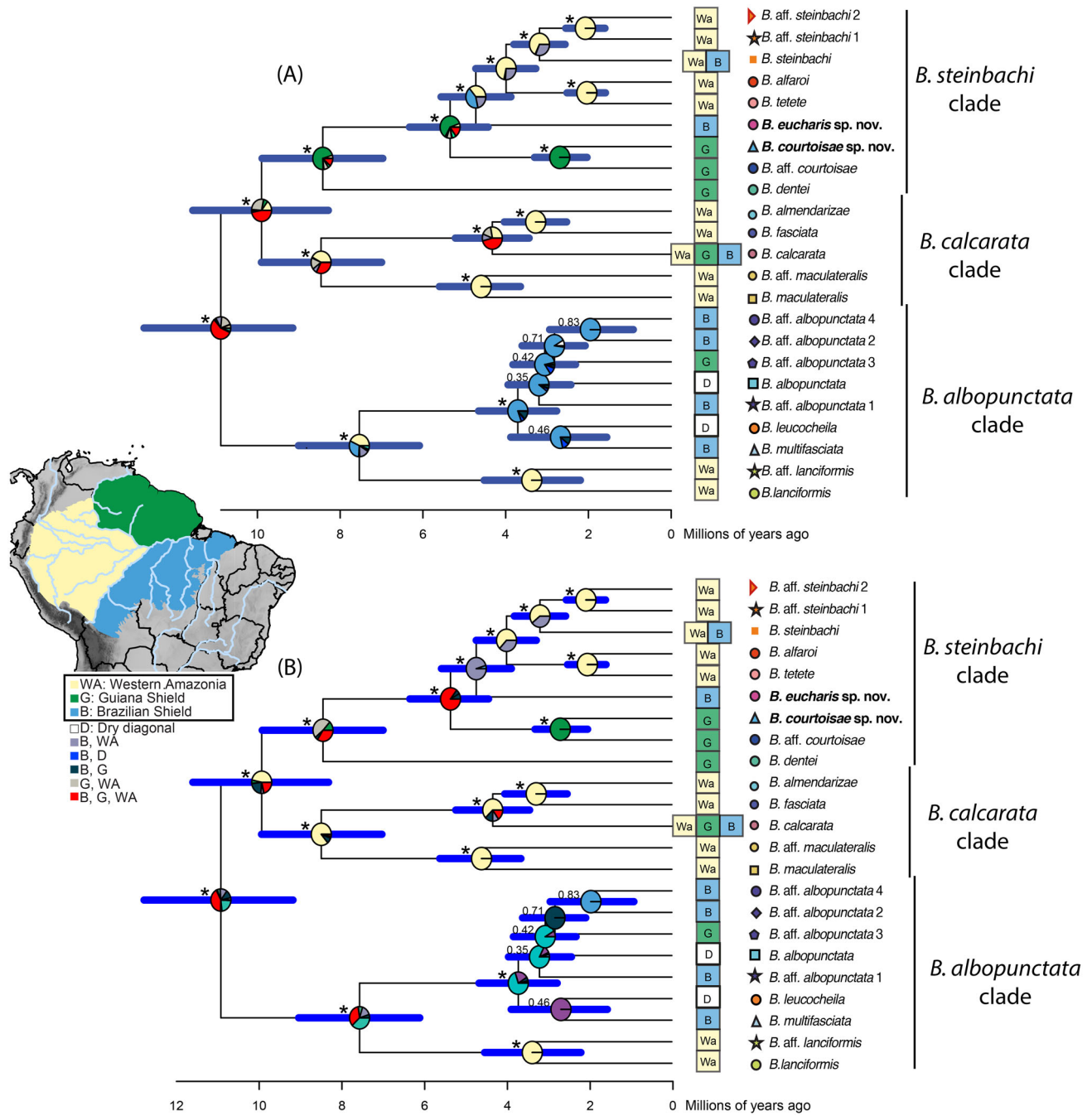


**Fig. 7.** Time calibrated tree inferred from the analysis of mitogenomic data in BEAST2. Nodes with maximum posterior probability (0.99 and 1) are indicated with an asterisk. Calibrated nodes are indicated with a red circle. The blue and the green circles point at major clades mentioned in the text. Node bars indicate the 95% highest posterior distributions of node dates. Symbols on the tips of the trees are the same as those used to indicate the geographic distribution of sampled species.

minute black dots on the dorsal surfaces of limbs and dorsum; flank white to light yellow with dark irregular blotches; venter white to cream; scattered brown flecks on the throat and chest, and bordering the lower lip; ventral surface of limbs pale yellow, with bright yellow granules; discs and webbing yellow; iris cream with an

undefined upper yellow band; limb bones (visible through skin) white.

**Variation.** Body size varies between 30.8 and 35.9 mm in males and between 43.0 and 45.9 mm in females (Supplemental Appendix 7). In life (Fig. 3e–f), dorsal



**Fig. 8.** Ancestral area reconstruction for the *Boana albopunctata* species group (*B. heilprini* and *B. raniceps* excluded) using BioGeoBears assuming (A) a DEC + J model and (B) a DEC model (Supplemental Appendix 9); most likely biogeographic scenarios plotted on the chronogram obtained with BEAST 2.5, (Numbers on branches are posterior clade probabilities, those  $> 0.95$  are indicated with an asterisk. Node bars indicate the 95% highest posterior distributions of node dates. Squares on the tips of the trees indicate the geographic distribution of extant species sampled in the phylogeny. Pie charts on nodes show the most likely reconstructions of ancestral areas, the size of each slice proportional to the maximum likelihood. Colours corresponding to the different geographic distributions are depicted on the left.

colouration varies from beige to bright yellow or orange brown, with faint brown transversal bands markings, purplish-brown to dark brown longitudinal lines and many scattered brown spots covering all over the dorsum. Throat, chest, and anterior belly varying from white to cream with

scattered spots covering the entire ventral surface in both sexes. Flank and groin with dark brown to black blotches on a bright yellow, bluish or white background. Ventral surface of hand, foot, and legs varying from bright yellow to dark grey, covered with black spots. Posterior surface of

thigh striped or spotted in black with a bright yellow or white background colour, sometimes with tints of blue particularly pronounced in females (individual MNHN-RA-2020.0006 has no blotches or dots on the posterior surface of thigh or spots on belly). Iris grey or cream, sometimes with a yellow upper band (the individual MNHN-RA-2020.0015 has also black markings surrounding the iris). Our collected individuals exhibit a dimorphic colouration between males and females. The ventral surface of thigh in males varies from bright yellow to greenish yellow. In females, the colouration of the ventral surface of thigh is blue, bluish grey or dark grey. The groin region, flank, and posterior surface of thigh have more tints of blue in females. In addition, flank and groin maculation is denser and more extensive in females. In preservative, the colours fade, becoming pale: the bright yellow tone sometimes present on dorsum is beige or brown with brown transversal bands and lines. Ventral surfaces of body, limbs, groin and flank are beige or pale cream. The blue tints on groin, flank and posterior surface of thigh completely vanish, as well as the dark blue or grey colours on ventral surfaces of female's thigh. Iris grey.

**Vocal repertoire.** Calls of five males were recorded from French Guiana and Suriname. The vocal repertoire of *B. courtoisae* is composed of two types of call (type 1:  $n = 13$  calls from five males; type 2:  $n = 5$  calls from three males) that are emitted sporadically at irregular intervals. The type 1 call (Fig. 4) lasts 140–240 ms, consisting of 3–4 nonpulsed notes that last 5–36 ms, separated by intervals of 20–69 ms. The rise time is at 7–94% of call duration. The minimum frequency ranges from 1335–1875, the maximum frequency from 2627–3101 Hz, and the dominant frequency from 1637–2455 Hz. The type 2 call consists of one note with poorly defined pulses. Notes last 39–79 ms. The rise time is at 39–82% of note duration. The minimum frequency ranges from 1464–1723 Hz, the maximum frequency from 2627–3144 Hz, and the dominant frequency from 1680–2412 Hz.

**Distribution and ecology.** *Boana courtoisae* is distributed throughout the eastern Guiana Shield in French Guiana, Suriname, Guyana and adjacent Brazilian Amazonia in the states of Amapá, Pará (Ávila-Pires *et al.*, 2010), and possibly Roraima (pending confirmation). The species could possibly occur in the state of Amazonas as well. The species is not abundant and found in scattered populations. Nevertheless, its range is extensive and encompasses numerous protected areas. Moreover, the species seems to tolerate habitat disturbance, indicated by the use of forest borders and clearings. Therefore, the conservation status of *B. courtoisae* might be classified as Least Concern (pending a formal

evaluation by IUCN team). It is a nocturnal species found in primary and secondary forest associated with the flooded zones of slow streams and medium-sized rivers. The males call perched at low height on the adjacent vegetation or even overlooking the water forming small groups of 2–10 individuals separated by a few metres from each other. A single clutch was observed at Mitaraka, French Guiana and contained approximately 1,100 beige eggs deposited directly in the water and forming a film on the surface.

**Etymology.** This species is dedicated to our friend Elodie Courtois, in honour of her invaluable contribution to field herpetology in French Guiana, notably the monitoring of populations of threatened species and discovery of previously undocumented species and many natural history observations.

### Mitogenomic phylogeny

The mitogenomic phylogeny is well resolved with the exception of the position of the clade formed by *B. cinerascens* + *B. punctata* and the position of *B. pellucens* within the genus (Fig. 7). Within the focal species group only the relationships among the different OTUs of the *B. albopunctata* clade remain ambiguous. *Boana heilprini* is strongly supported as the sister species of all other members of the *B. albopunctata* species group whose crown age is estimated to date back to 17.3 Ma (14.4–20.2). *Boana raniceps* forms a clade with all the other species of the group whose crown age is estimated to date back to 13.2 Ma (11.1–15.5). The remainder of species forms three main clades. The *B. albopunctata* clade diverges from the Amazonian species  $\sim 10.9$  Ma (9.1–12.7). The two Amazonian clades diverged  $\sim 9.9$  Ma (8.3–11.6).

### Biogeographic inferences

Model comparisons identified DEC + J as the best-fit model (Supplemental Appendix 9). According to both DEC and DEC + J models the ancestral range of the *B. albopunctata* species group remains largely ambiguous since each of the major lineages is widely distributed in Amazonia and even further for the *B. albopunctata* clade. However, the ancestral range of the *B. steinbachi* clade is supported to be located in the Guiana Shield by the DEC + J model (Fig. 8A); and while it remains ambiguous for the DEC model, all states with high likelihood encompass the Guiana Shield (Fig. 8B). This group probably dispersed southward to the Brazilian Shield  $\sim 5$  Ma, as suggested by the phylogenetic position and range of *B. eucharis* (Fig. 8). Subsequent dispersal from the Brazilian Shield toward western Amazonia is

suggested by the nested position of the clade formed by *B. tetete*, *B. alfaroi*, *B. steinbachi* and related OTUs. The occurrence of *B. steinbachi* in eastern Amazonia probably resulted from an even more recent dispersal toward the east (Pará state) from Western Amazonia (Fig. 8A, B). Mirroring that situation, the ancestral range of the *B. calcarata* clade is inferred in western Amazonia where it has exclusively diversified except for a single and recent dispersal of *B. calcarata* eastward throughout Amazonia.

The ancestral range of the *B. albopunctata* clade remains ambiguous since it also displays an east vs. west pattern with *B. lanciformis* occurring only in western Amazonia and the rest of the species of the clade occurring in the Cerrado and eastern Amazonia. However, this east-west divergence seems more recent in that group (7.5 Ma) than the *B. calcarata* vs. *B. steinbachi* divergence (9.9 Ma).

## Discussion

### Species richness and distribution

With 25 putative species and two taxa (*B. caiapo* and *B. paranaiba*) that could not be included in this study, the species richness of the *B. albopunctata* group may actually reach 27 species, i.e., 70% higher than currently recognized (16 valid species). Within the Amazonian clade, the actual number of species may be 44% higher than currently recognized (14 OTU for 10 described/valid species including the two species described herein). We could not gather sufficient phenotypic data for the OTU identified as *B. aff. maculateralis*, but we are confident that such data will contribute to its taxonomic resolution. Moreover, our genetic sampling remains limited, specifically in Colombia, Venezuela, and south-eastern Amazonia, and more species in the group probably remain undocumented.

The discovery of yet undescribed species in Amazonia is not surprising, since new species descriptions of squamates and anurans keep accumulating at a fast pace (e.g. Carvalho et al., 2020; Kok et al., 2018). In fact, almost all systematic investigation of broadly distributed groups in Amazonia led to the understanding that they actually represent species complexes, often hiding narrowly distributed and remotely diverging species within Amazonia (e.g., Fouquet et al., 2014; Vacher et al., 2020). This recurrent pattern is also illustrated herein in the *B. albopunctata* species group notably by *B. fasciata*, a taxon that was used to design populations throughout Amazonia until the conclusions of Caminer and Ron (2014). The extent of the actual diversity of anurans in Amazonia remains so speculative that it could be three to four times higher than the current

~600 species occurring in that region according to the IUCN (Vacher et al., 2020).

Relationships and distribution of the species we found within the Amazonian clade strikingly mirror those found in other groups of anurans, notably *B. semilineata* group (Caminer & Ron, 2020; Fouquet et al., 2016; Peloso et al., 2018); *Osteocephalus* (Jungfer et al., 2013); *Dendropsophus minutus* (Gehara et al., 2014); *Allobates* (Réjaud et al., 2020); *Amazophrynella* (Rojas et al., 2018); *Adenomera* (Fouquet et al., 2014) and, more broadly, matches a pattern of allopatry throughout Amazonia in which communities are spatially structured forming distinct bioregions (Vacher et al., 2020). However, the history of Pan-Amazonian diversification is relatively recent in the case of the Amazonian clade of *Boana* studied herein compared with other taxa, such as *Allobates* (Réjaud et al., 2020) or *Amazophrynella* (Rojas et al., 2018). A combination of historical and contemporary climatic heterogeneity as well as species-specific dispersal ability and niche breadth (Sheu et al., 2020) is probably responsible for these common and distinct spatio-temporal patterns across taxa. Our time-scaled phylogeny and biogeographic analyses provide some insights into the historical processes responsible for the diversification of the *B. albopunctata* species group in Amazonia.

### Biogeography

With nine OTUs the species richness in western Amazonia is confirmed to be higher than in the Guiana Shield (4 OTUs) and the Brazilian Shield (3 OTUs), suggesting that the climatic conditions and historical geomorphological dynamism in this region, notably hydrological changes, may have played a major role in *B. gr. albopunctata* diversification.

The initial diversification within the Amazonian clade between the *B. calcarata* clade in the west and the *B. steinbachi* in the east of Amazonia dates back to about 10 Ma. This estimate is relatively younger than those of Funk et al. (2012) and Duellman et al. (2016) partly because the divergences estimated by Feng et al. (2017), which were used as calibrations herein, are overall more recent than the nodes of those previous studies that were based on a lower amount of genomic data. Other east-west divergences, putatively simultaneous to the one found herein within the Amazonian clade of the *B. albopunctata* group, are documented in the *Adenomera heyeri* clade (Carvalho et al., 2020; Fouquet et al., 2014), in two instances within the *Allobates trilineatus* clade (Réjaud et al., 2020), in *Ameerega* (Guillory et al., 2020), and probably in many other lineages for which sampling and dating are still missing. This 10 Myr old node coincides with the end of the Pebas system and the transition from

a western watershed drained to the north to a Pan-Amazonian system drained to the east (Hoorn *et al.*, 2017). Available data indicate that the rise of the Vaupés Arch around 10 Ma completely separated the Western Amazon and Llanos basins (Hoorn *et al.*, 2010; Jaramillo *et al.*, 2017). We assume that this new configuration may have permitted the dispersal between the Guiana Shield and Western Amazonia and could be responsible for this 10 Myr old divergence in the *Boana* of the Amazonian clade and the other mentioned groups.

Subsequently, both groups have apparently diversified *in situ*, i.e., within Western Amazonia and within the Guiana Shield until some 5 Ma. This date coincides with the divergence between *B. courtoisae* and the other species of the *B. steinbachi* clade and suggests a dispersal from the Guiana to the Brazilian Shield across the trans-continental configuration of the Amazon River, which contradicts our expectation that such divergence would precede this configuration (9 Ma; Hoorn *et al.*, 2017). Temporally concordant north/south divergences are documented within *Allobates tapajos* and between *A. bacurau* and *A. sumtuosus* (Réjaud *et al.*, 2020), in *Chiasmocleis* (de Sá *et al.*, 2019) and most likely other lineages of terrestrial vertebrates for which the histories of diversification remain undocumented. The processes that may have fostered multiple trans-Amazon dispersals around 5 Ma remain highly speculative. Considerable uncertainty remains about the timing and amplitude of historical topographic, hydrological, and vegetational changes in Amazonia (Albert *et al.*, 2018a; Bicudo, Sacek, de Almeida, Bates, & Ribas 2019; Campbell, Frailey, & Romero-Pittman, 2006; Hoorn *et al.*, 2017; Latrubesse *et al.*, 2010). The sediment discharge in the Amazon fan was relatively modest until 5 Ma and vastly increased in the Pliocene-Pleistocene (Albert *et al.*, 2018a; Hoorn *et al.*, 2017). The lower course of the Amazon River may have become an impassable barrier for these taxa only from the Miocene-Pliocene boundary onward. Moreover, this period also coincides with vegetational and climatic changes, notably the expansion of grasslands not only in the Andes and the Cerrado but also within Amazonia (Kirschner & Hoorn, 2019).

Subsequently, i.e., the last 5 My, both lineages diversified extensively in Western Amazonia, notably along the foothills of the Andes. This diversification has probably been fostered by the combination of increasing availability of suitable *terra-firme* habitat due to the retreat of the lacustrine ecosystem and dynamic river capture (Albert *et al.*, 2018a). The ancestors of the *B. calcarata* clade have presumably diversified in the western Acre system, i.e., between the Andes and the Amazon rainforest in the east (Latrubesse *et al.*, 2010). Only recently, dispersal towards the east seems to have

occurred, notably in *B. calcarata*. This last species displays the largest range in the clade, which may be explained by the large diversity of rivers used by that species and the fact that large rivers do not represent efficient barriers for its dispersal.

The diversification of the *B. albopunctata* clade has taken place both in Amazonia and in the Cerrado and appears overall more recent than within the Amazonian clade. These lineages probably originated from transitional ecosystems between the Dry Diagonal and Amazonia. This diversification may be partly related to changes in climate and probable forest retreat in the eastern part of Amazonia during the Late Pliocene and Pleistocene (Cheng *et al.*, 2013; Ledru *et al.*, 2000; Pennington *et al.*, 2000; Van der Hammen & Hooghiemstra, 2000).

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## Supplemental data

Supplemental data for this article can be accessed here: <https://doi.org/10.1080/14772000.2021.1873869>.

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