

# Mind the gap!

## A review of Amazonian anurans in GenBank

MICHAEL MAYER, LUIS F. MARIN DA FONTE & STEFAN LÖTTERS

Department of Biogeography, Trier University, 54286 Trier, Germany

Corresponding author: STEFAN LÖTTERS, e-mail: loetters@uni-trier.de

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**Abstract.** We studied the knowledge gap in GenBank with regard to the ca. 600 anuran species from Amazonia. The markers 12S, 16S, COI and cytb were examined, on which information was available for about half of all species. Both the number of sample sites and the number of samples per species varied greatly (best studied each in 16S:  $4.85 \pm 10.37$ ;  $11.19 \pm 31.20$ ), and merely one fifth of all species had at least 5 sample sites. This suggests that a considerable portion of species is underrepresented in GenBank. Representativeness is especially difficult to assess in widespread species that at the same time could well represent cryptic allopatric species (i.e., with smaller distributions). This is a well-known phenomenon in Amazonian anurans considering that truly widespread species do exist. Moreover, limited sampling may not necessarily be the result of limited representativeness, as numerous species are known to occupy relatively small localised to regional ranges only. Our study furthermore revealed that in a geographic context, major portions of Amazonia have as yet been undersampled. That is, the total of 453 sample sites (most with more than one species sampled) are spatially clustered, often in areas with increased anthropogenic activity. We conclude that there is a large knowledge gap in terms of spatial sampling, resulting in taxonomic deficiencies.

Key words. ‘Missing areas’, mitochondrial markers, sampling, spatial distribution.

### Introduction

The Amazon Basin is one of the mega-diversity regions of the globe, and it has an iconic status in biogeographic and evolutionary research (HOORN et al. 2010, JENKINS et al. 2013). One of the most diverse animal groups in this region is amphibians. Of the worldwide more than 7,800 amphibian species known to date (AMPHIBIAWEB 2018), several hundreds have been recorded from Amazonia, of which most are members of the order Anura (IUCN 2017). Various studies aiming at a better understanding of Amazonian anuran diversity have to an increasing extent become available over the last two decades (e.g., NOONAN & GAUCHER 2005, SANTOS et al. 2009, WIENS et al. 2011, DUELLMAN et al. 2016, GEHARA et al. 2014). These modern approaches basically make use of molecular markers, and it is mandatory to most authors that data be stored and made public via online databases. Regarding amphibians, NCBI GenBank (BENSON et al. 2015) is broadly used for information storage (e.g., VENCES et al. 2005, VENCES & KÖHLER 2006, VIEITES et al. 2009, CHE et al. 2012).

Because anurans are so diverse in Amazonia and have been attracting ever-greater study interest, they have been proposed as suitable when studying more general research questions with regard to the genesis of Amazonian biota

(e.g., AZEVEDO-RAMOS & GALATTI 2002, BUCKLEY & JETZ 2007, ZEISSET & BEEBEE 2008, ANTONELLI et al. 2018). Concerning this potential role as a model group in biogeographic and evolutionary research, one may ask how well the various species are represented in GenBank, especially as more than a decade ago, Latin American amphibians were considered to be ‘under-represented’ in GenBank (VENCES & KÖHLER 2006). Assessing the biogeographic-taxonomic knowledge gap is especially relevant, as current studies do (e.g., PYRON & WIENS 2011), and forthcoming ones are expected to, increasingly make use of GenBank information.

We assessed Amazonian anuran species in GenBank with the focus on four markers and here provide information on the species included, the number of sample sites, and samples per species. We furthermore analyse the available data in a geographic context.

### Methods

#### Geographic focus

There is no universal definition of ‘Amazonia’ in the literature (GOULDING et al. 2003). In our study, we combined 25 global WWF Terrestrial Ecoregions as defined by OLSON

et al. (2001). This area, 5,959,751 km<sup>2</sup> in size (Fig. 1), encompasses all piedmont–lowland moist, 'várzea' and rain forest units plus two associated moist savannas within the Amazon and Tocantins river catchments and additionally incorporates parts of the Guiana Shield (Supplementary data 1). Geographical data were obtained from: [www.worldwildlife.org/publications/terrestrial-ecoregions-of-the-world](http://www.worldwildlife.org/publications/terrestrial-ecoregions-of-the-world) (accessed 25 November 2016). They were processed with ESRI ARCGIS 10.2.

#### GenBank search

We initially used the IUCN Red List of Threatened Species (IUCN 2017) and the GIS-ready shapefiles available from it ([www.iucnredlist.org/technical-documents/spatial-data](http://www.iucnredlist.org/technical-documents/spatial-data)) to identify anuran species native to Amazonia, as defined above. From these 609 species, we excluded those with less than 20% of their total distribution within Amazonia from

further analyses, i.e., we consider them 'non-Amazonian' (Supplementary data 2). This left us with 494 species that are partly or entirely distributed in Amazonia. Despite regular updates, the IUCN Red List lags behind the progress in taxonomy. Therefore, we used FROST (2017) and identified another 18 species described from our focal region between 2014 and 2017 and not yet considered by the IUCN (Supplementary data 3).

The combined list of 512 species names was used as an operational tool to run GenBank searches ([www.ncbi.nlm.nih.gov/genbank](http://www.ncbi.nlm.nih.gov/genbank)) for sequence availability via the 'nucleotide search' function. Because the IUCN Red List is behind taxonomic progress and taxonomic changes are not carried forward to GenBank at all, we also used both old (synonymous) and most recent names as available from FROST (2017). We aimed at four mitochondrial (mt) markers, widely used in Neotropical anuran research (e.g., VENCES et al. 2005, FOUQUET et al. 2007a, VIEITES et al. 2009, CHE et al. 2012, GEHARA et al. 2014, PELOSO et al.

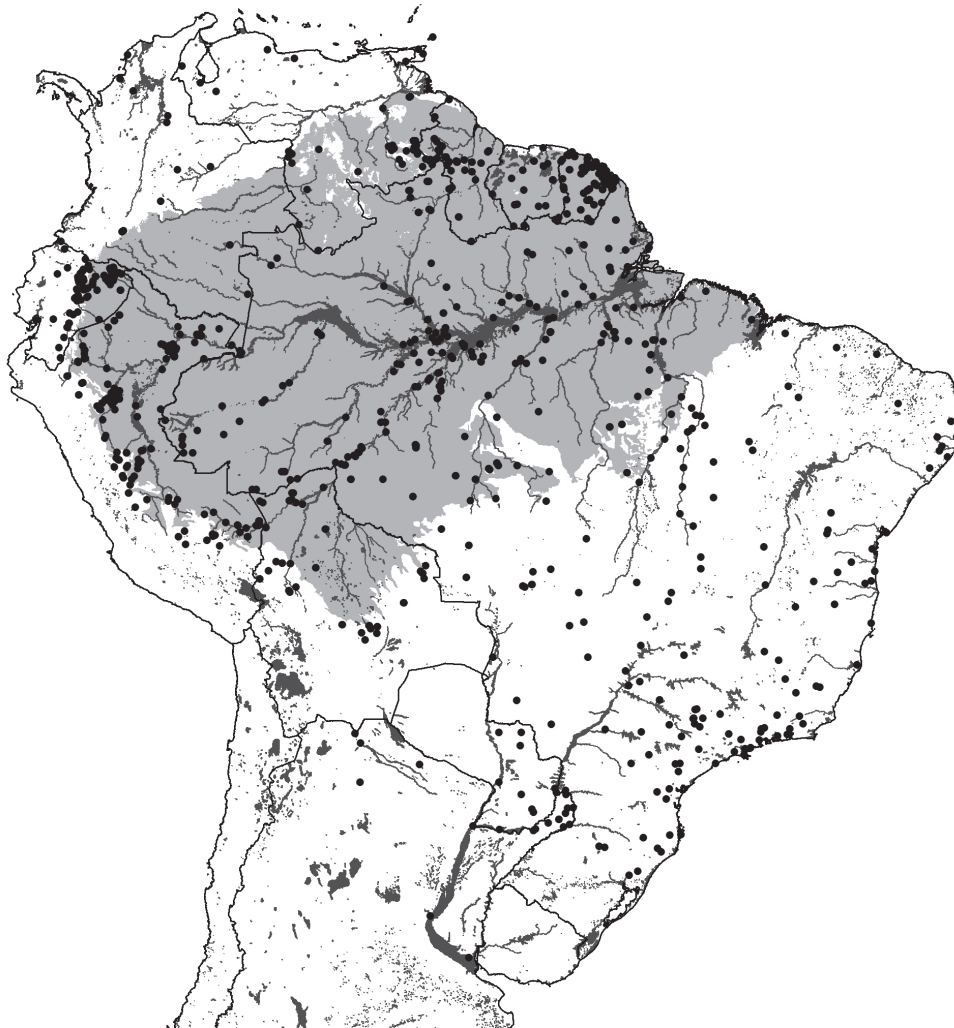


Figure 1. Delimitation of 'Amazonia' as a composite of 25 WWF Terrestrial Ecoregions highlighted in grey (Supplementary data 1). Dots represent 774 sample sites (453 of which in Amazonia) of anuran species that have at least 20% distribution overlap with 'Amazonia'.

Table 1. Quantitative data of four studied markers of Amazonian anurans in GenBank (for details see Supplementary data 4). Means are followed by standard deviations and ranges in parentheses.

Marker	Number of species	Number of sample sites per species	Number of samples per species
12S	254	3.40 ± 6.25 (1–54)	6.26 ± 14.12 (1–123)
16S	293	4.85 ± 10.37 (1–138)	11.19 ± 31.20 (1–394)
COI	117	1.83 ± 10.33 (1–147)	3.34 ± 23.57 (1–392)
cytb	151	1.91 ± 6.72 (1–77)	4.10 ± 19.80 (1–287)

2014, FERRÃO et al. 2016): ribosomal rRNA subunits 12S and 16S (12S and 16S, respectively), Cytochrome Oxidase 1 (COI), and Cytochrome b (cytb). Genetic data were available for a total of 348 entries from our operational list of species names (not necessarily for all four markers). Additionally, we recorded the number of samples (individuals) studied for each marker; data are provided in Supplementary data 4. In the process, currently valid names, as available from FROST (2017), were added to all names.

#### Geographic sample site allocation

For 40 of the 348 species names, locality information was lacking, or was given so imprecisely that spatial uncertainty was too great for integration into this study (e.g., referring to an entire river system of several hundred kilometres in length, or an entire country). For the resulting 308 species names (Supplementary data 4), geo-referenced locality data (latitude–longitude) were directly adopted from GenBank entries (listed in Supplementary data 5). Where not available, we searched for more precise locality information in the publications referred to in GenBank and used Google Earth 7. In this manner, a total of 1,558 geo-referenced records (i.e., sample sites) were obtained for the 308 species names. The elimination of duplicates left 774 unique sample sites (regardless of how many species were recorded from a single site). Of these, 453 (1,107 records) were located within the predefined region ‘Amazonia’, whereas the remaining ones were extralimital (Fig. 1).

#### Geographic data analysis

For an analysis of the resulting point pattern, a multi-distance spatial cluster analysis was performed using the 453 unique sample sites from Amazonia in terms of an L function using ArcGIS. The L function is a variance-stabilized derivative of Ripley’s K function (BESAG 1977) and uses a random point pattern following a Poisson distribution. If the observed function is greater than the function derived from the point pattern generated at random, the focal points (i.e., sample sites) are geographically clustered (HAASE 1995).

With the goal of explaining the geographic pattern, we assessed the Human Footprint Index (HFI) at sample sites within Amazonia in order to examine if their spatial dis-

tribution was influenced by increased anthropogenic activity. The HFI is based on population density, extents of infrastructure and agriculture, and other landscape features (SANDERSON et al. 2002). Grid-based HFI values in the range 0–100 (i.e., from ‘mostly wild’ to ‘high anthropogenic impacts’) are available at a 30 arc-sec resolution from the ‘Last of the Wild’ project (<http://sedac.ciesin.columbia.edu/wildareas>, last accessed 18 May 2017). Using ArcGIS, we extracted HFI values at sample sites and tested if their means were significantly different to those of all grid cells with no collection activity within the area previously defined as Amazonia (Mann-Whitney U-test for non-parametric data).

## Results

### Representation of Amazonian anurans in GenBank data

According to the most recent taxonomy, the 308 species names account for 305 species (Supplementary data 4). Our data search revealed that amongst these, the total number of georeferenced sample sites per species across their entire distributions ranged from 1–147 (mean  $5.07 \pm 11.98$ ). Within Amazonia only, the range was 1–74 (mean  $3.69 \pm 7.57$ ). About one third (i.e., 116) of all species had only 1, more than half (i.e., 170) had  $\leq 2$ , and merely about one fifth (i.e., 59) had  $\geq 5$  sample sites within Amazonia.

Regarding the individual four markers, information was available for 12S and 16S in 82.47 and 95.13% of all 308 species names, respectively; it was comparatively less for the other two markers (Table 1). Mean values of all markers were notably low (with a high standard deviation) in terms of both the number of sample sites and the number of samples. Sampling effort was high in a few species, however; this resulted in high upper ranges in these two parameters, which were especially high in 16S and COI (Table 1). Table 2 provides an overview of the 20 best-studied species over all four markers. For more comprehensive information see Supplementary data 4.

### Examination of geographic sampling effort

As is illustrated in Fig. 1, samples sites are unevenly distributed both within Amazonia and beyond. In accordance with this pattern, the L function analysis indicated that

Table 2. The 20 best sampled Amazonian anuran species in GenBank in alphabetical order. We here show the 10 highest values of the number of sample sites for each of the four genes in Supplementary data 4 and accumulated species names.

Species	Sample sites within Amazonia	Sample sites		Samples		Sample sites		Samples	
		12S	12S	16S	16S	COI	COI	cytb	cytb
<i>Adenomera andreae</i>	74	36	91	36	88	77	77	77	287
<i>Adenomera hylaedactyla</i>	43	13	27	16	27	69	79	69	76
<i>Allobates femoralis</i>	71	44	85	69	301	5	9	33	147
<i>Ameerega hahneli</i>	11	10	24	14	29	4	12	13	38
<i>Ameerega trivittata</i>	15	19	42	19	47	7	13	15	36
<i>Anomaloglossus baobatrachus</i>	19	26	81	26	82	1	4	1	4
<i>Atelopus flavescens</i>	2	3	3	3	3	4	15	4	18
<i>Dendrobates tinctorius</i>	15	4	5	4	49	2	2	16	34
<i>Dendropsophus minutus</i>	33	1	1	138	394	147	392	1	1
<i>Engystomops petersi</i>	23	30	123	30	123	1	1	0	0
<i>Leptodactylus fuscus</i>	8	23	25	23	47	1	4	1	1
<i>Leptodactylus mystaceus</i>	18	22	55	22	48	1	1	1	2
<i>Osteocephalus buckleyi</i>	14	16	34	10	24	12	19	3	5
<i>Osteocephalus taurinus</i>	33	54	80	58	111	14	30	18	19
<i>Pristimantis zeuctotylus</i>	20	23	59	20	48	0	0	0	0
<i>Ranitomeya imitator</i>	5	9	17	9	18	9	15	9	15
<i>Ranitomeya variabilis</i>	18	8	15	18	34	0	0	19	30
<i>Ranitomeya ventrimaculata</i>	20	19	44	19	44	14	24	19	36
<i>Rhinella marina</i>	13	5	15	15	60	15	65	15	17
<i>Scinax ruber</i>	13	22	22	28	28	0	0	18	18

sampling was significantly inhomogeneous across geographic space (Fig. 2). Within the study region, some clusters are obvious in Fig. 1, including for instance: the upper Amazon Basin in Ecuador and parts of Peru; the area around and south of Manaus (Brazil); the Guyana-Venezuela border area, and French Guiana. Proportionally well

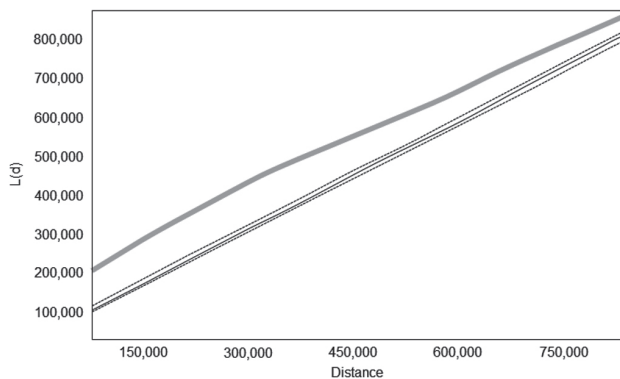


Figure 2. L functions showing that 453 sample sites from within Amazonia (cf. Fig. 1) are significantly clustered in geographic space. The observed function (bold grey line) runs above the confidence envelopes (hatched thin lines) of the expected function, derived from randomly distributed points (continuous thin black line).

sampling are parts of some major Amazonian rivers, most notably the Rio Madeira. In accordance with these findings, the HFI for Amazonia showed higher human influence at the sample sites (mean  $15.85 \pm 15.01$ ) than all the non-sampled area (mean  $7.56 \pm 8.04$ ; Fig. 3). The difference was highly significant at  $P < 0.001$ . On the other hand, huge areas of Amazonia are extremely poorly sampled; most notably a vast patch comprising eastern Colombia, western Brazil north of the Amazon River and western Venezuela.

## Discussion

### Biogeographic-taxonomic gap

Sampling efforts in terms of both the number of sample sites per species and the number of samples in total varied considerably, with a high proportion of species represented by only one or a few samples or sample sites. These pure data a priori suggest that Amazonian anurans are gravely underrepresented in GenBank. This is also supported by the 164 species names for which no GenBank entries were available (Supplementary data 4). However, sampling efforts have to be regarded in a geographic context. For instance, about one sixth of the species examined here are suggested to occupy geographic ranges of  $< 500 \text{ km}^2$  (Supplementary data 2). That is, a taxon is not necessarily underrepresented when only a small number of samples are available (Fig. 4A). Local and regional spatial range restric-

tions (micro-endemism) are a common phenomenon in certain Amazonian amphibian groups, such as dendrobatoïd frogs (LÖTTERS et al. 2007, BROWN et al. 2011).

Figures 2 and 3 illustrate that there is a clear spatial sampling gap with major portions of Amazonia having remained as yet unsampled, a phenomenon described as ‘missing areas’ by SANMARTÍN & RONQUIST (2002) in the context of area cladograms. ‘Missing areas’ might especially be responsible for the underrepresentation of species with large geographic ranges (Figs 4B, C). However, this has to be regarded with particular care. Often, widespread Amazonian anurans turn out to represent complexes of cryptic allopatric taxa when taxonomically studied using molecular genetics on the basis of broad sampling (e.g., FOUQUET et al. 2007a,b, 2012, 2014, 2016, BROWN et al. 2011, JUNGFER et al. 2013, PELOSO et al. 2014, GEHARA et al. 2014, FERRÃO et al. 2016). Due to the smaller distributions of these allopatrics, they are comparatively better sampled than. Hence, the assessment of how well a taxon is represented in GenBank is hampered in widespread species pending taxonomic clarification. A prime example is the poorly sampled *Atelopus spumarius*. It seems to have a relatively large geographic range across the Amazon Basin (Fig. 4B), but at the same time is suggested to represent a complex of various taxa based on bioacoustics, osteology, larval and adult morphology (LÖTTERS et al. 2002). On the other hand, some species (or widespread lineages within them) have been demonstrated to indeed occupy large geographic ranges, such as *Adenomera andreae*,

*Ameerega trivittata*, *Boana boans*, *B. calcarata*, *Chiasmocleis avilapiresae*, *C. bassleri*, *Lithobates palmipes*, *Osteocephalus taurinus*, or *Pipa pipa* (ROBERTS et al. 2006, FOUQUET et al. 2007a, 2014, ANGULO & ICOCHEA 2010, FUNK et al. 2011, PELOSO et al. 2014). An intriguing observation is that in part these are amongst the best-sampled species (Fig. 4D; Table 2). Moreover, some species might be truly widespread taxa, such as *Ceratophrys cornuta* (LYNCH 1982, DUELLMAN 2005), that are underrepresented in GenBank, however (Fig. 4C).

We conclude that a large knowledge gap exists for many Amazonian anurans species that are underrepresented in GenBank. This is not only due to spatial sampling, but also due to taxonomic deficiencies. It is not our goal to allocate particular species to certain categories of representativeness here (to avoid the definition of artificial limits), however, the following general patterns might apply:

(A) Species with local to regional distributions that are (a) taxonomically well understood and relatively well represented in GenBank (Fig. 4A); (b) taxonomically little understood and poorly represented in, or absent from, GenBank (Fig. 4A); (c) unknown but expected, especially when endemic to ‘missing areas’.

(B) Unconfirmed widespread species that might mask complexes of unidentified cryptic taxa that are poorly represented in GenBank (Fig. 4B).

(C) Species that are confirmed to be truly widespread and (a) are poorly represented in GenBank (Fig. 4C); or (b) adequately represented in GenBank (Fig. 4D).

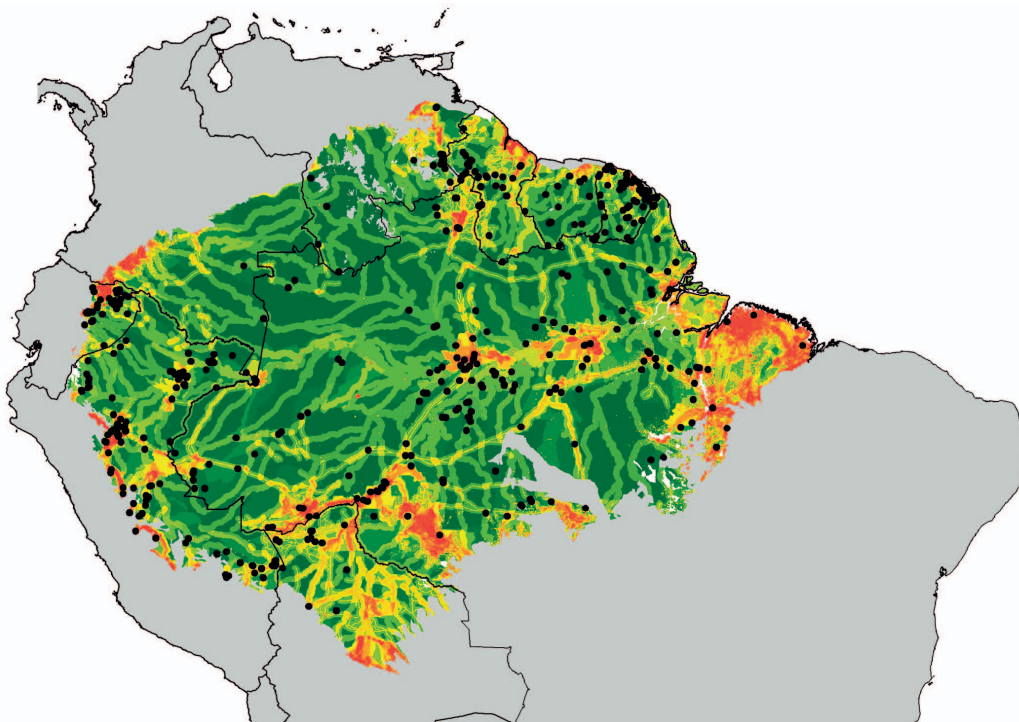


Figure 3. Human Footprint Index (HFI) values across Amazonia (from ‘most wild’ to ‘high anthropogenic impacts’, i.e., dark green to bright red). GenBank samples sites are indicated by black dots.

### Considerations on GenBank data

As may perhaps be expected, sampling effort was highest for 16S. In the past, the mt 16 rRNA gene has been suggested as a universal standard DNA barcoding marker in amphibians (VENCES et al. 2005) and was therefore favoured over COI in many studies. However, in more recent years, technical problems have been solved by the development of degenerate universal COI primers, and COI is on its way to 'overtake' 16S (CHE et al. 2012, PELOSO et al. 2014). This may be already reflected by our results for Amazonian anurans, as COI accounts for high numbers of samples and sample sites in some species, with the most prominent example being the *Dendropsophus minutus* species complex, which recently was the subject of comprehensive molecular studies by GEHARA et al. (2014).

There is no control mechanism for species names allocated to samples deposited in GenBank and names are not updated according to ongoing taxonomic changes. This

problem has repeatedly been pointed out before and is not particular to anurans (HARRIS 2003, SHEN et al. 2013). However, it is markedly relevant here, given the progress in Amazonian anuran taxonomy. It might be assumed that it is highly probable in the cases of Amazonian anuran GenBank samples that are not pooled under a valid name. This produces potential conflicts when adopting names from GenBank as has been done in this study.

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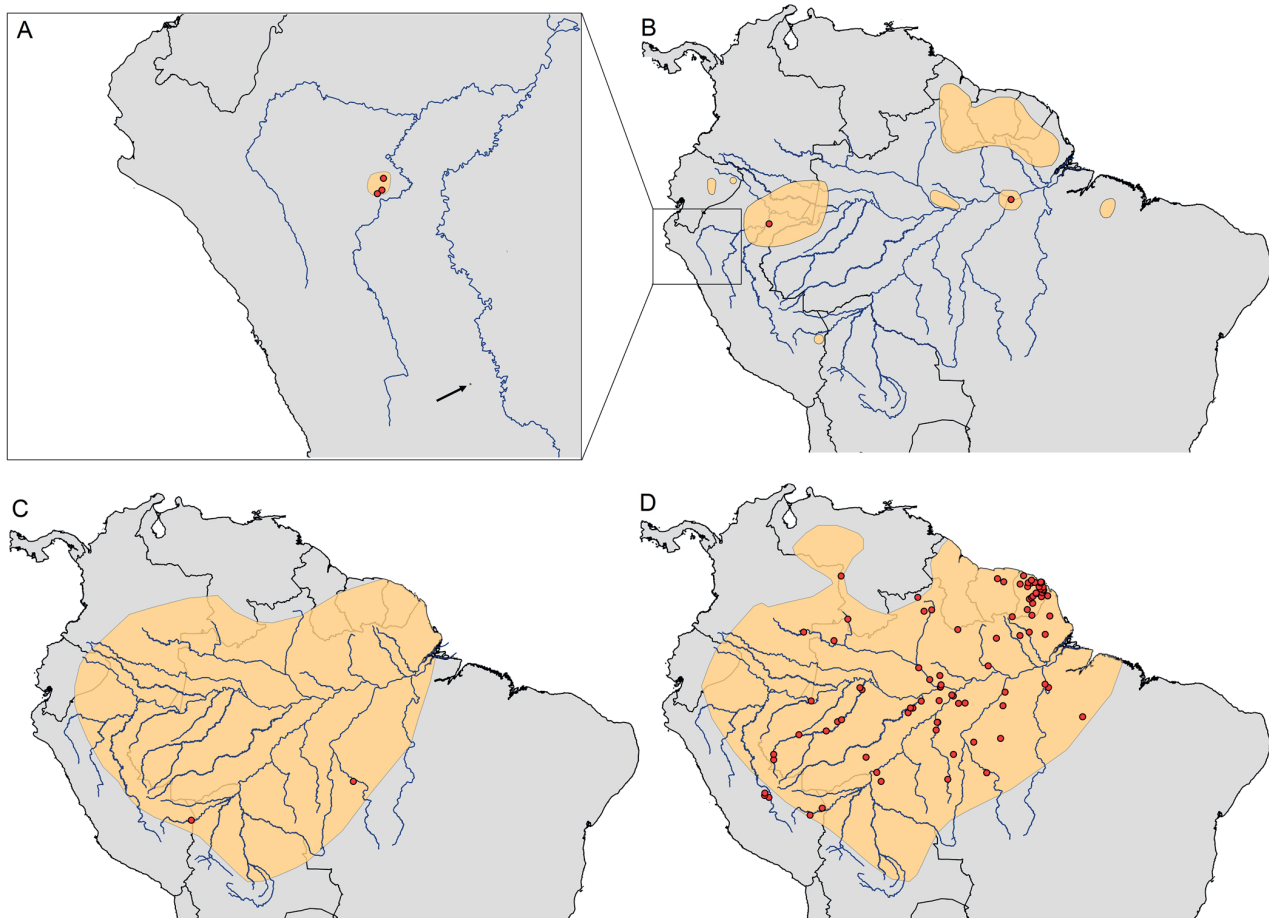


Figure 4. Geographic ranges and GenBank sample sites (red dots) of (A) two Peruvian species (*Hyloxalus azureiventris*, *H. patitae* (arrow)) with locally restricted distributions of which one is comparatively well and the other not represented in GenBank; (B) *Atelopus spumarius*, which occupies a relatively large geographic range, but is poorly sampled and likely represents of complex of distinct taxa; (C) *Ceratophrys cornuta*, an apparently truly widespread, but poorly sampled species; (D) the apparently truly widespread *Adenomera andreae*, which is among the best sampled of all Amazonian anuran species in GenBank. Distribution polygons were adopted from the IUCN Red List of Threatened Species, for details on sample sites see Supplementary data 4–5.

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### Supplementary data

The following Supporting Information for this article is available online:

Supplementary data 1. List of 25 WWF Terrestrial Ecoregions used to define 'Amazonia'.

Supplementary data 2. List of 609 anuran species with distributions overlapping with Amazonia, as available from the IUCN Red List of Threatened Species.

Supplementary data 3. List of recently described Amazonian anuran species in alphabetical order.

Supplementary data 4. Combined list of 512 Amazonian species names used for GenBank searches.

Supplementary data 5. Geo-referenced locality data for 308 Amazonian anuran species names.