Cryptic diversity in Amazonian frogs: Integrative taxonomy of the genus *Anomaloglossus* (Amphibia: Anura: Aromobatidae) reveals a unique case of diversification within the Guiana Shield

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ABSTRACT

Lack of resolution on species boundaries and distribution can hamper inferences in many fields of biology, notably biogeography and conservation biology. This is particularly true in megadiverse and under-surveyed regions such as Amazonia, where species richness remains vastly underestimated. Integrative approaches using a combination of phenotypic and molecular evidence have proved extremely successful in reducing knowledge gaps in species boundaries, especially in animal groups displaying high levels of cryptic diversity like amphibians. Here we combine molecular data (mitochondrial *16S* rRNA and nuclear *TYR*, *POMC*, and *RAG1*) from 522 specimens of *Anomaloglossus*, a frog genus endemic to the Guiana Shield, including 16 of the 26 nominal species, with morphometrics, bioacoustics, tadpole development mode, and habitat use to evaluate species delineation in two lowlands species groups. Molecular data reveal the existence of 18 major mtDNA lineages among which only six correspond to described species. Combined with other lines of evidence, we confirm the existence of at least 12 *Anomaloglossus* species in the Guiana Shield lowlands. *Anomaloglossus* appears to be the only amphibian genus to have largely diversified within the eastern part of the Guiana Shield. Our results also reveal strikingly different phenotypic evolution among lineages. Within the *A. degranvillei* group, one subclade displays acoustic and morphological conservatism, while the second subclade displays less molecular divergence but clear phenotypic divergence. In the *A. stepheni* species group, a complex evolutionary diversification in tadpole development is observed, notably with two closely related lineages each displaying exotrophic and endotrophic tadpoles.

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1. Introduction

Most of the extant terrestrial organisms are found in the tropical mountains and rain forests (Antonelli and Sanmartín, 2011; Dahl et al., 2009; Jenkins et al., 2013; McInnes et al., 2013). However, in vast regions largely covered with forests, like Amazonia, estimates of basic metrics of biodiversity, such as the number of species occurring at the regional scale as well as data on the distribution of these species, still remain very vague in many taxonomic groups (Bickford et al., 2007; Fouquet et al., 2007a; Vieites et al., 2009). Overcoming the lack of knowledge on species identities and distribution is

particularly challenging in groups with apparently high levels of cryptic species diversity (*i.e.*, two or more species classified as a single nominal species) because they are at least superficially morphologically indistinguishable (Bickford et al., 2007). Among vertebrates, amphibians are a group in which the occurrence of morphologically cryptic species appears to be rather common, as suggested by recent studies across the three extant orders (Díaz-Rodríguez et al., 2015; Fouquet et al., 2014, 2007b; Funk et al., 2012; Gehara et al., 2014; Kok et al., 2016a, 2016b; Nishikawa et al., 2012; Stuart et al., 2006; Vieites et al., 2009; Wielstra et al., 2013; Wielstra and Arntzen, 2016). This conservative trend in morphological evolution of amphibians is certainly promoted by the prominence of non-visual signals in their reproduction (calls, pheromones) (Bickford et al., 2007; Cherry et al., 1982; Emerson, 1988), and therefore some groups harbour few discernible morphological taxonomic descriptors. Integrative approaches, especially those combining morphological, bioacoustic and molecular data, have proved to be particularly useful to clarify the taxonomic status of lineages containing large cryptic species diversity (Padial and de la Riva, 2009; Simões et al., 2013; Vieites et al., 2009).

The genus *Anomaloglossus* is one of these challenging amphibian groups in terms of species delineation, due to large intraspecific morphological variability and the lack of morphological characters allowing easy diagnosis among species (Grant et al., 2006; Kok, 2010). Recent molecular analyses have revealed several deeply divergent lineages within currently recognized species (Fouquet et al., 2012b, 2007a; Kok et al., 2012), raising the possibility that these species may harbor multiple morphologically cryptic species. *Anomaloglossus* currently comprises 26 described species, and forms a clade of terrestrial frogs endemic to the Guiana Shield (GS) (Fouquet et al., 2015; Frost, 2016; Santos et al., 2009). Five additional species reported from the Chocó region in Ecuador, Colombia and Panama are still provisionally allocated in the genus even though they in fact do not form a clade with the GS species (Grant unpubl. data). While *Anomaloglossus* is endemic to the GS, most species (20) are found in the highlands of the Pantepui region, in the western GS, whereas the remaining six species are distributed outside the Pantepui region, mostly throughout the upland and lowland forests of the Eastern GS (EGS) (Barrio-Amorós et al., 2010; Kok and Kalamandeen, 2008; Lescure and Marty, 2000; Ouboter and Jairam, 2012). *Anomaloglossus* seems to be the only genus to have significantly diversified throughout the GS; all other groups restricted to the GS seem to have diversified more locally, either in Pantepui (e.g., *Oreophrynella, Stefania, Myersiohyla*) or only within the Amazonian lowlands. As a corollary, *Anomaloglossus* species in Pantepui generally have very small ranges, often restricted to one or few mountainous massifs (e.g., Barrio-Amorós et al., 2010; Barrio-Amoros and Santos, 2011; Kok et al., 2010, 2013; Señaris et al., 2014). This microendemic distribution pattern is also displayed by two species outside the Pantepui region, *A. apiau* (Serra do Apiaú in Roraima state, Brazil) and *A. leopardus* (Apalagadi Mountains in southern Suriname). In contrast, the other four species of the EGS are considered to have broader ranges. *Anomaloglossus degranvillei* occurs in most of French Guiana (Lescure and Marty, 2000), *A. baeobatrachus* in Suriname, French Guiana, and the states of Pará and Amapá in Brazil (Avilá-Pires et al., 2010; Fouquet et al., 2012b; Lescure and Marty, 2000; Ouboter and Jairam, 2012), *A. surinamensis* in Suriname and French Guiana (Fouquet et al., 2012b; Ouboter and Jairam, 2012), and *A. stepheni* from the Amazonas state in Brazil to Suriname (Avila-Pires et al., 2010; Fouquet et al., 2012b; Hoogmoed, 2013). However, our current understanding of the distribution of these large-range EGS species might be erroneous because several deeply divergent lineages have been uncovered among populations of *A. surinamensis, A. degranvillei*, and *A. baeobatrachus* (Fouquet et al., 2007a, 2012b).

Moreover, *Anomaloglossus* species display striking variation in reproductive modes: endotrophic and nidicolous in *A. stepheni* (Junca et al., 1994), endotrophic and phoretic in *A. degranvillei* (Lescure, 1975), exotrophic with maternal care in *A. beebei* (phytotelm-breeder), *A. kaiei*, and *A. roraima* (phytotelm-breeder) (Kok et al., 2006a, 2006b, 2013), and exotrophic and phoretic in other species (Grant et al., 2006).

To clarify the patterns of diversity, distribution, and reproductive traits in lowland *Anomaloglossus*, we tested species boundaries by combining molecular, morphometric, bioacoustic, and natural history data.

2. Material and methods

2.1. Collecting data in the field

We collected specimens during various trips to French Guiana and Suriname, as well as to the Roraima, and Amapá states in Brazil (see Supplementary Table S1 for details). Specimens were searched actively during the day, and caught by hand. They were euthanized by injection of a solution of lidocaine immediately after being photographed, fixed in 10% formalin for 24 h and then transferred in 70% ethanol for permanent storage. When possible, we recorded calling males before collection (see below *Bioacoustic data*), gathered data on habitat (*terra firme* forest vs. stream banks), and collected tadpoles to determine if they were endotrophic or exotrophic by examining the buccal morphology.

2.2. Molecular data

We extracted DNA from liver tissue of 258 samples using the Wizard Genomic extraction protocol (Promega; Madison, WI, USA), and we amplified a fragment of the *16S* rDNA of the mitochondrial DNA. PCR were conducted in a final volume of 25 μl each containing 2 μl of DNA template, 14.36 μl water, 5 μl of 10 × PCR Buffer, 1.25 μl of each primer, 1.67 μl of MgCL2, 0.5 μl of dNTPs, and 0.22 μl of GoTaq (Promega, Madison, Wisconsin, USA). The PCR conditions were as follows: 8 cycles of denaturation (45 s at 94 °C), annealing (60 s at 46 °C), and elongation (90 s at 72°), followed by 22 cycles of denaturation (45 s at 94 °C), annealing (60 s at 50 °C), and elongation (90 s at 72 °C). For *16S* rDNA, we used N16F and N16R primers (Salducci et al., 2005). Sanger sequencing of of *16S* rDNA of 183 samples was performed by Genoscreen (Lille, France). The 75 remaining *16S* rDNA sequences were obtained through MiSeq sequencing (Illumina, USA). We collated these sequences with all *16S* sequences of *Anomaloglossus* available from GenBank (n = 244). The final *16S* dataset contained sequences of 502 specimens of *Anomaloglossus* (see Supplementary Table S1 for GenBank accession numbers). We included all known species occurring in the lowlands of the Guiana Shield across their ranges, as well as 15 species from Pantepui. As we focused on the lowland species, having an incomplete dataset of Pantepui species did not hamper our analysis.

Additionally, we amplified and sequenced three protein-coding nuclear loci (*tyrosinase – TYR; proopiomelanocortin C – POMC*; and *recombination activating gene exon 1 – RAG1*). PCR conditions were as for the *16S* fragment. For *TYR*, we used tyrE dendro5 and tyrE dendro primers (Fouquet et al., 2012b), for *POMC* we used POMC-1 and POMC-2 primers (Wiens et al., 2005), and for *RAG1* we used MARTFL1 (Hoegg et al., 2004) and RAG1-AD2R (Fouquet et al., 2014) for the first fragment and RAG1-810F and RAG1-1240R (Fouquet et al., 2014) for the second fragment. We completed the dataset by adding 15 *TYR* sequences that were already available in GenBank. Novel sequences were deposited in GenBank and are listed in Supplementary Table S1.

2.3. Phylogenetic analyses

A first set of analyses was performed on 503 sequences of the *16S* rDNA dataset alone, which were aligned with MAFFT v.7 (Katoh and Standley, 2013) using default parameters (gap opening penalty = 1.53; gap extension penalty = 0.123; progressive method = FFT-NS-2). The resulting alignment was 418 bp long after exclusion of non-overlapping regions. An XML File was generated with BEAUti v.1.8.0 with the following settings: GTR + G + I substitution model, inferred as the best fitting model with PartitionFinder v.1.1.1 (Lanfear et al., 2012) with a Bayesian information criterion (BIC), empirical base frequencies, four gamma categories, birth-death process model, all codon positions partitioned with unlinked base frequencies and substitution rates. We then performed a Bayesian analysis using BEAST v.1.8.1 (Drummond et al., 2012), with an uncorrelated relaxed lognormal clock model under default parameters. The length of MCMC chain was 50,000,000 sampling every 5000. Trace files were evaluated with Tracer v1.6.0 (Rambaut et al., 2014). Maximum clade credibility trees with a 0.5 posterior probability limit, and node heights of target tree were constructed in TreeAnnotator v1.8.1 (Rambaut and Drummond, 2012).

A second set of molecular analyses was performed using the three nuclear protein-coding loci. These data were used to examine the congruence between mtDNA and nuDNA given that reciprocal monophyly of the same sets of individuals can be seen as evidence of reproductive isolation, particularly when there is an overlap among their ranges. We used 48 samples, and missing data were limited to only one locus per terminal (three terminals for POMC, five terminals for RAG1, and five terminals for TYR). MEGA v.7.0.16 (Kumar et al., 2016) was used to align sequences of each locus and to review amino acid translations to ensure correct alignment with respect to reading frame. We then used the program FASconCAT v.1.0 (Kück and Meusemann, 2010) to concatenate the three fragments because each locus was individually recovered as poorly informative in preliminary analyses. The resulting alignment comprised three partitions of a total length of 2524 bp (POMC 1–605, RAG1 606–2002, TYR 2003–2524). We inferred the best-fitting model of molecular evolution with PartitionFinder v.1.1.1 (Lanfear et al., 2012) with BIC, and conducted a ML analysis with RAxML v.8.2.4 using the GTR + Γ model. Support of nodes was investigated with 1000 bootstrap replicates using the fast bootstrapping algorithm. For both analyses, *Ameerega hahneli*, *Mannophryne collaris*, *Rheobates palmatus*, *Allobates femoralis*, *Allobates olfersioides*, and *Aromobates saltuensis* were used as outgroups (Santos et al., 2009). Mean pairwise *p*-distances were calculated among the main lineages with MEGA v.7.0.16 using pairwise deletion.

Computations were performed on EDB-Calc Cluster, which uses a software developed by the Rocks(r) Cluster Group (San Diego Supercomputer Center, University of California, San Diego and its contributors), hosted by the laboratory "Evolution et Diversité Biologique" (EDB).

2.4. Species delineation

Because our dataset was unbalanced in terms of number of specimens per species, we applied three different methods of DNA-based species delineation on the *16S* rDNA dataset: Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012), and two phylogeny-aware methods, General Mixed Yule Coalescent (GMYC) (Monaghan et al., 2009; Pons et al., 2006), and Poisson-Tree Process (PTP) (Zhang et al., 2013).

We ran the GMYC analyses with the *ape* (Paradis et al., 2004) and *splits* (Ezard et al., 2009) packages implemented in R v.3.2.4 (R Development Core Team, 2016).

PTP is similar to GMYC, but it does not require an ultrametric tree and is supposed to outperform GMYC when evolutionary distances between species are small (Zhang et al., 2013), a bias expected in our dataset (Fouquet et al., 2012b). As a maximum likelihood (ML) tree is required for this analysis, we subjected the *16S* rDNA alignment to phylogenetic inference using ML as implemented in RAxML v.8.2.4 (Stamatakis, 2014). We inferred the best-fitting model of molecular evolution with PartitionFinder v.1.1.1 (Lanfear et al., 2012) with a BIC. Support of nodes was investigated with 1000 bootstrap replicates using the fast bootstrapping algorithm, as it produces almost identical values as the standard bootstrap method but is faster (Stamatakis et al., 2008). *Mannophryne collaris*, *Rheobates pseudopalmatus*, and *Aromobates saltuensis* were used as outgroups (Santos et al., 2009). We then used the best ML tree (excluding the outgroups) obtained with RAxML as an input for a PTP analysis that we ran on the online PTP server (http://species.h-its.org/ptp/). We ran the PTP analysis using 100,000 MCMC generations, with a thinning value of 100, and a burn-in of 0.1.

We performed ABGD analyses from the source code with two different distance metrics (JC69 and simple *p*-distance) using default values for all parameters (Pmin: 0.001, Pmax: 0.1, steps: 10, Nb bins: 20).

We estimated the resolving power (quantitative approach) and reliability (qualitative approach) of the inferred species boundaries by these three methods with two indices, the *Relative Taxonomic Resolving Power Index* (R*tax*), and the *Taxonomic index of congruence* (C*tax*) (Miralles and Vences, 2013).

2.5. Morphometric data

We measured 89 male specimens assigned to the four nominal species of the *A. stepheni* species group (*A. apiau*, *A. baeobatrachus*, *A. leopardus*, *A. stepheni*) and 56 male specimens assigned to two species of the *A. degranvillei* species group (*A. degranvillei* and *A. surinamensis*). Two populations (Acari and Parú) were not included because specimens were not available. We measured 17 variables: snout-vent length (SVL); head length from corner of mouth to tip of snout (HL); head width at level of angle of jaws (HW); snout length from anterior edge of eye to tip of snout (SL); eye to naris distance from anterior edge of eye to centre of naris (EN); internarial distance (IN); horizontal eye diameter (ED); interorbital distance (IO); diameter of tympanum (TYM); forearm length from proximal edge of palmar tubercle to outer edge of flexed elbow (FAL); hand length from proximal edge of palmar tubercle to tip of finger (HAND); width of disc on Finger III (WFD); tibia length from outer edge of flexed knee to heel (TL); foot length from proximal edge of inner metatarsal tubercle to tip of toe IV (FL); width of disc on Toe IV (WTD); thigh length from vent opening to flexed knee (ThL); length of Finger I from inner edge of thenar tubercle to tip of disc (1FiL) following Fouquet et al. (2015), except TYM in species of the *A. degranvillei* species group because the tympanum is inconspicuous in these taxa. Specimens examined are listed in Supplementary Table S2. All measurements were taken on preserved specimens using a digital caliper to the 0.1 mm.

2.6. Bioacoustic data

We recorded specimens during various field trips in the EGS. Material used for call recording includes Olympus LS11 and Zoom H4N digital recorders, attached to a Sennheiser ME-66 supercardioid microphone powered with a K6P module. We analysed call recordings of 55 males assigned to three nominal species of the *A. stepheni* species group (*A. baeobatrachus, A. leopardus, A. stepheni*) and of 31 males assigned to two nominal species of the *A. degranvillei* group (*A. degranvillei* and *A. surinamensis*). *Anomaloglossus*

apiau was excluded from this analysis because this species displays a temporal call structure (long trills of paired notes) significantly different from the other species of the *A. stepheni* group (short trills of single notes). No call recording was available for populations from Acari and Parú. For species of the *A. stepheni* group, which emit a train of pulsed notes, we measured six call variables using Audacity v.2.1.1. Variables follow those standardized in Kok and Kalamandeen (2008): call rate (number of calls divided by their window duration), call length, note length, internote interval, note repetition rate (note rate: call duration divided by the number of notes in the call), and the dominant frequency. For the *A. degranvillei* group, which emits single-note calls, we considered three variables (note length, internote length, dominant frequency). For each variable per individual, we used the mean value calculated across four different calls. Recorded specimens are listed in Supplementary Table S3.

2.7. Data visualization and statistical analyses

We examined independently morphometric and bioacoustic data for the two species groups through principal component analysis (PCA), in order to visualize relationships among data (James and McCulloch, 1990). To control for variation in body-size among individuals, we additionally performed subsequent analyses on a size-corrected dataset obtained by linear-regressing the original morphometric measures of each variable with SVL (Strauss, 1985). For bioacoustic characters, we repeated the analyses considering solely the groups of individuals that were overlapping in the preliminary analyses.

In order to test if the variable "species" would explain the variance of the data, we performed a permutational non-parametric multivariate analysis of variance (Anderson, 2001) on each set of data (morphometrics and bioacoustics for both species groups). All analyses were conducted with the software R v.3.2.4 (R Development Core Team, 2016) with the packages *ade4* (Dray and Dufour, 2007) and *vegan* (Oksanen et al., 2016).

2.8. Integrative solution

In order to reach a diagnostic species delineation [*i.e.*, classify each candidate species (CS) as a confirmed candidate species (CCS), unconfirmed candidate species (UCS), or deep conspecific lineage (DCL)], we followed the framework presented by Padial et al. (2010). We considered as "confirmed" any CS for which there was at least one congruent difference in any other character than the primary molecular divergence criterion between close relatives, and as "unconfirmed" any CS for which additional data were lacking. When populations showed molecular genetic divergence but could not be distinguished by the measured morphometric or bioacoustic parameters, we considered by default these CS as "deep conspecific lineages".

3. Results

3.1. Phylogenetic analysis

Bayesian analysis of the *16S* rDNA resulted in a poorly resolved tree for deep divergences, but unravelled previously undocumented diversity within described species (Fig. 1A). Low resolution at the base of the tree probably explains the well-separated position of *Anomaloglossus stepheni* from the other lowland species. Letting *A. stepheni* apart, these lowland species form two clades (*A. apiau*, *A. leopardus*, and *A. baeobatrachus* vs. *A. surinamensis* and *A. degranvillei*).

Five allopatric and well-differentiated lineages currently assigned to *A. baeobatrachus* are recovered. These lineages are represented by the populations from Serra do Acari in Pará, Brazil (minimum *16S* p-distance = 6.8%), Parú in Pará, Brazil (3.9%), Mitaraka in French Guiana (3.4%), Bakhuis Mountains in Suriname (6%), and Brownsberg in Suriname (3.9%) (Fig. 1B, Supplementary Table S4). *Anomaloglossus baeobatrachus* is also recovered paraphyletic with respect to *A. leopardus*.

The *A. degranvillei* group is strongly supported (pp > 0.99), as are two subclades within it represented by *A. degranvillei* and *A. surinamensis* populations (respectively pp = 0.84 and pp ≥ 0.99). The divergences within *A. surinamensis* are deep, in particular for *A. surinamensis* 5 in Fig. 1A from Bakhuis Mountains (Suriname), which forms a well-differentiated lineage (minimum *16S* p-distance = 6.6%, see Supplementary Table S4) recovered as the sister group of all other representatives of this clade with good support. The remaining populations assigned to *A. surinamensis* form at least four well-differentiated lineages (2.7–6.4%, see Supplementary Table S4), among which *A. surinamensis* 2 includes topotypical material. These lineages are distributed allopatrically throughout Suriname and French Guiana (Fig. 1C). The divergences within *A. degranvillei* are lower, but three lineages are discriminated (1.9–2.6%, see Supplementary Table S4). These lineages are found only in French Guiana and on very localised massifs, except one lineage slightly more broadly distributed in north-eastern French Guiana (*A.* sp. "north FG" in Fig. 1C).

Even though less complete than the mtDNA dataset, the nuDNA data provide informative results about deeper relationships and species boundaries in the two species groups. Species from the Pantepui region and species from the EGS form two weakly supported clades, but the monophyly of both the *A. degranvillei* and *A. stepheni* groups is strongly supported (respectively 100% and 99% bootstrap support) (Fig. 2).

Within the *A. stepheni* group, most of the candidate species are also recovered as forming independent lineages. *Anomaloglossus stepheni* and *A. apiau* form a clade well differentiated from the rest of the species group. This remaining group forms a strongly supported clade. Within this clade, *A.* sp. "Bakhuis", *A.* sp. "Brownsberg", *A. leopardus*, and *A.* sp. "Mitaraka" represent clearly separated lineages. Populations assigned to *A. baeobatrachus* form the remaining clade.

Within the *A. degranvillei* group, the two subclades formed by populations assigned to *A. surinamensis* and *A. degranvillei* are also strongly supported (both with 100% bootstrap support). *Anomaloglossus degranvillei* and *A.* sp. "north FG" are also distinguished on nuDNA. Within *A. surinamensis*, the most divergent population, *A. surinamensis* 5 from the Bakhuis Mountains, is well differentiated from its relatives. However, *Anomaloglossus surinamensis* 1 is recovered paraphyletic with respect to *A. surinamensis* 2.

3.2. Species delineation

Using ABGD, a constant number of CS (31) is observed using initial partitions with a range of prior intraspecific divergence value (P = 0.0046–0.001 using *p-distance*, P = 0.0046–0.0028 using JC69) (Table 1). Recursive partitions were discarded as they provided unrealistic species delineation, notably with many singletons. For the GMYC analysis, both single and multiple threshold models outperformed the null model (Table 2), indicating the presence of several CS in our dataset. The result of the single threshold model (30 entities) was adopted, as the fit was not improved by the multiple threshold model (chi2 = 2.28, df = 6, *p* = 0.89). The tree resulting from PTP with best-fit ML recovered 29 clusters and four singletons, yielding in a total of 33 entities. The resolving power of PTP is the highest (Rtax = 0.88), which means that this method retrieved most of the species limits revealed by the two other
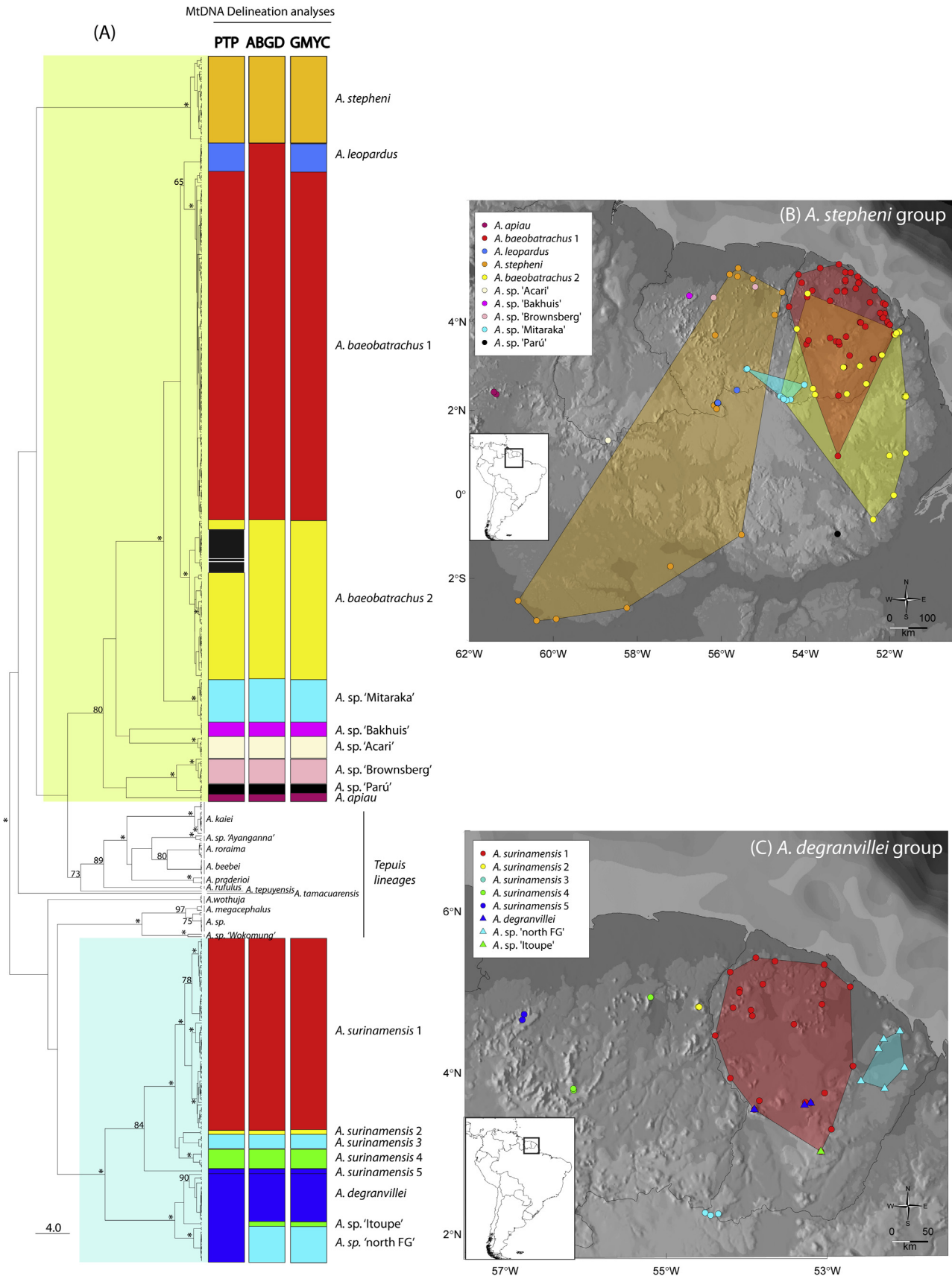
Fig. 1. (A) Maximum credibility clade tree obtained with BEAST using 400 bp of the *16S* rDNA. Posterior probabilities are indicated above nodes (* = 0.99 or 1; not indicated when <0.65), and results of *16S* rDNA analyses in ABGD, PTP, and GMYC (three columns from left to right). Maps of the eastern Guiana Shield showing the distribution records of the main lineages recovered from the phylogenetic analysis in the *A. stepheni* group (B) and in the *A. degranvillei* group (C).
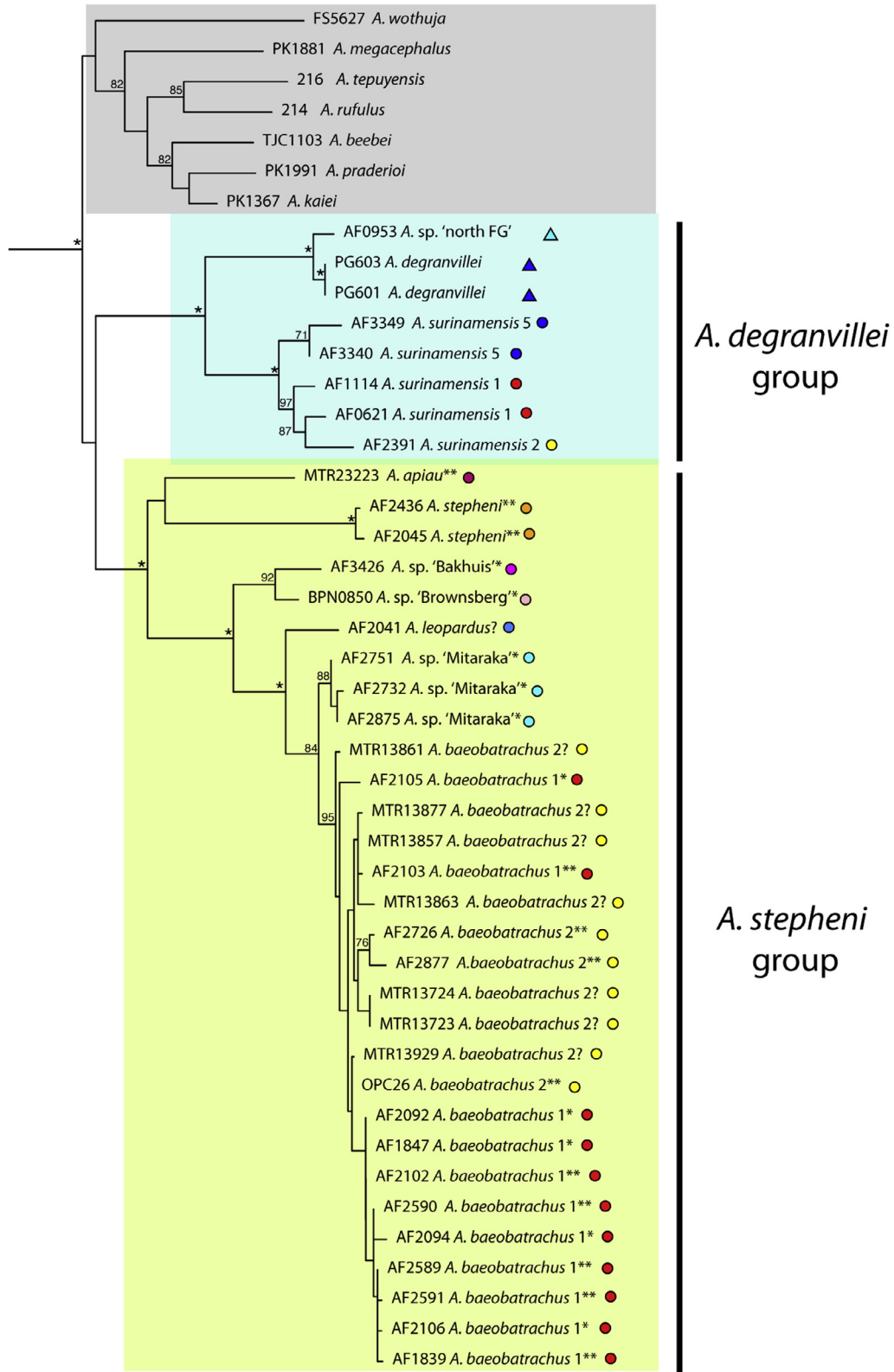
Fig. 2. Maximum likelihood tree obtained from concatenated nuDNA loci *POMC*, *RAG1*, and *TYR*. Colour codes denoted after labels correspond to the codes used in Fig. 1. Bootstrap values are indicated above nodes (* = ≥99%; not indicated when <70%). For the *A. stepheni* group, we indicated *exotrophic tadpole; **endotrophic tadpole; ? tadpole development mode not observed for the corresponding population. The coloured circles and triangle symbols correspond to the ones shown in the maps in Fig. 1B and C. Outgroups are not shown.

approaches, except for two CS. ABGD and GMYC have respectively a Rtax value of 0.82 an 0.79. The GMYC analysis was the most consensual (mean Ctax = 0.82), followed by AGBD (mean Ctax = 0.79),

and PTP (mean Ctax = 0.72), and will therefore be chosen for the comparison with the other lines of evidence. The results of these delineation approaches are summarized in Fig. 1.

Table 1
Number of delimited species resulting from the automatic barcode gap discovery analysis (ABGD) on the *16S* rDNA 400 bp fragment with different substitution models and initial or recursive partition. X = relative gap width.

Subst. model	X	Partition	Prior intraspecific divergence (P)							
			0.0359	0.0215	0.0129	0.0077	0.0046	0.0028	0.0017	0.001
Simple	1.5	Initial	0	13	13	16	31	31	31	31
		Recursive		14	18	20				
JC69	1.5	Initial	0	13	13	27	31	31	120	120
		Recursive		19	16	29	36	36		

Table 2
Results of the General Mixed Yule-coalescent (GMYC) analyses for the Bayesian tree under the birth-death process model applied on the *16S* rDNA 400 bp fragment. Clusters, OTUs delineated by GMYC with more than one specimen; Entities, clusters and singleton OTUs delineated by GMYC; CI, confidence interval; Likelihood$_{null}$, likelihood of the null model; Likelihood$_{GMYC}$, likelihood of the GMYC model; Threshold, the threshold between speciation and coalescence processes. Single, single-threshold model; Multiple, multiple-threshold model; ***$P < 0.001$.

Analysis	Clusters (CI)	Entities (CI)	Likelihood$_{null}$	Likelihood$_{GMYC}$	Likelihood ratio	Threshold
Single	25 (17–33)	30 (22–39)	2965.783	2990.261	48.95***	−2.37
Multiple	35 (18–36)	40 (22–43)	2965.783	2991.401	51.23***	−1.68; −0.79; −0.15

Anomaloglossus apiau, *A. stepheni*, and *A. leopardus* were identified as single CS in all analyses. However, populations currently assigned to *A. baeobatrachus* are identified as seven different CS (Fig. 1). Within these CS, two lineages of *A. baeobatrachus* remained indistinguishable using our nuDNA dataset (Fig. 2).

Eight CS were identified in the *A. degranvillei* group. Three of these CS are nested within a clade formed by populations currently assigned to *A. degranvillei*, all occurring in French Guiana (Fig. 1). The remaining five CS are found in a clade formed by populations currently assigned to *A. surinamensis*, and are distributed in Suriname and French Guiana (Fig. 1).

3.3. Morphological analyses

For both species groups, the raw morphometric data have limited discriminative power because most individual candidate species overlap with at least another one in the multidimensional space (Fig. 3). However, in many instances they revealed some differences in body size among pairs of closely related species. Analyses performed on the size-corrected dataset confirmed the overall lack of differences among groups in their body proportions, confirming that closely related species differ mainly in their body size.

PCA on data from the *A. stepheni* group showed that two components with eigenvalues >1.0 accounted for 81.97% of the total variation. Coefficients of the first component, which explains 67.53% of the variation (Fig. 3A), are highly and positively correlated (Fig. 3A, Supplementary Table S6). The second component explains 14.44% of the variation (Fig. 3A). Except *A. apiau*, which is well differentiated along the second axis, individuals are spread along the first axis segregating large-bodied (mean SVL > 17.4 mm) species (*A. leopardus*, *A.* sp. "Bakhuis", *A.* sp. "Brownsberg", and *A.* sp. "Mitaraka") from small-bodied (mean SVL < 17.4 mm) species (*A. stepheni*, *A. apiau*, and *A. baeobatrachus*). This was supported by the multivariate analysis of variance (MANOVA) that indicated that species identity explains 80% of the variance for morphometric variables (Adonis MANOVA R^2 = 0.8, p = 0.001), but only 55% when the data were corrected by body size (SVL) (Adonis MANOVA R^2 = 0.55, p = 0.001). When the data were corrected according to body size, all the groups largely overlap except *A. stepheni*, indicating that body proportions in that species differ from those observed in other species (see Suppl. Mat.). Interestingly, *A. baeobatrachus* is forming two different non-overlapping clusters of individuals differing in their body size. In fact, the distinction between large-bodied and small-bodied species seems to coincide with other traits, notably habitat and larval development (see below).

For the *A. degranvillei* group, two components accounted for 94.37% of the total variation. Coefficients of the first component, which explains 92.87% of variation (Fig. 3B), are highly and positively correlated (Supplementary Table S6). The second component explains 1.5% of variation (Fig. 3B), and has significant positive loading for IO and a significant negative one for WFD (Supplementary Table S6). The two subclades (*A. degranvillei* and *A. surinamensis* subclades) are well distinguished according to their body size. In the *A. degranvillei* subclade, *A.* sp. "north FG" is well segregated from the two closely related CS. However, the morphometric spaces of all CS within the *A. surinamensis* subclade broadly overlap. Additional analyses based on raw and size-corrected data focusing on the *A. surinamensis* subclade did not discriminate more groups. The multivariate analysis of variance (MANOVA) indicated that species identity explains 91% of the variance on morphometrics variables (Adonis MANOVA R^2 = 0.91, p = 0.001), but only 29% when the data were corrected by body size (SVL) (Adonis MANOVA R^2 = 0.29, p = 0.001).

3.4. Bioacoustics

Bioacoustic data showed a much greater discriminating power within the *A. stepheni* group than morphometric data, with no or limited overlap among CS across the multidimensional space. Two components with eigenvalues > 1.0 accounted for 82.62% of the total variation. Coefficients of the first component, which explains 51.66% of variation, have significant positive loadings for Internote length, Note length, and Call length, and significant negative ones for Call rate, Dominant frequency and Note rate (Fig. 4A, Supplementary Table S7). The second component explains 30.96% of the variation, and has significant positive loadings for call rate and note length, and significant negative ones for Dominant frequency and Call length (Fig. 4A, Supplementary Table S7). *Anomaloglossus baeobatrachus* contains individuals either with a slow trill call (note rate < 15 notes/s) or a rapid trill call (note rate > 15 notes/s). Two distinct clusters are recovered within *A. baeobatrachus*, corresponding to the results found using morphometric data. These two groups of individuals differ markedly in their calls. Together with morphometrics, these differences seem to reflect two distinct phenotypes within *A. baeobatrachus* that occur often in sympatry (see below). These results are in accordance with the MANOVA analysis, which indicated that species identity explains 93% of the variance on bioacoustics variables (Adonis MANOVA R2 = 0.93, p = 0.001).
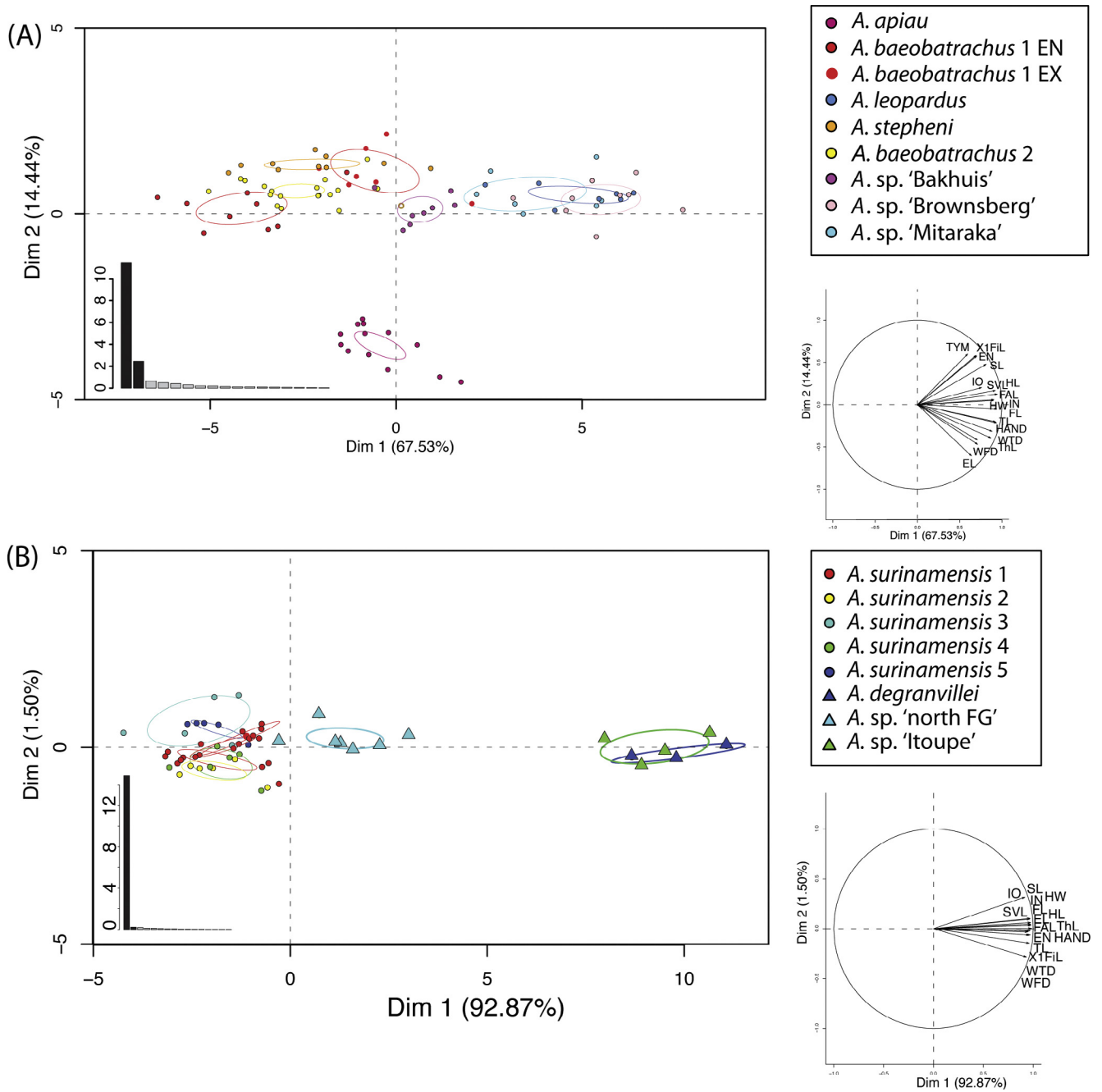
Fig. 3. Results of the PCA on raw morphometric variables with circle of correlations for (A) *A. stepheni* group and (B) *A. degranvillei* group. Symbols represent specimens on the first two principal components. The contribution of each axis for total variation is indicated in parentheses. The groups are delimited with coloured lines. Circles of correlations show the loadings of individual morphometric characters on the first two principal components (see Section 2.5 for character descriptions). EN = endotrophic; EX = exotrophic.

For the *A. degranvillei* group, two components accounted for 89.87% of the total variation. Coefficients of the variables have significant positive loadings for Note length and Internote length, and significant negative ones for Dominant frequency on the first component (Supplementary Table S7), which explains 62.69% of the variation (Fig. 4B). The second component explains 27.18% of the variation, and has significant positive loading for Internote length (Fig. 4B, Supplementary Table S7). The two subclades (*A. degranvillei* subclade and *A. surinamensis* subclade) are well segregated. In the *A. degranvillei* subclade, *A.* sp. "north FG", *A.* sp. "Itoupé", and *A. degranvillei* are all well separated from one another. These results are in accordance with the MANOVA analysis, which indi-

cated that species identity explains 85% of the variance on bioacoustics variables (Adonis MANOVA $R^2 = 0.85$, $p = 0.001$). However, as for the morphometric analysis, all CS within the *A. surinamensis* subclade completely overlap. Additional analysis focusing on these individuals failed to discriminate any additional CS.

3.5. Reproductive modes and habitat

In the *A. stepheni* group (excluding *A. apiau*), two reproductive modes have been observed, which seem to covary with habitat, call characteristics and body size (Table 3). The species in this group
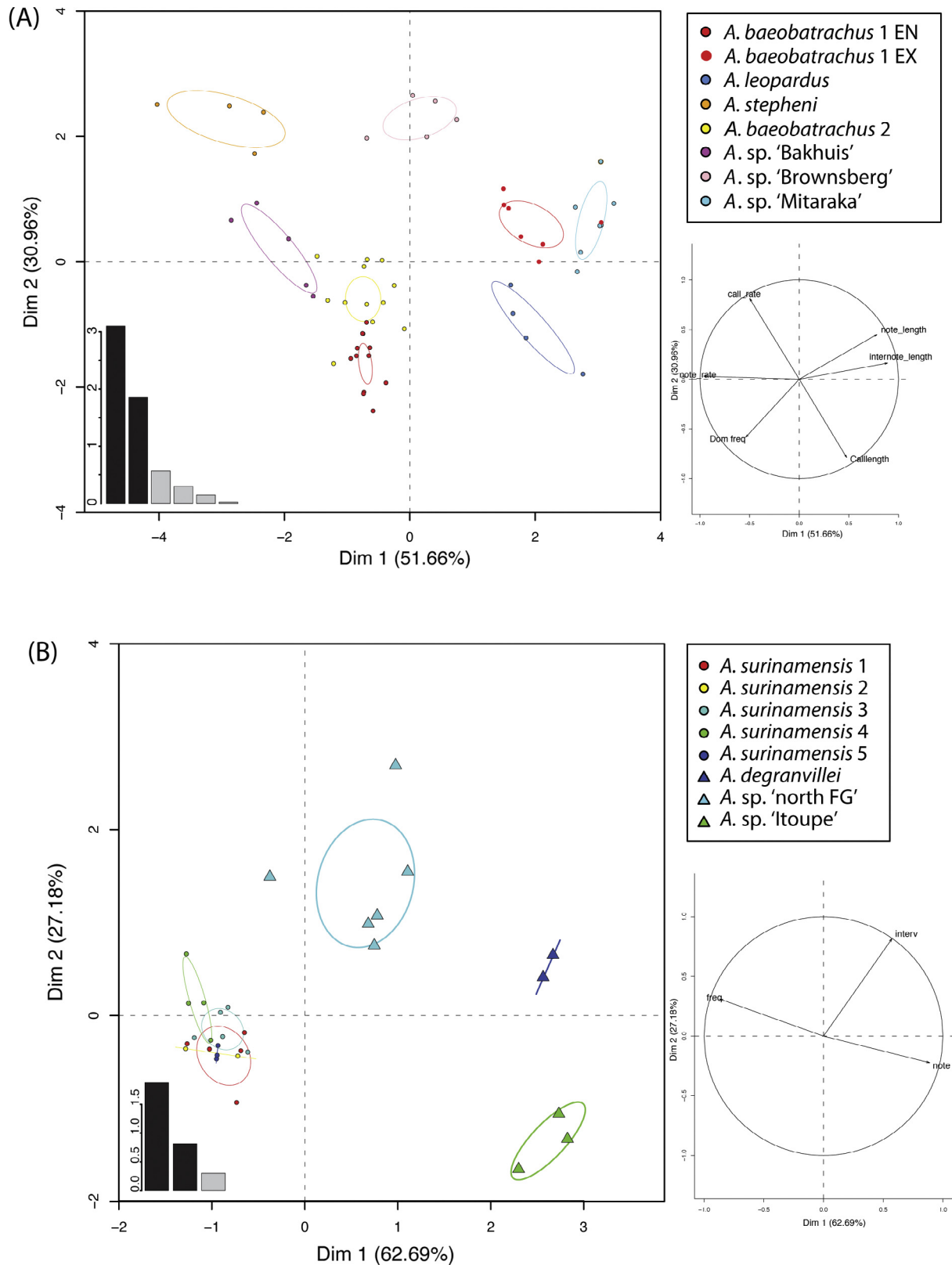
Fig. 4. Results of the PCA on raw bioacoustic variables with circle of correlations for (A) *A. stepheni* group and (B) *A. degranvillei* group. Symbols represent specimens on the first two principal components. The contribution of each axis for total variation is indicated in parentheses. The groups are delimited with coloured lines. Circles of correlations show the loadings of individual bioacoustic characters on the first two principal components (see Material and methods subsection *Bioacoustic data* for character descriptions). EN = endotrophic; EX = exotrophic.

have either nidicolous and endotrophic tadpoles (phenotype 2, Table 3) or exotrophic tadpoles with male phoresy (Phenotype 3, Table 3) (Fig. 5B). Endotrophic tadpoles are found in reduced number in the nest (≤4), have a reduced and bare mouth, large vitelline reserves, and complete their development in the nest. On the contrary, exotrophic tadpoles are in larger number (>4), have labial

teeth, less vitelline reserves, and are transported by the male to water bodies where they complete their development.

Anomaloglossus stepheni has an endotrophic and nidicolous tadpole occupying *terra firme* habitats (Juncá et al., 1994). In contrast, *Anomaloglossus* sp. "Acari", *A.* sp. "Bakhuis", *A.* sp. "Brownsberg", *A.* sp. "Mitaraka" all have exotrophic tadpoles, which males carry to water bodies. However, both phenotypes are observed in *A. baeobatrachus* (Fig. 5B). Indeed, northern populations of this species harbouring the *A. baeobatrachus* 1 mtDNA lineage are found in *terra firme* habitats and have endotrophic and nidicolous tadpoles, but some populations harbouring the same mtDNA lineage in the eastern and southern part of French Guiana are associated with streams, have an exotrophic tadpole and phoretic male. These populations are slightly larger in SVL and have a slower note rate. Although we could not gather totally unambiguous data, phoresy has been observed in Serra do Navio, Amapá state, Brazil, and slow-calling individuals have been observed along the Oyapock River (Brazilian margin in Mémora), Amapá state, Brazil (Grant et al., 2006). Given only the mtDNA lineage *A. baeobatrachus* 2 is occurring in this area, and that the sampled individuals along the Brazilian margin of the Oyapock were most likely of the endotrophic phenotype, we assume that both phenotypes are also found harbouring the *A. baeobatrachus* 2 mtDNA lineage.

Males carrying endotrophic tadpoles (reduced and bare mouth and large vitelline reserves) have been observed in seven populations of the *A. degranvillei* group assigned to different CS, thus documenting the mode of larval development and male behavior for most CS in that group (Table 3; Fig. 5A and C). All members of this clade live along streams, and we assume that they display phoresy until metamorphosis, or at least during a prolonged period of the larval development. Interestingly, *A. apiau*, despite being a member of the *A. stepheni* clade, is also associated with streams and also displays this reproductive mode with tadpoles having reduced and bare mouth and large vitelline reserves, and males transporting them until metamorphosis (Fig. 5C).

Data on the reproductive mode, body size, call, and habitat are completely missing for *A.* sp. "Parú", so we could not attribute a phenotype to this species. We also lack data on the reproductive mode of *A. leopardus*. However, this species is associated with streams, displays a large body size and a slow note rate, thus corresponding to phenotype 3 (Table 3). Therefore, it is likely that this species has exotrophic tadpoles transported by males. We summarized all these data in Fig. 6.

Interestingly, species of the *A. stepheni* group displaying phenotypes 2 and 3 co-occur in many places but in different combinations of CS in the *A. stepheni* group (Fig. 1). *Anomaloglossus stepheni* is found in sympatry with several exotrophic species in

Suriname (*A. leopardus*, *A.* sp. "Bakhuis", and *A.* sp. "Brownsberg") and in northern Pará, Brazil (*A.* sp. "Acari") (Fig. 5). The two phenotypes observed in the same lineage (*A. baeobatrachus*) occur in sympatry only in the north-eastern part of French Guiana (Route Nationale 2, a.k.a. RN2) (Fig. 5). This might also be the case in Amapá (Brazil), with two co-occurring phenotypes observed in the *A. baeobatrachus* lineage. However, in most cases, the two distinct co-occurring phenotypes are observed among different lineages. The phenotype 3 of *A. baeobatrachus* lineage 1 occurs in sympatry with the phenotype 2 *A. baeobatrachus* lineage 2 in the southern part of French Guiana (Fig. 5D), while in northern French Guiana, *A. baeobatrachus* displays phenotype 2. Similarly, *A.* sp. "Mitaraka", which displays phenotype 3, occurs in sympatry with phenotype 2 of *A. baeobatrachus* lineage 2 in south-western French Guiana. However, there is no co-occurrence of species sharing the same phenotype.

Within the *A. baeobatrachus* clade (*A. baeobatrachus* lineage 1 and 2), phenotype 2 seems to be distributed throughout FG and Amapá (Brazil), whereas phenotype 3 is apparently absent from north-western FG (west margin of the Approuague River north of Saül). Such distribution patterns are concordant for at least seven other frog species (*Allobates granti*, *Ameerega hahneli*, *Dendrobates tinctorius*, *Engystomops* sp., *Hyspiboas dentei*, *Pristimantis gutturalis*, and *Pristimantis* sp. 3 = *A. baeobatrachus* phenotype 2 distribution pattern) and four other frog species (*Amazophrynella* sp., *Leptodactylus longirostris*, *Pristimantis* sp. 1, and *Rhinella lescurei* = *A. baeobatrachus* phenotype 3 distribution pattern) (the authors pers. obs.).

4. Discussion

(1) *Anomaloglossus* represents a unique case of diversification within the Guiana Shield lowlands.

Our results highlight once more how far we still are from having a realistic view of the structure of the Amazonian biodiversity (Fouquet et al., 2007a; Funk et al., 2012). Underestimation of species richness within *Anomaloglossus* in the eastern Guiana Shield (EGS) had already been suggested in previous studies (Fouquet et al., 2012b, 2007a) based on more limited sampling. Our results indicate that these studies were still largely underestimating the actual species diversity of the genus. Indeed, the present study identified a total of 18 putative species within six currently recognized nominal species, out of which 11 are classified as Confirmed Candidate Species (CCS), six as Deep Conspecific Lineages (DCL) and one as Unconfirmed Candidate Species (UCS). These results show that *Anomaloglossus* represents the only documented group of frogs to have significantly diversified within the Guiana Shield

Table 3
Summary of the phenotypes that are observed within the EGS *Anomaloglossus* species and the characteristics attributed to each species and phenotype.

| Phenotype	Species	Development mode	Nidicoly/Phoresy	Body size	Habitat
Phenotype 1	*A. apiau*	Endotrophic	Phoresy	Large	Riparian
	A. degranvillei	Endotrophic	Phoresy	Large	Riparian
	A. sp. "Itoupé"	Endotrophic	Phoresy	Large	Riparian
	A. sp. "north FG"	Endotrophic	Phoresy	Small	Riparian
	A. surinamensis	Endotrophic	Phoresy	Small	Riparian
Phenotype 2	*A. stepheni*	Endotrophic	Nidicoly	Large	*Terra firme*
	A. baeobatrachus 1	Endotrophic	Nidicoly	Small	*Terra firme*
	A. baeobatrachus 2	Endotrophic	Nidicoly	Small	*Terra firme*
	A. leopardus	?	?	Large	Riparian
Phenotype 3	*A. baeobatrachus* 1	Exotrophic	Phoresy	Large	Riparian
	A. baeobatrachus 2	Exotrophic	Phoresy	?	Riparian
	A. sp. "Acari"	Exotrophic	Phoresy	?	Riparian
	A. sp. "Bakhuis"	Exotrophic	Phoresy	Large	Small water bodies
	A. sp. "Brownsberg"	Exotrophic	Phoresy	Large	Riparian/small water bodies
	A. sp. "Mitaraka"	Exotrophic	Phoresy	Large	Riparian
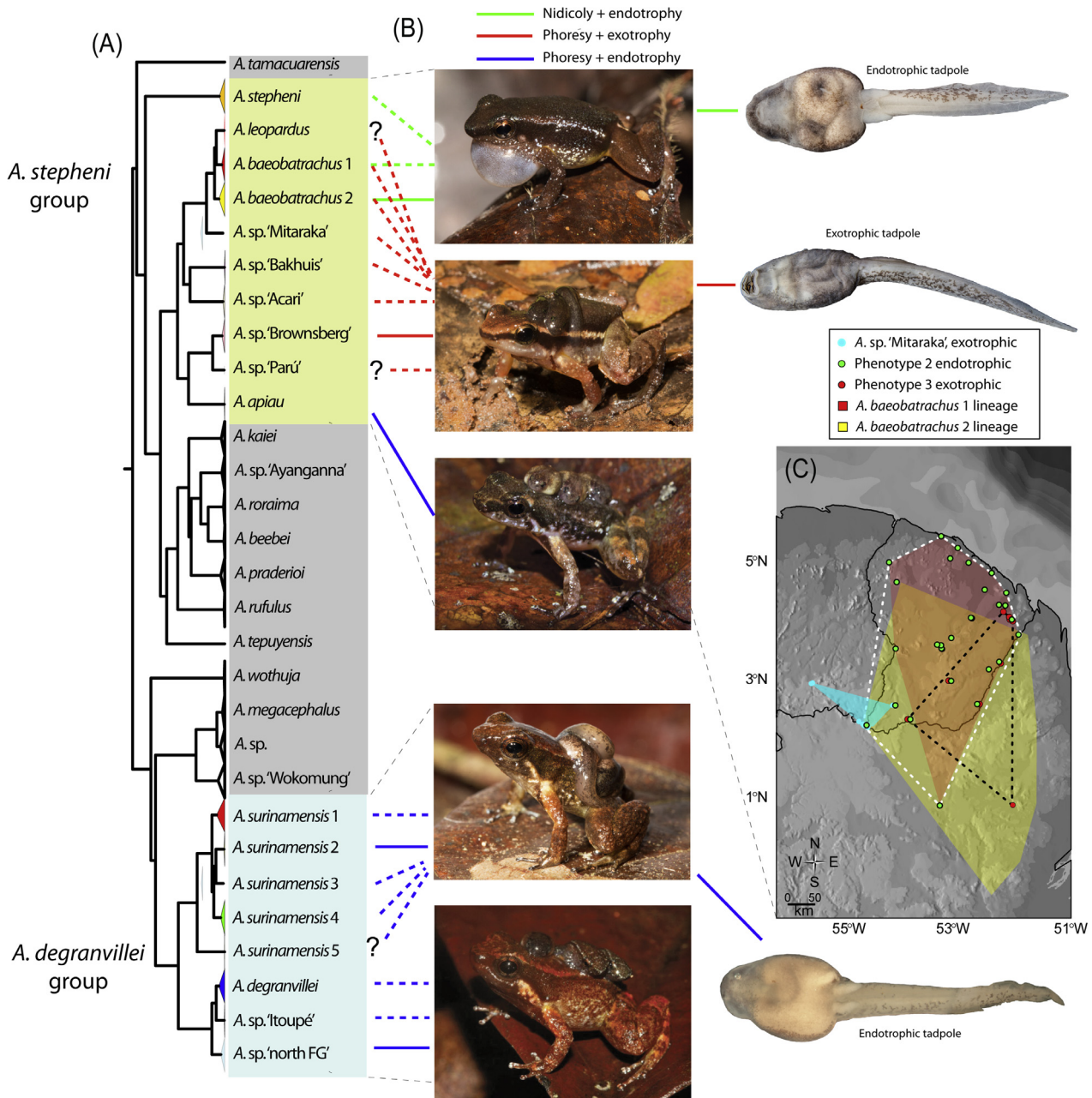
Fig. 5. Evidences for reproductive and larval developmental modes in the *Anomaloglossus* CS. (A) the topology obtained from analysis of the mtDNA used in Figs. 1 and 6. (B) Photographs of adult males of species representative of the different modalities (phoretic or nidicolous) found in *Anomaloglossus* [from top to bottom, the endotrophic and nidicolous *A. baeobatrachus* 2 from Mitaraka (French Guiana); the exotrophic and phoretic *A.* sp. "Brownsberg" from Brownsberg (Suriname); the endotrophic and phoretic *A. apiau* from Serra do Apiaú (Roraima, Brazil); the endotrophic and phoretic *A. surinamensis* 2 from Nassau (topotypic population in Suriname); the endotrophic and phoretic *A.* sp. "north FG" from Route Nationale 2 (French Guiana)]. Continuous lines indicate that the picture corresponds to the lineage while dashed lines indicate that the modality is found in the lineage. A question mark indicates when the modality has not been observed and is only assumed. Pictures of the three tadpoles representative of the endotrophic (reduced and non-functional mouth) or exotrophic (fully functional mouth) are also included (from top to bottom: *A. baeobatrachus* 2 from Mitaraka; *A.* sp. "Brownsberg"; *A. surinamensis* 2. The colours of the lines (blue, green, red) correspond to the three modalities of the reproductive traits found in the genus. (C) Distributions of the contrasting phenotypes in the *A. baeobatrachus* clade and the two mtDNA lineages. The white dashed line corresponds to the known distribution of phenotype 2, and the black dashed line corresponds to the distribution of phenotype 3.

lowlands. Given that many of these newly discovered, yet undescribed species, are microendemics, and that many massifs in the Guiana Shield remain virtually unexplored, it is likely that more undescribed species still remain to be discovered.

Within the two main groups that are restricted to the EGS, the *A. degranvillei* group is restricted to Suriname and French Guiana, with two species (*A. degranvillei* and *A.* sp. "Itoupé") having a very

restricted range (<500 km^2) in the southern part of the country (Fig. 1B). These two large-bodied species are associated with mountainous streams above 300 m a.s.l., while the smaller-bodied *A.* sp. "north FG" occurs at lower elevations but is also associated to massifs. In recent years, populations belonging to these three species seem to have drastically declined and some may have gone extinct (the authors pers. obs.). It is likely that these species
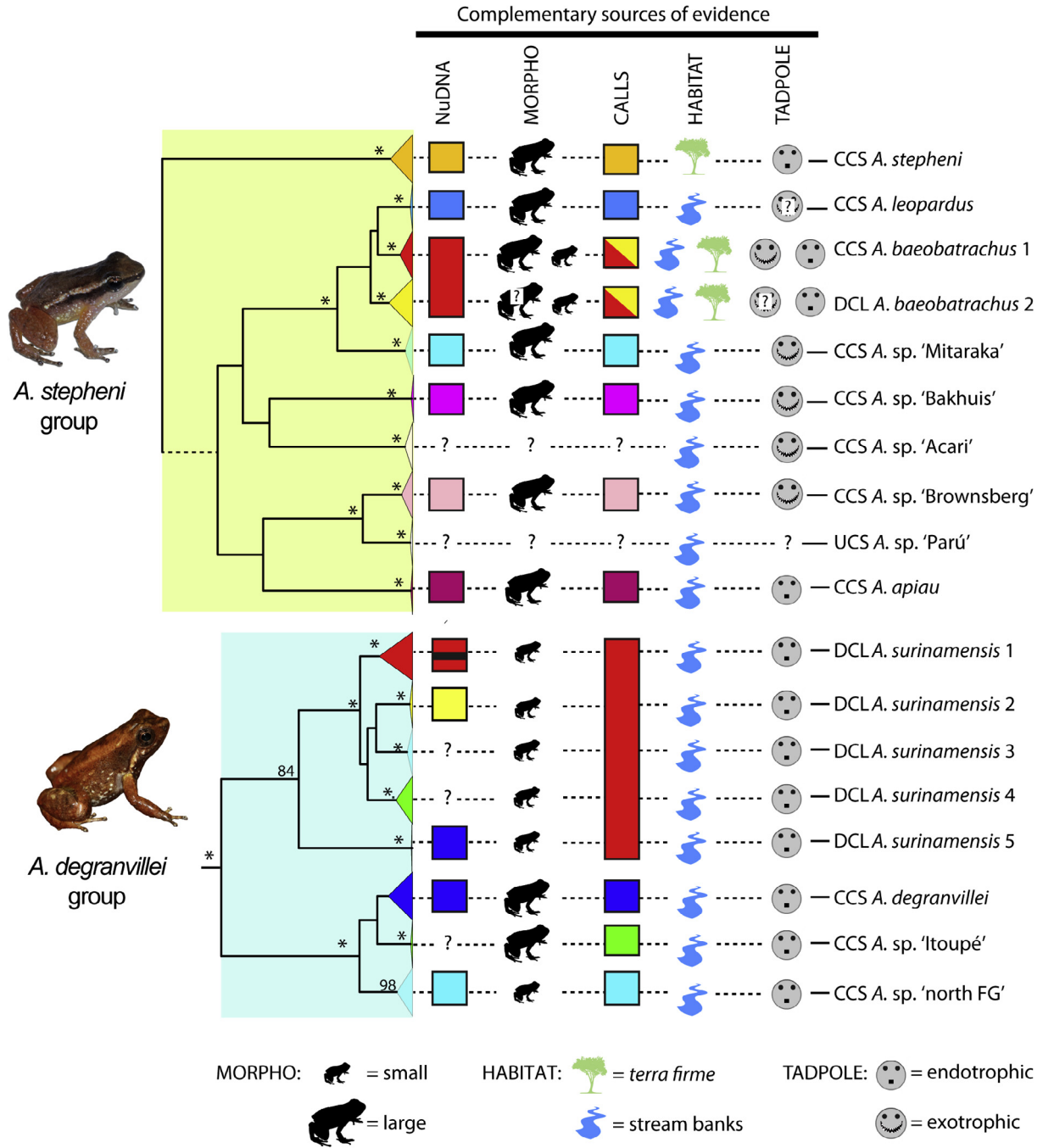
Fig. 6. Multiple evidence species delimitation of the two clades of *Anomaloglossus* of the eastern Guiana Shield lowlands. The terminals of the trees have been collapsed so they represent the results of *16S* rDNA delineation analyses in GMYC. Examined evidences are summarised in the first five columns and integrative species delimitation solution is shown in the last column. CCS = confirmed candidate species; UCS = unconfirmed candidate species; DCL = deep conspecific lineage. The phylogenetic position of *A. stepheni* (branching in dotted line) has been modified according to nuDNA results. The nuDNA data boxes are coloured according to the lineages recovered from the concatenated nuDNA loci. Morphological data have been simplified to only discriminate between large and small body-sized species. For the bioacoustic results, boxes are coloured according to the grouping found with the PCA. Habitat is represented by symbols depicting whether species are linked to stream banks (or other aquatic habitat) vs. *terra firme*. Larval development is indicated using "en" for endotrophic and "ex" for exotrophic; in one case (*A.* sp. "Acari"), the modality of this character is only assumed because one male was collected carrying tadpoles.

or additional ones occur in adjacent Suriname and Amapá state (Brazil), and given the conservation concerns raised above, they should be the focus of field surveys. The five DCL forming the *surinamensis* subclade also occur in allopatry, with three of them found in Suriname, and two others in French Guiana, but across larger areas than the *degranvillei* subclade, and no sign of decline has been detected among these populations yet. None of the species within each subclade have overlapping ranges, but one DCL from

the *surinamensis* subclade (*A. surinamensis* 1) occurs in sympatry with *A. degranvillei* and *A.* sp. "Itoupé" (Fig. 1B).

Within the other main group found in the EGS, the *A. stepheni* group, we were able to clarify the taxonomic status and range of *A. stepheni* and *A. baeobatrachus*. The first was recovered as diverging basally and has the widest distribution among the species group, occurring in the states of Amazonas and Pará (Brazil), and in Suriname. *Anomaloglossus baeobatrachus* is restricted to French

Guiana and Amapá, Brazil (Fig. 1C). Populations assigned to this species in Suriname (Ouboter and Jairam, 2012) correspond in fact to four different species: *A.* sp. "Bakhuis", *A.* sp. "Brownsberg", *A.* sp. "Mitaraka", or *A. stepheni.* Out of the 10 lineages in this group, seven are localised endemics or have at least narrow ranges in the EGS (*A. apiau, A. leopardus, A.* sp. "Acari", *A.* sp. "Bakhuis", *A.* sp. "Brownsberg", *A.* sp. "Mitaraka", and *A.* sp. "Parú"), even though species occurring in poorly documented areas such as northern Pará (Brazil) may have larger ranges. Finally, it is highly probable that additional data (bioacoustics and reproductive mode) would allow distinguishing *A.* sp. "Parú" from its close relatives and classifying it as a CCS.

Among the few genera that may have diversified in the GS lowlands, *Anomaloglossus* seems to be the only group of frogs to have diversified to such an extent. A few groups, *Otophryne* (de Sá et al., 2012), *Adelophryne* (Fouquet et al., 2012a), and the *Hypsiboas benitezi* group (Duellman et al., 2016) seem to represent ancient GS clades that have diversified both in the highlands and lowlands, but all display a small diversity in the lowlands compared to *Anomaloglossus.* On the opposite, many groups such as *Oreophrynella* (McDiarmid and Donnelly, 2005), *Stefania* (Kok, 2013; Kok et al., 2016b), *Myersiohyla* (Duellman, 1999), have diversified solely in the highlands of the Pantepui region, and most of the lowland lineages have apparently diversified throughout Amazonia or even larger areas throughout the continent (e.g., Fouquet et al., 2013).

(2) Contrasted divergence patterns within *Anomaloglossus*

As expected, morphometric data provided little discriminative power in our analysis, and most CCS were mainly distinguished by acoustic data. However, within the *Anomaloglossus* species groups, we found two sharply contrasting cases of molecular and phenotypic divergence that are worth discussing, even if possible explanations remain hypothetical at this stage.

The first concerns the five CS forming the *A. surinamensis* subclade. Despite deep genetic divergence among them (ranging from 2.7% to 6.4%, see Appendix 2), none of these populations can be discriminated with any morphological or bioacoustic variables. Moreover, even though sampling implied fewer individuals, congruent divergence is observed in the analyses based on nuDNA data. Since calls usually constitute strong discriminant characters among anurans (Vences and Wake, 2007), a lack of acoustic divergence between lineages diverging to such an extent is surprising. Given that they currently display an allopatric distribution pattern, one explanation could be that populations were isolated from each other without subsequent contacts, thus not promoting the evolution of premating isolation and therefore promoting call conservatism (Bogert, 1960; Hoskin et al., 2005). Although highly probable, this hypothesis is rather intriguing as an opposite pattern is observed within its sister group, the *A. degranvillei* subclade, which occurs in similar habitat and displays similar breeding mode. The three species that compose this clade have a comparatively low genetic divergence between them (1.9–2.6%, see Appendix 2), are also allopatric, but have well-differentiated calls. It is not clear which factor might have played a role in shaping these opposite patterns. However, we note that only the two largest species of the *A. degranvillei* subclade co-occur with *A. surinamensis* in French Guiana, while *A.* sp. "north FG" displays a similar body size as *A. surinamensis* but does not occur in sympatry with it (Fig. 1B). Phylogenetic relationships within the *A. surinamensis* subclade (even though deserving more investigation) demonstrated that French Guiana lineages are nested within Suriname lineages. This pattern supports the hypothesis that *A. surinamensis* could have secondarily dispersed to French Guiana and therefore came into contact with the ancestral *A. degranvillei* subclade. One hypothesis could be that niche overlap has fostered resource partitioning by charac-

ter displacement in *A. degranvillei* and *A.* sp. "Itoupé", thus evolving towards larger body size and a more specialized niche (Brown and Wilson, 1956). As a matter of fact, these two CS occur only in torrents above elevations of 300 m a.s.l., whereas *A. surinamensis* occurs from 0 to 800 m a.s.l. and *A.* sp. "north FG" from 0 to 300 m a.s.l.

The second contrasting case concerns *A. baeobatrachus,* which harbours two closely related mtDNA lineages that are distributed parapatrically. Both lineages display two phenotypes (phenotypes 2 and 3, Table 3) that are distinct in body-size, calls, habitat and larval development modes. Both phenotypes often co-occur and, at one exception (RN2, French Guiana), a given lineage harbours only one phenotype at a single location. To our knowledge, such a situation has never been observed in any other group of amphibian.

This pattern could result from at least four distinct scenarios that we enumerate below: (1) phenotypic plasticity; (2) ancient mtDNA divergence within the same species, and a single recent speciation; (3) ancient speciation and additional recent speciations phenotypically convergent, or (4) ancient speciation with recent secondary contact and introgression.

(1) Irrespective of the existence of either a single species displaying two mtDNA lineages or two distinct species, we can assume that their phenotypes could be determined by biotic or abiotic factors. We lack information to refute such a hypothesis. Nevertheless, it remains unlikely that such a large array of traits (call, habitat, body-size, larval development) would be plastic and result in only two phenotypic modalities and that at one exception, each phenotype harbours a single DNA lineage at each location.

(2) The second scenario would imply ancient divergence resulting in the two observed mtDNA lineages but the speciation itself occurred more recently resulting in the presence of both mtDNA lineages in each species. Such a hypothesis is weakened by the fact that under such circumstances, it would be expected to find the two mtDNA lineages harbouring the same phenotype at a given locality, which is never the case.

(3) The third scenario would suggest the existence of four distinct species each harbouring one of the two phenotypes. This scenario assumes a first ancient speciation corresponding to the mtDNA divergence and two additional speciation events that are too recent to be distinguished by our molecular data (mean *p-distances* = 0.3% within *A. baeobatrachus* and 0.8% within *A.* sp. 1). These last speciation events would have yielded converging phenotypes. Such a scenario implies an unlikely rapid evolution of the ecology, morphology, calls, and larval development mode. We lack fine scale genomic data to be able to refute this hypothesis.

(4) The last scenario would involve secondary contact between two ancestral and phenotypically distinct species. As in the previous scenario, the two lineages would have originated from historical isolation, and would have recently come into contact and hybridized. Given the striking phenotypical difference currently observed despite sympatry, we can assume that during the formation of a hybrid zone, selection against hybrids fostered premating isolation, yet allowing time for genomic exchanges, notably mitochondrial (Hoskin et al., 2005). Subsequently, gene flow would have allowed the spread of introgressive genomic material within each species.

Testing these four hypotheses deserves further investigation using population genomic data. It is noteworthy that either a rapid evolution of the larval development or the hybridization between

species differing in larval development suggests that the genetic architecture of this trait could be rather simple and worth further research.

(3) Biogeography and evolution of reproductive modes in *Anomaloglossus*

Our results suggest that *Anomaloglossus* represents an exceptional model to study speciation and diversification in the GS. The fact that all Pantepui species apparently all have exotrophic tadpoles, while in the EGS lowlands, the *A. degranvillei* species group has endotrophic tadpoles, and the other (*A. stepheni* group) in the same area contains both modes of tadpole development suggests a strong biogeographic signal. This also reveals that endotrophy evolved several times independently in the genus. Therefore, we hypothesize that evolution towards endotrophy in this genus probably allowed populations to colonize *terra firme* environments and to disperse into new niches in the lowland forests. As a matter of fact, species displaying nidicoly associated with endotrophic tadpoles (*A. baeobatrachus, A. stepheni*) have the widest distribution.

Even if endotrophy is common in anurans (McDiarmid and Altig, 1999), lineages of closely related populations or species that include both endotrophic and exotrophic developmental guilds are very rare (Anstis, 2010; McDiarmid and Altig, 1999). In the Neotropical genus *Allobates,* endotrophy has been reported in two species that are not closely related, *A. chalcopis* (Kaiser and Altig, 1994) and *A. nidicola* (Caldwell and Lima, 2003), thus suggesting independent evolution of endotrophy. Similarly, a striking pattern of evolution of larval developmental mode has been documented in the Malagasy *Gephyromantis* (Kaffenberger et al., 2012). A comparable pattern of intrageneric recurrent evolution of larval development is also known in *Adenomera* (*A. dyptix* and *A. thomei* being exotrophic in an otherwise endotrophic genus) (Fouquet et al., 2014). Nevertheless, to our knowledge, this is the first case of endotrophy/exotrophy evolution between species as closely related as in the *Anomaloglossus baeobatrachus* clade.

Delineating species is crucial for evaluating their threatened status (Bickford et al., 2007). Newly documented species can be formally described and their conservation status evaluated. Among the 21 species of *Anomaloglossus* currently listed in the IUCN Red List database, only six of them have been assessed for their conservation status. All the others are considered "Data deficient". Nonetheless, some authors highlighted conservation urgency for these frogs (Fouquet et al., 2015; Kok et al., 2013). Some *Anomaloglossus* are already considered threatened by extinction (*A. apiau*) or probably already extinct (*A. tepequem*) (Fouquet et al., 2015). The perception that so many undescribed (*A.* sp. "Bakhuis", *A.* sp. "Brownsberg", *A.* sp. "Itoupé") and nominal (*A. degranvillei, A. leopardus*) species are microendemics and that some of them are declining highlights the urgency for evaluating the conservation status of these species.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.04.017.

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