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ORIGINAL ARTICLE

Species delimitation, patterns of diversification and historical biogeography of the Neotropical frog genus *Adenomera* (Anura, Leptodactylidae)

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ABSTRACT

Aim For many taxa, inaccuracy of species boundaries and distributions hampers inferences about diversity and evolution. This is particularly true in the Neotropics where prevalence of cryptic species has often been demonstrated. The frog genus *Adenomera* is suspected to harbour many more species than the 16 currently recognized. These small terrestrial species occur in Amazonia, Atlantic Forest (AF), and in the open formations of the Dry Diagonal (DD: Chaco, Cerrado and Caatinga). This widespread and taxonomically complex taxon provides a good opportunity to (1) test species boundaries, and (2) investigate historical connectivity between Amazonia and the AF and associated patterns of diversification.

Location Tropical South America east of the Andes.

Methods We used molecular data (four loci) to estimate phylogenetic relationships among 320 *Adenomera* samples. These results were integrated with other lines of evidence to propose a conservative species delineation. We subsequently used an extended dataset (seven loci) and investigated ancestral area distributions, dispersal–vicariance events, and the temporal pattern of diversification within *Adenomera*.

Results Our conservative delineation identified 31 Confirmed Candidate Species (four remaining unconfirmed) representing a 94% increase in species richness. The biogeographical analysis suggested an Amazonian origin of *Adenomera* with as many as three dispersals to the DD and one to the AF during the Miocene. These dispersals were associated with habitat shifts from forest towards open habitats.

Main conclusions The DD played a major role in the history of *Adenomera* in limiting dispersal and favouring diversification of open-habitat lineages. Moreover, a forest bridge during the Miocene Climatic Optimum may have permitted dispersal from Amazonia towards the AF and subsequent diversification. Uncovering species boundaries and distributions might drastically change inferences based on currently perceived distribution patterns.

Keywords
Amazonia, Anura, Atlantic forest, biodiversity, biogeography, cryptic species, diversification, Neotropics, species delineation, tropical forests.

INTRODUCTION

Estimation of how many species exist on Earth and where they occur remains approximate (Mora *et al.*, 2011), not only for microorganisms (Quince *et al.*, 2008) or arthropods (Hamilton *et al.*, 2012), but also for some vertebrate groups such as tropical amphibians (e.g. in Amazonia: Jansen *et al.*, 2011; Fouquet *et al.*, 2007; Funk *et al.*, 2012). The consequences of such a prevalence of cryptic species can dramatically alter our perception of biodiversity structure (Bickford *et al.*, 2007).

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Basic understanding of biodiversity patterns are, however, necessary to investigate ecosystem functioning and to underpin conservation efforts (Sites & Marshall, 2003, 2004; Bini *et al.*, 2006). It is also mandatory to investigate the processes responsible for the origin of Neotropical diversity, a topic that has intrigued naturalists for more than a century (Wallace, 1852; Bates, 1863). Intricate geomorphological and climatic events of the Tertiary have been proposed as important causes of South American megadiversity, notably the uplift of the Andes cordillera during the Miocene–Pliocene (reviewed by Hoorn *et al.*, 2010 for Amazonia). Another striking feature of the South American biota is the north-east–south-west belt of open formations (Prado & Gibbs, 1993), also called the 'Dry Diagonal' (DD), which includes the Chaco, the Cerrado and the Caatinga, and which acts as a barrier to biotic exchange between Amazonia and the Atlantic Forest (AF) (Costa, 2003; Batalha-Filho *et al.*, 2013). Among the few studies to investigate the impact of the DD, some have revealed ancient divergences between forest-dwelling organisms on each side, dating back to the Oligocene/Miocene (Pellegrino *et al.*, 2011; Fouquet *et al.*, 2012a,b). Notwithstanding, some subsequent connections between these two forest blocks have been hypothesized to explain dispersals across Amazonia and the AF (Costa, 2003; Melo Santos *et al.*, 2007). In some other groups, lineages underwent habitat shifts, adapting to open habitats and dispersing through the continent. Ecological adaptations linked to historical contraction/expansion of different habitats have probably fed and enriched each major biome's species assemblages (Rheindt *et al.*, 2008; Valdujo *et al.*, 2013). Therefore, exploring historical biogeography and habitat shifts are probably key to understanding the processes that underlie the assembly and evolution of Neotropical biodiversity (Wiens & Donoghue, 2004).

The leptodactylid frog genus *Adenomera* includes 16 nominal species distributed almost throughout the lowlands of tropical South America east of the Andes. *Adenomera* species are generally omnipresent in their habitat, often representing the most abundant anurans of the leaf litter (Menin *et al.*, 2008). Nevertheless, they have remained relatively neglected and have puzzled frog systematists for decades (Heyer, 1973; Angulo *et al.*, 2003; Duellman, 2005). These dull-coloured, small-sized frogs typically display characteristics of a group rich in cryptic species, as indicated by bioacoustic data (Angulo *et al.*, 2003). Such a widespread and taxonomically complex taxon provides an excellent opportunity to investigate the role played by the DD in the diversification of South American groups. Fouquet *et al.* (2013) provided the first phylogeny to include a substantial number of species of *Adenomera*. This dated phylogeny estimated that diversification of *Adenomera* started in the Neogene (25 Ma, 95% confidence interval, CI: 29–21 Ma) and that AF and open-habitat species are nested within Amazonian lineages. This prompted us to question how many dispersal–vicariance events occurred between Amazonia, the DD and the AF, and when these events happened. We also investigated which geomorphological and climatic events of the Neogene could have driven these dispersals and promoted diversification within Amazonia, the DD and the AF.

Using one of the most geographically complete sampling efforts for any species-rich South American vertebrate genus studied so far (320 *Adenomera* individuals from 264 localities), we combined mitochondrial and nuclear data (3.3 kb) to produce a conservative species delineation within *Adenomera*. We subsequently used an extended dataset (6.6 kb for 34 terminals) to test whether dispersal–vicariance events occurred between Amazonia, the DD and the AF with associated habitat shifts from forest towards open habitats and associated patterns of diversification.

MATERIALS AND METHODS

Sampling and laboratory protocol

We gathered tissue samples for 320 *Adenomera* individuals from 264 localities, including all nominal species and encompassing the entire distribution of the genus. This material was preserved in absolute ethanol and was collected by the authors, colleagues or from loans of many institutions across the world (see Appendix S1a,e in Supporting Information). We followed the taxonomic nomenclature of Frost (2013).

Attribution of this material to currently recognized nominal species was often ambiguous. However, for 14 of the 16 nominal species (except *A. andreae* and *A. hylaedactyla*), we obtained tissues of topotypical specimens (or from nearby localities) that unambiguously matched the original descriptions (morphology and/or calls). Therefore, at least one sample could reliably be linked to each nominal species. Additionally, an array of other considerations (Appendix S2) was taken into account to cross-validate identifications.

Standard molecular protocols were used from DNA extraction to sequencing (Appendix S1b) following Fouquet *et al.* (2013). We targeted two mitochondrial loci [cytochrome *b* (cyt *b*), 667 bp; cytochrome *c* oxidase subunit I (*COI*), 657 bp] and two nuclear loci [recombination activating gene exon 1 (*RAG1*), 1422 bp; proopiomelanocortin C (*POMC*), 547 bp] and these were sequenced for all samples (primers detailed in Appendix S1a). Other selected species of Leptodactylidae and Centrolenidae were used as outgroups (Appendix S1c) following the relationships presented by Pyron & Wiens (2011) and Fouquet *et al.* (2013).

Bayesian analysis

The concatenated matrix comprised 3301 bp. We observed several codon insertion–deletions in *POMC* but none of them led to ambiguous alignment after checking the reading frame. We started by investigating phylogenetic relationships among samples. The best partition scheme was determined using PartitionFinder 1.0 (Lanfear *et al.*, 2012) with the Bayesian information criterion and considering each codon

position of each locus as a possible partition. Bayesian analyses followed Fouquet *et al.* (2013) (Appendix S1d).

Species delineation

The candidate species were primarily delimited from a threshold based on the mitochondrial DNA (mtDNA) distance between nominal species pairs. Genetic distances [uncorrected pairwise (*p*)-distances] were computed for the combined mitochondrial genes using MEGA 5.1 (Tamura *et al.*, 2011). The mean genetic distance found between nominal species recovered as sister species was used as a preliminary threshold (8%, see Results), considering that greater distances may indicate the existence of different species. We therefore flagged all the lineages diverging from their closest relatives by distances greater than this threshold as candidate species.

In a second step, we determined whether closely related candidate species display signs of reproductive isolation by examining nuclear DNA (nDNA) allele sharing and network cohesion in relation to range overlap. To determine the most probable alleles for individuals heterozygous for nDNA sequences we used PHASE (Stephens *et al.*, 2001; Stephens & Donnelly, 2003) implemented in DNASP 5 (Librado & Rozas, 2009). We divided the data by nDNA loci and by main species groups according to the topology recovered from the phylogenetic analysis. We performed five independent runs of 100 iterations each (which were sufficient to reach stationarity) after a burn-in of 100, and a thinning interval of 1; we used the default cut-off thresholds. Using these alignments we computed statistical parsimony networks using TCS 1.21 (Clement *et al.*, 2000) with a 95% connection limit.

We also considered additional lines of evidence such as bioacoustics, morphology and/or ecology from new and published data to reach a conservative and integrative species delineation. These case-by-case examinations are fully explained in Appendix S2 and summarized in Table 1. When evidence supported the delineation, the species were flagged as Confirmed Candidate Species (CCS). When no evidence was found, one of the species of the pair was flagged as an Unconfirmed Candidate Species (UCS). This approach was essentially adapted from Vieites *et al.* (2009).

Molecular dating

We selected one representative of each candidate species based on our species delineation (with the exception of *A.* sp. C for which no additional sequences could be gathered) and for each produced additional sequences in order to confidently estimate interspecific relationships and timing of divergences using BEAST 1.6.2 (Drummond & Rambaut, 2007). We used two approaches: (1) the concatenated dataset method (CM), and (2) the multilocus species tree method (MM) (*BEAST; Heled & Drummond, 2010). In addition to the abovementioned molecular data, we targeted the H-strand transcription unit 1 (H1, *c.* 2400 bp including *12S* and *16S*) and two additional nuclear loci: tyrosinase (*TYR*, 531 bp) and rhodopsin (*RHOD*, 316 bp) (Appendix S1). For the *12S–16S* fragment, we performed alignment using MAFFT 6 (Katoh *et al.*, 2002) under default parameters except for the use of the E-INS-i strategy, which is adapted to sequences with one conserved domain and long gaps. Along with previously analysed sequences, we obtained a final 6599 bp alignment.

This dataset was partitioned by codons for coding mtDNA and nDNA and one for *12S–16S*, and we used the software JMODELTEST 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) to select the substitution model that best fitted each of these partitions under the Akaike information criterion (Akaike, 1974). For the CM, we used the linked tree prior for all trees, and the MM was based on the five loci with unlinked tree prior for all trees (mtDNA, *RAG1*, *POMC*, *TYR* and *RHOD*). All partitions were considered under the estimated uncorrelated lognormal rates. The tree prior used the birth and death process, with a UPGMA (unweighted pair-group method using arithmetic averages) generated starting tree and the auto-optimize option for operators.

Both analyses were calibrated on the crown age of *Adenomera* (prior set as a normal distribution 25 Ma; SD = 3) and a maximum age of 80 Ma for Leptodactylidae according to results of Fouquet *et al.* (2013) based on a similar matrix but larger taxon sampling and more calibrations. We computed 2.0×10^8 generations, sampled every 1000 generations. We examined convergence on stationarity using TRACER 1.5 (Rambaut & Drummond, 2007). For both analyses effective sample sizes (ESS) were > 200 for all parameters except for a few substitution rates. Therefore, we computed an additional 2.0×10^8 generations run with the prior distribution of these rates changed from a gamma to a uniform distribution bounded between 10^{-5} and 1. This led to ESS > 300 for all parameters. The maximum clade credibility trees were computed with TREEANNOTATOR 1.6.2 from 10% of trees resampled.

Biogeographical reconstruction

We performed the Bayesian method of biogeographical and ancestral state reconstruction implemented in RASP 2.0 (Sanmartín *et al.*, 2008; Yu *et al.*, 2011). We used the obtained species tree, and species distribution areas were assigned to geographical regions as follows: A – Amazonia, B – DD (Cerrado, Chaco and Caatinga), and C – AF. The presence of *A. hylaedactyla* in the northern tip of the AF and in Amazonia resulted from recent dispersals (see Results). As this species is represented by one terminal only in the species tree, these secondary dispersals could not be inferred from the analysis. We therefore attributed *A. hylaedactyla* to region B. The Bayesian binary analysis was run with the fixed state frequencies model (Jukes–Cantor). Among-site variation was set to equal, and no outgroup was defined (root distribution was set to NULL). We ran the analysis for 5.0×10^6 generations, sampled every 1000, with 10 chains and two parallel runs from which a combined result was obtained. The first 1000 samples were discarded.

Table 1 Criterion used for candidate species delineation of *Adenomera*. Species pairs (in one case, a triplet) are in grey shaded cells. Species are eventually coded as Confirmed or Unconfirmed Candidate Species (CCS/UCS).

Clade	mtDNA divergence/ relative	Max *p*-distance within species	nDNA monophyly	No nDNA allele sharing and network cohesion	Sympatry with relative/ contact zone	Other lines of evidences vs. closest relative	Sampling (*n*)	Diagnose	Remark
A. andreae	> 10%	> **8%**	Yes PP = 1	Yes	Yes	Vocalization Forest type III (Angulo *et al.*, 2003)	Good (72)	CCS	Complex – over-conservative
A. sp. C	8.8%	< 8%	Yes PP = 0.97	Yes	Probable	Vocalization Forest type II (Angulo *et al.*, 2003)	Low (3)	CCS	
A. sp. T	8.8%	< 8%	Yes sister to *A.* sp. C	Yes	Probable	No data	Low (1)	UCS	May be conspecific with C
A. sp. E	> 10%	< 8%	No *A.* sp. D nested	Yes	Probable	Vocalization Forest type I (Angulo *et al.*, 2003)	Good (14)	CCS	
A. sp. D	> 10%	< 8%	No nested in *A.* sp. E	Yes	Probable	No data	Low (1)	UCS	May be conspecific with E
A. simonstuarti	> 10%	< 8%	No 2 clades	Yes	Yes	Vocalization (Angulo & Icochea, 2010)	Good (14)	CCS	Complex – over-conservative
A. diptyx	> 10%	< 8%	No 3 clades	Allele sharing and no cohesion	Yes	Exotrophic larvae (de la Riva, 1995)	Good (13)	CCS	Hybrid + complex
A. hylaedactyla	> 10%	< 8%	*A. diptyx* nested	Allele sharing and no cohesion	Yes	Endotrophic larvae (Menin *et al.*, 2009)	Good (77)	CCS	Hybrid + complex
A. martinezi	> 10%	< 8%	Yes PP = 1	Yes	No	Vocalization and morphology (de Carvalho & Giaretta, 2013)	Good (2)	CCS	
A. saci	> 10%	< 8%	Yes PP = 1	Yes	Probable	Vocalization and morphology (de Carvalho & Giaretta, 2013)	Good (10)	CCS	
A. sp. B	> 10%	< 8%	Yes sister to *A.* sp. A	Yes	Probable	No data	Low (1)	UCS	May be conspecific with *A. saci*
A. lutzi	> 10%	< 8%	Yes PP = 1	Yes	No	Vocalization and morphology (Kok *et al.*, 2007; Heyer, 1973)	Good (3)	CCS	
A. sp. P	> 10%	< 8%	Yes PP = 1	Yes	No	No bioacoustic data but habitat and morphology	Low (2)	CCS	
A. heyeri	> 10%	< 8%	Yes PP = 1	Yes	No	Vocalization and morphology (Boistel *et al.*, 2006)	Good (8)	CCS	
A. sp. F	> 10%	< 8%	Yes PP = 1	Yes	No	Vocalization and morphology	Good (9)	CCS	Complex – over-conservative
A. sp. G	> 10%	> **8%**	Yes PP = 1	Yes	No	Morphology	Good (6)	CCS	Complex – over-conservative
A. sp. H	> 10%	< 8%	Yes PP = 1	Yes	No	Vocalization	Low (5)	CCS	

Table 1 Continued

Clade	mtDNA divergence/ relative	Max *p*-distance within species	nDNA monophyly	No nDNA allele sharing and network cohesion	Sympatry with relative/ contact zone	Other lines of evidences vs. closest relative	Sampling (*n*)	Diagnose	Remark
A. sp. Q	> 10%	< 8%	Yes sister to *A. heyeri*	Yes	No	No bioacoustic data but habitat and morphology	Low (1)	CCS	
A. sp. I	> 10%	< 8%	Yes PP = 1	Yes	Yes	No data	Good (17)	CCS	Complex – over-conservative
A. sp. J	**8.30%**	< 8%	Yes PP = 0.99	Yes	Yes	Vocalization and morphology	Good (9)	CCS	
A. marmorata	**8.30%**	< 8%	*A.* sp. J nested	Yes	Yes	Vocalization and morphology	Good (13)	CCS	Complex – over-conservative
A. sp. S	9.5%	< 8%	Yes PP = 1	Allele sharing but cohesion	Probable	Vocalization	Good (6)	CCS	
A. ajurauna	9.5%	< 8%	S nested	Allele sharing but cohesion	Probable	Vocalization (Berneck *et al.*, 2008)	Good (5)	CCS	Complex – over-conservative
A. sp. K	> 10%	< 8%	2 lineages	Yes with *RAG1* not with *POMC*	Yes	No data	Low (2)	CCS	
A. sp. A	> 10%	< 8%	Yes	Yes	Probable	No data	Low(1)	CCS	
A. sp. O	9.3%	< 8%	Yes	Yes	Probable	No data	Low (1)	CCS	
A. sp. N	9.3%	< 8%	Yes	Yes	Probable	No data	Low (1)	CCS	
A. bokermanni	9.0%	< 8%	Yes PP = 1	Yes	Yes	Morphology (Heyer, 1973; Kwet, 2007) Vocalization	Good(2)	CCS	
A. engelsi	**7.6%**	< 8%	Yes PP = 1	Yes	Yes	Vocalization and morphology (Kwet *et al.*, 2009)	Good (4)	CCS	
A. nana	**7.6%**	< 8%	2 lineages	Yes	Yes	Vocalization and morphology (Kwet, 2007)	Low (3)	CCS	
A. sp. R	9.7%	< 8%	*A. araucaria* nested	No allele sharing but no cohesion	Probable	Vocalization (Kwet, 2006, 2007)	Low (2)	CCS	
A. araucaria	9.7%	< 8%	*A.* sp. R nested	No allele sharing but no cohesion	Probable	Vocalization (Kwet, 2006, 2007)	Low (3)	CCS	
A. thomei	9.4%	< 8%	Yes PP = 1	Yes	Probable	Vocalization, morphology and mode of reproduction (Almeida & Angulo, 2006)	Good (10)	CCS	
A. sp. M	9.2%	< 8%	*A.* sp. L nested	No allele sharing but no cohesion	Probable	Vocalization	Low (2)	CCS	May be conspecific with L
A. sp. L	9.2%	< 8%	*A.* sp. M nested	No allele sharing but no cohesion	Probable	Vocalization	Low (2)	UCS	
A. coca	0	0	**Nested in *A. hylaedactyla***	**No**	Yes	Vocalization (Angulo & Reichle, 2008)	Low (2)	UCS	May be conspecific with *A. hylaedactyla*

p-distance, pairwise distance; PP, posterior probability.

Diversification

To test whether changes in diversification rate occurred among clades, we used the model-based approach proposed by Rabosky *et al.* (2007). Using the MM tree (species tree), we first estimated the constant-rate model using the fitNDR_1rate.Rd function of the LASER package (Rabosky, 2006) under R 2.14.1 (R Development Core Team, 2011) and tested for shifts in diversification rate within the phylogeny by comparing likelihood of the chronogram under constant and rate-flexible diversification models (Rabosky *et al.*, 2007). The best-fitting model was determined using a likelihood ratio test (LRT) between the constant-rate and the flexible-rate models (nested), and by ΔAIC scores between the flexible-rate and rate-decrease models (not nested). All analyses were performed under two extremes of the relative extinction rate ($a = 0$ and $a = 0.99$) as a fixed parameter to determine the robustness of the results to variation in the extinction fraction (Raup, 1985). We also used the relative cladogenesis test implemented in the GEIGER package (Harmon *et al.*, 2008) to identify lineages characterized by unusually slow or rapid diversification rates.

RESULTS

Phylogenetic analysis

The phylogenetic tree obtained from Bayesian analysis of the 320 *Adenomera* terminals strongly supported *Adenomera* as monophyletic and as the sister group of *Lithodytes* (Fig. 1). The diversity within *Adenomera* was much higher than previously recognized, with highly divergent lineages that cannot be attributed to any nominal species (see below). On the contrary, *A. coca* was nested within *A. hylaedactyla* with a very short branch length.

Eight major clades can be delimited (Fig. 1), each predominately associated with one of the major South American biomes (Fig. 2). Only the *A. hylaedactyla* clade is widespread, occurring across Amazonia, the DD and even the northern tip of the AF. Up to five species co-occur at any single locality (Fig. 2). However, in most of Amazonia and the DD, typically three species co-occur at a single locality. Interestingly, this pattern contrasts with the AF situation, where species display a striking pattern of alloparapatry with very few cases of co-occurrence.

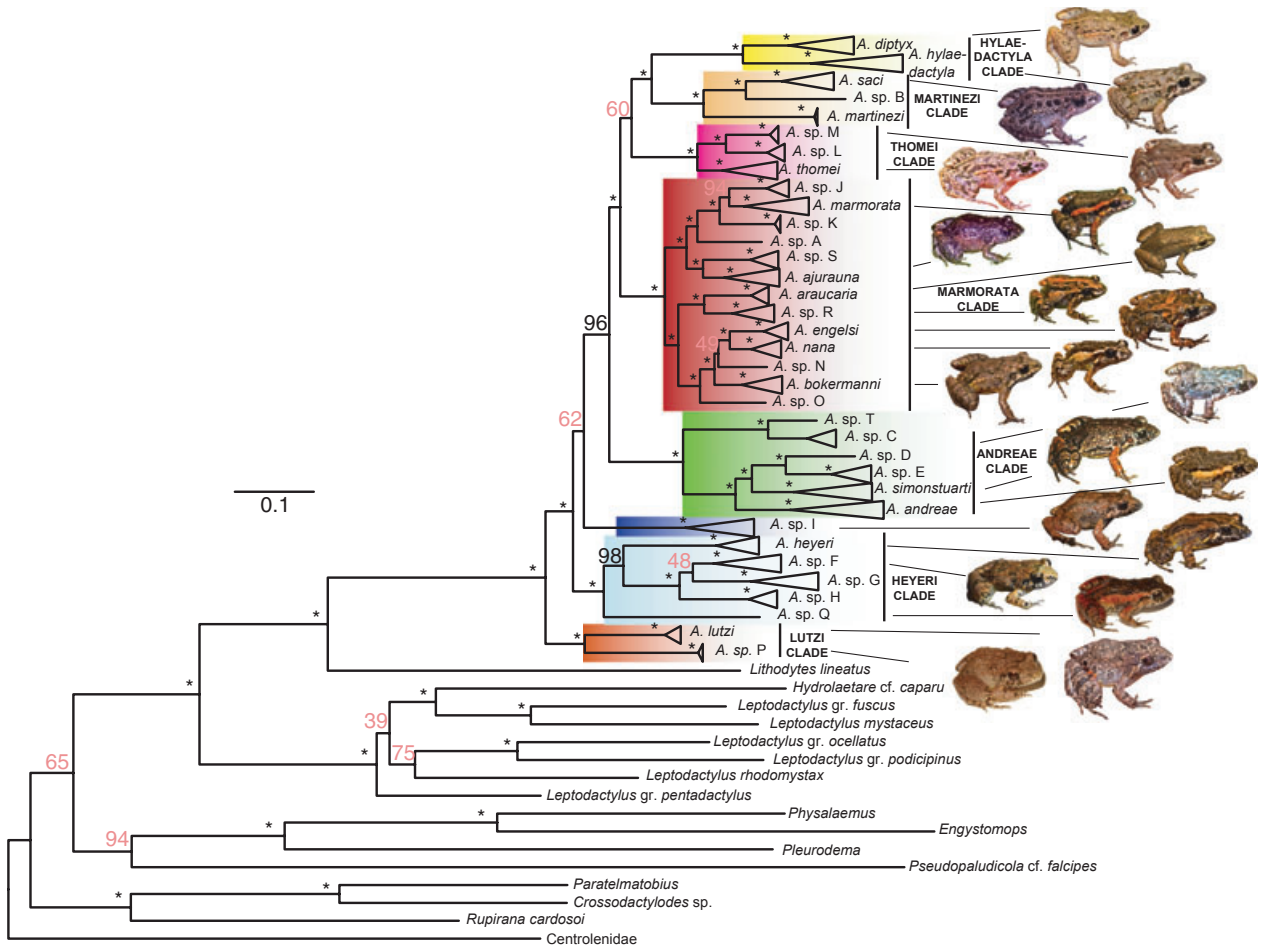

Figure 1 Phylogenetic reconstruction hypothesized from Bayesian analysis of 320 *Adenomera* rooted on Centrolenidae, using 3301 bp of concatenated mtDNA (cyt *b* + *COI*) and nDNA (*RAG1* + *POMC*). Posterior probabilities (PP × 100) are given near the nodes and branch scale is indicated in number of substitutions per site. Asterisks indicate PP > 0.95. For clarity, we collapsed branches within delimited species and indicate major clades with a colour code.

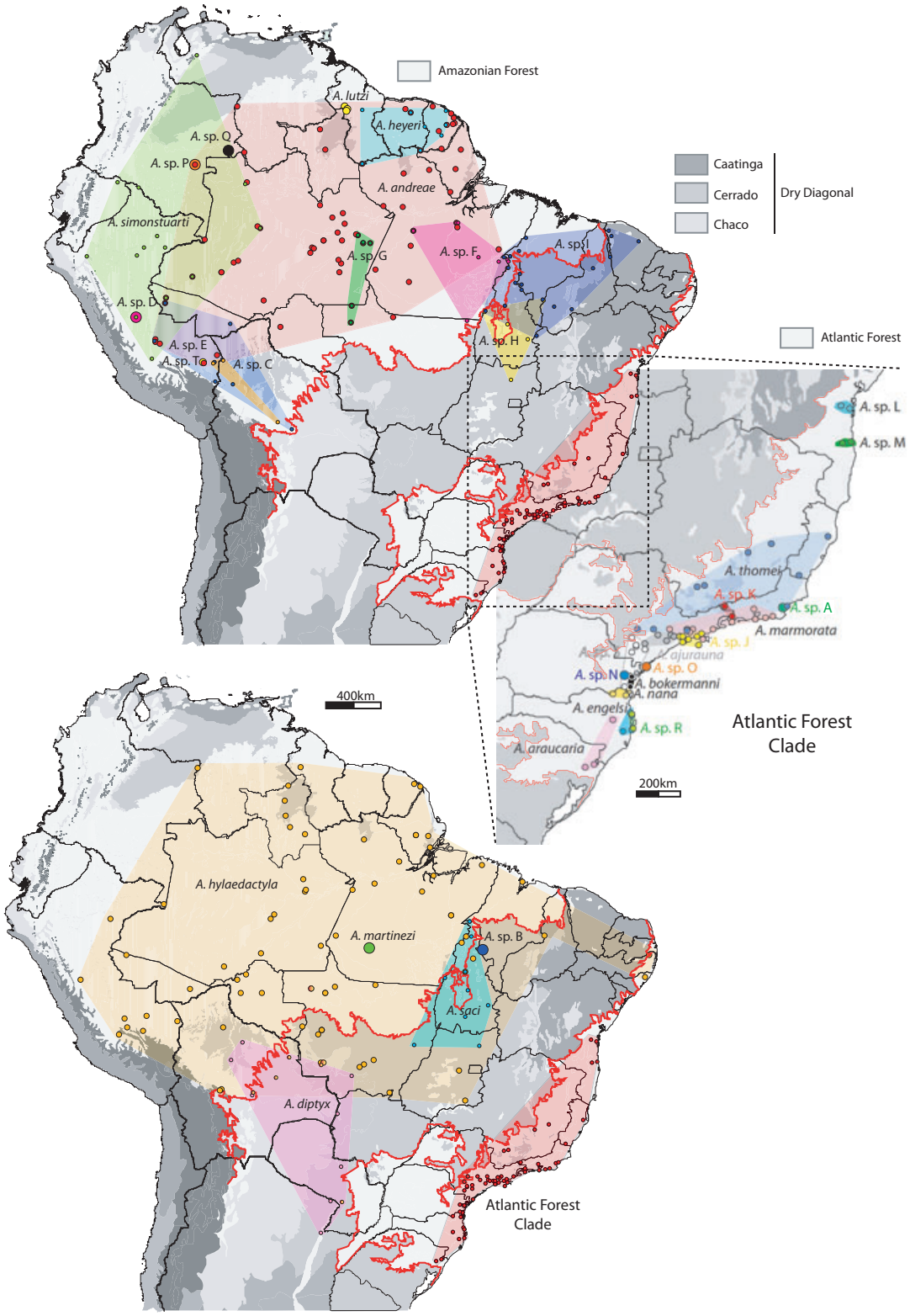

Figure 2 Distribution of the delimited species of *Adenomera* in relation to major South American biomes. Boundaries of the Dry Diagonal are highlighted in red.

Species delineation

One pair of sister nominal species was recovered (*A. engelsi* + *A. nana*) with mean *p*-distances of 7.6% (range: 7.1–8.3%). Therefore, we used a threshold of 8% that delimited 35 major mitochondrial lineages flagged as candidate species named from *A.* sp. A to *A.* sp. T. For reasons explained in Appendix S2, we departed in two cases (*A. andreae* and

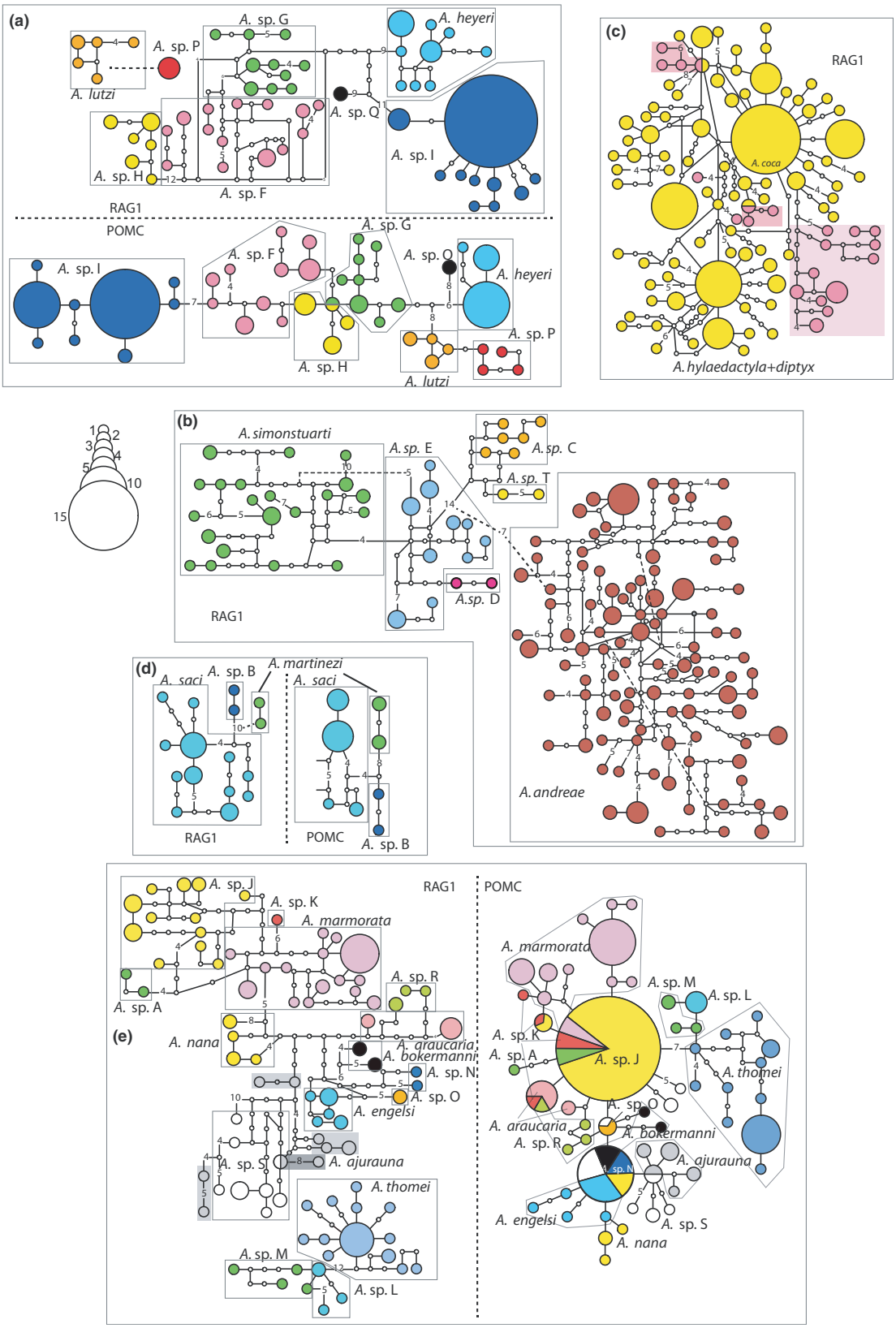

Figure 3 Statistical parsimony networks of phased nuclear loci (*RAG1* and *POMC*) for (a) the *Adenomera heyeri*, *A. lutzi* and *A.* sp. I clades; (b) the *A. andreae* clade, (c) the *A. hylaedactyla* clade, (d) the *A. martinezi* clade and (e) the AF clades. Haplotypes are shown as circles proportional in size to haplotype frequency. In two cases (*A. andreae* and *A. hylaedactyla* clades), the networks from the *POMC* locus were omitted because they were so anastomosed that a clear graphic was extremely difficult and would not provide any information.

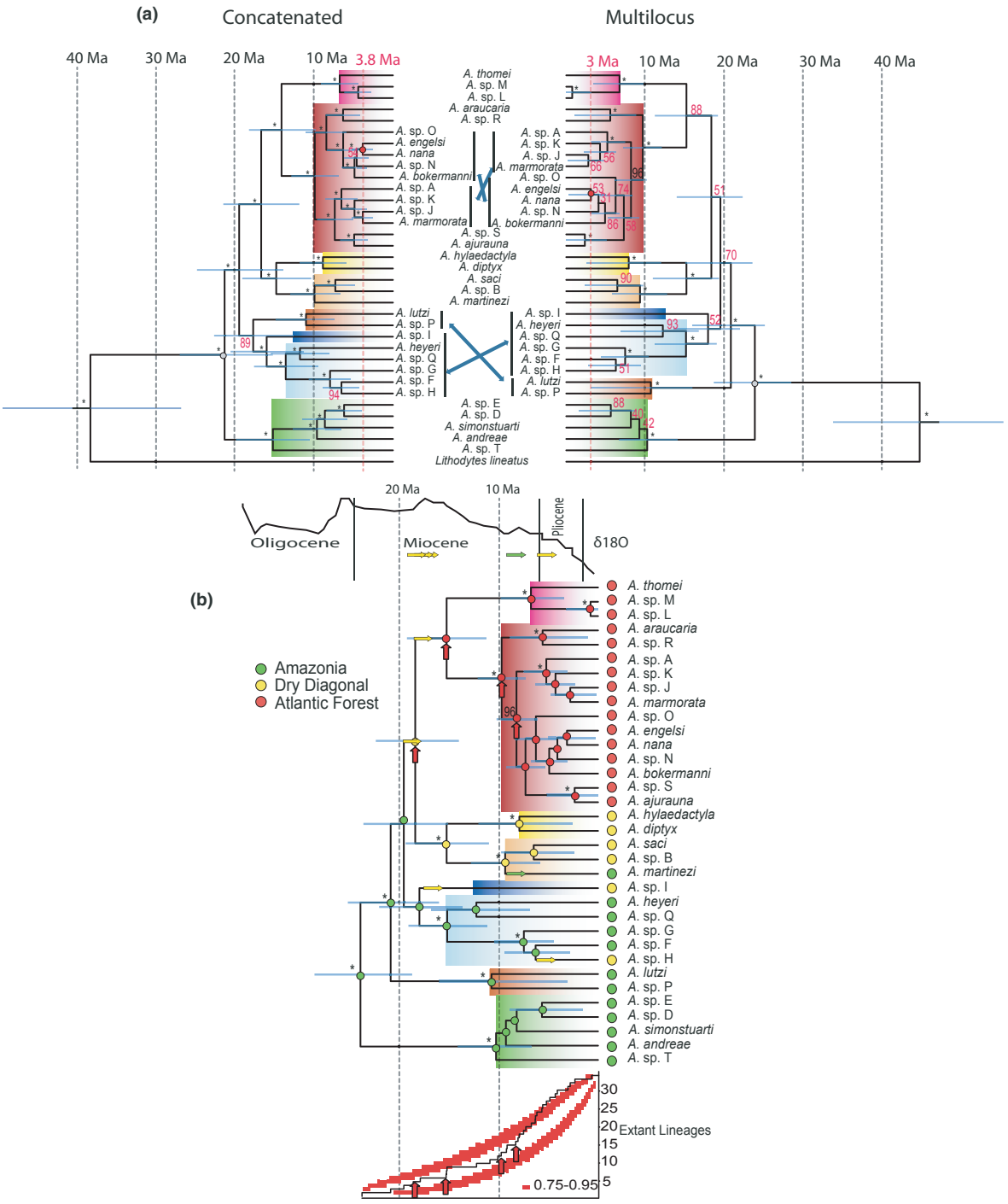

Figure 4 (a) Bayesian time-calibrated, maximum clade-credibility tree of *Adenomera* from 6599 bp using a concatenated partitioned dataset and a multilocus species tree (*BEAST). One internal calibration point is indicated by a yellow circle and the node of the pair of nominal species is indicated with a red circle. Posterior probabilities (× 100) are indicated near the nodes and are in red when < 0.95. 95% credibility intervals are indicated with blue bars. (b) Biogeographical analysis using the multilocus species tree showing only the most likely states at each node. Dispersal–vicariance events across biomes are indicated with arrows. The $\delta^{18}O$% variation across the Neogene, adapted from Zachos *et al.* (2001), is indicated on the top. A lineages-through-time (LTT) plot is illustrated at the bottom, with the 0.75–0.95 confidence interval indicated in red. Detected shifts in diversification trends are indicated with red arrows for increases in both tree and LTT plot.

A. sp. G which included divergences > 8%) in the strict application of this threshold.

Phased nDNA sequence networks provide information about potential reproductive isolation among species

(Fig. 3). Cohesion of *RAG1* networks without any allele sharing was overall encountered with only a few exceptions: between *A.* sp. S and *A. ajurauna*; *A.* sp. M and *A.* sp. L; and *A. hylaedactyla* and *A. diptyx*. For the first two pairs, geographical proximity among the individuals displaying allele sharing may indicate past or current hybridization. For *A. hylaedactyla* and *A. diptyx,* the pattern is complex (Fig. 3c) as the haplotypes attributed to *A. diptyx* clustered in five different positions embedded within *A. hylaedactyla*.

Examining bioacoustic, reproductive and morphological data as well as geographical distributions, most candidate species were supported and were therefore considered as CCS (details in Appendix S2 and summarized in Table 1). The distinction between *A. hylaedactyla* and *A. diptyx* is maintained given published evidence of distinct reproductive modes (de la Riva, 1995) but is, however, blurred by evidence of introgression between them and the probable existence of several species within each of these two candidate species. The existence of additional specific subdivisions is probable in six other candidate species (Appendix S2, Table 1), therefore highlighting the over-conservativeness of our species delineation.

Eventually, only four species pairs remained of ambiguous status. We recognized the four species with less data within each pair as UCS; 31 as CCS; and *A. coca* as UCS.

Molecular dating

The resulting trees from CM (concatenated) and MM (multiloci) were similar in topology and time estimates, even though the support values and the ages were generally lower for MM (Fig. 4). From MM, most of the defined species groups originated around 10 Ma, with the notable exceptions of *A. heyeri* (15 Ma, 95% CI: 11–19 Ma) and *A. thomei* clades (7 Ma, 95% CI: 3–11 Ma). The minimum time of divergence between the CCS pair is estimated between 3.8 Ma (95% CI: 2.5–5.2 Ma) (CM) and 3 Ma (95% CI: 0.3–5 Ma) (MM). Some discordances between CM and MM are noteworthy: (1) the estimates of the crown age of the *A. andreae* clade are markedly different (15 Ma, 95% CI: 11–20 Ma with CM vs. 10 Ma, 95% CI: 7–14 Ma with MM); (2) the position of the southern AF clade (*A. araucaria* + *A.* sp. R) is strongly sustained as the sister group to a south-central AF clade with CM but as the sister group to the rest of the *marmorata* clade with MM; and (3) one split is substantially younger in MM, i.e. between *A.* sp. L and M (0.8 Ma, 95% CI: 0–3.2 Ma) than with CM (4.4 Ma, 95% CI: 2.8–6.2 Ma).

Biogeography

The ancestral area reconstruction unambiguously supports an Amazonian origin of *Adenomera*, with three independent dispersal–vicariance events from Amazonia to the DD, one secondarily back to Amazonia, and one from Amazonia (or the DD) to the AF. These dispersals are estimated to have occurred recurrently between 20 and 7 Ma (Fig. 4). The cases of *A.* sp. I and *A.* sp. H are particularly interesting given that each one is a single species that has colonized the Cerrado from Amazonia at very different periods of time (18 vs. 7 Ma).

Two other events led to the emergence of a clade of open habitat dwellers (*A. hylaedactyla* and *A. martinezi* clades) and the AF clades (*A. thomei* and *A. marmorata* clades). Intriguingly, the emergence of the two major AF clades is estimated about 15 Ma (95% CI: 11–19 Ma from MM), but the bulk of its diversity appeared only in the last 10 Myr within the *A. marmorata* clade. The divergence between *A. martinezi*, which occurs in an isolated fragment of open vegetation within Amazonia, and its sister clade in the Cerrado, formed by *A. saci* and *A.* sp. B, is estimated to have occurred approximately 10 Ma (9.4 Ma, 95% CI: 6–13 Ma).

Table 2 Summary tables of the diversification analyses of the genus *Adenomera*. (a) Comparison of three models of diversification: a constant rate model (all lineages with equal diversification rate); a flexible-rate model (at least one lineage has significantly higher or lower diversification rate); and a rate decrease model (at least one lineage exhibits a clade-specific decrease from the ancestral diversification rate). (b) Congruence of models of diversification for the flexible rate model using the lowest extinction fraction and the relative cladogenesis (rc) test.

Model (a)	Constant rate	Flexible rate	Rate decrease
$a = 0.99$ ln$L$ (ΔAIC)	− 137.52 (0)	− 135.65 (0.26)	− 139.56 (7.83)
Parameters	$r = 0.003$	$r = 0.002$; rcl = 0.00	$r = 0.002$; rcl = 0.002
$a = 0$ ln$L$(ΔAIC)	− 131.802 (2.72)	− 128.44 (0)	− 133.703 (10.52)
Parameters	$r = 0.162$	$r = 0.126$; rcl = 0.245	$r = 0.126$; rcl = 0.120

Node (b)	Log$L$	r.1	r.2	P(LRT test)	P(rc test)
A. marmorata clade	-128.70	0.133	0.261	0.013	0.022
nested clade	-128.677	0.137	0.281	0.012	0.015

rcl, net diversification rate of the subtree partition (rate clade specific); *P*, probability; AIC, Akaike's information criterion; log*L*, log likelihood; LRT, likelihood ratio test.

Diversification

The constant-rate model is rejected in favour of the variable rate model when considering no extinction ($^*P < 0.05$ with $a = 0$), and is near rejection when considering a high extinction rate ($P = 0.06$ with $a = 0.99$). Additionally, the chronogram favoured the variable-rate model with diversification rate change in one or more lineage (Table 2) over an alternative of retained elevated ancestral diversification rate. Increases in diversification rates are detected in the AF and the *A. marmorata* clades. Using the relative cladogenesis test, increases of diversification were detected for the *A. marmorata* clade ($^*P < 0.05$).

DISCUSSION

Filling the species boundaries and species distributions gaps

Even a conservative estimate demonstrates how far from accurate our perception of species boundaries can be, mirroring or exceeding previous DNA-based attempts to evaluate actual species richness in tropical amphibians (Fouquet *et al.*, 2007; Vieites *et al.*, 2009; Jansen *et al.*, 2011; Funk *et al.*, 2012). Costello *et al.* (2012) predicted that approximately 30% more species remain to be described in terrestrial and marine ecosystems. Our estimation for *Adenomera* is far above that proportion (94% of additional species) and remains over-conservative in many cases. How many widespread Neotropical anurans also harbour such a number of undescribed species including sometimes localized endemics? Uncovering this diversity and its distribution might drastically change inferences made from currently perceived distribution patterns such as the estimation of species turnover (Condit *et al.*, 2002), the evaluation of exposure to climate change (Foden *et al.*, 2013) and conservation priorities in general (Jenkins *et al.*, in press). We acknowledge the necessity of describing the species uncovered here, given that naming species is necessary for species conservation (Angulo & Icochea, 2010). However, the level of diversity within *Adenomera* uncovered in this study clearly demonstrates that such an enterprise is beyond the scope of the current study. Nevertheless, this study leaves the door wide open for integrative taxonomic revisions of *Adenomera* (Dayrat, 2005; Padial *et al.*, 2010).

Our results profoundly alter our perception of the spatial distribution of *Adenomera* diversity. All 35 candidate species (except *A. hylaedactyla*) are circumscribed to one of the South American bioclimatic domains (Fig. 2). This demonstrates the existence, despite remarkable habitat shifts (see below), of a general strong habitat conservatism in the group. In terms of local diversity, we detected in south-western Amazonia and between Amazonia and the Cerrado a maximum of five species, but only two to three species generally co-occur. Overall, such alpha diversity is strikingly low compared to other frog genera such as *Leptodactylus*, *Hypsiboas* or *Dendropsophus*, which can reach more than 10 congeneric species in a single location. This may be due to the low dispersal ability, terrestrial habitat, and the mode of reproduction of *Adenomera*, which prevent trophic specialization for both adults and larvae and mirror the conserved morphology in the group.

Historical biogeography

Adenomera displays a striking pattern of historical diversification tied to major biotic modifications of the South American landscape during the Miocene, which can be divided into three categories: (1) from Amazonia towards the DD; (2) between Amazonia and the AF; and (3) within each major biome (Amazonia, DD and AF).

1. 'Out of Amazonia' dispersals towards the DD and associated habitat shifts have occurred during the middle and late Miocene. Despite such a long history, the DD seems to have been colonized only on its western half. This part of the Cerrado has been documented to harbour a strong Amazonian influence (Valdujo *et al.*, 2012). Moreover, *Adenomera* is also absent from the much drier Caatingas, except in a few relictual humid forests in coastal highlands. Such geographical restriction highlights the strong environmental heterogeneity of the DD, which represents a barrier even for species endemic to these biomes (Silva *et al.*, 2006; Diniz-Filho *et al.*, 2008; Valdujo *et al.*, 2012, 2013) and a remarkable historical component that seems to be influenced by the low dispersal ability, habitat conservatism (Valdujo *et al.*, 2013) and high ecophysiological constraints of anurans (Hillman *et al.*, 2009). Interestingly, two of these dispersals correspond to single speciation events (*A.* sp. H and *A.* sp. I), but one led to a diversified species group (*A. hylaedactyla* + *A. martinezi* clades). In fact, the habitats of the two former species correspond to the small patches of dry and gallery forests peppered through the Cerrado while the species of the *A. hylaedactyla* and *A. martinezi* clades are well adapted to grassland. Such an observation indicates that diversification trends subsequent to habitat shifts are probably linked with key eco-physiological adaptations.

The Middle Miocene Climatic Optimum (MMCO) (17–15 Ma) was a warm and wet period preceded and followed by periods of stronger seasonality associated with dry adapted ectotherm range expansions and extinction events (Böhme, 2003). Such environmental changes have probably been accompanied by forest expansion and contraction that may have isolated forest-adapted species within the DD, therefore favouring adaptation to new environmental conditions. Conversely, the open habitat adapted species of the *A. martinezi* clade were probably subsequently fragmented approximately 10 Ma, leading to the isolation of *A. martinezi* within Amazonia. This may be a compelling testimony of expansion/opening of Amazonia and of ancient stability of patches of open vegetation within this biome. Several open habitat species of other taxa are endemic to Serra do Cachimbo (plants: Zappi *et al.*, 2011; Squamata: Rodrigues, 1987; Anura: Faivovich *et al.*, 2005).

2. The emergence of the AF clade was concomitant with that of the DD clade. The divergence time between the AF clade and its closest relative could correspond to an ancient continuity between Amazonia and the AF during the warm and wet MMCO or dispersal through a supposedly already existing DD. Very little evidence is available to dissect these hypotheses, but Batalha-Filho *et al.* (2013) hypothesized a southern pathway between these biomes during the middle Miocene (Por, 1992; Costa, 2003). Widespread vegetation opening is documented subsequent to the MMCO (Flower & Kennett, 1994) with drastic climatic changes linked to major uplift of the Andes (Hoorn *et al.*, 2010) and sea-current modifications (Herold *et al.*, 2009; Le Roux, 2011). Moreover, this time window matches the periods of major diversification found in other groups isolated in Amazonia and AF such as

Adelophryne (Fouquet *et al.*, 2012a) and *Leposoma* (Pellegrino *et al.*, 2011), as well as birds (Batalha-Filho *et al.*, 2013). Ancestral state reconstruction suggests the AF clade dispersed from an open habitat ancestor, implying a reversion in ecological traits from open habitat back to forested habitat. Such a hypothesis implies a reversion in ecological traits from open habitat back to forested habitat. Given the general habitat conservatism in the group, the fact that only the 'Amazonian' part of the Cerrado is currently inhabited by *Adenomera* and that *A. hylaedactyla* colonized the AF domain via a northern route only recently (see below), we find this hypothesis less likely than a direct dispersal from Amazonia.

Interestingly, no other dispersal events between Amazonia and the AF occurred in *Adenomera*, which also mirrors the situation found in *Adelophryne* (Fouquet *et al.*, 2012a), *Allobates* (Santos *et al.*, 2009), *Dendrophryniscus/Amazophrynella* (Fouquet *et al.*, 2012b) and *Leposoma* (Pellegrino *et al.*, 2011) and strengthens the idea that only a little connectivity has subsequently occurred between these biomes, again highlighting the strong heterogeneity of the Cerrado (Werneck *et al.*, 2012; Valdujo *et al.*, 2013). Nevertheless, an exception is exemplified by *Adenomera hylaedactyla*, an extraordinarily widespread lineage. We hypothesized that this species only recently colonized the AF via a northern route. Such a route during Plio-Pleistocene is supported by biogeographical analysis of different groups, as well as by climatic and floristic evidence (Costa, 2003; Wang *et al.*, 2004; Melo Santos *et al.*, 2007; Cabanne *et al.*, 2008; Batalha-Filho *et al.*, 2013).

3. Most *Adenomera* diversification occurred subsequently to these dispersals within each biome, mostly during the last 10 Myr. In Amazonia, this coincides with a peak of diversity of plants, according to the pollen record, as well as with early diversification of some mammals, frogs and birds (Hoorn *et al.*, 2010). The biogeographical patterns and diversification trends are strikingly different across Amazonian *Adenomera* species groups (*A. andreae*, *A. heyeri*, *A. lutzi*). The diversification of the *A. andreae* clade seems to have taken place originally in western Amazonia some 10 Ma but with a subsequent burst of diversification, particularly within the widely distributed *A. andreae*. This is consistent with the establishment of the Acre system (11–7 Ma) and the subsequent origination of the Amazon drainage (Santos *et al.*, 2009; Hoorn *et al.*, 2010), isolating populations along the Andes, and both the Guiana and the Brazilian shields. Fouquet *et al.* (2012c) demonstrated that rivers constitute major barriers for *A. andreae* dispersal and this is likely to be the case for most *Adenomera* species. In the *A. heyeri* clade, two subclades occur on each sides of the Amazon River, corroborating that diversification is also likely to be linked to the origination of this drainage.

Within open biomes, diversification of *Adenomera* seems to have been reduced compared with that of the forest-dwelling groups, representing only 20% of the 35 delimited species and despite three independent origins, including two clades, being *c.* 18 Myr old and the large size of the area they occupy. Nevertheless, diversification *in situ* did occur between the Cerrado and the Chaco and within the northern part of the Cerrado. These speciation events occurred during the late Miocene, concomitantly with the dispersal of *A.* sp. H *c.* 7 Ma, a period with drier climate and spreading grasslands (Cerling *et al.*, 1997; MacFadden, 1997).

The diversification within the AF accounts for almost half of the species of the genus. This diversification is particularly striking given its more recent origin and given that it took place in a much smaller area than for the Amazonian or the DD groups. This may be related to sharper elevational and latitudinal gradients of the AF (Cadena *et al.*, 2011) than the two other regions, a hypothesis already formulated to explain the higher morphological diversity in *Dendrophryniscus* versus *Amazophrynella* (Fouquet *et al.*, 2012b). Nevertheless, our species delineation remained over-conservative, particularly in Amazonia, and incomplete sampling is supported by the final decrease seen on the lineages-through-time (LTT) plot (Fig. 4). Within the AF clade, we recovered four main discontinuities/contact zones all found in other AF vertebrates (de Mello Martins, 2011; Marques Silva *et al.*, 2012). The oldest one separates the *A. thomei* clade from the *A. marmorata* clade *c.* 15 Ma. The contact zone between these two clades is extensive but delimits the coastal mountains from the plateau and a division in northern Rio de Janeiro. Interestingly, this break matches the distribution of semi-deciduous forest (Carnaval *et al.*, 2009) and is also found concomitant in *Adelophryne* (Fouquet *et al.*, 2012a) and in *Gymnodactylus* (Pellegrino *et al.*, 2005). Other major breaks are found in Santa Catharina and in the south of São Paulo states between *c.* 10 and 8 Ma. Similar and concomitant genetic breaks were also found, for instance, in pitvipers (Grazziotin *et al.*, 2006) and toads (Thomé *et al.*, 2010; Amaro *et al.*, 2012). The causes of these genetic breaks are largely debated with non-mutually exclusive hypotheses that may explain these patterns (de Mello Martins, 2011). The overall congruence with the proposed AF Pleistocene refugia (see Carnaval & Moritz, 2008) is, however, striking and indicates that these genetic breaks could have resulted from past climatic oscillations.

CONCLUSIONS

This study provides evidence of a large species richness underestimation in the genus *Adenomera* with at least 94% more species than currently recognized and a better resolution in the estimation of the diversity and its geographical distribution in the group. The discovery of this pattern indicates that the DD played a major role in the history of the genus in limiting dispersal and favouring diversification of open-habitat adapted lineages. Moreover, a forest bridge between Amazonia and the Atlantic Forest during the Miocene Climatic Optimum is likely to have led to dispersal from Amazonia towards the AF, a feature strikingly mirrored by other taxa. This work also highlights that in taxonomically poorly defined groups, such as *Adenomera*, defining species boundaries and distributions is a necessary step

before diversification processes can be investigated. Crucial data are still lacking for a comprehensive taxonomic revision of such a diverse group as *Adenomera,* but the present work will undoubtedly facilitate such an enterprise.

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REFERENCES

Akaike, H. (1974) A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, **19**, 716–723.

Almeida, A.D.P. & Angulo, A. (2006) A new species of *Leptodactylus* (Anura: Leptodactylidae) from the state of Esprito Santo, Brazil, with remarks on the systematics of associated populations. *Zootaxa*, **1334**, 1–25.

Amaro, R.C., Carnaval, A.C., Yonenaga-Yassuda, Y. & Rodrigues, M.T. (2012) Demographic processes in the montane Atlantic rainforest: molecular and cytogenetic evidence from the endemic frog *Proceratophrys boiei*. *Molecular Phylogenetics and Evolution*, **62**, 880–888.

Angulo, A. & Icochea, J. (2010) Cryptic species complexes, widespread species and conservation: lessons from Amazonian frogs of the *Leptodactylus marmoratus* group (Anura: Leptodactylidae). *Systematics and Biodiversity*, **8**, 357–370.

Angulo, A., Cocroft, R.B. & Reichle, S. (2003) Species identity in the genus *Adenomera* (Anura: Leptodactylidae) in southeastern Peru. *Herpetologica*, **59**, 490–504.

Angulo, A. & Reichle, S. (2008) Acoustic signals, species diagnosis and species concepts: the case of a new cryptic species of *Leptodactylus* (Amphibia, Anura, Leptodactylidae) from the Chapare region, Bolivia. *Zoological Journal of the Linnean Society*, **152**, 59–77.

Batalha-Filho, H., Fjeldså, J., Fabre, P.-H. & Miyaki, C.Y. (2013) Connections between the Atlantic and the Amazonian forest avifaunas represent distinct historical events. *Journal of Ornithology*, **154**, 41–50.

Bates, H.W. (1863) *The naturalist on the River Amazons, a record of adventures, habits of animals, sketches of Brazilian and Indian life and aspects of nature under the Equator during eleven years of travel*. John Murray, London.

Berneck, B.V.M., Costa, C.O.R. & Garcia, P.C.A. (2008) A new species of *Leptodactylus* (Anura: Leptodactylidae) from the Atlantic Forest of São Paulo State, Brazil. *Zootaxa*, **1795**, 46–56.

Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K.L., Meier, R., Winker, K., Ingram, K.K. & Das, I. (2007) Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution*, **22**, 148–155.

Bini, L.M., Diniz, J.A.F., Rangel, T., Bastos, R.P. & Pinto, M.P. (2006) Challenging Wallacean and Linnean shortfalls: knowledge gradients and conservation planning in a biodiversity hotspot. *Diversity and Distributions*, **12**, 475–482.

Böhme, M. (2003) The Miocene Climatic Optimum: evidence from ectothermic vertebrates of Central Europe. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **195**, 389–401.

Boistel, R., de Massary, J.-C. & Angulo, A. (2006) Description of a new species of the genus *Adenomera* (Amphibia, Anura, Leptodactylidae) from French Guiana. *Acta Herpetologica*, **1**, 1–14.

Cabanne, G.S., d'Horta, F.M., Sari, E.H., Santos, F.R. & Miyaki, C.Y. (2008) Nuclear and mitochondrial phylogeography of the Atlantic forest endemic *Xiphorhynchus fuscus* (Aves: Dendrocolaptidae): biogeography and systematics implications. *Molecular Phylogenetics and Evolution*, **49**, 760–773.

Cadena, C.D., Kozak, K.H., Gómez, J.P., Parra, J.L., McCain, C., Bowie, R.B., Carnaval, A.C., Moritz, C., Rahbek, C., Roberts, T.E., Sanders, N., Schneider, C.J., VanDerWal, J., Zamudio, K. & Graham, C.H. (2011) Latitude, elevational climatic zonation, and speciation in New World vertebrates. *Proceedings of the Royal Society of London B: Biological Sciences*, **279**, 194–201.

Carnaval, A.C. & Moritz, C. (2008) Historical climate modelling predicts patterns of current biodiversity in the Brazilian Atlantic forest. *Journal of Biogeography*, **35**, 1187–1201.

Carnaval, A.C., Hickerson, M.J., Haddad, C.F.B., Rodrigues, M.T. & Moritz, C. (2009) Stability predicts genetic diversity in the Brazilian Atlantic forest hotspot. *Science*, **323**, 785–789.

de Carvalho, T.R. & Giaretta, A.A. (2013) Taxonomic circumscription of *Adenomera martinezi* (Bokermann, 1956) (Anura: Leptodactylidae: Leptodactylinae) with the recognition of a new cryptic taxon through a bioacoustic approach. *Zootaxa*, **3701**, 207–237.

Cerling, T.E., Harris, J.M., MacFadden, B.J., Leakey, M.G., Quade, J., Eisenmann, V. & Ehleringer, J.R. (1997) Global vegetation change through the Miocene/Pliocene boundary. *Nature*, **389**, 153–158.

Clement, M., Posada, D. & Crandall, K.A. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1660.

Condit, R., Pitman, N., Leigh, E.G., Chave, J., Terborgh, J., Foster, R.B., Núnez, P., Aguilar, S., Valencia, R. & Villa, G. (2002) Beta-diversity in tropical forest trees. *Science*, **295**, 666–669.

Costa, L.P. (2003) The historical bridge between the Amazon and the Atlantic Forest of Brazil: a study of molecular phylogeography with small mammals. *Journal of Biogeography*, **30**, 71–86.

Costello, M.J., Wilson, S. & Houlding, B. (2012) Predicting total global species richness using rates of species description and estimates of taxonomic effort. *Systematic Biology*, **61**, 871–883.

Dayrat, B. (2005) Towards integrative taxonomy. *Biological Journal of the Linnean Society*, **85**, 407–415.

Diniz-Filho, J.A.F., Bini, L.M., Vieira, C.M., Blamires, D., Terribile, L., Bastos, R., de Oliveira, G. & Barreto, B. (2008) Spatial patterns of terrestrial vertebrate species richness in the Brazilian Cerrado. *Zoological Studies*, **47**, 146–157.

Drummond, A.J. & Rambaut, A. (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology*, **7**, 214.

Duellman, W.E. (2005) *Cusco Amazónico: the lives of amphibians and reptiles in an Amazonian rainforest*. Comstock Publishing Associates, Cornell University Press, Ithaca, NY.

Faivovich, J., Haddad, C.F.B., Garcia, P.C.A., Frost, D.R., Campbell, J.A. & Wheeler, W.C. (2005) Systematic review of the frog family Hylidae, with special reference to Hylinae: phylogenetic analysis and taxonomic revision. *Bulletin of the American Museum of Natural History*, **294**, 6–228.

Flower, B.P. & Kennett, J.P. (1994) The middle Miocene climatic transition: East Antarctic ice sheet development, deep ocean circulation and global carbon cycling. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **108**, 537–555.

Foden, W.B., Butchart, S.H.M., Stuart, S.N., Vié, J.-C., Akçakaya, H.R., Angulo, A., DeVantier, L.M., Gutsche, A., Turak, E. & Cao, L. (2013) Identifying the world's most climate change vulnerable species: a systematic trait-based assessment of all birds, amphibians and corals. *PLoS ONE*, **8**, e65427.

Fouquet, A., Gilles, A., Vences, M., Marty, C., Blanc, M. & Gemmell, N.J. (2007) Underestimation of species richness in Neotropical frogs revealed by mtDNA analyses. *PLoS ONE*, **2**, e1109.

Fouquet, A., Loebmann, D., Castroviejo-Fisher, S., Padial, J.M., Dill Orrico, V.G., Lyra, M., Joventino, I., Kok, P., Haddad, C.F.B. & Rodrigues, M.T. (2012a) From Amazonia to the Atlantic Forest: molecular phylogeny of Phyzelaphryninae frogs reveals unexpected diversity and a striking biogeographic pattern that stress out conservation status. *Molecular Phylogenetics and Evolution*, **65**, 547–556.

Fouquet, A., Recoder, R., Teixeira, M., Cassimiro, J., Amaro, R.C., Camacho, A., Damasceno, R., Carnaval, A.C., Moritz, C. & Rodrigues, M.T. (2012b) Molecular phylogeny and morphometric analyses reveal deep divergence between Amazonia and Atlantic Forest species of *Dendrophryniscus*. *Molecular Phylogenetics and Evolution*, **62**, 826–838.

Fouquet, A., Ledoux, J.-B., Dubut, V., Noonan, B.P. & Scotti, I. (2012c) The interplay of dispersal limitation, rivers and historical events shapes the genetic structure of an Amazonian frog. *Biological Journal of the Linnean Society*, **106**, 356–373.

Fouquet, A., Blotto, B.L., Maronna, M.M., Verdade, V.K., Juncá, F.A., de Sá, R. & Rodrigues, M.T. (2013) Unexpected phylogenetic positions of the genera *Rupirana* and *Crossodactylodes* reveal insights into the biogeography and reproductive evolution of leptodactylid frogs. *Molecular Phylogenetics and Evolution*, **67**, 445–457.

Frost, D.R. (2013) *Amphibian species of the world: an online reference*. Version 5.6. American Museum of Natural History, New York. Available at: http://research.amnh.org/herpetology/amphibia/index.html.

Funk, W.C., Caminer, M. & Ron, S.R. (2012) High levels of cryptic species diversity uncovered in Amazonian frogs. *Proceedings of the Royal Society of B: Biological Sciences*, **279**, 1806–1814.

Grazziotin, F.G., Monzel, M., Echeverrigaray, S. & Bonatto, S.L. (2006) Phylogeography of the *Bothrops jararaca* complex (Serpentes: Viperidae): past fragmentation and island colonization in the Brazilian Atlantic Forest. *Molecular Ecology*, **15**, 3969–3982.

Guindon, S. & Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, **52**, 696–704.

Hamilton, A.J., Novotny, V., Waters, E.K., Basset, Y., Benke, K.K., Grimbacher, P.S., Miller, S.E., Samuelson, G.A., Weiblen, G.D., Yen, J.D.L. & Stork, N.E. (2012) Estimating global arthropod species richness: refining probabilistic models using probability bounds analysis. *Oecologia*, **171**, 357–365.

Harmon, L.J., Weir, J., Brock, C., Glor, R.E. & Challenger, W. (2008) GEIGER: investigating evolutionary radiations. *Bioinformatics*, **24**, 129–131.

Heled, J. & Drummond, A.J. (2010) Bayesian inference of species trees from multilocus data. *Molecular Biology and Evolution*, **27**, 570–580.

Herold, N., You, Y., Muller, R.D. & Seton, M. (2009) Climate model sensitivity to changes in Miocene paleotopography. *Australian Journal of Earth Sciences*, **57**, 377–379.

Heyer, W.R. (1973) Systematics of the *marmoratus* group of the frog genus *Leptodactylus* (Amphibia, Leptodactylidae). *Contributions in Science, Natural History Museum, Los Angeles County*, **251**, 1–50.

Heyer, W.R. (1975) *Adenomera lutzi* (Amphibia: Leptodactylidae), a new species of frog from Guyana. *Proceedings of the Biological Society of Washington*, **88**, 315–318.

Hillman, S.S., Withers, P.C., Drewes, R.C. & Hillyard, S.D. (2009) *Ecological and environmental physiology of amphibians*. Oxford University Press, New York.

Hoorn, C., Wesselingh, F.P., ter Steege, H., Bermudez, M.A., Mora, A., Sevink, J., Sanmartín, I., Sanchez-Meseguer, A.,

Anderson, C.L., Figueiredo, J.P., Jaramillo, C., Riff, D., Negri, F.R., Hooghiemstra, H., Lundberg, J., Stadler, T., Särkinen, T. & Antonelli, A. (2010) Amazonia through time: Andean uplift, climate change, landscape evolution, and biodiversity. *Science*, **330**, 927–931.

Jansen, M., Bloch, R., Schulze, A. & Pfenninger, M. (2011) Integrative inventory of Bolivia's lowland amphibians reveals hidden diversity. *Zoologica Scripta*, **40**, 567–583.

Jenkins, C.N., Pimm, S.L. & Joppa, L.N. (in press) Global patterns of terrestrial vertebrate diversity and conservation. *Proceedings of the National Academy of Sciences USA*.

Katoh, K., Misawa, K., Kuma, K. & Miyata, T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, **30**, 3059–3066.

Kok, P.J.R., Kokubum, M.N.C., MacCulloch, R.D. & Lathrop, A. (2007) Morphological variation in *Leptodactylus lutzi* (Heyer, 1975) (Anura: Leptodactylidae) with description of its advertisement call and notes on its courtship behavior. *Phyllomedusa*, **6**, 45–60.

Kwet, A. (2006) Bioacoustics in the genus *Adenomera* (Anura: Leptodactylidae) from Santa Catarina, southern Brazil. *Herpetologia Bonnensis II. Proceedings of the 13th Congress of the Societas Europaea Herpetologica September–2.October 2005* (ed. by M. Vences, J. Köhler, T. Ziegler and W. Böhme), pp. 77–80. Bonn, Germany.

Kwet, A. (2007) Bioacoustic variation in the genus *Adenomera* in southern Brazil, with revalidation of *Leptodactylus nanus* Muller, 1922 (Anura: Leptodactylidae). *Mitteilungenausdem Museum fur Naturkunde in Berlin, Zool. Reihe*, **83**, 56–68.

Kwet, A., Steiner, J. & Zillikens, A. (2009) A new species of *Adenomera* (Amphibia: Anura: Leptodactylidae) from the Atlantic rain forest in Santa Catarina, southern Brazil. *Studies on Neotropical Fauna and Environment*, **44**, 93–107.

Lanfear, R., Calcott, B., Ho, S.Y.W. & Guindon, S. (2012) PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, **29**, 1695–1701.

Le Roux, J.P. (2011) A review of Tertiary climate changes in southern South America and the Antarctic Peninsula. Part 1: Oceanic conditions. *Sedimentary Geology*, **247–248**, 1–20.

Librado, P. & Rozas, J. (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, **25**, 1451–1452.

MacFadden, B.J. (1997) Origin and evolution of grazing guild in New World terrestrial mammals. *Trends in Ecology and Evolution*, **12**, 182–187.

Marques Silva, S., Moraes-Barros, N., Ribas, C.C., Ferrand, N. & Morgante, J.S. (2012) Divide to conquer: a complex pattern of biodiversity depicted by vertebrate components in the Brazilian Atlantic Forest. *Biological Journal of the Linnean Society*, **107**, 39–55.

de Mello Martins, F. (2011) Historical biogeography of the Brazilian Atlantic forest and the Carnaval–Moritz model of Pleistocene refugia: what do phylogeographical studies tell us? *Biological Journal of the Linnean Society*, **104**, 499–509.

Melo Santos, A.M.M., Cavalcanti, D.R., da Silva, J.M.C. & Tabarelli, M. (2007) Biogeographical relationships among tropical forests in north-eastern Brazil. *Journal of Biogeography*, **34**, 437–446.

Menin, M., Waldez, F. & Lima, A.P. (2008) Temporal variation in the abundance and number of species of frogs in 10,000 ha of a forest in central Amazonia, Brazil. *South American Journal of Herpetology*, **3**, 68–81.

Menin, M., de Almeida, A.P. & Kokubum, M.N.C. (2009) Reproductive aspects of *Leptodactytus hylaedactylus* (Anura: Leptodactylidae), a member of the *Leptodactylus marmoratus* species group, with a description of tadpoles and calls. *Journal of Natural History*, **43**, 2257–2270.

Mora, C., Tittensor, D.P., Adl, S., Simpson, A.G.B. & Worm, B. (2011) How many species are there on earth and in the ocean? *PLoS Biology*, **9**, e1001127.

Padial, J.M., Miralles, A., de la Riva, I. & Vences, M. (2010) The integrative future of taxonomy. *Frontiers in Zoology*, **7**, 16.

Pellegrino, K.C.M., Rodrigues, M.T., Waite, A.N., Morando, M., Yassuda, Y.Y. & Sites, J.W., Jr (2005) Phylogeography and species limits in the *Gymnodactylus darwinii* complex (Gekkonidae, Squamata): genetic structure coincides with river systems in the Brazilian Atlantic Forest. *Biological Journal of the Linnean Society*, **85**, 13–26.

Pellegrino, K.C.M., Rodrigues, M.T., James Harris, D., Yassuda, Y.Y. & Sites, J.W., Jr (2011) Molecular phylogeny, biogeography and insights into the origin of parthenogenesis in the Neotropical genus *Leposoma* (Squamata: Gymnophthalmidae): ancient links between the Atlantic Forest and Amazonia. *Molecular Phylogenetics and Evolution*, **61**, 446–459.

Por, F.D. (1992) *Sooretama: the Atlantic rain forest of Brazil*. SPB Academic Publishing, The Hague.

Posada, D. (2008) jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution*, **25**, 1253–1256.

Prado, D.E. & Gibbs, P.E. (1993) Patterns of species distributions in the dry seasonal forest South America. *Annals of the Missouri Botanic Garden*, **80**, 902–927.

Pyron, R.A. & Wiens, J.J. (2011) A large-scale phylogeny of Amphibia with over 2,800 species, and a revised classification of extant frogs, salamanders, and caecilians. *Molecular Phylogenetics and Evolution*, **61**, 543–583.

Quince, C., Curtis, T.P. & Sloan, W.T. (2008) The rational exploration of microbial diversity. *The ISME Journal*, **2**, 997–1006.

R Development Core Team (2011) *R: a language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.R-project.org.

Rabosky, D.L. (2006) LASER: a maximum likelihood toolkit for detecting temporal shifts in diversification rates from molecular phylogenies. *Evol Bioinfo Online*, **2**, 257–260.

Rabosky, D.L., Donnellan, S.C., Talaba, A.L. & Lovette, I.J. (2007) Exceptional among-lineage variation in diversification rates during the radiation of Australia's most diverse

vertebrate clade. *Proceedings of the Royal Society of B: Biological Sciences*, **274**, 2915–2923.

Rambaut, A. & Drummond, A.J. (2007) *Tracer. Version 1.5*. Available at: http://tree.bio.ed.ac.uk/ software/tracer/.

Raup, D.M. (1985) Mathematical models of cladogenesis. *Paleobiology*, **11**, 42–52.

Rheindt, F.E., Christidis, L. & Norman, J.A. (2008) Habitat shifts in the evolutionary history of a Neotropical flycatcher lineage from forest and open landscapes. *BMC Evolutionary Biology*, **8**, 193.

de la Riva, I. (1995) A new reproductive mode for the genus *Adenomera* (Amphibia: Anura: Leptodactylidae): taxonomic implications for certain Bolivian and Paraguayan populations. *Studies on Neotropical Fauna and the Environment*, **30**, 15–29.

Rodrigues, M.T. (1987) Sistemática, ecologia e zoogeografia dos *Tropidurus* do grupo *torquatus* ao sul do rio Amazonas (Sauria, Iguanidae). *Arquivos de Zoologia*, **31**, 105–230.

Sanmartín, I., Mark, P.V.D. & Ronquist, F. (2008) Inferring dispersal: a Bayesian approach to phylogeny-based island biogeography, with special reference to the Canary Islands. *Journal of Biogeography*, **35**, 428–449.

Santos, J.C., Coloma, L.A., Summers, K., Caldwell, J.P., Ree, R. & Cannatella, D.C. (2009) Amazonian amphibian diversity is primarily derived from late Miocene Andean lineages. *PLoS Biology*, **7**, 448–461.

Silva, J.F., Fariñas, M.R., Felfili, J.M. & Klink, C.A. (2006) Spatial heterogeneity, land use and conservation in the cerrado region of Brazil. *Journal of Biogeography*, **33**, 536–548.

Sites, J.W., Jr & Marshall, J.C. (2003) Delimiting species: a renaissance issue in systematic biology. *Trends in Ecology and Evolution*, **18**, 462–470.

Sites, J.W., Jr & Marshall, J.C. (2004) Operational criteria for delimiting species. *Annual Review of Ecology, Evolution, and Systematics*, **35**, 199–227.

Stephens, M. & Donnelly, P. (2003) A comparison of Bayesian methods for haplotype reconstruction. *American Journal of Human Genetics*, **73**, 1162–1169.

Stephens, M., Smith, N. J. & Donnelly, P. (2001) A new statistical method for haplotype reconstruction from population data. *American Journal of Human Genetics*, **68**, 978.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, **28**, 2731–2739.

Thomé, M.T., Zamudio, K.R., Giovanelli, J.G., Haddad, C.F.B., Baldissera, F.A., Jr & Alexandrino, J. (2010) Phylogeography of endemic toads and post-Pliocene persistence of the Brazilian Atlantic Forest. *Molecular Phylogenetics and Evolution*, **55**, 1018–1031.

Valdujo, P.H., Silvano, D.L., Colli, G. & Martins, M. (2012) Anuran species composition and distribution patterns in Brazilian Cerrado, a neotropical hotspot. *South American Journal of Herpetology*, **7**, 63–78.

Valdujo, P.H., Carnaval, A.C. & Graham, C.H. (2013) Environmental correlates of anuran beta diversity in the Brazilian Cerrado. *Ecography*, **36**, 708–717.

Vieites, D.R., Wollenberg, K.C., Andreone, F., Kohler, J., Glaw, F. & Vences, M. (2009) Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. *Proceedings of the National Academy of Sciences USA*, **106**, 8267–8272.

Wallace, A.R. (1852) The monkeys of the Amazon. *Proceedings of the Zoological Society of London*, **20**, 107–110.

Wang, X., Auler, A.S., Edwards, R.L., Cheng, H., Cristalli, P.S., Smart, P.L., Richards, D.A. & Shen, C.-C. (2004) Wet periods in northeastern Brazil over the past 210 kyr linked to distant climate anomalies. *Nature*, **432**, 740–743.

Werneck, F.P., Gamble, T., Colli, G.R., Rodrigues, M.T. & Sites, J.W. (2012) Deep diversification and long-term persistence in the South American "Dry Diagonal": integrating continent-wide phylogeography and distribution modeling of geckos. *Evolution*, **66**, 3014–3034.

Wiens, J.J. & Donoghue, M.J. (2004) Historical biogeography, ecology and species richness. *Trends in Ecology and Evolution*, **19**, 639–644.

Yu, Y., Harris, A.J. & He, X.J. (2011) *RASP (Reconstruct Ancestral State in Phylogenies) 1.1*. Available at: http://mnh.scu.edu.cn/soft/blog/RASP>.

Zachos, J., Pagani, M., Sloan, L., Thomas, E. & Billups, K. (2001) Trends, rhythms, and aberrations in global climate 65 Ma to present. *Science*, **292**, 686–693.

Zappi, D.C., Sasaki, D., Milliken, W., Iva, J., Henicka, G.S., Biggs, N. & Frisby, S. (2011) Plantas vasculares da região do Parque Estadual Cristalino, norte de Mato Grosso, Brasil. *Acta Amazonica*, **41**, 29–38.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Samples, primers and information on models.
Appendix S2 Taxonomic considerations and species delineation explanations.
Appendix S3 Fully expanded subtrees and networks.

BIOSKETCH

Antoine Fouquet's main research interests are the diversity and evolution of Neotropical herpetofauna.

Author contributions: A.F. and C.C. designed the study; A.F., C.C., M.T.R. and C.F.B.H. carried out the fieldwork; A.F. and C.C. conducted the laboratory work; A.F. and N.P. analysed the data, A.F. wrote the first complete version of the manuscript; all authors read and improved the manuscript.

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