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).
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 northeast–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. hylaeactyla*), we obtained tissues of toptotypical 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

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**References**

- Wallace, 1852; Bates, 1863.
- Rheindt et al., 2008; Valdujo et al., 2013.
- Menin et al., 2008.
- Angulo et al., 2003; Duellman, 2005.
- Fouquet et al., 2012a,b.
- Fouquet et al., 2013.
- Pyron & Wiens (2011).
- Fouquet et al. (2013).
- PartionFINDER 1.0 (Lanfear et al., 2012).
- Wallance, 1852; Bates, 1863.
species 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 (Kato 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 MODELTEST 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 $2.0 \times 10^8$ 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 $2.0 \times 10^8$ 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 $5.0 \times 10^6$ 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).

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

<table>
<thead>
<tr>
<th>Clade</th>
<th>mtDNA divergence/relative</th>
<th>Max p-distance within species</th>
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<th>No nDNA allele sharing and network cohesion</th>
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<th>Sampling (n)</th>
<th>Diagnose</th>
<th>Remark</th>
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<tbody>
<tr>
<td>A. sp. Q</td>
<td>&gt; 10%</td>
<td>&lt; 8%</td>
<td>Yes sister to A. heyeri</td>
<td>Yes</td>
<td>No</td>
<td>No bioacoustic data but habitat and morphology</td>
<td>Low (1)</td>
<td>CCS</td>
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<td>&gt; 10%</td>
<td>&lt; 8%</td>
<td>Yes PP = 1</td>
<td>Yes</td>
<td>Yes</td>
<td>No data</td>
<td>Good (17)</td>
<td>CCS</td>
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<td>A. sp. J</td>
<td>8.30%</td>
<td>&lt; 8%</td>
<td>A. sp. J nested</td>
<td>Yes</td>
<td>Yes</td>
<td>Vocalization and morphology</td>
<td>Good (9)</td>
<td>CCS</td>
<td></td>
</tr>
<tr>
<td>A. marmorata</td>
<td>8.30%</td>
<td>&lt; 8%</td>
<td>A. sp. I</td>
<td>Yes</td>
<td>Yes</td>
<td>Vocalization and morphology</td>
<td>Good (13)</td>
<td>CCS</td>
<td></td>
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<tr>
<td>A. sp. S</td>
<td>9.5%</td>
<td>&lt; 8%</td>
<td>Yes PP = 1</td>
<td>Allele sharing but cohesion</td>
<td>Probable</td>
<td>Vocalization</td>
<td>Good (6)</td>
<td>CCS</td>
<td></td>
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<tr>
<td>A. ajurauna</td>
<td>9.5%</td>
<td>&lt; 8%</td>
<td>S nested</td>
<td>Allele sharing but cohesion</td>
<td>Probable</td>
<td>Vocalization (Bemeck et al., 2008)</td>
<td>Good (5)</td>
<td>CCS</td>
<td>Complex – over-conservative</td>
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<tr>
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<td>&gt; 10%</td>
<td>&lt; 8%</td>
<td>2 lineages</td>
<td>Yes</td>
<td>No</td>
<td>No data</td>
<td>Low (2)</td>
<td>CCS</td>
<td></td>
</tr>
<tr>
<td>A. sp. A</td>
<td>&gt; 10%</td>
<td>&lt; 8%</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No data</td>
<td>Low (1)</td>
<td>CCS</td>
<td></td>
</tr>
<tr>
<td>A. sp. O</td>
<td>9.3%</td>
<td>&lt; 8%</td>
<td>Yes</td>
<td>Yes</td>
<td>Probable</td>
<td>No data</td>
<td>Low (1)</td>
<td>CCS</td>
<td></td>
</tr>
<tr>
<td>A. sp. N</td>
<td>9.3%</td>
<td>&lt; 8%</td>
<td>Yes</td>
<td>Yes</td>
<td>Probable</td>
<td>No data</td>
<td>Low (1)</td>
<td>CCS</td>
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<tr>
<td>A. bockermanni</td>
<td>9.0%</td>
<td>&lt; 8%</td>
<td>Yes PP = 1</td>
<td>Yes</td>
<td>Yes</td>
<td>Morphology (Heyer, 1973; Kwet, 2007)</td>
<td>Good (2)</td>
<td>CCS</td>
<td></td>
</tr>
<tr>
<td>A. englesi</td>
<td>7.6%</td>
<td>&lt; 8%</td>
<td>Yes PP = 1</td>
<td>Yes</td>
<td>Yes</td>
<td>Vocalization and morphology (Kwet et al., 2009)</td>
<td>Good (4)</td>
<td>CCS</td>
<td></td>
</tr>
<tr>
<td>A. nana</td>
<td>7.6%</td>
<td>&lt; 8%</td>
<td>2 lineages</td>
<td>Yes</td>
<td>Yes</td>
<td>Vocalization and morphology (Kwet, 2007)</td>
<td>Low (3)</td>
<td>CCS</td>
<td></td>
</tr>
<tr>
<td>A. sp. R</td>
<td>9.7%</td>
<td>&lt; 8%</td>
<td>A. anuusca nested</td>
<td>No allele sharing but no cohesion</td>
<td>Probable</td>
<td>Vocalization (Kwet, 2007)</td>
<td>Low (2)</td>
<td>CCS</td>
<td></td>
</tr>
<tr>
<td>A. araucaria</td>
<td>9.7%</td>
<td>&lt; 8%</td>
<td>A. sp. R nested</td>
<td>No allele sharing but no cohesion</td>
<td>Probable</td>
<td>Vocalization (Kwet, 2007)</td>
<td>Low (3)</td>
<td>CCS</td>
<td></td>
</tr>
<tr>
<td>A. thomei</td>
<td>9.4%</td>
<td>&lt; 8%</td>
<td>Yes PP = 1</td>
<td>Yes</td>
<td>Probable</td>
<td>Vocalization, morphology and mode of reproduction (Almekl &amp; Angulo, 2006)</td>
<td>Good (10)</td>
<td>CCS</td>
<td></td>
</tr>
<tr>
<td>A. sp. M</td>
<td>9.2%</td>
<td>&lt; 8%</td>
<td>A. sp. L nested</td>
<td>No allele sharing but no cohesion</td>
<td>Probable</td>
<td>Vocalization</td>
<td>Low (2)</td>
<td>CCS</td>
<td>May be conspecific with L</td>
</tr>
<tr>
<td>A. sp. L</td>
<td>9.2%</td>
<td>&lt; 8%</td>
<td>A. sp. M nested</td>
<td>No allele sharing but no cohesion</td>
<td>Probable</td>
<td>Vocalization</td>
<td>Low (2)</td>
<td>UCS</td>
<td>May be conspecific with A. hylaedactyla</td>
</tr>
<tr>
<td>A. coca</td>
<td>0</td>
<td>0</td>
<td>Nested in A. hylaedactyla</td>
<td>No</td>
<td>Yes</td>
<td>Vocalization (Angulo &amp; Reichle, 2008)</td>
<td>Low (2)</td>
<td>UCS</td>
<td></td>
</tr>
</tbody>
</table>

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 \((a = 0 \text{ and } a = 0.99)\) 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 \(\times 100\)) 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.

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

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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.
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).

**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).

**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.

<table>
<thead>
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<th>Model (a)</th>
<th>Constant rate</th>
<th>Flexible rate</th>
<th>Rate decrease</th>
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<tr>
<td>a = 0.99 lnL (ΔAIC)</td>
<td>−137.52 (0)</td>
<td>−135.65 (0.26)</td>
<td>−139.56 (7.83)</td>
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<th>r = 0.003</th>
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<tr>
<td>a = 0 lnL(AAIC)</td>
<td>−131.802 (2.72)</td>
<td>−128.44 (0)</td>
<td>−133.703 (10.52)</td>
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</table>

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<th>rcl = 0.120</th>
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</thead>
</table>

<table>
<thead>
<tr>
<th>Node</th>
<th>LogL</th>
<th>r1</th>
<th>r2</th>
<th>P(LRT test)</th>
<th>P(rc test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. marmorata clade</td>
<td>-128.70</td>
<td>0.133</td>
<td>0.261</td>
<td>0.013</td>
<td>0.022</td>
</tr>
<tr>
<td>nested clade</td>
<td>-128.677</td>
<td>0.137</td>
<td>0.281</td>
<td>0.012</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Diversity 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 southwestern 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 refugial 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
Adenomera (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 (Carnaual 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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**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 delimitation 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. analyzed the data, A.F. wrote the first complete version of the manuscript; all authors read and improved the manuscript.

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