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Unexpected phylogenetic positions of the genera *Rupirana* and *Crossodactylodes* reveal insights into the biogeography and reproductive evolution of leptodactylid frogs

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ABSTRACT

Despite major progress in deciphering the amphibian tree of life by molecular phylogenetics, we identified two questions remaining to be answered regarding relationships within Hyloidea, the clade of South American origin that comprises most extant anuran diversity. A few genera like *Rupirana* and *Crossodactylodes* have enigmatic phylogenetic positions, and relationships among major lineages within some families like Leptodactylidae remain ambiguous. To resolve these specific questions we used two approaches (1) a complete matrix approach representing >6.6 kb, including most major Hyloidea lineages (61 terminals) combining different methods of phylogenetic reconstruction and measures of node support; and (2) a supermatrix approach >11.6 kb with a focus on Leptodactylidae. Both *Rupirana* and *Crossodactylodes* are unambiguously grouped with *Paratelmatobius* and *Scythrophrys*. The clade comprising these four genera is named Crossodactylodinae and embedded within Leptodactylidae. Crossodactylodinae is moderately supported as sister group of Leptodactylinae from (1) and as the sister group of the other Leptodactylidae from (2) with low support. Genera within Crossodactylodinae are scattered along a north–south axis in the Atlantic forest and their origins are very ancient (Paleocene). Such results stress the importance of the northern Atlantic forest in terms of conservation. Moreover, the position of *Pseudopaludicola*, which is well supported as the sister group to all other Leiuperinae, suggests that foam-nest building may have arisen independently in Leptodactylinae and Leiuperinae. Moreover, in spite of being of similar age, foam-nest builders are more widespread than nonfoam-nest breeders and have higher species diversity. Nevertheless, the bulk of the diversity within foam-nest breeders arose some 20 Myr later than the character itself.

1. Introduction

Molecular phylogenetics have revitalized taxonomy and systematics of most living groups, including amphibians (Faivovich et al., 2005; Frost et al., 2006; Grant et al., 2006; Pyron and Wiens, 2011). Furthermore, it has brought exciting new insights into the relationships and temporal/spatial patterns of diversification in amphibians (e.g. Roelants et al., 2007; Santos et al., 2009) often revealing otherwise cryptic evolutionary trends of amphibian morphology (e.g. Bossuyt and Milinkovitch, 2000; Wiens, 2008).

More than 90% of the current anuran species belong to Neobatrachia, and recent studies show that this clade has a Gondwanan origin and that its diversification began during Jurassic (Roelants et al., 2007). Neobatrachia consists of two well-supported clades: Ranoidea and Hyloidea; Ranoidea originated in Africa and India (Bossuyt et al., 2006) and Hyloidea (=Nobleobatrachia *sensu* Frost et al., 2006) in South America. Hyloidea has a relatively recent origin, 65–110 million years ago (Ma), considering that extant anurans started diversifying about 250 Mya (Marjanovic and Laurin, 2007; Roelants et al., 2007; San Mauro et al., 2005; Santos et al., 2009; Zhang et al., 2005). However, Hyloidea includes more than

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half of the neobatrachian species, and thus almost half of the species of the world.

Within Hyloidea, a few groups have been thoroughly investigated. Monophyly and relationships among most genera in Bufonidae (Pauly et al., 2004; Pramuk, 2006; Pramuk et al., 2007; Van Bocxlaer et al., 2010), Hylidae (Faivovich et al., 2005,2010; Wiens et al., 2005), Centrolenidae (Guayasamin et al., 2008, 2009), Terrarana (Hedges et al., 2008; Heinicke et al., 2007,2009), Hemiphractidae (Duellman et al., 2011; Wiens, 2011), and Dendrobatidae (Brown et al., 2011; Grant et al., 2006; Santos et al., 2009) are now relatively well-understood. The most recent contribution, with the largest composite dataset, is that of Pyron and Wiens (2011) based on 2871 terminals and 12712 bp, gathering sparse data for some neglected Hyloidea families like Leptodactylidae, Leiuperidae, Ceratophryidae, and Cycloramphidae, which were redefined by Pyron and Wiens (2011). However, despite Pyron and Wiens (2011) and preceding efforts in documenting amphibian tree of life (Frost et al., 2006; Heinicke et al., 2009), we identified two questions that remain to be answered: (1) the phylogenetic position of some enigmatic genera/species remains unresolved and these are considered as *incertae sedis* within Hyloidea, and (2) relationships of major clades within some families like Leptodactylidae remain ambiguous.

(1) Previous attempts remained taxonomically incomplete in genera included in several families. This is the case of *Rupirana*, *Crossodactylodes*, and *Zachaenus*, which were once included in Cycloramphidae (*sensu* Frost et al., 2006). These genera were actually considered by Pyron and Wiens (2011) as *incertae sedis* in Hyloidea, ignoring that a close affinity between *Zachaenus* and *Cycloramphus* was suggested by Maxson et al. (1981) based on immunological data and subsequently confirmed based on morphology (Verdade, 2005) and molecular data (Lourenço et al., 2008).

However, the phylogenetic position of *Rupirana* Heyer (1999) remains to be investigated. This monotypic genus is restricted to a mountain range in Bahia state, Brazil (Heyer, 1999; Juncá, 2005). The species is a stream dweller with aquatic eggs and free-swimming larvae (Juncá and Lugli, 2009). In the original description, Heyer (1999) included this genus as a member of Leptodactylidae (*sensu* Lynch, 1971) with affinities to *Thoropa* (now part of Cycloramphidae) given that both species are associated with streams and share other character states, but stressing that this was based on plesiomorphies. Therefore, no conclusive evidence was provided regarding the suggested relationship. Nevertheless, Frost et al. (2006) included *Rupirana* in Cycloramphidae, following Dubois (2005) who placed the genus in Cycloramphinae Bonaparte, 1850 without clear justification probably following Heyer (1975, 1999).

The history of the genus *Crossodactylodes*, which comprises three bromeliaceous nominal species distributed in the Atlantic forest of Brazil, is also ambiguous. The genus was erected by Cochran (1938), without relating it to any other anuran taxon. Later, in a reanalysis of the systematics of the "leptodactyloid" frogs, Lynch (1971) placed the genus in the leptodactylid tribe Grypiscini, together with *Cycloramphus* and *Zachaenus*, on the basis of morphology and breeding biology. Based on Lynch (1971), Frost et al. (2006) included *Crossodactylodes* in the tribe Cycloramphini of Cycloramphidae (also including *Cycloramphus*, *Zachaenus*, and *Rhinoderma*) without including sequences of *Crossodactylodes* in their analysis. Grant et al. (2006) raised the tribe Cycloramphini to subfamily (without modifying its content), within a redefined Cycloramphidae also without including sequences of *Crossodactylodes*.

Pyron and Wiens (2011) left *Crossodactylodes* and *Rupirana* as genera *incertae sedis* within Hyloidea. Reasons for this change were (1) the findings of a polyphyletic Cycloramphidae (*sensu* Grant et al., 2006), and (2) the fact that they did not include sequences of any exemplar of these genera. Nevertheless, as shown by Blotto et al. (in press) and Pyron and Wiens (2011), the former Cycloramphidae *sensu* Grant et al. (2006) needs to be thoroughly reevaluated, because of the inclusion of chimeric sequences of distantly related taxa, as well as several *Homo sapiens* contaminations. In fact, morphological data (Verdade, 2005; VKV pers. obs.) suggest that these two genera may be closer to Leptodactylidae. Cycloramphid frogs have *intermandibularis* and *submentalis* muscles adjacent or medially overlapping, corresponding to superficial throat muscles pattern 1 of Burton (1998), whereas *Leptodactylus*, *Physalaemus*, *Pseudopaludicola*, and *Paratelmatobius* (Leptodactylidae) have the *m. intermandibularis* overlapping the *m. submentalis* laterally, corresponding to pattern 2 (Burton, 1998). *Rupirana* and *Crossodactylodes* both present the pattern found in Leptodactylidae (Verdade, 2005; VKV pers. obs.).

(2) Similarly, relationships within Leptodactylidae (*sensu* Pyron and Wiens, 2011), a large family (186 species; Frost, 2012) distributed over all neotropical habitats, still remain ambiguous. Currently, the family consists of three subfamilies Leptodactylinae, Paratelmatobiinae, and Leiuperinae (Pyron and Wiens, 2011). Monophyly of Leiuperinae remains questionable given the alternative relationships of *Pseudopaludicola* in previous works (Frost et al., 2006; Faivovich et al., 2005, 2012; Grant et al., 2006; Santos et al., 2009; Pyron and Wiens, 2011). The position of Paratelmatobiinae is also versatile among previous studies; as the sister group to Leptodactylinae (Frost et al., 2006; Grant et al., 2006) or as the weakly supported sister group of Leiuperinae (Pyron and Wiens, 2011). Interestingly, Leptodactylinae and Leiuperinae build "foam nests" during breeding, except the genus *Pseudopaludicola* (Leiuperidae) and Paratelmatobiinae, a character not discussed by Pyron and Wiens (2011) in their support for a more inclusive definition of Leptodactylidae. Consequently, the homology of foam-nest building in Leptodactylidae is questionable (Faivovich et al., 2012) especially considering that it has evolved in many lineages unrelated to Leptodactylidae (Duellman and Trueb, 1986; Wells, 2007; Faivovich et al., 2012). Also, Paratelmatobiinae and *Pseudopaludicola* have fewer species and display more restricted ranges than do the foam-nest-building Leuiperinae and Leptodactylinae, suggesting that foam-nesting may be linked to the evolutionary success of these groups. Lynch (1971) suggested that foam-nesting in Leptodactylinae evolved during a period of increasingly dry climate, whereas Heyer (1975) argued that foam-nesting originated in wet forests and pre-adapted leptodactylines for later invasion of drier savanna habitats. We therefore need to investigate the relationships among the foam-nest builders and timing of their diversification in order to understand the evolution of this character.

Another important point concerning Leptodactylidae comes from the synonymisation by Frost et al. (2006) of *Adenomera*, *Lithodytes*, and *Leptodactylus* based on Heyer (1998) and Kokubum and Giaretta (2005), who suggested that *Adenomera* is phylogenetically grouped with the *Leptodactylus fuscus* species group. However, none of these publications was actually designed to investigate monophyly of these genera. Paraphyly of *Leptodactylus* with respect to *Adenomera* and *Lithodytes* was also suggested by Ponssa (2008) and Ponssa et al. (2010) based on morphology. Subsequently, Giaretta et al. (2011) erected "the unranked taxon *Spu-*

moranuncula (a name joining Latin words for foam/froth and tadpole) for the putative clade that includes those species of *Leptodactylus*" i.e., *Adenomera* + *Leptodactylus fuscus* species group that share at least the synapomorphy (sic.) of having tadpoles able to generate foam by themselves…". However, Pyron and Wiens (2011) and Fouquet et al. (2007,2012a) recovered *Leptodactylus* and *Adenomera* as monophyletic genera. Nevertheless, proper sampling within *Leptodactylus* and *Adenomera* as well as the integration of *Hydrolaetare* is still needed to test the monophyly of these groups within Leptodactylinae.

In order to fill these gaps, we investigate the phylogenetic position of *Rupirana*, *Crossodactylodes*, and the relationships among the main Hyloidea lineages in particular within Leptodactylidae using both (1) a complete matrix and (2) a supermatrix approach (de Queiroz and Gatesy, 2007). This allows investigating the evolution of foam-nest building in an explicit time frame. Previous studies used an incomplete character matrix and incomplete taxon sampling. Such missing data can, depending on multiple factors, have consequences for the resolution of the phylogeny (Lemmon et al., 2009; Wiens and Morrill, 2011; Wiens, 1998, 2003; Simmons, 2012) especially when estimating divergence time (Lemmon et al., 2009; Wiens and Morrill, 2011). Moreover, improving taxon sampling can resolve deep relationships by breaking long branches and increase accuracy of phylogenetic analyses (Graybeal, 1998; Heath et al., 2008; Hillis, 1998; Hillis et al., 2003; Rannala et al., 1998; Pollock et al., 2002; Zwickl and Hillis, 2002) notably the resolution for short internodes (e.g., Zwickl and Hillis, 2002). Therefore, by combining both approaches and improving both characters and taxon sampling, we partly circumvent these problems.

2. Materials and methods

We follow the family-level taxonomy of Pyron and Wiens (2011). The only modification regards the family allocation of *Batrachyla antartandica*, *B. taeniata*, and *Hylorina sylvatica*, which were transferred from Alsodidae to Batrachylidae (see Blotto et al. (in press) for a justification). We discuss and justify the most critical taxon sampling with regard to the allocation of *Crossodactylodes* and *Rupirana*; for the remaining selected taxa see Appendices S1 and S2.

2.1. Data matrix

2.1.1. Complete matrix

We targeted three mitochondrial loci [the *H-strand transcription unit 1* (*H1*, ~2400 bp including *12S* and *16S*); *cytochrome b* (*Cytb*, 605 bp); *cytochrome oxydase I* (*COI*, 658 bp)] and four nuclear loci [*recombination activating gene exon 1* (*RAG1*; 1244 bp), *pro-opiomelanocortin C* (*POMC*; 588 bp); *tyrosinase* (*TYR*, 531 bp), and *rhodopsin* (*RHOD*, 316 bp)] that were already partly available for main Hyloidea lineages and five outgroups (Appendix S1).

In order to fill data gaps we concatenated data from different species or even genera when monophyly of the group involved was unambiguous from literature. In only two cases, to represent the clades Australobatrachia (Calyptocephalellidae + Myobatrachidae) and *Sooglossus*/*Nasikabatrachus* (outgroups), we concatenated sequence data from different families (Appendix S1). The only early-diverging lineages within Hyloidea that were not represented were Ceuthomantidae and Rhinodermatidae (*Rhinoderma*, *Insuetophrynus*) considering that available data were too limited (RAG1, POMC, TYR missing) to include these terminals. Nevertheless, the former being supported as belonging to the Terrarana clade (Heinicke et al., 2009; Pyron and Wiens, 2011) and the second being unambiguously embedded, yet with undetermined position, within a clade gathering most former Cycloramphidae (*sensu* Frost et al. (2006)) (Blotto et al., in press; Pyron and Wiens, 2011), such omissions do not impede the analyses. Moreover, these terminals were included in the supermatrix (see below). Within Leptodactylidae we also omitted two genera for which available sequence data were considered too limited i.e. *Scythrophrys* and *Edalorhina*. Nevertheless, *Scythrophrys* is supported as the sister group of *Paratelmatobius* (Frost et al., 2006; Lourenço et al., 2008; Pyron and Wiens, 2011; Verdade, 2005) and *Edalorhina* as the sister group of *Engystomops* + *Physalaemus* (Faivovich et al., 2012; Frost et al., 2006; Pyron and Wiens, 2011); therefore, such omissions do not impede the analyses, and these terminals were also included in the supermatrix (see below). For *Adenomera* we included nine nominal and one undescribed species (seven are included here for the first time in a molecular phylogeny) and for *Leptodactylus* we collated sequences for six species groups for which monophyly was unambiguous. We used Blastn on each selected sequence and performed preliminary phylogenetic reconstructions for each locus to double-check potential errors in building the matrix or for erroneous sequences. Individually, each locus provided poor resolution for the deepest nodes (Hyloidea). Therefore, we focused our analyses on the concatenated dataset. *Rupirana cardosoi* and *Crossodactylodes* sp. (a newly discovered species being currently described M. Teixeira Jr. com. pers.) are also included, for the first time in any phylogenetic reconstruction. The final matrix comprised 61 terminals (Appendix S1).

We completed the molecular data directly from new biological material. For 46 terminals genomic DNA was extracted using Promega Wizard® Genomic DNA purification kit. Fragments were amplified by standard PCR techniques; detailed information is available in Appendix S3. Sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing kit and resolved on an automated sequencer at IQUSP and Genomic Engenharia corp. (São Paulo, Brazil). Sequences were edited and aligned with CodonCode Aligner v.3.5.2. Novel sequences were deposited in Genbank (Appendix S1).

We generated 172 new sequences and obtained an almost complete matrix. Missing data were limited to two complete loci for one terminal (*Allophryne COI* and *RAG1*a) and one locus for four terminals (*Hemiphractus TYR*, *Stefania COI*, *Melanophryniscus TYR*, and *Sooglossus*/*Nasikabatrachus POMC*). A 345 bp long portion of the *Cytb* fragment was also missing for four terminals (*Gastrotheca*, *Hemiphractus*, *Stefania*, *Allophryne*); ~600 bp of the *12S–16S* for one terminal (for *Leptodactylus mystaceus* group) and ~500 pb of *RAG1*a for *Craugastor*.

2.1.2. Supermatrix

We subsequently gathered most nominal species within Leptodactylidae available in GenBank for most shared loci available. This included many taxa previously omitted such as *Scythrophrys*, *Edalorhina*, and new terminals (e.g. *Rupirana*, *Crossodactylodes* sp. 2) for which we generated additional sequence data for some of them (see Appendix S2 for the specific terminals and sequence data included and Appendix S3 for primers used). In addition to the loci selected previously for the complete matrix, we included the following genes: *C-X-C chemokine receptor type 4* (*CXCR*) the nuclear, *histone 3a* (*H3A*), *sodium–calcium exchanger* (*NCX1*), *seven in absentia homolog 1* (*SIA*), and *solute-carrier family 8* (*SLC8A3*) (Appendix S2).

The matrix includes 162 terminals (160 species since two samples of *Crossodactylodes* sp. 2 and *Rupirana* were included). It contains data from 160 species for *12S* (100% of the species included), 160 for *16S* (100%), 97 for *Cytb* (60%), 94 for *RAG1* (59%), 74 for *TYR* (46%), 91 for *RHOD1* (57%), 66 for *SIA* (41%), 70 for *POMC* (44%), 39 for *H3A* (24%), 39 for *CXCR4* (24%), 31 for *NCX1* (19%), 23 for *SLC8A3* (14%), 64 for *ND1* (40%), 119 for *tRNAval* (74%), 40 for *tRNAile* (25%), 58 for *tRNAleu* (36%). The mean sequence length (based on the static matrix) per terminal is 5,376-bp (ca. 44% of the matrix length, 12,259 bp), with a range from 1104 bp (*Batrachyla antartandica*) to 11,630 bp (*Thoropa*). See Appendix S2 for GenBank numbers.

2.2. Data analyses

2.2.1. Complete matrix

2.2.1.1. Alignment. Most data consisted of coding regions and thus alignment was unambiguous. We observed the insertion of one codon in the *RAG1*a fragment for Hyloidea and several codon insertion/deletion in *POMC* but none of them led to ambiguous alignment after checking the reading frames.

We searched for the best alignment for the *H1* (*12S–16S*) fragment using the total concatenated dataset with MAFFT v6 (Katoh et al., 2009) and using default parameter except the use of the L-INS-i strategy, which is adapted to sequences with one conserved domain and long gaps. We obtained a final 6656 bp alignment (Appendix S4).

We used Bayesian analysis (BA) and Maximum Likelihood (ML) to investigate phylogenetic relationships among terminals.

2.2.1.2. Bayesian analyses. Bayesian analyses were conducted with Beast 1.6.2 (Drummond and Rambaut, 2007) using relaxed Bayesian molecular clock with uncorrelated lognormal rates. We divided the dataset into seven partitions: one for each codon position of the mtDNA and nuDNA coding genes and one for *H1*, with unlinked HKY+I+G substitution model and unlinked clock model but linked trees. This partitioning was chosen considering the coding nature of mtDNA (*Cytb*, *COI*) and nuDNA (*RAG1*, *POMC*, *TYR* and *RHOD*) loci and comparable magnitude of the rates of evolution (Fouquet et al., 2012c; Hoegg et al., 2004; Mueller, 2006; and also from preliminary analyses – results not shown). A more inclusive partitioning would have to join very different patterns of molecular evolution and more partitions would likely cause overparametrisation (Marshall, 2010). An alternative partitioning (10 partitions) inferred via PartitionFinder (Lanfear et al., 2012) was also used in a BA analysis that led to very similar topology and resolution as well as similar time estimates.

Previous large datasets studies using fossil or biogeographic calibrations to infer timing of diversification within anurans provided a good estimation of the crown age of some major groups. For the root of the tree (Neobatrachia) we used a uniform distribution bounded between 100 and 200 Ma whereas for Hyloidea we considered a uniform distribution bounded between 65 and 100 Ma based on the different estimates from Marjanovic and Laurin (2007). These two ranges fit all other studies (Igawa et al., 2008; Pramuk et al., 2007; Roelants et al., 2007; San Mauro et al., 2005; Wiens et al., 2005). We also bounded the TMRCA between Phyllomedusinae and Pelodryadinae between 35 and 65 Ma based on the evidence that the last connection between Australia and Antarctica was 35 Ma and previous molecular dating showing that maximum age cannot reasonably be older than 65 Ma. Finally, Bufonidae is a thoroughly investigated group whose origin can be reasonably bounded between 65 and 40 Ma.

The tree prior used the Birth and Death Process, with a randomly generated starting tree and default values were used with the "Auto Optimize" option. We computed 10^8 generations, sampled every 1000 generations. We examined convergence on stationarity using Tracer 1.5. The maximum clade credibility tree was computed with Tree Annotator 1.6.2. We considered relationships strongly supported when posterior probabilities were equal to or higher than 0.95 (Fig. 1). The convergence of the BA was quickly reached. Thus, initial burning step was set as 10% of the samples. All ESS were >500.

2.2.1.3. Maximum likelihood. We used GARLI 2.0 (Zwickl, 2006) to search for optimal phylogenetic tree on likelihood criteria, applying a HKY+I+G model (for consistency with BA the same evolutionary model i.e. 7 partitions and HKY is used for ML). The analysis consisted of 350 replicates, each starting with a random tree initially optimized with maximum parsimony criteria and full SPR tree search.

Supports for the recovered ML topology were estimated via PhyML 3.0.1-beta (Guindon et al., 2010; Anisimova et al., 2011) considering parametric aBAYES, aLRT and non-parametric SH-aLRT. We also estimated parametric aLRT and non-parametric SH-aLRT in addition to posterior probability for BA recovered tree on PhyML 3.0.1-beta (supports values were generated with the same analytic conditions as in ML topology search, except that PhyML 3.0.1-beta do not implement partitioned models). Values above 0.90 for parametric values and >0.8 for non-parametric values (Anisimova et al., 2011) were considered strongly supporting the node. Non-parametric support is sensitive to false negative values and parametric support to false positive values. We therefore combined the results from the different support methods in an explicit decision rule about the robustness of the nodes considering as (1) strongly supported, nodes having all values above the threshold, (2) moderately supported, nodes with one value below the thresholds (potential false negative and positive) and (3) weakly supported when more than one value was below the thresholds.

2.2.2. Supermatrix

The phylogenetic analyses using Direct Optimization were performed with POY4.1.1 (Varón et al., 2009, 2010), using equal weights for all transformations (substitutions and insertion/deletion events). Sequences of *H1* were preliminarily delimited in sections of putative homology (Wheeler et al., 2006), and protein-coding genes were considered as static alignments to accelerate the searches. For the protein coding-genes we employed the alignment provided by Pyron and Wiens (2011) with minor modifications. Searches with POY were performed using the command "Search", which implements a driven search composed of random addition sequence Wagner builds (RAS), Tree Bisection and Reconnection (TBR) branch swapping, Parsimony Ratcheting (Nixon, 1999), and Tree Fusing (Goloboff, 1999), storing the shortest trees of each independent run and performing a final round of Tree Fusing on the pooled trees. Two 96-h runs of Search were implemented in parallel at the American Museum of Natural History Cluster using 28 processors. The resulting trees were submitted to a final round of swapping using iterative pass optimization (Wheeler, 2003). We also performed a multiple alignment with MAFFT (Katoh et al., 2009). We then analyzed the competing alignment by performing searches with T.N.T Willi Hennig Society Edition v1.1 (Goloboff et al., 2008), keeping the alignments that yielded the lower tree length. For the regions of *12S* and *16S* we employed the alignments generated with Q-INS-i strategy (secondary structure of RNA is considered), while the alignments for the remaining fragments (*tRNAval*, *tRNAleu*, *tRNAile*, *ND1*) were generated with G-INS-i (global homology considered).

For the phylogenetic analysis we employed T.N.T v1.1, performing 1000 random addition sequences followed by a round of TBR swapping, and saving 10 trees per replicate. Two analyses were conducted, considering alternatively gaps as a fifth state and as missing data. Support estimation was done with New Technology search (which implements Sectorial Searches and Tree Fusing) hitting the minimum length two times per replicate, for a total of 1000 replicates of Parsimony Jackknife, with 0.36 of removal probability (Farris et al., 1996).

3. Results

3.1. Complete matrix

The topologies recovered across analyses are very similar; differences mostly lay with poorly sustained relationships among

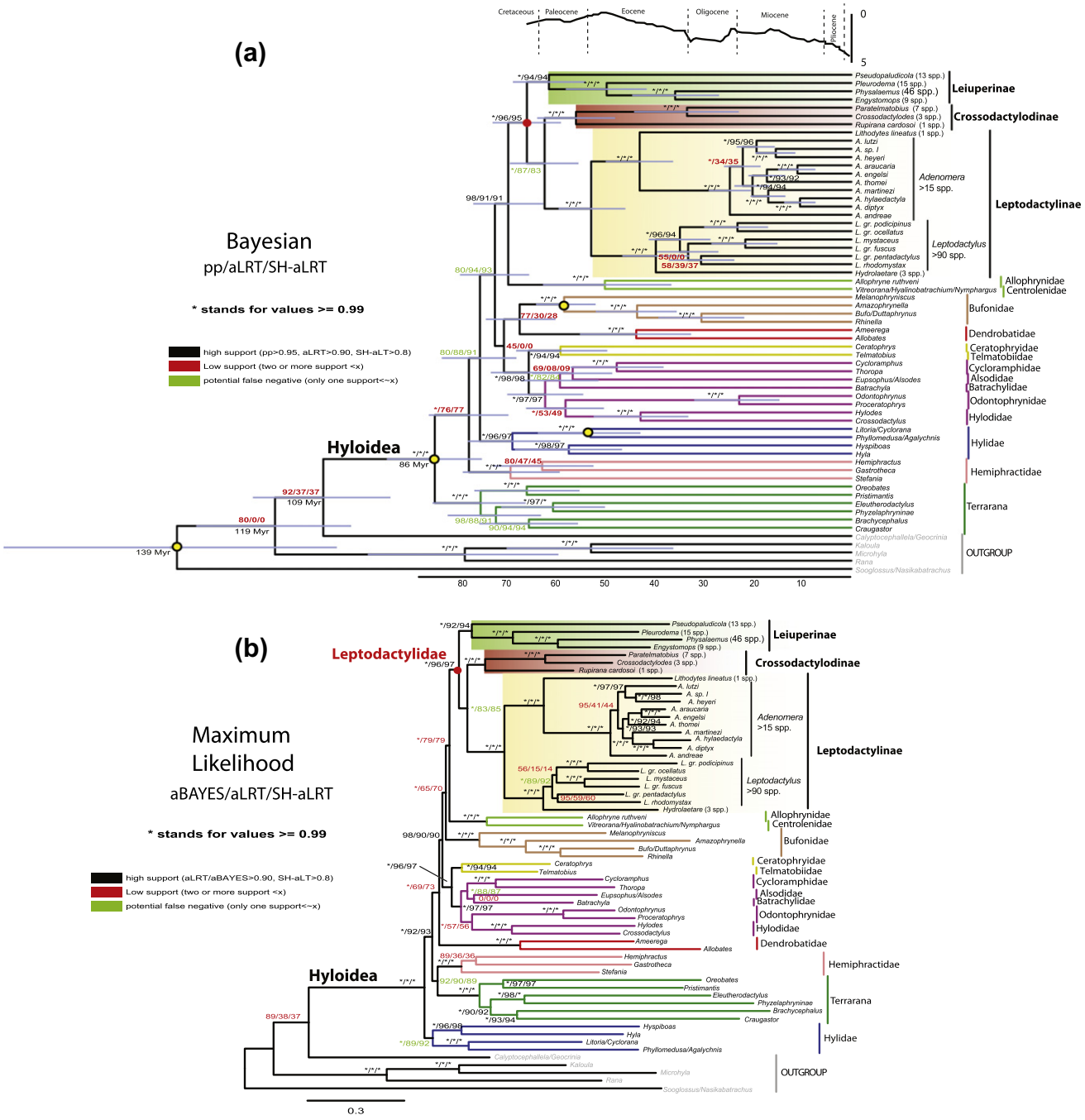

Fig. 1. (a) Bayesian time-calibrated, maximum clade-credibility tree using relaxed clock and selected terminals. Calibration points (see Text) are indicated with yellow circles. Posterior probabilities/aLRT/SH-aLRT are indicated near the nodes; 95% credibility intervals are indicated with blue bars. (b) Phylogenetic tree based on maximum likelihood method. Supports aBAYES/aLRT/SH-aLRT are indicated near the nodes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

families within Hyloidea, most notably due to alternative positioning of Hylidae and Dendrobatidae.

A clade comprising *Rupirana*, *Crossodactylodes*, and *Paratelmatobius* is strongly supported in all analyses (Fig. 1) and is unambiguously nested within Leptodactylidae. In agreement with previous works, Hyloidea is strongly supported as monophyletic, as well as major clades (Terrarana) and families. Leptodactylidae and the clade formed by Allophrynidae and Centrolenidae are also recovered as sister groups from BA and ML. However, some relationships have never been recovered with strong supports before: (1) Cycloramphidae + Alsodidae + Batrachylidae + Odontophrynidae + Hylodidae form a strongly supported clade mostly corresponding to a former definition of Cycloramphidae (*sensu* Frost et al., 2006) with BA and ML; (3) Ceratophryidae and Telmatobiidae form a strongly supported clade with BA and ML, furthermore (4) the latter two families are strongly supported forming a clade with most former Cycloramphidae (*sensu* Frost et al., 2006) with BA and ML.

Leptodactylinae is unambiguously recovered monophyletic and strongly supported in all analyses. The clades formed by *Rupirana*, *Crossodactylodes*, *Paratelmatobius* and by Leptodactylinae are recovered as sister groups. This clade is recovered in all methods

with strong supports except in SH-aLRT and aLRT with BA and SH-aLRT with ML where their relationship is moderately supported. Similarly, Leiuperinae is recovered monophyletic having *Pseudopaludicola* as the sister taxon of the other Leiuperinae in BA and ML, both with strong support, except aLRT with BA (moderate support). Within Leptodactylinae, *Adenomera* is strongly supported as monophyletic as well as *Leptodactylus*, having respectively as sister groups *Lithodytes* and *Hydrolaetare*.

Diversification of Leptodactylidae began about 68 Ma, with *Rupirana* diverging very early, about 58 Ma (Paleocene), from the clade formed by *Scythrophrys*, *Crossodactylodes* and *Paratelmatobius*. *Crossodactylodes* also displays an early divergence, some 34 Ma (Eocene/Oligocene boundary), from *Paratelmatobius* (and *Scythrophrys* by implication). Leptodactylinae started to diversify about 55 Ma (Paleocene/Eocene boundary) and *Leptodactylus* some 35 Ma while *Adenomera* diversified more recently, about 25 Ma (Oligocene/Miocene boundary).

A few discrepancies and diagnostic points should also be highlighted. Posterior probabilities from BA are $\geqslant$0.95 with 11 exceptions (Fig. 1a). These ambiguous relationships are: (1) the position of Hyloidea + Australobatrachia + Ranoidea; (2) the base of Hyloidea excluding Terrarana; (3) relationships among *Leptodactylus* main species groups, (4) within Hemiphractidae, and (5) between *Brachycephalus* and *Craugastor*, and (6) between Alsodidae and Cycloramphidae. Posterior probabilities >0.95 are generally accompanied by aLRT $\geqslant$ 0.90 and/or SH-aLRT $\geqslant$ 0.8 with a few exceptions, such as among the main clades within Leptodactylidae and basal relationships in *Adenomera*. These may represent potential false negative support values, given that similar topologies were found from ML. Other additional nodes displayed pp slightly < 0.95 but aLRT $\geqslant$ 0.90 and SH-aLRT $\geqslant$ 0.8, such as *Brachycephalus* + *Craugastor*, that we also considered to be potentially false negative supports. Other potentially false negatives display pp > 0.95 and either aLRT or SH-aLRT slightly below the threshold.

The topology obtained using ML is very similar to the one from BA with one sustained exception (Fig. 1b): Hylidae is recovered as the sister group to all the other Hyloidea with strong support and Terrarana to Hemiphractidae. In total 14 nodes (out of 59) are poorly or moderately supported and mostly match the ones poorly supported using BA. Among them three have support values interpreted as potential false negatives.

3.2. Supermatrix

The analysis using direct optimization with POY yielded three most parsimonious trees of length 60,329 (Fig. 2). The topology relevant for Leptodactylidae is overall similar to the one obtained from the complete matrix. *Crossodactylodes* is recovered as the sister taxon of *Paratelmatobius* with high support, while *Rupirana* is the sister taxon of *Scythrophrys* + *Crossodactylodes* + *Paratelmatobius* (with 100% jackknife support). Leiuperinae and Leptodactylinae are recovered monophyletic but the relationship among the main leptodactylid clades is different from the results obtained using the complete matrix, with the clade formed by *Rupirana* + *Scythrophrys* + *Crossodactylodes* + *Paratelmatobius* being the sister group to all other leptodactylids but with <50% jackknife support.

Cycloramphus was recovered paraphyletic with respect to *Zachaenus parvulus*, this species being the sister taxon to *C. boraceiensis* (with very low support), while the group composed of *Cycloramphus* + *Z. parvulus* is highly supported (0.99).

The analyses of the static matrix with TNT considering gaps as a fifth state or as missing data (results not shown) yielded identical results with respect to the above-mentioned relationships of Leptodactylidae and *Zachaenus parvulus*.

4. Discussion

4.1. An improved resolution among main Hyloidea lineages

The relationships inferred among main Hyloidea lineages (families and higher) from the complete matrix and the supermatrix are largely similar, particularly between BA of the complete matrix and the MP analysis of the supermatrix. The relative positions of Alsodidae, Batrachylidae, Odontophrynidae, Hylodidae, Cycloramphidae, Telmatobiidae and Ceratophryidae are notably similar across analyses (see later) but the deepest relationships between families remain weakly supported. Within Leptodactylidae, the inferred relationships are also similar across analyses. Even though the relative positions among subfamilies actually differ, the topology obtained via the supermatrix is weakly supported. The degree of node supports is in fact generally lower for the supermatrix, which is likely inherent to the use of MP.

Our results are also strikingly similar to those of Pyron and Wiens (2011), whose analysis was based on ML (GTR model) and provided only non-parametric bootstrap supports. However, they differ in some aspects particularly among genera previously embedded into Cycloramphidae and among Leptodactylidae subfamilies. Nevertheless, these few areas of disagreement were weakly supported in Pyron and Wiens (2011), whereas most are well supported herein. This is likely inherent to bootstrap calculation, which can be very sensitive to short internal branches, producing false negative values (Alfaro et al., 2003; Anisimova et al., 2011). The completeness of our matrix and the inclusion of additional taxa likely compensate our smaller matrix. Furthermore, the use of different analytical methods and different support estimates allow us better to evaluate the robustness of the inferred relationships.

It is also worth noticing that the estimated divergence times appear reliable given that they agree with most of the previous attempts to investigate timing of diversification among main lineages of Hyloidea. For example, we estimate the basal split within Dendrobatidae at about 45 Ma (as in Santos et al., 2009) and the basal split in Bufonidae at about 60 Ma (as in Van Bocxlaer et al., 2010). However, the different phylogenetic position found for Terrarana compared to Heinicke et al. (2007, 2009) implies an older divergence time for this clade (see below).

4.2. Leptodactylidae

The monophyly of this family including *Rupirana* and *Crossodactylodes* and the position of *Pseudopaludicola* as sister group to Leiuperinae, are highly supported from the complete matrix and moderately supported from the supermatrix. Phylogenetic methods based on morphological characters have not yet been employed to assess relationships among these groups; nevertheless, some characters support our results. Lynch (1971) stated that members of this family (as subfamily Leptodactylinae) share a bony style or an osseous plate in the sternum in opposition to the cartilaginous sterna of other Hyloidea frogs, that the frontoparietals are in medial contact and lack or present a reduced posterolateral process, and that the nasal bones are not in contact medially and are separated from the frontoparietals (Lynch, 1971; Trueb, 1973; Verdade, 2005). Burton's pattern 2 of the superficial throat musculature, shared by *Leptodactylus*, *Physalaemus*, *Pseudopaludicola*, *Paratelmatobius*, *Rupirana*, *Crossodactylodes* and *Scythrophrys*, is also a putative synapomorphy for Leptodactylidae (Burton, 1998; Verdade, 2005; VKV pers. obs.). *Pseudopaludicola* was traditionally considered closely related to Leiuperinae based on overall morphology (Cei, 1980). The genus shares with some species of *Physalaemus* a gap in the posterior row of marginal papillae of their

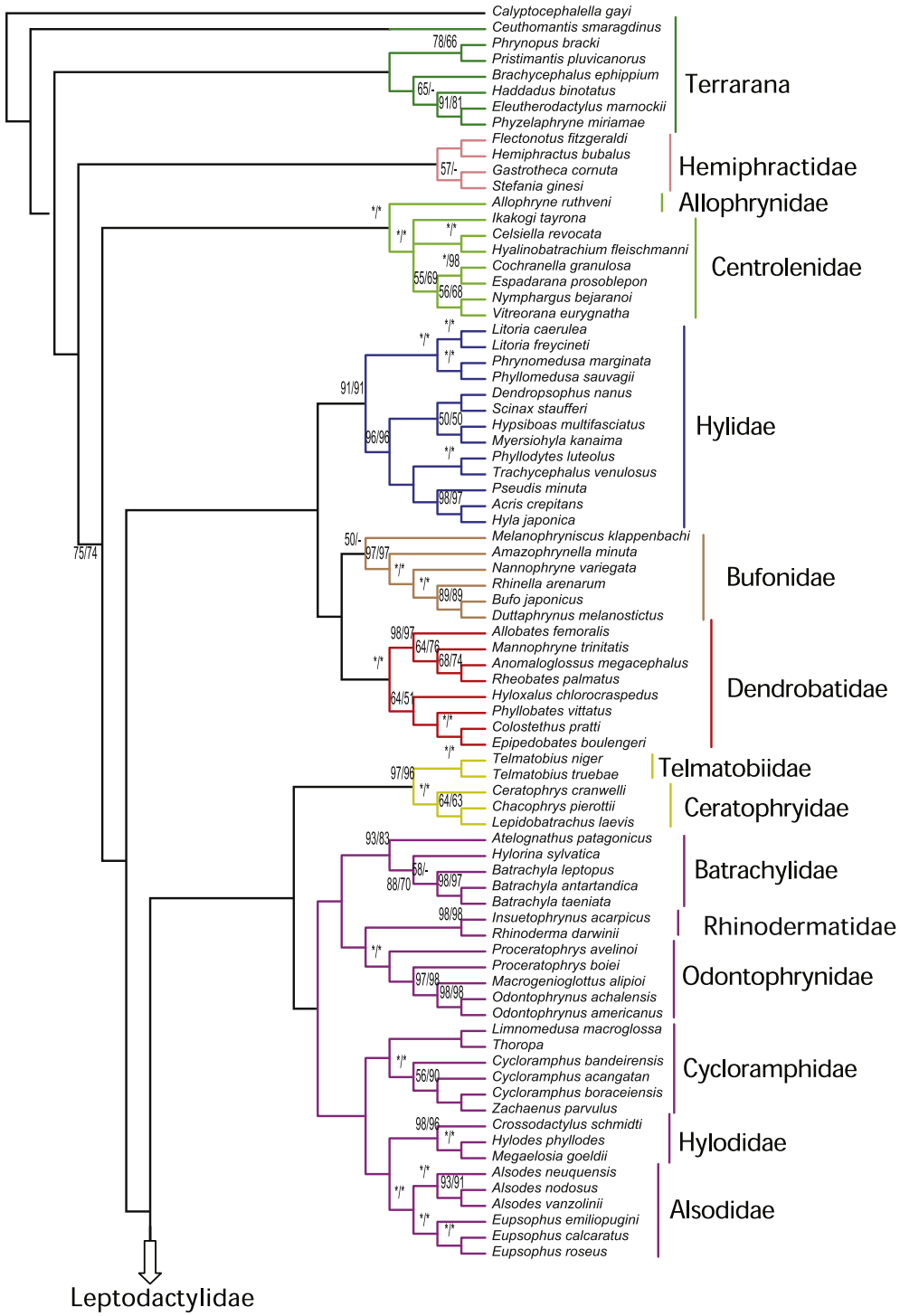

Fig. 2. Strict consensus from the super matrix approach of the three most parsimonious trees found (length 60,329) using direct optimization, under equal weights for all transformations (substitutions and insertion/deletion events). Numbers on nodes from left to right and separated by "/" indicate (i) parsimony jackknife absolute frequency estimated for the static alignment analyzed with parsimony in TNT with gap as fifth state; (ii) parsimony jackknife absolute frequency estimated for the static alignment analyzed with parsimony in TNT with gap as missing data. Asterisks indicate groups with ⩾99% of parsimony jackknife frequencies; "–" denotes groups not recovered in the analysis with the static alignment on TNT or with jackknife values <50%.

tadpoles (Giaretta and Facure, 2009). The monophyly of Leiuperinae is also supported by: (1) a double origin of *m. geniohyoideus lateralis* (1) from the anterior tip of the *maxillae* and (2) from the *fascia* covering the *m. submentalis* in *Physalaemus*, while this structure is restricted to the anterior tip of *maxillae* in *Crossodactylodes*, *Leptodactylus*, *Rupirana*, *Paratelmatobius*, and *Scythrophrys* (VKV, pers. obs.).

4.3. Taxonomical account

Rupirana and *Crossodactylodes* (for the first time included in any phylogenetic analysis) are unambiguously recovered nested within Leptodactylidae, and their association to *Paratelmatobius* is strongly supported in all analyses (Figs. 1 and 2b) as well as with *Scythrophrys* with the supermatrix (Fig. 2b). Paratelmatobiinae

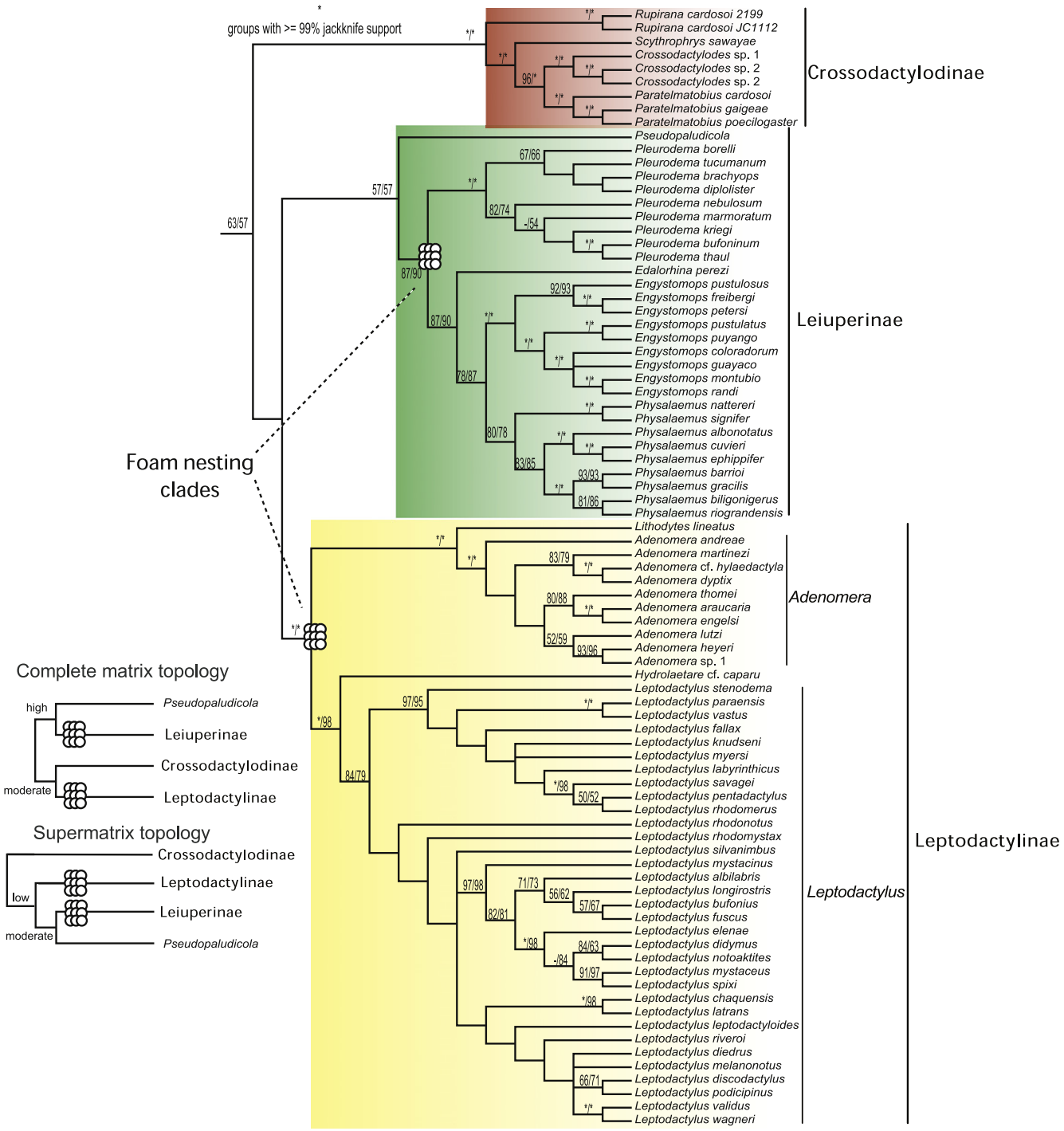

Fig. 2. (*continued*)

was erected by Pyron and Wiens (2011) to accommodate *Paratelmatobius* and *Scythrophrys*. However, Paratelmatobiinae, Pyron and Wiens, 2011, is a *nomen nudum* following the article 13.1 of ICZN (1999), since no description or definition is provided. To resolve the issue and considering the anteriority of *Crossodactylodes* over *Paratelmatobius*, we propose a new name for this subfamily as follows:

Crossodactylodinae subfam. nov.: Paratelmatobiinae, Pyron and Wiens, 2011 (*nomen nudum*).
Type genus: *Crossodactylodes* Cochran, 1938

Diagnosis: This subfamily is diagnosed by 73 transformations in nuclear and mitochondrial protein and ribosomal genes from the supermatrix. See Appendix S5 for a complete list of these molecular synapomorphies. We are not aware of any unambiguous morphological synapomorphy. Nevertheless, we discuss below some characters from morphology and reproductive biology, and discuss its taxonomic distribution, in order to establish potential synapomorphies and/or interesting characters to be evaluated more thoroughly in future studies. These are the presence/absence of columella and vomerine teeth, the morphology of the nuptial pads of males, and the oviposition site.

The diversity of reproductive modes is particularly striking in this subfamily as well as its range, which is fragmented along the Atlantic Forest domain (see below).

Content: *Crossodactylodes*, Cochran, 1938; *Paratelmatobius* Lutz and Carvalho, 1958; *Rupirana*, Heyer, 1999; *Scythrophrys*, Lynch, 1971.

The columella was reported absent in the three species of *Crossodactylodes* (Lynch, 1971; Gomes, 1988), and in *Paratelmatobius lutzii* (Lynch, 1971) but present in *P. cardosoi* (Verdade, 2005) and *Scythrophrys* (Verdade, 2005) as well as in *Rupirana* and other leptodactylids (e.g., Lynch, 1971; Heyer, 1999). Therefore, it may prove to be a putative synapomorphy of *Crossodactylodes*. The other character is the presence of vomerine teeth, which may be present or absent within Crossodactylodinae. It was reported as absent only in *Crossodactylodes izecksohni* and *C. pintoi*, while it is present in *C. bokermanni*, *Scythrophrys*, *Paratelmatobius* and *Rupirana* (Gomes, 1988; Heyer, 1999; Lynch, 1971; Peixoto, 1983 "1982"; Verdade, 2005). A phylogeny of *Crossodactylodes* would permit testing whether absence of vomerine teeth is a synapomorphy of *C. izecksohni* + *C. pintoi*.

Finally, the nature of the nuptial pads asperities and the oviposition site are putative synapomorphies of *Crossodactylodes*. Species of this genus present few well developed spines (3–4 in *C. bokermanni*, 9–12 in *C. izecksohni*, unreported in *C. pintoi*; Peixoto, 1983 "1982"). In the other genera of Crossodactylodinae, the pads are formed by numerous smaller spines, as in *Paratelmatobius* (Cardoso and Haddad, 1990; Garcia et al., 2009; Giaretta and Castanho, 1990; Pombal and Haddad, 1999; Verdade, 2005; Zaher et al., 2005), *Rupirana* (Heyer, 1999), and *Scythrophrys* (B. Blotto, pers. obs. on specimen CFBH 9369). *Crossodactylodes* is a phytotelmata breeder; it lays a few large eggs in bromeliads, and the tadpoles develop there (Lynch, 1971; Peixoto, 1983 "1982"; Peixoto, 1995). *Rupirana* and some *Paratelmatobius* lay their eggs in puddles in the bed of streams and ponds respectively (Garcia et al., 2009; Juncá and Lugli, 2009; Pombal and Haddad, 1999), while the clutch of *P. poecilogaster* is terrestrial, being deposited hanging on humid rocks above the water (Pombal and Haddad, 1999). *Scythrophrys* breeds, as do most *Paratelmatobius*, in forest temporary ponds (Garcia, 1996). The oviposition in bromeliads is therefore a putative synapomorphy of *Crossodactylodes*.

Crossodactylodinae and Leptodactylinae form a moderately supported clade using the complete matrix. This topology was also found by Frost et al. (2006) and Grant et al. (2006). However, Crossodactylodinae was recovered, with low support, as the sister group to Leiuperinae by Pyron and Wiens (2011) and as the sister group of other leptodactylids from the supermatrix approach but with low support. Compared to previous studies, breaking the Crossodactylodinae long-branches by the inclusion of *Rupirana* and *Crossodactylodes* and the completion of the matrix likely improved the accuracy of the analyses. Even though interrelationships among these three subfamilies remain ambiguous we argue that supports obtained from the complete matrix lead us to favor Leiuperinae (Crossodacylodinae + Leptodactylinae).

4.4. Crossodactylodinae biogeography and evolution

Crossodactylodinae, as defined in this paper, includes *Paratelmatobius*, *Scythrophrys*, *Rupirana* and *Crossodactylodes*. This reveals a striking biogeographic pattern, the clade being endemic to the Atlantic forest domain and the four genera having an allopatric distribution on a North–South gradient. Such pattern mirrors the one found in *Dendrophryniscus* (Fouquet et al., 2012b) with the earliest split separating *Rupirana* in the northern part of the Atlantic forest (Bahia) from all others and then the most recently diverging lineages (*Scythrophrys*, *Crossodactylodes* and *Paratelmatobius*) occurring in the southern part of the distribution of the clade. Such pattern matches the climatically stable areas previously suggested, the Bahia and São Paulo refugia (Carnaval et al., 2009). However, the inferred divergence time is older in Crossodactylodinae than in those previous studies. The comparison with *Dendrophryniscus* is also striking when examining reproductive behaviors. The early-diverging *Rupirana* and *D. proboscideus* breed in mountainous streams of Bahia, while *Crossodactylodes* and most *Dendrophryniscus* spp. occurring in the central and southern region are phytotelmic (Fouquet et al., 2012b). The use of phytotelmata as breeding sites and semi-arboreal habits by some *Dendrophryniscus* and *Crossodactylodes* may have been driven by the abundance of bromeliads and the rarity of lentic-water ponds in the steep Atlantic rainforest. Evolutionary shifts to bromeliad-breeding occurred recurrently and independently in several lineages of Atlantic Forest frogs (e.g., *Bokermannohyla astartea*, *Fritziana* spp., *Frostius* spp., *Phyllodytes* spp., *Scinax* spp. gr. *perpusillus*; Haddad and Prado, 2005), supporting that this strategy may be advantageous in coastal rainforest environments.

The occurrence of narrow endemic species in the Atlantic Forest, diverging some ~35 Ma (*Crossodactylodes*), is a testimony that some of these forest fragments remained relatively stable during most of the Tertiary and Quaternary, a much longer time period than that modeled by Carnaval and Moritz (2008). A similar pattern may be found in other Atlantic forest endemic frogs like *Brachycephalus*, *Holoaden*, *Ischnocnema*, *Phyllodytes*, *Fritziana*, *Aplastodiscus*, *Bokermanohyla*, *Scinax* gr. *catharinae* that remain to be explored. This understanding stresses the emergency of conservation efforts toward the amphibians of the Atlantic Forest, particularly on its northern range where too few areas are under protection. As a matter of fact, despite being flagged as a priority area some 15 years ago (Mittermeier et al., 1998) among the famous "biodiversity hotspots", the Atlantic forest of Brazil is still highly threatened, particularly in its northern area (Ribeiro et al., 2009).

4.5. Evolution of foam-nest building

Our results unambiguously support monophyly for *Adenomera* and *Leptodactylus* and imply the paraphyly of *Spumoranuncula* (Giaretta et al., 2011) and thus the homoplasic nature of endotrophy and tadpole foam-nest tissue structure in *Adenomera* and the *Leptodactylus fuscus* group. The genus *Adenomera* started to diversify some 25 Ma, while *Leptodactylus* about 35 Ma. Even though the actual diversity within each of these two groups and particularly within *Adenomera* (Angulo et al., 2003) and the *L. podicipinus* group (Fouquet et al., 2007) is largely underestimated, we argue that in these groups diversification has been particularly fast or less subject to extinction compared to other Leptodactylidae, especially those that do not build foam-nests, i.e., Crossodactylodinae and *Pseudopaludicola*.

Differences among clades in the probability of diversifying are the result of a combination of contingent historical events and clade intrinsic properties (Moore and Donoghue, 2007). Intrinsic characteristics (Moore and Donoghue, 2007; Phillimore et al., 2006) or a combination of life-history traits (Isaac et al., 2005) may constitute potential key innovations (reviewed by Heard and Hauser (1995)) associated with species richness. Hence, extrinsic factors may provide the opportunity for diversification, whereas intrinsic species characteristics may determine whether such opportunities lead to moderate or explosive diversifications, extinction, or evolutionary stasis. The relationships among foam-nest and nonfoam-nest builders within Leptodactylidae imply either independent origin of this trait in Leptodactylinae and Leiuperinae (70–50 Ma) or a unique origin (75–65 Ma) with subsequent independent loss in Crossodactylodinae and *Pseudopaludicola* (or only in *Pseudopaludicola* if Crossodactylodinae is in fact the

sister group to other leptodactylids). Giaretta and Facure (2009) as well as Faivovich et al. (2012) suggested that foam nest building in Leiuperidae is derived, i.e., *Pseudopaludicola* displays a plesiomorphic reproductive mode.

Interestingly, despite that Crossodactylodinae has an older crown age than foam nesting Leiuperinae (excluding *Pseudopaludicola*) and Leptodactylinae, it has fewer species and is restricted to highlands of the Atlantic forest domain with each species having a very restricted range (Fig. 3). However, foam-nesting Leiuperinae and Leptodactylinae have both many species and are widespread throughout the Neotropics. This striking opposition within Leptodactylidae strongly suggests that foam-nest building may have been advantageous for foam-nesting Leiuperinae and Leptodactylinae to adapt to a larger diversity of habitats and to disperse throughout the continent and therefore diversify.

Nevertheless, foam-nest building alone cannot explain the success of these groups considering: (1) its origin is likely to be much older (70–50 Ma) than the 35–25 Ma that have seen the bulk of the diversification of *Leptodactylus* and *Physalaemus*, (2) some genera like *Lithodytes* (1 spp.), *Hydrolaetare* (3 spp.), *Engystomops* (9 spp.), and *Edalorhina* (2 spp.) have more restricted distributions and fewer species than *Leptodactylus* (>90 spp.) and *Physalaemus* (>46 spp.) despite being foam-nest builders. Therefore, foam-nest building may not be equally related to the evolutionary success of these three genera. Instead, it is striking to note that all genera with many species are widespread throughout Amazonia, Cerrado, Chaco, and Atlantic Forest, whereas others foam-nest building genera are either restricted to one or the other. Therefore, propensity to disperse through the continent and thus the extent of the area and variety of climate, latitude, elevation, etc. may simply be the very reason for their diversity. This corresponds to the long-standing hypothesis that species richness increases with area (e.g., MacArthur and Wilson, 1967; Rosenzweig, 1995). Such propensity to disperse may be linked, but not exclusively, to foam-nest building.

The origination of the foam-nest building in Leptodactylinae and Leiuperinae can be estimated between 60 and 45 Ma if it is the result of independent origins and about 70 Ma if it has a less-probable single origin (i.e., secondarily lost in *Pseudopaludicola* and/or Crossodactylodinae). This time frame matches the Eocene thermal maximum. However, the 35–25 Ma window that corresponds to the *Adenomera*, *Leptodactylus*, and likely *Physalaemus* crown ages coincides with the Oligocene/Miocene transition. This transitional period corresponds to a cooling and mountain building that matches the diversification of the first modern Andean genera of plants and animals (Hoorn et al., 2010), such as the origin of the bufonid "range expansion phenotype", as coined by Van Bocxlaer et al. (2010), and the burst of diversification of bufonids. This mountain build-up had major impacts on Amazonia's hydrological system (Hoorn et al., 2010) and probably drove the spread of open vegetation at the expense of the rainforest that previously dominated the Southern continent (Roig Juñent et al., 2006; Romero, 1986). Therefore, it is intriguing that foam-nest building in leptodactylids may have originated during a warm period while their rapid diversification occurred during a cold and dry period. This scenario matches quite well the hypothesis formulated some 40 years ago by Heyer (1975). Nevertheless, an alternative hypothesis is that foam-nest building may have originated as a strategy to avoid predation in aquatic environments (Magnusson and Hero, 1991).

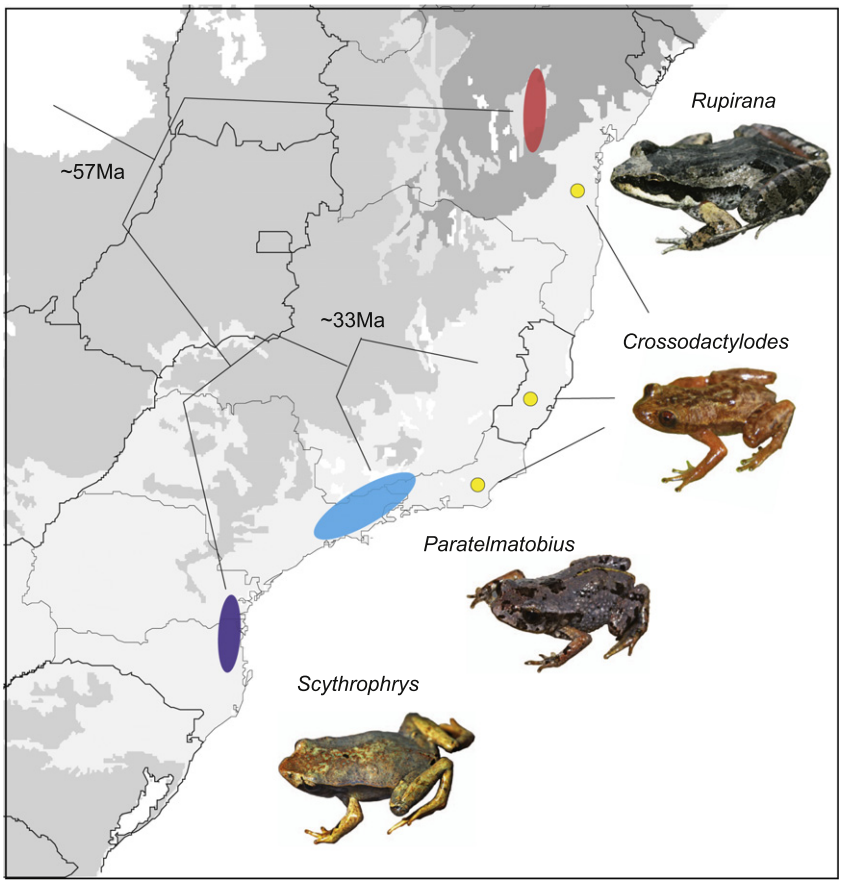

Fig. 3. Map of Crossodactylodinae distribution based on IUCN red lists including one additional record for *Crossodactylodes* sp. 1 extending the distribution northward.

4.6. Hyloidea higher clades

Only a few relationships among families were recurrently recovered across our analyses and in previous phylogenetic reconstructions, e.g., affinity between Allophynidae + Centrolenidae with Leptodactylidae (Frost et al., 2006; Guayasamin et al., 2009; Heinicke et al., 2009; Pyron and Wiens, 2011). The relationships among the other families remain virtually unknown. For example, relationships within Cycloramphidae and Ceratophryidae (*sensu* Frost et al., 2006) remained very unstable in previous works including Pyron and Wiens (2011). In order to stabilize the situation, Pyron and Wiens (2011) divided these groups into eight families corresponding to well-supported clades (except Alsodidae). Relationships among these families previously embedded within Cycloramphidae (*sensu* Frost et al., 2006) are relatively well resolved herein (with most of the internal relationships displaying high values of parametric and non-parametric supports). Additionally, *Zachaenus* was left *incertae sedis* in Hyloidea by Pyron and Wiens (2011). As noted earlier, they ignored previous results by Lourenço et al. (2008), where *Zachaenus parvulus* is recovered as the sister taxon of *Cycloramphus boraceiensis* (the only species of *Cycloramphus* included in that paper), with high bootstrap support. The results of Lourenço et al. (2008) are in accordance with ours from the supermatrix, where *Zachaenus parvulus* is nested within *Cycloramphus*. Although *Cycloramphus* is recovered here as paraphyletic with respect to *Zachaenus parvulus*, we prefer not to synonymize *Zachaenus* with *Cycloramphus* until a better sampling of *Cycloramphus* becomes available, including the other species currently allocated in *Zachaenus*. With these findings about the phylogenetic relationships of *Zachaenus*, in addition to the allocation of *Crossodactylodes* and *Rupirana*, the relationships of the three genera considered *incertae sedis* by Pyron and Wiens (2011) are resolved.

The support is relatively weak for the position of Terrarana as the sister group of the other Hyloidea given such placement is recovered with low support using BA (complete matrix) and ML (supermatrix) and not using ML (complete matrix). Such placement is in contradiction with most of the previous phylogenetic reconstructions except Pyron and Wiens (2011). The ambiguously positioned lineages are also Hylidae, Dendrobatidae, and Hemiphractidae considering the differences and lack of support from our results and in previous ML, BA and MP studies. We expect that additional sequence data for these lineages will hardly allow reaching more stable positions among the alternative phylogenetic positions as Heinicke et al. (2009) stated: "Most of the other basal branches in Nobleobatrachia are characterized by very short internodes which may confound efforts to resolve these early divergences even with increased gene sampling (Rokas and Carroll, 2006; Wiens, 2008)". Resolution and further stability of these branches is crucial to understand the processes of emergence of the most successful amphibian groups within Nobleobatrachia like Bufonidae, Dendrobatoidea, Leptodactylidae or Hylidae. However, given each locus provides independently very few information for these ancient internodes, unraveling the intricate gene histories to understand the genealogy of these groups remains a nut to be cracked. Actually, the primordial question may be more to put a precise time interval on the split of an ancestral lineage into multiple descendants simultaneously.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.02.009.

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