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Big, Bad, and Beautiful: Phylogenetic Relationships of the Horned Frogs (Anura: Ceratophryidae)

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Abstract. The horned frog family, Ceratophryidae, currently comprises three genera and 12 extant species, distributed from the Caribbean lowlands to the Pampean grasslands. Horned frogs are fossorial species that are remarkable in terms of their adult and larval morphology, karyotype, behavior, and other aspects of their biology. In this paper we present a molecular phylogenetic analysis with the goals of: (1) exploring the relationships among the species of Ceratophryidae; (2) studying the evolution of polyploidy; (3) studying the evolution of cocoon formation and larval development duration associated with surviving in semiarid environments; and (4) reviewing the ceratophryid fossil record that could be relevant as calibration points in molecular divergence estimations. The analysis included 11 of the 12 extant species and, when possible, multiple exemplars per species, as well as multiple outgroups. Sequence data were obtained on seven mitochondrial and six nuclear genes for up to 8200 bp per specimen. Our results indicate that the individual monophyly of *Ceratophrys* and *Lepidobatrachus* is well corroborated. The monotypic *Chacophrys* is recovered as the sister taxon of *Lepidobatrachus,* but with Jackknife frequency < 50%. *Lepidobatrachus asper* is the sister taxon of *L. laevis* + *L. llanensis*. Relationships within *Ceratophrys* are congruent with an earlier proposal, with a clade composed of the species possessing a dorsal bony shield (*Ce. aurita, Ce. cranwelli, Ce. joazeirensis,* and *Ce. ornata*), and another clade composed of *Ce. stolzmanni, Ce. calcarata,* and *Ce. cornuta.* Unlike earlier proposals, the octoploid species (*Ce. aurita, Ce. joazeirensis,* and *Ce. ornata*) are not monophyletic, as the diploid *Ce. cranwelli,* and *Ce. ornata* are sister taxa. This result implies an ambiguous optimization of ploidy levels, with either a single origin of octoploidy with a subsequent reversal to diploidy, or two independent origins of octoploidy being equally parsimonious; both alternatives are quite unusual from the perspective of chromosome evolution. Our results suggest that ceratophryids diversified in semiarid environments and three independent events resulted in three species subsequently occupying temperate or tropical humid areas. This early diversification in semiarid areas explains the retention of characteristics associated with these environments (like the production of a cocoon of dead skin during estivation, and possibly an accelerated larval period and development) in species present in humid areas. A revision of the fossil record of this family of frogs indicates that there are only two fossil remains that could serve as calibration points for molecular clock estimation, but a number of issues associated with them preclude their use.

Keywords. *Ceratophrys; Chacophrys;* Divergence time estimation; Fossil calibration; Hyloides; Karyotype evolution; *Lepidobatrachus;* Semiarid environments.

INTRODUCTION

> *"Irritandolo si avventa, e afferra con la sua larga e monstruossa bocca ció che gli si presenta, e fa sentire una grandissima, ma rauca voce."*
> First description of aggressive behavior in a ceratophryid frog (*Ceratophrys aurita*) by Giuseppe Raddi (1823).

Although not reaching the level of pop culture icons like the T-Rex or panda, horned frogs have gained a special place in the view of the general public: Their relatively large size and bizarre, and in some species colorful, aspect, coupled with their aggressiveness leave a long lasting impression on anyone that has ever had the chance to meet one alive, not to mention, get bitten by one.

The horned frog family, Ceratophryidae, currently comprises three genera and 12 extant species. *Ceratophrys* includes eight extant species that are the most colorful ceratophryids and collectively are distributed from the Caribbean lowlands in Colombia and Venezuela to central Argentina, including the arid coastal areas of northern Peru and southern Ecuador, the Amazon basin, the Caatinga in NE Brazil, the Cerrado in central Brazil, the Atlantic Forest, the Gran Chaco, and the Pampean grasslands. *Lepidobatrachus,* the Budgett's frogs, includes three species that are inhabitants of the Gran Chaco. *Chacophrys pierottii* is so far known only from the drier western section of the Gran Chaco.

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Interest in ceratophryids far exceeds their curious appearance. Among other aspects of their biology, their anatomy, larval morphology, ontogeny, diet, behavior, and karyology have been capturing the attention of herpetologists for a long time. The unique larval morphology in *Lepidobatrachus* and unusual larval morphologies of *Chacophrys* and *Ceratophrys* are well documented (Miranda-Ribeiro, 1923; Parker, 1931; Cei, 1968; Faivovich and Carrizo, 1992; Quinzio et al., 2006), as are several aspects of their anatomy and development (Fritzsch et al., 1987; Wassersug and Heyer, 1988; Ruibal and Thomas, 1988; Lavilla and Fabrezi, 1992; Ulloa Kreisel 2000, 2002; Vera Candioti, 2005; Fabrezi and Quinzio, 2008; Fabrezi and Lobo, 2009; Fabrezi, 2011; Bloom et al., 2013; Fabrezi and Cruz, 2014; Quinzio and Fabrezi, 2014), and a unique sound-producing mechanism in the larvae of *Ceratophrys* (Natale et al., 2011; Salgado Costa et al., 2014). The ontogeny of the postcranial skeleton and the massive skull have been well studied (Wild 1997, 1999; Fabrezi, 2011; Quinzio and Fabrezi, 2012; Ziermann et al., 2013), as has variation in the distinctive muscles employed in feeding and locomotion (Limeses, 1963; 1964; Reig and Limeses, 1963; Fabrezi and Lobo, 2009; Fabrezi et al., 2014); their carnivorous diet was recently reviewed by Schalk et al. (2014). The characteristic aggressive behavior of ceratophryids has been known since Raddi's (1823), Günther's (1882), and Hudson's (1892) pioneering observations, and described in detail by Barrio (1963). All ceratophryids are characterized by having a basic chromosome number of x = 13; however, interesting variation in ploidy level caught the attention of researchers in the 1960s (Beçak et al., 1967; Bogart, 1967; Morescalchi, 1967; Bogart, 1967; Barrio and Rinaldi de Chieri, 1970; Mercadal, 1981; Schmid et al., 1985; Soares-Scott et al., 1988; Vieira et al., 2006). Whereas *Chacophrys pierottii* and the three species of *Lepidobatrachus* are diploid (2n = 2x = 26), there are also diploid (2n = 2x = 26) and octoploid species (2n = 8x = 104) in *Ceratophrys,* yet no extant taxa are known to be tetraploid or hexaploid.

Ceratophryids are generally fossorial and are active during the rainy season regardless of whether they inhabit semiarid environments with a highly seasonal climate, humid grasslands, or rainforests (Fernández and Fernández, 1921; Vellard, 1948; Barrio, 1968a, b; Duellman and Lizana, 1994). When active, adults of *Lepidobatrachus* are mostly aquatic, whereas *Ceratophrys* and *Chacophrys* are more terrestrial. When humidity decreases, at least some ceratophryids are known to burrow into humid soil and produce a cocoon of dead skin that significantly decreases water loss in the dry soil during estivation (McClanahan et al., 1976, 1983; Bastos and Abe, 1998); according to an anecdotal report in captivity, they can persist in this situation for 20 months (Pisanó and Paz, 1954). Furthermore, most ceratophryids have an enlarged distal prehallical element that provides support for a heavily keratinized metatarsal spade used for digging (Fabrezi, 2001). Rapid larval development is known to occur in some species (Quinzio et al., 2006; Fabrezi, 2011).

A series of contributions by Barrio (1963), Limeses (1963, 1964, 1965a, b, 1968), Reig (1972), Reig and Cei (1963), Reig and Limeses (1963), and Lynch (1971) established the distinctiveness of the three genera currently included in Ceratophryidae on the basis of multiple sources of evidence. This distinct group received different ranks from tribe to family (Lynch, 1971, 1982; Heyer, 1975; Cei, 1980; Laurent, 1986), but its monophyly has never been questioned, possibly due to the number of diagnostic characters, several of which are today considered synapomorphies or putative synapomorphies advanced by the authors mentioned above.

The first quantitative analysis of the relationships of ceratophryids was Heyer's (1975) study of leptodactylid frogs using the monothetic group method, in which he included one exemplar each of *Ceratophrys* (*Ce. calcarata*) and *Lepidobatrachus* (*L. laevis*). Lynch (1982) presented a phylogenetic hypothesis focused on *Ceratophrys* on the basis of 20 phenotypic characters, with transformations polarized a priori. In a similar way, Mercadal (1986) presented a scheme of relationships for the species of *Ceratophrys* based on six characters. Maxson and Ruibal (1988) presented an analysis of albumin immunological distances among *Chacophrys,* one species of *Lepidobatrachus,* and five species of *Ceratophrys.*

The monophyly of Ceratophryidae was first tested in a modern, quantitative context using two exemplars (*Ceratophrys ornata* and *Lepidobatrachus laevis*) in the phylogenetic study of Haas (2003) on the basis of mostly larval morphology. Subsequent analyses of molecular data (e.g., Darst and Cannatella, 2004; Wiens et al., 2005, 2006: Supp. Data; Frost et al., 2006; Grant et al., 2006; Roelants et al., 2011: Supp. Data; Pyron and Wiens, 2011; Pyron, 2014: Supp. Data) using similar exemplars (or some including *Chacophrys pierottii* as well), corroborated the monophyly of this clade, always with high support. Fabrezi (2006) and Fabrezi and Quinzio (2008) obtained similar results using phenotypic characters of adults and larvae, with different datasets and taxonomic sampling.

Although species now included in *Chacophrys* and *Lepidobatrachus* have been considered species of *Ceratophrys* (Boulenger, 1919; Nieden, 1923; Parker, 1931; Vellard, 1948; Lynch, 1971), the limits of the latter have also changed, previously including a number of species now in the odontophrynid genus *Proceratophrys* (Boulenger, 1882; Cochran, 1955). The monotypic genus *Chacophrys* was considered first a synonym of *Ceratophrys* (Lynch, 1971) and then a putative hybrid between *Ce. cranwelli* and *L. llanensis* (Lynch, 1982). No support was found for this by Maxson and Ruibal (1988) or Faivovich and Carrizo (1992), and this hypothesis was rejected experimentally by Alt and Alt (1993).

Lynch (1971) presented the first comments on the complete composition of *Ceratophrys* as it is mostly considered today, whereby he included *Ce. aurita* (Raddi, 1823), *Ce. calcarata* Boulenger, 1890, *Ce. cornuta* (Linnaeus, 1768), *Ce. ornata* (Bell, 1843), *Ce. pierottii* Vellard, 1948, *Ce. stolzmanni* Steindachner, 1882, and *Ce. testudo* Andersson, 1945. Subsequently Barrio (1980) described *Ce. cranwelli,* and Mercadal de Barrio (1986) described *Ce. joazeirensis*. Lynch (1982) considered *Ce. testudo* a junior synonym of *Ce. cornuta,* whereas Mercadal de Barrio (1988) and Perí (1993) considered it a valid species. After an attempt to delimit the species of *Lepidobatrachus* by Reig and Cei (1963), Barrio (1968a, b) presented a thorough taxonomic review of the genus, clearly differentiating the three species currently included in the genus: *L. asper* Budgett, 1899, *L. laevis* Budgett, 1899, and *L. llanensis* Reig and Cei, 1963.

The fossil record assigned to Ceratophryidae is particularly abundant in comparison with the sparse fossil record of anurans in general (e.g., Sanchiz, 1998). Since the 19th century, several anuran fossil remains have been associated with this family (Günter, 1859; Ameghino, 1899), including remains from the Cretaceous (Báez and Perí, 1989; Evans et al., 2008, 2014) to the Holocene (Stoessel et al., 2008), and from Madagascar (Evans et al., 2008, 2014) to western Argentina (Casamiquela, 1963; Báez and Perí 1990; Contreras and Acosta 1998).

As summarized above, this small family of frogs has received relatively extensive attention; however, one aspect of the group that is essential for a complete understanding of their evolution is glaringly lacking: a robust phylogenetic hypothesis based on total evidence and broad taxon sampling. The abundance of information available on ceratophryids makes this group an ideal subject for a total evidence analysis. Furthermore, such a hypothesis is necessary in order to synthesize the vast amount of knowledge accumulated over decades of research on these fascinating frogs and to interpret their evolution in an explicitely historical framework. In anticipation of such a study, here we present the results of a molecular phylogenetic analysis of ceratophryids with the goals of: (1) exploring the relationships among its species, (2) studying the evolution of polyploidy, (3) studying the evolution of cocoon formation and larval development duration associated with surviving in semiarid environments, and (4) reviewing the ceratophryid fossil record that could be relevant as calibration points in molecular divergence time estimations.

MATERIALS AND METHODS

Taxon sampling

We included exemplars of *Chacophrys pierottii,* all three species of *Lepidobatrachus,* and seven of the eight species of *Ceratophrys*. The missing species, *Ce. testudo,* has not been collected since its original description and is known only from its holotype, a juvenile specimen (Andersson, 1945). Sequences for multiple specimens per species were available for all species except for *Ce. calcarata, Ce. ornata,* and *Ce. stolzmanni*.

Outgroups were selected on the basis of the results of Frost et al. (2006), Grant et al. (2006), Pyron and Wiens (2011), Fouquet et al. (2013), and Pyron (2014). Due to the incongruent or poorly supported position of Ceratophryidae in most of these analyses, we included as outgroups exemplars of the families in the clades recovered by the different analyses as closely related to Ceratophryidae. These include: Allophrynidae (*Allophryne*); Alsodidae (*Alsodes, Eupsophus,* and *Limnomedusa*); Bufonidae (*Amazophrynella, Bufo, Duttaphrynus, Melanophryniscus, Nannophryne,* and *Rhinella*); Centrolenidae (*Celsiella, Cochranella, Espadarana, Hyalinobatrachium, Ikakogi, Nymphargus,* and *Vitreorana*); Cycloramphidae (*Cyclorhamphus* and *Thoropa*); Hylodidae (*Crossodactylus* and *Hylodes*); Odontophrynidae (*Macrogenioglottus, Odontophrynus,* and *Proceratophrys*); and Rhinodermatidae (*Insuetophrynus* and *Rhinoderma*). Of special concern are Batrachylidae and Telmatobiidae, which were recovered as sister taxa of Ceratophryidae in several analyses with variable support (Faivovich et al., 2005; Frost et al., 2006; Grant et al., 2006; Fouquet et al., 2013; Blotto et al., 2013). From these we included one species of *Atelognathus,* the monotypic *Hylorina,* and one species of *Batrachyla*. We included just five species of *Telmatobius;* however, we consider this to be adequate considering that the levels of molecular diversity in this genus so far appear to be notably low (e.g., De la Riva et al., 2010; Sáez et al., 2014). We further included exemplars of other hyloid families and rooted the optimal trees with a hemiphractid, *Stefania evansi*.

Character sampling

The analysis included up to 8,200 bp per specimen. The mitochondrial gene sequences produced for this project include portions of cytochrome oxidase I (COI), cytochrome b, 12S, the intervening $tRNA^{Val}$, 16S, and a fragment including the complete upstream section of 16S, the intervening $tRNA^{Leu}$, NADH dehydrogenase subunit 1 (ND1), and $tRNA^{Ile}$ which was first incorporated by Wiens et al. (2005). The nuclear gene sequences produced include portions of seven in absentia homolog 1 (mistakenly called "Seventh in absentia" by Faivovich et al., 2005), exon 1 of rhodopsin, tyrosinase, recombination-activating gene 1 (RAG-1), proopiomelanocortin A gene (POMC; first employed by Wiens et al., 2005), and exon 2 of chemokine receptor 4 (CXCR4, first employed by Biju and Bossuyt, 2003). All the primers employed are the same as those employed by Faivovich et al. (2005),

with the addition of 16S-frog and tMet-frog (fragment of 16S + $tRNA^{Leu}$ + ND1 + $tRNA^{Ile}$; Wiens et al., 2005), CytbAR-H (used with MVZ15 to obtain a larger fragment of cytochrome b than the one employed by Faivovich et al., 2005; Goebel et al., 1999), POMC-1 and POMC-2 (Wiens et al., 2005), and CXCR4-C and CXCR4-G (Biju and Bossuyt, 2003). For COI we employed the primers AnF1-AnR1 designed by Mariana L. Lyra (ACHAAYCAYAAAGAYATYGG; CCRAARAATCARAADARRTGTTG).

DNA isolation and sequencing

Whole cellular DNA was extracted from ethanol-preserved tissues with the DNeasy (QIAGEN, Valencia, CA) isolation kit. Amplification was carried out in a 25-µl-volume reaction using Fermentas TAQ and reagents. For all the amplifications, the PCR program included an initial denaturing step of 30 s at 94°C, followed by 35 (mitochondrial gene fragments) or 45 (nuclear gene fragments) cycles of amplification (94°C for 30 s; 48–64°C for 30 s; 72°C for 60 s), with a final extension step at 72°C for 6 min. Polymerase chain reaction amplification products were cleaned using Exo I/SAP (Fermentas), and sequenced by a third party using fluorescent-dye labelled terminators (ABI Prism Big Dye Terminators v. 1.1 cycle sequencing kits; Applied Biosystems, Foster City, CA) with an ABI 3730XL (Applied Biosystems, Foster City, CA); all samples were sequenced in both directions to check for potential errors. Chromatograms obtained from the automated sequencer were read and contigs made using the sequence editing software Sequencher 3.0. (Gene Codes, Ann Arbor, MI). Complete sequences were edited with BioEdit (Hall, 1999). See Appendix S1 for a list of specimens and locality data, and Appendix S2 for GenBank numbers.

Phylogenetic analysis

The rationale for using parsimony as an optimality criterion was advanced by Farris (1983) and discussed, among others, by Goloboff (2003) and Goloboff and Pol (2005). The phylogenetic analyses included treatment of DNA sequences both as dynamic homologies and as static homology hypotheses. The consideration of sequences as dynamic homologies simultaneously with tree searches has been discussed and justified by Wheeler (1996, 2002, 2012), De Laet (2005), Kluge and Grant (2006), and Grant and Kluge (2009). Static alignments (multiple alignments) independent of tree searches are the most common procedure in molecular phylogenetics, regardless of the omnipresent and ignored problem of the lack of an optimality criterion to choose among competing alignments. Though our sympathies rest with direct optimization, we realize that many colleagues disagree, and so, with the objective of collegiality, we performed a multiple sequence alignment (see below) and analyzed it using both parsimony and Bayesian inference.

The phylogenetic analysis under direct optimization was performed with POY5.1.1 (Varón et al., 2010, 2011), using equal weights for all transformations (substitutions and insertion/deletion events). Sequences of 12S, 16S, $tRNA^{Val}$, $tRNA^{Leu}$, and $tRNA^{Ileu}$ were preliminarily delimited in sections of putative homology (Wheeler et al., 2006), and equal-length sequences of protein-coding genes were considered as static alignments to accelerate the searches.

Searches were performed using the command "Search". This command implements a driven search building Wagner trees using random addition sequences (RAS), Tree Bisection and Reconnection (TBR) branch swapping followed by Ratchet (Nixon, 1999), and Tree Fussing (Goloboff, 1999). The command (Search) stores the shortest trees of each independent run and does final tree fusing using the pooled trees as a source of topological diversity. The resulting topologies were submitted to a final round of TBR using iterative pass optimization (Wheeler, 2003).

Phylogenetic analyses using POY were executed in parallel using the Museu de Zoologia da Universidade de São Paulo's high-performance computing cluster Ace, which consists of 12 quad-socket AMD Opteron 6376 16-core 2.3-GHz CPU, 16 MB cache, 6.4 GT/s compute nodes (= 768 cores total), eight with 128 GB RAM DDR3 1600 MHz (16 × 8 GB), two with 256 GB (16 × 16 GB), and two with 512 GB (32 × 16 GB), and QDR 4X InfiniBand (32 GB/s) networking.

We also performed a multiple alignment with MAFFT v.7 (Katoh and Standley, 2013). For the regions of 12S, $tRNA^{Val}$, and 16S, and the fragment including the complete upstream section of 16S, the intervening $tRNA^{Leu}$, NADH dehydrogenase subunit 1 (ND1), and $tRNA^{Ile}$ we employed the alignments generated with Q-INS-i strategy (secondary structure of RNA is considered), whereas the alignments for the remaining genes were generated with G-INS-i (global homology considered). For the phylogenetic analysis using parsimony we employed T.N.T Willi Hennig Society Edition (Goloboff et al., 2008). Searches were done using the new technology search under search level 50, which included sectorial searches, tree drift and tree fusing (Goloboff, 1999), and requesting the driven search to hit the best length 100 times. Parsimony Jackknife absolute frequencies (Farris et al., 1996) were estimated using new technology as well requesting 10 hits with driven searches, for a total of 1,000 replicates. Trees were edited with FigTree (Rambaut, 2014).

For the bayesian analysis, models for each partition were chosen with jModelTest v0.1.1 (Posada, 2008), a modification of Modeltest (Posada and Crandall, 1998). First, second, and third codon positions were treated as

separate partitions for each protein-coding gene. The regions of 12S, $tRNA^{Val}$, 16S, $tRNA^{Leu}$ and $tRNA^{Ile}$ were treated as a single partition for model selection. The Akaike Information Criterion (AIC) was used to select the best fitting model for each gene (Pol, 2004; Posada and Buckley, 2004). Bayesian analyses were performed in MrBayes 3.2 (Ronquist et al., 2012) in the CIPRES web cluster (Miller et al., 2010). Analyses consisted of four runs, each consisting of two replicate Monte-Carlo Markov Chains. Each run used four chains and default settings of priors (Dirichlet for substitution rates and state frequencies, uniform for the gamma shape parameter and proportion of invariable sites, all topologies equally likely a priori, and branch lengths unconstrained: exponential). Two analyses running 60 million generations were performed (with a burn-in fraction of 0.20). Stabilization of resulting parameters was evaluated using Tracer (Rambaut et al., 2014). Uncorrected p-distances were calculated in PAUP* (Swofford, 2002).

RESULTS

The analysis using direct optimization resulted in 10 equally parsimonious tree/alignment combinations of 38,965 steps, swapping of these topologies under iterative pass resulted in no topological changes and a tree-length of 38,844 steps; one of the optimal topologies is shown in Fig. 1; see Figure S1 for outgroup topology. The best length was hit 354 times. The analysis of static parsimony resulted in six equally parsimonius trees of 39,780 steps. Relationships of Ceratophryidae recovered in the Bayesian analysis are the same as those of the direct optimization (see Figure S2) and are not further discussed. All conflict among equally parsimonius trees in the direct optimization and static parsimony analyses is restricted to internal relationships among exemplars of *Ceratophrys cranwelli, Chacophrys pierottii,* and *Lepidobatrachus llanensis*. The monophyly of Ceratophryidae, as well as the individual monophyly of *Ceratophrys* and *Lepidobatrachus* are supported in these analyses with 99–100% Jackknife frequency. The position of *Chacophrys pierottii* is poorly supported, being the sister taxon of *Lepidobatrachus* in the analysis using direct optimization, and the sister taxon of *Ceratophrys* + *Lepidobatrachus* in the static parsimony analysis. Other than this difference, all analyses recovered the same topology for Ceratophryidae. *Lepidobatrachus asper* is the sister taxon of a clade with 100% Jackknife frequency composed of *L. laevis* + *L. llanensis*.

Ceratophrys is composed of two main clades. One of these, with 60% Jackknife frequency, includes *Ce. stolzmanni* as the sister taxon of a clade composed of *Ce. calcarata* + *Ce. cornuta* (100% Jackknife frequency). The other clade (100% Jackknife frequency) includes *Ce. aurita* + *Ce. joazeirensis* as the sister taxon of *Ce. cranwelli* + *Ce. ornata*.

DISCUSSION

Outgroups

The inclusion of multiple outgroups was intended to present a stringent test of the monophyly of Ceratophryidae and its possible relationships with other clades. As such, we consider our results only in that context, and do not consider our analysis to constitute a test of previous hypotheses regarding relationships among other clades.

Our results recover relationships among outgroups that, overall, differ little from other studies in that exemplars of most currently recognized hyloid families (Centrolenidae, Hylidae, Dendrobatidae, Bufonidae, Odontophrynidae, Hylodidae, Batrachylidae, Alsodidae, and Rhinodermatidae) are monophyletic (Figure S1). Unlike other studies (Frost et al., 2006; Pyron and Wiens, 2011; Fouquet et al., 2013; Pyron, 2014: Supp. Data), our results failed to recover the monophyly of Leptodactylidae, obtaining instead a non-monophyletic Leiuperinae, distantly related to Leptodactylinae + Paratelmatobiinae. Like other studies, Cycloramphidae is not recovered monophyletic, and *Limnomedusa* is obtained in an alternative position. Relationships among most clades have jackknife frequencies < 50%. A sister group relationship between Telmatobiidae and Ceratophryidae has been obtained by some studies with different taxon sampling (Faivovich et al., 2005; Wiens et al., 2005; Grant et al., 2006; Blotto et al., 2013; Fouquet et al., 2013) but not in the re-analyses of GenBank sequences by Pyron and Wiens (2011) and Pyron (2014) or the present analysis, where these groups are only distantly related.

Ceratophryid relations

Our results highly support the individual monophyly of *Ceratophrys* and *Lepidobatrachus*, unlike recent analyses of GenBank sequences by Pyron and Wiens (2011) and Pyron (2014), in which *Ceratophrys* was found to be paraphyletic with respect to *Chacophrys* and *Lepidobatrachus*. We detected a number of problems with misidentified sequences in their analyses (See Appendix S3). However, these involve accidental chimeras among different species of *Ceratophrys,* a clade with a well-corroborated monophyly in our analysis, so they probably do not explain, by themselves, the non-monophyly of this genus found by these authors.

The phylogenetic position of *Chacophrys* among ceratophryids has been contentious. In the molecular analyses of Grant et al. (2006) and Frost et al. (2006), who included one exemplar of each genus, *Lepidobatrachus* was found to be the sister taxon of *Chacophrys* + *Ceratophrys*. Fabrezi (2006) used a diverse outgroup sampling, 80 phenotypic characters of adults and larvae, and exemplars

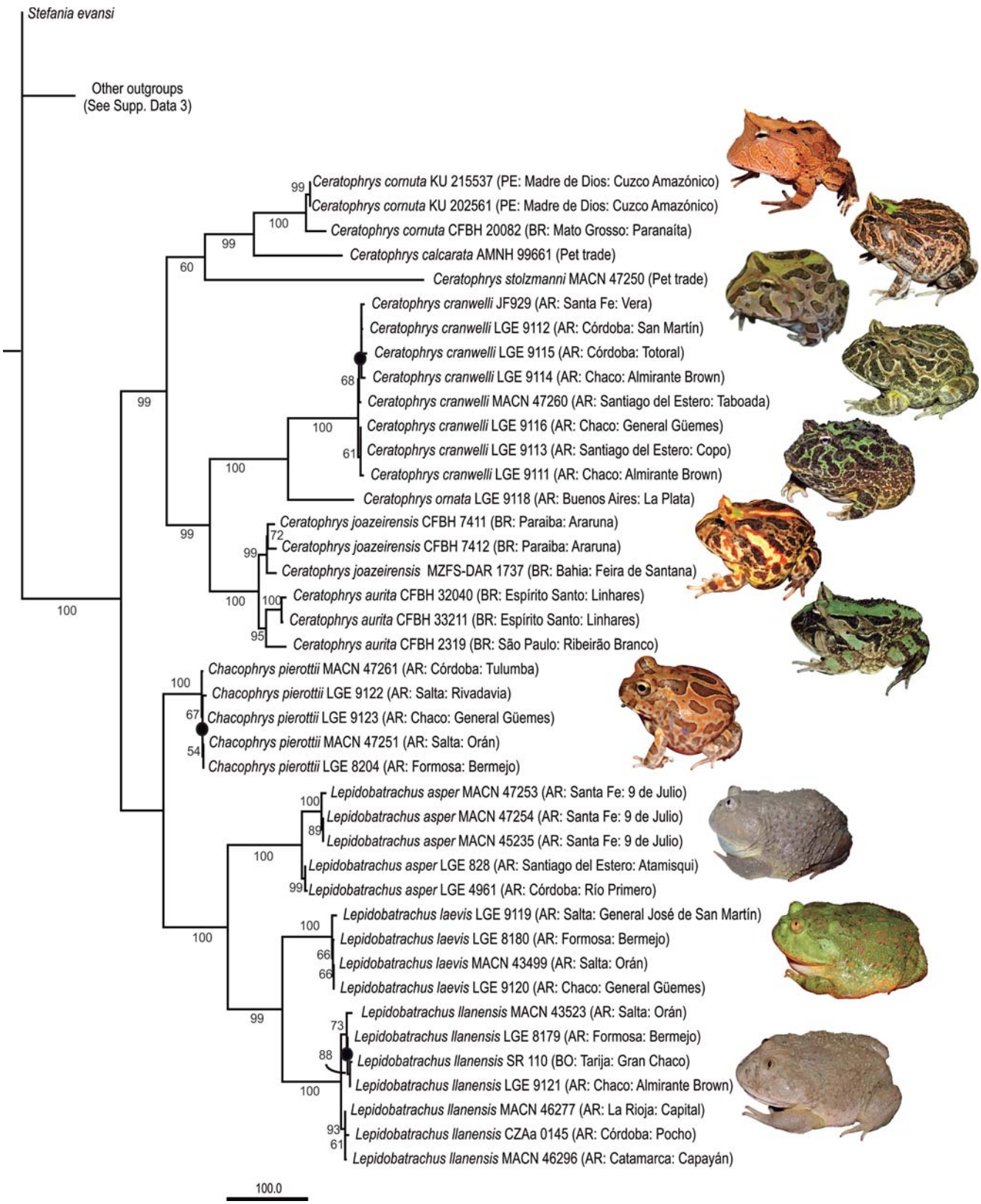

Figure 1. Phylogenetic relationships of Ceratophryidae as recovered in one of the 10 most parsimonious trees obtained with direct optimization (length 38,844 steps) under equal weights for all transformations. Black circles indicate nodes that collapse in the strict consensus. Values around nodes are parsimony jackknife frequencies estimated for the static alignment analyzed with parsimony in T.N.T. with gaps as fifth state. Nodes lacking values have < 50% jackknife frequencies. See Figure S1 for outgroup relationships. Institutional collection codes follow Sabaj Pérez (2014), with the exceptions noted in Appendix S1.

of Ceratophryidae *Chacophrys, Ce. cranwelli, L. laevis,* and *L. llanensis,* resulting in the topology *Ceratophrys* + (*Chacophrys* + *Lepidobatrachus*). Fabrezi and Quinzio (2008) greatly reduced the outgroup sampling of Fabrezi (2006), while expanding and modifying the characters, and found a topology that included *Chacophrys* + (*Ceratophrys* + *Lepidobatrachus*).

The present analysis is the first to combine a dense character sampling, with most known extant diversity of Ceratophryidae and extensive outgroup sampling of exemplars of most nobleobatrachian families. Despite these efforts, our results indicate an unsupported position of *Chacophrys* as the sister taxon of *Lepidobatrachus,* with a Jackknife frequency value < 50% (Fig. 1).

Considering that our taxon sampling of extant species in the family is nearly complete, this lack of supported resolution could have several explanations that mostly belong to speculation. It could be a simple methodological problem, such as the absence of informative characters for the relevant node resulting from insufficient character sampling. In this respect, we look forward to the combination of our data with a phenotypic dataset incorporating all the available informative variation in ceratophryids in a total evidence analysis that could further test the relationships of *Chacophrys* with *Ceratophrys* and *Lepidobatrachus*. On the other hand, we might also be missing important parts of the diversity that arose during the evolutionary history of the family, resulting in a depauperate extant diversity. Similar arguments could be raised for the 60% jackknife support for the monophyly of *Ce. stolzmanni* + (*Ce. calcarata* + *Ce. cornuta*).

Chacophrys

Chacophrys pierottii has a wide distribution in the western Chacoan region. It occurs in Alto Paraguay, Boqueron, and Presidente Hayes in Paraguay (Brusquetti and Lavilla, 2006); Chuquisaca, Santa Cruz and Tarija, in eastern Bolivia (De la Riva et al., 2000); and Santiago del Estero, Chaco, Formosa, and in the dry Chacoan areas of Córdoba, La Rioja, San Luis, San Juan, and Catamarca in Argentina. Our study includes specimens from Chaco, NW Córdoba, Formosa, La Rioja, and Salta. 16S p-distances among them are very low, 0.0–0.2% (Table S1.1). The biology of *Chacophrys pierottii* remains poorly known and is restricted to sparce information on natural history of their tadpoles (Faivovich and Carrizo, 1992; Quinzio et al., 2006), diet of juveniles (Pueta and Perotti, 2013), aggressive behavior, and advertisement and aggressive calls (Lescano, 2011).

Lepidobatrachus

Whereas there is extensive evidence supporting the monophyly of *Lepidobatrachus,* this hypothesis has not been tested in the context of an analysis that includes all the extant species. Previous studies (Fabrezi, 2006; Fabrezi and Quinzio, 2008) have included *L. laevis* and *L. llanensis,* or only *L. laevis* (Frost et al., 2006; Pyron and Wiens, 2011; Pyron, 2014), or only *L. llanensis* (Darst and Cannatella, 2004; as *Lepidobatrachus* sp., see Appendix S3).

Our sampling of *Lepidobatrachus asper* includes specimens from distant localities within the main area of distribution of this species in Argentina (Barrio, 1968a, b; Faivovich, 1994) in southern Santiago del Estero, along the salt flats of the Saladillo river (type locality of *L. salinicola* now considered a junior synonym of *L. asper*) to northeastern Córdoba, and northern Santa Fe and southwestern Chaco provinces, with two isolated records in western Corrientes. The species has also been recorded in a few localities in the Paraguayan Chaco (Faivovich, 1994), including its type locality, from which we lack samples. Barrio (1968a, b) noticed differences in pigmentation in adults from populations in northern Santa Fe and Santiago del Estero provinces, but considered this to be geographic variation. The sequences from our specimens differ minimally (0.2–0.5%, see Table S1.2). It is possible that populations from Argentina are actually continuous, as the westernmost known localities for *L. asper* in Santa Fe are not very distant from those in eastern Córdoba. Although *L. laevis* and *L. llanensis* have been studied extensively from different perspectives during the last 30 years (e.g., most papers dealing on *Lepidobatrachus* cited throughout this paper), *L. asper* remains the least known species of the genus.

Our sampling of *Lepidobatrachus laevis* and *L. llanensis* includes specimens from a few distant localities from throughout their known ranges, particularly in Argentina, but also from Bolivia (*L. llanensis*). There are two main areas of the distribution of *L. llanensis*. One includes the extreme western Chacoan plains, in Catamarca, Córdoba, and La Rioja, and the other includes the Chacoan plains of eastern Salta, northern Santiago del Estero, northern Chaco, and central-western Formosa. It is possible that the population known in the Bolivian Chaco (Reichle et al., 2004) belongs to the same area (16S p-distances between that sample and those from Salta and Chaco are 0.0–0.4%; Table S1.2), while there is an isolated record in northern Paraguay (Faivovich, 1994; Brusquetti and Lavilla, 2006). The 16S p-distance among our exemplars from both areas is 0.9% (see Table S1.2).

Lepidobatrachus laevis is known from a number of localities in eastern Salta, Chaco, central-western Formosa, and Santa Fe in Argentina, Chuquisaca, Santa Cruz and Tarija, in Bolivia (De la Riva et al., 2000), and Alto Paraguay, Boquerón, and Presidente Hayes, in Paraguay (Brusquetti and Lavilla, 2006). 16S p-distances among our exemplars from localities in Chaco, Salta, and Formosa, in Argentina are quite low (0.0–0.2%, see Table S1.2). A number of known areas of distribution for the three

species, particularly in Paraguay, have not been sampled. Although we know no reasons why these could be relevant for our phylogenetic analysis, they would provide a more complete idea of the level of intraspecific sequence variation.

Ceratophrys

Our optimal topology is mostly congruent with the non-quantitative phylogenetic proposal of Lynch (1982) for *Ceratophrys,* with the notable exception of the relationship between *Ce. cranwelli* and *Ce. ornata*. Lynch suggested that *Ce. aurita* and *Ce. ornata* were sister taxa on the basis of their octoploid karyotype (*Ce. joazeirensis* was not yet described at that time). In a non-quantitative phylogenetic hypothesis, Mercadal (1986) even considered *Ce. joazeirensis* to be the sister taxon of *Ce. ornata* on the basis of the octoploid karyotype, with this clade being considered the sister taxon of *Ce. cranwelli*. These hypotheses are not supported by our results. Lynch (1982) further recognized two subgenera in *Ceratophrys:* a nominal subgenus including *Ce. aurita, Ce. cranwelli,* and *Ce. ornata,* and the subgenus *Stombus* for *Ce. calcarata, Ce. cornuta,* and *Ce. stolzmanni*. Although our results are congruent with this proposal, these subgeneric names enjoyed no subsequent usage, so we are agnostic about them.

Our results show that our exemplars of *Ceratophrys aurita* from Espírito Santo and São Paulo are the sister taxon of the exemplars of *Ce. joazeirensis* from Bahia and Paraiba. The molecular divergence between the two species in the 16S fragment is 1.2–1.5% (see Table S1.3), only slightly greater than the intraspecific distances of *Ce. aurita* (0.9%). Curiously, the 16S distances between our specimen of *Ce. aurita* from southern São Paulo and those from Espírito Santo (0.9%) are only slight less than the 16S distance between that specimen and *Ce. joazeirensis* from Bahia (1.2%). *Ceratophrys joazeirensis* has been considered an endemic species of the semiarid Caatinga of northeastern Brazil. It is known from a few localities from Rio Grande do Norte southwards to northern Bahia and recently was recorded in the Cerrado in central Minas Gerais (Maciel et al., 2013). Since its original description there has been an emphasis on the morphological similarities between *Ce. joazeirensis* and *Ce. cranwelli* (Mercadal, 1986; Mercadal de Barrio and Barrio, 2002; Vieira et al., 2006), mostly their color pattern, and their distribution in semiarid areas. Our results strongly support the monophyly of *Ce. aurita* + *Ce. joazeirensis,* suggesting that the supposed similarity of *Ce. joazeirensis* and *Ce. cranwelli* should be reassessed. Furthermore, recently published photographs (Maciel et al., 2013: fig. 1A; Santana et al., 2014: fig. 2) show color patterns in specimens identified as *Ce. joazeirensis* that, if anything, resemble *Ce. aurita*. In fact, the dorsal patterns of non-captive bred individuals of the four species of *Ceratophrys* inhabiting semiarid areas, and *Chacophrys pierottii* in general, are remarkably similar.

Considering the low number of exemplars of both *Ceratophrys aurita* and *Ce. joazeirensis* and the intraspecific sequence divergence in *Ce. aurita,* a densely sampled phylogeographic study including exemplars of both of these species from throughout their distributions is needed. Such a study could shed light on the taxonomic status of *Ce. joazeirensis* and the history of apparent habitat switching in this clade (see below). The biology of both of these species remains remarkably poorly known.

Ceratophrys cranwelli and *Ce. ornata* have been repeatedly considered "diploid-octoploid counterparts" or a "diploid-octoploid cryptic species pair" (Bogart and Wasserman, 1972; Barrio, 1980; Mercadal, 1981, 1986; Mercadal de Barrio, 1987; Mercadal de Barrio and Barrio, 2002). However, this cannot necessarily be taken to mean that these two species are sister taxa, although this is recovered with high support in our analysis (Fig. 1). The meaning of the expression "diploid-octoploid counterparts" is ambiguous in a phylogenetic context, as it could be interpreted to mean sister species (e.g., Bogart and Wasserman, 1972) or be devoid of an explicit phylogenetic meaning (Beçak et al., 1970; Batistic et al., 1975; Beçak and Beçak, 1998; Martino and Sinsch, 2002). In the latter sense, Mercadal (1986) considers *Ce. cranwelli* and *Ce. ornata* a "diploid-octoploid cryptic species pair" yet suggests that *Ce. cranwelli* is the sister taxon of *Ce. joazeirensis*.

Ceratophrys cranwelli and *Ce. ornata* are the two better-known species in the genus and have been studied from multiple perspectives, many times as the sole exemplars of *Ceratophrys*. Their sister group relationship provides an explicit historical context for examining these numerous, varied studies.

Ceratophrys stolzmanni is a poorly known species that only recently has attracted attention (Ortiz et al., 2013). It is known to occur in xeric environments of the Pacific coastal dry shrub and deciduous forests around the gulf of Guayaquil in the province of Manabí, Ecuador, to northern Peru (Ortiz et al., 2013). Peters (1967) recognized populations from both areas as different subspecies, the nominal one in Peru and *Ce. stolzmanni scaphiopeza* in Ecuador; however it is unclear if the gaps separating these populations are real or the result of inadequate sampling. The diagnostic characters provided by Peters (1967) for both subspecies pertain mostly to skin texture and perceived skin thickness. We have no experience with this species in particular, but our experience with other ceratophryids indicates that the status of *Ce. stolzmanni scaphiopeza* requires serious reevaluation. The aspects of the reproductive biology of the Ecuadorian populations recently reported by Ortiz et al. (2013) is all that has been published on the biology of *Ce. stolzmanni,* and almost all of that was done in captivity.

Ceratophrys calcarata is another poorly known species that has been referred to marginally in the literature. Ruthven's (1922) brief report on its aggressiveness, La Marca's (1986) description of its tadpole, Murphy's (1976) observations on pedal luring behavior on a captive specimen, and Schalk et al.'s (2014) report on stomach contents remain the only information available about this species. Its geographic distribution also deserves some clarification, as both Rivero (1961) and Lynch (1982) referred to populations in the state of Apure, Venezuela, but Rueda et al. (2004) stated that these records require corroboration. Schalk et al. (2014) refer to a voucher specimen from the state of Amazonas, in Puerto Ayacucho. This would confirm that *Ce. calcarata* has a much broader distribution. An actual comparison of populations of the semiarid Caribbean lowlands, the main area where the species is known, with those of Amazonia, would be the minimum needed to assess if a taxonomic reevaluation of the latter is necessary.

Ceratophrys cornuta is the most widely distributed species of ceratophryid, being present in the Amazon basin, with records from Bolivia, Brazil, Colombia, Peru, Ecuador, and the Guyanas. Our samples include one exemplar from Cuzco Amazónico, Peru, and one from Mato Grosso, Brazil, separated by ca. 1200 km (airline). The 16S sequences differ in only 0.5–0.6% (Table S1.4). A full study on this species is necessary to better understand the extent of its variation. The biology of this species is relatively well known (Duellman and Lizana, 1994; Duellman, 2005; Pyke and Ray, 2006), although possibly less than would be expected for such a widely distributed species.

The only extant species of Ceratophryidae missing in our analysis is *Ceratophrys testudo*. This species is only known from its holotype, a juvenile specimen, and has been considered a valid species distinct from *Ce. cornuta* by Mercadal (1988) and Perí (1993a). These authors based their position on comparisons with a low number of juveniles of the latter (one available to each author), and therefore not taking into account possible intraspecific and geographic variation of the most widely distributed species of ceratophryid. The status of *Ce. testudo* should be carefully reassessed.

Dry habits die hard: Diversification in semiarid environments

A number of peculiar characteristics in ceratophryids, like the formation of a cocoon of dead skin to reduce water loss (McClanahan et al., 1976) and the short larval period and accelerated growth rates (Fabrezi, 2011; Fabrezi and Cruz, 2014), have been considered specializations associated with semiarid environments, where ephemeral pools dry fast and adults estivate during extensive dry periods.

The reduction of water loss to the surrounding dry soil in fossorial anurans is attained through a decrease in skin permeability or an increase in the osmolarity of body fluids (Shoemaker et al., 1992). *Lepidobatrachus llanensis* was well studied in this regard by McClanahan et al. (1976, 1983) and is known to produce a cocoon of up to 50 unshed layers of *stratum corneum*. The accumulation of the layers is accompanied by a rapid decrease in evaporative water loss in laboratory conditions, reaching a minimum after about a month (McClanahan et al., 1983). In natural conditions it has been believed that the cocoon functions by preventing water loss to the dry soil (Shoemaker et al., 1992), as recently demonstrated for *Litoria australis* (Reynolds et al., 2010).

Cocoon production has been observed to occur in the three species of *Lepidobatrachus* (McClanahan et al., 1976, 1983; J. Faivovich, pers. obs.; J. C. Stazzonelli, pers. comm.), *Chacophrys pierottii* (J. Lescano, pers. comm.), *Ceratophrys cranwelli* (as *Ce. ornata,* McClanahan et al., 1976), *Ce. joazeirensis* (C. Jared and M.M. Antoniazzi, pers. comm.), *Ce. aurita* (Bastos and Abe, 1998), *Ce. ornata* (Canziani and Cannata, 1980; Jared and Antoniazzi, pers. comm.; F. Kolenc, pers. comm.), and *Ce. stolzmanni* (P. Janzen, pers. comm.). There are no references to cocoon formation in *Ce. calcarata* and *Ce. cornuta*. Our results, however, suggest its occurrence in these species on the basis of parsimony, and that a cocoon was present as well in the hypothetical ancestor of Ceratophryidae. Interestingly, *Ce. aurita* and *Ce. ornata* inhabit the Atlantic Forest and the Pampean grasslands, respectively; although these areas present different degrees of seasonality, both have levels of humidity that far exceed those of the Chacoan region and the Caatinga (Bucher, 1982; McNaughton et al., 1993). Most other anurans known to produce cocoons are from semiarid or arid environments or subhumid regions with a prolonged dry season (for review see Hillman et al., 2009).

Short larval periods and accelerated growth rates have been documented in the wild by Fabrezi (2011) for *Chacophrys pierottii, Lepidobatrachus laevis,* and *L. llanensis* (15–18 days), and *Ceratophrys cranwelli* (20–24 days). Ruibal and Thomas (1988) reported 30 days to complete metamorphosis in captive-bred *L. laevis* and mentioned that for some larvae it took just 20 days. In captive-bred *Ce. stolzmanni,* metamorphosis was found to be completed in 20–32 days (Ortiz et al.,2012), whereas in captive-bred *Ce. ornata* metamorphosis was completed in 30 (Kollros and Bovbjerg, 1997) or 32–36 days (Honneger et al., 1985). No information is available about the length of larval periods of the other ceratophyids. Recently, Fabrezi and Cruz (2014) reported that *Ce. cranwelli, Ch. pierottii, L. laevis,* and *L. llanensis* show low activity of the thyroid glands during larval development and particularly during the metamorphic climax, contrary to what has been assumed to occur in anurans (Etkin, 1936).

In general, data on the duration of larval periods in anurans is scarce, and it seems reasonable that comparisons of larval periods should be done with precaution, particularly when some have been studied in the wild and others in captivity. Considering this, we are reluctant to assume that the developmental times in *Ceratophrys ornata* and *Ce. stolzmanni* are necessarily the same as in *Ce. cranwelli;* however, we should note that the overlapping (*Ce. stolzmanni*) or continuous ranges of development time (*Ce. ornata*) are quite suggestive of a common mechanism underlying their short developmental times and accelerated growth rates. This requires further testing, and this could be approached both with field observations and studies on the histology of the thyroid glands (e.g., Fabrezi and Cruz, 2014).

The optimization of habitats on our hypothesis indicates that most diversification in ceratophryids occurred in semiarid environments, with three independent transitions to different humid environments (Fig. 2). One of these is associated with the origin of *Ceratophrys cornuta* that is widespread in the Amazon basin. Another is associated with the origin of *Ce. aurita,* which occurs throughout the Atlantic Forest, and the other is associated with the origin of *Ce. ornata* in the humid Pampean grasslands. The inference that ceratophryids diversified primarily in semiarid environments provides an elegant phylogenetic explanation for the occurrence of a cocoon in at least *Ce. aurita* and *Ce. ornata* (Fig. 2). In the same way, if at least *Ce. ornata* is confirmed to have a short larval period and accelerated growth rates, our hypothesis would provide a historical explanation for that phenomenon as well.

The persistence of a plesiomorphic cocoon formation in species inhabiting humid areas might also occur in other anuran radiations associated with arid or semiarid environments. A potentially similar situation to our finding in ceratophryids might be that of the clade composed of the species of the pelodryadine hylid genus *Litoria* that were formerly placed in *Cyclorana*. At least two species

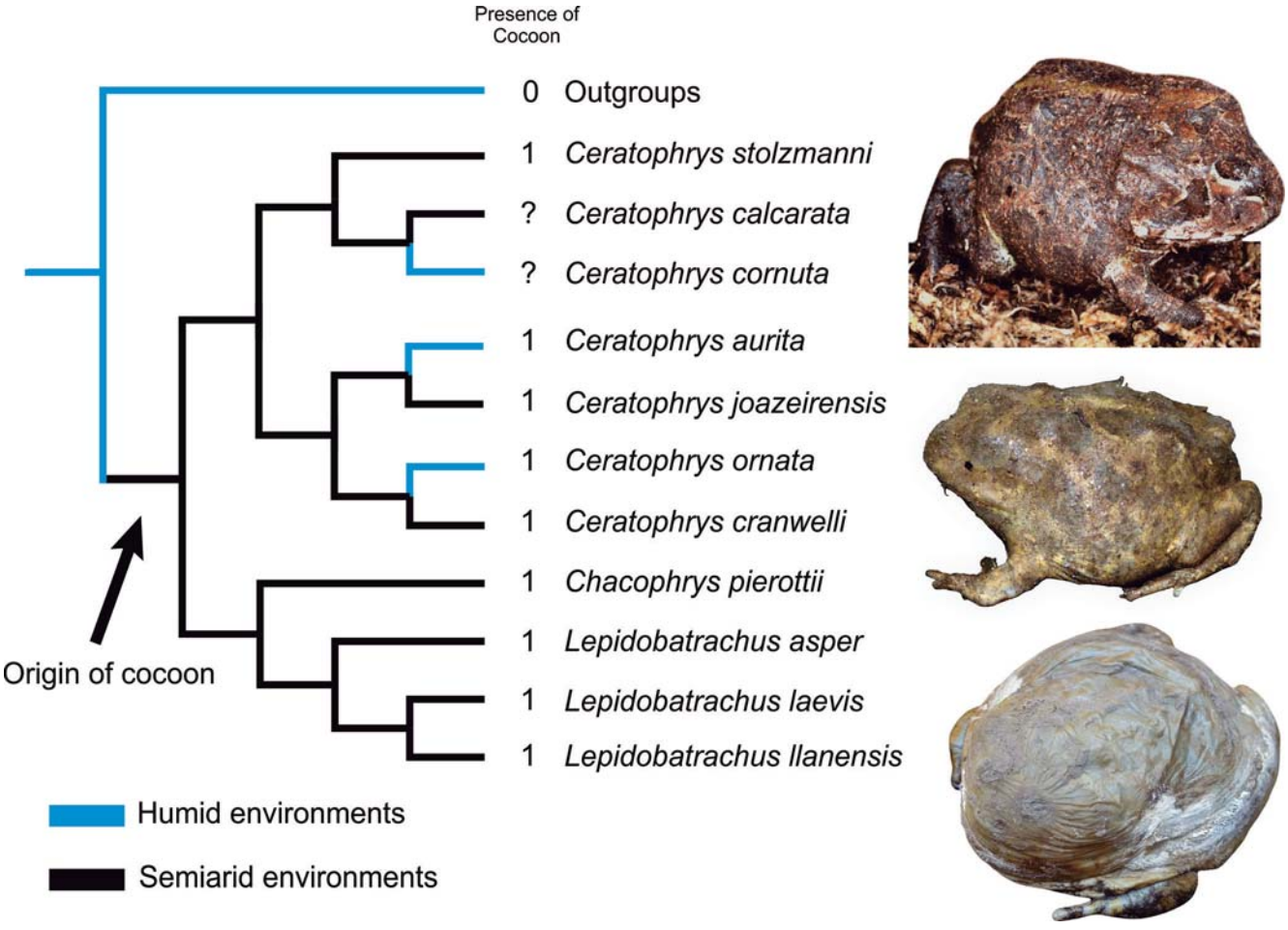

Figure 2. Transformations in the environments occupied by ceratophryid frogs during the evolutionary history of the group in relation to the occurrence of cocoon formation, a mechanism that prevents water loss during estivation. Our results indicate a primary diversification of ceratophryid frogs in semiarid environments (black), with at least three different events leading to the occupation of humid environments (blue), providing an explanation for the occurrence of cocoon formation in those species. The cocooned specimen on the upper right is *Chacophrys pierottii* (Photo by J. Lescano), the one in the center is *Ceratophrys stolzmanni* (Photo by P. Jenzen), and the one on the lower right is *Lepidobatrachus laevis* (Photo by J.C. Stazzonelli).

are known to estivate by forming cocoons in subhumid to wet tropical areas in northern Australia, as done by other closely related species in semiarid or arid environments (Withers, 1995, 1998; Withers and Thompson, 2000). Unfortunately the occurrence of estivation and cocoon formation is still unknown in several species of this clade, and although there are phylogenetic hypotheses available for this clade, the relationships among most of its species remain poorly supported and unstable in different analyses (Rosauer et al., 2009; Pyron and Wiens, 2011).

Groom et al. (2013) recently noticed that *Ceratophrys ornata* does not present a depressed metabolic rate during estivation, as occurs in other estivating frogs, adding that a limited response has been observed in *Ce. aurita* as well (Bastos and Abe, 1998) and generalizing that this might be the common condition in *Ceratophrys*. However, this is not what occurs in *Lepidobatrachus,* where McClanahan et al. (1983) reported metabolic depression in cocooned *L. llanensis* to 25% of the resting metabolic rate, as is known to occur in several estivating frogs from semiarid or arid environments (e.g., the limnodynastid genus *Neobatrachus* and some species of the hylid genus *Litoria* formerly included in *Cyclorana;* Withers, 1993). Neither of the species of *Ceratophrys* on which Groom et al. (2013) generalized to all *Ceratophrys* inhabit semiarid environments, so this generalization requires further testing. It could be that the species studied so far show modifications associated with estivation in less unpredictable environments than those of their close relatives inhabiting semiarid environments. This situation occurs in the clade of pelodryadines mentioned above. Whereas species from semiarid and subhumid areas are known to estivate and form cocoons, the metabolic rate depression is less pronounced in the few species studied from subhumid and tropical areas (Withers and Thompson, 2000).

Canziani and Cannata (1980) showed that the rate of evaporative water loss in *Ceratophrys cranwelli* (considering them as Chacoan populations of *Ce. ornata*) is lower than in *Ce. ornata,* whereas the rehydration rate is higher in the former than in the latter. They also conclude that bladder capacity is greater in *Ce. cranwelli* than in *Ce. ornata*. On the basis of our results, it is possible that the reduced rate of evaporative water loss, the lower rate of dehydration, and the larger bladder capacity of *Ce. cranwelli,* are actually ceratophryid plesiomorphies associated with their original diversification in semiarid environments.

Another characteristic present in ceratophryids that has been associated with the reduction of evaporative water loss is the presence of co-ossified dermis in the skull and dorsal bony shields (DeMar, 1966; Elkan, 1968; Trueb, 1970; Ruibal and Shoemaker, 1984). Cranial co-ossification has been shown in some hylids to greatly decrease evaporative water loss with respect to the non co-ossified skin (Seibert et al., 1974), and to greatly reduce overall water loss when associated with water conservation behavior (Andrade and Abe, 1997). The presence of a bony shield has been suggested as a possible mechanism of water retention (DeMar, 1966; Ruibal and Shoemaker, 1984), although there is still no experimental evidence. More recently, osteoderms and integumental dermal bone in general have been suggested to be involved in the buffering of CO_2 (Janis et al., 2012).

The calcified or Eberth-Katschenko (E-K) layer of the dermis, has also been related to reduction of water loss. The E-K layer is located in the dermis between the *stratum spongiosum* and the *stratum compactum*. It consists of glycosaminoglycans associated with mineral deposition as calcium. Its putative function as preventing water loss in anurans has been inferred mainly by its occurrence in terrestrial species and its absence in most aquatic species (Elkan, 1968, 1976; Toledo and Jared, 1993).

Our optimal topologies recover *Lepidobatrachus asper* as the sister taxon of *L. laevis* + *L. llanensis* (Fig. 1). This is an interesting result in that both *L. asper* and *L. llanensis* share the presence of a dorsal bony shield (Barrio, 1968a, b; Quinzio and Fabrezi, 2012). A dorsal bony shield also occurs in the four species of one of the two major clades of *Ceratophrys* (*Ce. aurita, Ce. cranwelli, Ce. joazeirensis,* and *Ce. ornata*), where it is a larger shield differing from that in *Lepidobatrachus* in being composed of several individual plates, instead of one or two medial elements (Lynch, 1982; Quinzio and Fabrezi, 2012). Furthermore, shields of *Lepidobatrachus* develop during premetamorphic stages, whereas shields of *Ceratophrys* develop postmetamorphically, sometime between juvenile and adult stages (Quinzio and Fabrezi, 2012). In the context of our results, the optimization of the sole presence of a dorsal shield indicates its independent origin within *Ceratophrys* and in *Lepidobatrachus*. Furthermore, its optimization is ambiguous in the latter genus, being explained as either an origin in the common ancestor of *Lepidobatrachus* and a subsequent loss in *L. laevis* or as two independent origins in *L. asper* and *L. llanensis*. Note, however, that this optimization is contingent on the poorly supported position of *Chacophrys* as the sister taxon of *Lepidobatrachus*.

Fabrezi (2006) referred to DeMar's (1966) hypothesis that the presence of a bony shield would reduce evaporative water loss through the skin in dissorophid temnospondyls and considered that it provided a reasonable explanation for the occurrence of a shield in some species of *Ceratophrys* and *Lepidobatrachus*. She also suggested that the occurrence in the mostly aquatic *L. llanensis* of a relatively smaller shield than the one occurring in some *Ceratophrys* might be related to a loss of selective advantage. This scenario is not supported by our results because the optimization indicates that bony shields are not homologues in both groups.

The E-K layer is known to occur in *Ceratophrys cranwelli, Ce. ornata, Ce. stolzmanni, Chacophrys pierottii, Lepidobatrachus asper, L. laevis,* and *L. llanensis* (Elkan, 1968,

Mangione et al., 2011; Quinzio and Fabrezi, 2012). Its occurrence is unknown in the other species of *Ceratophrys,* but in the context of our results its presence is predicted on the basis of parsimony. As this layer is, in general, absent in aquatic species (Elkan, 1968, 1976), the presence of the E-K layer in *Lepidobatrachus* deserves some comments. Although species of *Lepidobatrachus* are aquatic, they estivate. For this reason the presence of the E-K layer, if at all related to prevention of water loss, might be related to an aquatic mode of life interrupted by prolonged estivation, as might be the presence of other characters related with prevention of water loss (e.g., cocoon formation). Interestingly, *Litoria platycephala,* the most aquatic species of the former members of *Cyclorana* (Robinson and Cappo, 1989) parallels *Lepidobatrachus* in this sense. In *Litoria platycephala* the E-K layer is present (Bayomy et al., 2002) and during the dry season this species estivates and forms a cocoon (Withers, 1995). In any case, only experimental data can shed light on the function of the E-K layer.

The diversification of a clade that mainly inhabits semiarid environments In South America, such as Ceratophryidae, is also congruent with the inferred climatic history of the continent. During Mesozoic times, extensive areas with seasonally dry conditions developed on almost the entire surface of the extant South American territory (e.g., Parrish, 1987; Scotese et al., 1999; Hay and Floegel, 2012; Woodburne et al., 2014). Since the mid-Cretaceous and during the Cenozoic, the extension and particular conditions of these areas have varied, influenced by the break-up of Gondwana, the uplift of the Andes, and the succession of a series of marine transgressions that occurred during these times (e.g., Parrish, 1987; Scotese et al., 1999; Gregory-Wodzicki, 2000; Hartley, 2003; Hoorn et al., 2010; Hay and Floegel, 2012; Le Roux, 2012; Woodburne et al., 2014). Although the pattern of diversification of Ceratophryidae could be framed in several of these scenarios, the lack of a temporal context for this pattern prevents us from venturing a guess about the particular events that could have caused their diversification.

The strange turns of polyploidy

Polyploidy as a phenomenon in anurans has been reviewed several times from different perspectives (e.g., King, 1990; Beçak and Kobashi, 2004; Green and Sessions, 2007; Schmid et al., 2010; Mable et al., 2011; Evans et al., 2012). The most recent review (Evans et al., 2012) listed 61 cases of polyploid species. This figure reduces to 52 when ignoring cases of occasional triploids in normally diploid species, and to at least 17 independent occurrences in a phylogenetic context (data not shown). When compared with the more than 6,400 extant species of the group (Frost, 2014), it is fair to say that polyploidy is quite uncommon in anurans. An interesting situation is the reduced number of cases of multiple polyploid species in relatively restricted clades, regardless of whether polyploidy is explainable by common ancestry or not. One of these few clades in which polyploidy occurs in multiple species is Ceratophryidae, and in that regard, our results provide some points for discussion.

Ceratophryids include three polyploid species that, having 2n = 8x = 104, are among the few known cases of octoploidy in anurans: *Ceratophrys aurita, Ce. joazeirensis,* and *Ce. ornata* (Bogart, 1967; Beçak et al., 1967; Schmid et al., 1985; Soares-Scott et al., 1998; Vieira et al., 2006). All other ceratophryids are known or inferred to be diploids (Morescalchi, 1967; Bogart, 1967; Barrio and Rinaldi de Chieri, 1970; Mercadal, 1981).

The optimization of ploidy levels on our optimal topology shows that the strongly supported monophyly of *Ceratophrys cranwelli* and *Ce. ornata* implies an ambiguous optimization for the origin of the octoploid chromosome complement from a diploid complement (Fig. 3). Both interpretations, as a single origin in the common ancestor of the four species of that clade and a subsequent reversal to diploidy in *Ce. cranwelli* (Fig. 3A), *or* an independent origin of octoploidy in the common ancestor of *Ce. aurita* + *Ce. joazeirensis* and in *Ce. ornata* (Fig. 3B), are equally parsimonious. This situation has been suggested without additional comments by Mercadal (1986) and Vieira et al. (2006), when they considered that *Ce. joazeirensis* might be the sister taxon of *Ce. ornata*.

If polyploidy is uncommon in anurans, octoploidy is even more so, having been reported only in the three species of *Ceratophrys, Pleurodema cordobae* (Valetti et al., 2009), and eight species of *Xenopus* (Evans et al., 2012, and citations therein). Whereas *P. cordobae* is nested within a tetraploid clade (Faivovich et al., 2012), octoploid species of *Xenopus* are all hypothesized to have resulted from independent hybridization events among tetraploid parental species (Evans et al., 2005). The fact that polyploidy is so uncommon in anurans makes both equally parsimonious optimizations of polyploidy in ceratophryids most unusual. In the absence of any evidence of hybridization, the possible independent origin within a clade of four extant species of such a rare ploidy level for amphibians is perplexing. Alternatively, the reversion from an octoploid complement to a diploid complement is equally curious. A revision of available phylogenetic information on all polyploid anurans indicates that there is only a single known case of a transformation from a plesiomorphic polyploid complement to a derived diploid complement: the diploid *Silurana tropicalis* originates from a tetraploid ancestor (Evans et al., 2004).

Ceratophrys constitutes a unique instance among the anurans because no taxa with intermediate ploidy levels between diploid and octoploid (i.e., tetraploid and/

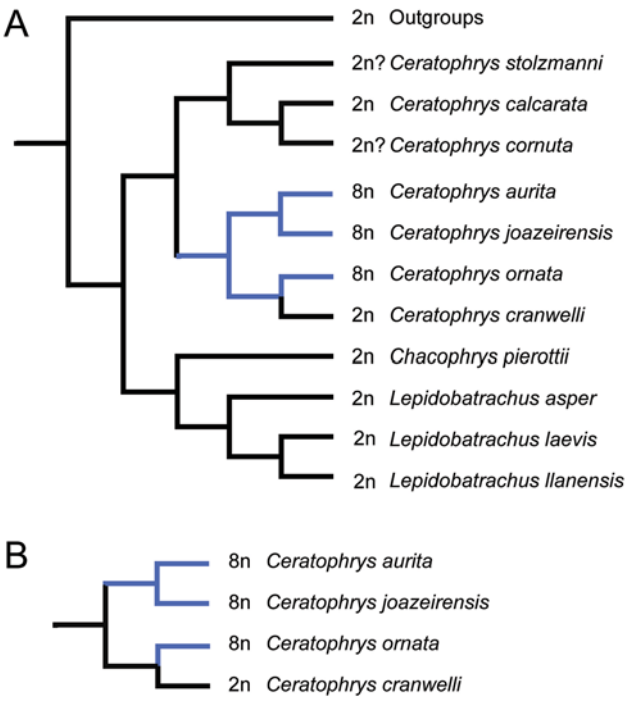

Figure 3. Evolution of ploidy levels in ceratophryid frogs. The optimization of ploidy levels on our optimal topology indicates an ambiguity involving the origin of octoploidy, with two equally parsimonious scenarios, involving either **(A)** a single transformation from diploidy (black) to octoploidy (blue), with a subsequent reversal to diploidy in *Ceratophrys cranwelli,* or **(B)** two independent origins in the common ancestor of *Ce. aurita* + *Ce. joazeirensis*, and in *Ce. ornata*. Both alternatives are remarkable in the context of our knowledge on amphibian chromosome evolution. See discussion for further comments. The diploid complements in *Ce. cornuta* and *Ce. stolzmanni* have been inferred on the basis of erythrocyte size by Mercadal (1981).

or hexaploid) are known. This situation raises the question of whether the octoploid species arose directly from diploid ancestors (which would be unique in vertebrates) or from taxa with intermediate ploidy levels. Mercadal de Barrio and Barrio (2002), using the technique developed by Reumer and Thiebaud (1987) to measure osteocyte lacunae, presented a survey of inferred ploidy levels in fossil remains associated with *Ceratophrys* in Argentina, from Pliocene to Holocene. Extrapolating from osteocyte lacunae of two specimens each of *Ce. cranwelli* and *Ce. ornata,* they inferred the occurrence of diploids, octoploids, and for the first time in *Ceratophrys,* tetraploids in the fossil specimens, some of which were from the same horizon and locality. Unfortunately, their survey did not include a detailed reassessment of the identity of each remain, most of which are listed as *Ceratophrys* sp. Therefore, the number of species involved in the analysis of Mercadal de Barrio and Barrio (2002) and their relationship to *Ce. cranwelli* + *Ce. ornata* remain unclear. The relationships of these fossil remains with the extant species of *Ceratophrys* require further study, as their putative intermediate ploidy levels would shed further light on the origin and evolution of polyploidy in this group of frogs.

Ceratophryid fossil record: More doubts than calibration points

A number of fossil anurans have been attributed to Ceratophryidae and considered evidence of its putative Mesozoic origin, early diversification, and wider, probably Gondwanan, past distribution (Casamiquela, 1963; Báez and Perí, 1989, 1990; Evans et al., 2008, 2014). However, the taxonomic placement of several of these remains is questionable, particularly the older ones, leading us to conclude that evidence is lacking to allow us to perform a relaxed molecular clock dating analysis of ceratophryids.

Two Cretaceous anurans have been attributed to Ceratophryidae: *Beelzebufo ampinga,* from Madagascar (Evans et al., 2008, 2014), and, tentatively, *Baurubatrachus pricei,* from Brazil (Báez and Perí, 1989; Sanchiz, 1998). The single known specimen of the latter was recently reprepared, showing new characters that allow questioning its ceratophryid affinities (A.M. Báez, pers. comm). Similarly, all ceratophryid synapomorphies recognized by Evans et al. (2008, 2014) in the fragmentary material of *Beelzebufo ampinga* seem to have been misinterpreted (A.M. Báez, pers. comm.; L. Nicoli, pers. obs.). A cursory examination of the morphological dataset employed by Evans et al. (2014) identifies a minimum of 25 errors in scoring (see Appendix S4) that suggest the need of a thorough reevaluation of their data set. Such an endeavor is beyond the scope of the present paper, but in the meantime we consider that there is no evidence associating *Beelzebufo* with ceratophryids. The immediately younger putative fossil ceratophryid, *Wawelia geroldhi,* from Miocene sediments of northern Patagonia (Casamiquela, 1963; Báez and Perí, 1990), has recently been reanalyzed and its ceratophryid affinities rejected (L. Nicoli et al., unpubl. data).

A fragmentary maxillary arcade from the Late Miocene of west-central Argentina has also been attributed to Ceratophryidae (Contreras and Acosta, 1998). However, this material was originally studied with a significant amount of sediment still adhered to the fossil. In addition, the material remains undescribed and the reasons for its association with Ceratophryidae have not been discussed. A recent revision of this material after a more extensive preparation allows it to be assigned to Ceratophryidae with some confidence, as it possess the synapomorphies proposed for the group that can be evaluated in the remains (i.e., non-pedicellate teeth; lack of distinguishable pars palatina on the anterior region of the maxilla, mentomeckelian indistinguishably fused to dentary; mentomeckelian forming large, acute, and robust medial fang; L. Nicoli et al., unpubl. data). However, this specimen shares some character states with different extant ceratophryids and possesses several character states that are unique among ceratophryids (i.e., a unique shape of articulation of maxilla and premaxilla involving several

character states), and thus, it is unknown at this time if it belongs to the crown or stem of the group.

All other fossil ceratophryids were assigned to extant genera, particularly *Ceratophrys* (for list see Nicoli, 2014). The only fossil record of *Lepidobatrachus* is a single specimen recently attributed to a new, fossil species of the genus (Tomassini et al., 2011; Nicoli, in press). This specimen was collected in Late Miocene-Early Pliocene sediments of the Farola Monte Hermoso locality, on the Atlantic coast of Buenos Aires province, Argentina. The oldest fossil material certainly attributed to *Ceratophrys,* the fossil species *Ce. ameghinorum,* was also collected in Farola Monte Hermoso in sediments deposited during the Late Miocene-Early Pliocene (Fernicola, 2001; Tomassini et al., 2013).

A series of specimens from Pleistocene sediments of Buenos Aires province were referred to the fossil species of *Ceratophrys ensenadensis* Rusconi, 1932, and *Ce. rusconi* Agnolin, 2005. The validity of the former has been questioned (Báez and Gasparini, 1977; Perí, 1993b), as it has been diagnosed on the basis of the shape of nasals, considered to be more robust and anteriorly projected than in extant *Ceratophrys,* and the structure of dermal ornamentation. Both characters, however, are included within the variation observed in *Ce. cranwelli* and *Ce. ornata* (which so far are osteologically indistinguishable). Similarly, *Ce. rusconi* has been diagnosed on the basis of proportions involving roofing bones and fenestrae of the skull, characters that are also included in the observed variation of *Ce. cranwelli* + *Ce. ornata* (L. Nicoli, pers. obs.). Several other Quaternary fossils were attributed to *Ceratophrys* without specific allocation (for list see Nicoli, 2014).

Two fossil skulls from the Quaternary of Lagoa Santa, Minas Gerais, Brazil, are the single record attributed to an extant ceratophryid species. Günther (1859) identified them as remains of *Ceratophrys cornuta;* however, photographs of these fossils (provided by the Natural History Museum, London, UK) indicate that they differ from *Ce. cornuta* in several character states. Among them, the presence of a series of conspicuous crests in the nasal, maxilla, and squamosal (absent in *Ce. cornuta*) and a subquadrangular *lamella alaris* of the squamosal, ending slightly posteriorly to the level of the occipital condyle (lanceolate, terminating far posterior to the level of the occipital condyles in *Ce. cornuta*). Although the latter character state is observed in several species of *Ceratophrys* (e.g., *Ce. aurita, Ce. joazeirensis, Ce. cranwelli, Ce. ornata*), the conspicuous crests on the nasal, maxilla, and squamosal are only present in *Ce. aurita* and *Ce. joazeirensis* (Pires-Gayer, 1984; Vieira et al., 2006).

This brief review indicates that the three oldest fossil remains that could had been used to constrain the minimum age of divergence of Ceratophryidae simply cannot be employed because they present inadequate evidence supporting a close relationship to Ceratophryidae. Considering that the phylogenetic position of the Late Miocene fossil from San Juan remains unknown, and that its age is within the range of that of the remains assignable to extant genera, it does not represent a relevant calibration point. This leaves *Ceratophrys ameghinorum* and the undescribed fossil species of *Lepidobatrachus,* both from the Late Miocene–Early Pliocene, as the two oldest calibration points for the crown group Ceratophryidae for a molecular clock exercise. At this point, however, we see a number of issues associated with this possibility.

First among these issues is the fact that none of the fossil species has been included in a phylogenetic analysis, and their exact position cannot be inferred based on available evidence. Whereas the ages of *Ceratophrys ameghinorum* and the new fossil species of *Lepidobatrachus* could be used to establish hard minimum bounds for prior parametric distributions for the most recent common ancestors of both *Ceratophrys* and *Lepidobatrachus,* we find it to be a poor substitute for actual phylogenetic knowledge and prone to induce errors in estimation, particularly for such a reduced clade. The inclusion of the relevant fossils for calibrating phylogenetic analyses has been strongly advised (Parham et al., 2012; Sterli et al., 2013), however this has not been the case in most approaches to amphibian temporal history.

The second issue deals with the chronostratigraphy of the geologic units in which the two possible calibrating fossils have been found. Neither numerical ages nor magnetostratigraphical studies exist for the Monte Hermoso Formation (MHF) exposed at Farola Monte Hermoso. Thus, the precise age of this unit cannot be established. The estimation of its age was done by the correlation of MHF with the presumably nearly contemporary formations with known ages. The chronological sequence of the different geological units is ideally determined by their physical succession (deeper units are older) in regions where several of these units are represented in the same sequence. Unfortunately, the Late Cenozoic units of the Buenos Aires province are lithologically uniform and, thus, geologically indistinguishable (Deschamps, 2005; Zarate, 2005). Each of these units is characterized, however, by its own paleontological remains, which consist of taxonomic assemblages of mammals and include a series of taxa with relative short biochrons. These characteristic assemblages define a series of biostratigraphic zones (Cione and Toni, 2005; Deschamps, 2005). Therefore, the chronological sequence of the different units could be determined by the known sequence of these biostratigraphic zones, which in some cases of the Late Cenozoic units of the Buenos Aires province are provided by sequences where these units are preserved superposed (Cione et al., 2007). In this way, the MHF has been considered to be deposited between the Late Miocene and the Early Pliocene (Cione et al., 2007). A recent attribute to this unit of a more defined lapse during the Early Pliocene (Tomasssini et al., 2013) is fundamentally based on the comparison of the "evolutionary stage" of the involved taxa, a rationale that has been questioned

specifically for the Late Cenozoic units of the Buenos Aires province (Cione and Toni, 1995).

Even if the hard minimum bounds for the prior parametric distributions of both calibrating points could be established as the minimum possible age of their chronostratigraphic provenance (Late Miocene), we see no reasonable way to establish the other parameters of the prior distribution curve (the mean and standard deviation, in the case of a prior lognormal distribution), a limitation noticed by several authors (e.g., Ho and Phillips, 2008; Lee and Skinner, 2011; Parham et al., 2012). In our specific case, the fact that the two calibration points are from the same locality and (uncertain) horizon—and could at least be associated with the respective most recent common ancestors of *Ceratophrys* and *Lepidobatrachus*—makes the selection of the soft maximum bound a matter of trying to set limits as to when the most recent common ancestor of Ceratophryidae could have occurred. Some authors have established soft maximum bounds on the basis of relatively rich and well-studied faunal associations in which the presence of outgroups serves as taphonomic-preservation controls using ecological/taxonomic equivalents (Bottjer and Jablonski, 1988), and where no remains of the group of interest could be found, therefore inferring its absence (e.g., Pérez and Pol, 2012). In our case, there are no well-documented Cenozoic fossil anuran faunas in South America where ceratophryids could be said to be absent (or for that matter, any other anuran group; Báez, 2000).

The lack of support in this and previous analyses for the relationships of Ceratophryidae with other hyloids is also problematic. Furthermore, the lack of relevant hyloid fossils that pass the criteria established by Parham et al. (2012) for fossil calibrations and that are clearly referable to any of the nodes of nobleobatrachians only complicates the establishment of a soft maximum bound. We also refrain from exporting calibrations from previous exercises with anurans, because we find that most of them have been based on very few paleontological calibrations (when not based on geotectonic events), for very large samplings, and with little if any meaningful discussion. For all the reasons above, we conclude that it would be premature to perform a relaxed molecular clock dating analysis for ceratophryids.

A final point that requires mention is that the uncorrected p-distances of the 16S fragment among ceratophryid species are relatively low when compared with other anurans (e.g., Fouquet et al., 2007; Padial et al., 2009), with sister species differing as little as 1.2–1.5% (*Ceratophrys aurita–Ce. joazeirensis;* Table S1.3) or 1.6–2.3% (*Lepidobatrachus laevis–L. llanensis;* Table S1.2) to 4.2–4.4% (*Ce. calcarata–Ce. cornuta;* Table S1.4). How this relates to the tempo of diversification of the group is difficult to establish at this time. Interestingly, observations in captivity (Honegger et al., 1985; Marangoni et al., 2009) indicate that sexual maturity is reached very early (a minimum of 158 days after metamorphosis in *Ce. ornata;* 301 days in *Ce. cranwelli*), suggesting short (annual?) generation times. However, Fabrezi and Quinzio (2008) reported 4–11 lines of arrested growth in adults of Chacoan ceratophryids, suggesting longer minimum generation times. The problem should be studied in detail.

Ceratophryidae is a fascinating frog clade. Knowledge on their phenotypic diversity and biology has been accumulating for a long time and at a particular fast pace in the last decade. Our study presents the first phylogenetic hypothesis for the whole group and provides the necessary historical framework for the study of its diversification and evolution.

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ONLINE SUPPORTING INFORMATION

The following Supporting Information is available for this article online:

Appendix S1. Locality data for the vouchers of the sequences produced for this study and relevant ceratophryid sequences downloaded from GenBank.

Appendix S2. List of GenBank accessions.

Appendix S3. Problems with GenBank Sequences employed in previous studies.

Appendix S4. Some of the scoring problems in the phenotypic dataset of Evans et al. (2014).

Figure S1. Topology of the outgroup taxa recovered in the ten most parsimonious trees obtained with direct optimization (length 38,965 steps) under equal weights for all transformations. Values around nodes are parsimony jackknife absolute frequencies estimated for the static alignment analyzed with parsimony in T.N.T. with gaps as fifth state. Nodes lacking values have < 50% jackknife frequencies. See Figure 1 for ceratophryid relationships.

Figure S2. Results of the Bayesian analysis using the static alignment. Values around nodes are Posterior Probabilities. Nodes with values < 0.5 are collapsed. See Appendix S1 for complete locality data.

Table S1. Genetic distances among 16S sequences of ceratophryids.