

A phylogenetic analysis of *Pleurodema* (Anura: Leptodactylidae: Leiuperinae) based on mitochondrial and nuclear gene sequences, with comments on the evolution of anuran foam nests

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Abstract

Species of the genus *Pleurodema* are relatively small, plump frogs that mostly occur in strong-seasonal and dry environments. The genus currently comprises 14 species distributed from Panama to southern Patagonia. Here we present a phylogenetic analysis of *Pleurodema*, including all described species and several outgroups. Our goals include testing its monophyly and the monophyly of the species groups that were historically proposed, and studying the evolution of some character systems, particularly macroglands and egg-clutch structure; this last point also provided the chance for a discussion of foam nest evolution in anurans. Our dataset includes portions of the mitochondrial genes *cytochrome b*, *12S*, *16S*, and the intervening *tRNA^{Val}*; the nuclear gene sequences include portions of *rhodopsin* exon 1 and *seven in absentia homolog 1*. Our results support a clade composed of *Pleurodema* and including the monotypic *Somuncuria* Lynch, 1978 nested within it. The latter genus is therefore considered a junior synonym of *Pleurodema* and its sole species is added to this genus. Furthermore, our results indicate the non-monophyly of several species groups proposed previously. We recognize four clades in *Pleurodema*: the *P. bibroni* clade (*P. bibroni*, *P. cordobae* and *P. kriegi*), the *P. thaul* clade (*P. bufoninum*, *P. marmoratum*, *P. somuncurensis* and *P. thaul*), the *P. brachyops* clade (*P. alium*, *P. borellii*, *P. brachyops*, *P. cinereum*, *P. dipolister* and *P. tucumanum*) and the *P. nebulosum* clade (*P. guayapae* and *P. nebulosum*). Our results further indicate the need for a taxonomic reassessment of *P. borellii* and *P. cinereum* (as did previous studies), *P. guayapae* and *P. nebulosum*, and the three species in the *P. bibroni* clade. *Pleurodema* shows a striking pattern of variation in presence/absence of lumbar glands. Our results indicate multiple losses or independent gains of this character associated with defensive displays. The reproductive modes of *Pleurodema* include four different egg-clutch structures. The optimization of these indicates that there are at least two independent transformations from the plesiomorphic mode of foam nests to egg-clutch structures involving gelatinous masses of different sorts (ovoid plates, masses, or strings). We hypothesize that these independent transformations could involve changes at the behavioural (the loss of foam beating behaviour by the parent) and/or structural level (transformations involving the pars convoluta dilata, the section of the oviduct where the foam-making substance is secreted). Finally, our study of foam nest evolution in *Pleurodema* is extended to the other groups of anurans where foam-nesting occurs, on the basis of available data and recent phylogenetic hypotheses. In the different hyloid groups where it occurs, foam-nesting evolved from clutches laid in water. However, in all ranoids in which foam-nesting occurs, it evolved from terrestrial clutches, with eggs laid hanging in vegetation, or, if the clutches are laid on a restricted volume of water, involving endotrophic development.

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The formerly enormous paraphyletic assemblage Leptodactylidae was first partitioned into several families by Frost et al. (2006), who restricted Leptodactylidae to most genera of the former subfamily Leptodactylinae (single exception *Limnomedusa*), on the basis of an analysis using several nuclear and mitochondrial genes and a morphological dataset restricted to some exemplars. The results of Grant et al. (2006) indicated the non-monophyly of the newly restricted Leptodactylidae, suggesting the need to further restrict it to a clade composed of the genera *Leptodactylus*, *Paratelmatobius*, *Scythrophrys* and *Hydrolaetare*, while resurrecting Leiuperidae for a clade composed of *Edalorhina*, *Engystomops*, *Eupemphix*, *Physalaemus*, *Pleurodema*, *Pseudopaludicola* and *Somuncuria*. More recently, Pyron and Wiens (2011) in a more comprehensive dataset again obtained the monophyly of Leptodactylidae as restricted by Frost et al. (2006), but recognized the subfamilies Leptodactylinae, the newly erected Paratelmatobiinae, and Leiuperinae, which retains the same content as Leiuperidae of Grant et al. (2006).

To the sampling of Leiuperinae of Frost et al. (2006) and Grant et al. (2006), Pyron and Wiens (2011) included sequences from Ron et al. (2006) and Correa et al. (2008). Internal relationships of the genera of Leiuperinae, however, have been the focus of several contributions using different approaches over the last 30 years. Relationships of *Pseudopaludicola* were first studied by Lynch (1989) and then by Lobo (1995). Relationships of *Engystomops* were studied by Cannatella and Duellman (1984), Cannatella et al. (1998) and Ron et al. (2006).

The species of *Pleurodema* are relatively small, plump frogs. They are discontinuously distributed over much of the Neotropical region, from Panama to southern Argentina and Chile. Most of their species are characteristic of strong-seasonal and dry environments, and several are explosive breeders (Fernández and Fernández, 1921; Barrio, 1964; León-Ochoa and Donoso-Barros, 1969; Hulse, 1979; Ceí, 1980; Peixoto, 1982; Hödl, 1992; Cardoso and Arzabe, 1993). This genus exhibits a striking variability in several features, for example occurrence of diploid and polyploid species (Barrio and Rinaldi de Chieri, 1970; Barrio, 1977; Valetti et al., 2009), multiple reproductive modes (foam nests, submerged egg strings, floating egg strings and floating egg masses; Ceí, 1962, 1980; Duellman and Veloso, 1977; Barrio, 1977; Hödl, 1992; Martori et al., 1994; Weigandt et al., 2004), cryptic and polymorphic species (Ceí and Capurro, 1957; Ceí, 1958; Vellard, 1960; Barrio, 1964; Laurent, 1975), and distribution across a broad altitudinal gradient (sea level to 5000 m a.s.l.; e.g. Seimon et al., 2007; Ferraro and Casagrande, 2009).

The current usage of *Pleurodema* was established by Parker (1927), who re-diagnosed it and included in it

most of the currently recognized species that had been described by then, plus some others that were subsequently transferred to other genera (Lynch, 1971). Parker (1927) distinguished *Pleurodema* from *Physalaemus* and *Pseudopaludicola* based mostly on the absence of the quadratojugal (then believed to be present in all *Physalaemus* and *Pseudopaludicola*).

In his study of the leptodactyloid frogs, Lynch (1971) provided a diagnostic definition using several phenotypic characters; however, it is not clear if any of these could be considered synapomorphic. Heyer (1975) conducted an analysis of Leptodactylidae (*sensu* Lynch, 1971) using the monothetic group method; although he assumed the monophyly of *Pleurodema* in his analyses, on the basis of the diversity in reproductive modes, he suggested that *Pleurodema* could be polyphyletic.

Duellman and Veloso (1977) presented a taxonomic review of *Pleurodema* and an early attempt to analyse the cladistic relationships of the genus using four characters: amplexus position, reproductive mode, occurrence of lumbar glands and basic chromosome number. Duellman and Veloso (1977) actually assumed the monophyly of *Pleurodema* and putative groups within the genus without testing it: reanalysis of their dataset results in an almost completely unresolved strict consensus (data not shown). These authors, however, presented an “...evolutionary scheme...with respect to reproductive biology and geological time...” (here reproduced as Fig. 1) that is quite different from their quantitative phylogenetic hypothesis and that they used as a basis to develop a biogeographical scenario for the diversification of the genus. Barrio (1977) corrected some observations of Duellman and Veloso (1977), added new information about some species and partly modified their “evolutionary scheme”, but restricted his scope to the group of species having lumbar glands (here reproduced as Fig. 2). Lynch (1978) presented a pre-cladistic analysis of the taxa then called “lower telmatobines”, in which he suggested that the new genus *Somuncuria* was the sister group of *Pleurodema* and “...a survivor of a transitional stage in the early evolution of the Leptodactylinae from the Telmatobiinae...”. The monophyly of *Pleurodema* was partially tested and corroborated by Pyron and Wiens (2011), who included sequences of *P. brachyops* (produced by Faivovich et al., 2005), *P. bufoninum*, *P. marmoratum* and *P. thaul* (produced by Correa et al., 2006, 2008).

The contents of *Pleurodema* have remained quite stable since the revision by Duellman and Veloso (1977). Nascimento et al. (2006) tentatively transferred *Eupemphix fuscomaculatus* from *Physalaemus* to *Pleurodema*, on the basis of the presence of vomerine teeth, which are absent in *Physalaemus* as redefined by Nascimento et al. (2005). However, critical examination of the available material by Kolenc et al. (2011) indicated that the holotype of *Eupemphix fuscomaculatus* is actually a

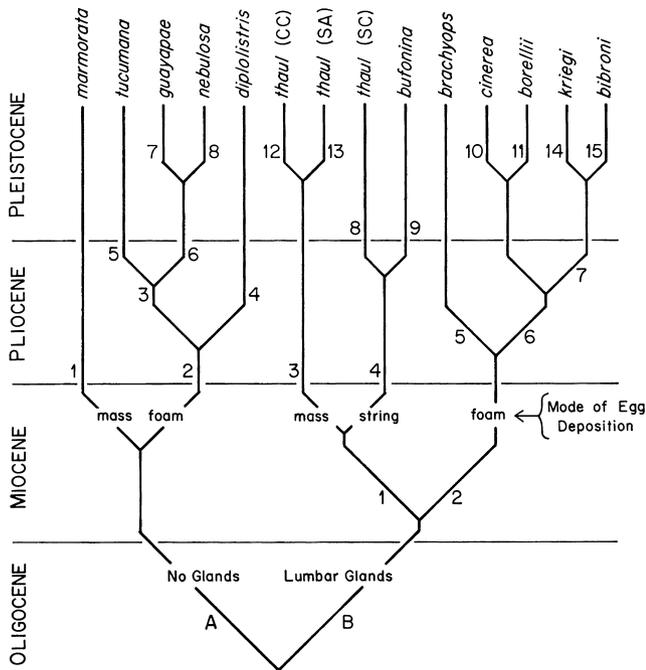


Fig. 1. Evolutionary scheme suggested for *Pleurodema* by Duellman and Veloso (1977) on the basis of reproductive biology and geological time. The authors also presented a quantitative approach on the basis of a reduced dataset whose resolution bears no resemblance to this scheme. Taken from Duellman and Veloso (1977).

synonym of *Physalaemus biligonigerus*. Specimens from Rio Apa, Paraguay, associated with this species by Cei (1990), are referable to *Pleurodema bibroni* (Kolenc et al., 2011). More recently, Valetti et al. (2009) described the octoploid *P. cordobae*, a cryptic species with *P. bibroni* and *P. kriegi* (both tetraploids). Finally, Bastos Maciel and Nunez (2010) described *P. alium*, formerly confused with *P. diplolister*, raising the number of described species to 14.

Duellman and Veloso (1977) recognized the following six species groups: (i) *P. marmoratum*, (ii) *P. diplolister*, *P. guayapae*, *P. nebulosum* and *P. tucumanum*, (iii) *P. thaul* from southern Argentina and central Chile, (iv) *P. thaul* from southern Chile and *P. bufoninum*, (v) *P. borellii*, *P. cinereum* and *P. brachyops*, and (vi) *P. bibroni* and *P. kriegi*. The monophyly of these groups was assumed but not tested. The evidence advanced by Duellman and Veloso (1977) *a priori* only supported the monophyly of *P. bibroni* and *P. kriegi* (44 chromosomes). Cei (1980) recognized, for the species present in Argentina and without further comments, a *P. cinereum* Group for *P. cinereum*, *P. borellii*, *P. thaul*, *P. bufoninum* and *P. kriegi*, a *P. nebulosum* Group for *P. nebulosum*, *P. guayapae* and *P. tucumanum*, and a monotypic *P. marmoratum* Group. Similarly, without any discussion Cei (1987) further recognized a monotypic *P. bufoninum* Group.

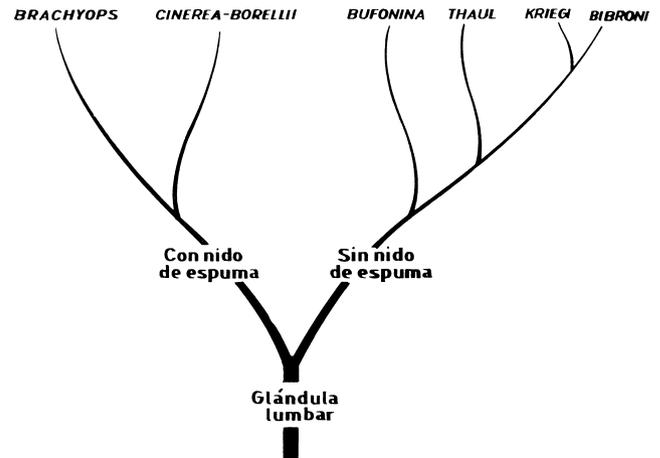


Fig. 2. Modifications to the scheme proposed by Duellman and Veloso (1977; here reproduced as Fig. 1) by Barrio (1977). This author corrected some observations of Duellman and Veloso (1977), and restricted his scope to the group of species having lumbar glands. Taken from Barrio (1977).

McLister et al. (1991) explored the relationships between five populations of *P. borellii* and *P. cinereum* by using both allozymes (analysed by UPGMA of Roger's distances) and advertisement calls. Their results emphasized the similarities among these species, providing no evidence that they are actually different.

Pleurodema thaul is a species broadly distributed along Chile but restricted in south-western Argentina (Cei, 1962; Correa et al., 2007, 2008; Ferraro and Casagrande, 2009). Following early reports of polymorphism in *P. thaul* by Cei and Capurro (1957) and Cei (1958), Duellman and Veloso (1977) added further evidence from amplexus position, egg-clutch structure, karyotypes and sternal morphology, and recognized three different groups of populations: (i) southern Chile, (ii) central Chile and (iii) southern Argentina. Victoriano et al. (1995) failed to find support for the existence of these three lineages using allozymes analysed through UPGMA clustering of Nei's genetic distances. Rosset et al. (2001) reported no significant morphometric differences among the different groups of populations. More recently, Correa et al. (2008) presented a phylogeographical analysis of insular (Robinson Crusoe Island) and mainland populations from Chile (none from Argentina). These authors recovered four main clades, two of which correspond to the southern and central Chilean groups of populations recognized by Duellman and Veloso (1977), while the two other are geographically intermediate.

The goals of the current study were (i) to test the monophyly of *Pleurodema* including all its described species, (ii) to test the monophyly of the species groups

that had been historically proposed, and (iii) to study the evolution of some character systems, particularly the lumbar glands and egg-clutch structure; this last point also provides the opportunity and elements for a general discussion of foam nest evolution in anurans.

Material and methods

Taxon sampling

On the basis of the results of Frost et al. (2006), Grant et al. (2006) and Pyron and Wiens (2011), it seems evident that knowledge of relationships among the fragments of Leptodactylidae as formerly defined by Lynch (1971), Heyer (1975) and Laurent (1986) still requires intensive research. We have included exemplars of all the other genera of Leiuperinae (*Edalorhina*, *Engystomops*, *Eupemphix*, *Pseudopaludicola* and *Somuncuria*), and all other genera of Leptodactylidae (as redefined by Pyron and Wiens, 2011). The latter included two exemplars of the major clades of *Leptodactylus*. We added as well exemplars of all major clades of Athesphatanura, as Ceratophryidae (*Ceratophrys*), Batrachylidae (*Batrachyla*), Telmatobiidae (*Telmatobius*), Cycloramphidae (*Cycloramphus*), Odontophrynidae (*Odontophrynus*), Bufonidae (*Melanophryniscus*), Hylodidae (*Crossodactylus*), Dendrobatoidea (*Allobates*), Allophrynidae (*Allophryne*), Centrolenidae (*Espadarana*) and Hylidae (*Litoria*). Overall, we included 25 outgroup taxa, including five species of *Physalaemus*, exemplars of five of the seven species groups recognized by Nascimento et al. (2005). The tree was rooted with *Allophryne ruthveni*. The analysis included all species of *Pleurodema*, often including multiple exemplars from different localities. Our sampling of *Pleurodema* was complemented with sequences available from GenBank. These included sequences produced by Darst and Cannatella (2004), Faivovich et al. (2005), Lehr et al. (2005), Correa et al. (2006, 2008), Frost et al. (2006), Grant et al. (2006) and Ron et al. (2006). Van Bocxlaer et al. (2009) published sequences assigned to *P. bibroni* with no precise locality data (“South America”, GenBank number FJ882760–61), and these were included by Pyron and Wiens (2011). Our preliminary analyses indicated that those sequences actually belong to *P. thaul*, a species for which the name *P. bibroni* was incorrectly applied until 1969 (see Donoso-Barros, 1969). Considering that we have several other samples of *P. thaul*, and that these sequences from Van Bocxlaer et al. (2009) lack locality data, we did not include them in our analyses. Authorities of all species named in both the text and figures are included in Supporting Information Appendix S1. Institutional abbreviations follow Sabaj Pérez (2010).

Character sampling

The mitochondrial gene sequences produced for this study include portions of *cytochrome b*, *12S*, *16S*, and the intervening *tRNA^{Val}*. The nuclear gene sequences produced include portions of *rhodopsin* exon 1 and *Seven in Absentia homolog I*. All primers are the same as those employed by Faivovich et al. (2005). Duellman and Veloso (1977) included a small dataset (four informative characters) of phenotypic characters. This dataset is not included in the present analysis, as these characters will be included and discussed in the context of a much larger phenotypic dataset in preparation by Daiana P. Ferraro.

DNA isolation and sequencing

Whole cellular DNA was extracted from ethanol-preserved tissues with the DNeasy (Qiagen, Valencia, CA, USA) isolation kit. Amplification was carried out in a 25- μ L reaction using Fermentas Master Mix. Polymerase chain reaction (PCR)-amplified products were desalted and concentrated using either a GE GFX PCR purification kit or EXO/SAP (Fermentas) and labelled with fluorescent-dye label terminators (ABI Prism Big Dye Terminators v. 3.1 cycle sequencing kits; Applied Biosystems, Foster City, CA, USA). The labelled PCR products were cleaned using cleanSEQ (Agencourt Biosciences, Beverly, MA, USA) or DyeEx (Qiagen). The products were sequenced with an ABI 3730XL (Applied Biosystems). All samples were sequenced in both directions. Chromatograms obtained from the automated sequencer were read and contigs made using the sequence editing software Sequencher 3.0 (Gene Codes, Ann Arbor, MI, USA). Complete sequences were edited with BioEdit (Hall, 1999). See Appendix S2 for GenBank accession numbers and specimen and locality data.

Phylogenetic analysis

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), 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 always ignored problem of the lack of an optimality criterion to choose among competing alignments. While our sympathies rest with direct optimization, we realize that many colleagues disagree, and with the objective of saving them from having to redo our analyses, we performed a multiple

sequence alignment (see below) and analysed it using both parsimony and Bayesian inference.

The rationale for using parsimony as an optimality criterion was advanced by Farris (1983) and recently discussed, among others, by Goloboff (2003) and Goloboff and Pol (2005). Within this framework, the phylogenetic analysis under direct optimization was performed with POY4.1.1 (Varón et al., 2009, 2010), using equal weights for all transformations (substitutions and insertion/deletion events). Sequences of *12S*, *16S*, and intervening *tRNA^{Val}* were preliminarily delimited in sections of putative homology (Wheeler et al., 2006), and equal-length sequences of nuclear 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 Fusing (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. Two 96-h runs of Search were implemented in parallel at the American Museum of Natural History Cluster using 32 processors. The resulting trees were submitted to a final round of swapping using iterative pass optimization (Wheeler, 2003a). Parsimony Jackknife (Farris et al., 1996) absolute frequencies were estimated from the implied alignment (Wheeler, 2003b) with TNT, Willi Hennig Society Edition (Goloboff et al., 2008), generating 50 RAS + TBR per replicate, for a total of 1000 replicates. Editing of trees was performed with Winclada (Nixon, 2002).

We performed a multiple alignment with Clustal-X (Thompson et al., 1997) under default parameters. For the phylogenetic analysis using parsimony we employed TNT. 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 were estimated by 50 RAS + TBR per replicate, keeping five trees, for a total of 1000 replicates. Trees were edited with Winclada (Nixon, 2002).

For the Bayesian analysis, models for each partition were chosen with jModelTest ver. 0.1.1 (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*, *16S* and *tRNA^{Val}* 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 3.1 (Huelsenbeck and Ronquist, 2001). Anal-

yses 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). Three analyses running 80 million generations were performed (with a burn-in fraction of 0.25). Stabilization of resulting parameters was evaluated using Tracer, ver. 1.5 (A. Rambaut and A.J. Drummond; <http://beast.bio.ed.ac.uk/Tracer>).

Results

The analysis with direct optimization resulted in nine optimal trees of 8412 steps (Figs 3 and 4). This length was hit multiple times during the 280 RAS + TBR followed by multiple rounds of Ratchet, Drift and Fusing. All conflicts among the multiple optimal trees involve internal relationships in the exemplars of *P. cinereum*, *P. diplolister* and *P. nebulosum*. Parsimony analysis using the static alignment found nine optimal trees of 8869 steps (Appendix S3). Most topological differences among optimal trees involve relationships among outgroups other than the sister taxon of *Pleurodema*, whose structure is stable. In the same way, the internal structure of *Pleurodema* differs only among trees and analyses in the relationships of *P. brachyops* and in the position of one specimen of *P. borelli* in the Bayesian analysis (see below, and Appendices S3 and S4).

Pleurodema is the sister taxon of a clade composed of the exemplars of *Edalorhina*, *Engystomops*, *Eupemphix* and *Physalaemus* (Fig. 3). *Eupemphix* is nested in *Physalaemus*, being the sister taxon of *P. signifer*, the only included exemplar of its species group.

In all analyses, *Pleurodema* as currently defined is paraphyletic due to the placement of the monotypic *Somuncuria* within it, being closely related to the exemplars of *P. bufoninum*. The strict consensus shows four main clades in *Pleurodema* (Fig. 4): (i) the *P. brachyops* clade, composed of *P. brachyops*, *P. diplolister*, *P. alium*, *P. tucumanum*, *P. cinereum* and *P. borellii*, (ii) the *P. bibroni* clade composed of the polyploid species, *P. bibroni*, *P. cordobae* and *P. kriegi*, (iii) the *P. nebulosum* clade composed of *P. guayapae* and *P. nebulosum*, and (iv) the *P. thaul* clade composed of *P. marmoratum*, *P. thaul*, *P. bufoninum* and *Somuncuria somuncurensis*. The *P. brachyops* clade is the sister taxon of the remaining clades of *Pleurodema*.

From the four non-monotypic species groups recognized by Duellman and Veloso (1977), only their group composed of (then) *P. kriegi* + *P. bibroni* is monophyletic in our analyses. Their group composed of *P. diplolister*, *P. guayapae*, *P. nebulosum* and

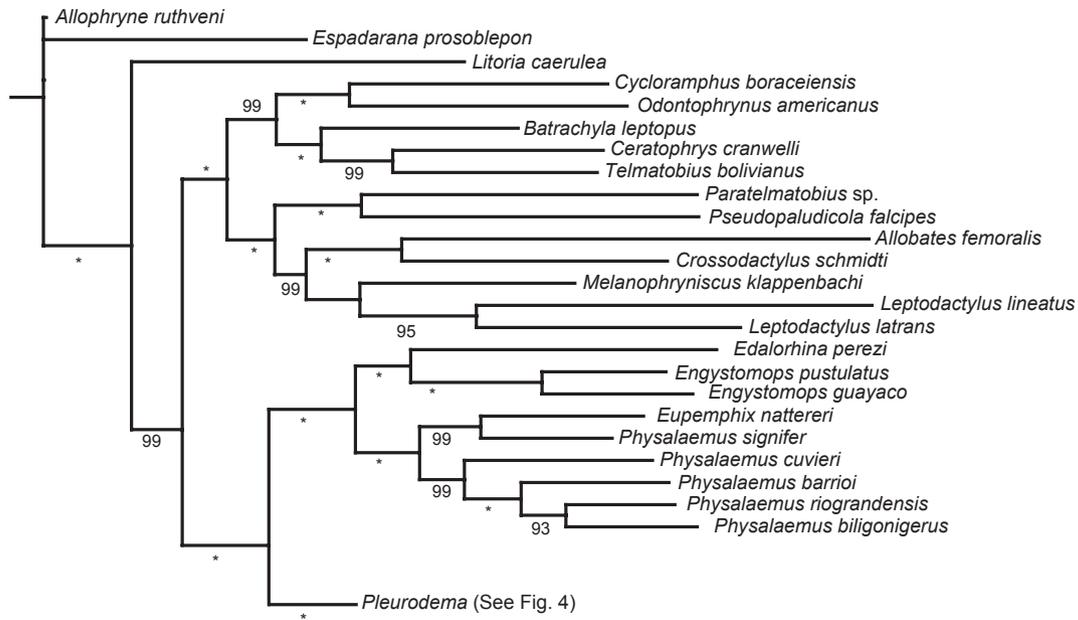


Fig. 3. Strict consensus of the nine equally parsimonious trees obtained using direct optimization. Only outgroups are shown. See Fig. 4 for relationships among species of *Pleurodema*. Relationships among outgroups differ from recent hypotheses, with the exception of the sister group of *Pleurodema*, which has been recovered in several recent analyses. Values around nodes are Parsimony Jackknife absolute frequencies estimated from the implied alignment using TNT. An asterisk (*) indicates 100% Parsimony Jackknife absolute frequency.

P. tucumanum is not monophyletic because *P. diplolister* and *P. tucumanum* are included in a clade with *P. brachyops* and *P. cinereum/P. borellii*, while *P. guayapae* and *P. nebulosum* are distantly related. The *P. cinereum* Group proposed by Cei (1980) is not monophyletic as its suggested members (*P. borellii*, *P. bufoninum*, *P. cinereum*, *P. kriegi* and *P. thaul*) are actually spread in three different clades, as are the members of his *P. nebulosum* Group (*P. guayapae*, *P. nebulosum* and *P. tucumanum*).

Discussion

Relationships among outgroups

The position of *Eupemphix*, nested inside *Physalaemus*, has also been obtained by Pyron and Wiens (2011), so there is reason to consider *Eupemphix* a junior synonym of *Physalaemus*. Other than these points of congruence, relationships among outgroups differ considerably from those of previous hypotheses (Frost et al., 2006; Grant et al., 2006; Pyron and Wiens, 2011). Most important among these differences is that Leiuperinae is not monophyletic, as the only exemplar of *Pseudopaludicola*, *P. falcipes*, is not related to the clade composed of all remaining leiuperines (Fig. 3). Relationships among hyloids still need a considerable amount of study effort. Our analysis was designed to study relationships of *Pleurodema* and does not constitute a strong test of previous results based on much more thorough taxon

and character sampling to study relationship at a higher level than we did. Considering these reasons, we maintain that relationships of *Pseudopaludicola* with Leiuperinae require an appropriate testing, and suggest continuing to consider it within Leiuperinae. Fortunately, the position of *Pseudopaludicola* in our study is immaterial to our discussions of character evolution in *Pleurodema*, as it lacks inguinal glands and does not produce foam nests (Barrio, 1954; Giaretta and Facure, 2009).

Pleurodema

The sister taxon relationship of *Pleurodema* and a clade composed of the exemplars of *Edalorhina*, *Engystomops*, *Eupemphix* and *Physalaemus* (Fig. 3) is congruent with several previous analyses (Faivovich et al., 2005; Frost et al., 2006; Grant et al., 2006; Pyron and Wiens, 2011). The four clades obtained for *Pleurodema* (Fig. 4) are not congruent with previous notions of relationships in the genus, with this discordance stemming partially from a complex scenario of multiple transformations involving lumbar glands and egg-clutch structure as implied by our results.

The *Pleurodema thaul* clade

The finding of *S. somuncurensis* nested within *Pleurodema* and, particularly, closely related to the exemplars of *P. bufoninum* deserves several comments.

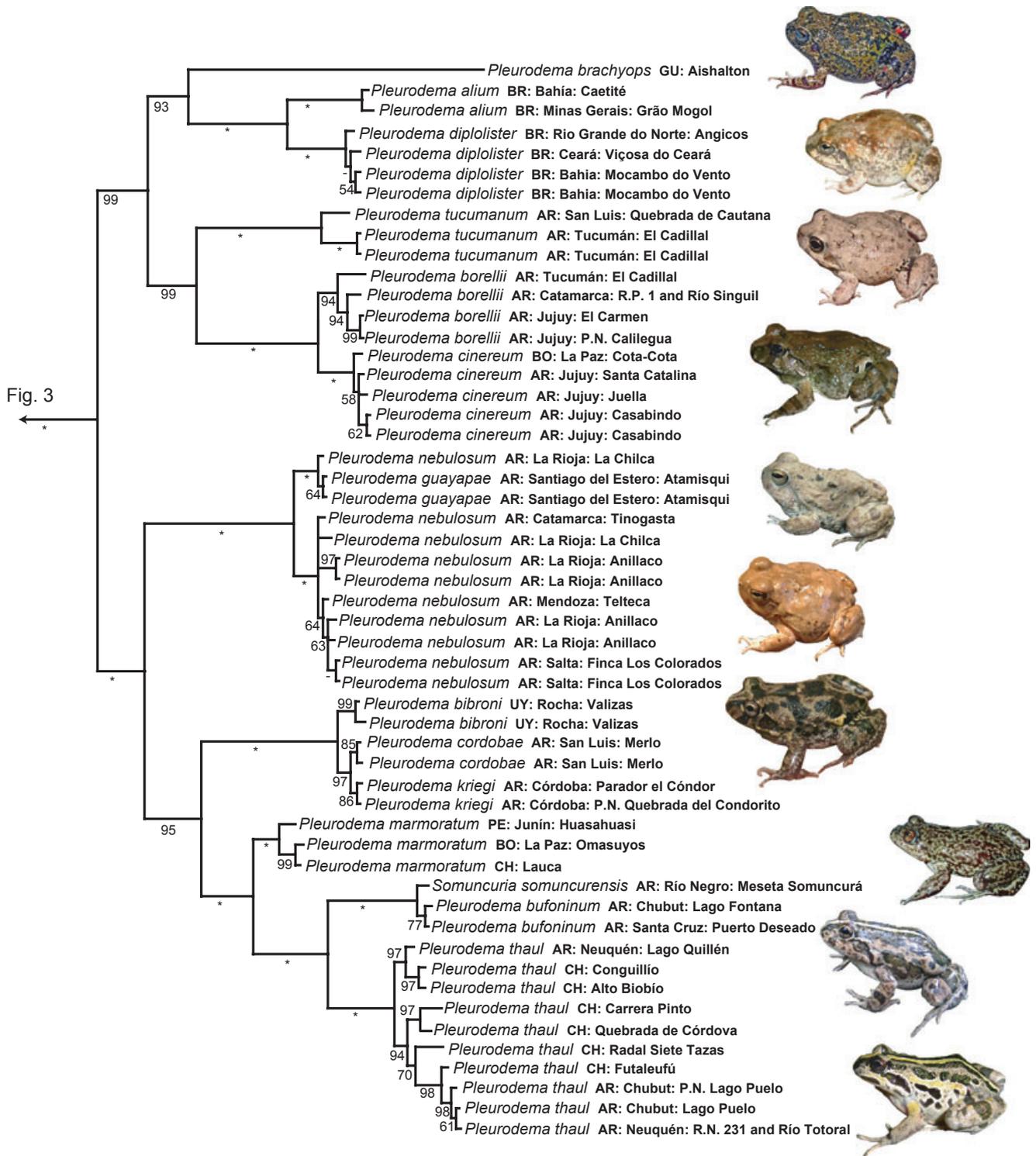


Fig. 4. Strict consensus of the nine equally parsimonious trees obtained using direct optimization. Only relationships among species of *Pleurodema* are shown. See Fig. 3 for relationships of outgroups. The only differences in topology for *Pleurodema* in analyses of a static alignment refer to the position of *P. brachyops* (basal taxon of the *P. brachyops* clade in the Bayesian analysis; sister taxon of *P. tucumanum* + *P. borellii* + *P. cinereum* or the same position obtained with direct optimization are equally parsimonious in the parsimony analysis) and the monophyly of the specimens of *P. borellii*. Values around nodes are Parsimony Jackknife absolute frequencies estimated from the implied alignment using TNT. An asterisk (*) indicates 100% Parsimony Jackknife absolute frequency; a dash (-) indicates values below 50%. See Appendix S2 for complete locality data (they are truncated on this figure) and GenBank numbers. See Appendix S3 for results of the parsimony analysis using the static alignment and Appendix S4 for results of the Bayesian analysis using the same alignment. Country abbreviations: AR, Argentina; BO, Bolivia; BR, Brazil; CH, Chile; GU, Guyana; PE, Peru; UY, Uruguay.

A sister-group relationships between *Somuncuria* and *Pleurodema* was first proposed by Lynch (1978) when erecting *Somuncuria*, on the basis of some character states of dubious polarity (e.g. quadratojugal lost, small nasals) and in the context of assuming the monophyly of *Pleurodema*.

Our results clearly indicate that *Somuncuria* Lynch, 1978 should be included in the synonymy of *Pleurodema* Tschudi, 1838. However, it is not immediately clear how to proceed with its single species, *Telmatobius somuncurensis* Cei, 1969a. *Pleurodema bufoninum* inhabits restricted areas in southern Chile but is widely distributed along Argentinean Patagonia, where a fair amount of phenotypic diversity has been described (Cei, 1972). Our results indicate that *S. somuncurensis* and our exemplars of *P. bufoninum*, including a topotype, have nearly identical sequences. Nevertheless, there are several characters reported by Cei (1969a,b, 1970) that differentiate the mostly aquatic, thermal spring inhabitant *S. somuncurensis* from the mostly terrestrial *P. bufoninum*. Our exemplars of *P. bufoninum* are an insufficient representation of the known diversity within this species, so at this point we do not have enough evidence to establish if the name *somuncurensis* could be applied to some populations currently assigned to *P. bufoninum*. Our finding has further implications, because the former *S. somuncurensis* has long been considered an endangered and endemic species from Meseta de Somuncurá (Central Patagonia of Río Negro province, Argentina), which is threatened by habitat destruction and introduction of predatory fishes (Ethridge in Frost, 1985; Lavilla et al., 2000; Úbeda and Lavilla in Stuart et al., 2008; Lavilla and Heatwole, 2010).

Given the uncertainty concerning the status of these species, we are reluctant to place *T. somuncurensis* in the synonymy of *Pleurodema bufoninum* and instead propose the combination *P. somuncurensis* (Cei, 1969b) comb. nov. A more thorough study of diversity within *P. bufoninum* is required to establish whether that name could be applied to some populations currently assigned to *P. bufoninum* or should be considered a junior synonym of the latter.

Pleurodema marmoratum, a high-altitude, wide-ranging species, was divided into three subspecies by Vellard (1960). Duellman and Veloso (1977) stressed that the levels of intra-population variations are frequently greater than inter-population variation and preferred not to recognize any subspecies within *P. marmoratum*. Our samples included one specimen from Bolivia (La Paz: Omasuyos: Huatajata), one from northern Chile (Region I: Lauca; sequences from Correa et al., 2006) and one from central Peru (Junín: Huasahuasi: Laguna Mamancocha; sequences from Lehr et al., 2005). Our results indicate a clade composed of the former two samples, sister taxon of the Peruvian specimen. Although the available samples are still a minimal

representation of the known variation and wide distribution of this species, our results, and the p-distances in *16S* between the central Peruvian sample and those of northern Chile and Bolivia (2%), highlight the need for a thorough study of this species throughout its range.

Correa et al. (2008) recovered four main clades of Chilean populations of *P. thaul*, including southern and central Chilean groups of populations (their clade Four and clades One and Two, respectively) recognized by Duellman and Veloso (1977), and a geographically intermediate clade (their clade Three). Our analyses included exemplars of the four clades recognized by Correa et al. (2008), plus other specimens from Argentina and Chile. All exemplars of *P. thaul* are monophyletic in our analyses, rejecting the suggestion of Duellman and Veloso (1977) that populations of *P. thaul* from southern Chile are more closely related to *P. bufoninum* than to *P. thaul* from central Chile and southern Argentina. This suggestion was based on the fact that, according to Duellman and Veloso (1977), *P. thaul* from southern Chile, like *P. bufoninum*, lays its eggs as short strings, while populations from southern Argentina and central Chile lay their eggs as gelatinous masses. Observations by Weigandt et al. (2004) indicate that *P. thaul* in southern Argentina lays eggs in short strings, not gelatinous masses. Curiously, the egg clutches and amplexus position in *P. thaul*, as well as its geographical variation, have not been treated in the literature since Duellman and Veloso (1977), even in those papers dealing with geographical distribution (Correa et al., 2007, 2010) or aspects of reproductive biology (Formas and Pugin, 1978; Díaz-Páez and Ortiz, 2001).

Our analyses clearly show that, as could be expected, the Chilean populations are not monophyletic, having trans-Andean populations from Argentina closely related to the two southern-most clades identified by Correa et al. (2008; their clades Three and Four). These results underline the need to avoid experimental designs constrained by political frontiers.

The Pleurodema brachyops clade

Although the monophyly of this clade is supported in our analyses, results differ as to the position of *P. brachyops*; it is the sister taxon of *P. alium* + *P. diploolister* in the direct optimization analyses, it is recovered alternatively on the same position or as sister taxon to *P. tucumanum* + *P. borellii* + *P. cinereum* in the parsimony analysis, and it is the sister taxon to all other species in the Bayesian analysis (see Appendices S3 and S4), leaving a basal polytomy inside the *P. cinereum* clade in the strict consensus of the optimal trees of that analysis. The alternative positions of *P. brachyops* in the direct optimization analysis and parsimony analysis of the static alignment have relatively low jackknife values,

as does the Bayesian support for the alternative position in the Bayesian analysis (see Appendix S4).

The taxonomy of *P. borellii* and *P. cinereum* has been contentious for a long time, with the former being considered a senior synonym of the latter by Parker (1927) and Barrio and Rinaldi de Chieri (1970) or as valid species by Gallardo (1968) and, even if tentatively, by subsequent authors (Duellman and Veloso, 1977; Cei, 1980, 1987; De la Riva et al., 2000; Lavilla and Cei, 2001). Currently, there are no known diagnostic characters, and altitudinal distribution has been the only, dubious, surrogate to differentiate both species. *Pleurodema cinereum* (type locality: “Juliaca at 12500 feet” Peru) is distributed from southern Peru to northern Argentina. In this study we included several specimens of *P. borellii* (from localities lower than 1970 m a.s.l.) from Argentina, and *P. cinereum* (from localities higher than 2600 m a.s.l.) from Argentina and one Bolivian locality. No topotypes or Peruvian samples were available, and our Bolivian sample (Departamento La Paz: south of La Paz city: Cota-Cota) was collected about 240 km (airline) south-east from the type locality of Juliaca (a town along the western coast of Lake Titicaca). Our results show two main clades, one composed of the exemplars identified as *P. borellii* from northern Argentina and one composed of all exemplars identified as *P. cinereum*. Within the latter clade, the extremely low level of sequence diversity (0.0–0.4% uncorrected p-distance in *16S*; Table 1) is remarkable, given that it includes samples from very distant localities (up to 750 km). In the clade of *P. borellii*, there is more internal divergence (0.7–1.4% uncorrected p-distance in *16S*; Table 1) than in the other clade.

However, in the Bayesian analysis one specimen assigned to *P. borellii* (Argentina: Tucumán: El Cadillal) is related to the remaining *P. cinereum* (see Appendix S4). Our results are not conclusive regarding to the recognition of two different species. Consequently, as no additional data from biology or morphology are known to support their differentiation (McLister et al., 1991; De la Riva et al., 2000), we are inclined to believe that

the topological result is more probably a result of coarse-grained phylogeographical pattern rather than species-level differentiation. In this way, we suggest that populations assigned to *P. borellii* and *P. cinereum* at least from the east coast of Lake Titicaca southwards to Argentina and Bolivia belong to the same species. The lack of material closer to the type locality of *P. cinereum* precludes us from formally considering this species a senior synonym of *P. borellii*.

Bastos Maciel and Nunez (2010) recently described *Pleurodema alium* from Grão Mogol, in the highlands of the Espinhaço mountain range in the State of Minas Gerais, a species closely related to *P. diplolister*. Our inclusion of sequences from a paratype (CFBH 10201) and one sample from the Espinhaço mountains about 250 km northwards in the State of Bahia (Caetitê) corroborates its specific status as compared with our samples of the wide-ranging *P. diplolister* (see Bastos Maciel and Nunez, 2010) from Rio Grande do Norte (Angicos), Ceará (Viçosa do Ceará) and northern Bahia (Mocambo do Vento).

Our sampling included a single exemplar of *Pleurodema brachyops* from Guyana. This is unfortunate, as this species has a wide and discontinuous distribution, with populations known to occur in northern Brazil, Venezuela, Guyana, Colombia, southern Panama, and the islands of Curaçao, Aruba, Bonaire and Klein Bonaire (Duellman and Veloso, 1977; Frost, 2011). The morphological variation throughout its range remains largely unstudied.

The Pleurodema nebulosum clade

Barrio (1964) described *P. guayapae*, differentiating it from *P. nebulosum* on the basis of their advertisement call and middle ear morphology. Our analyses resulted in two clades with maximum Jackknife frequency, one composed of most samples of *P. nebulosum* from Catamarca, Mendoza (topotype), Salta and La Rioja, and the other composed of two samples of *P. guayapae*, and one specimen morphologically assignable to *P. nebulosum* (MACN 38766, Argentina: La Rioja province:

Table 1

Uncorrected pairwise distances between *16S* sequences of available specimens of *Pleurodema borellii* and *P. cinereum*. See Fig. 4 for country abbreviations

	1	2	3	4	5	6	7	8	9
1. <i>P. borellii</i> MACN 42028 (AR: Catamarca: R.P. 1 and Río Singuil)	–								
2. <i>P. borellii</i> FML 13373 (AR: Tucumán: El Cadillal)	0.014	–							
3. <i>P. borellii</i> MLP 4648 (AR: Jujuy: El Carmen)	0.009	0.012	–						
4. <i>P. borellii</i> FML 9009 (AR: Jujuy: P.N. Calilegua)	0.007	0.011	0.002	–					
5. <i>P. cinereum</i> MACN 42034 (BO: La Paz: Cota-Cota)	0.021	0.028	0.023	0.021	–				
6. <i>P. cinereum</i> MLP A. 4684 (AR: Jujuy: Juella)	0.023	0.026	0.021	0.019	0.002	–			
7. <i>P. cinereum</i> MLP A. 4694 (AR: Jujuy: Casabindo)	0.025	0.025	0.023	0.021	0.004	0.002	–		
8. <i>P. cinereum</i> MLP A. 4699 (AR: Jujuy: Casabindo)	0.025	0.025	0.023	0.021	0.004	0.002	0	–	
9. <i>P. cinereum</i> MLP A. 4716 (AR: Jujuy: Santa Catalina)	0.025	0.028	0.023	0.021	0.004	0.002	0.004	0.004	–

Table 2

Uncorrected pairwise distances between *16S* sequences of available exemplars of the *Pleurodema nebulosum* clade. See Fig. 4 for country abbreviations

	1	2	3	4	5	6	7	8	9	10	11	12
1. <i>P. guayapae</i> FML 23186 (AR: Santiago del Estero: Atamisqui) –												
2. <i>P. guayapae</i> FML 23179 (AR: Santiago del Estero: Atamisqui) 0	–											
3. <i>P. nebulosum</i> MACN 38766 (AR: La Rioja: La Chilca)	0.002	0.002	–									
4. <i>P. nebulosum</i> FML 9090 (AR: La Rioja: Anillaco)	0.012	0.012	0.011	–								
5. <i>P. nebulosum</i> FML 9091 (AR: La Rioja: Anillaco)	0.012	0.012	0.011	0.004	–							
6. <i>P. nebulosum</i> MACN 42020 (AR: Catamarca: Tinogasta)	0.011	0.011	0.009	0.002	0.002	–						
7. <i>P. nebulosum</i> MACN 38767 (AR: La Rioja: La Chilca)	0.011	0.011	0.009	0.002	0.002	0	–					
8. <i>P. nebulosum</i> FML 9089 (AR: La Rioja: Anillaco)	0.012	0.012	0.011	0	0.004	0.002	0.002	–				
9. <i>P. nebulosum</i> FML 9094 (AR: Salta: Finca Los Colorados)	0.012	0.012	0.011	0.004	0	0.002	0.002	0.004	–			
10. <i>P. nebulosum</i> MACN 38993 (AR: Mendoza: Telteca)	0.012	0.012	0.011	0.004	0	0.002	0.002	0.004	0	–		
11. <i>P. nebulosum</i> FML 9095 (AR: Salta: Finca Los Colorados)	0.012	0.012	0.011	0.004	0	0.002	0.002	0.004	0	0	–	
12. <i>P. nebulosum</i> FML 9096 (AR: La Rioja: Anillaco)	0.014	0.014	0.012	0.005	0.002	0.004	0.004	0.005	0.002	0.002	0.002	–

General Lavalle: RN 40, 5 km south of La Chilca). The other specimen from the same locality (MACN 38767) is related to the remaining *P. nebulosum*. The minimum uncorrected p-distance of *16S* between samples in both clades is 1% (Table 2). Barrio (1964) mentioned the existence of hybrids between *P. guayapae* and *P. nebulosum* in areas surrounding the type locality, and stressed that these had the tympanum almost invisible externally. Our specimen has a perfectly externally visible tympanic ring with tympanic membrane. Unfortunately, the nuclear genes we included do not show any variation between *P. guayapae* and *P. nebulosum* to aid in this issue. This is a notable result, showing that these two species need further study.

Gonzales (1998) and De la Riva and Gonzales (1998) reported a population tentatively assigned to *Pleurodema guayapae* from Chacoan areas of Santa Cruz, Bolivia. Strikingly, these populations are separated by almost 1000 km (airline) from areas in Argentina where this species occurs. Unfortunately, we did not have Bolivian samples of *P. guayapae*; their inclusion is strongly desirable in future analyses.

The Pleurodema bibroni clade and the origin of polyploidy

Pleurodema bibroni, *P. cordobae* and *P. kriegi* comprise a clade of cryptic and polyploid species. Barrio and Rinaldi de Chieri (1970) studied karyotype morphology using standard giemsa-staining of several species of *Pleurodema*. Unlike all other species of *Pleurodema* that have $2n = 2x = 22$, they found that *P. bibroni* and *P. kriegi* have $2n = 4x = 44$. Furthermore, there is a lack of correspondence between the mitotic chromosome quartets that can be ordered in pairs, and only bivalents are formed during meiosis. These observations led Barrio and Rinaldi de Chieri (1970), Bogart and Wasserman (1972), Duellman and Veloso (1977) and Barrio (1977) to discuss the possible origin of these species; the alternatives are an allopolyploid origin or autopolyploidy followed by diploidization. The second alternative was convincingly contested

by Barrio (1977), who maintained that an allopolyploid origin is most likely. He even suggested, on the basis of cytogenetics, reproductive biology, morphology and biogeography, that either *P. bufoninum* or *P. thaul* could be one of the parental species. More recently, Valetti et al. (2009), when describing the octoploid species *P. cordobae*, did not report details on chromosome morphology or of the meiotic behaviour. Since Barrio (1977), no new evidence has been advanced on the problem of the putative hybrid origin of *P. bibroni* and *P. kriegi*.

On the basis of our results, the following facts are relevant:

1. All evidence of a hybrid origin comes from the ploidy levels ($4x$ and $8x$) coupled with the sole presence of bivalents in meiosis (as opposed to tetravalents) and the lack of correspondence between the mitotic chromosome pairs. Other than this, no other evidence from external morphology has ever been taken to indicate a hybrid origin. Meiosis of the octoploid *P. cordobae* has not been studied.

2. Unlike most polyploid anurans (for reviews, see Schmid et al., 2010; Mable et al., 2011), no diploid species “counterpart” is known for any of these polyploid species from which an origin by autopolyploidy could be postulated.

3. The phylogenetic hypothesis based only on mitochondrial data indicates that these sequences are different enough from all other mitochondrial sequences of *Pleurodema* so as to not support the polyploid clade as the sister taxon of any terminal species. This is taken to indicate that, if from a hybrid origin, the matrilineal parental species that provided the mitochondria is not among the currently known species of *Pleurodema*.

4. Our phylogenetic analysis using only nuclear sequences, although less resolved, is still congruent with the analysis using only mitochondrial sequences (results not shown).

From these facts, we conclude that, considering our taxon and sequence sampling, the possible hybrid origin of the polyploid clade is not a relevant issue in the search for alternative phylogenetic hypotheses of *Pleurodema* in

Table 3

Uncorrected pairwise distances between *16S* sequences of available exemplars of the *Pleurodema bibroni* clade. See Fig. 4 for country abbreviations

	1	2	3	4	5	6
1. <i>P. bibroni</i> MNHN 9453 (UY: Rocha: Valizas)	–					
2. <i>P. bibroni</i> LGE 1410 (UY: Rocha: Valizas)	0	–				
3. <i>P. cordobae</i> MACN 42299 (AR: San Luis: Junín: Merlo)	0.004	0.004	–			
4. <i>P. cordobae</i> MACN 42300 (AR: San Luis: Junín: Merlo)	0.004	0.004	0	–		
5. <i>P. kriegi</i> MACN 38738 (AR: Córdoba: Parador El Cóndor)	0.005	0.005	0.002	0.002	–	
6. <i>P. kriegi</i> MLP A. 4759 (AR: Córdoba: P.N. Quebrada del Condorito)	0.005	0.005	0.002	0.002	0	–

our study. Should a species be discovered that could be considered the parental species of this clade, our assessment should be re-evaluated.

Species in the *P. bibroni* clade have a highly disjunct geographical distribution comprising the Sierras Grandes in central Argentina (*P. cordobae* and *P. kriegi*), littoral and mountain areas in Uruguay and southern Brazil, and an isolated population in the mouth of the river Apa, in northern Paraguay (*P. bibroni*; Kolenc et al., 2009, 2011). The extremely low level of sequence divergence among the three currently recognized species of the *P. bibroni* clade (maximum 0.5% between both *P. cordobae* and *P. kriegi* and *P. bibroni* in *16S*, Table 3) is striking. This only adds weight to previous observations about the similar external morphology of adults, larvae, and vocalizations of these species (Barrio, 1977; Kolenc et al., 2009, 2011), and underlines the need for a taxonomic reassessment. These observations, coupled with the peculiar geographical distribution, have been considered as evidence of recent isolation of a formerly continuously distributed species (Barrio, 1977; Duellman and Veloso, 1977; Kolenc et al., 2009), in association with climatic fluctuations during the Pleistocene and Holocene. Kolenc et al. (2009) hypothesized that the recent phase of arid climate in South America extending from the late Pleistocene (≈ 18 ka) to the Lower Holocene (≈ 8.5 ka; van der Hammen, 1974; Ab'Sáber, 1977) might explain the distribution of the common ancestor of this clade. The subsequent phase of humid subtropical and tropical climate might have resulted in the fragmentation of a once widespread distribution. So far, this remains speculative, as no fossil calibration points are available to test this hypothesis.

The position of *P. cordobae* ($2n = 8x = 88$) as sister group of *P. kriegi* (tetraploid) and together forming the sister taxon of *P. bibroni* (tetraploid) would be compatible with an origin by autopolyploidy of *P. cordobae* from *P. kriegi*. Our sampling is still obviously inadequate to address this situation at a populational level and cytogenetic studies are required. However, it is notable that for other cases where polyploidy has been hypothesized, denser samplings showed it to be a persistent phenomena, in which autopolyploidy (e.g. *Neobatrachus*, Mable and Roberts, 1997) or allopolyploidy (e.g. Holloway et al., 2006) occurred several times independently during the evolutionary history of the clade.

Multiple transformations of the macroglands, flash coloration and deimatic behaviour

Macroglands are formed by the accumulations of serous glands in certain regions of the body (Toledo and Jared, 1995); in Leiuperinae, macroglands occur in the sacral region of the body and have been called inguinal or lumbar glands according to their position. These macroglands have been considered homologous on the basis of a similar histological structure, are not sexually dimorphic (Ferraro et al., in press) and are associated with defensive behaviour (see below). Macroglans occur in the leiuperines *Edalorhina*, *Engystomops* and many species of both *Physalaemus* and *Pleurodema*. Lumbar glands had an important historical role as a taxonomic character in *Pleurodema*. Duellman and Veloso (1977), Barrio (1977) and Cei (1980) based their species groups on the presence/absence of lumbar glands. While traditionally species of *Pleurodema* were considered either to have or to lack lumbar glands, Mangione and Lavilla (2004) showed that serous glands are more concentrated on the lumbar region than in other regions of the body when the lumbar glands are absent. It remains to be tested if these serous glands are the same as those that occur on the lumbar glands, considering the histological and histochemical characteristics of the latter (Ferraro et al., in press). The nature of secretions of these macroglands is almost unknown. Roseghini et al. (1986) reported two biogenic amines for *Pleurodema*: m-hydroxyphenyltrimethylammonium (Leptodactylin) from the skin of *P. borellii*, *P. guayapae*, *P. nebulosum*, *P. marmoratum*, *P. thaul* and *P. tucumanum*, and 5-hydroxytryptamine (serotonin) from the skin of *P. bufoninum*. While Erspamer et al. (1962, 1986) reported peptides (physalaemins, bombesins) in the skin of the species of *Engystomops* (*E. pustulosus*) and *Physalaemus* (*P. biligonigerus*, *P. centralis*, *P. cuvieri*, *P. nattereri*, *P. signififer*) available to them, they did not find peptides on *Pleurodema*. The occurrence of skin peptides in *Pleurodema* has not been studied since these early reports.

Macroglands in leiuperines are associated with a peculiar defensive display, traditionally named deimatic behaviour (reviewed by Toledo et al., 2011; who called it “body-raising with legs vertically stretched”). This behaviour is displayed when the animal perceives

a threat and consists of turning the body to face away from the predator, lowering the head, elevating the sacral region, and exposing the macroglands, thereby simulating a larger animal with a pair of very large eyes (Fig. 5a,b). Some species also expose hidden flash coloration while performing this behaviour. Deimatic behaviour occurs also in many anurans that lack conspicuous macroglands to correspond with the “eyes” of the deimatic position (e.g. Williams et al., 2000). This defensive behaviour has been described in *P. tucumanum* (Kolenc et al., 2009), in which macroglands are absent but the concentration of serous glands is greater in this

region and, although the exposed thigh is darker than the rest of the body, the contrasting hue pattern commonly associated with flash coloration is absent. In *Pleurodema* there are three conditions: (i) flash coloration and lumbar glands absent (*P. alium*, *P. diplolister*, *P. guayapae*, *P. marmoratum*, *P. nebulosum* and *P. tucumanum*), (ii) flash coloration and lumbar glands present (*P. borellii*, *P. brachyops*, *P. cinereum* and the *P. bibroni* clade; Fig. 5a), and (iii) flash coloration absent (skin surrounding lumbar glands with the same colour pattern as the dorsum) but lumbar glands present (*P. bufoninum*, *P. somuncurensis*, *P. thaul*; Fig. 5b).

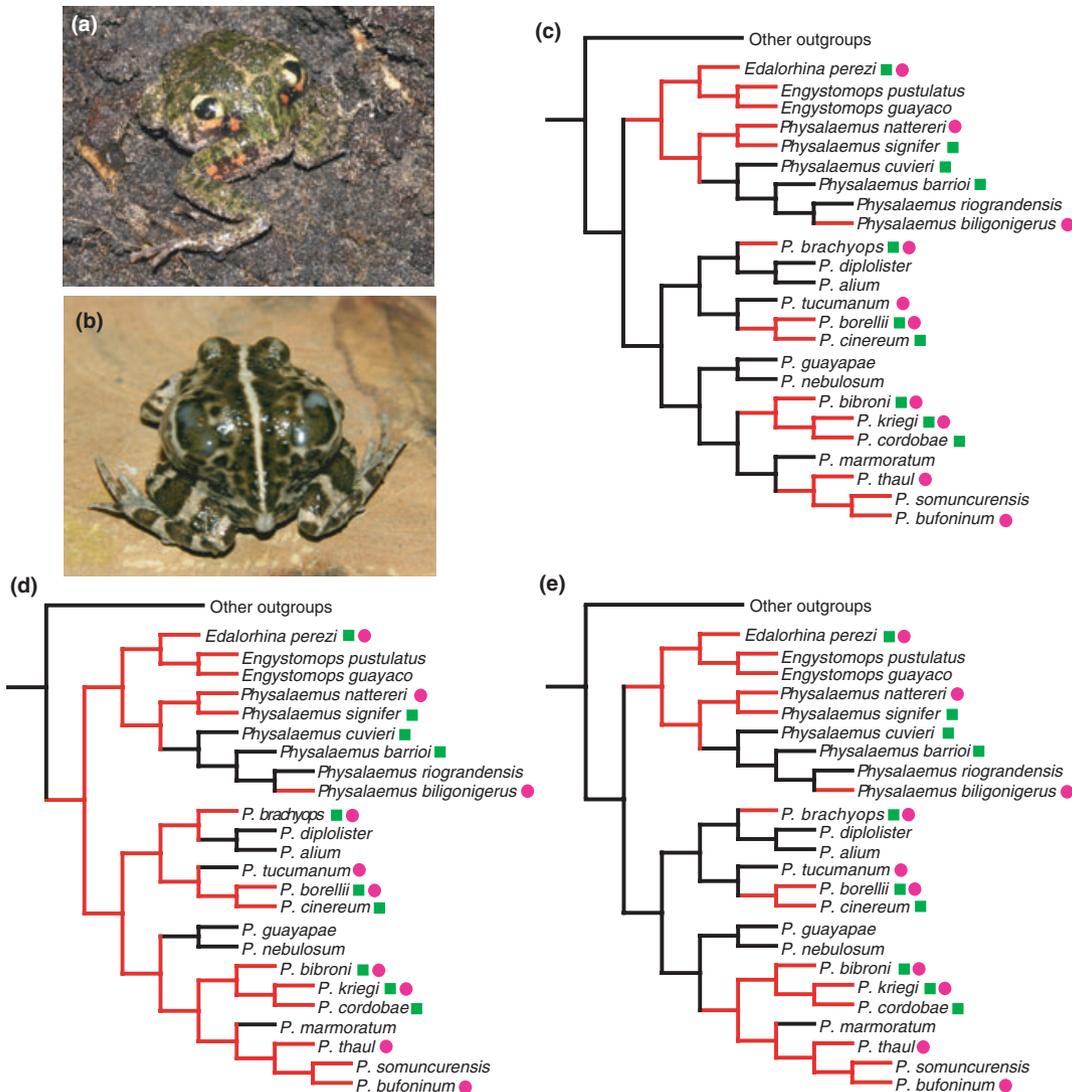


Fig. 5. The lumbar glands are prominently displayed with deimatic behaviour, here shown in a species with flash coloration (a, *Pleurodema bibroni*, photo by F. Kolenc) and in a species without flash coloration (b, *Pleurodema bufoninum*, photo by D. Baldo). The optimization of presence (red)/absence (black) of the macroglands requires at least seven steps, which could be explained as six independent origins and a reversal (c), as a single origin with subsequent losses and a reversal (d), or an intermediate scenario (e), among other equally costly alternatives. The green square indicates the presence of flash coloration on the hidden surfaces of thigh and shank, and/or inguinal region. The pink circle indicates the known occurrence of deimatic behaviour. The absence of a pink circle indicates that the occurrence of the behaviour is unknown, not necessarily absent.

From all these, deimatic behaviour is known to occur in *P. bibroni* (Vaz-Ferreira, 1984; Kolenc et al., 2009), *P. bufoninum* (Ceï, 1962), *P. brachyops* (Martins, 1989), *P. thaul* (Ceï, 1958) and *P. borellii*, *P. kriegi* and *P. tucumanum* (Kolenc et al., 2009).

Optimization of the presence/absence of macroglands on our phylogenetic hypothesis using direct optimization results in several alternative optimizations involving seven steps (Fig. 5c–e). These optimizations imply six independent origins and a reversion of the lumbar glands in *Pleurodema* and its sister taxon (Fig. 5c), or a single origin on its common ancestor with five subsequent independent losses and one regain (Fig. 5d), among several other combinations (e.g. Fig. 5e). Furthermore, the flash coloration in *Pleurodema* evolved three times independently, in the *P. bibroni* clade, in the common ancestor of *P. borellii* + *P. cinereum*, and in *P. brachyops*. When presence/absence of the macroglands is optimized on the static alignment topology, the pattern is not identical to the one obtained from direct optimization (and is identical to that obtained with Bayesian analysis: *P. brachyops* is the sister taxon to all other species of the *P. cinereum* clade), the optimization that is favoured is the one that involves a single origin on its common ancestor with five subsequent independent losses and one regain (data not shown). The occurrence of deimatic behaviour is unknown on several species of *Pleurodema*, where there are no experimental studies indicating that the behaviour is in fact absent. However, the fact that it occurs on species both with (e.g. *P. borellii*) and without macroglands (*P. tucumanum*) coupled with the scattered distribution of its known occurrence in the sister taxon of *Pleurodema* (e.g. Sazima and Caramaschi, 1986; Borteiro and Kolenc, 2007; Toledo et al., 2011) suggests that deimatic behaviour is most parsimoniously interpreted as a plesiomorphic condition in *Pleurodema* (Fig. 5c–e).

The variability in lumbar gland development and flash coloration in *Pleurodema* is striking, as is the extent of homoplasy suggested by our phylogenetic hypothesis. Could this be related to differences among potential visually oriented predators? Our results suggest that lumbar gland morphology is much more plastic than had previously been assumed, and in fact we are not aware of other cases in anurans where character states associated with antipredator mechanisms appear so plastic in such a restricted clade. Given the defensive role of these glands, this plasticity suggests variation in the selective pressure exerted by visually oriented predators and/or alternative responses to those predators. That is, the predators might vary, with different morphologies for each, or the predators may be the same but the species evolved different defensive responses. Diurnal activity was reported for some *Pleurodema* species when vocalizing (e.g. Ceï, 1956;

Agostini et al., 2007), including species with (*P. cinereum*) and without (*P. nebulosum*) both lumbar glands and flash coloration; consequently, it is not possible to suggest any hypothesis with regard to defensive behaviour in response to diurnal visually oriented predators. A more thorough understanding of this phenomenon is associated with the situation in its sister taxon, the clade composed of *Edalorhina*, *Engystomops* and *Physalae-mus*, where the morphology and taxonomic distribution of macroglands and flash coloration are as complex as in *Pleurodema*. While extensive variation has been described in both the external morphology and degree of development of the inguinal glands as well as in flash coloration in *Physalae-mus* (Nascimento et al., 2005), only three species were studied from a structural (histological) or ultrastructural perspective (Delfino et al., 1999; Alvarez et al., 2005; Lenzi-Mattos et al., 2005). Lenzi-Mattos et al. (2005) studied inguinal glands of *Physalae-mus nattereri*, finding that the histological structure is similar to that described by Delfino et al. (1999) for *P. biligonigerus*. Also, secretory products of ordinary serous glands of *P. albonotatus* and *P. biligonigerus* share similar ultrastructural traits in maturation paths (Delfino et al., 1999; Alvarez et al., 2005). Ron et al. (2004) described variation in the macroscopic structure of the acini of *Engystomops*, as exposed through superficial dissections. Macro-glands of *Edalorhina* remain unstudied.

Evolution of egg-clutch structure in Pleurodema: diversity arising from the foam

With only 15 species, the variation shown by *Pleurodema* in terms of reproductive modes is striking, as four egg-clutch structures are known to occur. With 53 species (Frost, 2011), its sister clade (*Physalae-mus* + *Engystomops* + *Edalorhina*) has so far a single egg-clutch structure, eggs laid in a foam nest (e.g. Budgett, 1899; Fernández and Fernández, 1921; Noble, 1927; Schlüter, 1990; Nascimento et al., 2005) with some variations as to where the nests are built (Haddad and Pombal, 1998; Pupin et al., 2010). The four different egg-clutch structures known to occur in *Pleurodema* are as follows:

1. Eggs laid in a floating foam nest built by the mating pair (oviductal secretions beaten by the male with his hindlimbs) during amplexus (Fig. 6b): *Pleurodema borellii*, *P. brachyops*, *P. cinereum*, *P. diplolister*, *P. guayapae* and *P. nebulosum* (Fernández and Fernández, 1921; Fernández, 1927; Barrio, 1964; Hoogmoed and Gorzula, 1979; Hulse, 1979; Hödl, 1992; Cardoso and Arzabe, 1993).

2. Eggs laid in gelatinous, ovoid plate-like masses in which the eggs are accommodated along a single plane (Fig. 6c): *P. tucumanum* (Martori et al., 1994). Earlier authors (Ceï, 1956; Barrio, 1964) reported eggs laid in a

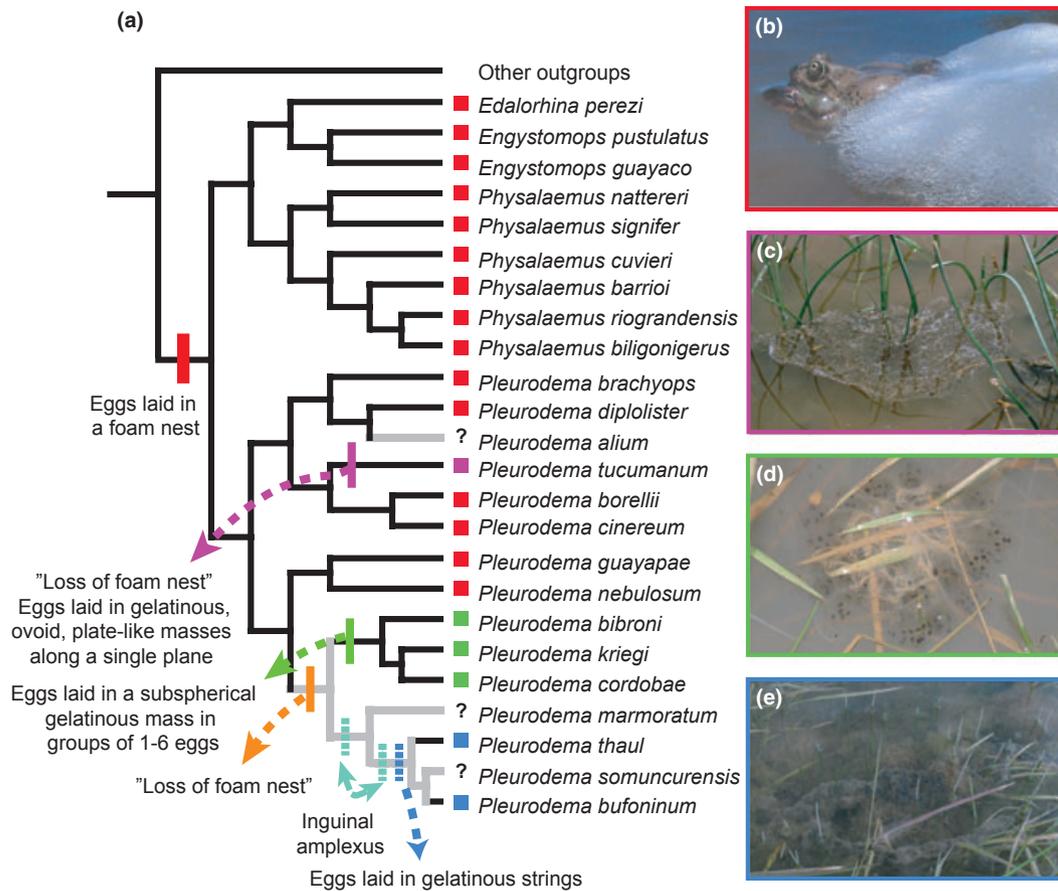


Fig. 6. (a) Presence/absence of foam nest and amplexus position of *Pleurodema* and its sister taxon, optimized on the simplified optimal topology resulting from our analysis for this clade. Note that the “loss of foam nest” is indicated only with the purpose of showing where in the tree has occurred a transformation leading to clutches without foam nest. We are uncertain as to the actual transformations involved, as these could simply be behavioural (a loss of foam beating behaviour in the male) and/or include transformations in the pars convoluta dilata (the caudal section of the oviduct before the ovisac, where the secretion that becomes the foam, is produced). This seems evident as judged by the two points where “losses” of foam had occurred, resulting in different spawn structures. See text for further discussion. The images on the right show the four different egg-clutch structures present in *Pleurodema* and its sister taxon. (b) Eggs laid in a floating foam nest built by the mating pair (oviductal secretions beaten by the male with his hind limbs) during amplexus (in this case, *Pleurodema cinereum*; photo by D. Baldo). (c) Eggs laid in gelatinous, ovoid plate-like masses in which the eggs are accommodated along a single plane (*Pleurodema tucumanum*; photo by R. Martori). (d) Eggs laid in subspherical gelatinous masses in groups of 1–6 eggs (in this case, *Pleurodema bibroni*; photo by F. Kolenc). (e) Eggs laid in gelatinous strings (in this case, *Pleurodema bufoninum*; photo by S. Nenda).

foam nest in this species, but recent observations by one of us (E.O. Lavilla, pers. obs.) confirm the description of Martori et al. (1994).

3. Eggs laid in subspherical gelatinous masses in groups of 1–6 eggs (Fig. 6d): *P. bibroni*, *P. cordobae* and *P. kriegi* (Barrio, 1977; Valetti et al., 2009).

4. Eggs laid in gelatinous strings (Fig. 6e): floating strings in *P. bufoninum*, and submerged strings in *P. thaul* (Fernández, 1927; Weigandt et al., 2004). The gelatinous strings had been referred in the literature as “short”, presumably to distinguish them from the extensive gelatinous strings occurring on several bufonids.

Duellman and Veloso (1977) and C. Diminich (pers. comm.), respectively, indicated that *P. marmoratum* and *P. somuncurensis* lay eggs in gelatinous masses, without

further detail. Consequently, it is unclear if these gelatinous masses have the same characteristics as those of the *P. bibroni* clade. Until more data become available, we consider them unknown (but consider the available information sufficient to discard the possibility of them being foam masses). Egg-clutch structure remains undescribed in *P. alium*.

The optimization of egg-clutch structure on our optimal hypothesis (Fig. 6a) requires some discussion as to their nature and homology. For this, we must consider aspects related to (i) oviductal anatomy, (ii) egg-clutch structure, and (iii) foam nest-making behaviour and amplexus position.

Foam nests in anurans are produced by the whipping of oviductal secretions by one or both parents. Although

some progress in the biochemical characterization of these secretions (Cooper et al., 2005; McMahon et al., 2006; Hissa et al., 2008; Fleming et al., 2009; Mackenzie et al., 2009) and in the anatomy and histochemistry of the secreting section of the oviduct (Coe, 1974; Kabisch et al., 1998; Alcaide et al., 2009; Furness et al., 2010) is slowly starting to take place, most studies have focused on the mechanics of foam production by the amplexic couple (Tyler and Davies, 1979; Hödl, 1986, 1990, 1992; Schlüter, 1990; Williams and Tyler, 1994; Altig and McDiarmid, 2007; Bastos et al., 2010; Dalgetty and Kennedy, 2010).

Anatomically, the production of foam nests has been associated with an enlarged portion of the posterior pars convoluta, anterior to the ovisac, called the pars convoluta dilata by Furness et al. (2010)—previous authors employed the term “foam gland” (Coe, 1974; Kabisch et al., 1994, 1998) or “albumin gland” (Alcaide et al., 2009) for the glandular tissue lining this region, or simply referred to it as a greatly dilated oviduct (Bhaduri, 1953); see also Amiet (1974). The general size of the pars convoluta dilata has been related to both body and clutch size, and the location of foam nests (Furness et al., 2010).

The diversity of oviductal anatomy and histochemistry in *Pleurodema* has not been as thoroughly studied as in *Leptodactylus* (Furness et al., 2010; and references therein). Bhaduri (1953) described a pars convoluta dilata in *P. cinereum*. Alcaide et al. (2009) noticed some unique histochemical properties in *P. borellii*, associated with the composition of the secreted product, which they associate with the general appearance of the foam once whipped (specifically, they mention their lax nature and large size of the air chambers). Furness et al. (2010) reported an interesting difference between the oviducts of the foam-nesting species *P. brachyops* and the oviduct of the egg-string layer *P. bufoninum*. While a pars convoluta dilata is present in the former, it is absent in the latter.

From the perspective of establishing homologies among components of the egg-clutch structure, the diversity in egg clutches cannot be logically reduced to the presence or absence of a foam nest. After all, in species that lay egg-clutches as gelatinous masses, the material of the gelatinous matrix must be secreted somewhere in the oviduct. It does not seem unreasonable to suggest that this material might be produced in the same area where the pars convoluta dilata occurs. In fact, Furness et al. (2010) note that the pars convoluta dilata might occur in species where foam nests are not produced but that have particularly gelatinous clutches, as Bhaduri (1953) reported in *Agalychnis dacnicolor*. From this perspective, the presence of a foam nest becomes a combination of a secreting region of the oviduct that produces a substance amenable to trap bubbles when beaten (or however the bubbles are

produced) and the foam-making behaviour produced by one member of the parental couple (or several, in the case of multi-male spawning; e.g. Hödl, 1992; Cardoso and Arzabe, 1993; Prado and Haddad, 2003; Agostini et al., 2007). With these factors in mind, when egg-laying within a clade “reverts” from foam-nesting to a different egg-laying mode, it could be explained as a transformation involving one or more of them. The identification of these factors would allow a refinement of the homology hypotheses.

Our and previous (Grant et al., 2006; Pyron and Wiens, 2011) results suggest that eggs laid in a foam nest built by the mating pair during amplexus is a synapomorphy for the clade composed of *Edalorhina*, *Engystomops*, *Physalaemus* and *Pleurodema*. However, in *Pleurodema* there are subsequent transformations from foam nest clutches (actually two independent transformations) to eggs laid in gelatinous structures, being subspherical masses, ovoid and plate-like, or egg strings (Fig. 6a). One transformation occurs in *P. tucumanum*, where eggs are placed in a gelatinous, ovoid plate-like mass in which the eggs are accommodated along a single plane. We suggest that this peculiar gelatinous matrix could be the result of a loss of the foam-making behaviour in the male; in this way the ovoid plate-like mass could be simply the unbeaten secretion from the pars convoluta dilata. This hypothesis could be easily tested with a histological study of the oviduct in *P. tucumanum*, and by artificially beating the secretion (see Haddad et al., 1990).

The other transformation occurs in the clade composed of the *P. bibroni* + *P. thaul* clades, where eggs are laid in gelatinous masses, each containing 1–6 eggs (*P. bibroni* clade), or in gelatinous strings (*P. bufoninum*, *P. thaul*). This clade is poorly supported in the parsimony analysis of the static alignment, and details of the egg-clutch structure of *P. marmoratum* and *P. somuncurensis* remain unknown; however, a few relevant points should be considered. First, even under a better scenario (increased support for the *P. bibroni* + *P. thaul* clades), the two egg-clutch structures in these two clades cannot be considered the same state simply for not including a foam nest. If anything, it could be reasonable to consider the possibility that one evolved from the other. However, at this point, we cannot establish the character polarity, partly because of the unknown egg-clutch structures of *P. marmoratum* and *P. somuncurensis* create an ambiguous optimization. The egg-clutch structure in the latter, however, is more parsimoniously predicted to be eggs in gelatinous strings (Fig. 6a). The second relevant point is that, unlike most New World hyloids, inguinal amplexus was reported in the *P. thaul* clade. It is known to occur in *Pleurodema bufoninum*, in *P. somuncurensis*, and at least in a population of *P. thaul* from southern Chile (Adam, 1954; Cei, 1958, 1972; Duellman and Veloso, 1977). The

mechanics of inguinal amplexus seem incompatible with foam beating by the male, whose rear limbs lay quite distant from the cloaca of the female. In fact, in limnodynastids where both foam-nesting and inguinal amplexus occur, the foam is beaten by the female with the help of expanded flanges on her fingers (Martin, 1967; Tyler and Davies, 1979). Our results suggest that in *Pleurodema* the inguinal amplexus evolved from axillary amplexus after the transformations leading to spawns without foam nests (Fig. 6a).

Evolution of foam-nesting in anurans: a phylogenetic perspective

Reproductive modes involving foam nests in anurans have evolved independently at least seven times during the evolutionary history of frogs, when considering the phylogenetic hypothesis of Frost et al. (2006), with the modifications in hyloid relationships by Grant et al. (2006) and Pyron and Wiens (2011): Hylidae (a single population of *Scinax rizibilis*; Haddad et al., 1990; Bastos et al., 2010), Hyperoliidae (*Opisthoxylax*; Amiet, 1974, 1991), Leiuperinae (all genera but *Pseudopaludicola*), Leptodactylinae (*Leptodactylus sensu* Frost et al., 2006; Heyer, 1969, 1973), Limnodynastidae (*Adelotus*, *Heleioporus*, *Lechriodus*, *Limnodynastes*, *Philoria* and *Platyplectrum*; Martin, 1967; Tyler and Davies, 1979), Microhylidae (*Stumpffia*; Glaw and Vences, 1994, 2007) and Rhacophoridae (*Chiromantis*, *Polypedates* and *Rhacophorus*; Wilkinson et al., 2002; Grosjean et al., 2008; Li et al., 2009). The peculiar aquatic bubble nest of the microhylid *Chiasmocleis leucosticta* (Haddad and Hödl, 1997) deserves more study in order to understand if it could be considered a variation of foam nest; in the meantime we do not consider it as such.

As in *Pleurodema*, a transformation from foam-nesting has been reported to occur as well in Rhacophoridae and Limnodynastidae. In Rhacophoridae, foam-nesting appears in the common ancestor of *Chiromantis*, *Feihyla*, *Ghatixalus*, *Polypedates*, *Rhacophorus* and *Taruga*. Li et al. (2009) found *Feihyla* to be nested within *Chiromantis*, with *C. vittatus* being their closest relatives. This species and one of the two species of *Feihyla* (*F. palpebralis*) are reported to produce a clutch contained in a mass of jelly described as containing some bubbles (Grosjean et al., 2008; Li et al., 2009). Pyron and Wiens (2011), however, recovered a different hypothesis of relationships for Rhacophoridae, where *F. palpebralis* is not nested within *Chiromantis*, and so it implies two different transformations from foam nest to a clutch contained in a mass of jelly.

Relationships of Limnodynastidae still require intensive study; hypotheses vary importantly between Frost et al. (2006) and Pyron and Wiens (2011), although in

both analyses the sampling density in this family is not substantially different; furthermore, in both studies support is, in general, low. The topology for this family obtained by Frost et al. (2006) implies alternative optimizations for the presence of foam nests, one in which foam-nesting appears once and is subsequently lost twice (in *Neobatrachus* and *Notaden*), or one in which foam-nesting appears once, is lost once (in the common ancestor of *Neobatrachus* + *Notaden* + *Lechriodus* + *Platyplectrum*), and reappears in the common ancestor of *Lechriodus* + *Platyplectrum*. The topology of Pyron and Wiens (2011) implies a single origin of the foam nest. In the context of this topology there are two subsequent transformations: *Philoria loveridgei* (Knowles et al., 2004), and certain populations of *Limnodynastes tasmaniensis*. Some populations of the latter species from south-east South Australia have been reported to produce a gelatinous mass within which the eggs are embedded, instead of a foam nest (Roberts and Seymour, 1989). Breeding females of these populations have a substantial reduction or absence in the finger flanges, a secondary sexual character that is used to produce the bubbles that will form the foam. Williams and Tyler (1994) have shown that in these populations the female still exhibits the stereotyped movements to produce the foam, albeit with some modifications that fail to conduct the bubbles to the groin. We find it remarkable that in all reviewed cases of transformations from a foam-nesting common ancestor, the clutches still retain as a common characteristic the gelatinous matrix. The relationship between this secretion, the structure of the pars convoluta, and the modifications of this part of the oviduct associated with a transformation from foam-nesting are as interesting a problem as the origin of foam-nesting itself.

Originally, the different places where foam nests are laid in hyloids (water surface, burrows on the side of water bodies, burrows without contact with water bodies) have been associated with gradually increased levels of terrestriality (Martin, 1967, 1970; Heyer, 1969; Prado et al., 2002). However, recent phylogenetic results (Frost et al., 2006; Grant et al., 2006; Pyron and Wiens, 2011) indicate complex patterns where the different places where foam nests are laid cannot be reduced to simple linear trends. In these studies the most basal group of *Leptodactylus* is the *L. marmoratus* Group (including the former genera *Adenomera* and *Lithodytes*), which shows increased levels of terrestriality. These include foam nests built in chambers on the sides of ponds (Lamar and Wild, 1995) or on sections of ant nests (Schlüter et al., 2009); the chambers subsequently are flooded and the exotrophic larvae that finish their development in a larger water body (De la Riva, 1995), or foam nests built in the same chambers that contain endotrophic larvae until they complete their development (Kokubum and Giaretta, 2005). The *L. marmoratus*

Group, on Pyron and Wiens (2011) integrative reanalysis, is the sister taxon of a clade containing all remaining species of *Leptodactylus*. The basal species of this clade is *L. rhodomystax*, a species that makes foam nests on the forest floor, between shrubs, roots, and fallen tree trunks some dozens of centimetres away from ponds, where tadpoles complete their development (Rodrigues et al., 2007). Only subsequently in the evolutionary history of *Leptodactylus* do foam nests appear as floating structures, suggesting, if anything, that the latter is derived from foam nests laid in more terrestrial contexts.

Foam nest building in the hyloid *S. rizibilis* occurs in a unique way. The female jumps over the eggs that are imbedded in the oviductal secretion; the impact of the body against the clutch allows the retention of air bubbles in the secretion, forming the foam nest after repeated jumps (Haddad and Hödl, 1997; Bastos et al., 2010). The clutch is laid on the surface of ponds (Pombal, 1997; Bastos et al., 2010), as occurs in the species with which *S. rizibilis* is most closely related, *S. aromothyella* and *S. berthae* (Barrio, 1962; Faivovich, 2005), in the phylogenetic analysis of Faivovich (2002), suggesting that, if anything, foam-nesting evolved from clutches laid in ponds.

In rhacophorids, it seems evident that foam-nesting evolved from terrestrial clutches, laid on vegetation or trunks overhanging water bodies. This occurs in the genera that are more relevant for the optimization of the oviposition site for the common ancestor of the foam-nesting clade (*Chiromantis*, *Feihyla*, *Ghatixalus*, *Polypedates* and *Rhacophorus*): *Gracilixalus* (e.g. Bain and Truong, 2004), *Kurixalus* (e.g. Ueda, 1986), *Liuixalus* (e.g. Smith, 1953), *Nyctixalus* (e.g. Inger, 1966) and *Theloderma* (e.g. Taylor, 1962). Bhaduri (1932, 1953) and subsequent authors (Hoffman, 1942; Bhaduri and Mondal, 1965; Liem, 1970; Grosjean et al., 2008) noticed a correlation between presence of convoluted wolffian ducts (as opposed to tubular) in males and foam-nesting behaviour in rhacophorids. However, since Bhaduri's discussion, there has not been any study indicating that the convoluted wolffian ducts do, in fact, provide material for the construction of the foam nest.

In the microhylid cophiline *Stumpffia*, foam nests are terrestrial and the embryos have endotrophic development (Glaw and Vences, 2007). In the phylogenetic hypotheses where species of this genus have been included (e.g. Wollenberg et al., 2008; Köhler et al., 2010; Pyron and Wiens, 2011), *Stumpffia* is nested in a large clade with endotrophic development (including the genera *Anodonthohyla*, *Cophila*, *Plethodontohyla*, *Platypelis* and *Rhombophryne*). Knowledge of the reproductive biology of this clade is still restricted to relatively few species; egg clutches are known to be laid in water (housed in tree holes or leaf axils) or nests on the ground (Glaw and Vences, 2007).

In the phylogenetic hypothesis of Pyron and Wiens (2011), the hyperoliid *Opisthoxylax immaculatus*, a species that produces foam nests, is in a clade that includes *Acanthixalus*, *Afrifaxalus*, *Heterixalus*, *Hyperolius*, *Morerella* and *Tachycnemis*. In this clade *Opisthoxylax* is in a basal grade that includes *Acanthixalus*, *Afrifaxalus* and *Morerella*, all of which lay clutches on leaves (Perret, 1962; Schiøtz, 1967, 1999; Rödel et al., 2009).

While relationships within hyloids are still poorly supported (Frost et al., 2006; Grant et al., 2006; Pyron and Wiens, 2011), current hypotheses suggest that in all cases where foam-nesting occurs, it evolved from spawns laid on the surface of water. In ranoids, however, foam nests apparently evolved under different conditions than in hyloids, as in two of the three groups where it occurs (Hyperoliidae and Rhacophoridae) it evolved from terrestrial spawns, as discussed above. In the third group (cophiline microhylids) even though it is still unclear if plesiomorphically the eggs are laid on water or not, foam-nesting still evolved in the context of an increased terrestriality as it arose after the origin of endotrophic development.

Future studies in Pleurodema

Although having a complete taxonomic (all known species of *Pleurodema*), and a relatively good character sampling (up to 3500 bp from mitochondrial and nuclear genes) there are some nodes of our phylogenetic hypothesis of *Pleurodema* that require further corroboration (e.g. the position of *P. brachyops* within the *P. cinereum* clade, and the sister-group relation of the *P. bibroni* and *P. thaul* clades). For this reason, we anticipate further research to provide a stronger test of the monophyly of each of these clades. In the same way, we have barely scratched the surface of the issue on the origin of the *P. bibroni* clade, and look forward to more fine-grained molecular and cytogenetic studies from most known populations of its species. The taxonomy of several species still deserve further attention, both as a consequence of our results (e.g. possible synonymy of *P. bufoninum* and *P. somuncuensis*, or limits of *P. guayapae* and *P. nebulosum*) or for being long known problems that our sampling was insufficient to address (e.g. possible synonymy of *P. borellii* and *P. cinereum*).

Pleurodema is among the few relatively small clades of frogs that has such a widespread distribution in the Neotropical region (from Panama to southern Patagonia), and for which now there is a phylogenetic hypothesis. Our results and the discussions built upon them dealt with some of the interesting biological problems associated with this genus, but by no means the only or necessarily the most relevant ones. In fact, there are interesting biogeographical inferences, such as

the independent colonization of the high Andes by *P. cinereum* and *P. marmoratum*, that we have chosen not to focus on in this contribution but that clearly would deserve the same attention that we have given to other topics. Other striking results, such as the multiple origins or losses of the lumbar glands in *Pleurodema* and its sister taxon, suggest that this clade could well become an amphibian model for the evolution of predation avoidance mechanisms involving both morphology and behavioural repertoires. In the same way, *Pleurodema* has a number of species from extreme terrestrial environments, including the harsh patagonian steppe (*P. bufoninum*), the high Andes up to 5200 m a.s.l. (*P. marmoratum*; Seimon et al., 2007), around salt flats in central-western Argentina (*P. nebulosum*), or the extremely dry Caatinga in north-east Brazil (*P. alium*, *P. diplolister*), with some of them (*P. bufoninum*, *P. cinereum*, *P. marmoratum*) being one of the two or three or sometimes even the only anuran living in certain areas. While environmental physiological studies on *Pleurodema* are still scarce (Ruibal, 1962; Carvalho et al., 2010), they have pointed to unusual water conservation strategies in arid and saline environments. As a whole, the group seems a promising clade for evolutionary studies of physiological responses to extreme environmental conditions.

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Supporting Information

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

Appendix S1. List of species mentioned in this paper with its taxonomic authority.

Appendix S2. Locality data and GenBank numbers of the sequences employed in this study.

Appendix S3. Results of the parsimony analysis using the static alignment.

Appendix S4. Results of the Bayesian analysis using the static alignment.

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