REVIEW

Evolution of macroglands and defensive mechanisms in Leiuperinae (Anura: Leptodactylidae)

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Anurans show a wide variety of anti-predator mechanisms, and the species of the Neotropical clade Leiuperinae display several of them. Most species of *Edalorhina*, *Physalaemus* and *Pleurodema* show eyespots, hidden bright colours, macroglands in a inguinal/lumbar position, defensive behaviours and/or chemical defence. We conducted a histological analysis of dorsal and lumbar skin and revised the colour patterns, defensive behaviours and glandular secretions to study the diversity and evolution of anti-predator mechanisms associated with macroglands. We describe 17 characters and optimize these in a phylogenetic hypothesis of Leiuperinae. In the most recent common ancestor of *Edalorhina* + *Engystomops* + *Physalaemus* + *Pleurodema*, a particular type of serous gland (the main component of macroglands) evolved in the lumbar skin, along with the absence of the Eberth–Katschenko layer. A defensive behaviour observed in leiuperines with macroglands includes four displays ('crouching down' behaviour, rear elevation, body inflation and eye protection), all present in the same ancestor. The two elements associated with aposematism (hidden bright colours and eyespots) evolved independently in several species. Our results provide phylogenetic evidence for the startle-first hypothesis, which suggests that behavioural displays arise as sudden movements in camouflaged individuals to avoid predatory attacks, before the origin of bright coloration.

ADDITIONAL KEYWORDS: aposematism – bright colour – deimatism – eyespots – phylogeny – predation – skin secretions.

INTRODUCTION

Adult and juvenile anurans are consumed by a great variety of predators (e.g. McCormick & Polis, 1982; Menin *et al.*, 2005; Toledo, 2005; Toledo *et al.*, 2007; Ferreira *et al.*, 2019a). Diverse anti-predator mechanisms have been described, such as crypsis, aposematism, a number of defensive behaviours, noxious skin secretions and defensive vocalizations (e.g. Dodd, 1976; Marchisin & Anderson, 1978; Toledo & Jared, 1995; Williams *et al.*,

2000; Toledo *et al.*, 2007, 2010, 2011; Ferreira *et al.*, 2019a). Crypsis or camouflage involves all mechanisms associated with concealment, including prevention of detection and recognition (*sensu* Stevens & Merilaita, 2009). Aposematism (= warning colour) refers to the use of bright colours by animals to denote unpalatability or toxicity or to direct the attention of a predator to non-vital body regions (Endler, 1991). Some animals, in response to predatory attacks, suddenly change their appearance or display bright colours that are concealed in the resting position (flash colours; e.g. Cott, 1940; Kang *et al.*, 2011; Umbers *et al.*, 2017). These sudden displays, known as 'deimatic displays' (Maldonado,

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1970; Edmunds, 1974), can cause predators to recoil reflexively. In consequence, deimatism can be interpreted as a combination of aposematism and camouflage, but including an element of surprise, which is absent in the other two (Umbers *et al.*, 2015).

There is an extensive database of anti-predator mechanisms in anurans, and new observations accumulate at a notable pace. However, the evolution of these mechanisms has been dealt with only in the most general terms (Ferreira *et al.*, 2019a). There are few specific cases of anuran groups where the evolution of defensive mechanisms has been studied. One of these involves the multiple studies of aposematic coloration in dendrobatoids in relationship to alkaloid sequestration (e.g. Daly *et al.*, 1987; Summers & Clough, 2001; Santos *et al.*, 2003; Vences *et al.*, 2003; Darst *et al.*, 2006; Grant *et al.*, 2006, 2017). Another case involves the genus *Uperoleia* Gray, 1841 (Myobatrachidae), in which some species have bright colours, and defensive postures were observed in species with and without these colour patterns (Torr, 1991; Brodie *et al.*, 1998). Based on these observations, Brodie *et al.* (1998) hypothesized that the presence of noxious secretions concentrated in glands 'preadapted' *Uperoleia* species for behaviours that avoid predators more efficiently. Also, they proposed that bright coloration evolved earlier, followed by a defensive posture that becomes more specialized to exhibit this coloration. More recently, Toledo *et al.* (2011), in a review of anuran defensive behaviours, proposed that macroglands evolved earlier than the behaviours that expose them directed towards the predators.

In more general terms, Umbers *et al.* (2017) advanced two hypotheses regarding the evolution of deimatism. The defence-first hypothesis suggests that deimatism evolves along a trajectory to aposematism. Owing to the enhanced detectability of aposematism, when it evolves the prey has a higher probability of surviving an attack if it can conceal its conspicuousness to reduce the chances of being detected. On the contrary, the startle-first hypothesis proposes that a hypothetical camouflaged ancestor first acquires the sudden movements that avoid predatory attacks and increase survival (Cott, 1940; Edmunds, 1974) and that aposematism evolves subsequently. If a conspicuous and unexpected colour pattern is suddenly revealed during an escape movement, the protective value of this movement is enhanced, and such coloration would be favoured by selection (Umbers *et al.*, 2017).

An appropriate model to study the evolution of defensive mechanisms in anurans is the Neotropical clade Leiuperinae (Leptodactylidae). This clade includes 100 species in five genera (Frost, 2020), in which several anti-predator mechanisms have been described (e.g. Cei, 1962; Sazima & Caramaschi, 1986; Martins, 1989; Borteiro & Kolenc, 2007; Kolenc *et al.*, 2009; Toledo *et al.*, 2011). Anuran skin has serous glands, which can occur singly or as large clusters. When conspicuous clusters are observed, these are considered macroglands and are named according to the region of the body where they occur, e.g. inguinal, parotoid or tibial glands (Toledo & Jared, 1995). Some leiuperines have macroglands in the inguinal or lumbar region (*Edalorhina* Jiménez de la Espada, 1870, *Physalaemus* Fitzinger, 1826 and *Pleurodema* Tschudi, 1838), whereas others have parotoid and flank glands (*Engystomops* Jiménez de la Espada, 1872; e.g. Lynch, 1970; Duellman & Morales, 1990; Cannatella *et al.*, 1998; Ron *et al.*, 2004). In some species, these macroglands are evident macroscopically [e.g. *Physalaemus nattereri* (Steindachner, 1863), *Pleurodema thaul*; (Schneider, 1799) Lenzi-Mattos *et al.*, 2005; Kolenc *et al.*, 2009), whereas in other species careful examination reveals slightly raised glands (e.g. in *Physalaemus riograndensis* Milstead, 1960). These macroglands have been associated with a defensive display considered to be a 'deimatic behaviour' (see reviews by Toledo *et al.*, 2011; Ferreira *et al.*, 2019a). This behaviour consists of lowering the head, closing the eyes (or not), inflating the lungs, elevating the sacral region and displaying (if present) macroglands, bright coloured areas and/or eyespots (e.g. Martins, 1989; Toledo *et al.*, 2011). In a few species of leiuperines, during the display, a glandular secretion is released passively over the macroglands (e.g. Kolenc *et al.*, 2009: fig. 7E). Skin secretions of Leiuperinae are known to include diverse biogenic amines and peptides (Erspamer *et al.*, 1962a, b, 1964a, b, 1986; Cei & Erspamer, 1966; Cei *et al.*, 1967; De Caro *et al.*, 1968; Cei, 1985; Roseghini *et al.*, 1986; Lenzi-Mattos *et al.*, 2005; Barbosa *et al.*, 2015; Marani *et al.*, 2015, 2017; Cancelarich *et al.*, 2020).

Particular colour patterns described in Leiuperinae include bright coloration and eyespots. Bright coloration was reported in *Edalorhina* and in some species of *Engystomops*, *Physalaemus* and *Pleurodema* (e.g. Haddad & Sazima, 2004: fig. 1; Kolenc *et al.*, 2009: fig. 7C; Toledo *et al.*, 2011: fig. 2E). Eyespots frequently occur in the lumbar region, usually associated with the macroglands of some species of *Edalorhina*, *Physalaemus* and *Pleurodema* (e.g. Lenzi-Mattos *et al.*, 2005: fig. 1; Faivovich *et al.*, 2012: fig. 5A).

The monophyly of Leiuperinae was corroborated in several recent analyses with different support values and taxon samplings (Grant *et al.*, 2006, 2017; Pyron & Wiens, 2011; Fouquet *et al.*, 2013; Veiga-Menoncello *et al.*, 2014; Lourenço *et al.*, 2015; Jetz & Pyron, 2018), but not in others (e.g. Lourenço *et al.*, 2008, Faivovich *et al.*, 2012). *Pleurodema* has been recovered repeatedly as the sister taxon of a clade composed of *Edalorhina* + *Physalaemus* + *Engystomops*

(Pyron & Wiens, 2011; Fouquet *et al.*, 2013; Veiga-Menoncello *et al.*, 2014; Lourenço *et al.*, 2015; Jetz & Pyron, 2018). *Pseudopaludicola* Miranda-Ribeiro, 1926 has an unstable position, being recovered as the sister taxon of *Edalorhina* + *Physalaemus* + *Engystomops* + *Pleurodema* (e.g. Pyron & Wiens, 2011; Fouquet *et al.*, 2013; Veiga-Menoncello *et al.*, 2014; Lourenço *et al.*, 2015; Jetz & Pyron, 2018) and as the sister taxon of other Hyloidea (e.g. Lourenço *et al.*, 2008; Faivovich *et al.*, 2012). Although the relationships at the species level have been studied in analyses including nearly complete taxonomic samplings (e.g. Ron *et al.*, 2006; Faivovich *et al.*, 2012; Veiga-Menoncello *et al.*, 2014; Lourenço *et al.*, 2015), these were not incorporated in recent large-scale analyses of anurans (Jetz & Pyron, 2018). Therefore, a comprehensive phylogenetic analysis of Leiuperinae is still lacking.

Considering the combination of morphological and behavioural characters associated with anti-predator mechanisms that occurs in Leiuperinae, and the several hypotheses regarding the evolution of defensive mechanisms, the goals of the present study were as follows: (1) to conduct a phylogenetic analysis of Leiuperinae; (2) to study the diversity and taxonomic distribution of macroglands, colour patterns, defensive behaviours and skin secretions in Leiuperinae; (3) to study the evolution of these anti-predator mechanisms in Leiuperinae; and (4) to test the hypotheses proposed by Brodie *et al.* (1998), Toledo *et al.* (2011) and Umbers *et al.* (2017) in the context of leiuperine frogs and other anuran clades.

MATERIAL AND METHODS

PHYLOGENETIC ANALYSES

To address the phylogenetic relationships of Leiuperinae, we used sequences from GenBank. The mitochondrial gene sequences included portions of 12S, 16S, the intervening tRNA^{Val} (12S-tRNAval-16S, ~2420 bp), cytochrome *b* (*Cytb*, 959 bp) and cytochrome oxidase I (*COI*, 656 bp), and the nuclear gene sequences included fragments of the recombination activating gene exon 1 (*RAG1*, 427 bp), rhodopsin (*RHOD*, 316 bp), seven in absentia homolog 1 (*SIAH1*, 395 bp), proopiomelanocortin (*POMC*, 575 bp) and tyrosinase (*TYR*, 531 bp). GenBank numbers, specimen localities and voucher data are available in the Supporting Information (Supplementary Data S1). When possible, we selected sequences from specimens collected at the same locality as the samples for histology (see next section of Material and Methods). If not, we selected samples from the nearest available localities.

Sequences were aligned using the online software MAFFT v.7 (Katoh & Toh, 2008) under the strategy E-INS-i (for the ribosomal gene 12S-tRNAval-16S) and G-INS-i (for the other genes), with default parameters for gap opening and extension. Final alignments were edited in BIOEDIT (Hall, 1999). Sequence files were concatenated with SEQUENCEMATRIX v.1.7 (Vaidya *et al.*, 2011). The dataset included 144 terminals, including 90 Leiuperinae and 54 outgroups.

The maximum parsimony (MP) analysis was done with TNT (Goloboff *et al.*, 2008) using equally weighted parsimony and considering gaps as a fifth state. Searches used the new technology search under level 50, which included sectorial searches, tree drifting and tree fusing (Goloboff, 1999), hitting the best length 500 times, and submitting the resulting trees to a final round of TBR branch swapping. Parsimony jackknife (Farris *et al.*, 1996) absolute frequencies were estimated from 1000 replicates, hitting the minimum length two times (search level 15) with new technology searches (Goloboff, 1999) in each replicate, because preliminary analyses of the original data matrix showed that the minimum length is hit with this search strategy.

We also performed a maximum likelihood (ML) analysis with IQ-TREE v.1.6.12 (Nguyen *et al.*, 2015). MODELFINDER (Kalyaanamoorthy *et al.*, 2017), which is implemented in IQ-TREE, was used to select the optimal partition scheme and substitution models for molecular characters. MODELFINDER implements a greedy strategy (Lanfear *et al.*, 2012) that starts with the full partition model and subsequently merges two genes until the model fit does not increase any further. The best partition scheme included three subsets. We considered the edge-linked-proportional partition model but separated substitution models and rate evolution between partitions (-spp option). The maximum likelihood tree was obtained with 1000 ultrafast bootstrap replicates (Minh *et al.*, 2013; Hoang *et al.*, 2018). The resulting tree was visualized and edited in FIGTREE v.1.4.3 (Rambaut, 2016). The partitions and models selected are detailed in the Supporting Information (Supplementary Data S2).

TAXON SAMPLING FOR HISTOLOGICAL STUDIES

We included species from all the major clades that were recovered in the phylogenetic analyses of the genera of Leiuperinae. We studied skin sections from 30 species of the five genera of Leiuperinae and ten more species from the literature (Supporting Information, Supplementary Data S3). *Engystomops* includes nine species in two clades: the Duovox and the Edentulus clades (Ron *et al.*, 2006). We included one species from the Duovox clade and two from the Edentulus clade. *Edalorhina* consists of two species, one of which was available for this study. *Physalaemus* includes 50 species. The phylogenetic analyses performed by Lourenço *et al.* (2015) recovered two major clades, the *Physalaemus signifer* and *Physalaemus cuvieri* clades;
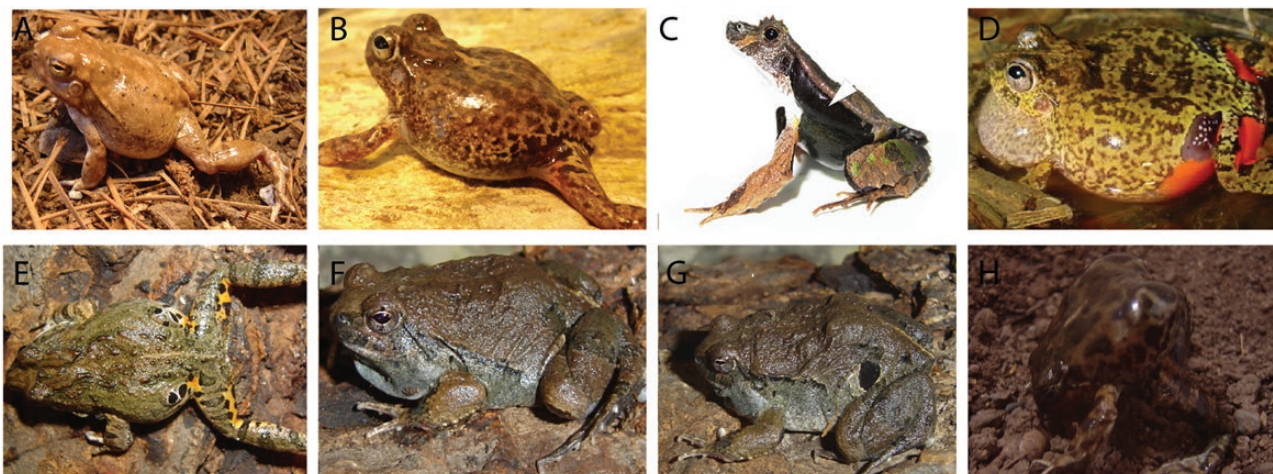
Figure 1. A, *Pleurodema nebulosum*. Absence of macrogland [character (char.) 0:0]; colour pattern similar to surrounding skin (chars 3:0; 4:0); and absence of eyespots (char. 5:0). B, *Pleurodema tucumanum*. Presence of patches of glandular tissue (char. 0:1); dark-brown colour pattern, contrasting with the surrounding skin (char. 3:2); colour pattern similar to surrounding skin in thighs (char: 4:0); and absence of eyespots (char. 5:0). C, *Edalorhina perezi*. Presence of flank gland (arrow; char. 2:1). Photograph: M. H. Yáñez-Muñoz. D, *Pleurodema brachyops*. Presence of macrogland in lumbar position (chars 0:2; 1:1); presence of bright colour (reddish) in lumbar region and thighs (chars 3:1; 4:1); and presence of one dark spot with small white pustules (char. 5:1). Photograph: J. Daza. E, *Pleurodema borellii*. Presence of macrogland in lumbar position (chars 0:2; 1:1); presence of bright colour (yellow) in lumbar region and thighs (chars 3:1; 4:1); and presence of various dark spots (char. 5:2). F, G, *Physalaemus biligonigerus*. Presence of macrogland in inguinal position (chars 0:2; 1:0); see the animal resting (F) and after being disturbed, starting the defensive display (G). H, *Pleurodema bufoninum* during full rear elevation (char. 12:2).

we included six species of the former and 14 of the latter. *Pleurodema* currently includes 15 species, and samples of skin from all but one species (*Pleurodema alium* Maciel & Nunes, 2010) were included. Finally, Veiga-Menoncello *et al.* (2014) recognized four major clades in *Pseudopaludicola* (25 species). However, given that neither macroglands nor a particular colour pattern was described in this genus, only two species were included. We also included species from most other genera of Leptodactylidae: *Crossodactylodes itambe* Barata, Santos, Leite & Garcias, 2013, *Paratelmatobius cardosoi* Pombal & Haddad, 1999 (Paratelmatobiinae), *Leptodactylus fuscus* (Schneider, 1799), *Leptodactylus laticeps* Boulenger, 1918 and *Leptodactylus latrans* (Steffen, 1815) (Leptodactylinae). Histological information from *Lithodytes lineatus* (Schneider, 1799) was coded from the study by Prates *et al.* (2012).

HISTOLOGICAL PROCEDURES

All examined specimens were fixed in 10% formalin and stored in 70% ethanol in herpetological collections (Supporting Information, Supplementary Data S3). Small strips (~5–10 mm²) of the dorsal skin (an area between the suprascapulae) and inguinal/lumbar skin (a posterolateral area on both sides of the sacrum)

were removed under a stereoscope. In the species with macroglands, the entire macrogland was extracted, i.e. flank and inguinal glands of *Edalorhina*, flank glands of *Engystomops*, inguinal glands of *Physalaemus* and lumbar glands of *Pleurodema*. *Leptodactylus laticeps* is the only examined species with red spots on the dorsal surface, and two sections of dorsal skin were examined, from red and pale brown regions.

Skin samples were dehydrated, cleared in butyl alcohol, paraffin embedded, sectioned in the transverse and sagittal planes (4–5 μm thick) and mounted onto microscope slides. Sections were stained with Haematoxylin and Eosin (Martoja & Martoja-Pierson, 1970), Masson's trichrome stain (Bancroft & Gamble, 2008) and Masson–Goldner trichrome stain (Martoja & Martoja-Pierson, 1970) and the following histochemical stains to characterize the secretory products of dermal glands: periodic acid–Schiff–Haematoxylin (PAS-H) for neutral carbohydrates, Alcian Blue (AB) 8GX (name of a cationic dye used to determine glycosaminoglycans) at pH 2.5 for primarily carboxylated acidic glycosaminoglycans and Ninhydrin–Schiff (NS) for proteins (Bancroft & Gamble, 2008). Finally, Von Kossa's method was used to test for the occurrence of the Eberth–Katschenko (EK) layer (Taylor *et al.*, 1966). Stained sections were

examined using a Nikon Eclypse200 microscope, and the images were captured using a Nikkon DS-Fi1 digital camera. Histological terminology of cutaneous glands followed that of Delfino *et al.* (2015).

Colour patterns, defensive behaviour and skin secretions

Occurrence of bright coloration, eyespots, defensive behaviour and skin secretions was coded based on direct observations and literature (Supporting Information, Supplementary Data S3 and S4).

Character definition and optimization

We coded the observed variation in 17 hypotheses of homology (Supporting Information, Supplementary Data S4). Ancestral character state reconstruction was done using Fitch's (1971) optimization algorithm as implemented in the software TNT v.1.5 (Goloboff *et al.*, 2008), on the basis of the maximum parsimony hypothesis.

RESULTS

Phylogenetic analysis

Parsimony analysis recovered 24 most parsimonious trees of 28 425 steps (Supporting Information, Supplementary Data S5). The consensus tree recovered Leptodactylidae as paraphyletic owing to the poorly supported position (< 50% jackknife) of a well-supported Allocentrolenidae (i.e. Allophrynidae + Centrolenidae; 99% jackknife), which is the sister clade of Leptodactylinae + Paratelmatobiinae (64% jackknife). This large clade is, in turn, sister to Leiuperinae. The three subfamilies of Leptodactylidae (i.e. Leiuperinae, Leptodactylinae and Paratelmatobiinae) are individually recovered as monophyletic (see obtained clades and support values in Supporting Information, Supplementary Data S5). Major topological differences among the most parsimonious trees include some internal clades or species of *Engystomops* and *Physalaemus*.

The monophyly of Leiuperinae is poorly supported (< 50% jackknife), but the individual monophyly of all its genera is well supported (> 98% jackknife). *Pseudopaludicola* is recovered as the sister taxon of the remaining Leiuperinae. *Pleurodema* resolves as the sister taxon of *Edalorhina* + (*Engystomops* + *Physalaemus*).

Maximum likelihood analysis (Supporting Information, Supplementary Data S6) recovers Leptodactylidae as monophyletic (82% bootstrap support). All subfamilies of Leptodactylidae are recovered as monophyletic, with high (Paratelmatobiinae and Leptodactylinae) or moderate (Leiuperinae) bootstrap support (100 and 88%, respectively). Within Leiuperinae, the main relationships (e.g. the intergeneric relationships) are largely congruent with those of the maximum parsimony analysis. The few differences between these hypotheses are discussed when relevant.

Hypotheses of homology

Based on the diversity in macroglands, colour patterns, defensive behaviours and skin secretions, 17 hypotheses of homology were defined and are discussed below (Supporting Information, Supplementary Data S4). These characters are optimized on the topology obtained from the maximum parsimony analysis. Character evolution is addressed in the Discussion.

Macroglands

0. **Macroscopic skin structure of inguinal/ lumbar region**: (0) glandular tissue or macrogland invisible externally (Fig. 1A); (1) slightly protuberant patches of glandular tissue, with diffuse boundaries (Fig. 1B); (2) macroglands developed, protruding from surrounding skin (Fig. 1C–H).
1. **Position of macrogland in inguinal/lumbar region**: (0) inguinal position (glands are partially/ completely hidden by hindlimbs in resting position; Fig. 1F, 1G); (1) lumbar position (glands are fully visible in resting position; Fig. 1D, 1E).
2. **Flank glands**: (0) absent; (1) present (Fig. 1C).

When inguinal/lumbar macroglands are present, they are prominent, protruding from the surrounding skin, observable to the naked eye and variable in size and shape. In *Pleurodema*, macroglands have a lumbar position (visible when the animal is resting; Fig. 1D, E). In *Edalorhina* and *Physalaemus*, they have an inguinal position (partly or completely covered when the frog is resting; Fig. 1F, G). In *Edalorhina perezi* Jiménez de la Espada, 1870, we also observed a flank gland, not previously described. This gland is ventrolateral to a longitudinal dermal ridge that extends from the eyelid to the inguinal macrogland (Fig. 1C). In *Engystomops*, there are parotoid glands and flank glands, but no inguinal/lumbar macroglands (Cannatella & Duellman, 1984). Macroglands in Leiuperinae are not sexually dimorphic.

Flank glands are considered independently from other macroglands because, topographically, they are placed in a different body region and, at least in *Ed. perezi*, flank glands and inguinal macroglands occur simultaneously.

COLOUR PATTERNS IN INGUINAL/LUMBAR REGION

3. **Coloration in hidden areas of inguinal/lumbar region**: (0) colour similar to the surrounding skin (Fig. 1A); (1) bright colour (yellowish, orange or reddish; Fig. 1D, E); (2) dull dark brown, contrasting to the surrounding skin (Fig. 1B).
4. **Coloration in hidden areas of thighs**: (0) colour similar to the surrounding skin; (1) bright colour (yellowish, orange or reddish; Fig. 1D, E).
5. **Eyespots in the inguinal/lumbar region**: (0) absent (Fig. 1A, B); (1) one dark spot (Fig. 1D, G); (2) various dark spots (Fig. 1E).

Some species have a lumbar colour pattern indistinguishable from the surrounding skin (Fig. 1A).

In one species (*Pleurodema tucumanum* Parker, 1927), there is a plain dark brown colour (Fig. 1B; Ferraro *et al.*, 2016: fig. 1A, B). In other species, there is bright coloration. This can be yellowish (e.g. Pimenta *et al.*, 2005), orange or reddish (Fig. 1D, E), or yellowish and orange/reddish simultaneously (documented only in *Physalaemus jordanensis* Bokermann, 1967). There are evident differences in the way in which bright colours have been described. For example, the flanks of *Engystomops petersi* Jiménez de la Espada, 1872 had been described, based on field notes of four herpetologists, as orange-brown, orange or reddish orange (Cannatella & Duellman, 1984). Considering this, we defined two characters to code all the species with bright colour in hidden areas of the inguinal/lumbar region and thighs. Duellman & Veloso (1977)
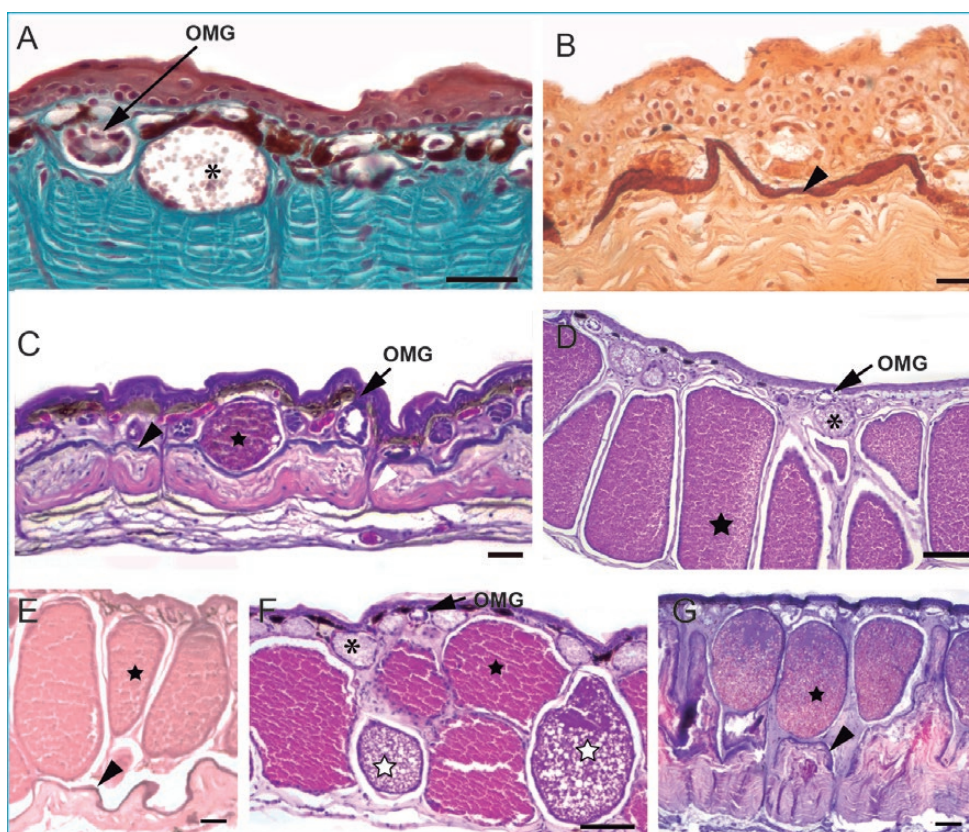

Figure 2. General features of skin of selected species. A, dorsal skin of *Engystomops freibergi*. Ordinary mucous glands (OMG) and type Ia serous glands (asterisk). Masson–Goldner trichrome stain. Scale bar: 20 µm. B, lumbar skin of *Physalaemus albifrons*. Eberth–Kastchenko (EK) layer in subglandular position (arrowhead). Von Kossa stain. Scale bar: 20 µm. C, lumbar skin of *Pleurodema tucumanum*. Ordinary mucous glands (OMG), type Ib serous glands (star) and EK layer (black arrowhead) only interrupted by smooth muscle cells (white arrowhead). Haematoxylin and Eosin (H&E). Scale bar: 50 µm. D, lumbar skin of *Physalaemus bokermanni*. Arrangement of type Ib serous glands (star), with contiguous acini, dorsoventrally elongated. Ordinary mucous glands and type Ia serous glands (asterisk) are also visible. H&E. Scale bar: 100 µm. E, lumbar skin of *Physalaemus aguirrei*, showing EK layer (arrow) developed underneath type Ib serous glands (star). Von Kossa stain. Scale bar: 20 µm. F, lumbar skin of *Engystomops pustulosus*, showing OMG, type Ia (asterisk), Ib (black star) and II serous glands (white stars). H&E. Scale bar: 100 µm. G, dorsal skin of *Leptodactylus laticeps*, showing type Ia serous glands (asterisk) and continuous EK layer (arrowhead).
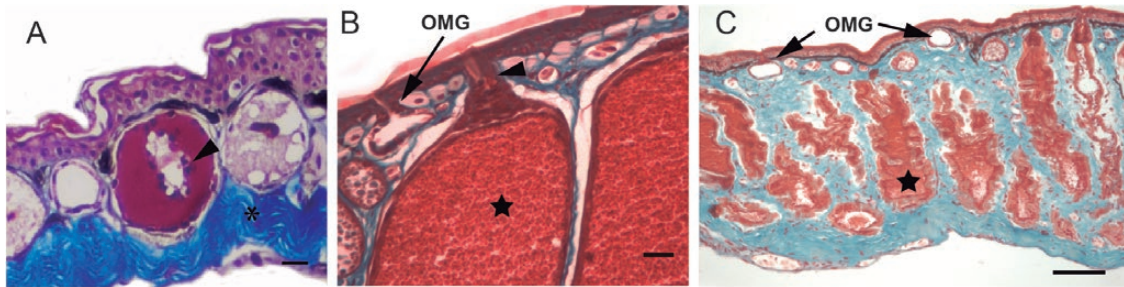
Figure 3. General features of skin of selected species. A, lumbar skin of *Crossodactylodes itambe*, showing type III serous glands (arrowhead). Masson–Goldner trichrome stain (M&G). Scale bar: 20 μm. B, lumbar skin of *Physalaemus nanus*, showing ordinary mucous glands (OMG) and type Ib serous gland (star) with its neck and duct (arrowhead). M&G. Scale bar: 20 μm. C, lumbar skin of *Physalaemus erythros*, showing the arrangement of type Ib serous glands (star), with their acini separated by connective tissue, with irregular profiles. Ordinary mucous glands are also indicated (arrows). M&G. Scale bar: 100 μm.

described a pink-lavender coloration for hidden areas of *Pleurodema borellii* (Peracca, 1895), but our field experience (Fig. 1E) and the literature (Akmentins *et al.*, 2014) indicate that at least Argentinean populations have yellow coloration, like its sister species (*Pleurodema cinereum* Cope, 1878).

Eyespots occur frequently in species with macroglands, although not exclusively. *Engystomops freibergi* (Donoso-Barros, 1969) and *En. petersi* have black spots on the inguinal/lumbar region, along with irregular spots on the belly, but lack inguinal/lumbar macroglands (Supporting Information, Supplementary Data S4). Different numbers and shapes of eyespots were also observed. In some species, there is one big spot [e.g. *Physalaemus biligonigerus* (Cope, 1861), Fig. 1G], whereas others have two or three smaller spots (e.g. *Pl. borellii*; Fig. 1E). In *Pleurodema brachyops* (Cope, 1869), on the single dark spot there are small white pustules (Fig. 1D), not observed in other species.

HISTOLOGICAL CHARACTERS

6. **Eberth–Katschenko layer between the stratum spongiosum and stratum compactum of inguinal/lumbar skin:** (0) absent (Fig. 2D); (1) present and discontinuous; (2) present and continuous (Fig. 2B).

The skin of the dorsal and lumbar regions has the typical configuration of anuran skin: an epidermis and an underlying dermis (Fig. 2A). In the epidermis, cytoplasmic processes of melanophores, flask cells (Whitear, 1975) and pillar cells (Linsenmair *et al.*, 1999) occur sporadically. In the dermis, the superficial stratum spongiosum consists of loose connective tissue that contains two types of cutaneous glands (mucous and serous glands; Figs 2A, 3B, C), pigment

cells (melanophores, iridophores and, less frequently, xanthophores) and blood vessels. The histochemical properties of the secretory products of the cutaneous glands are summarized in the Supporting Information (Supplementary Data S7).

The deeper stratum compactum consists of dense connective tissue formed by collagen fibres. Between both dermal strata, the EK layer (Elias & Shapiro, 1957; Toledo & Jared, 1993) is developed. In species without glandular tissue or macroglands in the inguinal/lumbar region, the EK layer is always present (Fig. 2B). In species with patches of glandular tissue, the EK layer is observed in the inguinal/lumbar skin of some species (*Pl. tucumanum*; Fig. 2C), but it is absent in others [*Ph. riograndensis* and *Pleurodema somuncurense* (Cei, 1969)]. Finally, when inguinal/lumbar macroglands occur, the EK layer is not observed (Fig. 2D), except in *Physalaemus aguirrei* Bokermann, 1966 (Fig. 2E) and *Physalaemus gracilis* (Boulenger, 1883).

7. **Occurrence of type II serous glands (*sensu* Delfino *et al.*, 2015) in lumbar skin:** (0) absent; (1) present (Fig. 2F).
8. **Occurrence of type Ib serous glands (*sensu* Delfino *et al.*, 2015) in lumbar skin:** (0) absent; (1) present (Fig. 2C–F).
9. **Occurrence of type Ib serous glands (*sensu* Delfino *et al.*, 2015) in dorsal skin:** (0) absent; (1) present (Fig. 2G).
10. **Occurrence of type III serous glands:** (0) absent; (1) present (Fig. 3A).

All serous glands have secretory portions larger than ordinary mucous glands, an intra-epidermal duct, a neck lined by a double row of epithelial cells (Fig. 3B) and a syncytial secretory layer surrounded by myoepithelial cells. We recognized four types of serous glands.
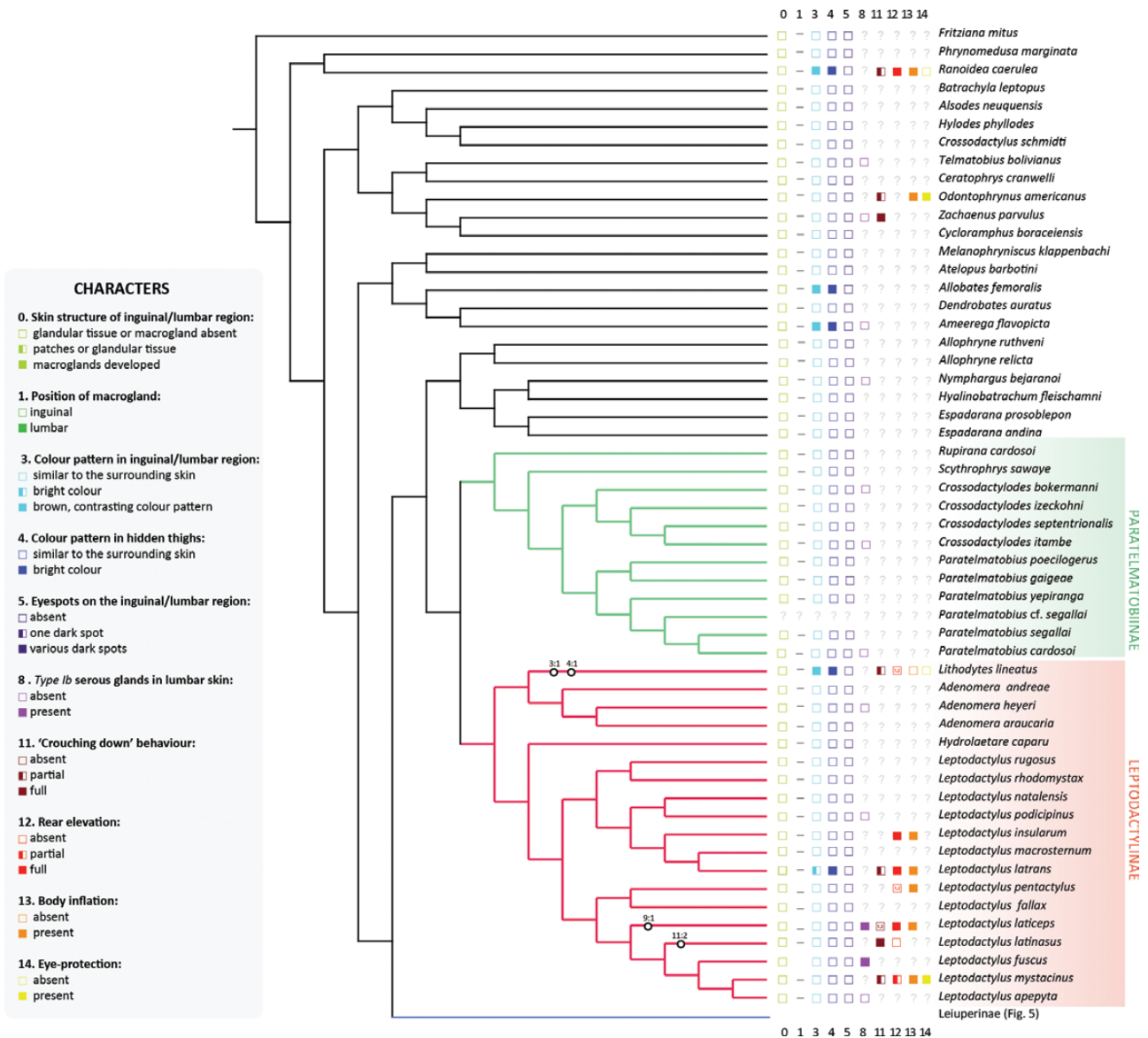
Figure 4. Phylogenetic hypothesis of Leiuperinae. Strict consensus of the most parsimonious trees, showing taxonomic distribution of selected characters and their optimization (common to all the most parsimonious trees) for the outgroups. Numbers above branches represent characters and states (see main text for character description).

Type Ia serous glands (*sensu* Delfino *et al.*, 2015; usually named ordinary serous glands) are placed in the upper third of the stratum spongiosum of the dermis of the dorsal and lumbar skin (Fig. 2A, D). These glands have a translucent, finely granular secretory product.

A second type of serous gland (type Ib serous glands *sensu* Delfino *et al.*, 2015) is the main secretory component of the macroglands. In species with macroglands, type Ib serous glands are larger than type Ia serous glands, oval in shape, with their major axes lying perpendicular to the epidermis, and usually they are densely packed (Fig. 2D–F).

The syncytium is filled with spherical granules. In some species, there is amorphous secretion. In *Physalaemus erythros* Caramaschi, Feio & Guimarães, 2003, *Ph. jordanensis* and *Physalaemus lateristriga* (Steindachner, 1864), the secretory portions of type Ib serous glands are not contiguous with each other, but they are separated by connective tissue; also, their profiles are irregular (Fig. 3C). In a few species without macroglands, type Ib serous glands were observed in dorsal skin in addition to the lumbar skin [*Engystomops coloradorum* (Cannatella & Duellman, 1984), *Engystomops pustulosus* (Cope, 1864) and *L. laticeps*; Fig. 2G]. In both *En. coloradorum* and
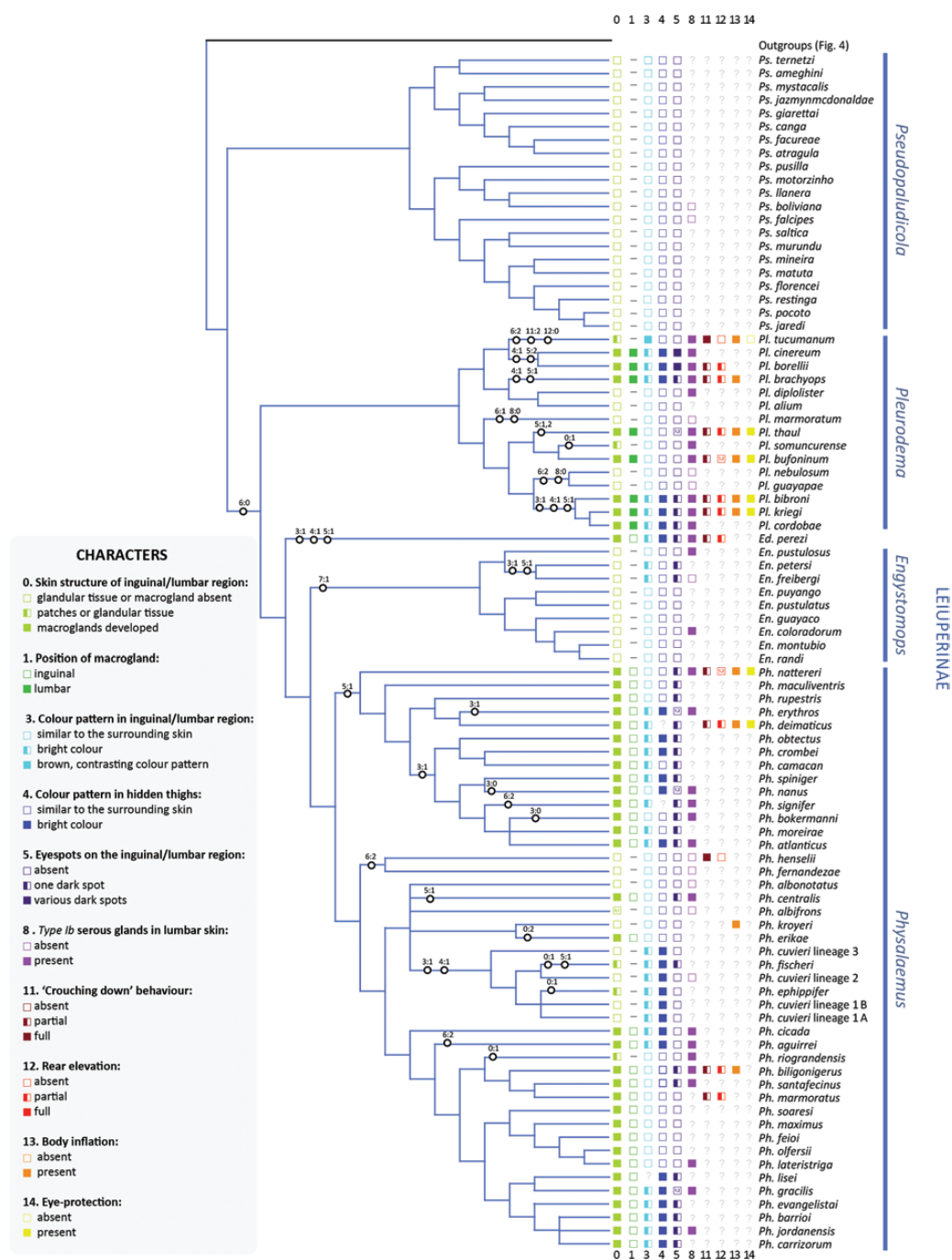
Figure 5. Phylogenetic hypothesis of Leiuperinae. Strict consensus of the most parsimonious trees, showing taxonomic distribution of selected characters and their optimization (common to all the most parsimonious trees) for the ingroup. Numbers above branches represent characters and states (see main text for character description).

En. pustulosus, type Ib glands are smaller and more scattered in the dorsal skin than in the lumbar skin. Finally, these glands were observed sporadically in the lumbar skin of species with patches of glandular tissue (e.g. *Pl. tucumanum*; Fig. 2C). Type Ib glands were described in Leiuperinae using different terminology:

'Giftdrüsen' (= venom glands in *Pl. thaul*; Adam, 1954), 'inguinal glands' (for *Ph. biligonigerus*; Delfino *et al.*, 1999), 'G2 glands' (for *Ph. nattereri*; Lenzi-Mattos *et al.*, 2005), 'lumbar serous glands' (for *Pleurodema* species with macroglands; Ferraro *et al.*, 2013) or 'type Ib serous glands' (observed in dorsal skin of

En. pustulosus; Delfino *et al.*, 2015). Given that the present study indicates that these glands also occur in the dorsal skin (see next character) and are not associated exclusively with macroglands, we modified the terminology previously adopted (i.e. lumbar serous glands; see Ferraro *et al.*, 2013).

Prates *et al.* (2012) reported that *Lithodytes lineatus* has serous glands concentrated across the two yellow bands on its dorsum. We interpreted these glands as type Ia serous glands, but we are not certain whether type Ib serous glands are absent. Consequently, we coded it as missing data (see Supporting Information, Supplementary Data S4).

A third type of serous gland (type II serous glands *sensu* Delfino *et al.*, 2015) occurs in low density in the three species of *Engystomops*. These glands, observed only in lumbar skin, have a vesicular secretion composed of a finely dispersed content (Fig. 2F).

A fourth type of serous gland (type III) was observed in dorsal and lumbar skin of *Crossodactylodes itambe*. These glands have a colloid-like content placed around a relatively circular lumen, positive to Ninhydrin. The ovoid-shaped, syncytial nuclei are arranged in a single peripheral row and surrounding the central lumen (Fig. 3A).

DEFENSIVE BEHAVIOURS

11. **'Crouching down' behaviour (modified from Marchisin & Anderson, 1978; Toledo *et al.*, 2011):** (0) absent; (1) partial (Kolenc *et al.*, 2009: fig. 7E); (2) full (Kolenc *et al.*, 2009: fig. 7H).
12. **Rear elevation (*sensu* Ferreira *et al.*, 2019a):** (0) absent; (1) partial (e.g. Lenzi-Mattos *et al.*, 2005: fig. 1B, C; Borteiro & Kolenc, 2007: fig. 5F, G; Faivovich *et al.*, 2012: fig. 5A, B; Ferreira *et al.* 2019a: fig. 13A); (2) full (Fig. 1H; Ferreira *et al.* 2019a: fig. 13B).
13. **Body inflation (Noble, 1931):** (0) absent; (1) present (Kolenc *et al.*, 2009: fig. 7E).
14. **Eye protection (*sensu* Toledo *et al.*, 2011):** (0) absent; (1) present (Kolenc *et al.*, 2009: fig. 7E).

A defensive behaviour observed in different species of Leiuperinae with inguinal/lumbar macroglands was described using different names: 'aposematic attitude' (Cei, 1858, 1962), 'deimatic behaviour' (e.g. Edmunds, 1974; Sazima & Caramaschi, 1986; Martins, 1989; Kolenc *et al.*, 2009), 'body-raising with legs vertically stretched' (Toledo *et al.*, 2011) and 'rear elevation behaviour' (Ferreira *et al.*, 2019a). This behaviour is displayed when the animal lowers the head, inflates the lungs and stretches its legs, meaning that the posterior region of the body is elevated. As advanced by Sazima & Caramaschi (1986), we consider that this complex behavioural display includes four different behaviours that we describe as characters (chars) 11–14.

'Crouching down' behaviour occurs when an animal flattens itself against the substrate (*sensu* Toledo *et al.*, 2011). However, during the defensive behaviour, the species of Leiuperinae with inguinal/lumbar macroglands have only the anterior body region in contact with the substrate, a position described for other species (e.g. Marchisin & Anderson, 1978). For this reason, we considered that 'crouching down' behaviour can be displayed in two ways: partial, in which the animal has the anterior body region (head and pectoral region) in contact with the substrate (Kolenc *et al.*, 2009: fig. 7E), or full, in which the animal has its head and belly in contact with the substrate (Kolenc *et al.*, 2009: fig. 7H).

Simultaneously with 'crouching down' behaviour, the leiuperines with inguinal/lumbar macroglands also stretch their legs, elevating the posterior region of the body; a behaviour termed 'rear elevation' (*sensu* Ferreira *et al.*, 2019a). This behaviour can also be displayed in two ways: partial, in which the animal partly stretches its legs (Ferreira *et al.*, 2019a: fig. 13A), or full, when the animal completely stretches its legs (Ferreira *et al.*, 2019a: fig. 13B). Full rear elevation was documented only in *Ph. nattereri* (Ferreira *et al.*, 2019a) and some species of *Leptodactylus* (see Supporting Information, Supplementary Data S4). We reported full rear behaviour for the first time in *Pleurodema* (*Pleurodema bufoninum* Bell, 1843; Fig. 1H). Finally, two other behaviours are displayed simultaneously with rear elevation: the animal inflates its lungs, puffing up the body ('body inflation'; Noble, 1931) and protects the eyes with its forearms ('eye protection'; Toledo *et al.*, 2011). Our observations on one individual of *Pl. tucumanum* (Kolenc *et al.*, 2009: fig. 7H) seem to indicate a partial inflation of the lungs; therefore, new observations are necessary to corroborate this.

The behavioural characters 11 and 12 were coded as unknown for all species for which data were not available. A few species, after being disturbed, did not display any behaviour: *Physalaemus rupestris* (Caramaschi *et al.*, 1991), *Pleurodema cordobae* Valetti, Salas & Martino, 2009 (under mechanical stimulus; J. Faivovich, pers. obs.) and *Pleurodema somuncurense* (under mechanical stimulus; M. Velasco, pers. comm.). Given that a protocol for proper testing of the behavioural response of anurans is lacking, we tentatively coded these species as missing data.

A 'deimatic posture' was described for two species without macroglands [*Leptodactylus latinasus* Jiménez de la Espada, 1875 and *Physalaemus henselii* (Peters, 1872); Borteiro & Kolenc, 2007: fig. 5D, G) and for a species with slightly protuberant patches of glandular

tissue (*Pl. tucumanum*; Kolenc *et al.*, 2009: fig. 7H). We interpreted this behaviour as full 'crouching down' behaviour, displayed when the animal has its head and belly touching the substrate and the back of the body is not elevated.

Skin secretions

15.   **Leptodactyline**: (0) absent; (1) present.
16.   **Physalaemin**: (0) absent; (1) present.

Different biogenic amines (*Pleurodema* and *Leptodactylus*) and peptides (*Physalaemus*) have been reported in the skin of leptodactylids (Erspamer *et al.*, 1962a, 1964a, b; Anastasi *et al.*, 1964; Cei & Erspamer, 1966; Cei *et al.*, 1967; De Caro *et al.*, 1968; Cei, 1985; Roseghini *et al.*, 1986). In recent decades, other bioactive peptides have been found in *Leptodactylus* and *Pleurodema* (e.g. Rollins-Smith *et al.*, 2005; Marani *et al.*, 2017; Gomes *et al.*, 2018; Cancelarich *et al.*, 2020). However, most of these papers dealt with only single species. For these reasons, we selected the two most frequently reported compounds from the skin of Leptodactylidae. These are (m-hydroxyphenyl)-trimethilammonium (= leptodactyline), a biogenic amine that displays potent nicotinic actions and strong curare-like effects of the depolarizing type (Roseghini *et al.*, 1986), and physalaemin, a peptide that potentially lowers blood pressure and stimulates extravascular smooth muscle (Erspamer *et al.*, 1962a, 1964a; Anastasi *et al.*, 1964).

DISCUSSION

Phylogenetic analyses

Leptodactylidae was recovered as non-monophyletic in our MP analysis [with Allocentroleniae (i.e. Centrolenidae + Allophrynidae) nested within it; see Figs 4, 5], but monophyletic in the ML analysis, although with low support (Supporting Information, Supplementary Data S6). Although some previous phylogenetic hypotheses recovered Leptodactylidae as monophyletic (e.g. Pyron & Wiens, 2011; Fouquet *et al.*, 2013; Lourenço *et al.*, 2015; Jetz & Pyron, 2018), others recovered this family as paraphyletic (e.g. Lourenço *et al.*, 2008; Veiga-Menoncello *et al.*, 2014) or even polyphyletic (e.g. Grant *et al.*, 2006, 2017; Faivovich *et al.*, 2012). Nevertheless, most of these relationships are poorly supported and should be explored further, considering additional evidence (e.g. considering a denser gene sampling with high-throughput sequencing and/or phenotypic data). Leptodactylinae and Paratelmatobiinae are recovered as sister clades in both analyses. Leiuperinae is recovered as the sister taxon of Allocentroleniae

+ Leptodactylinae and Paratelmatobiinae in the MP analysis and sister taxon of Leptodactylinae and Paratelmatobiinae in the ML analysis. Within Paratelmatobiinae, *Scythrophrys sawayae* (Cochran, 1953) was recovered as the sister taxon of *Crossodactylodes* + *Paratelmatobius* (in MP and ML analyses), in agreement with Fouquet *et al.* (2013) and Santos *et al.* (2020). However, Santos *et al.* (2019) recovered *S. sawayae* as the sister species of *Paratelmatobius*.

Although Leiuperinae was recovered as monophyletic in MP and ML analyses (Supporting Information, Supplementary Data S5 and S6), this clade has low support. *Pseudopaludicola* is the sister taxon of the remaining Leiuperinae, as in previous analyses (e.g. Pyron & Wiens, 2011; Fouquet *et al.*, 2013; de Sá *et al.*, 2014; Veiga-Menoncello *et al.*, 2014; Grant *et al.*, 2017; Jetz & Pyron, 2018). Both MP and ML analyses recovered, within *Pseudopaludicola*, the four main clades defined by Veiga-Menoncello *et al.* (2014).

The position of *Pleurodema* in MP and ML analyses as the sister taxon of the remaining three genera (*Edalorhina*, *Engystomops* and *Physalaemus*) is congruent with previous studies (e.g. Grant *et al.*, 2006; Pyron & Wiens, 2011; Faivovich *et al.*, 2012; Veiga-Menoncello *et al.*, 2014). Faivovich *et al.* (2012) recognized four clades within the genus, the *Pleurodema bibroni* Tschudi, 1838, *Pl. brachyops*, *Pleurodema nebulosum* (Burmeister, 1861) and *Pl. thaul* clades. In the MP analysis, the *Pl. brachyops* clade was recovered as the sister taxon of the remaining clades. However, the *Pl. thaul* clade is not recovered monophyletic, as *Pleurodema marmoratum* (Duméril & Bibron, 1841) (the sister taxon of the remaining species of the *Pl. thaul* clade of Faivovich *et al.* [2012]) is recovered as the sister taxon of the remaining species of the *Pl. thaul* clade plus the *Pl. nebulosum* and *Pl. bibroni* clades. In the ML analysis, *Pl. brachyops* was recovered as the sister species of *Pl. tucumanum* (*Pl. borellii* + *Pl. cinereum*), and the remaining three clades have a topology identical to those recovered by Faivovich *et al.* (2012). In our analyses, we included one species of *Edalorhina*, and it was recovered as the sister species of *Engystomops* + *Physalaemus*, as in other studies (e.g. Pyron & Wiens, 2011; Fouquet *et al.*, 2013). However, other analyses recovered *Edalorhina* as the sister taxon of *Engystomops* and these two genera as the sister clade of *Physalaemus* (e.g. Faivovich *et al.*, 2012; Veiga-Menoncello *et al.*, 2014; Lourenço *et al.*, 2015). Both positions of *Edalorhina* have good support in all these analyses; therefore, the relationships of these three genera are still uncertain.

Within *Engystomops*, in MP and ML analyses we recovered the same two major lineages defined by Ron *et al.* (2006), the Duovox and the Edentulus clades. The species of the Duovox clade have been recovered in the

Vivavox and Brevivox clades, as defined by Ron *et al.* (2006).

Finally, two major clades were recovered in *Physalaemus* in MP and ML analyses, as defined by Lourenço *et al.* (2015): the *Ph. signifer* clade and the *Ph. cuvieri* clade. Within both clades, the internal relationships of the species are congruent with those recovered by Lourenço *et al.* (2015). The incongruences are the relationship between the species of the *Ph. biligonigerus* group [although *Ph. biligonigerus* and *Physalaemus santafecinus* Barrio, 1965 are sister species in the study by Lourenço *et al.* (2015), we recovered *Ph. marmoratus* (Reinhardt & Lütken, 1862) and *Ph. santafecinus* as sister species] and the relationships within the *Ph. cuvieri* group in the MP analysis.

MACROGLANDS

The inguinal/lumbar macroglands that occur in leiuperines have a similar structure to other anuran macroglands (Fig. 2D), including the occurrence of type Ia serous glands (usually named ordinary serous glands) and other types of densely packed serous glands (e.g. Crook & Tyler, 1981; Toledo *et al.*, 1992; Jared *et al.*, 2009, 2011; O'Donohoe *et al.*, 2019). The ancestral character reconstruction indicates an ambiguity in the origin of macroglands in the inguinal/lumbar region (char. 0:2; Figs 4, 5), having 12 possible reconstructions. These imply an origin in the most recent common ancestor of *Pleurodema* + *Edalorhina* + *Engystomops* + *Physalaemus*, with multiple subsequent losses [e.g. in *Engystomops*, *Pleurodema guayapae* Barrio, 1964 + *Pl. nebulosum*, *Pl. marmoratum*, *Pl. alium* + *Pleurodema diplolister* (Peters, 1870), *Physalaemus fernandezae* (Müller, 1926) + *Ph. henselii*, a clade including several species of the *Ph. cuvieri* group], or up to nine independent origins.

A slightly protuberant patch of glandular tissue with diffuse limits evolved from a plesiomorphically present macrogland three times, in *Pl. somuncurense*, *Pl. tucumanum*, and *Ph. riograndensis*. In the *Ph. cuvieri* group, macroglands re-evolved independently in *Physalaemus centralis* Bokermann, 1962 and *Physalaemus erikae* Cruz & Pimenta, 2004, and a slightly protuberant patch of glandular tissue with diffuse limits evolved from a plesiomorphic state where there is no macroscopic evidence of glandular tissue or a macrogland in *Physalaemus fischeri* (Boulenger, 1890) and *Physalaemus ephippifer* (Steindachner, 1864). The position of the macrogland, as inguinal or lumbar, optimizes ambiguously, and no transformations between the two positions result from the ancestral character reconstruction.

The ancestral character reconstruction of flank glands (char. 2:1) is ambiguous, because it is equally parsimonious with a single origin in *Edalorhina* + *Engystomops* + *Physalaemus*, with a loss in *Physalaemus*, or with independent origins in *Edalorhina* and *Engystomops*. In *Ed. perezi*, this gland was not previously reported. We did not have access to specimens of *Edalorhina nasuta* Boulenger, 1912; therefore, it is unknown whether the two species of this genus share this character state. The flank glands of *Engystomops* have been used to diagnose the genus along with paratoid glands (Cannatella & Duellman, 1984). Both glands are usually distinct from each other, but different degrees of development have been described (e.g. Cannatella & Duellman, 1984; Ron *et al.*, 2004; Funk *et al.*, 2008). For example, in some species they are continuous (*Engystomops puyango* Ron, Toral, Rivera & Terán-Valdez, 2010; Ron *et al.*, 2010: fig. 1), whereas in other species the flank glands can extend posteriorly [e.g. *Engystomops randi* (Ron, Cannatella & Coloma, 2004); Ron *et al.*, 2004: fig. 4].

EBERTH–KATSCHENKO LAYER

Mangione & Lavilla (2004) described a double EK layer in *Pl. diplolister*, *Pl. nebulosum* and *Pl. tucumanum* (subepithelial and subglandular). In these three species, we observed the EK layer only in a subglandular position (Fig. 2C). Also, Mangione & Lavilla (2004) considered the EK layer to be absent in *Pl. marmoratum*, but we detected it in a subglandular position.

Absence of the EK layer in the lumbar skin is a synapomorphy of *Pleurodema* + *Edalorhina* + *Engystomops* + *Physalaemus* (char. 6:0; Fig. 5). The EK layer re-evolved in the lumbar skin during the evolutionary history of leiuperines once as a discontinuous layer (*Pl. marmoratum*) and ten times as a continuous layer: (1)–(4) *Pl. tucumanum*, *Ph. signifer* (Girard, 1853), *Physalaemus albonotatus* (Steindachner, 1864) and *Ph. aguirrei*; (5) *Pl. diplolister* (unknown in *Pl. alium*); (6) *Pl. guayapae* + *Pl. nebulosum*; (7) *En. freibergi* (unknown in *En. petersi*); (8) *Ph. fernandezae* + *Ph. henselii*; (9) *Ph. cuvieri* (Fitzinger, 1826; unknown in *Ph. ephippifer*, *Ph. fischeri* and other lineages associated with *Ph. cuvieri*); (10) *Ph. gracilis* (unknown in *Physalaemus lisei* Braun & Braun, 1977).

The EK layer in the lumbar region was lost in *Pleurodema* + *Edalorhina* + *Engystomops* + *Physalaemus*. In some ancestral character state reconstructions, this is the same node where macroglands evolved in this body region, whereas other reconstructions do not support this inference. This does not allow phylogenetic testing of the suggestion that the absence of the EK layer removes rigidity in the inguinal region, facilitating glandular compression and the release of glandular secretions (Lenzi-Mattos

et al., 2005). However, we note that the ancestral character reconstruction indicates that the EK layer re-evolved independently in the inguinal/lumbar region in two species that retain the plesiomorphic macrogland (*Ph. aguirrei* and *Ph. gracilis*). The study of the mechanics of secretion release in these species might shed light on the role of the EK layer in gland compression.

SEROUS GLANDS

The ancestral character reconstruction indicates that type Ib serous glands in the lumbar skin (char. 8:1) evolved in the most recent common ancestor of *Pleurodema* + *Edalorhina* + *Engystomops* + *Physalaemus*. In Leiuperinae, type Ib serous glands were lost independently at least four or five times in: (1) *Pl. marmoratum*; (2) *Pl. guayapae* + *Pl. nebulosum*; (3) *En. freibergi* (unknown in *En. petersi*); (4)–(5) the *Ph. cuvieri* clade. In this group, there is an ambiguous optimization, determined by the absence of the type Ib serous glands in the inguinal region of *Physalaemus albifrons* (Spix, 1824), *Ph. albonotatus* and *Ph. cuvieri* (lineage 2), its presence in *Ph. centralis* and its unknown occurrence in the other species of the group.

In the studied species without macroglands (char. 0:0) or with patches of glandular tissue in the inguinal/lumbar region (char: 0:1), when present, type Ib serous glands are not as densely packed as in species with macroglands. This is the case for *En. coloradorum*, *En. pustulosus*, *Ph. albonotatus*, *Pl. diplolister*, *Pl. somuncurense* and *Pl. tucumanum* (Delfino *et al.*, 2015; present work).

Type Ib serous glands were observed in the lumbar skin of *L. laticeps* and *L. latrans* and are likely to occur in some other species of Leptodactylinae. Given that these species lack a lumbar/inguinal macrogland, expanding the taxonomic sampling (i.e. including *Adenomera* Steindachner, 1867, *Hidrolaetare* Gallardo, 1963 and more species of *Leptodactylus*) could clarify whether the occurrence of type Ib serous glands in the lumbar/inguinal skin is more plesiomorphic than the origin of the macroglands. In a similar way, type Ib serous glands occur in the dorsal skin (in addition to the lumbar skin) in *En. coloradorum*, *En. pustulosus* and *L. laticeps* (char. 9:1).

Mangione & Lavilla (2004) studied the lumbar skin of six species of *Pleurodema* (*Pl. borellii*, *Pl. diplolister*, *Pl. guayapae*, *Pl. marmoratum*, *Pl. nebulosum* and *Pl. tucumanum*) and described one type of mucous and one type of serous gland in all of them (but in their fig. 10, type Ib serous glands can be recognized in *Pl. borellii*). Nevertheless, we observed two types of serous glands in *Pl. borellii* (Ferraro *et al.*, 2013), *Pl. diplolister* and *Pl. tucumanum* (type Ia and type Ib; Fig. 2C). Macroglands have been studied histologically

in only three species of *Physalaemus*: *Ph. albonotatus* (Alvarez *et al.*, 2005), *Ph. biligonigerus* (Delfino *et al.*, 1999) and *Ph. nattereri* (Lenzi-Mattos *et al.*, 2005). Our observations agree with these descriptions.

Studies of serous glands with light microscopy revealed different sizes of granules, spongeous granules, granules with repeating aggregation of subunits, vesicle-like products with an electron-transparent content (or faint density granules) or structureless granules (e.g. Alvarez *et al.*, 2005: table 1; Arifulova *et al.*, 2007: table 1). Despite this variability, some general patterns have been described in serous glands. In Leiuperinae (and other anurans), it was suggested that the products released by serous glands share a common biosynthetic machinery, and during post-Golgian (or maturational) processing the secretory products acquire a repeating substructure (Nosi *et al.*, 2013, and references therein). Also, a centripetal maturational gradient was described (Delfino, 1991; Delfino *et al.*, 1999, 2001; Alvarez *et al.*, 2005). This pattern, described under light microscopy in *Ph. albonotatus* (type Ia serous glands; Alvarez *et al.*, 2005) and *Ph. biligonigerus* (type Ia and type Ib serous glands; Delfino *et al.*, 1999) was not detected in our samples. Lenzi-Mattos *et al.* (2005) described type Ib serous glands in *Ph. nattereri*, with granules of heterogeneous size, electron density and content, but the centripetal pattern was not reported.

Delfino *et al.* (1999), based on transmission electron microscopy observations, reported identical immature granules of both type Ia and Ib serous glands in *Ph. biligonigerus* (i.e. they share the same repeating substructure). However, additional processes were observed. During a prolonged maturative process, discrete secretory deposits acquire their final appearance and composition (Delfino *et al.*, 1999; Alvarez *et al.*, 2005). The type Ia serous glands undergo a process of dilution (i.e. loss of any definite substructure), whereas type Ib serous glands undergo a process of condensation (Delfino *et al.*, 1999). Based on this evidence, Delfino *et al.* (1999) suggested that type Ib serous glands, specialized in storage of secretory products, are ontogenetically derived from type Ia serous glands. Our observations indicate that although ordinary serous glands (i.e. type Ia) occur in all the species that we studied and, for that matter, all studied anurans, type Ib glands occur in only a subset of the studied leptodactylids. We take this to indicate that, although type Ib serous glands might be derived ontogenetically from type Ia glands, there is an underlying evolutionary transition by which, in this group, some populations of type Ia glands are differentiated further into type Ib glands.

Delfino *et al.* (2015) recognized three types of serous cutaneous glands in the dorsal skin of *En. pustulosus*: type Ia (formed by granules with wide halos and

variable-density cores), type Ib (usually termed 'inguinal or lumbar serous glands', formed by high-density granules without halos) and type II (formed by vesicles containing a finely dispersed product). We observed type II serous glands (char. 7:1) in only the three studied species of *Engystomops*. Although these glands still require study in other species of *Engystomops*, the ancestral character reconstruction indicates that its occurrence is most parsimoniously interpreted as a synapomorphy of this genus, where they co-occur with type Ib glands.

COLOUR PATTERN

The colour pattern of an organism is influenced by a series of interrelated factors. Predator vision, predator hunting tactics, prey behaviour and background patterns affect the colour pattern of a species (Endler, 1978). Usually, the dorsal colour pattern of leiuperines resembles the background when the animal is resting (Ferreira *et al.*, 2019a: Supporting Information, Supplementary Data 1). The dorsum of limbs frequently has brown spots or stripes. Also, many *Physalaemus* species have a dark stripe extending from the postorbital area to the groin. This condition, in which an animal resembles the background, is termed crypsis (Poulton, 1890).

Cryptic colour patterns can be combined with disruptive coloration. Terrestrial aposematic species frequently use red, yellow and orange, often combined with black, to advertise their unpalatability to potential predators (Endler & Mappes, 2004). Predators learn to associate unpalatability with bright colours, and strong signals are detectable and memorable (e.g. Gittleman & Harvey, 1980; Sillén-Tullberg, 1985; Roper & Wistow, 1986). Although originally associated with advertisement through bright colours, some authors have considered the occurrence of eyespots as conspicuous or aposematic signals to predators (e.g. Stevens & Merilaita, 2009; Toledo & Haddad, 2009; Ferreira *et al.*, 2019a), whereas other authors consider eyespots as a case of mimicry (e.g. Umbers *et al.*, 2017). Ferreira *et al.* (2019a) classified the aposematism in anurans in two categories: exposed (e.g. *Brachycephalus*: Brachycephalidae) or hidden (e.g. all species coded as 3:1 and 4:1 in our data matrix). Hidden aposematism in leiuperines is exposed when frogs jump, vocalize floating on water or display 'rear behaviour'.

In *Engystomops*, *Edalorhina* and some species of *Physalaemus* and *Pleurodema*, brightly coloured areas are observed on hidden surfaces of the inguinal region and, sometimes, the thigh and shank. The ancestral character state reconstruction of the coloration on hidden surfaces of the inguinal region and thighs indicates that the plesiomorphic state in Leiuperinae is cryptic coloration similar to the surrounding skin

(chars 3:0; 4:0). The bright coloration in the inguinal region (char. 3:1) evolved between seven and 11 times during the evolutionary history of *Edalorhina* + *Engystomops* + *Physalaemus* + *Pleurodema*. In two cases, *Physalaemus bokermanni* Cardoso & Haddad, 1985 and *Physalaemus nanus* (Boulenger, 1888), there is a reversion from bright colours to cryptic coloration. The situations in the *Ph. biligonigerus* and *Physalaemus olfersii* (Lichtenstein & Martens, 1856) groups are ambiguous, with the hypotheses of reversions from bright colour to cryptic colour or homology with the plesiomorphic cryptic state in leiuperines being equally parsimonious. The contrasting dull dark brown evolved once (char. 3:2; *Pl. tucumanum*), but the plesiomorphic state is ambiguous, with a bright or a cryptic coloration being equally parsimonious.

The bright coloration in hidden areas of the thighs (char. 4:1) evolved between ten and 14 times during the evolutionary history of *Edalorhina* + *Engystomops* + *Physalaemus* + *Pleurodema*. The ancestral character reconstruction is similar to that of the bright coloration in the inguinal region. There are a few cases where this character state co-occurs with cryptically coloured hidden areas of the thighs. These are *En. freibergi* and *En. petersi*, *Physalaemus camacan* Pimenta, Cruz & Silvano, 2005, *Physalaemus deimaticus* Sazima & Caramaschi, 1988, *Ph. signifer* and *Physalaemus moreirae* (Miranda-Ribeiro, 1937). Conversely, *Ph. nanus* is the single case where brightly coloured hidden areas of thighs do not co-occur with a brightly coloured inguinal region.

In a similar manner to the bright coloration of hidden surfaces of the inguinal region and thighs, the occurrence of eyespots in the inguinal/lumbar region (char. 5) evolved in Leiuperinae 12 or 13 times during its evolutionary history, always from a plesiomorphically absent state. In five of these instances, the apomorphic state involves multiple eyespots, whereas in seven or eight cases it involves a single eyespot, considering that at least five species are polymorphic. Although eyespots are commonly associated with species with inguinal/lumbar macroglands, the ancestral character reconstruction indicates that those evolved later during the evolutionary history of *Edalorhina* + *Engystomops* + *Physalaemus* + *Pleurodema*. Interestingly, we find that with the exception of *En. freibergi* and *En. petersi*, the eyespots evolved only in species or clades where the macrogland was present. In the case of the sister species *Ph. gracilis* and *Ph. lisei*, two species with macroglands, eyespots have been described as present or absent (Milstead, 1960; Braun & Braun, 1977; Lema & Martins, 2011). Furthermore, the ancestral character state reconstruction shows that *Ph. centralis* and *Ph. gracilis* are the only cases where eyespots evolved along the same branch where macroglands re-evolved after being lost earlier in the evolutionary history of the *Ph. cuvieri* group.

Eyespots and the bright coloration evolved simultaneously in the same node in eight events. In two cases, the bright coloration evolved without the simultaneous or plesiomorphic occurrence of eyespots (*Ph. aguirrei* and *Physalaemus cicada* Bokermann, 1966). In four or five cases, eyespots evolved without the simultaneous or plesiomorphic occurrence of bright coloration (*Ph. centralis*, *Ph. biligonigerus*, *Physalaemus maculiventris* (Lutz, 1925), *Ph. nattereri* and *Ph. santafecinus*). In one case, the bright coloration evolved subsequent to the origin of eyespots (the *Ph. signifer* clade), and in only one case the eyespots evolved subsequent to the origin of bright coloration (*Ph. fischeri* and *Ph. ephippifer*, in the *Ph. cuvieri* group).

Historically, three major hypotheses have been postulated to explain the occurrence of eyespots (for reviews, see Ruxton *et al.*, 2004; Stevens, 2005). The 'intimidation hypothesis' argues that large eyespots intimidate predators, allowing the prey to escape (Blest, 1957). It was suggested that this occurs because eyespots resemble the eyes of the enemies of the predator (a case of mimicry; Kjernsmo & Merilaita, 2017). The 'conspicuous signal hypothesis' suggests that eyespots simply are highly conspicuous features (Marples & Kelly, 1999; Stevens & Merilaita, 2009). Behavioural experiments (none of which has included leiuperines) corroborated the hypothesis that paired, bilaterally symmetrical circular features increase the efficiency of visual aposematic displays (e.g. Scaife, 1976a, b; Jones, 1980; Forsman & Merilaita, 1999, 2003; Forsman & Herrström, 2004; Mukherjee & Kodandaramaiah, 2015). However, the mechanism(s) involved in its effectiveness are still discussed (Stevens & Ruxton, 2014). Finally, the 'deflection hypothesis' argues that eyespots attract predators towards themselves, thus deflecting predatory attacks away from the more vital parts of the prey (Poulton, 1890; Wourms & Wasserman, 1985).

In leiuperine species with eyespots, when broadly exposed during 'rear behaviour', they resemble, to the human eye, vertebrate eyes (e.g. Lenzi-Mattos *et al.*, 2005: fig. 1B; Kolenc *et al.*, 2009; fig. 7C, F), having been associated with snake eyes (e.g. Sazima & Caramaschi, 1986). Regardless of whether mimicry, intimidation or deflection is the mechanism involved in the anti-predator role of eyespots, their multiple independent origins are congruent with the hypothesis that a presumed selective agent for the diversity of this character state would be a visually oriented predator. Presumably, no colour vision would be necessary simply to distinguish the eyespots from the background coloration.

In a similar way, the multiple origins of bright coloration in the inguinal region and, sometimes, hidden surfaces of the thighs could be the result of selective pressure by a visually oriented predator with colour vision. However, the fact that on at least eight occasions bright coloration evolved simultaneously with eyespots and once evolved subsequent to the origin of these markings suggests that the combination of these character states is more complex in terms of the resulting visual effect during the encounter with a predator.

DEFENSIVE BEHAVIOURS

Our study of defensive behaviours in Leiuperinae is limited by the scarcity of observations for many species. However, there are detailed observations on some species, and these allow us to make several inferences that will be tested as more data become available.

The defensive behaviour observed in leiuperines in relationship to inguinal/lumbar macroglands has classically been termed 'deimatic behaviour'. This behaviour includes a series of movements that are usually displayed in the same order in different species. When this sequence of movements is performed, the macroglands, eyespots and/or bright colours are exhibited, and a passive secretion over the macrogland has been observed in a few species. Finally, in its maximum expression, there is eye protection with the forelimbs (Sazima & Caramaschi, 1986; Lenzi-Mattos *et al.*, 2005). Following Sazima & Caramaschi (1986), we evaluated this display in terms of the combination of four behaviours: partial crouching down (char. 11:1), rear elevation (char. 12:1,2), body inflation (char. 13:1) and eye protection (char. 14:1). These four behaviours have been discussed individually in the literature for several other anurans.

Crouching down, whether partial or full, when part or all of the body is flattened against the substrate, could avoid subjugation (Toledo *et al.*, 2011). Rear elevation increases the apparent body size of the individual and has been considered more defensive than inhibitory (Sazima, 1973) and also a possible mechanism to avoid subjugation (Toledo *et al.*, 2011). Body inflation occurs when an animal puffs up the body by inflating the lungs (e.g. Noble, 1931; Villa, 1969; Marchisin & Anderson, 1978; Torr, 1991; Toledo *et al.*, 2011; Ferreira *et al.*, 2019a). This behaviour was considered by Toledo *et al.* (2011) to avoid subjugation and by Ferreira *et al.* (2019a) to be intimidatory. Eye protection frequently occurs when an animal remains motionless, covering the head, eyes and/or the tympanum with its forearms, and Toledo *et al.* (2011) suggested that it avoids injuries during attempts at subjugation. Ferreira *et al.* (2019a) stated that this behaviour only reduces injury, and they did not consider it as an anti-predator mechanism.

The ancestral character state reconstruction of the four components that make up the 'deimatic display'

(i.e. crouching down, rear elevation, body inflation and eye protection) were already present in the most recent common ancestor of *Pleurodema* + *Edalorhina* + *Eupemphix* + *Physalaemus*. Although partial rear elevation evolves in the most recent common ancestor of this clade, the exact nodes where the other three behavioural components evolved before this node are ambiguous, and this is determined by the lack of observations of these behaviours in *Pseudopaludicola* and Paratelmatobiinae. To a certain extent, this could be the same explanation for the inference of homology of crouching down and body inflation as homologous with the same behaviours reported in at least some species of *Leptodactylus* (e.g. Cei, 1980; Borteiro & Kolenc, 2007; de Castro *et al.*, 2017). The limited available reports on Paratelmatobiinae and *Pseudopaludicola* (Garcia, 1999; Toledo *et al.*, 2010, 2011; Ferreira *et al.*, 2019b) did not mention any of these behaviours; even if these behaviours were actually absent in these groups, they would still not affect our inferences regarding the occurrence of these four behavioural components in the most recent common ancestor of *Pleurodema* + *Edalorhina* + *Eupemphix* + *Physalaemus*. During the evolutionary history of *Physalaemus*, rear elevation was lost, and 'crouching down' behaviour evolved from partial to full crouching down at least in *Ph. henselii* (character state unknown in its sister taxon, *Ph. fernandezae*). Faivovich *et al.* (2012) inferred that the 'deimatic display' was plesiomorphic in *Pleurodema*. This is congruent with our ancestral character state reconstruction of the four behavioural components of the 'deimatic display'.

SKIN SECRETIONS

There is a long history of research on bioactive peptides and biogenic amines in leptodactylids (e.g. Erspamer *et al.*, 1962a, 1964b, 1986; Cei & Erspamer, 1966; Cei *et al.*, 1967; De Caro *et al.*, 1968; Cei, 1985; Roseghini *et al.*, 1986). In the case of leiuperines, most research on peptides was focused initially on *Ph. biligonigerus*, with only a few other species of *Physalaemus* and *Pleurodema* prospected during that research cycle. More recently, a few species of these genera are being studied again (Lenzi-Mattos *et al.*, 2005; Barbosa *et al.*, 2015; Marani *et al.*, 2015, 2017; Cancelarich *et al.*, 2020). The peptide physalaemin was reported only in five species of *Physalaemus* (*Ph. biligonigerus*, *Ph. centralis*, *Ph. signifer* and, in lower concentrations, in *Ph. cuvieri* and *Ph. nattereri*). The ancestral character reconstruction indicates that the occurrence of physalaemin (char. 16:1) is at least a synapomorphy of this genus. However, it could have evolved earlier, because it is unknown in *Edalorhina* and *Engystomops*, but it is known to be absent in *Leptodactylus* and *Pleurodema* (the only other genera

of Leptodactylidae reported by Erspamer *et al.*, 1986). The inference of physalaemin as a synapomorphy of at least *Physalaemus*, added to the fact that there are no cases of absences reported for any species of the genus, allows us to predict that this peptide is present in most species. However, it should be noted that both macroglands and type Ib serous glands in the dorsal skin are absent in some species (*Ph. fernandezae* and *Ph. henselli*) that were not prospected for compounds.

Lenzi-Mattos *et al.* (2005) and Barbosa *et al.* (2015) reported 15 bradykinin-related peptides from *Ph. nattereri*, and Marani *et al.* (2015, 2017) and Cancelarich *et al.* (2020) reported several antimicrobial peptides from *Pl. thaul* and *Pl. somuncurense*. It is predictable that the prospection of most other leiuperines will lead to similar discoveries.

The presence of biogenic amines in Leiuperinae is limited to mostly low concentrations (compared with several species of *Leptodactylus*) of leptodactyline, and in a single case (and for that reason not included as a character), serotonin (5-hydroxytryptamine). The presence of leptodactyline (char. 15:1) is plesiomorphic for Leiuperinae, and its loss is a synapomorphy of the sister taxon of *Pleurodema* or of *Engystomops* + *Physalaemus*, with the ambiguity stemming from the unknown character state of *Edalorhina*. Furthermore, there was an independent loss in *Pl. bufoninum*. During the evolutionary history of *Engystomops* + *Physalaemus*, leptodactyline is known to have evolved independently at least in *Ph. biligonigerus*.

In *Ph. deimaticus*, *Ph. nattereri* and *Pl. bufoninum*, a secretion is released over the macrogland as part of the defensive display (Sazima & Caramaschi, 1986; Lenzi-Mattos *et al.*, 2005; Kolenc *et al.*, 2009). Cei & Erspamer (1966) reported the occurrence of serotonin concentrated in the lumbar macrogland of *Pl. bufoninum*. Barbosa *et al.* (2015) showed that the expression of bradykinin-related peptides in *Ph. nattereri* is ~30 000 times higher on the inguinal macroglands than on other parts of the skin. These data, together with the inferred plesiomorphic occurrence of leptodactyline in *Pleurodema* and the occurrence of physalaemin at least in *Physalaemus*, lends support to the notion that secretions from serous glands in the skin, and concentrated in macroglands if present, are indeed a chemical defence (e.g. Rada de Martínez & Finol, 1986; Sazima & Caramaschi, 1986; Toledo & Jared, 1995; Rodrigues & Oliveira Filho, 2004; Toledo *et al.*, 2011), although behavioural observations or experimental data are notably scarce.

DEIMATISM AND APOSEMATISM

The term 'deimatic reaction' was coined by Maldonado (1970) to describe a defensive behaviour observed in the praying mantis *Stagmatoptera biocellata*

Saussure, 1869 (Insecta: Mantodea). A series of independent and stereotyped signals were displayed by this insect under attack (Maldonado, 1970; Maldonado *et al.*, 1979). More recently, Umbers *et al.* (2015, 2017) defined 'deimatic displays' as a defensive, unexpected, sudden visual display performed by a prey in response to a predatory attack. Umbers *et al.* (2015, 2017) retained the term 'deimatic behaviour' as those displays that include other sensory modalities besides visual signals. In this sense, 'deimatic displays' are a combination of aposematism and camouflage, but including a startling element (Umbers *et al.*, 2015).

Skelhorn *et al.* (2016) proposed new definitions of deimatism and aposematism based on the mechanisms by which they prevent predation rather than the form taken by the displays. 'Deimatic displays' are defined as those displays that cause predators to fear for their immediate safety, not avoiding prey themselves, but avoiding an imminent danger. In contrast, 'aposematic displays' are those displays that cause the predator to classify a prey as an unprofitable prey item (owing to learned or unlearned aversions; Skelhorn *et al.*, 2016). From this perspective, differentiating these displays requires an experimental approach. Furthermore, it seems evident that the aposematic or deimatic nature of a display will depend on the predator.

'Deimatic displays' are thought to be performed as the predator approaches, avoiding or at least pausing its attack (Edmunds, 1974; Endler, 1991). However, it was demonstrated that at least some prey wait until the predator makes physical contact with them before performing their displays (Umbers & Mappes, 2015). Sazima & Caramaschi (1986) reported that *Ph. nattereri* displayed its defensive behaviour after being disturbed with both tactile and visual stimuli. More recently, Umbers *et al.* (2019) performed behavioural experiments using the katydid *Acripeza reticulata* (Guérin-Méneville, 1832) (Insecta: Tetiigoniidae) as prey, and they inferred that the efficacy of 'deimatic displays' depends on the experience of wild predators.

In the context of their definitions of deimatic display and behaviour, Umbers *et al.* (2017) proposed two hypotheses for the origin of deimatism; these are the defense-first hypothesis and the startle-first hypothesis. In the first hypothesis, in a camouflaged organism, the defences evolve first, followed by conspicuous colour patterns exposed at rest and, finally, by deimatism, when the cost of aposematism is offset by concealment at rest. In the second hypothesis, the behavioural components evolved as sudden movements in a camouflaged organism to avoid attacks, startling their predators. At this point, this hypothesis may or may not involve the evolution of defences. If it does, it enhances the protection during the display. In any case, the evolution of conspicuous coloration that is suddenly revealed might increase the protective value of the behaviour, also playing an aposematic role if defences are involved.

Three types of signals (changes in body shape through crouching down, body inflation and rear elevation; exposure of hidden eyespots; and exposure of bright colours) could be interpreted as the element of surprise necessary to defining a 'deimatic display' according to Umbers *et al.* (2015) or both the deimatic and aposematic displays of Skelhorn *et al.* (2016). Reports of these behaviours in Leiuperinae are mostly based on stimulation by tapping or manipulating the specimens (e.g. Martins, 1989; Kolenc *et al.*, 2009) or during field observations (e.g. Martins & Duarte, 2003).

Our ancestral character state reconstructions indicate that the four behavioural components of the deimatic display in *Pleurodema* + *Edalorhina* + *Engystomops* + *Physalaemus* were already present in the most recent common ancestor of this clade, having evolved in this ancestor or earlier (see discussion above). The inguinal/lumbar macroglands evolved simultaneously or subsequent to the origin of startling behaviour (this uncertainty arises from the alternative ancestral reconstructions for the macroglands and the poorly known defensive behaviours in *Pseudopaludicola*, Leptodactylinae and Paratelmatobiinae). The elements associated with aposematism, eyespots and the bright coloration in the inguinal/lumbar region and hidden areas of the thighs evolved much later, 12 or 13 times for the eyespots and at least ten to 14 times for the bright coloration.

The startle-first hypothesis for the origin of deimatism received recent support from experimental studies (Holmes *et al.*, 2018). The sequence of character evolution in leiuperines inferred from our results is congruent with the scenario of the startle-first hypothesis, providing phylogenetic evidence corroborating this hypothesis. Additional behavioural studies in Leptodactylinae, Paratelmatobiinae and *Pseudopaludicola* would establish whether the behavioural components evolved earlier than the macroglands, but this would only enrich the perspective on the evolution of this display in the group, without having a bearing on the support of the startle-first hypothesis.

Brodie *et al.* (1998) hypothesized that the presence of noxious secretions concentrated in the macroglands 'preadapted' *Uperoleia* species for behaviours that avoid predators more efficiently. Also, they hypothesized that bright colours evolved earlier, followed by defensive postures (similar to what has been called the 'deimatic display' in leiuperines; see Brodie *et al.*, 1998; Williams *et al.*, 2000) that become more specialized to exhibit this coloration. In the context of our results on leiuperines, this scenario is not supported, because macroglands

evolved simultaneously or subsequent to the origin of the behavioural components of the deimatic behaviour.

Toledo *et al.* (2011) suggested that macroglands evolved earlier than the behaviours that expose them directed towards the predator. Our results on leiuperines do not support this hypothesis, because the four behavioural components of the deimatic display were already present in the most recent common ancestor of *Pleurodema + Edalorhina + Engystomops + Physalaemus*; the inguinal/lumbar macroglands could have evolved in this same node or subsequently, multiple times.

We see a potential point for further exploration, but in that we are assuming in the present discussion that the evolution of the macroglands implies a qualitative enhancement in the chemical defences in the most recent common ancestor of *Pleurodema + Edalorhina + Engystomops + Physalaemus* compared with its sister taxon (*Pseudopaludicola*) and other leptodactylids. Current knowledge indicates that this might be correct with regard to *Pseudopaludicola* and Paratelmatobiinae (where no secretions of any kind have been reported; e.g. Erspamer *et al.*, 1986; Roseghini *et al.*, 1986), but certainly not for at least most species of *Leptodactylus*, several of which have a diverse array of biogenic amines, sometimes in high concentrations (e.g. Erspamer *et al.*, 1964b; Roseghini *et al.*, 1986).

EVOLUTION OF ANTI-PREDATOR MECHANISMS INVOLVING MACROGLANDS AND VISUAL SIGNALS IN ANURANS

The co-occurrence of macroglands, bright coloration and/or eyespots (apparently) in both sexes has also been described in Eleutherodactylidae and Myobatrachidae. Some species of the *Eleutherodactylus nitidus* group, from the western Caribbean clade, have protuberant macroglands (e.g. *Eleutherodactylus albolabris* Lynch & Lescure, 1980, *Eleutherodactylus angustidigitorum* Taylor, 1940, *Eleutherodactylus dilatus* Davis & Dixon, 1955, *Eleutherodactylus grandis* Dixon, 1957, *Eleutherodactylus maurus* Hedges, 1989, *Eleutherodactylus nitidus* Peters, 1870 and *Eleutherodactylus saxatilis* Webb, 1962), as does *Eleutherodactylus counouspeus* Schwartz, 1964, a basal species of the Eastern Caribbean clade. A diffuse glandular tissue in the inguinal region was described in *Eleutherodactylus campi* (Stejneger, 1915), *Eleutherodactylus cystignathoides* Cope, 1877, *Eleutherodactylus guttilatus* Cope, 1879, *Eleutherodactylus leprus* Cope, 1879, *Eleutherodactylus rubrimaculatus* Taylor & Smith, 1945, *Eleutherodactylus modestus* Taylor, 1942, *Eleutherodactylus pipilans* Taylor, 1940, *Eleutherodactylus verrucipes* Cope, 1885 and *Eleutherodactylus verruculatus* Peters, 1870 (e.g. Smith & Taylor, 1948; Dixon, 1957; Lynch, 1968). Also, *El. albolabris* has a reddish colour on the hidden portions of its thighs (Hedges *et al.*, 2008; Grünwald *et al.*, 2018). Other than the differences in terms of a compact macrogland and diffuse glandular tissue in the inguinal region, there is no information on the structural diversity of these glands. As far as we know, defensive behaviours associated with macroglands have not been reported in *Eleutherodactylus*, nor on chemical defences in this clade (subgenus *Syrrophus*). The most recent study on phylogenetic relationships of the *El. nitidus* species series (Grünwald *et al.*, 2018), although with poor resolution, indicates multiple origins of the macroglands during the evolutionary history of the group. A thorough study of the group is necessary to understand the taxonomic distribution of the diffuse glandular tissue in the inguinal region and its relationship with the macrogland, in order to obtain a clearer picture of the evolutionary history of this character.

The species of the myobatrachid genus *Uperoleia* (28 species; Frost, 2020) have parotoid, inguinal and coccygeal glands (Tyler *et al.*, 1981), and noxious secretions, mostly peptides, were recorded in the species that were prospected (Erspamer *et al.*, 1984). The distribution of these characters suggests a complex scenario, because there are species with macroglands and bright coloration, with macroglands but pale colour, with highly reduced macroglands and pale colour, and with highly reduced macroglands glands and bright colour (Tyler *et al.*, 1981; Anstis, 2013). No histological studies are available for the macroglands of *Uperoleia*. Body inflation and 'rear behaviour' are displayed by species with and without bright coloration and by species with prominent or reduced inguinal macroglands (e.g. Tyler *et al.*, 1981; Torr, 1991; Brodie *et al.*, 1998; Williams *et al.*, 2000; Doughty & Roberts, 2008; Catullo *et al.*, 2011, 2014; Clulow *et al.*, 2016). The phylogenetic information on myobatrachids (Jetz & Pyron, 2018: suppl. data) and *Uperoleia* (Catullo *et al.*, 2011) allows us to infer that the occurrence of inguinal and coccygeal glands are a synapomorphy of *Uperoleia*, having subsequently evolved into more reduced structures at some point during the evolutionary history of this genus. However, parotoid glands evolved earlier than inguinal and coccygeal glands. This could have happened in the most recent common ancestor of *Spicospina* Roberts *et al.*, 1997 and *Uperoleia* (with an independent origin in *Pseudophryne* Fitzinger, 1843) or in the most recent common ancestor of *Pseudophryne*, *Spicospina* and *Uperoleia* (with a subsequent loss in the clade including *Arenophryne* Tyler, 1976, *Metacrinia* Parker, 1940 and *Myobatrachus* Schlegel in Gray, 1850). The bright coloration in the inguinal region and thighs is also a synapomorphy of *Uperoleia*,

which was subsequently lost a few times. A defensive display has not been recorded in *Spicospina*, but one similar to that of *Uperoleia* was reported in *Pseudophryne* (Williams *et al.*, 2000). Although still unreported in *Spicospina*, the occurrence of at least some compounds of the toxic secretions seems to have evolved much earlier in myobatrachids; for example, the tachykinin peptide uperolein has been reported in *Pseudophryne* and *Taudactylus* Straughan & Lee, 1966 (Erspamer *et al.*, 1984), in addition to *Uperoleia*. These data allow us to infer that the secretions evolved earlier than the macroglands, being congruent with the scenario proposed by Brodie *et al.* (1998). The defensive behaviour was present at least in the most recent common ancestor of *Uperoleia* and might have evolved earlier. From this phylogenetic perspective, it is difficult to separate clearly the points where the defensive behaviour and the macroglands evolved. What is clear is that inguinal and coccygeal glands evolved after the parotoid glands and that the bright coloration evolved at the same time or after the origin of the defensive display, unlike the hypothesis of Brodie *et al.* (1998).

Although current knowledge in Eleutherodactylidae does not allow a comparison, the inferences on the evolution of macroglands, defensive display, secretions and bright colours in *Uperoleia* have some points in common with leiuperines. Macroglands were already present in the most recent common ancestor of *Uperoleia*, whereas this is ambiguous for *Pleurodema* + *Edalorhina* + *Engystomops* + *Physalaemus*. In the case of *Uperoleia*, the bright coloration evolved simultaneously with the inguinal and coccygeal macroglands, whereas in leiuperines the bright coloration and eyespots evolved independently many times. Although it is reasonable to infer that secretions evolved earlier than the macroglands in *Uperoleia*, the situation is ambiguous in leiuperines. In both clades, the defensive display was already present in the most recent common ancestors; although it is likely to be plesiomorphic for *Uperoleia* (if shown to occur in *Spicospina*, as it occurs in *Pseudophryne*), it remains ambiguous in Leiuperinae (because it was not recorded in *Pseudopaludicola* and is unknown in Paratelmatobiinae).

Finally, some microhylid frogs also have eyespots, such as *Cophixalus verrucosus* (Boulenger, 1898b), *Copiula oxyrhina* (Boulenger, 1898a), *Mysticellus franki* Garg & Biju, 2019, *Plethodontohyla ocellata* Noble & Parker, 1926, *Plethodontohyla bipunctata* (Guibé, 1974) and *Rhombophryne ellae* Scherz, 2020. In the case of *Mysticellus franki*, dark brown ventral spots expand dorsally, originating an eyespot when the animal is resting (Garg & Biju, 2019: fig. 1D–F); also, bright colours of the hidden areas of thighs

were described in *Cophixalus balbus* (Günther, 2003) and in at least seven species of *Callulops* Boulenger, 1888 (Kraus, 2019). The latter are also reported to have an aposematic/deimatic display (Kraus, 2019). Although there is no external evidence of macroglands in microhylids, the occurrence of a milky secretion during the display was reported for *Callulops* (Kraus, 2019) and *Plethodontohyla* (Glaw & Vences, 2007). As defensive behaviours and glandular structure become better known, it will be possible to integrate the sparse current knowledge of the group into the context of a broader discussion.

CONCLUSIONS

Our study of the evolution of macroglands and defensive mechanisms in Leiuperinae allows us to infer a complex scenario, in which macroglands in the inguinal/lumbar region evolved either in the most recent common ancestor of *Pleurodema* + *Edalorhina* + *Engystomops* + *Physalaemus* or multiple times during the evolutionary history of the group. This same hypothetical ancestor already showed the four behavioural components of the deimatic or aposematic display. Subsequently, eyespots and bright coloration in the inguinal/lumbar region and on the hidden surfaces of the thighs evolved multiple times. Macroglands in the inguinal/lumbar region also evolved three times into a protuberant patch of glandular tissue with diffuse limits and were lost at least seven times independently. This inferred scenario provides phylogenetic support for the startle-first hypothesis for the evolution of deimatic displays.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary Data S1. Voucher specimens and GenBank accession numbers for the taxa included in the phylogenetic analyses.

Supplementary Data S2. Best partition scheme and best-fitting models selected by MODELFINDER for the molecular data.

Supplementary Data S3. Specimens used for histological procedures.

Supplementary Data S4. Data matrix of the 17 coded characters.

Supplementary Data S5. Phylogenetic hypothesis of Leiuperinae. Strict consensus of the most parsimonious trees. Numbers above nodes indicate jackknife support values. Names of clades and species groups are those employed in published phylogenetic hypotheses for each genus of Leiuperinae (see main text).

Supplementary Data S6. Phylogenetic hypothesis of Leiuperinae. Maximum likelihood tree. Numbers above nodes indicate bootstrap support values.

Supplementary Data S7. Histochemical characteristics of mucous and serous glands of examined species.