

Morphometric Variations in the Skin Layers of Frogs: An Exploration Into Their Relation With Ecological Parameters in *Leptodactylus* (Anura, Leptodactylidae), With an Emphasis on the Eberth-Kastschenko Layer

MARÍA LAURA PONSSA,^{1*} J. SEBASTIÁN BARRIONUEVO,²
FRANCO PUCCI ALCAIDE,³ AND ANA PUCCI ALCAIDE⁴

¹Unidad Ejecutora Lillo. CONICET-Fundación Miguel Lillo, Tucumán, Argentina

²División Herpetología, Museo Argentino de Ciencias Naturales
“Bernardino Rivadavia”-CONICET, Tucumán, Argentina

³Instituto de Morfología Animal, Fundación Miguel Lillo, Tucumán, Argentina

⁴Facultad de Ciencias Naturales e Inst. Miguel Lillo, Universidad Nacional de Tucumán,
Tucumán, Argentina

ABSTRACT

Leptodactylus is a genus of frogs known to live in diverse habitats and to show both aquatic and terrestrial breeding habits. We studied 21 species of *Leptodactylus* to explore whether skin structure specialization relates to habitats and habit variation. Morphometric analyses of the skin thickness revealed that phylogeny has a strong influence on variations in the thickness of the epidermis, *stratum spongiosum*, Eberth-Kastschenko layer, and *stratum compactum*, while habitat and habits display no significant correlation. The optimization of the phylogenetic hypothesis suggested that a pattern of intermediate values for skin layer thickness are plesiomorphic for this group. Anat Rec, 00:000–000, 2017.

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Key words: anuran; skin; terrestrial habits; Eberth-Kastschenko layer; phylogeny

The integumentary system serves several crucial functions for amphibians, including mechanical protection, chemical defense, respiration, osmoregulation, water balance, and sensorial perception (e.g., Duellman and Trueb, 1994; Fox, 1994). The acquisition of a stratified integument with dermal glands was critical for the tetrapod's initial incursion into terrestrial environments. The basic skin structure of amphibians (and tetrapods in general) consists of two principal layers that have distinct embryological origins: the outer, ectoderm derived epidermis, and the inner, mesoderm derived dermis. The multilayered epidermis is composed of a thin superficial *stratum corneum* traversed by the ducts of the ectodermal glands embedded in the dermis. These glands play a fundamental role in skin function and are classified into two groups

based on secretion type: mucous and granular (Fox, 1986; Toledo and Jared, 1995; Clarke, 1997). The dermis is comprised of a superficial *stratum spongiosum*, made up of

Grant sponsors: CONICET, FONCyT; Grant numbers: PIP 1017; PICT 2015 1618.

*Correspondence to María Laura Ponssa, Unidad Ejecutora Lillo (UEL), CONICET-Fundación Miguel Lillo, Miguel Lillo 251, S. M. de Tucumán (4000), Tucumán, Argentina. E-mail: mlponssa@hotmail.com

Received 25 June 2016; Revised 11 January 2017; Accepted 16 January 2017.

DOI 10.1002/ar.23640

Published online 00 Month 2017 in Wiley Online Library (wileyonlinelibrary.com).

loose connective tissue that contains the cutaneous glands, blood vessels, and chromatophores, and a deeper *stratum compactum* layer composed of condensed organized connective tissue fibers. In some anuran amphibians, the calcified Eberth-Kastschenko (EK) layer is situated between these two strata (Elkan, 1968). The EK layer is an extremely hydrophilous matrix of amorphous substances, composed principally of glycosaminoglycans, proteoglycans and deposited minerals, such as calcium (Elkan, 1976; Toledo and Jared, 1993).

One of the principal challenges for the integumentary system of terrestrial amphibians is controlling evaporative water loss (EWL) resulting from both the functional loss during breathing in combination with a gradient-driven loss through the skin (Kobelt and Linsenmair, 1986), which loses water at an elevated rate (Withers et al., 1984). Nevertheless, specialized physiological mechanisms have independently evolved in unrelated anuran genera to address the reduction of cutaneous water loss. For example, some species of *Phyllomedusa* (Blaylock et al., 1976; McClanahan et al., 1978) and *Polydectes maculatus* (Lillywhite et al., 1997) have been described to have specialized lipid producing glands that coat the body surface as a barrier against water loss. An increased number of chromatophores with light-reflecting platelets (iridophores) has also been proposed as a water retention mechanism in *Chiromantis* (Drewes et al., 1977) although Withers et al. (1984) presented evidence that contradicts this hypothesis. Similarly, despite the fact that *Bokermannohyla alvarengai* is not considered a “waterproof” species, it has been suggested that a combination of mucosubstances and lipids compounds together with changes in skin coloration produced by the arrangement of iridophores, allow for heat acquisition without compromising water balance (Centeno et al., 2015). Histochemical differences between the glycosaminoglycan content in the skin of aquatic (*Xenopus laevis*) and semiterrestrial (*Bufo bufo*) species have also been suggested as an indicator of a relationship between habitat selection and skin content (Danguy and Genten, 1989; Toledo and Jared, 1995). In terrestrial amphibians, the cutaneous EWL and gas exchange cannot be dissociated as the processes effectively fluctuate in correlation (Shoemaker et al., 1992). Body regions with thinner skin, or those that lack specialized diffusion impeding morphological structures, exhibit an increased water loss (Kobelt and Linsenmair, 1986), as the skin requires a relatively thick layer between the environment and the capillaries to be adequately protective (Shoemaker et al., 1992). This suggests that the thickness of the skin, or of any of its layers, could be indicative of its capacity to minimize water loss.

The EK layer has been described in species in diverse families, including Discoglossidae, Hylidae, Microhylidae, Pelobatidae, Pipidae, Rhacophoridae (Elkan, 1968); Bufonidae (Elkan, 1968; Alves Azevedo et al., 2005; Almeida et al., 2007), Leptodactylidae (García and Cardozo, 2005; García et al., 2011; Ferraro et al., 2013), and Ranidae (Zepp, 1923; Elkan, 1968). This layer, which occurs frequently in terrestrial species and is more fully developed dorsally than ventrally (Elkan, 1968, 1976; Toledo and Jared, 1993), has been suggested as another protective mechanism against desiccation. Various investigators have concluded that the hydrophilic constitution of glycosaminoglycans lends evidence to the hypothesis

that the EK layer is related to the prevention of water loss (Elkan, 1968, 1976; Elkan and Cooper, 1980; Toledo and Jared, 1993; García et al., 2011). However, Drewes et al. (1977) questioned this putative function for the EK layer, arguing that the layer lies beneath the vascular vessels of the *stratum spongiosum*. Alternative functions have been proposed, including storage and release of calcium, metabolism of glucose, and sodium transportation (Moss, 1972; Baldwin and Bentley, 1981).

Previous studies have suggested that susceptibility to dehydration in amphibians is not necessarily phylogenetically conservative, as families with wide ecologic distribution also have wide ranges of tolerance (Shoemaker et al., 1992). Prates and Navas (2009) hypothesized that cutaneous resistance to EWL is correlated with ecological features and is influenced by taxonomic position.

Investigation of the *Leptodactylus* cutaneous apparatus presents an excellent case study for the exploration of skin specialization in the context of broad habitat and habit variability, as it contains species that inhabit both dry and wet environments, and exhibit both aquatic and terrestrial reproductive behavior. The genus includes 74 species divided into four species groups: *L. gr. latrans*, *L. gr. melanonotus*, *L. gr. pentadactylus*, *L. gr. fuscus* (Heyer, 1969) that are distributed throughout the Neotropics and the southern portion of the Nearctic region, from Texas to Argentina and certain Caribbean islands (Ponssa, 2008; de Sá et al., 2014). *Leptodactylus* species inhabit a wide variety of environments, from low-land dense rainforest to open habitats (e.g., Savanna, Catinga, Cerrado, Chaco, and Grasslands) (AbSáber, 1977; Joly et al., 1999; de Sá et al., 2014), including environments disturbed by cattle ranching and agriculture (Medina et al., 2016). Reproductively, *Leptodactylus* can be divided into two distinct modes: (i) species closely tied to aquatic habitats that oviposit in foam nests on water (e.g., species of the *L. latrans* group); and (ii) species with terrestrial habits. Terrestrial reproductive habits include either foam nest deposition basins (e.g., some species of the *L. melanonotus* and *L. pentadactylus* groups) or the construction of foam nest incubation chambers on land (e.g., species of the *L. fuscus* group) (Heyer, 1969).

Previous studies have addressed other morphological skin variations in *Leptodactylus* species. For example, the ultrastructure, histology and biochemistry of the granular (poison) glands were described for *L. chaquensis* (Alvarez et al., 2005), and a relationship between granular secretions and warning signals was suggested for *L. lineatus* (Prates et al., 2012). The EK layer has previously been analysed for six species of *Leptodactylus*: *L. pentadactylus* (Elkan, 1968), *L. elenae*, *L. fuscus*, *L. gracilis*, *L. latinasus* (García et al., 2011), and *L. mystacinus* (García and Cardozo, 2005), and both intra and interspecific EK layer width variations were found (García et al., 2011).

The objective of the current study is to analyse, within a phylogenetic framework, the correlation between morphometric skin layer variation, particularly the EK layer, and the ecology of the genus *Leptodactylus*.

MATERIALS AND METHODS

Morphological Analysis

Twenty eight skin samples were taken from 21 species of *Leptodactylus* selected to represent the main

TABLE 1. Species of *Leptodactylus* included in this study, its main habits and habitats.

Species	Habits	Literature	Specimen	Habitats (ecoregions)
<i>A. marmorata</i>	T	Almeida and Angulo (2002)	MNRJ28287	W: Serra do Mar coastal forests (moist forest)
<i>L. camaquara</i>	T ^a		MZUSP56842	D: Campos Rupestres/Cerrado
<i>L. colombiensis</i>	ST ^a		USNM148801	D: Cauca Valley Dry Forests
<i>L. colombiensis</i>	ST ^a		USNM148802	D: Cauca Valley Dry Forests
<i>L. fallax</i>	ST	Lescure (1979); Lescure and Letellier (1983); Gibson and Buley (2004)	USNM162244	W: Southern Caribbean (moist forest)
<i>L. furnarius</i>	T ^a		MZUSP83271	W: Guianan savanna/Guaianan Highland moist forest
<i>L. griseigularis</i>	ST ^a		USNM166023	W: Ucayali moist forests
<i>L. griseigularis</i>	ST ^a		USNM196024	W: Ucayali moist forests
<i>L. insularum</i>	A	Ponssa (2001)	MZUSP150743	D: Guajira-Barranquilla Xeric Scrub
<i>L. jolyi</i>	T	Barreto Pereira et al. (2015)	MZUSP47621	D: Cerrado
<i>L. leptodactyloides</i>	A	Downie (1996)	USNM247382	W: Southwest amazon moist forest
<i>L. longirostris</i>	T ^a		MZUSP65793	W: Purus Varzea (moist flooded forest)
<i>L. macrosternum</i>	A	Haddad and Prado, 2005; Barreto Pereira et al. (2015)	MNRJ213259	D: Cerrado
<i>L. melanotus</i>	ST ^a		USNM283471	W: Western Ecuador Moist Forests
<i>L. natalensis</i>	ST	Maranhão dos Santos and de Oliveira Amorim (2005)	MNRJ34988	W: Serra do Mar coastal forests (moist forest)
<i>L. natalensis</i>	ST	Maranhão dos Santos and de Oliveira Amorim (2005)	MNRJ27939	W: Serra do Mar coastal forests (moist forest)
<i>L. pentadactylus</i>	ST	^b	MZUSP56779	W: Purus Varzea (moist flooded forest)
<i>L. podicipinus</i>	ST	Prado et al. (2002)	ZUFMS0467-1	D: Pantanal
<i>L. podicipinus</i>	ST	Prado et al. (2002)	ZUFMS0478-2	D: Chiquitano dry Forest/Pantanal
<i>L. rhodonotus</i>	ST ^a		USNM19603	W: Ucayali Moist Forests
<i>L. rhodonotus</i>	ST ^a		USNM196009	W: Ucayali Moist Forests
<i>L. savagei</i>	ST	Heyer and Rand (1977), Vinton (1951), Valerio (1971), Heyer et al. (1975), Muedeking and Heyer (1976), Savage (2002), Hero and Galatti (1990), Lima et al. (2006)	USNM347152	W: Mesoamerica gulf caribbean mangroves
<i>L. spixi</i>	T ^a		MZUSP63671	W: Bahia coastal forest
<i>L. validus</i>	A	Downie (1996)	USNM167496	W: Trinidad and Tobago Moist Forests
<i>L. validus</i>	A	Downie (1996)	USNM192762	W: Trinidad and Tobago Moist Forests
<i>L. vastus</i>	ST	da Silva Vieira et al. (2007)	MZUSP50187	D: Cerrado
<i>L. ventrimaculatus</i>	T ^a		USNM196765	W: Northwestern Andean montane forests (moist forest)
<i>L. wagneri</i>	ST ^a		USNM36193	W: Japura-Solimoes-Negro Moist Forests
<i>L. wagneri</i>	ST ^a		USNM283837	W: Eastern Cordillera Real Montane Forests (moist forest)

Habits: T: terrestrial; ST: semiterrestrial; A: aquatic. Habitats: W: wet habitat; D: dry habitat. Museums acronyms: MNRJ: Museu Nacional do Rio de Janeiro; MZUSP: Museu de Zoologia da Universidade de São Paulo; USNM: United State Natural Museum; ZUFM: Coleção Zoológica de Referência da Universidade Federal de Mato Grosso do Sul.

^aAssignment according the phylogeny of de Sá et al. (2014) and the reconstruction of ancestral character of Barreto Pereira et al. (2015).

^bLiterature corresponding to *L. savagei*. Despite the extensive literature on the natural history of *L. pentadactylus*, much of it concerns the populations of Central America, which currently correspond to *Leptodactylus savagei* (Heyer, 2005). Consequently, the detailed descriptions of foam nest building found in Heyer and Rand (1977), Vinton (1951), Valerio (1971), Heyer et al. (1975), Muedeking and Heyer (1976), Savage (2002), Hero and Galatti (1990), Lima et al. (2006) correspond to *L. savagei*.

ecological habitats and reproductive habits of the genus (Table 1). One skin sample of the related species *Adenomera marmorata* (Ponssa, 2008; de Sá et al., 2014) was also included for comparison. A list of all the studied specimens with collection numbers and locality data are detailed in Appendix I.

A small strip of mid-dorsal skin (~5–10 mm²) was removed from each previously formalin-fixed specimen (Fig. 1). These skin samples were rinsed, dehydrated in a series of graded ethanols, cleared in xylene, and paraffin-embedded. Serial sections (4- to 6-µm thick)

were cut in a transverse plane, with a rotary microtome and microtome type sliding. The sections were stained with Hematoxylin-Eosin (HE) (Martoja and Martoja-Pierson, 1970), Alcian Blue (AB) and Periodic Acid Schiff (PAS) (Humason, 1962; Kiernan, 2010), pH 2.5 and 0.5, to detect mucous secretions and glycosaminoglycans (Kiernan, 2010). Specific calcium detection of the EK layer was performed with the Von Kossa staining method (Kiernan, 2010). This method allows for the observation of the calcium deposits by staining them dark brown or black. The soluble phosphates and

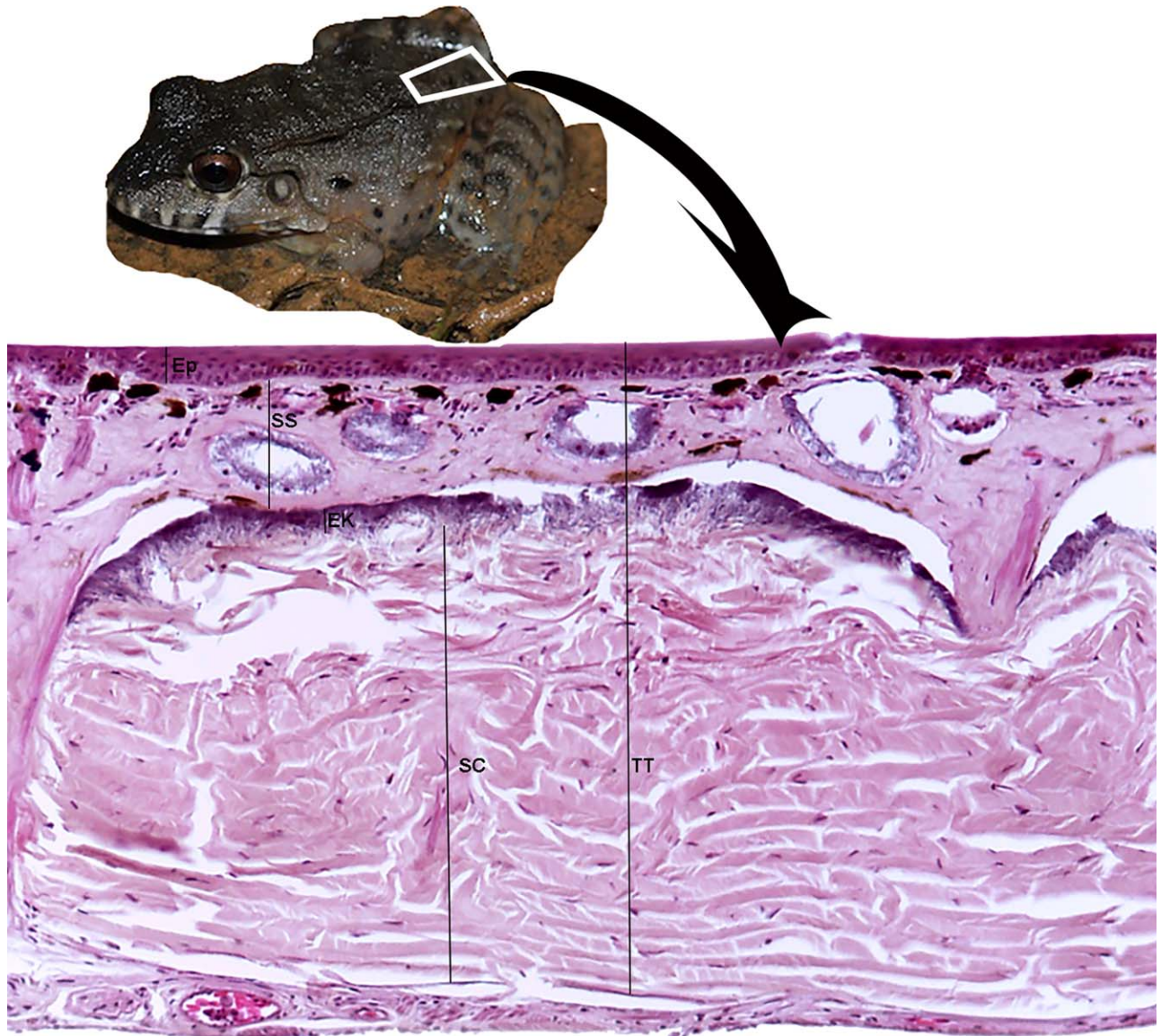


Fig. 1. *Leptodactylus vastus* (photo of F. Brusquetti), detail showing the skin section removed and processed, including the measurements of total thickness (TT) of the epidermis (Ep), *stratum spongiosum* (SS), EK layer (EK), and *stratum compactum* (SC). Scale bar 200 μ m.

carbonates are washed away and the calcium carbonates are retained, reacting with the silver. The positive reaction obtained in the Von Kossa test confirmed that the EK layer contains calcium phosphate. The controls for the histochemical techniques were the samples processed with Hematoxylin-Eosin (HE). Micrographs of the stained sections were captured using a ZEISS AxioCam ERc5s and a Leica ICC50HD camera. Measurements from the micrographs were taken at different magnifications using the software ZEISS ZEN 2012 (blue edition). The total thickness of the skin was measured, as well as each individual stratum: the epidermis, the *stratum spongiosum*, the EK layer, and the *stratum compactum* (Fig. 1). Measurements were repeated in five different sites per sample, except for the EK layer, which was measured in ten sites.

Statistical Analysis

To evaluate variations in skin layer thickness, we conducted three MANCOVA analyses using total skin thickness as a covariate and three different factors: (a) species, (b) habit, and (c) habitat. Post hoc Bonferroni tests are used for multiple comparisons. To categorize species' habit patterns, we referenced published data regarding reproduction mode and/or personal observations from field expeditions (MLP). When data were not available, the category of the closest relative was assigned following the phylogeny of de Sá et al. (2014) and the ancestral state reconstruction of Barreto Pereira et al. (2015) (Table 1). Habits were categorized according to breeding pattern: (1) aquatic; species that oviposit in floating foam nests; (2) semiterrestrial; species that produce foam nests in water accumulated in natural or constructed

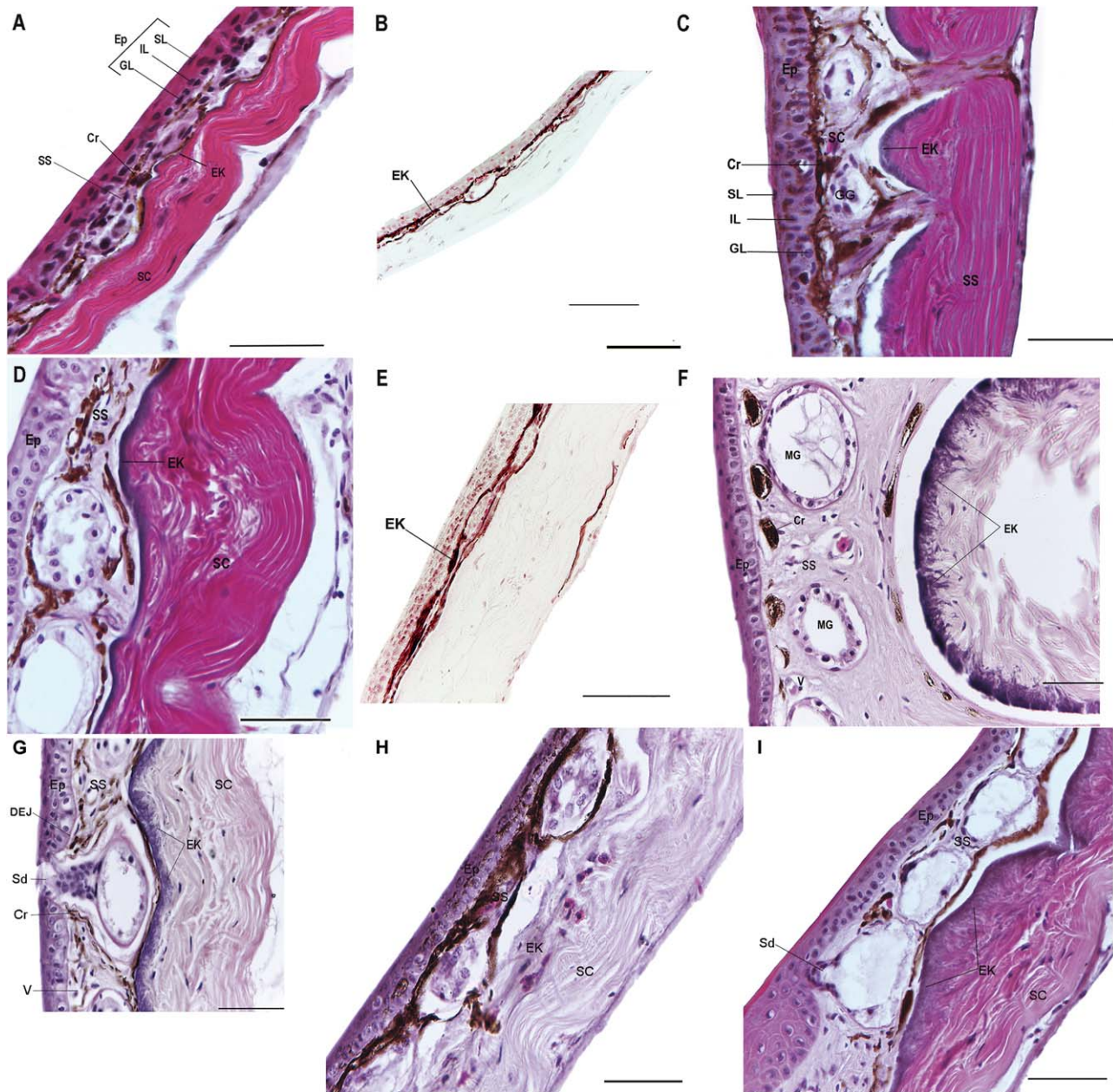


Fig. 2. Histological section of the dorsal skin of species of genus *Leptodactylus* and *Adenomera marmorata*. **A, B:** *Adenomera marmorata*. **C:** *Leptodactylus camaquara*. **D, E:** *L. colombiensis*. **F:** *L. fallax*. **G:** *L. griseigularis*. **H:** *L. jolyi*. **I:** *L. leptodactyloides*. The three strata of the epidermis are evident: the superficial, the intermediate and the geminativum. The dermal-epidermal junction shows evidence, by its reaction to PAS, of high content of neutral glycoconjugates. In the *stratum spongiosum* of the dermis, mucous glands (MG) and granular glands (GG) are recognized (C, D, F, G, H, I). Note the calcium deposits of the EK layer stained dark brown to black (B, E). Histological staining: Hematoxylin-Eosin (A, C, D, F, G, H, I), Van Kossa (B, E). Cr: chromatophores; DEJ: dermal-epidermal junction; EK: Eberth-Kastschenko layer; Ep: epidermis; GG: granular gland; GL: germinativum layer of the epidermis; IL: intermediate layer of the epidermis; MG: mucous gland; SC: *stratum compactum*; Sd: secretory duct; SL: superficial layer of the epidermis; SS: *stratum spongiosum*; V: vessel. Scale bar 50 μ m.

basins; (3) terrestrial; species with foam nests placed inside subterranean chambers. Habitats were assigned according to the characterization of the ecoregion (Olson et al., 2001) where each specimen was collected, and were grouped into two categories; (1) dry and (2) wet. The ecoregions considered in each category are listed in Table 1.

Values of all the morphometric traits were \log_{10} converted prior to analysis to meet the requirements of

normality and homoscedasticity (Zar, 1999) using the StatSoft, Inc. software package, version 7.0 (2004).

Ancestral State Reconstruction

The ancestral state reconstruction of four continuous characters, the thickness of the skin layers epidermis, *stratum spongiosum*, EK, and *stratum compactum* was run

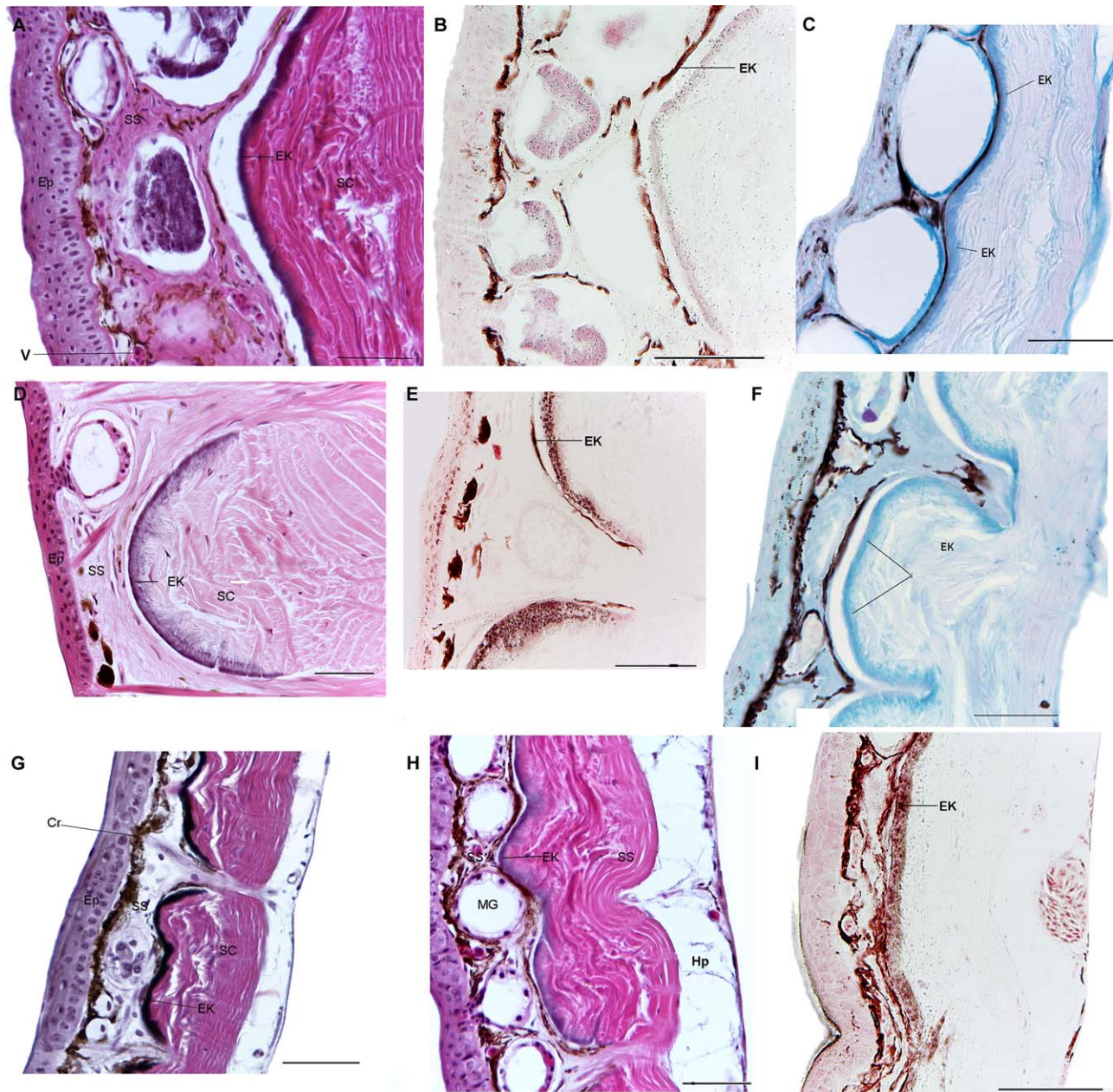


Fig. 3. Histological section of the dorsal skin of species of the genus *Leptodactylus*. **A, B:** *L. macrosternum*; **C:** *L. podicipinus*. **D, E:** *L. pentadactylus*; **F:** *L. rhodonotus*. **G:** *L. spixi*. **H, I:** *L. wagneri*. The three skin layers analyzed are visible: epidermis, dermis (*strata compactum* and *spongiosum*) and E-K layer. In the dermis, chromatophores (G), and blood vessels (A) are evident. Note the calcium deposits of the EK layer stained dark brown to black (B, E, I). Below the dermis, the hipodermis is present as loose connective tissue (H). Histological staining: Hematoxylin-Eosin (A, B, D, F, I); PAS-AB (C, F); Van Kossa (E, I). EK: Eberth-Kastschenko layer; Ep: epidermis; Hp: hipodermis; SC: *stratum compactum*; SD: secretory duct; SS: *stratum spongiosum*; V: vessel. Scale bar 50 μ m.

using Mesquite (Maddison and Maddison, 2015). The size effect was removed using the residuals of each log-transformed value over the total skin thickness. We employed the phylogenetic hypothesis proposed by de Sá et al. (2014) with parsimony and Brownian motion as models for evolutionary change (Felsenstein, 1985). Data regarding total skin thickness and the epidermis and EK layers of *Leptodactylus elenae*, *L. fuscus*, *L. gracilis*, and *L. latinasus* were taken from García et al. (2011) and were used for optimization. *Leptodactylus fuscus* is found in two relatively

distant clades in the phylogeny proposed by de Sá et al. (2014): clade *L. fuscus*12345 and clade *L. fuscus*6789. On the basis of the geographic proximity, we considered the samples analyzed here as belonging to the clade *L. fuscus*6789.

RESULTS

Integument Morphology

The epidermis of *Leptodactylus* is composed of various layers, the outermost of which is the *stratum corneum*, a

TABLE 2. Average thickness \pm standard error of each layer of the skin expressed in μm for each sample of skin of 21 species (29 specimens) of *Leptodactylus* and *Adenomera marmorata*

Species	ejemplar	Total	Epidermis	Compactum	Spongiosum	E-K
<i>A. marmorata</i>	MNRJ 28287	41.09 \pm 5.25	7.25 \pm 1.16	25.77 \pm 4.19	12.39 \pm 3.77	0.77 \pm 0.11
<i>L. camaquara</i>	MZUSP56842	94.26 \pm 9.54	14.63 \pm 1.88	41.29 \pm 5.15	34.41 \pm 13.64	1.78 \pm 0.21
<i>L. colombiensis</i>	USNM148801	47.7 \pm 2.31	9.79 \pm 0.48	29.23 \pm 3.06	8.4 \pm 2.11	1.36 \pm 0.35
<i>L. colombiensis</i>	USNM148802	92.34 \pm 16.56	15.65 \pm 0.78	42.24 \pm 2.73	23.8 \pm 3.07	2.15 \pm 0.43
<i>L. fallax</i>	USNM162244	397.81 \pm 12.07	13.20 \pm 1.6	305.55 \pm 9.15	84.08 \pm 9.24	9.68 \pm 1.49
<i>L. griseigularis</i>	USNM166023	101.41 \pm 5.24	13.95 \pm 1.55	67.67 \pm 4.9	45.87 \pm 19.72	3.78 \pm 0.27
<i>L. griseigularis</i>	USNM196024	66.94 \pm 3.71	6.69 \pm 0.71	49.18 \pm 6.07	17.81 \pm 4.57	2.64 \pm 0.38
<i>L. insularum</i>	MZUSP150743	121.59 \pm 2.16	14.43 \pm 1.3	95.31 \pm 3.22	23.81 \pm 4.36	3.55 \pm 0.47
<i>L. jolyi</i>	MZUSP47621	67.91 \pm 13.72	13.78 \pm 1.76	47.71 \pm 4.01	20.87 \pm 5.4	1.6 \pm 0.26
<i>L. leptodactyloides</i>	USNM247382	78.55 \pm 8.29	12.76 \pm 1.73	53.6 \pm 5.41	21.97 \pm 8.26	2.9 \pm 0.82
<i>L. furnarius</i>	MZUSP83271	42.66 \pm 4.88	11.45 \pm 2.6	19.92 \pm 3.11	15.05 \pm 6	1.36 \pm 0.35
<i>L. longirostris</i>	MZUSP65793	59.54 \pm 4.82	12.37 \pm 2.14	34.84 \pm 6.73	18.69 \pm 6.3	1.05 \pm 0.27
<i>L. macrosternum</i>	MNRJ213259	103.44 \pm 15.77	14.79 \pm 3.88	73.09 \pm 15.21	52.4 \pm 39.24	3.45 \pm 0.64
<i>L. melanonotus</i>	USNM283471	49.99 \pm 4.21	8.2 \pm 1.21	33.28 \pm 3.06	7.62 \pm 2.64	1.98 \pm 0.22
<i>L. natalensis</i>	MNRJ34988	69.49 \pm 5.28	13.82 \pm 1.65	40.59 \pm 2.96	21.27 \pm 7.66	1.62 \pm 0.11
<i>L. natalensis</i>	MNRJ27939	124.44 \pm 6.29	13.59 \pm 0.99	86.41 \pm 4.58	36.09 \pm 3.07	4.23 \pm 0.79
<i>L. pentadactylus</i>	MZUSP56779	145.76 \pm 8.84	5.4 \pm 0.79	122.92 \pm 6.73	16.7 \pm 5.96	2.83 \pm 0.43
<i>L. podicipinus</i>	ZUFMS 0467-1	68.19 \pm 5.54	11.43 \pm 1.29	40.45 \pm 9.12	16.42 \pm 7.55	1.52 \pm 0.37
<i>L. podicipinus</i>	ZUFMS 0478-2	78.28 \pm 7.89	7.1 \pm 1.88	53.57 \pm 19.49	19.58 \pm 5.88	2.6 \pm 0.5
<i>L. rhodonotus</i>	USNM19603	98.15 \pm 2.31	14.21 \pm 0.48	68.94 \pm 3.06	25.22 \pm 2.11	4.01 \pm 0.68
<i>L. rhodonotus</i>	USNM196009	126.92 \pm 6.21	11.64 \pm 0.69	91.71 \pm 9.01	31.61 \pm 7.46	3.55 \pm 0.44
<i>L. savagei</i>	USNM347152	189.27 \pm 8.43	12.86 \pm 0.72	151.65 \pm 8.24	19.97 \pm 4.72	6.13 \pm 0.96
<i>L. spixi</i>	MZUSP63671	62.07 \pm 2.89	14.79 \pm 1.39	31.66 \pm 3.72	19.18 \pm 4.16	1.84 \pm 0.32
<i>L. validus</i>	USNM167496	81.54 \pm 15.52	5.09 \pm 0.68	19.42 \pm 2.49	40.33 \pm 2.83	2.22 \pm 0.34
<i>L. validus</i>	USNM192762	47.02 \pm 4.36	10.09 \pm 0.62	33.81 \pm 3.63	7.72 \pm 1.15	1.58 \pm 0.34
<i>L. vastus</i>	MZUSP50187	130.09 \pm 1.82	6.77 \pm 0.72	100.72 \pm 2.89	24.02 \pm 2.45	5.92 \pm 1.02
<i>L. ventrimaculatus</i>	USNM196765	76.23 \pm 1.29	6.95 \pm 0.64	47.94 \pm 3.65	13.63 \pm 4.98	19.74 \pm 4.28
<i>L. wagneri</i>	USNM36193	104.74 \pm 5.74	14.83 \pm 2.4	67.7 \pm 3.75	18.96 \pm 4.1	3.17 \pm 0.57
<i>L. wagneri</i>	USNM283837	111.89 \pm 5.95	13.49 \pm 2.03	81 \pm 8.16	20.47 \pm 2.65	4.19 \pm 1.56

keratinized covering formed by two to five layers of acidophilic keratinized cells with an average total thickness of $\sim 11 \mu\text{m}$ (Figs. 2 and 3). Below the *stratum corneum*, an intermediate or transitional layer, consisting of one or two layers of cells; and below that a basal or germinativum layer (Fig. 2A,C). Underlying the epidermis is a thin dermal-epidermal junction with a high content of neutral glycoconjugates, as evidenced by its reaction to PAS (Fig. 2G).

The outer layer of the dermis, the *stratum spongiosum*, is made up of loose connective tissue, chromatophores (Fig. 2C,F), blood vessels (Fig. 3A) and two types of glands: mucous (Figs. 2F and 3H) and granular (Fig. 2C). The inner *stratum compactum* is formed of dense organized connective tissue with an abundance of collagen fibers and fibroblasts (Figs. 2 and 3). Below the dermis, the hypodermis is composed of loose connective tissue (Fig. 3H).

The EK layer was present in the dermis of all studied species, situated between the *stratum spongiosum* and *stratum compactum* (Figs. 2 and 3). The layer consists of a granular acellular matrix that stained upon exposure to Hematoxylin and AB pH 2.5, due to the content of

acidic mucopolysaccharides with carboxylated groups, phosphates and sialic acid. This layer is crossed by fibrous columns which extend from the hypodermis to the *stratum spongiosum* of the dermis (Fig. 2C).

Skin Thickness and Ecology

The measurements for each skin layer are detailed in Table 2. The multivariate analysis of covariance (MANCOVA), which was run using total skin thickness as the covariate, showed no significant difference among the 21 species of *Leptodactylus* ($F_{(80, 18)} = 0.88, P = 0.65$). Subsequent univariate F tests revealed significant differences only for the EK layer ($F_{(21,5)} = 5.5, P = 0.03$; Table 3). A post hoc test of multiple comparisons could not be performed as species were represented by only one or two specimens.

The results of the second MANCOVA using total skin thickness as a covariate and habits as factors (terrestrial vs. semi-terrestrial vs. aquatic) indicated non-significant skin layer differences ($F_{(8,44)} = 0.58, P = 0.78$). Tables 4 and 5 show the univariate effects and the Bonferroni post hoc test, respectively.

TABLE 3. Results of the analyses of covariance tests for differences in different measurements of the skin layer among 21 species of *Leptodactylus* and *Adenomera marmorata*

Epidermis		Compactum		Spongiosum		EK	
F	P	F	P	F	P	F	P
1.06	0.52	0.5	0.87	0.75	0.72	5.55	0.03*

*Significant difference among species.

TABLE 4. Results of the analyses of covariance tests for differences in different measurements of the skin layer, considering total thick skin as covariable, with the ecological habits (terrestrial vs. semiterrestrial vs. aquatic) of 21 species of *Leptodactylus* and *Adenomera marmorata* as factor

Epidermis		<i>Compactum</i>		<i>Spongiosum</i>		EK	
<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
0.29	0.59	0.0012	0.97	0.46	0.49	0.80	0.37

The third MANCOVA, which considered total skin thickness as a covariate and habitats as factors (wet vs. dry habitats) also produced non-significant skin layer differences ($F_{(4, 23)} = 0.28$, $P = 0.88$). Table 6 displays the univariate effects.

Ancestral State Reconstruction

The ancestral state reconstruction of the thickness of each skin layer showed clear evolutionary trends within *Leptodactylus* (Figs. 4 and 5). The four studied morphometric variables presented intermediate values of skin layer thickness for group ancestors, with independent higher or lower values on isolated terminals. The epidermal layer thickness (character 1) showed a tendency towards intermediate-low values: the lowest values were registered in the *L. pentadactylus* group (semi-terrestrial clade from wet habitats, except for *L. vastus*, which is found in dry habitats). Intermediate values appeared in the *L. fuscus* group (terrestrial clade from both dry and wet habitats). Within the *L. fuscus* clade, high values could be found at the base of the clade (*(L. mystaceus + L. fuscus) L. notoaktites*) *L. elenae*, with some of the highest values recorded for *L. elenae* (terrestrial from wet habitats) and the independent re-acquisition of intermediate-low values in *L. longirostris* and *L. furnarius* (both terrestrial species from wet habitats) (Fig. 4). The *stratum spongiosum* thickness (character 2) shows a prevalence for lower values, although they are scattered throughout the cladogram, with the lowest values in the clade *L. pentadactylus + L. savagei* (semi-terrestrial clade from wet habitats) and higher values in *L. macrosternum* (aquatic from dry habitats) (Fig. 4). The EK layer thickness (character 3) showed a trend of intermediate to lower values throughout the genus, independent of the species' ecological habits (terrestrial/semi-terrestrial or aquatic) or habitat (dry or wet). The only anomaly is *L. ventrimaculatus*, within the terrestrial *L. fuscus* group, whose wet habitat specimen displayed high values (Fig. 5). In general, the *stratum compactum* thickness (character 4) displayed intermediate values throughout the sampled species with the notable exception of decreased values in the terrestrial *L. fuscus* group, specifically in the clade *L.*

furnarius (wet habitats) + *L. camaquara* (dry habitats); the latter species displaying the lowest values. In the semi-terrestrial *L. pentadactylus* group, higher values were optimized in the clade *L. savagei + L. pentadactylus* (from wet habitats), and independently acquired in *L. vastus* (from dry habitats) and within the aquatic *L. latrans* group, in *L. insularum* (from dry habitats) (Fig. 5).

DISCUSSION

The current work is the first to address skin layer thickness as an independent phylogenetic factor. The skin traits tracked in previous phylogenies or proposed as synapomorphies have traditionally focused on specific skin structures, such as glands (e.g., Parsons and William, 1963; Ceriotti et al., 1989; Ford and Canatella, 1993; Maciel et al., 2003, 2006, 2010; Grant et al., 2006; Pramuk, 2006). The morphometric analyses of skin layer thickness in *Leptodactylus* performed in this study did not reveal significant differences in the epidermis, the *stratum spongiosum*, the EK layer, or the *stratum compactum* among species or between habit or habitat groups. Optimization of the phylogenetic hypothesis also failed to reveal a relationship between skin layer thickness and habits and/or habitats, but did seem to suggest a phylogenetically influenced pattern in which intermediate values are the plesiomorphic state for all four of the studied morphometric characters in *Leptodactylus*.

In the samples from *Leptodactylus* and *Adenomera*, the thickness of the epidermis layer varies between 5.09 μm (in *Leptodactylus validus*) and 29 μm (in *L. elenae*). The taxonomic distribution of variances in the epidermis layer thickness reveals an interesting phylogenetic pattern in both the *L. fuscus* and *L. pentadactylus* groups. In the clade (*(L. didymus, (L. mystaceus1, (L. elenae, ((L. mystaceus23, L. fuscus5789), L. notoaktites))*), (*L. tapiti, (L. cunicularis, (L. camaquara, L. furnarius)*), (*L. marambaie, L. plaumanni*))), (*(L. gracilis, (L. sertanejo, L. jolyi)*), *L. latinasus*)) belonging to the *L. fuscus* group, the ancestors show higher values of thickness with an increasing trend. In contrast, in the clade (*((L. knudseni (L. fallax, L. labyrinthicus), (L. myersi (L. peritoaktites (L. pentadactylus, L. savagei))*), (*L. vastus, L. paraensis*))) belonging to the *L. pentadactylus* group, the optimization suggests an opposite decreasing trend. The optimization run on the epidermis suggests that a thick epidermis is a putative synapomorphy for the *L. fuscus* group, with independent acquisitions in species of different groups: *L. insularum*, *L. macrosternum* (*L. latrans* group), *L. griseigularis*, *L. natalensis*, *L. validus* (*L. melanonotus* group), and *L. rhodonotus* (*L. pentadactylus* group). The optimization also implies that a thin epidermis may be a synapomorphy in the clade (*((L. knudseni (L. fallax, L. labyrinthicus), (L. myersi (L. peritoaktites (L. pentadactylus, L. savagei))*), (*L. vastus, L. paraensis*))). The epidermis thickness, however, has been shown to vary according to the body region and the

TABLE 5. Results of Bonferroni posthoc tests after ANCOVA, considering total thick skin as co-variable, with the habits as factor (terrestrial vs. semiterrestrial vs. aquatic) of 21 species of *Leptodactylus* and *Adenomera marmorata*

	Terrestrial	Semiterrestrial
Terrestrial		
Semiterrestrial	0.000001 ^a	
Aquatic	0.075619	0.008318 ^a

Cells show habit groups comparison.

^aSignificant difference among groups.

TABLE 6. Results of the analyses of covariance tests for differences in different measurements of the skin layer, considering total thick skin as covariable, with the habitats (wet vs. dry) of 21 species of *Leptodactylus* and *Adenomera marmorata* as factor

Epidermis		<i>Compactum</i>		<i>Spongiosum</i>		E-K	
<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
0.29	0.59	0.0012	0.97	0.46	0.49	0.80	0.37

presence or absence of tubercles (Elias and Shapiro, 1957). We attempted to minimize the influence of extraneous factors by consistently sampling from the same skin region and taking five measurements from each sample.

The *strata compactum* and *spongiosum* presented a remarkable pattern in the clade *L. pentadactylus* + *L. savagei*, in which the base of this clade indicates a change to a thicker *stratum compactum* and a thinner *stratum*

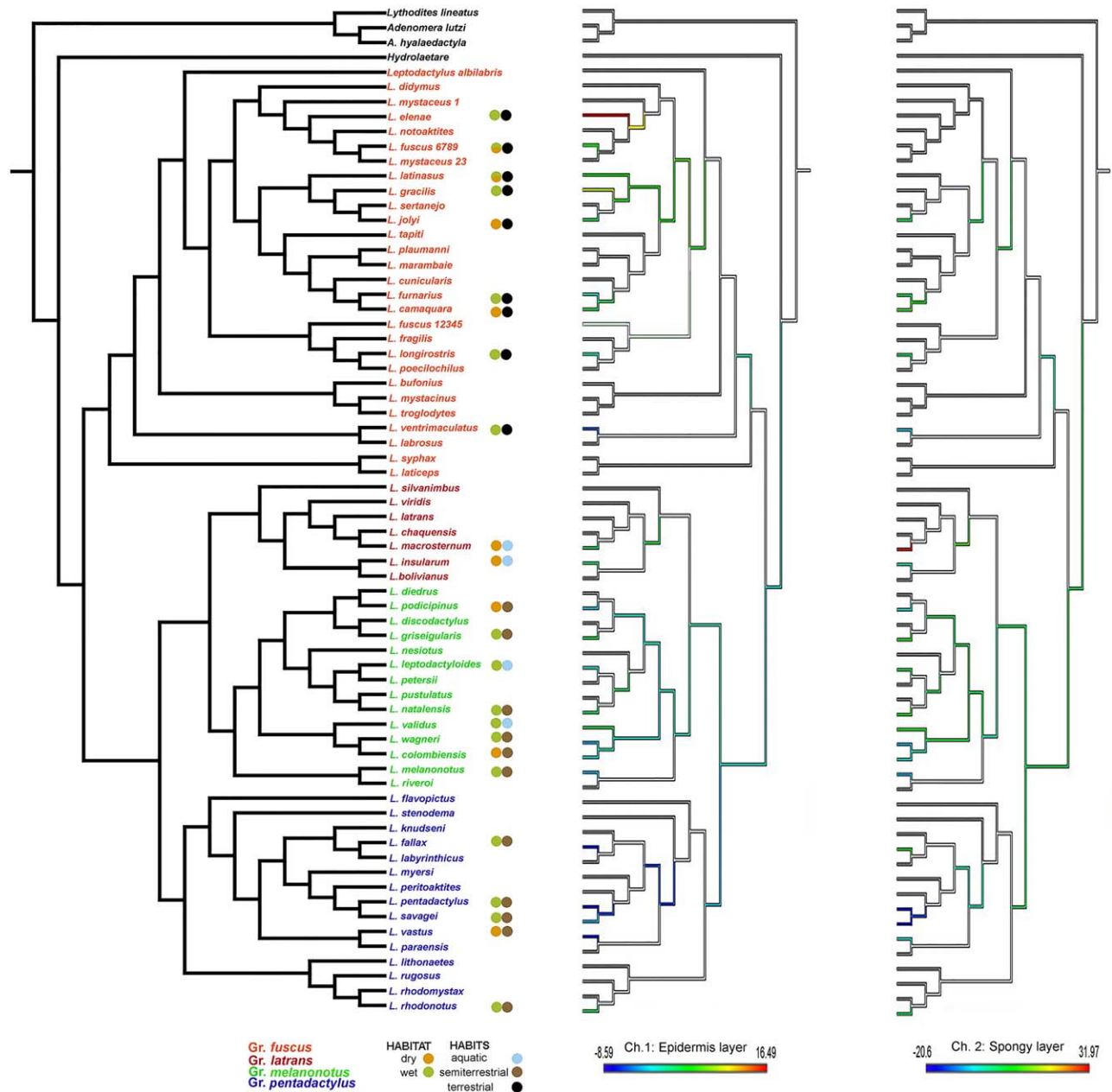


Fig. 4. *Leptodactylus* phylogeny by de Sá et al. (2014) with the relationships between the analyzed taxa illustrated. Colored circles indicate habitats and habits of the species included in the present study. The two trees on the right side show the changes of states in characters 1 (epidermis layer) and 2 (spongy layer).

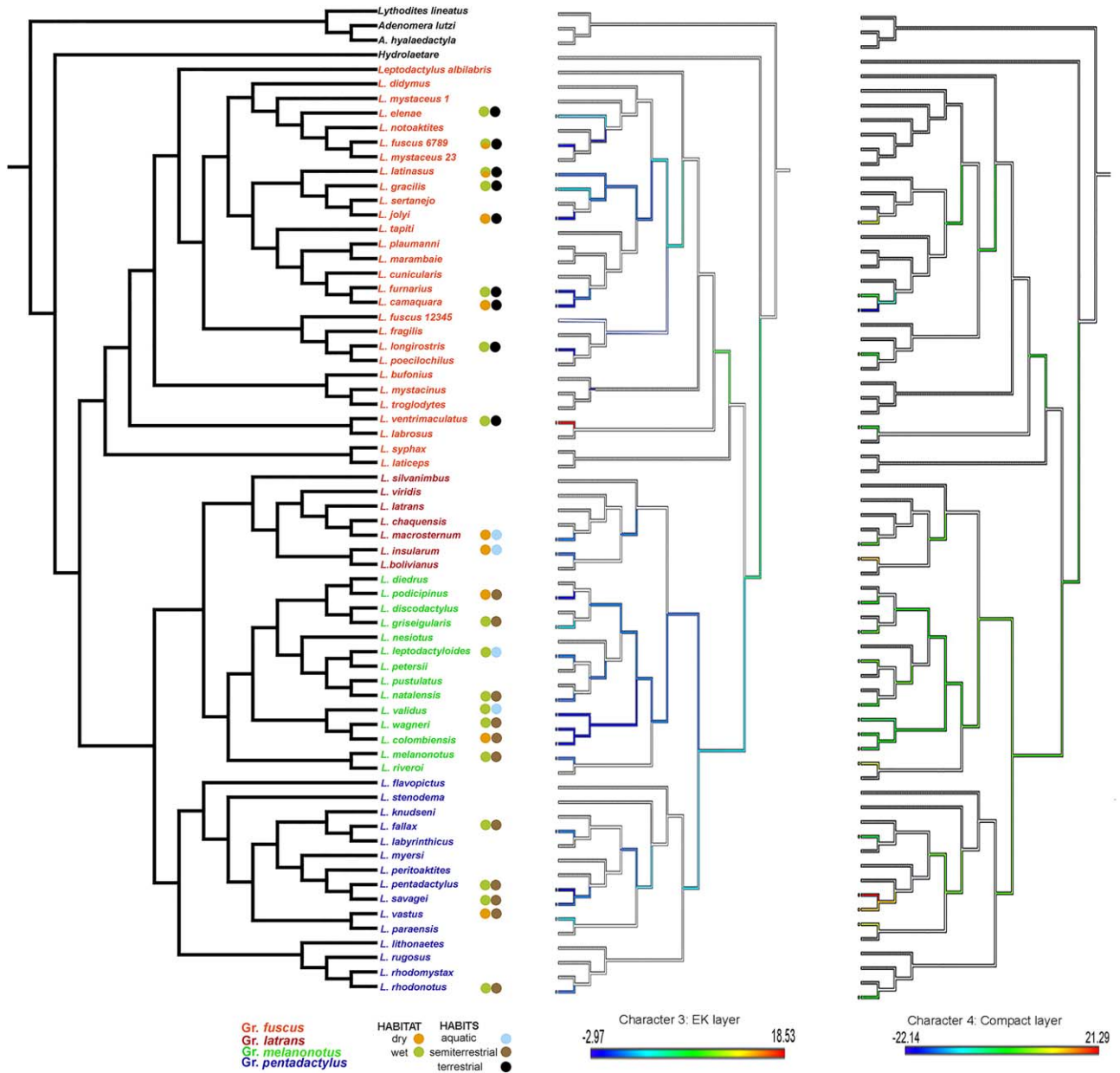


Fig. 5. *Leptodactylus* phylogeny by de Sá et al. (2014) with the relationships between the analyzed taxa illustrated. Colored circles indicate habitats and habits of the species included in the present study. The two trees on the right side show the changes of states in characters 3 (EK layer) and 4 (compact layer).

spongiosum. A thick *stratum compactum* and a thin *stratum spongiosum* could be confirmed as putative synapomorphies of this clade, but the character states of its relatives *L. myersi* and *L. peritoaktites* must be determined first.

The EK layer thicknesses measured in the current study varied between 0.77 μ (in *Adenomera marmorata*) and 19.75 μ m (in *Leptodactylus ventrimaculatus*). Elkan (1968) studied more than 100 anuran species (including *L. pentadactylus*) and recorded variations from 5 to 30 μ m. Within *Leptodactylus*, this character displays an interesting shift from intermediate to lower values in the base of the clade *L. fuscus* group, with a reversion to higher values in *L. ventrimaculatus*.

The EK layer has often been presented as a feature that could reduce or prevent water loss in anurans (Elkan, 1968, 1976; Toledo and Jared, 1993). Interestingly, in *Leptodactylus*, the EK layer shows a general decreasing tendency from the ancestor to the terminal taxa, regardless of the degree of terrestriality or habitat type. The lack of morphological and ecological association may be attributed to the unsuitability of the selected ecological categories. Habit types were defined according to a species' reproductive mode: foam nests deposited in water, in natural basins or in constructed incubation chambers (Heyer, 1969; Prado et al., 2002; Barreto Pereira et al., 2015). Other vital functions, such

as foraging, were not taken into account in this categorization. It is therefore important to determine exactly what factors are considered when categorizing a species as “aquatic” or “terrestrial.” Strictly aquatic anurans are defined as spending most of their lifetime in water and are, for example, skilled under water feeders (O’Reilly et al., 2002; Carreño and Nishikawa, 2010; Pethiyagoda et al., 2014; Barrionuevo, 2016). Many also exhibit morphological traits directly associated with aquatic existence, such as a lateral line, reduced tongue, and reduced lungs. While some aquatic *Leptodactylus* species have characters associated with aquatic life (i.e., toe webbing appropriate for swimming; Goldberg and Fabri, 2008; Ponssa, 2008; de Sá et al., 2014), their aquatic features are not extraordinary among anurans. Only a few anuran species can actually be considered aquatic *sensu stricto* (e.g., Pipidae, *Barbourula kalimantanensis*, *Lankanectes corrugatus*, and several species of *Telmatobius*) (Barrionuevo, in press).

Like the aquatic habit type definition, the terrestrial categorization within *Leptodactylus* is directly tied to the reproductive mode; specifically the deposition of nests in natural or constructed basins or mud chambers (Heyer, 1969). An important advantage of the terrestrial foam nest is the ability to mate and oviposit independent of standing water, allowing the eggs and first larval stages to develop safely within subterranean chambers (Heyer, 1969). During rains, the tadpoles then float to bodies of water and, having already developed functional mouthparts and enclosed gills, enjoy a temporal advantage over the other species that occupy the water body (Heyer, 1969). Features related to this mode of reproduction are a rigid chisel snout (Heyer, 1978) and the mineralization of the nasal region of the skull (Ponssa, 2008; Ponssa and Heyer, 2007; Ponssa and Medina, 2016). The skin of terrestrial *Leptodactylus* does not exhibit any extraordinary features in the EK layer, and the presence of the EK layer is shared by many anuran families, including discoglossids, pelobatids, ranids, bufonids, dendrobatids, microhylids, hylids, leptodactylids, and telmatobids (Taylor et al., 1966; Elkan, 1968; García and Cardozo, 2005; Vickaryous and Sire, 2009; García et al., 2011; Ferraro et al., 2013; Barrionuevo, in press). In *Telmatobius*, both ecological habits and habitat type are related to the development of the EK layer, which has been detected in 18 of 25 evaluated species (the genus contains 63 total species) (Barrionuevo, in press). The layer was found to be absent or thinned in lacustrine or strictly stream dwelling species, whereas it is more developed in semi-aquatic species living in arid habitats (Barrionuevo, in press). In other species related to *Telmatobius*, such as the *Atelognathus patagonicus* (lake dwelling species), the EK layer is also absent, but it is present in *A. reverberi*, a semi-aquatic species from the Patagonian steppe.

The absence of a correlation between habits and EK layer thickness also could be explained as the result of phylogenetic inertia. Elkan (1968) argued that the amount of ground substance or EK layer in anurans is more closely linked to ecology than to phylogenetic constraints. He concluded that the skin of *L. pentadactylus* was one of the most abundantly supplied with ground substance, together with *Rhinella marina* (as *Bufo marinus*) and *Conraua goliath* (as *Rana goliath*). However, in light of new data (García and Cardozo, 2005; García

et al., 2011; current study), the EK layer thickness values for *L. pentadactylus* are shown, in fact, to be comparatively low. An exhaustive analysis considering higher taxonomic levels may reveal that the putative intermediate values at the base of the *Leptodactylus* clade are actually relatively high or low, defining a pattern that supports the hypothesis of a correlation between ecology and the EK layer.

The EK layer was found to be present in *Leptodactylus* species inhabiting both wet and dry environments. Previous results had shown that the layer was absent in terrestrial anuran species (*Batrachyla*, *Eupsophus*, *Hylorina*, *Insuetophrynus*, *Rhinella margaritifera*) that live in hyper-humid environments (e.g., Valdivian Forest, Amazon basin) (Toledo and Jared, 1993; Barrionuevo, in press). A thickening of the EK layer, however, was actually observed in *L. ventrimaculatus*, which inhabits the north-western Andean cloud forests, one of the wettest regions on earth. The habitat classifications in this study were assigned based on the biogeographic region where the specimen was collected; however, it is possible that local microclimatic variations do not correspond with the general regional characterizations and could substantially affect the biological and morphological features of resident *Leptodactylus* species. For example, populations of *Leptodactylus fuscus*, typically found in the Yungas humid cloud forests of Parque Nacional Calilegua, are frequently located in the less humid microhabitats of the sugar cane plantations. Shelter microhabitats are already known to influence body temperature and govern dehydration rates in amphibians (Seebacher and Alford, 2002). Additional studies could reveal further subtle adaptations to microclimatic conditions.

Intraspecific EK layer variation has been previously described in relation to several factors: (1) Seasonality, e.g. *Rhinella arenarum* (Porto, 1936). Certain species of *Leptodactylus* inhabit regions characterized by marked seasonality. During the dry season, the exposure to intensified solar radiation, including UV and infrared rays, presents an ecological challenge to these amphibians as they must develop mechanisms to a) avoid dangerous overheating and b) minimize UV absorption (Kobelt and Linsenmair, 1986). (2) Location, e.g. *Leptodactylus fuscus* (García et al., 2011), *Telmatobius oxycephalus* (Barrionuevo, in press). (3) Physiological state, e.g. *Rana temporaria*, *Pseudis paradoxa* (Elkan, 1968). Calcium content in the EK layer may be related to physiological processes like bone formation, blood coagulation, capillary integrity, enzyme activity and the function of the nervous system, all of which depend on the participation of calcium ions (Brown, 1936). (4) Ontogeny, e.g. *Rana temporaria*, *Pseudis paradoxa* (Elkan, 1968), *Leptodactylus mystacinus* (García and Cardozo, 2005); the EK layer is present in adult specimens and thicker in older frogs (Elkan, 1968). While these four factors were not considered in the current study, further analyses could present a correlation between morphological and ecological features.

Other sources of possible variation are: (1) the fixation protocol, which affects EK layer preservation in paraffinized tissues. Integument fixed in Bouin’s liquid usually did not show the EK layer, although the same material from the same specimen fixed in the formaldehyde solution did preserve the EK layer (Alves Azevedo et al.,

2005); (2) body region. The dorsal and ventral skin demonstrated differences in water permeability (Withers et al., 1984) apparently as a consequence of a more abundant EK layer on the dorsal surface (Alves Azevedo et al., 2005). While both variables are important to bear in mind, they are not relevant to our study, as all the samples were formalin-fixed, and all were taken from the dorsal integument.

The data in the present study did not reveal a decisive pattern of morphometric association between skin layer thickness and habits and/or habitats in species of the genus *Leptodactylus*. The variability appears to be more directly driven by phylogenetic constraints, although a more in-depth analysis, taking into account an increased number of factors, such as dorsal and ventral skin variations, seasonality of the samples, etc., may reveal further morphological–ecological correlations.

ACKNOWLEDGEMENT

The authors thank M. Alcaide for his assistance with the interpretation of the histological data, and Jennifer Richardson and James Menzies for assistance with the English language edition.

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APPENDIX I

STUDIED SPECIMENS WITH COLLECTION NUMBERS AND LOCALITIES DATA. MNRJ: MUSEU NACIONAL RIO JANEIRO; MZUSP: MUSEU DO ZOOLOGIA DA UNIVERSIDADE DE SAO PAULO; USNM: UNITED STATES NATURAL MUSEUM; ZUFMS: COLEÇÃO DA UNIVERSIDADE FEDERAL DO MATO GROSSO DO SUL

Species	Specimen	Group	Locality	Ecoregion
<i>A. marmorata</i>	MNRJ 28287	MARMORATUS	Reciclagem, Parque Estadual Intervales, Ribeirão Grande, SP, Brazil.	Serra do Mar coastal forests
<i>L. camaquara</i>	MZUSP56842	FUSCUS	Serra do Cipó, MG, 2 km 127, MG, 1380–1450 m. Brazil.	Campos Rupestres/Cerrado
<i>L. colombiensis</i>	USNM 148802	MELANONOTUS	Valle de Cauca; Cali, 2 km S of Colombia.	Cauca Valley Dry Forests
<i>L. colombiensis</i>	USNM 148801	MELANONOTUS	Valle de Cauca; Cali, 2 km S of Colombia.	Cauca Valley Dry Forests
<i>L. fallax</i>	USNM162244	PENTADACTYLUS	West Indies. Dominica.	Southern Caribbean
<i>L. furnarius</i>	MZUSP83271	FUSCUS	Campo Grande da Serra, SP, Brazil	Guianan savanna/Guianan Highland moist forest
<i>L. griseigularis</i>	MZUSP 196023	MELANONOTUS	HUANUCO; Tingo Maria, Rio Huallaga, vicinity of Universidad Agraria de la Selva, 670 m. Peru.	Ucayali moist forests
<i>L. griseigularis</i>	USNM 196024	MELANONOTUS	HUANUCO; Tingo Maria, Rio Huallaga, vicinity of Universidad Agraria de la Selva, 670 m, 9° 18' S, 75° 59' W. Peru.	Ucayali moist forests
<i>L. insularum</i>	USNM 150743	OCELLATUS	Bolívar. Sincelajo to Tolu. Colombia	Guajira-Barranquilla Xeric Scrub
<i>L. jolyi</i>	MZUSP47621	FUSCUS	Chapada dos Veadeiros. Brazil.	Cerrado
<i>L. leptodactyloides</i>	USNM 247382	MELANONOTUS	MADRE DE DIOS; Puerto Maldonado, ca. 30 km (straight line) SSW of Tambopata Reserve, Explorer's Inn, 280m, 12 50S, 69 17w. Peru	Southwest amazon moist forest
<i>L. longirostris</i>	MZUSP65793	FUSCUS	Marco de Fronteira BV-8, Roraima. Brazil.	Purus Varzea (moist flooded forest)
<i>L. macrosternum</i>	MNRJ32259	OCELLATUS	Cuiabá, MT, Brazil	Cerrado
<i>L. melanonotus</i>	USNM 283471	MELANONOTUS	Pichincha; Santo Domingo de los Colorados, km 25 sobre la ruta a Chone, Rancho Santa Teresita. Ecuador.	Western Ecuador Moist Forests
<i>L. natalensis</i>	MNRJ34988	MELANONOTUS	Reserva Biológica de Sooretama, Sooretama/Linhares, ES -Brazil.	Serra do Mar coastal forests
<i>L. natalensis</i>	MNRJ27929	MELANONOTUS	Reserva Biológica de Duas Bocas (próximo à represa), Cariacica - ES, Brazil.	Serra do Mar coastal forests
<i>L. pentadactylus</i>	MZUSP56779	PENTADACTYLUS	Serrinha, Rio Jarupá. Amazonas. Brazil.	Purus Varzea
<i>L. podicipinus</i>	ZUFMS 0478–2	MELANONOTUS	Base de Estudos do Pantanal-BEP-, Corumbá. Mato Grosso do Sul, Brazil.	Chiquitano dry Forest/Pantanal
<i>L. podicipinus</i>	ZUFMS 0467–1	MELANONOTUS	Base de Estudos do Pantanal-BEP-, Corumbá. Brazil. Mato Grosso do Sul.	Pantanal
<i>L. rhodonotus</i>	USNM 196009	PENTADACTYLUS	HUANUCO; Leoncio Prado; Tingo Maria, Rio Huallaga, 670 m, 9 18 S, 75 59 W. Peru.	Ucayali Moist Forests
<i>L. rhodonotus</i>	USNM 19603	PENTADACTYLUS	HUANUCO; Tingo Maria, Rio Huallaga, vicinity of Universidad Agraria de la Selva, 670 m, 9° 18' S, 75° 59' W. Peru.	Ucayali Moist Forests
<i>L. savagei</i>	USNM347152	PENTADACTYLUS	S end of Isla Popa, 1 km E of Sumaco channel. Panama.	Mesoamerica Gulf Caribbean mangrooves
<i>L. spixi</i>	MZUSP63671	FUSCUS	Sao José (Fazenda Unacau), Bahia. Brazil.	Bahia coastal forest
<i>L. validus</i>	USNM 192762	MELANONOTUS		Trinidad And Tobago Moist Forests

APPENDIX I (Continued).

Species	Specimen	Group	Locality	Ecoregion
<i>L. validus</i>	USNM 167496	MELANONOTUS	St. John; Bloody Bay - Charlotteville Road, vicinity of Milepost 26, near Anse Fourmi (collectado de un pequeño charco). Tobago	Trinidad And Tobago Moist Forests
<i>L. vastus</i>	MZUSP 50187	PENTADACTYLUS	St. John; Roxborough - Parlatuvier Road, vicinity of Bloody Bay, at Bloody Bay River. Tobago.	Cerrado
<i>L. ventrimaculatus</i>	USNM196765	FUSCUS	PIAUI. Valença. Brazil.	Norwestern Andean montane forests
<i>L. wagneri</i>	USNM 283887	MELANONOTUS	Pichincha; Mindo, ca 18 km N of, on road to Puerto quito (11.5 km past Mindo turnoff) 1500m. Ecuador.	Eastern Cordillera Real Montane Forests
<i>L. wagneri</i>	MNRJ 36193	MELANONOTUS	MORONA-SANTIAGO; Sucua. Ecuador.	Japurá-Solimoes-Negro Moist Forests
<i>A. marmorata</i>	MNRJ 28287	MARMORATUS	Barcelos, AM (00°58'9,5"S; 62°55'33,8"W) Reciclagem, Parque Estadual Intervales, Ribeirão Grande, SP, Brazil.	Serra do Mar coastal forests