Molecular Phylogeny of the Lizard Clade Leiosaurae Endemic to Southern South America

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Abstract: The clade Leiosaurae currently includes 18 species in the genera *Diplolaemus, Leiosaurus,* and *Pristidactylus*. It is mainly distributed in Argentina from 30° latitude south in the northwestern region of the country, to 52° south in Patagonia, from 63° longitude in coastal areas to 73° along the Andean Cordillera, across multiple habitats and including a small area in Chile. Several morphological and molecular taxonomic studies on a subset of these species have been published, but no comprehensive phylogenetic hypothesis is available for the clade. The objective of this work is to present a molecular phylogenetic hypothesis for the majority of the described species in the clade. We sequenced two mitochondrial genes, five nuclear protein coding, and three anonymous nuclear loci, and implemented traditional concatenated analyses as well as a species-tree approach. All methods inferred very similar topologies. We found the genera *Diplolaemus* and *Leiosaurus* to be monophyletic, whereas *P. torquatus* was retrieved as a separate lineage from the other *Pristidactylus* species with strong statistical support. Within *Diplolaemus*, *D. darwinii* is a very distinct lineage with an estimated divergence time of ~14.74 million yr ago (mya). Based on an early Miocene Leiosaurae fossil mandible, we estimated the crown common ancestor of the genus *Leiosaurus* at ~9.24 mya, and *L. bellii* is the earliest divergent lineage within this genus. The Argentinean *Pristidactylus* species seem to have radiated relatively recently (4.02 mya). A combination of geological and climatic events during Middle and Late Miocene, and climatic changes associated with glaciations, most probably played a role in the divergence of the Leiosaurae clade. The diversification patterns of *Diplolaemus* and *Leiosaurus* seem to have followed a general south-to-north direction, while the Argentinean *Pristidactylus* may have diversified east-to-west and north-to-south. We suggest that morphological and thermo-physiological studies combined with palaeo-niche modeling analyses are needed to test these hypotheses and better understand the biogeographical history of this clade.

Key words: Concatenated tree; *Diplolaemus*; Leiosauridae; *Leiosaurus*; *Pristidactylus*; Species tree

Knowledge of many southern South American lizard taxa is still limited, and probably one of the most notorious examples is a group commonly known as "matuastos" in Argentina or "gruñidores" in Chile. Both common names include lizards of the genera *Diplolaemus, Leiosaurus,* and *Pristidactylus.* Prompted by E. E. Williams, Etheridge sketched out the relationships of all of the iguanid genera and hypothesized that these lizards formed a clade in what is now known as his "Old Tree" (subsequently published in a modified form in Paull et al. [1976] and Peterson [1984]). This clade was later corroborated by Etheridge and de Queiroz (1988), who applied the informal name "leiosaurs" to a slightly more inclusive group that incorporated some of the para-anoles. Frost et al. (2001) formally named the clade Leiosaurinae based in part on their decision to rank a more inclusive clade, the family Leiosauridae, containing also *Enyalius, Anisolepis,* and *Urostrophus,* which included more arboreal species from humid environments. Schulte et al. (2003:415) retained the traditional ranking scheme that recognized a single large family Iguanidae, and used the name Leiosaurae rather than Leiosaurinae "to minimize confusion related to the connotations concerning taxonomic rank and the position of these groups in their phylogenetic hypothesis." Later, Abdala et al. (2009) inferred Leiosaurae as monophyletic based on myological characters. Most recently, several papers addressing higher level relationships of Squamata based on extensive molecular data have been published (e.g., Wiens et al. 2012; Pyron et al. 2013; Reeder et al. 2015)—all of them support the monophyly of the clade Leiosaurae, and infer Enyaliinae as its sister group. In

particular, Pyron et al. (2013) included representatives of the three Leiosaurae genera and the five species included in Enyaliinae (*Anisolepis longicauda, Enyalius bilineatus*, *E. leechii, Urostrophus gallardoi,* and *U. vautieri*), and inferred these groups as sister to each other with high statistical support. Thus, there is firm evidence for the monophyly of Leiosaurae with Enyaliinae as its sister clade.

Lizards of the Leiosaurae clade are very distinctive in morphology and usually characterized by stout bodies and legs, robust heads, and short tails that usually do not autotomize (Cei 1986). With a few exceptions, the majority of the species occur in xeric habitats of southern South America (Fig. 1). Some species are considered as threatened because their distributions are restricted to small geographic areas in Argentina and Chile (Abdala et al. 2012).

Leiosaurus includes four species, three of which are distributed in northwestern Argentina (*L. catamarcensis, L. paronae, L. jaguaris*), whereas *L. bellii* is limited to the Monte or northern Patagonian steppes, or ecotonal areas between these biogeographic formations. There is little information about either the geographic distribution or species boundaries within *Leiosaurus*. The most recent revisionary study is from Gallardo (1961), with some information added by Cei (1973) on the geographic distribution of some species.

Diplolaemus is known from Bell's (1843) reports on collections made by Charles Darwin during the voyage of HMS Beagle, and includes four species: *D. bibronii* in coastal regions and arid plateaus of southern Patagonia, *D. darwinii* in the southernmost areas of Argentina and a small portion of Chile, *D. sexcinctus* in northern-central Patagonian Argentina, and *D. leopardinus* in the Andean slopes of

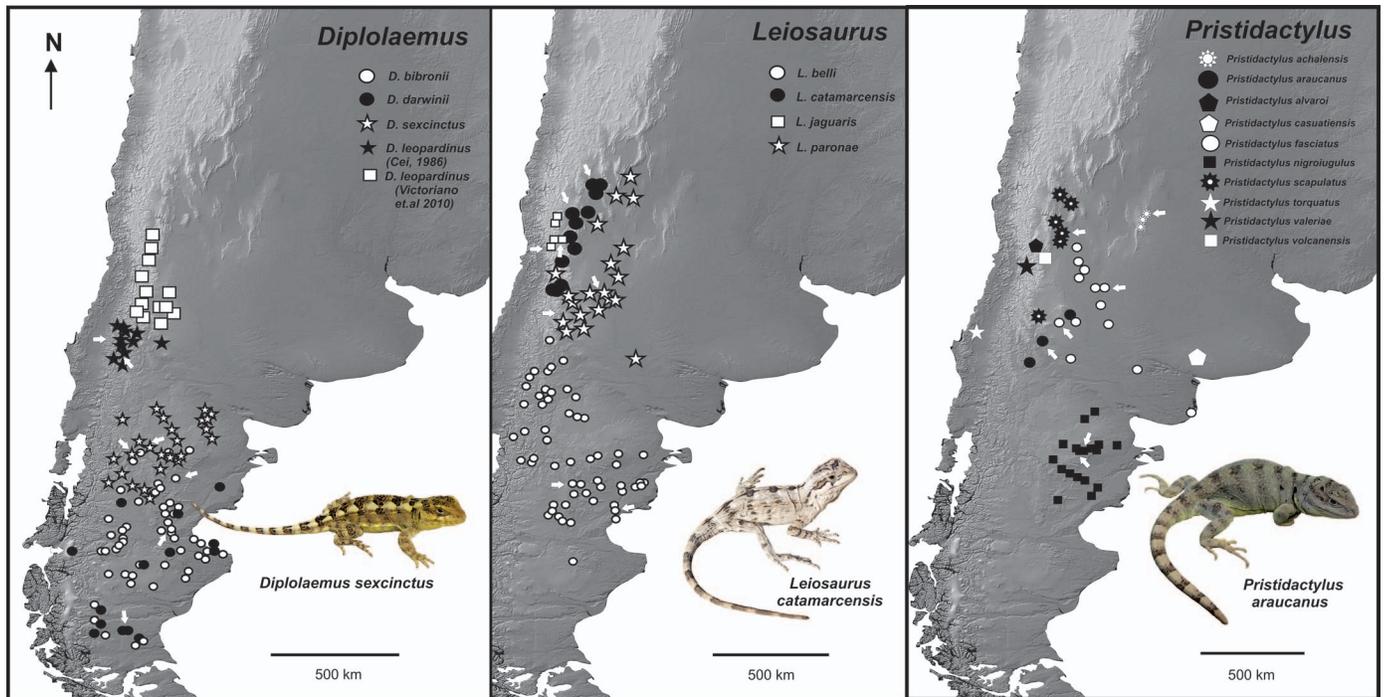[4] Correspondence: e-mail, morando@conicet-cenpat.gob.ar

Fig. 1.—Distribution map of all the Leiosaurae species in Argentina and Chile. Arrows = sampled localities.

northwestern Argentina and a small portion of Chile. Cei et al. (2003) published the last revision of the genus, and more recently Victoriano et al. (2010) addressed some nomenclatural problems related to northern populations.

Pristidactylus is the most diverse genus with 10 species: *Pristidactylus achalensis, P. alvaroi, P. araucanus, P. casuhatiensis, P. fasciatus, P. nigroiugulus, P. scapulatus, P. torquatus, P. valeriae,* and *P. volcanensis*. Of these, four are distributed in Chile, and six in Argentina (Etheridge and Williams 1991; Cei et al. 2001; Avila et al. 2003). Some species are endemic to single Pampean mountain ranges, including *P. casuhatiensis* in the Sierra de la Ventana, and *P. achalensis* in the Sierras de Córdoba. Other species (e.g., *P. nigroiugulus*) are found in Patagonian Steppe habitats, or the flatland dry habitats of the biogeographic region known as Monte (e.g., *P. fasciatus*). Etheridge and Williams (1991) and Cei et al. (2004) summarized the taxonomic history of this genus.

The understanding of life-history traits of the Leiosaurae species is limited, but these lizards tend to be diurnal, terrestrial, oviparous, and insectivorous. Of equal importance, taxonomic issues remain unresolved within each genus. Some phylogenetic hypotheses have been proposed based on morphological characters and mitochondrial DNA sequences (Fig. 2), but most of these studies were based on limited taxonomic and/or character sampling. The objective of our study is to present a multilocus molecular phylogeny of the Leiosaurae clade, including all described species of *Diplolaemus* and *Leiosaurus,* and 6 of the 10 species of *Pristidactylus*.

MATERIALS AND METHODS

Taxon Sampling

We included 28 terminals representing most of the described species in the phylogenetic analyses; the total sample size was 47 individuals, because for some species for some genes, we had to amplify different individuals from the same population or from a nearby locality. Missing taxa include three species of Chilean *Pristidactylus—valeriae, volcanensis,* and *alvaroi*—and one from Argentina—*casuhatiensis*. We used a 12S sequence from GeneBank (Accession number AF338325.1) for *Urostrophus gallardoi*. There is firm evidence both for the monophyly of Leiosaurae, and that *U. vautieri* from Brazil (the outgroup used here) is part of the sister clade Enyaliinae (Wiens et al. 2012; Pyron et al. 2013; Reeder et al. 2015).

Most of the ingroup taxa were sampled from Argentina, with a few individuals from Chile. Most species were represented by at least two individuals collected at the same or nearby localities; the exceptions were *Pristidactylus torquatus* and *P. achalensis,* for which we included one individual. The majority of the samples were obtained from the LJAMM–CNP tissue collection of the Centro Nacional Patagónico, Puerto Madryn, Argentina (CENPAT–CONICET, http://www.cenpat-conicet.gob.ar/coleccion-herpetologica-reptiles/), two were obtained from Chile from the Bean Life Science Museum's Herpetological collection, four were obtained from the San Juan University Herpetological Collection, and two were obtained from the Laboratory of Vertebrate Cytogenetics (IB–USP), Sao Paulo, Brazil (Table 1).

Gene Sampling

We collected sequences from two mitochondrial gene fragments: (1) cytochrome *b,* using the light-strand primers GluDGL and the heavy-strand primer Cyt-*b* 3 and Cyt-*b* 2 (Palumbi 1996), and the Cyt.F.1 primers (Whiting et al. 2003) were used as internal sequencing primers; and (2) 12S, using the primers of Wiens et al. (1999). Mitochondrial polymerase chain reaction (PCR) conditions followed Morando et al. (2003). We also sequenced eight nuclear genes, five of which are protein-coding loci (NPCL): (1) dmX-like protein 1 (DMXL1; Werneck et al. 2012), (2)
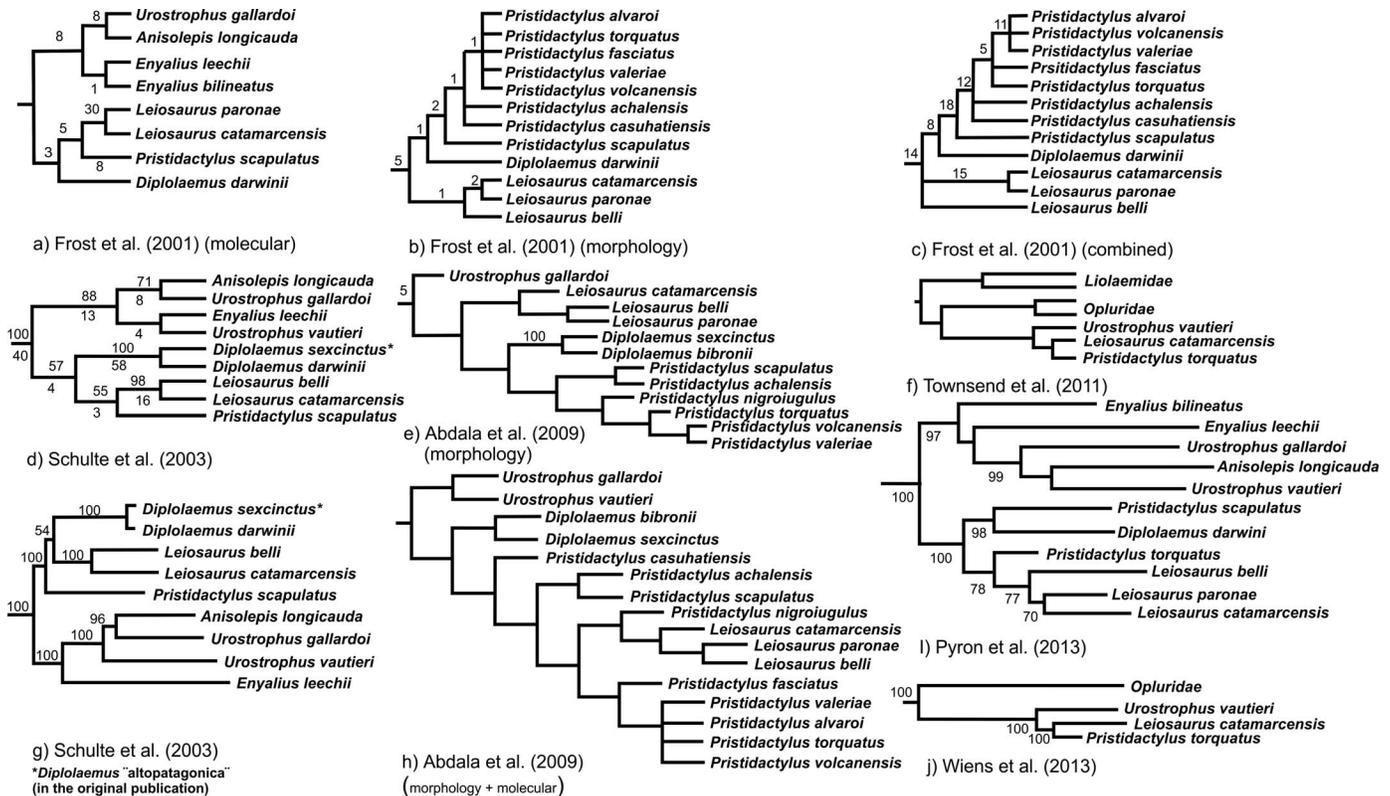
FIG. 2.—Previous phylogenetic hypotheses for the clade Leiosaurae (sensu Schulte et al. 2003; = Leiosaurinae, sensu Pyron et al. 2013). Each tree appears immediately above its cited source; the letters that follow correspond to the portions of the figure referenced in the text. (A) Frost et al. (2001; partially reproduced from Fig. 1, molecular data, 1040 bp mtDNA). (B) Frost et al. (2001; partially reproduced from Fig. 2, morphological data, 82 characters). (C) Frost et al. (2001; partially reproduced from Fig. 4, combined molecular and morphological data; numbers on branches are Bremer values). (D) Schulte et al. (2003; partially reproduced from Fig. 2, molecular data, 1838 bp mtDNA. Maximum Parsimony tree; numbers on top of branches are bootstrap values, below branches are decay indices). (E) Abdala et al. (2009; partially reproduced from Fig. 2, morphology only, 162 myological characters + 82 morphological characters from Frost et al. 2001). (F) Townsend et al. (2011; partially reproduced from Fig. 4, 29 nuclear genes). (G) Schulte et al. (2003; partially reproduced from Fig. 4, molecular data, 1838 bp mtDNA. Maximum Likelihood tree. Numbers on top of branches are posterior probability values). (H) Abdala et al. (2009; partially reproduced from Fig. 4, morphological data + molecular data from Frost et al. 2001). (I) Pyron et al. (2013; partially reproduced from Fig. 18, meta-analysis of molecular data available in GenBank). (J) Wiens et al. (2012; partially reproduced from Fig. 1, molecular data based on 44 nuclear genes. Numbers on branches are likelihood bootstrap values).

Moloney sarcoma oncogene (C-mos; Wiens et al. 1999), (3) Exophilin 5 (EXPH5; Townsend et al. 2008), (4) natural killer-tumor recognition sequence (NKTR; Townsend et al. 2011), and (5) intron 8 and flanking exon regions of RNA binding motif protein (RBMX; Gamble et al. 2011). The remaining three nuclear markers are from a nonpublished set of Anonymous Nuclear Loci (ANL; primers for loci ANL-D77, ANL-D90, and ANL-D92) developed by one of us (MM) from a genomic library (see Table 2 for details of each marker). To develop the ANLs, we assembled a genomic library from one individual of *Diplolaemus* following the general protocol of Noonan and Yoder (2009) with small modifications (detailed in Morando et al. 2014). We verified a subset of amplified fragments via Blast search on GenBank with megablast (highly similar sequences), and with more dissimilar sequences (discontiguous megablast) criteria. Sequences returned from a "nonsignificant similarity was found" output were considered anonymous markers. Primers were developed for 31 loci that met ANL criteria in these searches, and these were tested on a subset of two species per each of the three Leiosaurae genera. Of the 31 loci tested, we chose three for this project (ANL-D77_F: TCTGTCCCCTTGATCCTTTG; R: GGAAGCTGCACC-CATTTCTA; ANL-D90_F: GCTTCTCCATATCCCACCAG;

R: CATCCACAAACTCCAGGTCA; ANL-D92_F: TGATAG-CCTTTGGCTAAGCAC; R: CGCAGCATGTGGTTTTAA-TC) that met the following requirements: (1) they amplified without significant optimization; (2) they were variable between the two test species; and (3) they amplified/sequenced for the remaining samples.

Laboratory Procedures

Genomic DNA was extracted using the Qiagen® DNeasy® 96 Tissue Kit (Qiagen, Valencia, CA) for animal tissues following the protocol provided by the manufacturer. Protocols for PCR and sequencing procedures follow Morando et al. (2003, 2004) for 12S and cyt-*b*. The seven NPCL were amplified with one of the following standard touchdown cycles: 94°C for 2:45 min, 40× (94°C for 15 s, 51°C or 57°C for 20 s [−0.1°C/cycle], 72°C for 1 min), 72°C for 1 min, final rest at 4°C (called 1-touch51 or 1-touch57); or 95°C for 1:30 min, 10× (95°C for 35 s, 63°C for 35 s [−0.5°C/cycle], 72°C for 1 min), 10× (95°C for 35 s, 58°C for 35 s, 72°C for 1 min), 15× (95°C for 35 s, 52°C for 35 s, 72°C for 1 min; final rest at 10°C; called ANL63). The touchdown cycle described by Noonan and Yoder (2009), with standard reaction conditions (per sample: 2 μL dNTPs [1.25 mM], 2 μL 5× Taq buffer, 1 μL each primer [10 μM],

TABLE 1.—Information of all described Leiosaurae species: authority and year of description, type locality, sampled localities for this study, and voucher information.

Species	Study	Type locality	Sampled locality	Voucher numbers
Diplolaemus bibronii	Bell 1843	Argentina, Santa Cruz, Puerto Deseado	R.A. Chubut. Paso de Indios. Prov. Road 27, 78.1 km S El Sombrero and Prov. Road 53/Santa Cruz. Deseado. Prov. Road 43, 16 km E Las Heras	LJAMM–CNP 3895/ 13208
Diplolaemus darwinii	Bell 1843	Argentina, Santa Cruz, Puerto Deseado	R.A. Santa Cruz. Magallanes. Prov. Road 77, 66.6 km NW junction Prov. Road 25, 9.1 km SE Estancia Vega Grande/Chile. Región XI. Aysen	LJAMM-CNP 10025/ BYU48512
Diplolaemus leopardinus	Werner 1898	Chilean region of Lonquimay, Mari-Menu and Pino Hachado, located on western Andean slopes (Donoso-Barros 1966)/Santiago, Chile (in error fide Donoso-Barros 1965)/ Argentina (Patagonia, Neuquén)	Chile. VIII Región. Bío. Paso Pichachen, Road Antuco–Los Barros–Moncol, 10 km E de Los Barros/R.A. Neuquén. Picunches. Primeros Pinos./ Neuquén. Aluminé. Volcán Batea Mahuida./ Neuquén. Pehuenches. Auca Mahuida	LJAMM-CNP 14190–14191/ 14210/14202/ 14229
Diplolaemus sexcinctus	Cei et al. 2003	Argentina, Río Negro, Meseta Pedregosa 1100msnm, 10 km S Las Bayas, Río Negro (41°30′S, 70°38′W)	R.A. Río Negro. 25 de Mayo. Prov. Road 76, 23.5 km S junction National Road 23, S of Ingeniero Jacobacci/Río Negro. Ñorquinco. Prov. Road 6, 5 km NW Ojo de Agua/Río Negro. Valcheta. Meseta de Somuncura, Cañadon del Naciente/Río Negro. Ñorquinco. Prov. Road 6, 2.2 NE Ojo de Agua./Río Negro. 25 de Mayo. Prov. Road 72, 3 km W Puesto de Hornos	LJAMM-CNP 3550– 3551/3666/6089/ 3628/6255
Leiosaurus bellii	Duméril and Bibron 1837	Terra typica: Mexico (in error), Argentina, Santa Cruz, Chubut, Río Negro, Mendoza, Neuquén	R.A. Chubut. Rawson. Bahía Isla Escondida/Chubut. Paso de Indios. Prov. Road 40, 15 km NE junction Prov. Road 12/Chubut. Telsen. Prov. Road 61, 40.3 km junction Prov. Road 11, between Estancias Ranquilhuao and San Manuel	LJAMM-CNP 2451/ 8835/6151
Leiosaurus catamarcensis	Koslowsky 1898	Argentina, Provincia de Catamarca	R.A. Catamarca. Sta María. Prov. Road 40, 37 km W Punta de Balasto/La Rioja. Famatina. Road between Chañarmuyo and Campanas, 3 km N Chañarmuyo	LJAMM-CNP 4329/ 4742
Leiosaurus jaguaris	Laspiur et al. 2007	Argentina, San Juan, Jachal, Gualcamayo (29 49′ S, 68 45′ W)	R.A. San Juan. Calingasta. Prov. Road 412, junction with Prov. Road 425, 26 km N Villa Nueva/La Rioja. Felipe Varela. Parque Nacional Talampaya, Prov. Road 26, Km 139. 1303 m/San Juan. Ullum. Prov. Road 436, 6 km E La Ciénaga/La Rioja. RN 76, 13 km E Alto Jagüe, General Lamadrid	LJAMM-CNP 12521/ 1973/2383/ UNSJ31
Leiosaurus paronae	Peracca 1897	"Brasile," S Brazil, N Argentina (Mendoza), Paraguay	R.A. Mendoza. San Rafael. Prov. Road 153, 38.3 km S Aristides Villanueva, 7 km N Monte Coman, 1 km N junction Nat. Road 146/San Luis. Capital	LJAMM-CNP 12852/ 4537
Pristidactylus achalensis	Gallardo 1964	Argentina, Córdoba, Posta de Pampa de Achala	R.A. Córdoba. Posta de Pampa de Achala	LJAMM-CNP407FN
Pristidactylus alvaroi	Donoso-Barros 1975	Chile, Santiago, Cerro El Roble	—	
Pristidactylus araucanus	Gallardo 1964	Argentina, Neuquén, Laguna Blanca	R.A. Neuquén. Chos Malal. Prov. Road 37, 24.6 km N junction Nat. Road 40, Paraje Los Ranchos	LJAMM-CNP 10327–10328
Pristidactylus casuhatiensis	Gallardo 1968	Argentina, Bs. Sierra de la Ventana	—	
Pristidactylus fasciatus	D'orbigny and Bibron 1837	Argentina, Río Negro, Río Negro	R.A. La Pampa. Chical Co. Prov. Road 10, 9 km E Agua Escondida/Mendoza. Malargue. Reserva Provincial La Payunia, Pampas Negras	LJAMM-CNP 4123/ 7911
Pristidactylus nigroiugulus	Cei et al. 2001	Argentina, Chubut, Telsen. Foothills of the Meseta de Sierra Negra, 880 m	R.A. Chubut. Telsen. 10 km N Gan Gan/Gastre. Prov. Road 58, 3.2 N El Escorial/Telsen. Prov. Road 67, 16 Km N Gan Gan/Gastre. Aguada Oveja Muerta, Prov. Road 4, 40 km W Gan Gan/Río Negro. Valcheta. Road to Meseta de Somuncura, 14.5 km NW Puesto Luis Ovejero/Chubut. Telsen. Prov. Road 4, 2 km E Gan Gan/Río Negro. 25 de Mayo. Nat. Road 23, 14 km W Aguada de Guerra.	LJAMM-CNP 3841/ 12179/6904/6045/ 3308/5508/CNP– FN153
Pristidactylus scapulatus	Burmeister 1861	Argentina, Mendoza Sierra de Uspallata	R.A. Mendoza. Las Heras. Prov. Road 319, 8 km SW El Portezuelo. 2500 msnm/SJ. Iglesia. Prov. Road 436, Alto del Colorado/S.J. Sierra de la Invernada, Don Carmelo, Ullúm/Mendoza. Prov. Road 153, 20 km W Santa Clara, Las Heras	LJAMM-CNP 2736/ 2384/UNSJ138/ UNSJ109
Pristidactylus torquatus	Philippi and Landeck 1861	Chile, Near Concepción, Between 34th and 42nd degrees latitude	Chile, Cordillera de Nahuelbuta	BYU48263
Pristidactylus valeriae	Donoso-Barros 1966	Chile, Alhué	—	
Pristidactylus volcanensis	Lamborot and Díaz 1987	Chile, El Volcán, Cajón del Maipo, 33°49′S, 70°10′W, 1416 m, 60 km SE (roadline) Santiago	—	
Urostrophus vautieri	Duméril and Bibron 1837	Rio de Janeiro, Brazil (fide GUIBÉ 1954)	Brazil. São Roque, SP/Jundiaí, SP	LG 1153/487

TABLE 2.—Summary of each gene sampled from representatives of the clade Leiosaurae, with fragment length, and the best-fitting model of molecular evolution selected with JModelTest (using the corrected Akaike Information Criterion).

	Mitochondrial loci		Anonymous nuclear loci (ANL)			Nuclear protein coding loci (NPCL)					
	cyt-*b*	12S	D-ANL77	D-ANL90	D-ANL92	CMOS	DMLX	EXPH5	NKTR	RBMX	Total
Length	809 bp	905 bp	812 bp	587 bp	588 bp	522 bp	960 bp	889 bp	1062 bp	660 bp	7794 bp
Model	GTR+I+G	GTR+G	HKY	F81	SYM+G	HKY+I	HKY+I	GTR	HKY+G	HKY+I	

1 µL MgCl [25 mM], and 0.1 µL Taq DNA polymerase [5 U/ µL; Promega Corp., Madison, WI]; 14 µL total reaction volume) used for the ANL genes.

Sequencing reactions used the Big-Dye Terminator Cycle Sequencing Kit in a GeneAmp PCR 9700 thermal cycler (Applied Biosystems, Inc., Carlsbad, CA). Sequencing products were cleaned with Sephadex G-50 Fine (GE Healthcare Bio-Sciences AB, Piscataway, NJ) and sequenced in an ABI 3730xl DNA Analyzer (Applied Biosystems, Inc.) at the BYU DNA Sequencing Center. All sequences were edited using the program Sequencher v4. (Gene Codes Corporation Inc. 2007, Ann Arbor, MI), and aligned with MAFFT (Katoh and Standley 2013). We confirmed open reading frames in all protein-coding genes by translation into amino acids. In all cases, missing data were coded as "?", and sequences are deposited in GenBank (Accession numbers KT342881–343129; aligned matrices are available upon request to the first author).

Gene Tree and Species Tree Analyses

For each gene we selected the best-fitting model of evolution using JModelTest v0.1.1 (Posada 2008) using the Akaike Criterion Information (corrected AIC_c; Table 2). In all nuclear genes, recombination was tested using RDP: Recombination Detection Program v3.44 (Martin and Rybicki 2000; Heath et al. 2006).

Gene trees.—We conducted Bayesian analysis using MrBayes v3.2 (Ronquist and Huelsenbeck 2003) with four independent runs and two chains per run, for 5×10^6 generations of Markov chain Monte Carlo (MCMC) and sampled at intervals of 5000 generations; and for each gene, we used a burn-in of the first 25% of the generations. We also concatenated both mitochondrial loci (cyt-*b* and 12S) and ran a Bayesian analysis in BEAST v1.6.2. We ran 1×10^9 generations of MCMC and sampled at intervals of 100,000 generations with a burn-in of 10%. For all genes concatenated we also ran BEAST v1.6.2 for 5×10^7 generations of MCMC and sampled at intervals of 5000 (burn-in 10%).

TABLE 3.—Mutation rate estimates for each locus in sites per million years, summarized by means (left side of middle column) and standard deviations (right), and the HPD column represents the highest probability density (HPD) of 95% confidence interval.

Locus	Mutation rate (s/my)	HPD
12S	0.00578 ± 0.000009	0.00434–0.00749
CMOS	0.00035 ± 0.0000009	0.00020–0.00052
cyt-*b*	0.01294 ± 0.00002	0.00908–0.01698
D-ANL77	0.00069 ± 0.000001	0.00048–0.00094
D-ANL90	0.00075 ± 0.000002	0.00046–0.00107
D-ANL92	0.00106 ± 0.000002	0.00071–0.00144
DMLX	0.00023 ± 0.0000005	0.00014–0.00031
EXPH5	0.00066 ± 0.000001	0.00044–0.00092
NKTR	0.00044 ± 0.0000009	0.00030–0.00060
RBMX	0.00017 ± 0.0000007	0.00007–0.00030

Species trees.—We performed a coalescent species-tree analysis using *BEAST v1.6.1 (Drummond and Rambaut 2007; Heled and Drummond 2010) analysis using the 10 loci and 28 individuals. We ran 1×10^9 generations of MCMC and sampled at intervals of 100,000 generations (burn-in 10%). We also inferred a second species tree using only the eight nuclear loci. In this case, we ran 2×10^8 generation of MCMC and sampled at intervals of 5000 generations and we used a burn-in of the first 10% of the generations. Convergence was diagnosed by observation of Effective Sample Size values >200 in Tracer v1.5.0 (Rambaut and Drummond 2009).

Divergence Times and Mutation Rates Estimation

We estimated the mutation rates for the Leiosaurae clade (Table 3) based on the fossil described by Albino (2008). This fossil is a lower jaw that was identified as *Pristidactylus* sp. from the Colhuehuapense Formation (Early Miocene) of Gaiman (Chubut province, Argentina), based on several morphological characteristics, including more teeth (condition present in the fossil and in *P. torquatus, P. araucanus, P. scapulatus, P. fasciatus* and *P. nigroiugulus*) than are present in *Leiosaurus* and *Diplolaemus*. Given the topology of our species tree (see Results), conservatively this jaw can be placed on the node before the split of *P. torquatus,* implying that having more teeth is a derived condition within Leiosaurae and *Leiosaurus* maintains the primitive condition of fewer teeth. If having more teeth and several other dental characteristics present in *Pristidactylus* are the plesiomorphic state, then an alternative placement of this fossil could be at the base of the Leiosaurae species tree. This interpretation implies that *Leiosaurus* and *Diplolaemus* are convergent for tooth number and morphology (A. Albino, personal communication). The Colhuehuapense Formation has been dated at 20–20.4 million yr ago (mya; given its correspondence to the Gran Barraca, Sarmiento Formation, Early Miocene, Chubut province; Ré et al. 2010). Thus, conservatively, we assigned the fossil and the age of this formation to the node of the (*P. torquatus + Leiosaurus* + the remaining *Pristidactylus* species) clade. We used BEAST v1.6.2 software and model the calibration prior using a normal distribution with a mean of 20 mya (SD = 2, which leads to 23.29–16.71 95% confidence limit). We ran the analysis for 5×10^8 generations, sampled at intervals of 50,000 generations, and used a burn-in of the first 10% of the generations. This analysis also returned estimates of mutation rates for all genes.

RESULTS

Phylogenetic hypotheses for Leiosaurae species are depicted in the species tree inferred with *BEAST and in the concatenated Bayesian tree inferred with BEAST (Fig. 3). Individual Bayesian gene trees, concatenated mitochondrial and nuclear gene trees are included in Supplemental Materials available online (Appendix S1). The anonymous nuclear marker ANL 90 did not amplify
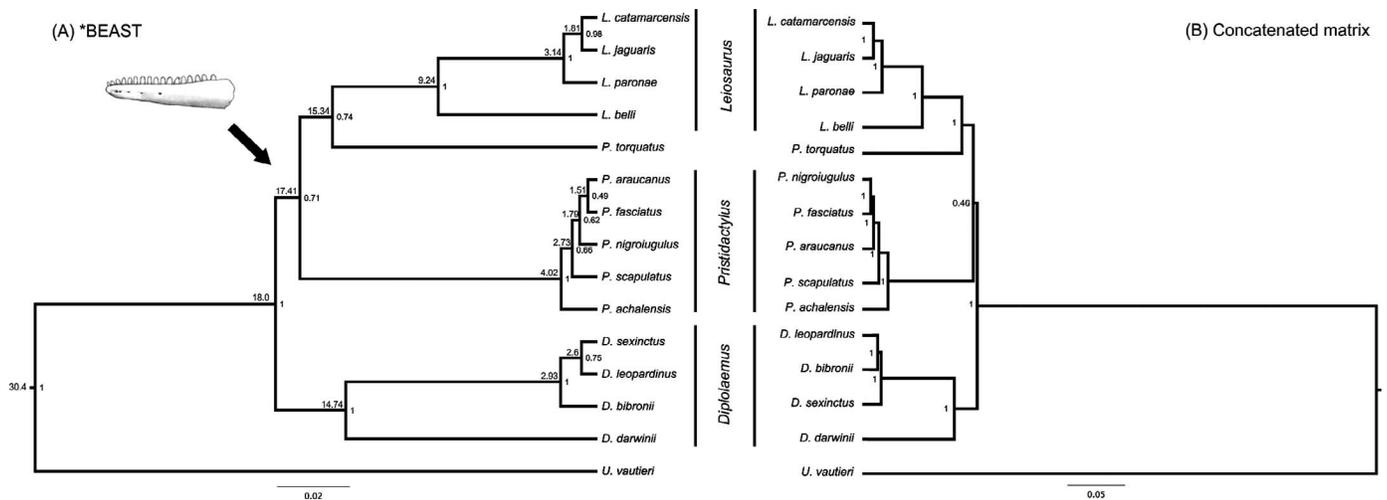
Fig. 3.—(A) Species tree inferred with *BEAST; numbers above nodes correspond to estimated divergence times in million years estimated with BEAST. Numbers to the right of the nodes are posterior probability values. Arrow indicates the node used to place the fossil for the calibration analyses. (B) Multilocus concatenated Bayesian tree (BEAST). Numbers on branches represent posterior probability values.

for any of the four *Leiosaurus* species and for *Urostrophus* (the outgroup taxa). For *P. torquatus*, we have missing data for one nuclear gene (RBMX), and for *Urostrophus* we also have missing data for one NPCL (EXPH5). Although the combined nuclear tree (Supplemental Materials available online, Appendix S1) inferred all three genera as monophyletic, the statistical support for *Leiosaurus* and *Diplolaemus* is very high (PP = 1), whereas there is relatively little statistical support for *Pristidactylus* (PP = 0.54). The combined mt gene inferred *P. torquatus* as sister to the *Leiosaurus* species, although with low statistical support (PP = 0.79). Based on these partial results, the position of *P. torquatus* is not resolved, but the combined results recovered it as sister to *Leiosaurus* (see below).

The monophyly of the Leiosaurae is strongly supported on several higher order systematic studies as well as the genus *Urostrophus* as part of its sister clade Enyaliinae (Frost et al. 2001; Schulte et al. 2003; Abdala et al. 2009; Wiens et al. 2012; Pyron et al. 2013; Reeder et al. 2015). Our combined results (Fig. 3) resolved the three main clades within Leiosaurae with strong statistical support, which generally correspond to the three described genera: *Diplolaemus*, *Leiosaurus* and *Pristidactylus*. The latter two genera appear to be sister clades, although there is little to no statistical support for that relationship (posterior probability on the species tree = 0.71, and posterior probability on the concatenated Bayesian tree = 0.46; Fig. 3). *Pristidactylus torquatus* was not included as part of the genus *Pristidactylus*, with both methods placing it as sister to *Leiosaurus* with moderate to high support (PP ranged from 0.74 to 1.0; Fig. 3).

Diplolaemus darwinii, one of the two most southerly distributed species of Leiosaurae (Fig. 1), was inferred as the sister taxon of the three other species (Fig. 3; PP = 1), and *D. bibronii* (PP = 1) as sister to (*D. sexinctus* + *D. leopardinus*) in the species tree (Fig. 3A); whereas in the Bayesian concatenated analyses, *D. sexinctus* was inferred as sister to (*D. leopardinus* + *D. bibronii*), also with high statistical support (PP = 1). *Leiosaurus bellii,* the most southerly distributed species of this genus, is strongly supported as the sister taxon of the other three species

(PP = 1). *Leiosaurus paronae* is sister to (*L. jaguaris* + *L. catamarcensis*), also with strong support (PP = 1; Fig. 3), and these two clades are the most northwestern distributed taxa of this genus. *Pristidactylus achalensis,* the most northerly distributed species of its genus, was strongly supported (PP = 1) as the earliest divergent taxon of the clade that includes all other congeneric species (except *P. torquatus*), and *P. scapulatus* is inferred as sister to the other three species with low species-tree and high Bayesian inference support (PP = 0.66 and 1, respectively; Fig. 3).

Our estimated divergence times calibrated with a Leiosaurae fossil give an approximate temporal window for the origin and diversification of this clade. Average divergence date estimates in million years (results obtained with BEAST) are given above the species-tree branches (Fig. 3A; results obtained with *BEAST). The first divergence within the crown group is estimated at ~18 mya, the origin of *Diplolaemus* has an average of 14.74 mya, and the divergence of *P. torquatus* is estimated at ~15.34 mya. The first *Leiosaurus* is estimated at ~9.24 mya, and the earliest divergence of Argentinean *Pristidactylus* is estimated at ~4.02 mya. Most divergences within these three genera are estimated between ~4 and ~1.51 mya, with most of the youngest speciation events occurring within *Pristidactylus*. Mitochondrial gene estimates have the higher mutation rates (cyt-*b* = 0.01294 and 12S = 0.00578), followed by the anonymous nuclear marker D-ANL92 (= 0.00106; Table 3). Lower mutation rates were estimated for the other nuclear markers, and the nuclear gene RBMX shows the lowest mutation rate (= 0.00017).

Discussion

Leiosaurae Phylogeny

We have presented a comprehensive molecular hypothesis for phylogenetic relationships of the clade Leiosaurae, including all described species of *Diplolaemus* and *Leiosaurus* and 6 out of 10 species of *Pristidactylus*. We used nine independent markers (eight nuclear and two mitochondrial genes) to infer trees based on two different approaches (concatenated trees with Bayesian Inference estimated in BEAST, and species trees estimated with *BEAST).

Including markers differing in modes of inheritance (mitochondrial and nuclear genes) and mutation rates (Table 3) seems to improve the outcome of the species-tree analyses (Camargo et al. 2012). Whereas both approaches provided well-resolved and largely concordant results, we consider the species tree as the best-supported hypothesis of phylogenetic relationships among species of the Leiosaurae clade because it accommodates the gene-tree/species-tree reality (Maddison 1997). Although it has been shown that these methods have greater accuracy than concatenated methods over a larger area of tree space (Leaché and Rannala 2011), a recent study reported that these two approaches have similar accuracy under a various conditions of simulation (Tonini et al. 2015).

Probably the most surprising result of our study is the topological position of *Pristidactylus torquatus,* a species endemic to temperate Chilean beech forests that is inferred as sister to *Leiosaurus* (with moderate to high support). This result is congruent with Pyron et al. (2013) but contrasts with that of Frost et al. (2001), who, on the basis of morphological and mtDNA data, found this species nested within other *Pristidactylus* species (partially reproduced in Fig. 2). There are several possible explanations for this discrepancy: (1) the taxon sampling for this genus is incomplete in both Frost et al. (2001) and in our study; (2) Frost et al. (2001) implemented Maximum Parsimony analyses whereas we used Bayesian and species-tree approaches; and (3) the *P. torquatus* samples used in both studies may have different geographic origins (specimen localities are not given in either Frost and Etheridge [1989] or Frost et al. [2001]). The *P. torquatus* sample we used in this study is from the Cordillera de Nahuelbuta, which harbors the native *Araucaria–Nothofagus* forests. This species of *Pristidactylus* is broadly distributed on the west side of the Andes from 35°S to 43°S (Etheridge and Williams 1985; Labra and Rosenmann 1994), and it has been suggested to have ancestral features associated with old Tertiary ecosystems persisting after the uplift of Andes (Cei 1986). These temperate forests harbor a number of endemic species and have been identified as one of the world's 25 top biodiversity hotspots (Myers et al. 2000). The strong association of *P. torquatus* with these forests in Chile makes it a sensitive species because commercial pine plantations have replaced most of its habitat.

The other three Chilean *Pristidactylus* species not included in our study have relictual distributions over the Coastal and Andean cordilleras (Lamborot and Díaz 1987), about 140 km north from the northernmost population of *P. torquatus* (but 650 km north from our sampled population), and are geographically close to *P. scapulatus* and *P. fasciatus* in Argentina (Fig. 1). These three Chilean species might be phylogenetically closely related to the Argentinean *Pristidactylus* species, with *P. torquatus* indeed representing an early divergent lineage within the Leiosaurae. Alternatively, because two of three species are also ecologically linked to forests (although to a lesser extent than *P. torquatus*), they could also be part of this distinctive lineage represented here by *P. torquatus.* Increased taxon, population, and gene sampling will be needed to distinguish among these alternative hypotheses for the correct placement of *P. torquatus* within the Leiosaurae clade.

The previous phylogenetic studies of the Leiosaurae clade were mostly centered on higher level relationships, and the monophyly of the Leiosaurae is strongly supported as well as the genus *Urostrophus* as part of its sister clade Enyaliinae (Frost et al. 2001; Schulte et al. 2003; Townsend et al. 2011; Wiens et al. 2012; Pyron et al. 2013; partially reproduced in Fig. 2). Within the past 5 yr, higher category studies have included only 2–6 species of the Leiosaurae, but recently Abdala et al. (2009) presented phylogenetic hypotheses for the majority of the clade based on new myological data (Fig. 2E) and combined data (reanalyses of molecular and morphological data from Frost et al. [2001] with the myological data, Fig. 2E,H). Regarding phylogenetic relationships between the three Leiosaurae genera, previous results represent all three possible hypotheses: (1) *Leiosaurus* as sister to *Pristidactylus* (Fig. 2A,D,H); (2) *Leiosaurus* as sister to *Diplolaemus* (Fig. 2G, but not statistically significant, 0.54 posterior probability); or (3) *Pristidactylus* as sister to *Diplolaemus* (Fig. 2B,C,E,I). Some of these phylogenies include only representatives of two genera (Fig. 2F,J), but in two of these trees, *Pristidactylus* is not monophyletic (Fig. 2H,I). Our results support the first of these hypotheses (moderate support on the species tree, Fig. 3A, and low support on the concatenated tree, Fig. 3B), but this relationship is in contrast to the alternative hypothesis (3) that was inferred in studies based mainly on morphological data, but also with a combined data set (Fig. 2C). In order to resolve the apparent conflict between the molecular and the morphological hypotheses, it is necessary to add more taxa and characters (molecular and morphological) and conduct new phylogenetic analyses. Recently, the placement of Iguania within the Squamata has been controversial because of conflicting morphological and molecular data (Gauthier et al. 2012; Wiens et al. 2012). Losos et al. (2012) suggested that some mechanisms of molecular evolution have not been accommodated in analytical methods, and that this omission could misinform molecular phylogenetic inference and lead to conflicting results with morphological data. Reeder et al. (2015) increased the data set related to this conflict, and found strong morphological support for the molecular hypotheses. We suggest that a similar issue might be relevant to phylogenetic analyses of the Leiosaurae, and because it represents a morphologically unique clade endemic to southern South America, an exhaustive morphological study should expand our understanding the evolutionary and biogeographic history of this clade.

Evolution and Diversification of Leiosaurae

The ancestor of the Leiosaurae clade was inferred to have originated ~18 mya, contemporaneous with the continuous uplift of Andean cordillera that during the next 10 million yr formed a barrier to the Pacific winds; and as a consequence, temperatures decreased and desertification of the eastern Patagonia began (Blisniuk et al. 2005, 2006; Folguera et al. 2011). These changes in turn might have caused the extinction of megathermal and nonseasonal plants in parallel with the diversification of xeric plants (Iglesias et al. 2011). Based on the *Pristidactylus* sp. fossil from the Colhuehuapense Formation (Albino 2008), we modeled the calibration using a normal distribution with a mean of 20 million yr on the node that represents the ancestor of (*P. torquatus* + *Leiosaurus* + the remaining Argentinean *Pristidactylus* species), with an estimated posterior average of 17.41 mya (the oldest split within the Leiosaurae). The disjunct

distribution of the *Pristidactylus* species on both sides of the Andes and the arboreal life style of most Chilean species (except *P. volcanensis,* which seems to have secondarily adapted to open rocky habitats; Etheridge and Williams 1985; Lamborot and Díaz 1987), led Cei (1986) to suggest that the genus might have originated before the uplift of the Andes in forested areas. Cei et al. (2001) and Scolaro et al. (2003) elaborated an ecogeographic speciation model for *Pristidactylus,* with a common ancestor inhabiting extensive forests available at that time and later adapting to more open, rocky, and dry habitats.

Etheridge and de Queiroz (1988) considered the arboreal Chilean *Pristidactylus* species as having plesiomorphic characteristics within *Pristidactylus*. In contrast, Frost et al. (2001) proposed that the ancestral condition for this genus was terrestrial, and arboreality was a derived feature. The paleo-environment for the Colhuehuapense Formation is characterized by forests and sand dune formations (Vucetich and Bond 1984; Vucetich and Verzi 1991); thus, the *Pristidactylus* Miocene fossils from central Patagonia could be assigned to an arboreal form within Leiosaurae (A. Albino, personal communication). The integration of this geological evidence with our results is generally concordant with the first hypothesis proposed by Cei (1986). Furthermore, the singularity of the *P. torquatus* lineage being restricted to forests supports the hypothesis that arboreality might be the ancestral condition within the crown group Leiosaurae. As the forests retreated toward northwestern areas on the Chilean side of the Andes, we suspect that some populations maintained their associations with these habitats, while the populations west of the Andes differentiated and adapted to drier climates that developed during the Miocene.

The first divergences within crown *Diplolaemus* and *Leiosaurus* are estimated at 14.74 mya and 9.24 mya, respectively (both mid-Miocene), with most of the subsequent divergence during the Pliocene. During the Middle and Late Miocene (10–5 mya), at least two extensive Atlantic marine transgressions took place into the low-lying basins of southern South America; these are collectively labeled the Paranean Sea (Ramos and Alonso 1995; Pascual et al. 1996; Lundberg et al. 1998; Hernández et al. 2005; Ruskin et al. 2011; see Fig. 3 in Morando et al. 2014). Further, multidisciplinary palaeo-environmental studies have shown a strong marine Miocene impact on this part of South America (Lundberg et al. 1998; Hovikoski et al. 2010; Cooke et al. 2011). *Diplolaemus* includes a very divergent species, *D. darwinii* (Fig. 3A). The topology inferred for *Diplolaemus* species, along with their geographic distributions, imply a south-to-north diversification pattern (Figs. 1 and 3). Based on the geological evidence and this divergence pattern, we hypothesize that the ancestor of *Diplolaemus* most probably inhabited the southern areas of Patagonia, and subsequently diversified toward the north sometime after the Paranean Sea retreated.

The ancestor of *Leiosaurus* was dated to ~9.24 mya (beginning of Late Miocene), which is contemporaneous with the end of the last major Andean uplift. This ancestor likely survived in the northwestern Patagonia area that was emerged land during marine ingressions (Ramos and Alonso 1995; Hernández et al. 2005). This divergence estimate is also congruent with the estimate for the first rapid radiation of the *Liolaemus montanus* section (Liolaemini, *Eulaemus* clade; Olave et al. 2015). Furthermore, the next divergence

within *Leiosaurus* was dated 5.81 mya, at the time a second rapid radiation was hypothesized for lizards of the *Liolaemus montanus* section (Olave et al. 2015). Based on our species tree (Fig. 3A), the diversification of this genus might have also followed a south-to-north direction, because *L. bellii* is the southernmost distributed species in the northern Patagonian steppe, whereas the other three are restricted to northwestern Argentina, and are mainly associated with the Monte biogeographic region. Alternatively, a vicariant event split northern from southern populations, with further diversification in the north. Apart from the marine ingressions, several other palaeoclimatic and geological events took place during this time (Rabassa et al. 2005) that shaped the biota of southern South America (Baéz and Scillato Yané 1979; Markgraft et al. 1995).

The Argentinean *Pristidactylus* species were the last to diverge from an ancestor dated at 4.02 mya. Its diversification may have followed an east-to-west and a north-to-south pattern, because *P. achalensis* and *P. scapulatus* are the northernmost distributed species, and the other three species have more southern distributions (Fig. 1). This pattern contrasts sharply with the two older genera.

In summary, we hypothesize that the ancestors of *Diplolaemus* and *Leiosaurus* might have been isolated by the Paranean Sea and most probably originated in southern Patagonia. Successive diversifications toward northern areas, mainly during the Pliocene, seem likely for both of these genera. The ancestor of the Argentinean *Pristidactylus* is more recent, and also might have followed a different general diversification pattern, toward western and southern areas beginning about 4 mya. It is necessary to develop and integrate palaeoclimatic niche models and physiological studies of the Leiosaurae clade to test these biogeographic hypotheses.

Supplementary Material

Supplementary material associated with this article can be found online at http://dx.doi.org/10.1655/Herpetologica-D-14-00067.S1.

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