Evolution of the cloacal and genital musculature, and the genitalia morphology in liolemid lizards (Iguania: Liolaemidae) with remarks on their phylogenetic bearing

Matías Quipildor[1,*], Virginia Abdala[2], Roy Santa Cruz Farfán[3], Fernando Lobo[1]

Abstract. In this study, we describe the intra- and interspecific anatomical variations of cloacal and related muscles of male and female genitalia in species of five iguanian genera (three liolemid: *Ctenoblepharys*, *Liolaemus*, and *Phymaturus* plus *Diplolaemus leopardinus* and *Tropidurus melanopleurus* as outgroups). We found variations (seventeen characters) in topology, origin and insertion areas, tendon morphology and size of the musculature of this region. We also describe the variations of hemipeneal morphology, which is especially notable for the hemipenis of *C. adspersa*, *D. leopardinus*, and *T. melanopleurus*, as this is first time they are described in the literature. Among the most significant findings are the identification of three new muscles, two of them inserted on the roof of the cloacal chamber (anterior and posterior cloacal retractor) and the third inserted superficially in the floor of the cloaca, just before anterior to the precloacal glands row (superficialis cloacalis retractor). We report sexual dimorphism in seven muscle characters. Musculature related to hemiclitoris is reduced in proportion to its size in comparison to the degree of development of male genitalia and associated musculature. The evolution of characters was traced on the known phylogenetic hypotheses of relationships among families. Characters taken from the cloacal/genital myology bring similar support to the liolemid tree even rooting the analysis using different outgroups. In addition, a phylogenetic study using only myological characters was performed. In this case, *C. adspersa* was found to be more related to *Liolaemus* species instead of being basal to *Liolaemus* plus *Phymaturus*.

Keywords: cloacal, hemiclitoris, hemipene, muscle.

Introduction

Liolaemidae is a group of iguanian lizards formed by three genera: *Ctenoblepharys*, *Liolaemus*, and *Phymaturus* (Frost and Etheridge, 1989; Etheridge, 1995; Lobo, Espinoza and Quinteros, 2010; Pyron et al., 2013), including 257 species (Abdala and Quinteros, 2014). Morphologically, these lizards have been studied for different reasons, including the search of characters for phylogenetic studies and taxonomic propositions like those involving skeleton (Keller and Krause, 1986; Lobo and Abdala, 2002; Gonzalez Marin and Hernando, 2016) and muscles (Abdala and Moro, 2003; Abdala, Abdala and Tulli, 2006). The musculature studies cited above were focused on cranial myology and limb muscles and in recent research some functional and adaptive subjects were analyzed (Tulli et al., 2009; Bonino et al., 2011). The most significant contribution to the knowledge of cloacal and hemipenial musculature across a diverse squamate lizards sample was Arnold (1984). He described thirty-seven characters associated with cloacal musculature, including seven related to opening and closure of cloaca and eversion/retraction of hemipenes. The main goal of Arnold (op.cit.) was to establish differences among the main lineages of Squamata. His references to species of liolemids are brief and few, restricted to *Ctenoblepharys adspersa*, *Phymaturus palluma*, and *Liolaemus multiformis* (= *L. signifer*). These observations are focused on differences among families, and the variation within Liolaemidae is not reported.

1 - IBIGEO (Instituto Bio y Geociencias del NOA), CONICET-unas, 9 de Julio 14, Rosario de Lerma, 4405 Salta, Argentina

2 - IBN (Instituto de Biología Neotropical), CONICET-UNT, Horco Molle s/n, Cátedra de Biología General, Facultad de Ciencias Naturales e IML, UNT, San Miguel de Tucumán, 4000 Tucumán, Argentina

3 - Museo de Historia Natural de la Universidad Nacional de San Agustín, Alcides Carrión s/n, Arequipa, Perú

*Corresponding author;
e-mail: amquipildor@gmail.com

Comparative studies of squamate cloacal musculature are limited, but there are many descriptions of comparative morphology of hemipenes (Dowling and Savage, 1960; Böhme, 1988; Grazziotin et al., 2012). The morphology of the hemipenes of Liolaemidae has been described for a few species (Cei, 1986, 1993; Böhme, 1988; Lobo, 2000). Cei (1986, 1993) published some observations on the hemipenes of *Liolaemus* (indicating fourteen species but without listing voucher materials), he remarked the lack of ornamentation in all species revisited, yet only presenting three species in one of his pictures.

Böhme (1988), in his monograph of the morphology of hemipenes of squamata families, described the hemipenis of *Phymaturus palluma*. Lobo (2000) reported the morphology of eighteen species of *Liolaemus* and *Phymaturus dorsimaculatus* (= *P.* cf. *palluma*), presenting main differences between both subgenera of *Liolaemus* (*Eulaemus* and *Liolaemus*). Recently, hemiclitores were found in females of *Phymaturus* and *Liolaemus* species (Valdecantos and Lobo, 2015). This was the first report for Liolaemidae, where significant variations in morphology, size and pigmentation were recorded.

The main goals of this paper are: 1) to describe the cloacal musculature of *Liolaemus irregularis* and how it varies among taxa (*Ctenoblepharys adspersa*, *Diplolaemus leopardinus*, *L. austromendocinus*, *Phymaturus palluma*, *P. patagonicus*, *P. laurenti*, and *Tropidurus melanopleurus*) 2) to describe the anatomy of three new muscles not reported before now 3) to analyze the evolutionary history of the different features related to those muscles and genitalia through an optimization of those traits, taking into account the intergeneric and interfamiliar relationships proposed most recently in the literature (Gauthier et al., 2012; Morando et al., 2013; Pyron, Burbrink and Wiens, 2013; Reeder et al., 2015; Zheng and Wiens, 2016), and finally 4) to analyze if there is a correlation in the degrees of development of male and female genitalia and their associated mus-

cles. We provide new morphological evidence related to the functional morphology of structures closely related to the reproductive behaviour of all these species (cloacal opening, hemiepenes/hemiclitoris motion, caudal autotomy and its effects on the rest of musculature, copula efficiency, etc.).

Materials and methods

Specimens from the following museum collections (acronyms given in parenthesis) were examined: Museo de Ciencias Naturales-Universidad Nacional de Salta (MCN) and Instituto de Bio y Geociencias del NOA, Salta, Argentina (IBIGEO) and Museo de Universidad de San Agustín, Arequipa, Perú (MUSA). Representatives of *Liolaemus* belonging to both subgenera: *Eulaemus* (*Liolaemus irregularis* MCN: 1885, 1884 males, 1880 female, San Antonio de los Cobres, Salta, Argentina) and *Liolaemus sensu stricto* (*L. austromendocinus*, MCN: 3686, male, Sierra del Nevado, Mendoza, Argentina) were selected. Within *Phymaturus*, we examined *P. palluma* (MCN: 2894 male, 2892 female, Valle Hermoso, Mendoza, Argentina) and *P. laurenti* (IBIGEO: 5179, 5179 male, 5174 female, Antofagasta de la Sierra, Catamarca, Argentina) as members of the *palluma* group and *P. patagonicus* (MCN: 3275, 1251 male, Telsen, Chubut, Argentina) as member of the *patagonicus* group, respectively (Lobo et al., 2010; Lobo, Abdala and Valdecantos, 2012; Morando et al., 2013). In addition, we studied specimens of the monotypic genus *Ctenoblepharys adspersa*, (MUSA: 4742, 4613 males, 4612 female, Reserva Nacional San Fernando, Ica, Perú). *Diplolaemus leopardinus* (Leiosauridae) (IBIGEO 5493 male, Primeros Pinos, Neuquén, Argentina) and *Tropidurus melanopleurus* (Tropiduridae) (IBIGEO: 5317, 5463 males, 5329 female, Aguas Blancas, Orán, Salta, Argentina) were studied and considered outgroup for phylogenetic reconstruction and mapping evolution.

Preparations of muscles, tendons, and fascia were made using a lugol staining solution (Bock and Shear, 1972). Musculature was described following the nomenclature of Arnold (1984). The muscles previously undefined in the literature were named according their topological position and function. Dissected specimens were studied using a stereoscopic microscope. Measurements were taken using a digital caliper (0.02 mm of precision) and an ocular micrometer. Hemipenes preparation and terminology follow Dowling and Savage (1960), Ziegler and Böhme (1999), and Zaher and Prudente (2003).

Phylogenetic analyses were performed with the software TNT (Goloboff et al., 2003). Given that our aim was to study the cladistic information provided by the anatomical characters tested, we performed two kinds of analyses. In the first case, we ran a data matrix by building trees using parsimony (TNT, traditional search, with 1000 replications and TBR with 100 trees saved per replication). In the second case, we optimized characters onto the most accepted topology of

the family. The continuous characters (2, 18, 22, 26 and 27) were coded as discrete to facilitate analysis and optimizations, states assigned were following Thiele (1993) and were coded according to their variation in: binary, multistate, and polymorphic.

Results

The following description is a composite made up of muscles, variation and hemipenes of *Liolaemus irregularis*, and later we provide comparisons with the other taxa studied.

Description of cloacal muscles of Liolaemus irregularis *(male)*

Transversus perinei (TP). Its fibers run transversely to the longitudinal axis of the body, fixed to the hypoischium and inserted into the ischial ligament located in the anterior apex of the cloacae (fig. 1A, C).

Superficialis cloacalis retractor (SCR). It is located anterior to the cloacal opening; its fibers pass obliquely, with its origin site in the
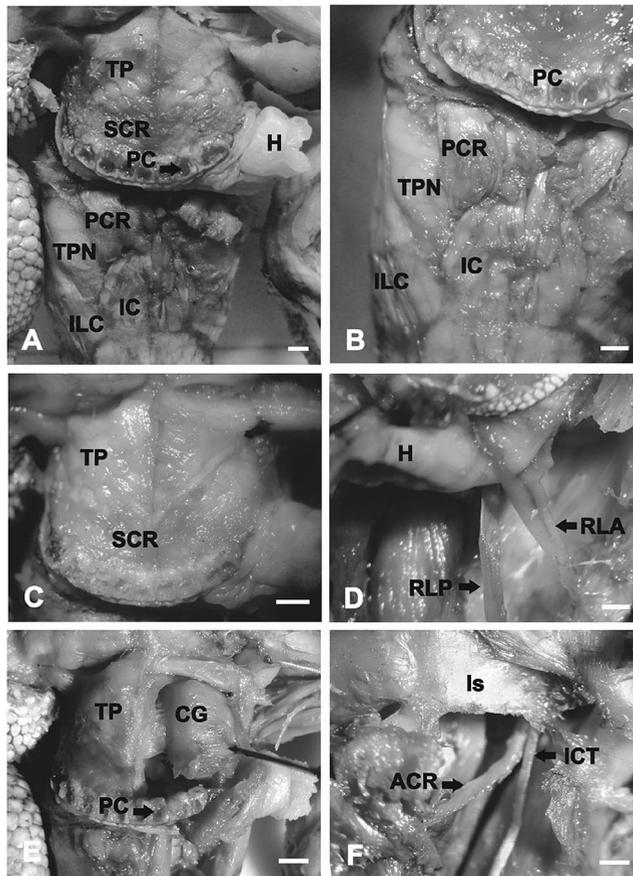

Figure 1. Ventral view of the cloacal region muscles in a male of *Liolaemus irregularis* (MCN 1885). The head of the specimen is located upwards, while the tail is located down. (A) General disposition of the superficial muscles. (B) Post cloacal superficial muscles. See the disposition of the posterior cloacal retractor, partially covering the transversus penis. (C) Pre-cloacal superficial muscles. (D) Anterior and posterior lateral retractor muscles. See the retractor lateral anterior division. (E) Compressor glandulae located dorsal to the transversus perinei (transversus perinei cut off from its position on the left side). (F) Disposition of the anterior cloacal retractor passing dorsal to the cloacal chamber and the origin of the ischiocaudalis tendon (superficial muscles dissected). Abbreviations as follows = H: hemipenis; Is: ischium; IC: ischiocaudalis; ILC: Iliocaudalis; PC: precloacal glands; PCR: posterior cloacal retractor; TP: transversus perinei; TPN: transversus penis; SCR: superficialis cloacal retractor; CG: compressor glandulae; ACR: anterior cloacal retractor; RLA: retractot lateral anterior; RLP: retractor lateral posterior; ICT: ischiocaudalis tendon. Scale = 0.5 mm.

transversus perinei fascia and its insertion in the floor of the cloacal chamber, anterior to the row of the precloacal glands (fig. 1A, C).

Sphincter cloacae. They surround anterior and posterior to the cloacal opening. They can be divided into two sections more or less independent anterior and posterior sphincter.

Protractor comissurae. Its muscle fibres run from the lateral extremity of the vent, obliquely outwards and forwards to attach to the ilioischial ligament, posterior to the insertion of the transversus perinei. It is confluent with the sphinter cloacae.

Iliocaudalis (ILC). It is a muscle located behind and lateral to the hemipenis and the transversus penis. It runs obliquely, originating at the iliac posterior tip, with fibers attached to the transverse processes of the first to eight caudal vertebrae. Some fibers at that posterior level attach to the lateral fascia of the ischiocaudalis (fig. 1A, B).

Ischiocaudalis (IC). This muscle is directed longitudinally with its origin in the external and dorsal margin of the ischium. It has a long tendon that passes dorsal to the cloacal chamber and reaches the chevrons of the third to eight caudal vertebrae (insertion area) (figs 1A, B; 2A, B, C, D).

Caudifemoralis (CF). It exhibits two divisions that become separated close to their origin, the caudifemoralis longus and brevis. The former is a massive muscle inserted from the first to the eighth caudal vertebrae, and the latter is smaller and lateral, inserted between the first to the third vertebrae. The origin of the CF is located in the femur close to its articulation with the pelvic girdle. Its tendon projects a ramus that is attached to the tibia, close to the knee (fig. 2A).

Compressor glandulae (CG). It is located dorsal to the transversus perinei. Its fibers are semicircularly arranged and transverse to the longitudinal axis of the animal. In the region of

contact with the digestive tract there is a dense fascia. Its fibers, like the transversus perinei, are attached to the hypoischium and to the ischial ligament. It is present only in males, and in its interior is a cloacae gland (fig. 1E).

Anterior cloacal retractor (ACR). It is an internal muscle, located ventral to the cloacae. Its fibers run obliquely from the dorsal part between the glenoid cavity and the pubes where it is fixed, to the fascia of the ventral region of the cloacae where it is inserted (fig. 1F).

Posterior cloacal retractor (PCR). It is located posterior to the cloacal opening. It is a superficial muscle, and its fibers pass obliquely to the anterior posterior axis of the body covering in part the transversus penis (figs 1A, B; 2A, C, D). This muscle connects a fascia located in the roof of the cloacal chamber with a fascia of the ischiocaudalis muscle.

Description of muscles associated to hemipenes

Transversus penis (TPN). It is arranged transversally to the hemipenis. Its semicircular fibers are located in the ventrolateral region, to the dorsomedial region. They are fixed to a dense fascia, which has two attachment points: one to the first chevron and another to the body of the sixth caudal vertebra. This muscle longitudinally covers the entire hemipenis and part of the penis magnus retractor (figs 1A, B; 2C, D).

Retractor lateral anterior (RLA). The fibers of this muscle run obliquely to be fixed in the transverse process of the fourth caudal vertebra. They insert in the anterior vertex of the cloacae (fig. 1D).

Retractor lateral posterior (RLP). Its fibers run obliquely. It is fixed in the transverse process of the sixth caudal vertebra. It has two points of insertion at the base of the hemipenes, one in the ventral region and another in the dorsal region (fig. 1D).

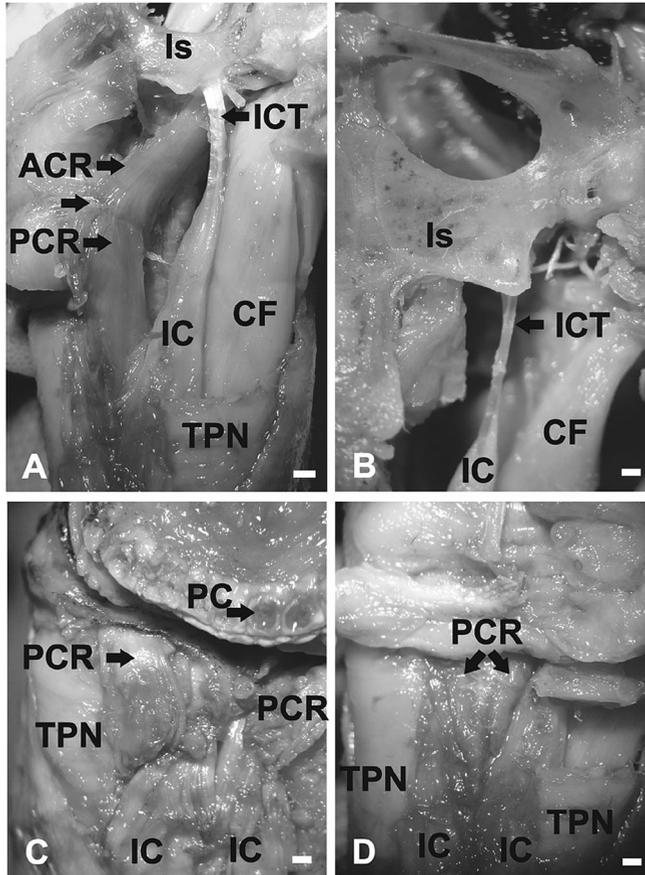*Retractor penis magnus (RPM).* Its fibers are longitudinally arranged. It is the main muscle

Figure 2. The head of the specimen is located upwards, while the tail is located down. (A) Internal muscles in a male of *Tropidurus melanopleurus* (IBIGEO 5317). See the origin of the ischiocaudalis tendon in the lateral margin of the ischium, the common insertion of anterior and posterior cloacal retractors in a fascia dorsal to the cloacal chamber. (B) Internal muscles in the male of *Phymaturus palluma* (MCN 2894). See the dorsal insertion of the ischiocaudalis tendon in the ischial bone. (C) See how the posterior cloacal retractor partially covers the transversus penis. (D) Posterior cloacal retractor located medially to the ischiocaudalis, not in contact or covering the transversus penis. Abbreviations as follows = Is: ischium; H: hemipenis; IC: ischiocaudalis; PC: precloacal glands; PCR: posterior cloacal retractor; TPN: transversus penis; ACR: anterior cloacal retractor; ICT: ischiocaudalis tendon; CF: caudofemoralis. Scale = 0.5 mm.

involved in the retraction of the hemipenes. It attaches to the vertebral bodies of the seventh and eighth caudal vertebrae, and inserts in the apex of hemipenis.

Variations among liolemid taxa and outgroups (muscles)

In *Ctenoblepahrys*, *Liolaemus*, and *Phymaturus* the ischiocaudalis originates in the dorsal side of ischium (fig. 2B), but in *Ctenoblepharys*, this tendon is absent. In *Diplolaemus leopardinus* and *Tropidurus melanopleurus* the IC origin is in the lateral region of the ischium (fig. 2A). The length of this tendon with respect to the SVL is quite homogeneous in all taxa analyzed with the exception of *L. austromendocinus* (14% of SVL versus 4-7, 5%).

A character shared exclusively by *C. adspersa* and both species of *Liolaemus* is the existence of medial rami of the ischiocaudalis inserted on the roof of the cloacae. The insertion of the ischiocaudalis can reach the seventh vertebra in *L. austromendocinus*, the eighth in *L. irregularis*, the ninth in *C. adspersa* and *P. pal-*

Table 1. Insertion of different muscles on caudal vertebrae of eight iguanian species. Abbreviations are the same cited in the text and indicated in figures.

Species	Caudal vertebrae number											
	1	2	3	4	5	6	7	8	9	10	11	12
Ctenoblepharys adspersa	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC			
	CF	CF	CF	CF	CF	CF	CF	CF	CF	CF		
				IC	IC	IC	IC	IC	IC			
					RLA	RLA	RPM	RPM				
					RLP	RLP						
Liolaemus irregularis	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC				
	CF	CF	CF	CF	CF	CF	CF	CF				
				IC	IC	IC	IC	IC				
				RLA		RLP	RPM	RPM				
Liolaemus austromendocinus	ILC	ILC	ILC	ILC	ILC	ILC	ILC	CF				
	CF	CF	CF	CF	CF	CF	CF	RPM				
				IC	IC	IC	IC					
				RLA	RLP	RLP						
							RPM					
Phymaturus patagonicus	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC		
	CF	CF	CF	CF	CF	CF	CF	CF	CF	CF		
			RLA	IC	IC	IC	IC	IC	IC	IC		
			RLP	RLP	RLP	RLP	RLP			RPM		
Phymaturus laurenti	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC		
	CF	CF	CF	CF	CF	CF	CF	CF	CF	CF		
				IC	IC	IC	IC	IC	IC	IC		
				RLA	RLA		RLP	RLP	RPM	RPM		
Phymaturus palluma	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC			
	CF	CF	CF	CF	CF	CF	CF	CF	CF	CF		
				IC	IC	IC	IC	IC	IC	RPM		
				RLA	RLP	RLP						
Diplolaemus leopardinus	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC	
	CF	CF	CF	CF	CF	CF	CF	CF	CF	CF	CF	CF
				IC	IC	IC	IC	IC	IC	IC	IC	RPM
				RLA	RLP	RLP	RLP				RPM	
				RLP								
Tropidurus melanopleurus	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC	ILC		
	CF	CF	CF	CF	CF	CF	CF	CF	CF	CF		
				IC	IC	IC	IC	IC	IC	IC		
										RPM		

luma, and the ninth and tenth in *P. laurenti*. In *P. patagonicus* and *T. melanopleurus*, its insertion reaches the tenth vertebra, while in *D. leopardinus* it reaches the eleventh (table 1).

Variations found of the iliocaudalis are limited to how far back in the tail this muscle can insert. In *L. austromendocinus*, it reaches the seventh caudal vertebra, in *L. irregularis* the eighth, in *C. adspersa* and *P. palluma* the ninth, while in *P. laurenti* the ninth and tenth. In *P. patagonicus* and *T. melanopleurus* the tenth, while in *D. leopardinus* the eleventh (table 1).

The superficialis cloacalis retractor is present only in *L. irregularis* (fig. 1A, C).

The Posterior cloacal retractor muscle is present in all taxa studied with the exception of *C. adspersa*. In both species of *Liolaemus* analyzed, the posterior cloacal retractor superficially covers part of the transversus penis (figs 1B, 2C). In the remaining species, this muscle is restricted to the medial region between both transverses penis (fig. 2D).

The Retractor laterals anterior and posterior are present in all species studied with the exception of *T. melanopleurus*. The origin of the retractor lateral anterior in *P. laurenti* reach the third caudal vertebra, in *L. irregularis* the fourth, in *P. palluma* third and fourth, in *D. leop-*

ardinus, *L. austromendocinus*, and *P. patagonicus*, the fifth, in *C. adspersa* the fifth and sixth (table 1). The origin of the retractor lateral posterior in *L. austromendocinus*, *P. palluma*, and *P. patagonicus*, is located in the sixth and seventh caudal vertebra; in *L. irregularis* in the sixth only; In *C. adspersa*, the fifth and sixth. In *D. leopardinus* it is located in four caudal vertebrae: the fifth, sixth, seventh and eight (table 1).

The retractor penis magnus (RPM) originates in different vertebrae for the various taxa analyzed. It reaches backward its most posterior site in the transverse process of the seventh caudal vertebra in *L. irregularis* and *L. austromendocinus*. In *C. adspersa* it originates in the seventh and eight caudal vertebrae. In *P. palluma* and *P. laurenti* it extends, reaching the ninth and tenth caudal vertebrae, while in *P. patagonicus* it goes backward reaching the eleventh. In *T. melanopleurus*, it reaches the tenth, and in *D. leopardinus* the twelfth caudal vertebra. The origin site is in a vertebra of the RPM in *L. austromendocinus*, in *C. adspersa* and *P. laurenti* it is found in the vertebral transverse process, while in *D. leopardinus*, *L. irregularis*, *P. palluma*, *P. patagonicus*, and *T. melanopleurus* its origin is restricted to the vertebral body (table 1).

The transversus penis (TP) fibers pass ventrally and superficially to the hemipenis. Its fibers extend backward, reaching the level of the fourth caudal vertebra in *C. adspersa*. In both species of *Liolaemus*, *P. palluma*, *P. laurenti*, and *T. melanopleurus* it goes beyond the fourth reaching the fifth, while in *P. patagonicus* it reaches the sixth. In *D. leopardinus*, it reaches the eighth vertebrae (table 1).

Muscles and hemiclitoris of Liolaemus irregularis

There is sexual dimorphism related to the presence or absence of certain muscles, and the degree of development of others. Among the reduced muscles are the retractor hemiclitoris magnus and the transversus hemiclitoris. The

RHM has a different origin than males, in a fascia located between the ischio and iliocaudalis muscles. On the other hand, in males its origin does not include a caudal vertebra. The transversus hemiclitoris exhibits few and thin weakly attached fibers that can be lost easily during the dissection process. The two retractor muscles (RLA and RLP) are also lost, in addition to the compressor glandulae, which is present only in males. In females, both tail muscles, the ilio and ischiocaudalis, are larger than in males (perhaps occupying the free space because of the absence of hemipenes and associated muscles that are well formed in males). In females there are fewer caudal pre-autotomic vertebrae. The general structure of females' hemiclitoris resembles that described for other Squamata species. They are smaller than the hemipenes and exhibit a sulcus spermaticus, without ornamentation.

Hemipenes of Liolaemus irregularis

In *Liolaemus irregularis* the lobes are ornamented by a fleshy hornlike structure. Both the asulcada and the sulcada faces are ornamented by a few fleshy plicae. The sulcus spermaticus is broad in the proximal region, with both lips thickened in this region (fig. 3A, B).

Variations among liolemid taxa and outgroups (hemipenis)

The general morphology of species studied here is easy to discriminate. The hemipenis of *C. adspersa* has a simple terminal ending without lobes. The *Phymaturus* species show slightly bilobed hemipenes, and this terminal separation is more evident in *Liolaemus* species (fig. 3). Both *Diplolaemus* and *Tropidurus melanopleurus* show strongly bilobed hemipenes (fig. 4). Hemipenis on its apex surface can exhibit ornamentation, with the exception of *C. adspersa*, which has a smooth surface lacking any structure or particular morphology. Both species of *Liolaemus* show expansions of its surface, similar to valves, while *Phymaturus* species present the lips of the apical region of the spermatic
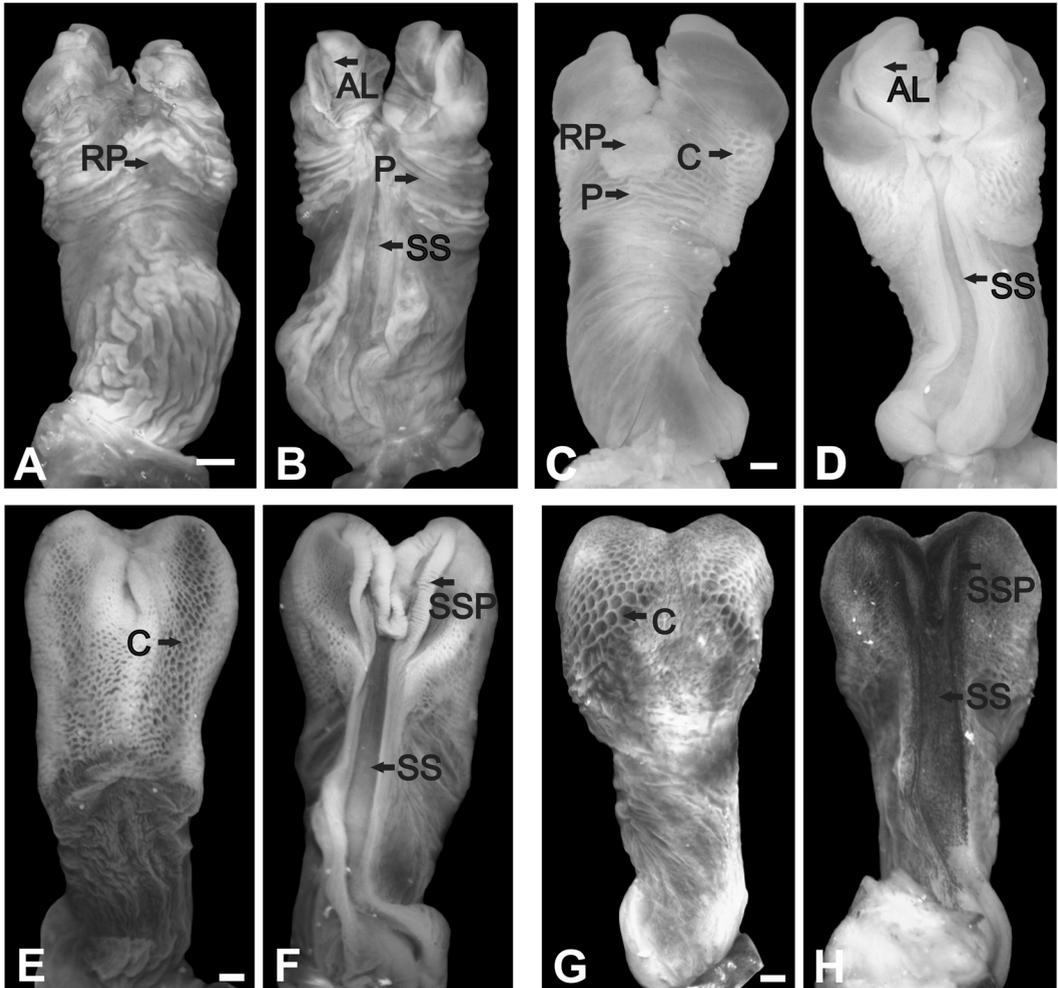
Figure 3. (A) Hemipenis asulcate face of *Liolaemus irregularis* (MCN 1884). (B) Hemipenis sulcate face of *L. irregularis*. (C) Asulcate face of *Liolaemus austromendocinus* (MCN 3686). (D) Sulcate face of *L. austromendocinus*. (E) Asulcate view of hemipenis of *P. patagonicus* (MCN 3275). (F) Sulcate face of the hemipenis of *P. patagonicus*. See dark pigmentation of the sulcus spermaticus in this species. (G) Asulcate face of the hemipenis of *P. palluma* (MCN 2894). (H) Sulcate face of the hemipenis of *P. palluma*. The arrow indicates the extended ornamentation in *P. patagonicus*. Abbreviations = C: calices; P: plicae; SS: sulcus spermaticus; AL: apical lobes; RP: round prominence; SSP: sulcus spermaticus plicae. Scale = 0.5 mm.

groove forming plicae. Both outgroups, *D. leopardinus* and *T. melanopleurus*, exhibit calyces in the majority of the surface of the apex. A distinctive round prominence is present in the asulcate side of the hemipenes of *C. adspersa* and in both species of *Liolaemus* (figs 3A, C and 4A), yet which is absent in the other genera.

The hemipenes are completely white, lacking any kind of pigmentation in most species, although only in *Phymaturus palluma* and *P. laurenti* there are some kind of pigmentation.

Phymaturus palluma shows dark scarce pigments along the sulcus spermaticus while *P. laurenti* exhibits a more melanistic hemipenis along the sulcus and also all over lobes surfaces (fig. 3H). On the sulcate side, the hemipenis of *C. adspersa* and *P. patagonicus* show no ornamentation (smooth); *L. irregularis* presents plicae, while the rest of species present calyces of different size and extension. The asulcate side *C. adspersa* and *L. irregularis* is ornamentated
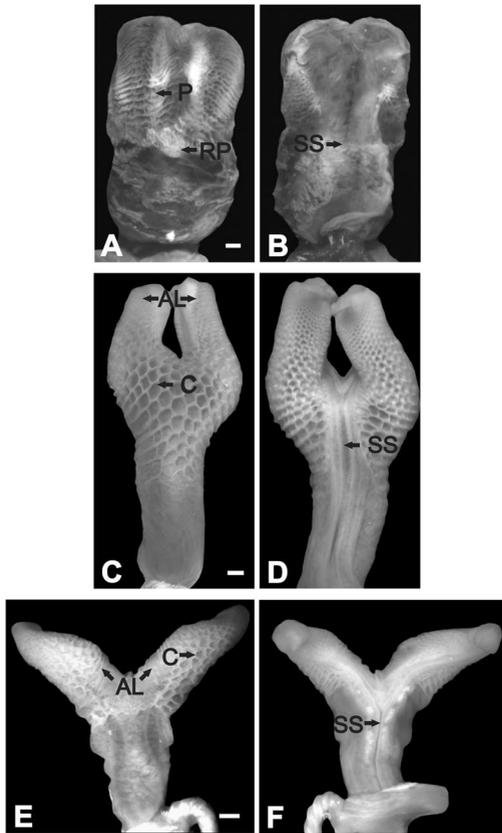
Figure 4. (A) Asulcate view of hemipenis of *Ctenoblepharys adspersa* (MUSA 4613). (B) Sulcate face of the hemipenis of *C. adspersa*. (C) View asulcate face of hemipenis of *Diplolaemus leopardinus* (IBIGEO 5493). (D) View sulcate face the hemipenis of *D. leopardinus*. (E) View asulcate face of hemipenis of *Tropidurus melanopleurus* (IBIGEO 5317). (F) Sulcate face the hemipenis of *Phymaturus T. melanopleurus*. Scale = 0.5 mm.

with plicae (17 in *C. adspersa* and 7 in *L. irregularis*). The three species of *D. leopardinus*, *Phymaturus*, and *T. melanopleurus* exhibit only calyces. Meanwhile, *L. austromendocinus* shows calyces and plicae. The hemipenes size and proportions are diverse. There is some variation in the length of the hemipenes. Our values are only illustrative because we lack a larger sample to get statistically confident results (*n* = 15). *Diplolaemus leopardinus* exhibits a hemipenis with a 16% of the SVL length, *L. austromendocinus* 10%, *P. patagonicus* and *L. irregularis* 9%, *T. melanopleurus* 8%, *P. palluma* 7% and *C. adspersa* 6%. The hemipenis width at its

half-length with respect to the hemipenis length is larger in *C. adspersa* (48%), *P. patagonicus* (47%) and *L. irregularis* (40%) with respect to the rest of species: *L. austromendocinus* (34%); *P. palluma* (25%), *P. laurenti* (26%); *D. leopardinus* (23%); *Tropidurus melanopleurus* (19%). Distal width of hemipenes at the level of lobes/hemipenis length ratio is as follows: *T. melanopleurus* 75%; *P. patagonicus* 76%; *C. adspersa* 75%; *P. laurenti* 49%; *P. palluma* 47%, *L. austromendocinus* 46%; *L. irregularis* 42%; *D. leopardinus* 25%.

The sulcus spermaticus looks wide and quite conspicuous in *C. adspersa* at a simple examination (20% of hemipenis length), while that of other species ranges between 3-10%.

Phylogenetic analyses

In the first case (fig. 5A, B) we run the data matrix building trees using parsimony (TNT). In the second case, we optimized characters onto the most accepted topology of the family (fig. 5C). Character numbers shown in the figure 5 are described in the character list and their corresponding coding in the online Supplementary material (table S1).

Independent analysis. Our set of characters was analyzed rooted in *Tropidurus melanopleurus*, recovering four most parsimonious trees (68 steps). Figure 5A shows the strict consensus tree. Liolaemidae is supported by five apomorphies: 1(1), 19(2), 22(1), 25(1), and 27(2). *Ctenoblepharys* and *Liolaemus* were recovered as sister taxa supported by several characters: 3(0, 1), 5(1), 8(1), 19(1), 20(0), 24(0), 25(2) and 26(1). *Phymaturus* was recovered as non-monophyletic. In figure 5B we changed the root of the analysis (now *Diplolaemus leopardinus*). There were no differences with the first analysis.

Character mapping. To find out how muscles and genitalia characters fit into the phylogeny of Liolaemidae, the relationships were forced (*Ctenoblepharys* (*Phymaturus* + *Liolaemus*)) (fig. 5C) as was proposed in the molecular
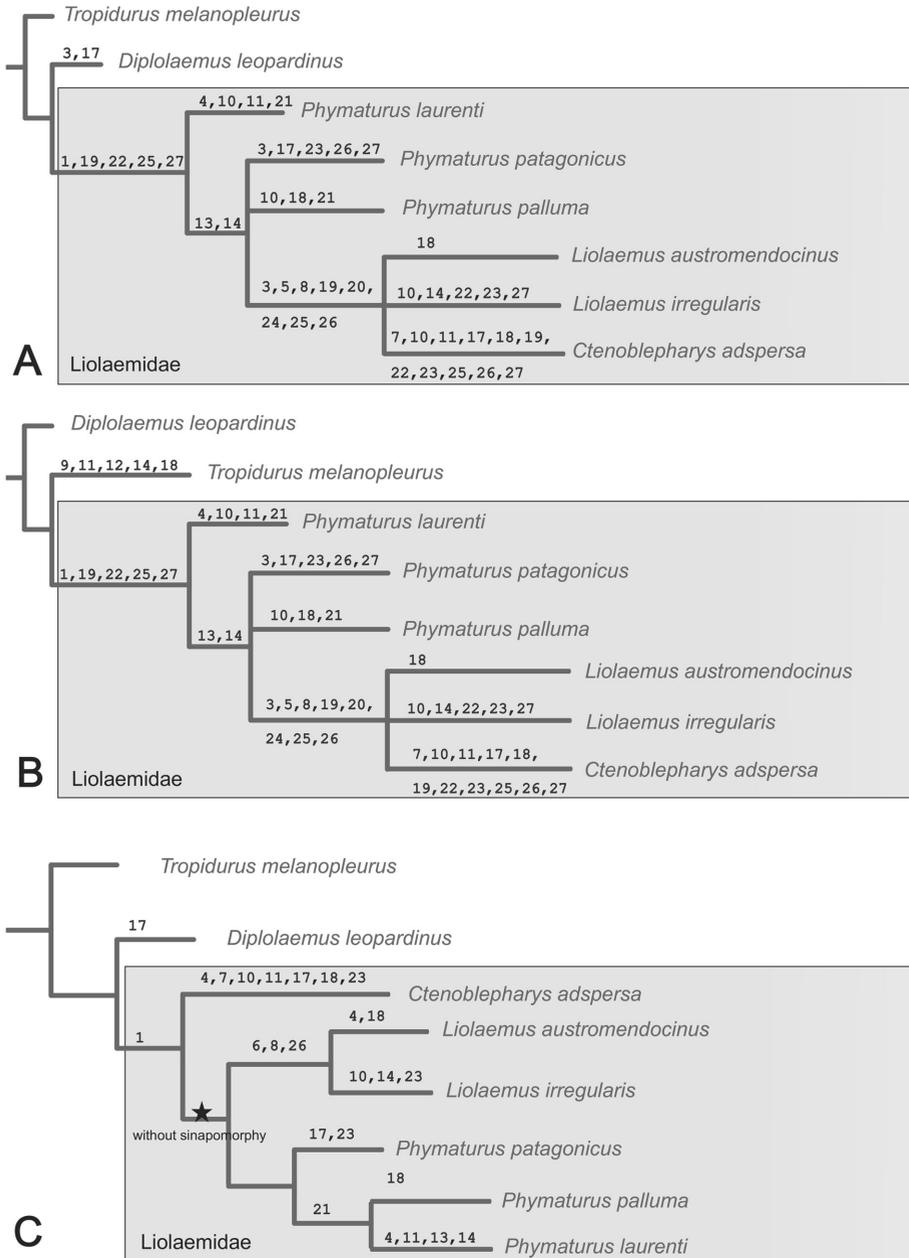
Figure 5. Trees obtained from the data set of this work. (A) Consensus tree recovered performing an analysis of the present anatomical data with the *Tropidurus melanopleurus* as outgroup. (B) Consensus tree of the same analysis but rooting the tree with *Diplolaemus leopardinus*. (C) Rebuilt tree with *Ctenoblepharys adspersa* as the basal taxon in the Liolaemidae family following relationships recovered molecular and morphological in previous studies. Numbers on branches (apomorphies) correspond to characters described in the character list (see also table S1).

analyzes of Gauthier et al. (2012); Pyron, Burbrink, and Wiens (2013); Morando et al. (2013); Reeder et al. (2015); and Zheng and Wiens,

(2016). Liolaemidae is supported by only one character 1(1). There are no apomorphies for the pair of genera *Liolaemus* + *Phymaturus*, or for

the genus *Phymaturus*. *Ctenoblepharys* exhibits several apomorphies: 4(1), 7(1), 10(3), 11(2), 17(0), 18(0) and 23(0).

Discussion

Comparisons with Arnold's study

Arnold (1984, fig. 4d) described a pattern for tropidurines that consists in a well-developed muscle surrounding the hemipenis 80-90%, due to its poorly developed fascia. In the species studied here, *T. melanopleurus*, the two species of *Liolaemus*, the three of *Phymaturus* and *D. leopardinus* exhibited other condition, where the muscle covers the lateral and ventral side of the hemipenis while the fascia is well extended covering the medial and dorsal sides of the organ. This disposition corresponds to the pattern described by Arnold (1984) in its figure 4b (character 19 of his table 1).

Arnold (1984, fig. 4c) describes the presence of dorsal accessories and ventral sheath muscles located under the transversus penis. He remarked that these structures are present or absent within tropidurines (unfortunately he gave no specific indication about the specimens of *Liolaemus*, *Phymaturus*, and *Tropidurus* examined). These muscle sheaths are absent in all of our studied species.

Arnold (1984) described that lateral retractors can be situated closely between each other at their origins (caudal vertebrae) or fused. In *Ctenoblepharys adspersa*, both lateral retractors originate in the same caudal vertebra, while *Liolaemus* and *Phymaturus* exhibit different areas of attachment (different vertebrae) since the retractor lateralis posterior is attached posterior to the retractor lateralis anterior.

Arnold (1984) indicates, in his table 2, a variation within tropidurines (present or absent) but information of his representative species is lacking and therefore we are not able to make comparisons. We provide the precise sites of the variations in the origin of that muscle among taxa. The origin areas of the retractor lateralis

posterior are also detailed in the present descriptions. We agree with Arnold's (1984) description regarding the origin of the retractor lateralis posterior without forming a tendon. The overall morphology, origins and insertions, size, and proportions of muscles, location, presence/absence, etc. are quite similar to the basic plan described by Arnold for squamate reptiles. Exceptions are due the presence of a couple of muscles not reported by him that we discuss below.

Arnold (1984) describes the ischiocaudalis and the iliocaudalis muscles as pairs of unique muscles, called the ilio-ischiocaudalis. Our observations allow us to discriminate them as independent muscles (in both origins and insertions) in all taxa studied. Ritzman et al. (2012) described this muscle in *Anolis caroliniensis* as originating mostly via fleshy fibers from the dorsal aspect of the ischium (such as the species studied here) and inserted in the ventral aspect of the transverse processes. The muscle ischiocaudalis is hypothesized to be a tail flexor when it contracts bilaterally, and a lateral flexor of the tail when it contracts unilaterally (Ritzman et al., 2012). We observed in both species of *Liolaemus*, three species of *Phymaturus*, *T. melanopleurus*, and *D. leopardinus*, that the ischiocaudalis is attached to the ischium by a strong rounded tendon (Haines, 1935; Arnold, 1984) whereas in *Ctenoblepharys adspersa* there is no tendon and the ischiocaudalis muscle has a fleshy insertion into the ischium. These morphological differences can be easily correlated to functional differences, perhaps functioning as spring stores and saving energy (Biewener, 1998). Short-fibered muscles also often transmit force via long tendons, which provide elastic energy savings that may further reduce metabolic cost (Biewener and Gillis, 1999). There is a trade-off however, in that although an increased tendon length favors greater elastic recovery, it constrains the muscle's ability to control changes in length (Biewener and Roberts, 2000). Lack of tendon in *Ctenoblepharys* could thus have relevance in

terms of muscle utilization and energy expenditure (Liebe, Brown, and Trestik, 1992; Fukunaga et al., 2002).

Previously unreported muscles

Posterior cloacal retractor (PCR).   This muscle is present in all species except in *C. aspersa*. It originates in the connective fascia that separates the ischiocaudalis from the transverse penis, in the proximity of the cloacal opening it turns deep, reaching the roof of the cloacal chamber. Its insertion is clearly the roof of the cloaca. In *L. irregularis*, this muscle is superficial in position, quite conspicuous in ventral view (figs 1A, B; 2C) but in other species it is covered partially by the transversus penis and less conspicuous as in *T. melanopleurus* (fig. 2D).

Anterior cloacal retractor (ACR).   Its origin is located in the pubis, in the margin of the acetabulum, and goes deep, reaching the area of insertion of the posterior retractor cloacalis (PCR) meeting together and separated by a thin sheet of connective tissue (fig. 2A).

Superficialis cloacalis retractor.   We were only able to find this muscle in *Liolaemus irregularis* (fig. 1A, C) (absent in *L. austromendocinus*, and the three species of *Phymaturus*, *T. melanopleurus*, and *D. leopardinus*). Without studying other species of *Liolaemus* we are not confident in identifying the presence of this particular muscle as an autapomorhy of *L. irregularis* or proposing it as apomorphy of a major clade (i.e. the *boulengeri* group). The disposition of this muscle connecting the posterior fascia of the transversus perinei and the floor of the cloaca under the precloacal glands could help the cloacal opening to facilitate the expulsion of feces and/or the eversion of hemipenis. Some influential motion could be transmitted to the precloacal glands, thus provoking its secretion. However, all these hypotheses should be tested with specific experiments.

Lereboullet (1851) describes two muscles in *Lacerta agilis*: a lateral and a lower dilator, the first one run along the lateral wall of the cloaca between the ischium and the ventral wall of the cloacae. The lower dilator shows its origin along the cartilaginous hipoischium and inserts to the ventral wall of the cloaca (floor). Because these particular arrangements are different from those described above in liolemids, *Diplolaemus*, and *Tropidurus*, we consider that the Lereboullet's muscles are not homologous to our retractor cloacae superficialis and the retractor anterior cloacae.

Haines (1935) described the cloacal muscle in *Iguana* and *Varanus* as a narrow slip, which arises from the inner aspect of the pelvis at the level of the acetabulum and extends back laterally to the cloaca to be inserted on the first haemal spine. Opposite to the cloaca, the fibers are interrupted by an intermediate tendon. This muscle resembles those described here as the retractor cloacal anterior and retractor cloacal posterior, but the latter is not inserted in the haemal spine as described by Haines (1935). In Liolaemidae, *Diplolaemus leopardinus*, and *Tropidurus melanopleurus* it is inserted in the fascia of the ischiocaudalis muscle.

Sexual dimorphism in genital and caudal musculature

Valdecantos and Lobo (2015) described the presence of hemiclitoris in *Liolaemus* and *Phymaturus* females. In this work not only did we corroborate the presence of hemiclitoris in *Liolaemus* and *Phymaturus*, but we also report them for *Ctenoblepharys adspersa*, *Diplolaemus leopardinus* and *Tropidurus melanopleurus*. We have observed that there is a sexual differentiation with respect to the degree of development of the musculature of the cloacal region, as has been mentioned previously (Arnold, 1984; Russell and Bauer, 1992; Barbadillo et al., 1995; Russell, Bergmann, and Barbadillo, 2001; Valdecantos and Lobo, 2015). Additionally, the females present a lower number of pre-autotomic vertebrae in all species (with the exception of *D. leopardinus* and *L.*

austromendocinus for which we have no skeletal preparations at this time), a feature already mentioned by Barbadillo et al. (1995) and Russell et al. (2001).

Associated with the beginning of the first caudal (pre-autonomic) vertebrae is the origin of the caudofemoralis muscle (Etheridge, 1967). According to Russell and Bauer (1992), Barbadillo et al. (1995), and Russell et al. (2001), in the autotomic species the origin of the caudofemoralis and the retractor hemipenis magnus muscle usually extend to the last pre-autotomic vertebra, while they are longer in non-autotomic species. Russell and Bauer (1992) worked with species belonging to different families of lizards, and observed that in the autotomic species the origin of the caudofemoralis muscle extends to the last preautotomic vertebra, with a correlation between the last non-autotomic vertebra and the most distal insertion of this muscle. They emphasize the importance of this muscle in the locomotion of the animal because autotomy could generally occur posterior to that level. In *Podarcis hispánica*, there is an overlap between the onset of the origin of the caudofemoralis longus muscle and the first autotomic vertebrae (Russell et al., 2001). These authors argue that the muscle does not originate from the bone or periosteum, but from a surrounding septal membrane, which envelops it along with the retractor penis muscle. Consequently, the caudofemoralis longus can be inserted into the new skeleton of cartilage when the tail is regenerated. In the studied species, we find the same correlation as described by Russell and Bauer (1992). In *C. adspersa* females, the autotomic vertebrae begins from the fifth caudal vertebra and their retractor muscle hemiclitoris magnus and caudofemoralis run until the fourth. In males of *C. adspersa*, these muscles are larger and project backward, reaching the seventh and eighth vertebrae (the autotomic vertebrae start at a more posterior level than in females). In males of *Ctenoblepharys*, the retractor penis magnus reaches the seventh or eighth caudal vertebra just at the level of the autotomic

vertebrae. A similar case was observed in the females of *L. irregularis*, which present caudal autotomy from the sixth caudal vertebrae, while in males from the seventh. Likewise, in males of *P. laurenti* the retractor penis magnus begins from the eleventh caudal vertebra while in females from the ninth. In *C. adspersa*, the iliocaudalis e ischiocaudalis reach the seventh caudal vertebra.

Compressor glandulae. his muscle was reported by Arnold (1984) for *Sphenodon punctatus*. In his Figure 8 it is shown related to an "anal gland" according to Günther (1867) or "scent gland" of Gadow (1887). Arnold (1984) reported both muscle and gland as larger in males than in females. Because he did not mention the variation of the compressor glandulae across Squamata, it is possible that he found it in all taxa studied. Here we found this gland and its associated muscle only present in males of the four genera. But there is a homology uncertainty at this time, because of the many different kinds of glands described for the proctodeum region, such as cloacal glands and paracloacal glands (Sanchez-Martinez et al., 2007) and anal glands for snakes (Gabe and Saint Girons, 1965). Apparently, those cloacal glands were proved to be important in chemical communication, like in *Eumeces* species (Cooper and Vitt, 1984, 1986). The gland with its associated muscle is found here with a lateral-ventral position, close to the corner of the cloacal opening. This gland was histologically described by Valdecantos, Martinez and Labra (2015) for *Liolaemus coeruleus*, *L. irregularis*, and *L. poecilochromus*, although its function remains unknown.

Hemipenes

The morphology of the hemipenis is particular for each studied genus. In *Liolaemus* and *Phymaturus*, it is consistent with the observations made by Lobo (2000). Thus, it is possible to differentiate both subgenera of *Liolaemus* by the presence of calices in the sulcate face in *Liolaemus* sensu stricto that, in some

cases, can be accompanied by plica, while in *Eulaemus* there are exclusively plicae. We agree with Böhme (1988), Lobo (2000) and Valdecantos and Lobo (2015) in that only *Phymaturus*, *Tropidurus*, and *Diplolaemus* present calices in both faces. The presence of pigments in the hemipenes separates the *palluma* clade of the *patagonicus* clade in which the pigmentation is absent (Valdecantos and Lobo, 2015). Pigmentation in the sulcus spermaticus or extended to the surface of the lobes was listed as a character in the recent phylogenetic analysis of the *palluma* group by Lobo et al. (2016).

The presence of calices in the sulcate face of the hemipenis is a plesiomorphic condition for liolemids because it is already found in *Tropidurus* and *Diplolaemus*. The formation of plicae in the same face of the hemipenis is a derived feature that arises in *Liolaemus* and *Ctenoblepharys*. If these genera are sister taxa, the presence of plicae was originated once in the evolution of liolemids. If we consider *Liolaemus* more closely related to *Phymaturus* then two scenarios are possible: plicae originated independently in *Ctenoblepharys* and *Liolaemus*, or it is a plesiomorphy of these two, and the character reverted to its primitive condition in *Phymaturus*, loosing plicae and forming calices as in *Diplolaemus* and *Tropidurus*.

Muscle anatomy and the information on relationships of and within Liolaemidae

The phylogenetic position of Liolaemidae in the family tree of squamate reptiles has changed in the last ten years. Traditionally, Liolemids were found related to tropidurids, and the category of subfamily or even tribe of Tropiduridae was assigned. Now, Leiosauridae and Opluridae are recovered as more closely related taxa to Liolaemidae (Gauthier et al., 2012; Wiens et al., 2012; Pyron et al., 2013; Reeder et al., 2015). To assess the evolutionary change of characters within Liolaemidae we used *T. melanopleurus* and *D. leopardinus* as outgroup. Unfortunately, we were unable to obtain any oplurid

species, and thus it is now an aim of our research in the future. Muscle and genital characters do not support the monophyly (or do so extremely weakly) of Liolaemidae, *Liolaemus* plus *Phymaturus* and the genus *Ctenoblepharys* (see fig. 5C). However, there is contradictory information; the anatomy studied here supports the family monophyly but relates *Ctenoblepharys* to *Liolaemus* and breaks the monophyly of *Phymaturus*. The relationship of *Ctenoblepharys* as sister taxon of *Liolaemus* was recovered by (Frost and Etheridge, 1989, pag. 26, fig. 14). They cited Arnold's character referring to the "fleshy" retractor lateralis posterior insertion. We observed the same muscle morphology and insertion in all genera studied. Here we find several other derived characters shared by these two genera (fig. 5A, B). The recovering of this relationship is not rare because in the past *C. adspersa* was related to other species now considered as *Liolaemus* (Etheridge, 1995). There is a great phenotypic similarity among species once named as *Phrynosaura* or even *Ctenoblepharys* (Etheridge, 1995) that inhabit northern Chilean deserts, not far from the type locality of *C. adspersa*. Current molecular analyses are not decisive since they show a polytomy among the three genera (Schulte, Valladares and Larson, 2003 and Pyron et al., 2013), performing maximum likelihood, found *C. adspersa* as basal. The most informative loci have not been used until now (12S, ND4, Cytb, COI, or even nuclear). From a biogeographic point of view, analyses were made by applying ancestral areas optimization methods that obtained some contradictory results: *C. adspersa* is found in coastal Peru deserts, while the rest of the basal groups of *Phymaturus* and *Liolaemus* inhabit southern South America, the Patagonia region (Díaz Gómez, 2009). Taking all the discussed evidence above into account, we believe further morphological and molecular studies are needed to solve this controversy.

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