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Structure, variation, and systematic implications of the hemipenes of liolaemid lizards (Reptilia: Liolaemidae)

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Draft

Abstract

The structure of copulatory organs is widely used in systematics for both differentiating species and for studying phylogenetic relationships. We describe the hemipenes of 42 species belonging to the genus *Liolaemus*, representing most of their internal groups. We reported 42 characters, the majority not published previously. We constructed a metatree based on previously proposed phylogenetic studies and optimized the hemipenial characters in this topology. Among the most informative characters are: presence or absence of flounces or calyces on the sulcate face, ornamentation of the apex, presence or absence of an asulcate face prominence and presence of a thickening on the proximal region of the asulcate face. Furthermore, we performed a phylogenetic analysis exclusively with the hemipenial characters, not with the intention of making a phylogeny based on this single set of characters, but rather to demonstrate their significance for the reconstruction of relationships in *Liolaemus*. The obtained results show that the main clades are recovered. We also compared the hemipenial morphology between closely related species to evaluate its taxonomic importance. We conclude that in *Liolaemus* the hemipenes can be used for both the differentiation of species and to provide additional evidence for establishing their phylogenetic relationships.

Key words: *Liolaemus*, Hemipenes, Morphology, Phylogeny, Male genitalia.

Introduction

In many groups of animals, copulatory organs provide a very significant proportion of the characters used in systematic studies. This especially applies to the intromittent organs of males, which are widely used in the taxonomy of, among others, beetles, butterflies and moths, grasshoppers, mollusks, some live-bearing fish and elasmobranchii, snakes, lizards and some mammal groups (Sharp and Muir 1912; Hamilton 1946; Gordon and Rosen 1951; Jaennell 1955; Tuxen 1970; Higgins 1975; Arnold 1986*a*; Fitzpatrick et al. 2012; Klaczlo et al. 2015; D'Angiolella et al. 2016). Indeed, genital features of invertebrates are so important that accounts of their variations numbering up to hundreds of pages have been produced (Sharp and Muir 1912; Jaennell 1955; Higgins 1975) as well as a substantial glossary of insect genitalia in general (Tuxen 1970). Big differences in male genitalia often allow us to easily distinguish between closely related species. Yet, in some cases, the organs are very uniform in certain aspects of their morphology and are characteristic of a whole genus or other higher taxa. At higher level taxonomic categories (genera, subfamily, etc.) different groups have copulatory organs that share similar derived features (Böhme 1988; Keogh 1999; Maduwage et al. 2008; Köhler 2009). The greater the phylogenetic distance between taxa does not always equal greater differences in genital morphology; it is common to find some markedly different genitalia between very closely related taxa (Böhme 1988; Arnold 1986*a*; Lobo 2000; Köhler et al. 2010; Köhler et al. 2012).

The genitalia of squamate males consist of a pair of intromittent organs called hemipenes which were recently proven to be homologous to the other phalluses of amniota (Gredler et al. 2014). The hemipenes are tubular structures that are retracted into the base of the tail when not in use but are everted during copulation. Each hemipenis surface contains a groove, the sulcus spermaticus, through which semen is transported. Hemipenial morphology varies extensively among squamate taxa, from cylindrical tubes to deeply bilobed structures ornamented with calyces, papillae, flounces, and spines (Dowling and Savage 1960), and thus has been used extensively for systematic studies on snakes

(Dowling and Duellman 1978; Zaher 1999; Myers and Donnelly 2001; Schargel and Castoe 2003; Schargel et al. 2005) and, to a lesser extent, on lizards (Arnold 1983, 1986a; Böhme 1988; Köhler et al. 2012; Nunes et al. 2012; Klaczko et al. 2015).

The first report of hemipenial morphology for liolaemids is a brief description of the hemipenes of *Phymaturus palluma* (Molina, 1782) by Böhme (1988). Structure and variation of male copulatory organs in *Liolaemus* lizards have been reviewed by Cei (1986, 1993) and Lobo (2000), yet lacking detailed descriptions. Cei (1986, 1993) showed some observations of the hemipenes of *Liolaemus* (14 species), where he pointed out a homogeneous morphology and the almost complete lack of ornamentation, only indicating three species in one of his pictures. Lobo (2000) reported the morphology of 18 species of *Liolaemus* and *Phymaturus dorsimaculatus* (Lobo and Quinteros, 2015) (*Phymaturus* is actually the sister genus of *Liolaemus*), where he described the organ for *L. chacoensis* (Shreve, 1948) and *L. chiliensis* (Lesson, 1830) and highlighted the main differences between both subgenera of *Liolaemus* (*Eulaemus* and *Liolaemus sensu stricto*).

The aim of this study is to provide an in-depth report of the hemipenial morphology of 42 species of the genus *Liolaemus*, including subgenera and subclades currently recognized within them. Furthermore, we emphasize the importance of this data set in the phylogeny and taxonomy of *Liolaemus*, thus providing extra validation of previous findings in the literature about the use of hemipenial characters as systematic tools (as in the citations above).

Materials and methods

All specimens are deposited in the Herpetological Collection of the Instituto de Bio y Geociencias del NOA (IBIGEO), the Herpetological Collection of the Fundación Miguel Lillo (FML), and Herpetological Collection of the Universidad Nacional de San Juan (UNSJ). Sample sizes vary from one hemipenis to four hemipenes per species. Small sample sizes are adequate for hemipenial

descriptions because intraspecific variation is generally slight, and the variation that may be present often only reflects artifacts of preservation (Arnold 1986 *a, b*; Böhme 1988; Keogh 1999). Details of specimens studied are listed in supplementary material (File S1).

We study hemipenes from representatives of 30 species of the subgenus *Eulaemus*: *L. montanus* section (*Sensu* Schulte et al. 2000): *L. boulengeri* series (17 species); *L. montanus* series (seven species); and *L. lineomaculatus* section (six species); and 11 species of the subgenus *Liolaemus sensu stricto*: *L. elongatus*–*petrophilus* group (three species); *L. pictus* group (one species); *L. alticolor*–*bibronii* group (seven species). Furthermore, we studied *Ctenoblepharys adspersa* (Tschudi, 1845) and three species of *Phymaturus* (*P. laurenti*, Lobo et al., 2010; *P. palluma*; and *P. patagonicus*, Koslowsky, 1898), considered to be an outgroup for phylogenetic and evolutionary interpretations of the anatomical characteristics found in *Liolaemus* (see supplementary material, file S1, for details).

We followed Zaher and Prudente (2003) protocol for preparing snake organs. One of the hemipenes from each fixed specimen was removed through a small incision at the base of the tail. The removed organ was immersed in a 2% KOH solution for 3–5 min or until it became translucent and flexible. The hemipenis was checked and everted manually using forceps to be sure the whole hemipenis was completely everted. Once fully everted, the organ was filled with colored vaseline to allow better visualization of ornamentation structures. We obtained digital images of hemipenes in sulcate, asulcate, and lateral view using a camera (Olympus DP25) attached to a stereomicroscope. Measurements were taken using a digital caliper (0.02 mm of precision) and an ocular micrometer. Terminology of hemipenial character states follow that of Dowling and Savage (1960), Savage (1997), Zaher (1999), Ziegler and Böhme (1999), and Zaher and Prudente (2003).

We constructed a matrix including 42 morphological characters taken from hemipenes. Phylogenetic analyses were performed with TNT v.1.5 (Tree analysis using New Technology Goloboff et al. 2003). We performed an analysis under implied weights (using the methodology proposed by

Mirande 2009). Discrete characters were coded according to their variation: binary or multistate. The continuous characters were also coded as such, following the methodology of Goloboff et al. (2006). Nodes support were measured with bootstrapping, performing 500 replicates. We included 45 terminal taxa: 41 species of *Liolaemus* representing our ingroup, and three species of *Phymaturus* and *Ctenoblepharys adspersa* used as outgroup (See Table S1 and S2).

In addition, we optimized the characters in the most recent phylogenetic topology within the family. Since there is no phylogeny for *Liolaemus* that includes all terminal taxa analyzed here, we reconstructed a metatree following Schulte et al. (2000), Espinoza et al. (2004), and Pyron et al. (2013) for the entire genus, Avila et al. (2006) and Abdala (2007) for the *L. boulengeri* series, and Lobo (2001, 2005), Avila et al. (2015) and Quinteros (2013) for the *Liolaemus* subgenus and the groups within.

Results

List of character states and descriptions are included in supplementary File S2. Variation of character states of continuous and discrete characters are listed in supplementary material (Table S1 and S2).

General descriptions of the hemipenes of the groups recovered in our phylogenetic analyses.

Hemipenes of *Liolaemus* show two kinds of ornamentations (flounces and calyces) which are located in both sulcate and asulcate face (Figure 1). The presence of one (flounces or calyces) does not exclude the presence of the other. Flounces are step-shaped structures, transversal to the hemipenis (Figure 1). On the other hand, calyces are reticulated structures. Generally, ornamentations are distributed in the upper third of both sulcate and asulcate faces of the hemipenis, although in some cases they can be extended all over the upper half of the organ, or even more. In the proximal region (base of hemipenis) the sulcus spermaticus shows a bag-shaped structure on one or both edges (Figures 1, 2 and 3). Species belonging to the subgenus *Eulaemus* exhibit their sulcate face of the hemipenis naked or

presenting flounces, but never calyces. On the other hand, the asulcate face can have both kinds of ornamentations at the same time (Figures 1 and 2). Within the *lineomaculatus* section of *Eulaemus*, species have a slenderer hemipenis than species of the *montanus* section. The apex shell-shaped show its inner edge thickened and with elliptical shape. Calyces always are present in the asulcate face while flounces are observed in some species. The proximal region (base of hemipenis) of the asulcate face shows a thickened area. The asulcate face prominence is absent. (Figures 1 and 2, A–C). Members of the *montanus* section show robust hemipenes with a cup-shaped apex. Their asulcate face have flounces, but never calyces. Their asulcate faces show a conspicuous prominence in the middle region of the truncus, never show a thickened area in the basal region as in the *lineomaculatus* section. (Figure 2, D–I). Species belonging to the *boulengeri* series of the *montanus* section exhibit an asulcate face their conspicuous prominence in the middle region of truncus, generally forward pronounced. The asulcate face has smooth flounces, not serrated. (Figure 2, D–F). Members of the *montanus* series show an asulcate face with their conspicuous prominence in the middle region of truncus rounded or upwardly pronounced, never forward pronounced. Flounces of the sulcate and asulcate face are always serrated. (Figure 2, G–I).

Hemipenes of the subgenus *Liolaemus sensu stricto* present calyces always in the asulcate face and flounces are observed in some species; the distal margin of the apex is elongated, either horn-shape or rounded. The flounces of the sulcate face are narrow and the edge of the calyces can be smooth or wavy (Figure 3). Within the subgenus *Liolaemus*, species of the *elongatus–petrophilus* group show a shell-shaped apex (different from that described above for *Eulaemus*) that we call from now “shell-shaped2”. Calyces in this group exhibit smooth edges. Ornamentations of the sulcate face are distributed only in the upper third of the truncus, never reaching the middle line. Flounces may be present in some species. (Figure 3, A–C). The *pictus* group, represented here only by *L. coeruleus* (Ceii and Ortiz-Zapata, 1983), shows hemipenes with a rounded apex and their calyces are evident on both

sulcate and asulcate faces. These calyces are deep with wavy edges. (Figure 3, D–F). Hemipenes of the *alticolor–bibronii* group show a horn-shaped apex. The apical lobules are well-developed and sulcate face has smooth calyces, never flounces. (Figure 3, G–I).

A phylogenetic hypothesis built using only the hemipenial characters (Figure 4)

We obtained a tree with 12,69 of fit. The numbers in brackets indicate character (see supplementary material File S2: character list for more details).

We recovered the genus *Phymaturus* as monophyletic, supported by the presence of uniform calyces over truncus and lobes of the asulcate face (Char. 37).

The *Liolaemus* genus is recovered as monophyletic supported by five synapomorphies: a higher ratio between the length of the hemipenis and the SVL (Char. 0). Lower ratio between the width of the sulcus spermaticus and the length of the hemipenis (Char. 3). Lower ratio between the width of the proximal region and the middle region with respect to hemipenis length (Chars. 4 and 5). The hemipenis of *Liolaemus* is more tubular than the hemipenis of *Ctenoblepharys* and *Phymaturus* (Char. 10).

The subgenus *Liolaemus sensu stricto* is recovered as monophyletic, supported by four synapomorphies: Lower ratio between the width of the proximal region and the middle region with respect to hemipenis length (Chars. 4 and 5). Calyces of the sulcate face occupy the region between the lobules (Char. 26). Calyces are not uniform (Char. 27). Species of the *elongatus–petrophilus* group are recovered basal inside the subgenus *Liolaemus*, but not forming a monophyletic group. The *alticolor–bibronii* group is recovered as monophyletic, supported by two synapomorphies: apex showing horn-shaped ornamentations (Char. 7), and well-developed lobules (Char. 11).

The *Eulaemus* subgenus is recovered as monophyletic, supported by two synapomorphies: flounces always present in the asulcate face (Char. 16) and 6–8 flounces in the sulcate face (Char. 22). The *lineomaculatus* section is recovered as monophyletic, supported by three synapomorphies: higher

ratio between the distance where the sulcus spermaticus bifurcate and the hemipenis length (Char. 2), the internal edge of the apex is elliptical (Char. 14) and the basal region of the truncus of the asulcate face is thickened (Char. 38). The *montanus* section is recovered as monophyletic, supported by five synapomorphies: Small bulge at the bifurcation of the sulcus spermaticus (Char. 23); only flounces shown on asulcate face (Char. 25); two flounces over lobules (Char. 34); five discontinuous flounces in the asulcate face of the truncus (Char. 35); and asulcate face shows a conspicuous prominence in the middle region of the truncus (Char. 40). The *boulengeri* series is paraphyletic, recovered within the species members of the *montanus* series. The *montanus* series is recovered as monophyletic, nested within the *boulengeri* series. It is supported by two synapomorphies: poorly-developed and joined lobules (Chars. 11 and 12).

Hemipenial character optimization on the known interspecific phylogenetic metatree (Figure 5).

Hemipenes were plotted on a metatree, constructed based on the most recent phylogenetic hypotheses for the *Liolaemus* genus. Relationships of terminal taxa studied here are shown in Figure 5. Numbers indicate character (see supplementary material File S2: character list).

The genus *Phymaturus* is supported by the presence of globular hemipenes, showing exclusively uniform and symmetric calyces over truncus and apex of the sulcate and asulcate face (Char. 37).

The genus *Liolaemus* is supported by the ratio between hemipenis length and SVL (Char. 0), lower ratio between the width of the sulcus spermaticus and hemipenis length (Char. 3), lower ratio between the width of the middle proximal region and middle distal region and hemipenis length (Char. 4 and 5), and the tubular shape of the hemipenis (Char. 10).

The subgenus *Liolaemus sensu stricto* is supported by the distribution of the calyces on the sulcate face; the calyces never reaching the lobules (Char. 26), the presence of non-uniform calyces (Char. 27), and the middle region of the truncus of the asulcate face shows a thickened area (Char. 39).

The *alticolor–bibronii* group is supported by the lower ratio between the width of the apex and the hemipenis length (Char. 6) and the presence of developed apical lobules (Char. 11). The *elongatus–petrophilus* group is supported by the presence of 4–5 flounces on the truncus of the asulcate face (Char. 22). The *pictus* group is supported by the higher ratio between hemipenis length and SVL (Char. 0), lower ratio between the width of the middle and proximal region of truncus and the hemipenis length (Chars. 4 and 5), the presence of a rounded apex (Char. 7), the presence of deep flounces on truncus of the sulcate face (Char. 20), and the calyces of the sulcate face are thick with wavy edges (Chars. 29 and 30).

The *Eulaemus* subgenus is supported by the presence of flounces, exclusively, on the truncus of the asulcate face (Char. 16). The *lineomaculatus* section is supported by the elliptical shape of the internal edge of the apex (Char. 14), deep flounces on the sulcate face (Char. 20), and the presence of a thick area in the proximal region of the truncus of the asulcate face (Char. 38). The *montanus* section is supported by the presence of a small asulcate face prominence in the bifurcation of the sulcus spermaticus (Char. 23) and the presence of a asulcate face prominence on the truncus of the asulcate face (Char. 40). Within the *montanus* section the *montanus* series is supported by the lower ratio between the sulcus spermaticus length and the hemipenis length (Char. 1), lower ratio between the distance where the sulcus spermaticus bifurcation begins and hemipenis length (Char. 2), presence of serrate flounces on the proximal region of the truncus of the asulcate face (Char. 32), thick flounces on truncus of the asulcate face (Char. 33), rough proximal region (base of truncus) on the asulcate face (Char. 37). The *boulengeri* series has no synapomorphies. Within the *boulengeri* series, the *melanops* group is supported by the lower ratio between the width of distal region of truncus and hemipenis length (Char. 6). Within the *melanops* group, the *telsen* group shows a higher ratio between hemipenis length and SVL (Char. 0) and rough proximal region of truncus of the asulcate face (Char. 37). Within the *melanops* group, the *goetschi* group is supported by the higher ratio between distance where the sulcus

spermaticus bifurcation begins and the hemipenis length (Char. 2) and developed apical lobules (Char. 11). The *goetschi* group is formed by two clades: 1) the *cuyanensis* clade supported by the lower ratio between hemipenis length and SVL (Char. 0), higher ratio between the length and width of the sulcus spermaticus and hemipenis length (Chars. 1 and 3), presence of 4–5 flounces on truncus of the sulcate face (Char. 22), and 1–2 continuous flounces on truncus of the asulcate face (Char. 36). and 2) the *fitzingerii* clade supported by thick flounces on truncus of the sulcate face (Char. 19), deep flounces on truncus of the sulcate face (Char. 20), flounces on truncus of the asulcate face are fleshy (Char. 33), and the presence of three flounces on the lobules of the asulcate face (Char. 34). The *laurenti* group has no synapomorphies, but we recovered synapomorphies of a group within it. The *wiegmannii* group is supported by 1–2 continuous flounces on the truncus of the asulcate face (Char. 36). The *darwinii* clade has no synapomorphies, but some subclades have. The *ornatus* clade is supported by the lower ratio between the hemipenis length and the SVL (Char. 0).

Importance of the hemipenial morphology in the diagnoses of species

We compared phylogenetically-close species with the aim to elucidate the role of hemipenial morphology as taxonomic characters. We made comparisons between sets of species from both subgenera of *Liolaemus*: *L. albiceps* (Lobo and Laurent, 1995) vs *L. irregularis* (Laurent, 1986); *L. ornatus* (Koslowsky, 1898), *L. crepuscularis* (Abdala and Diaz-Gomez, 2006), and *L. lavillai* (Abdala and Lobo, 2006); *L. multicolor* (Koslowsky, 1898), *L. molinai* (Valladares et al., 2012), and *L. poecilochromus* (Laurent, 1986); *L. vulcanus* (Quinteros and Abdala, 2011) vs *L. dorbignyi* (Koslowsky, 1898) (From *Eulaemus*); *L. puna* (Lobo and Espinoza, 2004) vs *L. pyriphlogos* (Quinteros, 2012), and *L. gracilis* (Bell, 1843) vs *L. tandiliensis* (Vega et al., 2008) (subgenus *Liolaemus*).

Liolaemus albiceps vs *L. irregularis* (Figure S1): the differences are that *L. albiceps* shows a higher ratio between the distance where the sulcus spermaticus bifurcation begins and the hemipenis length; a higher ratio between the middle and distal region width of truncus and the hemipenis length. These last character states made the hemipenis of *L. albiceps* more robust than that of *L. irregularis*. Moreover, the apex lobules are more developed in *L. albiceps*. The flounces are thick in *L. albiceps*, yet superficial in *L. irregularis*. Flounces on truncus of the asulcate face are serrated in *L. albiceps*, while smooth in *L. irregularis*. The hemipenes of *L. irregularis* are smaller than those of *L. albiceps* (ratio between the length of hemipenis and SVL is 0.09 vs. 0.06). The truncus of sulcate face of *L. irregularis*' hemipenes shows 4–5 flounces, whereas 6–8 in *L. albiceps*.

Liolaemus crepuscularis, *L. lavillai*, and *L. ornatus* (Figure S2). The difference is that *L. lavillai* and *L. crepuscularis* show 6–8 flounces on truncus of the sulcate face, whereas *L. ornatus* shows 9–10. The small asulcate face prominence located on the bifurcation of the sulcus spermaticus is rounded in *L. ornatus* and *L. lavillai*, yet triangular in *L. crepuscularis*. There are four non-continuous flounces on truncus of the asulcate face in *L. lavillai*, five in *L. crepuscularis*, and six in *L. ornatus*. The number of flounces on truncus of the asulcate face in *L. crepuscularis* and *L. lavillai* is 3–4, whereas in *L. ornatus* there are 5. Proximal region (base of the hemipenis) of the asulcate face is smooth in *L. lavillai* and *L. crepuscularis*, yet rugose in *L. ornatus*.

Liolaemus molinai, *L. multicolor*, and *L. poecilochromus* (Figure S3). The apical lobules are joined in *L. poecilochromus* and *L. molinai*, whereas in *L. multicolor* they are separated. In *L. molinai*, ornamentations of the sulcate face don't reach the middle line of truncus of the hemipenis, whereas in *L. multicolor* and *L. poecilochromus* they go beyond the middle line. The presence of serrated flounces on truncus of the asulcate face distinguish *L. molinai* and *L. multicolor* from *L. poecilochromus* (smooth flounces). The number of flounces on truncus of the asulcate face is 1–2 in *L. molinai*, 3–4 in *L. poecilochromus* and 5 in *L. multicolor*.

Liolaemus vulcanus vs *L. dorbignyi* (Figure S4). In *L. vulcanus* flounces on truncus of the sulcate face are deep, yet superficial in *L. dorbignyi*. The small asulcate face prominence on the bifurcation of the sulcus spermaticus is oval in *L. vulcanus*, while triangular in *L. dorbignyi*. Moreover, *L. vulcanus* shows five discontinuous and three continuous flounces on truncus of the asulcate face, but three and five (respectively) in *L. dorbignyi*. Furthermore, *L. vulcanus* has eight flounces on truncus of the sulcate face, yet six in *L. dorbignyi*.

Liolaemus puna vs *L. pyriphlogos*. The ratio between the width of middle and distal regions of truncus and the hemipenis length is higher in *L. puna* than in *L. pyriphlogos*. Based on this character, we can conclude that the general shape of the hemipenis in *L. puna* is more robust than in *L. pyriphlogos*. Ornamentations of the hemipenis in *L. pyriphlogos* are distributed in the upper third of truncus, whereas in *L. puna* they reach the middle line of truncus.

Liolaemus gracilis vs *L. tandiliensis* (Figure S5). These species differ in the ratio between the sulcus spermaticus length and hemipenis length, showing a lower ratio in *L. gracilis*. In addition, the ratio between the width of the proximal region of truncus and the hemipenis length is higher in *L. gracilis*. Furthermore, *L. gracilis* can exhibit calyces and flounces on truncus of the sulcate face, whereas *L. tandiliensis* only exhibits calyces. The number of calyces on truncus of the asulcate face in *L. gracilis* is 1–2, while 3–4 in *L. tandiliensis*.

Discussion

Hemipenis morphology

Cei (1986) studied the hemipenes of *L. fitzingerii* and *Vilcunia silvanae* (*L. silvanae*, Donoso-Barros and Cei, 1971), and Cei (1993) mentioned having studied the hemipenes of 14 species, yet only showed the hemipenes of *L. bibronii* (Bell, 1843), *L. irregularis*, and *L. salinicola* (Laurent, 1986), in which he argued the lack of ornamentation in all the species studied. In this study, all the hemipenes

showed some ornamentation. We agree with Lobo (2000) in the fact that the observations of Cei (1986, 1993) were probably due to an insufficient eversion.

Lobo (2000) studied several representatives of the *Liolaemus* subgenus: *L. bibronii* (Bell, 1843), *L. buergeri* (Werner, 1907), *L. ceii* (Donoso-Barros, 1971), *L. kriegi* (Müller and Hellmich, 1939), *L. lemniscatus* (Gravenhorst, 1838), *L. neuquensis* (Müller and Hellmich, 1939), *L. pictus argentinus* (Duméril and Bibron, 1837), and *L. tenuis* (Duméril and Bibron, 1837), where he described the hemipenis of *L. chiliensis* in detail, noting the presence of calyces and flounces on truncus of the sulcate face. Our results were consistent with his, where all specimens of the *Liolaemus* subgenus studied have calyces on truncus of the sulcate face, even though they can also exhibit some flounces. Lobo (2000), mentioned that in both *L. chiliensis* and *L. robertmertensi* (Hellmich, 1964) the calyces region goes beyond the middle region of truncus. These species are closely related to the *L. alticolor–bibronii* group in which the calyces reach the middle region of truncus. In our study, it was the members of the *Eulaemus* subgenus whose calyces region went beyond the middle region of the hemipenis.

For the *Eulaemus* subgenus, Lobo (2000) described the hemipenis of *L. chacoensis* (Shreve, 1948) in detail, mentioning that the truncus of sulcate face shows only flounces. In our study, all the specimens of the *Eulaemus* subgenus have flounces, but never show calyces.

Phylogenetic considerations

The obtained phylogeny, based exclusively on hemipenial morphology, recovered the main groups (until series level) recognized within *Liolaemus* as monophyletic. However, the relationships in lower level were not recovered (Figure 4). Our topology recovered the *L. elongatus–petrophilus* group as paraphyletic. The location of *L. coeruleus* as basal to the *L. elongatus–petrophilus* species plus the *L. alticolor–bibronii* group species, is inconsistent with other proposed phylogenies (Schulte et al. 2000; Lobo 2001, 2005; Lobo and Abdala 2002; Morando et al. 2007; Schulte 2013; Pyron et al. 2013). The

monophyly of the *L. alticolor–bibronii* group recovered here is consistent with previous studies (Lobo 2001, 2005; Morando et al. 2007; Pyron et al. 2013; Quinteros 2013; Schulte 2013).

The *Eulaemus* subgenus is recovered as monophyletic formed by the *L. lineomaculatus* section and the *L. montanus* section, and consistent with previous proposals (Schulte et al. 2000; Lobo et al. 2010; Pyron et al. 2013; Schulte 2013; Abdala and Quinteros 2014). Within the *L. montanus* section, the *L. boulengeri* series is recovered as paraphyletic, with some species more related to the *L. montanus* series nested within. This result is inconsistent with most of the previous proposals (Schulte et al. 2000; Lobo et al. 2010; Pyron et al. 2013; Schulte 2013; Abdala and Quinteros 2014) but agrees with a result found by Olave et al. (2014) who recovered the *L. montanus* series (represented by seven species) nested within the *L. boulengeri* series. The result of Olave et al. (2014) was based on two mitochondrial markers and several nuclear markers for 188 terminals (representing 70 species and several candidate species).

Optimization of hemipenial characters in the metatree constructed shows many synapomorphies of the groups below the series level (Figure 5). This result emphasizes the taxonomic and phylogenetic importance of this data set in the phylogenetic reconstructions, which could be added to other more traditional characters used (lepidosis, coloration, skeleton, and molecular) and helps to reconstruct the evolutionary history of the group.

In Etheridge (1995) *L. chacoensis* was included in the *chiliensis* group (actual subgenus *Liolaemus*); in Lobo (2000) its hemipenes was described as sharing the morphology of the Argentine group (current subgenus *Eulaemus*). In this study we found its morphology almost identical to that of *L. koslowskyi*; this fact is coherent with the last phylogenetic studies (Avila et al. 2006; Abdala 2007) which found *L. chacoensis* nested within the *L. darwinii* group.

Significance of hemipenial morphology for discriminating closely-related species

The significance of the hemipenial morphology in systematics has been highlighted by other researchers, such as those who have studied the genus *Anolis*. Köhler et al. (2007), Köhler and Smith (2008), Köhler and Sunyer (2008), Köhler (2009), Köhler et al. (2012), Köhler et al. (2010), and D'Angiolella et al. (2016) described many new species, adding hemipenial morphology to their formal descriptions. They emphasized the importance of this morphology in discriminating closely-related and/or cryptic species. They found differences in ornamentation (bilobed or unilobed; presence of calyces and/or flounces), sulcus spermaticus, and size and shape (bifurcate or not, large hemipenial, or smaller and thinner, etc.) among closely-related taxa, similarly to the way we describe the *Liolaemus* species in this study.

We chose a series of sister taxa (found in published phylogenetic analyses) for our comparisons due to the fact that out of the whole morphology studied only a few characters were used for their discrimination, mostly pattern color and continuous overlapping scale counting: *L. multicolor* vs *L. poecilochromus* in Laurent (1986); *L. albiceps* vs *L. irregularis*, in Lobo and Laurent (1995); *L. multicolor* vs *L. molinai* in Valladares et al. (2002); *L. ornatus* vs *L. crepuscularis* in Abdala and Díaz Gómez (2006); *L. ornatus* vs *L. lavillai* in Abdala and Lobo (2006); *L. gracilis* vs *L. tandiliensis* in Vega et al. (2007); *L. vulcanus* vs *L. dorbignyi* Quinteros and Abdala (2011); and *L. puna* vs *L. pyrrhologos*, in Quinteros (2012). Most of these taxa do not have DNA sequences available and it is not possible to estimate genetic distances at this time. For example, *L. albiceps* and *L. irregularis* show, in their distribution, a slender contact zone; furthermore, the ecological requirements are very similar, a fact that makes them interesting to analyze and evaluate their taxonomic status and potential mechanisms of reproductive isolation or/and speciation. Nevertheless, we can diagnose this pair of species (and the other pairs) based on hemipenial morphology. Our observations indicate that the amount of differences that facilitate the discrimination between these two species are equivalent to those found between a pair of species that are not sister taxa (within the genus). This pair of species

corresponds to a particular case, since their distributions show overlap. On the other hand, the other pairs (or set) of species which we take into account do not show a contact zone or overlapping area in their distribution. Differences in their hemipenial morphology are not as numerous as those found for *L. albiceps* and *L. irregularis*.

Klaczko et al. (2015) revealed that hemipenial morphology evolves faster than other morphological characters. Their analysis was restricted to a few characters including measurements related to size and shape: hemipenis length, apical and medial width. Contrasting their evolutionary change with other restricted sets of morphological characters (thighs, shank and dewlap size), they found that both sets of characters show phylogenetic structure. Be that as it may, their observations are restricted to this sole comparison, because the comparison of evolutionary rates of hemipenial characters can be made with the whole morphology known today (see Poe 2004 who showed 91 morphological characters); furthermore, conclusions can also change in cases where we include all hemipenial characters in the analysis, not only three measurements (for example those related to ornamentation, sulcus spermaticus, etc.). Unfortunately, we realize that it can be very difficult to study the hemipenial morphology in such a diverse genus such as *Anolis* due to its optimization in trees and test evolutionary rates. Furthermore, knowing only evolutionary rates of hemipenial traits does not provide enough evidence to hypothesize the role of this morphology in the evolution and diversification of the group. Genital morphology involves much more variation than that reported in evolutionary or phylogenetic analyses; in the case of *Anolis*, Poe (2004) didn't include any hemipenial character, and in our case, the analysis of the *Phymaturus palluma* group by Lobo et al. (2016) included only one character. Data on hemipenial morphology suggests much more variation and room for exploration; many more comparative studies of these genera are needed to better understand the evolution of this morphology and its significance in their diversification.

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Figure 1. General figure of a typical hemipenis of *Liolaemus. L. zullyae* (Cei and Scolaro, 1996) MCN 1543 shows the general regions used in the description of *Liolaemus*. A. asulcate face; B. lateral face; C. sulcate face; Ca: calyces; L: lobes (Apex); M: medial region of the truncus; B: base of the truncus; Fl: flounce; S: sulcus spermatucus; IE: inner edge; EE: external edge. Scale 2 mm.

Figure 2. Hemipenis of species representatives of *Eulaemus* subgenus. The pictures show the asulcate lateral and sulcate faces and the numbers correspond to hemipenial characters and their states in brackets (see below). A–C, *L. kingii* (Bell, 1843) (MCN 1546) represent *L. lineomaculatus* section. D–F, *L. Irregularis* (Laurent, 1986) (MCN 3429) represent *boulengeri* series of the *L. montanus* section. G–I, *L. multicolor* (Koslowsky, 1898) (IBIGEO 5253) represent *L. montanus* series of the *L. montanus* section. In C, the elliptical Shape of inner edge of apex is marked in red. In F, the small asulcate face prominence at the bifurcation of the sulcus spermatucus present and rounded shape are marked in red. In I, the oval shape of the small asulcate face prominence at the bifurcation of the sulcus spermatucus and inner edge of the thickened apex are marked in red. char 7: apex shape (1= shell-shaped; 2= cup-shaped); char 13: inner edge of the apex (0= thickened); char 14: shape of inner edge of apex (0= elliptical ; 1=not elliptical); char 16: ornamentations over sulcate face (1= flounces); char 21: sulcate face with flounces (0=serrate; 1=smooth); char 23: small bulge at the bifurcation of the sulcus spermatucus (0= present); char 24: shape of the small bulge at the bifurcation of the sulcus spermatucus (0= rounded; 2=oval); char 25: ornamentations on the asulcate face (0= flounces; 1= calyces); char 27: calyces of the asulcate face (0=uniform); char 32: flounces of the asulcate face (0= serrated); char 33: flounces of the asulcate face (0=thick; 1= thin); char 37: proximal region of the asulcate face (0= rough); char 38: proximal region of the asulcate face shows a thickened area (0= present); char 40: asulcate face shows a conspicuous prominence in the middle region (0=present); char 41: Shape of the asulcate face prominence (0= curved toward the apex; 1= rounded) see supplementary material File S2: Character list for details. Scale 2 mm.

Figure 3. Hemipenis of representatives of *Liolaemus sensu stricto* subgenus. The pictures show the asulcate lateral and sulcate faces and the numbers correspond to hemipenial characters and their states in brackets (see below). A–C *L. ceii* (Donoso-Barros, 1971) (MCN 3744) represent *L. elongatus* group, D–F *L. coeruleus* (Cei and Ortiz-Zapata, 1983) (MCN 3794) represent *L. pictus* group, G–I *L. yanalcu* (Martinez-Oliver and Lobo, 2002) (MCN 3409) represent *L. alticolor–bibroni* group. char 7: apex shape (0= shell-shaped; 2= round-shaped; 4= horn-shaped); char 13: inner edge of the apex (0= thickened; 1= not thickened); char 15: inner edge of the apex (0= elongated); char 16: ornamentations over sulcate face (1= flounces; 2= calyces); char 18: distribution of calyces in the sulcate face (0= over the lobes); char 25: ornamentations on the asulcate face (1=calyces); char 26: Distribution of calyces on the asulcate face (0= on the lobes of the apex; 1= never on the lobes); char 27: calyces of the asulcate face (1=not uniform); char 28: calyces of the asulcate face (0= superficial; 1= deep); char 29: calyces of the asulcate face (0= thin); char 30: calyces of the asulcate face (0= wavy); char 37: proximal region of the asulcate face (1= smooth) see supplementary material File S2: Character list for details. Scale 2 mm.

Figure 4. Tree most parsimonious recovered with the hemipenial character set. The top number of the branch corresponding to bootstraps support and the number below the branch corresponding to hemipenial characters and their states are indicated in brackets (see Supplementary Material File S2: Character list for details). The star indicates the corresponding species that bears the hemipenis represented by the figure.

Figure 5. Metatree constructed based on the most recent phylogenies of the reconstructed Liolaemidae family. The numbers corresponding to hemipenial characters and their states are indicated in brackets (see Supplementary Material File S2: Character list for details). The star indicates the corresponding species that bears the hemipenis represented by the figure.

Figure 1. General figure of a typical hemipenis of *Liolaemus*. *L. zullyae* (Ceï and Scolari, 1996) MCN 1543 shows the general regions used in the description of *Liolaemus*. A. asulcate face; B. lateral face; C. sulcate face; Ca: calyces; L: lobes (Apex); M: medial region of the truncus; B: base of the truncus; FI: flounce; S: sulcus spermatucus; IE: inner edge; EE: external edge. Scale 2 mm.

119x70mm (300 x 300 DPI)

Figure 2. Hemipenis of species representatives of Eulaemus subgenus. The pictures show the asulcate lateral and sulcate faces and the numbers correspond to hemipenial characters and their states in brackets (see below). A–C, *L. kingii* (Bell, 1843) (MCN 1546) represent *L. lineomaculatus* section. D–F, *L. Irregularis* (Laurent, 1986) (MCN 3429) represent *boulengeri* series of the *L. montanus* section. G–I, *L. multicolor* (Koslowky, 1898) (IBIGEO 5253) represent *L. montanus* series of the *L. montanus* section. In C, the elliptical shape of inner edge of apex is marked in red. In F, the small asulcate face prominence at the bifurcation of the sulcus spermaticus present and rounded shape are marked in red. In I, the oval shape of the small asulcate face prominence at the bifurcation of the sulcus spermaticus and inner edge of the thickened apex are marked in red. char 7: apex shape (1 = shell-shaped; 2 = cup-shaped; 3 = shell-shaped); char 13: inner edge of the apex (0 = thickened); char 14: shape of inner edge of apex (0 = elliptical; 1 = not elliptical); char 16: ornamentations over sulcate face (1 = flounces); char 21: sulcate face with flounces (0 = serrate; 1 = smooth); char 23: small bulge at the bifurcation of the sulcus spermaticus (0 = present); char 24: shape of the small bulge at the bifurcation of the sulcus spermaticus (0 = rounded; 2 = oval); char 25:

ornamentations on the asulcate face (0= flounces; 1= calyces); char 27: calyces of the asulcate face (0=uniform); char 32: flounces of the asulcate face (0= serrated); char 33: flounces of the asulcate face (0=thick; 1= thin); char 37: proximal region of the asulcate face (0= rough); char 38: proximal region of the asulcate face shows a thickened area (0= present); char 40: asulcate face shows a conspicuous prominence in the middle region (0=present); char 41: Shape of the asulcate face prominence (0= curved toward the apex;1= rounded) see supplementary material File S2: Character list for details. Scale 2 mm.

263x397mm (300 x 300 DPI)

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Figure 3. Hemipenis of representatives of *Liolaemus* sensu stricto subgenus. The pictures show the asulcate lateral and sulcate faces and the numbers correspond to hemipenial characters and their states in brackets (see below). A–C *L. ceii* (Donoso-Barros, 1971) (MCN 3744) represent *L. elongatus* group, D–F *L. coeruleus* (Ceí and Ortiz-Zapata, 1983) (MCN 3794) represent *L. pictus* group, G–I *L. yanalca* (Martínez-Oliver and Lobo, 2002) (MCN 3409) represent *L. alticolor-bibroni* group. char 7: apex shape (0= shell-shaped; 2= round-shaped; 4= horn-shaped); char 13: inner edge of the apex (0= thickened; 1= not thickened); char 15: inner edge of the apex (0= elongated); char 16: ornamentations over sulcate face (1= flounces; 2= calyces); char 18: distribution of calyces in the sulcate face (0= over the lobes); char 25: ornamentations on the asulcate face (1=calyces); char 26: Distribution of calyces on the asulcate face (0= on the lobes of the apex; 1= never on the lobes); char 27: calyces of the asulcate face (1=not uniform); char 28: calyces of the asulcate face (0= superficial; 1= deep); char 29: calyces of the asulcate face (0= thin); char 30: calyces of the asulcate face (0= wavy); char 37: proximal region of the asulcate face (1= smooth) see supplementary material File S2: Character list for details. Scale 2 mm.

271x383mm (300 x 300 DPI)

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Figure 4. Tree most parsimonious recovered with the hemipenial character set. The top number of the branch corresponding to bootstraps support and the number below the branch corresponding to hemipenial characters and their states are indicated in brackets (see Supplementary material File S2: Character list for details). The star indicates the corresponding species that bears the hemipenis represented by the figure.

206x159mm (300 x 300 DPI)

Figure 5. Metatree constructed based on the most recent phylogenies of the reconstructed Liolaemidae family. The numbers corresponding to hemipenial characters and their states are indicated in brackets (see Supplementary material File S2: Character list for details). The star indicates the corresponding species that bears the hemipenis represented by the figure.

203x156mm (300 x 300 DPI)