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**INTERNAL MORPHOLOGY REVEALS REPRODUCTIVE ISOLATION BETWEEN
TWO AMPHISBAENIAN CLOSELY-RELATED SPECIES (SQUAMATA:
AMPHISBAENIDAE)**

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AMPHISBAENIDAE)**

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Orientador: Sírnia Lisandra de Barcelos Ribeiro
Co-orientador: Amanda Frederico Mortati

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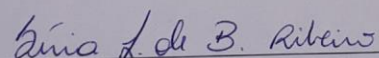


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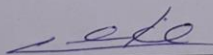
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Assim, aos 05 (cinco) dias do mês de fevereiro do ano de 2020 (dois mil e vinte), às 15h00min, na sala 304 Bloco Modular Tapajós, instalou-se a apresentação de seminário público da dissertação de mestrado da aluna Francesca Nicole Angiolani Larrea. Deu-se início a abertura dos trabalhos, onde o Professora Dra. Síría Lisandra de Barcelos Ribeiro, após esclarecer as normativas de tramitação da defesa e seminário público, de imediato solicitou à candidata que iniciasse a apresentação da dissertação, intitulada INTERNAL MORPHOLOGY REVEALS REPRODUCTIVE ISOLATION BETWEEN TWO AMPHISBAENIAN CLOSELY RELATED SPECIES (SQUAMATA: AMPHISBAENIDAE). Concluída a exposição, a orientadora comunicou à discente que a versão final da dissertação deverá ser entregue ao programa, no prazo de 30 dias; contendo as modificações sugeridas pela banca examinadora e constante nos formulários de avaliação da banca. A banca examinadora foi composta pelos examinadores professores doutores listados abaixo. Os pareceres assinados seguem em sequência.



Síría Lisandra de Barcelos Ribeiro
Orientadora



Francesca Nicole Angiolani Larrea
Discente

Para o futuro

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“Las cosas de las que uno está
completamente seguro nunca son
verdad. Ésa es la fatalidad de la fe y la
lección del romanticismo.”

Oscar Wilde

RESUMO

Comparar a morfologia do trato reprodutivo entre espécies intimamente relacionadas pode revelar mecanismos e processos de isolamento reprodutivo principalmente associados à divergência evolutiva. Embora o trato reprodutivo dos anfisbaenianos tenha sido descrito qualitativamente para algumas espécies, a variação interespecífica associada ao isolamento mecânico da reprodução tem sido pouco investigada por meio de hipóteses claramente definidas. Diferenças interespecíficas no trato reprodutivo podem ser particularmente interessantes nos anfisbaenianos, porque a fossorialidade causou assimetria bilateral nos órgãos internos. Neste estudo, comparamos a morfologia do trato reprodutivo entre duas espécies de Amphisbaenidae intimamente relacionadas (*Amphisbaena anaemariae* e *A. silvestrii*). Utilizamos abordagens multivariadas para testar a hipótese geral de que divergências interespecíficas em nove variáveis que quantificam o trato reprodutivo explicam mecanismos e processos de isolamento reprodutivo associados à divergência evolutiva. Nosso teste de hipótese foi focado na investigação dos níveis de dependência sexual e assimetria bilateral na divergência reprodutiva interespecífica. Encontramos assimetria bilateral na maioria das variáveis medidas e morfologia dependente de sexo do trato reprodutivo em ambos os sexos, apesar de esse achado ser menos evidente no sexo feminino. Nossos resultados estão associados principalmente a uma combinação de forças evolutivas que atuam na assimetria bilateral e na dispersão dependente do sexo. Por fim, este estudo fornece conhecimentos sobre processos evolutivos baseados em mecanismos de isolamento reprodutivo em organismos para os quais a amostragem é prejudicada pela fossorialidade.

Palavras-chave: Diferenças morfológicas. Assimetria bilateral. Fossório *Amphisbaena anaemariae*. *Amphisbaena silvestrii*.

ABSTRACT

Comparing reproductive tract morphology between closely related species may reveal mechanisms and processes of reproductive isolation mainly associated to evolutionary divergence. Although the reproductive tract of amphisbaenians has been qualitatively described for some species, interspecific variation associated with mechanical reproductive isolation has been poorly investigated through clearly defined hypotheses. Interspecific differences in reproductive tract may be particularly interesting in amphisbaenians, because fossoriality has caused bilateral asymmetry in internal organs. In this study we compared reproductive tract morphology between two closely related Amphisbaenidae species (*Amphisbaena anaemariae* and *A. silvestrii*). We used multivariate approaches to test the general hypothesis that interspecific divergence in nine variables that quantify the reproductive tract explain mechanisms and processes of reproductive isolation associated with evolutionary divergence. Our hypothesis testing was focused on investigating levels of sex-dependence and bilateral asymmetry on interspecific reproductive divergence. We found bilateral asymmetry in most of the variables measured, and sex-dependent morphology of the reproductive tract in both sexes, despite this finding was less evident in females. Our results are mainly associated with a combination of evolutionary forces acting on bilateral asymmetry and sex-dependent dispersal. Ultimately, this study provides insights into evolutionary processes based on reproductive isolation mechanisms in organisms for which sampling is hindered by fossoriality.

Key words: Morphological differences. Bilateral asymmetry. Fossorial. *Amphisbaena anaemariae*. *Amphisbaena silvestrii*.

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1. INTRODUÇÃO GERAL

Anfisbênios e suas diferenças morfológicas no trato reprodutivo ¹

O que é a pesquisa?

Os anfibênios são animais de hábitos fossoriais e pertencentes à um grupo de répteis chamado Squamata da qual fazem parte também seus representantes mais conhecidos as serpentes e os lagartos. No Brasil e em outros países esses animais são conhecidos popularmente como “cobra-cega” ou “cobra de duas cabeças” justamente por possuírem o corpo alongado como as cobras, por serem desprovidos de membros, e geralmente, apresentarem a cauda curta e arredondada, o que leva a cauda a ser confundida erroneamente com a cabeça.

Ao longo de sua história evolutiva os anfibênios sofreram uma série de adaptações, especializadas para o ambiente subterrâneo. A locomoção restrita por túneis embaixo do solo pode ter causado perda funcional de alguns órgãos, como por exemplo, o pulmão direito que é vestigial ou ausente em algumas espécies, e ainda, ter levado ao deslocamento de órgãos emparelhados como os rins e as gônadas, onde um dos lados tem uma posição mais próxima ao crânio em relação a cauda.

Diferenças interespecíficas no trato reprodutivo podem ser particularmente interessantes em anfibênios porque a fossorialidade (hábito subterrâneo) pode causar assimetria bilateral nos órgãos internos. Nesse estudo comparamos a morfologia interna do trato reprodutivo entre duas espécies de anfibênios que são geneticamente próximas, *Amphisbaena anaemariae* e *Amphisbaena silvestrii*. Além disso, investigamos se, entre as espécies, os níveis de simetria bilateral são dependentes do sexo.

¹Texto de comunicação científica formatado conforme as normas do “Canal Ciência - Portal de Divulgação Científica e Tecnológica”, do Instituto Brasileiro de Informação em Ciência e Tecnologia (Ibict).

Como a pesquisa foi realizada?

Nessa pesquisa utilizamos 20 indivíduos da espécie *A. anaemariae* e 20 indivíduos da espécie *A. silvestrii*, dos quais 10 foram machos e 10 fêmeas para cada espécie, para comparar as diferenças de tamanho entre nove variáveis do trato reprodutivos. Os indivíduos de *A. anaemariae* estavam depositados na coleção herpetológica (ou seja, coleção de anfíbios e répteis) da Pontifícia Universidade Católica de Goiás, Goiás, Brasil (CEPB-PUC Goiás) e os indivíduos de *A. silvestrii* estavam depositados na Coleção Zoológica da Universidade Federal do Mato Grosso, Brasil (CZUFMT-R).

Nós medimos o comprimento rostro-cloacal (medida que vai da ponta do focinho até a cloca), o diâmetro corporal, o tamanho dos rins, ovários, testículos, a porção posterior do oviduto e a porção do ducto deferente sem associação com os rins, o comprimento da gônada-cloaca o comprimento do conduto gonadal, sendo oviduto em fêmeas e ducto deferente em machos, o diâmetro da gônada e a superposição de rim e cloaca interna após dissociação. Finalmente só para fêmeas contamos o número de ovócitos. Todas variáveis foram testadas por meio de um Análise Discriminante de Componentes Principais (DAPC sigla em inglês) que é uma análise que permitiu determinar o nível de variação entre essas variáveis dependendo do lado do corpo, por sexo e por espécie.

Qual a importância da pesquisa?

Nossos resultados ampliam os dados científicos sobre os anfisbênios, répteis que devido seu hábito fossorial ainda possui déficit de informações sobre os mais variados aspectos tais como sua biologia, ecologia e morfologia. As descrições morfológicas têm sido tradicionalmente usadas para classificar organismos. Nesse caso, usamos uma visão mais profunda para inferir níveis de divergência entre espécies aparentadas geneticamente, com o objetivo de proporcionar ferramentas adicionais para obter dados de organismos dos quais as informações são escassas.

Apresentamos aqui uma nova abordagem para espécies fossoriais, como os anfisbênios, onde os níveis de divergência podem ser quantificados em espécies pouco detectáveis no meio ambiente. A fossorialidade tem sido um grande obstáculo para

determinar a biologia, ecologia e evolução de grupos com esse hábito e, portanto, existe uma necessidade de obter informações por meios indiretos.

Autores

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Crook, J.M., Parsons, T.S. (1980): Visceral anatomy of the amphisbaenia. *J. Morphol.* **163**: 99–133.

2. CAPITULO I

Sex-dependent divergence in asymmetrical reproductive tract morphology between two closely-related amphisbaenian species (Squamata: Amphisbaenidae)

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Sex-dependent divergence in asymmetrical reproductive tract morphology between two closely-related amphisbaenian species (Squamata: Amphisbaenidae)

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Article

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Abstract

Comparing reproductive tract morphology between closely related species may reveal mechanisms and processes of reproductive isolation mainly associated to evolutionary divergence. Although the reproductive tract of amphisbaenians has been qualitatively described for some species, interspecific variation associated with mechanical reproductive isolation has been poorly investigated through clearly defined hypotheses. Interspecific differences in reproductive tract may be particularly interesting in amphisbaenians, because fossoriality has caused bilateral asymmetry in internal organs. In this study we compared reproductive tract morphology between two closely related Amphisbaenidae species (*Amphisbaena anaemariae* and *A. silvestrii*). We used multivariate approaches to test the general hypothesis that interspecific divergences in nine variables that quantify the reproductive tract explain mechanisms and processes of reproductive isolation associated with evolutionary divergence. Our hypothesis testing was focused on investigating levels of sex-dependence and bilateral asymmetry on interspecific reproductive divergence. We found bilateral asymmetry in most of the variables measured, and sex-dependent morphology of the reproductive tract in both sexes, despite this finding was less evident in females. Our results are mainly associated with a combination of evolutionary forces acting on bilateral asymmetry and sex-dependent dispersal. Ultimately, this study provides insights into evolutionary processes based on reproductive isolation mechanisms in organisms for which sampling is hindered by fossoriality.

Key words:

Morphological differences. Bilateral asymmetry. Fossorial. *Amphisbaena anaemariae*. *Amphisbaena silvestrii*.

Introduction

Reproductive studies may potentially shed light on the mechanisms and processes driving diversification since reproductive isolation or segregation are effective ways by which evolution operates (Hedrick, 2007). Particularly, comparing reproductive tract morphology between closely-related species may reveal levels of reproductive isolation mainly associated to evolutionary divergence (Sánchez-Martínez, Ramírez-Pinilla, and Miranda-Esquivel, 2007; Eberhard, 2010). Comparative approaches have been relevant for determining species-specific characters and their level of divergence in several groups of animals (e.g. insects: Ehara, 1952; fish: Loir et al., 1989a; Carcupino et al., 2002; felines: Matthews, 1941 and primates: Hafez and Jaszczak, 1972; Dixson and Anderson, 2001). Specifically, in Squamata, reproductive studies have been largely focused on hemipenial morphology (e.g. Arribas, 2001; Böhme and Ziegler, 2009; Pinna, 2012; Klaczko, Ingram, and Losos, 2015; De-Lima et al., 2019).

Although internal reproductive morphology has been neglected, it has been described for Neotropical amphisbaenian species based on histological and topographical data of at least 32 species distributed throughout the world (Crook and Parsons, 1980; Rosenberg, Cavey, and Gans, 1991; Andrade, Nascimento, and Abe, 2006; Navega-Gonçalves, 2009; Balestrin, Cappellari, and Outeiral, 2010; Pinna, 2012; Santos, 2013; Aguirre, Ortiz, and Hernando, 2017). Overall, although these studies have generated very relevant information for the Amphisbaenian reproductive morphology, they have rarely used explicit hypothesis testing to compare closely-related species. This is a particularly relevant approach for the identification of evolutionary processes mediating reproductive isolation among lineages.

Qualitatively, the amphisbaenian reproductive system in females has paired organs and is composed by the ovaries connected to the oviducts by the mesovarium, which in turn connects all reproductive organs of the pleuroperitoneal cavity of the urogenital system. Inside the ovaries are

the follicles in different stages of development. Right and left ovaries are generally asymmetrical, the right ovary is more cranially positioned than the left ovary, and they may be overlaid. The oviduct is divided into three segments, the infundibulum, uterus and the posterior oviduct. The cranial limit of the ovary is distinguishable by the presence of follicles, which is easier to identify in reproductively mature females, through a dark-yellow colour. Once follicles (generally less than 11) are ready to be ovulated, they occupy almost the entire lower pleuroperitoneal cavity (Crook and Parsons, 1980; Vega, 2001; Navega-Gonçalves, 2009; Balestrin and Cappellari, 2011; Al-Sadoon, Kandeal, and Rodiny, 2014; Figure 1A). In males, testes are lobular and may vary interspecifically in shape, from elongated to reniform. The vas deferens connect the testes to the cloaca and are associated to the kidneys longitudinally. In both sexes, all organs are interconnected by a membrane called mesorchium (Crook and Parsons, 1980; Navega-Gonçalves, 2009; Santos, 2013; Figure 1B).

In this study we investigate whether interspecific differences in internal reproductive morphology can be detected when divergence is relatively recent. Reproductive isolation may be effectively demonstrated by comparing reproductive tract morphological traits between phylogenetically distinct species (Dixson and Anderson, 2001; Eberhard, 2010; Showalter, Todd, and Brennan, 2014). Closely-related species may lack specific mechanical mate recognition, present intraspecific coevolution in genitalia between males and females (Dixson and Anderson, 2001; Eberhard, 2010; Showalter, Todd, and Brennan, 2014), or generate infertile offspring when hybridized (Steyer and Nowak, 2017). All cases are often associated with intensity of gene flow, and levels of interspecific variation in reproductive tract morphology may reflect the cause or origin of cladogenetic processes. Cladogenesis in response to reproductive isolation has been readily demonstrated in the case of vicariance at spatial macroscales (Ribas et al., 2012) but it can also occur at finer scales, resulting in sympatric speciation (Barluenga et al., 2006). Therefore,

quantifying interspecific variation in reproductive tract morphology is useful for understanding the origin of biodiversity, and also for defining diagnostic traits that support the taxonomic status of sympatric phylogenetically distinct species (Showalter, Todd, and Brennan, 2014).

Amphisbaenians have been phylogenetically allocated as one of the 26 lineages of Squamate reptiles that present limb reduction and elongation of the body plan (Wiens, Brandley, and Reeder, 2006; Brandley, Huelsenbeck, and Wiens, 2008). Despite the adaptive advantages of these traits for fossoriality (in addition to modifications in head features), constricted locomotion through tight tunnels seems to have driven functional loss by reducing or displacing one side of paired organs (Gans, 1975; Crook and Parsons, 1980), such as lungs (Crook and Parsons, 1980) and gonads (Navega-Gonçalves, 2009). Therefore, as other limbless Squamate (Soldt et al., 2015; Lambertz, Arenz, and Grommes, 2018), Amphisbaenian lineages have evolved bilaterally asymmetrical internal morphology within a cylindrical body (Crook and Parsons, 1980; Navega-Gonçalves, 2009), although *A. alba* Linnaeus, 1758 shows symmetrical testis (Crook and Parsons, 1980). Bilateral asymmetry may have affected physiological, metabolic and reproductive processes. For instance, viviparity in squamates has independently evolved in several clades (Blackburn, 2015), although viviparity or embryo retention are rare in Amphisbaenians (Bons, 1963; Visser, 1967). In a scenario of bilateral asymmetry in the internal morphology of amphisbaenians, both sides of the body would be expected to be subject to different selective forces. Therefore, they should experiment different evolutionary rates, and interspecific differences in reproductive tract morphology should not be consistent between both sides of the body. Either functional or atrophied organs may evolve at rates different enough to generate interspecific variation (Shine et al., 2000).

In general, it has been speculated that body distention during pregnancy has been a key evolutionary consequence of body elongation in Amphisbaenians, since it may impose high costs

for locomotion, which reduces female dispersal capacity (Qualls and Shine, 1998; Pizzatto, Almeida-Santos, and Shine, 2007). Considering that dispersal capacity is affecting the evolution of morphological traits in a wide variety of organisms (Hedrick, 2007; Fraga et al., 2017), it is reasonable to hypothesize that sex-biased dispersal in amphisbaenians may cause distinct morphological traits under selective pressure between sexes. Additionally, the greater dispersal capacity in males should generate higher levels of gene flow, and therefore different levels of morphological variation compared to females (Trochet et al., 2016).

In this study, we analyse two phylogenetically closely-related sympatric amphisbaenian species, *Amphisbaena anaemariae* Vanzolini, 1999 and *A. silvestrii* Boulenger, 1902 (Pyron, Burbrink, and Wiens, 2013; Ribeiro et al., 2019), to investigate fine-scale interspecific variation in morphological variables associated to the reproductive tract. Vanzolini (1997) proposed the *Amphisbaena silvestrii* putative group where he allocated *A. silvestrii*, *A. anaemariae*, *A. crisae* Vanzolini, 1997 and *A. neglecta* Dunn & Piatt, 1936. Species within this group share relatively small adult sizes, body coloration of light and dark shades, no evident specializations on the cephalic and caudal shields, and two precloacal pores. Recently, Ribeiro *et al.* (2019) described *Amphisbaena mebengokre* as a sister species to *A. anaemariae*. Although *A. anaemariae* and *A. silvestrii* are phylogenetically closely related, they are not sister species and present a genetic distance of at least 18.9% (Ribeiro et al., 2019). Though this group is formed by five species, we did not include *A. crisae*, *A. mebengokre* and *A. neglecta* in our sample because of lack of data. The two sampled species are sympatric in central Brazil, although the distribution of *A. anaemariae* extends to South-eastern Brazil, and the distribution of *A. silvestrii* extends west to Bolivia (Ribeiro et al., 2019).

Fossoriality represents an environmental constraint that pushes squamates towards body elongation, and anatomical asymmetry on organ shape and positioning. Although many

amphisbaenian species (including *A. anaemariae* and *A. silvestrii*) show diagnostic characters from the external morphology, evidence of interspecific divergence based on internal morphology is rare. In this study we tested the hypotheses that (i) in the presence of bilateral asymmetry in the reproductive organs, both sides of the body should show different results regardless the levels of interspecific morphological divergence. (ii) Continuous (morphometric) and discrete (meristic) morphological traits of the reproductive tract differ between the species studied. (iii) Levels of divergence in reproductive tract morphology between species are sex dependent. This is consistent with the fact that differences in dispersal capacity between sexes cause sex-dependent gene flow (Greenwood, 1980; Trochet et al., 2016; Li and Kokko, 2019). Additionally, female locomotion might often be limited by offspring production due to mass increase, consequently affecting behaviour. All these hypotheses converge into the idea of reproductive isolation as a mechanism for lineage divergence.

Materials and methods

Morphological data

We measured 20 adult specimens (10 females, 10 males) of *A. anaemariae* from Luziânia, Goiânia and Minaçú, Goiás, deposited in the Coleção Herpetológica da Pontifícia Universidade Católica de Goiás, Goiânia, Goiás, Brazil (CEPB–PUC Goiás), and 20 specimens of *A. silvestrii* (10 females, 10 males) from UHE Guaporé, Vale de São Domingo of Mato Grosso, deposited in the Coleção Zoológica of Universidade Federal do Mato Grosso, Brazil (CZUFMT-R). These specimens belong to the Cerrado Biome and were obtained after wildlife rescue previous to construction activities and thus they do not possess specific coordinates for each specimen. We identified sexual maturity by the presence of developed gonads, usually presenting white-colour in females reproductively inactive or yellow in females reproductively active, as opposed to juveniles that present semi translucent organs. We identified mature males by the presence of coiled vas deferens (Navega-Gonçalves, 2009). We did not include immature specimens in our sample.

We measured one meristic and eight morphometrical variables to quantify the reproductive morphology of both species studied (Figure 1). Measurements were performed with a digital calliper and followed Navega-Gonçalves

(2009) for snout-vent length (SVL), body diameter (BD), and kidneys (K), ovaries and testis size (GS). We measured the Internal Cloaca (IntCL) in females as the ‘vaginal’ or ‘posterior oviduct’ section, considering the importance of tissue differences of the oviduct (Girling, 2002; Sánchez-Martínez, Ramírez-Pinilla, and Miranda-Esquivel, 2007; Siegel et al., 2014) and the posterior section of the vas deferens (portion of the vas deferens with no association to the kidneys) in males. Additionally, we measured gonad-cloaca length (GCL) as the position of gonads related to the gonadal conducts varied among specimens; gonadal conduct length referring to oviduct in females (OdL) and vas deferens in males (VDL); gonad diameter (GD); superposition of kidney and internal cloaca after dissociating (KIntCL); and for females number of oocytes (NO).

Bilateral asymmetry and between-sex differences

Species here used are closely-related, hence, we tested for size differences between species through a t-test applied to the SVL and BD. To discard allometric effect on analyses, we tested for correlation between SVL and BD and all internal anatomical variables separately by sex. We reduced the effects of SVL and BD on all variables measured by applying the formula $Y_i(LDR_0/LDR_i)^b$, where Y_i is the observed value, LDR_0 is the average body length-diameter ratio, LDR_i is the individual length-diameter ratio, and b is the slope of a linear regression between $\log Y_i$ and $\log LDR$ (Cundall et al., 2016). This approach was useful to reduce the effects of allometry in the morphological variation detected. Additionally, we scaled the transformed variables by subtracting the mean and dividing by the standard deviation. This approach was useful in reducing the effects of very different variances among morphological variables (Becker, Chambers, and Wilks, 1988). To determine bilateral asymmetry for each of the internal variables measured, we used paired t-tests between species and also separately by sex (Table 1, Supplementary material S2). This approach was useful in guiding us on testing interspecific differences in reproductive morphology with the complete dataset or separately by body side.

To test interspecific variation in reproductive tract morphology we used Discriminant Analysis of Principal Component (DAPC). This method has been indicated to identify clustering samples because the discriminant function optimize variation between clusters while minimize variation within clusters (Jombart, Devillard, and Balloux, 2010). As we used specimens in different stages of the reproductive cycle to maximize the amount of intraspecific variability, the morphological variation detected by DAPC is expected to be less biased by within-species variation than similar multivariate ordination methods, such as PCA (McCoy et al., 2006) making it a suitable assay for datasets with subtle variations. We implemented DAPC analyses in the adegenet R-package

(Jombart, 2008), using cross validation to define numbers of retained principal components axes. This method estimates proportions of success in predicting the cluster in which each specimen was allocated, based on the lowest mean squared error over a range of axis numbers of principal components (Jombart, Devillard, and Balloux, 2010). We ran DAPC models defining species as *a priori* clusters and controlled the quality of the clustering solution through posterior probabilities of the specimens being correctly allocated in each species. We obtained statistical support for the clustering observed based on mean differences in the scores produced by the first axis from each DAPC model, using ANOVA (Analysis of Variance) models which we set up with species as independent variable. All the ANOVA models were validated by normal distribution of residuals (Shapiro-Wilk $P < 0.05$ in all cases of significant differences).

Additionally, we used Permutational Multivariate Analysis of Variance (PERMANOVA) models to test for differences in pairwise Euclidean dissimilarities in morphological data between the species studied. We implemented PERMANOVA models in the vegan R-package (Oksanen et al., 2019) with 999 permutations. This approach was useful for testing interspecific variation in reproductive tract morphology considering intraspecific morphological variation.

Results

We found that *A. anaemariae* and *A. silvestrii* have interspecific differences seen by testing SVL and BD variables. There was interspecific differentiation in SVL (t-test $t_{19} = 3.67$, $P < 0.01$) and BD (t-test $t_{19} = 11.81$, $P < 0.01$), and there was high correlation of SVL and BD with tested variables (Table S1). Additionally, we found bilateral asymmetry in 50% of the variables measured in males, and 62.5% of the variables measured in females ($P < 0.01$ in all cases). These findings led us to conduct analyses on interspecific morphological variation separately by body side.

For the right side of females, the DAPC model retained six Principal Component axes, which captured 97% of the original variance observed. Graphically, we found two distinct clusters, which were consistent with the species studied (Figure 2A). This finding was supported by significant differences in DAPC scores between species (ANOVA $F_{1-18} = 4.65$, $P < 0.05$;

Figure 2B), and relatively high overall membership posterior probabilities (Figure 2C) for both *A. anaemariae* (70%) and *A. silvestrii* (60%). Between-species morphological variation was mainly associated to KIntCL (71%) and IntCL (10%). For the left side, the DAPC retained one Principal Component axis which captured 33.4% of the original observed variance. We found two overlapped clusters corresponding to the species studied (Figure 2D), which is consistent with indistinct DAPC scores ($F_{1-18} = 1.89$; $P = 0.18$; Figure 3E), and relatively low membership posterior probabilities (Figure 2F) for *A. anaemariae* (60%) and *A. silvestrii* (50%).

In males, the DAPC model based on data from the right-side organs retained one Principal Component axis, which captured 45.13% of the original variance observed. Graphically, two very distinct clusters were distinguishable (Figure 3A), which was supported by the ANOVA model ($F_{1-18} = 12.82$, $P < 0.01$; Figure 3B). The variables that most contributed to this differentiation were GCL (26.2%) and VDL (20.4%). Membership probabilities were high (Figure 3C) for both *A. anaemariae* (70%) and *A. silvestrii* (80%). For the left side, the DAPC retained three Principal Component axes that captured 85.2% of the original observed variance. We found clear graphical distinction of clusters (Figure 3D) supported by significant differences between DAPC scores ($F_{1-18} = 20.81$; $P < 0.001$; Figure 3E), and the variables that most contributed to the model were GS (33.3%), GD (24.3%) and VDL (20.4%). These findings were supported by high overall membership posterior probabilities (Figure 3F) for *A. anaemariae* (80%) and *A. silvestrii* (100%).

Females did not present interspecific differences between sides of the body after PERMANOVA test (PERMANOVA right side $F_{1-18} = 0.73$; $P = 0.63$; left side $F_{1-18} = 0.86$; $P = 0.54$). This finding suggests that morphological differences between species can be only detected when intraspecific variation is reduced. Contrarily, the PERMANOVA results were congruent with DAPC results in males, for which differences were significant in both body sides (right side $F_{1-18} = 4.72$; $P < 0.01$; left side $F_{1-18} = 4.72$; $P < 0.01$).

Discussion

Our results showed that divergence in reproductive tract morphology between two closely related species are sex dependent, independently of body side, despite morphological divergence was more evident in males. We showed that interspecific morphological divergence was mainly associated to position and size of posterior oviduct in females, and gonad shape and position, and vas deferens length in males. Our results suggest high levels of morphological divergence between species, although they are phylogenetically closely related. Our results have relevant implications for an understanding of the role of sex-dependent dispersal in the species studied.

In *A. anaemariae* and *A. silvestrii* males, bilateral asymmetry suggests independence of evolutionary processes selecting phenotypes on each body side. This was supported by a clear distinction between species based on several variables quantifying reproductive tract, which usually differed between body sides. In the left side, interspecific differences in gonad size and diameter are consistent with literature showing asymmetrical gonad disposition characterized by right side more cranial than the left side, and the left gonad usually smaller (Crook and Parsons, 1980; Navega-Gonçalves, 2009; Santos, 2013). For the right side, interspecific differences in GCL and VDL may indicate that the urogenital system is determined by space availability in the abdominal cavity, which differs between body sides. This is supported by mean differences in GS between body sides in both species, although GD was not significantly different between body sides. Overall, our results are consistent with other groups of elongated-body Squamata reptiles, for which testis, kidneys and hemipenis are larger on the right side of the body, which indicates reproductive success as a function of body side (Shine et al., 2000).

Although our multivariate models were efficient to show that the reproductive tract morphology differs among males of the studied species, our findings were less conclusive for females. Specifically, morphological divergence between species could only be identified when

intraspecific variation was arbitrarily neglected. This finding indicates slower evolutionary processes in females, probably resulting from lower dispersal capacity compared to males. Sex-biased dispersal has been considered as one of major factors driving Squamata evolution (Olsson and Shine, 2003; Chapple and Keogh, 2005; Rivera, Gardenal, and Chiaraviglio, 2006; Keogh, Webb, and Shine, 2007; Vercken et al., 2007; Dubey et al., 2008; Ujvari, Dowton, and Madsen, 2008), and in fossorial species such as amphisbaenians it may be associated to reduced dispersal capacity in pregnant females (Qualls and Shine, 1998; Shine, 2003; Bellini, Arzamendia and Giraudo, 2018). Considerable increase in body mass and volume during pregnancy is particularly critical for dispersal through tight underground tunnels. Additionally, greater dispersal in males generates comparatively high accumulated mutations over a given number of generations, which is optimized by longer reproductive seasons in males (Andrade, Nasciento, and Abe, 2006). Therefore, the inconsistencies between the statistical approaches we used are explained by a combination of dispersibility and reproductive rates accumulating mutations over generations.

Although our evidence is indirect, males apparently accumulate mutations faster than females due to their higher dispersal capacity. Under sexual-biased dispersal, high mutation rates generate relatively high probabilities of positive selection acting toward fitness optimization (reviewed by Ellegren and Parsch, 2007; Trochet et al., 2016; Li and Kokko, 2019). The influence of male-biased dispersal on the reproductive tract morphology may be readily understood from the perspective that males are the main mediators of gene flow. The assumption of positive selection acting differentially on male reproductive tract is supported by the fact that male amphisbaenians have homogametic chromosomes (Pokorná and Kratochvíl, 2009) and due to favoured dispersal capacity, they have access to a wider gene pool. Therefore, males tend to fix different phenotypes more rapidly than females (Ellegren and Parsch, 2007; Trochet et al., 2016; Li and Kokko, 2019). Consequently, differences in reproductive tract morphology tend to be

more evident in males, although we found divergence in females when we reduced intraspecific variation.

Greater morphological variation in males may also be associated with broader access to optimal thermoregulation sites or vertical migration compulsorily induced by rainwater infiltration. The latter option is supported by the fact that *A. anaemariae* and *A. silvestrii* inhabit in the Cerrado savannas in central Brazil, where temperature does not vary dramatically throughout the year, but rainfall is highly seasonal (Macena et al., 2008). As fossorial animals, amphisbaenians are subject to changes in soil saturation in water, and with high annual variability in levels of precipitation, and are probably destinating energy to vertically migrate in response to such changes. On the other hand, amphisbaenians are active thermoregulators especially in the reproductive season (López, Salvador, and Martín, 1998; Matias et al., 2018), but due to lower locomotor capacity during pregnancy, females may have restricted access to optimal thermoregulatory sites. Additionally, enlarged females need more time to gain or lose heat than nonpregnant females or males (López, Salvador, and Martín, 1998; Qualls and Shine, 1998; Matias et al., 2018). This is especially relevant to dispersal capacity as amphisbaenians thermoregulate by contact to warm surfaces (thigmothermal) and usually use enlarged rocks to thermoregulate (López, Salvador, and Martín, 1998; Matias et al., 2018), though this has been seen in species from southern Brazil and Spain, where seasonality is more pronounced than in the Cerrado savannas. Therefore, it is logical to assume that individuals with increased body mass, and possibly body diameter, will invest less time in dispersal and more time in thermoregulating (Qualls and Shine, 1998; Matias et al., 2018).

We presented a hypothesis-testing approach to compare reproductive tract morphology between two closely related fossorial species. Fossoriality has been a major obstacle for biodiversity sciences, because usually causes low detectability and consequently lack of data, as

is in amphisbaenians. However, we have shown that a combination of access to zoological collections and appropriate statistical treatment may reveal evolutionary aspects relevant to our understanding of the origins of biodiversity. We found that two closely related fossorial species show reproductive isolation mechanisms, which may be associated with sex-dependent dispersal and mutational rates. Although interspecific divergence has been widely demonstrated in amphisbaenians by external morphology and DNA sequences (Macey et al., 2004; Townsend et al., 2004; Vidal and Hedges, 2009; Pyron, Burbrink, and Wiens, 2013; Ribeiro et al., 2019), we showed that even the internal anatomy associated with reproduction may differ between species.

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Tables

Table 1. Mean of internal morphometric measurements (mm) of measured and counted variables expected to vary between species. SVL=body length, BD= body diameter, K=kidney size, GS = gonad size, IntCL=internal cloaca length, GCL=gonad-cloaca length, OdL= oviduct length, VDL= vas deferens length, GD= gonad diameter, KIntCL=superposition of kidney and internal cloaca, NO= number of oocytes. Variables marked with * show $p<0.05$ for bilateral symmetry, indicating differences between body sides.

	Sex	LEFT				RIGHT			
		<i>A. anaemariae</i>		<i>A. silvestrii</i>		<i>A. anaemariae</i>		<i>A. silvestrii</i>	
		Mean	SD	Mean	SD	Mean	SD	Mean	S
GCL*	F	35.91	4.64	33.1	6.86	46.77	9.04	45.49	7.91
	M	34.6	3.14	30.9	2.85	42.38	4	37.04	3.46
GS*	F	11.09	8.21	8.56	8.78	15.23	6.79	11.41	5.16
	M	6.69	1.83	5.09	1.61	7.09	1.91	5.96	2.19
OdL*	F	33.29	6.05	32.15	6.23	45.94	9.12	44.62	13.32
VDL*	M	33.2	3.26	28.14	2.33	38.79	5.8	34	3.03
IntCL	F	1.79	0.79	2.09	1.89	1.8	1.11	2.37	1.88
	M	0.92	0.17	0.77	0.28	0.8	0.41	0.94	0.35
GD	F	2.23	1.56	1.75	1.8	1.97	1.4	1.27	0.95
	M	1.85	0.62	1.31	0.47	1.61	0.55	1.27	0.34
KIntCL	F	0.66	0.21	1.39	1.44	0.83	0.35	1.28	1.6
	M	0.43	0.18	0.34	0.08	0.39	0.15	0.44	0.06
K*	F	14.68	2.64	13.82	1.51	15.44	2.83	15.11	1.98
	M	16.23	2.4	14.79	1.34	17.86	2.67	15.57	1.37
NO*	F	8.62	3.16	6.45	3.58	12.9	8.26	8.42	4.11

		<i>A. anaemariae</i>		<i>A. silvestrii</i>	
		Mean	SD	Mean	SD
SVL*	F	192.2	51.41	160.5	13.61
	M	197.95	35.86	153.09	17.27
BD*	F	6.37	0.68	4.66	0.62
	M	6.26	0.78	4.55	0.68

Figure legends

Figure 1. Internal reproductive morphology of A) Females and B) males of *Amphisbaena anaemariae* and *A. silvestrii*. Morphological variables are shown in red; kidneys are shown in dotted line in green and oviduct and vas deferens are shown in light blue. GS) gonad size, GD) gonad diameter, GCL) gonad-cloaca length, K) kidney length, VDL) vas deferens length, IntCL) internal cloaca length, KIntCL) Superposition of internal cloaca with kidney, OdL) oviduct length

Figure 2. Morphological differences between female *Amphisbaena anaemariae* (purple) and *Amphisbaena silvestrii* (green) based on eight morphological traits given in mm, and separate by right (A–C) and left (D–F) body sides. A and D) Show distributions of specimen densities along the first axis produced by a Discriminant Analysis of Principal Components (DAPC). The vertical lines in the bottom denote the positioning of individuals along the DAPC axis. B and E) show differences in the scores generated by the DAPC between body sides. Horizontal lines inside boxes are medians and whiskers show amplitudes. C and F) Probabilities that the specimens were correctly allocated in one of the species where each vertical bar shows a specimen. The more unicolor the bar, the more likely the specimen was correctly allocated by the DAPC in the a priori group (species).

Fig. 3. Morphological differences between male *Amphisbaena anaemariae* (purple) and *Amphisbaena silvestrii* (green) based on seven morphological traits given in mm, and separate by right (A–C) and left (D–F) body sides. A and D) Show distributions of specimen densities along the first axis produced by a Discriminant Analysis of Principal Components (DAPC). The vertical lines in the bottom denote the positioning of individuals along the DAPC axis. B and E) Differences in the scores generated by the DAPC between species. Horizontal lines inside boxes are medians and whiskers show amplitudes. C and F) Probabilities that the specimens were

correctly allocated in one of the species where each vertical bar shows a specimen. The more unicolor the bar, the more likely the specimen was correctly allocated by the DAPC in the a priori group (species).

Figures

Figure 1

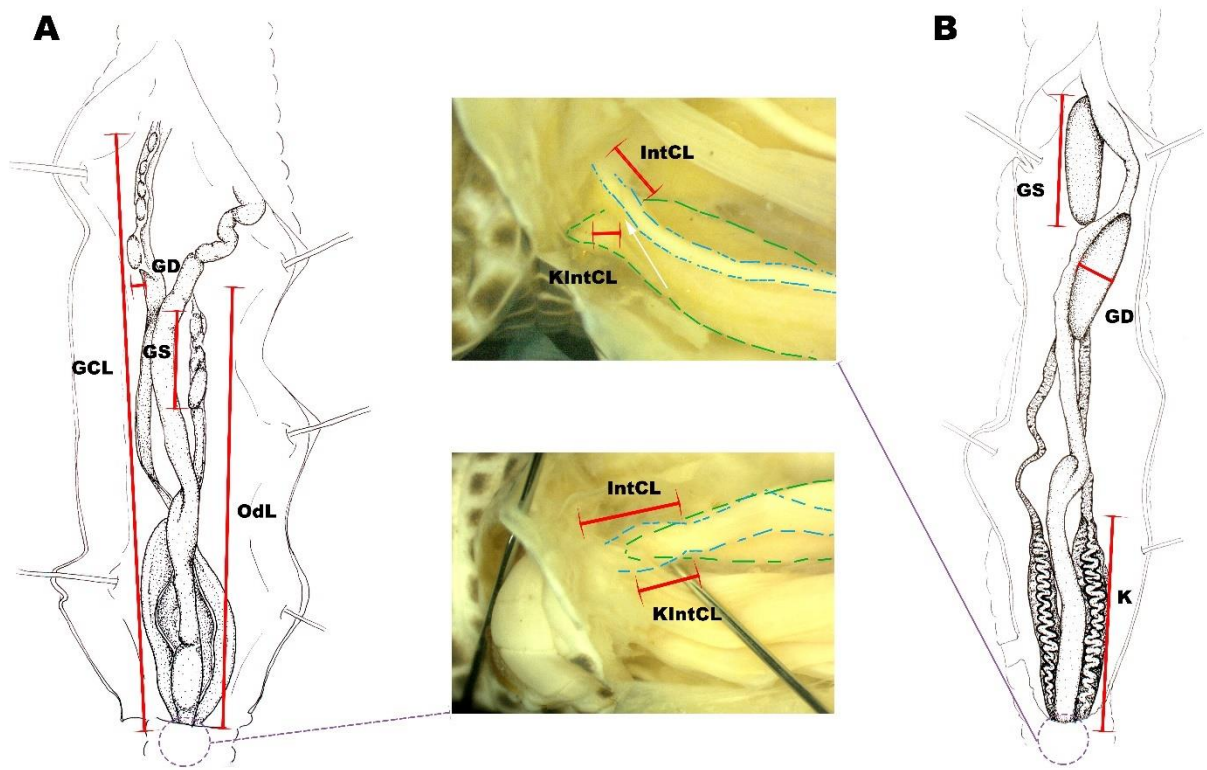


Figure 2

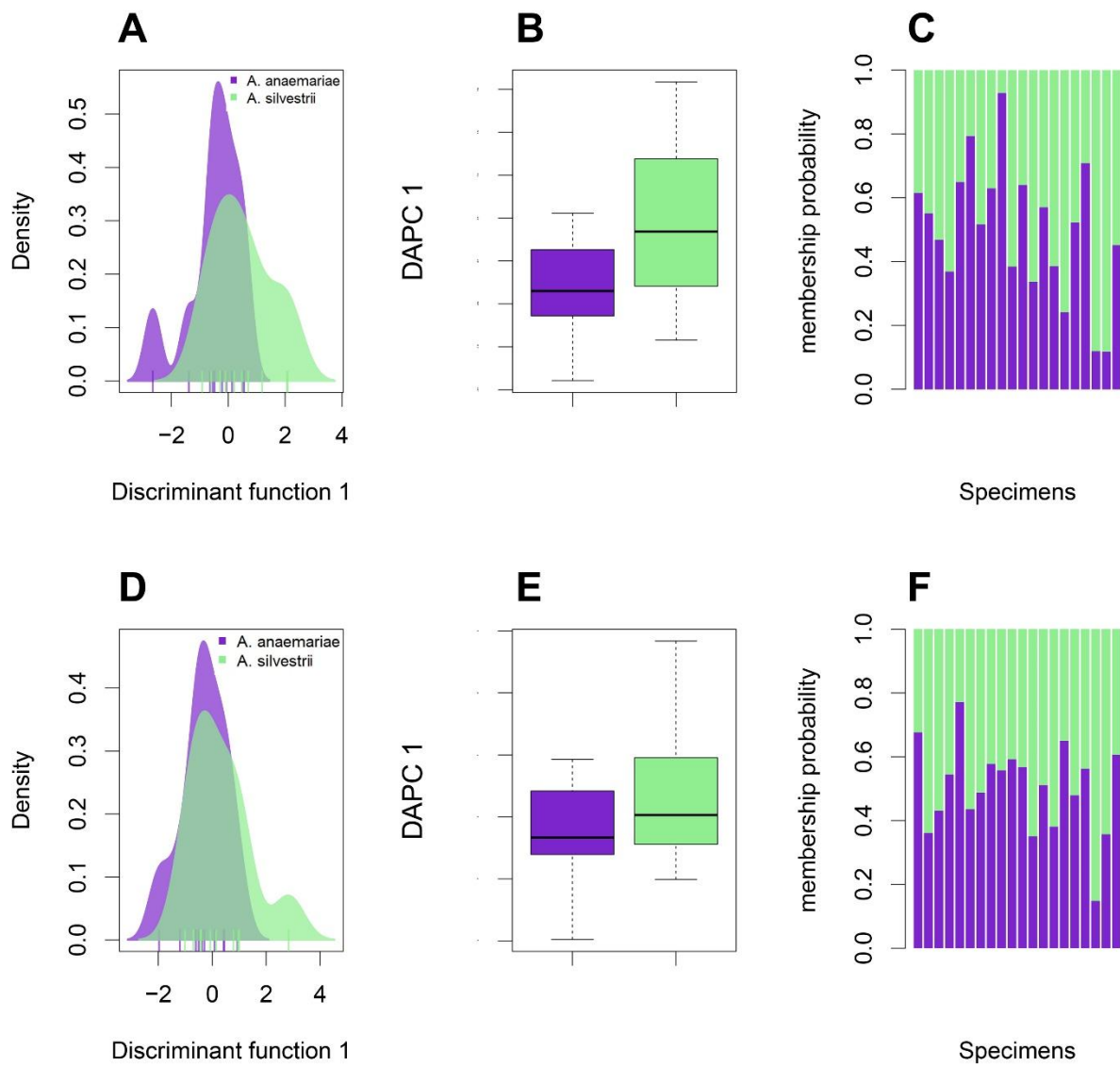
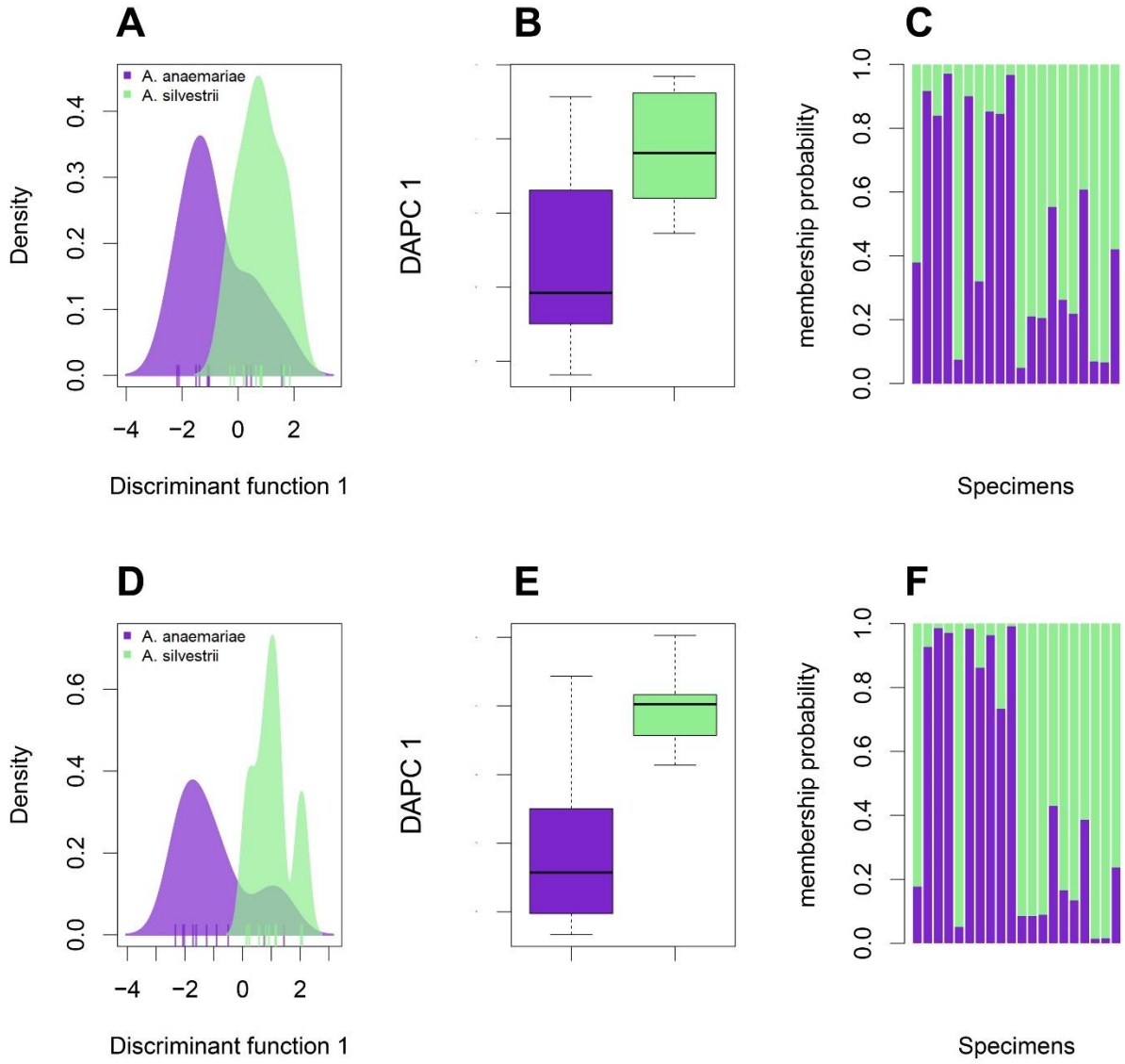


Figure 3



Comentários à coordenação do PPGBEES:

A dissertação está muito bem escrita e estruturada. Os resultados trazem dados novos e relevantes, e certamente é um trabalho com uma grande contribuição ao conhecimento da fauna neotropical.

Esse trabalho tem importância não só para o conhecimento biológico e anatômico do grupo, como também trás discussões evolutivas e sistemáticas sobre as anfisbenas, o que certamente terá impacto nas futuras discussões sobre esses animais.

Algumas normas da revista proposta para submissão do artigo não foram respeitadas. Por exemplo, a necessidade de ter linhas numeradas, exigência da cover letter, cover page, etc. Como as regras do PGBEES exigem que as regras de formatação sejam respeitadas, acredito que isso deva ser incluso na dissertação.

Sugiro aos autores tornar a introdução mais curta e mais linear. Por algumas vezes os tópicos acabam sendo discutidos mais de uma vez.

A seção do Material e métodos está adequada. Entretanto, eu acredito que os autores devam acrescentar ao final da dissertação uma lista dos animais examinados.

Os resultados e a discussão estão coerentes e claros com gráficos bem didáticos e bonitos.

A discussão do trabalho, embora esteja bem-feita e embasada, ainda tem algumas especulações que acredito que faltam mais embasamento em informações na literatura para torná-las robustas. Cuidado com especulações excessivas sobre os resultados e suas interpretações. Por exemplo, pouco se sabe sobre dispersão de jovens, e como isso impacta no fluxo gênico das espécies. Cuidado com este tipo de especulação.

Há diversas inconsistências de formatação nas referências, como nomes de espécies sem estar em itálico.

Recomendo a aprovação da dissertação com apenas algumas sugestões de correções. Nada que comprometa a qualidade final do trabalho.

Algumas dúvidas que tive e poderiam ser facilmente contornadas através de uma conversa com os autores eu incluí no pdf da dissertação.

As minhas sugestões para o texto estão inclusas no pdf em anexo.

Os autores citam que há material suplementar com informações extras, mas isso não estava presente no documento enviado para avaliação. Sugiro incluir no material suplementar as fotos que estavam presentes na qualificação.

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Comentários à coordenação do PPGBEES:

A presente dissertação intitulada "Internal Morphology Reveals Reproductive Isolation Between Two Amphisbaenian Closely Related Species (Squamata: Amphisbaenidae)" da discente Francesca Angiolani Larrea, ao meu ver esta apta a aprovação com correções mínimas feitas diretamente no corpo do texto.

A dissertação traz importantes contribuições para melhor compreensão de aspectos morfológicos e ecológicos sobre um grupo de répteis pouco conhecido, além de discutir os resultados com uma abordagem evolutiva.

A introdução possui revisão da bibliografia recente, além explicitar as hipóteses que foram testadas. Os dados e análises são apropriados para as perguntas, os resultados estão bem apresentados e a discussão é condizente com os resultados e possui uma abordagem evolutiva.

O manuscrito está de acordo com o scopus da revista a que foi submetida.

Sem mais a acrescentar considero a dissertação apta a aprovação com correções mínimas.

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