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Comparative Analysis of the Postcranial Skeleton of the South American Viperids (serpentes, Viperidae) *Bothrops* and *Crotalus* Using Two-Dimensional Geometric Morphometrics

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Abstract

The Viperidae is the most speciose family of Brazilian venomous snakes, with 33 known species. Although the family is well defined cladistically, there are few studies concerning the postcranial skeletal morphology, and only a single vertebral synapomorphy has been proposed. The paucity of knowledge on postcranial morphology poses challenges for the study of the Brazilian viper fossil record since most fossils consist of disarticulated and isolated vertebrae. Currently, *Bothrops* and *Crotalus* are the only vipers recognized in the Brazilian fossil record. Nonetheless, interspecific differentiation based on vertebral material is hampered due to the lack of comprehensive detailed anatomical data. We compared the trunk vertebrae of extant specimens of *Crotalus* and *Bothrops* using two-dimensional geometric morphometrics to obtain discriminant data about their vertebral morphology. We examined the trunk vertebrae of 20 vipers, 10 *Crotalus*, and 10 *Bothrops* and performed macroscopic analyses and measurements and landmark-based, two-dimensional geometric morphometric analyses. We sought to identify structural differences between the genera and to assess morphological variation along the spine. Most differences in the trunk vertebrae between *Crotalus* and *Bothrops* occurred in the length of the neural spine, the parapophyseal processes, the prezygapophyseal processes, and in the angle on the prezygapophyses. However, when we accounted for intracolumnar variation, differentiation is hampered. We expect our results will serve as a starting point for future studies of viperid vertebrae and aid paleontologists in accurately identifying fossil vipers.

Venomous snakes are animals of significant health and commercial interests, being potentially dangerous to humans. Currently, 760 venomous snakes are known worldwide (Uetz et al., 2022), of which 67 species are present in Brazil belonging to the Elapidae and Viperidae families (Costa et al., 2021). Between them, Viperidae (i.e., vipers) is the most diverse group, encompassing in Brazil around 33 known species of pit-vipers (subfamily Crotalinae), represented by the genera *Bothrops*, *Bothrocophias*, *Crotalus*, and *Lachesis* (Barbo et al., 2022; Costa et al., 2021; Dal Vechio et al., 2021; Uetz et al., 2022). Among the known viperids, the genera *Bothrops* and *Crotalus* are responsible for most of the venomous accidents recorded in Brazil (Bernarde, 2011).

With regard to morphology, the Viperidae is defined by numerous morphological synapomorphies (especially soft tissues; Zaher, 1999), and the most important one is the distinct and specialized solenoglyphous dentition (Vidal, 2002). For the postcranial skeleton, usually a combination of characters are used as identifiers of the family, such as long straight hypapophysis throughout the trunk region, depressed neural arches, and well-developed and ventrally oriented parapophyseal process (Szyndlar, 1984, 1991). However, only a single synapomorphy for the family is proposed in the postcranial skeleton, which is the well-developed and anteroventrally directed parapophyseal process in the vertebral column (Zaher, 1999). Nevertheless, the parapophyseal structure is present, although less developed, in other groups like natricids (Szyndlar, 1991; Zaher, 1999).

Despite efforts at anatomical descriptions of some viper genera based on fossil vertebrae (Albino & Montalvo, 2006; Camolez & Zaher, 2010; Georgalis et al., 2016; Szyndlar, 1991) and extant species (Walker, 2003), detailed studies that discriminate between extant Brazilian species of *Crotalus* and *Bothrops* and that properly describe the anatomy and intracolumnar variation in vertebrae of these species are lacking. Lack of information on vertebral morphology is a problem for paleontological studies in Brazil. The fossil record of vipers is composed mainly of disarticulated and isolated postcranial elements (Camolez & Zaher, 2010; Evans, 2003; Onary et al., 2017), which makes identification below the familial level (e.g., genera and species) difficult.

Based on the fossil record, *Bothrops* and *Crotalus* are the only viper genera that have been discovered in Brazil, especially from the Neogene and Quaternary of Ceará, Minas Gerais, Bahia, Goiás, and Acre states (Camolez & Zaher, 2010; Hsiou et al., 2012; Hsiou & Albino, 2011; Onary et al., 2017). Even though there are fossils attributed to these two genera, their taxonomic identification is contentious because no diagnostic vertebral characters have been proposed for the subgroups within Viperidae (Albino & Montalvo, 2006). Camolez and Zaher (2010) suggested some subtle differences between *Crotalus* and *Bothrops*, especially in height of the neural spine and zygosphene morphology

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in some specimens. Nonetheless, no detailed comparative study of *Crotalus* and *Bothrops* was provided. Therefore, it is necessary to investigate Brazilian vipers' vertebral morphology to better understand the fossil record and South American vipers. We hope that integration between qualitative description and geometric morphometrics (GMs) can be helpful for differentiation between these two genera of Brazilian viper. In this context, our study aimed to compare the vertebrae of extant specimens of *Crotalus* and *Bothrops* by applying two-dimensional GMs to obtain discriminating data about their vertebral morphology and contribute to the postcranial anatomic study of vipers which can be further used for future paleontological identification. Also, GM analyses are increasingly used to identify fossil species (Courtenay et al., 2019; Leshno Afriat et al., 2020; Marramà & Kriwet, 2017). Therefore, this study created a database that can be used for this purpose.

Materials and methods

To observe potential intra- and interspecific variation present in the trunk vertebrae of viper snakes, we analyzed specimens from the genera *Bothrops* and *Crotalus*. Skeletons were mechanically processed by applying the water maceration technique in the entire vertebral column, allowing soft tissue decomposition without osteological damage (Auricchio & Salomão, 2002).

Specimens were obtained from the Coleção Herpetológica de Ribeirão Preto (CHRP), managed by the Laboratório de Evolução e Biologia Integrativa (LEBI), at Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Brazil (FFCLRP- USP). Skeletons comprise adult individuals of *Bothrops* (*Bothrops moojeni*: CHRP1803, CHRP2068, CHRP2078, CHRP2079, CHRP2080; *Bothrops alternatus*: CHRP2070, CHRP2071, CHRP2072, CHRP2073, CHRP2082) and *Crotalus* (*Crotalus durissus*: CHRP1800, CHRP1801, CHRP1802, CHRP2065, CHRP2066, CHRP2067, CHRP2074, CHRP2075, CHRP2076, CHRP2077) .

To perform the osteological description, we first sampled trunk vertebrae from each specimen. One vertebra was sampled at 10 vertebrae intervals until the final trunk vertebra. Osteological nomenclature was based on Auffenberg, 1963; Lee & Scanlon, 2002; Rage, 1984; and Georgalis et al., 2021 (Fig.2). Qualitative description was made using a stereomicroscope and measurements were made using a caliper after LaDuke (1991a, 1991b). Only trunk vertebrae were used in this study.

For GM analysis, we separated the trunk vertebral column into three regions: anterior trunk, mid-trunk, and posterior trunk (Fig. 1). Anterior trunk vertebrae were defined as the 50 most anterior trunk vertebrae, posterior trunk vertebrae were the 50 most posterior, and mid-trunk were the remaining trunk vertebrae. Therefore, we separated the five more anterior of the vertebrae used in the osteological description (10th, 20th, 30th, 40th and 50th), then the five more posterior (the last, the penultimate, the antepenultimate, and so on until there were five posterior trunk vertebrae sampled), then five mid-trunk vertebrae were sampled with the remaining vertebrae that were closer to the middle. Thus, the analysis had 15 vertebrae (objects) from each specimen, totaling 100 objects for each vertebral area, and

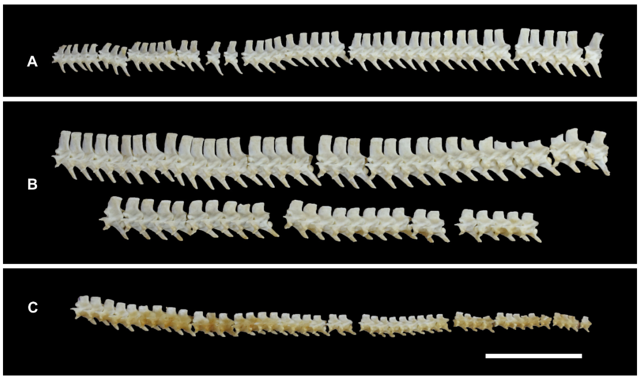

FIGURE 1. Longitudinal variation of the trunk vertebral column in Crotalus durissus (CHRP2076) in lateral view.

Division of regions minimized effects of longitudinal variation on ANOVA results. Anterior trunk vertebrae (A) were defined as the 50 most anterior vertebrae, posterior trunk vertebrae (C) were the 50 most posterior, and mid-trunk (B) were the remaining trunk vertebrae. Scale bar = 5 cm.

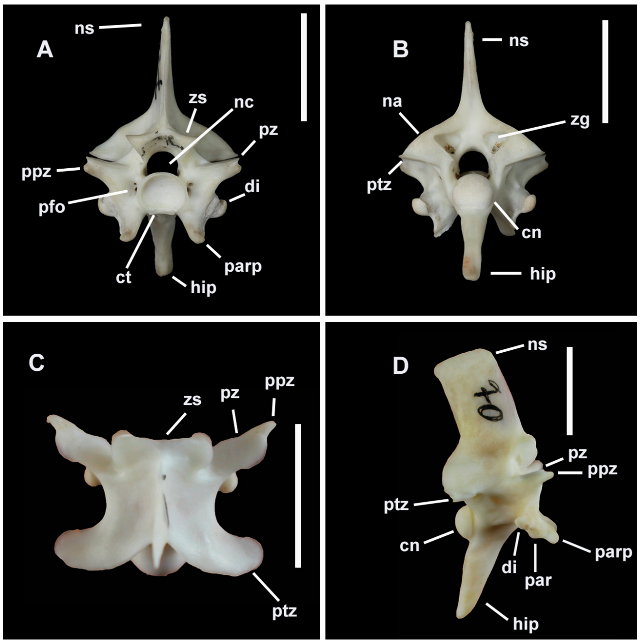

FIGURE 2. Isolated mid-trunk *Crotalus durissus* vertebrae showing the terminology adopted in (A) anterior, (B) posterior, (C) dorsal, and (D) lateral views.

Abbreviations: cn, condyle; ct, cotyle; di, diapophysis; hip, hypapophysis; na, neural arch; nc, neural canal; ns, neural spine; par, parapophysis; parp, parapophyseal process; pfo, paracotylar foramen; ppz, pre zygapophyseal process; ptz, postzygapophyses; pz, prezygapophyses; zg, zygantrum; zs, zygosphene.

those objects represented the entire trunk vertebral column. We could access each genus' intra-columnar variation and a generalized vertebral shape using five vertebrae from each trunk region.

Landmarks were selected to represent vertebral structures of interest that could be chosen repeatedly with minimal error. The plot of landmarks (Fig. 3) was conducted based on homology criteria (Zelditch et al., 2004) using the software TPSdig (Rohlf, 2021). We used the lateral and anterior views. Landmarks were imported into the software R 3.6.3 (R Development Core Team, 2022), superimposed us-

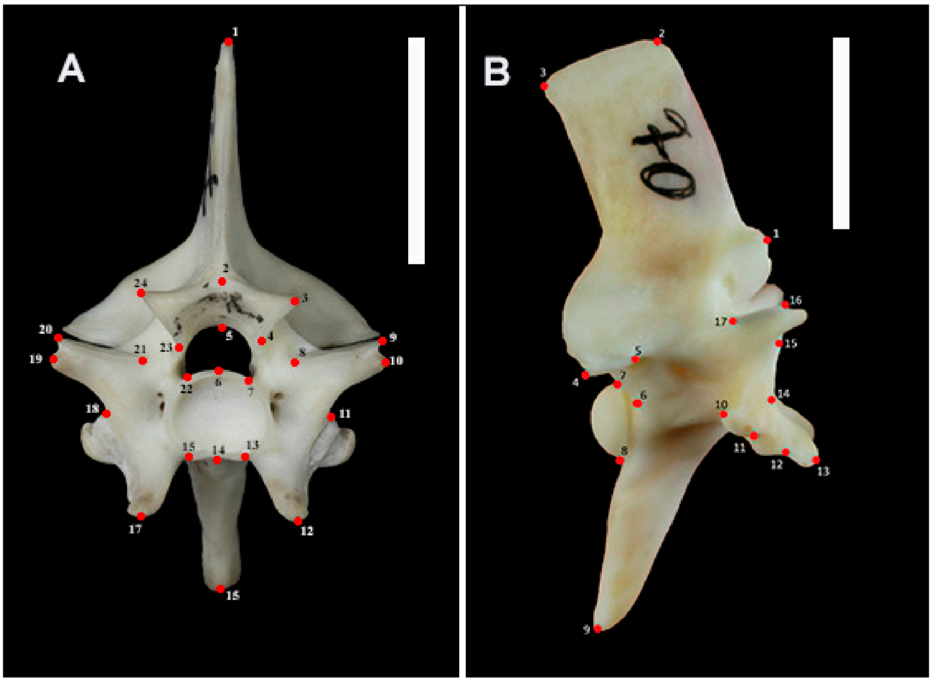

FIGURE 3. Landmarks used in (A) anterior and (B) lateral views are shown in two mid-trunk vertebrae of *Crotalus durissus*.

Numbers represent the order in which landmarks were selected. Scale bar = 1 cm.

ing a Procrustes alignment, and then submitted to a Non-parametric Procrustes ANCOVA to search for differences in shape between genera and the three trunk regions. Our analysis was conducted using the *geomorph* package (Adams & Otárola-Castillo, 2013). To account for the magnitude of the difference between the two genera and thus reduce the type I error, the effect size (Z) was estimated through a randomization and permutation procedure based on an F distribution, using the *RRPP* package (Collyer & Adams, 2018). Each region of the trunk vertebral column was tested separately so the longitudinal variation would not affect the results.

To assess intracolumnar variation, we performed a Principal Components Analysis (PCA) on all vertebrae. The PCA analysis was chosen because it exposes each vertebra's morphological variation, showing shape variation regardless of the genus.

We performed a Canonical Variate Analysis (CVA) on all vertebrae. The CVA was employed to identify which features best differentiate previously defined groups and tests whether the groups' CVA (Mahalanobis distance) is significant. For the PCA and CVA tests, we used the software MorphoJ (Klingenberg, 2011) to visualize shape variation through wireframes. Genera grouped the objects, and their mean shape was compared.

RESULTS

General Intracolumnar and Intraspecific Shape Differences.—Despite almost indistinguishable vertebral morphology of these Viperidae species, we found some differences that help distinguish *Crotalus* and *Bothrops* trunk vertebrae.

Crotalus.—Individual *Crotalus durissus* have around 167 trunk vertebrae with a very tall neural spine (sensu LaDuke, 1991b), especially in the anterior and mid-trunk regions. The neural spine becomes anteroposteriorly shorter and the vertebrae widen proportionally laterally on the posterior trunk vertebrae. In anterior view, the prezygapophyses of *Crotalus* are more horizontally oriented relative to the horizontal plane (0° to 18°) than the vertebrae of *Bothrops*. The more anterior trunk vertebrae have less inclined prezygapophyses while the more posterior trunk vertebrae have more inclined prezygapophyses. The vertebrae have a paracotylar foramen on each side of the cotyle of variable size throughout the column without an apparent pattern. The zygosphene is thin and dorsally elevated in anterior view in the more anterior vertebrae, becoming straighter regarding the horizontal plane on the mid-trunk and posterior vertebrae. The parapophyseal process is well developed and anteroventrally oriented, with little to no lateralization on all vertebrae (Fig. 4A, E, I).

In posterior view, the neural arch is triangular in almost all vertebrae, being more vaulted on the more anterior trunk vertebrae, vaulting ratio $\bar{x} = 0.49$ (sensu Georgalis et al., 2021), and becoming more depressed on the more posterior portion of the trunk, vaulting ratio $\bar{x} = 0.31$ (sensu Georgalis et al., 2021). In some anterior vertebrae, the neural arch is slightly arched. The postzygapophyses are more horizontally oriented. The zygantrum is wide and has a foramen in each articular facet (Fig. 4D, H, L). There are small pits immediately above the postzygapophyseal articular facets, usually they are near the more lateral portion of the postzygapophyses, though the quantity and placement of those pits is variable.

In lateral view, the hypapophysis is well developed throughout the whole trunk vertebral column, being proportionately shorter on the more posterior trunk vertebrae and projecting beyond the condyle on all vertebrae (Fig. 4B, F, J). The tip of the hypapophysis is pointed. The neural

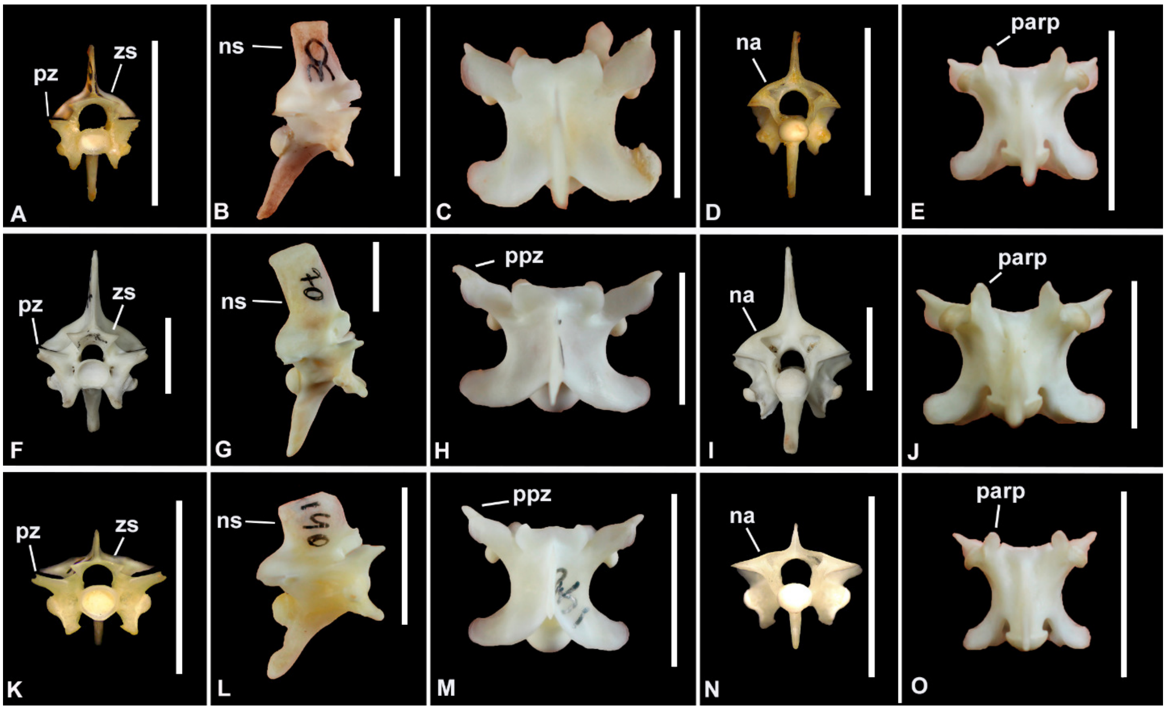

FIGURE 4. *Crotalus durissus* trunk vertebrae for each region in anterior (A, F, K), lateral (B, G, L), dorsal (C, H, M), posterior (D, I, N), and ventral (E, J, O) views. The first row (A-E) are anterior trunk vertebrae, the second row (F-J) are mid-trunk vertebrae, and the third row (K-O) are posterior trunk vertebrae.

Abbreviations: na (neural arch); ns (neural spine); parp (parapophyseal process); ppz (prezygapophyseal process); pz (prezygapophyses); zs (zygosphene). Scale bar = 1 cm.

spine is tall, especially in the anterior and mid-trunk vertebrae, corresponding to half the total vertebral height; it becomes shorter on the most posterior parts of the trunk. The lateral foramina are present, with one small foramen on each side of the vertebra.

In dorsal view, the prezygapophyses are anterolaterally oriented on the more anterior vertebrae and laterally oriented on the mid-trunk and posterior vertebrae. The prezygapophyseal process is thin and of medium length (sensu LaDuke, 1991b) in most vertebrae (Fig. 4C, G, K). The anterior margin of the zygosphene has multiple shapes across the column, ranging from the crenate morphology (sensu Auffenberg, 1963) to the straight/rectilinear or concave, i.e., “V” shaped anterior edge. The zygosphene is usually straight on the more anterior trunk vertebrae, becoming concave (“V” shape) on the following vertebrae, and it is usually crenated on the more posterior vertebrae.

In ventral view, there is usually one subcentral foramen on each side of the hypapophysis. The vertebral centrum has a slight triangular shape, being wider anteriorly. The parapophyseal processes are extremely well developed and anteriorly oriented. The postzygapophyseal articular facets have an elliptical shape.

Bothrops.—Snakes of the genus *Bothrops* have trunk vertebrae with a long neural spine (sensu LaDuke, 1991b) but are considerably shorter than those of *Crotalus durissus*. The neural spine becomes shorter on the more posterior vertebrae, and the vertebrae become proportionally laterally wider. *Bothrops moojeni* has around 186 trunk vertebrae, while *Bothrops alternatus* has around 173 trunk vertebrae.

In anterior view, the prezygapophyses are more diagonally inclined relative to the horizontal plane in the anterior view (0° to 24°30’) in relation to *Crotalus durissus*. The vertebrae have one or two foramina of varying size on each side of the cotyle. The zygosphene is thin and dorsoventrally arched in the more anterior trunk vertebrae, becoming straight in relation to the horizontal plane on the mid-trunk and posterior trunk vertebrae. The parapophyseal process is well developed and anterolaterally oriented (Fig. 5A, E, I).

In posterior view, the neural arch is triangular in almost all vertebrae, the more anterior trunk vertebrae are less depressed with vaulting ratio $\overline{x}$ = 0,40 (sensu Georgalis et al., 2021), and they become more depressed on the posterior portion of the trunk, with vaulting ratio $\overline{x}$ = 0,27 (sensu Georgalis et al., 2021). The postzygapophyses are diagonally oriented relative to the horizontal plane in comparison to the trunk vertebrae of *Crotalus durissus*. The zygantrum is wide and has a foramen in each articular facet (Fig. 5D, H, L). There are small pits right above the postzygapophyseal articular facets, usually near the more lateral portion of the postzygapophyses, although the quantity and placement are variable.

In lateral view, the hypapophysis is anteroposteriorly long, shorter on the column’s posterior trunk region, and projecting beyond the condyle on all vertebrae. In some individuals, the tip of the hypapophysis is blunted (i.e., hatchet-shaped) on the more anterior trunk vertebrae (Fig. 5B) and pointed on the remaining vertebrae. The neural spine is tall but comparatively lower than in *C. durissus*, with the height of the neural spine is usually less than half of the total vertebral height. The lateral foramina are present (Fig. 5B, F, J).

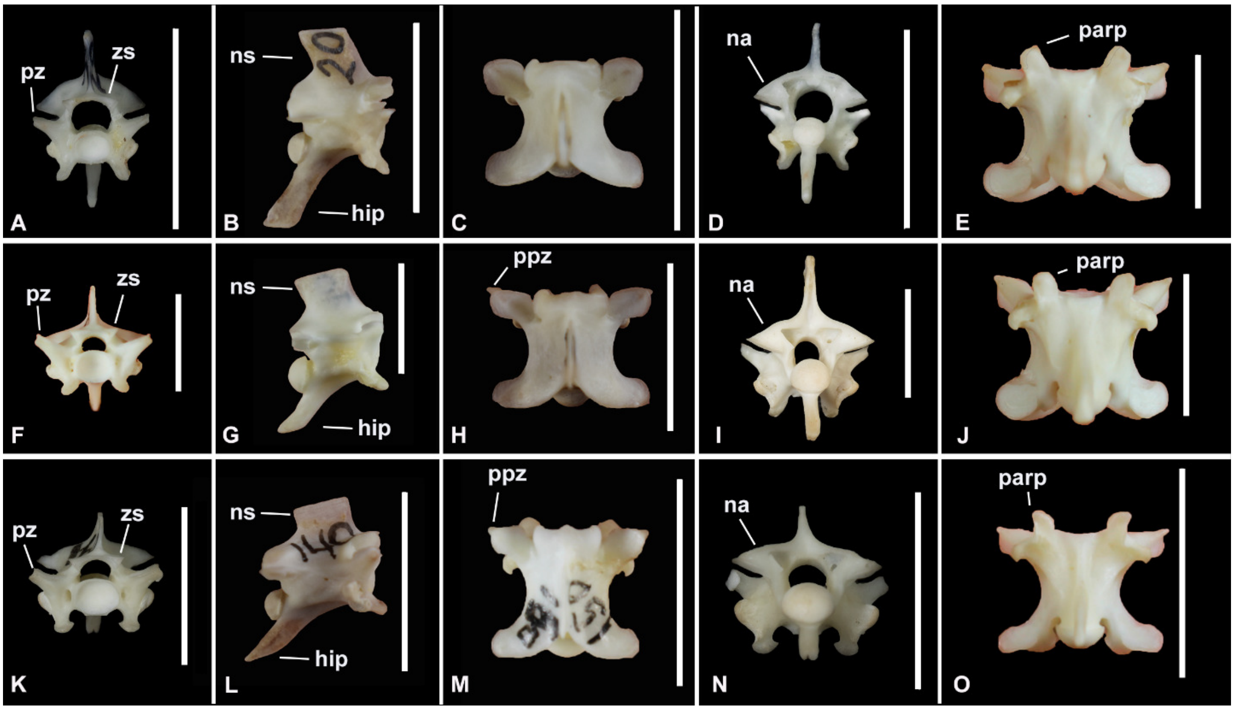

FIGURE 5. *Bothrops* trunk vertebrae for each region in anterior (A, F, K), lateral (B, G, L), dorsal (C, H, M), posterior (D, I, N), and ventral (E, J, O) views. The first row (A-E) are anterior trunk vertebrae, the second row (F-J) are mid-trunk vertebrae, and the third row (K-O) are posterior trunk vertebrae.

Abbreviations: na (neural arch); ns (neural spine); parp (parapophyseal process); ppz (prezygapophyseal process); pz (prezygapophyses); zs (zygosphene). Scale bar = 1 cm.

In dorsal view, the prezygapophyseal process is very short when visible (Figs 5C, G, K). The anterior margin of the zygosphene is variable, being either straight or crenated (sensu Auffenberg, 1963), with no pattern in this variation. In ventral view, there is one subcentral foramen on each side of the hypapophysis. The vertebral centrum is slightly triangular, being wider on the more anterior portion. In this view it is easier to notice the lateralization of the parapophyseal process.

Comparisons Between Crotalus *and* Bothrops—A qualitative description revealed some subtle differences between *Crotalus* and *Bothrops* (Table 1). The prezygapophyseal process is a valuable trait for differentiation between the genera. Individuals of *Crotalus* have short prezygapophyseal processes (in dorsal view) on the anterior trunk vertebrae, being elongated in the mid-trunk and posterior trunk vertebrae. In contrast, *Bothrops* tend to have much shorter prezygapophyseal processes regardless of the columnar region.

Another structure that can be useful for differentiating the genera is the neural spine. Considerably taller in *Crotalus,* the neural spine reaches half the total vertebral height in mid-trunk vertebrae, while in *Bothrops* it is much shorter. However, in posterior trunk vertebrae both genera have neural spines of similar heights.

In *Bothrops*, the zygosphene is usually crenate but sometimes straight. The “V” shape of the zygosphene anterior border is a common taxonomic trait used to identify *Crotalus* vertebrae (Camolez & Zaher, 2010). Still, according to our study, the “V” shape in the zygosphene may more likely represent individual variation than a diagnostic morphology consistently present in the genus. Among the vertebral variation of the zygosphene anterior border, we found vertebrae with crenate (Fig. 6A), straight zygosphene (Fig. 6B), and concave “V” shaped (Fig. 6C) morphotypes, with all three shapes occurring in the same *Crotalus durissus* individual.

We found that the pre- and postzygapophyses can be useful for differentiating the two genera. *Bothrops* tends to have more inclined pre- and postzygapophyses when compared to *Crotalus*. The neural arch is also slightly more depressed on *Bothrops*, vaulting ratio $\overline{x}$ = 0.31, than in *Crotalus*, vaulting ratio $\overline{x}$ = 0.39 (sensu Georgalis et al., 2021). Also, the postzygapophyses in *Bothrops* are visibly more inclined than those of *Crotalus*.

Nonparametric Procrustes ANCOVA.—Comparing shapes between the genera using nonparametric Procrustes ANCOVA showed a significant correlation between the genera and position of the vertebrae. The statistical tests in anterior and in lateral view were significant ($P < 0.05$) both between the genera and between the trunk column regions. However, because of the strong correlation between the two variables we cannot say there was a significant effect of the isolated variables (Tables 2, 4).

When comparing the trunk vertebrae more thoroughly in the pairwise comparison, there was a significant difference between *Crotalus* and *Bothrops* when comparing each trunk region separately (Table 3). In anterior view, anterior trunk vertebrae and mid-trunk vertebrae of *Bothrops* are different from *Crotalus*. Also, there was a significant difference between *Crotalus* anterior trunk vertebrae and *Bothrops* mid-trunk vertebrae. In lateral view (Table 5), there was a significant difference between *Crotalus* and *Bothrops* mid-trunk vertebrae. There was also a difference between *Bothrops* anterior trunk vertebrae and *Crotalus* posterior trunk verte-

TABLE 1. Summary of significant differences between *Crotalus* and *Bothrops* vertebrae.

		Zygosphene	Neural spine	Prezygapophyses mean angle	Prezygapophyseal processes	Hypapophysis
Crotalus	Anterior trunk	Straight	Tall	6,5	Short	Pointed
	Mid-trunk	Concave	Tall	11,6	Medium	Pointed
	Posterior trunk	Crenate	Short	15,7	Medium	Pointed
Bothrops	Anterior trunk	Crenate	Medium	10	Very short	Blunted
	Mid-trunk	Crenate	Medium	17,9	Short	Pointed
	Posterior trunk	Crenate	Short	19,1	Short	Pointed

TABLE 2. Nonparametric procrustes ANCOVA table of anterior view of vertebrae.

	Df	SS	MS	r^2	F-value	Z	P
Genera	1	0.18	0.18	0.03	30.66	2.89	< 0.05
Position	2	2.65	1.32	0.55	219.16	5.57	< 0.05
Genera:Position	2	0.13	0.06	0.02	10.81	5.74	< 0.05
Residual	294	1.77	0.006	0.37			
Total	299	4.74					

Df = degree of freedom; SS = sum of squares; MS = mean squares; r^2 = coefficient of determination; Z = effect size.

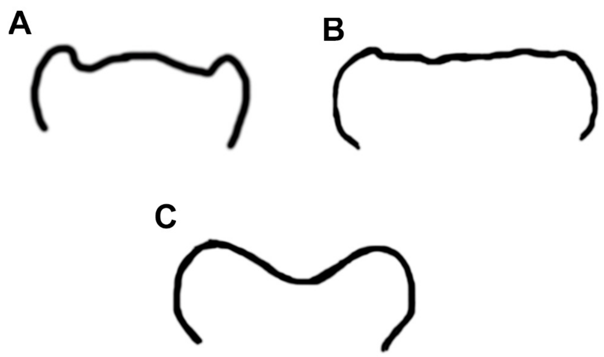

FIGURE 6. Zygosphene morphologies are present in *Crotalus durissus*, being (A) crenate; (B) straight; (C) concave.

brae, and *Crotalus* anterior trunk vertebrae and *Bothrops* mid-trunk vertebrae. On both views, the test showed significant differences between the trunk regions of *Crotalus*, but not between those of *Bothrops*. Also, in both views no significant difference was seen between the posterior trunk vertebrae of the two genera.

With regards to effect sizes (z-values) of the pairwise ANCOVA, in anterior view the anterior trunk vertebrae were those with the greatest differences between the genera. When comparing the different trunk regions of *Crotalus*, the anterior view shows relatively greater difference between anterior trunk and posterior trunk regions, while the lateral view shows greater differences between mid-trunk and posterior trunk vertebrae.

Principal Components Analysis.—The PCA in anterior and lateral views allowed us to assess morphological variation. We found variation among different regions of the vertebral column.

Members of the Viperidae do not show distinct regionalization of the vertebral column, as the hypapophysis is present throughout the trunk. However, PCA showed gradual longitudinal variation (Fig. 7). Most variation is present in the neural spine, the hypapophysis, and width of vertebrae (Prezygapophysis-Prezygapophysis). The first principal component (PC1) accounted for most of the variation, 66.57%. In this PC, it is possible to see the variation in height of the neural spine and width of vertebrae, with positive PCs (more posterior vertebrae) representing shorter and wider vertebrae and negative PCs (more anterior vertebrae) representing taller and narrower vertebrae. The second principal component (PC2) accounts for 8.23% of the variation. In this PC, it is possible to see variation in height of the neural spine and angle of the prezygapophyses, with positive PCs representing taller vertebrae with more oblique prezygapophyses and negative PCs representing shorter vertebrae with less oblique prezygapophyses. In both *Crotalus* and *Bothrops,* the more anterior trunk vertebrae have relatively longer neural spines, longer hypapophyses, and narrower vertebrae. In contrast, the posterior precloacal vertebrae show a shorter neural spine, more pos-

Table 3. Pairwise comparison table of anterior view of vertebrae.

	d	UCL (99%)	*Z*	*P*
Bothrops × *Crotalus*	0.04	0.05	−0.13	0.55
Anterior trunk× Mid-trunk	0.12	0.13	−0.08	0.52
Anterior trunk × *Posterior trunk*	0.22	0.23	−0.06	0.53
Mid-trunk × *Posterior trunk*	0.11	0.12	−0.05	0.52
Bothrops: anterior trunk **×** ***Crotalus: anterior trunk***	**0.07**	**0.06**	**3.76**	**< 0.05**
Bothrops: anterior trunk × *Bothrops: mid-trunk*	0.11	0.13	−0.64	0.73
Bothrops: anterior trunk × *Crotalus: mid-trunk*	0.08	0.10	−2.61	0.99
Bothrops: anterior trunk × *Bothrops: posterior trunk*	0.18	0.23	−5.07	1.00
Bothrops: anterior trunk × *Crotalus: posterior trunk*	0.19	0.20	1.22	0.11
Crotalus: anterior trunk **×** ***Bothrops: mid-trunk***	**0.18**	**0.17**	**2.92**	**< 0.05**
Crotalus: anterior trunk × *Crotalus: mid-trunk*	0.12	0.13	0.71	0.24
Crotalus: anterior trunk × *Bothrops: posterior trunk*	0.25	0.27	−1.42	0.92
Crotalus: anterior trunk **×** ***Crotalus: posterior trunk***	**0.26**	**0.23**	**5.17**	**< 0.05**
Bothrops: mid-trunk **×** ***Crotalus: mid-trunk***	**0.07**	**0.06**	**2.81**	**< 0.05**
Bothrops: mid-trunk × *Bothrops: posterior trunk*	0.08	0.13	−4.50	1.00
Bothrops: mid-trunk × *Crotalus: posterior trunk*	0.09	0.10	1.27	0.10
Crotalus: mid-trunk × *Bothrops: posterior trunk*	0.14	0.17	−1.69	0.96
Crotalus: mid-trunk **×** ***Crotalus: posterior trunk***	**0.15**	**0.13**	**4.31**	**< 0.05**
Bothrops: posterior trunk × *Crotalus: posterior trunk*	0.03	0.06	−2.99	0.99

d = Euclidian distance; UCL = upper confidence limit; *Z* = effect size.

Table 4. Nonparametric procrustes ANCOVA table of lateral view of vertebrae.

	Df	SS	MS	r^2	*F*-value	Z	*P*
Genera	2	0.55	0.55	0.07	50.24	3.20	< 0.05
Position	2	3.77	1.88	0.48	171.01	5.16	< 0.05
Genera:Position	2	0.15	0.07	0.01	6.85	6.32	< 0.05
Residual	294	3.24	0.01	0.42			
Total	299	7.72					

Df = degree of freedom; SS = sum of squares; MS = mean squares; r^2 = coefficient of determination; *Z* = effect size.

teriorly oriented hypapophysis, and wider vertebrae. Because this variation is gradual, it is noticeable that mid-trunk vertebrae are wider than anterior trunk vertebrae but narrower than posterior trunk vertebrae (Fig. 7A).

In lateral view, the overlap area of anterior trunk and mid-trunk vertebrae on the scatterplot is more pronounced (see the overlap area of green and red dots, Fig. 7B), suggesting that this view is not suitable for distinguishing the two regions. Posterior vertebrae do not show considerable overlap in values with other regions. Apparently, PC1, which accounts for 74.69% of total variation, shows differences in the neural spine, with positive PCs representing shorter vertebrae and negative PCs representing taller vertebrae, with anterior and mid-trunk vertebrae having similar proportions, taller than wide. In contrast, posterior vertebrae have very different ratios, being wider than tall. PC2 accounts for 4.76% of the variation and shows differences in the hypapophysis. Positive PCs represent vertebrae with more posteriorly pointed hypapophyses while negative PCs represents vertebrae with hypapophyses less posteriorly directed.

Canonical Variate Analysis.—Canonical Variate (CV) distances (Mahalanobis distance), in both anterior and lateral views, were significantly different between the genera (Fig. 8 and Table 2). The CVA showed that vertebrae of *Bothrops* possess (1) a shorter neural spine, (2) more oblique prezygapophyses, (3) accentuated lateralized parapophyseal process, (4) a slightly less elevated zygosphene, and (5) a more posteriorly oriented hypapophysis. On the other hand, individuals of *Crotalus* exhibit vertebrae that tend to have a taller neural spine, more horizontally oriented prezygapophyses, anteriorly oriented parapophyseal process, more dorsally arched zygosphene, and less posteriorly oriented hypapophysis.

TABLE 5. Pairwise comparison table of lateral view of vertebrae.

	d	UCL (99%)	*Z*	*P*
Bothrops × *Crotalus*	0.08	0.09	−0.10	0.55
Anterior trunk × *Mid-trunk*	0.10	0.11	−0.12	0.56
Anterior trunk x Posterior trunk	0.26	0.28	−0.05	0.50
Mid-trunk × *Posterior trunk*	0.17	0.18	−0.07	0.55
Bothrops: anterior trunk × *Crotalus: anterior trunk*	0.09	0.10	0.79	0.22
Bothrops: anterior trunk × *Bothrops: mid-trunk*	0.11	0.12	1.46	0.06
Bothrops: anterior trunk × *Crotalus: mid-trunk*	0.05	0.06	−2.28	0.99
Bothrops: anterior trunk × *Bothrops : posterior trunk*	0.24	0.28	−2.47	0.99
Bothrops: anterior trunk × ***Crotalus: posterior trunk***	**0.21**	**0.21**	**1.68**	**0.05**
Crotalus: anterior trunk × ***Bothrops: mid-trunk***	**0.20**	**0.19**	**2.13**	**< 0.05**
Crotalus: anterior trunk × *Crotalus: mid-trunk*	0.09	0.12	−1.26	0.89
Crotalus: anterior trunk × *Bothrops: posterior trunk*	0.33	0.36	−1.90	0.96
Crotalus: anterior trunk × ***Crotalus: posterior*** *trunk*	**0.29**	**0.28**	**2.82**	**< 0.05**
Bothrops: mid-trunk × ***Crotalus: mid-trunk***	**0.12**	**0.10**	**3.49**	**< 0.05**
Bothrops: mid-trunk × Bothrops: posterior trunk	0.13	0.19	−3.54	1.00
Bothrops: mid-trunk × Crotalus: posterior trunk	0.10	0.11	0.45	0.33
Crotalus: mid-trunk × Bothrops: posterior trunk	0.25	0.27	−0.24	0.59
Crotalus: mid-trunk × **Crotalus: posterior trunk**	**0.21**	**0.19**	**3.99**	**< 0.05**
Bothrops: posterior × Crotalus: posterior trunk	0.06	0.10	−2.63	0.99

d = Euclidian distance; UCL = upper confidence limit; Z = effect size.

TABLE 6. Mahalanobis distance and result of permutation test (1,000 rounds) for anterior and lateral views.

	Mahalanobis distance	Permutation test
Anterior view	3.267555	0.000999001
Lateral view	2.901712	0.000999001

DISCUSSION

We found distinct differences in trunk vertebral morphology between *Crotalus* and *Bothrops*. GM analysis corroborated some observations made in macroscopic examination of the same material. Morphometric and macroscopical analyses showed differences in the neural spine, prezygapophyses, and parapophyseal processes. Additionally, osteological description revealed a new trait that can be useful for differentiating *Bothrops* and *Crotalus*, a longer prezygapophyseal process in the latter genus. However, the GM analysis also showed that such an analysis is not particularly useful in identification of vertebrae of unknown trunk region.

Although GMs can help identify differences between the genera, traditional macroscopic comparison should not be overlooked. Some variation observed in osteological description is not noticeable by morphometry in anterior and lateral views, such as the zygosphene dorsal shape variation and differences in the tip of the hypapophysis. Also, GM analysis revealed that there is still much more to be explored. Pairwise ANCOVA showed that, using bidimensional GMs, it is not possible to differentiate *Bothrops* and *Crotalus* if the vertebral region is not known. Our results are consistent with those of Camolez and Zaher (2010) who concluded that *Crotalus* mid-trunk and *Bothrops* posterior trunk vertebrae are difficult to distinguish. If the trunk region of an isolated vertebra is unknown, analyses based on bidimensional GMs are not useful. In this case, a qualitative approach is a superior methodology. Therefore, GMs should not be the only analysis used in studies, but it is useful as a complementary analysis.

PCA showed gradual trunk intracolumnar variation, a similar result observed in the study of Sarris et al. (2012) for *Daboia russellii* (Viperidae), indicating no distinct regionalization of the vertebral column of Viperidae. Further studies incorporating more taxa of Viperidae should determine if this gradual variation is a common trait of the postcranial skeleton in the family.

Our study showed that the zygosphene shape in dorsal view is highly variable with multiple forms in the same genus and even in the same individual. Although only *C. durissus* showed a concave “V” shaped zygosphene, *C. durissus* also showed straight and crenated zygosphene morphologies. Therefore, this structure is helpful to differentiate the genera if combined with other vertebral features but should not itself be regarded as diagnostic. Some differences in size of the paracotylar foramina have been reported by Auffenberg (1963) and Albino and Montalvo (2006), with relatively small foramina in *Crotalus* and either large or small foramina in *Bothrops*. However, although we found

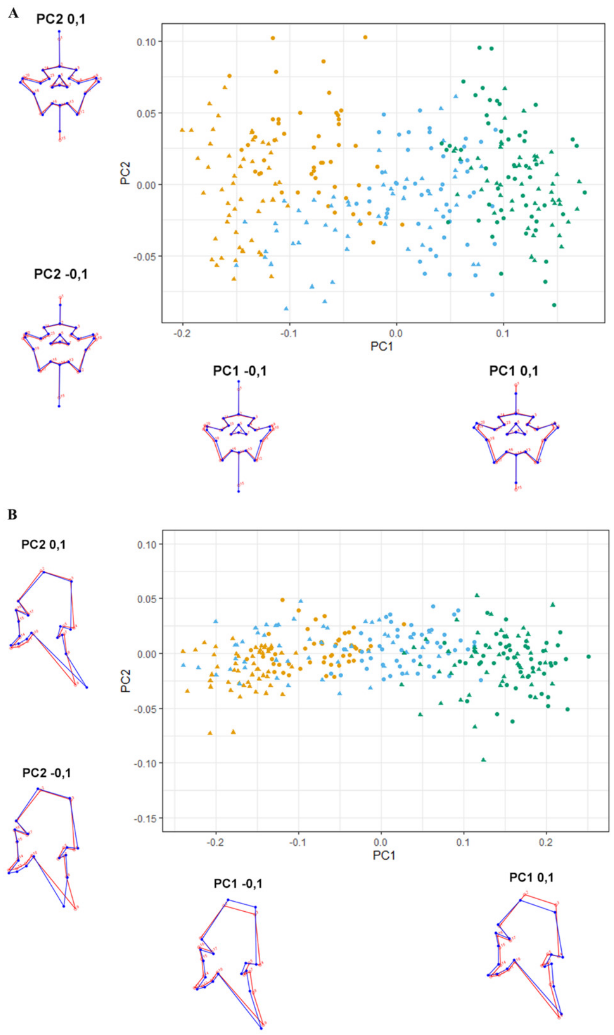

FIGURE 7. Principal Component Analysis charts of the Procrustes residues in anterior view (A) and lateral view (B).

The outlines in dark blue represent the shape variation for each extreme of the PC's axis, while the outlines in red are the mean shape (PC = 0). The yellow dots are the anterior trunk vertebrae, the light blue dots are the mid-trunk vertebrae, and the green dots are the posterior trunk vertebrae. The circles are the *Bothrops* trunk vertebrae and the triangles are the Crotalus trunk vertebrae.

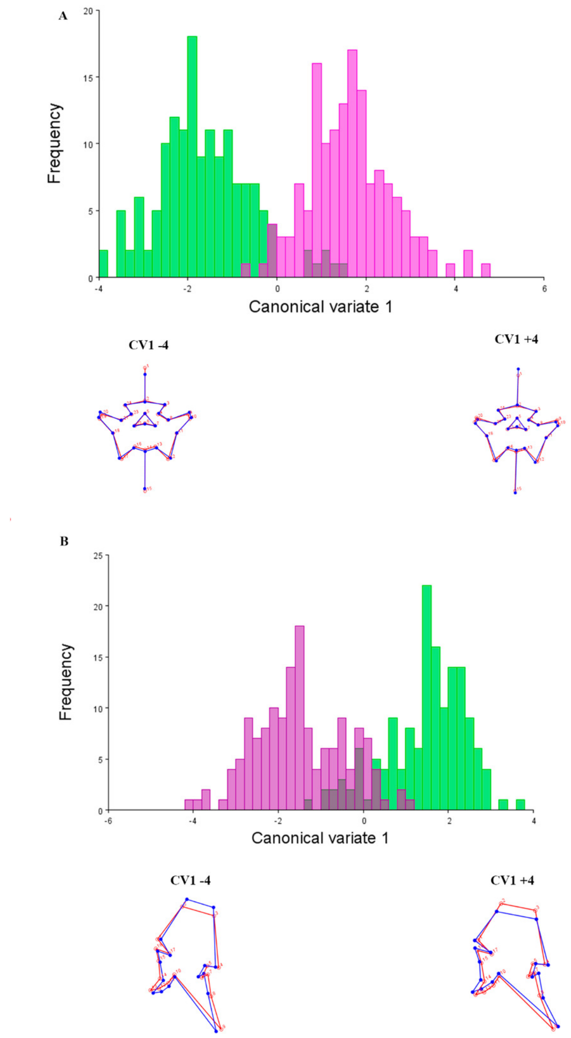

FIGURE 8. Canonical Variate Analysis chart for anterior view (A) and lateral view (B).

The outlines in dark blue represent the shape variation for each extreme of the CV1 axis, while the outlines in red are the mean shape (CV1 = 0). The green columns are the *Bothrops* specimens, and the pink columns are the *Crotalus* specimens.

that *Crotalus* foramina tended to be larger than in *Bothrops*, no solid pattern was recognized.

We hope these analyses can constitute an initial step in integrating qualitative and quantitative (i.e., GM and discriminant analyses) data of postcranial skeletons in South American vipers. We expect that results in both types of analyses can help paleontologists identify isolated fossil viper vertebrae. We also expect that future studies expanding these analyses can better differentiate the two genera. Analyses using extant and fossil specimens would be useful (e.g., 3-D geometric morphometrics) and could shed light on past diversity of vipers because similar statistical tests have been used previously to identify fossil and living taxa to the genus and species level (e.g., Courtenay et al., 2019; Leshno Afriat et al., 2020; Marramà & Kriwet, 2017). In the future, we intend to add *Lachesis* and *Bothrocophias* to these analyses for a more comprehensive view of the Brazilian vipers.

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