

## Morphological and Molecular Variation in the Pumpkin Toadlet, *Brachycephalus ephippium* (Anura: Brachycephalidae)

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**ABSTRACT.**—A recent study demonstrated marked variation in cranial shape between a population of *Brachycephalus ephippium* from Jundiá in São Paulo state and populations from Atibaia and São Francisco Xavier (São Paulo) and Macaé de Cima in the state of Rio de Janeiro in southeastern Brazil. This result contrasts with earlier work describing differences in cranial shape between the population from Rio de Janeiro and those from São Paulo. Here, we investigate the nature and extent of variation between populations of *B. ephippium* using two lines of evidence. First, we reevaluate patterns of morphological variation by incorporating semi-landmarks into the quantitative description of cranial shape and by using scanning electron microscopy (SEM) to describe qualitative skeletal features. Second, we assess molecular variation in the mitochondrial genes cytochrome *b*, 12S rRNA, and 16S rRNA, and the nuclear *Rag-1* gene, totaling 3,697 base pairs. Geometric analyses of cranial shape and SEM images of skeletal traits show that the population from Macaé de Cima and the populations from São Paulo differ markedly in morphology and diverge in the cytochrome *b*, 12S, and 16S rRNA genes by 9.7%, 2.8%, and 4.4%, respectively. Divergence in the nuclear gene *Rag-1* is, as expected, much lower (0.6%). These results clearly demonstrate heterogeneity between populations that are all currently diagnosed as *B. ephippium* and point to the need for further research to ascertain the true diversity currently hidden under the name of *B. ephippium*.

*Brachycephalus ephippium*, the Pumpkin Toadlet from the family Brachycephalidae (Hedges et al., 2008), is a remarkable example of the evolutionary process of miniaturization (Trueb and Alberch, 1985; Hanken, 1993). *Brachycephalus ephippium* shares with the other species of *Brachycephalus* features associated with miniaturization, including a snout-vent length less than 18 mm, the loss of phalanges in the manus and pes, a reduced number of functional toes, and the loss of skull bones such as the columella, palatine, and quadratojugal (Yeh, 2002; Frost, 2009). Hyperossification, an increased mineralization and excessive ossification of some skeletal elements, is also a prominent feature of the skeleton of *B. ephippium* (Trueb and Alberch, 1985; Hanken, 1993). Clemente-Carvalho et al. (2009) showed that under scanning electron microscopy (SEM) hyperossification of the skull in *B. ephippium* manifests itself as a marked exostosis in the region associated with the squamosals, exoccipitals, and in the bony shield lateral to the otic capsule (the parotic plate; Silva et al., 2007). Although hyperossification of the skull is typical of several species of *Brachycephalus*, the postcranial skeleton of *B. ephippium* is also hyperossified, displaying a novel bony dorsal shield, the paravertebral plate, lying above the presacral vertebrae and transverse processes (Hanken, 1993; Clemente-Carvalho et al., 2009; Campos et al., 2010). In none of the other 12 species of *Brachycephalus* described thus far is the paravertebral plate as developed as in *B. ephippium* (Clemente-Carvalho et al., 2009). Therefore, as currently understood, *B. ephippium* is uniquely diagnosed among the species of *Brachycephalus* by the presence of osseous, well-developed paravertebral plates.

Recently, Clemente-Carvalho et al. (2008) used anatomical landmarks and geometric-statistical methods to demonstrate the existence of significant geographical variation in cranial shape among populations assignable to *B. ephippium*. Their study revealed that population samples from the locality of Macaé de Cima in the state of Rio de Janeiro and the localities of Atibaia and São Francisco Xavier in the state of São Paulo were very similar in cranial shape, although they differed markedly from a sample from the locality of Jundiá, also in the state of São Paulo. This finding is particularly interesting because Miranda-Ribeiro (1920) remarked on the morphological uniqueness of the population from Macaé de Cima in the state of Rio de Janeiro, as compared to populations of *B. ephippium* from the state of São Paulo. In fact, Miranda-Ribeiro (1920) went so far as to name the population from Macaé de Cima as a new variety.

The objective of this study was to investigate the contrasting results of Miranda-Ribeiro (1920) and Clemente-Carvalho et al. (2008), as they relate to the nature and extent of variation between populations of *B. ephippium*. Two lines of evidence were pursued. First, we reexamine cranial shape variation in the population samples of *B. ephippium* previously analyzed by Clemente-Carvalho et al. (2008). These authors characterized variation in cranial shape with geometrically homolo-

gous anatomical landmarks. We also used anatomical landmarks but added semi-landmarks, which allow the sampling of outlines and surfaces, thus providing a more even coverage of the skull (Mitteroecker and Gunz, 2009). To further study the skeletal morphology, we examine qualitative cranial morphological variation in representative specimens of the four populations of *B. ephippium* using scanning electron microscopy. Second, we assess molecular variation in three mitochondrial genes, cytochrome *b*, 12S rRNA, and 16S rRNA and one nuclear gene (recombination activating protein 1; *Rag-1*), totaling 3,697 base pairs. The cytochrome *b* gene was chosen because it has been widely used in amphibian phylogenetic and phylogeographic studies (Liu et al., 2000; Rodríguez et al., 2010) and the 12S rRNA gene because it has been selected by the Amphibia Tree Consortium as a standard marker to describe molecular variation (Vences et al., 2005b; Fouquet et al., 2007a). The 16S rRNA was selected because variation in this gene has been recently used to define a threshold to distinguish between intraspecific and interspecific levels of divergence (Vences et al., 2005a,b; Fouquet et al., 2007b; Rodríguez et al., 2010). Finally, the nuclear *Rag-1* gene was included in the analysis because it has been increasingly used in amphibian systematic and evolutionary studies (Hedges et al., 2008; Rodríguez et al., 2010). Furthermore, these genes allow reliable and repeatable amplification and sequencing with universal primers and are informative with respect to evolutionary divergence (Vences et al., 2005b; Fouquet et al., 2007a,b). Our approach yielded conclusive results that are relevant for understanding patterns of variation and differentiation in *B. ephippium* in particular and in the genus *Brachycephalus* in general.

### MATERIALS AND METHODS

The following samples of *B. ephippium* were examined (Fig. 1): Macaé de Cima, Rio de Janeiro (22°28'50.16"S, 42°12'15.12"W; *N* = 11), Atibaia, São Paulo (23°7'0.84"S, 46°33'1.08"W; *N* = 11), São Francisco Xavier, São Paulo (23°10'45.84"S, 45°53'12.84"W; *N* = 8), and Jundiá, São Paulo (23°10'59.88"S, 46°52'0.12"W; *N* = 16). All specimens examined are listed in Appendix 1 and are deposited in the C. F. B. Haddad collection, Departamento de Zoologia, Universidade Estadual Paulista, Campus de Rio Claro, São Paulo, Brazil.

For all specimens in the four population samples, X-ray microradiograph images were obtained using the methodology described in Clemente-Carvalho et al. (2009). These images allowed all specimens to be unequivocally diagnosed as *B. ephippium* because of the presence of osseous, well-developed paravertebral plates. The individual X-ray images were used to define landmarks and semi-landmarks for the morphometric analyses.

To describe variation in skull shape, we used 10 anatomical landmarks defined by Clemente-Carvalho et al. (2008) and 43 semi-landmarks (Fig. 2A). Semi-landmarks are points on smooth curves or surfaces with estimated positions along the curve (Bookstein, 1997; Adams et al., 2004; Mitteroecker and Gunz, 2009). The differences in location, scaling, and orientation of landmarks and semi-landmarks

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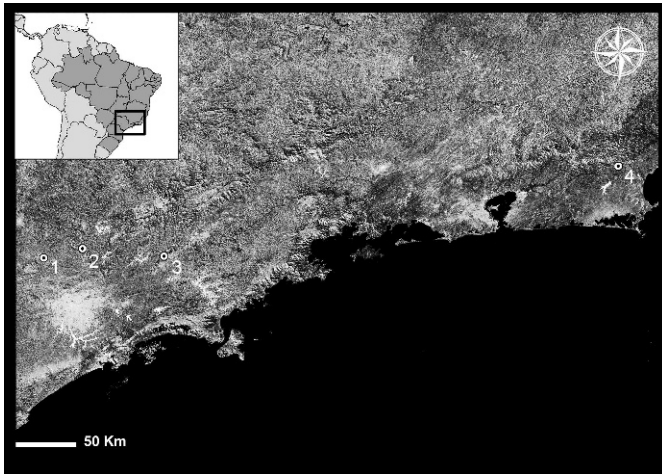


FIG. 1. Collection locations of *Brachycephalus ephippium* in south-eastern Brazil used for morphological and molecular analyses. 1. Jundiá, 2. Atibaia, and 3. São Francisco Xavier (state of São Paulo), and 4. Macaé de Cima (state of Rio de Janeiro).

were removed with generalized Procrustes analysis (Rohlf and Slice, 1990; Bookstein, 1991). In addition, the semi-landmarks were aligned by means of the sliding semi-landmark method proposed by Bookstein (1997). We used the tps Relw 1.44 software (Rohlf, 2007) to slide the semi-landmarks along their respective curves, to minimize the Procrustes distance between the subject and the reference (Bookstein et al., 2005). Aligned landmarks and semi-landmarks were subjected to relative warps analysis (i.e., a principal components analysis; Bookstein, 1991; Rohlf, 1993) to determine the principal directions of variation in cranial shape among population samples of *B. ephippium*. Cranial shape differences between populations were visualized as deformation grids (Rohlf et al., 1993; Adams et al., 2004).

The skeletons of two specimens (one male and one female) from each population of *B. ephippium* were prepared for scanning electron microscopy. These specimens were selected as representatives of the skeletal variation present in each population as determined by the examination of X-ray microradiographs from all samples. The specimens were immersed in a solution of sodium hypochlorite for removal of the soft tissues and then air dried as described in Clemente-Carvalho et al. (2009). The skeletons were then mounted onto metal stubs, coated with gold in a Sputter Coater Balzers SCD050 machine, and examined under a Jeol JSM 5800LV scanning electron microscope.

Genomic DNA was extracted from liver tissue preserved in 100% ethanol from four specimens of each population sample of *B. ephippium*. Tissue samples were digested with proteinase K and then followed a standard three-step phenol/chloroform extraction procedure (Sambrook et al., 1989). Sequences used in this study were obtained for three mitochondrial and one nuclear gene. Fragments of the mitochondrial cytochrome *b* and rRNA 12S genes were amplified using primers and parameters given in Goebel et al. (1999). A segment of the mitochondrial rRNA 16S gene was amplified using primers and specifications given in Darst and Cannatella (2004). Finally, a fragment of the nuclear gene *Rag-1* was amplified using primers and parameters given in Hedges et al. (2008). The amplification products were visualized on 1.0% agarose gels and purified using a GFX<sup>TM</sup> PCR DNA and Gel Band Purification kit (GE Healthcare). Purified PCR products were sent to Macrogen, Inc. in Seoul, South Korea, for sequencing (using the BigDye<sup>TM</sup> terminator kit and run on ABI 3730XL). Sequences were obtained in both directions with the same primers used for PCR amplification and submitted for BLAST searching (Altschul et al., 1997) in GenBank to verify that the required sequences had been amplified. Sequence traces were analyzed using the *phred* program (Ewing et al., 1998). Alignments were produced by ClustalX 2.0 (Larkin et al., 2007). Genetic distances between the population samples of *B. ephippium* were computed as uncorrected (*p*) distances, expressed as the number of nucleotide differences per site (Yang, 2006). All sequences obtained in this study have been deposited in GenBank (HM208304–HM208307; HM216359–HM216370).

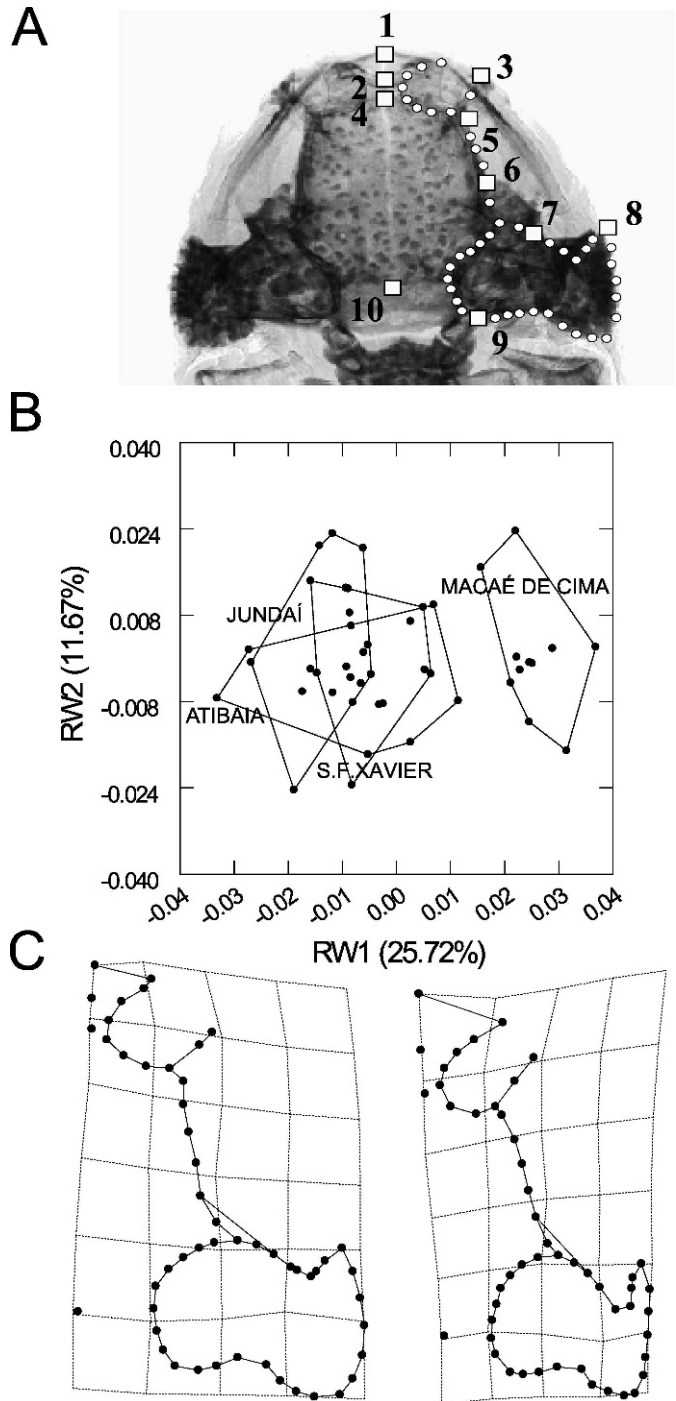


FIG. 2. (A) Morphological landmarks depicted as squares and semi-landmarks as circles on the dorsal view of X-ray microradiograph of skull of *Brachycephalus ephippium*. (B) Ordination of *Brachycephalus ephippium* population samples from Macaé de Cima in the state of Rio de Janeiro and Atibaia, Jundiá, and São Francisco Xavier from the state of São Paulo in the space of the first and second relative warps (RW). In these figures, each circle represents the position of an individual from a given sample and the limits of variation in cranial shape for each sample are defined as polygons. (C) Estimated changes in cranial shape are shown as deformations implied by the first relative warp for positive and negative deviations from the mean.

## RESULTS

*Variation in Cranial Shape and Osteology.*—The first two relative warps explain 37.7 of the total variation in skull shape (Fig. 2B). The convex polygons delimit the cranial shape variation for each of the samples of

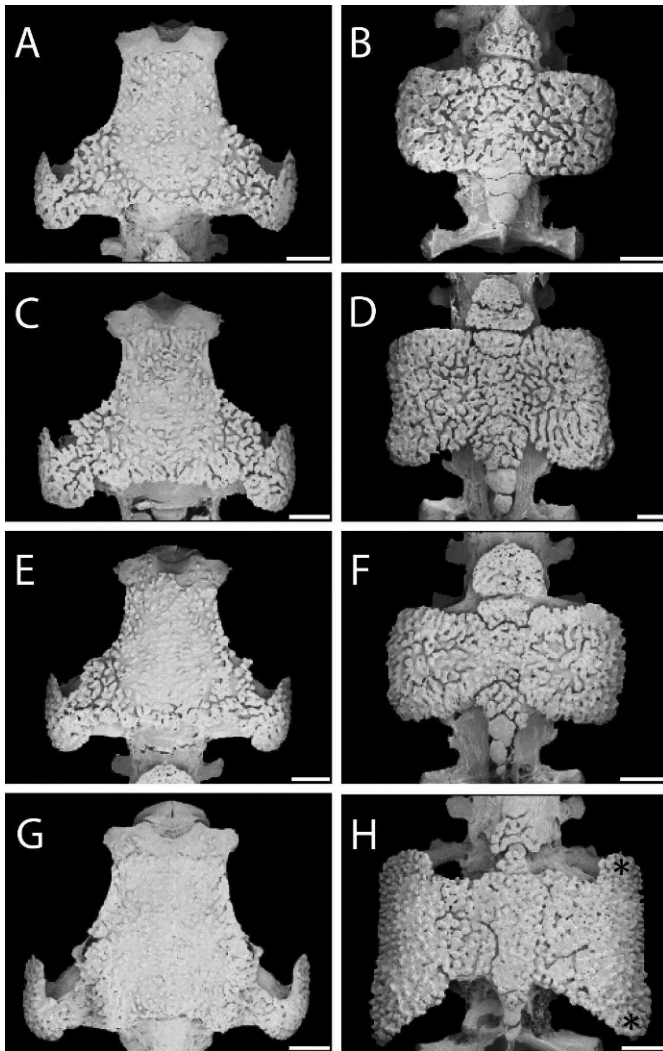


FIG. 3. Scanning electron microscopy images of the skull and paravertebral plates, respectively, of representative specimens of the *Brachycephalus ephippium* population samples. (A and B) Atibaia, São Paulo; (C and D) Jundiá, São Paulo; (E and F) São Francisco Xavier, São Paulo; (G and H) Macaé de Cima, Rio de Janeiro (asterisks denote anterior and posterior elongation of the parotic plates). Scale bar = 1 mm.

*B. ephippium* (Fig. 2B). Population samples from the localities of Atibaia, Jundiá, and São Francisco Xavier from the state of São Paulo are very similar in cranial shape, as indicated by the extensive overlap in the convex polygons (Fig. 2B). However, the population sample from Macaé de Cima in the state of Rio de Janeiro and the population samples from São Paulo differ markedly in cranial shape, as shown by the non-overlapping convex polygons along the first relative warp (Fig. 2B). Changes in cranial shape along the first relative warp are depicted in Figure 2C. The population samples from Atibaia, Jundiá, and São Francisco Xavier (São Paulo state) are characterized by wider nasal cavity and larger parotic plate. Conversely, the population sample from Macaé de Cima (Rio de Janeiro state) has a narrower nasal cavity and a smaller parotic plate (Fig. 2C). Procrustes distances, which measure differences in mean shape between samples, were calculated between the four population samples of *B. ephippium*. The average Procrustes distance between the populations of *B. ephippium* from Atibaia, Jundiá, and São Francisco Xavier in the state of São Paulo was 0.022. In contrast, the average Procrustes distance between the populations from São Paulo and the population from Macaé de Cima in the state of Rio de Janeiro was 0.036.

The skull and paravertebral plates in the four population samples of *B. ephippium* are hyperossified as revealed by the scanning electron microscopy images (Fig. 3). The skull in the population from Macaé de Cima in the state of Rio de Janeiro (Fig. 3G) differs from those populations from São Paulo (Fig. 3A,C,E) by the presence of ridges in

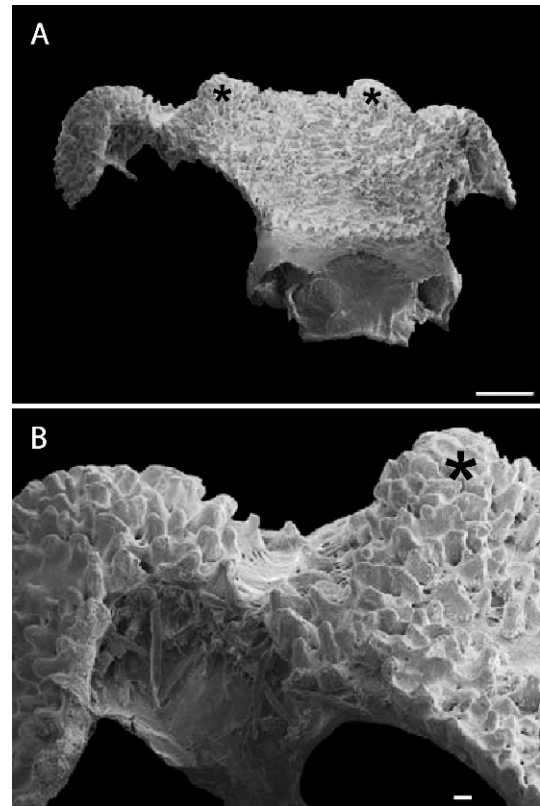


FIG. 4. Scanning electron microscopy images of the skull detailing the ridges in the posterior region of the skull (denoted by asterisks) of *Brachycephalus ephippium* from the locality of Macaé de Cima, state of Rio de Janeiro. (A) Scale bar = 1 mm. (B) Scale bar = 100  $\mu$ m.

the posterior region of the skull. These ridges are clearly seen in Figure 4 (A,B). The paravertebral plates of the population from Macaé de Cima (Fig. 3H) also differ markedly from those in the São Paulo populations (Fig. 3B,D,F). In the population from Macaé de Cima, the ends of the paravertebral plates are elongated anteriorly and posteriorly (Fig. 3H). Examination of differentially sized specimens from the population of Macaé de Cima provided insight into the ontogenetic changes of the ridges of the posterior region of the skull and the paravertebral plates (Fig. 5). The ridges are not visible in the smallest individual (SVL = 10.9 mm; Fig. 5A), which is a juvenile. The ridges then begin to become visible in the intermediate-sized individual (SVL = 13.6 mm; Fig. 5B), and are fully apparent in the adult individual (SVL = 18.6 mm; Fig. 5C). In the paravertebral plates, hyperossification starts at each end of the fourth and fifth vertebrae (10.9 mm; Fig. 5D), increasing laterally and inward (13.6 mm; Fig. 5E) until the hyperossification centers coalesce and generate the paravertebral plates (18.6 mm; Fig. 5F).

**Molecular Variation.**—The aligned sequences contained 893 base pairs (bp) of the cytochrome *b* gene, 832 bp of 12S, 1336 bp of 16S, and 636 bp of *Rag-1* for a total of 3,697 nucleotide sites. The observed number of point mutations between the samples from Atibaia, Jundiá, and São Francisco Xavier in the state of São Paulo was very low, ranging from zero to three. However, these populations and the population from Macaé de Cima in the state of Rio de Janeiro differed by 81, 24, 59, and 4 point mutations in the cytochrome *b*, 12S, 16S, and *Rag-1* genes, respectively. The observed (uncorrected) distance between the population from Macaé de Cima and the populations from Atibaia, Jundiá, and São Francisco Xavier is 9.7%, 2.8%, 4.4%, and 0.6% for the cytochrome *b*, 12S, 16S, and *Rag-1* genes, respectively.

#### DISCUSSION

Relative warps analysis and Procrustes distances of landmarks and semi-landmarks demonstrated marked differences in cranial shape between the population from Macaé de Cima in the state of Rio de Janeiro and the populations sampled from the localities of Atibaia, Jundiá, and São Francisco Xavier in the state of São Paulo. This result differs strikingly from that of Clemente-Carvalho et al. (2008) in which



FIG. 5. Scanning electron microscopy images of osteological characters of *Brachycephalus ephippium*. Individuals of different sizes (measured as snout-vent length, SVL) from the locality of Macaé de Cima, state of Rio de Janeiro. (A–C) Skull (asterisks denote the ridges of the posterior region of the skull). (D–F) Paravertebral plates. A and D from the same juvenile individual with SVL = 10.9 mm. B and E from the same juvenile individual with SVL = 13.6 mm. C and F from the same adult individual with SVL = 18.6 mm. Scale bar = 1 mm.

the population from Macaé de Cima is very similar in cranial shape to the populations from Atibaia and São Francisco Xavier, whereas these three populations differ markedly in cranial shape from the population from Jundiá. These results strongly indicate that the analysis of both landmarks and semi-landmarks more adequately portrays the geographic nature of morphological differentiation between the populations of *B. ephippium* than that based on landmarks alone (Clemente-Carvalho et al., 2008). The difference between the patterns of cranial shape variation in populations of *B. ephippium* obtained by Clemente-Carvalho et al. (2008), and those reported here is evidently attributable to the inclusion of semi-landmarks in the present study. The examination of semi-landmarks incorporates new information about morphological variation in the populations of *B. ephippium*, that is, information about curves, which allows for a finer sampling of the complexity of biological shape (Bookstein et al., 2005; Mitteroecker and Gunz, 2009). Such complexity may emerge as the result of evolutionary changes affecting larger areas instead of localized points of the skull, thereby allowing semi-landmarks to better capture patterns of morphological variation (Perez et al., 2006; Gunz et al., 2009). In fact, semi-landmarks have been recently used successfully to quantify shape differences in the human brain (Bookstein et al., 2005) and pelvis (Gonzalez et al., 2009) and also to describe cranial shape variation and test competing models of early human evolution (Gunz et al., 2009). However, this is the first report of semi-landmarks being used to study shape variation in amphibians.

Also, our qualitative analysis of skeletal variation demonstrates that the population of Macaé de Cima differs markedly from the populations from Atibaia, Jundiá, and São Francisco Xavier by the presence of ridges in the posterior region of the skull and also in the shape of the paravertebral plates (Figs. 3,4). These anatomical features of the skull and paravertebral plates of the population of *B. ephippium* from Macaé de Cima were first observed by Miranda-Ribeiro (1920). Miranda-Ribeiro (1920) also remarked that individuals of this population were all associated with bromeliads.

The sequences of the cytochrome *b*, 12S, 16S, and *Rag-1* genes revealed no or very low variation between the populations from the state of São Paulo. However, substantial variation between these populations and the population from Macaé de Cima in the state of Rio

de Janeiro were observed for the cytochrome *b*, 12S, and 16S genes. The nuclear gene *Rag-1* had the smallest uncorrected distance, an expected finding because this gene is well conserved and has been used to infer phylogenetic relationships at higher taxonomic levels (Chiari et al., 2009). Our finding that marked variation in the cytochrome *b* sequences exists between populations of *B. ephippium* is not surprising since recent studies have consistently uncovered molecular variation in Neotropical anurans (Elmer et al., 2007; Fouquet et al., 2007a,b; Padial and De la Riva, 2009).

The uncorrected *p*-distance calculated for the 16S rRNA gene between the population from Macaé de Cima in the state of Rio de Janeiro and the populations from the state of São Paulo is of particular significance here. Recently, Fouquet et al. (2007b) analyzed patterns of variation in the 16S rRNA gene in Neotropical frogs and have identified thresholds in genetic distances that define putative limits between levels of intra- and interspecific variation. According to Fouquet et al. (2007b; see also Vieites et al., 2009; Glaw et al., 2010), an uncorrected *p*-distance above the threshold of 0.03 (3%) signals lineages that may represent independent evolutionary units, that is, different species. Here, we estimated the uncorrected *p* distance for the rRNA 16S gene between the population from Macaé de Cima in Rio de Janeiro and those from São Paulo as 0.044 (4.4%)—a value larger than the threshold of 0.03 defined by Fouquet et al. (2007b).

The data on quantitative cranial shape, qualitative skeletal traits, and molecular sequences in *B. ephippium*, particularly for the 16S rRNA gene, clearly define concordant boundaries of variation between the population from Macaé de Cima in the state of Rio de Janeiro and the populations from Atibaia, Jundiá, and São Francisco Xavier in the state of São Paulo. Thus, it is quite conceivable that they could be considered two distinct species. However, our intent at this point is not to make formal nomenclatural changes. We believe that the next challenging step should be the sampling of *B. ephippium* along the complex chains of mountains in the states of Rio de Janeiro and São Paulo and the subsequent description of morphological, genetic, and ecological variation. Such an effort will most probably reveal widespread variation and thereby disclose the true diversity currently hidden under the name *B. ephippium*. Evidently, this search for diversity applies to the genus *Brachycephalus* as a whole because new species continue to be described

(Clemente-Carvalho et al., 2009; Haddad et al., 2010). The use of landmarks and semi-landmarks to describe quantitative shape variation, electron scanning microscopy to analyze skeletal qualitative traits, as well as molecular markers combined in an integrative approach will certainly contribute to uncover pattern and process in the evolutionary history of *Brachycephalus*.

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## LITERATURE CITED

- ADAMS, D. C., F. J. ROHLF, AND D. E. SLICE. 2004. Geometric morphometrics: ten years of progress following the "revolution." *Italian Journal of Zoology* 71:5–16.
- ALTSCHUL, S. F., T. L. MADDEN, A. A. SCHÄFFER, J. ZHANG, Z. ZHANG, W. MILLER, AND D. J. LIPMAN. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25:3389–3402.
- BOOKSTEIN, F. L. 1991. *Morphometric Tools for Landmark Data*. Cambridge University Press, New York.
- . 1997. Landmark methods for forms without landmarks: localizing group differences in outline shape. *Medical Image Analysis* 1:225–243.
- BOOKSTEIN, F. L., P. D. CONNOR, K. D. COVELL, H. M. BARR, C. A. GLEASON, R. W. SZE, J. A. MCBROOM, AND A. P. STREISSGUTH. 2005. Preliminary evidence that prenatal alcohol damage may be visible in averaged ultrasound images of the neonatal human *corpus callosum*. *Alcohol* 36:151–160.
- CAMPOS, L. A., H. R. SILVA, AND A. SEBEN. 2010. Morphology and development of additional bony elements in the genus *Brachycephalus* (Anura: Brachycephalidae). *Biological Journal of the Linnean Society* 99:752–767.
- CHIARI, Y., A. V. D. MEIJEN, O. MADSEN, M. VENCES, AND A. MEYER. 2009. Base composition, selection, and phylogenetic significance of indels in the recombination activating gene-1 in vertebrates. *Frontiers in Zoology* 6:32.
- CLEMENTE-CARVALHO, R. B. G., L. R. MONTEIRO, V. BONATO, H. S. ROCHA, G. R. PEREIRA, D. F. OLIVEIRA, R. T. LOPES, C. F. B. HADDAD, E. G. MARTINS, AND S. F. REIS. 2008. Geographic variation in cranial shape in the Pumpkin Toadlet (*Brachycephalus ephippium*): a geometric analysis. *Journal of Herpetology* 42:176–185.
- CLEMENTE-CARVALHO, R. B. G., M. ANTONIAZZI, C. JARED, C. F. B. HADDAD, A. C. R. ALVES, H. S. ROCHA, G. R. PEREIRA, D. F. OLIVEIRA, R. T. LOPES, AND S. F. REIS. 2009. Hyperossification in miniaturized toadlets of the genus *Brachycephalus* (Amphibia: Anura: Brachycephalidae): microscopic structure and macroscopic patterns of variation. *Journal of Morphology* 270:1285–1295.
- DARST, C. R., AND D. C. CANNATELLA. 2004. Novel relationships among hyloid frogs inferred from 12S and 16S mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution* 31:462–75.
- ELMER, K. R., J. A. DÁVILA, AND S. C. LOUGHEED. 2007. Cryptic diversity and deep divergence in an upper Amazonian leafhopper, *Eleutherodactylus ockendeni*. *BMC Evolutionary Biology* 7:247.
- EWING, B., L. HILLIER, M. C. WENDL, AND P. GREEN. 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Research* 8:175–185.
- FOUQUET, A., M. VENCES, M.-D. SALDUCCI, A. MEYER, C. MARTY, M. BLANC, AND A. GILLES. 2007a. Revealing cryptic diversity using molecular phylogenetics and phylogeography in frogs of the *Scinax ruber* and *Rhinella margaritifera* species groups. *Molecular Phylogenetics and Evolution* 43:567–582.
- FOUQUET, A., A. GILLES, M. VENCES, C. MARTY, M. BLANC, AND N. J. GEMMELL. 2007b. Underestimation of species richness in Neotropical frogs revealed by mtDNA analyses. *PLoS ONE* 2:e1109.
- FROST, D. R. 2009. *Amphibian Species of the World: An Online Reference* [Internet]. Vers. 3.0. New York: American Museum of Natural History [cited 2004 Aug 22]. Available from: <http://research.amnh.org/herpetology/amphibia/index.html>.
- GLAW, F., J. KÖHLER, I. DE LA RIVA, D. R. VIEITES, AND M. VENCES. 2010. Integrative taxonomy of Malagasy treefrogs: combination of molecular genetics, bioacoustics and comparative morphology reveals twelve additional species of *Boophis*. *Zootaxa* 2383:1–82.
- GOEBEL, A. M., J. M. DONNELLY, AND M. E. ATZ. 1999. PCR primers and amplification methods for 12S ribosomal DNA, the control region, cytochrome oxidase I, and cytochrome b in bufonids and other frogs, and an overview of PCR primers which have amplified DNA in amphibians successfully. *Molecular Phylogenetics and Evolution* 11:163–199.
- GONZALEZ, P. N., V. BERNAL, AND S. I. PEREZ. 2009. Geometric morphometric approach to sex estimation of human pelvis. *Forensic Science International* 189:68–74.
- GUNZ, P., F. L. BOOKSTEIN, P. MITTEROECKER, A. STADLMAYR, H. SEIDLER, AND G. W. WEBER. 2009. Early modern human diversity suggests subdivided population structure and a complex out-of-Africa scenario. *Proceedings of the National Academy of Sciences (USA)* 106:6094–6098.
- HADDAD, C. F. B., A. C. R. ALVES, R. B. G. CLEMENTE-CARVALHO, AND S. F. REIS. 2010. A new species of *Brachycephalus* from the Atlantic Rain Forest in São Paulo State, Southeastern Brazil (Amphibia: Anura: Brachycephalidae). *Copeia* 2010:410–420.
- HANKEN, J. 1993. Adaptation of bone growth to miniaturization of body size. In B. K. Hall (ed.), *Bone Growth—B*, pp. 79–104. CRC Press, Boca Raton, FL.
- HEDGES, S. B., W. E. DUELLMAN, AND M. P. HEINICKE. 2008. New World direct-development frogs (Anura: Terrarana): molecular phylogeny, classification, biogeography, and conservation. *Zootaxa* 1737:1–182.
- LARKIN, M. A., G. BLACKSHIELDS, N. P. BROWN, R. CHENNA, P. A. MCGETTIGAN, H. MCWILLIAM, F. VALENTIN, I. M. WALLACE, A. WILM, R. LOPEZ, J. D. THOMPSON, T. J. GIBSON, AND D. G. HIGGINS. 2007. Clustal W and Clustal X. Vers. 2.0. *Bioinformatics* 23:2947–2948.
- LIU, W., A. LATHROP, J. FU, D. YANG, AND R. W. MURPHY. 2000. Phylogeny of East Asian Bufonids inferred from mitochondrial DNA sequences (Anura: Amphibia). *Molecular Phylogenetics and Evolution* 14:423–435.
- MIRANDA-RIBEIRO, A. 1920. Os brachycephalideos do Museu Paulista. *Revista do Museu Paulista* 12:307–318.
- MITTEROECKER, P., AND P. GUNZ. 2009. Advances in geometric morphometrics. *Evolutionary Biology* 36:235–247.
- PADIAL, J. M., AND I. DE LA RIVA. 2009. Integrative taxonomy reveals cryptic Amazonian species of *Pristimantis*. *Zoological Journal of Linnean Society* 115:97–122.
- PEREZ, S. I., V. BERNAL, AND P. GONZALEZ. 2006. Differences between sliding semi-landmarks methods: implications for shape analyses of human populations. *Journal of Anatomy* 208:769–784.
- RODRÍGUEZ, A., M. VENCES, B. NEVADO, A. MACHORDOM, AND E. VERHEYEN. 2010. Biogeographic origin and radiation of Cuban *Eleutherodactylus* frogs of the *auriculatus* species group, inferred from mitochondrial and nuclear gene sequences. *Molecular Phylogenetics and Evolution* 54:179–186.
- ROHLF, F. J. 1993. Relative-warp analysis and an example of its application to mosquito wings. In L. F. Marcus, E. Bello, and A. Garcia-Valdecasas (eds.), *Contributions to Morphometrics*, pp. 131–159. Museo Nacional de Ciencias Naturales, Madrid, Spain.
- . tps series software [Internet]. Stony Brook (NY): State University of New York at Stony Brook, Department of Ecology and Evolution; 2007 [cited Dec 2008]. Available from: <http://life.bio.sunysb.edu/morph/>.
- ROHLF, F. J., AND D. SLICE. 1990. Extensions of the Procrustes method for the optimal superimposition of landmarks. *Systematic Zoology* 39:40–59.
- SAMBROOK, J., E. F. FRITSCH, AND T. MANIATIS. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, New York.
- SILVA, H. R., L. A. CAMPOS, AND A. SEBEN. 2007. The auditory region of *Brachycephalus* and its bearing on the monophyly of the genus (Anura: Brachycephalidae). *Zootaxa* 1422:59–68.
- TRUEB, L., AND P. ALBERCH. 1985. Miniaturization in the anuran skull: a case study of heterochrony. In H. R. Duncker and G. Fleischer (eds.), *Vertebrate Morphology*, pp. 113–121. Gustav Fisher Verlag, Stuttgart, Germany.
- VENCES, M., M. THOMAS, A. VAN DER MEIJEN, Y. CHIARI, AND D. R. VIEITES. 2005a. Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. *Frontiers in Zoology* 2:5.

- VENCES, M., M. THOMAS, R. M. BONETT, AND D. R. VIETIS. 2005b. Deciphering amphibian diversity through DNA barcoding: chances and challenges. *Philosophical Transactions of the Royal Society B-Biological Sciences* 360:1859–1868.
- VIETIS, D. R., K. C. WOLLENBERG, F. ANDREONE, J. KÖHLER, F. GLAW, AND M. VENCES. 2009. Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. *Proceedings of the National Academy of Sciences (USA)* 106: 8267–8272.
- YANG, Z. 2006. *Computational Molecular Evolution*. Oxford University Press, Oxford.
- YEH, J. 2002. The effect of miniaturized body size on skeletal morphology in frogs. *Evolution* 56:628–641.

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#### APPENDIX 1

*Specimens Examined*.—Macaé de Cima, Rio de Janeiro: CBFH CFBH 23564–23574. Atibaia, São Paulo: CBFH 16807–817. São Francisco Xavier, São Paulo: CBFH 16827–834. Jundiá, São Paulo: CBFH 16835–850.