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Boundaries of Morphological and Molecular Variation and the Distribution of a Miniaturized Froglet, *Brachycephalus nodoterga* (Anura: Brachycephalidae)

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Abstract.—**Most miniaturized froglets of the genus *Brachycephalus* occur in isolation in slopes of mountain ranges at elevations varying from 600 to 1,800 m in the Atlantic Forest of eastern Brazil. For organisms such as *Bracycephalus* with spatially discontinuous distributions, a fundamental task is to determine whether observed patterns of variation are consistent with geographic differentiation among allopatric populations within a single species or are suggestive of a potential species boundary. We address this problem by focusing on continental and island population samples potentially assignable to *Brachycephalus nodoterga* (Anura: Brachycephalidae) from the perspective of variation in qualitative and quantitative morphological traits and DNA sequences. Population samples from continental and island populations share color characteristics, qualitative traits, and multivariate patterns of variation and covariation in cranial metric traits. Comparative analysis of DNA sequences showed the magnitude of molecular distances between *B. nodoterga* and *Brachycephalus ephippium* to be 1 order of magnitude larger than molecular distances within *B. nodoterga* and *B. ephippium*. We interpret the combined morphological and molecular evidence to indicate that continental and island population samples examined here are conspecific. Therefore, by defining species boundaries for *B. nodoterga*, we also established minimal estimates of its distribution.**

Froglets of the genus *Brachycephalus* are a lineage of the Brachycephaloidea, a highly diverse clade of Neotropical direct-developing anurans including more than a thousand species (Padial et al., 2014). *Brachycephalus* is a remarkable example of the evolutionary process of miniaturization (Hanken, 1993), and all species in this genus have snout–vent length (SVL) less than 18 mm. Another conspicuous attribute of *Brachycephalus* is that most species occur in isolation at elevations varying from 600 to 1,800 m (Alves et al., 2006; Siqueira et al., 2013). Therefore, at any given mountain slope, the distribution will coincide with these altitudinal limits and populations within a species occurring in different slopes can be effectively allopatric (Clemente-Carvalho et al., 2008). *Brachycephalus* occurs in the Atlantic Forest of eastern Brazil ranging from the state of Bahia to the state of Santa Catarina, and currently 28 species have been described (Frost, 2014; Ribeiro et al., 2015). Of these 28 species, 22 species were described over the past 10 yr and most species of *Brachycephalus* still are known from their type locality only (Frost, 2014; Ribeiro et al., 2015). In a few cases, however, populations from different localities within a putative species have been sampled, allowing for the important task of delimiting species boundaries and distribution range.

One such case is *Brachycephalus nodoterga* (Anura: Brachycephalidae); this species is listed as data deficient in International Union for Conservation of Nature assessments because of uncertainties in taxonomy validity and lack of knowledge on the extent of occurrence (Silvano et al., 2004). *Brachycephalus nodoterga* was originally described as a variety of *B. ephippium* from Serra da Cantareira in the state of São Paulo in southeastern Brazil (Pombal, 2010). Later, Heyer et al. (1990) examined specimens from a different locality, Salesópolis, in the state of São Paulo, which they assigned to *B. nodoterga*. Heyer et al. (1990) nevertheless did not examine specimens from Serra da Cantareira. More recently, Sawaya (1999) discovered a population in Ilha de São Sebastião, a continental island off the coast of the state of São Paulo, which he tentatively assigned to *B. nodoterga*. The three populations assignable to *B. nodoterga* are spatially isolated from each other, as they occur at 600 m above sea level or higher. The mainland populations are further isolated from the continental island population by the São Sebastião channel, about 2 km wide and reaching depths between 4.5 and 50 m (Ângelo, 1989).

Patterns of variation among populations assignable to *B. nodoterga* have not yet been evaluated with the objective of defining species boundaries and its distribution. Mainland populations from Salesópolis and Serra da Cantareira occur at elevations of 800 m above sea level (Pombal, 2010), and the continental island population from Ilha de São Sebastião occurs at elevations between 600 and 1,000 m above sea level (Sawaya, 1999); therefore, the populations are effectively allopatric. The problem then is to determine whether the observed variation in qualitative and quantitative morphological traits and DNA sequences is consistent with geographic variation among allopatric populations within a single species or a boundary between species must be invoked. Insight into this question can be achieved by asking whether morphological variation is continuous or discontinuous in geographic space (Zapata and Jiménez, 2012), and whether the magnitude of molecular variation is associated with a threshold between geographic variation among populations and interspecific divergence (Glaw et al., 2010). In this context, continuous variation in

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FIG. 1. Representative specimens assignable to *Brachycephalus nodoterga* from Ilha de São Sebastião, Salesópolis, and Serra da Cantareira, São Paulo, Brazil. (A) Live specimens. (B) Specimens cleared and double stained with alizarin red and Alcian blue. (C) Electron scanning micrographs. Scale bars = 1 mm.

morphological traits associated with molecular variation below a quantitative threshold characteristic of species-level differences should lead to an interpretation of the existence of a single species. Otherwise, an interpretation of more than one species is appropriate. This line of reasoning is pursued in this paper.

MATERIALS AND METHODS

Population samples assignable to *B. nodoterga* were examined from Serra da Cantareira, Salesópolis, and Ilha de São Sebastião, all in the state of São Paulo in southeastern Brazil. Voucher numbers, locality coordinates, and samples sizes for morphometric and molecular analyses are given in Appendix 1. All specimens examined were adults and are deposited in the following herpetological collections: Célio F. B. Haddad collection (CFBH), Departamento de Zoologia, Universidade Estadual Paulista, Campus de Rio Claro, São Paulo, Brazil; Museu de Zoologia da Universidade de São Paulo (MZUSP), São Paulo, Brazil; Museu Nacional (MNRJ), Rio de Janeiro, Rio de Janeiro, Brazil; and Museu de História Natural da Universidade Estadual de Campinas, Campinas, São Paulo (ZUEC).

Live specimens from the three population samples were compared in color and warts on the skin. Information on variation in qualitative traits was obtained from specimens prepared for diaphanization and electron microscopy as follows. Two specimens from each population, one male and one female (when available), were cleared and double stained with alizarin red and Alcian blue (Taylor and van Dyke, 1985). The skeletons of two specimens from each population sample (one male and one female) were immersed in a solution of sodium hypochlorite for removal of the soft tissues and then air-dried (Clemente-Carvalho et al., 2009). The skeletons were then mounted onto metal stubs, coated with gold in a Sputter Coater Balzers SCD050 (Leica Microsystems Inc., Buffalo Grove, IL, USA) and examined under a JSM 5800LV scanning electron microscope (JEOL Ltd., Tokyo, Japan).

To explore the morphometric similarities and differences among the samples from Serra da Cantareira ($n = 13$), Salesópolis ($n = 7$), and Ilha de São Sebastião ($n = 20$), we measured the following 15 cranial and postcranial continuous traits: SVL; axilla–groin length (AGL); head length from tip of snout to angle of jaw (HL); head width, width of head between angles of jaw (HW); nostril diameter (ND); internostril distance, between inner margins of nostrils (IND); eye diameter (ED), interorbital distance, between anterior corners of eyes (IOD); eye–nostril distance, from anterior corner of the eye to posterior margin of nostril (END); thigh length (THL); tibia length (TBL); foot length, from the central region to the tip of the larger toe (FL); arm length (AL); forearm length (FAL); and hand length, from the wrist to the tip of the longer finger (HAL). The measurements were registered (in millimeters) by one of us (RBGC-C) to avoid interobserver error by using a micrometric ocular fitted to the stereomicroscope. The continuous traits were

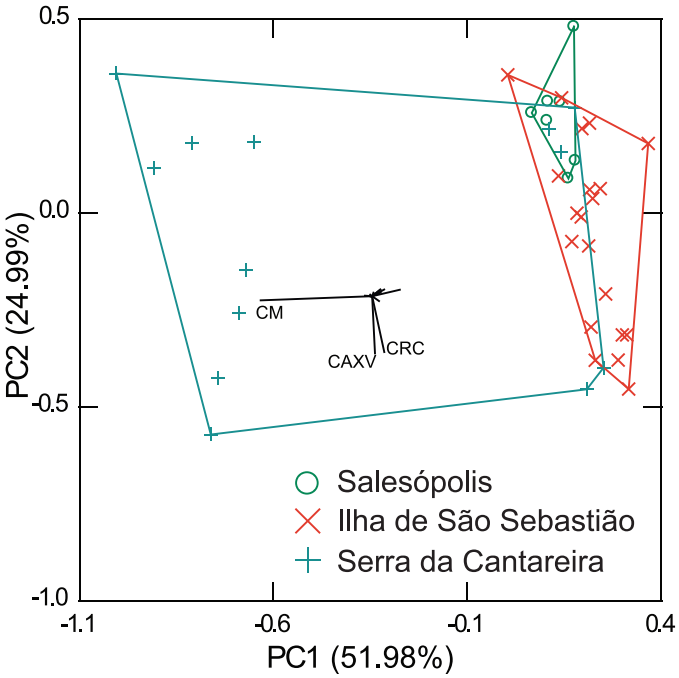


FIG. 2. Ordination of population samples assignable to *Brachycephalus nodoterga* from the localities of Salesópolis, Serra da Cantareira, and Ilha de São Sebastião in the state of São Paulo, Brazil, in the space of the first and second PCs. In these figures, each symbol represents the position of an individual from a given sample, and polygons define the limits of variation in cranial shape for each sample.

used to calculate Mosimann shape variables by dividing each original variable by the geometric mean, defined as the n^{th} root of the product of the n continuous traits (Jungers et al., 1995). Then, we calculated principal component (PC) scores based on the covariance matrix of the Mosimann shape variables (Jungers et al., 1995). PC scores describe the main trends in variation in body shape among samples and are interpreted as a low-dimensional representation of a Euclidean distance matrix among all sampled specimens.

Molecular variation was assessed for four mitochondrial genes—rRNA 12S, rRNA 16S, cytochrome *b* [Cyt *b*], and cytochrome c oxidase subunit I [COI]—and one nuclear gene, Rag-1, in population samples of *B. nodoterga* from Serra da Cantareira ($n = 4$ for rRNA 12, $n = 3$ for rRNA 16S, $n = 3$ for Cyt *b*, $n = 6$ for COI, and $n = 6$ for Rag-1); Salesópolis ($n = 7$); and Ilha de São Sebastião ($n = 8$). Genomic DNA was extracted from liver (or muscle) tissue preserved in 100% ethanol. Tissue samples were digested with proteinase K, and we then followed a standard three-step phenol–chloroform extraction procedure (Green and Sambrook, 2013). Amplification of the Cyt *b*, rRNA 12S, and COI genes followed Goebel et al. (1999). The rRNA 16S gene was amplified after Darst and Cannatella (2004), and the fragment of the Rag-1 was amplified according to Hedges et al. (2008). The amplification products were visualized on 1.0% agarose gels and purified using a QIAquick PCR Purification Kit (QIAGEN Inc., Venlo, The Netherlands). Purified PCR products were outsourced to Macrogen Inc. (Seoul, South Korea), for sequencing (using the BigDye® Terminator Kit and run on an ABI 3730xl DNA analyzer [Applied Biosystems, Inc., Grand Island, NY, USA]). Sequences were obtained in both directions with the same primers used for polymerase chain reaction amplification and subjected to BLAST searches (Altschul et al., 1997) in GenBank to verify the desired sequences had been amplified. Sequence traces were analyzed using the phred program (Ewing et al., 1998). We obtained 4,588 base pairs (bp) in total of which 882 bp were from Cyt *b*; 863 bp were from rRNA 12S; 822 bp were from COI; 1,385 bp were from rRNA 16S; and 636 bp were from Rag-1. The authenticity of Cyt *b*, COI, and Rag-1 genes was confirmed by amino acid translation. Sequences were aligned with Clustal X 2.0 (Larkin et al., 2007). The Rag-1 sequences were identical in all individuals in the population samples. Molecular distances between the population samples for the rRNA 12S, rRNA 16S, Cyt *b*, and COI genes were computed as uncorrected (*p*) distances, and are given as the number of nucleotide differences per site (Yang, 2006). Distances were calculated with MEGA6 software (Tamura et al., 2013).

To determine a quantitative threshold of between-species molecular variation, we compared the magnitude of genetic distances within population samples assignable to *B. nodoterga* with the magnitude of genetic distances within population samples of *B. ephippium*. Then, we compared these values with the magnitude of genetic distances between *B. nodoterga* and *B. ephippium*. We chose *B. ephippium* because *B. nodoterga* was originally described as a variety of *B. ephippium*. Molecular distances were calculated within population samples of *B. ephippium* by using sequences of rRNA 12S, rRNA 16S, Cyt *b*, and COI genes from the following localities in the state of São Paulo: Atibaia ($n = 10$), Serra do Japi ($n = 10$), Joaquim Egídio ($n = 10$), and São Francisco Xavier ($n = 9$ for rRNA 12S and rRNA 16S, $n = 10$ for Cyt *b*, $n = 10$ for COI, and $n = 10$ for Rag-1). The rRNA 16S gene was not available for the sample from Joaquim Egídio. The Rag-1 sequences were identical in all individuals in the population samples of *B. ephippium*. Molecular distances also were calculated within population samples assignable to *B. nodoterga*. Finally, molecular distances were calculated between samples assignable to *B. nodoterga* and *B. ephippium*. GenBank numbers, locality coordinates, and samples sizes for molecular analyses are given in Appendix 1.

Genealogical relationships among haplotypes were estimated for the three population samples assignable to *B. nodoterga* with a statistical parsimony network by using the rRNA 12S, rRNA 16S,Cyt *b*, and COI genes, selecting the 95% connection limit as the parsimony threshold (Clement et al., 2000). The analysis was run with the PopART software (http://popart.otago.ac.nz).

The same sequence data were used to estimate a genealogical tree with time of divergence by using the Bayesian phylogenetic inference implemented in *BEAST v1.6.1 (Drummond et al., 2006). BEAST does not call for any outgroup. Heled and Drummond (2010) pointed out that "there are no unrooted phylogenies—only phylogenies whose root position is uncertain." A substitution rate for the mitochondrial genes necessary to calibrate the BEAST inference was calculated using previously published dates; these dates are based on geologic and fossil evidence (Heinicke et al., 2007). Specifically, we estimated two substitution rates that were used to establish an interval by using the minimum and maximum dates of Heinicke et al. (2007) for the divergence of *Brachycephalus*. The best-fitting substitution model was estimated as the GTR by using JMODELTEST v0.1.1 (Posada, 2008). We used a log-normal relaxed clock and a Yule process model of speciation (Drummond et al., 2006). The length of the Markov chain Monte Carlo chain was set at 50,000,000, and samples were drawn every 5,000 steps. The convergence in the posterior distribution of trees was determined using the program Tracer v1.5 (Rambaut et al., 2013). The maximum clade credibility tree was estimated

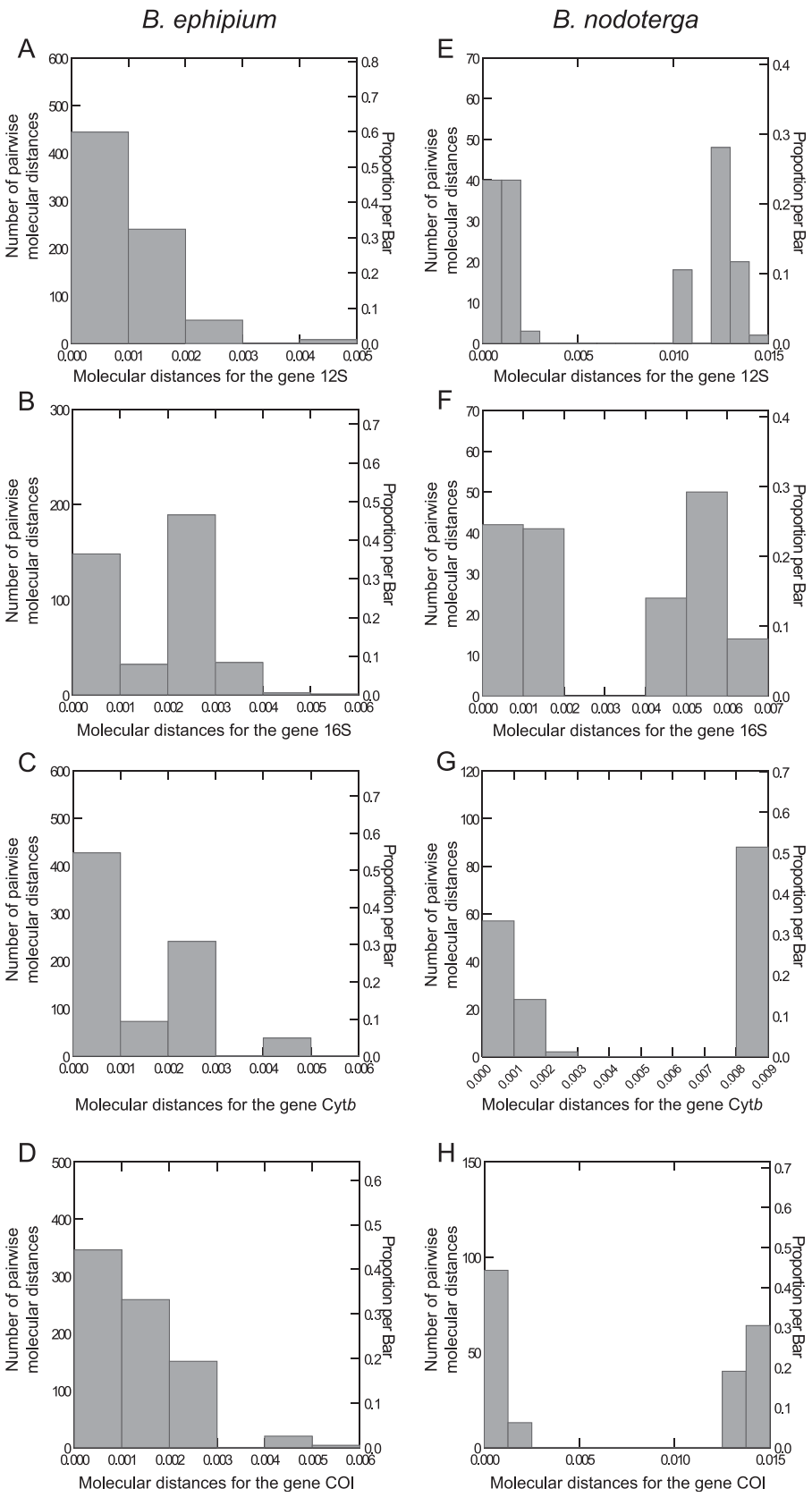
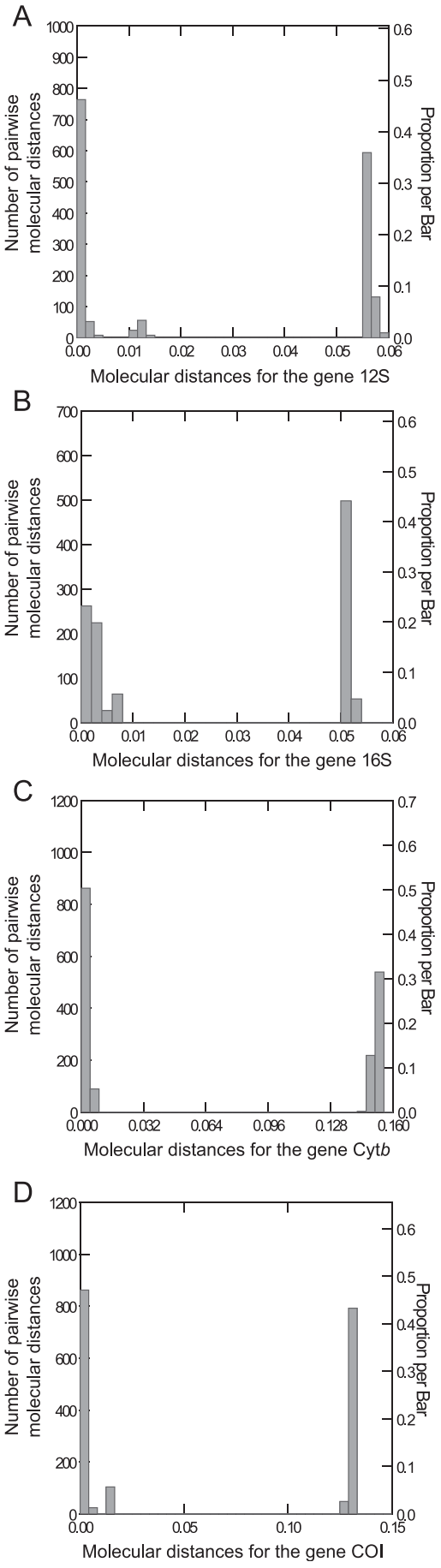


FIG. 3. Pairwise molecular sequence comparisons calculated within samples of *Brachycephalus ephippium* (A–D) and within population samples assignable to *B. nodogerga* (E–H) for the rRNA 12S, rRNA 16S, Cyt *b*, and COI genes.



using TreeAnnotator 1.4.8 (Drummond and Rambaut, 2007), excluding as burn-in the first 1,250 sampled trees.

Results

Qualitative and Quantitative Morphological Variation between Population Samples Assignable to B. nodoterga.—Live specimens from the population samples examined are greenish yellow to dark gray-green with yellow warts (Fig. 1A). The cleared and double-stained specimens revealed that warts are isolated bony structures imbedded in the skin (Fig. 1B). These individuals are representative of each population sample, and the density of the bony structures is lower in the sample from Salesópolis than in the samples from Serra da Cantareira and Ilha de São Sebastião (Fig. 1B). Scanning electron microscope images of representative specimens (Fig. 1C) showed the skull to be hyperossified (Clemente-Carvalho et al., 2009), although the degree of surface sculpturing varied among samples. Surface sculpturing of the skull was more prominent in the sample from Ilha de São Sebastião compared to the samples from Serra da Cantareira and Salesópolis (Fig. 1C).

Patterns of variation in the 15 continuous traits measured in the three population samples were evaluated with PC analysis by using the covariance matrix of the Mosimann shape variables (Fig. 2). The first two PCs explained approximately 77% of the variation. Samples from Salesópolis and Ilha de São Sebastião overlapped extensively, and both overlapped partially with the sample from Serra da Cantareira in the reduced space of the first two PCs (Fig. 2).

Comparison of Genetic Distances within and between Populations Assignable to B. nodoterga *and* B. ephippium.—Uncorrected molecular distances for the rRNA 12S, rRNA 16S, Cyt *b*, and COI genes were calculated within population samples assignable to *B. nodogerga* and within samples of *B. ephippium* (Fig. 3). Molecular distances were roughly similar in all four genes in the samples of *B. ephippium* and the distribution of distances was approximately continuous (Fig. 3A–D). In contrast, the range of molecular distances varied in the four genes in the samples assignable to *B. nodoterga* (Fig. 3E–H). Furthermore, contrary to *B. ephippium*, there was a discontinuity in the distribution of distances (Fig. 3E–H). The bars to the left of the graph are pairwise sequence comparisons between the two population samples assignable to *B. nodoterga* from the mainland (Cantareira and Salesópolis), whereas the bars to the right of the graph are pairwise sequence comparisons between the mainland samples and the sample from the continental island Ilha de São Sebastião (Fig. 3E–H).

Bars to the left of the graph (Fig. 4) are pairwise sequence comparisons within population samples assignable to *B. nodoterga* and samples of *B. ephippium*, whereas bars to the right of the graph are pairwise sequence comparisons between population samples assignable to *B. nodoterga* and *B. ephippium*. A marked discontinuity in the distribution of molecular distances between samples assignable to *B. nodoterga* and *B. ephippium* is evident (Fig. 4A–D). In fact, although molecular distances vary between 0 and 0.014 in comparisons within *B. nodoterga* or *B. ephippium*, the range of variation in molecular

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FIG. 4. Pairwise molecular sequence comparisons between population samples of *Brachycephalus ephippium* and population samples assignable to *B. nodoterga* for the rRNA 12S, rRNA 16S, Cyt *b*, and COI genes.

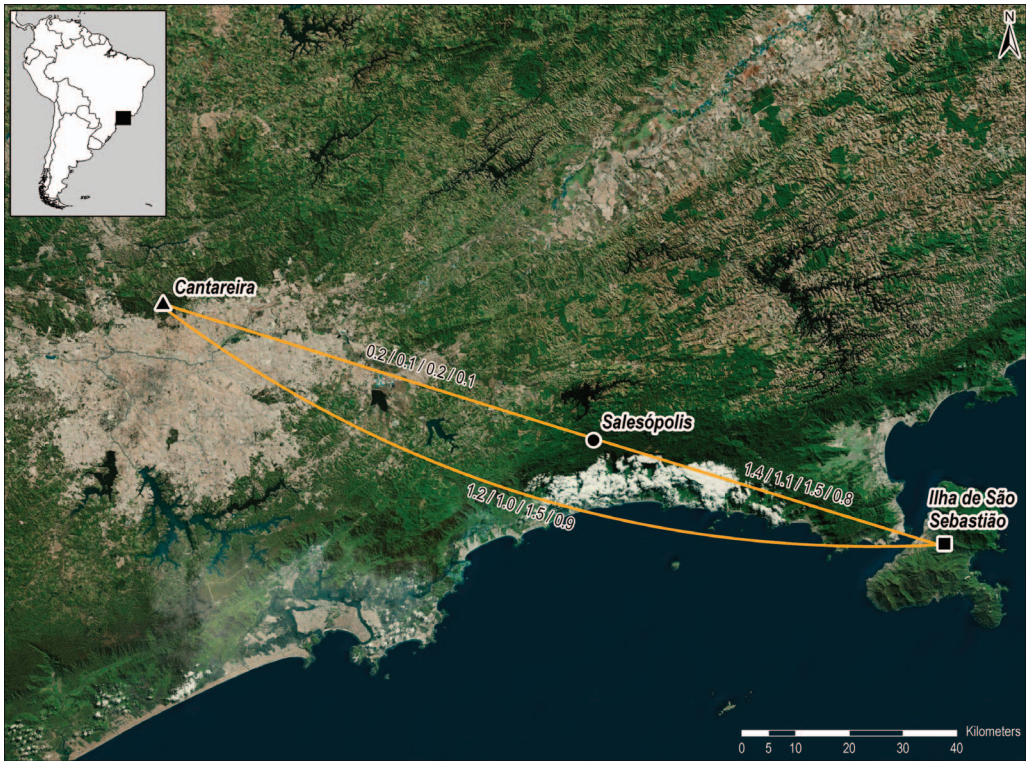

FIG. 5. Mean uncorrected molecular distances between population samples assignable to *Brachycephalus nodoterga* from the localities of Serra da Cantareira, Salesópolis, and Ilha de São Sebastião in the state of São Paulo, Brazil. Distances are given for the genes rRNA 12S, rRNA 16S, Cyt *b*, and COI.

distances between *B. nodoterga* and *B. ephippium* is 0.053–0.152. The latter numbers are estimates of uncorrected distances. Estimates of molecular distances corrected by the Tamura–Nei substitution model are, as expected, larger and range from 0.060 to 0.201.

Molecular distances between population samples assignable to *B. nodoterga* for the rRNA 12S, rRNA 16S, Cyt *b*, and COI genes were plotted and mapped (Fig. 5). Distances were lower between the mainland samples from Serra da Cantareira and Salesópolis (0.001–0.002) compared to that between samples from Serra da Cantareira and the continental island Ilha de São Sebastião (0.010–0.012) and samples from Salesópolis and Ilha de São Sebastião (0.012–0.014).

Genealogical Relationships and Estimation of Divergence Time between Population Samples Assignable to B. nodoterga.—The statistical parsimony network identified five haplotypes across the three localities for the 12S, 16S, and COI genes and four haplotypes for the Cyt *b* gene (Fig. 6A–D). The only case of haplotype sharing occurred between the mainland samples from Serra da Cantareira and Salesópolis for the Cyt *b* gene (Fig. 6C). In all four genes, the most divergent haplotype was from Ilha de São Sebastião, with an estimated minimum of 7 and a maximum of 13 mutational steps for the 16S and COI genes, respectively.

The most obvious feature of our genealogical tree is the partitioning of individual sequences into three lineages concordant with the geographic origin of the samples (Fig. 7A). The branching pattern of this genealogical tree displays a sister-group relationship of the mainland samples of Serra da Cantareira and Salesópolis with respect to the continental island sample of Ilha de São Sebastião, a pattern already suggested by the magnitudes of the molecular distances (Figs. 5, 7A). The genealogy (Fig. 7A) also shows the common ancestor of the mainland populations from Serra da Cantareira and Salesópolis lived at approximately 125 kyr (thousands of years before present; range between approximately 97 and 170 kyr). Further down in the genealogy, the most recent common ancestor (MRCA) of the extant mainland and continental island populations assignable to *B. nodoterga* existed at approximately 275 kyr (range between approximately 200 and 350 kyr; Fig. 7A).

The mainland and continental island localities currently are separated by the São Sebastião channel. Estimates of sea level fluctuations show global sea-level variation for the past 500 kyr as sea-level peaks and sea-level drops (Martin et al., 1986; Rohling et al., 1998; Fig. 7B). In particular, a sea-level drop of approximately 100 m below the present-day level occurred at approximately 275 kyr (Rohling et al., 1998), exposing the Atlantic shelf. The paleoshoreline at this time was reconstructed at a bathymetric level of 100 m, shown as a green-light contour (Fig. 8). Given that the São Sebastião channel has a maximum depth of 50 m, a land connection between present-day Ilha de São Sebastião and the mainland at approximately 275 kyr is evident.

DISCUSSION

We posed the question of whether variation in morphological traits and DNA sequence in population samples assignable to *B. nodoterga* is consistent with expected patterns of geographic variation among allopatric populations within a single species or is suggestive of a potential species boundary. In addressing this question, two issues become relevant. First, the spatial discontinuity of most species and populations of *Brachycephalus* is of primary significance (Clemente-Carvalho et al., 2008). Second, the definition of species boundaries is closely tied to the

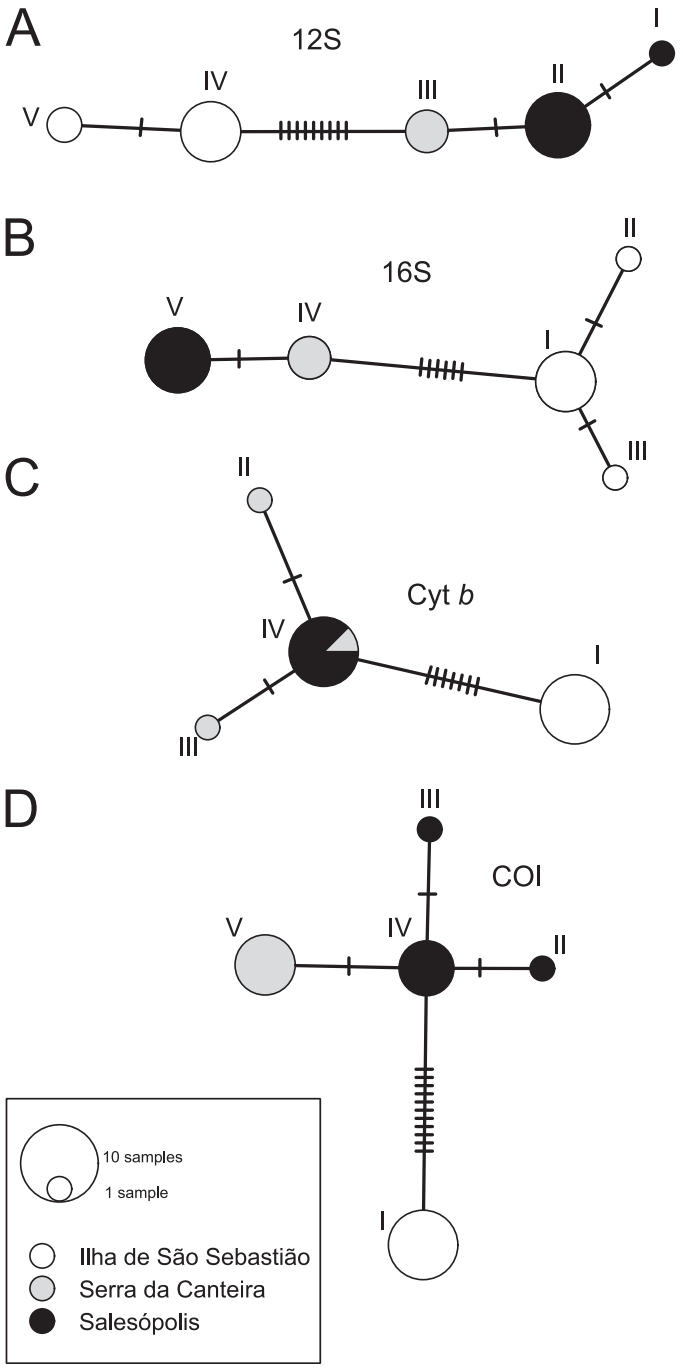

FIG. 6. Haplotype network for population samples assignable to *Brachycephalus nodoterga* for the genes (A) rRNA 12S, (D) rRNA 16S, (C) Cyt *b*, and (D) COI. Circle size is proportional to haplotype frequency. Each line represents one mutational step, and the cross-dashes are missing, that is, extinct or unsampled haplotypes.

definition of its distribution and our results do shed light on this problem as well.

Analyses of qualitative morphological traits showed that individuals in the three population samples assignable to *B. nodoterga* share similar color patterns and bony structures imbedded in the skin (dermal ossifications known as osteoderms; sensu Ruibal and Shoemaker, 1984). The three population samples also share patterns of variation and covariation in cranial metric traits. Indeed, there is morphometric variation between the population samples, because the polygons do not overlap completely; however, the absence of morphometric discontinuities between populations that are effectively allopatric provides evidence for morphologically cohesive populations. Therefore, the qualitative and quantitative morphological evidence suggests geographic variation within a single species rather than evidence for the existence of more than one species (Zapata and Jiménez, 2012).

Amphibians are known, however, for their lower rates of morphological evolution and consequently morphological conservatism (Wiens, 2008). Therefore, additional evidence, independent of morphological variation, is necessary to establish potential boundaries of intra- and interspecific variation. In this connection, molecular data are instrumental to define potential *quantitative* thresholds between levels of intra- and interspecific variation (Díaz-Rodriguez et al., 2015). This approach is inherently comparative and the magnitude of the discontinuity in molecular distances between populations assignable to *B. nodoterga* and *B. ephippium* is 1 order of magnitude larger than the discontinuity within populations assignable to *B. nodoterga* and *B. ephippium*. We interpret the molecular evidence combined with the morphological evidence to show that populations from the mainland localities of Serra da Cantareira and Salesópolis and the continental island of São Sebastião are conspecific.

Our statistical parsimony network and phylogenetic inference derived for all individuals by using the four genes indicated that the population from Ilha de São Sebastião diverged earlier from the populations from the mainland. The dated phylogeny showed the most recent common ancestor of the three samples existed approximately 275 thousand years before the present. The comparative analysis of molecular distances also demonstrated geographic variation in molecular distances between the mainland populations and the continental island population of *B. nodoterga*. Differentiation between populations inhabiting the mainland and several continental islands besides Ilha de São Sebastião off the coast of the state of São Paulo has been documented for many species of amphibians and reptiles (Marques et al., 2002; Brasileiro et al., 2007; Barbo et al., 2012). The climatic-environmental factor most likely to explain the observed differentiation is the sea-level fluctuation associated with global ice-volume changes during the Pleistocene (Marques et al., 2002; Brasileiro et al., 2007; Barbo et al., 2012). In this connection, the estimated time intervals for the MRCA node for the extant mainland and continental island populations of *B. nodoterga* overlap the time interval of a marked lowstand in global sea level. During glaciations, sea-level fell over 100 m (Rohling et al., 1998), and such marine regressions certainly established a land connection between present-day Ilha de São Sebastião and the mainland, given the maximum depth of the São Sebastião channel is 50 m (Ângelo, 1989). Also, during the glacial periods at which lowstands occurred, temperatures probably were lower. Therefore, the most recent common ancestor of *B. nodoterga*, which lived at the time of a lowstand, could have had a continuous distribution across this area. The population inhabiting present-day Ilha de São Sebastião then became isolated from the mainland in the next interglacial period when sea levels rose again.

The definition of species boundaries also is inextricably intertwined with the delimitation of a species' distribution (Kirkpatrick and Barton, 1997; Behrman and Kirkpatrick, 2011); and by defining species boundaries in *B. nodoterga*, we also have worked toward defining its potential geographic range. Evidently, this definition is conditional to our samples, and further sampling is necessary to establish the full distribution of

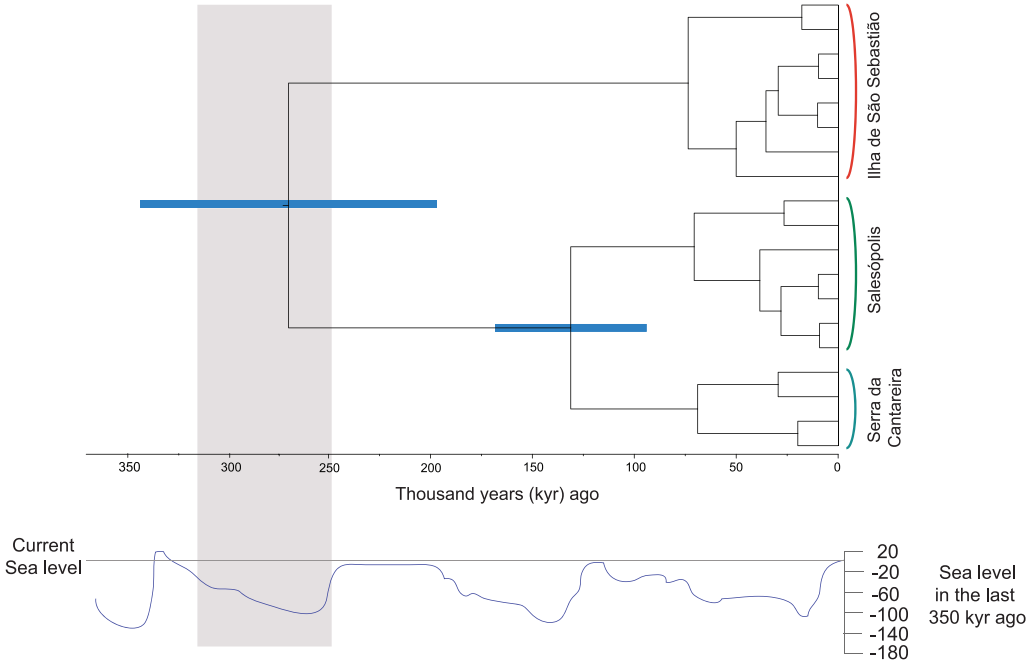

FIG. 7. (A) Molecular genealogy for the population samples assignable to *Brachycephalus nodoterga* from the localities of Salesópolis, Cantareira, and Ilha de São Sebastião in the state of São Paulo, Brazil. Blue horizontal bars represent the range for the node ages based on two different substitution rates for the mitochondrial genes obtained from previous date estimate (Heinicke et al., 2007). (B) Schematic global sea-level fluctuations during the past 350 kyr before present. Modified from Rohling et al. (1998).

B. nodoterga. This small sample is nevertheless informative because it shows that populations from the two most distant sampled localities, Serra da Cantareira and Ilha de São Sebastião, are separated by approximately 150 km. Evidently, the sampling of new localities can help increase the geographic range of *B. nodoterga*.

Recently, the size of the distribution of miniaturized species has become the topic of great interest, particularly due to the expectation of limited dispersal capabilities, leading to geographically restricted ranges and higher rates of diversification (Zimkus et al., 2012). Unfortunately, at this point, little or next to nothing can be said about the size of the distribution of most species of *Brachycephalus*, simply because we do not have the hard data. That is, we currently lack the geographical sampling for the majority of populations and species of *Brachycephalus* needed for such inference. Therefore, an important remaining

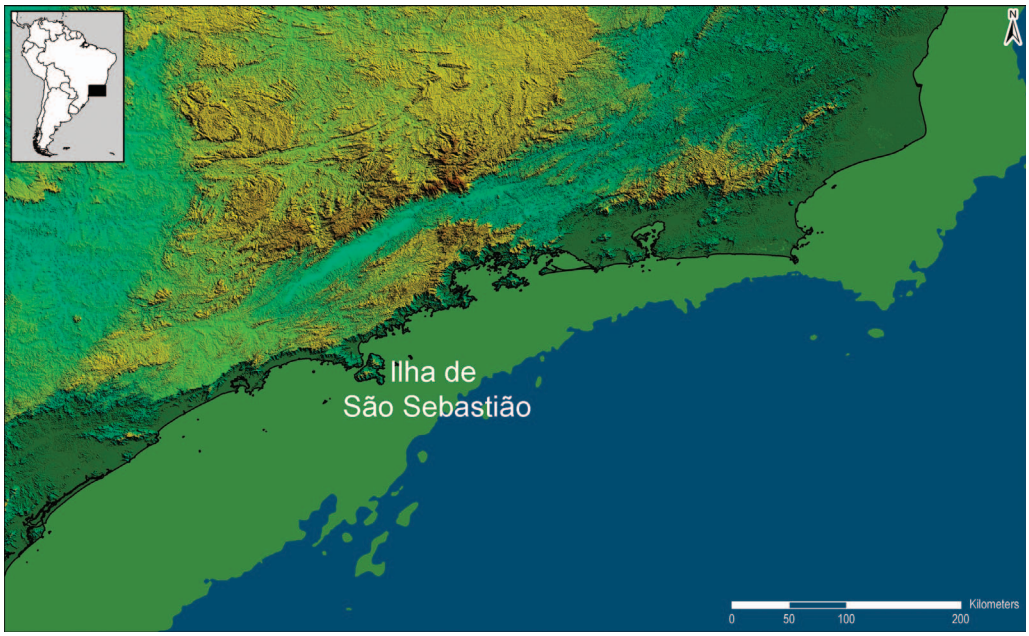

FIG. 8. Map showing present-day coastline, the continental island of Ilha de São Sebastião, and the adjacent mainland. The light-green contour represents the estimated landmass of the Atlantic shelf exposed by the sea-level drop (lowstand of 100 m) caused by glacial advance approximately 275 kyr ago.

task is the sampling of *Brachycephalus* across its geographic range. This sampling must be coupled with the integration of morphological, molecular, and ecological data to define accurate measures of the distribution. Only then will we be able to address meaningful questions of a potential association between the size of the distribution and rates of diversification in *Brachycephalus*. We hope the data and interpretations presented here are a first step to this endeavor.

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Literature Cited

Alves, A. C. R., L. F. Ribeiro, C. F. B. Haddad, and S. F. Reis. 2006. Two new species of *Brachycephalus* (Anura: Brachycephalidae) from the Atlantic forest in Paraná state, southern Brazil. Herpetologica 62: 221–233.

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.

Ângelo, S. 1989. Ilhas do Litoral Paulista, Série documentos. Secretaria do Meio Ambiente, São Paulo, Brazil.

Barbo, F. E., F. G. Grazziotin, I. Sazima, M. Martins, and R. J. Sawaya. 2012. A new and threatened insular species of lancehead from southeastern Brazil. Herpetologica 68:418–429.

Behrman, K. D., and M. Kirkpatrick. 2011. Species range expansion by beneficial mutations. Journal of Evolutionary Biology 24:665–675.

Brasileiro, C. A., C. F. B. Haddad, R. J. Sawaya, and Ivan Sazima. 2007. A new and threatened island-dwelling species of *Cycloramphus* (Anura: Cycloramphidae) from southeastern Brazil. Herpetologica 63:501–510.

Clement, M., D. Posada, and K. A. Crandall. 2000. TCS: a computer program to estimate gene genealogies. Molecular Ecology 9:1657–1659.

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 31:462–475.

Díaz-Rodríguez, J., H. Gonçalves, F. Sequeira, T. Sousa-Neves, M. Tejedo, N. Ferrand, and I. Martínez-Solano. 2015. Molecular evidence for cryptic candidate species in Iberian *Pelodytes* (Anura, Pelodytidae). Molecular Phylogenetics and Evolution 83:224–241.

Drummond, A. J., and A. Rambaut. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7:214.

Drummond, A. J., S. Y. W. Ho, M. J. Phillips, and A. Rambaut. 2006. Relaxed phylogenetics and dating with confidence. PLoS ONE 4(5): e88.

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.

Frost, D. R. 2014. Amphibian species of the world: an online reference. Version 5.6. 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.

Green, M. R., and J. Sambrook. 2013. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, USA.

Hanken, J. 1993. Adaptation of bone growth to miniaturization of body size. Pp. 79–104 in B. K. Hall (ed.), Bone Growth – B. CRC Press, USA.

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.

Heinicke, M. P., W. E. Duellman, and S. B. Hedges. 2007. Major Caribbean and Central American frog faunas originated by ancient oceanic dispersal. Proceedings of the National Academy of Sciences of the United States of America 104:10092–10097.

Heled, J., and A. J. Drummond. 2010. Bayesian inference of species trees from multilocus data. Molecular Biology and Evolution 27:570–580.

Heyer, W. R., A. S. Hand, C. A. G. Cruz, O. L. Peixoto, and C. E. Nelson. 1990. Frogs of Boracéia. Arquivos de Zoologia, São Paulo 31:231–410.

Jungers, W. L., A. B. Falsetti, and Ch. E. Wall. 1995. Shape, relative size, and size-adjustments in morphometrics. Yearbook of Physical Anthropology 38:137–161.

Kirkpatrick, M., and N. Barton. 1997. Evolution of a species' range. American Naturalist 150: 1–23.

Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, et al. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948.

Marques, O. A. V., M. Martins, and I. Sazima. 2002. A new insular species of pitviper from Brazil, with comments on evolutionary biology and conservation of the *Bothrops jararaca* group (Serpentes, Viperidae). Herpetologica 58:303–312.

Martin, L., N. A. Mörner, J. M. Flexor, and K. Suguio. 1986. Fundamentos e reconstrução de antigos níveis marinhos do Quartenário. Boletim do Instituto de Geociências, Publicação Especial 4:1–161.

Padial, J. M., T. Grant, and D. F. Frost. 2014. Molecular systematics of terraranas (Anura: Brachycephaloidea) with an assessment of the effects of alignment and optimality criteria. Zootaxa 3825:1–132.

Pombal, J. P. 2010. A posição taxonômica das "variedades" de *Brachycephalus ephippium* (Spix, 1824) descritas por Miranda-Ribeiro, 1920 (Amphibia, Anura, Brachycephalidae). Boletim do Museu Nacional (N.S.) Zoologia 526:1–12.

Posada, D. 2008. jModelTest: phylogenetic model averaging. Molecular Biology and Evolution 25:1253–1256.

Rambaut, A., M. A. Suchard, D. Xie, and A. J. Drummond. 2013. Tracer v1.5. Available from: http://beast.bio.ed.ac.uk/Tracer.

Ribeiro, L. F., M. R. Bornschein, R. Belmonte-Lopes, C. R. Firkowski, S. A. A. Morato, and R. Pie. Marcio. 2015. Seven new species of *Brachycephalus* (Anura: Brachycephalidae) from southern Brazil. PeerJ 3:e1011; DOI 10.7717/peerj.1011.

ROHLING, E. J., M. FENTON, F. J. JORISSEN, P. BERTRAND, G. GANSSEN, AND J. P. CAULET. 1998. Magnitudes of sea-level lowstands of the past 500,000 years. Nature 394:162–165.

RUIBAL, R., AND V. SHOEMAKER. 1984. Osteoderms in anurans. Journal of Herpetology 18:313–328.

SAWAYA, R. C. 1999. Diversidade, Densidade e Distribuição Altitudinal da Anurofauna de Serapilheira da Ilha de São Sebastião, S.P. Unpubl. M.S. thesis, Universidade de São Paulo, Brazil.

SILVANO, D., R, HEYER, AND U. CARAMASCHI. 2004. *Brachycephalus nodoterga*. 2013 IUCN Red List of Threatened Species. Version 2013.1. Available from: www.iucnredlist.org.

SIQUEIRA, C. C., D. VRCIBRADIC, AND C. F. D. ROCHA. 2013. Altitudinal records of data-deficient and threatened frog species from the Atlantic Rainforest of the Serra dos Órgãos mountains, in southeastern Brazil. Brazilian Journal of Biology 73:229–230.

TAMURA, K., G. STECHER, D. PETERSON, A. FILIPSKI, AND S. KUMAR. 2013. MEGA6: molecular evolutionary genetic analysis version 6.0. Molecular Biology and Evolution 30:2725–2729.

TAYLOR, W. R., AND G. C. VAN DYKE. 1985. Revised procedures for staining and clearing 390 small fishes and other vertebrates for bone and cartilage study. Cybium 391:107–119.

WIENS, J. J. 2008. Systematics and herpetology in the age of genomics. BioScience 58:297–307.

YANG, Z. 2006. Computational Molecular Evolution. Oxford University Press, England.

ZAPATA, F., AND I. JIMÉNEZ. 2012. Species delimitation: inferring gaps in morphology across geography. Systematic Biology 61:179–194.

ZIMKUS, B. M., L. LAWSON, S. P. LOADER, AND J. HANKEN. 2012. Terrestrialization, miniaturization and rates of diversification in African puddle frogs (Anura: Phrynobatrachidae). PLoS ONE 7: e35118.

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APPENDIX 1

Specimens Examined

All specimens examined are from the Brazil.

Brachycephalus nodoterga.—SERRA DA CANTAREIRA, SÃO PAULO (23°28′S, 46°38′W); CFBH 2226*, 28413*, TC140* (asterisks denote specimens used in the molecular analyses), MNRJ 73674–73675, MZUSP A112785–91, 809, 975. GenBank accession numbers: 12S rRNA, KJ 649759–62; 16S rRNA, KJ 649778–80; COI, KJ 649796–801; Cyt *b*, KJ 649817–19; Rag-1, KJ 649835–37. SALESÓPOLIS, SÃO PAULO (23°39′S, 45°53′W); TC 128*, TC132*, 136–138*, CFBH 34967*, 34968*. GenBank accession numbers: 12S rRNA, KJ 649752–58; 16S rRNA, KJ 649771–77; COI, KJ 649789–95; Cyt *b*, KJ 649810–16; Rag-1, KJ 649828–34. ILHA DE SÃO SEBASTIÃO, SÃO PAULO (23°52′S, 45°20′W); CFBH 07033, 07038, 07041–42, 07046–47, MNRJ 23633–36, 23638–40, TC 105–111, ZUEC 63.4*, 63.10*, 63.11*, 63.13–63.17*. GenBank accession numbers: 12S rRNA, KJ 649763–70; 16S rRNA, KJ 649781–88; COI, KJ 649802–09; Cyt *b*, KJ 649820–27; Rag-1, KJ 649838–45.

Brachycephalus ephippium.—ATIBAIA, SÃO PAULO (23°7′S, 46°33′W); CFBH 16807, 16809, AT 120–127. GenBank accession numbers: rRNA 12S, HM208305, HQ435679, KP999150–157; rRNA 16S, HM208306, HQ435693, KP999185–192; Cyt *b*, HM208304, HQ435706, KP999250–257; COI, KP999210–219; Rag-1, HM208307, HQ435721, KP999286–293. SERRA DO JAPI, SÃO PAULO (23°10′S, 46°52′W). GenBank accession numbers: rRNA 12S, HM216360, KP999158–166; rRNA 16S, HM216361, KP999193–201; Cyt *b*, HM216359, KP999258–266; COI, KP999221–229; Rag-1, HM216362, KP999294–302. JOAQUIM EGÍDIO, SÃO PAULO (22°53′S, 46°56′W); JE 1–2, JE6–13. GenBank accession numbers: rRNA 12S, KP999175–184; Cyt *b*, KP999276–285; COI, KP999240–249; Rag-1, KP999312–321. SÃO FRANCISCO XAVIER, SÃO PAULO (23°10′S, 45°53′W). GenBank accession numbers: rRNA 12S, HM216368, KP999167–174; rRNA 16S, HM216369, KP999202–209; Cyt *b*, HM216367, KP999267–275; COI, KP999230–239; Rag-1, HM216370, KP999303–311.