



## A cryptic palm-pitviper species (Squamata: Viperidae: *Bothriechis*) from the Costa Rican highlands, with notes on the variation within *B. nigroviridis*

TIFFANY M. DOAN<sup>1</sup>, ANDREW J. MASON<sup>1</sup>, TODD A. CASTOE<sup>2</sup>,  
MAHMOOD SASA<sup>3</sup> & CHRISTOPHER L. PARKINSON<sup>1,4</sup>

<sup>1</sup>Department of Biology, University of Central Florida, 4000 Central Florida Blvd, Orlando, FL 32816, USA.

E-mail: [tiffperu@yahoo.com](mailto:tiffperu@yahoo.com), [masonaj@knights.ucf.edu](mailto:masonaj@knights.ucf.edu), [parkinson@ucf.edu](mailto:parkinson@ucf.edu)

<sup>2</sup>Department of Biology, 501 S. Nedderman Drive, University of Texas at Arlington, Arlington, TX 76019, USA.

E-mail: [todd.castoe@uta.edu](mailto:todd.castoe@uta.edu)

<sup>3</sup>Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, and Palo Verde Biological Station, Organization for Tropical Studies, San José, Costa Rica. E-mail: [msasamarin@gmail.com](mailto:msasamarin@gmail.com)

<sup>4</sup>Corresponding author

### Abstract

Middle America is one of the most biodiverse regions in the world, harboring an exceptional number of rare and endemic species. This is especially true of Middle American cloud forests, where montane specialists occupy restricted, high-elevation ranges making them attractive candidates for investigating historical biogeography and speciation. One such highland-restricted species, the black speckled palm-pitviper (*Bothriechis nigroviridis*), occupies the Central, Tilarán, and Talamanca Cordilleras in Costa Rica and Panama. In this study, we investigate the genetic and morphological variation among populations of *B. nigroviridis* by inferring a multilocus phylogeny (21 individuals) and analyzing meristic scale characters with a principal component analysis (64 individuals). We find *B. nigroviridis sensu stricto* to be composed of two deeply divergent lineages, one with a restricted range in the northern and central Cordillera Talamanca and the other ranging throughout the Central, Tilarán, and Talamanca Cordilleras. Furthermore, these two lineages are morphologically distinct, with previously unrecognized differences in several characters allowing us to name and diagnose a new species *B. nubestris* **sp. nov.** We also examine the genetic and morphological variation within *B. nigroviridis* and discuss biogeographic hypotheses that may have led to the diversification of *Bothriechis* lineages.

**Key words:** *Bothriechis nubestris*, Costa Rica, Middle America, new species, Reptilia, snake, Squamata, taxonomy, Viperidae

### Resumen

Mesoamérica es una de las regiones con mayor biodiversidad en el mundo, que alberga un número excepcional de especies raras y endémicas. Esto es especialmente cierto de los bosques nubosos de Mesoamérica, donde los especialistas de montaña ocupan rangos restringidos de alta elevación siendo candidatos atractivos para la investigación de la especiación y la biogeografía histórica. Una de estas especies de montaña restringida, la lora pintada (*Bothriechis nigroviridis*), ocupa las Cordilleras Central, Tilarán y Talamanca en Costa Rica y Panamá. En este estudio, investigamos la variación genética y morfológica entre poblaciones de *B. nigroviridis* infiriendo una filogenia multilocus (21 individuos) y analizando caracteres merísticos de escamas con un análisis de componentes principales (64 individuos). Determinamos que *B. nigroviridis sensu stricto* está compuesto de dos linajes profundamente divergentes, uno con un área de distribución restringida en el norte y centro de la Cordillera de Talamanca y el otro que va a través de las Cordilleras Central, Tilarán y Talamanca. Además, estos dos linajes son morfológicamente distintos, con diferencias no reconocidos previamente en varios caracteres que nos permiten nombrar y diagnosticar una nueva especie *B. nubestris* **sp. nov.** También examinamos la variación genética y morfológica dentro de *B. nigroviridis* y discutimos hipótesis biogeográficas que pueden haber llevado a la diversificación de linajes de *Bothriechis*.

**Palabras claves:** *Bothriechis nubestris*, Costa Rica, Mesoamérica, nueva especie, Reptilia, Squamata, taxonomía, víbora, Viperidae

## Introduction

Middle America, defined here as the region extending from central Mexico to the northern border of Colombia, exhibits high species richness and biodiversity. Despite its relatively small total area, Middle America contains one of the world's highest concentrations of endemic species, with 5000 species of endemic plants and 1159 endemic vertebrate species (Myers *et al.* 2000). These high levels of endemism and biodiversity are the result of the region's complex geological history and climatic fluctuations, which have promoted differentiation, adaptation, and speciation at many taxonomic levels (Campbell 1999).

Much of Middle America's geologic complexity is the consequence of tectonic activity, particularly during the last 20–25 million years (Bagley & Johnson 2014). Formation and subduction of the Cocos plate beneath the Caribbean plate in the Miocene, beginning approximately 23 Ma, is responsible for a number of the morphotectonic features observed in Costa Rica and Panama (Marshall 2007). The continuing subduction of this plate led to increased volcanic activity and tectonic uplift approximately 17.5 Ma, leading to major geological features such as the Talamanca Cordillera (Gazel *et al.* 2009). The mid-Miocene saw the formation of the Nicaraguan Depression: a narrow geological depression running from northeastern Costa Rica to the Gulf of Fonseca that separates the highlands of northern and southern Middle America (Funk *et al.* 2009). Collision of the Cocos Ridge with western Costa Rica 5.5–3.5 Ma caused rapid uplift of the Talamanca Cordillera, which has since remained relatively stable geologically (Abratis & Wörner 2001; Driese *et al.* 2007; Dzierma *et al.* 2011). However, the continued subduction of the Cocos plate has promoted volcanic activity in northern Costa Rica, spawning the Cordillera Central (also known as Cordillera Volcánica Central) and the Tilarán Cordillera (Marshall *et al.* 2003; Marshall 2007). Furthermore, changes associated with the closing of the Isthmus of Panama, as well as Pleistocene glacial cycles have continued to alter climatic and ecological distribution patterns of Middle America (Lachniet & Seltzer 2002; Lunt *et al.* 2008; O'Dea *et al.* 2012; Bacon *et al.* 2015). These long-term geological and ecological dynamics have made Middle America an attractive system for testing regional biogeographic and phylogeographic hypotheses as lineages occupying the region often display clear phylogenetic structure with divergence patterns shared by independent lineages (Zamudio & Greene 1997; Arbogast & Kenagy 2001; Castoe *et al.* 2009; Daza *et al.* 2010).

One group that has been used to examine phylogeographic patterns in Middle America is the palm-pitvipers in the genus *Bothriechis*. This genus consists of 10 recognized species of arboreal pitvipers that range from southern Mexico to northern South America. Excluding *B. schlegelii*, the majority of *Bothriechis* are highland species, which typically inhabit restricted ranges (Campbell & Lamar 2004). In the case of *B. guifarroi*, *B. rowleyi*, and *B. supraciliaris*, these ranges may consist of a small number of known localities confined to a single highland region (Solórzano *et al.* 1998; Campbell & Lamar 2004; Townsend *et al.* 2013). Because of the limited ranges and disjunct distributions of these species, the evolutionary relationships of these snakes can provide insight into regional patterns of dispersal, diversification, and speciation. To this end, the phylogeny of *Bothriechis* has received a number of revisions over the past 25 years, often with biogeographic implications.

Crother *et al.* (1992) first established the phylogeny of *Bothriechis* based on morphological and allozyme data. An important finding of their work was the placement of *B. lateralis* as sister to *B. bicolor* within a northern Middle American highland clade containing ((*B. aurifer*, *B. rowleyi*), (*B. marchi*, (*B. bicolor*, *B. lateralis*))) (Crother *et al.* 1992). The range of *Bothriechis nigroviridis* overlaps with that of *B. lateralis* in southern Middle America, though *B. nigroviridis* typically occur at higher elevations and were found to be sister to northern highland *Bothriechis* species (Crother *et al.* 1992; Campbell & Lamar 2004). This suggested a complex evolutionary history of *Bothriechis* with diversification over a broad time span, and a southward invasion of Costa Rica and Panama (Crother *et al.* 1992). Taggart *et al.* (2001) expanded this phylogeny by adding mitochondrial DNA sequences of the 12S rRNA locus and performing independent and combined analyses. This study found *B. lateralis* to be sister to *B. nigroviridis*; however, the authors concluded that the mitochondrial data represented a case of incomplete lineage sorting (Taggart *et al.* 2001). Subsequent phylogenies using multilocus mitochondrial datasets have inferred *B. lateralis* as a sister lineage to the northern Middle American highland clade with *B. nigroviridis* as sister to all of the highland *Bothriechis* lineages (Castoe & Parkinson 2006; Castoe *et al.* 2009; Daza *et al.* 2010). Most recently, Townsend *et al.* (2013) reconstructed a phylogeny based on four mitochondrial loci in conjunction with the species description of *B. guifarroi* and found a (*B. nigroviridis* (*B. lateralis*, *B. guifarroi*)) clade sister to the northern Middle American highland clade. This suggested that divergence of the northern and southern Middle American highland *Bothriechis* groups may be more recent than previously estimated (Townsend *et al.* 2013).

The specific evolutionary relationships among *B. nigroviridis*, *B. lateralis*, and the northern Middle American highland clade provide implications for gene flow and divergence of *Bothriechis* through Middle America. However, our recent investigations of *B. nigroviridis* have indicated unexpected patterns of phylogeographic structure and evidence that *B. nigroviridis* appeared to be comprised of at least two distinct lineages. This phylogenetic structure within *B. nigroviridis* is of particular interest as this species' range is largely restricted to the higher elevations of the Talamanca Cordillera, the Tilarán Cordillera, and the Central Cordillera. These mountain ranges, especially the Talamanca Cordillera, have been hypothesized to act as “sky-islands”, that have driven *in situ* divergence of lineages (Savage 1982; Bagley & Johnson 2014). To explore patterns of divergence within *B. nigroviridis*, we used genetic and morphological analyses. Based on our molecular and morphological analyses, we recognize and describe a new *Bothriechis* species, sister to *B. nigroviridis*, discuss the variation within *B. nigroviridis*, and comment on the phylogeographic history of these groups.

## Materials and methods

**Genetic analysis.** We obtained tissue samples from 21 *Bothriechis nigroviridis sensu lato* individuals from localities in Costa Rica (Appendix 1). Additionally, we received tissue samples for three *B. guifarroi* individuals used in Townsend *et al.* (2013). Genomic DNA was isolated from these samples using the Qiagen DNeasy extraction kit with the manufacturer's suggested protocol (Qiagen) or using Serapure paramagnetic beads. We attempted to amplify three mitochondrial loci and a segment of one nuclear locus for all individuals: 16S rRNA (16S), NADH dehydrogenase subunit 4 (*ND4*), cytochrome B (*cytB*) and recombination-activating gene 1 (*Rag-1*). Amplification protocols followed those outlined in Castoe & Parkinson (2006) and Daza *et al.* (2009) using primers described in Parkinson (1999) (16S); Arévalo *et al.* (1994) (*ND4*); Burbrink *et al.* (2000) (*cytB*); and Groth & Barraclough (1999) (*Rag-1*). PCR products were sequenced on an ABI 3730XL (Applied Biosystems Inc.) by Eurofins Scientific. Sequences were visualized and edited manually in Sequencher 5.1 (Gene Codes Corp.).

These data were combined with existing sequence data retrieved from GenBank that included data for the remaining eight species of *Bothriechis* and outgroups (eight species) to create a matrix of 40 terminal taxa used for phylogenetic analyses (Appendix 1). All sequence data were aligned in MEGA v6.0 via the MUSCLE algorithm (Edgar 2004) and protein-coding sequences were checked for internal stop codons. New sequences generated are available on GenBank under the accession numbers given in Appendix 1.

We utilized three datasets for varying levels of phylogenetic reconstruction; the first used only the mitochondrial sequences and included all terminal taxa. Due to the faster evolutionary rate of mitochondrial loci, we expected this dataset to provide the most phylogenetic structure at inter- and intraspecific levels. To determine if latent nuclear structure existed within *B. nigroviridis sensu lato*, we used a second dataset that consisted of nuclear sequences and contained representatives of all *Bothriechis* species, 3 of the 5 outgroup taxa, and 13 of 21 *B. nigroviridis sensu lato* individuals. The final dataset combined mitochondrial and nuclear sequences and included all terminal taxa.

Previous studies have highlighted the importance of appropriate partitioning of datasets in generating robust phylogenetic results (Castoe *et al.* 2004; Brandley *et al.* 2005; Castoe & Parkinson 2006; Miller *et al.* 2009). Gene partitioning and nucleotide substitution models were determined using the greedy search algorithm in PartitionFinder v1.1 (Lanfear *et al.* 2012) with Bayesian Information Criterion (BIC) as the selection criteria. For all analyses gene sequences were partitioned by gene and codon position (in protein coding loci). For the combined mitochondrial-nuclear analyses, this resulted in a total of 10 partitions listed in Appendix 2.

Phylogenetic analyses were conducted using Bayesian Inference with Metropolis-Hastings coupled Markov chain Monte Carlo methods in MrBayes v3.2 with default priors, and a variable rate prior across partitions (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). MrBayes analyses consisted of two independent runs with one cold and three heated chains for 5 million generations and chains were sampled every 100 generations. The first 250,000 generations were discarded as burn-in as remaining samples produced ESS values >500 for all parameters. Stationarity of the chain was verified by plotting log-likelihood scores against generation in Tracer 1.6.0 (Rambaut *et al.* 2013).

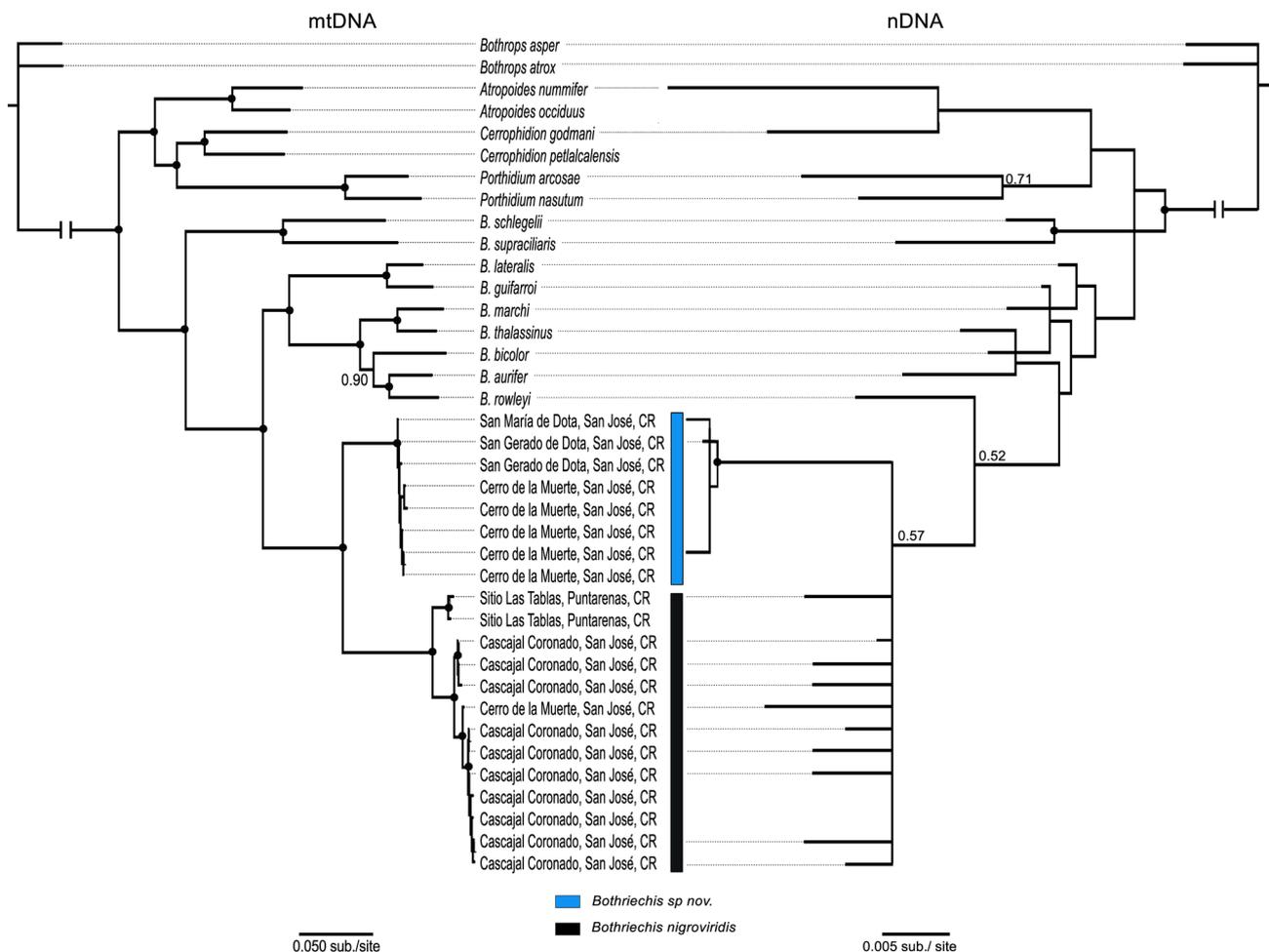
**Morphological analysis.** Preserved specimens of *Bothriechis nigroviridis sensu lato* were obtained from CAS, CM, KU, LACM, MCZ, MVZ, UCR, UMMZ, USNM, and UTA (Appendix 3). In total, 72 specimens were used in

morphological analysis, three of which (CAS 178120, CLPT 620, and UCR 11151) were also represented by tissues in the genetic analyses (Appendix 1). Morphological characters were measured with digital calipers (to the nearest 0.1 mm) or a meter stick (to the nearest mm). Scale terminology follows Gutberlet and Campbell (2001).

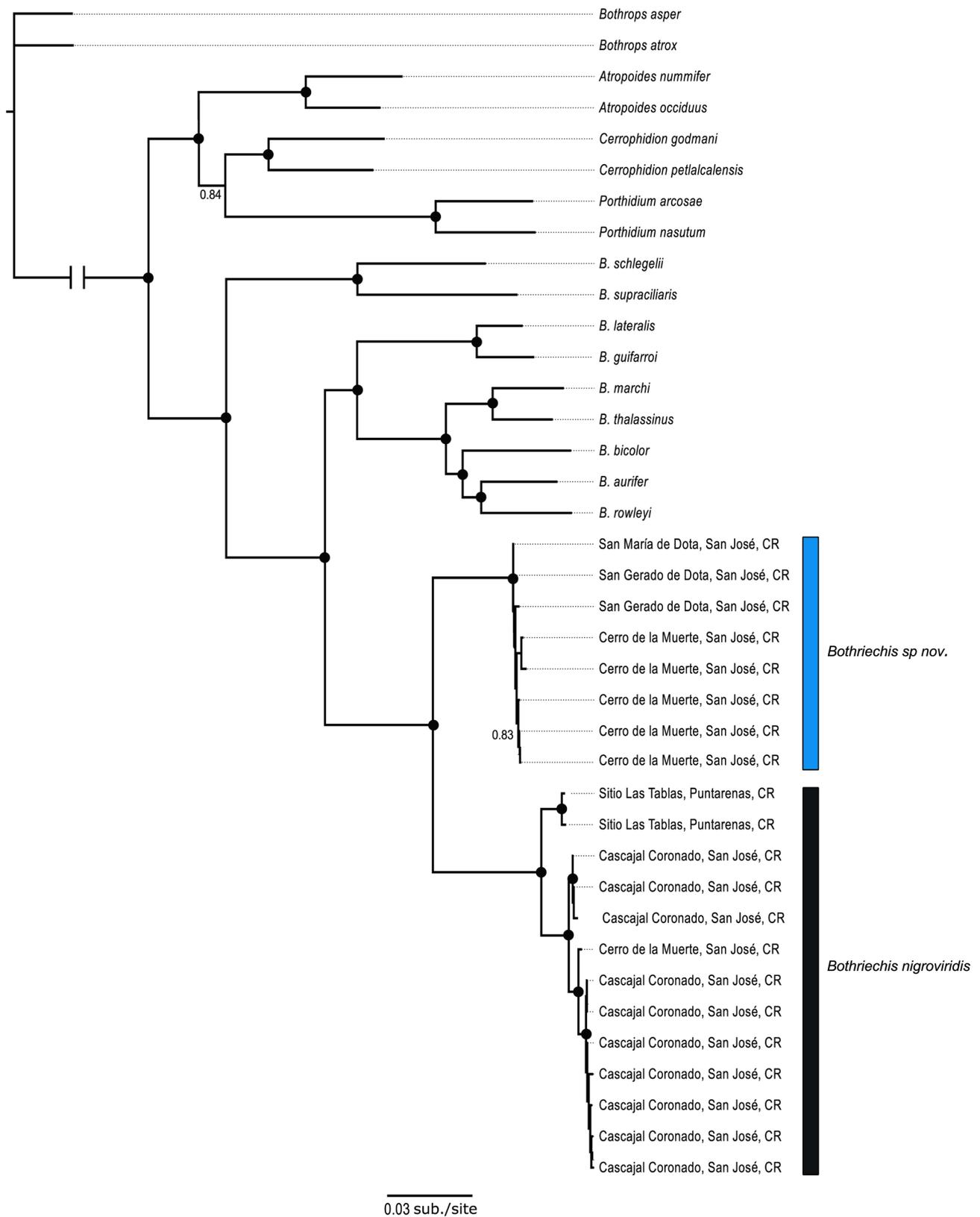
Morphological data were subjected to principal component analysis (PCA) on 15 scale characters using the `prcomp` command in R version 3.2 (R Core Team, 2015). Characters utilized in PCA included: first dorsals, second dorsals, third dorsals, ventrals, subcaudals, intercanthals, first row intersupraoculars, interriectals, interoculabials, loreals, suboculars, supralabials, infralabials, gulars (from genials to preventrals), and preventrals. Specimens for which data were missing or not applicable were omitted from analyses. Bilateral characters that were asymmetrical within an individual specimen were coded as the mean of the right and left counts. All variables were standardized to a mean of zero and unit variance prior to analysis in order to ensure equal weights.

## Results

The mitochondrial DNA dataset showed clear phylogenetic structure, including strong support for two distinct clades within *B. nigroviridis* (Fig. 1). In contrast, the nuclear DNA phylogeny displayed little phylogenetic structure (most nodes had support values < 0.50). One exception to this was the northern Talamanca Cordillera clade of *B. nigroviridis*, which was assigned very high support (posterior probability = 1) (Fig. 1). Thus, the presence of a divergent lineage within *B. nigroviridis* was detectable with both datasets.



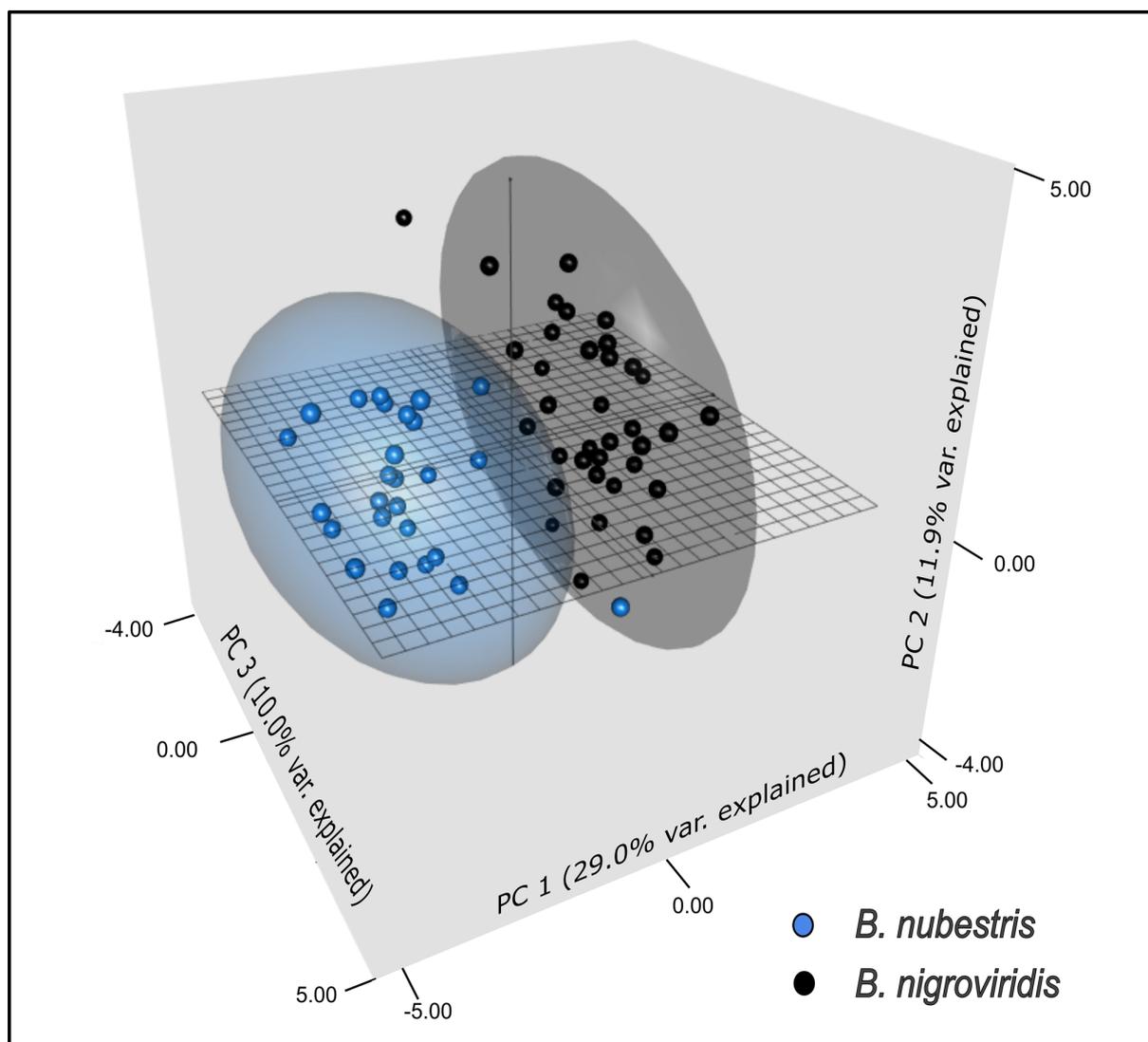
**FIGURE 1.** Phylogeny of the palm-pitviper genus *Bothriechis* based on separate mitochondrial and nuclear gene analyses. The mtDNA tree represents the Bayesian 50% majority-rule consensus resulting from analysis of three mitochondrial loci (*16S*, *cyt B*, *ND4*; 2091 base pairs total). The nDNA tree represents the Bayesian 50% majority-rule consensus resulting from analysis of the nuclear locus *Rag-1* (983 base pairs total). Posterior probabilities above 0.5 are shown; black dots represent nodes with posterior probabilities  $\geq 0.95$ .



**FIGURE 2.** Bayesian inference phylogeny of the palm-pitviper genus *Bothriechis*. The tree represents the 50% majority-rule consensus resulting from a concatenated data set of three mitochondrial loci and one nuclear locus (*16S*, *cyt B*, *ND4* and *Rag-1*; 3074 base pairs total). Posterior probabilities above 0.8 are shown; black dots represent nodes with posterior probabilities  $\geq 0.95$ .

The topology of our inferred phylogeny from the combined dataset was consistent with those of Castoe *et al.* (2009) and Daza *et al.* (2010) (Fig. 2), including a northern highland group containing ((*B. thalassinus*, *B. marchi*), (*B. bicolor*, (*B. aurifer*, *B. rowleyi*))). *Bothriechis lateralis* + *B. guifarroi* formed a clade sister to the northern highland clade, which differed from the findings of Townsend *et al.* (2013). We found *B. nigroviridis sensu lato* to comprise two deeply divergent lineages: one containing individuals from the Central Cordillera plus the Talamanca Cordillera, and the other containing individuals from the northern Talamanca Cordillera (Fig. 2). Snake samples from the middle portion of the Talamanca Cordillera (Sitio Las Tablas) clustered with *B. nigroviridis* from the Central Cordillera, though with notable genetic divergence. In contrast, snakes from the northern Talamanca Cordillera showed little genetic divergence from one another.

In total, 64 specimens were included in the PCA of meristic scale data (some of the examined specimens could not be used in the PCA because of incomplete data). The first three axes of the PCA accounted for 50.9% of the total variation in morphological characters. All variables were negatively correlated with the first principal component, which explained 29.0% of the variation. For PC1, first dorsals, second dorsals, third dorsals, interrials, and ventrals were assigned high factor loadings. The second principal component explained 11.9% of the variation, with prefrontals, supralabials, and loreals receiving high factor loadings. The third principal component explained an additional 10.0% of the variation, with intercanthals, intersupraoculars and gulars designated as high factor loadings.



**FIGURE 3.** Principal component scores of 64 specimens of *Bothriechis nigroviridis* and *B. nubestris* on the first three factors; PC1 and PC2 together explain 40.9% of the total variance and PC3 explains an additional 10.0% of the variance. *B. nigroviridis* are represented by black dots; blue dots represent the new species. Ellipses represent the 95% confidence interval for each group.

The first principal component axis (PC1) for morphological characters shows substantial separation of the same two geographically-distinct populations of *B. nigroviridis* that genetic analyses indicate are distinct lineages (Fig. 3). The second and third principal components largely captured variation occurring within each of these two distinct clusters; the variation observed across these axes was notably higher for *B. nigroviridis sensu stricto* than for the northern Talamancan snakes (Fig. 3). Thus, the northern Talamancan group appears morphologically distinct from the wide-ranging *Bothriechis nigroviridis sensu stricto* throughout the Tilarán, Central, and Talamanca Cordilleras of Costa Rica and Panama. The two forms also differ in several previously unrecognized characters, which support the presence of a second, cryptic species within the nominal *B. nigroviridis*. Accordingly, below we recognize this cryptic lineage as a new species.

## Systematic account

### Genus *Bothriechis* Peters, 1859

#### *Bothriechis nubestris* new species

Talamancan Palm-Pitviper

Figures 4–5.

*Bothriechis nigroviridis* Peters 1859:278. [In part.]

**Holotype.** UTA R-9637 (Campbell and Lamar, 2004: fig. 97B), an adult female, from San Isidro de El General, Province of San José, COSTA RICA; approximately 3000 m; collected in October 1973 by Peter Seigfried.

**Paratypes.** All from province of San José, COSTA RICA: UTA R-2801, 2808, 2850, 7327, 7463, 9635, 9636, 10433, all from San Isidro de El General District; UCR 5727, 5728, 5758, 11151, 15420, 15423, 15424, 15432, all from San Gerardo de Dota; UCR 12356, from División, Páramo District.

**Referred specimens.** From province of San José, COSTA RICA: UCR 5757, from San Gerardo de Dota; UTA R-6799, 9364, 9365, 10432, all from San Isidro de El General District; CM 145874, 148063, 148064, LACM 154553, Cerro de la Muerte near San Gerardo; UCR 16726, Providencia; UCR 15422, 15429, Alto Palma, Parque Nacional Braulio Carrillo, Bajo la Hondura (locality possibly in error); UCR 15428, Las Nubes (locality possibly in error); UCR 3951, Naranjo, Río Naranjo; UMMZ 117734, no precise locality data. From province of Cartago: MVZ 24228. From unknown Costa Rican locality: UCR 15439. From province of Limón: UCR 22428, Talamanca, Telire, Cerro Uthyum.

**Diagnosis.** (1) a medium-sized slender arboreal pitviper; (2) dorsum green with heavy black mottling; (3) iris blackish; (4) superciliary scales absent; (5) interrials 22–29; (6) supraoculars thin, usually kidney-shaped; (7) intersupraoculars 6–10; (8) partial rows or two rows of irregular scales between suboculars and supralabials; (9) infralabials 9–12; (10) first dorsals usually 21 (75.9%); (11) second dorsals usually 21 (58.6%); (12) third dorsals usually 17 (85.7%); (13) ventrals 150–160; (14) subcaudals 52–64; (15) tail prehensile.

Specimens of *Bothriechis nubestris* differ from *B. schlegelii* and *B. supraciliaris* by lacking superciliary scales (present in *B. schlegelii* and *B. supraciliaris*). *Bothriechis nubestris* differs from all other *Bothriechis* species except *B. nigroviridis* by having green dorsal coloration with heavy black mottling and a blackish iris. *Bothriechis nubestris* differs from *B. nigroviridis* (see Table 1 for summary) by the combination of having 150–160 ventral scales (136–149 in *B. nigroviridis*), thin, often kidney-shaped supraoculars with a wide intersupraocular space (*B. nigroviridis* usually have wider supraoculars, never kidney-shaped, with narrow intersupraocular space), and higher average counts of interrials, dorsals, and subcaudal scales than *B. nigroviridis*.

**Description of holotype.** An adult female; SVL 779 mm; tail length 132.2 mm, comprising 14.51% of total; head length 38.9 mm; maximum head width 23.8 mm; rostral scute broader than high (4.2 X 3.2 mm); nasal divided above and below nostril, nasal fused with first supralabial on right side; loreal 1/1, contacting canthal, upper preocular, supralacunal, prelacunal, and two prefoveals; prefoveals 6/8; subfoveals absent; postfoveals 3/4; prelacunal large, contacting third supralabial on right side, second and third supralabials on left side; sublacunals 2/2; postlacunal absent because supralacunal and second sublacunal in contact; preoculars 3/3, upper large, middle about half size of upper, lower small and rounded; suboculars 2/2, anterior scale long; postoculars 3/3; loreal pit large, directed anteriorly, center of pit located slightly below line drawn from center of eye to naris and

approximately halfway between center of eye and naris; supralabials 11/11 (including fusion of nasal with first supralabial on right side); infralabials 10/11, first pair meet posteriorly. Mental broader than long (3.6 X 2.9 mm); three pairs of chin shields flanking mental groove, first two pairs contacting infralabials; five gulars between chin shields and prementals; three prementals. Internasals 3 anteriorly; canthals 2/2, anterior above nasal, posterior contacting loreal and upper preocular; 1/2 small scales between posterior canthal and supraocular; three scales between anterior canthal pair; four scales between posterior canthal pair; superciliaries absent; supraoculars narrow, approximately three times longer than broad; intersupraoculars nine; scales in parietal region small and keeled; interrials 23. Dorsal scale rows 21-21-17; ventrals 156; cloacal entire; subcaudals 53, first divided; tail spine short, blunt, 1 ½ times as long as preceding subcaudal scale, covered by scales; tail prehensile.

**TABLE 1.** Comparison of morphological characters of *Bothriechis nigroviridis* (n = 41) and *B. nubestris* (n = 27) (some specimens could not have all characters scored because of damage). The format is Range; Mode; Mean.

| Character                  | <i>B. nigroviridis</i> | <i>B. nubestris</i>  |
|----------------------------|------------------------|----------------------|
| Dorsal scales--First Third | 17–21; 19; 18.90       | 19–23; 21; 20.93     |
| Dorsal scales--Midbody     | 17–21; 19; 18.90       | 17–23; 21; 20.21     |
| Dorsal scales--Last Third  | 15–19; 15; 15.82       | 15–17; 17; 16.71     |
| Ventral scales             | 136–149; 141; 142.73   | 150–160; 155; 154.64 |
| Subcaudal scales           | 48–64; 50; 52.79       | 52–64; 59; 57.41     |
| Supralabials               | 9–11; 10; 9.76         | 9–11; 10; 9.62       |
| Intersupraoculars          | 3–10; 6; 6.34          | 6–10; 8; 7.83        |
| Interrictals               | 19–26; 22; 21.90       | 22–29; 24; 25.07     |

**Coloration in preservative.** Dorsum of head and body black and pale green mottled; dorsal surface of tail less mottled, grey-green speckled with black, last third yellowish brown lightly speckled with black; postocular stripe extending to rictus, above stripe faint black longitudinal striping, below stripe yellowish with some black blotches on upper lip; ventral surface of head and body yellow, becoming more yellowish green posteriorly. Subcaudal region grey-green with black speckling fading to brown with faint black speckling near tip. Coloration in life of holotype unknown.

**Variation.** Dorsals were most commonly arranged in 21–21–17 (42.9% of specimens), whereas each of the following combinations were also observed: 23–23–17, 23–21–17, 21–21–15, 21–20–17, 21–19–17, 21–19–15, 19–21–17, 19–19–17, 19–17–17, and 19–17–15.

Ventral scales range 151–157 (mean = 153.7) in males and 152–160 in females (mean = 155.4). Subcaudal scales range 53–62 (mean = 58.6) in males and 52–64 (mean = 56.2) in females. Tail length comprises 14–17% (mean = 15.2%) of total length in males; tail comprises 14–16% (mean = 15.0%) of total length in females.

Head scale variation is as follows: prefoveals 4–7; subfoveals 0–1 (present in one specimen only, all others lack subfoveals); postfoveals 1–4; suboculars 1–4; postoculars 2–3; supralabials 9–11; infralabials 9–12; internasals 2–4; intercanthals 4–7; interrials 22–29 (Table 1).

Coloration in preservative varied among specimens and consisted of two rather distinct morphs, with few specimens showing intermediate coloration. Twenty-six percent of the specimens had nearly identical coloration to the holotype. Sixty percent of the specimens displayed a darker color morph with dorsal green being replaced by dark grey and the yellowish venter being replaced by beige, fading to grey-green on the tail. The same ventral pattern is present in both morphs but the yellow is replaced by beige in the darker morph. In two specimens, the dorsum is the “darker morph” but the venter is like the holotype’s with yellow. Recently-born juveniles had brown and black dorsums with beige-brown venters and the tail tips were completely brownish yellow. Coloration in life of three known specimens is nearly identical to the holotype coloration in preservative.

**Hemipenes.** Paratype UCR 11151 had fully everted hemipenes. The total length of the right hemipenis was equal to the length of the first five subcaudal scales. The two lobes of the organ bifurcate at an approximate length of two subcaudals and the sulcus spermaticus divides at a distance of approximately ½ the length of a subcaudal. The base of the organ is spinous, whereas the distal portion is covered in calyces. Spines are dense and evenly dispersed; some spines are quite large. Calyces begin approximately 1 ½ subcaudal scale lengths from the tip of the organ.



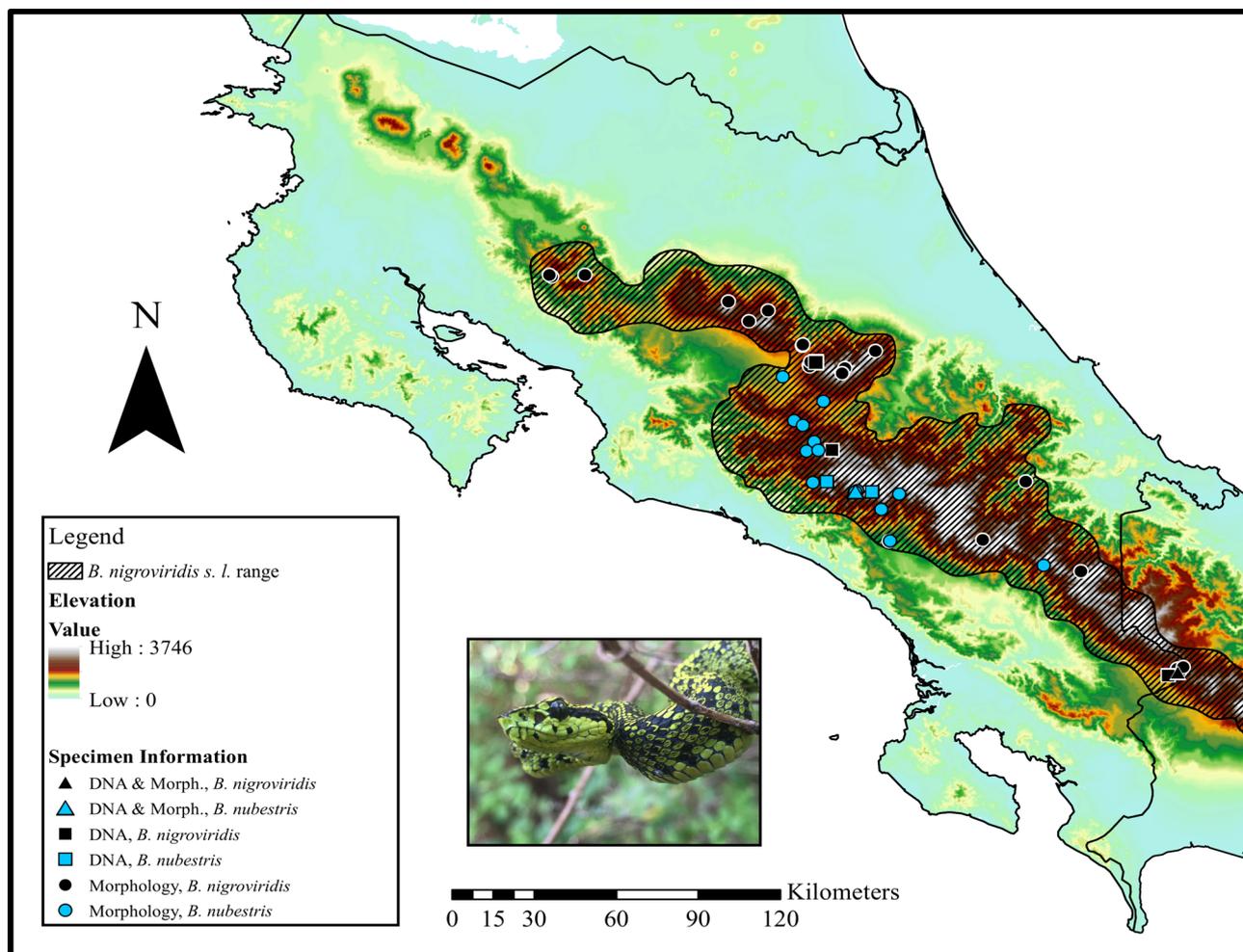
**FIGURE 4.** Photographs of *Bothriechis nubestris* sp. n. in life. Top photographer: John Tashjian; middle and bottom photographer Wayne van Devender.



**FIGURE 5.** Dorsal and lateral views of the head of male paratype UCR 11151 from San Gerardo, Costa Rica. Scale bar = 20 mm. The diagnostic kidney-shaped supraoculars and partial scale rows between the supralabials and suboculars are highlighted.

**Distribution and natural history.** *Bothriechis nubestris* is known from the northern and central portions of the Cordillera de Talamanca of Costa Rica in the provinces of San José, Cartago, and Limón (see Fig. 6). Three specimens (UCR 15422, 15428, 15429) have locality data that indicate they are from the Cordillera Central, but they were snakes donated by locals to the Instituto Clodomiro Picado and it is possible that the locality data were recorded in error. We exclude these localities from consideration here, but acknowledge that if these localities are correct, the species also occurs in the Cordillera Central. The species has been recorded from 2400 m on Cerro de la Muerte to over 3000 m in San Gerardo de Dota. *Bothriechis nubestris* is an arboreal species usually found in the transition zone between cloud forest and montane rainforest.

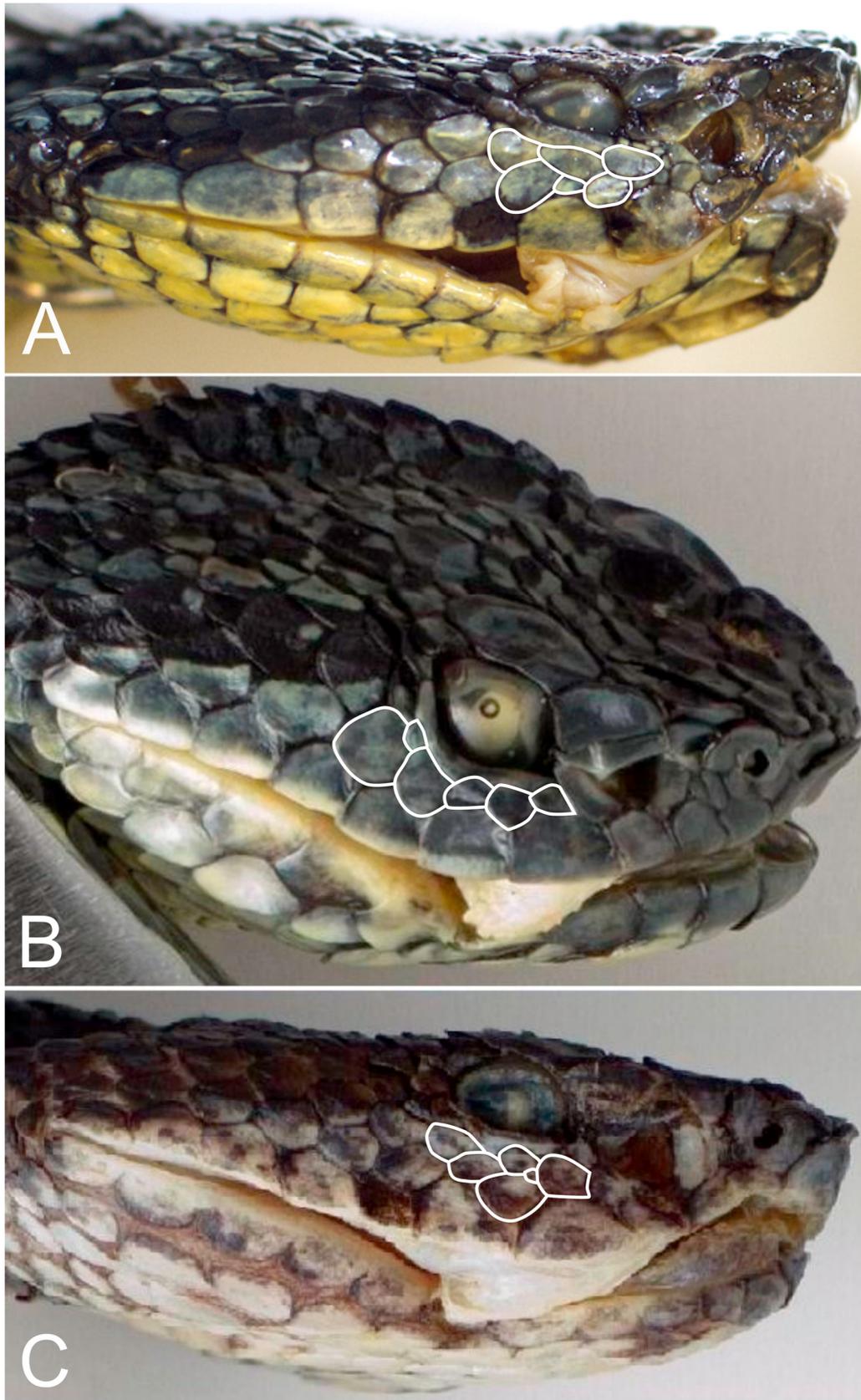
**Etymology.** The specific epithet means ‘belonging to the clouds’. It is derived from the Latin noun *nubes*, *-is*, meaning cloud, and the Latin suffix *-estris*, meaning belonging to. This name alludes to the fact that this species inhabits cloud forests. The common name Talamancan Palm-Pitviper refers to its range in the Cordillera de Talamanca.



**FIGURE 6.** Topographical map indicating localities of *Bothriechis nigroviridis* (black symbols) and *B. nubestris* sp. n. (blue symbols) used in this study: 72 used in morphological analyses (circles), 21 used in genetic analyses (squares), and 3 used in both (triangles).

## Discussion

**Evolution and Biogeography.** Evidence from phylogenetic and morphological analyses corroborated the existence of a previously unrecognized species, *Bothriechis nubestris*, which is closely related to, but morphologically and genetically distinct from, *B. nigroviridis*. The divergence in the *B. nubestris*+*B. nigroviridis* clade suggests that a common ancestor of this lineage may have once spanned the Talamanca and Central Cordilleras, and underwent a deep ancient divergence that led to endemic diversification of these two lineages in these two montane regions. Our phylogeny also recovered a *B. lateralis*+*B. guifarroi* clade sister to the northern Middle American highland *Bothriechis*, in contrast to a *B. nigroviridis*+*B. lateralis*+*B. guifarroi* clade recovered by Townsend *et al.* (2013), and supports inferences of Castoe *et al.* (2009) and Daza *et al.* (2010) of a Mid-Late Miocene divergence of the northern and southern Middle American highland groups.



**FIGURE 7.** A–C. Variation among interocular scales between supralabials and suboculars. (A) *Bothriechis nubestris* (UCR 12356) displaying two complete rows of interocular scales between supralabials and suboculars. (B) *Bothriechis nigroviridis* (UCR 16518) displaying a single row of scales between supralabials and suboculars. (C) *Bothriechis nigroviridis* (UCR 15428) displaying partial rows of scales between supralabials and suboculars.

The phylogeographic structure of *B. nigroviridis sensu lato* is of particular interest as they are primarily restricted to montane cloud forests and relatively high elevations in Costa Rica and northern Panama (Savage 2002; Campbell & Lamar 2004), a region that contains a considerable number of endemic taxa (García-Paris *et al.* 2000; Savage & Lahanas 2008; García-Rodríguez *et al.* 2012). The rise of the Talamanca, Central, and Tilarán Cordilleras has been implicated in the divergence of several native montane taxa, which may have “ridden” the mountains during their rapid uplift, promoting *in situ* speciation (Savage 1982; García-Paris *et al.* 2000; Bagley & Johnson 2014). The high peaks of the Talamanca Cordillera may also act as “sky-islands” separated by diverse lowland habitats, which may have promoted speciation in some groups while providing refuge during climatic and glacial cycles for others (Savage 1982, 2002; Bagley & Johnson 2014). Although no instances of sympatry between the two species have been recorded, the ranges of *B. nigroviridis* and *B. nubestris* appear to reach close proximity in the southern portions of the San José and Limón provinces. It is an open question as to whether the two lineages diverged in allopatry in the two mountain ranges, or whether ecological specialization and divergence may have contributed to divergence and parapatric speciation.

Another alternative hypothesis is that the observed phylogeographic structure of this group is a result of differential dispersal across the landscape. Little is known about the dispersal abilities of the *B. nigroviridis* and *B. nubestris*, but anecdotal evidence suggests that they may be environmentally sensitive as they quickly disappear from degraded habitat (Campbell & Lamar 2004). Thus, the dispersal of these species may largely depend on the availability of suitable habitat. The Talamanca Cordillera exhibits great environmental heterogeneity across its range, including variable patterns of climate and plant regimes. This heterogeneity has been implicated in the speciation of other groups, especially environmentally sensitive amphibian species, by limiting dispersal and gene flow (García-Paris *et al.* 2000; Streicher *et al.* 2009). If the environmental heterogeneity of the region facilitated the dispersal of *B. nigroviridis* along the Atlantic slopes of the Talamanca Cordillera while limiting southern dispersal of *B. nubestris*, then we would expect evolutionary relationships similar to those produced by our phylogenetic analysis.

Unfortunately, the forces governing speciation of this group currently remain enigmatic. While this work represents the largest study of the *B. nigroviridis*+*B. nubestris* group to date, parts of these species ranges are poorly sampled and many localities are not represented in our analyses. This paucity of information across the groups’ range constrains our scope of inference and leaves many questions unanswered. Further sampling throughout the range of both species may shed light on their evolutionary history and speciation processes in the future.

**Variation within *B. nigroviridis*.** In addition to discovering the cryptic species within *B. nigroviridis* we examined the variation within *B. nigroviridis sensu stricto*. The species has been recorded in all of the provinces of Costa Rica and the Chiriquí and Bocas del Toro provinces of Panama. These snakes range through the Tilarán, Central, and Talamanca Cordilleras of Costa Rica and Panama. A single specimen was listed by Savage (2002) as occurring in the Guanacaste Cordillera, but its locality data are questionable and the specimen appears to have been lost, so we exclude it from the known range of the species. Although *B. nigroviridis* is easily diagnosable through a variety of characters, some interesting variation exists within the species. Compared to its sister species *B. nubestris*, *B. nigroviridis* displays much greater morphological and molecular variation. One character that displays substantial variation is the number of interocular rows between the supralabials and suboculars. *Bothriechis nubestris* always has either partial scale rows, as in the holotype (Fig. 5), or two complete scale rows (Fig. 7A). *Bothriechis nigroviridis* most often has a single complete scale row between the supralabials and suboculars (Fig. 7B), but some specimens have partial rows or two rows, as in *B. nubestris* (Fig. 7C).

As discussed above, specimens for which we have molecular data from extreme southeastern Costa Rica diverge from the other specimens from the Central Cordillera. However, morphologically these specimens match well with the other specimens of *B. nigroviridis*, but they are two of the four specimens we encountered with irregular scale rows between the suboculars and supralabials (which are otherwise indicative of *B. nubestris*). On the other hand, the three specimens examined from the Cordillera de Tilarán have high numbers of subcaudals. In addition, a single specimen from Chiriquí, Panama has the unique combination of 19-19 dorsal scales, with no reduction along the body. It appears that snakes from the northern (Tilarán) and southern extents of the *B. nigroviridis* range in the Talamancas have exceptional morphological character states. The much wider geographic range of *B. nigroviridis* correlates with its wider range of character states, whereas the small geographic range of *B. nubestris* demonstrates little morphological or molecular divergence. These trends of morphological and molecular

variation, along with the geographic distribution data known for these two species, suggest that the effective population sizes of these two sister species are quite different, with *B. nigroviridis* having substantially larger populations than *B. nubestris*.

**Conservation.** This description of *Bothriechis nubestris* is part of a growing body of work identifying cryptic lineage diversity in a long established group. Recognition of cryptic species has become increasingly common with the spread of molecular-based phylogeographic studies (Martin & Bermingham 2000; Sanders *et al.* 2006; Venegas-Anaya *et al.* 2008). This has been especially true in Middle American taxa where the dynamic geologic history and climate have limited dispersal and caused frequent vicariance and isolation of many taxa (Venegas-Anaya *et al.* 2008; Köhler *et al.* 2010; Jadin *et al.* 2012; Bagley & Johnson 2014). Identification of these unique genetic lineages is vital to conservation efforts as unrecognized instances of cryptic speciation cause underestimations of true biological diversity and result in inadvertently harmful conservation policies (Bickford *et al.* 2007). This is particularly true in the case of *B. nubestris*, which is naturally uncommon, occupies a restricted range, and may be particularly sensitive to habitat loss and degradation. These characteristics predispose *B. nubestris* to a high risk of extinction, and thus measures to preserve this species should be a high priority.

**Medical implications.** Like other *Bothriechis* species, *B. nubestris* is a venomous viperid snake and therefore is medically relevant. However, marked variations in venom composition and function are known to exist among even closely related and cryptic species (Sanders *et al.* 2006). Comparative proteomics of other Costa Rican *Bothriechis* (*B. schlegelii*, *B. lateralis*, and *B. nigroviridis*) venoms has shown high variability in the venom composition of these species (Fernandez *et al.* 2010). The venom of *B. nigroviridis* is of particular interest as it displays high neurotoxicity, largely a result of a crotoxin-like PLA2 (nigroviriditoxin), which is the first instance of a toxin of this type in a New World viperid species outside of rattlesnakes (Lomonte *et al.* 2015).

Whether *B. nubestris* venom exhibits a proteomic profile and biological activity similar to that of *B. nigroviridis* remains to be tested. Given the diversity observed in venoms of other closely related *Bothriechis* species and the unexplained venom variation observed in several other pitviper species, it is possible that *B. nubestris* venom is notably divergent from that of *B. nigroviridis*. Investigation into the composition and function of *B. nubestris* venom phenotype could potentially yield valuable insight into venom evolution and adaptation.

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**APPENDIX 1.** Localities, voucher numbers, and GenBank accession numbers for taxa used in the molecular analysis. Novel sequences are shown in bold. Country codes are as follows:

CR= Costa Rica, EC=Ecuador, GT=Guatemala, HN= Honduras, MX= Mexico.

| Taxon                           | Locality  | Voucher           | GenBank Accession Numbers |                 |                 |  | Rag-1           |
|---------------------------------|---|-------------------|---------------------------|-----------------|-----------------|--|-----------------|
|                                 |   |                   | 16S                       | cyt b           | ND4             |  |                 |
| <i>Atropoides nummifer</i>      | MX: San Andres Tziuluan, Puebla                           | ENS 10515         | DQ305445                  | DQ61195         | DQ061220        |  | <b>KU234642</b> |
| <i>Atropoides occidius</i>      | MX: Finca El Rosario Vista Hermosa, UTA-R-29680 Escuintla | UTA-R-29680       | DQ305446                  | AY220315        | AY220338        |  | -               |
| <i>Bothriechis aurifer</i>      | GT  | UTA-R35031        | DQ305448                  | DQ305466        | DQ305483        |  | <b>KU234643</b> |
| <i>Bothriechis bicolor</i>      | GT  | UTA-R34156        | DQ305449                  | DQ305467        | DQ305484        |  | -               |
| <i>Bothriechis bicolor</i>      | -   | DPL 2899          | -                         | -               | -               |  | <b>KU234644</b> |
| <i>Bothriechis guifarroii</i>   | HN: Texiguat, Atlántida                                   | USNM 579873       | KC847262                  | KC847274        | KC847286        |  | <b>KU234639</b> |
| <i>Bothriechis guifarroii</i>   | HN: Texiguat, Atlántida                                   | USNM 579874       | KC847263                  | KC847282        | KC847288        |  | <b>KU234640</b> |
| <i>Bothriechis guifarroii</i>   | HN: Texiguat, Atlántida                                   | USNM 579875       | KC847264                  | KC847281        | KC847287        |  | <b>KU234641</b> |
| <i>Bothriechis lateralis</i>    | CR: Acosta, San José                                      | MZUCR11155        | AF057258                  | AY223588        | U41873          |  | <b>KU234645</b> |
| <i>Bothriechis marchi</i>       | HN: Cerro del Mono, Zacapa                                | UTA-R52959        | DQ305451                  | DQ305469        | DQ305486        |  | -               |
| <i>Bothriechis marchi</i>       | -   | San Antonio Zoo 5 | -                         | -               | -               |  | <b>KU234646</b> |
| <i>Bothriechis nigroviridis</i> | CR: Cascajal de Coronado, San José                        | ICP-1043          | -                         | <b>KU203333</b> | -               |  | -               |
| <i>Bothriechis nigroviridis</i> | CR: Cascajal de Coronado, San José                        | ICP-1063          | -                         | <b>KU203332</b> | <b>KU215604</b> |  | <b>KU234626</b> |
| <i>Bothriechis nigroviridis</i> | CR: Cascajal de Coronado, San José                        | ICP-1064          | <b>KU176464</b>           | <b>KU203335</b> | <b>KU215605</b> |  | <b>KU234627</b> |
| <i>Bothriechis nigroviridis</i> | CR: Cascajal de Coronado, San José                        | ICP-1066          | <b>KU176465</b>           | <b>KU203336</b> | <b>KU215606</b> |  | <b>KU234628</b> |
| <i>Bothriechis nigroviridis</i> | CR: Cascajal de Coronado, San José                        | ICP-1067          | <b>KU176466</b>           | <b>KU203337</b> | <b>KU215607</b> |  | <b>KU234629</b> |
| <i>Bothriechis nigroviridis</i> | CR: Cascajal de Coronado, San José                        | ICP-1068          | <b>KU176467</b>           | <b>KU203338</b> | <b>KU215608</b> |  | -               |
| <i>Bothriechis nigroviridis</i> | CR: Cascajal de Coronado, San José                        | ICP-1065          | <b>KU176468</b>           | <b>KU203339</b> | <b>KU215609</b> |  | <b>KU234630</b> |
| <i>Bothriechis nigroviridis</i> | CR: Cerro de la Muerte, San José                          | CLPT 616          | <b>KU176476</b>           | <b>KU203348</b> | <b>KU215616</b> |  | <b>KU234637</b> |
| <i>Bothriechis nigroviridis</i> | CR: Las Tablas, Coto Brus, Puntarenas                     | CLPT 617          | <b>KU176477</b>           | <b>KU203349</b> | <b>KU215617</b> |  | <b>KU234638</b> |
| <i>Bothriechis nigroviridis</i> | CR: Cascajal de Coronado, San José                        | CLPT 624          | <b>KU176474</b>           | <b>KU203345</b> | <b>KU215615</b> |  | <b>KU234635</b> |
| <i>Bothriechis nigroviridis</i> | CR: Cascajal de Coronado, San José                        | CLPT 626          | <b>KU176469</b>           | <b>KU203341</b> | <b>KU215610</b> |  | <b>KU234631</b> |
| <i>Bothriechis nigroviridis</i> | CR: Cascajal de Coronado, San José                        | CLPT 628          | <b>KU176471</b>           | <b>KU203343</b> | <b>KU215612</b> |  | <b>KU234633</b> |
| <i>Bothriechis nigroviridis</i> | CR: Las Tablas, Puntarenas                                | CAS 178120        | <b>KU176472</b>           | <b>KU203344</b> | <b>KU215613</b> |  | -               |

.....continued on the next page

APPENDIX 1. (Continued)

| Taxon                               | Locality                              | Voucher     | GenBank Accession Numbers |          |          | Rag-1    |
|-------------------------------------|---------------------------------------|-------------|---------------------------|----------|----------|----------|
|                                     |                                       |             | 16S                       | cyt b    | ND4      |          |
| <i>Bothriechis nubestris</i> sp. n. | CR: Cerro de la Muerte, San José      | CLPT 618    | -                         | KU203349 | KU215601 | -        |
| <i>Bothriechis nubestris</i> sp. n. | CR: Cerro de la Muerte, San José      | CLPT 620    | KU176473                  | KU203347 | KU215602 | KU234634 |
| <i>Bothriechis nubestris</i> sp. n. | CR: Cerro de la Muerte, San José      | CLPT 621    | -                         | KU203331 | KU215603 | -        |
| <i>Bothriechis nubestris</i> sp. n. | CR: Cerro de la Muerte, San José      | CLPT 622    | KU176463                  | KU203334 | KU215600 | -        |
| <i>Bothriechis nubestris</i> sp. n. | CR: Cerro de la Muerte, San José      | CLPT 623    | -                         | KU203340 | -        | -        |
| <i>Bothriechis nubestris</i> sp. n. | CR: San Gerardo de Dota, San José     | CLPT 627    | KU176470                  | KU203342 | KU215611 | KU234632 |
| <i>Bothriechis nubestris</i> sp. n. | CR: Santa Maria de Dota, San José     | CLPT 625    | KU176475                  | KU203346 | KU215614 | KU234636 |
| <i>Bothriechis nubestris</i> sp. n. | CR: San Gerardo de Dota, San José     | MZUCR 11151 | AF057259                  | AY223589 | AY223635 | -        |
| <i>Bothriechis rowleyi</i>          | MX: Cerro El Baúl, Oaxaca             | UTA R-22243 | DQ305450                  | DQ305468 | DQ305485 | KU234647 |
| <i>Bothriechis schlegelii</i>       | CR: Cariblanco de Sarapiquí, Heredia  | MZUCR 11149 | AF057260                  | AY223590 | AY223636 | KU234648 |
| <i>Bothriechis supraciliaris</i>    | CR: San Vito de Coto Brus, Puntarenas | -           | DQ305452                  | DQ305470 | DQ305487 | KU234649 |
| <i>Bothriechis thalassinus</i>      | GT: Zacapa                            | UTA-52958   | DQ305447                  | DQ305465 | DQ305482 | -        |
| <i>Bothriechis thalassinus</i>      | GT: Izabal                            | UTA-46526   | -                         | -        | -        | KU234650 |
| <i>Bothrops asper</i>               | CR: Quebrada Ganado, Puntarenas       | MZUCR 11152 | AF057265                  | AY223599 | U41876   | KU234651 |
| <i>Bothrops atrox</i>               | -                                     | WWW-743     | AY223672                  | AY223598 | AY223641 | KU234652 |
| <i>Cerrophidion godmani</i>         | CR: Las Nubes de Coronado, San José   | MZUCR 11153 | AF057250                  | AY223578 | U41879   | KU234653 |
| <i>Cerrophidion petlalcalensis</i>  | MX: Orizaba, Veracruz                 | ENS10528    | DQ305443                  | DQ061202 | DQ061227 | -        |
| <i>Porthidium arcosae</i>           | EC                                    | WWW750      | AY223668                  | AY223582 | AY223631 | KU234654 |
| <i>Porthidium nasutum</i>           | CR: Penshurst, Limón                  | MZUCR11150  | AF057251                  | AY223579 | U41887   | KU234655 |

**APPENDIX 2.** Partitioning scheme determined *a priori* for *Bothriechis* phylogenetic analysis. Determined in PartitionFinder v1.1.

| <i>Bothriechis</i> Phylogeny Partition | Number of Characters | AIC Model       |
|--|----------------------|-----------------|
| 16S                                    | 521                  | HKY+I+ $\Gamma$ |
| Cyt B position 1                       | 237                  | HKY+I+ $\Gamma$ |
| Cyt B position 2                       | 237                  | HKY+I+ $\Gamma$ |
| Cyt B position 3                       | 237                  | GTR+I+ $\Gamma$ |
| ND4 position 1                         | 286                  | HKY+I+ $\Gamma$ |
| ND4 position 2                         | 286                  | HKY+I+ $\Gamma$ |
| ND4 position 3                         | 286                  | GTR+I+ $\Gamma$ |
| Rag-1 position 1                       | 328                  | HKY+ $\Gamma$   |
| Rag-1 position 2                       | 328                  | HKY+ $\Gamma$   |
| Rag-1 position 3                       | 327                  | HKY+ $\Gamma$   |

### APPENDIX 3. Specimens Examined.

*Bothriechis nigroviridis*: COSTA RICA: Alajuela: San Juan, Sabanilla: UCR 15427, Brisas, Santa Rosa: UCR 16518, Volcán Poas: UTA R-21852; Cartago: Santa Cruz de Turrialba, B. de Bonilla: UCR 3079, Casamata: UCR 8098, Cerro de la Muerte, 3.2 Km S El Empalme: LACM 154554, El Empalme: UTA R-24841, Patio de Agua: UCR 15446, Volcán Irazú: UTA R-16075, UTA R-16077–16078, UCR 15425–15426; Heredia: Vara Blanca: UTA R-21926, Santo Domingo del Roble, Los Cartagos: UCR 14344, Vara Blanca, Parque Nacional Braulio Carrillo: UCR 13409; Limón: Río Telire: UCR 15444, Cerro Kamuk: USNM 32580–32581, no further locality data: UMMZ 131330; Talamanca: Telire: Valle del Dúrika: UCR 21679, UCR 21811; San José: Alto Palma: UCR 15431, UCR 15433, UCR 15445, Bajo La Hondura: UTA R-21853, Cascajal de Coronado: UTA R-21851, UCR 15421, Las Nubes: UCR 15430, UCR 15452, San Pedro de Dota, Quebradilla: UCR 6325, Piedra Alta, Estación Biológica Cuerici: UCR 11080; Puntarenas: Sabalito, Cerro Pando UCR 8276, Monteverde Cloud Forest Reserve: MVZ 215983, LACM 154555, UTA R-32458, Sabalito, Sitio Las Tablas: CAS 178120, LACM 154552, UCR 10876. PANAMA: Bocas del Toro: N slope Cerro Pando: KU 112598; Chiriquí: Chiriquí-Viejo River: MCZ R-39655, no further locality data: UMMZ 147776.

*Bothriechis nubestris*: COSTA RICA: Cartago: specific locality unknown: MVZ 24228; Limón: Talamanca: Telire: Cerro Uthyum 2 km S: UCR 22428; San José: specific locality unknown: UMMZ 117734; Cerro de la Muerte near San Gerardo: CM 145874, CM 148063–148064, LACM 154553; Páramo: División: UCR 12356; San Gerardo: UCR 5727–5728, UCR 5757–5758, UCR 11151, UCR 15420, UCR 15423–15424, UCR 15432, CM 145874, CM 148063–148064; San Isidro de El General: UTA R-2801, UTA R-2808, UTA R-2850, UTA R-6799, UTA R-7327, UTA R-7463, UTA R-9634–9637, UTA R-10432–10433; Santa María de Dota, Naranjo, Río Naranjo: UCR 3951; Providencia de Dota: UCR 16726; Bajo la Hondura, Alto Palma, Parque Nacional Braulio Carrillo (possibly in error, actual locality uncertain): UCR 15422, UCR 15429; Las Nubes (possibly in error, actual locality uncertain): UCR 15428; Unknown locality: UCR 15439.