



Genetic divergence and diversity in the Mona and Virgin Islands Boas, *Chilabothrus monensis* (*Epicrates monensis*) (Serpentes: Boidae), West Indian snakes of special conservation concern



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ABSTRACT

Habitat fragmentation reduces the extent and connectivity of suitable habitats, and can lead to changes in population genetic structure. Limited gene flow among isolated demes can result in increased genetic divergence among populations, and decreased genetic diversity within demes. We assessed patterns of genetic variation in the Caribbean boa *Chilabothrus monensis* (*Epicrates monensis*) using two mitochondrial and seven nuclear markers, and relying on the largest number of specimens of these snakes examined to date. Two disjunct subspecies of *C. monensis* are recognized: the threatened *C. m. monensis*, endemic to Mona Island, and the rare and endangered *C. m. granti*, which occurs on various islands of the Puerto Rican Bank. Mitochondrial and nuclear markers revealed unambiguous genetic differences between the taxa, and coalescent species delimitation methods indicated that these snakes likely are different evolutionary lineages, which we recognize at the species level, *C. monensis* and *C. granti*. All examined loci in *C. monensis* (sensu stricto) are monomorphic, which may indicate a recent bottleneck event. Each population of *C. granti* exclusively contains private mtDNA haplotypes, but five of the seven nuclear genes assayed are monomorphic, and nucleotide diversity is low in the two remaining markers. The faster pace of evolution of mtDNA possibly reflects the present-day isolation of populations of *C. granti*, whereas the slower substitution rate of nuDNA may instead mirror the relatively recent episodes of connectivity among the populations facilitated by the lower sea level during the Pleistocene. The small degree of overall genetic variation in *C. granti* suggests that demes of this snake could be managed as a single unit, a practice that would significantly increase their effective population size.

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1. Introduction

Threatened and endangered species are often characterized by isolated, small and/or declining demes (Frankham et al., 2009). Habitat fragmentation, whether due to natural or anthropogenic factors, is among the most important causes of population and species declines and extinctions (Gibson et al., 2013; Wilcox and Murphy, 1985). Fragmentation reduces the extent and connectivity of suitable habitats, and thus can affect the sizes of local

populations, their demography, migration rates, and the geographic distribution of demes (Groom et al., 2006; Moreno-Arias and Urbina-Cardona, 2013; Pérez-Espona et al., 2012). Consequently, a frequent outcome of habitat fragmentation is reduced population size and increased isolation of conspecific demes (Gottelli et al., 2013).

Habitat fragmentation can also lead to pronounced changes in population genetic structure. Limited gene flow among isolated demes and small effective population sizes often result in increased genetic divergence among populations, and decreased genetic diversity within demes (Chan et al., 2005; Draheim et al., 2012; Joyce and Pullin, 2003; Lacy, 1997). Low genetic diversity can further lead to diminished population fitness, due to faster fixation of deleterious mutations and reduced evolutionary potential, including the ability of demes to evolve to cope with

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environmental change caused by either native or introduced competitors, parasites, predators, new or evolved diseases, global climate change, or other factors (Avolio et al., 2012; Frankham and Kingsolver, 2004; Lacy, 1997; Ouborg, 2010; Tolson, 1991).

The herpetofauna of the West Indies includes a small radiation of morphologically and ecologically diverse, endemic boid snakes. These snakes have traditionally been placed in the genus *Epicrates* Wagler 1830, together with the *Epicrates cenchria* complex from Central and South America (McDiarmid et al., 1999). However, recent studies using molecular sequence data indicated that *Epicrates* is paraphyletic with respect to the genus *Eunectes* Wagler 1830 (South American anacondas; Burbrink, 2005; Noonan and Chippindale 2006; Rivera et al., 2011). A detailed systematic assessment of the West Indian boas confirmed this finding (Reynolds et al., 2013), and led the authors to propose restricting *Epicrates* to the five continental species (Passos and Fernandes, 2008; Rivera et al., 2011), and transferring the ten currently recognized Caribbean lineages to the genus *Chilabothrus* Duméril and Bibron 1844, a nomenclatural recommendation that we herein follow.

Chilabothrus monensis (Zenneck 1898) is a small (usually <1 m snout-to-vent length), nocturnal, semi-arboreal snake that has a fragmented distribution on Mona Island and the Puerto Rican Bank, in the eastern Caribbean Sea. The Puerto Rican Bank comprises the Greater Antillean island of Puerto Rico, its outlying keys and islands (of which the largest are Vieques and Culebra), the United States Virgin Islands (Saint Thomas, Saint John; but not Saint Croix, which belongs to the Saint Croix Bank), the British Virgin Islands (Tortola, Virgin Gorda, Anegada), and more than 180 associated small islets and cays (Heatwole and MacKenzie, 1967; Thomas, 1999). Two disjunct subspecies of *C. monensis* are traditionally recognized. The Mona Island Boa, *Chilabothrus m. monensis* (Zenneck 1898; Fig. 1A), is endemic to Mona, a small (55 km²) island situated 66 km west of Puerto Rico, in the Mona Passage, a ca. 130 km wide strait between Hispaniola on the west and Puerto Rico on the east (Fig. 2). Mona is not part of the Puerto Rican Bank, and has never been connected to Puerto Rico (Heatwole and MacKenzie, 1967). However, its herpetofauna is phylogenetically related to Puerto Rican forms and thus the product of overwater colonization events from the east after Mona became emergent (Grazziotin et al., 2012; Rivero, 1998; Rodríguez-Robles et al., 2007; Thomas, 1999; Williams, 1969). The Virgin Islands Boa (sometimes called the Puerto Rican Bank Tree Boa), *Chilabothrus m. granti* (Stull 1933; Fig. 1B), is known to occur in a single locality in northeastern Puerto Rico, and on various cays and islands of the Puerto Rican Bank, namely Cayo Diablo, Culebra, Saint Thomas, Jost van Dyke, Tortola, Great Camanoe, and perhaps Guana Island (Mayer, 2012; Fig. 2). All these islands have been periodically connected into a single landmass during glacial periods (when eustatic sea level was more than 100 m below its present level), and fragmented during interglacial periods of the Quaternary (2.6 Mya – present; Barker et al., 2012; Heatwole and MacKenzie, 1967; Fig. 2). These sea level oscillations likely produced changes in the size, configuration, and isolation of terrestrial habitats that affected the connectivity of *C. m. granti* populations.

The taxonomic status of the Mona Island Boa and the Virgin Islands Boa is unclear. The two taxa have been traditionally considered different subspecies since the first detailed systematic revision of the genus *Epicrates* (Sheplan and Schwartz, 1974). Nevertheless, the snakes exhibit noticeable phenotypic and behavioral differences (Schwartz and Henderson, 1991; Sheplan and Schwartz, 1974; pers. observ.), and some authors have recently treated them as different species (Mayer, 2012; Platenberg and Harvey, 2010). Morphological, behavioral, and ecological criteria used to distinguish allopatric species generally serve as proxies for reproductive potential or lineage status, and are therefore



(A)



(B)

Fig. 1. (A) Adult female *Chilabothrus monensis monensis* from Mona Island. Photograph by Peter J. Tolson. (B) Adult female *Chilabothrus monensis granti* from Tortola, British Virgin Islands. Photograph by Father Alejandro J. Sánchez Muñoz.

difficult to measure objectively, which makes delimiting allopatric species a challenging task (Fujita et al., 2012). Coalescent species delimitation methods reduce the inherent subjectivity required by the application of such proxies, and provide objective, robust, replicable measures for identifying distinct evolutionary lineages (Fujita et al., 2012; Myers et al., 2013; Shirley et al., 2014).

The Virgin Islands Boa and the Mona Island Boa are taxa of special conservation concern. Due to its scarcity and restricted distribution, *E. m. granti* was listed as Endangered (Federal Register, October 13, 1970, 35:16047), and *E. m. monensis* was listed as Threatened (Federal Register, February 3, 1978, 43:4618) under the United States' Endangered Species Act. In 2004, the Department of Natural and Environmental Resources of Puerto Rico designated *E. m. granti* as Critically Endangered, and *E. m. monensis* as Endangered under the Regulation to Govern the Endangered and Threatened Species in the Commonwealth of Puerto Rico (Departamento de Recursos Naturales y Ambientales, 2004). In 2011, *Epicrates monensis* (sensu lato) was listed as Endangered in the Red List of Threatened Species of the International Union for Conservation of Nature (IUCN, 2014). *Epicrates m. monensis* and *E. m. granti* are also given international protection under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, Appendix I).

Molecular surveys of threatened and endangered taxa are particularly important, as these assessments provide the necessary information for setting conservation priorities, that is, for proposing evidence-based legislation for preserving genetic diversity,

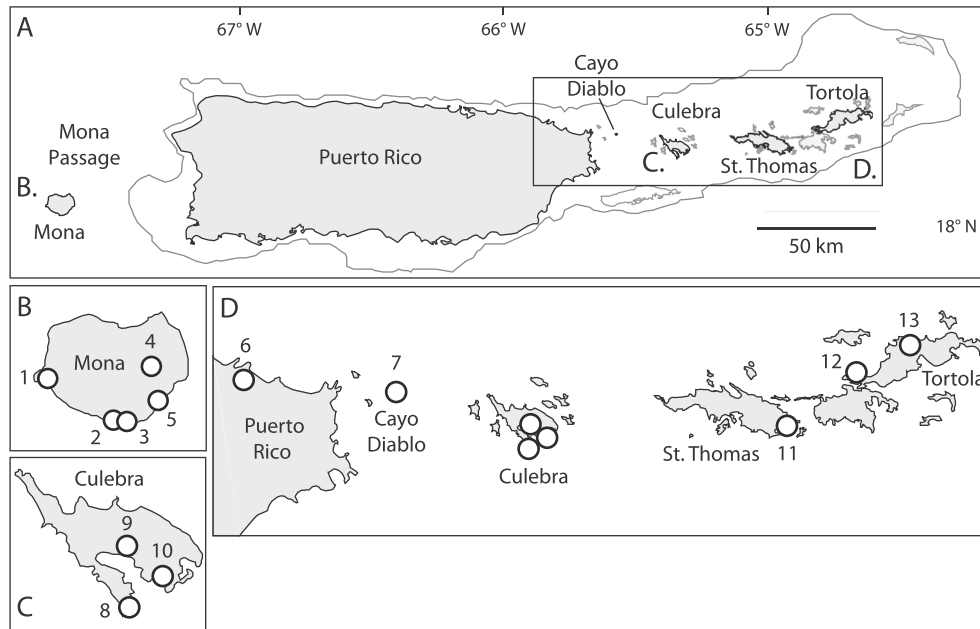


Fig. 2. Map of Mona Island and the Puerto Rican Bank, in the eastern Caribbean Sea. The outermost line in A indicates the approximate land configuration at maximum sea level lowering (~120 m; Siddall et al., 2003) during the Last Glacial Maximum (ca. 26.5–19 Kya). Circles indicate the approximate locations of the specimens of *Chilabothrus monensis monensis* from (B) Mona Island, and of *C. m. granti* from (C) Culebra, (D) Puerto Rico, Cayo Diablo, Saint Thomas (U.S. Virgin Islands), and Tortola (British Virgin Islands) included in this study.

the foundation for all biological diversity (Ehrlich and Wilson, 1991). In this study we conducted a range-wide genetic survey of *C. monensis* s.l. to characterize levels of divergence and diversity among and within populations of this snake using mitochondrial (mtDNA) and nuclear (nuDNA) markers. We quantified genetic divergence between *C. m. monensis* and *C. m. granti*, and determined levels of diversity within each taxon, and within individual populations of *C. m. granti*. We also applied a probabilistic, coalescent-based species delimitation model (Yang and Rannala, 2010) to test whether *monensis* and *granti* occupy different evolutionary trajectories. Collectively, this knowledge is essential to assess which populations of these snakes may exhibit low levels of genetic diversity due to small size and/or limited gene flow and therefore be at risk of extinction; to adopt a systematic treatment that reflects the phylogenetic history of the Mona Island and the Virgin Islands Boas; and to identify appropriate management units for these imperiled snakes (Frankham et al., 2009).

2. Materials and methods

2.1. Taxon sampling, genetic markers, and laboratory methods

We obtained scale clips and/or tail fragments from 10 individuals of *C. m. monensis* from Mona Island, and 40 specimens of *C. m. granti* from Puerto Rico, Cayo Diablo, Culebra, Saint Thomas, and Tortola (Table 1; Fig. 2). We also obtained samples from closely-related taxa of *C. monensis* s.l. (Reynolds et al., 2013): *C. fordii* ($n = 2$), *C. gracilis* ($n = 1$), *C. inornatus* ($n = 3$), and *C. striatus* ($n = 2$).

We isolated total genomic DNA from tissue samples using the DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA). For all taxa, we amplified two mitochondrial (mtDNA) markers, cytochrome b (Cyt b), and the nicotinamide adenine dinucleotide dehydrogenase subunit 4 and adjacent tRNAs (tRNA^{His}, tRNA^{Ser}, tRNA^{Leu}) (hereafter referred to as “ND4”). For *C. fordii*, *C. inornatus*, and *C. monensis* s.l., we also amplified seven nuclear markers (nuDNA): brain-derived neurotrophic factor (BDNF), dynein axonemal heavy chain 3 (DNAH3), 3'-nucleotidase (NT3), ornithine decarboxylase 1

(ODC1), prolactin receptor (PRLR), ribosomal protein L9 (RPL9), and sodium channel, voltage-gated, type V, alpha subunit (SCN5A). Amplification primers are listed in Table S1, Supporting Information.

We carried out PCR reactions in 12.5 volumes consisting of 1 μ l of template DNA, 0.5 μ l of each primer (10 μ M), 6.25 μ l of Takara Ex Taq™ Polymerase Premix (Takara Mirus Bio Inc., Madison, WI), and 4.25 μ l of ddH₂O. DNA was denatured initially at 95 °C for 2.5 min, and then 40 cycles of amplification were performed under the following conditions: denaturation at 95 °C for 1 min, marker-specific annealing temperature for 1 min (Table S1, Supporting Information), and extension at 72 °C for 1 min, followed by a final 5 min elongation at 72 °C. Three microliters of all PCR products were electrophoresed on a 0.8% agarose gel stained with ethidium bromide to verify product band size. We cleaned the double-stranded PCR products with ExoSap-IT® (USB Corporation, Cleveland, OH). We sequenced all fragments using the same primers used for amplification. We used the Big Dye Terminator Ready Reaction Kit 1.1 or 3.1 (Applied Biosystems, Foster City, CA) for cycle sequencing, and ran the sequences on an ABI 3130 automated sequencer. For each nuclear marker we initially sequenced a subset of 12–18 representative samples of *C. monensis* s.l. If we did not detect any variation among the samples processed we designated the marker as “uninformative,” and did not obtain additional sequences for that particular gene region. The final dataset comprised 1925 bp of mtDNA and 3545–3556 bp of nuclear markers (Table 2). Sequences were deposited in GenBank under accession numbers KP746416–KP746740 (Table S2, Supporting Information).

2.2. Phylogenetic analyses, divergence dating, and median-joining network

We used the program Mega (version 5; Tamura et al., 2011) to calculate the number of variable sites for each marker, genetic diversity within populations, and mean genetic distance between *C. m. monensis* and *C. m. granti*, and within *C. m. granti*. We relied

Table 1

Taxon, island (population number), specific locality, and number of individuals used in this study.

Taxon	Island-locality number	Specific locality	Number of individuals
Outgroup			
<i>Chilabothrus fordii</i>	Hispaniola	Dominican Republic, southern Azúa Province	2
<i>Chilabothrus gracilis</i>	Hispaniola	Dominican Republic, Hato Mayor Province	1
<i>Chilabothrus inornatus</i>	Puerto Rico	Municipality of Utuado	1
	Puerto Rico	Specific locality unknown	1
	Puerto Rico	Municipality of Carolina	1
<i>Chilabothrus striatus</i>	Hispaniola	Dominican Republic, specific locality unknown	2
Ingroup			
<i>Chilabothrus m. monensis</i>	Mona 1	Western Plateau	5
	Mona 2	Littoral forest, southcentral region	1
	Mona 3	Littoral forest, southcentral region	1
	Mona 4	Central Plateau	1
	Mona 5	Littoral forest, southeastern region	2
<i>Chilabothrus m. granti</i>	Puerto Rico 6	Municipality of Río Grande	5
	Cayo Diablo 7	—	14
	Culebra 8	Southcentral region	2
	Culebra 9	Central region	6
	Culebra 10	Southeastern region	1
	Saint Thomas (U.S. Virgin Islands) 11	Area surrounding Red Hook Bay	7
	Tortola (British Virgin Islands) 12	West End	1
	Tortola (British Virgin Islands) 12	Zion Hill	1
	Tortola (British Virgin Islands) 13	Area surrounding the Briercliffe-Davis Observatory, Skyworld, Ridge Road	1
	Tortola (British Virgin Islands)	Specific locality unknown	2

Table 2Characterization of the mtDNA (Cyt b, ND4) and nuDNA (BDNF, DNAH3, NT3, ODC1, PRLR, RPL9, SCN5A) sequences used in this study, including number of sequences obtained per gene per taxon, number of variable sites within *Chilabothrus monensis* (sensu lato), and *p*-distance between *C. m. monensis* and *C. m. granti*. See Section 2 for the complete names of genes.

Gene	Coding region	Number of samples (<i>C. m. monensis</i> , <i>C. m. granti</i> , outgroups)	Number of base pairs	Number of variable sites within <i>C. monensis</i> (sensu lato): synonymous, non synonymous substitutions	Divergence (<i>p</i> -distance) between <i>C. m. monensis</i> and <i>C. m. granti</i>
Cyt b	Yes	10, 40, 8	1059	40, 6	0.03
ND4	Yes	10, 40, 8	866	37, 5	0.035
BDNF	Yes	4, 9, 0	546	None	0
DNAH3	Yes	10, 38, 4	693	2, 0	0.003
NT3	Yes	8, 11, 0	477	None	0
ODC1	Yes	4, 8, 0	384	None	0
PRLR	Yes	4, 8, 0	350	None	0
RPL9	No	10, 39, 4	530	3, N/A	0.002
SCN5A	No	10, 36, 2	564–575 ^a	2, N/A	0.002

^a indel variation among *C. fordii*, *C. inornatus*, and *C. monensis* s.l.

on the software DnaSP (version 5.10; [Librado and Rozas, 2009](#)) to collapse the combined mtDNA (Cyt b, ND4, tRNAs) and nuDNA sequences of *C. monensis* s.l. into unique haplotypes and alleles, respectively. We conducted Bayesian Inference (BI) and divergence dating analyses using the unique mtDNA haplotypes of *C. monensis* s.l. and the outgroup taxa. We did not include the nuclear markers in these calculations due to the very low variation among nuDNA sequences (Table 2). We partitioned the mtDNA dataset by gene (Cyt b, ND4, tRNAs), and the coding Cyt b and ND4 further by codon (1st. and 2nd. codon positions combined; 3rd. codon position). We identified the best-fitting model of nucleotide substitution for each partition using jModelTest (version 2.1.4; [Darriba et al., 2012](#)). Hierarchical likelihood ratio tests and Akaike Information Criteria identified HKY + G for the 1st. and 2nd. codon positions of Cyt b and ND4, and HKY for the 3rd. codon position of both genes and for the tRNAs as the most appropriate models of nucleotide substitution for these partitions.

We assessed tree topology and clade support for the combined mtDNA dataset using the BI analysis in the program MrBayes (version 3.1.1; [Ronquist and Huelsenbeck, 2003](#)). We initiated the BI analyses from a random starting tree with uniform (uninformative) priors ([Brandley et al., 2006](#)). We produced posterior probability distributions by allowing four Monte Carlo Markov Chains (MCMC) to proceed for ten million generations each, with samples

taken every 100 generations, a procedure that yielded 100,000 trees. We ran the Bayesian analyses twice to ensure that the chains were not trapped on local optima. After visual evaluation in Tracer (version 1.4.1; [Rambaut et al., 2008](#)), we discarded the first 10,000 trees as “burn-in,” and combined the remaining samples to estimate tree topology, posterior probability values, and branch lengths.

We estimated divergence dates between *C. m. monensis* and *C. m. granti* from the combined mtDNA dataset using the Bayesian divergence time estimation analysis in the program Beast (version 1.7.5; [Drummond and Rambaut, 2007](#)). We used two calibration points for this analysis: a mean divergence of 10.0 Mya (standard deviation [SD] = 5 Mya) between *C. monensis* s.l. and *C. inornatus*, and a mean divergence of 19.2 Mya (SD = 4 Mya) for the root of the tree, which included *C. fordii*, *C. gracilis*, *C. inornatus*, *C. monensis* s.l., and *C. striatus*, following the estimates of [Reynolds et al. \(2013\)](#). We used the Yule Process tree prior and lognormal relaxed clock for the divergence analysis ([Drummond and Bouckaert, 2014](#)). We ran the analysis for 100 million MCMC chains, which we sampled every 10,000 iterations. We imported the resulting log file into Tracer, and used the effective sample sizes to evaluate the estimates of posterior distributions. After discarding the first 25% of trees as burn-in, we generated the summary of output trees with TreeAnnotator (version 1.4.8; [Drummond and Rambaut, 2007](#)).

We constructed median-joining networks for the combined mtDNA dataset and the three variable nuclear markers (DNAH3, RPL9, SCN5A) of *C. monensis* using the program Network (Bandelt et al., 1999). Network analysis is suitable for shallow phylogenies where multifurcations occur and ancestral genotypes are still present. Nuclear markers that did not contain heterozygotes were analyzed as haplotypes. The RPL9 nuclear gene contained heterozygotes, and therefore these sequences were first phased into alleles using the software Phase (as implemented in DnaSP; Stephens and Donnelly, 2003; Librado and Rozas, 2009), and then imported into Network in bi-allelic form. For the mtDNA network we only used data for *C. monensis* s.l., whereas for the nuDNA networks we included data for *C. inornatus* and *C. fordii*, as the nuclear markers showed low levels of interspecific variation.

2.3. Species delimitation analyses

We used Bayesian Phylogenetic and Phylogeography (BP&P version 2.13; Yang and Rannala, 2010) to assess the systematic status of the taxa *inornatus*, *monensis*, and *granti*. [*C. inornatus* is sympatric with *C. monensis granti* in northeast Puerto Rico and in Culebra (A. R. Puente-Rolón, pers. comm.), and the two taxa incontrovertibly are distinct evolutionary lineages (Kluge, 1989; Reynolds et al., 2013; Schmidt, 1928; Sheplan and Schwartz, 1974; Stejneger, 1904).] We conducted these analyses using the mtDNA (Cyt b, ND4) and nuDNA (DNAH3, RPL9, SCN5A) sequences associated with the different matrilineal lineages identified for *inornatus* ($n = 3$), *monensis* ($n = 1$), and *granti* ($n = 8$). To test the parameter space under different demographic scenarios (Leaché and Fujita, 2010), we ran four analyses with different combinations of effective population size (θ ; large theta $\sim \text{Gamma}(1, 10)$, small theta $\sim \text{Gamma}(2, 2000)$) and tree length (deep divergence $\sim \text{Gamma}(1, 10)$, shallow divergence $\sim \text{Gamma}(2, 2000)$). We used the guide tree (*inornatus*, (*granti*, *monensis*)) (Reynolds et al., 2013), and conducted the analysis using the algorithm 0 of Yang and Rannala (2010). Each analysis was run for 50,000 generations, using a burn-in of 5000 trees.

3. Results

3.1. Phylogenetic relationships, divergence estimates, and genetic diversity

The combined mtDNA dataset (Cyt b, ND4, tRNAs) inferred that *C. monensis* s.l. is monophyletic, and that it is the sister taxon of the Puerto Rican *C. inornatus*, a relationship previously suggested by Burbrink (2005) and Reynolds et al. (2013). The mitochondrial data also indicated that *C. monensis* s.l. contains two well supported clades that correspond to the two traditionally recognized subspecies, *C. m. monensis* and *C. m. granti* (Fig. 3). The divergence between these two taxa was estimated to have occurred 3.27 Mya, with 95% highest posterior densities ranging from 2.22 to 4.54 Mya. The basal split within *C. m. granti* (i.e., the separation between two haplotypes from Culebra and Tortola and six other haplotypes from Puerto Rico, Cayo Diablo, Culebra, Saint Thomas, and Tortola) was calculated to have taken place 0.92 Mya, with 95% highest posterior densities ranging from 0.53 to 1.44 Mya (Fig. 3).

The mtDNA markers did not show nucleotide variation within *C. m. monensis*. Our samples of *C. m. granti* from Puerto Rico, Cayo Diablo, and Saint Thomas originated from a single population on each island. Each locality possessed a different haplotype, but there was no mtDNA diversity within any of these three populations (Fig. 4A). We sampled individuals of the Virgin Islands Boa from three localities in Culebra and two in Tortola (Fig. 2), and identified private mtDNA haplotypes at each location (Fig. 3). The three

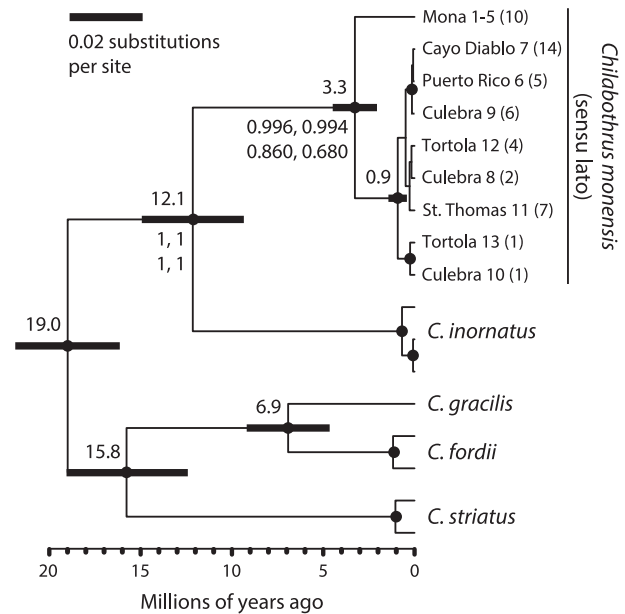


Fig. 3. Bayesian Inference tree inferred for 17 unique mtDNA (Cyt b and ND4) haplotypes of *Chilabothrus monensis* (sensu lato) and four outgroup taxa. Mean divergence times are indicated above each node, with the black bars representing the 95% highest posterior density. Nodes labeled with a black dot received $\geq 95\%$ posterior probability support in Bayesian Inference assessments performed with the Beast (Drummond and Rambaut, 2007) and MrBayes (Ronquist and Huelsenbeck, 2003) programs. For *C. monensis* s.l., labels refer to the island where each haplotype occurs, and numbers in parentheses indicate haplotype frequency. Numbers below nodes indicate the speciation probability (sensu Leaché and Fujita, 2010) of the split between *C. inornatus* and *C. monensis* s.l., and between *C. m. monensis* and *C. m. granti* estimated using the Cyt b, ND4, DNAH3, RPL9, and SCN5A datasets (see Section 2 for further details). Speciation probabilities were calculated for four different combinations of effective population size and divergence depth, and are listed as follows: upper left: small population, long branches; upper right: small population, short branches; lower left: large population, long branches; lower right: large population, short branches.

haplotypes from Culebra were recovered from a circular area with a diameter of ca. 4.3 km, whereas the two mitochondrial types from Tortola are separated by a linear distance of ca. 10 km. The average, uncorrected mtDNA p -distance within *C. m. granti* is 0.47%.

Of the seven nuclear genes examined, four markers (BDNF, NT3, ODC1, PRLR) did not vary within *C. monensis* s.l. One gene (DNAH3) showed nucleotide variation between *C. m. monensis* and *C. m. granti*, whereas two (RPL9, SCN5A) exhibited variation between the two subspecies, as well as within *C. m. granti* (Table 2; Fig. 4B–D). For each of the three variable nuDNA genes (DNAH3, RPL9, SCN5A), *C. m. monensis* exhibited a single, private allele. One DNAH3 allele was shared between *C. m. granti* and *C. inornatus* (Fig. 4B). As stated, only two of the seven nuDNA genes exhibited variation within the Virgin Islands Boa, RPL9 (four alleles) and SCN5A (two alleles). Two different RPL9 alleles occurred on four islands (Puerto Rico, Cayo Diablo, Culebra, Tortola; and Puerto Rico, Culebra, Saint Thomas, Tortola); another allele was restricted to Saint Thomas; and a fourth one to Tortola. Only one snake from Puerto Rico possessed the second SCN5A allele. The number of individuals per taxon assayed for each marker, number of base pairs and of variable sites for each gene, and genetic distances between *C. m. monensis* and *C. m. granti* are listed in Table 2.

3.2. Species delimitation analyses

In all analyses, the speciation probability (sensu Leaché and Fujita, 2010) of the split between *inornatus* and (*monensis*, *granti*)

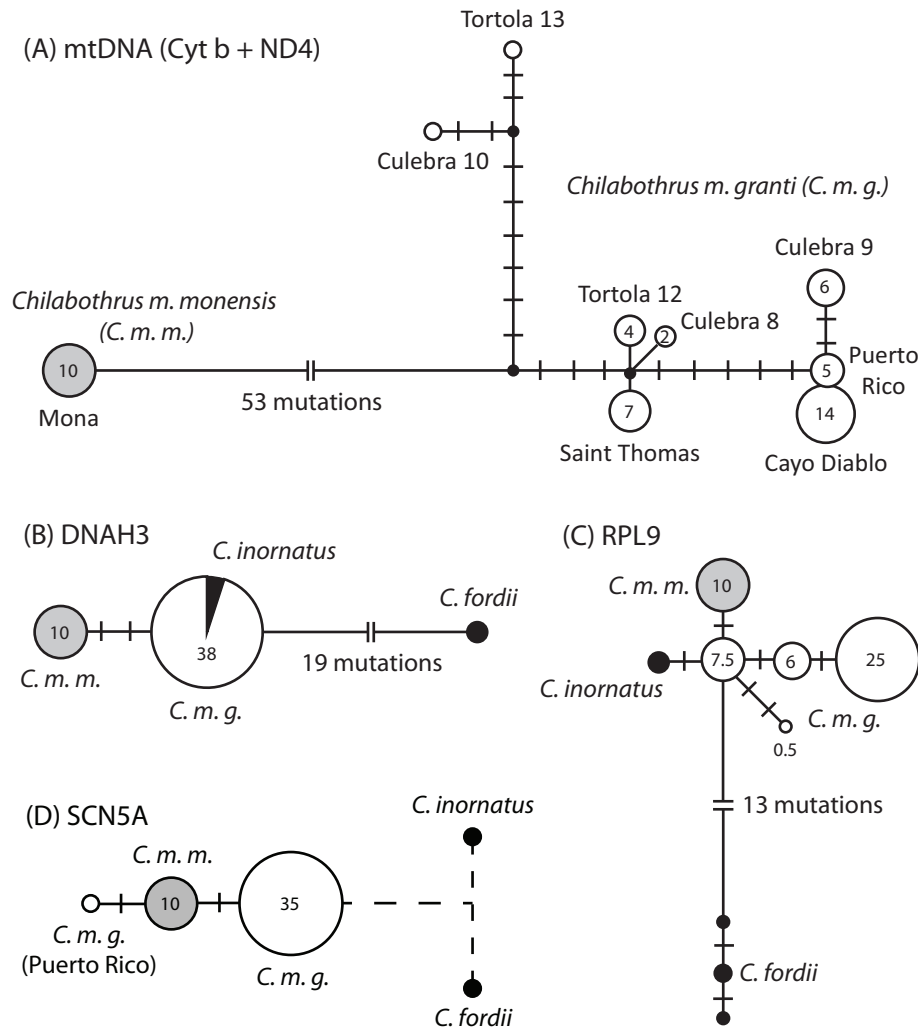


Fig. 4. Median-joining networks representing the relationships among (A) mitochondrial haplotypes (estimated from the combined mtDNA dataset) and alleles of the (B) DNAH3, (C) RPL9, and (D) SCN5A nuclear genes of *Chilabothrus m. monensis* (light grey circles) and *C. m. granti* (white circles). Circle size is proportional to genotype frequencies, with the number of sequences indicated within each circle. Circles without a number inside represent a single genotype. Branch length is proportional to the number of mutational steps, which are indicated with tick marks (for shorter branches) or actual numbers (for longer branches). Sequences of the outgroup taxa *C. fordii* and *C. inornatus* are included for the three nuclear genes (B, C, D) for comparative purposes. The dashed lines between *C. m. granti* and *C. fordii* and *C. inornatus* in (D) represent uncertainty in the number of mutational steps, as these species are differentiated by an indel event.

was always 1, as expected. Under conditions of small effective population sizes, the speciation probability supporting *monensis* and *granti* as separate species was 0.996 when considering deep divergences (long branches), and 0.994 when considering shallow divergences (short branches). However, assuming large effective population sizes, the speciation probability supporting *monensis* and *granti* as separate species was smaller: 0.86 when considering deep divergences and 0.68 when considering shallow divergences. This latter case (large theta and shallow divergences) is the most conservative scenario in which speciation by neutral processes (e.g., allopatry) will be difficult to identify.

4. Discussion

4.1. Genetic variation between *Chilabothrus m. monensis* (Mona Island Boa) and *C. m. granti* (Virgin Islands Boa)

Organelle and nuclear data indicate that *C. m. monensis* and *C. m. granti* can be diagnosed genetically. The Mona Island Boa is characterized by matrilineal lineages distinctive from those present in populations of the Virgin Islands Boa. Further, the taxa

do not share nuDNA sequences that show variation within *C. monensis* s.l., as *C. m. monensis* has private DNAH3, RPL9, and SCN5A alleles. Therefore, our genetic data suggest that *C. m. monensis* and *C. m. granti* should be considered demographically independent from one another at the present time, and that the geographic separation between populations of these snakes constitutes an effective barrier to gene flow. In fact, we estimated that the two taxa have been on separate evolutionary trajectories for approximately 3.3 Mya.

The Mona Island Boa could have evolved in situ on Mona, after its ancestors colonized the island from Puerto Rico. Alternatively, the divergence between *C. m. monensis* and *C. m. granti* could have originally occurred on mainland Puerto Rico, and after colonizing Mona, *C. m. monensis* ultimately became extinct on Puerto Rico. Mona lies in its own bank, and is believed to have never been connected to Puerto Rico or to any other island (Kaye, 1959). Consequently, the terrestrial fauna of Mona is ultimately the product of successful colonization events. At present, water currents sweep around the southwestern corner of Puerto Rico and travel northward through the Mona Passage (Heatwole and MacKenzie, 1967). If the major trends of ocean currents have been

similar since the Pliocene (ca. 5 Mya), they could have facilitated overwater dispersal from Puerto Rico to Mona. It has been hypothesized that the ancestors of *Anolis monensis* (Mona Island Anole) and *Sphaerodactylus monensis* (Mona Island Dwarf Gecko) colonized Mona from southwestern Puerto Rico via waif dispersal (Díaz-Lameiro et al., 2013; Rodríguez-Robles et al., 2007), and *Chilabothrus* boas could have also reached Mona in this manner.

The disjunct range of *C. monensis* s.l. (Fig. 2) suggests that these boas were once more widely distributed on the Puerto Rican Bank, but likely experienced extirpation on localities in Puerto Rico and offshore islands and cays, perhaps as the result of habitat fragmentation (Nellis et al., 1983), or more recently, anthropogenic activities. Relatively brief periods (ca. 11–18 Kya) of higher sea level during interglacial episodes (such as the present one) divided the Puerto Rican Bank to an extent similar to its current configuration at least three times in the past 250,000 years (Dutton et al., 2009; Muhs et al., 2011). Therefore, the higher sea level fragmented and reduced the size of littoral habitats in the Puerto Rican Bank (Heatwole and MacKenzie, 1967; Renken et al., 2002), leading to population isolation and smaller population sizes, changes that may have facilitated local extirpation (Barker et al., 2012; Pregill and Olson, 1981). Deforestation through urbanization and land development for residential and commercial purposes, particularly in northeastern Puerto Rico (pers. observ.), Culebra, and Saint Thomas (Renata Platenberg, pers. comm.) has reduced even more the available habitat for *C. m. granti*, and further isolated populations of this snake.

4.2. Genetic variation within *Chilabothrus m. monensis* and *C. m. granti*

We did not detect any variation in mtDNA or nuDNA within *C. m. monensis*. In other words, all loci are monomorphic, despite the fact that we sampled individuals from five locations across Mona Island. The lack of genetic diversity among Mona Island Boas may be the result of a founder effect, reflect a low effective population size, a slowdown in the mutation rate, or indicate a relatively recent selective sweep (Jovelín et al., 2014; Pyhäjärvi et al., 2007; Ray et al., 2004). We estimated that *C. m. monensis* diverged from its relatives on the Puerto Rican Bank 3.3 Mya, and thus the event(s) that presumably led to the circumstances that account for the present-day absence of genetic variability in this boa probably occurred subsequent to the colonization of Mona Island.

Our genetic assessment documented geographic structuring in *C. m. granti*. Each of the five islands sampled (Puerto Rico, Cayo Diablo, Culebra, Saint Thomas, Tortola) exclusively contains private mtDNA haplotypes of the Virgin Islands Boa. For the two islands (Culebra, Tortola) for which we obtained individuals from more than one site, each location is characterized by a different mitochondrial type. The degree of population subdivision in *C. m. granti* indicates that gene flow is minimal or nonexistent among populations of this snake, even within the same island. In terms of habitat utilization, the Mona Island Boa and the Virgin Islands Boa exhibit clear preferences for vegetational continuity, that is, interlocking of the canopy that facilitates movement without descending to the ground, as well as encounters with *Anolis* lizards sleeping on leaves and branches, a major prey item of the boas (Chandler and Tolson, 1990; Tolson, 1988). This habitat preference may account for the genetic structuring of *C. m. granti* within Culebra and Tortola, as the sampled locations on these islands are not interconnected.

Despite this degree of population subdivision in *C. m. granti*, the overall level of genetic diversity in this snake is low. Specifically, there is zero mtDNA variation within any unique sampling locality. Further, five of the seven nuclear markers examined are monomorphic for the Virgin Islands Boa. Two nuDNA genes display variation,

although only one snake possesses the second SCN5A allele. Two RPL9 alleles are restricted to a single island, but contrary to the pattern exhibited by the mtDNA, demes from four islands share two RPL9 alleles. Still, nucleotide diversity for both markers is low (RPL9, $\pi = 0.0008097$; SCN5A, $\pi = 0.0001970$). Interestingly, all individuals of *C. m. granti* examined ($n = 40$) shared one DNAH3 allele with *C. inornatus* (Fig. 4B). Given the marked differences in morphology and body size (Rodríguez-Robles and Greene, 1996; Schwartz and Henderson, 1991) between these two sister taxa, this allele sharing likely represents an instance of incomplete lineage sorting.

The biogeographic context of *C. m. granti* may clarify the pattern of genetic diversity displayed by this snake. Changes in sea level during the Pleistocene Epoch (2.6 million years ago – 10,000 years ago) have caused the Puerto Rican Bank to oscillate between a single landmass and its present configuration consisting of approximately 200 islands, islets, and cays. The lower sea level during glacial periods led to the formation of a land-bridge connection among Puerto Rico and the islands off Puerto Rico's eastern coast, uniting the Puerto Rican Bank into a single entity (Barker et al., 2012; Heatwole and MacKenzie, 1967; Renken et al., 2002; Rohling et al., 2009). Glacial periods were associated with drier climates, leading to xeric environments in coastal lowlands (Renken et al., 2002), the type of habitat where Virgin Island Boas are typically found. More arid conditions would have resulted in larger patches of suitable habitat, and presumably higher population density and connectivity among *C. m. granti* demes. This scenario would have led to a greater effective population size and greater overall genetic diversity, but lower genetic divergence among populations. On the contrary, opportunities for endemic lineages to develop probably occurred after the higher sea level during interglacial periods (such as the present) isolated populations that may have been connected in the past. This fragmentation would have caused a decline in the effective population size and the erosion of genetic diversity in those demes. The faster pace of evolution of mtDNA possibly reflects the present-day isolation of the populations of *C. m. granti* examined, whereas the slower substitution rate of nuDNA may instead mirror the relatively recent episodes of connectivity among these populations. This scenario implies that gene flow and reduction in effective population size and genetic diversity caused by habitat loss and fragmentation likely account for the overall low levels of genetic diversity in the Virgin Islands Boa. As previously stated, depressed nucleotide diversity can also result from a slowdown in the mutation rate, or from a selective sweep. All these mechanisms can contribute to the pattern of genetic diversity documented for the Virgin Islands Boa, although the biogeographic hypothesis may be the most parsimonious explanation for our findings.

4.3. Taxonomic implications

Since the first detailed systematic revision of the genus *Epicrates* (Sheplan and Schwartz, 1974), the Mona Island Boa (*C. m. monensis*) and the Virgin Islands Boa (*C. m. granti*) have traditionally been treated as different subspecies. However, Henderson and Powell (2009:350) stated that “the two taxa likely are distinct at the species level”. More recently, Platenberg and Harvey (2010) used the name *Epicrates granti*, and Mayer (2012) used the names *Epicrates monensis* and *Epicrates granti*, but no justification was provided for this treatment. Similarly, the online database *Caribherp* (Hedges, 2015) lists the two taxa as different species, *C. monensis* and *C. granti*, based on morphological differences between them (S. Blair Hedges, pers. comm.). Available evidence does indicate the existence of distinct phenotypic (i.e., adult body size, morphology, coloration pattern, behavior; Rivero, 1998; Sheplan and Schwartz, 1974; pers. observ.) and genetic differences

(this study) between the Mona Island Boa and the Virgin Islands Boa, suggesting that the taxa may constitute independent lineages.

We performed coalescent species delimitation analyses to assess the systematic status of *monensis* and *granti*. We used four combinations of priors to encompass different demographic scenarios (small effective population size, deep divergence; small effective population size, shallow divergence; large effective population size, deep divergence; large effective population size, shallow divergence). Because of the stochasticity of the coalescent, large effective population sizes and shallow divergence make species delimitation more difficult than scenarios of small effective population size and longer tree branches. The Mona Island Boa and the Virgin Islands Boa likely encompass demographics closer to the latter scenario. On Mona, *monensis* may be locally abundant in restricted habitats (Tolson et al., 2007), but overall the snake is rare (sensu Gaston, 1994). In most surveyed populations, *granti* densities are so low that conducting mark-recapture studies is not practical; instead, abundance is better described in terms of unit effort: 0.14 snakes/person/hour on Puerto Rico, 0.22 s/p/h on Culebra, and 0.025 s/p/h on Saint Thomas (Peter J. Tolson and Miguel A. García, unpubl. data). Further, we estimated that the divergence between *monensis* and *granti* occurred 3.3 Mya. Available evidence thus suggests that the posteriors for small population size and a deeper divergence are applicable to our study system. Consequently, the coalescent analyses indicated that with very high probability, the Mona Island Boa and the Virgin Islands Boa are different species, a finding in agreement with the distinct phenotypic differences between the taxa. In conclusion, we agree with the recent suggestions to recognize the snakes as two different species, *Chilabothrus monensis* and *Chilabothrus granti*, as this treatment more accurately reflects the evolutionary history of these boas.

4.4. Conservation implications

The Mona Island Boa and the Virgin Islands Boa receive individual protection under local, federal, and international regulations, laws, and treaties (see Introduction). The two snakes are independently managed by the Department of Natural and Environmental Resources of Puerto Rico, the United States Virgin Islands Department of Planning and Natural Resources, the U.S. Fish and Wildlife Service, and by the Department of Conservation and Fisheries of the British Virgin Islands. Our genetic assessment indicates that the Mona Island Boa and the Virgin Islands Boa should continue to be managed separately, because the two snakes are demographically independent and occupy distinct evolutionary trajectories, as indicated by mtDNA and nuDNA markers.

As previously stated, the ten individuals of *C. monensis* (sensu stricto) included in this study did not show variability in any of the two mtDNA and seven nuDNA markers assayed. This lack of genetic variation threatens population viability, as it is expected to result in lower individual fitness and reduced adaptive potential. Mona Island is a natural reserve administered by Puerto Rico's Department of Natural and Environmental Resources. The island has no permanent settlements, and levels of anthropogenic disturbance are low and concentrated around camping grounds, circumstances that favor the persistence of *C. monensis*. Nevertheless, feral Domestic Cats (*Felis catus*) inhabit Mona, and are known to predate on snakes, geckos and other squamate reptiles, and birds on the island (García et al., 2001; Tolson, 1996). Long-term control or preferably, eradication of these introduced predators, is recommended to reduce or eliminate their impact on Mona's wildlife (García et al., 2001).

Given the relatively low levels of overall genetic diversity within *C. granti*, our assessment supports the development of initiatives to manage the various populations of this snake as a single unit. This

practice would markedly increase the effective population size of the Virgin Islands Boa, and may be the most effective action to significantly increase the long-term survival of the species. Conservation geneticists have provided contrasting recommendations for using hybridization between isolated populations as a management tool, with some advocating an active approach (Frankham et al., 2011), and others urging to proceed cautiously, because of the risks of outbreeding depression (Edmands, 2007). The low genetic variability among the populations of *C. granti* documented in this study and the similarity of the habitats where the snakes occur (Tolson, 1996) suggest that the likelihood of outbreeding depression in these boas is small. Further, if these populations are experiencing inbreeding and genetic drift, interpopulation hybridization among Virgin Islands Boas may counteract the detrimental effects of these genetic phenomena, and yield long-lasting fitness benefits for the snakes (Pekkala et al., 2012).

Cayo Diablo is an uninhabited cay (0.05 km²) off the northeastern tip of Puerto Rico (Fig. 2) that harbors a dense population of Virgin Islands Boas (Chandler and Tolson, 1990). Black Rats (*Rattus rattus*) and Domestic Cats (*Felis catus*), introduced mammals known to prey on snakes (García et al., 2001; Tolson, 1996), do not occur on Cayo Diablo, and the absence of these exotics relaxes the predation pressure on the snakes. The mtDNA and nuDNA datasets indicate that there is greater haplotype and allele diversity in boas from Puerto Rico and Culebra than in those on Cayo Diablo. Unfortunately, boa populations on the two former islands are increasingly imperiled by habitat alteration and destruction due to introduced predators and urbanization. Authorities should consider releasing snakes from Puerto Rico and Culebra on Cayo Diablo, in an effort to increase genetic diversity on this cay, which could potentially become at least a temporary genetic reservoir for *C. granti*.

Global climate change, particularly sea level rise, represents an additional threat to the long-term persistence of the various populations of the Virgin Islands Boa that occur at low elevations (Tolson, 1988, 1991). Between 1901 and 1990, global mean sea level rose 1.2 ± 0.2 mm per year, a rate that increased to 3.0 ± 0.7 mm per year between 1993 and 2010 (Hay et al., 2015), and projections of global sea level rise by 2100 range from 20 cm to 2 m (Bamber and Aspinall, 2013; Willis and Church, 2012). The processes that influence the rising seas, thermal expansion of the oceans and glacier and ice-sheet mass loss, have a tremendous inertia. Therefore, even if global-mean surface temperatures were to stabilize by the end of the 21st century, the seas would go on rising for centuries before stabilizing again (Levermann et al., 2013; Willis and Church, 2012). Increased sea level will inundate low-lying cays and low elevation locations on larger Caribbean islands (Bellard et al., 2014), displacing, fragmenting even more, or perhaps leading to the extirpation of the various populations of Virgin Islands Boas that inhabit these localities. In fact, the ocean swell caused by Hurricane Hugo on 18 September 1989 (U.S. Department of Commerce, 1990) nearly flooded a significant portion of the central region of Cayo Diablo (maximum elevation: 15 m above sea level; Departamento de Recursos Naturales y Ambientales, 2010; Peter J. Tolson and Miguel A. García, pers. observ.), and the consensus is that anthropogenic warming will lead to more intense tropical cyclones (Knutson et al., 2010). The combination of sea level rise and projected increased intensity of tropical storms due to global climate change will likely decrease the likelihood of survival of at least some *C. granti* populations. The single known Puerto Rican population of *C. granti* occurs inland, at an elevation of 64 m above sea level, highlighting the importance of appropriately managing this deme to ensure its long term survival.

Knowledge of taxon boundaries is essential for accurately documenting biodiversity, estimating abundance, assessing threats, and

determining whether conservation efforts are required (Wheeler et al., 2004). Although nomenclature is central to systematics and conservation, information about evolutionary relationships is of greater importance than names or nomenclatural procedures for the protection of endangered taxa (Leslie, 2015). Herein we followed the taxonomic recommendation of Reynolds et al. (2013) by using the genus *Chilabothrus* for the West Indian boas traditionally placed in *Epicrates*. The purpose of this rearrangement was to make the classification of West Indian boas better reflect our current understanding of the evolutionary relationships of these snakes to their closest relatives. We emphasize that adopting this nomenclatural change does not call into question, much less erodes, the conservation protections that the United States' Endangered Species Act and the Regulation to Govern the Endangered and Threatened Species in the Commonwealth of Puerto Rico grant to *C. monensis* and *C. granti*.

To summarize, we performed a genetic assessment of the Mona Island Boa and the Virgin Islands Boa, relying on the largest number of specimens of these snakes examined to date. We uncovered unambiguous genetic differences between the two taxa, and coalescent species delimitation methods indicated that the snakes in all likelihood are different evolutionary entities, which we recognize at the species level, *Chilabothrus monensis* and *C. granti*. In contrast with their interspecific differentiation, we documented low levels of intraspecific diversity in both serpents, which we believe exemplify how distinct biogeographic events, that is, isolation versus connectivity, can shape patterns of spatial genetic variation, particularly in *C. granti*. Finally, we made evidence-based recommendations for the management of the endangered *C. granti*, as various populations of this snake are under severe pressure from anthropogenic activities. We exhort the various government agencies responsible for the conservation of different *C. granti* populations to initiate discussions to develop a cohesive strategy to ensure the preservation of this distinct evolutionary lineage.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2015.03.019>.

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