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Origin and phylogenetic position of the Lesser Antillean species of *Bothrops* (Serpentes, Viperidae): biogeographical and medical implications

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Synopsis. We use mitochondrial DNA sequences to infer the origin and phylogenetic position of the Lesser Antillean species of the pitviper genus *Bothrops*, *B. caribbaeus* and *B. lanceolatus*. The two species form a monophyletic group, which in turn forms the sister clade to the *Bothrops asper-atrox* complex. High levels of sequence divergence among the Caribbean species, and between them and the nearest mainland relatives, suggest a relatively ancient origin of these snakes. The hypothesis that the Lesser Antillean *Bothrops* are the result of a recent colonisation event from within the South American *B. atrox* complex is rejected, as is the hypothesis that they were introduced to their island habitats by aboriginal humans. The high level of morphological apomorphy displayed by *B. lanceolatus* suggests a stepping-stone colonisation, St. Lucia being colonised first and then Martinique from St. Lucia. The medical implications of these findings are discussed: a recent case of envenoming from Saint Lucia suggests that *Bothrops caribbaeus* causes the same thrombotic syndrome of envenoming as *B. lanceolatus*.

INTRODUCTION

The genus *Bothrops* Wagler, 1824 contains most of the pitviper fauna of South America. The genus (including the arboreal species sometimes assigned to *Bothriopsis*) contains approximately 36 species, with a wider variety of body shapes and natural history traits than in any other New World pitviper genus. This greater diversity has been ascribed to the fact that *Bothrops* was the first group of pitvipers to reach the South American continent, thus giving ample opportunity for adaptive radiation (Wüster *et al.*, in press).

Two species of *Bothrops* occur in the Lesser Antilles: *Bothrops caribbaeus* (Garman, 1887) on St. Lucia, and *Bothrops lanceolatus* (Lacepède, 1789) on Martinique. The status and origin of these forms has been the subject of much debate. Long considered to be conspecific with *Bothrops atrox*, *B. lanceolatus* was revalidated by Hoge (1952), and the validity of *B. lanceolatus* and *B. caribbaeus* confirmed by Lazell (1964). This latter interpretation has been followed by most authors since then (e.g., Campbell & Lamar, 1989). However, Sandner Montilla (1981, 1990) regarded the Lesser Antillean *Bothrops* as conspecific with each other, as well as with mainland *Bothrops asper* and the northern Venezuelan populations of the *B. asper-atrox* complex.

The origin of the Antillean *Bothrops* has been the subject of much speculation and mythology. This includes popular tales that the snakes were originally introduced by Carib Indians in their attempts to gain control of the islands from resident Arawaks (Dowling, 1965), and the notion that dispersal from the South American mainland is common and ongoing (Sandner Montilla, 1981).

The reptile fauna of the Lesser Antilles is primarily the result of long-distance dispersal by individual species, as these islands have not been linked to the South American mainland or any other landmass at any time in their history (Thorpe *et al.*, in press; Malhotra and Thorpe 1999). This means that some species present in these islands represent long-standing endemic lineages (Thorpe *et al.*, in press; Malhotra and Thorpe 2000), whereas others appear to be the result of relatively recent dispersal events from well-defined source populations or taxa in South America, as is the case for the genus *Corallus* (Henderson & Hedges, 1995).

Compared to morphological data, molecular markers such as mitochondrial DNA (mtDNA) sequence data have the advantage that they can give an estimate of phylogeny reasonably free of the confounding effects of differing natural selection pressures on the external phenotype. Moreover, molecular sequence data also have the advantage that they can give at least an approximate estimate of times of divergence between lineages, although the interpretation of molecular clocks is subject to various analytical and empirical problems (Hillis *et al.*, 1996).

Several recent mtDNA-based phylogenetic analyses of the genus *Bothrops* have included the Antillean species. Salomão *et al.* (1997,

PLATE 1

Bothrops caribbeus © R.S. Thorpe

Bothrops lanceolatus © D. Warrell

1999), using 580 b.p. of cytochrome *b* sequence, found *B. caribbaeus* and *B. lanceolatus* to the sister species of the South American populations of the *B. atrox* complex. However, the study included only a limited sampling of South American members of the *B. atrox* complex, and did not include representatives of *B. asper* from Central America.

The aim of this paper is to explore in more depth the origin of the Antillean *Bothrops*, and its implications for other fields, using an expanded dataset of more sequence information from a larger number of potentially related species.

MATERIALS AND METHODS

We obtained tissue (ventral scale clippings or tail tips) and/or blood samples from species representing the principal clades within the genus *Bothrops* (including *Bothriopsis*), as well as the closely related *Bothrocophias* – see Wüster *et al.* (in press). We also included samples of the *B. asper-atrox* complex from around the coast of South and Central America, as these have been considered to be potential founder populations from which the ancestor of Antillean *Bothrops* could have arisen. For outgroup rooting, we used sequences of *Bothrops alternatus* and *Bothrocophias microphthalmus*. Two regions of the mitochondrial DNA molecule were amplified using the polymerase chain reaction (PCR): a 767 base pair (bp) section of the gene for cytochrome *b* (cyt*b*), and a 900 bp region of the gene for NADH dehydrogenase subunit 4 (ND4). Details of primers and laboratory protocols are given in Pook *et al.* (2000).

Sequences were aligned by eye against published *Bothrops* sequences (Puorto *et al.*, 2001). In order to test for the presence of saturation of certain categories of substitution, we calculated Tamura–Nei distances between all samples. This takes into account deviations from equal base compositions and differences in substitution rates among nucleotides. We then plotted unadjusted p-distances for transitions and transversions, and for the three codon positions separately, against Tamura–Nei distances. A decline in the rate of accumulation of individual categories of substitution with increased Tamura–Nei distances indicates saturation of that substitution category.

We checked all sequences for insertions, deletions or the presence of stop codons. Any of these would have indicated that the sequences represent nuclear insertions of the mitochondrial genes (Zhang and Hewitt, 1996). The sequence data were assayed for the presence of a significant phylogenetic signal by means of the g1 tree skewness statistic (Hillis and Huelsenbeck, 1992), calculated from 100,000 trees randomly generated by PAUP* 4.0b8 (Swofford, 2001). Sequence divergences between clades were estimated using the program Phyltest (Kumar, 1996).

We analysed our sequence data using both maximum parsimony (MP) and maximum likelihood (ML) as optimality criteria. Using multiple optimality criteria should identify those parts of a phylogenetic tree that are supported independently of the optimality criterion used. Such nodes should inspire greater confidence than nodes that are unstable and vary depending on method of analysis. All analyses were carried out using the program PAUP* 4.0b8 (Swofford, 2001).

For MP analyses, we selected *Bothrops alternatus* and *B. microphthalmus* as outgroups. We employed the heuristic search algorithm of PAUP* 4.0b8, using TBR branch swapping and 100 random addition sequence replicates. The analysis was carried out on the unweighted data only.

The extent to which individual nodes on the tree were supported by the data was assessed using bootstrapping and Bremer (1994) branch support. Non-parametric bootstrap was implemented using heuristic searching, 1000 replicates, TBR branch swapping and 10 random-addition-sequence replicates per bootstrap replicate. Bremer branch support for individual nodes was calculated through the use of the converse constraint option of PAUP*.

For ML analyses, we identified the most appropriate model of sequence evolution through the use of the MODELTEST software (Posada & Crandall, 1998). A first ML search was run, using heuristic searching, a neighbour-joining starting tree and TBR branch swapping, and the sequence evolution parameters identified by the Modeltest software. These parameters were then re-estimated from the resulting ML tree, and a further search run using these re-estimated parameters. This was repeated until further estimates yielded no further changes of parameter values or tree likelihood scores. Bootstrap analysis involved 100 replicates, using NJ starting trees and NNI branch swapping.

An important consideration of any proposed scientific hypothesis is whether the data supporting it can reject alternative hypotheses with statistical significance. In other words, do the data allow us to reject the null hypothesis that differences in tree optimality between the optimal tree and trees consistent with alternative hypotheses are due to random chance? In the case of the Antillean *Bothrops*, we tested the following alternative phylogenetic hypotheses: (i) non-monophyly of the Antillean *Bothrops*, i.e., the Antillean populations of *Bothrops* result from separate colonisation events; (ii) non-

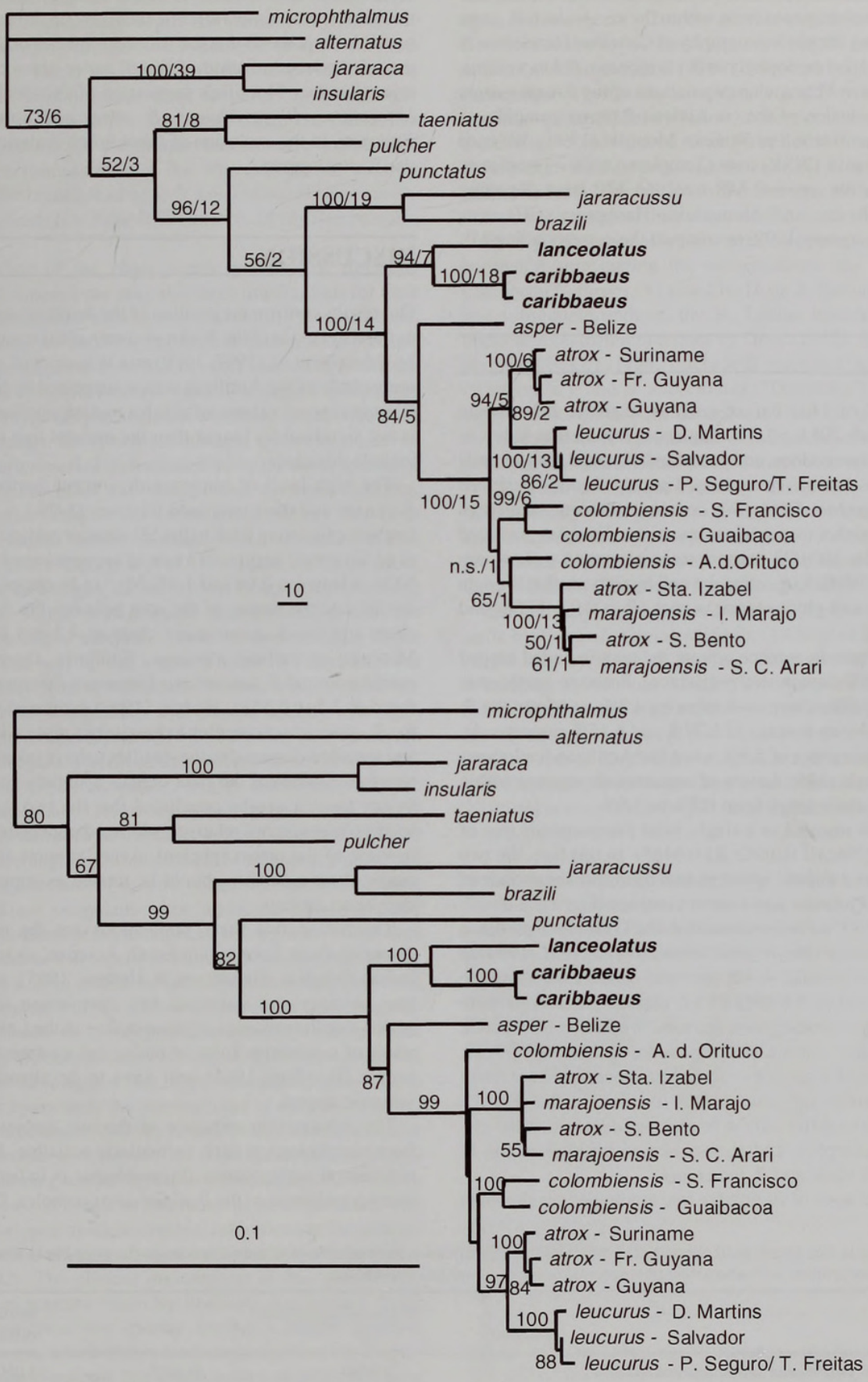

Fig. 1 Maximum parsimony (top) and maximum likelihood (bottom) estimates of the phylogeny of *Bothrops*. In the MP tree, numbers before the slash refer to bootstrap support, numbers after the slash indicate Bremer support. In the ML tree, numbers on nodes indicate bootstrap support.

monophyly of the *B. asper-atrox* complex, i.e., that the Antillean populations originate from within the *B. asper-atrox* complex; (iii) non-monophyly of the South American *B. atrox* complex, i.e., that the Antillean species originate from within the cis-Andean *B. atrox* complex, paralleling the phylogeography of *Corallus* (Henderson & Hedges, 1995); and (iv) monophyly of *B. caribbaeus*, *B. lanceolatus*, *B. asper* and northern Venezuelan populations of the *B. asper-atrox* complex to the exclusion of the cis-Andean *B. atrox* complex, as implied by the classification of Sandner Montilla (1990). We used Wilcoxon signed-ranks (WSR) tests (Templeton tests – Templeton, 1983) to compare the optimal MP tree and MP trees depicting alternative hypotheses, and Shimodaira–Hasegawa (SH) tests (Shimodaira & Hasegawa, 1999) to compare the corresponding ML trees.

RESULTS

We aligned a total of 1401 b.p. of mtDNA sequence information (ND4: 693 b.p.; cyt*b*: 708 b.p.). The sequences included no indels or stop or other nonsense codons, and contained the usual bias towards transitions and substitutions concentrated into third codon positions typical of mitochondrial DNA. We conclude that our sequences represent mtDNA rather than nuclear insertions. Samples are listed in Appendix 1. The 100,000 random trees generated a skewness statistic of g1 = –0.599403, rejecting the null hypothesis that the data contain no significant phylogenetic signal ($P < 0.01$; Hillis and Huelsenbeck, 1992).

Levels of sequence divergence among the taxa included ranged from 0.3% to 13.65% (unadjusted p-distance). *Bothrops caribbaeus* and *B. lanceolatus* differ from each other by 4.3%, and from the *B. asper-atrox* group by an average of 5.77% and 6.15% respectively, with an average divergence of 5.9% when the Antillean haplotypes are treated as a single clade. Levels of sequence divergence within the *B. asper-atrox* clade range from 0.3% to 5.5%

The MP analysis resulted in a single most parsimonious tree of 1030 steps (CI 0.5398; HI 0.4602; RI 0.6465). In this tree, the two Antillean taxa formed a clade, which in turn forms the sister clade of all samples of the *Bothrops asper-atrox* complex (Fig. 1).

The MODELTEST software identified the GTR+I+G model, a submodel of the general time-reversible model (Yang *et al.*, 1994) as optimal for the data at hand. A ML tree was constructed using the parameters calculated by MODELTEST, and the parameters were recalculated from the resulting tree, and used in a further ML search, which resulted in a tree with the likelihood score -ln(L) = 6652.69122.

Further estimates of sequence evolution parameters did not result in any change of parameter values or tree likelihood score (Fig. 1). The MP and ML trees differ only in branching order within the cis-Andean *B. atrox* complex, and in the relative position of the *B. jararacussu-brazili* clade and *B. punctatus*.

The results of our tests of alternative tree topologies are shown in Table 1. Neither the WSR nor the SH test significantly reject the possibility that the two Antillean species may be the result of separate colonizations of the Lesser Antilles, although the result of the SH test was almost significant. They do, however, significantly reject the hypothesis that the Antillean species originate from within the cis-Andean radiation of the *B. asper-atrox* complex, and also reject Sandner Montilla's suggestion of conspecificity between *B. lanceolatus*, *B. caribbaeus*, *B. asper* and northern Venezuelan *Bothrops*, to the exclusion of other South American populations of the *B. atrox* group.

DISCUSSION

Our results confirm the position of the Antillean species of *Bothrops* as the sister clade of the *Bothrops asper-atrox* complex, as suggested by Salomão *et al.* (1997, 1999) and Wüster *et al.* (1997, 1999). The monophyly of the Antillean taxa is supported by high bootstrap and Bremer support values, although a tree supporting this arrangement is not significantly longer than the optimal tree constrained not to include this clade.

The high level of sequence divergence between the Antillean *Bothrops* and their mainland relatives (5.9%) is consistent with a lineage split dating back to the Miocene or earliest Pliocene. Wüster *et al.* (in press) suggested a rate of sequence evolution for cyt*b* and ND4 of between 0.66 and 1.4% My^{-1} in Neotropical pitvipers. This would date the timing of the split between the Antillean *Bothrops* clade and the *B. asper-atrox* clade at 4.2–8.9 Mya, i.e., the late Miocene or earliest Pliocene. Similarly, the split between *B. caribbaeus* and *B. lanceolatus* (sequence divergence: 4.3%) can be dated to 3.1–6.5 Mya. Hedges (1996) estimated the divergence of the *B. asper-atrox* complex to have taken place within the last 4 My, and assumed dispersal to the Antilles to have taken place during that timeframe, whereas our data suggest a slightly earlier lineage split. In any case, it can be concluded that the two Antillean *Bothrops* species represent two relatively old, independent lineages. Obviously, in view of the errors inherent in any attempt at molecular clock usage, these estimates should be treated as approximations rather than exact timings.

The notion that these populations are the result of a recent dispersal event from within South America, as is the case in West Indian *Corallus* (Henderson & Hedges, 1995), is refuted by both tree topology and statistical tree comparison tests. Equally, the notion that the presence of these snakes in the Lesser Antilles is the result of a primitive form of biological warfare among aboriginal people (Dowling, 1965) will have to be abandoned, despite its romantic appeal.

The colonisation sequence of the two species can be resolved from morphological data, particularly scalation. In terms of dorsal and ventral scale counts, *B. caribbaeus* is indistinguishable from many populations of the *B. asper-atrox* complex. On the other hand,

Table 1 Differences in tree length or likelihood, statistics, and their significance, between the most parsimonious or the most likely trees, and trees constrained to be compatible with alternative phylogenetic or biogeographical hypotheses.

		Wilcoxon signed-ranks		Shimodaira–Hasegawa	
	d(steps)	– z	P	d(lnL)	P
Non-monophyly of *B. caribbaeus* and *B. lanceolatus*	7	1.4000	0.1615	15.05075	0.054
Non-monophyly of *B. asper-atrox* complex	5	1.1471 – 1.5076	0.1317–0.2513	3.15866	0.181
Non-monophyly of cis-Andean *B. atrox* complex	15	2.4019	0.0163*	20.06423	0.018*
Monophyly of *B. caribbaeus*, *lanceolatus*, *asper* and northern Venezuelan populations	18	2.9200 – 3.0870	0.002 – 0.0035*	24.34213	0.005*

B. lanceolatus has higher ventral and dorsal scale row counts than practically all populations of the *B. jararacussu-punctatus-atrox* clade. This suggests that the extreme scale counts found in *B. lanceolatus* represent an autapomorphy compared to *B. caribbaeus* and mainland *Bothrops*. This makes a hypothesis of dispersal from the mainland to St. Lucia, and then a further dispersal event to Martinique, more parsimonious than dispersal to Martinique followed by further dispersal to St. Lucia. Since St. Lucia lies between South America and Martinique, this scenario is also more geographically parsimonious than the alternative. The slightly greater length of the branch leading to *B. lanceolatus* is also consistent with this hypothesis (De Salle & Templeton, 1988; Thorpe *et al.*, 1994).

An understanding of the phylogenetic position of *Bothrops caribbaeus* and *B. lanceolatus* may also have implications for their venom composition and the treatment of snakebite in the Caribbean. *Bothrops lanceolatus* envenoming has been documented to produce a unique syndrome different from that of other species of *Bothrops*. In addition to local symptoms such as pain, swelling, bleeding at the site of the bite, ecchymosis and necrosis, which are common to most crotaline envenomings, the systemic bothropic syndrome observed in Central and South America is characterised by the development of consumption coagulopathies and spontaneous systemic bleeding, depending on venom components which affect clotting factors as well as haemorrhagins which damage vascular endotheliums (Barrantes *et al*, 1985; Kamiguti *et al*, 1991). On the other hand, apart from similar local signs, the severity of systemic envenoming by *Bothrops lanceolatus* in Martinique was correlated with the development of multiple cerebral infarctions and/or other major vessel occlusion that may appear within 8 hours to 7 days after the bite in approximately 30 to 40% of cases (Thomas *et al*, 1995, 1998). Infarctions can develop in patients who present initially with signs of moderate envenoming with normal blood clotting and low serum levels of venom antigens. The infarction process can involve several small vascular territories altogether, and is associated with the development of an isolated thrombocytopenia. Bogarin *et al.* (1999) demonstrated that *Bothrops lanceolatus* venom, obtained from 20 specimens collected at different locations in Martinique, is devoid of thrombin-like enzymes and of *in vitro* coagulant and defibrinating activities, and is not coagulant when added to human citrated plasma, even at concentration as high as 100 µg/mL. These data suggest that thromboses observed in human *B. lanceolatus* envenoming result from a toxin-linked vasculitis process rather than from a systemic procoagulant effect. However, the exact thrombogenic mechanism responsible for these thromboses remains unexplained.

The monophyly of *Bothrops lanceolatus* and *B. caribbaeus* leads to the prediction that these snakes may share venom properties, which may in turn be of importance for the treatment of patients bitten by these snakes. In particular, do bites by *B. caribbaeus* result in a similar thrombotic syndrome as observed in *B. lanceolatus*? *Bothrops caribbaeus* envenoming was poorly documented until now. However, the case of a 32 year old man who was bitten in Saint Lucia and who subsequently developed multiple cerebral infarctions in the anterior and posterior cerebral artery territories was recently published (Numeric *et al*, 2002). The clinical presentation of this patient was identical to that of patients bitten by *Bothrops lanceolatus*. Thus, envenomings from these two species develop a unique systemic thrombotic syndrome, which differs fundamentally from the defibrination and bleeding syndrome that characterizes all other *Bothrops asper-atrox* complex envenomations. This example suggests that, at least in some cases, an understanding of the phylogeny of medically important snakes can help predict the syndrome of envenoming to be expected from a hitherto undocumented species.

Our results also have implications for the conservation of the Antillean *Bothrops*. Our data show that both *B. caribbaeus* and *B. lanceolatus* represent relatively old, independent evolutionary lineages, and not recent offshoots of widespread South American taxa. Conservation policy on their respective islands needs to take this into account. Although Lazell (1964) described both *B. lanceolatus* and especially *B. caribbaeus* as common (and Dowling, 1965, reported similar experiences for the latter), more recent workers have reported these snakes to be harder to find (Powell & Wittenberg, 1998). These observations indicate that *B. caribbaeus* and *B. lanceolatus* may have suffered a decline in population numbers over the last few decades, and that a reassessment of their conservation status should be a priority.

Finally, this paper also represents an opportunity to clarify some confusion surrounding the nomenclature and synonymy of the Caribbean *Bothrops*. As noted by Hoge & Romano Hoge (1978/79) and subsequent authors, the St. Lucian lancehead was described under several different names by Gray (1842). Species of *Bothrops* described by Gray (1842) include *B. cinereus* ('America'), *B. sabinii* ('Demerara'), and *B. subscutatus* ('Demerara'). Gray (1849) also described *B. affinis* ('Demerara' and 'Berbice').

The types of *B. sabinii* and *B. subscutatus* were the specimens collected by Capt. (later Col.) Sabine discussed by Underwood (1993), and are unquestionably assignable to *B. caribbaeus* (Underwood, 1993; pers. obs.), of which the names *B. subscutatus* and *B. sabinii* therefore represent senior synonyms. However, the precedence of Garman's well-established name *B. caribbaeus* over Gray's disused names was formally established by Wüster (2000).

The female type specimen of *Bothrops cinereus*, considered *incertae sedis* by Peters & Orejas-Miranda (1970) and conspecific with *B. caribbaeus* by Hoge & Romano Hoge (1978/79) and Powell & Wittenberg (1998), has 31 scale rows at midbody and 224 ventral scales. These counts are consistent with *B. lanceolatus*, but not with *B. caribbaeus; B. cinereus* is thus a junior synonym of *B. lanceolatus*. The syntypes of *B. affinis* are assignable to *B. atrox*, and are consistent with Guyanan populations of that species based on both scalation (24–27 dorsal scale rows, 189–200 ventrals) and colour pattern.

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Appendix 1

Origin and vouchers of samples sequenced in this study. Institutional Codes for vouchers: IB = Instituto Butantan, São Paulo, Brazil, Herpetological Collection. FHGO = Fundación Herpetológica Gustavo Orcés, Quito, Ecuador. INHMT = Instituto de Higiene y Medicina Tropical 'L. Izquieta Pérez', Guayaquil, Ecuador. ROM = Royal Ontario Museum, Toronto. WW = Wolfgang Wüster collection. Collection numbers refer to preserved specimens unless otherwise stated. Photographs and/or morphological data for many unvouchered specimens are available from the first author.

Bothrocophias microphthalmus: ECUADOR: Zamora Chinchipe: Cuenca del Río Jamboe: Pumbami. FHGO 2566. *Bothrops alternatus*: BRAZIL: Paraná: Pinhão. IB 55314. *B. asper*: Belize: Mile 38, Western Highway. WW 264. *B. atrox*: BRAZIL: Pará: Santa Izabel. WW 735. Maranhão: São Bento: WW 723. FRENCH GUYANA: Mana. WW 554. GUYANA: NorthWest District: Baramita. ROM 22848. SURINAME: Coronie District: 7.5 km E. Totness. WW 537. *B. brazili*: ECUADOR: Morona Santiago: Macuma. FHGO 982. *B. caribbaeus*: SAINT LUCIA: Grande Anse. WW 144 WW 148. *B. colombiensis*: VENEZUELA: Guárico: Altagracia de Orituco. WW 74. Falcón: San Francisco. J.L. Yrausquin, live coll. Guaibacoa. J.L. Yrausquin, live coll. *B. insularis*: BRAZIL: São Paulo: Ilha da Queimada Grande. Released after sampling. *B. jararaca*: BRAZIL: Paraná: Piracuara. WW 926. *B. jararacussu*: BRAZIL: São Paulo: Cananéia. IB 55313. *B. lanceolatus*: MARTINIQUE. Not vouchered. *B. leucurus*: BRAZIL: Bahia: Porto Seguro. IB 55480–1; Salvador. IB 55478. Espírito Santo: Domingos Martins. IB 55557. *B. marajoensis*: BRAZIL: Pará: Ilha de Marajó: 10 km NW Camará. WW 80. Santa Cruz do Arari: WW 943. *B. pulcher*: ECUADOR: Zamora Chinchipe: Estación Científica San Francisco. FHGO live coll. 2142. *B. punctatus*: ECUADOR: Pichincha: Pedro Vicente Maldonado. FHGO live coll. 2166. *B. taeniatus*: ECUADOR: Morona Santiago: Macuma. FHGO 195.