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Phylogeography of the bushmaster (*Lachesis muta*: Viperidae): implications for neotropical biogeography, systematics, and conservation

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We used mitochondrial gene sequences to reconstruct phylogenetic relationships among subspecies of the bushmaster, *Lachesis muta*. These large vipers are widely distributed in lowland tropical forests in Central and South America, where three of four allopatric subspecies are separated by montane barriers. Our phylogeny indicates that the four subspecies belong to two clades, the Central American and South American lineages. We use published molecular studies of other taxa to estimate a 'reptilian mtDNA rate' and thus temporal boundaries for major lineage divergences in *Lachesis*. We estimate that the Central and South American forms diverged 18–6 Mya, perhaps due to the uplifting of the Andes, whereas the two Central American subspecies may have diverged 11–4 Mya with the uprising of the Cordillera de Talamanca that separates them today. South American bushmasters from the Amazon Basin and the Atlantic Forest are not strongly differentiated, perhaps due to episodic gene flow during the Pleistocene, when suitable habitat for this species was at times more continuous. Our results agree with previous evidence that genetic divergence among some neotropical vertebrates pre-dated Pleistocene forest fragmentation cycles and the appearance of the Panamanian Isthmus. Based on morphological, behavioral, and molecular evidence, we recognize three species of *Lachesis*. In addition to *L. muta*, the widespread South American form, the Central American forms are treated as distinct species (*L. melanocephala* and *L. stenophrys*), each deserving of special conservation status due to restricted distribution and habitat destruction.

ADDITIONAL KEY WORDS:—molecular clock – species concepts – vicariance – genetic differentiation – conservation – Serpentes.

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INTRODUCTION

Systematists have demonstrated patterns of strong differentiation between Central and South American biotas, involving species as well as higher taxa (e.g. Savage, 1966, 1982; Rosen, 1975; Wake & Lynch, 1976; Duellman, 1979; Gentry, 1982a; Cadle, 1985; Crother, Campbell & Hillis, 1992). Such analyses of individual lineages might corroborate previous biogeographic models as well as generate novel hypotheses, the predictions of which are testable with phylogeographic studies of additional groups with similar distributional patterns. Congruent phylogenetic patterns among diverse groups, interpreted in a geological context, can then be used to infer a generalized history of the area under study (e.g. Kluge, 1989; Cracraft, 1994). Such general historical explanations for patterns of differentiation within and among taxa require estimates of the temporal framework for the separation of lineages within each group, because a common temporal framework may not apply to all groups with similar geographic distributions (Cadle, 1985). Thus, detailed studies of specific taxa should include, whenever possible, a temporal estimate independent of that assumed for the underlying biogeographic model.

Although a number of workers have focused on major lineage divergences within large radiations of neotropical organisms (e.g. Cadle, 1984a,b,c; Prance, 1987; Cracraft & Prum, 1988; Ayres & Clutton-Brock, 1992), few investigators have addressed more recent differentiation among widespread species or populations (but see Patton, da Silva & Malcolm, 1996; Patton, in press). Here we use mtDNA sequences to infer phylogenetic relationships among populations of four allopatric subspecies of a widespread neotropical pitviper, the bushmaster (*Lachesis muta*), then use a molecular clock calibrated for 'reptilian rates' of mtDNA evolution to estimate temporal boundaries for major divergence events within this lineage. Our objectives here are first to elucidate the evolutionary history of a prominent component of the Central and South American herpetofauna, assess its relevance to neotropical biogeography and climatic history, and thereby contribute to the emerging rapprochement of paleontological and neontological perspectives on neotropical biotas (e.g. Cadle & Greene, 1993; Webb & Rancy, 1996; Lundberg, 1997; Patton, in press). Then, based on our phylogenetic hypothesis, published morphological and behavioral differences, and the allopatric distributions of distinctive population

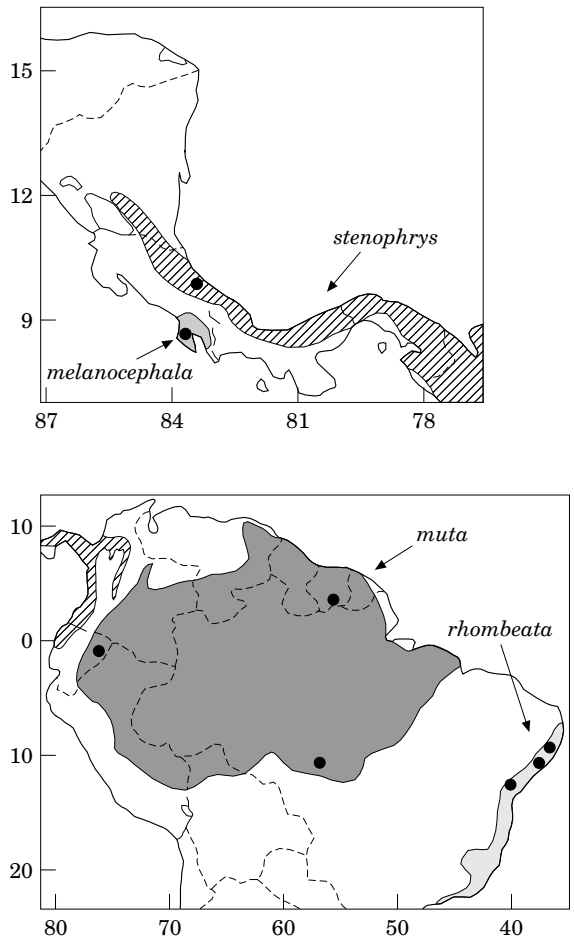

Figure 1. Geographic distributions of the four subspecies of *Lachesis muta* in Central and South America (modified from Campbell and Lamar, 1989). Collection localities for specimens included in our study are denoted by solid dots.

groups, we revise species boundaries for these snakes. Finally, we assess the conservation status of bushmasters in light of our findings.

MATERIAL AND METHODS

Population sampling and laboratory protocols

We obtained ventral scale clips of *Lachesis muta* from animals in private and public animal collections. Our tissue samples include all four subspecies of *L. muta* (Fig. 1) and two New World pitviper outgroups, *Atropoides nummifer* and *Agkistrodon contortrix*. We sequenced genes of 16 individuals of *L. muta* from 10 localities throughout Central

TABLE 1. Unique mtDNA lineages of *Lachesis* used for phylogenetic reconstruction. The subspecies, number of individuals with each haplotype, localities of origin, and the sources of tissue samples are listed for reference

mtDNA haplotype	Subspecies	Number	Localities	Source
Muta 1	*L. m. muta*	3	Tepoe, Surinam (2); Surinam, exact locality unknown (1)	D. Ripa Dallas Zoo, U.S.A.
Muta 2	*L. m. muta*	1	Surinam, exact locality unknown	Dallas Zoo, U.S.A.
Muta 3	*L. m. muta*	1	Napo Waimo River area, Ecuador	Dallas Zoo, U.S.A.
Muta 4	*L. m. muta*	3	Ribeirão Cascalheira (3), Mato Grosso, Brazil	Instituto Butantan, Brazil
Muta 5	*L. m. muta*	1	Pontes e Lacerda, Mato Grosso, Brazil	Instituto Butantan, Brazil
Rhombeata 1	*L. m. rhombeata*	2	Sao José do Lage, Alagoas, Brazil (1); São Paulino, Bahia, Brazil (1)	Instituto Butantan, Brazil D. Ripa
Rhombeata 2	*L. m. rhombeata*	1	Recife, Pemambuco, Brazil	Instituto Butantan, Brazil
Melanocephala 1	*L. m. melanocephala*	2	Rincon, Peninsula de Osa, Costa Rica (2)	D. Ripa
Stenophrys 1	*L. m. stenophrys*	2	Bri-Bri (1) and Chiroles (1), Costa Rica	D. Ripa

and South America (Table 1), including all subspecies as well as geographically distant localities from throughout the range of the widespread Amazon Basin subspecies (*L. m. muta*). Although the small number of samples limits interpretation of geographic genetic structuring within subspecies, they proved sufficient to elucidate phylogenetic relationships among bushmaster subspecies and their biogeographical history.

Total cellular DNA was isolated from frozen tissue samples by standard proteinase K extraction, followed by phenol/chloroform purifications (Maniatis, Frisch & Sambrook, 1982). Two segments of the mitochondrial genome were amplified with the polymerase chain reaction (PCR; Saiki *et al.*, 1988) and two pairs of primers. The regions sequenced correspond to 252 bases of the ND4 gene and 276 bases of the cytochrome *b* gene (cytb). The ND4 gene segment was amplified using primers ND4 (5′-CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC-3′) and LEU (5′-CAT TAC TTT TAC TTG GAT TTG CAC CA-3′) (Arévalo, Davis & Sites, 1994). Amplification conditions for the ND4 fragment consisted of 30 thermal cycles: 1 min denaturation at 93°C, 30 sec annealing at 56°C, and 2 min extension at 72°C, followed by a 5 min extension at 72°C. The cytb fragment was amplified using primers MVZ05 (5′-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3′) and MVZ 04′ (5′-GTA GCA CCT CAG AA[C/G/T] GAT ATT TG-3′). Amplification conditions for the cytb fragment consisted of 30 thermal cycles: 1 min denaturation at 94°C, 1 min annealing at 45°C, and 2 min extension at 72°C, followed by a 5 min extension at 72°C. In every case, one primer was marked with a biotinylated 5′ end. Four microliters of the resulting PCR products were eletrophoresed on a 1% agarose gel and visualized with ethidium bromide staining to verify product band size. Single-stranded template for sequencing was obtained directly from the remaining amplified product by use of Streptavidin-coated magnetic beads (according to manufacturer's protocol, Dynal, Inc.). The bead/DNA solution was used directly in dideoxy chain-termination sequencing (Sanger, Nicklen &

Coulson, 1977) with Sequenase Version 2.0 (U.S. Biochemicals) and ^{35}S labelled dATP. Sequences were obtained for only one direction, using primers ND4 and MVZ04′ in the sequencing reactions.

Data analyses

Sequences were read from one strand and aligned by eye to each other and to published sequences of *Xenopus* (Roe *et al.*, 1985). Pairwise sequence comparisons to determine the distribution and amount of variation, and levels of saturation by codon position were performed using the Molecular Evolutionary Genetics Program (MEGA, Version 1.01; Kumar, Tamura & Nei, 1993). Phylogenetic analysis was performed using aligned sequences for both gene regions combined (total 528 nucleotides). We used only unique mtDNA lineages for phylogenetic reconstruction (Table 1), so our final data set, including the two outgroup species, is composed of 11 unique mtDNA haplotypes. All mtDNA sequences included in this study have been entered in the GenBank/EMBL databases under accession numbers U96015-U96034.

We used maximum likelihood (ML; Felsenstein, 1981, 1993) and maximum parsimony analysis (Swofford, 1997), in combination with various weighting schemes, for phylogenetic inference. Each base position was treated as an unordered character with four alternative states. Trees were rooted by outgroup comparisons with sequences of two New World pitvipers (*Akgistrodon contortrix* and *Atropoides nummmifer*). We reconstructed and evaluated maximum likelihood trees using the DNAML program in Phylip 3.5 (Felsenstein, 1993). In ML we used equal-weighting, where all substitutions are weighted equally regardless of type or codon position, and three differential transitions/transversion weighting schemes (ts/tv = 1/5, ts/tv = 1/10, and ts/tv = 1/15). Sequence of taxon entry in phylogenetic reconstructions can bias species position in the resulting tree (Maddison, 1991), so we used ten repeated randomized input orders for all ML analyses. Maximum parsimony phylogenies were estimated using the exhaustive search option in PAUP* 4.0 (Swofford *et al.*, 1996). We searched for most parsimonious trees by using four weighting schemes: one assuming equal weights for every codon position and the others downweighting only third-position transitions relative to all other substitution types (by a factor of 5, 10, and 15). For each weighting scheme, we also performed bootstrap analyses as a relative measure of clade support (Felsenstein, 1985; Hillis & Bull, 1993); these were based on 1000 replicates, each using the branch and bound algorithm. Parsimony and ML results were compared across all weighting methods for congruence of tree topologies.

Estimating evolutionary divergence times in Lachesis

Studies of mtDNA evolution among various vertebrate lineages indicate a mutation rate of approximately 2% sequence divergence per million years (Upholt & Dawid, 1977; Brown, George & Wilson, 1979), and this 'standard' rate has been used to date divergences in numerous other taxa (e.g. Meyer *et al.*, 1990; Thorpe *et al.*, 1994; Riddle, 1995). Recent evidence underscores variation in the rate of mtDNA evolution among vertebrates (Avise *et al.*, 1992; Martin, Naylor & Palumbi, 1992;

Rand, 1993, 1994), implying that rate calibrations for one group may not be appropriate for others. In particular, absolute rate-heterogeneity is associated primarily with body size and metabolic rate (Martin & Palumbi, 1993; Rand, 1994), such that endotherms and ectotherms exhibit distinct relationships, and rate of mtDNA evolution and an organism's body size are negatively correlated within each group. Rates and associated errors of clocks should thus be calibrated for a specific taxonomic group under study, based on the fossil record or vicariant events, and interpreted with caution (Rand, 1994). To more accurately estimate the temporal scale of diversification in bushmasters, we calibrated the rate of mtDNA evolution for reptiles based on other published studies in this group. The requirements for inclusion in our rate estimate were that the ectotherm should be roughly similar in mass to *Lachesis*, large adults of which weigh 3–5 kg (Greene, unpublished data), and that the 'known' divergence date (from fossils or geologic evidence) was at least 5 Mya (to avoid biases associated with very recent divergences). We then used the highest and lowest rates observed in the published studies to define boundaries of divergence times during the evolutionary history of *Lachesis*.

Five studies meet our criteria for estimating a 'reptile mtDNA rate' (Table 2). Lamb, Avise & Gibbons (1989) reported divergences and rates of evolution within and between species of tortoises based on RFLP analysis of the entire mtDNA genome; because they included more than one individual from within each species or lineage, we corrected for within-lineage sequence divergences (according to Avise *et al.*, 1992). Thorpe *et al.* (1994) applied a standard vertebrate clock (2%/my) to estimate colonization times for *Gallotia galloti* in the Canary Islands from two mainland ancestors; we combined their sequence data for cytochrome *b*, cytochrome oxidase subunit I, and 12S rRNA to estimate a mitochondrial divergence rate, assuming colonization occurred from ancestral populations at the time of the origin of the islands. We averaged the resulting rates from both putative ancestors and did not correct for within-lineage divergences, because the authors reported sequences for only one individual from each species. We estimated a mitochondrial divergence rate for xantusiid lizards from the cytochrome *b* and rRNA 12S sequence-based phylogeny of Hedges, Bezy & Maxson (1991), by assuming that the Cuban endemic *Cricosaura* diverged from other xantusiids as the proto-Antilles drifted from their original Middle American position, approximately 70–60 Mya (Crother & Guyer, 1996; Hedges, 1996). Finally, we used sequences for Galapagos iguanas and immediate outgroup taxa (Rassmann, 1997) and estimated their divergence rate, assuming speciation began shortly after geologic origin of the islands. In this study, our estimated rate combined 16S and 12S gene fragments and was averaged across both species.

RESULTS

Genetic differentiation

We obtained sequences of 528 base pairs (coding for 176 amino acids) from 16 individuals of *Lachesis muta* and one individual each of *Agkistrodon contortrix* and *Atropoides nummifer*. No substitutions causing frameshifts were present, and sequences from both gene segments were combined in the final analysis. Levels of uncorrected

TABLE 2. mtDNA evolution rates for reptile species or lineages. The evolutionary event, possible vicariant events, % sequence divergence, method of divergence estimation, and approximate divergence rate are listed for the five reptiles used in this study

Evolutionary event	Vicariant event (time at divergence, Mya)	% sequence divergence	Method of estimation	Divergence rate (%/my)	References
Xerobates, east/west	Bouse embayment (5.5)	5.3[a]	RFLP (entire mtDNA)	0.95	Lamb *et al.*, 1989
Gopherus/*Xerobates*	None[b] (23–15)	11.2[a]	RFLP (entire mtDNA)	0.48–0.75	Lamb *et al.*, 1989
Gallotia galloti, from ancestral populations	Origin/colonization of island (15.7)	12.5[c]	Sequence (cytb, COI, 12S averaged)	0.80	Thorpe *et al.*, 1994
Cricosaura/other xantusiids	Fragmentation of island arc (60–70)	32.6[c]	Sequence (cytb and 12S averaged)	0.47–0.50	Hedges *et al.*, 1991
Amblyrhyncus and *Conolophus*/mainland sister species	Origin/colonization of island (9–5)	6.6[c]	Sequence (12S and 16S averaged)	0.73–1.32	Rassmann (in press)

[a] % sequence divergence corrected for within lineage divergences: $p_{corr}=p_{xy}-0.5(p_x+p_y)$, where p_{xy} is the mean pairwise genetic distance between individuals in populations x and y, and p_x and p_y are nucleotide diversities within regions or populations (Avise *et al.*, 1993).

[b] Divergence time of genera estimated from fossil record.

[c] Average of uncorrected % sequence divergence between sister species.

TABLE 3. Percent sequence divergences (uncorrected) among all unique mtDNA *Lachesis* haplotypes and two outgroups (*Agkistrodon contortrix* and *Atropoides nummifer*). Above diagonal: mean pairwise sequence divergences; below diagonal: absolute nucleotide differences (for both gene segments combined)

		1	2	3	4	5	6	7	8	9	10	11
1	Stenophrys 1	—	0.053	0.074	0.080	0.078	0.076	0.083	0.076	0.078	0.146	0.167
2	Melanocephala 1	28	—	0.087	0.089	0.091	0.089	0.091	0.089	0.091	0.142	0.170
3	Muta 1	39	46	—	0.006	0.011	0.009	0.013	0.008	0.009	0.144	0.159
4	Muta 2	42	47	3	—	0.013	0.011	0.015	0.009	0.011	0.146	0.161
5	Muta 3	41	48	6	7	—	0.002	0.013	0.011	0.009	0.138	0.159
6	Muta 4	40	47	5	6	1	—	0.011	0.009	0.011	0.140	0.161
7	Muta 5	44	48	7	8	7	6	—	0.013	0.015	0.146	0.161
8	Rhombeata 1	40	47	4	5	6	5	7	—	0.002	0.144	0.163
9	Rhombeata 2	41	48	5	6	5	6	8	1	—	0.142	0.161
10	*A. contortrix*	77	75	76	77	73	74	77	76	75	—	0.148
11	*A. nummifer*	88	90	84	85	84	85	85	86	85	78	—

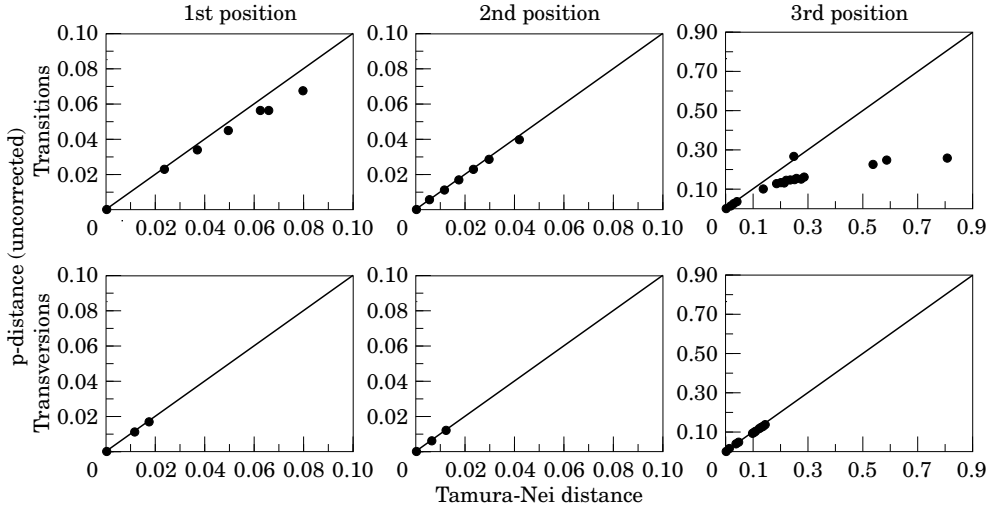

Figure 2. Saturation graphs for transitions and transversions at 1st, 2nd, and 3rd codon positions. Pairwise comparisons between all *Lachesis* haplotypes and two outgroups using uncorrected proportional distances and Tamura-Nei corrected distances are plotted for all six substitution categories. Deviations from the isometric line indicate that changes in that particular class of mutation are possibly biased due to 'multiple hits' at any one nucleotide position.

sequence divergence among the nine unique *Lachesis* haplotypes ranged from 0.2% (among samples of the South American forms) to 9.1% (between *L. m. melanocephal*a and South American forms; Table 3). Uncorrected sequence divergences between *Lachesis* and the outgroup taxa ranged from 13.8 to 17.0%. Of the total 528 characters, 138 were variable and 69 were phylogenetically informative.

To assess levels of saturation of base substitutions at each codon position, we plotted uncorrected percent sequence divergences against Tamura-Nei estimates of relative sequence divergence for transitions and transversions at 1st, 2nd, and 3rd codon positions (Fig. 2; modified from Moritz, Schneider & Wake, 1992; Villablanca,

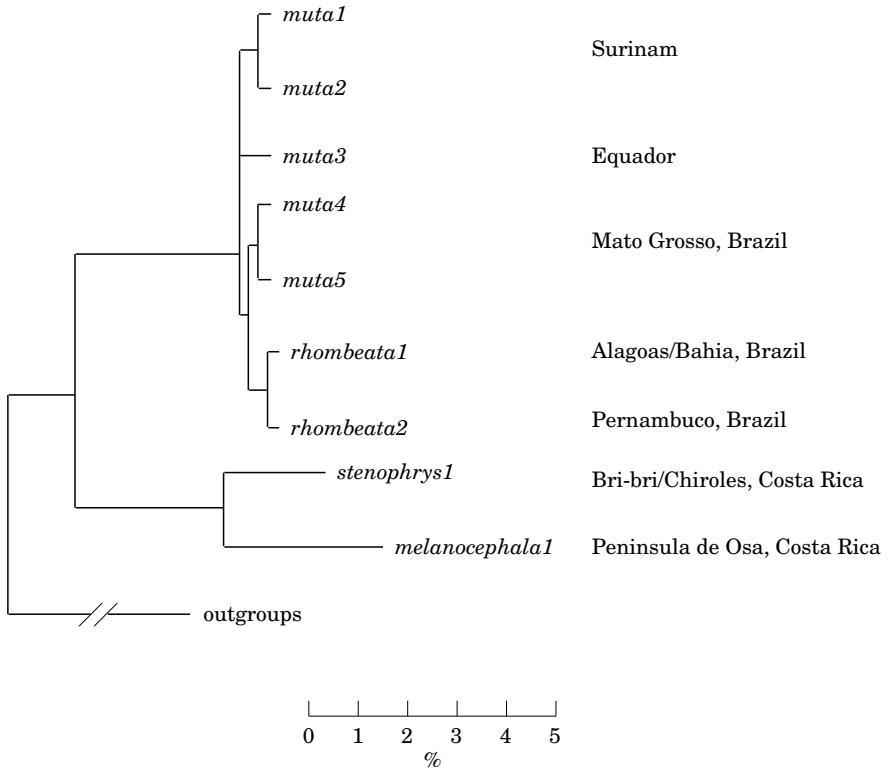

Figure 3. Maximum likelihood phylogeny for nine unique haplotypes of *Lachesis* with all characters weighted equally. The tree was rooted by the two outgroups sequenced in this study (*Atropoides nummifer* and *Agkistrodon contortrix*). Reconstructions with transitions and transversions weighted differentially are identical in topology to the tree shown here. Except where indicated, branches are drawn proportional to branch lengths estimated by the Maximum Likelihood algorithm and a % scale is included for reference.

1993). Non-isometric plots indicate increasing saturation of transitions or transversions at each codon position; third position transitions are potentially saturated and thus may possibly bias phylogenetic reconstruction because of 'multiple hits'. We therefore explored a number of different weighting schemes in our reconstructions including equal-weighting, downweighting of third position transitions relative to other substitutions, and differential weighting of transitions relative to transversions.

Phylogenetic relationships

All weighting schemes in parsimony resulted in three most parsimonious trees, and the strict consensus of the three trees is represented in Figure 4. Parsimony reconstruction under equal weighting resulted in three most parsimonious trees that were 183 steps in length (CI = 0.842, RI = 0.736). All differential weighting schemes resulted in 3 parsimonious trees that varied in length: L = 459 (for 3rd position transitions downweighted 1:5), L = 804 (1:10), and L = 1149 (1:15), but were consistent in other measures of fit (CI = 0.806, RI = 0.731). Maximum likelihood reconstructions

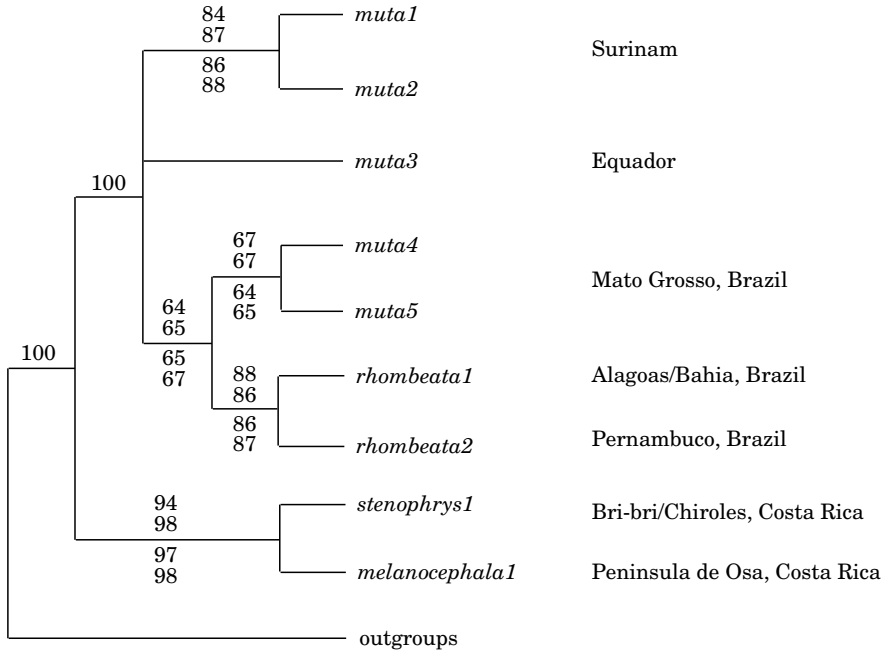

Figure 4. Strict consensus of three most parsimonious phylogenies for nine unique haplotypes of *Lachesis*. Numbers along the branches are bootstrap values from the four weighting schemes used in parsimony reconstruction. Bootstrap values were estimated from 1000 replicates and are listed (from top to bottom) for equal-weighting, and for third position transitions downweighted by a factor of 5, 10, and 15 relative to other substitution types. A single number is listed at nodes where bootstraps were identical for all weighting schemes.

yielded identical topologies to those obtained in parsimony. Ten independent ML reconstructions with equal weighting resulted in one tree (Fig. 3; LnL = −1608.4). Multiple ML runs with differential weighting of transitions and transversions resulted in identical topologies: LnL = −1577.5 for a ts/tv of 1:5, LnL = −1588.2 for ts/tv of 1:10, and LnL = −1598.3 for ts/tv of 1:15. These results suggest that the transition bias evident in third codon positions in our data does not affect phylogenetic reconstruction.

Maximum likelihood (Fig. 3) and maximum parsimony (Fig. 4) methods, under all weighting assumptions, yielded identical phylogenetic trees for populations of *Lachesis*. A single basal divergence separates the four allopatric subspecies of *L. muta* into South and Central American pairs. Further differentiation is present in the Central American forms: the unique mtDNA haplotypes of *L. m. stenophrys* and *L. m. melanocephala* from either side of the Central American Cordillera exhibit clear genetic differentiation. Divergence in the South American pair is less evident, in that the Amazon Basin (*L. m. muta*) and Atlantic Forest forms (*L. m. rhombeata*) are closely related and form a polytomy in our reconstruction.

Maximum parsimony bootstrap analyses and maximum likelihood branch lengths indicate the relative support for all clades in our phylogeny (Figs 3 and 4). The monophyly of both the South and Central American clades is supported by high bootstrap values (ranging from 94 to 100%), and long branches are indicative of

TABLE 4. Upper and lower time estimates for major divergences within *Lachesis* using the reptile mtDNA divergence rates. Overall sequence divergences are corrected for within-lineage variability according to Avise (1992)

Evolutionary divergence	Sequence divergence (%)	mtDNA clock rate (%/my)	Estimated time (Mya) (upper and lower)
Between South and Central America	8.44	0.47	17.9
		1.32	6.4
Between *stenophrys* and *melanocephala*	5.30	0.47	11.0
		1.32	4.0
Between *muta* and *rhombeata*	0.40	0.47	0.8
		1.32	0.3

deep differentiation between the two sister pairs. Divergence between the two Central American subspecies is also well supported, as is evident from the long branches in the ML reconstruction (Fig. 3). Our sampling allows for only tentative interpretation of divergences within the South American lineage, but most branches are relatively short and bootstrap resampling in the parsimony analysis offers limited support for geographic differentiation within this clade. Although the haplotypes representing the Atlantic Forest *L. m. rhombeata* are distinct and form their own clade (supported by bootstraps >85%), their phylogenetic placement is uncertain; there is some suggestion that *L. m. rhombeata* may be more closely related to particular populations of *L. m. muta* in southern regions of its distribution (e.g. Mato Grosso, Brazil). In any case, differentiation among the South American samples is less pronounced than between the Central American subspecies.

Rates of reptile mtDNA evolution and divergences in Lachesis

The reptilian mtDNA rates we estimated vary from 0.47 to 1.32%/my (Table 3) and, although the particular mtDNA genes used in the five published studies were different than those we used for *Lachesis*, all estimates are lower than the 2%/my commonly used 'vertebrate rate' (based primarily on data for mammals). Our rates are simply high and low point estimates based on five appropriate studies. Each of these estimates is only an approximate calibration, because they do not include corrections for sequence errors or saturation of changes at most variable codon positions. We are well aware of the difficulty of applying molecular clock estimates (e.g. Collins, 1996; Hillis, Mable & Moritz, 1996), and regard our 'ballpark' estimates of mtDNA divergence rates only as a useful starting point in formulating biogeographic hypotheses for small to medium-sized ectotherms. Accordingly, a lineage including *Lachesis* split from our outgroup pitvipers roughly 36–10 Mya, by the mid-Miocene and perhaps much earlier. Divergence between South and Central American *Lachesis* might have occurred 18.0–6.5 Mya, the split between Central American *L. m. melanocephala* and *L. m. stenophrys* perhaps took place 11–4 Mya, and

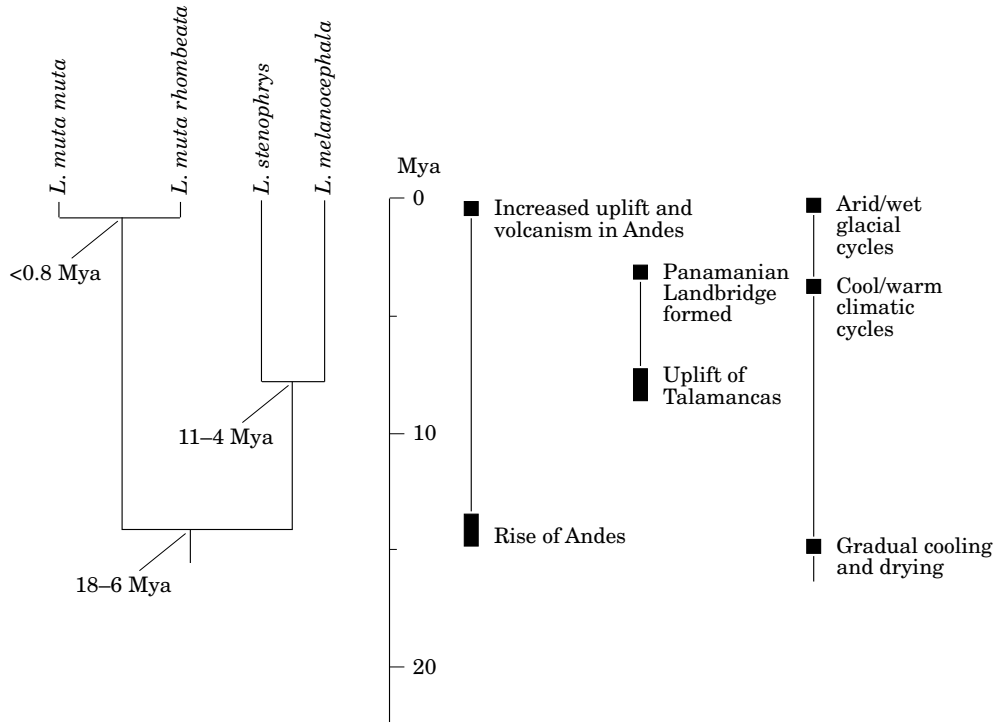

Figure 5. Summary diagram of evolutionary history of *Lachesis* populations. The current phylogeny of *Lachesis* (left) is drawn according to the geological scale and the time ranges estimated by our calibration are listed for each node. To the right of the scale are the relevant abiotic changes in Central and South America during this time period (modified from Potts and Behrensmeyer, 1992).

differentiation among the South American lineages happened only 300 000 to 800 000 years ago (Table 4 and Fig. 5).

DISCUSSION

Biogeographical implications

Morphological and molecular evidence thus far offers limited insights on the origin of *Lachesis*. Briefly, pitvipers probably diverged from other vipers in Eurasia during the early Tertiary, and invaded the New World via a Bering land bridge no later than the Miocene (Cadle, 1987; Kraus, Mink & Brown, 1996). Studies to date suggest that bushmasters are not basal to all other New World pitvipers or even to all other predominantly neotropical lineages, and there is as yet no strong evidence linking *Lachesis* with any particular other pitviper lineages (Kraus, Mink & Brown, 1996; Vidal *et al.*, 1997). Therefore, although these snakes are often assigned to South American faunal assemblages in biogeographic analyses, from the perspective of vicariance biogeography, we cannot at this point exclude the hypothesis that Central American *Lachesis* are remnants of initial colonization of the tropics (from the north) rather than more recent immigrants from South America.

The allopatric ranges of current subspecies, subdivided by major montane axes (the Andes and the Cordillera de Talamanca), suggest that vicariant geologic events underlie differentiation in these snakes. Bushmasters typically occur at elevations below 1000 m in moist tropical forests (Vial & Jímenez-Porras, 1967); the only exception is *L. m. melanocephala* in Costa Rica, which inhabits forests up to at least 1500 m (Solórzano & Cerdas, 1986). In addition to the mountain ranges currently separating *Lachesis* subspecies, our current understanding of Central and South American geological history suggests a dynamic picture of tectonic movement interspersed by temporary links between the two continents over the last 150 million years (Gentry, 1982a; Estes & Baez, 1985; Räsänen, Salo & Kalliola, 1987; Pindell & Barrett, 1990; Hoorne, 1993, 1994). Much of this history probably predates the evolution of *Lachesis* or its ancestors; however, given uncertainty about the first appearance of vipers in the New World and about the age of *Lachesis* (Cadle, 1987), we review several geologic events which may have occurred during the evolution of the bushmasters and, thus, may explain patterns of differentiation among populations in this species.

Most interpretations of geologic data infer two connections between Central and South America during the last 100 million years. The first occurred during the late Cretaceous or early Tertiary (90–60 Mya) and was not a continuous land bridge, but rather a series of volcanic arcs connecting North and South America (also referred to as the proto-Antilles; Gentry, 1982a; Pindell & Barrett, 1990; Crother & Guyer, 1996; Hedges, 1996). A northeastward drift of this system fragmented the distributions of taxa across this island bridge at the beginning of the Tertiary ($\sim$80 Mya). There was in fact faunal exchange between the two continents (including by dinosaurs, crocodilians, lizards, and primitive snakes) at the Cretaceous-Tertiary boundary (Estes & Baez, 1985). Both continents were separated to at least some degree by a marine barrier for much of the Tertiary, and this vicariant event has been implicated in the diversification of various groups of organisms, including some frogs, colubrid snakes, and angiosperms (Gentry, 1982a; Savage, 1982; Cadle, 1985).

The second proposed connection is the Pliocene formation of the Isthmian Link at approximately 3.5 Mya, during which extensive volcanism led to the uplift of islands that eventually coalesced into today's Isthmus of Panama (Coates & Obando, 1996). This re-establishment of a dispersal route between North and South America heavily influenced present distributions of a variety of organisms, particularly land mammals (Marshall *et al.*, 1982; Webb, 1991); however, although some taxa dispersed wholesale during this interchange (references in Stehli & Webb, 1985), current distributional patterns indicate minimal interchange for Central and South American amphibians and reptiles (Cadle, 1985; Vanzolini & Heyer, 1985). For those latter groups, dispersal subsequent to complete closure of the marine Panamanian Portal seems to have been limited to a few species which favor drier habitats (e.g. the neotropical rattlesnake *Crotalus durissus*), those conditions having predominated in the area at that time (Cadle, 1987). Despite the lack of a continuous dispersal route during most of the Tertiary, fossil and recent phylogeographic evidence suggests terrestrial faunal exchanges did occur between the continents during most of this time (Cadle & Sarich, 1981; Estes & Baez, 1985). Reptiles in particular must have moved between the northern and southern land masses, perhaps by means of shifting island chains that formed in the area occupied today by lower Central America.

Geological or climatic events with potentially major importance for neotropical species during the Cenozoic included the uplift of the Andes, the uplift of the Central

American highlands, and the advent of Pleistocene climatic fluctuations associated with glacial advances and retreats at higher latitudes. The Andean orogeny is complex. It is well established that certain parts of the Andes already existed during the Cretaceous (Van der Hammen, 1961; Kroonenberg, Bakker & Van der Wiel, 1990), although at much lower elevations. Mid-Miocene activity in the Andean axis uplifted much of the northern Cordillera to elevations above 1000 m approximately 14–11 Mya (Potts & Behrensmeyer, 1992; Guerrero, 1993), and this was followed by a second more dramatic uplift during the Pliocene and Pleistocene (Potts & Behrensmeyer, 1992) when the mountains reached their present elevations above 4000 m. The initial mid-Miocene uplift of the Andes is associated with wide-scale change in the Neotropical flora (Gentry, 1982a; Van der Hammen, 1989) and major physiographic changes in the Amazon Basin (Räsänen *et al.*, 1987; Hoorne, 1994). Our estimated time span for divergence between Central and South American clades of *Lachesis* (Table 4) overlaps that of a major uplift of the northern Andes (to above 1000 m) and the development of high montane vegetation in the mid Miocene (15–12 Mya).

A second major physiographic development, the uplift of the Central American highlands, might also have been an important vicariant event for bushmasters. Central American orogeny seems to have occurred from north to south, with montane habitats first forming during the Miocene (Savage, 1982; Coates & Obando, 1996). The uplift of the mountains of lower Central America (including the Cordillera de Talamanca, which presently separates the two Central American bushmasters in Costa Rica) occurred in the late Miocene or early Pliocene (8–5 Mya) and culminated in the Pliocene closure of the Panamanian Portal (Coates & Obando, 1996). This uplift fragmented a homogeneous lowland Central American herpetofauna into allopatric Atlantic and Pacific lowland assemblages (e.g. Savage, 1982; Crother *et al.*, 1992). Today the Atlantic lowlands are composed primarily of humid evergreen forests while the Pacific Versant, with the exception of southeastern Costa Rica, encompasses subhumid to semi-arid deciduous or thorn forests. Moist tropical forest habitat, inhabited by Pacific Coast *Lachesis*, is found on the Osa Peninsula and adjacent Golfo Dulce region. Our molecular data are consistent with a hypothesis that *Lachesis m. melanocephala* and *L. m. stenophrys* diverged during the late Miocene or early Pliocene, and their differentiation was at least broadly contemporary with uplift of the Cordillera de Talamanca, the range of mountains that now separates those taxa.

A final climatic event relevant to differentiation in *Lachesis* is the onset of temperature-glacial variations and global cooling in the Cenozoic. Global cooling accelerated in the late Neogene, with numerous reversals on all continental masses (Potts & Behrensmeyer, 1992), and culminated in large amplitude climatic oscillations over the last million years. Tropical climates during the Quaternary were unstable (Van der Hammen & Absy, 1994) and Pleistocene climatic cycles have received considerable attention as factors underlying regional areas of high endemism in a wide variety of Amazonian taxa, including birds (Haffer, 1969), lizards (Vanzolini & Williams, 1970), angiosperms (Prance, 1982, 1987), and butterflies (Brown, 1982). Proponents of the 'forest refugia' hypothesis suggest that lowland forest was fragmented into isolated patches during Pleistocene glacial cycles, resulting in patterns of differentiation observed today. This paleoclimatic speciation model has been widely critiqued (e.g. Cracraft & Prum, 1988; Bush, 1994; Colinvaux *et al.*, 1996; Vitt & Zani, 1996) and is to a certain extent untestable by studies of differentiation

in extant taxa, because there is no explicit expectation of area relationships imbedded within the model (Patton, in press). Nonetheless, available paleoenvironmental, climatic, and organismal evidence offers a complex scenario for tropical South America during the Pleistocene, and the possible effects of environmental changes on the genetic differentiation of tropical lowland taxa should be considered. In fact, the two South American bushmasters, *Lachesis m. rhombeata* and *L. m. muta*, are not strongly differentiated and evidently experienced gene flow in the recent past. Currently, those two weakly differentiated taxa are separated by an expanse of dry and unsuitable habitat between coastal Atlantic Forest and the Amazon Basin, and thus Pleistocene climatic and vegetational changes might underlie their differentiation.

Our results indicate that the oldest genetic divergences within *Lachesis* reflect vicariant events that isolated groups of populations in regions occupied by three of the four subspecies today (Fig. 5). Given early divergences between Central and South American clades, the ancestral *Lachesis* probably was continuously distributed in Amazonian-Pacific lowlands before fragmentation by the mid-Miocene uplift of the Andes. Molecular evidence also implies that the ancestral lineages of Central and South American *Lachesis* differentiated prior to formation of a continuous Panamanian Isthmus. Our temporal estimates of divergences also refute the forest refugia hypothesis for speciation in *Lachesis*, in that the deepest branching within this clade occurred much earlier in the Tertiary rather than during Pleistocene climatic cycles. The genetic imprint of Pleistocene events might be present in recent divergences among South American populations of *L. muta*, and a more detailed study within and between those subspecies will probably reveal diversification not evident in our results. Finally, our conclusion that initial divergence within bushmasters predates the Pliocene closing of the Panamanian portal underscores a continuing enigma in Middle American biogeography (see e.g. Hanken and Wake, 1982, for salamanders; Cadle, 1985, for other snakes), the interchange of terrestrial organisms across what is usually portrayed as a marine barrier. Although some vipers occasionally disperse over water (e.g. Lazell, 1964), there is no evidence that bushmasters do so. These large snakes are absent, for example, from the seemingly habitable Bocas del Toro archipelago although present on adjacent mainland Panama (R.I. Crombie, pers. comm.); their presence on Trinidad presumably reflects prior residency on that continental shelf island (cf. Henderson & Hedges, 1995).

Species concepts and bushmaster taxonomy

Throughout this century bushmasters have been regarded as a single polytypic species (e.g. Peters & Donoso-Barros, 1970; Hoge & Romano-Hoge, 1978; Campbell & Lamar, 1989), in keeping with a widely prevalent 'inertial species concept' (Good, 1994: 194): taxa are "treated as conspecific because herpetologists are used to them being conspecific, not because evidence for or against conspecificity has been rigorously examined." As Ripa (1994) noted, *Lachesis m. muta, L. m. melanocephala*, and *L. m. stenophrys* are substantially distinctive among themselves, there are no confirmed zones of intergradation or of overlapping occurrence (but see below), and no explicit justification exists for the current taxonomy of these vipers. Boulenger (1896) simply sunk Cope's (1875) *L. stenophrys* into *L. muta* without comment, and Solórzano and Cerdas (1986) described *L. m. melanocephala* without defending their decision to treat it as a subspecies rather than a distinct species.

Morphological and behavioral differences among the subspecies of *Lachesis muta* remain poorly explored, but parallel our molecular results (Table 5). The Atlantic Forest bushmaster (*L. m. rhombeata*) resembles the widespread Amazonian subspecies (*L. m. muta*) in scalation, head and body shape, and behavioral response to danger, being weakly differentiated only by head colour pattern. Both Central American taxa are distinct from the South American bushmasters in scalation, head and body shape, and colour pattern. The Central American bushmaster (*L. m. stenophrys*) is distinct from the other three subspecies in scalation, palatine bone shape, and colour pattern, as is the black-headed bushmaster (*L. m. melanocephala*) in scalation, colour pattern, and defensive behaviour; the latter resembles South American bushmasters in certain morphological attributes, whereas the former is derived in those respects.

Our studies confirm that the four allopatric subspecies of bushmasters are morphologically and biochemically distinct. The concordance between morphological, behavioral, and molecular markers is evidence that at least three of these allopatric population groups are on separate evolutionary trajectories, likely having been isolated for long periods of time, and therefore are distinct evolutionary species (*sensu* Frost, Kluge & Hillis, 1992). Accordingly, we propose that they should be known as *Lachesis muta*, the South American bushmaster; *L. stenophrys* (as first described by Cope, 1875), the Central American bushmaster; and *L. melanocephala* (Solórzano & Cerdas, 1986; new combination), the black-headed bushmaster. Conversely, the Atlantic Forest bushmaster is weakly differentiated morphologically and molecularly, and our mtDNA data suggest that some populations of Amazonian *L. m. muta* might be more closely related to the Atlantic *L. m. rhombeata* than to other populations of *L. m. muta* (likely on geographic grounds as well). The Atlantic bushmaster will continue to be recognized as a subspecies by those who feel that category fills a useful role in systematics, but we see no reason to upgrade that taxon to species status.

The bushmasters of eastern Panama and the Pacific lowlands of Colombia and Ecuador remain problematic. Previous studies of other taxa have demonstrated a close affiliation between species in the Chocó lowlands of northwestern South America and those in Central America (Haffer, 1967; Gentry, 1982b; Chapman, 1917; Brumfield & Capparella, 1996). Campbell & Lamar (1989) believed that Chocó populations of *Lachesis* are probably referable to the widespread Central American taxon (*L. stenophrys*), although Martínez & Bolaños (1982) regarded a specimen from eastern Panama as *L. m. muta*, based on its high ventral count. We think that interbreeding in nature between Central and South American bushmasters is highly unlikely, given the extent of unsuitable habitat in the Andes and the deep mtDNA divergence between those clades. Nevertheless, a range-wide analysis of morphological and molecular variation in bushmasters with particular emphasis on northwestern South American will clearly be relevant to hypotheses about the derivation of organisms in the Chocó region (Chapman, 1917; Haffer, 1967; Brumfield & Capparella, 1996).

Conservation

Our findings have immediate implications for bushmaster conservation, in that they underscore the distinctiveness of each of the Central American forms as well as their precarious status. Rather than weakly differentiated subspecies of a widespread

TABLE 5. Morphological and behavioral variation among bushmasters

Characters	*L. m. muta*	*L. m. rhombeata*	*L. melanocephala*	*L. stenophrys*	Source
Ventral scales	213–230				Boulenger, 1896; Roze,
Males	>214	>214, 223–225	211–216	198–204	1966; Peters & Donoso-
Females	>225	>226	209–216	199–209	Barros, 1970; Solórzano & Cerdas, 1986
Caudal scales				49, 36–37	Cope, 1875; Boulenger,
Males		34–37	44		1896; Solórzano & Cerdas,
Females	35–36				1986
Dorsal scales				35–37	Boulenger, 1896;
Males		35	36–38	33–36	Solórzano & Cerdas, 1986
Females	35		36–40	33–38	
Prenasal scales	Enlarged, protruberant, triangular	Enlarged, protruberant, triangular	Reduced, flat, rounded	Reduced, flat, rounded	Ripa, 1994
Internasal scales	Enlarged	Enlarged	Reduced	Reduced	Ripa, 1994
Canthal scales	Elongate, distinct, upraised	Elongate, distinct, upraised	Oval, indistinct, flattened	Oval, indistinct, flattened	Ripa, 1994
Supralabials	8–11	8–11	7–9	7–9	Ripa, 1994
Head pattern	Small distinct spots, narrow postocular stripe, white border	Large distinct spots, wide postocular stripe, no white border	Black	Unspotted	Peters & Donoso-Barros, 1970; Ripa, 1994
Anterior lateral blotches	Rhomboid	Rhomboid	Vertical bars	Vertical bars	Ripa, 1994
Head shape	Small, thin	Small, thin	Large, blunt	Large, blunt	Ripa, 1994
Body shape	Round	Round	Laterally compressed	Laterally compressed	Ripa, 1994
Anterior surface of palatine bone	Concave	Unknown	Concave	Straight	Solórzano & Cerdas, 1986; Greene, unpublished data
Defensive behaviour	Usually calm	Usually calm	Aggressive	Usually calm	Solórzano & Cerdas, 1986; Ripa, 1994

Amazonian snake, these are well differentiated lineages, the result of an ancient divergence from South American populations and subsequent diversification within Central America. *Lachesis stenophrys* and especially *L. melanocephala* have extremely small overall distributions (Campbell & Lamar, 1989; Greene & Campbell, 1992), and both are restricted to relatively undisturbed tropical wet forests (Vial & Jímenez-Porras, 1967; Solórzano & Cerdas, 1986). Each species occurs within the Costa Rican National Parks system (e.g. *L. stenophrys* at the La Selva Biological Preserve and adjacent lower reaches of Braulio Carillo National Park, *L. melanocephala* in Corcovado National Park), but outside of those and other reserves within their distributions, most remaining low and middle elevation forest has been converted to agriculture (e.g. Monge-Nájera, 1994). The range of each of these snake taxa is already severely fragmented by habitat destruction, and each species is undoubtedly subject to persecution by humans (e.g. wanton killing, commercial collecting). Bushmasters clearly warrant special consideration from wildlife agencies in Costa Rica, Nicaragua, Panama, and perhaps elsewhere.

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