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Tracing an invasion: landbridges, refugia, and the phylogeography of the Neotropical rattlesnake (Serpentes: Viperidae: *Crotalus durissus*)

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Abstract

Pleistocene fragmentation of the Amazonian rainforest has been hypothesized to be a major cause of Neotropical speciation and diversity. However, the role and even the reality of Pleistocene forest refugia have attracted much scepticism. In Amazonia, previous phylogeographical studies have focused mostly on organisms found in the forests themselves, and generally found speciation events to have predated the Pleistocene. However, molecular studies of open-formation taxa found both north and south of the Amazonian forests, probably because of vicariance resulting from expansion of the rainforests, may provide novel insights into the age of continuous forest cover across the Amazon basin. Here, we analyse three mitochondrial genes to infer the phylogeography of one such trans-Amazonian vicariant, the Neotropical rattlesnake (*Crotalus durissus*), which occupies primarily seasonal formations from Mexico to Argentina, but avoids the rainforests of Central and tropical South America. The phylogeographical pattern is consistent with gradual dispersal along the Central American Isthmus, followed by more rapid dispersal into and across South America after the uplift of the Isthmus of Panamá. Low sequence divergence between populations from north and south of the Amazon rainforest is consistent with mid-Pleistocene divergence, approximately 1.1 million years ago (Ma). This suggests that the Amazonian rainforests must have become fragmented or at least shrunk considerably during that period, lending support to the Pleistocene refugia theory as an important cause of distribution patterns, if not necessarily speciation, in Amazonian forest organisms. These results highlight the potential of nonforest species to contribute to an understanding of the history of the Amazonian rainforests themselves.

Keywords: Amazonia; biogeography; *Crotalus durissus*, Isthmus of Panamá, phylogeography, Pleistocene refugia

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Introduction

The Neotropical region is one of the most biologically diverse parts on Earth. Three major biogeographical events have been highlighted as key factors in causing present-day patterns of distribution and diversity: the uplift of the Andes, the emergence of the Isthmus of Panamá, and Pleistocene climatic and vegetational fluctuations in tropical South America.

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The final uplift of the Andes in the late Tertiary (Gregory-Wodzicki 2000) created a formidable barrier between the Pacific and Amazonian lowlands of South America, separating the flora and fauna of the Pacific lowlands from that of the eastern versant and the Amazonian lowlands on the eastern side.

The late-Tertiary uplift of the Isthmus of Panamá above sea level led to the final closure of the Central American Seaway approximately 3.5–3 million years ago (Ma) (Coates & Obando 1996). This connected the North and South American landmasses, previously isolated through much of the Cainozoic, and resulted in a large-scale dispersal of

terrestrial faunal elements, a phenomenon referred to as the great American biotic interchange (GABI) (Marshall *et al*. 1979; Stehli & Webb 1985; Webb & Rancy 1996). This phenomenon was particularly marked in mammals, where much of the evidence suggests two phases of faunal interchange, beginning approximately 2.5 Ma. In contrast, in reptiles, there is evidence that many taxa, including several groups of pit vipers, dispersed from North to South America (or vice versa) throughout the late Cainozoic, from the Oligocene onwards (Estes & Báez 1985; Zamudio & Greene 1997; Wüster *et al*. 2002).

Despite the intensity of discussion of the GABI, there have been few detailed phylogeographical studies of organisms likely to have participated in the interchange. No published phylogeographical studies of Neotropical organisms have shown the pattern that might be predicted for such a colonization event: a paraphyletic and highly divergent set of mitochondrial DNA (mtDNA) haplotype clades in the continent of origin, and a monophyletic set of haplotypes (or several in case of multiple colonization events) in the colonized continent nested within them, with levels of sequence divergence consistent with late Pliocene cladogenesis.

The third major event hypothesized to have affected Neotropical diversity patterns is the series of global climatic fluctuations that occurred throughout the late Pliocene and Pleistocene (Potts & Behrensmeyer 1992). However, both their nature and their importance in affecting Neotropical biodiversity remain hotly contested. The Pleistocene refugia hypothesis states that drier climatic phases caused a fragmentation of the Amazonian rainforest into forest refugia isolated by savannahs, leading to increased diversity due to allopatric speciation among forest species (Haffer 1969; Prance 1973). However, the nature, significance, and even reality of Pleistocene rainforest fragmentation remain highly controversial (Colinvaux *et al*. 2000, 2001; Haffer & Prance 2001; Hooghiemstra 2001). In particular, critics of the hypothesis state that, at least during the Last Glacial Maximum (LGM), palynological evidence provides little support for widespread savannah vegetation in Amazonia (e.g. Colinvaux *et al*. 1996, 2001; Kastner & Goñi 2003), speciation in many widespread Neotropical species complexes predates the Pleistocene climatic fluctuations, often by a considerable margin (reviewed by Moritz *et al*. 2000), and the expected genetic consequences of postrefugial Quaternary range expansion, so widespread in temperate biota (e.g. Hewitt 2004), have not been detected in Neotropical forest mammals, at least in the southwestern Amazon (Lessa *et al*. 2003).

Most previous studies seeking to elucidate the biogeographical history of the Amazonian rainforests by means of genetic methods understandably focused on species found within these forests, and dates of speciation in them (Moritz *et al*. 2000), and generally ignored species occurring outside the rainforests (Pennington *et al*. 2000). However, taxa occuring both north and south of Amazonia, but not inside, can potentially provide more definitive information on rainforest fragmentation than the rainforest species themselves (Pennington *et al*. 2004). Pre-Pleistocene speciation in rainforest taxa only indicates that Pleistocene fragmentation did not cause the speciation event, but cannot disprove its occurrence. However, vicariant nonrainforest taxa separated by the Amazonian forest must have had a contiguous distribution at some point in the past. The time of this vicariance represents the maximum age for the last genetic exchange across the Amazon: the rainforest must have been fragmented at that time, and continuous forest cover cannot predate that time. Thus, evidence of recent continuous distributions in presently vicariant nonrainforest organisms would represent unambiguous evidence of recent rainforest fragmentation or contraction. The times of such vicariance events can be inferred using molecular methods, a line of research that has undergone considerable development in recent years (e.g. Sanderson 2002, 2003; Yang & Yoder 2003).

A promising organism for a phylogenetic study of the effects of both the GABI and possible Pleistocene rainforest fragmentation in the Amazon Basin is the *Crotalus durissus* (Neotropical rattlesnake) complex. These snakes occur in seasonally dry formations from Mexico to northern Argentina, but are absent from the Central American and Amazonian rainforests, resulting in a highly disjunct distribution (Campbell & Lamar 1989, 2004). Rattlesnakes are a primarily Nearctic clade that probably originated in north central Mexico (Place & Abramson 2004), the *C. durissus* complex being the only representative to extend south of Mexico. Post-isthmian dispersal by *C. durissus* from Central to South America has been assumed by various authors (e.g. Estes & Báez 1985; Vanzolini & Heyer 1985), and corroborated by a preliminary phylogeographical study (Wüster *et al*. 2002).

The highly discontinuous distribution of *C. durissus* in South America, including open habitats both north and south of the Amazon rainforest as well as isolated open formations within it, but avoiding the forest itself, adds considerable interest to a phylogeographical study of the complex. Large rattlesnakes are unlikely candidates for aeolian dispersal, and oceanic dispersal around the coast of northeastern South America is equally unlikely, as the prevailing North Brazil current runs southeast to northwest, against the direction of dispersal. Consequently, the discontinuous distribution of *C. durissus* is most likely due to vicariance, the timing of which can be inferred using molecular methods. Here, we present an mtDNA-based phylogeographical study of the *C. durissus* complex, with the specific objective of elucidating the timing of the vicariance between populations from north and south of the Amazon rainforests.

Materials and methods

Sampling, molecular methods

We obtained tissue (ventral scale clippings), blood samples, or shed skins from specimens covering most of the range of the *Crotalus durissus* complex, as well as of the related *C. molossus-basiliscus* group (Murphy *et al*. 2002): reported hybridization between that group and *C. durissus* (Campbell & Lamar 1989) suggests that these taxa are closely related and not necessarily reciprocally monophyletic. Sampling localities are shown in Fig. 1.

Total DNA was extracted by standard methods (Sambrook *et al*. 1989). Three regions of mtDNA molecule were amplified using polymerase chain reaction (PCR): a 767-base pair (bp) section of the gene for cytochrome *b* (cyt*b*), a 900-bp region of NADH dehydrogenase subunit 4 (*ND4*), and a 1067-bp region of NADH dehydrogenase subunit 2 (*ND2*). Primers for cyt*b* and *ND4* are given in Pook *et al*. (2000), the *ND2* primers were L4437b (Kumazawa *et al*. 1998) and tRNA-trpR (Ashton & de Queiroz 2001). For PCR conditions, see Pook *et al*. (2000) and Wüster *et al*. (2005). Automated sequencing was carried using BigDye Terminator Ready Reaction Mix (ABI), followed by analysis on an ABI Prism 377 DNA sequencer according to the manufacturer's instructions.

Cyt*b* and *ND4* were sequenced for all available specimens, and combined with sequences already available from GenBank. We then sequenced the *ND2* gene for representative specimens in order to increase resolution and statistical support for critical nodes. *Crotalus viridis* and *C. tigris* were selected as outgroups, based on the results of Murphy *et al*. (2002) showing them to lie outside the *C. durissus* group. See Appendix for details.

Data analysis

Sequences were aligned by eye against the published sequence of *Dinodon semicarinatus* (Kumazawa *et al*. 1998), and checked for indels or stop codons, which would indicate the presence of nuclear copies (Zhang & Hewitt 1996). Because mtDNA evolves as a single linkage unit, we concatenated the different gene sequences for each specimen and analysed the data jointly, after testing for incongruence between gene trees by means of the incongruence length difference (ILD) test (Farris *et al*. 1994), with 1000 homogeneity replicates with 10 random addition sequence replicates each, using PAUP* 4.0b10 (Swofford 2002). We analysed two sets of data: a two-gene data set consisting of cyt*b* and *ND4* for all our samples plus sequences available from GenBank, and a three-gene data set, which also included the *ND2* sequences, but from fewer samples. We assayed

Fig. 1 Sampling localities for the *Crotalus durissus* complex. Locality numbers correspond to Figs 2 and 3. For details, see Appendix.

for the presence of a significant phylogenetic signal by means of the g1 tree skewness statistic (Hillis & Huelsenbeck 1992), calculated from 100 000 random trees generated by PAUP*.

The phylogeny of the complex was inferred using maximum parsimony (MP), maximum likelihood (ML), and Bayesian approaches. MP and ML analyses were performed using PAUP*, and Bayesian analysis using MRBAYES version 3.0 (Ronquist & Huelsenbeck 2003).

MP analysis involved heuristic searching with tree bisection-reconnection (TBR) branch swapping and 10 000 random addition sequence replicates. Internal support for nodes was assessed using nonparametric bootstrap analysis (Felsenstein 1985) under exclusion of uninformative characters, using 1000 bootstrap replicates with five random addition sequence replicates each and TBR (three genes) or SPR (two genes) branch swapping. Branch support values (Bremer 1994) were calculated through the converse constraint option of PAUP*.

For ML analyses, the appropriate model of sequence evolution was estimated using MODELTEST 3.0 (Posada & Crandall 1998). We selected the model giving the highest likelihood score, and fixed the parameters in a heuristic ML search, using a neighbour-joining (NJ) starting tree and TBR branch swapping. We then re-estimated the parameters from the resulting ML tree, and ran another heuristic search using these new settings and 10 random addition sequence replicates. ML bootstrapping involved 500 (three genes) or 100 (two genes) replicates, NJ starting trees, and nearest neighbour-interchange (NNI) branch swapping.

For Bayesian analysis, we used the model of sequence evolution estimated by MODELTEST, while allowing the analysis to estimate the relevant parameter values. Searches were run using four chains, over 2 million (2-gene data) or 5 million (3-gene data) generations, sampling every 1000th tree. Burn-in, the time taken for the parameters to reach stationarity, was estimated by plotting tree log-likelihood score against generation number, and determining the number of generations required to reach an asymptote. Trees sampled before completion of burn-in and a buffer period of nine times the burn-in period were discarded. The searches were run five times with random starting trees and results compared.

Because sequence information was available for more *C. durissus* specimens and other taxa for cyt*b* and *ND4* than for *ND2*, we used the two-gene data for the dating of biogeographically relevant nodes on the tree. Dating the nodes of a tree requires calibration points. Since there are no well-dated nodes that could serve as calibration points in the *Crotalus* phylogeny, we re-estimated the model of sequence evolution and re-ran the 2-gene ML analysis under inclusion of additional taxa that furnished appropriate calibration points:

1 We included sequences from three South American populations of the genus *Porthidium*, which postdate the uplift of the Isthmus of Panamá (Wüster *et al*. 2002), and fixed their basal divergence at 3.5 Ma.
2 Based on palaeontological evidence (Szyndlar & Rage 1990), we included sequences of two Asiatic cobras (*Naja naja* and *Naja kaouthia*) and two African cobras (*Naja nigricollis* and *Naja nivea*), and constrained the divergence between the Asian species and their African sister group to a minimum age of 16 million years (Myr).
3 The divergence between the Elapidae and the Viperidae was constrained to 95 Ma or later, based on the earliest colubroid fossils from the Cenomanian (Rage *et al*. 2003).

To assess the age of relevant nodes, we calculated estimates of time of divergence with confidence limits using the Langley–Fitch (LF) method (Langley & Fitch 1974), which reconstructs divergence times under the assumption of a molecular clock, and the semiparametric, penalized likelihood (PL) method (Sanderson 2002), which makes no assumptions of clock-like sequence evolution. Both methods used the truncated Newton (TN) algorithm, and were implemented in the program R8S (Sanderson 2003).

Results

Sequence analysis

For the two-gene analysis, we aligned 1332 bp (657 bp of *ND4* and 675 bp of cyt*b*) from 82 specimens (including outgroups), which yielded 59 unique haplotypes. For the three-gene analysis, we aligned an additional 671 bp of *ND2* gene sequence, resulting in 39 haplotypes from 41 specimens. Sample, haplotype and GenBank Accession nos are in Appendix. The sequences contained no nonsense codons, indels or frameshifts. High transition:transversion ratios and the expected excess of third codon position substitutions confirmed their mitochondrial origin.

The two-gene data contained 459 variable and 365 parsimony-informative positions, the three-gene data contained 615 variable and 417 parsimony-informative positions. The 100 000 random trees generated from the two-gene and three-gene data showed a highly skewed distribution (g1 = −0.4705 and g1 = −0.8579), indicating a significant phylogenetic signal ($P < 0.01$; Hillis & Huelsenbeck 1992). The ILD test provided no indication of incongruence between the three genes ($P = 0.892$), validating the joint analysis of all three gene sequences.

Phylogenetic analysis and dating

The two-gene MP analysis yielded 601 equally most parsimonious trees (EMPTs) of 1066 steps (c.i. = 0.4747,

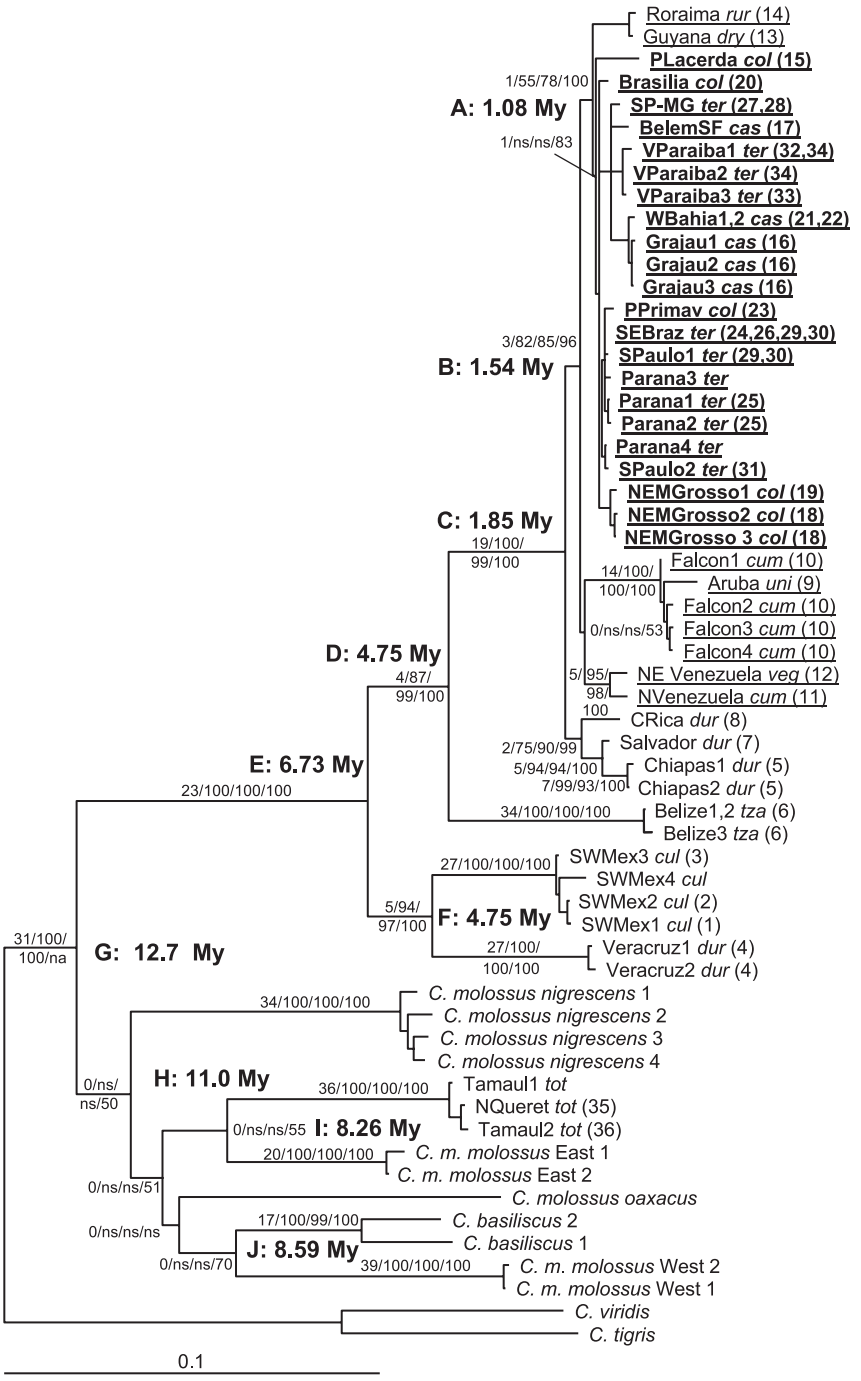

Fig. 2 Maximum-likelihood phylogram of the two-gene analysis (–ln L = 7091.51851). Tip labels are as follows: haplotype name (see Appendix), 3-letter subspecies code for the *Crotalus durissus* complex, following Campbell & Lamar (1989) (*cas*, *cascavella*; *col*, *collilineatus*; *cul*, *culminatus*; *cum*, *cumanensis*; *dry*, *dryinas*; *dur*, *durissus*; *ter*, *terrificus*; *tza*, *tzabcan*; *uni*, *unicolor*; *veg*, *vegrandis*), and, in brackets, locality code (numbers correspond to Fig. 1). Samples from South America are underlined, bold type indicates haplotypes from south of the Amazon, for details, see Appendix. Numbers along branches indicate Bremer support/MP bootstrap support/ML bootstrap support/percentage Bayesian posterior probability. Dates of nodes are penalized likelihood estimates (see Table 1). For clarity, support is only shown for important nodes; ns, not supported, na, not applicable.

r.i. = 0.8329), and the three-gene analysis 64 EMPTs of 1201 steps (c.i. = 0.5167; r.i. = 0.7627). For ML analysis, the MODELTEST program selected the GTR + I + Γ model as optimal for both data sets. The resulting trees are shown in Figs 2 and 3, and the distribution of clades is mapped in Fig. 4. MP and ML analyses agreed on all robustly supported nodes, differences being restricted to minor rearrangements within the major clades and lack of resolution in the *totonacus-basiliscus-molossus* group in the case of MP.

Bayesian analyses completed burn-in after approximately 10 000 generations for both data sets, but we conservatively discarded the first 100 000 generations. Multiple random starting tree replicates yielded virtually identical likelihood estimates and tree topologies.

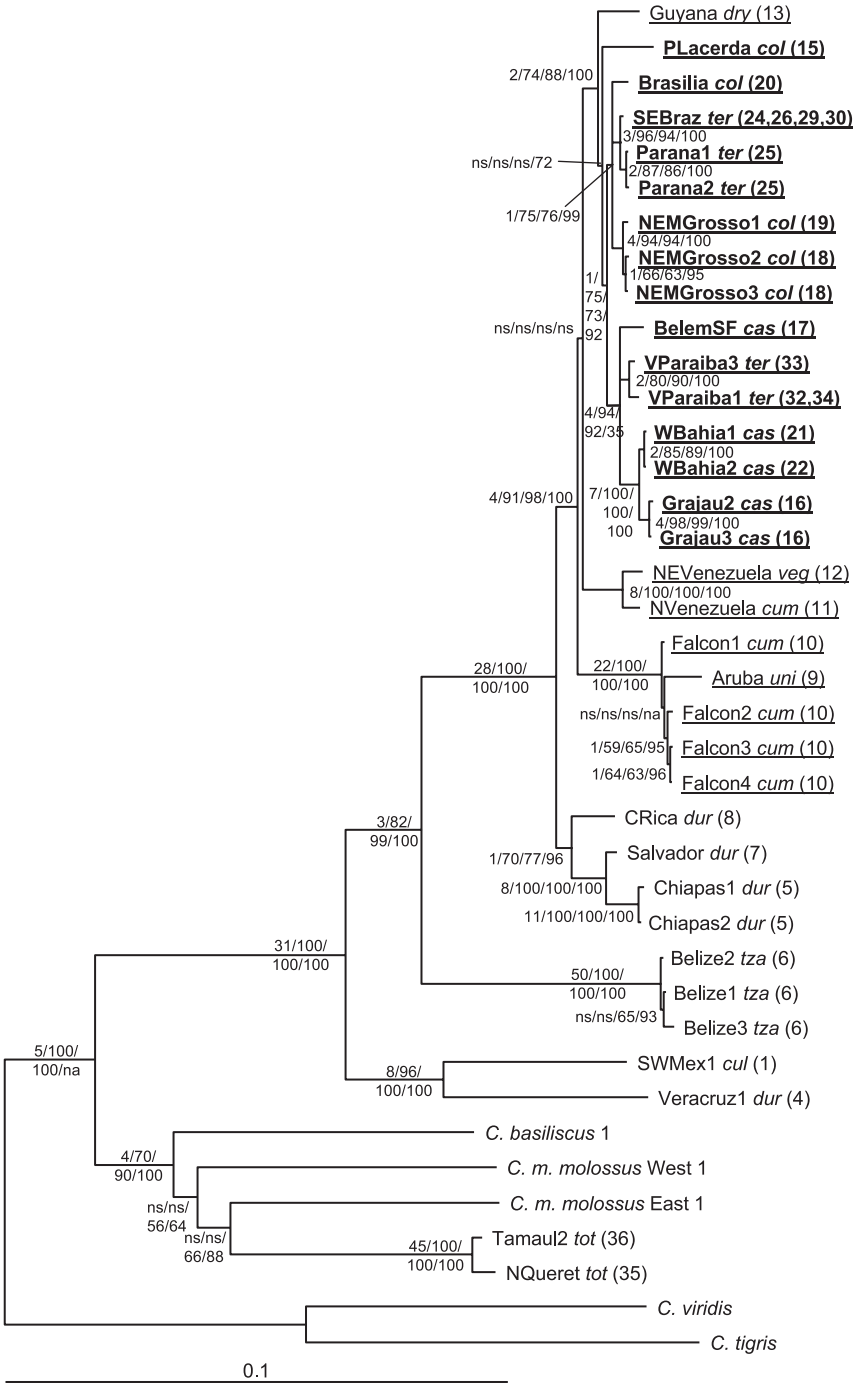

Fig. 3 Maximum-likelihood phylogram of the three-gene analysis. –ln L = 8616.50208. See Fig. 2 for explanations.

All trees agreed on the following biologically relevant aspects of the phylogeny of the complex:

1 The basic pattern consists of the sequential hierarchical nesting of southern haplotype clades within paraphyletic northern groups across South and Central America (strongly supported).

2 Monophyly and low levels of sequence divergence within South America, contrasted with paraphyly and high levels of divergence in Central America (strongly supported).

3 Monophyly of the populations from south of the Amazon (weakly supported), nested within a paraphyletic northern South American assemblage (strongly supported in ML and Bayesian analyses).

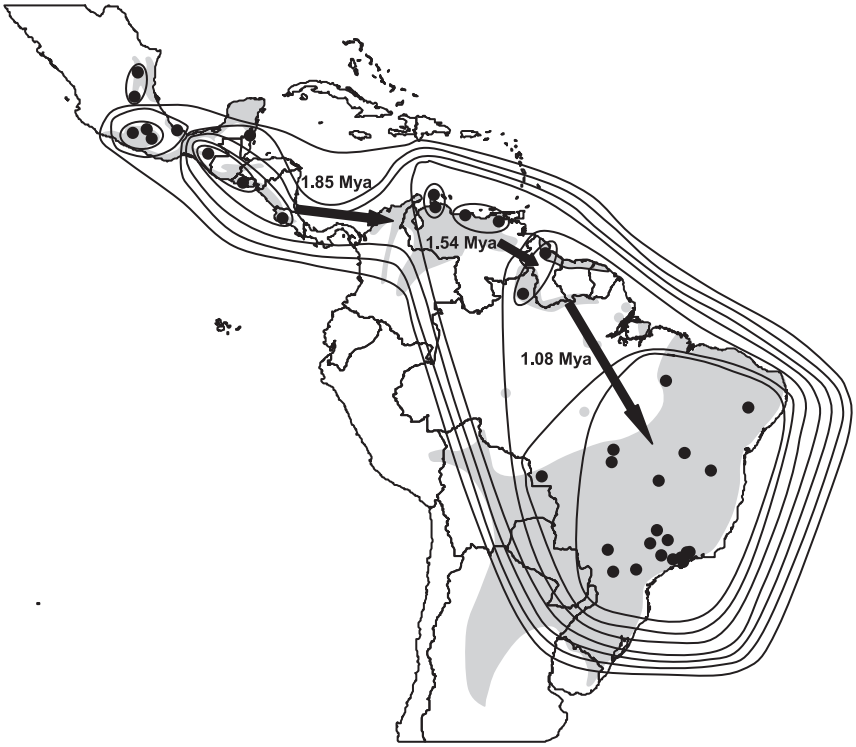

Fig. 4 Clade distribution, major dispersal events and their estimated dates in the *Crotalus durissus* complex. Solid lines indicate haplotype clades, grey shading the range of the complex.

Table 1 Estimated ages and confidence limits of important tree nodes. Some confidence limits could not be calculated because of lack of a crossover point

Node label in Fig. 2	Node description	Est. age in Myr (confidence limits), Langley–Fitch	Est. age in Myr (confidence limits), penalized likelihood
A	Trans-Amazon vicariance	1.16 (0.79–1.54)	1.08 (0.74–1.51)
B	Basal S. American divergence	1.67 (1.35–2.08)	1.54 (1.2–1.96)
C	South America vs. Central America	1.97 (1.63–2.45)	1.85 (1.47–2.31)
D	Yucatán vs. Central and South America	4.78 (n/a)	4.75 (n/a)
E	Basal cladogenesis in *C. durissus*	6.45 (n/a)	6.73 (n/a)
F	SW. vs. E. Mexico	4.48 (n/a)	4.75 (n/a)
G	*C. durissus* vs. *C. molossus* group	11.26 (10.32–12.53)	12.70 (11.09–14.84)
H	Basal cladogenesis in *C. molossus* group.	9.35 (8.26–10.53)	11.03 (9.39–13.07)
I	*C. totonacus* vs. eastern *C. m. molossus*	6.72 (5.76–7.84)	8.27 (6.52–10.13)
J	*C. basiliscus* vs. western *C. m. molossus*	7.16 (6.37–8.19)	8.59 (n/a)

4 Polyphyly of the *Crotalus durissus* complex, in that the *Crotalus durissus totonacus* haplotypes are nested among sequences of *Crotalus molossus* and *Crotalus basiliscus*, not with other *C. durissus*.

The ML tree of the two-gene data with additional taxa for the analysis of times of divergence (not shown) corresponded to the existing 2-gene tree. The results of the dating analyses are shown in Table 1.

Discussion

Biogeography

Our results reveal a classical pattern of stepwise colonization progressing from a northern centre of origin in Mexico to northern South America, and across the Amazon Basin. The pattern consists of a set of nested clades, in which any southern clade is nested within a

paraphyletic group consisting of more northerly haplotype clades. Thus, the monophyletic southern South American populations are nested within in a paraphyletic northern South American group; the South American clade is nested within a paraphyletic Central American group; and a clade including specimens from South America and southern/nuclear Central America is nested within a paraphyletic northern Central American group.

Deep divergences between the clades inhabiting Mexico and Central America suggest ancient cladogenesis: the four major Central American clades (*Crotalus durissus culminatus*, northern *Crotalus durissus durissus*, *Crotalus durissus tzabcan* and southern *C. d. durissus*) diverged from each other during the late Miocene/early Pliocene (Table 1). On the other hand, divergences within South America are small and indicative of relatively recent cladogenesis: in particular, the divergence between the populations south of the Amazon and their Guyanan sister group occurred approximately 1.1 Ma (Table 1; Node A, Fig. 2). The biogeographical scenario represented by these data is one of slow, progressive stepping-stone colonization from Mexico down the Central American peninsula towards South America, followed by much more rapid and recent dispersal first into northern South America, and then across the Amazon Basin into southern South America (Fig. 4).

Webb & Rancy (1996), in an insightful comment, described Central America as playing 'a particularly important role as a tropical staging area in which northern groups became adapted to tropical American conditions, especially to tropical savannahs'. This pattern is very evident in *C. durissus*: the presence of multiple older Central American lineages suggests a pattern of gradual range expansion south and east, corresponding to the phase of adaptation suggested by Webb & Rancy (1996).

The pattern of colonization of South America by *C. durissus* is consistent with our understanding of late Pliocene and Quaternary climate and vegetational change (Burnham & Graham 1999) and the pattern of colonization described for South American mammals (Webb & Rancy 1996). Webb & Rancy note two phases of biotic interchange between Central and South America: an early phase involving primarily savannah animals (large grazers) followed by a later exchange of forest animals. This is related to the postulated presence of savannah vegetation in Isthmian Central America in the early Quaternary, followed by its displacement in the later part of the Pleistocene. Webb & Rancy (1996) note that those grazing species that spread from northern temperate to southern temperate latitudes had completed this process by the middle Pleistocene. In *C. durissus*, the basal South American cladogenesis appears to date back to the early Pleistocene, and trans-Amazonian vicariance to the middle Pleistocene (Table 1), a time frame that corresponds approximately to that observed in savannah mammals.

The middle Pleistocene timing of the trans-Amazonian vicariance of South American *C. durissus* estimated by both the clock-based LF algorithm and the nonclock-dependent PL analysis has profound implications for our understanding of the history of the rainforest cover of South America: the Amazonian forests must have shrunk or become fragmented sufficiently to establish a continuous corridor of more seasonal or open vegetation from the northern coast of South America to the open formations south of Amazonia at least once during the Pleistocene. Moreover, the present-day distribution of *C. durissus* suggests that the extent of rainforest fragmentation was considerable: the remnant populations of *C. durissus* along the coasts of the Guyanas, Amapá, and Marajó Island could be explained by limited shrinking of the rainforests, but those isolated deep inside Amazonia (Humaitá, Santarém, Serra do Cachimbo: Campbell & Lamar 2004; Fig. 4) suggest more extensive fragmentation, as proposed by the refugia hypotheses. Future phylogeographical studies including these isolated populations, and additional gene sequences for increased resolution, could provide additional evidence of dispersal routes of *C. durissus* across the Amazon, and thus reveal patterns of rainforest fragmentation.

There have been few other phylogeographical studies of open formation vicariants from north and south of the Amazon. Salazar-Bravo *et al*. (2001) estimated a time of divergence at 8.2 Ma between the northern and southern lowland sister clades of field mice of the genus *Calomys*, thus precluding Pleistocene events as a causal factor, but matching the age of many older Amazonian sister species across a variety of taxa (Moritz *et al*. 2000). Similarly, Pennington *et al*. (2004) did not detect any Pleistocene trans-Amazonian sister-group relationships in dry-forest plants, but noted that phylogeographical analyses of single species found in multiple dry-forest enclaves may prove revealing. Eberhard & Bermingham (2004) found mtDNA sequence divergences consistent with early mid-Pleistocene vicariance in the parrot *Amazona ochrocephala*, paralleling the results of this study. Palaeontological evidence of primarily grazing mammals in western Amazonia, as well as the apparent passage of early, savannah-adapted northern immigrant mammals across Amazonia to southern South America (Webb & Rancy 1996) are equally supportive of a hypothesis of middle Pleistocene forest fragmentation.

The suggested early to mid Pleistocene time for trans-Amazonian vicariance may be important, as much of the palynological evidence used to discredit the Pleistocene refugia hypothesis by its critics stems only from the late Pleistocene (e.g. Colinvaux *et al*. 1996; Kastner & Goñi 2003). Extensive global climatic fluctuations with high-latitude glaciation started occurring at least 2.4 Ma (Shackleton *et al*. 1984; Hooghiemstra & Cleef 1995), so rainforest fragmentation in the Amazon may not have occurred during the last one or two glacial maxima, but could have

occurred in the early or middle Pleistocene or indeed even earlier (Haffer 1997), which is plausible in view of the documented occurrence of Milankovitch cycles long before the beginning of Quaternary glaciations (Bartlein & Prentice 1989). Indeed, one prediction of vicariance dating back to the LGM would be identical or virtually identical mtDNA haplotypes shared by the nonforest vicariants, as found in populations of the elapid snake *Oxyuranus scutellatus* in Australia and New Guinea, separated by rising sea levels after the LGM (Wüster *et al*. 2005), but not in this study. A greater age of the last instance of rainforest fragmentation may be one reason why Lessa *et al*. (2003) did not detect the expected genetic traces of recent range expansion in SW Amazonian rainforest rodents. Again, the inclusion of isolated Amazonian populations of *C. durissus* in phylogeographical studies would shed further light on the timing of forest contraction or fragmentation.

In conclusion, our results provide new evidence of substantial rainforest fragmentation in the Amazon basin in the early or middle Pleistocene, and illustrate the potential usefulness of phylogeographical studies of nonrainforest species in the elucidation of the history of the forests themselves.

Systematic implications

In addition to their biogeographical implications, our results provide a phylogenetic background to the resolution of long-standing taxonomic problems affecting the *C. durissus* complex. Campbell & Lamar (2004) split *C. durissus*, previously regarded as a single polytypic species, into three species, *C. totonacus* (northeastern Mexico), *C. simus* (Mexico and C. America) and *C. durissus* (South America). Our results support species status for *C. totonacus*, which appears to be an ancient lineage more closely related to *C. molossus* and *C. basiliscus* than to *C. durissus*. Our data also demonstrate that *C. molossus* is highly heterogeneous, falling into four clades dating back to the Miocene, suggesting that it may also represent a species complex.

Crotalus simus (*sensu* Campbell & Lamar 2004) is both paraphyletic and highly heterogeneous. The taxa *culminatus* (southwestern Mexico) and *tzabcan* (Yucatán Peninsula) form highly distinct clades dating from the earliest Pliocene, and are morphologically distinct (Klauber 1952, 1972). We therefore consider them separate evolutionary species, *Crotalus culminatus* and *Crotalus tzabcan*, although we recognize the need for additional work on their status. (See Campbell & Lamar 2004 for photographs, descriptions, and distribution maps.) *Crotalus simus simus* (*C. d. durissus*, *sensu* Campbell & Lamar 1989) is polyphyletic: the specimens from northeastern Mexico (Veracruz) constitute the sister taxon of *C. culminatus*, whereas the remaining Central American populations are the sister group of the South American representatives of the complex. Klauber (1952) noted subtle differences between specimens from Veracruz and elsewhere in Central America, but in the absence of additional data, we prefer not to propose any formal reclassification based on mtDNA alone (see Puorto *et al*. 2001). Recognition of these distinct lineages is important for conservation assessment and management, particularly as many populations appear to be declining rapidly (Campbell & Lamar 2004).

Within South America, extensive superficial variation in pattern has led to the description of a plethora of often ill-defined subspecies or even species (summarized in Vanzolini & Calleffo 2002 and Campbell & Lamar 2004). Our data show all South American populations of the *C. durissus* complex to be phylogenetically closely related. South of the Amazon, the poorly defined phylogeographical pattern suggests that subspecific distinctions are unwarranted, and we consider *Crotalus durissus cascavella* and *Crotalus durissus collilineatus* to be synonymous with *Crolatus durissus terrificus*. North of the Amazon, the populations from Aruba (*unicolor*) and northwestern Venezuela (*vegrandis*), often recognized as distinct species (e.g. Klauber 1972), are placed among the other South American populations of *C. durissus*. In northern South America, incongruence between morphological variation and phylogeographical patterns, and substantial morphological differences between geographically proximal rattlesnake populations suggest that the delimitation of species here will require the analysis of multiple unlinked character systems.

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This study forms part of a wide-ranging collaborative research program into the evolutionary biology, phylogeny, and biogeography of venomous snakes involving WW, CEP, MGS and RST. The sequence data were largely generated as part of postgraduate research by JEF and JAQM.

Appendix

Haplotype, locality and taxon details. Haplotype codes are as in Figs 2 and 3, locality codes are as in Fig. 1, the taxonomy follows Campbell & Lamar (1989)

Taxon	Locality	Loc no. in Fig. 1	Voucher/ sample number	Haplotype	GenBank Accession nos: cyt*b*, *ND4*, *ND2*
C. durissus culminatus	Morelos, Mexico	1	3291	SW Mex 1	AY704830, AY704880, AY704785
C. durissus culminatus	El Aguacate, Puebla, Mexico	2	004	SW Mex 2	AY704828, AY704878
C. durissus culminatus	Sierra de Puebla, Puebla, Mexico	3	RA	SW Mex 3	AY704829, AY704879
C. durissus culminatus	Mexico		ZSL, live collection	SW Mex 4	AY704827, AY704877
C. durissus durissus	Catemaco, Veracruz, Mexico	4	001	Veracruz 1	AY704831, AY704881, AY704786
C. durissus durissus	Los Tuxtlas, Veracruz, Mexico	4	CDD	Veracruz 2	AY704832, AY704882
C. durissus durissus	Estación Ecológica Selva Lacandona, Chiapas, Mexico	5	2065	Chiapas 1	AY704833, AY704883, AY704787
C. durissus durissus	Estación Ecológica Selva Lacandona, Chiapas, Mexico	5	2067	Chiapas 2	AY704834, AY704884, AY704788
C. durissus tzabcan	Near Xaibe, Corozal District, Belize	6	255 — Peter Singfield, live collection	Belize 1	AY704806, AY704856, AY704791
C. durissus tzabcan	Near Xaibe, Corozal District, Belize	6	258 — Peter Singfield, live collection	Belize 2	AY704806, AY704856, AY704792
C. durissus tzabcan	Near Xaibe, Corozal District, Belize	6	259 — Peter Singfield, live collection	Belize 3	AY704836, AY704886, AY704793
C. durissus durissus	Surroundings of San Salvador, El Salvador	7	Sal 1, Sal 3, Sal 6	Salvador	AY704803, AY704853, AY704789
C. durissus durissus	Bahía de Pájaros, Puntarenas, Costa Rica	8	1097	C Rica	AY704835, AY704885, AY704790
C. durissus unicolor	Aruba, Netherlands Antilles	9	211, 212 — ZSL, live collection	unicolor	AY704805, AY704855, AY704777
C. durissus cumanensis	Distrito Colina, Falcón, Venezuela	10	781	Falcon 1	AY704820, AY704870, AY704778
C. durissus cumanensis	Curimagua, Falcón, Venezuela	10	788	Falcon 2	AY704821, AY704871, AY704779
C. durissus cumanensis	Las Ventosas, Falcón, Venezuela	10	789	Falcon 3	AY704822, AY704872, AY704780
C. durissus cumanensis	Las Ventosas, Falcón, Venezuela	10	790	Falcon 4	AY704823, AY704873, AY704781
C. durissus cumanensis	La Guaira, Distrito Federal, Venezela	11	775	N Venezuela	AY704825, AY704875, AY704783
C. durissus vegrandis	Venezuela	12	833 — ZSL, live coll.	vegrandis	AY704824, AY704874, AY704782
C. durissus dryinas	Guyana	13	1092 — SBS-UWB live collection	Guyana	AY704826, AY704876, AY704784
C. durissus ruruima	Boa Vista, Roraima, Brazil	14	unknown	Roraima	AY196656, AY255083
C. durissus collilineatus	Pontes e Lacerda, Mato Grosso, Brazil	15	IB live coll. 4610	P Lacerda	AY704813, AY704863, AY704769
C. durissus cascavella	Grajau, Maranhão, Brazil	16	IB 56419	Grajau 1	AY704816, AY704866
C. durissus cascavella	Grajau, Maranhão, Brazil	16	IB 56420	Grajau 2	AY704817, AY704867, AY704773
C. durissus cascavella	Grajau, Maranhão, Brazil	16	IB 56421	Grajau 3	AY704818, AY704868, AY704774
C. durissus cascavella	Belém do São Francisco, Pernambuco, Brazil	17	IB live coll. 4112	BelemSF	AY704819, AY704869, AY704775
C. durissus collilineatus	Alto Boa Vista, Mato Grosso, Brazil	18	IB 58460	NE MGrosso 2	AY704811, AY704861, AY704767
C. durissus collilineatus	Alto Boa Vista, Mato Grosso, Brazil	18	IB 58466	NE MGrosso 3	AY704812, AY704862, AY704768
C. durissus collilineatus	Ribeirão Cascalheira, Mato Grosso, Brazil	19	IB live coll. 4826	NE MGrosso 1	AY704814, AY704864, AY704770

Appendix *Continued*

Taxon	Locality	Loc no. in Fig. 1	Voucher/ sample number	Haplotype	GenBank Accession nos: cyt*b*, *ND4*, *ND2*
C. durissus collilineatus	Brasília, Distrito Federal, Brazil	20	IB live coll. 4826	Brazilia	AY704815, AY704865, AY704771
C. durissus cascavella	São Desidério, Bahia, Brazil	21	IB live coll. 4872	W Bahia 1	AY704804, AY704854, AY704772
C. durissus cascavella	Guanambi, Bahia, Brazil	22	IB live coll. 4127	W Bahia 2	AY704804, AY704854, Y704776
C. durissus collilineatus	Porto Primavera, São Paulo, Brazil	23	Unknown	P Primav	*AY196652,*AY196631
C. durissus collilineatus	Porto Primavera, São Paulo, Brazil	23	Unknown	P Primav	*AY196651, *AY196630
C. durissus collilineatus	Porto Primavera, São Paulo, Brazil	23	Unknown	P Primav	*AY196650, *AY196629
C. durissus terrificus	Campo Mourão, Paraná, Brazil	24	Unknown	SE Braz	*AY196645, *AY196621
C. durissus terrificus	Campo Mourão, Paraná, Brazil	24	Unknown	SE Braz	*AY196644, *AY196620
C. durissus terrificus	Arapoti, Paraná, Brazil	25	907	Parana 1	AY704809, AY704859, AY704765
C. durissus terrificus	Arapoti, Paraná, Brazil	25	908	Parana 2	AY704810, AY704860, AY704766
C. durissus terrificus	Colômbia, São Paulo, Brazil	26	IB 55553	SE Braz	AY704801, AY704851
C. durissus terrificus	Patrocínio Paulista, São Paulo, Brazil	27	Unknown	SP-MG	*AY196647, *AY196624
C. durissus terrificus	Patrocínio Paulista, São Paulo, Brazil	27	Unknown	SP-MG	*AY196646, *AY196623
C. durissus terrificus	Machado, Minas Gerais, Brazil	28	Unknown	SP-MG	*AY196642, *AY196615
C. durissus terrificus	Machado, Minas Gerais, Brazil	28	Unknown	SP-MG	*AY196641, *AY196614
C. durissus terrificus	Machado, Minas Gerais, Brazil	28	Unknown	SP-MG	*AY196640, *AY196613
C. durissus terrificus	Itu, São Paulo, Brazil	29	Unknown	S Paulo	*AY196648, *AY196626
C. durissus terrificus	Itu, São Paulo, Brazil	29	Unknown	SE Braz	*AY196649, *AY196628
C. durissus terrificus	Pindamonhangaba, Vale do Paraiba, São Paulo, Brazil	30	IB 55600	S Paulo	AY704802, AY704852
C. durissus terrificus	Pindamonhangaba, Vale do Paraiba, São Paulo, Brazil	30	IB 55601	SE Braz	AY704801, AY704851, AY704762
C. durissus terrificus	Roseira, São Paulo, Brazil	31	IB 55522	Roseira	AY704808, AY704858
C. durissus terrificus	São Luiz do Paraitinga, Vale do Paraiba, São Paulo, Brazil	32	IB 55552	V Paraiba 1	AY704800, AY704850, AY704764
C. durissus terrificus	Guaratinguetá, Vale do Paraiba, São Paulo, Brazil	33	IB 555521	V Paraiba 3	AY704807, AY704857, AY704763
C. durissus terrificus	Lorena, Vale do Paraiba, São Paulo, Brazil	34	Unknown	V Paraiba 1	*AY196655, *AY196634
C. durissus terrificus	Lorena, Vale do Paraiba, São Paulo, Brazil	34	Unknown	V Paraiba 2	*AY196654, *AY196633
C. durissus terrificus	Lorena, Vale do Paraiba, São Paulo, Brazil	34	Unknown	V Paraiba 2	*AY196653, *AY196632
C. durissus terrificus	Paraná State, Brazil		Unknown	Parana 3	*AY196638, *AY196611
C. durissus terrificus	Paraná State, Brazil		Unknown	Parana 4	*AY196639, *AY196612
C. durissus terrificus	Paraná State, Brazil		Unknown	Parana 4	*AY196637, *AY196610
C. durissus terrificus	Paraná State, Brazil		Unknown	SE Braz	*AY196636, *AY196609
C. durissus terrificus	Paraná State, Brazil		Unknown	SE Braz	*AY196635, *AY196608
C. durissus totonacus	Tomajo, Querétaro, Mexico	35	3102	NQueret	AY704838, AY704888, AY704795
C. durissus totonacus	Tamaulipas, Mexico		SD	Tamaul1	AY704837, AY704887, AY704794
C. durissus totonacus	30 km SW Ciudad Victoria, Tamaulipas, Mexico	36	SA	Tamaul2	AY704839, AY704889
C. molossus molossus	Las Uvas Mountains, New Mexico, USA		C135	*C. m. molossus* East 1	AY704848, AY704898, AY704798
C. molossus molossus	El Paso, Texas, USA		CLP66	*C. m. molossus* East 2	*AY223607, *AY223645
C. molossus molossus	Payson, Arizona, USA		C134	*C. m. molossus* West 1	AY704847, AY704897, AY704797

Appendix *Continued*

Taxon	Locality	Loc no. in Fig. 1	Voucher/ sample number	Haplotype	GenBank Accession nos: cyt*b*, *ND4*, *ND2*
C. molossus molossus	Sonoyta, Sonora, Mexico		CMSON	*C. m. molossus* West 2	AY704846, AY704896
C. molossus nigrescens	El Pedregal, Valle de Mexico, Mexico		CMN 4	*C. molossus nigrescens* 1	AY704849, AY704899
C. molossus nigrescens	El Pedregal, Valle de Mexico, Mexico		CMN 3	*C. molossus nigrescens* 2	AY704840, AY704890
C. molossus nigrescens	Cadereita, Querétaro, Mexico		1566	*C. molossus nigrescens* 3	AY704841, AY704891
C. molossus nigrescens	San Luis Potosí, Mexico		2643	*C. molossus nigrescens* 4	AY704842, AY704892
C. molossus oaxacus	Zapotitlán Salinas, Puebla, Mexico		3014	*C. molossus oaxacus*	AY704843, AY704893
C. basiliscus	Guadalajara, Jalisco, Mexico		Zoológico de Guadalajara, live coll.	*C. basiliscus* 1	AY704845, AY704895, AY704796
C. basiliscus	San Blas, Nayarit, Mexico		822	*C. basiliscus* 2	AY704844, AY704894
C. tigris	Pima County, Arizona, USA		CLP 169 (ND4, cytb), MVZ150244 (ND2)	*C. tigris*	*AF156574, *AY223606, *AY016240
C. virids viridis	Sherman County, Texas, USA		UTEP 15872	*C. viridis*	*AF147869, *AF194160, AY704799
Porthidium nasutum	Zapallo Grande, Esmeraldas, Ecuador		FHGO live coll. 517	*P. nasutum*	*AF 292574, *AF 292612
Porthidium arcosae	Salango, Manabí, Ecuador		FHGO live coll. 738	*P. arcosae*	*AF 292575, *AF 292613
Porthidium lansbergii rozei	San Antonio, Falcón, Venezuela		J.L. Yrausquin, live coll.	*P. l. rozei*	AY713375, *AF393623
Naja naja	Nepal		579	*N. naja*	AY713376, AY713378
Naja kaouthia	Ayeyarwady Division, Myanmar		CAS 206602	*N. kaouthia*	*AF217835, *AY058982
Naja nigricollis	Lara, Kaélé, Cameroon		Latoxan live coll. 9735002	*N. nigricollis*	*AF399745, AY713377
Naja nivea	unknown		none	*N. nivea*	*AF217827, *AY058983
Acrochordus granulatus	Unknown		none	Acrochordus	*AF217841, *U49296

Institutional Codes for specimens: CAS, California Academy of Sciences; CLP, Christopher L. Parkinson, private collection; FHGO, Fundación Herpetológica Gustavo Orcés, Quito, Ecuador; IB, Instituto Butantan, São Paulo, Brazil; Latoxan: Latoxan, Valence, France; MVZ, Museum of Vertebrate Zoology, Berkeley, California; SBS-UWB, School of Biological Sciences, University of Wales, Bangor, UK; UTEP, University of Texas, El Paso; ZSL, Zoological Society of London. Other numbers indicate DNA or tissue sample numbers. *Asterisks denote sequences previously available on GenBank.