



Phylogeographic patterns of trans-Amazonian vicariants and Amazonian biogeography: the Neotropical rattlesnake (*Crotalus durissus* complex) as an example

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ABSTRACT

Aim To investigate the phylogeography and execute a historical-demographic analysis of the Neotropical rattlesnake, *Crotalus durissus*, thereby testing the hypothesis of a Pleistocene central Amazon corridor of dry forest or savanna that partitioned the Amazonian rain forest into western and eastern portions.

Location South America.

Methods Using sequences of three mitochondrial genes, we estimated the phylogeography, gene and nucleotide diversity across the South American range of *C. durissus*. Tree topology tests were used to test alternative biogeographical hypotheses, and tests of population genetic structure and statistical parsimony networks and nested clade phylogeographic analysis (NCPA) were used to infer connectivity and historical population processes on both sides of the Amazon basin.

Results Tree topology tests rejected the hypothesis of a coastal dispersal in favour of a central corridor scenario. Gene diversity was similar on both sides of the Amazon basin. Nucleotide diversity indicated that the populations from north of the Amazon basin represented ancestral populations. Analysis of molecular variance (AMOVA) showed that intra-population molecular variation was greater than between regions. Historical-demographic statistics showed significant population expansion south of the Amazon, and little differentiation in the north, indicating moderate past gene flow between north and south of the Amazon. The parsimony network connected clades from the Roraima and Guyana populations with Mato Grosso, suggesting an Amazonian central corridor, and NCPA supported allopatric fragmentation between north and south of the Amazon.

Main conclusions The distribution of *C. durissus* on both sides of the Amazon basin is evidence of changes in the distribution of rain forest vegetation during the Pleistocene. Our results suggest a formerly continuous distribution of this rattlesnake along a central Amazonian corridor during the middle Pleistocene. Allopatric fragmentation inferred from NCPA is consistent with vicariance resulting from a subsequent closure of this habitat corridor. This study emphasizes the potential of trans-Amazonian open formation species to inform the debate on the past distribution of rain forests in the Amazon Basin.

Keywords

Amazonia, *Crotalus durissus*, molecular phylogeny, Neotropical biogeography, phylogeography, Pleistocene, trans-Amazon vicariance.

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INTRODUCTION

Many historical hypotheses have been proposed to explain the extraordinary species richness of the Amazon basin (Haffer, 1969, 1997; Bush, 1994; Moritz *et al.*, 2000; Cheviron *et al.*, 2005; Wüster *et al.*, 2005a). These hypotheses generally invoke vicariance events in explaining the origin of such diversity (but cf. Endler, 1982; Schneider *et al.*, 1999 regarding the ecological gradient hypothesis). The hypothesized causes of vicariant events include Andean uplift, riverine barriers, palaeoclimatic shifts resulting in forest refugia, and marine transgressions (Chapman, 1917; Sick, 1967; Haffer, 1969; Nores, 1999, 2004). To a large degree, all these hypotheses were formulated based on geographical distribution patterns of selected taxa.

The most controversial vicariance hypothesis has been the Pleistocene refugia hypothesis (PRH), first formulated by Haffer (1969). This hypothesis postulates that the area of Amazon forest was reduced during the dry periods due to global climatic fluctuations occurring throughout the late Pliocene and Pleistocene. In its earlier formulations (Haffer, 1969; Vanzolini & Williams, 1970; Prance, 1973), the PRH envisaged extensive fragmentation of the rain forests into isolated pockets, or refugia, separated by expanses of savanna or other open, dry formations (Haffer, 1969, 1997; Potts & Behrensmeyer, 1992; Vrba, 1993; Pennington *et al.*, 2000). The savanna and seasonally dry forests are assumed to have functioned as barriers separating populations of rain forest species, facilitating allopatric speciation (Haffer, 1969, 1997; Prance, 1973; Haffer & Prance, 2001).

Although the PRH was initially widely accepted, it has received increasing criticism in more recent years. The main debate about the PRH has focused on the fact that palynological evidence provides little support for widespread savanna vegetation in Amazonia, at least during the last glacial maximum and late Pleistocene (the past 18,000–50,000 years) (Bush, 1994; Colinvaux *et al.*, 1996, 2000, 2001; Haberle & Maslin, 1999; Kastner & Goñi, 2003). However, pollen records from the Early and Middle Pleistocene are lacking, so a hypothesis of rain forest fragmentation in the Middle or Early Pleistocene can be neither rejected nor confirmed by the palynological evidence currently available (Wüster *et al.*, 2005a,b). Moreover, while palynological data can reject widespread savanna vegetation at the time of the last glacial maximum, the pollen profiles resulting from dry, deciduous forests are much more difficult to distinguish from rain forest pollen (Pennington *et al.*, 2000). Recent synthetic analyses using palaeoclimatological, palynological and palaeoecological data suggest that Pleistocene rain forest fragmentation remains a possible scenario, and does not necessarily contradict the notion that the forests have been largely stable through the last glacial maximum (Haffer, 1997; Hooghiemstra & van der Hammen, 1998).

In addition to the lack of palynological evidence for widespread Pleistocene savannas, molecular phylogenetic studies of a variety of Amazonian rain forest taxa indicate that most divergences between sister species of rain forest

vertebrates significantly predate the Pleistocene (Hass *et al.*, 1995; Moritz *et al.*, 2000; Patton *et al.*, 2000; Salazar-Bravo *et al.*, 2001; Cheviron *et al.*, 2005; Ribas *et al.*, 2005), including some of the taxa used during the early formulations of the PRH (Glor *et al.*, 2001). Furthermore, the genetic fingerprints of Quaternary range expansion in temperate biota (Hewitt, 2004) have not been detected in Amazonian biota (Lessa *et al.*, 2003; Cheviron *et al.*, 2005). However, it is important to note that pre-Pleistocene speciation in rain forest taxa indicates only that Pleistocene rain forest fragmentation did not cause the speciation event, and cannot disprove its occurrence (Wüster *et al.*, 2005a). The lack of evidence of range expansion comes from taxa distributed in the western Amazon, which is more likely than eastern and central parts of the basin to have retained a continuous cover of tropical rain forest.

Savanna and seasonal dry forests are located around the periphery of the Amazon basin, with isolated enclaves within the rain forest (Eiten, 1972; Pennington *et al.*, 2000; Cardoso da Silva & Bates, 2002). Several savanna vertebrate taxa occur on both sides of the basin and in the enclaves (Fig. 1). Of these taxa with a disjunct distribution, the most studied are birds (Eberhard & Bermingham, 2004), but other taxa show similar distribution patterns (Ávila-Pires, 1995; Cardoso da Silva & Bates, 2002; Campbell & Lamar, 2004; Courtenay & Maffei, 2004; Frost, 2004; Wüster *et al.*, 2005a). Phylogeographic studies of trans-Amazonian taxa may have the potential to provide stronger evidence on changes in rain forest cover and the existence of dry corridors than studies of forest species, since evidence of recent connectivity between open formations north and south of the Amazon constitutes strong evidence of past rain forest fragmentation, much more so than lack of Quaternary speciation in forest species provides evidence of lack of fragmentation (Pennington *et al.*, 2000, 2004; Wüster *et al.*, 2005a).

Three main corridors connecting northern and southern savanna regions have been proposed (Fig. 2): the Andean Corridor connects the southern block of savannas directly with the Colombian and Venezuelan llanos and the savannas of Roraima through the Andean slopes; the Central Amazonian Corridor connects the southern block of savannas with patches of savannas north of the Amazon basin (Monte Alegre, Parú and Roraima); the Coastal Corridor connects the southern and northern blocks through savanna patches (e.g. Marajó and Amapá as well as the coastal savannas of the Guyanas) and is located close to the Atlantic coast (Webb, 1991; Haffer, 1997; Cardoso da Silva & Bates, 2002). The distributional patterns of some savanna vertebrate taxa are congruent with the Andean and Coastal corridor, but to date there has been less distributional evidence supporting the Central Amazonian corridor (Silva, 1995; Ávila-Pires, 1995; Pinto-Henriques & Oren, 1997; Silva *et al.*, 1997; Cardoso da Silva & Bates, 2002).

However, relying only on distributional patterns without incorporating their phylogenetic component may hide intra-specific population-level connections that can reflect past effects of corridors. A phylogeographic approach, relating the phylogeny of mitochondrial haplotypes to the geographical

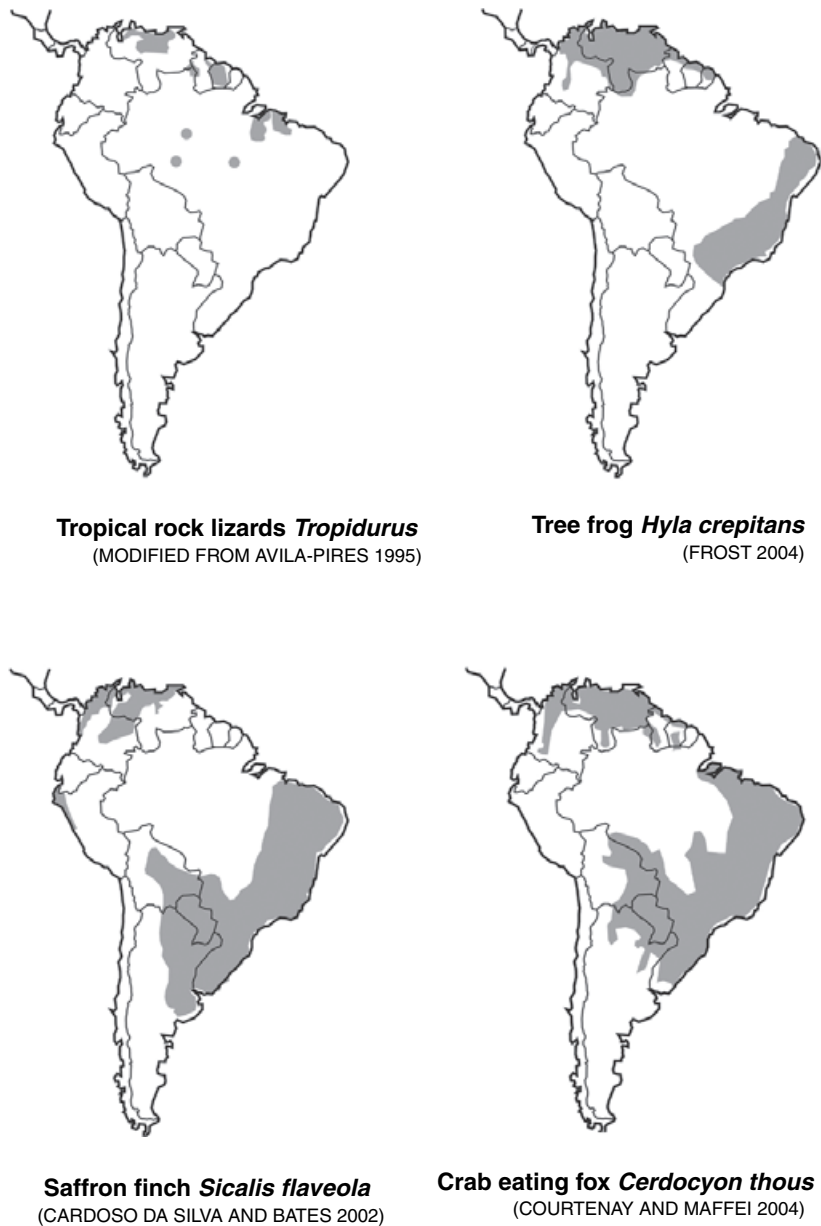


Figure 1 Distribution patterns of trans-Amazonian vicariant taxa. The diversity of taxa involved and similarity of distributional patterns suggests that changes in dry vegetation (seasonal forests and savannas) and the development of the Amazon basin affected regional biota in a similar way. Maps are based on Ávila-Pires (1995), Cardoso da Silva & Bates (2002), Frost (2004) and Courtenay & Maffei (2004).

distribution of this genetic variation, can help reconstruct the biogeographical history of the taxa involved (Avice, 2000). In addition to a simple phylogenetic approach, different biogeographical events (long-distance dispersal, range expansion, range fragmentation) all leave different genetic traces. This means that different hypothesized events lead to different predictions of genetic variation in the populations affected, and these predictions can be tested by comparison with observed patterns. New phylogeographic and population approaches such as measurements of nucleotide diversity (Hewitt, 1996, 2004; Zink *et al.*, 2000), statistical parsimony and nested clade phylogeographic analysis (NCPA) (Templeton, 2004), and neutrality tests (Fu, 1997; Ramos-Onsins & Rozas, 2002; Cheviron *et al.*, 2005) can be used to infer genetic connections and population-level processes. In particular, haplotype networks constitute one of the most promising

methods to infer intraspecific phylogenetic relationships by identifying multiple possible genetic connections between haplotypes (unique combination of DNA polymorphisms in a sample, inherited as a unit). They thus provide more realistic representations of phylogenetic relationships within populations than traditional phylogenetic methods, and can be used to determine the genetic composition of ancestral populations (Posada & Crandall, 2001).

Among trans-Amazonian open-formation taxa, the Neotropical rattlesnake, *Crotalus durissus*, is one of the best studied (Wüster *et al.*, 2002, 2005a,b; Campbell & Lamar, 2004). This species is found only in savanna and dry seasonal forests, but not in tropical rain forests (Campbell & Lamar, 2004; Wüster *et al.*, 2005a,b), although it is quick to colonize anthropogenically deforested areas within the rain forest zone (Sazima & Haddad, 1992; Marques *et al.*, 1998; Bastos *et al.*, 2005).

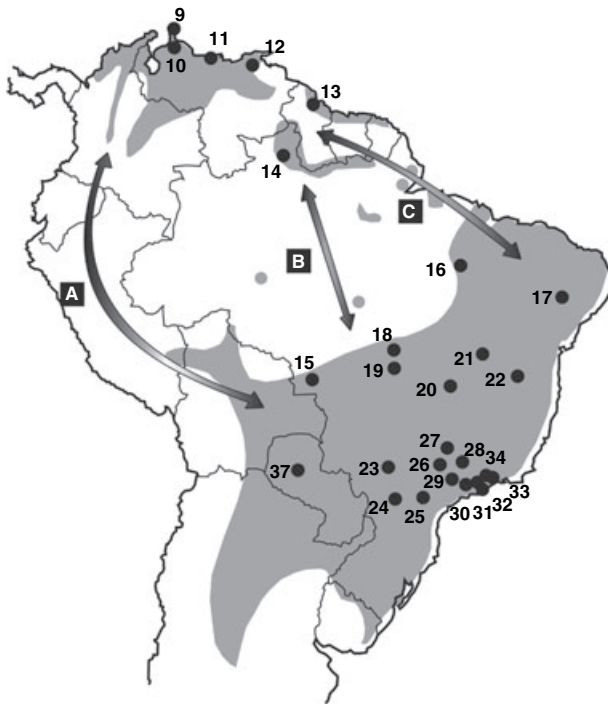


Figure 2 Sampling localities for the *Crotalus durissus* complex in South America. Numbers for localities correspond to Wüster *et al.* (2005a) and are as discussed in the text and in Figs 4, 5 and 8. Arrows represent major hypothesized corridors connecting northern and southern blocks of South American open formations (Cardoso da Silva & Bates, 2002): (a) Andean corridor; (b) central Amazonian corridor; (c) Coastal corridor.

Recent studies on rattlesnakes from temperate deserts provide evidence for a mixture of genetic tracks of lineage differentiation caused by tectonic events and climate cycles during the late Tertiary and Quaternary (Pook *et al.*, 2000; Douglas *et al.*, 2006; Castoe *et al.*, 2007), demonstrating the potential of these snakes for unravelling the Pleistocene history of regional biota. These genetic signatures of dispersal, vicariance and range expansion can be investigated in the phylogeographic pattern of the Neotropical rattlesnake (Wüster *et al.*, 2005a).

In a previous study, we described and dated the gradual dispersal of *C. durissus* from its centre of origin in Mexico and Central America through the Central American Isthmus and, after the uplift of the Isthmus of Panamá, the more rapid dispersal into and across South America (Fig. 2; Wüster *et al.*, 2005a), demonstrated by the monophyly of the South American populations. The low sequence divergence between populations from north and south of the Amazon rain forest was consistent with mid-Pleistocene divergence *c.* 1.08 Ma. Based on this, we postulated that the Amazonian rain forests must have become fragmented or at least shrunk considerably during that period, allowing rattlesnakes to cross the Amazon Basin and to colonize more southerly parts of South America (Wüster *et al.*, 2005a). This idea provoked some controversy on the basis of alternative explanations of the phylogeographic pattern (Gosling & Bush, 2005; Wüster *et al.*, 2005b).

Our previous work (Wüster *et al.*, 2005a) focused solely on the interpretation of phylogenetic trees derived from mitochondrial DNA sequences, and the dating of important nodes on the tree. Here we use a number of additional molecular genetic methods in a more explicitly hypothesis-testing framework, with the aim of testing alternative hypotheses for the history of the current, discontinuous distribution of *C. durissus* in South America. Our approach is founded on the basis that different biogeographical histories predict different genetic and phylogeographic structures for these populations, and these predictions can be tested with appropriate analytical methods. For instance, long-distance dispersal over existing barriers will result in a different genetic structure than will a vicariant event separating a formerly continuous population, even though the resulting distribution may be the same. Moreover, these methods can allow us to infer the location of past connections between presently isolated populations.

METHODS

The sampling, laboratory protocols, mtDNA sequence data and GenBank accession codes used in this study are given in Wüster *et al.* (2005a). For most analyses in this study, we used the better sampled two-gene data set of Wüster *et al.* (2005a), consisting of 1332 base pairs (bp) (657 bp of *ND4* and 675 bp of cytochrome *b*) of aligned sequences. We also used the three-gene data set, including an additional 671 bp of the *ND2* gene for additional phylogenetic analyses, and in particular tests of alternative tree topologies. For phylogenetic analyses of the two-gene data set, we included sequence data of an additional specimen from Filadelfia, Paraguay (GenBank accession number DQ899736 for cytochrome *b* and DQ899735 for *ND4*).

Phylogeography and tree topology tests

Our original paper (Wüster *et al.*, 2005a) was based on a phylogeographic approach (the phylogenetic analysis of a mitochondrial gene tree), but did not use an explicitly statistical approach to test and compare alternative biogeographical hypotheses. Different biogeographical hypotheses predict different mitochondrial gene trees. Using statistical methods such as the Wilcoxon signed ranks test (Templeton, 1983) and the Shimodaira–Hasegawa (SH) test (Shimodaira & Hasegawa, 1999), the actual, observed gene tree recovered from the animals can be compared statistically with the trees predicted by alternative biogeographical scenarios. A statistically significant difference indicates that the observed data reject the gene tree predicted by the alternative biogeographical scenario.

Here we used tree topology comparisons to test the hypothesis that the populations of *C. durissus* south of the Amazon Basin are a result of dispersal along a past coastal corridor. This hypothesis predicts that north-eastern Brazil would have been the first part of southern South America to be colonized, and thus that the most basal haplotypes south of the Amazon should be found there, whereas haplotypes from

southern and south-western South America should be monophyletic (Fig. 4).

We reconstructed the phylogeny of the South American populations, using haplotypes from Belize and El Salvador (haplotypes Belize 3 and Salvador from Wüster *et al.*, 2005a) as outgroups. All phylogenetic analyses were carried out in PAUP 4.0b10 (Swofford, 2002) unless stated otherwise. Maximum parsimony (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 (two-gene data) or 10,000 replicates with 10 random addition sequence replicates each (three-gene data) and TBR branch swapping. For maximum likelihood (ML) analyses, the appropriate model of sequence evolution was estimated using MODELTEST 3.0 (Posada & Crandall, 1998). We selected the model favoured under the Akaike information criterion, and fixed the parameters in a heuristic ML search using a neighbour-joining (NJ) starting tree and TBR branch swapping. Bootstrapping involved 100 replicates with NJ starting trees and nearest-neighbour interchange (NNI) (two-gene data) or subtree pruning-regrafting (SPR) (three-gene data) branch swapping.

To test whether the uncovered phylogeographic pattern is consistent with the coastal corridor hypothesis, we then used the same methods to generate constraint trees in which haplotypes from southern and south-western South America were constrained to be monophyletic to the exclusion of haplotypes from north-eastern Brazil (states of Bahia, Maranhão and Pernambuco) (Fig. 4). We compared these constrained trees with the optimal trees using Wilcoxon signed ranks tests under the MP criterion (Templeton, 1983) and using the SH test (Shimodaira & Hasegawa, 1999), run in PAUP* using the full option and 1000 bootstrap replicates, under the ML criterion.

Population structure

Historical events such as dispersal and vicariance leave genetic ‘footprints’ that can be revealed and interpreted with appropriate statistical tools. Several studies have suggested that higher levels of nucleotide diversity are indicative of population ancestry (Hewitt, 1996, 2000, 2004; Zink *et al.*, 2000; Cheviron *et al.*, 2005). By comparing these parameters in different populations of *C. durissus* in South America, we aimed to distinguish ancestral areas from more recently colonized areas. We used analytical methods often used to describe population structure from nucleotide sequence information (reviewed by Schneider *et al.*, 2000). Gene diversity and nucleotide diversity (π) are equivalent measures of the degree of polymorphism within a population. In this study we inferred ancestral vs. derived populations based on π .

Analysis of molecular variance (AMOVA) is similar to hierarchical analysis of variance in that it separates and tests

levels of genetic diversity: among groups of populations, among populations within groups, and among individuals within a population. AMOVA differs from analysis of variance in that it can accommodate different evolutionary assumptions without modifying the basic structure of the analysis. Hypotheses are tested using permutational methods that involve few assumptions about the statistical properties of the data (Excoffier *et al.*, 1992; Schneider *et al.*, 2000). AMOVA results provide information regarding the degree of genetic differentiation among populations compared with within-population variation.

For analyses of population structure, we compared the populations from north of the Amazon with those from south of the Amazon. The northern group consisted of populations on the northern periphery of the Amazon rain forest, including Aruba island, the Venezuelan savannas, and populations from coastal Guyana and Roraima state, northern Brazil (populations 9–14; Fig. 2). The southern group encompasses all the 18 populations from the southern border of the Amazon rain forest to far southern Paraguay (populations 15–25, 27–29, 31, 33, 34, 37; Fig. 2). Estimates of population structure for the regions were based on gene diversity and AMOVA among the two regions using ARLEQUIN 2.0.6 (Schneider *et al.*, 2000). Levels of nucleotide diversity (π) were calculated within each region using standard equations (Nei, 1987) using DnaSP 4.10.3.5 (Rozas & Rozas, 1999).

Nested clade phylogeographic analysis

Whereas conventional phylogeographic approaches focus primarily on the visual synthesis of a gene tree and the geographic distribution of haplotypes, NCPA (Templeton & Sing, 1993; Templeton, 1998, 2004) uses nested networks of haplotypes to make statistical inferences on the causes of observed patterns. It thus has potential for discriminating between the different causal hypotheses for observed patterns, such as range expansion, long-distance waif dispersal and vicariance. Moreover, NCPA includes an explicit test of sampling adequacy as part of the procedure, thus guarding against false interpretations caused by sampling gaps, and the procedure has been validated extensively by testing with existing data with strong *a priori* expectations of the biogeographical processes involved (Templeton, 2004). By establishing the most parsimonious connections between haplotypes, NCPA also has the potential to reveal past connections between currently separated sets of populations.

As a first step during NCPA, a parsimony-based haplotype network is obtained, which takes account of multiple connections between haplotypes. In a statistical parsimony network, the most parsimonious connections are evaluated with a confidence level (Posada & Crandall, 2001). Based on the parsimony network thus obtained, NCPA defines a series of nested clades following a proposed nesting procedure (Templeton & Sing, 1993; Clement *et al.*, 2000; Templeton, 2004). Then a permutation analysis is used to test statistically

for phylogeographic associations based on the combination of nested design and haplotype sampling locations.

After phylogeographic structure is detected, NCPA uses the geographical distances within and among nested clades (clade distance, D_c and nested clade distance, D_n) to make inferences about the causal mechanisms of the statistically significant phylogeographic associations. The biological interpretations of the results are made using an explicit inference key (Templeton, 2004). If sample size is adequate, the practical inference procedure yields many insights into restricted but recurring gene flow, isolation by distance, allopatric fragmentation, contiguous range expansion, long-distance colonization, and historical factors that can contribute directly to the process of population differentiation (Templeton, 2004).

In order to infer statistical parsimony networks, we used the TCS 1.13 program (Templeton *et al.*, 1992; Clement *et al.*, 2000). The program collapses the sequences into haplotypes and calculates the haplotype frequencies in the sample (Clement *et al.*, 2000). Haplotypes differing by up to 15 mutational steps had > 95% probability of being connected parsimoniously; TCS was used to construct networks for these haplotypes. In addition, we allowed a 93% probability of parsimonious connection in order to include the marginal connection between the Venezuelan clade and the Southern clades. As we stated in our previous paper (Wüster *et al.*, 2005a), Venezuelan populations represent the ancestral clades of the clades from South of the Amazon. With this in consideration, we included those clades with a marginal probability. These networks were used to define a nested cladogram following the nesting rules described by Templeton & Sing (1993).

We used GEODIS 2.0 (Posada *et al.*, 2000) to calculate D_c , D_n , and comparisons of these measures among interior and tip clades using the derived nested cladogram. Then distance between individuals and clade centre was calculated using great circle distances. Comparisons of these measures were calculated using 100,000 random permutations of clades against sampling locality to test the null hypothesis of no geographic association among haplotypes or clades at each nesting level. Geographic association was based on contrasts between large or small clade distances or interior–tip. The inference key given by Templeton (2004) was used to infer the process most consistent with the observed statistically significant pattern at those nesting levels.

Historical demographic analysis

In order to infer past demographic changes, we used a set of analytical methods to measure deviations of mtDNA polymorphisms from molecular neutrality. These neutrality tests provide statistical estimates of historical changes in population size (Fu, 1997), which can test inferences derived from phylogeographic patterns. Evolutionary and biogeographical hypotheses can thus be tested by combining the findings from the application of neutrality tests, NCPA and other phylogeographic methods (Cheviron *et al.*, 2005).

In this study, we performed Tajima's D and Fu's F_s tests (Fu, 1997) to detect past population changes based on the nucleotide composition between north and south Amazon, and undertook further analyses of clades and subclades inferred from NCPA. Significant positive values indicate long-term isolation, and negative values indicate recent population expansion. Significance was determined based on 10,000 coalescent simulations under a model of population growth–decline size.

Mismatch distributions (Rogers, 1995) were also calculated to compare the demographic histories of *C. durissus* populations, as smooth Poisson mismatch distributions are characteristic of rapid demographic expansion. The distribution is usually multimodal in samples drawn from populations at demographic equilibrium, as it reflects the highly stochastic shape of gene trees, but is usually unimodal in populations having passed through a recent demographic expansion (Rogers, 1995). The frequency of pairs of randomly chosen individuals differing by a given number of nucleotide substitutions, and expected frequencies under a model of rapid demographic expansion, were calculated using DnaSP 4.10.3.5 (Rozas & Rozas, 1999). These expected frequencies were then overlaid onto the observed frequencies. The raggedness index (Harpending, 1994) was used to measure the smoothness of the observed distributions, and the significance of raggedness indices was calculated using 10,000 coalescent simulations. We compared the value of these indexes at different hierarchical levels and among nested clades, beginning with the all-South America clade and then performing the analysis at nested clade levels.

RESULTS

Phylogeography and tree topology tests

Maximum parsimony analyses yielded 44 equally most parsimonious trees of 259 steps (CI = 0.7220) for the two-gene data, and four trees of 323 steps (CI = 0.7833) for the three-gene data (Fig. 3). Maximum likelihood analyses revealed a single optimal tree for both the two-gene and three-gene data ($-\ln L = 3232.60517$ and 4475.23181 , respectively).

The optimal trees for the South American *C. durissus* complex were broadly similar to those published previously (Wüster *et al.*, 2005a), with the important difference that the populations from south of the Amazon were no longer monophyletic (except in the three-gene ML analysis), haplotypes PLacerda from Mato Grosso (Wüster *et al.*, 2005a) and Paraguay instead forming a sister group to the Guyanan and Roraima haplotypes. However, bootstrap support for this relationship was weak in all cases, as was the case for the monophyly of the southern haplotypes in previous analyses (Wüster *et al.*, 2005a).

The trees obtained above differ both qualitatively and statistically from the tree topology predicted by a hypothesis of dispersal along a coastal corridor (Fig. 4). In the case of the two-gene data, the difference between the observed tree and

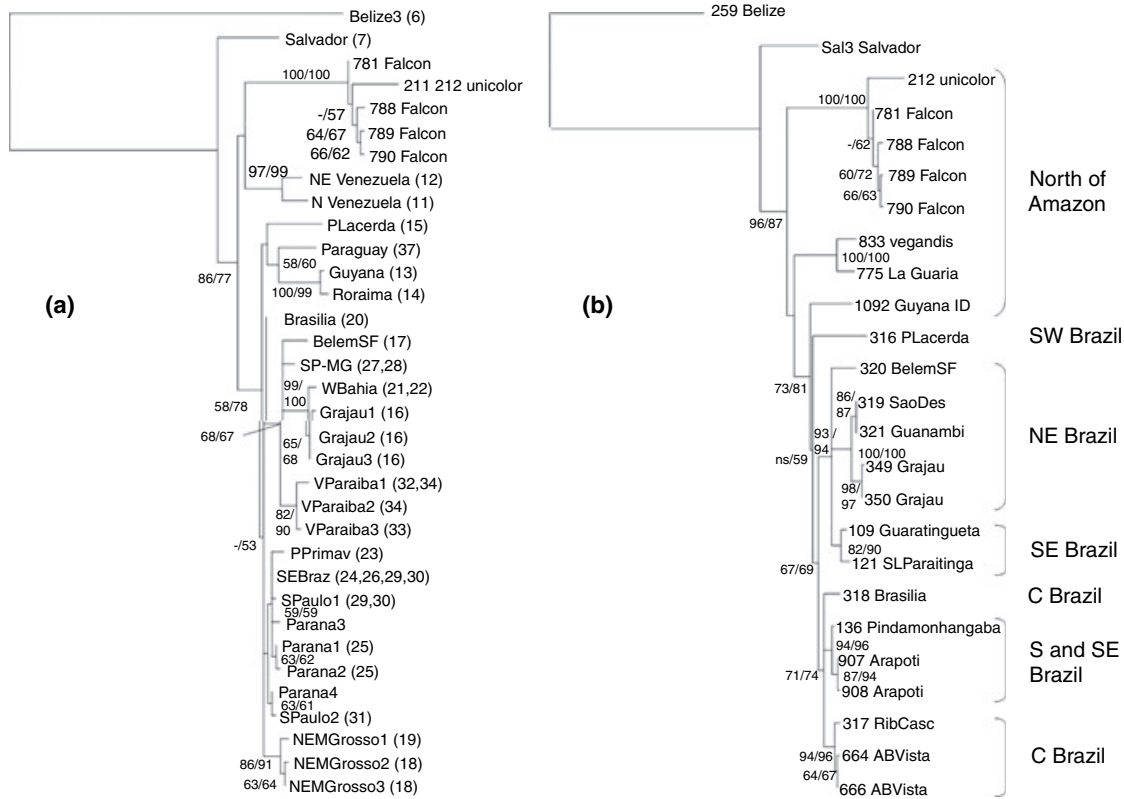


Figure 3 Maximum likelihood phylogenetic trees of (a) two-gene data; (b) three-gene data. Tip labels correspond to haplotype codes; locality codes from Wüster *et al.* (2005a) are given in parentheses. Support values are given as MP bootstrap/ML bootstrap support. –, Support < 50%; ns, node contradicted in the relevant analysis.

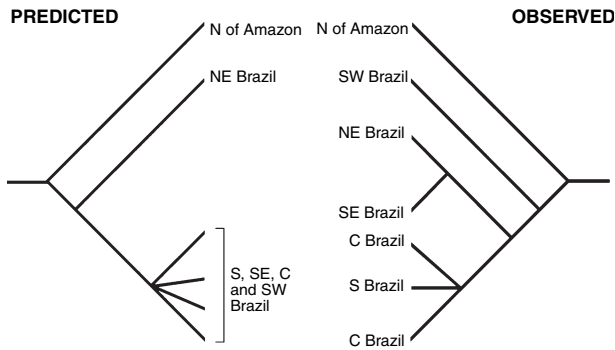


Figure 4 Phylogeographic pattern predicted under the hypothesis of dispersal along coastal corridor: (a) north-eastern Brazilian haplotypes are expected to be basal to a monophyletic group of haplotypes from southern Brazil; (b) schematic representation of observed phylogeographic pattern of *Crotalus durissus* in South America (three-gene data; Fig. 3). South-western Brazilian haplotypes are basal, north-eastern haplotypes are nested deeply among other more southerly haplotypes.

that predicted by the coastal corridor hypothesis was not significant in the MP analysis (Wilcoxon signed ranks test, $Z = -1.5000$ to -1.8974 , $P > 0.05$), but the SH test revealed a significant difference between the optimal ML tree and the constrained tree ($d(-\ln L) = 18.85746$, $P = 0.047$). In the case of the three-gene data, the difference between the optimal and constrained trees was significant in the MP and ML analyses (Wilcoxon signed ranks test, $Z = -2.1106$ or -2.3333 , $P = 0.0196$ or 0.0348 ; SH test, $d(-\ln L) = 19.42484$, $P = 0.016$).

Nucleotide diversity and AMOVA

There were striking differences between the patterns of gene and nucleotide diversity indexes (Table 1). The populations north of the Amazon displayed the highest nucleotide diversity (π), indicating that these areas represent ancestral populations. This trend contrasted with gene diversity indices, which showed similar values on both sides of the Amazon, suggesting

Table 1 Summary of population structure and historical demographic analysis of *Crotalus durissus* in South America.

Region	<i>n</i>	Gene diversity	Nucleotide diversity (π) ($\times 100$)	Raggedness (<i>P</i>)	Fu's F_s (<i>P</i>)	Tajima's <i>D</i> (<i>P</i>)
North Amazon	9	1.00 \pm 0.052	2.00 \pm 0.28	0.0679 (0.49)	1.048 (0.182)	0.5403 (0.773)
South Amazon	23	0.97 \pm 0.020	0.84 \pm 0.09	0.106 (0.009)	-5.961 (0.025)	-1.371 (0.082)
Total	32	0.99 \pm 0.012	1.45 \pm 0.12	0.0071 (0.023)	-8.222 (0.012)	-1.092 (0.1324)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	<i>P</i>
Among regions	1	76.616	5.45569 V_a	44.07	< 0.001
Within region	30	200.803	6.92424 V_b	55.93	< 0.001
Total	31	277.419	12.37994		

Significance level (*P*) was determined by a random permutation test with 10,000 replicates.

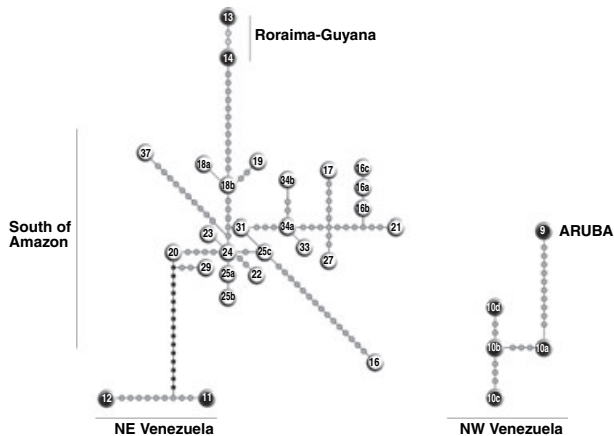


Figure 5 Networks inferred by the TCS 1.13 program representing parsimonious connections between haplotypes within the limits of statistical parsimony (95%, maximum 15 mutational steps). Numbers in circles, sampled haplotypes; grey circles, north-of-Amazon haplotypes. Dots along connecting lines indicate number of mutational steps connecting haplotypes; black dots along connecting lines are mutational steps of connections above the significance level (93%, 17 mutational steps).

a comparable degree of differentiation on each side (Fig. 6). AMOVA analysis showed that 44% of molecular variation was between populations and 56% within populations ($F_{st} = 0.4407$, $P < 0.001$), indicating moderate differentiation between both regions and more variation within populations in a given region (Table 2).

Statistical parsimony and NCPA

Three haplotype networks were recognized by the TCS program (95% confidence, Fig. 5). The average number of mutational steps connecting haplotypes was five ($X = 4.846 \pm 0.869$ SE). The largest network included clades from both north and south of the Amazon, which were connected by 12 mutational steps (e.g. Roraima and Guyana, northern populations 13–14 with South Amazon populations 18–19). Marginal parsimonious connections (93% confidence) connected other northern populations with South Amazon populations (populations 11–12 with 29–24). These connections suggest the existence of a past central Amazon corridor connecting Roraima with Mato Grosso.

We performed the NCPA in the largest network due to the higher nesting clade resolution obtained (Fig. 6). In a few of

Table 2 Summary of AMOVA results comparing north and south Amazon populations of *Crotalus durissus*.

the clades analysed the NCPA rejected the null hypothesis of no geographical associations of nesting clades (Table 3), indicating phylogeographic structure in these clades. For lower-level clades, NCPA indicated restricted gene flow and range expansion, particularly in one-step clades nested in clade 2-2 (southern Brazil). Within higher-level clades the analysis indicated allopatric fragmentation between clades north and south of the Amazon (Table 4). Support for this inference resulted from analysis of clades 4-1, 4-2 and 4-7, corresponding to the Venezuelan and Guyana–Roraima clades, and the clades south of the Amazon. The Venezuela and Guyana–Roraima clades are separated from the other clades by the Amazon rain forest. These clades display non-overlapping distributions and are connected to one another by a larger-than-average number of mutational steps. According to Templeton's inference key, this is consistent with allopatric fragmentation. Similarly, NCPA indicated allopatric fragmentation between clades of Guyana and Roraima. NCPA analysis suggests a continuous distribution in the past, and is consistent with a discontinuous Amazonian rain forest scenario during the early to middle Pleistocene. Haplotype network distribution, probable corridor connections, and our preferred scenario are shown in Fig. 8.

Historical demographic analysis

Comparisons using several neutrality tests between north and south of the Amazon basin show striking differences (Table 1).

Mismatch distributions between north and south of the Amazon differed on obvious evidence of demographic expansion (Fig. 7). The haplotypes from south of the Amazon presented a smooth Poisson distribution, which is supported by a significant raggedness index ($r = 0.0091$, $P = 0.01$), in contrast to populations from north of the Amazon ($r = 0.0679$, $P = 0.51$). The significantly smooth Poisson distribution south of the Amazon suggests population expansion in contrast to north of the Amazon, where the non-significant raggedness suggests constant population size.

Historical demographic analysis of the entire South American *C. durissus* rattlesnake clade is consistent with a dispersal event in the past, followed by limited population differentiation and vicariance. Mismatch distribution of the entire South America clade showed a slightly smooth Poisson distribution with a non-significant raggedness index ($r = 0.0086$, $P = 0.056$). Additionally, values for Tajima's and Fu's indices were incongruent ($D = -0.992$, $P = 0.14$; $F_s = -7.455$, $P = 0.01$), but most consistent with a population

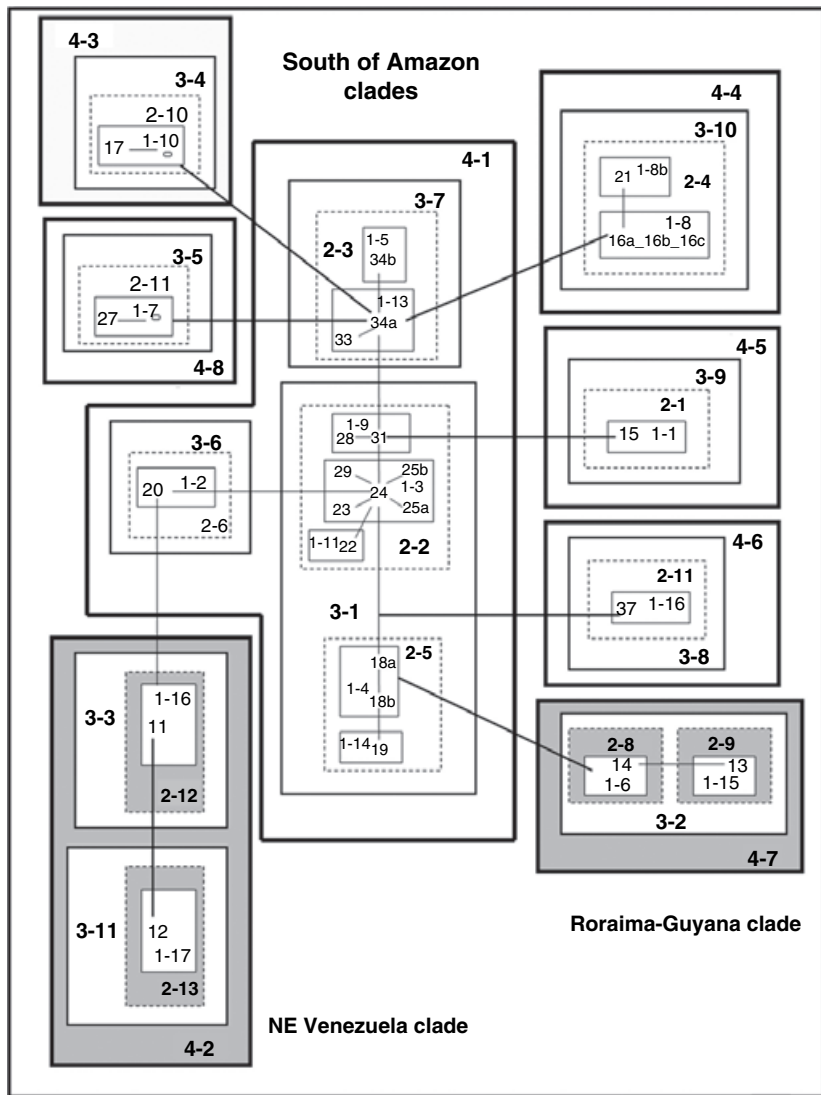


Figure 6 Nested cladogram for the *Crotalus durissus* data set based on the network of Fig. 4. Clades in grey represent north-of-Amazon populations.

Table 3 Results of permutational contingency test for clades analysed by nested clade phylogeographic analysis.

Clade	χ^2	P
1-3	6.0	0.354
1-13	3.0	0.328
2-2	12.38	0.008
2-3	3.0	0.333
2-4	7.0	0.047
3-1	12.00	0.007
3-2	14.0	0.001
4-1	16.0	0.001
4-2	4.0	0.31
Total cladogram	94.11	0.0001

expansion (negative values). Further analysis of clades and sub-clades suggested an incipient trend towards population expansion and differentiation. Tajima's *D* and Fu's *F_s* statistics showed non-significant negative values in clades distributed south of the Amazon, but positive values in the north.

Particularly, clade 4-1, which includes ancestral subclades south of the Amazon, presented significant Fu's and raggedness indices ($F_s = -5.462, P = 0.007; D = -0.952, P = 0.174; r = 0.0154, P = 0.004$), indicating that this clade was the result of population growth and expansion.

DISCUSSION

The data presented in this study shed additional light on the distributional history of the Neotropical rattlesnake in South America and the Amazon. Previously, we inferred (Wüster *et al.*, 2005a) that the populations of this species from open formations south of the Amazon basin originated from mid-Pleistocene dispersal across the Amazon basin, and interpreted this as supporting a hypothesis of Pleistocene fragmentation of the Amazonian rain forests. The question of how much modification of forest cover in the Amazon this allows us to infer, and in particular the question whether the phylogeography of *C. durissus* suggests fragmentation of the rain forests, or whether limited shrinkage around the edges can account for

Table 4 Nested clade phylogeographic analysis results for *Crotalus durissus* in South America.

Haplotypes			One-step clades			Two-step clades			Three-step clades			Four-step clades		
No.	D_c	D_n	No.	D_c	D_n	No.	D_c	D_n	No.	D_c	D_n	No.	D_c	D_n
23	0.0	104.16	1–9	200.60	395.45	2–2	573.65*	629.06	3–1	648.72	657.28	4–1	601.39**	1474.01
29	0.0	298.84	1–3	0.0*	490.20	2–5	0.0	644.21	3–7	0.0	371.132**	4–2	686.40	2459.65
24	0.0	104.16	1–11	0.0	806.27	<i>I – T</i>	–	–	<i>I – T</i>	648.72	286.15	4–7	112.07*	1539.44
25	0.0	104.16	<i>I–T</i>	75.21	–351.60*							<i>I – T</i>	–	–
<i>I – T</i>	0.0	–48.67												
			1-2-11-YES			1-2-11-12-NO			1-2-3-5-6-7-YES			1-2-3-4-9-NO		
			Range expansion			Contiguous range expansion			Restricted gene flow/ dispersal but with some long-distance dispersal			Allopatric fragmentation		
			Implies range expansion in clades from south western Sao Paulo area			Implies range expansion from south-west area to north-east and Mato Grosso, especially evident in clade 2-2			Implies a dispersal event between clades from the Sao Paulo area to north-east Brazil (Fig. 7)			Indicated by allopatric distribution between Venezuelan and Guyana–Roraima–South Amazon clades separated by the Amazon rain forest		
33	0.0	89.84	1–8	0.0*	416.33	2–8	0.0**	2976.8	3–3	0.0	861.00			
34	0.0	313.16	1-8b	0.0	312.15*	2–9	0.0	119.56	3–11	0.0	570.03			
<i>I – T</i>	0.0	313.16	<i>I – T</i>	0.0	104.17	<i>I – T</i>	0.0	1490.5**	<i>I – T</i>	0.0	290.97			
			1-2-3-5-6-7-8-YES			1-19-NO								
			Restricted gene flow/ dispersal but with some long-distance dispersal			Allopatric fragmentation								
			Implies a dispersal event between populations 21 and 16 (Fig. 7)			Indicated by allopatric separation between Guyana and Roraima clades								

D_c = clade distance; D_n = nested clade distance; *I – T* = Internal minus tip clades in D_c and D_n values. Inferred processes are in bold, followed by the implication for the phylogeographic pattern of *C. durissus*.

* $P < 0.05$, ** $P < 0.001$.

the observed pattern, was subject to subsequent debate (Gosling & Bush, 2005; Wüster *et al.*, 2005b). Here we show that the phylogeographic pattern of *C. durissus* is best explained by a Pleistocene trans-Amazonian corridor of continuous distribution: past savanna or dry forest vegetation.

Our analyses do not support the hypothesis of a coastal corridor, which only suggests limited shrinkage of the rain forests. This hypothesis would predict that the most basal haplotypes south of the Amazon should be found in north-eastern Brazil, whereas haplotypes from further south and south-west in southern South America should be monophyletic. Instead, the most basal and divergent haplotypes south of the Amazon originate from western-central Brazil (western Mato Grosso) and Paraguay, whereas the north-eastern haplotypes are nested deeply among haplotypes from southern and central-western Brazil. The phylogeny predicted by the coastal corridor hypothesis, involving basal north-eastern haplotypes, is rejected by significant tree topology tests. This finding suggests that the populations of *C. durissus* south of the Amazon had their origin in the western part of southern South America, which is consistent with a corridor through the central Amazon basin, but not with the coastal corridor hypothesis.

Using haplotype networks, we found a statistically significant parsimonious genetic connection between rattlesnake populations from Roraima and Mato Grosso, but not with populations from north-eastern Brazil. Furthermore, the nested clade analysis supports range fragmentation, rather than long-distance dispersal, as the cause of the trans-Amazonian break in the distribution of *C. durissus*. In other words, the genetic structure of these populations bears the hallmarks of a past continuous distribution. Gosling & Bush (2005) suggested that the current distribution may be due to dispersal ‘through a series of staging areas as different regions altered in response to different climate changes’, for instance due to dispersal corridors opening and closing asynchronously in different parts. However, this ‘shifting mosaic’ model suggests extensive isolation of a relatively small dispersing population. As a result, the resulting population structure of the southern populations would probably have been more akin to that resulting from long-distance dispersal, rather than that expected from vicariance in a formerly continuous distribution.

The ancestral haplotypes for the southern populations appear to be located in southern Brazil, not north-eastern Brazil. This suggests that *C. durissus* dispersed from present-

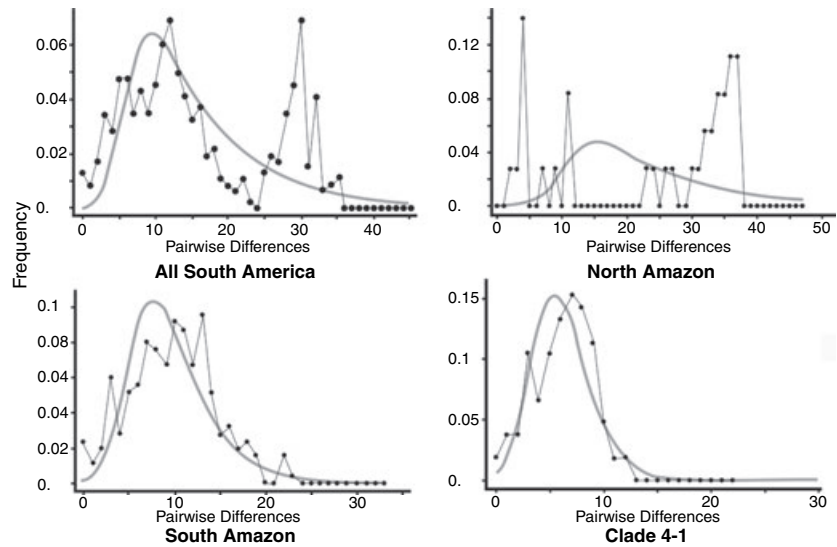


Figure 7 Mismatch distributions based on the frequency distribution of pairwise nucleotide difference between *Crotalus durissus* individuals within populations in regions. The expected frequency is based on a population growth–decline model, determined using the DnaSP 4.10.3 5 program (Rozas & Rozas, 1999), and is represented by a continuous line. The observed frequency is represented by a dotted line.



Figure 8 Distribution of clades inferred in the present study (Table 3; Fig. 6). Numbers represent sampled localities. Solid arrows represent dispersal routes and our inferred central corridor based on statistical parsimony connections (95% significance). Dashed arrow represents 93% significance multi-step connection.

day Roraima to Mato Grosso and south-eastern Brazil, then from there to other locations south of the Amazon Basin. The populations of *C. durissus* in north-eastern Brazil thus appear to be the result of dispersal from south-eastern or central Brazil, not from the Guyanas along the Atlantic coast. Again, these genetic patterns suggest dispersal across the centre of the Amazon Basin, suggesting the presence of a savanna and/or dry forest corridor across the centre of the Amazon basin. This contention is supported further by neutrality tests and nucleotide diversity indices.

Our biogeographical interpretations based on topology tests, haplotype network connections and neutrality tests are supported by NCPA. However, there are some limitations regarding the conclusions of this analysis. First, our data set consisted of a limited sample size per locality. NCPA is quite sensitive to sample size and low geographical variation (e.g. few individuals and localities sampled), which can lead to an inconclusive inference (Templeton, 2004). Nevertheless, despite our limited sampling, we were able to follow Templeton’s inference key and to find allopatric fragmentation as the

most likely process at higher nesting levels. In particular, the NCPA indicates allopatric fragmentation, not long-distance dispersal, as the explanation for genetic structure in the Venezuelan and Guyana–Roraima–South Amazon clades, which are separated by the Amazon rain forest and are connected to one another by a larger-than-average number of mutational steps. Despite the limited sample size, our data thus suggest that allopatric fragmentation of the range of *C. durissus* is the result of the mid-Pleistocene closure of a habitat corridor that once connected populations from north and south of the Amazon Basin, as a result of expansion of the Amazon rain forest. The accuracy of NCPA has previously been questioned, based on simulations and comparing the outcomes of the inference key (Knowles & Maddison, 2002; Masta *et al.*, 2003), the main criticism being that NCPA can lead to a high rate of false-positive conclusions. In order to resolve these problems, Templeton (2004) provided a revised and more conservative version of the inference key. We therefore believe that the accuracy of our results based on NCPA is robust, especially taken in conjunction with the other analyses, which all provide congruent results.

In summary, all our analyses reveal phylogeographic and genetic patterns most consistent with dispersal along a central Amazonian corridor, followed by later vicariance due to expanding rain forests, and dispersal into north-eastern Brazil. It is theoretically possible that this pattern may have been caused by an initial coastal dispersal, followed by extinction of north-eastern Brazilian populations, and later recolonization from the south. Consistent with this hypothesis, there is evidence of Pleistocene increases in forest cover across north-eastern Brazil, connecting the Atlantic forest and the Amazon Basin (Lynch, 1988; De Oliveira *et al.*, 1999; Puerto *et al.*, 2001; Costa, 2003). However, several lines of evidence weaken the extinction–recolonization hypothesis for north-eastern Brazil:

1. the high degree of endemism in the Atlantic forest and the often basal position of Atlantic forest organisms in phylogeographic studies of tropical South American organisms (Patton *et al.*, 2000) all suggest that rain forest cover in north-eastern Brazil is very unlikely to have been complete enough to lead to extinction of *C. durissus* in that region;
2. an explanation of colonization via north-eastern Brazil, followed by extinction and recolonization, is less parsimonious than one of dispersal across the central Amazon and then up to north-eastern Brazil;
3. the divergence between the western Mato Grosso haplotype (PLacerda) and all other southern populations followed very rapidly after the divergence between Guyanan and southern populations: the molecular dating analysis of Wüster *et al.* (2005a) estimated the divergence between the Guyanan and southern populations at 1.08 Ma, and the divergence between PLacerda and other southern haplotypes at 0.95 Ma.

Moreover, in our analysis here the monophyly of the southern populations is supported only weakly by the three-gene data and not at all by the two-gene data. This suggests that genetic diversification began in south-western Brazil

immediately after the crossing of the Amazon. A coastal dispersal route followed by extinction and recolonization would predict a greater hiatus between the initial north–south divergence and later divergence in south-western Brazil.

We did not explicitly test the possibility of an Andean corridor for *C. durissus*, as the observed distribution and phylogeographic pattern make this hypothesis extremely unlikely. Dispersal along an Andean corridor would predict that the haplotypes closest to those of the southern populations should be found in north-western South America (western Venezuela, Colombia), and predict the existence of remnant populations along the western edge of the Amazon Basin or within the Andes. In fact, as shown earlier, the north-western haplotypes are the most basal and most distant from the southern haplotypes, and relict populations are found in the central Amazon (Santarém, Roraima, Humaitá, Cachimbo), whereas there are none along the western edge of the Amazon or in the Andes.

In summary, our analyses suggest that dispersal across the central Amazon Basin is a more likely explanation for the observed phylogeographic pattern than alternative scenarios involving coastal dispersal followed by multiple extinction and dispersal events.

Gosling & Bush (2005) criticized our interpretation of a phylogeographic analysis of *C. durissus* as supporting a hypothesis of Pleistocene fragmentation of the Amazonian rain forests. They offered alternative scenarios to explain the occurrence of *C. durissus* in some of the enclaves, and suggested peripheral changes in the rain forest cover and niche-habitat shift in *C. durissus*. As previously outlined, there is no evidence for historical shifts in the habitat requirements of *C. durissus* (Wüster *et al.*, 2005b). The data presented here refute Gosling & Bush's scenarios on the basis of phylogenetic population-level analyses, which have identified the most parsimonious corridors and dispersal routes. The most parsimonious explanation for the pattern observed is dispersal and a subsequent continuous distribution across the central Amazon (Fig. 6), followed by later vicariance. Again, data from isolated populations within the Amazon basin, particularly those isolated deep inside Amazonia and in the path of a likely trans-Amazonian dispersal corridor, such as Humaitá, Santarém and Serra do Cachimbo (Campbell & Lamar, 2004), would provide powerful additional evidence on the distributional history of *C. durissus* in South America.

Many critics of the PRH argue that the hypothesis oversimplifies the process of speciation in the Amazon (e.g. Bush, 1994, 2005). They rightly argue that the high degree of Amazonian species diversity is due to a complex suite of mechanisms. The data presented here and previously (Wüster *et al.*, 2005a) lead us to argue that some kind of fragmentation of the Amazonian rain forests is very likely to have been one of the mechanisms explaining some aspects of Amazonian diversity, or at least affecting distributional and population genetic patterns. The precise nature of this disruption remains unclear. The more extreme scenarios calling for semi-arid

formations and extensive savannas have been contradicted by palynological evidence, at least for the late Pleistocene (Colinvaux *et al.*, 2001; Mayle *et al.*, 2004). Alternative suggestions have included deciduous forests (which are more difficult to differentiate palynologically from tropical rain forests and thus are much easier to reconcile with the existing palynological evidence; Pennington *et al.*, 2000) or at least reduced canopy density in many parts of the basin, including a corridor across the middle of the Amazon basin (Cowling *et al.*, 2001; Maslin *et al.*, 2005). These scenarios are in accord with modelling of the distribution of forest species during the climatic conditions of the last glacial maximum (Bonaccorso *et al.*, 2006). The degree of perturbation of the Amazonian forests required to account for dispersal and subsequent vicariance by species such as *C. durissus* and co-distributed taxa remains unclear in the absence of a better understanding of the fundamental niches of these species (Bush, 2005).

The role of possible Pleistocene rain forest fragmentation or perturbation in causing speciation among Amazonian forest taxa remains unclear. Many speciation events in the Amazon clearly pre-date the Pleistocene by a considerable margin (Moritz *et al.*, 2000; Patton *et al.*, 2000; Glor *et al.*, 2001; Ribas *et al.*, 2005). However, high speciation rates in rain forest taxa have occurred during the Pleistocene, for instance in tree genera such as *Ruprechtia* and *Inga*, the phylogenetic patterns of which reflect an outstanding diversification in the Amazon rain forest during the Pleistocene (Richardson *et al.*, 2001). On a smaller geographical scale, patterns of molecular diversification in mammals (Steiner & Catzeflis, 2004), amphibians (Noonan & Gaucher, 2006; Noonan & Wray, 2006) and snakes in the Atlantic forest (Grazziotin *et al.*, 2006) have also demonstrated the genetic footprint of Pleistocene range perturbation. We believe that other taxa may show a similar mixture of processes in their diversification patterns, and that allopatric differentiation due to historical climate change has to be considered as a factor in the complex model of Amazonian speciation (Noonan & Wray, 2006; Rull, 2006).

The results of this study re-emphasize the potential for open-formation taxa with disjunct trans-Amazonian distributions as ideal organisms for detecting the effects of past climate changes on Amazon forest fragmentation and the presence of refugia. Savanna and seasonal dry forests might themselves now be refugia for species adapted to drier habitats (Pennington *et al.*, 2000). Diverse taxa share this distributional pattern (Pennington *et al.*, 2000, 2004; Fig. 1), and it can be hypothesized that they share a common biogeographical history, a hypothesis that can be tested using similar approaches to that exemplified here and by Wüster *et al.* (2005a). These species thus have the potential to provide a rich source of evidence on the Pleistocene history of the Amazonian forests.

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