DOCTOR OF PHILOSOPHY

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Molecular Phylogeography of the Neotropical Rattlesnake *Crotalus durissus*

A thesis submitted to the University of Wales, Bangor by

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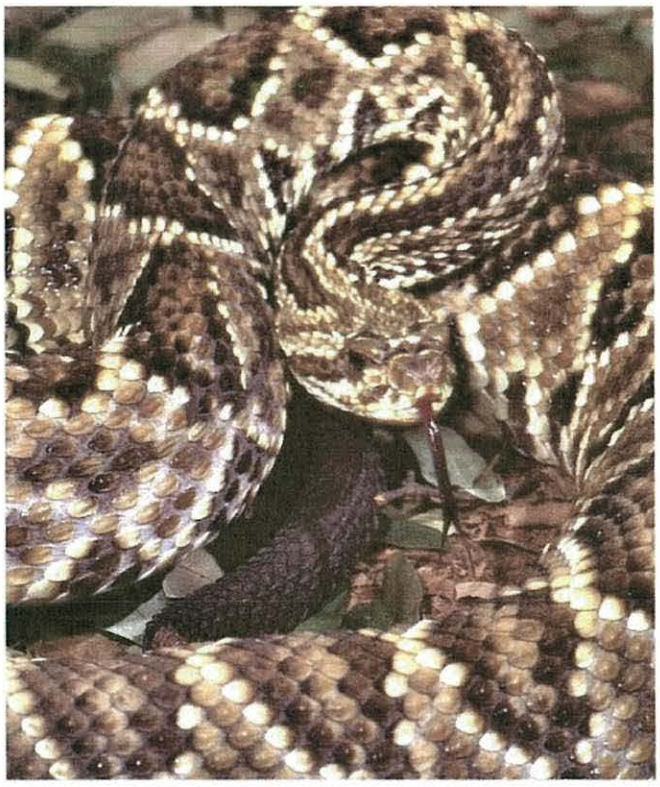

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SUMMARY

This study analyzes the phylogenetic and phylogeographic relationships of the rattlesnake, *Crotalus durissus*, throughout its geographical range using several molecular markers. The Cyt b and ND4 fragments of the mtDNA were sequenced and analyzed. The results support the monophyly of all South American *C. durissus* populations, including populations that had previously been considered as separate species. The Cyt-b and ND4 analysis indicates that the Central American and Mexican *C. durissus* populations are paraphyletic.

The nuclear intron 7 of β-fibrinogen was also studied. However, it did not aid in resolving the phylogenetic relationships of the populations analyzed. Amplified fragment length polymorphism (AFLP) analysis suggested phylogenetic patterns similar to those indicated by mtDNA analysis, except for small inconsistencies in the topologies. Pairwise and partial Mantel tests showed a significant correlation between AFLP and mtDNA phylogenies, taking into consideration the geographic distance effect.

This study reveals that *C. durissus* recently dispersed into South America. The timing of the dispersal event is consistent with the hypothesis that this species invaded South American 1-3 Mya after the uplift of the Panamá land bridge. The Central American lineages are much older and divergent, the products of orogenic evolution in Mexico. The implications for systematics include a reconsideration of the status of some taxa (*C. unicolor* and *C. vegrandis*) that clearly appear conspecific of South American *C. durissus*. The Mexican lineages are regarded as full evolutionary species.

The phylogeography of *C. durissus* in South America shows a stepwise colonisation 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 northernly haplotype clades. Low sequence divergence between populations from north and south of the Amazon rainforest is consistent with mid-Pleistocene divergence, approximately 1.08 million years ago. This suggests that the Amazonian rainforests must have become fragmented or considerably constricted during that period.

Correction Sheet

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I dedicate this effort to the memory of Guadalupe Lopez, Maria Socorro Mascareñas, Benito Hernandez, and Marcos Mascareñas

Chapter 1. Introduction to Phylogeography and the geographical variation in *Crotalus durissus*

1.1. Phylogeography

Phylogeography is a subdiscipline of biogeography that takes into account the geographic distribution of taxa (generally species and populations) in establishing their phylogeny. Phylogeography describes and seeks to understand the geographic patterns that result from divergence, leading to speciation (Avise *et al.* 1987; Avise 2000). The main goal of phylogeography is to draw inferences about organisms based on gene phylogenies and their distributions (Avise 2000).

Despite that phylogeography as a science is still quite young (less than 20 years, Avise *et al.* 1987), the explosive amount of empirical research devoted to understanding phylogeographic patterns does not have precedent in the history of the biology (Avise 2000). Among the reasons of this success, is that fact that phylogeography offers a testable arena where species distribution patterns can be evaluated using evolutionary processes as competing hypothesis (e.g. dispersal versus vicariance) at any level of the phylogeny of the target taxon. On the other hand, phylogeography is a unifying science, incorporating the views of other biological disciplines, making it a multidisciplinary approach in evolutionary research. Likewise, phylogeography has contributed to a better understanding of the systematic relationships in several taxa, and has made a landmark in the species concept issue, introducing alternative concepts based on the homology of gene lineages (Avise *et al.* 1987; Avise 2000; Moore 1995; Templeton 2001).

In the present thesis, I explore the origin of the Neotropical rattlesnake, *Crotalus durissus*, and test the hypothesis that *C. durissus* orginated in Mexico and Central America, and then dispersed to South America. I approach this problem using several molecular markers and different phylogenetic algorithms. Finally, I explore the implications of this study for the systematics of the *C. durissus* complex.

1.2. Introduction to methods in phylogenetic inference

There are different approaches for using molecular sequence data in the reconstruction of the evolutionary relationships among genes and organisms. The most useful is the reconstruction of such relationships in the form of a phylogram or phylogenetic tree (Swofford *et al.* 1996; Page and Holmes 1998; Holder and Lewis 2003; Hall 2004). The steps necessary to build a phylogenetic tree from molecular data are:

a) Alignment of sequences. The nucleotide or protein sequences from different taxa can be aligned using determined criteria of homology. In some cases, the homology of different sequences can be visualized and then the alignment can be done by hand. In others, multiple sequences offer some difficulties. Fortunately, there are several programs capable of producing alignments of many sequences simultaneously. The most popular is CLUSTAL which has been integrated as part of the phylogenetic package MEGA (Kumar *et al.* 2003). Previous to tree building, the final alignment should be carefully scrutinized in order to find congruence with previous and independent phylogenetic evidence and other assumptions of structure and function. Once one proceeds to tree-building, the computer generated alignment will be blind to any errors in alignment (Page and Holmes 1998; Holder and Lewis 2003; Hall 2004).

b) Phylogenetic signal. Some aligned sequences are almost identical and are not suitable for phylogenetic comparisons. Others are so divergent that sequence comparisons show lack of homology. Useful aligned sequences for phylogenetic reconstruction are those that fall in between. These sequence alignments will have a mixture of conserved and random positions and will be the most useful in phylogenetic inference (Hillis 1993).

c) Choice of the tree building method. Once the alignment is complete, the next steps in phylogenetic inference are to decide the most appropriate tree building method for a set of sequences.

In order to build a phylogram or a phylogenetic tree, there are several classifications of phylogenetic methods based on different criteria. This section will describe briefly the methods available and comment on some of their advantages and. For more detailed information on these and other methods see Holder and Lewis (2003), perhaps the best review on traditional and Bayesian methods.

Commonly, the tree building methods are classified as distance based and character based methods. Distance based methods compute pairwise distances according to a set of characters (e.g. sequences or morphological characters). In the process, the actual data is transformed to fixed distances, which are used in the building of the tree. In contrast, character based methods use the actual data without any transformation, using all the character information, and assuming an evolutionary mode of character change to build the tree (Swofford *et al.* 1996; Page and Holmes 1998; Holder and Lewis 2003; Hall 2004).

These methodologies are classified on the basis of the approach used to infer phylogeny. For example, some methods focus more on the algorithms and while others focus on the phylogenetic criterion used in the analysis (Swofford 1996). An algorithm-based method generates a phylogeny by following a series of steps, whereas criterion-based methods define an optimality criterion for comparing alternative phylogenies and deciding which one is better. In criteria-based methods, every tree is scored and used to rank the resultant phylogenies to provide information about the strength of support for that tree. A limitation of the criteria-based methods is that these methods do not produce exact results because of the large number of alternative phylogenetic solutions. The algorithmic methods are computationally much faster than the criteria-based methods because they do not evaluate the trees generated.

In practice, for both the algorithm or the phylogenetic approaches, there are four methodologies currently used for inference and phylogenetic reconstruction (Holder and Lewis 2003): a) distance methods, b) parsimony methods, c) likelihood based methods, and d) Bayesian methods.

a) Distance methods. These are based on the estimation of a pairwise-genetic distance matrix from the sequences under study, so that original data is transformed into distance measurements. One of these methods, the Neighbour-joining (NJ) performs a cluster algorithm allowing unequal rates of molecular evolution among branches. This is performed by adding at each step of the analysis, a transformed distance matrix, adjusting the branch lengths between each pair of nodes, which is based on the mean divergence compared to all the nodes. One obvious disadvantage of this methodology is that a considerable amount of phylogenetic information is lost in the process. However, the fast

calculation of the distances and trees made these methods very popular in the past, but they currently are regarded as preliminary methods of phylogenetic reconstruction (Page and Holmes 1998; Holder and Lewis 2003; Hall 2004).

b) Parsimony methods. These are character-based and use information on changes in the nucleotides at every site. Maximum Parsimony (MP) searches for the phylogeny that minimises the number of evolutionary steps required to explain the original data (Page and Holmes 1998; Holder and Lewis 2003; Hall 2004). The basic principle is that shared common characters are a consequence of common ancestry, and the simplest pattern is two descendents from one ancestor. Homoplasy arises from any violation of this assumption (Swofford *et al.* 1996; Page and Holmes 1998; Hall 2004). In MP the minimum number of steps to explain the data is the criterion for choosing the best tree among many possible. An algorithm is used to determine the minimum number of steps necessary for any given tree to be consistent with the data. That number is the score for the tree, and the tree with the lowest score is the most parsimonious tree among the universe of possible trees (Hall 2004). The main disadvantage of MP is its simplicity. In some cases, different molecular evolutionary modes can produce the same set of sequences.

c) Maximum Likelihood (ML). In order to infer a phylogeny, ML evaluates the probability that a chosen evolutionary model will have generated the observed sequence data. Phylogenies are then inferred by finding those trees that show the highest likelihood. The supposition is that a phylogenetic tree with a higher probability of reaching the observed data is preferred to one with a lower probability. A basic difference between Parsimony and ML is that parsimony attempts to minimize the amount of

evolutionary change required to explain the sequence data, while ML estimates the actual amount of change in sequences according to an evolutionary model (e.g. a molecular model of sequence substitution). The advantage of ML over other methods is that it is statistically well founded, performing evaluations of different tree topologies and using all the sequence information. Two relative disadvantages of ML are the computational effort required for many sequences, and the high dependence on the model of evolution used. Of available programs, Model Test is currently the most popular because it provides the user 56 models from which to chose the best fit for the sequence data (Posada and Crandall 1998; Holder and Lewis 2003).

d) Bayesian inference. The relatively new Bayesian methods are part of the Likelihood methods family of phylogenetic analysis (Huelsenbeck *et al.* 2001; Holder and Lewis 2003; Hall 2004), and are becoming quite popular because the methods produce both tree estimates and measures of uncertainty for the clades on the tree. Bayesian inference is quite similar to ML in that it uses a likelihood function and an explicitly stated model of nucleotide substitution. The preferred phylogenetic hypothesis is the one that maximizes the posterior probability. The posterior probability for a hypothesis is proportional to the likelihood multiplied by the prior probability of that hypothesis. For a better theoretical background see Huelsenbeck *et al.* (2001), Holder and Lewis (2003) and Hall (2004). Prior probabilities of different hypotheses are based on an expected distribution of data. The prior probability distribution describes the probability of different trees given previous expectations of the data; the posterior probability distribution describes the probability of trees considering the prior distribution, the evolutionary model, and the sequence data. In most cases, the distribution of prior probabilities are specified as uninformative (e.g. uniform or flat priors), therefore most of

the differences in the posterior probability of hypotheses can be attributable to different likelihood values. Among the advantages of Bayesian inference compared to other methods, is the fact that it provides measures of clade support faster than ML bootstrapping. Like ML, Bayesian methods allow complex models of sequence evolution to be implemented using all the information of sequence data. The likelihood functions for phylogenetic models are too complex to integrate analytically, so Bayesian analysis is based on an algorithm, the Markov chain Monte Carlo (MCMC) (Metropolis *et al.* 1953; Hastings 1970;). Bayesian inference utilizes the MCMC simulation in combination with a chosen evolutionary model (e.g. mode of sequence evolution) and the sequence data to produce a posterior probability distribution of trees. The distribution of trees is the main product of Bayesian phylogenetic analyses. Thus the values seen in Bayesian phylograms are the posterior probabilities for a particular clade (some times along with MP and ML bootstrap values), the probability that such a clade is the most likely given the priors, model, and data (Huelsenbeck *et al.* 2001; Holder and Lewis 2003; Hall 2004).

Bootstrapping

Once the phylogram has been built, we need to know the realiability of the obtained tree, in other words, the extent to which the sequence data supports each of the relationships depicted in the phylogram. For many years, the statistical method known as bootstrapping was used for this problem (Efron 1979; Felsenstein 1985; Goldman *et al.* 2000, Swofford *et al.* 1996; Page and Holmes 1998; Holder and Lewis 2003). Bootstrapping attempts to estimate confidence levels of inferred relationships. The method consists of resampling the original data matrix with replacement of the characters. An algorithm is performed on each of these replicate data sets. This process is repeated until a new pseudoreplicate is produced with the same size as the original. Some characters will be sampled more than

once. The process is repeated many times and trees are re-built each time. When the bootstrap procedure is finished, a majority rule consensus tree is obtained from the optimal tree from each bootstrap sample. The bootstrap support for any internal branch is the number of times it was recovered during the bootstrapping procedure and is usually depicted at the node of each clade. A general assumption in bootstrapping is that each of the sites in the original data is independent of the others. Some researches consider bootstrap values as conservative measures of support (Hillis and Bull 1993; Zharkikh and Li 1992). Bootstrapping is a measure of the effect of the perturbation in sequence data sampling, so bootstrap values predict whether the same result would be seen if more data were collected, rather then if the bootstrap result is correct (Holder and Lewis 2003).

1.3. Molecular Markers: mtDNA and AFLPs

Mitochondrial DNA

The use of mitochondrial DNA (mtDNA) in phylogenetic studies has clarified the relationship between gene genealogies and the phylogeny of organisms. The usefulness of mtDNA analysis has forced the addition of a phylogenetic perspective to studies of intraspecific evolutionary process and as a result, has provided an empirical and conceptual bridge between the traditionally separate disciplines of systematics and population genetics (Moritz *et al.* 1987; Avise *et al.* 1987).

Mitochondrial DNA has become increasingly popular in phylogenetic and population genetic studies with the development of a methodology for mtDNA isolation, the use of restriction enzymes to detect nucleotide differences, the development of PCR methodology and universal primers for amplification of mtDNA (Lansman *et al.* 1981;

Kocher *et al.* 1989). Since the development of PCR amplification (Saiki *et al.* 1988) and direct sequencing techniques, the use of mtDNA to infer phylogenetic relationships has increased dramatically (Moritz *et al.* 1987; Edwards and Wilson 1990; Meyer *et al.* 1990; Carr and Marshall 1991; reviewed in Avise 2000).

The mtDNA marker is currently used widely, even though the assumption of selective neutrality of molecular evolution (Kimura 1983; Gillespie 1991) has been questioned (Nigro and Prout 1990; Singh and Hale 1990; Malhotra and Thorpe 1997). Substitution rates vary within a codon of protein-coding regions of the mitochondrial genome, suggesting the operation of selective constraints (Palumbi and Kessing 1991), although the extent to which selection influences substitutions at silent positions is not clear (Gillespie 1991).

Several characteristics of mtDNA make it highly useful for phylogenetic analysis: First, the rapid pace of mtDNA nucleotide substitution offers advantages for phylogenetic analysis at the microevolutionary level that can not be matched by any other nuclear gene. The approximate mutation rate in mtDNA is 10^{8}/site/year (Brown *et al.* 1979, Ferris *et al.* 1983, De Salle *et al.* 1987) compared to 10^{9}/site/year in nuclear genes. Most differences between mtDNA sequences are point mutations, with a strong bias for transitions over transversions (Brown *et al.* 1982).

Second, mitochondrial genes are inherited as one linkage group in the absence of recombination (Hayashi *et al.* 1985, Hoech *et al.* 1991). mtDNA is haploid and uniparentally inherited (with some exceptions, see below) and thus the variability is introduced by mutations alone. Compared to diploid nuclear autosomal genes with

biparental transmission, the effective population size of mtDNA is one quarter of that for nuclear autosomal genes (Moore 1995). Therefore, an mtDNA tree is more likely to be congruent with a species tree due to a high probability of coalescence even when speciation events have occurred within short time-periods.

AFLPs

The amplified fragment length polymorphism (AFLP) technique takes advantage of PCR to amplify a limited set of DNA fragments from a DNA sample (Vos *et al.* 1995; Blears *et al.* 1998). The technique consists of a series of steps to screen genetic variation as a pattern of polymorphic bands and to analyze the resulting information using multivariate and grouping algorithms or phylogenetic tools. The first step consists of digesting the whole genomic DNA using 6 cutter restriction enzymes followed by a PCR-preamplification using preselective primers. Then a set of anonymous specific primers is added and a final PCR amplification is performed where the polymorphic bands are screened in a polyacrilamide gel or automatic sequencer (Vos *et al.* 1995; Kardolus *et al.* 1998; Seehausen *et al.* 2003; Ogden and Thorpe 2002; Creer *et al.* 2004; Sullivan *et al.* 2004).

AFLPs are quickly becoming popular for the study of genetic variation. Since the AFLP technique can be applied to a wide variety of organisms with no prior sequence information, the technique has the potential to become a universal DNA fingerprinting tool. AFLP can be used to distinguish even very closely related organisms, including near isogenic lines (Ogden and Thorpe 2002; Creer *et al.* 2004; Sullivan *et al.* 2004). The differences in fragment lengths generated by this technique can be traced to base changes in the restriction-adapter site, or to insertions or deletions in the body of the

DNA fragment (Vos *et al.* 1995; Kardolus *et al.* 1998). Most importantly, AFLPs have been shown to be reproducible and reliable (Vos *et al.* 1995; Bakkeren *et al.* 2000; Hodkinson *et al.* 2000; Kanzaki *et al.* 2000; Giannasi *et al.* 2001; Parsons and Shaw 2001; Buntjer *et al.* 2002; Allender *et al.* 2003; Seehausen *et al.* 2003; Ogden and Thorpe 2002; Creer *et al.* 2004; Sullivan *et al.* 2004). This is because limited sets of generic primers are used and these are annealed to the target under stringent hybridization conditions. AFLP data must be treated as dominant markers, since homo and heterozygotes cannot be distinguished unless pedigree studies are undertaken to determine the inheritance patterns of each band. However, the large number of bands gives a good estimate of variation across the entire genome (Giannasi *et al.* 2001; Parsons and Shaw 2001; Buntjer *et al.* 2002; Allender *et al.* 2003; Seehausen *et al.* 2003; Ogden and Thorpe 2002; Creer *et al.* 2004; Sullivan *et al.* 2004).

1.4. The *Crotalus durissus* complex: Biogeography

Any attempt to understand the phylogeographic processes that govern the amount of current variation in selected taxa must take into account the historical biogeography of the taxa in question. The genus *Crotalus* originated somewhere in Mexico and Central America (Armstrong and Murphy 1979; Klauber 1972; Greene, 1997; Place and Abramson 2004); some authors propose the highlands of Mexico as the centre of rattlesnake diversification (Place and Abramson 2004). Probably the ancestor of the *C. durissus* complex was part of the vicariance events that occurred as consequence of the orogenesis of central Mexico and the Isthmus of Tehuantepec (Flores 1993; Graham 1993). The event that favoured the dispersal of *C. durissus* in South America occurred over the past 3-3.5 Mya and is known as the Great American Interchange (Marshall *et al.*

1979; Stehli and Webb 1985; Bermingham and Martin 1998). This process started with the formation of the isthmian land bridge of Panamá and occasioned an extensive intercontinental exchange of flora and fauna between North and South America. This event has been widely studied in geologic and biogeographic contexts and offers the opportunity to analyze the times of diversification and differentiation of the different lineages throughout the range of *C. durissus*.

Based on its present time distribution, *C. durissus* could have evolved in North America and subsequently dispersed into Central and South America after the formation of the lower Central American land bridge (Duellman 1978; Campbell and Lamar 1989; Bermingham and Martin 1998). The morphological differentiation in the South American rattlesnakes could be the consequence of forest contraction and isolation during the cycles of wet and dry periods of the Tertiary and Quaternary (Haffer 1997). Because the great forests of South America may not have existed until the late Pliocene-Pleistocene (Haffer and Prance 2001; but see Colinvaux *et al.* 2000), it is possible that *C. durissus* was present in the region prior to this time and that most of the vicariance events of *C. durissus* resulted from the emerging Amazonian forests.

Among rattlesnakes, the members of the Neotropical group, the *C. durissus* complex, are the most widely distributed rattlesnakes and exhibit a considerable amount of geographic variation (Campbell and Lamar 1989, 2004), leading some authors to claim full species recognition for some *C. durissus* populations in South America (Klauber 1972; Campbell and Lamar 1989). Additionally, some authors include *C. molossus*, *C. basiliscus*, *C. horridus*, as part of the complex, and *C. enyo* as a sister species (reviewed in Murphy *et al.* 2002). The thirteen subspecies of *C. durissus* recognized by Campbell and Lamar

(1989) have a discontinuous and scattered range distribution throughout Mexico, Central and South America, although most of subspecies are in South America (Fig 1). The fact that *C. durissus* is the only rattlesnake in Central and South America, strongly suggests a recent dispersal event from the northern region of its distribution (Duellman 1978; Campbell and Lamar 1989). The Amazon basin disrupts the South American *Crotalus* distribution; there are rattlesnakes north and south of the basin, but no rattlesnakes in the forests of the Amazon basin. They are found in the Amazon drainage in savannah enclaves, e.g., Humaita, Amapa, Marajo, Serra do Cachimbo (Campbell and Lamar 1989; 2004).

The traditionally recognized subspecies (Campbell and Lamar 1989, Fig 1.1) are as follows: *C.d. tectonics, C.d. culminates, C.d. tracing, C.d. durissus* in Mexico and Central America; *C.d. humanness* in Venezuela and Colombia; *C.d. unicolor, C.d. vegrandis, C.d. curium, C.d. dryings, C.d. marajoensis, C.d. cascavella, C.d. collilineatus,* and *C.d. terrificus* in the rest of South America. Recently (Campbell and Lamar 2004) modified the taxonomy of the group. In this thesis, I evaluate the modifications suggested by Campbell and Lamar (2004).

The South American *C. d. unicolor* and *C. d. vegrandis* have been accorded full species status by other authors (Klauber 1972; Murphy *et al.* 2002). Both of these forms appear to be recently derived from an ancestor shared with the northern South American populations of *C. durissus*. Thus recognizing *C. d. unicolor* and *C. d. vegrandis* as full species would make *C. durissus* a paraphyletic species group. In order to maintian *C. durissus* as a monophyletic group, Campbell and Lamar (1989) consider *C. d. unicolor* and *C. d. vegrandis* to be subspecies of *C. durissus.*

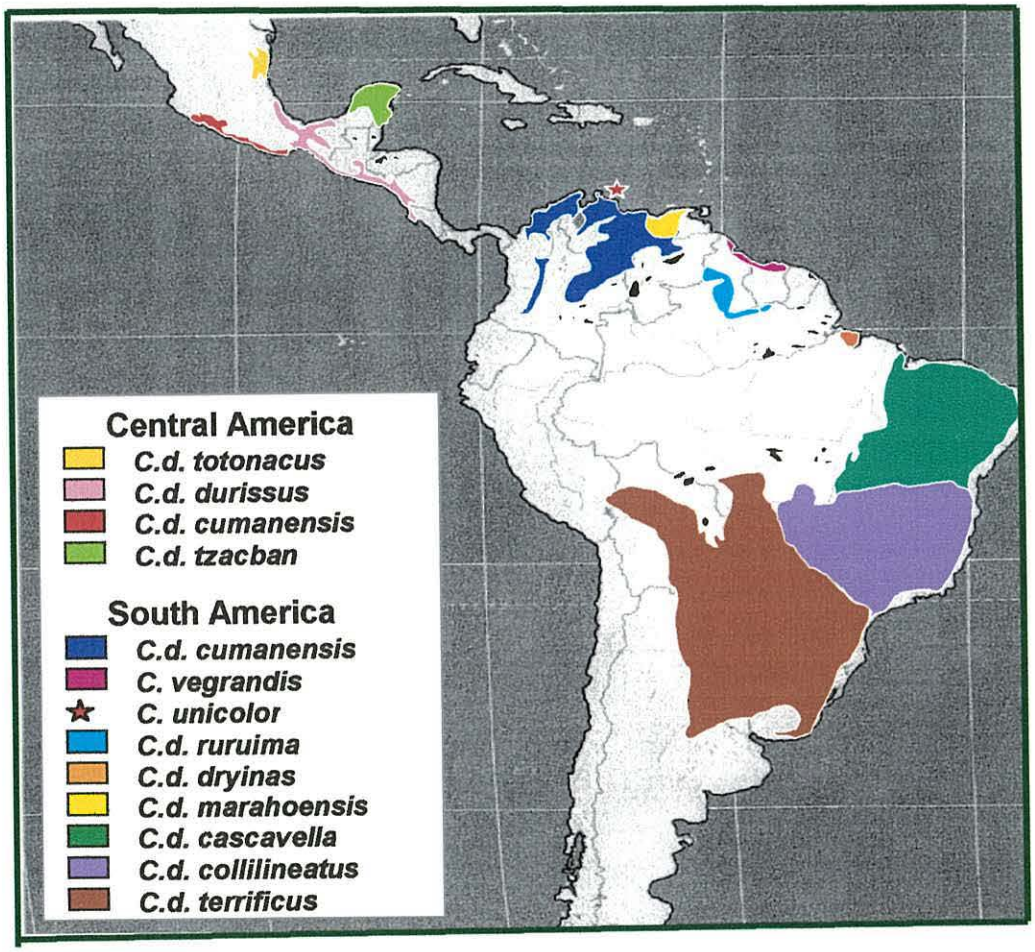

Fig.1.1. Geographic distribution of *C. durissus* in Central and South America. Approximate ranges of the recognized subspecies are shown (Campbell and Lamar 1989).

1.5. The *C. durissus* complex: Habitat

C. durissus is usually found in semi-arid regions, including dry to very dry tropical forest and thorn woodlands, but also in relatively dry open areas within mesic forests (Campbell and Lamar 1989; 2004). Other habitats include tropical deciduous forests, pine-oak forest, arid tropical scrub, grass pine or palm savannas, and less frequently natural breaks in cloud forests. *C. durissus* is encountered infrequently in tropical dense forest, and it is largely absent from rainforest.

Activity patterns vary according to latitude and habitat. In North and Central America, activity depends largely on season, due to hibernation in winter (Klauber 1972; Salomão *et al.* 1997). The rattlesnakes' prey consists mainly of rodents and birds. Some degree of ontogenetic change in diet has been observed in the North and Central America populations, but in South America this pattern is absent (Salomão *et al.* 1997).

1.6. The *C. durissus* complex: Morphological and colour pattern variation

For the most part, the various subspecies of *C. durissus* have been distinguished on the basis of size, colour, and to a lesser extent, scale pattern (reviewed in Klauber 1972, and Campbell and Lamar 1989). Body size varies geographically. The rattlesnake commonly reaches about 1000 mm (and much more) in length in most of the known distribution, but some South American populations apparently are dwarfed, including those in the inland savannas and highlands of Venezuela and Guyana and on the island of Aruba, where the

largest specimens do not exceed 1 m in length. *C. durissus* exhibits a considerable amount of ground colour variation, which hinders the taxonomy of this species.

As a broad generality, the ground colour of snakes from densely forested regions, such as the Pacific lowlands of Guatemala or parts of northern South America, tends to be considerably darker than in snakes from more arid regions, such as the Rio de las Balsas or Rio Motagua valleys in Mexico and Guatemala, respectively (Klauber 1972; Campbell and Lamar 1989). Other characters like body blotches, head pattern, paravertebral stripes, and head scutellation exhibit variation, but the extent and amount by which these characters help to distinguish the different populations of *C. durissus* must be explored.

The morphological differentiation of the populations in South America could be a consequence of the contraction and isolation of forest blocks near areas of surface relief in the periphery of early Amazonia during the cycles of wet-dry climatic periods of the Tertiary and Quaternary (Haffer 1997). Because the great forests of South America did not exist until the late Pliocene-Pleistocene, it is possible that *C. durissus* was present in the region prior to this time and that most of the vicariance events of *C. durissus* resulted from the emerging Amazon forests.

1.7. The *C. durissus* complex: Venom variation

In general, snake venom varies at the species and subspecies level and several studies have shown intraspecific variation in New World Crotalines (for reviews see: Daltry *et al.* 1997; Chippaux *et al.* 1991; Warrell 2004). This is of considerable importance to snake bite treatment because venom therapy depends greatly on the antivenom, so the

antivenom prepared for one variant might not provide adequate protection against a bite by another (Daltry *et al.* 1997; Theakston 1997; Warrell 2004).

Several types of venom variation in the Neotropical rattlesnakes have been reported, including geographic variation (Schenberg 1959; Jimenes-Porras 1964; Warrell *et al.* 1997; Daltry *et al.* 1996; Francischetti *et al.* 2000; Saravia *et al.* 2002), ontogenetic variation (Theakston and Reid 1979; Gutierrez *et al.* 1990; 1991; Minton and Weinstein 1986; Saravia *et al.* 2002), and sexual variation (Gutierrez *et al.* 1990; 1991).

The venom of the rattlesnake *C. durissus* has been reported to have a myotoxic and neurotoxic action characterized by the release of myoglobin from damaged skeletal muscle into serum and urine accompanied by intravascular haemolysis (Cupo *et al.* 1988). In South American, variation is evident in the symptoms caused by *C. durissus* bites (Barrio and Brazil 1951; Saravia *et al.* 2002). Two of the components of *C. durissus* venom, Crotamine and Crotoxin, have been studied extensively. Crotamine is a phospholipase myotoxin that induces paralysis and myonecrosis in skeletal muscle cells (Oguiur *et al.* 2000; Warrell 2004). Crotoxin is a neurotoxin consisting of a phospholipase and an acidic A component (crotapotin), which has a triphasic action, initially inhibiting, then facilitating, and finally blocking the release of acetylcholine in the cell membrane (Marlas and Bon 1982; Warrell 2004).

Several studies have demonstrated that there is geographical variation in the presence of Crotamine and Crotoxin *C. durissus* venoms (Schenberg 1959; Jimenes-Porras 1964; Warrell *et al.* 1997; Daltry *et al.* 1996; Francischetti *et al.* 2000; Saravia *et al.* 2002). Schenberg (1955) analyzed the distribution of the Crotamine in South America.

Crotamine is absent from populations in north and eastern Brazil, present in northwestern Sao Paulo State, and adjacent areas of Parana, and also present in Argentina. Some rattlesnake populations in south-eastern Sao Paulo State and Minas Gerais exhibit Crotamine while some do not, indicating a great degree of variation (Schenberg 1955; Warrell 1997).

Saravia *et al.* (2002) performed a comparative study of the electrophoretic profiles of the venoms of adult specimens and newborns of *C. durissus*, from Guatemala, Costa Rica, Venezuela and Brazil, and found variation in the presence of Crotamine. Using experimental mice, they found that venoms of *C. d. terrificus, C. d. cumanensis* (Brazil and Venezuela) and newborn *C. durissus* (Costa Rica) induced higher lethal and myotoxic effects than those of adult *C. durissus* (Guatemala and Costa Rica). In contrast, adult *C. d. durissus* and *C. d. cumanensis* venoms induced haemorrhage, whereas venoms of *C. d. terrificus* and newborn *C. durissus* lacked this effect. All venoms showed a coagulant effect in plasma, the highest activity caused by the venom of newborn *C. d. durissus.*

The high toxicity of South American and newborn *C. durissus* venoms is related to the presence of high concentrations of Crotoxin (Saravia *et al.* 2002; Warrell 2004). Crotaxin is less variable than Crotamine (Rangel-Santos *et al.* 2004). The main toxic characteristics of Crotoxin (CTX) and CB fraction were evaluated for three subspecies, *C. d. cascavella, C. d. collilineatus, C. d. terrificus*. The venoms presented similar chromatographic profiles, indicating no intraspecific variation in Crotoxin (Rangel-Santos *et al.* 2004).

Ontogenetic changes in venom have been detected in the electrophoretic patterns of *C. durissus* (Gutierrez *et al.* 1991; Saravia *et al.* 2002). Costa Rican populations of *C. durissus* show ontogenetic changes from neurotoxic venom in juveniles to hemotoxic venom in adults. In contrast, both juveniles and adults in the southern Brazil have neurotoxic venom (Gutierrez *et al.* 1990; Minton and Weinstein, 1986). Toxicity commonly decreases with increasing size, leveling off around maturation, and coagulant activity generally decreases with size (Gutierrez *et al.* 1990, 1991; Saravia *et al.* 2002; Warrell 2004). Finally, proteolytic activity has been observed to increase with snake body size (Gutierrez *et al.* 1991).

1.8. Research goals

The goals of this research are: 1) To provide a phylogeographic explanation for the geographic distribution of the *C. durissus* complex, using several molecular markers and analytical tools. 2) To determine the biogeographic events that produced the present time geographic distribution of *C. durissus.* 3) To compare the phylogenetic information provided by different molecular markers.

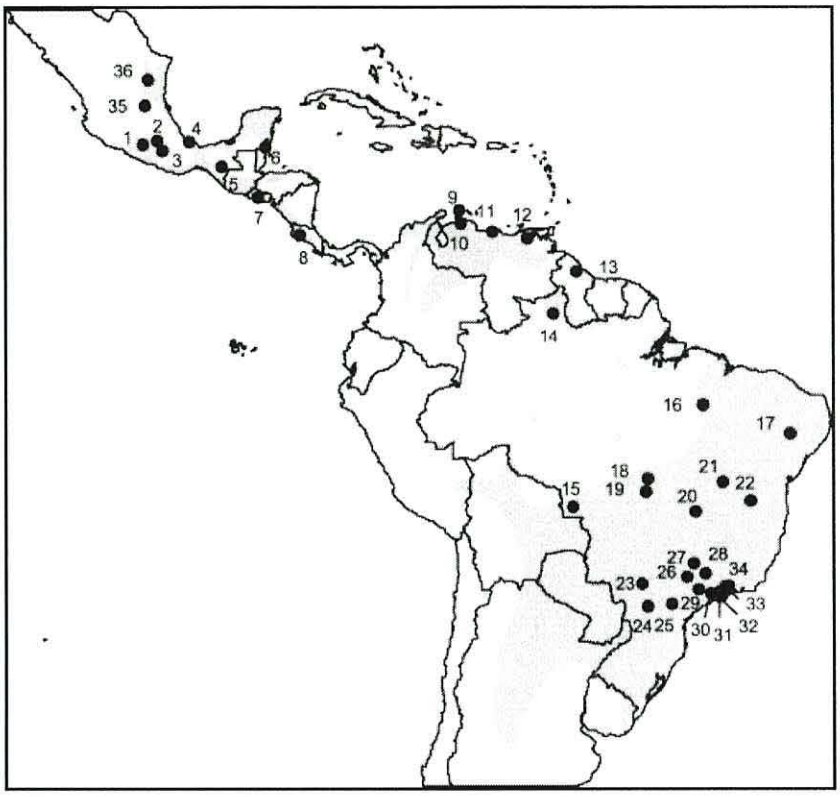

Fig 1.2. Sampling localities for the *C. durissus* complex (See the published paper in Appendix 1 for further details).

Chapter 2. Molecular phylogeography using mitochondrial Cytb and ND4 sequences

2.1. Introduction

The use of mitochondrial DNA (mtDNA) genes to infer phylogenies and resolve systematic problems in the sub family Crotaline has recently been a subject of intense research interest (e.g., Kraus *et al.* 1996; Parkinson 1999; Vidal *et al.* 1997, 1999; Wüster *et al.* 2002). Many studies have focused on the analysis of restricted groups and at the intraspecific level (Salomão *et al.* 1997, 1999; Wüster *et al.* 1997, 1999, 2002; Zamudio and Greene 1997; Pook *et al.* 2000; Creer *et al.* 2001; Puorto *et al.* 2001). Using the cytochrome-b (Cyt-b) mitochondrial gene, a recent study of South American pitvipers suggested that the continent was invaded by at least 4 independent lineages: *Bothriechis schlegelii, Porthidium, Crotalus durissus*, and the ancestor of *Bothrops* (Wüster *et al.* 2002). The genus *Bothrops* diversified in South America, while the remaining genera were the consequence of more recent colonisations. The low levels of sequence divergence in the South American *Crotalus* populations led the authors to hypothesize that the species had recently invaded South American (Wüster *et al.* 2002). This had to have happened after the uplift of the Panamá land bridge, estimated to have occurred approximately 3.5 Mya (Coates and Obando 1996).

In this chapter, the phylogenetics and phylogeography of *C. durissus* is investigated using two mitochondrial genes, Cyt-b and ND4, and by sampling numerous *C. durissus* populations throughout its geographic range. The benefit of analyzing two or more genes

is the greater number of informative characters, which increases the accuracy of the algorithms and the resolution of the phylogenetic reconstruction. The following questions are answered:

- Are the South American *C. durissus* populations monophyletic?

- Did *C. durissus* recently disperse in a southern direction from Mexico and Central America into South America?

- Are *C.d. unicolor* and *C.d. vegrandis*, often regarded as separate species, distinct historical lineages? How they are related to other members of the *C. durissus* complex?

- How does the phylogeographic structure of *C. durissus* in South America compare to the phylogeographic structure within Central America?

- Is there genetic differentiation between the populations north and south of the Amazon basin?

2.2. Materials and Methods

Fieldwork

A set of 120 *C. durissus* blood aliquot samples were assembled. The samples came from several sources, including samples I personally collected in the field, and samples obtained from zoos and private collections (Fig 1.2). In addition, *C. molossus* and *C.*

basiliscus samples were collected in the field and obtained from zoos and other sources. See Appendix 2 for a complete list of samples, locations, and sources.

For field sampling, the snakes were collected using a herpetological hook and then transported to the field camp in cloth bags. The blood was obtained by a tail puncture using a syringe. Approximately 0.1-0.2 ml of blood was extracted and then flushed into a cell-lysis solution (100 µl of blood in 2-3 mls 2 % SDS, 100 mM Tris, 100mM EDTA). After the blood samples were extracted, the snakes were released at the point of capture.

DNA isolation

Whole genomic DNA was extracted using a standard proteinase K protocol (Sambrook *et al.* 1989). The proteinase K samples were incubated at 60 °C overnight and then transferred to ice. The samples were then incubated with RNase for 30 min. at 37 °C, and then transferred to ice. Ice-cold 5M ammonium acetate and centrifugation were used to precipitate, and then pellet the proteins. DNA was then precipitated from the resulting supernatant with ice-cold isopropanol, and centrifuged to form a pellet, which was washed with 70% ethanol, air-dried and then dehydrated with TE (Buffone 1985). Extracts were visualised by UV radiation on circular 25ml, 1.4% agarose plates incorporating 3µl ethidium bromide.

PCR

The Cyt-b and ND4 fragments of the mitochondrial DNA were amplified from 1-2 ml DNA extracts using modified primers. For the Cyt-b fragment (ca 600 bp), the primers correspond to positions 14977 and 15735 of the total mtDNA sequence of Dinodon

semicarinatus (Kumazawa *et al.* 1998). The primer sequences were 5'-TCA AAC ATC TCA ACC TGA TGA AA-3' (L-strand modified from Kocher *et al.* 1989), and 5'-GGC AAA TAG GAA GTA TCA TTC TG-3' (H-strand, a modified version of primer MVZ 16 of Moritz *et al.* (1992). For the ND4 fragment (ca 500bp), the following primers were used: ND4 (5'-CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA-3'), and Leu (5'-ACC ACG TTT AGG TTC ATT TTC ATT AC-3') of Arevalo *et al.* (1994).

The 50 ml PCR reactions were performed using a 50 ng template, 0.52 mM primers, 20 mM tris-HCl, 0.5 mM MgCl, 0.4 mM dNTP, 2 units of Taq DNA polymerase, and 0.5% DMSO. Amplification conditions involved an initial denaturation step of 4 minutes at 94 °C, 35 cycles of denaturation for 1 minute at 94 °C, primer annealing for 1 minute at 50 °C, extension for 2 minutes at 72 °C, and finally, an extra extension step for 3 minutes at 72 °C, cooling to 4 °C. The PCR products were concentrated by pooling 2-3 products per sample, and purified using the GenElute TM (Supelco) nucleic acid purification kit.

Sequencing

A single stranded automated sequencing method was carried out for both fragments using 10 µl reactions containing a 50 ng PCR template, 0.16 pmol of 5' primer, and the Big Dye Terminator Ready Reaction Mix (ABI). The reaction mix was cycled in a PE-ABI 9700 Thermal Cycler for 30 seconds at 94 °C, 50 cycles of 10 seconds at 96 °C, 5 seconds at 50 °C, 4 minutes at 60 °C, and then cooled to 4 °C. The samples were precipitated using 2M sodium acetate and 100% ethanol, and the resulting pellet was washed in 70% ethanol and air dried. The samples were suspended in 4 µl of a dextran blue EDTA/formamide loading buffer, and 1 µl of this mixture was loaded into a 5% long ranger gel in 1x TBE running buffer. The samples were analysed on an ABI 377

DNA Sequencer, generating a chromatogram and a text sequence. The sequences were read and aligned by eye to one another using the Chromas 1.51 package (Technelsyum Pty Ltd, 1988).

Preliminary sequencing analysis

Pairwise sequence comparisons to determine the distribution and amount of variation in the sequences of a distance based phylogeny were made for the Cyt-b and ND4 data sets separately using the Molecular Evolutionary Genetics Program (MEGA, Kumar *et al.* 1993). Levels of saturation at the first, second, and third codon positions were assessed from plots of uncorrected pairwise sequence divergences against Tamura–Nei (Tamura and Nei 1993). Pairwise divergences for transitions and transversions, in which deviations from the isometric lines represent a qualitative measure of degree of saturation were obtained (Zamudio and Greene1997).

Skewness (g1) statistics were calculated for both the Cyt-b and ND4 sequences from randomly generated trees in PAUP 4.0 (Swofford, 1998) to distinguish phylogenetic signals from random noise in the sequences. This was based on the assumption that the distribution of tree lengths of all tree topologies provides a sensitive measure of the phylogenetic signal. Data matrices with phylogenetic signals produce tree-length distributions that are strongly statistically skewed to the left, whereas those composed of random noise are more symmetrical (Hillis and Huelsenbeck 1992).

Aligned sequences for both Cyt-b and ND4 were subjected separately to parsimony analyses and then combined in an analysis using PAUP 4.0. Bootstrap analysis was carried out for all the parsimony analyses (Felsenstein 1985). An assessment of the

branch support and tree stability of the generated topologies was performed using the Bremer (1994) method, basically a count of the number of steps necessary to disassemble a clade, creating a polytomy. Bremer values were calculated using the Autodecay program (Eriksson 2001).

In order to calculate the best model of DNA substitution for the different mtDNA regions, and to test whether the combined Cyt-b/ND4 modes of substitution were congruent, an analysis with MODELTEST was performed (Posada and Crandall 1998). This program compares the different nested models of DNA substitution in a hierarchical hypothesis-testing framework. Due to computational limitations, small sets of sequences were chosen for both the Cyt-b and ND4 regions separately. Matrices of likelihood scores were obtained using PAUP 4.0b2 and then analyzed with MODELTEST.

Once the substitution model was found, Maximum Likelihood analysis (ML) was carried out in PAUP 4.0 for the combined Cyt-b/ND4 sequences. Addition of sequences was random with three replicates for ML. Because of the amount of time it required to perform bootstraps on the trees using ML analysis, confidence values to support the different branches were calculated by the MrBayes program (Huelsenbeck and Ronquist 2001). MrBayes performs Bayesian estimations of phylogenies, based on the posterior probability distribution of trees. MrBayes program uses simulation Markov chain Monte Carlo (MCMC) to approximate the posterior probabilities (Huelsenbeck and Ronquist 2001). The MrBayes gives posterior probability values that are comparable to bootstrap values for every consensus tree that is obtained by the program (Hall 2001). The trees obtained by the PAUP 4.0's maximum likelihood search for each tree data set were specified in the execution file and constraints were assigned to the different groups. By

specifying the tree and constraints, MrBayes executed the search together with the best tree obtained with PAUP. A tree with the same topology but with now with posterior probability values was obtained at the end of the search.

Wilcoxon signed-ranks tests (Templeton 1983) were used to test whether the cladograms predicted by alternative phylogenetic hypotheses were significantly different from the most-parsimonious tree, or whether differences in topology were a result of chance. Heuristic searches were performed on the un-weighted, combined Cyt-b and ND4 dataset, constraining the analysis to retain only the most parsimonious trees compatible with the alternative phylogenetic hypothesis to be tested. Differences in tree length between each of the constrained trees and the most-parsimonious trees obtained from the unconstrained analysis were tested for significance using Wilcoxon signed-ranks test.

The following hypotheses were tested (table 2.2):

a) The South American populations of *C. durissus* are monophyletic.

b) The conventional subspecies of *C. durissus* are monophyletic

c) There is genetic differentiation between the populations north and south of the Amazon basin.

2.3. Results

A total of 120 clear sequences of the Cyt-b (62 sequences) and ND4 (58 sequences) regions were aligned. No stop codons were found in either region, and the levels of saturation at the first, second and third codons for transversions and transitions were

similar (Fig 2.1). Tree length distribution, determined from a random sampling of un-weighted trees, was significantly skewed to the left in both regions (Cyt-b, g1 = -0.6492, $p < 0.01$; ND4, g1 = -0.6262, $p < 0.01$), indicating phylogenetic signal in the data (Hillis and Huelsenbeck 1992). The analysis included 36 *C. durissus* populations that represented the species' geographic range and eleven recognised subspecies. Sequences from *C. molossus, C. basiliscus, C. cerastes, C. scutulatus*, and *C. viridis* were also included.

For the analysis of the sequences, 614 bp were aligned, including the out-groups *C. cerastes* and *C. v. cerberus*, of which 213 bp (30.9%) were variable among the taxa, and 172 sites (28.01%) were parsimony informative. Sequence divergences based on the Kimura 2-parameter ranged between 1.03% -10.63% among in-group taxa, 5.8% between *C. molossus* populations, 6.69-5.66% between *C. molossus* and *C. basiliscus*, and 1.03 % - 9.78% between the *C. durissus* populations (Table 2.3).

For the ND4 sequences, 501 bp were aligned, including the out-groups *C. scutulatus* and *C. viridis*, of which 184 sites (36.72%) were variable among the taxa, and 142 (28.38%) were parsimony informative. Sequence divergence ranged between 1.05% - 9.84% among in-group taxa, 5.6% between *C. molossus* populations, 5.7-5.8% between *C. molossus* and *C. basiliscus*; and 1.02 % and 9.50% between the *C. durissus* populations.

I was unable to obtain both Cyt-b and ND4 sequences from all the sampled populations. For the distance and parsimony analysis where I analyzed Cyt-b and ND4 combined, I was only able to include the 36 populations where I had been able to obtain both

sequences. Sequence divergence ranged between 0.0% and 0.04% in the Cyt-b region and 0.0% and 0.08% in the ND4 region among individuals of the same location.

The analysis of the combined sequences aligned 1115 bp including the out-groups *C. scutulatus* and *C. viridis*, of which 375 sites (33.63%) were variable among the taxa, and 322 (28.87%) were parsimony informative. The combined sequences showed that sequence divergence ranged between 1.6%-11.09% among in-group taxa, 6.86% between *C. molossus* populations, 5.69-5.8% between *C. molossus* and *C. basiliscus*, and 1.02 %-11.27% within the *C. durissus* populations (Table 2.3). In the South American taxa, the sequence divergence of Cyt-b and ND4, both individually and combined, was consistently low, with a maximum pairwise divergence of 1.4%. On the other hand, divergence among the Central American lineages ranged up to 8.0%.

The distance based tree using the neighbour-joining method and Tamura-Nei analysis grouped all the South American populations and most of Mexican *C. durissus* populations into two clades. The *C. totonacus* sequences were grouped with *C. molossus* and *C. basiliscus*. This was true for the Cyt-b and ND4 sequences both individually and combined (Figs. 2.2-2.7). The bootstrap analysis for the separate regions strongly supports the existence of a single South American *C. durissus* clade. From the bootstrap, it is quite clear that the Central American and Mexican *Crotulas* taxa are paraphyletic (Figs. 2.2-2.7).

The branch and bound analysis using maximum parsimony as criterion generated a total of 12 equally parsimonious trees for Cyt-b (tree length = 380; CI =0.6658; HI= 0.3342; RI =0.7617; RC = 0.5071). The analysis of ND4 produced 20 equally parsimonious trees

(tree length =434; CI =0.5115; HI= 0.4885; RI =0.8203; RC = 0.4196). The bootstrap of the combined consensus tree (the tree with both Cyt-b and ND4) is shown in Fig. 2.6. The existence of a single South American clade is strongly supported by this analysis. Less supported were the *C. durissus*- *C. culminatus*, the *C. totonacus*- *C. molossus*, and the *C. tzabcan* South American clades, suggesting that the South American *Crotalus* are indeed monophyletic.

The Bremer method in the maximum parsimony analysis also supports the monophyly of South American *Crotalus*. The relationships among the South American rattlesnake populations were weak, strongly supporting a single South American clade (Fig 2.6). In Central America and Mexico, the Bremer method supports the idea of *C. culminatus*-*C. durissus* and *C. totonacus*- *C. molossus* clades, but the relationships inside these clades are still relatively unclear.

The MODELTEST program identified as optimal the HKY85 model with gamma distribution for the ML analysis. The HKY85 model does not assume equal base frequencies and accounts for the difference between transitions and transversions with one parameter (Hasegawa-Kishino-Yano 1985). Bootstrap values for the tree obtained by this analysis are consistent with the general pattern of monophyly in South America and paraphyly in Central America (Fig 2.7). Again, a single South America clade is supported, but in this case, the Central America clades had weak bootstrap values.

The Wilcoxon signed-ranks tests supported the idea of a monophyletic the South American clade as compared to the paraphyletic populations of Central America (Z= -0.06, P>0.05). The tests do not support the conventional species and subspecies

categorization of the South American rattlesnakes ($Z= -5.2$, $P<0.003$), indicating that there is only one South American species. Furthermore, the tests indicate that the rattlesnake populations north and south of the Amazon Basin are genetically similar, or monophyletic ($Z= - 4.8$, $P<0.005$).

Table 2.1. Parsimony informative positions of the Cyt-b and ND4 combined regions of the mtDNA of *C. durissus*, *C. molossus*, *C. basiliscus* and the outgroups *C. scutulatus* and *C. viridis*.

	111	222	223	344	444	455	555	555	555	666	666	666	666	666	666
	145	249	012	250	803	335	834	456	678	889	000	222	233	444	555	577
	008	469	820	467	201	377	434	705	670	696	127	036	925	147	036	914
CscutulNM	CAA	CTC	ACA	CTT	CCC	ACC	TTA	CAA	TTA	TTA	AAT	AAA	CTT	CAA	GCT	CTC
CvdsCO	...	...	...	...	...	...	.C.	...	...	C..	...	...	...	...	...	...
CvdsAZ	...	...	...	...	...	...	.C.	...	...	C..	...	...	...	...	...	...
CDtotoTamp	T.G	..T	...	.C.	...	C..	CC.	...	C..	C..	G..	.G.	.C.	A..	.T.	AC.
CDtotoQuet	T.G	..T	...	.C.	...	C..	CC.	...	C..	C..	G..	.G.	.C.	A..	.T.	AC.
CDculPuebl	T.G	.CT	.T.	.C.	T..	T..	CC.	.T.	CC.	CA.	G..	GGG	.CC	AGG	AT.	GC.
unicolor	T.G	ACT	...	TCC	T..	C..	CCG	..G	C.G	C..	G..	GGG	...	TGG	AT.	AC.
vegrandis	T.G	ACT	...	TCC	T..	C..	CCG	..G	C.G	C..	G..	G..	...	TGG	AT.	ACT
CDDcateMX	T.G	ACT	.T.	.C.	TT.	...	CC.	AC.	CC.	CA.	G..	G..	T..	.GG	AT.	AC.
CBBguadal	.TG	..T	..G	TC.	...	C?.	CC.	...	...	A..	...	.CG	.A.	T..	AT.	.C.
CDcolBABoaBZ	T.G	ACT	...	TCC	T..	C..	C.G	..G	C.G	C..	G..	G..	...	TGG	AT.	AC.
CDTGuarBZ	T.G	ACT	G..	TCC	T..	CT.	C.G	T.G	C.G	C..	G..	G..	.C?	TGG	AT.	AC.
CDTSLParBZ	T.G	ACT	G..	TCC	T..	C..	CCG	..G	C.G	C..	G..	G..	.C.	TGG	AT.	AC.
CDTRosBZ	T.G	ACT	...	TCC	T..	C..	CCG	..G	C.G	C..	G..	G..	...	TGG	AT.	AC.
CDTPindBZ	T.G	ACT	...	TCC	T..	C..	CCG	..G	C.G	C..	G..	G..	...	TGG	AT.	AC.
CMMaricpAZ	T.G	..T	..G	.CC	T..	C.T	.CG	..G	...	C.T	G.C	..G	...	T..	ATC	AC.
CMSnBernAZ	T.G	..T	...	TCC	...	CTT	.CG	..G	...	C.T	G.C	.GG	...	T..	ATC	AC.
CMLUvasNM	T.G	..T	...	TCC	...	CTT	.CG	..G	...	C..	GGC	.GG	...	T..	ATC	AC.
CMQuertMX	T.G	...	...	TC.	T..	...	.C.	?..	C..	C..	GGC	GGT	...	T..	.T.	.C.
CDculIxtMX	T.G	.CT	.T.	.C.	T.T	...	CC.	AC.	CC.	CA.	G..	GG.	.?.	TGG	AT.	AC.
CDDChisMX	T.G	ACT	.T.	.C.	TT.	...	CC.	AC.	CC.	CA.	G..	G..	T..	.GG	AT.	AC.
CDtzBez	T.G	.CT	...	TC.	T..	C..	CC.	...	C..	C.G	G..	GGG	...	TGG	.T.	AC.
CMOOaxMX	T.G	...	...	TC.	T..	...	CC.	...	...	C..	GGC	..T	..C	T..	.T.	.C.
CDPLaBZ	T.G	ACT	...	TCC	T..	C..	CCG	..G	C.G	C..	G..	...	...	TGG	AT.	AC.
CDRCasBZ	T.G	ACT	...	TCC	T..	C..	CCG	..G	C.G	C..	G..	G..	...	TGG	AT.	AC.
CDBelmBZ	T.G	ACT	...	TCC	T..	C..	CCG	..G	C.G	C..	G..	G..	.C.	TGG	AT.	AC.
CDGuanBZ	T.G	ACT	...	TCC	T..	C..	CCG	..G	C.G	C..	G..	G..	.C.	TGG	AT.	AC.
CDculMorMX	T.G	.CT	.T.	.C.	T.T	T..	CC.	.T.	C.G	C..	G..	GGG	.CC	AGG	AT.	GC.
CDcasGrajBZ	T.G	ACT	...	TCC	T..	C..	CCG	..G	C?G	C..	G?.	G..	.C.	TGG	AT.	AC.
CDcolABoaBZ	T.G	ACT	...	T.C	T..	C..	C.G	..G	C.G	C..	G..	G?.	.??	TGG	AT.	AC.
CDcuman	T.G	A.T	...	TCC	T..	C..	CCG	..G	C.G	C..	G..	G..	...	TGG	AT.	ACT
CBBNaytMX	.TG	..T	...	TC.	..T	C..	CC.	...	...	A..	...	.CG	.A.	T..	AT.	.C.
CDTrArapBZ	T.G	ACT	...	TCC	T..	C..	CCG	..G	C.G	C..	G..	G..	...	TGG	AT.	AC.
CMEdomexMX	T.G	...	...	TC.	T.T	.TT	.CG	..G	...	C..	GGC	GGT	...	T..	...	.C.
ElSalvador	T..	ACT	.T.	.C.	TT.	...	CC.	AC.	CC.	CA.	G..	G..	...	TGG	AT.	AC.
CDdryGuyana	T.G	A.T	...	TCC	T..	C..	CCG	..G	C.G	C..	G..	G..	...	TGG	AT.	ACT

Table 2.1 (Cont.)

	666 789 732	666 999 458	777 000 147	777 111 035	777 112 692	777 223 584	777 445 392	777 567 540	777 778 692	777 889 581	788 900 709	888 113 250	888 334 398	888 666 036	889 780 242	999 001 581
CscutulNM	GTC	TCA	CCA	GAT	TAC	CCC	GCG	ACC	TTC	TAC	AAC	CCA	CGC	ACC	CCA	TCG
CvdsCO	...	...	...	...	...	...	...	...	...	...	...	..G	...	...	...	...
CvdsAZ	...	...	...	...	...	...	...	...	...	...	...	..G	...	...	...	...
CDtotoTamp	C.T	.T.	TT.	A..	CGT	.A.	A.A	.TT	.C.	.G.	...	.TC	...	..T	T..	CTA
CDtotoQuet	C.T	GG.	TT.	A..	C.T	.G.	A.A	.TT	.C.	.G.	...	.TC	...	..G	T..	CTA
CDculPuebl	A.T	?G.	.T.	...	C.T	TG.	ATA	GT.	...	.G.	...	.TC	A.T	.TT	.A.	CTA
unicolor	A..	.TG	.TG	.GC	C..	.G.	A.A	G..	...	C.T	..T	TTC	AA.	GT.	.AG	CTA
vegrandis	A.T	.T.	.T.	.GC	C..	.G.	A.A	...	CC.	CG.	...	TTC	A..	...	...	CTA
CDDcateMX	A.T	.T.	.TG	...	C..	TG.	A.A	G..	...	.G.	...	.TC	A..	G..	.A.	CTA
CBBguadal	ACT	.T.	.TG	...	CG.	..T	A.A	..T	CC.	.G.	.G.	TTC	A..	...	...	CTA
CDcolBABoaBZ	...	.T.	.TG	...	C..	TG.	A.A	...	..T	...	...	.TC	AA.	GT.	.A.	CTA
CDTGuarBZ	A..	.T.	.TG	...	C..	TG.	A.A	...	..?	...	...	.TC	AA.	GT?	.A.	CTA
CDTSLParBZ	A..	.T.	.TG	...	C..	TG.	A.A	...	..T	...	...	.TC	AA.	GT.	.A.	CTA
CDTRosBZ	A..	.T.	.TG	...	C..	TG.	A.A	...	..T	...	...	.TC	AA.	GT.	.A.	CTA
CDTPindBZ	A..	.T.	.TG	...	C..	TG.	A.A	...	..T	...	...	.TC	AA.	GT.	.A.	CTA
CMMaricpAZ	A.T	.T.	...	...	CG.	.A.	A.A	G..	.C.	...	...	.TC	...	...	...	CTA
CMSnBernAZ	A.T	.T.	...	...	CG.	.A.	A.A	G..	.C.	...	...	.TC	...	...	...	CTA
CMLUvasNM	A.T	.T.	...	A..	CG.	.A.	A.A	G..	.C.	...	...	.TC	...	...	...	CTA
CMQuertMX	ACT	.T.	.T.	...	CG.	.A.	AT.	G.T	.C.	C.G	G..	.TC	...	T.G	.A.	CTA
CDculIxtMX	ACT	GT.	.T.	...	C.T	TG.	ATA	GT.	...	.G.	...	.TC	A.T	.TT	.A.	CTA
CDDChisMX	A.T	.T.	.TG	...	C..	TG.	A.A	G..	...	.G.	...	.TC	A..	G..	.A.	CTA
CDtzBez	..T	CT.	.T.	...	C..	TA.	A.A	..T	.C.	.G.	...	.TC	...	..?	.A.	CTA
CMOOaxMX	A.T	.T.	.T.	.G.	...	.A.	A.A	...	.C.	C..	...	.TC	AA.	...	...	CTA
CDPLaBZ	A..	.T.	.TG	...	C..	TG.	A.A	...	..T	..T	...	.TC	AA.	GTT	.A.	CTA
CDRCasBZ	...	.T.	.TG	...	C..	TG.	A.A	...	..T	...	...	.TC	AA.	GT.	.A.	CTA
CDBelmBZ	A..	.T.	.TG	...	C..	TG.	A.A	...	..T	...	...	.TC	AA.	GT.	.A.	CT.
CDGuanBZ	A..	.T.	.TG	...	CG.	TG.	A.A	...	..T	...	...	?TC	AA.	GT.	.A.	CTA
CDculMorMX	A.T	GG.	.T.	...	C.T	TG.	ATA	GT.	...	.G.	...	.TC	A.T	.TT	.A.	CTA
CDcasGrajBZ	A..	.T.	.T.	...	CG.	TG.	A.A	...	..T	...	...	.TC	AA.	GT.	.A.	CTA
CDcolABoaBZ	...	.T.	.TG	...	C..	TG.	A.A	...	..T	...	...	.TC	AA.	GT.	.A.	CTA
CDcuman	A.?	.TG	.T.	.GC	C..	.G.	A.A	G..	...	C.T	..T	TTC	A..	GT.	.AG	CTA
CBBNaytMX	ACT	.T.	.TG	...	CG.	..T	A.A	..T	CC.	.G.	.G.	TTC	A..	...	...	CTA
CDTrArapBZ	A..	.T.	.TG	...	C..	TG.	A.A	...	..T	...	...	.TC	AA.	GT.	.A.	CTA
CMEdomexMX	ACT	.T.	TT.	...	CG.	.A.	AT.	G.T	.C.	C.T	G..	.TC	...	T.G	.A.	CTA
ElSalvador	A..	.T.	.TG	...	C..	TG.	A.A	G..	.C.	.G.	...	.TC	A..	GT.	.A.	CTA
CDdryGuyana	A.?	.TG	.T.	.GC	C..	.G.	A.A	G..	...	C.T	..T	TTC	A..	GT.	.AG	CTA

Table 2.1 (Cont.)

	999 122 706	999 333 258	999 445 470	999 566 325	999 688 806	999 889 898	111 000 011 736	111 000 122 915	111 000 233 814	111 000 344 736	111 000 445 892	111 000 667 140	111 000 777 368	111 000 888 258	111 000 999 147	1 1 0 0
CscutulNM	ATA	ACC	AAC	CAC	GAA	TAT	CCA	CCA	CCT	CCA	TTA	ACA	CAC	GTT	ACA	A
CvdsCO	G..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.
CvdsAZ	G..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.
CDtotoTamp	.C.	.T.	G..	...	...	C.A	..C	.T.	T.A	..G	...	...	.G.	.C.	.T.	.
CDtotoQuet	.C.	.T.	G..	...	...	C.C	..C	.T.	TTA	..G	...	...	.G.	.C.	.T.	.
CDculPuebl	GC.	.TA	G..	...	A..	C..	..C	A..	.TA	...	...	GT.	T.T	AC?	...	G
unicolor	.C.	.TA	...	..T	A..	C.C	..C	..T	.TA	...	..G	GT.	..T	AC.	.G.	.
vegrandis	.C.	.TA	...	..T	A..	C.C	..C	.TT	.TA	...	..G	GT.	..T	.C.	.T.	.
CDDcateMX	GC.	.TA	...	.GT	A..	C.C	..C	..T	.TA	...	...	GT.	..T	.C.	.T.	.
CBBguadal	.CG	.T.	...	T..	A..	C.C	..C	...	.TA	.T.	.C.	.T.	...	.CC	GT.	.
CDcolBABoaBZ	GC.	.TA	...	..T	A..	C.C	.TC	..T	.TA	.A.	...	GT.	..T	.C.	...	.
CDTGuarBZ	GC.	.TA	...	..T	A..	C.?	..C	..T	.TA	GA.	..G	GT.	..T	.C.	...	.
CDTSLParBZ	GC.	.TA	...	..T	AG.	C.C	..C	.TT	.TA	.A.	..G	GT.	..T	.C.	...	.
CDTRosBZ	GC.	.TA	...	..T	A..	C.C	..C	..T	.TA	.A.	...	GT.	..T	.C.	...	.
CDTPindBZ	GC.	.TA	...	..T	A..	C.C	..C	..T	.TA	.A.	...	GT.	..T	.C.	...	.
CMMaricpAZ	.C.	.T.	..A	...	...	.GC	..T	T..	.TA	.AG	...	...	...	.CC	GT?	.
CMSnBernAZ	.C.	GT.	..A	...	...	.GC	..T	T..	.TA	.AG	...	...	...	.CC	GTT	.
CMLUvasNM	.C.	GT.	..A	...	...	..C	..T	T..	.TA	.AG	.C.	...	...	.CC	.TT	.
CMQuertMX	.C.	.T.	...	...	A.G	C.C	T.C	.TT	TTA	.AG	...	G..	...	ACC	...	.
CDculIxtMX	GC.	.TA	G..	...	A..	C..	..C	A..	.TA	...	...	GT.	T.T	ACC	...	G
CDDChisMX	GC.	.TA	...	.GT	A..	C.C	..C	..T	.TA	...	...	GT.	..T	.C.	.T.	.
CDtzBez	.C.	.TA	.G.	...	A..	C..	..C	T..	.TA	A..	...	GTG	..T	ACC	...	.
CMOOaxMX	.C.	GTA	GG.	...	...	C.C	..C	T..	.TA	A.G	...	.T.	...	AAC	.G.	.
CDPLaBZ	GC.	.TA	...	..T	A..	C.C	..C	.TT	.TA	.A.	...	.T.	..T	.C.	...	.
CDRCasBZ	GC.	.TA	...	..T	A..	C.C	?TC	..T	.TA	.A.	...	GTG	..T	.C.	...	.
CDBelmBZ	GC.	.TA	...	..T	A..	C.C	..C	..T	.TA	.A.	..G	GT.	..T	.C.	...	.
CDGuanBZ	GC.	.TA	...	..T	A..	C.?	.TC	..T	.TA	.A.	..G	GT.	..T	AC.	...	.
CDculMorMX	GC.	.TA	G..	...	A..	C..	..C	A..	.TA	...	...	GT.	T.T	AC?	...	.
CDcasGrajBZ	GC.	.TA	...	..T	A..	C.C	..C	..T	.TA	.A.	..G	GT.	..T	AC.	...	.
CDcolABoaBZ	GC.	.TA	...	..T	A..	C.C	..C	..T	.TA	.A.	...	GT.	..T	.C.	...	.
CDcuman	GC.	.TA	...	..T	A..	C.C	..C	T.T	.TA	?..	A.G	GT.	..T	.C.	.TG	.
CBBNaytMX	.CG	.T.	...	T..	A..	C.C	..C	...	.TA	.T.	.C.	.T.	...	.CC	GT.	.
CDTrArapBZ	GC.	.TA	...	..T	A..	C.C	..C	..T	.TA	.A.	...	GT.	..T	.C.	...	.
CMEdomexMX	.C.	.T.	...	...	AGG	C.C	T.C	T.T	TTA	.AG	...	T..	...	ACC	...	.
ElSalvador	GC.	.TA	...	.GT	A..	C.C	..C	..T	.TA	...	...	GT.	..T	.C.	...	.
CddryGuyana	GC.	.TA	...	..T	A..	C.C	..C	T.T	.TA	...	A.G	GT.	..T	.C.	.TG	...

Table 2.2. Constraints of the hypothesis implemented in PAUP 4.0 for the Wilcoxon test.

i) Monophyly of the South American populations of *C. durissus*.

(CscutulNM, CvdsCO, CvdsAZ ((CMO_OaxMX)
(CBBguadal,CBB_NaytMX)((CM_MaricpAZ, CM_SnBernAZ, CM_LUvasNM)
(CM_QuertMX,CM_EdomexMX))((CDtoto_Tamp, CDtoto_Quet)(CDtz_Bez)(
CDcul_Puebl, CDcul_MorMX,
CDcul_IxtMX)(ElSalvador,CDD_cateMX,CDD_ChisMX)(Unicolor,
vegrandis,CDcolB_ABoaBZ,CDT_GuarBZ,CDT_SLParBZ,CDT_RosBZ,
CDT_PindBZ,CD_PLaBZ,CD_RCasBZ,CD_BelmBZ,CD_GuanBZ,CDcas_GrajBZ
, CDcol_ABoaBZ,CDcuman,CDTr_ArapBZ,CDdry_Guyana)))

ii) Conventional subspecies of *C. durissus* are monophyletic

(CscutulNM, CvdsCO, CvdsAZ ((CBBguadal,CBB_NaytMX)) ((CMO_OaxMX)
(CM_MaricpAZ, CM_SnBernAZ, CM_LUvasNM)
(CM_QuertMX,CM_EdomexMX))((CDtoto_Tamp, CDtoto_Quet)(CDtz_Bez
)(CDcul_Puebl,CDcul_MorMX,CDcul_IxtMX)(ElSalvador,CDD_cateMX,CDD_C
hisMX)(Unicolor)(vegrandis) (CDcolB_ABoaZ,
CDcol_ABoaBZ)(CDT_GuarBZ,CDT_SLParBZ,CDT_RosBZ,
CDT_PindBZ,CD_PLaBZ,CD_RCasBZ,CD_BelmBZ,CD_GuanBZ,CDTr_ArapBZ
)(CDcas_GrajBZ)(CDcuman)(CDdry_Guyana)))

iii) Genetic differentiation between the North and South populations in the border of the Amazon basin

(CscutulNM, CvdsCO, CvdsAZ ((CBBguadal,CBB_NaytMX)) ((CMO_OaxMX)
(CM_MaricpAZ, CM_SnBernAZ, CM_LUvasNM)
(CM_QuertMX,CM_EdomexMX))((CDtoto_Tamp, CDtoto_Quet)(CDtz_Bez
)(CDcul_Puebl,CDcul_MorMX,CDcul_IxtMX)(ElSalvador,CDD_cateMX,CDD_C
hisMX)(Unicolor,vegrandis,CDcuman,CDdry_Guyana) (CDcolB_ABoaZ,
CDcol_ABoaBZ,CDT_GuarBZ,CDT_SLParBZ,CDT_RosBZ,CDT_PindBZ,CD_P
LaBZ,CD_RCasBZ,CD_BelmBZ,CD_GuanBZ,CDTr_ArapBZ,CDcas_GrajBZ)))

Table 2.3. Pairwise sequence divergence data (average%+ SE) between Central America and South America populations of *C. durissus*. The distance measure used is the Kimura 2-parameter distance. Sequence divergence in the *C. molossus-C. basiliscus* group was included for comparison purposes.

Group	ND4	Cyt-b	ND4-Cyt-b
Central America	7.96 ± 0.82	7.49 ±0.79	7.41± 0.52
South America	2.32 ± 0.36	1.19 ± 0.24	1.85± 0.23
C. molossus - C. basiliscus	7.43± 0.81	7.18± 0.59	7.20± 0.51
Total	7.86 ± 0.75	7.73± 0.51	7.73± 0.52

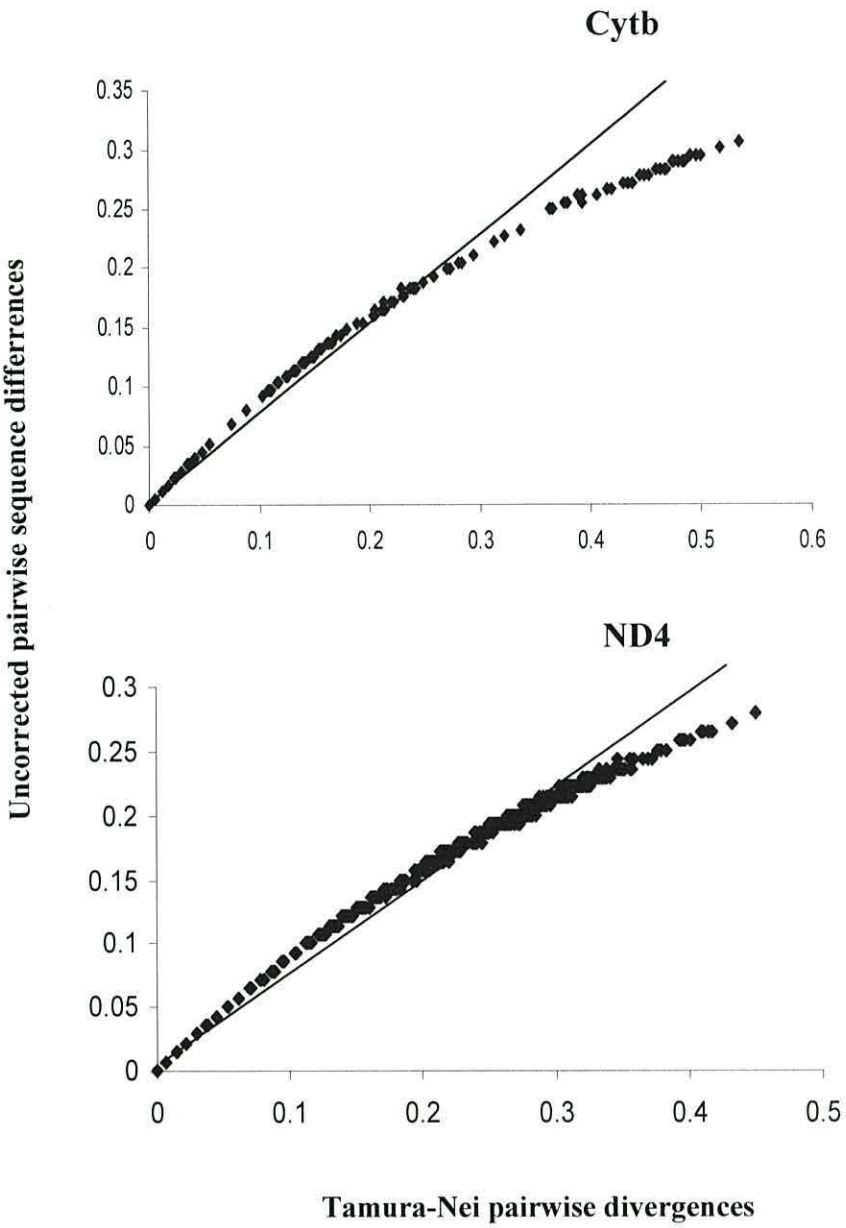

Fig. 2.1. Plots of the levels of saturation at 3rd codon position transitions in the mtDNA sequences of *C. durissus* for both Cyt-b and ND4 regions. The plots were quite similar for the 1st and 2nd codon position.

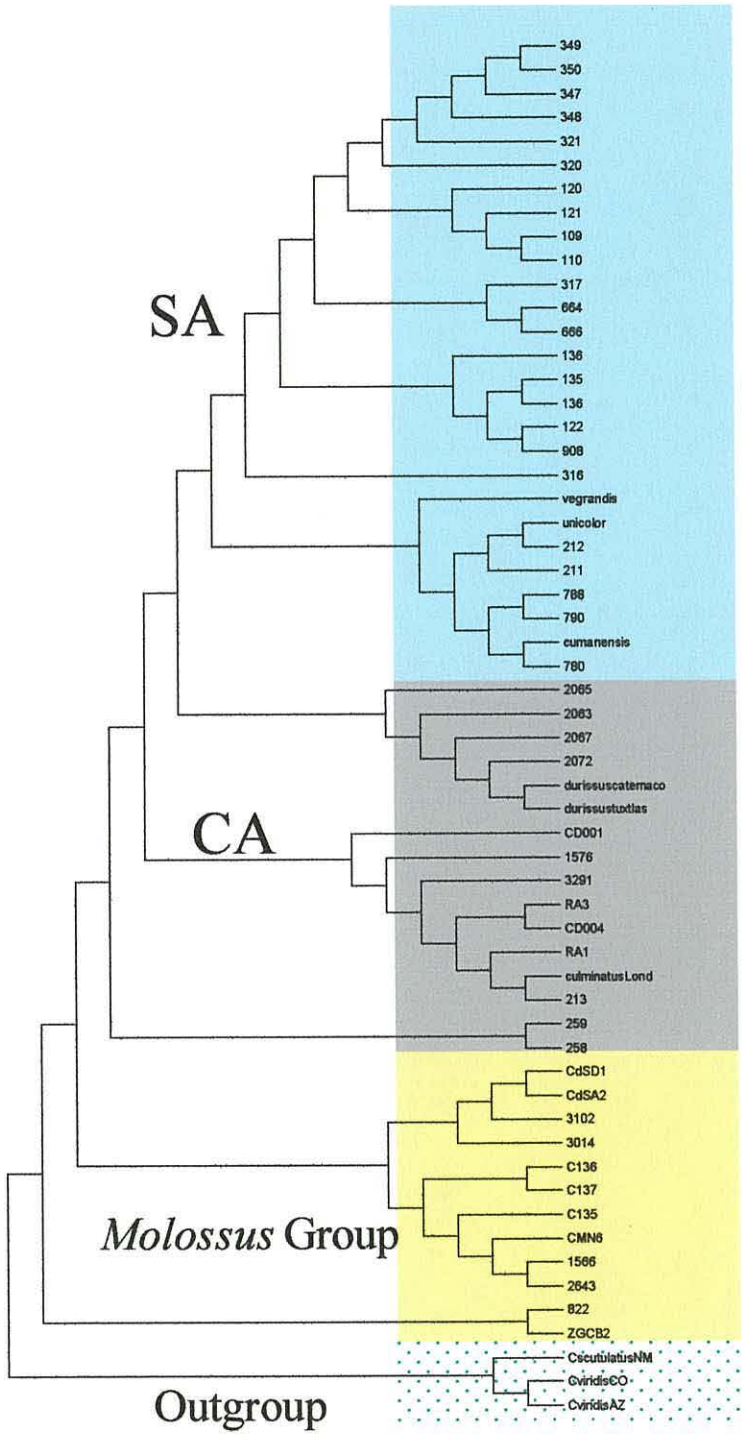

Fig 2.2. Neighbour joining tree using maximum parsimony for the Cyt-b sequences of *C. durissus*. SA South America (blue), CA Central America (grey), *Molossus* group (yellow), Outgroup (dotted).

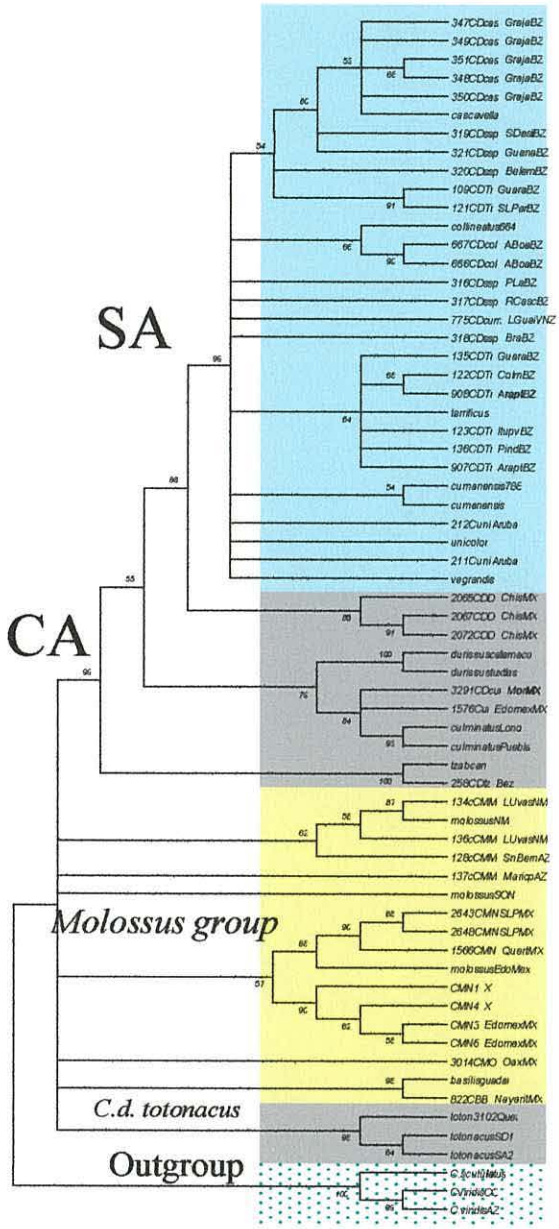

Fig 2.3. Bootstrap 50% majority-rule NJ consensus tree of the Cyt-b sequences of *C. durissus*. SA South America (blue), CA Central America (grey), *Molossus* group (yellow), Outgroup (dotted).

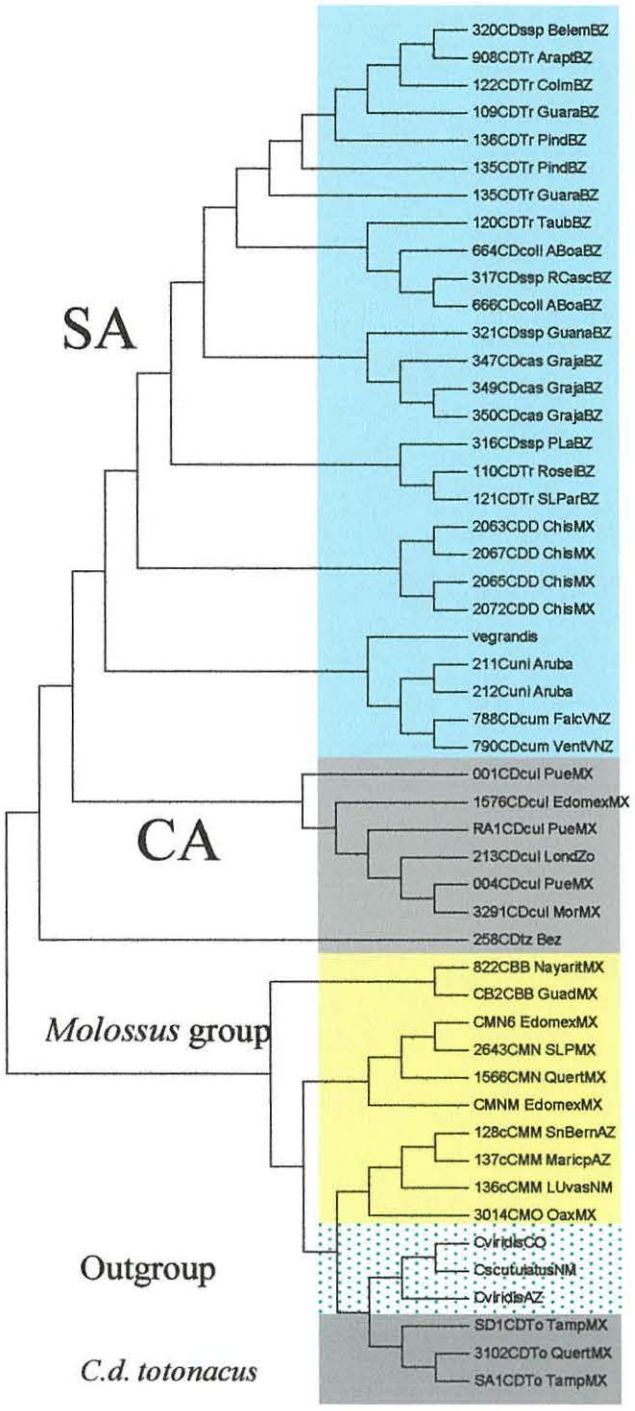

Fig 2.4. Neighbour joining tree for the ND4 sequences of *C. durissus*. SA South America (blue), CA Central America (grey), *Molossus* group (yellow), Outgroup (dotted).

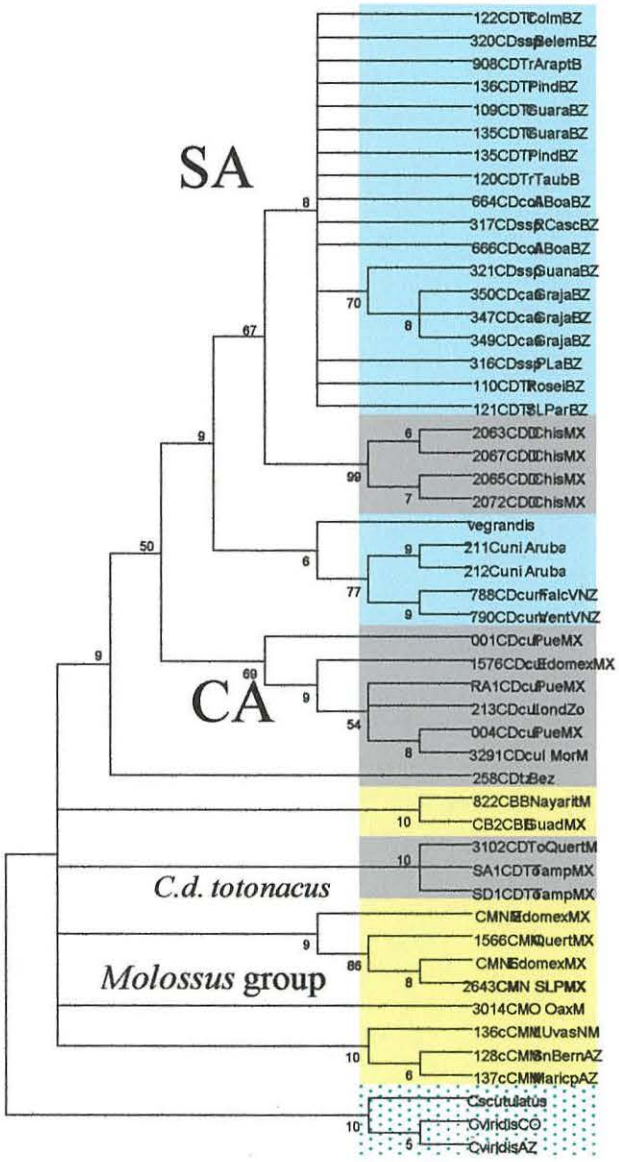

Fig 2.5. Bootstrap 50% majority-rule NJ consensus tree of the ND4 sequences of *C. durissus*. SA South America (blue), CA Central America (grey), *Molossus* group (yellow), Outgroup (dotted).

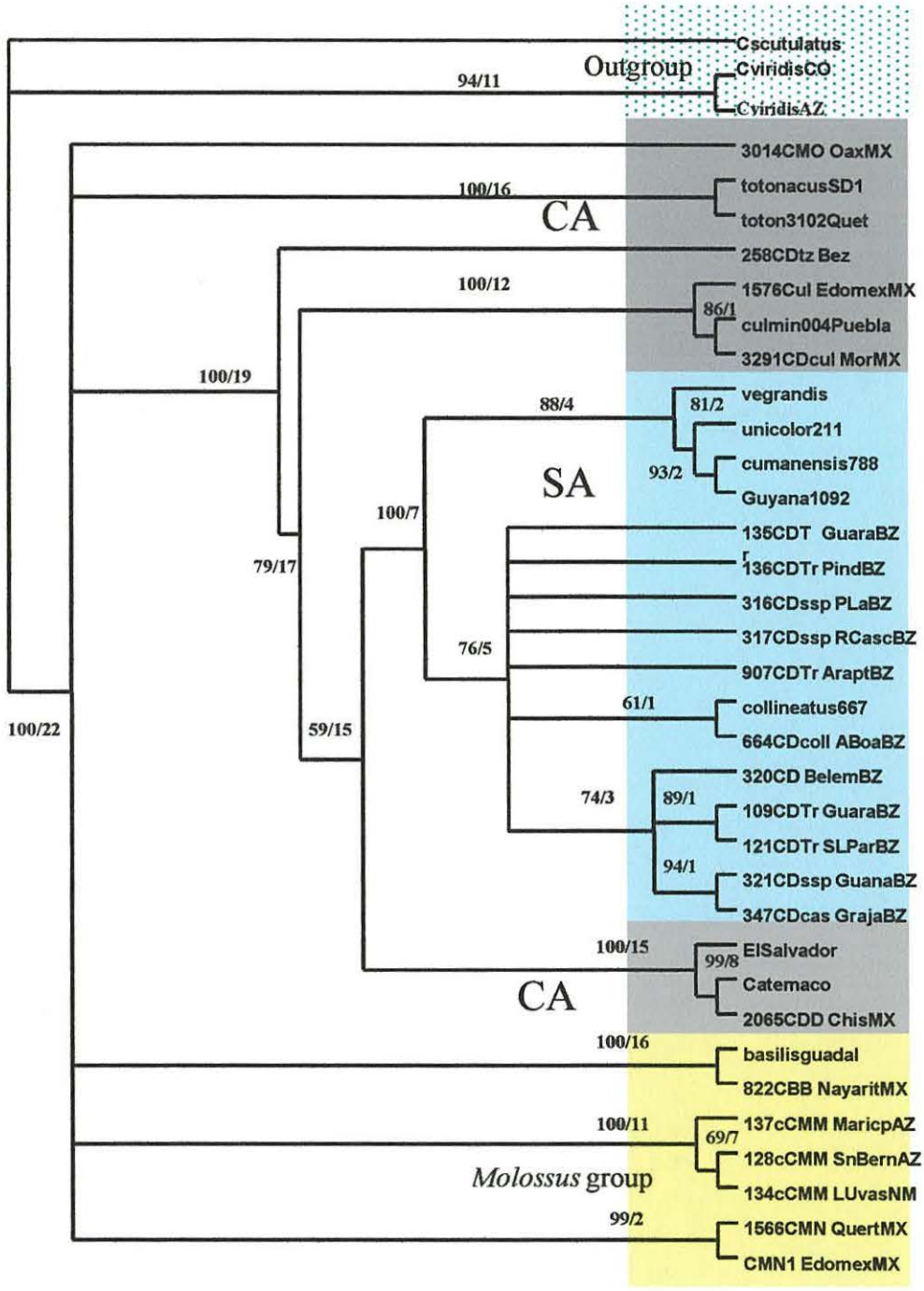

Fig 2.6. Bootstrap 50% majority-rule NJ consensus tree of the Cytb-ND4 sequences of *C. durissus*, showing bootstrap support and Bremer decay index respectively. SA South America (blue), CA Central America (grey), *Molossus* group (yellow), Outgroup (dotted).

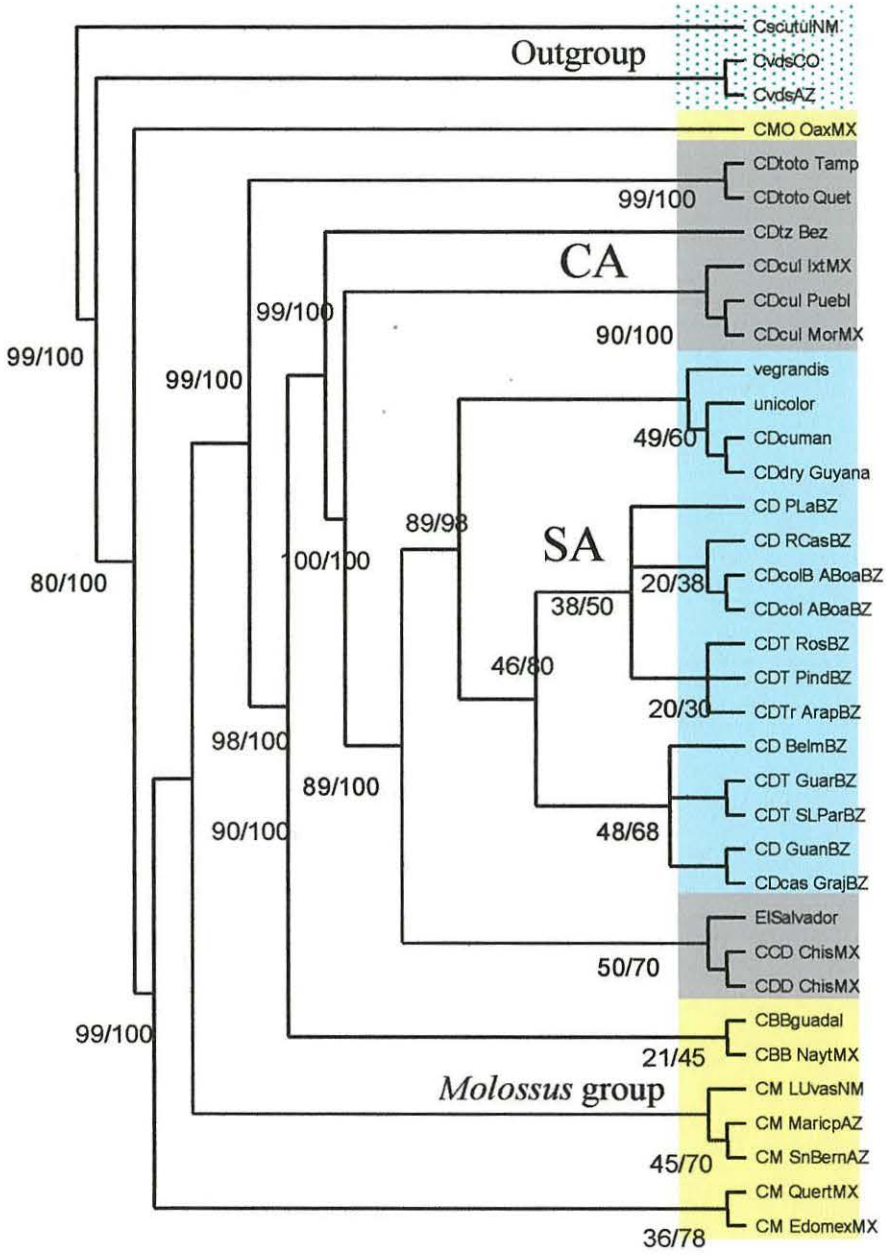

Fig 2.7. Maximum-likelihood tree with bootstrap values and Bayesian posterior probability (ML/B) for the combined Cyt-ND4 sequences of *C. durissus* obtained using MrBayes approach (Huelsenbeck and Ronquist, 2001). SA South America (blue), CA Central America (grey), *Molossus* group (yellow), Outgroup (dotted).

2.4. Discussion

The results of this study strongly support the hypothesis that *C. durissus* dispersed into South America from Central America. Furthermore, the results indicate that while the South American populations are one species, the Central American rattlesnake populations should be considered as belonging to several different species. Thus a systematic revision of several Central American *Crotalus* forms is needed. The data suggest that *C. totonacus* and *C. d. tzabcan* be recognized as separate species, and that the status of *C. durissus* (sensu Campbell and Lamar 1989) be revised. Several different analytical methods to analyze the combined ND4 and Cyt-b sequences were used. All methods produced similar topologies, indicating that the results and derived conclusions are robust.

Both bootstrap methods and the Bremer branch support index show strong support for the monophyly of South American forms, separating them from the Central American forms. The topology of the trees generated by the combined sequences suggests that at least four lineages evolved in Central America. These correspond to the subspecies categories currently recognised as *C. durissus*, *C. d. culminatus*, *C. d. tzabcan* and *C. totonacus*. The separation of *C. totonacus* from both the South American and Central American clades, and its position relative to *C. basiliscus* and *C. molossus*, strongly suggests that it belongs to a very different evolutionary lineage and that it is not conspecific with *C. durissus*. The distance and parsimony methods grouped *C. totonacus* with *C. basiliscus* and *C. molossus*, suggesting a shared lineage. This is consistent with the objections that some authors have raised to placing *C. totonacus* in *C. basiliscus* or *C. durissus* (Taylor 1951; Golay *et al.* 1993).

Previous studies have claimed full species status for some South American subspecies of *C. durissus* (e.g. Klauber 1972). However, this study of the Cyt-b and ND4 phylogenies reveals that *C. durissus* has a unique evolutionary lineage with relatively little genetic differentiation among the recognised South American forms, including little genetic variation between *C. d. unicolor* and *C. d. vegrandis*. The fact that some forms are morphologically different from others, suggests that ecogenetic adaptation to recent ecological conditions, and phylogenetic evolution have operated in different ways to produce sharp differences in colour and pattern (Thorpe *et al.* 1996; Malhotra and Thorpe 1997). The genetic consequences of past and present relative isolation have influenced the morphological variation in these populations without driving them towards lineage differentiation. In striking contrast, there is very little morphological variation in the Central American populations, but as mentioned above, sufficient genetic variation to warrant species-level categorization for many groups previously considered as sub-species.

Comparative phylogeography with other crotalines

The Neotropical rattlesnake, which dispersed through the Panamá Isthmus to South America, was only one species of a dynamic guild of vertebrates crossing from North America to South during the Great American Interchange. This species interchange had an enormous influence on the biogeography of the Mid and South American biota about 3-3.5 Mya (Marshall *et al.* 1979; Stehli and Webb 1985; Bermingham and Martin 1998; Wüster *et al.* 2002).

An outstanding question regarding the vicariance or dispersal events of the Crotalines is the timing of the events. Inferring the timing of events from comparative analysis of sequence divergence in the taxa provides an opportunity for a proximate calibration of the molecular

clock (Parkinson *et al.* 2002). Wüster *et al.* (2002) presented an alternative calendar for the arrival of Crotaline lineages into South America and for their evolution. In this paper, we suggested that molecular clock calculations could be interpreted as upper and lower boundaries of the timeframe (Wüster *et al.* 2002). Here, I synthesize the patterns and conclusions of this analysis, and discuss how the results reported in this chapter, further elucidate the dispersal of *C. durissus* in South America.

Central America was the centre of multiple vicariance events during the Tertiary period (Savage 1982; Parkinson *et al.* 2002), which had an enormous impact on the distribution and cladogenesis of many vertebrate taxas. Among the New World pitvipers, Parkinson *et al.* (2002) suggest a basal dichotomy between a Nearctic clade, including rattlesnakes and *Agkistrodon*, and a Neotropical pitviper clade, including the remaining New World pitviper genera. This corresponds to the dispersal of northern taxa into Central America, followed by vicariance between central and northern clades (Savage 1982). At some point during the Tertiary, in Central America, the ancestral clade of all the Neotropical pitviper genera originated. At least four separate colonisations of the South American mainland must have taken place from within the Neotropical clade: an early colonisation by the ancestor of *Bothrops*, and much later, colonisations by *Porthidium, Bothriechis*, and *C. durissus* (see Parkinson 1999; Vidal *et al.* 1999; Parkinson *et al.* 2002; Wüster *et al.* 2002).

Explanations for the origin of *Lachesis*, the largest crotaline species, are still to be resolved (Werman 1992; Zamudio and Greene 1997; Vidal *et al.* 1999; Wüster *et al.* 2002). Most studies of *Lachesis* indicate it to be the sister taxon of *Bothrops* (Gutberlet 1998; Werman 1992; Vidal *et al.* 1999). But even if *Lachesis* is the sister taxon of all *Bothrops*, it does not

preclude a Central American origin, as *Lachesis* may be the sister to the ancestor of the first pitviper to colonize South America.

Although the levels of sequence divergence found within the major clades are similar, the genus *Bothrops* contains considerably more sequence divergence than the South American representatives of other clades. The South American *Bothrops* are paraphyletic with respect to species found in Central America, and diversification of the genus appears to have taken place in South America. This suggests that the common ancestor of *Bothrops* was the first viper to colonize South America, sometime during the Miocene, 10-23 Mya. A single species, *Bothrops asper*, reinvaded Central America much later, and remains the only widespread species of *Bothrops* there (Wüster *et al.* 2002).

The ancestor of *Bothrops* clearly occupied South America long before the emergence of the Isthmus of Panamá, and the available data for *Lachesis* and *Bothrops schlegelii* are consistent with pre Isthmian divergence between Central and South American populations. Some biogeographic patterns suggest that there was a land connection between Central and South America in the late Middle Miocene, 12.9-11.8 Mya (Iturralde-Vinent and MacPhee, 1999), producing a faunal exchange between Central and South America prior to final emergence of the Isthmus of Panamá. Depending on rates of sequence divergence, the time of this land connection would correspond either to the first cladogenesis of *Bothrops* in South America, or the split between the Central and South American *Lachesis* and *B. schlegelii* (Wüster *et al.* 2002).

Present data suggest that South American populations of *Porthidium lansbergii* and *Porthidium nasutum* form a monophyletic group representing a single invasion from Central

America to South America. The *Lachesis*-clock places this event in the late Miocene, 7.7-6.6 Mya, whereas the *Porthidium*-clock places the invasion immediately after the emergence of the Isthmus of Panamá (3.5 Mya) as its calibration point (Wüster *et al.* 2002).

C. durissus is clearly a recent occupant of the South American continent, as noted by Vanzolini and Heyer (1985). The low levels of sequence divergence among South American populations of *C. durissus* are consistent with the hypothesis that this species invaded South America during the Pleistocene, 1-2 Mya, after the uplift of the Panamá land bridge. On the other hand, the Central American lineages (*C. totonacus*, *C. d. culminatus* and *C. d. tzabcan*) are clearly much older. In summary, my sequence data suggests that the colonisation of South America by *C. durissus* can be unambiguously attributed to overland dispersal after final emergence of the Isthmus of Panamá. Details about the colonisation process of the South American continent may require more definition and resolution in the most external branches, especially for those populations on both sides of the Amazon forest.

A different methodological approach is need in order to paint a better picture of the vicariance and dispersal events that produced a divergence between the Central and South American *C. durissus*. Some mtDNA genes are promising; among them ND2 and the D-loop show good resolution in studies of other rattlesnakes (Ashton and de Queiroz 2001). It will be necessary to thoroughly sample the main lineages identified in this study. Sample size is critical for most of the analytical tools available to detect historical processes among populations (Avise 2000; Templeton *et al.* 1995; Templeton 1998; Posada *et al.* 2000). One of the most promising approaches to elucidate the phylogeographic history of the different major lineages of *C. durissus* is the nested clade analysis developed by Templeton and co-workers (Templeton *et al.* 1995; Templeton 1998; Posada *et al.* 2000). Nested clade

analysis is used to screen the evolution of populations by combining a phylogram of the relationships among haplotypes and their geographic distribution (Templeton *et al.* 1987). Using nested clade analysis, population structure can be separated from population history, so we can differentiate restricted gene flow from past fragmentation, colonisation, or range expansion (Templeton 1998). Haplotypes are first linked in a cladogram, which normally is a spanning network that describes the mutational steps connecting all the population or taxa under study. This has stimulated phylogeographic investigations of mitochondrial DNA in Crotalines (e.g., Creer *et al.* 2001) and could be the next step in phylogeographic studies of the *Crotalus durissus* complex.

Chapter 3. Testing variation and phylogenetic information in the fibrinogen intron using *C.durissus* populations

3.1. Introduction

Estimation of phylogenies from DNA sequence data has been the main methodology of molecular phylogenetics. Most studies have focused on the mitochondrial DNA genome (Avise 1994). Relying solely on mtDNA analysis may be problematic because mitochondria do not undergo recombination, meaning that animal mitochondrial genes are inherited as single units. Therefore the phylogenies derived from mtDNA genes are not necessarily independent estimates of the phylogeny of the organism (Avise 1987; Palumbi and Baker 1994; Thorpe *et al.* 1994; Moore 1995; Page 2000). An alternative method without theses drawbacks is to sequence additional non-mitochondrial genes, representing distinct linkage groups. The resultant gene trees therefore provide independent estimates of the species' phylogenetic tree (Wu 1991; Giannasi *et al.* 2001). It is necessary to use gene sequences which contain enough phylogenetic information to resolve relationships between closely related species or populations. Nuclear-encoded introns are ideal because they evolve more rapidly than exons (Prychitko and Moore 1997; Weibel and Moore 2002).

In some taxa, nuclear intron sequences are useful markers to screen phylogenetic relationships (Weibel and Moore 2002 and references therein). Introns evolve rapidly and substitution rates are relatively uniform over the length of the sequence because they do not typically undergo natural selection. A difficulty of using introns or other nuclear markers is identifying orthologous sequences for phylogenetic analysis (Giannasi *et al.* 2001). Nuclear

introns should come from a single copy gene in which the intron arrangement is conserved across a wide range of organisms. The introns should be long enough to reduce the probability of random error (Weibel and Moore 2002).

Recently, intron 7 of the β-fibrinogen gene has been used to determine phylogenetic relationships. The utility of this gene segment has been successfully explored at different taxonomic levels in studies of birds (Johnson and Clayton, 2000; Moyle, 2004; Prychitko and Moore, 1997, 2000, 2003; Weibel and Moore, 2002) and reptiles (Creer *et al.*, 2003; Giannasi *et al.*, 2001) The nucleotide substitutions are randomly distributed along the length of the β-fibrinogen intron, suggesting selective neutrality. Additionally, in the taxa studied, the intron phylogenetic signal is as strong as the mitrochondrial Cyt-b signal, and the topology of the β-fibrinogen tree is quite similar to that of the Cyt-b tree (Prychitko and Moore 1997; Weibel and Moore 2002).

3.2. Materials and methods

Nineteen samples, a subset of the samples from the *C. durissus* populations in the mitochondrial study, were used in the β-fibrinogen study. The samples represented the species' geographical range.

DNA isolation

Whole genomic DNA was extracted using a standard proteinase K protocol (Sambrook *et al.* 1989). The samples with the proteinase K were incubated overnight at 60 °C and then transferred to ice. They were then incubated with RNase for 30 min. at 37 °C and then transferred to ice. Ice-cold 5M ammonium acetate and centrifugation was used to

precipitate, and then pellet the proteins. DNA was then precipitated from the resulting supernatant with ice-cold isopropanol, and centrifuged to form a pellet which was washed with 70% ethanol, air-dried, and then dehydrated with TE (Buffone 1985). Extracts were visualised by UV radiation on circular 25ml, 1.4% agarose plates with 3µl ethidium bromide.

PCR

The 525 bp fragment of intron 7 of the β-fibrinogen region was amplified from 1-2 µl DNA extracts using modified primers (Prychitko and Moore 1997): Fib-B17U, 5'- GGA GAA AAC AGG ACA ATG ACA-3' and Fib-B17L, 5'-TCC CCA GTA GTA TCT GCC ATT – 3'. The 50 µl PCR reactions were performed with a 50 ng template, 0.52 µM primers, 20 mM tris-HCl, 0.5 mM MgCl, 0.4 µM dNTP, 2 units Taq DNA polymerase, and 0.5% DMSO. Amplification conditions involved an initial denaturation step of 4 minutes at 94 °C, 35 cycles of denaturation of 1 minute at 94 °C, primer annealing of 1 minute at 50 °C, extension of 2 minutes at 72 °C, and at the end, an extra extension step for 3 minutes at 72 °C, cooling to 4 °C. The PCR products were concentrated by pooling 2-3 products per sample, and purified using the GenElute TM (Supelco) nucleic acid purification kit.

Sequencing and data analysis

A single stranded automated sequencing method was carried out using 10 µl reactions containing 50 ng PCR template, 0.16 pmol 5' primer, and BigDye Terminator Ready Reaction Mix (ABI). The reaction mix was cycled in a PE-ABI 9700 Thermal Cycler for 30 seconds at 94 °C, 50 cycles of 10 seconds at 96 °C, 5 seconds at 50 °C, 4 minutes at 60 °C, and then cooled to 4 °C. The samples were precipitated using 2M sodium acetate and

100% ethanol, and the resulting pellet was washed in 70% ethanol and air dried. The samples were suspended in 4 µl of a dextran blue EDTA/formamide loading buffer, and 1 µl of this mixture was loaded onto a 5% longranger gel in 1x TBE running buffer. The samples were analysed on an ABI 377 DNA Sequencer, generating a chromatogram and a text sequence. The sequences were read and aligned by eye to each other using the Chromas 1.51 package (Technelsyum Pty Ltd, 1988).

The uncorrected p-distance was used to obtain pairwise sequence comparisons from the neighbour joining approach (Saitou and Nei 1987). This was performed using the Molecular Evolutionary Genetics Program (MEGA, Kumar *et al.* 1993). Aligned sequences were subjected to parsimony analyses using PAUP 4.0. Branch and bound searches and bootstrap (Felsenstein 1985) were carried out for all the parsimony analyses. Assessments of the branch support and tree stability of the generated topologies were performed using the Bremer (1994) method. To do this, branch searches were repeated, retaining successively longer trees until all nodes were collapsed. In order to distinguish phylogenetic signal from random noise in the sequences, skewness (g1) statistics were calculated from randomly generated trees in PAUP 4.0 (Hillis and Huelsenbeck 1992; Swofford 1998).

3.3. Results

A very low degree of differentiation was observed among the sequences of the β-fibrinogen intron. Pairwise distances ranged from 0 -1.2% in the in-groups and 1.64% in the out-groups, indicating a very conserved fragment with very low resolution of the inner nodes.

Tree length distribution, determined from random sampling of unweighted trees, was slightly skewed to the left ($g1 = -0.492$, $p < 0.02$), indicating poor phylogenetic signal in the data (Hillis and Huelsenbeck 1992). Bootstrap values are shown in Fig 3.1. There was very poor resolution in the relationships of the sequences analyzed. However, there was a small difference between interspecific samples. For example, the species categorization of *C. molossus* and *C. basiliscus* was better supported than the species categorization of *C. durissus*. In this analysis, *C.d. totonacus, C.d. tzabcan* and the *C. durissus* from El Salvador were grouped. Finally, the monophyly of South America *C. durissus* populations was well supported.

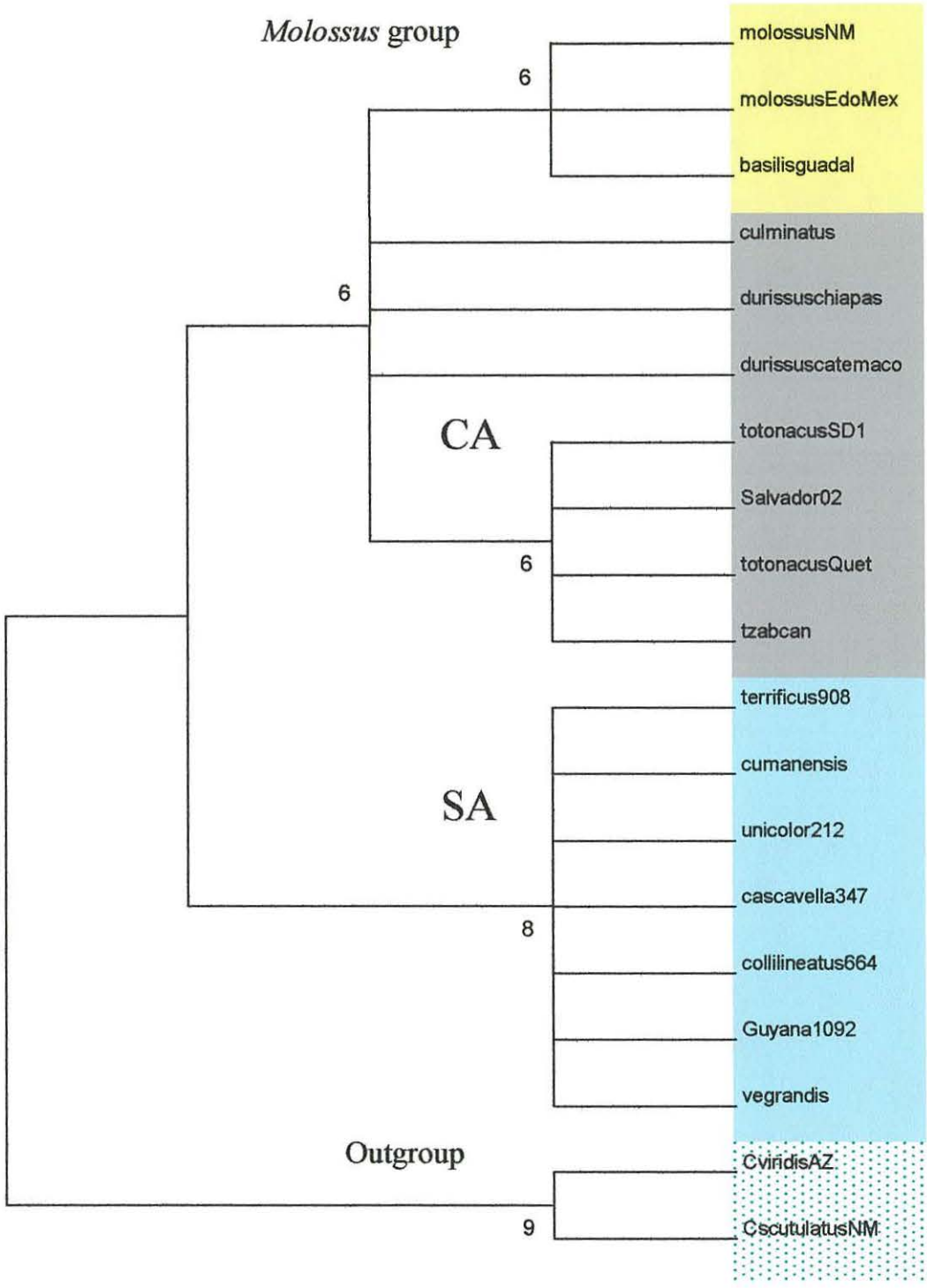

Fig 3.1. Bootstrap 50% majority-rule consensus tree of the sequences of the intron 7 of the β-fibrinogen gene in selected samples of *C. durissus*. Internal labels: CA, Central America clade; SA, South America clade.

3.4. Discussion

Despite the low resolution of the sequences analyzed, there are some aspects to be considered before judging the appropriateness of intron 7 of the β-fibrinogen region to resolve snake phylogenies. The fact that only a 525 bp fragment was sequenced suggests that much of the information available in this region could have been missed. Several studies report that the size of the β-fibrinogen region is approximately 1100 bp, considerably longer than the fragment amplified here (Prychitko and Moore 1997; Weibel and Moore 2002). Is quite likely that the primers used did not flank the complete region and that a conserved region was amplified. On the other hand, some studies indicate that certain introns are particularly useful for resolving phylogenies at higher levels, such as species and genera, but difficult for lower taxonomic levels (Moore 1995). Several papers have successfully used intron sequences in phylogenetic studies. In particular, studies of actin in cetaceans (Palumbi and Baker 1994), and fibrinogen (Prychitko and Moore 1997), aldolase, glyceraldehyde-3 phosphate dehydrogenase, alpha enolase, and lamin in birds (Friesen *et al.* 1997). However, the slow rate of intron sequence evolution observed (Moore 1995) has limited the usefulness of these markers to deep taxonomic splits (i.e. between relatively divergent species and genera). Intron sequences are probably not suitable for population and species level studies.

Chapter 4. Phylogenetic relationships among *C. durissus* populations based on amplified fragment length polymorphism (AFLP) analysis.

4.1. Introduction

The most commonly used molecular markers for phylogenetic differentiation are mtDNA markers followed by nuclear markers (Avise 2000; Mueller and Wolfenbarger 1999). These methods have some drawbacks. In mtDNA analyses, the lack of mitochondrial recombination means that genes are inherited as single units, and that therefore the phylogenies derived mtDNA genes are not necessarily independent estimates of the phylogeny of the organism (Avise 1987; Palumbi and Baker 1994; Thorpe *et al.* 1994; Moore 1995; Page 2000; Avise 2000). Few nuclear genes have demonstrated sufficient variation to make them useful for phylogenetic analysis (Mueller and Wolfenbarger 1999). Amplified Fragment Length Polymorphism (AFLP) analysis samples the entire genome, therefore screening for more genetic variation (Vos *et al.* 1995; Kardolus *et al.* 1998; Labra *et al.* 1999; Baayen *et al.* 2000; Bakkeren *et al.* 2000; Hodkinson *et al.* 2000; Kanzaki *et al.* 2000; Giannasi *et al.* 2001; Parsons and Shaw 2001; Buntjer *et al.* 2002; Allender *et al.* 2003; Seehausen *et al.* 2003 Ogden and Thorpe 2002; Creer *et al.* 2004; Sullivan *et al.* 2004).

The AFLP technique uses multiple restriction fragments screened on a single polyacrylamide gel (Vos *et al.* 1995). The technique involves restriction digestion of genomic DNA with rare and frequent enzyme cutters. Following adapter ligations, restriction fragments from a total digest of genomic DNA are selectively amplified and separated by gel electrophoresis.

AFLPs may be the ideal marker system for resolving genetic relatedness among individuals, populations, and species (Mueller and Wolfenbarger 1999; Buntjer *et al.* 2002; Allender *et al.* 2003; Seehausen *et al.* 2003 Ogden and Thorpe 2002; Creer *et al.* 2004; Sullivan *et al.* 2004). The incorporation of PCR allows for rapid and efficient marker generation. Furthermore, the cost and development time are low compared to other markers (reviews summarized by Mueller and Wolfenbarger 1999). AFLP is less problematic than other techniques where in order to avoid spurious polymorphisms, the laboratory conditions for PCR amplification must be highly controlled (Mueller and Wolfenbarger 1999; Buntjer *et al.* 2002; Allender *et al.* 2003; Seehausen *et al.* 2003; Ogden and Thorpe 2002; Creer *et al.* 2004; Sullivan *et al.* 2004). In addition, extensive screening to identify useful primers is not required, the levels of polymorphisms are generally higher, and the number of markers generated is virtually unlimited (e.g. Barker *et al.* 1999). A limitation of AFLPs is that heterozygous alleles are difficult to detect because the marker is co-dominant (Vos *et al.* 1995; Buntjer *et al.* 2002; Allender *et al.* 2003; Seehausen *et al.* 2003 Ogden and Thorpe 2002; Creer *et al.* 2004; Sullivan *et al.* 2004).

The purpose of this chapter is to analyse the phylogenetics of *C. durissus* using AFLPs as a molecular marker. A further goal is to compare the phylogenetic history suggested by the AFLP analysis with the history suggested by the mtDNA analysis (Chapter 2).

4.2. Materials and Methods

In order to compare the mitochondrial and AFLP techniques, the same blood aliquot samples were used for both analyses (see Chapter 2). Whole genomic DNA was extracted using a standard proteinase K protocol (Sambrook *et al.* 1989). The samples with the proteinase K were incubated at 60 °C overnight and then transferred to ice. Then the samples were incubated with RNase for 30 minutes at 37 °C and transferred to ice. Ice-cold 5M ammonium acetate and centrifugation was used to precipitate, and then pellet the proteins. DNA was then precipitated from the resulting supernatant with ice-cold isopropanol, and centrifuged to form a pellet, which was washed with 70% ethanol, air-dried and then dehydrated with TE (Buffone 1985).

Extracts were visualised by UV radiation on circular 25ml, 1.4% agarose plates with 3ml ethidium bromide. AFLP analysis was performed using the AFLP Analysis System 1' kit (GIBCO BRL, cat. no. 10544-013, http://www.invitrogen.com), developed by Vos *et al.* (1995). The protocol followed the manufacturer's instructions that three S3-nucleotide selective primer extension combinations be used for the forward and reverse primers. Each primer combination was comprised of an EcoR-1 radio labelled (33P) primer, E-AGG, with one of three Mse-1 primers (M-CTT, M-CAT, M-CTA). These will subsequently be referred to as follows: Primer Pair A (E-AGG + M-CTT), Primer Pair B (E-AGG + M-CAT), and Primer Pair C (E-AGG + M-CTA).

AFLP markers were scored using automated sequencing, which produced a matrix of presence-absence bands per size category as assigned by the sequencing software. Data from the three primer pairs was analysed collectively by combining matrices. Using Principal Components Analysis (PCA), eigenvectors can be obtained from correlation and dissimilarity matrices. A dissimilarity matrix was generated by Gower's general

similarity coefficient using the program MVSP Version 3.11c (Kovach 1999). As only binary data were being used in this analysis, Gower's general similarity coefficient (GGScij) was equivalent to Jaccard's coefficient (Jcij):

$$GGScij = Jcij = a/(a+b+c)$$

where a is the number of bands shared by individuals j, and I b is the number of bands present in i, but not in j and c, the number in j but not in i (Sneath and Sokal 1963). A Q-mode Principal Coordinate Analysis (PCOA) was then performed on the dissimilarity matrix. The resulting PCA scores were plotted to discriminate between species and populations.

A goal of this chapter was to test the congruence between the AFLP and mtDNA topologies. Because genetic distance can easily be confounded by geographic distance, it was necessary to control for genetic distance (Mantel 1967; Thorpe 1991). To do so, three congruent matrices were constructed, one of geographic distances, one of genetic distances according to AFLP analysis, and the third of distances based on mtDNA analyses. The matrices were constructed as follows:

For the AFLP matrix, the presence/absence data for the band patterns was converted to a similarity matrix based on the mean character differences using PAUP 4.0 (Swofford 1998). Then an mtDNA matrix was constructed from the Tamura-Nei 2 distances (Chapter 2). Finally a geographic matrix was generated from the latitude and longitude coordinates of the locations sampled. Great Circle Distances (GCD) were calculated using the following Microsoft Excel algorithm:

GCD=RadiusEarth*ACOS(COS(RADIANS(90-(Lat1*24)))*COS(RADIANS(90-(Lat2*24)))+SIN(RADIANS(90-(Lat1*24)))*SIN(RADIANS(90-(Lat2*24)))*COS(RADIANS(24*(Long1-Long2))))

Where, Lat1 is the latitude of point 1, entered as DD: MM: SS, Long1 is the longitude of point 1, entered as DD: MM: SS, Lat2 is the latitude of point 2, entered as DD:MM:SS, Long2 is the longitude of point 2, entered as DD: MM: SS, and RadiusEarth is the radius of the earth (3,963 miles or 6,377 kilometres).

The effect of geographic distance in the phylogenetic relationships for both AFLP and mtDNA data sets was controlled using partial Mantel tests. Mantel tests evaluate the association between the observed phylogeographic structure and patterns predicted by different hypotheses, while simultaneously controlling for the confounding effect of geographic distance (Thorpe *et al.* 1994). Associations between the AFLP, mtDNA and geographic distance matrices were examined with pairwise and partial Mantel tests using 10,000 permutations with programs developed by Liedloff (1999) and Goudet (2002).

4.3. Results

The PCOA plot using the Gower general similarity index screened the different *C. durissus* populations and the resulting pattern was consistent with the geographic origin of the populations (Fig 4.1). The South American rattlesnakes were grouped separately from the Central America populations. In the PCOA plot, the Central American populations appeared as independent clusters, which were consistent with different

Crotalus lineages identified by the mtDNA study (Chapter 2). The cumulative eigenvalue percentage for the first three PCOA axes was higher for the Central American populations than the South American group, indicating more genetic differentiation in Central America (Table 4.1).

A total of 248 characters (polymorphic bands) were obtained from the AFLP analysis, of which 229 were parsimony informative. A significant phylogenetic signal in the data set was indicated by the skewness parameter ($g1 = 0.68$, $P < 0.001$) for the number of characters and taxa involved (Hillis and Huelsenbeck 1992). The branch and bound analysis using maximum parsimony as criterion generated a total of 20 equally most parsimonious trees (tree length = 380; CI =0.1741; HI=0.8259; RI =0.7263; RC =0.1264).

Five clades were recognized from the bootstrap analysis including the *molossus-basiliscus* clade. Bootstrap support throughout the tree was generally high for the internal branches but particularly low in the South America clade (Fig.4.2). In the combined mtDNA and AFLP analysis, there was a closer relationship between the Belize *C. d. tzabcan* populations and the South American clade than was indicated solely by the mtDNA analysis (Chapter 2). The relationship of the Belize clade to the South American group was highly supported by both the bootstrap values and the Bremer decay index.

Pairwise correlation coefficients showed a clear correlation between the mtDNA and AFLP patterns (Fig 4.4). The Mantel tests controlling for geographic distance showed that distance had little effect on the correlation of the mtDNA and AFLP patterns, while

there was a strong correlation between the mtDNA and AFLP data sets. With both the mtDNA and AFLP data sets, the geographic distance effect was weak, but it was slightly stronger in the AFLP data (Table 4.2).

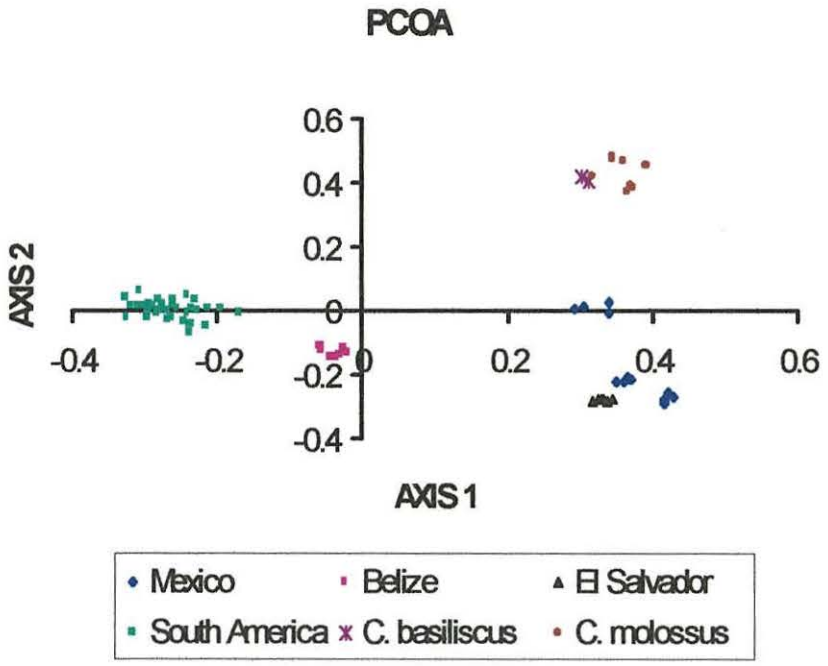

Fig. 4.1. Principal coordinate plot using Gower general similarity index showing the AFLP groups according to the geographical origin.

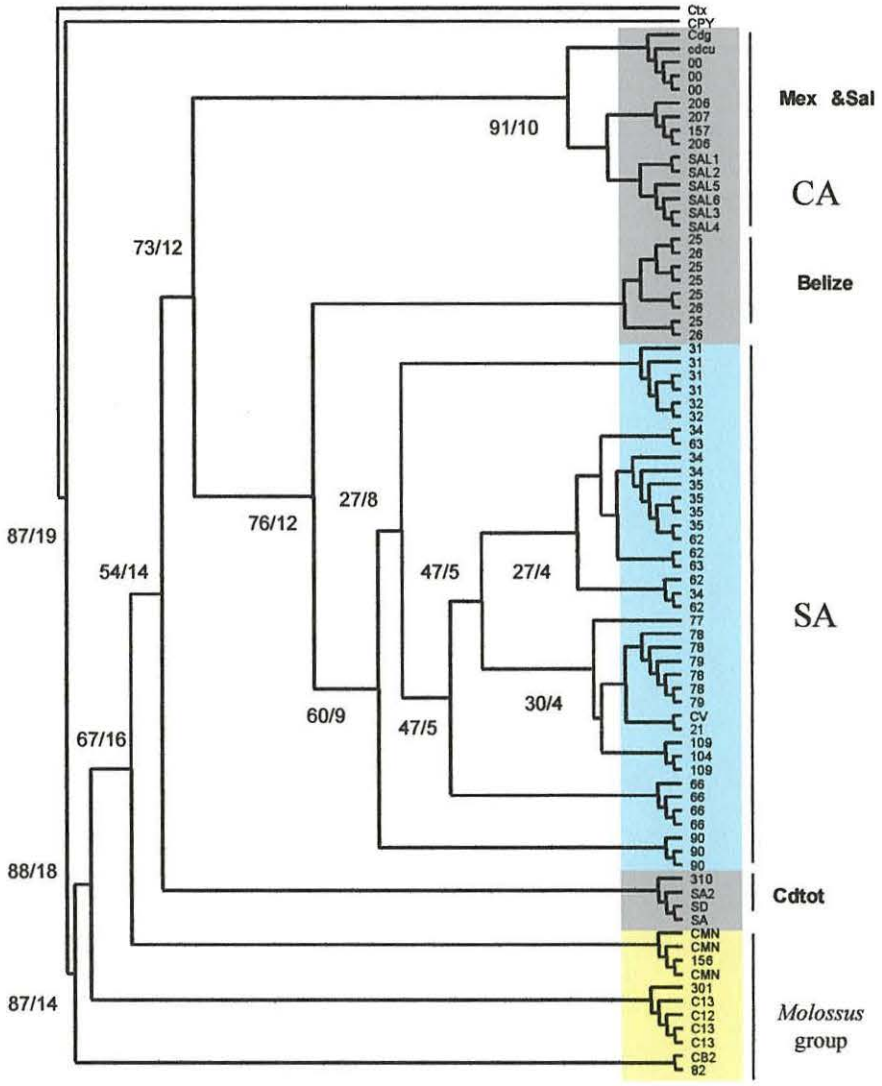

Fig 4.2. Bootstrap values and Bremer decay index of the AFLP patterns for the *C. durissus* populations showing five main clades. Mex & Sal, populations from Mexico and El Salvador; Belize, *C.d. tzacban; C.d. toto, C.d. totonacus; Molossus* group, *C. molossus* and *C. basiliscus*. SA South America (blue), CA Central America (grey), *Molossus* group (yellow), Outgroup (dotted).

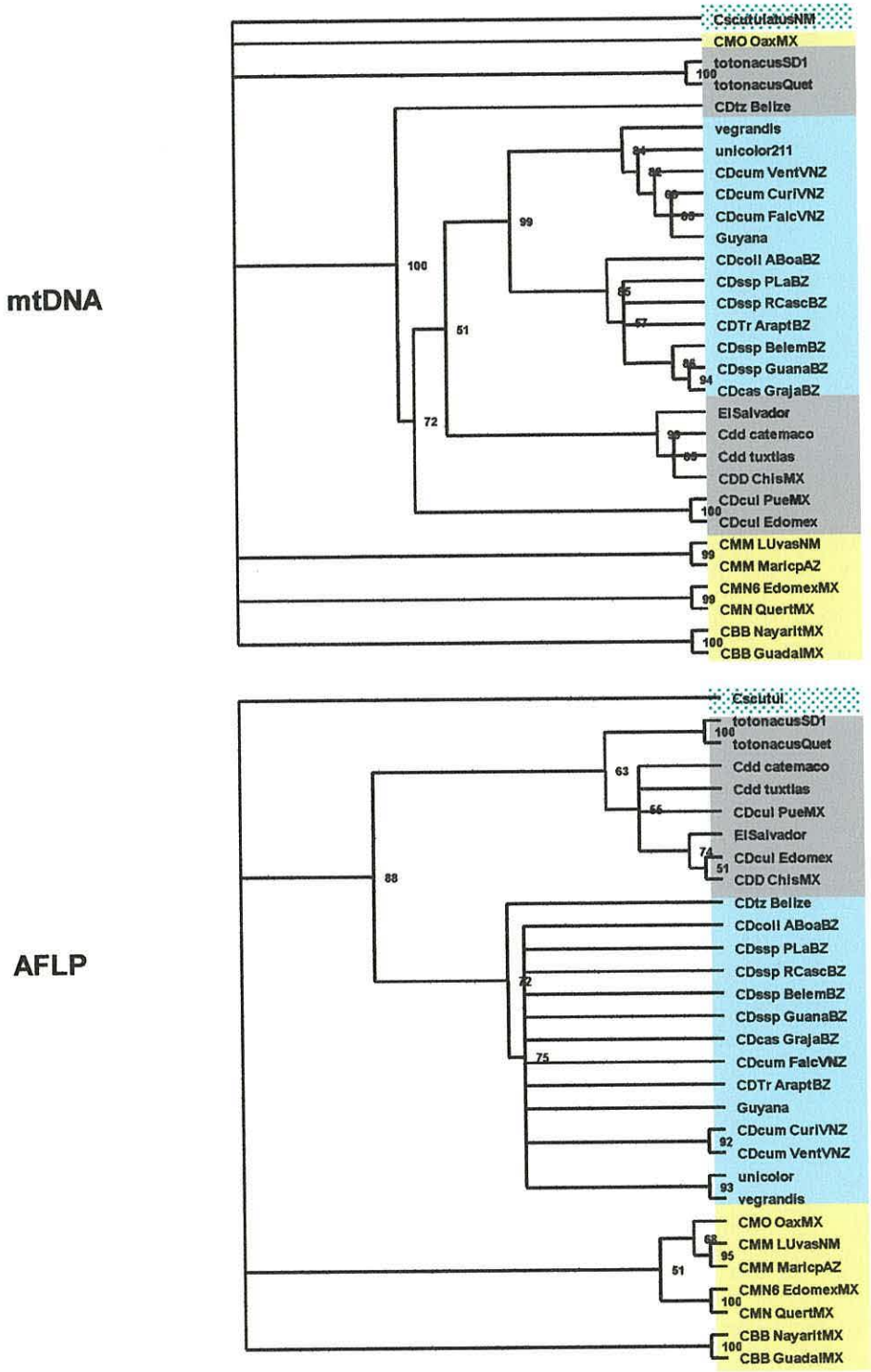

Fig.4.3. Bootstrap 50% majority-rule consensus trees of the mtDNA sequences and AFLP patterns of *C. durissus*. For comparison purposes the same samples and localities were used in the analysis. SA South America (blue), CA Central America (grey), Molossus group (yellow), Outgroup (dotted).

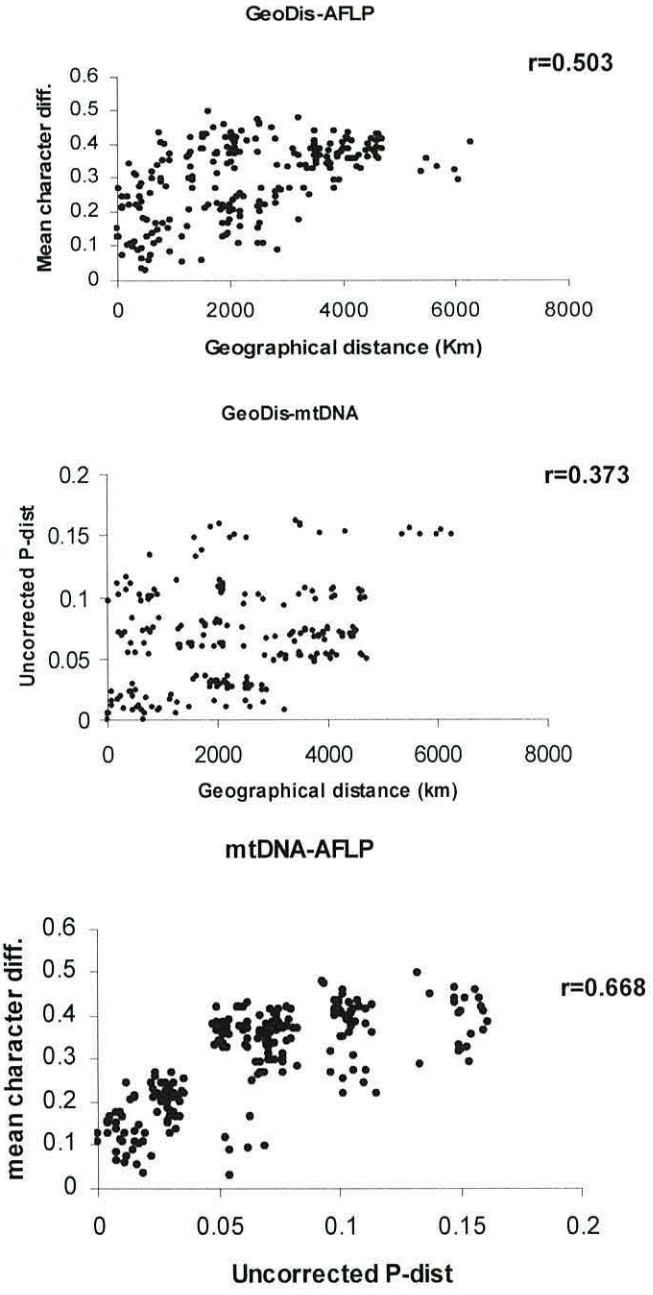

Fig 4.4. Plots of the Mantel pairwise correlation (r) between AFLP (mean character differences), mtDNA (Kimura 2-parameter) and geographic distance (Km) for the *C. durissus* populations.

Table 4.1 Cumulative eigenvalue percentage on the first three principal coordinate axes using principal coordinate analysis for the *C. durissus* populations and related species based on the polymorphic AFLP bands obtained.

principal cordinate analysis	**porcentage of variation on axis1**	**porcentage of variation on axis 2**	**porcentage of variation on axis 3**
Central America	44.96	47.23	64.34
South America	38.5	47.52	66.1
C. molossus	13.39	31.07	35.74

Table 4.2. Pairwise and partial Mantel test results of the variables Geographical distances, mtDNA p-distances, and AFLP mean character distance for both all the taxa (*C. durissus*, *C. scutulatus*, *C. molossus*, and *C. basiliscus*) and the *C. durissus* populations analyzed.

Pairwise Mantel test	**g**	**Z**	**r**	**P**
All taxa analyzed				
mtDNA, AFLP	18.603	15.892	0.9116	<0.005
mtDNA, GeoDis	3.1328	102398.99	0.1839	<0.050
AFLP, GeoDis	3.0908	398994.4	0.1787	<0.050
Only *C. durissus*				
mtDNA, AFLP	6.414	11.0144	0.668	<0.005
mtDNA, GeoDis	3.5507	80251.148	0.3727	<0.050
AFLP, GeoDis	6.3102	54441.828	0.5031	<0.005
Partial Mantel test	**Partial r**	**β**	**Sum. Squares**	
Geographic distance	0.3030	0.0021	0.5228	<0.050
mtDNA p-distances	0.5660	1.675716	0.9151	<0.001

4.4. Discussion

This chapter uses the AFLP technique to infer the phylogenetic relationships among the *C. durissus* populations in Central and South America. The parsimony based phylogenetic analysis and other molecular statistics (e.g. skewness g1 statistics) demonstrate that AFLP data are suitable for phylogenetic reconstruction. Additionally, similar results are obtained when the data set is subjected to ordination analysis like PCOA. This analysis showed that the AFLP *C. durissus* phylogenies were consistent with those obtained by mtDNA analysis with biogeographic and systematic conclusions alike. The AFLP and mtDNA phylogenies showed similar topologies and patterns. This study supports the use of the AFLP marker for phylogenetic inference (e.g. Giannasi *et al.* 2001; Ogden and Thorpe 2002; Creer *et al.* 2004; Sullivan *et al.* 2004).

AFLP requires no prior knowledge of sequence information, does not depend on the specific flanking of molecular regions, and can be applied in any genome. Not only do these markers aid the reconstruction of gene evolution, but they are also very useful for reconstructing the evolutionary history of species and populations. However, because the AFLP technique surveys the whole genome by digesting with restriction enzymes, each AFLP fragment is anonymous, its position on a functional region and the nature of its coding properties are not identifiable (Mueller and Wolfenbarger 1999; Buntjer *et al.* 2002; Allender *et al.* 2003; Seehausen *et al.* 2003 Ogden and Thorpe 2002; Creer *et al.* 2004; Sullivan *et al.* 2004).

The AFLP analysis supports the previous chapters' conclusions that the South America *C. durissus* populations are monophyletic, with a weak differentiation in those

populations currently considered as separate species. The bootstrap values and decay indexes of the internal nodes in the South America clade do not support the species status given by some authors to *C.d. unicolor* and *C.d. vegrandis*, (Campbell and Lamar 1989). These populations should be considered as conspecific of *C. durissus*. The AFLP supports the paraphyly of Mexican and Central America based on mtDNA.

The AFLP-generated phylogeny revealed two details about the evolutionary history of *C. durissus* that were not immediately evident from the mtDNA phylogenies. First, the AFLP analysis grouped the Belize populations with the South American ones, suggesting a genetic connection between the Central and South American populations. The Belize and Yucatan rattlesnakes may belong to the same lineage from which the South American clade originated. Second, the AFLP analysis grouped *C. totonacus* closer to the *C. durissus* complex than to the *C. molossus* group as was suggested by the mtDNA analysis. The reasons for this discrepancy are unclear, although it could be due to introgression between both complexes. What is clear is that *C. totonacus* belong to a very deep lineage. The present data supports the consideration of *C. d. tzabcan* as separate species.

This study is an example of how the same phylogenetic or biogeographic question may be approached using different molecular markers. There are a number of different approaches available to construct phylogenetic trees when using more than one data source. These approaches can be placed into two categories, first, those that combine the data, and second, those that keep the data separate and combine the inference of the resulting trees. Combining sequence data of a known genomic location (e.g. mtDNA) with molecular data of an unknown location presents an analytical challenge. In this

study, we followed the second approach, using Mantel tests to compare the phylogenetic trees of two different markers.

Conclusion: the utility of AFLP to infer phylogenetic relationships

Compared to other techniques, AFLP have some advantages and some disadvantages as a source of characters for phylogenetic analysis. For instance, AFLP shows higher reproducibility and a lower incidence of non independence than random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphisms (RFLP). On the other hand, AFLP share some characteristics of these other markers that are problematic like the high probability of parallel losses of characters relative to gains (Backeljau *et al.* 1995; Swofford *et al.* 1996; Mueller and Wolfenbarger 1999). However, phenomena like homoplasy, nonindependence, and asymmetry in character state change, are potential sources of error for molecular and morphological characters (Malhotra and Thorpe 2004). The best evaluation of AFLP as phylogenetic markers will continue to be congruence studies between AFLP derived topologies and other from molecular and morphological characters.

The present study on the phylogenetic relationships in the *C. durissus* complex, joins a growing consensus of others that indicate that these multi-locus markers can resolve relationships among closely related species and populations, groups for which sequence data have often been inconclusive or problematic, or to incorporate nuclear data parallel to mtDNA.

Chapter 5. General Discussion

5.1. Outcomes

In this study, I present several well-supported phylogenies of the Neotropical rattlesnake, *C. durissus,* using different molecular markers and methods. Using these phylogenies, I investigate hypotheses about the dispersal of *C. durissus* into South America, and test the systematic status of the Central American forms.

The topologies of the phylogenetic trees obtained by different methods were significantly similar. The differences in the phylogenetic trees were mostly due to the nature of the molecular markers used and their modes of inheritance, and perhaps to the experimental limitations of using the fibrinogen region and AFLPs. Bootstrap methods and the Bremer branch support index show strong support for several phylogenetically important nodes, especially for those clades that demonstrate the monophyly of South American forms and separate them from the Central American forms. Using these results, I interpret the biogeographic events which occasioned dispersal of *C. durissus* into South America, suggest a reclassification of some *Crotalus* species, discuss the implications of this research for conservation, and suggest future research.

5.2. The origin of the *C. durissus* complex in Mexico and north Central America

The rattlesnakes (*Crotalus* and *Sistrurus*) are part of the pitviper monophyletic group in the New World (Parkinson 1999; Parkinson *et al.* 2002; Murphy *et al.*, 2002; Wüster *et al.* 2002). Approximately 30 species of rattlesnakes are currently recognized (McDiarmid

et al. 1999; Campbell and Lamar 2004). Brattstrom (1964) suggests that rattlesnakes originated in the central plains of North America. He argues that the *Lachesis-Crotalus-Sistrurus* "stock" diverged soon after the arrival of an *Agkistrodon contortrix*-like ancestor in the New World via the Bering Land Bridge. The demonstrated monophyly of the New World temperate group (*Agkistrodon, Crotalus*, and *Sistrurus*) and the tropical group of the bothropoid genera and *Lachesis* is inconsistent with Brattstrom's hypothesis (Parkinson *et al.* 2002; Wüster *et al.* 2002). Brattstrom (1964) proposed that *Agkistrodon contortrix* was an Old World migrant and that *Crotalus* and *Sistrurus* were closely related to *Lachesis*. Recent molecular data indicate that *Agkistrodon* is of New World origin, and *Lachesis* is more closely related to a bothropoid genus than to rattlesnakes (Zamudio and Greene 1997; Parkinson *et al.*, 2002; Wüster *et al.* 2002).

There is general consensus that rattlesnakes originated in Mexico (Armstrong and Murphy 1979; Klauber 1972; Greene 1997; Place and Abramson 2004), primarily because several morphologically primitive species of rattlesnakes occur in Mexico. Some authors specify the Mexican Plateau as the ancestral area of the rattlesnakes. Only recently, Place and Abramson (2004), using a cladistic method of inferring the ancestral area of a taxon (Bremer 1992), established the Sierra Madre Occidental of Mexico as the most probable ancestral area of all the rattlesnakes (*Crotalus* and *Sistrurus*). Furthermore, they inferred that the vegetation of the ancestral area was pine-oak forest. They provide the first quantitative assessment of the ancestral area of rattlesnakes and assessments of the ancestral habitats of rattlesnakes, supporting the hypothesis that the ancestral area of rattlesnakes is in North-Central Mexico (Place and Abramson 2004).

The historical factors providing the conditions for the evolution of the older lineages of *C. durissus* in Mexico and north Central America can be explained by the geological history of the region (Graham 1993; Flores 1993; Ferrari *et al.* 1999). In eastern and southeastern Mexico, the Sierra Madre Oriental has provided upland habitats for the diversification of the biota since the last Cretaceous-Palaeocene (Graham 1993; Flores 1993; Ferrari *et al.* 1999). The Trans-Mexican Volcanic Belt began to develop during the early Tertiary but underwent its principal uplift and deformation during Miocene and Quaternary periods (Ferrari *et al.* 1999).

The Cenozoic era was also a time of significant climatic change, with documented effects on the biota of southern Mexico. There was a decline in global temperatures beginning during the mid-Miocene. Such physiographic and climatic fluctuations not only affected speciation rates through vicariance but also provided a diversity of habitats for the perpetuation of new forms of reptiles (Flores 1993). These conditions probably produced speciation events that produced the *molossus-basiliscus-totonacus* clades. The isolation of the Yucatan peninsula, due to the Tehuantepec-Gulf of Mexico channel, produced the vicariance event for the origin of the *C. d. tzabcan* clade. Further studies could attempt to calibrate the molecular clock with the geological events described above.

In addition, southern Mexico, located at the confluence of the two migration routes through Central America and the Antilles, has been important throughout the Tertiary for the diversification of rattlesnakes. An extensive new habitat area to the south became increasingly available as South America moved closer to North America and the Panamá land bridge became established. By the end of the Tertiary, southern Mexico was accessible to temperate biota from the north, with introductions facilitated by the cooling

climates of the late Eocene through the Pleistocene, and to tropical biota from the south, with introductions progressively increasing during the warm climates of the Paleocene, early and middle Eocene, Oligocene, and early Miocene (Flores 1993). These biogeographic conditions probably produced the *C. durissus* lineages of Pacific Mexico and Central America. Further studies are required of the biogeography of the Mexican lineages of the *C. durissus* complex and other rattlesnake species are necessary in order to chronologically map the evolution of the rattlesnake fauna at its centre of diversification. Special emphasis should be focus on the "germinal" taxa of Central Mexico in a comparative phylogeographic context.

5.3. The Great American Interchange and the dispersal of *C. durissus* into South America

The rise of the Isthmus of Panamá was the most important event to structure the biogeography of the former islands of North America and South America. This event produced the "Great American Interchange," the migration and intermingling of terrestrial lineages that had been separated since the Cretaceous (Simpson 1980; Marshall *et al.* 1979; Stehli and Webb 1985; Pindell and Barrett 1990). The dating and calibration of the moment when the rise of the Isthmus occurred, inferred from rates of molecular evolution, has been the subject of much research interest. Most of the focus has been on germinate species or trans-isthmus sister-species pairs of taxa in different organisms (Knowlton *et al.* 1993; Knowlton & Weigt 1998; Lessios 1998; Bermingham *et al.* 1997). These studies provide evidence that gene flow in some taxa was a gradual process over millions years instead of a simultaneous event for different organisms. This is consistent with geological data indicating a gradual transformation of the early Isthmus from an

archipelago system, in the middle to late Miocene, to its completion as a terrestrial corridor about 3 Mya (Bermingham and Lessios 1993; Knowlton *et al.* 1993; Coates and Obando 1996; Coates *et al.* 2003). These findings imply that the dispersal of some taxa during the trans-isthmus interchange may have happened in either a gradual fashion, producing different lineages crossing the Isthmus (i.e. the island-hopping hypothesis; Simpson 1950), or may have occurred in one event, which would be reflected by one or a very few lineages crossing the Panamá land-bridge.

The data presented here suggest that *C. durissus* dispersed towards South America shortly after the final uplift of the Panamá land bridge. In accordance with Wüster *et al.* (2002) and the data presented here, the very low levels of sequence divergence among the South American populations are consistent with the hypothesis that this species invaded the South American continent 2.3 - 2.0 Mya after the uplift of the Panamá land bridge, estimated to have occurred approximately 3.5 Mya (Marshall *et al.* 1979; Bermingham and Martin 1998; Coates and Obando, 1996; Wüster *et al.* 2002). The rapid spread of rattlesnakes into South America in a relatively short time makes it difficult to accept the island-hopping hypothesis for the dispersal of *C. durissus* into South America as is suggested for other pitvipers (Wüster *et al.* 2002).

Prior to the dispersal event, the Central American lineages of *C. durissus* (including *C. totonacus*) could have evolved, and the lineage that dispersed into South America could have been present near the primitive Panamánian land bridge (probably in the Yucatan Peninsula), and crossed during the Pliocene-Pleistocene period, when presumably a savannah corridor was established throughout the isthmus (Duellman 1978; Vanzolini and Heyer 1985).

Among the vipers, *C. durissus* was the last migrant crossing the Isthmus towards South America. There are few candidates of pitvipers with which to compare phylogeographic patterns of *C. durissus* and to test the dispersal mechanisms involved. Among the genus *Bothrops*, the species *B. asper* apparently reinvaded Central America and Mexico (Wüster *et al.* 2002). Further studies on the phylogeography of *B. asper* may find parallel phylogenetic patterns with *C. durissus*.

5.4. The South American Invasion

Short after the post-Isthmian dispersal of *C. durissus* into South America, the species rapidly invaded dry savannah habitats throughout the continent in a relatively short period of time, and established both north and south of the Amazon forest. This discontinuity and the low genetic differentiation of the South American rattlesnake populations provide an opportunity to discuss the role of the Amazon forest in the dispersal of *C. durissus*.

During the late Pliocene and Pleistocene, a series of global climatic fluctuations affected patterns of Neotropical diversity (Potts and Behrensmeyer 1992). The Pleistocene refugia hypothesis argues that drier climatic phases caused a fragmentation of the Amazonian rainforest into forest refugia isolated by savannahs, leading to increased allopatric speciation among forest species (Haffer 1969; Prance 1973). However, the notion of Pleistocene rainforest fragmentation remains highly controversial (Colinvaux *et al.* 2000, 2001; Haffer and Prance 2001; Hooghiemstra 2001). The available palynological evidence provides little support for the hypothesis of savannah vegetation

in Amazonia (e.g. Colinvaux *et al.* 1996, 2001; Kastner and Goñi 2003). On the other hand, speciation in many widespread Neotropical species complexes predates the Pleistocene climatic fluctuations, often by a considerable margin, and the expected genetic pattern of post-refugia Quaternary range expansion has not been demonstrated for Neotropical forest mammals (Hewitt 2004; Moritz *et al.* 2000; Lessa *et al.* 2003).

Based on the cytochrome-b, ND4 and AFLP's phylogenies, the pattern of evolution of the South American populations of *C. durissus* suggests that wet and dry cycles and Amazon forest contraction probably did occur. The lack of genetic differentiation between rattlesnake populations from north and south of the Amazon (currently separated by unsuitable rainforest habitat) supports the hypothesis that the Amazon forests have gone through at least one cycle of fragmentation during the Pleistocene since the colonisation of South America by *C. durissus*. Furthermore, alternative molecular divergence times indicates that the Trans-Amazon Vicariance occurred 1.08 -1.2 Mya, thus the dispersal across the Amazon Basin can be dated to the early to mid-Pleistocene (Wüster *et al.* 2005, see Appendix 1).

Kastner and Goni (2003) analyzed sediment samples from the Amazon deep sea fan, and reported a remarkable constancy in different parameters and similitude to modern Amazon River suspended sediments. They concluded that the vegetation of the Amazon Basin did not change significantly during the last 70,000 years (Last Glacial Maximum, late Pleistocene) and found no evidence for the development of large savannas, which are indicators of increased glacial aridity in Amazonia (Haffer 1997; Haffer and Prance 2001). However, these findings are limited the time since the Last Glacial Maximum and do not take into account that extensive global climatic cycles with glaciations in the

higher latitudes occurred earlier (ca 2.4 May, Hooghiemstra and Cleef 1995). The dispersal dates of *C. durissus* as a consequence of the uplift of Panamá Isthmus (about 1-1.2 Mya) match with these events of global climate cycles and may indicate that the Amazon barrier has limited the genetic flow of the Neotropical rattlesnake for the last 70,000 years.

5.5 Implication for *Crotalus* systematics

Understanding phylogenetic patterns is necessary to better understand the Evolutionary Species Concept (ESC). The ESC is often preferred over other species concepts because it represents the most general and universal notion of a species matching a more objective set of criteria (de Queiroz, 1998). The criteria used in the ESC are (1) exclusivity, i.e. the monophyly of the collection of DNA sequences representing the group, (2) geographic isolation, (3) differentiation of characters other than the mtDNA sequences, and (4) degree of sequence differentiation. The Mexican and Central America rattlesnake lineage, as indicated by the mtDNA and AFLP evidence, is in accordance with these criteria. The South America taxa are not monophyletic even though there is a considerable amount of morphological variation. This indicates the South America forms, traditionally considered separate species (*C. vegrandis* and *C. unicolor;* Klauber 1972; Murphy *et al.* 2002), fail on the criteria above described and therefore are not species under the ESC.

Contrary to previous opinions about the systematics of the South American forms, (some authors claimed full species status recognition for some subspecies of *C. durissus*, e.g. Klauber 1972), the combined Cyt-b, ND4 and AFLP phylogenies analyzed in this study

reveal a unique evolutionary lineage with poor differentiation among the recognised forms, including *C. d. unicolor* and *C. d. vegrandis*.

The topology of the trees generated by the different methods suggest that at least four rattlesnake lineages evolved in Central America, and these correspond to the subspecies categories recognised, *C. totonacus*, *C. d. culminatus*, *C. d. tzabcan* and *C. simus*. However, the remarkable separation of *C. totonacus* and *C. d. tzabcan* from both the South American and Central American clades, and their position relative to *C. basiliscus* and *C. molossus*, strongly suggest that they belong to very different evolutionary lineages.

The relationship between the *C. durissus* complex, and *C. basiliscus* and *C. molossus* remains poorly resolved based on these phylogenies, indicating the need for further analysis with more samples from *C. molossus* and *C. basiliscus* and possibly other markers from nuclear regions. The distance, maximum likelihood, and parsimony methods grouped *C. totonacus* as a sister taxon of *C. basiliscus* and *C. molossus*, as indicated their shared lineage. This pattern is less clear in the AFLPs but could explain the previous difficulties of placing *C. totonacus* in *C. basiliscus* or *C. durissus* (Taylor 1951; Golay *et al.* 1993). A noteworthy finding in all the phylogenies is the relatively strong differentiation between the *C. molossus* populations, which all supposedly belonged to the same subspecies, *C. m. molossus*.

Recently Campbell & Lamar (2004) separated *Crotalus durissus* into three species based only on taxonomic rearrangements and historical evidence. They recognised the populations from Tamaulipas and adjoining parts of NE Mexico (formally *C.d.*

totonacus) as a full species, a finding also supported by the results of this study. The same authors divided the Central American and South American members of the complex into separate species as they considered the likely type locality of *C. durissus* to have been the Guyana region of South America, not Mexico, and they reject Smith and Taylor's (1966) restriction of the type locality to Jalapa, Veracruz, Mexico. As a consequence, all the South American forms of the complex are treated as subspecies of *C. durissus*, and the populations from coastal Guyana, Suriname and French Guyana, formerly known as *C. durissus dryinas,* become *C. durissus durissus*. The oldest available name for the Central American populations is *Crotalus simus* (Latreille 1801), so that all Central American populations are treated as subspecies of *C. simus*. For example, those formerly treated as *C. durissus durissus* are now treated as *C. simus simus*.

In the present study, the taxonomic validity of *C. simus* as a monophyletic group is newly questioned, because the mtDNA and AFLP phylogenies suggest that the Yucatan populations of the former *C. d. tzabcan* deserve full species. According to Campbell and Lamar (1989), the taxa *C. vegrandis* and *C. unicolor* should be treated as subspecies of *C. durissus*; this is consistent with their phylogenetic position within *C. durissus* as demonstrated by Wüster *et al.* (2002) and this study (Fig 5.1).

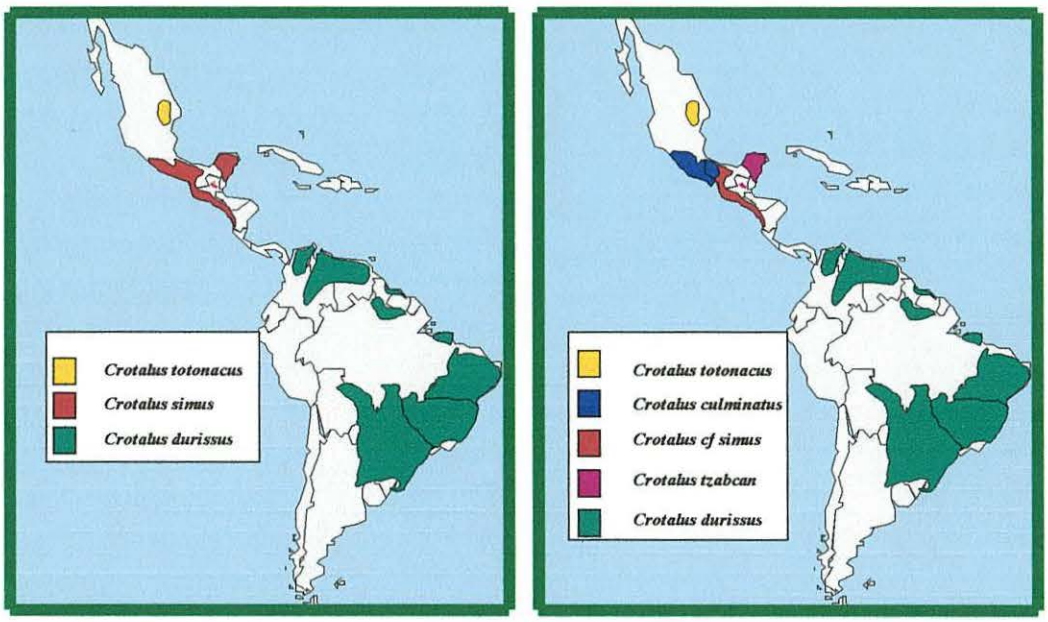

Fig 5.1. Proposed changes in the systematics of the *C. durissus* complex. Left, taxonomic changes proposed by Campbell and Lamar (2004). Right, proposed arrangements according to the results of this study.

5.6. The status of the *C. durissus* complex as a species group

Taxonomically, the genus *Crotalus* has been classified into several species groups according to morphological and molecular characters (reviewed by Murphy *et al.* 2002). Several authors have proposed four to eight species groups, and consistently the *C. durissus* group appears in these classifications (Gloyd 1940; Brattstrom 1964; Klauber 1972; Foote and MacMahon 1977; Murphy et. al 2002). Despite the recognition of the *C. durissus* group in the early stages of the rattlesnake classification (Gloyd 1940), the suggested group has varied substantially in terms of composition and number of species included. For instance Klauber (1972), using morphological, behavioural, and habitat classification approaches, included *C. basiliscus*, *C. cerates*, *C. durissus*, *C. enyo*, *C. horridus*, *C. molossus*, *C. unicolor* and *C. vegrandis*. Other authors included and excluded other species; however Klauber's opinion has been consistent with all morphological classifications.

Recently the use of molecular markers has challenged the classification of species groups and changed their composition. In the case of the *C. durissus* group, the most recent classification, based on sequences of five mtDNA genes (Murphy *et al.* 2002), recognized seven species: *C. durissus*, *C. vegrandis*, *C. unicolor*, *C. enyo*, *C. basiliscus*, *C. estebanensis*, and *C. molossus*. The phylogenetic analyses presented here indicate that the *C. durissus* group is more complex in composition than was previously supposed. The increased sampling effort, which considered most of the rattlesnake's geographic distribution, and improved molecular resolution, has improved the topology of phylogeny of the *C. durissus* complex.

The *C. durissus* group is integrated by at least 12 evolutionary lineages that deserve species status recognition (according to the ESC) and further studies may increase the number of species in the membership of the group. This study proposes a new arrangement and composition of the *C. durissus* group, consisting of the following evolutionary species: *C. durissus*, *C. totonacus*, *C. culminatus*, *C. tzabcan*, *C. molossus*, *C. basiliscus*, *C. estebanensis* and *C. enyo*. The Baja California rattlesnake *C. enyo* is included on the basis of morphological similarity to the *C. durissus* group (Klauber 1972; Brattstrom 1964) and molecular data that indicates that *C. enyo* is the sister taxon of the *C. durissus* clade (Murphy *et al.* 2002). On the other hand, this study suggests that *C. molossus* is composed of at least four evolutionary lineages, which is likely to increase the membership of the *C. durissus* group in future studies.

5.7. Remarks on venom variation

The finding that all *C. durissus* populations in South America are monophyletic and that the populations in Mexico and Central America have multiple, deep lineages, raises new questions regarding the evolution and variation in some venom protein compounds. It is very well known that some components of venom in *C. durissus* vary at the intraspecific level while others do not (Schenberg 1959; Jimenes-Porras 1964; Warrell *et al.* 1997; Daltry *et al.* 1997; Francischetti *et al.* 2000; Saravia *et al.* 2002; Rangel-Santos *et al.* 2004).

The causes of intraspecific variation are complex due the multi-loci nature of venom expression in snakes and its response to several factors (Fry and Wüster 2004; Warrell 2004). Among these factors are phylogenetic constraints, natural selection by prey type,

and geographic distance (Daltry *et al.* 1997; Creer *et al.* 2003; Fry *et al.* 2003; Warrell 2004).

At the present, there is a need for systematic studies on venom variation in *C. durissus*. Many studies of other vipers have analyzed geographical variation in detail and elucidated some of the causal factors of the variation (e.g. Daltry *et al.* 1996; Creer *et al.* 2003). These studies provide clues about the factors that may affect *C. durissus* venom variation. However there is not a single study that explores the evolutionary factors producing strong geographic differentiation in the chemical composition of *C. durissus* venom.

Many papers suggest that venom variation in the Neotropical rattlesnakes may be influenced by several causal factors, including geographic location (Schenberg 1959; Jimenes-Porras 1964; Warrell *et al.* 1997; Daltry *et al.* 1996; Francischetti *et al.* 1997; Francischetti *et al.* 2000; Saravia *et al.* 2002), ontogenetic variation (Theakston and Reid 1979; Gutierrez *et al.* 1990; 1991; Minton and Weinstein 1986; Saravia *et al.* 2002), and sexual variation (Gutierrez *et al.* 1990; 1991). We now have a phylogenetic background that allows for testing evolutionary hypotheses that may explain the patterns of venom variation. However, more data on venom variation is still needed.

In the past, standard electrophoresis and characterization of the venom protein by cDNA cloning made it difficult to analyze a large number of samples (Creer *et al.* 2003). The development of techniques such as matrix-assisted laser de-sorption ionization time-of-flight mass spectrometry offer an accurate and efficient alternative to past methods. This technique directly measures the molecular weight of proteins from complex mixtures,

allowing for the analysis of large numbers of samples (Mirgorodskaya *et al.* 2000). This technique has already been used to identify the masses of key components in spider venom and in the viper *Trimeresurus stejnegeri* (Escoubas *et al.* 1999; Mirgorodskaya *et al.* 2000; Creer *et al.* 2003).

5.8. Implications for Conservation

Rattlesnakes in general, and the *C. durissus* complex in particular, are threatened throughout their range distribution. These snakes have faced a long-term conflict with human activities and settlements (reviewed in Campbell and Lamar 2004). Despite protection efforts, many people are still prejudiced against rattlesnakes. Rattlesnakes are still subject to constant persecution and killing. Fortunately in Mexico, the centre of rattlesnake diversification, most conventional rattlesnake taxa are protected under government law (SEMARNAP ECO-089 2001), but they still suffer from habitat destruction and illegal pet trade (Flores *et al.* 1998; CONABIO 2002).

There do exist international efforts to protect rattlesnakes, but some of the most common lists of endangered species do not include many rare species of pitvipers (e.g. IUCN 2003; CITES 2001). This suggests that compared to other reptiles, conservation efforts are biased against rattlesnakes.

Some populations of the *C. durissus* complex have been the object of conservation interest based on their rareness and the human impact on their populations. The only rattlesnakes that appear on lists of endangered species are *C. d. unicolor*, *C. d. vegrandis,* and the populations of *C. durissus* in Honduras (IUCN 2002; CITES 2001). In Mexico,

the members of the species group *C. durissus*, *C. molossus*, *C. basiliscus*, and *C. enyo* are protected (SEMARNAP ECO-089 2001). Due the taxonomic changes that have been proposed by Campbell and Lamar (2004) and this study, these lists should be up-dated.

The systematics and recognition of old and delimited lineages of the *C. durissus* complex offer the opportunity to discuss the importance of the Evolutionary Significant Units (ESU) in the conservation (Ryder 1986; Waples 1991; Moritz 1994; Dimmick *et al.* 1999). The ESU relies on the Evolutionary Species Concept, and has profound implications in the selection of priority taxa for conservation. ESUs have been defined as lineages that are "reciprocally monophyletic for mtDNA genes and show significant divergence of allele frequencies at nuclear loci" (Moritz 1994). This concept has implications in conservation decisions that give protection priority to those lineages that show clear delimitation and are unique or rare (Ryder 1986; Waples 1991; Moritz 1994; Dimmick *et al.* 1999; Avise 2002).

The Mexican and Central America rattlesnake lineages are examples of ESU, as their monophyly is evident in both mtDNA and AFLP analyses (which includes several anonymous nuclear loci). *C. totonacus* and *C. tzabcan* should be a conservation priority because their habitats are suffering fast degradation (Campbell and Lamar 2004).

Conservation programs for South American populations, such as *C. d. unicolor*, should be undertaken carefully. Currently, an effort is being made in order to protect *C. d. unicolor* (e.g. Reinert *et al.* 2005). Given that the results of this study suggest the population currently known as *C. d. unicolor* is not a separate species, it is important that conservationists be clear that they are protecting a unique population, but not an

endangered species. In contrast to *C. d. unicolor*, which is just one population, very little is being done to conserve the entire species of the ancient, endemic, and rare *C. totonacus*. Future conservation programs must consider the phylogeography of the *C. durissus* complex, when seeking to protect the rare lineages of Neotropical rattlesnakes.

REFERENCES

Allender CJ, Seehausen O, Knight ME, Turner GF, Maclean N (2003) Divergent selection during speciation of Lake Malawi cichlid fish inferred from parallel radiations in nuptial coloration. *Proc. Natl. Acad. Sci. USA*.102, 5448-5453

Anderson, JB, Kohn LM (1998) Genotyping, gene genealogies and genomics bring fungal population genetics above ground. *TREE* 13:444-449.

Anderson SG, Gutierrez JM, Ownby CL (1993) Comparison of the immunogenicity and antigenic composition of ten Central American snake venoms. *Toxicon* 31(8): 1051-1059.

Ankel-Simons F, Cummins JM (1996) Misconceptions about mitochondria and mammalian fertilization: implications for theories on human evolution. *Proc Natl Acad Sci USA* 93: 13859-13863.

Apostol BL, Black WC, Reiter P, Miller BR (1994) Use of randomly amplified polymorphic DNA amplified by polymerase chain reaction markers to estimate the number of *Aedes aegypti* families at oviposition sites in San Juan, Puerto Rico . *American Journal of Tropical Medicine and Hygiene* 51: 89-97.

Apostol BL, Black WC, Reiter P, Miller BR (1996) Population genetics with RAPD-PCR markers: the breeding structure of *Aedes aegypti* in Puerto Rico . *Heredity* (Edinburgh), 76: 325-334.

Aquadro CF, Greenberg BD (1983) Human mitochondrial DNA variation and evolution: analysis of nucleotide sequences from seven individuals. *Genetics* 103: 287-312.

Arbogast BS, Slowinski JB (1998) Pleistocene speciation and the mitochondrial DNA clock. *Science* 282: 1955

Arevalo E, Davies SK, Sites JW (1994) Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of the *Sceloporus grammicus* complex (Phrynosomatidae) in Central Mexico. *Syst. Biol.* 43:387-418.

Aris-Brosou S, Yang Z (2002) Effects of models of rate evolution on estimation of divergence dates with special reference to the metazoan 18S ribosomal RNA phylogeny. *Syst. Biol.* 51: 703–714.

Armstrong B L, Murphy J B (1979) The natural history of Mexican rattlesnakes. *Univ. Kansas Museum Natural History Special Publication.* No. 5. vii.

Ashton KG, A de Queiroz (2001) Molecular Systematics of the western rattlesnake, *Crotalus viridis* (Viperidae), with comments on the utility of the D-Loop in phylogenetic studies of snakes. *Molecular Phylogenetics and Evolution* 21:176-189.

Avise JC (2002) Genetics in the Wild. *Smithsonian Institute* (240 pp).

Avise JC (2000) Phylogeography. The history and formation of species. *Harvard University Press*, Cambridge, MA.

Avise JC, Alisauskas RT, Nelson WS and Ankney CD (1992) Matriarchal population structure in an avian species with female natal philopatry. *Evolution* 46: 1084-1096.

Avise JC , Walker D (1998) Pleistocene phylogeographic effects on avian populations and the speciation process. *Proc R Soc Lond* B 265: 457-463.

Avise JC (1994) Molecular Markers, Natural History and Evolution. *Chapman and Hall*, New York, NY.

Avise JC, Walker D, Johns GC (1998) Speciation durations and Pleistocene effects on vertebrate phylogeography. *Proc R Soc Lond* B 265: 1707-1712.

Avise JC, Wollenberg K (1997) Phylogenetics and the origin of species. *Proc. Natl. Acad. Sci.* USA 94: 7748–7755.

Avise JC, Reeb CA, Saunders NC (1987) Geographic population structure and species differences in mitochondrial DNA of mouthbrooding catfishes (Aridae) and demersal spawing toadfishes (Batrachoidae). *Evolution* 41: 991–1002.

Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Neigel JE, Reeb CA, Saunders NC (1987) Intraspecific phylogeography: The mitochondrial DNA bridge between population genetics and systematics. *Ann Rev Ecol Syst* 18: 489-522

Avise JC, Giblin-Davidson C, Laerm J, Patton JC, Lansman RA (1979) Mitochondrial DNA clones and matriarchal phylogeny within and among geographic populations of the pocket gopher, *Geomys pinetis*. *Proceedings of the National Academy of Sciences* USA, 7: 6694-6698.

Awadalla P, Eyre-Walker A, Smith JM (1999) Linkage disequilibrium and recombination in hominid mitochondrial DNA. *Science* 286: 2524-2525.

Baayen, R P, O'Donnell K, Bonants PJM, Cigelnik E, Kroon L, Roebroeck EJA, Waalwijk C (2000) Gene genealogies and AFLP analyses in the *Fusarium oxysporum* complex identify monophyletic and nonmonophyletic formae speciales causing wilt and rot disease. *Phytopathology* 90:891–900.

Backeljau T, De Bruyn L, De Wolf H, Jordaens K, Van Dongen S, Verhagen R, Winnepenninckx B (1995) Random amplified polymorphic DNA (RAPD) and parsimony methods. *Cladistics* 11:119–130.

Bakkeren, G, Kronstad JW, Levesque CA (2000) Comparison of AFLP fingerprints and ITS sequences as phylogenetic markers in Ustilaginomycetes. *Mycologia* 92:510–521.

Baldwin JD, Bass AL, Bowen BW, Clark WH (1998) Molecular phylogeny and biogeography of the marine shrimp *Penaeus*. *Molecular Phylogenetics and Evolution*, 10: 399-407.

Barker JH, Matthews M, Arnold GM, Edwards KI, Ahman KI, Larsson S, Karp A (1999) Characterization of genetic diversity in potential biomass willows (*Salix* spp.) by RAPD and AFLP analyses. *Genome* 42:173–183.

Barrio A, Brazil OV (1951) Neuromuscular action of *Crotalus terrificus terrificus* poisons. *Acta Physiol. Latinoamericana* 1: 291-308.

Bartlein PJ, Prentice IC (1989) Orbital variations, climate and paleoecology. *Trends in Ecology and Evolution*, 4: 195–199.

Bartlett AS, Barghoorn ES (1973) Phytogeographic history of the Isthmus of Panama during the past 12 000 years, a history of vegetation, climate and sea-level change, In: *Vegetation and Vegetational History of Northern Latin America* (ed. Graham A), pp. 203-299. Elsevier, New York

Becker J, Vos P, Kuiper M, Salamini F, Heun M (1995) Combined mapping of AFLP and RFLP markers in barley. *Molecular and General Genetics*. 249: 65-73.

Belt P J, Malhotra A, Thorpe R S, Warrell D A, Wüster W (1997) Russell's viper in Indonesia: snakebite and systematics. In*: Venomous Snakes, Ecology and Evolution*, eds. RS Thorpe, W Wüster, and A Malhotra. Symp. zool. Soc. Lond. 70: 219-234.

Bermingham E, Avise JC (1986) Molecular zoogeography of freshwater fishes in the southeastern United States. *Genetics*, 7: 939-965.

Bermingham E, Lessios HA (1993) Rate variation of proteins and mitochondrial DNA evolution as revealed by sea urchins separated by the Isthmus of Panamá. *Proceedings of the National Academy of Sciences of the USA*, 90: 2734-2738.

Bermingham E, McCafferty S, Martin A (1997) Fish biogeography and molecular clocks: perspectives from the Panamanian Isthmus. In: *Molecular Systematics of Fishes* (eds Kocher T, Stepien C), pp. 113-128. Academic Press, San Diego, California.

Bermingham E, Rohwer S, Freeman S, Wood C (1992) Vicariance biogeography in the Pleistocene and speciation in North American wood warblers, a test of Mengels model. *Proceedings of the National Academy of Sciences USA*, 7: 6624-6628.

Bermingham E, Seutin G, Ricklefs RE (1996) Regional approaches to conservation biology: RFLPs, DNA sequences, and Caribbean birds. In: *Molecular Genetic Approaches in Conservation* (eds Smith TB, Wayne RK), pp. 104-124. Oxford University Press, NY.

Bermingham E, Martin A P (1998) Comparative mtDNA phylogeography of neotropical freshwater fishes: testing shared history to infer the evolutionary landscape of lower Central America. *Molecular Ecology* 7: 499-517.

Bernatchez L, Guyomard R, Bonhomme F (1992) DNA sequence variation of the mitochondrial control region among geographically and morphologically remote European brown trout *Salmo trutta* populations. *Molecular Ecology* 1: 161-173.

Blears, M J, De Grandis SA, Lee H, Trevors JT (1998) Amplified fragment length polymorphism (AFLP): a review of the procedure and its applications. *J. Industrial Microbiol Biotech.* 21:99-114.

Bolaños R, Marin O, Mora-Medina E, Alfaro E A (1981) El accidente ofidico por cascabela (*Crotalus durissus*) en Costa Rica. *Act. Med. Cost.* 24 (3): 211-214

Bonatto SL, Salzano FM (1997) A single and early migration for the peopling of the Americas supported by mitochondrial DNA sequence data. *Proceedings of the National Academy of Sciences of the USA.* 94: 1866-1871.

Boom R, Sol CJA, Salimans MMM (1990) Rapid and simple method for purification of nucleic acids. *Journal of Clinical Microbiology*. 28: 495-503.

Brattstrom B H (1964) Evolution of the pitvipers. *Transactions of the San Diego Society of Natural History* 13:185–268.

Brede EG, Thorpe RS, Arntzen JW, Langton TES (2000) A morphometric study of a hybrid newt population (*Triturus cristatus/T.carnifex*): Beam Brook Nurseries, Surrey, UK. *Biological Journal of the Linnean Society*. 70: 685-695.

Bremer K (1994) Branch support and tree stability. *Cladistics* 10: 245-304.

Bremer K (1992) Ancestral areas: a cladistic reinterpretation of the center of origin concept. *Systematic Biology* 41:436–445.

Brown GG, Gadaleta G, Pepe G, Saccone C, Sbisá E (1986) Structural conservation and variation in the D-loop-containing region of vertebrate mitochondrial DNA. *J Mol Biol* 192: 503-511.

Brown WM, George M Jr., Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. *Proc Acad Natl Sci USA* 76: 1967-1971.

Brown WM, Prager EM, Wang A, Wilson AC (1982) Mitochondrial DNA sequences of primates: Tempo and mode of evolution. *J Mol Evol* 18: 225-239.

Brundin LZ (1988) Phylogenetic biogeography. In: *Analytical Biogeography: An Integrated Approach to the Study of Animal and Plant Distributions* (eds Myers AA, Giller PS), pp. 343-370. Chapman and Hall, London.

Buffone G J (1985) Isolation of DNA from biological specimens without extraction with phenol. *Clin. Chem*. 31:164-165

Buntjer J B , Otsen M, Nijman I J, Kuiper M T, Lenstra J A (2002) Phylogeny of bovine species based on AFLP fingerprinting. *Heredity* 88: 46-51, 2002.

Bussing WA (1976) Geographic distribution of the San Juan ichthyofauna of Central America with remarks on its origin and ecology. In: *Investigations of the ichthyofauna of Nicaraguan lakes* (ed. Thorson TB), pp. 157-175. University of Nebraska, Lincoln, Nebraska.

Bussing WA (1985a) Patterns of distribution of the Central American ichthyofauna. In: *The Great American Biotic Interchange* (eds Stehli FG, Webb SD), pp. 453-473. Plenum Press, NY.

Bussing WA (1985b) *Roeboides ilseae*, n. sp., a new scale-eating characid fish from Costa Rica. *Revista Biologica Tropical*, 7, 45-50.

Caetano-Anolles G, Gresshoff PM (1994) DNA amplification fingerprinting of plant genomes. *Methods in Mol. Cell. Biol.* 5:62-70.

Campbell JA , Lamar WW (1989) The venomous reptiles of Latin America. *Comstock Pub. Ass.*

Campbell JA , Lamar WW (2004) The Venomous Reptiles of the Western Hemisphere. *Comstock, Ithaca and London.*

Cann RL, Brown WM, Wilson AC (1984) Polymorphic sites and the mechanism of evolution in human mitochondrial DNA. *Genetics* 106:479-499.

Carlin B P, Louis T A (2000) Bayes and Empirical Bayes Methods for Data Analysis (*Chapman and Hall/CRC, Boca Raton*).

Carr SM, Marshall HD (1991) A direct approach to the measurement of genetic variation in fish populations: applications of the polymerase chain reaction to studies of Atlantic cod, *Gadus morhua* L.. *J. Fish Biol.* 39 (Suppl. A):101-107.

Cavalli-Sforza LL, Menozzi P, Piazza A (1994) The history and geography of human genes. *Princeton University Press.*

Cavilli-Sforza LL, Edwards A W F (1967) Phylogenetic analysis: models and estimation procedures. *Evolution* 21: 550-570.

Chang D, Clayton D (1985) Priming of human mitochondrial DNA replication occurs at the light-strand promoter. *Proc Natl Acad Sci USA* 82: 351-355.

Chernoff B (1982) Character variation among populations and the analysis of biogeography. *American Zoologist.* 7: 425-439.

Chippaux J P , Williams V, White J (1991) Snake venom variability: methods of study, results and interpretation. *Toxicon.* 29 (11) :1279-1303.

CITES (2001) Checklist of CITES Species / Lista de la Especies CITES / Liste des Especies CITES (English/French) 335pp

Coates AG, Obando JA (1996) The geologic evolution of the Central American isthmus. In: *Evolution and Environment in Tropical America* (eds Jackson J, Budd AF, Coates AG), pp. 21-56. Chicago University Press, Chicago, Ill.

Coates AG, Aubry M-P, Berggren WA, Collins LS, Kunk M (2003) Early Neogene history of the Central American arc from Bocas del Toro, western Panamá. *Geological Society of America Bulletin.* 115: 271-287.

Coates AG, Jackson JBC, Collins LS (1992) Closure of the Isthmus of Panama, the near-shore marine record of Costa Rica and western Panama. *Bulletin of the Geological Society of America.* 7: 814-828.

Colinvaux PA (1996) Quaternary environmental history and forest diversity in the Neotropics. In Jackson JB.C, Budd A F, and Coates AG (Eds). *Evolution and Environment in Tropical America.* The University of Chicago Press. 425 pp.

Colinvaux PA, De Oliveira PE, Bush MB (2000) Amazonian and Neotropical plant communities on glacial timescales: the failure of the aridity and refuge hypotheses. *Quaternary Science Reviews*. 19: 141–169.

Colinvaux PA, De Oliveira PE, Moreno JE, Miller MC, Bush MB (1996) A long pollen record from lowland Amazonia: forest and cooling in glacial times. *Science* 274: 85–88.

Colinvaux PA, Irion G, Räsänen ME, Bush MB, Nunes de Mello JAS (2001) A paradigm to be discarded: geological and paleoecological data falsify the Haffer and Prance refuge hypothesis of Amazonian speciation. *Amazoniana*,16: 609–646.

Collins LS (1996) Environmental changes in Caribbean shallow waters relative to the closing tropical American seaway. In: *Evolution and Environment in Tropical America* (eds Jackson JBC, Budd AF, Coates AG), pp. 130-167. Chicago University Press, Chicago.

CONABIO (2002) Estrategia Nacional sobre biodiversidad de México. *Semarnap, Conabio*, México

Cornell HV, Lawton JH (1992) Species interactions, local and regional processes, and limits to the richness of ecological communitites, a theoretical perspective. *Journal of Animal Ecology*, 7: 1-12.

Creer S, Malhotra A, Thorpe RS (2003) Assessing the phylogenetic utility of four mitochondrial genes and a nuclear intron in the Asian pitviper genus *Trimeresurus*: separate, simultaneous, and conditional data combination analyses. *Mol Biol Evol* 20: 1240-1251.

Creer S, Malhotra A, Thorpe RS, Stocklin R, Favreau P, Chou W-H (2003) Genetic and ecological correlates of intraspecific variation in pitviper venom composition detected using matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) and isoelectric focusing. *J Mol Evol* 56: 317-329.

Creer S, Malhotra A, Thorpe RS, Chou W-H (2001) Multiple causation of phylogeographical pattern as revealed by nested clade analysis of the bamboo viper (*Trimeresurus stejnegeri*) within Taiwan. *Molecular Ecology* 10: 1967–1981.

Creer S, Thorpe R S, Malhotra A, Chou W-H, Stenson AG (2004) The utility of AFLPs for supporting mitochondrial DNA phylogeographical analyses in the Taiwanese bamboo viper, *Trimeresurus stejnegeri. J. Evol. Biol.* 17: 100-107

Croizat L, Nelson G, Rosen DE (1974) Centers of origin and related concepts. *Sytematic Zoology*, 7, 265-287.

Cronin TM, Dowsett HF (1996) Biotic and oceanographic response to the Pliocene closing of the Central American Isthmus. In: *Evolution and Environment in Tropical America* (eds Jackson J, Budd AF, Coates AG), pp. 76-104. Chicago University Press, Chicago, Ill.

Crother B I (2001) Scientific and standard English names of amphibians and reptiles of North America north of Mexico, with comments regarding confidence in our understanding. *Committee of Standard English and Scientific Names, Society for the Study of Amphibians and Reptiles, Herpetological Circular 29* (as supplemented in *Herp. Review* 32: 152-153.

Cupo P, Azevedo-Marques M M, Hering S E (1988) Clinical and laboratory features of South American rattlesnake (*Crotalus durissus terrificus*) envenomation in children. *Trans. Roy. Soc. Trop. Med. Hyg.* 82: 924-929.

Daltry J, Ponnudurai G, Shin CK, Tan NH, Thorpe RS, and Wüster W (1996) Electrophoretic profiles and biological activities: Intraspecific variation in the venom of the Malayan pit viper (*Callosellasma rhodostoma*). *Toxicon.* 34:67-79.

Daltry J C, Wuster W (2002) A new species of wolf snake (Serpentes: Colubridae: *Lycodon*) from the Cardamom Mountains, southwestern Cambodia. *Herpetologica* 58(4): 498-504.

Daltry JC, Wuster W, Thorpe RS (1997) The role of ecology in determining venom variation in the Malayan pitviper, *Calloselasma rhodostoma*. In: *Venomous Snakes, Ecology, Evolution and Snakebite*. Eds. RS Thorpe, W Wuster, and A Malhotra. Symp. zool. Soc. Lond. 70: 155-171.

Daltry JC, Wüster W, Thorpe R S (1996) Diet and snake venom evolution. *Nature*. 379: 537-540.

Davies N, Villablanca FX, Roderick GK (1999) Determining the source of individuals: multilocus genotyping in nonequilibrium population genetics. *TREE* 14:17-21.

de Knijff P, Denkers F, van Swelm ND, Kuiper M (2001) Genetic affinities within the herring gull *Larus argentatus* assemblage revealed by AFLP genotyping. *Journal of Molecular Evolution* 52: 85-93.

de Queiroz K (1998) The general lineage concept of species, species criteria, and the process of speciation: A conceptual unification and terminological recommendations. In: D J Howard and S H Berlocher (eds.), *Endless Forms: Species and Speciation*, pp. 57–75. Oxford University Press, Oxford, England.

DeSalle R, Freedman T, Prager EM, Wilson AC (1987) Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila. J Mol Evol* 26: 157-164.

Dimmick W W , Ghedotti M J, Grose M J, Maglia A M, Meinhardt D J, Pennock D S (1999) The importance of systematic biology in defining units of conservation: *Conservation Biology*, Vol 13, p. 653–660.

Douglas M E, Douglas MR, Schuett GW, Porras LW, Holycross AT (2002) Phylogeography of the western rattlesnake (*Crotalus viridis*) complex, with emphasis on the Colorado Plateau, pp. 11-50 in GW Schuett, M Höggren, ME Douglas and H W Greene (eds.), *Biology of the Vipers*. Eagle Mountain Publ., Eagle Mountain, UT.

Duellman W E (1978) The South American herpetofauna: Its origin, evolution, and dispersal. *Mus. Nat. Hist. Univ. Kansas* Monogr. 7:1-485

Eberhard JR, Bermingham E (2004) Phylogeny and biogeography of the *Amazona ochrocephala* (Aves: Psittacidae) complex. *Auk*, 121: 318–332.

Edwards SV, Wilson AC (1990) Phylogenetically informative length polymorphism and sequence variability in mitochondrial DNA of Australian songbirds (*Pomatostomus*). *Genetics* 126: 695-711.

Efron B (1979) Bootstrap methods: another look at the jackknife. *Annals Stat*. 7: 1–26.

Efron B, Halloran E, Holmes S (1996) Bootstrap confidence levels for phylogenetic trees. *Proc. Natl Acad. Sci. USA* 93: 13429–13434.

Eizirik E, Bonatto SL, Johnson WE (1998) Phylogeographic patterns and mitochondrial DNA control region evolution in two Neotropical cats (Mammalia, Felidae). *Journal of Molecular Evolution* 47: 613 624.

Eriksson T (2001) AutoDecay ver. 5.0 (program distributed by the author). *Bergius Foundation, Royal Swedish Academy of Sciences, Stockholm.*

Escoubas JM, Briant L, Montagnani C, Hez S, Devaux C, Roch P (1999) Oyster IKK-like protein shares structural and functional properties with its mammalian homologues. *FEBS Lett*. 453:293-8.

Estes R, Báez A (1985) Herpetofauna of North and South America during the late Cretaceous and Cenozoic: evidence for interchange? In: *The Great American Biotic Interchange* (eds Stehli FG, Webb SD), pp. 139–197. Plenum Press, New York.

Excoffier L, Smouse PE (1994) Using allele frequencies and geographic subdivision to reconstruct gene trees within a species: molecular variance parsimony. *Genetics*, 136: 343-359.

Excoffier L, Smouse P, Quattro J (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, 131: 479-491.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131: 479-491.

Eyre-Walker A, Smith NH, Maynard Smith J (1999) How clonal are human mitochondria? *Proc R Soc Lond* B 266: 477-483.

Farris JD, Källersjö M, Kluge AG, Bult C (1994) Testing significance of incongruence. *Cladistics*, 10: 315–319.

Felsenstein J (1981) Evolutionary trees from DNA sequences: A maximum likelihood approach. *J. Mol. Evol.* 17: 368-376.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 39: 783-791.

Felsenstein J (1992) Estimating effective population size from samples of sequences: inefficiency of pairwise and segregating sites as compared to phylogenetic estimates. *Genetical Research*, 7: 139-147.

Felsenstein J (1993) PHYLIP 3.5 . *Washington University*, Seattle.

Felsenstein, J, Kishino H (1993) Is there something wrong with the bootstrap on phylogenies? A reply to Hillis and Bull. *Syst. Biol.* 42: 193–200.

Felsenstein J (1996) Inferring phylogenies from protein sequences by parsimony, distance, and likelihood methods. *Meth. in Enzym.* 266: 419-427.

Ferrari L, Pasquarè G, Venegas-Salgado S, Romero-Rios F (1999) Geology of the western Mexican Volcanic Belt and adjacent Sierra Madre Occidental and Jalisco block,

in Delgado-Granados, H, Aguirre-Díaz, G, and Stock, J M, eds., *Cenozoic Tectonics and Volcanism of Mexico*: Boulder, Colorado, *Geological Society of America* Special Paper 334.

Ferris SD, Sage RD, Prager EM, Ritte U, Wilson AC (1983) Mitochondrial evolution in mice. *Genetics* 105: 681-721.

Fisher RA (1930) The genetical theory of natural selection. *Clarendon Press*, Oxford.

Flores O (1993) Herpetofauna of México: distribution and endemism. In: TP Ramamoorthy, R Bye, A Lot y J Fa (eds.), *Biological Diversity of Mexico. Orígins and distribution. Oxford University Press*

Foote R, MacMahon J (1977). Electrophoretic studies of rattlesnake (*Crotalus* and *Sistrurus*) venom: Taxonomic implications. *Comp. Biochem. Physiol.* 57B: 235–241.

Francischetti IM, Gombarovits ME, Valenzuela JG, Carlini CR, Guimaraes JA (2000) Intraspecific variation in the venoms of the South American rattlesnake (*Crotalus durissus terrificus*). Comp *Biochem Physiol and Toxicol Pharmacol.* 2000 Aug; 127 (1): 23-36.

Francischetti I M, Saliou B, Leduc M, Carlini C R, Hatmi M, Randon J, Faili A, Bon C (1997) Convulxin, a potent platelet-aggregating protein from *Crotalus durissus terrifricus* venom, specifically binds to platelets. *Toxicon* 35: 1217–1228.

Friesen H, Hepworth SR, Segall J (1997) An Ssn6-Tup1-dependent negative regulatory element controls sporulation-specific expression of DIT1 and DIT2 in Saccharomyces cerevisiae. *Mol. Cell. Biol.* 17:123-134.

Fry BG, Wüster W (2004) Assembling an arsenal: origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences. *Molecular Biology and Evolution* 21(5): 870–883.

Fry B G, Wickramaratna JC, Hodgson WC, Alewood PF, Kini RM, Ho H, Wüster W (2002) Electrospray liquid chromatography/mass spectrometry fingerprinting of *Acanthophis* (death adder) venoms: taxonomic and toxinological implications. *Rapid Communications in Mass Spectrometry* 16: 600-608.

Fry BG, Lumsden NG, Wüster W, Wickramaratna JC, Hodgson WC, Kini RM (2003) Isolation of a neurotoxin (alpha-colubritoxin) from a 'non-venomous' colubrid: evidence for early origin of venom in snakes. *Journal of Molecular Evolution*, 57(4): 446-452.

Fry B G, Wüster W, Kini RM, Brusic V, Khan A, Venkataraman D, Rooney AP (2003) Molecular evolution and phylogeny of snake venom three finger toxins. *Journal of Molecular Evolution* 57(1): 110-129

Fu Y-X (1994) Estimating effective population size or mutation rate using the frequencies of mutations of various classes in a sample of DNA sequences. *Genetics*, 7: 1375-1386.

Furtado MFD, Santos MC, Kamiguti A (2003) Age-related biological activity of South American rattlesnake (*Crotalus durissus terrificus*) venom. *Journal Venom. Anim. Toxins incl. Trop. Dis.* Botucatu-SP., 9 (2): 186-201.

Ghivizzani SC, Madsen CS, Nelen MR, Ammini CV, Hauswirth WW (1994) In organello footprint analysis of human mitochondrial DNA: Human mitochondrial transcription factor A interactions at the origin of replication. *Mol Cell Biol* 14: 7717-7730.

Giannasi N, Thorpe RS, Malhotra A (2001) The use of amplified fragment length polymorphism in determining species trees at fine taxonomic levels: analysis of a medically important snake, *Trimeresurus albolabris*. *Molecular Ecology*, 10: 419-426.

Gillespie JH (1991) The causes of molecular evolution. *Oxford Univ. Press*, Oxford

Glenn TC, Glenn SJ (1994) Rapid elution of DNA from agarose gels using polyester plug spin inserts (PEPSIs). *Trends in Genet* 10: 344.

Gloyd H K (1940) The Rattlesnakes, Genera *Sistrurus* and *Crotalus*: A Study in Zoogeography and Evolution. Special Publication No. 4. *Chicago Academy of Sciences*, Chicago.

Golay P, Smith HM, Broadley DG, Dixon JR, Mc Carthy C, Rage JC, Schatti E, Toriba M (1993) Endoglyphs and other major venomous snakes of the world. A checklist. *Azemiops S.A.*, Herpetological data center.478 pp.

Goldman, N, Anderson, J P and Rodrigo, A G (2000) Likelihood based tests of topologies in phylogenetics. *Syst. Biol.* 49: 652–670.

Goldstein DB, Roemer GW, Smith DA (1999) The use of microsatellite variation to infer population structure and demographic history in a natural model system. *Genetics*, 151: 797-801.

Golik A (1968) History of the holocene transgression in the Gulf of Panama. *Journal of Geology*, 7: 497-507.

Goudet J (1995) FSTAT, version 1.2: a computer program to calculate F-statistics. *Journal of Heredity*, 86: 485-486.

Goudet J (2002). Fstat Vers. 2.9.3.2. *Inst. Of Ecology*, Swtzerland.

Graham MA (1993) Historical Factors and Biological Diversity in Mexico. In: TP Ramamoorthy, R Bye, A Lot y J Fa (eds.), *Biological Diversity of Mexico. Orígins and distribution. Oxford University Press*

Graham M A (1987) Tropical American Tertiary floras and paleoenvironments: Mexico, Costa Rica, and Panama. *American Journal of Botany* 74:1516–1531.

Graham MA (1989) Late Tertiary paleoaltitudes and vegetational zonation in Mexico and Central America. *Acta Botanica Neerlandica* 38:417–424.

Grande L (1985) The use of paleontology in systematics and biogeography and a time control refinement for historical biogeography. *Paleobiology*, 7: 234-243.

Gray MW (1989) Origin and evolution of mitochondrial DNA. *Annu Rev Cell Biol* 5: 25-50.

Greenberg BD, Newbold JE, Sugino A (1983) Intraspecific nucleotide variability surrounding the origin of replication in human mitochondrial DNA. *Gene* 31: 33-49.

Greene HW (1997) Snakes: The Evolution of Mystery in Nature. *Univ. of California Press*, Berkeley.

Gregory-Wodzicki KM (2000) Uplift history of the central and northern Andes: a review. *Geological Society of America Bulletin*, 112: 1091–1105.

Griffiths CS (1997) Correlation of functional domains and rates of nucleotide substitution in cytochrome b . *Mol Phyl Evol* 7: 352-365.

Griffiths R, Orr K (1999) The use of amplified fragment length polymorphism (AFLP) in the isolation of sex-specific markers. *Mol. Ecol.* 8:671-674.

Griffiths R, Orr KJ, Adam A, Barber I (2000) DNA sex identification in the three-spined stickleback. *J. Fish Biology* 57: 1331-1334.

Guo S, Thompson E (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics*, 48: 361-372.

Gutberlet Jr RL (1998) The phylogenetic position of the Mexican black-tailed pitviper (Squamata: Viperidae: Crotalinae). *Herpetologica* 54: 184-206.

Gutierrez JM, Chaves F, Bolazos R (1980). Estudio comparativo de venenos de ejemplares recien nacidos y adultos de *Bothrops asper*. *Revta Biol. trop.* 28: 341-353.

Gutierrez JM, dos Santos MC, Furtado MF, Rojas G (1991). Biochemical and pharmacological similarities between the venoms of newborn *Crotalus durissus durissus* and adult *Crotalus durissus terrificus rattlesnakes*. *Toxicon* 10: 1273-1277.

Gutiérrez JM, Rojas G, Lomonte B, Gené JA, Chaves F, Alvarado J and Rojas E (1990) Standardization of assays for testing the neutralizing ability of antivenoms. *Toxicon* 28: 1127-1129.

Gyllesten UB, Wharton D, Josefsson A, Wilson AC (1991) Paternal inheritance of mitochondrial DNA in mice. *Nature* 352: 255-257.

Hackett SJ (1996) Molecular phylogenetics and biogeography of tanagers in the genus *Ramphocelus* (Aves). *Mol Phyl Evol* 5: 368-382.

Haffer J, Prance GT (2001) Climatic forcing of evolution in Amazonia during the Cenozoic: on the refuge theory of biotic differentiation. *Amazoniana*, 16: 579–607

Haffer J (1969) Speciation in Amazonian forest birds. *Science* 165: 131-137.

Haffer J (1997) Alternative models of vertebrate speciation in Amazonia: an overview. *Biodiversity and Conservation* 6: 451-476.

Hagelberg E, Goldman N, Lió P, Whelan S, Sciefenhövel W, Clegg JB, Bowden DK (1999) Evidence for mitochondrial recombination in a human population of island Melanesia. *Proc R Soc Lond* B 266: 485-492.

Haig SM, Rhymer JM, Heckel DG (1994) Population differentiation in randomly amplified polymorphic DNA of red-cockaded woodpeckers *Picoides borealis*. *Molecular Ecology*, 3: 581 595.

Haldane JBS (1932) The causes of evolution. *Longmans and Green*, London.

Hall BG (2001) Phylogenetic Trees Made Easy. A how-to manual for molecular biologist. *Sinauer Associates*, Sunderland, Mass.USA

Hall BG (2004) Phylogenetic Trees Made Easy: A How-To Manual for Molecular Biologists, 2nd edition. *Sinauer Associates*, Sunderland, Mass.USA

Han TH, van Eck HJ, De Jeu MJ, Jacobsen E (1999) Optimization of AFLP fingerprinting of organisms with a large-sized genome: a study on *Alstroemeria* spp. *Theor. Appl. Genet.* 98:465-471.

Hasegawa M, Kishino H, Yano T (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, 7: 160-174

Hasekawa M, Kishino H, Yano T (1985) Dating the human ape split by a molecular clock of mitochondrial DNA. *J Mol Evol* 22: 160-174.

Hastings W K. (1970) Monte Carlo sampling methods using Markov chains and their applications. *Biometrika* 57: 97–109.

Hayashi JI, Tagashira Y, Yoshida MC (1985) Absence of extensive recombination between inter- and intraspecies mitochondrial DNA in mammalian cells. *Exp Cell Res* 160: 387-395.

Hershkovitz MA, Leipe DD (1998) Phylogenetic analysis. In *Bioinformatics: A practical guide to the analysis of genes and proteins.* (Baxevanis, AD and Ouellette, BFF eds.). Wiley Interscience, New York, 1998.

Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. *Biol J Linn Soc* 58: 247-276.

Hewitt GM (2000) The genetic legacy of the Quaternary ice ages. *Nature* 405: 907-913.

Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society of London.* Series B, Biological Sciences, 359, 183–195.

Hill M, Witsenboer H, Zabeau M, Vos P, Kesseli R, Michelmore R (1996) PCR-based fingerprinting using AFLPs as a tool for studying genetic relationships in *Lactuca* spp. *Theor. Appl. Genet.* 93:1202-1210.

Hillis DM, Huelsenbeck JP (1992) Signal, noise, and reliability in molecular phylogenetic analyses. *Journal of Heredity* 83: 189-195.

Hillis DM, Bull JJ (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology*, 42: 182-192.

Hillis DM, Moritz C, Mable BK (1996) Molecular systematics. 2nd edition. *Sinauer*, MA.

Hillis DM, Bull J J (1993) An empirical test of bootstrapping as a methods for assessing confidence in phylogenetic analysis. *Syst. Biol.* 42: 182–192.

Hillis DM, Allard MW, Miyamoto MM (1993) Analysis of DNA sequence data: phylogenetic inference. *Meth. In Enzym.* 224: 456-487.

Hodkinson TR, Renvoize SA, NíChonghaile G, Stapleton CMA, Chase MW (2000) A comparison of ITS nuclear rDNA sequence data and AFLP markers for phylogenetic studies in *Phyllostachys* (Bambusoideae, Poaceae). *Journal of Plant Research*, 113: 259-269.

Hoech WR, Blakley KH, Brown WM (1991) Heteroplasmy suggests limited biparental inheritance of *Mytilus mitochondrial* DNA. *Science* 251: 1488-1490.

Holder MT, Lewis PO (2003) Phylogeny estimation: traditional and Bayesian approaches. *Nature Reviews Genetics* 4:275-284.

Hooghiemstra H (2001) The continuing debate on the history of the Amazonian rain forest. *Amazoniana*, 16: 653–656.

Hooghiemstra H, Cleef AM (1995) Pleistocene climatic change and environmental and generic dynamics in the north Andean montane forest and paramo. In: *Biodiversity and Conservation of Neotropical Montane Forests* (eds Churchill SP, Balslev H, Forero E, Luyeyn JL), pp. 35–49. *New York Botanical Garden*, New York.

Hudson RR (1991) Gene trees, species trees and the segregation of ancestral alleles. *Genetics*. 1991 127(2):429-35.

Hudson RR, Slatkin M, Maddison WP (1992) Estimation of levels of gene flow from DNA sequence data. *Genetics*, 7: 583-589.

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*. Vol 17:754-755.

Huelsenbeck J P, Larget B, Swofford DL (2000) A compound Poisson process for relaxing the molecular clock. *Genetics* 154: 1879–1892.

Huelsenbeck J P, Ronquist F, Nielsen R, Bollback JP (2001) Bayesian inference of phylogeny and its impact on evolutionary biology. *Science* 294: 2310–2314.

Humphries CJ, Parenti L (1986) Cladistic Biogeography. *Clarendon Press*, Oxford.

Ingman M, Kaessmann H, Pääbo S, Gyllensten U (2000) Mitochondrial genome variation and the origin of modern humans. *Nature* 408: 708-713.

Innan H, Terauchi R, Kahl G, Tajima F (1999) A method for estimating nucleotide diversity from AFLP data. *Genetics* 151:1157-1164.

Iturralde-Vinent M A, McPhee RDE (1999) Paleogeography of the Caribbean region: Implications for Cenozoic biogeography. *Bull. Amer. Mus. Nat. Hist.* 238: 1-95.

IUCN (2003) Guidelines for Application of IUCN Red List Criteria at Regional Levels: Version 3.0. *IUCN Species Survival Commission*. IUCN, Gland, Switzerland and Cambridge, UK.

Jackson JBC, Jung P, Coates AG, Collins LS (1993) Diversity and extinction of tropical American mollusks and emergence of the Isthmus of Panamá. *Science,* 260: 1624-1626.

Jimenez-Porras JM (1964) Intraspecific variations in composition of venom of the jumping viper, Bothrops nummifera. *Toxicon.* 2: 187-195.

Jin L, Nei M (1990) Limitations of the evolutionary parsimony method of phylogenetic analysis. *Molecular Biology and Evolution*, 7: 82-102.

Johnson WE, OBrien SJ (1997) Phylogenetic reconstruction of the Felidae using 16S rRNA and NADH-5 mitochondrial genes. *Journal of Molecular Evolution*, 44: 34-67 (Suppl. 1).

Johnson WE, Culver M, Iriarte A et al. (1998) Tracking the evolution of the elusive Andean Mountain Cat (*Oreailurus jacobita*) from mitochondrial DNA. *Journal of Heredity*, 89: 227-232.

Johnson WE, Dratch PA, Martenson JS, OBrien SJ (1996) Resolution of recent radiations within three evolutionary lineages of Felidae using mitochondrial restriction fragment length polymorphism variation. *Journal of Mammalian Evolution*, 3: 97-120.

Johnson WE, Pecon-Slattery J, Eizirik E et al. (1999) Disparate phylogeographic patterns of mitochondrial DNA variation in four closely related South American small cat species. *Molecular Ecology*, 8: 79-94.

Johnson KP, Clayton DH (2000) Nuclear and mitochondrial genes contain similar phylogenetic signal for pigeons and doves (Aves: Columbiformes). *Molecular Phylogenetics and Evolution* 14:141-151.

Jordan DS (1908) The law of geminate species. *American Naturalist*, 42: 73-80.

Jorde PE, Palm S, Ryman N (1999) Estimating genetic drift and effective population size from temporal shifts in dominant gene marker frequencies. *Molecular Ecology*, 8: 1171-1178.

Joseph L, Moritz C, Hugall A (1995) Molecular support for vicariance as a source of diversity in rainforest. *Proceedings of the Royal Society of London* B, 7: 177-182.

Jouquand S, Cheron A, Galibert F (1999) Microsatellite analysis using a two-step procedure for fluorescence labeling of PCR products. *Biotechniques* 26: 902-905.

Kambhampati S, Black WC, Rai KS (1992) Random amplified polymorphic DNA of mosquito species and populations (Diptera: Culicidae): techniques, statistical analysis, and applications. *Journal of Medical Entomology*, 29: 939-945.

Kanzaki S, Yonemori K, Sato A, Yamada M, Sugiura A (2000) Analysis of the genetic relationships among pollinationconstant and non-astringent (PCNA) cultivars of persimmon (*Diospyros kaki* Thunb.) from Japan and China using amplified fragment length polymorphism (AFLP). *J. Jpn. Soc. Hortic. Sci.* 69: 665–670.

Kardolus J P, van Eck HJ, van den Berg RG (1998) The potential of AFLPs in biosystematics: a first application in Solanum taxonomy (Solanaceae). *Plant Syst. Evol.* 210: 87–103.

Karp A, Seberg O, Buiatti M (1996) Molecular techniques in the assessment of botanical diversity. *Ann. Bot.* 78: 143-149.

Kastner TP, Goñi MA (2003) Constancy in the vegetation of the Amazon basin during the late Pleistocene: evidence from the organic matter composition of Amazon deep sea fan sediments. *Geology*, 31: 291–294.

Keigwin LD (1978) Pliocene closing of the Isthmus of Panama based on biostratigraphic evidence from nearby Pacific Ocean and Caribbean Sea cores. *Geology*, 7: 630-634.

Keigwin LD (1982) Isotopic paleoceanography of the Caribbean and east Pacific, Role of Panama uplift in Late Neogene time. *Science*, 7: 350-353.

Kimura M (1968) Evolutionary rate at the molecular level. *Nature* 217: 624-626.

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16: 111-120.

Kimura M (1987) The neutral theory of molecular evolution. *Cambridge University Press*, Cambridge.

Kimura M (1983) The neutral theory of molecular evolution. *Cambridge University Press*, Cambridge.

Kishino H, Hasegawa M (1989) Evaluation of the maximum likelihood estimate of the evolutinary tree topologies from DNA sequence data, and the branching order in Hominoidea. *Journal of Molecular Evolution*, 7: 170-179.

Kishino H, Thorne J L, Bruno W J (2001) Performance of a divergence time estimation method under a probabilistic model of rate evolution. *Mol. Biol. Evol.* 18: 352–361.

Klauber LM (1952) Taxonomic studies of the rattlesnakes of mainland Mexico. *Bulletins of the Zoological Society of San Diego*, 26: 5–143.

Klauber LM (1972) Rattlesnakes: Their Habits, Life Histories, and Influence on Mankind. Abridged ed. by KH McClung. *Univ. of Calif. Press, Berkeley*. Publ. for Zoological Soc., San Diego.

Klicka J, Zink RM (1997) The importance of recent Ice Ages in speciation: A failed paradigm. *Science* 277: 1666-1669.

Knowlton N, Weigt LA (1998) New dates and new rates for divergence across the Isthmus of Panamá. *Proceedings of the Royal Society of London*, Series B, 265: 2257-2263.

Knowlton N, Weigt LA, Solorzano LA, Mills DK, Bermingham E (1993) Divergence in proteins, mitochondrial DNA, and reproductive compatability across the Isthmus of Panama. *Science*, 7: 1629-1632.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, Wilson AC (1989) Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proc Natl Acad Sci USA* 86: 6196-6200.

Kondo R, Satta Y, Matsuura ET, Ishiwa H, Takahata N, Chigusa SI (1990) Incomplete maternal transmission of mitochondrial DNA in Drosophila. *Genetics* 126: 657-663.

Kovach WL (1999) A Multivariate Statistical Package for Windows,Ver 3.1. *Kovach Computing Services*, Pentraeth, UK.

Kraus F, Mink DG, Brown WM (1996) Crotaline intergeneric relationships based on mitochondrial DNA sequence data. *Copeia* 1996:763-773.

Krauss SL (2000) Accurate gene diversity estimates from amplified fragment length polymorphism (AFLP) markers. *Molecular Ecology* 9:1241-1245.

Kreitman M, Akashi H (1995) Molecular evidence for natural selection. *Annual Review of Ecology and Systematics*, 26: 403-422.

Kumar S, Hedrick P, Dowling T, Stoneking M (2000) Questioning evidence for recombination in human mitochondrial DNA. *Science* 288: 1931.

Kumar S, Tamura K, Nei M (1993) MEGA: Molecular Evolutionary Genetics Analysis, Version 1.0. *Penn State University*, University Park.

Kumar S, Tamura K, Nei M (2003) MEGA3: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequence Alignment. *Briefings in Bioinformatics* 5:150-163.

Kumar S, Tamura K, Nei M (1993) MEGA, Version 1.1 . *The Pennsylvania State University, University* Park, PA.

Kumazawa Y, Ota H, Nishida M, Ozawa T (1998) The complete nucleotide sequence of a snake (*Dinodon semicarinatus*) mitochondrial genome with two identical control regions. *Genetics* 150: 313-329.

Labra M, Failla O, Fossati T, Castiglione S, Scienza A, Sala F (1999) Phylogenetic analysis of grapevine cv. Ansonica growing on the island of Giglio, Italy, by AFLP and SSR markers. *VITIS Band*/Volume 38 (1999): 814–825.

Lande R (1988) Genetics and demography in biological conservation. *Science* 241: 1455-1460.

Lander ES, Green P (1987) Construction of multilocus genetic maps in humans. *Proc. Natl. Acad. Sci.* USA 84: 2363-2367.

Langley CH, Fitch W (1974) An estimation of the constancy of the rate of molecular evolution. *Journal of Molecular Evolution*, 3: 161–177.

Lansman R A, Shade RO, Shapira JE, Avise JC (1981) The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations III. Techniques and potential applications. *Journal of Molecular Evolution* 17: 214-226.

Lapointe FJ, Legendre P (1992) Statistical significance of the matrix correlation coefficient for comparing independent phylogenetic trees. *Systematic Biology*, 7: 378-384.

Larget B, Simon DL (1999) Markov Chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Mol. Biol. Evol.* 16 : 750–759.

Latreille P An X (1801a) Tome III in CS Sonnini and P Latreille, *Histoire naturelle des Reptiles*. Seconde partie: Serpens. Paris: Deterville. 335p.

Latreille P An X (1801b) Tome IV in CS Sonnini et P Latreille, *Histoire Naturelle des Reptiles*. Seconde partie, Serpens. Paris: Deterville. 410p.

Lessa EP, Cook JA, Patton JL (2003) Genetic footprints of demographic expansion in North America, but not Amazonia, during the Late Quaternary. *Proceedings of the National Academy of Sciences of the United States of America*, 100: 10331–10334.

Lessios HA (1998) The first stage of speciation as seen in organisms separated by the Isthmus of Panamá. In: *Endless Forms* (eds Howard DJ, Berlocher SH), pp. 186-201. Oxford University Press, Oxford.

Lewis PO (1998) A genetic algorithm for maximum likelihood phylogeny inference using nucleotide sequence data. *Mol. Biol. Evol.* 15(3): 277-283.

Li S, Pearl D K, Doss H (2000) Phylogenetic tree construction using Markov Chain Monte Carlo. *J. Am. Stat. Assoc.* 95: 493–508.

Lin JJ, Kuo J (1995) AFLPTM: a novel PCR-based assay for plant and bacterial DNA fingerprinting . *Focus*, 17: 66-70.

Lin J-J, J Ma, J Kuo (1999) Chemiluminescent detection of AFLP markers. *Biotechniques* 26: 344-348.

Liu ZJ, Li P, Kucuktas H et al. (1999) Development of AFLP markers for genetic linkage mapping analysis using channel catfish and blue catfish interspecific hybrids. *Transactions of the American Fisheries Society*, 128: 317-327.

Liu ZJ, Nichols A, Li P, Dunham RA (1998) Inheritance and usefulness of AFLP

markers in channel catfish (*Ictalurus punctatus*), blue catfish (I.furcatus), and their F1, F2 and backcross hybrids. *Molecular and General Genetics*, 258: 260-268.

Liu Z, Furnier GR (1993) Comparison of allozyme, RFLP, and RAPD markers for revealing genetic variation within and between trembling aspen and bigtooth aspen. *Theor. Appl. Genet.* 87: 97-105.

Lockhart PJ, Steel MA, Hendy MD, Penny D (1994) Recovering evolutionary trees under a more realistic model of sequence evolution. *Molecular Biology and Evolution*, 7: 605-612.

Lopes M, Rainieri S, Henschke PA, Langridge P (1999) AFLP fingerprinting for analysis of yeast genetic variation. *Int. J. Syst. Bacteriology.* 49: 915-924.

Lu J, Knox MR, Ambrose MJ, Brown JKM, Ellis THN (1996) Comparative analysis of genetic diversity in pea assessed by RFLP- and PCR-based methods. *Theoretical and Applied Genetics*, 93: 1103-1111.

Lu J, Knox MR, Ambrose MJ, Brown JKM, Ellis THN (1996) Comparative analysis of genetic diversity in peas assessed by RFLP and PCR-based methods. *Theor. Appl. Genet.* 93: 1103-1111.

Lunt DH, Hyman BC (1997) Animal mitochondrial DNA recombination. *Nature* 387: 247.

Lunt DH, Whipple LE, Hyman BC (1998) Mitochondrial DNA variable number tandem repeats (VNTRs): utility and problems in molecular ecology. *Molecular. Ecology* 7: 1441-1455.

Lynch M, Milligan BG (1994) Analysis of population genetic structure with RAPD markers. *Molecular Ecology*, 3: 91-99.

Lyons-Weiler J, Hoelzer GA (1997) Escaping from the Felsenstein Zone by detecting long branches in phylogenetic data. *Mol. Phyl. Evol.* 8(3): 375-384.

Magro AJ, Da Silva RJ, Ramos PRR, Cherubini AL, Hatayde MR (2001) Intraspecific Variation in the venom electrophoretic profile of recently captured *Crotalus durissus terrificus* (Laurenti, 1768) snakes. *J. Venom. Anim. Toxins* v.7 n.2, Botucatu Dic. 2001.

Malhotra A, Thorpe RS (1994) Parallels between island lizards suggests selection on mitochondrial DNA and morphology. *Proceedings of the Royal Society of London* Series B, 257: 37-42.

Malhotra, A, Thorpe RS (2004) A phylogeny of four mitochondrial gene regions suggests a revised taxonomy for Asian pit vipers (*Trimeresurus* and *Ovophis*). *Mol Phylo Evol.* 32: 83-100.

Malhotra A, Thorpe RS (1997) New perspectives on the evolution of south-east Asian pitvipers (Genus *Trimeresurus*) from molecular studies. In: *Venomous snakes, ecology, evolution and snakebite*. Eds. RS Thorpe, W Wuster, and A Malhotra. *Symp. Zool. Soc. Lond.* 70. 115-128.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research*, 7: 209-220.

Marko PB (2002) Fossil calibration of molecular clocks and the divergence times of geminate species pairs separated by the Isthmus of Panamá. *Molecular Biology and Evolution*, 19: 2005-2021.

Marlas G, Bon C(1982) Relationship between the pharmacological action of crotoxin and its phospholipase activity. *Eur J Biochem* 1982 125: 157-165.

Marshall LG (1988) Land mammals and the Great American Interchange. *American Scientist*, 7: 380-388.

Marshall LG, Butler RF, Drake RE, Curtis GH, Tedford RH (1979) Calibration of the Great American Interchange. *Science*, 7: 272-279.

Mattison C (1996) Rattler! A Natural History of Rattlesnakes. *Blandford, London.*

Mau B, Newton MA, Larget B (1999) Bayesian phylogenetic inference via Markov Chain Monte Carlo methods. *Biometrics*. 55: 1-12.

Mayden R L (1997). A hierarchy of species concepts: The denouement in the saga of the species problem. In *Species: The Units of Biodiversity* (M F Claridge, H A Dawah, and M R Wilson, Eds.), pp. 381–424. Chapman and Hall, London.

McDiarmid RW, Campbell JA, Touré TA (1999) Snakes species of the world. Vol. 1. Washington, D.C: *The Herpetologists' League*. xi 509p.

Meksem K, Leister D, Peleman J, Zabeau M, Salamini F, Gebhardt C (1995) A high-resolution map of the vicinity of the R1 locus on chromosome V of potato based on RFLP and AFLP markers. *Molecular and General Genetics*, 249: 74-81.

Metropolis, N, Rosenbluth AW, Rosenbluth MN, Teller AH, Teller E (1953) Equation of state calculations by fast computing machines. *J. Chem. Phys*. 21: 1087–1092.

Metz EC, Gomez-Guitierrez G, Vacquier VD (1998) Mitochondrial DNA and bindin gene sequence evolution among allopatric species of the sea urchin genus Arbacia. *Molecular Biology and Evolution*, 15: 185-195.

Meyer, A, Kocher TD, Basasibwaki P, Wilson AC (1990) Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequences. *Nature* (London) 347: 550-553.

Michalakis Y, Excoffier L (1996) A generic estimation of population subdivision using distances between alleles with special reference to microsatellite loci. *Genetics*, 142: 1061-1064.

Minton SA, Weinstein, SA (1986) Geographic and Ontogenetic variation in venom of the western diamondback rattlesnake (*Crotalus atrox*). *Toxicon* 24 (1): 71-80.

Mirgorodskaya OA, Kozmin YP, Titov MI, Körner R, Sönksen C, Roepstorff P (2000) Quantitation of peptides and proteins by matrix-assisted laser desorption/ionization mass spectrometry using 18O-labeled internal standards, *Rapid Communications in Mass Spectrometry*, 14: 1226-1232.

Mishler B D, Donoghue M J (1982) Species concepts: A case for pluralism. *Syst. Zool.* 31: 491–503.

Miyashita, NT, Kawabe A, Innan H (1999) DNA variation in the wild plant *Arabidopsis thaliana* revealed by amplified fragment length polymorphism analysis. *Genetics* 152: 1723-1731.

Moore WS (1995) Inferring phylogenies from DNA variation: Mitochondrial-gene trees versus nuclear-gene trees. *Evolution* 49: 718-726.

Moritz C, Dowling T, Brown W (1987) Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Annual Review of Ecology and Systematics*, 7: 269-292.

Moritz C, Patton JL, Schneider CJ, Smith TB (2000) Diversification of rainforest faunas: an integrated molecular approach. *Annual Review of Ecology and Systematics*, 31: 533-563.

Moritz C (1994) Applications of mitochondrial DNA analysis in conservation: a critical review. *Mol. Ecol.* 3: 401-411.

Moritz C (1994) Defining "Evolutionarily Significant Units" for conservation. *Trends in Ecology and Evolution* 9: 373-375.

Moyle RG (2004) Phylogenetics of barbets (Aves: Piciformes) based on nuclear and mitochondrial DNA sequence data. *Molecular Phylogenetics and Evolution* 30: 187-200.

Mueller UG, Wolfenbarger LL (1999) AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution*, 14: 389-394.

Mueller UG, Lipari SE, Milgroom MG (1996) Amplified fragment length polymorphism (AFLP) fingerprinting of symbiotic fungi cultured by the fungus-growing ant *Cyphomyrmex minutus*. *Molecular Ecology*. 5: 119-122.

Murphy R W, Fu J, Lathrop A, Feltham JV, Kovac V (2002) Phylogeny of the rattlesnakes (*Crotalus* and *Sistrurus*) inferred from sequences of five mitochondrial DNA genes. In: G W Schuett, M Höggren, M E Douglas, and H W Greene (eds.). *Biology of the Vipers*. Pp. 69-92. Eagle Mountain Publishing, LC, Eagle Mountain, UT.

Myers GS (1938) Fresh-water fishes and West Indian zoogeography. *Smithsonian Report*, 1937: 766-773.

Nei M, Jin L (1989) Variances of the average numbers of nucleotide substitutions within and between populations. *Molecular Biology and Evolution*, 6: 290-300.

Nei M (1987) Molecular evolutionary genetics. *Columbia University Press*, New York.

Nei M, Kumar S, Takahashi K (1998) The optimization principle in phylogenetic analysis tends to give incorrect topologies when the number of nucleotides or amino acids used is small. *Proc. Natl. Acad. Sci. USA*. 95: 1239-12397.

Nigro L, Prout T (1990) Is there Selection on RFLP differences in Mitochondrial DNA? *Genetics*. 80: 87-91.

Nijman J, Bradley DG, Hanotte O, Otsen M, Lenstra JA (1999) Satellite DNA polymorphisms and AFLP correlate with Bos indicus-taurus hybridization. Animal *Genetics*, 30: 265-273.

Ogden R, Thorpe RS (2002a) Molecular evidence for ecological speciation in tropical habitats. *Proceedings of the National Academy of Sciences of the USA*, 99: 13612-13615.

Ogden R, Thorpe RS (2002b). The usefulnes of amplified fragment length polymorphism markers for taxon discrimation across graduated fine evolutionary levels in Caribbean *Anolis* lizards. *Mol. Ecol.* 11: 437-445.

Oguiur N, Camargo ME, da Silva AR, Horton DS (2000) Quantification of crotamine, a small basic myotoxin, in South American rattlesnake (*Crotalus durissus terrificus*) venom by enzyme-linked immunosorbent assay with parallel-lines analysis. *Toxicon.* 2000 Mar; 38(3): 443-448.

Ohta T, Kimura M (1969) Linkage disequilibrium at a steady state determined by random genetic drift and recurrent mutation. *Genetics*, 63: 229 238.

Ohta T (1982) Linkage disequilibrium due to random genetic drift in finite subdivided populations. *Proceedings of the National Academy of Sciences of the USA*, 79: 1940-1944.

Olsen GI, Matsuda H, Hagström R, Overbeek R (1994) FastDNAml: A tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comput Applic Biosci* 10: 41-48.

Ovilo C, Cervera MT, Castellanos C, Martinez-Zapater JM (2000) Characterisation of Iberian pig genotypes using AFLP markers. *Animal Genetics*, 31: 117-122.

Page RDM (2000) Extracting species trees from complex gene trees: reconciled trees and vertebrate phylogeny. *Molecular Phylogenetics and Evolution*, 14: 89–106.

Page RDM (1991) Clocks, clades, cospeciation, comparing rates of evolution and timing of cospeciation events in host-parasite assemblages. *Systematic Zoology*, 7: 188-198.

Page RDM (1993) COMPONENT , version 2.0. *The Natural History Museum*, London.

Page RDM (1996) Temporal congruence revisited: comparison of mitochondrial DNA sequence divergence in cospeciating pocket gophers and their chewing lice. *Systematic Biology*, 7: 151-167.

Page RDM, Holmes EC (1998) Molecular Evolution. A Phylogenetic Approach. *Blackwell Science*.

Palumbi SR, Baker CS (1994) Contrasting population structure from nuclear intron sequences and mtDNA of humpback whales. *Molecular Biology and Evolution*, 11: 426–435.

Palumbi, S R, Kessing BD (1991) Population biology of the trans-Arctic exchange: mtDNA sequence similarity between Pacific and Atlantic sea urchins. *Evolution* 45: 1790–1805.

Parkinson CL (1999) Molecular systematics and biogeographical history of the pitvipers as determined by mitochondrial ribosomal sequences. *Copeia*, 1999: 576–586.

Parkinson,CL, Campbell JA, Chippindale PT (2002) Multigene Phylogenetic Analysis of Pitvipers, with Comments on Their Biogeography. In *Biology of the Vipers* (GW Schuett, M Höggren, ME Douglas and HW Greene, eds.), pp. 111-128. *Eagle Mountain Publishing*, Eagle Mountain, Utah.

Parsons YM, Shaw KL (2001) Species boundaries and genetic diversity among Hawaiian crickets of the genus Laupala identified using amplified fragment length polymorphism. *Mol. Ecol.* 10: 1765–1772.

Patton J, da Silva MNF, Malcolm JR (1994) Gene genealogy and differentiation among spiny arboreal rats (Rodentia: Echimyidae) of the Amazon basin: a test of the riverine barrier hypothesis. *Evolution*, 7: 1314-1323.

Paul, S, FN Wachira, W Powell, R Waught (1997) Diversity and genetic differentiation among populations of Indian and Kenyan tea (Camellia sinensis (L.) O Kuntze) revealed by AFLP markers. Theor. Appl. Genet. 94: 255-263.

Pennington RT, Lavin M, Prado DE, Pendry CA, Pell SK, Butterworth CA (2004) Historical climate change and speciation: Neotropical seasonally dry forest plants show patterns of both Tertiary and Quaternary Diversification. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 359: 515–538.

Pennington RT, Prado DE, Pendry CA (2000) Neotropical seasonally dry forests and Quaternary vegetation changes. *Journal of Biogeography*, 27: 261–273.

Perkin-Elmer (1995) AFLP Plant Mapping Kit Protocol. (P/N 402083)

Pindell JL, Barrett SF (1990) Geological evolution of the Caribbean region: a plate-tectonic perspective. In: *The Caribbean Region, the Decade of North American Geology*, Vol. H (eds Dengo G, Case JE), pp. 405-432. Geol. Soc. of America, Boulder, CO.

Place AJ, Abramson CI (2004) A quantitative analysis of the ancestral area of rattlesnakes. *Journal of Herpetology*, 38: 152–156.

Pook CE, Wüster W, Thorpe RS (2000) Historical biogeography of the western rattlesnake (Serpentes: Viperidae: *Crotalus viridis*), inferred from mitochondrial DNA sequence information. *Molecular Phylogenetics and Evolution* 15: 269-282.

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* 14: 817-818.

Posada D, Crandall KA, Templeton AR (2000) GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, 9: 487-488.

Potts R, Behrensmeyer AK (1992) Late Cainozoic terrestrial ecosystems. In: *Terrestrial Ecosystems Through Time: Evolutionary Paleoecology of Terrestrial Plants and Animals* (eds Behrensmeyer AK, Damuth JD, DiMichele WA, Potts R, Sues H-D, Wing SL), pp. 419–541. *University of Chicago Press*, Chicago.

Prance G T (1973) Phytogeographic support for the theory of Pleistocene forest refuges in the Amazon Basin, based on evidence from distribution patterns in Caryocaraceae, Chrysobalanaceae, Dichapetalaceae and Lecythidaceae. *Acta Amazonica* 3: 5-28.

Prychitko TM, Moore WS (1997) The utility of DNA sequences of an intron from the β-fibrinogen gene in phylogenetic analysis of woodpeckers (Aves: Picidae). *Molecular Phylogenetics and Evolution*, 8: 193-204.

Prychitko TM, Moore WS (2000) Comparative evolution of the mitochondrial cytochrome b gene and nuclear β-.brinogen intron 7 in woodpeckers. *Molecular Biology and Evolution*, 17: 1101–1111.

Prychitko TM, Moore WS (2003) Alignment and phylogenetic analysis of β-brinogen intron 7 sequences among avian orders reveal conserved regions within the intron. *Molecular Biology and Evolution*, 20: 762–771.

Puorto G, Salomao MG, Theakston RDG, Thorpe RS, Warrell DA, Wüster W (2001) Combining mitochondrial DNA sequences and morphological data to infer species boundaries: phylogeography of lanceheaded pitvipers in the Brazilian Atlantic forest, and the status of *Bothrops pradoi* (Squamata: Serpentes: Viperidae). *Journal of Evolutionary Biology*, 14(4): 527-538.

Qamaruz-Zaman F, Fay MF, Parker JS, Chase MW (1997) The use of AFLP fingerprinting in conservation genetics: a case study of *Orchis simia* (Orchidaceae). Preliminary report. *Royal Botanica Gardens, Kew*.

Rage J-C, Bajpai S, Thewissen JGM, Tiwari BN (2003) Early Eocene snakes from Kutch, western India, with a review of the Palaeophiidae. *Geodiversitas*, 25: 695–716.

Ranamukhaarachchi DG, Kane ME, Guy CL, Li PB (2000) Modified AFLP technique for rapid genetic characterization in plants. *BioTechniques* 29(4): 858-866.

Rangel-Santos A., Dos-Santos EC, Lopes-Ferreira M, Lima C, Cardoso DF, Mota I (2004) A comparative study of biological activities of crotoxin and CB fraction of

venoms from *Crotalus durissus terrificus*, *Crotalus durissus cascavella* and *Crotalus durissus collilineatus*. *Toxicon* 43: 801–810.

Rannala B, Yang Z (1996) Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. *J. Mol. Evol.* 43: 304–311.

Rannala B, Mountain JL (1997) Detecting immigration by using multilocus genotypes. *PNAS* 94: 9197-9201.

Reich DE, Feldman MW (1999) Statistical properties of two tests: the use of multilocus data sets to detect population expansions. *Mol. Biol. Evol.* 16: 453-466.

Reinet HK, Lauretta MB, Odum RA (2005) The ecology and conservation biology of the Aruba Island Rattlesnake, *Crotalus unicolor*. *Biology of the Rattlesnakes, Symposium*. Loma Linda University, California.

Rodriquez R, Oliver JL, Marin A, Medina JR (1990) The general stochastic model of nucleotide substitution. *J Theor Biol* 142: 485-501.

Rogers JS, Swofford DL (1998) A fast method for approximating maximum likelihoods of phylogenetic trees from nucleotide sequences. *Syst. Biol.* 47: 77-89.

Roman BL, Pham VN, Bennett PE, Weinstein BM (1999) Non-radioisotopic AFLP method using PCR primers fluorescently labeled with CyTM5." *Biotechniques* 26: 236-238.

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics, 19: 1572–1574.

Russo CAM, Takezaki, N, Nei, M (1996) Efficiencies of different genes and different tree-building methods in recovering a known vertebrate phylogeny. *Mol. Biol. Evol.* 13(3): 525-536.

Ryder OA (1986) Species conservation and systematics: the dilemma of subspecies. *Trends in Ecology and Evolution*, 1: 9-10.

Saiki RK, Gelfland DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*. 239: 487-491.

Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406-425.

Salazar-Bravo J, Dragoo JW, Tinnin DS, Yates TL (2001) Phylogeny and evolution of the Neotropical rodent genus *Calomys*: inferences from mitochondrial DNA sequence data. *Molecular Phylogenetics and Evolution*, 20: 173–184.

Salomão MG, Wüster W, Thorpe RS (1999) MtDNA evolution in neotropical pitvipers of the genus *Bothrops* (Squamata: Serpentes: Viperidae). *Kaupia* 8: 127-134.

Salomão M G, Wüster W, Thorpe RS, Touzet J-M (1997) DNA evolution of South American pitvipers of the genus *Bothrops* (Reptilia: Serpentes: Viperidae). Pages 89-98 in *Venomous snakes: Ecology, evolution and snakebite*. (R S, Thorpe, Wüster, W and A Malhotra, eds). *Symposia Zoological Society of London* No. 70., Oxford University Press.

Sambrook J, Fritsch EF, Maniatis TE (1989) Molecular Cloning: a Laboratory Manual. 2nd edn. *Cold Spring Harbor Laboratory Press*, New York.

Sambrook J Fritsh EF, Maniatis TE (1982) Molecular cloning: a laboratory manual. *Cold Spring Harbour*, New York.

Sanderson MJ (2002) Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. *Molecular Biology and Evolution*, 19: 101–109.

Saravia, P Rojas-Umaña, E Arce, V Guevara, C López, JC Chaves-Olarte, E Velásquez, R Rojas-Céspedes, G Gutiérrez-Gutiérrez, JM (2002) Geographic and ontogenic variability in the venom of the neotropical rattlesnake *Crotalus durissus*: Pathophysiological and therapeutic implications. *Revista de Biología Tropical*. 50 (1): 337-346.

Savage JM (1982) The enigma of the Central American herpetofauna: dispersals or vicariance? *Annuals Missouri Botanical Gardens*, 7: 464-547.

Schenberg S (1959) Geographical pattern of crotamine distribution in the same rattlesnake subspecies. *Science* 129: 1361-1363.

Schneider S, Kueffer JM, Roessli D, Excoffier L (1997) ARLEQUIN, Version 1.1. University of Geneva, Switzerland. (http://lgb.unige.ch/arlequin).

Schupp, JM, Price LB, Klevytska A, Keim P (1999) Internal and flanking sequence from AFLP fragments using ligation-mediated suppression PCR. *Biotechniques* 26: 505-511.

Seehausen O, Allender Charlotte J, Knight, Mairi E, Turner, George F, Maclean, Norman (2003) Divergent selection during speciation of Lake Malawi cichlid fishes inferred from parallel radiations in nuptial colouration. *Proceedings of the National Academy of Sciences of the USA* 100: 14074-14079

SEMARNAP ECO-089 (2001) Lista de especies amenazadas en Mexico. Semarnat, Gob Mexico.

Shackleton NJ, Backman J, Zimmerman H et al. (1984) Oxygen isotope calibration of the onset of ice-rafting and history of glaciation of the North Atlantic region. *Nature*, 307: 620–623.

Sharma, SK, Knox MR, Ellis THN (1996) AFLP analysis of the diversity and phylogeny of Lens and its comparison with RAPD analysis. *Theor. Appl. Genet.* 93: 751-758.

Shaw, DV, Kahler AL, Allard RW (1981) A multilocus estimator of mating system parameters in plant populations. *Proc. Natl. Acad. Sci.* 78: 1298-1302.

Simpson GG (1980) Splendid Isolation, the Curious History of South American Mammals. *Yale University Press*, New Haven, CT.

Simpson G G (1950) History of the Fauna of Latin America. *American Scientist* 38: 361-389.

Simpson GG (1951). The species concept. *Evolution* 5: 285–298.

Simpson GG (1961) Principles of Animal Taxonomy. *Columbia Univ. Press*, New York.

Singh RS, Hale LR (1990) Are mitochondrial DNA variants selectively non-neutral? *Genetics* 124: 995-997

Slatkin M, Madison WP (1989) A cladistic measure of gene flow from the phylogenies of alleles. *Genetics*, 7: 603-613.

Slatkin M (1989) Detecting small amounts of gene flow from phylogenies of alleles. *Genetics*, 7: 609-612.

Smith HM, Taylor EH (1966) Herpetology of Mexico-Annotated checklists and keys to the amphibians and reptiles: A Reprint of Bulletins 187, 194 and 199 of the US National Museum with a List of Subsequent Publications. 1st ed. Ashton: Eric Lundberg.

Sokal R R, Sneath PHA (1963) Principles of Numerical Taxonomy. *W H Freeman*, San Francisco.

Solórzano-López A, Cerdas-Fallas L (1988) Reproductive biology of the Central American rattlesnake, *Crotalus durissus durissus* (Serpentes: Viperidae). *Revista de Biologia Tropical*. 36 (2a): 221-226.

Soltis PS, Gitzendanner MA (1999) Molecular systematics and the conservation of rare species. *Cons Biol* 13: 471-483.

Sorenson MD, Fleischer RC (1996) Multiple independent transpositions of mitochondrial DNA control region sequences to the nucleus. *Proc Natl Acad Sci USA* 93: 15239-15243.

Stehli FG, Webb SD (1985) The Great American Biotic Interchange. *Plenum Press*, New York.

Stillman JH, Reeb CA (2001) Molecular phylogeny of eastern Pacific porcelain crabs, genera *Petrolisthes* and *Pachycheles*, based on the mtDNA 16S rDNA sequence: phylogeographic and systematic implications. *Molecular Phylogenetics and Evolution*, 19: 236-245.

Suazo AH, Hall G (1999) Modification of the AFLP protocol to Honey Bee (Apis mellifera L.) DNA. *Biotechniques* 26:704-709.

Sullivan JP, Lavoue S, Arnegard ME, Hopkins CD (2004) AFLPs resolve phylogeny and reveal mitochondrial introgression within a species flock of African electric fish (Mormyroidea : Teleostei). *Evolution* 58: 825-841.

Swofford DL (1993) PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1. *Illinois Natural History Survey*, Champaign.

Swofford DL (1998) PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods), Version 4.0. *Sinauer Associates*, Sunderland, Massachusetts.

Swofford, D L et al. (2001) Bias in phylogenetic estimation and its relevance to the choice between parsimony and likelihood methods. *Syst. Biol.* 50: 525–539.

Swofford DL, Olsen GJ (1990) Phylogeny reconstruction. Pp. 411-501 in *Molecular Systematics*, D.M. Hillis and C. Moritz, eds. Sinauer Associates, Sunderland, MA.

Szyndlar Z, Rage J-C (1990) West Palearctic cobras of the genus *Naja* (Serpentes: Elapidae): interrelationships among extinct and extant species. *Amphibia-Reptilia*, 11: 385–400.

Takahata N (1993) Allelic geneology and human evolution. *Molecular Biology and Evolution*, 7: 2-22.

Tamura K, Nei M (1993) Estimation of the Number of Nucleotide Substitutions in the Control Region of Mitochondrial-DNA in Humans and Chimpanzees. *Molecular Biology and Evolution* 10: 512-526.

Tautz D (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Res.* 17: 6463-6471.

Tautz D, Rentz M (1984) Simple sequences are ubiquitous repetitive elements of eukaryote genomes. *Nucleic Acids Res.* 12: 4127-4138.

Taylor EH (1951) A brief review of the snakes of Costa Rica. *Univ. Kansas. Sci. Bull.* 34: 1-188.

Templeton AR (1983) Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. *Evolution*, 37: 221–244.

Templeton AR (1998) Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecolology*, 7: 381-397.

Templeton AR (2001) Using phylogenetic analyses of gene trees to test species status and processes. *Molecular Ecology*, 10: 779–791.

Templeton AR, Boerwinkle E, Sing CF (1987) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. I. Basic theory and an analysis of alcohol dehydrogenase activity in *Drosophila*. *Genetics* 117: 343 351.

Templeton AR, Routman E, Phillips CA (1995) Separating population structure from population history: A cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the Tiger salamander, *Ambystoma tigrinum*. *Genetics* 140: 767-782.

Theakston RDG (1997) The kinetics of snakebite envenoming and therapy. In: *Venomous Snakes Ecology, Evolution and Snakebite* (eds Thorpe RS, Wüster W, Malhotra A), pp. 251–257. Clarendon Press, Oxford.

Theakston RD, Reid HA (1979) Enzyme-Linked immunosorbent assay (ELISA) in assessing antivenom potency. *Toxicon* 17: 511.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673-80.

Thorne J L, Kishino H, Painter IS (1998) Estimating the rate of evolution of the rate of molecular evolution. *Mol. Biol. Evol.* 15: 1647–1657.

Thorpe RS (1980) A comparative study of ordination techniques in numerical taxonomy in relation to racial variation in the ringed snake *Natrix natrix* (L.). *Biological Journal of the Linnean Society*, 13: 7-40.

Thorpe RS, Malhotra A, Stenson A, Reardon JT (2002) Adaptation and speciation in lesser Antillean Anoles. In: *Adaptive Speciation* (eds Deickmann, H, Metz, HAJ, Doebeli, M, Tautz, D), Cambridge University Press, Cambridge,.

Thorpe R S , Black H, A Malhotra A (1996) Matrix correspondence tests on the DNA phylogeny of the Tenerife Lacertid elucidates both historical causes and morphological adaptation. *Systematic Biology*. 45: 335-343.

Thorpe RS, McGregor DP, Cumming AM, Jordan WC (1994) DNA evolution and colonization sequence of island lizards in relation to geological history: MtDNA RFLP,

cytochrome b, cytochrome oxidase, 12srRNA sequence and nuclear RAPD analysis. *Evolution* 48: 230–240.

Thorpe RS, Brown RP, Malhotra A, Wüster W (1991) Geographic variation and population systematics: distinguishing between ecogenetics and phylogenetics. *Boll. Zool.* 58: 329-335.

Travis SE, Maschinski J, Keim P (1996) An analysis of genetic variation in Astragalus cremnophylax var. cremnophylax, a critically endangered plant, using AFLP markers. *Mol. Ecol.* 5: 735-745.

van der Wruff, AWG, Chan YL, van Straalen NM, Schouten JT (2000) TE-AFLP: Combining rapidity and robustness in DNA fingerprinting. *Nucleic Acids Research* 28(24): e105

VanToal, TT, Peng J, St. Martin SK (1997) Using AFLP markers to determine the genomic contribution of parents to populations. *Crop Science* 37: 1370-1373.

Vanzolini PE, Heyer RW (1985) The American herpetofauna and the Interchange. In: *The Great American Biotic Interchange* (eds. Stehli FG, Webb SD): pp. 475-487. Plenum Press, New York.

Vanzolini PE, Calleffo MEV (2002) A taxonomic bibliography of the South American snakes of the *Crotalus durissus* complex (Serpentes, Viperidae). *Anais Da Academia Brasileira de Ciências*, 74: 37–83.

Vermeij GJ (2001) Distribution, history, and taxonomy of the *Thais* clade (Gastropoda: Muricidae) in the Neogene of tropical America. *Journal of Paleontology*, 75: 697-705.

Vidal, N, Lecointre G, Vie JC, Gasc JP (1997) Molecular systematics of pitvipers: Paraphyly of the Bothrops complex. C.R. Acad. Sci. Paris, *Sciences de la vie/Life Sciences* 320: 95-101.

Vidal, N, Lecointre G, Vié JC, Gasc, JP (1999) What can mitochondrial gene sequences tell us about intergeneric relationships of pitvipers? *Kaupia* (Darmstadt) (8): 107-112.

Vos, P, Hogers R, Bleeker M, Rijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Kuiper M, Zabeau M (1995) AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res.* 23: 4407-4414.

Wallis GP (1999) Do animal mitochondrial genomes recombine? *Trends Ecol Evol* 14: 209-210.

Waples RS (1991) Definition of "species" under the Endangered Species Act: Application to Pacific salmon. *U.S. Dep. Commer., NOAA* Tech. Memo. NMFS F/NWC-194, 29 p.

Warrell DA (2004) Snakebites in Central and South America: Epidemiology, Clinical features, and Clinical Management pages 708-791 In Campbell, JA and WW Lamar (2004) *The Venomous Reptiles of the Western Hemisphere*. Comstock, Ithaca and London.

Warrell DA (1997) Geographical and intraspecies variation in the clinical manifestations of envenoming by snakes. In: *Venomous Snakes, Ecology, Evolution and Snakebite*. Eds. RS Thorpe, W Wüster, and A Malhotra. Symp. zool. Soc. Lond. 70: 155-171.

Webb SD, Rancy A (1996) Late Cenozoic evolution of the Neotropical mammal fauna. In: *Evolution and Environment in Tropical America* (eds Jackson JBC, Budd AF, Coates AG), pp. 335–358. University of Chicago Press, Chicago.

Weibel AC, Moore WS (2002) Test of a Mitochondrial gene-based phylogeny of woodpeckers (genus *Picoides*) using an independient nuclear gene, β-fibrinogen intron 7. *Molecular Phylogenetics and Evolution* . 22(2): 247-257.

Werman SD (1992) Phylogenetic relationships of Central and South American pitvipers of the genus *Bothrops* (sensu lato): Cladistic analyses of biochemical and anatomical

characters. Pages 21-40 in *Biology of the pitvipers* (Campbell, J A and E D Brodie, Jr., eds). Selva, Tyler, Texas.

Wiley E O (1978) The evolutionary species concept reconsidered. *Syst. Zool.* 27: 17–26.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18: 6531-6535.

Wright S (1931) Evolution in Mendelian populations. *Genetics* 16: 97-159.

Wu C-I (1991) Inferences of species phylogeny in relation to segregation of ancient polymorphisms. *Genetics*, 127: 429–435.

Wüster W, Dumbrell AJ, Hay C, Pook CE, Williams DJ, Fry BG (2005) Snakes across the Strait: trans-Torresian phylogeographic relationships in three genera of Australasian snakes (Serpentes: Elapidae: *Acanthophis*, *Oxyuranus* and *Pseudechis*). *Molecular Phylogenetics and Evolution*, 34: 1–14.

Wüster W, Ferguson JE, Quijada-Mascareñas JA, Pook CE , Salomão MG, Thorpe RS (2005) Tracing an invasion: landbridges, refugia and the phylogeography of the Neotropical rattlesnake (Serpentes: Viperidae: *Crotalus durissus*). *Molecular Ecology* 14(4): 1095-1108.

Wüster W, Yrausquin JL, Mijares-Urrutia A (2001) A new species of indigo snake from northwestern Venezuela (Serpentes: Colubridae: *Drymarchon*). *Herpetological Journal* 11: 157-165.

Wüster W, Salomão MG, Duckett GJ, Thorpe RS (1999) Mitochondrial DNA phylogeny of the *Bothrops atrox* species complex (Squamata: Serpentes: Viperidae). *Kaupia* 8: 135-144.

Wüster W, Salomao MG, Quijada-Mascareñas JA, Thorpe RS (2002) Origin and evolution of the South American pitviper fauna: evidence from mitochondrial DNA

sequence analysis. In *Biology of the Vipers* (GW Schuett, M Höggren, ME Douglas and HW Greene, eds.), pp. 111-128. *Eagle Mountain Publishing*, Eagle Mountain, Utah.

Wüster W, Thorpe RS, Salomao MG, Thomas L, Puorto G, Theakson RDG, Warrell DA (2002) Origin and phylogenetic position of the Lesser Antillean species of *Bothrops* (Serpentes: Viperidae): biogeographical and medical implications. *Bulletin of the Natural History Museum London* (Zoology), 68: 101-106.

Wüster W, Salomão MG, Thorpe RS, Puorto G, Furtado MFD, Hoge SA, Theakston RDG, Warrell DA (1997) Systematics of the *Bothrops atrox* complex: New insights from multivariate analysis and mitochondrial DNA sequence information. Pages 99-113 in *Venomous snakes: Ecology, evolution and snakebite*. (R S, Thorpe, Wüster, W and A Malhotra, eds). *Symposia Zoological Society of London* No. 70, Oxford University Press.

Yan, G, Romero-Severson J, Walton M, Chadee DD, Severson DW (1999) Population genetics of the yellow fever mosquito in Trinidad: comparisons of amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) markers. *Mol. Ecol.* 8:951-963.

Yang ZY, Yoder AD (2003) Comparison of likelihood and Bayesian methods for estimating divergence times using multiple gene loci and calibration points, with application to a radiation ofcute-looking mouse lemur species. *Systematic Biology*, 52: 705–716.

Yang ZH, Rannala B (1997) Bayesian phylogenetic inference using DNA sequences: a Markov Chain Monte Carlo method. *Mol. Biol. Evol.* 14: 717–724.

Yee E, Kidwell KK, Sills GR, Lumpkin TA (1999) Diversity among selected *Vigna angularis* (Azuki) accessions on the basis of RAPD and AFLP markers. *Crop Science* 39: 268-275.

Zabeau M, Vos P (1993) Selective restriction fragment amplification: a general method for DNA fingerprinting. *European Patent Publication 92402629* (Publication no. EP0534858A1).

Zamudio KR, Greene HW (1997) Phylogeography of the bushmaster (*Lachesis muta*: Viperidae): implications for neotropical bigeography, systematics and conservation. *Biol. J. Linn. Soc.* 62: 421-442.

Zhang DX, Hewitt GM (1996) Nuclear interactions: challenges for mitochondrial DNA markers. *Trends Ecol Evol* 11: 247-251.

Zharkikh A, Li W-H (1992) Statistical properties of bootstrap estimation of phylogenetic variability from nucleotide sequences. I. Four taxa with a molecular clock. *J. Mol. Evol.* 9: 1119–1147.

Zhivotovsky LA (1999) Estimating population structure in diploids with multilocus dominant DNA markers. *Mol. Ecol.* 8: 907-913.

Appendix 2 List of samples used in the present study

Label	Taxon	Locality	Geographical cordinates
Cd001	*C. durissus durissus*	Catemaco, Veracruz	24 16 00 N, 98 41 00 W
Cd002	*C. durissus culminatus*	El aguacate, Puebla	18 45 00 N, 98 11 00 W
Cd003	*C. durissus culminatus*	El aguacate, Puebla	18 45 00 N, 98 11 00 W
Cd004	*C. durissus culminatus*	El aguacate, Puebla	18 45 00 N, 98 11 00 W
Cd005	*C. durissus culminatus*	El aguacate, Puebla	18 45 00 N, 98 11 00 W
CdSA	*C.durissus totonacus*	30Km SW Cd. Victoria, Tamaulipas	23 44 00 N, 98 48 00 W
CdSA2	*C.durissus totonacus*	30Km SW Cd. Victoria, Tamaulipas	23 44 00 N, 98 48 00 W
CdSD	*C.durissus totonacus*	Tamaulipas	23 36 00 N, 98 38 00 W
RA	*C. durissus culminatus*	Sierra de Puebla, Puebla	18 16 00 N, 97 58 00 W
RA3	*C. durissus culminatus*	Sierra de Puebla,	18 16 00 N, 97 58 00 W
Ctx1	*C. atrox*	Hermosillo, Sonora	29 06 00 N, 110 56 00 W
Csct	*C. scutulatus*	Hermosillo, Sonora	29 06 00 N, 110 56 00 W
CM1	*C. molossus molossus*	Sonoyta, Sonora	31 58 00 N, 113 18 00 W
CM2	*C. molossus molossus*	Sonoyta, Sonora	31 58 00 N, 113 18 00 W
1HFC	*C. molossus nigrescens*	El Pedregal, Valle de Mexico	19 21 00 N, 99 09 00 W
2HFC	*C. molossus nigrescens*	El Pedregal, Valle de Mexico	19 21 00 N, 99 09 00 W
4HFC	*C. molossus nigrescens*	El Pedregal, Valle de Mexico	19 21 00 N, 99 09 00 W
5HFC	*C. durissus culminatus*	Sierra de Puebla, Puebla	18 45 00 N, 98 11 00 W
ZG1Cdd	*C. durissus durissus*	Los Tuxtlas, Veracruz	18 35 00 N, 95 19 00 W
ZG2Cdd	*C. durissus durissus*	Selva Lacandona, Chiapas	17 31 00 N, 91 46 00 W
ZG3Cdd	*C. durissus durissus*	Paso del Toro, Veracruz	18 59 00 N, 96 33 00 W
ZGCB1	*C. basiliscus*	Guadalajara, Jalisco	20 37 00 N, 103 24 00 W
ZGCB2	*C. basiliscus*	Guadalajara, Jalisco	20 37 00 N, 103 24 00 W
ZGCB3	*C. basiliscus*	Guadalajara, Jalisco	20 37 00 N, 103 24 00 W
ZGCC1	*C. durissus culminatus*	Oaxaca	17 44 00 N, 97 44 00 W
ZGCC2	*C. durissus culminatus*	Oaxaca	17 44 00 N, 97 44 00 W
ZGCC3	*C. durissus culminatus*	Oaxaca	17 44 00 N, 97 44 00 W
CTSON	*C. tigris*	Hermosillo, Sonora	29 06 00 N, 110 56 00 W
CTSON2	*C. tigris*	Hermosillo, Sonora	29 06 00 N, 110 56 00 W
1567	*C.molossus nigrescens*	Queretaro, El Internado	20 45 00 N, 100 19 00 W
1576	*C.durissus culminatus*	Edo. Mexico, Ixtapan	18 40 00 N, 99 31 00 W
2063	*C. durissus durissus*	Chiapas, La Lacandona	17 31 00 N, 91 46 00 W
2065	*C. durissus durissus*	Chiapas, La Lacandona	17 31 00 N, 91 46 00 W
2067	*C. durissus durissus*	Chiapas, La Lacandona	17 31 00 N, 91 46 00 W
2072	*C. durissus durissus*	Chiapas, La Lacandona	17 31 00 N, 91 46 00 W
3102	*C. durissus totonacus*	Queretaro, Tomajo	21 17 00 N, 99 51 00 W
cdculB	*C.durissus culminatus*	El aguacate, Puebla	18 45 00 N, 98 11 00 W
Cdg1a	*C.durissus durissus*	Los Tuxtlas, Veracruz	18 35 00 N, 95 19 00 W
1566	*C.molossus nigrescens*	Queretaro, Cadereita	20 27 00 N, 100 19 00 W

822	*C. basiliscus*	Nayarit, San Blas	21 36 00 N, 105 12 00 W
C128	*C.molossus molossus*	San Bernandino, California	34 07 00 N, 116 34 00 W
C135	*C.molossus molossus*	Las Uvas, New Mexico	33 04 00 N, 107 43 00 W
C136	*C.molossus molossus*	Las Uvas, New Mexico	33 04 00 N, 107 43 00 W
C137	*C.molossus molossus*	Maricopa, AZ	33 14 00 N, 112 06 00 W
CMN3	*C.molossus nigrescens*	El Pedregal, Valle de Mexico	19 21 00 N, 99 09 00 W
CMN4	*C.molossus nigrescens*	El Pedregal, Valle de Mexico	19 21 00 N, 99 09 00 W
CMN6	*C.molossus nigrescens*	El Pedregal, Valle de Mexico	19 21 00 N, 99 09 00 W
3014	*C. molossus oaxacus*	Zapotitlan Salinas, Puebla	18 24 00 N, 97 27 00 W
108	*Crotalus durissus terrificus*	Jacarei SP	23 18 00 S, 46 03 00 W
109	*Crotalus durisuis terrificus*	Guaratinguet<	22 48 00 S, 45 12 00 W
110	*Crotalus durissus terrificus*	Roseira SP	22 54 00 S, 45 21 00 W
120	*Crotalus durissus terrificus*	Taubat9 SP	23 01 00 S, 45 33 00 W
121	*Crotalus durissus terrificus*	Svo Luiz do Paraitinga	23 15 00 S, 45 27 00 W
122	*Crotalus durissus terrificus*	Colombia SP	20 05 00 S, 48 58 00 W
123	*Crotalus durissus terrificus*	Itupeva SP	23 06 00 S, 47 02 00 W
135	*Crotalus durissus terrificus*	Pindamonhangaba	22 55 00S, 45 29 00 W
136	*Crotalus durissus terrificus*	Pindamonhangaba	22 55 00S, 45 29 00 W
137	*Crotalus durissus terrificus*	Roseira SP	22 54 00 S, 45 21 00 W
211	*Crotalus unicolor*	Aruba Island - London Zoo C1695	12 32 00 N, 69 56 00 W
212	*Crotalus unicolor*	Aruba Island - London Zoo C1693	12 32 00 N, 69 56 00 W
213	*Crotalus d. culminatus*	London Zoo C229	
254-262	*Crotalus durissus tzabcan*	Xaibe, Corozal, Belize	18 27 00 N, 88 25 00 W
303	*Crotalus durissus ssp.*	Alto Paraiso GO -	15 17 00 N, 47 38 00 W
316	*Crotalus durissus ssp.*	Pontes e Lacerda MT	15 44 00 S, 59 50 00 W
317	*Crotalus durissus ssp.*	Ribeirvo Cascalheira MT	14 35 00 S, 51 23 00 W
318	*Crotalus durissus ssp.*	BrasPlia DF	15 46 00 S, 47 47 00 W
319	*Crotalus durissus ssp.*	Svo Desid9rio BA	12 11 00 S, 44 54 00 W
320	*Crotalus durissus ssp.*	Bel9m do Svo Francisco	08 41 00 S, 38 57 00 W
321	*Crotalus durissus ssp.*	Guanambi BA	14 08 00 S, 42 42 00 W
346-353	*Crotalus d. cascavella*	Grajau, MA	05 44 00 S, 46 07 00 W
626-635	*Crotalus d. cascavella*	Grajau, MA,	05 44 00 S, 46 07 00 W
664-667	*Crotalus d. collilineatus*	Alto da Boa Vista, MT	22 13 00 S, 43 32 00 W
775	*Crotalus d.*	La Guaira, D.F., Venezuela	10 34 00 N, 67 02 00 W

	cumanensis		
	Crotalus d.		
780	*cumanensis*	Distrito Colina, Falc.∴n, Venezuela	11 19 00 N, 69 37 00 W
	Crotalus d.		
781	*cumanensis*	Distrito Colina, Falc.∴n, Venezuela	11 19 00 N, 69 37 00 W
	Crotalus d.		
782	*cumanensis*	Paraguan<, Falc.∴n, Venezuela	11 54 00 N, 69 59 00 W
	Crotalus d.		
788	*cumanensis*	Curimagua, Falc.∴n, Venezuela	11 12 00 N, 69 45 00 W
	Crotalus d.		
789	*cumanensis*	Las Ventosas, Falc.∴n, Venezuela	11 27 00 N, 69 33 00 W
	Crotalus d.		
790	*cumanensis*	Las Ventosas, Falc.∴n, Venezuela	11 27 00 N, 69 33 00 W
	Crotalus d.		
791	*cumanensis*	Las Ventosas, Falc.∴n, Venezuela	11 27 00 N, 69 33 00 W
833	*Crotalus vegrandis*	London Zoo female	
906	*Crotalus d. terrificus*	Arapoti,PR	24 08 00 S, 49 04 00 W
907	*Crotalus d. terrificus*	Arapoti, PR	24 08 00 S, 49 04 00 W
908	*Crotalus d. terrificus*	Arapoti, PR	24 08 00 S, 49 04 00 W
SAL01	*C. durissus durissus*	San Salvador, El Salvador	13 48 00 N, 89 10 00 W
SAL02	*C. durissus durissus*	San Salvador, El Salvador	13 48 00 N, 89 10 00 W
SAL03	*C. durissus durissus*	San Salvador, El Salvador	13 48 00 N, 89 10 00 W
SAL04	*C. durissus durissus*	San Salvador, El Salvador	13 48 00 N, 89 10 00 W

APPENDIX 3. Matrix of pairwise differences (average + SE) using Kimura 2-parameter for the combined Cytb-ND4 sequences.

	1	2	3	4	5	6	7	8	9	10
C scutulatus	-									
C. viridisCO	0,0108	-								
C. viridisAZ	0,0117	0,0027	-							
CDtoto_Tamp	0,1130	0,1121	0,1112	-						
CDtoto_Quet	0,1176	0,1167	0,1158	0,0206	-					
CDcul_Puebl	0,1333	0,1324	0,1315	0,1062	0,1117	-				
C. unicolor	0,1429	0,1438	0,1429	0,1141	0,1214	0,0812				
C. vegrandis	0,1341	0,1349	0,1341	0,0998	0,1089	0,0795	0,0306	0,0000		
CDD_cateMX	0,1283	0,1274	0,1265	0,1040	0,1104	0,0666	0,0629	0,0620	0,0000	
CBBguadal	0,1117	0,1117	0,1108	0,0703	0,0794	0,1040	0,1056	0,0939	0,1027	0,0000
CDcolB_ABoaBZ	0,1332	0,1341	0,1332	0,1062	0,1126	0,0740	0,0279	0,0316	0,0540	0,0994
CDT_GuarBZ	0,1447	0,1456	0,1447	0,1127	0,1201	0,0828	0,0365	0,0420	0,0655	0,1106
CDT_SLParBZ	0,1399	0,1390	0,1381	0,1085	0,1158	0,0801	0,0315	0,0333	0,0610	0,1054
CDT_RosBZ	0,1320	0,1311	0,1302	0,1041	0,1096	0,0802	0,0315	0,0360	0,0593	0,0992
CDT_PindBZ	0,1318	0,1309	0,1300	0,1049	0,1113	0,0720	0,0252	0,0306	0,0511	0,0991
CM_MaricpAZ	0,1106	0,1097	0,1106	0,0737	0,0846	0,1083	0,1054	0,0965	0,1043	0,0659
CM_SnBernAZ	0,1089	0,1080	0,1089	0,0828	0,0955	0,1094	0,1064	0,1011	0,1116	0,0732
CM_LUvasNM	0,1125	0,1098	0,1107	0,0773	0,0891	0,1075	0,1073	0,0992	0,1071	0,0768
CM_QuertMX	0,1089	0,1089	0,1098	0,0783	0,0856	0,1012	0,1046	0,0992	0,1017	0,0786
CDcul_IxtMX	0,1373	0,1365	0,1356	0,1120	0,1184	0,0235	0,0842	0,0787	0,0587	0,1079
CDD_ChisMX	0,1283	0,1274	0,1265	0,1040	0,1104	0,0666	0,0629	0,0620	0,0000	0,1027
CDtz_Bez	0,1303	0,1294	0,1303	0,1033	0,1061	0,0767	0,0747	0,0712	0,0728	0,0984
CMO_OaxMX	0,1140	0,1131	0,1122	0,0877	0,0806	0,1153	0,1106	0,1043	0,1103	0,0907
CD_PLaBZ	0,1327	0,1318	0,1327	0,1014	0,1104	0,0756	0,0306	0,0360	0,0565	0,1009
CD_RCasBZ	0,1316	0,1316	0,1307	0,1063	0,1136	0,0750	0,0271	0,0316	0,0541	0,1004
CD_BelmBZ	0,1329	0,1320	0,1311	0,1050	0,1114	0,0721	0,0261	0,0315	0,0539	0,1000
CD_GuanBZ	0,1375	0,1366	0,1357	0,1045	0,1128	0,0758	0,0301	0,0365	0,0564	0,1023
CDcul_MorMX	0,1365	0,1356	0,1347	0,1077	0,1123	0,0108	0,0783	0,0765	0,0709	0,1055
CDcas_GrajBZ	0,1370	0,1360	0,1351	0,1054	0,1135	0,0759	0,0298	0,0361	0,0567	0,1049
CDcol_ABoaBZ	0,1349	0,1358	0,1349	0,1078	0,1142	0,0746	0,0272	0,0336	0,0543	0,1009
CDcuman	0,1416	0,1407	0,1398	0,1109	0,1182	0,0833	0,0163	0,0235	0,0640	0,1069
CBB_NaytMX	0,1175	0,1175	0,1166	0,0753	0,0808	0,1054	0,1087	0,0971	0,1058	0,0171
CDTr_ArapBZ	0,1320	0,1311	0,1302	0,1050	0,1114	0,0721	0,0252	0,0306	0,0512	0,0992
CM_EdomexMX	0,1193	0,1184	0,1166	0,0906	0,0933	0,1135	0,1070	0,1061	0,1139	0,0839
ElSalvador	0,1292	0,1283	0,1274	0,1076	0,1140	0,0666	0,0611	0,0612	0,0081	0,1036
CDdry_Guyana	0,1401	0,1392	0,1383	0,1114	0,1187	0,0821	0,0153	0,0243	0,0647	0,1055

11	12	13	14	15	16	17	18	19	20	21	22	23

0,0000												
0,0164	0,0000											
0,0153	0,0137	0,0000										
0,0144	0,0237	0,0162	0,0000									
0,0063	0,0173	0,0117	0,0090	0,0000								
0,0993	0,1113	0,1061	0,0954	0,0989	0,0000							
0,1038	0,1142	0,1107	0,1027	0,1035	0,0208	0,0000						
0,1029	0,1132	0,1098	0,1009	0,1025	0,0289	0,0190	0,0000					
0,1011	0,1113	0,1044	0,1027	0,1008	0,0686	0,0740	0,0741	0,0000				
0,0761	0,0850	0,0822	0,0804	0,0750	0,1105	0,1124	0,1115	0,0979	0,0000			
0,0540	0,0655	0,0610	0,0593	0,0511	0,1043	0,1116	0,1071	0,1017	0,0587	0,0000		
0,0676	0,0809	0,0773	0,0737	0,0674	0,0982	0,1037	0,1045	0,0974	0,0770	0,0728	0,0000	
0,1098	0,1181	0,1166	0,1086	0,1085	0,0789	0,0844	0,0826	0,0789	0,1175	0,1103	0,1033	0,0000
0,0135	0,0237	0,0170	0,0180	0,0090	0,0971	0,1017	0,1007	0,1008	0,0786	0,0565	0,0701	0,1103
0,0054	0,0201	0,0126	0,0117	0,0054	0,1003	0,1049	0,1039	0,1012	0,0771	0,0541	0,0659	0,1108
0,0090	0,0146	0,0090	0,0135	0,0045	0,1017	0,1054	0,1045	0,1026	0,0760	0,0539	0,0701	0,1112
0,0137	0,0212	0,0154	0,0191	0,0109	0,1040	0,1078	0,1049	0,1031	0,0796	0,0564	0,0701	0,1107
0,0711	0,0799	0,0772	0,0772	0,0691	0,1098	0,1117	0,1108	0,1027	0,0253	0,0709	0,0774	0,1168
0,0154	0,0211	0,0171	0,0207	0,0135	0,1020	0,1066	0,1030	0,1013	0,0789	0,0567	0,0703	0,1099
0,0046	0,0175	0,0154	0,0163	0,0081	0,1016	0,1053	0,1044	0,1027	0,0767	0,0543	0,0681	0,1113
0,0298	0,0366	0,0325	0,0325	0,0279	0,1013	0,1040	0,1049	0,1050	0,0827	0,0640	0,0786	0,1118
0,1026	0,1146	0,1085	0,1023	0,1022	0,0719	0,0774	0,0818	0,0819	0,1075	0,1058	0,0979	0,0895
0,0063	0,0173	0,0117	0,0090	0,0000	0,0990	0,1036	0,1027	0,1009	0,0750	0,0512	0,0674	0,1086
0,1062	0,1155	0,1112	0,1077	0,1058	0,0647	0,0657	0,0675	0,0270	0,1075	0,1139	0,1043	0,0841
0,0504	0,0610	0,0565	0,0548	0,0484	0,1052	0,1125	0,1080	0,1008	0,0560	0,0081	0,0719	0,1103
0,0306	0,0383	0,0332	0,0333	0,0288	0,1018	0,1037	0,1036	0,1054	0,0833	0,0647	0,0801	0,1133

24	25	26	27	28	29	30	31	32	33	34	35	36

0,0000												
0,0117	0,0000											
0,0117	0,0081	0,0000										
0,0182	0,0128	0,0082	0,0000									
0,0727	0,0721	0,0692	0,0728	0,0000								
0,0207	0,0172	0,0108	0,0082	0,0740	0,0000							
0,0154	0,0100	0,0100	0,0165	0,0717	0,0155	0,0000						
0,0333	0,0290	0,0289	0,0348	0,0804	0,0317	0,0301	0,0000					
0,1040	0,1036	0,1032	0,1055	0,1051	0,1081	0,1041	0,1100	0,0000				
0,0090	0,0054	0,0045	0,0109	0,0692	0,0135	0,0081	0,0280	0,1023	0,0000			
0,1076	0,1063	0,1078	0,1083	0,1131	0,1063	0,1078	0,1055	0,0861	0,1059	0,0000		
0,0538	0,0505	0,0512	0,0528	0,0709	0,0532	0,0498	0,0622	0,1067	0,0485	0,1130	0,0000	
0,0341	0,0298	0,0297	0,0355	0,0810	0,0325	0,0309	0,0027	0,1087	0,0288	0,1060	0,0629	

APPENDIX 4. Pairwise distances of the AFLP patterns between taxa.

Below diagonal: Total character differences; Above diagonal: Mean character differences

Label	Locality	1	2	3	4
Ctx	Sonora,MX	-	0,06855	0,42742	0,42742
CPY	Michoacan,MX	17	-	0,39113	0,39113
Cd001	Veracruz,MX	106	97	-	0,01613
Cd002	Puebla,MX	106	97	4	-
Cd004	Puebla,MX	107	98	3	3
cdcul	Puebla,MX	104	95	8	8
Cdg1	Veracruz,MX	104	95	8	6
SD	Tamaulipas,MX	124	111	66	70
SA	Tamaulipas,MX	126	117	70	68
Cd 3102	Queretaro, MX	110	101	56	54
Cd 1566	Queretaro, MX	86	89	86	84
Cd 1576	Ixtapan, MX	111	106	27	27
Cd 2065	Chiapas, MX	113	104	23	21
Cd 2067	Chiapas, MX	109	104	27	27
Cd 2072	Chiapas, MX	114	105	24	22
Cd 255	Corozal, Belize	92	81	64	66
Cd 259	Corozal, Belize	104	93	76	76
SAL1	El Salvador	120	111	42	42
SAL2	El Salvador	114	107	44	46
SAL6	El Salvador	119	110	39	39
Cd 316	Pontes e Lacerda BZ	79	66	81	81
Cd 317	Ribeirvo Cascal. BZ	82	69	88	88
Cd 318	BrasPlia, BZ	93	80	93	93
Cd 319	Svo Desid9rio BZ	91	76	93	93
Cd 320	Bel9m S. Francis. BZ	80	65	88	86
Cd 321	Guanambi BZ	72	59	90	92
Cd 346	Grajau, BZ	85	72	93	93
Cd 349	Grajau, BZ	88	73	88	88
Cd 352	Grajau, BZ	101	86	101	101
Cd 353	Grajau, BZ	95	82	87	87
Cd 630	Grajau, BZ	85	72	85	85
Cd 635	Grajau, BZ	86	73	90	90
Cd 667	Alto da Boa Vista, BZ	83	68	75	75
Cd 775	La Guaira, Venezuela	85	70	83	81
Cd 780	Falc.·.n, Venezuela	90	77	86	86
Cd 781	Falc.·.n, Venezuela	91	76	81	81
Cd 906	Arapoti,BZ	95	84	89	89
Cd 907	Arapoti,BZ	101	88	95	95
Cd 908	Arapoti,BZ	97	86	95	95
Cd 1043	SE Guyana	104	89	86	86
Cd 1092	SE Guyana	99	84	85	85
Cd 1095	SE Guyana	88	73	78	78
C.vegrand	Venezuela	109	94	93	93
Cd 212	Aruba I.	96	85	86	86

5	6	7	8	9	10	11
0,43145	0,41935	0,41935	0,5	0,50806	0,44355	0,34677
0,39516	0,38306	0,38306	0,44758	0,47177	0,40726	0,35887
0,0121	0,03226	0,03226	0,26613	0,28226	0,22581	0,34677
0,0121	0,03226	0,02419	0,28226	0,27419	0,21774	0,33871
-	0,02823	0,03629	0,27016	0,27823	0,21371	0,34274
7 -		0,03226	0,25806	0,28226	0,21774	0,35484
9	8 -		0,27419	0,27419	0,21774	0,33871
67	64	68 -		0,08065	0,10484	0,41935
69	70	68	20 -		0,09677	0,41129
53	54	54	26	24 -		0,3629
85	88	84	104	102	90 -	
26	29	27	79	83	65	87
22	23	21	79	79	63	83
26	29	27	79	79	61	83
23	24	22	78	78	62	86
63	64	62	100	104	96	106
77	78	76	100	100	100	108
41	42	38	88	90	70	86
43	44	42	88	90	68	88
40	41	39	85	89	73	87
82	85	81	99	103	91	107
89	92	86	102	102	94	108
94	95	95	107	105	95	113
94	93	91	109	109	97	111
87	86	82	106	110	96	108
89	90	88	102	108	92	110
94	95	93	101	105	105	107
89	90	84	108	108	102	112
102	99	95	111	115	113	133
88	91	85	103	105	105	113
86	89	85	105	107	105	107
91	94	90	110	114	108	112
76	79	75	97	101	93	107
84	83	83	103	99	91	105
87	88	86	104	100	102	108
82	83	77	101	99	95	105
90	91	87	97	105	99	111
94	95	91	103	111	105	121
96	97	93	107	115	103	117
87	86	88	100	104	98	118
86	87	85	95	99	97	111
77	76	76	96	102	94	114
94	99	93	117	113	115	123
85	86	86	108	114	106	112

12	13	14	15	16	17	18
0,43952	0,45968	0,37097	0,41935	0,45968	0,45968	0,47984
0,41935	0,42339	0,32661	0,375	0,41532	0,43145	0,44355
0,10887	0,09677	0,25806	0,30645	0,29032	0,17742	0,15726
0,10887	0,08871	0,26613	0,30645	0,29032	0,18548	0,15726
0,10484	0,09274	0,25403	0,31048	0,29435	0,17339	0,16129
0,11694	0,09677	0,25806	0,31452	0,31452	0,17742	0,16532
0,10887	0,08871	0,25	0,30645	0,29839	0,16935	0,15726
0,31855	0,31452	0,40323	0,40323	0,43548	0,35484	0,34274
0,31855	0,31452	0,41935	0,40323	0,42742	0,3629	0,35887
0,24597	0,25	0,3871	0,40323	0,42742	0,27419	0,29435
0,33468	0,34677	0,42742	0,43548	0,42742	0,35484	0,35081
0,03226	0,03629	0,29435	0,31855	0,34274	0,10887	0,12097
0,03226	0,0121	0,28629	0,31048	0,33468	0,10887	0,09677
-	0,02823	0,30242	0,32661	0,35887	0,10887	0,12903
7 -		0,29032	0,29839	0,33871	0,1129	0,10887
75	72 -		0,09677	0,12903	0,26613	0,27016
81	74	24 -		0,16129	0,28226	0,27016
25	22	64	66	82	0,03226	0,04435
27	28	66	70	86 -		0,05242
32	27	67	67	81	13 -	
92	89	55	61	69	85	80
93	94	52	60	66	90	89
96	99	67	69	73	87	88
102	99	67	69	75	89	90
95	92	52	62	66	84	85
93	98	54	70	74	84	91
102	101	63	59	77	97	90
97	92	58	58	74	82	79
112	109	65	73	75	103	100
102	97	61	61	67	95	88
98	97	61	61	73	89	84
107	102	62	66	72	94	91
92	87	51	57	61	85	78
98	91	65	65	73	89	84
99	94	56	54	62	90	85
96	91	59	61	71	83	82
98	93	63	67	79	87	86
104	99	61	71	71	93	90
102	99	63	67	75	95	94
101	94	66	64	78	92	89
102	97	67	67	73	93	88
95	90	52	60	68	86	85
104	101	67	71	73	93	90
95	94	62	74	78	92	93

19	20	21	22	23	24	25	26
0,31855	0,33065	0,375	0,36694	0,32258	0,29032	0,34274	0,35484
0,26613	0,27823	0,32258	0,30645	0,2621	0,2379	0,29032	0,29435
0,32661	0,35484	0,375	0,375	0,35484	0,3629	0,375	0,35484
0,32661	0,35484	0,375	0,375	0,34677	0,37097	0,375	0,35484
0,33065	0,35887	0,37903	0,37903	0,35081	0,35887	0,37903	0,35887
0,34274	0,37097	0,38306	0,375	0,34677	0,3629	0,38306	0,3629
0,32661	0,34677	0,38306	0,36694	0,33065	0,35484	0,375	0,33871
0,39919	0,41129	0,43145	0,43952	0,42742	0,41129	0,40726	0,43548
0,41532	0,41129	0,42339	0,43952	0,44355	0,43548	0,42339	0,43548
0,36694	0,37903	0,38306	0,39113	0,3871	0,37097	0,42339	0,41129
0,43145	0,43548	0,45565	0,44758	0,43548	0,44355	0,43145	0,45161
0,37097	0,39919	0,40323	0,41935	0,36694	0,38306	0,41129	0,375
0,35484	0,375	0,39516	0,40323	0,36694	0,39113	0,40323	0,36694
0,37097	0,375	0,3871	0,41129	0,38306	0,375	0,41129	0,39113
0,35887	0,37903	0,39919	0,39919	0,37097	0,39516	0,40726	0,37097
0,22177	0,20968	0,27016	0,27016	0,20968	0,21774	0,25403	0,23387
0,24597	0,24194	0,27823	0,27823	0,25	0,28226	0,2379	0,23387
0,33468	0,3629	0,35081	0,35887	0,33065	0,35484	0,375	0,33065
0,34274	0,3629	0,35081	0,35887	0,33871	0,33871	0,39113	0,33065
0,32258	0,35887	0,35484	0,3629	0,34274	0,36694	0,3629	0,31855
-	0,06048	0,12903	0,1129	0,06048	0,05242	0,1129	0,125
15 -		0,14113	0,125	0,08065	0,07258	0,125	0,14516
32	35 -		0,16129	0,125	0,14113	0,20161	0,20565
28	31	40 -		0,125	0,11694	0,16935	0,17339
15	20	31	31 -		0,06452	0,14919	0,12903
13	18	35	29	16 -		0,13306	0,1371
28	31	50	42	37	33 -		0,08468
31	36	51	43	32	34	21 -	
42	47	56	52	43	43	38	33
36	41	58	48	41	43	28	29
26	31	44	42	33	33	16	17
27	36	51	45	34	34	19	22
24	27	42	36	29	29	26	31
30	35	42	36	35	39	36	37
29	32	47	39	34	36	33	38
34	39	52	42	37	41	42	43
44	49	62	56	51	51	42	45
38	41	58	56	39	41	52	51
38	41	56	48	41	41	50	45
43	52	61	45	50	52	47	52
40	43	54	44	43	45	46	47
31	34	45	37	32	36	37	36
56	55	66	66	61	63	62	55
51	60	61	69	54	54	55	58

27	28	29	30	31	32	33	34
0,40726	0,38306	0,29435	0,32661	0,33468	0,3629	0,36694	0,43548
0,34677	0,33065	0,23387	0,26613	0,27419	0,31048	0,30645	0,375
0,40726	0,35081	0,32661	0,32661	0,30242	0,34677	0,32661	0,34677
0,40726	0,35081	0,31855	0,33468	0,30242	0,34677	0,32661	0,34677
0,41129	0,35484	0,33065	0,33065	0,30645	0,35081	0,33065	0,35081
0,39919	0,36694	0,32661	0,33468	0,31855	0,35484	0,33468	0,35484
0,38306	0,34274	0,31048	0,33468	0,30242	0,34677	0,31048	0,33871
0,44758	0,41532	0,42339	0,39919	0,39113	0,41935	0,40726	0,41129
0,46371	0,42339	0,43952	0,41532	0,40726	0,40323	0,39919	0,43548
0,45565	0,42339	0,39113	0,39113	0,375	0,41129	0,38306	0,40323
0,53629	0,45565	0,43145	0,41532	0,43145	0,43548	0,42339	0,5
0,44355	0,39516	0,3629	0,37903	0,35484	0,40726	0,3871	0,38306
0,43548	0,3871	0,35484	0,35484	0,34677	0,38306	0,3629	0,36694
0,45161	0,41129	0,37903	0,3871	0,37097	0,39919	0,3871	0,39113
0,43952	0,39113	0,35887	0,36694	0,35081	0,37903	0,36694	0,37097
0,2621	0,24597	0,20565	0,20565	0,20565	0,22581	0,2379	0,25806
0,29435	0,24597	0,24597	0,2379	0,22984	0,21774	0,24597	0,26613
0,41532	0,375	0,32661	0,32661	0,33468	0,3629	0,32661	0,35484
0,41532	0,38306	0,33468	0,33468	0,34274	0,3629	0,33468	0,37097
0,40323	0,35484	0,31452	0,32258	0,31452	0,34274	0,33065	0,34274
0,16935	0,14516	0,06452	0,08871	0,09677	0,11694	0,1371	0,18952
0,18952	0,16532	0,10081	0,10887	0,10887	0,12903	0,15726	0,20968
0,22581	0,23387	0,15323	0,16129	0,16935	0,18952	0,20968	0,2379
0,20968	0,19355	0,12903	0,15323	0,14516	0,15726	0,16935	0,18952
0,17339	0,16532	0,06855	0,10887	0,11694	0,1371	0,14919	0,19355
0,17339	0,17339	0,08468	0,10887	0,11694	0,14516	0,16532	0,20968
0,15323	0,1129	0,10484	0,10484	0,10484	0,13306	0,16935	0,20565
0,13306	0,11694	0,09274	0,13306	0,125	0,15323	0,17339	0,16935
-	0,15323	0,14516	0,18548	0,18548	0,18145	0,21774	0,20565
38 -		0,12097	0,14516	0,14516	0,15726	0,16935	0,18952
40	28	0,08871	0,10484	0,08871	0,14113	0,15323	0,19758
37	25	0,08468	0,09274	0,10081	0,1371	0,15726	0,19355
46	36	14	16 -		0,10081	0,12097	0,17339
54	42	22	28	30	0,10887	0,12097	0,15726
45	39	27	29	25 -		0,10887	0,15323
54	42	26	32	30	27 -		0,14919
60	52	44	42	46	53	50	59
54	48	44	44	46	55	56	69
56	54	42	44	44	57	58	63
59	49	41	41	45	36	35	44
54	44	34	34	36	29	32	37
47	35	25	29	27	28	27	32
58	52	48	52	50	51	52	53
63	59	47	51	45	52	53	54

35	36	37	38	39	40	41	42
0,41129	0,40726	0,39113	0,41935	0,39919	0,35484	0,43952	0,3871
0,35081	0,35484	0,34677	0,35887	0,33871	0,29435	0,37903	0,34274
0,37097	0,38306	0,38306	0,34677	0,34274	0,31452	0,375	0,34677
0,37097	0,38306	0,38306	0,34677	0,34274	0,31452	0,375	0,34677
0,375	0,37903	0,3871	0,35081	0,34677	0,31048	0,37903	0,34274
0,37903	0,38306	0,39113	0,34677	0,35081	0,30645	0,39919	0,34677
0,37097	0,36694	0,375	0,35484	0,34274	0,30645	0,375	0,34677
0,41129	0,41532	0,43145	0,40323	0,38306	0,3871	0,47177	0,43548
0,42742	0,44758	0,46371	0,41935	0,39919	0,41129	0,45565	0,45968
0,41935	0,42339	0,41532	0,39516	0,39113	0,37903	0,46371	0,42742
0,44355	0,4879	0,47177	0,47581	0,44758	0,45968	0,49597	0,45161
0,42339	0,41129	0,40323	0,40726	0,40323	0,375	0,42742	0,375
0,39919	0,39516	0,39516	0,38306	0,3871	0,35887	0,40323	0,38306
0,43145	0,41935	0,41129	0,40726	0,41129	0,38306	0,41935	0,38306
0,41129	0,39919	0,39919	0,37903	0,39113	0,3629	0,40726	0,37903
0,28226	0,24597	0,25403	0,26613	0,27016	0,20968	0,27016	0,25
0,29032	0,28629	0,27016	0,25806	0,27016	0,24194	0,28629	0,29839
0,37903	0,36694	0,375	0,35484	0,375	0,33871	0,38306	0,3629
0,37903	0,375	0,38306	0,37097	0,375	0,34677	0,375	0,37097
0,35081	0,3629	0,37903	0,35887	0,35484	0,34274	0,3629	0,375
0,16532	0,15323	0,15323	0,17339	0,16129	0,125	0,22581	0,20565
0,18548	0,16532	0,16532	0,20968	0,17339	0,1371	0,22177	0,24194
0,24597	0,23387	0,22581	0,24597	0,21774	0,18145	0,26613	0,24597
0,21371	0,22581	0,19355	0,18145	0,17742	0,14919	0,26613	0,27823
0,20161	0,15726	0,16532	0,20161	0,17339	0,12903	0,24597	0,21774
0,20161	0,16532	0,16532	0,20968	0,18145	0,14516	0,25403	0,21774
0,18145	0,20968	0,20161	0,18952	0,18548	0,14919	0,25	0,22177
0,20968	0,20565	0,18145	0,20968	0,18952	0,14516	0,22177	0,23387
0,2379	0,21774	0,22581	0,2379	0,21774	0,18952	0,23387	0,25403
0,20565	0,19355	0,21774	0,19758	0,17742	0,14113	0,20968	0,2379
0,20565	0,19355	0,19355	0,19758	0,18548	0,13306	0,20968	0,21371
0,20968	0,19758	0,19758	0,18548	0,18145	0,1371	0,22177	0,21774
0,17339	0,18548	0,17742	0,18145	0,14516	0,10887	0,20161	0,18145
0,125	0,25	0,23387	0,125	0,12097	0,11694	0,20161	0,21371
0,14516	0,22177	0,22984	0,14516	0,11694	0,1129	0,20565	0,20968
0,125	0,22581	0,23387	0,14113	0,12903	0,10887	0,20968	0,21371
53	0,16129	0,1371	0,18145	0,22581	0,19758	0,27419	0,27823
61 -		0,10484	0,20565	0,21774	0,18952	0,26613	0,27016
63	26 -		0,18145	0,20968	0,20565	0,28226	0,30242
38	51	45 -		0,13306	0,12097	0,25403	0,28226
37	54	52	33 -		0,09274	0,20968	0,2379
36	47	51	30	23 -		0,18952	0,20161
57	66	70	63	52	47 -		0,18145
52	67	75	70	59	50	45 -	