## Bangor University

## DOCTOR OF PHILOSOPHY

## Molecular phylogeography of the neotropical rattlesnake Crotalus durissus

## Quijada Mascarenãs., Jesus Adrián

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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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## 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 $\beta$-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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# 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 treebuilding, 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 ( $\mathrm{NJ} \mathrm{)} \mathrm{performs} \mathrm{a} \mathrm{cluster}$ 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 PCRpreamplification 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 (RangelSantos 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 $0.1-0.2 \mathrm{ml}$ of blood was extracted and then flushed into a cell-lysis solution ( $100 \mu \mathrm{l}$ of blood in $2-3 \mathrm{mls} 2 \%$ SDS, 100 mM Tris, 100 mM 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 $60{ }^{\circ} \mathrm{C}$ overnight and then transferred to ice. The samples were then incubated with RNase for 30 min . at $37^{\circ} \mathrm{C}$, and then transferred to ice. Ice-cold 5 M 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 $25 \mathrm{ml}, 1.4 \%$ agarose plates incorporating $3 \mu \mathrm{l}$ ethidium bromide.

PCR

The Cyt-b and ND4 fragments of the mitochondrial DNA were amplified from $1-2 \mathrm{ml}$ 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 ( $5^{\prime}$-CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA-3'), and Leu ( $5^{\prime}$ 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 $-\mathrm{HCl}, 0.5 \mathrm{mM} \mathrm{MgCl}, 0.4 \mathrm{mM}$ dNTP, 2 units of Taq DNA polymerase, and $0.5 \%$ DMSO. Amplification conditions involved an initial denaturation step of 4 minutes at 94 ${ }^{\circ} \mathrm{C}, 35$ cycles of denaturation for 1 minute at $94^{\circ} \mathrm{C}$, primer annealing for 1 minute at 50 ${ }^{\circ} \mathrm{C}$, extension for 2 minutes at $72^{\circ} \mathrm{C}$, and finally, an extra extension step for 3 minutes at $72{ }^{\circ} \mathrm{C}$, cooling to $4^{\circ} \mathrm{C}$. 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 $10 \mu \mathrm{l}$ reactions containing a 50 ng PCR template, 0.16 pmol of $5^{\prime}$ 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 $94{ }^{\circ} \mathrm{C}, 50$ cycles of 10 seconds at $90^{\circ} \mathrm{C}, 5$ seconds at $50{ }^{\circ} \mathrm{C}, 4$ minutes at $60{ }^{\circ} \mathrm{C}$, and then cooled to $4^{\circ} \mathrm{C}$. The samples were precipitated using 2 M sodium acetate and $100 \%$ ethanol, and the resulting pellet was washed in $70 \%$ ethanol and air dried. The samples were suspended in $4 \mu$ of a dextran blue EDTA/formamide loading buffer, and $1 \mu \mathrm{l}$ 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 Greene 1997).

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 4.0 b 2 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 unweighted trees, was significantly skewed to the left in both regions (Cyt-b, $\mathrm{gl}=-0.6492$, $\mathrm{p}<0.01 ;$ ND4, $\mathrm{g} 1=-0.6262, \mathrm{p}<0.01$ ), 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 $213 \mathrm{bp}(30.9 \%)$ 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 $=380 ; \mathrm{CI}=0.6658 ; \mathrm{HI}=0.3342$; $\mathrm{RI}=0.7617 ; \mathrm{RC}=0.5071$ ). The analysis of ND4 produced 20 equally parsimonious trees
(tree length $=434 ; \mathrm{CI}=0.5115 ; \mathrm{HI}=0.4885 ; \mathrm{RI}=0.8203 ; \mathrm{RC}=0.4196$ ). 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 ( $\mathrm{Z}=-$ $0.06, \mathrm{P}>0.05$ ). The tests do not support the conventional species and subspecies
categorization of the South American rattlesnakes $(\mathrm{Z}=-5.2, \mathrm{P}<0.003)$, 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 ( $\mathrm{Z}=-4.8, \mathrm{P}<0.005$ ).

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.


## Table 2.1 (Cont.)

$\begin{array}{lllllllllllllllllllllllllll}666 & 666 & 777 & 777 & 777 & 777 & 777 & 777 & 777 & 777 & 788 & 888 & 888 & 888 & 889 & 999\end{array}$ $\begin{array}{lllllllllllllllllllllllll}789 & 999 & 000 & 111 & 112 & 223 & 445 & 567 & 778 & 889 & 900 & 113 & 334 & 666 & 780 & 001\end{array}$ $\begin{array}{lllllllllllllllllllll}732 & 458 & 147 & 035 & 692 & 584 & 392 & 540 & 692 & 581 & 709 & 250 & 398 & 036 & 242 & 581\end{array}$ Cscutulnm GTC TCA CCA GAT TAC CCC GCG ACC TTC TAC AAC CCA CGC ACC CCA TCG
CvdsCO
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 | 999 | 999 | 999 | 999 | 999 | $\begin{array}{ll} 111 & 111 \\ 000 & 000 \end{array}$ | $\begin{aligned} & 111 \\ & 000 \end{aligned}$ | $\begin{aligned} & 111 \\ & 000 \end{aligned}$ | $\begin{aligned} & 111 \\ & 000 \end{aligned}$ | $111$ | $\begin{aligned} & 111 \\ & 000 \end{aligned}$ | $\begin{aligned} & 111 \\ & 000 \end{aligned}$ | 111 |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | 122 | 333 | 445 | 566 | 688 | 889 | 011122 | 233 | 344 | 445 | 667 | 777 | 888 | 999 |
|  | 706 | 258 | 470 | 325 | 806 | 898 | 736915 | 814 | 736 | 892 | 140 | 368 | 258 |  |
| Cscutulnm | ATA | ACC | AAC | CAC G | GAA | TAT C | CCA CCA C | ССT | CCA T | TTA A | ACA | CAC | TT | CA |
| CvdsCO | G |  |  |  |  |  |  |  |  |  |  |  |  |  |
| CvdsAz | G |  |  |  |  |  |  |  |  |  |  |  |  |  |
| CDtotoTamp | . C. | .T. |  |  |  | A | C | . A | G |  |  | G. | C | T. |
| CDtotoQuet | . C | . T. | . |  |  | C | C . T. | TTA | G |  |  | G. | C | T. |
| CDculpuebl | GC. | . TA | G. . |  |  | . | C | . TA |  |  | T. | T.T | $A C$ ? |  |
| unicolor | . | TA |  | T |  | C . | C | . TA |  | . G | GT. | T | AC. | G. |
| vegrandis | . C . | . TA |  | T |  | C | C . TT | . TA |  | G | GT. | .T | C. | T. |
| CDDcateMX | GC. | . TA |  | . GT A | A. . | . C | C . . T | . TA |  |  | GT. | . T | C. | .T. |
| CBBguadal | . CG | .T. |  | T.. |  | C . | . C | . TA | . T. | . C. | T |  | CC | GT. |
| CDcolBABoabZ | GC. | . TA |  | T |  | C. | TC . . T | TA | .A. |  | T. | T | C. |  |
| CDTGuarBZ | GC. | . TA |  | T | A. . | ? | C .. T | . TA | GA. | . G | GT. | . T | C. |  |
| CDTSLParBZ | GC | . TA |  | T A | AG. | C | . TT | . TA | .A. . | G | GT. | T | C. |  |
| CDTRosBZ | GC. | . TA |  | T |  | C | T | . TA | . |  | 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 | . TA | . AG |  |  |  | CC | GTT |
| CMLUvasNM | . C | GT. | A |  |  | C | T | . TA | . AG | . C. |  |  | CC | TT |
| CMQuertMX | . C. | .T. |  |  | . G | C.C T | T.C.TT | TTA | . AG |  | G. . |  | ACC |  |
| CDculIxtMX | GC. | . TA | G. |  |  |  | C A.. . | . TA |  |  | T. | .T | ACC |  |
| CDDChismX | GC. | . TA |  | . GT |  | . C | C . . T | . TA |  |  | GT. . | . .T | C. | T. |
| CDtzBez | . C | . TA | . G . |  |  |  | C T.. . | . TA |  |  | GTG | . T | ACC |  |
| CMOOaxMX | . C | GTA | GG. |  |  |  |  | TA | A.G |  | . T . |  | AAC | G. |
| CDPLaBZ | GC. | . TA |  |  |  |  | . TT | . TA | . $A$ |  | .T. . | . T | C. |  |
| CDRCasBZ | GC. | . TA |  | T |  | C ? | ?TC .. T | . TA | . A . |  | GTG | . T | C. |  |
| CDBelmBZ | GC | A |  | T |  |  | T | . TA | . A. | G | GT. |  | C |  |
| CDGuanBZ | GC. | . TA |  | T |  | ? . | . TC . . T | . TA | . A. | . . G | GT. | . . T | $A C$. |  |
| CDculmormX | GC. | . TA | G. . |  |  | . | C A. | . TA |  |  | GT. | T.T | $A C$ ? |  |
| CDcasGrajBZ | GC | . TA |  | T |  | C | . C ...T . | .TA | . A . | G | GT. |  | AC. |  |
| CDColABoabZ | GC. | . TA |  | T |  | 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.. | . | . C | C | . TA | .T. | . C . | . 1. |  | . CC | GT. |
| CDTrArapBZ | GC. | . TA |  | T | A. . | . C | C .. T | . TA | . A . |  |  |  | C. |  |
| CMEdomexMX | . C. | .T. |  | A | AGG | C.C T | T.C T.T T | TTA | . AG |  | T. |  | ACC |  |
| ElSalvador | GC. | . TA |  | . GT A | A. . | C. C | . . C . . T | . TA |  |  | GT. | $\ldots \mathrm{T}$ | . C . |  |
| CddryGuyana | GC. | . TA |  | . .T A | 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)(EISalvador,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)(EISalvador,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 | $7.96 \neq 0.82$ | $7.49 \neq 0.79$ | $7.41 \neq 0.52$ |
| South America | $2.32 \neq 0.36$ | $1.19 \neq 0.24$ | $1.85 \neq 0.23$ |
| C. molossus - C. basiliscus | $7.43+0.81$ | $7.18+0.59$ | $7.20 \neq 0.51$ |
| Total | $7.86 \neq 0.75$ | $7.73 \neq 0.51$ | $7.73 \neq 0.52$ |



Tamura-Nei pairwise divergences
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 1 st and 2 nd 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, 7.76.6 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 Dloop 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 coworkers (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 $\beta$-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 $\beta$-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 $\beta$-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 $\beta$-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 $60^{\circ} \mathrm{C}$ and then transferred to ice. They were then incubated with RNase for 30 min . at $37{ }^{\circ} \mathrm{C}$ and then transferred to ice. Ice-cold 5 M 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 $25 \mathrm{ml}, 1.4 \%$ agarose plates with $3 \mu \mathrm{l}$ ethidium bromide.

## PCR

The 525 bp fragment of intron 7 of the $\beta$-fibrinogen region was amplified from 1-2 $\mu \mathrm{l}$ DNA extracts using modified primers (Prychitko and Moore 1997): Fib-B17U, 5'- GGA GAA AAC AGG ACA ATG ACA-3' and Fib-B17L, $5^{\prime}$-TCC CCA GTA GTA TCT GCC ATT $3^{\prime}$. The $50 \mu \mathrm{PCR}$ reactions were performed with a 50 ng template, $0.52 \mu \mathrm{M}$ primers, 20 mM tris- $\mathrm{HCl}, 0.5 \mathrm{mM} \mathrm{MgCl}, 0.4 \mu \mathrm{M} \mathrm{dNTP}, 2$ units Taq DNA polymerase, and $0.5 \%$ DMSO. Amplification conditions involved an initial denaturation step of 4 minutes at 94 ${ }^{\circ} \mathrm{C}, 35$ cycles of denaturation of 1 minute at $94^{\circ} \mathrm{C}$, primer annealing of 1 minute at $50^{\circ} \mathrm{C}$, extension of 2 minutes at $72{ }^{\circ} \mathrm{C}$, and at the end, an extra extension step for 3 minutes at 72 ${ }^{\circ} \mathrm{C}$, cooling to $4{ }^{\circ} \mathrm{C}$. 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 $10 \mu \mathrm{l}$ 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 $94^{\circ} \mathrm{C}, 50$ cycles of 10 seconds at $96^{\circ} \mathrm{C}, 5$ seconds at $50^{\circ} \mathrm{C}, 4$ minutes at 60 ${ }^{\circ} \mathrm{C}$, and then cooled to $4^{\circ} \mathrm{C}$. The samples were precipitated using 2 M sodium acetate and
$100 \%$ ethanol, and the resulting pellet was washed in $70 \%$ ethanol and air dried. The samples were suspended in $4 \mu$ l of a dextran blue EDTA/formamide loading buffer, and 1 $\mu \mathrm{l}$ 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 $\beta$-fibrinogen intron. Pairwise distances ranged from $0-1.2 \%$ in the in-groups and $1.64 \%$ in the outgroups, 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 $(\mathrm{g} 1=-0.492, \mathrm{p}<0.02)$, 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 $\beta$-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 $\beta$-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 $\beta$-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 $60^{\circ} \mathrm{C}$ overnight and then transferred to ice. Then the samples were incubated with RNase for 30 minutes at $37^{\circ} \mathrm{C}$ and transferred to ice. Ice-cold 5 M ammonium acetate and centrifugation was used to precipitate, and then pellet the proteins. DNA was then precipitated from the resulting supernatant with icecold 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 3 ml ethidium bromide. AFLP analysis was performed using the AFLP Analysis System I' 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, EAGG, 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 $(\mathrm{E}-\mathrm{AGG}+\mathrm{M}-\mathrm{CAT})$, and Primer Pair C $(\mathrm{E}-\mathrm{AGG}+\mathrm{M}-\mathrm{CTA})$.

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 $=\mathrm{Jcij}=\mathrm{a} /(\mathrm{a}+\mathrm{b}+\mathrm{c})$
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 Qmode 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-(Lat 1*24)))*SIN(RADIANS(90-(Lat2*24)))*
$\operatorname{COS}($ 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 $(\mathrm{g} 1=0.68, \mathrm{P}<0.001)$ 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 $=380 ; \mathrm{CI}=0.1741 ; \mathrm{HI}=0.8259 ; \mathrm{RI}=0.7263 ; \mathrm{RC}$ $=0.1264$ ).

Five clades were recognized from the bootstrap analysis including the molossusbasiliscus 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).

## PCOA



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 $(\mathrm{Km})$ 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 <br> analysis | porcentage of <br> variation on axis1 | porcentage of <br> variation on axis 2 | porcentage of <br> 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 | $\mathbf{g}$ | $\mathbf{Z}$ | $\mathbf{r}$ | $\mathbf{P}$ |
| :---: | :---: | :---: | :---: | :---: |
| 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 $\mathbf{r}$ | $\boldsymbol{\beta}$ | 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-CrotalusSistrurus "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$1.2 \mathrm{Mya})$ 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-offlight 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.

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## Appendix 2 List of samples used in the present study

| Label | Taxon | Locality |
| :---: | :---: | :---: |
| Cd001 | C. durissus durissus | Catemaco, Veracruz |
| Cd002 | C. durissus culminatus | El aguacate, Puebla |
| Cd003 | C. durissus culminatus | El aguacate, Puebla |
| Cd004 | C. durissus culminatus | El aguacate, Puebla |
| Cd005 | C. durissus culminatus | El aguacate, Puebla |
| CdSA | C.durissus totonacus | 30 Km SW Cd. Victoria, Tamaulipas |
| CdSA2 | C.durissus totonacus | 30 Km SW Cd. Victoria, <br> Tamaulipas |
| CdSD | C.durissus totonacus | Tamaulipas |
| RA | C. durissus culminatus | Sierra de Puebla, Puebla |
| RA3 | C. durissus culminatus | Sierra de Puebla, |
| Ctx 1 | C. atrox | Hermosillo, Sonora |
| Csct | C. scutulatus | Hermosillo, Sonora |
| CMI | C. molossus molossus | Sonoyta, Sonora |
| CM2 | C. molossus molossus | Sonoyta, Sonora |
| 1 HFC | C. molossus nigrescens | El Pedregal, Valle de Mexico |
| 2HFC | C. molossus nigrescens | El Pedregal, Valle de Mexico |
| 4HFC | C. molossus nigrescens | El Pedregal, Valle de Mexico |
| 5HFC | C. durissus culminatus | Sierra de Puebla, Puebla |
| ZGICdd | C. durissus durissus | Los Tuxtlas, Veracruz |
| ZG2Cdd | C. durissus durissus | Selva Lacandona, Chiapas |
| ZG3Cdd | C. durissus durissus | Paso del Toro, Veracruz |
| ZGCB1 | C. basiliscus | Guadalajara, Jalisco |
| ZGCB2 | C. basiliscus | Guadalajara, Jalisco |
| ZGCB3 | C. basiliscus | Guadalajara, Jalisco |
| ZGCCI | C. durissus culminatus | Oaxaca |
| ZGCC2 | C. durissus culminatus | Oaxaca |
| ZGCC3 | C. durissus culminatus | Oaxaca |
| CTSON | C. tigris | Hermosillo, Sonora |
| CTSON2 | C. tigris | Hermosillo, Sonora |
|  | C. molossus |  |
| 1567 | nigrescens | Queretaro, El Internado |
| 1576 | C. durissus culminatus | Edo. Mexico, Ixtapan |
| 2063 | C. durissus durissus | Chiapas, La Lacandona |
| 2065 | C. durissus durissus | Chiapas, La Lacandona |
| 2067 | C. durissus durissus | Chiapas, La Lacandona |
| 2072 | C. durissus durissus | Chiapas, La Lacandona |
| 3102 | C. durissus totonacus | Queretaro, Tomajo |
| cdculB | C. durissus culminatus | El aguacate, Puebla |
| Cdg1a | C. durissus durissus | Los Tuxtlas, Veracruz |
|  | C. molossus |  |
| 1566 | nigrescens | Queretaro, Cadereita |

Geographical cordinates

241600 N, 984100 W $184500 \mathrm{~N}, 981100 \mathrm{~W}$ $184500 \mathrm{~N}, 981100 \mathrm{~W}$ $184500 \mathrm{~N}, 981100 \mathrm{~W}$ $184500 \mathrm{~N}, 981100 \mathrm{~W}$
$234400 \mathrm{~N}, 984800 \mathrm{~W}$
$234400 \mathrm{~N}, 984800 \mathrm{~W}$ $233600 \mathrm{~N}, 983800 \mathrm{~W}$ 181600 N, 975800 W 181600 N, 975800 W 290600 N, 1105600 W 290600 N, 1105600 W 315800 N, 1131800 W 315800 N, 1131800 W 192100 N, 990900 W 192100 N, 990900 W 192100 N, 990900 W 184500 N, 981100 W 183500 N, 951900 W 173100 N, 914600 W 185900 N, 963300 W 203700 N, 1032400 W 203700 N, 1032400 W 203700 N, 1032400 W 174400 N, 974400 W $174400 \mathrm{~N}, 974400 \mathrm{~W}$ $174400 \mathrm{~N}, 974400 \mathrm{~W}$ 290600 N, 1105600 W 290600 N, 1105600 W
$204500 \mathrm{~N}, 1001900 \mathrm{~W}$ $184000 \mathrm{~N}, 993100 \mathrm{~W}$
$173100 \mathrm{~N}, 914600 \mathrm{~W}$ 173100 N, 914600 W $173100 \mathrm{~N}, 914600 \mathrm{~W}$ $173100 \mathrm{~N}, 914600 \mathrm{~W}$ 211700 N, 995100 W $184500 \mathrm{~N}, 981100 \mathrm{~W}$ 183500 N, 951900 W

202700 N, 1001900 W

| 822 | C. basiliscus | Nayarit, San Blas | 213600 N, 1051200 W |
| :---: | :---: | :---: | :---: |
| C128 | C.molossus molossus | San Bernandino, California | $340700 \mathrm{~N}, 1163400 \mathrm{~W}$ |
| C135 | C.molossus molossus | Las Uvas, New Mexico | $330400 \mathrm{~N}, 1074300 \mathrm{~W}$ |
| C136 | C. molossus molossus | Las Uvas, New Mexico | 330400 N, 1074300 W |
| C137 | C.molossus molossus | Maricopa, AZ | 331400 N, 1120600 W |
|  | C.molossus |  |  |
| CMN3 | nigrescens | El Pedregal, Valle de Mexico | $192100 \mathrm{~N}, 990900 \mathrm{~W}$ |
|  | C.molossus |  |  |
| CMN4 | nigrescens | El Pedregal, Valle de Mexico | $192100 \mathrm{~N}, 990900 \mathrm{~W}$ |
|  | C.molossus |  |  |
| CMN6 | nigrescens | El Pedregal, Valle de Mexico | 192100 N, 990900 W |
| 3014 | C. molossus oaxacus | Zapotitlan Salinas, Puebla | 182400 N, 972700 W |
|  | Crotalus durissus |  |  |
| 108 | terrificus | Jacarei SP | $231800 \mathrm{~S}, 460300 \mathrm{~W}$ |
|  | Crotalus durisuis |  |  |
| 109 | terrificus | Guaratinguet< | 224800 S, 451200 W |
|  | Crotalus durissus |  |  |
| 110 | terrificus | Roseira SP | 225400 S, 452100 W |
|  | Crotalus durissus |  |  |
| 120 | terrificus | Taubat9 SP | 230100 S, 453300 W |
|  | Crotalus durissus |  |  |
| 121 | terrificus | Svo Luiz do Paraitinga | 231500 S, 452700 W |
|  | Crotalus durissus |  |  |
| 122 | terrificus | Colombia SP | $200500 \mathrm{~S}, 485800 \mathrm{~W}$ |
|  | Crotalus durissus |  |  |
| 123 | terrificus | Itupeva SP | 230600 S, 470200 W |
|  | Crotalus durissus |  |  |
| 135 | terrificus | Pindamonhangaba | $225500 \mathrm{~S}, 452900 \mathrm{~W}$ |
|  | Crotalus durissus |  |  |
| 136 | terrificus | Pindamonhangaba | $225500 \mathrm{~S}, 452900 \mathrm{~W}$ |
|  | Crotalus durissus |  |  |
| 137 | terrificus | Roseira SP | 225400 S, 452100 W |
|  |  | Aruba Island - London Zoo |  |
| 211 | Crotalus unicolor | C1695 | 123200 N, 695600 W |
|  |  | Aruba Island - London Zoo |  |
| 212 | Crotalus unicolor | C1693 | 123200 N, 695600 W |
| 213 | Crotalus d. culminatus | London Zoo C229 |  |
|  | Crotalus durissus |  |  |
| 254-262 | tzabcan | Xaibe, Corozal, Belize | 182700 N, 882500 W |
| 303 | Crotalus durissus ssp. | Alto Paraiso GO- | $151700 \mathrm{~N}, 473800 \mathrm{~W}$ |
| 316 | Crotalus durissus ssp. | Pontes e Lacerda MT | $154400 \mathrm{~S}, 595000 \mathrm{~W}$ |
| 317 | Crotalus durissus ssp. | Ribeirvo Cascalheira MT | $143500 \mathrm{~S}, 512300 \mathrm{~W}$ |
| 318 | Crotalus durissus ssp. | BrasPlia DF | $154600 \mathrm{~S}, 474700 \mathrm{~W}$ |
| 319 | Crotalus durissus ssp. | Svo Desid9rio BA | 121100 S, 445400 W |
| 320 | Crotalus durissus ssp. | Bel 9 m do Svo Francisco | $084100 \mathrm{~S}, 385700 \mathrm{~W}$ |
| 321 | Crotalus durissus ssp. | Guanambi BA | $140800 \mathrm{~S}, 424200 \mathrm{~W}$ |
| 346-353 | Crotalus d. cascavella | Grajau, MA | $054400 \mathrm{~S}, 460700 \mathrm{~W}$ |
| 626-635 | Crotalus d. cascavella | Grajau, MA, | $054400 \mathrm{~S}, 460700 \mathrm{~W}$ |
| 664-667 | Crotalus d. collilineatus | Alto da Boa Vista, MT | $221300 \mathrm{~S}, 433200 \mathrm{~W}$ |
| 775 | Crotalus d. | La Guaira, D.F., Venezuela | 103400 N, 670200 W |


|  | cumanensis <br> Crotalus d. <br> 780 <br> cumanensis <br> Crotalus d. <br> 781 <br> cumanensis <br> Crotalus $d$. | Distrito Colina, Falc.:n, Venezuela |
| :--- | :--- | :--- |$\quad 111900 \mathrm{~N}, 693700 \mathrm{~W}$

# APPENDIX 3. Matrix of pairwise differences (average + SE) using Kimura 2parameter 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 |
| EISalvador | 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 |

```
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
```



```
0,0711 0,0799 0,0772 0,0772 0,0691 0,1098 0,1117 0,1108 0,1027 0,0253 0,0709 0,0774 0,1168
```



```
0,0046 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
```

```
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}00,1036 0,1032 0,1055 0,1051 0,1081 0,1041 0,1100 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}00,0505 0,0512 0,0528 0,0709 0,0532 0,0498 0,0622 0,1067 0,0485 0,1130 0,0000
```



## 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 |
| 5 |  |  |  |  |  |  |  |


| 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 | 0,45655 | 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 |
|  |  | 4 |  |  |  |  |  |


| 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 | 0,3 |
| 0,37097 | 0,38306 | 0,38306 | 0,34677 | 0,34274 | 0,31452 | 0,375 | 0,34677 |
| 0,375 | 0,37 | 0,38 | 0,35081 | 0,34677 | 0,31048 | ,37903 | 0,34274 |
| , 37903 | 0,38306 | 0,39113 | 0,34677 | 0,35081 | 0,30645 | 0,39919 | 0,34677 |
| 0,37097 | 0,36694 | 0,375 | 0,3548 | 0,34274 | 0,30645 | 0,375 | 0,34677 |
| 0,41129 | 0,41532 | 0,43145 | 0,40323 | 0,38306 | 0,3871 | 177 | 0,43548 |
| 0,4 | 0,44758 | 0,46 | 0,41935 | 0,39919 | 0,41129 | 0,45565 | 59 |
| 0,41935 | 0,42339 | 0,41532 | 0,39 | 0,39 | 0,37903 | 0,46371 | 0,427 |
| 0,44355 | 0,4879 | 0,47177 | 0,47581 | 0,44758 | 0,45968 | 0,49597 | 0,451 |
| 0,42339 | 0,41129 | 0,40323 | 0,40726 | 0,40323 | 0,375 | 0,42742 | ,37 |
| 0,3991 | 0,3951 | 0,39516 | 0,38306 | 0,3 | 0,35887 | 323 | 0,3830 |
| 0,43145 | 0,41935 | 0,41129 | 0,40726 | 0,41129 | 0,38306 | 0,41935 | 0,38306 |
| 0,41129 | 0,399 | 0,39 | 0,37 | 0,39113 | 0,3629 | 0,40726 | ,37903 |
| 0,28226 | 0,24597 | 0,25403 | 0,26 | 0,27016 | 0,20 | 0,27016 | ,2 |
| 0,29032 | 0,28629 | 0,27016 | 0,25806 | 0,27016 | 0,24194 | 0,28629 | 0,29839 |
| 0,37903 | 0,36694 | 0,375 | 0,35 | 0,37 | 0,33871 | 0,38306 | 0,3629 |
| 0,37903 | 0,37 | 0,38306 | 0,37097 | 0,375 | 0,34677 | 0,375 | 0,3709 |
| 0,35081 | 0,3629 | 0,37903 | 0,3588 | 0,35484 | 0,34274 | 0,3629 | ,37 |
| 0,16532 | 0,15323 | 0,15323 | 0,17 | 0,16129 | 0,125 | 0,22581 | 0,2056 |
| 0,18548 | 0,16532 | 0,16532 | 0,20968 | 0,17339 | 0,1371 | 0,22177 | 0,2 |
| 0,24597 | 0,23387 | 0,22581 | 0,24597 | 0,21774 | 0,18145 | 0,26613 | 0,2 |
| 0,2 | 0,22 | 0,19355 | 0,18 | 0,17742 | 0,14919 | 0,26613 | 0,2 |
| 0,20161 | 0,15726 | 0,16532 | 0,20 | 0,17339 | 0,12903 | 0,24597 | 0,2177 |
| 0,20 | 0,165 | 0,16532 | 0,20968 | 0,18145 | 0,14516 | 0,25403 |  |
| 0,18145 | 0,20968 | 0,20161 | 0,18952 | 0,18548 | 0,14919 | 0,25 | 0,2217 |
| 0,20968 | 0,20565 | 0,18145 | 0,20968 | 0,18952 | 0,14516 | 0,22177 | 0,2338 |
| 0,2379 | 0,21 | 0,22581 | 0,2379 | 0,21774 | 0,18952 | 0,23387 | 0,2 |
| 0,20565 | 0,19355 | 0,21774 | 0,19758 | 0,17742 | 0,14113 | 0,20968 | 0,237 |
| 0,20565 | 0,19355 | 0,19355 | 0,19758 | 0,18548 | 0,13306 | 0,20968 | 0,2137 |
| 0,20968 | 0,19758 | 0,19758 | 0,18548 | 0,1 | 0,1371 | 0,22177 | 0,2177 |
| 0,17339 | 0,18548 | 0,17742 | 0,18145 | 0,14516 | 0,10887 | 0,20161 | 0, |
| 0,125 | 0,25 | 0,23387 | 0,125 | 0,12097 | 0,11694 | 0,20161 |  |
| 0,14516 | 0,22177 | 0,22984 | 0,14516 | 0,11694 | 0,1129 | 0,20565 | 0,2096 |
| 0,125 | 0,22581 | 0,23387 | 0,14113 | 0,12903 | 0,10887 | 0,20968 | 0,213 |
| 53 | 0,16129 | 0,1371 | 0,18145 | 0,22581 | 0,19758 | 0,27419 | 0,270 |
| 61 - |  | 0,10484 | 0,20565 | 0,21774 | 0,18952 | 0,26613 | 0,270 |
| 63 | 26 - |  | 0,18145 | 0,20968 | 0,20565 | 0,28226 | 0,302 |
| 38 | 51 | 45 - |  | 0,13306 | 0,12097 | 0,25403 | 0,282 |
| 37 | 54 | 52 | $33-$ |  | 0,09274 | 0,20968 | 0,23 |
| 36 | 47 | 51 | 30 | 23. |  | 0,18952 | 0,20 |
| 57 | 66 | 70 | 63 | 52 | 47 |  |  |
| 52 | 67 | 75 | 70 | 59 | 50 | 45. |  |

