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Biogeography And Diversification In The Neotropics: Testing Macroevolutionary Hypotheses Using Molecular Phylogenetic Data

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BIOGEOGRAPHY AND DIVERSIFICATION IN THE NEOTROPICS: TESTING MACROEVOLUTIONARY HYPOTHESES USING MOLECULAR PHYLOGENETIC DATA

by

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ABSTRACT

Lineage diversification in the Neotropics is an interesting topic in evolutionary biology and one of the least understood. The complexity of the region precludes generalizations regarding the historical and evolutionary processes responsible for the observed high diversity. Here, I use molecular data to infer evolutionary relationships and test hypotheses of current taxonomy, species boundaries, speciation and biogeographic history in several lineages of Neotropical snakes. I comprehensively sampled a widely distributed Neotropical colubrid snake and Middle American pitvipers and combined my data with published sequences. Within the colubrid genus *Leptodeira*, mitochondrial and nuclear markers revealed a phylogeograhic structure that disagrees with the taxonomy based only on morphology. Instead, the phylogenetic structure corresponds to specific biogeographic regions within the Neotropics. Molecular evidence combined with explicit divergence time estimates reject the hypothesis that highland pitvipers in Middle America originated during the climatic changes during the Pleistocene. My data, instead, shows that pitviper diversification occurred mainly during the Miocene, a period of active orogenic activity. Using multiple lineages of Neotropical snakes in a single phylogenetic tree, I describe how the closure of the Isthmus of Panama generated several episodes of diversification as opposed to the Motagua-Polochic fault in Guatemala where a single vicariant event may have led to diversification of snakes with different ecological requirements. This finding has implications for future biogeographic studies in the region as explicit temporal information can be readily incorporated in molecular clock analyses. Bridging the gap between the traditional goals of historical biogeography (i.e., area relationships) with robust statistical methods, my research can be applied to multiple levels of the biological hierarchy (i.e., above species level), other regional systems and other sub-disciplines in biology such as medical research, evolutionary ecology, taxonomy and conservation.

To the one who left too early, my father

And the one that came as a bless, my daugther

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CHAPTER 1 – INTRODUCTION

The role of molecular phylogenetics in historical biogeography

Historical biogeography, the subdiscipline of biology that aims to understand the historical processes that led to present–day diversity, has experienced massive transformation in methodologies over the last 30 years (reviewed in Crisci et al., 2003; Posadas et al., 2006). The first significant advance occurred in the late 1970's when biogeography incorporated the philosophy of Karl Popper, plate tectonics theory and cladistic methods (Ball, 1975; Nelson, 1974; Platnick and Nelson, 1978; Rosen, 1978). This research program used phylogenetic methods to discover area relationships and then used vicariance as the main process to explain biogeographic patterns (Nelson and Platnick, 1981). In the 1980's, a paradigm shift occurred, bridging the gap between population genetics (microevolution) and phylogenetics (macroevolution) by using molecular markers (in particular mitochondrial DNA), and population genetics theory to infer the evolution of genetic lineages among closely related species (Avise et al., 1987; Avise et al., 1979; Avise et al., 1983; Neigel and Avise, 1986). Advances in computational biology and availability of inexpensive and fast methods for obtaining molecular data at the intra–specific level shifted historical biogeography to a more "microevolution" oriented research program (Avise, 1998). Thus, interest changed from a broad continental and temporal scale to an intra–specific regional scale, focused mainly on recent historical events that impacted biodiversity such as the glacial periods during the Pleistocene.

In historical biogeography, vicariance and dispersal are still considered the major forces that determine the divergence and geographic distribution of new lineages (Crisci et al., 2003; McDowall,

2004; Posadas et al., 2006). Neither process, however, can be easily extracted from a single phylogenetic pattern (Ebach and Humphries, 2003; Ebach et al., 2003; Humphries, 2000). Using coalescent models and the genetic structure, data can be tested against specific historical, demographic scenarios, which in turn can be used to suggest either a vicariant or dispersal event (Avise, 2000; Drummond et al., 2005; Kuhner, 2009; Ramakrishnan et al., 2005; Rosenberg and Nordborg, 2002; Strimmer and Pybus, 2001; Templeton, 2008). Such robust statistical approaches, however, are designed for addressing questions associated with shallow phylogenetic trees, mostly at the intra–specific level, where haplotype relationships represent gene genealogies and not necessarily species trees (Avise, 2000; Riddle and Hafner, 2004, 2007).

The biogeographic history of more ancient cladogenetic events (i.e., relationships among higher lineages or entire biotas), continues to be part of "traditional" historical biogeography methods. Therefore, a wide variety of methods from ancestral area reconstruction (Bremer, 1992; Bremer, 1995; Ronquist, 1994, 1995), discovery–based methods such as Dispersal–Vicariance and Brooks Parsimony Analysis (Brooks and McLennan, 2001; Brooks et al., 2001; Ronquist, 1997; van Veller et al., 2000), panbiogeography (Craw et al., 1999; Heads, 2005), and traditional event–based methods (Humphries and Parenti, 1986; Humphries and Parenti, 1999; Nelson and Ladiges, 1991; Nelson and Platnick, 1981; Page, 1993, 1994; Wiley, 1988), represent the inferential tools by which a biogeographic pattern and its underlying mechanisms (vicariance vs. dispersal) can be discovered. Despite the significant number and types of analytical strategies, consensus has not been reached as to which method is preferred (Brooks, 2004; Brooks et al., 2004; Brooks and Veller, 2003; Nelson and Ladiges, 2001; Nelson and Platnick, 1978; Platnick and Nelson, 1988; van Veller, 2000; Van Veller and Brooks, 2001; Van Veller et al., 2003; van Veller et al., 2002). As a consequence, more recent efforts stress the need for an integrative approach that includes population genetics, GIS

information, divergence times, and molecular phylogenetics to investigate the patterns and processes in historical biogeography (Andersson, 1996; Avise, 2004; Brooks, 2005; Crisci, 2001; Donoghue and Moore, 2003; Posadas et al., 2006; Riddle, 2005, 2009).

The Neotropics as an excellent setting

The geographic region that spans from Mexico south to northern Argentina is considered one of the most interesting natural experiments in evolution (Jackson et al., 1996; Prance, 1982; Whitmore and Prance, 1987). Its geological history with long periods of isolation, transient landmass connections and a complex orogenic history (Marshall, 2006) have led to a rich fauna and flora that challenges evolutionary biologists to identify the patterns and processes of geographic speciation. However, recent molecular phylogenies and comprehensive sampling throughout the region is providing new evidence as to the mechanisms that generated biodiversity. For example, studies conducted in Middle America have revealed a long history of isolation and divergence, which contrasts with the traditional view that Pleistocene climatic fluctuations generated species diversity in this region (e.g., Prance, 1982). Instead, new evidence suggests that more ancient events dating back to the Miocene, or previous to this period, were critical in shaping Mesoamerican lineages (Castoe et al., 2009; Crawford et al., 2007; Crawford and Smith, 2005; Perdices et al., 2002; Perdices et al., 2005; Smith et al., 2007). Likewise, the South American biota appears to have a more complex evolutionary history caused mostly by the Andean uplift and the drainage shift of the entire Amazon basin during the Miocene, combined with the periodic climatic and eustatic changes of sea level during the Pliocene and Pleistocene (Brumfield and Capparella, 1996; Burnham and Graham, 1999; Hubert and Renno, 2006; Lovejoy et al., 1998; Tuomisto, 2007). Thus, the significant role of Pleistocene climatic fluctuations as the main factor for geographic speciation and present–day species distribution (e.g.,

Hooghiemstra and van der Hammen, 1998; and references therein) is being challenged by molecular evidence and divergence time estimation, which suggests more continued and ancient events impacted diversification across multiple lineages (Rull, 2006; 2008; this study)

Snakes as model in evolutionary biology

The study of evolution most often relies on model organisms which are used to describe biological patterns and mechanisms and from this then make inferences on other organisms and systems. Naturally, an ideal "model organism" would depend on the questions and hypotheses to be addressed so different models will fit different research programs. In historical biogeography, snakes can be a good model system to understand not only the historical and ecological processes that generated the diversity and distribution in this group but hopefully the processes occurring in entire communities (see Chapter 4). Snakes with more than 3,100 species represent around 36 percent of the diversity of all non-avian reptiles (www.reptile-database.org, accessed March 2010), and almost 15 percent of the entire vertebrates. This high diversity is at some extent the product of higher speciation rates observed in Alethinophidians (advanced snakes) during the last 150 million years (Ricklefs et al., 2007; Vidal et al., 2009). Therefore, snakes represent an excellent opportunity to study the historical, climatic, and morphological factors that might have led to such diversification (Burbrink and Castoe, 2009). In the Neotropics, snakes are an important community component with high levels of endemism but also with lineages that expand the entire region and temperate zones (Cadle, 1985). There are representatives of old lineages such as Boids and Scolecophidians (blind snakes) with an ancient evolutionary history dating back to the Gondwana break up (Adalsteinsson et al., 2009; Noonan and Chippindale, 2006). The other major components include the majority of species (colubroids, vipers and elapids) and are hypothesized to be of a more recent

colonization, most likely dating back to the early Miocene (Cadle, 1985; Castoe et al., 2009; Parkinson et al., 2002; see Chapters 3 and 4).

I will be using three main snake lineages from the Neotropical region: Crotalinae, a monophyletic group within vipers, is highly diverse in Tropical America ranging from sea level to high mountains and inhabiting a wide variety of habitats, from tropical rainforest to deserts (Campbell et al., 2004). The other two groups of snakes addressed during this study includes the Dipsadids with more than 400 species (sensu Zaher et al., 2009), and coral snakes (genus *Micrurus*) with more than 80 species (Campbell et al., 2004). These two lineages are almost exclusively endemic to the region comprising Mexico to northern Argentina.

Goals of this study

Here, I use molecular data to infer evolutionary relationships and then test hypotheses of current taxonomy, species boundaries, speciation and biogeographic history in one of the most biodiverse regions on the planet. My study increases our understanding of the processes and mechanisms of species formation and how these factors have shaped the rich biodiversity in the Neotropical region. I begin with a widely-distributed Neotropical lineage of snakes (*Leptodeira*) and show how molecular phylogenetics and morphological evidence disagree, and how detailed phylogeographic data can reveal hidden genetic diversity that in turn is useful for taxonomic and conservation decisions. I test specific biogeographic hypotheses that reveal the evolutionary history of *Leptodeira* and highlight the different roles of ecology and geology in shaping its speciation throughout the Neotropics. Next, I use venomous snakes to test a specific hypothesis regarding highland speciation in Middle America. I demonstrate how comprehensive sampling and robust molecular analysis falsifies the previous

hypothesis that climatic fluctuation during the Pleistocene drove species formation in highland taxa. Lastly, I develop a hypothetical framework where multiple independent biogeographic studies are combined to make general inferences about geographic speciation and how these strategies can provide hypotheses testable with independent lineages. My approach, combines the traditional goals in historical biogeography with robust statistical methods that model evolutionary processes, that in turn, can be applied at multiple levels of the biological hierarchy (i.e., above species level) and in systems other than the Neotropical region.

The implications of my research go beyond historical biogeography and will benefit other disciplines in Neotropical biology. For example, given that snake venom can evolve under different conditions in different populations (lineages) of snakes, production of specific anti–venom is critical for snakebite treatments (Daltry et al., 1996a; Daltry et al., 1996b; Wüster, 1996). Therefore, delimiting geographic evolutionary lineages of venomous snakes will help direct medical research to specific geographic and genetic lineages. This will reduce costs and research efforts, a limited resource in the developing countries that these snakes inhabit. In addition, the phylogeographic characterization of the biotic component in a highly diverse but still poorly explored region will impact conservation decisions, by putting efforts in endangered, genetically unique, and ecologically constrained evolutionary lineages.

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CHAPTER 2 – COMPLEX EVOLUTION IN THE NEOTROPICS: THE ORIGIN AND DIVERSIFICATION OF THE WIDESPREAD GENUS *LEPTODEIRA* (SERPENTES: COLUBRIDAE)[1]

Introduction

Inferring patterns of species diversification is among the most interesting topics in evolutionary biology because it may provide key insight into the processes that have led to current biodiversity. This is especially true in the Neotropics, given the extreme geological complexity and the high diversity and endemicity in this region (Prance 1982; Cracraft and Prum 1988; Graham 1997; Burnham and Graham 1999). This extreme intricacy of historical processes, however, has hampered a consensus regarding the historical and ecological processes responsible for the observed diversity. One particularly important means of developing a strong hypothesis for broad and general biogeographic patterns is the simultaneous analysis and comparison of multiple independent lineages that are codistributed throughout a region (Nelson and Platnick 1981; Lomolino et al. 2006; Castoe et al. 2009). This approach is particularly difficult to apply in the Neotropical region because the spatial and temporal dimensions of a majority of lineages in this area remain poorly known. To overcome this problem, a more realistic approach is to investigate phylogenetic patterns of independent lineages and then to test specific hypotheses regarding the historical and ecological processes that have shaped the species diversity (Beheregaray 2008; Riddle et al. 2008). The cat–eyed snakes, *Leptodeira,* range through nearly the entire Neotropical region, making this group excellent to

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investigate the effects of historical and ecological processes across different biogeographic provinces on lineage diversification.

The genus *Leptodeira* is a member of the subfamily Dipsadinae, a group that originated in Middle–America but now inhabits Tropical and Subtropical America (Duellman 1958a; Cadle 1984; Zaher 1999). It is the most widely distributed genus of the subfamily, ranging from the southern U.S.A. to northern Argentina and Paraguay, the east coast of Brazil and the islands of Aruba, Margarita, Tobago and Trinidad (Duellman 1958a). Several hypotheses regarding the diversification in the Mexican transition zone (*sensu* Halffter 1987), in lower Central America and the interchange between Central and South America can be explored through the phylogeography of different lineages of *Leptodeira*. Nevertheless, several recognized species are morphologically similar and the overlapping in color patterns makes distinction among species difficult. Thus, comprehensive molecular phylogenetic analyses of these morphologically complex groups are necessary to elucidate their evolutionary and biogeographic history. Lastly, *Leptodeira* ranges from very dry areas in Mexico and northern South America to mesic and evergreen humid forests in Middle America and the Amazon basin. This extraordinary ecological distribution provides further insight into the environmental factors that may affect gene flow, diversification and geographic distribution of the lineages within the genus.

Phylogenetic hypotheses regarding the genus *Leptodeira* have not been addressed comprehensively. Duellman (1958a) proposed that the genus *Hypsiglena* was the sister group to *Leptodeira*. (Dowling and Jenner 1987) inferred the phylogenetic relationships among several Xenodontines (Dipsadines) related to *Leptodeira*, but were unable to resolve which lineages are the closest relatives of *Leptodeira*. (Vidal et al. 2000) placed *Leptodeira* within the subfamily Dipsadinae but again they provided no

insight into what taxon may be its sister lineage. Recent molecular phylogenetic analyses have hypothesized the genus *Imantodes* as the sister taxon to *Leptodeira* (Pinou et al. 2004; Mulcahy 2007). Mulcahy (2007) examined the phylogenetic relationships among *Leptodeira* and tested the monophyly of the Leptodeirini (*sensu* Cadle 1984). The monophyly of *Leptodeira* was not supported under his parsimony analysis but received moderate support using maximum likelihood and Bayesian inference. The only comprehensive taxonomic study within *Leptodeira* was conducted five decades ago by Duellman (1958a). Four species groups were recognized and one species, *Leptodeira discolor*, was considered *incertae sedis*. Few taxonomic changes have been made since Duellman (1958a), except that *L. discolor* and *L. latifasciata* have been allocated to the monotypic genera *Tantalophis* and *Pseudoleptodeira,* respectively (Duellman 1958b; Smith and Smith 1976). Taylor (1951) recognized *L. rubricata* as a separate species, but it was synonymized with *L. annulata* by Duellman (1958a). Currently, *L. rubricata* is considered a valid species, although no quantitative evidence has been shown to support this (Savage 2002). In general, the subspecies proposed by Duellman (1958a) are still recognized today (e.g., Savage 2002; Köhler 2003).

The spatial and temporal diversification of *Leptodeira* has not been addressed comprehensively. Duellman (1958a) proposed a tentative biogeographic scenario from which phylogenetic relationships and the spatial and temporal diversification may be extracted (Figure 2.1). His reconstruction placed the origin of *Leptodeira* in the Miocene, followed by a diversification into the different species and subspecies throughout the Miocene and Pliocene with some subspecies originating during the Pleistocene. Dowling and Jenner (1987) also suggested a Miocene origin. Duellman (1958a) and Mulcahy (2007) both hypothesized that *Leptodeira* originated in Mexico with at least two dispersal events into South America directly after the closure of the Isthmus of Panama in

the Late Pliocene. These dispersal events involved the independent colonization of South America by the species *L. annulata* and *L. septentrionalis*.

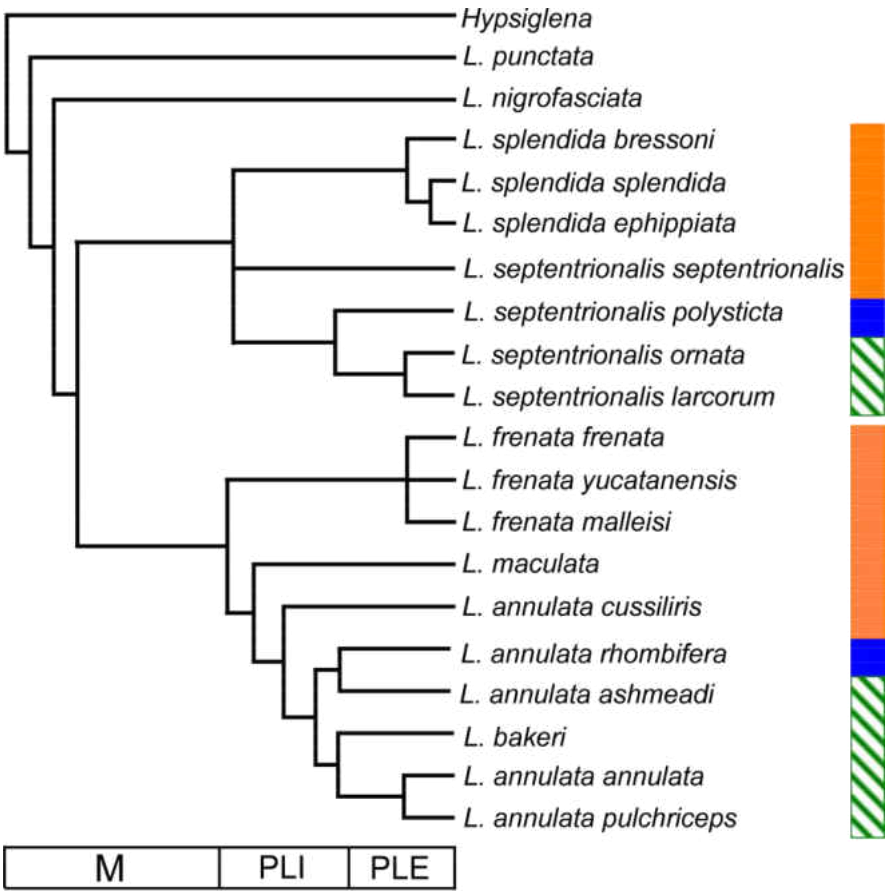

Figure 2.1. Hypothesis for the spatio–temporal diversification of *Leptodeira* in the Neotropics based on Duellman (1958a). Time periods (not drawn to scale) as follows: M = Miocene, PLI = Pliocene, PLE = Pleistocene.

In this study, we use sequences from mitochondrial and nuclear genes and extensive taxon sampling to investigate the following questions surrounding the evolution and biogeography of *Leptodeira*: 1) do nuclear and mitochondrial sequence data yield congruent phylogenetic inferences for the relationships among the dipsadines and the inter– and intra–relationships within *Leptodeira,* 2) is the monophyly of the genus *Leptodeira* supported, 3) is the current morphological classification consistent with the molecular phylogenetic estimates, and 4) is the spatial and temporal

diversification of *Leptodeira* congruent with Duellman's hypotheses? In addition to these questions, we apply our phylogenetic and phylogeographic data, together with estimates of divergence times, to develop hypotheses for the historical patterns and processes that have shaped lineage diversity in *Leptodeira* and which may be broadly informative about patterns of Neotropical diversification in general.

Methods

Taxon sampling

We combined previously published DNA sequences with new sequences from this study to create a matrix with a total of 135 terminals including taxa outside *Leptodeira* (Table 1.1). We followed the taxonomic classification of Duellman (1958a) except for *L. latifasciata* and *L. discolor*, which are considered *Pseudoleoptodeira latifasciata* and *Tantalophis discolor* respectively. Although *L. rubricata* was synonymized with *L. a. rhombifera* (Duellman 1958a), we sequenced one specimen to explore its phylogenetic position and species status (see Savage 2002). Within the genus *Leptodeira*, our dataset included 89 individuals representing all nine species, and nine of the 15 subspecies. Our geographic sampling spanned the entire known distribution for the genus (Fig. 2.2). Outgroups were chosen based on two criteria. First, we included 27 members from the subfamilies Dipsadinae, Xenodontinae, Natricinae and Colubrinae to determine the phylogenetic position of *Leptodeira* within Dipsadinae and to gain further insight into the relationships within the subfamily Dipsadinae. Second, because Mulcahy (2007) did not recover *Leptodeira* as a well–supported clade (86% posterior probability), we included 16 samples of the genus *Imantodes* (inferred as the sister taxon to *Leptodeira* by Mulcahy, 2007) to test the monophyly of *Leptodeira*. Finally, to estimate divergence times, we included three representatives of the family Viperidae for calibration purposes.

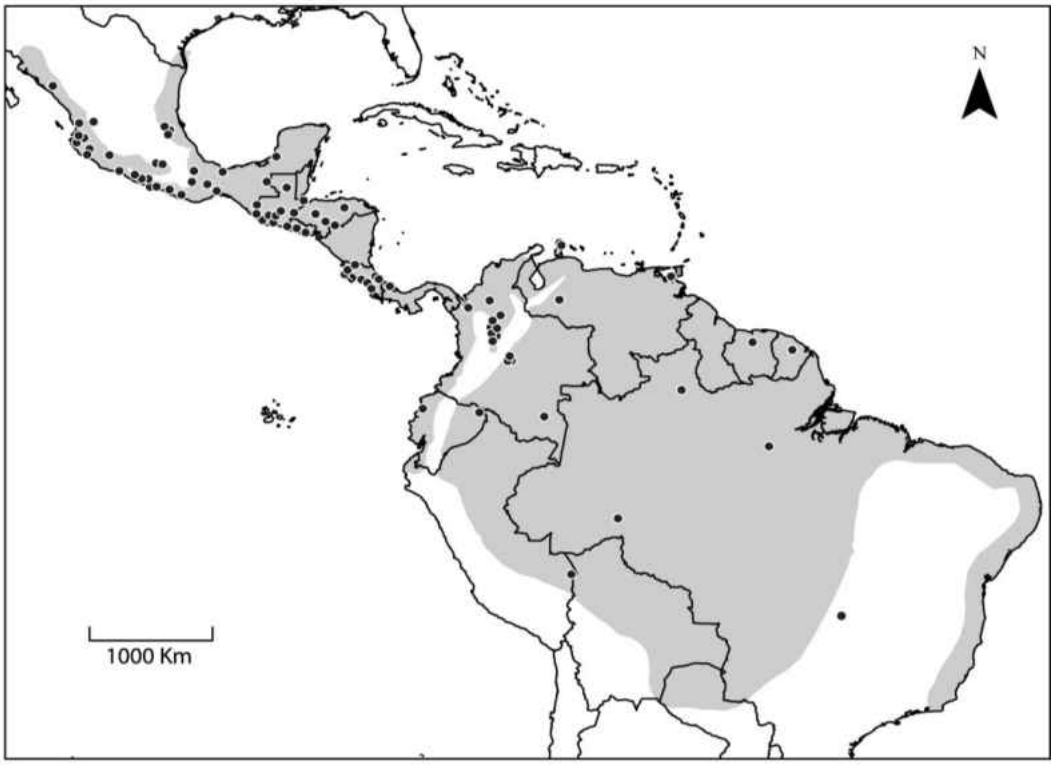

Figure 2.2. Geographic distribution of the genus *Leptodeira* based on Duellman (1958a). Dots represent localities sampled in this study.

Laboratory protocols

Total genomic DNA was extracted from tissue samples (liver, muscle or skin shed) using the Qiagen DNeasy kit (QIAGEN). Two regions of the mitochondrial genome, including genes encoding Cytochrome b (*cyt–b*), NADH dehydrogenase subunit 4 (*ND4*) and the tRNA's His, Ser and Leu were amplified via PCR. Additionally, we amplified 24 terminals for the nuclear protein-coding genes neurotrophin 3 (*NT3*) and dynein, axonemal, heavy chain 3 (*DNAH3*). These terminals represent the main clades recovered with the mitochondrial dataset. *Cyt-b* was amplified using the primers Gludg, AtrCB3, and H16064 (Burbrink et al. 2000; Parkinson et al. 2002). *ND4* plus the adjacent tRNA region was amplified using the primers ND4 and LEU (Arévalo et al. 1994). *NT3* was amplified with the primers NT3-F3 and NT3-R4 (Noonan and Chippindale 2006a, b), and

Table 2.1. Sequences used in this study, with Genbank numbers and voucher information. Sequences added specifically in this study are indicated in bold.

Taxon [a]	Locality	Voucher [b]	Cytb	ND4	DNAH3	NT3
Alsophis portoricensis	Unknown	No voucher	AF471085	U49308		
Amastridium sapperi	**Guatemala, Izabal**	**UTA R-46905**	**GQ334479**	**GQ334580**	**GQ334557**	**GQ334663**
Arrhyton exiguum	USA, Puerto Rico	CAS 200732	AF471071			
Atractus wagleri	**Colombia, Antioquia**	**MHUA 14368**	**GQ334480**	**GQ334581**	**GQ334558**	**GQ334664**
Carphophis amoenus	USA, Illiniois	CAS 160710	AF471067			
Coluber constrictor	USA, California	CAS 212760, SDSU 3929	EU180467	AY487041	EU402743	EU390914
Coniophanes fissidens	El Salvador, San Salvador	KU 289798	EF078586,	EF078538		
Contia tenuis	Unknown	No voucher	AF471095	DQ364666		
Crtotalus tigris	**USA, Arizona, Pima Co.**	**CLP 169**	AY223606	AF156574		**GQ334665**
Cryophis hallbergi	**Mexico, Oaxaca**	**UTA R-12272**	**GQ334481**	**GQ334582**	**GQ334559**	**GQ334666**
Diadophis punctatus	Unknown	No voucher	AF471094	DQ364667		
Dipsas catesbyi	Peru, Madre de Dios	KU 214851	EF078585,	EF078537		
Dipsas pratti	**Colombia, Antioquia**	**MHUA 14278**	**GQ334482**	**GQ334583**	**GQ334560**	**GQ334667**
Eridiphas slevini	Mexico, Baja California	MVZ 234613	EF078547,	EF078499		
Farancia abacura	USA, Florida	CAS 184359	U69832	DQ902307		
Gloydius shedaoensis	China, Liaoning	ROM-20468	AY223566	AY223623		
Gonyosoma frenatum	Unknown	No voucher	DQ902110	DQ902290		
Helicops angulatus	Trinidad & Tobago	LSUMZ 3346	AF471037	U49310		
Heterodon simus	USA, Florida	CAS 195598	AF217840	DQ902310		
Hydrops triangularis	Peru, Loreto	LSUMZ 3105	AF471039			
Hypsiglena torquata	**USA, California**	**CAS 206502**	**GQ334483**	**GQ334584**		
Imantodes cenchoa	Brazil, Para	MPEGLJV 5763	EF078556,	EF078508		
Imantodes cenchoa	**Colombia, Antioquia**	**MHUA 14290**	**GQ334484**	**GQ334585**	**GQ334561**	**GQ334668**
Imantodes cenchoa	**Colombia, Antioquia**	**MHUA 14500**	**GQ334485**	**GQ334586**		
Imantodes cenchoa	**Colombia, Choco**	**JMD 1616**	**GQ334486**	**GQ334587**		
Imantodes cenchoa	Costa Rica, Limon	MVZ 149878	EF078553,	EF078505		
Imantodes cenchoa	Guatemala, Izabal	UTA R-42360	EF078554,	EF078506		
Imantodes cenchoa	Panama, Cocle	SIUC R-03724	EF078555,	EF078507		
Imantodes gemmistratus	**Guatemala, San Marcos**	**UTA R-45922**	**GQ334487**	**GQ334588**		
Imantodes gemnistratus	Mexico, Sinaloa	UTA R-51979	EF078557,	EF078509		
Imantodes gemnistratus	Mexico, Sonora	LSUMZ 39541	EF078558,	EF078510		
Imantodes inornatus	**Colombia, Antioquia**	**MHUA 14540**	**GQ334488**	**GQ334589**	**GQ334562**	**GQ334669**
Imantodes inornatus	**Costa Rica**	**ASL 307**	**GQ334489**	**GQ334590**		
Imantodes inornatus	Costa Rica, Heredia	MVZ 204110	EF078560,	EF078512		
Imantodes inornatus	Costa Rica, Cartago	MVZ 204109	EF078559,	EF078511		
Imantodes lentiferus	Brazil, Amazonas	MPEGLJV 6880	EF078561,	EF078513		
Imantodes lentiferus	Brazil, Para	MPEGLJV 5581	EF078562,	EF078514		
Leptodeira a. annulata	**Brazil, Amazonas**	**LSU-H 14016**	**GQ334494**	**GQ334595**		
L. annulata annulata	**Brazil, Goias**	**No voucher**		**GQ334599**		
L. annulata annulata	**Brazil, Para**	**LSU-H 14438**	**EF078564**	**EF078516**		
L. annulata annulata	**Brazil, Roraima**	**LSU-H 12442**	**GQ334495**	**GQ334596**		
L. annulata annulata	**Colombia, Meta**	**UTA T-55-G5**	**GQ334490**	**GQ334591**		

Taxon [a]	Locality	Voucher [b]	Cytb	ND4	DNAH3	NT3
L. annulata annulata	**Colombia, Meta**	**UTA T-55-G6**	**GQ334491**	**GQ334592**		
L. annulata annulata	**Colombia, Meta**	**UTA T-55-G7**	**GQ334492**	**GQ334593**		
L. annulata annulata	**Ecuador, Sucumbios**	**LSU-H 12755**	**GQ334496**	**GQ334597**		
L. annulata annulata	**French Guyana**	**Vidal et al., 2000**	**GQ334497**	**GQ334598**		
L. annulata annulata	Peru, Madre de Dios	KU 214878	EF078563	EF078515		
L. annulata annulata	**Suriname, Para**	**BPN 963**	**GQ334493**	**GQ334594**	**GQ334563**	**GQ334670**
L. annulata ashmeadi	Trinidad, St. Patrick	USNM 314700	EF078565	EF078517		
L. annulata ashmeadi	**Venezuela, Barinas**	**MHNLS-X516**	**GQ334498**	**GQ334600**		
L. annulata cussiliris	**Guatemala, Huehuetenango**	**UTA R-42220**	**GQ334499**	**GQ334601**		
L. annulata cussiliris	**Guatemala, San Marcos**	**UTA R-53305**	**GQ334501**	**GQ334603**	**GQ334564**	**GQ334671**
L. annulata cussiliris	Mexico, Guerrero	JAC 21939	EF078568	EF078520		
L. annulata cussiliris	Mexico, Hidalgo	ITAH 912	EF078566	EF078518		
L. annulata cussiliris	Mexico, Hidalgo	ITAH 913	EF078567	EF078519		
L. annulata cussiliris	**Mexico, Oaxaca**	**ENEPI 6546**	**GQ334500**	**GQ334602**		
L. annulata cussiliris	**Mexico, Oaxaca**	**UTA R-52630**	**GQ334502**	**GQ334604**		
L. annulata cussiliris	**Mexico, Veracruz**	**EBUAP UOGV 188**	**GQ334503**	**GQ334605**		
L. annulata rhombifera	**Costa Rica**	**ICP 1280**	**GQ334505**	**GQ334607**		
L. annulata rhombifera	**Costa Rica, San Jose**	**MSM 130**	**GQ334514**	**GQ334616**		
L. annulata rhombifera	**El Salvador, San Salvador**	**MUHNES C-30-1351**	**GQ334506**	**GQ334608**		
L. annulata rhombifera	**El Salvador, Usulutan**	**KU 289913**	**GQ334507**	**GQ334609**		
L. annulata rhombifera	**Guatemala, Baja Verapaz**	**UTA R-42456**	**GQ334508**	**GQ334610**		
L. annulata rhombifera	**Guatemala, Baja Verapaz**	**MSM 705**		**GQ334617**		
L. annulata rhombifera	**Guatemala, Escuintla**	**UTA R-44713**	**GQ334513**	**GQ334615**		
L. annulata rhombifera	**Guatemala, Zacapa**	**UTA R-42393**	**GQ334512**	**GQ334614**		
L. annulata rhombifera	**Honduras, Comayagua**	**UNAH-MSM 456**	**GQ334511**	**GQ334613**		
L. annulata rhombifera	**Honduras, El Paraiso**	**UTA R-41255**	**GQ334509**	**GQ334611**	**GQ334565**	**GQ334672**
L. annulata rhombifera	**Honduras, Francisco Morazan**	**JHT 2004**	**GQ334504**	**GQ334606**		
L. annulata rhombifera	**Honduras, Olancho**	**UNAH-ENS 8766**	**GQ334510**	**GQ334612**		
L. bakeri	**Aruba**	**Avid 023783888**	**GQ334516**	**GQ334619**		
L. bakeri	**Aruba**	**Avid 023851115**	**GQ334517**	**GQ334620**		
L. bakeri	**Aruba**	**Avid 023858355**	**GQ334515**	**GQ334618**	**GQ334566**	**GQ334673**
L. bakeri	**Aruba**	**Avid D**	**GQ334518**	**GQ334621**		
L. bakeri	**Aruba**	**Avid E**	**GQ334519**	**GQ334622**		
L. frenata	Mexico, Campeche	LSUMZ 38200	EF078580	EF078532		
L. frenata	Mexico, Guerrero	LSUMZ 39524	EF078579	EF078531		
L. maculata	Mexico, Guerrero	MZFC 19477	GQ334520	GQ334623		
L. maculata	Mexico, Jalisco	MZFC 17434	GQ334523	GQ334626		
L. maculata	Mexico, Jalisco	UTA R-53323	GQ334521	GQ334624	GQ334567	GQ334674
L. maculata	Mexico, Jalisco	UTA R-53324	GQ334522	GQ334625		
L. maculata	Mexico, Jalisco	UTA R-53322	GQ334524	GQ334627		
L. nigrofasciata	Costa Rica	ASL 190	GQ334525	GQ334628	GQ334569	
L. nigrofasciata	Costa Rica	MSM 706	GQ334526	GQ334629		
L. nigrofasciata	Mexico, Guerrero	MVZ 241573	EF078581	EF078533		
L. nigrofasciata	Mexico, Oaxaca	UTA R-52634		GQ334630	GQ334568	GQ334681

Taxon [a]	Locality	Voucher [b]	Cytb	ND4	DNAH3	NT3
L. punctata	Mexico, Sinaloa	UTA R-51974	EF078577	EF078529		
L. punctata	Mexico, Sinaloa	UTA R-51976	EF078578	EF078530		
L. punctata		**UTA R-53503**			**GQ334571**	**GQ334682**
L. rubricata	**Costa Rica**	**ASL 304**	**GQ334527**	**GQ334631**		
L. septentrionalis ornata	**Colombia, Antioquia**	**MHUA 14291**	**GQ334530**	**GQ334634**		
L. septentrionalis ornata	**Colombia, Antioquia**	**MHUA 14292**	**GQ334531**	**GQ334635**		
L. septentrionalis ornata	**Colombia, Antioquia**	**MHUA 14403**	**GQ334528**	**GQ334632**		
L. septentrionalis ornata	**Colombia, Antioquia**	**MHUA 14404**	**GQ334529**	**GQ334633**		
L. septentrionalis ornata	**Colombia, Antioquia**	**MHUA 14419**	**GQ334535**	**GQ334639**		
L. septentrionalis ornata	**Colombia, Antioquia**	**MHUA 14423**	**GQ334532**	**GQ334636**	**GQ334572**	**GQ334676**
L. septentrionalis ornata	**Colombia, Antioquia**	**MHUA 14449**	**GQ334537**	**GQ334642**		
L. septentrionalis ornata	**Colombia, Antioquia**	**MHUA 14476**	**GQ334534**	**GQ334638**		
L. septentrionalis ornata	**Colombia, Antioquia**	**MHUA 14495**		**GQ334640**		
L. septentrionalis ornata	**Colombia, Antioquia**	**MHUA 14541**	**GQ334533**	**GQ334637**		
L. septentrionalis ornata	**Colombia, Antioquia**	**MHUA 14653**	**GQ334536**	**GQ334641**		
L. septentrionalis ornata	**Colombia, Caldas**	**JMD-T44**	**GQ334538**			
L. septentrionalis ornata	**Costa Rica**	**ASL 308**	**GQ334541**	**GQ334646**	**GQ334574**	**GQ334678**
L. septentrionalis ornata	**Costa Rica, Limon**	**ICP 1089**	**GQ334540**	**GQ334645**		
L. septentrionalis ornata	**Costa Rica, Punta Arenas**	**ICP 1108**		**GQ334643**		
L. septentrionalis ornata	**Costa Rica, Punta Arenas**	**MSM PH 90**	**GQ334539**	**GQ334644**	**GQ334573**	**GQ334677**
L. septentrionalis ornata	Ecuador, Manabi	KU 218419	EF078576	EF078528		
L. septentrionalis ornata	Panama, Bocas del Toro	USNM 347357	EF078575	EF078527		
L. septentrionalis polysticta	**El Salvador, Ahuachapan**	**MUHNES C-30-1352**	**GQ334544**	**GQ334649**		
L. septentrionalis polysticta	**Guatemala, Escuintla**	**UTA R-46878**	**GQ334545**	**GQ334650**	**GQ334570**	**GQ334675**
L. septentrionalis polysticta	**Guatemala, Guatemala**	**UTA R-45878**	**GQ334546**	**GQ334651**		
L. septentrionalis polysticta	**Guatemala, Izabal**	**UTA R-39558**	**GQ334542**	**GQ334647**		
L. septentrionalis polysticta	**Guatemala, Peten**	**UTA R-46125**	**GQ334547**	**GQ334652**	**GQ334575**	**GQ334679**
L. septentrionalis polysticta	Guatemala, Peten	UTA R-50312	EF078572	EF078524		
L. septentrionalis polysticta	Guatemala, Suchitepequez	UTA R-52284	EF078571	EF078523		
L. septentrionalis polysticta	Mexico, Guerrero	MVZ 164942	EF078570	EF078522		
L. septentrionalis polysticta	**Mexico, Oaxaca**	**MZFC 16548**		**GQ334653**		
L. septentrionalis polysticta	**Mexico, Oaxaca**	**ENEPI 6819**	**GQ334543**	**GQ334648**		
L. septentrionalis polysticta	**Mexico, Oaxaca**	**MZFC 16915**	**EF078574**	**EF078526**		
L. septentrionalis polysticta	**Mexico, Sinaloa**	**UTA R-51978**	**EF078573**	**EF078525**		
L. splendida bressoni	**Mexico, Jalisco**	**MZFC 17240**	**GQ334548**	**GQ334654**	**GQ334576**	**GQ334680**
L. splendida bressoni	**Mexico, Jalisco**	**UTA R-53409**	**GQ334550**	**GQ334656**		
L. splendida bressoni	**Mexico, Jalisco**	**UTA R-53410**	**GQ334551**	**GQ334657**		
L. splendida bressoni	**Mexico, Nayarite**	**UTA R-53595**	**GQ334549**	**GQ334655**		
L. splendida splendida	**Mexico, Morelos**	**UTA R-51738**	**GQ334552**	**GQ334658**		
L. splendida splendida	**Mexico, Puebla**	**EBUAP 2060**	**EF078569**	**EF078521**		

Taxon [a]	Locality	Voucher [b]	Cytb	ND4	DNAH3	NT3
Micrurus fulvius	USA, Florida	CAS 21347, YPM 14096	EF137413	EF137405	EU402760	EU390929
Natrix natrix	Spain, Catalonia	MVZ 200534	AY487756	AY487800	EU402762	EU390931
Ninia atrata	**Colombia, Caldas**	**MHUA 14452**	**GQ334553**	**GQ334659**	**GQ334577**	**GQ334683**
Oxyrhopus petola	**Guatemala, Izabal**	**UTA R-46698**	**GQ334554**	**GQ334660**	**GQ334578**	**GQ334684**
Pseudoleptodeira latifasciata	**Mexico**	**EBUAP ENS 10549**	**GQ334555**	**GQ334661**		
Rhadinaea fulvivittis	Mexico, Veracruz	MVZ 231852	EF078539	EF078587		
Sibon nebulatus	**Colombia, Antioquia**	**MHUA 14511**	**GQ334556**	**GQ334662**	**GQ334579**	**GQ334685**
Sistrurus catenatus	**USA, Texas, Haskel Co.**	**Moody-502**	AY223610	AY223648		**GQ334686**
Tantalophis discolor	Mexico, Oaxaca	EBUAP 1853	EF078589	EF078541		

[a] Taxonomy of *Leptodeira* based on Duellman (1958a).
[b] Voucher information: ASL = Alejandro Solórzano (private collection, Serpentario Nacional, Costa Rica); Avid = Pieter Barendsen (private collection); BPN = Brice P. Noonan (field number, UTA); CAS = California Academy of Sciences, Herpetological Collection, USA; EBUAP = Escuela de Biología de la Universidad Autónoma de Puebla, Mexico; ENEPI = Escuela Nacional de Estudios Profesionales Ixtacala, Distrito Federal, Mexico; ENS = Eric N. Smith (field number, UTA); ICP = Instituto Clodomiro Picado, Costa Rica; ITAH = Instituto Technológico Agropecuario de Hidalgo, Mexico; JAC = Jonathan A. Campbell (field number, UTA); JHT = Joshua H. Townsend (field number, UF); JMD = Juan M. Daza (field number, MHUA); KU = University of Kansas, Museum of Natural History, Division of Herpetology, USA; LJV = Laurie J. Vitt (field number, OU); LSU H = Lousiana State University Tissue Collection, USA; LSUMZ = Lousiana State University, Museum of Zoology, USA; MHNLS = Museo de Historia Natural La Salle, Caracas, Venezuela; MHUA = Museo de Herpetología, Universidad de Antioquia, Colombia; MPEG = Museu Paraense Emilio Goeldi; MSM = Mahmood Sasa Marin (private collection); MUHNES = Museo de Historia Natural de El Salvador, San Salvador; MVZ = Museum of Vertebrate Zoology, University of California, USA; MZFC = Museo de Zoología Facultad de Ciencias, UNAM, Mexico; SDSU = San Diego State University Museum, USA; SIUC = Southern Illinois University Carbondale, USA; UNAH = Universidad Nacional Autónoma de Honduras, Tegucigalpa; UTA = University of Texas at Arlington, Amphibian and Reptile Diversity Research Center, USA; YPM = Yale Peabody Museum, USA.

DNAH3 was amplified using the primers DNAH3-f1 and DNAH3-r6 (Townsend et al. 2008). All PCR products were sequenced directly in both directions using the amplification primers on an ABI 3730 DNA Analyzer. Raw sequence chromatographs were edited using Sequencher 4.7 (Gene Codes) and aligned manually using GeneDoc 2.6 (Nicholas and Nicholas 1997). All sequences generated in this study were deposited in GenBank (Table 1.1).

Phylogenetic reconstruction

Maximum likelihood (ML) and Bayesian Inference using Metropolis-Hasting coupled Markov chain Monte Carlo methods (BI) were used to infer phylogenies. For the phylogenetic analyses, we used two different datasets, one that was entirely mitochondrial and included all terminals. The second, included both mitochondrial and nuclear genes, was a reduced dataset with only the well supported

haplotype clades inferred in the prior analysis. First we inferred phylogenetic relationships using 130 terminals with the two mitochondrial genes. This extensive sampling included, in many cases, intraspecific sampling for several *Leptodeira* subspecies. By using model-based phylogenetic reconstruction methods, we assumed that mtDNA would have a strong phylogenetic signal to determine relationships both at the intra and interspecific level. To avoid potential problems in phylogenetic reconstruction with only mtDNA (i.e. saturation or introgression), we added two slow evolving genes from the nuclear genome that have been suggested as good candidates for phylogenetic reconstruction (Townsend et al. 2008). Therefore, for the second strategy of analyses, we reduced the dataset to 24 terminals representing the well supported clades recovered in the first analysis. This dataset included several outgroup species and one representative from each clade within *Leptodeira* recovered with the large mitochondrial dataset. The reduced dataset was analyzed in two ways: using the nuclear gene dataset exclusively, and including the mtDNA sequences in a combined analysis.

We used partitioned model analyses for all datasets because numerous studies have shown that partitioning models based on gene and codon position may be important for obtaining precise phylogenetic inferences (Castoe et al. 2004; Brandley et al. 2005; Castoe and Parkinson 2006), even at inter-specific levels of divergence (Castoe et al. 2005). We determined the best partition scheme by calculating the Bayes factor between two competing partition strategies (Nylander et al. 2004); results not shown). The mitochondrial dataset was partitioned by gene and codon position while the nuclear dataset was partitioned by gene and each gene was partitioned in two: one partition for first and second codon positions, and a second partition for third codon positions. The best substitution model for each partition was determined using the Akaike Information Criterion (AIC) with the programs Modeltest 3.7 (Posada and Crandall 1998) for the ML analyses and MrModeltest 2.3

(Nylander 2004) for the BI analyses (Table 2.2). The model likelihood values for each partition were calculated with PAUP* 4.0b10 (Swofford 2003) and then AIC scores were determined in Modeltest and MrModeltest.

Table 2.2. Substitution models obtained with Modeltest and MrModeltest for the different partitions. The mitochondrial dataset was partitioned by gene and codon position while the nuclear dataset was partitioned by gene and each gene was partitioned in two: one partition for first and second codon positions, and a second partition for third codon positions.

Partition	Maximum likelihood (Treefinder)	Bayesian inference (MrBayes)
1Cytb	GTR[Optimum, empirical]: GI[Optimum]:4	GTR+G+I
2Cytb	GTR[Optimum, empirical]: I[Optimum]:4	GTR+I
3Cytb	GTR[Optimum, empirical]: G[Optimum]:4	GTR+G
1ND4	GTR[Optimum, empirical]: GI[Optimum]:4	GTR+G+I
2ND4	GTR[Optimum, empirical]: GI[Optimum]:4	GTR+G+I
3ND4	HKY[Optimum, empirical]: G[Optimum]:4	HKY+G
tRNA	GTR[Optimum, empirical]: GI[Optimum]:4	GTR+G+I
12NT3	HKY[Optimum, empirical]: GI[Optimum]:4	HKY+G+I
3NT3	HKY[(4,1,1,1,1,4),(1,1,1,1)]: G[Optimum]:4	K80+I
12DNAH3	HKY[Optimum, empirical]: I[Optimum]:4	HKY+I
3DNAH3	HKY[Optimum, empirical]: G[Optimum]:4	HKY+G

Maximum likelihood analyses were conducted in Treefinder (Jobb 2008). Model parameters for each partition are described in Table 2.2. We allowed the program to estimate the best rate for each data partition. To estimate the relative support of nodes for the ML analysis, we conducted 500 non-parametric bootstrap pseudoreplicates in Treefinder. Bayesian analyses were conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Two independent MCMC runs were initiated with random starting trees and using one cold and three heated chains for 20 x 10^6 generations, sampling every 1000 steps. Model parameters were estimated independently for each partition using the *unlink* option in MrBayes. Stationarity of chains was verified for each analysis by plotting the chain likelihoods against generations using Tracer 1.4

(Rambaut and Drummond 2007). Three million generations were discarded as burn-in as the remaining samples resulted in ESS values larger than 1000 for all parameters. A consensus phylogram with posterior probabilities was determined by combining the remaining posterior samples from the two independent runs.

Divergence time estimation

We inferred divergence times among lineages using the combined reduced dataset (nDNA + mtDNA). Relaxed clock methods for divergence time estimation are preferred when the assumption of rate constancy is violated (Arbogast et al. 2002). Using the log likelihood ratio test, we rejected the null hypothesis of rate constancy ($p < 0.001$). Therefore, we used a stochastic model within a Bayesian approach that allows the estimation of rates and dates without the assumption of a molecular clock. We used two different approaches to check for congruence in the time estimates. First, we used Beast v1.4.7, which estimates the phylogeny and divergence times simultaneously, permitting more complex models of evolution and topological uncertainty during the optimization of divergence times (Drummond et al. 2006; Drummond and Rambaut 2007). We implemented the lognormal relaxed clock option with a Yule prior for the speciation pattern and again partitioned the dataset in a similar way as used in the ML and BI analyses. Second, we used the topology obtained with Treefinder and MrBayes and estimated divergence times using the package Multidistribute (Thorne et al. 1998; Thorne and Kishino 2002). For this second analysis, we partitioned the molecular data by gene. Using *baseml* (PAML Package; Yang 1997), model parameters for each partition were estimated under the F84 + Γ model. Branch lengths and the variance-covariance matrix were calculated using the program *estbranches*. Divergence times were then estimated using the program *multidivtime*. The priors used for analyses in *multidivtime* included: rttm = 3.9, rttmsd = 0.3, rtrate = 0.3, rtratesd = 0.3, brownmean = 0.7, brownsd = 0.7, and bigtime = 10.0. The remaining

priors used in *multidivtime* analyses were set to the program's defaults. For both approaches (Beast and *multidivtime*), we used a reduced data set for three reasons. First, we were interested in determining divergence times only at the interspecific level and among the main clades in the *Leptodeira annulata* / *septentrionalis* group. Second, intraspecific relationships do not correspond to a Yule process of speciation, which was the prior utilized in Beast. Third, intraspecific divergences show very short internodes, affecting the performance of branch length optimization in the program *estbranches* and, thus, producing unrealistic divergence time estimates.

Calibration points

The earliest fossil record of the Dipsadinae is very limited and difficult to interpret based only on osteology (Holman 2000), making the inferred placement of fossils onto a tree very imprecise (Graur and Martin 2004). In addition, most well-confirmed records for Dipsadinae come from very recent geological layers, obscuring the deeper origins of lineages (see Holman 2000). Therefore, we added three viperid species and one representative of Elapidae, Natricinae and Colubrinae to the dataset to constrain the root of the tree. Based on the oldest colubrid fossil found, the split between Viperidae and Colubridae is estimated to have occurred before 40 Ma (Rage et al. 1992; Head et al. 2005). We used a value of 40 ± 16 Ma for the program *multidivtime* and a lognormal prior of the root height of the tree with a lognormal mean = 3.7 and lognormal SD=0.3 for the program Beast. We used wide uniform priors and constrained the divergence between the New World and Old World Crotalinae to be older than 16 Ma and less than 32 Ma (Holman 2000; Guiher and Burbrink 2008; Castoe et al. 2009) and the origin of *Sistrurus* to be older than 9 Ma and less than 32 Ma (Parmley and Holman 2007). Finally, we constrained the origin of Natricinae to be older than 30 Ma (Rage 1988) and used a lognormal mean = 3.42 and a lognormal SD=0.3.

Ancestral area reconstruction

We tested the biogeographic hypothesis of Duellman (1958a) that states that the genus *Leptodeira* originated in Mexico with a directional north-to-south expansion. We reconstructed the ancestral distribution within *Leptodeira* using DIVA (Ronquist 1997). This event-based method does not require information about the area relationships and instead optimizes ancestral areas for nodes in a phylogenetic tree using a parsimony algorithm giving costs to dispersal and extinction scenarios. Even though taxon sampling may affect the ancestral area reconstruction (Ronquist 1997), our inferred ancestral areas for *Leptodeira* were not affected by the areas we used for the tips outside *Leptodeira* and *Imantodes* (results not shown). We assigned lineages to the three main biogeographic regions found in the Neotropics: Mexico that includes the tropical and subtropical region west of the Isthmus of Tehuantepec, Middle America that goes from the Isthmus of Tehuantepec to the Isthmus of Panama and South America that goes from eastern Panama to Brazil.

Results

Alignment and sequence variation

The total alignment for the mitochondrial dataset comprised 1933 bp (*Cyt-b* = 1083 bp, *ND4* = 681 bp, and *tRNA*'s = 169 bp). For the nuclear dataset, it was 1266 bp (*DNAH3* = 741 bp and *NT3* = 525 bp). The alignment was straightforward for protein coding genes, as no internal stop codons were detected. The mitochondrial dataset had 916 parsimony-informative sites (47.4%) for the large dataset and 659 parsimony-informative sites (34.1%) for the reduced dataset. On the other hand, the nuclear dataset had 70 (5.5%) parsimony-informative sites. The largest uncorrected percent genetic distance (P), using the mitochondrial dataset, was found between *Oxyrhopus petola* and *Leptodeira*

nigrofasciata (23.7%). Similarly, the largest P distance, using the nuclear dataset, was found between *Leptodeira septentrionalis* and *Oxyrhopus petola* (6.7%). Within *Leptodeira*, the largest genetic distance was found between *L. nigrofasciata* and *L. septentrionalis* for both the mitochondrial and the nuclear datasets (20.5% and 3.6%, respectively).

Phylogenetic reconstruction

Both the ML and BI analyses recovered well-supported clades and nearly identical topologies with some minor differences in nodal support, regardless of the dataset analyzed (Figs 2.3–2.4). The genera *Pseudoleptodeira, Hypsiglena*, and *Eridiphas* formed a well-supported clade, as did a cluster of other genera including *Cryophis*, *Atractus*, *Sibon*, *Ninia* and *Dipsas*; the sister-group relationship between these two clades was not well supported, however. *Leptodeira* and *Imantodes* formed a clade with 100% support in both ML and BI analyses. *Leptodeira* was inferred to be monophyletic, with relatively high support (bootstrap = 81% PP = 92%, Fig. 2.3). In contrast, *Imantodes* was found to be paraphyletic, with a clade containing *I. lentiferus*, *I. gemmistratus* and *I. cenchoa* being the sister taxon to *Leptodeira*, and *I. inornatus* the sister taxon to both. Within *Leptodeira*, there was a ladderized pattern, with *L. nigrofasciata* diverging the earliest, followed by *L. frenata*. *Leptodeira punctata* formed a clade with *L. splendida*, with moderate support (bootstrap = 69%, PP = 94%) and their sister clade is composed of members of the *L. septentrionalis* and *L. annulata* groups (*sensu* Duellman, 1958a).

Intraspecific sampling recovered all *Leptodeira* species as monophyletic except the species *L. annulata* and *L. septentrionalis*. Samples assigned to *L. septentrionalis* were found in three distantly related clades (Fig. 2.4). Although samples assigned to *L. s. polysticta* formed a monophyletic group, such was not the case for *L. s. ornata*. A similar polyphyletic pattern was observed in *L. annulata*, in which four independent clades were recovered. Only the subspecies *L. a. rhombifera* was found to be

monophyletic. Each *L. annulata* clade recovered was the sister taxon to either *L. septentrionalis*, *L. maculata*, or *L. bakeri* (Fig. 2.4). Overall, sister-taxon relationships were found between geographically contiguous lineages rather than between traditionally recognized subspecies (Fig. 2.4).

The analysis of the combined dataset (nDNA + mtDNA) produced essentially the same topology as the one recovered with the large mtDNA dataset. The phylogenetic signal of the nuclear dataset alone was sufficient to infer the relationships among the main clades that were obtained with the large mitochondrial dataset (around 50% of the nodes were resolved with high support; Fig. 2.5). The supports for the ML and BI analyses of the nuclear gene data were relatively high for the intergeneric relationships (bootstrap > 70%, PP > 95%). Again, *Leptodeira* and *Imantodes* clustered to form a well-supported clade within the Dipsadinae (100% support for both analyses), although there was a polytomy among major lineages of *Imantodes* and *Leptodeira* that rendered the *Leptodeira* monophyly unresolved (Fig. 2.5). Overall, the resolution of phylogeny estimated from the nuclear data was in excellent agreement with that of the mitochondrial data (Figs. 2.3–2.5).

Divergence times and ancestral area reconstruction

Analyses with Beast and *multidivtime* produced similar divergence time estimates (Table 2.3, Fig. 2.6), and hereafter we refer specifically to the Beast results. Mutation rates varied among branches and between mitochondrial and nuclear markers. The average mutation rate for mitochondrial genes was 1.34% per million years ($CI_{95\%}$ = 0.99–1.70%) and for nuclear genes was 0.14% per million years ($CI_{95\%}$ = 0.10–0.18%). The origin of Dipsadinae was inferred to be approximately 28.4 Ma ($CI_{95\%}$ = 19.9–37.3). Most of the diversification of the Dipsadinae was estimated as having occurred during the first half of the Miocene (~11–20 Ma; Fig 2.7), while the

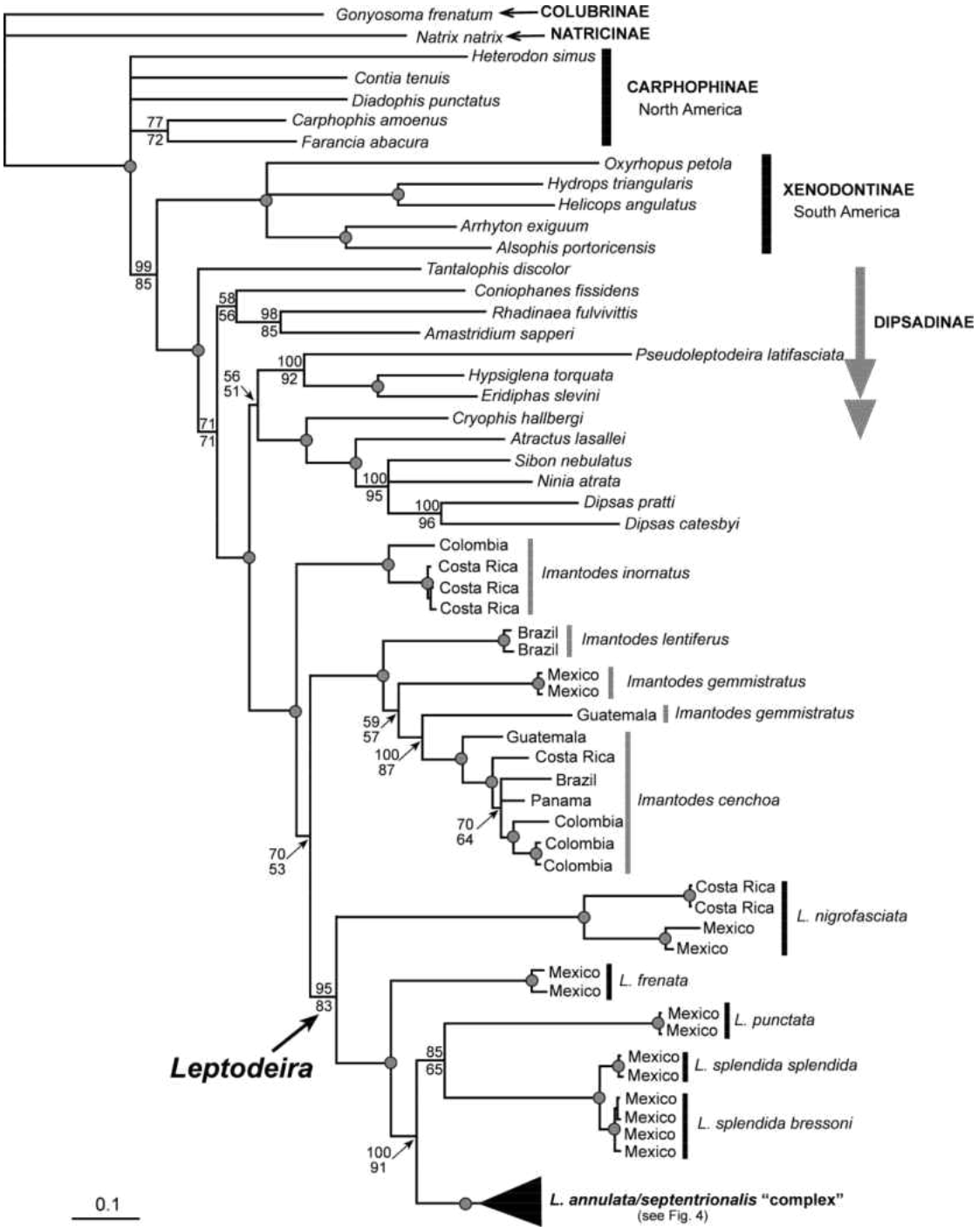

Figure 2.3. Phylogenetic estimate of relationships within the Dipsadinae, and among the major groups of *Leptodeira*. The tree represents the Bayesian 50% majority-rule consensus phylogram from a partitioned analysis of mitochondrial gene sequences (*Cyt-b*, *ND4*, and *tRNA*'s; total of 1933 bp). Grey circles represent nodes with > 95% support obtained via maximum likelihood (bootstrap values) and Bayesian (posterior probabilities) analyses. Numbers above nodes are posterior probabilities and numbers below nodes are maximum likelihood bootstrap support.

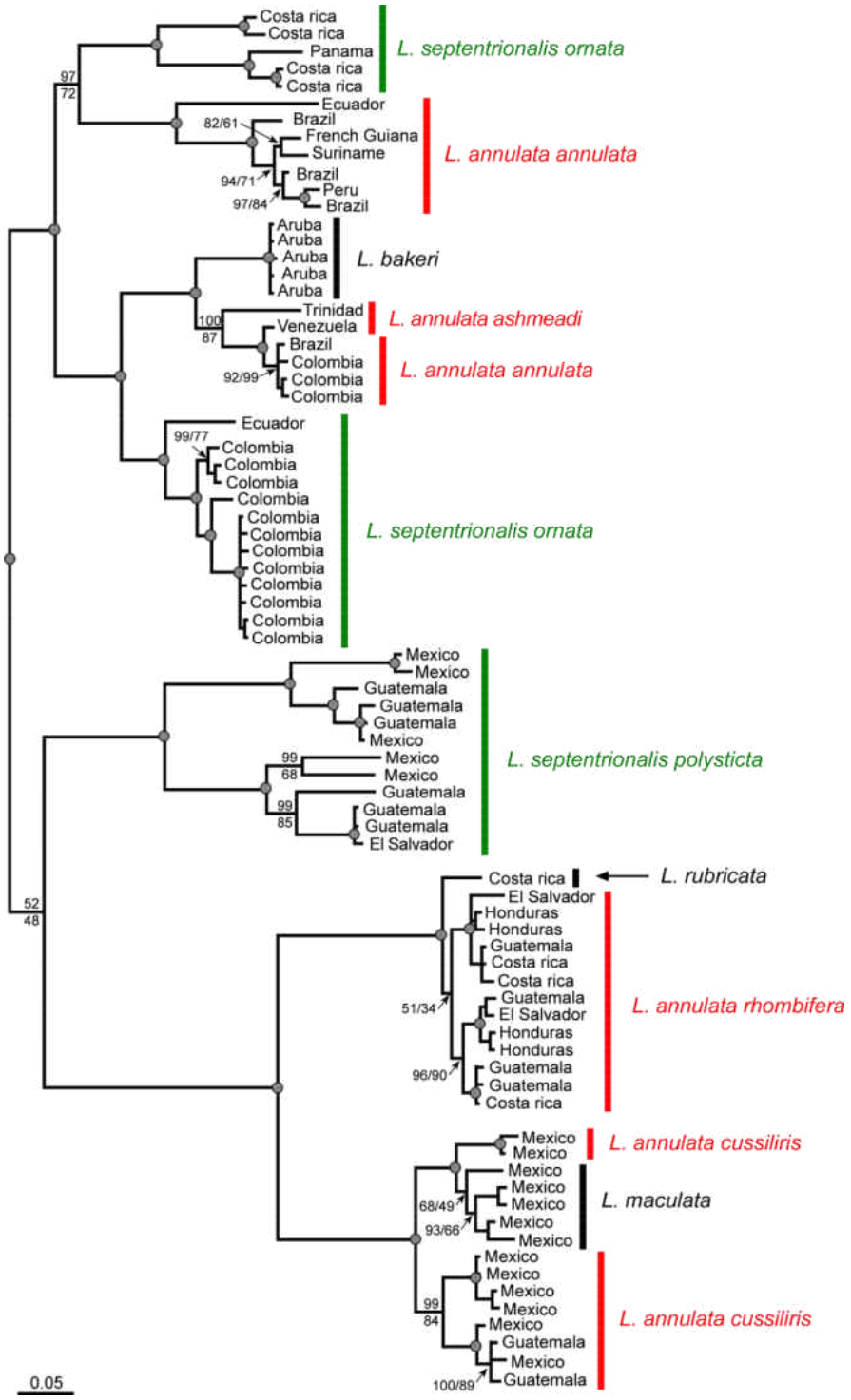

Figure 2.4. Expanded view from Fig. 2.3 depicting the phylogenetic relationships of the *Leptodeira annulata* and *L. septentrionalis* species complex.The tree represents the Bayesian 50% majority-rule consensus phylogram from a partitioned analysis of mitochondrial gene sequences (*Cyt-b*, *ND4*, and *tRNA*'s; total of 1933 bp). Grey circles represent nodes with > 95% support obtained via maximum likelihood (bootstrap values) and Bayesian (posterior probabilities) analyses. Numbers above nodes are posterior probabilities and numbers below nodes are maximum likelihood bootstrap support.

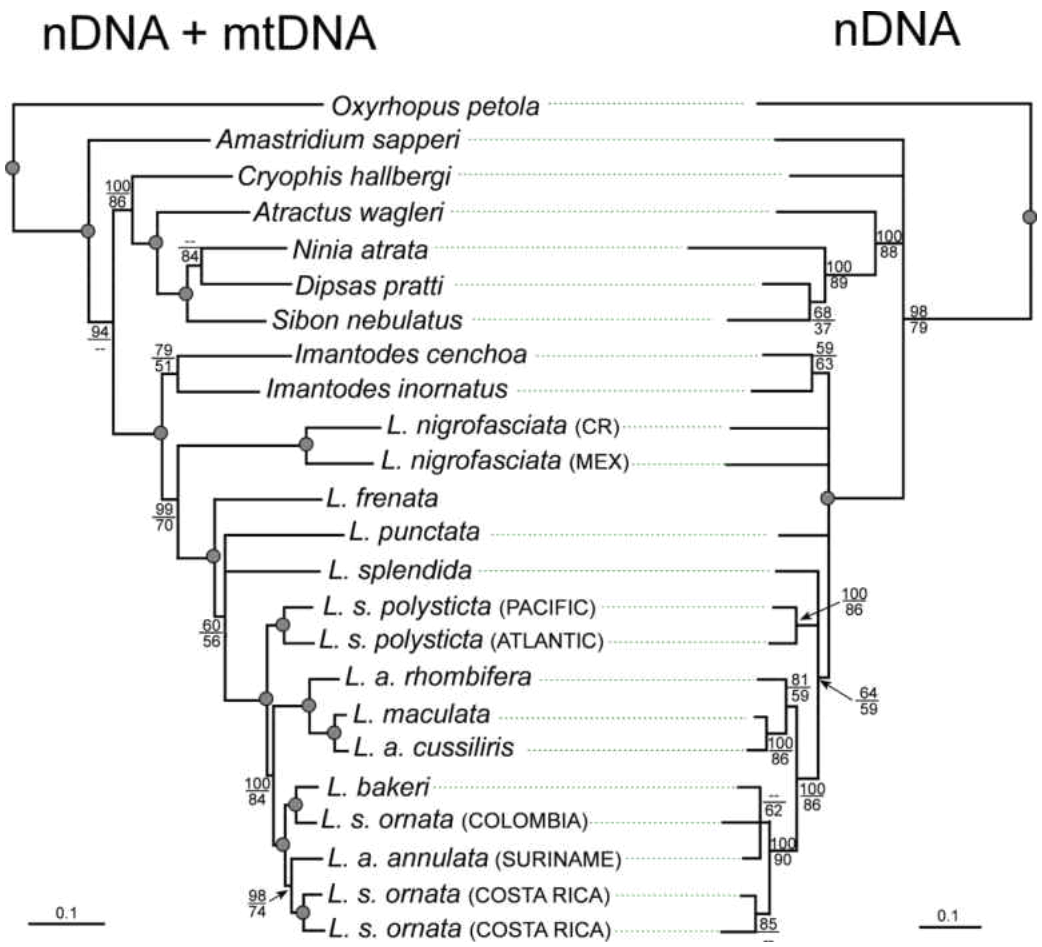

Figure 2.5. Phylogenetic relationships of *Leptodeira* and relatives. A) Bayesian 50% majority-rule consensus phylogram from a partitioned analysis of the mitochondrial and nuclear combined data (total of 3199 bp). B) Bayesian 50% majority-rule consensus phylogram from a partitioned analysis including only the nuclear genes *DNAH3* and *NT3* (total of 1266 bp). Grey circles represent nodes with > 95% support obtained via maximum likelihood (bootstrap values) and Bayesian (posterior probabilities) analyses. Numbers above nodes are posterior probabilities and numbers below nodes are maximum likelihood bootstrap support. Dashes represent nodes that were not recovered with either Bayesian or maximum likelihood analysis.

Table 2.3. Statistics for the divergence times estimates obtained from two different programs. Node numbers from Fig. 6. Lower and upper boundaries for the 95% Credibility Intervals are shown.

	multidivtime			Beast		
Node	**Mean**	**Lower**	**Upper**	**Mean**	**Lower**	**Upper**
30	13.68	9.37	20.90	10.62	9.13	13.43
31	24.40	17.42	34.10	19.95	16.00	25.69
32	3.59	2.24	5.49	3.49	2.06	5.17
33	2.64	1.60	4.12	2.44	1.36	3.58
34	2.19	1.25	3.50	1.89	1.05	2.82
35	3.45	2.19	5.25	3.26	2.12	4.60
36	4.18	2.72	6.20	3.91	2.61	5.38
37	1.14	0.59	1.90	1.07	0.58	1.66
38	3.19	1.92	4.93	2.99	1.88	4.27
39	5.53	3.67	8.14	5.47	3.73	7.44
40	5.89	3.93	8.63	6.02	4.12	8.12
41	8.74	5.64	12.74	8.61	5.49	11.97
42	10.28	7.12	14.44	10.37	7.02	13.83
43	11.14	7.77	15.71	11.48	7.83	15.38
44	5.97	3.35	9.42	6.37	3.86	9.22
45	13.46	9.71	18.58	14.80	10.37	19.66
46	12.70	9.02	17.62	13.55	8.90	18.80
47	14.69	10.83	20.19	16.09	11.40	21.36
48	9.97	6.93	14.24	9.15	6.42	15.05
49	10.85	7.67	15.38	9.99	6.43	13.80
50	12.95	9.32	18.12	13.18	8.84	17.86
51	15.32	11.30	20.97	16.31	11.23	21.88
52	18.37	14.12	24.48	19.73	13.93	25.90
53	19.70	15.35	26.21	21.33	15.22	28.12
54	28.13	22.74	36.54	28.40	19.85	37.26
55	31.79	26.83	40.89	32.44	23.44	42.96
56	34.29	30.14	43.76	35.24	25.43	46.22
57	37.64	31.73	48.18	40.24	29.15	53.19
58	41.12	33.80	52.40	43.58	31.47	56.34

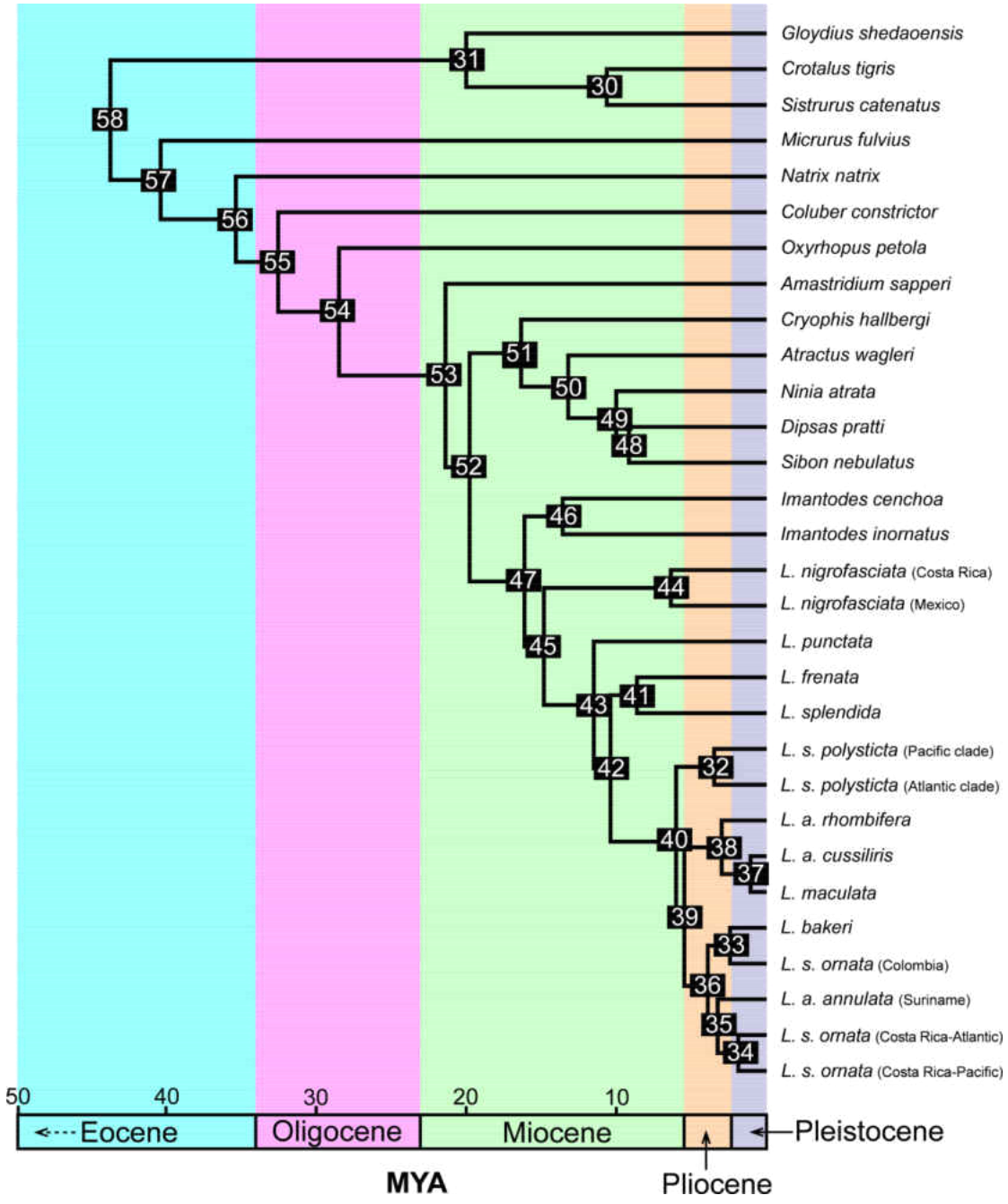

Figure 2.6. Maximum clade credibility tree obtained with Beast. Mean and 95% Credibility Limits for numbered nodes are shown in Table 2.3.

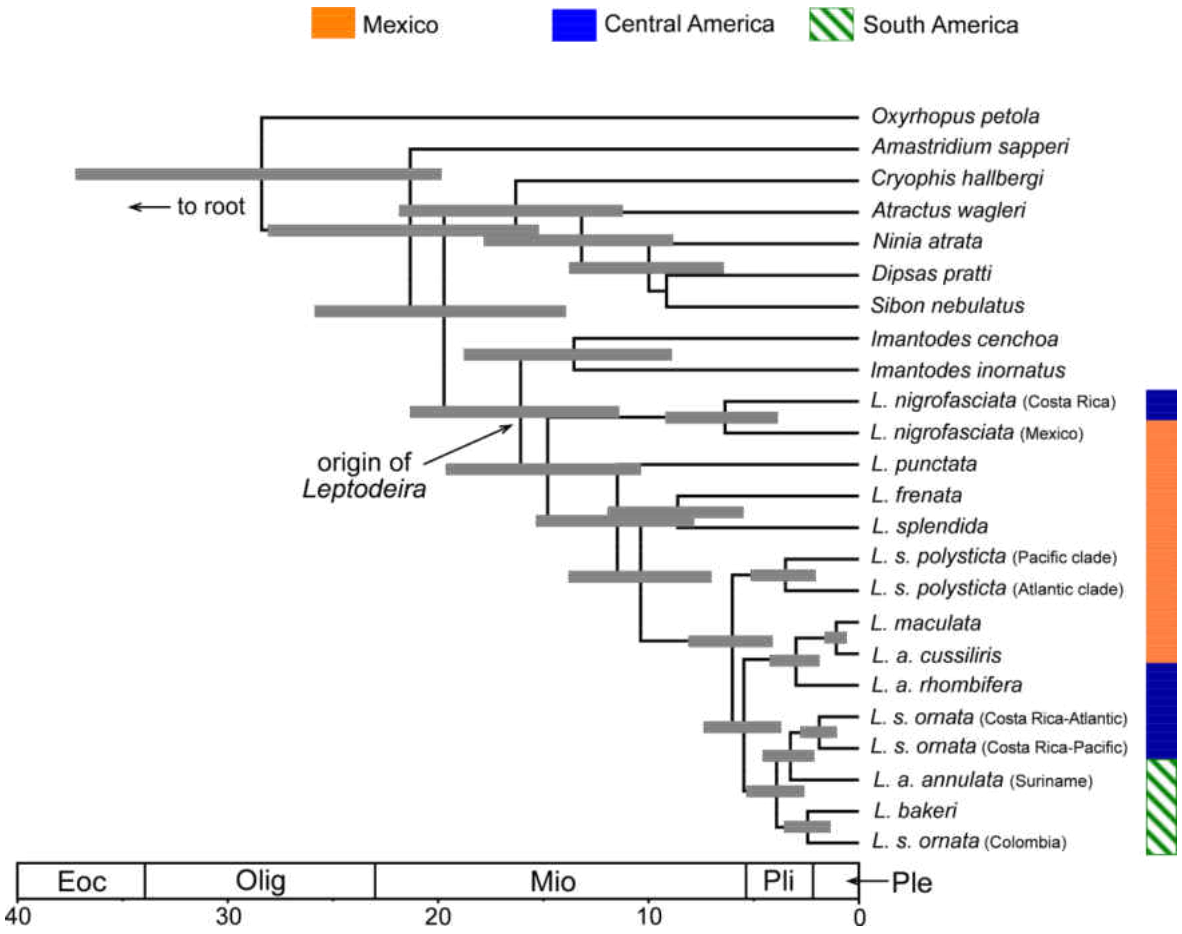

Figure 2.7. Divergence time estimates of *Leptodeira* and relatives inferred with Beast 1.4.7. Grey bars represent the 95% credibility intervals for node heights. Time periods as follows: Olig = Oligocene, Mio = Miocene, Pli = Pliocene, Ple = Pleistocene.

origin of *Leptodeira* was estimated to be 16.1 Ma ($CI_{95\%}$=11.4–21.6 Ma). Speciation within *Leptodeira* appears to be mostly from the second half of the Miocene, although certain lineages originated both during the Pliocene and as recently as the Pleistocene (Fig. 2.7). Regarding the geographic speciation of *Leptodeira*, lineage diversification in the Mexican transition zone occurred from the Miocene to the Pleistocene, and the diversification of species distributed in Central and South America occurred in a narrower window of time during the Pliocene.

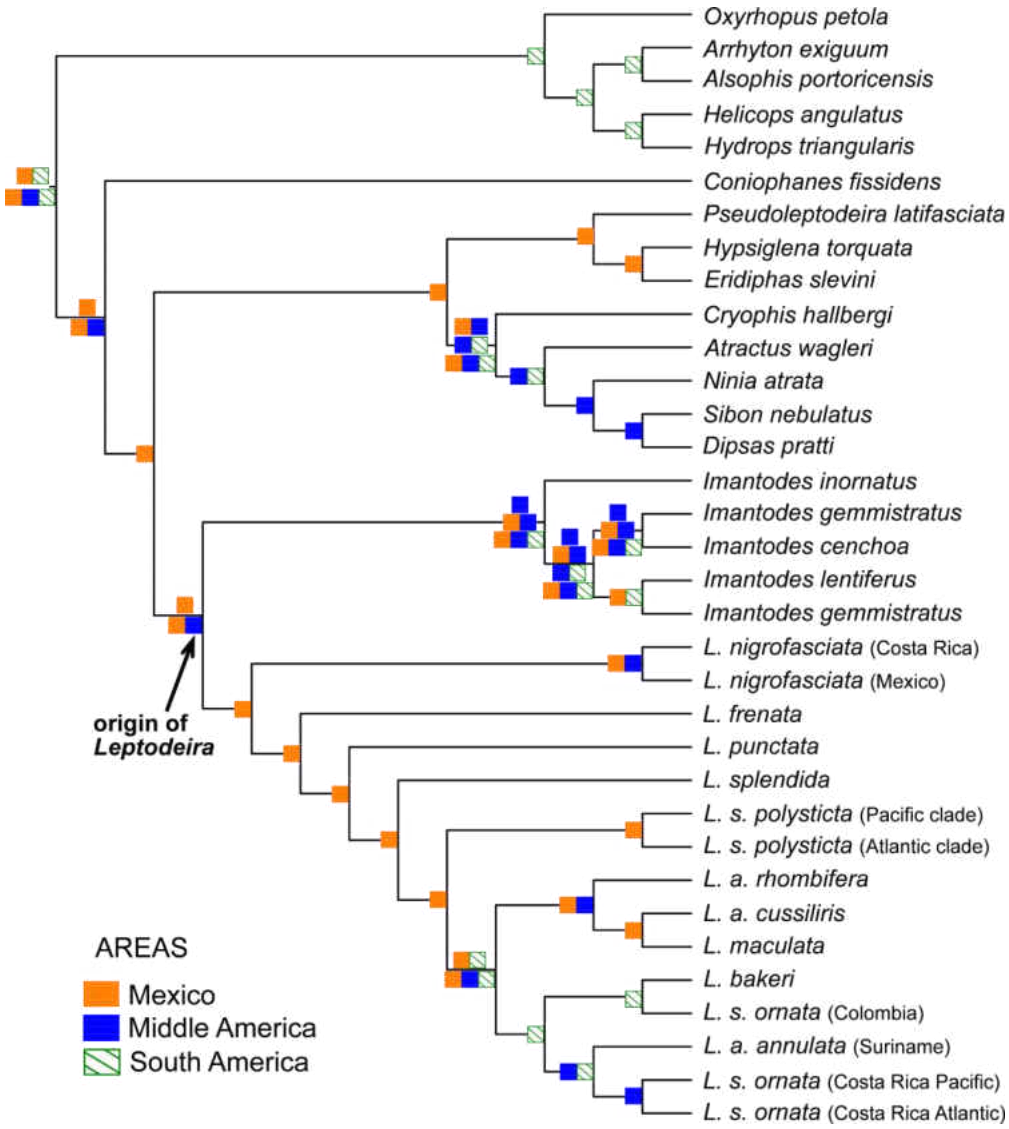

Figure 2.8. Ancestral area reconstruction of Dipsadinae lineages using DIVA. More than one reconstruction for the same node indicates ambiguity.

According to DIVA, the geographic origin of *Leptodeira* could not be resolved unambiguously (Fig. 2.8). The ancestral area for the nodes leading to *Leptodeira* and the first split within *Leptodeira* may have been either Mexico or an area comprising Mexico and Middle America. The ancestral area for the species *L. frenata*, *L. punctata* and *L. splendida* and the subspecies *L. septentrionalis polysticta* was estimated to be Mexico. A general pattern of north-to-south colonization from Mexico to South America was observed within *Leptodeira* (Fig. 2.8).

Discussion

Phylogenetic and biogeographic patterns obtained during this study highlight the spatial and temporal complexity of biological diversification in the Neotropics. Given its broad distribution throughout this region, *Leptodeira* appears to be an excellent model through which to understand the historical patterns of lineage diversification in one of the most biodiverse regions in the world. Our results challenge both the current phylogenetic and taxonomic status of the genus *Leptodeira*, and the traditional use of morphology to delimit evolutionary units in Neotropical snakes. Patterns of lineage diversification within *Leptodeira* also reveal much about the historical processes that have shaped the genus evolution, and probably many other lineages throughout the Neotropics since the Miocene.

Phylogenetic relationships within Dipsadinae

The subfamily Dipsadinae has been hypothesized to represent a monophyletic group, although roughly 50% of the putative genera have not been analyzed (Zaher 1999; Vidal et al. 2000; Pinou et al. 2004; Lawson et al. 2005). To increase our understanding of Dipsadinae relationships, we included the genera *Amastridium* and *Ninia*. We found that the two genera should be included within the Dipsadinae (Fig. 2.3). The monotypic genus *Tantalophis* was previously considered a member of *Leptodeira*, but evidence has repeatedly shown *Tantalophis* to be a very distinct lineage (Duellman 1958b; Mulcahy 2007). Our data confirm this idea, as well as the hypothesis of *Tantalophis* as a member of the Dipsadinae, as opposed to Lawson et al. (2005) who defined the genus as *incertae sedis*. The subfamily Dipsadinae has more than 400 extant species and future phylogenetic studies are required to elucidate the patterns and mechanisms by which its fascinating diversity was accomplished.

Monophyly of Leptodeira

The first species of the genus *Leptodeira* was described by (Linnaeus 1758) as *Coluber annulatus*, but (Fitzinger 1843) later allocated this species to its own genus, *Leptodeira*. Since then, several species currently in *Leptodeira* have been assigned to other genera of Central American dipsadines (e.g., *Sibon*, *Hypsiglena*; Duellman 1958a). Mulcahy (2007) examined the monophyly of the genus, and even though he did not include all the species assigned to *Leptodeira*, two main results can be highlighted from his work. First, *Leptodeira* appeared to be non-monophyletic in the parsimony analysis (see his Fig. 4) but monophyletic with moderate support, in the Bayesian analysis (PP = 86%). Second, regardless of the reduced taxon sampling, some species groups and subspecies appeared to be paraphyletic.

Using a combined analysis of four genes, we inferred a strongly supported clade that includes all species of *Leptodeira* (Fig. 2.5). The nuclear dataset alone, however, did not infer a monophyletic *Leptodeira* but rather a polytomy including *Imantodes* and *Leptodeira* species was recovered. This lack of resolution is likely due to the low numbers of informative characters in the nuclear dataset (see results). The two nuclear genes resolved the relationships among different genera of Dipsadinae and even within *Leptodeira*, but they did not support the monophyly of the genus (Fig. 2.5). It is also plausible that the divergence between *Imantodes* and *Leptodeira* occurred in a narrow window of time and therefore a high degree of nuclear polymorphism in the ancestor of these genera did not have enough time to coalesce between splitting of population lineages, resulting in a lack of phylogenetic signal (Moore 1995; Rosenberg 2002).

The present results suggest *Imantodes* as monophyletic, based on both combined nuclear and mitochondrial data or nuclear alone. In addition to the increased character sampling, including

intraspecific sampling of *Imantodes inornatus* and *Imantodes cenchoa* (both from Central America and northern South America) has provided evidence of previously unexpected genetic diversity. This diversity should be further examined to elucidate phylogeographic patterns that might parallel the co-distributed genus *Leptodeira*. The paraphyly of *I. gemmistratus*, the uncertain phylogenetic position of *I. tenuissimus* and *I. phantasma* (species not included in this study), and the observed genetic diversity within *I. cenchoa* further justify a broader biogeographic study for this widely distributed group.

Leptodeira *species groups and alpha taxonomy*

Current taxonomic classification of *Leptodeira* is based entirely on morphology. Duellman (1958a) defined species groups and alpha taxonomy on hemipenial morphology, color pattern and geographic distribution. Our study, in addition to Mulcahy's (2007) work, supports the idea that current species groups in *Leptodeira* do not represent natural groupings. None of the species groups proposed by Duellman were recovered as monophyletic (Figs 2.3–2.6). Consequently, the previously employed species group assignments need to be removed from the systematics of this genus, and species and subspecies status should be reassessed to reflect our new views of the evolutionary history of *Leptodeira*.

We obtained strong support for *Leptodeira nigrofasciata* being the sister taxon to a clade comprising all other species of the genus. Interestingly, uncorrected genetic distance between *L. nigrofasciata* and the remaining species of *Leptodeira* was as high as that found between *L. nigrofasciata* and *Imantodes* (about 16–17%; see also Mulcahy 2007). Even though we examined only four individuals of *L. nigrofasciata*, our results present two very divergent allopatric lineages with a fairly ancient divergence; the first

lineage includes populations from the pacific coast of Mexico and the second populations from northern Guatemala to northwestern Costa Rica. The deep genetic divergence, the strong morphological difference (Smith and Taylor 1945; Taylor 1954; Shannon and Humphrey 1964), and the allopatric distribution provide evidence for potential species recognition of these two divergent lineages after analyzing samples from the intervening land, El Salvador, Honduras, and Nicaragua.

The sister-taxon relationship between *L. splendida* and *L. punctata*, as suggested by Mulcahy (2007), was not recovered in our combined analysis using nuclear and mitochondrial markers, but it was recovered by the mitochondrial dataset alone. The nuclear dataset, although with low support, suggests that *L. punctata* may be the sister taxon to a clade including *L. splendida* and members of the *L. septentrionalis* and *L. annulata* groups. Whether the mitochondrial or nuclear datasets separately infer the true phylogeny, our results highlight the importance of adding independent phylogenetic markers and more individuals to estimate the species tree from gene trees (Maddison and Knowles 2006). Regarding the subspecies status within *L. splendida*, we did find reciprocal monophyly between *L. s. splendida* and *L. s. bressoni*. Based on these preliminary results, in addition to the morphological evidence given by Duellman (1958a), we suggest maintaining the subspecies status within *L. splendida* until additional evidence is gathered and phylogeographic boundaries can be discovered (see below).

Leptodeira annulata-septentrionalis *"complex"*

The most striking result of this study is the polyphyly of the species *L. annulata* and *L. septentrionalis* (Fig. 2.4). These two groups are the most widely distributed species of the genus, and given the morphological and geographic variation, five subspecies of *L. annulata* and four of *L. septentrionalis* are currently recognized (Duellman 1958a). Our results detailing excessive polyphyly of these two species, however, are not entirely surprising given the high degree of morphological variability in

both species that often overlaps between species. It thus appears that morphological parallelism has likely precluded previous taxonomic efforts to accurately identify evolutionary units in this complex. (Sasa-Marin 2000) investigated the phylogeography of *L. annulata* in the dry forests of Central America. His *L. annulata* includes those belonging to *L. a. cussiliris* in the Pacific coast of Oaxaca and western Guatemala and the dry Grijalva Valley of Mexico and Guatemala, and *L. a. rhombifera* from the eastern Pacific coast and interior valleys of Guatemala to northwestern Costa Rica. Both forms represent relatively short and terrestrial forms. Herein we confirm his deep division in Guatemala, between the two subspecies, and find *L. a. rhombifera* also in two main clades located north and south of the Comayagua valley of Honduras.

Several "variants" allied to *L. annulata* have been elevated to species level (*L. rubricata*, *L. maculata*, *L. bakeri*). For instance, Savage (2002) refers to an unpublished work that "convincingly" suggests keeping *L. rubricata* as a distinct species after Duellman (1958a) synonymized it with *L. annulata*. Our analyzed sample of *L. rubricata* was not found to be genetically distinct from members of *L. a. rhombifera* as its sequence divergence was equivalent to that among members of the subspecies (Fig 4.) While genetic distance should not be the sole criterion for species diagnosis (Wiens and Servedio 2000; Sites and Marshall 2004; Esselstyn 2007), this finding warrants further studies to determine if *L. rubricata* is a distinct lineage deserving species status.

As predicted by Duellman (1958a), *L. bakeri* was closely related to the mainland form, *L. a. ashmeadi* (Fig. 2.4). Given the small geographic distribution of *L. bakeri*, and the monophyly observed we hypothesize that this is most likely the result of a single population lineage that colonized the island of Aruba. In addition to the phylogenetic results, its morphological distinctiveness from the mainland clade and its allopatric distribution (Mijares-Urrutia et al. 1995) support its recognition as a

distinct evolutionary unit (*sensu* Wiens and Penkrot 2002). Based on geographic gradients of the number of dorsal blotches, Duellman (Duellman 1958a; Duellman 1966) also recognized *L. maculata* as a different species from *L. annulata cussiliris* and suggested sympatry as unlikely. These two species are not easily diagnosable based on the characters given by Duellman (E. Smith, *pers. comm.*; see also (Shannon and Humphrey 1964). Our phylogenetic results (both mitochondrial and nuclear) suggest the same mixed pattern. Individuals from Guerrero and Oaxaca considered *L. a. cussiliris* are phylogenetically nested within *L. maculata*, instead of being nested with the remaining *L.a. cussiliris* (Figs. 2.4 and 2.7). This result, in addition to the morphological similarity between the two groups, suggests that *L. maculata* is a geographic variant of the widespread *L. a. cussiliris* and should therefore be synonymized (contra Duellman 1966).

Leptodeira septentrionalis, as currently recognized, can be distinguished phylogenetically as three distantly related clades: one in northern Central America (Mexico and Guatemala), another clade in lower Central America (Costa Rica and Panama), and a third in northwestern South America (Colombia and Ecuador). Each of these three lineages is the sister group to a clade of *L. annulata,* and all are allopatric except for the presence of sympatric *L. s. polysticta* with *L. a. cussiliris* in Mexico and *L. a. rhombifera* in Central America, from Guatemala to, probably, Costa Rica. Similarly, *L. annulata* consists of five independent clades that intermix with *L. septentrionalis* clades, *L. maculata* or *L. bakeri*. Collectively, these findings underscore the need for numerous taxonomic changes regarding these two species, as well as *L. maculata* and *L. bakeri*. Species delimitation and description is, however, outside the scope of this study, and taxonomic changes will be treated elsewhere using additional lines of evidence, such as morphological and ecological modeling data.

(Campbell 1998) elevated *L. septentrionalis polysticta* to species status based on morphological evidence. Our phylogenetic evidence strongly supports his claim as this group represents a monophyletic group, highly divergent from *L. s. ornata* or the other subspecies examined (Fig. 2.4). More interesting is the fact that *L. s. polysticta* had the greatest within-species genetic structure within the genus. Two divergent clades, which appear candidates for species status, were recovered with high support from both mitochondrial and nuclear datasets (Figs. 2.4 and 2.5); one clade represents the humid forests in the Atlantic versant of Mexico and Guatemala while the other clade corresponds to the dry regions of the Pacific coast of Mexico, Guatemala and El Salvador (Fig. 2.9). Our lack of sampling in Honduras and the Mosquitia region of Nicaragua preclude any further confirmation of the southern extent of *L. s. polysticta* or the northern extent of *L. s. ornata*. According to Duellman (1958a) the first form should occur all the way south to northeastern Costa Rica, and *L. s. ornata* should have its northern limit near de Costa Rica-Panama border.

Diversification and biogeography

Lineage diversification within *Leptodeira* corresponds largely to the major biogeographic provinces in the Neotropics. Well-recognized biogeographic regions, such as the Mexican transition zone, lower Central America and northwestern South America, played a critical role in shaping the diversity of *Leptodeira*. In contrast, the Amazon basin did not appear to be a major factor for lineage diversification. Understanding the phylogenetic relationships and the time of cladogenetic events within *Leptodeira* will help us to identify the importance of historical events occurring in these provinces and to highlight their contributions to the Neotropical diversity.

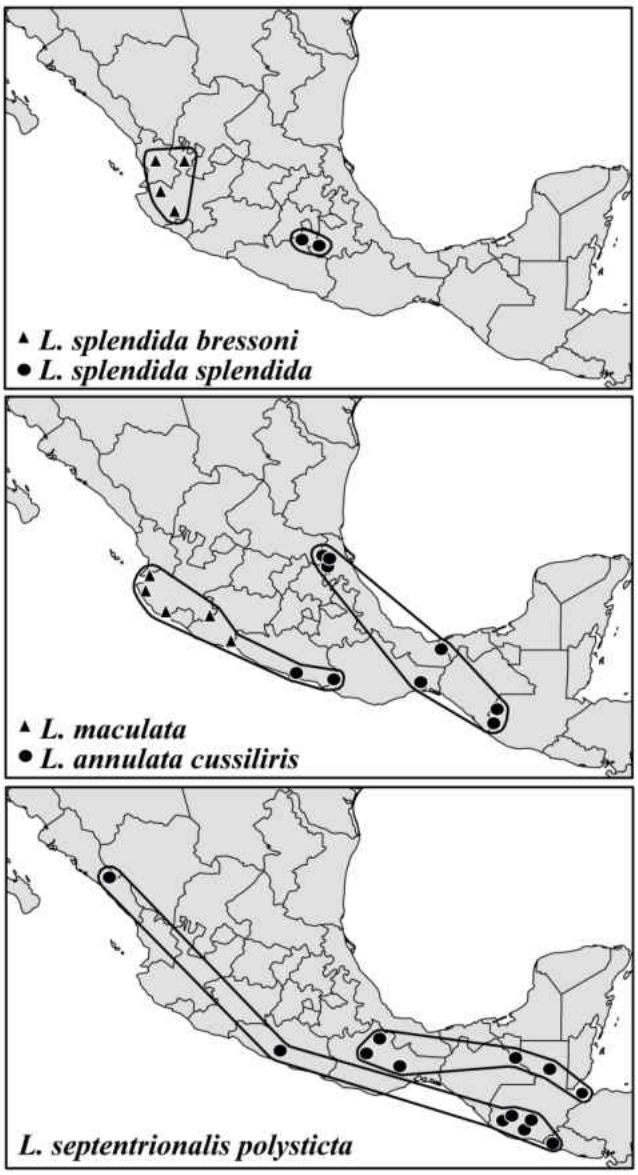

Figure 2.9. Intraspecific phylogeographic structure of *Leptodeira* species in the Mexican transition zone. Lines delimit the clades recovered with the mtDNA dataset, and dots represent sampled localities.

The Mexican transition zone

The Mexican transition zone (*sensu* Halffter 1987) is one of the most complex regions in the Americas, with a dynamic geological evolution since the Cretaceous period (Coney 1982; Ortega and Arita 1998). The importance of its *in situ* diversification and the interchange between the Neartic and

the Tropical region has been addressed by many authors (Marshall and Liebherr 2000; Morrone and Márquez 2001; Escalante et al. 2004; Huidobro et al. 2006); and references therein). It has been hypothesized that the origin of *Leptodeira* occurred in Mexico (Duellman 1958a; Mulcahy 2007). This hypothesis is largely based on the observation that the majority of species, many separated by the deepest phylogenetic splits of the genus, occur there. Using an explicit method for ancestral area reconstruction (DIVA), we could not resolve unambiguously the area where the *Leptodeira* + *Imantodes* ancestor may have originated. This lack of resolution is likely due to *Imantodes,* the sister taxon to *Leptodeira*, having a widespread distribution. Instead, we did find evidence that the early and most important lineage diversification of *Leptodeira* occurred in the Mexico (Fig. 2.8). Using explicit methods to estimate divergence times, we also inferred that this diversification began during the middle Miocene and spanned throughout the Pleistocene. Duellman (1958a) proposed a similar temporal frame, using geological and geographic information (compare Figs. 2.2 and 2.6). Most likely, the recurrent orogenic events across the Mexican transvolcanic axis and the Isthmus of Tehuantepec during the Miocene severed gene flow between Atlantic and Pacific populations to give rise to *L. frenata* on the Atlantic and *L. nigrofasciata*, *L. splendida* and *L. punctata* on the Pacific versant. The diversification of lowland species within western Mexico is less obvious but could be related to either the formation of the main river basins or to Miocene climatic changes (Devitt 2006; Espinosa et al. 2006; Bryson et al. 2008); and references therein). During more recent times, Pleistocene climatic changes and sea level fluctuations in the Isthmus of Tehuantepec might have severed gene flow among Mexican populations, generating the phylogeographic patterns observed at the intraspecific level (Fig. 2.9).

The bridge between Central and South America

Lower Central America harbors one of the most diverse biota per square kilometer on the planet (Savage 2002). The tremendous *in situ* diversification and the role as the final bridge between South America and the Neartic region during the Pliocene allowed multiple lineages to colonize both continents (Marshall et al. 1979; Webb 1997). Current phylogenetic and biogeographic evidence shows that this interchange occurred several times, even prior to the Pliocene, a time for which evidence of land connection between the two regions is missing (Marshall et al. 1979; Bermingham and Martin 1998; Pennington and Dick 2004; Koepfli et al. 2007); and references therein). Our DIVA results show that *Leptodeira* reached South America via the Panama Isthmus in a single colonization. Later on, an event of dispersal from South America back to Lower Central America (Fig.S2) is predicted. If the expansion of *Leptodeira* into South America was gradual and monotonic, we would expect to see sister-taxon relationships between adjacent regions. Instead, *L. septentrionalis* from Costa Rica is the sister taxon to the clade in the Amazon basin, and the Colombia + Ecuador + Venezuela clade is the sister taxon to the Costa Rica + Amazon basin clade. Ancestors of *Leptodeira* colonized northern South America around 4 Ma prior to the closure of the isthmus. We hypothesize that after the closure, around 3.4 Ma, a second colonization event occurred, this time from South America back to Lower Central America. It is interesting to note that cat-eyed snakes from humid forests in Costa Rica resemble the ones in the Amazon basin in their arboreal-semiarboreal habits, whereas *Leptodeira* from Colombia and northern Venezuela are mostly terrestrial (Duellman 1958a; Savage 2002; pers. obs.). Given these phylogenetic patterns and the ecological distribution of *Leptodeira* in South America, we hypothesize that fluctuations in vegetation cover allowed range expansion and severed gene flow affecting the arid and mesic clades differently (Crawford et al. 2007; Peterson and Nyári 2008; Wang et al. 2008). Finally, the divergence between

the Chocó-Magdalena clade and the northern Colombia-Venezuela clade during the Pleistocene might have been mediated by climatic fluctuations and eustatic sea level changes, isolating and severing gene flow among the different populations (Nores 2004).

The Amazon basin

In contrast with other biogeographic provinces where *Leptodeira* is distributed, the Amazon basin clade did not show strong genetic structure, despite having the largest distribution (Fig. 2.10). Lack of genetic structure in the Amazon basin, attributed to Quaternary expansion, has been observed in other groups (Zamudio and Greene 1997; Dick et al. 2004; Nyári 2007; Peterson and Nyári 2008). It has been documented that climatic fluctuations in the Amazon basin were drastic during the Pleistocene, expanding and contracting dry and humid habitats, which might have led to speciation or intraspecific phylogeographic patterns (Prance 1982; Hooghiemstra and van der Hammen 1998; Quijada-Mascarenas et al. 2007; Rull 2008; but see Colinvaux et al. 2000). Although our results suggest that *Leptodeira* did not respond to these dramatic changes in the Amazon, it is also possible that the Amazonian clade was never fragmented and persisted in a more stable environment (Colinvaux et al. 2000). Sampling from the southernmost part of the *Leptodeira* distribution (*L. annulata annulata* and *L. a. pulchriceps*) and from the Atlantic forests of Brazil might reveal hidden phylogeographic structure, which has been observed in other codistributed species (Wüster et al. 2005; Grazziotin et al. 2006; Martins et al. 2007).

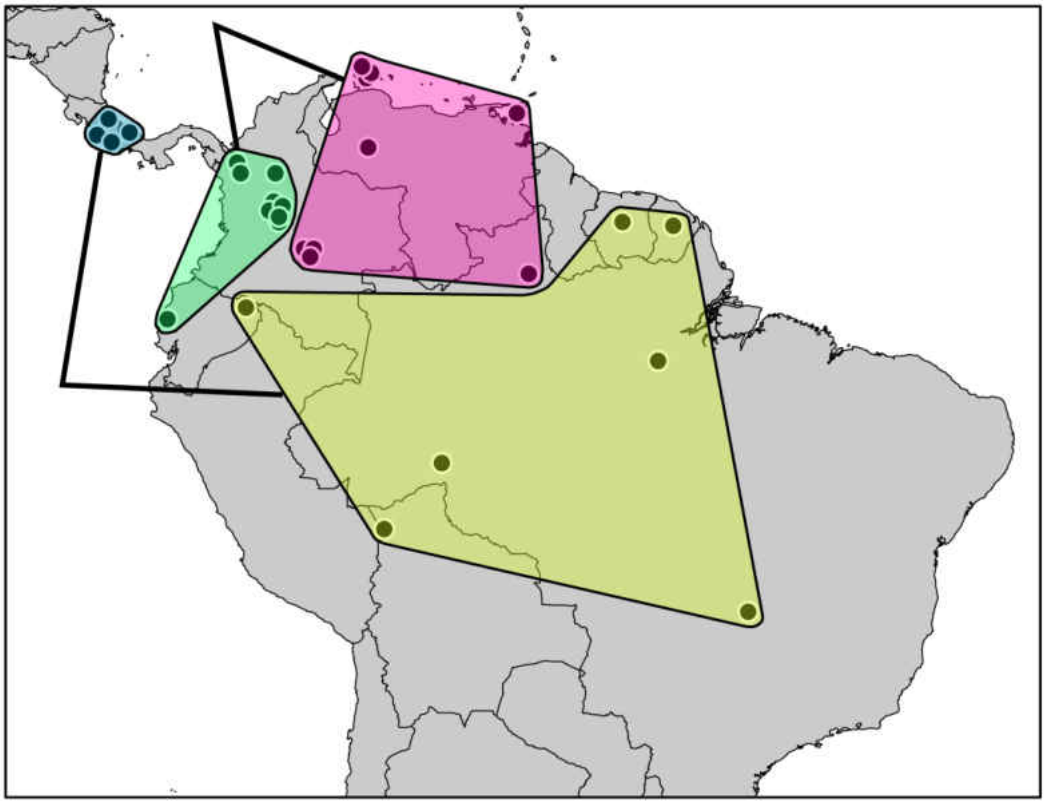

Figure 2.10. Phylogeographic structure of *Leptodeira* species in Lower Central America and South America. Lines represent the clades recovered with the mtDNA dataset, and dots represent sampled localities. Lines connecting clades indicate sister relationships.

Conclusion

The present study highlights the complex evolutionary history of the widespread genus *Leptodeira* across the entire Neotropical region. Current species and subspecies recognition is not consistent with our phylogenetic results. Our inferred lineages correspond to biogeographic provinces rather than to previous classifications based solely on morphology. We concur with Duellman (1958a) in recognizing that geological and climatic changes since the Miocene determined the lineage diversification within *Leptodeira*. Such observation regarding spatial and temporal diversification in

the Neotropical region, evidenced in the genus *Leptodeira*, should be tested with other widely codistributed lineages. Increasing taxon sampling in some areas (southern USA, northeastern Mexico, eastern Paraguay and southeastern Brazil) might uncover new phylogeographic patterns that, in turn, will provide us with a better picture of lineage diversification of populations inhabiting the limits of the Neotropical region. Finally, current taxonomy of *Leptodeira* warrants dramatic changes so that a new classification will reflect the evolutionary and biogeographic history of the genus.

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CHAPTER 3 – COMPARATIVE PHYLOGEOGRAPHY OF PITVIPERS SUGGESTS A CONSENSUS OF ANCIENT MIDDLE AMERICAN HIGHLAND BIOGEOGRAPHY[2]

Introduction

Phylogenetic inferences coupled with robust estimates of divergence times can provide tremendous insight into the patterns and underlying causes of the historical diversification of lineages. Despite the power of such inferences, however, it is difficult to deduct to what extent any single biogeographic example may be broadly representative of the patterns exhibited by diverse biotic components of a region or ecosystem. By comparing and contrasting phylogeographic scenarios from co-distributed lineages, comparative phylogeography (Bermingham and Martin 1998; Bermingham and Moritz 1998; Avise 2000; Sullivan et al. 2000; Lapointe and Rissler 2005; Hickerson et al. 2006) provides further understanding by identifying biogeographical patterns and the extent to which these apply to various taxa. If multiple lineages appear to be subject to spatially and temporally congruent patterns of divergence, a more powerful inference of the major events that have broadly impacted multiple lineages of co-distributed species can be made (Rosen 1978; Nelson and Platnick 1981). Deductions from comparative phylogeographic analyses are particularly important and enlightening for areas with either vague geological or tectonic information, or where little historical consensus is available (Arbogast and Kenagy 2001; Riddle and Hafner 2006).

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Middle America, the zone extending from central Mexico through Panama (Fig. 3.1), is extremely biodiverse and a large component of this diversity is endemic (Savage 1982; Campbell 1999). Although this region spans ~16 degrees of latitude, the landmass is fairly small (about 2.5 million km^2), rendering its high endemicity most impressive (Campbell 1999). The exaggerated topography, the inter–digitation of diverse habitats, and the dynamic tectonic and climatic history of the region have synergistically contributed to its high endemicity and diversity (Whitmore and Prance 1987; Jackson et al. 1996; Campbell 1999). Middle America has experienced a complex tectonic and geological history, and lies at the active junction of four major tectonic plates and several tectonic blocks (Iturralde-Vinent 2006; Marshall 2006). Deciphering the events that have historically shaped present–day biological diversity is complicated due to the continual physiographic reshaping of the region since the Cretaceous. Despite substantial progress over the past several decades, the details of much of the tectonic history of Middle America remain fragmentary and controversial (Coney 1982; Iturralde-Vinent 2006; Mann et al. 2006).

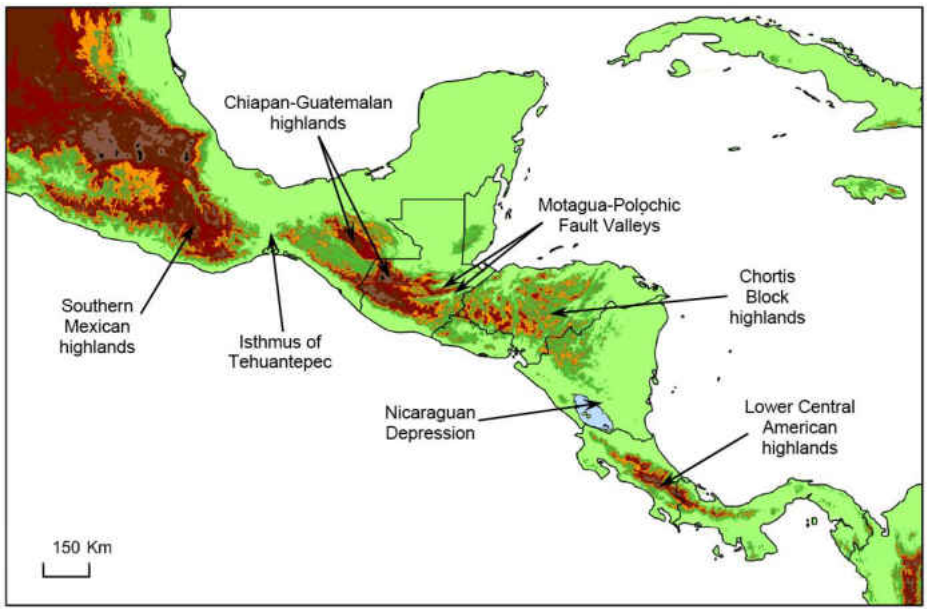

Figure 3.11. Map of Middle America showing the main highland regions and putative biogeographic barriers for highland taxa (based on NASA Shuttle Radar Topography Mission).

A majority of biogeographical studies concerning Middle America have been focused on understanding this region's role in biotic dispersal between North and South America, in many cases neglecting endemic patterns of Middle American biodiversity. Accordingly, most studies have dealt with biogeographical patterns in the late Pliocene–Pleistocene relating to the establishment of the final land connection with South America (Stehli and Webb 1985; Hafner 1991; Webb 1997), and relatively few have investigated earlier patterns in the Miocene and early Pliocene using contemporary phylogenetic data and analyses (Bermingham and Martin 1998; Parra-Olea et al. 2004; Pennington and Dick 2004; Crawford and Smith 2005; Ribas et al. 2005; Barker 2007; Crawford et al. 2007; Heinicke et al. 2007).

Several early broad–scale studies on the biogeographical history of Middle American fauna have shaped current perceptions of the historical patterns and processes that impacted the regional fauna (Dunn 1931; Duellman 1966; Savage 1966; Stuart 1966; Savage 1982). In particular, Savage (2002) proposed a model for highland speciation in Middle America in which highland species diversity was primarily the result of climatic cycles beginning in the late Pliocene and extending through the Pleistocene. Savage proposed that subsequent to the dispersal of Nearctic lineages to Middle America in the Miocene–Pliocene, speciation in the highlands occurred as a combination of mountain uplift and fluctuations in climate during Pleistocene glacial periods (see also Savage 2002: 830). These studies focusing on Middle American biogeography have been disadvantaged by lacking: 1) recent geological and tectonic insights into the region's history, 2) robust and detailed phylogenetic estimates, and 3) explicit estimates of divergence times independent of the assumptions of a strict molecular clock.

Pitvipers represent an ideal model system for investigating historical patterns of Neotropical diversification. This large group of venomous snakes has a relatively well–known phylogeny (e.g., Parkinson et al. 2002; Malhotra and Thorpe 2004; Castoe and Parkinson 2006), extensive fossil record in the USA (reviewed in Holman 2000), and appears to have dispersed into the New World as a single lineage from Asia during the Miocene (Kraus et al. 1996; Parkinson 1999; Parkinson et al. 2002; see also Holman 2000). Pitvipers are also good models for comparative phylogeography because several distinct and diverse lineages are broadly co–distributed, and extrinsic temporal constraints for divergence time estimates are available. Furthermore, because relaxed clock inferences of the relative divergence times within a single tree are particularly robust to the assumptions of calibration points (Thorne and Kishino 2005), pitvipers are ideal for testing hypotheses of coincident divergence among multiple lineages.

Several studies have examined biogeographical hypotheses for Neotropical pitviper lineages (e.g., Crother et al. 1992; Zamudio and Greene 1997; Parkinson et al. 2000; Wüster et al. 2002; Gutberlet and Harvey 2004; Werman 2005), but have resulted in little explicit consensus. Most of these studies provided brief comments on biogeography (e.g., Kraus et al. 1996; Parkinson 1999; Parkinson et al. 2002) or employed limited phylogenetic or phylogeographic data with no explicit temporal component (Crother et al. 1992; Castoe et al. 2003; Werman 2005), or with temporal estimates derived from a strict molecular clock (Zamudio and Greene 1997; Wüster et al. 2002). In this study, we compare historical biogeographical patterns simultaneously across three lineages of Neotropical pitvipers that are broadly co–distributed across the highlands of Middle America. These include members of the genera *Cerrophidion* (the montane pitvipers), *Atropoides* (the jumping pitvipers), and *Bothriechis* (the palm pitvipers).

To test the highland speciation model proposed by Savage (2002) and previous hypotheses of Middle American biogeography/phylogeography, we used a large molecular phylogenetic dataset for pitvipers that includes a dense (including intra–specific) sampling of members of the three genera of interest. We added new DNA sequences from members of the genera *Atropoides* and *Cerrophidion* to the data available for Neotropical pitvipers. We also estimated lineage divergence times based on multiple flexible approaches to provide a robust and probabilistic temporal component, avoiding assumptions of a strict molecular clock. We synthesize these inferences to address four questions: 1) Is the Savage speciation model supported by highland pitviper phylogeography? This model predicts that Middle American highland species diverged from one another primarily during the late Pliocene and Pleistocene when dramatic fluctuations in temperature may have affected highland habitat connectivity. 2) Is there evidence that temporal and geographic patterns of divergence are shared among multiple co–distributed highland lineages, and is there evidence of underlying geological or climatic causes? 3) Is there phylogeographic signal apparent from highland pitvipers that can be used to formulate an explicit model of Middle American highland speciation? 4) What effects did glacial cycles (in the late Pliocene – Pleistocene) have on lineage diversification in highland pitvipers of Middle America?

Methods

Taxon sampling and laboratory methods

Because our goals included inferences of biogeographical patterns ranging from ancient (i.e., Miocene) to recent in multiple pitviper lineages, we incorporated a large mitochondrial DNA sequence dataset (including 178 terminals) designed to provide accurate phylogenetic and divergence time estimates across this range of time. We combined mitochondrial DNA sequences from several

studies (Parkinson 1999; Malhotra and Thorpe 2000; Parkinson et al. 2002; Castoe et al. 2003; Malhotra and Thorpe 2004; Castoe et al. 2005; Castoe and Parkinson 2006) to include representatives of Old World pitvipers, and extensive sampling of all major New World lineages. The dataset included sequences of four mitochondrial gene fragments: portions of the 12S and 16S rRNA genes and the protein coding genes NADH dehydrogenase subunit four (ND4) and cytochrome–b (cyt–b), for a total of 2,306 aligned nucleotide positions. This included sequences for all four genes for a vast majority of species, and essentially all major lineages, although some intra–specific samples only included sequences of the two protein coding genes ND4 and cyt–b (1,386 bp; Table 3.1).

We included all inter and intra–specific sampling available from previous studies for the three genera of interest: *Atropoides*, *Bothriechis*, and *Cerrophidion*. All taxonomic references in this study follow Campbell and Lamar (2004). We also added new sequences for 20 samples of *Atropoides* and *Cerrophidion* (Table 3.1). Laboratory methods for generating new sequences followed Parkinson et al. (2002), Castoe et al. (2005), and Castoe and Parkinson (2006), as did the sequence alignment methods.

Table 3.4. Sequences used in phylogenetic and divergence time estimation, with Genbank numbers and voucher information. Sequences added specifically in this study are indicated in bold.

Sample Identifier	Voucher	Locality	Genbank Accession Numbers per Gene Fragment			
			12S	16S	Cyt–b	ND4
Agkistrodon bilineatus	WWL	Costa Rica, Guanacaste	AF156593	AF156572	AY223613	AF156585
Agkistrodon contortrix	Moody 338	USA, Ohio, Athens Co.	AF057229	AF057276	AY223612	AF156576
Agkistrodon piscivorus	CLP–30	USA, South Carolina	AF057231	AF057278	AY223615	AF156578
Agkistrondon taylori	CLP–140	Mexico, Tamaulipas	AF057230	AF057230	AY223614	AF156580
Atropoides mexicanus Cartago CR	UTA–R–12943	Costa Rica: Cartago: Pavones de Turrialba	-----	-----	AY220312	AY220335
Atropoides mexicanus Puntarenas CR	MSM	Costa Rica: Puntarenas: San Vito	-----	-----	AY220313	AY220336
Atropoides mexicanus SanJose CR	CLP–168	Costa Rica: San Jose	AF057207	AF057254	AY223584	U41871
Atropoides mexicanus AltaVerapaz GUA	UTA–R–46616	Guatemala: Alta Verapaz: Finca San Juan	-----	-----	AY220306	AY220329
Atropoides mexicanus BajaVerapaz GUA	UTA–R–35942	Guatemala: Baja Verapaz: Nino Perdido	-----	-----	AY220037	AY220330
Atropoides mexicanus Huehetenango GUA	UTA–R–32746	Guatemala: Huehetanango: Finca Chiblac	-----	-----	AY220308	AY220331
Atropoides mexicanus Izabal GUA	UTA–R–35944	Guatemala: Izabal: Puerto Barrios	-----	-----	AY220309	AY220332
Atropoides mexicanus Peten GUA	UTA–R–32419	Guatemala: Petén: San José El Espinero	-----	-----	AY220310	AY220333
Atropoides mexicanus Quiche GUA	UTA–R–43592	Guatemala: Quiché: Mountains West of El Soch	-----	-----	AY220311	AY220334
Atropoides nummifer Hidalgo MEX	UTA–R–24842	Mexico: Hidalgo: vic. Huejutla	-----	-----	AY220314	AY220337
Atropoides nummifer Puebla MEX	ENS–10515	Mexico: Puebla: San Andres Tziaulan	DQ305422	DQ305445	DQ061195	DQ061220
***Atropoides nummifer* Veracruz 1 MEX**	**ENS–10516**	**Mexico: Veracruz, Cordoba**	**-----**	**-----**	**EU684271**	**EU684288**
***Atropoides nummifer* Veracruz 2 MEX**	**ENS–10523**	**Mexico: Veracruz, Ixhuatlan del Café**	**-----**	**-----**	**EU684272**	**EU684289**
***Atropoides nummifer* Veracruz 3 MEX**	**ENS–10515**	**Mexico: Veracruz, northern Veracruz**	**-----**	**-----**	**EU684273**	**EU684290**
Atropoides occiduus Sonsonate ELS	KU–289807	El Salvador: Sonsonate	-----	-----	AY220318	AY220341
Atropoides occiduus Escuintla GUA	UTA–R–29680	Guatemala: Escuintla: S. slope Volcán de Agua	DQ305423	DQ305446	AY220315	AY220338
Atropoides occiduus Guatemala GUA	UTA–R–24763	Guatemala: Guatemala: Villa Nueva	-----	-----	AY220316	AY220339
Atropoides occiduus Solola GUA	UTA–R–46719	Guatemala: Sololá: San Lucas Tolimán	-----	-----	AY220317	AY220340
Atropoides sp Olancho HND	ENS–10630	Honduras: Olancho: Sierra de Botaderos	-----	-----	DQ061194	DQ061219
Atropoides olmec BajaVerapaz GUA	UTA–R–34158	Guatemala: Baja Verapaz: Niño Perdido	-----	-----	AY220319	AY220342
Atropoides olmec Chiapas1 MEX	ENS–10510	Mexico: Chiapas: Mapastepec	-----	-----	DQ061196	DQ061221
***Atropoides olmec* Chiapas 2 MEX**	**ENS–10511**	**Mexico: Chiapas: Mapastepec**	**-----**	**-----**	**EU684274**	**EU684291**
Atropoides olmec Oaxaca MEX	JAC–9745	Mexico: Oaxaca: Cerro El Baúl	-----	-----	AY220320	AY220343
Atropoides olmec Veracruz1 MEX	UTA–R–25113	Mexico: Veracruz: Sierra de los Tuxtlas	AY223656	AY223669	AY220321	AY220344
Atropoides olmec Veracruz2 MEX	UTA–R–14233	Mexico: Veracruz: Sierra de los Tuxtlas	-----	-----	AY220322	AY220345
Atropoides picadoi Alajuela CR	CLP–45	Costa Rica: Alajuela: Varablanca	AF057208	AF057255	AY223593	U41872
Atropoides picadoi SanJose CR	UTA–R–23837	Costa Rica: San José: Bajo la Hondura	-----	-----	AY220324	AY220347
Atropoides picadoi SanJose2 CR	MSM–10350	Costa Rica: San José: Bajo la Hondura	-----	-----	DQ061197	DQ061222

Sample Identifier	Voucher	Locality	Genbank Accession Numbers per Gene Fragment			
			12S	16S	Cyt-b	ND4
Bothriechis aurifer	UTA–R35031	Guatemala	DQ305425	DQ305448	DQ305466	DQ305483
Bothriechis bicolor	UTA–R34156		DQ305426	DQ305449	DQ305467	DQ305484
Bothriechis lateralis	MZUCR–11155	Costa Rica, Acosta	AF057211	AF057258	AY223588	U41873
Bothriechis marchi	UTA–R52959	Guatemala: Zacapa: Cerro del Mono	DQ305428	DQ305451	DQ305469	DQ305486
Bothriechis nigroviridis	MZUCR–11151	Costa Rica, San Gerondo de Dota	AF057212	AF057259	AY223589	AY223635
Bothriechis rowleyi	JAC 13295	Mexico: Cerro Baúl	DQ305427	DQ305450	DQ305468	DQ305485
Bothriechis schlegelii	MZUCR–11149	Costa Rica, Cariblanco de Sarapiquí	AF057213	AF057260	AY223590	AY223636
Bothriechis supercilliaris		San Vito, Costa Rica	DQ305429	DQ305452	DQ305470	DQ305487
Bothriechis thalassinus	UTA–R52958	Guatemala: Zacapa	DQ305424	DQ305447	DQ305465	DQ305482
Bothriopsis bilineata		Colombia, Letícia	AF057214	AF057261	AY223591	U41875
Bothriopsis oligolepis	LSUMZ–41037	Peru, Pasco Dept.	DQ305430	DQ305453	DQ305471	DQ305488
Bothriopsis taeniata		Suriname	AF057215	AF057262	AY223592	AY223637
Bothrocophias hyoprora		Colombia, Letícia	AF057206	AF057253	AY223593	U41886
Bothrocophias microphthalmus	LSUMZ H–9372	Peru, Pasco Dept.	AY223657	AY223670	AY223594	AY223638
Bothrops alternatus	DLP–2879		AY223660	AY223673	AY223601	AY223642
Bothrops ammodytoides	MVZ–223514	Argentina, Neuguen	AY223658	AY223671	AY223595	AY223639
Bothrops asper	MZUCR–11152	Costa Rica	AF057218	AF057265	AY223599	U41876
Bothrops atrox	WWW–743		AY223659	AY223672	AY223598	AY223641
Bothrops cotiara	WWW	Brazil	AF057217	AF057264	AY223597	AY223640
Bothrops erythromelas	RG–829	Brazil, Algóóas, Piranhas	AF057219	AF057266	AY223600	U41877
Bothrops insularis	WWW	Brazil, São Palo, Iiha Queimada Grande	AF057216	AF057263	AY223596	AF188705
Bothrops jararacussu	DPL–104		AY223661	AY223674	AY223602	AY223643
Bothrops diporus	PT3404	Argetina: La Rioja: Castro Barros	DQ305431	DQ305454	DQ305472	DQ305489
Calloselasma rhodostoma	UTA–R22247		AF057190	AF057237	AY223562	U41878
Cerrophidion godmani SanJose1 CR	MSM	Costa Rica: San José	-----	-----	AY220328	AY220351
Cerrophidion godmani SanJose2 CR	MSM	Costa Rica: San José: Goicochea	-----	-----	DQ061199	DQ061224
Cerrophidion godmani SanJose3 CR	MSM	Costa Rica: San José: Goicochea	-----	-----	DQ061200	DQ061225
***Cerrophidion godmani* SanJose4 CR**	**MSM**	**Costa Rica: San José**	**-----**	**-----**	**EU684275**	**EU684292**
***Cerrophidion godmani* SanJose5 CR**	**MSM**	**Costa Rica: San José**	**-----**	**-----**	**EU684276**	**EU684293**
***Cerrophidion godmani* SanJose6 CR**	**MSM**	**Costa Rica: San José**	**-----**	**-----**	**EU684277**	**EU684294**
Cerrophidion godmani SanJose7 CR	MZUCR–11153	Costa Rica: San Jose	AF057203	AF057250	AY223578	U41879
***Cerrophidion godmani* SantaAna ES**	**SMF–81323**	**El Salvador: Santa Ana, Montecristo**	**-----**	**-----**	**EU693494**	**-----**
***Cerrophidion godmani* Guatemala GUA**		**Guatemala: Guatemala**	**-----**	**-----**	**EU684278**	**EU684295**
***Cerrophidion godmani* Guatemala2 GUA**		**Guatemala: Guatemala**	**-----**	**-----**	**EU684279**	**EU684296**
***Cerrophidion godmani* GUA**	**JAC–10458**	**Guatemala**	**EU684303**	**EU684304**	**EU684280**	**EU684297**

Sample Identifier	Voucher	Locality	Genbank Accession Numbers per Gene Fragment			
			12S	16S	Cyt-b	ND4
Cerrophidion godmani BajaVerapaz GUA	UTAR–40008	Guatemala: Baja Verapaz: La Union Barrios	DQ305419	DQ305442	AY220325	AY220348
***Cerrophidion godmani* BajaVerapaz2 GUA**	**UTA–R–32421**	**Guatemala: Baja Verapaz, Sierra de las Minas**	**-----**	**-----**	**EU684281**	**EU684298**
***Cerrophidion godmani* Huehuetenango GUA**	**UTA–R–42237**	**Guatemala: Huehuetenango, La Democracia**	**-----**	**-----**	**EU684282**	**EU684299**
***Cerrophidion godmani* Quetzaltenango GUA**	**ENS–8350**	**Guatemala: Quetzaltenango**	**-----**	**-----**	**EU684283**	**EU684300**
Cerrophidion godmani Quiche GUA	ENS–8195	Guatemala: Quiché	-----	-----	DQ061198	DQ061223
Cerrophidion godmani SanMarcos GUA	UTA–R–42247	Guatemala: San Marcos, Esquipulas Palo Gordo	-----	-----	AY220327	AY220350
***Cerrophidion godmani* Ocotepeque1 HND**	**SMF–77768**	**Honduras: Ocotepeque, San Antonio de las Ojas**	**-----**	**-----**	**EU684284**	**-----**
***Cerrophidion godmani* Ocotepeque2 HND**	**SMF–78424**	**Honduras: Ocotepeque, El Pital**	**-----**	**-----**	**EU684285**	**-----**
Cerrophidion godmani Ocotepeque3 HND	ENS–10631	Honduras: Ocotepéque: Güisayote	-----	-----	DQ061201	DQ061226
***Cerrophidion godmani* Fmorazan HND**	**ENS–10632**	**Honduras: Francisco Morazan, La Tigra**	**-----**	**-----**	**EU684286**	**EU684301**
Cerrophidion godmani Oaxaca MEX	JAC–15709	Mexico: Oaxaca: Cerro El Baúl	-----	-----	AY220326	AY220349
***Cerrophidion godmani* Oaxaca2 MEX**	**JAC–15708**	**Mexico: Oaxaca: Cerro El Baúl**	**-----**	**-----**	**EU684287**	**EU684302**
Cerrophidion petlalcalensis	ENS–10528	Mexico, Veracruz, Orizaba	DQ305420	DQ305443	DQ061202	DQ061227
Cerrophidion tzotzilorum Chiapas1 MEX	ENS–10529	Mexico: Chiapas: Las Rosas	-----	-----	DQ061203	DQ061228
Cerrophidion tzotzilorum Chiapas2 MEX	ENS–10530	Mexico: Chiapas: Zinacantán	-----	-----	DQ061204	DQ061229
Crotalus adamanteus	CLP–4	USA, Florida, St. Johns Co.	AF057222	AF057269	AY223605	U41880
Crotalus aquilus	ROM–18117	Mexico, San Luis Potosi	AF259232	AF259125	AF259162	-----
Crotalus atrox	CLP–64	USA, Texas, Jeff Davis Co.	AF0572225	AF057272	AY223608	AY223646
Crotalus basiliscus	ROM–18188	Mexico, Nyarit	AF259244	AF259136	AF259174	-----
Crotalus catalinensis	ROM–18250, BYU–34641–42	Mexico, Baja California Sur, Isla Santa Catalina	AF259259	AF259151	AF259189	-----
Crotalus cerastes	ROM–FC–20099, ROM–19745	USA, California, Riverside Co.	AF259235	AF259128	AF259165	-----
Crotalus durissus	ROM–18138	Venezuala	AF259248	AF259140	AF259178	-----
Crotalus enyo	ROM–FC411, ROM13648	Mexico, Baja California Sur	AF259245	AF259137	AF259175	-----
[1]Crotalus "exsul"	BYU–34753–54	Mexico, Baja California, Isla de Cedros	AF259260	AF259152	AF259190	-----
Crotalus horridus (AR)	UTA–R14697	USA, Arkansas	AF259252	AF259144	AF259182	-----
Crotalus horridus (NY)	ROM–18132–33	USA, New York	AF259251	AF259143	AF259181	-----
Crotalus intermedius	ROM–FC223, ROM–18164	Mexico, Veracruz	AF259238	AF259131	AF2589205	-----
Crotalus lepidus	ROM–18128	Mexico, Chihuahua	AF259230	AF259123	AF259160	-----
Crotalus mitchelli	ROM–18178	USA, California, Imperial Co.	AF259250	AF259142	AF259180	-----
Crotalus molossus	CLP–66	USA, Texas, El Paso Co.	AF057224	AF057271	AY223607	AY223645
Crotalus polystictus	ROM–FC263, ROM–18139	Mexico, Districto Federal	AF259236	AF259129	AF259166	-----
Crotalus pricei	ROM–FC2144, ROM–18158	Mexico, Nuevo Leon	AF259237	AF259130	AF259167	-----
Crotalus pusillus	ROM–FC271	Mexico, Michoacan	AF259229	AF259122	AF259159	-----
Crotalus ravus	UTA–live	Mexico, Puebla, Zapotitlán	AF057226	AF057273	AY223609	AY223647
Crotalus ruber	ROM–18197–98, ROM18207	USA, California, Riverside CO.	AF259261	AF259153	AF259191	

Sample Identifier	Voucher	Locality	Genbank Accession Numbers per Gene Fragment			
			12S	16S	Cyt-b	ND4
Crotalus scutulatus	ROM–18210, ROM–18218	USA, Arizona, Mojave Co.	AF259254	AF259146	AF259184	-----
Crotalus tigris	CLP169	USA, Arizona, Pima Co.	AF057223	AF057270	AY223606	AF156574
Crotalus tortugensis	ROM–18192, ROM–18195	Mexico, Baja California Sur, Isla Tortuga	AF259257	AF259149	AF259187	-----
Crotalus transversus	KZ–shed skin	Mexico	AF259239	AF259206	AF259169	-----
Crotalus triseriatus (LG)	ROM–18114	Mexico, Districto Federal, Llano Grande	AF259231	AF259124	AF259161	-----
Crotalus triseriatus (TO)	ROM–18121	Mexico, Districto Federal, Toluca	AF259233	AF259126	AF259163	-----
Crotalus triseriatus (XO)	ROM–18120	Mexico, Districto Federal, Xochomiko	AF259234	AF259127	AF259164	-----
Crotalus unicolor	ROM–18150	Aruba Island	AF259246	AF259138	AF259176	-----
[2]*Crotalus "vegrandis"*	ROM–18261	Venezuela	AF259247	AF259139	AF259177	-----
Crotalus viridis	ROM–19656		AF259253	AF259145	AF259183	-----
Crotalus willardi (2575)	HWG–2575	USA, Arizona, Coshise Co.	AF259242	AF259134	AF259172	-----
Crotalus willardi (413)	ROM–FC363, KZ–413	USA, Arizona, Santa Cruz Co.	AF259241	AF259133	AF259171	-----
Crotalus willardi (ROM)	ROM–18183, ROM–18185	Mexico, Sonora	AF259240	AF259132	AF259170	-----
Deinagkistrodon acutus	CLP–28	China	AF057188	AF057235	AY223560	U41883
Garthius chaseni	AM B306	Malaysia, Sabah	AY352791	AY352729	AY352760	AY352825
Gloydius halys		Kazakhstan	AF057191	AF057238	AY223564	AY223621
Gloydius shedaoensis	ROM–20468	China, Liaoning	AF057194	AF057241	AY223566	AY223623
Gloydius strauchi	ROM–20473	China, Jilin, Waqie Sichuan	AF057192	AF057239	AY223563	AY223620
Gloydius ussuriensis	ROM–20452	China, Jilin, Kouqian	AF057193	AF057240	AY223565	AY223622
Hypnale hypnale	CLP–164	Sri Lanka, Columbo	AF057189	AF057236	AY223561	U41884
Lachesis muta	Cadle 135	Peru	AF057221	AF057268	AY223604	AY223644
Lachesis stenophrys		Costa Rica, Limón	AF057220	AF057267	AY223603	U41885
Ophryacus melanurus	UTA–R34605	Mexico	AF057210	AF057257	AY223587	AY223634
Ophryacus undulatus	CLP–73	Mexico	AF057209	AF057256	AY223586	AY223633
Ovophis monticola (A87)	AM A87	Taiwan	AY059545	AY059561	AF171907	AY059582
Ovophis monticola (JBS)	CAS215050	China, Yunnan Prov., Nu Jiang Prefecture	DQ305416	DQ305439	DQ305462	DQ305480
Ovophis monticola (MAK)	NTNUB200800		DQ305417	DQ305440	DQ305463	DQ305481
Ovophis monticola (ROM)	ROM–7798	Vietnam	AY223652	AY223665	AY223572	AY223626
Ovophis okinavensis (162)	CLP–162	Japan, Okinawa	AF057199	AF057246	AY223573	U41895,
Ovophis okinavensis (FK)	FK		DQ305418	DQ305441	DQ305464	U41895
Porthidium arcose	WWW–750	Ecuador: Manabí: Salango	AY223655	AY223668	AY223582	AY223631
Porthidium dunni	ENS–9705	Mexico: Oaxaca: near San Pedro Pochutla	AY223654	AY223667	AY223581	AY223630
Porthidium dunni Pd4	MS	Mexico: Chiapas: Guardiania	-----	-----	DQ061217	DQ061243
Porthidium lansbergi Panama	MSM	Panama: Darién	-----	-----	DQ061206	DQ061231
Porthidium lansbergi Venezuela	WES	Venezuela: Isla Margarita	-----	-----	DQ061205	DQ061230

Sample Identifier	Voucher	Locality	Genbank Accession Numbers per Gene Fragment			
			12S	16S	Cyt-b	ND4
Porthidium nasutum	MZUCR–11150	Costa Rica	AF057204	AF057251	AY223579	U41887
Porthidium nasutum Alajuela	MSM	Costa Rica: Alajuela: Río Cuarto de Grecia	-----	-----	DQ061210	DQ061235
Porthidium nasutum AVerapaz	UTA–R–44749	Guatemala: Alta Verapaz: Cobán	-----	-----	DQ061207	DQ061232
Porthidium nasutum CR1	MSM	Costa Rica: Cartago: Guayacán de Turrialba	-----	-----	DQ061208	DQ061233
Porthidium nasutum CR4	MSM	Costa Rica: Cartago: Guayacán de Turrialba	-----	-----	DQ061209	DQ061234
Porthidium nasutum Ecuador	FGO–live–517	Ecuador: Esmeraldas: Zapallo Grande	-----	-----	AF292612	AF29574
Porthidium ophryomegas	UMMZ–210276	Costa Rica, Guanacaste	AF057205	AF057252	AY223580	U41888
Porthidium ophryomegas Hond	UTA–R–52580	Honduras: Gracias a Dios: Mocorón	-----	-----	-----	DQ061240
Porthidium ophryomegas Zacapa	MSM–23	Guatemala: Zacapa	-----	-----	DQ061216	DQ061241
Porthidium porrasi Punt 2	MSM	Costa Rica: Puntarenas: Sierpe	-----	-----	DQ061211	DQ061236
Porthidium porrasi Punt 3	MSM	Costa Rica: Puntarenas: San Pedrillo	-----	-----	DQ061212	DQ061237
Porthidium porrasi Punt 4	MSM	Costa Rica: Puntarenas: Golfito	-----	-----	DQ061213	DQ061238
Porthidium porrasi Punt 5	MSM	Costa Rica, Puntarenas	DQ305421	DQ305444	DQ061214	DQ061239
Porthidium yucatanicum PY1	JAC–24438	Mexico: Yucatán: Car. Yaxcabá-Tahdzibichen	-----	-----	DQ061215	DQ061244
Protobothrops cornutus	ZFMK75067	Vietnam, Phong Nha- Ke NP	AY294272	AY294262	AY294276	AY294267
Protobothrops elegans	UMMZ–199970	Japan, Ryuku Is., Ishigaki	AF057201	AF057248	AY223575	U41893
Protobothrops flavoviridis	UMMZ–199973	Japan, Ryuku Is., Tokunoshima	AF057200	AF057247	AY223574	U41894
Protobothrops jerdonii	CAS215051	China, Nu Jiang, Yunnan	AY294278	AY294269	AY294274	AY294264
Protobothrops mucrosquamatus (2717)	ROM–2717	Vietnam	AY223653	AY223666	AY223577	AY223629
Protobothrops mucrosquamatus (B106)	AM B106	Vietnam, Vin Phuc Prov.	AY294280	AY294271	AY294275	AY294266
Protobothrops tokarensis	FK–1997	Japan, Ryuku Is., Takarajima	AF057202	AF057249	AY223576	AY223628
Sistrurus catenatus	Moody–502	USA, Texas, Haskel Co.	AF057227	AF057274	AY223610	AY223648
Sistrurus miliarus	UTA–live	USA, Florida, Lee Co.	AF057228	AF057275	AY223611	U41889
Trimeresurus gracilis (A86)	AM A86	Taiwan	AY352789	AY352728	AF171913	AY352823
Trimeresurus gracilis (NTUB)	NTNUB 200515		DQ305415	DQ305438	DQ305460	DQ305478
Zhaoermia mangshanensis	AM B300	China, Hunan Prov.	AY352787	AY352726	AY352758	AY352821

Phylogenetic analysis

Aside from the relatively small number of new intra–specific sampling added in this study, the data used here essentially represents the combination of datasets from Castoe et al. (2005) and Castoe and Parkinson (2006) with the exclusion of some fine–scale sampling of Old World pitvipers. To infer phylogeny in this study, we applied the partitioning scheme and partition–specific models identified in Castoe and Parkinson (2006). The Bayesian Markov–chain Monte Carlo (BMCMC) estimate of the phylogeny was inferred using MrBayes 3.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) with default priors. As per the defaults, two parallel BMCMC runs were executed simultaneously and each was run for 5×10^6 generations. Parameters among partitions were unlinked, as was the rate of evolution (using the *ratepr* = *variable* command). Based on diagnostics described in Castoe and Parkinson (2006), both runs appeared stationary prior to 10^6 generations, and we conservatively excluded the first 1.5×10^6 generations of each run as burn–in. All post burn–in estimates (sampled every 1000 generations) were combined, and phylogeny and parameter estimates were summarized from this combined posterior distribution. We also tested the alternative phylogenetic placement of *Bothriechis lateralis* as the sister lineage to *B. bicolor* (Crother et al. 1992) using the SH test (Shimodaira and Hasegawa 1999) and the AU test (Shimodaira 2002) implemented in the program Consel (Shimodaira and Hasegawa 2001).

Divergence time estimation

We used two relaxed clock methods to estimate divergence times across the pitviper phylogeny, the penalized likelihood (PL) method implemented in r8s (Sanderson 1997, 2002, 2003) and the Bayesian relaxed clock method implemented in the program Multidistribute (Thorne et al. 1998; Thorne and Kishino 2002). For the PL estimate, we estimated divergence times using r8s and then obtained confidence intervals on these dates using bootstrapped versions of the dataset. To estimate PL confidence intervals, 1000 bootstrap replicates were generated using the program Bootseq (Felsenstein 2005). Branch lengths for each replicate dataset were estimated using the GTR+Γ+I model in PAUP v4.10b (Swofford 2003). Trees (and branch lengths) from the bootstrapped datasets were run in r8s and confidence intervals were summarized from this distribution using the Perl scripts provided at http://www.bergianska.se/index_kontaktaoss_torsten.html.

For the Bayesian inference of divergence times in Multidistribute, we partitioned the molecular data by gene (four partitions) for all analyses. Using the program *baseml* (PAML package; Yang 1997), model parameters were estimated using the model F84+Γ for each partition. From this, branch lengths and the variance–covariance matrix were calculated using the program *estbranches*. Estimates of evolutionary rates and divergence times were then estimated using the program *multidivtime*. The priors used for analyses in *multidivtime* included: *rttm* = 1.6, *rttmsd* = 0.2, *rtrate* = 0.3, *rtratesd* = 0.3, *brownmean* = 0.5, *brownsd* = 0.5, and *bigtime* = 3.0. The remaining priors used in *multidivtime* analyses were set to the program's default. Because the performance of divergence time estimation approaches utilized here rely heavily on accurate branch length estimation, divergence estimates are extremely sensitive to short internodes that may have estimation variance that includes negative values of length or time. To avoid this potential problem, only subsets of the entire phylogenetic

dataset were used for r8s and Multidistribute analyses. For both analyses, the topology was pruned to include only phylogenetically distinct lineages, thereby excluding lineages or samples that were associated with extremely small (near zero) branch lengths (as per the suggestions of both programs).

Calibration points

Because branch lengths represent the product of evolutionary rate and time, calibration points are necessary to separate these two underlying parameters and obtain an estimate of divergence times (Thorne and Kishino 2005). We used four calibration points as minimum constraints to obtain date estimates for the pitviper phylogeny. In both the PL and Bayesian divergence analyses, we constrained the minimum ages of two temperate North American lineages based on fossil data: the origin of *Sistrurus* at 9 my (Parmley and Holman 2007) and the origin of *Agkistrodon piscivorus* at 4.7 my (Holman 2000). Because the PL method requires the age of one node to be fixed, for PL we fixed the age of the divergence between the two North American rattlesnake species *Crotalus ruber* and *C. atrox* at 3.2 my (Castoe et al. 2007). The divergence between these two species is thought to have occurred due to a well–dated Pliocene marine incursion of the Sea of Cortez, and is generally well corroborated across other taxa (see Castoe et al. 2007 for discussion). In the PL analyses, we also constrained the split between New World and Old World pitvipers as a minimum age at 16 my based on two sources of evidence: the oldest fossil of a viper found in the New World (Holman 1977, 2000) and the end of the thermal optimum in the Miocene (Bohme 2003; see also Burbrink and Lawson 2007). For the Bayesian estimates of dates, the split between Old and New World pitvipers was used as the prior *rttm*; based on the evidence mentioned above, the *rttm* prior was set to 16 my and the standard deviation for that prior (*rttmsd*) to ± 4 my. The *Crotalus atrox/ruber* split was also added as a constraint to the Bayesian estimation, set as 2.9–3.5 my.

Results

Phylogenetic estimate

Our estimate of pitviper phylogeny is extremely similar to recent studies (Wüster et al. 2002; Castoe et al. 2005; Castoe and Parkinson 2006), which was expected because a majority of the data and analytical approaches are the same. To maintain focus on groups of interest, we show summarized relationships among New World genera (Fig. 3.2) as well as detailed results only for genera of interest (Figs. 3.2–3.4). We found strong support for the monophyly of all New World pitvipers (posterior probability or PP = 100), as well as a clade representing the temperate genera *Crotalus*, *Sistrurus*, and *Agkistrodon* (PP = 96; Fig. 3.2). Inter–generic relationships among Neotropical lineages match that of Castoe and Parkinson (2006), and include a large South American group (*Bothrops*, *Bothriopsis*, and *Bothrocophias*) strongly supported as the sister clade of the Middle American *Porthidium* group (*Atropoides*, *Cerrophidion*, and *Porthidium*). As in previous studies, *Bothriechis* was inferred to be the sister group to this Middle and South American assemblage (Fig. 3.2).

Monophyly of each genus of interest was inferred, with strong support for *Cerrophidion* and *Bothriechis* (PP = 100), and weaker support for *Atropoides* (PP = 66; Fig. 3.2). Relationships among all nominal species and major lineages within each of these genera were well–resolved, with strong support in most cases. The new sequences of *Atropoides* and *Cerrophidion* added in this study illuminate substantial genetic structure within species. In *Atropoides* (Fig. 3.3), all species except *A. picadoi* and *Atropoides* sp. appear to contain substantial genetic diversity below the species level. We found substantial genetic structure within *Cerrophidion godmani,* consisting of at least four distinct and divergent clades (C1 through C4; Fig. 3.4) that correspond to four main geographic components of the range of this species (Fig. 3.4B). Like Castoe and Parkinson (2006), we found strong support for

Bothriechis lateralis forming the sister group to the northern Central American highland *Bothriechis* species, counter to the estimate that *B. lateralis* is the sister lineage to *B. bicolor* (Crother et al. 1992; Taggart et al. 2001). The sister–lineage relationship between *B. lateralis* and *B. bicolor* was also rejected by SH tests ($p < 0.001$) and AU tests ($p < 0.001$). These results provide strong evidence in support of the topology with *B. lateralis* as the sister group to all northern Central American highland *Bothriechis* species (Fig. 3.2A).

Divergence times

Estimates of divergence times were generally similar between the two divergence dating methods used (Table 3.2). The most notable contrast between the two sets of estimates was a substantial difference in confidence intervals, with the PL intervals being narrower and symmetrically distributed around the mean, whereas the Bayesian estimates had broader confidence intervals that were asymmetric and skewed towards more ancient divergence times. This contrast between Bayesian and PL estimates has been noted elsewhere, and some have suggested that the current method of obtaining bootstrap–based intervals in PL can produce confidence interval distributions that are improperly uniform and overly narrow (Burbrink and Pyron 2008). Thus, the credible intervals of Bayesian estimates are thought to be more accurate in their breadth and skew in contrast to PL bootstrap–based intervals. To circumvent this potential bias in the PL estimates, we report results primarily based on the Bayesian estimates and 95% credible intervals, and comment on the PL estimates where relevant; direct comparisons of the results of both methods are given in Table 3.2.

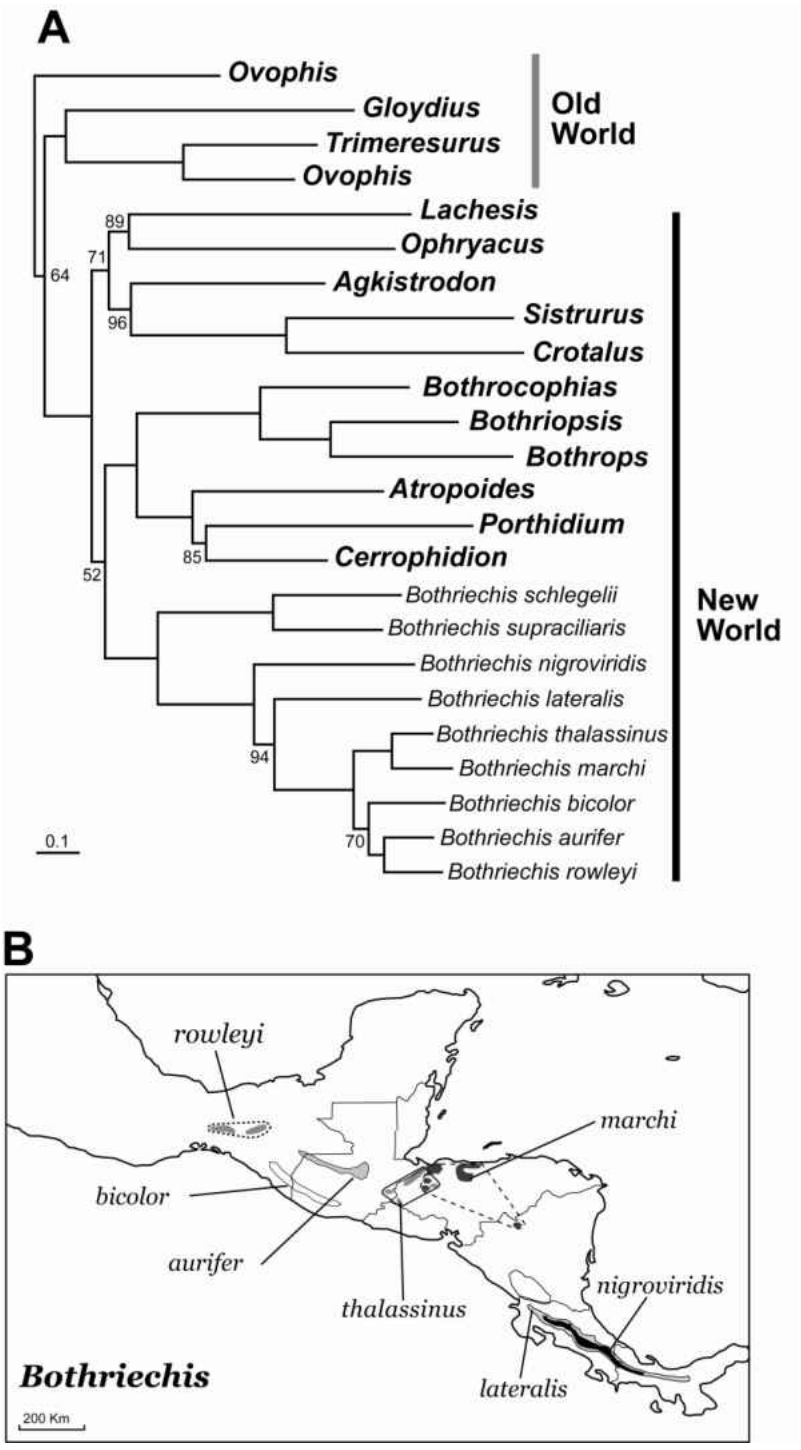

Figure 3.12. A) Summary of Bayesian phylogenetic estimates of relationships among New World pitviper genera and relationships among species of the genus *Bothriechis*. All shown nodes received Bayesian posterior probabilities of 100% unless otherwise annotated on the tree. B) Geographic distribution of Middle American highland species of the genus *Bothriechis* based on Campbell and Lamar (2004).

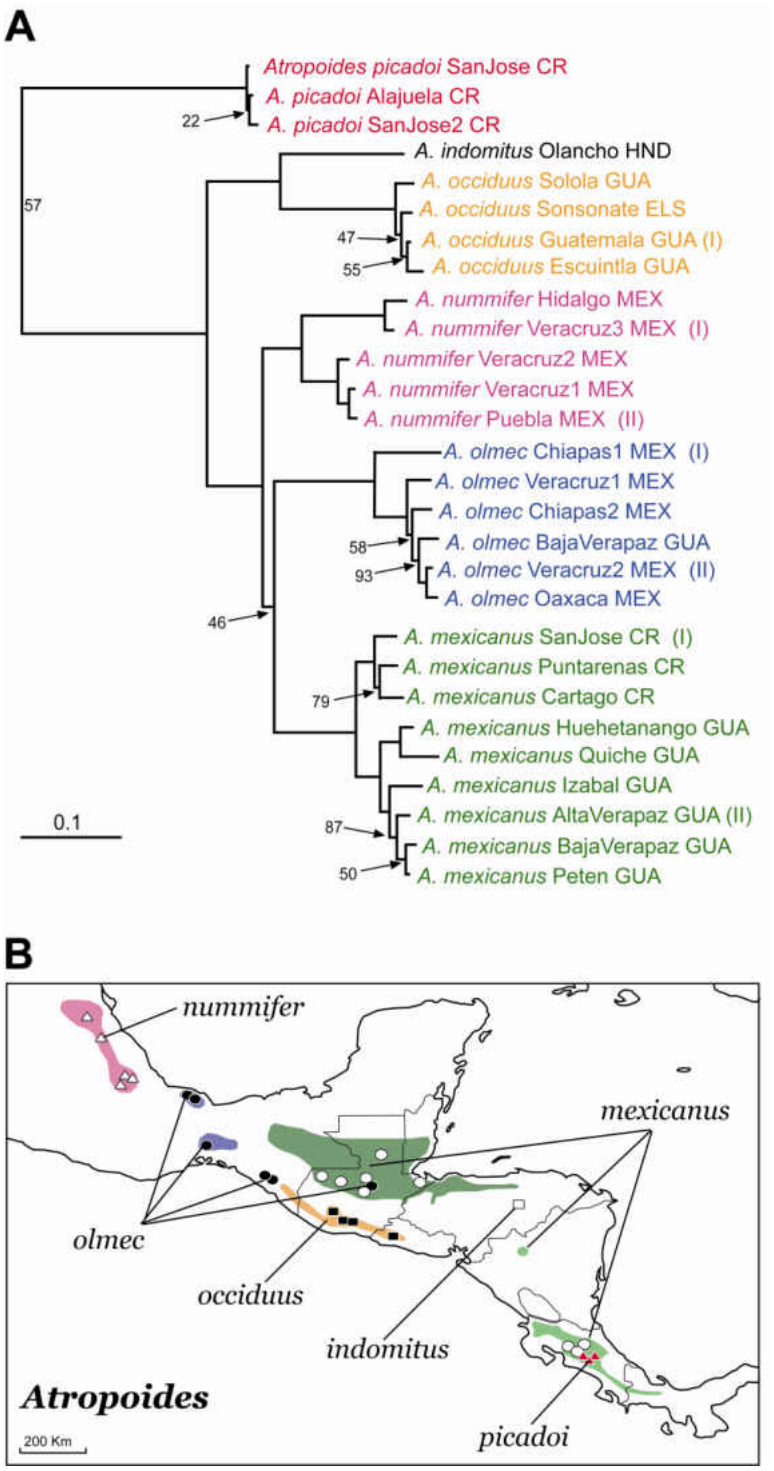

Figure 3.13. A) Bayesian phylogenetic estimate of relationships among members of the genus *Atropoides*. All shown nodes received Bayesian posterior probabilities of 100% unless otherwise annotated on the tree. Roman numerals to the right of taxon names indicate individuals used for divergence dating, and correspond with Figure 3.5. B) Geographic distribution of *Atropoides* species. Shaded areas represent the known distribution for each species based on Campbell and Lamar (2004); dots correspond to the geographic origin of samples used for the molecular analyses.

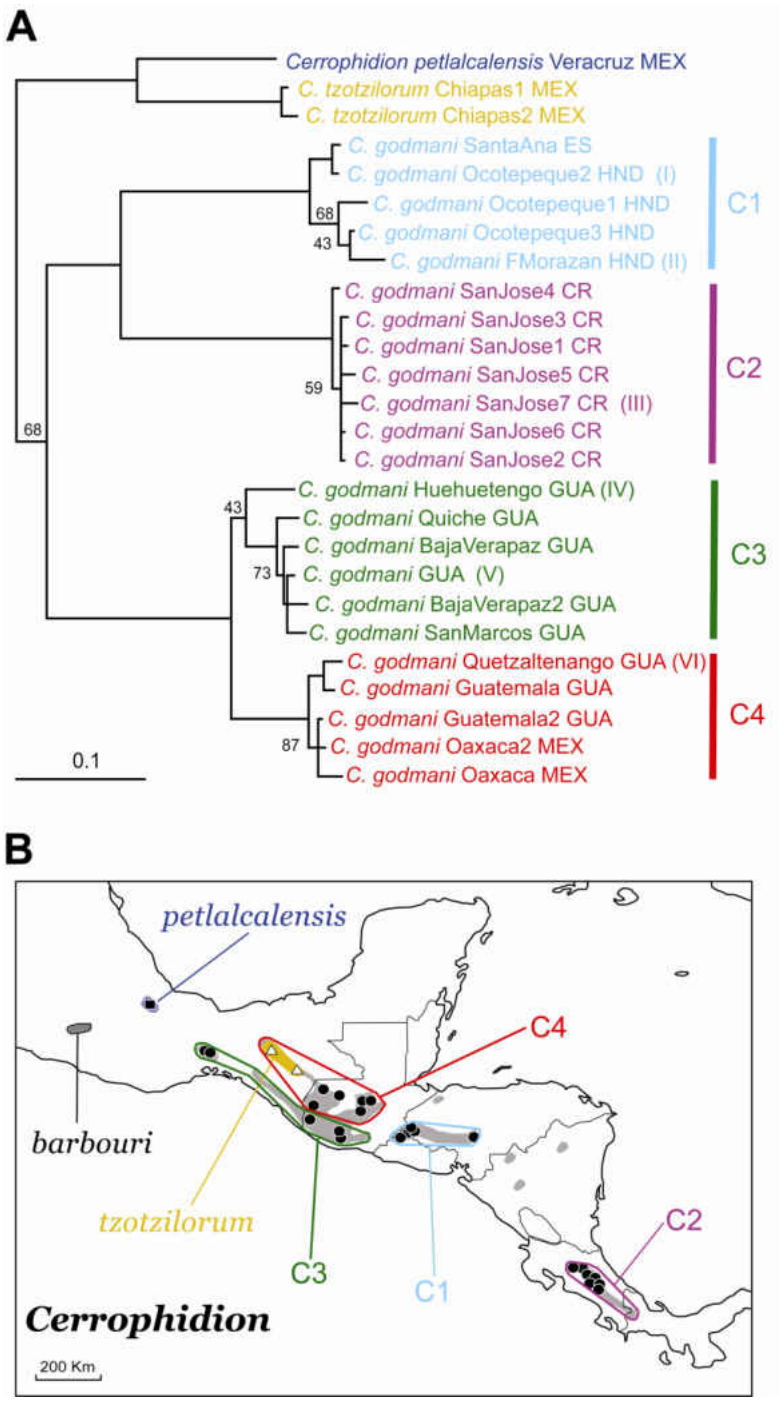

Figure 3.14. A) Bayesian phylogenetic estimate of relationships among members of the genus *Cerrophidion* included in this study. All shown nodes received Bayesian posterior probabilities of 100% unless otherwise annotated on the tree. Roman numerals to the right of taxon names indicate individuals used for divergence dating, and correspond with Fig. 3.5. B) Geographic distribution of *Cerrophidion* species in Middle America. Shaded areas represent the distribution for each species based on Campbell and Lamar (2004); dots correspond to the geographic origin of samples used for the molecular analyses. Major phylogeographic lineages within *C. godmani* are labeled C1–C4 (A and B), and are indicated by polygons on the map (B).

All inter–generic divergences within the New World pitvipers are estimated to have occurred during the Miocene, and the New World lineage is estimated to have diverged from Old World pitvipers in the early Miocene, between 14 and 18 mya (Table 3.2, Fig. 3.5). The majority of cladogenetic events that gave rise to the current genera and most of the species occurred in the middle–late Miocene and early Pliocene. The three genera we focus on here are inferred to have arisen from the middle to late Miocene (Table 3.2, Fig. 3.5). All nominal species of highland pitvipers appear to have diverged prior to the late Pliocene, predominantly from late Miocene to middle Pliocene (Fig. 3.5). Major divergences within highland pitviper species occurred over a broad period of time (early Pliocene – Pleistocene; Fig. 3.5). Phylogroups within the wide–ranging species *C. godmani* began to diverge in the late Miocene (~5.7 mya) and continuing through the Pliocene and Pleistocene, before the divergence of many other lineages of Neotropical pitviper species diverged from their sister groups (Fig. 3.5). Intraspecific phylogroups within *Atropoides* species diverged at the end of the Pliocene and the Pleistocene (2.1–0.9 mya; Fig. 3.5).

Three major phylogeographic divergence events that have occurred in each of the three genera of interest show different levels of temporal correspondence; these are labeled as 1–3 in Fig. 3.6. For the first phylogeographic break at the Nicaraguan Depression (labeled split 1; Fig. 3.6), *Bothriechis* and *Atropoides* lineages show strong overlapping temporal divergence (Table 3.2; Fig. 3.6) in the middle–late Miocene, whereas the corresponding geographic split in *Cerrophidion* is substantially later in the early–middle Pliocene (Figs 3.5–3.6; Table 3.2). The posterior probability distributions of divergence times in the first two genera broadly overlap, but show almost no overlap with that of *Cerrophidion* (Fig. 3.6), suggesting that *Atropoides* and *Bothriechis* had undergone an essentially coordinated divergence that was not shared with *Cerrophidion*. For the second major divergence

event, across the Motagua–Polochic Fault, there is strong evidence for the shared divergence between *Atropoides* and *Bothriechis*, also with moderate evidence of this divergence being shared by *Cerrophidion* (Table 3.2, Figs. 3.5–3.6). Posterior probability distributions of divergence times for all three genera do largely overlap across the period of ~4–5.5 Ma, providing evidence that they experienced a mostly simultaneous divergence at the Motagua–Polochic Fault in the late Miocene – early Pliocene (Fig. 3.6).

Table 3.5. Estimates of divergence times for major events in New World pitviper lineages. Mean estimates of divergence times based on Bayesian inference (BI) and Penalized likelihood (PL) are given with the corresponding upper and lower bounds of the 95% credibility (BI) or confidence intervals (PL) for each estimate.

	BI			PL		
Node	**Mean**	**Lower**	**Upper**	**Mean**	**Lower**	**Upper**
Origin of New World pitvipers	**16.08**	14.33	17.99	**17.35**	16.15	18.55
Origin of Bothropoid group	**12.82**	10.67	15.15	**14.15**	13.13	15.17
Origin of *Atropoides*	**9.95**	8.13	12.02	**10.76**	9.98	11.54
Origin of *Bothriechis*	**14.1**	11.99	16.29	**15.24**	14.25	16.23
Origin of *Cerrophidion*	**9.43**	7.66	11.47	**10.41**	9.65	11.17
(1) Nicaragua						
Atropoides	**8.56**	6.77	10.61	**9.28**	8.47	10.09
Bothriechis	**7.67**	5.73	9.87	**8.04**	7.35	8.74
Cerrophidion	**4.39**	3.06	6.03	**4.03**	3.54	4.53
(2) Motagua–Polochic						
Atropoides	**4.82**	3.55	6.35	**4.69**	4.25	5.13
Bothriechis	**4.56**	3.3	6.03	**4.5**	4.08	4.92
Cerrophidion	**5.73**	4.31	7.37	**5.51**	4.97	6.04
(3) Tehuantepec						
Atropoides	**3.05**	2.18	4.15	**3.29**	2.96	3.63
Bothriechis	**3.49**	2.44	4.72	**3.05**	2.68	3.42
Cerrophidion	**3.31**	2.16	4.67	**2.94**	2.54	3.35

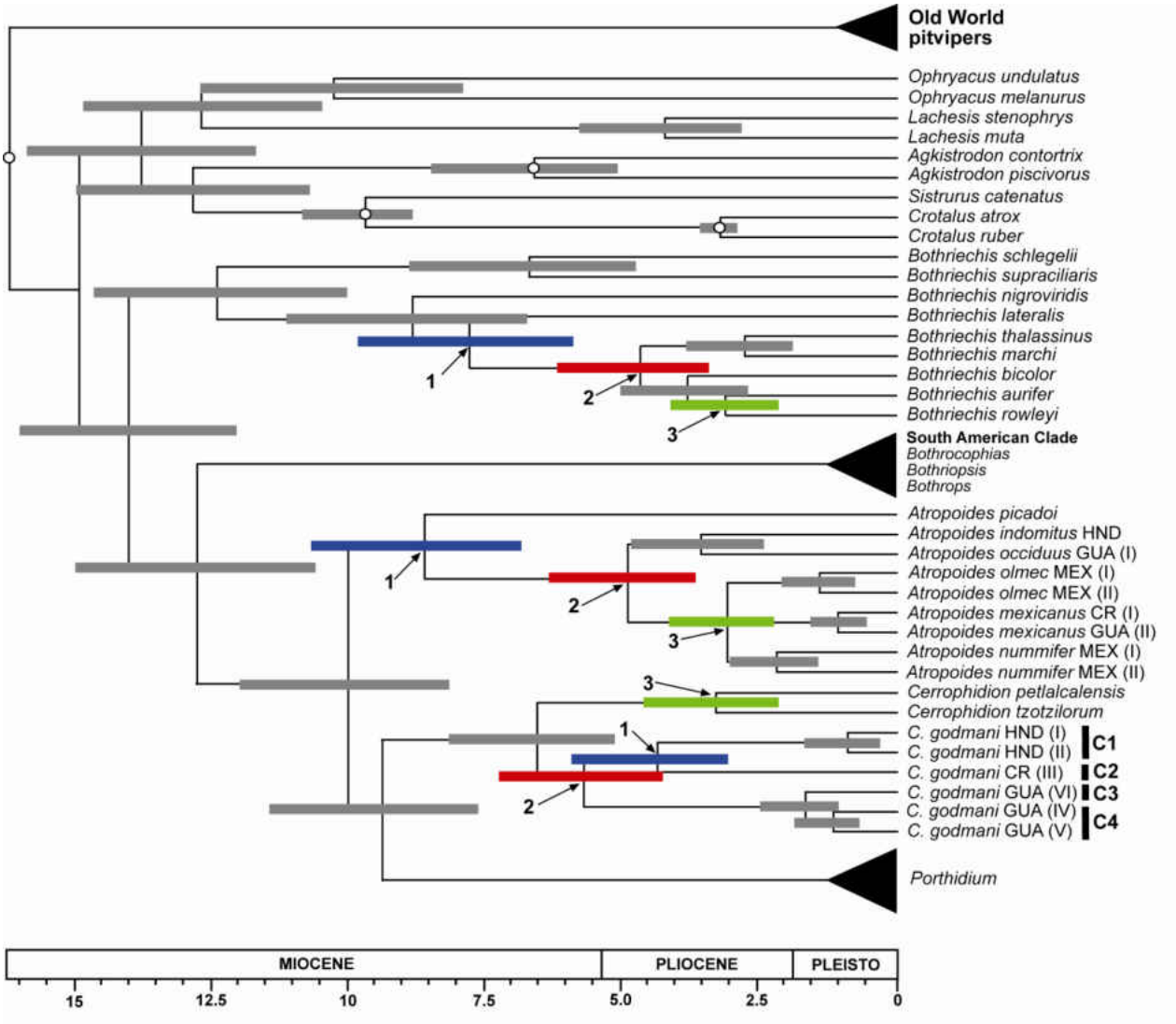

Figure 3.15. A) Bayesian estimates of divergence times for the pitviper phylogeny. The mean estimate is represented by the node and grey bars represent 95% credibility intervals for divergence estimates; open circles represent calibration points described in the text. Numbers on nodes (1–3) correspond to the biogeographic breaks for highland taxa: 1) Nicaragua Depression, 2) Motagua–Polochic Fault valleys, and 3) Isthmus of Tehuantepec. Roman numerals are used to cross reference (with Figs. 3.3 and 3.4) individuals per species used in divergence estimation.

The third major phylogeographic break, across the Isthmus of Tehuantepec, provides particularly strong evidence of a shared simultaneous divergence across the three genera in the middle Pliocene (Table 3.2, Figs. 3.5–3.6). The posterior probability distributions of divergence time estimates are nearly identical between *Atropoides* and *Cerrophidion*, which show a divergence at the geographically defined Isthmus of Tehuantepec. Although *Bothriechis* does not occur north of the geographic Isthmus, the divergence of *B. rowleyi* (from *B. aurifer*) directly adjacent to the isthmus shows nearly perfect temporal correspondence with the breaks in the other two genera (Fig. 3.6). Below we elaborate on geological evidence suggesting that the break observed in *Bothriechis* adjacent to the Isthmus of Tehuantepec may be geologically tied to the events leading to divergence in the other two genera in this region.

Discussion

A consensus of ancient Middle American highland speciation

Glacial climatic cycles during the late Pliocene – Pleistocene, subsequent to establishment of the late Pliocene land connections between Middle and South America, have been viewed as the predominant processes that have generated substantial Middle American species diversity, particularly for highland taxa (Savage 2002 and references therein). In general, this has also been the dominant hypothesis for explaining highland pitviper speciation – both Crother et al. (1992) and Castoe et al. (2003) focused on the period from the middle Pliocene and later, and on climatic fluctuations, as having hypothetically generated a majority of the species diversity in *Bothriechis* and *Atropoides*, respectively. Despite consensus in the identification of major biogeographical boundaries that have shaped the region's biodiversity (Savage 1982; Morrone 2001), there has been little quantitative insight as to when these barriers may have led to diversification and in what temporal

order. This study contributes three new important findings that reject previous hypotheses and clarify historical biogeographical patterns in Middle American highland taxa.

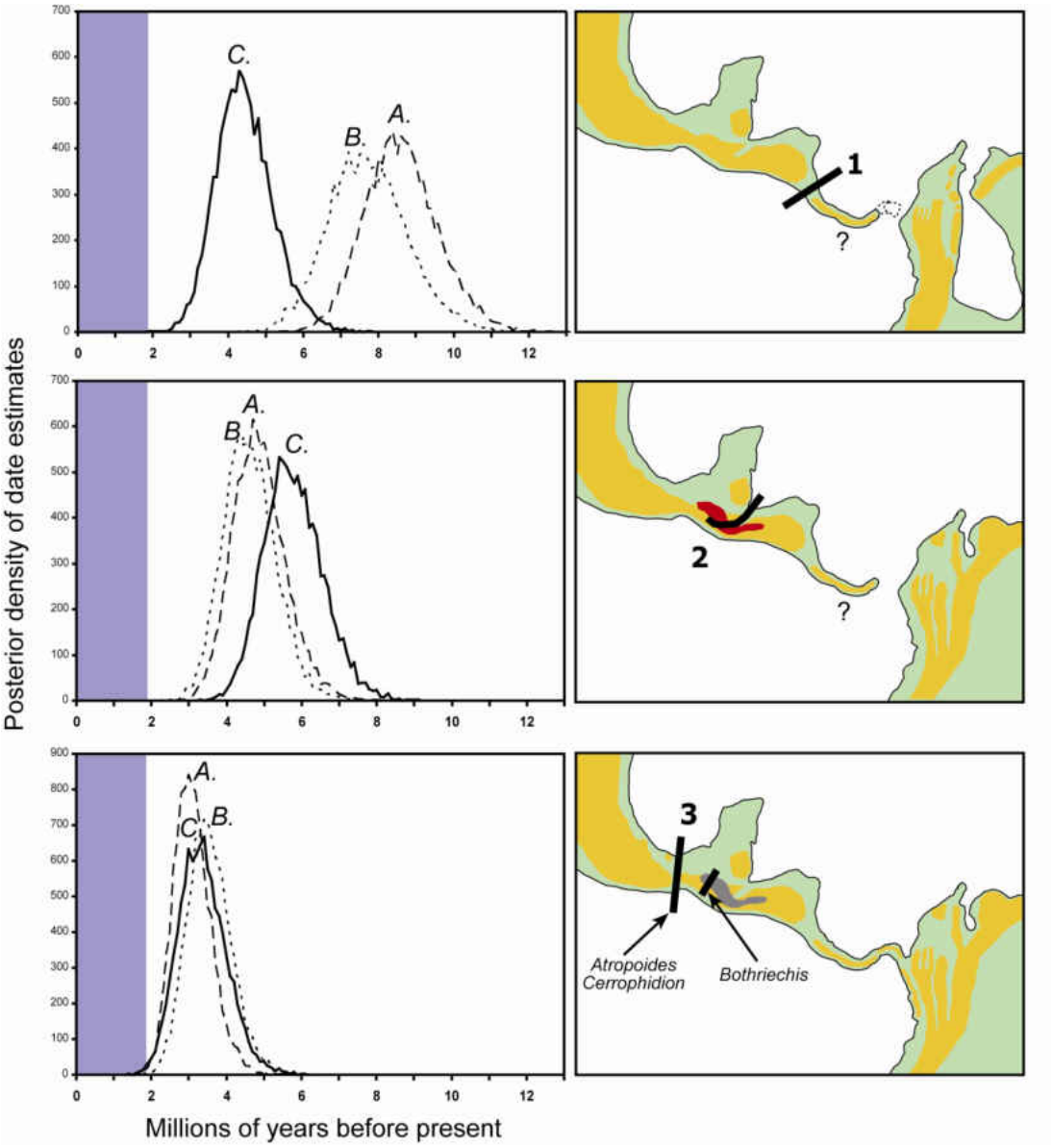

Figure 3.16. Bayesian posterior densities for divergence time estimates of the three highland genera across three major biogeographic breaks. Letters over the distributions indicate the three genera (*A.* = *Atropoides*, *B.* = *Bothriechis*, *C.* = *Cerrophidion*). The shaded region in the three graphs represents the period of glacial cycles in the late Pliocene through the Pleistocene. The figures on the right show the biogeographic break and the potential approximate palaeogeographic reconstruction at that time; gray shading indicates major highland masses in palaeogeographic reconstructions. Palaeogeographic reconstruction in Lower Central America based on Kirby and MacFadden (2005; see discussion for other reconstructions).

First, estimates of pitviper phylogeny and divergence times for Middle American highland lineages reject Savage's model of highland speciation in which late Pliocene and Pleistocene climatic changes are major determinants of current species diversity (Savage 2002). Instead, our results suggest that Miocene – Pliocene tectonic activity played a dominant role in generating regional highland species biodiversity. This conclusion contrasts with the majority of previous suggestions by taxon–specific studies on pitvipers (Crother et al. 1992; Castoe et al. 2003; Werman 2005), plants alone (Burnham and Graham 1999), and plants, insects and fish (Marshall and Liebherr 2000). This and other recent studies highlight the significance of pre–Pliocene diversification in Middle America (Smith et al. 2007; Wiens et al. 2007), together with ancient faunal interchange between Middle and South America (Bermingham and Martin 1998; Barraclough and Vogler 2002; Wüster et al. 2002; Parra-Olea et al. 2004; Pennington and Dick 2004; Steppan et al. 2004; Crawford and Smith 2005; Concheiro-Pérez et al. 2007; Koepfli et al. 2007; Wahlberg and Freitas 2007).

Second, there is evidence for a congruent temporal pattern of divergence across three different lineages of Middle American highland pitvipers, corresponding to major geographic breaks among Middle American highland masses. This, to our knowledge, is the first evidence of a clear pattern of temporal and spatial congruence in divergence patterns across multiple highland lineages of any taxa in Middle America. This example therefore provides one of the first explicit predictive models for speciation in this heavily studied epicenter of biodiversity. These biogeographical break points are obvious contemporary barriers for highland species and have been the focus of previous biogeographical attention (Savage 1982, 1987; Campbell 1999; Duellman 1999; Sullivan et al. 2000; Morrone 2001), but no clear evidence or consensus for when and how these regions have broadly shaped biodiversity has previously emerged. It is also significant to bear in mind an important strength of our analyses – regardless of the exact estimates of absolute divergence times, our

inference of relative temporal congruence among lineage divergences is particularly robust because all estimates are derived from a single, large dated tree (Thorne and Kishino 2005). Thus, the evidence in this study regarding the relative correspondence of divergence times across multiple lineages of pitvipers is robust and fairly independent of the accuracy of the absolute estimates of divergence times.

Third, we do find evidence that climatic changes associated with the onset of glacial cycles in the late Pliocene – Pleistocene may have led to lineage diversification in Middle American highland pitvipers, but only among populations within species. This evidence is consistent with glacial climatic cycles contributing to the fragmentation of once contiguous highland habitats, leading to the subsequent divergence among populations of *Atropoides* and *Cerrophidion*. These inferences provide new insight into corridors of highland habitat that at one time facilitated gene flow that may have been fragmented due to climatic changes in the late Pliocene and Pleistocene.

Below we first discuss evidence from this study for three shared ancient (Miocene – Pliocene) divergences across Middle American highland pitviper lineages, and the underlying tectonic and biogeographical hypotheses surrounding these divergences. Next we focus on the intraspecific sampling of *Atropoides* and *Cerrophidion* and evidence for late Pliocene – Pleistocene effects on lineage diversification, and we examine previous biogeographical hypotheses for *Bothriechis* species.

Shared divergence (1): the Nicaraguan Depression

The lowland area known as the Nicaraguan Depression is the geological result of a backarc formation that has continued to evolve for the last 10 million years (Rogers et al. 2002; Marshall 2006). This region separates two highland masses, the Chortis block highlands (Honduras and Nicaragua) to the north, and the Lower Central American highlands of Costa Rica and Panama.

Evidence suggests a marine gap existed between the Chortis and Lower Central American highlands during the Miocene and a majority of the Pliocene (Coates and Obando 1996; Iturralde-Vinent and MacPhee 1999; Iturralde-Vinent 2006). Alternatively, Kirby and MacFadden (2005) have suggested that a narrow landmass connected modern–day Honduras and Costa Rica during this time. The Nicaraguan Depression has been identified as a major phylogeographic break for many taxa, including frogs (Savage 1987; Campbell 1999), salamanders (Parra-Olea et al. 2004), lizards (E.N. Smith, *in litt.*), snakes (Savage 1982; Cadle 1985), birds (Pérez-Emán 2005), and plants, insects, and fish (Marshall and Liebherr 2000; Halas et al. 2005).

Middle American highland pitvipers also provide strong support for this region representing a major historical barrier to gene flow. We found evidence for temporal congruence of highland pitviper divergence across this break in two of the three pitviper lineages. *Bothriechis* and *Atropoides* show broadly overlapping divergence estimates across this break in the middle–late Miocene, approximately 7.7–8.6 mya (Bayesian confidence intervals, or BCIs = 5.7–9.9 and 6.8–10.6, respectively, Fig. 3.6). Although estimates of these two genera appear to indicate a fairly coincident divergence at the depression, the third genus, *Cerrophidion*, appears to have diverged across this region much later in the early–middle Pliocene, approximately 4.4 mya (BCI = 3.1–6.0 mya, Fig. 3.6). The posterior probability distribution of *Cerrophidion* divergence times shows very little overlap with that of the other two genera (Fig. 3.6) and strongly suggests a unique biogeographical scenario for *Cerrophidion* divergence across this barrier.

The apparent lack of temporal correspondence of divergences between *Cerrophidion* and the other two genera may indicate that *Cerrophidion* has different dispersal capabilities or that members of this genus may not have been distributed across the depression in the middle–Miocene. Of the three

genera, *Cerrophidion* tends to inhabit the highest elevations (up to ~2700 m; Campbell and Lamar, 2004), and it has been suggested that high elevation habitats may not have existed in lower Central America until the Pliocene (Coates and Obando 1996). The estimate of more recent cladogenesis within *Cerrophidion* that is not observed in either *Atropoides* or *Bothriechis* is intriguing, and suggests that dispersal and vicariance of highland lineages across the Nicaraguan Depression has occurred multiple times in the Miocene – Pliocene. It is notable that these estimates of divergence times are collectively consistent with the model of Kirby and MacFadden (2005), corroborating their suggestion of a narrow landmass across the Nicaraguan Depression during the Miocene and Pliocene.

Shared divergence (2): the Motagua–Polochic Faults

The Motagua–Polochic Fault represents the contact zone between the Maya and Chortis tectonic blocks (Marshall 2006). The eastward motion of the Chortis block that has continued since the Cretaceous is responsible for the generation of a majority of the mountain building across southwestern Mexico and Nuclear Central America (Rogers et al. 2002). Numerous studies have suggested this physiographic barrier leading to phylogeographic breaks in different taxa (Humphries 1982; Perdices et al. 2002; Halas et al. 2005; Perdices et al. 2005; Devitt 2006; Concheiro-Pérez et al. 2007). For lowland–inhabiting snakes, Devitt (2006) estimated a cladogenetic event in this region at 7.7 mya, and Perdices et al. (2005) found that freshwater eel–like synbranchid fishes diverged around 11.2 mya. In contrast, our estimates suggest divergence of highland lineages of pitvipers later in the Miocene and/or early Pliocene (Figs. 3.5–3.6).

Our divergence time estimates show a geographically congruent, nearly simultaneous diversification scenario in the late Miocene, centered around 4.1–5.0 mya, for the three highland lineages of

pitvipers (Figs. 3.5–3.6). The correspondence between divergence times for *Atropoides* and *Bothriechis* is excellent (4.3 and 4.1 mya, respectively), and it appears that *Cerrophidion* may have diverged slightly earlier (5.0 mya, Fig. 3.5–3.6). This result is consistent with the expectation that, because *Cerrophidion* is restricted to higher elevation habitats, gene flow may have been severed slightly earlier in this group compared to the other two lineages. It is interesting that there is fairly strong evidence for simultaneous divergence across highland lineages at this fault zone that contrasts substantially with more ancient divergence estimates for lowland groups (Perdices et al. 2005; Devitt 2006). The extensive mountain building and physiographic reshaping of the region makes historical inferences difficult, but these results may indicate that this region has contributed to the divergence of lineages with different habitat requirements in markedly different ways over an extended period of time.

Shared divergence (3): the Isthmus of Tehuantepec

Geographically, the Isthmus of Tehuantepec is the narrow lowland region that separates the highlands of southern Mexico (Sierra Madre Oriental and Sierra Madre del Sur) from the Chiapan–Guatemalan highlands of Nuclear Central America. This region is well known as a major biogeographical node where historical events have formed a transition between the Nearctic and the Neotropical biogeographical zones (Halffter 1987; Marshall and Liebherr 2000; Morrone and Márquez 2001). Biogeographical studies on specific taxa have found the Isthmus of Tehuantepec to be a phylogeographic barrier for highland species (Chippindale et al. 1998; Sullivan et al. 2000; Leon-Paniagua et al. 2007). More recent studies on lowland species have similar phylogeographic structure separating lineages on both sides of the Isthmus (Hasbun et al. 2005; Devitt 2006; Mulcahy et al. 2006).

Tectonically, the Isthmus represents a visible marker for the three–way junction of tectonic plates that have remained extremely active in shaping the regional landscape since the Cretaceous. It is thought that a highland corridor spanning the Isthmus in the Miocene was subsequently destroyed due to extreme tectonic activity relating to the subduction of the Cocos Plate (Barrier et al. 1998; Manea and Manea 2005). Tectonic markers distributed both on the Isthmus of Tehuantepec and on surrounding upland areas show massive down–dropping of the Chiapan–Guatemalan region with respect to the areas to the north and west during the late Miocene – early Pliocene associated with faulting occurring across the short axis of the Isthmus, resulting in a significant reduction in elevation and subsequent marine inundations (Barrier et al. 1998).

Atropoides and *Cerrophidion* each show clear phylogeographic breaks centered around the geographic Isthmus of Tehuantepec, and estimates of divergence times between these two genera show remarkable temporal congruence over this boundary. Our results suggest these two genera experienced a simultaneous divergence across this zone in the Pliocene, around 3.1–3.5 mya (Fig. 3.6), consistent with geological evidence for a tremendous tectonic event in which highlands at the Isthmus were reduced to a submarine embayment over a short period of time in the Pliocene (Barrier et al. 1998).

Unlike the other two genera, *Bothriechis* does not occur west of the Isthmus of Tehuantepec, although one species, *B. rowleyi*, is endemic to northwest Chiapas adjacent to the Isthmus (Fig. 3.2). *Bothriechis rowleyi* is distributed only in the mountain region of northern Chiapas, a recent geological formation called the Modern Chiapas Volcanic Front (Manea and Manea 2005). Around 3 mya, the continued slab subduction of the Cocos plate generated extensive orogenic changes not only at the Isthmus proper, but also in surrounding regions that led to the uplift of the Modern Chiapas

Volcanic Front (Manea and Manea 2005). It is thus reasonable to infer that the final formation of the Chiapas highlands during the Pliocene, associated with tectonic activity at the triple plate junction at the Isthmus, led to the vicariance between the ancestors of *B. rowleyi* and its sister species *B. aurifer* (Fig. 3.6). The temporal congruence between this divergence in *Bothriechis* and that of the other two genera at almost exactly 3 mya is impressive and suggests that these vicariant events were nearly simultaneous and possibly driven by the same tectonic activity surrounding the Isthmus. Although strongly supported by geological data, this is the first evidence of which we are aware that demonstrates potential temporal (and tectonic) link between evolutionary vicariance events at the Isthmus of Tehuantepec and in the neighboring Chiapan highlands. Future research to increase the resolution of biogeographical analysis in the Isthmus region may provide tests of this hypothesis, while further illuminating the complex role of this biogeographical node in shaping historical gene flow between the Nearctic and Neotropical regions.

Intra–specific phylogeography of Atropoides *and* Cerrophidion

Intra–specific sampling of *Atropoides* and *Cerrophidion* highlights substantial genetic structuring within species (Figs. 3.3–3.4) estimated to have occurred during the Pliocene and Pleistocene. Within *Atropoides* species, Pleistocene divergences are estimated 1) within the Sierra Madre Occidental in eastern–central Mexico (*A. nummifer*), 2) across the Nicaraguan Depression (*A. mexicanus*), and 3) across the Isthmus of Tehuantepec (*A. olmec*). Like *Atropoides*, there is evidence that some among–population gene flow in *C. godmani* may have been affected by glacial climatic cycles in the Pleistocene. The divergence of phylogroups C3 and C4 (Fig. 3.4), representing the separation of Northeastern from Southwestern Guatemalan highlands, appears to have occurred at the temporal boundary between the Pliocene and Pleistocene (Fig. 3.5). Further divergences across highlands in

eastern Honduras (within C1), and among interior Guatemalan highlands (within C4) may also have been associated with Pleistocene climatic change (Fig. 3.5).

New sampling within *Atropoides* and *Cerrophidion* also provides insight into previous biogeographical and taxonomic hypotheses. Castoe et al. (2003) hypothesized that a recent corridor for gene flow extended across the Isthmus of Tehuantepec to explain the close relationship between populations of *A. olmec* in Veracruz, Mexico and Baja Verapaz, Guatemala; new *Atropoides* samples from Chiapas, Mexico are associated with *A. olmec* further support this. Our new sampling of *C. godmani* demonstrates an extensive amount of ancient genetic structure, which has generally been suggested previously (Castoe et al. 2003; Castoe et al. 2005). Estimates of divergence times also suggest that the species *C. godmani* began to diversify prior to some major clades of *Atropoides* (all except *A. picadoi*) and *Bothriechis* (all northern highland species; Fig. 3.5). Our results indicate future research is needed to evaluate whether major phylogeographic clades of *C. godmani* may warrant recognition as distinct species, which we are currently undertaking.

Alternative hypotheses for Bothriechis *biogeography*

The phylogeny of *Bothriechis* is controversial (Crother et al. 1992; Taggart et al. 2001; Castoe and Parkinson 2006) largely because a previous study (Taggart et al. 2001) had suggested that conflicting phylogenetic estimates from morphology plus allozymes versus mitochondrial gene sequences indicated that mitochondrial introgression and/or incomplete lineage sorting may confound mitochondrial gene phylogenies of the group. Based on allozyme and morphological data, Crother et al. (1992) suggested that *B. lateralis* was phylogenetically nested within northern Middle American highland lineages (sister to *B. aurifer*), rather than our phylogenetic placement of *B. lateralis* as the sister group to all northern highland species (Fig. 3.2). Based on our mitochondrial dataset, SH and

AU tests of the former hypothesis strongly rejected this ($p << 0.001$) in favour of the relationships recovered in our tree (Fig. 3.2). Our mitochondrial phylogeny and that of the combined data of Taggart et al. (2001: his Fig 6B) are almost exactly the same. Both place *B. lateralis* as the sister lineage to the northern highland species. The conclusion of Taggart et al. (2001), however, was that the mitochondrial tree was incorrect because it differed from the tree based on a relatively small set of morphological and allozyme characters. In unpublished analyses, we have analyzed multiple nuclear genes, and sampled intraspecificly using mitochondrial gene sequences for each lineage of highland *Bothriechis*. These data suggest that the nuclear gene tree is consistent with our mitochondrial tree. Also, intraspecific sampling of mtDNA found no instances of incomplete lineage sorting or hybridization (Parkinson, Castoe, and Daza, unpublished data). While the phylogeny of *Bothriechis* remains somewhat of an open question, we expect that our mitochondrial phylogeny estimate for *Bothriechis* is reasonably accurate and representative of the underlying nuclear and species tree.

Our biogeographical hypothesis for *Bothriechis* is similar to Crother et al. (1992) in suggesting the pre–Pleistocene vicariance of the group, and identifies essentially the same set of geographic boundaries and associated geologic and tectonic events underlying phylogenetic splits. However, their phylogeny estimate places *B. lateralis* nested within the northern highland species, thus they argue for a recent dispersal event for the ancestor of *B. lateralis* from northern Middle America to Costa Rica. In contrast, our phylogeographic model essentially depicts a more simplistic South–to–North progression of vicariance that requires no inference of dispersal and is more compatible with patterns observed in *Atropoides* and *Cerrophidion*. Unlike the other two genera in this study, *Bothriechis* appears to have diversified (into *B. nigroviridis* and *B. lateralis*) early within Lower Central America during the middle–late Miocene. This divergence is also associated with a shift in altitudinal habitat

as *B. lateralis* typically occupies lower elevations than does *B. nigroviridis* (Campbell et al. 2004). Despite this uniqueness, temporal and phylogeographic patterns strongly coincide between *Bothriechis* and *Atropoides*, and to a lesser extent *Cerrophidion*.

Conclusions

The species–level biodiversity of Middle American highland pitvipers, as currently recognized, appears to have been predominantly generated by tectonic events occurring during the Miocene and Pliocene, independent of Pleistocene climatic fluctuations. We do, however, find evidence that the onset of glacial cycles may have impacted highland pitviper lineage diversity, but only within species. Although future taxonomic changes (i.e., in *Cerrophidion godmani*) may alter this broad conclusion, the conclusion that the current high taxonomic diversity of pitvipers in the region owes its origins to events predating the Pleistocene is both significant and impressive. We have identified several major historical events, each of which appears to have resulted in the simultaneous vicariance and diversification of multiple highland lineages in Middle America. This finding suggests that Miocene and Pliocene events may have broad predictive power across entire communities of highland–distributed organisms. Inferences from highland pitviper lineages show a strong underlying pattern of South to North, Miocene – Pliocene pattern of vicariance across highland masses that can be explicitly examined as a null hypothesis for other taxa. This new evidence suggesting the existence of an underlying and unifying model of Middle American biogeography is a strong motivation for future comparative phylogeographic work in the region, and it suggests that a cohesive hypothesis of the region's history may eventually be unveiled through the comparative phylogeography of its biodiversity. The complex and controversial geological and tectonic history of Middle America has

posed a substantial challenge for palaeogeographic and biogeographical research. Further comparative biogeographical research may provide tremendous potential for both generating and testing hypotheses leading to the formulation of a synthetic physical and biotic inference of the region's history and evolution.

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CHAPTER 4 – USING REGIONAL COMPARATIVE PHYLOGEOGRAPHIC DATA FROM SNAKE LINEAGES TO INFER HISTORICAL PROCESSES IN MIDDLE AMERICA[3]

Introduction

Historical biogeography, conservation biology, evolutionary ecology, and global climate change biology all require information about how historical patterns and processes have shaped lineage diversification at various spatial and temporal scales. It is important to understand how specific historical processes, and specific biogeographic boundaries, may have differentially impacted lineages or various components of biotic assemblages. The convergence of molecular phylogeographic datasets with robust approaches for estimating lineage divergence times has enabled an outgrowth of comparative phylogeographic research that may address such questions about differential biological responses of lineages. It is becoming increasingly clear that large comparative phylogeographic datasets may provide an excellent way to use multiple independent lineages simultaneously to infer models of historical divergence across landscapes (Arbogast and Kenagy, 2001; Bermingham and Moritz, 1998; Hickerson and Meyer, 2008). These, in turn, may represent broad and generalizable models for projection onto other unstudied taxonomic groups, and even larger biotic assemblages. This insight from comparative analyses are particularly important for areas with either vague geological or tectonic information, or where little historical consensus is available (Castoe et al., 2009; Riddle et al., 2008)

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In historical biogeography, vicariance and dispersal are considered the major forces that determine the divergence and geographic distribution of lineages (Nelson and Platnick, 1981; Ree and Sanmartín, 2009; Ree and Smith, 2009). Neither of these two processes are, however, easily extracted from any single phylogeographic or phylogenetic pattern. Using coalescent models and the geographic structure of genetic data, it is possible to test the data against specific historical demographic scenarios that invoke vicariance or dispersal (Hickerson and Meyer, 2008; Knowles and Carstens, 2007; Richards et al., 2007). Such statistical approaches, however, are designed to address data associated with shallow phylogenetic trees, mostly at the intraspecific level. For deeper evolutionary events, different biogeographic methods are preferred . The most commonly used methods for such deep historical inferences search for evidence of congruence among different lineages and then explain this congruence (or lack of congruence) with vicariance/dispersal scenarios (Nelson and Platnick, 1981; Ree and Sanmartín, 2009; Ree and Smith, 2008; Ronquist, 1997).

Here we explore the application of comparative phylogeography beyond the intraspecific level to interpret regional historical processes in Middle America, and formulate new hypotheses to describe spatial–temporal lineage diversification on this broad regional scale. The core concept is that a biogeographic boundary may represent a spatial context over which a large number of lineage divergences may be temporally mapped (Leaché et al., 2007). For a given area, or axis of vicariance, the distribution of divergence times across lineages holds important biological information which can be used to interpret historical scenarios, and also predict the breadth of impact of historical processes on other components of biological communities (Hickerson et al., 2006a; Hickerson and Meyer, 2008; Hickerson et al., 2006b). Given the overlap of divergence time estimates for multiple related lineages, common patterns can be identified which may represent deep–reaching historical processes. These can be contrasted with patterns unique to particular lineages or groups of lineages.

Using related lineages, such that a single phylogenetic tree can be used for the entire analysis (as in the current study), allow the predictions of temporal congruence to be largely independent of errors in calibration points (required for absolute time estimation). This is because estimates of relative time within a single dated tree are particularly robust , making such systems particularly ideal for testing for temporal correspondence of events among lineages (regardless of the accuracy of calibration points).

We applied this comparative approach to patterns of lineage diversification in snakes of Middle America – the tropical region between Mexico and northwestern South America. A fairly large number of lineages of snakes that range throughout Middle America have been sampled for the same mitochondrial loci, making them a good system for the current study. The exaggerated relief, diversity of habitats, and the dynamic tectonic and climatic history of the Middle America have all contributed to its high endemicity and diversity (Jackson et al., 1996; Whitmore and Prance, 1987). Middle America has experienced a complex tectonic and geological history, and lies at the active junction of four major tectonic plates and several tectonic blocks (Iturralde-Vinent, 2006; Marshall, 2006). Deciphering the events that have historically shaped present-day biological diversity is complicated due to the continual physiographical reshaping of the region since the Cretaceous. Details of most of the tectonic history of Middle America still remain fragmentary and controversial (Coney, 1982; Iturralde-Vinent, 2006; Mann et al., 2006). This region has been the subject of intense biogeographic study for more than 40 years, although the geological and climatic complexities of the region have precluded any clear consensus model describing the historical processes that generated its high taxonomic diversity (Campbell, 1999; Savage, 1982). For this reason, Middle America is an ideal setting for applying comparative phylogeographic data to infer patterns of lineage diversification, and the degree to which divergences are temporally coincident.

While many previous studies largely agree in identifying major biogeographic boundaries across Middle America (Castoe et al., 2009; Crawford et al., 2007; Devitt, 2006; Marshall and Liebherr, 2000; Perdices et al., 2005), there is no consensus of when these boundaries may have been relevant in splitting lineages. Furthermore, there is even less resolution on how many times, through history, these boundaries were effective in dividing lineages. Thus, our two aims were to (1) determine the degree to which these ecologically diverse lineages appear to share overlapping divergence times over the same biogeographic break, and (2) to estimate the number of discrete times in history each boundary may have led to lineage diversification. To address these questions, we examined Bayesian posterior distributions of divergence time estimates for a total of five major biogeographic boundaries across Middle America that are shared by multiple snake lineages, totaling 28 individual phylogeographic breaks. We also used an approximate Bayesian computation approach, using a hierarchical coalescent model, to infer the discrete number of divergence episodes for the same biogeographic breaks (Hickerson et al., 2007; Hickerson et al., 2006b). We use these results to infer how the distributions of divergence times may be related to an interpretation of historical biogeographic events that have broadly impacted the fauna in the region.

Methods

Target taxa

Our phylogenetic sampling includes multiple clades of snakes, including viperids and elapids, as well as non-venomous colubrids, that contain lineages distributed throughout Middle America. Previously, we had conducted a more restricted comparative study including three lineages of mesic highland-inhabiting viperid snakes in Middle America, and found evidence for shared divergences

across three biogeographic boundaries in Middle America (Castoe et al., 2009). The current study includes expanded sampling of a greater ecological diversity of lineages, such as lowland groups (e.g., *Micrurus, Bothriechis schlegelli, Porthidium, Leptodeira*), habitat or dietary specialists (*Micrurus* spp., *Leptodeira nigrofasciata*) and habitat or dietary generalists (*Bothrops asper*, *Leptodeira septentrionalis*). Despite all lineages being snakes and thus sharing somewhat similar dispersal characteristics and life history traits, the lineages sampled do contain a diverse sampling of ecological groupings, and should be capable of providing a much broader perspective on co-diversification and speciation in Middle America than the previous study (Castoe et al., 2009).

We assembled a single combined data set, incorporating 28 nodes that correspond to clear phylogeographic breaks across Middle America (Fig. 4.17; Castoe et al., 2009; Castoe et al., 2007a; Daza et al., 2009; Devitt, 2006). The first major lineage comprises the subfamily Crotalinae. This group of venomous snakes is particularly diverse in the Neotropical region, and their phylogenetic relationships have been studied extensively (Castoe et al., 2009; Castoe and Parkinson, 2006; Castoe et al., 2005; Parkinson et al., 2002; Parkinson et al., 2000). Sequences for all relevant nodes of pitvipers were obtained from several published trees: *Agkistrodon*, (Parkinson et al., 2000), *Bothriechis schlegelii* (Wüster et al., 2002), *Crotalus durissus* (Wüster et al., 2005); *Lachesis* (Zamudio and Greene, 1997), and highland pitvipers (Castoe et al., 2009; Castoe et al., 2005). The second lineage includes members of the family Elapidae, and specifically includes representatives of the monadal and triadal coralsnake lineages (Castoe et al., 2007a). Finally, we compiled phylogenetic results of Neotropical colubrids from two sources: Devitt (2006) and Daza et al. (2009). The first includes the major lineages of the genus *Trimorphodon* (Colubrinae) and the second includes the major lineages of the genus *Leptodeira* (Dipsadinae).

Phylogenetic reconstruction

We assembled a molecular dataset that includes two mitochondrial protein–coding genes sequences from cytochrome b and NADH dehydrogenase subunit 4 (Table 4.6). Alignment of each gene was accomplished using Clustal W (Larkin et al., 2007) and corrected manually using GeneDoc 2.6 (Nicholas and Nicholas, 1997). The dataset was partitioned by gene and codon position, and a different GTRΓI model for each partition was implemented (as selected by MrModeltest 2.3 using AIC, Nylander, 2004). We used the package Beast 1.4.8, a Bayesian approach to estimate simultaneously the phylogeny and both relative and absolute divergence times (Drummond and Rambaut, 2007).

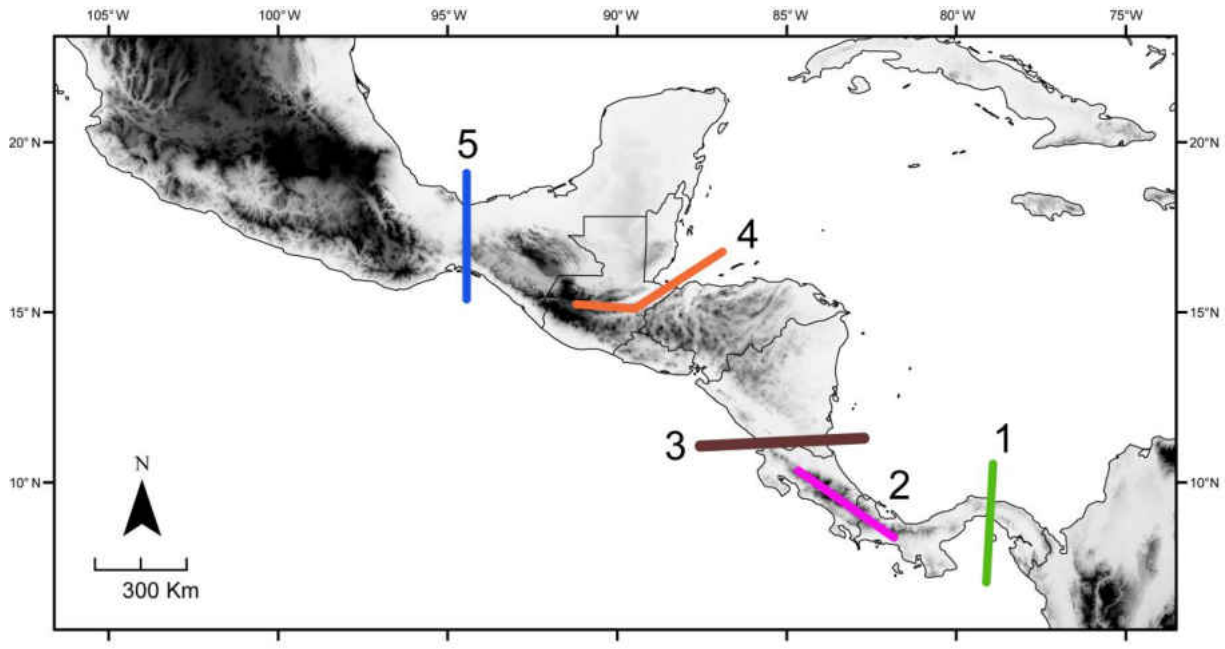

Figure 4.17. Map of Middle America showing the five major biogeographic boundaries analyzed in this study. [1] Middle America–South America transition, [2] Talamanca Cordillera, [3] Nicaraguan Depression, [4] Motagua–Polochic river valleys, [5] Isthmus of Tehuantepec.

Divergence time estimation

We estimated divergence times using two different approaches. First, we estimated relative divergence times (RT analysis here) so that we could examine temporal congruence among nodes regardless of absolute time (and the calibration assumptions that accompany absolute time estimation). Second, we calibrated the molecular phylogenies using fossil and other calibrations metrics to obtain absolute estimates of divergence dates (AT analysis). The strength of this approach is that we can first optimize rates using a Bayesian approach and obtain an ultrametric tree that relies only on the evolutionary process (and fitting of the relaxed clock model) and is unaffected by the uncertainty of the fossil record and other calibrations (Graur and Martin, 2004; Heads, 2005). This non-calibrated tree can be used to infer congruence in divergence time among lineages even when no nodal calibrations exist, and further used to evaluate the impact of adding calibration points on the correspondence of divergence time across nodes. Once inferences of temporal congruence are made, calibration points can then be added to estimate the absolute time scale of divergence events.

We implemented the Bayesian relaxed molecular clock method with uncorrelated lognormal rates among branches (Drummond et al., 2006), assuming a birth–death process for the speciation model. For the RT analysis we set the treeModel.rootHeight parameter to be 1 using a normal distribution with a mean=1.0 and SD=0.1 and used the program's default priors. For the AT analysis we used a lognormal prior for the treeModel.rootHeight parameter with a mean=3.7 and SD=0.3, and the following additional constraints: for the tMRCA of *Crotalus atrox* and *C. ruber* we used a uniform prior between 2.5 and 4.5 Ma; for the tMRCA of *Sistrurus*+*Crotalus* we used a uniform prior between 9.0 and 32.0; for the tMRCA of *Agkistrodon contortrix* we used a uniform prior between 5.0 and 32.0. The remaining priors were set to the program defaults for the AT analysis.

To ensure convergence of our estimates, we initiated four independent runs in Beast with random starting trees, and ran each for 10 million generations. Chains were sampled every 1000 generations, and convergence and stationarity were verified by examining likelihood scores and parameter estimates using Tracer 1.4 (Rambaut and Drummond, 2007). Based on examination of trial runs in Tracer (which burned in prior to 2 million generations), the conservative burnin period of three million generations was used for final runs, and we combined the posterior samples from all four runs, and report the results of this combined posterior sample. We used the program TreeStat 1.2 (Rambaut and Drummond, 2008) to summarize the Markov chain results for posterior divergence date estimates, and used an R script to create posterior density plots for nodes of interest.

Table 4.6. Genbank sequences utilized in this study.

Taxa	Locality	Voucher	Cyt-b	ND4
Agkistrodon bilineatus	Costa Rica, Guanacaste	WWL	AY223613	AF156585
Agkistrodon contortrix	USA, Ohio, Athens Co.	Moody 338	AY223612	AF156576
Agkistrodon piscivorus	USA, South Carolina	CLP-30	AY223615	AF156578
Agkistrondon taylori	Mexico, Tamaulipas	CLP-140	AY223614	AF156580
Atractus lasallei	Colombia, Antioquia	MHUA 14368	GQ334480	GQ334581
Atropoides indomitus	Honduras, Olancho	ENS-10630	DQ061194	DQ061219
Atropoides mexicanus	Costa Rica, San Jose	CLP-168	AY223584	U41871
Atropoides nummifer	Mexico, Puebla,	ENS-10515	DQ061195	DQ061220
Atropoides occiduus	Guatemala, Escuintla	UTA-R-29680	AY220315	AY220338
Atropoides olmec	Mexico, Veracruz	UTA-R-14233	AY220322	AY220345
Atropoides picadoi	Costa Rica, Alajuela, Varablanca	CLP-45	AY223593	U41872
Bothriechis aurifer	Guatemala	UTA-R35031	DQ305466	DQ305483
Bothriechis bicolor		UTA-R34156	DQ305467	DQ305484
Bothriechis lateralis	Costa Rica, Acosta	MZUCR-11155	AY223588	U41873
Bothriechis marchi	Guatemala, Zacapa, Cerro del Mono	UTA-R52959	DQ305469	DQ305486
Bothriechis nigroviridis	Costa Rica, San Gerondo de Dota	MZUCR-11151	AY223589	AY223635
Bothriechis rowleyi	Mexico, Cerro Baúl	JAC 13295	DQ305468	DQ305485
Bothriechis schlegelii	Costa Rica, Cariblanco de Sarapiquí	MZUCR-11149	AY223590	AY223636
Bothriechis schlegelii	Ecuador, Pichincha	FHGO Live coll.	AF292573	AF292611
Bothriechis supraciliaris	San Vito, Costa Rica		DQ305470	DQ305487
Bothriechis thalassinus	Guatemala, Zacapa	UTA-R52958	DQ305465	DQ305482
Bothriopsis taeniata	Suriname		AY223592	AY223637
Bothrops asper	Costa Rica, Limon	WW 1318	EU624301	EU624210
Bothrops asper	Costa Rica, San Jose	MZUCR-11152	AY223599	U41876
Bothrops atrox		WWW-743	AY223598	AY223641

Taxa	Locality	Voucher	Cyt-b	ND4
Cerrophidion godmani	Costa Rica, San Jose	MZUCR-11153	AY223578	U41879
Cerrophidion godmani	Guatemala, Huehuetenango	UTA-R-42237	EU684282	EU684299
Cerrophidion godmani	Guatemala, Quetzaltenango	ENS-8350	EU684283	EU684300
Cerrophidion godmani	Honduras, Francisco Morazan	ENS-10632	EU684286	EU684301
Cerrophidion petlalcalensis	Mexico, Veracruz, Orizaba	ENS-10528	DQ061202	DQ061227
Cerrophidion tzotzilorum	Mexico, Chiapas, Las Rosas	ENS-10529	DQ061203	DQ061228
Crotalus atrox	USA, Texas, Jeff Davis Co.	CLP-64	AY223608	AY223646
Crotalus durissus	Venezuela	ROM 18138	AF259178	
Crotalus durissus collilineatus	Brazil, Mato Grosso	IB 58460	AY704811	AY704861
Crotalus durissus culminatus	Mexico, Morelos	3291	AY704830	AY704880
Crotalus durissus durissus	Mexico, Chiapas	2065	AY704833	AY704883
Crotalus durissus durissus	Mexico, Veracruz	1	AY704831	AY704881
Crotalus durissus tzabcan	Belize, Corozal	255, P. Singfield live coll.	AY704806	AY704856
Crotalus ruber	USA, California, Riverside CO.	ROM18207	AF259191	
Crotalus tigris	USA, Arizona, Pima Co.	CLP169	AY223606	AF156574
Dipsas pratti	Colombia, Antioquia	MHUA 14278	GQ334482	GQ334583
Gloydius shedaoensis	China, Liaoning	ROM-20468	AY223566	AY223623
Gloydius ussuriensis	China, Jilin	ROM-20452	AY223565	AY223622
Imantodes cenchoa	Colombia, Antioquia	MHUA 14290	GQ334484	GQ334585
Imantodes inornatus	Colombia, Antioquia	MHUA 14540	GQ334488	GQ334589
Lachesis melanocephala	Costa Rica		U96018	U96028
Lachesis muta	Peru	Cadle 135	AY223604	AY223644
Lachesis stenophrys	Costa Rica, Limon	UMMZ 176987	AY223603	U41885
Leptodeira annulata annulata	Suriname, Para	BPN 963	GQ334493	GQ334594
Leptodeira annulata cussiliris	Guatemala, San Marcos	UTA R-53305	GQ334501	GQ334603
Leptodeira annulata rhombifera	Honduras, El Paraiso	UTA R-41255	GQ334509	GQ334611
Leptodeira bakeri	Aruba	Avid 023858355	GQ334515	GQ334618
Leptodeira frenata	Mexico, Guerrero	LSUMZ 39524	EF078579	EF078531
Leptodeira maculata	Mexico, Jalisco	UTA R-53323	GQ334521	GQ334624
Leptodeira nigrofasciata	Costa Rica	ASL 190	GQ334525	GQ334628
Leptodeira nigrofasciata	Mexico, Guerrero	MVZ 241573	EF078581	EF078533
Leptodeira punctata	Mexico, Sinaloa	UTA R-51974	EF078577	EF078529
Leptodeira septentrionalis ornata	Colombia, Antioquia	MHUA 14423	GQ334532	GQ334636
Leptodeira s. ornata	Costa Rica, Limon	ICP 1089	GQ334540	GQ334645
Leptodeira s. ornata	Costa Rica, Punta Arenas	MSM PH 90	GQ334539	GQ334644
Leptodeira s. polysticta	Guatemala, Escuintla	UTA R-46878	GQ334545	GQ334650
Leptodeira s. polysticta	Guatemala, Peten	UTA R-46125	GQ334547	GQ334652
Leptodeira splendida bressoni	Mexico, Nayarite	UTA R-53595	GQ334549	GQ334655
Leptomicrurus narducci	Ecuador, Napo	KU 202955	EF137412	EF137404
Micrurus fulvius	USA, Florida, Liberty Co.	CAS-214347	EF137413	EF137405
Micrurus mipartitus	Panama, Cocle	CH-5377	EF137414	EF137406
Micrurus surinamensis	Brazil, Rondonia	OMNH-37596	EF137415	EF137407
Ninia atrata	Colombia, Caldas	MHUA 14452	GQ334553	GQ334659
Ophryacus melanurus	Mexico	UTA-R34605	AY223587	AY223634
Ophryacus undulatus	Mexico	CLP-73	AY223586	AY223633
Ovophis monticola	China, Yunnan	CAS 215050	DQ305462	DQ305480

Taxa	Locality	Voucher	Cyt-b	ND4
Porthidium arcose	Ecuador, Manabí	WWW-750	AY223582	AY223631
Porthidium dunni	Mexico, Oaxaca	ENS-9705	AY223581	AY223630
Porthidium hespere	Mexico, Michoacan	MZFC 19742	EU017534	EU016098
Porthidium lansbergi	Venezuela, Isla Margarita	WES	DQ061205	DQ061230
Porthidium nasutum	Costa Rica	MZUCR-11150	AY223579	U41887
Porthidium ophryomegas	Costa Rica, Guanacaste	UMMZ-210276	AY223580	U41888
Porthidium porrasi	Costa Rica, Puntarenas, Sierpe	MSM	DQ061211	DQ061236
Porthidium yucatanicum	Mexico, Yucatán	JAC-24438	DQ061215	DQ061244
Rhinocerophis alternatus		DLP-2879	AY223601	AY223642
Sinomicrurus kelloggii		ROM-37080	EF137417	EF137409
Sinomicrurus mcclellandi		ROM-35245	EF137418	EF137410
Sistrurus catenatus	USA, Texas, Haskel Co.	Moody-502	AY223610	AY223648
Trimorphodon biscutatus	Mexico, Oaxaca	JAC 24309		DQ497525
Trimorphodon lyrophanes	USA, California, Inyo Co.	JMM 79		DQ497506
Trimorphodon lyrophanes	Mexico, Baja California Sur	ROM 34073		DQ497514
Trimorphodon paucimaculatus	Mexico, Sinaloa	UTA-R 52929		DQ497498
Trimorphodon paucimaculatus	Mexico, Jalisco	UTA-R 52654		DQ497494
Trimorphodon quadruplex	Guatemala, Zapaca	ENS 10800		DQ497541
Trimorphodon vilkinsonii	USA, Texas, Presidio Co.	TLJ 338		DQ497492

Calibration points

We used four calibration points to obtain absolute date estimates for the molecular phylogeny. We constrained the origin of *Sistrurus* to be at least 9.0 Ma (Parmley and Holman, 2007), and the origin of *Agkistrodon contortrix* to be at least 5.0 Ma (Holman, 2000). We also constrained the divergence between the species *Crotalus ruber* and *C. atrox* to be between 2.5 and 4.5 Ma based on phylogeographic information on the vicariance between mainland and Baja California peninsula desert regions (Castoe et al., 2009; Castoe et al., 2007b). Finally, based on the oldest colubrid fossil known, the root of the tree (the tMRCA of Colubroidea) was set to have occurred before 40 Ma (Head et al., 2005; Rage et al., 1992).

Shared divergence

To make inferences about the degree to which lineage divergences were coordinated in time we used msBayes (Hickerson et al., 2006a) to estimate the number of independent/discrete lineage divergence times per biogeographic break. MsBayes implements an approximate Bayesian computation approach using a hierarchical coalescent model where hyper-parameter estimation is utilized to discriminate the differences between time of divergence among pairs of taxa and variance in coalescent times (Hickerson et al., 2007; Hickerson et al., 2006b). For these analyses we included only the nodes that had more than two samples per taxon pair, based on the requirements of the program. For each analysis (corresponding to each break) we drew one million samples from the hyper-prior and, using the hierarchical approximate Bayesian computation acceptance/rejection algorithm, constructed the hyper-posterior from 2000 samples (tolerance=0.002).

We contrasted the results obtained with msBayes and those based on posterior distributions of divergence dates and 95% credibility intervals obtained with Beast. Additionally, from posterior densities of individual lineage divergence times (from the Beast divergence dating analyses), we assemble pooled posterior densities for divergence times by combining data from multiple lineages (for a particular biogeographic break). For these pooled posterior densities, we summed the lineage–specific posterior density per unit time, across all lineages for each break. These distributions can be interpreted as the probability of divergence pooled over all lineages examined, and we discuss in the text how these may be useful particularly as informed priors for future studies. For interpreting co-divergence, however, these pooled posteriors may be somewhat misleading in that they may obscure multi-modal divergence posteriors of different lineages.

Results

Our estimate of phylogeny is consistent with recent studies that have specifically analyzed phylogenetic relationships among the taxa included here (Fig. 4.18, Castoe et al., 2009; Castoe et al., 2007a; Daza et al., 2009; Devitt, 2006; Wüster et al., 2005; Wüster et al., 2002). The ultrametric trees we obtained with the RT and AT analyses yielded similar results (Fig. 4.19). When standardizing the root of the RT tree to be the absolute date obtained with the AT analysis, we did not find any difference in the relative timing of phylogenetic events between the two trees. In other words, adding calibration points did not affect our inferences of relative divergence times, as compared between lineages/nodes of codistributed lineages.

The AT analysis resulted in a tree with an overall depth of 41.8 Ma (95% Credibility Interval=30.9–55.69). The divergence between Colubridae and Elapidae was estimated to be 38.8 Ma and the split between Old World and New World Elapids was inferred at 21.5 Ma and the same divergence but within crotalines was estimated at 19.4 Ma. Divergence times were consistent with those from Kelly et al. (2009), Sanders and Lee (2008), Castoe et al. (2009) and Daza et al. (2009). In contrast, our estimated divergence times were younger than those from Burbrink and Pyron (2008), Devitt (2006), Vidal et al. (2009), and Wüster et al. (2008).

The eight splits identified in the Middle–South America transition spanned from the early Miocene to the Pleistocene ($CI_{95\%}$ = 0.8 – 22.8 Ma). The three lineage divergences across north and south areas of the Talamanca Cordillera occurred between 2.5 and 3.9 Ma ($CI_{95\%}$ = 1.4 – 5.4). The divergences across the Nicaraguan Depression spanned from 4.1 to 8.8 Ma ($CI_{95\%}$ = 2.4 – 11.9). The divergences across the Motagua–Polochic faults were estimated to have occurred between 3.8 and

6.8 Ma ($CI_{95\%}$ = 2.4 – 9.9). Lastly, the five cladogenetic events identified across the Isthmus of Tehuantepec were estimated to be between 2.8 and 7.35 Ma ($CI_{95\%}$ = 1.5 – 10.1). Out of the five phylogeographic breaks analyzed, three of them showed a strong correspondence in divergence times among multiple lineages (Fig. 4.20). Across the Isthmus of Tehuantepec break, with the exception of a single divergence estimate (for *Porthidium* species), the lineages appeared to have diverged around the same time. The cladogenetic events occurring at the other biogeographic breaks were not entirely coincident in time, although as we discuss in detail below, a number of strong patterns of congruence are evident.

The summary of estimated parameters using the Approximate Bayesian Computation algorithm is shown in Table 4.7. According to the msBayes results, the Talamanca Cordillera and the Motagua–Polochic Faults have likely undergone a single vicariant event. The pooled posterior distributions in these two breaks also showed a single peak, and the widely overlapping 95% CIs further supports a shared divergence (Figs 4.20 and 4.21). Small values of Ω, a parameter that measures the incongruence among divergence times along the same barrier, were found for these two biogeographic boundaries. In contrast, Ω value was highest for the Middle American – South American transition (Ω=3.46), followed by the divergences along the Nicaraguan Depression and the Isthmus of Tehuantepec (Table 1). Similarly, non-overlapping 95% CIs and multimodal pooled posterior distribution of dates were observed in these three phylogeographic breaks (Fig 4.20).

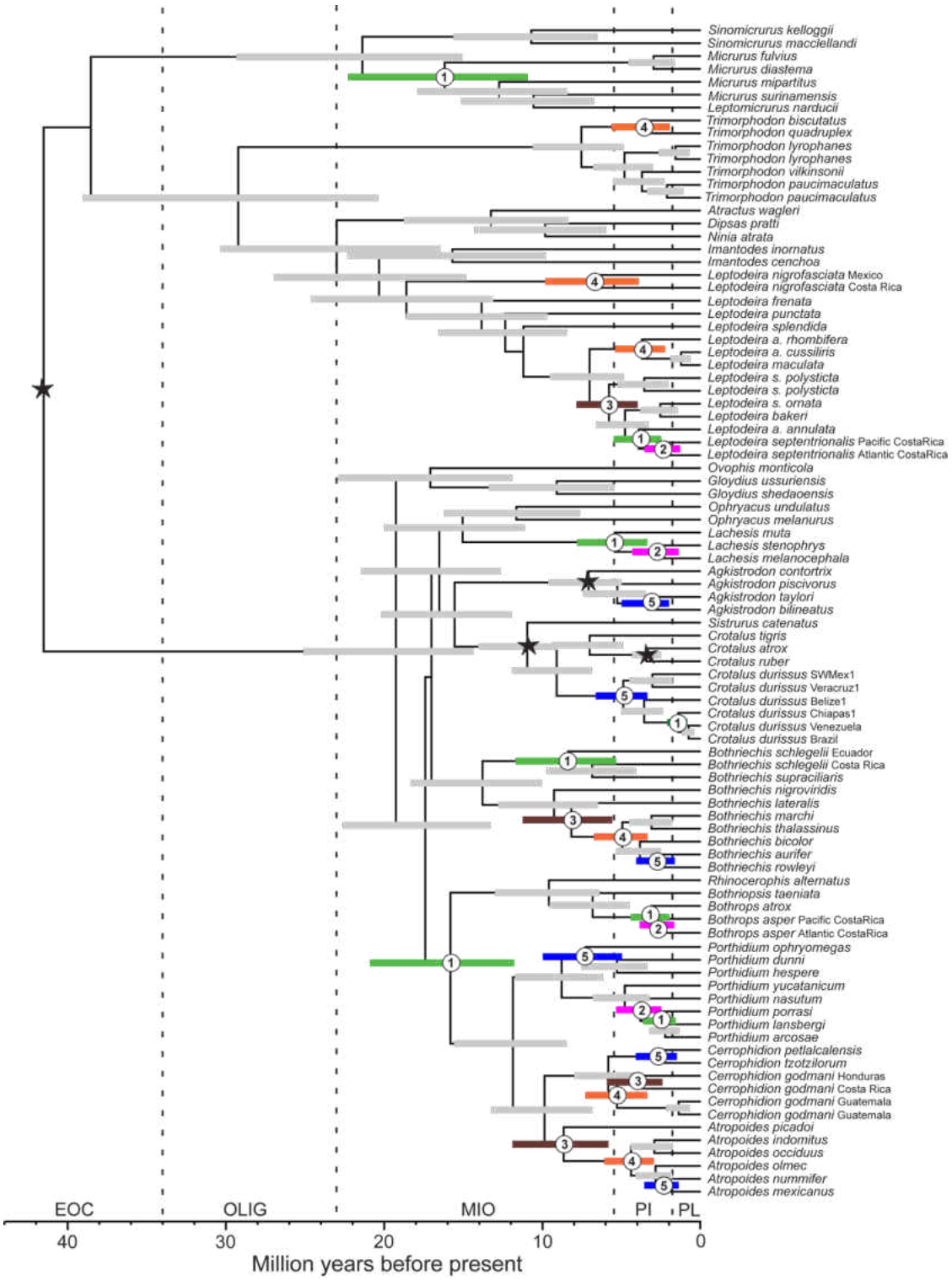

Figure 4.18. Dated tree obtained using the relaxed molecular clock method using Beast. Node heights represent mean node ages (based on the combined posterior of four independent runs). Bars on nodes represent the 95% credibility interval of divergence times. Stars depict calibration points and numbers indicate nodes (see Fig. 4.1) utilized in the congruence tests.

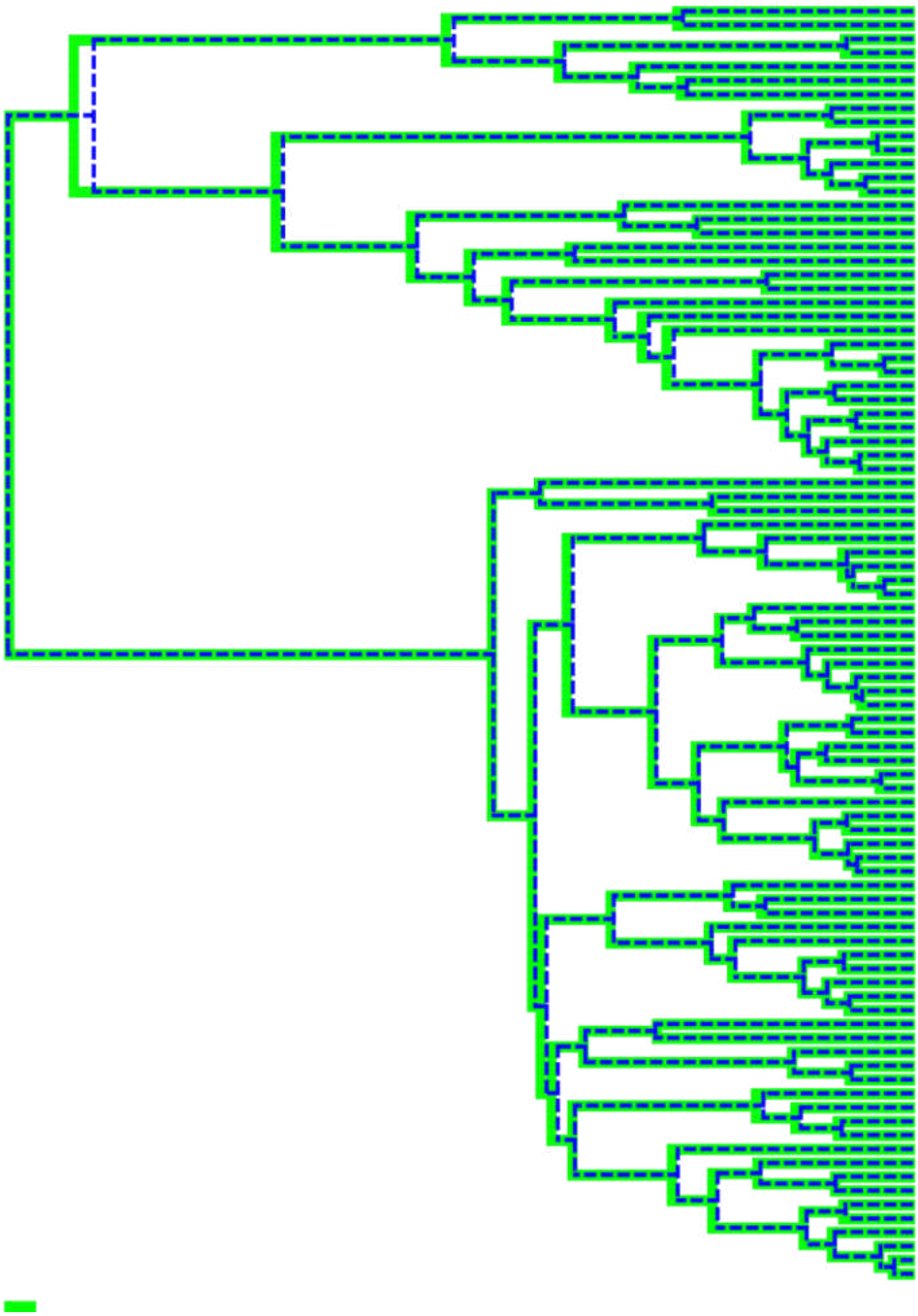

Figure 4.19. Ultrametric trees obtained with Beast. The green solid lines represent the tree with fossil constraints (AT analysis) and the blue dashed lines represents the tree without fossil constraints (RT analysis). The RT tree was standarized to the root of the AT analysis.

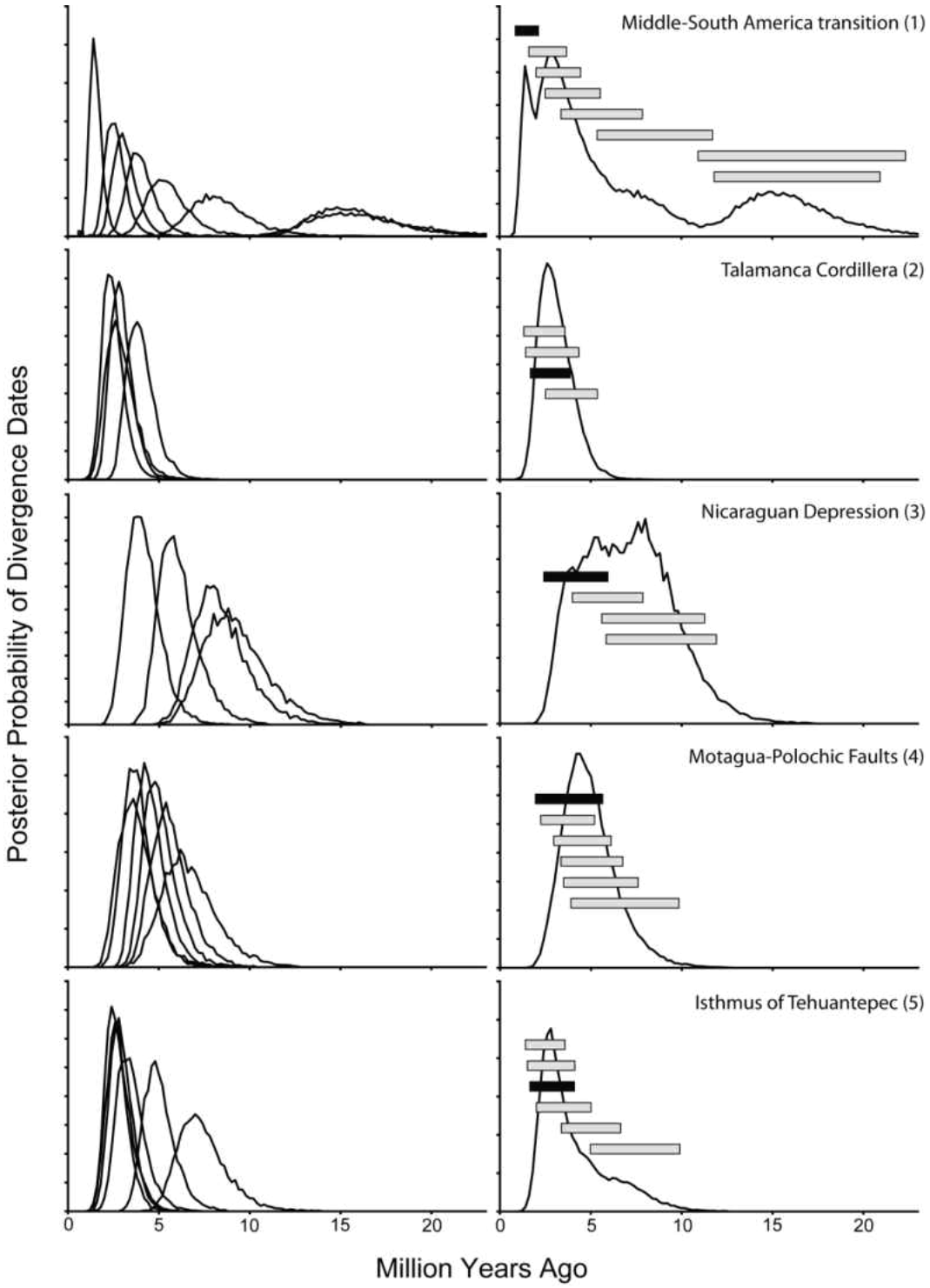

Figure 4.20. Left: Posterior density plots of divergence times of various lineages across five biogeographic boundaries of Middle America. Right: Pooled posterior distribution of divergence times for each biogeographic barrier. Bars indicate the 95% Credibility Intervals of divergence times. Black bars represent the lineage-pair that was not included in the msBayes analysis.

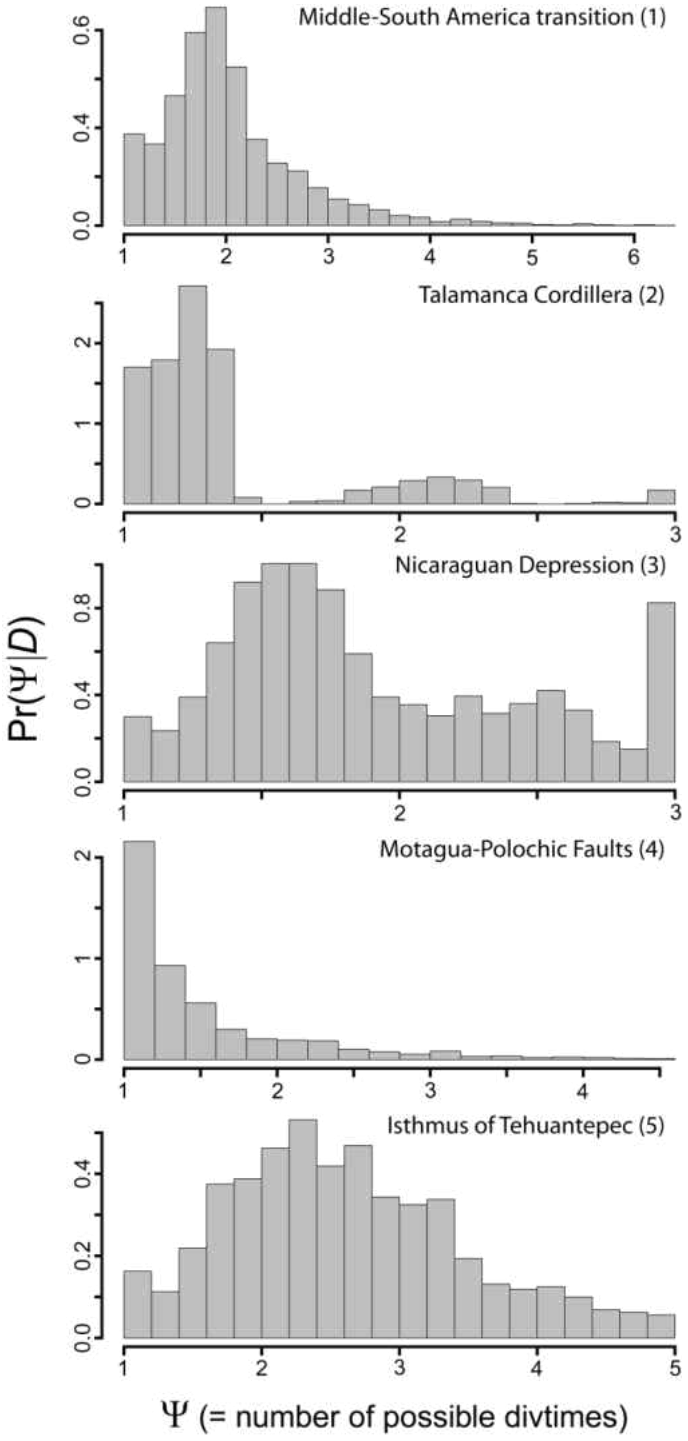

Figure 4.21. Posterior distribution of the number of divergence times for snake lineages across five biogeographic boundaries in Middle America.

Table 4.7. Statistics summary from the msBayes runs. n=number of lineage pairs, Ψ=number of possible divergence times, Ω=parameter indicating the degree of discordance among divergence times.

	Phylogeographic break	n	Ψ mode	Ψ mean	Ψ $CI_{95\%}$	Ω mean
(1)	Middle–South America transition	7	1.87	2.03	1.00–3.88	3.46
(2)	Talamanca Cordillera	3	1.27	1.39	1.00–2.36	0.12
(3)	Nicaraguan Depression	3	1.58	1.91	1.07–3.00	0.59
(4)	Motagua–Polochic Faults	5	1.01	1.49	1.00–3.29	0.13
(5)	Isthmus of Tehuantepec	5	2.23	2.62	1.12–4.56	1.15

Discussion

Emerging hypotheses for Middle American speciation patterns

Despite consensus in the identification of major biogeographic boundaries that have shaped Middle America's biodiversity (e.g., Marshall and Liebherr, 2000; Morrone, 2001; Savage, 1982), there has been little quantitative insight as to when these barriers may have led to diversification, in what temporal order, and especially the degree to which divergences were temporally coordinated. In total, our dataset included 28 individual cladogenetic events that span five biogeographic boundaries, bringing a fair amount of evidence to bear on inferences of regional diversification. Analysis of this dataset contributes new findings that appear to reject previous hypotheses of temporal diversification and further clarify historical biogeographic patterns in Middle American taxa. It thus presents an encouraging example of how such a comparative spatio–temporal approach may yield insight into the historical processes that have shaped a previously well studied yet poorly understood region.

Our results show that a surprising majority of divergences across these diverse snake lineages appeared to be essentially coincident in time and space (Fig. 4.20). These findings suggest coordinated vicariance as a dominating force in speciation in the Middle American snake lineages

studied. We found that some boundaries show great synchrony among diverse lineages (breaks in Talamanca, Motagua-Polochic and Tehuantepec). Other biogeographic breakpoints show evidence of multiple divergence time periods, evidenced by comparisons of credibility intervals and from the Approximate Bayesian Computation analyses; these multi-modal periods of divergences appear to characterize the breaks in Panama and Nicaragua.

Since the Miocene, Middle America has continually endured extensive terrain dynamics powered by tectonic activity, and we interpret our results as indicating that this dynamic process has been the dominant force in lineage diversification, and that such tectonically-driven vicariance explains the remarkably high degree of synchronization among such ecologically distinct lineages. In some cases, however, we do find evidence that intrinsic factors (e.g., dispersal and ecological features) may have also played roles in lineage divergence times, rather than purely extrinsic (e.g., tectonic) forces. Examples of this include divergences along the Isthmus of Panama, the divergence of *Bothriechis* across the Talamanca cordillera and the Nicaraguan Depression, and the divergence of *Porthidium* along the Isthmus of Tehuantepec.

Estimates of relative and absolute divergence times

Comparative phylogeographic data coupled with divergence time estimates can illuminate much about a region's history. When divergence time estimates from independent studies are compared, however, we expect that substantial error in absolute divergence time estimates may often exist, due largely to differences in dating approaches and interpretations of the fossil and geological record (Heads, 2005). In such comparative studies the precise absolute divergence times are often much less important than the estimates of the relative coordination of divergence events across lineages. This is particularly the case when inferring the number of discrete temporal windows of divergence,

such as in the current study. To circumvent this issue here, we assembled multiple related lineages into a single dataset, and use this large combined dataset for jointly estimating divergence times and instances of co–divergence. Because the same calibration assumptions are applied to the entire tree, and also because relative divergence time estimates are highly robust within a tree, this approach can provide precise estimates of the relative timing of divergence across lineages.

Our absolute dates are consistent with our previous work with these snakes (e.g., Castoe et al., 2009; Daza et al., 2009), most likely because of the very similar divergence dating strategies and calibrations, and they are also consistent with other independent studies (Kelly et al., 2009; Sanders and Lee, 2008). A few studies, however, on particular lineages we included in our dataset have estimated older node ages than we have here, particularly for deeper nodes. We interpret these discrepancies in two ways. First, fossil snakes are extremely scarce for certain taxonomic groups and usually the available and non-ambiguous ones are used as calibrations for fairly recent cladogenetic events since most of the fossils come from the Pliocene and Pleistocene (see Holman, 2000); using recent calibrations points to estimate older nodes has been identified as a potential source of error previously (Ho et al., 2008). Second, discrepancies are likely to occur when different calibrations points are used. For example Devitt (2006) and Wüster et al. (2005; 2008) incorporated geological information (the emergence of the Mexican transvolcanic axis and the Isthmus of Panama, respectively) instead of fossil data (as in our case) for dating *Trimorphodon* and *Crotalus* divergences, respectively. Given the uncertainty in the fossil and geological record we would not necessarily expect multiple studies converge to the same dates (given the use of different calibrations). Because of the potential biases that different choices of calibrations may impose on estimates of shared divergence, our combination of all data into a single dataset, and our ability to rely on highly accurate

inferences of relative divergence time across lineages (rather than calibration points), we expect our results of shared divergence to be particularly robust.

Divergence across the Middle America – South America transition (1)

The area between southern Honduras and northwestern Colombia is biogeographically important because it represents the intermediate land connection between the two main continental landmasses of the Western Hemisphere, as well as the division between two oceans. The details of the dynamic connections between these landmasses from the Miocene onward, however, remain controversial. Recent phylogenetic and biogeographic evidence has uncovered complex patterns that suggest that biotic interchange between terrestrial fauna may have entailed multiple dispersal and vicariant events that occurred across a fairly broad time scale, far broader than the time surrounding the final closure of the Isthmus of Panama around 3.5 Ma (Bermingham and Martin, 1998; Collins et al., 1996; Koepfli et al., 2007; Pennington and Dick, 2004).

Our analyses indicate that recurrent diversification has occurred since the middle Miocene (Figs. 4.20, 4.21). MsBayes suggests two main episodes of diversification, although there is no strong demarcation between these two episodes based on the 95% CIs of divergence times. Although this study is limited in taxonomic scope, it is the first to include explicit temporal evidence across multiple terrestrial lineages, showing evidence (independent of assumptions of fossil calibrations, etc.) for multiple episodes of lineage divergence among the continents. A similar disparate pattern has been recently found for divergences between marine geminate species on either side of the isthmus (Hurt et al., 2009; Marko, 2002), suggesting that both terrestrial and marine species responded in a similar broad temporal fashion. Collectively, our data and others' raise the question of whether pre–final closure dispersal/vicariant events of terrestrial lineages were all based on

overwater dispersal, or instead, multiple transient land–connections joined parts of Lower Central America and South America prior to the final isthmus closure. Given the number of Pliocene and Miocene divergences associated with this region, the early transient land bridges hypothesis seems more likely, and warrants further evaluation with additional comparative data.

It is notable that the final closure date for the Panamanian Isthmus at ~3.5 Ma has been commonly used as a regional calibration point for previous marine and terrestrial biogeographic studies (Bermingham et al., 1997; Wüster et al., 2005; Wüster et al., 2008; Wüster et al., 2002). In the case of terrestrial studies, this practice is unsound because this time period probably represents a period of dispersal, rather than having any direct relevance to vicariance (and is thus not particularly useful in applying to divergence time estimates). More importantly, based on our results, we find evidence from multiple lineages that divergence times across this boundary appear almost completely independent of this 3.5 Ma closure date (Fig. 4.20). Therefore, of all the biogeographic breaks we have examined here, this event represents one of the most problematic choices for use as a calibration point. Furthermore, recent evidence has shown that marine geminate species across both sides of the Isthmus diverged in a temporally staggered manner since the Miocene (Hurt et al., 2009), suggesting that this region represents a poor calibration point for both marine and terrestrial divergence times estimates.

Divergence across the Talamanca Cordillera (2)

The Talamanca mountain range and associated cordilleras running down the spine of Costa Rica and Northwestern Panama represent a composite of Neogene and Quaternary mountains with an active geomorphological history since the Miocene (MacMillan et al., 2004; Marshall, 2006; Marshall et al., 2003). Phylogenetically, lineages along the Pacific slope of Costa Rica/Panama and those in

Northern South America tend to be more closely related than are lineages on either side of the Talamanca ridge (Castoe et al., 2005; Crawford et al., 2007; Daza et al., 2009; Weigt et al., 2005). Combining the results from msBayes and the pooled posterior distributions of divergence times, our results favor a single vicariant event centered around 3.9 Ma (Fig. 4.20). The timing of this event near the final closure of the isthmus of Panama raises the question of whether this event was driven by the final tectonic uplifts of the Talamancan ridge (MacMillan et al., 2004) or possibly the large-scale changes in habitat distributions brought about through changes in ocean currents and weather patterns accompanying the closure of the isthmus of Panama.

Divergence across the Nicaraguan Depression (3)

The Nicaraguan Depression is a lowland corridor running from the Caribbean to the Pacific near the border between Costa Rica and Nicaragua. Marine sediments indicate that a seaway existed multiple times here during the Pliocene, separating regions to the north and south (Coates and Obando, 1996). There is also evidence implying that a continuous peninsular landmass connected Honduras with modern day Costa Rica during the Miocene (Kirby et al., 2008; Kirby and MacFadden, 2005), contrasting a hypothesis that this region comprised a set of islands interconnected by shallow waters during the Miocene (Coates and Obando, 1996).

Two lineages of highland pitvipers (*Atropoides* and *Bothriechis*) show largely overlapping early divergences over this area, whereas a third highland pitviper lineage (*Cerrophidion*) and the lowland lineage (the colubrid *Leptodeira septentrionalis*) show substantially later divergences. The posterior distributions cluster in a staggered manner that broadly extends from ~4–10 Ma (Fig. 4.20), countering a hypothesis of a single coordinated divergence event. This multi-modal pattern of divergence is also evident in the msBayes results that show diffuse posterior density across a broad

range of discrete divergence events from one to five, although a majority of posterior density is centered over 2 events (Table 1, Fig. 4.21). A reasonable *a priori* expectation for divergences across this boundary may include rapid and highly coordinated divergence across multiple lineages due to the geo–tectonic model including seaway formation in the Pliocene. Instead, our data point to multiple periods (or one long broad period) of vicariance (and probably also dispersal) across the Nicaraguan Depression, rejecting a model centered on a single discrete barrier to gene flow coordinating divergences across lineages. Our data do fit an alternative model, that of Kirby and MacFadden (2005), which suggests a dynamic landmass may have transiently existed across the Nicaraguan Depression during the second half of the Miocene. This particular example highlights the important synergistic role in generating and testing hypotheses that comparative phylogeographic studies can have in conjunction with geological–tectonic data.

Divergence across the Motagua–Polochic Faults (4)

Recent studies have uncovered a sharp phylogeographic break along the axis where the Maya and Chortis tectonic blocks (in northern Middle America) come in contact and form a long NE–SW trending basin along the Motagua–Polochic Fault zone (Concheiro-Pérez et al., 2007; Devitt, 2006; Perdices et al., 2005). The continued tectonic activity uplifting highlands on either side of this basin, and its further entrenchment, appears to have generated divergence events in both lowland and highland species. Based on the pooled posterior distribution of divergence times, credibility intervals and msBayes results (Figs 4.20 and 4.21), we find a clear pattern of concentrated temporal divergence across multiple species that span this area, suggesting that this zone acted as a barrier to many different lineages over this period from ~3–8 Ma (Fig. 4.20). Our phylogeographic analysis suggests the primarily lowland snake genera, *Trimorphodon* and *Leptodeira,* diverged across this barrier in near concert with the highland lineages *Bothriechis*, *Atropoides* and *Cerrophidion* (Figs. 4.18 and 4.20).

Terrestrial fossil information for Middle America is scarce, therefore the regional calibration for dating purposes needs to rely either on the fossil record from relatively distant lineages, or be based on estimated evolutionary rates. Here, we find evidence that the Motagua–Polochic Fault phylogeographic break may be a reasonably sound calibration point when no other information for regional calibrations is available. For example, the results of our pooled posterior distribution for the shared divergence across this break (Fig. 4.20) could be readily incorporated as a prior distribution for species divergence times in a Bayesian analysis when other useful calibration points are lacking, or a null hypothesis for other statistical tests in future studies.

Divergence across the Isthmus of Tehuantepec (5)

Mexico's Isthmus of Tehuantepec has long been considered a biogeographic break for both highland and lowland species (Marshall and Liebherr, 2000; Morrone and Márquez, 2001; Parkinson et al., 2000). Geological evidence suggests that from the late Miocene through late Pliocene, an extensive downdropping of the eastern block along the Tehuantepec fault zone resulted in a considerable reduction of the highlands and probably a marine embayment (Barrier et al., 1998). Given the cumulative evidence of diversification across multiple lineages on both sides of the Isthmus, a broad–reaching vicariant event during the Pliocene has been suggested as being responsible for the divergence of numerous lineages (Castoe et al., 2009; Hasbún et al., 2005; Marshall and Liebherr, 2000; Mulcahy et al., 2006).

Our posterior distributions for divergence times stongly support this model, inferring a highly constrained temporal window at the end of the Pliocene when a majority of diversification events (4 of 6) occurred (Fig. 4.20). This window is consistent with proposals that events during the Pliocene

severed gene flow among lineages straddling the isthmus (Hasbún et al., 2005; León-Paniagua et al., 2007; Mulcahy et al., 2006). However, the 95% credibility intervals (Fig 4.20) and the msBayes results (Fig. 4.21) suggest that a second period of divergence also occurred earlier in the Miocene across the isthmus. Two genera, *Crotalus* and *Porthidium,* apparently diverged earlier, suggesting that a different geological/climatic event at the end of the Miocene (e.g., vegetation shifts; Cerling et al., 1997) may have been responsible for divergence in these two arid–adapted groups. Our data are thus consistent with hypotheses of broad vicariance across the isthmus due to Pliocene downdropping and seaway formation across the isthmus, but further suggest a more ancient divergence here affecting at least arid-adapted species.

Conclusion

In this study we investigated Middle American regional historical biogeography by focusing on particular spatial areas known to be major biogeographic boundaries, and characterizing these boundaries by synthesizing information about how multiple lineages temporally diverged across them. The large number of independent lineage diversification events examined provides new data for testing existing hypotheses of regional patterns of lineage diversification, and further evidence for generating new hypotheses of Neotropical diversification.

We expect that our estimates of divergence, and the degree of synchronization, represent sound testable hypotheses for unstudied taxa or communities, certainly in cases where we found divergence to be highly correlated across lineages. Combining ABC statistical methods for inferring the coordination of divergences across lineages (Hickerson and Meyer, 2008; Hickerson et al., 2007; Hickerson et al., 2006b; Leaché et al., 2007) with analyses of posterior distributions of divergence times based on robust probabilistic methods from a combined phylogenetic dataset provided an

ideal complementary strategy for dissecting shared divergence patterns. Additionally, in the absence of any other information about lineage divergence, our empirical pooled posterior distributions of divergence times could be use as an *a priori* expectation of divergence time for unstudied species, or even as a Bayesian prior in analyses; these would be especially valuable when calibration points are otherwise scarce.

Advances in estimation and comparison of divergence times, coupled with the growing interest in phylogeographic research, will surely continue to illuminate new understanding of the roles that historical processes have played in generating the planet's biodiversity. We found widespread evidence for a surprisingly high number of lineages showing coordinated divergence, and these divergences often fit previous expectations based on geological and tectonic evidence. In other cases, however, (e.g., Nicaraguan Depression) we found substantial evidence supporting one geological model (dynamic transient land connections) over other models. Overall, our findings are highly encouraging, and strongly implicate the existence of an underlying and unifying model of Middle American biogeography that is tractable to assemble and eventually comprehend. The level of detailed information emerging from comparative phylogeographic studies, augmented with information from the fossil, geological, tectonic, and climatic records, hold great promise for accelerating insight into how biodiversity was established on the planet, and also how it may be shaped by climate change and anthropogenic disturbance.

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CHAPTER 5 – CONCLUSION

Biological diversification in the Neotropics

From explaining geographic distributions to testing specific biogeographic hypotheses, the tropical region from Mexico to northern Argentina has been under intense biogeographic scrutiny for more than 40 years (Jackson et al., 1996; Prance, 1982; Whitmore and Prance, 1987). Although biogeographic studies concluded that the tremendous geological and biological complexity of this region precludes broad generalizations over the entire biota, very distinct biogeographic boundaries have been proposed. The Isthmus of Panama, the Talamanca cordillera in Costa Rica, the Nicaraguan Depression, the Motagua-Polochic Fault in Guatemala and the Isthmus of Tehuantepec are considered the main geographic features that shaped the biodiversity in Middle America (Savage, 1982). Here, I demonstrated how a particular group of organisms (i.e., snakes), are an excellent model to gain insights about the underlying mechanisms that shaped the neotropical biodiversity as a whole. Although snakes represent only one lineage that may respond differentially to climatic and geological changes than other organisms, the analytical approach I developed suggests general processes of Neotropical speciation that can be extended to other groups.

My study highlights several aspects regarding the spatial and temporal diversification of snakes across the entire Neotropical region:

[1] Highland speciation in the tropics is not necessarily related to Pleistocene climatic fluctuations. Instead, I showed how Miocene events (probably orogeny-related events) drove the diversity and present–day distribution of highland pitvipers.

[2] Molecular phylogenetics represents a very powerful tool that, combined with geographic information, can inform evolutionary differentiation in Neotropical species. For example, the phylogeography of *Leptodeira* and *Cerrophidion* illustrate that morphology can be misleading and thus affect further inferences (e.g., ecology, biogeography, conservation).

[3] Evolution of the widespread genus *Leptodeira* occurred in an extended temporal window that began in the middle Miocene. The colonization of the entire Neotropical region appeared to occur in a continuous sequence from north to south, with final colonization of the Amazon basin during the Pleistocene.

[4] The concerted geographic diversification observed in multiple lineages of Neotropical snakes highlights the importance of using a comparative biogeographic approach to identify underlying mechanisms of geographic speciation that can similarly affect multiple organisms with a wide variety of life histories. Under the premise that life and earth evolve together, a comparative approach will illuminate the common mechanisms that shaped present–day biological diversity.

[5] Speciation in Middle America has been particularly intense in the last 15 million years, generating high levels of phylogenetic diversity both in lowland and highland taxa. Although recent climatic fluctuations during the Pleistocene do not appear to be responsible for diversity above the species level, phylogeographic evidence from Middle American snakes indicates that these climatic changes severed gene flow in some populations, increasing sub–regional genetic diversity.

Comparative phylogeography beyond the species level

The single most detailed record of historical events is stored in the phylogeographic structure of extant lineages. In fact, it has been suggested that phylogenetic nodes may be more important in biogeographic studies than the areas where species are distributed (Fattorini, 2008; Hovenkamp, 1997, 2001). Nodes from phylogenetic trees represent cladogenetic events, and, when coupled with geographic information (i.e., current distributions of terminal lineages), can help illustrate the mechanisms responsible for divergence. Thus, the combination of spatial and temporal evidence compared and contrasted over multiple codistributed lineages can provide unparalleled insight into underlying diversification processes.

Congruence in spatial and temporal diversification across multiple lineages is commonly viewed as evidence for shared vicariance (Crisp and Cook, 2007; Nelson and Platnick, 1981; Wiley, 1988; Williams et al., 2008). When multiple lineages are inferred to have diverged at different times across a biogeographic barrier, then alternative hypotheses need to be proposed and evaluated.
The most common cause suggested is dispersal, given its stochastic nature that can generate disparate patterns of lineage divergence. However, when using molecular data to generate and test biogeographic hypotheses, incongruent cladogenetic patterns can arise from factors other than dispersal (Fig. 5.1). For example, errors in phylogenetic reconstruction can lead to spurious cladogenetic events and thus affect inferences regarding such nodes. Also, when dealing with recent phylogeographic patterns, differences in coalescent times among different genetic markers and populations can generate incongruent diversification patterns (Edwards and Beerli, 2000; Hickerson et al., 2006a; Hickerson and Meyer, 2008; Hickerson et al., 2007; Hickerson et al., 2006b). In addition, ecological and behavioral differences among populations in colonizing new areas can

generate incongruent patterns despite the geological or climatic events involved in the divergence occurring in a narrow time period. Lastly, geological events such as barrier formations do not necessarily occur in a constrained period of time and as a consequence can generate a staggered cladogenesis in codistributed biota (see Chapter 4).

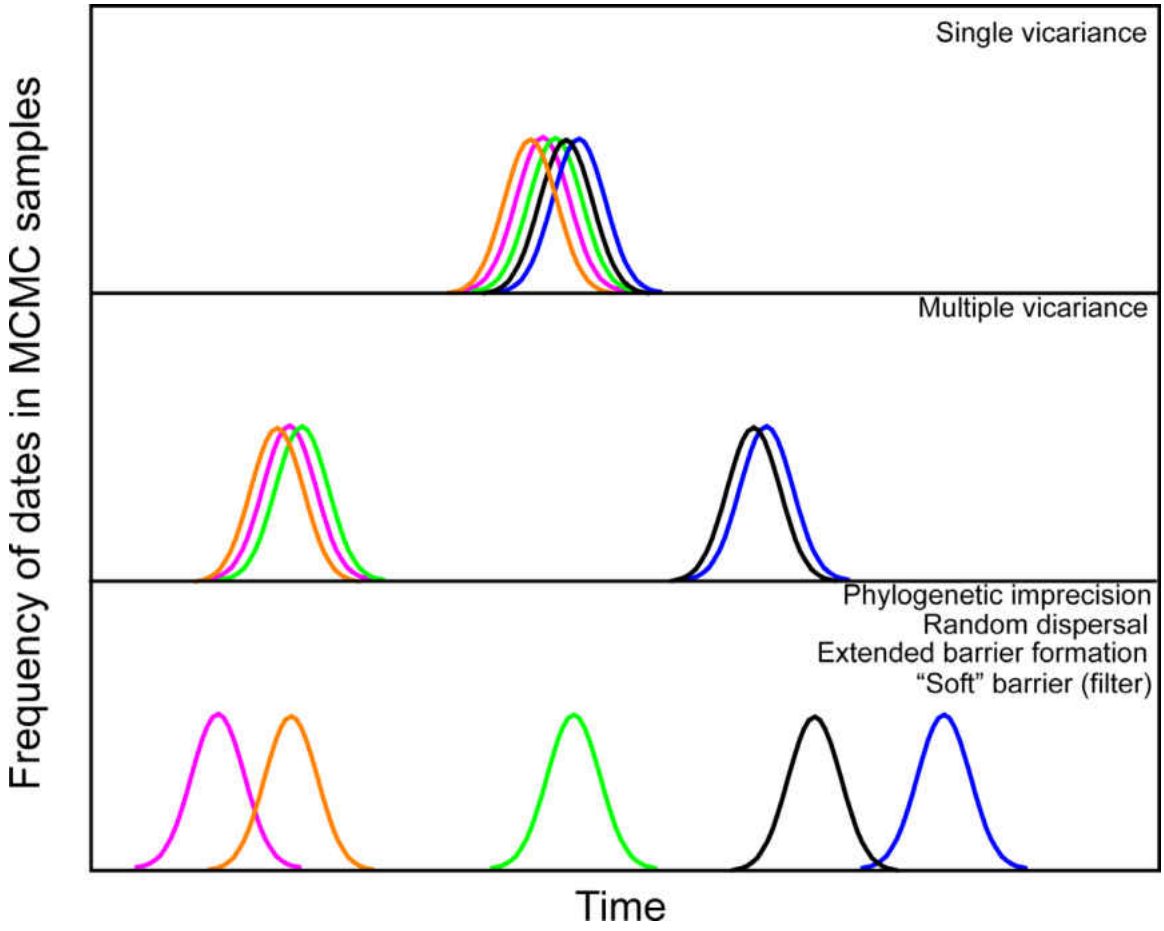

Figure 5.22. Predictions of divergence time distributions and their underlying mechanisms when multiple lineages are incorporated.

When coupled with divergence time estimates, cladogenic events of single taxa are compared with geological or climatic evidence, and a historical process (i.e., vicariance or dispersal) is inferred according to whether the origin of the putative barrier to gene flow (mountain, river, etc.) is known to be younger, contemporaneous or older to the divergence time estimates (Arbogast et al., 2002;

Hunn and Upchurch, 2001; Upchurch and Hunn, 2002). This approach has several problems. First, divergence time estimates are prone to estimation error and if no measure of confidence is given, any inference regarding the historical process that generated such divergence becomes speculative (Graur and Martin, 2004; Lee et al., 2009; Shaul and Graur, 2002). Second, divergence time estimates should be considered minimum ages for divergence and, thus, any geological or climatic event previous to the age estimated with the phylogenetic tree could be responsible for generating such cladogenetic event (Heads, 2005). Third, the fossil record, which is the most common strategy to calibrate phylogenetic trees, is quiet incomplete and its availability is not random across the tree of life (Benton and Donoghue, 2007; Donoghue and Benton, 2007). Identity and placement of a fossil on a tree can be very ambiguous so many phylogeographic studies lack proper calibration points,which affects the accuracy of absolute dates (Lee et al., 2009). Lastly, for very recent evolutionary events, divergence time estimation depends on the degree of ancestral polymorphisms, effective population size and the substitution rate process (Edwards and Beerli, 2000; Hickerson et al., 2003; Ho and Larson, 2006; Ho et al., 2005; Ho et al., 2007).

I circumvented some of these problems by combining a large dataset including several lineages of snakes and determining spatio–temporal congruence across a regional scale. The main conclusions derived from my research regarding estimation of diversification times in biogeographic studies are:

[1] Biogeographic inferences can be made beyond the population level (i.e., species, genus, family) and across a larger regional scale if we combine multiple phylogenetic studies into one single tree and estimate relative divergence times (free of calibration errors).

[2] The final closure of the Isthmus of Panama (a long standing calibration point) does not represent a good proxy for molecular clock calibration given the fairly unpredictable temporal diversification in both marine and terrestrial organism.

[3] Using only phylogenetic information, the spatio–temporal congruence in cladogenesis across multiple lineages represents strong evidence for vicariance. On the other hand, dispersal, the other historical process in biogeography, still relies heavily on external evidence other than the phylogenies (i.e., geological evidence of when a barrier was formed).

[4] Given the nature of Bayesian inference, the use of priors is fundamental to molecular biogeography. Here I demonstrate how the Motagua–Polochic fault formation in Guatemala can be used to calibrate molecular clocks better than the traditional closure of the Isthmus of Panama.

[5] Deductions from comparative phylogeographic analyses coupled with divergence time estimation are particularly important and enlightening for areas with either vague geological or tectonic information, or where little historical consensus is available

Molecular phylogenetics and the future of the Neotropical biodiversity

The field of molecular phylogenetics has shifted from only estimating evolutionary relationships to a broader goal informing many other sub–disciplines in biology (Harvey et al., 1996; Wiens, 2008). Thus, fields as diverse as genetics, biogeography, molecular evolution, development and conservation now benefit from the use of molecular phylogenies. Coupled with robust analytical approaches, phylogenies give insights about the origin, function, and evolution of biological systems whether we refer to genes, organs, populations, species or entire regions.

Robust estimates of what happened biologically and geologically in the past are critical for understanding and interpreting the present, and the prediction of the future to preserve the evolutionary legacy in the highly diverse Neotropical region. For instance, knowing the geographic origin and the time that lineages have been evolving is a good indicator of phylogenetic diversity, which in turn can be used to direct conservation efforts (Grehan, 1993; Moritz et al., 2001; Prance, 2000; Richardson, 2005; Whittaker et al., 2005). Therefore, identifying natural boundaries and the tempo of evolution of Neotropical snakes may be used to identify regions defined by political boundaries with more or less phylogenetic diversity that can be used to evaluate conservation priorities (Fig. 5.2).

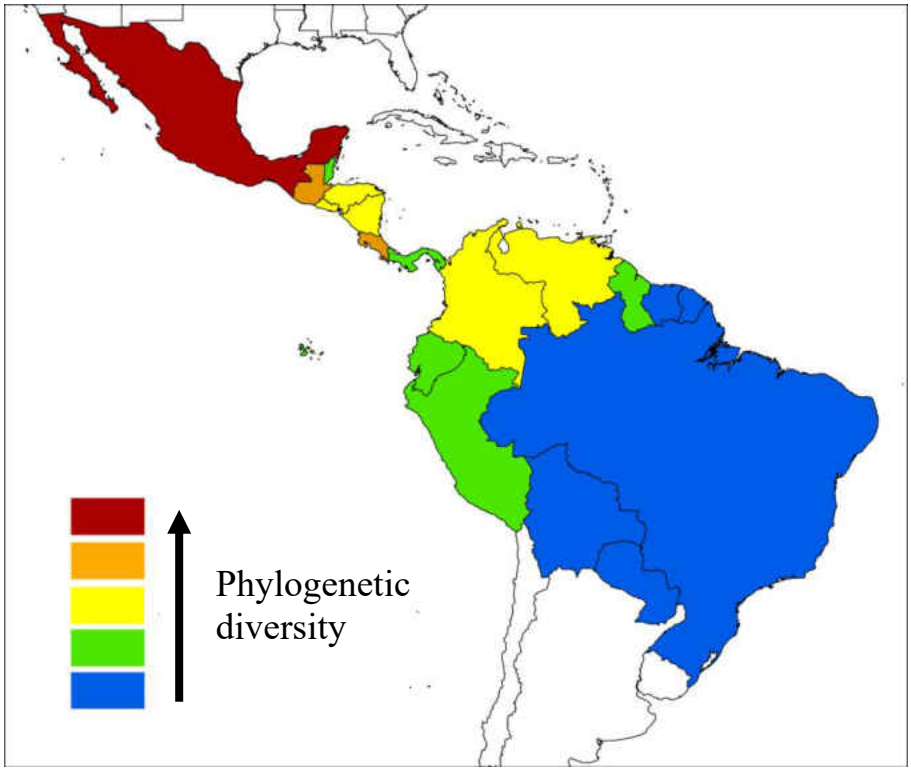

Figure 5.23. Phylogenetic diversity (number of clades) in the genus *Leptodeira* within political boundaries across the entire Neotropical region. The phylogeographic groups were identified during this study (see Chapter 2).

The next decade will see an increase in phylogeographic studies across multiple organisms and throughout the entire Neotropics. Therefore, we need to create robust models such that we can combine independent studies to disentangle historical processes (e.g., dispersal vs. vicariance), and determine the different roles of extrinsic causes vs. intrinsic ones (i.e., orogeny, river formation vs. ecological constraints, movement capability), and methodological difficulties (i.e., deep coalescences, taxonomic error). Accomplishing this will reveal the evolutionary history and its present and future consequences for lineage persistence in this highly diverse biogeographic region.

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