The Pennsylvania State University

The Graduate School

Department of Biology

MOLECULAR PHYLOGENETICS AND BIOGEOGRAPHY OF THREADSNAKES (SERPENTES: LEPTOTYPHLOPIDAE)

A Thesis in

Biology

by

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ABSTRACT

The threadsnakes (family Leptotyphlopidae) are one of the most poorly known families of terrestrial vertebrates. The two included genera, *Leptotyphlops* and *Rhinoleptus* include over one hundred species, which have a primarily West Gondwanan distribution. In this study, DNA sequencing and molecular phylogenetic methods were used to investigate the diversity, evolutionary history, and biogeography within Leptotyphlopidae. Four mitochondrial genes (12S, tRNA-val, 16S, and cytochrome *b*) were sequenced for 92 taxa (2971 sites) and an additional five nuclear genes (AMEL, BDNF, C-*mos*, NT3, RAG1) were sequenced for a subset of 24 taxa (5563 sites).

Phylogenetic reconstructions showed that the genus *Rhinoleptus* is nested within *Leptotyphlops*, and that the family can be divided into two major clades: a mostly New World clade (which includes *Rhinoleptus koniagui* and *Leptotyphlops bicolor*, both African species); and an Old World clade, which is further divided into a Southern African clade, and a mostly West African clade (with the exception of *L. longicaudus*, from South Africa, and *L. blanfordii*, from Yemen).

Taxon sampling included representatives of nearly all morphology-based species groups. Groups determined from molecular data were compared with classical morphological groups, and radiations among major groups were dated. Most New World species groups were supported, but two major Old World species groups will require revision. The divergence time between Old World and New World leptotyphlopids was estimated to be 91–94 million years ago (95% Credibility Interval: 74–119 Ma), suggesting that the split resulted from the break up of West Gondwana.

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Chapter 1

INTRODUCTION

Leptotyphlopidae, a family of blindsnakes of the infraorder Scolecophidia, includes the world's smallest snakes, and is distributed throughout tropical and sub-tropical North and South America, the West Indies, Africa, and southwest Asia (Figure 1-1). The family consists of two genera, *Leptotyphlops* and *Rhinoleptus*, which are both poorly known. The genus *Leptotyphlops* includes more than one hundred species, while *Rhinoleptus* is monotypic. Members of the family Leptotyphlopidae live a fossorial lifestyle, and have greatly reduced features. Most publications on Leptotyphlopidae focus on the descriptions of new species rather than the evolutionary history within the family. Analyses of morphological characters have been used to define species groups (Broadley & Broadley 1999; Broadley & Wallach 1997a; Broadley & Wallach 1997b; Broadley & Wallach 2007; Broadley & Watson 1976; Cei 1986; Cei 1994; Hahn 1978; Hahn & Wallach 1998; Hoogmoed 1977; Klauber 1931; Klauber 1940; Kretzschmar 2006; Miranda 1966; Miranda & Tio Vallejo 1985; Orejas Miranda 1967; Orejas-Miranda 1966; Orejas-Miranda 1969; Peters & Orejas Miranda 1970; Peters & Orejas-Miranda 1970; Taylor 1939; Taylor 1940; Thomas 1965; Thomas et al. 1985) and, in one study, to build species group-level phylogenies (Wallach 1998). However, these species are often misidentified due to their small size and reduced features, and accurate conclusions based on morphological comparisons are difficult to make. As a result, these snakes remain poorly understood in terms of their level of diversity, evolutionary history, and biogeography.

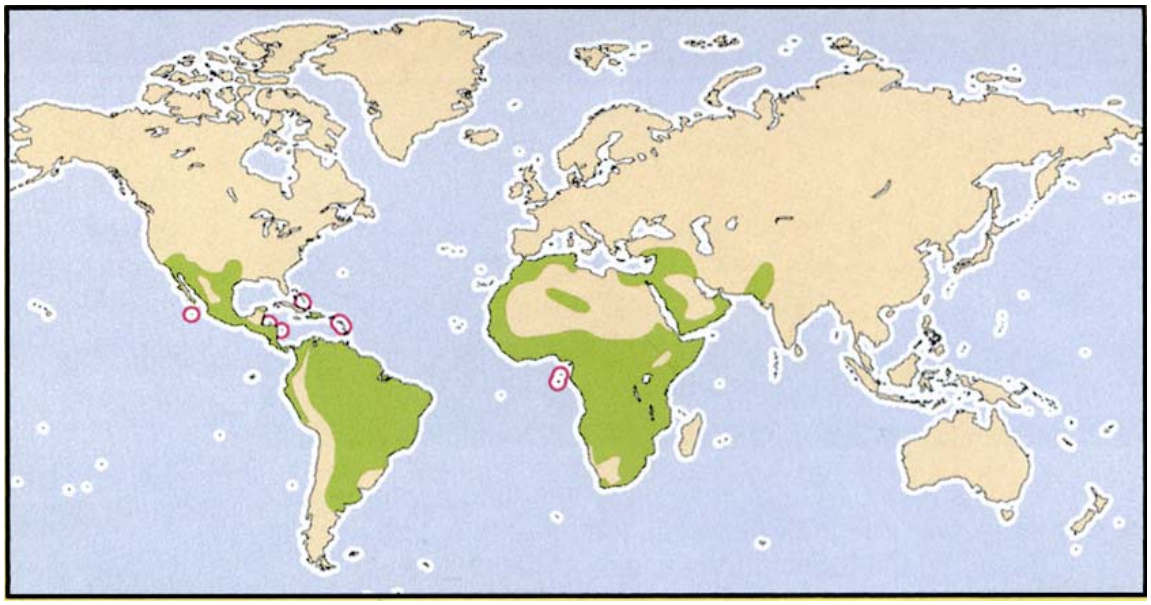


Figure **1-1**: Global distribution of Leptotyphlopidae. Shaded areas indicate the presence of leptotyphlopids in these regions. Source: Kley 2003.

Leptotyphlopidae

Leptotyphlopidae is classified as part of the infraorder Scolecophidia within Serpentes. The scolecophidians (Leptotyphlopidae, Typhlopidae, and Anomalepidae) are generally considered to be monophyletic (Vidal et al. 2009; Wallach 1998). Leptotyphlopids are distinguished from other scolecophidians in having 14 (all but one *Leptotyphlops* species) or 16 (*L. parkeri* and *R. koniagui*) scale rows around the middle of the body, small teeth present only in the lower jaw, and a single anal shield (Cei 1986; Kley 2003). Relatively little is known of the natural history of leptotyphlopids. Most species are thought to eat termites and/or insect larvae (Greene 1997; Webb et al. 2000), and they are generally burrowing animals. Gehlbach (1968) found that *L. dulcis* have defenses against army ant attacks to help them locate and feed on their broods. Another species, *L. humilis* significantly increases the survival of owlets by feeding on parasites in Eastern Screech-Owl nests (Gehlbach & Baldridge 1987). Collectors often encounter

leptotyphlopids under rocks or in leaf litter, or on ground surfaces at night and in wet conditions (Klauber 1940), although some species have been found in trees (Das & Wallach 1998; Gehlbach & Baldridge 1987; Vanzolini 1970).

Though the fossorial lifestyle seems to be common to all leptotyphlopids, these snakes are found in a wide variety of habitats and elevations. Leptotyphlopids are found in arid, desert-like regions, tropical rainforests, wetlands, and savannas. Furthermore, the Californian *L. humilis* is found in Death Valley below sea-level (Kay 1970), while *L. tricolor* and *L. macrops* are found around 3250 meters (m) elevation in the Andes (Zug 1977) and 2285 m elevation in the Kenyan highlands (Broadley & Wallach 2007), respectively. Given their small size and burrowing lifestyle, leptotyphlopids may not seem capable of dispersal over long distances. However, these same traits may make them more likely to be moved over water on floating vegetation (flotsam), and leptotyphlopids have also been known to be able to survive extreme conditions (Baz 1953; Dunn & Saxe 1950). Many leptotyphlopids occur on islands, and dispersal by rafting may have been an important factor in the family's biogeographic history.

Morphological Species Groups

Most publications on leptotyphlopids are descriptions of new species or revisions of other authors' work describing a species. Much confusion still exists about the identity and even validity of certain species. The *L. albifrons* holotype was lost in a fire (Smith & List 1958), and several authors have attempted redescriptions of this species (Orejas Miranda 1967; Orejas-Miranda 1967; Taylor 1940). Another species, *L. parkeri*, was described based on one specimen, which had its skull removed (Broadley 1999), and no other *L. parkeri* specimens have been collected since (Broadley & Wallach 2007). Several authors have attempted revisions of the genus *Leptotyphlops* in various regions (Broadley & Broadley 1999; Broadley & Wallach 1997a;

Broadley & Wallach 1997b; Broadley & Wallach 2007; Broadley & Watson 1976; Hahn 1978; Kretzschmar 2006; Miranda & Tio Vallejo 1985; Orejas-Miranda 1966; Orejas-Miranda 1967). In the course of these analyses, species groups have been defined on the basis of certain morphological characters including scale counts and the arrangement of head scales.

Rhinoleptus koniagui was placed in its own genus within Leptotyphlopidae after some deliberation. This species was first assigned to *Typhlops* in the family Typhlopidae based on its snout shape and body size before it was renamed as a *Leptotyphlops* species because of its 1:1 scale: vertebrae ratio, mid-body scale rows, teeth on the lower mandible, and supralabial scale arrangement (Guibe et al. 1967). Upon reexamination of the holotype (Orejas-Miranda et al. 1970), enough differences were recognized between *koniagui* and all other *Leptotyphlops* to erect a separate genus for this species.

Within the genus *Leptotyphlops*, differences in hemipenial morphology and testes segments between Old World and New World species have been noted (Branch 1986; Wallach 1998). Among New World species, the following main species groups are recognized: *L. dulcis, L. albifrons, L. tesselatus, L. bilineatus, L. septemstriatus*. Orejas-Miranda (1967) described a *L. macrolepis* group and a *L. dimidiatus* group in a review of Amazonian leptotyphlopids, however both species and their allies were more recently included in the *L. dulcis* group (Passos et al. 2006). The *L. dulcis* group includes species that have small supraoculars (generally smaller than frontal and postfrontal), which do not contact the supralabials, and these species may have two or three supralabials (Passos et al. 2006). The *L. macrolepis* group was distinguished by the presence of three supralabials, two being anterior to the ocular (Orejas-Miranda 1967). The same review (Orejas-Miranda 1967) defined the *L. dimidiatus* group by having supraoculars smaller than the frontal and post frontal, and only two supralabials. The *L. albifrons* group is defined by the following characteristics: medium supraoculars, which are larger than the frontal but not in contact with the supralabials; two supralabials; ten rows of scales around the tail; and generally

less than 260 dorsal scales (Freiberg & Orejas-Miranda 1968; Cei 1993; Kretzschmar 2006). Though the characters of the group have been defined several times, Smith and List (1958) pointed out that the original description of *L. albifrons* (Wagler 1824) was not detailed enough to warrant using the name for any new specimens. Species included in the *L. tesselatus* group have supraoculars of normal size, and two supralabials, the anterior of which are in strong contact with the supraoculars (Orejas-Miranda 1967). The *L. tesselatus* group is the only unrepresented New World species group in this study. The *L. bilineatus* group species share a unique trait among leptotyphlopids; they have two subocular, supralabial scales, preventing the ocular scale from contacting the upper lip (Thomas 1965; Thomas et al. 1985). The *L. septemstriatus*, or *Siagonodon* group is defined by the lack of supraoculars (Taylor 1940). This character has been used to establish *Siagonodon* as a distinct genus (Peters 1881; Wallach 1998), though it remains unclear whether these species form a group deserving generic status (Klauber 1931; Kretzschmar 2006; Orejas-Miranda 1967). Table 1-1 summarizes the New World morphological species group classifications, and indicates which species are included in the present study. Note that this table does not include all recognized species, as not all have been assigned to a group, and Kretzschmar (2006) placed *L. weyrauchi* and *L. melanotermus* in the synonymy of *L. albipunctus*. Additionally *L. columbi*, a species included in this study, is not in table 1-1 because of its uncertain relationship with other leptotyphlopids. It has been suggested that *L. columbi* may be more closely related to *L. albifrons* than to *L. dulcis* (Klauber 1939), yet affinities with either the *L. bilineatus* and *L. albifrons* groups have also been disputed (Thomas 1985).

Table **1-1**: Morphology-based New World *Leptotyphlops* species groups. Species included in this study are in bold. *indicates species that may be included in an *L. macrolepis* species group. ** indicates species that display an intermediate condition between *L. albifrons* and *L. tesselatus* species groups.

Species Groups	**Members of Species Groups**			
dulcis	*L. affinis*	*L. anthracinus**	*L. bressoni*	*L. brevissimus**
	L. dimidiatus	***L. dissectus***	*L. dugandi**	***L. dulcis***
	L. fulginosus	***L. humilis***	*L. joshuai**	*L. koppesi**
	L. macrolepis*	*L. maximus*	*L. salgueiroi**	*L. unguirostris*
albifrons	***L. albifrons***	*L. albipunctus*	*L. amazonicus*	*L. australis*
	L. borapeliotes	*L. collaris*	*L. diaplocius***	***L. goudotii***
	L. melanotermus	*L. munoai*	*L. peruvianus*	*L. rufidorsus***
	L. subcrotillus	*L. tenelllus***	*L. vellardi*	*L. weyrauchi*
tesselatus	*L. alfredschmidti*	*L. diaplocius***	*L. rubrolineatus*	*L. rufidorsus***
	L. teaguei	*L. tenellus***	*L. tesselatus*	*L. tricolor*
bilineatus	***L. asbolepis***	*L. bilineatus*	***L. breuili***	*L. calypso*
	L. carlae	***L. leptepileptus***	***L. pyrites***	
septemstriatus	*L. cupinensis*	*L. borrichianus*	*L. brasiliensis*	***L. septemstriatus***

Among Old World species, the following groups have been described within *Leptotyphlops*: *L. longicaudus*, *L. rostratus*, *L. reticulatus*, *L. bicolor*, *L. nigricans*, *L. scutifrons*, and *L. parkeri*. *Leptotyphlops macrorhynchus*, and *L. blanfordii* have been recognized as distinct species groups (Hahn 1978), but in the most recent revision of Old World leptotyphlopids (Broadley & Wallach 2007), *L. macrorhynchus* and *L. blanfordii* were included as members of *L. longicaudus*. By extension their allies (Hahn 1978) are considered to be in the *L. longicaudus* group. Broadley & Wallach (2007) also described *L. reticulatus*, a species that used to be part of the *L. boulengeri* group (Wallach 1996), as a distinct species group that now includes *L. boulengeri* and its former group members.

Species of the *Leptotyphlops longicaudus* group are considered to be the most primitive, with these characters: a discrete frontal shield; small anterior supralabial; moderate supralabial; semilunate cloacal shield (Broadley & Wallach 2007). They also have primitive skull characters such as paired (as opposed to fused) parietal bones, and in eastern members of the group, an

unossified braincase roof (Broadley & Wallach 2007). Broadley & Broadley (1999) suggested that this group may have led to the origin of the *L. rostratus* group in southwest arid regions. The *L. rostratus* group is the dominant group of southwestern Africa, and these species have their frontal and rostral shields fused, and their cloacal shield shape may be semilunate to subtriangular (Broadley & Broadley 1999). *Leptotyphlops reticulatus* group species share the following traits: moderate anterior supralabial; thorn-like or conical terminal spine; robust skull with fused parietal bones; semilunate cloacal shield; and a low number of middorsals (<248) (Broadley & Wallach 2007). Species in the *L. bicolor* group are unique among Old World leptotyphlopids in having three supralabials (Broadley 2004), a trait shared with certain New World species (Orejas-Miranda 1967). *Leptotyphlops bicolor* species also have the following traits in common: twelve midtail scale rows; small anterior supralabials; and a frontal shield larger than the supraoculars (Wallach & Hahn 1997).

Both the *Leptotyphlops nigricans* and *L. scutifrons* groups have enlarged, sub-triangular cloacal shields, and the species usually have fused parietal bones, which are thought to be derived traits (Broadley & Broadley 1999). The *L. nigricans* group species have: a discrete frontal shield, small anterior supralabials; large posterior supralabials, reaching eye level; ten mid-tail scale rows; and a robust skull (Broadley & Wallach 2007). The *L. scutifrons* group is the dominant group of southern Africa, and it is distinguished from other groups by having: a robust skull; a frontal shield fused to the rostral; and variation in the height of the posterior supralabial (Broadley & Wallach 2007). Only one specimen has ever been known of *L. parkeri*, and though its skull has been removed and lost, it appears different enough from all other leptotyphlopids to constitute its own species group. Its characters are considered the most primitive of the genus: an undifferentiated cloacal shield; 16 scale rows around the body; fragmented head shields; and 14 mid-tail scale rows (Broadley & Broadley 1999; Broadley & Wallach 2007). The former two conditions are shared only with one other member of the family, *Rhinoleptus koniagui*; and the

latter condition is shared only with *R. koniagui* and one South American species, *L. cupinensis*, a constituent of the *L. septemstriatus* group. A summary of Old World *Leptotyphlops* species groups can be found in Table 1-2. The table does not include all Old World species, and it represents the most recent consensus on species groups from a review of the literature. Species included in the present study are in bold.

Table **1-2**: Morphology-based Old World *Leptotyphlops* species groups. Species included in this study are in bold.

Species Groups	Members of Species Groups
longicaudus	***L. adleri*** *L. albiventer* ***L. algeriensis*** ***L. blanfordii*** ***L. boueti*** *L. braccianii* *L. burii* *L. cairi* *L. erythraeus* *L. filiformis* *L. ionidesi* ***L. longicaudus*** ***L. macrorhynchus*** *L. macrurus* *L. nursii* *L. tanae* *L. wilsoni* *L. yemenicus*
nigricans	*L. aethiopicus* *L. emini* *L. howelli* *L. jacobseni* ***L. kafubi*** *L. keniensis* *L. macrops* *L. mbanjensis* *L. monticolus* ***L. nigricans*** *L. pembae*
scutifrons	***L. conjunctus*** ***L. distanti*** ***L. incognitus*** *L. latirostris* ***L. nigroterminus*** *L. pungwensis* ***L. scutifrons*** ***L. sylvicolus*** *L. telloi*
rostratus	*L. gracilior* *L. labialis* ***L. occidentalis*** *L. rostratus* *L. latifrons*
bicolor	***L. bicolor*** *L. broadleyi* *L. greenwelli* *L. sundewalli*
reticulatus	*L. boulengeri* *L. drewesi* *L. narirostris* *L. reticulatus*
parkeri	*L. parkeri*

Using a large data set of visceral characters, Wallach (1998) carried out the only comprehensive phylogenetic analysis done until now to determine the relationships among the *Leptotyphlops* species groups.

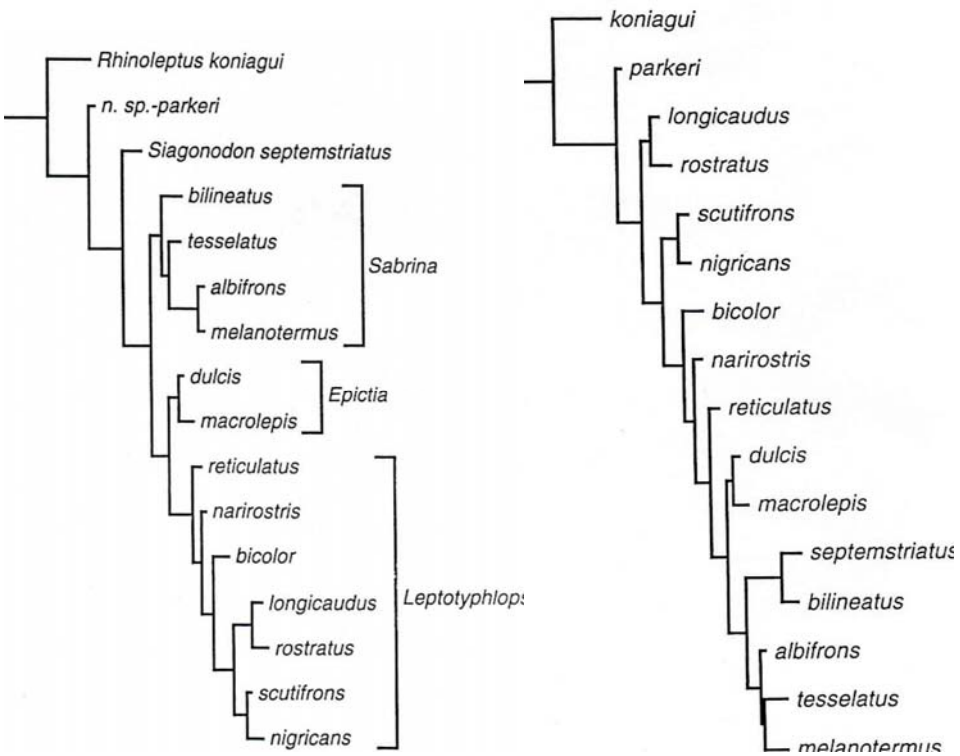


Figure **1-2**: Leptotyphlopidae species group level phylogeny: Preferred (left) and Alternative (right) hypotheses, constructed from visceral character data. Source: Wallach 1998 PhD thesis

Species groups described in the literature were confirmed or revised by Wallach (1998), and once groups were established, a species group level phylogeny was built by him. Figure 1-2 was determined to be the preferred hypothesis for leptotyphlopid phylogenetics. In both reconstructions, *R. koniagui* and *L. parkeri* were the most basal species of the family. The preferred hypothesis (Fig 1-2) placed all New World species groups basal to a monophyletic *Leptotyphlops* Old World clade (excluding *L. parkeri*), while the alternative hypothesis presented a derived, monophyletic New World clade. Generic names were resurrected for certain New World species groups based on the preferred hypothesis. The *L. septemstriatus* group was assigned to *Siagonodon*, while the Caribbean *L. bilineatus* group and the South American *L. tesselatus, L. albifrons,* and *L. melanotermus* were placed in *Sabrina*. *Leptotyphlops melanotermus* has since been recognized as conspecific with *L. albipunctus*, a member of the *L.*

albifrons group (Kretzschmar 2006). The *L. dulcis* and *L. macrolepis* groups were assigned to the genus *Epictia*. All Old World taxa except *L. parkeri* retained the *Leptotyphlops* genus name because they were determined to be monophyletic. Biogeographic hypotheses were presented for the distribution of leptotyphlopids, which will be discussed in Chapter 3 in the context of timing results and their implications for leptotyphlopid biogeography (Wallach 1998).

Visceral character data have been useful in some phylogenetic studies (see list in (Broadley & Wallach 2007); however, morphological characters such as scutellation may be problematic for inferences about evolution. The number of dorsal scales, a typical diagnostic character, may increase with latitude (as summarized in Murphy 1975), creating additional difficulties with understanding evolution through morphology. Phenotypic evolutionary trends described among leptotyphlopids such as increased fusion of head scales (Wallach 2003) appear to be influenced by the degree of fossoriality. Though all leptotyphlopids are most likely burrowing to some extent, there is no evidence to suggest that the most derived or "advanced" forms are the most fossorial. Furthermore, the level of head scale fusion seems more likely to be influenced by ecological factors including the type of soil substrate, temperature, and humidity in a species' range. Therefore, molecular data are used here to investigate the systematics and historical biogeography of Leptotyphlopidae.

Chapter 2

MATERIALS AND METHODS

Taxon Sampling

The sample group was assembled from various collectors, herpetologists, and museums in the Americas and Africa. Appendix A provides a list of the specimens included, their museum vouchers, and the localities where they were collected. Material was contributed from the following museums and collectors: Aaron M. Bauer (AMB), Villanova University; American Museum of Natural History (AMNH); Eric N. Smith (ENS), University of Texas at Arlington; Jonathan A. Campbell (JAC), University of Texas at Arlington; Louisiana State University Museum of Zoology (LSUMZ); (MB); (MBUR); Museum of Comparative Zoology (MCZ), Harvard University; (MSM), University of Texas at Arlington; Museum of Vertebrate Zoology (MVZ), University of California, Berkeley; Port Elizabeth Museum (PEM), South Africa; Royal Ontario Museum (ROM), Canada; S. Blair Hedges (SBH), Pennsylvania State University; Jean-Francois Trape (TR), Senegal; National Museum of Natural History (USNM), Smithsonian Institute.

Field and laboratory research was approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University (#17632). Specimens in the sample group represent both genera, four out of the five recognized New World *Leptotyphlops* species groups, and five out of seven Old World *Leptotyphlops* species groups based on morphology. The South American *L. tesselatus* group and the African *L. reticulatus* and *L. parkeri* groups are the only unrepresented species groups in this study. Tables 1-1 and 1-2 provide summaries of species groups and the species representing each one in this study. All specimen tissue samples were

either frozen in liquid nitrogen or preserved in 75% ethanol and stored in the laboratory at -86°C. DNA was extracted using a Qiagen DNEasy Tissue extraction kit, following a modified version of the manufacturer's protocol.

Data Collection

DNA Extraction

Ten to twenty-five milligram sections of tissue were cut from specimen tissue samples using sterile razor blades. Tissues that had been stored in ethanol were soaked for thirty minutes in distilled, de-ionized water at 4°C. Tissues were placed in 180 microliters of ATL buffer in labeled 1.5mL microcentrifuge tubes. Twenty microliters of Proteinase K were added, and mixtures were vortexed for ten to fifteen seconds. All tubes were then placed in a 56°C water bath until tissues were fully lysed. Mixtures were vortexed again for fifteen seconds. Buffer AL (200 μL) was added, the tubes were vortexed for another ten seconds, and then incubated at 70°C for ten minutes. A 97% ethanol solution (200 μL) was added, and the mixtures were vortexed for ten seconds.

Each mixture was pipetted into DNeasy Mini Spin Columns, which were in 2mL collection tubes. These tubes were centrifuged at 8,000 rpm for one minute, and the collection tubes with flow-through were discarded. Buffer AW1 (500 μL) was added to each spin column, which was placed in a new collection tube, and centrifuged at 8,000 rpm for one minute. Collection tubes and flow-through were discarded. Buffer AW2 (500 μL) was added to each spin column, which was placed in a new collection tube, and centrifuged at 14,000 rpm for three minutes. The flow-through was discarded; spin columns and collection tubes were centrifuged at 14,000 rpm for one minute. Spin columns were placed in labeled 1.5 mL microcentrifuge tubes,

buffer AE (100 μL) was pipetted directly onto the DNeasy membranes, and the tubes were incubated at room temperature for five minutes. Tubes were centrifuged at 8,000 rpm for one minute. Spin columns remained in the same 1.5 mL microcentrifuge tubes, buffer AE (50 μL) was pipetted onto DNeasy membranes, and the tubes were incubated at room temperature for five minutes. Tubes were centrifuged at 8,000 rpm for one minute. Spin columns were discarded, and the products (150 μL) were stored in the labeled 1.5 mL microcentrifuge tubes in the laboratory at 4°C.

DNA Amplification

Polymerase chain reactions (PCR) were carried out to amplify desired genes or gene fragments of the extracted DNA. Mitochondrial 12S and 16S ribosomal RNA genes were amplified for all samples in five to seven overlapping fragments; the region connecting 12S and 16S genes coding for tRNA valine was amplified in the process and included in the sequence alignment of ~2.5kb in total length. Cytochrome b (~830bp) was amplified and sequenced for all samples in either one or two overlapping fragments, depending on the quality of the sample. Preliminary phylogenetic tree construction from mitochondrial DNA sequences identified a number of well-supported monophyletic species groups, but their relations to each other were not all resolved. To determine these higher-level relationships, slower-evolving nuclear genes were amplified and sequenced for representative samples of each species group. *Leptotyphlops albifrons* (*L. albifrons* group); *L. columbi* (uncertain affinities); *L. septemstriatus* (*L. septemstriatus* group); *L. dulcis* (*L. dulcis* group); and *L. pyrites, L. leptepileptus, L. asbolepis, L. sp. nov.* (*L. bilineatus* group) were the sequenced New World group representatives. Among Old World groups, *L. incognitus, L. conjunctus, L. sylvicolus, L. distanti, L. nigroterminus* (*L. scutifrons* group); *L. kafubi* (*L. nigricans* group); *L. occidentalis* (*L. rostratus* group); *L.*

longicaudus, *L. algeriensis*, *L. boueti*, *L. rouxestevae*, *L. blanfordii*, *L. adleri*, *L. macrorhynchus* (*L. longicaudus* group); and *L. bicolor* (*L. bicolor* group) had nuclear genes amplified and sequenced in addition to mitochondrial ones. *Rhinoleptus koniagui* also had both mitochondrial and nuclear gene data collected.

Nuclear genes amplified and sequenced include: recombination activating gene-1, RAG1 (~513 bp); brain-derived neurotrophic factor, BDNF (~670 bp); neurotophin-3, NT3 (~495 bp); oocyte maturation factor Mos, C-mos (~566 bp); and amelogenin, AMEL (~323 bp). All nuclear genes were amplified in one fragment except for RAG1 and NT3, which had to be amplified in two overlapping fragments for several samples. Primers (and their sequences) used for amplification and sequencing are listed in Appendix B. Similar protocols were followed for all mitochondrial and nuclear DNA amplification. Reagent volumes, the use of DMSO, and DNA volume differed between mitochondrial and nuclear protocols. Overall, the main variations were in annealing temperature and the number of cycles used in the polymerase chain reactions. The following is a description of the protocol used for mitochondrial DNA amplification, with nuclear DNA protocol differences in brackets.

A mixture was prepared for each sample consisting of: 20–23μL [30.2μL] water; [10μL DMSO]; 20μL [5μL] dNTP; 5μL [5μL] PCR Buffer; 1μL of each 50μM primer or 2.5μL of each 20μM primer [1μL for both 50μM primers]; and 0.2μL [0.5μL] Taq DNA polymerase. The mixtures were pipetted into labeled PCR tubes and 1μL [5–10μL] extracted DNA was added to each tube. A negative control was always run consisting of all the above reagents except for the extracted DNA. Polymerase chain reactions were run using Genamp® PCR Systems 2400 and 2700 thermocyclers. At the beginning of each run there was a hold at 94ºC for 150 seconds. Thermocyclers then ran samples through 40–48 cycles consisting of: melting at 94ºC for 30 seconds; annealing at 42-53ºC for 45 seconds, and extending at 72ºC for 45 seconds.

Amplified fragments were checked using gel electrophoresis. Gels were prepared by dissolving high-melting agarose powder (0.6g) into TBE solution (50 mL), adding ethidium bromide (3μL), and allowing the mixture to harden in a mold for approximately 20 minutes. When the gel had solidified, PCR products (5μL) mixed with 6x dye (1μL) were loaded into its wells. A 1000-bp DNA ladder (5μL) was loaded in an adjacent well so that fragment lengths could be determined. Electric current (140V) was run through TBE solution surrounding the gel, and amplicons were separated by size as they moved across the gel. Bands were viewed under UV light, and assessed according to brightness, clarity, and size. If bands were faint, smeared, or absent, PCR was repeated for those samples with altered conditions or reagents.

Purification and Sequencing

Sufficiently bright, clear amplicons were purified using a vacuum filtration system with Millipore Multiscreen filters or a gel extraction kit with Millipore Ultrafree-DA gel filters. In general, the highest quality bands were purified via vacuum filtration. Amplicons were pipetted into the wells of the Millipore plate and placed on a vacuum until the filter membrane appeared dry. Distilled water (100 μL) was added to each well, and the plate was placed on the vacuum until filters were dried. The plate was washed with distilled water (100 μL) again, and placed back on the vacuum until the filters were dried. DNA was re-suspended by adding 50 μL distilled water, and putting the plate on a shaker for ten minutes. Each sample was pipetted into a labeled 1.5-mL microcentrifuge tube and stored in the laboratory freezer.

Products with weak or multiple bands were purified with Millipore Ultrafree-DA gel filters. A gel was prepared by melting 1.2g low-melting agarose gel into TAE solution (100mL), adding ethidium bromide (10 μL), and allowing the gel to solidify in a mold for 30 to 40 minutes. Dye (3μL) was added to each amplicon, which were loaded into wells of the prepared gel. At

140V, samples were run across the gel in a solution of TAE. After approximately 30 minutes, the gel was removed from the electrophoresis machine. Bands were viewed under UV light, cut out of the gel, and placed into labeled Millipore Ultrafree-DA gel filter tubes. Samples were filtered in the tubes through centrifugation at 8,000 rpm for 10 minutes. The resulting filtered products were stored in the laboratory freezer.

Ninety-six well plates were prepared in the laboratory and sent to the Penn State Nucleic Acids Facility in University Park, PA for sequencing. Plates were prepared by adding purified template (2 μL) and diluted primer (2 μL at 1μM) to each well. Both strands were sequenced from each fragment on an ABI Hitachi 3730XL DNA Analyzer.

Phylogenetic Analyses

Sequences were downloaded and edited by hand using MEGA 4.0 (Tamura et al. 2007). Fragments and their complements were aligned, and primer sequences were deleted from final sequences. Overlapping sequences were aligned and spliced together to assemble full sequences in instances when multiple fragments of one gene were sequenced for each specimen.

Cytochrome b, tRNA-valine, RAG1, BDNF, NT3, C-mos, and AMEL sequences were aligned using ClustalW (Thompson et al. 1994) in MEGA 4.0 (Tamura et al. 2007). Ribosomal RNA genes 12S and 16S were aligned according to secondary structure using an alignment of squamate sequences from the European ribosomal RNA database (Wuyts et al. 2004) in Muscle (Edgar 2004). To eliminate hypervariable loop regions, these alignment files were run through GBlocks (Castresana 2000) using default parameters with the least stringent settings: 1. Allow smaller final blocks; 2. Allow positions with gaps within the final blocks; 3. Allow less strict flanking positions. Approximately 80% of sequence data could be retained using such settings.

The resulting alignment files were concatenated with $tRNA^{val}$, and treated as one gene of 12S-$tRNA^{val}$-16S in phylogenetic analyses.

Two data sets were used in subsequent analyses and tree-building. The first was a concatenation of mitochondrial data (12S, $tRNA^{val}$, 16S, Cytochrome b) for all 92 taxa (~2,971 bp), which will be referred to as the 'species-rich data set.' The second was a concatenation of mitochondrial data (12S, $tRNA^{val}$, 16S, Cytochrome b) and nuclear data (AMEL, BDNF, C-*mos*, NT3, RAG1) for 24 taxa representing species groups (~5,563 bp), which will be referred to as the 'gene-rich data set.' Four outgroups were included in these alignments and analyses; outgroup taxa included *Ramphotyphlops braminus*, *Boa constrictor*, sample Elapidae (*Naja naja* or *Dendroaspis*), and *Python regius*. Table 2-1 contains information on these outgroup sequences and their sources. *Heloderma suspectum* is included since it was added later for timing analysis.

Table **2-1**: Outgroup, GenBank, and rRNA database sequences and their sources. Taxa 1-7 were outgroups in phylogenetic analyses. *Leptotyphlops humilis* was incorporated in the species-rich data set. *Heloderma suspectum* was added as an additional outgroup in timing analyses. *Plestiodon, Dinodon*, and *Chelonia* sequences were obtained from the European ribosomal RNA database for secondary structure alignments.

Taxon	**Genes**	**Source/ GenBank Accession Number**
Boa constrictor	12S, $tRNA^{val}$, 16S, cytochrome *b*	NC_007398
Boa constrictor	amel, BDNF, C-mos, NT3, RAG1	Vidal et al. 2009
Dendroaspis (Elapidae)	amel, BDNF, C-mos, NT3, RAG1	Vidal et al. 2009
Naja naja (Elapidae)	12S, $tRNA^{val}$, 16S, cytochrome *b*	NC_010225
Python regius	12S, $tRNA^{val}$, 16S, cytochrome *b*	NC_007399
Python regius	amel, BDNF, C-mos, NT3, RAG1	Vidal et al. 2009
Ramphotyphlops	amel, BDNF, C-mos, NT3, RAG1	Vidal et al. 2009
Leptotyphlops humilis	12S, $tRNA^{val}$, 16S, cytochrome *b*	NC_005961
Heloderma suspectum	12S, $tRNA^{val}$, 16S, cytochrome *b*	NC_008776
Heloderma suspectum	amel, BDNF, C-mos, NT3, RAG1	Vidal et al. 2009
Plestiodon egregius	12S, 16S	AB016606
Dinodon semicarinatus	12S, 16S	AB008539
Chelonia mydas	12S, 16S	AB012104

Certain sequence fragments were unable to be amplified or sequenced from particular specimens due to poor sample quality or lack of proper primers. The following specimens were missing specific gene fragments in final alignments: *L. blanfordii* (375bp BDNF, 495bp NT3,

248bp RAG1); *L. algeriensis* (566 C-mos); *L. kafubi* (495 NT3, 513 RAG1); *L. albifrons*-1,2 (424bp cytochrome *b*); *L. goudotii*-4 (274bp 12S). Missing sites comprised 2.3% of the gene-rich data set, and 0.4% of the species-rich data set. When all sites in both data sets were considered together, missing data accounted for 0.97%.

Models of sequence evolution were determined for each gene (Table 3-1) using Modeltest 3.06 (Posada & Crandall 1998) in PAUP4b10 (Swofford 2003), and incorporated in tree-building. Maximum likelihood trees were constructed using RAxML-VI-HPC v2 (Stamatakis 2006) through the San Diego Supercomputing Center, and Bayesian analyses were run using Mr.Bayes v3.2 (Ronquist & Huelsenbeck 2003) in the laboratory. RAxML-VI-HPC uses the general time reversible distance model, which was favored with a gamma parameter for the majority of genes in ModelTest (Table 3-1). Each tree was built from 100 alternative runs under the GTRGAMMA model. Likelihood trees were built from both data sets using a variety of partitions and outgroup combinations to assess the robustness of tree topology. Unpartitioned and partitioned alignments were used. The gene-rich data set was partitioned into seven genes (12S, $tRNA^{val}$, and 16S were treated as one gene), and in another analysis, partitioned into two genes to consider mitochondrial and nuclear DNA separately. Nuclear data were considered alone, and partitioned into five genes. The total sequence alignment was also partitioned into seven genes, and then according to codon position in each. Trees were also built using the seven-gene partition with and without outgroups, and with and without *R. koniagui* and *L. bicolor* to determine the influence of these taxa on bootstrap values. The same methods were applied to the species-rich data set, but gene partitions were done between only the ribosomal RNA and cytochrome *b*. Nodal support for final trees was provided from non-parametric bootstrapping done in 2000 replicates, following (Hedges 1992).

Neighbor-joining trees built with MEGA 4.0 (Tamura et al. 2007) used the most complex distance model available, Tamura-Nei, with a gamma parameter averaged from those calculated

by ModelTest. MEGA trees were built and compared using pairwise and complete deletions for both data sets. Bayesian analyses for both data sets were done using four Markov chains started at random trees that were run for one million generations each, and sampled every 100 generations. The gene-rich data set was partitioned into seven genes, with appropriate models (table 3-1) set for each partition. Another tree was built from an unpartitioned gene-rich data set under the GTR + *I* + Γ model to compare topologies and support values. Bayesian analyses for the species-rich data set were performed on an unpartitioned, two gene partitioned (12S, $tRNA^{val}$, 16S; and cytochrome *b*), and four gene partitioned (12S; $tRNA^{val}$; 16S; cytochrome *b*) alignments, all under the GTR + *I* + Γ model of evolution. A comparison of tree topologies and nodal support from various methods can be referenced in Appendix C.

Divergence Time Estimates

Bayesian time estimates were determined using MultiDivtimeT3 (Thorne & Kishino 2002; Yang & Yoder 2003). Each gene in both data sets was analyzed in PAML 3.14 (Yang 1997) to determine model parameters, and in estbranches (Thorne et al. 1998) to estimate branch lengths. Both programs were run with default parameters, using a forced topology from the favored likelihood trees. Estbranches output files were used in MultiDivTime analyses, which were run using a variety of calibrations, RTTMs, and rtrates. Two *Leptotyphlops* fossils known from the Pleistocene-Holocene boundary (van Devender & Mead 1978; van Devender & Worthington 1977) were far too young to provide useful calibrations. Another outgroup, *Heloderma suspectum*, was added to use a fossil calibration (100–94 million years ago, Ma), constraining the divergence between two outgroups, Boidae and Elapidae, at a minimum of 94 Ma (Rage & Werner 1999). All other calibrations were maximums corresponding to geologic dates when West Indian islands became habitable (rose above sea-level). In both data sets, the

nodes uniting *L. pyrites* and *L. leptepileptus* were constrained at a maximum of 10 Ma for the Hispaniolan south island (Huebeck & Mann 1985), where both species occur. In the species-rich data set, the node joining the two groups of *L. breuili* population specimens was constrained to a maximum of 3 Ma, when northern St. Lucia emerged above sea-level (Maury et al. 1990). Also in the species-rich data set, the node uniting all taxa in the *L. bilineatus* group was constrained at a maximum of 37.2 Ma for the West Indies (Iturralde-Vinent & MacPhee 1999). Analyses were run with RTTMs set at the highest, 159.9 Ma (Vidal et al. 2009), and lowest, 102.3 Ma (Sanders & Lee 2008) estimates for the alethinophidian-scolecophidian divergence. Values for rttmsd, rtrate, rtratesd, brown mean and brownmeansd were set according to the RTTM used. Both data sets had the Markov chain sampled 10,000 times, with 100 cycles between samples, and the first sample taken after 10,000 cycles.

Table **2-2**: Dates used to estimate divergence times in MultiDivTimeT3.

	Date (Ma)	Reference
RTTM: alethinophidian-scolecophidian divergence	159.9	(Vidal et al. 2009)
RTTM: alethinophidian-scolecophidian divergence	102.3	(Sanders & Lee 2008)
Fossil calibration: Boide-Elapidae divergence	94	(Rage & Werner 1999)
Geologic calibration: emergence of West Indies	37.2	(Iturralde-Vinent & MacPhee 1999)
Geologic calibration: emergence of southern island of Hispaniola	10	(Huebeck & Mann 1985)
Geologic calibration: emergence of northern St. Lucia	3	(Maury et al. 1990)

Saturation may be problematic for timing analyses when fast-evolving genes are used (Halanych & Robinson 1999). Therefore, mitochondrial and nuclear genes were tested for saturation by plotting the ratio of transitions/transversions against the corresponding pairwise

differences. The plot for cytochrome *b* indicated that this gene had become saturated. Therefore, cytochrome *b* was excluded from final divergence time estimates on both data sets.

Chapter 3

RESULTS AND DISCUSSION

Phylogenetics

Hypervariable sites identified and removed with G-Blocks (Castresana 2000) left 870 of the original 1,095 and 892 of the original 1,059 aligned 12S sites for the species-rich and gene-rich data sets, respectively. For the 16S alignments, G-Blocks outputs retained 1,212 of 1,488 and 1,219 of 1,421 sites for the species-rich and gene-rich data sets, respectively. The final, 4-gene species-rich alignment contained 2,971 sites, and the 9-gene gene-rich data set consisted of 5,563 total sites. Results obtained from ModelTest are shown below in table 3-1. The general time reversible model was selected for AMEL, BDNF, RAG1, and 12S, $tRNA^{val}$, and 16S. Amelogenin and C-*mos* were the only genes for which the model selected did not include a gamma parameter.

Table **3-1**: Models and parameters determined for each gene using Modeltest

Gene	Model selected	p-invar	gamma	freqA	freqC	freqG	freqT
AMEL	GTR+I	0.3284	equal rates for all sites	0.3216	0.3305	0.128	0.2198
BDNF	GTR+I+Γ	0.457	0.9527	0.2926	0.2106	0.2675	0.2292
C-*mos*	HKY+I	0.4254	equal rates for all sites	0.2747	0.2078	0.232	0.2854
NT3	K80+Γ	0	0.6505	=	=	=	=
RAG1	GTR+Γ	0	0.3392	0.3565	0.1687	0.2245	0.2503
cytochrome *b*	TVM+I+Γ	0.2845	0.5629	0.3886	0.3805	0.0773	0.1536
12S,tRNA,16S	GTR+I+Γ	0.2792	0.7009	0.3997	0.2851	0.1578	0.1574

The maximum likelihood (ML) trees shown in figures 3-1, 3-2 display branch lengths, bootstrap (BS) values and Bayesian posterior probabilities (PP) for corresponding nodes. Trees were built from a variety of partitions using different programs, the results of which can be seen

in Appendix C. Trees from both data sets demonstrate a monophyletic Leptotyphlopidae family, but a paraphyletic *Leptotyphlops*, since *Rhinoleptus koniagui* clusters within all other *Leptotyphlops* taxa (94% BS, 1.0 PP from the gene-rich data set). *Rhinoleptus koniagui* and *L. bicolor* are in a basal position to the rest of the family in a likelihood tree with *Ramphotyphlops* as the only outgroup and in unpartitioned and 2-gene partitioned Bayesian trees constructed from the species-rich data set. A Neighbor-Joining (NJ) tree constructed in MEGA 4.0 from the gene-rich data set also displays this basal placement of *R. koniagui* and *L. bicolor*. However, support values for the clades are not well-supported (<65% BS; <.70 PP). Furthermore, the ML tree from the species-rich data set and the NJ tree form the gene-rich data set both show *L. bicolor* as basal to *R. koniagui*, suggesting that the status of this taxon as a monotypic genus is inappropriate regardless of its relationship to the rest of the family. The topology of the gene-rich data set trees was favored because it included more slowly-evolving nuclear genes, 5,563 sites compared to 2,971, and it was also supported by all but one ML trees built from the species-rich data set.

There is a deep divergence between a mostly New World clade and an Old World clade. Notably, *Rhinoleptus koniagui* and *Leptotyphlops bicolor*, two species that occur in West Africa, cluster within the New World clade, but appear to be basal to all other New World species. The placement of *R. koniagui* and *L. bicolor* in the New World is reasonably supported from the species group alignment (94% BS; 1.0 PP), though without these two taxa, the support for a monophyletic New World group is significant (>95% BS). One reconstruction in a previous study (Wallach 1998) demonstrated a monophyletic New World group, though this was not the preferred phylogeny of the study. The Old and New World clades, excluding *R. koniagui* and *L. bicolor*, are supported by morphology (Branch 1986; Wallach 1998). Only one of these studies (Wallach 1998) considered *R. koniagui* and *L. bicolor* in its sample group, though most of the literature has treated old and New World taxa separately. For the sake of discussion, these two

groups will be referred to as the Old and New World clades, although the New World clade also includes these two Old World species.

A trend evident in both trees is the pattern of diversification; there appears to have been a rapid series of divergences among New World species groups, while speciation in the Old World seems to have been more gradual. These patterns are also reflected in the resolution attained for each clade. The relationships among species groups in the Old World are generally highly supported. The branching order of *L. kafubi* and *L. nigroterminus* clades varied among certain trees, though the majority of analyses placed the *L. kafubi* branch basal to the *L. nigroterminus* one (<95% BS; <.85 PP). Although there is high resolution and support for the relationships among New World species, no consensus is reached on the order of diversification among certain species groups. All trees except for a NJ tree from the gene-rich data set show *L. septemstriatus* branching basally to the *L. albifrons/L. columbi* clade (<95% BS, 1.0 PP), yet the positions of the *L. bilineatus* and *L. dulcis* groups are more variable. All trees built from the species-rich data set place these two in a clade together that is basal to the *L. septemstriatus/L. albifrons* groups, however neither node is significant in ML trees (<80% BS). Species group trees built from unpartitioned data sets or alignments with outgroups removed place the *L. dulcis* group basal to *L. septemstriatus/L. albifrons*, and then the *L. bilineatus* group basal to the rest of these; yet ML trees do not provide significant support for this branching order either (<80% BS). The ML tree built from a partitioned species group data set (Figure 3-1) places the *L. bilineatus* group basal to *L. septemstriatus/L. albifrons* and *L. dulcis* basal to the rest of these, but also lacks significant support. Additional slow-evolving nuclear gene data will be required to resolve the branching order among *L. bilineatus, L. dulcis,* and *L. septemstriatus/L. albifrons* species groups.

Systematics/ Species Groups

The placement of *Rhinoleptus koniagui* and *Leptotyphlops bicolor* in a monophyletic, mostly New World clade must be considered in a future taxonomic revision. Perhaps the primitive traits of *R. koniagui* (Guibe et al. 1967) are plesiomorphic, similar to those of an original proto-leptotyphlopid, which probably resembled a Typhlopidae ancestor. Morphology indicates that *L. parkeri* has similar primitive traits (Broadley & Wallach 2007), so future genetic studies should address the origin of this taxon. The *L. bicolor* group shares a unique trait of three supralabials with *L. macrolepis* and related South American species (Broadley 2004; Orejas-Miranda 1967), perhaps supporting its placement among New World taxa.

A number of genetically-distinct taxa are presented here under the same species name. All taxa are labeled in this study according to identifications made by collectors and museums based on the current state of valid species in the genus. From genetic data alone, it is difficult to determine which are cryptic species or are simply the result of a misidentification. However, particular specimens' localities suggest the revalidation of certain species. *Leptotyphlops magnamaculatus* was described from Isla de Utila, Honduras (Taylor 1940), and later was synonymized with *L. goudotii* (Peters & Orejas-Miranda 1970). The specimen *L. goudotii-7* was collected on Isla de Utila, and is genetically distant from Mexican *L. goudotii* specimens, suggesting that *L. magnamaculatus* is a valid species. The unidentified *L. sp-1* from Jalisco, Mexico in the *L. dulcis* group may be *L. humilis dugesii*, one of the few leptotyphlopids definitively known from Jalisco (Klauber 1940; Taylor 1940). If this is the true identity, then *L. dugesii* should be given full-species status. Other complexes of species exist among *L. goudotii*, *L. macrolepis*, *L. humilis*, *L. occidentalis*, *L. scutifrons*, and *L. nigricans* specimens. Additionally, *L. rouxestevae* specimens do not form a monophyletic group, but it is unknown whether *L. boueti* is conspecific or was misidentified. Similarly, *L. incognitus* and *L. conjunctus*

appear to be almost genetically-identical, but morphological data should be considered before synonymizing these species. A more extensive morphological analysis of these specimens and comparison with literature will be needed to make taxonomic corrections. *Leptotyphlops sp. nov.* is labeled as such because the morphological analysis has been done to confirm it, though this species has yet to be formally described (Hedges and Thomas, in prep).

All New World morphological species groups are supported by genetic data given the sample group. The status of the *L. septemstriatus* group cannot be confirmed, but this species branches independently from other species groups, and the *L. albifrons* group appears to be derived from it (<95% BS, 1.0 PP). Because *L. septemstriatus* is nested within the New World clade, which remains in *Leptotyphlops*, it does not seem fit to erect the genus *Siagonodon* for this taxon and its allies. The species complex identified as *L. goudotii* is allied with *L. albifrons*, and these have been considered conspecific in the past (Roze 1952). *Leptotyphlops columbi* is sister to a *L. goudotii*-related species from Isla de Utila, Honduras (100% BS, 1.0 PP), and part of the *L. albifrons* group (>95% BS, 1.0 PP), as suggested from past observations (Klauber 1940). The monophyly of the *L. dulcis* and *L. bilineatus* species groups is also strongly supported (100% BS, 1.0 PP) in all trees. As proposed (Orejas-Miranda 1967; Wallach 1998), the South American *L. macrolepis* branches independently from North American taxa in the group.

In the Old World, the *Leptotyphlops longicaudus* group (100% BS, 1.0 PP) consists of very deep-branching nodes, and the taxa included in it are consistent with morphological analyses (Broadley & Wallach 2007). As discussed earlier, the *L. boueti* taxon may have been misidentified, but *L. rouxestevae* is clearly a member of the *L. longicaudus* group. *Leptotyphlops longicaudus* seems to be the most basal taxon of the group (95% BS in gene-rich tree, 1.0 PP), which is supported by its morphology (Broadley & Broadley 1999). It seemed difficult to resolve long branches in the group with mitochondrial data alone, and although few nodes had significant support, there was a greater consensus among trees built from the gene-rich data set (see

Appendix C). The *L. longicaudus* group diverges from another clade, which has *L. occidentalis* in a basal position in all reconstructions (100% BS, 1.0 PP). *Leptotyphlops occidentalis* is the only representative from the *L. rostratus* group, but it branches independently from other groups. The *L. rostratus* group is the dominant group of southwest Africa, and it has been suggested (Broadley & Broadley 1999) that it was derived from the *L. longicaudus* group.

The relative positions of *Leptotyphlops kafubi* and *L. nigroterminus* are less resolved (<95% BS), yet both groups are always basal to the *L. scutifrons* and *L. nigricans* complex that occurs in the most derived position in the Old World clade. Genetic data do not support recent reviews (Broadley & Broadley 1999; Broadley & Wallach 2007) that included *L. kafubi* and *L. nigroterminus* in the *L. nigricans* and *L. scutifrons* species groups, respectively. *Leptotyphlops kafubi* appears to form its own group. A specimen identified as *L. scutifrons merkeri* appears closely related to *L. nigroterminus*, however *L. nigroterminus* and this *L. scutifrons merkeri* specimen are distant from all other *L. scutifrons* specimens.

A diagnostic characteristic of both the *L. nigricans* and *L. scutifrons* groups is fused parietals (Broadley & Broadley 1999; Broadley & Wallach 2007), yet *L. kafubi* has paired parietals (Broadley & Broadley 1999; List 1966), which is regarded as a primitive trait. *Leptotyphlops incognitus* also exhibits the paired parietal condition (Broadley & Broadley 1999), and clusters within the *L. scutifrons* group in the trees (>95% BS, 1.0 PP). It is possible, however, that this specimen was misidentified since it is almost identical to *L. conjunctus.* *Leptotyphlops nigroterminus* has a unique skull in having a rhombic postparietal bone stuck between its parietals and supraoccipitals (Broadley & Wallach 2007), yet the parietal condition of *L. s. merkeri* (its close relative in the phylogenetic trees) is unclear. Nonetheless, genetic data seem to support the view (Broadley 2004) that, in terms of morphology, a better way to categorize leptotyphlopid species groups is through analysis of dorsal skull bones. Skull

characters may be less ecologically-influenced and thus less biased than head scutellation for inferring phylogenetics.

The *L. nigricans* taxa appear to be several distinct species, yet they are all nested within a clade currently recognized as part of the *L. scutifrons* group (Broadley & Wallach 2007). Either the *L. nigricans* species group should be redefined to include *L. sylvicolus* and *L. conjunctus/incognitus* without *L. kafubi*, or *L. nigricans* should be incorporated into a *L. scutifrons* group without *L. nigroterminus*.

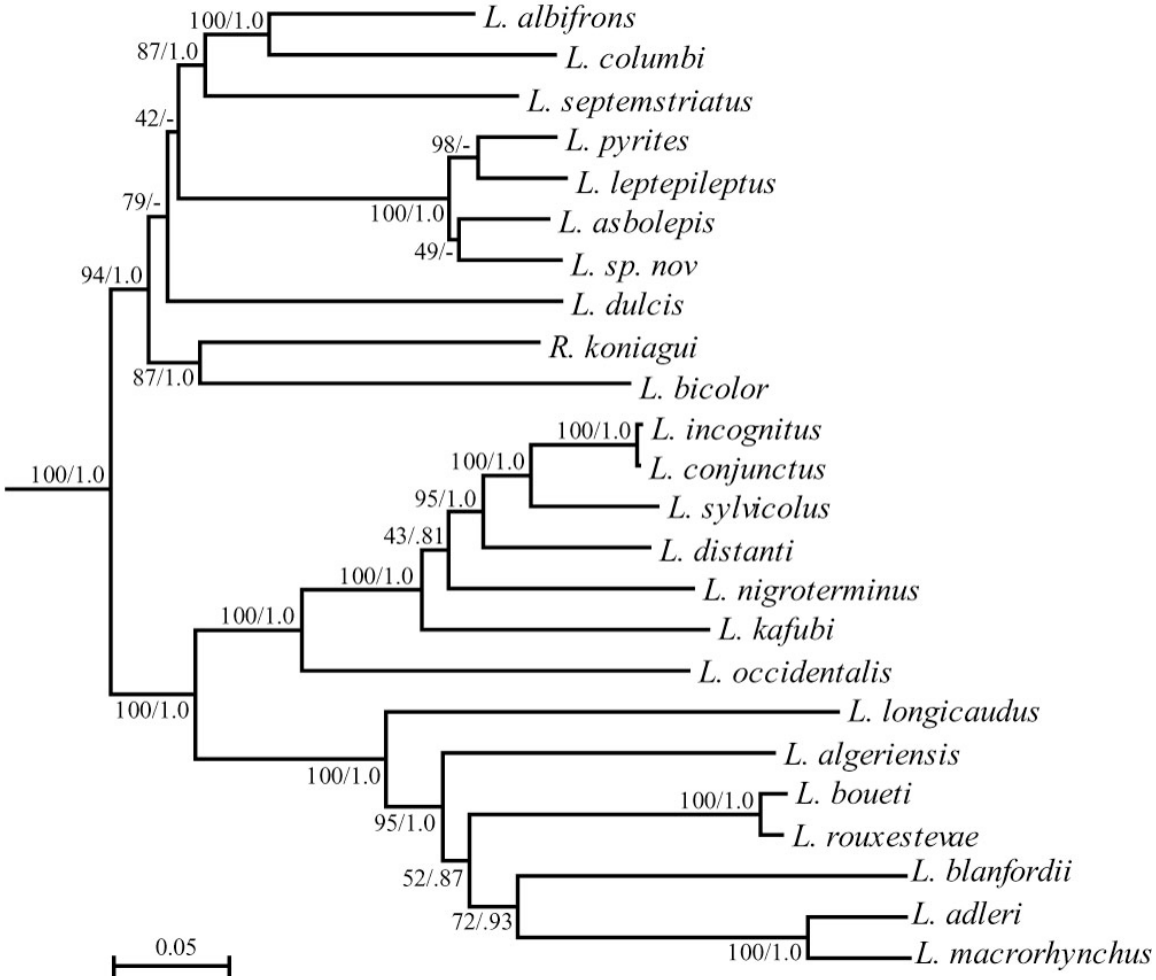


Figure **3-1**: Species group level phylogeny. Maximum likelihood tree with bootstrap support and Bayesian posterior probabilities from gene-rich data set (24 taxa, 5563 sites). Taxa from *L. bicolor* and above are the "New World" clade; from *L. incognitus* and below represent the "Old World" clade. Outgroups not shown: *Ramphotyphlops*, *Python*, Elapidae (*Dendroaspis* and *Naja*)

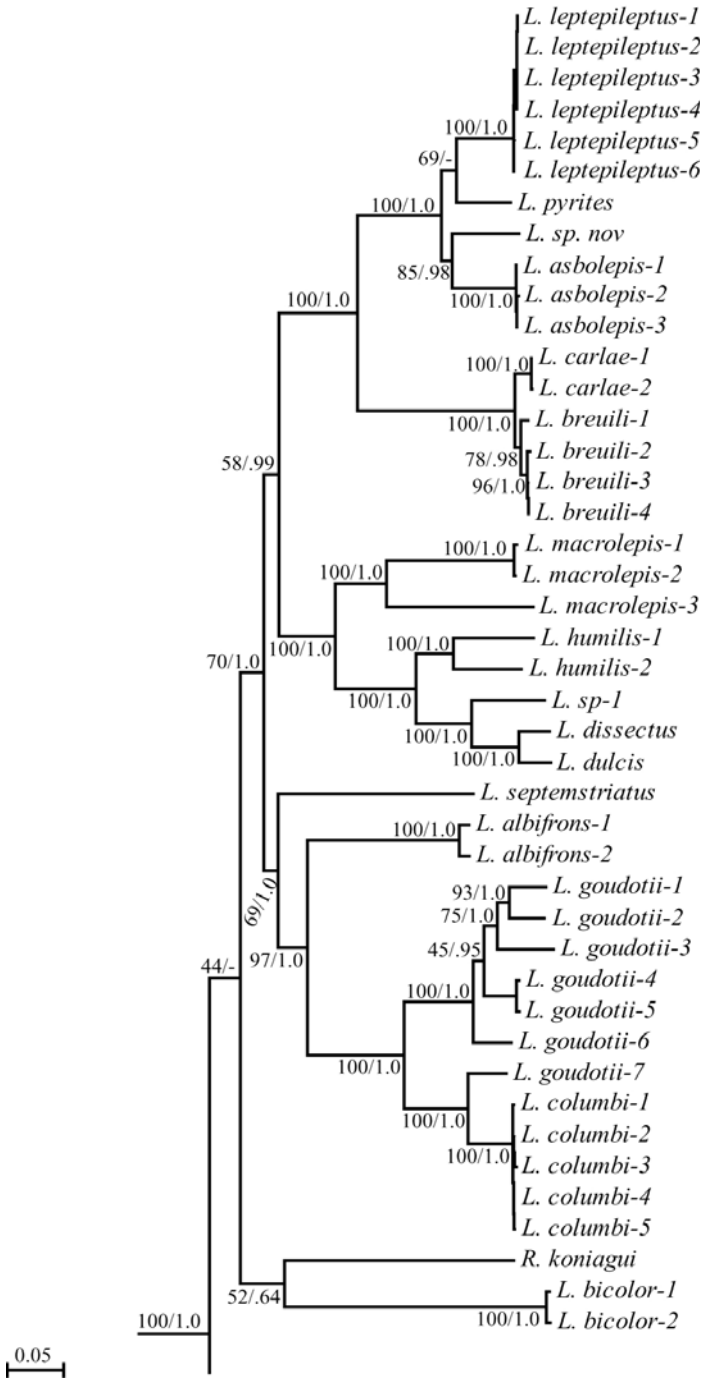


Figure **3-2**: Phylogeny of Leptotyphlopidae: "New World" clade. Maximum likelihood tree with bootstrap support and Bayesian posterior probabilities from species-rich data set (92 taxa, 2971 sites). Outgroups not shown: *Ramphotyphlops*, *Python*, Elapidae (*Dendroaspis* and *Naja*)

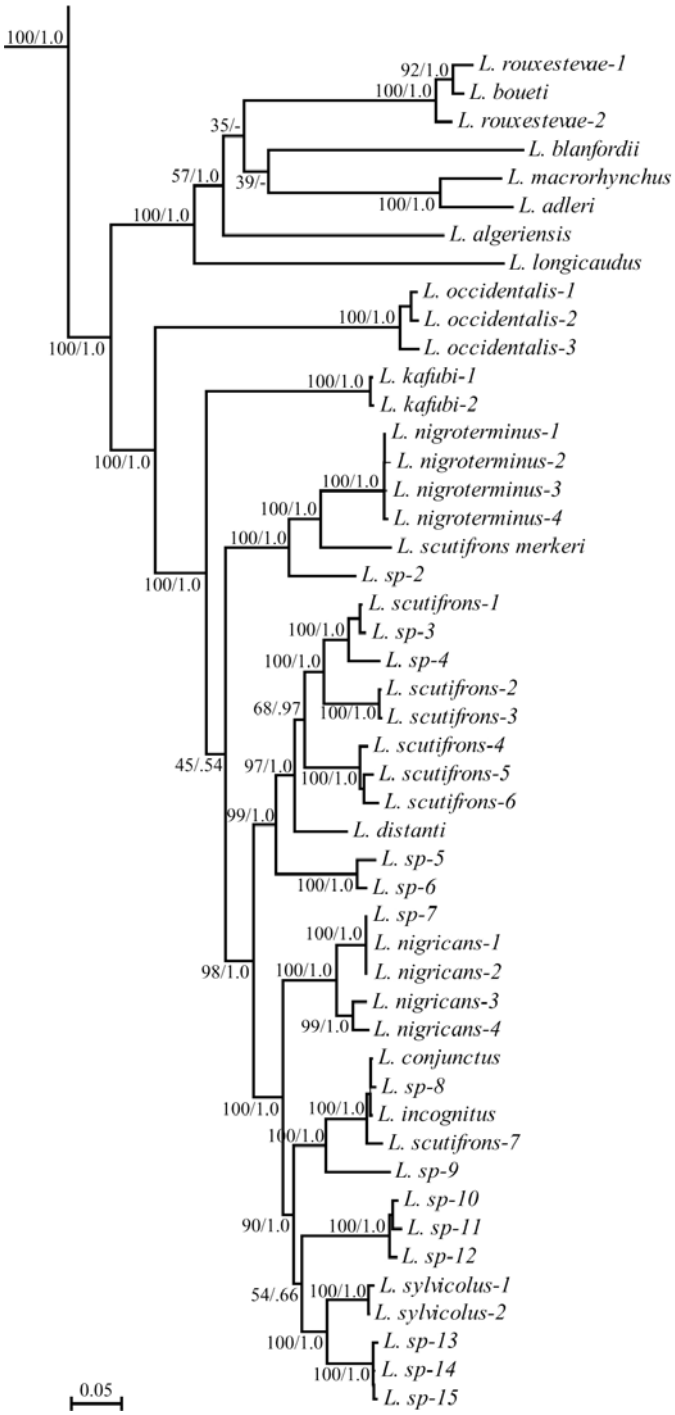


Figure **3-3**: Phylogeny of Leptotyphlopidae: "Old World" clade. Maximum likelihood tree with bootstrap support and Bayesian posterior probabilities from species-rich data set (92 taxa, 2971 sites). Outgroups not shown: *Ramphotyphlops, Python,* Elapidae (*Dendroaspis* and *Naja*)

Biogeography

Results of timing analyses with 159.9 Ma and 102.3 Ma used as RTTMs were not significantly different. Point estimates for most nodes varied by less than two percent. For each data set, timing results from both analyses (RTTM=159.9 Ma and RTTM=102.3 Ma) were averaged, including the upper and lower credibility interval bounds. Tables 3-2 and 3-3 display these averaged results. Time estimates obtained from nuclear data alone are displayed in table 3-2 for comparison, yet the dates discussed here are from the two main data sets, excluding cytochrome *b*: gene-rich (12S, $tRNA^{val}$, 16S, AMEL, BDNF, C-mos, NT3, RAG1) and species rich (12S, $tRNA^{val}$, 16S). Time estimate ranges discussed are point estimates from both data sets and 95% credibility intervals (CI) are the widest ranges of dates when CIs of both data sets are considered together.

According to timing from both data sets, Leptotyphlopidae diverged from Typhlopidae at around 133–146 Ma (CI: 109–169 Ma), which is supported by a recent study (Vidal et al. 2009) that placed this divergence at 151.9 Ma, though one of the same calibration points was used (Rage & Werner 1999). Unlike Typhlopidae, Leptotyphlopidae is confined to a West Gondwanan distribution, and West Gondwana rifted from East Gondwana between 166–116 Ma (Ali & Aitchison 2008). Leptotyphlopidae may have been derived from Typhlopidae subsequent to the isolation of West Gondwana.

The breakup of West Gondwana began around 133 Ma (Ogg et al. 2004), and continued until South America and Africa were completely separated at about 100 Ma (Pitman III et al. 1993). The first major split within Leptotyphlopidae, between the mainly Old and New World clades, occurred at around 91–94 Ma, and the credibility intervals (74–119 Ma) span the 100 Ma

date for the isolation of South America from Africa. Since this date is not significantly different from the time when West Gondwana split, vicariance cannot be rejected. Therefore, an early leptotyphlopid living throughout West Gondwana may have led to two distinct lineages as a result of the tectonic divide. Recent molecular studies have found vicariance due to the rifting between South America and Africa to be the cause of major divergences among placental mammals (Murphy et al. 2007), alethinophidian vs. aniliid/tropidopiid snakes (Vidal et al. 2007), and lungfish (Heinicke & Hedges 2009). Under this scenario, the earliest South American leptotyphlopid gave rise to an ancestor of *R. koniagui* and *L. bicolor*, which diverged from the remaining New World taxa at around 77–79 Ma (CI: 60–101). This ancestral species could have rafted back to West Africa, which was not yet widely separated from Eastern Brazil, and would be congruent with modern ranges of *R. koniagui* and *L. bicolor*. By 68–71 Ma (CI: 49–93), a speciation event in West Africa led to the origins of *R. koniagui* and *L. bicolor*.

Because the two major leptotyphlopid clades are not exclusively Old and New World clades, alternative explanations are also considered. If the divergence at node 4 (figs. 3-4, 3-5, 3-6) occurred on Africa rather than as a result of continental vicariance, the New World clade (excluding *R. koniagui* and *L. bicolor*) could have arisen from a trans-Atlantic dispersal event from Africa to South America around 77–79 Ma (CI: 60–101). Since *R. koniagui* and *L. bicolor* occur in West Africa, it is possible that an ancestral species from the same region could have floated to South America. This scenario, however, depends upon an ancient speciation event on Africa around 92–93 Ma (CI: 77–109 Ma). Given the date of this first major leptotyphlopid divergence and the large-scale tectonic activity at that time, the vicariance and subsequent dispersal of an ancestral *R. koniagui* / *L. bicolor* species back to West Africa is the most parsimonious explanation. Furthermore, any scenario other than the two described above would require at least two trans-Atlantic dispersal events.

The earliest divergence in the Old World, between the *Leptotyphlops longicaudus* group and all other African leptotyphlopids, occurred at 79–84 Ma (CI: 64–109). Notably all species that cluster within this group are from West Africa, except for *L. longicaudus* from South Africa, and *L. blanfordii* from Yemen. This may be a sampling bias, as the *L. longicaudus* group is often labeled as North African. However, West Africa became isolated from the rest of Sub-Saharan Africa beginning at the Cenomanian-Turonian boundary by a shallow seaway that connected the south Atlantic Ocean to the eastern Tethys Sea (Russell & Paesler 2003) through the Benue Trough (Maisey 2000). During this time sea-levels were, on average, 250m higher than they are today, and up to 40% of most continents was under water (Hallam 1992). The early divergence of this group may be explained by this seaway, which could have been an effective barrier between populations. Alternatively, this seaway may have isolated the *R. koniagui / L. bicolor* ancestor before its dispersal to South America.

The modern range of *Leptotyphlops longicaudus* extends from South Africa to Kenya and Somalia (Broadley & Watson 1976). *Leptotyphlops longicaudus* diverged from related species at 47–59 Ma (CI: 37–84), so perhaps this species dispersed south after West Africa became connected to the rest of the continent just after the Cretaceous–Paleogene boundary (Scotese 2002) and was out-competed in its original West African range. The divergence between *L. blanfordii* and other *L. longicaudus* group members is 35–44 Ma (CI: 26–67). An ancestral *L. blanfordii* may have dispersed northward after the epicontinental seaway disappeared and once Africa and southwest Asia were linked, which may have been as early as the Middle Eocene (Gheerbrant & Rage 2006). Wallach (1998) hypothesized a northward dispersal within this group before the Miocene. With lower sea levels and more emergent land area, early forms of North African and southwest Asian *L. longicaudus* group members could have dispersed from West Africa in the late Eocene and Oligocene.

Most members of the recently-described *Leptotyphlops nigricans* group (Broadley & Wallach 2007) occur in central to southern Africa, and many occupy high elevation habitats. It has been suggested (Wallach 1998) that species in this group represent isolated relict populations of an early species distributed on former Mesozoic cratons. While this may be true in parts of the range, the earliest divergences within both the *L. nigricans* and *L. scutifrons* groups (as defined in Broadley & Wallach 2007) are between northern and southern forms. *Leptotyphlops kafubi* (previously in the *L. nigricans* group) from the Democratic Republic of the Congo is more divergent from *L. nigricans* in South Africa than *L. nigricans* is from South African *L. scutifrons*. Also in a basal position to the *L. nigricans/L. scutifrons* species complexes are *L. nigroterminus, L. scutifrons merkeri* (members of *L. scutifrons* group) and *L. sp* from central and eastern Africa. The more northern *L. kafubi* and *L. nigroterminus* groups may represent old forms from the ancient Eastern Arc mountain chain distinct from the southern center (Kingdon 1989), perhaps separated from *L. nigricans* and *L. scutifrons* by the Limpopo River in Mozambique. The Limpopo and other ancient drainage systems have flowed from west to east, emptying into the Indian Ocean through Mozambique since the break up of Gondwana (Moore & Larkin 2001). Other species in central Africa and along the eastern arc may cluster with *L. kafubi* or *L. nigroterminus* instead of with *L. scutifrons* or *L. nigricans*. The intergradation between *L. scutifrons merkeri* and *L. scutifrons scutifrons* in southeastern Tanzania (Broadley & Wallach 2007) may represent a secondary contact zone following range expansion or erosion of the eastern and southern mountains in the species' ranges.

A radiation in southern Africa (after isolation from more northern central African and Eastern Arc species) seems to have produced the derived *L. scutifrons*/*L. nigricans* complex. Taxa in this complex seem to be ecologically differentiated and spread across South Africa, Swaziland, Mozambique, and parts of southern Bostwana and Zimbabwe. Coastal forests, fynbos, and grasslands are the main habitat for *L. nigricans*, while *L. scutifrons* occurs mostly in eastern

savannas (Broadley & Broadley 1999), but both are distributed across west, central, and eastern South Africa. Woodland species include *L. incognitus*, whose range extends from northeastern South Africa to Zambia and Malawi; *L. sylvicolus* in South Africa's eastern coastal forests; and *L. distanti* in northeastern South Africa (Broadley & Broadley 1999). Studies of herpetofauna along the Eastern Arc and southern Africa have discovered significant species turnover from northern to southern regions and lowland to highland habitats (Poynton 1996; Poynton et al. 2007). Sampling along altitudinal gradients and genetic comparisons between Eastern Arc and South African leptotyphlopids could provide more insight into the origins and direction of dispersal between these two groups.

The most derived forms occur in South Africa, indicating that either this region has been colonized independently at least three times (*L. longicaudus, L. occidentalis, L. nigricans/scutifrons*), or there have been several major dispersal events from South Africa (other *L. longicaudus* group taxa, and the *L. kafubi* and *L. nigroterminus* clades). There has been a lot of recent leptotyphlopid speciation in South Africa, which may not be unique to this region because the majority of the African samples in this study are from South Africa. Nonetheless, southern Africa also has been a center for recent speciation in other groups of organisms because of its various altitudinal habitats and north-south barriers (Clancey 1994; Poynton 1996). Eastern South Africa has a wide variety of forest, alpine, and savanna habitats, which have expanded and contracted during glaciation events, causing increased speciation (Lawes 1990; Lawes et al. 2007).

In the New World clade, a common ancestor of *R. koniagui* and *L. bicolor* diverged from all other New World species around 77–79 Ma (CI: 60–101), and a period of rapid diversification among other New World groups occurred from about 63–71 Ma. Within this time frame, the *L. bilineatus* group branched off, and *L. septemstriatus*, a species that occurs on the tepuis in Brazil, diverged from the *L. albifrons* group. Point estimates and credibility intervals spanning the K-Pg

boundary suggest that the Chicxulub impact and the mass extinction that followed may have promoted speciation among leptotyphlopids. Radiations following the K-Pg boundary have been identified using molecular data among other taxa such as amphibians (Roelants et al. 2007), xenarthran mammals (Delsuc et al. 2004), marsupial mammals (Nilsson et al. 2003), alcid birds (Pereira & Baker 2008), and beetles (McKenna & Farrell 2006). Without dinosaurs dominating the landscape, predation may have been less of a pressure for Leptotyphlopidae; although many birds, mammals, and other reptiles survived the impact and probably also fed on leptotyphlopids. Insect radiations at the time among ants and other prey species (McKenna & Farrell 2006; Moreau et al. 2006) also may have promoted leptotyphlopid speciation.

The majority of species group-level New World divergence times are close to the K-Pg boundary, and Old World speciation seems to have been less frequent at this time. This may be an artifact of the sample group, and increased Old World sampling from more regions may show a pattern that mirrors the New World topology; however, the majority of Old World species groups are represented here. It is also possible that the impact on the Yucatan Peninsula had greater influence on regional New World taxa. Of the taxa that also underwent radiations listed above, amphibians, xenarthran mammals, and beetles all occupied South America during/after the K-Pg boundary (Roelants et al. 2007).

As previously suggested (Thomas et al. 1985), the monophyletic *L. bilineatus* group seems to have originated from one dispersal to the West Indies. The Caribbean islands had not been permanently established when the group split from others (63–71 Ma; CI: 49–85), suggesting that this speciation event was continental. The origins of the group before Caribbean dispersal, however, cannot be determined without better resolution at the species group level. Timing indicates that speciation on the Greater and Lesser Antilles began around 34 Ma, yet this node was already constrained at a maximum of 37.2 Ma (Iturralde-Vinent & MacPhee 1999). The divergence within the *L. bilineatus* group indicates separate radiations on Hispaniola and the

Lesser Antilles. The clade including *L. carlae* and *L. breuili,* which are endemic to Barbados and St. Lucia, respectively, represents the Lesser Antillean radiation (3 Ma; CI: 2–5). This date seems slightly too old since Barbados is thought to have emerged only 1 Ma (Speed 1994). However, a species not included in the sample group, *L. bilineatus*, occurs on Martinique, and this three million year old divergence may represent the split between *L. breuili* and *L. bilineatus* if *L. carlae* is derived from *L. bilineatus*. It is unclear whether *L. bilineatus* is more closely related to *L. breuili* or to *L. carlae* (Hedges 2008*)*. Speciation on Hispaniola seems to have begun between 12–13 Ma (CI: 9–16), though the divergence between *L. pyrites* and *L. leptepileptus* was constrained at a maximum of 10 Ma (Huebeck & Mann 1985).

Leptotyphlops columbi presents another interesting biogeographical relationship; it is endemic to San Salvador, Bahamas, yet its sister species is *L. magnamaculatus* (a relative of *L. goudotii*) from off the coast of Honduras. Leptotyphlopids do not occur in the southeastern United States, so *L. columbi* likely originated from a mainland Central American leptotyphlopid related to the Isla de Utila *L. goudotii* (*L. magnamaculatus*). There would be greater odds for dispersal from a larger, mainland population. Ocean currents in the Caribbean flow north along the eastern coast of Central America, turn east above Cuba, and continue past the southern tip of Florida (Hedges 2006); this current could have provided the route for rafting Central or South American organisms to reach the Bahamas. The sample group does not include mainland leptotyphlopids from Honduras, but such a specimen may be even more similar to *L. columbi*.

Both the *L. albifrons* group and the *L. dulcis* group show deep divergences between North and South American constituents (50–57 Ma; CI: 38–78), indicating two independent dispersal events from South America to North America. One dispersal, within the *L. albifrons* group occurred around 50–57 Ma (CI: 40–78). The basal *L. albifrons* is found in northern South America, and the derived members of the group, from the *L. goudotii* species complex, were collected from localities throughout Mexico and northern Guatemala. The second South-North

American dispersal was in the *L. dulcis* group. The *L. macrolepis* taxa in the group are restricted to South America, but the more derived species have increasingly northern ranges. It was hypothesized that dispersal to Mexico and the southwestern United States from South America occurred since the Pliocene (Wallach 1998), yet timing from these molecular clocks suggest a much older dispersal event. This dispersal also appears to have been at around 50 Ma (CI: 38–67), however there is no evidence of a continuous land bridge between South and North America at this time (Hedges 2006), so both dispersals likely occurred at least partially over water.

Alternatively, these divergences (50–57 Ma; CI: 38–78) may indicate initial speciation events prior to dispersal, as in the split of the *L. bilineatus* group from other species groups. Periods of uplift and deformation of the Andes mountains occurred from ~53–40 Ma, which influenced mammalian speciation in the area (Delsuc et al. 2004; Marshall & Sempere 1993). Since divergences in both groups happened at similar times, perhaps barriers created in the Northern Andes influenced leptotyphlopid speciation in the *L. dulcis* and *L. albifrons* groups during the Eocene, and dispersal occurred later. The delayed dispersal scenario is more plausible in the *L. albifrons* group since northern South American forms remain very similar morphologically, and have even been considered the same species as North American forms (Dunn & Saxe 1950; Roze 1952); the type specimen for *L. goudotii* (specimens in this study mostly from Mexico) is from Colombia. The divergence within the *L. dulcis* group is well defined from morphology, as South American group members have distinguishing characteristics (Orejas-Miranda 1967; Wallach 1998), thus North and South American taxa have probably been isolated longer.

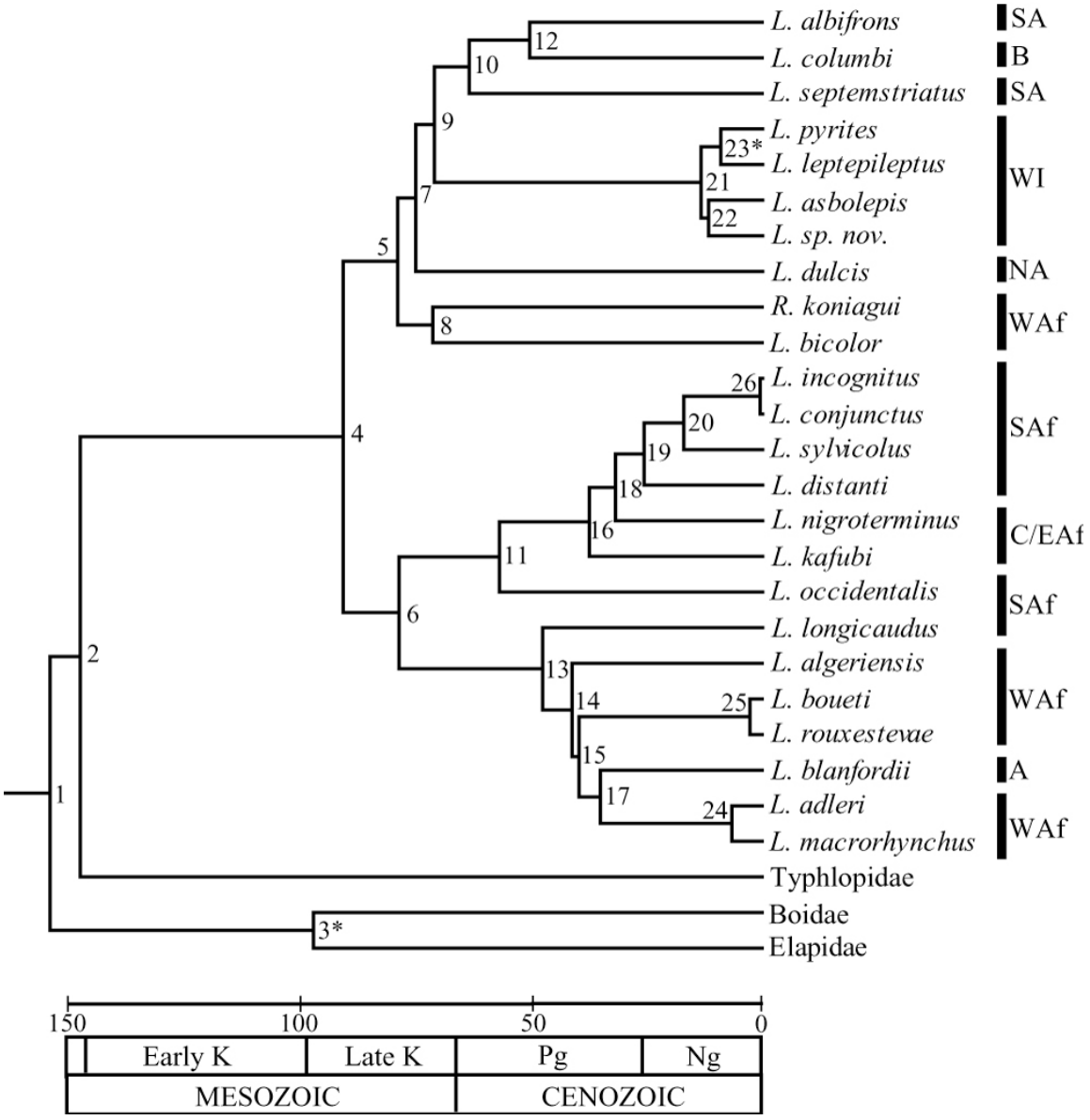


Figure **3-4**: Time tree of gene-rich data set. Asterisks indicate nodes calibrated with fossil or geologic dates. Abbreviations are as follows: SA-South America; B-Bahamas; WI-West Indies; NA-North America; WAf-West Africa; SAf-South Africa; C/EAf- Central/East Africa; A-Arabia. Time scale is in millions of years before present. Outgroup not shown: *Heloderma suspectum.*

Table **3-2**: Times (in millions of years ago) from two analyses of the gene-rich data set, excluding cytochrome *b*. Point estimates and Credibility Interval bounds have been averaged from analyses using 159.9 and 102.3 as RTTMs. Nodes correspond to those on the time tree in fig. 3-4. CI represents the 95% Credibility Interval.

GR	All genes except cytochrome *b*			Five nuclear genes		
Node	Time	CI		Time	CI	
1	**152.7**	135 -	175	**189.5**	146 -	265
2	**146.2**	128 -	169	**180.4**	137 -	253
3	**97.8**	94 -	108	**112.4**	94 -	158
4	**90.5**	76 -	107	**95.0**	67 -	138
5	**78.9**	66 -	94	**84.7**	59 -	125
6	**78.5**	64 -	94	**73.4**	50 -	109
7	**75.0**	62 -	89	**78.2**	54 -	116
8	**71.3**	58 -	87	**78.3**	54 -	116
9	**71.0**	59 -	85	**71.5**	48 -	108
10	**63.5**	51 -	77	**62.7**	41 -	96
11	**57.0**	45 -	70	**52.6**	34 -	81
12	**50.4**	40 -	62	**45.4**	28 -	72
13	**47.4**	37 -	59	**41.6**	26 -	65
14	**41.1**	32 -	52	**35.6**	22 -	56
15	**39.5**	30 -	50	**32.5**	20 -	51
16	**37.6**	29 -	48	**32.8**	19 -	53
17	**34.9**	26 -	45	**28.1**	16 -	45
18	**31.9**	24 -	41	**26.2**	15 -	43
19	**25.8**	19 -	34	**21.2**	12 -	36
20	**17.2**	12 -	23	**14.2**	7 -	25
21	**13.3**	10 -	16	**9.3**	4 -	16
22	**11.6**	9 -	15	**6.6**	1 -	13
23	**9.1**	7 -	10	**5.9**	1 -	10
24	**6.5**	4 -	9	**4.2**	0 -	10
25	**2.6**	2 -	4	**2.2**	0 -	6
26	**0.5**	0 -	1	**2.8**	0 -	8

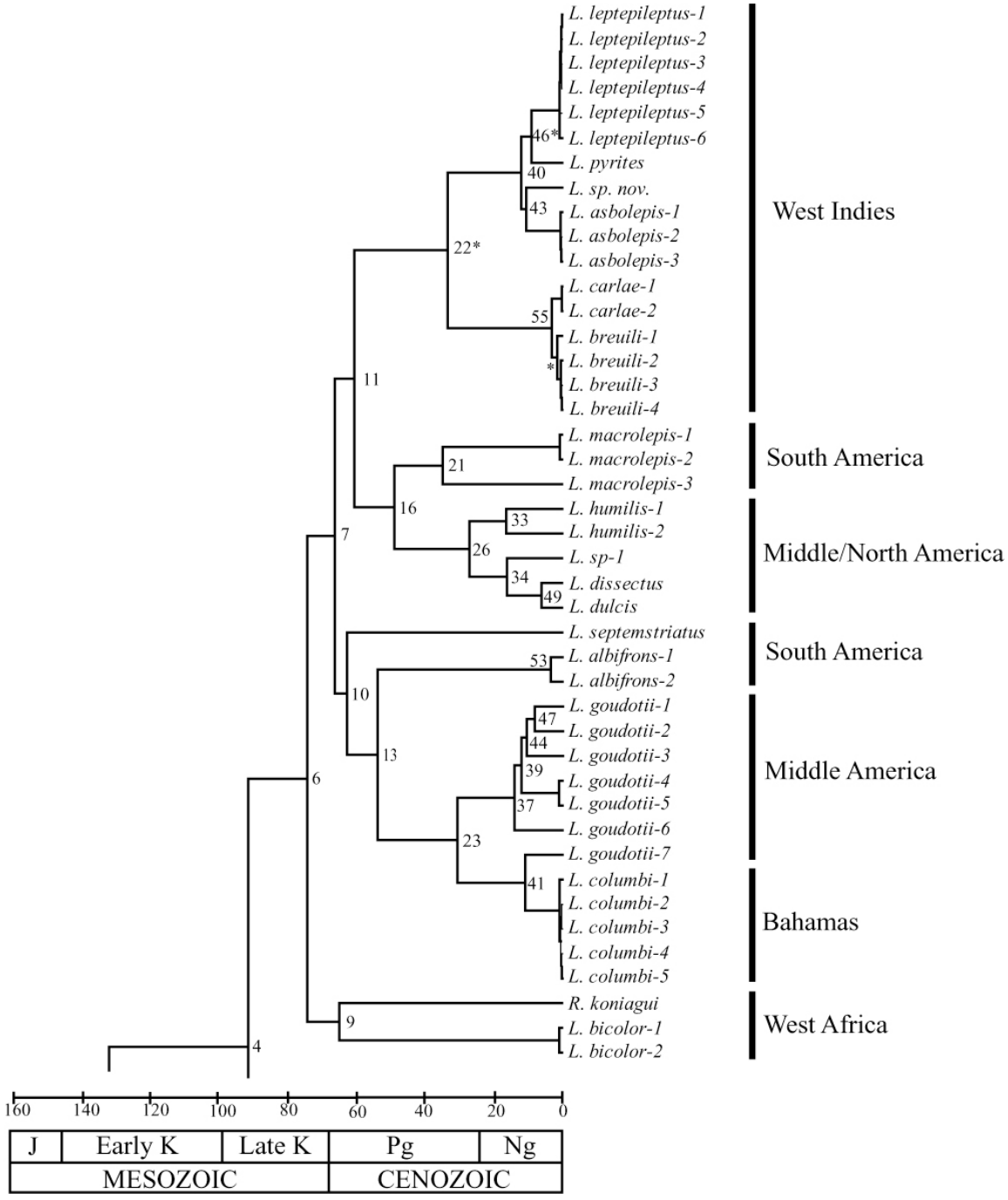


Figure **3-5**: Time tree ("New World" clade) from species-rich data set. This clade includes New World taxa + *R. koniagui* and *L. bicolor* (of West Africa). Asterisks indicate nodes calibrated with geologic dates. Timescale is in millions of years before present. Outgroup not shown: *Heloderma suspectum.*

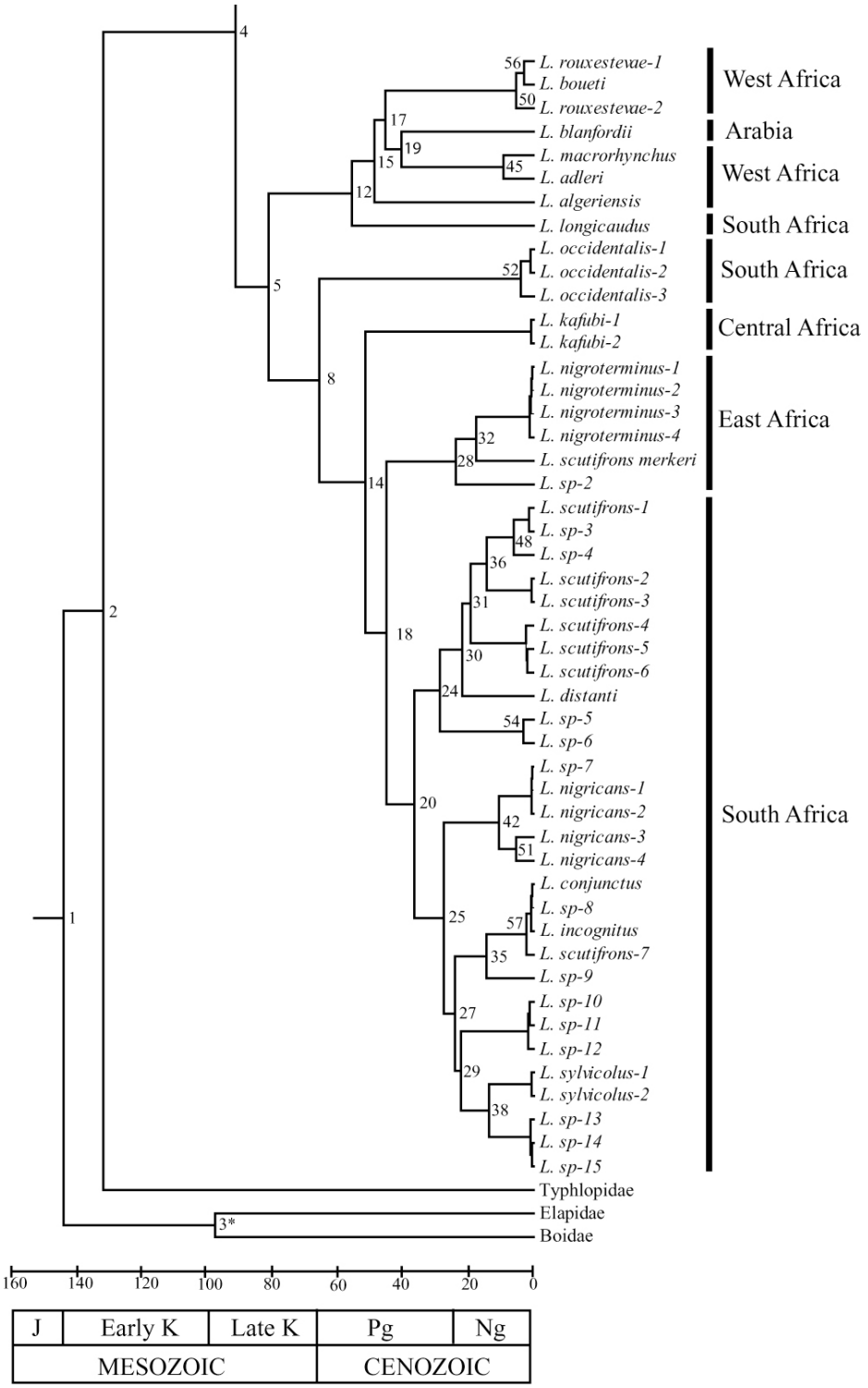


Figure **3-6**: Time tree ("Old World" clade) from species-rich data set. This clade includes all Old World taxa except *R. koniagui* and *L. bicolor*, which cluster among New World taxa. Asterisks indicate nodes calibrated with fossil dates. Timescale is in millions of years before present. Outgroup not shown: *Heloderma suspectum.*

Table **3-3**: Times (in millions of years ago) from the species-rich data set, excluding cytochrome *b* data. Point estimates and Credibility Interval bounds have been averaged from analyses using 159.9 and 102.3 as RTTMs. Nodes correspond to those on time trees in figs. 3-5 and 3-6. CI represents 95% Credibility Interval.

	12S, tRNAval, 16S; all taxa					
Node	**Time**	**CI**		**Node**	**Time**	**CI**
1	**144.0**	122 - 172		30	**26.0**	15 - 49
2	**132.5**	109 - 161		31	**23.1**	13 - 45
3	**99.4**	94 - 114		32	**20.8**	11 - 40
4	**94.2**	74 - 119		33	**17.3**	11 - 29
5	**84.2**	64 - 109		34	**16.9**	11 - 28
6	**77.2**	60 - 101		35	**18.1**	9 - 38
7	**69.1**	54 - 91		36	**17.6**	9 - 37
8	**69.4**	50 - 96		37	**16.3**	9 - 32
9	**67.7**	49 - 93		38	**17.5**	9 - 38
10	**65.5**	50 - 87		39	**13.7**	8 - 27
11	**62.8**	49 - 82		40	**12.1**	9 - 15
12	**59.1**	42 - 84		41	**13.6**	7 - 31
13	**56.7**	42 - 78		42	**14.1**	6 - 34
14	**55.9**	38 - 83		43	**10.6**	8 - 14
15	**52.2**	36 - 77		44	**11.8**	7 - 23
16	**50.4**	38 - 67		45	**10.9**	6 - 23
17	**48.8**	34 - 73		46	**9.1**	7 - 10
18	**49.6**	33 - 76		47	**9.2**	5 - 18
19	**43.8**	29 - 67		48	**8.1**	3 - 22
20	**41.2**	26 - 67		49	**6.4**	3 - 12
21	**36.4**	26 - 52		50	**7.0**	3 - 19
22	**33.9**	28 - 37		51	**7.4**	3 - 21
23	**33.7**	22 - 54		52	**6.5**	2 - 24
24	**33.2**	20 - 58		53	**4.9**	2 - 17
25	**32.0**	19 - 57		54	**4.5**	2 - 15
26	**28.1**	19 - 42		55	**3.1**	2 - 5
27	**28.4**	17 - 52		56	**3.8**	2 - 11
28	**27.7**	16 - 51		57	**3.9**	1 - 14
29	26.5	15 - 50				

Chapter 4

SUMMARY AND CONCLUSIONS

Leptotyphlopidae arose on West Gondwana and subsequently diverged into two major predominantly Old and New World clades in the late Cretaceous. Estimates of the divergence times indicate that this major split may have been vicariant, caused by the rifting of South America from Africa. A vicariant scenario suggests that one taxon dispersed back to Africa, which would be the first known case of dispersal from South America to Africa. Alternatively, a monophyletic New World clade may have been derived from trans-Atlantic dispersal of a West African form. All morphology-based New World species groups are supported by molecular data, but Old World *L. nigricans* and *L. scutifrons* species groups require revision. There may be two distinct, previously unrecognized, central and eastern African species groups.

Speciation patterns vary in the Old and New World clades. Most early New World divergences are centered around the Mesozoic-Cenozoic boundary. With the exception of one early divergence, Old World taxa began to diverge in the late Paleocene to early Eocene. There have been at least two independent dispersals from South to North America and two additional independent dispersals across the Caribbean. A Bahaman taxon appears to be derived from a Central American lineage, while other West Indian taxa represent a monophyletic radiation from an undetermined New World location.

An early Old World divergence may have been caused by an epicontinental seaway that isolated West Africa. Subsequent splits seem to have occurred gradually through time. Southern and eastern African speciation occurred possibly along altitudinal gradients, but first from a regional northern-southern divide. Future studies should incorporate more detailed altitudinal sampling of leptotyphlopids in these areas.

Morphological studies should be conducted on a number of specimens included in the present study. Genetic data support the recognition of at least fifteen new species, which may be cryptic or previously described but requiring revalidation. Incorporating morphological analyses with the genetic and biogeographic analyses here would produce the first complete revision of the family Leptotyphlopidae.

Appendix A

List of Specimens and Localities

Museum or Tissue Voucher	Species Name	Latitude (if known)	Longitude (if known)	Locality
TR7750	adleri	12˚ 32' N	12˚ 19' W	Senegal: Bandafassi
ROM22487	albifrons (1)			Guyana: Baramita
ROM20503	albifrons (2)			Guyana: Kurpukari
TR115	algeriensis	18˚ 48' N	11˚ 41' W	Mauritania: Rachid
SBH160213	asbolepis (1)			Dominican Republic: Barahona; Canoa, 0.3km S, 13.5km E
SBH160212	asbolepis (2)			Dominican Republic: Barahona; Canoa, 0.3km S, 13.5km E
SBH160211	asbolepis (3)			Dominican Republic: Barahona; Canoa, 0.3km S, 13.5km E
TR2219	bicolor (1)	8˚ 41' N	0˚ 46' E	Togo: Fazao
TRNIGER01	bicolor (2)	13˚ 31' N	2˚ 7' E	Niger: Niamey Airport
MVZ 236621 or 236622	blanfordii	12˚ 9575" N	44˚ 9291667" E	Yemen: Lahij; Bir Nasr Farm, 3km SW Sabir
TR3305	boueti	14˚ 30' N	9˚ 39' W	Mali: Bouyanga
SBH267734	breuili (1)	13° 56.080' N	61° 02.950' W	St. Lucia: Anse Galet, 5m elevation
SBH267733	breuili (2)	13° 43.430' N	60° 55.897' W	St. Lucia: Maria Major Island, slope on N side, 60m elevation
SBH267738	breuili (3)	13° 52.875' N	60° 53.418' W	St. Lucia: 1.6km N Praslin, 40m elevation
SBH267737	breuili (4)	13° 52.875' N	60° 53.418' W	St. Lucia: 1.6km N Praslin, 40m elevation
USNM564818	carlae (1)	13° 11.196' N	59° 32.445' W	Barbados: Bonwell, 280m elevation

USNM564819	carlae (2)	13° 11.196' N	59° 32.445' W	Barbados: Bonwell, 280m elevation
SBH192878	columbi (1)			Bahamas: San Salvador; Little Fortune Hill, NE corner
SBH192936	columbi (2)			Bahamas: San Salvador; Little Fortune Hill, NE corner
SBH192979	columbi (3)			Bahamas: San Salvador; Little Fortune Hill, NE corner
SBH192980	columbi (4)			Bahamas: San Salvador; Little Fortune Hill, NE corner
SBH192981	columbi (5)			Bahamas: San Salvador; Little Fortune Hill, NE corner
PEMR17410	conjunctus	27˚ 59272" S	32˚ 24876" E	South Africa: KwaZulu-Natal; Mkhuze Game Reserve, Mixed Bushveld
LSUMZ H-9314	dissectus			USA: Arizona; Chochise Co, 1.4mi by rd SW Portal
MBUR0874	distanti			South Africa: Mpumalanga; Phalaborwa
MVZ230602	dulcis			USA: Texas; Crane Co., 1.5mi W of junction with Farm Road 1601
from Sandy Echternacht, UT-Knoxville	goudotii (7)			Honduras: Isla de Utila
MSM373	goudotii (4)			Guatemala: Huehuetenango
JAC23296	goudotii (6)			Mexico: Veracruz; Municipio Catemaco, vicinity of La Victoria
JAC23977	goudotii (1)			Mexico: Michoacan
JAC24210	goudotii (3)			Mexico: Oaxaca
JAC24222	goudotii (2)			Mexico: Oaxaca
ENS9932	goudotii (5)	16˚ 28' 41" N	96˚ 3' 7" W	Mexico: Oaxaca; San Isidro Manteca
ROM45259	humilis (1)			Mexico: Baja California Norte; Vizcaino
MVZ190030	humilis (2)	23.989444˚ N	110.15444440˚ W	Mexico: Baja California, 3.8 mi N (via Mexico Hwy. 1), San Pedro
	incognitus			South Africa: KwaZulu-Natal; Lembombo Mountains

PEMR17439	kafubi (1)	10˚ 38’ 07.7” N	25˚ 55’ 54.9” E	Democratic Republic of the Congo: HautKatangaProvince; Kalakundi
PEMR17441	kafubi (2)	10˚ 39' 43.6" S	25˚ 55' 35.8" E	Democratic Republic of the Congo: HautKatangaProvince; Kalakundi
SBH103599	leptepileptus (6)			Haiti: l'Quest; Soliette, N of Fond Verrettes, along border with Dominican Republic
SBH103600	leptepileptus (5)			Haiti: l'Quest; Soliette, N of Fond Verrettes, along border with Dominican Republic
SBH103601	leptepileptus (2)			Haiti: l'Quest; Soliette, N of Fond Verrettes, along border with Dominican Republic
SBH103602	leptepileptus (4)			Haiti: l'Quest; Soliette, N of Fond Verrettes, along border with Dominican Republic
SBH103603	leptepileptus (3)			Haiti: l'Quest; Soliette, N of Fond Verrettes, along border with Dominican Republic
SBH103604	leptepileptus (1)			Haiti: l'Quest; Soliette, N of Fond Verrettes, along border with Dominican Republic
AMB8302	longicaudus	29˚ 49’ 40” E	22˚ 42’ 19” S,	South Africa: Limpopo Province; near Waterpoort
ROM28367	macrolepis (3)			Guyana: Paramakatoi
LSUMZ H-14220	macrolepis (1)	3˚ 8' 47.2" S	54˚ 50' 32.3" W	Brazil: Para; Agropecuaria Treviso LTDA, ca 101km S and 18km E Santarem
LSUMZ H-14449	macrolepis (2)	3˚ 8' 18.6" S	54˚ 50' 29.6" W	Brazil: Para; Agropecuaria Treviso LTDA, ca 101km S and 18km E Santarem
LSUMZ H-20102	macrorhynchus			Ghana: Northern region; 2.5km SW Buipe
PEM R17392	nigricans (3)			South Africa: Sardinia Bay; Port Elizabeth
AMB4822, PEM R12556	nigricans (1)			South Africa: Western Cape province; Cape Hangklip, Caledon
AMB6028	nigricans (2)			South Africa: Western Cape province; Cape Hangklip, Caledon
MCZF38479	nigricans (4)			South Africa

PEM R17330	nigroterminus (1)	01˚ 50' 05.8"S	35˚ 14' 46.3"E	Tanzania: NW Serengeti; Klein's Camp Lodge area, Loliondo Game Controlled Area
PEM R17346	nigroterminus (4)	01˚ 50' 05.8"S	35˚ 14' 46.3"E	Tanzania: NW Serengeti; Klein's Camp Lodge area, Loliondo Game Controlled Area
PEM R17347	nigroterminus (3)	01˚ 50' 05.8"S	35˚ 14' 46.3"E	Tanzania: NW Serengeti; Klein's Camp Lodge area, Loliondo Game Controlled Area
PEM R17348	nigroterminus (2)	01˚ 50' 05.8"S	35˚ 14' 46.3"E	Tanzania: NW Serengeti; Klein's Camp Lodge area, Loliondo Game Controlled Area
AMNH AMCC 105532	occidentalis (3)			South Africa
AMB4680, PEM R11906	occidentalis (2)	28° 25' 31" S	17° 00' 06" E	South Africa: Northern Cape Province; 3.2km from Koebus, Richtersveldt National Park, Namaqualand
AMB4503, PEM R11915	occidentalis (1)	28° 15' 38" S	16° 56' 18" E	South Africa: Northern Cape Province; Hellskloof Gate, Richtersveldt National Park, Namaqualand
SBH102591	pyrites			Dominican Republic: Pedernales; 6.4km SW of Las Mercedes
TR7757	Rhinoleptus koniagui			Senegal: Ibel
TR?	rouxestevae (1)	12˚ 12' N	8˚ 42' W	Mali: 3.286km N of Sebekourani
TR7760	rouxestevae (2)	12˚ 31' N	12˚ 23' W	Senegal: Ibel
AMB8390	scutifrons (5)	24˚ 04’ 02” S	28˚ 26’ 16” E	South Africa: Limpopo Province; Kgama, Tshukudu Lodge area
MCZF38884	scutifrons (6)			South Africa
AMB8414	scutifrons (4)	24˚ 19’ 58” S	28˚ 23’ 05” E	South Africa: Limpopo Province; 33.1 km S Kgama on gravel road to Molimolle
MB21438	scutifrons (1)	27°07' 49" S	23° 59' 42" E	South Africa: North West Province; near Dithakong, 65k NE Kuruman
	scutifrons (7)			South Africa: KwaZulu-Natal Province; Phinda PGR
MB327	scutifrons (3)			South Africa: Limpopo; Blouberg

MB393	scutifrons (2)			South Africa: Limpopo; Blouberg
PEM R17862	scutifrons merkeri	45 32 86	9611968	Kenya: Tana Hills; Sagalla
LSUMZ H-12312	septemstriatus			Brazil: Roraima; Fazenda Nova Esperanca, 47km W. BR-174 on BR-210
JAC23308	sp (1)			Mexico: Jalisco
AMB6026	sp (7)			South Africa
MBUR00107	sp (10)			South Africa: Mpumalanga Province; approx 40km S Lydenburg, in stomach of Psammophis brevirostris
MBUR00127	sp (12)			South Africa: Mpumalanga Province; approx 40km W Nelspruit in mountains
MBUR00143	sp (11)			South Africa: Mpumalanga Province; approx 40km W Nelspruit in mountains
MBUR00435	sp (14)	30˚ 32' 08" S	28˚ 49' 38" E	South Africa: Eastern Cape Province; Matatiele Dist, Fever Village, 79 km SW Cedarville, Transkei
MBUR00438	sp (15)	30˚ 32' 08" S	28˚ 49' 38" E	South Africa: Eastern Cape Province; Matatiele Dist, Fever Village, 79 km SW Cedarville, Transkei
MBUR00932	sp (4)	22˚ 37' 46" S	30˚ 24' 42" E	South Africa: Limpopo Province; E of Tsipise
MBUR01104	sp (8)	26˚ 56' 10" S	31˚ 59' 58" E	South Africa: KwaZulu-Natal Province; Lebombo, Manyiseni region
PEMR17418	sp (9)	27˚ 59' 38.3" S	32˚ 24' 02.9" E	South Africa: KwaZulu-Natal Province; Mkhuze Game Reserve, Mixed Bushveld
PEMR17420	sp (5)	27˚ 60' 96.7" S	32˚ 17' 16.6" E	South Africa: KwaZulu-Natal Province; Mkhuze Game Reserve, Lebombo foothills
MB20939	sp (3)	28˚ 50' 07" S	22˚ 34' 21" E	South Africa: Northern Cape Province; Fm Black Ridge, E of Langeberge, NEE of Groblershoop, near Upington
MBUR00439	sp (13)	30˚ 32' 08" S	28˚ 49' 38" E	South Africa: Eastern Cape Province; Matatiele Dist, Fever Village, 79 km SW Cedarville, Transkei

MBUR01235	sp (6)			South Africa: KwaZulu-Natal Province; Lebombo, Manyiseni region
PEM R5577	sp (2)	1°25' 32.9" S	30°29' 31.7" E	Rwanda: L'Akagera National Park; between Gabiro and the Tanzanian border
SBH266699	sp.nov.			Domincan Republic: Independencia; La Zurza
	sylvicolus (1)			South Africa: KwaZulu-Natal Province; Xilonde Transect, coll. July 2004
PEM R17343	sylvicolus (2)			South Africa: KwaZulu-Natal Province; Xilonde Transect, 1km S of Mozambique border, coll. Dec 2003

Appendix B

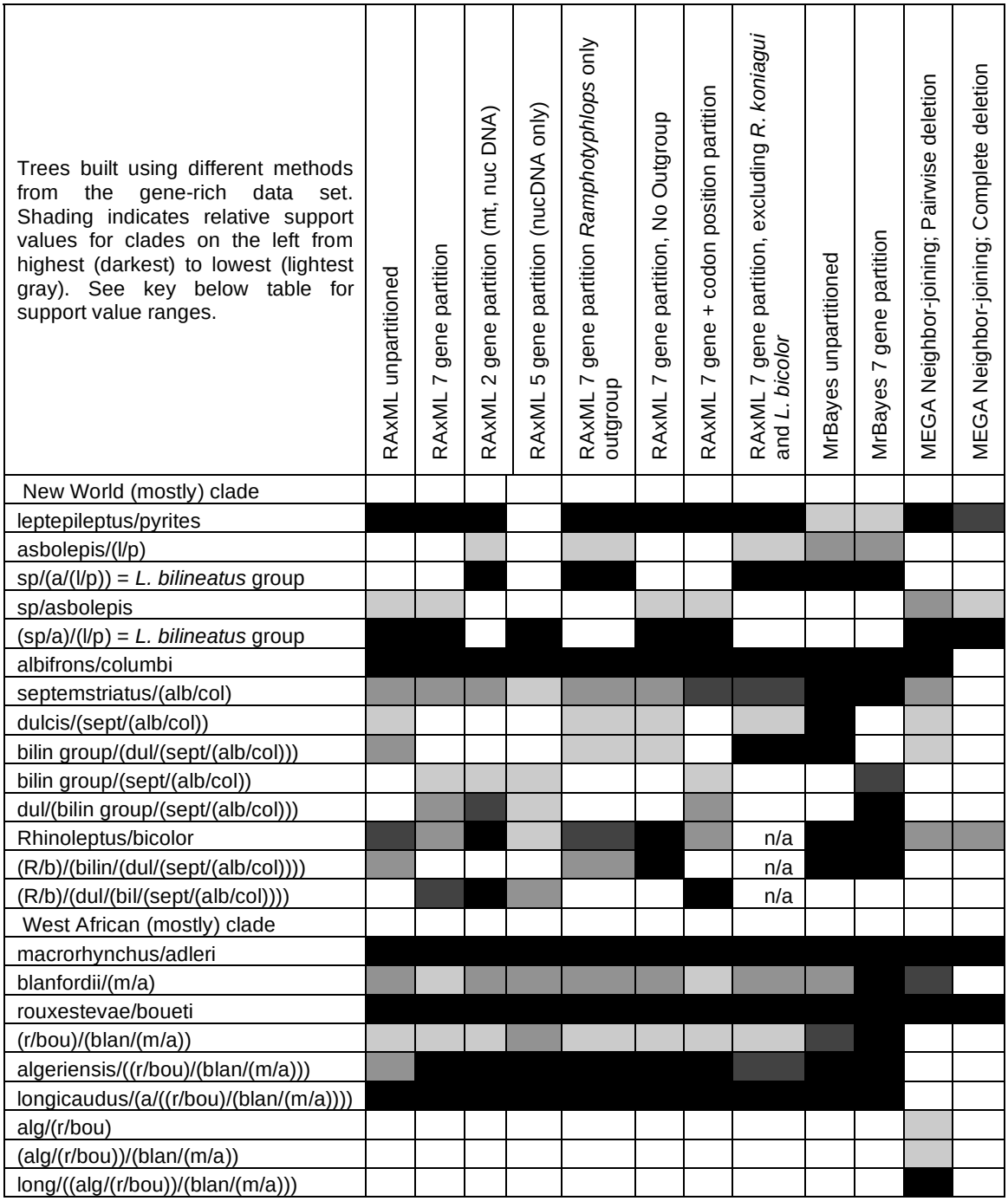
Primers

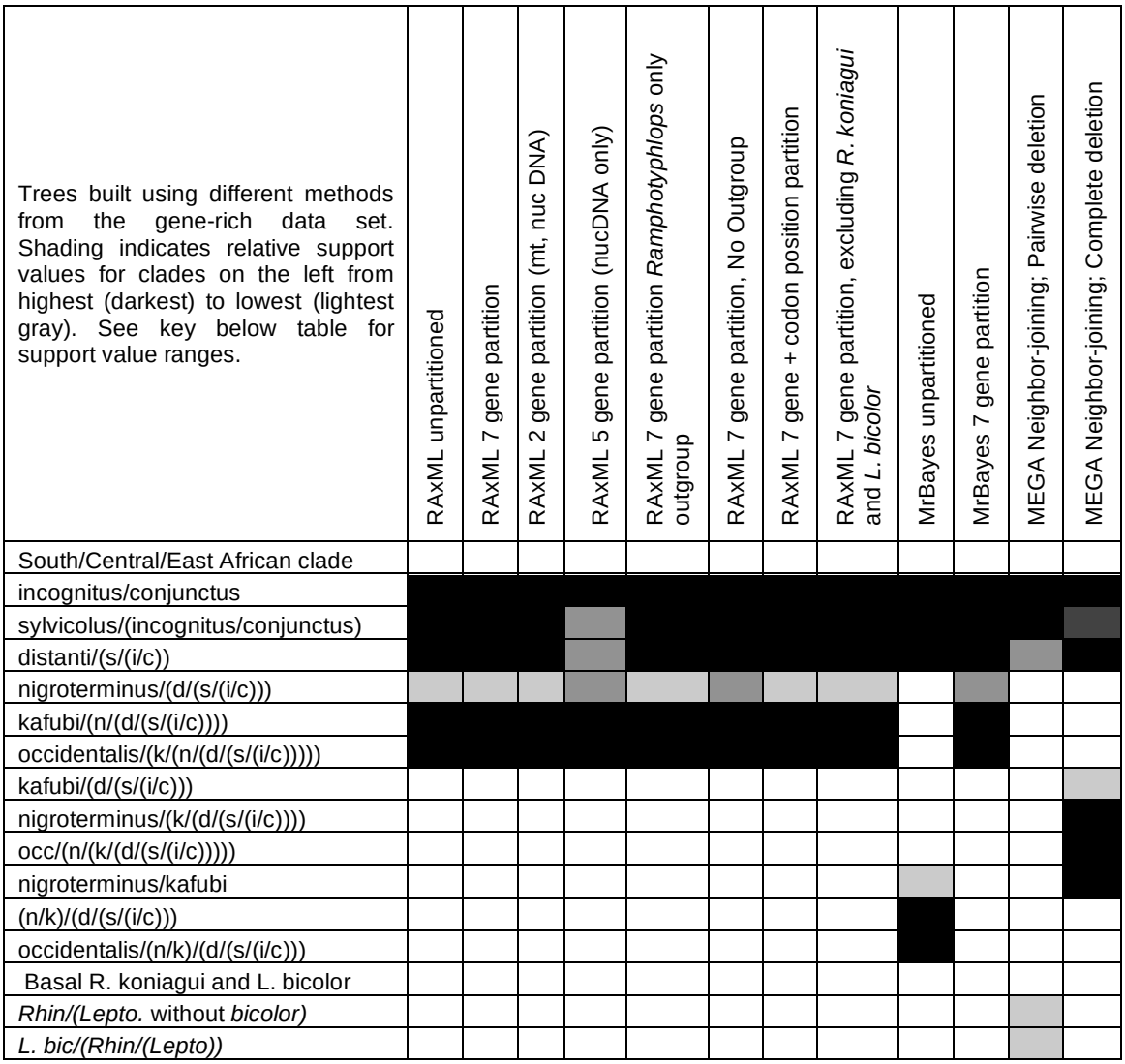
Gene	Primer name	Sequence (5'–3')
12S	12L2	AAAGCAWRGCACTGAARATGCTWAGATG
12S	12L31	AAAGTSTTGGTCCTRAACCT
12S	12L16	AAAGCATGGCACTGAAGATGCCAAGAYGG
12S	12H3	CTAGAGGAGCCTGTTCTYTAATCGATKKCCRCG
12S	12L17	CAAACTAGGATTAGATACCCTACTATGC
12S	12L24	CAAACTRGGATTAGATACCCYACTAT
12S	12L5	GATTAGA TACCCCACTA TGC
12S	12H11	CACTTTCCAGTACGCTTACCATGTTACG
12S	12H40	CGTAACATGGTAAGCGTACTGGAAAGTG
12S	12H10	AAGTCGTAACAYGGTAARYGYACYGGAARGTG
12S	12H4	CGYACAC ACCGCCCGTC ACCCT
12S-tRNAval-16S	12L3	TGARGCRCGYACACACCGCCCGTCACCCTC
12S-tRNAval-16S	12L7	GAAGGWGGATTTAGYAGTAAA
12S-tRNAval-16S	12L14	ACTAAWACGTCAGGTCAAGGTGYAGC
12S-tRNAval-16S	12L23	CTATATACCGCCGTCGRAAGTTCA
12S-tRNAval-16S	12L13	AAAGAAGA GGAAAG TCGTAACA TGGTA
12S-tRNAval-16S	16L3	AGCAAAGAYYAAMCCTYGTACCTTTTGC AT
12S-tRNAval-16S	16L26	GTRCCGYAAGGGAMYAATGAAA
12S-tRNAval-16S	16H22	GTAGGCCYTAAAGCAGCCAYCAAWAA
12S-tRNAval-16S	16H27	GTRGRCCTYTAARCMGCCAMCAAAAAYA
12S-tRNAval-16S	16H21	GTACCTHTTGCATCATGGTYYAGCDAG
16S	16L44	CCCGAAACCRRGTGAGCTAC
16S	16L10	AGTGGGCCTAAAAGCAGCCA
16S	16L20	TGAAAASCCWAMCGARCYTGRTGATAGCTG
16S	16L16	AACCCKTCTCTGTKGCAAAAGAGTGRGA
16S	16H24	ACGGCCGCGGTAYMCTAACCGTGCGAAGGTA
16S	16H17	GCWRRRGGRKATGTTTTTGGTAAACA
16S	16L39	CTGTTTACCAAAAACATAGCCTTTAG
16S	16H1	CCTACGTGATCTGAGTTCAGACCGGAG
cytochrome b	S1L	GAAAAA CCG CYR TTG TWW TTC AAC TA
cytochrome b	Ltyph3L	CATATATCGGACAAACTCTTGTCA
cytochrome b	Ltyph5L	GCCACMGTMATCACYAAYCT
cytochrome b	H16064	CTTTGGTTTACAAGAACAATGCTTTA
cytochrome b	Ltyph4R	GTGTTAATGTGGCGTTGTTTACTGA
cytochrome b	Ltyph2R	AGYTTGTTTGGGATKGCTCGTAGRAT
cytochrome b	Ltyph6R	AGAAYCGKGTTARDGTGGCGT
AMEL	LAMSQ	ATGGGAGGATGGATGCACCA
AMEL	LAM2N	TATCCACGTTATGGCTATGAACC
AMEL	HAMSQ	TGGCCATGRTTCAAGAGGYGTAT

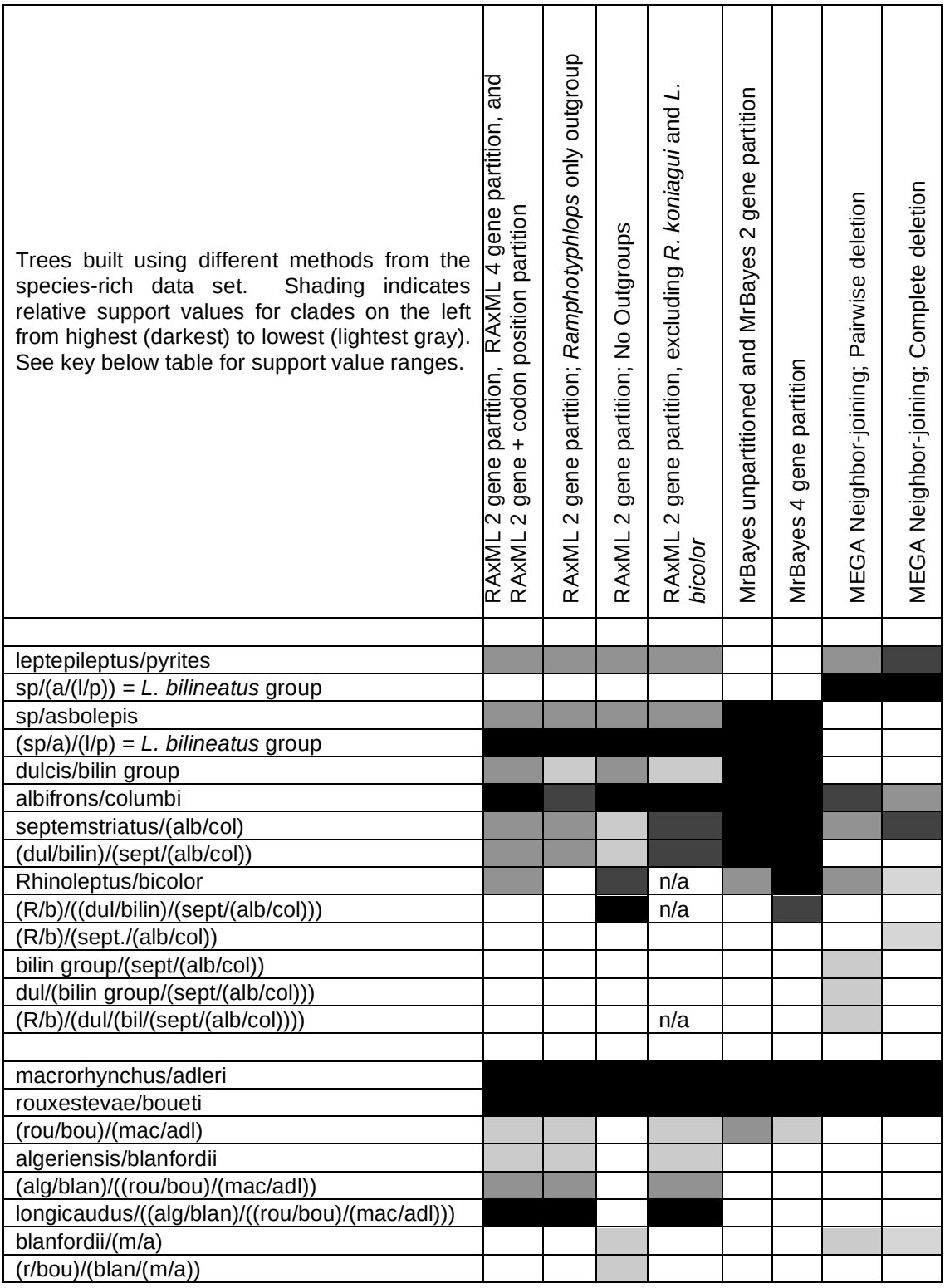
BDNF	F	GACCATCCTTTTCCTKACTATGGTTATTTCATACTT
BDNF	R	CTATCTTCCCCTTTTAATGGTCAGTGTACAAAC
C-*mos*	F4	AATGHACRTCCMTGYAGYAGCCCTTTGGTCTGT
C-*mos*	G74	TGAGCATCCAAAGTCTCCAATC
NT3	F1	ATGTCCATCTTGTTTTATGTGATATTT
NT3	F3	ATATTTCTGGCTTTTCTCTGTGGC
NT3	R1	ACRAGTTTRTTGTTYTCTGAAGTC
NT3	R4	GCGTTTCATAAAAATATTGTTTGACCGG
RAG-1	L2408	TGCACTGTGACATTGGCAA
RAG-1	Ltyph2L	AGAGAATTAATGGACCTTTA
RAG-1	H2920	GCCATTCATTTTYCGAA
RAG-1	Ltyph1R	ATCTCCATACTGGTTTCATC

Appendix C

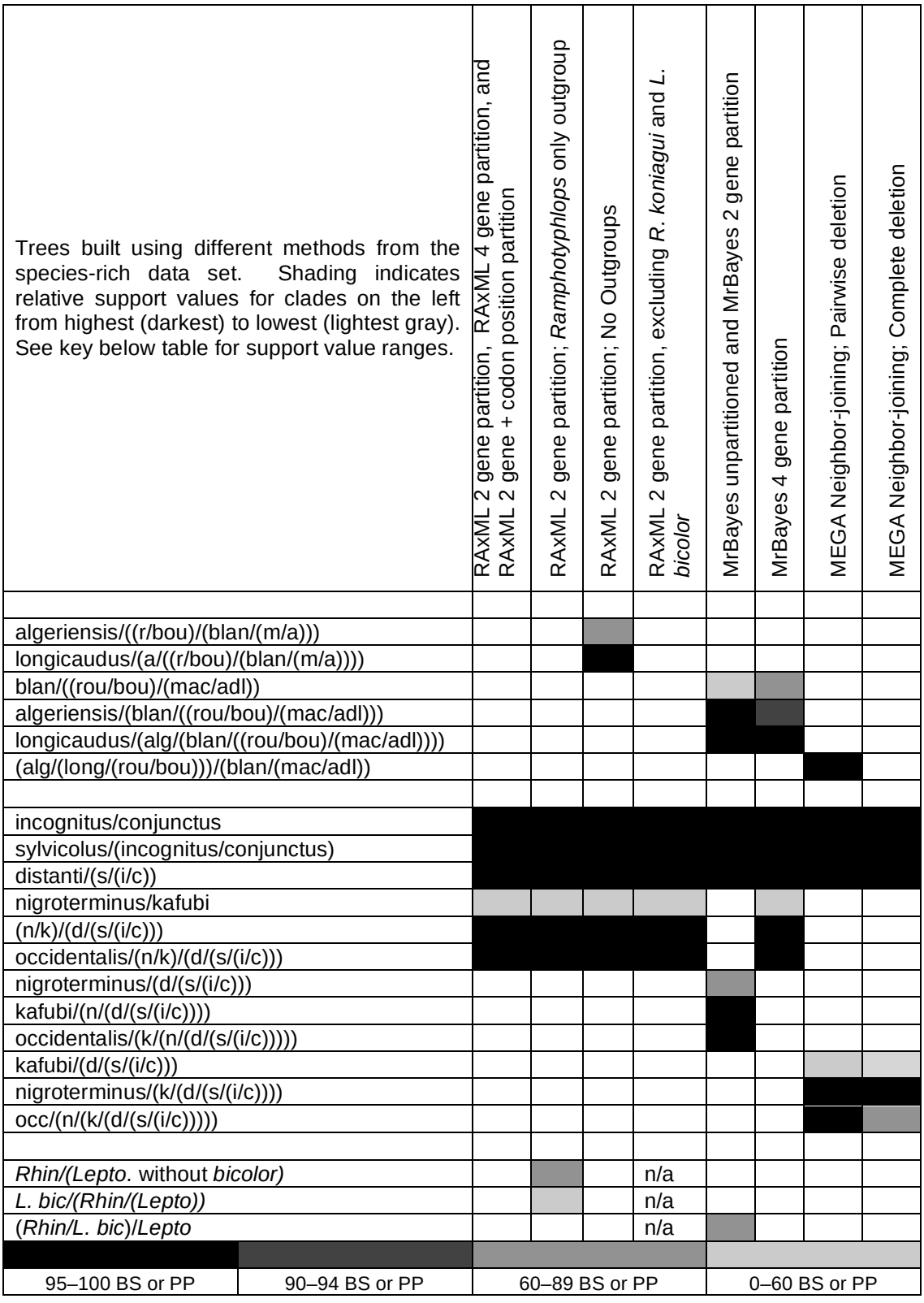
Tree Topologies




Trees built using different methods from the gene-rich data set. Shading indicates relative support values for clades on the left from highest (darkest) to lowest (lightest gray). See key below table for support value ranges.
RAxML unpartitioned
RAxML 7 gene partition
RAxML 2 gene partition (mt, nuc DNA)
RAxML 5 gene partition (nucDNA only)
RAxML 7 gene partition Ramphotyphlops only outgroup
RAxML 7 gene partition, No Outgroup
RAxML 7 gene + codon position partition
RAxML 7 gene partition, excluding R. koniagui and L. bicolor
MrBayes unpartitioned
MrBayes 7 gene partition
MEGA Neighbor-joining; Pairwise deletion
MEGA Neighbor-joining; Complete deletion
South/Central/East African clade
incognitus/conjunctus
sylvicolus/(incognitus/conjunctus)
distanti/(s/(i/c))
nigroterminus/(d/(s/(i/c)))
kafubi/(n/(d/(s/(i/c))))
occidentalis/(k/(n/(d/(s/(i/c)))))
kafubi/(d/(s/(i/c)))
nigroterminus/(k/(d/(s/(i/c))))
occ/(n/(k/(d/(s/(i/c)))))
nigroterminus/kafubi
(n/k)/(d/(s/(i/c)))
occidentalis/(n/k)/(d/(s/(i/c)))
Basal R. koniagui and L. bicolor
Rhin/(Lepto. without bicolor)
L. bic/(Rhin/(Lepto))
95–100 BS or PP
90–94 BS or PP
60–89 BS or PP
0–60 BS or PP


Trees built using different methods from the species-rich data set. Shading indicates relative support values for clades on the left from highest (darkest) to lowest (lightest gray). See key below table for support value ranges.
RAxML 2 gene partition, RAxML 4 gene partition, and RAxML 2 gene + codon position partition
RAxML 2 gene partition; *Ramphotyphlops* only outgroup
RAxML 2 gene partition; No Outgroups
RAxML 2 gene partition, excluding *R. koniagui* and *L. bicolor*
MrBayes unpartitioned and MrBayes 2 gene partition
MrBayes 4 gene partition
MEGA Neighbor-joining; Pairwise deletion
MEGA Neighbor-joining; Complete deletion
leptepileptus/pyrites
sp/(a/(l/p)) = *L. bilineatus* group
sp/asbolepis
(sp/a)/(l/p) = *L. bilineatus* group
dulcis/bilin group
albifrons/columbi
septemstriatus/(alb/col)
(dul/bilin)/(sept/(alb/col))
Rhinoleptus/bicolor
(R/b)/((dul/bilin)/(sept/(alb/col)))
(R/b)/(sept./(alb/col))
bilin group/(sept/(alb/col))
dul/(bilin group/(sept/(alb/col)))
(R/b)/(dul/(bil/(sept/(alb/col))))
n/a
n/a
n/a
macrorhynchus/adleri
rouxestevae/boueti
(rou/bou)/(mac/adl)
algeriensis/blanfordii
(alg/blan)/((rou/bou)/(mac/adl))
longicaudus/((alg/blan)/((rou/bou)/(mac/adl)))
blanfordii/(m/a)
(r/bou)/(blan/(m/a))

Trees built using different methods from the species-rich data set. Shading indicates relative support values for clades on the left from highest (darkest) to lowest (lightest gray). See key below table for support value ranges.



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