To the Graduate Council:

I am submitting herewith a dissertation written by Philip Joseph Heise entitled "Phylogeny and Biogeography of Galápagos Lava Lizards (*Microlophus*) Inferred From Nucleotide Sequence Variation in Mitochondrial DNA." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Ecology and Evolutionary Biology.

Linda Maxson

Linda Maxson, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School

PHYLOGENY AND BIOGEOGRAPHY OF GALÁPAGOS LAVA LIZARDS (*MICROLOPHUS*) INFERRED FROM NUCLEOTIDE SEQUENCE VARIATION IN MITOCHONDRIAL DNA

A Dissertation
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Philip Joseph Heise
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I would also like to express my deep gratitude to my parents and to Lauren for always supporting my decisions, regardless of what those might have been.

Abstract

The genus *Microlophus* has representatives in South America and in the Galápagos Islands. Although the phylogenetics of the seven endemic Galápagos species have been previously investigated, no consensus exists as to the relationships among the island species or of the relationships of the island species to the mainland taxa. In this study, variation in the nucleotide sequence in portions of the mitochondrial cytochrome *b* and 16S ribosomal RNA genes is analyzed and used to propose hypotheses regarding the phylogenetic relationships and biogeographic history of the Galápagos species of *Microlophus*. The major findings of this study are:

(1) the present-day species of Galápagos lava lizards are not a monophyletic assemblage; there are two main lineages, with the first including *M. bivittatus* and *M. habeli* while the second is comprised of all other island species;

(2) *M. bivittatus* and *M. habeli* are more closely related to *M. occipitalis* from mainland South America than they are to the other Galápagos species;

(3) the ancestor of the remaining island species could not be unequivocally resolved but, of the mainland South American taxa examined, *M. theresiae, M. thoracicus,* and *M. tigris* were identified as possible sister taxa;

(4) of those populations previously recognized as *M. albemarlensis*, four groups consistently emerge; *M. indefatigabilis* is resurrected for the populations from Santa Cruz, Baltra, and Daphne islands and *M. jacobi* is resurrected for the populations from Santiago and Bartolomé islands;

(5) *M. delanonis* is the most basal species within the main lineage, followed by *M. grayi*; *M. pacificus* is the sister taxon to *M. albemarlensis*; *M. indefatigabilis* is the sister species to *M. jacobi*; *M. duncanensis* is the closest relative to the two latter species;

(6) the Galápagos lava lizards are almost certainly derived from South American species and dispersal to the islands most likely occurred via rafting on mats of vegetation swept out to sea;

(7) at least two colonization events are necessary to account for the present-day distribution of the Galápagos lava lizards;

(8) the lineages of *Microlophus* inhabiting the Galápagos Islands diverged from their closest mainland relatives approximately 7.1 - 8.1 million years ago (mya) and the Galápagos species separated from each other from 2.1 - 6.6 mya;

(9) the estimates of divergence times for the Galápagos species pre-date the age of the current Galápagos Islands;

(10) the combination of morphological data, cytochrome *b* data, and 16S rRNA data support the proposition that *Plesiomicrolophus* be synonymized with *Microlophus*.

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Chapter 1

Review of Lava Lizard Systematics and Biogeography

A. Introduction

The Galápagos Archipelago is, as it has often been stated, "a natural laboratory for evolution." Discovered in 1535 and named for the giant land tortoises which inhabit them, the islands lie on the equator off the western coast of South America (Fig. 1.1). The archipelago includes thirteen large islands over 10 km^2, six smaller islands, more than forty officially named islets, and numerous other islets and rocks which do not have names (Jackson, 1985). The largest island, Isabela, has an area of 4,588 km^2. Four other islands are larger than 500 km^2, but most of the islands are smaller than 60 km^2. The total land area is approximately 8,000 km^2 and is spread out over 45,000 km^2 of ocean. The nearest land mass, mainland Ecuador, is approximately 960 km to the east. Cocos Island and Costa Rica lie 720 and 1,100 km, respectively, to the northeast. Guatemala is 1,600 km to the north whereas Easter and San Felix islands are about 3,200 km to the south.

The isolation of the Galápagos Archipelago has allowed for diversification in many groups of both plants and animals. Of the approximately 550 species of native plants found in the archipelago, 34% are endemic (Porter, 1984) as are 61% of the 85 native species of terrestrial vertebrates (Jackson, 1985). Among the vertebrates, reptiles show the highest degree of endemism, with 20 of the 22 described species found only in the Galápagos. Many of the reptiles are further restricted, occurring on only a single

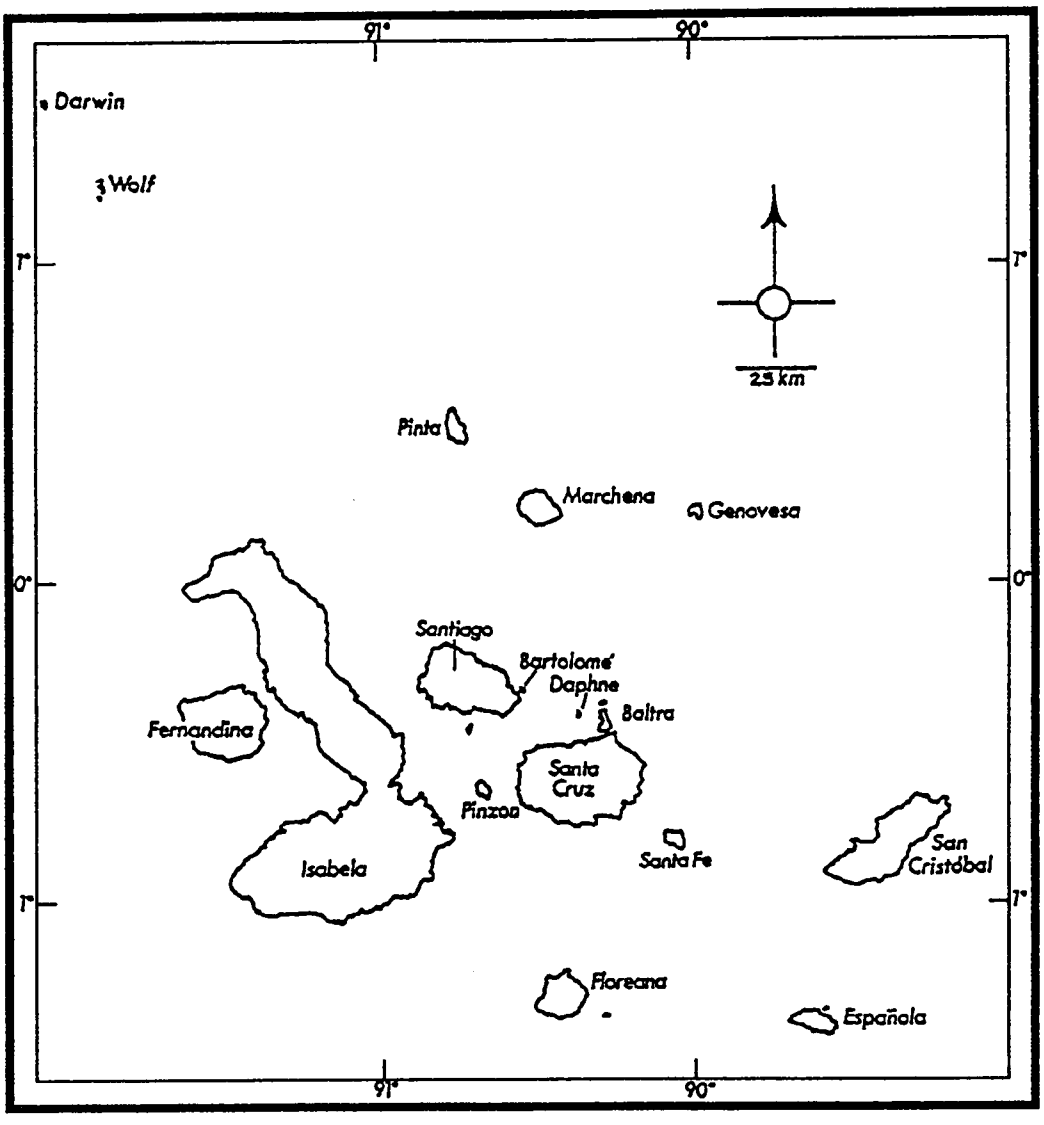


Figure 1.1. Map of the Galápagos Archipelago. (Map taken from Wright, J. W. 1983. The evolution and biogeography of the lizards of the Galapagos Archipelago: evolutionary genetics of *Phyllodactylus* and *Tropidurus* populations. Pp. 123-155 *in* R. I. Bowman, M. Berson, and A. E. Leviton (eds.), Patterns of evolution in Galapagos organisms. Pacific Division of the American Association for the Advancement of Science, San Francisco.)

island. These species include three large iguanas, seven geckos, three snakes, one giant land tortoise (with ten surviving subspecies), one sea turtle, and seven lava lizards.

Galápagos lava lizards are small (less than 30 cm total length), diurnal, omnivorous lizards which inhabit the arid lowlands of most islands in the archipelago. The Galápagos species all are placed in the family Tropiduridae as members of the genus *Microlophus* (Frost, 1992). The 19 species comprising *Microlophus* are limited in distribution, occurring in South America west of the Andes from southern Ecuador to northern Chile, east of the Andes only in the Huancabamba Depression Region of northern Peru, and in the Galápagos Archipelago (Frost, 1992); seven species are endemic to the Galápagos. With the exceptions of Genovesa, Wolf, and Darwin islands, *Microlophus* is found on most of the approximately sixty named islands and islets that constitute the archipelago (Jackson, 1985). *Microlophus albemarlensis* occurs on all of the central and western islands whereas six of the more peripheral islands each has its own distinct species (Fig. 1.2).

B. Lava Lizard Systematics

The Class Reptilia is divided into four orders: Crocodylia (crocodiles and alligators), Testudines (turtles and tortoises), Sphenodontida (tuataras), and Squamata (squamates). Within the squamates, two major lineages are recognized: the Iguania and the Autarchoglossa (Gauthier et al. 1988). The Iguania is comprised of those taxa traditionally placed in the Iguanidae, Agamidae, and Chamaeleonidae whereas the Autarchoglossa includes all other squamates (including various lizard lineages, snakes

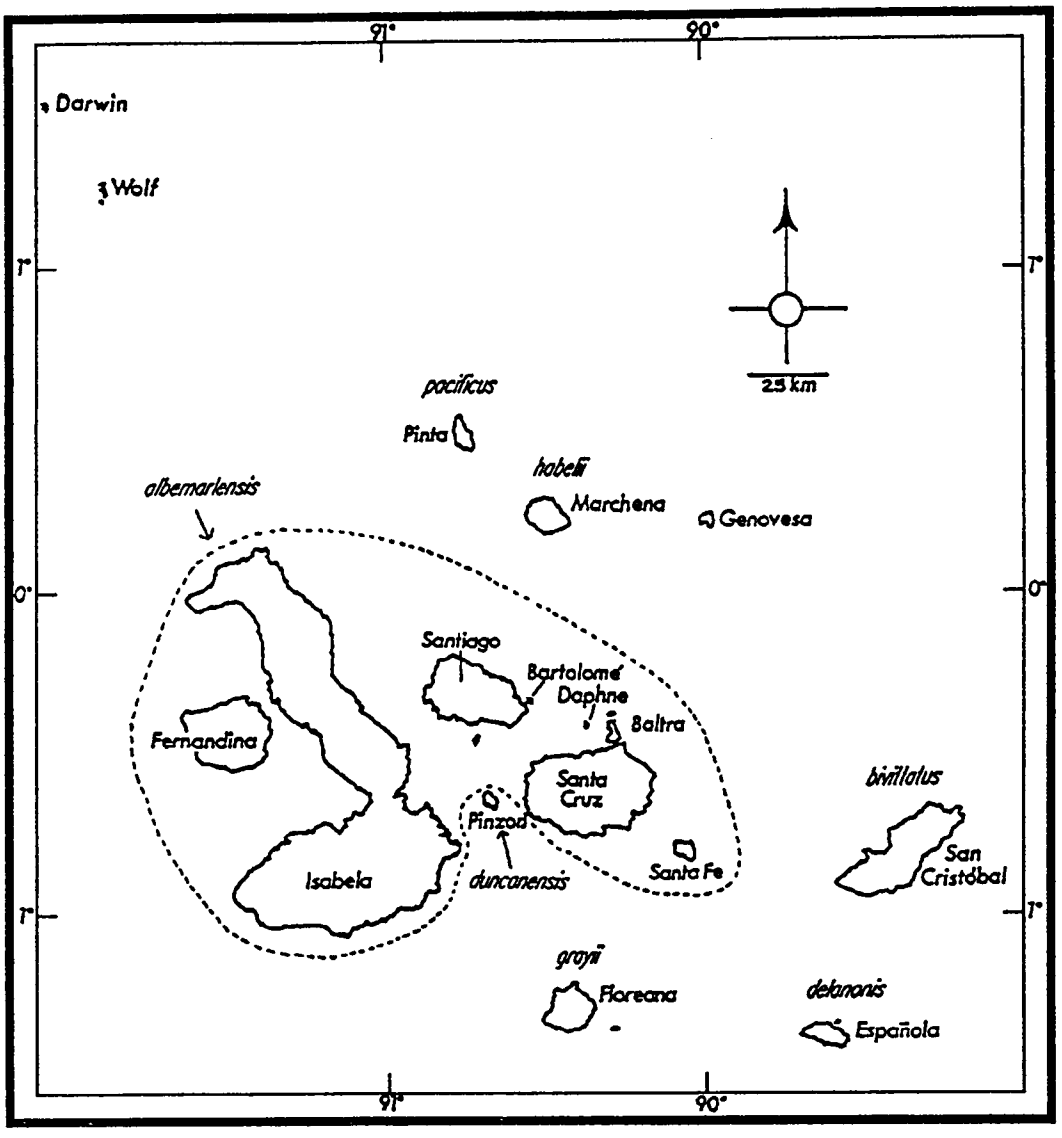


Figure 1.2. Distribution of *Microlophus* in the Galápagos Archipelago. (Map adapted from Wright, J. W. 1983. The evolution and biogeography of the lizards of the Galapagos Archipelago: evolutionary genetics of *Phyllodactylus* and *Tropidurus* populations. Pp. 123-155 *in* R. I. Bowman, M. Berson, and A. E. Leviton (eds.), Patterns of evolution in Galapagos organisms. Pacific Division of the American Association for the Advancement of Science, San Francisco.)

and amphisbaenians). The Iguanidae is defined on the basis of plesiomorphies and, thus, its monophyly has been questioned (Estes et al. 1988; Etheridge and de Queiroz, 1988; Gauthier et al. 1988). Frost and Etheridge (1989) analyzed a broad suite of morphological characters and split the Iguanidae into eight families; the newly erected families showed strong concordance to previously recognized monophyletic groups within the Iguanidae (*sensu* Etheridge and de Queiroz, 1988). One of these families, the Tropiduridae, is comprised of thirteen genera from South America, the West Indies, and the Galápagos Islands (Table 1.1). These genera are placed in three subfamilies: the Liolaeminae, the Leiocephalinae and the Tropidurinae.

Bell (1843) named the first Galápagos lava lizard (from material collected by Charles Darwin) as *Leiocephalus grayi*[1]. Peters (1871) moved *grayi* to *Tropidurus*, named the second of the Galápagos species, *bivittatus*, and placed both species in the subgenus *Craniopeltis*. Steindachner (1876 in Frost, 1992) named *Tropidurus* (*Craniopeltis*) *pacificus pacificus* and *T.* (*C.*) *p. habeli* from the Galápagos and considered *bivittatus* as a synonym of *grayi*. Both *pacificus* and *grayi* (including *bivittatus*) were returned to *Leiocephalus* by Günther (1877). This revision was reversed by Boulenger (1885), who placed both species back in *Tropidurus*. Cope (1889) named an additional species from the Galápagos, *Tropidurus lemniscatus*.

[1] According to Article 31, Paragraph a, Subsection ii of the International Code of Zoological Nomenclature (Ride et al. [eds.], 1985), a species-group name formed directly from a modern personal name is to be formed by adding *-i* to the stem of the name when the personal name is that of a man. For this reason, *grayi* is used in preference to *grayii*, *habeli* in preference to *habelii*, etc., regardless of the original spelling.

Table 1.1. Sub-families and genera of the Tropiduridae (*sensu* Frost and Etheridge, 1989)[1]

Sub-family	Genera	Distribution
Leiocephalinae	*Leiocephalus*	West Indies
Liolaeminae	*Ctenoblepharys* *Liolaemus* *Phymaturus*	South America
Tropidurinae	*Ophryoessoides*[2] *Proctotretus*[2] *Stenocercus*[2] *Plica*[3] *Strobilurus*[3] *Tapinurus*[3] *Tropidurus*[3] *Uracentron*[3] *Uranoscodon*[3]	South America and Galápagos

[1] *Source:* Frost, D. R., and R. Etheridge. 1989. A phylogenetic analysis and taxonomy of iguanian lizards (Reptilia: Squamata). Misc. Publ. Mus. Nat. Hist. Univ. Kansas **81:**1-65.

[2] *Stenocercus* group.

[3] *Tropidurus* group.

Baur (1890) named five new Galápagos species: *albemarlensis, indefatigabilis, delanonis, duncanensis,* and *abingdonensis*. Boulenger (1891) reviewed the taxonomy of the Galápagos *Tropidurus* and recognized only three species: *bivittatus* (including *lemniscatus* as a synonym), *pacificus* (including *abingdonensis* in synonymy), and *grayi* (including *albemarlensis, indefatigabilis, delanonis,* and *duncanensis* as synonyms). Baur (1892) responded by recognizing *albemarlensis, bivittatus, delanonis, duncanensis, grayi, indefatigabilis*, and *pacificus* as valid species, raising *T. pacificus habeli* to species status, and naming two new taxa, *T. jacobi* and *T. barringtonensis*. Heller (1903) described a new subspecies, *T. grayi magnus*, placed *barringtonensis* as a subspecies of *grayi*, and considered *abingdonensis* as a synonym of *pacificus*. He also recognized the following species as valid: *duncanensis, delanonis, bivittatus,* and *habeli*.

Van Denburgh and Slevin (1913) recognized seven species of Galápagos lava lizards: *albemarlensis, bivittatus, delanonis, duncanensis, grayi, habeli,* and *pacificus*. Of the other species named by previous workers, Van Denburgh and Slevin considered *lemniscatus* as a synonym of *bivittatus* and *abingdonensis* as a synonym of *pacificus*. The remaining taxa were sunk into *albemarlensis* as members of one of two subspecies: *a. albemarlensis* (for *indefatigabilis, jacobi,* and *grayi magnus*) and *a. barringtonensis* (for *barringtonensis*). Van Denburgh and Slevin's treatment has not been challenged and is the currently accepted taxonomic arrangement for this group.

Frost (1992) reviewed the taxonomy of the *Tropidurus* group of lizards. This group is one of two monophyletic lineages recognized within the Tropidurinae (the other being the *Stenocercus* group; Table 1.1) and includes all of the Galápagos lava lizards

(Etheridge and de Queiroz, 1988; Frost and Etheridge, 1989). Using a maximum parsimony analysis of morphological characters, Frost (1992) reconstructed a phylogenetic tree in which *Tropidurus* formed two groups, the "Western *Tropidurus*" (which includes the Galápagos species) and the "Eastern *Tropidurus*"; these two groups were not each other's closest relatives (Fig. 1.3). Based on this tree, several taxonomic changes were proposed. A monotypic genus, *Plesiomicrolophus*, was erected for *Tropidurus koepckeorum*. The genus *Microlophus* was resurrected for all species of the former "Western *Tropidurus*", excluding *Plesiomicrolophus koepckeorum*. *Tropidurus* was redefined to include all former *Tropidurus* east of the Andes; in addition, *Plica*, *Strobilurus*, *Tapinurus*, and *Uracentron* were placed in synonymy with *Tropidurus* (Table 1.2). Geographic distributions for these genera are shown in Fig. 1.4.

C. Previous Phylogenetic Studies of Galápagos Lava Lizards

Only two previous studies have specifically investigated the phylogenetic relationships among Galápagos lava lizards. Interestingly, both of these studies have utilized molecular, rather than morphological, data. The first was an analysis of allozyme variation (Wright, 1983) in a total of 231 lava lizards, representing 18 localities from 15 islands. Wright analyzed the variation in 16 proteins encoded by 23 presumptive loci and obtained Roger's coefficients of genetic similarity between populations ranging from 0.293 to 0.990 (Fig. 1.5). From these data, Wright constructed a phenogram which showed two major lineages of lava lizards (Fig. 1.6). One of these lineages consisted of *bivittatus* and *habeli* whereas the other contained the remaining five species. The

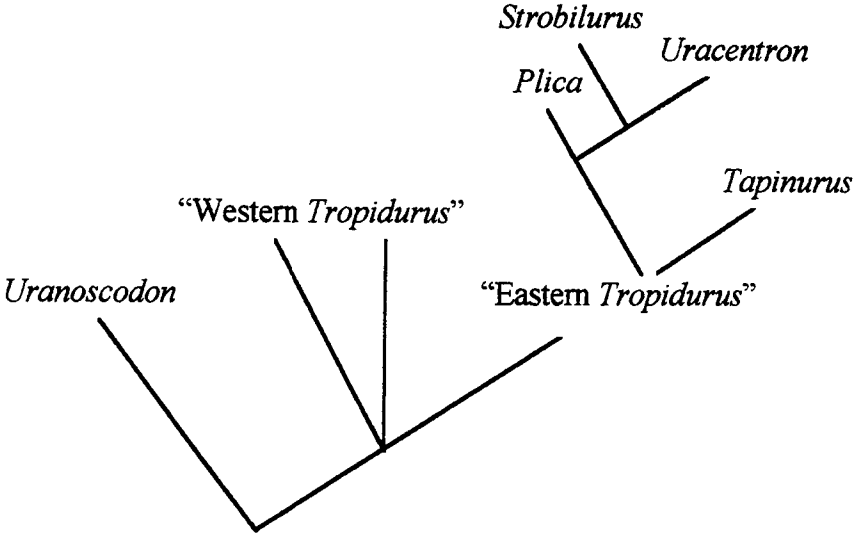


Figure 1.3. Phylogenetic relationships of the *Tropidurus* group of lizards as determined by Frost (1992). (Adapted from Frost, D. R. 1992. Phylogenetic analysis and taxonomy of the *Tropidurus* group of lizards (Iguania: Tropiduridae). American Museum Novitates **3033:**1-68.)

Table 1.2. Frost's (1992) taxonomic designations of the *Tropidurus* group of lizards[1]

Genus	Species		
Uranoscodon	*superciliosus*		
Plesiomicrolophus	*koepckeorum*[2]		
Microlophus	*albemarlensis*[2,3]	*heterolepis*[4]	*tarapacensis*[4]
	atacamensis[4]	*occipitalis*[2]	*theresiae*[4]
	bivittatus[2,3]	*pacificus*[2,3]	*theresioides*[4]
	delanonis[2,3]	*peruvianus*[4]	*thoracicus*[4]
	duncanensis[2,3]	*quadrivittatus*[4]	*tigris*[4]
	grayi[2,3]	*stolzmanni*[2]	*yanezi*[4]
	habeli[2,3]		
Tropidurus	*amathites*	*hispidus*[6]	*oreadicus*[6]
	azureus[5]	*hygomi*[6]	*pinima*[7]
	bogerti[6]	*insulanus*[6]	*plica*[8]
	cocorobensis[6]	*itambere*[6]	*psammonastes*[6]
	divaricatus	*lumarius*[8]	*semitaeniatus*[7]
	erythrocephalus[6]	*melanopleurus*	*spinulosus*
	etheridgei[6]	*montanus*[6]	*strobilurus*[9]
	flaviceps[5]	*mucujensis*[6]	*torquatus*[6]
	helenae[7]	*nanuzae*	*umbra*[8]

[1] *Source:* Frost, D. R. 1992. Phylogenetic analysis and taxonomy of the *Tropidurus* group of lizards (Iguania: Tropiduridae). American Museum Novitates **3033:**1-68.

[2] Former member of Frost's (1992) *Tropidurus occipitalis* group.

[3] Galápagos species.

[4] Former member of Frost's (1992) *T. peruvianus* group.

[5] Former member of *Uracentron.*

[6] Former member of Frost's (1992) *T. torquatus* group.

[7] Former member of *Tapinurus.*

[8] Former member of *Plica.*

[9] Formerly *Strobilurus torquatus.*

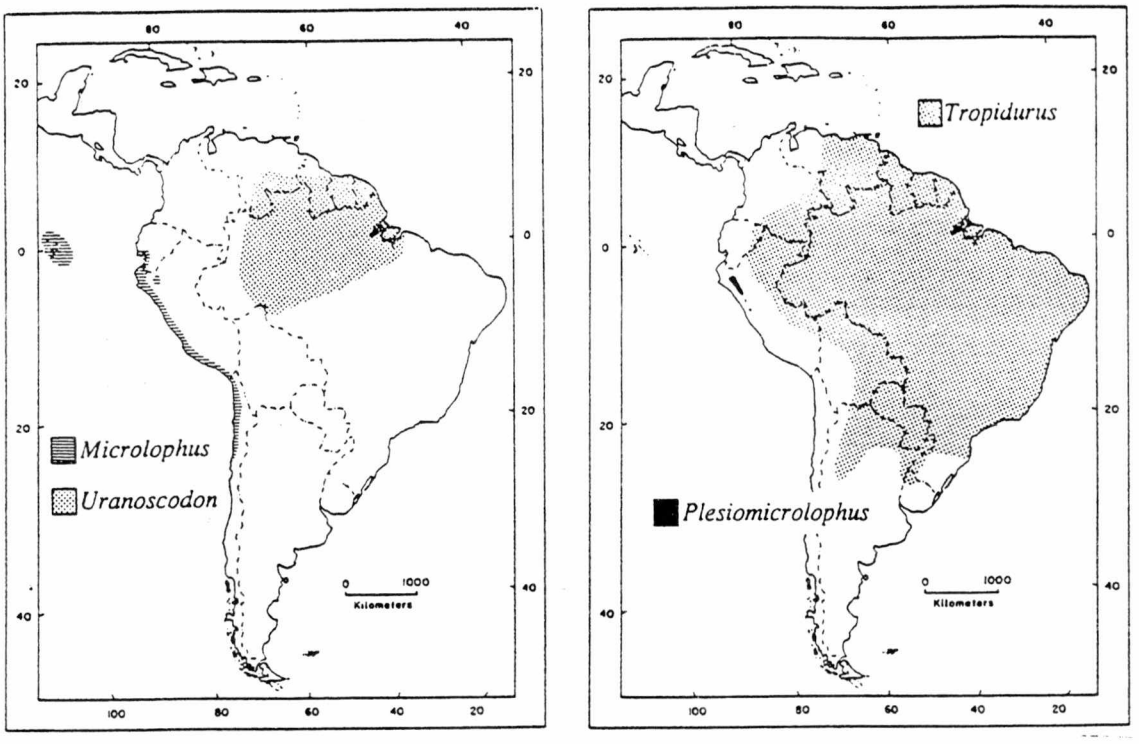


Figure 1.4. Geographic distribution of selected genera of the *Tropidurus* group of lizards. (Maps from Frost, D. R. 1992. Phylogenetic analysis and taxonomy of the *Tropidurus* group of lizards (Iguania: Tropiduridae). American Museum Novitates **3033:**1-68.)

	AB	CB	BA	DA	JB	BR	SB	CR	BC
Academy Bay (AB)	1.000	0.920	0.934	0.942	0.847	0.826	0.832	0.861	0.847
Conway Bay (CB)		1.000	0.969	0.961	0.846	0.857	0.841	0.844	0.845
Baltra (BA)			1.000	0.977	0.864	0.874	0.858	0.856	0.854
Daphne (DA)				1.000	0.872	0.863	0.861	0.865	0.863
James Bay (JB)					1.000	0.961	0.979	0.950	0.966
Bartolomé (BR)						1.000	0.964	0.934	0.947
Sullivan Bay (SB)							1.000	0.939	0.958
Cartago Bay (CR)								1.000	0.970
Black Cove (BC)									1.000
Fernandina (FE)									
Santa Fé (SF)									
Pinzón (PI)									
Wreck Bay (WB)									
Marchena (MA)									
Gardner (GA)									
Española (ES)									
Floreana (FL)									
Pinta (PN)									

Figure 1.5. Coefficients of genetic similarity (Roger's) among populations of Galápagos *Microlophus*. (*Source:* Wright, J. W. 1983. The evolution and biogeography of the lizards of the Galapagos Archipelago: evolutionary genetics of *Phyllodactylus* and *Tropidurus* populations. Pp. 123-155 *in* R. I. Bowman, M. Berson, and A. E. Leviton (eds.), Patterns of evolution in Galapagos organisms. Pacific Division of the American Association for the Advancement of Science, San Francisco.)

Figure 1.5 (continued)

	FE	SF	PI	WB	MA	GA	ES	FL	PN
Academy Bay (AB)	0.856	0.816	0.796	0.390	0.443	0.682	0.688	0.761	0.794
Conway Bay (CB)	0.889	0.857	0.817	0.362	0.416	0.696	0.701	0.780	0.820
Baltra (BA)	0.895	0.860	0.820	0.371	0.419	0.701	0.707	0.786	0.819
Daphne (DA)	0.886	0.858	0.818	0.376	0.422	0.690	0.696	0.771	0.817
James Bay (JB)	0.942	0.897	0.823	0.385	0.465	0.797	0.803	0.880	0.864
Bartolomé (BR)	0.957	0.912	0.831	0.371	0.458	0.819	0.824	0.906	0.871
Sullivan Bay (SB)	0.938	0.893	0.818	0.380	0.460	0.807	0.813	0.881	0.858
Cartago Bay (CR)	0.948	0.903	0.836	0.390	0.470	0.789	0.795	0.861	0.866
Black Cove (BC)	0.953	0.906	0.836	0.394	0.476	0.794	0.800	0.874	0.868
Fernandina (FE)	1.000	0.952	0.870	0.378	0.460	0.794	0.799	0.874	0.903
Santa Fé (SF)		1.000	0.918	0.335	0.420	0.749	0.754	0.829	0.876
Pinzón (PI)			1.000	0.378	0.383	0.745	0.751	0.789	0.872
Wreck Bay (WB)				1.000	0.772	0.297	0.293	0.367	0.370
Marchena (MA)					1.000	0.347	0.343	0.460	0.464
Gardner (GA)						1.000	0.990	0.786	0.798
Española (ES)							1.000	0.785	0.797
Floreana (FL)								1.000	0.913
Pinta (PN)									1.000

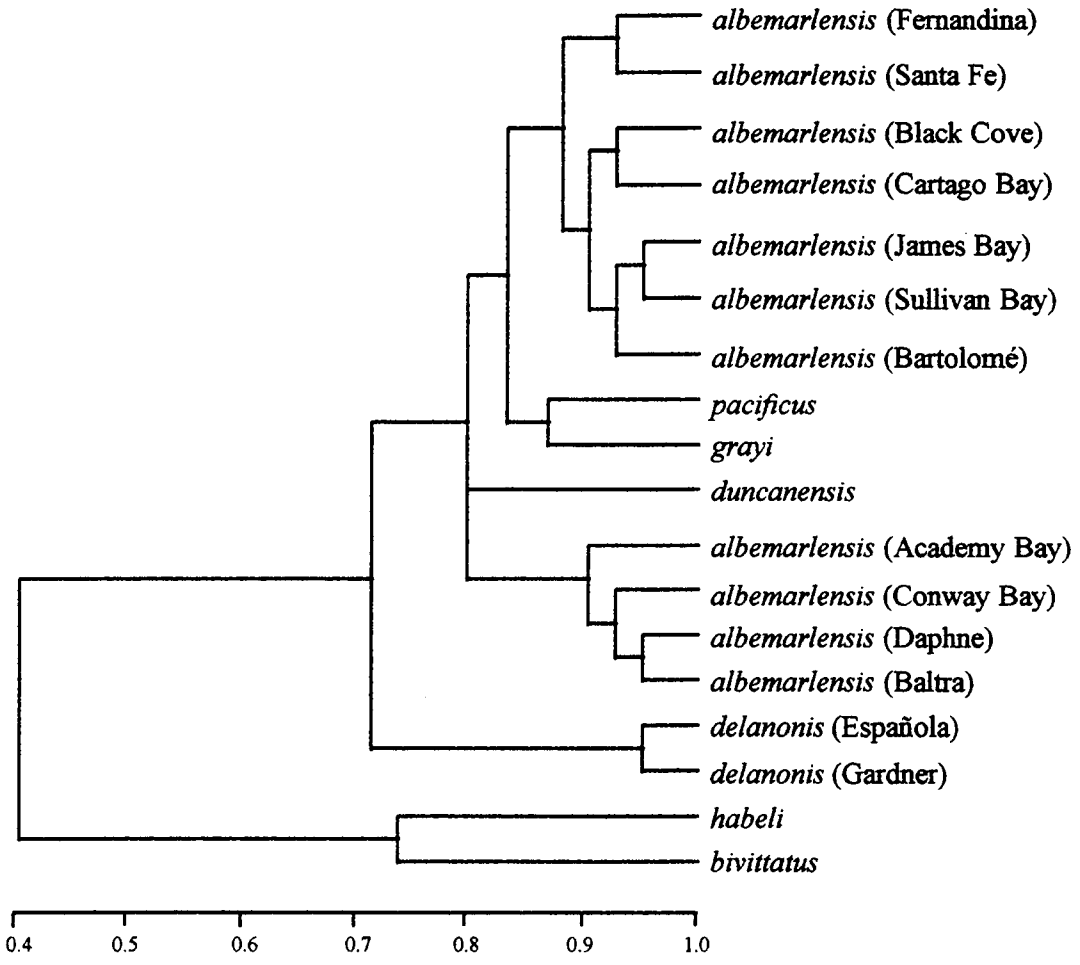


Figure 1.6. Phenogram for Galapágos *Microlophus* populations produced by UPGMA clustering of Roger's genetic similarity coefficients in Figure 1.5. (Adapted from Wright, J. W. 1983. The evolution and biogeography of the lizards of the Galapagos Archipelago: evolutionary genetics of *Phyllodactylus* and *Tropidurus* populations. Pp. 123-155 *in* R.I. Bowman, M. Berson, and A. E. Leviton (eds.), Patterns of evolution in Galapagos organisms. Pacific Division of the American Association for the Advancment of Science, San Francisco.)

populations of *bivittatus* and *habeli* were genetically more similar to three (unspecified) mainland species than to other Galápagos species. In the second major lineage of Wright's phenogram, the *delanonis* populations formed the most basal group. The 11 *albemarlensis* populations examined did not form a single monophyletic group, but rather were placed in two distinct clades. The first contained the populations from Santa Cruz, Baltra, and Daphne; the second consisted of the Fernandina, Santa Fe, Isabela, Santiago, and Bartolomé populations. The latter *albemarlensis* clade was placed as the sister group to a pairing of *grayi* and *pacificus*. This group was in turn placed in an unresolved trichotomy with *duncanensis* and the clade composed of the other *albemarlensis* populations. Based on these data, Wright (1983) postulated that the present-day populations were the result of two colonization events from South America.

The most recent phylogenetic treatment of Galápagos lava lizards by Lopez et al. (1992) utilized the quantitative immunological technique of micro-complement fixation (MCF). Albumins from 19 species of *Microlophus, Tropidurus, Stenocercus, Uranoscodon*, and *Sceloporus* (the outgroup) were used to calculate immunological distances (ID), where one ID unit is approximately equal to one amino acid substitution between the albumins of the taxa compared. For seven species, reciprocal immunological distances were obtained (Table 1.3) and used to construct a phylogenetic tree (Fig. 1.7). The island species *albemarlensis* and *pacificus* were placed as sister taxa and grouped with a clade consisting of *habeli* (an island species) and *occipitalis* (a mainland species). These four species were placed as the sister taxa to the mainland species *peruvianus*. Basal to this group was the island species *delanonis*. These data

Table 1.3. Reciprocal immunological distances among *Microlophus* species and *Sceloporus spinosus*[1]

	Immunological distance to albumin antiserum of:						
	AL	PA	HA	OC	PE	DE	SC
Antigen:							
M. albemarlensis (AL)	0	11	25	15	11	17	63
M. pacificus (PA)	9	0	36	11	32	30	80
M. habeli (HA)	22	19	0	8	37	17	82
M. occipitalis (OC)	26	19	13	0	33	16	80
M. peruvianus (PE)	30	36	24	28	0	28	87
M. delanonis (DE)	38	65	5	42	43	0	71
S. spinosus (SC)	77	79	84	98	74	81	0

[1] *Source:* Lopez, T. J., E. D. Hauselman, L. R. Maxson, and J. W. Wright. 1992. Preliminary analysis of phylogenetic relationships among Galapagos Island lizards of the genus *Tropidurus*. Amphibia-Reptilia **13**:327-339.

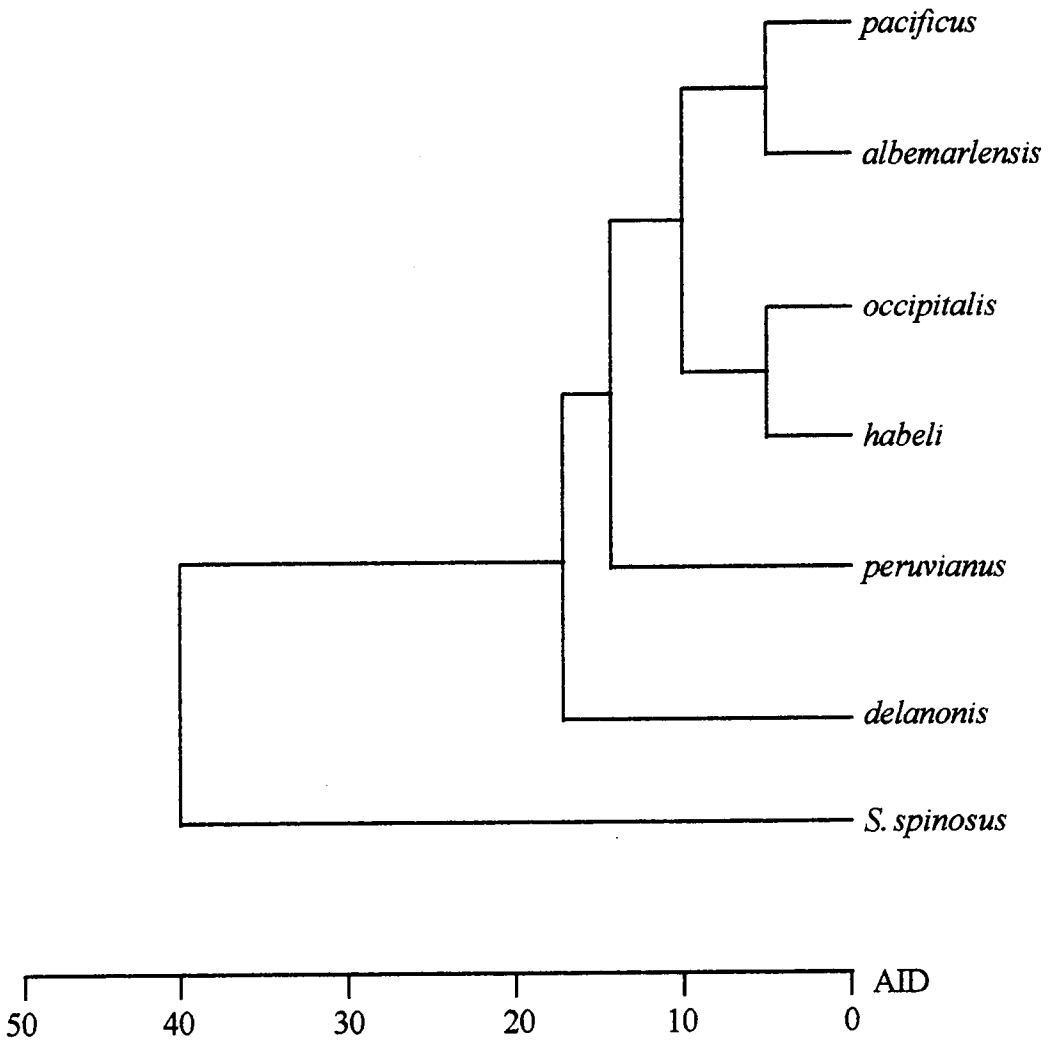


Figure 1.7. Phylogenetic relationships of *Microlophus* constructed by modified Wagner technique of averages of reciprocal immunological distances in Table 1.3. (Adapted from Lopez, T. J., E. D. Hauselman, L. R. Maxson, and J. W. Wright. 1992. Preliminary analysis of phylogenetic relationships among Galapagos Island lizards of the genus *Tropidurus*. Amphibia-Reptilia **13:**327-339.)

were interpreted as evidence that at least three separate invasions from South America were necessary to explain the present distribution and patterns of relatedness of lava lizards in the Galápagos Islands (Lopez et al. 1992). Additional one-way comparisons were made for taxa for which antisera were not available. Among these, seven populations of *albemarlensis* were compared to the albumin antiserum of an *albemarlensis* population from Academy Bay, Santa Cruz. The distances obtained ranged from 0 (for a second Santa Cruz population) to 20 ID units (for a Fernandina population; Table 1.4). Some distances measured were greater than those seen between populations which are recognized as separate species (Table 1.5). These data concur with the finding of Wright (1983) that the various populations of *albemarlensis* are not each others' closest relatives and do not form a monophyletic assemblage.

D. Biogeography of Galápagos Lava Lizards

Interpretation of the biogeographic history of the Galápagos Islands depends on the model assumed for the origin of the islands. Although various authors have proposed models suggesting that the islands were at one time connected to the mainland, either directly or through a series of "stepping-stone" islands (Van Denburgh and Slevin, 1913; Croizat, 1958; Rosen, 1975), it is now generally accepted that the islands have never experienced such a connection. The Galápagos Archipelago is of volcanic origin and the present-day islands comprise the top of the Carnegie Ridge, a submarine mountain chain which extends eastward almost to the mainland of South America. The Carnegie Ridge, in turn, rests on the Nazca Plate which is moving eastward at a rate of 50 km per million

Table 1.4. Comparisons of albumins of *Microlophus albemarlensis* populations to *M. albemarlensis* from Academy Bay, Santa Cruz[1]

Island	ID
Santa Cruz (Academy Bay)	0
Santa Cruz (Charles Darwin Research Station)	0
Sante Fé	2
Santiago	6
Isabela (Tagus Cove)	7
Bartolomé	12
Fernandina	20

[1] *Source:* Lopez, T. J., E. D. Hauselman, L. R. Maxson, and J. W. Wright. 1992. Preliminary analysis of phylogenetic relationships among Galapagos Island lizards of the genus *Tropidurus*. Amphibia-Reptilia **13:**327-339.

Table 1.5. One-way comparison of *Microlophus* and related species[1]

Species	ID to albumin antiserum of:					
	AL[2]	DE	HA	PA	PE	OC
M. grayi	6	11	17	8	30	10
M. bivittatus	20	26	7	17	35	9
M. duncanensis	12	21	18	11	33	17
M. theresiae	15	23	18	25	13	27
M. thoracicus	10	23	29	20	14	29
M. tigris	28	34	35	32	9	29
M. stolzmanni	16	22	28	24	26	23
Plesiomicrolophus koepckeorum	15	26	15	15	21	8
Tropidurus hispidus (French Guiana)	52	37	52	42	37	38
T. hispidus (Venezuela)	60	49	58	39	49	39
T. umbra	75	50	51	98	90	60
Stenocercus iridescens	67	57	69	65	65	71
Uranoscodon superciliosus	48	48	40	32	55	60

[1] *Source:* Lopez, T. J., E. D. Hauselman, L. R. Maxson, and J. W. Wright. 1992. Preliminary analysis of phylogenetic relationships among Galapagos Island lizards of the genus *Tropidurus*. Amphibia-Reptilia **13:**327-339.

[2] Abbreviations as in Table 1.3.

years (Cox, 1983). Origin of the islands is presumed to be the result of the movement of the Nazca Plate over a "hot spot" or plume of magma rising from the deep upper mantle (Wilson, 1963; Holden and Dietz, 1972; Cox, 1983; Sinton et al. 1996). As the plate moves over the hot spot, volcanic eruptions on the ocean floor build a topographic ridge capped by volcanic islands. The age of these islands increases with increasing distance from the center of active volcanism. Holden and Dietz (1972) concluded, based on relative plate motions of the Nazca Plate, that the age of the hot spot is about 40 million years. More recent compositional studies and plate reconstructions link portions of the Caribbean Plate to the initiation of Galápagos volcanism (Duncan and Hargraves, 1984; Sen et al. 1988; Hauff et al. 1997) and suggest that the hot spot may be as old as 80-90 million years. However, unlike a "simple" hot spot, such as Hawaii, where the active volcanism is confined to a relatively restricted area, the Galápagos hot spot exhibits a widespread and dispersed pattern of volcanism (White et al. 1993; Sinton et al. 1996); at least nine widely distributed volcanoes have been historically active in the archipelago.

Christie et al. (1992) presented evidence that suggested that islands may have existed through the entire history of the Galápagos hot spot. Basalt cobbles were dredged from submerged seamounts and other sites "downstream" of the hot spot which showed evidence of erosion at or near sea-level. Chemical composition of the basalts were consistent with an origin at the Galápagos hot spot. Radiometric dating of these cobbles ranged from 5.3 to 9.1 million years, with age increasing with increasing distance from the hot spot. These data were taken as evidence that islands have existed for at least 9 million years (and probably much longer) over the hot spot.

The ages of the present-day islands have been determined through radiometric dating, magnetic polarity reversals, and marine stratigraphy and paleontology (Cox, 1983, and references within; Hickman and Lipps, 1985). The oldest islands are in the eastern portion of the archipelago and are estimated to be approximately 3.4 to 4.5 million years old. Some of the western islands (e.g. Isabela and Fernandina) have been dated at less than 700,000 years old.

Organisms may have been present in the archipelago for much longer than the ages of the present islands would allow. An immunological study investigating the relationships between Galápagos land and marine iguanas calculated a divergence time between these taxa of 15-20 million years (Wyles and Sarich, 1983). More recently, analysis of sequence divergence in two mitochondrial genes suggest at least ten million years of separation between these taxa (Rassmann, 1997). In their study of lava lizards, Lopez et al. (1992) estimated an average time of divergence of Galápagos species from mainland species of 35 million years ago and times of divergence among Galápagos species ranging up to 34 million years. These ages support the theory that the present-day islands do not represent the complete geologic history of the archipelago, but are merely the most recent emergent islands in a long chain.

Wright (1983) proposed a scenario for the dispersal of *Microlophus* to the Galápagos which involved two founder events whereas Lopez et al. (1992) proposed at least three founding events. The two models also differ in the timing and pattern of colonization. These differing explanations for the arrival of *Microlophus* to the Galápagos underscore the need for further investigation into the patterns of colonization

of the region. Analysis of molecular data has been used repeatedly to illuminate patterns of biogeography (Wright, 1983; Maxson, 1984; Maxson and Roberts, 1984; Bermingham and Avise, 1986; Riddle and Honeycutt, 1990; Hass and Hedges, 1991; Hedges et al. 1991; Lopez et al. 1992; Hedges et al. 1993*b*). By obtaining a more direct measure of the genetic relatedness between mainland and Galápagos species of *Microlophus*, it should be possible to propose a hypothesis regarding the biogeographical history of this group. This study uses direct sequencing of two mitochondrial genes to obtain such data.

E. Utility of DNA Sequence Analysis

The development of the polymerase chain reaction (PCR) to amplify regions of DNA (Saiki et al. 1988) has provided the means for obtaining substantial quantities of DNA template. This has allowed nucleotide sequencing to become a rapid and efficient method for generating sequence data for analysis. The PCR can produce millions of copies of a specific region of DNA in less time, more easily, and at a lower cost than can be obtained by standard cloning methods. Another advantage of the PCR is that, because of its sensitivity, one can utilize almost any tissue sample, provided at least some DNA remains. A very small tissue sample, such as diluted plasma (Hedges et al. 1990), or preserved tissue (Pääbo, 1989) is usually sufficient to enable the PCR to produce large quantities of DNA. Following amplification, standard sequencing procedures (Sanger et al. 1977; Hedges et al. 1991) are utilized to determine nucleotide sequences.

DNA sequence comparison and analysis has become a useful and powerful research method and has been used to investigate the phylogeny and systematics of a

wide range of taxa, from prokaryotes to plants to fungi to the vertebrates (Kocher et al. 1989; Hedges et al. 1990, 1991; Albert et al. 1992; Bowman et al. 1992; Cunningham et al. 1992; Hedges et al. 1992, 1993*a*, 1993*b*; Kuhnert et al. 1996). The routine use of DNA sequence analysis is due (in part) to its ability to provide a direct measure of genetic relationships. In contrast, the amount of morphological change observed between taxa may be completely uncorrelated with the level of genetic differentiation between them (Wallace et al. 1971; Maxson and Wilson, 1974; Meyer et al. 1990; Sturmbauer and Meyer, 1992). In a similar manner, allozyme studies can only identify amino acid substitutions that result in changes in protein mobility while amino acid changes that do not involve a change in charge often go undetected. MCF detects alterations in antigenic sites due to changes in the amino acid sequence of a protein molecule but, similar to allozyme studies, cannot detect synonymous nucleotide substitutions. By directly sequencing DNA, both synonymous and non-synonymous substitutions can be detected and a more accurate measure of the genetic divergence between taxa may be obtained. This level of sensitivity makes DNA sequence data a powerful and valuable tool for the determination of the phylogenetic relationships of Galápagos lava lizards.

F. Advantages of DNA Sequence Data

Much of the systematic research on *Microlophus* has utilized morphological characters (see references above). Although this approach is useful and yields valuable information, it has limitations. One of the drawbacks in relying solely on morphological data is that not all organisms have the same morphological characters. It is also often

difficult to assign the proper polarity to morphological characters, which can detract from the efficiency in recovering the correct phylogeny. Another challenge lies in establishing the homology of a structure. The similarity of a morphological feature between taxa may be due to one of two reasons: it may be derived from the same structure in a common ancestor, or it could have arisen independently in the lineages under examination. For example, Russell (1976) has shown that similar toe pad structure, a common morphological feature used to decipher gekkonid lizard relationships, can arise in phylogenetically distant lineages due to parallel evolution. Parallel and/or convergent evolution has often made the illumination of phylogenetic relationships difficult (Russell, 1976; Kluge, 1977) since a phylogenetic reconstruction using a non-homologous morphological character may erroneously assign a closer relationship between taxa than is warranted.

Molecular data can be valuable in the resolution of the problem of parallel evolution. Analyses of DNA sequences are effectively resistant to the effects of convergence because of the complexity of the DNA molecule and the enormous number of possible configurations for a given nucleotide sequence (see Patterson, 1988, for a thorough discussion). Furthermore, morphological evolution has been shown to proceed independently of evolution at the molecular level (Wallace et al. 1971; Maxson and Wilson, 1974). By using molecular methods, phylogenetically informative data may be gained from taxa that, due to convergence, lack obvious morphological differentiation yet are genetically distinct from one another.

Another useful aspect of DNA sequence data is that different genes evolve at different rates (Miyata et al. 1980; Li et al. 1985; Hillis and Dixon, 1991). Through careful choice of the gene to sequence, one can ensure that the data set will contain appropriate levels of variation to yield phylogenetically useful information. Certain nuclear ribosomal RNA (rRNA) genes evolve extremely slowly, enabling examination of the most ancient evolutionary events (Hillis and Dixon, 1991). The mitochondrial (mt) genome has an overall rate of evolution that is much faster than that of the nuclear genome (Brown et al. 1982), yet individual mt genes exhibit sufficient variation in rates to allow a wide array of questions to be addressed. Analyses of sequences from the mt 12S and 16S rRNA genes have been used to investigate relationships between organisms that diverged up to 400 million years ago (Hedges et al. 1990, 1991, 1993*a*, 1993*b*), whereas the mt cytochrome *b* gene exhibits approximately 2.0 - 2.5% sequence divergence per million years (Irwin et al. 1991; Hedges et al. 1992) and thus can be used effectively for studies of organisms that have diverged relatively recently. By sequencing different regions of the mt genome, this study will attempt to discern the relationships of the Galápagos lava lizards to each other as well as to their mainland relatives.

Chapter 2

Materials and Methods

A. Taxa Examined

The focus of this study is the Galápagos lava lizards; all seven species are represented, totaling thirty-six populations from twenty-four islands and islets. In addition, twelve mainland species have been sampled. These taxa include representatives from the tropidurid genera *Leiocephalus*, *Microlophus*, *Plesiomicrolophus*, *Stenocercus*, *Tropidurus*, and *Uranoscodon* as well as the phrynosomatid genus *Sceloporus* (as the outgroup taxon). The sampled taxa include all of the genera re-designated by Frost (1992) as members of the Tropidurinae (*Microlophus*, *Plesiomicrolophus*, *Stenocercus*, *Tropidurus*, and *Uranoscodon*) and a single representative from the Leiocephalinae (*Leiocephalus*). *Sceloporus* was chosen as the outgroup for two reasons: (1) "sceloporine" lizards have been suggested to be the sister group to the tropidurines (Frost and Etheridge, 1989), and (2) the data already was present in GenBank, alleviating the need to obtain it firsthand. Appendix A lists the species examined and the sources from which tissues were obtained.

B. Laboratory Procedures

1. Tissue preparation and DNA extraction. Total genomic DNA was extracted from liver, tissue homogenates, red blood cells or whole toes clipped from living animals as part of a mark-recapture study. With the exception of the clipped toes, which were

stored in a desiccant at room temperature, tissue was stored at either -20°C or -70°C. DNA was extracted directly from frozen tissue if available. Clipped toes were placed in a 0.5 ml eppendorf tube with 500 microliter (µl) sterile distilled water and soaked for twelve to twenty-four hours. The toes were then cut into fragments using a sterile razor blade, added to 500 µl DNA extraction buffer (see below) in a 1.5 ml eppendorf tube, and ground with a pestle. DNA extraction then proceeded as detailed below.

Extraction of total DNA from tissue samples was achieved using a modification of the procedure of Kocher et al. (1989). An approximate ten µl volume of tissue (or 25-50 µl aliquot of fluid sample) was added to 500 µl DNA extraction buffer (Appendix B) in a 1.5 ml eppendorf tube. The sample was then incubated at 37°C for two to three hours under constant agitation (Thermolyne Rotomix Type 50800) to allow for Proteinase K digestion. After this period, two extractions with an equal volume of buffered phenol (Appendix B) were performed to remove proteinaceous fractions. Any residual phenol was removed by two extractions with a chloroform:isoamyl alcohol (24:1) solution. After each extraction, the aqueous fraction was transferred to a new 1.5 ml eppendorf tube. Two volumes of -20°C absolute ethanol and one tenth total volume (combined volume of aqueous fraction and ethanol) of 3 M sodium acetate were added to the final aqueous fraction and stored overnight at -70°C for DNA precipitation. DNA was recovered by 10 minutes of centrifugation at 14,000 RPM. The aqueous solution was removed and the DNA pellet was washed with 25 µl 70% ethanol to remove excess salts. The pellet was vacuum-dried for 30 minutes, resuspended in 10 µl sterile distilled water or a Tris-EDTA solution (TE; Appendix B), and stored at -20°C. The concentrated

DNA was diluted with sterile distilled water for use in PCR reactions; dilutions ranged from 1:10 to 1:1000, depending on the size and quality of the original DNA pellet.

2. Polymerase chain reaction and DNA amplification. The desired gene fragments were amplified from the dilutions of total genomic DNA by standard procedures of PCR (Saiki et al. 1988). All PCR reactions were conducted in a Perkin-Elmer-Cetus DNA Thermal Cycler. Double-stranded reactions were performed in 25 µl volumes in 0.5 ml eppendorf tubes (see Appendix B for specific description of reagents involved). Typical thermal regimes for double-stranded amplifications were one minute at 95°C for duplex strand denaturation, one minute at 50°C for primer annealing, and two and a half minutes at 72°C for DNA synthesis. Usually thirty cycles were sufficient to produce double-stranded PCR products; however, this value ranged from twenty-five to forty cycles, depending on the sample involved, to optimize the quality of the amplified DNA (as determined by visual inspection of the fragment; see below). Following amplification, 10 µl of each PCR reaction was visualized with 1% ethidium bromide after electrophoresis on a 2% agarose gel (Appendix B); this allowed one to inspect template quality, to ensure that the appropriate fragment size had been obtained, and to detect any contamination.

After a successful double-stranded amplification, the target template had to be isolated. Ten µl of the double-stranded PCR product was electophoresed on a 2% low-melting-point agarose gel (Nusieve, *BRL*; Appendix B), and a portion of each band was removed and dissolved in 500 µl sterile distilled water. One µl of this solution then served as the template in an unbalanced PCR reaction (Gyllensten and Erlich, 1988) to generate single-stranded DNA. This reaction differed from that used to amplify double-

stranded DNA in that reaction volumes were 50 µl, rather than 25 µl, and the annealing temperature was 60°C, rather than 50°C. Thirty cycles of amplification were typically performed, although this number ranged from twenty-five to forty. As before, 10 µl of the PCR product was visualized with 1% ethidium bromide after electrophoresis on a 2% agarose gel to inspect single-stranded DNA quality, to ensure appropriate fragment size, and to check for contamination.

After single-stranded DNA amplification, the PCR products were purified and reduced to a final volume of 7 - 8 µl by centrifuging in Millipore Ultrafree-MC filters (30,000 molecular weight retention). This solution was used as the template for DNA sequencing.

3. DNA sequencing. Dideoxy chain termination sequencing followed a modification of the method of Sanger et al. (1977). The protocol is outlined in Appendix C. Polyacrylamide sequencing gels (Appendix B) were dried in a Bio-Rad Model 583 Gel Dryer and autoradiograph film was exposed to the dried gel. This film was developed after two to twenty-one days of exposure, depending on the quality of the template used in the sequencing reactions. Each gene fragment was sequenced in both directions to ensure that the entire nucleotide sequence internal to the oligonucleotide primer pairs was obtained.

C. Genes Sequenced

Portions of the mt cytochrome *b* gene and the mt 16S rRNA gene were amplified and sequenced (Fig. 2.1). The oligonucleotide primers used for the cytochrome *b* region

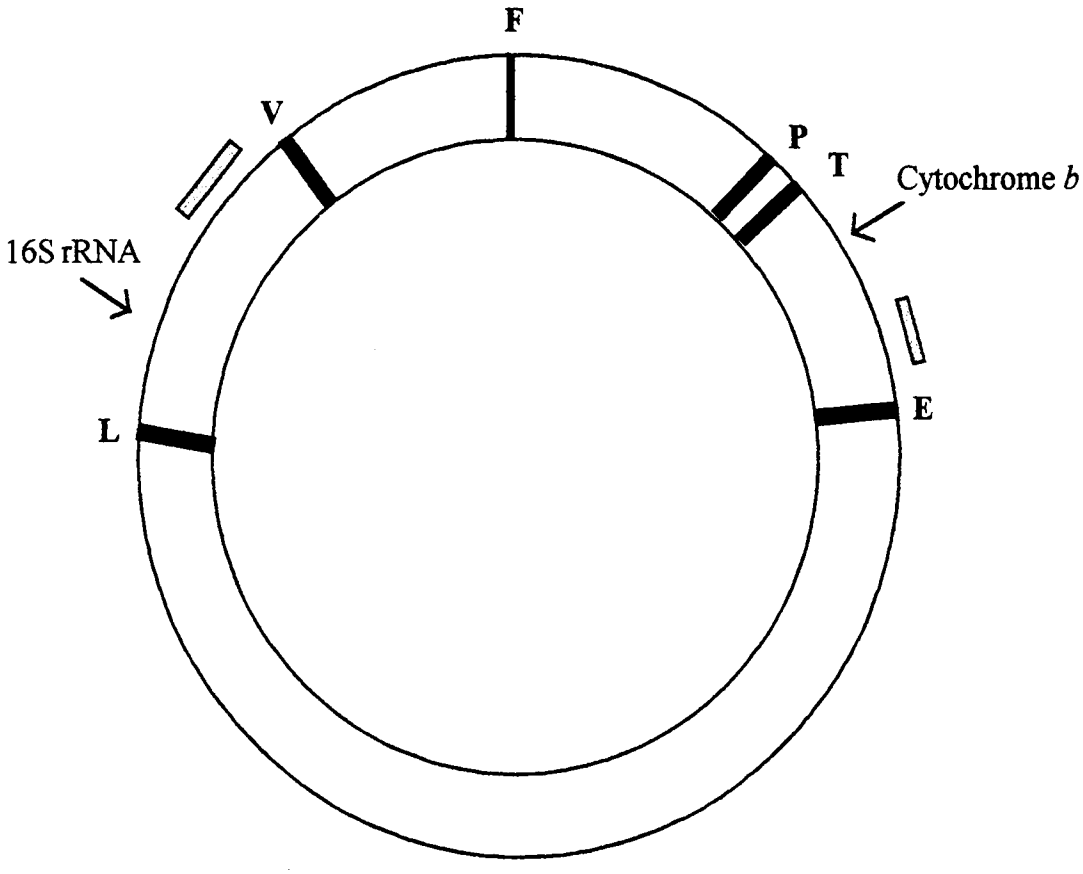


Figure 2.1. The generalized vertebrate mitochondrial genome. The gray-shaded bars indicate the relative sizes and postions of the regions of the mitochondrial genes which were amplified and sequenced. The bold single letters correspond to transfer RNA genes. (Adapted from Wallace, D. C. 1992. Mitochondrial genetics: a paradigm for aging and degenerative diseases? Science **256:**628-632.)

of interest were those designed by Kocher et al. (1989) for vertebrates and the 16S rRNA primers were designed by Hedges et al. (1993*b*) for amphibians; the nucleotide sequences for these primers are shown in Table 2.1. The regions between the primers include a 307 base pair (bp) portion of the cytochrome *b* gene and an approximately 469 bp portion of the 16S rRNA gene corresponding to positions 16,347 - 16,653 and 4,079 - 4,547, respectively, of the published *Xenopus laevis* mt genome (Roe et al. 1985).

D. Data Analysis

1. Alignment methods. The tonal digitizing program GELIN (S. W. Schaeffer, Pennsylvania State University) was used to create a computer file of the DNA sequences from autoradiograph films. In some instances, because of poor quality of the film, sequences were typed in by hand. Each segment of sequence was read twice and the individual readings were compared to check for errors. After a consensus sequence was determined, overlapping segments were joined to create a single sequence for a particular taxon. Alignment of multiple sequences was performed by eye using the manual alignment program Eyeball Sequence Editor (ESEE; Cabot and Beckenbach, 1989). Alignment of multiple sequences was simplified by matching the oligonucleotide primer sequences. In the 16S rRNA sequences, the number of gaps inserted to optimize alignments and account for insertion/deletion events was minimized.

2. Methods of phylogenetic analysis. DNA sequence data can be treated either as character data or as distance data. If character data are chosen, each nucleotide position of a particular sequence is an individual character with four possible character states

Table 2.1. Oligonucleotide primers used for DNA amplification and sequencing

Primer designation	Nucleotide sequence
CYTBL	5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3'
CYTBH	5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3'
16S1L	5'-CTGACCGTGCAAAGGTAGCGTAATCACT-3'
16S1H	5'-CTCCGGTCTGAACTCAGATCACGTAGG-3'

corresponding to the four possible nucleotides (Swofford and Olsen, 1990). This method requires not only that the molecules but the nucleotide positions be homologous (Patterson, 1988; Titus and Frost, 1996). Character data have several advantages over distance data:

1. It is relatively easy to add information on new taxa to a data set.
2. Different sources of data (i.e., other molecules or types of attributes) can be easily combined for analysis.

DNA sequences can also be treated as distance data, resulting in estimates of genetic relatedness between pairs of taxa (Swofford and Olsen, 1990). Two reasons for this approach are of importance:

1. A sequence alone has little or no meaning. It is the relationship between sequences that provide information, not the sequences themselves.
2. As mutations accumulate in a given sequence, the chance of multiple changes occurring at a single position increases. By use of assumptions concerning the nature of evolutionary change, a correction for these multiple substitutions can be obtained. These corrections may be applied to a table of distances between pairs of sequences, but not to the sequences themselves. In the same manner, corrections for codon usage bias also can be incorporated into a distance matrix.

Different algorithms exist for inferring phylogenetic trees from different types of data. Maximum parsimony utilizes character data, whereas neighbor-joining, maximum likelihood, and minimum evolution use distance data. Two computer programs were

used to perform the phylogenetic analyses in this study. MEGA (Molecular Evolutionary Genetics Analysis) Version 1.02 can be used to analyze DNA, RNA, or amino acid sequence data with distance algorithms or parsimony methods (Kumar et al. 1994). Trees can be constructed by neighbor-joining, maximum parsimony, or UPGMA (unweighted pair group method using arithmetic means). Branch lengths and their standard errors can be computed for the trees. For maximum parsimony, both branch-and-bound and heuristic search options are available. Numerous measures of genetic distance (e.g. Jukes-Cantor, Kimura 2-parameter correction for transition/transversion bias, gamma distance) can be calculated in MEGA for use with the distance-based algorithms. MEGA also can perform the bootstrap and confidence probability tests on reconstructed trees.

PAUP (Phylogenetic Analysis Using Parsimony) Version 3.1.1 (Swofford, 1993) can be used to perform discrete character analyses of nucleotide and amino acid sequences. Parsimony methods use only those nucleotide sites which exhibit synapomorphies when reconstructing a phylogeny; the topology (or topologies) with the fewest number of substitutions required to explain the data is (are) chosen as the best estimation of the true phylogeny

Numerous studies have tried to determine which algorithm is most likely to yield the closest approximation to the true phylogeny (Saitou and Nei, 1987; Saitou and Imanishi, 1989; Nei, 1991; Hillis et al. 1992, 1994; Tateno et al. 1994). When a constant rate of nucleotide substitution is assumed, neighbor-joining shows a greater efficiency of recovering the correct topology than UPGMA, distance Wagner, modified Farris,

maximum likelihood, and transformed distance methods (Nei, 1991). This holds true whether the expected number of nucleotide substitutions per site is small or large. Maximum parsimony is less likely to obtain the correct tree than neighbor-joining when the expected number of substitutions per site is large, but is as good as neighbor-joining when this value is small, provided a large number of nucleotide sites is examined.

If a varying rate of substitution is assumed, the neighborliness method, neighbor-joining and the minimum evolution method are nearly equally efficient and show a better performance than other methods (Nei, 1991; Tateno et al. 1994). Neighbor-joining produces the same tree as minimum evolution in most cases and, when it does not, the tree obtained is usually very close to the minimum evolution tree. However, neighbor-joining requires much less computer time. Neighborliness and neighbor-joining seem to be equally efficient in obtaining the correct tree; neighbor-joining has the advantage in that it gives not only the topology but also estimates of the branch-lengths of the tree produced.

For this study, the neighbor-joining (NJ) algorithm (Saitou and Nei, 1987) was used to reconstruct phylogenetic trees. A number of different methods were available to estimate the genetic distance between nucleotide sequences and correct for multiple substitution events at a single nucleotide site. The method of Jukes and Cantor (1969) is a single parameter method which assumes that the rate of nucleotide substitution is the same for all pairs of the four nucleotides; it gives a maximum likelihood estimate of the number of substitutions between two sequences. Kimura's (1980) 2-parameter distance allows for differing rates of transitional versus transversional nucleotide substitutions.

Tajima and Nei's (1984) model accounts for situations where the four nucleotide frequencies deviate substantially from 0.25. Tamura (1992) developed a maximum likelihood method in which both unequal nucleotide frequencies and differing rates of transitional versus transversional substitutions are considered in calculating the genetic distance between two sequences. Tamura and Nei's (1993) model corrects for transition-transversion and G+C-content biases. All of the distance measures described so far assume that the rate of nucleotide substitution is the same for all nucleotide sites. However, this assumption may not be valid; numerous studies have suggested that the rate varies from site to site according to the gamma distribution (Uzzell and Corbin, 1971; Kocher and Wilson, 1991; Tamura and Nei, 1993; Wakeley, 1993, 1994). Gamma distances can be calculated using the Jukes-Cantor, Kimura 2-parameter, and Tamura-Nei corrections to correct for any such rate heterogeneity.

Maximum likelihood methods for constructing phylogenetic trees were not used in this study due to the prohibitively large computational demands required by available algorithms when dealing with large numbers of taxa. However, Tateno et al. (1994) suggested that NJ can perform at least as efficiently as maximum likelihood methods and that, when used with gamma distances, NJ can be more efficient than maximum likelihood methods. Therefore, several gamma distances were calculated and used to construct NJ phylogenies.

Numerous simulation studies have found that the NJ, maximum likelihood, and minimum-evolution algorithms are, in many circumstances, superior to maximum parsimony (MP) for reconstructing a "known" phylogeny (Saitou and Imanishi, 1989;

Nei, 1991; Hillis et al. 1992; Tateno et al. 1994). These methods may be less affected by substitution biases or unequal rates of substitutions among lineages than is MP. It should be noted that in many cases where NJ supports a certain tree topology, MP gives similar results (Hillis et al. 1992; Tateno et al. 1994). Both NJ and MP methods were used to reconstruct phylogenetic trees in this study.

3. Statistical tests. All results were subjected to the standard error and/or bootstrap tests to estimate statistical confidence limits of the nodes in the tree topologies. In the standard error test, once a neighbor-joining tree has been obtained, the branch lengths of the tree are re-estimated by using the ordinary least squares method, and the standard errors of the estimates are computed (Rzhetsky and Nei, 1992, 1993; Kumar et al. 1994). Each interior branch is then tested via a *t*-test to determine if its length is significantly different than zero. MEGA converts the complement of the probability of Type I error (1-a) into a confidence probability for the reliability of any given branch length. Recent computer simulations have shown that the standard error test is statistically more reliable than is bootstrapping (Sitnikova et al. 1995).

Bootstrapping is a method by which data is sampled with replacement a number of times (Felsenstein, 1985). The value of the bootstrap value is often debated. Hillis and Bull (1993) conclude that the bootstrap is usually a conservative measure of the accuracy of a phylogenetic tree (with accuracy being defined as "the probability that a specified group is contained in the true phylogeny") and so should be used only as an relative ranking of the degree of support for the various groups recovered in a given analysis. Sanderson (1989) argued that such rankings are superior to other measures and

thus are of value. Felsenstein and Kishino (1993) propose the following interpretation for bootstrap *p*-values. For a group with a bootstrap *p*-value of x, one should regard 1 - x as a conservative estimate of the probability of getting that much data supporting the group if it is not present. For example, if a group is seen to have a *p*-value of 0.85, then that much evidence supporting the group would be expected less than 15% of the time if that group were not present in the true phylogeny. Hedges (1992) indicates that at least 2000 cycles of bootstrapping are required to determine statistically significant confidence values.

Chapter 3

Cytochrome B Sequence Variation and Phylogenetic Relationships of Galápagos Lava Lizards

A. Introduction

In 1836, after leaving the Galápagos, Charles Darwin wrote in his Ornithology Notes "the zoology of Archipelagoes will be well worth examination" (Barlow, 1963). This prediction has been well supported. The study of island biogeography has contributed much to evolutionary biology, from understanding how organisms colonize new habitats to discerning how isolation contributes to the formation of new species. Indeed, many distinguished pioneers of evolutionary biology (e. g. Darwin, Alfred Russell Wallace and Joseph Hooker) were greatly influenced by their work on islands. Wallace spent a number of years collecting specimens in the Malay Archipelago whereas Hooker visited New Zealand, Tasmania, and Kerguelen Island. But, arguably, the most famous set of islands visited by any biogeographer is the Galápagos Archipelago. Although Darwin was impressed most by the variation he observed in mockingbirds, numerous other groups of organisms exhibit similar patterns of inter-island differences; among these are the lava lizards. Through a more thorough knowledge of the phylogenetic and biogoegraphic history of these lizards, a better understanding of the evolutionary process may be obtained.

To investigate the phylogenetic relationships between the various island populations of Galápagos lava lizards as well as to assess their relatedness to the mainland species, I examined the variation in DNA sequences of a portion of the mt cytochrome *b* gene. Sequence data were chosen in preference to morphological or indirect molecular data for the reasons discussed in Ch. 1.

B. Materials and Methods

The taxa sampled included all seven of the Galápagos lava lizards (*Microlophus albemarlensis, M. bivittatus, M. delanonis, M. duncanensis, M. grayi, M. habeli,* and *M. pacificus*). Whenever possible, sequence was obtained for more than one population of each species. In addition, *M. albemarlensis* was more thoroughly sampled than the other Galapágos taxa, because it is the only species to occur on more than a single island. This resulted in data for a total of thirty-six populations from sixteen islands in the Galápagos. The remaining taxa for which sequence data were obtained are mostly mainland South American species; they include five *Microlophus* species (*occipitalis, stolzmanni, theresiae, thoracicus,* and *tigris*), the monotypic *Plesiomicrolophus koepckeorum*, two species of *Tropidurus* (*torquatus* and *umbra*), and *Leiocephalus melanochlorus*. All of these taxa are members of the family Tropiduridae (*Leiocephalus* in the subfamily Leiocephalinae and the others in the subfamily Tropidurinae). The outgroup taxon was *Sceloporus undulatus*. Appendix A lists all taxa included in the study.

The species were chosen to represent the major species groups within the Tropidurinae as recognized by Frost (1992). The *occipitalis* group is comprised of the

seven Galápagos species as well as *M. occipitalis* and *M. stolzmanni* whereas the *peruvianus* group contains ten species and is represented in this study by *M. theresiae, M. thoracicus,* and *M. tigris*. The "eastern *Tropidurus*" group is represented by *T. torquatus* and *T. umbra*. *Plesiomicrolophus koepckeorum* was previously placed within the *occipitalis* group but its affiliations are now uncertain (Frost, 1992). It is included here both to ensure complete sampling of all putative species groups as well as in the hopes of resolving its phylogenetic position within the Tropidurinae.

DNA amplification and sequencing were performed as outlined in Ch. 2 and Appendix C. Both the heavy and light strands of the 307 bp region of the mt cytochrome *b* gene described in Ch. 2 were amplified for all taxa. For one sample of *Microlophus grayi* (gray 1; Appendix A), repeated attempts at sequencing the heavy strand were unsuccessful. As a result, only 234 bp of sequence were obtained for this population.

Numerous evolutionary distance methods were used to estimate nucleotide sequence divergence and to calculate genetic distances. These methods include the Jukes-Cantor, Kimura 2-parameter, Tajima-Nei, Tamura, and Tamura-Nei distances; gamma distances using the Jukes-Cantor, Kimura 2-parameter, and Tamura-Nei correction models were calculated to account for possible site-specific variation in the rates of substitution (Tamura and Nei, 1993; Wakeley, 1993; Kumar et al. 1994). Evolutionary distances were determined after discarding all sites for which there were missing or ambiguous data.

C. Results

1. Sequence variation. Figure 3.1 shows the cytochrome *b* sequences (307 bp) for forty-five tropidurid and one outgroup taxa. These sequences varied greatly across taxa (approximately 50% of all sites were variable) and 74 sites exhibited two or more nulceotides. Nucleotide composition for these sequences were approximately 26.6% A, 29.0% T, 27.8% C, and 16.5% G. The relatively low percentage of G residues is even more extreme if just the third codon position is examined; only 5.7% of third position nucleotides are G, compared to 26.1% and 17.7%, respectively, for first and second positions. However, numerous other studies of the mt cytochrome *b* gene show a similar low percentage of G residues in such diverse taxa as mammals (Irwin et al. 1991), toads (Graybeal, 1993), snakes (Lopez, 1994), and rodents, birds, and fishes (Kocher et al. 1989).

For the forty-six populations in this study, there were 154 variable sites within the 307 bp region examined (Fig 3.1); of these, 130 were informative under the conditions of parsimony. The mean pairwise sequence difference among the forty-six taxa was 24.9%. Avise et al. (1994) concluded that, in the mt cytochrome *b* molecule, pairwise sequence variation of 20% is the upper limit for obtaining robust phylogenetic results. Values above this ceiling may suggest patterns of relatedness but rarely provide statistically supported analyses. The value for the present data set may suggest that accurate resolution of relationships is not possible but this is not necessarily true. Four of the samples (alb 1, alb 6, alb 7, and alb 8) were noticeably divergent from all other samples and cluster together outside all other ingroup taxa (see trees in Appendix D). When these

Figure 3.1. Mitochondrial cytochrome *b* sequences. A question mark (?) indicates an ambiguous or missing nucleotide. A dash (-) indicates a gap introduced into the alignment. A dot (.) indicates identity to the nucleotide in the first sequence. Sites 1 and 2 are the last two bases of the 5' (upstream) primer and were excluded from analysis. Site 1 corresponds to position 16,345 of the published *Xenopus laevis* mitochondrial genome (Roe et al. 1985). Sample abbreviations are given in Appendix A.

Figure 3.1 (continued)

```
                  1 1111111112 2222222223 3333333334 4444444445
         1234567890 1234567890 1234567890 1234567890 1234567890
alb1     AACTTCGGCT CACTCCTTGG CGCCTGCCTG ATCCTCCAAA TCACCACAGG
alb2     ........A. .C.....A.. ACT.....  ..TG.G.... .TCT......
alb3     ........A. .C?....A.. ACT.....  ..TG.G.... .TCT......
alb4     ........G. .C.....A.. ACT......A ..TG...... .TCT...T..
alb5     ........?. .C.....A.. ACT......A ..TG...... .TCT...T..
alb6     ..T..T..T. ....AT.A.. A.TG.....A ..TA.T.... ..CT......
alb7     ..T..T..T. ....AT.A.. A.TG.....A ..TA.T.... ..CT......
alb8     ..T..T..T. ....?T.A.. A.TG.....A ..TA.T.... ..CT......
alb9     ........A. .C.....A.. ACT......A G.TG.T.... .TCT......
alb10    ........G. .C.....A.. ACT......A ..TG...... .TCT...T..
alb11    ........A. .C.....A.. ACT......A ..TA...... .TCT...C..
alb12    ........G. .C.....A.. ACT......A ..TG...... .TCT...T..
alb13    ........A. .C...?.A.. ACT.....  ..TG.G.... .TCT......
alb14    ........A. .C.....A.. ACT......A G.TG.T.... .TCT......
alb15    ?.......A. .C.....A.. ACT......A G.TG.T.... .TCT......
alb16    ........A. .C.....A.. ACT......A ..TA...... .TCT...C..
alb17    ........A. .C.....A.. ACT.....  ..TG.G.... .TCT......
alb18    ........A. .C...?.A.. ACT.....  ..TG.G.... .TCT......
alb19    ........A. .C.....A.. ACT.....  ..TG.G.... .TCT......
alb20    ?.......A. .C.....A.. ACT......A G.TG.T.... .TCT......
alb21    ........G. .C.....A.. ACT......A ..TG...... .TCT...T..
biv1     .....T..A. .C..G..A.. ACTT...T.A ...A...... ..CTT.....
biv2     .....T..A. .C..G..A.. ACTT...T.A ...A...... ?.CTT.....
biv3     .....T..A. .?..G..A.. ACTT...T.A ...A...... ..CTT.....
del1     ........A. .C.....A.. ACTA.....A G.AA...... ..TTA.....
del2     ?????...A. .C.....A.. ACTA.....A G.AA...... ..TTA.....
del3     ........A. .C.....A.. ACTA.....A G.AA...... ..TTA.....
del4     ?????????? ??.....A.. ACTA.....A G.AA.T...? ..TTA.....
del5     ........A. .C...?.A.. ACTA.....A G.AA...... ..TTA.....
del6     ........A. .C.....A.. ACTA.....A G.AA...... ..TTA.....
dunc     ........G. .T.....A.. -CTA.....A ..TA.T.... .TCT...C..
gray1    ........A. .C.....A.. -CTT.....A ..TA...... .TCT......
gray2    ........A. .C.....A.. -CTT.....A G.TG...... .TCT......
gray3    ........A. .C.....A.. -CTT.....A ..TA...... .TCT......
habe     .....T..A. .C..A..A.. ACTT...T.A ...A...... ..CTT.....
paci     ........G. .C.....A.. -CT......A ..TA...... .TCT...T..
occi     ........A. .C..AT.A.. ACT......A ..AA.T.... ..CT......
stol     .....T..A. .C..AT.A.. ACTT.....A ...A...... .TCTT.....
ther     .....?A.T. .?..A..A.. ACT....T.A G.AA...... ...T......
thor     ........A. .CT.A..A.. GCTT...T.A C.A..T.... ..CT......
tigr     ........A. .C..A..A.. GCT....T.A ..AA.T.... ..TTA.....
Leio     ..T.....A. .C..G..... TCTT.....T ..TA.....G .TTTA.....
Plesio   ........G. .T..A..A.. ACTT.....? ..AA...... ..CT......
Ttor     .....T.... .T..A..A.. .CT....T.A ..TA.A...G ..CTA.....
Tumb     .....T.... ....T..A.. ACTA..TT.A ...G.A.... ..CT......
Scel     .....T.... .T..T..A.. AA.......A ..TA.T...G .ACTT..T..
```

Figure 3.1 (continued)

```
                                                                      1
           5555555556 6666666667 7777777778 8888888889 9999999990
           1234567890 1234567890 1234567890 1234567890 1234567890
alb1       ACTATTCCTA GCCATGCACT ACTCACCAGA CGCCTCAACC GCCTTTTCAT
alb2       ...G...... ..A..A.... .TA.CG.... TATT..C.TA .....C....
alb3       ...G...... ..A..A.... .TA.CG.... TATT..C.TA .....C....
alb4       C..G...... ..A..A..T. ..A.CG.... .ATT..C.TG .....C....
alb5       C..G...... ..A..A..T. ..A.CG.... .ATT..C.TA .....C....
alb6       CT.....T.. ..A..A..T. .TA..T.T.. TA.A.T.... ..A.......
alb7       CT.....T.. ..A..A..T. .TA..T.T.. TA.A.T.... ..A.......
alb8       CT.....T.. ..A..A..T. .TA..T.T.. TA.A.T.... ..A.......
alb9       C......... ..A..A.... ..A.CG.... TATT..C.TA .....C....
alb10      C..G...... ..A..A..T. ..A.CG.... .ATT..C.TA .....C....
alb11      C..G..T... ..A..A..T. ..A.CG.... .ATT..C.TA .....C....
alb12      C..G...... ..A..A..T. ..A.CG.... .ATT..C.TA .....C....
alb13      ...G...... ..A..A.... .TA.CG.... TATT..C.TA .....C....
alb14      C......... ..A..A.... ..A.CG.... TATT..C.TA .....C....
alb15      C......... ..A..A.... ..A.CG.... TATT..C.TA .....C....
alb16      C..G..T... ..A..A..T. ..A.CG.... .ATT..C.TA .....C....
alb17      ...G...... ..A..A.... .TA.CG.... TATT..C.TA .....C....
alb18      ...G...... ..A..A.... .TA.CG.... TATT..C.TA .....C....
alb19      ...G...... ..A..A.... .TA.CG.... TATT..C.TA .....C....
alb20      C......... ..A..A.... ..A.CG.... TATT..C.TA .....C....
alb21      C..G...... ..A..A..T. ..A.CG.... .ATT..C.TA .....C....
biv1       .T....T..G ..A....... .TA.CG.... .AT...C.TA .....C....
biv2       .T...?T..G ..A....... .TA.CG.... .AT...C.TA .....C....
biv3       .T....T..G ..A....... .TA.CG.... .AT...C.TA .....C....
del1       C......... ..A..A.... ..A.CG.... .ATT..C.TA .....C....
del2       C......... ..A..A.... ..A.CG.... .ATT..C.TA .....C....
del3       C......... ..A..A.... ..A.CG.... .ATT..C.TA .....C....
del4       C......... ..A..A.... ..A.CG.... .ATT..C.TA .....C....
del5       C......... ..A..A.... ..A.CG.... .ATT..C.TA .....C....
del6       C......... ..A..A.... ..A.CG.... .ATT..C.TA .....C....
dunc       C......... ..A..A.... ..A.CG.... TATT..C.TA ..........
gray1      C..G...... ..A..A.... ..A.TG.... .ATT..C.TA .....C....
gray2      C..G...... ..A..A.... ..A.TG.... .ATT..C.TA .....C....
gray3      C..G...... ..A..A.... ..A.TG.... .ATT..C.TA .....C....
habe       .T....T..G ..A....... .TA.CG.... .AT...C.TA .....C....
paci       C..G...... ..A..A.... ..A.CG.... .ATT..C.TA .....C....
occi       .T....TT.. ..A..A.... ..A.CG.... TATT..T.TA .....C....
stol       .T........ ..A..A.... ..A.CG.... TAT...C.TA .....C....
ther       G.....T... ..A..A.... ..A.CG.... .AT...C.TA .....C....
thor       C.....T... ..A..A.... ..A.CG.... TAT.....TA .....C....
tigr       ......T... ..A..A..T. ..A.TG.... .AT...C.TA ..T.....C.
Leio       CT.......T .....A.... ..A..G.... .AT....T.A ..A.......
Plesio     ...T..T... .......... ..A.TG.C.. .AT...C.TA .....C....
Ttor       ...C...... .....A.... ..A..G.... .AT.A...TA .....C....
Tumb       CT.....T.. .....A.... ..A..G.... ..T.....TA .....C....
Scel       C......T.. .....A.... ..A..G.T.. .AT....T.A ..T..C....
```

Figure 3.1 (continued)

```
          1111111111 1111111111 1111111111 1111111111 1111111111
          0000000001 1111111112 2222222223 3333333334 4444444445
          1234567890 1234567890 1234567890 1234567890 1234567890
alb1      CAATCGCCCA CATCACTCGA GACGTAAATT ATGGCTGAAT CATCCGCTAC
alb2      .CG.G..... ....TGC... ......C.G. .C......C. AC.T..AA..
alb3      .CG.G..... ....TGC... ......C.G. .C......C. AC.T..AA..
alb4      .CG.A..... ....TGC... ......C.A. .C......T. AC.T..AA.T
alb5      .CG.A..... ....TGC... ......C.A. .C......T. AC.T..AA.T
alb6      ..G.A..... T..TTGC... ........C. .C..A...C. T.....AA.T
alb7      ..G.A..... T..TTGC... ........C. .C..A...C. T.....AA.T
alb8      ..G.A..... T..TTGC... ........C. .C..A...C. T.....AA.T
alb9      .CG.G..... ....TGC... ......C.A. .C......C. AC....AA..
alb10     .CG.A..... ....TGC... ......C.A. .C......T. AC.T..AA.T
alb11     .CG.A..... ....TGC... ......C.A. .C......T. AC.T..AA.T
alb12     .CG.A..... ....TGC... ......C.A. .C......T. AC.T..AA.T
alb13     .CG.G..... ....TGC... ......C.G. .C......C. AC.T..AA..
alb14     .CG.G..... ....TGC... ......C.A. .C......C. AC....AA..
alb15     .CG.G..... ....TGC... ......C.A. .C......C. AC....AA..
alb16     .CG.A..... ....TGC... ......C.A. .C......T. AC.T..AA.T
alb17     .CG.G..... ....TGC... ......C.G. .C......C. AC.T..AA..
alb18     .CG.G..... ....TGC... ......C.G. .C......C. AC.T..AA..
alb19     .CG.G..... ....TGC... ......C.G. .C......C. AC.T..AA..
alb20     .CG.G..... ....TGC... ......C.A. .C......C. AC....AA..
alb21     .CG.A..... ....TGC... ......C.A. .C......T. AC.T..AA.T
biv1      .TG.A..... ....TGC... ..T..CC.A. ....T...C. T.....AA..
biv2      .TG.A..... ....TGC... ..T..CC.A. ........C. T.....AA..
biv3      .TG.A..... ....TGC... ..T..CC.A. ....T...C. T.....AA..
del1      .CG.A..... ....TGC... ..T...C.A. .C......C. AC....AA..
del2      .CG.A..... ....TGC... ..T...C.A. .C......C. AC....AA..
del3      .CG.A..... ....TGC... ..T...C.A. .C......C. AC....AA..
del4      .CG.A..... ....TGC... ..T...C.A. .C......C. AC....AA..
del5      .CG.A..... ....TGC... ..T...C.A. .C......C. AC....AA..
del6      .CG.A..... ....TGC... ..T...C.A. .C......C. AC....AA..
dunc      .CG.A..... ....TGC... ......C.A. .C.....GC. AC.T..AAG.
gray1     .CG.G..... ....TGC... ......C.A. .C.....GC. AC....AA..
gray2     .CG.G..... ....TGC... ......C.A. .C.....GC. AC....AA..
gray3     .CG.G..... ....TGC... ......C.A. .C.....GC. AC....AA..
habe      .TG.A..... ....TGC... ..T..CC.A. ........C. T.....AA..
paci      .CG.A..... ....TGC... ......C.A. .C......C. AC.T..AA.T
occi      .CG.G..... ....TGC... ..T..CC.A. ....T...C. G.....AA..
stol      .CG.A..... ...TTGC... .....TC.A. .C..A...C. ...T..AA..
ther      .??.G..?.. ....TGC.?? .....CC.A. .C..GC..C. .......A..
thor      ..G.A..... ....TGC... ......C.A. .C..G...C. A..T..AA..
tigr      .CG.A..... ....TGC... ......C.A. .C......C. ......AA..
Leio      ..G.A..A.. ....TGC... ..T...C.A. ........C. A..T..AA.T
Plesio    .CG.A..... T..TTGC... ..T...C.A. .C..A...C. T.....AA..
Ttor      .T..TT.... ....TGC... ..T..CC.A. ....A...C. ...T..GA..
Tumb      .C..TA.A.. ...TTGC..T ..T..TC.A. ....A...C. A.....AA..
Scel      ..G....... ....TGC... .....CC.A. .C......C. ......AA.T
```

Figure 3.1 (continued)

```
          1111111111 1111111111 1111111111 1111111111 1111111112
          5555555556 6666666667 7777777778 8888888889 9999999990
          1234567890 1234567890 1234567890 1234567890 1234567890
alb1      CTTCACGCCA ATGGCGCCTC AATATTCTTT ATCTGCCTCT TCCTACACAT
alb2      A.C..T.... .C..A..T.. .......... ........A. A.........
alb3      A.C..T.... .C..A..T.. .......... ........A. A.........
alb4      A....T.... .C..A..T.. .......... ..T..TT.A. A.........
alb5      A....T.... .C..A..T.. .......... ..T..TT.A. A.........
alb6      A.C..T.... .C..A..A.. T......... ..A.....T. ....T..TG.
alb7      A.C..T.... .C..A..A.. T......... ..A.....T. ....T..TG.
alb8      A.C..T.... .C..A..A.. T......... ..A.....T. ....T..TG.
alb9      A.C..T.... .C..G..T.. G......... .....T..A. A.........
alb10     A....T.... .C..A..T.. .......... ..T..TT.A. A.........
alb11     A....T.... .C..A..T.. .......... ..T..TT.A. A.........
alb12     A....T.... .C..A..T.. .......... ..T..TT.A. A.........
alb13     A.C..T.... .C..A..T.. .......... ........A. A.........
alb14     A.C..T.... .C..G..T.. G......... .....T..A. A.........
alb15     A.C..T.... .C..G..T.. ?......... .....T..A. A.........
alb16     A....T.... .C..A..T.. .......... ..T..TT.A. A.........
alb17     A.C..T.... .C..A..T.. .......... ........A. A.........
alb18     A.C..T.... .C..A..T.. .......... ........A. A.........
alb19     A.C..T.... .C..A..T.. .......... ........A. A.........
alb20     A.C..T.... .C..G..T.. G......... .....T..A. A.........
alb21     A....T.... .C..A..T.. .......... ..T..TT.A. A.........
biv1      ........A. .C..T..A.. ......T..C .....T..A. A...T.....
biv2      ........A. .C..T..A.. ......T..C ........A. A...T.....
biv3      ........A. .C..T..A.. ......T..C .....T..A. A...T.....
del1      ..A..T.... .C..G..T.. .........C ..T...T.A. A...G.....
del2      ..A..T.... .C..A..T.. .........C ..T...T.A. A...G.....
del3      ..A..T.... .C..A..T.. .........C ..T...T.A. A...G.....
del4      ..A..T.... .C..A..T.. .........C ..T...T.A. A...G.....
del5      ..A..T.... .C..A..T.. .........C ..T...T.A. A...G.....
del6      .....T.... .C..A..T.. .........C ..T...T.A. A...G.....
dunc      A.C..T.... .C..A..T.. ...G...... .....T..A. A.........
gray1     A.C..T.... .C..A..T.. .......... ..T.....A. A...T.....
gray2     A.C..T.... .C..A..T.. .......... ..T.....A. A...T.....
gray3     A.C..T.... .C..A..T.. .......... ..T.....A. A...T.....
habe      ........A. .C..T..A.. ......T..C ........T. A...C.....
paci      A....T.... .C..A..T.. .......... ..T..T..A. A...G.....
occi      A.C.....A. .C.....A.. ......T..C ..T.....A. AT..T.....
stol      T.A..T..A. .C.....T.. C........C ........A. AT..T.....
ther      T.?....... .C..A..... .C........ ..T.....A. AT..T.....
thor      ..A..T.... .C..A..... C.....T..C ........A. A.........
tigr      ..A....... .C..A..... C........C ........A. A.........
Leio      A....T..A. .C........ C.....T... ......A.A. A.T....TG.
Plesio    ..C..T..A. .C..G..... .........C ..T.....A. AT..T.....
Ttor      ..C..T.... .C.....T.. .......... .....TA.T. ATT.......
Tumb      .....T..T. .C..G..... C........C ..T...A.T. AT..C.....
Scel      G.C....... .C....?... .......... .....TA.T. A.T.G..TG.
```

Figure 3.1 (continued)

```
          2222222222 2222222222 2222222222 2222222222 2222222222
          0000000001 1111111112 2222222223 3333333334 4444444445
          1234567890 1234567890 1234567890 1234567890 1234567890
alb1      CGGGCGAGGC CTATATTACG GATCATTTCT CTACTCAGAA ACCTGAAACA
alb2      ...A..G... ........T. .G..C.AC.. A.T.AA.... ..A.......
alb3      ...A..G... ........T. .G..C.AC.. A.T.AA...G ..A.......
alb4      ...A..G... ..G.....T. ....C.AC.. ..TTAA.... ..A.......
alb5      ...A..G... ..G.....T. ....C.AC.. ..TTAA.... ..A.......
alb6      A..A.....A A.T..C..T. ......A... T...AA.... ..A.....T.
alb7      A..A.....A A.T..C..T. ......A... T...AA.... ..A.....T.
alb8      A..A.....A A.T..C..T. ......A... T...AA.... ..A.....T.
alb9      ...A...... ..G..C..T. .G..T.AC.. G.T.AA.... ..A.......
alb10     ...A..G... ..G.....T. ....C.AC.. ..TTAA.... ..A.......
alb11     ...A..G... ..G....... ....T.AC.. ..TTAA.... ..A.......
alb12     ...A..G... ..G.....T. ....C.AC.. ..TTAA...G ..A.......
alb13     ...A..G... ........T. .G..C.AC.. A.T.AA...G ..A.......
alb14     ...A...... ..G..C..T. .G..T.AC.. G.T.AA.... ..A.......
alb15     ...A...... ..G..C..T. .G..T.AC.. G.T.AA.... ..A.......
alb16     ...A..G... ..G....... .G..T.AC.. ..TTAA.... ..A.......
alb17     ...A..G... ........T. .G..C.AC.. A.T.AA...G ..A.......
alb18     ...A..G... ........T. .G..C.AC.. A.T.AA.... ..A.......
alb19     ...A..G... ........T. .G..C.AC.. A.T.AA.... ..A.......
alb20     ...A...... ..G..C..T. .G..T.AC.. G.T.AA.... ..A.......
alb21     ...A..G... ..G.....T. ....C.AC.. ..TTAA.... ..A.......
biv1      T..A..T... ..T..C.... ......AC.. G.T.AA.... ..A.......
biv2      T..A..T... ..T..C.... ......AC.. G.T.AA.... ..A.......
biv3      T..A..T... ..T..C.... ......AC.. G.T.AA.... ..A.......
del1      ...A...... ..C..C.... ....T.ACT. A.TTAA.... ..A.......
del2      ...A...... ..C..C.... ....T.ACT. A.TTAA.... ..A.......
del3      ...A...... ..C..C.... ....T.ACT. A.TTAA.... ..A.......
del4      ...A...... ..C..C.... ....T.ACT. A.TTAA.... ..A.......
del5      ...A...... ..C..C.... ....T.ACT. A.TTAA.... ..A.......
del6      ...A...... ..C..C.... ....T.ACT. A.TTAA.... ..A.......
dunc      ...A...... ..G..C..T. ....T.AC.. G.T.AA.... ..A.......
gray1     ...A...... ..C..C..T. .G..C.?CT. ..TT?????? ??????????
gray2     ...A...... ..C..C..T. .G..C.AC.. ..TTAA.... ..A.......
gray3     ...A...... ..C..C..T. .G..C.ACT. ..TTAA.... ..A.......
habe      T..A..T... ..T..C.... ......AC.. G.T.AA.... ..A.......
paci      ...A..G... ..G.....T. .G..T.AC.. A.TTAA.... ..A.......
occi      ...A..C..T ..T..C.... ....T.AC.. A.TTAA.... ..A.......
stol      T..A..C... .....C.... ....C.AC.. A.TTAA.... ..A.......
ther      T..A...... .....C.... ......ACT. A.TTAA...G ..A.....??
thor      ...A...... ..T..C.... ....G.AC.. T.TTAA.... ..A.......
tigr      T..C...... ..C....... ....C.ACT. G.TTAA.... ..A.....T.
Leio      A..A...... A.C..C.... ....G.ACA. A.TTAA...G ..T.....T.
Plesio    ...A...... ..C....... .G....A... A.TTAA.... ..A......G
Ttor      T.....C..A ..C..C..T. ....G.AC.. A.TTAT.... ..A.....T.
Tumb      T..A...... ..T..C..T. .G..C.AC.. A.TTAA.... .........C
Scel      A..A..C..G ..C....... .C....A.A. A.TTAA.... ........T.
```

Figure 3.1 (continued)

```
          2222222222 2222222222 2222222222 2222222222 2222222223
          5555555556 6666666667 7777777778 8888888889 9999999990
          1234567890 1234567890 1234567890 1234567890 1234567890
alb1      TCGGCATTAT CCTCCTGCTT GCAACTATAG CAACAGCCTT CATAGGCTAT
alb2      .A...G.C.. TT.A.....A CTCGTA.... .G........ ...G..A...
alb3      .A...G.C.. TT.A.....A CTCGTA.... .......... ...G..A...
alb4      .G...G.... ...A..A..G CT.GTA.... .......... ......A..C
alb5      .G...G.... ...A..A..G CT.GTA.... .......... ......A..C
alb6      .T..AG.... ...A..A..A A..GT?.... .T..?..A.. .G.T.....C
alb7      .T..AG.... ...A..A..A A..GT..... .T..T..A.. .G.T.....C
alb8      .T..AG.... ...A..A..A A..GT..... .T..?..A.. .G.T.....C
alb9      .G...G.C.. T..A..A..A CTTGTA.... .......... ......A...
alb10     .G...G.... ...A..A..G CT.GTA.... .......... ......A..C
alb11     .G...G.... ...A..A..G CT.GTA.... .......... ......A..C
alb12     .G...G.... T..A..A..G CT.GTA.... .......... ......A..C
alb13     .A...G.C?. TT.A.....A CTCGTA.... .......... ...G..A...
alb14     .G...G.C.. T..A..A..A CTTGTA.... .......... ......A...
alb15     .G...G.C.. T..A..A..A CTTGTA.... ....??.... ......A...
alb16     .G...G.... ...A..A..G CT.GTA.... .......... ......A..C
alb17     .A...G.C.. T..A.....A CTCGTA.... .G...???.. ...G..G...
alb18     .A...G.C.. TT.A.....A CTCGTA.... .G...???.. ...G..A...
alb19     .A...G.C.. TT.A.....A CTCGTA.... .G...???.. ...G..A...
alb20     .G...G.C.. T..A..A..A CTTGTA.... ....?..... ......A...
alb21     .G...G.... ...A..A..G CT.GTA.... .......... ......A..C
biv1      .T..T..C.. ...A..A..A TT.GTA.... .C........ .........C
biv2      .T..T..C.. ...A..A..A TT.GTA.... .C........ .........C
biv3      .T..T..C.. ...A..A..A TT.GTA.... .C........ .........C
del1      .G...G.... ...A..A..C CTCGTA.... .......... ......A...
del2      .G...G.... ...A..A..C CTCGTA.... .......... ......A...
del3      .G...G.... ...A..A..C CT.GTA.... .......... ......A...
del4      .G..???... ...A..A..C CTCGTA.... .......... ......A...
del5      .G...G.... ...A..A..C CTCGTA.... .......... ......A...
del6      .G...G.... ...A..A..C CT.GTA.... .......... ......A...
dunc      .A...G.... T..A..A..A CTCGTA.... .......T.. ......A...
gray1     ?????????? ?????????? ?????????? ?????????? ??????????
gray2     .G...G.... T..A..A..A CTCGTA.... .......... ......A...
gray3     .G...G.... T..A..A..A CTCGTA.... .......... ......A..C
habe      .T..T..C.. ...T..A..A TT.GTA.... .C........ .........C
paci      .G...G.... ...A.....G CT.GTA.... .......T.. ......A..C
occi      .T........ ...A..AT.. CT.GTG.... .C........ T.....T...
stol      .A.....C.. ......AT.A CTCGTA.... .......T.. T.....A...
ther      .A..AG.AG. ...A..A..A CTT.TA.... .......?.. ......A...
thor      .G........ ......T..A CTCGTA.... .......... ...G.....C
tigr      .T..A...G. A.....C..C CT.GTA.... ....T..T.. T.....A...
Leio      ....AG..G. A.....A... CT.GT..... .C.....A.. TG.C......
Plesio    .A........ ...T..A..C CT.GTA.... ....C..... ...G......
Ttor      .T..AG.C.. T..T..A..A TT.GTG.... .C.....T.. .G....A..C
Tumb      .A?.T?.GG. .T.AT.AT.. CTGGT..... .C.....T.. .G....A..C
Scel      .T..TG.AG. ...T..T..C TT.GTA.... .C..T..G.. TG........
```

Figure 3.1 (continued)

```
          333333333
          000000000
          123456789
alb1      GTCCTCCCG
alb2      ..G..G..C
alb3      ..G..G..C
alb4      ..A..G..C
alb5      ..A..G..C
alb6      ..A..A..A
alb7      ..A..A..A
alb8      .?A..A..?
alb9      ..A..G..C
alb10     ..A..G..C
alb11     ..G..A..C
alb12     ..A..G..C
alb13     ..G..G..C
alb14     ..A..G..C
alb15     ..A..G..C
alb16     ..G..A..C
alb17     ..A..G..C
alb18     ..G..G..C
alb19     ..G..G..C
alb20     ..A..G..C
alb21     ..A..G..C
biv1      .....G..T
biv2      .....G..T
biv3      .....G..T
del1      ..A..A..T
del2      ..A..A..T
del3      ..A..A..T
del4      ..A..A..T
del5      ..A..A..T
del6      ..G..A..T
dunc      ..G..G..C
gray1     ?????????
gray2     .....G..C
gray3     .....G..C
habe      .....G..C
paci      ..A..G..C
occi      ........C
stol      .....G..A
ther      ..A..A..A
thor      ..A..G..A
tigr      ...T.A..A
Leio      ...T.A..A
Plesio    .....A..T
Ttor      ........A
Tumb      ........A
Scel      ........A
```

four samples are removed, the mean pairwise sequence difference drops to 20%, the suggested limit for this region of the mt genome. This value drops even further with the exclusion of the outgroup taxon. Therefore, the mean pairwise sequence divergence of the ingroup populations is within the recommended limits for accurate topology resolution. Figure 3.2 lists the number and percentage of nucleotide sites which differ betweeen each pair of samples.

When translated using the mammalian mitochondrial genetic code, 37 sites (12%) exhibited nonsynonymous substitutions, with seven of these involving amino acid substitutions in only a single sample (Fig. 3.3). Only 25 of the variable sites were parsimoniously informative; this low number precluded analysis using amino acid substitutions.

Five samples (dunc, gray 1, gray 2, gray 3, and paci) required the introduction of a single bp gap at position 21 in their respective sequences in order to maintain proper alignment (Fig. 3.1). In order to verify that this was not a sequencing artifact, these samples were extracted, amplified and sequenced three or four times each; in every attempt, the deletion was present. This would appear problematic because cytochrome *b* is one of the 9 or 10 proteins that make up complex III of the mitochondrial oxidative phosphorylation system (Hatefi, 1985). Complex III is involved in the transfer of electrons from dihydroubiquinone to cytochrome *c*, and this reaction in turn is linked to the translocation of protons across the mitochondrial inner membrane (Hatefi, 1985). Cytochrome *b* contains both redox centers (Q_o and Q_i) involved in electron transfer. Any insertion/deletion events in the sequence of this gene would result in a shift in the reading

	alb1	alb2	alb3	alb4	alb5	alb6	alb7	alb8	alb9
alb1	--	0.2697	0.2697	0.2697	0.2697	0.2697	0.2697	0.2697	0.2659
alb2	72	--	0.0075	0.1086	0.1049	0.2397	0.2397	0.2397	0.0787
alb3	72	2	--	0.1086	0.1049	0.2434	0.2434	0.2434	0.0787
alb4	72	29	29	--	0.0037	0.2247	0.2247	0.2247	0.0936
alb5	72	28	28	1	--	0.2247	0.2247	0.2247	0.0899
alb6	72	64	65	60	60	--	0.0000	0.0000	0.2210
alb7	72	64	65	60	60	0	--	0.0000	0.2210
alb8	72	64	65	60	60	0	0	--	0.2210
alb9	71	21	21	25	24	59	59	59	--
alb10	72	28	28	1	0	60	60	60	24
alb11	72	32	32	8	7	61	61	61	28
alb12	74	28	26	3	2	62	62	62	24
alb13	72	2	0	29	28	65	65	65	21
alb14	71	21	21	25	24	59	59	59	0
alb15	71	21	21	25	24	59	59	59	0
alb16	73	31	31	9	8	62	62	62	27
alb17	72	4	4	29	28	63	63	63	21
alb18	72	0	2	29	28	64	64	64	21
alb19	72	0	2	29	28	64	64	64	21
alb20	71	21	21	25	24	59	59	59	0
alb21	72	28	28	1	0	60	60	60	24
biv1	70	51	52	51	50	61	61	61	45
biv2	68	49	50	51	50	60	60	60	45
biv3	70	51	52	51	50	61	61	61	45
del1	71	40	40	31	30	64	64	64	27
del2	71	39	39	30	29	63	63	63	28
del3	70	40	40	29	28	62	62	62	28
del4	72	39	39	31	30	62	62	62	27
del5	71	39	39	30	29	63	63	63	28
del6	70	39	39	30	29	63	63	63	29
dunc	73	25	25	27	26	58	58	58	16
gray2	71	24	24	23	22	61	61	61	17
gray3	72	26	26	23	22	59	59	59	21
habe	68	51	52	53	52	61	61	61	47
paci	72	25	25	10	9	60	60	60	22
occi	73	49	50	50	49	62	62	62	43
stol	72	37	37	43	42	65	65	65	38
ther	67	47	45	47	46	68	68	68	39
thor	67	39	39	38	37	57	57	57	33
tigr	64	49	49	46	45	67	67	67	45
Leio	72	62	61	57	56	62	62	62	58
Plesio	65	48	48	48	47	60	60	60	47
Ttor	72	52	53	52	51	69	69	69	49
Tumb	76	56	57	56	55	67	67	67	52
Scel	68	65	66	59	58	61	61	61	59

Figure 3.2. Sequence divergence in a portion of the mitochondrial cytochrome *b* gene among populations of Galápagos and South American *Microlophus* and related taxa. Values above the diagonal are proportion sequence divergence and values below the diagonal are number of nucleotide changes between samples. Sample abbreviations are given in Appendix A.

Figure 3.2 (continued)

	alb10	alb11	alb12	alb13	alb14	alb15	alb16	alb17	alb18
alb1	0.2697	0.2697	0.2772	0.2697	0.2659	0.2659	0.2734	0.2697	0.2697
alb2	0.1049	0.1198	0.1049	0.0075	0.0787	0.0787	0.1161	0.0150	0.0000
alb3	0.1049	0.1198	0.0974	0.0000	0.0787	0.0787	0.1161	0.0150	0.0075
alb4	0.0037	0.0300	0.0112	0.1086	0.0936	0.0936	0.0337	0.1086	0.1086
alb5	0.0000	0.0262	0.0075	0.1049	0.0899	0.0899	0.0300	0.1049	0.1049
alb6	0.2247	0.2285	0.2322	0.2434	0.2210	0.2210	0.2322	0.2360	0.2397
alb7	0.2247	0.2285	0.2322	0.2434	0.2210	0.2210	0.2322	0.2360	0.2397
alb8	0.2247	0.2285	0.2322	0.2434	0.2210	0.2210	0.2322	0.2360	0.2397
alb9	0.0899	0.1049	0.0899	0.0787	0.0000	0.0000	0.1011	0.0787	0.0787
alb10	--	0.0262	0.0075	0.1049	0.0899	0.0899	0.0300	0.1049	0.1049
alb11	7	--	0.0337	0.1198	0.1049	0.1049	0.0037	0.1273	0.1198
alb12	2	9	--	0.0974	0.0899	0.0899	0.0375	0.0974	0.1049
alb13	28	32	26	--	0.0787	0.0787	0.1161	0.0150	0.0075
alb14	24	28	24	21	--	0.0000	0.1011	0.0787	0.0787
alb15	24	28	24	21	0	--	0.1011	0.0787	0.0787
alb16	8	1	10	31	27	27	--	0.1236	0.1161
alb17	28	34	26	4	21	21	33	--	0.0150
alb18	28	32	28	2	21	21	31	4	--
alb19	28	32	28	2	21	21	31	4	0
alb20	24	28	24	21	0	0	27	21	21
alb21	0	7	2	28	24	24	8	28	28
biv1	50	48	52	52	45	45	49	51	51
biv2	50	48	52	50	45	45	49	49	49
biv3	50	48	52	52	45	45	49	51	51
del1	30	28	32	40	27	27	29	40	40
del2	29	27	31	39	28	28	28	39	39
del3	28	26	30	40	28	28	27	40	40
del4	30	28	32	39	27	27	29	39	39
del5	29	27	31	39	28	28	28	39	39
del6	29	25	31	39	29	29	26	41	39
dunc	26	25	26	25	16	16	26	27	25
gray2	22	27	22	24	17	17	26	25	24
gray3	22	25	22	26	21	21	24	27	26
habe	52	50	54	52	47	47	51	51	51
paci	9	12	11	25	22	22	11	25	25
occi	49	45	51	50	43	43	46	49	49
stol	42	43	44	37	38	38	44	38	37
ther	46	43	46	45	39	39	44	45	47
thor	37	37	39	39	33	33	38	38	39
tigr	45	42	46	49	45	45	43	50	49
Leio	56	54	55	61	58	58	55	60	62
Plesio	47	43	49	48	47	47	42	48	48
Ttor	51	52	51	53	49	49	53	53	52
Tumb	55	59	57	57	52	52	58	59	56
Scel	58	58	60	66	59	59	58	65	65

Figure 3.2 (continued)

	alb19	alb20	alb21	biv1	biv2	biv3	del1	del2	del3
alb1	0.2697	0.2659	0.2697	0.2622	0.2547	0.2622	0.2659	0.2659	0.2622
alb2	0.0000	0.0787	0.1049	0.1910	0.1835	0.1910	0.1498	0.1461	0.1498
alb3	0.0075	0.0787	0.1049	0.1948	0.1873	0.1948	0.1498	0.1461	0.1498
alb4	0.1086	0.0936	0.0037	0.1910	0.1910	0.1910	0.1161	0.1124	0.1086
alb5	0.1049	0.0899	0.0000	0.1873	0.1873	0.1873	0.1124	0.1086	0.1049
alb6	0.2397	0.2210	0.2247	0.2285	0.2247	0.2285	0.2397	0.2360	0.2322
alb7	0.2397	0.2210	0.2247	0.2285	0.2247	0.2285	0.2397	0.2360	0.2322
alb8	0.2397	0.2210	0.2247	0.2285	0.2247	0.2285	0.2397	0.2360	0.2322
alb9	0.0787	0.0000	0.0899	0.1685	0.1685	0.1685	0.1011	0.1049	0.1049
alb10	0.1049	0.0899	0.0000	0.1873	0.1873	0.1873	0.1124	0.1086	0.1049
alb11	0.1198	0.1049	0.0262	0.1798	0.1798	0.1798	0.1049	0.1011	0.0974
alb12	0.1049	0.0899	0.0075	0.1948	0.1948	0.1948	0.1198	0.1161	0.1124
alb13	0.0075	0.0787	0.1049	0.1948	0.1873	0.1948	0.1498	0.1461	0.1498
alb14	0.0787	0.0000	0.0899	0.1685	0.1685	0.1685	0.1011	0.1049	0.1049
alb15	0.0787	0.0000	0.0899	0.1685	0.1685	0.1685	0.1011	0.1049	0.1049
alb16	0.1161	0.1011	0.0300	0.1835	0.1835	0.1835	0.1086	0.1049	0.1011
alb17	0.0150	0.0787	0.1049	0.1910	0.1835	0.1910	0.1498	0.1461	0.1498
alb18	0.0000	0.0787	0.1049	0.1910	0.1835	0.1910	0.1498	0.1461	0.1498
alb19	--	0.0787	0.1049	0.1910	0.1835	0.1910	0.1498	0.1461	0.1498
alb20	21	--	0.0899	0.1685	0.1685	0.1685	0.1011	0.1049	0.1049
alb21	28	24	--	0.1873	0.1873	0.1873	0.1124	0.1086	0.1049
biv1	51	45	50	--	0.0075	0.0000	0.1648	0.1648	0.1610
biv2	49	45	50	2	--	0.0075	0.1573	0.1573	0.1536
biv3	51	45	50	0	2	--	0.1648	0.1648	0.1610
del1	40	27	30	44	42	44	--	0.0037	0.0075
del2	39	28	29	44	42	44	1	--	0.0037
del3	40	28	28	43	41	43	2	1	--
del4	39	27	30	45	43	45	2	1	2
del5	39	28	29	44	42	44	1	0	1
del6	39	29	29	43	41	43	3	2	1
dunc	25	16	26	48	48	48	31	30	31
gray2	24	17	22	48	46	48	25	24	25
gray3	26	21	22	46	44	46	25	24	25
habe	51	47	52	5	3	5	44	44	43
paci	25	22	9	48	48	48	27	26	25
occi	49	43	49	33	33	33	39	39	38
stol	37	38	42	35	34	35	37	37	38
ther	47	39	46	43	42	43	33	32	32
thor	39	33	37	38	37	38	32	31	32
tigr	49	45	45	44	42	44	34	33	32
Leio	62	58	56	56	54	56	49	49	48
Plesio	48	47	47	39	38	39	33	34	33
Ttor	52	49	51	45	46	45	51	51	50
Tumb	56	52	55	50	49	50	47	48	48
Scel	65	59	58	59	59	59	58	58	57

Figure 3.2 (continued)

	del4	del5	del6	dunc	gray2	gray3	habe	paci	occi
alb1	0.2697	0.2659	0.2622	0.2734	0.2659	0.2697	0.2547	0.2697	0.2734
alb2	0.1461	0.1461	0.1461	0.0936	0.0899	0.0974	0.1910	0.0936	0.1835
alb3	0.1461	0.1461	0.1461	0.0936	0.0899	0.0974	0.1948	0.0936	0.1873
alb4	0.1161	0.1124	0.1124	0.1011	0.0861	0.0861	0.1985	0.0375	0.1873
alb5	0.1124	0.1086	0.1086	0.0974	0.0824	0.0824	0.1948	0.0337	0.1835
alb6	0.2322	0.2360	0.2360	0.2172	0.2285	0.2210	0.2285	0.2247	0.2322
alb7	0.2322	0.2360	0.2360	0.2172	0.2285	0.2210	0.2285	0.2247	0.2322
alb8	0.2322	0.2360	0.2360	0.2172	0.2285	0.2210	0.2285	0.2247	0.2322
alb9	0.1011	0.1049	0.1086	0.0599	0.0637	0.0787	0.1760	0.0824	0.1610
alb10	0.1124	0.1086	0.1086	0.0974	0.0824	0.0824	0.1948	0.0337	0.1835
alb11	0.1049	0.1011	0.0936	0.0936	0.1011	0.0936	0.1873	0.0449	0.1685
alb12	0.1198	0.1161	0.1161	0.0974	0.0824	0.0824	0.2022	0.0412	0.1910
alb13	0.1461	0.1461	0.1461	0.0936	0.0899	0.0974	0.1948	0.0936	0.1873
alb14	0.1011	0.1049	0.1086	0.0599	0.0637	0.0787	0.1760	0.0824	0.1610
alb15	0.1011	0.1049	0.1086	0.0599	0.0637	0.0787	0.1760	0.0824	0.1610
alb16	0.1086	0.1049	0.0974	0.0974	0.0974	0.0899	0.1910	0.0412	0.1723
alb17	0.1461	0.1461	0.1536	0.1011	0.0936	0.1011	0.1910	0.0936	0.1835
alb18	0.1461	0.1461	0.1461	0.0936	0.0899	0.0974	0.1910	0.0936	0.1835
alb19	0.1461	0.1461	0.1461	0.0936	0.0899	0.0974	0.1910	0.0936	0.1835
alb20	0.1011	0.1049	0.1086	0.0599	0.0637	0.0787	0.1760	0.0824	0.1610
alb21	0.1124	0.1086	0.1086	0.0974	0.0824	0.0824	0.1948	0.0337	0.1835
biv1	0.1685	0.1648	0.1610	0.1798	0.1798	0.1723	0.0187	0.1798	0.1236
biv2	0.1610	0.1573	0.1536	0.1798	0.1723	0.1648	0.0112	0.1798	0.1236
biv3	0.1685	0.1648	0.1610	0.1798	0.1798	0.1723	0.0187	0.1798	0.1236
del1	0.0075	0.0037	0.0112	0.1161	0.0936	0.0936	0.1648	0.1011	0.1461
del2	0.0037	0.0000	0.0075	0.1124	0.0899	0.0899	0.1648	0.0974	0.1461
del3	0.0075	0.0037	0.0037	0.1161	0.0936	0.0936	0.1610	0.0936	0.1423
del4	--	0.0037	0.0112	0.1086	0.0936	0.0936	0.1685	0.1011	0.1423
del5	1	--	0.0075	0.1124	0.0899	0.0899	0.1648	0.0974	0.1461
del6	3	2	--	0.1124	0.0936	0.0936	0.1610	0.0974	0.1423
dunc	29	30	30	--	0.0861	0.0861	0.1873	0.0899	0.1760
gray2	25	24	25	23	--	0.0150	0.1835	0.0824	0.1648
gray3	25	24	25	23	40	--	0.1760	0.0749	0.1648
habe	45	44	43	50	49	47	--	0.1873	0.1348
paci	27	26	26	24	22	20	50	--	0.1685
occi	38	39	38	47	44	44	36	45	--
stol	38	37	38	37	36	36	36	40	34
ther	33	32	33	46	38	38	45	43	41
thor	30	31	33	34	35	35	38	36	41
tigr	32	33	32	44	43	41	43	46	45
Leio	50	49	48	55	54	53	55	55	54
Plesio	35	34	33	50	38	38	39	42	40
Ttor	51	51	50	52	51	49	44	50	49
Tumb	48	48	48	58	49	49	48	54	45
Scel	57	58	57	63	61	60	57	53	55

Figure 3.2 (continued)

	stol	ther	thor	tigr	Leio	Plesio	Ttor	Tumb	Scel
alb1	0.2697	0.2509	0.2509	0.2397	0.2697	0.2434	0.2697	0.2846	0.2547
alb2	0.1386	0.1760	0.1461	0.1835	0.2322	0.1798	0.1948	0.2097	0.2434
alb3	0.1386	0.1685	0.1461	0.1835	0.2285	0.1798	0.1985	0.2135	0.2472
alb4	0.1610	0.1760	0.1423	0.1723	0.2135	0.1798	0.1948	0.2097	0.2210
alb5	0.1573	0.1723	0.1386	0.1685	0.2097	0.1760	0.1910	0.2060	0.2172
alb6	0.2434	0.2547	0.2135	0.2509	0.2322	0.2247	0.2584	0.2509	0.2285
alb7	0.2434	0.2547	0.2135	0.2509	0.2322	0.2247	0.2584	0.2509	0.2285
alb8	0.2434	0.2547	0.2135	0.2509	0.2322	0.2247	0.2584	0.2509	0.2285
alb9	0.1423	0.1461	0.1236	0.1685	0.2172	0.1760	0.1835	0.1948	0.2210
alb10	0.1573	0.1723	0.1386	0.1685	0.2097	0.1760	0.1910	0.2060	0.2172
alb11	0.1610	0.1610	0.1386	0.1573	0.2022	0.1610	0.1948	0.2210	0.2172
alb12	0.1648	0.1723	0.1461	0.1723	0.2060	0.1835	0.1910	0.2135	0.2247
alb13	0.1386	0.1685	0.1461	0.1835	0.2285	0.1798	0.1985	0.2135	0.2472
alb14	0.1423	0.1461	0.1236	0.1685	0.2172	0.1760	0.1835	0.1948	0.2210
alb15	0.1423	0.1461	0.1236	0.1685	0.2172	0.1760	0.1835	0.1948	0.2210
alb16	0.1648	0.1648	0.1423	0.1610	0.2060	0.1573	0.1985	0.2172	0.2172
alb17	0.1423	0.1685	0.1423	0.1873	0.2247	0.1798	0.1985	0.2210	0.2434
alb18	0.1386	0.1760	0.1461	0.1835	0.2322	0.1798	0.1948	0.2097	0.2434
alb19	0.1386	0.1760	0.1461	0.1835	0.2322	0.1798	0.1948	0.2097	0.2434
alb20	0.1423	0.1461	0.1236	0.1685	0.2172	0.1760	0.1835	0.1948	0.2210
alb21	0.1573	0.1723	0.1386	0.1685	0.2097	0.1760	0.1910	0.2060	0.2172
biv1	0.1311	0.1610	0.1423	0.1648	0.2097	0.1461	0.1685	0.1873	0.2210
biv2	0.1273	0.1573	0.1386	0.1573	0.2022	0.1423	0.1723	0.1835	0.2210
biv3	0.1311	0.1610	0.1423	0.1648	0.2097	0.1461	0.1685	0.1873	0.2210
del1	0.1386	0.1236	0.1198	0.1273	0.1835	0.1236	0.1910	0.1760	0.2172
del2	0.1386	0.1198	0.1161	0.1236	0.1835	0.1273	0.1910	0.1798	0.2172
del3	0.1423	0.1198	0.1198	0.1198	0.1798	0.1236	0.1873	0.1798	0.2135
del4	0.1423	0.1236	0.1124	0.1198	0.1873	0.1311	0.1910	0.1798	0.2135
del5	0.1386	0.1198	0.1161	0.1236	0.1835	0.1273	0.1910	0.1798	0.2172
del6	0.1423	0.1236	0.1236	0.1198	0.1798	0.1236	0.1873	0.1798	0.2135
dunc	0.1386	0.1723	0.1273	0.1648	0.2060	0.1873	0.1948	0.2172	0.2360
gray2	0.1348	0.1423	0.1311	0.1610	0.2022	0.1423	0.1910	0.1835	0.2285
gray3	0.1348	0.1423	0.1311	0.1536	0.1985	0.1423	0.1835	0.1835	0.2247
habe	0.1348	0.1685	0.1423	0.1610	0.2060	0.1461	0.1648	0.1798	0.2135
paci	0.1498	0.1610	0.1348	0.1723	0.2060	0.1573	0.1873	0.2022	0.1985
occi	0.1273	0.1536	0.1536	0.1685	0.2022	0.1498	0.1835	0.1685	0.2060
stol	--	0.1386	0.1348	0.1536	0.1910	0.1348	0.1685	0.1648	0.2172
ther	37	--	0.1423	0.1423	0.2322	0.1498	0.1948	0.1985	0.2247
thor	36	38	--	0.1386	0.1910	0.1423	0.1910	0.1873	0.2285
tigr	41	38	37	--	0.1835	0.1461	0.1910	0.2210	0.1985
Leio	51	62	51	49	--	0.2022	0.1985	0.2210	0.1760
Plesio	36	40	38	39	54	--	0.1948	0.1723	0.2097
Ttor	45	52	51	51	53	52	--	0.1610	0.1798
Tumb	44	53	50	59	59	46	43	--	0.2285
Scel	58	60	61	53	47	56	48	61	--

Figure 3.3. Cytochrome *b* amino acid sequences. These amino acid sequences were inferred from the nucleotide sequences of Fig. 3.1. A question mark (?) indicates an ambiguous or missing amino acid. A dot (.) indicates identity to the amino acid in the first sequence. A dash (-) indicates those codons which exhibited a nucleotide deletion in Fig. 3.1. The first amino acid codon contains primer bases and is marked as unknown. Site 1 corresponds to the amino acid codon beginning with position 16,345 of the published *Xenopus laevis* mitochondrial genome (Roe et al. 1985). Sample abbreviations are given in Appendix A.

Figure 3.3 (continued)

```
                  1 1111111112 2222222223 3333333334 4444444445
          1234567890 1234567890 1234567890 1234567890 1234567890
alb1      ?FGSLLGACL ILQITTGLFL AMHYSPDAST AFSSIAHITR DVNYGWIIRY
alb2      .......L.. .V..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb3      ....?..L.. .V..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb4      .......L.. .V..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb5      ..?....L.. .V..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb6      .......V.. .I..L..... ....TS.TL. ....V...C. ......L..N
alb7      .......V.. .I..L..... ....TS.TL. ....V...C. ......L..N
alb8      ....?..V.. .I..L..... ....TS.TL. ....V...C. ......L..N
alb9      .......L.. VV..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb10     .......L.. .V..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb11     .......L.. .I..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb12     .......L.. .V..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb13     .....?.L.. .V..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb14     .......L.. VV..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb15     .......L.. VV..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb16     .......L.. .I..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb17     .......L.. .V..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb18     .....?.L.. .V..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb19     .......L.. .V..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb20     .......L.. VV..L..... ....TA.I.M ....V...C. ..Q...LL.N
alb21     .......L.. .V..L..... ....TA.I.M ....V...C. ..Q...LL.N
biv1      .......L.. .I..L..... ....TA.I.M ....V...C. ..Q...L..N
biv2      .......L.. .I.?L...?. ....TA.I.M ....V...C. ..Q...L..N
biv3      ...?...L.. .I..L..... ....TA.I.M ....V...C. ..Q...L..N
del1      .......L.. VI..L..... ....TA.I.M ....V...C. ..Q...LL.N
del2      .?.....L.. VI..L..... ....TA.I.M ....V...C. ..Q...LL.N
del3      .......L.. VI..L..... ....TA.I.M ....V...C. ..Q...LL.N
del4      .???...L.. VI.?L..... ....TA.I.M ....V...C. ..Q...LL.N
del5      .....?.L.. VI..L..... ....TA.I.M ....V...C. ..Q...LL.N
del6      .......L.. VI..L..... ....TA.I.M ....V...C. ..Q...LL.N
dunc      ......-L.. .I..L..... ....TA.I.M ....V...C. ..Q...LL.S
gray1     ......-L.. .I..L..... ....TA.I.M ....V...C. ..Q...LL.N
gray2     ......-L.. VV..L..... ....TA.I.M ....V...C. ..Q...LL.N
gray3     ......-L.. .I..L..... ....TA.I.M ....V...C. ..Q...LL.N
habe      .......L.. .I..L..... ....TA.I.M ....V...C. ..Q...L..N
paci      ......-L.. .I..L..... ....TA.I.M ....V...C. ..Q...LL.N
occi      .......L.. MI..L..... ....TA.I.M ....V...C. ..Q...L..N
stol      .......L.. .I..L..... ....TA.I.M ....V...C. ..Q...L..N
ther      .?S?...L.. VI..I..... ....TA.I.M ...???..C? ..Q..RL..N
thor      .......L.. L...L..... ....TA.I.M ....V...C. ..Q...L..N
tigr      .......L.. MI..L..... ....TA.I.M ....V...C. ..Q...L..N
Leio      .......L.. .I.VL..... ....TA.I.S ....V...C. ..Q...L..N
Plesio    .......L.? MI..L..... ....TA.I.M ....V...C. ..Q...L..N
Ttor      .......L.. .M.VL..... ....TA.ITM .....S..C. ..Q...L..N
Tumb      .......L.. .V..L..... ....TA.V.M .....T..C. ..Q...L..N
Scel      .......T.. .I.VL..... ....TA.I.S ....V...C. ..Q...L..N
```

Figure 3.3 (continued)

```
                                                           1
         5555555556 6666666667 7777777778 8888888889 9999999990
         1234567890 1234567890 1234567890 1234567890 1234567890
alb1     LHANGASMFF ICLFLHIGRG LYYGSFLYSE TWNIGIILLL ATMATAFMGY
alb2     I......... ...Y...... .....Y.FK. ...M.V.... LV........
alb3     I......... ...Y...... .....Y.FK. ...M.V.... LV........
alb4     I......... ...Y...... .....Y.FK. ...M.V.... LV........
alb5     I......... ...Y...... .....Y.FK. ...M.V.... LV........
alb6     I......... M.....V... I....Y..K. .....V.... T?..?..V..
alb7     I......... M.....V... I....Y..K. .....V.... TV.....V..
alb8     I......... M.....V... I....Y..K. .....V.... TV..?..V..
alb9     I......... ...Y...... .....Y.FK. ...M.V.... LV........
alb10    I......... ...Y...... .....Y.FK. ...M.V.... LV........
alb11    I......... ...Y...... .....Y.FK. ...M.V.... LV........
alb12    I......... ...Y...... .....Y.FK. ...M.V.... LV........
alb13    I......... ...Y...... .....Y.FK. ...M.V?... LV........
alb14    I......... ...Y...... .....Y.FK. ...M.V.... LV........
alb15    I.....?... ...Y...... .....Y.FK. ...M.V.... LV..??....
alb16    I......... ...Y...... .....Y.FK. ...M.V.... LV........
alb17    I......... ...Y...... .....Y.FK. ...M.V.... LV...?....
alb18    I......... ...Y...... .....Y.FK. ...M.V.... LV...?....
alb19    I......... ...Y...... .....Y.FK. ...M.V.... LV...?....
alb20    I......... ...Y...... .....Y.FK. ...M.V.... LV..?.....
alb21    I......... ...Y...... .....Y.FK. ...M.V.... LV........
biv1     .......... ...Y...... .....Y.FK. .......... LV........
biv2     .......... ...Y...... .....Y.FK. .......... LV........
biv3     .......... ...Y...... .....Y.FK. .......... LV........
del1     .......... ...Y...... .....Y.FK. ...M.V.... LV........
del2     .......... ...Y...... .....Y.FK. ...M.V.... LV........
del3     .......... ...Y...... .....Y.FK. ...M.V.... LV........
del4     .......... ...Y...... .....Y.FK. ...M??.... LV........
del5     .......... ...Y...... .....Y.FK. ...M.V.... LV........
del6     .......... ...Y...... .....Y.FK. ...M.V.... LV........
dunc     I......... ...Y...... .....Y.FK. ...M.V.... LV........
gray1    I......... ...Y...... .....?FF?? ?????????? ??????????
gray2    I......... ...Y...... .....Y.FK. ...M.V.... LV........
gray3    I......... ...Y...... .....YFFK. ...M.V.... LV........
habe     .......... ...Y...... .....Y.FK. .......... LV........
paci     I......... ...Y...... .....Y.FK. ...M.V.... LV........
occi     I......... ...Y...... .....Y.FK. .........F LV........
stol     .......... ...Y...... .....Y.FK. ...M...... LV........
ther     ?......L.. ...Y...... .....Y.FK. ..??.VV... LM...?....
thor     .......... ...Y...... .....Y.FK. ...M...... LV........
tigr     .......... ...Y...... .....Y.FK. ......V... LV........
Leio     I......... ..MY..V... I....YMFK. .....VV... LV.....V..
Plesio   .......... ...Y...... .....Y.FK. ...V...... LV........
Ttor     .......... ..IY...... .....Y.FM. .....V.... LV.....V..
Tumb     .......... ..IY...... .....Y.FK. ...L??V..F LV.....V..
Scel     V....?.... ..IY..V... .....YMFK. .....VV... LV.....V..
```

Figure 3.3 (continued)

```
          111
          000
          123
alb1      VLP
alb2      ...
alb3      ...
alb4      ...
alb5      ...
alb6      ...
alb7      ...
alb8      ?.?
alb9      ...
alb10     ...
alb11     ...
alb12     ...
alb13     ...
alb14     ...
alb15     ...
alb16     ...
alb17     ...
alb18     ...
alb19     ...
alb20     ...
alb21     ...
biv1      ...
biv2      ...
biv3      ...
del1      ...
del2      ...
del3      ...
del4      ...
del5      ...
del6      ...
dunc      ...
gray1     ???
gray2     ...
gray3     ...
habe      ...
paci      ...
occi      ...
stol      ...
ther      ...
thor      ...
tigr      ...
Leio      ...
Plesio    ...
Ttor      ...
Tumb      ...
Scel      ...
```

frame when the DNA is translated. However, the position at which the deletion occurs in the present data set is located adjacent to a region noted as a "hypervariable residue" by Irwin et al. (1991). This amino acid position is located in a transmembrane portion of the cytochrome *b* molecule (Howell, 1989) and is not a component of either the Q_o or Q_i redox centers. This could free it from any functional constraint and allow for its removal by post-transcriptional processing without altering the function of the cytochrome *b* molecule; in fact, Vrana et al. (1994) report that this amino acid residue is missing from the walrus mitochondrial genome. The only known cytochrome *b* amino acid insertion in mammals (in the African elephant) also occurs in a transmembrane region (Irwin et al. 1991). It may be that the transmembrane regions of the molecule serve mostly as "place-holders" and maintain the correct orientation of the Q_o and Q_i redox centers. Indeed, most of the variable amino acid positions in mammalian cytochrome *b* are located in transmembrane regions while the redox centers exhibit very little variability (Irwin et al. 1991).

2. Phylogenetic analyses. Neighbor-joining trees were constructed using a number of different algorithms (discussed above). Because of the high level of nucleotide sequence variation, the most appropriate estimators of genetic distance are those of Kimura (1980) and Tamura and Nei (1993). The Kimura 2-parameter distance corrects for differing rates of transition versus transversion substitutions whereas the Tamura-Nei distance accounts for both transition-transversion and G + C biases. Kumar et al. (1994) suggest that these methods be used when the following criteria are met: (a) the estimated number of nucleotide substitutions per site is between 0.05 and 0.3, (b) the transition-transversion

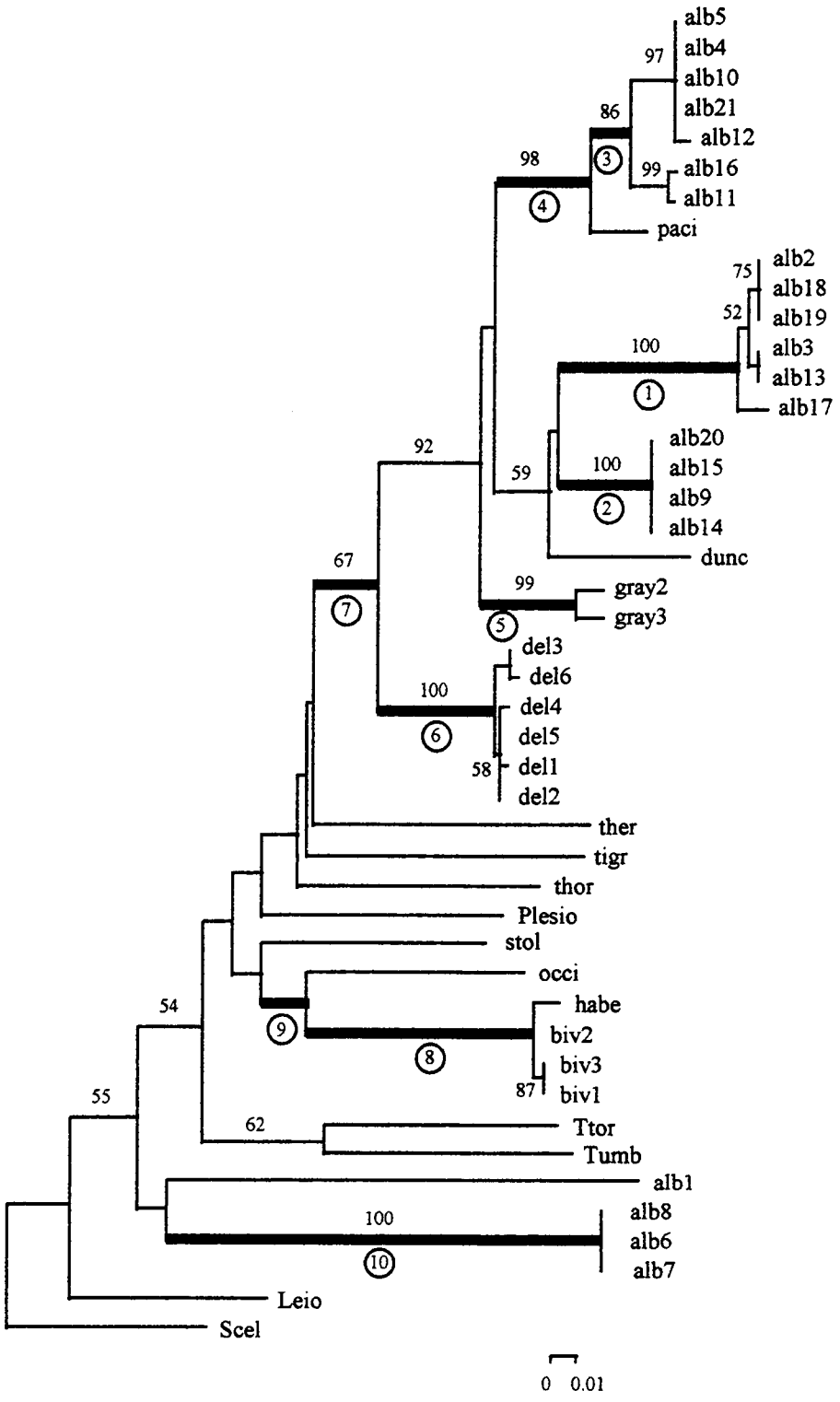
ratio is greater than 2.0, and (c) the frequencies of the four nucleotides (A, T, C, G) deviate substantially from equality. The present data set meets all three of these conditions. Neighbor-joining trees constructed with these two methods had nearly identical topologies (see below). Multiple maximum parsimony analyses were performed with the following transition/transversion ratios: 1:1, 3:1, and 10:1.

As noted above, sequence for the entire 307 bp region of the mt cytochrome *b* gene was not obtained for one sample of *M. grayi* (gray 1). However, because all MEGA analyses were performed using the option whereby sites with missing or ambiguous data in even a single sample were deleted, this would decrease the size of the data set by approximately 24% (from 307 bp to 234 bp). Therefore, trees were reconstructed without this sample, in order to have as many bp as possible to analyze. Subsequent analyses were performed which included this population; tree topology did not significantly change (see below).

Twenty-two trees were reconstructed and are included in Appendix D. For ease of reference, Fig. 3.4 shows one of the twenty-two phylogenies examined. There is a great deal of concordance in the reconstructed phylogenies, with ten clades appearing in all (or nearly all) of the trees. These clades generally reflect the geographic origin of the samples comprising them. The ten clades are designated as follows:

(1) a "Santa Cruz" clade (includes samples from Santa Cruz [alb 2, alb 3, alb 13, and alb 17], Baltra [alb 18], and Daphne [alb 19]; refer to Appendix A for exact localities);

Figure 3.4. A phylogeny of Galápagos lava lizards. This phylogeny was reconstructed using nucleotide variation in a 307 bp portion of the mitochondrial cytochrome *b* gene. It was built by the neighbor-joining algorithm using the Kimura 2-parameter model for estimating evolutionary distance and is one of 22 phylogenies appearing in Appendix D. The branches printed in bold identify the ten clades which consistently appear throughout the phylogenies in Appendix D. Numbers on the branches indicate the bootstrap *p*-values from 2000 replicates (only values greater than 50% are shown). Circled numbers correspond to the clades indentified in the text. The scale bar indicates relative branch length (measured in number of substitutions per nucleotide site). Sample abbreviations are listed in Appendix A.


alb5
alb4
alb10
alb21
alb12
alb16
alb11
paci
alb2
alb18
alb19
alb3
alb13
alb17
alb20
alb15
alb9
alb14
dunc
gray2
gray3
del3
del6
del4
del5
del1
del2
ther
tigr
thor
Plesio
stol
occi
habe
biv2
biv3
biv1
Ttor
Tumb
alb1
alb8
alb6
alb7
Leio
Scel
97
86
98
99
75
52
100
92
59
100
67
99
100
58
54
55
62
87
100
0 0.01

(2) a "Santiago" clade (includes samples from Santiago [alb 9, alb 15, and alb 20] and Bartolomé [alb 14]);

(3) an "Isabela/Fernandina" clade (includes samples from Isabela [alb 4, alb 10, alb 11, alb 12, and alb 16] and Fernandina [alb 5 and alb 21]);

(4) an "Isabela/Fernandina + Pinta" clade (includes all the samples included in the "Isabela/Fernandina" clade plus the taxon from Pinta [paci]);

(5) a "Floreana" clade (includes samples from Floreana [gray 2, and gray 3]);

(6) an "Española" clade (includes samples from Española [del 1, del 2, del 3, del 4, del 5, and del 6]);

(7) a "Central Islands" clade (includes all the samples from the previous six clades plus the taxon from Pinzón [dunc]);

(8) a "San Cristóbal + Marchena" clade (include samples from San Cristóbal [biv 1, biv 2, and biv 3] and Marchena [habe]);

(9) a "San Cristóbal + Marchena + *occipitalis*" clade (includes all the samples included in the "San Cristóbal + Marchena" clade plus *Microlophus occipitalis* from mainland South America);

(10) a "Guy Fawkes" clade (includes samples from the Guy Fawkes islets [alb 7 and alb 8] and Santa Cruz [alb 6]).

These ten groupings occurred in all (or nearly all) of the phylogenies shown in Appendix D, regardless of the distance-correction algorithm or method of tree-building used.

Table 3.1 lists the statistical occurrence of the ten recurrent clades. For trees reconstructed by neighbor-joining, the distance-correction algorithm and gamma distance

Table 3.1. Tree statistics for recurrent clades. The following statistics are given for each clade: bootstrap *p*-values from 2000 replications (= BP) and/or confidence probability values (= CP) for each clade in the distance-based NJ phylogenies, the frequency of appearance of a clade in equally parsimonious trees, and bootstrap values for maximum parsimony trees based on 100 bootstraps (nearest-neighbor interchange). The samples comprising each clade are as follows: clade 1 = alb 2, alb 3, alb 13, alb 17, alb 18, and alb 19; clade 2 = alb 9, alb 14, alb 15, and alb 20; clade 3 = alb 4, alb 5, alb 10, alb 11, alb 12, alb 16, and alb 21; clade 4 = clade 3 and paci; clade 5 = gray 2 and gray 3; clade 6 = del 1, del 2, del 3, del 4, del 5, and del 6; clade 7 = clades 1 through 6 and dunc; clade 8 = biv 1, biv 2, biv 3, and habe; clade 9 = clade 8 and occi; and clade 10 = alb 6, alb 7, and alb 8. For each distance-based tree, the distance algorithm used is indicated (Kimura's 2-parameter, Tamura-Nei's, Jukes-Cantor's, Tajima-Nei's or Tamura's) and whether bootstrap *p*-values or confidence probability values were calculated. If a gamma distance was used, the alpha value is shown (a = 0.3, 0.5, 1.0, or 2.0). For maximum parsimony (MP) trees, the transversion to transition ratio used in the analysis is indicated (1:1, 3:1, or 10:1) and whether the analysis was bootstrapped (= bootstraps). A dash (-) indicates a clade does not appear in a particular analysis.

Analysis			Clade 1	Clade 2	Clade 3	Clade 4	Clade 5	Clade 6	Clade 7	Clade 8	Clade 9	Clade 10
Kimura		BP	100	100	86	98	99	100	67	100	47	100
Kimura		CP	99	99	79	99	99	99	88	99	65	99
Tamura-Nei		BP	100	99	86	98	98	100	64	100	43	100
Jukes-Cantor		BP	100	100	86	99	99	100	68	100	51	100
Jukes- Cantor		CP	99	99	79	99	99	99	89	99	67	99
Tajima-Nei		BP	100	100	85	98	99	100	65	100	46	100
Tamura		BP	100	99	86	98	99	99	67	100	46	100
Jukes-Cantor	a=0.5	BP	100	99	88	98	98	99	63	100	48	100
Jukes-Cantor	a=1.0	BP	100	99	88	98	98	100	66	100	50	100
Jukes-Cantor	a=2.0	BP	100	100	86	98	99	100	67	100	51	100
Kimura	a=0.5	BP	100	99	87	97	96	99	58	100	39	100
Kimura	a=1.0	BP	100	99	87	98	98	100	64	100	43	100
Kimura	a=2.0	BP	100	99	85	98	98	100	66	100	44	100
Tamura-Nei	a=0.3	BP	99	94	86	90	86	93	-	97	23	99
Tamura-Nei	a=0.5	BP	99	99	87	95	95	99	48	99	29	100
Tamura-Nei	a=1.0	BP	100	99	88	98	98	99	57	100	37	100
MP	1:1		100	100	100	100	100	100	100	100	100	100
MP	3:1		100	100	100	100	100	100	100	100	100	100
MP	10:1		100	100	100	100	100	100	100	100	100	100
MP	1:1	bootstraps	100	98	76	89	100	99	73	100	-	100
MP	3:1	bootstraps	100	99	86	95	96	100	86	99	72	100
MP	10:1	bootstraps	100	99	84	87	99	96	83	73	64	100

alpha value (if used) are indicated as well as the bootstrap *p*-values (BP) from 2000 replicates or confidence probability values (CP) generated by the standard error test. For maximum parsimony trees, no single most parsimonious tree was recovered; the frequency of appearance of a clade in equally parsimonious trees or bootstrap values from 100 bootstraps (nearest-neighbor interchange) are shown. Only 100 bootstraps were performed on the MP trees due to the prohibitively large computational demands presented by the current data set.

In the reconstructed phylogenies, confidence limits of a number of nodes (as determined by bootstrapping, the standard error test, or frequency of a clade in equally parsimonious trees) are high enough to be considered statistically significant (i.e. 95% or greater). There are, however, values lower than 95% for certain nodes. These lower values are due to the large number of taxa included in the trees relative to the number of nucleotides sites examined. Nei (1991) has shown in simulation studies that from 760 to 8,200 base pairs of sequence are required to achieve bootstrap values of 95% or higher for all nodes in a tree with as few as 4 or 5 taxa. The present data set contains ~300 bp from each of 46 taxa; thus one would not expect significant confidence levels except for those clades comprised of the most closely related taxa. Therefore it is reassuring to see so many groups consistently supported by confidence values of 95% or greater. Furthermore, it has been found that in phylogenies involving over 20 taxa and more than 200 bp, bootstrap confidence levels of 30% may be associated with significant branch lengths (J. Archie, pers. comm., in Lopez, 1994). Although I do not suggest that all confidence values ≥ 30% reported here are significant, this conclusion lends additional

support for those groups with confidence values which are high (greater than 75%) but less than 95% .

D. Discussion

1. Galápagos *Microlophus*. One recurrent feature of the phylogenies in Appendix D is the presence of three distinct groups of Galápagos *Microlophus*: (1) the "San Cristóbal + Marchena" clade, (2) the "Central Islands" clade, and (3) the "Guy Fawkes" clade (Fig. 3.4). The division of the island samples into these groups is consistent and appears in every phylogeny examined, whether based upon parsimony or distance analyses.

The "San Cristóbal + Marchena" clade, consisting of *M. bivittatus* and *M. habeli* populations, is strongly supported in all neighbor-joining trees, with BP or CP values (depending on the specific analysis) of 97% or greater (Table 3.1). Indeed, in the majority of trees, this clade is supported with values of 100%. Parsimony analysis yielded similar results, with five of the six MP trees supporting this clade at 99% or greater (measured by the percentage of equally parsimonious trees in which the clade appears or the bootstrap value obtained after 100 bootstrap replications). Within this group, two samples (biv 1 and biv 3) consistently cluster together to the exclusion of the other samples (with BP or CP values ranging from 77% to 88% in the neighbor-joining trees and 81% to 100% in the parsimony trees). Both of these animals were sampled from the island of San Cristóbal; biv 2 was collected from Islote Lobos, a small islet off the coast of San Cristóbal, and the remaining sample of this clade (habe) is from the

island of Marchena. The relationships indicated, therefore, are concordant with both the geographic origin of the samples as well as previously recognized patterns of relatedness.

The present data set shows Kimura 2-parameter genetic distances of only 0.0165 to 0.0233 between *M. bivittatus* and *M. habeli*, implying a close relationship between the two species. Wright (1983) noted a high degree of similarity in the allozymes of these species and considered them sister taxa not closely related to the other island species (Fig. 1.6). In like manner, Lopez et al. (1992) concluded on the basis of immunological comparisons of serum albumins that the albumin of *M. bivittatus* was most similar to that of *M. habeli.*

The sister taxon to the "San Cristóbal + Marchena" clade is *M. occipitalis*, a species from western mainland South America (Fig. 3.4). This relationship is supported in nearly every phylogeny, although the mean level of statistical support does not approach significant values (Table 3.1). The sister taxa status of the "San Cristóbal + Marchena" clade and *M. occipitalis* has been hypothesized previously. Wright (1983; p.146) mentions unpublished data suggesting that the populations on San Cristóbal and Marchena are genetically more similar to three mainland species than they are to the other Galápagos populations; *M. occipitalis* is identified as the sister taxon to the populations from San Cristóbal and Marchena (J. Wright, pers. comm.). In the study of Lopez et al. (1992), analysis of reciprocal immunological distances place *M. habeli* as the sister taxon to *M. occipitalis* (Fig. 1.7).

The phylogenetic relationship between the "San Cristóbal + Marchena" clade and *M. occipitalis* is important in that it implies that the current assemblage of Galápagos

Microlophus is not monophyletic. The "San Cristóbal + Marchena" clade containing two of the Galápagos species of *Microlophus* is not the sister group to the "Central Islands" clade, which contains the remaining five Galápagos species. This phylogeny suggests that multiple colonization events are needed to account for the present-day distribution and patterns of relatedness among Galápagos lava lizards. Similar conclusions were reached by both Wright (1983) and Lopez et al. (1992). Using allozyme data, Wright (1983) proposed two independent colonizations of the Galápagos by lava lizards; Lopez et al. (1992) interpreted the immunological data as supporting at least three colonization events (see Ch. 4 for a discussion of biogeography and models of colonization).

The majority of Galápagos samples form a large monophyletic group (the "Central Islands" clade) which appears in every phylogeny examined (Fig. 3.4; Appendix D). This clade is supported by statistical values ranging from 48% to 89% and 73% to 100% in the distance and parsimony analyses, respectively (Table 3.1). The grouping of these taxa into a monophyletic assemblage concurs with the relationships proposed by Wright (1983). His data supported the separation of Galápagos lava lizards into two main lineages, with the populations from San Cristóbal and Marchena comprising one lineage and the remaining populations comprising the second (Fig. 1.6). Unfortunately, the data of Lopez et al. (1992) are limited in that reciprocal distances could not be obtained for all Galápagos taxa; as such, they cannot be used to reach conclusions regarding the phylogenetic status of the taxa comprising the "Central Islands" clade.

Within the "Central Islands" clade, numerous monophyletic groups can be successively identified. Basal to the other taxa within the clade is the "Española" clade

(Fig. 3.4). This group consists of six populations of *M. delanonis* from Española and surrounding islets. Very low levels of genetic divergence exist among this group, with pairwise distances ranging from 0.0034 to 0.0138 (calculated using Kimura's [1980] 2-parameter correction); the values obtained using the correction of Tamura and Nei (1993) are almost identical (0.0034 to 0.0139). Statistical support for the monophyly of this group is very high in both distance and parsimony analyses, with all but two values reaching 99% or greater (Table 3.1).

The placement of *M. delanonis* as the sister taxon to the other "Central Islands" taxa agrees with previous work. Wright (1983) concluded that, within the lineage containing the majority of Galápagos taxa, the populations from Española and its satellite island Gardner have the most divergent allozymes and occupy the basal position in that lineage (Figs. 1.5 and 1.6). Lopez et al. (1992) obtained average immunological distances between *M. delanonis* and three other Galápagos species (*M. albemarlensis, M. habeli*, and *M. pacificus*) which were greater than those between the latter three species and *M. peruvianus*, a mainland South American species (Table 1.3). When these data were used to reconstruct phylogenetic relationships, *M. delanonis* was placed as the basal species within the ingroup (Fig. 1.7). The agreement of three independent measures of the genetic relationship of *M. delanonis* relative to the other "Central Islands" taxa gives strong support for the phylogenetic placement of this species.

The remaining "Central Islands" taxa form a clade whose monophyly is well supported (Fig 3.4; Appendix D). In the neighbor-joining trees, the statistical values of the node leading to this clade range from 88% to 98%; all but one of the parsimony trees

gave values of 88% or greater, with four reaching 96% or greater (Appendix D). The populations of *M. grayi* (the "Floreana" clade) cluster together and form the sister group to the remaining "Central Islands" taxa (the populations of *M. albemarlensis, M. duncanensis*, and *M. pacificus*). Statistical support for the "Floreana" clade ranges from 86% to 99% in the distance trees (with the majority of values at 95% or greater) and from 96% to 100% in the parsimony trees. These results vary slightly from those of Wright (1983); in that phenogram, *M. grayi* is the sister taxon to *M. pacificus* (Fig. 1.6).

Within the remaining taxa of the "Central Islands" clade, four distinct lineages are present; they are, in successive branching order, the "Isabela/Fernandina + Pinta" clade (consisting of populations of *M. albemarlensis* and *M. pacificus*), the sample from Pinzón (*M. duncanensis*), the "Santiago" clade and the "Santa Cruz" clade (the latter two both consisting of *M. albemarlensis* populations) (Fig. 3.4). These groups appear in every phylogeny, whether based on distance or parsimony analysis (Table 3.1; Appendix D). The order of their appearance is also very consistent, with only minor variations in a few trees. Some of these variations are due to phylogeny reconstruction using unrealistic estimators of genetic distance. For instance, the phylogeny obtained after calculating gamma distances with Tamura and Nei's (1983) correction and an alpha value of 0.3 is quite different from the other phylogenies (Appendix D). The results are most likely due to the use of an alpha value which drastically over-estimates site-specific variation in the rate of substitution (S. Kumar, pers. comm.). When a more realistic alpha value of 1.0 is used, the topology of the tree is concordant with those obtained in other analyses (Appendix D).

The "Isabela/Fernandina + Pinta" clade is basal to the three other lineages (Fig. 3.4). This clade consists of seven populations of *M. albemarlensis* from the islands of Isabela and Fernandina (the "Isabela/Fernandina" clade) and a single population of *M. pacificus* from Pinta. The average genetic distance between *M. pacificus* and the taxa comprising the "Isabela/Fernandina" clade is 0.0376, whether determined using Kimura's (1980) 2-parameter or Tamura and Nei's (1993) correction. Statistical support for the grouping of these taxa is high, ranging from 90% to 99% in the neighbor-joining trees and 87% to 100% in the parsimony trees (Table 3.1). Within the clade, *M. pacificus* is the sister taxon to a monophyletic "Isabela/Fernandina" clade; although this relationship appears in every tree examined, statistical support is somewhat lower than for the previous clade (Table 3.1). The sister taxa relationship of the "Isabela/Fernandina" clade with *M. pacificus* conflicts with the findings of Wright (1983). That study grouped *M. pacificus* and *M. duncanensis* as each other's closest relative, the population from Isabela closest to one from Santa Fe, and those from Fernandina closest to those from the islands of Santiago and Bartolomé (Fig. 1.6). The phylogeny of Lopez et al. (1992) placed *M. pacificus* as the sister taxon to a population of *M. albemarlensis* from Santa Cruz; however, they had reciprocal immunological distances for only a single population of *M. albemarlensis* and thus direct comparisons cannot be made between their findings and those of the present study.

Within the "Isabela/Fernandina" clade, the Kimura (1980) and Tamura-Nei (1993) genetic distances between the populations are identical, with a range from 0.0000 to 0.0337 and a mean value of 0.0150. Two of the populations (albe 11 and albe 16)

group together in every phylogeny examined with high statistical support (97% or greater in the distance trees and 92% or greater in the parsimony trees) (Appendix D). The other five samples also cluster together repeatedly with high support (92% or greater in all but one of the distance trees and 82% or greater in the parsimony trees). Samples albe 11 and albe 16 are from either side of the narrowest point of Isabela (Elizabeth Bay and Cartago Bay, respectively); the other samples originate from northern Isabela, offshore islets, or northern Fernandina. Thus, the sister group relationship of albe 11 and albe 16 is most likely due to the close geographic proximity of these taxa.

Of the remaining populations of the "Central Islands" clade, *M. duncanensis* (from Pinzón) is basal to a grouping of the "Santiago" and "Santa Cruz" clades (Fig. 3.4). This relationship appears in most (but not all) of the phylogenies examined, at intermediate levels of statistical support (46% to 83% and 58% to 100% in distance and parsimony trees, respectively). The nucleotide sequence of *M. duncanensis* is quite distinct from the other two clades; the average genetic distance between it and the populations of the "Santiago" clade is of 0.0637 and 0.0649 (Kimura 2-parameter and Tamura-Nei, respectively). The differentiation between *M. duncanensis* and the "Santa Cruz" clade is even greater, with average genetic distances of 0.1023 and 0.1048 (Kimura 2-parameter and Tamura-Nei, respectively). Previously, the phylogenetic placement of *M. duncanensis* had been questionable, with Wright (1983) placing it in an unresolved polytomy (Fig. 1.6) whereas Lopez et al. (1992) find it to be immunologically similar to both *M. albemarlensis* and *M. pacificus*.

The "Santiago" clade is comprised of four populations from the islands of Santiago and Bartolomé, appears in every phylogeny in Appendix D, and is strongly supported. Statistical values in the distance trees are 95% or greater; parsimony analyses yield even stronger support, with values of 98% or greater (Table 3.1). The four populations exhibit no nucleotide substitutions relative to each other (Fig. 3.1), resulting in genetic distances of 0.0000. The close phylogenetic relationship of these populations has also been demonstrated by the allozyme data of Wright (1983); those data produced Roger's coefficients of genetic similarity of 0.961 or greater (Fig. 1.5), and so their grouping here is not surprising.

The "Santa Cruz" clade consists of six populations: four from Santa Cruz and one each from Baltra and Daphne (Fig. 3.4). This clade has perhaps the strongest statistical support of any group in this study; it appears in every phylogeny, whether based on distance or parsimony analysis, at values of 99% or greater (Table 3.1). The populations which comprise it are very similar genetically, with average pairwise distances of 0.0072 (this value is the same for both the Kimura 2-parameter and Tamura-Nei distances). In a like manner, these populations exhibit low levels of genetic differentiation in their allozymes, with Roger's coefficients of genetic similarity ranging from 0.920 to 0.977 (Wright, 1983; Fig 1.5).

The phylogenies obtained from the various distance algorithms and methods of tree reconstruction are very consistent with respect to the "Central Islands" clade (Appendix D). However, several alternative topologies were obtained. The first, which appeared in only four trees, involved the relationships among *M. duncanensis* and the

"Santa Cruz" and "Santiago" clades. In these trees, the "Santa Cruz" clade was placed as the sister group to a pairing of *M. duncanensis* and the "Santiago" clade. The statistical support for this arrangement was fairly weak, with values ranging from 39% to 47%. All four trees involved gamma distances whose alpha values most likely were not appropriate for the given data set.

A second alternative topology also involved the placement of *M. duncanensis*. In two of the parsimony trees (in both of which a 10:1 transition/transversion weighting was used), this taxon was identified as the sister group to the "Isabela/Fernandina + Pinta" clade (Appendix D), and this group was placed in an unresolved polytomy with the "Santa Cruz" and "Santiago" clades. The observed topology may be the result of using a 10:1 transition/transversion ratio; the actual ratio calculated from all pairwise comparisons is approximately 2.4:1. This ratio is similar in value to the 3:1 ratio used in a subset of the parsimony analyses. Interestingly, the topology of the trees obtained using a 3:1 ratio is nearly identical to that obtained in the distance-based trees (Appendix D).

As noted above, for one sample of *M. grayi* (gray 1), the nucleotide sequence for the entire 307 bp region of the mt cytochrome *b* gene was not obtained. However, all nucleotide sites with missing data were removed prior to tree reconstruction. Therefore, to prevent a severe reduction in the size of the data set (from 307 bp to 234 bp), this sample was excluded from analysis. This was also done to circumvent the statistical problems which can arise when determining confidence probability values from a data set in which each pairwise comparison does not involve the same number of nucleotides (S. Kumar, pers. comm.). However, to investigate the effects of gray 1 on the results,

phylogenetic trees were reconstructed with it included. The topology which is obtained shows similarities to those trees which exclude gray 1 but differ in several noticeable points. All of the clades listed in Table 3.1 are still present. Within the "Central Islands" clade, the "Española" clade is still basal to the other groups. The gray 1 population clusters with the other *M. grayi* populations as part of the "Floreana" clade, which forms the sister group to the remaining "Central Islands" samples.

To this point, the topologies of the trees with and without gray 1 are in agreement. However, no further similarities are present. In those trees in which gray 1 is included, a group consisting of *M. duncanensis* and the "Santiago" clade forms the sister group to the "Santa Cruz" and "Isabela/Fernandina + Pinta" clades; in trees lacking gray 1, the "Isabela/Fernandina + Pinta" clade is basal to a group comprised of *M. duncanensis* and the "Santa Cruz" and "Santiago" clades. Despite the overall similar topologies, the trees reconstructed without gray 1 are most likely closer approximations of the true phylogenetic history of the Galápagos lava lizards, due to the prohibitively small size of the data set when gray 1 is included.

The remaining group of Galápagos taxa form the "Guy Fawkes" clade (Fig. 3.4). This group included two populations from the Guy Fawkes rocks (a collection of small rocks off the northwest coast of Santa Cruz) and one from Conway Bay, Santa Cruz, immediately south of the Guy Fawkes rocks. This clade appears in every phylogeny examined, with high statistical support; three of the values shown for this clade in Table 3.1 are 99% and the rest are 100%. When the nucleotide sequences of these populations are examined, they are identical to one another yet quite divergent from the remaining

taxa (Fig. 3.1). The average Kimura 2-parameter genetic distance between the "Guy Fawkes" populations and the other samples is 0.2749, a much higher value than that seen between most other populations in this study.

One possible explanation for the extreme genetic differentiation between the "Guy Fawkes" populations and the other clades is the effect of the small size of the Guy Fawkes rocks. These rocks are quite small and do not support a large number of individual lizards. Due to their small size, the Guy Fawkes populations would be affected by genetic drift to a greater degree than would larger populations (Holgate, 1966; Nei et al. 1975; Chakraborty and Nei, 1977). In addition, the inability of these populations to rebound to a large size would further increase any founder effect. Through these and other stochastic events, the genomes of this clade would become more divergent than might otherwise be expected.

In most of the phylogenies in Appendix D, the sample alb 1 (a population of *M. albemarlensis* from Islote Santa Fe, a small islet off the coast of Santa Fe) is placed as the sister taxon to the "Guy Fawkes" clade. This is surprising when one considers the geographic distance between these populations (Fig. 1.1). As with the "Guy Fawkes" clade, the nucleotide sequence of alb 1 is quite distinct from all other populations (Fig. 3.1) and its extreme genetic divergence is most likely due to stochastic events relating to the founder effect. The average Kimura 2-parameter distance between it and all other taxa is 0.3303; the average genetic distance between alb 1 and the "Guy Fawkes" clade is even greater, 0.3377. The apparent phylogenetic relationship between these taxa is probably the result of long-branch attraction. This occurs when there are many changes

along a branch(es) in a phylogeny, causing artifactual grouping among these longer branches (Hendy and Penny, 1989; Miyamoto and Boyle, 1989; Wheeler, 1990). While the true phylogenetic relationships of these populations cannot be resolved with the present data set, the previous work of Wright (1983) and Lopez et al. (1992) suggests that both the Santa Fe population and the "Guy Fawkes" populations are closest to those samples included here in the "Santa Cruz" clade.

2. Populations of *Microlophus albemarlensis*. In the Galápagos, *M. albemarlensis* is the only species of lava lizard which occurs on more than a single island (Fig. 1.2). Several of the numerous populations currently assigned to this species have at various times been accorded specific status based on morphological differences (see the discussion of the taxonomic history in Ch. 1).

More recent work examining differences at the genetic level have also documented the presence of distinct clades within *M. albemarlensis*. Wright's (1983) allozyme study identified two lineages containing populations of *M. albemarlensis*; the first was comprised of the populations from Santa Cruz, Daphne, and Baltra islands and the second contained the populations from Isabela, Fernandina, Santiago, and Bartolomé (Fig. 1.6). Roger's coefficients of genetic similarity between these lineages are in many cases smaller than those between populations recognized as distinct species (Fig. 1.5). In addition, immunological data exhibit marked differences among the *M. albemarlensis* populations (Lopez et al. 1992). One-way immunological distances between the albumin of a population from Academy Bay, Santa Cruz, to other *M. albemarlensis* populations

range from 0 to 20 ID units (Table 1.4). In contrast, values of comparisons between recognized species can be as low as 6 ID units (Table 1.5).

In the present study, populations of *M. albemarlensis* appear as three distinct clades within the reconstructed phylogenies (Fig. 3.4; Appendix D), if one disregards the taxa comprising the "Guy Fawkes" clade for the reasons discussed above. These clades are the "Santa Cruz", "Santiago", and "Isabela/Fernandina" clades. The first two groups are placed as each others' sister group, whereas *M. pacificus* from Pinta is the sister taxon to the "Isabela/Fernandina" clade. In this aspect, the present data are in concordance with the earlier studies.

The genetic distances between these clades and the other taxa support the hypothesis that the populations of *M. albemarlensis* are not each others' closest relatives and do not form a monophyletic assemblage. The "Isabela/Fernandina" clade has an average genetic distance of 0.0394 to *M. pacificus*, whether calculated using the Kimura (1980) 2-parameter or Tamura-Nei (1993) correction. In comparison, the average Kimura 2-parameter genetic distance between the "Isabela/Fernandina" clade and the "Santa Cruz" and "Santiago" clades is 0.1207 and 0.1032, respectively. These "intra-specific" values are much higher than those observed for many comparisons involving taxa recognized as distinct species. For example, the genetic distance between *M. duncanensis* and *M. pacificus* is 0.0983, that between *M. grayi* and *M. pacificus* is 0.0840, and that between *M. duncanensis* and *M. grayi* is 0.0926.

In addition, the average level of genetic divergence between the "Santa Cruz" and "Santiago" clades is also much higher than would be expected if these populations were

conspecific. The average Kimura 2-parameter genetic distance between these clades is 0.0791. As comparison, the average distance between *M. duncanensis* (the sister taxon to the "Santa Cruz" + "Santiago" clade; Fig. 3.4; Appendix D) is 0.1036 and 0.0634, respectively. Although the populations of these two *M. albemarlensis* clades are currently recognized as belonging to the same species, the "Santiago" clade is demonstrably distinct from the "Santa Cruz" clade and is genetically more similar to *M. duncanensis*.

Based on the results of the current data set, as well as the patterns of genetic differentiation noted in the previous studies of Wright (1983) and Lopez et al. (1992), I am proposing that the populations of the "Isabela/Fernandina", "Santa Cruz", and "Santiago" clades each be considered separate species. They are as genetically distinct from each other as any of the currently designated Galápagos species and warrant recognition at the level of species, rather than subspecies or population.

Microlophus albemarlensis was originally described from the island of Isabela (Baur, 1890) and so those populations of the "Isabela/Fernandina" clade will retain that species designation. The populations comprising the "Santa Cruz" clade are re-designated *M. indefatigabilis*, having been described as such previously (Baur, 1890). The "Santiago" clade populations are re-designated *M. jacobi*, in accordance with Baur's (1892) description of individuals from this island.

The specific status of these populations is more than an academic exercise. Conservation of threatened, endangered, and unique organisms has become increasingly important in recent years as expanding human populations and destruction of the

environment increase the pressures exerted on the natural world. It is probable that, due to the destruction of their habitat, untold numbers of species are being driven to extinction before they are described. Accurate systematic knowledge of the Earth's organisms is required in order to most efficiently preserve this biodiversity. Imprecise taxonomy can have dangerous consequences. The tuatara of New Zealand exemplifies this problem. Until recently, the genus *Sphenodon* was considered monotypic. However, recent allozyme work revealed the presence of two species, one with two subspecies (Daugherty et al. 1990). The "rediscovered" species is found on only a single island. Had it not been for this reinstatement of the second species, protection of its habitat may not have been considered and, due to the pressures of an increasingly urbanized world, another irreplaceable animal might have gone extinct.

Similar pressures may be present in the Galápagos Islands. In addition to the growing human population in the archipelago, numerous exotic species have been introduced. These invaders include eleven species of mammals, including dogs, cats, pigs, goats, and black rats (Hoeck, 1984); all have had a negative impact on the native flora and fauna, either through competition for resources or predation upon the native species (MacFarland et al. 1974; Kruuk and Snell, 1981; Hoeck, 1984).

To possess a clear view of the degree to which the Galápagos need to be protected, it is necessary to have an accurate knowledge of exactly how many species inhabit the islands as well as their status. The Galápagos National Park Service and the Charles Darwin Research Station were created to monitor and preserve the unique biota of the archipelago. In addition to eradicating feral species, these organizations maintain

captive breeding programs to provide the necessary animals for repatriation. Currently these programs focus on the giant land tortoises and the land iguanas, perhaps the two groups most impacted by feral species.

As yet, the populations of lava lizards have not been incorporated into the captive breeding efforts. However, it is imaginable that the time will come when active conservation of the lava lizards will be necessary. In order to ensure the most efficient expenditure of limited resources (both human and financial), a clear understanding of the status of lava lizard populations is imperative. The proposed taxonomic changes indicated in this study should assist in conserving the biological wealth and uniqueness of an incomparable locality by aiding the decisions regarding future conservation plans.

3. Relationships of Galápagos *Microlophus* to mainland taxa. The lava lizards of the Galápagos almost certainly are derived from the *Microlophus* species of western South America. Numerous studies using either morphological (Van Denburgh and Slevin, 1913; Frost, 1992) or biochemical characters (Wright, 1983; Lopez et al. 1992) have suggested that the closest relatives to the Galápagos taxa are found on the mainland west of the Andes. It is less clear, however, exactly which of the mainland species is the ancestor of the island forms.

Figure 3.4 represents one hypothesis as to the sister taxa of the Galápagos species of lava lizards. As noted above, the "San Cristóbal + Marchena" clade is most closely related to *M. occipitalis*; this relationship has been noted before (Wright, 1983; Lopez et al. 1992). This group is then placed in a sister group relationship with another mainland species, *M. stolzmanni*. All four of these species had previously been placed within the

occipitalis group of Frost (1992); the only other members of this group were the remaining Galápagos species. As such, the *occipitalis* group is not a monophyletic assemblage and should no longer be recognized.

The ancestral species of the "Central Islands" clade is not as easily identified. Several potential ancestors are identified in the trees in Appendix D, three of which appear in the majority of trees. However, none of the alternative topologies receives strong statistical support. The species most commonly placed as the sister taxon to the "Central Islands" clade is *M. theresiae* (Fig. 3.4). This relationship appears in 7 of the 22 trees, but statistical support is never greater than 56%. In five of the trees, *M. thoracicus* is placed as the sister taxon to the "Central Islands" clade. While the support for this topology is weak in the distance-based trees in which it appears (26% - 31%), in the parsimony analyses it is supported in 100% of the equally parsimonious trees. A less frequently occurring topology is that in which the "Central Islands" clade is grouped with a clade containing *M. theresiae* and *M. tigris* (Appendix D). This is the most weakly supported topology, with statistical values of only 10%.

Using the present data set, the mainland species of *Microlophus* which gave rise to the "Central Islands" clade cannot be clearly identified. However, three species are identified as potential candidates (*M. theresiae*, *M. thoracicus*, and *M. tigris*). Further phylogenetic research is warranted in order to fully resolve the evolutionary history of the lava lizards of the Galápagos Islands.

E. Summary

Results of analyses of nucleotide sequence variation in a portion of the mitochondrial cytochrome *b* gene from Galápagos and South American species of tropidurine lizards concur with earlier studies of their patterns of genetic relatedness (Wright, 1983; Lopez et al. 1992). The present-day species of Galápagos lava lizards are not a monophyletic assemblage. There are two main lineages; the first includes those populations from San Cristóbal and Marchena (*M. bivittatus* and *M. habeli*, respectively) while the second is comprised of all other island populations (representing *M. albemarlensis, M. delanonis, M. duncanensis, M. grayi, M. indefatigabilis, M. jacobi,* and *M. pacificus*). The lizards from San Cristóbal and Marchena are more closely related to *M. occipitalis* from mainland South America than they are to the other Galápagos species. The ancestor of the remaining island species could not be unequivocally resolved but, of the mainland South American taxa examined, *M. theresiae, M. thoracicus,* and *M. tigris* were identified as possible sister taxa.

The populations of *M. delanonis* from Española and surrounding islands form the most basal group within the main lineage, followed by the *M. grayi* populations from Floreana. *Microlophus pacificus* (from Pinzón) is the sister taxon to the populations from the islands of Isabela and Fernandina. Populations from Santa Cruz, Baltra, and Daphne group as a monophyletic clade and form the sister group to a monophyletic assemblage of populations from Santiago and Bartolomé. *Microlophus duncanensis* from the island of Pinzón is the closest relative to these two groups.

Of those populations recognized as *M. albemarlensis*, four groups consistently emerged. One of these is formed by populations from the Guy Fawkes rocks and Islote Santa Fe. The observed levels of genetic divergence among these taxa are most likely attributable to genetic drift caused by the founder effect and small population size. The other three groups are distinct lineages with levels of genetic differentiation comparable to those exhibited by taxa historically recognized as valid species. Therefore, the populations from Santa Cruz, Baltra, and Daphne islands are raised to the specific level as *M. indefatigabilis* (Baur, 1890) and the populations from Santiago and Bartolomé islands are re-named *M. jacobi* (Baur, 1892). Future conservation plans should be altered accordingly so that these unique species are not lost due to habitat loss and/or effect of introduced biota.

Chapter 4

Biogeography of Galápagos Lava Lizards

A. Introduction

The study of the geographic distribution of plants and animals has played an important role in the development of evolutionary biology. Island biogeography has been especially important. Charles Darwin was struck by the uniqueness of the organisms inhabiting the Galápagos Islands and the inter-island variation he observed. Alfred Russel Wallace also was influenced by the time he spent on islands, specifically those of the Malay Archipelago. Such experiences aided in the independent formulation by these two workers of the theory of the origin of species.

Islands are particularly informative because their (generally) limited area and their isolation combine to make patterns of evolution more obvious. In general, islands support fewer species and therefore fewer species interactions. This results in a simplified ecosystem which enables evolutionary biologists to more easily comprehend the mechanisms at work. Darwin's (1859) *The Origin of Species* and MacArthur and Wilson's (1967) *The Theory of Island Biogeography* are two of many works which resulted from the contemplation of islands.

The Galápagos offer numerous examples from which to learn about island biogeography. Differing explanations for the arrival of *Microlophus* to the Galápagos (Wright, 1983; Lopez et al., 1992) underscore the need for further investigation into the patterns of colonization of the region. Analysis of molecular data has been used

repeatedly to illuminate patterns of biogeography (Wright, 1983; Maxson, 1984; Maxson and Roberts, 1984; Bermingham and Avise, 1986; Riddle and Honeycutt, 1990; Hass and Hedges, 1991; Hedges et al., 1991; Lopez et al., 1992; Hedges et al., 1993b). Through analysis of the genetic relatedness between mainland and Galápagos species of *Microlophus*, combined with an examination of the geologic history of the archipelago, this study will propose a hypothesis regarding the biogeographical history of this group.

B. Previous Studies on the Biogeography of the Galápagos Islands

Numerous studies have investigated the distribution patterns of the species inhabiting the Galápagos (Grant, 1981, 1984; Porter, 1983; Patton, 1984*a*). Surprisingly, however, few studies have offered models explaining the migration patterns of organisms in the archipelago. For many groups, including the finches and numerous plants, only the number of colonization events necessary to account for observed species diversity have been proposed. These studies have neglected to explain the subsequent movement of taxa among the islands. Species for which colonization models have been proposed include the giant tortoises, land iguanas, geckos, and weevils.

Marlow and Patton (1981) used allozyme data to estimate genetic relatedness among seven of the fifteen recognized subspecies of giant tortoise (*Geochelone elephantopus*). They proposed that the Galápagos tortoises are descended from a single mainland species. A single initial colonization event resulted in the establishment of tortoises on San Cristóbal. This population then served as the source for both Española

and Santa Cruz. Santa Cruz tortoises gave rise to all other sampled populations, either directly or indirectly through other islands.

A similar pattern was suggested for the two species of land iguanas (*Conolophus pallidus* and *C. subcristatus*). Using morphological characters, Snell et al. (1984) hypothesized a single colonization event on either Santa Cruz or Santa Fe. Subsequently, Santa Cruz was colonized by animals from Santa Fe (or vice versa, depending on which island was initially invaded from the mainland). Santa Cruz animals then served as the source population for Plaza Sur, Baltra, and Isabela (which in turn served as the source for Fernandina).

In contrast to the giant tortoises and land iguanas which are believed each to have arisen from a single colonization event, the six species of Galápagos leaf-toed geckos (*Phyllodactylus*) probably are the result of at least three founding events (Wright, 1983). One lineage of mainland geckos colonized San Cristóbal, which provided founders for the populations on Española. The population on Santa Cruz was derived from animals from either San Cristóbal or Española, but more likely the latter. The population on Santa Cruz gave rise to the lizards on Santa Fe (and perhaps to those on Fernandina). Later, the Santa Cruz population provided founders for the populations on Isabela, Pinzón, and Santiago and then to those of its satellite islands. The Santiago population served as the source of the founders for the Marchena population. A second lineage of mainland gecko colonized Isla Wolf and a third (perhaps inadvertently introduced by humans) resulted in a second colonization of San Cristóbal.

Weevils of the genus *Galapaganus* occur on most of the islands. Lanteri (1992) suggested that this group colonized the archipelago three times. One event led to the establishment of populations on the islands of Tower, Wolf, and Darwin. A second colonization event occurred on Santa Cruz. The third event gave rise to the populations on San Cristóbal, which then served as the source for both Española and Floreana. Santiago and Isabela were then colonized by animals from Floreana.

All of these proposed models are similar in that the eastern and central islands are colonized first and then serve as sources for the western islands. This sequence is concordant with the geologic evidence which suggests that the age of the islands decreases in a east-to-west direction (Cox, 1983; Hickman and Lipps, 1985). It is also concordant with the hypothesis that South America served as the source of immigrants. The Humboldt Current flows north along the western coast of South America before deflecting west to the Galápagos and is the likeliest route by which terrestrial, non-flying organisms were dispersed to the islands.

C. Biogeography of the Galápagos Lava Lizards

The presence of lava lizards in the Galápagos is undeniable. However, the manner in which they arrived is a matter of uncertainty. The first step in understanding the biogeography of Galápagos lava lizards is determining how they initially colonized the islands. Oceanic islands like the Galápagos start off without any native organisms and must gain their biota through long-distance dispersal (Carlquist, 1966*a*, *b*; 1981). The most likely means by which a small lizard would reach the Galápagos is as a

passenger on a floating raft of vegetation swept out to sea. A raft could be as small as a single log or tree trunk but can be much larger (up to a few hectares). Such rafts have been recorded and a number of them have had animals on them. In 1911, a floating mat of vegetation 30 meters square, and with 9 meter high trees on it, was reported traveling as far as one thousand miles in the North Atlantic (Thornton, 1971). Thornton also mentions a boa constrictor, which in 1827, arrived on the island of St. Vincent in the West Indies, wrapped around the trunk of a cedar tree.

An alternative method of dispersal involves pumice, which floats and has been recorded traveling great distances over water. In 1952, the Barcena volcano in the Revillagigedo Islands off the west coast of Mexico erupted. Richards (1958) recorded pumice ejected from this volcano drifting as far as Hawaii, Johnston Island, and even the Marshall Islands (an astounding 8700 km away!). While most of the pieces were relatively small, blocks approximately 3 feet square were observed and numerous fragments had small corals or barnacles attached to them. In a similar incident, Sutherland (1965) reported the arrival in Tasmania and western Australia of pumice which originated from an eruption in the South Sandwich Islands.

McBirney and Williams (1969) note that large quantities of pumice from the volcanoes of Central America and the Andes have been carried into the Pacific Ocean and propose the following scenario. Pumice, having lain for some time on river banks and beaches, accumulates soil and vegetation along with seeds, eggs, and the young of various plants and animals. This "populated pumice" is then washed out into the ocean where the prevailing currents carry it to the Galápagos Islands. However, while seeds or

insects may have reached the Galápagos in this fashion, it seems unlikely that larger animals would have arrived in such a manner. Nevertheless, floating lumps of pumice may be a feasible mechanism for movement among the islands, once an organism such as a lava lizard had become established in the archipelago via alternative methods.

The Humboldt Current flows from the western coast of South America to the Galápagos and is the likeliest route by which rafts would be carried to the islands. Based on the speed of the current between South America and the Galápagos, as well as the prevailing wind speeds, Thornton (1971) calculated that a vegetation raft would take about two weeks to reach the islands. Reptiles are probably more likely than mammals to survive a long trip on such a raft due to their often low food and water requirements, which is aided by their relatively water-impermeable skin. Peterson (1996) documented desert tortoises in the Mojave Desert that survived sixteen months between drinks. Numerous species of reptiles can withstand a considerable degree of dehydration (e.g. a loss of over 50% of initial body weight) without ill effects (Cloudsley-Thompson, 1991). While not all species of reptiles can endure such extreme conditions, such findings are indicative of their generally high tolerance to water loss, a trait which would contribute to their successful dispersal via rafting.

This model for colonization of the islands pre-supposes that the terrestrial plants and animals of the Galápagos have their origin in South America. This hypothesis has been widely investigated. Darwin (1845) was the first naturalist to note that the organisms of the Galápagos had affinities with South America. Porter (1983) demonstrated that most of the terrestrial vascular plants have relatives in South America.

The available evidence points to a South American origin for most of the insect taxa (see Lanteri, 1992, for review). The giant land tortoises were probably derived in comparatively recent times from a now extinct western South American species of *Geochelone* (Marlow and Patton, 1981). Patton (1984*b*) reviews the evidence which suggests the native rat species of the Galápagos were derived from the oryzomyine and thomasomyine groups of rodents from mainland South America.

Many of the mainland taxa examined in the present study are found in xeric coastal habitats (Dixon and Wright, 1975), including the putative ancestral species of the Galápagos taxa. A scenario such as the following may explain a colonization event. Heavy rains along the dry regions of the coastal plain could cause flash flooding, as the dry earth prevents the excess moisture from being quickly absorbed. The flooding rips a vegetation raft from the sides of a gully or small canyon. The raft, along with any lizards on it, is carried out to sea and into the Humboldt Current. Eventually the raft makes landfall on one of the Galápagos islands, where the lizards disembark and found a breeding population. Indeed, the coastal regions of the Galápagos islands (where lava lizards are most abundant) are characterized by having a arid climate, and so the ecological "shock" of colonization by a mainland lizard from a similar environment would be lessened. In addition, the original colonists would not have encountered ecologically related competitors.

Once the proposal that the lava lizards arrived from South America via rafting has been accepted, one must still deduce the pattern of their colonizations of the various islands. Wright (1983) proposed one scenario for the dispersal of *Microlophus* to the

Galápagos, and another was put forth by Lopez et al. (1992). The two models differ on a number of points. The model suggested by Wright (1983) involves two founder events, one each to San Cristóbal and Española (Fig 4.1). San Cristóbal served as the source for Marchena and no other islands were derived from this lineage. The Española population gave rise to the lizards on Santa Cruz, which then served as the source for the colonization of Santa Fe, Pinzón, and Santiago. Santiago in turn provided propagules for Pinta and Isabela, from which the populations on Fernandina and Floreana are descended.

Lopez et al. (1992) proposed that at least three founding events occurred (Fig. 4.2). One was to Española and a second gave rise to the (eventual) colonization of Pinta, Santa Cruz, Santiago, Santa Fe, Isabela, Bartolomé, and Fernandina. The third and most recent event established the lineage on Marchena. Unfortunately, no hypothesis is advanced to explain the colonization of San Cristóbal, Floreana or Pinzón.

The relationships among the Galápagos lava lizards as indicated by the cytochrome *b* data (Fig. 3.4), when combined with the geologic history of the islands, suggest the following biogeographic model. One founding event from the mainland colonized San Cristóbal and this population then served as the source for the colonization of Marchena (Fig. 4.3). A second founding event occurred on Española. Floreana was colonized from Española, and then Santa Cruz from Floreana. Lizards from Santa Cruz then colonized Pinta. At some later time, the population on Pinzón was established from Santa Cruz, followed by the colonization of Santiago (also from Santa Cruz). The islands of Isabela and Fernandina were colonized rather recently by animals from Pinta.

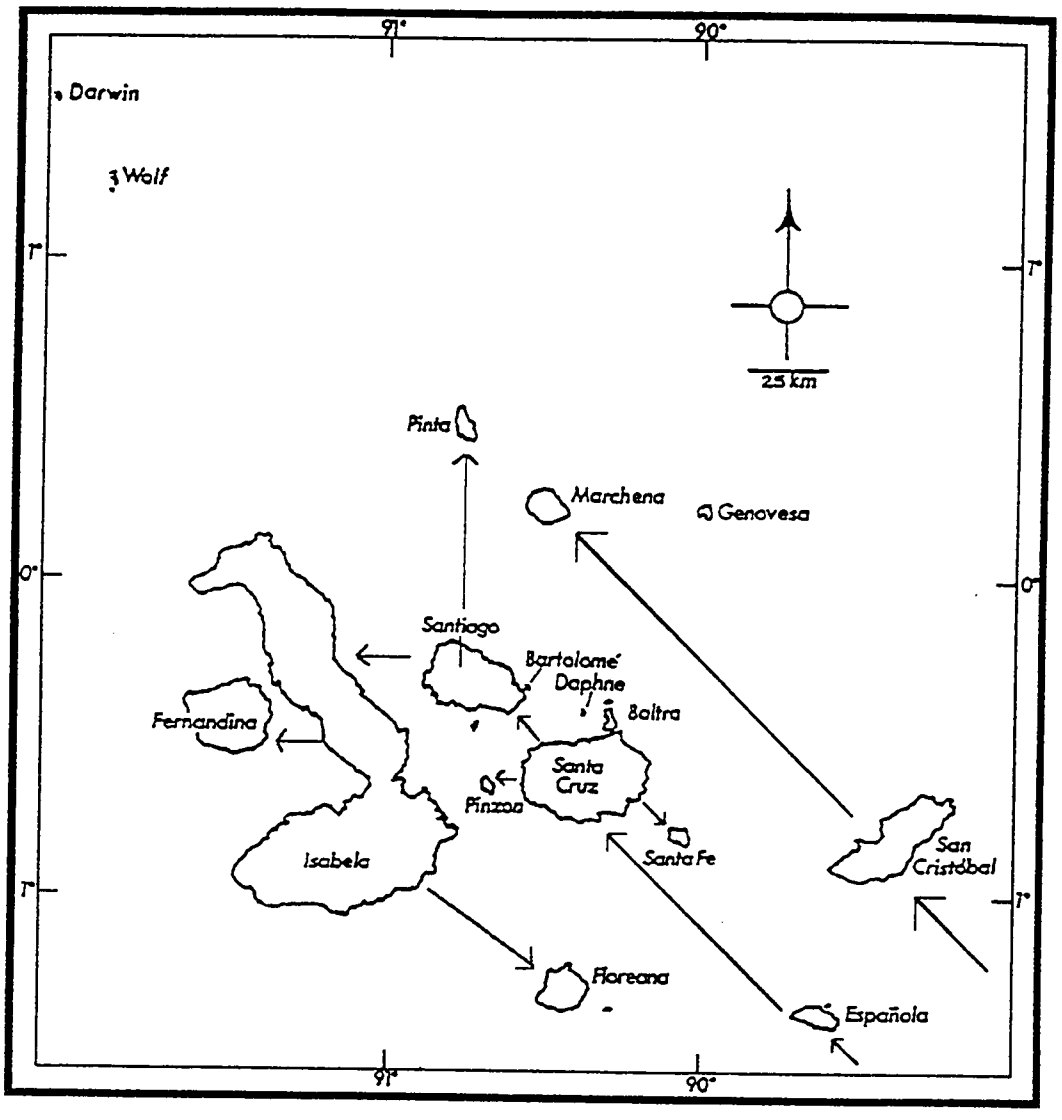


Figure 4.1. Colonization model proposed for *Microlophus* in the Galápagos Islands by Wright (1983).

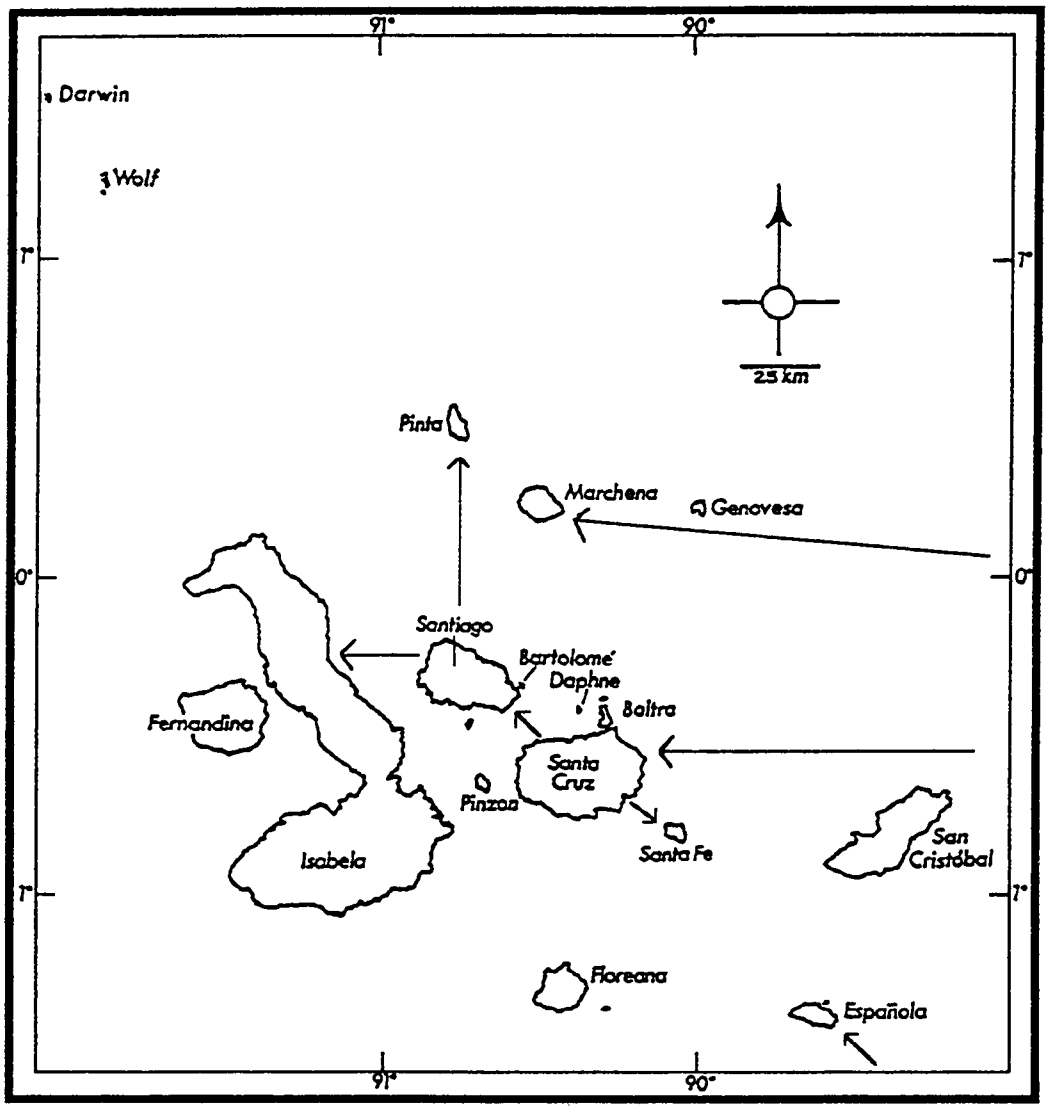


Figure 4.2. Colonization model proposed for *Microlophus* in the Galápagos Islands by Lopez et al. (1992).

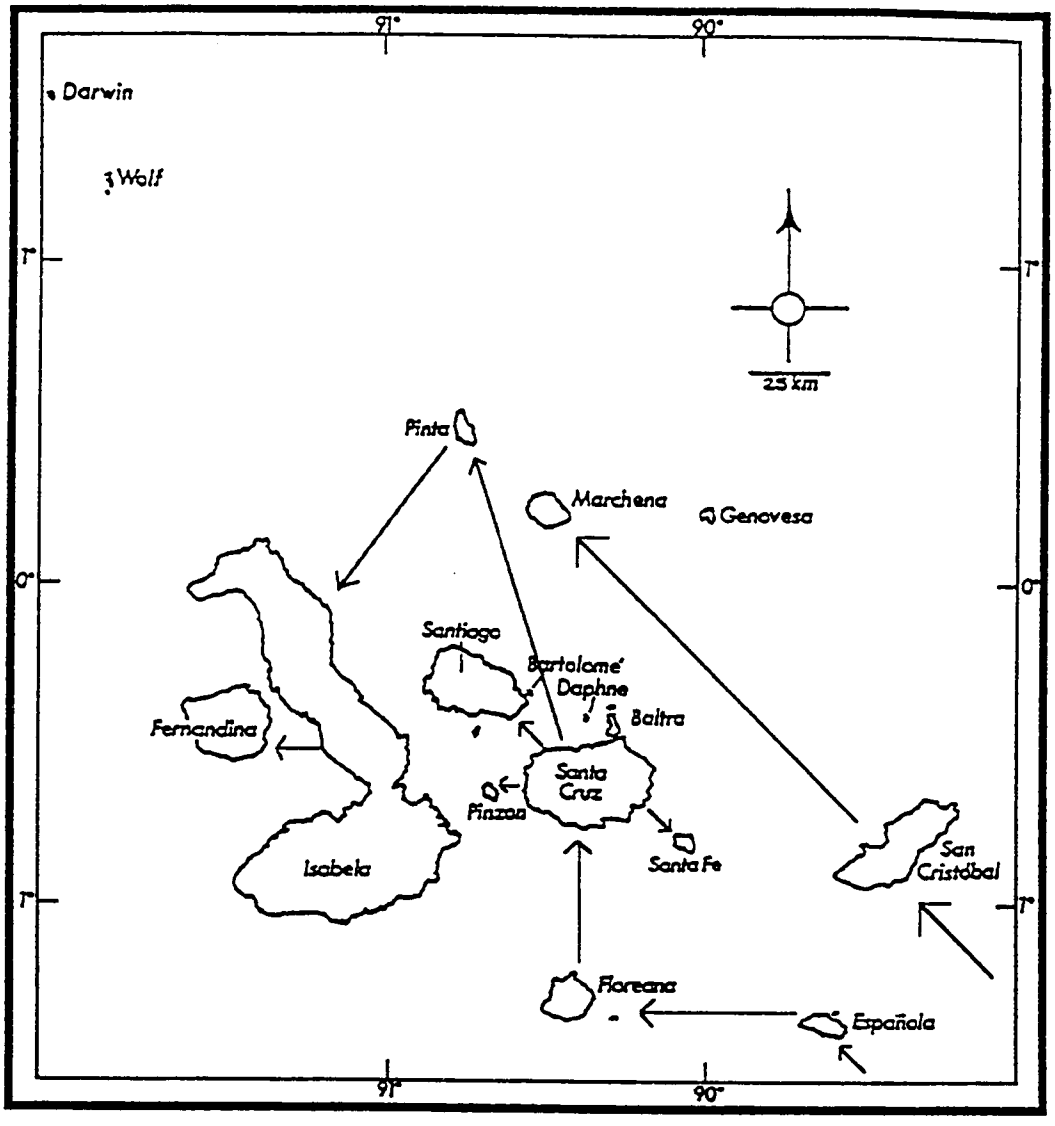


Figure 4.3 Colonization model proposed for *Microlophus* in the Galápagos Islands by analysis of sequence variation in the mitochondrial cytochrome *b* gene.

An alternative sequence of island colonizations could also be proposed. The founding of the populations on San Cristóbal, Marchena, Española, and Floreana proceed as described above. Floreana then serves as the source for the colonization of Pinzón, which in turn provides animals for the founding of the Pinta population. After this event, Santa Cruz is colonized from Pinzón, and then Santiago from Santa Cruz. Most recently, the Isabela and Fernandina populations were founded from Pinta. Both of the models proposed here are in agreement with the recovered tree topology (Fig 3.4; Appendix D); however, the current data do not allow one to determine which model is a closer approximation to the true biogeographic history.

Both models proposed here are similar but not identical to the model proposed by Wright (1983). All three models propose that San Cristóbal was colonized directly from the mainland by one lineage of lizards which then served as the source for Marchena. The models agree in that the second lineage originally colonized Española. It is also agreed that Santa Cruz served as the source (either directly or indirectly) for most of the central and western islands. One discrepancy between the models concerns the island of Floreana. Wright's model proposes that Floreana was colonized from Isabela relatively late in the sequence, and that no other islands were derived from the Floreana population. In contrast, the models proposed here suggest that Floreana was colonized fairly early from Española, and that it served as the source for the colonization of Santa Cruz (either directly or indirectly via Pinzón).

Once the various species of lava lizards have reached their respective islands, one must explain why there is only a single species per island. Even if over-water inter-island movement occurs at extremely low frequencies, there was still the possibility of movement during periods of lowered sea level (such as during glacial periods). Within the last 125,000 years, the sea level may have been lowered as much as 120 meters (Dawson, 1992). Such a change in sea level would connect Isabela and Fernandina and greatly reduce the distance between the islands of Isabela, Santiago, Santa Cruz, Santa Fe, and Pinzón. A change in sea level of 200 meters would connect all these islands and alter the coastlines of the remaining islands to the point that almost any inter-island distance would be less than 19 km. Such an altered topography of the archipelago would allow for the direct contact of several species and greatly reduce the geographic barriers between the remaining species.

With the removal of physical barriers, the most likely reason explaining the "one island - one lava lizard" phenomenon is competitive exclusion. This has been demonstrated in other taxa. In areas where they occur in sympatry, yellow-headed blackbirds will drive out red-winged blackbirds from marsh habitats, thus denying them access to the resources (i.e. food, nesting locations, etc.) contained within the marsh (MacArthur, 1972). Wilson (1971) examined the competitive exclusion of one ant species by another in a coconut plantation in Tanzania. Two species of warblers in New Guinea limit the elevational distribution of the other through resource competition (Diamond, 1972 in MacArthur, 1972). On the Caribbean island of St. Lucia, *Anolis*

extremus has eliminated the endemic *A. luciae* from numerous localities through interspecific competition (Gorman, 1976).

Lava lizards on the various islands have very similar life styles. They inhabit the arid regions of the islands where they feed primarily on flies, beetles, moths, grasshoppers, ants, spiders and scorpions (Jackson, 1985). Plant material, such as berries and flower petals, are occasionally eaten; this habit is more common during the dry season and on the more arid islands, especially Bartolomé. Although no studies have looked at competition between different species of lava lizards, it is probable that this factor is responsible for the occurrence of only a single species on any given island.

An analogy may be drawn with the *Anolis* lizards of the Caribbean. Roughgarden (1995) has shown that anoles in the West Indies are limited primarily by food. Two separate species cannot co-exist on an island unless their snout-vent lengths differ by at least a factor of 1.3, thus allowing partitioning of food resources and a reduction in competition. Unfortunately, such basic data as the mean body size of Galápagos lava lizards on different islands is not currently available. This paucity of information precludes any investigation of the possible interaction between the relative size of different lava lizards and competitive exclusion. However, given the similarities between anoles and lava lizards (both are relatively small, diurnal, active, and insectivorous), it seems likely that comparable pressures affect their interactions with closely related congeners.

D. Timing of Colonizations

Once one decides *how* the lava lizards colonized the Galápagos, it is still necessary to determine *when*. Although the current islands are geologically young (less then 5 million years old) (Cox, 1983, and references within; Hickman and Lipps, 1985), it is probable that islands have existed over the Galápagos hot spot for much longer times (Holden and Dietz, 1972; Duncan and Hargraves, 1984; Sen et al. 1988; Christie et al. 1992; Hauff et al. 1997). This situation increases the difficulty in assigning accurate times of colonizations, because one cannot assume that the lineages inhabiting the Galápagos are younger than the islands themselves.

Ideally, one could use the fossil record to determine the minimum time of divergence between the mainland South American and Galápagos species and between the various island taxa. Unfortunately, being volcanic in origin, the Galápagos Islands have a limited vertebrate fossil record, and that which does exist extends back no earlier than the Holocene (Steadman, 1982, 1985; Steadman et al. 1991). This limits the usefulness of the fossil record for establishing times of divergence for Galápagos species.

An alternative to fossils for determining the age of a particular lineage involves the use of a molecular clock. This idea was first put forth by Zuckerkandl and Pauling (1965) who suggested that genes and their protein products might evolve at rates constant enough that estimates of molecular divergence could be used to calibrate a "molecular clock." Numerous studies have supported this hypothesis and have used molecular data to estimate dates of divergence (Bowen et al. 1989; Hedges et al. 1991, 1992; Vigilant et al. 1991; Wayne et al. 1991; Kumar and Hedges, 1998).

One molecule commonly used in immunological studies, albumin, has been shown to approximate a molecular clock (Maxson, 1992). Work has shown that the serum albumin protein accumulates approximately one amino acid substitution every 0.6 million years (Wallace et al. 1971; Maxson and Wilson, 1975; Maxson and Maxson, 1986; Wilson et al. 1987; Maxson, 1992). Lopez et al. (1992) used serum albumin to approximate the times of divergence among Galápagos *Microlophus*. Based on the immunological distances obtained, they estimated that *M. delanonis* diverged from the other species tested approximately 20 million years ago (mya). *Microlophus habeli* diverged from *M. occipitalis* and *M. pacificus* separated from *M. indefatigabilis* about the same time (6 mya). Thus the immunological data suggest that the speciation among Galápagos *Microlophus* preceded the emergence of the present-day islands.

A similar situation has been reported for the Galápagos land and marine iguanas (Wyles and Sarich, 1983). Analysis of albumin differences between these species indicated their monophyly relative to other iguanines, but suggested a divergence time of fifteen to twenty mya. More recently, Rassmann (1997) sequenced portions of two mitochondrial rRNA genes from all exant genera of the family Iguanidae (in which the land and marine iguanas are placed). Her results indicate that the Galápagos land and marine iguanas are sister taxa and that they have been separated between ten and twenty million years. The combined results of these studies support the theory that the present-day islands do not fully represent the geologic history of the archipelago.

In order to estimate the times of divergence among Galápagos lava lizards, it is necessary to calibrate the rate of substitution for the portion of the mitochondrial

cytochrome *b* gene examined. Lacking Galápagos fossils of the appropriate age with which to calibrate the clock, an alternative source was used: the timing of the uplift of the Andes Mountains. This uplift would result in the separation of the South American tropidurine lizards into cis-Andean (east of the Andes) and trans-Andean (west of the Andes) assemblages. The date of this event can be used to calibrate the rate of sequence divergence in the mitochondrial cytochrome *b* gene between the cis- and trans-Andean groups. This rate can then be used to estimate times of colonization of the Galápagos Islands by trans-Andean species.

The Andes are divided into three regions: the Northern, Central, and Southern Andes (Zeil, 1979; Aguirre, 1985; Baldock, 1985; Cobbing, 1985). Each region has an independent geologic history and so it is necessary to determine which area is the most appropriate for use in this study. Based on the geographic distribution of the species sampled in this study (Fig 1.4), the Central Andes (located in Peru and Bolivia) are the most likely to have been involved in a vicariance event affecting the ancestors of the present-day Galápagos species.

Radiometric (K-Ar) dating of the Central Andes was performed by Farrar and Noble (1976) and Megard and Philip (1976). According to these authors, folding began in the Central Andes between 17 and 15 mya but subsequent uplift did not commence until 10.5 mya. Therefore, the separation of the cis- and trans-Andean species of tropidurine lizards will be assumed to have occurred no later than 10.5 mya; dispersal across the Andes is unlikely due to the low tolerance to cold temperatures exhibited by these ectothermic lizards. It is possible that the lineages had diverged prior to this date

but the lack of independent evidence suggesting such a scenario prohibits the use of an earlier date.

The mean pairwise divergence between the cis-Andean species (*Tropidurus torquatus* and *T. umbra*) and the trans-Andean species (*Plesiomicrolophus koepckeorum, M. occipitalis, M. stolzmanni, M. theresiae, M. thoracicus*, and *M. tigris*) is 18.63% (Fig. 4.4). When combined with the age of the Central Andes, a rate of sequence divergence of 1.77% per million years is obtained. Due to possible errors in assigning an exact age to the uplift of the Andes (and thus to the separation of the cis- and trans-Andean species), the value obtained is an estimate of the rate of substitution in the cytochrome *b* gene and should not be assumed to be an absolute value. However, the calculated rate of 1.77% per million years is similar to the 2 - 2.5% per million years that is often cited as the substitution rate for the mitochondrial cytochrome *b* gene (Brown et al. 1979; Meyer et al. 1990; Irwin et al. 1991; Hedges et al. 1992).

Another potential source of error when attempting to determine the actual sequence of events is the estimates of the age of the islands. Attempts to date the islands have encountered several difficulties: (1) younger lava flows tend to cover the older ones, (2) erosion in most parts of these arid islands is too slow to cut through to the older rocks, (3) potassium, the element whose radioactive decay to argon is most commonly measured to determine age, is present in only very small quantities in most Galápagos rocks, and (4) the dry, "rubbly" lava flows that constitute so much of the islands have prevented the discovery of the islands' oldest rocks (Simkin, 1984). Therefore, the actual

	alb1	alb2	alb3	alb4	alb5	alb6	alb7	alb8	alb9
alb1	0.0000	0.2697	0.2697	0.2697	0.2697	0.2697	0.2697	0.2697	0.2659
alb2		0.0000	0.0075	0.1086	0.1049	0.2397	0.2397	0.2397	0.0787
alb3			0.0000	0.1086	0.1049	0.2434	0.2434	0.2434	0.0787
alb4				0.0000	0.0037	0.2247	0.2247	0.2247	0.0936
alb5					0.0000	0.2247	0.2247	0.2247	0.0899
alb6						0.0000	0.0000	0.0000	0.2210
alb7							0.0000	0.0000	0.2210
alb8								0.0000	0.2210
alb9									0.0000
alb10									
alb11									
alb12									
alb13									
alb14									
alb15									
alb16									
alb17									
alb18									
alb19									
alb20									
alb21									
biv1									
biv2									
biv3									
del1									
del2									
del3									
del4									
del5									
del6									
dunc									
gray2									
gray3									
habe									
paci									
occi									
stol									
ther									
thor									
tigr									
Leio									
Plesio									
Ttor									
Tumb									
Scel									

Figure 4.4. Proportion of sequence divergence in a portion of the mitochondrial cytochrome *b* gene among populations of Galápagos and South American *Microlophus* and related taxa. Sample abbreviations are given in Appendix A.

Figure 4.4 (continued)

	alb10	alb11	alb12	alb13	alb14	alb15	alb16	alb17	alb18
alb1	0.2697	0.2697	0.2772	0.2697	0.2659	0.2659	0.2734	0.2697	0.2697
alb2	0.1049	0.1198	0.1049	0.0075	0.0787	0.0787	0.1161	0.0150	0.0000
alb3	0.1049	0.1198	0.0974	0.0000	0.0787	0.0787	0.1161	0.0150	0.0075
alb4	0.0037	0.0300	0.0112	0.1086	0.0936	0.0936	0.0337	0.1086	0.1086
alb5	0.0000	0.0262	0.0075	0.1049	0.0899	0.0899	0.0300	0.1049	0.1049
alb6	0.2247	0.2285	0.2322	0.2434	0.2210	0.2210	0.2322	0.2360	0.2397
alb7	0.2247	0.2285	0.2322	0.2434	0.2210	0.2210	0.2322	0.2360	0.2397
alb8	0.2247	0.2285	0.2322	0.2434	0.2210	0.2210	0.2322	0.2360	0.2397
alb9	0.0899	0.1049	0.0899	0.0787	0.0000	0.0000	0.1011	0.0787	0.0787
alb10	0.0000	0.0262	0.0075	0.1049	0.0899	0.0899	0.0300	0.1049	0.1049
alb11		0.0000	0.0337	0.1198	0.1049	0.1049	0.0037	0.1273	0.1198
alb12			0.0000	0.0974	0.0899	0.0899	0.0375	0.0974	0.1049
alb13				0.0000	0.0787	0.0787	0.1161	0.0150	0.0075
alb14					0.0000	0.0000	0.1011	0.0787	0.0787
alb15						0.0000	0.1011	0.0787	0.0787
alb16							0.0000	0.1236	0.1161
alb17								0.0000	0.0150
alb18									0.0000
alb19									
alb20									
alb21									
biv1									
biv2									
biv3									
del1									
del2									
del3									
del4									
del5									
del6									
dunc									
gray2									
gray3									
habe									
paci									
occi									
stol									
ther									
thor									
tigr									
Leio									
Plesio									
Ttor									
Tumb									
Scel									

Figure 4.4 (continued)

	alb19	alb20	alb21	biv1	biv2	biv3	del1	del2	del3
alb1	0.2697	0.2659	0.2697	0.2622	0.2547	0.2622	0.2659	0.2659	0.2622
alb2	0.0000	0.0787	0.1049	0.1910	0.1835	0.1910	0.1498	0.1461	0.1498
alb3	0.0075	0.0787	0.1049	0.1948	0.1873	0.1948	0.1498	0.1461	0.1498
alb4	0.1086	0.0936	0.0037	0.1910	0.1910	0.1910	0.1161	0.1124	0.1086
alb5	0.1049	0.0899	0.0000	0.1873	0.1873	0.1873	0.1124	0.1086	0.1049
alb6	0.2397	0.2210	0.2247	0.2285	0.2247	0.2285	0.2397	0.2360	0.2322
alb7	0.2397	0.2210	0.2247	0.2285	0.2247	0.2285	0.2397	0.2360	0.2322
alb8	0.2397	0.2210	0.2247	0.2285	0.2247	0.2285	0.2397	0.2360	0.2322
alb9	0.0787	0.0000	0.0899	0.1685	0.1685	0.1685	0.1011	0.1049	0.1049
alb10	0.1049	0.0899	0.0000	0.1873	0.1873	0.1873	0.1124	0.1086	0.1049
alb11	0.1198	0.1049	0.0262	0.1798	0.1798	0.1798	0.1049	0.1011	0.0974
alb12	0.1049	0.0899	0.0075	0.1948	0.1948	0.1948	0.1198	0.1161	0.1124
alb13	0.0075	0.0787	0.1049	0.1948	0.1873	0.1948	0.1498	0.1461	0.1498
alb14	0.0787	0.0000	0.0899	0.1685	0.1685	0.1685	0.1011	0.1049	0.1049
alb15	0.0787	0.0000	0.0899	0.1685	0.1685	0.1685	0.1011	0.1049	0.1049
alb16	0.1161	0.1011	0.0300	0.1835	0.1835	0.1835	0.1086	0.1049	0.1011
alb17	0.0150	0.0787	0.1049	0.1910	0.1835	0.1910	0.1498	0.1461	0.1498
alb18	0.0000	0.0787	0.1049	0.1910	0.1835	0.1910	0.1498	0.1461	0.1498
alb19	0.0000	0.0787	0.1049	0.1910	0.1835	0.1910	0.1498	0.1461	0.1498
alb20		0.0000	0.0899	0.1685	0.1685	0.1685	0.1011	0.1049	0.1049
alb21			0.0000	0.1873	0.1873	0.1873	0.1124	0.1086	0.1049
biv1				0.0000	0.0075	0.0000	0.1648	0.1648	0.1610
biv2					0.0000	0.0075	0.1573	0.1573	0.1536
biv3						0.0000	0.1648	0.1648	0.1610
del1							0.0000	0.0037	0.0075
del2								0.0000	0.0037
del3									0.0000
del4									
del5									
del6									
dunc									
gray2									
gray3									
habe									
paci									
occi									
stol									
ther									
thor									
tigr									
Leio									
Plesio									
Ttor									
Tumb									
Scel									

Figure 4.4 (continued)

	del4	del5	del6	dunc	gray2	gray3	habe	paci	occi
alb1	0.2697	0.2659	0.2622	0.2734	0.2659	0.2697	0.2547	0.2697	0.2734
alb2	0.1461	0.1461	0.1461	0.0936	0.0899	0.0974	0.1910	0.0936	0.1835
alb3	0.1461	0.1461	0.1461	0.0936	0.0899	0.0974	0.1948	0.0936	0.1873
alb4	0.1161	0.1124	0.1124	0.1011	0.0861	0.0861	0.1985	0.0375	0.1873
alb5	0.1124	0.1086	0.1086	0.0974	0.0824	0.0824	0.1948	0.0337	0.1835
alb6	0.2322	0.2360	0.2360	0.2172	0.2285	0.2210	0.2285	0.2247	0.2322
alb7	0.2322	0.2360	0.2360	0.2172	0.2285	0.2210	0.2285	0.2247	0.2322
alb8	0.2322	0.2360	0.2360	0.2172	0.2285	0.2210	0.2285	0.2247	0.2322
alb9	0.1011	0.1049	0.1086	0.0599	0.0637	0.0787	0.1760	0.0824	0.1610
alb10	0.1124	0.1086	0.1086	0.0974	0.0824	0.0824	0.1948	0.0337	0.1835
alb11	0.1049	0.1011	0.0936	0.0936	0.1011	0.0936	0.1873	0.0449	0.1685
alb12	0.1198	0.1161	0.1161	0.0974	0.0824	0.0824	0.2022	0.0412	0.1910
alb13	0.1461	0.1461	0.1461	0.0936	0.0899	0.0974	0.1948	0.0936	0.1873
alb14	0.1011	0.1049	0.1086	0.0599	0.0637	0.0787	0.1760	0.0824	0.1610
alb15	0.1011	0.1049	0.1086	0.0599	0.0637	0.0787	0.1760	0.0824	0.1610
alb16	0.1086	0.1049	0.0974	0.0974	0.0974	0.0899	0.1910	0.0412	0.1723
alb17	0.1461	0.1461	0.1536	0.1011	0.0936	0.1011	0.1910	0.0936	0.1835
alb18	0.1461	0.1461	0.1461	0.0936	0.0899	0.0974	0.1910	0.0936	0.1835
alb19	0.1461	0.1461	0.1461	0.0936	0.0899	0.0974	0.1910	0.0936	0.1835
alb20	0.1011	0.1049	0.1086	0.0599	0.0637	0.0787	0.1760	0.0824	0.1610
alb21	0.1124	0.1086	0.1086	0.0974	0.0824	0.0824	0.1948	0.0337	0.1835
biv1	0.1685	0.1648	0.1610	0.1798	0.1798	0.1723	0.0187	0.1798	0.1236
biv2	0.1610	0.1573	0.1536	0.1798	0.1723	0.1648	0.0112	0.1798	0.1236
biv3	0.1685	0.1648	0.1610	0.1798	0.1798	0.1723	0.0187	0.1798	0.1236
del1	0.0075	0.0037	0.0112	0.1161	0.0936	0.0936	0.1648	0.1011	0.1461
del2	0.0037	0.0000	0.0075	0.1124	0.0899	0.0899	0.1648	0.0974	0.1461
del3	0.0075	0.0037	0.0037	0.1161	0.0936	0.0936	0.1610	0.0936	0.1423
del4	0.0000	0.0037	0.0112	0.1086	0.0936	0.0936	0.1685	0.1011	0.1423
del5		0.0000	0.0075	0.1124	0.0899	0.0899	0.1648	0.0974	0.1461
del6			0.0000	0.1124	0.0936	0.0936	0.1610	0.0974	0.1423
dunc				0.0000	0.0861	0.0861	0.1873	0.0899	0.1760
gray2					0.0000	0.0150	0.1835	0.0824	0.1648
gray3						0.0000	0.1760	0.0749	0.1648
habe							0.0000	0.1873	0.1348
paci								0.0000	0.1685
occi									0.0000
stol									
ther									
thor									
tigr									
Leio									
Plesio									
Ttor									
Tumb									
Scel									

Figure 4.4 (continued)

	stol	ther	thor	tigr	Leio	Plesio	Ttor	Tumb	Scel
alb1	0.2697	0.2509	0.2509	0.2397	0.2697	0.2434	0.2697	0.2846	0.2547
alb2	0.1386	0.1760	0.1461	0.1835	0.2322	0.1798	0.1948	0.2097	0.2434
alb3	0.1386	0.1685	0.1461	0.1835	0.2285	0.1798	0.1985	0.2135	0.2472
alb4	0.1610	0.1760	0.1423	0.1723	0.2135	0.1798	0.1948	0.2097	0.2210
alb5	0.1573	0.1723	0.1386	0.1685	0.2097	0.1760	0.1910	0.2060	0.2172
alb6	0.2434	0.2547	0.2135	0.2509	0.2322	0.2247	0.2584	0.2509	0.2285
alb7	0.2434	0.2547	0.2135	0.2509	0.2322	0.2247	0.2584	0.2509	0.2285
alb8	0.2434	0.2547	0.2135	0.2509	0.2322	0.2247	0.2584	0.2509	0.2285
alb9	0.1423	0.1461	0.1236	0.1685	0.2172	0.1760	0.1835	0.1948	0.2210
alb10	0.1573	0.1723	0.1386	0.1685	0.2097	0.1760	0.1910	0.2060	0.2172
alb11	0.1610	0.1610	0.1386	0.1573	0.2022	0.1610	0.1948	0.2210	0.2172
alb12	0.1648	0.1723	0.1461	0.1723	0.2060	0.1835	0.1910	0.2135	0.2247
alb13	0.1386	0.1685	0.1461	0.1835	0.2285	0.1798	0.1985	0.2135	0.2472
alb14	0.1423	0.1461	0.1236	0.1685	0.2172	0.1760	0.1835	0.1948	0.2210
alb15	0.1423	0.1461	0.1236	0.1685	0.2172	0.1760	0.1835	0.1948	0.2210
alb16	0.1648	0.1648	0.1423	0.1610	0.2060	0.1573	0.1985	0.2172	0.2172
alb17	0.1423	0.1685	0.1423	0.1873	0.2247	0.1798	0.1985	0.2210	0.2434
alb18	0.1386	0.1760	0.1461	0.1835	0.2322	0.1798	0.1948	0.2097	0.2434
alb19	0.1386	0.1760	0.1461	0.1835	0.2322	0.1798	0.1948	0.2097	0.2434
alb20	0.1423	0.1461	0.1236	0.1685	0.2172	0.1760	0.1835	0.1948	0.2210
alb21	0.1573	0.1723	0.1386	0.1685	0.2097	0.1760	0.1910	0.2060	0.2172
biv1	0.1311	0.1610	0.1423	0.1648	0.2097	0.1461	0.1685	0.1873	0.2210
biv2	0.1273	0.1573	0.1386	0.1573	0.2022	0.1423	0.1723	0.1835	0.2210
biv3	0.1311	0.1610	0.1423	0.1648	0.2097	0.1461	0.1685	0.1873	0.2210
del1	0.1386	0.1236	0.1198	0.1273	0.1835	0.1236	0.1910	0.1760	0.2172
del2	0.1386	0.1198	0.1161	0.1236	0.1835	0.1273	0.1910	0.1798	0.2172
del3	0.1423	0.1198	0.1198	0.1198	0.1798	0.1236	0.1873	0.1798	0.2135
del4	0.1423	0.1236	0.1124	0.1198	0.1873	0.1311	0.1910	0.1798	0.2135
del5	0.1386	0.1198	0.1161	0.1236	0.1835	0.1273	0.1910	0.1798	0.2172
del6	0.1423	0.1236	0.1236	0.1198	0.1798	0.1236	0.1873	0.1798	0.2135
dunc	0.1386	0.1723	0.1273	0.1648	0.2060	0.1873	0.1948	0.2172	0.2360
gray2	0.1348	0.1423	0.1311	0.1610	0.2022	0.1423	0.1910	0.1835	0.2285
gray3	0.1348	0.1423	0.1311	0.1536	0.1985	0.1423	0.1835	0.1835	0.2247
habe	0.1348	0.1685	0.1423	0.1610	0.2060	0.1461	0.1648	0.1798	0.2135
paci	0.1498	0.1610	0.1348	0.1723	0.2060	0.1573	0.1873	0.2022	0.1985
occi	0.1273	0.1536	0.1536	0.1685	0.2022	0.1498	0.1835	0.1685	0.2060
stol	0.0000	0.1386	0.1348	0.1536	0.1910	0.1348	0.1685	0.1648	0.2172
ther		0.0000	0.1423	0.1423	0.2322	0.1498	0.1948	0.1985	0.2247
thor			0.0000	0.1386	0.1910	0.1423	0.1910	0.1873	0.2285
tigr				0.0000	0.1835	0.1461	0.1910	0.2210	0.1985
Leio					0.0000	0.2022	0.1985	0.2210	0.1760
Plesio						0.0000	0.1948	0.1723	0.2097
Ttor							0.0000	0.1610	0.1798
Tumb								0.0000	0.2285
Scel									0.0000

age of many of the islands is still unclear, and the reported dates may represent only the minimum possible ages.

Using a rate of sequence divergence of 1.77% per million years, the timing of the colonizations of the Galápagos Islands may be estimated. The "San Cristóbal + Marchena" clade diverged from *M. occipitalis* approximately 7.1 mya (Fig. 4.4); this estimate is similar to the 6 million years proposed by Lopez et al. (1992). As this date is older than the calculated age of the islands of San Cristóbal and Marchena (3.7 - 4.5 and 0.3 million years, respectively; Cox, 1983), it suggests that these species originated before the emergence of the islands. Two scenarios could account for this finding: (1) the lineages separated on the mainland of South America before colonizing the present-day islands, or (2) the colonization occurred on an island which has since subsided but which served as the source for the present-day islands. The current data cannot determine which of these scenarios is more likely, but do indicate that the lineages of lava lizards inhabiting San Cristóbal and Marchena are older than the islands themselves.

Of the taxa examined in this study, the two species most closely related to the "Central Islands" clade are *M. theresiae* and *M. thoracicus*. The average percentage sequence divergence between each and the "Central Islands" clade is 15.04% and 13.28%, respectively (Fig. 4.4). These values correspond to 8.7 and 7.5 million years of divergence. As with the "San Cristóbal + Marchena + *occipitalis*" clade, the date of divergence between the island and mainland lineages is older than the present-day islands.

The "Central Islands" clade collectively occurs on the islands of Española, Fernandina, Floreana, Isabela, Pinta, Pinzón, Santiago, Santa Cruz, and Santa Fe. The oldest of these islands, Española, has been dated at approximately 3.3 million years old (Bailey, 1976; Cox, 1983; Hall, 1983). Santa Fe is the next oldest at 2.6 - 2.8 million years old (Bailey, 1976; Cox, 1983) followed by Santa Cruz at 2.2 million years. The remaining islands have rocks dated in the 0.7 and 1.5 million years age range (Swanson et al. 1974; Cox, 1983; Simkin, 1984). For the reasons mentioned above, these estimates may represent only the minimum age of the islands and not their absolute ages.

This study indicates that *M. delanonis* diverged from the other "Central Islands" taxa approximately 6.6 million years ago (Fig. 4.4). This age is considerably younger than the 20 million years proposed by Lopez et al. (1992). A possible explanation for this discrepancy could be the rate of evolution in the serum albumin of *M. delanonis*. Lopez et al. (1992) noted that this species exhibited a more rapid change in its albumin than did the other species in their study. A higher rate of albumin evolution could result in over-estimating the time of divergence between this species and the other Galápagos taxa and may explain the differences between the immunological data and the present study.

The next separation was that of *M. grayi* from the remaining "Central Islands" species (*M. albemarlensis, M. duncanensis, M. indefatigabilis, M. jacobi,* and *M. pacificus*). This was followed by the separation of the clade containing *M. albemarlensis* and *M. pacificus* from the *M. duncanensis/indefatigabilis/jacobi* group (Fig 3.4). When the rate of 1.77% per million years is applied, it yields approximate divergence dates of 4.8 million years for the former split and 6.0 million years for the latter. This would

appear to be contradictory. However, a relative rate test indicated that the rate of sequence evolution in *M. grayi* was slower in comparison to that of the other species. Therefore, fewer substitutions would accumulate in the *M. grayi* sequence, which in turn would result in a decrease in the estimated age of this lineage.

The divergence of *M. duncanensis* from the *indefatigabilis/jacobi* pair and the splitting of the latter pair occurred at around the same time, 4.6 million years for the former and 4.4 million years for the latter (Fig 4.4). The most recent event occurred approximately 2.1 million years ago when *M. albemarlensis* separated from *M. pacificus*.

The dates suggested by the present data concur with the dates obtained from the immunological data (Lopez et al. 1992). They calculated divergences of 6 mya for both the separation of *M. habeli* from *M. occipitalis* and of *M. indefatigabilis* from *M. pacificus*. The dates obtained here for these separations are 7 mya and 5.3 mya, respectively. Given the potential errors involved in pin-pointing these ages, the agreement between the two data sets is encouraging.

All of the divergence dates given here are older than the present-day islands and suggest that there has been inhabitable land in the archipelago for much longer times than is usually hypothesized. The current findings are supported by the report of submerged seamounts "downstream" from the Galápagos hot spot which show evidence of subaerial erosion and suggest that islands have existed for at least 9 million years (and probably much longer) over the hot spot (Christie et al. 1992). The times of divergence calculated from the current data concur with previous studies which have suggested ages for various Galápagos taxa which are greater than the present islands (Wyles and Sarich,

1983; Lopez et al. 1992; Rassmann, 1997). Although the Galápagos Islands themselves are geologic young, the organisms inhabiting them appear to be much older.

E. Summary

The Galápagos lava lizards are almost certainly derived from South American species. Dispersal to the islands most likely occurred via rafting on mats of vegetation swept out to sea. Once in the Humboldt Current, as little as two weeks may have been necessary to reach the archipelago. The hardships of such a crossing would be lessened due to the physiologic resistance to dehydration and food deprivation exhibited by many reptiles. Once established on a single island, dispersal to other islands could result from crossing over land during times of lowered sea levels or by rafting on chunks of floating pumice ejected by volcanic eruptions.

At least two colonization events are necessary to account for the present-day distribution of the Galápagos lava lizards. One occurred on San Cristóbal, which served as the source for the colonization of Marchena. The second founding event was to Española, from which Floreana was colonized. The next island colonized (from Floreana) was either Pinzón or Santa Cruz; if it was the former, it then served as the source for the latter, and vice versa. The Pinta lineage was derived from whichever of the two previous islands was originally colonized from Floreana. Santa Cruz served as the source population for Santiago. Isabela and Fernandina were colonized from Pinta.

The lineage of *Microlophus* inhabiting the islands of San Cristóbal and Marchena diverged from their closest mainland relative approximately 7.1 million years ago (mya).

The other Galápagos species separated from their mainland ancestor 8.1 mya. *Microlophus delanonis* diverged from the remaining taxa 6.6 mya, the *M. albemarlensis/pacificus* clade split off around 6.0 mya, and *M. grayi* 4.8 mya. *Microlophus duncanensis* diverged from *M. indefatigabilis* and *M. jacobi* around 4.6 mya and the latter pair separated 4.4 mya. The time of divergence between *M. albemarlensis* and *M. pacificus* is approximately 2.1 mya.

The estimates of divergence times for the Galápagos species pre-date the age of the current Galápagos Islands. Either the lineages separated on the mainland of South America prior to their colonizations of the islands or they colonized islands which have since subsided below the surface of the ocean. Regardless of which scenario actually happened, the Galápagos lava lizards are older than the Galápagos Islands.

Chapter 5

Taxonomic Status of *Plesiomicrolophus koepckeorum*

A. Introduction

Modern lizards belong to the Order Squamata. Within this order, two major lineages are recognized: the Iguania and the Autarchoglossa (Gauthier et al. 1988). The Iguania is comprised of those taxa traditionally placed in the Iguanidae, Agamidae, and Chamaeleonidae. Galápagos lava lizards had been placed in the Iguanidae. However, Frost and Etheridge (1989) analyzed a broad suite of morphological characters and split the Iguanidae into eight families; Galápagos lava lizards were placed in one of newly erected families, the Tropiduridae.

A further taxonomic analysis of the Tropiduridae was performed by Frost (1992). Numerous revisions were made, including resurrecting the genus *Microlophus* for the species of *Tropidurus* west of the Andes (including the Galápagos species), with the exception of *T. koepckeorum*. On the basis of a suite of morphological characters, Frost erected a new monotypic genus for this species, *Plesiomicrolophus*.

The phylogenetic placement of *Plesiomicrolophus* was not resolved, with it being placed in a trichotomy with *Microlophus* and *Tropidurus*. On its taxonomic status, Frost (1992, p.42) wrote:

> One might argue that because *T. koepckeorum* is the likely sister taxon of other species of western *Tropidurus*, it would be more prudent to place it in a collective, *Microlophus** (a metataxon sensu Gauthier, 1986), defined solely by plesiomorphy with respect to its presumptive sister taxon, *Tropidurus*. The problem with this approach is that it does not

invite additional evaluation of the proposition of monophyly of western *Microlophus* + *T. koepckeorum* and is merely an easy bookkeeping convention.

Thus, the taxonomic status of *P. koepckeorum* was left in some uncertainty.

Analysis of variation in the mitochondrial cytochrome *b* gene placed *P. koepckeorum* well within a clade composed of species belonging to *Microlophus* (Fig 3.4; Appendix D). In order to further investigate the phylogenetic relationships of this species, a portion of the mitochondrial 16S ribosomal RNA (rRNA) gene was sequenced.

B. Materials and Methods.

The taxa sampled included seven of the nine Galápagos lava lizards (*Microlophus albemarlensis, M. delanonis, M. duncanensis, M. grayi, M. indefatigabilis, M. jacobi,* and *M. pacificus*). Repeated attempts to obtain sequence for the two remaining species (*M. bivittatus* and *M. habeli*) were unsuccessful. Other taxa for which sequence data were obtained are mostly mainland South American species; they include five *Microlophus* species (*occipitalis, stolzmanni, theresiae, thoracicus,* and *tigris*), *Plesiomicrolophus koepckeorum, Tropidurus umbra, Stenocercus roseiventris, Uranoscodon superciliosa,* and *Leiocephalus melanochlorus.* All of these taxa are members of the family Tropiduridae (*Leiocephalus* in the subfamily Leiocephalinae and the others in the subfamily Tropidurinae). The outgroup taxon was *Sceloporus undulatus.* Appendix A lists all taxa included in the study.

DNA amplification and sequencing were performed as outlined in Ch. 2 and Appendix C. Both the heavy and light strands of the approximately 469 bp region of the mt 16S rRNA gene described in Ch. 2 were amplified for all taxa.

Alignment of the sequences was performed visually in the ESEE program (Cabot and Beckenbach, 1989). When needed, gaps were introduced to optimize alignment and to account for insertion/deletion mutations. In most instances, gaps were needed to account for mutation in only a single sample, simplifying the process. However, one highly variable 33 bp region (sites 158 - 190 in Fig. 5.1) could not be aligned with confidence. Therefore, these nucleotides were omitted from analysis.

Numerous evolutionary distance methods were used to estimate nucleotide sequence divergence and to calculate genetic distances. These methods include the Jukes-Cantor, Kimura 2-parameter, Tajima-Nei, Tamura, and Tamura-Nei distances; gamma distances using the Jukes-Cantor, Kimura 2-parameter, and Tamura-Nei correction models were calculated to account for possible site-specific variation in the rates of substitution (Tamura and Nei, 1993; Wakeley, 1993; Kumar et al. 1994). Evolutionary distances were determined after discarding all sites for which there were missing or ambiguous data.

C. Results

Figure 5.1 shows the 16S rRNA sequences for eighteen tropidurid and one outgroup taxa. A total of 447 nucleotide sites was aligned; with the removal of the 33 bp region whose alignment was uncertain, 414 sites were analyzed. These sequences exhibited sufficient variation to provide phylogenetic analysis (approximately 35% of analyzed sites were variable) and 93 sites were informative under the conditions of

```
                1 1111111112 2222222223 3333333334 4444444445 5555555556
       1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
del    TGTCCTCTAA ATAAGGACTA GTATGAATGG CTAAATGAGG GCCAACCTGT CTCCCCAAAC
albe   .......... ...G...... .......C.. .......... .......... ......T...
inde1  .......... ...G...... .......C.. .......... .......... ......TG..
inde2  .......... ...G...... .......C.. .......... .......... ......TG..
jacob  .......... .......... .......C.. ...G...... .......... ......T...
dunc   .......... ...G..?... .......C.. .......... .......... ......T.G.
gray   ???....... ...G...... .......C.. .......... .......... ......T.G.
Plesio .......... .......... .......C.. .......... ..T....... ....T.T..T
occi   C......... ........C. .......C.. .......... A....A?... ...TT.T..T
paci   .......... .......... .......C.. .......... .......... ......T...
stol   .......... .......... .......C.. .......... .TT....... ....T.T...
ther   ???????... .........?. .......... ..........- A......... ....T.T...
thor   ?????????? ?????????? .......... .......... .......... ......T.GT
tigr   ?????????? ????????AG .......... .......... .......... ....T.T...
Tumb   .......... ........C. .......C.. .......... .......... ....TTT...
Sten   .......... .......... .......... ..T....... ATT.G..... ...TTTTG..
Uran   ..C.TC.... ...GA.G... .......C.. .......... A.T....... ....TTT...
Leio   ?????..... -..-....CT .......-C.. .......... ATT....... ....TTT...
Scel   .....CT... ...G....C. .......... .......... ATA....... ....TTT...
```

Figure 5.1. Mitochondrial 16S ribosomal RNA sequences. A question mark (?) indicates an ambiguous or missing nucleotide. A dash (-) indicates a gap introduced into the alignment. A dot (.) indicates identity to the nucleotide in the first sequence. Sites 158 - 190 were excluded from analysis. Site 1 corresponds to position 4,079 of the published *Xenopus laevis* mitochondrial genome (Roe et al. 1985). Sample abbreviations are as follows: del, *Microlophus delanonis*; albe, *M. albemarlensis*; inde1, *M. indefatigabilis*, Guy Fawkes Rocks; inde2, *M. indefatigabilis*, Santa Cruz; jacob, *M. jacobi*; dunc, *M. duncanensis*; gray, *M. grayi*; Plesio, *Plesiomicrolophus koepckeorum*; occi, *M. occipitalis*; paci, *M. pacificus*; stol, *M. stolzmanni*; ther, *M. theresiae*; thor, *M. thoracicus*; tigr, *M. tigris*; Ttumb, *Tropidurus umbra*; Sten, *Stenocercus roseiventris*; Uran, *Uranscodon superciliosa*; Leio, *Leiocephalus melanochlorus*; and Scel, *Sceloporus undulatus*.

Figure 5.1 (continued)

```
                                                          1 1111111111 1111111111
          6666666667 7777777778 8888888889 9999999990 0000000001 1111111112
          1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
del       CCATCAGTGA AACTGATCTT CCAGTCCAAA AGCTGGTATA ACCCCATAAG ACGAGAAGAC
albe      .......... .........A .....T.... .......... ..TA...... ..........
indel     .......... .........A .....T.... .......... .......... ..........
inde2     .......... .........A .....T.... .......... .......... ..........
jacob     .......... .........A .....T.... .......... ...A...... ..........
dunc      .......... .........A .......... .......... ...A...... ..........
gray      .......... .........A .....T.... .......... ...A...... ..........
Plesio    ?AG....... .........A .....A.... .....A.... C......... ..........
occi      .T...GA... .........C T....A.... .....A.... TA........ ..........
paci      .......... .........A .....T.... .......... ..TA...... ..........
stol      TA........ .........A .....A.... .......... TA....C... ..........
ther      TA........ .........A .....A.... .......... .A........ ..........
thor      .T........ .........G .....A.T.. .....AC... TG........ ..........
tigr      .A........ .........A .....A.... .......... .G.A...... ..........
Tumb      .T........ .........A .....A.... .......... G...T..... ..........
Sten      .TG....... .........A .....A.... .......... .AA....... ..........
Uran      .A....A... .........A ......A... .......... .AA....... ..........
Leio      TAG.T..... .......... .....A.... ......C... .AGA...... ..........
Scel      AA........ .........A .....A.... .......... .A........ ..........


          1111111111 1111111111 1111111111 1111111111 1111111111 1111111111
          2222222223 3333333334 4444444445 5555555556 6666666667 7777777778
          1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
del       CCTGTGGAGC TTTAAATCAA AGATCAACAC AAAGCCAACC CACAACCCAC A--TAT---G
albe      .......... .......... GA........ ..G.....A. A..T.AA.CT CACGCAT...
indel     .......... .......... TA........ ..G......T A..T.AG.CT CAC.CAT...
inde2     .......... .......... TA........ ..G....... A..T.AG.CT CAC.CAT...
jacob     .......... .......... GA........ ..G....... A..CTAA.CT CACGCAT...
dunc      .......... .......... .A........ ..G....... A..TTAA.CT CCC.CAT...
gray      .......... .......... GA........ ..G....... A..C.AA.CT CACACAT...
Plesio    .......... .......... .A.....A.. ..TCT..C.. GCT..TT.CT GAT.--...-
occi      .......... ..C.....T. .A-..... ..GATT.CTA ACA.T.A... .CT---....
paci      .......... .......... GA........ .TTAGGCCAA .CA.CTAA.. CTCACGCAT.
stol      .......... ..C....... .A........ ..GCT..--- --.C.AA.TA TGCCCATTGA
ther      .......... ..A....... .A........ G..C.TCCA. ..--CTAA.. TTTC.ACCT.
thor      .......... .......... .......... .GCTA.C... ATAGCTA.CA -CT---....
tigr      .......... .......... .A........ C.CCAT.CA. ....CAA..A .CT---....
Tumb      .......... ..A...CT.T ..TCACCA.A .CCCT.T.AT AT..TTAGC. CT.---....
Sten      .......... ........G. .CCCA..T.A C.CTAA.C.A T.ACTTA--- -..---.AAT
Uran      .......... ..C..GCTTT GATCA.CA.T CC.C.AC..A .CACG...T- -..---....
Leio      .......... .......T.T T.-....ACA ...TAAGCA. ..A.C.GTCA .T.---....
Scel      .......... .......TTT .A-C...GCA .CTAA.G--. A.-.-.T--. .GC-CAA.T.
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Figure 5.1 (continued)

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          1111111111 1111111112 2222222222 2222222222 2222222222 2222222222
          8888888889 9999999990 0000000001 1111111112 2222222223 3333333334
          1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
del       ATTTAAATGA TTTTCAGTTG GGGCGACTTC GGAACAAAAA CTAACTTCCG AGAAAA-AGA
albe      ...A.CCC.. .......... .......... .......... AC........ ..C.C.....
indel     ...A...C.. .......... .......... .........G AC........ ..C.C.....
inde2     ...A...C.. .......... .......... .........G AC........ ..C.C.....
jacob     ...A...C.. .......... .......... .......... AC........ ..C.C.....
dunc      ...A...C.. .......... .......... .........G A......... ..C.C.....
gray      ...A--GC.. .......... .......... .........G AC........ ..C.C.....
Plesio    -..A..-... .......... .......... .....T.... AC........ ..C.C.....
occi      ......-C.. ....T..... .......... .......... AC........ ..C.C.....
paci      ...A..CC.. .......... .......... .......... AC........ ..C.C.....
stol      T....G.CC. .......... .......... .......... AC........ ..C.T.....
ther      ......-C.. .......... .......... .......... AC.......A ..C.C.....
thor      ......T-.. .......... .......... ....A.C... AC........ ..C.......
tigr      ....CG.C.. .......... .......... .......... AC........ ..C.C.....
Tumb      ..C...TA.- .......... .........T ...GTC...C AAGC.C...A ..C.......
Sten      TGCC.CCC.. .......... .......... ...GT....C AC...C.... ..C.T.A...
Uran      ..CC.C.AAG C...A..... .......... ....AC...C .A........ ..C.C.....
Leio      ...A.C.A-- ....G..... .......... .........T AA.......A ..C.C.G...
Scel      GC.A...A-- C...A..... .......... ....AT...C .A........ ..C.T.....


          2222222222 2222222222 2222222222 2222222222 2222222222 2222222223
          4444444445 5555555556 6666666667 7777777778 8888888889 9999999990
          1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
del       CCCACTCTTA CCA----AGA CCCACAAGTC TAAACAAATA TTGACCCAGT AA-CACTGAC
albe      T......... ..C....... ..A....... C.......C. .......... ..........
indel     T......... ..T....... ..A....... C.......C. .......... ..........
inde2     T......... ..T....... ..A....... C.......C. .......... ..........
jacob     T......... T.C....... ..A....... ........C. .......... ..........
dunc      T......... .......... ..A....... ........C. .......... ..........
gray      T......... ..C....... ..A....... C.......C. .......... ..........
Plesio    .T........ T.C....... ..T....... ........AT .......... C.........
occi      TTT.T...C. TT........ .TA....... A......TAT .......... C.........
paci      T......... ..C....... ..A....... C.......C. .......... ..........
stol      ..TGT...C. ..T....... ..A....... C..G....A. .......... C........T
ther      ..T..C..CT TT.CAC.... ..A....... --.......AC .......... .CA.......
thor      ..T..C..CT TA.CCCT... ..A...C... --.....C.AT .......... CT.A.....T
tigr      .TT..A..CT TT.AC..... ..A....... A...-----T .......... CCA......T
Tumb      ..T.AAA.CT TTCACAC... ..T....... ACT------T .......... ...-.....T
Sten      ---CAC..CT .ACCA....G AAT.....C. A..G...... .......G.C CT.T..C..T
Uran      ..ACAG-.CT AACCAC.-.. .TA...T... ..G.T-.TA. .......... .T.......T
Leio      A.T..A.CCT .A.TCA...G ..A.T...C. A.G.TT..AC .......... ...T.....T
Scel      ..T..G-.CT TACCA..... ..T....... A..G.T..A. .......... .C........
```

Figure 5.1 (continued)

```
          3333333333 3333333333 3333333333 3333333333 3333333333 3333333333
          0000000001 1111111112 2222222223 3333333334 4444444445 5555555556
          1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
del       CAACGAACCA AGTTACCCCA GGGATAACAG CGCAATCTTC TTCAAGAGTC CATATCGACA
albe      .......... .......... .......... .......... .......... .C........
indel     .......... .......... .......... .......... .......... .C........
inde2     .......... .......... .......... .......... .......... .C........
jacob     .......... .......... .......... .......... .......... .C........
dunc      .......... .......... .......... .......... .......... .C........
gray      .......... .......... .......... .......... .......... .C........
Plesio    .......... .......... .......... .......... .......... ..........
occi      .......... .......... .......... .......... .......... ..........
paci      .......... .......... .......... .......... .......... .C........
stol      .......... .......... .......... .......... .......... ..........
ther      .......... .......... .......... .......... .......... ..........
thor      .......... .......... .......... .......... ........C. ..........
tigr      .......... .......... .......... .......... ........A. ..........
Tumb      .......... .......... .......... .......... .......... .C........
Sten      A......... .......... .......... .......... .......... ..........
Uran      A......... .......... .......... .......... .........T .C........
Leio      ....A..... .......... .......... ...C...... ..T....... ..........
Scel      A.C....... .......... .......... .......... .......... .C........


          3333333333 3333333333 3333333333 3333333334 4444444444 4444444444
          6666666667 7777777778 8888888889 9999999990 0000000001 1111111112
          1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
del       AGAAGGTTTA CGACCTCGAT GTTGGATCA- GGACACCCAA TTGGTGCAGC CGCTATTAA-
albe      .......... .......... .......... .......... .......... ..........
indel     .......... .......... .......... .......... .......... ..........
inde2     .......... .......... .......... .......... .......... ..........
jacob     .......... .......... .......... .......... .......... ..........
dunc      .......... .......... .......... .......... .......... ..........
gray      .......... .......... .......... .......... .......... ..........
Plesio    .......... .......... .........A ........T. A......... ..........
occi      .......... .......... .......... .......... .......... ..........
paci      .......... .......... .......... .......... .......... ..........
stol      .......... .......... .......... .......... .......... ..........
ther      .......... .......... .......... .......... .........? ..........
thor      .......... .......... .......... .......... .......... ..........
tigr      .......... .......... .......... .......... .......... .........T
Tumb      .......... .......... .......... ....C...C. A......... ..........
Sten      .......... ........TA .......... .......... A......... ..........
Uran      G......... .......... .......... .......... .......... ..........
Leio      .......... .......... .......... ........C. A.....T... ..........
Scel      .......... ..T....... ????.??... ..?.....C? ?......... ..........
```

Figure 5.1 (continued)

```
          4444444444 4444444444 4444444
          2222222223 3333333334 4444444
          1234567890 1234567890 1234567
del       GGGTTCGTTT GTTCAACGAT TAACAGT
albe      .......... .......... .......
inde1     .......... .......... .......
inde2     .......... .......... .......
jacob     .......... .......... .......
dunc      .......... .......... .......
gray      .......... .......... .......
Plesio    .......... .......... .......
occi      A......... ....?-.?.. ...T...
paci      .......... .......... .......
stol      A......... .......... .......
ther      A....A?G.. T????????? ???????
thor      A......... .......... .......
tigr      ......???? ?????????? ???????
Tumb      A......... .......... ...T...
Sten      C......... .......... .......
Uran      A......... .......... .......
Leio      A......... .......... ...T...
Scel      A......... .......... .......
```

parsimony. Nucleotide composition for these sequences were approximately 33.5% A, 22.4% T, 24.9% C, and 19.2% G.

Phylogenetic trees were reconstructed using the distance correction algorithms mentioned above. Figure 5.2 shows the tree recovered when using Kimura's (1980) 2-parameter correction. All distance-based trees were nearly identical in topology, with only minor exceptions (such as the branching order within the *theresiae/thoracicus/tigris* clade). The statistical significance of each node was determined by calculating either the bootstrap *p*-values (BP) from 2000 replicates or confidence probability values (CP) from the standard error test. Multiple maximum parsimony analyses were performed with the following transition/transversion ratios: 1:1, 3:1, and 10:1. The significance of nodes in these trees were assessed by the frequency of appearance of a clade in equally parsimonious trees or bootstrap values from 100 bootstraps (nearest-neighbor interchange).

D. Discussion

The seven "Central Islands" Galápagos species form a monophyletic assemblage (Fig. 5.2); this topology is well supported, with statistical values reaching 98% or greater in all but two of the trees. As such, the 16S rRNA data are in agreement with the cytochrome *b* data, which also grouped these species together. Within this clade the two samples of *M. indefatigabilis* group together with statistical support of 95% or greater in all recovered trees. Of the "Central Islands" species, the most basal is *M. delanonis*. The phylogenetic placement of this species concurs with the cytochrome *b* data.

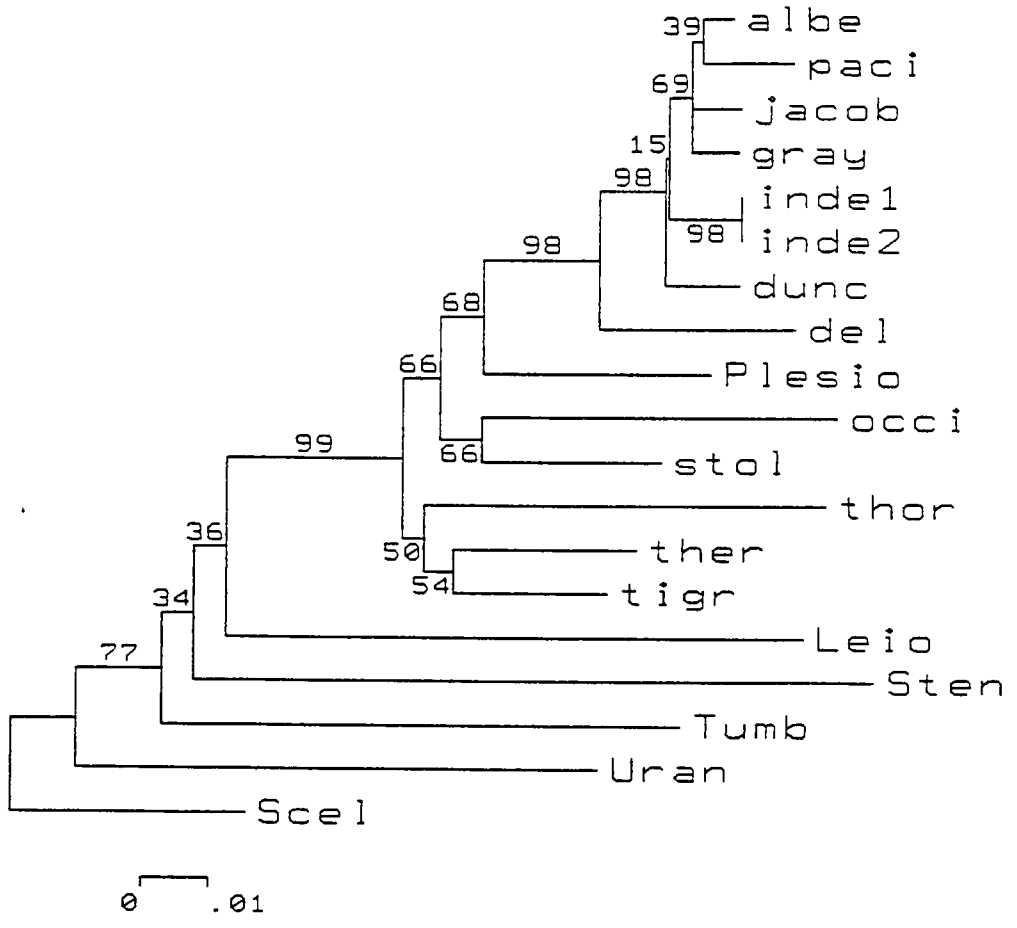

Figure 5.2. A phylogeny of Galápagos lava lizards. This phylogeny was reconstructed using nucleotide variation in a 477 bp portion of the mitochondrial 16S ribosomal RNA gene. It was built by the neighbor-joining algorithm using the Kimura 2-parameter model for estimating evolutionary distance. Numbers on the branches indicate the confidence probability values generated by the standard error test. The scale bar indicates relative branch length. Sample abbreviations are given in Figure 5.1.

Plesiomicrolophus koepckeorum is placed as the sister taxon to the clade composed of the "Central Islands" species (Fig. 5.2). Five *Microlophus* species (*occipitalis, stolzmanni, theresiae, thoracicus,* and *tigris*; all are mainland south American taxa) are placed basal to the *Plesiomicrolophus* + Galápagos clade. This topology appears in every distance-based tree and in three of the six maximum parsimony trees, although the statistical support does not reach significant levels in most of the trees.

In the three maximum parsimony trees with alternative branching patterns, *Plesiomicrolophus* is placed in an unresolved polytomy with a clade composed of the Galápagos species and some (or all) of the mainland South American *Microlophus* species. However, in no tree is *Plesiomicrolophus* placed outside of the *Microlophus* species. The monophyly of *Microlophus* + *Plesiomicrolophus* is strongly supported, with statistical values in the majority of trees of 96% or greater.

Both the 16S rRNA and cytochrome *b* data sets place *Plesiomicrolophus* well within the sampled species of *Microlophus* and no change is observed when the two data sets are combined and analyzed together. Therefore, it is proposed here that *Plesiomicrolophus* be synonymized with *Microlophus*. Frost's (1992) study on these taxa identified only a single diagnostic character which differentiated these genera, the presence or absence of apical disks on the hemipenes (present in *Microlophus*, absent in *Plesiomicrolophus*). Indeed, Frost (1992, pp. 45-46) wrote:

> My conjecture is that future work will show [*Plesiomicrolophus*] to be the sister taxon of *Microlophus*, in which case it could justifiably be considered a junior synonym of *Microlophus*.

Hence, neither the genetic nor the morphological characters of this taxon warrant its placement in a monotypic genus.

The sister taxa of the *Microlophus* + *Plesiomicrolophus* clade are *M. occipitalis* and *M. stolzmanni* (Fig. 5.2). This is in disagreement with the cytochrome *b* data which identified either *M. theresiae* or *M. thoracicus* as the most likely ancestor to the "Central Islands" clade of Galápagos species (Fig. 3.4). There are two possibilities for this apparent discrepancy. The first involves the statistical support given to the two alternative topologies by the respective data sets which support them. With the 16S rRNA data, the statistical values supporting the sister taxa status of *M. occipitalis* + *M. stolzmanni* relative to the *Microlophus* + *Plesiomicrolophus* clade average less than 55%. Likewise, in the cytochrome *b* data set, statistical values supporting *M. theresiae* or *M. thoracicus* as the sister taxon do not approach significant levels. Thus, the South American species ancestral to the "Central Islands" clade cannot be resolved with the present data.

The 16S rRNA data are not used to more closely examine the relationships among the Galápagos species because 16S sequences were not obtained for all taxa (stemming from logistical constraints of time and money). If a data set and tree inference technique were perfect, then any two subsets of taxa would yield congruent trees. However, this is rarely the case. Simulation studies have shown that, due to both systematic and random error, an inferred branching order can be dependent on the taxa included in the analysis (Swofford et al. 1996 and references therein). Therefore, to avoid these problems, the

16S data were used as additional support for the systematic placement of *Plesiomicrolophus* but not to investigate the relationships among Galápagos lava lizards.

E. Summary

Results of analyses of nucleotide sequence variation in a portion of the mitochondrial 16S ribosomal RNA gene from Galápagos and South American species of tropidurine lizards support earlier studies (Wright, 1983; Frost, 1992; Lopez et al. 1992) as well as the cytochrome *b* data reported in this study (Ch. 3). The Galápagos species of lava lizards (excluding *M. bivittatus* and *M. habeli*) form a monophyletic assemblage. Within this clade, *M. delanonis* is the most basal taxon.

Plesiomicrolophus koepckeorum is closely related to *Microlophus*. In the majority of analyses, its phylogenetic placement is well within the *Microlophus* species examined. The combination of the prior morphological assessment of this taxon (Frost, 1992), the cytochrome *b* data, and the 16S rRNA data support the proposition that *Plesiomicrolophus* be synonymized with *Microlophus*.

The 16S rRNA data set conflicts with the cytochrome *b* data with regards to which of the South American species of *Microlophus* is ancestral to the species of Galápagos lava lizards. Further phylogenetic research should be conducted on this group of lizards in order to fully resolve their relationships.

Chapter 6

Summary

Results of analyses of nucleotide sequence variation in a portion of the mitochondrial cytochrome *b* gene from Galápagos and South American species of tropidurine lizards concur with earlier studies of their patterns of genetic relatedness (Wright, 1983; Lopez et al. 1992). The present-day species of Galápagos lava lizards are not a monophyletic assemblage. There are two main lineages; the first includes those populations from San Cristóbal and Marchena (*M. bivittatus* and *M. habeli*, respectively) while the second is comprised of all other island populations (representing *M. albemarlensis, M. delanonis, M. duncanensis, M. grayi, M. indefatigabilis, M. jacobi,* and *M. pacificus*). The lizards from San Cristóbal and Marchena are more closely related to *M. occipitalis* from mainland South America than they are to the other Galápagos species. The ancestor of the remaining island species could not be unequivocally resolved but, of the mainland South American taxa examined, *M. theresiae, M. thoracicus,* and *M. tigris* were identified as possible sister taxa.

The populations of *M. delanonis* from Española and surrounding islands form the most basal group within the main lineage, followed by the *M. grayi* populations from Floreana. *Microlophus pacificus* (from Pinzón) is the sister taxon to the populations from the islands of Isabela and Fernandina. Populations from Santa Cruz, Baltra, and Daphne group as a monophyletic clade and form the sister group to a monophyletic

assemblage of populations from Santiago and Bartolomé. *Microlophus duncanensis* from the island of Pinzón is the closest relative to these two groups.

Of those populations previously recognized as *M. albemarlensis*, four groups consistently emerge. One of these is formed by populations from the Guy Fawkes rocks and Islote Santa Fe. The observed levels of genetic divergence among these taxa are most likely attributable to genetic drift caused by the founder effect and small population size. The other three groups are distinct lineages with levels of genetic differentiation comparable to those exhibited by taxa historically recognized as valid species. Therefore, the populations from Santa Cruz, Baltra, and Daphne islands are raised to the specific level as *M. indefatigabilis* (Baur, 1890) and the populations from Santiago and Bartolomé islands are re-named *M. jacobi* (Baur, 1892). Future conservation plans should be altered accordingly so that these unique species are not lost due to habitat loss and/or effect of introduced biota.

The Galápagos lava lizards are almost certainly derived from South American species. Dispersal to the islands most likely occurred via rafting on mats of vegetation swept out to sea. Once in the Humboldt Current, as little as two weeks may have been necessary to reach the archipelago. The hardships of such a crossing would be lessened due to the physiologic resistance to dehydration and food deprivation exhibited by many reptiles. Once established on a single island, dispersal to other islands could result from crossing over land during times of lowered sea levels or by rafting on vegetation or chunks of floating pumice ejected by volcanic eruptions.

At least two colonization events are necessary to account for the present-day distribution of the Galápagos lava lizards. One occurred on San Cristóbal, which served as the source for the colonization of Marchena. The second founding event was to Española, from which Floreana was colonized. The next island colonized (from Floreana) was either Pinzón or Santa Cruz; if it was the former, it then served as the source for the latter, and vice versa. The Pinta lineage was derived from whichever of the two previous islands was originally colonized from Floreana. Santa Cruz served as the source population for Santiago. Isabela and Fernandina were colonized from Pinta.

The lineage of *Microlophus* inhabiting the islands of San Cristóbal and Marchena diverged from their closest mainland relative approximately 7.1 million years ago (mya). The other Galápagos species separated from their mainland ancestor 8.1 mya. *Microlophus delanonis* diverged from the remaining taxa 6.6 mya, the *M. albemarlensis/pacificus* clade split off around 6.0 mya, and *M. grayi* 4.8 mya. *Microlophus duncanensis* diverged from *M. indefatigabilis* and *M. jacobi* around 4.6 mya and the latter pair separated 4.4 mya. The time of divergence between *M. albemarlensis* and *M. pacificus* is approximately 2.1 mya.

The estimates of divergence times for the Galápagos species pre-date the age of the current Galápagos Islands. Either the lineages separated on the mainland of South America prior to their colonizations of the islands or they colonized islands which have since subsided below the surface of the ocean. Regardless of which scenario actually happened, the Galápagos lava lizards are older than the Galápagos Islands.

Results of analyses of nucleotide sequence variation in a portion of the mitochondrial 16S ribosomal RNA gene from Galápagos and South American species of tropidurine lizards support earlier studies (Wright, 1983; Frost, 1992; Lopez et al. 1992) as well as the cytochrome *b* data reported in this study. The Galápagos species of lava lizards (excluding *M. bivittatus* and *M. habeli*) form a monophyletic assemblage. Within this clade, *M. delanonis* is the most basal taxon.

Plesiomicrolophus koepckeorum is closely related to *Microlophus*. In the majority of analyses, its phylogenetic placement is well within the *Microlophus* species examined. The combination of the prior morphological assessment of this taxon (Frost, 1992), the cytochrome *b* data, and the 16S rRNA data support the proposition that *Plesiomicrolophus* be synonymized with *Microlophus*.

The 16S rRNA data set conflicts with the cytochrome *b* data with regards to which of the South American species of *Microlophus* is ancestral to the species of Galápagos lava lizards. Further phylogenetic research should be conducted on this group of lizards in order to fully resolve their relationships.

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APPENDICES

APPENDIX A

TAXA SAMPLED

Appendix A

Taxa Sampled

Tissue samples were obtained from the following sources: LM = L. R. Maxson, Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, TN; HS = H. L. Snell, Department of Biology, University of New Mexico, Albuquerque, NM; JW = J. W. Wright, Los Angeles County Museum of Natural History, Los Angeles, CA; and USNM = United States National Museum Field Series. Each sample is identified by a number and/or letter code. The most precise known localities are given following each identification number. Codes in quotation marks are those used to identify samples in the phylogenetic trees.

GALÁPAGOS *MICROLOPHUS*

albemarlensis

HS 7-249	"alb1"	Islote Sante Fe (near Isla Santa Fe)
HS 8.1-199	"alb2"	Cerro Colorado, Isla Santa Cruz
HS 8.3-238	"alb3"	Punta Roca Fuerte, Isla Santa Cruz
HS 11.2-26	"alb4"	Tagus Cove, Isla Isabela
HS 12-37	"alb5"	Punta Espinoza, Isla Fernandina
HS 33-119	"alb6"	South Punta Bowditch, Conway Bay, Isla Santa Cruz
HS 34-68	"alb7"	Guy Fawkes
HS 37-129	"alb8"	Guy Fawkes

HS 40-157	"alb9"	Sombero Chino, Isla Santiago
HS CB1-18	"alb10"	Beagle Crater Islet, Isla Isabela
HS EZ-26	"alb11"	Elizabeth Bay, Isla Isabela
HS SML-56	"alb12"	Southern Marielas, Elizabeth Bay, Isla Isabela
JW G-18	"alb13"	Isla Santa Cruz
JW G-213	"alb14"	Isla Bartolomé
JW G-232	"alb15"	Isla Santiago
JW G-271	"alb16"	Cartago Bay, Isla Isabela
JW G-285	"alb17"	Conway Bay, Isla Santa Cruz
JW G-301	"alb18"	Isla Baltra
JW G-323	"alb19"	Isla Daphne
JW G-350C	"alb20"	James Bay, Isla Santiago
LM 2396	"alb21"	Isla Fernandina
bivittatus		
HS 3-257	"biv1"	Isla San Cristóbal
HS 25-568	"biv2"	Islote Lobos (near Isla San Cristóbal)
JW G-42	"biv3"	Isla San Cristóbal
delanonis		
HS 6-848	"del1"	Isla Española
HS 14-389	"del2"	Islote Gardner (near Isla Española)
HS 22-397	"del3"	Islote Osborn, Gardner Bay, Isla Española
HS 23-266	"del4"	Islote Xaifra, Gardner Bay, Isla Española

HS 24-266	"del5"	West Islet, Gardner Bay, Isla Española
HS LA-59	"del6"	Gardner Bay, Isla Española
duncanensis		
JW G-269	"dunc"	Isla Pinzón
grayi		
HS CHP-29	"gray1"	Islote Champion (near Isla Floreana)
HS END-38	"gray2"	Islote Enderby (near Isla Floreana)
LM 2398	"gray3"	Isla Floreana
habeli		
JW G-209	"habe"	Isla Marchena
pacificus		
JW G-194	"paci"	Isla Pinta

NON-GALÁPAGOS *MICROLOPHUS*

occipitalis		
JW G-4-80	"occi"	Ecuador
stolzmanni		
LM 2392	"stol"	Peru
theresiae		
LM 2253	"ther"	Peru
thoracicus		
LM 2389	"thor"	Peru

tigris

LM 2390	"tigr"	Peru

OTHER TROPIDURID TAXA

Leiocephalus melanochlorus

USNM 103721	"Leio"	Haiti: Grand Anse; ca. 3 km N Bois Sec

Plesiomicrolophus koepckeorum

LM 2391	"Plesio"	Peru

Tropidurus torquatus

LM 2257	"Ttor"	Venezuela

T. umbra

LM 2183	"Tumb"	Cuzco Amazonico, Peru

Stenocercus roseiventris

LM 1767	"Sten"	Cuzco Amazonico, Peru

Uranoscodon superciliosa

LM 2796	"Uran"	Brazil

OUTGROUP TAXON

Sceloporus undulatus	"Scel"
cytochrome *b* sequence	uncatalogued sequence from lab of L. Maxson
16S rRNA sequence	GenBank (accession number L28075)

APPENDIX B

SOLUTIONS

Appendix B

Solutions

Most of the solutions listed below are common laboratory solutions. However, some of them were modified for specific uses in the lab of L. R. Maxson (by S. B. Hedges, T. J. Lopez, and other lab personnel).

TBE Buffer

This buffer is used as an electrophoretic buffer for agarose and sequencing gels.

Tris (hydroxymethylaminomethane)	216.0 g
boric acid	110.0 g
ethylenediaminetetraacetate (EDTA)	18.6 g

Pour components into a 20 l container, add approximately 10 l deionized H_2O, and let dissolve; *q. v.* to 20 l.

For 10X or 5X TBE buffer, smaller amounts may be made as follows.

10X	5X
108.0 g Tris	54.0 g Tris
55.0 g boric acid	27.5 g boric acid
9.3 g EDTA	4.7 g EDTA

Dissolve in 500 ml hot deionized H_2O; *q. v.* to 1 l.

DNA Extraction Buffer (modified from Kocher et al., 1989)

Fifty ml of this buffer is prepared in a sterile test tube and divided into 0.5 ml aliquots, which are placed in 1.5 ml eppendorf tubes. This results in final concentrations of 100 mM Tris-HCL, 10 mM EDTA, 12% 100 mM NaCl, 0.1% SDS, 50 mM DTT, and 500 ng/ml proteinase K.

1 M Tris (pH 8.0)	5000 µl
0.5 M EDTA	1000
5 M NaCl	1000
20% sodium dodecylsulfate (SDS)	250
0.5 M dithiothreitol (DTT)	5000
Proteinase K (1 mg/ml)	250
sterile distilled H_2O	37,500

Buffered Phenol

This protocol follows directions enclosed with UltraPURE phenol, Life Technologies, Gaithersburg, MD.

1. Melt the phenol in a 65°C water bath.
2. Add an equal volume of 1 M Tris (pH 8.0) directly into the phenol bottle.
3. Shake vigorously and let settle for approximately two hours.
4. Remove the aqueous layer.
5. Add an equal volume of 0.1 M Tris (pH 8.0).
6. Shake vigorously and let settle for approximately one half hour.

7. Remove the aqueous layer.

8. Add an equal volume of 0.1 M Tris (pH 8.0).

9. Shake vigorously and let settle for approximately one half hour. DO NOT remove aqueous layer.

10. Add 0.1 g 8-hydroxyquinoline and 0.2 ml b-mercaptoethanol.

11. Shake and let sit overnight before use.

NOTE: this preparation will remain usable for several months when stored at 4°C. When the organic layer turns form light yellow to brown, the solution is no longer usable for protein extractions.

Tris-EDTA (TE)

Five thousand µl of this solution are prepared, aliquotted into five 1.5 ml eppendorf tubes and stored at -20°C.

1 M Tris pH 7.5	50 µl
0.5 M EDTA pH 7.5	10 µl
sterile distilled H_2O	4940 µl

PCR Solutions

1. Double-stranded (ds) PCR Reaction Buffer

A solution containing all necessary components is prepared in advance to perform numerous PCR reactions. Each reaction buffer is a 24 µl volume containing:

67 mM Tris

6.7 mM $MgSO_4$

16.6 mM $(NH_4)SO_4$

10 mM beta-mercaptoethanol

0.1 mM dideoxyadenine triphosphate

0.1 mM dideoxycytosine triphosphate

0.1 mM dideoxyguanine triphosphate

0.1 mM dideoxythymidine triphosphate

1 μM primer 1

1 μM primer 2

0.5 International Units (IU) *Thermus aquaticus* (*Taq*) DNA polymerase

Typically, for *n* templates, solution sufficient for *n* + 1 reactions is prepared and aliquotted to *n* + 1 0.5 ml eppendorf tubes. One μl of each DNA dilution is added to the proper amplification tubes; the remaining tube serves as a negative control. After PCR amplification (see Chapter 3), a 10 μl aliquot is added to 2 μl Load Dye (2.5 mg/ml Bromophenol Blue, in 30% glycerol). Ten μl of this mixture is loaded onto a 2% agarose gel containing 5 X 10^{-7} % ethidium bromide (5.0 μl of 1% ethidium bromide solution per 100 ml gel) and run at 100 volts for approximately 30 minutes. Double-stranded PCR products were visualized and photographed under ultraviolet light; this procedure acted as a check against any contamination of the PCR solution.

2. Single-stranded (ss) PCR Reaction Buffer

Single-stranded PCR reactions were performed in 50 µl volumes. The buffer used was identical to the double-stranded PCR buffer with the exception that one of the primers was at a concentration of 0.01 mM and the other was at a concentration of 1 mM. This follows the unbalanced PCR method of Gyllensten and Erlich (1988) which allows for a few cycles of double-stranded amplification, followed by many cycles of amplification of primarily one strand. Single-stranded PCR products were visualized and photographed under ultraviolet light in an identical manner as double-stranded PCR products (see above).

Agarose Gels

Agarose gels were made with either one or two grams of agarose (depending on the size of gel needed) in sufficient TBE buffer to produce a final agarose concentration of 2%. Typically, 1.0 g of agarose was measured into a tared 250 ml ehrlenmeyer flask. Sixty ml of TBE buffer was added and the flask was microwaved for approximately two and one half minutes, or until the agarose was completely dissolved. The boiling reduced the volume of buffer to about 50 ml, resulting in a 2% agarose concentration. The flask was removed from the microwave and 2.5 µl of a 1% ethidium bromide solution was added. The mixture was swirled briefly and cooled by placing the flask under running tap water for about ten seconds. The agarose was then poured into a gel box with two prepositioned combs and allowed to cool completely before running the templates. When required, a larger gel was prepared by the same method, with the following

adjustments: a 500 ml ehrlenmeyer flask was used, 2.0 g agarose was added to 110 ml of TBE buffer, and 5.0 µl of 1% ethidium bromide was used.

Low Melting Point Agarose Gels

Low melting point agarose (Nu-Seive, *BRL*) is used to isolate double-stranded templates generated in dsPCR reactions. Gel preparation is identical to that used for normal agarose gels, except that the gel is prepared and run in a walk-in cold room. In addition, a lower voltage is used, typically from 32 to 45 volts, and only every other lane is loaded (this procedure helps prevent any cross-contamination of templates).

1% Ethidium Bromide

10 mg ethidium bromide per1.0 ml sterile distilled H_2O

Polyacrylamide Sequencing Gels

Two different solutions are prepared and then mixed together to produce sufficient volume for six 40 cm X 60 cm sequencing gels. Each resulting gel is approximately 6% acrylamide and has an urea concentration high enough to insure that DNA fragments remain denatured during electrophoresis.

The first solution is prepared in a 1 l flask and contains:

375 g urea

280 ml distilled H_2O

75 ml 10X TBE buffer

This solution is placed on a hotplate, and stirred until dissolved under very low heat. As the first solution is dissolved, the second is prepared in a 500 ml flask:

43.7 g acrylamide

2.3 g bis-acrylamide

80 ml distilled H_2O

Add the second solution to the first and swirl once. Vacuum-filter this mixture and pour into six squirt bottles (~120 ml each). These should be stored in darkness and used within one month. Immediately prior to use, two catalysts (TEMED and ammonium persulfate) are added to the squirt bottle; the exact procedure is detailed in the sequencing protocol (Appendix C).

5X *Taq* Annealing Buffer

Five thousand µl of this solution are prepared, aliquotted into five 1.5 ml eppendorf tubes and stored at -20°C.

sterile distilled H_2O	3575 µl
1 M Tris (pH 8.0)	1250
1 M $MgCl_2$	175

Labeling Mix/dH_2O

Approximately 10 ml of this solution are prepared, aliquotted into fifteen 1.5 ml eppendorf tubes and stored at -20°C.

dH_2O	10 ml

1 mM dCTP	5.0 µl
1 mM dGTP	5.0
1 mM dTTP	5.0

Taq Dilution Buffer

Five thousand µl of this solution are prepared, aliquotted into five 1.5 ml eppendorf tubes and stored at -20°C.

sterile distilled H_2O	4859 µl
1 M Tris (pH 8.0)	125
0.5 M EDTA	1.0
Tween 20	7.5
Nonidet P-40 (=NP40)	7.5

Termination Mixes

To each 1.5 ml eppendorf tube, add:

10 mM dATP	2.0 µl
10 mM dCTP	2.0
10 mM dGTP	2.0
10 mM dTTP	2.0

To the "A" tube, add:

5 mM ddATP	160 µl
sterile distilled H_2O	832

To the "C" tube, add:

5 mM ddCTP	80 µl
sterile distilled H_2O	912

To the "G" tube, add;

5 mM ddGTP	12 µl
sterile distilled H_2O	980

To the "T" tube, add:

5 mM ddTTP	160 µl
sterile distilled H_2O	832

Store at -20°C. ddNTPs are supplied from Pharmacia or PROMEGA at 5 mM concentration.

Stop Dye

Formamide	860 µl
0.2 M EDTA (pH 7.2)	50
Xylene cyanol (2%)	40
Bromophenol blue (2%)	40
sterile distilled water	10

Store at -20°C.

APPENDIX C

SEQUENCING PROTOCOLS FOR USE WITH

TAQ DNA POLYMERASE

Appendix C

Sequencing Protocol For Use

With *Taq* DNA Polymerase

A. Polyacrylamide Gel Preparation.

1. Place one short (43 X 38 cm) and one long (45 X 38 cm) glass plate on bench paper.
2. Clean the inside of each plate with 95% ethanol (EtOH) and paper towels.
3. Turn the plates over, and clean the outside of both plates with 95% EtOH. Use a razor blade to remove any tape remnants.
4. Turn the plates over, and clean the inside of both plates twice more with 95% EtOH.
5. Clean two wedge-shaped spacers by wiping with paper towel wetted with 95% EtOH.
6. Coat the inside of the short plate with a siliconizing agent (Sigmacote or Gel Slick) according to manufacturers' directions.
7. Clean the inside of both plates with distilled water and paper towels. Be sure to remove any dust or particulate matter from the surface of the plates.
8. Position the spacers on the inside of the short plate. The thick end (0.8 mm) should be even with the bottom of the plate.

9. Place the long plate on top of the short plate, aligning the bottoms. The thin ends (0.4 mm) of the spacers should protrude past the top of the short plate.
10. Invert the plates and place them onto a 20 X 20 X 2 cm styrofoam block. Center the block under the plates. Orient the plates so that the tops and bottoms face to either side.
11. Using wide adhesive tape (5.1 cm), seal down one side of the plates, making sure to pull out any wrinkles and pressing for a secure seal. Continue across the bottom of the plates, and up the remaining side.
12. Tape once around the bottom of the plates. Repeat Step 11, beginning and ending no higher than one third up each side.
13. Place three large (5 cm wide) binder clips along each side of the plates, directly over the spacers.
14. Clean a plastic shark's tooth comb with 95% EtOH.
15. Place a squirt bottle of 6% polyacrylamide gel mix on a stir plate. Add a 1.5 cm magnetic stir bar, and turn stir plate to low speed.
16. Add 0.12 g of ammonium persulfate and 36 µl of TEMED to the gel mix. Stir until the ammonium persulfate crystals go into solution (approximately one minute).
17. Holding the plates at approximately a 60 degree angle, pour the gel mix carefully between the plates. Pour as quickly as is possible, but avoid introducing small bubbles between the plates.

18. Place the plates back onto the styrofoam block and carefully insert the comb, with the teeth sticking out away from the gel. The comb should be centered and inserted until the side flanges are even with the top of the short plate. Be sure there are no bubbles along the inside edge of the comb.

19. Use three clips, evenly spaced, to clamp over the comb lip.

20. Let the gel stand for at least one hour before using. Typically, the gel is poured the evening before the gel is to be run. In these instances, plastic wrap is loosely placed over the open end of the plates and comb to prevent any dust from adhering to the top of the gel.

Depending on the number of samples which are to be run, either one or two gels need to be prepared. One gel is sufficient if the number of samples is small enough to allow the gel to be double-loaded (see below). If not, two gels are needed, one each for the three-hour and eight-hour electrophoretic runs (see below).

B. Sequencing Reactions

These reactions typically were performed the night before the gel was to be run. Alternatively, it can be performed in the morning, if immediately followed by electrophoresis. If the sequencing reactions are completed the evening before, the microtitre plate should be stored at -20°C overnight. The protocol below describes the methods used for performing sequencing reactions using ssPCR products and the dideoxy-termination method of Sanger et al. (1977).

1. Turn on the DNA Thermal Cycler and set a water bath to 70°C (used to stop the extension reactions).
2. Remove the following components from the freezer and thaw: 5X *Taq* annealing buffer, primers (20 μM), ssDNA samples, label mix/dH_2O, *Taq* dilution buffer, *Taq* DNA polymerase, isotope (^{35}S dATP), termination mixes (A, C, G, and T), and stop dye. Single-stranded DNA samples should be in 0.5 ml eppendorf tubes.
3. Label a microtitre plate with the sample numbers above the appropriate columns and with "A"," C"," G", or "T" along the top four rows of wells.
4. Add 2 μl 5X *Taq* annealing buffer and 1 μl primer to the side of each reaction tube. Spin tubes in microcentrifuge for 15 seconds and place into the thermal cycler for three minutes @ 70°C, then ten minutes @ 42°C.
5. While the reaction tubes are in the thermal cycler, make the labeling reaction mix (LRM) in a 1.5 ml eppendorf tube. Prepare enough for $n + 2$ samples. For each sample, the LRM must contain:

label mix/dH_2O	5.0 μl
^{35}S dATP	0.5
Taq dilution buffer	1.6
Taq DNA polymerase	0.4

6. When the reaction tubes are finished heating in the thermal cycler, remove them and spin briefly in a microcentrifuge. Add 7.0 μl of LRM to each tube. Spin briefly and return to thermal cycler for five minutes @ 42°C.

7. Vortex and briefly spin the termination mixes. Add 4.0 µl of the A termination mix to the appropriate wells of the microtitre plate (place the drop on one side). Repeat with the C, G, and T mixes. Lightly place adhesive cover over microtitre plate.
8. When the reaction tubes are finished heating in the thermal cycler, remove them and spin briefly in a microcentrifuge. Add 4.0 µl of each sample to the appropriately numbered wells of the microtitre plate. Place the drops on the opposite side of the well from the termination mix so that the two drops do not come into contact. Seal the microtitre plate with the adhesive cover and **gently** tap the plate against the bench top to mix the two drops.
9. Float the microtitre plate in the 70°C water bath for five minutes. Remove the plate from the bath and carefully pull back the cover.
10. Add 4.0 µl of stop dye to each well. Place the drop on the side of the well. Reseal the plate and gently tap to mix the stop dye with the sequencing reaction.
11. Load the samples onto the sequencing gel immediately or store at -20°C if loading the next morning.

C. Polyacrylamide Gel Mounting

1. Remove the clips and tape from the plates. Use distilled water to remove any crystallized gel mixture from the plates and to loosen the comb. Remove

the comb by carefully pulling it straight out. Using a razor blade and distilled water, thoroughly clean the comb, both sides of the plates, the pocket formed between the short and long plates, and the bottom margin of the gel.

2. Apply a thin layer of petroleum jelly to the rubber gasket on the electrophoresis rig and to two small (1 X 2 cm) rubber bumpers.
3. Place the plates and gel into the electrophoresis rig. The short plate should face into the rig. The rubber bumpers will help form a leakproof seal for the upper buffer reservoir. Align the plates and bumpers with the rig and lock them into place. Seal the outer edges of the bumpers with more petroleum jelly.
4. Fill the upper reservoir with TBE buffer to within 1/2 cm of the top of the long plate. Using a 60 cc syringe, clean the space at the top of the gel by squirting buffer from the upper reservoir into the space between the two plates. If any debris remains in this space, carefully dislodge it and flush the space with the syringe. Make sure not to damage the top of the gel.
5. Check for leaks around the top gasket and the rubber bumpers. Use additional petroleum jelly as needed to seal the plates. Fill the bottom reservoir with TBE buffer until the bottom of the plates and gel are submerged.
6. Connect the rig to the E-C 600 power supply and start the current. Allow the gel to warm for at least one half of an hour. The power supply settings should be approximately 1250 volts, 65 watts, and 50 milliamperes.

7. Check the rig for leaks.

D. Loading the Polyacrylamide Gel

This procedure is performed after the gel had been mounted and allowed to warm for at least thirty minutes (see above).

1. Float the microtitre plate containing the samples in a 80°C water bath for 3-4 minutes. During this time, turn off the power to the electrophoretic rig and, using a 60 cc syringe, clean the space at the top of the gel by flushing buffer through it. This removes excess urea from this area.
2. Using one's fingers and a small amount of petroleum jelly, lightly coat the teeth of the comb.
3. Gently insert the comb between the plates until the teeth press lightly into the surface of the gel. This will form the wells into which the DNA samples are loaded.
4. Flush the top of the gel one more time with the syringe. Be careful not to damage the teeth of the comb.
5. Using a Drummond pipettor, load 2.0 µl of a sample to a well. The order should be A/C/G/T. Make sure to rinse the pipettor after each well. If the tip becomes clogged with petroleum jelly, clear it by briefly immersing the tip into a hot water bath. Load four wells (totalling one sample) and then skip the next well before loading the next sample.

6. If nine to eighteen samples are being run, two gels will be needed. Load the first gel, turn on the power, and load the second gel. After loading the final sample on the second gel, there should be a small amount of fluid left in each well of the microtitre plate. Use the pipettor to draw a total of 2.0 µl from a number of wells and load this onto the extreme right side of the gel. Repeat for the second gel.This will serve as a orientation marker once the gel is dried and exposed to a radiograph film (see below). Run one gel for three hours and the second for seven hours (350 bp fragments) or eight hours (500 bp fragments).
7. If eight or fewer samples are being run, a single gel may be double-loaded. Load each sample once, starting at the left side of the gel, turn on the power and return the microtitre plate to the freezer. After four or five hours (depending on the size of the fragments), turn off the power. Following the procedure outlined above, load what remains of the samples making sure to start to the right of the previously loaded samples. Turn on the power and run for three hours.

E. Gel Fixation and Autoradiography

1. When the gel is finished running, turn off and disconnect the power supply. Drain the buffer from the reservoirs.
2. Turn on the vacuum pump and condenser. Fill the water trap casement with ice.

3. Remove the gel from the electrophoretic rig and place on bench paper with the long plate down. Carefully remove the comb by pulling straight out.
4. Loosen the plates by carefully inserting the tip of a spatula between the plates and remove the wedge spacers by pulling them straight out. Using the spatula as a lever, carefully remove the short plate (the gel should remain against the long plate). Make sure not to tear the gel.
5. Soak the gel in fixative (a 5% methanol - 5% acetic acid solution) on the long plate for 10-15 minutes. Make sure the gel is completely covered with fixative. Carefully remove the plate from the fixative. If needed, the fixative can be drained from the reservoir before the plate is lifted. Tilt the plate to drain any remaining fixative off the gel. Be very careful not to let the gel slip off the plate nor to introduce wrinkles into the gel.
6. Place the plate on a flat surface. Center a large sheet (46 X 57 cm) of filter paper over the gel. Smooth the filter paper over the gel, making sure not to wrinkle or tear the gel. Carefully lift the filter paper; the gel should stick to it. Place the paper down (gel-side up) on a flat surface.
7. Cover the gel with plastic wrap and smooth out any wrinkles. Trim the gel to fit into the gel dryer; usually, this will require trimming approximately one cm from each side and ten cm from the top of the gel. Do NOT trim any from the bottom of the gel.

8. Place the gel in the gel dryer, close the vacuum system, and turn on the gel dryer. The thermal profile should be a spike heat to 92°C, followed by a slow decay to 80°C. Hold at 80°C for two hours.
9. Once the gel is dry, it is removed from the dryer and the plastic wrap is pulled away. Measure the activity of the middle of the gel with a geiger counter to insure that the fragments have travelled properly. Lightly spread a small amount of talcum powder across the gel. This will help prevent the gel from sticking to the autoradiograph film.
10. In a photographic dark room, load the gel into a X-ray film cassette and place an autoradiograph film directly in contact with the gel. After allowing sufficient time for the gel to expose the film (a few hours to several days, depending on the activity of the gel), the film is developed in an X-omat Developer (Kodak).

APPENDIX D

PHYLOGENETIC TREES ANALYZED IN CHAPTER 3

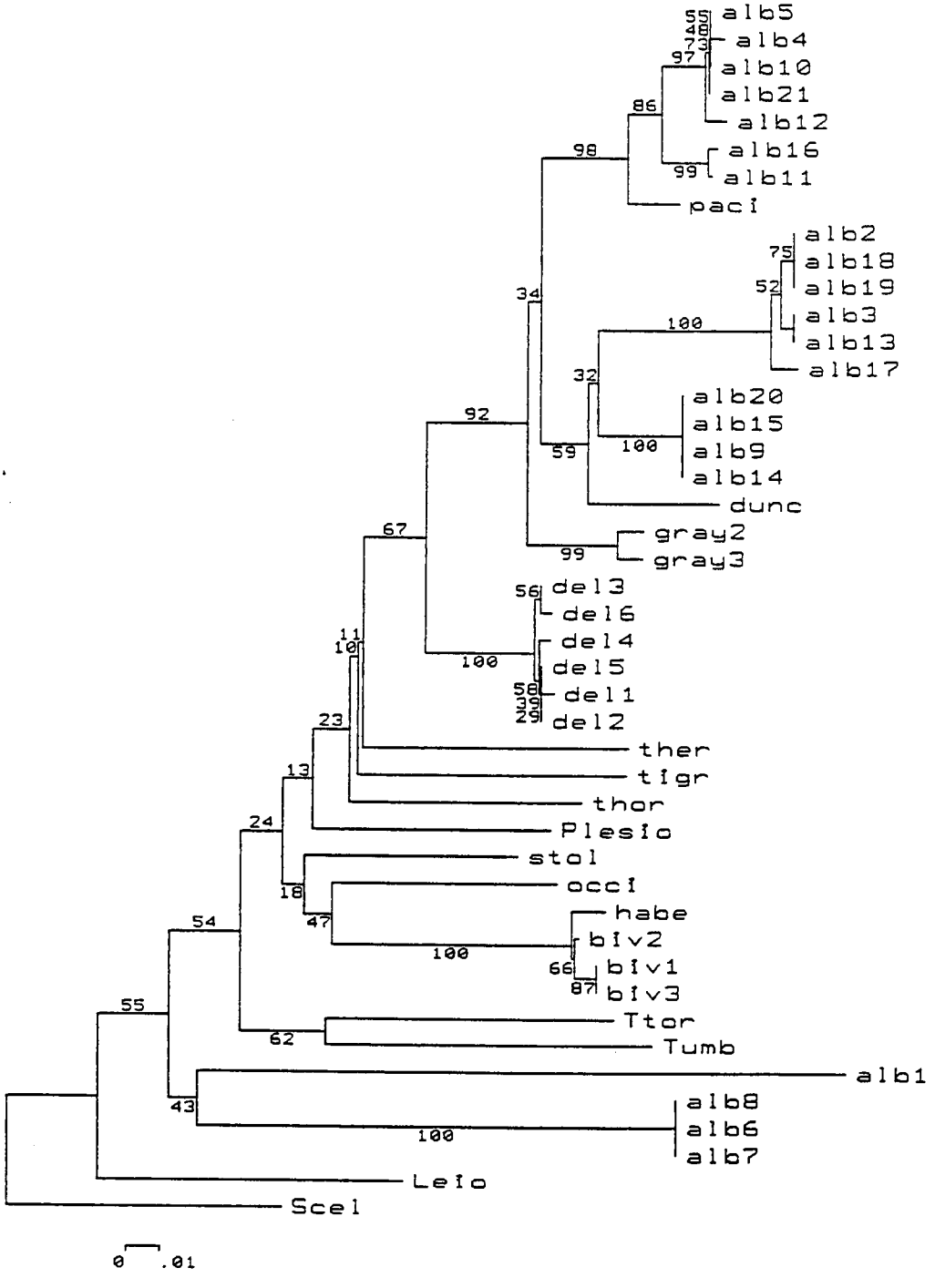
Appendix D

Phylogenetic Trees Analyzed in Chapter 3

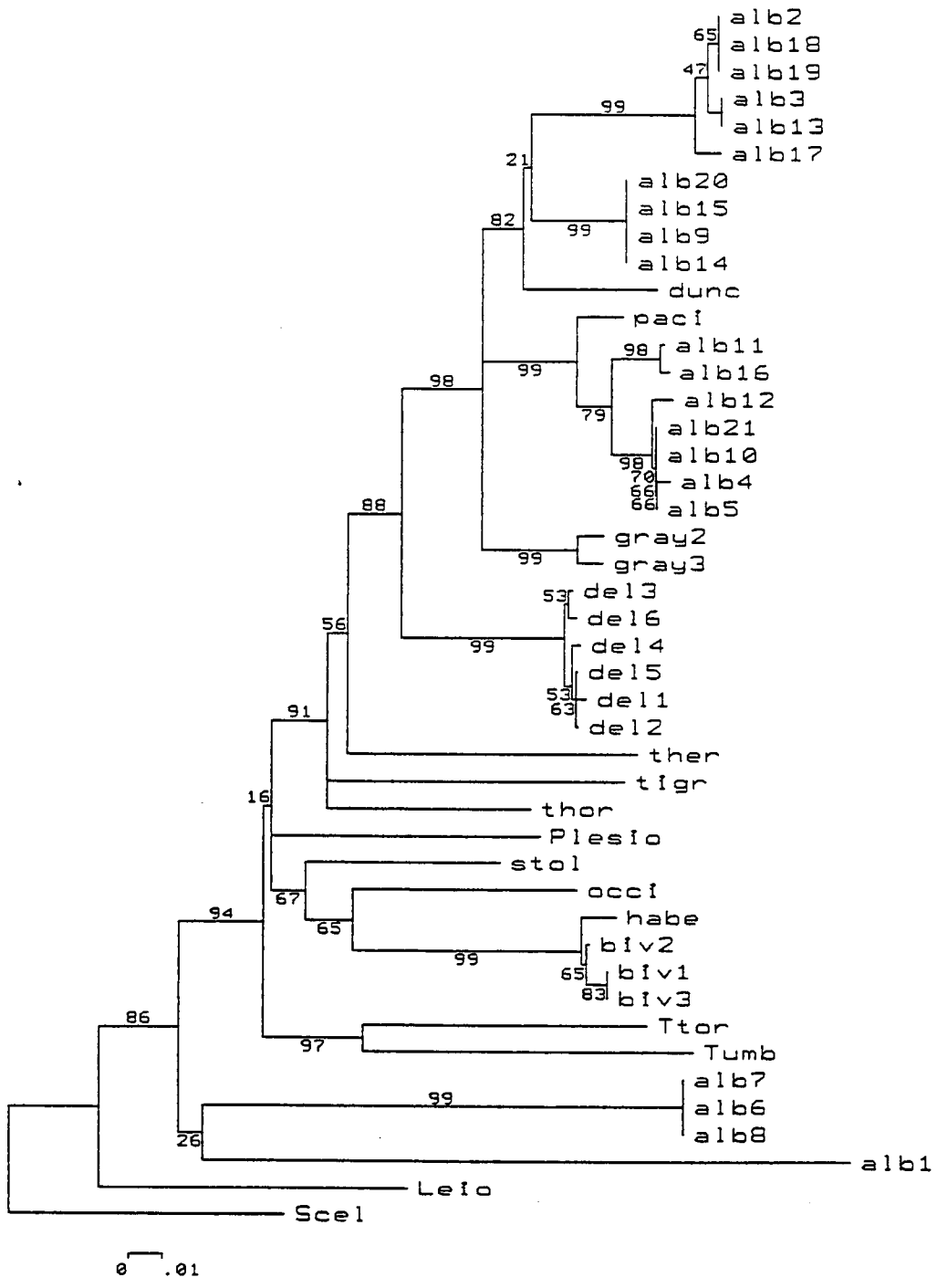
The following 22 phylogenies were reconstructed using the nucleotide sequence variation in a 307 bp portion of the mitochondrial cytochrome *b* gene from 45 tropidurine species and one outgroup taxon. The order of appearance of these phylogenies corresponds to the order in which they are listed in Table 3.1.

For neighbor-joining trees, branch lengths are proportional to evolutionary distance; a scale bar provides a measure of relative length. The key below each phylogeny identifies the algorithm used to estimate distances and whether the values on the branches are bootstrap *p*-values or confidence probability values.

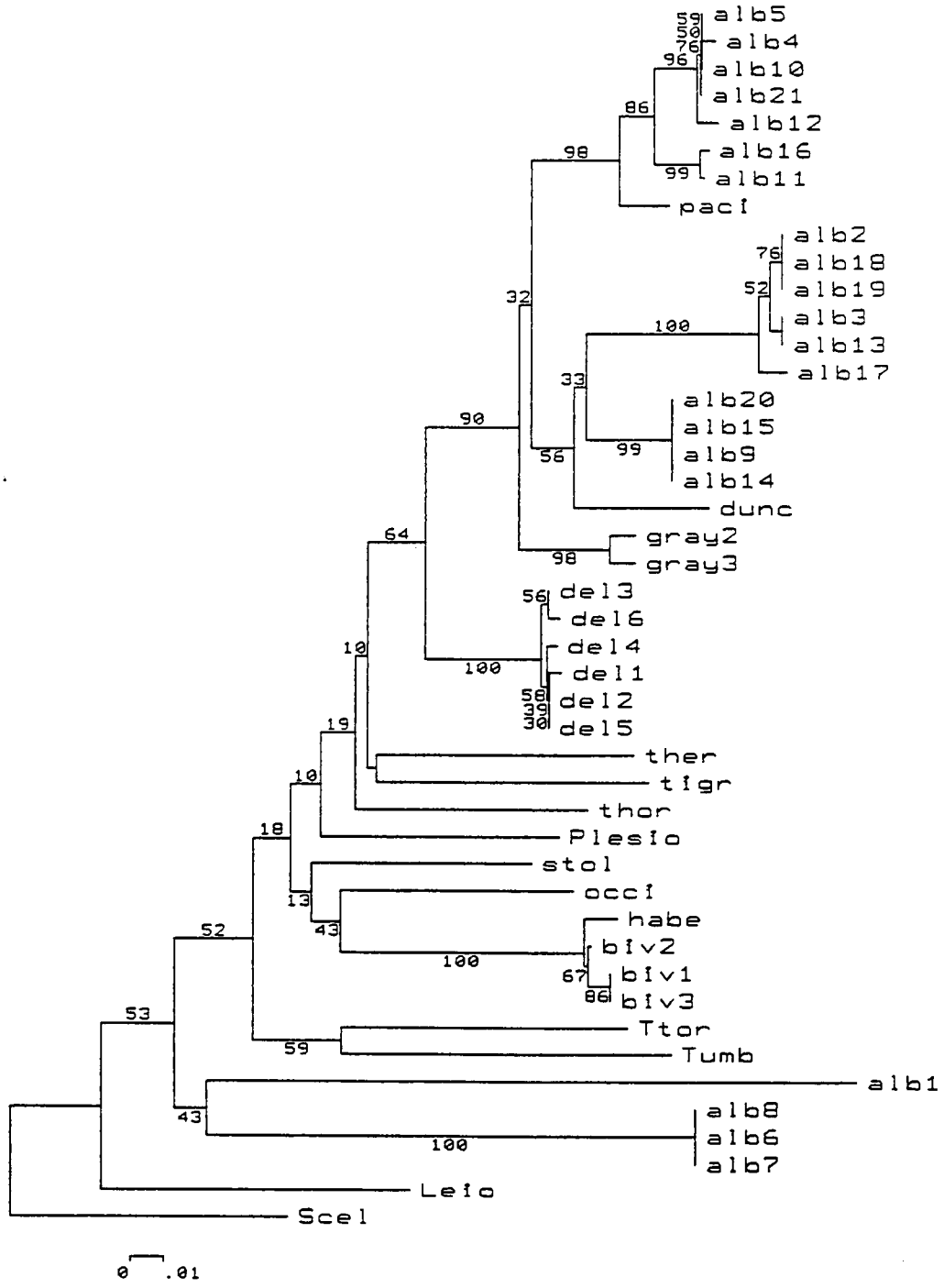
For parsimony trees, the key identifies the number of equally parsimonious trees generated by the heuristic search and whether the values on the branches are the percentage of trees in which a clade appears or the bootstrap value from 100 replicates (nearest-neighbor interchange).



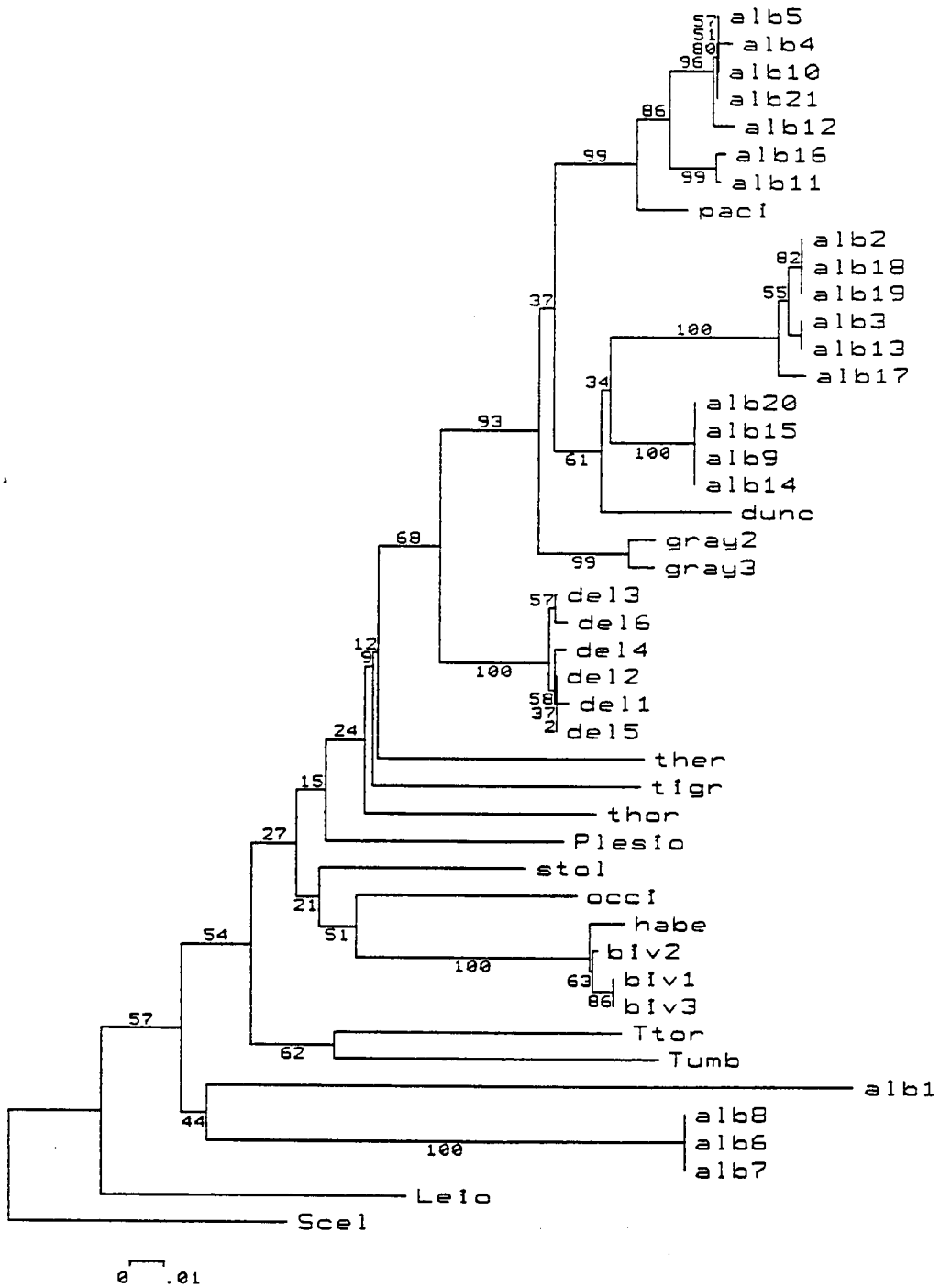
Kimura 2-parameter distance with bootstrap *p*-values for 2000 replicates



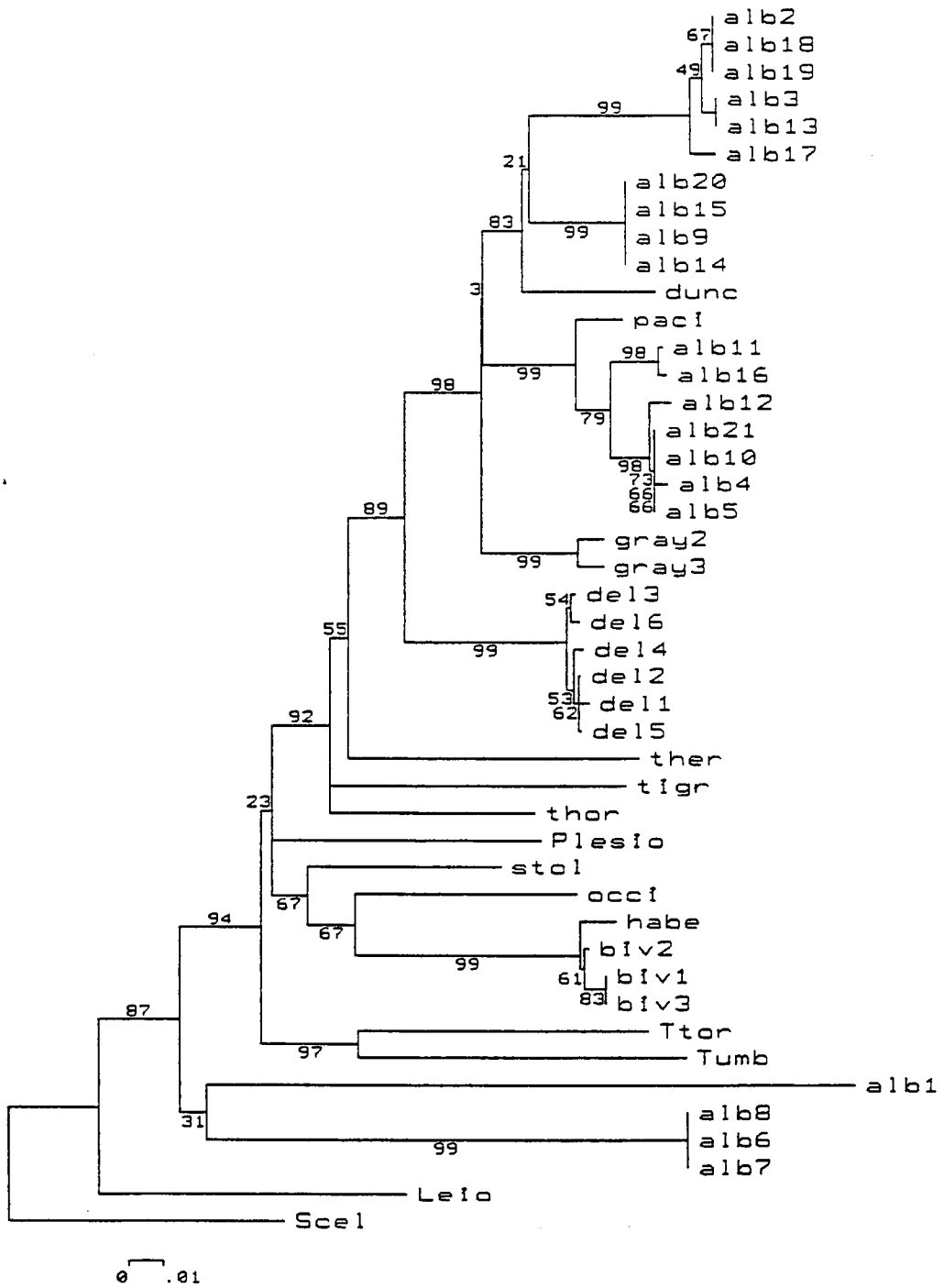
Kimura 2-parameter distance with confidence probability values from standard error test



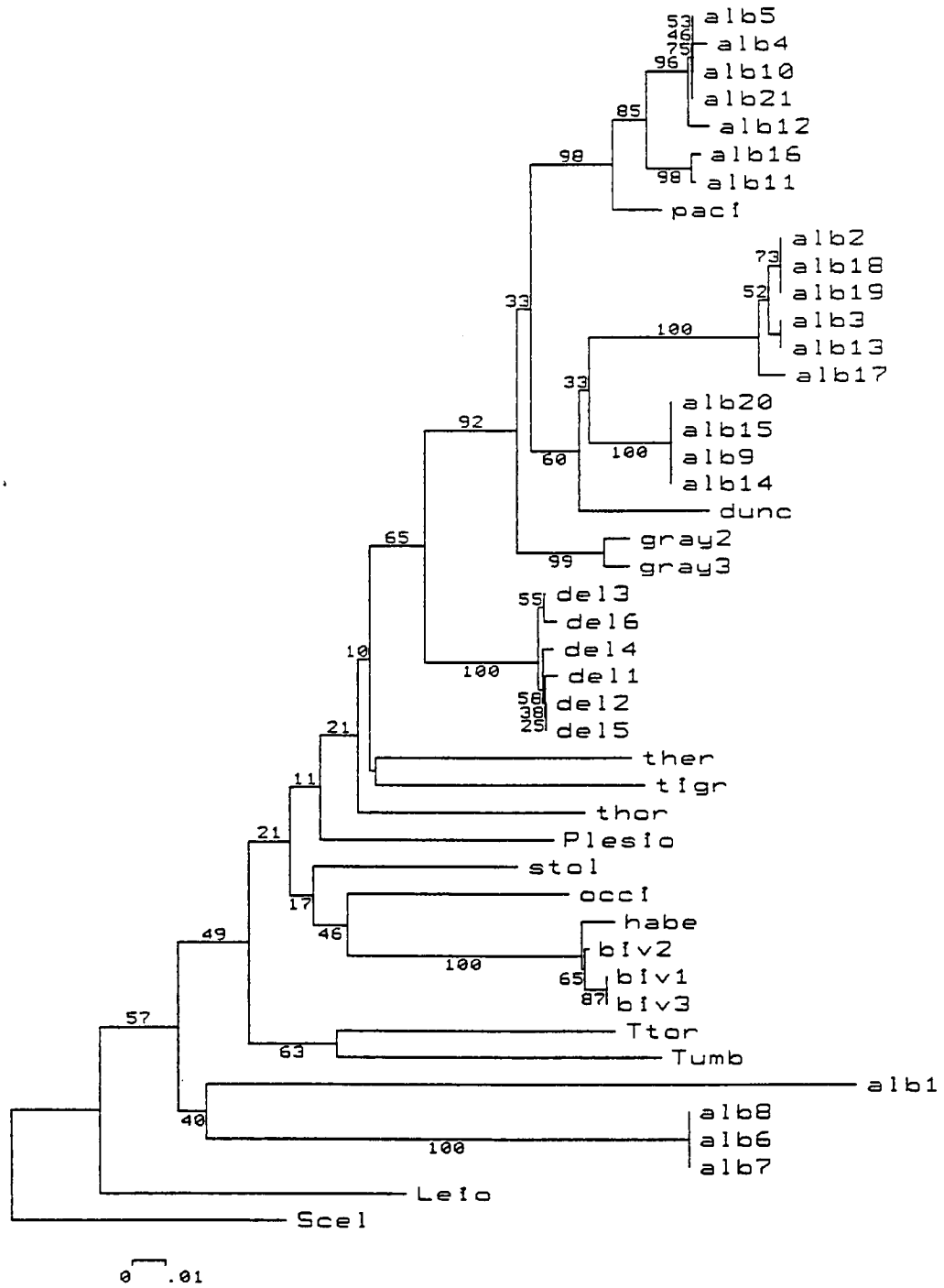
Tamura-Nei distance with bootstrap *p*-values for 2000 replicates



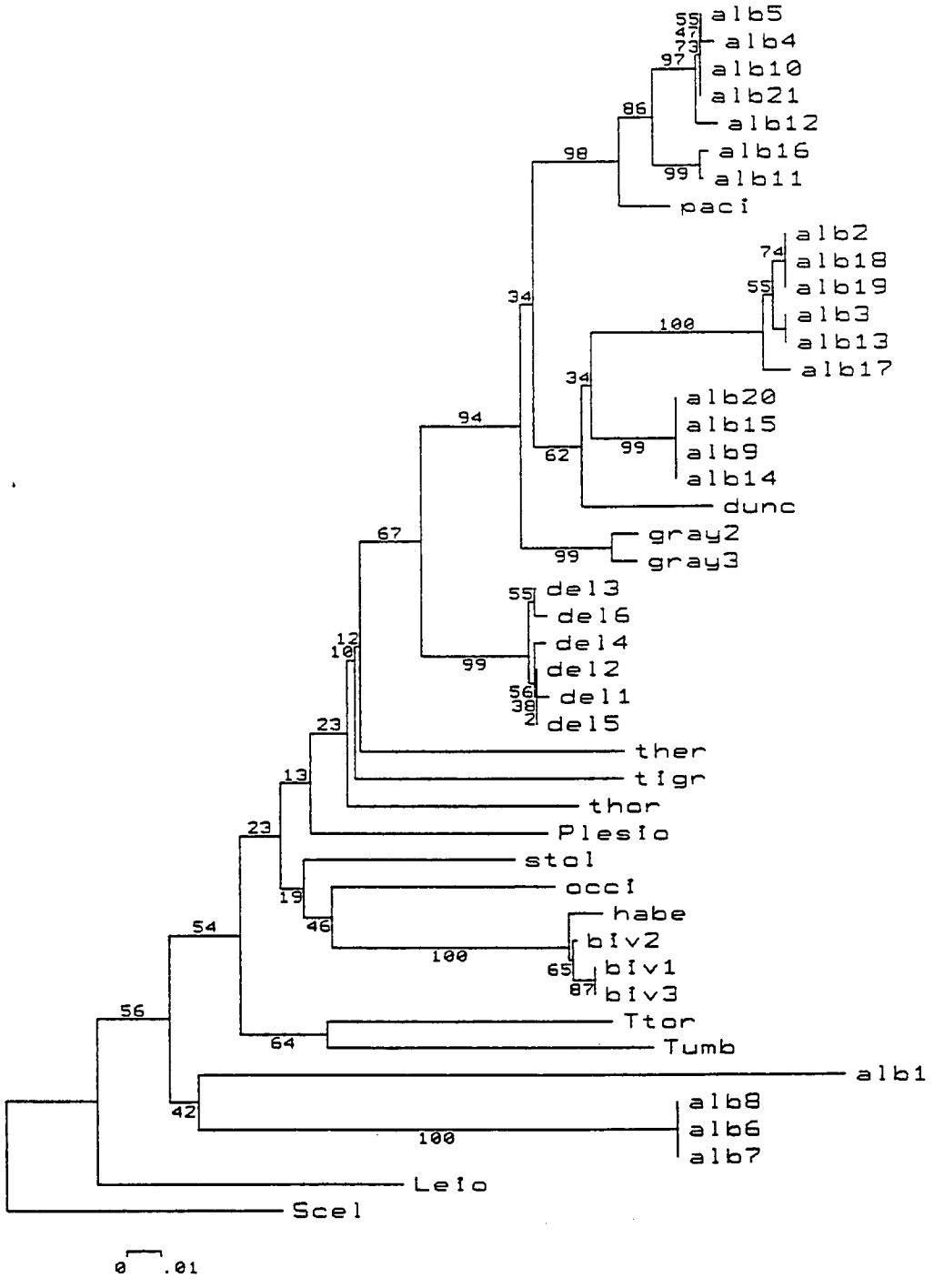
Jukes-Cantor distance with bootstrap *p*-values for 2000 replicates



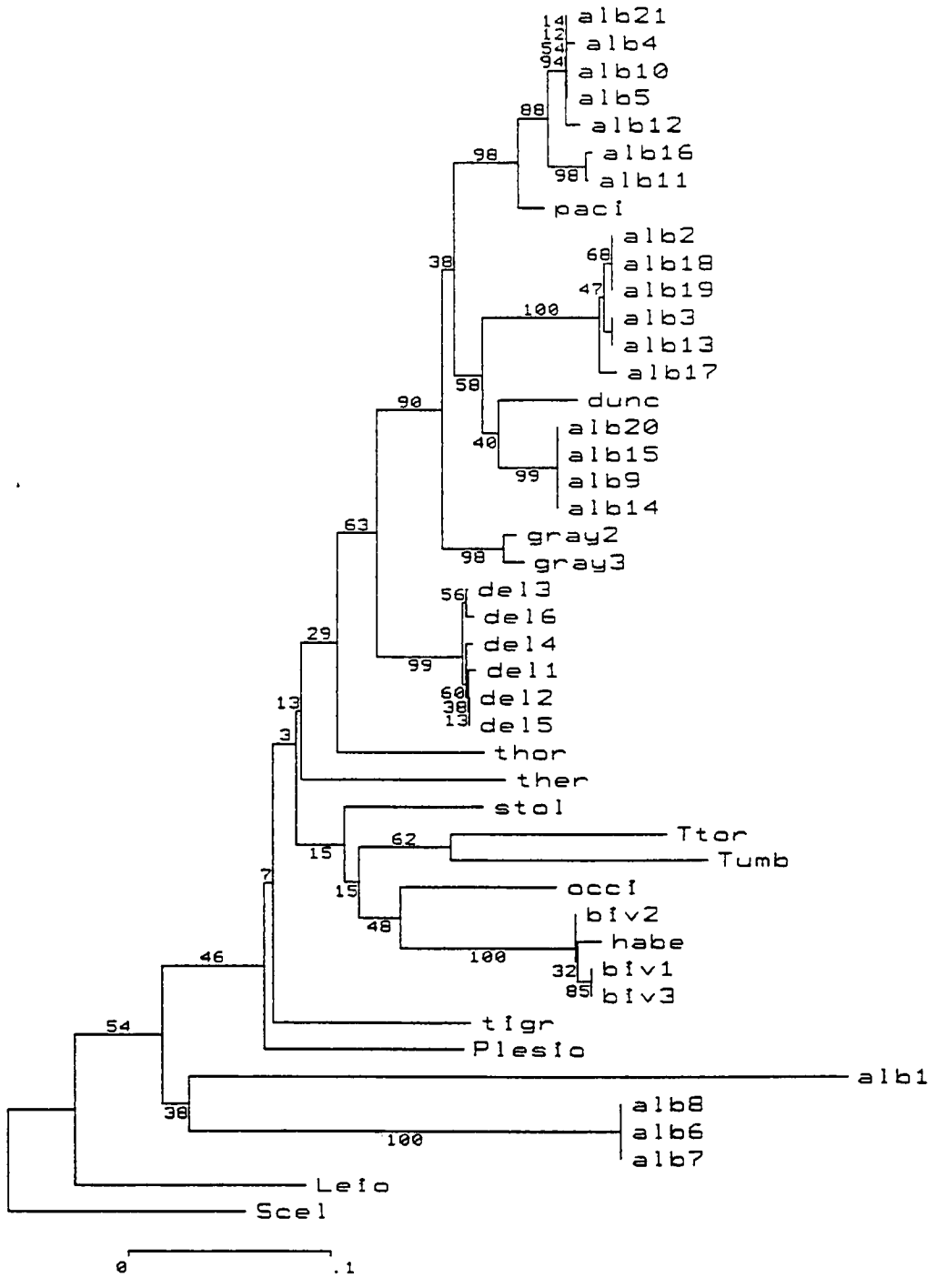
Jukes-Cantor distance with confidence probability values from standard error test



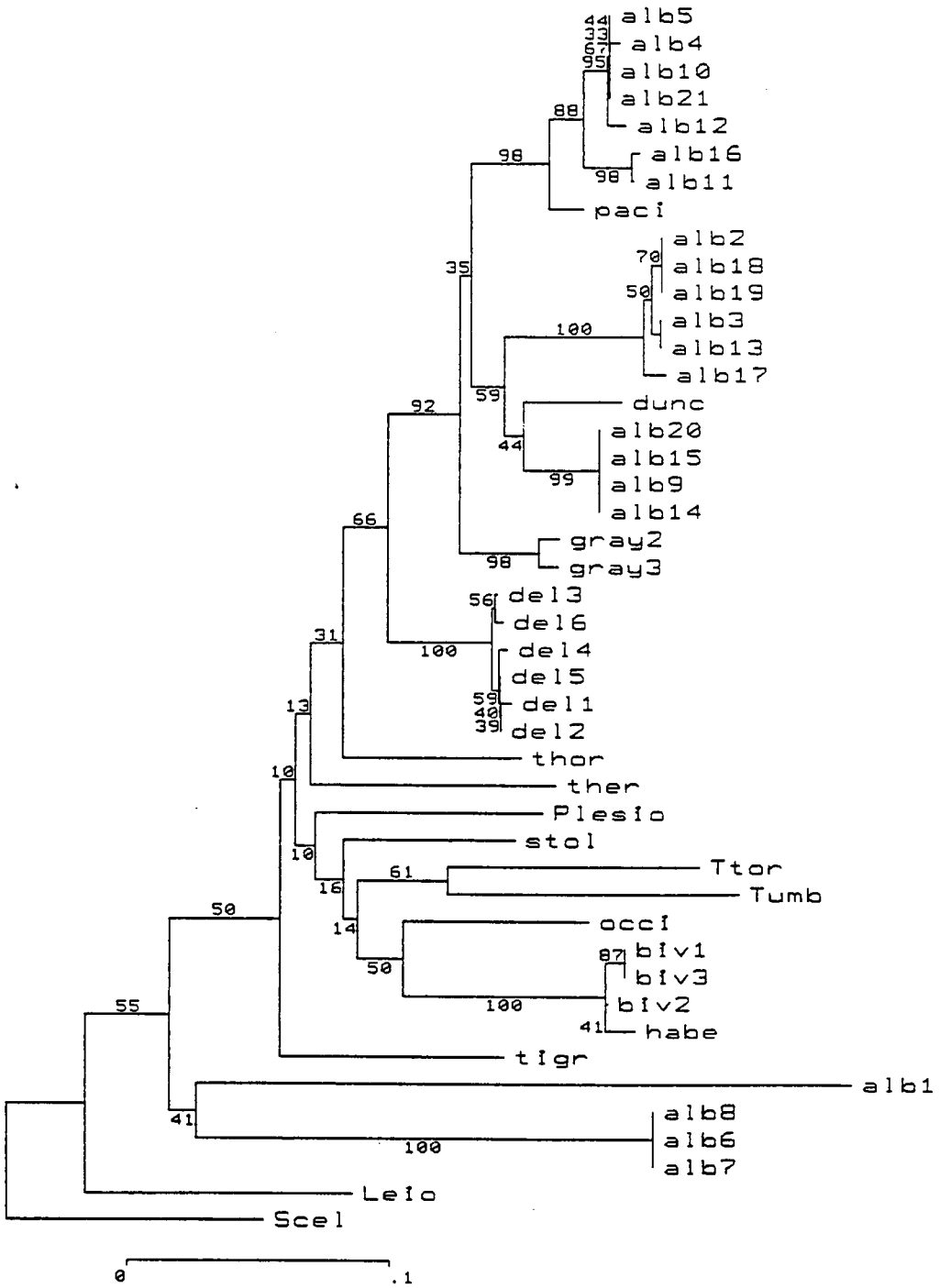
Tajima-Nei distance with bootstrap *p*-values for 2000 replicates



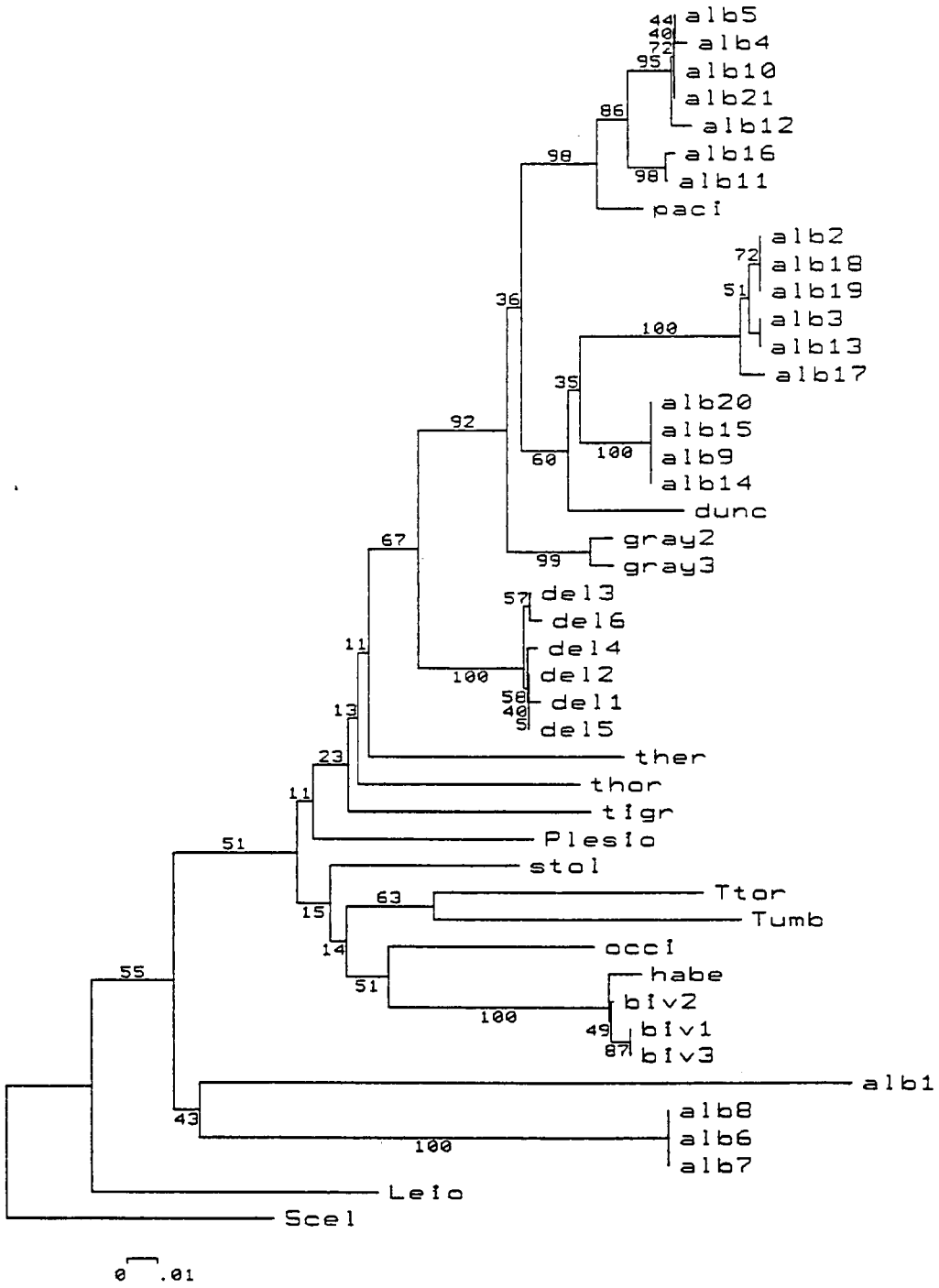
Tamura distance with bootstrap *p*-values for 2000 replicates



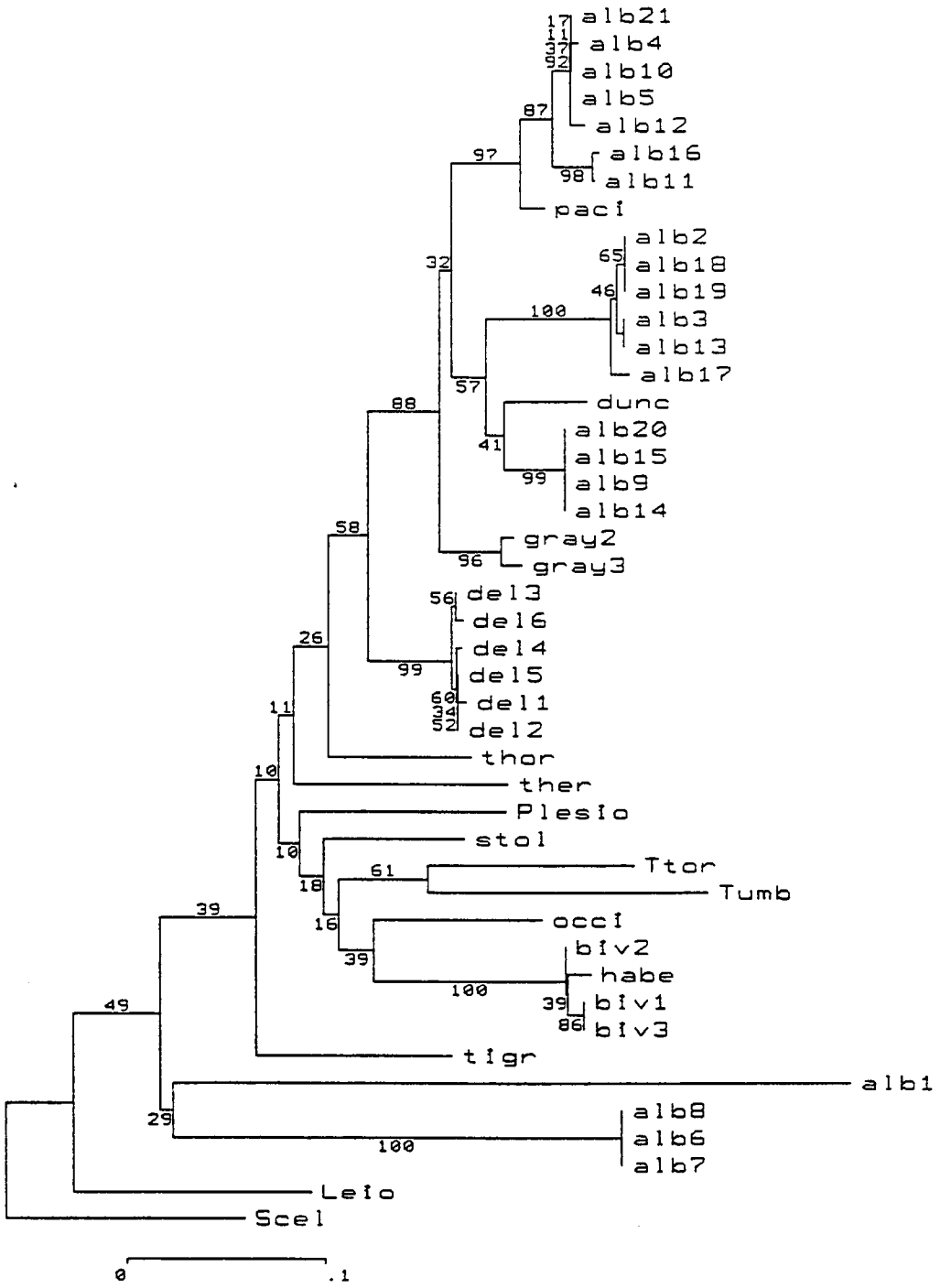
Jukes-Cantor gamma distance (a = 0.5) with bootstrap *p*-values for 2000 replicates



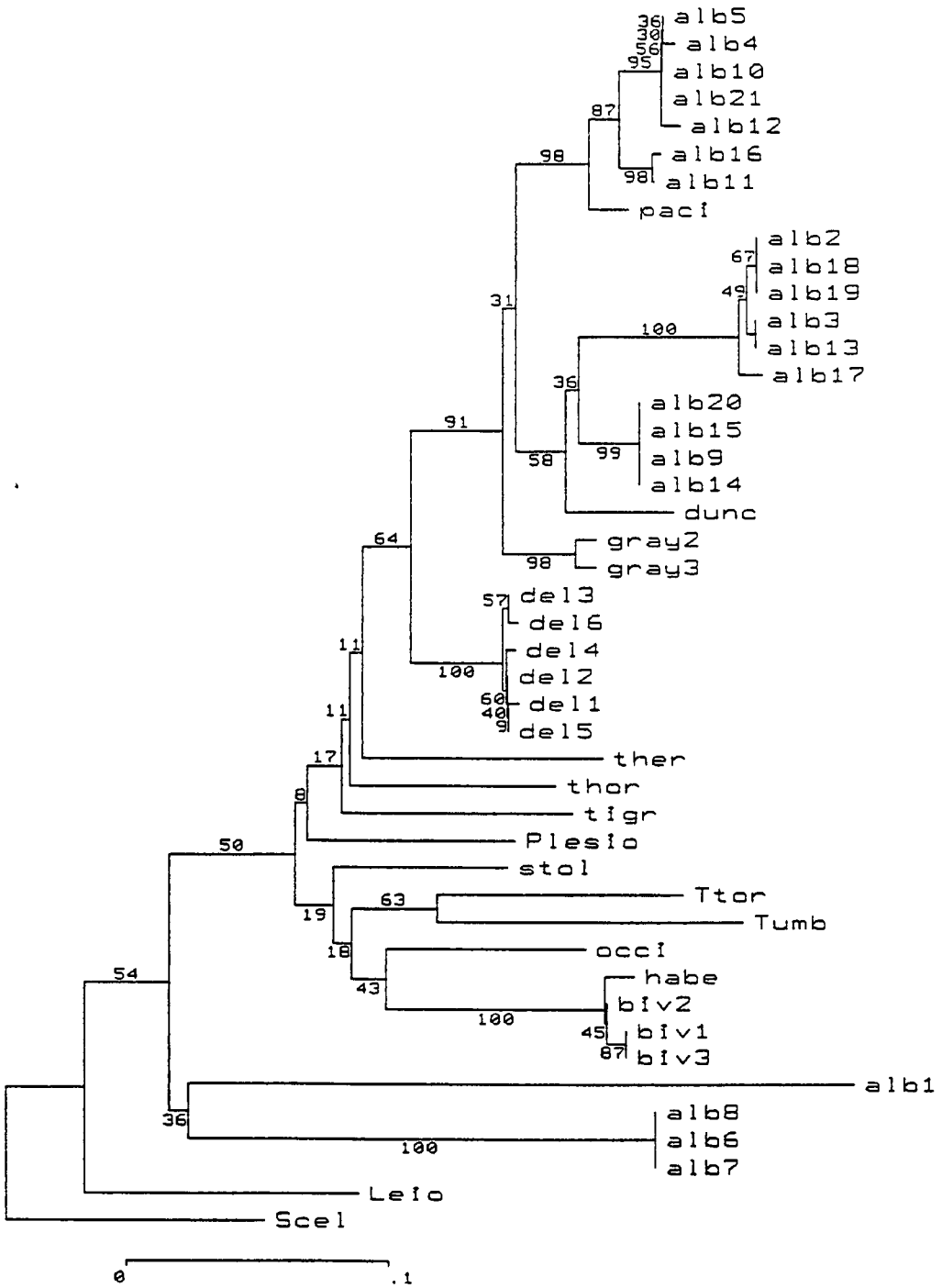
Jukes-Cantor gamma distance (a = 1.0) with bootstrap *p*-values for 2000 replicates



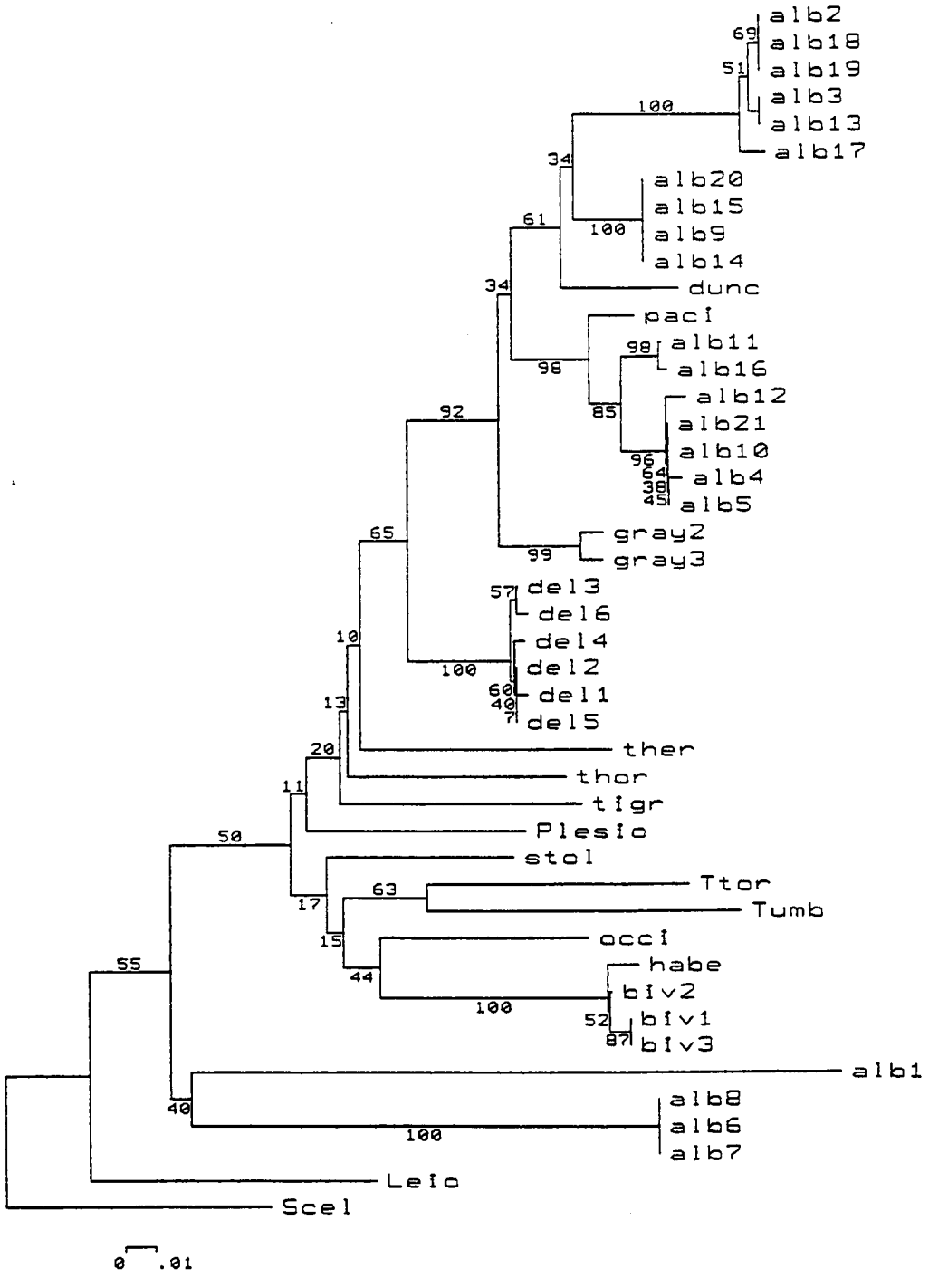
Jukes-Cantor gamma distance (a = 2.0) with bootstrap *p*-values for 2000 replicates



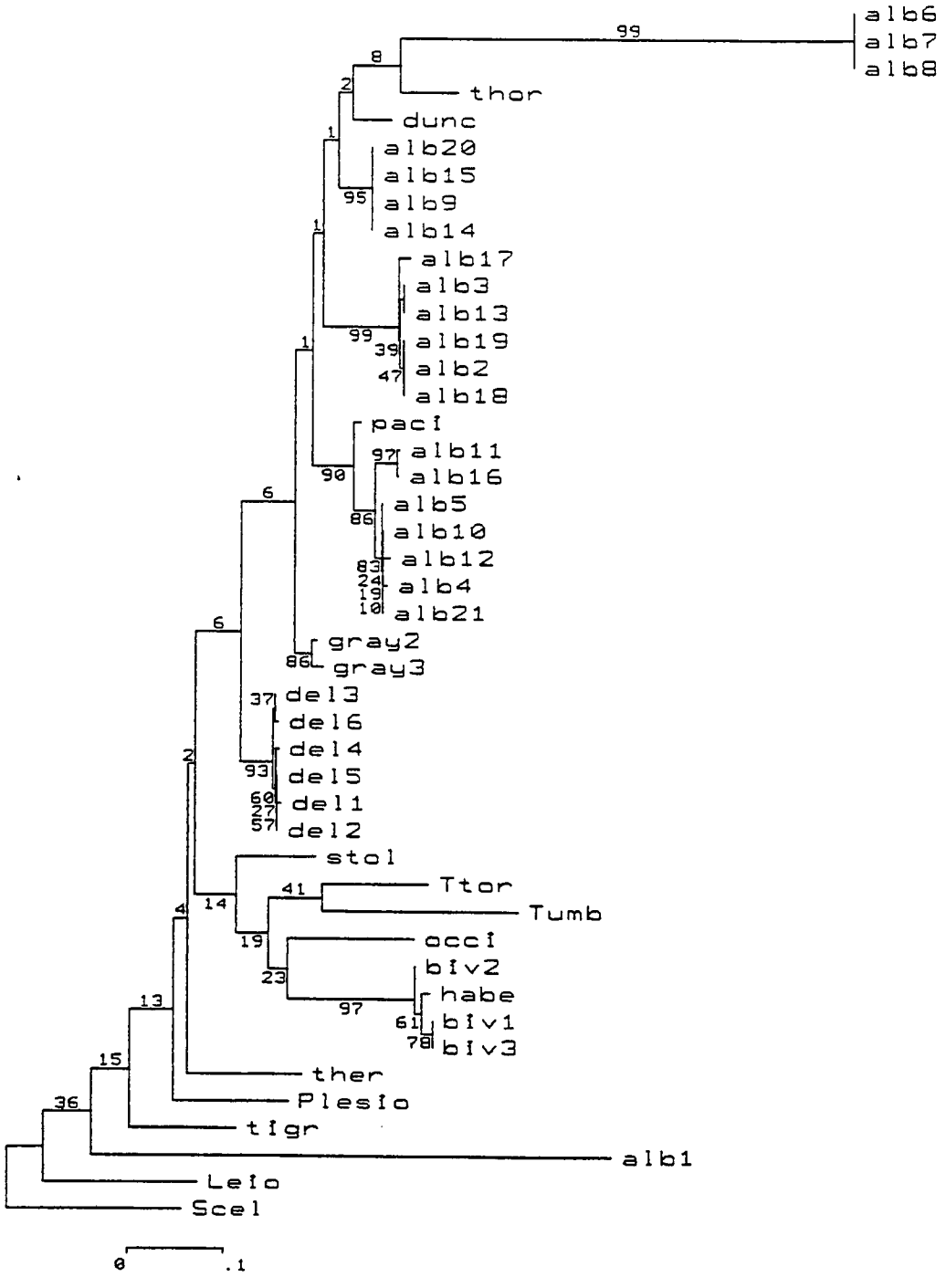
Kimura 2-parameter gamma distance (a = 0.5) with bootstrap *p*-values for 2000 replicates



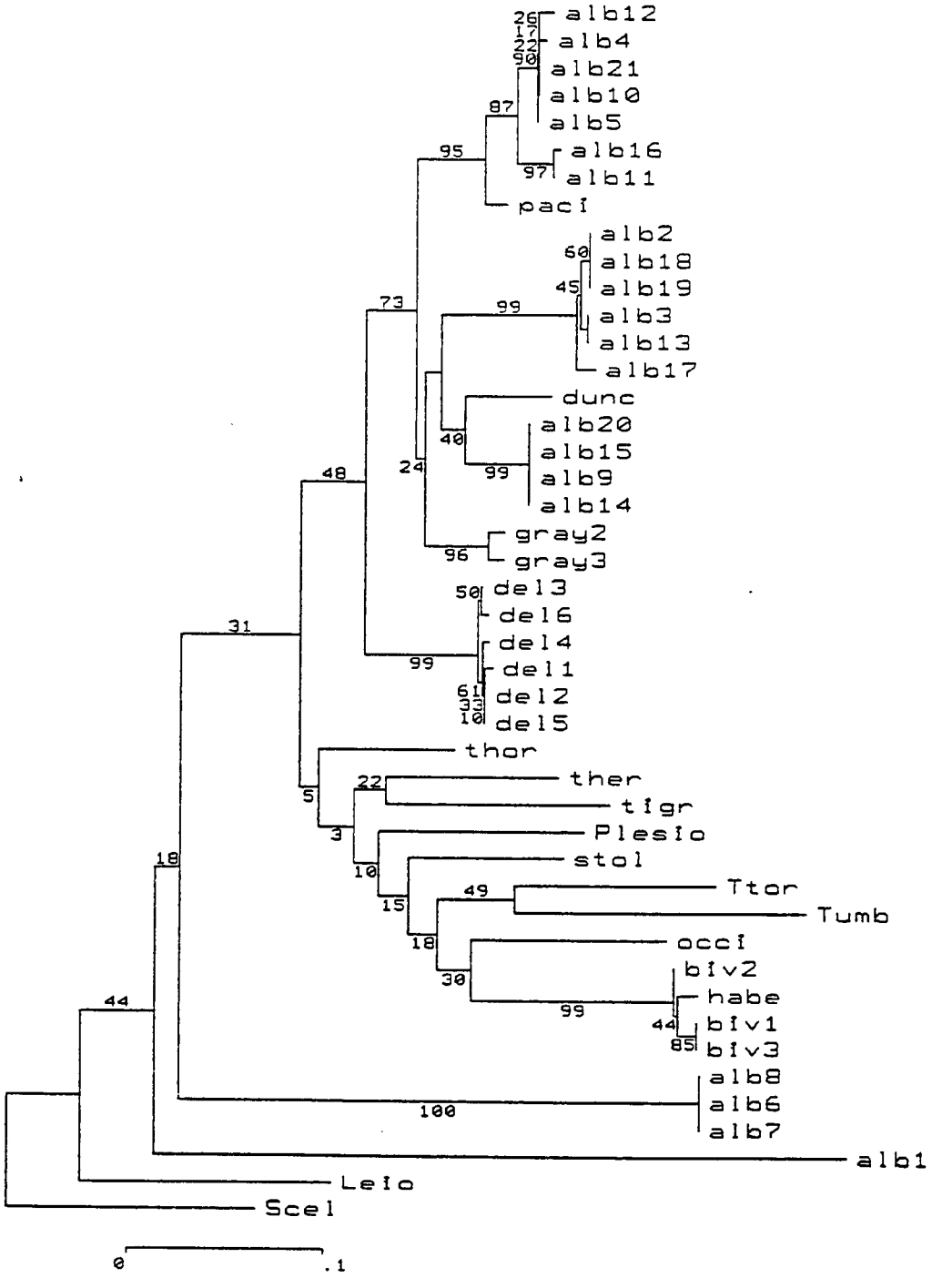
Kimura 2-parameter gamma distance (a = 1.0) with bootstrap *p*-values for 2000 replicates



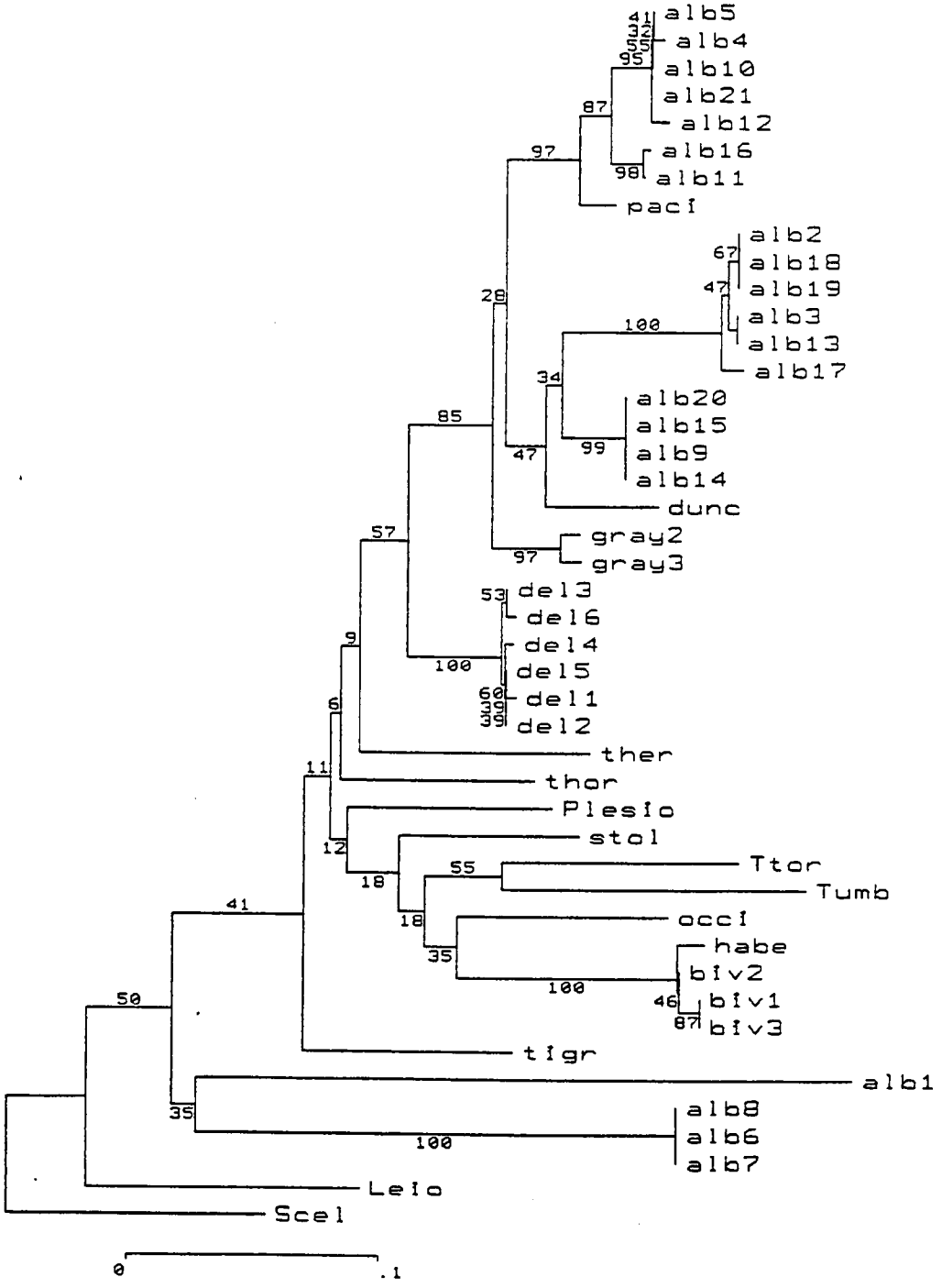
Kimura 2-parameter gamma distance (a = 2.0) with bootstrap *p*-values for 2000 replicates



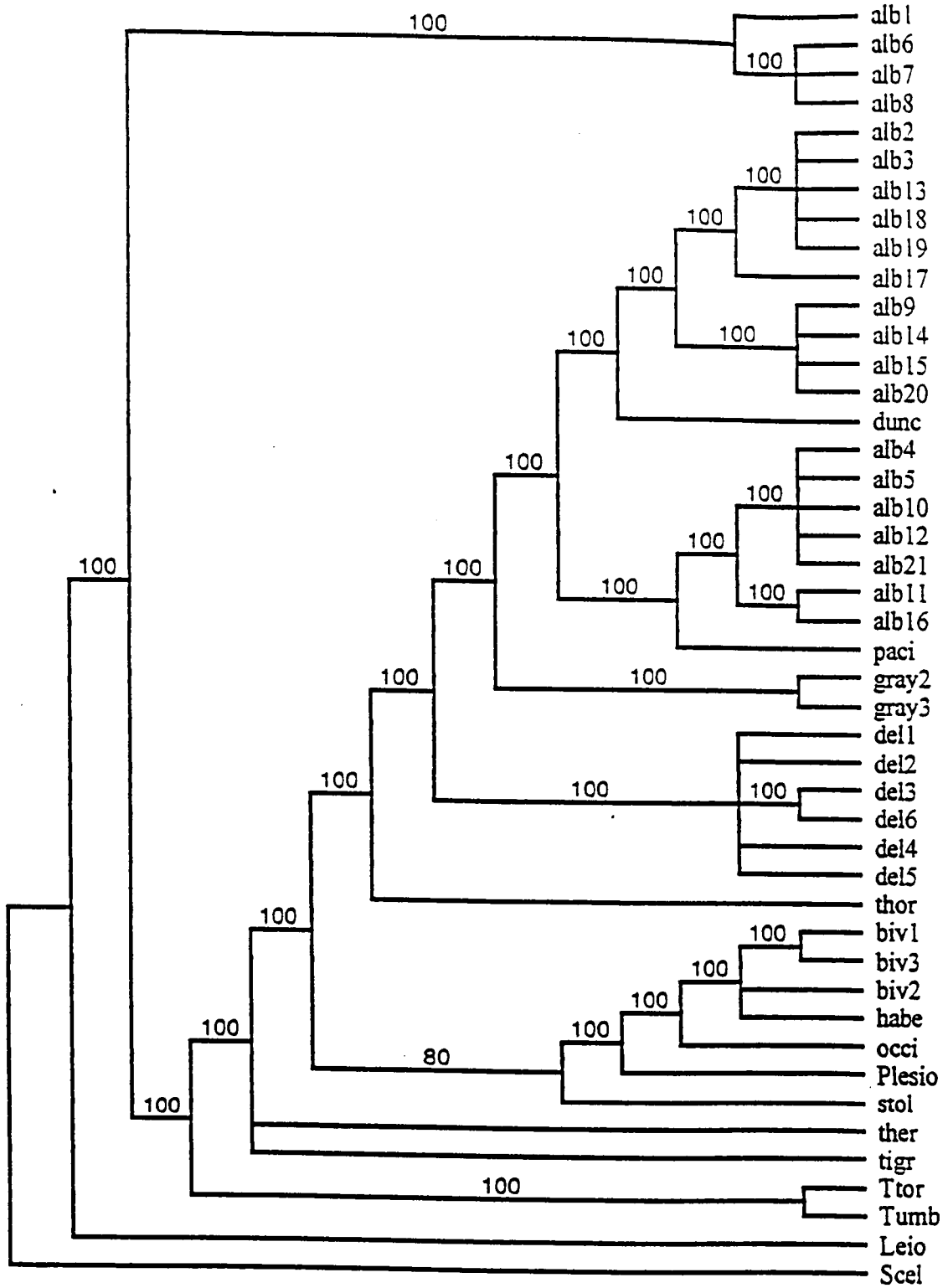
Tamura-Nei gamma distance (a = 0.3) with bootstrap *p*-values for 2000 replicates



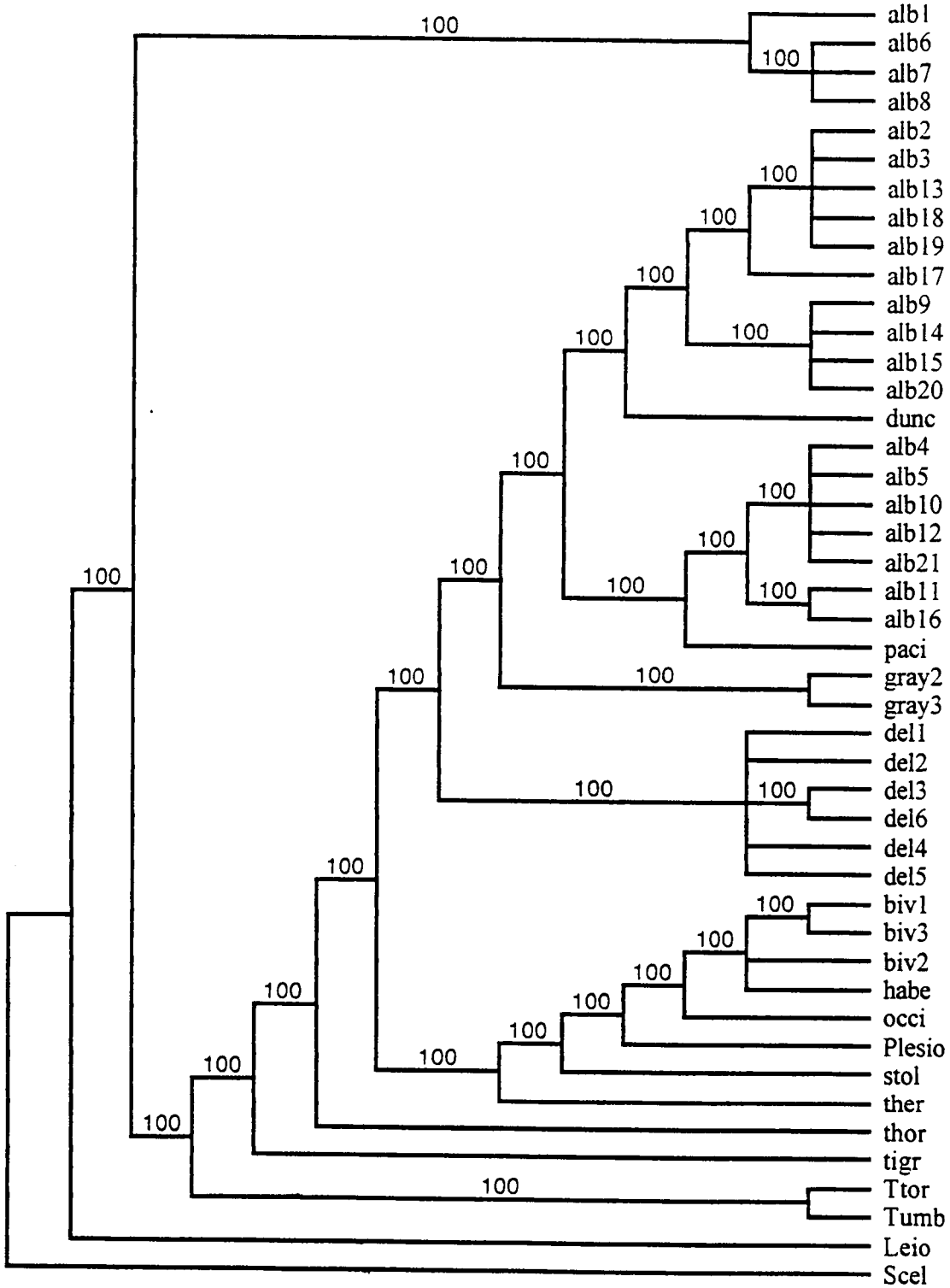
Tamura-Nei gamma distance (a = 0.5) with bootstrap *p*-values for 2000 replicates



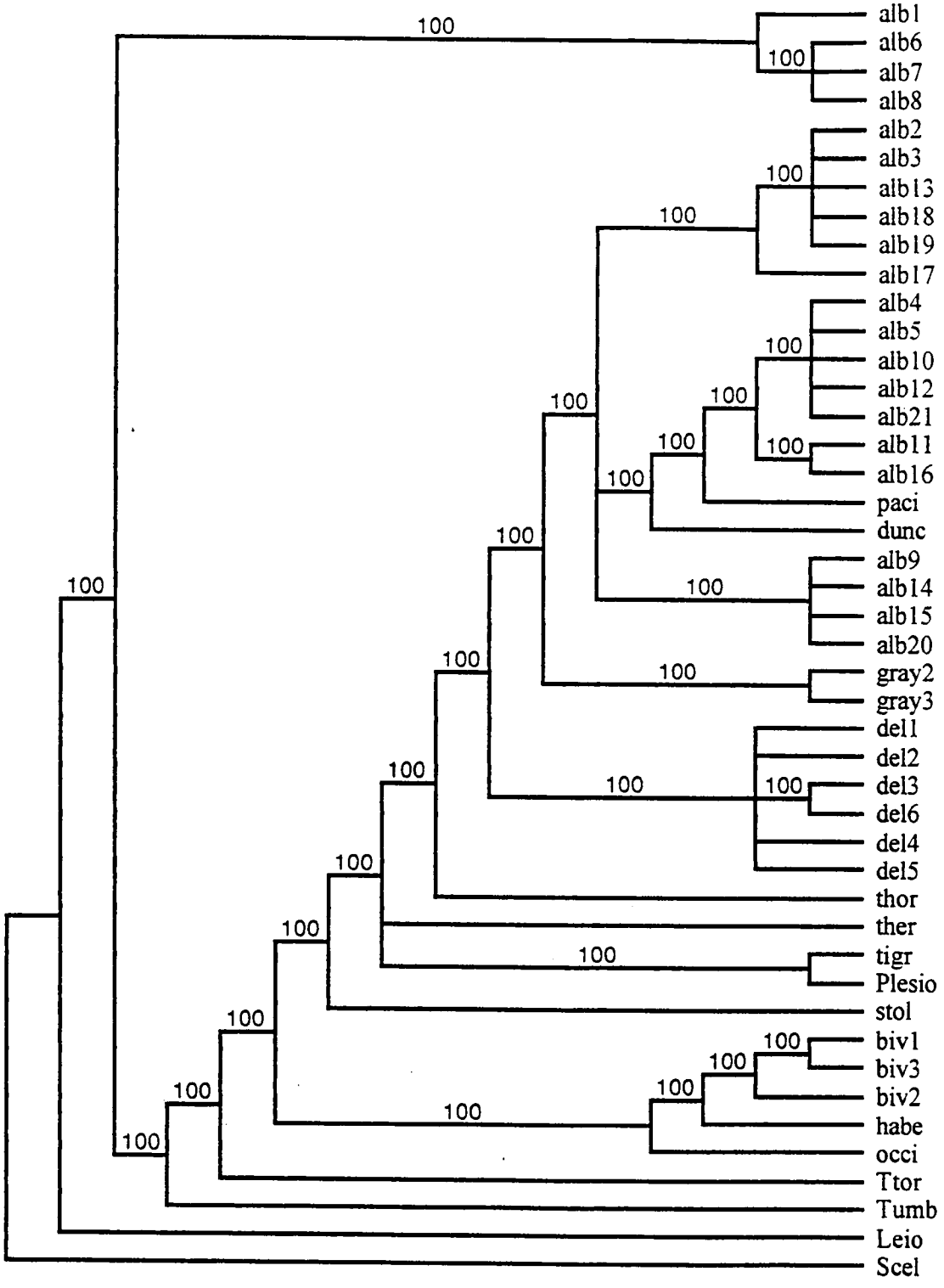
Tamura-Nei gamma distance (a = 1.0) with bootstrap *p*-values for 2000 replicates



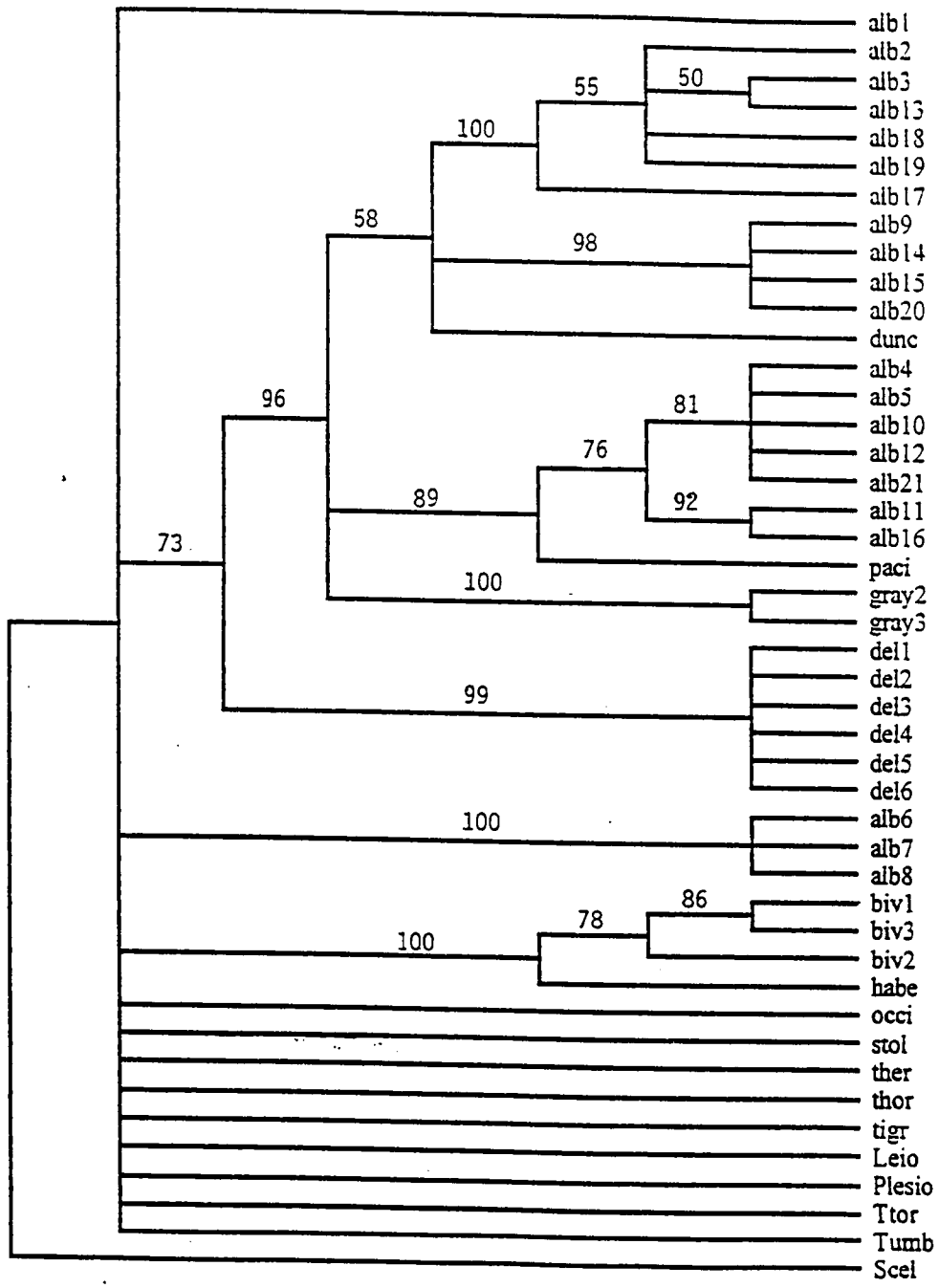
Consensus (75% majority rule) of 179 equally parsimonious trees of 598 steps (1:1 transition/transversion ratio)



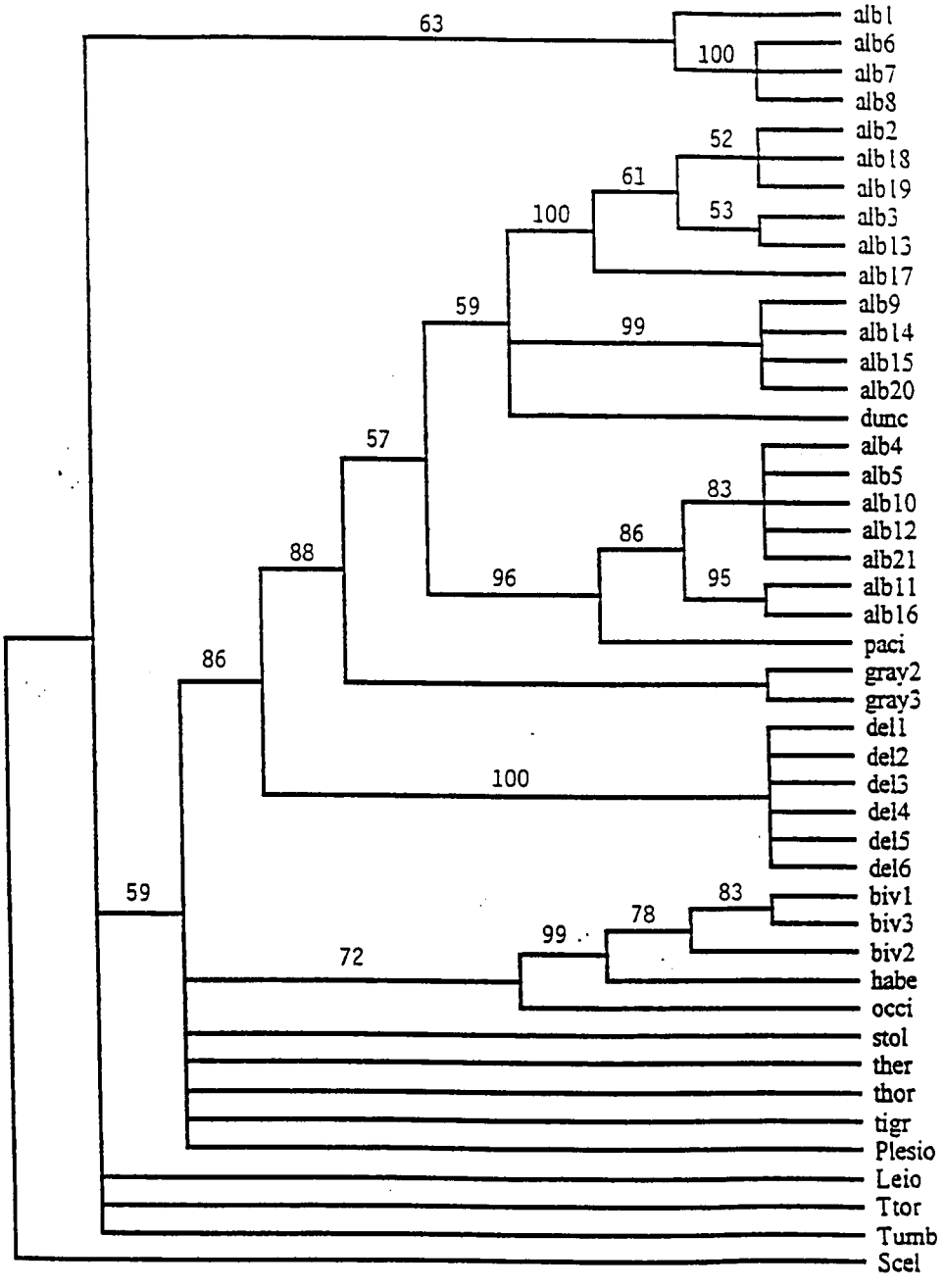
Consensus (75% majority rule) of 71 equally parsimonious trees of 1056 steps (3:1 transition/transversion ratio)



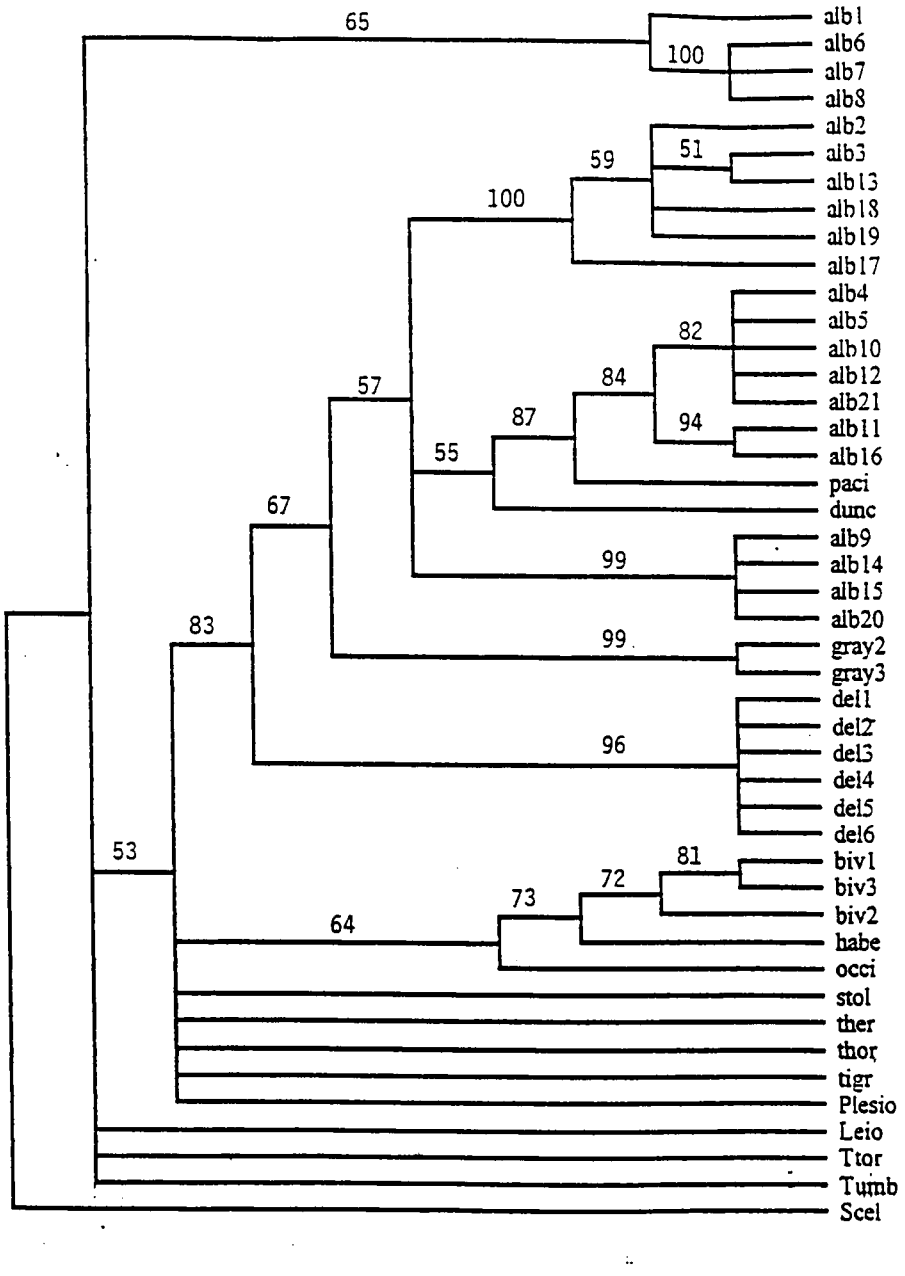
Consensus tree (75% majority rule) of 108 equally parsimonious trees of 2616 steps (10:1 transition/transversion ratio)



Bootstrap values for 100 replicates with nearest-neighbor interchange (1:1 transition/transversion ratio)



Bootstrap values for 100 replicates with nearest-neighbor interchange (3:1 transition/transversion ratio)



Bootstrap values for 100 replicates with nearest-neighbor interchange (10:1 transition/transversion ratio)

VITA

Philip Heise was born in Albuquerque, New Mexico on September 29, 1966. He attended schools in the public system of Albuquerque, New Mexico, the Albuquerque Public School System, where he graduated from Eldorado High School in May, 1984. He entered the University of New Mexico (in Albuquerque) during August of 1984 where in December, 1990 he received the Bachelor of Science in Biology (with distinction). He then began his graduate studies at the Pennsylvania State University (in University Park), where in August of 1995, he received the Master of Science in Biology. In August of 1995, he entered the University of Tennessee, Knoxville, where he received the Doctorate of Philosophy in Ecology and Evolutionary Biology in August, 1998.

He is currently living in Knoxville with his wife, Lauren, and is working as laboratory coordinator in the laboratory of Dr. Edward Schilling, Jr., in the Department of Botany, University of Tennessee, Knoxville.