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Molecular Phylogenetics and Mitochondrial Genomic Evolution in the Chamaeleonidae (Reptilia, Squamata)

Ted Townsend[1] and Allan Larson

Department of Biology, Washington University, St. Louis, Missouri 63130

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A phylogenetic hypothesis for the lizard family Chamaeleonidae is generated from 1503 aligned base positions (883 parsimony-informative) of mitochondrial DNA for specimens representing 59 species (57 ingroup and two outgroup). Sequences are reported for a genomic segment encoding eight transfer RNAs, NADH dehydrogenase component 2 (ND2), and portions of NADH dehydrogenase component 1 (ND1) and cytochrome *c* oxidase subunit 1 (COI). Newly reported genomic rearrangements and duplications support the hypothesis that mitochondrial gene order and content are destabilized by phylogenetic loss of a functional origin for light-strand replication between the genes encoding $tRNA^{Asn}$ and $tRNA^{Cys}$. A novel gene order characterizes all sampled *Brookesia* except *B. nasus*. *Brookesia nasus*, the apparent sister taxon of a clade formed by all other *Brookesia*, has the ancestral gene order but contains a large tandem duplication. An apparently noncoding 220 base pair insertion between the genes encoding ND2 and $tRNA^{Trp}$ is reported for *Bradypodion tavetanum*. Phylogenetic analysis identifies nine clades whose ancestral lineages diverged early in chamaeleonid evolutionary history: (1) *Brookesia* (possibly excluding *B. nasus*), (2) *Chamaeleo* subgenus *Chamaeleo* (excluding *C. namaquensis*), (3) *Chamaeleo* subgenus *Trioceros*, (4) viviparous *Bradypodion*, (5) oviparous *Bradypodion*, (6) genus *Furcifer* (except *F. balteatus*), and (7–9) three distinct clades of *Calumma*. *Chamaeleo namaquensis*, *Brookesia nasus*, *Furcifer balteatus*, *Rhampholeon brevicaudatus*, and *R. spectrum* represent ancient lineages dating to approximately the same time. Multiple independent losses and a possible secondary gain of horns are inferred for *Trioceros*. Viviparity has at least two separate origins in chameleons, one in *Bradypodion* and one in *Trioceros*.

Key Words: **Chamaeleonidae; gene organization; mitochondrial DNA; phylogenetics; Reptilia; Sauria; tRNA.**

INTRODUCTION

Acrodont lizards comprise two large families, Agamidae and Chamaeleonidae. Extensive molecular phylogenetic work on agamids reveals an ancient Gondwanan origin for the group and extensive variation in mitochondrial genomic structure (Macey *et al.*, 2000a, 2000b).

Three structural peculiarities of the mitochondrial genome constitute synapomorphies for acrodont lizards (Macey *et al.*, 1997a): (1) lack of a D-stem in the cysteine transfer RNA ($tRNA^{Cys}$) gene, (2) lack of a recognizable replication origin for the light strand between the genes encoding $tRNA^{Asn}$ and $tRNA^{Cys}$, and (3) a novel ordering of the tRNA genes located between the genes encoding NADH dehydrogenase component 1 (ND1) and NADH dehydrogenase component 2 (ND2). The mutational mechanism of slipped-strand mispairing (reviewed by Levinson and Gutman, 1987) is invoked to explain these anomalies (Macey *et al.*, 1997b, 1998a), with tandem duplication of genes inferred as an intermediate step in the evolution of a novel gene order. Macey *et al.* (1997c) report a phylogenetic association between loss of a recognizable origin for light-strand replication and changes in mitochondrial gene order, which predicts structural instability of the mitochondrial genome in chamaeleonids. Major structural changes are considered unlikely to show homoplasy (Macey *et al.*, 2000a), although parallel evolution has been reported for some mitochondrial genomic rearrangements (Mindell *et al.*, 1998; Bensch and Härlid, 2000). Changes in the secondary structure of tRNAs (Wolstenholme, 1992; Macey *et al.*, 1997b, 2000a) and in mitochondrial gene order (e.g., Sankoff *et al.*, 1992; Macey *et al.*, 1997a, 1997c, 2000b; Boore, 1999; Boore *et al.*, 1995, 1998) have great potential utility as phylogenetic characters.

Monophyly of Chamaeleonidae (=Chamaeleoninae of Frost and Etheridge, 1989) is strongly supported by distinctive morphological features uniquely shared by all members of the group (Klaver, 1981; Hillenius, 1986; Frost and Etheridge, 1989); however, evolutionary relationships *within* Chamaeleonidae are uncer-

[1] To whom correspondence should be addressed at Box 1137, Dept. of Biology, Washington University, St. Louis, MO 63130. Fax: 3143-935-4432. E-mail: townsend@biology.wustl.edu.

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TABLE 1

Summary of Previous Phylogenetic Hypotheses for Chamaeleonidae[a]

	Origins of Chamaeleonidae	Basal Cladogenesis[b]	Generic/subgeneric (Sg.) scheme	Comments
Hillenius (1959, 1963, 1986)	East Africa, post-Africa–Madagascar split	Subgenus *Chamaeleo*	None. Found support for monophyletic (*Brookesia* + *Rhampholeon*)	Proposed multiple trans-oceanic migrations
Klaver (1981); Klaver and Böhme (1986, 1997)	Madagascar, Cretaceous (pre-Africa–Madagascar split)	*Brookesia* (+/− *Rhampholeon*)	Genus *Brookesia* Genus *Rhampholeon* Genus *Calumma* Genus *Furcifer* Genus *Bradypodion* Genus *Chamaeleo* Sg. *Chamaeleo* Sg. *Trioceros*	Malagasy/African distribution result of vicariant event. *Bradypodion* included both S. African dwarf chameleons as well as several east African species.
Hofman *et al.* (1991)	Cretaceous	Did not address	None. Found weak support for many of Klaver and Böhme's (1986) groups	Supported vicariance scenario. No support for (*Brookesia* + *Rhampholeon*) clade or *Bradypodion*.
Rieppel (1987)	Madagascar, post-Africa–Madagascar split	*Brookesia*	None	No support for *Furcifer*, *Calumma*, or the subgenera of *Chamaeleo*. Supported (*Rhampholeon* + *Chamaeleo*) clade.
Rieppel and Crumly (1997)	Madagascar, post-Africa–Madagascar split	*Brookesia*	None	Supported (*Bradypodion* + *Chamaeleo*) clade. No support for monophyly of *Rhampholeon* or *Bradypodion*.

[a] All hypotheses are described using current terminology (Klaver and Böhme, 1997), although the terminology was not yet available when many of the hypotheses were first proposed.
[b] Denoted group is hypothesized to be the sister taxon to a clade containing all other chameleons.

tain. The most recent morphological phylogenetic analysis of Chamaeleonidae is by Klaver and Böhme (1986, 1997), who divide the group into six genera. The African chameleons are divided into three genera largely by hemipenal characters: *Rhampholeon* (African leaf chameleons), *Bradypodion* (including the viviparous South African dwarf chameleons and several oviparous east African species), and *Chamaeleo*, which contains subgenera *Chamaeleo* and *Trioceros*. Malagasy chameleons are classified into three genera: *Brookesia* (Malagasy leaf chameleons), *Calumma*, and *Furcifer*. *Calumma* and *Furcifer* were considered part of *Chamaeleo* prior to Klaver and Böhme (1986).

Morphological (Klaver and Böhme, 1986; Rieppel, 1987; Rieppel and Crumly, 1997) and molecular (Hofman *et al.*, 1991) phylogenetic studies provide hypotheses of relationships among these taxa. Rieppel's (1987) osteological study suggests the pattern (*Brookesia (Bradypodion (Rhampholeon* + *Chamaeleo*))). This work does not support monophyly of Klaver and Böhme's (1986) *Calumma*, *Furcifer*, or their subgenera of *Chamaeleo*. Rieppel (1987) places *Brookesia* as the sister taxon to all other Chamaeleonidae. Rieppel and Crumly (1997) find *Bradypodion* and *Chamaeleo* to be sister taxa. *Brookesia* is still supported as the sister taxon of all other chameleons, but their data contradict the monophyly of both *Rhampholeon* and *Bradypodion*. Hofman *et al.* (1991), using protein electrophoresis and microcomplement fixation, find weak support for several of Klaver's (1981) and Klaver and Böhme's (1986) groups. However, *Chamaeleo* appears paraphyletic with respect to *Brookesia* and *Rhampholeon*. No support is found for Klaver and Böhme's (1986) grouping of oviparous and viviparous *Bradypodion*. Major systematic work on Chamaeleonidae is reviewed in Table 1.

A major historical biogeographic problem is to explain occurrence of chameleons on different Gondwanan land masses, particularly Africa and Madagascar. Hillenius (1959, 1963, 1986) proposes that chameleons originated in east Africa after the separation of Africa and Madagascar and that subsequent diversification involved three dispersals across the Mozambique Channel: (1) an ancestor of the Malagasy species rafted across the channel, with subsequent divergence giving rise to the large Malagasy species, (2) a member of the *Calumma nasuta* group of Madagascar dispersed to Africa, giving rise to *Rhampholeon*, and (3) a *Rhampholeon* dispersed to Madagascar giving rise to *Brookesia*. Alternatively, Klaver (1981) and Klaver and Böhme (1986) hypothesize that Chamaeleonidae originated during the Cretaceous, when Africa and Madagascar still formed a continuous land mass.

Vicariant fragmentation of Africa and Madagascar subsequently separated their chameleon faunas, with no further chameleon dispersal hypothesized between these land masses.

We present the first molecular phylogenetic study of the Chamaeleonidae using mitochondrial DNA sequences, and use this phylogeny to examine mitochondrial genomic structure, morphology, and historical biogeography of chameleons.

MATERIALS AND METHODS

Sampling of Taxa

Monophyly of the acrodonts has been corroborated by morphological (Frost and Etheridge, 1989) and molecular (Macey *et al.*, 1997a) evidence, and some support has been found for reciprocal monophyly of Agamidae and Chamaeleonidae within Acrodonta (Honda *et al.*, 2000, but see Frost and Etheridge, 1989). We therefore used *Uromastyx acanthinurus* and *Leiolepis belliana* as outgroups to 57 ingroup species. Ingroup species were sampled to cover all currently recognized genera and subgenera. We used Klaver and Böhme's (1997) taxonomy for our taxon-sampling decisions because it is the most widely accepted classification and because the genera and subgenera it defines are the least inclusive of the competing hypotheses (i.e., if taxon sampling is adequate under this scheme, it will be appropriate also for competing schemes). Table 2 compares numbers of recognized species with numbers of species sampled for chameleon genera and subgenera. See Appendix for museum numbers (when available) and GenBank accession numbers for specimens included in this study.

TABLE 2

Taxon Sampling

Taxonomic group[a]	No. of recognized species[a]	No. of species sampled
Genus *Brookesia*	24	9
Genus *Furcifer*	19	7
Genus *Calumma*	19	10
Genus *Rhampholeon*	11	2
Genus *Bradypodion*	15	7[b]
Genus *Chamaeleo*		
Subgenus *Chamaeleo*	14	7
Subgenus *Trioceros*	32	15
Total	134	57

[a] Following Klaver and Böhme (1997).

[b] This number includes four taxa designated as subspecies of *Bradypodion pumilum* by Klaver and Böhme (1997).

Laboratory Protocols

For most specimens, genomic DNA was extracted from muscle, liver, or skin using the Qiagen QIAamp tissue kit; others were received as preextracted DNA suspended in water. Genomic DNA was amplified using an initial denaturation at 94°C for 2 min, then a denaturation at 94°C for 35 s, annealing at 45–53°C for 35 s, and extension at 70°C for 150 s with 4 s added to the extension time per cycle, for 30 cycles. Amplified products were purified on 1.8% Nusieve GTG agarose gels and reamplified under similar conditions, except that annealing temperature was consistently 45°C. Reamplified double-stranded products were purified on 2.5% acrylamide gels (Maniatis *et al.*, 1982). Template DNA was passively eluted from acrylamide fragments over at least 3 days using Maniatis elution buffer (Maniatis *et al.*, 1982). Cycle sequencing reactions were run using the Promega fmol DNA sequencing system with an initial denaturation at 95°C for 2 min, then a denaturation at 95°C for 35 s, annealing at 45–61°C for 35 s, and extension at 70°C for 1 min for 30 cycles. Sequencing–reaction fragments were run on Long Ranger sequencing gels for 5–12 h at 38–40°C.

Primers used in this study are listed in Table 3. Both heavy and light strands were sequenced for all specimens across the entire genomic region used for this study. Sequences included approximately 70 base pairs (bp) of the ND1 (subunit 1 of NADH dehydrogenase) gene, the three tRNA genes for glutamine, isoleucine, and methionine ($tRNA^{Gln}$, $tRNA^{Ile}$, and $tRNA^{Met}$, respectively), the entire ND2 (subunit 2 of NADH dehydrogenase) gene, the five tRNA genes for tryptophan, alanine, asparagine, cysteine, and tyrosine ($tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, and $tRNA^{Tyr}$, respectively), the stem-and-loop structure representing a degenerate origin for light-strand replication (O_L), and 30 bp of the COI (subunit I of cytochrome *c* oxidase) gene.

Alignments and Phylogenetic Analyses

All tRNA gene sequences were aligned manually following the structural models of Kumazawa and Nishida (1993). Length-variable loops that could not be aligned confidently were excluded from all analyses. Protein-coding regions were converted to amino acids and aligned manually. Regions encoding amino acid sequences that were not sufficiently conserved to allow confident alignment were excluded from all analyses. All gaps were treated as missing data.

PAUP* (Swofford, 1998) was used to estimate phylogenetic trees by maximum-parsimony and maximum-likelihood criteria. Parsimony analyses were conducted using a heuristic search with 100 random-addition replicates. Bootstrap resampling was applied to assess heuristic support for individual nodes (Felsenstein, 1985b) using 1000 bootstrap replicates with 25 random additions of sequences per replicate. Decay indices (Donoghue *et al.*, 1992; = "branch support" of Bremer, 1994) were calculated as heuristic support measures for all resolved internal branches of the tree. Decay indices were calculated by first con-

TABLE 3

Primers Used in This Study

Human position[a]	Gene	Sequence	Reference
L3914	ND1	5′-GCCCCATTTGACCTCACAGAAGG-3′	Macey *et al.*, 1998b
L4178	ND1	5′-CAACTAATACACCTACTATGAAA-3′	Macey *et al.*, 1997c
L4160	ND1	5′-CGATTCCGATATGACCARCT-3′	Kumazawa and Nishida, 1993
H4419a	$tRNA^{Met}$	5′-GGYATGGGCCCAACTGCTT-3′	Macey *et al.*, 2000b
H4419b	$tRNA^{Met}$	5′-GGTATGAGCCCAATTGCTT-3′	Macey *et al.*, 1997c
L4437	$tRNA^{Met}$	5′-AAGCAGTTGGGCCCATRCC-3′	Macey *et al.*, 1997a
L4882	ND2	5′-TGACAAAAAATTGCNCC-3′	Macey *et al.*, 2000b
H4980	ND2	5′-ATTTTTGCTAGTTGGGTTTGRTT-3′	Macey *et al.*, 1997c
H4995	ND2	5′-GATGAGTATGCTATTARTTTTCG-3′	This study
L1032[b]	ND2	5′-AATACTGCCTGCCCTAGCA-3′	This study
L5556b	$tRNA^{Trp}$	5′-GCCTTCAAAGCCCTAAA-3′	Macey *et al.*, 1997c
L5550	$tRNA^{Trp}$	5′-AACCARAGGCCTTCAAAGC-3′	Macey *et al.*, 2000b
L5549	$tRNA^{Trp}$	5′-AACCRARGRCCTTCAAAG-3′	This study
L5638a	$tRNA^{Ala}$	5′-CTGAATGCAACYCAGAYATTTT-3′	Macey *et al.*, 1997c
L5638b	$tRNA^{Ala}$	5′-CTGAATGCAACTCAGACACTTT-3′	Macey *et al.*, 1997c
H5617a	$tRNA^{Ala}$	5′-AAAATRTCTGRGTTGCATTCAG-3′	Macey *et al.*, 1997c
H5617b	$tRNA^{Ala}$	5′-AAAGTGTCTGAGTTGCATTCAG-3′	Macey *et al.*, 1997c
H5692a	$tRNA^{Asn}$	5′-TTGGGTGTTTAGCTGTTAA-3′	Macey *et al.*, 1997c
H5692b	$tRNA^{Asn}$	5′-TTGGGCRGCTAGCTGTTAA-3′	This study
H5934	COI	5′-AGRGTGCCAATGTCTTTGTGRTT-3′	Macey *et al.*, 1997c

[a] Primer names reflect the position of the 3′ end with respect to the human mitochondrial genome (Anderson *et al.*, 1981). H and L designate primers whose extension produces the heavy and light strands, respectively. Mixed bases are designated by the standard one-letter codes: R = A or G, Y = C or T, N = G, A, C, or T.

[b] L1032 was designed from a region near the 5′ end of the heavy-strand-encoded ND2 gene of *Brookesia nasus*, and is named for its position in the ND2 gene of this particular taxon. This region is extremely variable in length and base composition, and homology is difficult or impossible to determine even within chameleons; therefore, no reference to the human mitochondrial genome is possible.

straining the phylogenetic topologies *not* to contain a particular node of interest using MacClade (Maddison and Maddison, 1992). A heuristic search with 100 random-addition replicates using PAUP* (Swofford, 1998) then found the shortest tree under this constraint. The difference in number of steps between this shortest constrained tree and the overall shortest tree is the decay index for the node in question.

Statistical support for individual nodes in the shortest unconstrained tree was assessed using the nonparametric Wilcoxon signed-ranks test (Felsenstein, 1985a; Templeton 1983). This test is statistically conservative relative to other criteria for assessing branch support (Lee, 2000). The shortest trees compatible with alternative hypotheses tested were obtained by imposing constraints using MacClade (Maddison and Maddison, 1992), and these trees were then compared to the shortest unconstrained tree using the "Tree Scores" option of PAUP* (Swofford, 1998). For regions of the parsimony tree showing numerous short, poorly supported branches, we use the method of Jackman *et al.* (1999) based on four-taxon subsamples to ask whether the hypothesis of a hard polytomy (simultaneous evolutionary branching of numerous lineages) can be statistically rejected by the data.

The model of evolution and all maximum-likelihood parameters were estimated for the full data set using Modeltest (Posada and Crandall, 1998). Maximum-likelihood analyses were conducted using the heuristic search option of PAUP* (Swofford, 1998) and a neighbor-joining tree as the starting tree for branch swapping. Statistical support was assessed for each branch in the maximum-likelihood tree by comparing the maximum-likelihood tree to the tree obtained by collapsing the branch to form a polytomy, and applying a likelihood-ratio test with one degree of freedom and a significance level of 0.05. Alternative phylogenetic hypotheses were tested using the likelihood-based test of Shimodaira and Hasegawa (1999; see also Goldman *et al.*, 2000) as implemented in PAUP* (Swofford, 1998). The methodology for producing these alternative topologies was essentially the same as that used for the parsimony-based Templeton tests except that the optimality criterion was likelihood.

RESULTS

Sequence Alignments and Homology

Sequences correspond to positions 4419 to 5933 on the human mitochondrial genome (Anderson *et al.*, 1981). The complete, aligned data file (with excluded positions marked as such) is available at http://www.biology.wustl.edu/≈townsend. Alignment of the protein-coding ND1 gene is unambiguous because no length variation occurs among ingroup species for the

segment reported, whereas the sequences for *Leiolepis* and *Uromastyx* contain nine and 12 more bp, respectively, at the 3′ end of the gene (all references to gene polarity are to the reported light-strand sequence, regardless of which strand is the sense strand for a particular gene). These positions (73–84 in the aligned data file) are excluded from phylogenetic analyses. The sequence for *Brookesia nasus* has a 3-bp deletion corresponding to codon three of ND2 (positions 334–336). In this same gene, the *Bradypodion tavetanum* sequence has a 6-bp deletion corresponding to codons 146 and 147 (positions 763–768), and the *B. tavetanum* and *B. fischeri* sequences share a probable synapomorphy in a 3-bp insertion at codon 339 (positions 1342–1345). Additionally, the *Furcifer campani* sequence has a 3-bp deletion at codon 327 (positions 1306–1308). The 3′ end of the ND2 gene is extremely variable among acrodonts, making determination of homology between outgroup and ingroup sequences (even when amino acid translations were used) impossible for the last approximately 30 codons of the gene. Therefore, these positions, as well as the stop codon and any noncoding sequence preceding the $tRNA^{Trp}$ gene, are excluded from all phylogenetic analyses (positions 1282–1393). For the 30 bp of COI sequence included in this study, the only length variation is a 3-bp deletion at codon three (positions 1879–1881) in *Chamaeleo namaquensis*.

Several loop regions of tRNA genes are excluded from phylogenetic analyses because length variation makes positional homologies ambiguous. T-loops are excluded for $tRNA^{Ile}$ (positions 226–233), $tRNA^{Met}$ (positions 307–313), $tRNA^{Trp}$ (positions 1447–1454), $tRNA^{Ala}$ (positions 1493–1500), $tRNA^{Cys}$ (positions 1685–1692), and $tRNA^{Tyr}$ (positions 1812–1818). Variable-loop sequence is excluded for $tRNA^{Asn}$ (positions 1591–1595), $tRNA^{Cys}$ (positions 1698–1703), and $tRNA^{Tyr}$ (positions 1824–1828). The D-loops of $tRNA^{Gln}$ (positions 135–142), $tRNA^{Ile}$ (positions 187–194), $tRNA^{Met}$ (positions 268–274), $tRNA^{Trp}$ (positions 1407–1415), and $tRNA^{Tyr}$ (positions 1851–1857) also are excluded. The D-arm replacement loop (positions 1721–1732) in the $tRNA^{Cys}$ gene (Macey *et al.*, 1997b) is excluded because of ambiguous alignment. Noncoding sequences between $tRNA^{Gln}$ and $tRNA^{Ile}$ (positions 156–173), between $tRNA^{Ile}$ and $tRNA^{Met}$ (positions 247–254), between $tRNA^{Met}$ and *ND2* (positions 326–327), between $tRNA^{Trp}$ and $tRNA^{Ala}$ (positions 1467–1479), between $tRNA^{Ala}$ and $tRNA^{Asn}$ (positions 1551–1565), and between $tRNA^{Cys}$ and $tRNA^{Tyr}$ (positions 1740–1798) cannot be confidently aligned, nor can the stem-and-loop structure representing the degenerate O_L. All are therefore excluded from phylogenetic analyses.

Procedures for accommodating mitochondrial genomic duplications and rearrangements in the phylogenetic analyses are described below.

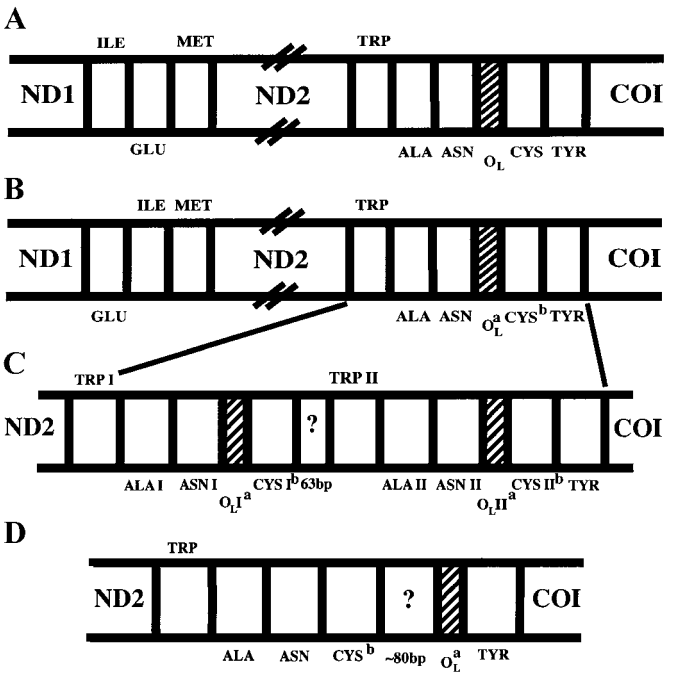

FIG. 1. Gene order variations for the mtDNA region used in this study. ND1, ND2 = genes for subunits 1 and 2 of NADH dehydrogenase, O_L = origin of light-strand replication, COI = gene for subunit one of cytochrome *c* oxidase. tRNA-encoding genes are denoted by the standard three-letter abbreviations of their respective amino acids. (A) Typical vertebrate gene order. (B) Typical Chamaeleonidae gene order. As in other acrodonts, the $tRNA^{Gln}$ gene precedes the $tRNA^{Ile}$ gene, the O_L is presumably nonfunctional, and $tRNA^{Cys}$ has a D-arm replacement loop. (C) Gene order in *Brookesia nasus*. Four of the five tRNA genes between *ND2* and *COI*, as well as the O_L, have been tandemly duplicated, and an apparently noncoding sequence 63 bp in length occurs between the duplicated fragments. (D) Gene order in all other sampled *Brookesia*. No recognizable duplications are found, but the O_L is now situated between $tRNA^{Cys}$ and $tRNA^{Tyr}$, and is immediately preceded by an apparently noncoding sequence roughly 80 bp in length. [a] Presumably nonfunctional; [b] D-stem absent, D-arm replacement loop present instead.

Mitochondrial Genomic Structure

The typical vertebrate gene order for the mitochondrial region used in this study is *ND1*, $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, *ND2*, $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, O_L, $tRNA^{Cys}$, $tRNA^{Tyr}$, and *COI* (Fig. 1A; Macey *et al.*, 1997c). In Acrodonta, a rearrangement switches positions of the $tRNA^{Gln}$ and $tRNA^{Ile}$ genes (Macey *et al.*, 1997a,c, 2000a; Fig. 1B). In *Brookesia nasus*, a large tandem duplication of four tRNA genes and the degenerate O_L occurs between the ND2 and COI genes (Fig. 1C). An apparently noncoding segment 63 bp in length lies between the duplicated regions. This fragment contains no recognized start codon or discernable homology to any tRNA genes used in this study, and BLAST searches of GenBank find no match to it. The duplicated regions are nearly identical in sequence, with the only differences occurring between the $tRNA^{Ala}$ genes. The first copy of this gene ($tRNA^{Ala}$ *I*) has a substitution at one site in the D-stem

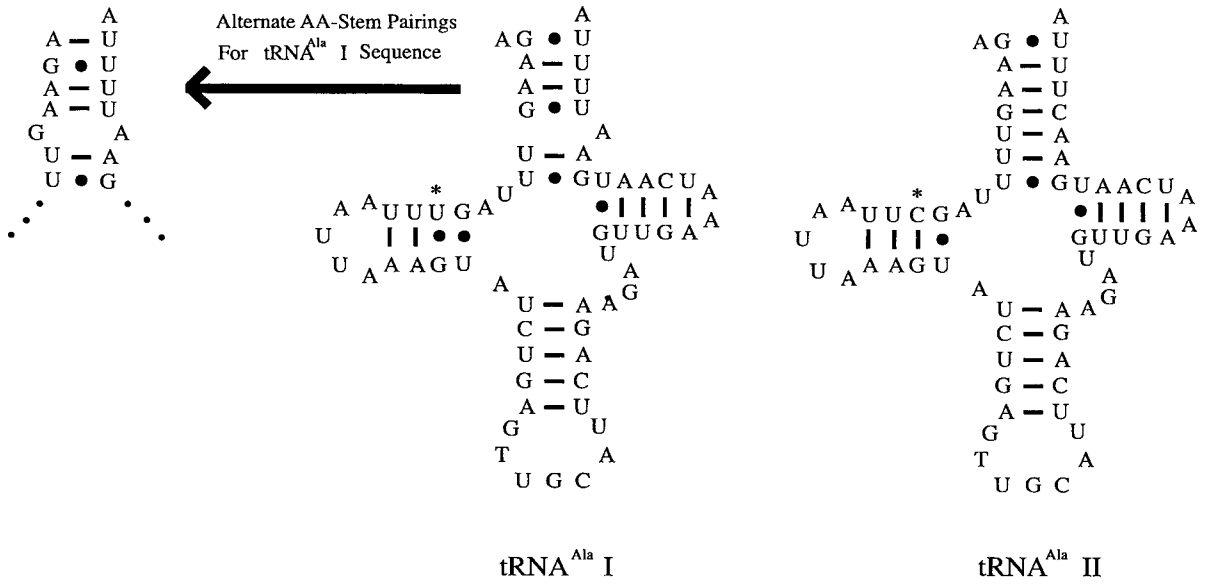

FIG. 2. Nucleotide sequence and secondary structure of both copies of the light-strand encoded tRNA^{Ala} in *Brookesia nasus*. The first copy of the tRNA^{Ala} gene (labelled ALA I in Fig. 1) codes for tRNA^{Ala} I, which has an apparent substitution in the D-stem. Sequences from all other chameleons used in this study, as well as the other copy of the tRNA^{Ala} gene (ALA II in Fig. 1) from this same specimen, code for a tRNA having a cytosine at position 10 (from the 5′ end of the tRNA, and denoted by an asterisk), while tRNA^{Ala} I contains a uracil in this position. In addition, a one-base deletion appears to have occurred in the AA stem of tRNA^{Ala} I, and the two tRNA copies differ at one other site in the AA stem. The two most likely secondary structures for tRNA^{Ala} I are illustrated in the middle and left side of the figure. Neither option produces the stem stability present in tRNA^{Ala} II (illustrated at right), and homology is uncertain.

(Fig. 2). This substitution reduces the number of full bonds in the D-stem from three to two, a condition shared by only one other species in this study, *Calumma nasuta*. The other differences occur in the AA stem, where a base substitution and an apparent deletion in *tRNA^{Ala} I* lessen both the strength and the number of bonds within this stem relative to that of the second copy (*tRNA^{Ala} II*), and make positional homologies uncertain (Fig. 2). We therefore exclude the entire first copy of the duplicated region and the 63-bp noncoding region from all phylogenetic analyses.

All remaining species of *Brookesia* sampled lack the large tandem duplication seen in *B. nasus*. Instead, they have a gene rearrangement in the same region such that the tRNA^{Cys} gene and the degenerate O_L have switched positions (Fig. 1D). In addition, a roughly 80-bp insertion precedes the O_L. The inserted fragment appears noncoding by the same criteria discussed above for the 63-bp segment in *B. nasus*. To align homologous sequences for phylogenetic analyses, the gene order among members of this clade is returned to the order found in other chameleons, and the noncoding fragment preceding the O_L is excluded.

An unalignable 220-bp fragment was found in *Bradypodion tavetanum* between apparently normal copies of the ND2 and tRNA^{Trp} genes. This sequence has no matches on BLAST searches of GenBank files and has no apparent homology with any part of the mitochondrial genome used for this study. The second and third positions of the stop codon for ND2 typically overlap the beginning of the tRNA^{Trp} gene. In the two other species from this study in which this overlap does *not* occur (*Brookesia nasus* and *B. peyrierasi*), the ND2 stop codon is completed in a short intervening sequence not more than 16 bases long. The first two bases of the 220-base fragment in *Bradypodion tavetanum* also complete a functional stop codon (TAA). The intervening fragment contains no recognized start codon, and five stop codons result from attempts to group the entire fragment into codons. All 220 bp are excluded from phylogenetic analyses.

Phylogenetic Analyses

A single shortest tree results from parsimony analysis of the 1503 aligned base positions (883 parsimony informative; see Table 4) for all 59 species (Fig. 3). Substitutional saturation is evident only for silent transitions in plots of maximum-likelihood distances against percentage sequence differences between haplotypes (not shown). Parsimony analysis conducted with silent transitions removed increases support for some nodes in the tree (Fig. 3) but many other nodes, especially more nested ones, lose support. Useful phylogenetic information, especially with regard to more recent branching events, appears to occur even in saturated positions, a view corroborated by recent work (Broughton *et al.*, 2000). Further analyses therefore use the full data set.

The favored model of evolution for maximum-likelihood analysis is a general time reversible model with rate variation among sites and a proportion of invariable sites. Base frequencies as estimated by Modeltest are 0.4097 (A), 0.2898 (C), 0.0758 (G), and 0.2247 (T), and relative substitution rates are 0.6486 (A–C), 6.0527 (A–G), 0.7838 (A–T), 0.3558 (C–G), 6.0527 (C–T), and 1.0 (G–T). The proportion of invariable sites and the gamma-distribution shape parameter are estimated to be 0.2261 and 0.7460, respectively.

Maximum-likelihood analysis yields a tree similar in topology (Fig. 4) to the parsimony tree, with all differences confined to nodes poorly supported in the parsimony analysis. All but seven nodes are statistically supported by likelihood-ratio tests comparing dichotomous branching to the alternative hypothesis of a polytomy (Fig. 4).

Several nodes are well supported by all analyses. Monophyly of chameleons as a taxonomic family to the exclusion of agamid outgroups is clear (bootstrap 100%, decay index 60). Within Chamaeleonidae, a clade containing all sampled *Brookesia* with the exception of *B. nasus* is supported by heuristic and statistical measures (Figs. 3 and 4; Table 5). *Brookesia nasus* is weakly supported as the sister taxon of this group by heuristic measures (Fig. 3) and a log-likelihood test (Fig. 4); however, nonmonophyly of *Brookesia* cannot be rejected by the other statistical tests (Table 5).

Klaver and Böhme's (1986) subgenus *Chamaeleo* (the *C. chamaeleon* group of Hillenius, 1959) is represented here by *C. dilepis, C. quilensis, C. gracilis, C. africanus, C. chamaeleon, C. calyptratus*, and *C. namaquensis*. All sampled species of this group except *C. namaquensis* form a strongly supported clade (Figs. 3 and 4; Table 5). Although there is no support for placing *C. namaquensis* in this group, its inclusion is not statistically rejected (Table 5). Two well-supported subclades of subgenus *Chamaeleo* are identified (Figs. 3 and 4; Table 5).

Klaver and Böhme's (1986) subgenus *Trioceros* is represented here by *Chamaeleo deremensis, C. jacksonii, C. hoehnelii, C. rudis, C. sternfeldi, C. ellioti, C. fuelleborni, C. johnstoni, C. melleri, C. quadricornis, C. weidersheimi, C. pfefferi, C. cristatus, C. montium*, and *C. feae*. This clade receives moderate heuristic support in the parsimony analysis (Fig. 3) and statistical support from likelihood analyses (Fig. 4; Table 5). Within subgenus *Trioceros, C. jacksonii, C. hoehnelii, C. rudis, C. sternfeldi*, and *C. fuelleborni* form one of the two viviparous clades of chameleons (Necas, 1999). This clade received high heuristic (Fig. 3) and statistical support from likelihood-ratio tests (Fig. 4). *Chamaeleo johnstoni* and *C. melleri* form a clade, as does the *C. cristatus* group (*C. quadricornis, C. weidersheimi, C. pfefferi, C. cristatus, C. montium*, and *C. feae*, Klaver and Böhme 1992), which itself contains two well-supported clades (Fig. 3). Monophyly of the *C. cristatus* group is supported by all statistical criteria (Table 5).

Our results neither support nor statistically reject monophyly of *Bradypodion* (Figs. 3 and 4; Table 5), which forms two major subclades. One major subclade contains the viviparous dwarf chameleons of South Africa (Necas, 1999), represented here by *B. occidentale, B. ventrale, B. transvaalense*, and *B. pumilum*. This grouping is strongly supported by heuristic and statistical criteria (Figs. 3 and 4; Table 5). Within this clade, *B. occidentale, B. ventrale*, and *B. transvaalense* form a clade with support from heuristic measures (Fig. 3) and the likelihood-ratio test (Fig. 4), although other statistical tests are not quite significant (Table 5); *B. pumilum* is the sister taxon of this group (Figs. 3 and 4). The second major clade of *Bradypodion* contains the three oviparous, east African species *Bradypodion adolffriderici, B. fischeri*, and *B. tavetanum*, but this grouping is supported only by the likelihood-ratio test (Fig. 4).

Klaver and Böhme's (1986) Malagasy genus *Furcifer* is represented here by *F. labordi, F. lateralis, F. belalandaensis, F. oustaleti, F. verrucosus, F. campani*, and *F. balteatus*. All of these species, except *F. balteatus*, form a clade strongly supported by heuristic and statistical measures (Figs. 3 and 4; Table 5). Although *Furcifer* is not monophyletic in the parsimony tree, the shortest tree constrained to maintain monophyly of *Furcifer* requires only four extra steps and is not significantly longer (Table 5). The maximum-likelihood analysis places *F. balteatus* as the sister taxon of all other *Furcifer* (Fig. 4).

Three distinct clades of the Malagasy genus *Calumma* appear strongly supported by parsimony analysis but do not collectively form a monophyletic group (Fig. 3): (1) *C. globifer, C. parsonii*, and *C. oshaughnessyi*; (2) *C. gastrotaenia* and *C. furcifer*; and (3) *C. brevicornis, C. cucullata, C. hilleniusi, C. nasuta*, and *C. boettgeri*. Statistical tests based on parsimony do not reject monophyly of *Calumma* (Table 5), however, and maximum-likelihood analysis supports grouping clades 2 and 3 (Fig. 4).

Monophyly of the African leaf chameleons, genus *Rhampholeon*, represented here by *R. spectrum* and *R. brevicaudatus*, is neither supported nor statistically rejected (Figs. 3 and 4; Table 5). Haplotypes from these species are highly divergent from each other and from other chameleons (Table 6). Parsimony and likelihood analyses agree in placing each of these species as relatively basal branches, although their exact phylogenetic position differs between these analyses (Figs. 3 and 4).

Many of the most basal nodes in the tree are not well supported (Figs. 3 and 4), and their support does not change significantly when silent substitutions are excluded. Fifteen separate lineages appear to originate nearly simultaneously early in the history of the

TABLE 4

Distribution of Phylogenetically Informative and Variable Sites[a]

	ND1 Codon positions			ND2 Codon positions			COI Codon positions		
	1	2	3	1	2	3	1	2	3
Informative	15	7	23	194	116	305	4	3	7
Variable	17	8	24	233	164	317	8	6	9

	$tRNA^{Glu}$		$tRNA^{Ile}$		$tRNA^{Met}$		$tRNA^{Trp}$	
	Stem	Loop	Stem	Loop	Stem	Loop	Stem	Loop
Informative	22	7	19	5	18	3	18	3
Variable	26	11	26	8	27	6	27	3

	$tRNA^{Ala}$		$tRNA^{Asn}$		$tRNA^{Cys}$		$tRNA^{Tyr}$	
	Stem	Loop	Stem	Loop	Stem	Loop	Stem	Loop
Informative	23	3	20	5	31	1	29	2
Variable	33	5	33	10	31	1	34	4

Total	Protein-coding genes codon positions			tRNA		
	1	2	3	Stem	Loop	Complete analyzed sequence
Informative	213	126	335	180	29	883
Variable	258	178	350	237	48	1071

[a] Numbers for tRNAs represent only those positions included in the analysis.

Chamaeleonidae (Fig. 3). The hypothesis that these 15 lineages form a hard polytomy is rejected using the methodology of Jackman *et al.* (1999). Approximately half (50–51%) of the four-taxon subsamples show significant phylogenetic structure, greatly exceeding the 5% critical value. The likelihood analysis (Fig. 4) is consistent with this result in supporting dichotomous branching among some of these lineages while failing statistically to resolve a large polytomy near the base of the tree.

DISCUSSION

Mitochondrial Genomic Structure and Evolution

Our results add 56 species to the acrodont lizards known to share three major synapomorphies in mitochondrial genomic structure: loss of a functional O_L between the $tRNA^{Cys}$ and $tRNA^{Ala}$ genes, a rearrangement of the $tRNA^{Gln}$ and $tRNA^{Ile}$ genes, and formation of a D-arm replacement loop in the $tRNA^{Cys}$ gene. Phylogenetic stability of these characteristics in the Acrodonta is now supported by results from 127 species with no known exceptions.

Furthermore, our results confirm the prediction by Macey *et al.* (1997c) that loss of a functional O_L between the $tRNA^{Asn}$ and $tRNA^{Cys}$ genes increases the incidence of mitochondrial genomic structural changes caused by slipped-strand mispairing. The large tandem duplication observed in the mitochondrial genome of *B. nasus* is such a change; duplicate copies differ by only two base substitutions, indicating a recent evolutionary origin of this duplication. *Brookesia lolontany*, a close relative of *B. nasus* (Raxworthy and Nussbaum, 1995), should be examined to clarify the evolutionary timing of this genomic duplication. This duplication seems unrelated to the genomic rearrangement that forms a synapomorphy for all other sampled *Brookesia*. The duplication in *B. nasus* is too recent, and at least three separate deletions would be required to convert the duplicated segment of *B. nasus* to the gene order characteristic of other *Brookesia*. The most parsimonious explanation of the genomic rearrangement observed in all *Brookesia* except *B. nasus* involves slipped-strand duplication of the segment O_L-$tRNA^{Cys}$-$tRNA^{Tyr}$ followed by separate deletions of the first O_L and the second $tRNA^{Cys}$ gene, and mutational formation of a pseudogene from the first $tRNA^{Tyr}$ gene.

The 220-bp fragment that follows the ND2 gene in *Bradypodion tavetanum* probably results from slipped-strand duplication of the $tRNA^{Trp}$-$tRNA^{Ala}$-$tRNA^{Asn}$ gene region, followed by mutations producing pseudogenes from the adjacent first copies of the three genes. This sequence appears to be noncoding and has no recognizable homology with either the ND2 gene or the tRNA genes that follow it, but it has the length ex-

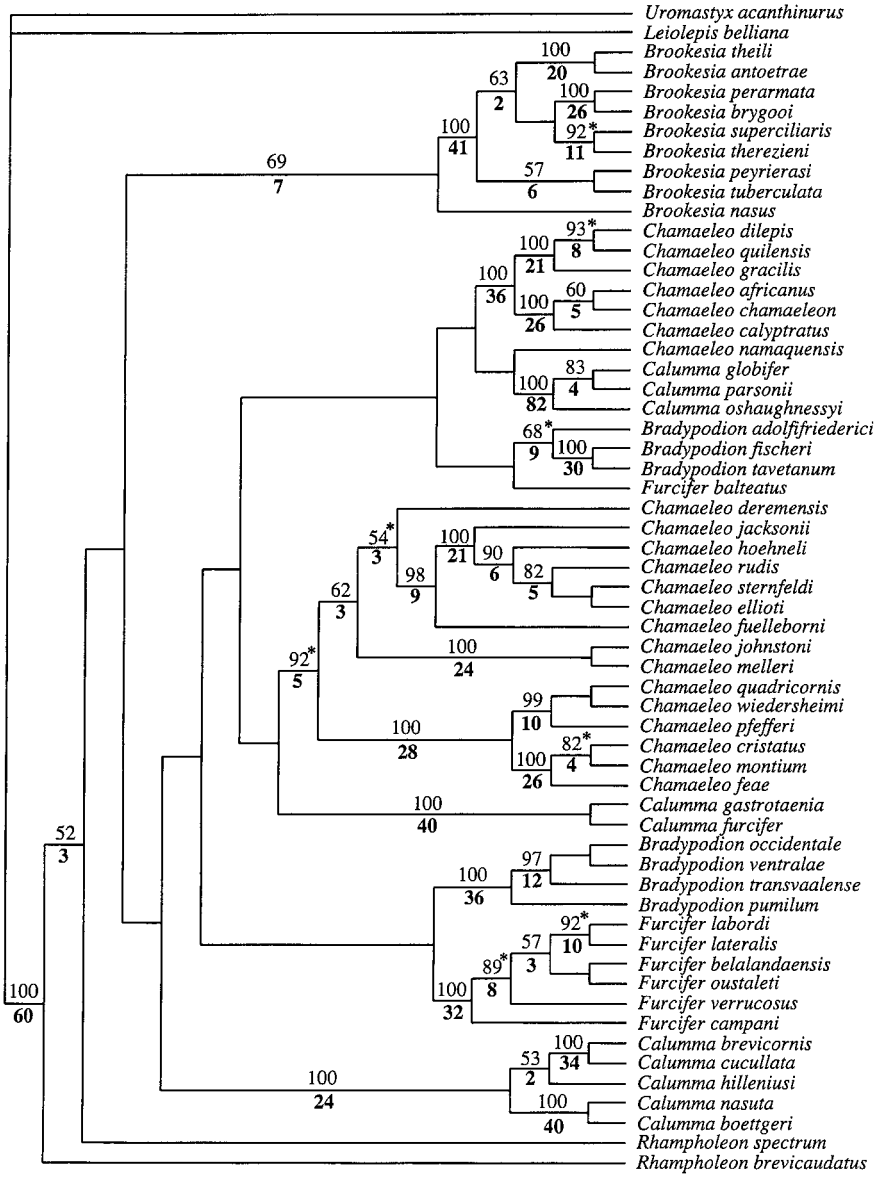

FIG. 3. Single most-parsimonious tree produced from analysis of the 1503 aligned (883 phylogenetically informative) positions. Bootstrap values are shown above branches and decay indices in bold below branches. Nodes for which bootstrap support is increased when silent transitions are removed are denoted with an asterisk.

pected for duplication of three adjacent tRNA genes. Many east African *Bradypodion* species remain to be sampled, which permits a test of the hypothesis that the 220-bp fragment observed in *B. tavetanum* is a degenerate copy of tandemly duplicated genes produced initially by slipped-strand mispairing.

Instability of mitochondrial genomic structure in chameleons is similar to that observed in other acrodonts (Macey *et al.*, 2000a) and contrasts with the genomic stability observed in their sister taxon, the Iguanidae (Macey *et al.*, 1997a, 2000b), which retains the structure of a functional O_L between the $tRNA^{Asn}$ and $tRNA^{Cys}$ genes.

Evolutionary History of Chamaeleonidae

Our sampling of Chamaeleonidae reveals nine clades whose ancestral lineages appear to have diverged early in the history of the group: (1) *Brookesia* (possibly excluding *B. nasus*); (2) *Chamaeleo* subgenus *Chamaeleo* (excluding *C. namaquensis*); (3) *Chamaeleo* subgenus *Trioceros*; (4) viviparous *Bradypodion*; (5) oviparous *Bradypodion*; (6) genus *Furcifer* (except *F. balteatus*); and (7–9) three distinct clades of *Calumma*. In addition, five species (*Chamaeleo namaquensis*, *Brookesia nasus*, *Furcifer balteatus*, *Rhampholeon brevicaudatus*, and *R. spectrum*) represent ancient lin-

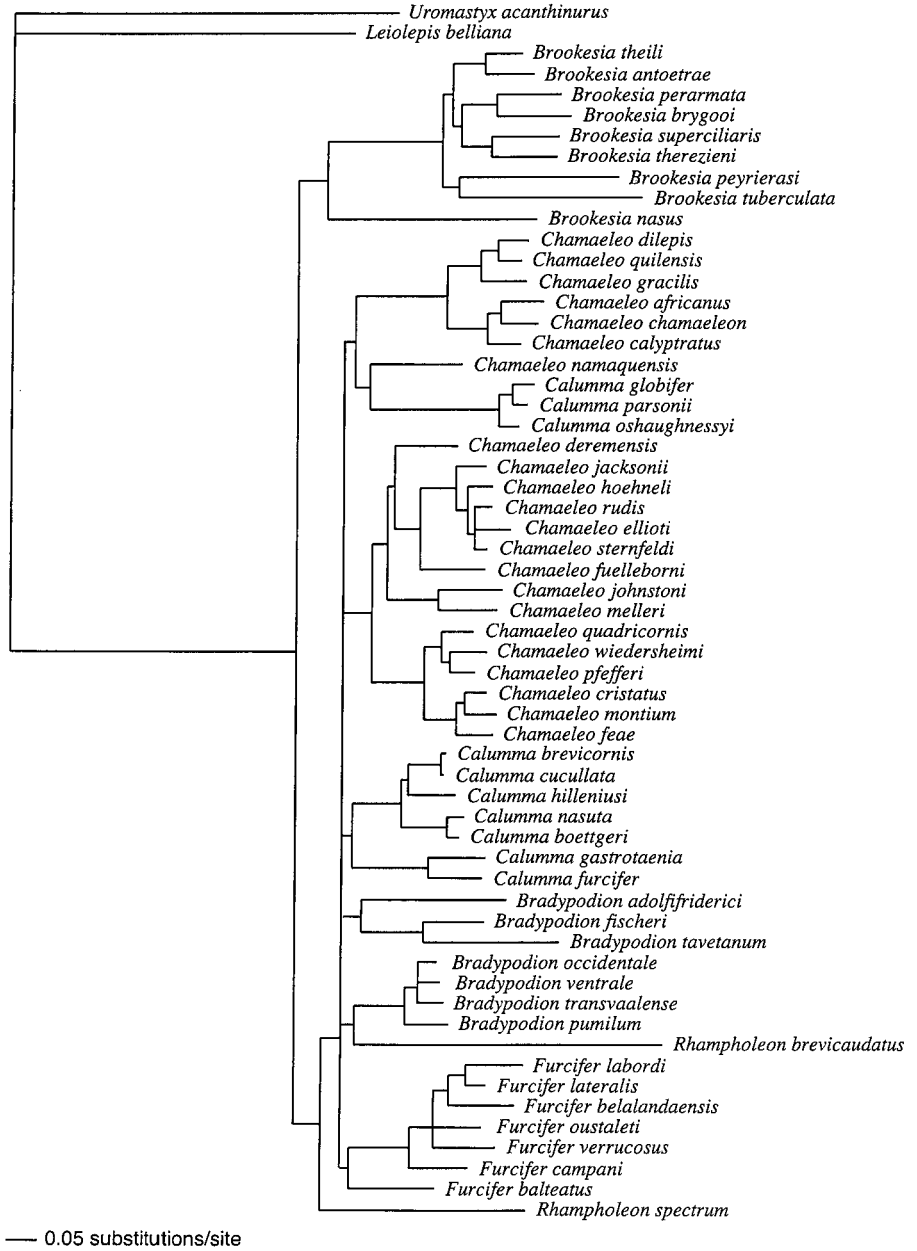

FIG. 4. Maximum-likelihood topology. Collapsed branches are not statistically supported by likelihood-ratio tests. Topological conflicts between this tree and Fig. 3 generally involve deep, weakly supported branches connecting well-supported groups (Table 5).

eages dating to approximately the same time. Average haplotype difference among these 14 clades and lineages is 21.1% (Table 6), comparable to levels of divergence observed among species of the widespread African agamid genus *Agama* (Macey *et al.*, 2000b). This level of divergence is compatible with an African or Malagasy origin of the Chamaeleonidae, and about 10% lower than expected for cladogenesis associated with early fragmentation of Gondwanan land masses (Macey *et al.*, 2000b). Maximum-likelihood distances between these groups (Table 6) suggest that the oldest divergences sampled are no older than Paleocene, with most comparisions more compatible with Eocene cladogenesis (Weisrock *et al.*, 2001). The current distribution of chameleons therefore requires overseas dispersal between Africa and Madagascar, and terrestrial dispersal from Africa to Asia and Europe. Vicariance associated with the original fragmentation of Gondwana does not account for observed divergences between African and Malagasy chameleons.

Among the major clades, *Brookesia* shows the greatest within-clade sequence divergences, with an average of 18.3% among species. The East African oviparous *Bradypodion* also form an old clade, with 15.9% se-

TABLE 5

Results of Statistical Tests of Topology

Alternative hypotheses tested[a]	Templeton test		SH test
	N[b]	P[c]	P[c]
1. Nonmonophyly of *Brookesia*	246	0.3100	0.354
	243	0.3032	
	253	0.3146	
2. Nonmonophyly of *Brookesia* (excluding *B. nasus*)	214	0.0055*	<0.001*
	203	0.0044*	
3. Monophyly of the subgenus *Chamaeleo*	101	0.4805	0.374
4. Nonmonophyly of the subgenus *Chamaeleo* (excluding *Chamaeleo namaquensis*)	180	0.0059*	0.030*
	194	0.0086*	
5. Nonmonophyly of the subgenus *Trioceros*	33	0.1921	0.011*
6. Nonmonophyly of the *Chamaeleo cristatus* group	120	0.0086*	<0.001*
	105	0.0058*	
7. Monophyly of *Furcifer*[d]	115	0.3593	—
8. Nonmonophyly of *Furcifer*[d]	—	—	0.483
9. Nonmonophyly of *Furcifer* (excluding *F. balteatus*)	163	0.0160*	0.036*
	153	0.0139*	
10. Monophyly of *Calumma*	150 to 188	0.3220 to 0.3408	0.374
11. Monophyly of *Rhampholeon*	208 to 253	0.2515 to 0.3146	0.346
12. Monophyly of *Bradypodion*	97	0.2245	0.211
13. Nonmonophyly of the viviparous South African *Bradypodion*	143	0.0034*	0.001*
	131	0.0025*	
	124	0.0018*	
	112	0.0012*	
14. Nonmonophyly of the viviparous subclade within *Trioceros*	97	0.1393	0.221
	41	0.0497*	
15. Monophyly of all viviparous species	137 to 180	0.0716 to 0.1315	0.002*
16. *Bradypodion occidentale, B. ventrale,* and *B. transvaalense* do not form a monophyletic sister group to *B. pumilum*	28 to 166	0.0041* to 0.1779	0.095
17. *Chamaeleo dilepis, C. quilensis,* and *C. gracilis* do not form a monophyletic sister group to *C. africanus, C. chamaeleon,* and *C. calyptratus*	37	0.0006*	0.005*

Note. Templeton tests were performed using parsimony topologies and SH tests were performed using maximum-likelihood topologies.

[a] A significant result means that the stated alternative hypothesis is rejected. See http://www.biology.wustl.edu/~ townsend for phylogenetic topologies used in tests.

[b] Number of characters differing in minimum number of changes on paired topologies.

[c] Asterisks indicate that the favored tree is significantly shorter or more likely than the alternative tree. One-tailed probabilities are shown.

[d] *Furcifer* is monophyletic in the maximum-likelihood topology but not in the maximum-parsimony topology.

quence divergence between *B. fischeri* and *B. tavetanum* (if *B. adolfifriederici* is included, the average increases to 20.1%). Substitution rates at this level are known to be nonlinear due to saturation effects (Moritz *et al.*, 1987). Nonetheless, Macey *et al.* (Macey *et al.* 1999, Table 4), using the same genes reported here, found roughly these same divergences between two subfamilies of anguid lizards, the Anguinae and the Gerrhonotinae, and argued for the formation of the Atlantic Ocean 50 MYBP (million years before present) as the isolating event between these two groups. Divergences within *Brookesia* and the oviparous *Bradypodion* probably are about the same age. Most of the other major clades show lower average sequence divergences within them, ranging from 5.7% (the *Calumma parsonii* group) to 12.3% (the subgenus *Trioceros*).

Subgenus *Chamaeleo* appears to have originated in the sub-Saharan region of Africa with later geographic expansion producing the northern African and Arabian radiations. Significant structure exists within subgenus *Chamaeleo,* with species divided into two clearly monophyletic groups (Fig. 3). One of these groups (*C. dilepis, C. quilensis,* and *C. gracilis*) is widely distributed over tropical and southern Africa, with *C. gracilis* found as far north as Somalia, Ethiopia, and Sudan. The second clade contains one sub-Saharan African species (*C. africanus*), another species found in southern Europe, northern Africa, and Arabia (*C. chamaeleon*), and a third species restricted to Arabia (*C. calyptratus*). Statistical tests (Table 5) confirm that the sub-Saharan region harbors species from either side of the deepest split within the clade, implying that the

TABLE 6

Average Sequence Divergence between Major Lineages and Clades[a,b]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	—	0.243	0.244	0.227	0.255	0.257	0.257	0.224	0.239	0.236	0.228	0.234	0.231	0.258	0.275
2	0.614	—	0.240	0.227	0.247	0.241	0.241	0.221	0.237	0.235	0.246	0.237	0.230	0.237	0.274
3	0.628	0.619	—	0.174	0.206	0.204	0.204	0.180	0.191	0.201	0.186	0.198	0.186	0.226	0.241
4	0.539	0.533	0.313	—	0.174	0.194	0.196	0.165	0.174	0.235	0.159	0.178	0.165	0.211	0.235
5	0.674	0.604	0.422	0.315	—	0.210	0.219	0.182	0.198	0.194	0.196	0.205	0.184	0.228	0.267
6	0.679	0.589	0.405	0.368	0.433	—	0.209	0.183	0.200	0.202	0.189	0.211	0.190	0.238	0.250
7	0.682	0.609	0.414	0.382	0.481	0.400	—	0.187	0.204	0.204	0.189	0.209	0.191	0.234	0.254
8	0.515	0.487	0.322	0.271	0.324	0.33	0.346	—	0.172	0.167	0.158	0.166	0.155	0.204	0.226
9	0.595	0.570	0.368	0.303	0.394	0.378	0.397	0.299	—	0.179	0.167	0.186	0.176	0.214	0.240
10	0.597	0.576	0.405	0.327	0.389	0.393	0.416	0.314	0.336	—	0.173	0.189	0.168	0.225	0.234
11	0.541	0.529	0.353	0.273	0.393	0.348	0.349	0.260	0.289	0.319	—	0.176	0.157	0.205	0.235
12	0.583	0.595	0.405	0.336	0.426	0.434	0.436	0.279	0.362	0.379	0.325	—	0.180	0.218	0.246
13	0.532	0.546	0.343	0.275	0.349	0.340	0.359	0.245	0.314	0.288	0.258	0.333	—	0.201	0.241
14	0.678	0.552	0.514	0.423	0.508	0.540	0.520	0.388	0.448	0.488	0.404	0.476	0.389	—	0.256
15	0.799	0.792	0.575	0.536	0.672	0.609	0.630	0.483	0.556	0.561	0.519	0.607	0.559	0.626	—

[a] Uncorrected sequence divergence is shown in the upper triangle, maximum-likelihood divergence in the lower triangle. See text for maximum-likelihood parameters for the full data set.

[b] 1 = *Brookesia*, excluding *B. nasus*, 2 = *B. nasus*, 3 = *Chamaeleo (Chamaeleo)*, excluding *C.* (*C.*) *namaquensis*, 4 = *C. (C.) namaquensis*, 5 = *Calumma globifer* group, 6 = *Bradypodion adolfifiriderici*, 7 = *Bradypodion* (oviparous), 8 = *Furcifer balteatus*, 9 = *Chamaeleo (Trioceros)*, 10 = *Calumma gastrotaenia* group, 11 = *Bradypodion* (viviparous), 12 = *Furcifer*, excluding *F. balteatus*, 13 = *Calumma brevicornis* group, 14 = *Rhampholeon spectrum*, 15 = *Rhampholeon brevicaudatus*.

ancestor to the group had a sub-Saharan distribution. This result fits well with previous biogeographic hypotheses for both this group and chameleons as a whole (Hillenius, 1959, 1963).

Similar reasoning suggests that South African *Bradypodion* originated in southwestern coastal areas of that country, and that the northwestern radiation occurred later. *Bradypodion pumilum*, found in southern and southwestern montane and coastal areas of South Africa, is the sister taxon to a clade containing two other species from this region (*B. ventrale, B. occidentale*) and a fourth species (*B. transvaalense*) restricted to wet montane areas in northeastern South Africa. The basal split within this clade receives strong heuristic support (Fig. 3), although statistical tests are not definitive (Table 5). Sampling of additional *Bradypodion* species is needed to test our hypothesis of a southwestern African origin for viviparous *Bradypodion*.

Viviparous chameleons of the genera *Bradypodion* and *Chamaeleo* form separate clades that are only distantly related (Table 6), suggesting at least two separate origins of viviparity in Chamaeleonidae. The hypothesis that all viviparous chameleons form a monophyletic group is rejected by the likelihood-based SH test (Table 5).

Horn-bearing species of *Trioceros* do not form a monophyletic group. Two equally most-parsimonious optimizations of horn evolution, each requiring five separate evolutionary events, are obtained (Fig. 5). One optimization postulates acquisition of horns on the lineage ancestral to *Trioceros* followed by four independent losses, one loss within the east African *Trioceros* (involving the common ancestor to the *C. bitaeniatus* group) and three separate losses on terminal branches within the *C. cristatus* group. The alternative optimization is similar except that the lineage ancestral to *C. cristatus, C. montium*, and *C. feae* lost the horns, and *C. montium* regained them (see Fig. 5). Chameleon horns are used in combat between males (Bustard, 1958; Hillenius, 1959), and may provide species-recognition cues (Rand, 1961). Repeated loss of rostral horns is consistent with Schluter and Price's (1993) argument that evolutionary change in female preference for male-display traits often leads to evolutionary reduction or loss of such characters (Burns, 1998; Wiens *et al.*, 1999). Given these phylogenetic results, chameleons of subgenus *Trioceros* constitute a good study system for behavioral ecological studies on evolution of male displays.

Haplotypic divergences among all species sampled in this study (see complete pairwise distance matrix at http://www.biology.wustl.edu/~townsend) are sufficiently large to indicate that these species represent separate evolutionary lineages whose most recent common ancestry is at least 3 million years old and often much older. Particularly noteworthy are divergences observed between pairs of species that recently have been synonymized (Broadley and Howell, 1991; Raxworthy and Nussbaum, 1995; Klaver and Böhme, 1997): (1) the South African *Bradypodion* species sampled (5.1–8.5% sequence difference), (2) *Brookesia tuberculata* and *B. peyrierasi* (21.6% sequence difference), (3) *B. theili* and *B. antoetrae* (9.2% sequence difference), and (4) *Chamaeleo rudis* and *C. sternfeldi*

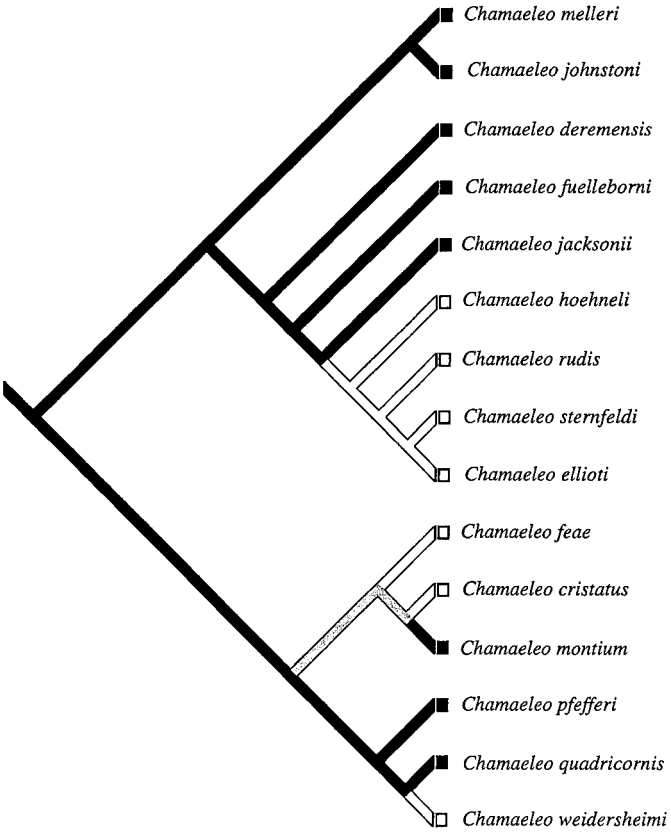

FIG. 5. Horn evolution within the subgenus *Trioceros* reconstructed on the shortest estimate of phylogeny, which requires a minimum of five evolutionary events (including the original acquisition of horns at the root of the tree). Black lines indicate presence of bony, keratinized horn tissue in one or both sexes. White lines indicate absence of horns, and gray lines are equivocal.

(4% sequence difference). Evolutionary calibration of the mitochondrial genomic segment used in our comparisons suggests 1.3% divergence per million years, with this estimate being conservative for differences exceeding 10% (see Weisrock *et al.*, 2001, for review). Current taxonomy therefore probably underestimates species diversity of the Chamaeleonidae.

Although our results question the monophyly of several genera (*Bradypodion, Calumma, Furcifer, Rampholeon*), we will not make taxonomic changes at this time because monophyly is not statistically rejected. These genera might be considered metataxa to denote their uncertain taxonomic status (Schulte *et al.*, 1998).

APPENDIX

Origins of specimens from which sequence was included for this study are presented below in phylogenetic order (alphabetical within genera). Acronyms are AMB, Aaron M. Bauer's field collection numbers (specimens not yet catalogued); CAS, California Academy of Sciences, San Francisco; FM, Field Museum of Natural History, Chicago; ZFMK, Zoologisches Forschungsinstitut and Museum A. Koenig, Bonn; MV, private collection of Miguel Vences. TMT-series voucher specimens are in the senior author's collection and were originally obtained from the St. Louis Zoo. BM 24, Kian 117, Mor 24 and 46, Rano 96, 109, and 114, and Zah 20 and 75 represent specimens from which blood and/or tissue samples were taken during the senior author's field studies, and abbreviated collection localities are given in each case. GenBank accession numbers are given in parentheses following the specimen numbers.

Bradypodion: 1. *Bradypodion adolfifriderici* (catalogued as *Chamaeleo adolfifriderici*), Uganda, Kabale Dist. (CAS 201593) (AF448727). 2. *Bradypodion fischeri*, Tanzania, Tanga Region (CAS 168965, reported as *Chamaeleo fischeri* by Macey *et al.*, 1997a). 3. *Bradypodion occidentale*, Kleinzee, Northern Cape Province, South Africa (AMB 5618) (AF448728). 4. *Bradypodion pumilum*, Stellenbosch, Western Cape Province, South Africa (AMB 5683) (AF448729). 5. *Bradypodion taveta-num* (MV 9) (AF448730). 6. *Bradypodion transvaalense*, Tzaneen, Northern Province, South Africa (AMB 6154) (AF448731). 7. *Bradypodion ventrale*, South Africa (AMB 4251) (AF448732).

Brookesia: 1. *Brookesia antoetrae*, Ranomafana National Park, Madagascar (Rano 114) (AF448773). 2. *Brookesia brygooi*, Kirindy near Morondava, Madagascar (ZFMK 66707) (AF448774). 3. *Brookesia nasus*, Ranomafana National Park, Madagascar (Rano 96) (AF448775). 4. *Brookesia perarmata* (TMT 50) (AF448776). 5. *Brookesia peyrierasi*, Nosy Mangabe, Madagascar (ZFMK 66670) (AF448777). 6. *Brookesia superciliaris*, Manombo Special Reserve, Madagascar (BM 24) (AF448778). 7. *Brookesia theili*, Ambohitantely Special Preserve, Madagascar (FM 13949) (AF448780). 8. *Brookesia therezieni*, Zahamena Strict Natural Reserve, Madagascar (Zah 20) (AF448779). 9. *Brookesia tuberculata*, Madagascar (MV 15) (AF448781).

Calumma: 1. *Calumma boettgeri*, Madagascar (ZFMK 68612) (AF448733). 2. *Calumma brevicornis*, Ambohitantely Special Preserve, Madagascar (FM 13715) (AF448734). 3. *Calumma nasuta*, Ambohitantely Special Preserve, Madagascar (FM 13722) (AF448740). 4. *Calumma cucullata*, Madagascar (ZFMK 68606) (AF448735). 5. *Calumma furcifer*, Zahamena Strict Natural Reserve, Madagascar (Zah 75) (AF448736). 6. *Calumma gastrotaenia*, Ambohitantely Special Preserve, Madagascar (FM 13711) (AF448737). 7. *Calumma globifer*, Madagascar (ZFMK 68615) (AF448738). 8. *Calumma hilleniusi*, Madagascar (ZFMK 68611) (AF448739). 9. *Calumma oshaughnessyi*, Ranomafana National Park, Madagascar (Rano 109) (AF448741). 10. *Calumma parsonii*, Ambohitantely Special Preserve, Madagascar (FM 13723) (AF448742).

Chamaeleo: 1. *Chamaeleo africanus*, Chad (ZFMK 65568) (AF448743). 2. *Chamaeleo chamaeleon*, captive-bred specimen (breeding stock from southern Portugal)

(ZFMK 68644) (AF448745). 3. *Chamaeleo calyptratus* (TMT 56) (AF448744). 4. *Chamaeleo cristatus* (ZFMK 68627) (AF448746). 5. *Chamaeleo deremensis*, Tanzania, Tanga Region (CAS 168888) (AF448747). 6. *Chamaeleo dilepis*, Tanzania, Tanga Region (CAS 168922, Macey *et al.*, 2000b). 7. *Chamaeleo ellioti*, Uganda, Rukungiri Dist (CAS 201722) (AF448748). 8. *Chamaeleo feae*, Ecuatorial Guinea, Bioko Island (CAS 207681) (AF448749). 9. *Chamaeleo fuelleborni* (ZFMK 66746) (AF448750). 10. *Chamaeleo gracilis*, pet trade animal from Togo (ZFMK 68645) (AF448751). 11. *Chamaeleo hoehnelii* (ZFMK 68622) (AF448752). 12. *Chamaeleo jacksonii*, Kenya, Nairobi Province (CAS 199070) (AF448753). 13. *Chamaeleo johnstoni*, Uganda, Kabale Dist. (CAS 201596) (AF448754). 14. *Chamaeleo melleri* (ZFMK 68607) (AF448755). 15. *Chamaeleo montium* (TMT 54) (AF448756). 16. *Chamaeleo namaquensis*, 33 km E of Luderitz, Namibia (AMB 5863) (AF448757). 17. *Chamaeleo pfefferi* (MV20) (AF448758). 18. *Chamaeleo quadricornis* (TMT 52) (AF448759). 19. *Chamaeleo quilensis* (MV19) (AF448760). 20. *Chamaeleo rudis*, Uganda, Kabale Dist. (CAS 201711) (AF448761). 21. *Chamaeleo sternfeldi* (MV18) (AF448762). 22. *Chamaeleo weidersheimi* (ZFMK 68632) (AF448763).

Furcifer: 1. *Furcifer balteatus*, Kianjavato, Madagascar (Kian 117) (AF448764). 2. *Furcifer belalandaensis*, Madagascar (ZFMK 68604) (AF448765). 3. *Furcifer campani*, Madagascar (ZFMK 68610) (AF448766). 4. *Furcifer labordi*, Madagascar (ZFMK 68616) (AF448767). 5. *Furcifer lateralis*, Ambohitantely Special Preserve, Madagascar (FM 13721) (AF448768). 6. *Furcifer oustaleti*, Morondava, Madagascar (Mor 24) (AF448769). 7. *Furcifer verrucosus*, Morondava, Madagascar (Mor 46) (AF448770).

Rhampholeon: 1. *Rhampholeon brevicaudatus*, Uluguru Mtns., Tanzania (ZFMK 68487) (AF448771). 2. *Rhampholeon spectrum*, Ecuatorial Guinea, Bioko Island (CAS 207688) (AF448772).

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