

Evolution and Phylogenetic Information Content of Mitochondrial Genomic Structural Features Illustrated with Acrodont Lizards

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Abstract.—DNA sequences from 195 squamate reptiles indicate that mitochondrial gene order is the most reliable phylogenetic character establishing monophyly of acrodont lizards and of the snake families Boidae, Colubridae, and Viperidae. Gene order shows no evidence of evolutionary parallelisms or reversals in these taxa. Derived secondary structures of mitochondrial tRNAs also prove to be useful phylogenetic characters showing no reversals. Parallelisms for secondary structures of tRNAs are restricted to deep lineages that are separated by at least 200 million years of independent evolution. Presence of a stem-and-loop structure between the genes encoding tRNA^{Asn} and tRNA^{Cys}, where the replication origin for light-strand synthesis is typically located in vertebrate mitochondrial genomes, is found to undergo at least three and possibly as many as seven evolutionary shifts, most likely parallel losses. This character is therefore a less desirable phylogenetic marker than the other structural changes examined. Sequencing regions that contain multiple genes, including tRNA genes, may be preferable to the common practice of obtaining single-gene fragments for phylogenetic inference because it permits observation of major structural changes in the mitochondrial genome. Such characters may occasionally provide phylogenetic information on relatively short internal branches for which base substitutional changes are expected to be relatively uninformative. [Acrodonta; gene organization; mitochondrial DNA, phylogenetics; replication; Reptilia; tRNA.]

Mitochondrial DNA sequences are currently the most widely used source of phylogenetic information among vertebrates. Most phylogenetic investigations use complete sequences of a single gene or partial sequences of a few genes, genes that typically comprise regions encoding rRNA (12S and 16S) or proteins (most often, cytochrome *b*, COI, COII, COIII, and ND4). Major structural features of the vertebrate mitochondrial genome that are known to show variation are (1) gene organization, (2) position of the replication origin for light-strand synthesis (O_L), and (3) secondary structures of encoded tRNAs. These structural features are rarely incorporated into phylogenetic studies because regions containing multiple genes must be sequenced to evaluate them.

We have evaluated the evolution and phylogenetic utility of these three major structural features in acrodont lizards and related squamate reptiles. The region analyzed includes one-third of the gene junctions in the vertebrate mitochondrial genome and extends from the protein-coding gene ND1 (subunit one of NADH dehydrogenase) through the genes encoding

tRNA^{Gln}, tRNA^{Ile}, tRNA^{Met}, ND2, tRNA^{Trp}, tRNA^{Ala}, tRNA^{Asn}, tRNA^{Cys}, and tRNA^{Tyr} to the protein-coding gene COI (subunit I of cytochrome *c* oxidase) (Fig. 1). This region shows three derived characters shared by all acrodont lizards: a unique tRNA gene order, lack of a recognizable O_L , and a derived secondary structure of encoded tRNA^{Cys} (Macey et al., 1997a, 1997b, 1997c, 1998a).

The evolutionary patterns of these structural characteristics indicate that they can be a useful source of phylogenetic characters that is not often exploited in phylogenetic studies.

MATERIALS AND METHODS

See Macey et al. (2000) for GenBank accession numbers of the DNA sequences examined here, and the museum numbers and localities for voucher specimens. Additional sequences that include the tRNA^{Asn} gene, a stem-and-loop structure, and the tRNA^{Cys} gene are reported here for three species of *Ceratophora*. Data for these specimens are as follows: *Ceratophora karu*, Sri Lanka (WHT 2259, AF128520); *Ceratophora*

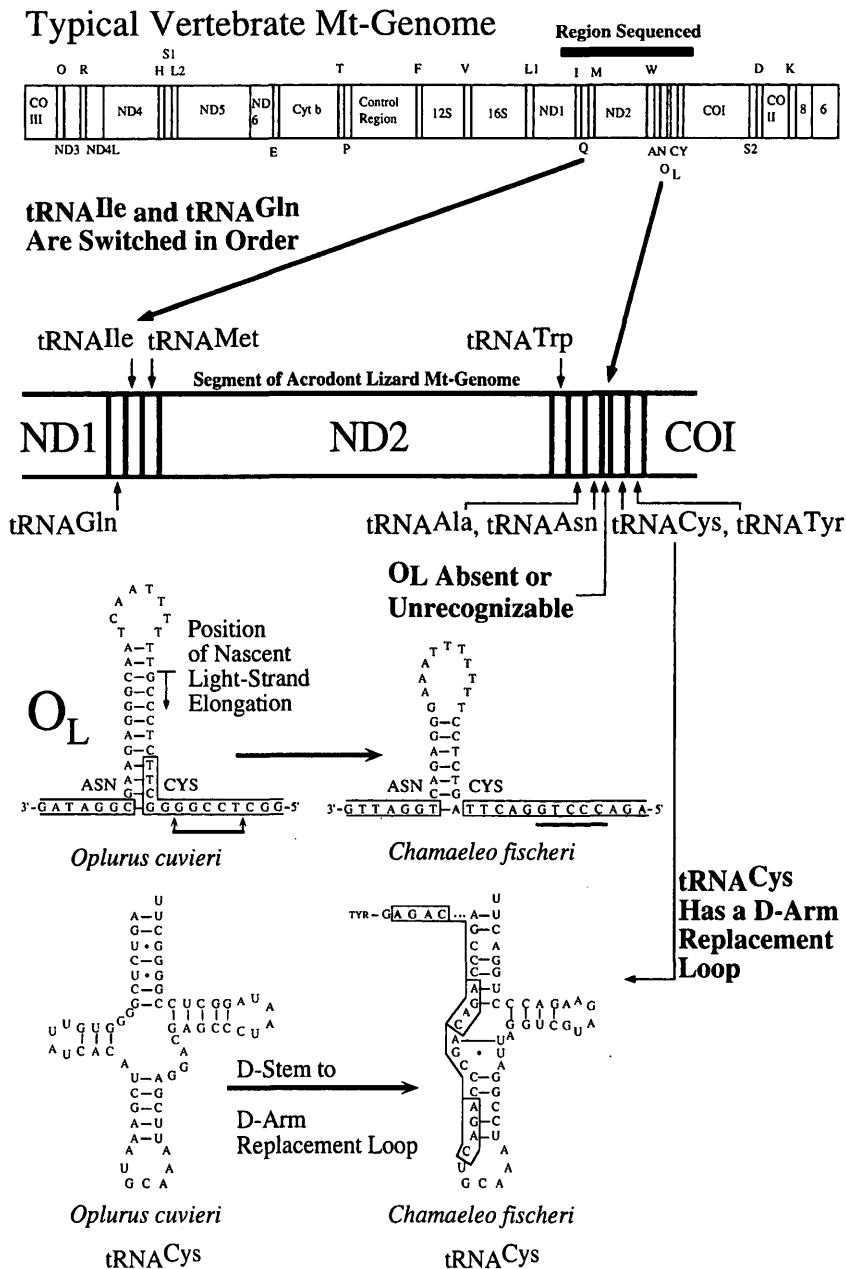


FIGURE 1. Gene order and major structural features of a segment of the mitochondrial genome from acrodont lizards. The gene order observed in most vertebrates is presented in linear fashion at the top of the figure; the segment sequenced for acrodont lizards is enlarged for detail. Three unusual structural characteristics of the acrodont mitochondrial genome occur in this segment. Among the Acrodonta, the tRNA^{Gln} gene precedes the tRNA^{Ile} gene, the OL is absent or unrecognizable, and the encoded tRNA^{Cys} has a D-arm replacement loop instead of a D-stem. Comparison of sequences from the iguanid lizard, *Oplurus cuvieri*, which has the mitochondrial genomic characteristics typical for vertebrates in this region, and the acrodont lizard, *Chamaeleo fischeri*, illustrates the changes in OL and the encoded tRNA^{Cys}. The *Oplurus* OL has a 3'-GCC-5' heavy-strand sequence in the stem region identified as the point of light-strand elongation in mouse (Brennicke and Clayton, 1981) and a heavy-strand sequence related to the 3'-GGCCG-5' sequence (underlined with arrows) required for in vitro replication of human mitochondrial DNA (Hixson et al., 1986). The *Chamaeleo* OL lacks a 3'-GCC-5' heavy-strand sequence in the stem region but does have a heavy-strand sequence related to the 3'-GGCCG-5' sequence (underlined) downstream from the stem region. In the encoded tRNA^{Cys}, *Chamaeleo* lacks a D-stem and instead contains a D-arm replacement loop. Note the noncontiguous repeats, boxed and outlined, which are postulated to have formed by slipped-strand mispairing during replication (Macey et al., 1997b). All three unusual characteristics of the acrodont mitochondrial genome may be evolutionarily coupled (Macey et al., 1997c, 1998b).

tennentii, Sri Lanka (WHT 1633, AF128521); *Ceratophora erdeleni*, Sri Lanka (WHT1808, AF128522). The acronym WHT is for the Wildlife Heritage Trust, Colombo, Sri Lanka, where the voucher specimens are deposited. Detailed locality data are archived in the GenBank accessions.

Secondary structures of tRNAs are inferred from sequences of the genes encoding them based on the model of Kumazawa and Nishida (1993). The criteria for inferring an O_L between the tRNA^{Asn} and tRNA^{Cys} genes include the presence of a possible stem-and-loop structure, a 3'-GCC-5' heavy-strand sequence identified as the point of light-strand elongation in mouse (Brennicke and Clayton, 1981), and a heavy-strand sequence related to the 3'-GGCCG-5' sequence required for in vitro replication of human mitochondrial DNA (Hixson et al., 1986).

Average homoplasy for variable nucleotide positions was estimated by using MacClade (Maddison and Maddison, 1992) as follows. The minimum possible number of evolutionary steps for each variable character, which is equal to one less than the total number of character states observed, was tabulated and summed across all characters. This sum was subtracted from the length of the three equally most-parsimonious trees recovered from phylogenetic analysis of all sites combined (see Macey et al., 2000) in PAUP* beta version 4.0b1 (Swofford, 1998) to yield the total amount of homoplasy. This total homoplasy was divided by the number of variable sites to obtain the average homoplasy per variable site.

Some changes in major structural features occur on branches that appear not to be well supported in phylogenetic analyses of nucleotide substitutions. A conservative estimate of character evolution was obtained by plotting bootstrap values against decay indices. Branches that do not meet the 95% bootstrap criterion and corresponding decay index for substitutional changes are allowed to vary to minimize inferred changes in the major structural character being studied. Evolution of the character was then evaluated on both the overall shortest tree and the less-resolved tree that minimized evolutionary changes in the character being studied.

RESULTS

Genomic Organization

All 70 acrodont lizard species sampled contain a derived gene order in which tRNA^{Gln} precedes tRNA^{Ile} instead of the typical vertebrate gene order of ND1, tRNA^{Ile}, tRNA^{Gln}, tRNA^{Met}, ND2, tRNA^{Trp}, tRNA^{Ala}, tRNA^{Asn}, O_L , tRNA^{Cys}, tRNA^{Tyr}, and COI (Macey et al., 1997a, 1997c, 1998a). In addition, *Sitana ponticeriana* from the South Asian clade contains an extra sequence between the ND2 and tRNA^{Trp} genes; this sequence appears to be a highly modified, nonfunctional duplicate of the tRNA^{Trp} gene.

Origin for Light-Strand Replication

Acrodont lizards show variation for the presence of a stem-and-loop structure in the typical vertebrate position for the O_L between the tRNA^{Asn} and tRNA^{Cys} genes. None of these structures have the characteristics identified as necessary for function in a mammalian O_L (Brennicke and Clayton, 1981; Hixson et al., 1986). Therefore, these structures are interpreted as nonfunctional.

Some acrodont lizards [*Uromastix* (Macey et al., 1997c), *Physignathus cocincinus* (Macey et al., 1997c), all Australian taxa (except *Amphibolurus*), all New Guinean taxa, and *Otocryptis*] have no more than a few bases between the tRNA^{Asn} and tRNA^{Cys} genes, where O_L is typically located in vertebrates. Other taxa [*Amphibolurus*, *Hydrosaurus*, *Sitana*, *Laudakia stellio*, *Phrynocephalus* (Macey et al. 1997c), *Pseudotrapelus*, and *Agama*] have in this location a sequence that cannot form the stable secondary structure typical for O_L among vertebrates. Many taxa [*Chamaeleo* (Macey et al., 1997c), *Leiolepis* (Macey et al., 1997c), all South Asian taxa sampled (except *Hydrosaurus*, *Physignathus cocincinus*, *Otocryptis*, and *Sitana*), all *Laudakia* (except *L. stellio*), and *Trapelus*] contain stem-and-loop structures in the typical vertebrate position for O_L between the tRNA^{Asn} and tRNA^{Cys} genes, but these sequences do not have the functional characteristics of O_L identified in studies of mammalian mitochondrial replication (Brennicke and Clayton, 1981; Hixson et al., 1986).

Among taxa found to have stem-and-loop structures in the typical vertebrate po-

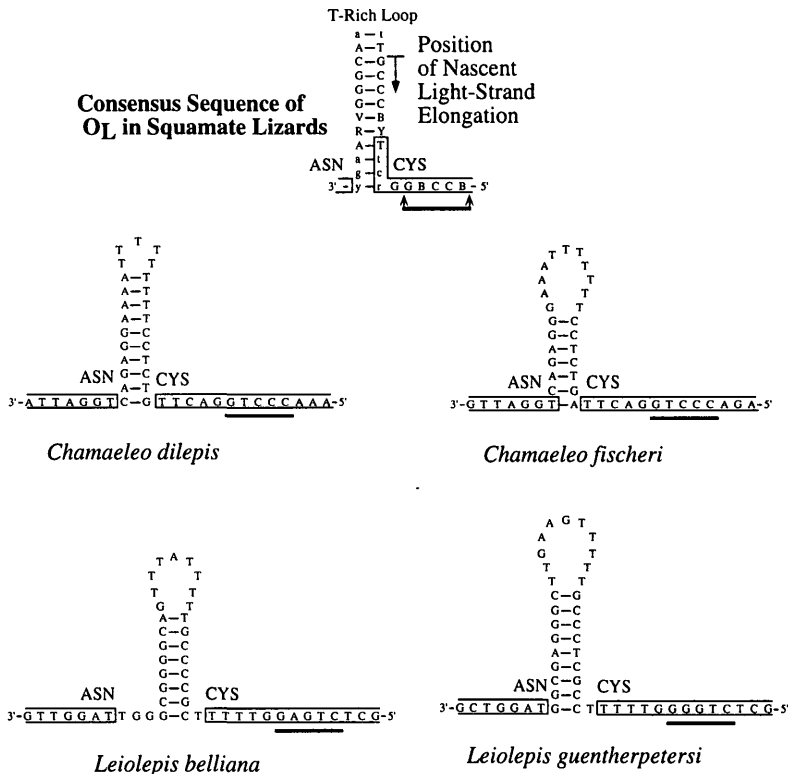


FIGURE 2. Stem-and-loop structures in the typical position for O_L between the tRNA^{Asn} and tRNA^{Cys} genes among basal acrodont lizards. The top figure shows a consensus heavy-strand sequence of putative O_L from lizards. This structure is based on representatives from the Iguanidae (nine genera), Gekkonidae (*Teratoscincus*), Dibamidae (*Dibamus*), Lacertidae (*Eremias*), Teiidae (*Cnemidophorus*), Cordylidae (*Platysaurus*), Anguidae (*Elgaria*), Xenosauridae (*Xenosaurus*), and Varanidae (*Varanus*), (Macey et al., 1997a, 1997c). Bases in capitals are conserved pairings and downstream sequence. Bases in lower case are often paired. Variable positions are labeled with their standard one-letter codes: R = G or A; Y = C or T; B = G, C, or T; and V = G, C, or A. The 3'-GCC-5' heavy-strand template sequence identified as the point of light-strand elongation in mouse (Brennicke and Clayton, 1981) is indicated (arrow). The heavy-strand sequence 3'-GGCC-5' or 3'-GBCCB-5' in the tRNA^{Cys} gene related to the 3'-GGCCG-5' sequence found to be required for in vitro replication of human mitochondrial DNA (Hixson et al., 1986) is underlined with arrows. Below the top figure are stem-and-loop structures in the typical position for O_L between the tRNA^{Asn} and tRNA^{Cys} genes among acrodont lizards. None of these structures has both functional characteristics.

sition for O_L between the tRNA^{Asn} and tRNA^{Cys} genes, only the two *Leiolepis* species sampled have in the stem region a potential 3'-GCC-5' heavy-strand sequence, identified as the point of light-strand elongation in mouse (Brennicke and Clayton, 1981) (Fig. 2). The two *Leiolepis* species do not have a heavy-strand sequence downstream of the stem region related to the 3'-GGCCG-5' sequence required for in vitro replication of human mitochondrial DNA (Hixson et al., 1986). Among lizards, this sequence is found to vary (Fig. 2): 3'-GBCCB-5', where B is G, T, or C (Macey et al., 1997a, 1997c).

Both *Chamaeleo* species sampled have a heavy-strand sequence downstream of the stem region related to the 3'-GGCCG-5' sequence required for in vitro replication of human mitochondrial DNA (Hixson et al., 1986). This sequence is present in both taxa as 3'-GTCCC-5'. Most South Asian taxa sampled that have stem-and-loop structures contain the potential heavy-strand sequence, 3'-GBCCB-5', downstream of the stem region. This sequence is conserved across most taxa as 3'-GTCCT-5' but deviates in *Calotes calotes*, *Ceratophora* species, and *Aphaniotis*. Among taxa sampled from Africa and West Asia that were found to

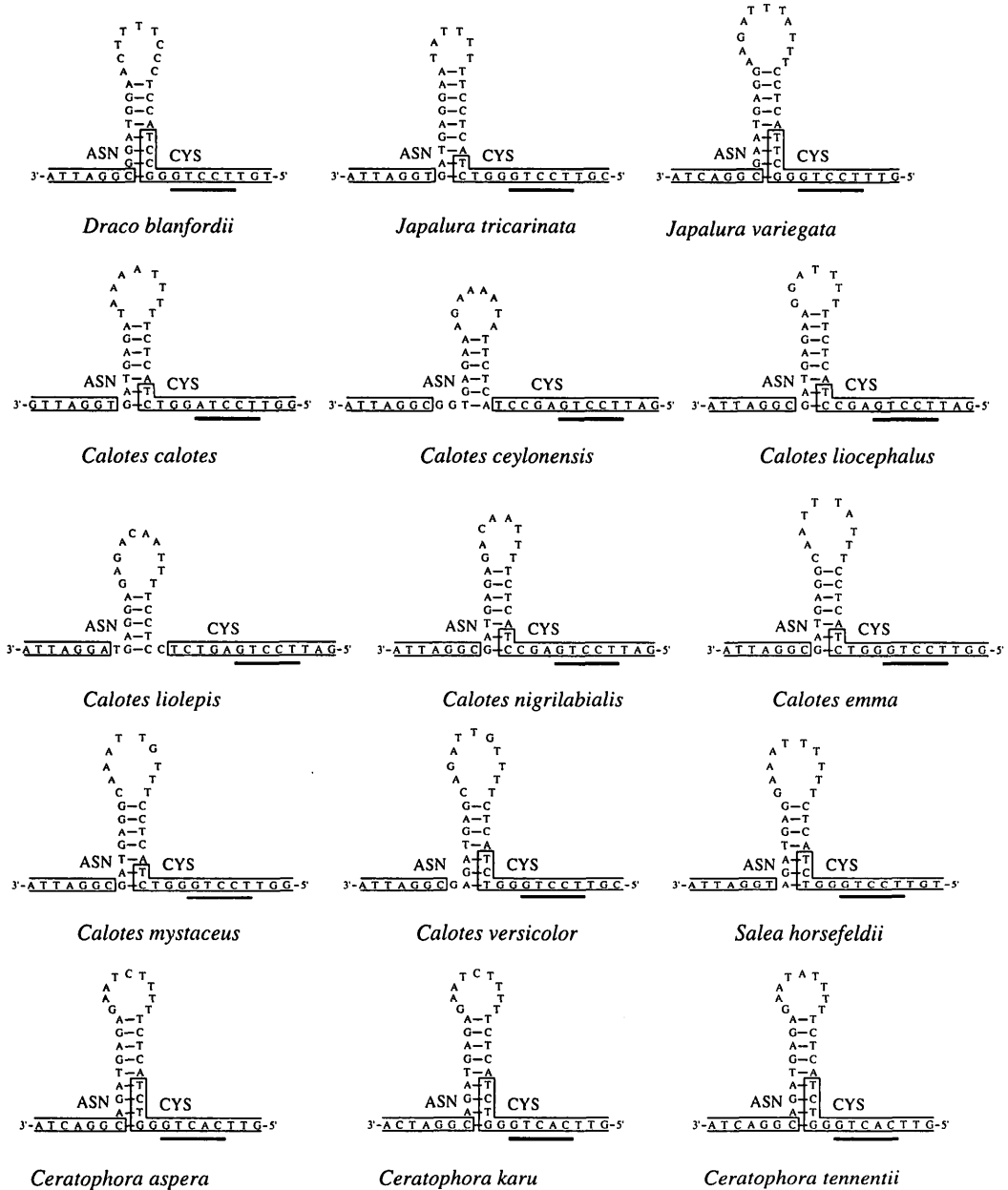


FIGURE 2. Continued

have stem-and-loop structures in the typical vertebrate position for O_L between the tRNA^{Asn} and tRNA^{Cys} genes, no potential downstream heavy-strand sequence related to the 3'-GGCCG-5' sequence could be identified.

A minimum of three evolutionary events is required to explain absence of stem-and-loop structures in the typical vertebrate po-

sition for O_L among acrodont lizards (Figs. 3 and 4). Both the South Asian and the African/West Asian clades are variable for the presence of stem-and-loop structures. Either a loss of stem-and-loop structures occurred in the ancestor of each clade with subsequent regains of stem-and-loop structures nested within the clades, or independent losses of stem-and-loop structures oc-

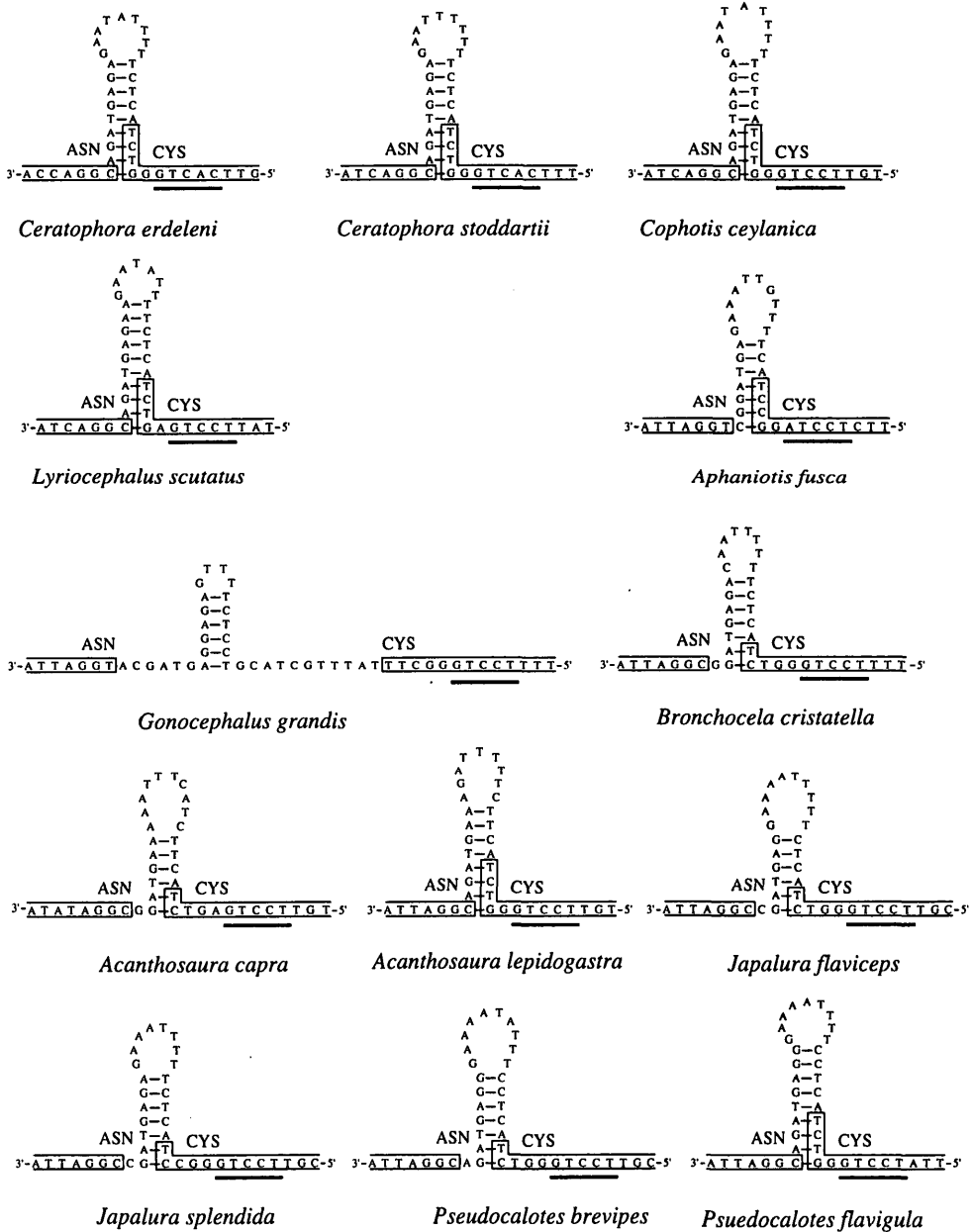


FIGURE 2. Continued

curred among taxa in each clade and in other acrodont groups (*Uromastix*, *Hydrosaurus*, and the *Physignathus cocincinus* and Australian-New Guinean clade). In addition, when this character is plotted on the shortest estimate of phylogeny, seven evolutionary events are required, either seven gains of stem-and-loop structures or seven losses. The hypothesis of seven parallel

losses is favored because it seems unlikely that stem-and-loop structures would reappear in exactly the same place after they were lost.

The loss of a stem-and-loop structure between the tRNA^{Asn} and tRNA^{Cys} genes is shared between *Physignathus cocincinus* and taxa occurring on the Australia–New Guinea Plate and is postulated to predate

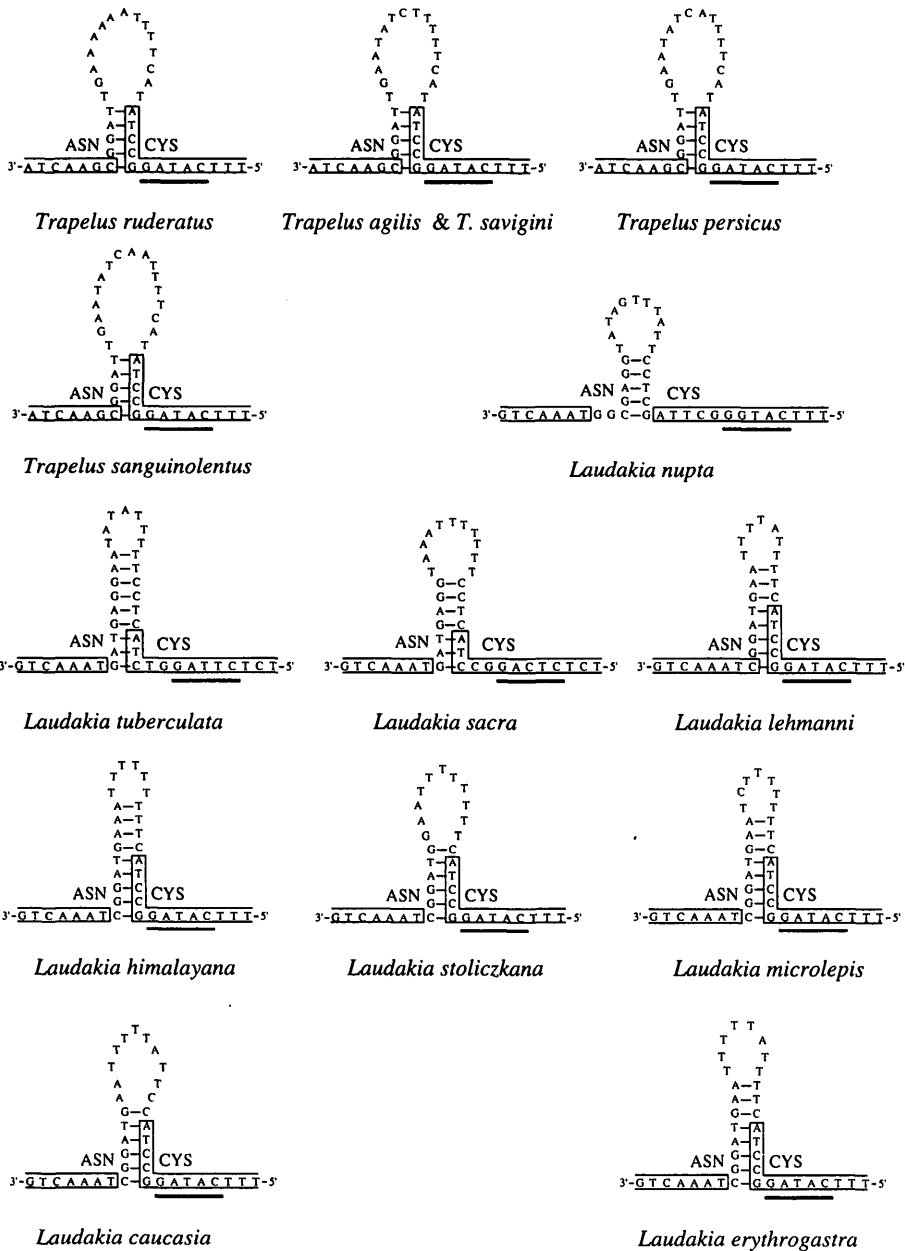


FIGURE 2. Continued

the separation of Southeast Asia from the Australia–New Guinea Plate, hundreds of MYBP (Metcalf, 1996). Other losses may have occurred more recently.

tRNA Secondary Structure

tRNA^{Cys}.—The *tRNA^{Cys}* gene of all acrodont lizards sampled encodes a tRNA

that lacks a D-stem (Fig. 1; Macey et al., 1997a, 1997b, 1997c, 1998a) and this loss is postulated to have occurred in the Jurassic between 210 and 160 MYBP (Macey et al., 1997b, 1997c). The inferred secondary structure of *tRNA^{Cys}* is highly variable (Fig. 5). Some species have *tRNA^{Cys}* gene sequences that encode a D-arm replacement loop, whereas others do not. Transfer RNAs with

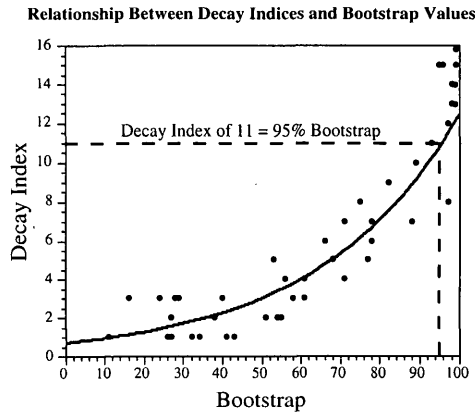


FIGURE 3. Plot of the relationship between bootstrap values and decay indices. Nodes with a bootstrap value of 100% are not plotted; all of these nodes had decay indices between 15 and 84, which overlap with decay indices observed on nodes with bootstrap values of 94%, 95%, and 99%. A direct relationship is observed with the plot being fitted to an exponential curve (r^2 value of 0.78). In this data set, a branch can be considered well supported if it has a decay index of at least 11 and a bootstrap value of at least 95%.

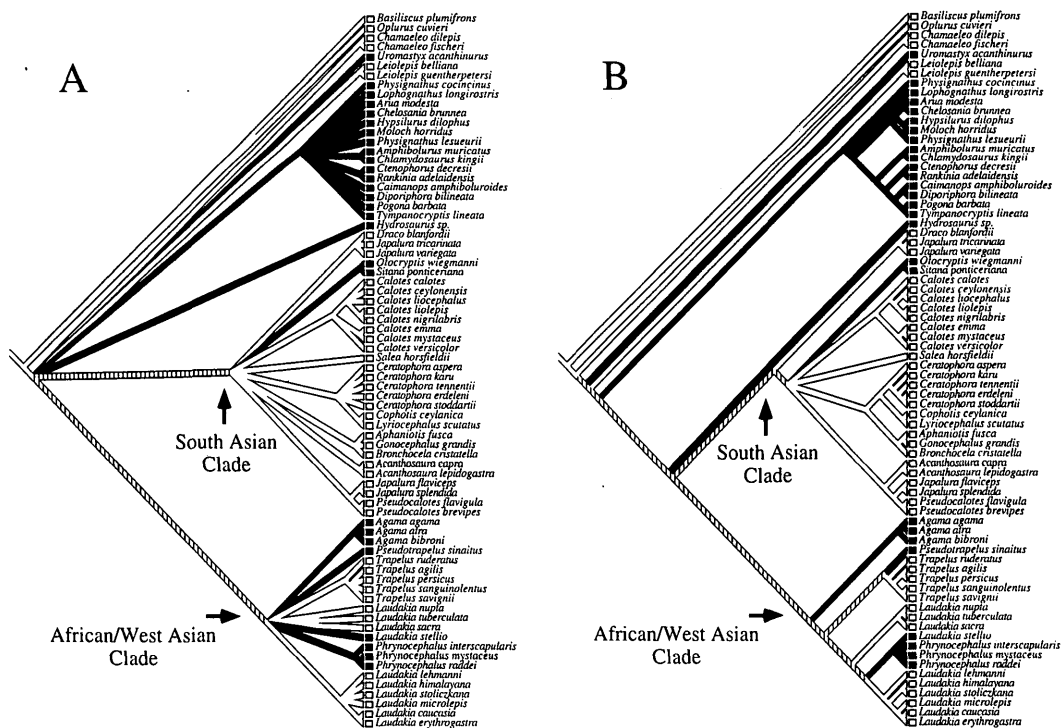


FIGURE 4. Evolution among the Acrodonta of stem-and-loop structures in the typical vertebrate position for the O_L between the $tRNA^{Asn}$ and $tRNA^{Cys}$ genes. (A) A conservative estimate of character evolution based on the criterion of well-supported branches identified in Figure 3. The phylogenetic topology is based on nodes that received support from 95% of bootstrap replicates and decay indices of 11 or greater (Macey et al., 2000). Black lines indicate taxa in which no stem-and-loop structure could be identified between the $tRNA^{Asn}$ and $tRNA^{Cys}$ genes, whereas open lines denote presence of this structure and cross-hatched lines are equivocal. Note that both the South Asian clade and the African-West Asian clade are variable, and that the evolution of stem-and-loop structures requires a minimum of three events. (B) Character evolution on the shortest estimate of phylogeny, which requires a minimum of seven evolutionary events. Parallelisms are favored because it is unlikely that stem-and-loop structures would evolve in exactly the same place after they were lost.

D-arm replacement loops also have a standard variable loop of three to five bases. As pairing increases between the D-arm replacement and variable loops, the AC-stem is extended beyond the five bases normally observed in standard tRNAs. In some cases, both the D-arm replacement and the variable loops are completely paired. Tremendous variation is observed also in the encoded AA- and T-stems. If the T-stem shifts, it can provide additional bases to both the variable loop and the AA-stem, with a net reduction in size of the T-stem and loop.

The tertiary structure of mitochondrial tRNAs that lack a D-stem differs from that of standard tRNAs (Steinberg et al., 1994). Transfer RNAs lacking a D-stem may extend their AA- or AC-stems (or both) to avert the problem of having a shorter mitochondrial tRNA that must function with protein-synthetic machinery suited to full-sized tRNAs (Steinberg et al., 1994). The acrodon tRNA^{Cys} appears to be extending the number of pairings in the AC- and AA-stems from 12 to as many as 18. A standard tRNA has a total of 12 paired positions in the AA- and AC-stems. Almost all acrodonts have tRNA^{Cys} gene sequences that encode a tRNA with >12 pairings in the AA- and AC-stems.

Taxa that lack a stem-and-loop structure in the typical position for O_L between the tRNA^{Asn} and tRNA^{Cys} genes also tend to have tRNA^{Cys} gene sequences that encode a tRNA with the highest number of pairings in the AA- and AC-stems. For example, *Uromastix* has 18 pairings, and the average among Australian and New Guinean taxa is 16.4 pairings. In contrast, the smallest number of pairings (average 13.9) is observed in the South Asian clade, which usually has a stem-and-loop structure in the typical position for O_L.

Because both the stem-and-loop structure in the typical position for O_L between the tRNA^{Asn} and tRNA^{Cys} genes and the tRNA^{Cys} gene have considerable secondary structure, the net increase in secondary structure between the two regions of DNA may be in equilibrium due to constraints on replication. Detailed sampling within other groups of squamate reptiles reported to contain tRNA^{Cys} genes that do not encode a D-stem (Macey et al., 1997b; Macey et al.,

1999b) would be an independent way of confirming our observation of longer AA- and AC-stems in taxa that lack a stem-and-loop structure in the typical position for O_L between the tRNA^{Asn} and tRNA^{Cys} genes.

tRNA^{Asn}.—Four potentially novel secondary structures are identified for tRNA^{Asn} (Fig. 6). In *Acanthosaura capra*, a base appears to have been inserted in the AA-stem, placing three bases between the AA- and D-stems instead of the two normally observed. This alternative secondary structure for tRNA^{Asn} appears to be an autapomorphy in *Acanthosaura capra* because it appears in no other taxon.

A second potentially novel secondary structure for tRNA^{Asn} is identified between the AC- and D-stems where two bases are observed instead of the single base normally found in that position (Kumazawa and Nishida, 1993). *Physignathus cocincinus* and all taxa sampled from Australia and New Guinea have this secondary structural deviation for tRNA^{Asn}. These taxa form a monophyletic group, and our sampling suggests that the addition of an extra base between the AC- and D-stems occurred in the ancestor of all Australian and New Guinean taxa prior to the separation of Southeast Asia, where *Physignathus cocincinus* occurs, hundreds of MYBP. This extra base appears to have been gained independently in the ancestor of the Sri Lankan genus *Ceratophora*, a member of the South Asian clade that may have an origin with the Indian Subcontinent (Macey et al., 2000).

A third novel secondary structure for tRNA^{Asn} is observed in an Australian species, *Arua modesta*, where only a single base was observed between the AA- and D-stems instead of the two bases normally found in that position (Kumazawa and Nishida, 1993). This structure is an autapomorphy for this taxon and may have evolved fairly recently, certainly after the separation of Southeast Asia, hundreds of MYBP.

A fourth novel secondary structure for tRNA^{Asn} has been reported in the iguanid lizard *Basiliscus plumifrons* (Figure 2 of Macey et al., 1997c). In this instance the variable loop is enlarged to seven bases instead of the normal three to five bases and appears to be an autapomorphy for this taxon.

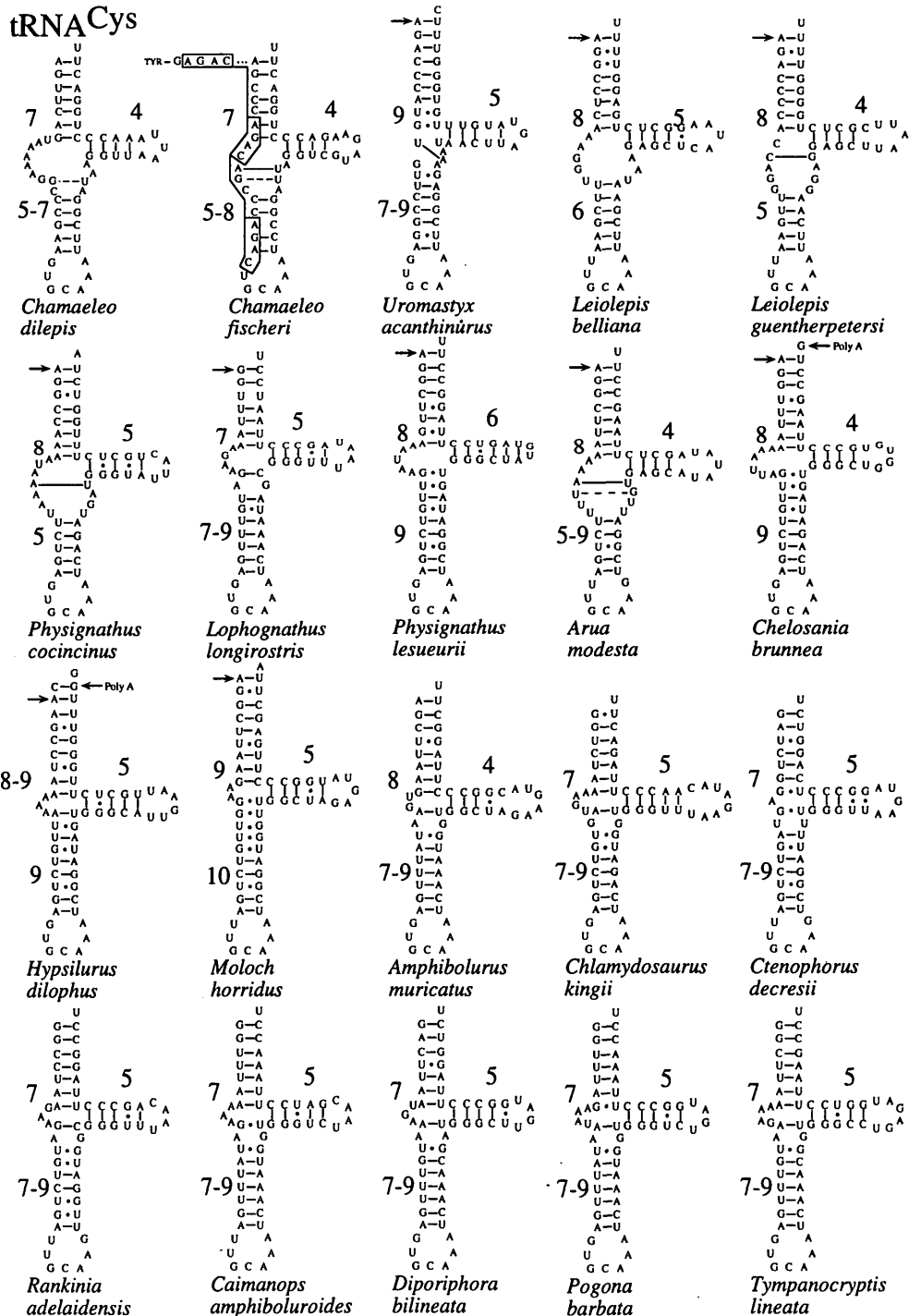


FIGURE 5. Secondary structure of tRNA^{Cys} encoded in the mitochondrial genome of acrodont lizards. Transfer RNAs are arranged in phylogenetic order (Macey et al., 2000). Dashed lines and dots depict potential G-U wobble pairings. Note that as the AA- and AC-stems get longer, the D-arm replacement and variable loops get smaller because of base pairing. Also note that if the T-stem shifts, it may contribute one base to the AA-stem and one base to the variable loop. See Figure 6 for the names of stems and loops in a standard tRNA. Arrows point to sequences that overlap with adjacent genes; sequences to the left overlap with the tRNA^{Tyr} gene, and those to the right overlap with the tRNA^{Asn} gene. Overlapping sequences with the tRNA^{Asn} gene might not be encoded but instead may be formed posttranscriptionally by polyadenylation (Börner et al., 1997). The first pairing in the

tRNA^{Cys}

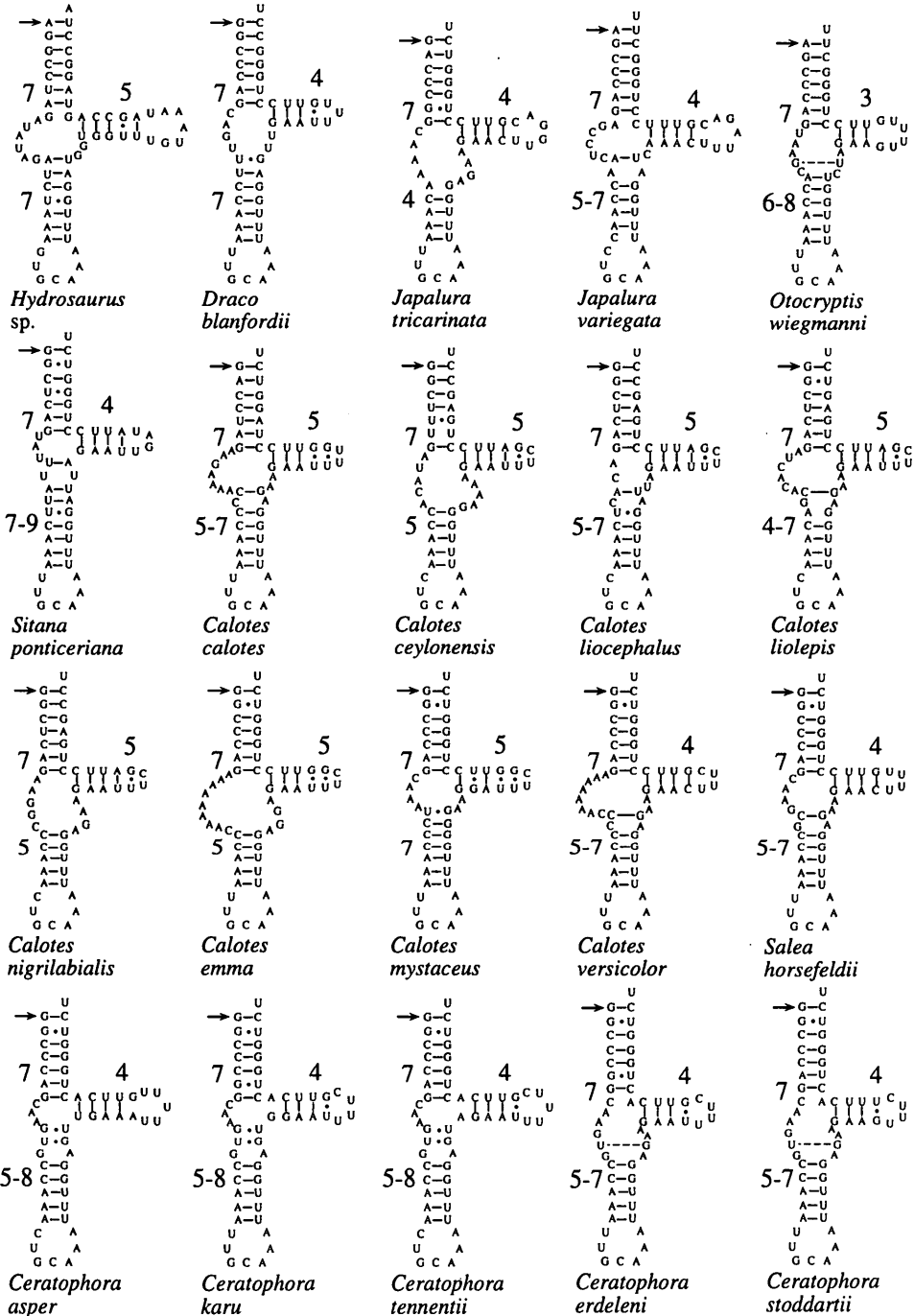


FIGURE 5. (Continued) AA-stem of the encoded tRNA^{Cys} in *Hypsilurus dilophus*, which overlaps adjacent tRNA genes, may not be encoded in the tRNA^{Cys} gene, according to sequences from other taxa in the clade containing *Physignathus cocininus* and Australian-New Guinean species. The tRNA^{Cys} genes encoding the longest AA- and AC-stems are found in *Uromastyx* and the clade containing *Physignathus cocininus* and Australian-New Guinean species. These taxa lack a stem-and-loop structure in the typical position for O_L between the tRNA^{Asn} and tRNA^{Cys} genes. The tRNA^{Cys} genes encoding the shortest AA- and AC-stems are found in the South Asian clade and have a stem-and-loop structure in the typical position for O_L between the tRNA^{Asn} and tRNA^{Cys} genes.

tRNA^{Cys}

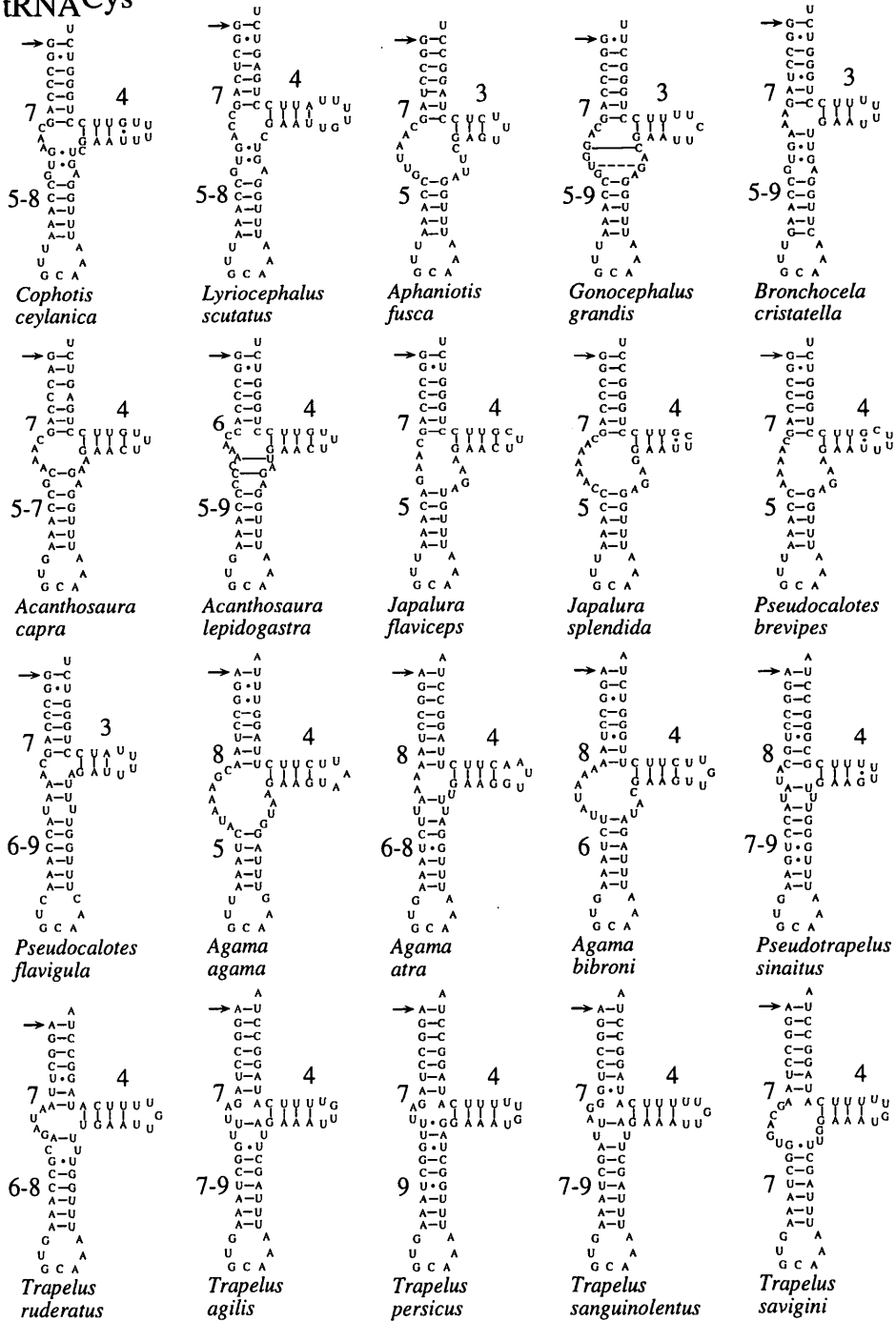


FIGURE 5. Continued

tRNA^{Cys}

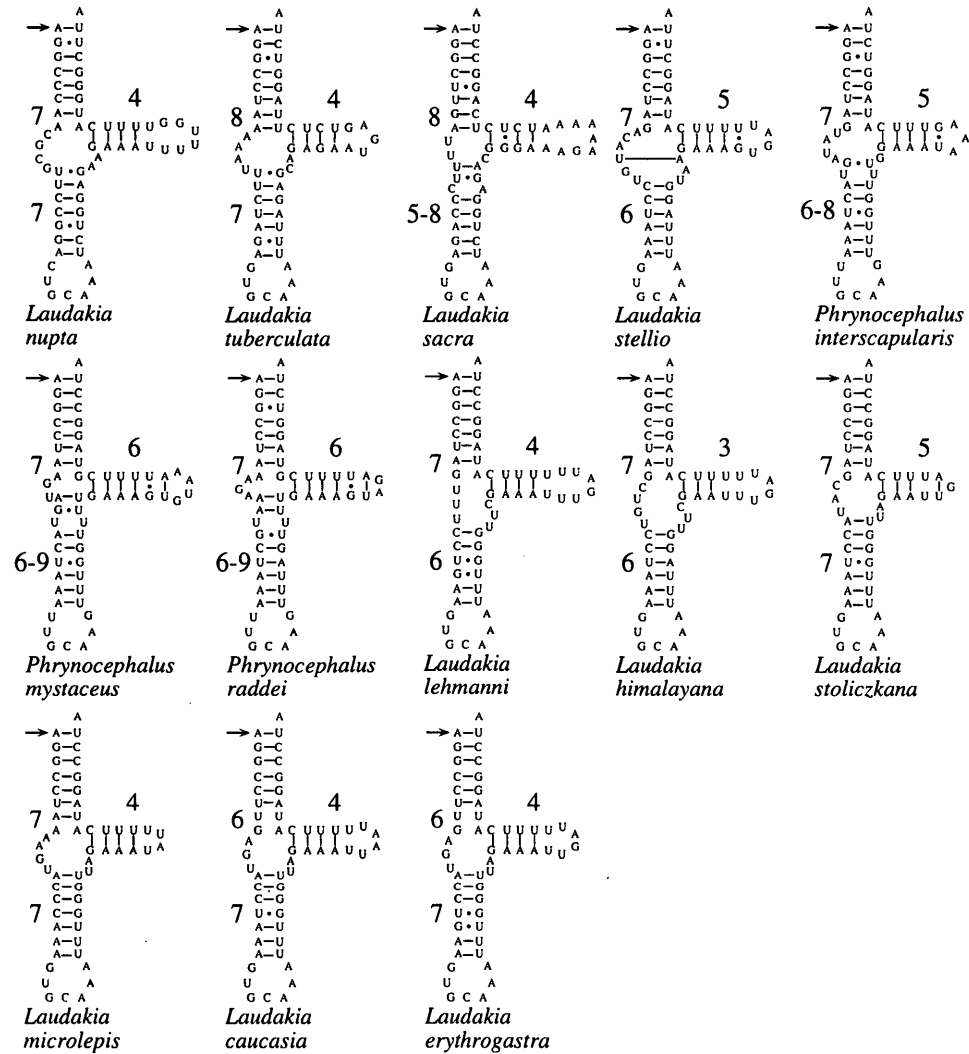


FIGURE 5. Continued

tRNA^{Tyr}.—In the *tRNA^{Tyr}* gene, both *Acanthosaura* species sampled have a gene that encodes a tRNA with a truncated T-stem of three base pairs instead of the usual five pairs (Fig. 6). The formation of a truncated T-stem in *tRNA^{Tyr}* among the two *Acanthosaura* species that occur entirely outside the Indian Subcontinent may have evolved after the Indian collision, 50 MYBP. Interestingly, the formation of a truncated T-stem in *tRNA^{Tyr}* has been reported also in the gene sequence of the snake, *Ovophis okinavensis* (Kumazawa et al., 1996).

tRNA^{Gln}.—The *tRNA^{Gln}* gene of *Aphanio-tis fusca* appears to encode a tRNA that has an AA-stem of only six pairs instead of seven (Fig. 6). This observation is highly unusual, requiring deletions on both sides of the AA-stem. It is an autapomorphy for this taxon.

Phylogenetic analysis of these seven derived secondary-structural features of tRNA gene sequences is compatible with our estimate of phylogeny with the observation of a single homoplasy (Fig. 7). Among the seven characters, three are parsimony in-

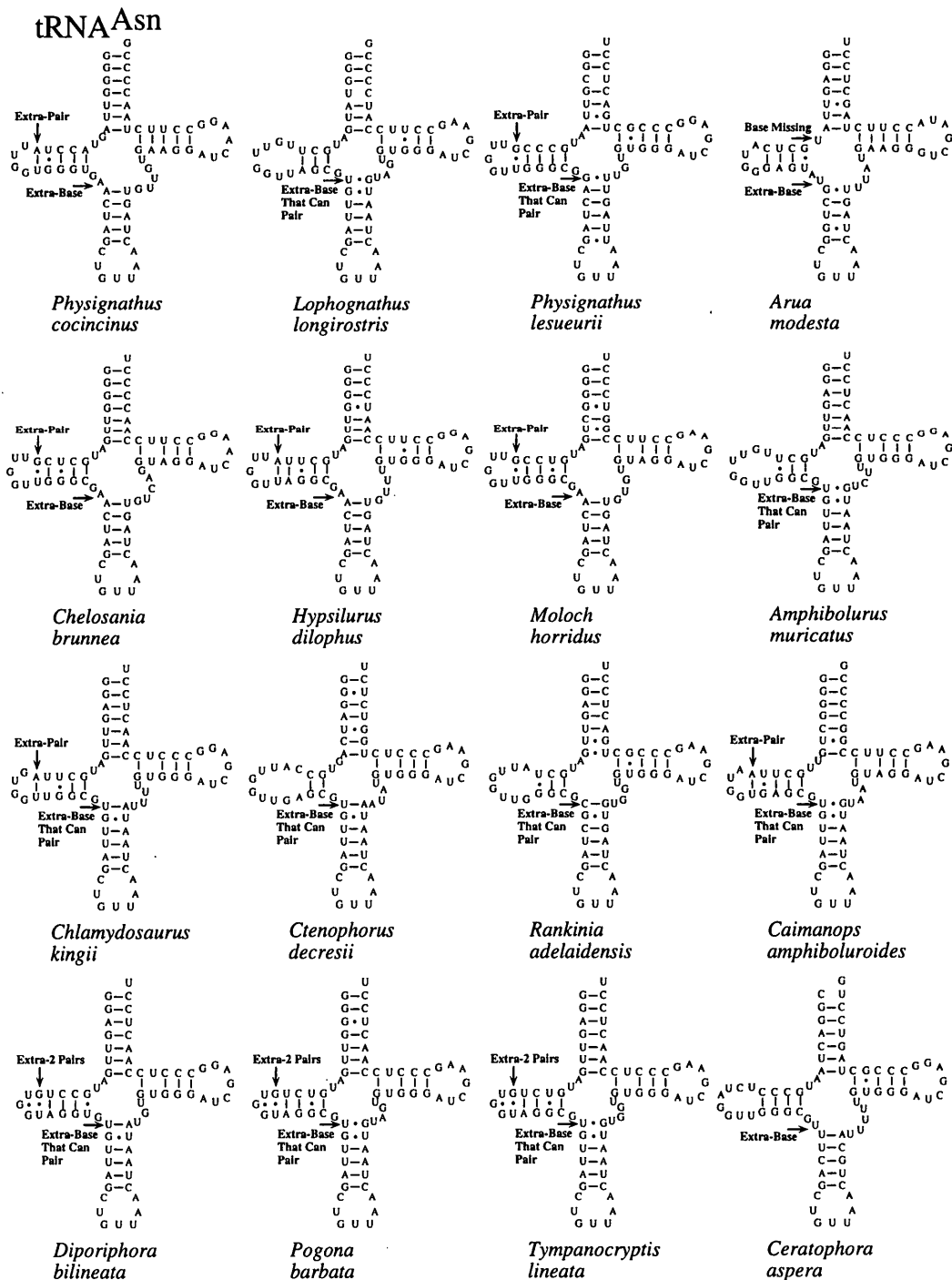


FIGURE 6. Variation in tRNA secondary structure suggested by gene sequences encoding tRNA^{Asn}, tRNA^{Tyr}, and tRNA^{Gln}. The last encoded tRNA^{Asn} shown has a typical tRNA secondary structure with a seven-base AA-stem, four-base D-stem, five-base AC-stem, three- to five-base variable loop, and a five-base T-stem. Between the AA- and D-stems are normally two bases, and between the AC- and D-stems is normally a single base. The three regions in which length variation is permitted are depicted with a large L in the D-loop, the variable loop, and the T-loop. Among tRNA^{Asn} gene sequences, *Acanthosaura capra* has a base inserted in the AA-stem. Base-shifting results in an extra base between the AA- and D-stems. In all other tRNAs, an extra base is inserted between the AC- and D-stems. Only a single base is observed between the AA- and D-stems in *Arua*. One other deviation in tRNA

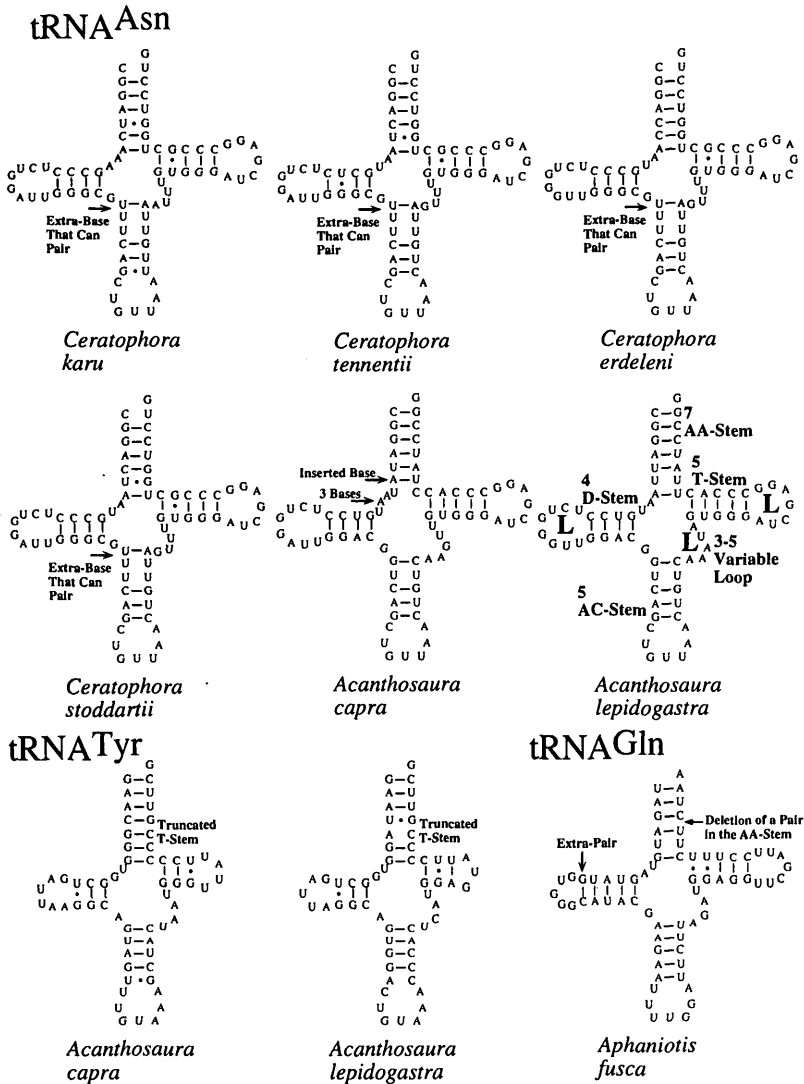


FIGURE 6. (Continued) secondary structure for the encoded tRNA^{Asn} has been reported in *Basiliscus plumifrons*, in which the variable loop is enlarged to seven bases instead of the normal five (Fig. 2 of Macey et al., 1997c). Variation in the number of potential base pairings is observed in the D-stem, which may have from three to six pairs instead of the standard four pairs. This observation is not highly unusual among mitochondrial tRNAs because there is always more than the minimal 12 bases between the AA- and AC-stems (Macey et al., 1997b). Two nonstandard tRNAs are encoded by sequences of the tRNA^{Tyr} gene in *Acanthosaura* species. Each encoded tRNA has a truncated T-stem that contains less than the 11 bases required to form a normal five-base stem and a minimal one-base loop. Each encoded tRNA has only three base pairings in the stem instead of the normal five pairs. In the sequence encoding tRNA^{Gln} of *Aphaniotis fusca*, a base pair from the middle of the AA-stem is deleted.

formative and four are autapomorphies, although more detailed examination may reveal that these characters are synapomorphies for groups not sampled extensively (i.e., *Basiliscus* or the Corytophaninae, *Acanthosaura capra*, *Aura*, and *Aphaniotis*). We estimate an average amount of homo-

plasy among the 1,137 variable nucleotide positions used for phylogenetic inference by Macey et al. (2000) of 8.4 homoplastic changes per site. Although rare, tRNA secondary structural changes show considerably less homoplasy than aligned base positions in nucleotide sequences.

Phylogenetic Tree of the Acrodonata
with tRNA Secondary Structural Characters

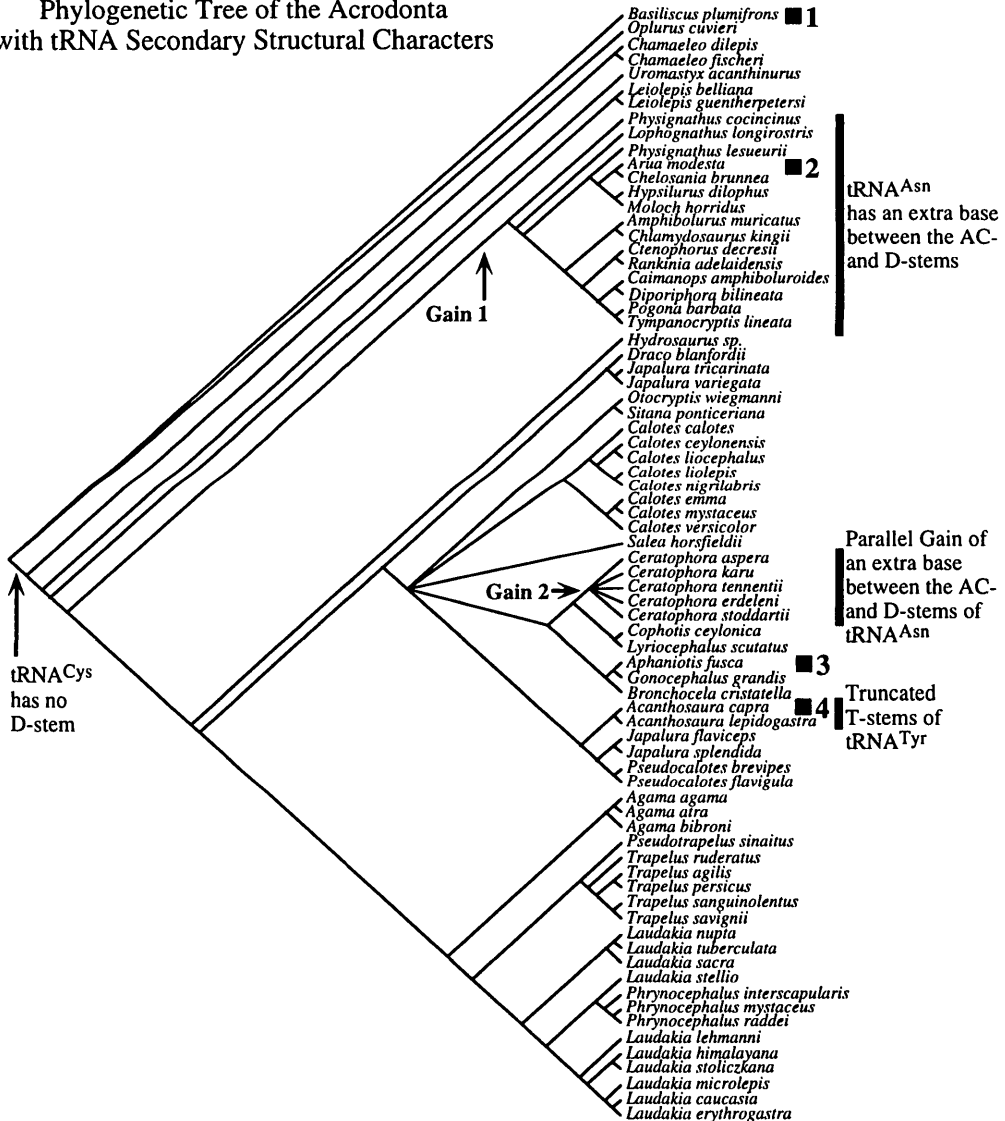


FIGURE 7. Phylogenetic tree of the Acrodonata showing changes in secondary structure of tRNAs. Seven deviations from the standard secondary structure of tRNAs are known in the Iguania. Four of these characters are autapomorphies, labeled with a square and numbered: (1) enlargement of the variable loop beyond five' bases, (2) an extra base between the AA- and D-stems in the sequence encoding tRNA^{Asn}, (3) loss of a base pair from the middle of the AA-stem in the sequence encoding tRNA^{Gln}, and (4) loss of a base between the AA- and D-stems in the sequence encoding tRNA^{Asn}. Three characters are synapomorphies for well-supported clades (Macey et al., 2000) and are indicated with arrows: (1) Monophyly of the Acrodonata (bootstrap 100%, decay index 79; Macey et al., 2000) is supported by the absence of a D-stem in the sequence encoding tRNA^{Cys}, (2) monophyly of *Acanthosaura* (bootstrap 100%, decay index 53) is supported by a truncated T-stem in the sequence encoding tRNA^{Tyr}, (3) a clade containing *Physignathus cocincinus* and all taxa sampled from Australia and New Guinea (bootstrap 100%, decay index 42) is supported by the presence of an extra base between the AC- and D-stems in the sequence encoding tRNA^{Asn} (labeled gain 1). This same character evolves independently in the common ancestor of *Ceratophora* species (bootstrap 97%, decay index 8) found in Sri Lanka (labeled gain 2). Within the Iguania, one homoplasy exists for the seven characters (Macey et al., 1997a, 1997c, 1998a; Schulte et al., 1998, 2000). Note the occurrence of synapomorphies at different levels of phylogenetic divergence: the Acrodonata, the Southeast Asian genus *Acanthosaura*, a clade from South Asia, and a clade from Southeast Asia, Australia and New Guinea.

DISCUSSION

Changes in major structural features of the mitochondrial genome, including gene order, displacement of a recognizable O_L , and secondary structures of tRNAs, are potentially important sources of phylogenetic information (Kumazawa and Nishida, 1995; Macey et al., 1997a; Wolstenholme, 1992). Our data suggest that at least some of these characters may be less subject to homoplasy than simple base-substitutional changes.

Because major structural features of the mitochondrial genome are known to vary among iguanian lizards, a thorough sampling of the Acrodonta provides a test of the evolutionary stability and phylogenetic utility of these features. Phylogenetic hypotheses derived from analysis of base-substitutional changes are used as a framework for studying evolutionary changes in major structural features of the mitochondrial genome. The major structural changes studied here are found not to undergo evolutionary reversal, making them useful markers at various levels of phylogenetic divergence.

Mitochondrial gene order appears particularly unlikely to undergo homoplastic evolution (Macey et al., 1997a). We reported previously (Macey et al., 1997c) that a switch in gene order of the genes encoding tRNA^{Ile} and tRNA^{Gln} in the mitochondrial genome is a reliable synapomorphy for the Acrodonta. Mechanistic considerations (Fig. 8; Macey et al., 1997a) predict that this feature is very stable evolutionarily and unlikely to undergo reversal or to evolve in parallel. A recent challenge (Mindell et al., 1998) to this idea suggests that gene order may be subject to parallelism in birds. However, two phylogenetic hypotheses on which this conclusion is based show little congruence, suggesting that a more definitive test of avian phylogeny is needed to establish parallelism for mitochondrial gene arrangement in birds. A more recent phylogeny based on complete mitochondrial genomes (Mindell et al., 1999) samples two of the groups that have the secondarily derived arrangement and cannot reject a single origin of the arrangement for these two groups. Furthermore, because Mindell et al. (1998) sequenced gene junctions rather than

1. AB > **ABAB** > AB No Rearrangement
Single deletion of the first two tRNA genes
2. AB > **ABAB** > AB No Rearrangement
Single deletion of the second two tRNA genes
3. AB > **ABAB** > AB No Rearrangement
Single deletion of the middle two tRNA genes
4. AB > **ABAB** > BA Gene Rearrangement
Separate deletions of the two end tRNA genes

FIGURE 8. Gene reorganization under a model of tandem duplication of genes as illustrated by the rearrangement of two adjacent genic segments, A and B (typically two tRNA genes). After tandem duplication of genic segments is produced, multiple deletions of redundant genes occur (Moritz et al., 1987). If a single deletion occurs, the gene order will return to the previous state (cases 1–3). Only when multiple deletions occur does a rearrangement result (case 4). Hence, shifts in gene order require more than one deletion event, making parallelisms and reversals unlikely. This mechanism explains most mitochondrial genomic rearrangements among vertebrates (Macey et al., 1997a, 1998b).

the entire rearranged segment, further study may reveal that the arrangements observed in different taxa are not identical.

Results presented here increase our sampling of the mitochondrial genes known to undergo rearrangement to 70 acrodont species, representing all seven major acrodont groups that have been evolving independently for at least 160 million years (see Macey et al., 1997c). Sampling across 125 other squamate reptiles shows no evidence of parallelism, but instead indicates that a duplicated Control Region is also a reliable phylogenetic character for the snake families Boiidae, Colubridae, and Viperidae (Table 1). In addition, a third gene arrangement is an autapomorphy in the snake family Leptotyphlopidae (Kumazawa and Nishida, 1995). The novel gene order of ND1, tRNA^{Gln}, tRNA^{Ile}, and tRNA^{Met} appears completely stable in all acrodont lineages and does not show evidence of parallelism, confirming predictions that it is a highly stable feature and a reliable synapomorphy for the Acrodonta.

Displacement of a recognizable O_L from the typical vertebrate position between the tRNA^{Asn} and tRNA^{Cys} genes is known to evolve in parallel among vertebrates (Macey et al., 1997a). The larger sampling of the Acrodonta reported here also con-

TABLE 1. Summary of 195 squamate reptile taxa that have been sampled for all gene junctions between the ND1 and COI genes.

Squamate taxon	Sampled species	Gene order ^a	Reference
Iguanidae			
Corytophaninae	1	Plesiomorphic	Macey et al., 1997c
Crotaphytinae	2	Plesiomorphic	Macey et al., 1997c
Hoplocercinae	1	Plesiomorphic	Macey et al., 1997c
Iguaninae	2	Plesiomorphic	Macey et al., 1997c; Schulte et al., 1998
Oplurinae	1	Plesiomorphic	Macey et al., 1997c
Phrynosomatinae	8	Plesiomorphic	Kumazawa and Nishida, 1995; Macey et al., 1997c; Schulte et al., 1998
Polychrotinae	1	Plesiomorphic	Macey et al., 1997c
Tropidurinae	61	Plesiomorphic	Macey et al., 1997c; Schulte et al., 1998, 2000
Acrodonta			
Agamidae			
Uromastycinae	1	Derived (ND1, QIM)	Macey et al., 1997a
Leiolepidinae	2	Derived (ND1, QIM)	Macey et al., 1997c; this study
Amphibolurinae	15	Derived (ND1, QIM)	Macey et al., 1997c; this study
Hydrosaurinae	1	Derived (ND1, QIM)	this study
Draconinae	27	Derived (ND1, QIM) ^b	this study
Agaminae	22	Derived (ND1, QIM)	Macey et al., 1997c, 1998a, this study
Chamaeleonidae	2	Derived (ND1, QIM)	Macey et al., 1997c; this study
Gekkonidae			
Eublepharinae	2	Plesiomorphic	Macey et al., 1999b; Kumazawa and Nishida, 1995
Gekkoninae	5	Plesiomorphic	Macey et al., 1997a, 1999b
Pygopodidae	1	Plesiomorphic	Unpublished data ^c
Xantusiidae	1	Plesiomorphic	Macey et al., 1997a
Lacertidae	1	Plesiomorphic	Macey et al., 1997a
Teiidae	1	Plesiomorphic	Macey et al., 1997a
Cordylidae	1	Plesiomorphic	Macey et al., 1997a

firmly uniform absence of a recognizable O_L at the typical vertebrate position between the genes encoding tRNA^{Asn} and tRNA^{Cys}. Loss of the stem-and-loop structure is inferred to have occurred at least three times in parallel and may have been lost in seven separate events. Although this feature appears not to be subject to reversal, it has been lost in parallel among the Acrodonta and other vertebrate lineages (Macey et al., 1997a); therefore, it is a less reliable phylogenetic character than gene order.

Within iguanian lizards, seven unique secondary structures are observed for mitochondrial encoded tRNAs. These structures include loss or reduction of D- or T-stems, insertion of extra bases between adjacent stems, and the deletion of pairings in the

middle of stems. Only a single homoplasy is observed for these secondary structural changes within iguanian lizards (sampled here and in Macey et al., 1997a, 1997b, 1997c, 1998a, Schulte et al., 1998, 2000). Thus, a phylogenetic tree for the Acrodonta derived strictly from tRNA secondary structure is nearly compatible with, although less resolved than, the phylogenetic estimate obtained from DNA substitutional data (Fig. 7; Macey et al., 2000). We calculate that base positions in the 70 analyzed acrodont sequences show an average of 8.4 homoplastic changes, substantially more than observed for tRNA secondary structural characters (0.14 homoplastic changes per character). Hence, novel secondary structures may be useful phylogenetic

TABLE 1. Extended

Squamate taxon	Sampled species	Gene order ^a	Reference
Scincidae	2	Plesiomorphic	Kumazawa and Nishida, 1995, 1999; Macey et al., 1997a
Dibamidae	1	Plesiomorphic	Macey et al., 1997a ^b
Anguinae			
Diploglossinae	6	Plesiomorphic	Macey et al., 1999a
Anguinae	6	Plesiomorphic	Macey et al., 1999a
Gerrhonotinae	9	Plesiomorphic	Macey et al., 1997c, 1999a
Anniellidae	2	Plesiomorphic	Macey et al., 1999a
Helodermatidae	1	Plesiomorphic	Macey et al., 1999a
Shinisauridae	1	Plesiomorphic	Macey et al., 1999a
Varanidae	1	Plesiomorphic	Macey et al., 1997a
Xenosauridae	1	Plesiomorphic	Macey et al., 1997a
Amphisbaenia	1	Plesiomorphic	Macey et al., 1997a
Serpentes			
Leptotyphlopidae	1	Derived (IM, ND2, WQA)	Kumazawa and Nishida, 1995
Boiidae	1	Derived (ND1, I, CR, LQ) ^d	Kumazawa and Nishida, 1995; Kumazawa et al., 1996
Viperidae	2	Derived (ND1, IP, CR, FLQ) ^d Derived (ND1, IP, CR, LQ) ^d	Kumazawa et al., 1996
Colubridae	1	Derived (ND1, IP*, CR, LQ) ^e	Kumazawa et al., 1996, 1998

^aThe plesiomorphic condition of the genomic segment reported includes, in the following order, genes encoding NADH dehydrogenase subunit 1 (ND1), tRNA^{Ile} (I), tRNA^{Gln} (Q), tRNA^{Met} (M), NADH dehydrogenase subunit 2 (ND2), tRNA^{Trp} (W), tRNA^{Ala} (A), tRNA^{Asn} (N), tRNA^{Cys} (C), tRNA^{Tyr} (Y), and cytochrome c oxidase subunit I (COI). For rearrangements, the rearranged portion of the genome is indicated in parentheses. Some rearrangements also include genes encoding tRNA^{Phe} (F), tRNA^{Leu} (L), and tRNA^{Pro} (P).

^b*Sitana ponticeriana* contains an extra sequence between the ND2 and tRNA^{Trp} genes; this sequence appears to be a highly modified, nonfunctional duplicate of the tRNA^{Trp} gene.

^cThe sequence reported in Macey et al. (1997a) as *Lialis jicari* is actually that of *Dibamus novaeguineae*. An unpublished sequence of *Lialis jicari* confirms its gene order as plesiomorphic.

^dNot sampled for the gene junctions from the tRNA^{Met} through COI genes; CR represents a duplicated Control Region.

^eAsterisk denotes a pseudogene.

markers independent of simple base-substitutional data.

Several novel secondary structures are identified in the sequences encoding tRNA^{Asn}, tRNA^{Cys}, tRNA^{Gln}, and tRNA^{Tyr} (Table 2). Secondary structures are most variable in the sequences encoding tRNA^{Cys}. These structures are hard to classify and, because the T-stem is shifting and the AA- and AC-stems are extending, homologous sites are hard to determine (Macey et al., 1997b). The D-stem is known to have been lost in parallel nine times among squamate reptiles (Macey et al., 1997b, 1999b). Although this character is prone to parallel loss, it may be useful at shallower levels of divergence. Within the Iguania, loss of the D-stem defines the Acrodonta and no homoplasy is observed (Fig. 7;

Macey et al., 1997b, 1997c, 1998a; Schulte et al., 1998, 2000).

Among the sequences encoding tRNA^{Gln} and tRNA^{Asn}, four novel secondary structures appear as autapomorphies in iguanian lizards. *Aphaniotis fuscus* has a base pair deleted from the AA-stem of tRNA^{Gln}. *Basiliscus plumifrons* has an enlarged variable loop in tRNA^{Asn}. *Acanthosaura capra* has a base inserted in the AA-stem of tRNA^{Asn}, which causes a structural rearrangement resulting in three bases between the AA- and D-stem. *Arua modesta* has a single base located between the AA- and D-stems of tRNA^{Asn}.

An additional novel secondary structure for tRNA^{Asn} characterizes two separate clades of iguanian lizards. All species sampled from the clade comprising *Physis-*

TABLE 2. Secondary structural deviations from standard tRNAs and their phylogenetic utility.

Character	Rate of occurrence	Phylogenetic pattern
1. Loss of D- or T-stem	Uncommon	Parallel loss ^a
2. Loss of base between AA- and D-stems	Uncommon	Parallel loss ^b
3. Insertion of base between AA- and D-stems	Rare	Autapomorphic ^c
4. Insertion of base between AC- and D-stems	Rare	Rare parallelism ^d
5. Deletion of paired bases in the middle of the AA-stem	Rare	Autapomorphic ^e
6. Extension or reduction in number of base pairings adjacent to the D- or T-loops	Common	Highly homoplastic
7. Reduction in the D- or T-stem and loop to <12 bases in the D-stem and loop, and <11 bases in the T-stem and loop	Uncommon	Parallel loss ^f
8. Enlargement of the variable loop beyond 5 bases	Rare	Autapomorphic ^g

^aLoss of the sequence encoding a D-stem from the tRNA^{Cys} gene occurs at least nine times among squamate reptiles.

^bLoss of a base between AA- and D-stems is observed in the sequence encoding the tRNA^{Asn} from *Arua modesta* and has been reported in other mitochondrial tRNAs (Janke et al., 1994).

^cThe only case of an extra base between the AA- and D-stems is that observed in the sequence encoding tRNA^{Asn} of *Acanthosaura capra*.

^dThe only two cases of an extra base between the AC- and D-stems occur in sequences encoding tRNA^{Asn} of (1) *Physignathus cocincinus* and all acrodont taxa sampled from Australia and New Guinea and (2) the genus *Ceratophora*.

^eThe only case of deletion of paired bases in the AA-stem occurs in the sequence encoding tRNA^{Gln} of *Aphaniotis fusca*.

^fThe reduction of the D- or T-stem and loop to <12 bases in the D-stem and loop (Macey et al., 1997b), and <11 bases in the T-stem and loop is rare (Kumazawa et al., 1996; Macey and Verma, 1997) but parallel reduction in the T-stem of mitochondrial tRNAs is observed between the snake (*Ovophis okinavensis*; Kumazawa et al., 1996) and *Acanthosaura* species for sequences encoding tRNA^{Tyr}.

^gThe only case of an enlargement of the variable loop beyond five bases is observed in *Basiliscus plumifrons* for sequences encoding the tRNA^{Asn} (Macey et al., 1997c).

nathus cocincinus and all taxa from Australia and New Guinea have an extra base between the AC- and D-stems. This same character occurs in the five species of *Ceratophora* from Sri Lanka. Although two independent origins are inferred for this derived character, we estimate that the first one occurred before the split of the south-east Asian and Australian–New Guinean plates hundreds of MYBP, whereas the second one, associated with the Indian Plate, probably occurred much more recently. At a shallower level, the truncated T-stem in tRNA^{Tyr} characterizes *Acanthosaura* species, and the only parallelism for this character is observed in a single snake (*Ovophis*; Kumazawa et al., 1996).

The scoring of major structural changes in the mitochondrial genome is enhanced by sequencing long, multigenic regions of the mitochondrial genome as illustrated by this study. We predict that structural changes of the kind analyzed here would be reported much more frequently if sequencing of multigenic regions were more common in the systematic literature. Too many systematic studies rely entirely on reporting DNA sequences from fragments of sin-

gle genes, usually those encoding cytochrome *b*, cytochrome *c* oxidases, or the two major subunits of ribosomal RNA. Phylogenetic information from major structural features may occasionally be able to resolve branches deep in a phylogenetic tree, where relationships are hard to resolve by using nucleotide-substitutional variation alone.

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