Phylogenetic relationships among amphisbaenian reptiles based on complete mitochondrial genomic sequences

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Abstract

Complete mitochondrial genomic sequences are reported from 12 members in the four families of the reptile group Amphisbaenia. Analysis of 11,946 aligned nucleotide positions (5797 informative) produces a robust phylogenetic hypothesis. The family Rhineuridae is basal and Bipedidae is the sister taxon to the Amphisbaenidae plus Trogonophidae. Amphisbaenian reptiles are surprisingly old, predating the breakup of Pangaea 200 million years before present, because successive basal taxa (Rhineuridae and Bipedidae) are situated in tectonic regions of Laurasia and nested taxa (Amphisbaenidae and Trogonophidae) are found in Gondwanan regions. Thorough sampling within the Bipedidae shows that it is not tectonic movement of Baja California away from the Mexican mainland that is primary in isolating \textit{Bipes} species, but rather that primary vicariance occurred between northern and southern groups. Amphisbaenian families show parallel reduction in number of limbs and \textit{Bipes} species exhibit parallel reduction in number of digits. A measure is developed for comparing the phylogenetic information content of various genes. A synapomorphic trait defining the Bipedidae is a shift from the typical vertebrate mitochondrial gene arrangement to the derived state of \textit{trnE} and \textit{nad6}. In addition, a tandem duplication of \textit{trnT} and \textit{trnP} is observed in \textit{Bipes biporus} with a pattern of pseudogene formation that varies among populations. The first case of convergent rearrangement of the mitochondrial genome among animals demonstrated by complete genomic sequences is reported. Relative to most vertebrates, the Rhineuridae has the block \textit{nad6}, \textit{trnE} switched in order with the block \textit{cob}, \textit{trnT}, \textit{trnP}, as they are in birds.

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1. Introduction

Amphisbaenians are fossorial reptiles that inhabit tropical and semitropical regions of the world (Fig. 1). The vast majority of species occur on land masses of Gondwanan origin in South America, Africa, and Arabia. However, two of the four families are restricted to regions associated with Laurasia, the Bipedidae, with three species in Mexico (Fig. 2), and the Rhineuridae, with a single species in Florida. These taxa have pleurodont dentition (Gans, 1968) and were originally placed in the family Amphisbaenidae which now primarily includes taxa distributed in Gondwanan regions of South America and Africa (Gans, 1978). The fourth family of Amphisbaenia, the Trogonophidae, is the only one to have acrodont dentition and is restricted to Gondwanan plates of North Africa, Arabia, and the island of Socotra. By superimposing a phylogeny of these groups on their distribution relative to the breakup of these major
land masses, it is possible to test hypotheses about their origins. For example, monophyly of taxa found in Laurasia or Gondwana may reflect their isolation stemming from the breakup of Pangaea 200 million years before present (MYBP) (Figs. 3C and D).

Among the four families of Amphisbaenia, all taxa are limbless except the Bipedidae, which contains reptiles with two forelimbs. If gradual reduction in limbs were to have occurred from an ancestor with both hind- and forelimbs, then the Bipedidae, with its presumed intermediate state, would be thought to be the sister taxon to the remaining families as has been suggested by morphological data (Kearney, 2003; Fig. 3B).

Within the Bipedidae, species are variable for the number of digits on a limb (Papenfuss, 1982). The Baja California taxon, Bipes biporus, always has five digits on each limb. In mainland Mexico, the more northern taxon, Bipes canaliculatus, is variable, with either four or five digits, while the more southern species, Bipes tridactylus, always has three digits on each limb. If digits are being gradually lost from an ancestor with five digits then B. biporus is predicted to be the sister taxon to the other two species (Fig. 3E). This phylogenetic hypothesis is consistent with an initial splitting of Bipes by the separation of peninsular Baja California from the west coast of Mexico (12–14 MYBP; Ferrari, 1995), with the two mainland species later diverging. Alternatively, tectonic activity along the west coast of Mexico in the Cretaceous to Paleocene (Ferrari, 1995) may have isolated the more northern taxa, B. biporus and B. canaliculatus, from B. tridactylus to the south (Fig. 3F).

Mitochondrial gene order has often been found to be a robust phylogenetic character for reconstructing evolutionary relationships among animals (Boore, 1999; Boore and Brown, 1998; Macey et al., 1997a, 2000). Although no character can be asserted to be completely...
free from the possibility of homoplasious change, parallelisms or reversals of gene rearrangements seem, in principle, to be unlikely, and have not been observed to date in sampling approximately 300 complete mtDNA sequences. A common mode of mitochondrial genomic rearrangement appears to be a tandem duplication of genes with random loss of extra copies (Boore, 2000; Macey et al., 1997a; Moritz et al., 1987). Hence, the pattern of pseudogene formation in a tandemly duplicated segment is causative in establishing novel gene orders.

Amphisbaenian reptiles are known to have several novel mitochondrial genomic features. *Bipes biporus* has an unusual stem and loop structure atypical of vertebrates between *trnN* and *trnC* (Macey et al., 1997a) where light-strand replication usually initiates. This character has been linked to mitochondrial genomic rearrangement among vertebrates (Macey et al., 1997a). In this same taxon, *trnC* encodes a transfer RNA that lacks a dihydrouridine (D) arm and instead contains a D-arm replacement loop (Macey et al., 1997b). In addition, this taxon has a tandem duplication of *trnT* and *trnP* with a pattern of pseudogene formation that does not result in mitochondrial genomic rearrangement (Macey et al., 1998).

In addition, each complete mtDNA sequence includes 37 genes whose sequences can be compared (about 16kb in total). Recent studies have shown that comparisons of complete mtDNA sequences give much more robust phylogenetic reconstructions than when using smaller portions of the mtDNA (Ingman et al., 2001).

The complete mitochondrial genomic sequence is newly reported from 12 specimens representing all four

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**Fig. 3. Alternative phylogenetic hypotheses tested.**

Hypotheses (A) through (D) concern relationships among amphisbaenian families. (A) Monophyly of taxa with pleurodont “P” as opposed to acrodont “A” dentition (Gans, 1968). (B) Monophyly of limbless taxa (Kearney, 2003) “0” as opposed to having two forelimbs “2.” (C and D) Monophyly of Laurasian “L” vs. monophyly of Gondwanan “G” taxa. Note that hypothesis (A) is consistent with (C), and hypothesis (B) is consistent with (D). Hypotheses (E and F) concern relationships among the three species in the Bipedidae where on the trees “B” represents Baja California and “M” represents mainland Mexico. The number of digits in forelimbs are also plotted on the trees. (E) Monophyly of the two mainland *Bipes* taxa which have reduced numbers of digits (3 and usually 4; Papenfuss, 1982). (F) Monophyly of the more northern *Bipes* taxa, which suggests independent loss of digits in the two mainland species.
families of the Amphisbaenia (Fig. 1). The single species of Rhineuridae, *Rhineura floridana*, is sampled from Florida. In the Bipedidae, samples from several localities in each of the three species are sequenced (Fig. 2). *Diplometapon zarudnyi* is sampled from Iran on the Arabian Shield to represent the Trogonophidae. In the Amphisbaenidae, *Amphisbaena schmidti* from Puerto Rico is used as a representative of the New World radiation and *Geocalamus acutus* from Tanzania is sampled to represent the Old World. Two diverse lizard taxa are used as outgroups with data from the literature, an iguanian, *Iguana iguana* (AJ278511, Janke et al., 2001), and a sceleglossan, *Eumeces egregius* (AB016606, Kumazawa and Nishida, 1999).

2. Materials and methods

The alignment of sequences is deposited at Elsevier electronic supplementary material to this article.

2.1. Specimen information

All voucher specimens are deposited at the Museum of Vertebrate Zoology, University of California at Berkeley (MVZ), or Universidad Nacional Autónoma de México (UNAM). Full locality data are listed in GenBank files. Species names, followed by corresponding museum voucher specimen numbers, then GenBank accession numbers are as follows: *Rhineura floridana*, Florida (MVZ 233342, AY605473); *Diplometapon zarudnyi*, Iran (MVZ 234273, AY605474); *Amphisbaena schmidti*, Puerto Rico (MVZ 232754, AY605475); *G. acutus*, Tanzania (MVZ 232838, AY605476); *Bipes tridactylus*, 13km south of Rio Tecpan, Guerrero, Mexico (MVZ 236261, AY605477); *B. tridactylus*, south bank Rio Tecpan, Guerrero, Mexico (MVZ 236262, AY605478); *B. tridactylus*, north bank Rio Tecpan, Guerrero, Mexico (MVZ 236305, AY605479); *B. biporus*, Vizcaino, northern Baja California, Mexico (MVZ 236257, AY605480); *B. biporus*, La Paz, southern Baja California, Mexico (MVZ 236258, AY605481); *B. candidiculatus*, Las Cañas, Michoacan, Mexico (MVZ 233341, AY605482); *B. candidiculatus*, Mezcala, Guerrero, Mexico (MVZ 240725, AY605483); and *B. candidiculatus*, Petalcalco, Guerrero, Mexico (UNAM-TP27893, AY605484).

2.2. Laboratory protocols

Genomic DNA was extracted from liver or muscle using the Qiagen QIAamp tissue kit. Amplification of genomic DNA was conducted using rTh long PCR enzyme (Applied Biosystems) with a denaturation at 94°C for 35s, annealing at 50°C for 35s, and extension at 70°C for 150s with 4s added to the extension per cycle, for 38 cycles. Negative controls were run on all amplifications to check for contamination. Initial amplifications were conducted using primers described in Macey et al. (1997a, 1998). Perfectly matching primers were then constructed for each taxon based on the DNA sequence of this fragment to complete the amplification of each mtDNA.

Amplification products were sheared randomly into fragments of approximately 1.5 kb by repeated passage through a narrow aperture using a Hydrashear device. After end-repair, the sheared DNA was gel purified and ligated into pUC18 vector to construct a library of random fragments, and then transformed into bacterial cells. Automated colony pickers introduced single clones into bacterial broth in 384-well format. These plasmid clones were processed robotically through rolling circle amplification (Dean et al., 2001; Hawkins et al., 2002), sequencing reactions, and reaction cleanup using SPR1 (Elkin et al., 2002). Sequences were determined using ABI3730xl DNA sequencers and then assembled to form deep contigs using Phrp or Sequencer.

2.3. Phylogenetic analysis

DNA sequences were aligned manually. Positions encoding proteins were translated to amino acids using MacClade 4.03 (Maddison and Maddison, 2001) for confirmation of alignment. Alignments of sequences encoding tRNAs were constructed based on secondary structural models (Kumazawa and Nishida, 1993; Macey and Verma, 1997). Secondary structures of tRNAs were inferred from primary structures of the corresponding tRNA genes using these models. Unalignable regions (see Section 3) were excluded from phylogenetic analyses. Non-coding and rRNA gene sequences were not included due to ambiguity of alignment.

Phylogenetic trees were inferred by parsimony using PAUP* beta version 4.0b8 (Swofford, 2001) with the branch and bound search option, which guarantees an exact solution. Bootstrap resampling (Felsenstein, 1985a) was applied to assess support for individual nodes using 1000 branch and bound replicates. Decay indices (=“branch support” of Bremer, 1994) were calculated for all internal branches using branch and bound searches that retained suboptimal nodes.

Alternative phylogenetic hypotheses (Fig. 3) were tested using the most parsimonious phylogenetic topologies compatible with each. To find the most parsimonious tree(s) compatible with a particular phylogenetic hypothesis, topologies were first constructed using MacClade (Maddison and Maddison, 2001), providing input as constraint trees to PAUP* (Swofford, 2001) for subsequent branch and bound searches. Wilcoxon-signed-ranks tests (Templeton, 1983) were used to examine statistical significance of the shortest tree relative to alternative hypotheses. This test determines whether the most parsimonious tree is significantly shorter than an
alternative or whether their differences in length are statistically indistinguishable. Wilcoxon signed-ranks tests were conducted as two-tailed tests (Felsenstein, 1985b). Tests were conducted using PAUP* (Swofford, 2001), which incorporates a correction for tied ranks.

3. Results

3.1. Alignment

Mitochondrial genomic sequences range in size from 16,196 to 17,423 nucleotides. Nearly all nucleotides coding for transfer RNAs or proteins are deemed alignable. In tRNA genes, the D-loop is excluded from trnI, A, N, Y, and H. The D-arm replacement loop and V(variable)-loop are excluded from trnSl. In trnLJ the D- and V-loops are excluded. The D- and T-loops are excluded from trnF, V, M, W, C, D, K, R, E, T, and P. This also included the D-stem in trnC because some taxa contain a D-arm replacement loop. The D-, V-, and T-loops are excluded from trnG. This resulted in the exclusion of 271 positions among tRNA genes. In protein coding regions, a total of 258 amino acid positions (774 nucleotide sites) are excluded as follows with the number in parentheses a total of 258 amino acid positions (774 nucleotide sites) are excluded from tRNA and protein coding regions.

3.2. Phylogenetic information content

The genic regions included in the phylogenetic analysis have different levels of phylogenetic information (Table 1). To compare regions that were sequenced for different numbers of bases, we standardized these values as the ratio of the number of informative sites over the number of bases sequenced in a region, then multiply by 1000. We term this the standardized information content (SIC). The SIC value for the total data set analyzed is 485. The tRNA genes combined, and each of the protein genes nad1, cox1, cox2, cox3, and cob have SIC values below that for the total data set, while nad2, atp8, atp6, nad3, nad4L, nad4, nad5, and nad6 have SIC values higher than that for the total data set. The transfer RNA genes collectively have the lowest SIC value of 352 while contributing 8% of the total phylogenetic information. Nad4L has the highest SIC value of 621 while only contributing 3.1% of the total phylogenetic information. It is interesting that the protein encoding genes that are generally the most well conserved (e.g., cox1 and cob), the most amenable to study by PCR amplification based on having well-conserved regions for designing primers, and that are most commonly employed in phylogenetic studies that use only one or a few genes, are among the lowest in SIC. Perhaps this is due to their having high among-site rate variation, i.e. a low fraction of their sites are free to change, so that undetected multiple substitutions occur at some sites, while many other sites remain constant, hence reducing phylogenetic signal.

3.3. Phylogenetic relationships

The alignment of 11,946 nucleotide positions contains 5797 that are phylogenetically informative. A single most parsimonious tree is produced and all phylogenetic relationships are well supported (Fig. 4). Each branch of the tree has bootstrap support of 100% and very high decay indices with the exception of the monophyly of the Amphisbaenidae (Amphisbaena and Geocalamus) with a

Table 1. Phylogenetic information by genic region in the common vertebrate gene order.

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<th>Genic Region</th>
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<th>Non-informative variable sites</th>
<th>Phylogenetically informative sites</th>
<th>Percent of total informative sites (%)</th>
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<td>485</td>
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a Calculated as the ratio of phylogenetically informative positions and the total number of characters in a data partition, standardized per kilobase by multiplying by 1000.
3.4. Statistical evaluation of alternative hypotheses

The phylogenetic results provide an area cladogram and a framework to examine the evolution of dentition and limbs in amphisbaenian reptiles. The Wilcoxon-signed-ranks test (Felsenstein, 1985b; Templeton, 1983) is applied to compare the most parsimonious tree from these nucleotide sequences with alternative hypotheses (Fig. 3).

(1) Trogonophids have acrodont dentition, but the family is nested within taxa having pleurodont dentition (Amphisbaenidae, Bipedidae, and Rhineuridae), leading to the inference that the acrodont condition is derived for this lineage. The shortest alternative tree that unites taxa with pleurodont dentition (Fig. 3A) requires 312 extra steps and is rejected in favor of the unconstrained shortest tree ($n=1030$, $Z=9.7216$, $P<0.0001$).

(2) Bipedids have forelimbs, but the family is nested within limbless taxa, leading to the inference that loss of limbs occurred multiple times, minimally twice, once in the Rhineuridae and once in the common ancestor of Trogonophidae and Amphisbaenidae. The alternative, that limbs were lost once in the common ancestor of amphisbaenians, then regained in bipedids, seems very unlikely, but see Greene and Cundall (2000). The shortest alternative tree that unites limbless taxa as monophyletic (Fig. 3B) requires 187 extra steps and is rejected in favor of the unconstrained shortest tree ($n=523$, $Z=8.1769$, $P<0.0001$).

(3) The Gondwanan Amphisbaenidae and Trogonophidae appear as monophyletic in the unconstrained shortest tree (Fig. 3D), with the Laurasian Rhineuridae and Bipedidae (Bipes) represent successive, paraphyletic basal lineages indicating that amphisbaenian reptiles predate the breakup of Pangaea 200MYBP.

(4) The unconstrained shortest tree unites the northern Bipes species (B. biporus and B. canaliculatus) to the exclusion of B. tridactylus rather than phylogenetically separating the mainland Mexican species from those on the Baja peninsula (Fig. 3F). The shortest alternative tree that unites mainland Mexican Bipes, each of which has reduced numbers of digits, as monophyletic (Fig. 3E) requires 116 extra steps and is rejected in favor of the unconstrained shortest tree ($n=448$, $Z=5.4805$, $P<0.0001$).

3.5. Mitochondrial structural features

Amphisbaenian mtDNAs have several atypical structural features. Relative to the gene arrangement
commonly found for vertebrate mtDNAs, and most parsimoniously the ancestral condition for the Amphisbaenia, the Rhineuridae has the block \textit{nad6}, \textit{trnE} switched in order with the block \textit{cob}, \textit{trnT}, \textit{trnP} (Fig. 5). This gene order is the same as that observed in all birds sampled to date, but not their sister taxon, the crocodilians, and so represents the first case of convergent gene order evolution found in comparisons of complete mtDNA sequences. [Some have suggested that there is a convergent gene rearrangement within the birds (Mindell et al., 1998), but only non-coding regions have moved; all studied bird mtDNAs actually have the same arrangement of all genes.]

All species of the Bipedidae share the synapomorphy of having \textit{nad6} and \textit{trnE} switched in order. There is a small region of non-coding sequence between \textit{nad6} and \textit{cob}, where \textit{trnE} typically is found among vertebrate mtDNAs, perhaps as a vestige of the original \textit{trnE} that formerly resided there. Interestingly, this sequence is conserved in length with \textit{B. tridactylus} and \textit{B. canaliculatus} populations having sequences of 72 bases while the northern \textit{B. biporus} has 77 bases and the southern \textit{B. biporus} has 79 bases. This is within the range typically observed for tRNA genes, but there is no obvious sequence similarity to \textit{trnE}. In addition, \textit{Bipes biporus} has a tandem duplication of the block \textit{trnT}, \textit{trnP}, but with some appearing to be pseudogenes. The pattern of pseudogene formation is different between the two \textit{B. biporus} populations with the southern sample retaining function of \textit{trnT1} and \textit{trnP2} and the northern sample retaining function of \textit{trnT2} and \textit{trnP2}. In all Amphisbaenia except the Rhineuridae, \textit{trnC} encodes a transfer RNA that lacks a D-stem and instead contains a D-arm replacement loop (Macey et al., 1997b).

Species of the Rhineuridae, Amphisbaenidae, and Trogonophidae retain the potential for a strong stem–loop structure between \textit{trnN} and \textit{trnC} where light-strand replication is thought to usually initiate for vertebrate mtDNAs, whereas members of the Bipedidae have a short atypical stem–loop at this position. In particular, the 3’-GCC-5’ heavy-strand template sequence identified as the point of light-strand elongation in mouse (Brennicke and Clayton, 1981) is not present in these structures for the Bipedidae. \textit{B. tridactylus} has an eight base stem and the other two species have a seven base stem, which is less than that normally found among squamate reptiles and these stem regions show little base compositional similarity with that observed across other squamates (Macey et al., 1997a).

Fig. 5. Evolution of mtDNA structural features among amphisbaenian reptiles. At the base of the tree, the gene arrangement commonly observed between \textit{nad5} and the Control Region (CR) among vertebrates is depicted. The Rhineuridae has the block \textit{nad6}, \textit{trnE} switched in order with \textit{cob}, \textit{trnT}, \textit{trnP}, which is convergent with that observed in birds. The Bipedidae has the synapomorphic trait of having \textit{nad6} and \textit{trnE} switched in order. Also there is a small region of non-coding sequence (nc) between \textit{nad6} and \textit{cob} where \textit{trnE} typically is found. \textit{B. biporus} has a tandem duplication of the block \textit{trnT}, \textit{trnP}. The pattern of pseudogene formation is different in the two \textit{B. biporus} populations (see text). In all amphisbaenians except the Rhineuridae, \textit{trnC} encodes a transfer RNA that lacks a D-stem and instead contains a D-arm replacement loop. The Bipedidae has an atypical stem and loop structure between \textit{trnN} and \textit{trnC} where light-strand replication usually initiates among vertebrates.
4. Discussion

4.1. Historical biogeography

Our phylogenetic analysis shows that taxa found in Laurasian regions (Rhineuridae and Bipedidae) are basal and that the more broadly distributed Gondwanan groups (Amphisbaenidae and Trogonophidae) are nested within. This indicates that the most basal divergences of Amphisbaenia predate the geological split of Pangaea 200 MYBP. This event is likely responsible for the isolation of the ancestor of Amphisbaenidae and Trogonophidae in Gondwana.

Old and New World Amphisbaenidae, represented by Geocalamus and Amphisbaena, respectively, are monophyletic, so that this family dates back at least to the opening of the Atlantic Ocean 80 MYBP. Hence, the divergence between the sister taxa Amphisbaenidae and Trogonophidae in the Old World must have occurred even earlier.

The earliest divergence in the family Bipedidae from Mexico is between northern and southern species rather than between mainland and peninsular. The transversional distance between Old and New World Amphisbaenidae representing the opening of the Atlantic Ocean 80 MYBP is 12.6%. If transversions are accumulating substitutions in a linear fashion then the average transversional distance observed between B. tridactylus and the other two Bipes species of 10.9% is approximately equivalent to 69 MYBP. This is consistent with known Cretaceous to Paleocene tectonic activity along the west coast of Mexico (Ferrari, 1995) and inconsistent with the tectonic split of Baja California (12–14 MYBP; Ferrari, 1995). The transversional distance between the sister taxa B. canaliculatus from the Mexican mainland and B. biporus from peninsular Baja California is 8.6% which corresponds to a divergence 55 MYBP. The Cape Region of Baja California separated from Jalisco on the Mexican mainland 12–14 MYBP (Ferrari, 1995) far to the north of B. canaliculatus. Therefore, the divergence of B. canaliculatus and B. biporus is likely to be related to early tectonic activity as Baja California slid northward along the west coast of Mexico prior to oceanic separation.

4.2. Limb evolution

The Bipedidae, with two forelimbs, is nested within the remaining limbless taxa. Because the ancestor must have had four legs, the most parsimonious reconstruction requires independent loss of limbs in the Rhineuridae, and the lineage leading to the Amphisbaenidae and Trogonophidae. A less likely alternative is that the forelimbs in the Bipedidae have arisen secondarily, perhaps as an atavism mediated by genes regulating leg morphogenesis, such as those of the hox family (reviewed in Greene and Cundall, 2000).

Species within the Bipedidae vary in the number of digits on their forelimbs, B. tridactylus, with three digits, is the sister taxon to B. canaliculatus and B. biporus. While B. biporus always has five digits, B. canaliculatus is variable, usually having four, but sometimes five, digits (Papenfuss, 1982). It appears that there has been independent reduction of digits in B. tridactylus and B. canaliculatus from an ancestor with five digits.

4.3. Evolution of the amphisbaenian mitochondrial genome

Mitochondrial gene rearrangement and duplication has occurred in separate lineages of amphisbaenians, the Rhineuridae and Bipedidae (Fig. 5). Considering their distant placement on the phylogenetic tree, it appears that these two lineages acquired novel mitochondrial gene orders independently via different mechanisms, presumably involving replicational errors. However, surprisingly, these events occurred in the same genomic region.

Mitochondrial gene rearrangements seem to commonly evolve through tandem duplication of genes followed by loss of extra copies (Boore, 2000; Moritz et al., 1987). For faithful replication of a circular genome, termination must be made to exactly coincide with the leading strand’s initiation point, whereas duplications can result from that strand’s extension beyond an alternative termination (see Boore, 2000; Macey et al., 1997a). In the mtDNAs of vertebrates, the origin of replication for the heavy-strand (O H) is thought to coincide with the leading strand’s initiation point, whereas duplications result from that strand’s extension beyond an alternative termination (see Boore, 2000; Macey et al., 1997a). In the mtDNAs of sea lamprey (Petromyzon marinus; Lee and Kocher, 1995), birds (Desjardins and Morris, 1990), crocodilians (Kumazawa and Nishida, 1995), tuatara (Sphenodon punctatus; Rest et al., 2003), accrodon lizards (reviewed in Macey et al., 2000), and the Texas blind snake (Leptotyphlops dulcis; Kumazawa and Nishida, 1995). As an alternative to the stem–loop at O L, other structures formed in the displaced strand may potentially serve this function (Macey et al., 1997a).
including those of tRNA genes (Clayton, 1982). It may then be possible that having alternative origins, perhaps with suboptimal regulatory signals, increases the likelihood of gene duplications followed by gene rearrangements. These rearrangements could potentially occur at various sites in the mtDNA depending on the location of the alternate O_L. It can be reasoned that the position of O_I would be much more labile than that of O_H since the latter regulates replication initiation, controls D-loop formation, and serves a dual role as a transcription initiation site (Clayton, 1991). In addition, light-strand synthesis requires heavy-strand replication to produce a single stranded stem–loop template typically 11kb from O_H, therefore it could potentially initiate at alternative sites along this strand throughout the mitochondrial genome.

The mitochondrial gene order observed in *Rhineura* is identical to that of birds, where the block *nad6, trnP* is switched in order with *cob, trnT, trnP* relative to the arrangement commonly found for vertebrates. This is the first parallelism of gene rearrangement fully documented by complete mitochondrial genome sequences. To our knowledge, there have been only two cases (Dowton and Austin, 1999; Flook et al., 1995) of purported parallelism suggested even from partial sequences. Unlike all other amniotes that have rearranged mitochondrial gene orders, including birds, *Rhineura* has an apparently functional O_L in the typical location. Considering this, and that the rearranged genes flank the Control Region, the gene rearrangement observed in *Rhineura* appears to be derived from errors of heavy—rather than light-strand replication.

Surprisingly, the gene rearrangement in *Bipes* involves the same gene region. However, we suggest that this gene rearrangement, where *trnE* and *nad6* are switched in order, is produced by error at a light-strand replicational origin. This taxon lacks a recognizable stem–loop at the typical location of O_L, so some other region(s) of the mtDNA must perform this function. *Bipes* appears to be yet another case having the loss of O_L for its typical location accompanying gene rearrangement.

The typical O_L has functionally important, overlapping regions with the adjacent gene encoding tRNA(C) (reviewed in Macey et al., 1997a). All sampled amphisbaenians except *Rhineura* have a tRNA(C) lacking a D-stem and instead containing a D-arm replacement loop (Macey et al., 1997b). Macey et al. (1997c) proposed a cascade model suggesting that changes to the secondary structure of *trnC* may destroy function of O_L in its typical vertebrate location which, in turn, may facilitate shifts in gene order by enabling the utility of alternative sites for initiation and termination of light-strand replication (see above). It seems to be reasonable that *Bipes* constitutes an additional example in support of this model.

Additionally, *B. biporus* mtDNA contains a tandem duplication of *trnT* and *trnP*. We cannot confidently speculate whether this has been induced by light-strand or heavy-strand replicational error because, on the one hand, all *Bipes* have an atypical stem–loop structure where O_I commonly is located, but on the other hand, this duplication is adjacent to the Control Region. Interestingly, although the two *B. biporus* sampled retain function of different copies of *trnT*, no gene rearrangement will occur in either population because both taxa retain function of the second copy of *trnP* which is preceded by both copies of *trnT*. In order for a gene rearrangement to result from a tandem duplication of two genes, non-adjacent copies of both genes must lose function (Macey et al., 1997a), which, in this case, is the first copy of *trnT* and the second copy of *trnP* (Macey et al., 1998).

While heavy-strand induced gene rearrangements are restricted to the vicinity of the Control Region, as observed for large, tandem duplications among lizards (Moritz and Brown, 1986, 1987), light-strand induced rearrangements can occur throughout the mitochondrial genome. Hence, mitochondrial genomic rearrangements caused by heavy-strand replication are less reliable phylogenetic characters than rearrangements produced by light-strand replication, which are known to be located throughout the genome and identified by loss of a recognizable O_L in the typical vertebrate location (Macey et al., 1997a).

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**Appendix A. Supplementary material**

Supplementary data associated with this article containing the aligned DNA sequences as a Nexus file, can be found, in the online version, at doi:10.1016/j.ympev.2004.05.003.

**References**

