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**The complete mitochondrial genome of a gecko and the phylogenetic position
of the Middle Eastern *Teratoscincus keyserlingii***

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Running head: Middle Eastern Gecko Mt-Genome

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1. Introduction

Squamate reptiles are traditionally divided into six groups: Iguania, Anguimorpha, Scincomorpha, Gekkota (these four are lizards), Serpentes (snakes), and Amphisbaenia (the so-called worm lizards). Currently there are complete mitochondrial genomes from two representatives of the Iguania (Janke et al., 2001; Kumazawa, 2004), three from the Anguimorpha (Kumazawa, 2004; Kumazawa and Endo, 2004), two from the Scincomorpha (Kumazawa and Nishida, 1999; Kumazawa, 2004), two from Serpentes (Kumazawa et al., 1998; Kumazawa, 2004) and 12 from Amphisbaenia (Macey et al., 2004). The only traditional group of Squamata from which a complete mitochondrial genome has not been sequenced is the Gekkota.

Here we report the complete mitochondrial genome of *Teratoscincus keyserlingii*, a Middle Eastern representative of the Gekkota. The gekkonid lizard genus *Teratoscincus* is distributed throughout the deserts of central and southwest Asia as shown in figure 1, with five species currently recognized (Macey et al. 1997a, 1999b). Included in this figure are the positions of mountain ranges discussed in the text; see also figure 1 in Macey et al. (1999b). Two species, *T. bedriagai* and *T. microlepis*, are restricted to Southwest Asia south of the Kopet Dagh and Hindu Kush in Iran, Afghanistan, and Pakistan (Anderson, 1999). Two species are found in the deserts of western China and Mongolia, with *T. przewalskii* occurring in the Taklimakan and lowland Gobi deserts, and *T. roborowskii* restricted to the Turpan Depression. The fifth species, *T. scincus*, is sometimes considered to be restricted to the Caspian Basin in Kazakhstan, Kyrgyzstan, Tadjikistan, Turkmenistan and Uzbekistan. Alternatively, *Teratoscincus* populations in Southwest Asia, primarily on the Iranian Plateau, situated directly north of the

Arabian Plate, are sometimes considered to be a subspecies of *T. scincus* or, otherwise, to constitute a sixth species, *T. keyserlingii*.

Macey et al. (1999b) assessed the phylogenetic relationships of four *Teratoscincus* species with mitochondrial DNA sequences from a ~1800 base-pair segment spanning from *nad1* to *cox1*. Phylogenetic analysis places *T. microlepis* in a basal position to a clade containing *T. scincus*, *T. przewalskii* and *T. roborowskii*, with the later two as sister taxa. This phylogenetic arrangement suggests that tectonic plate movements in Southwest Asia and western China due to the Indian and Arabian collisions caused speciation among *Teratoscincus* species. No molecular phylogenetic study has included the putative species *T. keyserlingii*.

2. Materials and methods

2.1. Specimen Information

The sample of *T. keyserlingii* from which DNA was extracted is deposited in the Museum of Vertebrate Zoology, University of California at Berkeley as MVZ 243455. The collection locality of this specimen is elevation 740 feet, 30° 26.741' N 57° 49.967' E, east side of Shahdad, Kerman Province, Iran. The complete mitochondrial genome sequence from this specimen of *T. keyserlingii* is deposited in GenBank as accession number AY753545.

This sample of *T. keyserlingii* is selected because it is close to the type locality of *T. keyserlingii* in the Lut Desert of eastern Iran. The original type locality is given as Seri-Tschah, eastern Iran by Strauch (1863) but is most likely Sar-I-Chah, 32° 16' N 58° 52' E, Khorasan (Blanford, 1876; also see Anderson, 1999).

2.2. Laboratory Protocols

Genomic DNA was extracted from liver using the Qiagen QIAamp tissue kit. Amplification of the mtDNA was conducted using *rTth* long PCR enzyme (Applied Biosystems) with a beginning denaturation at 94°C for 45 sec, then followed by 37 cycles of a denaturation at 94°C for 15 sec, annealing at 50°C for 20 sec, and extension at 68°C for 9 min, with a final extension at 72°C for 12 min after the last cycle. Negative controls were run on all amplifications to check for contamination. Initial amplifications were conducted using primers described in Macey et al. (1997b, 1999b). Perfectly matching primers were then constructed based on the DNA sequence of this fragment to complete the amplification of each mtDNA in two additional long PCR fragments.

Amplification products were sheared randomly into fragments of approximately 1.5 kb by repeated passage through a narrow aperture using a Hydroshear device (GeneMachines). After end-repair, the sheared DNA was gel purified and ligated into pUC18 vector to construct a library of random fragments, 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 clean up using SPRI (Elkin et al., 2002). Sequences were determined using ABI3730xl or MegaBace 4000 DNA sequencers, then assembled to form a deep, contiguous sequence using Phrap (Green, 1996) or Sequencher 3.0 (Gene Codes Co., Ann Arbor, MI).

2.3. Phylogenetic Analysis

The phylogenetic analysis is based on nucleotide sequences for the same region of the mitochondrial genome from *nad1* to *cox1* as presented in Macey et al. (1999b). DNA sequences for protein- and tRNA-encoding genes were aligned manually as in Macey et al. (1999b). 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). Unalignable regions totaling 82 positions were excluded from phylogenetic analyses as in Macey et al. (1999b). These positions are situated at the end of *nad1*, in O_L , and the D- and T-loops of *trnW* and *-trnC* (see figure 2 in Macey et al., 1999b).

The region analyzed in Macey et al. (1999b) from *nad1* to *cox1* corresponds to positions 3558-5330 of the complete mitochondrial genome of *T. keyserlingii* and has a length of 1773 bases. To align this new sequence with the data of Macey et al. (1999b) a total of 42 gaps are introduced. These gaps are after the following positions on the complete mitochondrial genome of *T. keyserlingii* with the number of gaps introduced in parenthesis if more than one: 3668 (7), 3797, 3828, 3860, 3864, 3881, 4927 (3), 4942 (7), 4976, 4980, 4993 (6), 5010, 5049, 5136 (2), 5141, 5156 (2), 5163, 5181, 5230, 5243, and 5281. Sequence divergences based on this alignment are reported as uncorrected pairwise divergences.

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.

In order to test specific, alternative phylogenetic hypotheses, we first built incompletely resolved constraint trees using MacClade (Maddison and Maddison, 2001). These were provided as input into PAUP* (Swofford, 2001) for branch and bound searches to determine the most parsimonious tree compatible with each alternative hypothesis. We then compared these to the unconstrained most parsimonious tree using Wilcoxon signed-ranks tests (Templeton, 1983). This test determines whether the most parsimonious tree is significantly shorter than each alternative or whether their differences in length are statistically indistinguishable. Wilcoxon signed-ranks tests were conducted as two-tailed tests (Felsenstein, 1985b) using PAUP* (Swofford, 2001), which incorporates a correction for tied ranks.

3. Results

3.1. Mitochondrial genomic structure

The complete mitochondrial genome of *T. keyserlingii* is 17,199 base pairs in length. This genome contains the same 37 genes common among animals and they are ordered as is most commonly found for vertebrates (Boore, 1999). The two replication origins are located in the typical vertebrate locations as well. This order as in *T. keyserlingii* is *trnF*, *rrnS*, *trnV*, *rrnL*, *trnL (UUR)*, *nad1*, *trnI*, *-Q*, *M*, *nad2*, *trnW*, *-A*, *-N*, *-C*, *O_L*, *-trnY*, *cox1*, *-trnS (UCN)*, *D*, *cox2*, *trnK*, *atp8*, *atp6*, *cox3*, *trnG*, *nad3*, *trnR*, *nad4L*, *nad4*, *trnH*, *S (AGY)*, *L (CUN)*, *nad5*, *-nad6*, *-trnE*, *cob*, *trnT*, *-trnP*, and Control Region. The noncoding region typically observed between *-trnP* and *trnF*, presumed to be the Control Region, is 2,117 base pairs in length. This region has seven near identical repeats of 48 nucleotides adjacent to *-trnP*.

Some squamate reptiles are known to contain atypical vertebrate mitochondrial genomes. Three main observations have been made: (1) gene rearrangements, (2) duplicated genes, and (3)

duplicated Control Regions. Gene rearrangements are known in amphisbaenian reptiles (Macey et al., 1994), Acrodonta including the lizard families Agamidae and Chamaeleonidae (Macey et al. 1997b, c, 2000), the snake family Leptotyphlopidae (Kumazawa and Nishida, 1995; Kumazawa and Endo, 2004), "advanced snakes" (Kumazawa and Nishida, 1995; Kumazawa et al., 1998), and the lizard family Varanidae (Kumazawa and Endo, 2004). Duplicated genes are known in *Cnemidophorus* lizards in the family Teiidae (Moritz and Brown, 1986, 1987), *Heternotia* geckoes in the family Gekkonidae (Moritz, 1991), the amphisbaenian species *Bipes biporus* (Macey et al. 1998, 2004), and the lizard family Cordylidae (Kumazawa, 2004). Duplicated Control Region sequences are independently (see Townsend et al., 2004) associated with gene rearrangements in "advanced snakes" (Kumazawa and Nishida, 1995; Kumazawa et al., 1998), and the lizard family Varanidae (Kumazawa and Endo, 2004). In addition, the loss of the stem-loop structure implicated as the light-strand origin for replication from its typical location between *-trnN* and *-C* is associated with the majority of gene rearrangements among vertebrates and many squamate reptiles (reviewed in Boore et al., 2005; Macey et al. 1997b,c, 2000, 2004). All of these features are observed as the typical vertebrate state in *T. keyserlingii*, and not as just outlined for some exceptions among squamate reptiles.

3.2. Phylogenetic relationships

Phylogenetic analysis of the 1733 aligned positions (381 informative) produces a single most parsimonious tree (Fig. 2). All relationships are well supported by a bootstrap value of 100%. The monophyly of *Teratoscincus* receives further support from a decay index of 64. The monophyly of *T. scincus*, *T. keyserlingii*, *T. przewalskii*, and *T. roborowskii*, to the exclusion of *T. microlepis* is supported by a decay index of 35. *Teratoscincus scincus* and *T. keyserlingii* are

sister taxa (decay index 20), and *T. przewalskii* and *T. roborowskii* are sister taxa (decay index 24). The shortest alternative tree that does not unite *T. scincus* and *T. keyserlingii* requires 20 extra steps and is rejected by the Wilcoxon-signed-ranks test (Felsenstein, 1985b; Templeton, 1983) in favor of the unconstrained shortest tree ($n = 45$, $Z = 3.0861$, $P < 0.002$).

The phylogenetic results provide an area cladogram for *Teratoscincus* (Fig. 2). The Chinese-Mongolian deserts (Taklimakan, Gobi and Turpan) are monophyletic and the Caspian Basin is sister to the Iranian Plateau. Collectively these regions are monophyletic to the exclusion of the Afghan-Pakistan deserts (Helmand Basin and Makran).

4. Discussion

4.1. Sequence divergences and geologic history

Four geologic events can be mapped onto the phylogenetic tree [Fig. 2, also see figure 5 in Macey et al. (1999b) for a tectonic map of central and southwest Asia]. The rise of the Hindu Kush of central Afghanistan has been previously hypothesized to be responsible for vicariant separation of *T. microlepis* from the ancestor of *T. scincus*, *T. keyserlingii*, *T. przewalskii*, and *T. roborowskii* (Macey et al., 1999b). The initial rise of the Hindu Kush is coupled with the Karakorum Range and are approximately 20 million years old (Le Fort, 1998; Searle, 1991). The Tien Shan separates *T. scincus* of the Caspian Basin and *T. keyserlingii* of the Iranian Plateau from *T. przewalskii* and *T. roborowskii* of western China and Mongolia, and is well dated at 10 MYBP (million years before present; Abdrakhmatov et al., 1996). The rise of the Kopet-Dagh along the Iran-Turkmen border is coupled with intense Miocene activity along the Red Sea rift causing deformation in the Iranian Plateau by the Arabian collision (Girdler, 1984). While uplift of the Kopet-Dagh was most intense in the Middle Pliocene 3-4 MYBP (Sborshchikov et al.,

1981), a conservative estimate of minimum age is at least 5 MYBP at the time of major activity along the Red Sea rift (Girdler, 1984), and may correspond to the separation of *T. scincus* and *T. keyserlingii*. The Kuruk Tagh separates *T. przewalskii* in the Tarim Basin and Gobi Desert from *T. roborowskii* endemic to the Turpan Depression, and is coupled with east-west fault movements at the Mio-Pliocene boundary 5 MYBP (Tapponnier and Molnar, 1979; Windley et al., 1990).

The region of mitochondrial DNA examined here spanning from *nad1* to *cox1* has been shown to evolve at a rate of 1.3% per million years (MY) for uncorrected pairwise comparisons (Macey et al., 1998a). This calibration has been shown to be robust across numerous amphibian and reptile taxa (reviewed in Weisrock et al., 2001). Using the four geologic events that separate *Teratoscincus* species independently to calculate divergence rates (Table 1), a close calibration is made using the Tien Shan and Kuruk Tagh events (1.18% and 1.31% per MY). The Kopet-Dagh is a little higher (1.7%) and the Hindu Kush lower (0.82%). The Kopet-Dagh may be older than 5 MYBP due to earlier movement along the Red Sea rift (Girdler, 1984), and mitochondrial DNA is known to begin to accumulating multiple substitutions at the same site beyond 10 million years (Moritz et al., 1987) suggesting that a linear relationship between sequence divergence and time may not be expected. Previous studies have not examined divergence rates of transversional changes across this region of the mitochondrial genome. Examination of transversional divergences across these four geologic barriers yields a remarkably similar rate of change per million years (0.21-0.31%). Although our calculations are not exactly the same, there is good evidence that these sequences are evolving in a clock-like manner.

4.2. Taxonomic considerations

A large amount of comparative sequence data are available in the literature for the segment of mitochondrial DNA sequence examined here from *nad1* to *cox1*. The amount of sequence divergence observed across the Kopet-Dagh between *T. scincus* and *T. keyserlingii* is 8.5%. This is larger than that typically observed between species pairs of amphibians and reptiles (Table 2). Because *T. keyserlingii* is completely allopatric, isolated for millions of years, and is highly divergent from *T. scincus*, we recommend specific status for this taxon. A complete synonymy for Iranian populations is presented in Anderson (1999), and the oldest name available is *T. keyserlingii*.

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Table 1

Sequence divergences across taxa and dated geologic events with rates per million years^a

	1	2	3	4	5	6	7
1. <i>Eublepharus</i>	—	35.52%	31.02%	30.83%	31.36%	30.30%	30.59%
2. <i>Gecko</i>	20.31%	—	30.70%	32.05%	32.53%	31.97%	32.45%
3. <i>T. microlepis</i>	18.01%	16.20%	—	15.97%	16.79%	15.91%	16.56%
4. <i>T. scincus</i>	17.26%	16.93%	5.17%	—	8.52%	11.12%	11.59%
5. <i>T. keyserlingii</i>	17.32%	16.99%	5.64%	1.52%	—	12.41%	12.12%
6. <i>T. przewalskii</i>	17.32%	16.68%	5.76%	2.93%	3.16%	—	6.55%
7. <i>T. roborowskii</i>	17.38%	16.86%	5.76%	2.99%	3.22%	1.05%	—
		Sequence		Rate		TV Sequence	
Mtn. Range	Age	Divergence		per MY		Divergence	per MY
Hindu Kush	20 MY	16.31%		0.82%		5.58%	0.28%
Tien Shan	10 MY	11.81%		1.18%		3.08%	0.31%
Kopet-Dagh	5 MY	8.52%		1.70%		1.52%	0.30%
Kuruk Tagh	5 MY	6.55%		1.31%		1.05%	0.21%

^aValues above the dashed line are uncorrected pairwise distances and those below are uncorrected transversional distances. Abbreviations are MY = million years and TV = transversions.

Table 2

Comparative pairwise sequence divergences between species of amphibians and reptiles^a

Family	Genus	Taxa Compared	Pairwise Sequence Divergences	Reference
Bufo	<i>Bufo</i>	<i>B. andrewsi</i> and <i>B. gargarizans</i>	6.0–6.9%	Macey et al., 1998b
Rana	<i>Rana</i>	<i>R. aurora</i> , <i>R. cascadae</i> and <i>R. muscosa</i>	7.0–8.4%	Macey et al., 2001
Salamandridae	<i>Salamandra</i>	<i>S. infraimmaculata</i> and <i>S. salamandra</i>	7.4–7.5%	Weisrock et al., 2001
Agamidae	<i>Laudakia</i>	<i>L. caucasia</i> and <i>L. erythrogastra</i>	4.2–5.3%	Macey et al., 1998a
Anguillidae	<i>Elgaria</i>	<i>E. kingii</i> to the clade containing <i>E. multicarinata</i> , <i>E. panamintina</i> and <i>E. paucicarinata</i>	4.8–5.9%	Macey et al., 1999a
Gekkonidae	<i>Teratoscincus</i>	<i>T. przewalskii</i> and <i>T. roborowskii</i>	6.5%	Macey et al., 1999b

^aSequence divergences are calculated for the same segment of mitochondrial DNA spanning from *nad1* to *cox1*. Bufonid frogs include only the first half of this segment (from *nad1* to *nad2*).

Figure legends

Fig. 1. Map showing the distribution of the five *Teratoscincus* species examined in this study. One additional taxon, *T. bedriagai*, that occurs on the Iranian Plateau and Helmand Basin but not examined here is not shown. Major mountain ranges dividing species and discussed in the text are depicted.

Fig. 2. The single most parsimonious tree resulting from analysis of the 1733 (381 informative) aligned sites which is 1363 steps in length. Bootstrap values appear above branches and decay indices are presented below in bold. Geologic events hypothesized to have caused speciation are plotted on nodes.

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