The complete mitochondrial genome of an agamid lizard from the Afro-Asian subfamily Agaminae and the phylogenetic position of *Bufoniceps* and *Xenagama*

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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). The Iguania is recognized as having two major lineages the Iguanidae and Acrodonta (Agamidae and Chamaeleonidae). Currently there are complete mitochondrial genomes from three Anguimorpha (Kumazawa, 2004; Kumazawa and Endo, 2004), two from the Scincomorpha (Kumazawa and Nishida, 1999; Kumazawa, 2004), one from Gekkota (Macey et al., 2005) two from Serpentes (Kumazawa et al., 1998; Kumazawa, 2004) and 12 from Amphisbaenia (Macey et al., 2004). In addition, two representatives of the Iguanian family Iguanidae (Janke et al., 2001; Kumazawa, 2004) have been sequenced. Its sister taxon, the Acrodonta, constists of seven monophyletic groups the family Chamaeleonidae and six distantly related subfamilies of the family Agamidae (Macey et al., 2000b). Currently the only acrodont lineage sequenced for the complete mitochondrial genome is *Pogona vitticepes* from the Australasian agamid subfamily Amphibolurinae (Amer and Kumazawa, 2005).

Here we report the complete mitochondrial genome of *Xenagama taylori*, a North African representative of the agamid subfamily Agaminae and compare it to *P. vitticepes*. The agamid lizard genus *Xenagama* is distributed in a restricted region of the Horn of Africa in northwestern Somalia and adjacent eastern Ethiopia as shown in figure 1, with two species currently recognized (Moody, 1980; Wermuth, 1967). In addition, we report a segment of the mitochondrial genome of *Bufoniceps laungwalansis* spanning from *nad1* to *cox1*. The monotypic genus *Bufoniceps* is restricted to the Thar Desert, Jaisalmer District, Rajasthan State, India and adjacent Pakistan (Fig. 1).

Both *Bufoniceps* and *Xenagama* belong to the subfamily Agaminae and are poorly understood phylogenetically. These genera were not represented in the most recent molecular systematic study of the Agamidae (Macey et al., 2000b). *Bufoniceps* was originally described as a member of the West Asian genus *Phrynocephalus* (Sharma, 1978), and later placed in its' own genus (Arnold, 1992) because morphological data suggested it is the sister taxon to *Phrynocephalus* (Arnold, 1999). *Xenagama* was previously considered part of the *Agama* complex before the allocation of its member species to several genera (see Moody, 1980).

2. Materials and methods

2.1. Specimen Information

The sample of *Bufoniceps laungwalansis* from which DNA was extracted is deposited in the Raffles Museum of Biodiversity Research, National University of Singapore as ZRC 2.5681. The collection locality of this specimen is elevation 192 m, 26.50.26' N 70.32.24' E, vicinity of Sam, Rajasthan State, India. The mitochondrial segment spanning from *nad1* to *cox1* is deposited in GenBank as accession number DQ008214. The sample of *Xenagama taylori* from which DNA was extracted is deposited in the California Academy of Sciences, San Francisco as CAS 225502. The collection locality of this specimen is elevation 1140 m, 9.670000' N 44.207500' E, 21 km ENE of the center of Hargeysa on Berbera Rd., then 4 km N on dirt road, Waqooyi Galbed Region, Somalia. The complete mitochondrial genome sequence from this specimen of *X. taylori* is deposited in GenBank as accession number DQ008215.

2.2. Laboratory Protocols

Genomic DNA was extracted from liver using the Qiagen QIAamp tissue kit. For *B. laungwalansis* amplification of genomic DNA was conducted using a denaturation at 94°C for 35 sec, annealing at 50°C for 35 sec, and extension at 70°C for 150 sec with 4 sec added to the extension per cycle, for 30 cycles. Primers used are described in Macey et al. (1997a,c, 2000b). Negative controls were run on all amplifications to check for contamination. Amplified products were purified on 2.5% Nusieve GTG agarose gels and reamplified under the conditions described above to increase DNA yield for downstream sequencing reactions. Reamplified double-stranded products were purified on 2.5% acrylamide gels and template DNA was eluted passively over three days with Maniatis elution buffer (Maniatis et al., 1982) or purified using the QIAquick PCR purification kit. Cycle-sequencing reactions were run using the ABI Prism Big Dye Terminator DNA Sequencing Kit (Perkin-Elmer) with a denaturation at 95°C for 15 sec, annealing at 50°C for 1 sec, and extension at 60°C for 4 min for 35-40 cycles. Sequencing reactions were run on an ABI 373 Genetic Analyzer or MJ Research Basestation sequencers.

For *X. taylori* amplification of the mtDNA was conducted using rT*th* 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. (1997a). Perfectly matching primers were then constructed based on the DNA sequence of this fragment to complete the amplification of the mtDNA. 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 DNA sequencers, then assembled to form a deep, contiguous sequence using Phrap or Sequencher.

2.3. Phylogenetic Analysis

DNA sequences for protein- and tRNA-encoding genes were aligned manually as in Macey et al. (2000b). 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). Of the 1965 characters, unalignable regions totaling 561 positions were excluded from phylogenetic analyses as in Macey et al. (2000b).

The region analyzed in Macey et al. (2000b) from *nad1* to *cox1* corresponds to positions 3495-5193 of the complete mitochondrial genome of *X. taylori* and has a length of 1699 bases. To align the new sequences with the 72 sequences analyzed in Macey et al. (2000b) a total of 268 gaps are introduced in the *B. laungwalansis* sequence and 266 gaps in the *X. taylori* sequence. These gaps are after the following positions on the *B. laungwalansis* sequence with the number of gaps introduced in parenthesis if more than one: 86 (18), 99, 145 (2), 162 (13), 181 (2), 218 (4), 229 (11), 246 (2), 280 (4), 295 (3), 298 (12), 319 (3), 340 (3), 1105 (3), 1318 (9), 1321 (82), 1343 (3), 1369, 1380 (4), 1393 (10), 1412, 1448, 1461 (13), 1490 (3), 1507, 1515,

1528, 1553 (13), 1570 (5), 1602 (6), 1609 (6), 1625 (3), 1634, 1661 (2), and 1674 (11). These gaps are after the following positions on the complete mitochondrial genome of *X. taylori* with the number of gaps introduced in parenthesis if more than one: 3579 (19), 3592, 3638 (2), 3652 (16), 3670 (3), 3706 (5), 3718 (10), 3736, 3769 (6), 3782 (4), 3785 (9), 3809 (3), 3830 (3), 4595 (3), 4808 (9), 4812 (81), 4833 (4), 4859, 4870 (4), 4883 (10), 4900 (3), 4936, 4949 (13), 4978 (3), 4995, 5003, 5016, 5041 (13), 5057 (6), 5088 (6), 5096 (6), 5111 (4), 5120, 5147 (2), and 5160 (11). 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 heuristic searches featuring 100 random additions of sequences. Bootstrap resampling (Felsenstein, 1985a) was applied to assess support for individual nodes using 500 heuristic searches featuring 100 random additions of sequences per replicate. Decay indices (= "branch support" of Bremer, 1994) were calculated for all internal branches using heuristic searches featuring 100 random additions of sequences in 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 heuristic searches featuring 100 random additions of sequences 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 one-tailed tests (Felsenstein,

1985b) using PAUP* (Swofford, 2001), which incorporates a correction for tied ranks. Felsenstein (1985b) showed that one-tailed probabilities are close to the exact probabilities for this test but not always conservative. The two-tailed probabilities are simply double the one-tailed probabilities and the two-tailed test is always conservative (Felsenstein, 1985b).

3. Results

3.1. Mitochondrial genomic structure

The complete mitochondrial genome of *X. taylori* is 16,220 base pairs in length. This genome contains the same 37 genes common among animals but differs from the ordered as is most commonly found for vertebrates (Boore, 1999). As previously reported for Acrodonta (Agamidae and Chamaleonidae), both *B. laungwalansis* and *X. taylori* have *trnI* and *Q* switched to yield the order *nad1, trnQ, I,* and *M* (Macey et al. 1997a, c, 2000a). Both taxa have *trnC* that encodes a transfer RNA which lacks a D-stem and instead contains a D-arm replacement loop (Macey et al., 1997b), as is typical for the Acrodonta. In addition, these taxa have atypical stem-loop structures between *trnN* and *trnC* where light-strand replication is thought to usually initiate for vertebrate mtDNAs, which is also observed in other members of the Agaminae clade (Macey et al., 2000b). 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. *Bufoniceps laungwalansis* has a five base stem with a 16 base loop, whereas *X. taylori* has a nine base stem with an eight base loop. As observed in most other vertebrates the mt-genome of *X. taylori* has a large noncoding region presumed to be the Control Region (CR) of 1449 base pairs in length. This differs from that of the amphibolurine *P. vitticeps* which has a near identical second non-coding region inserted between *nad5* and *nad6* (Amer and Kumazawa,

2005). Unlike previously reported mt-genomes among vertebrates (but see McKnight and Shaffer, 1997) the noncoding region in *X. taylori* is between *trnT* and *trnP* and not between *trnP* and *trnF,* as is typical and observed for one copy in *P. vitticeps* (Amer and Kumazawa, 2005). An additional 26 bases separate *trnP* and *trnF* in *X. taylori*. Therefore, *X. taylori* has the complete mt-order of *trnF, rrnS, trnV, rrnL, trnL(taa), nad1, -trnQ, I, M, nad2, trnW, -A, -N, -C,* stem-loop, *-trnY, cox1, -trnS(tga), D, cox2, trnK, atp8, atp6, cox3, trnG, nad3, trnR, nad4L, nad4, trnH, S(tct), L (tag), nad5, -nad6, -trnE, cob, trnT,* CR, and *-trnP*.

3.2. Phylogenetic relationships

Phylogenetic analysis of the 1434 aligned positions (1046 informative) for the 72 taxa in Macey et al. (2000b) and the two newly reported sequences from *nad1* to *cox1* produces seven equally most parsimonious trees (Fig. 2). The tree is largely the same as that reported by Macey et al. (2000b) with the exception of a few weak nodes that are collapsed in this analysis because of the four additional equally parsimonious trees. Here we concentrate on the phylogenetic relationships of the Agaminae, which is monophyletic with a bootstrap of 100% and decay index of 27. The strict consensus tree yields nine lineages in the Agaminae labeled A-I in figure 2. The African genus *Agama* (*sensu stricto*) appears monophyletic with strong support (A in Fig. 2, bootstrap 100%, decay index 97). *Pseudotrapelus sinaitus* of Arabia, Egypt and Libya groups with *X. taylori* from the Horn of Africa and is well supported (B in Fig. 2, bootstrap 99%, decay index 97). *Bufoniceps laungwalansis,* restricted to the Indian Subcontinent, forms the sister taxon to the wide-ranging genus *Trapelus* with considerable support (C in Fig. 2, bootstrap 100%, decay index 22). *Laudakia sacra* of Tibet forms its own lineage (D in Fig. 2). *Laudakia nupta* of the Iranian Plateau groups weakly with *L. tuberculata* of the Himalaya (E and F in Fig. 2,

bootstrap 57%, decay index 2). *Laudakia stellio* of Anatolia and the Levant forms its own lineage (G in Fig. 2). The Asian genus *Phrynocephalus* is well supported (H in Fig. 2, bootstrap 100%, decay index 41). A well supported clade of *Laudakia* ranging from the Iranian Plateau to Mongolia is present (I in Fig. 2, bootstrap 100%, decay index 20).

The Wilcoxon-signed-ranks test (Felsenstein, 1985b; Templeton, 1983) is applied to compare the most parsimonious tree from these nucleotide sequences with alternative hypotheses. The genus *Bufoniceps* has been previously suggested to be either in the genus *Phrynocephalus* or the sister taxon to *Phrynocephalus*. The seven shortest alternative trees that unites *Bufoniceps* with *Phrynocephalus* require 55 extra steps and are rejected in favor of the unconstrained shortest trees ($P < 0.0046$). The genus *Xenagama* has been previously suggested to be related to *Agama*. The two shortest alternative trees that unite *Xenagama* with *Agama* require 30 extra steps and are not rejected in favor of the unconstrained shortest trees ($P < 0.0861$).

4. Discussion

4.1. Biogeography and the breakup of Gondwana

Acrodont lizards (Agamidae and Chamaeleonidae) are of Gondwanan origin (Macey et al. 2000b). Clades of agamid lizards rafted with alternative fragments of Gondwana which collided with the southern margin of Asia. The subfamily Agaminae arrived in Asia either with the Indian Subcontinent 50 MYBP (million years before present) or with Afro-Arabia 18 MYBP.

Bufoniceps is found to be the sister taxon to *Trapelus* and is statistically rejected as the sister taxon to *Phrynocephalus* as previously suggested (Arnold, 1999). The genus *Trapelus* ranges from North Africa across Arabia, through the Iranian Plateau and Caspian Basin to the

western edge of the Indian Subcontinent in the vicinity of the range of *Bufoniceps*. *Bufoniceps* is restricted to a small region of the Thar Desert on the western edge of the Indian Subcontinent. The tree presented in Macey et al. (2000b) was suggestive of an Afro-Arabian origin for the Agaminae but the alternative of an origin in the Indian Subcontinent could not be rejected. The analysis presented here is equivocal for an origin in either region because of a basal polytomy. The fact that the sister taxon to a major clade of the Agaminae is an Indian endemic raises the question of a possible origin of the Agaminae in the Indian Subcontinent. Further work is needed to resolve this issue, perhaps with phylogenetic analysis of complete mitochondrial genomes.

Xenagama is found to be the sister taxon to *Pseudotrapelus* and not to the African genus Agama. *Pseudotrapelus* occurs in Arabia and adjacent regions of Egypt and Libya. *Xenagama* is restricted to a small region of the Horn of Africa in Somalia and Ethiopia directly across the Red Sea from Arabia where *Pseudotrapelus* is found. The Afro-Arabian Plate began to divide along the Red Sea rift 40 MYBP but accelerated 5-10 MYBP and there have been periodic connections between the Horn of Africa and Arabia (Girdler, 1984).

4.2. Sequence divergences and age

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 for uncorrected pairwise comparisons in agamine lizards (Macey et al., 1998). This calibration has been shown to be robust across numerous amphibian and reptile taxa (reviewed in Weisrock et al., 2001). In addition, we calibrated a transversional rate of 0.98% per million years by comparing divergences of *Laudakia* from the Pamir (*L. lehmanni, L. himalayana*, and *L. stoliczkana*) with those from the

Iranian Plateau (*L. microleps, L. caucasia,* and *L. erythrogastra*). The rise of the Pamir-Tien Shan is well dated at 10 MYBP (million years before present; Abdrakhmatov et al., 1996).

Pairwise sequence divergences are presented in Table 1. Using straight uncorrected pairwise distances we calculate a 17.6 MYBP separation of *Xenagama* and *Pseudotrapelus*, and using the trasversional pairwise distances a 19.2 MYBP separation. Although mitochondrial DNA is known to begin to accumulate 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, our two estimates are quite similar. These dates fit well with earlier vicariant separation of Africa and Arabia across the Red Sea rift and do not fit with the more recent activity 5-10 MYBP.

Using straight uncorrected pairwise distances we calculate a 15.7 MYBP separation of *Bufoniceps* and *Trapelus*, and using the trasversional pairwise distances a 16.8 MYBP separation. No obvious geologic barrier currently exists between *Bufoniceps* and *Trapelus*. Indeed, the *Trapelus agilis* complex has been found in sympatry with *B. laungwalansis* at Sam, Rajasthan, India. Perhaps along the western margin of the Indian Subcontinent, tectonic activity, coupled with the second phase of Tibetan uplifting 20 MYBP (Le Forte, 1998; Searle, 1991), these taxa were divided. This would have been followed by subsequent dispersal of *Trapelus* back into the Indian Subcontinent. Clearly, Miocene events are responsible for divergences of the Agaminae.

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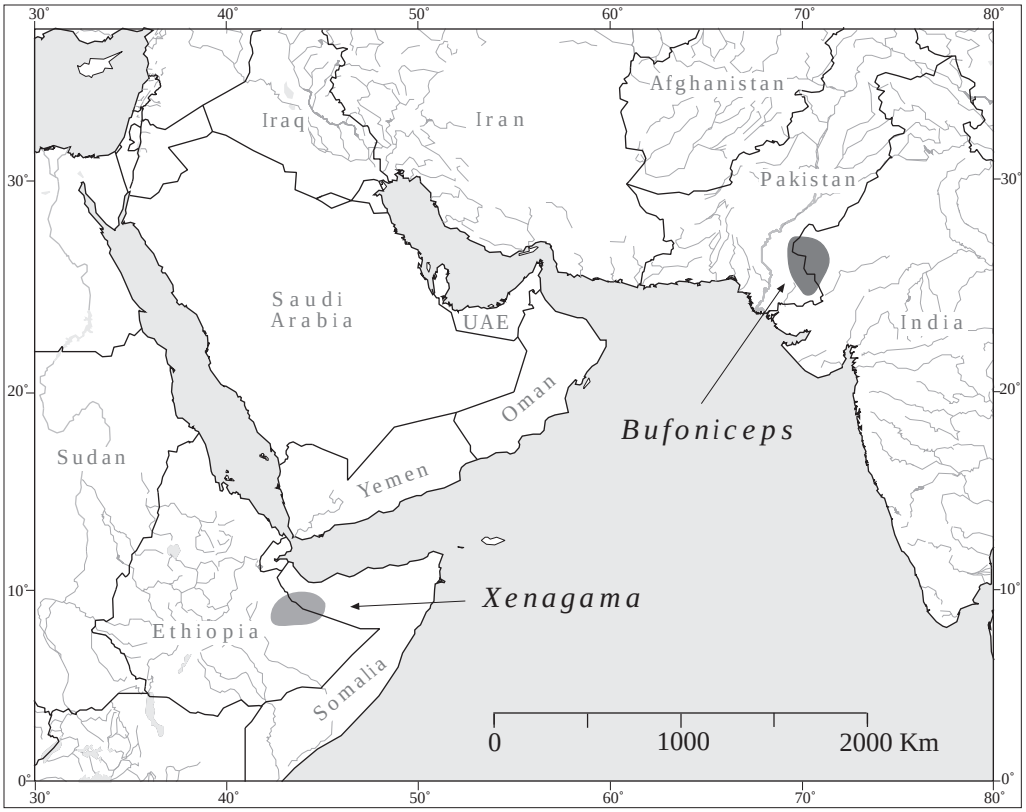
Figure legends

Fig. 1. Map showing the distribution of *Bufoniceps laungwalansis* and *Xenagama taylori*. Each taxon has a limited distribution with *Bufoniceps laungwalansis* restricted to the Thar Desert in western India and adjacent Pakistan. The two species of *Xenagama* are restricted to the Horn of Africa in Somalia and adjacent Ethiopia. Other members of the Agaminae range from North Africa through Arabia, Southwest Asia to Central Asia and Tibet (not shown).

Fig. 2. The strict consensus of seven most parsimonious trees resulting from analysis of the 1434 (1046 informative) aligned sites which is 12,236 steps in length. Bootstrap values appear above branches and decay indices are presented below. Note the analysis includes all 72 taxa from Macey et al. (2000b) and the two newly reported sequences but only taxa in the subfamily Agaminae are shown here. The new taxa, *Bufoniceps laungwalansis* and *Xenagama taylori* are depicted in bold as is the support for their placement in the Agaminae. The nine major lineages of the Agaminae are delineated to the right as A-I.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50°
60°
70°
80°
30°
20°
10°
0°
Afghanistan
Iraq
Iran
Pakistan
Saudi
Arabia
UAE
India
Oman
Bufoniceps
Sudan
Yemen
Xenagama
Ethiopia
Somalia
0
1000
2000 Km

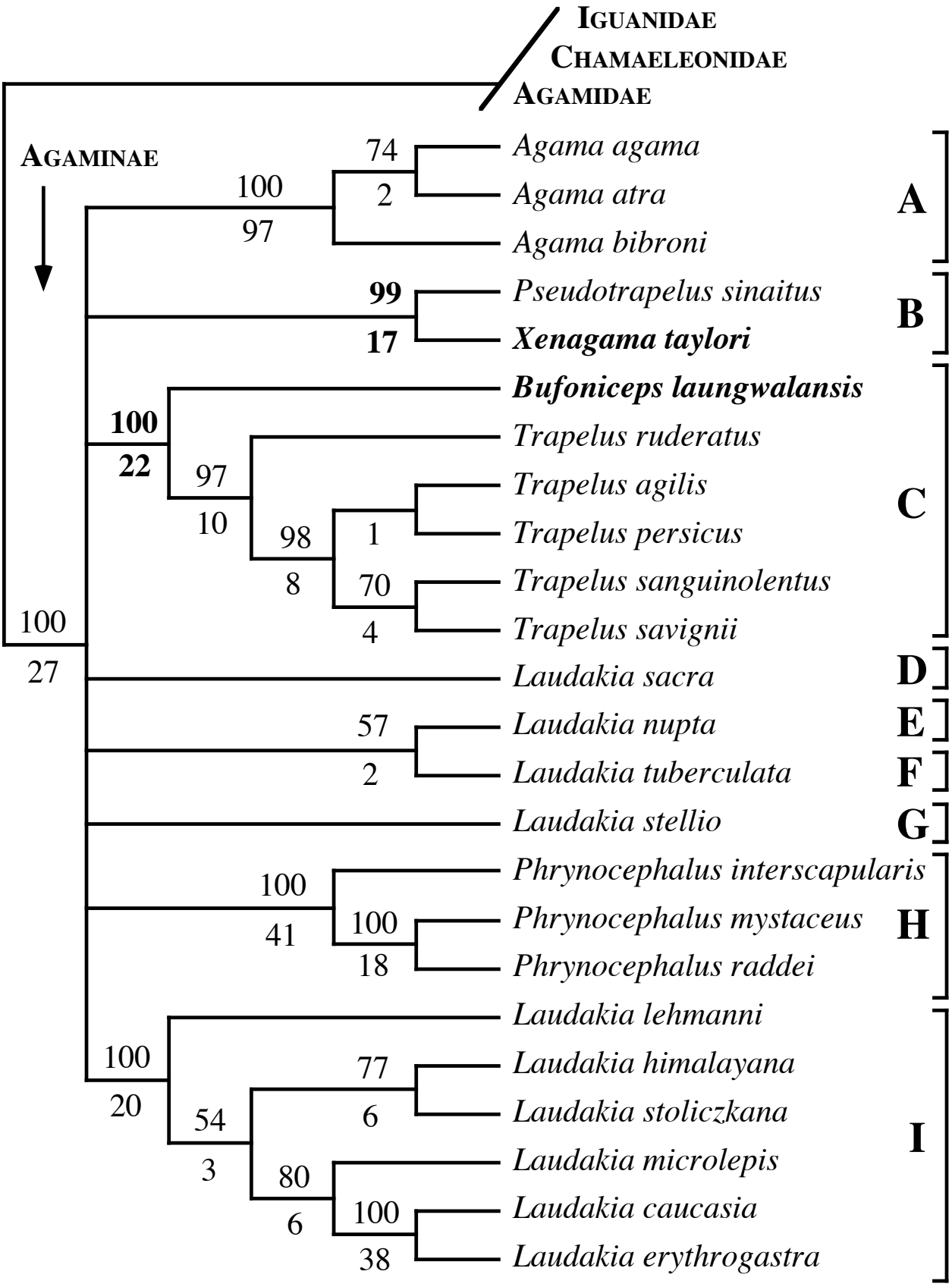
IGUANIDAE
CHAMAELEONIDAE
AGAMIDAE
AGAMINAE
74
2
100
97
Agama agama
Agama atra
Agama bibroni
A
99
17
Pseudotrapelus sinaitus
Xenagama taylori
B
Bufoniceps laungwalansis
100
22
97
10
98
8
1
70
4
Trapelus ruderatus
Trapelus agilis
Trapelus persicus
Trapelus sanguinolentus
Trapelus savignii
C
100
27
Laudakia sacra
D
57
2
Laudakia nupta
Laudakia tuberculata
E
F
Laudakia stellio
G
100
41
100
18
Phrynocephalus interscapularis
Phrynocephalus mystaceus
Phrynocephalus raddei
H
Laudakia lehmanni
100
20
54
3
77
6
80
6
100
38
Laudakia himalayana
Laudakia stoliczkana
Laudakia microlepis
Laudakia caucasia
Laudakia erythrogastra
I

Table 1

Sequence divergences across taxa[a]

	1	2	3	4	5	6	7	8
1. *P. sinaitus*	—	22.86%	28.90%	28.17%	27.96%	28.73%	27.60%	27.81%
2. *X. taylori*	18.83%	—	25.85%	25.20%	23.99%	24.49%	24.28%	24.49%
3. *B. laungwalansis*	23.58%	20.75%	—	20.40%	20.54%	20.96%	20.04%	20.11%
4. *T. ruderatus*	22.78%	19.96%	16.01%	—	15.57%	17.06%	16.35%	16.49%
5. *T. agilis*	22.36%	19.67%	17.00%	12.88%	—	12.24%	10.90%	10.97%
6. *T. persicus*	22.15%	19.60%	17.07%	14.37%	11.25%	—	14.15%	13.09%
7. *T. sanguinolentus*	21.30%	19.04%	16.36%	14.08%	10.26%	12.88%	—	11.25%
8. *T. savignii*	21.16%	10.04%	15.94%	13.94%	9.98%	11.75%	10.19%	—

[a]Values above the dashed line are uncorrected pairwise distances and those below are uncorrected transversional distances. Note genera are abbreviated as *P.* = *Pseudotrapelus, X.* = *Xenagama, B.* = *Bufoniceps*, and *T.* = *Trapelus*.