

Rostral Horn Evolution among Agamid Lizards of the Genus *Ceratophora* Endemic to Sri Lanka

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Received April 10, 2001; revised July 9, 2001

The first phylogenetic hypothesis for the Sri Lankan agamid lizard genus *Ceratophora* is presented based on 1670 aligned base positions (472 parsimony informative) of mitochondrial DNA sequences, representing coding regions for eight tRNAs, ND2, and portions of ND1 and COI. Phylogenetic analysis reveals multiple origins and possibly losses of rostral horns in the evolutionary history of *Ceratophora*. Our data suggest a middle Miocene origin of *Ceratophora* with the most recent branching of recognized species occurring at the Pliocene/Pleistocene boundary. Haplotype divergence suggests that an outgroup species, *Lyriocephalus scutatus*, dates at least to the Pliocene. These phylogenetic results provide a framework for comparative studies of the behavioral ecological importance of horn evolution in this group. © 2002 Elsevier Science

Key Words: reptilia; squamata; Iguania; Agamidae*; *Ceratophora*; mitochondrial DNA; phylogenetics; character evolution.

INTRODUCTION

Squamate reptiles exhibit substantial variation in body ornamentation, including crests, frills, spines, casques, dewlaps, and horns. Rostral protuberances or “horns” are rare and all occurrences are in Iguania. Within the iguanian family Iguanidae, horns are confined to three species of *Anolis* from the Amazonian Andes (Williams, 1979). Within Acrodonta, horns occur only in the family Chamaeleonidae and the agamid subfamily Draconinae (Macey *et al.*, 2000b). Phylogenetic analysis of Chamaeleonidae suggests two evolutionary origins and three losses of horns in this group (Townsend and Larson, 2002).

The agamid lizard genus *Ceratophora* contains five species endemic to Sri Lanka, most exhibiting modifications of the rostrum in the form of a rhinoceros-like

horn (see color plates in Pethiyagoda and Manamendra-Arachchi, 1998). Horn morphology is radically different for each species (Table 1). Only two other monotypic genera within Agamidae* (asterisk denotes a metataxon, whose monophyly is uncertain) contain a prominent, horn-like rostral appendage: *Harpesaurus*, which has a forked appendage, and *Thaumatorhynchus*, which has a single, cylindrical horn (Manthey and Schuster, 1996).

As originally described, *Ceratophora* contained three species, *C. aspera*, *C. stoddartii*, and *C. tennentii*, each exhibiting prominent rostral appendages. *Ceratophora aspera*, a ground-dwelling species, and the subarboreal *C. stoddartii* occupy the largest geographic area of the described species, whereas the arboreal species, *C. tennentii*, is found only in the Knuckles mountain range. Recently, Pethiyagoda and Manamendra-Arachchi (1998) reviewed the taxonomic content of *Ceratophora* and described two additional species, *C. erdeleni* and *C. karu*. The subarboreal *C. erdeleni* has a rudimentary or nonexistent rostral appendage and is otherwise very similar morphologically to *C. stoddartii*, whereas *C. karu* is ground-dwelling and the only fast-moving lizard containing a rostrum composed of numerous pointed scales. The rostrum of *C. karu* lacks the fleshy protuberance characteristic of horned *Ceratophora*, however. Both of these species have restricted distributional ranges in the Morningside Forest Reserve of southeastern Sri Lanka and surrounding areas. Species of *Ceratophora* are allopatric to each other, except *C. erdeleni* and *C. karu*, whose ranges overlap in the Morningside Forest Reserve.

Phylogenetic relationships of all species of *Ceratophora* are examined using 1670 aligned positions (472 informative) of mitochondrial DNA sequence. The region sequenced encodes part of ND1 (NADH dehydrogenase subunit 1), tRNA^{Gln}, tRNA^{Ile}, tRNA^{Met}, ND2 (NADH dehydrogenase subunit 2), tRNA^{Trp}, tRNA^{Ala}, tRNA^{Asn}, replication origin for the light strand (O_L), tRNA^{Cys}, tRNA^{Tyr}, and part of COI (cytochrome *c* ox-

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TABLE 1
List of Specimens Included in This Study

Species	Microhabitat	Rostral appendage	Locality	Museum number	GenBank number
<i>Aphaniotis fusca</i>	Arboreal	None	Selangor, Malaysia	TNHC 57874	AF128495
<i>Japalura flaviceps</i>	Subarboreal	None	Sichuan Prov., China	MVZ 216622	AF128500
<i>Cophotis ceylanica</i>	Arboreal	None	Nagrak Div., Sri Lanka	WHT 2061	AF128493
<i>Lyriocephalus scutatus(1)</i>	Arboreal	Round; bulbous complex of scales	Puwakpitiya, Sri Lanka	WHT 2196	AF128494
<i>Lyriocephalus scutatus(2)</i>	Arboreal	Round; bulbous complex of scales	Kottawa, Sri Lanka	WHT 1828	AF364052
<i>Ceratophora aspera</i>	Ground-dwelling	Cylindrical; covered with pointed scales	Kottawa, Sri Lanka	WHT 1825	AF128491
<i>Ceratophora karu</i>	Ground-dwelling	Rudimentary or absent; small, triangular rostral scales	Rakwana, Sri Lanka	WHT 2259	AF128520
<i>Ceratophora tennentii</i>	Arboreal	Laterally compressed, elliptical; covered in granular scales	Knuckles Mtn., Sri Lanka	WHT 1633	AF128521
<i>Ceratophora erdeleni</i>	Subarboreal	Rudimentary or absent	Rakwana, Sri Lanka	WHT 1808	AF128522
<i>Ceratophora stoddartii(1)</i>	Subarboreal	Pointed; restricted to rostral scale alone	Sita Eliya, Sri Lanka	WHT 1682	AF364053
<i>Ceratophora stoddartii(2)</i>	Subarboreal	Pointed; restricted to rostral scale alone	Tangamalai, Sri Lanka	WHT 1512	AF128492
<i>Ceratophora stoddartii(3)</i>	Subarboreal	Pointed; restricted to rostral scale alone	Namunukula, Sri Lanka	WHT 1511	AF364054

Note. Brief descriptions of microhabitat, specimen localities, museum voucher number, and GenBank accession numbers are provided. Rostral appendages (when present) are described in terms of horn shape and scale morphology, respectively (Pethiyagoda and Manamendra-Arachchi, 1998). Museum codes: TNHC (Texas Memorial Museum, Austin, TX), MVZ (Museum of Vertebrate Zoology, University of California at Berkeley), and WHT (Wildlife Heritage Trust, Colombo, Sri Lanka).

dase subunit I). The previously published sequence of *C. aspera* (Macey *et al.*, 2000b) is compared to a new sequence from the same population. Three populations of *C. stoddartii* are examined to assess phylogenetic divergence within this wide-ranging species. Phylogenetic inferences are used to generate hypotheses of horn evolution within *Ceratophora*.

A recent revision of Agamidae* by Macey *et al.* (2000b) placed *Ceratophora* within the subfamily Draconinae. In their analysis, *Lyriocephalus scutatus* and *Cophotis ceylonica* formed a clade strongly supported as the sister group to *Ceratophora*. Sequences from *Cophotis* and *Lyriocephalus*, including an additional population of *Lyriocephalus scutatus*, are used here as closest outgroups. In addition, two other southeast Asian members of Draconinae, *Japalura flaviceps* and *Aphaniotis fusca*, are selected as outgroups based on the phylogenetic hypothesis of Macey *et al.* (2000b). All four of these sequences are reported in Macey *et al.* (2000b). Sequences for *Ceratophora karu*, *C. tennentii*, and *C. erdeleni* that were previously reported for approximately 300 bp (Macey *et al.*, 2000c) have been extended for the full 1741-bp region analyzed here.

MATERIALS AND METHODS

Specimen Information

See Table 1 for museum numbers, localities of voucher specimens from which DNA was extracted,

and GenBank accession numbers for DNA sequences. Complete locality data are deposited in GenBank files.

Laboratory Protocols

Genomic DNA was extracted from liver or muscle using Qiagen QIAamp tissue kits. Amplification of genomic DNA was conducted using a denaturation at 94°C for 35 s, annealing at 50°C for 35 s, and extension at 70°C for 150 s with 4 s added to the extension per cycle for 30 cycles. 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. Reamplified double-stranded products were purified on 2.5% acrylamide gels (Maniatis *et al.*, 1982). Template DNA was eluted from acrylamide passively over 3 days with Maniatis elution buffer (Maniatis *et al.*, 1982). Cycle-sequencing reactions were run using the Promega fmol DNA sequencing system with a denaturation at 95°C for 35 s, annealing at 45–60°C for 35 s, and extension at 70°C for 1 min for 30 cycles. Sequencing reactions were run on Long Ranger sequencing gels for 5–12 h at 38–40°C.

Two primer pairs were used to amplify genomic DNA from the ND1 gene to the COI gene: L3914 and H4980 and L4437 and H5934. Both strands were sequenced using L3914, L4178b, L4160, L4437, L4831, L4882b, H5617b, L5638b, and H5934. Primers L4831, L4178b, L4831, H4980, L5638b, and H5934 are from Macey *et al.*

al. (1997a). L3914 is from Macey *et al.* (1998b) which is erroneously listed as L3878. L4160 is from Kumazawa and Nishida (1993). L4437 and H5617b are from Macey *et al.* (1997b). L4882b is from Macey *et al.* (2000b). Primer numbers refer to the 3' end on the human mitochondrial genome (Anderson *et al.*, 1981), where L and H denote extension of light and heavy strands, respectively.

Phylogenetic Analysis

DNA sequences were aligned manually. Positions encoding part of ND1, all of ND2, and part of COI were translated to amino acids using MacClade (Maddison and Maddison, 1992) for confirmation of alignment. Alignment of sequences encoding tRNAs was 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 were excluded from phylogenetic analyses (see Results). Alignment has been deposited in GenBank.

Phylogenetic trees were estimated using PAUP* beta version 4.0b4a (Swofford, 2000) with branch-and-bound searches. Bootstrap resampling (Felsenstein, 1985a) was applied to assess support for individual nodes using 1000 bootstrap replicates with branch-and-bound searches. Decay indices ("branch support" of Bremer, 1994) were calculated for all internal branches of the tree using branch-and-bound searches that retained suboptimal trees. We obtained the decay index by tabulating the minimum increase in number of steps resulting from removal of the node of interest from the overall shortest tree.

Evolution of rostral horns among *Ceratophora* species was reconstructed using MacClade (Maddison and Maddison, 1992) on the single overall most parsimonious tree. The outgroup species *Aphaniotis fusca*, *Japalura flaviceps*, and *Cophotis ceylonica* do not contain a rostral protuberance; however, the outgroup species *Lyriocephalus scutatus* does contain a knob-like rostral structure.

Wilcoxon signed-ranks tests (Templeton, 1983; Felsenstein, 1985b) were used to examine statistical significance of the shortest tree relative to alternative hypotheses. All tests were conducted as two-tailed tests (Felsenstein, 1985b). Tests were performed using PAUP* (Swofford, 2000), which incorporates a correction for tied ranks.

Alternative phylogenetic hypotheses were tested using the most parsimonious phylogenetic topologies compatible with them. To find the most parsimonious tree(s) compatible with a particular phylogenetic hypothesis, phylogenetic topologies were constructed using MacClade (Maddison and Maddison, 1992) and analyzed as constraints using PAUP* (Swofford, 2000) with branch-and-bound searches.

RESULTS

Assessment of Homology and Sequence Alignment

Protein-coding genes are alignable except at the C-terminal end of ND1, which is excluded because of questionable alignment (positions 82–86). Gaps are placed in the ND2 gene sequence at nucleotide positions 1252–1254 in the outgroup *J. flaviceps*.

Among tRNA genes, several loop regions are unalignable as are noncoding regions between genes. The dihydrouridine (D) loops for genes encoding tRNA^{Ile} (positions 176–182) and tRNA^{Trp} (positions 1345–1354) are excluded from analyses. Part of the variable loop for the gene encoding tRNA^{Asn} (positions 1516–1518) is not used. The loop of the origin for light-strand replication (O_L, positions 1563–1585) between the tRNA^{Asn} and tRNA^{Cys} genes is not alignable and therefore not used for phylogenetic analysis. The TΨC (T) loop and part of the T-stem for the gene encoding tRNA^{Cys} (positions 1597–1605) and the T-loop for the gene encoding tRNA^{Tyr} (positions 1656–1659) are excluded from analyses. Noncoding sequences between the tRNA^{Ala} and tRNA^{Asn} genes (positions 1479–1488) are not used. Excluded regions comprise 4% of aligned sequence positions (71 of 1741 positions).

Several observations suggest that DNA sequences reported are from the mitochondrial genome and not nuclear-integrated copies of mitochondrial genes (see Zhang and Hewitt, 1996). Protein-coding genes do not contain premature stop codons, and sequences of tRNA genes appear to code for tRNAs with stable secondary structures, indicating functional genes. In addition, all sequences show strong strand bias against guanine on the light strand (G = 11.4–13.4%, A = 32.6–36.7%, T = 21.9–23.7%, and C = 28.6–32.0%), which is characteristic of the mitochondrial genome but not the nuclear genome (Macey *et al.*, 1997a; McGuire and Heang, 2001).

Genetic Variation

Variation in phylogenetically informative positions (parsimony criterion) is observed among all tRNA and protein-coding genes (Table 2). Phylogenetically informative sites are predominately from protein-coding regions (83% of informative sites) with most of the variation observed in third codon positions (48%). However, first and second codon positions, as well as stem and nonstem regions of tRNA genes, together contributed over half of the phylogenetically informative sites (21, 13, 13, and 4%, respectively). Therefore, no single set of characters dominates the phylogenetic analysis.

Phylogenetic Relationships

A single overall most parsimonious tree is produced from analysis of 12 aligned DNA sequences containing

TABLE 2

Distribution of Phylogenetically Informative and Variable Positions

	ND1 codon positions ^a			tRNA ^{Gln}		tRNA ^{Ile} ^a		tRNA ^{Met}	
	1st	2nd	3rd	Stem	Nonstem	Stem	Nonstem	Stem	Nonstem
Informative sites	6	2	22	12	7	7	2	9	0
Variable sites	11	6	25	16	11	14	6	14	5
	ND2 codon positions ^a			tRNA ^{Trp} ^a		tRNA ^{Ala}		tRNA ^{Asn}	
	1st	2nd	3rd	Stem	Nonstem	Stem	Nonstem	Stem	Nonstem
Informative sites	92	58	203	5	5	5	2	10	1
Variable sites	174	110	293	7	6	13	4	17	4
	tRNA ^{Cys} ^a		tRNA ^{Tyr} ^a		COI codon positions			Noncoding regions ^b	
	Stem	Nonstem	Stem	Nonstem	1st	2nd	3rd		
Informative sites	1	3	12	1	3	1	3	0	
Variable sites	8	7	20	2	5	1	8	5	
Total	Protein-coding codon positions			tRNA		Noncoding regions ^b	All aligned sequence		
	1st	2nd	3rd	Stem	Nonstem				
Informative sites	101	61	228	61	21	0	472		
Variable sites	190	117	326	109	45	5	792		

^a Not including unalignable positions excluded from phylogenetic analysis.

^b Noncoding region one is between the tRNA^{Gln} and tRNA^{Ile} genes and contains three variable positions. Noncoding region two is between the tRNA^{Trp} and tRNA^{Ala} genes and contains two variable positions.

1670 base positions, 472 of which are phylogenetically informative (Fig. 1, Table 2). Phylogenetic relationships are well resolved, receiving bootstrap values above 95% and decay indices greater than 5 for all nodes except two. A clade comprising *Lyriocephalus*, *Cophotis*, and *Ceratophora* is well supported (bootstrap 100%, decay index 42). The sister-taxon relationship of *Lyriocephalus* and *Cophotis* is well supported (bootstrap 99%, decay index 17). Monophyly of *Ceratophora* is strongly supported by a bootstrap value of 97% and a decay index of 13. The small, ground-dwelling species *C. aspera* is the sister taxon to a moderately supported group containing the remaining species (bootstrap 76%, decay index 4), and the two *C. aspera* sequences from the same population are identical. The small, ground-dwelling species *C. karu* is the sister taxon to a group containing *C. tennentii*, *C. erdeleni*, and all populations of *C. stoddartii* (bootstrap 100%, decay index 17). The large-bodied, subarctic species *C. erdeleni* and *C. stoddartii* form a well-supported, monophyletic group (bootstrap 100%, decay index 48). The three populations of *C. stoddartii* form a moderately supported monophyletic group to the exclusion of the morphologically similar *C. erdeleni* (bootstrap 86%, decay index

4). Within *C. stoddartii*, populations from Tangamalai and Namunukula are strongly supported as a group (bootstrap 96%, decay index 5).

We investigated whether presence of horns in *Ceratophora* arose once or multiple times. When occurrence of horns is mapped onto the overall shortest tree using MacClade (Fig. 2), multiple origins or losses of horns are depicted on the overall shortest tree (Fig. 1). To test the hypothesis of multiple evolutionary events involving horns, the Wilcoxon signed-ranks test is applied. When the overall shortest tree from analysis of the DNA sequence data is compared to the shortest alternative tree (A in Appendix 1) in which all species containing prominent horns from a monophyletic group, this alternative is rejected in favor of the overall shortest tree ($n = 122$, $T_s = 5.98$, $P < 0.0001^{**}$). When the overall shortest tree from analysis of the DNA sequence data is compared to the shortest alternative tree (B in Appendix 1) in which the two species of *Ceratophora* lacking prominent horns form a clade, this alternative is rejected in favor of the overall shortest tree ($n = 98$, $T_s = 6.87$, $P < 0.0001^{**}$). These results indicate that horns either arose multiple times or were lost multiple times among the five species of *Ceratophora*.

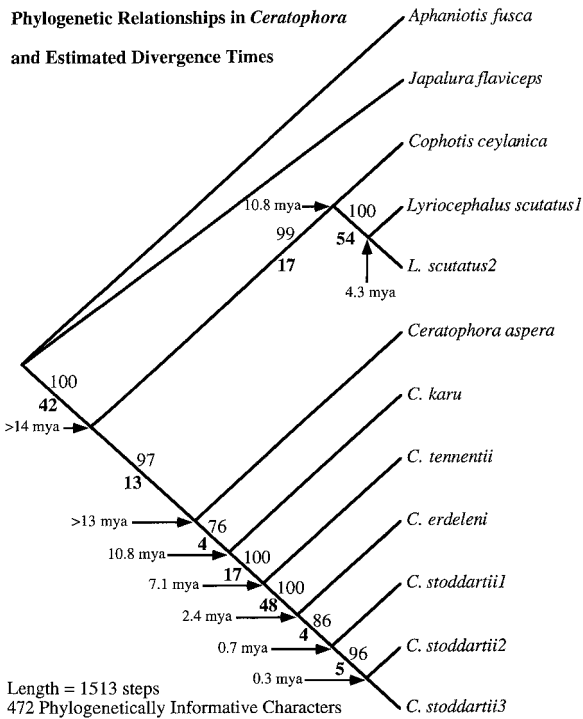


FIG. 1. Phylogenetic relationships among *Ceratophora* species. Single most parsimonious tree obtained from analysis of 1670 aligned base positions (472 phylogenetically informative) of mitochondrial DNA (length = 1513 steps, consistency index 0.716). Bootstrap values are presented above branches and decay indices are shown in bold below branches. Times of divergence for each node are denoted with arrows at that particular node (mya represents million years ago) based on the pairwise sequence divergence rate of 1.3% per million years (Macey *et al.*, 1998a).

DISCUSSION

Horn Evolution in Ceratophora

This study provides the first phylogenetic hypothesis for the agamid horned-lizard genus *Ceratophora* and reveals multiple evolutionary events involving horns among the five species. In the parsimony reconstruction (Fig. 2), horns have evolved either three times in *C. aspera*, *C. tennentii*, and *C. stoddartii*, or once at the base of the *Ceratophora* tree followed by separate losses in *C. karu* and *C. erdeleni*. This latter hypothesis appears less likely given the radically different morphologies of horns among *Ceratophora* species (Pethiyagoda and Manamendra-Arachchi, 1998). If horns have a single evolutionary origin, horn morphology should be similar among all horned species.

Unfortunately, relatively little is known about the function of horns in *Ceratophora*. Two primary hypotheses have been proposed for the function of ornamentation in iguanian lizards. One hypothesis is that horns are important for species recognition (Rand, 1961).

This hypothesis was suggested because closely related species of African chameleons have very different ornamentation types when occurring in sympatry; this is unlikely for *Ceratophora*, because the only two sympatric species in this genus do not have horns.

Another hypothesis is that horns function in intraspecific communication, primarily during male–male interactions. The agamid frillneck lizard of Australia, *Chlamydosaurus kingii*, displays more elaborate head-bobs, push-ups, mouth-openings and full erection of the frill during male–female interactions (Shine, 1990). However, partial erection of the frill is observed during male–male interactions and possible predator interactions. Watkins (1998) demonstrates that the crest in males of the iguanid lizard *Microlophus occipitalis* is relatively larger and is elevated only during interactions between males, never during male–female encounters. This latter hypothesis is the most plausible explanation for horns in *Ceratophora*. In general, horns of male *Ceratophora* are larger than female horns and are movable (Ilangakoon, 1990; Pethiyagoda and Manamendra-Arachchi, 1998). More detailed ecological work is necessary to determine function of horns in *Ceratophora*.

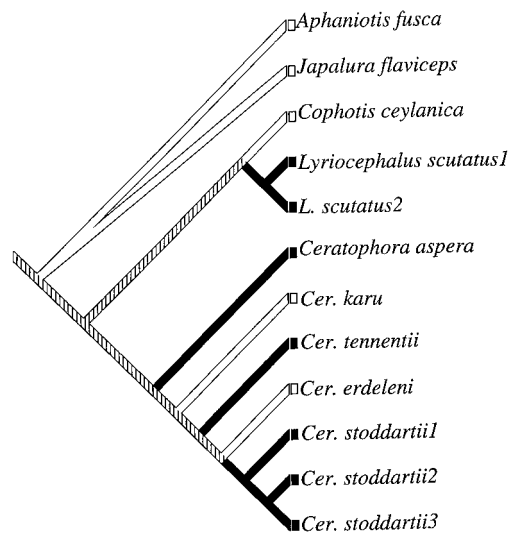


FIG. 2. Parsimony reconstruction of the presence of horns in the agamid lizard genus *Ceratophora*. The overall shortest tree as shown in Fig. 1 is presented. Light branches represent lineages that do not contain a prominent extension of the rostrum, dark branches are those lineages with a rostral protuberance, and hatched branches are lineages equivocal for the presence of rostral structures. Based upon large morphological differences among horns of different species, we favor four separate origins of horns in *C. aspera*, *C. stoddartii*, *C. tennentii*, and *L. scutatus*.

TABLE 3

Percent Sequence Divergences between Haplotypes (above Diagonal) and Maximum-Likelihood Corrected Distances (GTR + I + Γ Model; Yang, 1994) Reported as Percentages (below Diagonal)

	<i>Aphaniotis fusca</i>	<i>Japalura flaviceps</i>	<i>Cophotis ceylanica</i>	<i>Lyr. scutatus1</i>	<i>Lyr. scutatus2</i>	<i>Ceratophora aspera</i>	<i>Cer. karu</i>	<i>Cer. tennentii</i>	<i>Cer. erdeleni</i>	<i>Cer. stoddartii1</i>	<i>Cer. stoddartii2</i>	<i>Cer. stoddartii3</i>
<i>Aphaniotis fusca</i>	—	25.52	23.77	24.31	23.26	25.00	24.14	24.75	24.14	24.01	24.25	24.43
<i>Japalura flaviceps</i>	68.59	—	24.19	24.92	24.36	25.67	25.45	24.39	24.54	24.05	24.18	24.30
<i>Cophotis ceylanica</i>	56.12	55.20	—	14.56	13.61	19.27	17.23	16.63	16.97	15.95	16.31	16.43
<i>Lyr. scutatus1</i>	62.23	61.60	22.68	—	5.56	19.71	18.92	18.75	19.09	18.18	18.49	18.61
<i>Lyr. scutatus2</i>	57.62	58.52	20.63	6.31	—	19.24	18.70	17.98	18.26	17.78	18.08	18.20
<i>Ceratophora aspera</i>	64.72	65.72	35.55	37.89	36.68	—	17.55	15.87	17.07	15.92	15.86	15.86
<i>Cer. karu</i>	59.42	63.12	28.32	34.15	33.44	30.02	—	13.45	14.35	14.23	14.23	14.29
<i>Cer. tennentii</i>	61.07	59.97	27.42	33.67	31.78	26.09	19.61	—	10.13	8.93	8.93	9.05
<i>Cer. erdeleni</i>	59.72	58.97	27.74	34.87	32.60	28.54	21.49	13.12	—	2.95	3.31	3.25
<i>Cer. stoddartii1</i>	58.16	56.99	25.53	32.32	31.13	25.84	21.06	11.23	3.13	—	0.96	0.90
<i>Cer. stoddartii2</i>	59.40	57.32	26.37	33.10	31.89	25.65	21.09	11.26	3.54	0.98	—	0.42
<i>Cer. stoddartii3</i>	59.82	57.48	26.59	33.34	32.13	25.70	21.15	11.43	3.47	0.92	0.42	—

Dating Horn Evolution and Cladogenesis in *Ceratophora*

Rates of molecular evolution for this mitochondrial DNA segment are reported in the agamid lizard genus *Laudakia* (Macey *et al.*, 1998a, 2000a) as 0.65% change per million years per lineage. This approximate calibration has been corroborated for fishes (0.65%), bufonid frogs (0.69%), and gekkonid lizards (0.57%) (Bermingham *et al.*, 1997; Macey *et al.*, 1998a, 2000a). After 10 million years, mitochondrial DNA is expected to saturate (Moritz *et al.*, 1987); therefore, a linear relationship of nucleotide substitutions and time is not anticipated. If this rate of molecular evolution is approximately correct for other agamid lizards, then divergence times in the subfamily Draconinae and within *Ceratophora* are expected to exceed 23 million and 13 million years, respectively (Table 3). All branching events among the endemic Sri Lankan genera *Ceratophora*, *Lyriocephalus*, and *Cophotis* are at least 14 million years (~18% sequence divergence; Fig. 1; Table 3).

Our data suggest a Miocene origin of *Ceratophora* with the most recent branching of recognized species occurring at the Pliocene/Pleistocene boundary. The first divergence among *Ceratophora* lineages is estimated to be approximately 12.6 MYA (~16% sequence divergence; Table 3) between *C. aspera* and the common ancestor of the remaining four species. Two million years later at ~10.8 MYA (Fig. 1; Table 3) the lineage leading to *C. karu*, without a prominent horn, split from the lineage ancestral to the remaining species. *Ceratophora tennentii* separated at ~7.1 MYA (Fig. 1; Table 3) from a lineage giving rise to *C. erdeleni* and *C. stoddartii*. At approximately 2.4 MYA (Fig. 1; Table 3) divergence occurred between ancestral lineages of *C. erdeleni*, which lacks prominent horns, and *C. stoddartii*, which has a unique, singly scaled, pointed horn. Divergence of the two populations of

Lyriocephalus, between which relatively little morphological divergence has occurred, is approximately 4.3 MYA (Fig. 1; Table 3).

The large morphological differences observed among horn structures of *C. aspera*, *C. stoddartii*, and *C. tennentii* and the fact that these species do not form a clade suggest three separate origins of horns in *Ceratophora*. Our results suggest that horns in *C. aspera* and *C. tennentii* arose no earlier than middle Miocene and that horns of *C. stoddartii* arose no earlier than late Pliocene. More detailed studies of relationships among populations within *Ceratophora* species and their ecological attributes are needed to generate more detailed hypotheses regarding horn evolution in this group.

APPENDIX I

Trees Used in Wilcoxon Signed-Ranks Tests

The shortest alternative trees recovered from phylogenetic analyses imposing constraints are listed with tree lengths in parentheses.

A. The most parsimonious tree depicting the alternative hypothesis that *Ceratophora* with prominent horns form a monophyletic group (1579 steps): (*Aphaniotis fusca*, (*Japalura flaviceps*, ((*Cophotis ceylanica*, (*Lyriocephalus scutatus1*, *L. scutatus2*)), (((*Ceratophora aspera*, *C. tennentii*), (*C. stoddartii1*, (*C. stoddartii2*, *C. stoddartii3*))), *C. erdeleni*), (*C. karu*))).

B. The most parsimonious tree depicting the alternative hypothesis that *Ceratophora* without prominent horns form a monophyletic group (1581 steps): (*Aphaniotis fusca*, (*Japalura flaviceps*, ((*Cophotis ceylanica*, (*Lyriocephalus scutatus1*, *L. scutatus2*)), (*Ceratophora aspera*, (((*C. karu*, *C. erdeleni*), (*C. stoddartii1*, (*C. stoddartii2*, *C. stoddartii3*))), *C. tennentii*))).

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Foundation (predoctoral fellowship to J.A.S.; DEB-9726064 to A.L., J.R.M., and Theodore J. Papenfuss; and DEB-9318642 and DEB-9982736 to J. B. Losos, K. de Queiroz, and A.L.). This work is LBNL-48056 and was partially performed under the auspices of the U.S. Department of Energy by the University of California, Lawrence Berkeley National Laboratory under Contract DE-AC03-76SF00098.

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