Phylogenetic Relationships of Eublepharid Geckos (Reptilia: Squamata): A Molecular Approach

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ABSTRACT—Phylogenetic analyses were carried out for representatives of all eublepharid genera and a few other gekkonoid taxa using sequence data for 879 base pairs of mitochondrial 12S and 16S ribosomal RNA genes. Neighbor-joining (NJ) distance analysis of the data suggested independent great divergences of Coleonyx and Aeluroscalabotes, and monophyly of the remainder within Eublepharidae (bootstrap proportion [BP]=76%). Of the latter, the two African genera, Hemitheconyx and Holodactylus, were almost certainly monophyletic altogether (BP=99%), whereas their sister-group relationship with Eublepharis received a weaker, but still substantial support (BP=68%). Within Goniurosaurus kuroiwae, G. k. splendens first diverged from the remainder (BP=100%), followed by G. k. kuroiwae from the northern part of Okinawajima (BP=100%): G. k. kuroiwae from the southern part of Okinawajima and G. k. orientalis, differing from each other at only seven bases, diverged finally (BP=99%). Parsimony analysis yielded results consistent with those of NJ analysis with respect to the monophyly of the two African genera and relationships within G. kuroiwae, but retained the other relationships within Eublepharidae unresolved. Our results, while showing no serious discrepancies with the relationships among eublepharid genera hypothesized from morphological data, cast a serious doubt to the currently accepted population systematics within G. kuroiwae. Furthermore, results of both analyses suggested a closer affinity of Diplodactylinae (as represented by Rhacodactylus trachyrhynchus) with Eublepharidae, rather than with Gekkoninae. Our study lends a robust support to the Laurasian origin of the family Eublepharidae.

INTRODUCTION

Kluge (1987), based on the cladistic analysis of morphological characters, recognized "eyelid geckos" classified to the subfamily Eublepharinae of the family Gekkonidae to that date (Kluge, 1967), as constituting a single primitive lineage distant from all other members of the microorder Gekkota (Fig. 1). He thus revalidated family Eublepharidae Boulenberg, 1883, to accommodate all eyelid geckos that sporadically occur in Eurasia, Africa and North America.

Also on the basis of the cladistic analysis of morphological characters, Grismer (1988), while confirming the monophyly of the eublepharid geckos, formulated a detailed, comprehensive phylogenetic hypothesis for the family (Fig. 2) and revised the infrafamilial classification accordingly. Through this and two subsequent taxonomic works (Grismer, 1989, 1991), 18 species belonging to six genera of two subfamilies have been recognized for the family Eublepharidae.

A few other studies demonstrated considerable chromosomal and eco-physiological variations among some eublepharid geckos (e.g., Murphy, 1974; Ota et al., 1987; Dial and Grismer, 1992), and these predict the effectiveness of approaches other than the external and anatomical analyses to the eublepharid phylogeny. For the improvement of a given phylogenetic hypothesis formulated on the basis of a certain set of characters, testing by using a different character set would be highly effective. Nevertheless, no approaches other than the above-mentioned morphological ones have ever been attempted toward the comprehensive phylogeny of this family or its relationship with other gekkotan groups.

We partially sequenced mitochondrial DNA (mtDNA) for representatives of all eublepharid genera and a few of other major groups of Gekkota (subfamilies Gekkoninae and Diplodactylinae of the family Gekkonidae), and analysed

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Fig. 1. Cladistic relationships of the major groups of Gekkota (exclusive of Pygopodidae) hypothesized by Kluge (1987). He also argued for (and depicted) the sister-group relationship of Pygopodidae with Diplodactylinae, but this account is not fully accepted (e.g., Estes et al., 1988).

Fig. 2. Cladistic relationships of the eublepharid genera hypothesized by Grismer (1988).

resultant sequences phylogenetically. In this paper, we compare our results with results of morphological analyses by previous authors, and discuss their systematic and biogeographical implications.

MATERIALS AND METHODS

A total of 17 specimens (three subspecies and seven species) representing all of the six eublepharid genera were used. Of these, six specimens of Goniurosaurus kuroiwaie and two specimens of Aeluroscalabotes felinus were sampled in the field. The other specimens were obtained from pet dealers in life or in fresh dead state. These included two obviously conspecific eublepharid specimens of unknown origins that showed morphological features characteristic of Goniurosaurus (as defined by Grismer [1988]), but were distinct from either of the two known species of the genus (i.e., G. kuroiwaie and G. lichtenfelder: Grismer, 1988) (Fig. 3). We henceforth refer to

Fig. 3. Apparently undescribed species of Goniurosaurus used in this study.
these specimens as G. sp. One diplodactyline (*Rhacodactylus trachyrhynchos*) and two gekkonine geckos (*Gekko gecko* and *Cyrtodactylus intermedius*) of the family Gekkonidae were also subjected to this study. As outgroups, two scincids (*Eumeces laticutatus* and *Lygosoma bowringii*) and one iguanid species (*Iguana iguana*) were chosen as representatives of Autarchoglossa (an extant sistergroup of Gekkota) and a more basal lineage (Estes *et al.*, 1988), respectively. Voucher specimens are deposited in the Department of Zoology, Kyoto University (KUZ: see Appendix-1).

Livers, removed from anesthetized or dead specimens and stocked at –80°C, were used for DNA extraction. Each sample was processed for sequencing of 12S and 16S ribosomal RNA (rRNA) genes of mitochondrial DNA (mtDNA) following the method by Wada *et al.* (1992) as modified by Honda *et al.* (1999). These genes, functionally important and thus are supposedly fairly conservative, are expected to be informative for divergence back to about 300 million years ago (MYA) at maximum (Mindell and Honeycutt, 1990). Thus, sequence variation in these genes seems to be suitable for the phylogenetic reconstruction of extant Gekkota, which divergence is considered to have initiated some 180 MYA (Kluge, 1987).

Alignments for DNA sequences were determined based on maximum nucleotide similarity. Using the aligned sequences, we eliminated gap sites and prepared a pairwise matrix of distance by Kimura’s (1980) two-parameter model for transition/transversion bias. The matrix was then subjected to the analysis by the neighbor-joining (NJ) method (Saitou and Nei, 1987) using Clustal W (Thompson *et al.*, 1994). Degrees of supports for internal branches of each tree were assessed by 1,000 bootstrap pseudoreplications (Felsenstein, 1985). Parsimony analysis was also performed using the Heuristic algorithm of PAUP (version 3.11: Swofford, 1993). In this analysis, each nucleotide base was regarded as a character and four kinds of salts as different character states. The result of this analysis was expressed as a 50% majority-rule consensus tree. In both analyses, each specimen was set as constituting a single OTU by itself.

The interpretation of bootstrap proportions (BPs) is still controversial (e.g., Felsenstein and Kishino, 1993; Hillis and Bull, 1993). We tentatively postulated BPs≥90% as strong support, 70≤BPs<90% as moderate support, 50≤BPs<70% as constituting limited evidence of monophyly, and BPs<50% as insufficient support for monophyly, following Shaffer *et al.* (1997).

**RESULTS**

Aligned sequences from two mtDNA genes are presented in Appendix-2. The 12S rRNA fragment consisted of 406 total aligned sites, of which 253 (62.3%) were variable. For the

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**Fig. 4.** NJ dendrogram deriving from Kimura’s (1980) distance matrix from 12S and 16S rRNA sequence data. Numbers at branches indicate bootstrap proportions in 1,000 bootstrap pseudoreplications. Of the ingroup taxa, those belonging to the family Eublepharidae, and subfamilies Gekkoninae and Diplodactylinae of the family Gekkonidae are denoted by circles, triangles and a rectangle, respectively. N and S following G. k. *kuroiwae* indicate that those specimens were from the northern and the southern parts of Okinawajima, respectively (see Appendix-1 for further details). For each of other species/subspecies, different individuals, when examined, are discriminated by arabic numerals (1, 2). Scale bar equals 0.1 of the distance.
16S rRNA fragment, there were 473 total sites, of which 259 (54.8%) were variable. Nucleotide replacements of the eublepharids with a diplodactyline and gekkonines varied from 174 base pairs (bp) (Holodactylus africanus vs Rhacodactylus trachyrhynchus) to 200 bp (Goniurosaurus sp. vs R. trachyrhynchus), and from 202 bp (H. africanus vs Cyrtodactylus intermedius) to 236 bp (G. kuroiwa kuroiwa vs Gekko gecko), respectively, whereas those between the latter two groups were from 197 bp (C. intermedius vs G. gecko) to 239 bp (R. trachyrhynchus vs G. gecko). Within Eublepharidae, the intergeneric and intrageneric replacements ranged from 137 bp (Hemitheconyx caudicinctus vs Holodactylus africanus) to 200 bp (Aeluroscalabotes felinus vs H. africanus), and from 7 bp (G. k. kuroiwa vs G. k. orientalis) to 159 bp (G. kuroiwa vs G. sp.), respectively.

Fig. 4 shows the NJ dendrogram derived from the OTU-distance matrix. In this analysis, no sufficient supports were obtained for the monophyly of the Eublepharidae, because of the inclusion of R. trachyrhynchus (Diplodactylinae of Gekkonidae) to the otherwise eublepharid cluster. Within Eublepharidae, Coleonyx and Aeluroscalabotes showed great, independent divergences, whereas the others appeared monophyletic (BP=76%). Sister-group relationships of the two African genera (Hemitheconyx and Holodactylus) of the latter was strongly supported (BP=98%), whereas their sister-group relationships with Eublepharis received a weaker support (BP=68%). On the other hand, monophyly of Goniurosaurus was even more weakly supported (BP=56%). All conspecific/consubspecific combinations received 98–100% BP supports. Of G. kuroiwa subspecies/populations examined here, G. k. splendens first diverged, and then did G. k. kuroiwa from the northern part of Okinawajima: G. k. kuroiwa from the southern part of Okinawajima and G. k. orientalis constituted a minimum monophyletic unit with high BP value (99%).

Results of the parsimony analysis showed no apparent inconsistency with the relationships suggested by the NJ dendrogram. As are depicted in Fig. 5, relationships resulting from this analysis weakly but still substantially supported the closer diplodactyline affinity to eublepharids than to gekkonines (BP = 64%), and strongly supported the monophyly of African eublepharids (97%), greatest divergence of G. k. splendens among the conspecifics, and closer affinity of G. k. kuroiwa from southern Okinawajima to G. k. orientalis than to G. k. kuroiwa from northern Okinawajima (BP=100% for both).

Fig. 5. The majority-rule consensus tree deriving from parsimony analysis using nucleotide bases of 12S and 16S rRNA genes as characters and the four kinds of salts as different character states. See Fig. 4 for explanations of denotations.
DISCUSSION

As far as the intergeneric phylogeny within Eublepharidae is concerned, results of the present analyses showed no serious contradictions with the previous hypothesis formulated on the basis of a cladistic analysis of morphological data (Grismer, 1988) (Fig. 2). Our results, nevertheless, did not support the initial, most fundamental divergence of *Aeluroscalabotes* from the remaining eublepharids, which let Grismer (1988) establish the monotypic subfamily Aeluroscalabotinae against Eublepharinae. Further, more comprehensive analysis is needed to verify the validity of these subfamilies from the phylogenetic view point.

The relatively poor support for the closest affinity between the two *Goniurosaurus* species examined may be attributable to their relatively long isolation as is also implied by the highly relict distribution of the genus (Ota, 1998). This *ad hoc* assumption needs verifications by incorporating data for the other *Goniurosaurus* species, *G. lichtenfelderi*, into future analyses.

The relationship among populations of *G. kuroiwae* did not agree with their assignments to morphologically defined subspecies (Gresper *et al.*, 1994), because it strongly suggests the genealogical proximity of the southern Okinawajima population of *G. k. kuroiwae* with *G. k. orientalis* rather than with the consubspecific population in the northern part of the island. The habitat of *G. kuroiwae* is largely confined to areas covered with humid vegetation, such as primary and well-recovered secondary forests (Matsui and Ota, 1999). Thus, the broad, dry vegetational zone in the middle of Okinawajima (AJLSA, 1994), from which no *G. kuroiwae* have ever been found (Matsui and Ota, 1999), might have possibly been intervening the gene flow between populations on both sides. The small, but still substantial genetic divergence between the *G. k. kuroiwae* populations from the northern and southern parts of the island seems to reflect a certain duration of such an isolation. Considerably close affinity of the southern Okinawajima population of *G. k. kuroiwae* with *G. k. orientalis* is puzzling, because, as long as the current geography is concerned, locality of the sample of *orientalis* examined here (lejima) is closer to the northern part of Okinawajima. This may reflect a closer geohistorical relationship of lejima with the southern part of Okinawajima than with the northern part.

The closer relationship of a diplodactyline species (*R. trachyrhynchus*) with eublepharids than with gekkonines (*Gekko gecko* and *Cyrtoptictylus intermedius*), suggested by both NJ and parsimony analyses, is also puzzling because such a relationship needs to presume occurrences of either parallel derivations of a number of advanced character states shared by the diplodactyline and gekkonine gekcoks, or reversals of a number of characters to primitive states in the eublepharid lineage (Kluge, 1987; Grismer, 1988). Further analyses using more samples especially of representatives of Sphaerodactylinae (the other gekkonid subfamily; see Fig. 1), as well as of additional gekkonine and diplodactyline species, are strongly desired to confirm such diplodactyline relation.

Kluge (1967) assumed that Southeast Asia was the center of origin of all extant gekkonan lineages and that diplodactyline gekcoks, having emerged there from eublepharid ancestor, dispersed into Australia through the Indo-Australian Archipelago during the late Mesozoic. With the subsequent general acceptance of the “mobile continents” and the revision of the cladistic hypothesis of Gekko, he formulated a completely different scenario, in which divergences of eublepharids and diplodactylines from the others are, respectively, attributed to the breakup of Pangaea into Laurasia and Gondowanaland in 180 MYA, and more tentatively to the Africa-India and Australia-New Zealand separation of the latter in 85 MYA (Kluge, 1987). The secondary dispersal is assumed to explain the current occurrence of a few eublepharid genera in the Gondowana region (i.e., *Hemitheconyx* and *Holodactylus* in Africa) in this hypothesis. In the same year, King (1987a, b), based on the chromosomal and immunodistance data, and the fact that all major extant lineages occur in the Gondowana region, argued for the Gondowan origin of the whole Gekkonidae in his use. He considered the current Laurasian eublepharids (e.g., *Aeluroscalabotes* in Southeast Asia and *Coleonyx* in North America) as deriving from the secondary dispersal.

Based on a few morphological characters, Borner (1981) argued that *Holodactylus* in Africa is most primitive among the eublepharid genera. A fact account seems to lend some supports to King’s (1987a, b) hypothesis. However, our results, along with results of morphological analysis by Grismer (1988), strongly suggest the most primitive status for the Laurasian *Aeluroscalabotes* and *Coleonyx*, and, contrary to the view of Borner (1981), most recent divergence of the Gondowan *Holodactylus*, as well as of *Hemitheconyx*. These strongly suggest the Laurasian origin of the eublepharid gekcoks as was hypothesized by Kluge (1987). Closer affinity of *Rhacodactylus* with eublepharids suggested in our results may be indicative of the past dispersal of the diplodactyline ancestor to the Australia-New Zealand region directly from Laurasia (contra Kluge [1987] and King [1987a, b]).

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*Goniurosaurus kuroiwae* has been designated as a natural monument of Okinawa Prefecture and the handling of this species is strictly...
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APPENDIX-1. Specimens examined in this study.

Iguana iguana: A South American species, from pet dealer, KUZ 37209*.

Eumeces latiscutatus: Kyoto City, Japan, KUZ 46592*.

Lygosoma bowringii: Khao Chong, Thailand, KUZ 37884*.

Gekko gecko: Kaeng Krachan, Thailand, KUZ 35397*.

Cyrtodactylus intermedius: Chanthaburi, Thailand, 32823*.

Rhacodactylus trachyrhynchus: A New Caledonian species, from pet dealer, KUZ 47440*.

Coleonx mitratus: A central American species, from pet dealer, KUZ 47330, 47331*.

Aeluroscalabotes felinus: Sarawak, Malaysia, KUZ 27181*; one uncatalogued specimen.

Goniurosaurus sp.: From pet dealer (localities unknown), KUZ 34556, 34557*.

G. kuroiwae kuroiwae: Seiha, Chinen, southern part of Okinawajima, KUZ 8040*; Yona, Kunigi, northern part of Okinawajima, KUZ 21014*.

G. k. orientalis: Shiroyu, Iejima, KUZ 47418, 47441*.

G. k. splendens: Tete, Amagi, Tokunoshima, KUZ 33025*, 33030.*

Eublepharis macularius: A Southwest Asian species, from pet dealer, KUZ 33169, 45197*.

Holodacyctus africanus: An African species, from pet dealer, KUZ 45191, 45192*.

Hemiteconyx caudicinctus: An African species, from pet dealer, KUZ 32574*.

* Specimens, for which sequences are given in Appendix-2.

APPENDIX-2.

Aligned sequences of the mitochondrial DNA segment investigated. The initial 406 bp in each row correspond to the 12S rRNA gene sequence, whereas the remaining 473 bp, marked with an asterisk at the beginning, correspond to the 16S rRNA gene sequence. Dot indicates an identity with the first sequence; dash denotes a gap. Abbreviations are: II, Iguana iguana; EL, Eumeces latiscutatus; LB, Lygosoma bowringii; GG, Gekko gecko; CI, Cyrtodactylus intermedius; RT, Rhacodactylus trachyrhynchus; CM, Coleonx mitratus; AF, Aeluroscalabotes felinus; GU, Goniurosaurus sp.; GS, G. kuroiwae splendens; GKN, G. k. kuroiwae from the northern part of Okinawajima; GKS, G. k. kuroiwae from the southern part of Okinawajima; GO, G. orientalis; EM, Eublepharis macularius; HA, Holodacyctus africanus; HC, Hemiteconyx caudicinctus. Except for G. k. kuroiwae, each taxon is represented by a single specimen here (see Appendix-1).