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# A MOLECULAR PHYLOGENETIC STUDY OF THE OLD WORLD TREEFROG FAMILY RHACOPHORIDAE

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A phylogenetic tree is presented for the Old World treefrog family Rhacophoridae and other ranoid frogs that that have an Africa-Madagascar-Asia distribution. The tree was inferred from parts of the mitochondrial ribosomal 12S and 16S genes and the $tRNA^{val}$ gene sequences with the Microhylidae as outgroup. The tree indicates that the rhacophorids are a monophyletic group composed of a Madagascar clade and an Asian-African clade. When endemic Madagascar mantellids were added to the tree, they also were part of the Madagascar rhacophorid clade, but the support for this assignment is weak. *Tomopterna labrosa*, a ranid endemic to Madagascar, appears more closely related to the Madagascar rhacophorids than it does to the ranids included in the analysis. Support for this relationship is strong enough to merit reinvestigation of the morphology and extension of the molecular data set.

## INTRODUCTION

Ever since spending a sabbatical year in Africa in 1972, CMR has had an interest in the African reedfrog family Hyperoliidae, first in genetic and developmental problems (Richards, 1976, 1981, 1982; Richards & Schiotz, 1977; Richards, Carlson & Rogers, 1975; Richards, Carlson & Connelly, 1977) and more recently in the molecular systematics of the family (Richards & Moore, 1996). Richards & Moore (1996) showed that the 3' portion of the 12S mtDNA plus the $tRNA^{val}$ (a total of 606 nucleotides) were able to ascertain with some certainty the relationships among the genera of the family Hyperoliidae. This led to an investigation of the other Old World family of treefrogs, the Rhacophoridae, which has a single genus, *Chiromantis*, in Africa, two genera (*Boophis* and the monotypic *Aglyptodactylus*) in Madagascar, while the remaining seven genera are distributed across Asia. It also led to an interest in other African frogs which share this distribution. These land masses, so disconnected today, had a long shared history before the breaking up of Pangea.

Work in progress shows that the same amount of sequence data that resolved the Hyperoliidae is not enough to resolve the relationships among the Asian and African rhacophorids. The deep branches of the tree are very short, so that there are not enough differences in the Asian groups to distinguish among genera with confidence. Consequently, a 475 bp sement of 16S rDNA was added to the data set. The combined data set (1081bp) gave rise to a strongly supported tree for the Rhacophoridae and some related Madagascar species.

The species sequenced (Table 1) included representatives of all rhacophorid genera except *Nyctixalus* and *Theloderma*. Mantellids were also included because Liem (1970) included them in his morphological study of the Rhacophoridae, and because their taxonomic and phylogenetic relations to other families are disputed (see discussion).

## MATERIALS AND METHODS

### GENE AMPLIFICATION AND SEQUENCING

The isolation of DNA from frozen and alcohol-fixed specimens followed standard procedures (Richards & Moore, 1996). All DNA sequencing was done from Polymerase Chain Reaction (PCR) products. Standard protocols of Kocher, Thomas, Meyer, Edwards, Paabo, Villablanca & Wilson (1989) were used with minor modifications for the specific genes being amplified (Richards & Moore, 1996). The primers listed in Richards & Moore (1996) were used to amplify a 606bp segment of 12S rDNA and $tRNA^{val}$; primers 16L8 (Hedges, 1994) and 16H9 (Ruvinsky & Maxson, 1996) were used to amplify a 475bp region of the 16S gene.

Other modifications in the Kocher protocol were directed at reducing the chance of amplifying contaminant sequences (see Derr, Davis, Wooley & Wharton, 1992; Hackett, Griffiths, Bates & Klein, 1995; Moore & DeFilippis, 1997 for discussion). Aerosol resistant pipette tips were used in all procedures involving isolation and PCR. DNA isolation and PCR mixes were exposed to short-wave UV light to destroy any contaminant DNA before adding DNA template (Cimino *et al.*, 1990; Moore & DeFilippis, 1997). Where possible, two specimens of each species were sequenced to assure that the sequences are indeed the target species. Where only a single specimen was available, the gene was sequenced in both directions.

All PCR products were cleaned using WizardTM PCR Preps (Promega) and quantified before running sequencing reactions. Sequencing was done both on a Pharmacia ALF and an ABI automated sequencer. For the ALF, cycle-sequencing reactions were run on PCR products using fluorescein-labeled primers and Thermo Sequenase kits (Amersham).

TABLE 1. List of species sequenced. Museums: UMMZ, University of Michigan Museum of Zoology; USNM, U. S. National Museum; AMNH, American Museum of Natural History; FMNH, Field Museum of Natural History; TTV, Texas Tech University.

| Species name | Museum No. | Location |
|---|---|---|
| Rhacophoridae | | |
| *Aglyptodactylus madagascariensis* | UMMZ 37571 | Madagascar |
| *Boophis erythrodactylus* | USNM 336403 | Madagascar |
| *Boophis tephraeomystax* | USNM 59146 | Madagascar |
| *Chiromantis xerampelina* | UMMZ 210197 | Kenya |
| *Chiromantis* sp. | AMNH A153250 | Tanzania |
| *Chirixalus eiffingeri* | UMMZ 190578 | Taiwan |
| *Chirixalus idiooticus* | UMMZ 5732 | Taiwan |
| *Philautus mjobergi* | FMNH 18084 | Malaysia |
| *Philautus petersi* | FMNH 18164 | Malaysia |
| *Polypadetes megacephala* | UMMZ 189969 | Taiwan |
| *Polypadetes leucomystax* | USNM 498993 | Philippines |
| *Rhacophorus moltrechti* | UMMZ 190564 | Taiwan |
| *Rhacophorus arboreus* | TTV-R-11748 | Japan |
| *Buergeria japonica* | UMMZ 190060 | Taiwan |
| *Buergeria robusta* | UMMZ 189974 | Taiwan |
| Mantellinae | | |
| *Mantidactylus grandidieri* | UMMZ 42628 | Madagascar |
| *Mantella* sp. | UMMZ 46479 | Madagascar |
| Ranidae | | |
| *Arthroleptides martiensseni* | AMNH A151339 | Tanzania |
| *Ptychadena mascareniensis* | UMMZ 213491 | Madagascar |
| *Tomopterna labrosa* | UMMZ 43541 | Madagascar |
| Microhylidae | | |
| *Probreviceps macrodactylus loveridgei* | AMNH A153248 | Tanzania |
| *Scaphiophryne gottlebei* | UMMZ 53423 | Madagascar |
| *Scaphiophryne breviceps* | UMMZ 53430 | Madagascar |

### ALIGNMENT AND TREE CONSTRUCTION

The sequences were aligned by eye using the computer program ESEE (version 3, Cabot & Breckenbach, 1993) with careful attention to the stem and loop secondary structure of the RNA molecule as explained in detail in Kjer (1995) and Richards & Moore (1996). Hypervariable regions whose alignments were uncertain were omitted from the analysis because homology of the bases was not assured. Of the 1081 bases in the combined sequences, 913 were used in the analysis. Sequences have been submitted to Genbank (Accession numbers AF 026341-79).

Test version 4.0d55 of PAUP*, written by David L. Swofford was used to construct a Neighbor Joining tree using the gamma distribution in conjunction with the Tamura-Nei divergence model. Both transitions and tranversions were used. Ribosomal sequences have different rates of change at different positions (Van de Peer, Jansen, De Rijk & De Wachter, 1997). A gamma distibution is most appropriate for this kind of data (Gu, Fu & Li, 1995; Yang, 1996; Hillis, Moritz & Mable, 1996). A value of $\alpha = 0.4$ was estimated by Greg Spicer for this data set using PAUP*. Bootstrap values are for 500 replicates. The tree created in Paup* was imported into TREEVIEW (Page, 1996) and edited in Microsoft Power Point 4.0.

### OUTGROUP SELECTION

Liem (1970), Drewes (1984) and Channing (1989) provide arguments for the selection of the Ranidae as the most appropriate outgroup for the Rhacophoridae. The Madagascan species *Tomopterna labrosa* (subfamily Raninae) and the African *Arthroleptides martiensseni* (subfamily Petropetedinae) were selected to sequence, because Africa has been postulated as the site of the major radiation of ranids (Duellman & Trueb, 1986) and to increase the species diversity of the outgroup, as suggested by Smith (1994). *Tomopterna labrosa* is endemic to Madagascar; its closest relatives are species of the same genus in Africa and two species in India-Sri Lanka. The subfamily Petropedetinae is found only in sub-Saharan Africa. We originally in-

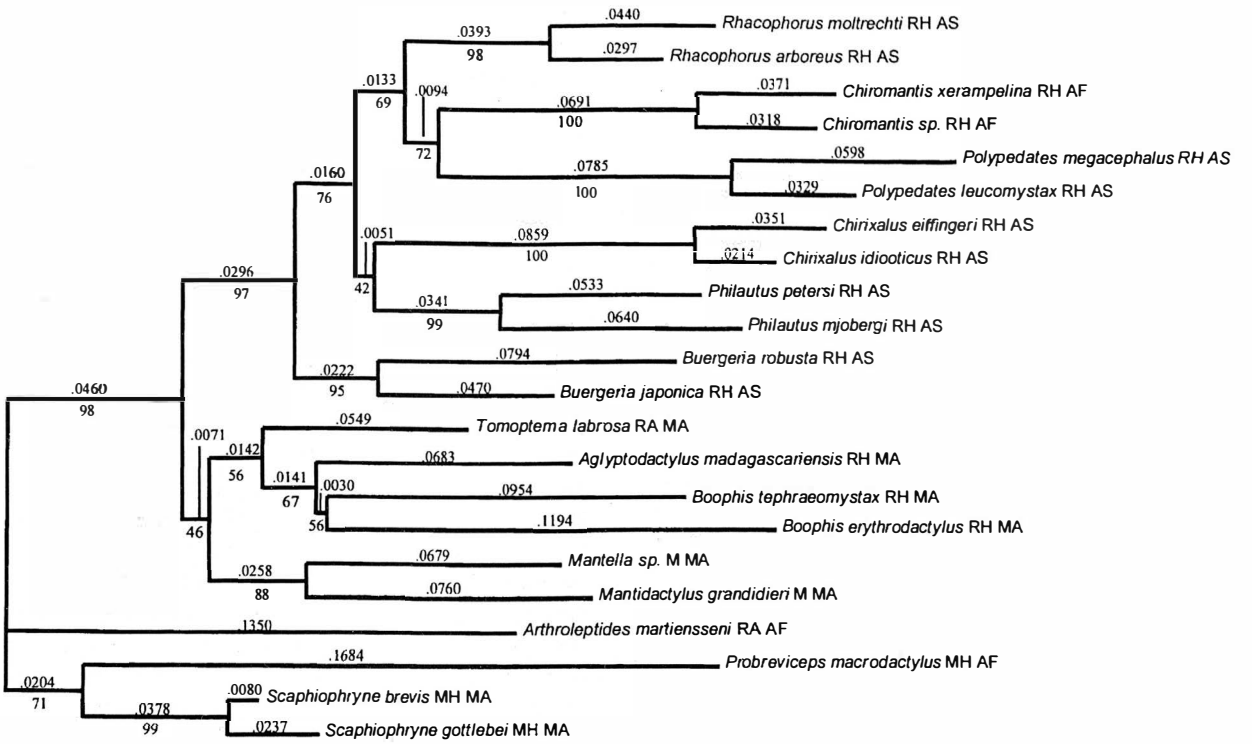


FIG. 1. Neighbor Joining tree for the species in Table 1. Families: RH, Rhacophoridae; M, Mantellidae; RA, Ranidae; MH, Microhylidae. Continent: AF, Africa; MA, Madagascar; AS, Asia. Numbers above a line are distances; below, the bootstrap value for 500 bootstraps.

tended to include *Ptychadena mascareniensis*, a non-endemic ranid found on Madagascar as well as in Africa, but the sequence contained some peculiarities that caused us to exclude it (see results and discussion)

In addition, species from the Microhylidae were used as the outgroup for the entire Ranoidea. Wu (1994), in his morphological analysis of the Microhylidae, found that they were sister group to the Ranoidea and therefore most appropriate to be used here. Species selected were the Madagascar *Scaphiophryne* and the African *Probreviceps,* representatives of the subfamilies Scaphiophryninae and Brevicipinae.

## RESULTS

The Neighbor Joining phylogenetic tree constructed from these data, using the criteria described in materials and methods, is shown in Fig. 1. When the ranid *Arthroleptides* and three microhylids are used as outgroup species, the Rhacophoridae and associated Madagascar genera form a strongly supported clade. This clade, in turn, is composed of a strongly supported Asian-African clade and a more weakly supported (bootstrap value 46) clade that includes the Madagascar rhacophorids, the mantellids and the "ranid" *Tomopterna* (see below and discussion). If a tree is constructed for the rhacophorids alone (without the mantellids and *Tomopterna*), the bootstrap values for the African and Asian clade and a Madagascar clade are 97 and 95, respectively (data not shown). Clearly, the family Rhacophoridae consists of at least two distinct clades.

It is also clear that the African *Chiromantis* is very closely related to the Asian genera. The bootstrap support for the Asian *Polypedates* as sister group to *Chiromantis* is 72. These two genera form a clade with *Rhacophorus,* and this clade is sister to a clade including *Chirixalus* and *Philautus,* with *Buergeria* as the basal genus to all the other Asian and African species. Bootstrap support for an African-Asian clade is a very strong 97%.

In the tree in Fig. 1, *Tomopterna labrosa* is sister group to the Madagascar rhacophorids, but with only modest bootstrap support and does not group with the ranid *Arthroleptides* as would be expected. Using the branch swapping facility of MacClade (Maddison & Maddison, 1992), making *Tomopterna* sister group to the mantellids gave an equally parsimonious tree, but making it sister group to *Arthroleptides* produced a tree that is 21 steps longer. Thus, the most parsimonius tree supports the placement of *Tomopterna labrosa* in the Rhacophoridae.

The mantellids are the sister group to the Madagascar rhacophorids. However, the support for this position is also weak, and using the branch swapping facility of MacClade to make the mantellids sister group to the entire rhacophorid clade results in a tree that is only two steps longer, an insignificant difference. However, making the mantellid branch sister group to the Ranidae or the Microhylidae results in a tree that is 18-21 steps longer.

In the course of this work, it was discovered that the branch length of *Ptychadena* was extraordinarily long.

This is an indication of many differences between it and even its closest relatives. Surprisingly, in the 12S segment it has nine autapomorphies in characters that are invariant in some 90 ranoid frogs we have sequenced. In addition to these many differences, there is a 2bp insertion in the loop between stems 38 and 39 (positions 183-184 in the alignment), a 16bp insertion in the loop between stem 45' and stem 47 (positions 304-319) and the loop region between the 12S gene and the adjacent tRNAval gene has been reduced to a single nucleotide (position 533). See Richards & Moore (1996) for loop numbers. These are not artifacts of sequencing or of misidentification of specimens as two individuals from different localities in Madagascar and one from mainland Africa were sequenced (see Table 1), and they all contained these same insertions and deletions. Since we felt that there was something unusual about these long branch sequences, they were removed from the analysis (see discussion). The 16S sequence showed no such anomalies.

## DISCUSSION

The results presented above reflect the rhacophorid relationships suggested by morphology. Liem (1970), in his morphological study, found the African *Chiromantis* among the Asian rhacophorids and *Buergeria* at the base of the African and Asian clade, but the level of resolution of the Asian genera was not great. These relationships are now corroborated at the molecular level. However, Liem placed *Chiromantis* near *Buergeria* at the base of the clade while this study shows *Polypedetes* as the closest relative. Channing's reanalysis of Liem's data (Channing, 1989) presents a somewhat different phylogenetic interpretation of the data where he finds *Buergeria* at the base of all the rhacophorids including *Mantidactylus*, a conclusion not supported by the molecular data. Channing also moved *Chiromantis* to a position higher in the Asian clade rather than at the base. This placement is supported by the molecular data.

Liem also included *Mantidactylus* in his study and found, as here, that it was related to the Madagascar rhacophorids, *Aglyptodactylus* and *Boophis* with all three outside the Asian and African clade. The relationships of the mantellids to other groups has long been disputed. They have variously been assigned to the Ranidae (Blommers-Schlosser, 1979; Frost, 1985; Duellman & Trueb, 1986), to the Rhacophoridae (Liem, 1970; Channing, 1989) and to their own family, the Mantellidae (Blommers-Schlosser & Blanc, 1991; Duellman, 1993). This study suggests that the mantellids belong to the Rhacophoridae and not the Ranidae.

It is not surprising that the Madagascan rhacophorid species are differentiated from the Asian ones, given the biogeographic history of the land masses involved. Madagascar and India separated 88 million years ago (Storey, Mahoney, Saunders, Duncan, Kelley & Coffin, 1995; Storey, 1995) and the species have been evolving separately for a very long time. After separation from Madagascar, India floated northward until it joined the Asian continent. The progenitors of the Asian rhacophorids then spread out over a vast continent with empty treefrog niches, so it can be speculated that they speciated quite rapidly, a speculation that is perhaps supported by the short branch lengths deep in the tree. The fact that the African *Chiromantis* is placed deep in the Asian rhacophorid clade would suggest that the progenitor stock of this genus did not remain behind in Africa when India-Madagascar broke away but rather that it was originally an Asian genus that migrated westward overland and thence entered Africa.

*Tomopterna* is a genus with the same geographic distribution as the Rhacophoridae: Africa, Madagascar and Asia. It is a burrowing form with the morphological adaptations suited to a fossorial life and it seems slightly fanciful for it to be even remotely related to treefrogs with their own suite of morphological adaptations that enable them to climb so well. Nevertheless, the molecular data thus far available are strong enough to be an indication that this matter needs clarification at both the molecular and morphological levels. At present, there are just four molecular character states linking *Tomopterna* to the Madagascar rhacophorids. More sequence data, either additional ribosomal sequence or sequence from another gene, are needed to expand the data presented here. The morphology should also be examined even more closely to determine whether fossorial adaptations are obscuring true phylogenetic relationships. Channing's group (personal communication) has sequenced the same 12S region in South African *Tomopterna* species, and these do show a close relation to the Ranidae.

Details about the sequence of *Ptychadena* are included here to point out that the peculiarities of the 12S sequence that make it so very different from all the other ranoids sequenced may prove to be exceedingly valuable characters in resolving the phylogeny of the Ranidae. The Ranidae is an extremely large and diverse family with a world-wide distribution. The only attempt at creating a phylogeny was the morphological study of Clarke (1981) who worked only with the African members of the subfamily Raninae. Other mitochondrial sequence peculiarities have been found in the genus *Rana* (Macey, Larson, Ananjeva, Fang & Papenfuss, 1997; Yoneyama, 1987). In *Rana catesbeiana* and *Rana limnocharis*, four of the mitochondrial tRNA's have been rearranged relative to the common vertebrate mitochondrial gene order found in species as diverse as fish, *Xenopus* and mammals (Macey *et al.*, 1997), but the order among these four genes differs in the two species.

Alternately, since the *Ptychadena* sequence is very different from that of other ranoid frogs, the possiblity must be entertained that it is a mitochondrial sequence that has been transposed to the nucleus (Zhang & Hewitt, 1996). This could explain the large number of nucleotide changes in positions that are conserved in the mitochondria of almost all ranoids. If this were a nonfunctional nuclear pseudogene evolving at a differ-

ent rate and under different constraints, such conserved positions could be free to change. In either case, this sequence can be used as a phylogenetic marker provided it is compared with truly orthologous sequences.

Species groups that have radiated rapidly and consequently have very short branches in the deep portions of the tree are going to require longer sequences of ribosomal genes or sequence from additional genes to resolve the phylogenetic relationships in the Rhacophoridae and to establish firmly the phylogenetic position of the mantellids with some degree of confidence. If not, another gene, probably a nuclear protein gene, will be selected in an effort to gather sufficient data for firm resolution.

## NOTE ADDED IN PROOF

At the Third World Congress of Herpetology in Prague (August 1997), Glaw, Vences & Böhme reported two new species of *Aglyptodactylus*. An analysis using 18 phylogenetically informative characters showed a closer relationship between *Aglyptodactylus* and *Tomopterna* than between *Aglyptodactylus* and *Boophis*, the other rhacophorid on Madagascar. On the basis of this, they propose removing *Aglyptodactylus* from the Rhacophoridae and placing it in the Ranidae despite the presence of intercalary phalangeal elements in *Aglyptodactylus* which are unknown in ranines (Glaw, Vences & Böhme, in press).

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