Evolution of reproduction in the Rhacophoridae (Amphibia, Anura)

S. Grosjean, M. Delorme, A. Dubois and A. Ohler

Abstract

Rhacophorid treefrogs have different reproductive modes: some go through a tadpole stage and some have direct development, and the adults of some species produce foam nests. Philautus is the only genus characterized by direct development. The production of foam nests has been reported in the genera Polypedates, Rhacophorus, Chiromantis and Chirixalus. Recent molecular studies did not provide a robust hypothesis concerning the origin of these reproductive modes in the Rhacophoridae. In order to better understand the evolution of these reproductive modes, we tried to clarify relationships within this group, using DNA sequencing. Our data set consists in a large number of new sequences (1676 base pairs corresponding to three genes) for five outgroup ranoids and 48 Rhacophoridae, including 16 undescribed species from Sri Lanka and southern India, and all homologous data available in Genbank. After the inclusion of Philautus from India, our data show that the separation of Philautus into clades does not coincide with their geographic distribution. Our data point to the existence of a clade, including the genera Rhacophorus, Polypedates, Chiromantis and Chirixalus, which confirms the results of Wilkinson et al. (2002) and suggests that the ability to produce foam nests has emerged only once in the Rhacophoridae, as already stated by these authors.

Key words: Amphibia – Anura – Rhacophoridae – 12S – 16S – Rhodopsin – phylogeny – direct development – foam nests – reproductive modes

Introduction

Frogs are known to have a wide variety of reproductive modes that can also differ within families (Duellman and Trueb 1985). Rhacophorid treefrogs, that are either considered as a subfamily of the Ranidae Rafinesque, 1814 or as a family (option retained here), are a striking example in this respect. Whereas some species show a typical frog development through a free-feeding tadpole stage and metamorphosis, others have ‘direct development’ with lecithotrophy (Wourms 1981; Dubois 2004), in other words, feeding through the vitellus of the egg until hatching as a young frog similar to the adult. In addition, the adults of numerous species with a tadpole stage produce aerial foam nests into which they lay their eggs.

Direct development with lecithotrophy is a remarkable feature that has evolved in several anuran families (Lamotte and Lescure 1977). Among the Ranoids (sensu Frost et al. 2006), it is known in the Ceratobatrachidae Boulenger, 1884 (Boulenger 1886; Brown and Alcala 1983) and Dicroglossidae Anderson, 1871 (Taylor 1962; Ohler et al. 1999), in the Mantellidae Laurent, 1946 (Glaw and Vences 1994), and in the Rhacophoridae Hoffman, 1932 (Dring 1979; Brown and Alcala 1994; Bahir et al. 2005). Recent molecular phylogenetic studies (Richards and Moore 1998; Marmayou et al. 2000; Wilkinson et al. 2002; Delorme et al. 2005; Frost et al. 2006). Our data set consists of type-species of possible valid genera (eleven recognized by Frost et al. 2006). In this paper, we focus on the Rhacophoridae, a subfamily of the Ranidae that includes 177 species. Many of these species have a free-feeding tadpole stage and metamorphosis, while others have direct development. The production of foam nests has emerged only once in the Rhacophoridae, as already stated by these authors.

Key words: Amphibia – Anura – Rhacophoridae – 12S – 16S – Rhodopsin – phylogeny – direct development – foam nests – reproductive modes

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Materials and Methods

We sequenced 1676 base pairs, i.e. four portions of DNA, corresponding to the three genes: the mitochondrial genes for 12S and 16S rRNA and the exon 1 of the nuclear gene coding for rhodopsin.

Tissue samples (muscle or liver; either fresh or preserved in 98% ethanol) were available from 53 ranoid species (including 48 Rhacophoridae) and *Bufo melanostictus* Schneider, 1799. The collection number of the voucher specimens is given in the Appendix, Table S1. Unfortunately, the abbreviations of the institutions where the vouchers are deposited are as follow: DNP field number of Indraneil Das, University of Malaysia, Sarawak; FMNH Field Museum of Natural History, Chicago; IEBR Institute for Ecology and Biological Research, Hanoi; KUHE Graduate School of Human and Environmental Studies, Kyoto; MNHN Musée National d’Histoire Naturelle, Paris; TBGRI Botanic Garden and Research Institute of Trivandrum, Trivandrum; UADBA Université d’Antananarivo, Département de Biologie Anima l, Antananarivo; WHT World Heritage Trust, Colombo; ZSM Zoologische Staatssammlung München, Munich. The type-species are always identified by an asterisk. DNA was extracted using the protocol followed by Delorme (2004) and Delorme et al. (2004). We amplified two fragments of 12S rRNA gene (417 pb and 470 pb) using primers of Delorme et al. (2004), except for *Polypedates leucomystax* (Gravenhorst, 1829), *Polypedates megacephalus* Hallowell, 1861 and *Polypedates dates mutus* (Smith, 1940) for which we used a particular primer for the light chain (L43: 5’–GAC ACA TGC AAG TMT CA–3’). We followed the PCR conditions as given in Venes et al. (2000) and the PCR products were purified and sequenced using automated sequencers of the EQ 2000 Beckmann. The sequences (see Appendix, Table S1 for Genbank accession numbers) were aligned using the program Se-Al (Rambaut 1995), and by taking into consideration the secondary structure of molecules (Kjer 1995, 1997).

We also included data present in Genbank for these three genes for 35 congeners in our data matrix; however, these data were not complete for all species, and the complete supermatrix therefore included numerous missing data. Hence, we performed two separate analyses, one with the complete data matrix, and a second with only the taxa for which complete sets of sequences were available. Furthermore, it was not always possible to get the sequences of these three genes from a single individual. For some species, data concern in fact several individuals.

To assess whether the different gene fragments could be used in a combined analysis, we tested all possible combinations using the partition homogeneity test (parsimony method of Farris et al. 1995), as implemented in PAUP*, version 4.08 (Swoford 2001). Prior to phylogenetic reconstruction, we explored which substitution model best fits our sequenced data using the program MODELTEST (Posada and Crandall 1998).

Phylogenetic analyses were carried out using PAUP* with which we calculated maximum parsimony (MP) and maximum likelihood (ML) trees. In the MP analyses we conducted heuristic searches with initial trees obtained by simple stepwise addition, followed by branch swapping using the TBR (tree bisection-reconnection) routine implemented in PAUP*. Ten random addition sequence replicates were carried out. All characters-state changes were equally weighted and the gaps were treated as missing data. Following Hedges (1992), 2000 bootstrap replicates (Felsenstein 1985) were run in the MP analysis. The ML trees were obtained using heuristic searches, using the substitution model proposed by MODELTEST.

We used Bayesian inference in the program MrBayes 2.01 (Huelsenbeck and Ronquist 2001) for another ML analyses for which we ran four simultaneous Metropolis-coupled Monte Carlo Markov chains for 500 000 generations, sampling a tree every ten generations. The initial set of generations needed before convergence on stable likelihood values (‘burnin’) was set at 50 000 (10%) based on empirical evaluation. Furthermore Wolffian ducts were observed on 18 Rhacophoridae species (Appendix, Table S2). An extensive incision was effected on the left flank of the specimens in order to allow a good observation of the Wolffian ducts. The relationship between species and production of foam nests was obtained by direct observation in the field or a search of the literature. All terms relating to tadpoles are in agreement with those employed in McDairmid and Aliq (1999). Diagnostic characters for the tadpoles of the genera *Polypedates* and *Rhacophorus* are given. The examined species are the following: *P. megacephalus* (Sa Pa, Lao Cai province, Vietnam, collection numbers MNHN 2000.4722, 2000.4753), *Polypedates maculatus* hainanensis Annandale, 1912 (Nepal, MNHN 1989.1393, 1998.8944–8945), *P. miniatus* (Ben En National Park, Thanh Hoa province, Vietnam, MNHN 1998.6816, 1998.6818–6819, 1998.6835–6836, 1998.6855), *Polypedates sp.* (Phang Nga province, Thailand, MNHN 2000.8283–8284), and *Rhacophorus* sp. 1, R. sp. 2, R. sp. 3, R. sp. 4, R. sp. 5 (all collected at Sa Pa, Lao Cai province, Vietnam, collection numbers MNHN 2000.7535–7536, MNHN 2000.7601–7602, MNHN 2000.7546–7547, MNHN 2000.7199–7201, MNHN 2000.7252–7253 respectively). All these specimens were deposited in the herpetological collection of the Museum of natural History from Paris. On the 93 characters scored from the external morphology and buccopharyngeal anatomy (43 for the external morphology alone) six are synapomorphies and so diagnostic for *Polypedates* tadpoles (Grosjean 2004).

Results

The partition homogeneity test did not reject the null hypothesis of congruence of the included gene fragments (1000 replicates, p > 0.89), thus not contradicting their suitability for combination in the phylogenetic analyses. Among the 1676 characters included in the analyses, 679 were constant, 259 variable but parsimony-uninformative and 538 variable and parsimony-informative.

The maximum parsimony analysis of the complete data matrix (included taxa with incomplete sequences) found 417 equally parsimonious trees (6211 steps; consistency index 0.27, retention index 0.50). MODELTEST proposed a (GTR + I + G) model with an alpha shape parameter of 0.589, a proportion of invariable sites of 0.255, and user-defined substitution rates (A–C, 3.883; A–G, 11.354; A–T, 4.706; C–G, 1.342; C–T, 2.450; G–T, 1.000) and base frequencies (A = 0.346, C = 0.233, G = 0.167, T = 0.255). The ML analysis ran on PAUP using the settings proposed by MODELTEST. The Bayesian analysis resulted in the tree shown in Fig. 1.

Taking into account only those species for which complete sequences of all four DNA fragments were available (including *Chirixalus vitatus* for which we have only three portions), the maximum parsimony analysis found one most parsimonious tree (4402 steps; consistency index 0.33, retention index 0.47). MODELTEST proposed a (GTR + I + G) model with a gamma shape parameter of 0.540, a proportion of invariable sites of 0.281, and user-defined substitution rates (A–C, 4.436; A–G, 11.444; A–T, 5.832; C–G, 1.342; C–T, 27.939; G–T, 1.000) and base frequencies (A = 0.339, C = 0.239, G = 0.171, T = 0.251). The ML analysis ran on PAUP using
the settings proposed by MODELTEST. The Bayesian analysis resulted in the tree shown in Fig. 2.

The structures of the maximum parsimony trees are similar to the likelihood and Bayesian trees, the differences regarding only weakly supported nodes. In the analyses of the complete data matrix, only three relationships between genera appeared clearly supported: (1) the close relationship between the African genus *Chiromantis* and the species *Chirixalus doriae* (type-species of *Chirixalus*); (2) the close relationship between *Kurixalus* and two *Aquixalus* species (*A. idiootocus* and *A. verrucosus*); and (3) the close relationship between *Nyctixalus* and *Theloderma* according to the maximum likelihood and Bayesian analyses. Additional results relate to the relationships within genera. In this sample, the included species of *Buergeria*, *Nyctixalus*, *Theloderma*, *Chiromantis* and *Polypedates* form monophyletic groups, but in some cases with only low support. The numerous species of *Philautus* studied are distributed in two groups: a clade of all *Philautus* from Sri Lanka and India (all new species included) and a second clade with all *Philautus* of the Sundas islands.

The tree including only the species for which complete sequence sets (Fig. 2) were available has stronger support for many more nodes when compared with the first tree (Fig. 1). The monophyly of the included rhacophorids is robustly supported, and *Mantella betsileo* appears as their sister group.

Within the Rhacophoridae, the basal position of *Buergeria* is
well supported. *Nyctixalus pictus* is close to *Theloderma*. A group of four genera (*Chiromantis, Chirixalus, Polypedates* and *Rhacophorus*) is well supported in the maximum likelihood and the Bayesian analyses. Surprisingly, this relationship is not supported by maximum parsimony, but no strong bootstrap values for a contradicting hypothesis were observed. Among these four genera, the African genus *Chiromantis* is consistently the sister taxon of *Chirixalus*, and the genera *Rhacophorus* and *Polypedates* are sister to each other. The position of the *Philautes* from the Sunda Islands relative from those of Sri Lanka and India is not solved. The positions of *Kurixalus* and *Aquixalus* are not solved as well.

Concerning the Wolffian ducts, three shapes are recognized:
1. They are convoluted in *Chirixalus doriae*, *Chiromantis rufescens*, *Chiromantis xerampelina*, *Polypedates leucomystax*; *P. megacephalus*, *Rhacophorus dennyi*, *Rhacophorus reinwardtii* and *Rhacophorus schlelegeli*.
2. They are simple tubes in *Aguixalus carinensis*, *Aquixalus gracilipes*, *Aquixalus odontotarsus*, *Buergeria japonica*, *Kurixalus eiffingeri*, *Nyctixalus pictus*, *Philautus aurifasciatus*, *Philautus microtymanus* and *Thelodermata bicolor*.
3. They show irregular bulges in *Chirixalus vittatus*.

We can remark that *Kurixalus eiffingeri* does not have convoluted Wolffian ducts, as opposed to *Liem* (1970).

A similar division appears when we study the production of foam nests: the species with convoluted Wolffian ducts produce foam nests, the species with simple Wolffian ducts do not produce foam nests and *Chirixalus vittatus* lays its eggs in jelly with some bubbles.

The genera *Polypedates* and *Rhacophorus* are close phylogenetically. The boundaries of each genus are vague (Dubois 1987) and attribution of species to one of these genera has often confronted systematists with problems (Wilkinson et al. 2002), as shown by the frequent generic changes of species of these genera. Whereas the morphological differences of adults of both genera are few, the larval forms show great morphological differences that allow a generic allocation without doubt. Indeed, whereas the tadpoles of *Rhacophorus* resemble greatly those of *Aquixalus*, the tadpoles of *Polypedates* show six morphological differences with those of *Rhacophorus* (Grosjean, 2004): an ovoid body (body wide almost equal to body height) versus a depressed body, a regular snout slope versus a concavity on the snout in profile view, a white point versus a depressed body, a regular snout slope (Grosjean, 2004): an ovoid body (body wide almost equal to body height) versus a concavity on the snout in profile view, a white point versus a depressed body, a regular snout slope (Grosjean, 2004): an ovoid body (body wide almost equal to body height) versus a concavity on the snout in profile view, a white point.

### Discussion

Our data support the monophyly (homophyly) of rhacophorids as in previous studies (Richards and Moore 1998; Richards et al. 2000; Meegaskumbura et al. 2002a; Wilkinson et al. 2002; Roelants et al. 2004; Delorme et al. 2005; Frost et al. 2006) and provide new information on relationships within this subfamily. After the inclusion of *Philautes* from India, the separation of *Philautes* into two clades does not really follow their geographic distribution. Indeed, the Indian species appear to be paraphyletic relative to the Sri-Lankan clade, as a Sri-Lankan species (*Philautes* sp. WHT 3421) is nested within an Indian clade. This could be explained by dispersals between southern India and Sri Lanka that occurred about 9000 years ago (Meegaskumbura et al. 2002b). The maximum likelihood analysis alone is in agreement with a common phylogenetic origin for all direct-developing *Philautes*, the question being unsolved with the maximum parsimony and the Bayesian analyses. Although different clades with high support exist within the clade of the Sri-Lankan *Philautes*, none morphological synapomorphy could be found as all these species are very different morphologically (Delorme, pers. obs.). This group seems to have been subject to a rapid morphological differentiation.

The clade including the genera *Rhacophorus, Polypedates, Chiromantis* and *Chirixalus* has only be, until now, emphasized by Wilkinson et al. (2002). These four genera are mostly characterized by the capacity of the adults to produce foam nests. Without *Chirixalus* in their sample, Richards and Moore (1998) emphasized only the close relationships between *Rhacophorus, Polypedates* and *Chiromantis*. Recently Frost et al. (2006) put *Chirixalus* in synonymy with *Chiromantis* on the basis of the paraphyly of the former genus with respect to *Chiromantis*. External morphological characters support the clades (*Rhacophorus, Polypedates*) and (*Chiromantis, Chirixalus*) (Delorme 2004). The synapomorphies for the clade (*Chiromantis, Chirixalus*) are the apposition of fingers I and II with the fingers III and IV, the presence of anal glands in semi-circle above the cloaca and the presence of webbing between fingers not extending more than half-length of fingers, those for the clade (*Rhacophorus, Polypedates*) are vomerine teeth in contact with choanae, an internal metatarsian tubercle inferior or equal to 1/4 of toe I and the presence of a folding of skin along toe V. The study of the Wolffian ducts confirms the close relationships between these four genera already assumed by these previous authors. The convolution of the Wolffian ducts is certainly correlated with the production of foam nests, as already assumed by several authors (Bhaduri 1932, 1953; Bhaduri and Mondal 1965; Liem 1970); *Chirixalus vittatus* lays its eggs in a jelly with some bubbles but its Wolffian ducts are not simple tubes like in the other species without foam nests, as they show irregular bulges. We have no data concerning the production of foam nests in *Thelodermata bicolor* but it has no irregular Wolffian ducts, and *T. asperum*, *T. stellatum*, *T. gordonii* and *T. corticale* do not produce foam nests (Orlov 1997). The presence of foam nests in *T. horridum* reported by Boulenger (1903) should be verified.

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**Fig. 3. Photos of *Rhacophorus* sp.**

(a): stage 35; total length = 39.2 mm and *Polypedates maculatus*

(b): stage 37; total length = 40.7 mm


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The clade containing *Theloderma* and *Nyticixalus* is characterized by a singular reproductive behaviour not unusual for anurans: these species deposit their small clutches in tree holes, at 30 cm above water (Taylor 1962; Orlov 1997; Malkmus et al. 2002). However, this particular reproductive mode is also shared by *Kurixalus eiffingeri* (Ueda, 1986; Kam et al. 1996) which stands in another clade in the tree (see below). An analysis of external morphological characters of adults also supported a close relationship of these two genera (Delorme 2004): they have warts with or without horny granules all over the back, and a short supratympanic fold formed by very small warts. *Kurixalus* seems close to *Aquixalus*, a new genus erected by Delorme et al. (2005) for several species previously placed in *Philautus* but whose development goes through a tadpole stage. Even if the molecular data support this relationship only weakly, the study of larvae strongly clusters them (Grosjean 2004). As in previous studies (Wilkinson et al. 2002; Delorme et al. 2005; Frost et al. 2006) two species initially included in the genus *Aquixalus* (Delorme et al. 2005), *A. idioofoicus* and *A. verrucosus*, form a clade with *Kurixalus eiffingeri*. We agree with Frost et al. (2006) that these two species must be included in the genus *Kurixalus*.

Our data allow two further notes on the work of Wilkinson et al. (2002). First, the genera *Polyypedates* and *Rhacophorus*, which are difficult to delimit from each other on the basis of adult morphology, are also not clearly delimited in our molecular analysis. However, if the genus *Polyypedates* is restricted to the species of the former ‘leucomytstax’ group’ (Dubois 1987), the tadpoles allow a clear distinction between these two genera. Secondly, in our analysis and in contrast with Wilkinson et al. (2002), ‘*Chirixalus palpebralis* appears close to other *Chirixalus*. Frost et al. (2006) erected a new genus for this species, *Feihyla*, which phylogenetically distinct from other ‘*Chirixalus*’ in previous studies (Wilkinson et al. 2002; Delorme et al. 2005). In our results, this species is nested with other *Chirixalus* and *Chiromantis* although basal in position and so do not deserve a new generic status at this state of our knowledge, waiting for a clarification of the status of this species by further studies.

We propose to recognize two subfamilies and two tribes in the family Rhacophoridae, the two subfamilies being first recognized by Channing (1989) although this author included also the Mantellinae within the Rhacophoridae:

1. Buergeriinae: the basal position of the insular genus *Buergeria* is supported by a simple reproductive behaviour widespread among the Anura: the simple deposit of many eggs in water.

2. Rhacophorinae: it includes all remaining genera. It is itself divided in two tribes, the *Nyctixalini* and the *Rhacophorini*.

3. Nyctixalini new tribe. Type genus: *Nyctixalus* Boulenger, 1882: it includes two genera, *Theloderma* and *Nyticixalus*. This tribe is supported by one morphological apomorphy: the presence of numerous dense glands of every sizes on the eyelid.

4. Rhacophorini: this tribe includes the following genera: *Aquixalus*, *Chiromantis*, *Kurixalus*, *Philautus*, *Polyypedates* and *Rhacophorus*.

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Résumé

Evolution des modes de reproduction chez les Rhacophoridae (Amphibia, Anura)

Les rainettes de la famille des Rhacophoridae ont différents modes de reproduction: certaines ont un stade têtard alors que d'autres ont un développement direct, et les adultes de certaines espèces fabriquent des nids d'écumé. *Philautus* est le seul genre caractérisé par un développement direct. La production de nids d'écumé est connue dans les genres *Polyypedates*, *Rhacophorus*, *Chiromantis* et *Chirixalus*. Les récentes études moléculaires n'ont pas donné d'hypothèses robustes concernant l'origine de ces modes de reproduction chez les Rhacophoridae. Afin de mieux comprendre l'évolution de ces modes de reproduction, nous avons essayé de clarifier les relations phylogénétiques à l'intérieur de ce groupe à l'aide de séquences d'ADN. Notre jeu de données consiste en un grand nombre de nouvelles séquences (1676 paires de bases correspondant à trois génomes) pour cinq extra-groupes de Ranaoida et 48 Rhacophoridae, incluant 16 espèces non décrites du Sri Lanka et du sud de l'Inde, et toutes les séquences homologues disponibles sur Genbank. Après l'inclusion des *Philautus* d'Inde, nos résultats montrent que la séparation de *Philautus* en clades ne coïncide pas avec leur distribution géographique sauf entre la région indienne et les îles de la Sonde. Nos résultats mettent en évidence un clade regroupant les genres *Polyypedates*, *Rhacophorus*, *Chiromantis* et *Chirixalus*. Ce point suggère que la capacité à produire des nids d'écumé n'est apparue qu'une seule fois chez les Rhacophoridae. Une nouvelle taxinomie des Rhacophoridae est proposée, avec une tribu nouvelle, *Nyticixalini*, réunissant les espèces des genres *Theloderma* et *Nyticixalus*.

References


Boulenger GA (1903) Report on the Batrachians and Reptiles of the Solomon Islands. Transact Zool Soc 12:48:


**Supplementary Material**

The following supplementary material is available for this article online:

Appendix Table S1. Collection number, Genbank accession numbers for each gene part and geographic origin of specimens examined.
Appendix Table S2. Collection number of species of which Wolffian ducts were observed and sources of data concerning the making of foam nests.

<table>
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<th>Species</th>
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<td>Species 2</td>
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