

Exploring the potential of life-history key innovation: brook breeding in the radiation of the Malagasy treefrog genus *Boophis*

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Abstract

The treefrog genus *Boophis* is one of the most species-rich endemic amphibian groups of Madagascar. It consists of species specialized to breeding in brooks (48 species) and ponds (10 species). We reconstructed the phylogeny of *Boophis* using 16S ribosomal DNA sequences (558 bp) from 27 species. Brook-breeders were monophyletic and probably derived from an ancestral pond-breeding lineage. Pond-breeders were paraphyletic. The disparity in diversification among pond-breeders and brook-breeders was notable among endemic Malagasy frogs, although it was not significant when considering *Boophis* alone. Sibling species which have different advertisement calls but are virtually indistinguishable by morphology were common among brook-breeders; genetic divergence between these species was high (modal 8% total pairwise divergence). Substitution rates in brook-breeding species were significantly higher than in pond-breeders. Speciation of pond-breeders may be hindered by their usually more synchronous reproduction and a higher vagility which enhances gene flow, while a higher potential of spatial segregation and speciation may exist along brooks.

Keywords: Amphibia, Madagascar, Mantellidae, phylogeny, sibling species

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Introduction

Adaptive radiations have received particular attention as sources of organismal diversity. One major topic has been the exploration of why some lineages gave rise to higher levels of diversity than others. As an explanation, the influence of key innovations has often been invoked (Sanderson & Donoghue 1996). These have been defined as morphological, physiological, or behavioural traits that enable a lineage to colonize new adaptive zones and thereby confer the ability to undergo rapid speciation and diversification (Simpson 1953; Liem 1973).

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Recent theoretical and empirical evidence suggests that rapid diversification processes may in part take place through sympatric rather than allopatric speciation. This process is triggered by ecological specialization (Dieckmann & Doebeli 1999) or sexual selection (Higashi *et al.* 1999; Gavrillets 2000; Boughman 2001). The latter model highlights the importance of optical and other mate recognition mechanisms for speciation (Barraclough *et al.* 1995).

Treefrogs of the genus *Boophis* are one of the most spectacular radiations within the endemic Malagasy family Mantellidae (Bossuyt & Milinkovitch 2000; Vences & Glaw 2001). Taking into account recent descriptions, more than 40 nominal and about 18 undescribed *Boophis* species are attributed to seven phenetic species groups (Blommers-Schlösser & Blanc 1991; Glaw & Vences 1994, 2000). Among anurans, the genus *Boophis* stands out for its large number of cryptic sibling species. By morphology alone these are

virtually indistinguishable, and they are usually diagnosed only by their advertisement call or by differences in coloration (Blommers-Schlösser 1979; Glaw & Vences 2000).

Speciation through call differentiation has been suggested for certain insect groups in which females recognize their mates by species-specific advertisement calls (Henry 1994). Similarly, in *Boophis* the bioacoustically divergent cryptic species may also be examples for this type of speciation. If this were the case, a low genetic differentiation among many *Boophis* species would be predicted, as was documented in other instances of incipient speciation (e.g. Meyer *et al.* 1990; Schliewen *et al.* 1994; Freeland & Boag 1999; Wilson *et al.* 2000).

In this study, we sequenced fragments of the 16S ribosomal RNA (rRNA) gene from 27 *Boophis* species to investigate their phylogenetic relationships, ages and rates of speciation among brook and pond-breeding species. We used the mitochondrial data to test the hypotheses of (i) low genetic divergence between sibling species and (ii) reproductive mode as possible key innovation and mechanism for diversification.

Material and methods

DNA sequencing

DNA was extracted using QIAmp tissue extraction kits (Qiagen) from muscle tissue samples preserved in pure ethanol. We used the primers 16SA-L (light chain; 5'-CGC CTG TTT ATC AAA AAC AT-3') and 16SB-H (heavy chain; 5'-CCG GTC TGA ACT CAG ATC ACG T-3') of Palumbi *et al.* (1991) to amplify a section [ca. 550 base pairs (bp)] of the mitochondrial 16S ribosomal RNA gene. We chose this gene instead of a more variable marker, such as cytochrome *b* or the control region, based on preliminary data which indicated a high amount of genetic divergence between *Boophis* species. Polymerase chain reaction (PCR) conditions followed Vences *et al.* (2000b). PCR products were purified using QIAquick purification kits (Qiagen) and were sequenced using an automatic DNA sequencer (ABI 377). Sequences (see Appendix I for GenBank accession numbers) were aligned using the computer program SEQUENCE NAVIGATOR 2.0 (Applied Biosystems), taking secondary structures into account (Kjer 1995). The alignment needed single gaps at four positions, double gaps at two positions, and three or four consecutive gaps at two additional positions. Of the latter two cases, one was due to insertions in only four species, while the second was within a hypervariable region of 53 bp (positions 257–309 of the alignment).

In addition to our own data we also re-analysed the five *Boophis* sequences from the work of Richards *et al.* (2000) who studied higher-level relationships in a more inclusive sample of Malagasy frogs.

Phylogenetic analyses

Phylogenetic analyses were carried out using PAUP*, version 4b8 (Swofford 2001). Prior to phylogenetic reconstruction, we explored which substitution model fitted our sequence data the best. We applied a hierarchical likelihood method to test the goodness-of-fit of nested substitution models, using the program MODELTEST (Posada & Crandall 1998). The substitution model estimated as best fitting our data (see Results) was used to obtain maximum likelihood (ML) trees using the heuristic search option, and a random addition-sequence with 10 replicates. Maximum parsimony (MP) and neighbour-joining (NJ) trees were also calculated.

In the MP analysis we conducted two heuristic searches, coding each gap (also in multiple gap sequences) either as fifth state or as missing character, with branch swapping using the tree bisection-reconnection (TBR) routine. To identify multiple islands of equally most parsimonious trees, 100 random addition-sequence replicates were performed. Only minimal-length trees were saved and zero-length branches were collapsed. In the NJ analyses we used LogDet distances, which are robust against possible variation of sequence evolution among lineages (Lockhart *et al.* 1994). All MP and NJ analyses were repeated after exclusion of the hyper-variable region (53 bp).

All available *Boophis* sequences were included in a first data exploration. To avoid the masking of cladogenetic patterns in the tests of tree shape and in diversity comparisons by inclusion of multiple sequences of the same species, we conducted further analyses with a reduced data set of only a single sequence per species. The excluded sequences differed by a maximum of only three substitutions from the included sequences.

Two thousand bootstrap replicates (Felsenstein 1985) were run in all analyses except ML (only 100 bootstrap replicates due to computational constraints). The robustness of nodes was tested by Shimodaira–Hasegawa tests as implemented in PAUP*. Sequences were checked for clock-like behaviour using the program TREE-PUZZLE 5.0 (Schmidt *et al.* 2000).

Tests of relative molecular substitution rates (Takezaki *et al.* 1995) were conducted using PHYLTEST (Kumar 1996); of the different substitution models implemented in this program, we used Jukes–Cantor distances, uncorrected *P*-distances of transversion only, Kimura two-parameter distances, and Kimura two-parameter distances of transversions only.

Tests of diversification rate and tree imbalance

Although disparities in species numbers among lineages may seem obvious, the differences are not significant in many cases. Under a Markovian model of cladogenesis a stochastic difference in initial rates can lead to a much higher diversity in one of the lineages (Slowinski & Guyer 1989). Careful

examination of the initial data is therefore needed to assess possible differences in species diversity (Sanderson & Donoghue 1996). Our approach largely follows Cook & Lessa (1998) who applied a number of different statistics to a similar problem (the assumed high rates of diversification in subterranean rodents). Standard null model tests (Slowinski & Guyer 1989, 1993) were used to compare standing diversity in brook-breeding and pond-breeding *Boophis*. The more sensitive three-taxon tests, as implemented in LRDIIVERSE (Sanderson & Donoghue 1994; Sanderson & Wojciechowski 1996), were performed with 1000 Monte Carlo replicates, keeping the relative age of the internal node as unknown.

We used the Colless index (Colless 1982) and confidence intervals as provided by Kirkpatrick & Slatkin (1993) as a measure of tree imbalance. This index was calculated by hand from the topology of the ML tree. The program END-EPI (Rambaut *et al.* 1997) was used to estimate relative cladogenesis. This statistic calculates the probability of the existence of a given number of tips (terminal taxa) of a lineage through time in comparison with the actual number of tips, and identifies branches with higher than expected rates of cladogenesis (Nee *et al.* 1996). As a basis for calculation of relative cladogenesis (see below) we built a tree using the nonparametric rate-smoothing algorithm in the r8s program

(Sanderson 1997). POWELL algorithm was chosen and the unit for branch lengths was defined as per site.

Results

Phylogeny and substitution rates

In the preliminary data exploration using all 37 available *Boophis* sequences and three outgroups, and coding gaps as fifth state, of 558 characters included in the analysis, 328 were constant, 44 were variable but parsimony-uninformative, and 186 were parsimony-informative. MODELTEST suggested a general time-reversible substitution model with a gamma distribution shape parameter of 0.713, a proportion of invariable sites of 0.498 and empirical base frequencies (A: 0.341; C: 0.207; G: 0.196; T: 0.256) and substitution rates (A-C, 3.65; A-G, 17.030; A-T, 7.938; C-G, 2.380; C-T, 39.395; G-T, 1.000). These settings were used for ML analyses.

In the ML tree (Fig. 1), with the exception of the *Boophis majori* group, pairs and trios of sibling species were grouped as monophyletic units. Species assigned to the same phenetic species group formed monophyletic groups in some cases (*B. goudoti* group; *B. luteus* group) but appeared to be paraphyletic in several other instances (*B. majori* group; *B. rappiodes* group; *B. tephraeomystax* group). The included

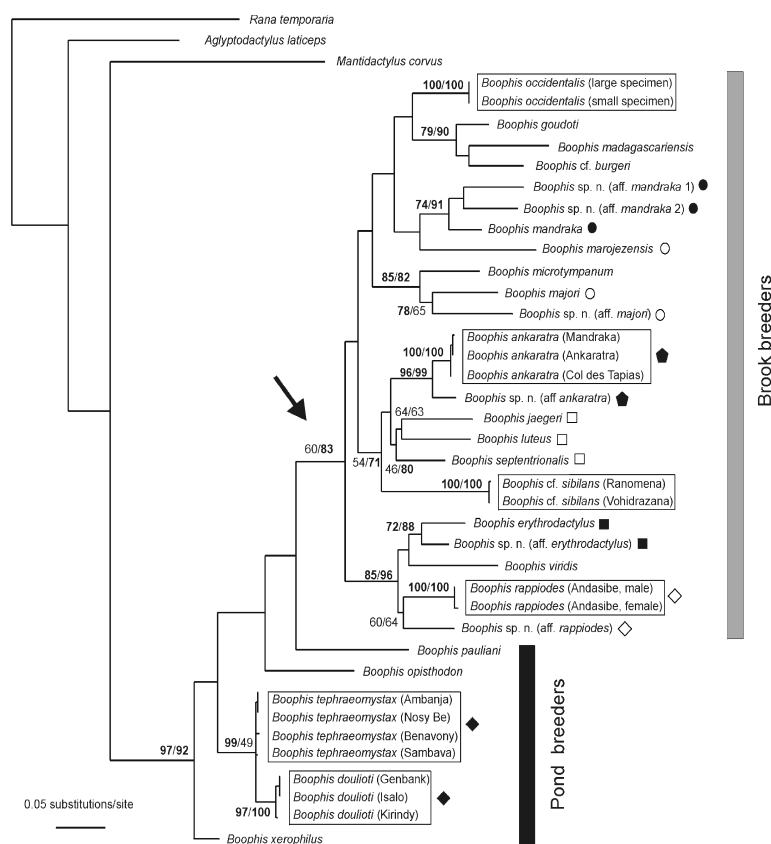


Fig. 1 Maximum likelihood phylogram based on all available sequences of the 16S rRNA gene (558 bp). *Rana temporaria* was used as outgroup. Numbers are bootstrap values in per cent (2000 replications) for MP and NJ analyses, respectively. No values are given for nodes which received support below 50% in both analyses. Values above 70% are printed in bold. The arrow marks the support for monophyly of the brook-breeding clade. Different symbols mark pairs or trios of sibling species which are not or are almost not distinguishable morphologically after preservation (Table 1). Sequences of conspecific specimens are included in boxes.

Table 1 Classification of pairs and trios of sibling species (not or almost not distinguishable morphologically after preservation) included in the present study relative to their distribution pattern and bioacoustic differences

Allopatric species with weak call differences	Altitudinally separated species with weak call differences	(Potentially) syntopic species with strong call differences	Allopatric species with strong call differences
<i>B. luteus</i> – <i>B. septentrionalis</i>	<i>B. ankaratra</i> – <i>B. aff. ankaratra</i>	<i>B. marojezensis</i> – <i>B. majori</i> – <i>B. aff. majori</i>	<i>B. jaegeri</i> – <i>B. luteus</i>
<i>B. tephraeomystax</i> – <i>B. doulioti</i>		<i>B. erythroductylus</i> – <i>B. aff. erythroductylus</i> <i>B. rappiodes</i> – <i>B. aff. rappiodes</i> <i>B. mandraka</i> – <i>B. aff. mandraka 1*</i> – <i>B. aff. mandraka 2*</i>	<i>B. jaegeri</i> – <i>B. septentrionalis</i>

**B. mandraka* 1 and 2 refer to two undescribed species which both are morphologically similar to *B. mandraka*.

brook-breeding *Boophis* were grouped in one clade, whereas the pond-breeding species were arranged paraphyletically at the basis of the cladogram. Sequences from specimens which *a priori* were considered to be conspecific were grouped with high bootstrap support as monophyletic units in all cases except for *B. tephraeomystax*.

These results were also confirmed by the different NJ and MP analyses performed.

(i) NJ, both with all characters and after exclusion of the hypervariable region, supported the same patterns outlined above; bootstrap values for the analysis including all characters are given in Fig. 1, those after exclusion of the hypervariable sites did not show relevant differences.

(ii) MP, with gaps coded as fifth state and all characters included, yielded 12 equally most parsimonious trees [1042 steps, consistency index (CI) 0.378, retention index (RI) 0.610]; these were not unequivocal regarding brook-breeder monophyly, since a second alternative, placing basally a clade containing *Boophis rappiodes*, *B. viridis* and *B. erythroductylus* was also equally parsimonious. However, the bootstrap value (60%) and a majority rule consensus tree (67%) did support monophyly of brook-breeding *Boophis*. Excluding the hypervariable region did not alter these results; 333 equally most parsimonious trees were obtained (684 steps; CI 0.393, RI 0.627). Brook-breeder monophyly was not apparent from a strict consensus tree but from a majority rule tree (85%) and from the bootstrap analysis (65%). The monophyly of the included species of the *B. luteus* group and *B. goudoti* group and paraphyly of the *B. rappiodes* group, *B. majori* group and *B. tephraeomystax* group, and the sister-group relationships of sibling species, were confirmed by each of the analyses.

(iii) MP, with gaps coded as missing data and including all characters, yielded 28 equally most parsimonious trees (993 steps; CI 0.363, RI 0.605). These did not unambiguously

support brook-breeder monophyly, but in a 50% majority consensus tree brook-breeding *Boophis* were monophyletic (79%). The same was true in a bootstrap analysis (59%). Exclusion of the hypervariable region resulted in 200 equally most parsimonious trees (657 steps, CI 0.385, RI 0.627). In a strict consensus of these the brook-breeding species did form a monophyletic group, which received a bootstrap value of 79%. Monophyly/paraphyly of *Boophis* species groups and relationships of sibling species were as in the former analyses.

In the reduced set of 27 *Boophis* sequences (one sequence per species), coding gaps as fifth character and not including the hypervariable region, 328 out of 558 characters were constant, 54 were variable but parsimony-uninformative, and 176 were parsimony-informative. The topology of the ML tree as shown in Fig. 2 totally agreed with the results of the preliminary data exploration. As in the previous analyses, all MP and NJ searches (with hypervariable region included or excluded) agreed upon the monophyly/paraphyly of the different *Boophis* species groups. Monophyly of brook-breeding species was supported by the NJ trees, MP and NJ bootstrap analyses (68–69%), and MP 50% majority rule consensus trees, while the MP strict consensus trees were equivocal in this respect.

The sequences of Richards *et al.* (2000) included four species of brook-breeding *Boophis* (*B. albilabris*, *B. erythroductylus*, *B. luteus*, *B. madagascariensis*) and one pond-breeder (*B. doulioti*, as *B. tephraeomystax*). Re-analysis of these sequences using *Aglyptodactylus* as outgroup (results not shown) yielded bootstrap supports of 74%, 85% and 73% (ML, MP, NJ) for the monophyly of a group composed by the four brook-breeders.

A tree obtained enforcing a molecular clock with TREE-PUZZLE was rejected on a significance level of $P < 0.05$ by a likelihood ratio test when compared with the ML tree. Tests

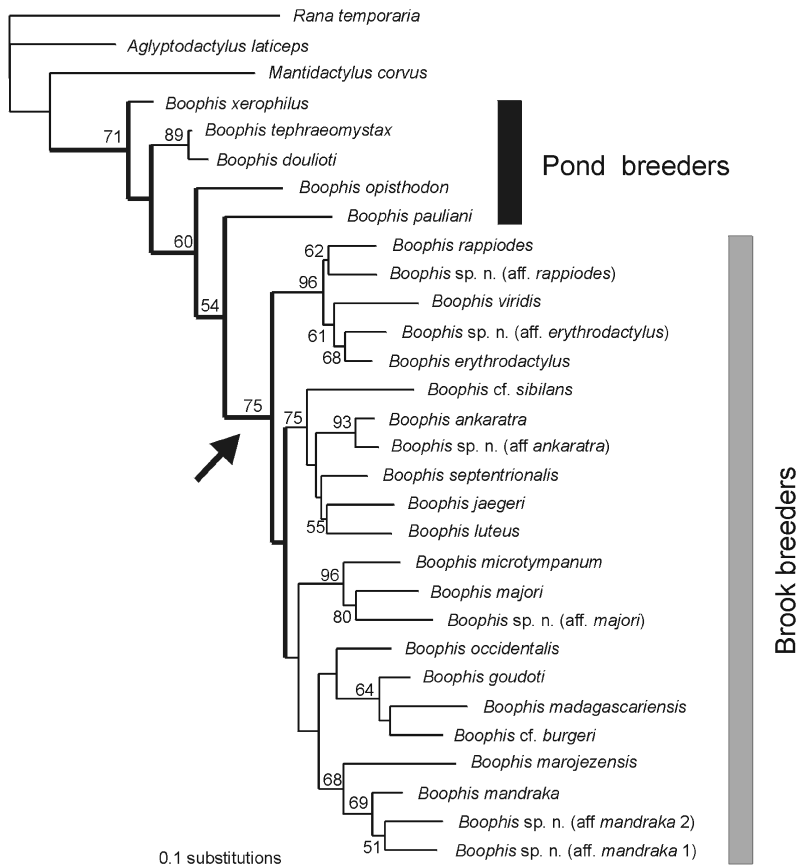


Fig. 2 Maximum likelihood phylogram of analysis of a reduced data set (one sequence per species only). Numbers are bootstrap values in per cent (100 replications). Thicker lines indicate lineages that were found to have statistically higher than expected rates of cladogenesis ($P < 0.01$) after nonparametric rate smoothing of the tree. The arrow marks the support for monophyly of the brook-breeding clade.

of relative nucleotide substitution rates using PHYLTEST significantly rejected rate constancy when comparing the brook-breeding lineage separately with each pond-breeder. This result was obtained independently from the substitution model used, except for a single comparison considering transversions only. In all cases the branch lengths of brook-breeders were longer, indicating a faster substitution rate. The results were similar when comparing the pond-breeding *B. doulioti* with the four brook-breeders of the data set of Richards *et al.* (2000), but in these analyses the comparisons based on transversions only did not yield significant differences.

Diversification rates

Three taxon tests with the program LR DIVERSE, using the ML tree topology (Fig. 2), indicated a significantly higher speciation rate in brook-breeders. For instance, using the pond-breeders closest to the brook-breeding lineage (*Boophis opisthodon* as outgroup, *B. pauliani* as first ingroup and the brook-breeders as second ingroup – species diversities 1, 1 and 22), only the model assuming different speciation rates of the brook-breeding lineage received P -values smaller than 0.98 and was therefore selected ($P < 0.005$).

However, the nonmonophyly of pond-breeders was only weakly indicated by the phylogenetic results, and a more

conservative approach assuming their monophyly was therefore also used. This LR DIVERSE analysis did not indicate significant differences in speciation rate between pond-breeding and brook-breeding *Boophis* when the respective standing diversities were compared and *Aglyptodactylus* (with three species) was used as outgroup (P -value for null model = 0.863). Such a conservative assumption was also used for tests of standing diversity (Table 2). These did not provide evidence for an increased diversification of brook-breeding *Boophis*. Cumulative tests also failed to indicate such a pattern when the second large group of Malagasy frogs with brook specialists, the mantellines, were included in a combined analysis (Table 2).

The Colless index of the ML tree (0.391) was above the high cut-off for the corresponding confidence interval (Kirkpatrick & Slatkin 1993) and thus demonstrated a significant tree asymmetry.

Analysis of the clock-like ML tree with END-EPI (Rambaut *et al.* 1997) showed significantly higher than expected rates of cladogenesis at almost all basal positions of the tree, starting with the split between the outgroup and *Boophis*, and including the origin of the main brook-breeding lineages (not shown). The same pattern was found after application of nonparametric rate smoothing using the R8S program (Fig. 2).

Table 2 Species diversity in Malagasy anurans and *P*-values for two-taxon tests of imbalance (Slowinski & Guyer 1989, 1993) and the cumulative test (Slowinski & Guyer 1993) across pairs of taxa

	Pond-breeding	Brook-breeding	<i>P</i> -value
Mantellidae			
Boophinae (<i>Boophis</i>)	10	48	0.175
Laliostominae (<i>Aglyptodactylus</i> + <i>Laliostoma</i>)	4	0	—
Mantellinae A (<i>Mantidactylus</i> + <i>Mantella</i>)*†	20	38	0.351
Mantellinae B (<i>Mantidactylus</i> + <i>Mantella</i>)*‡	27	50	0.355
Hyperoliidae			
Hyperoliinae (<i>Heterixalus</i>)	10	0	—
Microhylidae§			
Scaphiophryninae	9	0	—
Dyscophinae	3	0	—
Cumulative tests			
Boophinae + Mantellinae A			0.234
Boophinae + Mantellinae B			0.235

The phylogenetic basis of the groupings is mainly based on Richards *et al.* (2000). Following the NJ tree presented by these authors, the subgenus *Spinomantis* (represented in the phylogeny by *Mantidactylus aglavei*) is here considered as part of the brook-breeding lineage, and brook-breeding mantellines are considered as a monophyletic group. The species numbers are recent estimates (e.g. Glaw & Vences 2000) that include nominal species and already identified but not yet formally described species (F. Glaw and M. Vences, personal observation).

*A few deviating species (brook-breeders in the pond-breeding lineage) were excluded: *Mantella cowani* group, *Mantidactylus grandisonae*.

†Tree-hole or phytotelmic-breeders in the pond-lineage excluded; all species from the brook-lineage which probably have no free-swimming tadpoles excluded.

‡Tree-hole and phytotelmic-breeders included in the pond lineage; species without free-swimming tadpoles but which regularly reproduce along brooks included in the brook-lineage.

§Microhylids of the subfamily Cophylinae are not regarded here; this endemic and speciose radiation (about 50 identified species) has nonfeeding tadpoles and reproduces in terrestrial nests or tree-holes.

An overall comparison of the native frog fauna in Madagascar (Table 2) showed that only two out of six subfamilies include brook-breeding lineages. For one of these, the Boophinae (genus *Boophis*), our data indicated monophyly of brook-breeders as opposed to pond-breeders. The second group including both reproductive strategies, the subfamily Mantellinae (*sensu* Vences & Glaw 2001), also appears to be divided into two main lineages that largely correspond to brook and pond breeders (Richards *et al.* 2000; Vences *et al.* 2002a). If vicariance scenarios apply to explain the origin of Malagasy anurans (Bossuyt & Milinkovitch 2001), each anuran group was present on Madagascar since its separation from Gondwana and should have undergone a similar biogeographic history. A direct comparison of brook-breeding and pond-breeding lineages may therefore be justified and supports a significantly higher diversity of pond-breeders as compared with brook-breeding lineages (Mann–Whitney *U*-test; $P < 0.05$).

Levels of divergence

We assessed levels of divergence using two models: pairwise total sequence divergence in per cent (Fig. 3), which allows for a direct comparison with values in other animal groups

(e.g. Avise *et al.* 1998), and pairwise ML distances, which better account for the specific substitution model in our data set. We calculated divergences (i) between *Boophis* and other mantellid genera (*Mantidactylus* and *Aglyptodactylus*), (ii) between pond- and brook-breeding *Boophis*, (iii) between brook-breeding species groups, and (iv) within species groups. In the ML distance calculations the mean intergeneric values were distinctly higher than the intrageneric *Boophis* values (0.42 vs. 0.28, 0.22 and 0.17). This trend was less distinct in the total divergence calculations (16% vs. 13%, 12% and 10%; Fig. 3), probably indicating the start of saturation. Total divergences below 9% were always found between species *a priori* assigned to the same phenetic species group. Most of these (and all below 6%) referred to species pairs considered as sibling species. Divergences between sibling species ranged from 2 to 13%, with a modal value of 8%. Species with strong differences in advertisement calls (whether syntopic, potentially syntopic, or allopatric) had divergences of 5–13%. The two identified divergences lower than 5% referred to allopatrically separated species pairs with weak call differences (*B. aff. ankaratra* – *B. ankaratra*; *B. tephraeomystax* – *B. doulioti*). Conspecific specimens consistently showed a very low degree of sequence differentiation (0–3 substitutions; 0–0.5% divergence).

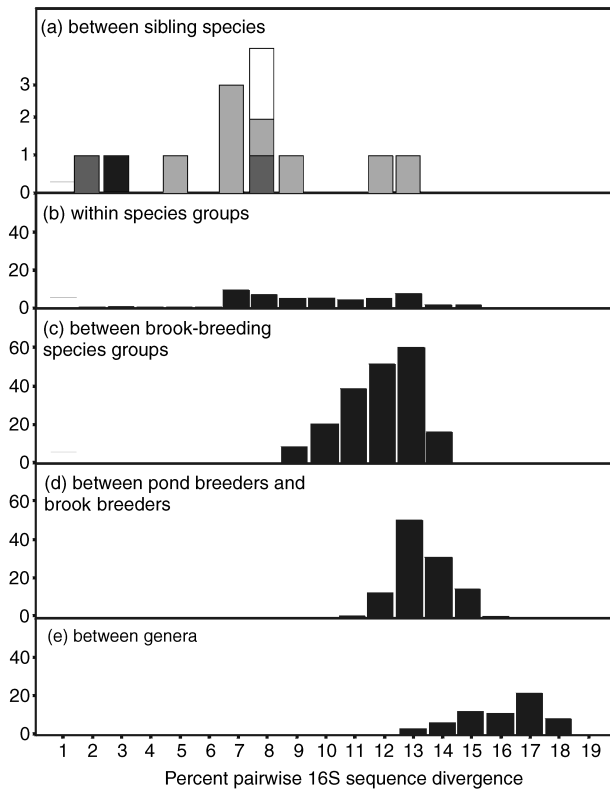


Fig. 3 Distribution of pairwise total divergences in per cent among 558 bp fragments of the 16S rRNA gene: (a) between different types of *Boophis* sibling species (as listed in Table 2): white bars, latitudinally separated species with strong call differentiation; black bars, latitudinally separated species with weak call differentiation; dark grey bars, altitudinally differentiated species with weak call differentiation; light grey bars, syntopic species with strong call differentiation; (b) between brook-breeding *Boophis* included in the same species group (the sibling pairs are a subset of these data); (c) between brook-breeding *Boophis* included in different species groups; (d) between species of the brook-breeding lineage and the pond-breeding assemblage in *Boophis*; and (e) between species of *Boophis* and outgroup species of *Mantidactylus* and *Aglyptodactylus*. Based on the reduced data set (one sequence per species).

Discussion

Monophyly and diversity of brook-breeding Boophis

The present study provides moderate support (bootstrap values 60–83%) for the monophyly of the assemblage of brook-breeding frogs of the genus *Boophis*. Similar values were found by re-analysis of the sequences from Richards *et al.* (2000) in a reduced taxon set (73–85%). Although Shimodaira-Hasegawa (SHI) tests could not exclude significantly all alternative topologies (results not shown), we consider the monophyly of brook-breeders as relatively well established since it agrees with two relevant osteological characters: (i) representatives of all brook-breeding species

groups lack the anterolateral hyoid process which is present in the pond-breeding *B. tephraeomystax* and *B. doulioti*; and (ii) all brook-breeders are characterized by the apomorphic state of two tarsal elements while there are three at least in one pond-breeder (*B. doulioti*) (Vences *et al.* 2002a).

Our sample of species (five pond-breeders vs. 22 brook-breeders; 19% vs. 81%; including taxa from all phenetic species groups) was representative of the overall species diversity so far identified in *Boophis* (Table 2; 17% vs. 83%). As our sample included representatives of all species groups we suppose that our phylogenetic conclusions can also be extended to the remaining species.

Four pond-breeders (*B. opisthodon*, *B. doulioti*, *B. tephraeomystax*, *B. xerophilus*) are known to occur at least partly outside densely forested areas; at least the latter three occur outside rainforest. In the brook-breeding lineage, only *B. goudoti* lives outside forested areas in the Malagasy highlands (Blommers-Schlösser 1979) and *B. laurenti*, *B. microtympnum* and *B. williamsi* are specialized to montane heathlands (Glaw & Vences 1994). A few other species, such as *B. luteus*, are also regularly found in largely degraded areas. The percentage of forest species (which depend at least on stretches of continuous gallery forest) is thus below 70% among pond-breeders and about 90% among brook-breeders, indicating a possible association of brook-breeding with rainforest habitat.

High levels of divergence between siblings

We herein provide the first survey of genetic divergence in a representative sample of pairs of sibling species in Malagasy frogs. The high genetic divergence observed between most species pairs contrasts with the low intraspecific variation. In six species in which more than one specimen was examined (*B. ankaratra*, *B. occidentalis*, *B. rappiodes*, *B. cf. sibilans*, *B. tephraeomystax*, *B. doulioti*), divergence between sequences did not amount to more than 0.5% (three substitutions). The modal divergence of 8% between sibling species was much higher than would be expected in the case of recent Pleistocene–Holocene speciation events (Klicka & Zink 1997; Avise *et al.* 1998). We did not identify a single example of a very recently diverged pair of sibling species which differ by advertisement call. Instead, all syntopic species with important bioacoustic differentiation had high sequence divergences, and the lowest levels of divergence were found between allopatric species. Our data therefore do not provide evidence for a recent rapid sympatric speciation by sexual selection in *Boophis*. If the same factors influencing *Boophis* speciation have been active over the evolutionary history of the genus, our data would rather favour allopatric speciation as a prevailing mechanism in this group, as it is assumed for tropical salamanders (García-Paris *et al.* 2000).

Patterns of diversification in *Boophis*

Using the molecular phylogeny of *Boophis* as the basis, the three taxon test supports a significant disparity in diversification rates between brook- and pond-breeding *Boophis*. Also, the ML tree (Fig. 2) was found to be significantly asymmetric.

In contrast, other analyses led to less unequivocal results. Higher than expected rates of cladogenesis were found in the initial radiation of brook-breeding *Boophis*, but also characterized all other basal splits in the genus, including pond-breeding species. This was true when enforcing a clock-like tree, but also after nonparametric rate smoothing which has been shown to provide more accurate estimates of divergence times in the case of nonclock-like substitution rates (Sanderson 1997).

Despite the obvious differences between species numbers of brook-breeding and pond-breeding *Boophis* species, the comparisons of standing diversities were not significant in a conservative approach that assumed monophyly of pond-breeders as an alternative hypothesis. The crucial point is the supposed paraphyly of pond-breeders, a question which cannot be unequivocally solved with the present data.

Future analyses of larger data sets are needed to understand the relevance of the indications of a higher diversification rate of brook-breeding *Boophis* compared to pond-breeders. The tendencies of an overall higher species diversity of brook-breeding clades among Malagasy frogs, however, appear to correlate with a lower mitochondrial nucleotide substitution rate in two pond-breeding lineages: *Boophis* and the microhylid genus *Scaphiophryne* (Vences *et al.* 2002b). Reproduction in ponds is usual in open savannah landscapes in which permanent brooks are less frequent. Lentic waters are often temporary, and a highly synchronous 'explosive' breeding behaviour is therefore shown by pond-breeding *Boophis* in arid environments (Andreone *et al.* in press). Furthermore, savannah-breeders are also vagile since ponds may form at different sites each year. Such natural history patterns contribute to a high degree of gene flow within and between populations. This may prevent sympatric and allopatric speciation and fixation of mutations, thereby causing a lower species diversity and slower substitution rates in pond-breeders.

Slowinski & Guyer (1993) explicitly distinguish between intrinsic causes of diversity (i.e. morphological novelties that can be seen as key innovations) and extrinsic causes (i.e. environmental changes or tectonic activity). If brook-breeding adaptations were partly responsible for *Boophis* diversity, they certainly could be defined as key innovations. The fact that four out of six lineages of Malagasy frogs did not colonize brooks indicates that important modifications of mating and egg-laying behaviour as well as embryonal and larval development are necessary for an original pond-breeder to reproduce successfully in brooks. However, brook-breeding has also an extrinsic aspect. Occurrence of small

permanent brooks in Madagascar is closely related to rainforests. Furthermore, most Malagasy rainforests are on relatively steep slopes where few lentic waters occur. It is plausible to assume that rainforests were a novelty for the ancestors of the extant Malagasy amphibian fauna: In vicariance scenarios (Duellman & Trueb 1986; Bossuyt & Milinkovitch 2001) ancestral mantellids were present on Madagascar already in the Early Cretaceous when probably a rather seasonal and partly xeric climate prevailed (Spicer *et al.* 1994). In dispersal scenarios (Krause *et al.* 1997) they reached the Malagasy west coast in the Cenozoic from Africa via waif dispersal, and had to be adapted to aridity to survive under the conditions encountered (Vences *et al.* 2000a). Hence, the wealth of new ecological niches in rainforest opened up for these ancestral frogs, and only some of them succeeded in colonizing this new habitat. Those which switched to reproduction in brooks rather than ponds were relieved of the need of synchronous breeding. Temporal and spatial segregation of subpopulations reduced effective population sizes and favoured fixation of mutations, thereby accelerating DNA substitution rates and probably promoting speciation.

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This work is part of a long-term study of the diversity and biogeography of the amphibians and other vertebrates of Madagascar. Miguel Vences is head of the vertebrate section of the Zoological Museum, University of Amsterdam and assistant professor; until April 2002 he was a postdoctoral fellow of the Deutsche Forschungsgemeinschaft in the laboratory of Axel Meyer at the University of Konstanz, using molecular methods to contribute to the understanding of speciation mechanisms in frogs. While his taxonomic and ecological work on the Malagasy herpetofauna is largely carried out in cooperation with Frank Glaw and Franco Andreone, a part of the molecular work has so far been performed in the laboratory of Michael Veith at the University of Mainz, in cooperation with Joachim Kosuch and Hans-Christian Schaefer.

Appendix I

Specimens examined and GenBank accession numbers of sequences. All localities are in Madagascar except that of *Rana temporaria* (Germany)

Species group	Species	Origin	Voucher	Genbank accession
—	<i>Rana (Rana) temporaria</i>	Koblenz	ZFMK 69883	AF124135
—	<i>Aglyptodactylus laticeps</i>	Kirindy	ZFMK 64135	AF215329
—	<i>Mantidactylus corvus</i>	Isalo	ZFMK 70494	AF215320
<i>luteus</i> group	<i>Boophis ankaratra</i>	Mandraka	ZSM 400/2000	AJ315909
	<i>Boophis ankaratra</i>	Manjakatampo	ZSM 367/2000	AJ315911
	<i>Boophis ankaratra</i>	Col des Tapias	ZSM 399/2000	AJ315910
	<i>Boophis</i> sp. n. (aff. <i>ankaratra</i>)	Ranomafana	ZFMK 62907	AJ315912
	<i>Boophis jaegeri</i>	Berara	ZSM 413/2000	AJ315914
	<i>Boophis luteus</i>	Andasibe	UADBA 2000.063	AJ315916
	<i>Boophis septentrionalis</i>	Ambre	ZSM 493/2000	AJ315915
	<i>Boophis</i> cf. <i>sibilans</i>	Ranomafana	ZFMK 62797	AF215338
	<i>Boophis</i> cf. <i>sibilans</i>	Vohidrazana	ZSM 327/2000	AJ315913
<i>goudoti</i> group	<i>Boophis</i> cf. <i>burgeri</i>	Marojejy	ZFMK 59905	AF215336
	<i>Boophis goudoti</i>	Col des Tapias	not preserved	AJ315917
	<i>Boophis madagascariensis</i>	An'Ala	ZFMK 62265	AF215337
<i>microtypanum</i> group	<i>Boophis microtypanum</i>	Col des Tapias	ZSM 393/2000	AJ315918
<i>albilabris</i> group	<i>Boophis occidentalis</i> (large)	Berara	MRSN A2000	AJ314820
	<i>Boophis occidentalis</i> (small)	Berara	MRSN uncat.	AJ314819
<i>majori</i> group	<i>Boophis majori</i>	Vohiparara	ZFMK 62672	AF215340
	<i>Boophis</i> sp. n. (aff. <i>majori</i>)	Andasibe	ZSM 313/2000	AJ315922
	<i>Boophis marojezensis</i>	Vohidrazana	ZSM 326/2000	AJ315923
<i>rappiodes</i> group	<i>Boophis erythrodactylus</i>	Mandraka	ZSM 324/2000	AJ314814
	<i>Boophis</i> sp. n. (aff. <i>erythrodactylus</i>)	Andasibe	ZFMK 62888	AF215339
	<i>Boophis mandraka</i>	Mandraka	ZSM 346/2000	AJ315921
	<i>Boophis</i> sp. n. (aff. <i>mandraka</i> 1)	Vohidrazana	ZSM 310/2000	AJ315919
	<i>Boophis</i> sp. n. (aff. <i>mandraka</i> 2)	Ambavaniasy	NMBE 1046008	AJ315920
	<i>Boophis rappiodes</i> (male)	Andasibe	ZSM 347/2000	AJ314815
	<i>Boophis rappiodes</i> (female)	Andasibe	UADBA 2000.59	AJ314816
	<i>Boophis</i> sp. n. (aff. <i>rappiodes</i>)	Andasibe	ZSM 344/2000	AJ314817
	<i>Boophis viridis</i>	Andasibe	ZSM 338/2000	AJ314818
<i>tephraemystax</i> group	<i>Boophis doulioti</i> *	unknown*	USNM 59146	AF026360
	<i>Boophis doulioti</i>	Isalo	ZFMK 70495	AF215332
	<i>Boophis doulioti</i>	Kirindy	ZFMK 66690	AF215334
	<i>Boophis opisthodon</i>	Cap Est	ZFMK 70480	AF215331
	<i>Boophis pauliani</i>	Andasibe	ZSM 345/2000	AJ315924
	<i>Boophis tephraemystax</i>	Ambanja	ZSM 489/2000	AJ312115
	<i>Boophis tephraemystax</i>	Nosy Be	ZSM 458/2000	AJ312114
	<i>Boophis tephraemystax</i>	Cap Est	ZFMK 66685	AF215333
	<i>Boophis tephraemystax</i>	Sambava	UADBA 2000.379	AJ312116
<i>Boophis xerophilus</i>	Kirindy	ZFMK 66705	AF215335	

Collection abbreviations are: MRSN, Museo Regionale di Scienze Naturali, Torino; NMBE, Naturhistorisches Museum Bern; UADBA, Université d'Antananarivo, Département de Biologie Animale (numbers are provisional field numbers of F. Glaw and M. Vences); USNM, United States National Museum; ZFMK, Zoologisches Forschungsinstitut und Museum A. Koenig, Bonn; ZSM, Zoologische Staatssammlung, München.

*The *B. doulioti* sequence marked with a star was obtained from GenBank (published by Richards *et al.* 2000).