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Molecular Systematics and Biogeography of the Fanged Frogs of Southeast Asia

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Our analysis of parts of the mitochondrial ribosomal 12S and 16S genes from 39 populations of Southeast Asian ranid frogs confirms that the fanged frogs are a monophyletic clade. This group, properly called *Limnonectes*, appears to have arisen in the early Tertiary at a time when free faunal exchange was possible among Southeast Asia, Borneo, Sumatra, Java, and, probably, Sulawesi. Four species groups are tentatively identified within the clade. Part of group 1 includes species related to *L. kuhlii* that occur in Borneo. Another part of group 1 includes species from Malay Peninsula and Thailand that are related to *L. pileata*. Species group 2, *L. leporina*, occurs only in Borneo. Species group 3 is restricted to species distributed in Sulawesi and the Philippines. Species group 4 includes *L. blythii* and relatives. There is a lack of compatibility between phylogenetic hypotheses generated from molecular and morphological data sets. These differences are related, in large part, to whether some species of *Limnonectes* have secondarily lost fangs or whether lack of fangs represents the primitive condition. © 2000 Academic Press

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The family Ranidae is one of the most speciose groups of living frogs (Duellman and Trueb, 1986). Presumed to have had an African origin sometime in the early Tertiary (Savage, 1973), ranids currently show areas of high species richness in both Southeast Asia and Africa. The Southeast Asian species are of special interest as they occur throughout the Malay Archipelago—a region with a well-studied and particularly complex biogeographic history. Nonetheless, few studies have examined phylogenetic relationships among these frogs.

Among the Southeast Asian ranids is a cluster of species variously characterized by unusual, derived, sexually dimorphic features, including fangs (outgrowths of the lower jaw bone), male voicelessness, males being larger than females, and male parental care. Many of these taxa show great phenotypic similarity (Kiew, 1978, 1984; Inger, 1954, 1958, 1966) and even recognizing individual species has proven to be a challenge (e.g., Emerson and Ward, 1998).

Dubois (1987) placed the fanged frogs and their relatives in one tribe, two genera, and five subgenera, later (Dubois, 1992) redistributing them into three tribes, four genera, and three subgenera but without any type of systematic analysis (Inger, 1996). Emerson and Berrigan (1993), using a morphological data set (40 characters) performed a preliminary cladistic analysis on 18 species. The results of that study (1) confirmed that the fanged frogs were a monophyletic group, (2) recognized at least two main species groups within the clade, and (3) contradicted the taxonomic classification of Dubois (1987, 1992). The morphological data set could not resolve the majority of species relationships within the clade, but further work (Emerson and Ward, 1998) using mitochondrial ribosomal DNA sequences delineated species boundaries and relationships among 18 taxa within one of the major species groups. In this paper we present the results of an even broader molecular study in which mitochondrial ribosomal DNA sequences were investigated to examine the relationships among a larger number of species, including representatives of all the species groups of fanged frogs (sensu Dubois, 1987, 1992).

MATERIALS AND METHODS

Molecular sequence data were collected from individuals of 39 populations of Southeast Asian ranids. For convenience we choose to retain use of the generic name *Rana* for most of these frogs, pending the outcome of this analysis. Samples include *Rana cancrivora, Rana limnocharis* (two populations), *Occidozyga laevis, Rana blythii* (five localities, including Sumatra, Thailand, Vietnam, Borneo, and peninsular Malaysia), *Rana ingeri, Rana leporina* (three populations), *Rana paramacrodon, Rana magna, Rana mac-*

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rodon, Rana macrocephala, Rana modesta, Rana acanthi, Rana microtympanum, Rana malesiana (two populations), *Rana grunniens, Rana ibanorum, Rana kuhlii* (three populations), *Rana pileata, Rana laticeps, Rana adspersus, Rana ibanorum, Rana finchi, Rana palavanensis, R. limborgii,* and *Rana leytensis.* Tissue samples of three unamed species from Sulawesi were also included. Molecular data were also taken from the African ranid *Hoplobatrachus occipitalis. H. occipitalis, R. limnocharis, R. cancrivora,* and *O. laevis* are the outgroup taxa (Dubois, 1992; Emerson and Berrigan, 1993; Emerson and Ward, 1998).

The above taxa include representatives of all of the groups of fanged frogs identified by Dubois (1992). Dubois recognized four subgenera in the genus *Limnonectes: Hoplobatrachus, Fejervarya, Bourretia,* and *Limnonectes.* He placed most of the fanged frogs into a single subgenus, *Limnonectes,* and broke the subgenus into three groups: the *grunniens* group, the *kuhlii* group and the *microdiscus* group. Representative species of all major subgenera of the genus *Limnonectes* as well as members of the three species groups of the subgenus *Limnonectes* are included in the present analysis. Dubois (1992) placed two species of fanged frogs with direct development in a different tribe and genus than the other fanged frogs. One of these species, *R. limborgii,* is also included in this analysis.

Collection localities for the tissue samples are presented in Appendix 1. Voucher specimens are deposited at the University of Malaya; The University of Malaysia, Sarawak; Sabah Museum, Kota Kinabalu; the Field Museum of Natural History; the United States National Museum; The Royal Ontario Museum; the Texas Natural History Museum; the Museum Zoologicum Bogoriense; and the Zoological Reference Collection at the National University of Singapore (Appendix 1). One or two individuals were examined for each taxon or location after preliminary work on several individuals of *R. blythii* from Sabah Malaysia indicated negligible intrapopulation, interindividual variation.

Mitochondrial DNA sequences (1160 bp) were obtained for portions of the 12S and 16S ribosomal RNA genes. A phenol–chloroform extraction was used to obtain DNA from frozen muscle and liver tissue. Biotinylated primers for 12S and 16S were made, using primer sequences from Hedges *et al.* (1993). The DNA was amplified by PCR (94°C 1 min, 58°C 1 min, 72°C 2 min) for 35 cycles. In all but three cases, the biotinylated strand was isolated for single-strand sequencing. For three taxa, the PCR products were cloned into the pCR-II vector using the TA cloning system (Invitrogen) and sequenced after selection of single bacterial colonies containing the correct inserts. This sequencing was performed on an ABI Prism 377 automated DNA sequencer using *Taq* FS DNA Polymerase and the custom oligonucleotides (DNA Sequencing Facility at the University of Utah, under the direction of Margaret Robertson).

Sequence alignments were made by eye using the conserved-motif (Hickson *et al.,* 1996) and secondary-structure (Kjer, 1995, 1997) approaches. Special attention was given to aligning stems and loops according to the latest models for RNA secondary structure (e.g., Richards and Moore, 1996). Gaps were coded as 0/1 character states or as missing. There were a total of 15 gap characters. Regions that could not be aligned with reasonable certainty were excluded from the analysis (90 sites). Sequences are cataloged as GenBank Accession Nos. U55262–U55275, U66110–U66139, AF183123–AF183138, AF261244–45, AF261251, AF261262–63, and AF261269.

Data analysis was done with the PAUP computer software package (version 3.1.1.; Swofford, 1993) and test version 4. 0b2 of PAUP*. Genetic distances between any two taxa were calculated as their uncorrected pairwise sequence divergence. Of the 1160 bases in the 12S/16S sequences, 1070 were used in the actual analysis along with the 15 gap characters. Both parsimony and maximum-likelihood analyses were performed. In the parsimony analysis, transitions and transversions were weighted equally because recent work has indicated that traditional transversional weighting may not work well for ribosomal genes (Simon *et al.,* 1994; Kjer, 1995; Reeder, 1995). Regions of sequence that were conservative from *Escherichia coli* to mammals, but had base substitutions in the frogs under study (three sites), were more heavily weighted by a factor of 5. Analyses were run scoring (1) gaps as missing characters and (2) gaps as a fifth character. Sequences were analyzed using the heuristic search option and 10 replicate searches with random addition of taxa (PAUP 3.1.1). Nonparametric bootstrap analyses with 100 replicates were run to evaluate the strength of the groupings. Additional parsimony analyses were also run keeping all trees one and two steps back from the shortest trees to examine decay rates for various nodes (after Bremer, 1988).

We performed the maximum-likelihood analysis on the 12S/16S data set using PAUP* 4.0b2. To establish parameters for the likelihood model a preliminary parsimony analysis was done and the resulting trees were saved to file. From the data set and the parsimony trees it was possible to obtain estimates of substitution rates, nucleotide frequencies, the number of sites assumed invariable, and alpha. Ribosomal RNA has different rates of change at different positions and it appears that a gamma distribution is most appropriate for this kind of data (Hillis *et al.,* 1996). The obtained estimates confirmed that a two-parameter model distinguishing only between transitions and transversions was too simple. A gamma correction for among-site variation with a general rate reversible model was significantly better than simpler models. We also in-

cluded topological constraints to speed the analysis. Nodes that had been supported by bootstrap values of at least 90% in parsimony analyses were constrained in the maximum-likelihood analysis.

Initially we ran two likelihood analyses, one with all the taxa in the data set including five outgroup taxa and one using only the two closest outgroup taxa, *H. occidentalis* and *O. laevis,* as determined by the parsimony analysis. The results were similar and below we report the model parameters for the analysis with only the two outgroup taxa. Nucleotide frequencies were A = 0.3402, C = 0.2331, G = 0.1832, and T = 0.2435. The data indicated six substitution types, which were input from the R matrix estimated from the initial parsimony tree. Proportion of invariant sites = 0.207. Rates for variable sites were assumed to follow a gamma distribution with alpha = 0.482, estimated from the initial parsimony tree. A heuristic search with 10 random stepwise additions with tree bisection and reconnection branch swapping (TBR) was run.

To test for constancy of DNA sequence rate change we used a likelihood-ratio test. A clock-like tree (null hypothesis) is a special case of the non-clock-like tree. If there is a constant rate of change, then a clock-like tree will be no worse than an non-clock-like tree of the same topology (Goldman, 1993). We compared the maximum-likelihood tree that was obtained (with the general rate reversible model and gamma correction) when the molecular clock was not enforced with the likelihood score for that tree with the molecular clock enforced. The test statistic is twice the difference between the log likelihood scores of the two trees distributed as a χ^2 with $n - 2$ degrees of freedom, where n is the number of taxa. As we used the data set with two outgroup taxa but didn't include *R. limborgii* in the analysis, there were 31 degrees of freedom.

Previous work (Emerson and Berrigan, 1993) had resulted in a phylogenetic hypothesis of relationship for 18 taxa based on morphological data. Unfortunately, morphological data were not available for all taxa included in the present study. Yet, it was still of interest to understand the relationship between the previous morphological study and those phylogenies that would be the outcome of the present analysis of molecular data. For example, it might be that, even though the morphological and molecular data strongly supported different trees, one or both of the data sets might not conflict with the other data's suboptimal tree. To test this possibility, Templeton's tests were used (Templeton, 1983). This is a Wilcoxon signed-ranks test of the differences in lengths of characters when a data set is optimized on one tree versus another.

To perform Templeton's tests, only those taxa for which there were both morphological and molecular data were used. Tests were applied that examined the compatibility of the morphological data set to the most-parsimonious tree generated from the molecular data and, reciprocally, the compatibility of the molecular data set to the most-parsimonious tree generated from the morphological data. A two-tailed test was used to test for significance.

RESULTS

Parsimony analysis. Parsimony analysis of the 12S/16S data set with gaps scored as missing resulted in two equally parsimonious trees of 2285 steps (Fig. 1). The CI (excluding uninformative characters) of the most-parsimonious trees is 0.3264. Three hundred eighty-eight of the 1175 characters were parsimony informative. The results of the analysis suggest that there are at least four major species groups within the clade. The two trees varied most in the relationships among the basal taxa (see below). Similar trees were obtained from the analysis in which gaps were treated as a fifth character, but the bootstrap values for these trees were slightly higher than those for the trees produced from data for which gaps were treated as missing. Nonetheless, the results of the analyses were so similar that no further mention will be made of the analysis coding gaps as a fifth character.

Figure 1 shows one of the two most-parsimonious trees. In this tree, *R. kuhlii* (Taiwan), *R. pileata, R. laticeps,* and *R. limborgii* constitute the single, most basal group of fanged frogs. Bootstrap support for this clade is 41%. Also in this tree, *R. kuhlii* (2 populations) and *R. asperata* from Borneo form another basal group (group 1b). Bootstrap support for this group is 99%. In contrast, the other equally parsimonious tree (not shown) places the two *R. kuhlii* populations and *R. asperata* with *R. kuhlii* (Taiwan), but with bootstrap support of less than 25%. In this second tree, *R. laticeps, R. pileata,* and *R. limborgii* (species group 1a) together constitute the most basal group of fanged frogs. Both trees show the population of *R. kuhlii* from Java as representing yet another separate clade.

In both trees, a second species group includes the Bornean taxa that have been called *R. blythii* (Emerson and Ward, 1998) but should be reassigned to *R. leporina* (Inger and Tan, 1996; Emerson and Ward, 1998). This clade shows 100% bootstrap support.

A third group with 100% bootstrap support includes all the Sulawesan and Philippine species: *R. sp. nov., R. microtympanum, R. magna, R. macrocephala, R. modesta, R. leytensis,* and *R. acanthi.* In addition, two other undescribed species from Sulawesi for which only 12S data are available also cluster in this clade when an analysis is done including these taxa but scoring the 16S data as missing. Three of the species within this third species group, *R. magna, R. macrocephala,* and *R. modesta* cluster together (85% bootstrap value). Relationships among the other species in the group remain largely unresolved. The parsimony analysis

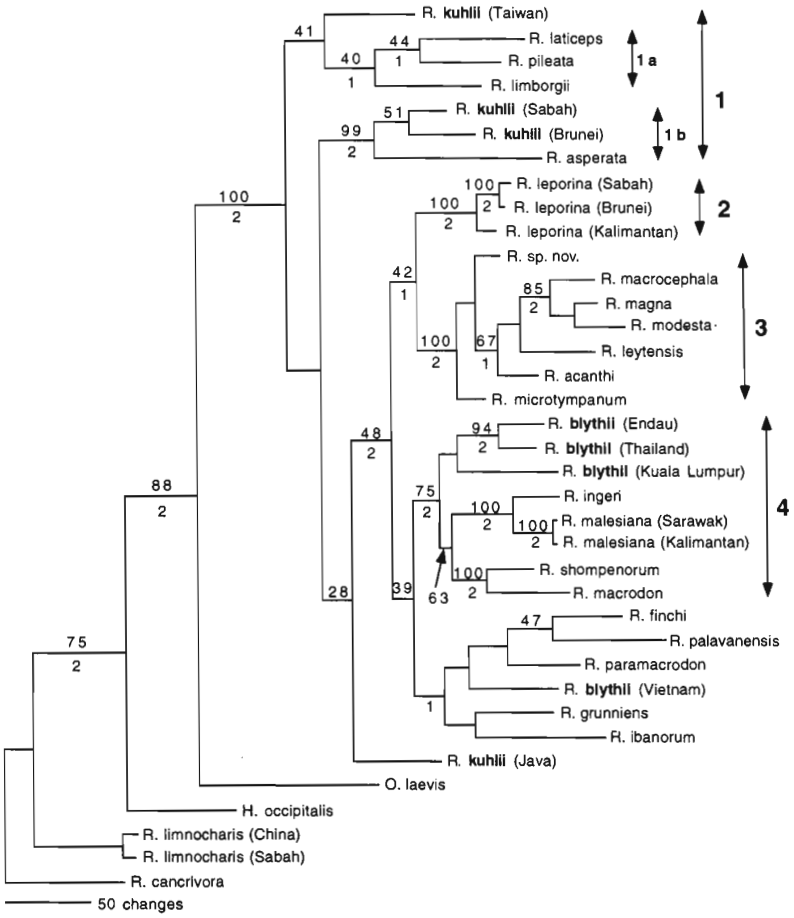

FIG. 1. A phylogram, one of two equally parsimonious trees, showing the relationships among the fanged frogs based on a parsimony analysis. Numbers above the lines indicate bootstrap values higher than 25%. Numbers below the line indicate whether or not that node is retained in trees 1 or 2 steps longer than the shortest tree. Arrows and numbers at the right indicate the four species groups in the fanged frog clade. Names of paraphyletic taxa are printed in boldface.

shows *R. leporina* from Borneo as the sister group to the Sulawesi and Philippine species but with fairly low bootstrap support (42%).

The fourth group includes various populations of *R. blythii* from the Malay Peninsula, *R. macrodon* from Java, *R. shompenorum* (another *R. blythii*-like population) from Sumatra, *R. ingeri* from Borneo, and *R. malesiana* that occurs both in Borneo and on the Malay Peninsula. This group shows moderate bootstrap support (75%). In addition to the four apparent species groups there are a number of taxa that seem most closely related to the fourth group (39% bootstrap value) but whose precise relationships with that group and each other remain unresolved.

The decay analysis showed 18 trees one step back and 68 trees two steps back from the shortest trees. The strict consensus trees from both one and two steps back retain species groups 2, 3, and 4, but most deeper nodes have collapsed. One step back, the grouping of *R. pileata, R. laticeps, and R. limborgii* (species group 1a) and *R. kuhlii* (Taiwan) has collapsed with *R. kuhlii* (Taiwan) now forming a separate offshoot. Species groups 2 and 3 are still grouped together one step back but that node collapses in the strict consensus tree two steps longer.

Maximum-likelihood analysis. Maximum-likelihood analysis using two outgroups, *H. occipitalis* and *O. laevis,* resulted in one tree with a score of 10353.508. The maximum-likelihood analysis produced a tree that differed from the parsimony trees in those areas where bootstrap values were low for the parsimony tree. In the maximum-likelihood tree, *R. limborgii* is shown as the most basal species of the fanged frogs. *R. kuhlii*

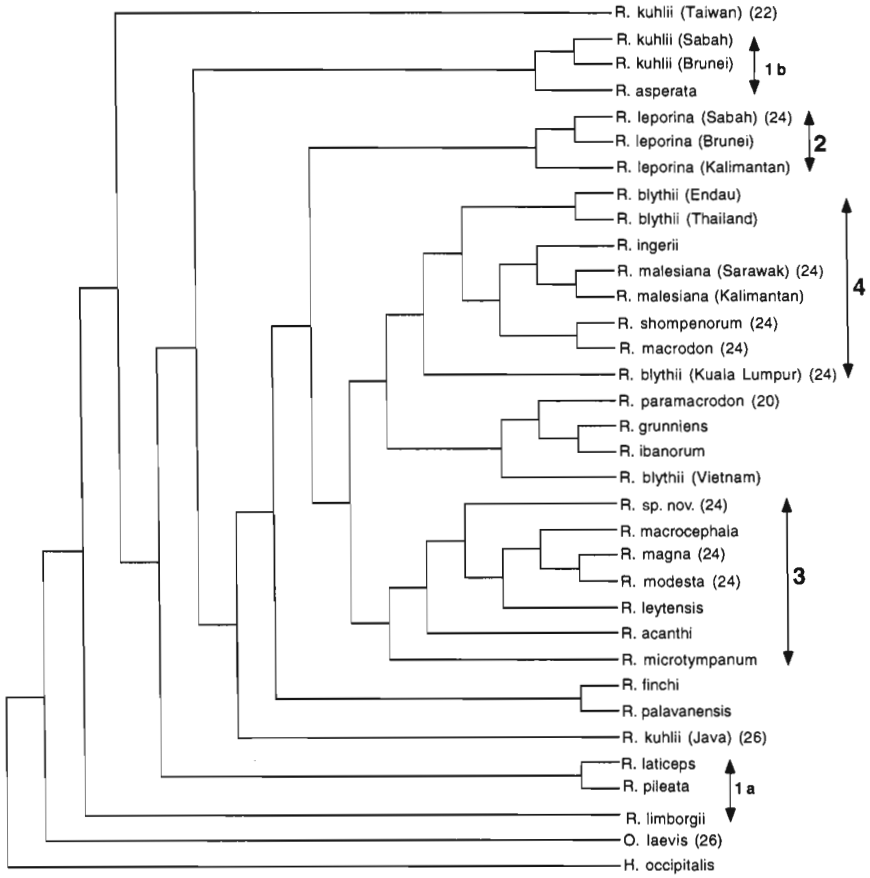

FIG. 2. A phylogenetic hypothesis for the relationships among the fanged frogs based on a maximum-likelihood analysis. Chromosome numbers for taxa are given in parentheses. Species groups are indicated by lines with arrows.

(Taiwan) is also a separate, basal lineage. *R. laticeps* and *R. pileata* remain sister taxa as do the Bornean species in group 1b (Fig. 2).

Three of the four groups appear in the maximum-likelihood tree, although the relationships among the species in the third group vary from those in the parsimony analysis (Fig. 2). Additionally, groups 2 and 3 are not sister taxa in the maximum-likelihood tree. Rather, species group 2 is the sister group to all the other species in the *R. blythii* complex as well as the Sulawesi and Philippine taxa.

R. finchi and *R. palavanensis* are not grouped with *R. paramacrodon* as is the case in the parsimony-generated trees. Additionally, *R. finchi* and *R. palavanensis* are not as closely related to species group 4 as indicated by the parsimony analysis (Fig. 2). In the maximum-likelihood tree, *R. finchi* and *R. palavanensis* constitute the sister group to species groups 2, 3, and 4.

The likelihood ratio test rejected the hypothesis that the DNA sequences were evolving at a constant rate of change ($\chi^2 = 159.47$, $P < 0.005$).

Data compatibility. Templeton tests indicate that the morphological data partition is incompatible with both the parsimony and the maximum-likelihood molecular trees and vice versa (Table 1). A comparison of the trees generated by morphological and molecular

TABLE 1

Results of Templeton's Tests

Tree	Length	Rank sums	N	z	P
Morphology data					
Morphology	55	75.0	12	−2.8858	0.0039
Molecular	73	−3.0			
Molecular data					
Morphology	1161	2099.5	76	−3.4935	0.0005
Molecular	1116	−826.5			

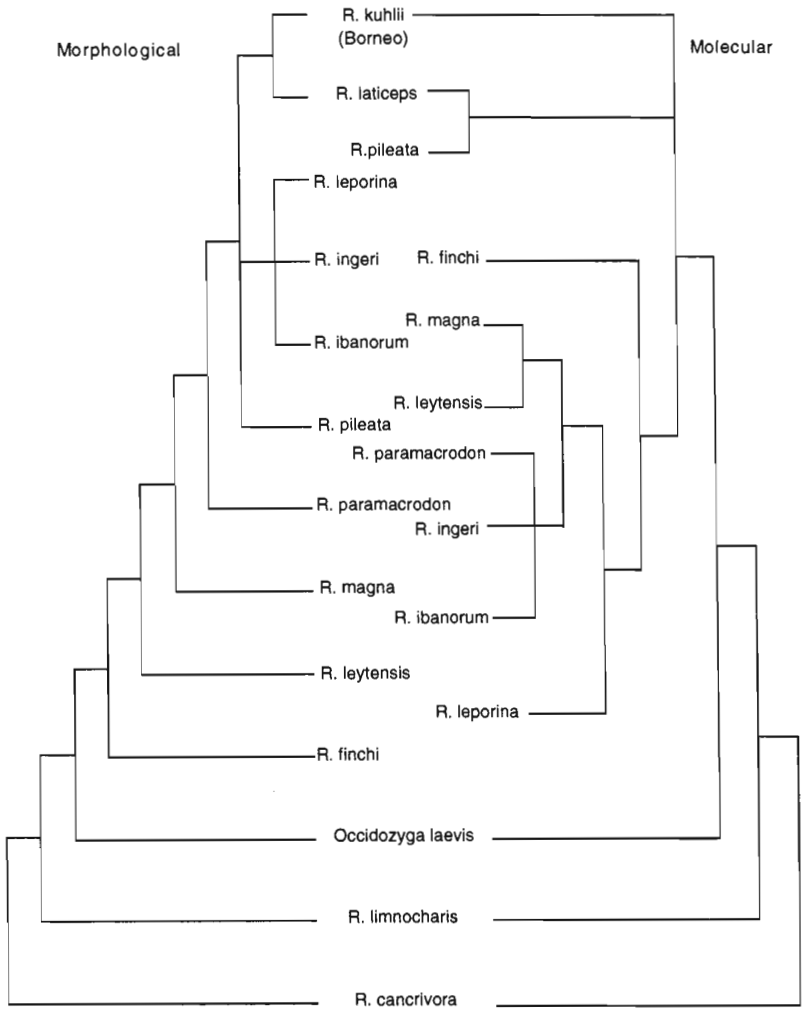

FIG. 3. A comparison of the phylogenetic hypotheses for the relationships among fanged frogs generated by morphological data by and molecular data.

data (Fig. 3) shows (1) differences in the relationships of *R. finchi* and *R. leytensis* to each other and to the rest of the fanged frogs, (2) differences in whether *R. pileata* and *R. kuhlii* are basal species of fanged frogs, and (3) further identification and ordering of the relationships among the major species groups in the molecular tree.

DISCUSSION

The molecular analysis fully supports the finding from the previous morphological study that the fanged frogs constitute a monophyletic group. Thus, it seems appropriate, in the future, to refer to these frogs as members of the genus *Limnonectes*. The groupings and relationships hypothesized by the results of the present analysis are not congruent with those proposed previously by Dubois (1987, 1992). It does not seem advisable to include either *R. cancrivora* or *R. limnocharis* as part of the genus *Limnonectes* at this time (contrary to Dubois, 1992), as neither taxon represents the actual sister group to the fanged frogs in this analysis. Additionally, both the parsimony- and the maximum-likelihood-generated phylogenies have Dubois' three species groups of the subgenus *Limnonectes* as paraphyletic. These results make it important to once again mention the problems of classifications that are not based on the principle of common descent (see also Inger, 1996; Emerson and Ward, 1998).

An interesting aspect of the evolution of the fanged frogs is the shift in chromosome number that has taken

place. Most frogs, including the outgroup species of the fanged frogs are characterized by having 26 chromosomes (Kuramoto, 1990). The fanged frog species *L. kuhlii* from Java retains 26 chromosomes (D. Iskandar, unpublished) but all the other fanged frogs for which there is information have a reduced number of chromosomes. Most of the species have 24 chromosomes, but *L. paramacrodon* has 20 (Yong and Ng, 1994) and the *L. kuhlii* population from Taiwan and the closely related *L. namiyei* have 22 (Kuramoto, 1983).

The present study identifies at least four major groups within the *Limnonectes* clade. Whereas the relationships among these groups are not well resolved, there is strong support for the integrity of three of these groupings. The fourth cluster, group 1, which includes the most basal species of the clade, may not be monophyletic, but information for additional species is necessary before that hypothesis can be adequately tested (see next paragraph). For the present time, two subgroups (1a and 1b) are recognized. Although the maximum-likelihood analysis divides species group 1a by placing *L. limborgii* basal to *L. laticeps* and *L. pileata* (Fig. 2), the three species share an extremely unusual, derived feature—the zygomatic ramus of the squamosal is in contact with the pars facalis of the maxillary bone—providing additional morphological support for their monophyly (Emerson and Berrigan, 1993).

Additional species of fanged frogs exist (Dubois, 1987; Emerson and Berrigan, 1993 and references therein), but tissue samples were not available for this study. These include species allied to *L. finchi* and *L. palavanensis* (Iskandar and Tjan, 1996; Inger, 1954), as well as a number of other species from Thailand and the Malay Peninsula which also have the zygomatic ramus of the squamosal in contact with the maxillary bone and thus are probably related to *L. pileata* and part of group 1 (Berry, 1975; Taylor, 1962; Inger, 1954; Emerson and Berrigan, 1993). The addition of these data may help define the relationships within and among groups more clearly. Alternatively, it is possible that these major species groups evolved very rapidly and it will be difficult to recover the exact sequence of evolutionary events even with additional data or further analysis.

Recent work (Emerson and Ward, 1998) documented that the wide-ranging *L. blythii* is actually a number of different species (see also Fig. 1). A similar situation exists with *L. kuhlii.* This analysis demonstrates that there are at least four species subsumed under that name: two in Borneo, one on Java, and one on Taiwan. The distinctiveness of the *kuhlii* populations is also supported by their karyotypes. Recall from the discussion above that *L. kuhlii* on Taiwan has 22 chromosomes (Kuramoto, 1983), whereas *L. kuhlii* on Java has 26 chromosomes (D. Iskandar, unpublished). Chromosome number is not known for the Bornean populations.

An interesting finding of this study is the lack of compatibility between the morphological data and the molecular phylogenies and vice versa. These differences are the result, in part, of the placement of *L. finchi* and its relationships with other taxa. *L. finchi* and the closely related *L. palavanensis* both lack fangs. In the morphological analysis this was considered the primitive condition and these taxa were found to be the basal members of the *Limnonectes* clade (Emerson and Berrigan, 1993). The molecular phylogenies do not show *L. finchi* and *L. palavanensis* as basal members of the clade but rather more interior and more closely related to *blythii*-like frogs, in which case fanglessness has to be secondarily derived. Fangs are secondary sexual characteristics, used in male intrasexual competition by at least some species of *Limnonectes* (Emerson and Voris, 1992; Orlov, 1997; Tsuji and Kuang, 1998). Their presence is correlated with male territorial combat in these species. In contrast, in *L. finchi* and *L. palavanensis,* the males carry tadpoles on their backs, providing prolonged parental care, and the breeding biology has shifted away from aggressive, territorial interactions (Inger and Voris, 1988). Theoretical work on sexual selection (Arnold and Duvall, 1994) predicts less intense sexual selection on males in such situations and this would be consistent with the (secondary) loss of fangs in the males of *L. finchi* and *L. palavanensis.*

Limnonectes, as defined in this paper, is a group of primarily forest-dwelling frogs, occupying a vast region of high annual rainfall extending from southern China and Burma through the Malay Archipelago to the Philippines and the Moluccas (Fig. 4). The majority of the species, for which the natural history is known, breed at or around streams and almost all have free-swimming larvae associated with flowing water. Some species, for example, *L. palavanensis* and *L. finchi,* have a terrestrial breeding pattern, laying their eggs on land, but retaining aquatic larvae that develop in small ponds. Although none has an exclusively montane distribution, the majority of species are restricted to hilly terrain with streams having moderate currents. These topographic conditions are widespread within the range of the group. Only a few species, for example, *L. malesiana* and *L. paramacrodon,* are found frequently in peat swamps or other flat, low-lying forests. The pattern of reproductive behavior and habitat selection of the fanged frogs sets limits on the types of terrain through which these species can disperse and, given the geologic history of their area of occurrence, sets limits on the times of likely faunal exchange.

The geographic range of this group of species is a region with a complicated geologic history in the Tertiary. The most recent summary of that history (Hall, 1998) concludes that the block encompassing present-

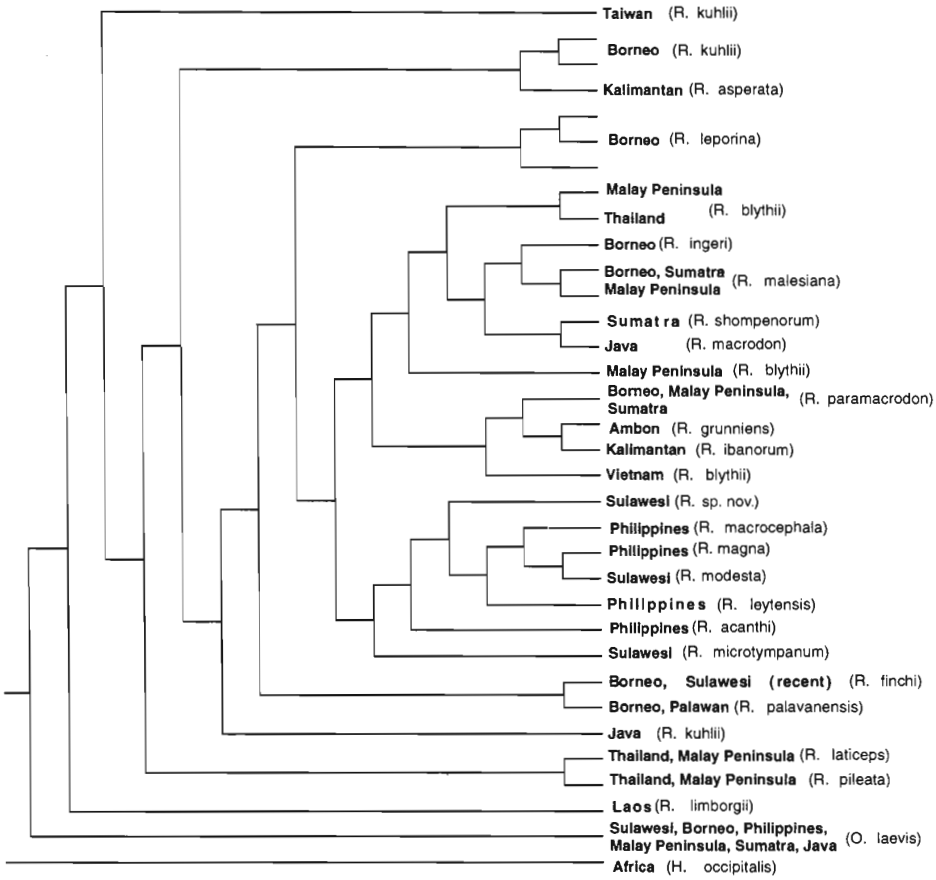

FIG. 4. The present-day geographical distribution of species of fanged frogs shown on the maximum-likelihood phylogenetic hypothesis in Fig. 2. (Taxon names are in parentheses).

day Indochina, Burma, and the Malay Peninsula was a continental area from early Eocene (ca. 50 Ma) onward. This Southeast Asian land mass included the southwestern two-thirds of Borneo at least from the early Eocene (ca. 50 Ma) into the Pliocene (ca. 5–10 Ma). Sumatra and Java were included in this continental area early in the Tertiary until the end of the Oligocene (ca. 50–25 Ma) and then again from late Miocene into the Pliocene (5 Ma). In the early Tertiary (ca. 50–30 Ma), no portion of the Philippines was emergent; the Palawan–Mindoro chain was relatively close to the southern coast of China and the remainder of the Philippines was much farther east and south of its present position. In the early Eocene (ca. 50 Ma) Sulawesi did not exist as an assemblage of peninsulas; only a portion of West Sulawesi was emergent and probably connected with eastern Borneo at this time (Moss and Wilson, 1998). Block faulting and subsidence in the middle Eocene created the Makassar Strait (Moss and Wilson, 1998) that has continued to separate Borneo and Sulawesi until the present.

In the middle Miocene (15 Ma) Sumatra and Java were inundated by shallow seas and reduced to a string of small volcanic islands. At the same time, the several parts of Sulawesi were nearly assembled into a partly emergent land area. Palawan–Mindoro had moved much closer to its present position but still a bit to the north and was adjacent to or part of a narrow emergent area; the Luzon block had shifted northward almost to its present position but was not emergent; the remainder of the Philippines and the Moluccas were still far to the east of their present positions and not emergent. However, a string of volcanoes apparently extended northeastward from the tip of Borneo and the eastern tip of Sulawesi, the precursors of the Sulu and Talaud archipelagoes, respectively. During the late Miocene (10 Ma), the central and southern Philippine Islands were close to the northeastern part of Sulawesi and included portions of emergent land.

By the early Pliocene (5 Ma), most islands in the Philippines had reached their present positions, though emergent portions were probably smaller than

now. The area of the South China Sea had expanded in the Pliocene, narrowing the connection among the Malay Peninsula, Sumatra, and Borneo. Hall (1998) cautions that his estimates of emergent land areas are probabilistic statements; some areas that he suggests were emergent may have been shallow seas and vice versa.

During the Pleistocene broader land connections among the Malay Peninsula, Borneo, Sumatra, and Java and among some islands of the Philippines were established at intervals coinciding with periods of northern glaciation (Molengraaff and Weber, 1921), with the last such connections ending less than 20 Ka. Although the gap between Borneo and Sulawesi was narrowed during the Pleistocene, it was apparently never eliminated. The gap between Borneo and the Palawan chain may have been eliminated at times in the Pleistocene. In summary, there clearly were a number of times during the Cenozoic when exchange of terrestrial faunas between the continent and its fringing islands would have been possible, provided that climate, vegetation, and topography were conducive to dispersal.

The present distribution of *Limnonectes* includes both seasonal and essentially aseasonal climates. Presumably from the Oligocene through the late Miocene, when the area connecting Indochina and the Malay Peninsula with Borneo was relatively broad, the climate of this expanded continent may have been more strongly seasonal than at present, resembling more the climate of northeastern Thailand and Vietnam than the present climate of Sumatra, Borneo, and the Malay Peninsula. If this is correct, either evergreen or seasonally deciduous forest should have existed. There is no evidence suggesting semiarid grassland. Consequently, movement of forest-dwelling *Limnonectes* between, say, Indochina and the Malay Peninsula, on the one hand, and Borneo or Sumatra, on the other, should have been possible during the mid-Tertiary and at several intervals during the Pleistocene.

One hypothesis concerning the timing and pattern of phyletic divergences and speciation events for this lineage of frogs is that the events occurred during the Pleistocene. Fluctuations in sea level and land connections among the major land masses during the Pleistocene provided both periods of isolation fostering genetic divergence and periods of broad terrestrial interconnections allowing dispersal of newly differentiated species to other land areas (Heany, 1991). Nonetheless, this hypothesis seems unlikely for several reasons. The varying levels of genetic distance separating pairs of species of fanged frogs suggest variation in the timing of divergence. The genetic distances themselves, ranging in value from 6–20%, suggest divergence times that would substantially predate the Pleistocene. Additionally, the hypothesis requires either (a) that this very successful lineage invaded Southeast Asia and Sundaland no earlier than the Pliocene or (b) that, if present in the area long before the Pliocene, it did not experience significant genetic subdivision during previous periods, such as the Miocene, when parts of the area were fragmented and isolated. Alternative (a) seems at odds with the fact that most species of *Limnonectes* occur in Southeast Asia and Sundaland. If fragmentation of land area was the driving mechanism for speciation in the Pleistocene, it should have had the same effect in pre-Pleistocene times, making alternative (b) untenable. We propose therefore that the divergences within this lineage occurred prior to the Pleistocene.

A conventional calibration for evolutionary rate of change for endothermic animal mtDNA is about 2% sequence divergence per million years (Avise, 1994), assuming a constant rate of change. Recent work suggests that the pattern of evolution of ribosomal DNA in frogs is dramatically slower, perhaps as little as 2% sequence divergence per nine million years (Graybeal, 1997). The genetic distance between the basal fanged frogs and their sister outgroup is around 20%. The log-likelihood test indicates that in the case of the fanged frogs the assumption of constancy of rate change is violated, and it is inappropriate to apply a standard divergence rate across taxa. Nonetheless, the 20% sequence divergence does lend support for the possibility that the fanged frog clade may have originated as much as 50 or more million years ago. This approximate timing is also consistent with the available geological data.

Free faunal exchange would have been possible among Southeast Asia, Borneo, Sumatra, and Java in the early Tertiary and as late as late Oligocene (25 Ma) (Hall, 1998). We might therefore assume the presence of ancestral stock in all the above areas. The more basal species of fanged frogs still show that distribution pattern, ranging from Indochina through the Malay Peninsula, Borneo, Sumatra, and Java (Inger and Tan, 1996).

The postulated connection between west Sulawesi and eastern Borneo during the early Eocene and the subsequent continuous separation of these two areas beginning in the middle Eocene (ca 42 Ma) (Moss and Wilson, 1998) suggest both early entry into Sulawesi and long isolation. Long-duration isolation in Sulawesi is also supported by additional observations: (1) at least seven endemic species of *Limnonectes* occur in Sulawesi and they show a wide diversity of behavior and size (Iskandar and Tjan, 1996) and (2) no species of *Limnonectes* occurs in both Sulawesi and Borneo (Inger and Tan, 1996; Inger, 1999) (*L. finchi* in Sulawesi is thought to be a recent invasion). The speciation of *Limnonectes* in Sulawesi is plausible, given Sulawesi's Tertiary history of frequent fragmentation and consolidation (Moss and Wilson, 1998).

The relatively close approximation of the Philip-

pines with Sulawesi in the late Miocene may explain why the species from these two areas form species group 3. On the other hand, *L. acanthi* is a geographically anomalous member of species group 3; it occurs in Palawan, whose amphibian species distribution has a much stronger similarity to Borneo than to the remainder of the Philippines (Inger, 1999). At times, Palawan has been geographically as close or closer to Borneo than to the rest of the Philippines (Inger, 1999).

Populations of the Bornean form *L. leporina* (species group 2, Fig. 1 of this paper and "Bornean *Rana blythi*" in Emerson and Ward, 1998) are distinct both morphologically (R. F. Inger, D. Iskandar, and S. B. Emerson, unpublished) and genetically (Emerson and Ward, 1998) from species groups 3 and 4. The divergence of this group from the remainder may have occurred in the early or middle Miocene (15–20 Ma), when Sumatra and Java were isolated from Borneo and the Malay Peninsula. However, as the last two land masses were apparently broadly connected during the Miocene, the unanswered question is: By what means did *L. leporina* become genetically divergent from its continental relative? Little is known of the topography of the Malay Peninsula/Borneo area of that time, although Hall's (1998) maps show highland regions in Borneo. If the land between what is now Borneo and the Malay Peninsula was low-lying and flat, it could have constituted a barrier to gene flow. The geographic fragmentation and isolation of Sumatra and Java in the Miocene (15–20 Ma) could have provided an opportunity for local differentiation and speciation of the *L. blythii* populations in those two areas. The divergence of *L. shompenorum* (Fig. 1) and *L. macrodon* could date from this period.

The species whose present distributions most strongly indicate Pleistocene movements are *L. malesiana* and *L. paramacrodon.* Both occur in Borneo, Sumatra, and the Malay Peninsula in peat swamps and other low-lying, flat forests. Habitat characteristics of these species would have enabled them to disperse through the relatively flat areas of the South China Sea emergent at intervals during the Pleistocene.

This study provides the most complete analysis to date of the relationships among species of a diverse and interesting group of Southeast Asian ranids. The group, properly called *Limnonectes,* appears to have arisen in the Tertiary at a time when free faunal exchange was possible among Southeast Asia, Borneo, Sumatra, Java, and, probably, Sulawesi. There are four species groups that are tentatively identified within the clade. Nonetheless, many questions about the systematics of *Limnonectes* remain unanswered. Future research encompassing an increased number of taxa and longer molecular sequences is necessary to clarify, even further, the relationships among the fanged frogs.

APPENDIX 1

Locality Data

Hoplobatrachus occipitalis (FMNH 257224—Field Museum of Natural History). Ivory Coast, Comoe National Park.

Occidozyga laevis (SBE 072—University of Malaya). Malaysia, Selangor Dist., Gombak Field Study Centre.

Rana acanthi (TNHC 54922—Texas Natural History Museum). The Philippines, Mindoro Island, Midoro Oriental Province, Tamarau Falls.

Rana asperata (FMNH 252416—Field Museum of Natural History). Indonesia, S. Kalimantan, Palangkaraya.

Rana blythii Vietnam (ROM25130—Royal Ontario Museum). Vietnam, Gia Lai Province, Ankhe Dist., Buoenloy. Malaysia (ZRC1.3571—Zoological Reference Collection at the National University of Singapore). Malaysia, Pahang; base camp at Kuala Jasin and Marong River, Endau Rompin Park. (SBE066—no voucher specimen). Malaysia, Selangor Dist., Gombak Field Study Centre. Thailand (FMNH 950701—Field Museum of Natural History). Thailand, Uthai Thani, Huai Kha Khang Wildlife Sanctuary.

Rana sp. nov. = *R. duboisi* (Emerson and Ward, 1998) (Museum Zoologicum Bogoriense). Indonesia, C. Sulawesi, Kamarora, Lore Lindu National Park.

Rana finchi (FMNH 242870—Field Museum of Natural History). Malaysia, Sabah, Sipitang Dist., Medolong, km 6.8.

Rana grunniens (Museum Zoologicum Bogoriense). Indonesia, Haruku, Saparua.

Rana ibanorum (FMNH 251721—Field Museum of Natural History). Indonesia, West Kalimantan, Bentuang Karimum National Park.

Rana ingeri (FMNH 251722—Field Museum of Natural History). Malaysia, Sarawak, 7th Division, Belaga Dist., Wong Tebah Camp, Sg. Pejuan.

Rana kuhlii Brunei (FMNH 248357—Field Museum of Natural History). Brunei, Belait Dist., Labi, Mendaram. Malaysia (FMNH 230302—Field Museum of Natural History). Malaysia, Sabah, Lahad Datu Dist., Danum Valley Research Centre. Taiwan (FMNH 257133—Field Museum of Natural History). Taiwan, Wulai. Java (Museum Zoologicum Bogoriense). Indonesia, Java, Cibodas, Mt. Gede.

Rana laticeps (SBE 071—University of Malaya). Malaysia, Selangor Dist., Gombak Field Study Centre.

Rana leporina Brunei (FMNH 248228—Field Museum of Natural History). Brunei, Tutong Dist., Tasek Merimbun, Sg. Merimbun. Malaysia (FMNH 230212—Field Museum of Natural History). Malaysia, Sabah, Lahad Datu Dist., Danum Valley Research Centre.

Indonesia (DJI S 18—Museum Zoologicum Bogoriense). Indonesia, S. Kalimantan, Barito Ulu, Mentaya Hulu.

Rana leytensis (USNM Field No. 222546—National Museum of Natural History). Philippines, Samar Island, Samar province, Bagacay Mine, Bagacay. One third the distance across the trans-island road connecting Wright and Taft.

Rana limborgii (FMNH 255382—Field Museum of Natural History). Laos, Khammouane Province, Nakai Dist., Phou Hin Poun National Biodiversity Conservation Area.

Rana limnocharis China (HKV 37049—Field Museum of Natural History). China, Sichuan, Hongya Xian, Bing Ling. Malaysia (SBE 098—Sabah Museum of Natural History). Sabah, Kota Kinabalu.

Rana macrocephala (FS054563—National Museum of Natural History). Philippines, Luzon, Cagayan Province, Baggao Municipality, Barrio Via, hot springs area of Intal River.

Rana macrodon (FMNH 257159—Field Museum of Natural History). Indonesia, West Java, Tarogong, Garut.

Rana magna (USNM222570—National Museum of Natural History). Philippines, Samar Island, Samar Province, Bagacay Mine, Bagacay.

Rana malesiana Indonesia (Museum Zoologicum Bogoriense). Indonesia, S. Kalimantan, Mentaya Hulu. Malaysia (RFI 49534—University of Malaysia, Sarawak). Malaysia, Sarawak, Samarahan Div., Dimunjan Dist., Simunjan Forest Reserve.

Rana microtympanum (Museum Zoologicum Bogoriense). Indonesia, S. W. Sulawesi, Loka.

Rana modesta (Museum Zoologicum Bogoriense). Indonesia, North Sulawesi, Manado.

Rana palavanensis (FMNH 230800—Field Museum of Natural History). Malaysia, Sabah, Lahad Datu Dist., Danum Valley Research Centre.

Rana paramacrodon (FMNH 248283—Field Museum of Natural History). Brunei, Tutong Dist., Tasek Merimbun, Sg. Merimbun.

Rana pileata (PWRC 002—Phuluang Wildlife Research Centre Museum). Thailand, Loei, Phuluang Wildlife Research Centre.

Rana shompenorum (DJI S18—Museum Zoologicum Bogoriense). Indonesia, West Sumatra, Malibou Anai, Anai Valley, 60 km from Padang.

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