

TAXONOMIC CHAOS IN ASIAN RANID FROGS: AN INITIAL PHYLOGENETIC RESOLUTION

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The taxonomy of ranid frogs is in a state of chaos, and Asian ranids are no exception. We undertook an investigation of the phylogenetic relationships of most major groups of Asian ranids using mitochondrial DNA sequences from the 12S, tRNA^{Val} and 16S genes. The resulting phylogenetic hypothesis had varying correspondence with the current taxonomy of the frogs at the subfamilial and generic levels. In order to maintain a taxonomy that reflects phylogenetic history, a number of taxonomic changes are proposed. Within subfamily Raninae, we recognize the genera *Rana*, *Amolops*, *Hylarana*, *Odorrana* and *Nidirana*. Recognition of *Huia* is not supported by our data and the recognition of *Pseudorana* is equivocal. Tribe Limnonectini is elevated to subfamily Limnonectinae and it contains *Limnonectes*, *Hoplobatrachus* and *Nanorana*. Membership in Genus *Limnonectes* is redefined. Recognition of genera *Paa* and *Chaparana* results in a paraphyletic taxonomy.

Key words: Anura, Asia, mtDNA, phylogeny, Ranidae

INTRODUCTION

For many years following the major revisions of Boulenger (1882, 1918, 1920), the taxonomy of ranid frogs was stable. Now it is in a state of chaos. Numerous generic and subgeneric shifts have been proposed, usually without an examination of phylogenetic relationships. Dubois (1986 (1987)) recognized six tribes within the Raninae (=Ranidae by most authorities). Among ranids, his Ranini included the genera *Altirana*, *Amolops*, *Batrachylodes*, *Micrixalus*, *Nanorana*, *Stauroids*, and *Rana*, with the subgenera *Ametia*, *Hylarana*, *Paa*, and *Strongylopus*, and *Rana*. Dubois' Tomopternini only included the genus *Tomopterna*. The tribe Ptychadenini had *Ptychadena* and *Hildebrandtia*. His Dicroglossini contained *Ceratobatrachus*, *Conraua*, *Discodeles*, *Limnonectes* (with five subgenera: *Limnonectes*, *Bourretia*, *Fejervarya*, *Hoplobatrachus*, and *Taylorana*), *Occidozyga* (with two subgenera: *Occidozyga* and *Euphlyctis*), *Palmatorappia*, *Phrynoglossus*, *Platymantis*, and *Ingerana* (with *Ingerana* and *Liurana* as subgenera). The fifth tribe, Pyxicephalini, included *Pyxicephalus*. Finally, tribe Ranixalini had *Ranixalus*, *Nannophrys*, and *Nyctibatrachus*.

Higher taxonomy has continued to change. Dubois (1992) raised the tribe Dicroglossini to the subfamily level, Dicroglossinae. He recognized four tribes in this

subfamily: (1) Ceratobatrachini (*Ceratobatrachus*, *Discodeles*, *Ingerana*, *Palmatorappia*, *Platymantis*, and *Taylorana*), (2) Conrauaiini (*Conraua*), (3) Dicroglossini (*Euphlyctis*, *Occidozyga*, and *Phrynoglossus*), and (4) Limnonectini (*Hoplobatrachus* and *Limnonectes*). These arrangements have been controversial. Inger (1996) noted that tribe Limnonectini was demonstrably paraphyletic with respect to the Ceratobatrachini, Conrauiini, and Dicroglossini. No evidence supported the monophyly of Limnonectini. It may be paraphyletic with respect to the Mantellinae and through the mantellines to the Rhacophoridae. Laurent (1951, 1979) and Ford (1993) questioned the monophyly of the Ranidae (*sensu stricto*) with respect to the family Rhacophoridae.

Recently, Chinese authorities have proposed numerous other changes, in particular generic reallocations. Fei *et al.* (1990 [1991]) described or erected a number of new generic combinations for many Chinese species. Further generic changes were made by Ye *et al.* (1993) and Fei (1999). These changes were made in the absence of a phylogenetic evaluation. Thus, we undertook an investigation of ranid relationships, particularly for representative south-east Asian genera and species. When we initiated this study, no phylogenetic evaluation of the group had been attempted at a higher taxonomic level, although one distance-based evaluation had been made (Wallace *et al.*, 1973). Subsequently, four other phylogenetic studies have reported on the relationships of ranid frogs, as discussed below.

TABLE 1. Primers used for amplifying and sequencing fragments of RNA genes in the subfamily Raninae. Sequence position indicates the starting position of the primer in the *Xenopus laevis* genome and is preceded by the amplification direction as indicated by (H) heavy or (L) light strand.

Name	Sequence 5' to 3'	Sequence position	Reference
12S1L	CAAACCTGGGATTAGATACCCCACTAT	L2484	Kocher <i>et al.</i> (1989)
12S2H	AGGGTGACGGGCGGTGTGT	H2897	Kocher <i>et al.</i> (1989)
12S2L	ACACACCGCCCGTCACCCTC	L2917	Fu (1999)
16S3H	GTAGCTCACTTGATTTCCGGG	H3341	Fu (pers. comm.)
16S3L	CCCGAAATCAAGTGAGCTAC	L3362	Fu (pers. comm.)
16S1H	GGCTATGTTTTTGGTAAACAG	H3958	Modified from Palumbi (1996)
16S5H	CTACCTTTGCACGGTTAGGATACCGCGGC	H4040	Fu (2000)
16S1M	CCGACTGTTTACCAAAAACAT	L3955	Fu (1998)
16S2H	CCGGATCCCCGGCCGGTCTGAACTCAGATCAGC	H4552	Palumbi (1996)

MATERIALS AND METHODS

SPECIMENS EXAMINED

Forty-five individuals, most of them south-east Asian ranines, were sequenced for three mitochondrial DNA genes. Additional sequence data from GenBank were used for the following species: *Rana pipiens* (X86247, X86318), *R. catesbeiana* (M57572), *R. temporaria* (Y11977), and *Xenopus laevis* (M10217). We used *X. laevis* as our initial outgroup taxon, and included an Asian treefrog (family Rhacophoridae, subfamily Rhacophorinae), *Polypedates megacephalus* (AF026350, AF026367), and an African mantelline (family Rhacophoridae, subfamily Mantellinae), *Laliostoma labrosum* (AF026354, AF026374), in our study to evaluate the monophyly of the family Ranidae as questioned by Laurent (1951, 1979) and Ford (1993). GenBank accession numbers, collection locality and voucher data for specimens sequenced in this study are given in an electronic Appendix downloadable from the Journal's website (<http://biology.bangor.ac.uk/~bss166/HJ/>). These tissue samples and most voucher specimens are preserved in the Royal Ontario Museum (ROM), or in the tissue collections of Jinzhong Fu (JF) and James P. Bogart (JPB) (Department of Zoology, University of Guelph). We also incorporated sequences from an analysis of fanged ranids (Emerson & Ward, 1998). These species include *Limnectes acanthi* (U66120-21), several populations of *L. blythii* (U55262-3, U55269-70, U66114-15, U66126-27, U66130-31, U66134-37), *L. grunniens* (U66124-25), *L. ibanorum* (U66122-23), *L. ingeri* (U55268, U55275), *L. limnocharis* (U55265, U55272), *L. macrocephala* (66116-17), *L. macrodon* (U66132-33), *L. magna* (U66118-19), *L. paramacrodon* (U55267, U55274), *Limnectes* sp. ("duboisii," a nomen nudum; Dubois, 1999) (U66112-13), and *Occidozyga laevis* (U66138-39). Taxonomic assignment of examined species generally follows Frost (2004).

DNA AMPLIFICATION AND SEQUENCING

Three ribosomal RNA genes, 12S, 16S, and tRNA^{Val} from the mitochondrial genome were selected to reconstruct the phylogeny. Total genomic DNA was extracted from frozen or alcohol preserved tissue sam-

ples of muscle or liver by digestion with proteinase K for 5-12 hr, and purified three times with phenol-chloroform-isoamyl alcohol (PCI), and then once with chloroform-isoamyl alcohol (CI). The mtDNA region of 12S through 16S was sequenced using the following method. Double stranded fragments were amplified in 33 cycles of the polymerase chain reaction (PCR; 92°C for 30 sec, 45-55°C for 30 sec 72°C for 1.5 min) performed in 25 µl reactions. Annealing temperatures were changed from 45°C to 55°C as needed in order to improve the quality of PCR products. Usually, PCR reactions amplified the entire fragment from 12S1L to 16S2H. Subsequently, several internal primers were used for sequencing. Infrequently, amplification of the larger fragment was not possible and thus the following primers were used: 12S1L, 12S2H, 16S3H, 16S3L, 16S5H, 16SML and 16S2H (Table 1). After amplification, the 25 l product was separated by electrophoresis on an agarose gel and stained with ethidium bromide. The bands containing DNA were excised and the DNA was eluted using Gene Clean II kit (Bio101) and suspended in distilled, deionized water. The cleaned DNA was sequenced directly with Thermo Sequenase 33P-labeled terminator cycle sequencing kit (Amersham). Locations of the primers are shown in Fig 1.

The products of the sequence reactions were resolved in a polyacrylamide 7M urea gel that was then dried and visualized on autoradiograph films (Kodak) within 24-48 hr. A few sequences were resolved using an ABI 377 automated DNA sequencer using the manufacturer's protocols.

DNA SEQUENCE ANALYSIS

Sequences were initially aligned using ClustalW (Thompson *et al.*, 1994) with gap-open and gap-exten-

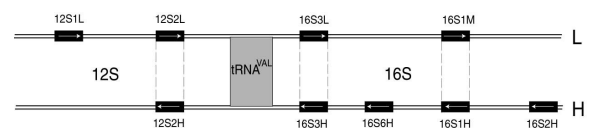


FIG 1. Schematic drawing of the 12S, tRNA^{Val}, and 16S mitochondrial gene and the relative positions of the primers used in this study. Hatched lines refer to primers that are complements to each other; L and H denote light and heavy strands, respectively. Specific primers are listed in Table 1.

TABLE 2. Summary of RNA genes sequenced from the ingroup and outgroup taxa. NT = Total number of taxa analyzed; TS = total number of homologous sites resolved; AS = number of ambiguous sites removed; NSR = number of homologous sites retained; NVS = number of variable sites; NPPIS = number of potentially phylogenetically informative sites; NMPTs = number of most parsimonious trees resolved; LMPTs = Length of most parsimonious solution; CI = consistency index; RI = retention index. Trees for the tRNA^{Val} gene were not calculated (n/a) owing to the limited number of characters (37) available to resolve nodes among the 52 taxa in the analysis.

Gene	NT	TS	AS	NSR	NVS	NPPIS	NMPTs	LMPTs	CI	RI
12S rRNA	53	546	29	517	335	236	6	1664	0.276	0.524
tRNA ^{Val}	52	72	2	70	48	37	n/a	n/a	n/a	n/a
16S rRNA	69	1509	25	1484	910	728	16	6279	0.237	0.530
All RNAs	70	2127	56	2071	1301	1012	3	8512	0.239	0.527

sion penalties set to 5. Subsequently, minor adjustments to the computer alignments were made by eye in BioEdit (ver. 5.0.9; Hall, 2001) and MacClade (ver. 4.0.5; Maddison & Maddison, 2002). Sites with ambiguous alignment were excluded from the phylogenetic analysis because the homology cannot be confidently assumed (Hillis *et al.*, 1991). Only potentially cladistically informative sites were maintained for the analysis in PAUP* (ver. 4.0b8a; Swofford, 2001).

All multistate characters were evaluated as unordered because there is no *a priori* reason to assume order of evolutionary change between nucleotide bases adenine (a), cytosine (c), guanine (g), or thymine (t) (Swofford *et al.*, 1996). The phylogenetic analysis using PAUP* employed an heuristic search, with random addition sequence, 500 replicates, retaining minimal trees only, using tree bisection reconnection branch swapping with steepest descent and collapsing zero length branches. The two genes were initially analyzed separately, because different genes may experience different evolutionary pathways. Second, a combined data analysis was conducted. Ratios of transitions to transversions were calculated in MacClade.

Nodal support was assessed for the combined data sets. Bootstrap proportions (BSP; Felsenstein, 1985) used 1000 replicates calculated in PAUP*. We also performed decay analyses (DI; Bremer, 1988) using AutoDecay (ver 4.0.2; Eriksson, 1999).

RESULTS

Forty-five specimens were sequenced for 12S through 16S RNA genes. In total, 546 sites were sequenced for 12S, 72 for tRNA^{Val}, and 1509 for 16S for a total of 2127 aligned sites. Among these sites, 56 were ambiguously aligned and 1012 were potentially phylogenetically informative (Table 2). All sequences were deposited in GenBank (12S = AF206072-AF206116; tRNA^{Val} = AF206117-206161; 16S rRNA = 206453-206497).

PARSIMONY EVALUATION

For 12S, analysis of the 236 potentially informative sites yielded six most parsimonious trees (MPTs, Table 2). We did not attempt a separate phylogenetic analysis of the tRNA^{Val} gene because there were too few potentially phylogenetically informative sites for a

meaningful analysis. For 16S, 728 potentially informative sites resulted in 16 cladograms, the differences constrained to one subclade. Because of similarities in nucleotide proportions and levels of site divergence, all RNA gene sequence data were combined for a total evidence analysis.

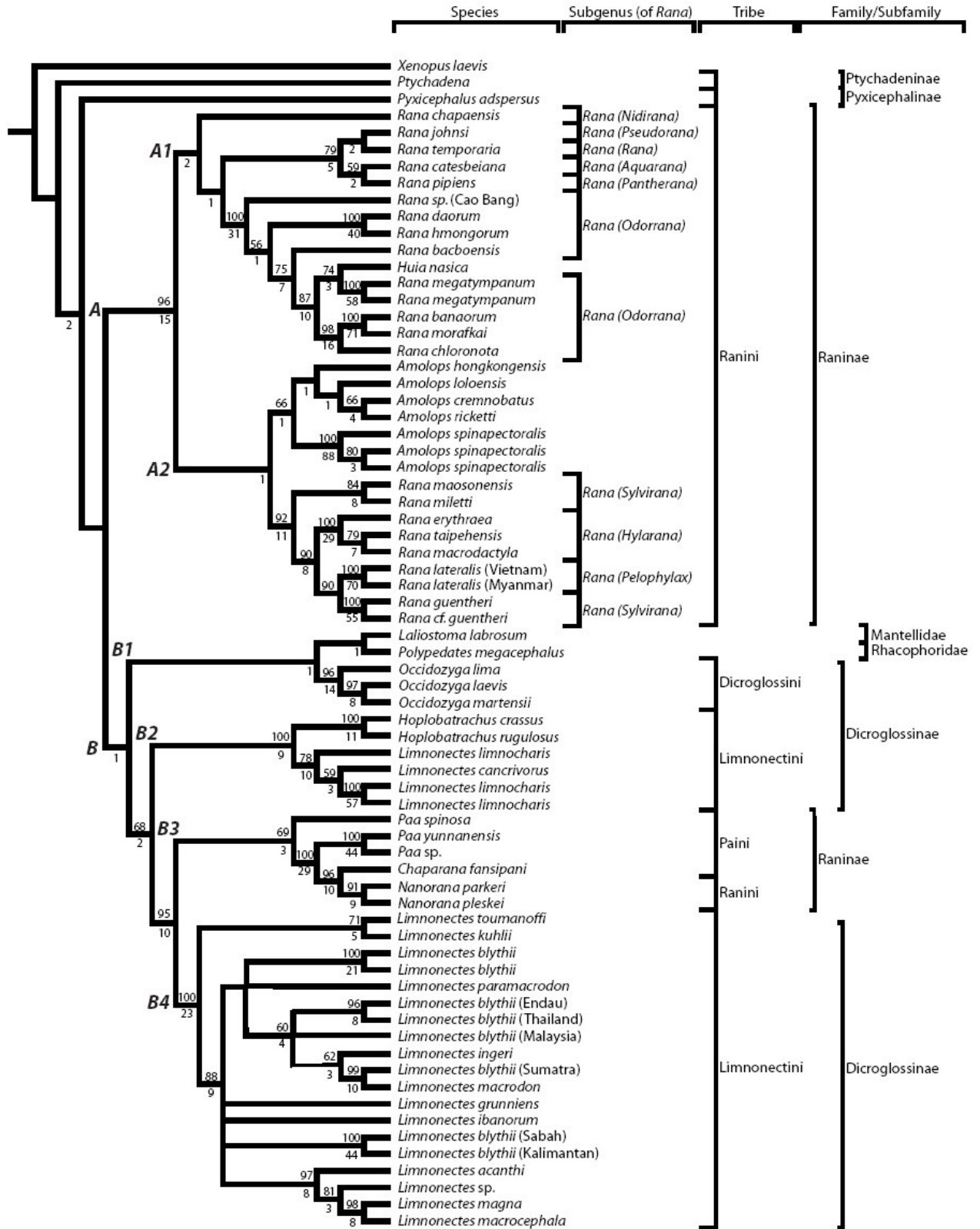
Combining all RNA gene sequence data into a single data set resulted in 1012 potentially cladistically informative characters. Analysis of these data yielded three most parsimonious trees (8512 steps in length, CI=0.24, RI=0.53). *Ptychadena* was resolved as the sister group of *Pyxicephalus* plus two major speciose clades (A and B) of *ranids* (Fig. 2): Clade A, included *Amolops*, *Hylarana*, *Nidirana*, *Odorrana*, and *Rana*; Clade B was composed of *Chaparana*, *Hoplobatrachus*, *Limnonectes*, *Nanorana*, *Occidozyga*, and *Paa*, plus a mantelline, *Laliostoma*, and rhacophorine, *Polypedates*.

Clade A was treated as having two major subclades, A1 and A2. *Nidirana chapaensis* was resolved as the sister group to all other members of clade A1, including specimens of *Odorrana* and *Rana*. *Rana (Pantherana) pipiens* was resolved as the sister species of *R. (Aquarana) catesbeiana*. Their sister group contained *R. (Pseudorana) johnsi* plus *R. (Rana) temporaria*. The clade containing *Rana* formed the sister group to a clade composed of *Amolops (Huia) nasica* and a paraphyletic *Odorrana*.

In Clade A2, a monophyletic *Amolops (Amolops)* was the sister group to a clade of *Rana* including subgenera *Hylarana* and *Pelophylax*, and a paraphyletic subgenus *Sylvirana*.

Clade B was treated as having four major subclades, B1-B4. In Clade B1, the mantelline, *Laliostoma labrosum*, and the rhacophorine, *Polypedates megacephalus*, were resolved as sister taxa. Together they formed the sister group of the three species of *Occidozyga*. Clade B1, in turn, was the sister group of clades B2-B4. Clade B2 consisted of *Hoplobatrachus crassus*, *H. rugulosus*, *Limnonectes cancrivorus*, and a paraphyletic *L. limnocharis*. It was the sister group of clades B3 and B4. Clade B3 contained *Paa* (as a paraphyletic taxon), *Chaparana* and *Nanorana*. The sister group of Clade B3 was B4, which consisted of *Limnonectes blythii* and its relatives, with *L. blythii* being resolved as paraphyletic (Fig. 2).

FIG 2. The strict consensus tree of the two most parsimonious explanations of mtDNA sequence data for south-east Asian ranids. *Xenopus laevis* was used to root the tree. Taxonomy reflects current usage. Taxonomic groupings proposed by Dubois, 1992 appear to the right of the tree. Numbers above the line are bootstrap proportions (50) and those below are Bremer decay indices examined up to six steps longer than the most parsimonious trees.



ASSESSING NODAL STABILITY

Values of nodal support are indicated on the tree (Fig. 2). Bootstrapping (BS) trials supported 43 nodes with a consistency greater than 70%. Decay analyses revealed that many nodes required a considerable number of additional steps to collapse, except those not generally supported by high BS proportions.

DISCUSSION

Because different portions of the mtDNA genome evolve at different rates, cladograms from different genes for the same set of organisms may differ. The relatively slowly evolving 12S and 16S rRNA genes seem appropriate for resolving older divergences, perhaps as old as 150 Ma (Mindell & Honeycutt, 1990).

The two mitochondrial genes evolved in similar ways. We assume that this conciliation is due to their phylogenetic history. Our phylogenetic analysis of the combined data resulted in three MPTs. Independent analyses for each gene revealed compatible branching patterns.

PREVIOUS STUDIES

The phylogenetic relationships of ranid frogs have been investigated in three recent molecular studies. Marmayou *et al.* (2000) evaluated a short, 305 bp segment of mtDNA 12S for 28 species of ranid and rhacophorid frogs using maximum parsimony, transversion weighting, and phenetic neighbour joining. Their unweighted parsimony evaluation resolved *Occidozyga* and *Phrynoglossus* as sister taxa, which together formed the sister group of all other ranids plus rhacophorids. The remaining taxa clustered into four groups whose relationships to each other were not resolved. Representative rhacophorids, including species of *Buergeria*, *Philautus*, *Polypedates* and *Chirixalus*, formed one monophyletic group. *Amolops* and *Rana chalconota* formed another group. The genera *Limnonectes*, *Fejervarya*, *Hoplobatrachus*, *Sphaerotecca*, and *Taylorana* formed a third clade and several species of *Rana* formed the fourth cluster. In this taxonomy, *Rana* was paraphyletic. Transversion weighting and the phenetic evaluation resolved paraphyly in *Philautus*, *Limnonectes*, and an additional example of paraphyly with respect to *Rana*. Given the small numbers of characters analyzed, it is not surprising that most nodes received low levels of branch support.

Bossuyt & Milinkovich (2000) evaluated 2692 bp of mitochondrial and nuclear homologous DNA sequence sites, excluding third position codon sites for cytochrome b. They constructed trees using maximum likelihood and BS consensus methods based on maximum parsimony. Because the initial outgroup was very divergent it was dropped from the analysis and Madagascan ranids and rhacophorids were used to root the network of Asian ranids and rhacophorids combined, and vice versa. Unfortunately, bootstrapping is problematic (Kluge & Wolf, 1993) and consensus methods

themselves have long been known to be suspicious (Miyamoto, 1985; Miyamoto & Fitch, 1995). Maximum likelihood analyses are philosophically problematic (Kluge, 1997; Siddall & Kluge, 1997). This puzzle is exemplified, in part, by "Brooks' conundrum" (D. R. Brooks, Univ. of Toronto, pers. comm., 2002): "Do you believe that evolution occurs in a most parsimonious manner? If not (which is demonstrably true-homoplasy exists), then why try to force a model of maximum parsimony on the analysis of your data, which is exactly what maximum likelihood does?" Maximum parsimony should be used as a criterion for selecting among all possible trees, and not as a model of evolution. Regardless, the basal relationships in the bootstrap consensus tree of Bossuyt and Milinkovich were unresolved. Asian treefrogs were monophyletic, as was a clade containing representative species of *Fejervarya*, *Hoplobatrachus*, *Nanophrys*, *Euphlyctis*, Asian *Tomopterna* (*Sphaerotecca*) and some *Limnonectes*.

Kosuch *et al.* (2001) investigated the monophyly of tiger frogs, *Hoplobatrachus*, which occur in both Asia and Africa. They evaluated 34 ranids using a total of 903 homologous nucleotide sites from 16SrRNA and 12SrRNA with 281 sites being potentially phylogenetically informative. Though their focus was on the biogeographical relationships of Asian and African *Hoplobatrachus*, representatives of *Fejervarya*, *Limnonectes*, *Nannophrys*, *Occidozyga*, *Phrynoglossus*, *Ptychadena* and several species of *Rana* were also included. Support was found for a monophyletic *Hoplobatrachus*, which was resolved as the sister group to *Fejervarya*. Subfamily Dicroglossinae was not resolved as a monophyletic grouping in either of the two trees presented.

More recently, Roelants *et al.* (2004) evaluated DNA sequences of several groups of ranid frogs, though their focus was on the biogeography of these frogs rather than taxonomy. The taxonomic implications of their study are summarized below.

PATTERNS OF RELATIONSHIPS

Although the relationships we resolved among the putative subfamilies of ranid frogs were not entirely consistent with previous taxonomies, lower taxonomic groupings were congruent in a number of ways with those proposed by Dubois (1986[1987], 1992). However, our analysis discovered several problematic associations. For example, the genus *Rana* was not resolved as a monophyletic taxon and *Limnonectes limnocharis* appears to be paraphyletic with respect to *L. cancrivorus*.

MONOPHYLY OF THE RANIDAE AND RELATIONSHIPS AMONG SUBFAMILIES

Family Ranidae was resolved as a paraphyletic taxon with respect to rhacophorids. Therefore, recognizing Family Rhacophoridae as a subfamily within family Ranidae, as suggested by Dubois (1992) and Blommers-Schlösser (1993), provides an acceptable solution.

Alternatively, in order to avoid having an extremely speciose Ranidae, multiple families could be recognized. The problem requires further investigation using sequences from more conserved genes and a broader array of taxa, especially African ranids and rhacophorids.

RANINAE, CLADE A

Clade A consisted of five potential genera of ranid frogs: *Amolops*, *Hylarana*, *Nidirana*, *Odorrana*, and *Rana*, although group membership did not mirror current taxonomy. These genera were distributed amongst two subclades (A1 and A2).

CLADE A1

Genus Rana (part), subgenus Nidirana: One species, *R. (Nidirana) chapaensis*, was used to represent this subgenus of *Rana*. It was resolved as the sister taxon to the following two subclades of Clade A1:

Genus Rana (part), subgenera Aquarana, Pantherana, Rana, and Pseudorana: One species each was used to represent four relatively speciose subgenera of *Rana*. The two North American species, *R. (Pantherana) pipiens* and *R. (Aquarana) catesbeiana*, were resolved as sister taxa. Their sister group contained the Asian species, *R. (Pseudorana) johnsi*, and its sister group represented by the European *R. (Rana) temporaria*.

Genus Rana, subgenus Odorrana: The group containing *Odorrana* and *Amolops (Huia)* forms the sister group to the clade containing *Rana catesbeiana* and *R. pipiens* plus *R. johnsi* and *R. temporaria*.

The large, odoriferous ranids sometimes referred to the genus *Odorrana* formed a paraphyletic lineage with respect to *Amolops (Huia) nasica*. *Amolops (Huia)* was resolved within a group of *Odorrana*, and not with other *Amolops* with which it is usually associated (Yang, 1991). *Amolops (Huia)* differs from *Odorrana* by its non-glandular skin and the absence of enlarged toe discs.

Our data also support the finding that *Odorrana chloronota* is a species complex (Murphy *et al.*, 1997; Bain *et al.*, 2003). As cryptic species are identified, the number of species of *Odorrana* will likely increase significantly.

In some regions, like the Khe Moi River, Nghe An Province, Vietnam, three large species of this clade occur in sympatry (Bain *et al.*, 2003). Some sympatric species are derived from distant lineages, such as the co occurrence of *O. chloronota* and a similar species, *O. bachoensis*. However, other sympatric species appear to be much more closely related, such as *O. chloronota* and *O. morafkai*. This pattern of sympatry repeats in most other areas in Vietnam, although the species composition changes.

CLADE A2

Genus Amolops, subgenus Amolops: the sampled species are monophyletic, and a larger survey of species

is currently underway. The two most anatomically similar species included in this analysis, *A. ricketti* and *A. torrentis*, formed a terminal sister relationship, followed basally by *A. loloensis*, and the geographically more distant, but anatomically similar *A. hongkongensis*. *Amolops spinapectoralis* was resolved as the sister group of these species. *Amolops* formed the sister group of the remaining subclade containing *Rana maasonensis* and *R. erythraea*.

Genus Rana, subgenera Hylarana, Pelophylax, and Sylvirana: this clade includes a paraphyletic assemblage of subgenera within the genus *Rana*. The association of subgenera is as follows: (*Sylvirana*((*Sylvirana*, *Pelophylax*)(*Hylarana*))).

RANINAE/RHACOPHORIDAE, CLADE B

The second major group of ranines contains relatively stocky, largely edible Asian frogs. Frogs within this clade belong to several genera, possibly reflecting, in part, their economic significance (and, hence, greater attention) and a greater amount of anatomical divergence. The frogs within clade B clustered into four serially arranged clades as follows: (B1, (B2, (B3, and B4))).

CLADE B1

Genera Occidozyga, Polypedates, and Laliostoma: The two representative rhacophorids, *Polypedates megacephalus*, a rhacophorine, and *Laliostoma labrosum*, a mantelline, were resolved as sister taxa. These taxa formed the sister group to a monophyletic *Occidozyga*. The sister group to this clade contains the microglossine frogs of the genera *Fejervarya*, *Hoplobatrachus*, and *Limnonectes*, separated by the ranine frogs *Chaparana*, *Nanorana*, and *Paa* (Fig. 2).

CLADE B2

Genera Hoplobatrachus, Limnonectes (part) and Fejervarya: This subclade, sometimes considered to be three genera, has been particularly problematic. Kosuch *et al.* (2001) examined the biogeographic relationships of *Hoplobatrachus*, and found a monophyletic *Hoplobatrachus* to be the sister group to *Fejervarya*. We also found a monophyletic *Hoplobatrachus*, with *H. crassus* plus *H. rugulosus* being the sister group to the remainder of the clade.

The rice frog, *L. limnocharis*, is resolved as paraphyletic with respect to *L. cancrivorus*. A considerable amount of allozyme work in other parts of its extensive range suggests that it is a composite of many cryptic species (e.g. Dubois, 1984; Toda *et al.*, 1994, 1998a,b). Our data and cladogram support this conclusion.

CLADE B3

Genera Paa, Chaparana, and Nanorana: This clade is a paraphyletic assemblage of genera. *Paa spinosa* is resolved as the sister group of a clade containing two other

species of *Paa* plus *Chaparana fansipani*, *Nanorana parkeri* and *N. pleskei*. Thus, the genus *Paa* is paraphyletic with respect to *Nanorana* and *C. fansipani*. The association of these species is particularly interesting, given that, though *Chaparana* and *Nanorana* are heavy-set, they are not large frogs like *Paa*. This association does not appear to be spurious since all nodes within this clade received substantial support. This clade, in turn, is resolved as the sister group to the remaining ranine clade.

CLADE B4

Genus *Limnonectes* (*part*): The third subclade of Asian edible frogs includes species placed in this genus. Within this clade, paraphyly is the rule rather than exception. Populations of *L. blythii* are variously associated with *L. macrodon*, *L. ingeri*, and *L. paramacrodon*. The clades have a greater correspondence to geographic location than taxonomy. Sister taxa co-occur on a single island. Some species appear to be large complexes of morphologically similar species. For example, Inger *et al.* (1999) noted several anatomical differences between *L. blythii* from the Malay Peninsula and southern Vietnam. Thus, as with *L. limnocharis*, the taxonomy of this group needs to be revised as it undoubtedly represents far more species than previously thought. Our arrangement differs from that of Roelants *et al.* (2004) who resolved this group as the sister of clade B2+B3. However, both studies found weak support at the conflicting nodes. Whereas we included 19 specimens, Roelants *et al.* (2004) sequenced two representatives.

TAXONOMIC IMPLICATIONS

Type species of Rana Linnaeus 1758: Before undertaking revisions, it is first necessary to establish the relationships of the type species of the genus *Rana*. Fleming (1822) designated *Rana temporaria Linnaeus, 1758* as the type species of *Rana*. This species is the name-bearer of the genus, subgenus, tribe, subfamily, and family. Genus *Rana sensu* Frost (2004) has more than 240 species divided into 22 subgenera. It is one of the most speciose groups of vertebrates and contains many independent lineages. Taxonomically, recognition of these major lineages as genera would better summarize their phylogenetic history.

Taxonomic chaos: At virtually every hierarchical level, taxonomic problems exist. For example, tribe Ranini is a paraphyletic assemblage of genera with respect to the genus *Rana* and at the subfamilial level with the genus *Nanorana* Günther 1869.

The taxonomy of these frogs has been unstable. Not exhaustive, Table 3 briefly summarizes some of the changes for Asian groups from 1985 onward for many of the species included in this study. For most species, placement in one group or another has remained relatively stable, but the taxonomic rank accorded to the groups has been quite unstable. For example, the crab-eating frog, *Limnonectes cancrivorus*, was placed in

Genus *Rana*, subgenus *Euphlyctis* Fitzinger 1843 by Frost (1985), then into genus *Limnonectes* Fitzinger 1843, subgenus *Hoplobatrachus* Peters 1863 by Dubois (1986 [1987]). Subsequently, it was assigned to genus *Euphlyctis* by Fei *et al.* (1990), then to genus *Hoplobatrachus*, subgenus *Fejervarya* Bolckay 1915 by Dubois (1992). Most recently, the species was placed in Genus *Fejervarya* (Fei, 1999). Yet others (e.g. Inger, 1996; Nguyễn & Ho, 1996; Zhao, 1994; Zhao & Adler, 1993) have left the species in the genus *Rana*. Much of this taxonomic instability is due to the absence of a reasonable phylogeny upon which to identify membership within particular clades.

A phylogenetically based taxonomy reflects the greatest amount of information within a hierarchical system (Farris, 1967; Wiley, 1980; Brooks & McLennan, 1991, 2002). Below, we review the taxonomy of these frogs and make taxonomic changes that directly reflect phylogenetic history, albeit conservatively.

Subfamily Dicroglossinae, tribe Dicroglossini: This group was represented by three of 12 species from the genus *Occidozyga* Kuhl et Hasselt 1882: *O. laevis* and *O. lima*, and *O. martensii*. *Occidozyga laevis* and *O. martensii* have been placed in the genus *Phrynoglossus* Peters 1867 by many authorities (e.g., Peters, 1867; Smith & Chasen, 1931; Taylor, 1962; Dubois, 1986 [1987]). Our data do not refute this placement but recognition of *Phrynoglossus* could result in a paraphyletic *Occidozyga*.

Subfamily Raninae: The subgenus *Nidirana* Dubois 1992 contains seven species: *R. (Nidirana) adenopleura*, *R. (N.) caldwelli*, *R. (N.) chapaensis*, *R. (N.) daunchina*, *R. (N.) lini*, *R. (N.) pleuraden*, and *R. (N.) psaltes*. It has been resolved as the sister taxon to the clade containing *Rana temporaria* plus *Odorrana* Fei Ye et Huang 1991. Within the genus *Rana*, *R. johnsi* forms the sister group of the type species, *R. temporaria* and the two North American representatives of the subgenera *Pantherana* Dubois 1992 and *Aquarana* Dubois 1992. Our data neither refute recognition of Fei's genus *Pseudorana* Fei Ye et Huang 1991 nor support it. Given the lack of additional specimens from this group and arguments for its rejection (Tanaka-Ueno *et al.*, 1998), we believe it preferable not to recognize *Pseudorana* until sufficient evidence exists.

Genus Nidirana Dubois 1992: In order to maintain recognition of genus *Odorrana* and not render genus *Rana* paraphyletic, subgenus *Nidirana* must be elevated to generic status for *N. adenopleura*, *N. caldwelli*, *N. chapaensis*, *N. daunchina*, *N. lini*, *N. pleuraden*, and *N. psaltes*.

Genus Hylarana Tschudi 1838: The genus *Rana* is paraphyletic with respect to *Amolops* Cope 1865. In order to maintain the genus *Amolops*, another ranine genus must be recognized. The group of ranids that form the sister group of *Amolops* contains the subgenera *Hylarana* Tschudi 1838, *Pelophylax* Fitzinger 1843, *Sylvirana* Dubois 1992, and *Tenuirana* Fei Ye et Huang 1991. On the basis of priority, we recognize genus

TABLE 3. A representative summary of the history of names applied to some of the Asian species of ranid frogs investigated in this study.

Specific epithet	Frost 1985	Dubois 1987 "1986"	Fei et al. 1991 "1990"	Dubois 1992	Ye et al. 1993	Fei 1999
<i>hongkongensis</i>	<i>Amolops</i>	-	<i>Amolops</i>	<i>A. (Amolops)</i>	<i>Amolops</i>	<i>Amolops</i>
<i>loloensis</i>	<i>Amolops</i>	-	<i>Amolops</i>	<i>A. (Amolops)</i>	<i>Amolops</i>	<i>Amolops</i>
<i>ricketti</i>	<i>Amolops</i>	-	<i>Amolops</i>	<i>A. (Amolops)</i>	<i>Amolops</i>	<i>Amolops</i>
<i>torrentis</i>	<i>Amolops</i>	-	-	<i>R. (Hylarana)</i>	-	-
<i>erythraea</i>	<i>R. (Hylarana)</i>	-	<i>H. (Hylarana)</i>	<i>R. (Sylvirana)</i>	<i>H. (Hylarana)</i>	<i>Hylarana</i>
<i>guentheri</i>	<i>R. (Hylarana)</i>	-	<i>Amolops</i>	<i>A. (Huia)</i>	-	<i>Amolops</i>
<i>nasica</i>	<i>Amolops</i>	-	<i>Pseudorana</i>	<i>R. (Pseudorana)</i>	<i>Pseudorana</i>	<i>Pseudorana</i>
<i>johnsi (as sauteri)</i>	<i>R. (Hylarana)</i>	-	<i>Odorrana</i>	<i>R. (Eburana)</i>	<i>Odorrana</i>	<i>Odorrana</i>
<i>livida</i>	<i>R. (Hylarana)</i>	-	<i>Hylarana (Tenuirana)</i>	<i>R. (Hylarana)</i>	<i>H. (Tenuirana)</i>	<i>H. (Tenuirana)</i>
<i>macrodactyla</i>	<i>R. (Hylarana)</i>	-	-	<i>R. (Sylvirana)</i>	-	-
<i>maosonensis</i>	<i>R. (Hylarana)</i>	-	-	<i>R. (Sylvirana)</i>	-	-
<i>milleti</i>	<i>R. (Hylarana)</i>	-	<i>Hylarana (Tenuirana)</i>	<i>R. (Hylarana)</i>	<i>H. (Tenuirana)</i>	<i>H. (Tenuirana)</i>
<i>taipehensis</i>	<i>R. (Hylarana)</i>	-	-	<i>R. (Pelophylax)</i>	-	-
<i>lateralis</i>	<i>Rana</i>	<i>Rana (Rana)</i>	-	<i>R. (Pantherana)</i>	-	-
<i>pipiens</i>	<i>R. (Rana)</i>	<i>R. (Rana)</i>	-	<i>R. (Pantherana)</i>	-	-
<i>catesbeiana</i>	<i>R. (Rana)</i>	<i>R. (Rana)</i>	-	<i>R. (Aguarana)</i>	<i>R. (Aguarana)</i>	<i>R. (Aguarana)</i>
<i>fansipani</i>	<i>Rana (Paa?)</i>	-	-	<i>C. (Chaparana)</i>	-	-
<i>kuhlii</i>	<i>R. (Limnonectes)</i>	<i>L. (Limnonectes)</i>	<i>Limnonectes</i>	<i>L. (Limnonectes)</i>	<i>Limnonectes</i>	<i>Limnonectes</i>
<i>toumanoffi</i>	<i>R. (Euphlyctis)</i>	<i>L. (Bourretia)</i>	-	<i>L. (Bourretia)</i>	-	-
<i>blythii</i>	<i>R. (Euphlyctis)</i>	<i>L. (Limnonectes)</i>	-	<i>L. (Limnonectes)</i>	-	-
<i>rugulosus</i>	<i>R. (Euphlyctis)</i>	<i>L. (Hoplobatrachus)</i>	<i>Tigrina</i>	<i>Hoplobatrachus</i>	<i>Hoplobatrachus</i>	<i>Hoplobatrachus</i>
<i>cancrivorus</i>	<i>R. (Euphlyctis)</i>	<i>L. (Hoplobatrachus)</i>	<i>Euphlyctis</i>	<i>L. (Fejervarya)</i>	<i>Euphlyctis</i>	<i>Fejervarya</i>
<i>limnocharis</i>	<i>R. (Euphlyctis)</i>	<i>L. (Fejervarya)</i>	<i>Euphlyctis</i>	<i>L. (Fejervarya)</i>	<i>Euphlyctis</i>	<i>Fejervarya</i>
<i>labrosa</i>	<i>Tomopterna</i>	<i>T. (Sphaerotecla)</i>	-	<i>T. (Sphaerotecla)</i>	-	-
<i>yunnanensis</i>	<i>R. (Paa) phrynoides</i>	<i>R. (Paa)</i>	<i>Paa (Paa) phrynoides</i>	<i>Paa (Gyandropaa)</i>	<i>Paa (Paa)</i>	<i>Paa (Paa)</i>
<i>parkeri</i>	<i>Altirana</i>	-	<i>Altirana</i>	<i>N. (Altirana)</i>	<i>Altirana</i>	<i>Nanorana</i>
<i>pleskei</i>	<i>Nanorana</i>	-	<i>Nanorana</i>	<i>N. (Nanorana)</i>	<i>Nanorana</i>	<i>Nanorana</i>
<i>chapaensis</i>	<i>Rana (Hylarana)</i>	-	-	<i>R. (Nidirana)</i>	-	-
<i>spinosa</i>	<i>R. (Paa)</i>	<i>R. (Paa)</i>	<i>Paa</i>	<i>Paa (Quasipaa)</i>	<i>R. (Paa)</i>	<i>Paa (Paa)</i>
<i>laevis</i>	<i>Occidozyga</i>	<i>Occidozyga</i>	-	-	-	-
<i>lima</i>	<i>Occidozyga</i>	<i>Phrynoglossus</i>	-	-	-	-

Hylarana. It contains those species associated with the subgenera *Hylarana*, *Sylvirana*, *Tenuirana*, and *Pelophylax*. The type species of *Hylarana*, *H. erythraea*, was included in our evaluation.

Recognition of the subgenera within *Hylarana* requires a phylogeny and the current taxonomy results in paraphyletic groupings (Fig. 2). For example, Dubois (1992) included *H. guentheri*, *H. maosonensis* and *H. milleti* in genus *Rana*, subgenus *Sylvirana*. However, whereas *H. guentheri* is the sister group of subgenus *Pelophylax*, *R. maosonensis* plus *R. milleti* is the sister group of the clade containing *R. guentheri* (subgenus *Sylvirana* in part), subgenus *Hylarana*, and subgenus *Pelophylax*.

The subgenus *Tenuirana* is also a puzzle. *Tenuirana* contains only *R. taipehensis* and *R. macrodactyla*. Although these two species are sister taxa, recognition of this subgenus results in the paraphyly of other subgenera. Thus, *Tenuirana* should not be elevated to generic status as it leaves *Hylarana* a paraphyletic taxon.

Given the large number of species in *Hylarana*, the apparent polyphyly within the subgenus *Sylvirana*, and the problems surrounding the recognition of *Tenuirana*, recognition of these or any other subgenera or genera is premature in the absence of a more complete phylogeny.

GENUS RANA LINNAEUS 1758

Rana temporaria is a member of the clade consisting of *R. johnsi* and the American frogs, *R. catesbeiana* and *R. pipiens*. Dubois (1992) included *R. johnsi* (as *R. sauteri*) in the subgenus *Pseudorana*, and *R. pipiens* in subgenus *Pantherana*. He placed *R. temporaria* in subgenus *Rana*, and *R. catesbeiana* in subgenus *Aquarana*. This subgeneric arrangement is phylogenetically acceptable from the perspective of our data. Taxonomically, these species have been closely associated with one another.

Genera Odorrana Fei Ye et Huang 1991 and Huia Yang 1991: The usually large, odoriferous frogs referred to the genus *Odorrana* are the sister group to *Rana*. The type species for the genus *Odorrana* is *Rana margaretae* Liu, 1950 by original designation. Unfortunately, we did not have tissue samples from this species and no sequences exist in GenBank. Nevertheless, for the moment, we recognize genus *Odorrana* and include within it *O. bacboensis*, *O. banaorum*, *O. chloronota*, *O. daorum*, *O. hmongorum*, *O. megatympnum*, *O. morafkai*, and *O. nasica*. This list of species is not exclusive and at least 13 additional species could belong to the genus, including: *O. andersonii*, *O. anlungensis*, *O. exiliversabilis*, *O. grahami*, *O. hainanensis*, *O. jingdongensis*, *O. huangwuensis*, *O. livida*, *O. lungshengensis*, *O. margaretae*, *O. nasuta*, *O. schmackeri*, and *O. swinhoana*.

Genus Amolops Cope 1865: Few have questioned the validity or membership of genus *Amolops*, though our data reveal that *Amolops (Huia) nasica* occurs within the clade containing *Odorrana chloronota*. Con-

sequently, membership in one genus or another may be uncertain for many of the larger species referred to as either *Amolops (Huia)* or *Odorrana* (see above).

Subfamily Limnonectinae (new content/combination): Dubois placed genus *Paa* Dubois 1975 in subfamily Raninae, tribe Paini. However, subfamily Raninae is a paraphyletic group. Consequently, tribe Paini must be moved from subfamily Raninae and placed in subfamily Dicroglossinae, tribe Limnonectini along with the genera *Hoplobatrachus* and *Limnonectes*. However, doing so still leaves subfamily Dicroglossinae a paraphyletic group with respect to the Rhacophorinae and Mantellinae. Thus, to avoid paraphyly, tribe Limnonectini must be elevated to subfamily Limnonectinae. Recognition of the families Rhacophoridae and Mantellidae will necessitate recognition of the family Limnonectidae.

Limnonectinae has three distinctive lineages (Fig. 3). One lineage contains genus *Hoplobatrachus* and some species of genus *Limnonectes* referred to genus *Fejervarya* by Fei (1999). These frogs are placed in the tribe Hoplobatrachini (new combination). Another lineage, tribe Paini, contains the genera *Chaparana* Bourret 1939, *Nanorana* and *Paa* (but see below). Finally, tribe Limnonectini contains genus *Limnonectes* excluding those species previously referred to *Fejervarya*.

Genus Hoplobatrachus Peters 1863: This genus was represented by the species *H. crassus* and *H. rugulosus*. Kosuch *et al.* (2001) found this genus to be monophyletic. Our data support their conclusion.

Genus Fejervarya Bolckay 1915: *Fejervarya* is represented, in this clade, by the two species *F. limnocharis* and *F. cancrivora*. However, *F. limnocharis* is paraphyletic with respect to *F. cancrivora*. Though both species are generally assigned to genus *Limnonectes*, Fei (1999) included both species in the genus *Fejervarya*. Our data and cladogram support this conclusion.

Genera Chaparana Bourret 1939, Nanorana Günther 1896, and Paa Dubois 1975: The subclade containing *Paa* also contains members of the genera *Chaparana* and *Nanorana*. The genus *Paa* contains more than 29 species (Frost, 2004) of which two were included in our study plus one undescribed species. The genus is paraphyletic. The genera *Nanorana* and *Chaparana* fall out as sister taxa within the genus *Paa*. Among the available generic names, *Nanorana* (type species *N. pleskei* by original designation) is the oldest available name having priority over *Altirana* Stejneger, 1927 (type species *N. parkeri* by original designation), *Chaparana* (type species *Rana (Chaparana) fansipani* by original designation), and *Paa* (type species *Rana liebigii* Günther, 1860, [named originally as a subgenus of *Rana*] by original designation). Paraphyletic relationships preclude retention of the subgenera within *Nanorana*. In addition to species already included in the genus *Nanorana*, we add those species previously recognized as *Paa*, as well as *Nanorana fansipani*, *Nanorana aenea*, *N. delacouri*, *N. quadrans*, *N.*

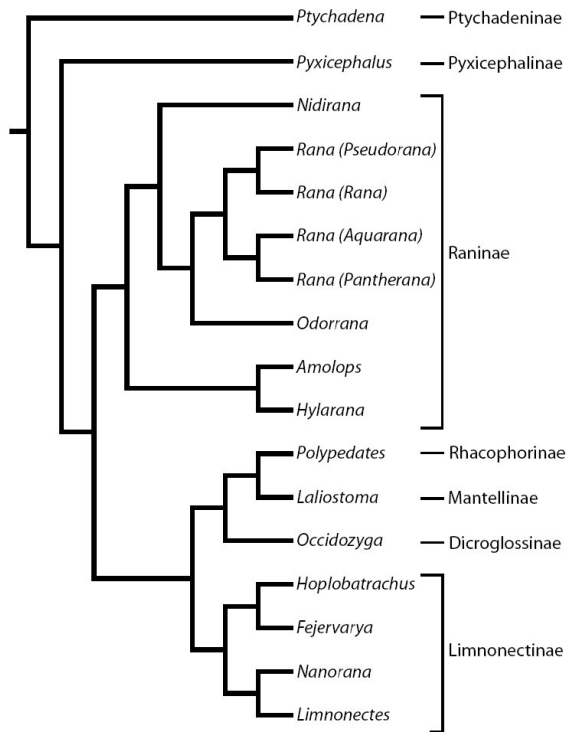


FIG 3. A reduced cladogram with genera as terminal taxa depicting nomenclatorial adjustments. To the right of the tree are the subfamilial taxonomic groupings supported by this study. Taxonomy reflects the recommendations of this manuscript.

sikimensis, *N. unculuanus*, *N. parkeri*, *N. pleskei* and *N. ventripunctata*.

Genus *Limnonectes* Fitzinger 1843: The type species of *Limnonectes* is *L. kuhlii* by original designation. We recognize *Limnonectes* for the following species included in our study, *L. acanthi*, *L. blythii*, *Limnonectes* sp. (“*duboisii*”), *L. grunniens*, *L. ibanorum*, *L. ingeri*, *L. kuhlii*, *L. macrocephala*, *L. macrodon*, *L. magna*, *L. paramacrodon*, and *L. toumanoffi*, and exclusive of *Fejervarya limnocharis* and *F. cancrivora*. The tree of Roelants *et al.* (2004) does not conflict with this new taxonomy.

Although our analysis contains a small number of ranid frogs, major Asian groups are represented herein. No doubt the genus *Rana* remains a “megataxon” in that it is a paraphyletic assemblage of species. Our evaluation revealed that most assemblages of species contained paraphyletic grades of species, and not monophyletic assemblages. Consequently, in the interest of nomenclatorial stability we believe that further divisions of ranid frogs in the absence of a phylogenetic hypothesis will only result in additional confusion in an already incredibly complex history of names and species. We have initiated further biochemical studies on some genera, particularly *Amolops*, *Odorrana* and *Paa*, but also including Vietnamese species in the genus *Hylarana*. Future investigations using gene sequences from 12S and 16S rRNA of smaller subsets of species should

prove equally fruitful for resolving relationships among the genera of ranid frogs.

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