Phylogeny of East Asian Bufonids Inferred from Mitochondrial DNA Sequences (Anura: Amphibia)

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We investigated the relationships of Asian bufonids using partial sequences of mitochondrial DNA genes. Twenty-six samples representing 14 species of Bufo from China and Vietnam and 2 species of Torrentophryne from China were examined. Three samples of Bufo viridis from Armenia and Georgia were also sequenced to make a comparison to its sibling tetraploid species B. danatensis. Bufo americanus, from Canada, was used as the outgroup. Sequences from the 12S ribosomal RNA, 16S ribosomal RNA, cytochrome b, and the control region were analyzed using parsimony. East Asian bufonids were grouped into two major clades. One clade included B. andrewsi, B. bankorensis, B. gargarizans, B. tibetanus, B. tuberculatus, its sister clade B. cryptotympanicus, and the 2 species of Torrentophryne. The second clade consisted of B. galeatus, B. himalayanus, B. melanostictus, and a new species from Vietnam. The placement of three taxa (B. raddei, B. viridis, and its sister species, B. danatensis) was problematic. The genus Torrentophryne should be synonymized with Bufo to remove paraphyly. Because B. raddei does not belong to the clade that includes B. viridis and B. danatensis, it was removed from the viridis species group. The species status of B. bankorensis from Taiwan is evaluated.

Key Words: Bufonidae; Bufo; Torrentophryne; East Asia; phylogeny; mitochondrial genes.

The family Bufonidae, with more than 350 species distributed among 33 genera, is one of the most species-rich amphibian families. Members of this family occur throughout the world, except for Madagascar, New Guinea and adjacent islands, and the Arctic regions (Frost, 1985; Duellman and Trueb, 1986; Duellman, 1993). The number of species in bufonid genera varies significantly. One genus, Bufo, contains over half the species, while most of the remaining genera contain fewer than 10 species each. This distribution of species reflects the preponderance of phenotypic diversity among bufonids and the degree to which the group has been studied. However, the current taxonomy is likely a poor reflection of the phylogenetic relationships within the family (Graybeal, 1993). Within bufonids, the genus Bufo is the most problematic group. Evidence suggests that Bufo is paraphyletic with respect to either some or all of the remaining bufonid genera (Maxson, 1984; Lynch and Renjifo, 1991; Graybeal, 1993).

Because bufonids are a major component of the amphibian fauna of East Asia, they have been the subject of many investigations. The majority of previous studies have focused on anatomical characters. Inger (1972) recognized five species groups in East Asia. Hu et al. (1984) reviewed Chinese Bufo and formed two additional species groups: the cryptotympanicus and the galeatus groups. Recently, Yang et al. (1996) described a new genus, Torrentophryne, based on the morphology of both adults and in particular the tadpoles. Despite these contributions, the phylogenetic relationships among East Asian bufonids are far from resolved. Because of the paucity of phylogenetically informative anatomical characters, due to an apparent conservation of the bufonid body plan, many questions remain open.

Karyological studies using conventional and banded chromosomes reveal that the karyotypes of Asian bufonids are highly conservative (Bogart, 1972; Moreschalchi, 1973; Schmid, 1978; Yang, 1983; Kuramoto, 1990; Liu and Yang, 1997). All species, except for Bufo danatensis, have a karyotype consisting of 22 chromosomes, including six large and five small pairs. Bufo danatensis is a tetraploid species that evolved from a 22-chromosome ancestor (Pisannitz, 1978). Although the karyological studies have contributed to resolving some questions of species identity in Asian bufonids, the data have been phylogenetically uninformative at higher taxonomic levels.

Recently, mitochondrial DNA sequences have been used to reconstruct the history of bufonids. Graybeal (1997) examined species that represent most of the geographic and taxonomic groups, as an effort to provide the overall pattern of the family. Another study...
focused on the *Bufo bufo* species group from the eastern Tibetan Plateau (Macey et al., 1998). In this study, we used mitochondrial DNA sequences to investigate the evolutionary relationships of East Asian bufonids. Our samples represent two genera, at least four species groups, and multiple populations of three widely distributed species. Consequently, we sequenced a range of genes, from slowly evolving ribosomal RNA genes to the rapidly evolving control region. In addition, because of the diverse selection of genes employed, we evaluated the variation in evolutionary rates observed among the different genes.

**MATERIALS AND METHODS**

**Specimens Examined**

Twenty-nine individuals, representing 15 East Asian species of bufonids were examined. Among these species, *B. gargarizans, B. melanostictus,* and *B. viridis* are broadly distributed. Consequently, we collected tissue samples from a variety of localities to evaluate divergence within these species. *Bufo americanus* was chosen as the outgroup (Graybeal, 1997). Data for specimens examined are given in Table 1 and a map of collecting localities is given in Fig. 1. Tissue samples were derived from either skeletal muscle or liver tissues that were preserved in ethanol. Voucher specimens are preserved in herpetological collections of the Kunming Institute of Zoology, Chinese Academy of Sciences (KIZ), the Royal Ontario Museum (ROM), and the American Museum of Natural History (AMNH) (deposited in the Institute of Ecology and Biological Resources, Hanoi, Vietnam). Taxonomic assignment of examined species follows Frost (1985) and Zhao and Adler (1993).

**DNA Amplification and Sequencing**

Total genomic DNA was extracted from alcohol-preserved tissue samples of muscle or liver by digestion with proteinase K for about 5–12 h and purified three times with phenol–chloroform–isoamyl alcohol (PCI) and once with chloroform–isoamyl alcohol (CI). Two regions of 16S and 12S mitochondrial rRNA genes, two portions of mitochondrial cytochrome *b*, and the left domain of the control region were sequenced using the following scheme. Double-stranded fragments were amplified in 33 cycles of the polymerase chain reaction (PCR; 92°C for 30 s, 45–55°C for 30 s, 72°C for 1.5 min) performed in a 25-µl reaction. Annealing temperatures were changed from 45 to 55°C as needed to improve the quality of PCR products. The PCRs were accomplished with the primer pairs 12S-1/12S-2, 16S-1M/16S-2, CytbB2L/ControlB, and CytbA/ControlK. Primer sequences are listed in Table 2. After amplification, the 25-µl product was separated by electrophoresis on 1.5% agarose gel and stained with ethidium bromide. The bands containing DNA were excised and the DNA was sequenced using the primer pairs 12S-1/12S-2, 16S-1M/16S-2, CytbB2L/ControlB, and CytbA/ControlK. Primer sequences are listed in Table 2. After amplification, the 25-µl product was separated by electrophoresis on 1.5% agarose gel and stained with ethidium bromide. The bands containing DNA were excised and the DNA was sequenced using the primer pairs 12S-1/12S-2, 16S-1M/16S-2, CytbB2L/ControlB, and CytbA/ControlK.

### Table 1

<table>
<thead>
<tr>
<th>Taxon number</th>
<th>Species</th>
<th>Voucher no.</th>
<th>Locality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Bufo americanus</em></td>
<td>ROM 21664</td>
<td>Ontario, Canada</td>
</tr>
<tr>
<td>2</td>
<td><em>B. andrewsi</em></td>
<td>KIZ-95L004</td>
<td>Kunming, Yunnan Prov., China</td>
</tr>
<tr>
<td>3</td>
<td><em>B. bankorensis</em></td>
<td>KIZ-97L366</td>
<td>Taiwan</td>
</tr>
<tr>
<td>4</td>
<td><em>B. cryptotympanicus</em></td>
<td>AMNH 13198</td>
<td>Pan-Si-Pan; Lao Cai, Vietnam</td>
</tr>
<tr>
<td>5</td>
<td><em>B. danatensis</em></td>
<td>KIZ-96L196</td>
<td>Hutubi Co.; Xining; China</td>
</tr>
<tr>
<td>6</td>
<td><em>B. galeatus</em></td>
<td>ROM 33281</td>
<td>Ko Rong; Gia Lia Prov., Vietnam</td>
</tr>
<tr>
<td>7</td>
<td><em>B. gargarizans</em></td>
<td>KIZ-95LN011</td>
<td>Zhuanghe Co.; Liaoning Prov., China</td>
</tr>
<tr>
<td>8</td>
<td><em>B. gargarizans</em></td>
<td>KIZ-96L078</td>
<td>Xi'an; Shaanxi Prov., China</td>
</tr>
<tr>
<td>9</td>
<td><em>B. himalayanus</em></td>
<td>KIZ-95L010</td>
<td>Gongshan Co.; Yunnan, China</td>
</tr>
<tr>
<td>10</td>
<td><em>B. melanostictus</em></td>
<td>KIZ-97H001</td>
<td>Linghui Co.; Hainan, China</td>
</tr>
<tr>
<td>11</td>
<td><em>B. melanostictus</em></td>
<td>KIZ-95L001</td>
<td>Pingbian Co.; Yunnan, China</td>
</tr>
<tr>
<td>12</td>
<td><em>B. melanostictus</em></td>
<td>KIZ-97L118</td>
<td>Mengla Co.; Yunnan, China</td>
</tr>
<tr>
<td>13</td>
<td><em>B. melanostictus</em></td>
<td>KIZ-97L202</td>
<td>Lunic Co.; Yunnan, China</td>
</tr>
<tr>
<td>14</td>
<td><em>B. melanostictus</em></td>
<td>KIZ-92L080</td>
<td>Tengchong Co.; Yunnan, China</td>
</tr>
<tr>
<td>15</td>
<td><em>B. melanostictus</em></td>
<td>ROM 33162</td>
<td>Ko Rong; Gia Lia Prov., Vietnam</td>
</tr>
<tr>
<td>16</td>
<td><em>B. melanostictus</em></td>
<td>ROM 32540</td>
<td>Ko Rong; Gia Lia Prov., Vietnam</td>
</tr>
<tr>
<td>17</td>
<td><em>B. melanostictus</em></td>
<td>ROM 33163</td>
<td>Ko Rong; Gia Lia Prov., Vietnam</td>
</tr>
<tr>
<td>18</td>
<td><em>B. melanostictus</em></td>
<td>ROM 33855</td>
<td>Yok Don; Dac Lac Prov., Vietnam</td>
</tr>
<tr>
<td>19</td>
<td><em>B. melanostictus</em></td>
<td>ROM 33861</td>
<td>Yok Don; Dac Lac Prov., Vietnam</td>
</tr>
<tr>
<td>20</td>
<td><em>B. melanostictus</em></td>
<td>KIZ-97L372</td>
<td>Taiwan</td>
</tr>
<tr>
<td>21</td>
<td><em>B. rodeni</em></td>
<td>KIZ-96G003</td>
<td>Gulong Co.; Gansu, China</td>
</tr>
<tr>
<td>22</td>
<td><em>B. tibetanus</em></td>
<td>KIZ-97L004</td>
<td>Zhongdian Co.; Yunnan, China</td>
</tr>
<tr>
<td>23</td>
<td><em>B. tuberculatus</em></td>
<td>KIZ-97L005</td>
<td>Zhongdian Co.; Yunnan, China</td>
</tr>
<tr>
<td>24</td>
<td><em>B. viridis</em></td>
<td>ROM 23777</td>
<td>Vedi, Armenia</td>
</tr>
<tr>
<td>25</td>
<td><em>B. viridis</em></td>
<td>ROM 26852</td>
<td>Nozaduz, Armenia</td>
</tr>
<tr>
<td>26</td>
<td><em>B. viridis</em></td>
<td>ROM 26853</td>
<td>Achaldaba, Georgia</td>
</tr>
<tr>
<td>27</td>
<td><em>Bufo sp.</em></td>
<td>ROM 32471</td>
<td>Yok Don; Dac Lac Prov., Vietnam</td>
</tr>
<tr>
<td>28</td>
<td><em>Torrentophryne aspinia</em></td>
<td>KIZ-93A011</td>
<td>Yangbi Co.; Yunnan, China</td>
</tr>
<tr>
<td>29</td>
<td><em>Torrentophryne tuberospina</em></td>
<td>KIZ-91A089</td>
<td>Yunnan, China</td>
</tr>
</tbody>
</table>
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). The regions between CytbA and ControlK, 12S-1 and 12S-2, and 16S-1M and 16S-2 were sequenced in both directions. Unfortunately, it was not possible to sequence the regions between CytB2L and CytbA and between ControlK and ControlB in both directions (Fig. 2).

The products of the sequence reactions were resolved in 6% polyacrylamide–7 M urea gel that was then dried and visualized on autoradiograph films (Kodak) within 24–48 h.
DNA Sequence Analysis

Clustal W (ver. 1.6; Thompson et al., 1994) was used for sequence alignment, and minor modifications were made by eye to correct the computer-aligned sequences using the program ESEE (ver. 3; Cabot and Beckenbach, 1989). Using MacClade, (ver. 3.04; Maddison and Maddison, 1992), we excluded the tandem repeats in the control region from further analysis because the large length variation and the presence of highly variable regions resulted in questionable alignments. Only phylogenetically informative sites were maintained for the analysis in PAUP* (ver. 4.0b1; Swofford, 1998).

Hillis et al. (1993) suggested that prior to an in-depth analysis, the presence of phylogenetic signal (character covariance) should be tested. One method to test for character covariance is the permutation tail probability test (PTP; Faith and Cranston, 1991). Each character state was randomly reallocated to different populations using the permutation test option in PAUP*. One-thousand randomizations were performed without randomization of the outgroup states. A second method for analyzing character covariance in the data is the g1 statistic (Huelsenbeck, 1991; Hillis and Huelsenbeck, 1992). Ten-thousand randomly chosen trees were used in PAUP* to generate the g1 statistic that tested for significance against the critical value.

In our analysis, 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, 50 replicates, retaining minimal trees only, using tree bisection–reconnection branch swapping with steepest descent, and collapsing zero-length branches. Ratios of transitions to transversions were calculated in MacClade.

Nodal support was assessed for each gene individually and for the analysis that combined all data sets. Sankoff matrices (Sankoff and Rousseau, 1975) were used for differentially weighting transitions and transversions. Bootstrap proportions (BSP; Felsenstein, 1985) using 1000 replicates were calculated in PAUP* and then compared to the resulting tree(s). Templeton’s test (1983) was used to evaluate the most-parsimonious solutions in light of alternative phylogenies. We also performed PTP evaluation (Faith and Cranston, 1991; Archie, 1989) and decay analysis (Bremer, 1988), in which the latter involved saving trees up to 10 steps longer than the most-parsimonious solution. The occurrence and location of significantly covaried characters are shown in the phylogenetic trees generated.

TABLE 2

Primers Used for Amplifying and Sequencing Fragments of Mitochondrial DNA Genes in Toads, Genera Bufo and Torrentophryne

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’–3’</th>
<th>Sequence position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>12S1</td>
<td>CAACCTGGGAAATGACAT-</td>
<td>L2484</td>
<td>Kocher et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>CCCACTAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12S2</td>
<td>ACACACCCGCGTCACCCCTC</td>
<td>H2997</td>
<td>Kocher et al. (1989)</td>
</tr>
<tr>
<td>16S1M</td>
<td>CCGACTGTTTACCAAAAAAT-</td>
<td>L3955</td>
<td>Fu (1999)</td>
</tr>
<tr>
<td></td>
<td>CAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S2</td>
<td>CCGGGATCCCCGGCCTCCT-</td>
<td>H4552</td>
<td>Palumbi (1996)</td>
</tr>
<tr>
<td></td>
<td>GAACCTGACGCCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyth2L</td>
<td>TGAGGCAATAATATCTCTTCT-</td>
<td>L16654</td>
<td>Fu (1999)</td>
</tr>
<tr>
<td></td>
<td>GAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CythA</td>
<td>GAATGACGGGACCAGTAGAACCTGACCC</td>
<td>L17258</td>
<td>Goebel et al. (1999)</td>
</tr>
<tr>
<td>ControlK</td>
<td>AATGGTTGAAATGCT-</td>
<td>H474</td>
<td>Goebel et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>GAGATTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ControlB</td>
<td>GTCTAGGGGAGTTA-</td>
<td>H857</td>
<td>Goebel et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>GATCTACCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note. Sequence position indicates the starting position of the primer in the Xenopus laevis mitochondrial genome (Roe et al., 1985) and is preceded by the amplification direction as indicated by (H) heavy or (L) light strand.

FIG. 2. Schematic drawing of the regions sequenced and the relative positions of the primers used in this study. The bars above indicate the regions that were sequenced. Grey areas indicate regions that were sequenced in both directions, and cross-hatched area indicates the region of tandem repeats.
RESULTS

Sequence Variation in East Asian Bufonids

The sequences have been deposited in GenBank (Accession Nos.: AF160761–160797, AF171190–171214, 174500–174526, 190229–190255). The number of variable sites is given in Table 3.

For each of the 16 species, 380 and 572 bp of aligned nucleotides were sequenced from the 12S and 16S rRNA genes, respectively. When compared, 71 sites from 12S and 144 sites from 16S sites varied among Asian bufonids (23% of total sites). In 12S, only a single site was variable among populations of the same species, whereas up to 37 sites differed among species. Similarly, for 16S rRNA, only 5 sites were variable among populations of a single species, but up to 77 sites were variable among species. The ratios of transitions to transversions (TS:TV) were 3.5:1 and 2:1 for 12S and 16S, respectively.

Two sections from the latter half of the mtDNA cytochrome b gene were sequenced for each of the samples. These included a section of 518 bp (beginning with primer CytB2), as well as a 100-bp fragment at the end of the Cyt b. A total of 242 sites were variable (39% of total sites); 39 changes occurred in first codon position, 41 in the second position, and 162 in the third (Table 3). Thus, as in other protein-coding genes, the third position was most variable. Pairwise comparisons revealed that up to 47 sites were variable among populations of a species, while 122 sites were variable among species. The TS:TV ratio was 1.8:1.

Insertions of tandem repeats were predominant in the first portion of the control region; this variation created substantial differences in the lengths of the fragments. This region was not included in the analysis because of a lack of phylogenetic utility (See Fig. 2).

The Number of Variable Sites and Substitution Ratios for Partial Sequences of Mitochondrial DNA Genes Sequenced in the Analysis of East Asian Bufonids

<table>
<thead>
<tr>
<th>Gene</th>
<th>Total bp</th>
<th>Variable sites (informative sites)</th>
<th>Variable sites by codon position (informative sites)</th>
<th>TS:TV</th>
</tr>
</thead>
<tbody>
<tr>
<td>12S rRNA</td>
<td>376</td>
<td>77 (45)</td>
<td>NA</td>
<td>3.5:1</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>571</td>
<td>144 (87)</td>
<td>NA</td>
<td>2:1</td>
</tr>
<tr>
<td>Cytochrome b</td>
<td>613</td>
<td>242 (194)</td>
<td>72 (31)  15 (5)  182 (158)</td>
<td>1.8:1</td>
</tr>
<tr>
<td>Control region</td>
<td>649</td>
<td>358 (287)</td>
<td>NA</td>
<td>1:1</td>
</tr>
</tbody>
</table>

Note. Among the variable sites, numbers in parentheses indicate informative characters.

Except for Bufo sp. and B. galeatus from Vietnam, 649 bp of the first half of control region were determined for all species. A total of 358 sites (55%) were found to be variable. Up to 52 sites were variable among populations, whereas up to 218 sites were variable among pairs of species. The TS:TV ratio was very low, 1:1.

Phylogenetic Evaluation

The analysis of ribosomal genes (12S and 16S) resulted in eight trees (336 steps in length, CI = 0.566, RI = 0.760; Fig. 3), excluding uninformative characters. The strict consensus shows three major clades: the melanostictus clade ((melanostictus, himalayanus) galeatus, Bufo sp.) with B. raddei as its sister group, the gargarizans–cryptotympanicus clade, and the viridis clade. Ambiguities exist in the intraspecific relationships of B. melanostictus and in the placement of the B. viridis clade (B. viridis and B. danatensis) as the sister species to either the melanostictus clade or the gargarizans–cryptotympanicus clade. All resolved nodes on the strict consensus tree received high BSPs (e.g., =0.70; Hillis and Bull, 1991), except the node at the base of the melanostictus–raddei clade. Weighting transversions to transitions 2:1 had no effect on the topology.

Thirty-six trees were recovered from the analysis of the cytochrome b data (127 steps, CI = 0.549, RI = 0.824). The topology was very similar to the topology derived from the rRNA genes, except the intraspecific relationships of B. melanostictus and B. viridis were not resolved (Fig. 4). Furthermore, B. bankorensis formed a polytomy with the southern populations of B. gargarizans. Among the 16 nodes that appear on the strict consensus tree, 13 nodes received high BSPs. Using only first and second positional substitutions resulted in 75 trees, in which the overall topology was very similar to that of the initial cytochrome b analysis, but the relationships within B. melanostictus and B. viridis were even less resolved.

The control region sequences yielded a single MPT (736 steps, CI = 0.618, RI = 0.860), in which all nodes were resolved, except among populations of B. viridis, in which no variation occurred (Fig. 5). The viridis clade was the sister group to the melanostictus clade, and B. raddei was the sister to that clade. Bufo bankorensis lies among the populations of B. gargarizans, rendering it paraphyletic.

Among the resulting topologies from the independent analyses, those nodes that had high BSPs were not in conflict. Because of this, a combined analysis was deemed appropriate (Doyle, 1992; Hulsenbeck et al., 1996) in an attempt to find further support for some of the weaker nodes (viz., raddei, viridis clade, and bankorensis with respect to gargarizans).

The global unweighted parsimony evaluation resulted in three MPTs (1590 steps in length, CI = 0.578, RI = 0.829); the strict consensus is given in Fig. 6.
Using PTP, randomization of the data did not produce a tree shorter than that resulting directly from the data. The shortest tree generated from the randomized data was 3838 steps (2000+ steps longer than MPTs from nonrandom data). The distribution of trees from 3838–3909 steps had a mode and mean of 3877 steps (homoplasy excess ratio = 0.62 [Archie, 1989]), indicating that our data were significantly different from random data.

The strict consensus tree had two major clades: the melanostictus–viridis clade and the gargarizans–cryptotympanicus clade. All populations of a given species clustered together. However, the genus Torrentophryne was nested within the other species of Bufo, and B. cryptotympanicus was placed within Torrentophryne, thus rendering both genera paraphyletic.

Assessing Nodal Stability

The results of the tests for nodal support are indicated on the consensus tree (Fig. 6). Similar ranking was found by both DI and BSP. For example, all nodes except 1, that received BSPs of 100, also had DIIs that were greater than 10. Decay indices revealed that most nodes required a considerable number of additional steps to collapse, except those that were not supported by either significant values from n-PTP or high BSPs. Intraspecific clades were among the first to collapse, some disappearing after adding only one or two steps. All but 6 nodes received a BSP greater than 70%. The n-PTP evaluations revealed that 17 of the 28 nodes were supported by significant character covariation (PTP \#0.05), and 3 nodes received no support. Significant n-PTP values were strongly correlated with BSPs of 95% or higher. Eleven nodes received significant support from Templeton's test. These were among the nodes that were supported by n-PTP and received the highest BSP (100%) and DI (>10). We found that Templeton's test is the most conservative method of nodal support evaluation.

DISCUSSION

Different portions of mtDNA evolve at different rates. Thus, the phylogenetic trees reconstructed from different genes for the same set of organisms may differ. The
slow-evolving 12S and 16S ribosomal RNA genes have been shown to be appropriate for resolving older divergences (ca. 150 m.y.a.) (Mindell and Honeycutt, 1990). The protein-encoding cytochrome \( b \) gene has been found to be informative for intermediate levels of divergence, as well as deep (old) and shallow (recent) events (review by Graybeal, 1993). The rapidly evolving control region (D-loop) sequences have been used for recent divergences, such as at the population level (Yang et al., 1994). The evolutionary rates for each gene from our analysis are summarized in Fig. 7 for each level of divergence: within populations (shallow), among populations (middle), and between the ingroup and outgroup taxa (deep).

Variation in the rRNA gene sequences (12S and 16S) was very low for the 16 species of bufonids included in this analysis. Among 571 bp sequenced from the 16S rRNA gene, 63 sites were variable between ingroup species and \( B. \text{americanus} \), and 31 sites of 376 bp varied in the 12S rRNA gene. Genetic differentiation between species was represented by an average of 5% (12S) and 8% (16S) sequence difference. Levels of intraspecific variation were extremely low (0–1%), precluding resolution of relationships among populations. Although, some relationships near the base of the tree were resolved, in particular the \( \text{melanostictus-raddei} \) clade, these branches did not receive high values by any method that tests for nodal support. The number of unambiguous changes at these nodes was surprisingly low (five to nine characters) in comparison to some of the more terminal branches. Similar genetic divergence for these rRNA genes have been found among

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**FIG. 4.** Strict consensus tree from the analysis of cytochrome \( b \) that resulted in 25 MPTs. Numbers on the nodes represent BSPs $\geq 70$. Numbers before the taxon name correspond to taxon numbers in Table 1.
other species of bufonids (Graybeal, 1997). As expected, the transition:transversion ratio was notably higher than the nearly 1:1 ratio reported by Graybeal (1997) for both 12S and 16S genes.

Sequences from the control region and cytochrome \( b \) exhibited substantial intraspecific variability (0–8%), although these sequences were less variable than Graybeal’s (1997) data for the \( B. \) boreas species group. Similarly, we found the transition:transversion proportion to be 1:1.8 in cytochrome \( b \) and 1:1 for the more variable control region (Table 3). Only 2% of the sites (16 sites) were variable in the control region among populations; ranging from 0% among \( B. \) viridis and some Vietnamese populations of \( melanostictus \) to 9% among Yunnan and southern Vietnamese populations of \( B. \) melanostictus.

Most of the unambiguous changes occur among the deeper nodes. As noted by Graybeal (1993) for cytochrome \( b \), such divergence (15–20%; Graybeal, 1997) can preclude the resolution of basal relationships. Most were silent changes (third codon position). When third positional changes were excluded from the analysis of cytochrome \( b \), the topology did not change. There were 31 and 5 potentially phylogenetically informative characters among the first and second codon positions, respectively. Translating the sequences to amino acids produced 17 variable and potentially informative characters. The number of amino acid changes was higher

FIG. 5. The single most-parsimonious tree from the analysis of the control region. Numbers on the nodes represent BSPs ≥70. Numbers before the taxon name correspond to taxon numbers in Table 1.
than that reported by Graybeal (1993), as she found no informative character changes among or within species (mid-shallow divergences). Nearly all unambiguous changes occur among the basal nodes, with only one amino acid change among populations; one each from the *B. melanostictus* population from Hainan and the *B. gargarizans* population from Liaoning.

Our phylogenetic analyses of the combined data resulted in three MPTs. The strict consensus tree had most nodes supported by significant character covariation and high BSP and decay values. Independent gene analyses revealed a similar pattern; where incongruences occurred, these nodes were not well supported in the combined analysis. In particular, the placements of *Bufo raddei* and the *B. viridis* clade did not receive support.

**Populations of Bufo melanostictus**

Significant character covariation occurred among the sequences at the population level. In the combined analysis, the *B. melanostictus* clade had 6 of the 10 nodes supported by significant character covariation, and all but 1 node had high BSPs. In all analyses the Vietnamese populations consistently clustered together. However, the interrelationships among these populations were not corroborated across data sets, and
although the BSPs were high, they did not receive support from the n-PTP analyses.

Two distinct groups occurred among *B. melanostictus* (Figs. 4 and 5). These groups do not fully reflect geographic proximity (Fig. 1). Two distant populations of *B. melanostictus*, those from Taiwan and Hainan Island, clustered together. This finding was unexpected and may reflect a concomitant introduction of toads from Hainan Island to Taiwan with the movement of agricultural products, rather than a reflection of geographic history. The relatively close association of the Hainan Island toads with Vietnam, exclusive of Yunnan Province, is expected. During the Pleistocene, Hainan Island formed a continuous land mass with Vietnam and southeast China. Consequently, Hainan Island shares many species with Vietnam and Guangxi Province, China that is exclusive of Yunnan Province. For example, geckos of the genus *Goniurosaurus* occur only in Hainan Island, Guangxi Province, and northeastern Vietnam. The aquatic skink, *Tropidophorus hainanensis*, occurs both on Hainan Island and in northeastern Vietnam. The sunbeam snake, *Xenopeltis hainanensis*, has an identical distribution, as do several other species of amphibians and reptiles. Consequently, the genealogical associations of the toads, as revealed in our maximum parsimony evaluation, provide cladogenic evidence of a history of faunal sharing between Hainan Island and Vietnam, exclusive of neighboring Yunnan Province.

**Populations of Bufo gargarizans**

*Bufo gargarizans* is distributed throughout China, except for the extreme west and south (Zhao and Adler, 1993). The three samples used in this analysis are from localities across its broad distribution—one sample each from northern China (LN = Liaoning Province), southern China (FJ = Fujian Province), and northwestern China (XN = Shaanxi Province). Among the populations of *B. gargarizans*, the genetic variation appears to be very low; only 1–4% of the total sites were variable. Consequently, relationships between these populations could not be resolved.

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**FIG. 7.** Evolutionary rates among the four genes used in this study. Plotted are the averages for percentage of sequence difference among the three hierarchical levels with the ranges in the table below. Statistics are corrected for data not included; regions of 12S and 16S that could not be aligned and tandem repeats from the control region are omitted.
Taxonomic Implications

The taxonomy of Eurasian *Bufo* has had a long, complex history. The result has been a vague interpretation of the relationships among more than 35 species. Previous studies have yielded phenetic clusterings that may not reflect phylogenetic history (Inger, 1972; Matsui, 1986). These groupings will likely change as additional data and taxa become available. Phylogenetic analyses are a good way to examine the validity of taxa and are useful for questions of specific status and for establishing species groups that reflect the genealogical history.

The *melanostictus* group is widely distributed throughout much of the Indian region, southern China, and all of Southeast Asia, including Indonesia. Previous ideas about the members of this group have included five species (Inger, 1972). One, *B. melanostictus*, is widely distributed. *Bufo himalayanus* has a more restricted distribution. Our findings support the association of these two species.

The *galeatus* group has been problematic. Inger (1972), for a lack of data, was unable to place *galeatus* within a species group. Hu et al. (1984) considered *B. galeatus* to be the only member of its own group but did not provide justification. Our data support an association of *B. galeatus* with its Vietnamese sister species (*Bufo* sp.). These two species consistently clustered as the sister group to the *melanostictus–himalayanus* clade.

The *viridis* group is broadly distributed across all of Eurasia, roughly between 60° and 28° N and southward into northern parts of Africa. Inger (1972) included *B. viridis* and *B. raddeti* in this group, among other forms. The lack of nodal support makes the placement of *B. viridis* and *B. raddeti* among the clade of East Asian bufonids problematic. Our results consistently indicated that these taxa neither were sister species nor clustered within a monophyletic group that could be considered the *viridis* group (Figs. 3–6). The *viridis* group should include, based on our results, *B. viridis* and its tetraploid sister species, *B. danatensis*.

The *bufo* group has historically been one of the most problematic groups in terms of identifying membership. This is likely because it contains some of the more widely distributed species of Eurasian *Bufo* (*Bufo bufo* and *B. gargarizans*), and the morphological variation within these taxa across their distributions is poorly understood. Our analysis indicated that this group should include *B. gargarizans*, *B. bankorensis*, and *B. andrewsi*, as well as *B. tuberculatus* and its sister species, *B. tibetanus*. Macey et al. (1998) also suggested the close affiliation of *B. gargarizans* and *B. andrewsi*.

*Bufo bankorensis* has undergone several revisions that have either synonymized it with *B. bufo* (Pope and Boring, 1940; Liu and Hu, 1961) or recognized it as a distinct species (Frost, 1985; Matsui, 1986; Zhao and Adler, 1993). Matsui (1986) found that, morphologically, *B. bankorensis* was very similar to allopatric populations of *B. andrewsi* but distinct from adjacent populations of *B. gargarizans*. With reservation, he recognized *B. bankorensis* as, at least, a subspecies of *B. gargarizans*, and because it was allopatric with populations of *andrewsi*, he considered it a distinct species. Our analyses did not provide justification for the recognition of *B. bankorensis*. Our data (cytochrome *b* and control region) indicated that Fujian *B. gargarizans* were more closely related to *B. bankorensis* than to other populations of conspecifics. In the combined analysis, *bankorensis* was the sister to all populations of *B. gargarizans*. However, the node uniting *B. gargarizans* was not supported by significant n-PTP, BSPs, or decay analysis (the node collapsed when 1 step was added to the tree).

The position of *Torrentophryne* within *Bufo* renders the latter genus paraphyletic. Similarly, *B. cryptotympanicus* was the sister species to *T. aspinia*, and their sister species was *T. tuberospinia*. Therefore, the genus *Torrentophryne* was itself paraphyletic. Significantly covared character states, as well as high BSPs, support the clade consisting of these three species. Recognition of *Torrentophryne* is based on the occurrence of sucker-like abdominal disks on the tadpoles and the absence of the tympanum and columella—structures associated with the auditory system (Yang et al., 1996). These traits are also found in at least one other bufonid genus, *Atelopus*. The tadpoles of *Bufo cryptotympanicus* are not known. However, the morphology of the adults supports this grouping, as its name indicates (hidden tympanum). *B. cryptotympanicus* also has a reduced auditory system. All of these species occur at higher elevations in northern Vietnam and Yunnan and Guangxi Provinces, China, and it is possible that the tadpoles of *B. cryptotympanicus* may also possess the derived sucker disks. These modifications are likely special adaptations to living near loud, fast-moving streams. Regardless of the association of these three species, in the interest of nomenclatural stability, it is best not to recognize the genus *Torrentophryne* because it forces *Bufo* to be paraphyletic.

For the classification of bufonids to reflect monophyletic assemblages, we would need to include *B. cryptotympanicus* in the clade containing *Torrentophryne*. Although it would be more phylogenetically informative to construct a classification of multiple genera for the more than 350 species of *Bufo*, such is premature, given that representation of species is very small. In order to avoid taxonomic chaos, we prefer to avoid making such changes, pending additional data from a greater representation of species.
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