

Phylogenetic Relationships of a Chinese Frog, *Rana zhengi* Zhao, 1999 Inferred from Mitochondrial Cytochrome b Gene Sequences (Amphibia, Ranidae)

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Abstract: In order to assess phylogenetic relationships of a Chinese frog, *Rana zhengi* Zhao, 1999, the sequences of 587 base pairs of the mitochondrial cytochrome b gene are compared with six species of Japanese (*R. pirica*, *R. ornativentris*, *R. japonica*, *R. okinavana*, *R. tagoi*, and *R. tsushimensis*), one species of Taiwanese (*R. sauteri*), one species of Russian (*R. amurensis*), and two species of European brown frogs (*R. temporaria* and *R. dalmatina*). The resultant phylogenetic tree suggests monophyly of *R. zhengi* and these brown frogs, and the earliest divergence of *R. zhengi* among all the ingroup species examined. Monophyly of *R. zhengi* and *R. sauteri* was rejected. For this reason, placement of *R. zhengi* within the brown frogs of the *R. temporaria* group requires further study, and separation of *R. zhengi* and *R. sauteri* as a distinct genus or subgenus *Pseudorana* is not supported.

Key words: Brown frog; Cytochrome b; Phylogeny; *Pseudorana*; *Rana zhengi*; *R. sauteri*; China

INTRODUCTION

Rana zhengi was described from Zhangcun Village, Hongya Co., Sichuan Province, China (Zhao, 1999). Zhao (2000a) considered this species to be related to members of the genus (Fei et al., 1990) or subgenus (Dubois, 1992) *Pseudorana*, which includes *R. sauteri* from Taiwan (Fei et al., 1990). This species, however, has

been proven to include cryptic species (Chou and Lin, 1997) and is considered to form a species complex (Tanaka-Ueno et al., 1998a). In our previous study, we showed that two populations of *R. sauteri* (sensu Chou and Lin, 1997), while being distant from each other phylogenetically, form sister groups with some Japanese brown frogs, and that the genus or subgenus *Pseudorana* is invalid (Tanaka-Ueno et al., 1998a).

From these lines of previous information, *R. zhengi* might be a member of the brown frogs of the *R. temporaria* group (Boulenger, 1920), but no studies have ever been

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1 **50** **100**
X. laevis CAAATCATTACAGATTATTCTTAGCTATACATTATACAGCAGACACTTAGCCTTCTCATCAGTAGCCCAATTTGGCCGTACGTTAACTATGGAT
R. catesbeianaG-C-T-C.....C.....C.....C.....C.....T-CC-T-A.....TA-C-T-C.....T.....TA-C-C
R. zhengiGC-T-CC.....TC-C.....C.....G-C.....C-CC-T-A.....C-CA-C-T.....C.....A-C-C
R. temporariaGCC-C-C.....T-G.....C.....C-T-C-CC-C-A.....TA-C.....C-T-G-T.....TA-C-C
R. dalmatinaG-T-CC.....C-T.....G-C-C.....C.....G-CC-C-A-T.....TA-T-T-C-C.....TA-C
R. sauteri (Sanyi)GCC-C-CC.....C-A.....C-C.....T.....T-C-G-A-T.....TA-C.....C.....T.....TA-C
R. sauteri (Wulai)GCC-C-CC.....C-G-C.....C-G-T.....C-CC-A-T-C-T-C.....C.....T.....A-C-C
R. amurensisTGC-T-C.....C.....C.....C-G-T-T-T-C-G-A.....TA-C-A-C.....A-T.....TA-C
R. tagoiGCC-C-CC.....C-G.....C-G-C-T-T-CC-C-A.....TA-C-C-C.....C-T-A-TA-C
R. okinavanaGCC-C-CC.....C-T-C.....C-G-T-T-T-CC-A-T-T-TA-T-C-C.....C-T-A-A-C
R. tsushimensisGCC-C-CC.....C-G-C.....C-G-T.....C-CC-A-T-C-TA-T-C-C.....C-T-A-C-C
R. japonicaG-GCC-C-C.....TC-G-C.....C-C.....T-T-T-CC-A-T.....TA-C.....C-T-C-A-C-C
R. ornativentrisGCC-G-C.....C.....C.....T-T-C.....A-T.....TA-T-C-C.....A-T-A-A-C-C
R. piricaGCC-C-GC.....TC.....C.....G-T-T-C.....G-T.....CA-C.....C-C-T-A-T-A-TA-C-C

101 **150** **200**
X. laevis GATTAATTCGCAATCCATGCCAATGGAGCCCTATTCTTCTTCTTTCATTCACATCGGACGAGGGTGTACTACGGCTCTTCTTATATAA
R. catesbeianaC-CC-A-A.....C.....A.....T-T-C-T-T-TT-C.....C-CC-C.....C-A-C-T.....
R. zhengiC-CC-C.....C-T.....C-C-A.....T.....T-C-T-T-T-C.....T.....G-AC-C-T-T.....A-A-C-C
R. temporariaC-CC-T-T-T.....C-T-A.....T.....T-C-T-T-C.....G-CC-T-T.....A-A-C-C-C
R. dalmatinaGC-CC-C.....C-C-A.....T.....T-T-T-T-C.....C.....CC-T-T.....ATC-C
R. sauteri (Sanyi)C-CC-C.....C-C-G-A.....C.....T-C-T.....G-CC-T-T.....C-A-C-C-C
R. sauteri (Wulai)C-CC-C.....C-C-C-A.....T.....T-T-C.....G-CC-T-T.....C-A-C-C
R. amurensisGC-CC-A.....T-C.....C-C-A.....T.....C-T-T-T-T-G.....TC-T-T.....C-A-C-C
R. tagoiGC-CC-C.....C-T-C.....C-C-A.....T.....C-T-T-T-T-T.....TC-T-T.....C-A-C-C
R. okinavanaGC-CC-C.....C-C-C-A.....C.....T-T-T-T-T-T.....TC-T-T.....C-A-C-C
R. tsushimensisC-CC-C.....C-C-C-A.....T.....T-T-C-T.....G-CC-T-T.....C-A-C-C
R. japonicaC-CC-T.....C-C-C.....T.....C-C-T-T.....G-CC-T-T.....A-A-C-C-C
R. ornativentrisC-TC.....C-C-C.....C-C-A.....T-C-T-T-T-C.....CC-T-T-T-T-C-A-C-C
R. piricaC-TC-C.....C.....C-C-A.....T-T-C.....T-T-C.....G-G-CC-T-T-T.....A-C-T

201 **250** **300**
X. laevis AGAAACATGAAATTTGGTGTGATCTTCTATTTTAGTTATAGTACAGCATTGTAGGATATGTTCTCCATGAGGACAAATATCTTTTGGAGGGCT
R. catesbeianaC.....A-A-G.....A-C.....T.....T-C.....C.....C-C-C-C-C-C.....C.....A-C.....T-C
R. zhengiG.....G.....A.....C-C-CC-G.....C.....C-C-C-C-C-C.....C.....A-C.....T-C
R. temporariaG.....C-C-A-A-C-CT.....G.....C.....T.....C-C-C-C-C-C.....T-G.....G-A-C.....T-C
R. dalmatinaC.....C-A-A-C-CT-G-CC-G-G-C.....C.....G-C.....T.....G.....A-C.....T-C
R. sauteri (Sanyi)T.....C-C-A-T.....T-C-C-C-G.....C.....C-C-C-AT-G-G.....G-C-C.....C-C
R. sauteri (Wulai)G-C-C-G-T-C-C-C-C-A-G-C.....C.....G-C-C-G-G.....A-C-C-C
R. amurensisG.....T-C-T.....A-A.....C-C-C-C-C-A-G.....C.....C-A-G.....A-G-C-C
R. tagoiC-C-A.....C-T.....A-G-C.....T-C-C-C.....T.....C.....G-A-C.....C-C
R. okinavanaC-C-A-C-C-C-CC-A.....C-G-T-C-G-T-C-C-G-G.....C.....G-A-C.....T
R. tsushimensisG-G-C-C-G-T-C-C-C-A-G-C.....C.....G-C-C-G-G.....A-C.....C-C
R. japonicaG.....C-A-A-C-C-C-A.....C.....T.....C-CT.....C.....G-C-C.....C
R. ornativentrisG.....C-A-A-C-C-C-A.....C.....T.....C-GT.....C.....C-C-C.....C-C
R. piricaG-G-C.....A-A-C-C-G-C.....G.....C-T.....C.....T-G.....C-G.....C-C

301 **350** **400**
X. laevis ACAGTAATTACTAATCTTCTTCTGCTATTCCGTACATCGGAAACGTACTAGCCTCAATGAATTTGAGGAGGTTCTCTGTAGATAACGCCACTTTAACCC
R. catesbeianaC.....A-C-GGCC-A.....TTC-AC-T-T.....G.....T-A.....C.....CC-T
R. zhengiC.....C-A-GCC-T-G.....CT-AC-T.....G.....G-C-A.....C.....CC-C-T
R. temporariaC.....A-CGCC-C.....T-CT-AC-C.....G-C.....A-A-C-T-T-C
R. dalmatinaC.....C-A-CGCC-A.....CC-A-AC-C-T.....C.....C-A-C-T-A-C-C
R. sauteri (Sanyi)C-C-C-C-A-CGCC-C.....CTC-AAC-C.....G-C.....G-C-A-C-C-T
R. sauteri (Wulai)C-C-C-C-C-A-CGCC-C.....CC-AC-T.....G-C.....G-C-A-G-C-A-CC-G
R. amurensisC-C-C-C-C-A-CGCC-C.....CTCT-AC-C.....C-G-G.....A-C-C-T
R. tagoiC-C-C-C-C-A-CGCC-T.....CTC-AAC.....G-C.....G-C.....A-C-C
R. okinavanaT-C-C-C-C-C-A-CGCC-C-G.....TC-AC-C.....G-C.....G-C.....T-CC-C-T
R. tsushimensisC-C-C-C-A-CGCC-C.....TC-AC-C.....G-C.....G-C.....A-CC-C
R. japonicaC-C-C-C-A-CGCC-C-T.....CTC-AC-C-T.....G-C.....A-CC-C
R. ornativentrisC-A.....C-A-CGCC-C.....CTTT-ACT-T.....G-C-G.....A-A-CC-C
R. piricaC-C-A-CGCC-C.....CTTT-ACT-G.....C-G.....C.....G.....C.....T-CC-C

401 **450** **500**
X. laevis GATTCCTCCGATTTCACTTCTCTCTCTTATTATGCGGGAGCTAGCATCTCCACTTTTATTTCACGAACTGGATCAACAAACCCAACTGG
R. catesbeianaTA-T.....A.....C-G.....C-A-C-A-T-GA.....C-GC-T-C.....TC.....C.....A
R. zhengiA-C.....C-A.....T-CCATA-T-AA-T.....C.....T-TC.....A-G-CT.....T-A
R. temporariaA-C.....TA-T.....G.....C-T-CT-TG.....AA-T.....C.....A-C-A-G-T-T-C
R. dalmatinaTA-G-C.....C-A.....C-C-T-CTATG.....AA-T-C-C-G-C-A-TC.....A.....T-C-T-C
R. sauteri (Sanyi)G.....A-T-C-A.....C-C-T-CT-C-G-A-T-C-CC-G.....T-TC-G-A.....T-C-A
R. sauteri (Wulai)T-TA-G-C.....C-C-C-C-T-CTATA.....GA-C-CC-C-G-TC.....C-G-C-A
R. amurensisG.....TA.....C-A.....C-C-CC-T.....C-AA-T.....C-T-A-TC.....A-T-T-C-G
R. tagoiA-C.....A.....C-C-G-CT-TA.....A-T.....CC-G-T-A-C-A-C-T-T-C-A
R. okinavanaG-T-TA.....C-A-T-C-C-C-T-CG-A.....GA-C-C-C-G-T-C-G-G-T-C-A
R. tsushimensisT-TA-G-C.....A-C-C-G-C-T-CTATA.....GA-C-CC-C-G-TC.....C-A
R. japonicaTA-T-C.....C-A.....C-CTATA.....AA-T-C-CC-C-G-TC.....C-C-CT-C-A
R. ornativentrisTA.....TA-T-C-A-C-TA-T-AA-T-C-CC-T-TC.....C-G-T-C
R. piricaT-TA.....C.....A-C-G-C-C-C-A-CTATA-T-AA-T-C-C-G.....T-C-A-C-T-C-A

501 **550**
X. laevis ATTAAACTCAGACCCAGATAAAGTACCTTTCCACCCTACTTCTCTTACAAGACCTTTTAGCCTTCTTATTACTTACAGCACT
R. catesbeianaCC-C-T-A-TT.....G-CT-C-T-T.....C.....T-A-C.....AC.....GG-CT
R. zhengiCC-C-T-CA-TT.....CA-C-T.....T.....C-T.....G-C-C.....AGGCCTG
R. temporariaC-C.....TA-T-T-C.....CT-A.....T.....C.....G-T.....GGT
R. dalmatinaC-C.....TA-T-C.....CT-A.....G-T.....C.....G-CT.....GGT
R. sauteri (Sanyi)GC.....TA-TT-C.....CT-A.....C.....T.....C-C-G-CC.....GGG-C-T
R. sauteri (Wulai)TC-CGG-CA-T.....A-CT-A.....T.....C.....T.....TG-GG-CT.....CGCC-C
R. amurensisC-C.....TA-T-C.....TT-C-T.....C.....T.....T-C-T.....GGG-G
R. tagoiC-CG-TA-T.....CT-A.....T.....C.....T-T-TG-A-CC.....CGG-T
R. okinavanaC-T.....TA-T-C.....C-A.....C.....C-T-T-TG-GGCC-G-CGG
R. tsushimensisTC-C-G-CA-TG.....A-CT-A.....T.....C.....C-T-T-TG-GGCC-G-CGG
R. japonicaC-T.....TA-T-C.....CT-A.....C.....T-C.....TG-A-CC.....GGTA-C
R. ornativentrisC-T.....CA-T-T-C.....CT-A.....C.....C-T.....G-A-CC-T.....GGT-C
R. piricaC-C.....CA-T.....CT-G.....T-T-T-C-T.....T-C.....TG-A-CC-T.....GGT-C

made to confirm this idea. In order to assess the taxonomic position of *R. zhengi* and further ascertain the invalidity of *Pseudorana*, we tried in the present study to clarify the phylogenetic relationships of *R. zhengi* with ten species of brown frogs from Japan, Taiwan, Russia, and Europe on the basis of sequence variation in the mitochondrial cytochrome b gene.

MATERIALS AND METHODS

We extracted DNA from small amounts (less than 50 mg) of ethanol preserved tissue samples from liver and muscles of one *R. zhengi* from Sichuan, China. Tissues from specimens of *R. temporaria* and *R. dalmatina* from Czech and Austria were also used for the analyses (see appendix). We amplified a part of the mtDNA cytochrome b gene using the polymerase chain reaction (PCR) and sequenced approximately 600 bp of this gene following Tanaka et al. (1994, 1996). Primers used for the amplification and sequencing were: L14850-1 (5'-TCTCCGCATGATGAAACTTCGGCTC-3': Tanaka et al., 1994), L14850-2 (5'-TTCATCCTGATGAAACTTTGGCTC-3': Tanaka et al., 1996), and H15502 (5'-GGATTAGCTGGTGTGAAATTGTCTGGG-3': Tanaka-Ueno et al., 1998b). We adopted the sequence numbering system of the human sequence (Anderson et al., 1981).

We estimated genetic distance among haplotypes based on the pairwise matrix of nucleotide sequence divergences calculated by Kimura's (1980) two-parameter method with the DNADIST program in PHYLIP Version 3.5c (Felsenstein, 1993). A distance tree was generated with the neighbor-joining algorithm (Saitou and Nei, 1987) in the NEIGHBOR program of the PHYLIP package (Felsenstein, 1993). For maximum

likelihood (ML) analysis, we employed Felsenstein's (1993) DNAML algorithm with empirical base frequencies and an expected transition-transversion bias of 2.0. Degrees of support for internal branches in each tree were assessed by 1000 bootstrap pseudoreplications (Felsenstein, 1985). Maximum parsimony (MP) analysis of unweighted, unordered character-state data was performed with the heuristic search option using the tree bisection-reconnection (TBR) branch-swapping in PAUP* version 4.0b (Swofford, 1998). Confidence in each node was assessed by 1000 bootstrap replications.

For comparisons, we used the published sequences of two populations of *R. sauteri* (sensu Chou and Lin, 1997) from Taiwan, six species from Japan (*R. pirica*, *R. ornativentris*, *R. japonica*, *R. okinavana*, *R. tagoi*, and *R. tsushimensis*), one species from Russia (*R. amurensis*), and an outgroup taxon, *R. catesbeiana* (Tanaka-Ueno et al., 1998a, b). We also incorporated a published sequence of *Xenopus laevis* (Dunon-Bluteau et al., 1985) into the analysis as that for another outgroup.

RESULTS

We could constantly obtain nucleotide sequence data of 587 bp for all samples (Fig. 1). Samples of *R. dalmatina* from Czech and Austria showed an identical sequence. In the neighbor-joining tree (Fig. 2A), monophyly of *R. zhengi* and the brown frogs was supported in high iterations of 88.1%. Nevertheless, *R. zhengi* exhibited the earliest divergence among the ingroup taxa and the monophyly of the brown frogs was also supported in high iterations of 84.0%.

Among the brown frogs, two European species formed a cluster and diverged next

FIG. 1. Aligned sequences of a 587-bp segment of the cytochrome b gene. Dots indicate identity with the sequence of *Xenopus laevis*.

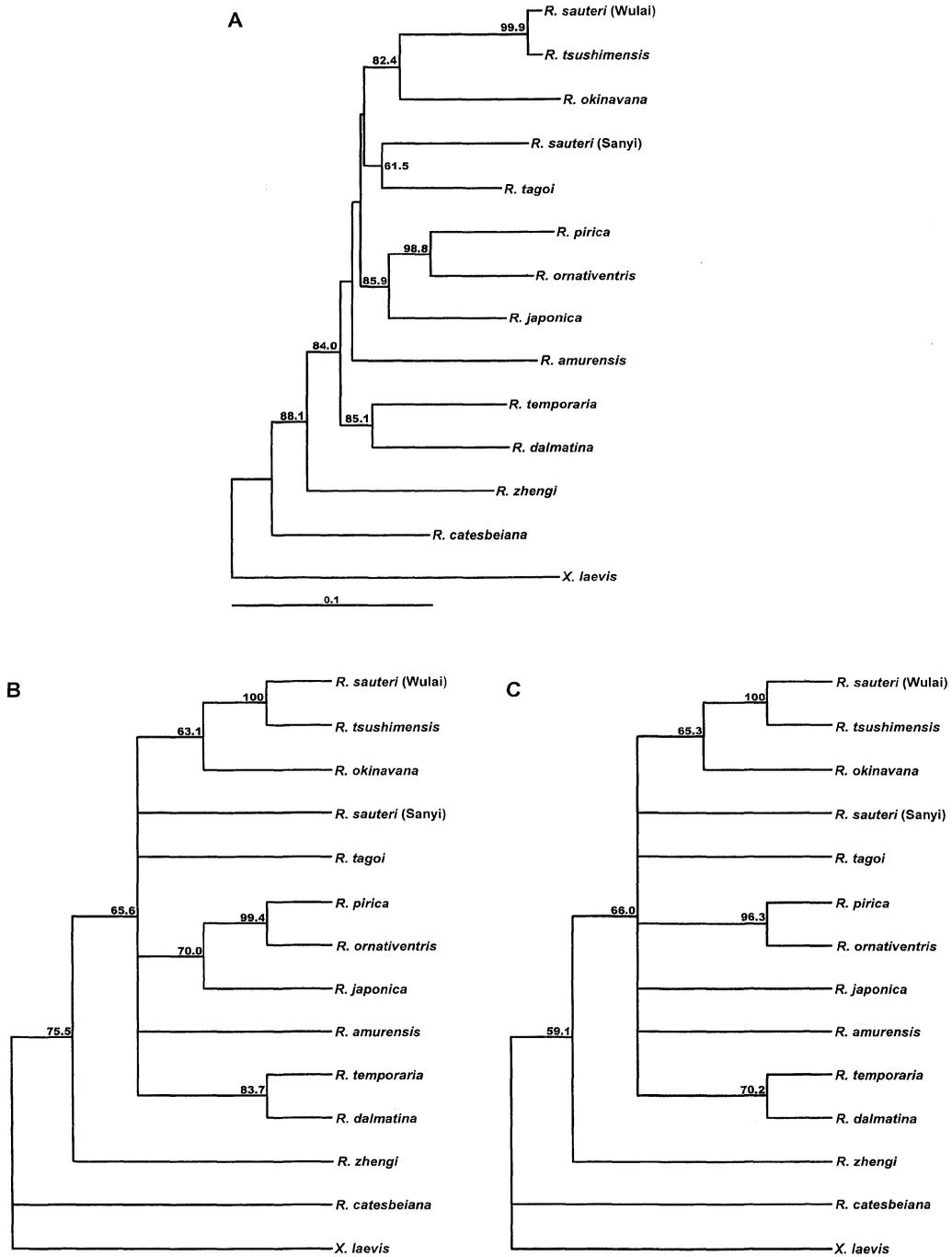


FIG. 2. A neighbor-joining tree rooted at the midpoint of the longest path (A), a maximum-likelihood tree (B), and a bootstrapped parsimony tree (C). Nodal values in all trees indicate percent support for branches in 1,000 bootstrap replicates (BP). BP values less than 50% are omitted.

to *R. zhengi*. The order of divergence among the remaining Asian brown frogs was the same as that reported by Tanaka-Ueno et al. (1998b). However, most of these branches were supported in very low iterations of less than 50%, and the relationships within the brown frogs were viewed as polytomous including five clusters. First, two European species formed a cluster (Group 1) supported in iterations of 85.1%. Then, *R. amurensis* diverged from all the remaining brown frogs (Group 2).

The latter consisted of two major clusters, of which one, supported by iterations of 85.9%, contained *R. japonica*, *R. pirica*, and *R. ornativentris* (Group 3). Within this group, sister relationships of *R. pirica* and *R. ornativentris*, both characterized by $2n=24$ chromosomes against $2n=26$ in most other brown frogs including *R. japonica* (Tanaka et al., 1996), was supported in high iterations of 98.8%.

The other major cluster contained two clusters, one consisting of *R. tagoi* and *R. sauteri* from Sanyi (Group 4: supported in 61.5% iterations), and the other of *R. okinavana*, *R. tsushimensis*, and *R. sauteri* from Wulai (Group 5: supported in 82.4% iterations). In Group 5, a sister relationship of *R. tsushimensis* and *R. sauteri* from Wulai was supported in very high iterations of 99.9%.

In this way, *R. zhengi* was found to be greatly diverged from the brown frogs including European species, and was not close to *R. sauteri* from Sanyi or Wulai.

In the maximum-likelihood tree (Fig. 2B), bootstrap supports of monophyly of *R. zhengi* and the brown frogs (75.5%), and of the brown frogs (65.6%) were weaker than in the neighbor-joining tree. Brown frogs exhibited polytomous relationships and the monophyly of *R. tagoi* and *R. sauteri* from Sanyi (Group 4 of the neighbor-joining tree) were supported in low iterations of 47.7%. Supports of monophyly of Groups 3 (70.0%) and 5 (63.1%) recognized in the neighbor-joining

tree were also weaker, while those of the other groups were nearly the same as in the neighbor-joining tree.

In the maximum-parsimony tree (Fig. 2C), monophyly of *R. zhengi* and the brown frogs, and of the brown frogs were supported as in the neighbor-joining tree, although iterations were lower (59.1% and 66.0%, respectively). This analysis resulted in a polytomous consensus tree encompassing seven clades, and monophyly were supported only for Group 1 (supported by 70.2% iterations), Group 5 (65.3%), and the *R. pirica*-*R. ornativentris* clade of Group 3 (96.3%). The sister relationship of *R. tsushimensis* and *R. sauteri* from Wulai was supported by 100% iterations, whereas that of *R. japonica* with the species with 24 chromosomes (*R. pirica* and *R. ornativentris*) was not supported, unlike in the neighbor-joining tree.

DISCUSSION

From the results of the present study, monophyly of *R. zhengi* and the brown frogs, and of the brown frogs were supported. Nevertheless, relationships among the latter were not sufficiently resolved, and only sister relationships of (1) two European species (*R. temporaria* and *R. dalmatina*), (2) *R. tsushimensis* and *R. sauteri* from Wulai, (3) *R. okinavana* and the (2) species, and (4) *R. pirica* and *R. ornativentris* were unequivocally confirmed.

Also, it is highly probable that (5) *R. japonica* and the species with 24 chromosomes (*R. pirica* and *R. ornativentris*) are phylogenetically very close, and that *R. tagoi* is not remote from *R. sauteri* from Sanyi. On the other hand, relationships of *R. amurensis* with other brown frogs remain unclear, although the order of divergence among the Asian brown frogs in the neighbor-joining tree was identical with our previous results (Tanaka-Ueno et al., 1998b).

In all the three trees obtained, *R. zhengi* was located outside of the brown frogs

examined, and this clearly indicates an early divergence of the ancestral form of *R. zhengi* from the common ancestor of the brown frogs from both Europe and Asia, including *R. sauteri* from Taiwan (Chou and Lin, 1997; Tanaka-Ueno et al., 1998b).

We have already rejected Fei et al.'s (1990) and Dubois' (1992) idea of regarding *R. sauteri* from Taiwan as a member of a distinct genus or subgenus *Pseudorana*, and suggested that the species be included in the subgenus *Rana* of the genus *Rana* (Dubois, 1992) in our previous studies of the Taiwanese, Japanese, and Russian brown frogs (Tanaka-Ueno et al., 1998a, b). The present study again demonstrated a monophyly of the brown frogs including *R. sauteri*, and parallel divergences of this species in the course of brown frog evolution.

Rana sauteri is surely divergent from other brown frogs in having unique larval characteristics of complicate dentition on a large mouth and abdominal sucker in adaptation to a torrential life (Chou and Lin, 1997), but these characteristics can be viewed as results of convergent evolution (Tanaka-Ueno et al., 1998a).

Zhao (1999) first gave only a brief diagnosis of *R. zhengi* without providing pictures, and later redescribed it in longer form and showing a color photograph of two specimens that are not clearly designated as type series (Zhao, 2000a). In the latter paper, Zhao (2000a) also compared this species with *R. sangzhiensis* and *R. johnsi* under an implicit presumption of their close affinities to *R. zhengi*. These latter two species have been assigned to members of *Pseudorana* (Fei et al., 1990; Dubois, 1992).

Zhao (2000a) also described tadpoles of *R. zhengi* with rather complicate dentition (mostly I:4-4/4-4:I), but never mentioned the presence or absence of an abdominal sucker. *Rana johnsi*, which is now regarded as a distinct species (e.g., Inger et al., 1999), was first described as a subspe-

cies of *R. sauteri* (see Smith, 1921) and is superficially similar to the latter species in adult morphology. However, larvae of this species lack an abdominal sucker or large mouth parts, although dentition is complicate (I:5-5/4-4:I; Matsui's observation of the type series in the Natural History Museum, London). Most probably, this species belongs to a lineage distant from brown frogs of the *R. temporaria* group.

In the same journal (Sichuan Journal of Zoology) as he described *R. zhengi* (Zhao, 1999, 2000a), Zhao (2000b) referred to convergent properties of *R. sauteri* with torrent-dwelling *Amolops* species in the structure of larval ventral sucker without mentioning *R. zhengi*. He should have described this type of modification in *R. zhengi* if it was ever present in this species.

Thus, both morphologically and genetically, *R. zhengi* is considered to be remote from *R. sauteri*. In order to clarify the exact phylogenetic position of *R. zhengi* in the genus *Rana*, it is necessary to examine biochemically representatives of many subgenera proposed by Dubois (1992), as well as morphologically related species such as *R. johnsi* and *R. sangzhiensis*.

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APPENDIX

Material examined.—Sources of tissue samples from a total of four frogs are stored at the Graduate School of Human and Environmental Studies, Kyoto University (KUHE).

Rana zhengi Zhao, 1999 (N=1): Zhangcun Village, Hongya Co., Sichuan Province, China (KUHE 27951).

Rana temporaria Linne, 1758 (N=1): Frahi Lazhe, Czech (KUHE 28259).

Rana dalmatina Bonaparte, 1840 (N=2): U Soukupu, the Czech Republic (KUHE 28260); Vienna, Austria (KUHE 26568).

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