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Phylogenetic Relationships of the Chinese Brown Frogs (Genus *Rana*) Inferred from Partial Mitochondrial 12S and 16S rRNA Gene Sequences

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Based on partial sequences of the 12S and 16S ribosomal RNA genes, we estimated phylogenetic relationships among brown frogs of the *Rana temporaria* group from China. From the phylogenetic trees obtained, we propose to include *Rana zhengi* in the brown frogs. Monophyly of the brown frogs was not unambiguously supported, but four well-supported clades (A, B, C, and D) always emerged, although relationships among them remained unresolved. Clade A contained brown frogs with 24 chromosomes and was split into two distinct subclades (Subclade A-1: *R. chensinensis* and *R. huanrenensis*; Subclade A-2: *R. dybowskii*). Polytomous relationships among populations of *R. chensinensis* and *R. huanrenensis* suggested the necessity of further taxonomic assessment. *Rana kunyuensis* proved to be the sister group to *R. amurensis*, and these two species formed Clade B. Clade C was composed of *R. omeimontis* and *R. chaochiaoensis*, and Clade D included *R. sauteri*, which has been placed in other ranid genera. These relationships did not change after adding published data, and monophyly of Subclade A-1, A-2, and other East Asian brown frogs with 24 chromosomes (*R. pirica* and *R. ornativentris*) was ascertained, though their relationships were unresolved. Clade C, together with *R. japonica* and *R. longicrus*, also formed a monophyletic group. Brown frogs related to Clades A and C were estimated to have dispersed from continental Asia to adjacent regions through multiple events.

Key words: brown frogs, China, *Rana*, phylogeny, biogeography

INTRODUCTION

Ranine brown frogs of the *Rana temporaria* group (Boulenger, 1920) are characterized by the possession of a prominent dorsolateral fold and dark temporal mask, and absence of horizontal grooves at the digital tips (Liu and Hu, 1961); they encompass about 30 species widely occurring in Eurasia (Dubois, 1992; Frost, 2004). The main center of diversification, however, is in East Asia, where about 20 species are recognized, including recently described species (Ye *et al.*, 1995; Lu and Li, 2002) and the *R. sauteri* species complex (Chou and Lin, 1997), which is sometimes placed in other ranid genera (Frost, 2004, but see Matsui *et al.*, 2006).

Brown frogs are notoriously difficult to identify because of their close morphological similarities. However, it has long been known that they include two groups that are characterized by different number of chromosomes (2n=24 and 26). Species with 24 chromosomes include several East Asian and a European species (*R. arvalis* Nilsson, 1842), and independent phylogenetic relationships of these Asian and European members were clarified by isozyme and karyotype analyses (Green and Borkin, 1993). More recently, relationships among these 2n=24 species and the 2n=26 species were clarified through DNA analyses (*e.g.*, Matsui *et al.*, 1998; Tanaka-Ueno *et al.*, 1998b; Sumida *et al.*, 2003). These studies, however, were based mainly on species from outside China, and reports on Chinese species in particular are meager (Jiang and Zhou, 2001; Yang *et al.*, 2001). This paucity of comprehensive analyses on Chinese brown frogs hinders our understanding of the evolutionary history of this group in East Asia. Moreover, previous molecular phylogenetic works on East Asian brown frogs were based chiefly on the cyt b gene (Tanaka *et al.*, 1996; Tanaka-Ueno *et al.*, 1998a, b, c, 1999; Matsui *et al.*, 1998; Yang *et al.*, 2001; Kim *et al.*, 2002) and analyses from other gene partitions are much more limited (Jiang and Zhou, 2001, 2005; Sumida *et al.*, 2003).

In this study, we reconstructed the phylogeny of Chinese brown frogs using 12S and 16S rRNA gene sequences, with the objectives of (1) testing the monophyly

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of Chinese brown frogs with 24 chromosomes, and (2) providing a robust phylogenetic hypothesis to assess and revise the current classification of Chinese brown frogs based on morphological studies. Additionally, we studied the phylogenetic relationships among brown frogs from the whole of East Asia by combining our data with those already published.

MATERIALS AND METHODS

Specimens

Twenty-one specimens from 15 populations, representing seven brown frog species, were examined in this study (Table 1, Fig. 1). Of these, Taiwanese *R. sauteri* Boulenger, 1909 is very difficult to identify, because it is morphologically very similar to its sister species, *R. multidenticulata* Chou and Lin, 1997 (*cf*. Tanaka-Ueno *et al.*, 1998a). Our identification relied on the locality where the specimen was collected (Kaohsiung). These samples cover all Chinese brown frogs, exception for *R. chevronta* Hu and Ye *In* Hu, Fei, and Ye, 1978, *R. zhenhaiensis* Ye, Fei, and Matsui, 1995, and *R. multidenticulata*. We chose *R. zhengi* Zhao, 1999, *R.* (*Aquarana*) *catesbeiana* Shaw, 1802, and *R.* (*Pelophylax*) *shuchinae* Liu, 1950 as outgroup taxa based on the result of Matsui *et al.* (2001) and our own unpublished data. Because there are disagreements in ranid classification at the specific and generic levels, we basically followed the taxonomical scheme proposed by Frost (2004).

Extraction, amplification, sequencing, and alignment

Muscles or liver-tissue samples were collected and stored in 95% or 100% ethanol. DNA was extracted using the standard 3-step phenol/chloroform method. Partial sequences of the mitochondrial 16S rRNA and 12S rRNA genes were chosen as target fragments and amplified with published primers. Primers FS01 (5'-AAC GCT AAG ATG AAC CCT AAA AAG TTC T-3') and R16 (5'-ATA GTG GGG TAT CTA ATC CCA GTT TGT TTT-3') were used for amplification and sequencing of an approximately 410 bp segment of 12S (Sumida and Ogata 1998, Sumida *et al.* 2000a, b). Primers F51 (5'-CCC GCC TGT TTA CCA AAA ACA T-3') and R51 (5'-GGT CTG AAC TCA GAT CAC GTA-3') were used for an approximately 550 bp segment of 16S (Sumida *et al.*, 2002). Amplification was performed in 50-μl reaction volumes under the following conditons for both the 12S and 16S fragments: initial denaturation step for 4 min at 95°C; 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C; and a final extension for 10 min at 72°C. PCR products were gel-purified and directly sequenced using the BigDye V3.0 protocols with the same primers as used for PCR amplification, and an ABI 3700 DNA sequencer (Applied Biosystems). All PCR products were sequenced from both directions. All sequences were aligned with ClustalX 1.8 (Thompson *et al.*, 1997) and verified by eye based on their secondary structures.

Phylogenetic Analysis

Base compositional information was estimated using MEGA 3.0 (Kumar *et al.*, 2004). Prior to phylogenetic analysis, we used Modeltest 3.06 (Posada and Crandall, 1998) to select a best-fit substitution model by hierarchical likelihood rate test (hLRT). Three different methods of phylogenetic reconstruction were employed: (i) maximum parsimony (MP), with gaps treated as missing data, equal weighting for transitions and transversions, heuristic search with TBR branch-swapping, and 1,000 random-addition replicates; (ii) maximum-likelihood (ML) analysis based on the substitution model and phylogenetic parameters identified as optimal by Modeltest 3.06; and (iii) Bayesian inference using the same substitution model as ML, performed in two separate runs with four Markov chains. Each run was conducted for 15,000,000 generations and sampled every 100 generations. The log-likelihood scores were found to stabilize after 400,000 generations (4,000 samples) of each run. Therefore, the initial 4,000 samples were discarded as burn-in, and the remaining samples were used to estimate a consensus tree and to calculate Bayesian posterior probabilities. The MP and ML analyses were conducted with PAUP* 4.0b10a (Swofford, 2003), and the Bayesian analyses were performed using MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). Robustness of MP and ML tree topologies was estimated by bootstrap analyses (Felsenstein, 1985), with 1,000 replicates for MP and 100 replicates for ML. Only bootstrap values 70% or greater were regarded as sufficiently supporting topologies in MP and ML (Huelsenbeck and Hillis, 1993). For the Bayesian analyses, posterior probabilities 95% or greater were considered significant (Leaché and Reeder, 2002).

RESULTS

A total of 959 sites was obtained in the combined alignment, including 544 sites for 16S and 415 sites for 12S. All these sequences are deposited in GenBank (accession nos. DQ289078–DQ289127). Among the sites, 202 were variable and 138 were parsimony-informative. A total of 25 haplotypes were recognized among 10 species, including outgroups.

The best substitution model derived from MODELTEST was TrN+I+G selected by hLRT, with the gamma shape parameter estimated as 0.5101 and the proportion of invariant sites estimated as 0.5276. Bayesian inference and ML produced almost identical topologies, and only the Bayesian tree is shown in Fig. 2a. MP yielded one most parsimonious tree (*L*=361, CI=0.651, RI=0.793; Fig. 2b). These three analyses indicated the following relationships, when statistical reliability was taken into consideration (Fig. 2):

(i) Monophyly of brown frogs and *R. zhengi* with respect to the outgroup taxa *R. catesbeiana* and *R. shuchinae* was supported (100%, 94%, and 97% support for Bayesian posterior probability and ML and MP bootstrap values, respectively).

(ii) Monophyly of brown frogs was strongly supported by Bayesian analysis (99%), but weakly by ML (75%) and not by MP (55%).

(iii) Four distinct clades (Clades A–D) were recognized, but their relationships remained unresolved.

(iv) Species of brown frogs with 24 chromosomes formed a monophyletic group (Clade A; 100%, 98%, and 82% support).

(v) Within Clade A, *R. chensinensis* David, 1875 from Neimenggu, Sichuan, Gansu, and Shaanxi, and *R. huanrenensis* Fei, Ye, and Huang, 1991 "1990" composed a well supported subclade (Subclade A-1; 100%, 99%, and 100% support) that was the sister group to *R. chensinensis* from Jilin and Heilongjiang (Subclade A-2; 100% support).

(vi) Within Subclade A-1, populations of *R. chensinensis* from Sichuan and Gansu formed a monophyletic group (99%, 95%, and 93% support), but their relationships with *R. chensinensis* from Neimenggu and Shaanxi and *R. huanrenensis* were not resolved.

(vii) *Rana amurensis* Boulenger, 1886 and *R. kunyuensis* Lu and Li, 2002 were grouped as sister species (Clade B; 100%, 99%, and 97% support).

(viii) Two species with 26 chromosomes (*R. omeimontis* Ye and Fei in Ye, Fei, and Hu, 1993 and *R. chaochiaoensis* Liu, 1946) formed a monophyletic group (Clade C; 100% support).

Table 1. Samples used in this study, and GenBank accession numbers.

Species	Locality*	Voucher**	Accesion number	
			12S	16S
Rana amurensis	China: Harbin, Heilongjiang (1)	SYNU040004	DQ289085	DQ289110
Rana chaochiaoensis	China: Zhaojue, Sichuan (2)	SCUM0405170CJ	DQ289082	DQ289107
	China: Zhongdian, Yunnan (3)	SCUM045098WD	DQ289080	DQ289105
		SCUM045096WD	DQ289081	DQ289106
Rana chensinensis	China: Huxian, Shaanxi (4)	KIZ-RD05SHX001	DQ289094	DQ289119
		KIZ-RD05SHX002	DQ289095	DQ289120
		KIZ-RD05SHX003	DQ289096	DQ289121
	China: Hohhot, Neimenggu (5)	SCUM0502WJW	DQ289093	DQ289118
	China: Maowen, Sichuan (6)	SCUM0405202YJ	DQ289087	DQ289112
	China: Huixian, Gansu (7)	SCUM04GP1	DQ289088	DQ289113
		SCUM04GP2	DQ289092	DQ289117
	China: Hongyuan, Sichuan (8)	SCUM045013CJWD	DQ289089	DQ289114
	China: Zoigê, Sichuan (9)	KIZ-RD05REG001	DQ289090	DQ289115
		SCUM045101WD	DQ289091	DQ289116
	China: Tonghua, Jilin (10)	SCUM05JLCJ96	DQ289098	DQ289123
		SCUM05JLCJ98	DQ289100	DQ289125
	China: Shangzhi, Heilongjiang (11)	SYNU040002	DQ289099	DQ289124
Rana huanrenensis	China: Huanren, Liaoning (12)	SYNU040006	DQ289097	DQ289122
Rana kunyuensis	China: Mt. Kunyu, Shandong (13)	CIB-HUI040001	DQ289086	DQ289111
Rana omeimontis	China: Zhangcun, Hongya, Sichuan (14)	SCUM0405196CJ	DQ289083	DQ289108
Rana sauteri	China: Kaohsiung, Taiwan (15)	SCUM0405175CJ	DQ289084	DQ289109
Rana catesbeiana	China: Chengdu, Sichuan	SCUM0405176CJ	DQ289102	DQ289127
Rana shuchinae	China: Zhaojue, Sichuan	CIB-HUI040009	DQ289101	DQ289126
Rana zhengi	China: Zhangcun, Hongya, Sichuan	SCUM0405190CJ	DQ289078	DQ289103
		SCUM0405189CJ	DQ289079	DQ289104

* Locality numbers correspond to those in Fig. 1.

** SCUM=Zoological Museum of Sichuan University, China; SYNU=Shenyang Normal University, China; CIB = Chengdu Institute of Biology, the Chinese Academy of Sciences, Chengdu; KIZ=Kunming Institute of Zoology, the Chinese Academy of Sciences, Kunming.

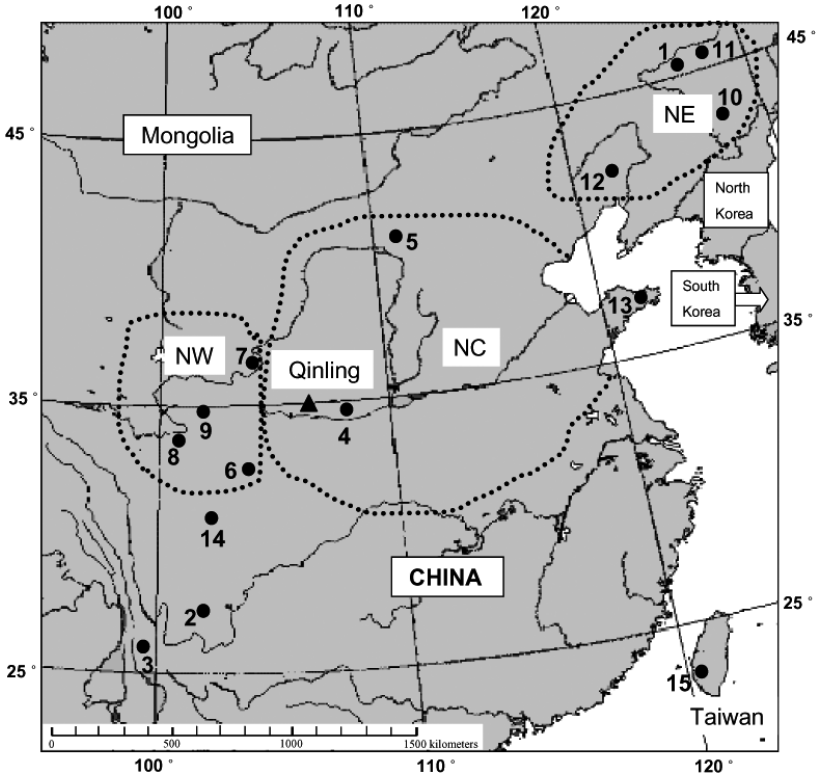

Fig. 1. Map showing sampling localities of Chinese brown frogs used in the present study. For the locality numbers, refer to Table 1. For convenience, the sampling sites were grouped into three units based mainly on geographical topology: northwest (NW), north and central (NC), and northeast (NE) China.

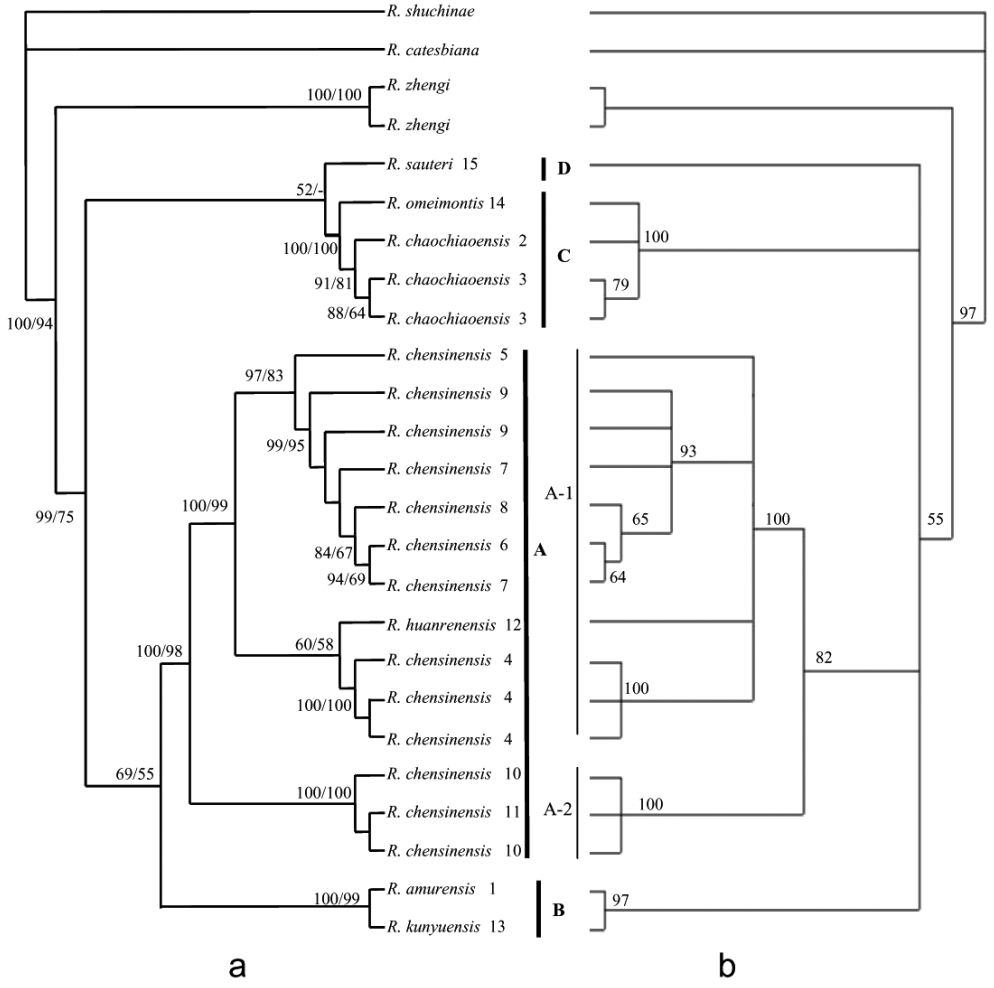

Fig. 2. **(a)** Bayesian and **(b)** MP trees based on a 959-bp sequence of the 12S and 16S rRNA genes for Chinese brown frog species. Numbers near nodes represent Bayesian posterior probability/bootstrap support for ML (100 replicates) inference (a), and bootstrap support for MP (1,000 replicates) (b), for the respective clade (only≥50% retained).

DISCUSSION

Phylogenetic relationships of *Rana zhengi* and brown frogs

Monophyly of brown frogs was only ambiguously supported in our results, with relatively low nodal support in the MP tree. Instead, brown frogs constituted a robust clade including *R. zhengi*, which was initially chosen as an outgroup taxon. *Rana zhengi* was relatively recently described (Zhao, 1999), and its systematic status remained unclear. Fei and Ye (2001) relegated it as a synonym of *R. johnsi* Smith, 1921 and placed it in the genus *Pseudorana* (Fei *et al.*, 1991 "1990"), which was treated as a subgenus of *Rana* by Dubois (1992). Tanaka-Ueno *et al.* (1998a) and Matsui *et al.* (2001), however, rejected the valid generic/subgeneric status of *Pseudorana* from analyses of cyt b sequences. Our present result from 12S and 16S rRNA sequences agrees with that of Matsui *et al.* (2001), who showed a close relationships of *R. zhengi* to brown frogs. Furthermore, our preliminary molecular analysis (Che *et al.*, unpublished data) indicated *R. zhengi* to be remote from *R. weiningensis* Liu, Hu, and Yang, 1962, the type species of *Pseudorana* (Fei *et al.*, 1991 "1990"; Dubois, 1992). In external and anatomical characters, other than the presence of horizontal grooves at the ends of the toes, *R. zhengi* is actually similar to brown frogs (Zhao, 2000; G. Wu, personal communication).

Rana sauteri, long considered a member of the brown frog group (Frost, 1985), was also moved to *Pseudorana* at the generic level by Fei *et al.* (1991 "1990") and the subgeneric level by Dubois (1992) because of the presence of digital discs and horizontal grooves. Furthermore, because of the unique morphology of its larvae, *R. sauteri* was elected as the type species of *Pseudoamolops* (Jiang *et al.*, 1997). However, studies by Tanaka-Ueno *et al.* (1998a) from cyt b sequences, and Matsui *et al.* (2006) from 12S and 16S rRNA sequences indicated this species to belong in *Rana* as a brown frog. Our present results from the 12S and 16S rRNA genes also support this view, though the relationships of *R. sauteri* (Clade D) to other brown frogs (Clades A, B, and C) remained unresolved. Although brown frogs were once defined by the absence of digital discs and horizontal grooves (*e.g.*, Liu and Hu, 1961), we propose here to include *R. zhengi*, like *R. sauteri*, as a member of brown frogs on the basis of molecular evidence. Digital discs, sometimes equipped with horizontal grooves, are found in various frog lineages and are considered to act as adhesive organs (Liu

and Hu, 1961). These organs could have evolved convergently as a result of adaptation to habitats that require climbing. Thus, the absence of digital discs and horizontal grooves at the tips of the toes should not be considered as synapomorphies for brown frogs.

Relationships of Chinese brown frogs with 24 chromosomes

Though the relationships among Chinese species traditionally included among the brown frogs were not resolved from our data, four well-supported clades, A, B, C, and D were consistently recognized (Fig. 2). Clade A contained species with 24 chromosomes (*R. chensinensis* and *R. huanrenensis*), while the other three clades contained species with 26 chromosomes. These latter clades, Clade B, C, and D, included *R. amurensis*+*R. kunyuensis*, *R. omeimontis*+*R. chaochiaoensis*, and *R. sauteri*, respectively. Clear monophyly of brown frogs with 24 chromosomes, as found among the Chinese populations, was concordant with previous studies using species mainly from outside China. For example, Nishioka *et al.* (1992) analyzed allozymes of Japanese, Russian, and some Chinese brown frogs and obtained a UPGMA tree in which brown frogs with 24 chromosomes formed a single group. However, the method of analysis they employed yielded only a phenogram, which does not always indicate phylogenetic relationships. From analyses of isozyme and karyotype variation, Green and Borkin (1993) proposed monophyly of the East Asian brown frogs with 24 chromosomes and their separation from European *R. arvalis*, which also has a 2n=24 karyotype. More recent studies based on DNA sequences (*e.g.*, Tanaka-Ueno *et al.*, 1998b; Sumida *et al.*, 2003) also indicated the monophyly of East Asian brown frogs with 24 chromosomes.

In contrast, the results of Jiang and Zhou (2001) and Yang *et al.* (2001) for Chinese species were inconsistent with these previous results, as well as our result. In the study of Jiang and Zhou (2001) based on 12S rRNA, monophyly of brown frogs with 24 chromosomes was not supported, because *R. amurensis* (with 26 chromosomes) and *R. chensinensis* from Heilongjiang formed a clade, which was the sister group to the cluster of *R. chensinensis* from Gansu and *R. huanrenensis*. On the other hand, the result of Yang *et al.* (2001) based on cyt b indicated a sister-group relationship of *R. huanrenensis* to all other Chinese species with 24 and 26 chromosomes.

In our results, the clade of frogs with 24 chromosomes (Clade A) was clearly split into two subclades (A-1 and A-2). Subclade A-1 contained populations of *R. chensinensis* from northwestern China (NW: northwestern Sichuan and Gansu), north and central China (NC: Neimenggu and Shaanxi), and *R. huanrenensis*, while Subclade A-2 consisted solely of *R. chensinensis* from northeastern China (NE: Jilin and Heilongjiang). We consider these two subclades as separate species (see below about the Hashima frog). In Subclade A-1, however, the topotypic population of *R. chensinensis* from Shaanxi was not the sister group to any of the conspecific populations from NW China and Neimenggu (NC), nor to *R. huanrenensis* (also topotypic). Relevant to this result, Xie *et al.* (2000) recently separated NW China populations of *R. chensinensis* from NC China populations and resurrected the name *R. kukunoris* Nikolskii, 1918 for the former (confined to east of Xizang, Qinghai, northwest of Sichuan, and Gansu). According to Xie *et al.* (2000), *R. kukunoris* is morphologically distinct from *R. chensinensis* from Shaanxi and Beijing. However, from our own observations, it is not easy and is sometimes impossible to distinguish NW China populations (Huixian of Gansu and the northwest of Sichuan) from other populations in China, including the Shaanxi (type locality) population.

In Subclade A-1 in the trees obtained, *R. chensinensis* was not the sister group to *R. huanrenensis*, although they were very close to each other, as already suggested by Jiang and Zhou (2001). Our result thus contradicted the opinion of Yang *et al.* (2001), who considered *R. huanrenensis* to be a distinct evolutionary lineage among brown frogs. Characters that diagnose *R. huanrenensis* include primarily morphological, distributional, and ecological ones. *Rana huanrenensis* usually breeds in medium-sized mountain rivers with slowly flowing water and many large stones on the bottom, and females attach eggs to the upper surfaces of stones (Liu *et al.*, 2004). This breeding habit differs from that of most other brown frogs, including *R. chensinensis*, in which eggs are laid on the bottom of stagnant waters such as open pools and ponds. Morphologically, *R. huanrenensis* is diagnosed by the absence of vocal sacs. However, some specimens of *R. chensinensis* from Sichuan have been found to lack vocal sacs (G. Wu, personal communication). Thus, the known differences between *R. huanrenensis* and *R. chensinensis*, at least with regard to morphology, probably represent intraspecific polymorphisms.

Generally, remote relationships obtained by phylogenetic analyses suggest taxonomic distinction among the taxa in question, but not vice versa. For example, a biologically distinct bufonid, *Bufo torrenticola* Matsui, 1976, is nested within populations of *B. japonicus* Temminck and Schlegel, 1838 (Igawa *et al.*, 2006). Also, several pairs of distinct ranid species in the genus *Amolops* Cope, 1865 are very close to each other in terms of genetic distance, compared to other congeneric species (Matsui *et al.*, 2006). Therefore, we refrain from further discussion and only emphasize the necessity of future taxonomic study of the brown frogs of Subclade A-1.

What seems to be clearer is the distinct species status of Subclade A-2, which contained NE China (Jilin and Heilongjiang) populations of *R. chensinensis*. Liu and Hu (1961) first noted that the NE China population of *R. chensinensis* is called the “Hashima-frog” and has characteristic oviducts that swell very large in preservative. This phenomenon has never been observed in populations from other regions. From the similarity of this oviductal feature, Matsui *et al.* (1993) suggested the Hashima frog to be more closely related to Japanese *R. pirica* than to “true” *R. chensinensis* from Shaanxi. As discussed below in comparisons of brown frogs from inside and outside China, the Hashima frog forms a clade with a brown frog from the Maritime territory of Russia identified as *R. dybowskii* Günther, 1876 [type locality Abrek Bay, near Wladiwostok (=Vladivostok), Russia] by Matsui *et al.* (1998) and Kuzmin and Maslova (2005), and this name should be applied to Subclade A-2. This conclusion is consistent with the opinion of Xie *et al.* (1999) based on the results of morphological analyses.

Relationships of other Chinese brown frogs

In our results, *R. amurensis* and *R. kunyuensis* exhibited a sister-group relationship with high support and formed distinct Clade B. In contrast, Jiang and Zhou (2001, 2005) found *R. amurensis* to group within brown frogs with 24 chromosomes. This discrepancy might have resulted from misidentification of specimens sampled by Jiang and Zhou (2001). When their 16S data (AF315133) are compared with corresponding data from other sources (our own and those of Sumida *et al.*, 2003), their *R. amurensis* does not nest within other conspecific populations (see below). As already suggested by Liu and Hu (1961), brown frogs from China are highly polymorphic and difficult to identify, and misidentification could have occured.

Rana kunyuensis was recently described by Lu and Li (2002) and is known only from Mt. Kunyu in Shandong, China. To date, the species is very poorly known, and only a preliminary observation on postembryonic development is available (Sun *et al.*, 2003). Lu and Li (2002) associated this species with *R. chensinensis* on the basis of the similarly curved dorsolateral fold, but they also noted its similarity to *R. huanrenensis* and *R. amurensis* in the absence of vocal sacs and linea masculina. Sun *et al.* (2003) furthermore noted a similar labial-tooth formula between tadpoles of *R. kunyuensis* and those of *R. amurensis* (mostly I:I-I/III). Our present analyses showed *R. kunyuensis* to be closest to *R. amurensis*, rather than to *R. chensinensis* or *R. huanrenensis*.

Of the two species in Clade C, *R. chaochiaoensis* was once treated as a subspecies of Chinese *R. japonica* (Liu and Hu, 1961), which is now split into *R. omeimontis* (Ye *et al.*, 1993) and *R. zhenhaiensis* (Ye *et al.*, 1995; not available for our study). Our results agree well with this taxonomic history.

Comparison with brown frogs from outside China

Because we could examine only representative populations of Chinese brown frogs, we combined our own data with GenBank data for 17 sets of 12S and 16S rRNA gene sequences representing 12 species (Sumida *et al.*, 2003) and analyzed phylogenetic relationships (Fig. 3). These sequences include Russian *R. amurensis* (Maritime: AB058868, AB058886), *R. asiatica* Bedriaga, 1898 (Kirghizia: AB058866, AB058884), *R. chensinensis* (Maritime: AB058852, AB058870), and *R. temporaria* Linnaeus, 1758 (St. Petersburg: AB058864, AB058882); European *R. arvalis* (Luxembourg: AB058865, AB058883); Mongolian *R. amurensis* (AB058867, AB058885); Japanese *R. japonica* Günther, 1859 (Hiroshima: AB058858, AB058876 and Iwate: AB058859, AB058877), *R. dybowskii* (Tsushima: AB058855, AB058873), *R. ornativentris* Werner, 1903 (Hiroshima: AB058856, AB058874 and Aomori: AB058857, AB058875), *R. okinavana* Boettger, 1895 (Okinawa: AB058861, AB058879), *R. pirica* Matsui, 1991 (Hokkaido: AB058854, AB058872), and *R. tsushimensis* Stejneger, 1907 (Tsushima: AB058860, AB058878); and Chinese *R. longicrus* Stejneger, 1898 (Taipei: AB058863, AB058881), *R. chensinensis* (Beijing: AB058853, AB058871), and *R. amurensis* (NE China: AB058869, AB058887).

The results were less convincing than those obtained from Chinese frogs only, though essentially identical with regard to relationships among the Chinese populations. Brown frogs and *R. zhengi* again grouped as monophyletic (100%, 98%, and 98% support), but the monophyly of brown frogs was not supported (99%, <50%, and <50% support), resulting in nine clades, including *R. zhengi*. These included the four clades recognized in the analysis of Chinese frogs and five additional clades, four of which contained only a single species (*R. arvalis*, *R. asiatica*, *R. temporaria*, and *R. zhengi*). In the remaining clade, *R. okinavana* and *R. tsushimensis* exhibited a sister-group relationship (100%, 97%, and 98% support).

After including additional populations, East Asian species of brown frogs with 24 chromosomes still formed a monophyletic group (100%, 95%, and 93% support) and clearly split from European *R. arvalis*. This result is identical with that previously reported (Sumida *et al.*, 2003; Veith *et al.*, 2003), and paraphyly of brown frogs with 24 chromosomes between East Asia and Europe is almost certain. Reduction the diploid chromosome number from 26 to 24 in the two lineages is likely the result of parallel evolution. This time, however, the clade showed polytomy into five subclades. Two subclades recognized in Chinese frogs (A-1 and A-2) were again supported (100%, 100%, and 99% support, and all 100% support, respectively), but in addition to these, each of three Japanese species (*R. ornativentris*, *R. pirica*, and *R. dybowskii* from Tsushima) represented its own subclade. *Rana chensinensis* from Beijing grouped in Chinese Subclade A-1, but was not a sister group to any other population.

On the other hand, Russian *R. chensinensis* from the Maritime region and Chinese Subclade A-2 (Hashima frog) formed a monophyletic group (all 100% support). As already discussed above for the Hashima frog, the brown frog with 24 chromosomes from the Maritime region should be called *R. dybowskii*. From this result, the Hashima frog from NE China is again ascertained to belong to *R. dybowskii*. However, *R. dybowskii* was paraphyletic with the population from Tsushima, which formed its own subclade. The status of the Tsushima population of *R. dybowskii* as a distinct species has already been suggested by Tanaka-Ueno *et al.* (1998b) based on analyses of the cyt b gene. Populations genetically almost identical with the Tsushima population also occur in Korea (Matsui *et al.*, 1998; Kim *et al.*, 1999), and taxonomic study of these frogs is now underway (Matsui, unpublished).

Rana amurensis from the Maritime region, Russia, was very close to conspecific Chinese populations, and Clade B recognized in Chinese samples was again strongly supported (100%, 100%, and 99% support). As already discussed above, Jiang and Zhou's (2001) *R. amurensis* did not cluster within this clade (data not shown). Although previous studies suggested the early divergence of *R. amurensis* among East Asian brown frogs (Matsui *et al.*, 1998; Tanaka-Ueno *et al.*, 1998b, c; Veith *et al.*, 2003), our data neither supported nor rejected this hypothesis, although our trees tended to indicate a closer relationship of Clade B to Clade A than to Clade C.

Japanese *R. japonica* and Taiwanese *R. longicrus*, together with Clade C recognized in Chinese species, proved to be monophyletic (100%, 97%, and 99% support), and two populations of *R. japonica* (100%, 97%, and 91% support) were the sister clade to the group of other species

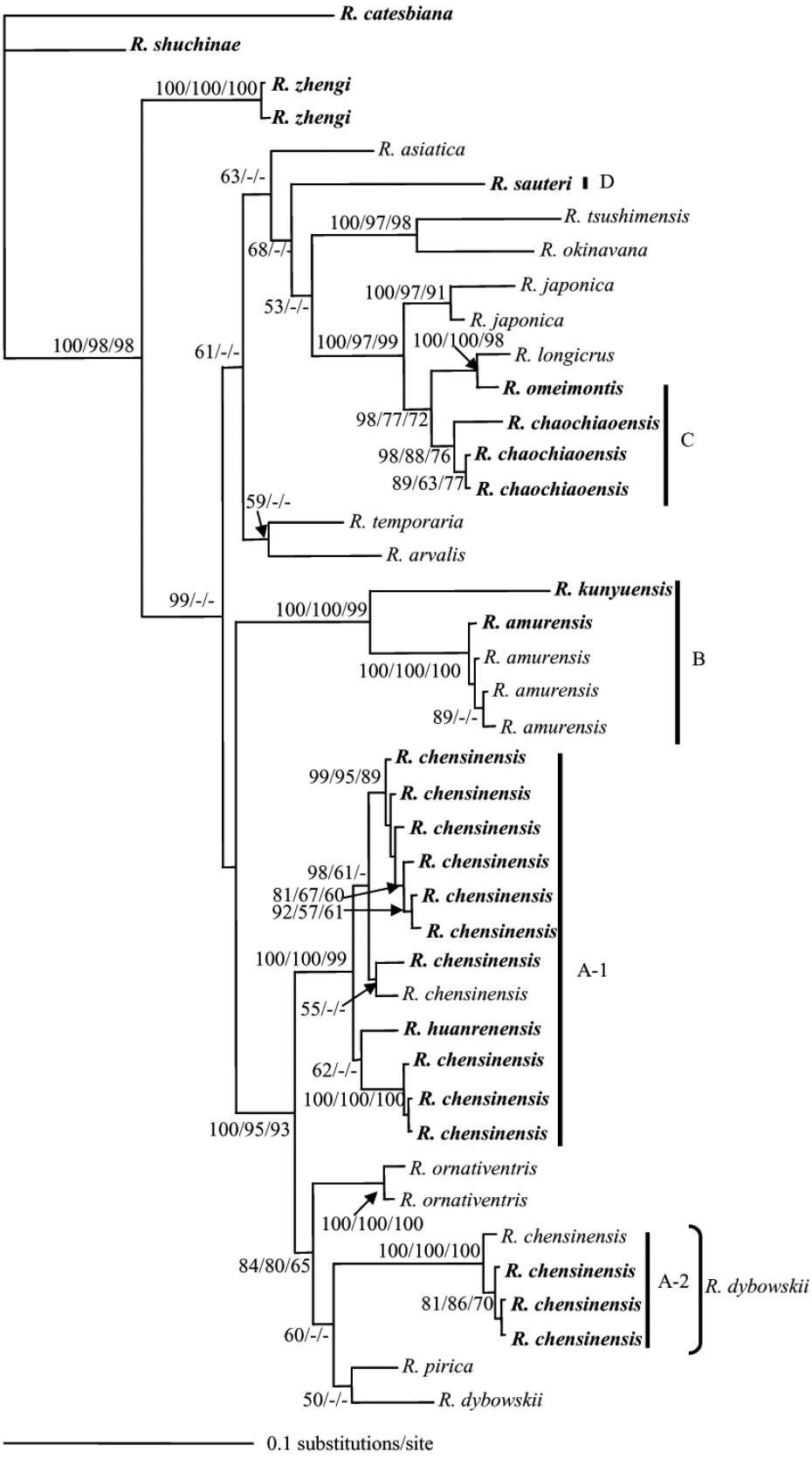

Fig. 3. Bayesian tree of a 962-bp sequence of the 12S and 16S rRNA genes for species of brown frogs from China and other Eurasian regions. Numbers near nodes represent Bayesian posterior probability/bootstrap support for ML (100 replicates)/MP (1,000 replicates) inference (only≥50% retained). Specimens for which we obtained sequences in the present study are shown in bold font.

(98%, 77%, and 72% support) in this new clade. Chinese *R. omeimontis* was the sister group to *R. longicrus* (100%, 100%, and 98% support), and these were the sister group to populations of *R. chaochiaoensis* (98%, 88%, and 76% support). As mentioned above, all these species with 26 chromosomes were once considered as conspecific with or subspecies of *R. japonica* (Liu and Hu, 1961) due to their morphological similarities. Our result is consistent with not only these previous views from morphology, but also with more recent results from molecular analyses (Jiang and Zhou, 2005, but see Tanaka-Ueno *et al.*, 1998a). It is almost certain that frogs previously associated with *R. japonica* are monophyletic. Finally, the sister-group relationship of *R. tsushimensis* and *R. okinavana* observed is identical with that reported previously (Sumida *et al.*, 2003).

In conclusion, *R. zhengi* and brown frogs of the *R. temporaria* group, including *R. sauteri*, form a monophyletic group, and placement of *R. zhengi* and *R. sauteri* in distinct genera or subgenera (Fei *et al.*, 1991 "1990"; Dubois, 1992; Jiang *et al.*, 1997; Fei and Ye, 2001) is rejected. As discussed above, we prefer to include these two species in the *R. temporaria* group of Boulenger (1920), enlarging the definition of the group (Liu and Hu, 1961). However, phylogenetic relationships among brown frogs were not well resolved, as in previous studies (Tanaka-Ueno *et al.*, 1998a; Sumida *et al.*, 2003; Veith *et al.*, 2003), particularly the complex nature of the relationships among the *R. chensinensis* populations. Further extensive sampling of populations from inside and outside China and sequencing of additional genes are needed for a more reliable reconstruction of the relationships among Chinese brown frogs.

Under these circumstances, we can infer very little about the phylogeography of Chinese brown frogs at present. What we can briefly discuss are possible dispersal events from continental China to adjacent islands. From our results, combined with the results from several previous studies (Matsui *et al.*, 1998; Tanaka-Ueno *et al.*, 1998a; Sumida *et al.*, 2003), it is almost certain that each of Chinese Clades A (represented by *R. chensinensis* and *R. dybowskii*) and C (represented by *R. omeimontis* and *R. chaochiaoensis*) have close relatives in adjacent regions. Combined phylogenetic analysis failed to elucidate the relationships of members related to Clade A, but relatives of this clade occur in Korea (*R.* sp.), on Tsushima (*R.* sp.), and on Hokkaido (*R. pirica*) and the other main islands of Japan (*R. ornativentris*). In contrast, closer relationships of members of Clade C to the species occurring on the main islands of Japan exclusive of Hokkaido (*R. japonica*) and on Taiwan (*R. longicrus*) are evident. Clearly, the patterns of distribution differ between these two groups. Relatives of Clade A occur mainly in the northern part, while those of Clade C occur in the southern part around the Japan Sea. These different distribution patterns between the two groups indicate their different dispersal histories from continental China. Reverse dispersals in these groups, i.e., from outside China to the continent, cannot be precluded, but seem to be less likely, judging from the wider distributions and more pronounced divergence patterns on the continent.

The ancestral stock that led to Clade A may have invaded adjacent islands via Korea and Sakhalin, while that that led to Clade C invaded via a more southern route. In the latter clade, occurrence of a relative in Taiwan (*R. longicrus*) can be easily explained by geographic proximity and known geohistorical events (Ota, 1998). However, the distribution of another clade on the main islands of Japan (*R. japonica*) is not easily explained, because relatives are absent from Korea, Tsushima, or the Ryukyu archipelago, which probably connected the main islands of Japan with the continent or Taiwan in the past. Instead, endemic brown frogs of different lineages (*R. coreana* in Korea, *R. tsushimensis* on Tsushima, and *R. okinavana* in the Ryukyus) occur in these intermediate regions (Tanaka-Ueno *et al.*, 1998a; Song *et al.*, 2006). Recent geological studies suggest the past presence of a land bridge between continental China and the southwestern part of the main islands of Japan (present-day East China Sea and Yellow Sea regions; Koizumi, 2000; Kitamura *et al.*, 2001), and ancestral *R. japonica* might have used this route for dispersal.

At present, we cannot infer further the phylogeography of the East Asian brown frogs from our limited data and information available from other literature sources, because clear phylogenetic relationships among the distinct clades recognized have not been resolved (Tanaka *et al.*, 1996; Matsui *et al.*, 1998; Tanaka-Ueno *et al.*, 1998b, c; Sumida *et al.*, 2003). However, it seems certain that there were multiple migrations, at least two of them, from continental Asia to adjacent islands by brown frogs with different evolutionary histories.

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