

Validity and Systematic Position of *Rana altaica* (*Rana*: Ranidae): Results of a Phylogenetic Analysis

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Abstract: In order to evaluate the phylogenetic position and validity of *Rana altaica*, we investigated the phylogeny of brown frogs in Eurasia by Bayesian Inference and Maximum Parsimony analyses of a fragment from the mitochondrial DNA gene Cytochrome *b*. Both analyses resolved *R. altaica* as nesting deeply within *R. arvalis*. Most samples of the nominal *R. altaica* from the Altai region and specimens from Central Siberia shared a haplotype with *R. arvalis* based on the network analysis. The matrilineal relationships suggested that *R. altaica* should be considered as a junior synonym of *R. arvalis*. Furthermore, our study suggested that the species group division of Chinese brown frogs should be re-evaluated within a phylogenetic context.

Key words: *Rana altaica*; *Rana arvalis*; *Rana arvalis altaica*; Cytochrome *b*; Demographic expansion

阿尔泰林蛙的物种有效性及其分类地位——来自系统发育分析的证据

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摘要: 为检验阿尔泰林蛙(*Rana altaica*)的系统发育地位及其物种有效性, 该文运用线粒体细胞色素 *b* 基因, 应用贝叶斯分析和最大简约方法构建了欧亚大陆分布的部分林蛙的系统发育关系。两种分析方法均支持阿尔泰林蛙在田野林蛙(*R. arvalis*)这一分支的内部。单倍型网络图显示来自阿尔泰地区和中西伯利亚地区所谓的阿尔泰林蛙与田野林蛙有共享单倍型。通过该文母系遗传发育分析结果显示阿尔泰林蛙种级地位不成立, 是田野林蛙的同物异名。另外, 该文实验分析结果提示, 对中国分布的林蛙内部种组划分应建立在系统进化关系的基础上重新进行评估。

关键词: 阿尔泰林蛙; 田野林蛙; 田野林蛙阿尔泰亚种; 细胞色素 *b*; 群体扩张

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Brown frogs of the *Rana temporaria* group distributed in Eurasia (Frost, 2009). Great morphological (Boulenger, 1920) comprise about 36 species widely similarity can make it difficult to identify species (Che et

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al, 2007a). China harbors 17 species of brown frogs (Frost, 2009). Most Chinese studies on this group of frogs focus on alpha taxonomy. Reconstructions of their evolutionary history and taxonomic status from a phylogenetic perspective remain to be explored. Recent studies use few gene markers and limited sampling (e.g., Yang et al, 2001; Jiang et al, 2001; Che et al, 2007a). The political boundary between China and neighboring countries often hampers systematic studies and the validity of some species distributed near the borders remains uncertain. *Rana altaica*, described in China by Ye et al (1981), represents such a case.

Kashchenko (1899) described a new subspecies of brown frog, *R. arvalis altaica*, from four localities in the Altai region of Russia south of Biisk Town. Subsequently, Kashchenko (1909) used the new combination *R. altaica* for brown frogs from Issyk-Kul Lake (Kyrgyzstan). In the first half of the 20th century, the epithet *altaica* was used to designate subspecies of *R. arvalis* or even a species (e.g., Nikolsky, 1905, 1918; Terentjev, 1927). Its distribution included Altai, Tuva, western and central Siberia, Kazakhstan and Kyrgyzstan. Terentjev (1927) described an infrasubspecific entity under the name *R. arvalis altaica* nation *issaitschikovi* from Arkhangelsk Town, Russia. This taxon was later considered to be a subspecies.

Stugren (1966) reports that typical *R. arvalis* coexist in the eastern areas with *R. altaica*. Morphological analyses of several thousand brown frogs from different areas fail to separate *R. arvalis* and *R. altaica* (Ishchenko, 1978). In overviews of the amphibians of the former Soviet Union, neither *R. altaica* (or *R. arvalis altaica*) nor *R. arvalis issaitschikovi* are considered as valid taxa (Banninov et al, 1977; Kuzmin, 1999; Kuzmin & Semenov, 2006). Populations from northern and central Kazakhstan are sometimes identified as *R. arvalis*, while those from southern Kazakhstan and Kyrgyzstan are referred to as *R. asiatica*. Recent studies on the genome size in *R. arvalis altaica* and *R. arvalis issaitschikovi* report that differences among these taxa and *R. arvalis arvalis* fall within the interpopulation variability typical for amphibians (Litvinchuk et al, 2008).

The brown frogs known as *R. altaica* have been found beyond the borders of the former USSR relatively recently. Ye et al (1981) discovered such populations in the Altai area of Xinjiang, China. These authors first used the name *R. altaica* (Kashchenko, 1899) for the frogs in this area, a determination accepted by most Chinese herpetologists (Fei et al, 1990, 2009; Fei, 1999;

Zhao & Adler, 1993). These frogs remain unstudied genetically, and molecular data might serve to identify the brown frogs from Altai.

China has three major species groups of brown frogs including the *R. longicrus* group, the *R. chensinensis* group, and the *R. amurensis* group (Fei et al, 2009). *Rana altaica* is placed in the *R. amurensis* group.

Herein we present a phylogeny of most species of Eurasian brown frogs. The tree is used to address taxonomic status of *R. altaica*. Our own mtDNA sequence data are combined with those of Tanaka-Ueno et al (1998), Babik et al (2004), and Zhou et al (unpublished data).

1 Materials and Methods

1.1 Specimens examined

Twenty-five samples representing 22 species were used as ingroup taxa for the phylogenetic reconstruction. The outgroup consisted of *R. shuchinae*, *Lithobates catesbeiana*, *L. palustris*, and *L. sylvatica* based on Che et al (2007b). Locality and voucher data for new sequences used in our analysis are given in Tab. 1. We also obtained sequences from Babik et al (2004) (GenBank Nos. AY522383 – AY522428) and Tanaka-Ueno et al (1998) for *R. asiatica*, *R. tagoi*, *R. ornativentris*, *R. pirica*, and *R. japonica* (not shown in this study).

For population history reconstruction, we used 11 samples of *R. altaica* from the Altai area including 10 from Xinjiang and one from Kazakhstan (Tab. 1), as well as 44 haplotypes recovered by Babik et al (2004). We also used *R. arvalis* from Krasnoyarskii Krai in central Siberia, an area included in the distribution range of *R. altaica* in the first half of the 20th Century. *Rana temporaria* and *R. asiatica* were used as outgroups, as Babik et al (2004) suggested.

1.2 DNA extraction, amplification, and sequencing

DNA was extracted from muscle or liver tissue samples stored in 95% or 100% ethanol using a standard 3-step phenol/chloroform extraction procedure. The primers used for PCR amplification and sequencing of the mitochondrial Cytochrome *b* (Cyt *b*) gene were conducted using primers ralu1 (5'-AACCTTATGACC-CCAACAATACG-3') (Bos & Sites, 2001) and H15502 (5'-GGGTTAGCTGGTGTAAAATTGTCTGGG-3') (Tanaka-Ueno et al, 1998). Amplification was performed in a 25 volume reaction with the following procedures: initial denaturation step with 5 min at 95°C, 35 cycles of denaturation 45 s at 95°C, annealing for 45 s at 45°C,

extension for 45 s at 72°C. Final extension at 72°C was conducted for 10 min. PCR products were purified with Gel Extraction Mini Kit (Watson BioTechnologies, Shanghai). The purified product was used as the template DNA for cycle sequencing reactions performed using

BigDye Terminator Cycle Sequencing Kit (version 2.0, Applied Biosystems), and sequencing was conducted on ABI PRISM 3730 (Applied Biosystems) automatic DNA sequencer.

1.3 Data analyses

Tab. 1 Sampling information including voucher specimens, localities and GenBank accession numbers for species used in this study

| Species | Voucher | Locality | Haplotype | GenBank No. | Sequences resources |
|----------------------------|--|---|-----------|-------------|------------------------------|
| Ingroup | | | | | |
| | KIZ04238 | Haba River region, Xinjiang, China | A2 | HM116919 | This study |
| | KIZ04239 | Haba River region, Xinjiang, China | C2 | HM116918 | This study |
| | KIZ04240 | Haba River region, Xinjiang, China | A2 | HM116917 | This study |
| | KIZ04241 | Haba River region, Xinjiang, China | A2 | HM116916 | This study |
| | KIZ04242 | Haba River region, Xinjiang, China | A2 | HM116915 | This study |
| <i>Rana altaica</i> | KIZ04243 | Haba River region, Xinjiang, China | A2 | HM116914 | This study |
| | KIZ04244 | Haba River region, Xinjiang, China | A2 | HM116913 | This study |
| | KIZ04245 | Haba River region, Xinjiang, China | A2 | HM116912 | This study |
| | KIZ04247 | Haba River region, Xinjiang, China | A2 | HM116911 | This study |
| | KIZ04248 | Haba River region, Xinjiang, China | C1 | HM116910 | This study |
| | ZMMU-A-4290 | Markakol lake environs, Altai, Kazakhstan | A2 | HM116920 | This study |
| | ZMMU-A-4291-1 | Mirnoe, Krasnoyarskiy kray, Russia | A2 | HM116923 | This study |
| | ZMMU-A-4291-2 | Mirnyi, Krasnoyarskiy kray, Russia | A2 | HM116922 | This study |
| | ZMMU-A-4291-3 | Mirnyi, Krasnoyarskiy kray, Russia | A2 | HM116921 | This study |
| <i>Rana arvalis</i> | no voucher, tissue ID: MSUZP-SLK-RUS-3 | Kipenevshchina, Kirovskaya Pov., Russia | R1 | HM116927 | This study |
| | no voucher, tissue ID: MSUZP-SLK-RUS-4 | Kipenevshchina, Kirovskaya Prov., Russia | A2 | HM116926 | This study |
| | no voucher, tissue ID: MSUZP-SLK-RUS-5 | Kipenevshchina, Kirovskaya Prov., Russia | A7 | HM116925 | This study |
| | no voucher, tissue ID: MSUZP-SLK-RUS-6 | Kipenevshchina, Kirovskaya Prov., Russia | A7 | HM116924 | This study |
| <i>Rana amurensis</i> | SYNU040003 | Harbin, Heilongjiang, China | | | Zhou et al, unpublished data |
| <i>Rana asiatica</i> | KIZ-YP07060251 | 47tuan, Xinjiang, China | | | Zhou et al, unpublished data |
| <i>Rana chaochiaoensis</i> | SCUM0405170CJ | Zhaojue, Sichuan, China | | | Zhou et al, unpublished data |

(to be continued)

(continued)

| Species | Voucher | Locality | Haplotype | GenBank No. | Sequences resources |
|-------------------------------|---|---|-----------|-------------|------------------------------|
| <i>Rana chensinensis</i> | KIZ-RD05SHX001 | Huxian, Shaanxi, China | | | Zhou et al, unpublished data |
| <i>Rana dybowskii</i> | no voucher, tissue ID: MSUZP-IVM-1d | Kedrovaya pad NR, Khasanskii district, Primorye region, Russia | | | Zhou et al, unpublished data |
| <i>Rana graeca</i> | ZMMU-A-4293-1 | Kapa Moracka, Kapetanovo lake, environs of Niksic, Crna Gora (Montenegro) | | | Zhou et al, unpublished data |
| <i>Rana huanrenensis</i> | SYNU040006 | Huanren, Liaoning, China | | | Zhou et al, unpublished data |
| <i>Rana kukunoris</i> | KIZ-CJ06102001 | Qinghai lake, Qinghai, China | | | Zhou et al, unpublished data |
| <i>Rana kunyuensis</i> | no voucher, tissue ID: KIZ-HUI040001 | Mt.Kunyu, Shandong, China | | | Zhou et al, unpublished data |
| <i>Rana longicrus</i> | NMNS15022 | Xiangtian lake, Miaosu, Taiwan, China | | | Zhou et al, unpublished data |
| <i>Rana macrocnemis</i> | no voucher, tissue ID: MSUZP-LFM-12 | Burshang environs, Daghestan, Agulskiy district, Russia | | | Zhou et al, unpublished data |
| <i>Rana omeimontis</i> | SCUM0405196CJ | Hongya, Sichuan, China | | | Zhou et al, unpublished data |
| <i>Rana zhenhaiensis</i> | IOZCAS2869 | near Wuyi Shan, Fujian, China | | | Zhou et al, unpublished data |
| <i>Rana multidenticulata</i> | NMNS15108 | Wulaitong, Taibei, China | | | Zhou et al, unpublished data |
| <i>Rana sauteri</i> | SCUM0405175CJ | Kaohsiung, Taiwan, China | | | Zhou et al, unpublished data |
| Outgroup | | | | | |
| <i>Lithobates catesbeiana</i> | SCUM0405176CJ | Emei, Sichuan, China | | | Zhou et al, unpublished data |
| <i>Lithobates palustris</i> | ROM21658 | Middleburg, New York, USA | | | Zhou et al, unpublished data |
| <i>Lithobates sylvatica</i> | no voucher, tissue ID: MSUZP-SUNY-R-4-3 | Cranberry Lake BS, Adirondack mts., New York, USA | | | Zhou et al, unpublished data |
| <i>Rana shuchinae</i> | no voucher, tissue ID: KIZ-HUI040009 | Zhaojue, Sichuan, China | | | Zhou et al, unpublished data |

KIZ, Kunming Institute of Zoology, the Chinese Academy of Sciences, China; ROM, Royal Ontario Museum, Toronto, Canada; ZMMU, Zoological Museum of Moscow State University, Russia; SCUM, Sichuan University Museum, China; NMNS, 0HNational Museum of Natural Science, Taichung, Taiwan, China. IOZ, Institute of Zoology, the Chinese Academy of Sciences, China. SYNU, Shenyang Normal University, China.

DNA sequences were edited using Lasergene7.0. Nucleotide sequences were aligned using Clustal X 1.81 (Thompson et al, 1997) with default parameters, and then optimized by eye in MEGA 4.0 (Tamura et al, 2007).

Phylogenetic analyses among haplotypes were conducted using Bayesian Inference (BI) and Maximum Parsimony (MP). BI analyses were performed in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Each

analysis used four heated Markov chains (using default heating values) that were run for 4 million generations. Trees were sampled every 1000 generations and calculating a consensus tree was calculated after omitting the first 1 000 trees as burn-in. MP analyses were implemented using PAUP* 4.0b10a (Swofford, 2003). The heuristic MP searches were executed for 1 000 replicates with all characters treated as unordered and

equally weighted. Tree searching used tree bisection reconnection (TBR) branch swapping. To assess nodal reliabilities, bootstrap analysis (BBP) was conducted using 1000 replicates.

A median-joining network (Bandelt et al, 1999) was drawn using the program Network 4.5.1.0 (Bandelt et al, 1999) to investigate the possible relationships among haplotypes between *R. arvalis* and *R. altaica*.

2 Results

2.1 MtDNA variation and sequence characteristics

Twenty-five samples representing 22 species were used to reconstruct the brown frogs phylogeny. Among the 836 aligned ingroup nucleotide sites of Cyt *b*, 308 among 342 variable sites were potentially phylogenetically informative.

Among 62 sequences from *R. arvalis* and *R. altaica*, 47 haplotypes were identified. These sequences contained 66 variable sites, of which 32 were potentially phylogenetically informative. A total of 44 haplotypes were recovered by Babik et al (2004), and three new haplotypes were found here, named with C1, C2, and R1 (Tab. 1). No insertions, deletions and premature stop codons occurred. All haplotype sequences were submitted to GenBank under Accession Nos. HM116910 – HM116927.

2.2 Phylogenetic relationships of brown frogs

Our analysis supported the monophyly of brown frogs (Fig. 1). The BI and MP analyses found virtually identical sets of relationships except at some weak or unresolved nodes. As expected, the BI analyses obtained relatively higher nodal support than the MP analyses.

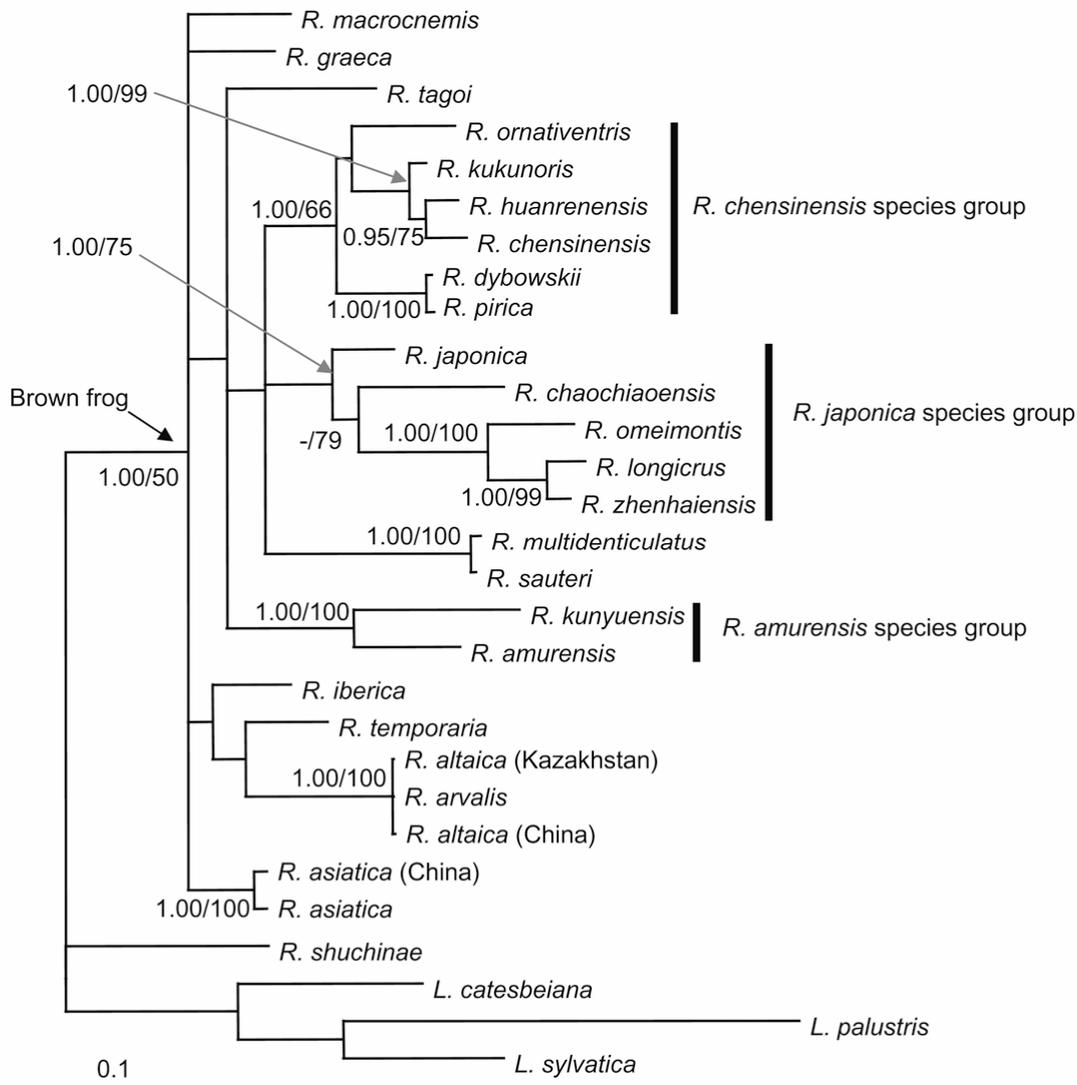


Fig. 1 The phylogenetic hypothesis derived from Cytochrome *b* gene using Bayesian Inference. Numbers around the branches are Bayesian posterior probabilities (>90) and Parsimony bootstrap support (>50).

The three species groups, *R. chensinensis*, *R. japonica*, and *R. amurensis* were generally confirmed (Fig. 1). All specimens of *R. altaica* and *R. arvalis* formed a monophyletic group that fell out as the sister group of European *R. temporaria* (Fig. 1).

2.3 Genealogical reconstruction and haplotype network of *R. arvalis* and *R. altaica*

Comparing those haplotypes with previously well defined lineages A (I, II) and B (Babik et al, 2004), our analyses (Fig. 2a) recovered the same topology with two major clades (A, B). Clades A and B were strongly

supported statistically. Within A, two subclades, AI and AII were recovered. Clade AII was strongly supported only by BBP, and Clade AI received weak statistical support (not shown). The median-joining network analyses (Fig. 2b) identified three internal haplogroups (A1, A2, and A7). Each terminal group was usually separated from a corresponding interior by only one mutational step. Eleven samples from the Altai region (A2, C1, and C2) and three samples from central Russia (Krasnoyarskii Krai) were assigned to internal group A2 (Fig. 2b, Tab. 1).

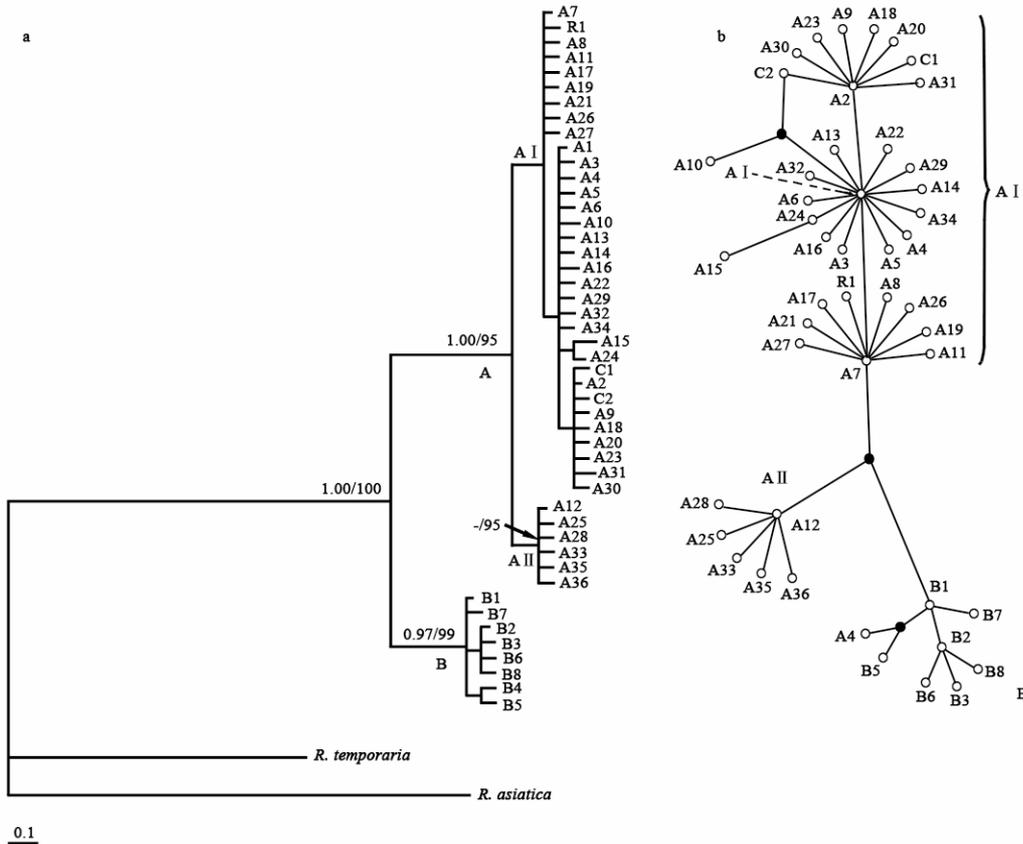


Fig. 2 Phylogeny tree and network of the haplotypes of *Rana arvalis*

(a) A Bayesian tree of *Rana arvalis* haplotypes; (b) A Median-joining network. Numbers near branches are given as Bayesian posterior probabilities (>90) / Parsimony nonparametric bootstrap support (>50).

3 Discussion

3.1 The taxonomic status of *R. altaica*

Though the phylogenetic relationships among Eurasian brown frogs were not well resolved based on *Cyt b* alone, our results obtained some new information. We used samples of nominal *R. altaica* from Altai Mountains which are geographically near the type locality: the Biisk Town in the Russian part of the Altai. Morphological studies (Ishchenko, 1978) failed to

morphologically differentiate populations of *R. arvalis* from Altai and Siberia. We found no evidence for distinct mitochondrial lineages associated independently with either *R. altaica* or *R. arvalis* (Fig. 1, 2). Our analysis from *Cyt b* data corresponded with the conclusions that *R. altaica* should not be recognized taxonomically (e.g., Kuzmin, 1999; Litvinchuk et al, 2008). The name *R. altaica* is junior synonym of *R. arvalis* by the principle of priority. Morphological diversity within *R. arvalis* seems to be largely a result of variable ecological

conditions as suggested by Babik & Rafiński (2000).

Babik et al (2004) recovered 44 haplotypes for *R. arvalis* throughout the species' geographical range. Most of the nominal *R. altaica* from Altai region had the same haplotype, A2, as other *R. arvalis* (Fig. 2B). This matriarchal lineage has a wide distribution ranging eastwards from Western Europe to Yakutia and south-eastwards to the Eastern Altai.

3.2 Higher taxonomic inferences

The three species groups of Chinese brown frogs suggested by Fei et al (2009) largely correspond to three of our clades (Fig. 1). However, the assignment of *R. altaica* to the *R. amurensis* group (Fei et al, 2009), and thus by association *R. arvalis*, was not supported by our analyses. *Rana arvalis* did not cluster with any East Asian species. Veith et al (2003) discovered that *R. arvalis* was associated phylogenetically with European *R. temporaria*. Our result was consistent with this conclusion, although with weak nodal support.

Consequently, we removed *R. arvalis* from the *R. amurensis* group.

The basal relationships of the Eurasian brown frogs were poorly resolved by our limited data. Regardless, our analyses hinted at the presence of a *R. temporaria* group. Similarly, *R. asiatica* fell out inside the *R. chensinensis* group (Fig. 1) as placed by Fei et al (2009). As a Central Asian species, the systematic position of *R. asiatica* should be further explored using additional gene markers.

Our analysis highlights the importance of a phylogeny when considering the validity of species and the formation of species groups. Studies will be more repeatable when done in a phylogenetic context involving both morphological and molecular data. Certainly, independent genes and, especially from the nuclear genome, should be explored to understand evolutionary history and to verify gene flow within species.

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封面图片说明

物种名：叶城沙蜥 (*Phrynocephalus axillaris*)。

该图片是郭宪光博士 2008 年 6 月于新疆吐鲁番沙漠植物园采样时拍摄。生境为长有部分沙生植物——沙拐枣（图片中树枝为沙拐枣的枝条）的固定沙丘。沙蜥的身体姿态使之有利于吸收更多的热量，快速升高体温。但由于当时（约为正午 12 时）沙面温度较高，叶城沙蜥并未以四肢全部着地，而是主要以对侧前后肢接触地面，保持身体平衡。此外，红色腋斑和白色尾尖是该种区别于沙蜥属其他物种的主要鉴别特征。