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A New Record of *Bufo gargarizans* Complex (Bufonidae, Anura) from Truong Son Mounts, Ha Tinh and Ha Giang Provinces, Vietnam Based on Molecular Evidence with a Description of a New Species

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Abstract: Based on a combination of molecular and morphological data, we herein report a new species within the bufonid *Bufo gargarizans* species complex. This is a widespread species complex with distribution from eastern Russia and the Korean Peninsula to China and the Ryukyu Islands of Japan. Records of this species have been documented in the Guangxi and Yunnan Provinces near the border with Vietnam and, for the first time from Vietnam, in Ha Giang Province. The new record of *Bufo* cf. *gargarizans* from Vietnam is from Ha Tinh Province. This species has never been reported from Vietnam so far south, about 550 km south from the previously known locality in Ha Giang Province. The female specimen was found in the Ha Tinh Province, Vu Quang National Park of central Vietnam and two specimens (male and female) were found Ha Giang Province. They are clearly distinguished from *B. gargarizans* and all the mentioned species by a specific color pattern on the belly and creamy-yellowish throat with large, bright red speckles. Genetic divergences of three Vietnam specimens from Ha Giang and Ha Tinh Provinces in the ND2 gene sequences between the *B.* sp. nov. and all other congeners ranged from 4.3% (with *B. andrewsi*) to 7.0% (with *B. stejnegeri*). We give a description of the morphological characters and coloration of the new record and provide an expanded diagnosis.

Keywords: *Bufo* sp. nov.; Vietnam; morphology; molecular evidence; DNA analysis

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1. Introduction

Genus *Bufo* Garsault, 1764 is a genus of anuran amphibians of the family Bufonidae inhabiting temperate Eurasia and south Japan to north Africa, the Middle East, northeastern and western Myanmar and through China to northern Vietnam. Taxonomic study of this genus has a long history, and herpetologists have presented several different and controversial ideas about its composition and phylogenetic position [1]. According to contemporary ideas [1], it contains 25 species: *Bufo ailaoanus* Kou, 1984; *Bufo andrewsi* Schmidt, 1925; *Bufo aspinius* (Rao and Yang, 1994); *Bufo bankorensis* Barbour, 1908; *Bufo bufo* (Linnaeus, 1758); *Bufo cryptotympanicus* Liu and Hu, 1962; *Bufo eichwaldi* Litvinchuk, Borkin, Skorinov, and Rosanov, 2008; *Bufo exiguus* Qi, Lyu, Song, Wei, Zhong, and Wang, 2023; *Bufo formosus* Boulenger, 1883; *Bufo gargarizans* Cantor, 1842; *Bufo luchunnicus* (Yang and Rao, 2008); *Bufo menglianus* (Yang, 2008); *Bufo minshanicus* Stejneger, 1926; *Bufo pageoti* Bourret, 1937; *Bufo praetextatus* Boie, 1826; *Bufo sachalinensis* Nikolskii, 1905; *Bufo spinosus* Daudin, 1803; *Bufo stejnegeri* Schmidt, 1931; *Bufo tibetanus* Zarevskii, 1926 "1925"; *Bufo torrenticola* Matsui, 1976; *Bufo tuberculatus* Zarevskij, 1926; *Bufo tuberospinius* (Yang, Liu, and Rao, 1996);

Bufo verrucosissimus (Pallas, 1814); *Bufo yongdeensis* (Rao, Liu, Ma, and Zhu, 2022 "2020"); *Bufo yunlingensis* Rao, Hui, Zhu, and Ma, 2022 "2020". Most of species of this genus (18) are known to occur in China China [2]. The first record of *B. gargarizans* in Vietnam was registered in 2016 [3]. *Bufo sachalinensis* Nikolsky, 1905, which was considered earlier as *B. gargarizans*, is distributed in the Amur River Basin of Liaoning, Jilin and Heilongjiang, China; adjacent Russia, including Sakhalin; and the Korean Peninsula [1,4]. In Vietnam, three species were registered [3,5]: *Bufo gargarizans* Cantor, 1842; *B. luchunnicus* (Yang et Rao, 2008); and *B. pageoti* Bourret, 1937.

Bufo gargarizans Cantor, 1842 is a widespread species with distribution from northeastern China south through central and eastern China south to high elevations of Guangxi and Yunnan and west through Sichuan and Qinghai to eastern Xizang; Dibang Valley of Arunachal Pradesh, India; Ha Giang Province, Vietnam; Miyakojima, Ryukyu Is., Japan; and introduced onto the islands of Kita Daitojima and Minami Daitojima and into the northern part of Okinawa [1–3]. The northeastern records of frogs of this species complex that were earlier considered as *Bufo gargarizans* Cantor, 1842, have been recently assigned to the distinct species *B. sachalinensis* Nikolskii, 1905 and its subspecies [4].

During our fieldwork in central Vietnam in Vu Quang National Park, Ha Tinh Province of Vietnam (October 2019), we collected a specimen that has been assigned to the *Bufo gargarizans* species group (Figure 1). Subsequent results of the detailed morphological and molecular analyses confirm the allocation of this specimen to the genus *Bufo* and show that it is similar to other specimens collected in Ha Giang Province both genetically and morphologically but cannot be assigned to any currently known species of this genus. Thus, we describe these toad specimens as a new species. Species of the *B. gargarizans* complex have never been reported so far south in Vietnam.

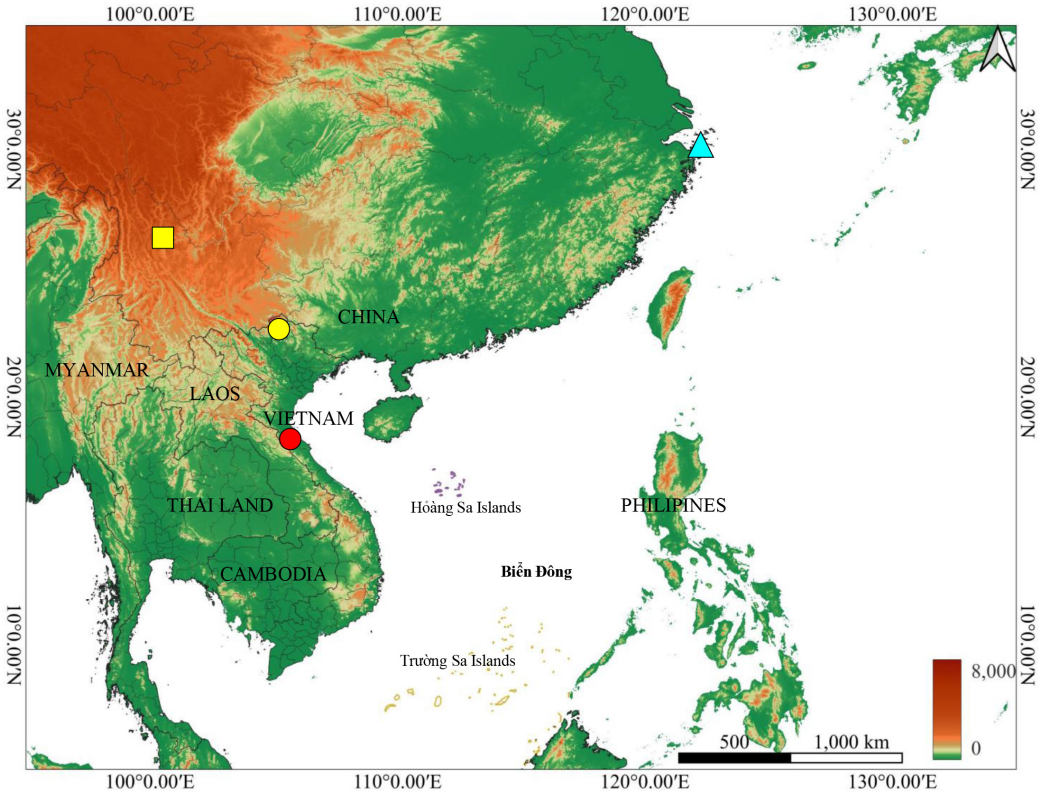

Figure 1. Type localities of *Bufo rubroventromaculatus* sp. nov. and related species in Vietnam and China: Red circle—type locality in Ha Tinh Province, Vu Quang National Park; yellow circle—locality of the paratypes in Ha Giang Province, Quan Ba district.; light blue triangle—type locality of *B. gargarizans*, Chusan Island, East China Sea, off northeastern coast of Zhejiang, China. Yellow square—type locality of *B. andrewsi*, Likiang, 8500 feet altitude, Yunnan "[Province]", China.

2. Materials and Methods

2.1. Sampling

Fieldwork was conducted in Truong Son Mounts, Vietnam, in Vu Quang National Park, Ha Tinh Province. A female specimen was collected in October 2019 by Orlov N.L, Manh Van Le, Tao Thien Nguyen and Iogansen L.K. Together with two specimens from Ha Giang Province, collected in June 2019, it composed the type series used for description. We also collected comparative material of *Bufo sachalinensis* from Primorsky Krai in July 2021. After the frogs were photographed alive, three specimens of the new species (IGR 10497, ZISP 15117–15118) and *B. sachalinensis* (ZISP 15119–15121) were anaesthetized and euthanized in a closed vessel with a piece of cotton wool containing ethyl acetate, preserved in 80% ethanol for 5 h, and then transferred to 70% ethanol for permanent storage. They are deposited at the Institute of Genome Research, Vietnam Academy of Science and Technology (IGR) and the Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia (ZISP).

Bufo sp. (cf. *gargarizans*), female, IGR 10497—Vietnam, Ha Tinh Province, Vu Quang NP., 18°16′12.7′ N., 105°21′35.8 E. 16 October 2019; 525 m a.s.l. 16 October 2019, Leg et Det. Huy Quoc Nguyen, Orlov N., Manh Van Le, Chung Van Hoang, Iogansen L.

Bufo sp. (cf. *gargarizans*), male, ZISP 15117—Vietnam, Ha Giang province, Quan Ba district, Tung Vai, 23°4′35″ N 104°53′12″ E. 11 June 2019. Leg et Det: Orlov N.L.

Bufo sp. (cf. *gargarizans*), female, ZISP 15118—Vietnam, Ha Giang province, Quan Ba district, Tung Vai, 23°4′35″ N 104°53′12″ E. 11 June 2019. Leg et Det: Orlov N.L.

Bufo sachalinensis, ZISP 15119, subad.,—Russia, Verkhne-Ussuriysk forest station, Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch of the Russian Academy of Sciences, Bulyga-Fadeevo, Chuguevsky district, Primorsky Krai. 44°02′ N. 133°52′ E. 18 July 2021. Leg et Det: N. Orlov, Iogansen L.K.

Bufo sachalinensis ZISP 15120, female, Russia, Verkhne-Ussuriysk forest station, Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch of the Russian Academy of Sciences, Bulyga-Fadeevo, Chuguevsky district, Primorsky Krai. 44°02′ N. 133°52′E. 18 July 2021. Leg et Det: Orlov N.L., Iogansen L.K.

Bufo sachalinensis ZISP 15121, female, Russia, Verkhne-Ussuriysk forest station, Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch of the Russian Academy of Sciences, Bulyga-Fadeevo, Chuguevsky district, Primorsky Krai. 44°02′ N. 133°52′ E. 18 July 2021. Leg et Det: Orlov N.L., Iogansen L.K.

2.2. Molecular and Phylogenetic Analyses

The genetic dataset of our study included six species of east Asia toads (*B. gargarizans*, *B sachalinensis*, *B. andrewsi*, *B. stejnegeri*, *B. japonicus*, *B.* sp. nov.) and consisted of 97 samples for the mtDNA *ND2* gene and 67 samples for the nuDNA *RAG-1* gene. Accession numbers of the GenBank of used samples are given in Table 1 and Appendices A.1 and A.2.

Whole genomic DNA was extracted from tissue using the phenol–chloroform method [6]. We used two molecular genetic markers—mtDNA fragment of the NADH dehydrogenase 2 (*ND2*) and the nuDNA gene fragment recombination activating gene 1 (*RAG-1*). The fragment of *ND2* and its flanking tRNAs (536 bp) was amplified using primer pairs L-int, 5′-AGC ATC CTA CCC ACG ATT TCG-3′ [7], and H4980, 5′-ACT TTT CGG ATT TGA GTT TGG TT-3′ [8], with modifications according to the complete mitochondrial genome of *B. gargarizans* NC_008410. The *RAG-1* gene (642 bp) was amplified using primer pairs snoBGRAG1F, 5′-TGA GAA ACG CAG AGA AAG CCC-3′, and snoBGRAG1R, 5′-GAC GGG TGG CAT CAC AAA GAG-3′ [4]. The reaction conditions were an initial denaturation of 3 min at 95 °C, followed by 32 cycles of denaturation (30 s at 95 °C), annealing (30 s at 58 °C), and extension (60 s at 72 °C). The PCR products were analyzed using electrophoresis in 6% PAAG with subsequent staining with ethidium bromide and visualization under UV light. PCR products were sequenced with same primers using Evrogen Joint Stock Company commercial services (Moscow, Russia).

Table 1. Specimens, localities and GenBank accession numbers of *Bugo gargarizans* complex species used in this study.

Collector	Date	Locality	Coordinates	Voucher Number	*RAG-1* (nuDNA) GenBank Number	*ND2* (mtDNA) GenBank Number
N. Orlov, Manh Van Le, Tao Thien Nguyen, L. Iogansen.	16 October 2019	Vietnam, Ha Tinh Province, Vu Quang NP.	18.16 N 105 21 36 E	IGR 10497, female	OR113666	OR113672
N.Orlov	11 June 2019	Vietnam, Ha Giang, Quan Ba, Tung Vai	23.06 N 104.92 E	ZISP:15117 male	OR113664	OR113670
N.Orlov	11 June 2019	Vietnam, Ha Giang, Quan Ba, Tung Vai	23.06 N 104.92 E	ZISP:15118 female	OR113665	OR113671
N. Orlov, L. Iogansen	18 July 2021	Primorskiy kray, Bulyga-Fadeevo	44.04 N 133.88 E	ZISP:15119 subad.	OR113661	OR113667
N. Orlov, L. Iogansen	18 July 2021	Primorskiy kray, Bulyga-Fadeevo	44.04 N 133.88 E	ZISP:15120, female	OR113662	OR113668
N. Orlov, L. Iogansen	18 July 2021	Primorskiy kray, Bulyga-Fadeevo	44.04 N 133.88 E	ZISP:15121, female	OR113663	OR113669

We performed a maximum likelihood (ML) phylogenetic analysis of the unpartitioned *ND2* sequences in IQ-TREE [9] using its online web interface W-IQ-TREE [10]. The best fit model of sequence evolution according to Bayesian information criterion (BIC) scores was TN + F + G4 [11]. Branch support estimates result from a Shimodaira–Hasegawa-like approximate likelihood ratio test (SH-aLRT [12]), an approximate aBayes branch test (aBayes [13]), and an ultrafast bootstrap test (UFBoot; [14]) (SH-aLRT/aBayes/UFBoot). SH-aLRT $\geq$ 80%, aBayes $\geq$ 0.95, and UFBoot $\geq$ 95% were defined as robust statistical supports. Haplotype networks of *RAG-1* gene were constructed using the algorithm of TCS [15] implemented in PopART software [16]. Heterozygous individuals were identified based on the presence of two peaks of approximately equal height at a single-nucleotide site. We used PHASE [17] as implemented in DnaSP v.5 [18] to infer haplotypes from set of *RAG-1* sequences, based on alignments that were trimmed to ensure that all sequences have the same length. The genetic distance matrices (*p*-distances) were calculated in MEGA7 software [19]. The obtained sequences were deposited in the GenBank database (accession numbers *ND2*: OR113667–OR113672; *RAG-1*: OR113661–OR113666).

2.3. Morphological Analysis

All measurements were taken with digital calipers to the nearest 0.01 mm and rounded to 0.1 mm. All measurements provided in text are in mm. The following abbreviations of characters used are as follows:

SVL: snout–vent length; HL: head length (from the back of mandible to tip of snout); HW: maximum head width (across angle of jaws); SNL: snout length from anterior corner of eye to the tip of snout; MN: mandible–nostril distance; MFE: distance from the back of mandible to the front of the eye; MBE: distance from the back of mandible to the back of the eye; ED: horizontal diameter of the eye; UEW: maximum width of upper eyelid; IN: internasal space; IOD: interorbital distance; DAE: distance, front of the eyes; DPE: distance, back of the eyes; NS: nostril–snout distance; EN: eye–nostril distance; FLL: forelimb length, axilla–elbow; HAL: hand length, elbow–tip of Finger III; FHL: forelimb hand length; IPT: inner palmar tubercle length; OPT: outer palmar tubercle length; finger I length; finger II length; finger III length; finger IV length.

The type specimens are deposited at the Institute of Genome Research, Vietnam Academy of Science and Technology (IGR) and the Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia (ZISP).

3. Results

We here describe the new, most southerly record of *B. gargarizans* from central Vietnam. We studied all the known specimens from Vietnam (Ha Giang and Ha Tinh Provinces) using molecular genetics and morphological characterization. It allows us to reveal a new crypic species of the *B. gargarizans* complex.

The ML analysis resulted in generally well-resolved topology only within three unsupported nodes (Figure 2). We suggest the following set of genealogical relationships: The first branching events in the Asiatic toad radiation separated *B. japonicus* and *B. stejnegeri*. Further, a large but poorly supported clade (72/0.99/87; hereafter, node support values are given as SH-aLRT/aBayes/UFBoot, respectively) is separated, formed from two pairs of supported sister species, *B. gargarizans* and *B. sachalinensis* (89/1/90) on the one hand and *B. andrewsi* and *B. rubroventromaculatus* sp. nov (85/1/95) on the another. Clade *B.* sp. nov., which includes specimens from north Vietnam and south China, is strongly supported (97/1/98) (Figure 2). The only specimen from south China (CIB ZYC668) used in phylogeographic analysis of the *Bufo gargarizans* species complex [7] using a mitochondrial *ND1–ND2* fragment originated from Jiangkou County, Guizhou Province, N 27°53.772′, E 108°42.918′, elevation 810 m, collected by Dr. Yuchi Zheng in 2001 (Fu, personal data).

Genetic divergences in the *ND2* gene sequences between the *B. rubroventromaculatus* sp. nov. and all other congeners ranged from 4.3% (with *B. andrewsi*) to 7.0% (with *B. stejnegeri*) (Table 2). Genetic differences in the *RAG-1* gene of nuDNA are weak (0.2–1.2%); however, as in the case of the *ND2*, minimal differences were noted between the *B. rubroventromaculatus* sp. nov. and *B. gargarizans* and *B. andrewsi* (0.4–0.5%) (Table 2). On the *RAG-1* haplotype network, specimens from north Vietnam and south China are isolated, but do not form a separate phylogroup (Figure 3).

Table 2. Average uncorrected *p*-distances % between members of *Bufo* in east Asia calculated from *ND2* (below the diagonal) and *RAG-1* (above the diagonal) gene fragment sequences.

Species	sp. nov.	*Andrewsi*	*Gargarizans*	*Sachalinensis*	*Stejnegeri*	*Japonicus*
sp. nov.	–	0.5	0.4	1.0	1.2	0.9
Andrewsi	4.3	–	0.2	0.7	0.9	0.7
Gargarizans	5.4	5.1	–	0.7	0.9	0.7
Sachalinensis	6.0	5.5	4.2	–	1.0	1.0
Stejnegeri	7.0	7.2	8.0	7.9	–	1.0
Japonicus	6.2	6.0	7.1	6.1	7.3	–

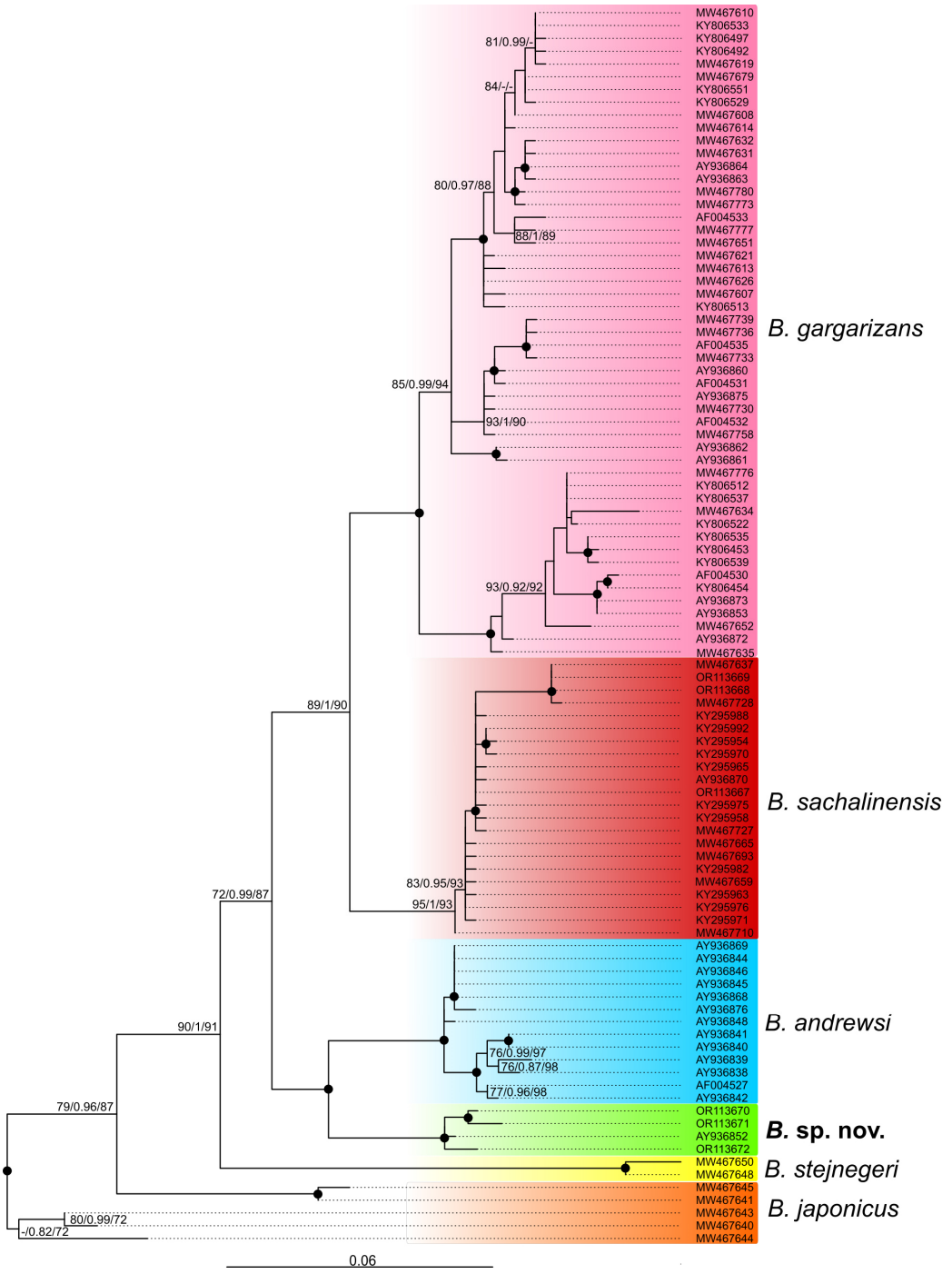

Figure 2. Maximum likelihood phylogenetic tree of *Bufo* in east Asia, inferred with IQ-TREE based on the *ND2* gene. SH-aLRT/approximate aBayes support/ultrafast bootstrap support values are shown beside the respective nodes. Nodes with black circles indicate triple high support values of SH-aLRT $\geq$ 80, approximate aBayes support $\geq$ 0.95, and ultrafast bootstrap support $\geq$ 95. The scale bar represents expected number of nucleotide substitutions per site. Outgroup not shown.

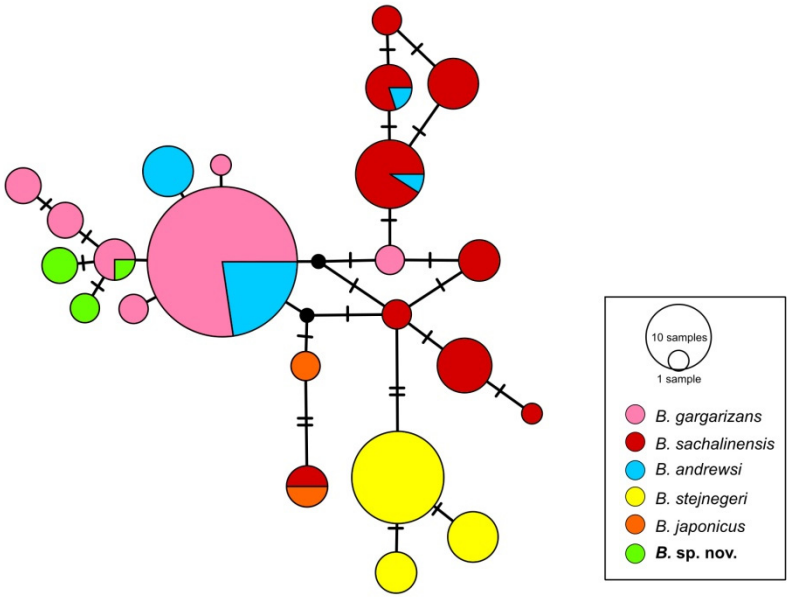

Figure 3. The haplotype network for *Bufo* in East Asia based on RAG-1 gene. The numbers of transverse strokes on the branches correspond to the number of nucleotide substitutions.

Taxonomic Account

Bufo rubroventromaculatus sp.nov. Figures 4 and 5.

Figure 4. Holotype of *Bufo rubroventromaculatus* sp.nov. IGR 10497, female: (**A**) in the wild; (**B**) dorsal view; (**C**) ventral view; (**D**) palmar surface of the right forelimb.

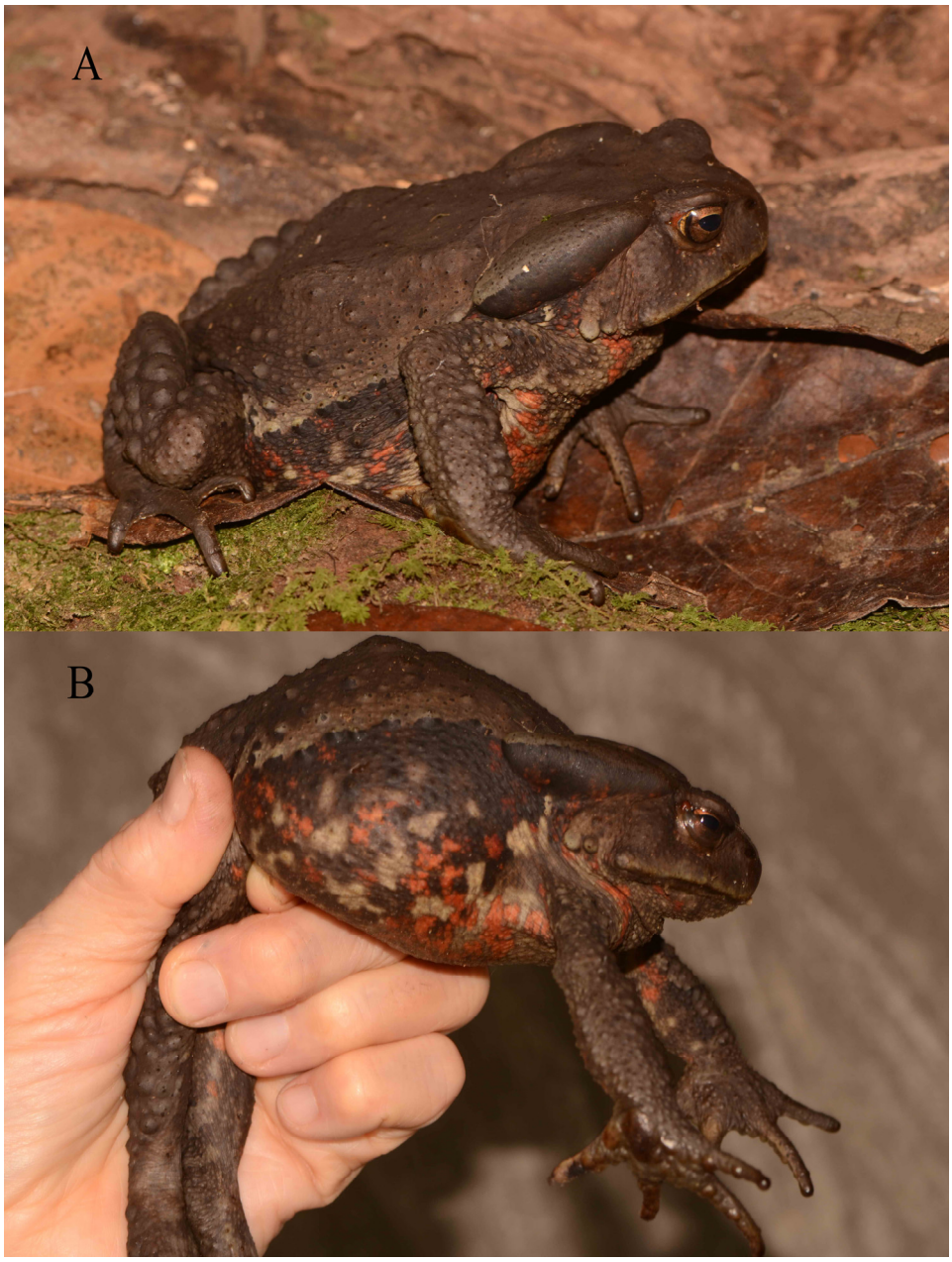

Figure 5. Paratype of *Bufo rubroventromaculatus* sp. nov. ZISP 15118. (**A**) lateral view; (**B**) lateral view a part of belly with bright red large blotches.

https://zoobank.org:act:EF7782F5-A31D-4CE1-AF82-E569FCC5CE08

Holotype: IGR 10497, adult female (Figure 4). Vietnam, Ha Tinh Province, Vu Quang National Park, N 18°16′12.7″, E 105°21′35.8″, 16 October 2019; 525 m a.s.l. Collected on 16 October 2019 by Orlov N.L, Manh Van Le, Tao Thien Nguyen, and Iogansen L.K.

Paratypes: ZISP 1511 7, adult male and ZISP 15118 (Figure 5), adult female; Vietnam, Ha Giang Province, Quan Ba district, Tung Vai village, 23°4′35″ N 104°53′12″ E. Collected on 11 June 2019 by Orlov N.L, Tao Thien Nguyen, and Iogansen L.K.

Type locality: Vietnam, Ha Tinh Province, Vu Quang National Park, Quan Ba district, Tung Vai village, 18°16′12.7′ N., 105°21′35.8 E.; 525 m a.s.l.

Diagnosis: Three specimens of toads were assigned to the genus *Bufo* based on molecular phylogenetic analyses. *Bufo rubroventromaculatus* sp. nov. is distinguished from other species in the genus *Bufo* by specific coloration with red speckles on the belly (Figures 4 and 5). Maximal size known for this species (SVL 125.45 mm) (Table 3); head longer than width (maximal

HW 60.32 mm, HL 65.42 mm), snout obtuse, protruding in profile; canthus rostralis distinct; pupil horizontally oval; loreal region flat and oblique; snout length greater than eye horizontal diameter (HL 13.7 mm, ED 10.6 mm, ED/SNL 0.77); parotoid gland well developed, elongated; tympanum distinct, small and round; vomerine teeth present; tongue not notched posteriorly.

Table 3. Measurements (in mm) and ratios of the series of *Bufo rubroventromaculatus* sp. nov. For abbreviations, see Section 2.

Characters	ZISP 15117 Male Paratype	ZISP 15118 Female Paratype	IGR 10497 Female Holotype
SVL	65.5	73.5	125.4
HL	23.3	25.2	65.4
HW	20.4	29.0	60.3
MN	15.1	16.5	50.3
MFE	12.3	14.0	43.4
MBE	12.0	13.4	35.7
SNL	10.7	11.8	13.7
ED	7.6	8.0	10.6
ED/SNL	0.7	0.7	0.8
UEW	6.5	7.0	8.6
IN	5.6	5.8	8.5
IOD	10.9	12.3	12.5
DAE	18.5	18.9	20.5
DPE	26.5	27.5	31.5
NS	5.16	5.4	6.7
EN	6.0	6.3	7.8
FLL	18.2	19.0	23.6
HAL	37.8	39.0	59.8
FLL/HAL	0.5	0.5	0.4
FHL	27.7	28.6	31.8
IPT	4.2	4.8	5.9
OPT	4.7	5.0	6.6
Finger I	8.6	9.0	11.6
Finger II	6.5	7.0	9.9
Finger III	12.7	12.9	15.7
Finger IV	9.5	10.0	11.5

Description of the holotype: Measurements of the holotype are provided in Table 3. Coloration is reddish-brown above and the lower side of the body is creamy-yellowish with bright red large blotches (Figure 4).

Coloration in life: The dorsal surface of the body and head is reddish-brown; the dorsal surface of the fore and hind limbs is dark brown. Along the lateral line, there is a dark, almost black stripe with red speckles, which extends onto the lateral surface of the parotids. The belly and throat are creamy-yellowish with bright red large blotches. The iris of the eye is golden-red. Sexual dimorphism and individual variability in coloration were not detected on the available material identified using a DNA marker.

Coloration in preserve: The color of the body above is faded, light brown; the ventral surface is yellowish with dark irregular spots in the lower abdomen.

Comparison: The comparison was made based on original species descriptions and our own research in China and Indochina with species found in Vietnam and members of the *B. gargarizans* species complex. The well-known difficulty of searching for diagnostic characters in a taxonomically complex group with cryptic diversity makes it difficult to identify morphometric characters based on a type series consisting of specimens of different ages. However, the new species is clearly different from all comparable species in its larger body size. *Bufo luchunnicus* is a medium-sized toad with a male snout–vent length of 55–61 mm [20] (n = 2) compared to 65.52 in *B. rubroventromaculatus* sp.nov; males of small-sized toad *B. sachalinensis* have a body size up to 47 mm (n = 15) [21]; females of *Bufo pageoti* are smaller (SVL to 67 mm); males SVL 43.7–50.0 mm (n = 7) [22] compared to 125.4 in *B. rubroventromaculatus* sp.nov. *Bufo andrewsi* is a medium-sized toad with SVL 63.71 ± 0.77–82.57 ± 6.10 (355 males from 14 populations) [23]. *Bufo gargarizans*, a large-sized toad, ranges in snout–vent length from 56 to 102 mm [24,25]. The new species differs from all large-sized species of the complex (*B. gargarizans, B. tibetanus, B. sachalinensis,* and *B. minshanicus*) by significantly smoother spikes on the outgrowths of the body. It differs by a significantly wider band extending from the parotid gland to the side of the body. The tympanum is covered with skin. The upper side of the body is brighter (reddish-brown); the lower side of the body is the brightest of all species of complex (Figure 6). Currently, a more accurate approach including the use of DNA markers for the cryptic toad species of this complex and especially *Bufo gargarizans—B. rubroventromaculatus* sp. nov. is required to clarify their distribution in China and Vietnam. This will allow for us to identify diagnostic characters, including dimensional ones, which allow for clarifying the delimitation of the species of the complex. Genetic divergences in the *ND2* gene sequences between the *B. rubroventromaculatus* sp. nov. and all other congeners ranged from 4.3% (with *B. andrewsi*) to 7.0% (with *B. stejnegeri*) (Table 2).

Genetic divergences in the *ND2* gene sequences between the *B. rubroventromaculatus* sp. nov. and all other congeners ranged from 4.3% (with *B. andrewsi*) to 7.0% (with *B. stejnegeri*) (Table 2).

Etymology: The specific name *rubroventromaculatus* originates from the type of characteristic coloring of the belly with numerous large red spots of irregular shape.

Distribution: The type locality of *B. gargarizans* Cantor, 1842 sensu stricto is Zhoushan "Chusan Island", East China Sea, off northeastern coast of Zhejiang, China. With respect to the description of a new *Bufo* species, the question of the distribution of these two forms becomes particularly important. The only specimen from south China (CIB ZYC668) used in phylogeographic analysis of the *Bufo gargarizans* species complex [7] using mitochondrial *ND1–ND2* gene originated from Jiangkou County, Guizhou Province, N 27°53.772′, E 108°42.918′, elevation 810 m a.s.l. It was collected by Yuchi Zheng in 2001. *B. gargarizans* is widespread in China from the northeastern regions through central and eastern China south, to high elevations of Guangxi and Yunnan, west through Sichuan and Qinghai, to eastern Xizang [1]. The authors of the first record of *B. gargarizans* in Vietnam [2] wrote that this specimen (IEBR A.2015.62, GenBank accession number LC155912) is genetically about 0.97% divergent from the specimen from Yunnan, China (GenBank accession number FJ882843, unspecified location). The authors used fragments containing 16S rRNA, ~500 bp long. Unfortunately, we were unable to find more information about the storage location and morphological characteristics of this specimen from Yunnan [3].

Our research has shown that *B. gargarizans* sensu stricto is found in China even at a very short distance from the localities of *B. rubroventromaculatus* sp. nov. (Appendix A; Genbank AY936852). For example, the location data and haplotype designation from the Table 1 [7] demonstrate that Suiyang 28°13.581 N, 107°09.593 E 1350 1 22A/13C AY924359 1 22A Genbank number AY936875 of *B. gargarizans* included in our analysis (see Attachment) is located at a short distance (about 150 km) from Jiangkou 27°53.772 N, 108°42.918 E 810 1 23A AY924343 1 23A AY936852. All three specimens from Vietnam (Figure 1) collected by us and used in our study belong to the new species. *B. rubroventromaculatu.* sp. nov., unlike the species of this complex, except only *Bufo pageoti* Bourret, 1937 (Vietnam: Lao Cai, Nghe

An, Ha Tinh, and Quang Nam provinces), penetrates far into the tropics. Future research will investigate whether they represent an allopatric or parapatric species in southern China and Vietnam.

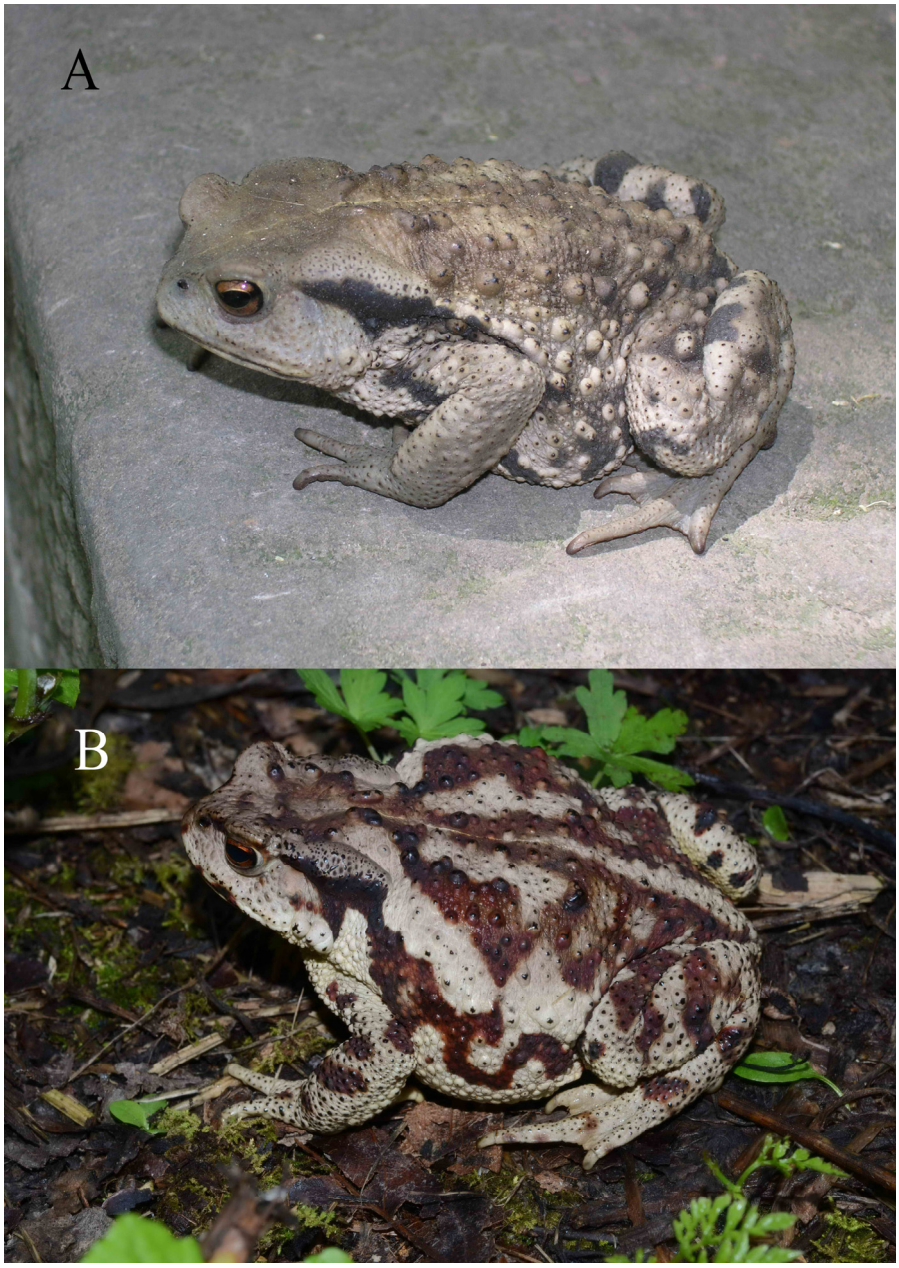

Figure 6. *Bufo gargarizans*, Chengdu (**A**) *Bufo sachalinensis*, Ussuri region (**B**).

Natural History: In Ha Tinh Province, Vu Quang National Park *Bufo rubroventromaculatus* sp. nov. found on a forested mountain slope at an altitude of 525 m a.s.l. and in Ha Giang Province, Quan Ba district, in the forest on the karst, 800 m a.s.l. All toads were found in the forest on the karst during twilight and nighttime, from approximately 18 p.m. to 1 a.m. In the same biotope, the following sympatric species were noted: *Leptobrachella sungi*, *Leptobrachium chapaense*, *Duttaphrynus melanostictus*, *Occidozyga lima*, *Hylarana macrodactyla, H. nigrovittata, H. maosonensis*, and *Gracixalus gracilipes.* The actual distributional range should be confirmed in further studies. Given the available information, we suggest this species be considered as Data Deficient following IUCN's Red List categories (IUCN 2023).

4. Discussion

According to modern views on the phylogeny and taxonomy of the family, the genus *Bufo* is restricted to members of the *B. bufo* group, as earlier authors removed most of the species of former "*Bufo*" to other genera [1]. Phylogenetic relationships between Old World bufonids (genera *Bufo, Bufotes, Strauchbufo*, and others) are under extremely intensive research. The results of these studies were summarized and discussed in fundamental publications [4,26,27], which hypothesize the dispersal of the *Bufo* genus path in the Palearctic. They integrate phylogeographic and taxonomic research, underlying that they are two rapidly evolving fields in an exciting era of new species discoveries. Within the genus, the "Western" *Bufo* complex and the east Asian *Bufo gargarizans* complex are distinguished [3,7]. The *Bufo gargarizans* species complex includes a number of species: the mainland Asiatic toad, *B. gargarizans* sensu lato (which includes *B. tibetanus*, *B. andrewsi*, and *B. minshanicus*), as well as the Taiwanese Bankor toad, *B. bankorensis* [3,7,8,26–30]. *Bufo gargarizans* s.s., *B. sachalinensis*, and *B. andrewsi* host Pleistocene diversifications, but appear too young (<2 Myr) to underly additional speciation events [26,28,29]. Our phylogenetic tree agreed well with earlier phylogenies of the genus *Bufo* in East Asia [3,4,7,20,21].

In Japan, bufonid populations belong to *Bufo* cf. *japonicus*, a complex that primarily diversified into a western and eastern clade, assigned to the morphological subspecies *B. j. japonicus* Schlegel, 1838 and *B. j. formosus* Boulenger, 1883, respectively [1]. We used the samples of *B. japonicus* for better resolution of clades in phylogeny; they are named as *B. japonicus* in GenBank. However, the paraphyly (the absence of a common clade for five samples of *B. japonicus*) is present in the Figure 2. It is highly likely that under the name "*japonicus*", both "*japonicas*" and "*formosus*" are "hiding" in the GenBank. According to Othman et al. [4], they are probably distinct species. Moreover, ND2 for *formosus* are absent in the GenBank, which is why, in our opinion, we cannot divide them into two different names and show them on the tree with different colors.

The problem of critical diversity in taxonomically complex groups of animals has been widely discussed in the scientific community for a long time. It goes beyond the scope of this study. Moreover, the studies of recent years cited in our work [26,27,30–36], especially [4,31], are specifically devoted to this topic. Two or more distinct species classified previously as a single species currently have different taxonomic status. These days, this is facilitated by a broad routine using DNA sequencing, which has given biologists a new tool for detecting and differentiating morphologically similar species. Because morphological diagnostic characters are unreliable, most of these forms have morphological differences, which are minimal and often insufficient.

All the authors underlined that taxonomy of the group is debatable because of its genetic complexity [4], and the status of these forms can be changed [1]. It was shown that *B. bankorensis*, *B. gargarizans*, *B. tibetanus*, and *B. tuberculatus* were clustered into the *B. gargarizans* complex with low intrapopulation genetic differentiation, but due to the lack of samples of topotype and low support at some nodes, the taxonomic placements of these four species require more evidence to determine [1,3]. These authors also underline that the record from Vietnam requires further clarification.

Study of the phylogenetic relationship between Bufonidae with emphasis on East Asian *Bufo* lineage inferred on partial 16S rRNA gene fragment [4] led to some important conclusions. Among them, the fact that the gray shaded box in the Figure 4—figure supplement 2—highlights the paraphyletic of the placement of *Bufo gargarizans* sampled in Vietnam (Clade C) in a different subclade segregated from the low-elevated *B. gargarizans* clades from the eastern mainland (Clade A) and northeast Asia (Clade B). The new species, unlike the species of this complex, except only *Bufo pageoti* Bourret, 1937, penetrates far into the tropics. This corresponds to our conclusions about the independence of the "southern lineages" of the *B. gargarizans* species complex and supports the conclusion that this widely studied cryptic species complex, from a phylogeographic perspective, is still confusing taxonomically [5,31].

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Institutional Review Board Statement: This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of the Zoological Institute Russian Academy of Sciences (protocol No.1-3-15-06-2021, 15 June 2021) and Vietnam Academy of Since and Technology – Institute of Genome research (protocol No. 115/QĐ-NCHG, 4th July 2021, based on the Vietnam Museum of Nature - Academy of Sciences and Vietnam Technology in Document No. 639/BTTNVN dated September 12, 2019 and proposal of the Department of Foreign Affairs of Ha Tinh province in Document No. 657/SNgV-LSBG dated October 4, 2019 (after obtaining consensus from relevant units and localities).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data from the study is available upon request from authors.

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Appendix A. Accession Numbers of the GenBank of Used Samples

Appendix A.1. ND2 (n = 97)

B. gargarizans (n = 51): AF004530–33, AF004535, AY936853, AY936860–64, AY936872–73, AY936875, KY806453–54, KY806492, KY806497, KY806512–13, KY806522, KY806529, KY806533, KY806535, KY806537, KY806539, KY806551, MW467607–08, MW467610, MW467613–14, MW467619, MW467621, MW467626, MW467631–32, MW467634–35, MW467651–52, MW467679, MW467730, MW467733, MW467736, MW467739, MW467758, MW467773, MW467776–77, MW467780.

B. stejnegeri (n = 2): MW467648, MW467650.

B. sachalinensis (n = 22): AY936870, KY295954, KY295958, KY295963, KY295965, KY295970–71, KY295975–76, KY295982, KY295988, KY295992, MW467637, MW467659, MW467665, MW467693, MW467710, MW467727, MW467728, OR113667–69.

B. japonicus (n = 5): MW467640–41, MW467643–45.

B. andrewsi (n = 13): AF004527, AY936838–42, AY936844–46, AY936848, AY936868–69, AY936876.

B. sp. nov. (n = 4): AY936852, OR113670–72.

The sequences of common toad (*B. bufo*, DQ629612; MK014039) and Caucasian toad (*B. verricossimus*, AF004526) were used as an outgroup.

Appendix A.2. RAG1 (n = 76)

B. gargarizans (n = 27): KF666177, MW489967–76, MW489978, MW489991, MW490014–16, MW490024, MW490028–37.

B. stejnegeri (n = 15): MK031634, MK031636–37, MK031639, MK031643–47, MK031649, MK031652–53, MW489983–85.

B. sachalinensis (n = 19) MW489979, MW489989–90, MW489994, MW489999, MW490001–04, MW490006–10, MW490012, MW490027, OR113661–63.
B. japonicus (n = 2): MW489980–81.
B. andrewsi (n = 10): MW490013, MW490017–23, MW490025–26.
B. sp. nov. (n = 3) OR113664–66.

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