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# Resolving the taxonomic equivocacy and the population genetic structure of *Rana uenoi* – insights into dispersal and demographic history

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**Abstract.** *Rana uenoi*, a common brown frog inhabiting South Korea and the Japanese Tsushima Island, has a long history of taxonomic discussion. Although *R. uenoi* was described in 2014 as being distinct from *R. dybowskii*, the taxonomic status and distribution of the South Korean clade of *R. uenoi* has remained uncertain, particularly with respect to the related species *R. dybowskii*. Considering the species' importance as a biological indicator of climate change, resolving the taxonomic equivocacy and clarifying its genetic structure is a matter of urgency. To do this, we included samples from across its distribution in South Korea, sequenced and analyzed Cytochrome *b* sequences, and developed novel microsatellite markers for population genetic analyses. Our phylogenetic analyses verified the absence of *R. dybowskii* or another species in South Korea and confirmed the genetic divergence between *R. uenoi* and *R. dybowskii*. Population genetic analyses showed two distinct groups within *R. uenoi*, one on the Korean mainland and Japanese Tsushima Island and the second on Jeju Island, with the likely scenario being the Jeju Island population originating from mainland Korea during the Pleistocene. Demographically, we found evidence of population bottleneck events. Based on these results, we propose the English common name "Korean large brown frog" and a Korean common name "큰산개구리" [keun-san-gaeguri] for *R. uenoi*. These results provide an important baseline data for understanding future climate change impacts.

Key words. Amphibia, Anura, Ranidae, *Rana uenoi*, *R. dybowskii*, brown frogs, taxonomic review, population genetic analysis, bottleneck, Jeju Island.

## Introduction

Protruding from Northeast Asia into the North Pacific Ocean, the Korean Peninsula functioned as a glacial refugium for numerous species during the Pleistocene glacial periods (Chung et al. 2018, Lee et al. 2018), especially for amphibians (Zhang et al. 2008, Borzée et al. 2017, Jeon et al. 2021). This function resulted in the peninsula harbouring today a significant biodiversity and cryptic endemism relative to its size (Baek et al. 2011, Chung et al. 2018). In this respect, there has been a long-lasting discussion about the cryptic diversity and taxonomic status of a common brown frog species in South Korea, *Rana uenoi*, particularly in relation to *R. dybowskii* (Matsui 2014, Song & Chang 2018).

Since the establishment of binomial nomenclature and the description of the first brown *Rana* frog from Northeast Asia, the scientific name of the South Korean clade of *R. uenoi* has changed several times. This South Korean clade was first treated as a species of the *Rana temporaria* Linnaeus, 1758 group that was presumed to be distributed across the Eurasian continent (Stejneger 1907). The clade was later assigned to *R. dybowskii* Günther, 1876 (Günther 1876), followed by the its designation as a subspecies, *R. temporaria ornativentris* (Okada 1928, Matsui 2014) in 1928, and later changed to *R. t. temporaria* (Shannon 1956). The taxonomic status of the clade in South Korea subsequently underwent a period of back-and-forth, oscillating between two subspecies of the *Rana temporaria* group: *R. t. dybowskii,* and *R. t. chensinensis*. The name *R. dybowskii* was used for the clade

in South Korea for the first time in 1978 (Yang & Yu 1978) and was also confirmed as a species-level taxon in 1983 owing to different chromosome numbers compared to *R. temporaria* (Green 1983, Frost 1985).

Since then, *R. dybowskii* became the recognized name of the South Korean clade (Kim et al. 1999, 2002, Lee et al. 2011) until Matsui (2014) assigned clades from the Japanese Tsushima Island and South Korea to the newly described *R. uenoi*. In doing so, Matsui (2014) split the new species from *R. dybowskii* from northern regions in China and Russia. Despite the description of the new species *R. uenoi*, this name has not yet gained general acceptance, and the denomination *R. dybowskii* is still frequently used in Korea (e.g., Lee & Park 2016, Do et al. 2018, National Institute of Biological Resources 2019, The Korean Society of Herpetologists 2020, Park et al. 2021), with some authors treating *R. uenoi* as a synonym of *R. dybowskii* (e.g., Lee & Park 2016, National Institute of Biological Resources 2019, The Korean Society of Herpetologists 2020). The absence of a Korean common name for *R. uenoi* is another reason for this taxonomic equivocacy, and the Korean common name of *R. dybowskii* has been used for both species to date, despite the fat that there are even two Korean common names for it: 북방산개구리 [bukbang-san-gaeguri] and 산개구리 [san-gaeguri] (Song & Chang 2018). Additionally, some herpetologists also suspect the presence of *R. dybowskii* and an undescribed cryptic species in South Korea due to the morphological variations within the South Korean clade of *R. uenoi* (Lee & Park 2016).

*Rana uenoi* is a common brown frog in South Korea, but its distribution is limited to the Korean Peninsula and nearby islands, including the South Korean Jeju Island and the Japanese Tsushima Island. As a result of overharvesting for food and the loss of breeding habitat, its population has been shrinking and it was consequently added to the list of species whose capture is prohibited in South Korea (National Institute of Biological Resources 2019). In addition, this species was listed as a biological indicator of climate change by the Korean government (National Institute of Biological Resources 2019), due to its breeding season shifting towards an earlier date, putatively due to global warming and increased winter temperatures (Kwon et al. 2020). In Korea, the genetic structure of *R. uenoi* populations has been studied using mitochondrial and allozyme data, but these studies are limited in their geographic scopes (Kim et al. 1999, 2002). Further population genetic studies are necessary to establish a baseline and monitor changes before the species might become significantly impacted by future climate change (e.g., mortality of egg masses due to abnormal temperature fluctuations during warmer-than-normal winters).

Here, we review the taxonomic status of *R. uenoi* using a DNA barcode gene Cytochrome *b* (Cyt *b*), define its population structure using Cyt *b* and nine newly developed microsatellite markers, and clarify the presence or absence of *R. dybowskii* and any other related species in South Korea. Contrary to previous studies using a limited number of samples (Kim et al. 1999, 2002, Matsui 2014), here we analyzed data from samples collected across South Korea.

## Materials and methods

### Sample collection

Putative *Rana uenoi* samples were collected between 2006 and 2020 from ten geographic populations in South Korea. By including GenBank sequences from the type locality (Tsushima, Japan), we covered the whole distribution range of this species (Fig. 1 and Table 1). For tissues, we clipped a toe at the height of the last joint for adults or captured tadpoles (from separated schools) and stored them in 70% EtOH until DNA extraction. Each adult was released at the point of capture immediately after sample collection. Sampling was permitted by local governments according to the Wildlife Protection and Management Act of the Korean Ministry of Environment. The tissue sampling protocol conformed to the guidelines of Seoul National University Institutional Animal Care and Use Committee on ethical animal experimentation. We collected a total of 239 samples for this study (see Table 1 for the detailed sample sizes of each analysis).

### Laboratory protocols

We extracted Genomic DNA with the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's manual. Extracted DNA quantity and quality were evaluated using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA). We uniformly di-

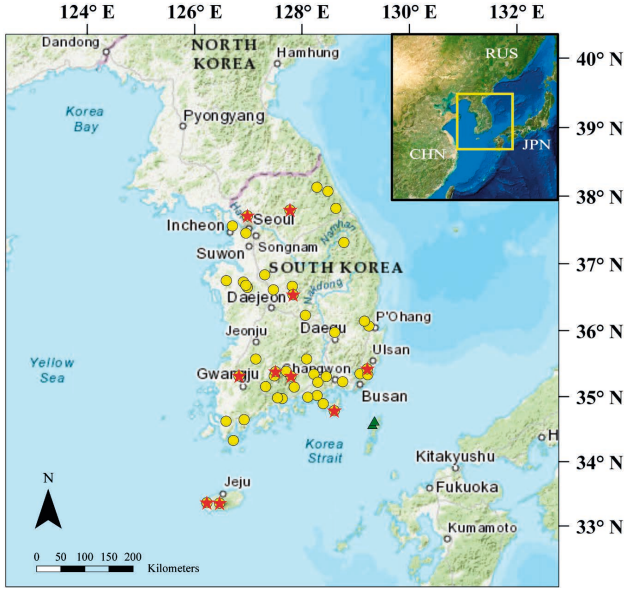


Figure 1. Sampling localities of *Rana uenoi* used in this study throughout South Korea. Copyright of the base map belongs to OpenStreetMap contributors and the GIS User Community. Yellow dots indicate sampling sites for mitochondrial data, while red stars indicate sampling sites for microsatellite data. Green triangles indicate approximated localities for mitochondrial data of individuals from Yang et al. 2017.

Table 1. Sample sizes for mitochondrial DNA and microsatellite analyses of *Rana uenoi* populations in South Korea and the type locality (Tsushima, Japan; sequences from GenBank).

| Category | Province | City / County | Sample size | | |
|---|---|---|---|---|---|
| | | | Phylogenetic Tree | Haplotype Network | Microsatellite Analyses |
| Mainland | Gyeonggi | Yangju | 6 | 6 | 14 |
| | | Seoul | 4 | 4 | – |
| | | Incheon | 5 | 3 | – |
| | Gangwon | Chuncheon | 2 | 2 | 20 |
| | | Yangyang | 1 | 1 | – |
| | | Inje | 3 | 3 | – |
| | | Gangneung | 1 | 1 | – |
| | | Jeongseon | 2 | 2 | – |
| | North Chungcheong | Goesan | 4 | 4 | – |
| | | Cheongju | 1 | 1 | – |
| | | Boeun | 4 | 4 | 13 |
| | South Chungcheong | Asan | 2 | 2 | – |
| | | Cheonan | 1 | 1 | – |
| | | Seosan | 3 | 3 | – |
| | | Yesan | 2 | 2 | – |
| | | Gongju | 1 | 1 | – |
| | North Gyeongsang | Pohang | 2 | 2 | – |
| | | Gimcheon | 1 | 1 | – |
| | | Daegu | 1 | 1 | – |
| | | Gyeongju | 1 | 1 | – |
| | South Gyeongsang | Hapcheon | 1 | 1 | – |
| | | Hamyang | 2 | 2 | – |
| | | Ulsan | 6 | 5 | 16 |
| | | Yangsan | 6 | 6 | – |
| | | Uiryeong | 2 | 2 | – |
| | | Sancheong | 3 | 3 | 9 |
| | | Haman | 1 | 1 | – |
| | | Gimhae | 1 | 1 | – |
| | | Jinju | 3 | 3 | – |
| | | Busan | 7 | 7 | – |
| | | Hadong | 2 | 2 | – |
| | | Sacheon | 1 | 1 | – |
| | | Goseoung | 3 | 3 | – |
| | | Tongyeong | 3 | 3 | – |
| | | Geoje | 5 | 5 | 18 |
| | North Jeolla | Namwon | 9 | 8 | 17 |
| | | Jeonju | 1 | 1 | – |
| | South Jeolla | Jangseong | 2 | 2 | 17 |
| | | Gurye | 1 | 1 | – |
| | | Gokseoung | 2 | 2 | – |
| | | Gwangyang | 2 | 2 | – |
| | | Suncheon | 1 | 1 | – |
| | | Jangheung | 2 | 2 | – |
| | | Haenam | 1 | 1 | – |
| | | Wando | 1 | 1 | – |
| Island | Jeju | Jeju | 3 | 3 | 20 |
| | | Seogwipo | 2 | 2 | 19 |
| Japan | Tsushima | Sago | 1 | 1 | – |
| | | Shitaru | 2 | 2 | – |
| Total | | 49 | 123 | 119 | 163 |

luted successfully extracted DNA samples to 10–20 ng/μl and stored at -20°C until the genetic analysis.

We amplified the partial mitochondrial Cyt *b* region using the primers Cytb 981F and Cytb 981R (Min et al. 2004). The PCR mixture consisted of total volume of 30 μl containing 1X Taq buffer with 2 mM $MgCl_2$, 0.2 mM dNTP mixture, 1 μl of 10 μM forward and reverse primers each, 1 U Ex Taq polymerase (Takara Bio, Shiga, Japan), and 1 μl of template DNA using a TaKaRa PCR Thermal Cycler Dice Gradient. The PCR cycling procedure started with 5 min at 95°C for initial denaturation; followed by 35 cycles of 40 sec at 95°C for denaturation, 40 sec at 45°C for primer annealing, 1 min at 72°C for extension; finishing with 7 min at 72°C for final extension. PCR products were sequenced on an AB 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA) by Cosmo Genetech Inc. (Seoul, South Korea).

Novel microsatellite markers were developed for *Rana uenoi*. We outsourced whole genome paired-end sequences of *Rana uenoi* (see Suk et al. 2021 for mitochondrial whole genome information), using the Illumina MiSeq platform (Illumina, San Diego, CA, USA), to Macrogen Inc. The adaptor sequences were removed from reads, then sequences were scanned to identify repeat DNA sequences using RepeatModeler (Smit et al. 2014) (http://www.repeatmasker.org/). We used SSR Finder (Stieneke & Eujayl 2019) to annotate reads containing simple sequence repeats. A total of 40 candidate markers (10 tetra-, 15 tri- and 15 dinucleotide repeats) were tested (amplification and polymorphism) on eight individuals (two samples from each of four geographic populations). Finally, a total of 13 microsatellite markers were chosen to genotype all the remaining samples. The microsatellite PCR mixture consisted of 1X Taq buffer with 2 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.5 μl of 10 μM forward and reverse primers each, 1 U i-StarTaq polymerase (iNtRON Biotechnology, Seongnam, South Korea) and 1 μl of template DNA in a total volume of 20 μl. PCR cycling was conducted using a Takara PCR Thermal Cycler Dice Gradient (Takara Bio) with touchdown-PCR conditions consisting of an initial denaturing at 94°C for 5 min, 20 cycles of denaturing at 94°C for 20 sec, touchdown annealing at 60–50°C for 20 sec, and an extension at 72°C for 20 sec; followed by 20 cycles of denaturing at 94°C for 20 sec, annealing at 50°C for 20 sec, and an extension at 72°C for 20 sec; followed by a final extension at 72°C for 7 min. We outsourced the genotyping of amplified PCR products on an ABI 3730xl platform to NICEM Inc. (Seoul, South Korea). Microsatellite and mitochondrial sequences used in this paper have been deposited at GenBank (Accession Nos. MW448298–448330, MW58891–58902, MW626913–626915).

### Cyt *b* phylogenetic tree and haplotype network

We checked the quality and edited Cyt *b* sequence data using Geneious Prime (https://www.geneious.com). The sequences were aligned in MEGA-X (Kumar et al. 2018) using the Clustal-W (Thompson et al. 1994) method under the default setting. We generated Cyt *b* haplotype data using DnaSP 5.10.01 (Librado & Rozas 2009) and deposited the haplotype sequences at GenBank (Accession Nos. MW448298–448330, MW58891–58902, MW626913–626915; Supplementary Table S1).

For the phylogenetic tree reconstruction, we included Cyt *b* sequences of *Rana* frogs from GenBank, mostly Eurasian species, including three *R. uenoi* samples from the type locality (27 species, 35 sequences), and used *Pelophylax nigromaculatus* from GenBank as an outgroup (Supplementary Table S1). The substitution model was selected according to the Bayesian Information Criterion computed by the greedy algorithm (Lanfear et al. 2012) using Partition Finder 2.1.1 (Lanfear et al. 2017) before tree reconstruction. We reconstructed the Bayesian inference (BI) phylogenetic tree in MrBayes 3.2.6 (Ronquist & Huelsenbeck 2003), treating missing values as non-contributing. The software conducted two independent runs of $10^7$ Metropolis Coupled Markov Chain Monte Carlo (MCMC) generations using four chains (three heated and one cold chain; temperature set to 0.1) per run. Trees were sampled every 500 generations and summarized after discarding 25% as burn-in. We visualized the final tree in FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

We reconstructed the haplotype network computed from the Cyt *b* sequences of *R. uenoi* using the median-joining approach in NETWORK 10.0.0.0 (Bandelt et al. 1999) (http://www.fluxus-engineering.com). The network calculation relied on variable sites within the 731 bp of the Cyt *b* region from a total of 116 sequences, including three *R. uenoi* samples from Tsushima Island.

### Population genetic analyses using microsatellites

We checked the quality of genotyping and peak calling using Geneious Prime. We used Micro-Checker 2.2.3 (van Oosterhout et al. 2004) to detect experimental errors, such as null alleles, large allelic dropouts, and scoring errors. We examined the likelihood of linkage disequilibrium between the 13 selected marker loci and the Hardy-Weinberg equilibrium using Fisher's exact test as incorporated in GENEPOP 4.7.2 (Rousset 2008) with default settings. We used GenAlEx 6.503 (Peakall & Smouse 2006) to calculate summary statistics for each locus and geographic population, such as number of alleles (N), number of effective alleles ($N_a$), observed heterozygosity ($H_O$), expected heterozygosity ($H_E$), and fixation index ($F_{IS}$) for each locus and population. Pairwise-$F_{ST}$ (Wright 1965) and -$R_{ST}$ (Slatkin 1995) values between populations were calculated with 1,000 permutations using ARLEQUIN 3.5.2.2 (Excoffier et al. 2005). We used GenAlEx to carry out a Principal Coordinate Analysis (PCoA) with a covariance-standardized approach to identify genetic clusters among populations based on the distribution of individuals. We used STRUCTURE 2.3.4 (Pritchard et al. 2000) to infer the Bayesian population genetic structure from 10 in-

dependent runs of $10^5$ MCMC generations (including 10% burn-in) from K = 1 to K = 10. The optimal K was determined based on the Evanno method (Evanno et al. 2005) on STRUCTURE HARVESTER web 0.6.94 (Earl & Vonholdt 2012). Lastly, we searched for signs of bottleneck events using the mode-shift indicator and Wilcoxon signed rank test in BOTTLENECK 1.2.02 (Cornuet & Luikart 1997) with 1,000 iterations at TPM (0% SMM, 36% variance; as recommended for most microsatellites by the software authors) and a Garza-Williamson index (M-ratio; Garza & Williamson, 2001), which is lower than 0.68 if populations have undergone population declines, using ARLEQUIN.

For further analyses, we divided the Korean samples into two groups: one limited to Jeju Island (here referred to as "Island"), and one restricted to the Korean mainland and associated islands that used to be in direct contact with the mainland until a few thousand years ago (Li et al. 2014) (referred to as "Mainland"). The Japanese samples were not used in this analysis. To understand the demographic history of Mainland and Island populations, two dispersal scenarios (Scenario 1 and Scenario 2) were compared using the approximate Bayesian computation incorporated in DIYABC 2.1.0 (Cornuet et al. 2014) with microsatellite data. Scenario 1 describes 'from Mainland to Island' dispersal and Scenario 2 'from Island to Mainland' dispersal. The parameter settings for the two groups and the two scenarios were based on preliminary analyses that had followed the user manual. To test our scenarios, we applied a generalized stepwise mutation model with the generation time set to 2–3 years. Ranges of prior distributions of parameters were adjusted from preliminary runs.

## Results

### Phylogenetic tree using Cytochrome *b*

We recovered a 802-bp aligned segment including sites with missing characters for the phylogenetic tree analysis and a 731-bp aligned segment without missing characters for the haplotype network analysis. Among the 120 *Rana uenoi* individuals sampled in South Korea, 45 haplotypes were detected with DnaSP when including sites with missing characters and 33 haplotypes when excluding sites with missing values. The best partitioning scheme deduced from these haplotypes was a 3-partition scheme according to codon positions: F81 (Felsenstein 1981) + I for the first position, TRNEF (Tamura & Nei 1993) + I + G for the second position, and TIM + G for the third position of codons. In the BI tree, all three *R. uenoi* individuals from Tsushima Island clustered with the Mainland populations of South Korea, having the same single haplotype as individuals from Busan and Yangsan (in South Gyeongsang Province) which are the regions of the Korean Peninsula closest to Tsushima Island. There were no particular groupings within the Mainland populations in relation to geographic locations (Fig. 2). However, the Island populations in Jeju Island clustered separately from the Mainland populations (Fig. 2).

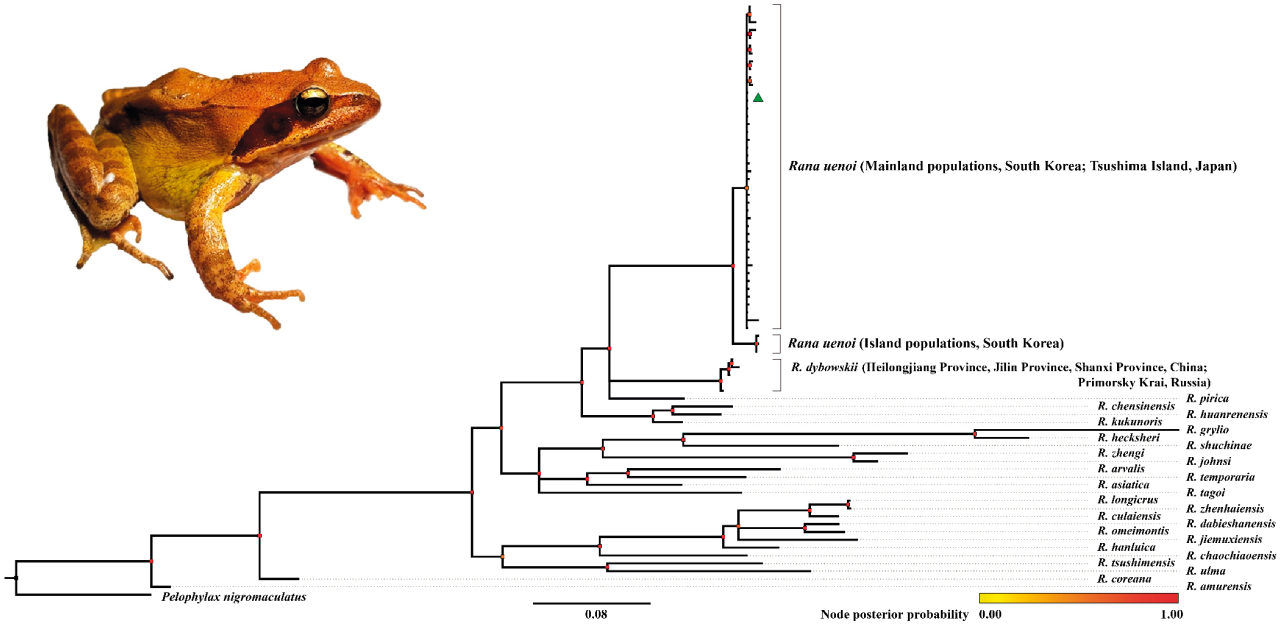


Figure 2. Bayesian phylogenetic tree of *Rana uenoi* and other species in the family Ranidae based on a fragment of the mitochondrial Cytochrome *b* gene. The Bayesian posterior probability (BPP) of each node is indicated by the gradient bar below the tree, where dark red represents a higher BPP. The green triangle indicates the position of the haplotype including Tsushima Island samples. Detailed information on populations of *Rana uenoi* and outgroup species can be found in Table 1 and Supplementary Table S1.

The haplotype network indicated a pattern concordant with that of the phylogenetic tree; The network did not highlight any particular grouping based on geographic location, but had one major haplotype including individuals from all eight administrative provinces of South Korea and all derivative haplotypes clustered around it (Fig. 3). Individuals from Tsushima Island were characterized by the same haplotype as individuals from Busan, and northern Gyeongsang, southern Gyeongsang, and southern Jeolla Provinces. The cluster of haplotypes from Jeju Island individuals was separated from Mainland haplotypes by 11 mutational steps (Fig. 3).

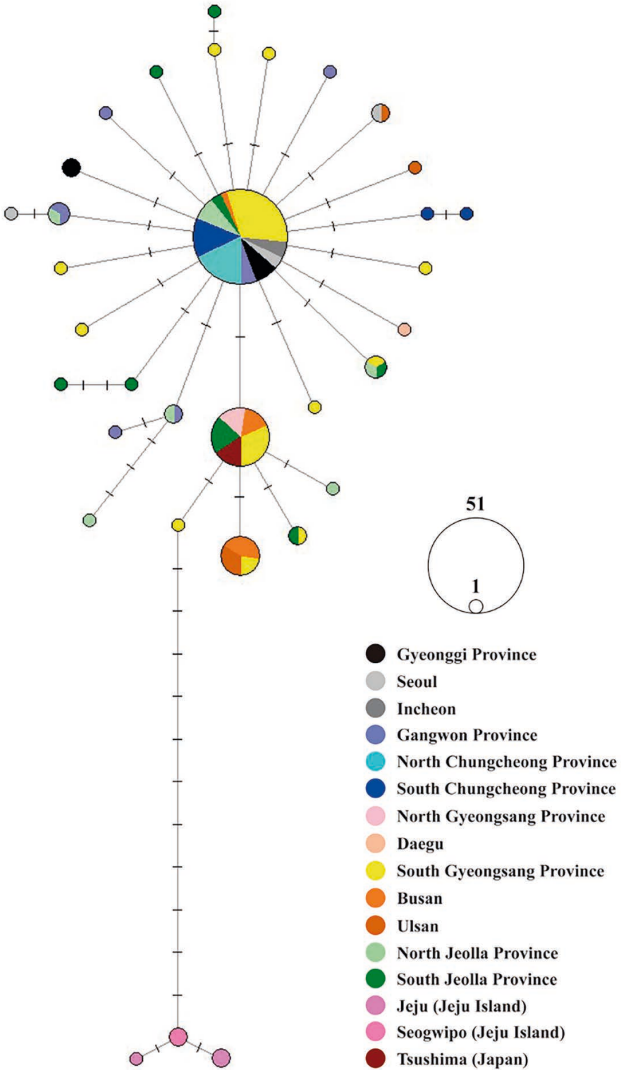


Figure 3. The haplotype network of populations of *Rana uenoi* based on a fragment of the mitochondrial Cytochrome *b* gene. Circle sizes are proportional to the number of samples included in the haplotype analyses. Sites were grouped into provinces and each province was colour-coded. Detailed information on sample sizes from each locality, localities included in each province, and localities included in each haplotype analysis can be found in Table 1 and Supplementary Table S1.

Table 2. Descriptive indices of the nine microsatellite markers newly developed for *Rana uenoi* in South Korea. Na = the number of alleles, Ne = the effective number of alleles, $H_O$ = observed heterozygosity, $H_E$ = expected heterozygosity, and $F_{IS}$ = fixation index of each microsatellite marker.

| Locus | Repeat motif | Allele range | Na | Ne | $H_O$ | $H_E$ | $F_{IS}$ |
|---|---|---|---|---|---|---|---|
| RuMic_4 | ATAC | 70–162 | 21 | 6.638 | 0.509 | 0.849 | 0.400 |
| RuMic_7 | AGAT | 172–484 | 49 | 27.604 | 0.761 | 0.964 | 0.211 |
| RuMic_10 | AAAG | 172–336 | 19 | 10.662 | 0.595 | 0.906 | 0.343 |
| RuMic_17 | ATA | 189–252 | 21 | 11.475 | 0.778 | 0.913 | 0.148 |
| RuMic_21 | TG | 104–250 | 43 | 24.669 | 0.558 | 0.959 | 0.419 |
| RuMic_28 | GT | 146–178 | 12 | 5.583 | 0.521 | 0.821 | 0.365 |
| RuMic_34 | TAT | 120–201 | 17 | 7.761 | 0.407 | 0.871 | 0.532 |
| RuMic_35 | TAA | 205–262 | 18 | 6.505 | 0.613 | 0.846 | 0.275 |
| RuMic_39 | AT | 101–143 | 12 | 4.417 | 0.571 | 0.774 | 0.262 |

## Population genetic analyses using microsatellites

Thirteen microsatellite markers (7 tetra-, 3 tri-, and 3 dinucleotides) were developed out of the 40 candidates (deposited in GenBank, accession Nos. MW273296–MW273308; Supplementary Table S2). Among the 13 microsatellite markers, nine pairs exhibited evidence of linkage disequilibrium in GENEPOP (Supplementary Table S2), resulting in the exclusion of four markers (RuMic_2, 3, 6, and 9). The remaining nine markers (3 tetra-, 3 tri-, 3 dinucleotides) were used for further analyses. All markers had moderate levels of null alleles based on the results provided by MICRO-CHECKER, but we kept all nine markers because phylogenetically similar species are known to show similar characteristics (Martínez-Solano et al. 2005, Zhang et al. 2010). When we detected stuttering, peak calling was manually verified. Allelic dropout was not detected.

Detailed information and descriptive indices for the 13 microsatellite markers are summarized in Table 2 and Supplementary Table S2. The number of alleles ranged from 12 (RuMic_28) to 49 (RuMic_7). Observed heterozygosity ranged from 0.407 (RuMic_34) to 0.778 (RuMic_17) and expected heterozygosity ranged from 0.774 (RuMic_39) to 0.964 (RuMic_7). These discrepancies between $H_O$ and $H_E$ led to high levels of fixation indices. Descriptive indices of populations computed from these microsatellite markers are summarized in Table 3. The range of number of alleles spanned from 5.444 (Jeju) to 12.111 (Namwon). Observed heterozygosity ranged from 0.519 (Sancheong to 0.634 (JS) and expected heterozygosity ranged from 0.514 (Jeju) to 0.859 (Boeun). The discrepancy between $H_O$ and $H_E$ for all populations, with the exception of Jeju and Seogwipo that had little discrepancy, led to high-level fixation indices.

Pairwise-$F_{ST}$ and $R_{ST}$ followed a similar pattern. Generally, moderate to high levels of genetic differentiation were identified, and the populations on Jeju Island (Jeju and Seogwipo) consistently exhibited higher levels of genetic differentiation than any of the other Mainland popula-

Table 3. Descriptive indices of ten *Rana uenoi* populations in South Korea analyzed using microsatellites. Data shown for each population are the samples size (N), the number of alleles (Na), the effective number of alleles (Ne), observed heterozygosity ($H_O$), expected heterozygosity ($H_E$), unbiased expected heterozygosity ($uH_E$), fixation index ($F_{IS}$), mode-shift indicator, and Wilcoxon Test P (one-tailed probability for heterozygosity excess) from BOTTLENECK, and M-ratio.

| Population | N | Na (SE) | Ne (SE) | $H_O$ (SE) | $H_E$ (SE) | $uH_E$ (SE) | $F_{IS}$ (SE) | Mode-shift Indicator | Wilcoxon Test P | M-ratio (SE) |
|---|---|---|---|---|---|---|---|---|---|---|
| Yangju | 14 | 8.000 (1.093) | 5.953 (0.865) | 0.624 (0.081) | 0.799 (0.032) | 0.829 (0.033) | 0.235 (0.095) | L-shaped | 0.00293 | 0.214 (0.178) |
| Chuncheon | 20 | 11.444 (1.396) | 7.043 (1.014) | 0.571 (0.051) | 0.837 (0.020) | 0.859 (0.020) | 0.312 (0.065) | L-shaped | 0.10156 | 0.262 (0.162) |
| Boeun | 13 | 10.111 (0.841) | 7.495 (0.629) | 0.620 (0.055) | 0.859 (0.011) | 0.894 (0.011) | 0.275 (0.067) | L-shaped | 0.00098 | 0.167 (0.086) |
| Ulsan | 16 | 10.778 (1.402) | 6.935 (1.253) | 0.604 (0.040) | 0.812 (0.034) | 0.838 (0.035) | 0.250 (0.050) | L-shaped | 0.12500 | 0.218 (0.141) |
| Sancheong | 9 | 8.111 (0.716) | 5.547 (0.938) | 0.519 (0.069) | 0.781 (0.030) | 0.827 (0.032) | 0.326 (0.092) | L-shaped | 0.63281 | 0.185 (0.119) |
| Geoje | 18 | 8.556 (0.868) | 5.629 (0.660) | 0.617 (0.081) | 0.800 (0.025) | 0.823 (0.026) | 0.224 (0.099) | L-shaped | 0.00293 | 0.165 (0.065) |
| Namwon | 17 | 12.111 (1.918) | 8.174 (1.563) | 0.612 (0.064) | 0.835 (0.031) | 0.860 (0.032) | 0.262 (0.078) | L-shaped | 0.08203 | 0.255 (0.128) |
| Jangseong | 17 | 10.444 (1.215) | 6.965 (1.041) | 0.634 (0.065) | 0.832 (0.022) | 0.857 (0.023) | 0.238 (0.076) | L-shaped | 0.00195 | 0.256 (0.168) |
| Jeju | 20 | 5.444 (1.192) | 3.550 (0.954) | 0.561 (0.128) | 0.514 (0.115) | 0.528 (0.118) | -0.083 (0.048) | L-shaped | 0.27344 | 0.186 (0.093) |
| Seogwipo | 19 | 7.667 (1.944) | 4.292 (1.297) | 0.532 (0.121) | 0.553 (0.112) | 0.568 (0.115) | 0.112 (0.130) | L-shaped | 0.82031 | 0.214 (0.083) |

Table 4. Pairwise-$F_{ST}$ and $R_{ST}$ among 11 *Rana uenoi* populations in South Korea analyzed using nine microsatellites. Estimates of $F_{ST}$ appear above the diagonal and estimates of $R_{ST}$ below diagonal. All estimates significantly deviated from zero ($p < 0.05$) except those denoted by 'NS'. Abbreviations – YJ: Yangju, CC: Chuncheon, BE: Boeun, US: Ulsan, SC: Sancheong, GJ: Geoje, NW: Namwon, JS: Jangseong, JJ: Jeju, SGP: Seogwipo.

| | YJ | CC | BE | US | SC | GJ | NW | JS | JJ | SGP |
|---|---|---|---|---|---|---|---|---|---|---|
| YJ | | 0.035 | 0.053 | 0.046 | 0.081 | 0.075 | 0.048 | 0.035 | 0.277 | 0.247 |
| CC | 0.005$^{NS}$ | | 0.037 | 0.045 | 0.048 | 0.062 | 0.031 | 0.038 | 0.262 | 0.233 |
| BE | 0.046$^{NS}$ | 0.027$^{NS}$ | | 0.054 | 0.057 | 0.056 | 0.018$^{NS}$ | 0.031 | 0.256 | 0.230 |
| US | 0.013$^{NS}$ | 0.027$^{NS}$ | 0.006$^{NS}$ | | 0.051 | 0.076 | 0.030 | 0.058 | 0.264 | 0.233 |
| SC | 0.177 | 0.150 | 0.071$^{NS}$ | 0.133 | | 0.081 | 0.027$^{NS}$ | 0.046 | 0.288 | 0.261 |
| GJ | 0.271 | 0.252 | 0.191 | 0.267 | 0.122 | | 0.042 | 0.031 | 0.260 | 0.238 |
| NW | 0.232 | 0.188 | 0.118 | 0.183 | -0.033$^{NS}$ | 0.072 | | 0.021 | 0.275 | 0.241 |
| JS | 0.153 | 0.121 | 0.073$^{NS}$ | 0.125 | -0.006$^{NS}$ | 0.033$^{NS}$ | -0.020$^{NS}$ | | 0.247 | 0.221 |
| JJ | 0.091 | 0.176 | 0.129 | 0.084 | 0.244 | 0.301 | 0.270 | 0.190 | | 0.041 |
| SGP | 0.117 | 0.219 | 0.177 | 0.117 | 0.336 | 0.423 | 0.369 | 0.283 | 0.012$^{NS}$ | |

tions. In the PCoA, Jeju and Seogwipo populations were clearly separated from all the other Mainland populations by principal coordinate 1 (Fig. 4). Similarly, the STRUCTURE analysis identified two genetic clusters according to the delta K method (Evanno et al. 2005), which distinguished Island from Mainland populations (Fig. 5).

Different signs of demographic history were indicated by the mode-shift indicator and Wilcoxon signed rank test and M-ratio (Table 3). The mode-shift indicator did not suggest any population size reduction in contrast to the M-ratio that indicated population size reduction for all populations, as an M-ratio lower than 0.68 is considered to indicate a reduction in population size (Garza & Williamson 2001). On the other hand, the Wilcoxon signed rank test detected significant bottlenecks in four populations: Yangju, Boeun, Geoje, and Jangseong.

From the result of DIYABC, Scenario 1, 'from Mainland to Island' dispersal, was supported slightly better than Scenario 2 by both the direct approach and the logistic regression results (Supplementary Tables S3, S4 and Fig. S1).

The divergence dates back to 19,700 generations (39,400–59,100 years, based on a generation time of 2–3 years).

## Discussion

In this study, we review the molecular taxonomic status of *Rana uenoi*, a common brown frog in South Korea. We build on the data from previous studies (Matsui 2014, Yuan et al. 2016, Yang et al. 2017) by including samples from across Korea, and verify the absence of *R. dybowskii* from South Korea. Through the development of new microsatellite markers for *R. uenoi*, we are able to characterize the genetic diversity and population structure of this species in South Korea.

Our sampling included *R. dybowskii* samples from China (Changbai Mountain in Jilin Province) and Russia (Primorsky Krai), regions proximate to the Korean Peninsula, and the samples of the South Korean/Japanese *R. uenoi* clade proved distinct from them. This is consistent with Borzée et al. (2021) from which *R. uenoi* is known to be present as far north as Pyongyang in North Korea. *Rana dybowskii* and *R. uenoi* are estimated to have diverged in the Miocene due to the orogenesis of the Changbai Mountain Range approximately 11.01–8.55Ma (Yuan et al. 2016, Yang et al. 2017). Our results, are consistent with this hypothesis, as all samples from south of the Changbai Mountain Range in our analyses were referable to *R. uenoi*. In addition, we did not find evidence for the presence of a species other than *R. uenoi* in Korea and *R. dybowskii*. The fact that all three *R. uenoi* individuals from Tsushima Island were grouped together within the Mainland populations strongly supports the status and distribution of *R. uenoi* as suggested by Matsui (2014).

The Korean Society of Herpetologists still lists *R. uenoi* and *R. dybowskii* under the same Korean common name, and based on our results we suggest to establish a new Korean common name for *R. uenoi*, replacing the current name 북방산개구리 [bukbang-san-gaeguri] where 북방 [bukbang] means northern distribution (which is more relevant to *R. dybowskii*) and 산개구리 [san-gaeguri] refers to the genus *Rana*, with a new name: 큰산개구리 [keun-san-gaeguri]; where 큰 [keun] means 'large' and reflects the species' relatively large body size compared to other Korean brown frog species. In addition, as Matsui (2014) did not define an English common name for *R. uenoi*, we suggest the use of "Korean large brown frog", considering its primary distribution on the Korean Peninsula.

The genetic diversity of the South Korean clade of *R. uenoi* was higher in Mainland populations than in Island populations, but the inbreeding coefficient showed a contrary pattern. This implies that geographic populations on the Korean mainland have been altogether genetically more isolated and diverse than the populations on Jeju Island, perhaps reflecting complex geographic barriers on the Korean mainland (Jeon et al. 2021). Regarding the species' population genetic structure, it is worth noting that while individuals from the Mainland populations are grouped together with individuals from Tsushima Is-

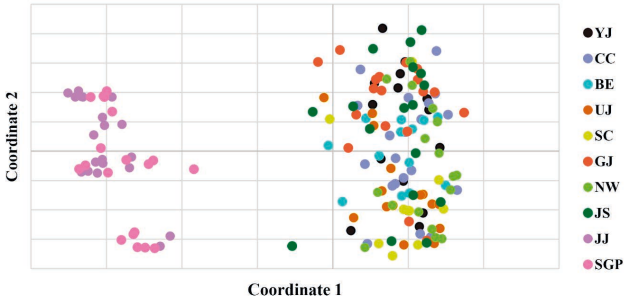


Figure 4. The principal coordinate analysis plot based on microsatellite $F_{ST}$ values for populations of *Rana uenoi*. Coordinates 1 and 2 explain 13.75 and 4.93% of the genetic differentiation between populations, respectively. Abbreviations – YJ: Yangju, CC: Chuncheon, BE: Boeun, US: Ulsan, SC: Sancheong, GJ: Geoje, NW: Namwon, JS: Jangseong, JJ: Jeju, SGP: Seogwipo. JJ and SGP refer to populations on Jeju Island, while all others are from the Korean mainland. Colour coding of localities follows their province's colour in Figure 3 (e.g., Black for YJ in this figure and Gyeonggi Province in Figure 3).

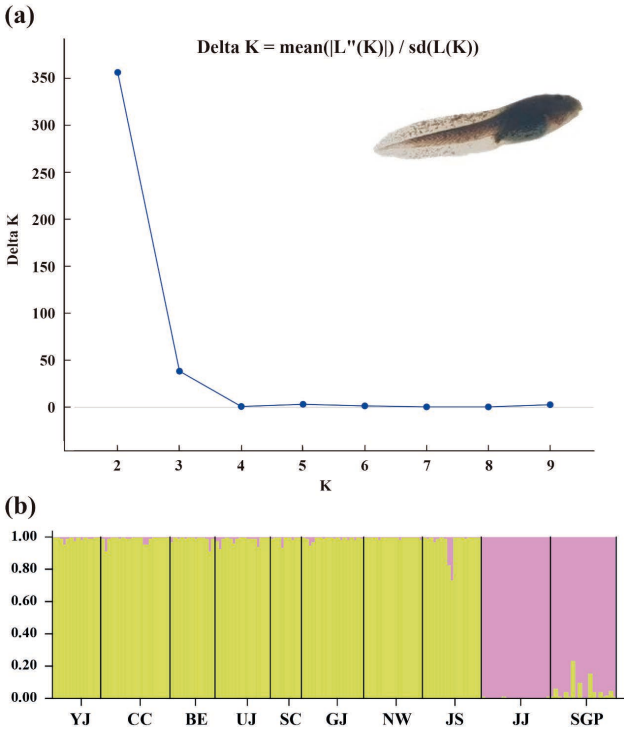


Figure 5. The delta K graph (a) and bar plot (K = 2) from the STRUCTURE result (b) based on microsatellite data, showing genetic differentiation between populations of *Rana uenoi*. The delta K graph indicates that K = 2 is the optimal number of populations. Abbreviations – YJ: Yangju, CC: Chuncheon, BE: Boeun, US: Ulsan, SC: Sancheong, GJ: Geoje, NW: Namwon, JS: Jangseong, JJ: Jeju, SGP: Seogwipo. JJ and SGP refer to populations on Jeju Island, while all others are from the Korean mainland.

land, individuals from the Island population form a cluster that is differentiated from the Mainland/Tsushima. This result is consistent throughout all analyses: pairwise-$F_{ST}$/$R_{ST}$, phylogenetic tree, haplotype network, PCoA, and STRUCTURE result. The DIYABC analysis inferred that the differentiated Island population was the result of immigration from the mainland, which began before the Last Glacial Maximum when the mainland and the islands were connected, between 33,000 and 14,000 years ago (Clark et al. 2009). Temporal changes of connectivity between the mainland and the islands are likely related to the pattern of sea level change in the Yellow Sea. When the continental shelf was maximally emerged circa 21,000 years ago (Li et al. 2014), it facilitated connectivity between amphibian populations (Borzée et al. 2020). However, Jeju Island was separated from the Korean Peninsula by the combined large palaeorivers, creating a boundary difficult to cross for small amphibian species (Angelone & Holderegger, 2009; Le Lay et al. 2015), as is also seen in the Vistula River in Poland, marking the boundary between *Hyla arborea* and *H. orientalis* (Stöck et al. 2012).

Similar patterns of genetic isolation or subspeciation on Jeju Island have been reported for other animals, too, including for mammals (Siberian Roe Deer [*Caproulus pygargus*; Koh & Randi 2001; Koh et al. 2013; Park et al. 2014; Lee et al. 2015, 2016], Lexmann's Shrew [*Sorex caecutiens*; Koh et al. 2012], Striped Field Mouse [*Apodemus agrarius*; Yoon et al. 2004; Oh et al. 2013] and Asian Lesser White-toothed Shrew [*Crocidura shantungensis*; Lee et al. 2018]), and other amphibians (Jeju Salamander [*Hynobius quelpaertensis*; Suk et al. 2020] and Japanese Tree Frog [*Dryophytes japonicus*; Jang et al. 2011]). Faunal divergence between mainland Korea and Jeju Island has been attributed to the formation of a temporary land bridge between the Korean Peninsula and Jeju Island during the Pleistocene glacial epochs (Yoon et al. 2004, Oh et al. 2013, Park et al. 2014, Lee et al. 2018), and it is hypothesized that individuals of *Rana uenoi* dispersed from the Korean mainland to Jeju Island when a land bridge formed (Woo et al. 2013, Lee et al. 2018), became isolated when this bridge disappeared, and the Mainland and Island populations began to diverge genetically (Johnson et al. 2000, Jang et al. 2011, Park et al. 2014) by random genetic drift and/or adaptation to the island's environmental conditions (Jo et al. 2012).

Signs of bottlenecks in all geographic populations were detected by the Wilcoxon sign ranked test and M-ratio, but not by the mode-shift indicator. This incongruence between bottleneck indices has also been noted in several other empirical studies (Hundertmark & Van Daele 2010, Swatdipong et al. 2010, Gong et al. 2015) and could be attributed to different calculation methods and the resultant time scales (Cornuet & Luikart 1997, Luikart et al. 1998, Garza & Williamson 2001). The M-ratio compares the number of alleles to the range of alleles and generally resolves along a timescale of several hundred generations (Garza & Williamson 2001). On the other hand, the Wilcoxon signed rank test is a non-parametric test to check for a significant loss of alleles and it reflects two to four times the 'effective population size ($N_e$)' generations (Luikart et al. 1998, Valenzuela-Quiñonez et al. 2014). Lastly, the mode-shift indicator examines allele frequency distribution with the idea that bottlenecks reduce the occurrence of low-frequency alleles and increases that of intermediate- and high-frequency alleles (Luikart et al. 1998), which may occur over a few dozen generations (Valenzuela-Quiñonez et al. 2014). Considering that this species' $N_e$ should be much higher than a few dozen individuals (Supplementary Table S3), the mode-shift indicator reflected only the most recent demographic history. Overall, it seems more likely that *R. uenoi* populations, and especially the Mainland populations, suffered from population size reduction a much longer time ago than a few dozen generations and have since recovered. This is supported by a star-shaped haplotype network, a common signature of rapid population expansion after population size reduction (Slatkin & Hudson 1991, Hewitt 2004). The smaller population size of the Island than on the Mainland in DIYABC may be due to the founder effect or the difference in the number of samples used for Mainland and Island (124 Mainland samples, 39 Island samples).

The characteristics of the markers developed in this study could be affected by this bottleneck event. A wide range of alleles compared to the number of alleles, as detected by the M-ratio, was found in three markers (e.g. RuMic_7, 10, and 21; in Table 2). The relatively low levels of observed heterozygosity (Table 2) may have resulted from genetic drift or inbreeding that have increased the number of homozygotes during population expansions after population size reductions (Cornuet & Luikart 1997, Kleinhans & Willows-Munro 2019). The nine marker pairs in linkage disequilibrium also suggest the presence of previous bottleneck events, since reduced effective population size can cause superficial linkages between unlinked loci (Hill 1981, Ledig et al. 1999, Freeland et al. 2011). Similar genetic consequences were identified in the sister taxon, *R. dybowskii* (Zhang et al. 2010), and in *R. iberica*, a species once restricted to a refugium and now endemic to the Iberian Peninsula (Martínez-Solano et al. 2005).

We reviewed the taxonomic status of *Rana uenoi* using Cyt *b* sequences and microsatellites to investigate the population genetic structure of this common and large brown frog in South Korea. From the phylogenetic analyses, we verified the absence of *R. dybowskii* and other species in South Korea. Previous studies (Matsui 2014, Yang et al. 2017, Suk et al. 2021) have identified *R. uenoi* and *R. dybowskii* as distinct species, which we confirm after using a larger dataset including samples from across South Korea. Based on our results, we recommend the full replacement of instances of "*Rana dybowskii*" with "*Rana uenoi*" in the species lists of the Korean government and academic societies (National Institute of Biological Resources 2019, The Korean Society of Herpetologists 2020). We also suggest changing the Korean common name of *R. uenoi* to 큰산개구리 [keun-san-gaeguri] and establishing "Korean large brown frog" as its English common name, both of which were previously lacking. From the population ge-

netic analyses, we conclude that Mainland and Island populations are differentiated, and the Mainland populations have experienced a population bottleneck in the Pleistocene.

The establishment of future management strategies for *R. uenoi* should be based on the taxonomy and genetic results of this study. As an indicator species of climate change designated by the Korean Ministry of Environment, we should monitor for future ecological and genetic changes, not only for the management of this species due to its high inbreeding level, but also for that of other wildlife on the Korean Peninsula. In this regard, our results provide important baseline data for comparison with future studies. Similarly, commercial imports of *R. dybowskii* into Korea should now be outlawed, as it is not a native species of Korea. Continued imports of *R. dybowskii* may result in the establishment of invasive populations that potentially have negative ecological impacts.

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## Supplementary data

The following data are available online:

Supplementary Figure S1. The outputs of the Approximate Bayesian Computation (ABC) analysis as incorporated in DIYABC for identifying the most likely pattern of historical divergence between populations.

Supplementary Table S1. GenBank accession numbers and geographical information for each Cytochrome *b* haplotype used in this study.

Supplementary Table S2. GenBank accession numbers and detailed information for each microsatellite marker developed in this study.

Supplementary Table S3. Parameter estimation of two DIYABC scenarios between two *Rana uenoi* clusters based on microsatellites.

Supplementary Table S4. Confidence evaluation of two DIYABC scenarios between two *Rana uenoi* clusters based on microsatellites.