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Molecular and Morphological Evidence for *Rana kunyuensis* as a Junior Synonym of *Rana coreana* (Anura: Ranidae)

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ABSTRACT.—**We investigate the relationship between *Rana coreana* and *Rana kunyuensis* using morphological and molecular data. Morphological comparisons showed these two species to be identical in terms of body measurements and qualitative characteristics. Phylogenetic analyses found that *R. kunyuensis* and *R. coreana* are nested within a single clade and exhibited low divergence across several mitochondrial and nuclear genes. All evidence indicates that *R. kunyuensis* and *R. coreana* are the same species, making *R. kunyuensis* a junior synonym of *R. coreana*. This study stresses the importance of comprehensive taxon sampling, especially in neighboring countries.**

The *Rana temporaria* group (Boulenger, 1920), commonly known as "Brown Frogs," is distributed widely from the Western Palearctic to Northeast Asia (Frost et al., 2006; Frost, 2013). The true species diversity in this group is unclear because of substantial intraspecific morphological variation, high levels of interspecific morphological similarity, and logistic challenges such as obtaining material to compare between countries (Che et al., 2007). The suspected result may be both an under-estimation of diversity, because of the presence of unrecognized or "cryptic" species masquerading in widely distributed species, and additional cases of over-estimation of diversity because of conspecific populations recognized in neighboring countries as different species.

To understand species diversity in the *Rana temporaria* group, it is necessary to incorporate molecular data and comprehensive taxon sampling (Che et al., 2007; Yang et al., 2010; Yan et al., 2011). We compare the morphology and genetics (mitochondrial and nuclear DNA) of two closely related Brown Frogs, *Rana coreana* and *Rana kunyuensis*, and use this as an example of how multinational collaboration in species comparison can help clarify species classification.

Rana coreana is restricted to, but distributed widely across, the Korean Peninsula (Fig. 1). This species was first described as a subspecies of *R. temporaria*: *Rana temporaria coreana* (Okada, 1928) and was later placed as a subspecies of *Rana amurensis* (*Rana amurensis coreana*) because of the short hind legs and the lack of vocal sacs (Shannon, 1956). In 2006, *R. coreana* was recognized as a distinct species based on morphological and genetic data (Song et al., 2006). However, this study did not include samples of the then recently described species *R. kunyuensis* from Kunyu Mountain, Shandong, China (Lu and Li, 2002), which also belongs to the *R. amurensis* group (Fei et al., 2009). To date, the relationship between *R. coreana* and *R. kunyuensis* remains unclear. Herein, we compare the morphology and DNA of *R. coreana* and *R. kunyuensis* to help clarify their systematic relationship.

MATERIALS AND METHODS

Genetic and Phylogenetic Analyses.—We obtained five gene fragments including three partial mitochondrial DNA (mtDNA) sequences (~410 base pairs [bp] of the 16S ribosomal RNA gene [16S], ~750 bp of the cytochrome-*b* gene [Cyt*b*], and ~560 bp of the cytochrome c oxidase subunit 1 gene [COI]) and two partial nuclear DNA (nuDNA) sequences (~810 bp of the recombination-activating protein 2 gene [RAG2] and ~1,010 bp of the ATP-dependent DNA ligase IV gene [LIG4]). A total of 19 specimens of six Brown Frog species were used for phylogenetic analyses: *R. kunyuensis* ($n = 7$), *R. coreana* ($n = 4$), *R. amurensis* ($n = 5$), *Rana chensinensis* ($n = 1$), *Rana zhenhaiensis* ($n = 1$), and *Rana hanluica* ($n = 1$) (Table 1). Among them, seven mtDNA sequences of three specimens were downloaded from GenBank (Yang et al., 2010; Jeong et al., 2013). *Babina daunchina* was selected as an outgroup taxa based on the results of Frost et al. (2006).

Liver and muscle tissues were preserved in 95% ethanol and stored at −75°C before use. Total DNA was extracted using the TIANamp Genomic DNA Kit (Tian Gen Biotech

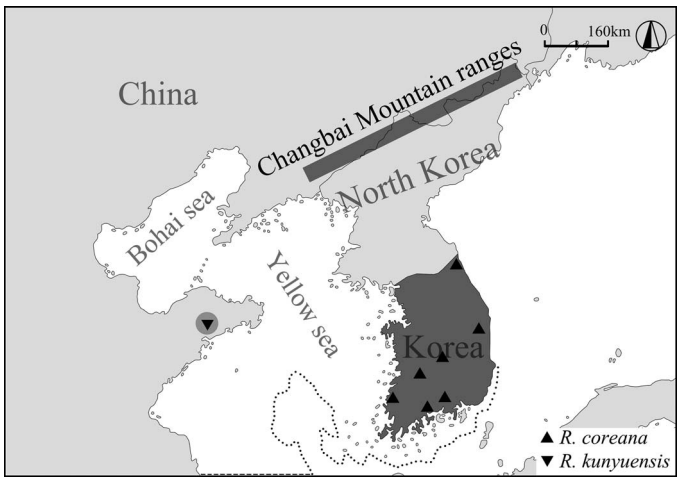

FIG. 1. Distribution map of sampling locations of *R. coreana* and *R. kunyuensis*. The dotted line indicates ocean areas of sea level depth less than 80 m that were dry land during lower sea levels of the Late Quaternary.

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TABLE 1. Specimens and GenBank accession numbers of DNA sequences used in this study. The sequences with superscript "a" and "b" in GenBank accession numbers are referenced from Jeong et al., 2013 and from Yang et al., 2010, respectively. For Korean locality information, levels of administrative regions are kept in the Korean language. The following is translation of the levels: "-do" = province, "-si" = city, "-gun" = county, "-myeon" = township.

				GenBank accession numbers				
				Mitochondrial gene			Nuclear gene	
Species name	Voucher No.	Locality	Sample name	16S	Cyt*b*	CO1	RAG2	LIG4
R. amurensis	SYNU11100268	Harbin, Heilongjiang, China	amu.1	KF020588	KF020617	KF020602	KJ371974	KJ371957
	SYNU11100267	Harbin, Heilongjiang, China	amu.2	KF020589	KF020618	KF020603	KJ371975	KJ371958
	SYNU11100271	Harbin, Heilongjiang, China	amu.3	KF020590	KF020619	KF020604	KJ371976	KJ371959
	SYNU11100272	Harbin, Heilongjiang, China	amu.4	KF020591	KF020620	KF020605	KJ371977	KJ371960
	SYNU07050056	Zhangwu, Liaoning, China	amu.5	KF020592	KF020621	KF020606	KJ371969	KJ371950
R. coreana	GRKOAM0000100650	Jeollabuk-do, Jinan-gun, Seongsu-myeon, Korea	cor.1	JQ815309[a]	JQ798762[a]	JQ844534[a]	-	-
	NIBRAM0000000737	Gyeongsangbuk-do, Yeongyang-gun, Korea	cor.2	JQ837967[a]	JQ837980[a]	JQ844532[a]	-	-
	mms3976	Jeollanam-do, Gwangyang-si, Korea	cor.3	KF020593	KF020622	KF020607	KJ371978	KJ371961
	mms4407	Gangwon-do, Goseong-gun, Korea	cor.4	KF020594	KF020623	KF020608	KJ371979	KJ371962
R. kunyuensis	SYNU07050112	Kunyu Mountain, Shandong, China	kun.1	KF020595	KF020624	KF020609	-	KJ371951
	SYNU07050130	Kunyu Mountain, Shandong, China	kun.2	KF020596	KF020625	KF020610	-	KJ371952
	SYNU08090633	Kunyu Mountain, Shandong, China	kun.3	KF020597	KF020626	KF020611	-	KJ371953
	SYNU13030005	Kunyu Mountain, Shandong, China	kun.4	KJ371937	KJ371945	KJ371941	KJ371980	KJ371965
	SYNU13030011	Kunyu Mountain, Shandong, China	kun.5	KJ371938	KJ371946	KJ371942	KJ371981	KJ371966
	SYNU13030022	Kunyu Mountain, Shandong, China	kun.6	KJ371939	KJ371947	KJ371943	KJ371982	KJ371963
	SYNU13030029	Kunyu Mountain, Shandong, China	kun.7	KJ371940	KJ371948	KJ371944	KJ371983	KJ371964
R. chensinensis	SYNU-hld1	Huludao, Liaoning, China		KF020598	KF020627	KF020612	KJ371973	KJ371956
R. zhenhaiensis	SYNU08040100	Hangzhou, Zhejiang, China		KF020599	KF020628	KF020613	KJ371970	KJ371954
R. hanluica	SYNU0700490	Mt. Yangmingshan, Hunan, China		HQ228158[b]	KF020629	KF020614	KJ371968	KJ371949
B. daunchina	SYNU12050567	Hangzhou, Zhejiang, China		KF020600	KF020630	KF020615	KJ371971	KJ371955
	SYNU12050568	Hangzhou, Zhejiang, China		KF020601	KF020631	KF020616	KJ371972	KJ371967

TABLE 2. Inter- and intraspecific pairwise Kimura's two-parameter genetic distance among (1) *R. kunyuensis*, (2) *R. coreana*, and (3) *R. amurensis* using the separate and combined mitochondrial datasets.

	16S			Cyt*b*			COI			Combined dataset		
	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
(1)	0.001			0.002			0.001			0.001		
(2)	0.001	0.001		0.009	0.007		0.007	0.009		0.005	0.006	
(3)	0.047	0.048	0.002	0.147	0.149	0.008	0.124	0.119	0.005	0.103	0.101	0.005

[Beijing] Co., Ltd., no. DP304). The mtDNA was amplified using a standard polymerase chain reaction (PCR) protocol using the primers F51/R51 (Sumida et al., 2002) for 16S, Cytba/Cytbs (Zhou et al., 2012) and L14850/H15502 (Tanaka-Ueno et al., 1998) for Cyt*b*, and Chmf4/Chmr4 (Che et al., 2012) and L-turtCOIc/H-turtCOIc (Stuart and Parham, 2004) for COI. Amplification of the nuDNA (RAG2 and LIG4) was conducted with a nested PCR strategy (Shen et al., 2013). All PCR products were purified and sequenced by the Beijing Genomics Institute.

The Cyt*b* and COI nucleotide sequences were translated into amino acids to assess the presence of nuclear DNA pseudogenes. To examine sequences homology, all the sequences were subjected to a BLAST search in GenBank. Sequences were aligned using ClustalX 1.81 (Thompson et al., 1997). Inter- and intraspecific mtDNA sequence divergences of Kimura's two-parameter (K2P) model (Kimura, 1980) were calculated using Mega5 (Tamura et al., 2011) with the ratio of transitions:transversions equally weighted. An analysis of molecular variance (AMOVA) for the mtDNA dataset was implemented using Arlequin Ver3.5 (Excoffier and Lischer, 2010).

We performed separate phylogenetic analyses for four different datasets: each nuclear gene (RAG2 and LIG4, separately), concatenated mtDNA (16S + CO1 + Cyt*b*), and combined data (mtDNA + nuDNA). Bayesian inference (BI) and maximum likelihood (ML) methods were used to estimate phylogenies. The best-fit nucleotide substitution model for each gene was selected using MrModeltest version 2.3 (Nylander, 2004) based on the Akaike information criterion (AIC). Partitioned BI analyses were implemented in MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck, 2003) with the GTR+G model for each mtDNA gene, the GTR model for RAG2, and the GTR+I model for LIG4. Markov chain Monte Carlo (MCMC) sampling was run for 10 million generations. Trees were sampled every 1,000 generations. Stationarity was checked graphically by plotting log-likelihood scores in Tracer ver. 1.4.1 (Rambaut and Drummond, 2007). The first one million generations were discarded as burn-in and the remaining trees were used to build a consensus tree. Partitioned ML analysis was executed using a rapid-hill-climbing algorithm in RAxML ver. 7.0.4 (Stamatakis, 2006) under the GTRGAMMA model. First, the best-scoring ML tree was inferred with 100 replicates, followed by a nonparametric bootstrap analysis of 1,000 replicates to evaluate node robustness.

Morphological Observation.—Morphological observations and measurements were taken on newly collected material. Ten specimens of *R. kunyuensis* (five males, five females) collected from the type locality (Kunyu Mountain, Wendeng County, Shandong Province, China) were examined alongside 10 specimens of *R. coreana* (five males, five females) from across Korea. *Rana kunyuensis* samples were deposited in the Institute of Amphibians and Reptiles of Shenyang Normal University (SYNU), while *R. coreana* were deposited in the Conservation Genome Resource Bank for Korean Wildlife (CGRB), College of Veterinary Medicine, Seoul National University. Examinations of qualitative characteristics were documented with a CCD color camera (60D, Canon Co.) and compared by eye. The five morphological characteristics compared were color variation, smoothness of skin, toe webbing, pattern of dorsolateral folds, and tubercles on dorsum. Mensural characters were taken using vernier calipers (±0.02 mm) and compared between groups using Welch two-sample *t*-tests in R v.3.0.1 (R Core Team, 2013). Three datasets were tested statistically: males only, females only, and all samples (males + females). The 11 mensural characters examined were snout–vent length (SVL), head length (HL), snout length (SL), diameter of eye (ED), tympanum diameter (TD), head width (HW), internarial distance (IND), interorbital distance (IOD), tibia length (TL), foot length (FL), and hindlimb length (HLL).

RESULTS

Genetic and Phylogenetic Analyses.—A total of 3,528 bp were obtained in the combined sequence alignment including 1,717 bp of mtDNA (409 bp of 16S, 750 bp of Cyt*b*, and 558 bp of COI) and 1,811 bp of nuDNA (807 bp of RAG2 and 1,004 bp of LIG4). All sequences were deposited in GenBank (accession numbers shown in Table 1). For the mtDNA data, 372 sites were variable and 319 were parsimony informative. For the nuDNA data, RAG2 had 62 variable and 44 parsimony informative sites, while LIG4 had 79 variable and 63 parsimony informative sites. Of the 12 mtDNA haplotypes recognized from 19 specimens of Brown Frogs, six specimens of *R. kunyuensis* (kun.1, kun.2, kun.3, kun.5, kun.6, and kun.7) shared an identical haplotype. For the 16S gene, these specimens of *R. kunyuensis* and three specimens of *R. coreana* (cor.1, cor.3, and cor.4) shared an identical haplotype.

We calculated inter- and intraspecific sequence divergence between *R. amurensis*, *R. coreana*, and *R. kunyuensis* using the separate and combined mtDNA dataset. Intraspecific K2P distances were small (*R. kunyuensis*: 0.000–0.002; *R. coreana*: 0.002–0.011; *R. amurensis*: 0.002–0.009), while interspecific comparisons of *R. amurensis* to *R. coreana* and *R. kunyuensis* revealed relatively large K2P distances of 0.092–0.101 and 0.096–0.100, respectively. In contrast, interspecific comparison of *R. coreana* and *R. kunyuensis* produced much smaller distances (0.003–0.012); values were similar to the intraspecific range calculated for *R. coreana* (Table 2). The results of the AMOVA analysis of the mtDNA showed that the genetic variation between *R. coreana* and *R. kunyuensis* was small (F_{ST}

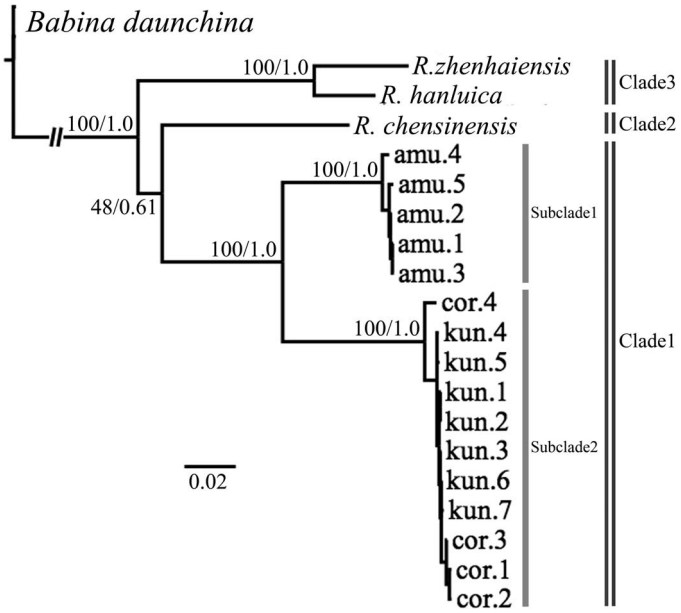

FIG. 2. The maximum likelihood (ML) phylogeny based on the combined dataset (mtDNA + nuDNA). The nodal support values are marked as ML bootstrap proportions/Bayesian posterior probabilities.

$= 0.1242$), and the genetic differentiation was not significant ($P > 0.05$).

The results of the phylogenetic analyses of nuDNA (Fig. S1), concatenated mtDNA (Fig. S1), and combined dataset (mtDNA + nuDNA; Fig. 2) were similar and indicated that the ingroup of Brown Frogs were divided into three major clades (Clade 1: *R. amurensis*, *R. coreana*, and *R. kunyuensis*; Clade 2: *R. chensinensis*; Clade 3: *R. zhenhaiensis* and *R. hanluica*). The phylogenetic relationships among the three clades were poorly supported in the phylogeny (Fig. 2). Clade 1 is composed of two subclades: 1) *R. amurensis* and 2) *R. coreana* and *R. kunyuensis*. The monophyly of these subclades was strongly supported by posterior probabilities (1.00) and ML bootstraps (100).

Morphological Observation.—Morphological comparisons of *R. coreana* and *R.* kunyuensis revealed similarities (Fig. 3). Our examination of color variation between *R. coreana* and *R. kunyuensis* revealed the same colors—light orange dorsum and white venter. Both species possess dark brown spots and two dorsolateral folds. The skin was predominantly smooth with few or no tubercles present. A distinguishing feature identical in both *R. kunyuensis* and *R. coreana* specimens was a white line on the upper lip. Toe webbing on the hindfoot of *R. coreana* and *R. kunyuensis* was moderate and did not reached the joint connection of the second subarticular tubercles between the third and fourth toes and between the fourth and fifth toes. The results from the mensural characters showed there were no significant differences ($P > 0.05$) in any of the measurements between *R. coreana* and *R. kunyuensis* for all group comparisons (Table 3).

DISCUSSION

Rana coreana and *R. kunyuensis* are geographically isolated, with *R. coreana* found throughout the Korean Peninsula and *R. kunyuensis* restricted to Kunyu Mountain in Shandong Province, China. *Rana kunyuensis* was described as a new species based on morphological comparisons with *R. amurensis*, *R. chensinensis*, and *R. huanrenensis* but not with *R. coreana* (Lu and Li, 2002). Additionally, *R. kunyuensis* has been used in large-scale phylogenetic studies of anurans (e.g., Wiens et al., 2009) and was found to be the sister group to *R. amurensis*, but again *R. coreana* was not included. Our study is the first to include both *R. kunyuensis* and *R. coreana* in a single study, comparing the morphology and DNA of these two species. Our morphological analyses and specimen comparisons reveal that *R. kunyuensis* and *R. coreana* are indistinguishable. The two species show almost identical characteristics in coloration, smoothness of skin, pattern of dorsolateral fold, tubercles on dorsum, and toe webbing (Fig. 3). Meanwhile, none of the statistical comparisons of the mensural characters were statistically significant (Table 3).

The results from genetic analyses mirror morphological results, showing high similarity between *R. coreana* and *R. kunyuensis*. The degree of interspecific sequence divergence between *R. kunyuensis* and *R. coreana* (0.003–0.012) is similar to intraspecific genetic divergence within *R. coreana* (0.002–0.011; Table 2). Similarly, results from an AMOVA analysis indicated that most of the genetic variation (87.6%) came from within populations, namely within species. The genetic divergence between *R. coreana* and *R. kunyuensis* was not significant. In all phylogenetic analyses, *R. kunyuensis* and *R. coreana* formed a monophyletic group with strong support.

All evidence from both genetic and morphological data indicates that *R. kunyuensis* and *R. coreana* are the same species. Based on the priority of names designated by the International Code of Zoological Nomenclature (ICZN), *R. kunyuensis* should be considered a junior synonym of *R. coreana*.

The recognition of *R. kunyuensis* as a junior synonym of *R. coreana* presents an interesting biogeographic pattern in Northeastern Asia. The Yellow Sea currently separates the Korean Peninsula from Shandong Province, China, resulting in geographically isolated populations of *R. coreana*. The Yellow Sea is a typical epicontinental sea, possessing a flat and broad sea floor with shallow water depths averaging 55 m and less than 100 m at all points (Alexander et al., 1991; Kim et al., 1999). The rising and falling of the sea level during the Late Quaternary resulted in alternating intervals of connected and fragmented land areas in the region (Fig. 1). Based on geological data, populations of *R. coreana* in Korea and China were probably connected as far back as the Late Quaternary, when sea levels were as much as 120 m lower than present levels and the entire Yellow Sea basin was exposed (Butenko et al., 1985; Voris, 2000). As there is no current or historical evidence of *R. coreana* existing in the areas between Shandong and the Korean Peninsula (Hebei, Liaoning, and Jilin Provinces), it is most likely the rise of sea levels to the present state separated and interrupted gene flow between these populations. We conclude that the present distribution pattern of *R. coreana* in Korea and China was formed by both dispersal and isolation events occurring during the Late Quaternary.

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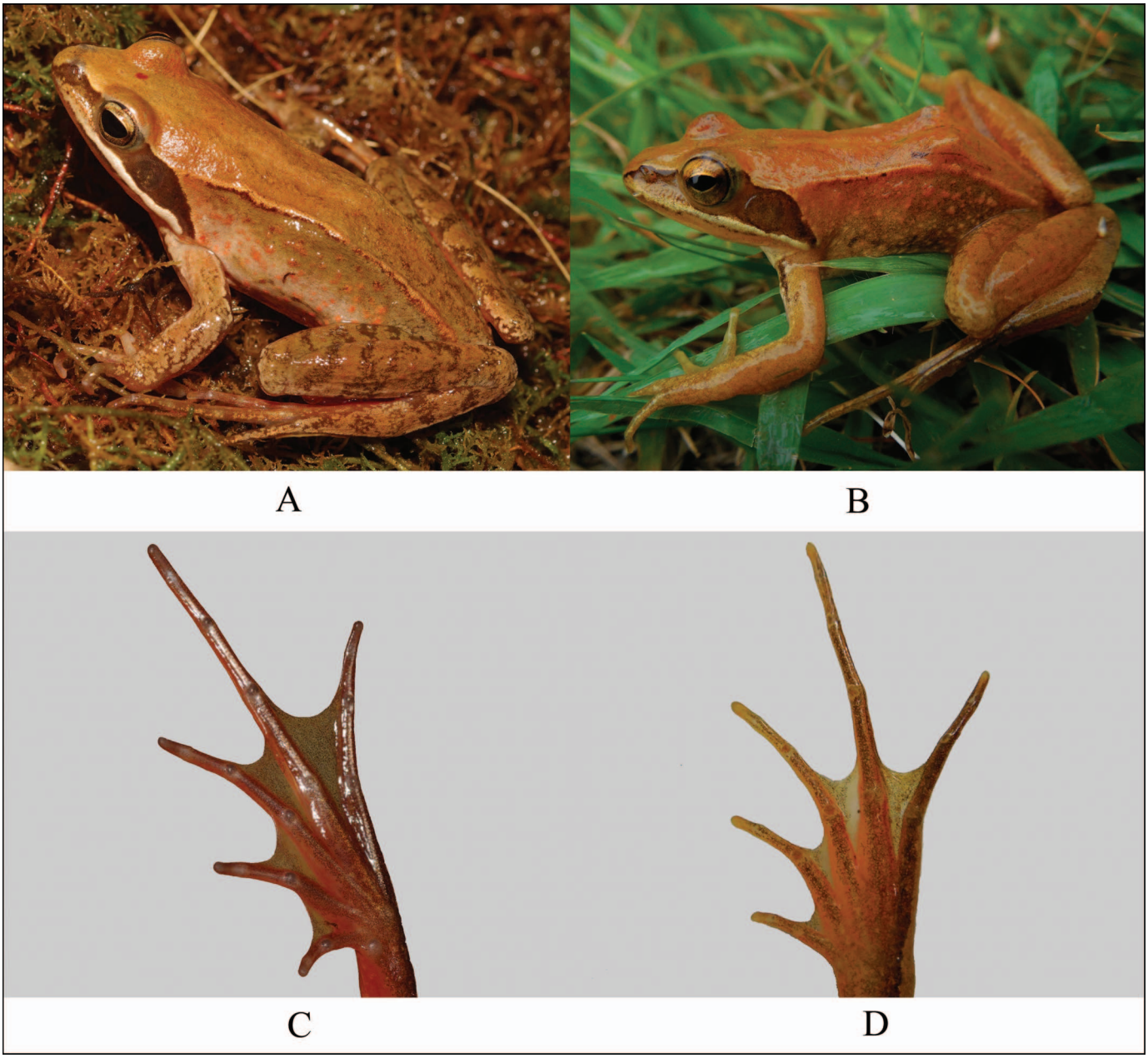

FIG. 3. Dorsal surface of (A) *R. kunyuensis* and (B) *R. coreana* and plantar surface of the left foot of (C) *R. kunyuensis* and (D) *R. coreana*.

TABLE 3. Measurements of *R. coreana* and *R. kunyuensis*. The *t*-tests of all measurements of *R. coreana* and *R. kunyuensis* were not significant ($P > 0.05$). All measurements are in millimeters.

	Mean ± SD					
	Rana coreana ($n = 10$)			*Rana kunyuensis* ($n = 10$)		
Mensural characters	Female	Male	Combined	Female	Male	Combined
Snout–vent length (SVL)	41.47 ± 5.72	42.58 ± 2.23	42.03 ± 4.14	44.13 ± 2.47	41.58 ± 3.28	42.86 ± 3.05
Head length (HL)	12.03 ± 1.34	12.59 ± 0.53	12.31 ± 1.01	12.62 ± 0.98	12.38 ± 1.14	12.50 ± 1.01
Snout length (SL)	5.26 ± 0.96	5.18 ± 0.33	5.22 ± 0.68	5.27 ± 0.72	5.33 ± 0.34	5.30 ± 0.54
Diameter of eye (ED)	4.07 ± 0.22	4.00 ± 0.22	4.04 ± 0.21	4.18 ± 0.44	4.05 ± 0.29	4.11 ± 0.36
Tympanum diameter (TD)	1.83 ± 0.37	2.12 ± 0.48	1.98 ± 0.43	1.92 ± 0.22	2.23 ± 0.25	2.08 ± 0.28
Head width (HW)	12.20 ± 1.29	12.53 ± 0.70	12.36 ± 1.00	13.19 ± 1.04	12.70 ± 1.13	12.95 ± 1.21
Internarial distance (IND)	2.45 ± 0.49	2.49 ± 0.25	2.47 ± 0.37	2.49 ± 0.34	2.71 ± 0.44	2.60 ± 0.39
Interorbital distance (IOD)	2.44 ± 0.19	2.49 ± 0.25	2.47 ± 0.21	2.72 ± 0.23	2.65 ± 0.26	2.69 ± 0.24
Tibia length (TL)	19.73 ± 2.59	21.19 ± 1.20	20.46 ± 2.05	20.29 ± 0.87	22.29 ± 0.73	21.29 ± 1.30
Foot length (FL)	21.81 ± 3.77	25.18 ± 1.25	23.50 ± 3.19	22.09 ± 2.00	25.34 ± 2.48	23.72 ± 2.73
Hindlimb length (HLL)	61.59 ± 9.32	66.17 ± 4.22	63.88 ± 7.23	66.40 ± 5.08	69.41 ± 3.49	67.91 ± 4.40

Supplementary Data

Supplementary data associated with this article may be found online at http://dx.doi.org/10.1670/13-111.s1.

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