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수의학석사 학위논문

Phylogeography and taxonomic status
of Korean *Glandirana emeljanovi*
(Anura: Ranidae)

한국 음개구리 (*Glandirana emeljanovi*)의
분류학적 고찰 및 계통지리

2021년 2월

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A Dissertation for the Degree of Master

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Abstract

Phylogeography and taxonomic status of Korean *Glandirana emeljanovi* (Anura: Ranidae)

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Wrinkled frogs belong to the genus *Glandirana* that includes five species: *Glandirana emeljanovi*, *Glandirana rugosa*, *Glandirana tientaiensis*, *Glandirana minima*, and *Glandirana susurra*. Even though only a single species of wrinkled frogs is known to inhabit South Korea, there has been confusion on the exact classification of the species; it is described as *G. rugosa* or *G. emeljanovi* depending on the authors' preference without sound evidence. According to original records, *G. rugosa* inhabiting Japan were designated as syntype and *G. emeljanovi* discovered at Imienpo station, Heilongjiang Province, China, were designated as holotype. In previous studies, South Korean wrinkled frogs are divided into two sublineages; they are genetically distinct from *G. rugosa* and are monophyletically related to *G. emeljanovi*. Nevertheless, because of the limitations in comparing South Korean wrinkled frog populations with a few individuals from China or Japan, accurate phylogenetic relations could not be determined. Therefore, this study used phylogenetic methods to compare and

analyze samples from three wrinkled frog populations of South Korea, China, and Japan representing most of their range and attempted to infer the phylogeographic divergence and evolutionary history of South Korean wrinkled frogs. In this study, *G. tientaiensis* of the same genus and *Rana huanrensis*, *Sylvirana guentheri*, and *Pelophylax nigromaculata* of the same suborder Raninae were used as outgroups.

We used the sequences of five mitochondrial loci (cytochrome *b*, 817 bp; D-loop, 765–774bp; 12S rRNA, 823–828bp; tRNA–val, 69–71bp; 16S rRNA, 62 bp). In total, we collected 319 wrinkled frog specimens; 304 individuals from 93 localities in South Korea; 2 from 1 locality in China; 13 from 3 localities in Japan. In addition, a sequence (KU641020) from one individual in Jilin, China, and four sequences (LC536281–LC536284) from NCBI GenBank were used as a homoeotype of *G. emeljanovi* and representatives of each regional group in Japan, respectively.

Comparative analyses performed using a total of 2630 bp sequences from the South Korean, Chinese, and Japanese wrinkled frog populations revealed that the South Korean and Chinese populations formed a monophyletic taxon and both were genetically differentiated from the Japanese populations (*G. rugosa*). A total of 198 haplotypes were discovered in all the populations, of which haplotype 9 was the most common haplotype and was detected only at 21 localities in South Korea and in Liaoning and Jilin Provinces of China. When the results of this and previous studies were compared, South Korean wrinkled frogs were found to be identical to Chinese *G. emeljanovi*; hence, the nomenclature of South Korean wrinkled frogs as *G. emeljanovi* was judged to be reasonable. Furthermore, populations of *G. rugosa* were shown to be paraphyletic with *G. tientaiensis*.

Phylogenetic analyses also revealed that wrinkled frogs in South Korea were divided into two sublineages, A1 and A2. Sublineage A1 was distributed in the north central region of Gyeongsang Province and was separated from the distribution of sublineage A2 in South Korea. A few sympatric areas were detected in the southern part of Gyeongsang Province. The results of barrier test showed the presence of genetic barriers between regions where allopatry was established between the two sublineages. As per this test, the Sobaek Mountain Range and the southern Sudo and Biseul ridge lines were assumed to function as geographic barriers for two sublineages. We performed MANTEL test to determine the dispersal history by comparing four hypotheses based on dispersal mechanisms. Wrinkled frogs in South Korea had significantly positive correlation with dispersal via independent freshwater streams (hypothesis 2) and paleostreams (hypothesis 3). Conversely, low level of correlation was found for the other hypotheses suggesting unlikely dispersal via coastlines (hypothesis 1) and direct dispersal (hypothesis 4). The time of population expansion was about 46,000 years ago (YA) for sublineage A1 and 58,000 YA for sublineage A2. Thus, population expansion of the two sublineages in South Korea was estimated to have occurred during the late Pleistocene of Quaternary.

We attempted to infer the evolution history of South Korean wrinkled frogs by combining the results of previous historical geologic studies focusing on the Korean Peninsula. South Korean wrinkled frogs diverged into two sublineages owing to geographic barriers. These barriers are formed by the Sobaek Mountain Range that stretches northeast to southwest of the southern Korean Peninsula and the Sudo and Biseul ridge lines dividing the middle region of South Gyeongsang Province. Geological topology was formed by weathering and erosion.

The formation of freshwater streams in the Korean Peninsula was shown to be mostly affected by mountain range formation during the Miocene, and its pattern was stabilized during the Pleistocene. In particular, Nakdong watershed basin isolated by the Sobaek Mountain Range could affect the divergence of the two sublineages of South Korean wrinkled frogs that depend on dispersal within independent freshwater streams. Moreover, the formation of land and freshwater streams on the East Sea near Tsushima Island, the Yellow Sea, and the South Sea were assumed to influence not only the speciation of *G. emeljanovi* and *G. rugosa*, but also the formation of sublineages within each species.

In conclusion, we revealed that South Korean wrinkled frogs were clearly differentiated as distinct species from Japanese *G. rugosa* and formed a monophyletic lineage with Chinese *G. emeljanovi*. We showed that South Korean wrinkled frogs dispersed by independent freshwater streams and then diverged into two allopatric sublineages due to the changes in climate and sea level. Thus, this study suggests the rational basis of using *G. emeljanovi* as the species name for South Korean wrinkled frogs and provides information on their evolutionary history by inferring the dispersal and divergence processes of two sublineages of South Korean wrinkled frogs and three sublineages of Japanese wrinkled frogs. However, further studies are required for detailed genetic monitoring of the subpopulation in sympatric areas that were discovered in Chupungryeong and Yangsan fault in our study.

Keywords: Northeastern Asia, Raninae, Wrinkled frogs, mitochondrial DNA, *Rana emeljanovi*, *Rana rugosa*, *Rugosa rugosa*

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General Introduction

Nomination of Wrinkled Frogs

Temminck and Schlegel (1838) reported the names of not only the Japanese wrinkled frog *Rana rugosa* but also all amphibians and reptiles from Korea, China, and Japan. Herein, we use the common names used for these species in the 1800's. In their paper, Temminck and Schlegel used the French term "Grenouille ridée" which means wrinkled frog, for *R. rugosa*. The common names were introduced in the list of philology: In China, 黑蝦蟆 (He hai ma) and written together with Korean pronunciation 흑하마 (Houk hama); in Japan, ツチカエル (Tsoutsi kaherou) and クロヒキ (Kouro fiki). During the fourth year of King Hunjong of Joseon Dynasty (1838 AD), no specific common name was used for Korean wrinkled frogs, and all amphibians were recorded as 개구리 (Kai kouli), 두텁이 (Tou theupi), and 두새비 (Tou saipi) in Korea.

In 1913, the Russian researcher Nikolsky (1913) reported a Chinese wrinkled frog, *Rana emeljanovi* from Manchuria, China. The paper was written in two languages, French and Russian. Nikolsky recorded a type specimen by comparing the morphological characteristics between *R. emeljanovi* and *R. rugosa*. The type locality was Il'yampo (=Yimianpo) Station, Chinese Eastern Railway, Manchuria, China. Hence, *R. emeljanovi* is also called Imienpo (=Yimianpo) Station frog. During the Japanese colonial period (1910-1945), many independence movement organizations were established in Manchuria. After the end of the Japanese colonial period, Korea suffered again from the Korean War (1950-1953). During the war, Frederick A. Shannon who was stationed in Korea as a surgeon for the 194th Engineer Combat Battalion investigated and reported 37 species or subspecies of Korean reptiles

and amphibians (Shannon, 1956). He collected 60 specimens from Kapyong, Kumhwa, Ch'orwon, Inje, Pusan, Kaesong, Nanam, Tongnae, and Ch'uja Do and recorded them as *R. rugosa*.

Subsequently, Dr Hong Gu Won from North Korea first used the Korean word “옴개구리 (Om gae goo ri)” for Korean wrinkled frog (Won, 1971). Dr Won was a respectful biologist who organized Korean wildlife species of birds, mammals, reptiles, and amphibians after the Korean War (lifetime: 1888–1970). In South Korea, Korean wrinkled frog “옴개구리 (Om gae goo ri)” was cited as *R. rugosa*, and the other amphibian species were organized by Yang and Yu (1978). Dr Suh Yung Yang was another respectful biologist who reestablished Korean herpetology in phylogeny and taxonomy (lifetime: 1934–2008). Recently, wrinkled frogs are called “옴개구리 (Om gae gu ri)” in both South and North Korea (*Amphibians in Korea*, 2020; Kim, 2009), “东北粗皮蛙 (dōngběi cū pí wā)” in China (Fei et al., 1999), “Бугорчатая лягушка (bugornaya leguska)” in Russian (Кузьмин, 2012), “ツチガエル (tsuchigaeru)” in Japan, and wrinkled frog or rough-skinned frog in English.

Morphology and Life Cycle of Wrinkled Frogs

The major morphological characteristic of wrinkled frogs is granulated skin as the name represents. The dorsal side of the body is covered with rough skin, and the roughness becomes intense like tubers toward the limbs. The dorsal side is black or blackish brown, with the rare presence of a vague single stripe stretching from the snout to vent. The ventral side is smooth and has white to yellow coloration; the density, size, and range of black spots varies across individuals. This color variation was analyzed at the preliminary level to determine the relations between color and phylogenetic lineage as

well as color and regional distribution (Kim et al., 2012). however, no relations were found.

In the original report of *R. rugosa* (Temminck and Schlegel, 1838), the species description was provided without the actual type specimen. From one type specimen of *R. emeljanovi* (Nikolsky, 1913), the size of each body part was measured: snout-vent length (SVL) was 35 mm, head and SVL ratio was 3:8, length of tibia was 19 mm, anterior leg was 21 mm, and posterior leg was 54 mm. The SVL of *R. emeljanovi* was five or two times greater than that of *R. rugosa*, but the tympanum of *R. emeljanovi* was smaller than that of *R. rugosa*.

However, recent skeletochronological studies have yielded contrasting results. The maximum SVL of South Korean wrinkled frogs was reported to be 51.11 mm in females and 40.97 mm in males (Lee et al., 2009). Japanese wrinkled frogs had SVL of 62.44 mm in females and 46.20 mm in males (Khonsue et al., 2001). These studies also estimated the longevity and sexual maturity. Sexual maturity was reached at 3 years in South Korean wrinkled frogs and at 2–3 years (female) or 1–2 years (male) in Japanese wrinkled frogs. Longevities were 6–8 years and 5 years (female) or 4 years (male) in South Korean and Japanese wrinkled frogs, respectively. In addition, the annual cycle of Korean wrinkled frogs includes a hibernation period (October to March), an active period (April and September), and a breeding period (May to August) (Lee et al., 2011). According to these results, the life cycle of Korean wrinkled frogs starts with eggs that hatch into tadpoles. Tadpoles undergo metamorphosis the same year, but those that hatch late undergo metamorphosis the following year. The annual cycle repeats every year for 6 to 8 years for adult individuals.

Behavior of Wrinkled Frogs

Korean wrinkled frogs prefer habitats abundant in rocks, pebbles, and sand because it is related to their unique behavior. Feeding activities are frequently observed on the ground at watersides. Korean wrinkled frogs prefer small-sized insects such as ants, true bugs, or beetles that have low flying ability, high hovering tendency, and appropriate size to eat. However, there are no specific prey preferences (Han et al., 2015). Another behavior related to rocks and pebbles is widely known as the defense mechanism camouflage when the frog crouches appear like a pebble or part of a rock (Lee et al., 2011). Male Korean wrinkled frogs have also developed defense calling mechanisms to deceive their body size. Small male individuals have low call frequency (Hz) and increased pulse and call duration, but reduced dominant frequency. This technique allows them to pretend to be bigger in size. This call has a relatively wider modulated range of dominant frequency than that in the other species in Korea. Defense call is for not only defending the territory from predators or other competing males, but also attracting females. Calling season starts in April to August corresponding to the breeding season. The pace of the breeding season is affected by temperature. If the temperature rises earlier during the breeding season, the number of calling individuals and their frequency are increased, which leads to an earlier spawning season. The observed daily calling patterns have no relation with temperature. Whereas only a few individuals try to call during the daytime, group calls start to increase after sunset and reach the maximum frequency between 9 and 10 pm (Jung, 2001).

Physiology of Wrinkled Frogs

Korean wrinkled frogs secrete toxins through their skin. The toxic substance has not been identified and is diagnostically known as digitalis compounds (Yonhap, 1997). Digitalis compounds are inhibitors of Na^+/K^+ -ATPase, which leads to the accumulation of calcium inside cells. Calcium accumulation throughout the body can cause heart failure (Klabunde, 2015). The toxin in the skin glands of Korean wrinkled frogs functions as a defense mechanism against predators.

In 1994, six peptides named “gaegurins” derived from the Korean word “개구리 (Gaeguri, meaning frog)” were newly reported. The antimicrobial peptides extracted from the skin of Korean wrinkled frogs that act against gram-positive and gram-negative bacteria, fungi, and protozoa (Park et al., 1994). Antimicrobial peptides can be categorized into an α -helix family and a defensin family. The antimicrobial peptide gaegurin contains the α -helical structure. Electrostatic interaction between helical peptides and lipid membranes are the major factors of antibacterial action (Suh et al., 1999). Recent studies have been focusing on developing a new antibiotic and anticancer agent using the mechanisms and characteristics of a 24-residue membrane-active antimicrobial peptide, gaegurin 5 (Won et al., 2006).

General Purpose of Research

After *R. rugosa* was first reported by Temminck and Schlegel (1838), other species of the genus *Glandirana* were continuously discovered, and a total of five species have been reported since then up to recently. Wrinkled frogs are one of the most interesting species for studies because of their predominant regional variations. Recently, *Glandirana susurra*, an endemic species found in Sado Island of Japan, was reported as a new species. In addition, wrinkled frogs have unique behavioral characteristics such as call deceiving and defensive camouflage as well as have specific physiological characteristics such as toxin and antimicrobial peptide production. These fascinating features attract researchers from various academic fields.

In this study, we considered the taxonomic status of wrinkled frogs and attempted to trace the patterns of their historical distribution in South Korea. The taxonomic status analysis can support the use of Korean wrinkled frogs' name *Glandirana emeljanovi* instead of the species name of Japanese wrinkled frog, *Glandirana rugosa*. Simultaneously, tracing the patterns of historical distribution can reveal the composition of sublineages. These analyses assume relations to Chinese and Japanese wrinkled frogs and environmental factors affecting their dispersal or evolution history. We expect that this study will contribute to the improvement in taxonomic considerations and allow the understanding of the dispersal history of wrinkled frogs in South Korea.

CHAPTER 1. The taxonomic status of South Korean wrinkled frogs of the Genus *Glandirana* (Anura: Ranidae)

1-1. Introduction

The genus *Glandirana* currently includes five species: *G. emeljanovi* (Nikolsky, 1913), *G. rugosa* (Temminck and Schlegel, 1838), *G. tientaiensis* (Chang, 1933), *G. minima* (Ting and Ts'ai, 1979), and *G. susurra* (Sekiya et al., 2012). This genus was newly recorded in China in the “*Key to Chinese Amphibia*” (Fei et al., 1990) as the subgenera of *Rana* with 279 species or subspecies, new subfamily Occidozyginae, five new genera (*Pseudorana*, *Rugosa*, *Tigrina*, *Glandirana*, and *Odorrana*), three new subgenera [*Paa* (*Quadrana*), *Paa* (*Unculuanus*), and *Hylarana* (*Tenuirana*)], and six new species. However, only *R. minima* was included in the genus *Glandirana*, and two other species *R. emeljanovi* and *R. tientaiensis* were included in the genus *Rugosa*. This classification had been used until they were combined into the genus *Glandirana* by Frost et al. (2006). Subsequently, Che et al. (2007) performed phylogenetic analysis of the genus *Raninae* and found that *Rana* (*Rugosa*) *emeljanovi*, *Rana* (*Rugosa*) *tientaiensis*, and *Rana* (*Glandirana*) *minima* are independent monophyletic lineages; they suggested a distinguished subgenera *Glandirana* by comparing their results with those of previous studies (Dubois, 1992, 2005; Fei, 2005; Fei et al., 1990; Frost et al., 2006). The polyphyly of genus *Glandirana* was reported by Wiens et al. (2009), because an *everetti* group was located between *G. minima* and three other species *G. emeljanovi*, *G. rugosa*, and *G. tientaiensis*. However, we considered this result with low

branch support (52%) invalid. Thus, in this study, we used the genus name *Glandirana* following the classifications of Frost and Che.

Previous studies performed in South Korea suggested the divergence of the phylogenetic lineages between populations of South Korea and Japan. In 1999, the mitochondrial cytochrome *b* sequence of Korean wrinkled frogs was used for analysis. The result of that study revealed distinctively diverged lineages between Korean and Japanese wrinkled frogs (Lee et al., 1999). Subsequently, nationwide sampling of 543 adults from 29 localities was conducted, and the samples were analyzed using the isozyme method; the study discussed the detailed divergence pattern with biogeographic distribution that suggested the subdivision of the Kyoto (Japan) population (Yang et al., 2000).

Studies conducted in Japan also suggested that the relations between Korean and Japanese wrinkled frog populations were highly diverged and included several groups. Japanese wrinkled frogs were revealed to have two types of sex chromosomes, XX/XY and ZZ/ZW (Nishioka et al., 1993). Subsequent studies found new groups by comparing the Kanto and XY groups (Nishioka et al., 1994), Kanto and west Japan groups (Ogata et al., 2003), and ZW and Neo-ZW groups (Ogata et al., 2008). In all, five groups were revealed. In 2012, a new species of Japanese wrinkled frogs, *G. susurra*, was reported, and phylogenetic relationships found among the five Japanese groups were consistent with those reported previously (Sekiya et al., 2012). A recent study proposed more subdivision—East (East Japan), Central (XY), West Central (Neo-ZW), North (ZW), West (West Japan), and se-Kyushu (new)—by analyzing eggs, morphology, molecular phylogeny, calls, and sex-linked DNA markers (Oike et al., 2020).

The results obtained from studies performed in Korea, China, and Japan suggest that the Chinese population formed one group and the South Korean population was divided into two subgroups. The Japanese population was divided into five to six subgroups. These phylogenetic patterns are obtained by considering the Korean and Chinese populations as *G. emeljanovi* and the Japanese population as *G. rugosa*. However, although a recent phylogenetic study proposed that the species name *G. emeljanovi* should be used for Korean wrinkled frogs (Eo et al., 2019), analysis of type specimen records, more detailed consideration of phylogeographic relations, and inclusion of more specimens and localities will provide support to use the species name, *G. emeljanovi*.

Thus, to clarify the taxonomic nomenclature and to assume phylogeographic reconstruction, we determined the genetic differences and phylogenetic relations between *G. emeljanovi* and *G. rugosa* by using five mitochondrial loci (cytochrome *b*, d-loop, 12S, tRNA-val, and 16S) from three individuals from near type locality–Northeast China–of *G. emeljanovi* (Nikolsky, 1913), 17 individuals from syntype locality–Japan–of *G. rugosa* (Temminck and Schlegel, 1838), and 304 individuals from Korea. We could not use specimens from the original type locality, Yimianpo Station, Shangzhi, Harbin, Heilongjiang Province, reported by Nikolsky (1913) who recorded the new species *G. emeljanovi*, because the species were not been found in Heilongjiang Province for a long time (Zhao, 2008). Instead of the specimens from the original type locality, one sequence from GenBank (KU641020) from Jilin Province (Liu et al., 2017) was included as a homoeotype.

1-2. Material and Methods

Sampling Sites and Collection

Muscle and skin tissues of wrinkled frogs were obtained from 324 total individuals; 304 individuals from South Korea (93 sites); 13 from Japan (3 sites); 5 from Zhejiang Province, China (1 site); 2 from Liaoning Province, China (1 site). DNA sequences from GenBank for *G. emeljanovi* (KU641020), *G. rugosa* (LC536281-LC536284), and *G. tientaiensis* (KJ941041, KF771342) were used as ingroups, and those for *Pelophylax nigromaculata* (NC002805), *Rana huanrensis* (NC028521), and *Sylvirana guentheri* (NC024748) were used as outgroups. Sampling localities were marked on the maps (South Korea: Figure 2, foreign countries: Figure 3). Appendix 1 lists the collection data, and each locality was recategorized by current streams and paleostreams of the Last Glacial Maximum (LGM) (Yoo et al., 2016). Tissues were obtained from live individuals by clipping the toe (Perry et al., 2011) or from preserved individuals via dissection. Both tissues were either preserved in 80% ethanol or frozen at -70°C . DNA was extracted from the tissue samples by using the DNeasy Blood and Tissue Kit (69504; QIAGEN, Germany), following manufacturer's instructions.

DNA Extraction, polymerase chain reaction, and Sequencing

The five mitochondrial DNA loci (cytochrome *b*, d-loop, 12S, tRNA-val, and 16S) were amplified using polymerase chain reaction (PCR) by using the primers listed in Appendix 2. Each PCR mixture contained 50ng of template DNA, 1x PCR buffer (iNtRON Biotechnology), 0.2mM dNTPs, 1U of Taq polymerase (i-StarTaq; iNtRON Biotechnology), and 0.3~0.4 μM of primers. The thermal cyclers

conditions were as follows: 94°C for 4min, followed by 35 cycles of 52°C for 1min, 72°C for 1.5min, and 94°C for 45s, and a final extension at 72°C for 7min. PCR products were sequenced in both directions by using PCR primers on an ABI 3730xl DNA analyzer (Applied Biosystems) at the National Instrumentation Center for Environmental Management (NICEM) and Cosmogenetech Co., Ltd.

Sequence Alignments and Phylogenetic Analysis

Forward and reverse sequences for each locus from each individual were assembled and checked by eye to produce concatenated sequences by using Geneious Prime (Drummond et al., 2011). Concatenated sequences were aligned using MAFFT version 7 (Kato et al., 2017) with default options. We determined partitions and substitution models by using PartitionFinder version 2.1.1 (Lanfear et al., 2012) on XSEDE (Miller et al., 2010) for Bayesian and maximum likelihood (ML) analyses. Partitioned Bayesian analysis was performed using Mr. Bayes on XSEDE v. 3.2.7a (Ronquist et al., 2012); sampling was performed for every 1,000 generations until the value of standard deviation was under 0.02 with the number of chains set to 8 and heating parameter (temp) of 0.2. Partitioned ML analysis was performed using the IQ tree web server (Trifinopoulos et al., 2016), with 5,000 ultrafast bootstrap alignments (Minh et al., 2013) and SH-aLRT branch test with 1,000 replicates (Guindon et al., 2010). FigTree v. 1.4.2 (Rambaut, 2012) was used to visualize the trees.

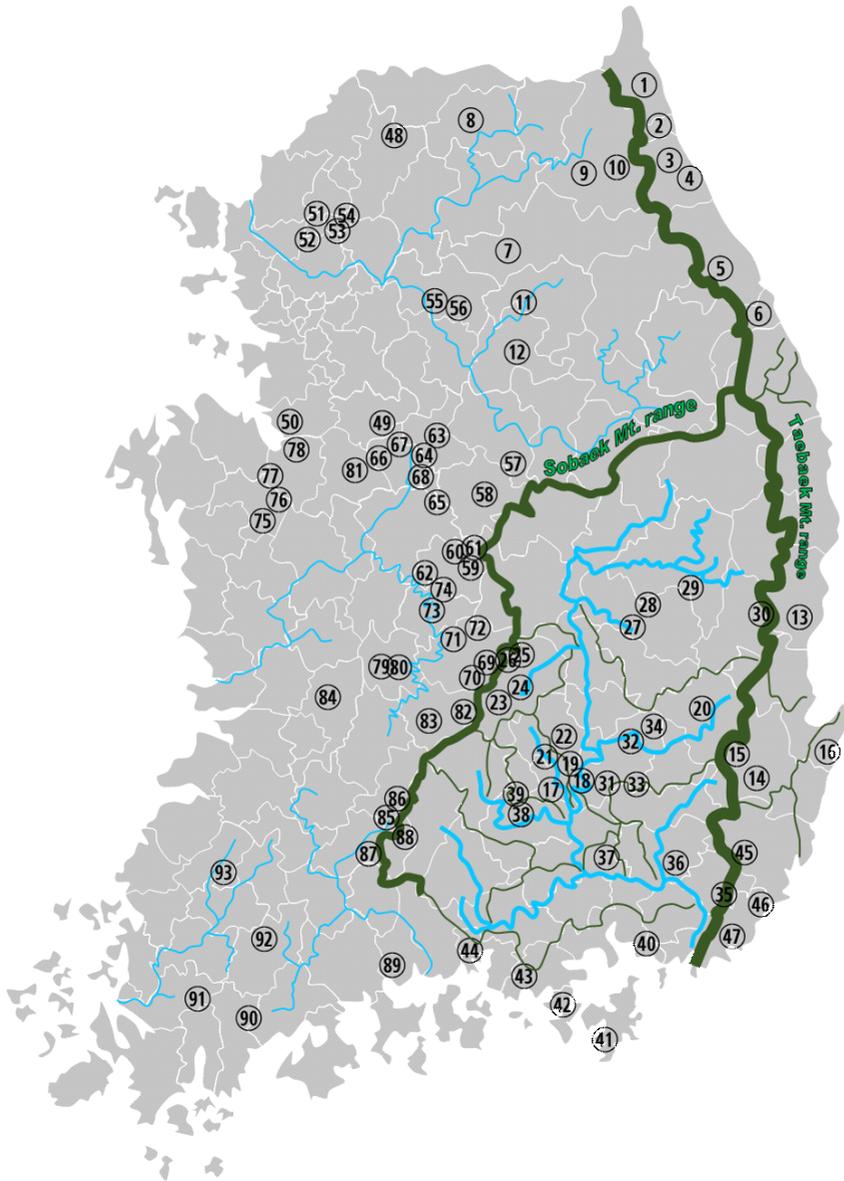


Figure 2. Sampling localities in South Korea.



Figure 3. Foreign sample distribution map.

1-3. Results

Phylogenetic lineage compositions

Sequences of 817 base pairs (bp) of cytochrome *b*, 765~774 bp of D-loop, 823~828 bp of 12S, 69~71 bp of tRNA-val, and 62 bp of 16S genes were aligned (Figure 4). The concatenated sequence was partitioned using the best-fit partitioning scheme [TVMEF+I+G:(cyt *b*_pos1), K81UF+I:(cyt *b*_pos2), GTR+G: (cyt *b*_pos3), TVM+G: (d-loop), GTR+I+G:(12S), HKY+G:(tRNA-Val), and HKY+G:(16S)] with AIC model selection criteria (Table 1).

From the concatenated sequences of 324 individuals, a total of 198 haplotypes were defined by 724 polymorphic sites, and the genetic status of each group classified by the lineages revealed the population parameters (Table 2). Haplotype diversity of lineage A was higher than that of lineage B. Both lineages of Korean wrinkled frogs had higher haplotype diversity (Lineage A1=0.9694, Lineage A2=0.9790) than that of another lineage used in this study (Lineage B=0.9490). Conversely, Japanese wrinkled frogs had more polymorphic sites ($S=581$), nucleotide substitutions ($NS=674$), and nucleotide diversity ($\pi=0.07917$).

Inferred Bayesian and ML trees were highly consistent (Figure 5 and 6). Two major lineages (A and B) and their several sublineages were recognized. Lineage A was a monophyletic taxon and was composed of two sublineages. Sublineage A1 was distributed in South Korea and China, and sublineage A2 was distributed only in South Korea as an endemic lineage. Haplotype 9 was the most common haplotype and was found in 23 sampling regions in South Korea and China (Figure 7) and included 36 individuals (15.7%) of sublineage A1 (Table 3).

Lineage B included the species *G. rugosa* that inhabits Japan and had a paraphyletic topology with three sublineages. Sublineage B1 from western Japan (including Okayama and Hiroshima), sublineage B2 from northern Japan (including Niigata), and sublineage B3 were a paraphyletic taxon from Eastern Japan (including Shizuoka and Chiba; Figure 5 and 6). Lineage B consisted of 13 haplotypes from 17 samples, and no sympatric areas were detected.

Table 1. The best partitioning schemes of information criteria.

criteria	$-\ln L$	score	subsets & models
BIC	16401.323	36267.53097	GTR+I+G:(cyt <i>b</i> pos1, 12S, tRNA-val), K81UF+I:(cyt <i>b</i> pos2), TIM+G:(cyt <i>b</i> pos3), HKY+G:(d-loop, 16S)
AIC	16352.014	33632.02710	TVMEF+I+G:(cyt <i>b</i> pos1), K81UF+I:(cyt <i>b</i> pos2), GTR+G:(cyt <i>b</i> pos3), TVM+G:(d-loop), GTR+I+G:(12S), HKY+G:(tRNA-Val), HKY+G:(16S)
AICc	16361.334	33823.54138	K80+I+G:(cyt <i>b</i> pos1), K81UF+I:(cyt <i>b</i> pos2), GTR+G:(cyt <i>b</i> pos3), TVM+G:(d-loop), GTR+I+G:(12S, tRNA-Val), HKY+G:(16S)

Table 2. Genetic status of all lineages: (n) sample size, HT (number of haplotypes), S (polymorphic sites), NS (number of nucleotide substitutions), h (haplotype diversity) and π (nucleotide diversity).

Clade	(n)	HT	S	NS	h	π
A1 (Korea, China)	230	132	204	210	0.9694	0.00330
A2 (Korea)	77	53	114	116	0.9790	0.00577
A	307	185	280	303	0.9775	0.02061
B (Japan)	17	13	581	674	0.9490	0.07917
A+B	324	198	724	889	0.9771	0.03035

Table 3. The compositions of the most common haplotype 9.

Sites codes	City, County	Haplotype 9
OGB	Geumsan	1
KWB	Wonju	2
KKA	Gangneung	3
KIC	Inje	3
KHA	Hongcheon	2
KGA	Goseong	3
KEA	Hoengseong	1
KDB	Donghae	2
KCB	Sokcho	1
HOC	Okcheon	1
HKA	Goesan	1
HGG	Jincheon	2
HGB	Jincheon	1
HEB	Eumseong	1
HCA	Chungju	1
HBD	Boeun	1
GYA	Yangju	1
GPA	Pyeongtaek	1
GOA	Yangpyeong	2
GFA	Pocheon	1
GAA	Anseong	2
CNL	Huanren County, China	2
KU641020	Fusong County, China	1
	Sum	36
	No. of sublineage A1 individuals	230

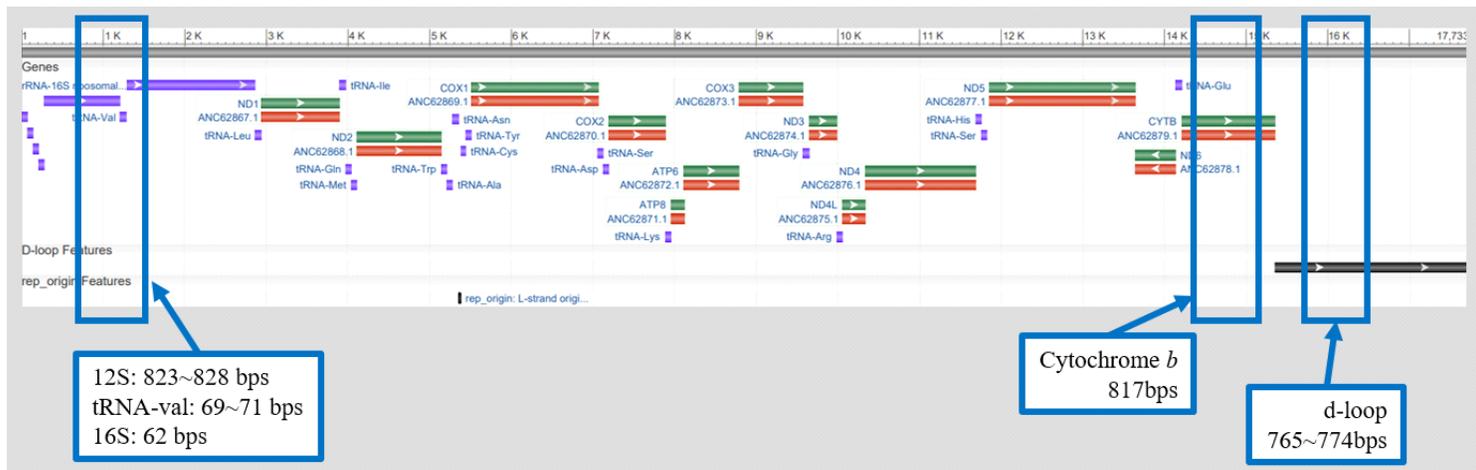


Figure 4. Five mitochondrial DNA loci used in this study.

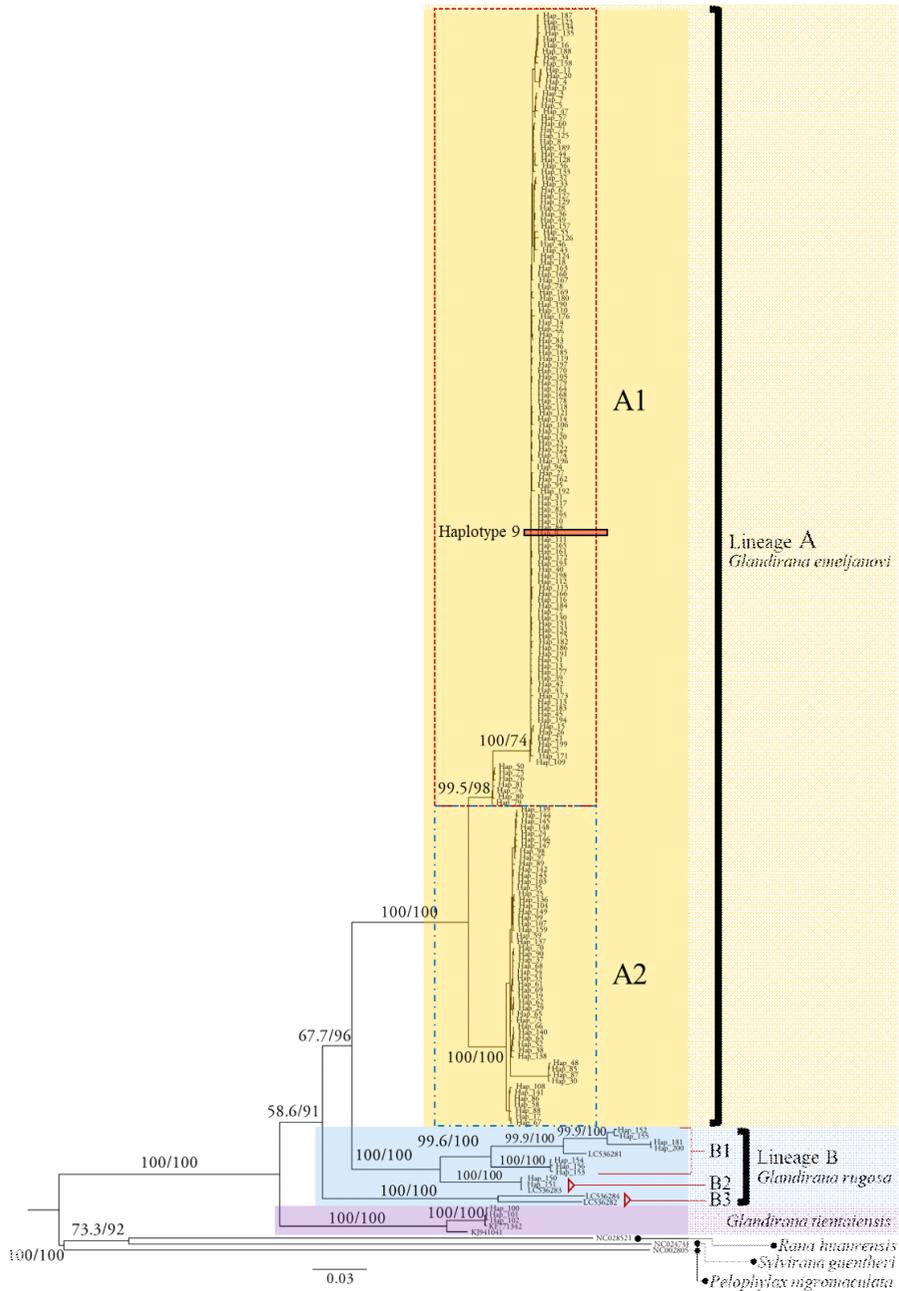


Figure 4. Tree reconstruction by using maximum likelihood of genus *Glandirana*. Each lineage and subdivided lineage of the genus is marked. SH-aLRT and ultrafast bootstrap values are shown on the main branch.

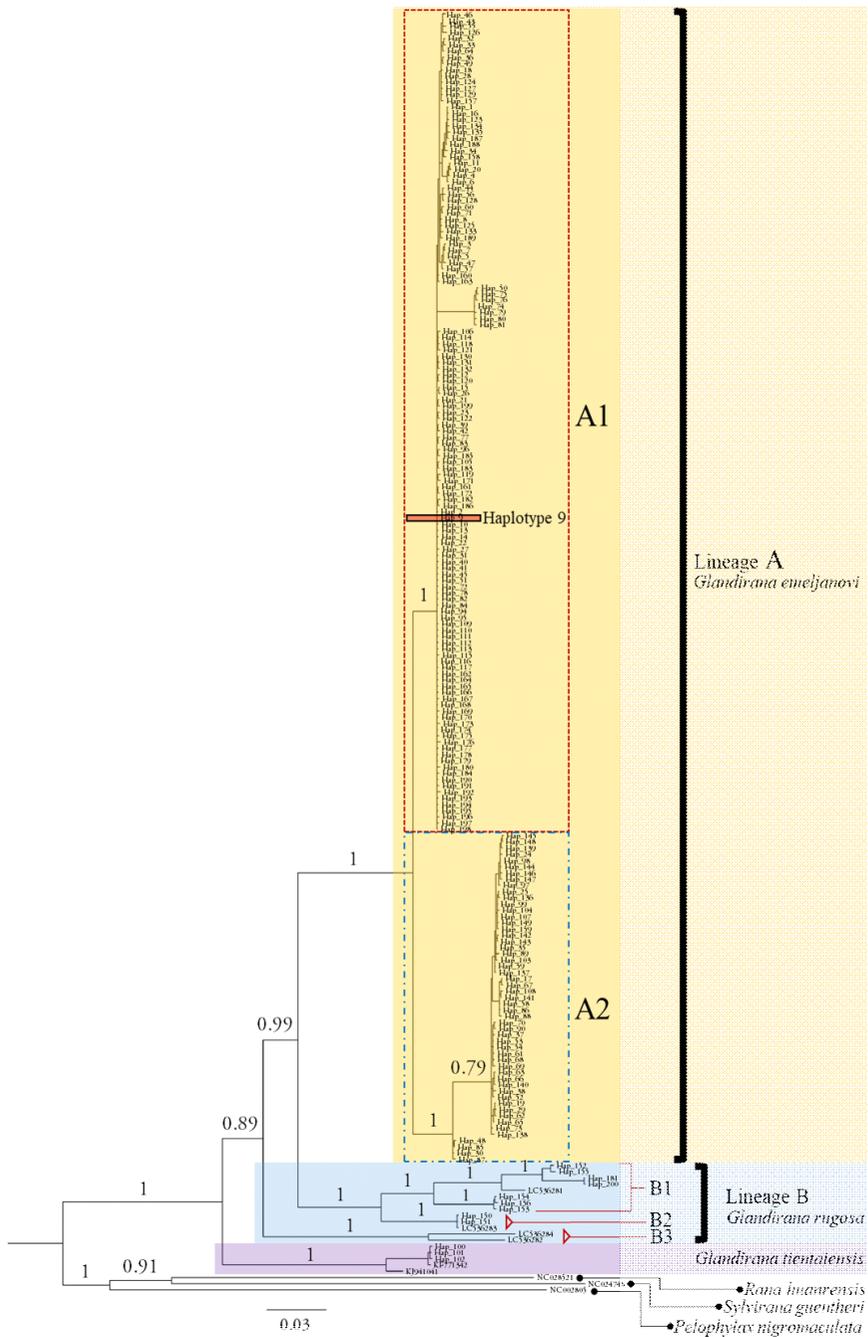


Figure 6. Tree reconstruction by using Bayesian inference for the genus *Glandirana*. Each lineage and subdivided lineage of the genus is marked. Posterior probability values are shown on the main branch.

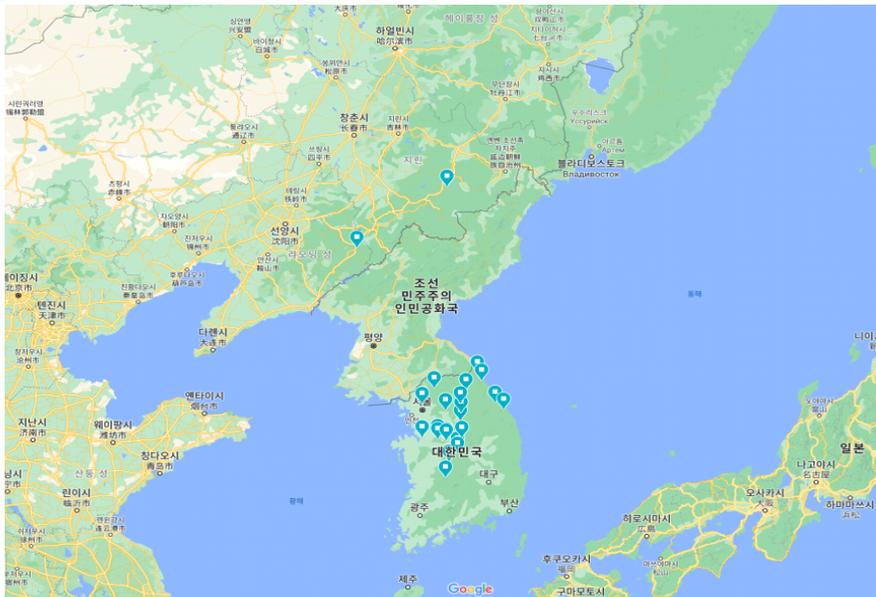


Figure 7. The distributions of the most common haplotype 9.

1-4. Discussion

The results of this study showed two major mitochondrial lineages of the genus *Glandirana* (Figure 5 and 6). Both lineages A and B have been previously nominated and regarded as one species, *G. rugosa*. Herein, these two lineages were compared for taxonomic purposes by using data for individuals from original reports from type localities, China and Japan.

Lineage A was composed of all haplotypes from the southern part of the Korean Peninsula and Liaoning and Jilin Provinces, China. In particular, a common haplotype 9, including individuals from South Korea and China, was one of the most widespread haplotype from lineage A1. Regional coverage was greater in Gangwon, Gyeonggi, and Northern Chungcheong Provinces and one site of Southern Chungcheong Province in South Korea, and one site each of Liaoning Province and Jilin Province in China. Although sublineage A2 was an endemic lineage in South Korea, speciation evidence was not sufficient because of the presence of sympatric areas. However, as a monophyletic taxon, phylogenetically, lineage A was *G. emeljanovi*.

Lineage B was composed of all haplotypes from the eastern, western, and northern parts of the Japanese Archipelago; was known as *R. rugosa*; and was originally introduced by Temminck and Schlegel (1838). Lower haplotype diversity and higher nucleotide diversity than those of Lineage A might be attributed to the limited sampling size relative to that of the highly diverged lineages reported previously. This study showed that Lineage B was subdivided into lineages B1, B2, and B3, and each sublineage indicated a regional group in western, northern, and eastern Japan, respectively. The paraphyletic pattern of lineage B from this study is consistent with polyphyly with

G. susurra and *G. tientaiensis* reported in previous studies (Sekiya et al., 2012). Moreover, previous studies showed genetic divergence (Lee et al., 1999; Ogata et al., 2008; Oike et al., 2017); therefore, introduction of new species needs to be challenged (Bickford et al., 2007), such as a recent study introduced a new species *G. susurra* (Sekiya et al., 2012), which was formally known as *G. rugosa*.

Thus, lineage A was deduced to be *G. emeljanovi*, and lineage B was *G. rugosa*, because the monophyly of lineage A corresponded with the homoeotype (Liu et al., 2017) of the type locality records. With regard to the sublineages of lineage B, further studies are required to reveal the divergence tendency and paraphyly.

CHAPTER 2. Dispersal history of wrinkled frogs in South Korea (*Glandirana emeljanovi*) determined using phylogeographic inference

2-1. Introduction

The genus *Glandirana* is distributed across east and northeast China, Korea, and Japan around the Yellow Sea where it was affected by sea level oscillations in the Quaternary. According to phylogeographic patterns, the Quaternary era most influenced speciation and endemism (Araújo et al., 2008; Carnaval and Bates, 2007; Sandel et al., 2011). The Yellow Sea was also impacted by the repeated reclaiming of land from the sea (Lindberg, 1972; Ota, 1998), and the land bridge between the Korean Peninsula and Japanese archipelago appeared several times (Yonekura et al., 2001) owing to the sea level oscillation at glaciation stages (Feng, 1983). Thus, we consider that multiple refugia existing as current distributions might have been affected by the Quaternary glaciation and regional climates.

One phylogenetic study that analyzed macro-mitochondrial DNA of Korean wrinkled frogs by using restriction enzymes in the early 1990's indicated the presence of Eastern and Western groups (Lee et al., 1992). In addition, previous karyological and restriction fragment length polymorphism studies indicated mtDNA size variation among populations of Korean wrinkled frogs (Lee and Park, 1991; Park, 1990). With technological development, rapid sequence determination was became possible by using PCR and direct sequencing. A study analyzing the sequence of mitochondrial cytochrome *b* of Korean wrinkled frogs revealed two distinct groups supporting the findings of former studies (Lee et al., 1999).

Korean wrinkled frogs inhabit rivers, creeks, streams, valleys, and reservoirs where rocky, gravelly, or sandy areas exist; they also inhabit peripheral regions of rice paddy or farm waterways (Lee et al., 2011). The Korean Peninsula has a west-tilting topology, and thus most of the freshwater streams flow toward the Yellow Sea. Freshwater streams branch from mountain range formations that originated by tectonic movements. Geological studies indicate that the Taebaek and Sobaek Mountain Ranges were formed during 7~20 million years ago (MYA) during the Miocene (Kim et al., 2008; Lee, 1987). The Taebaek Mountain Range directly affected the west-tilting topology. In contrast, the Sobaek Mountain Range formed freshwater streams flowing southward. Because the Sobaek Mountain Range originated from the Taebaek Mountain of the Taebaek Mountain Range stretching from northeast to southwest, it separated Gyeongsang Province (Nakdong River watershed region) from the rest of the Korean Peninsula (Chu et al., 2017; Kwon et al., 2016). Thus, the divergence of freshwater streams might have affected the divergence of Korean wrinkled frogs in freshwater stream habitats. Moreover, the altitude of mountain ranges can act as barriers. Korean wrinkled frogs are mostly distributed at elevations between 83.7 and 434m (mean: 259.1m) (Song and Lee, 2009). Both the mean altitudes of the Taebaek Mountain Range (800m) and Sobaek Mountain Range (500m) are higher than the mean distribution range of Korean wrinkled frogs.

To reconstruct the dispersal history of wrinkled frogs in South Korea, we estimated the geographic barriers and expansion history of Korean wrinkled frog populations. Hypotheses stating that water stream linkages or terrestrial isolations can be affected by geographic barriers and expansion history during the Quaternary were tested. The findings of our study will not only focus on phylogenetic history

reconstruction, but also improve the understanding of the importance of climatic and tectonic changes in the Quaternary that have led to and force to maintain the biodiversity of East Asia.

2-2. Material and Methods

Sampling and DNA extraction

Muscle and skin tissues of wrinkled frogs were obtained from a total of 324 individuals; 304 individuals from South Korea (93 sites); 13 from Japan (3 sites); 5 from Zhejiang Province (1 site); 2 from Liaoning Province, China (1 site). DNA sequences from GenBank for *G. emeljanovi* (KU641020), *G. rugosa* (LC536281~LC536284), and *G. tientaiensis* (KJ941041, KF771342) were used as ingroups and those of *Pelophylax nigromaculata* (NC002805), *Rana huanrensis* (NC028521), and *Sylvirana guentheri* (NC024748) were used as outgroups. Sampling localities were marked on the maps (South Korea: Figure 2, foreign countries: Figure 3). Appendix 1 lists the collected data, and each locality was re-categorized by current streams and paleostreams of the LGM (Yoo et al., 2016). Tissues were obtained from live individuals by clipping the toe (Perry et al., 2011) or from preserved individuals via dissection. Both tissues were either preserved in 80% ethanol or frozen at -70°C. DNA was extracted from tissue samples by using the DNeasy Blood and Tissue Kit (69504; QIAGEN, Germany), following manufacturer's instructions.

Polymerase chain reaction and Sequencing

Five mitochondrial DNA loci (cytochrome *b*, d-loop, 12S, tRNA-val, and 16S) were amplified using polymerase chain reaction (PCR) by using the primers listed in Appendix 2. Each PCR mixture contained 50ng of template DNA, 1x PCR buffer (iNtRON Biotechnology), 0.2mM dNTPs, 1U of Taq polymerase (i-StarTaq, iNtRON Biotechnology), and 0.3~0.4 μ M of primers. The thermal cycler conditions were as follows: 94°C for 4min, followed by 35 cycles of 52°C for 1min, 72°C for 1.5 min, and 94°C for 45s, and a final extension at 72°C for 7min. PCR products were sequenced in both directions by using PCR primers on an ABI 3730xl DNA analyzer (Applied Biosystems) at the National Instrumentation Center for Environmental Management (NICEM) and Cosmogenetech Co., Ltd.

Demographic History

Neutrality tests of Tajima's D (Tajima, 1989) and Fu's F_S (Fu, 1997) were performed using Arlequin 3.5 (Excoffier and Lischer, 2010) to confirm the evidence of population expansion. We also created mismatch distribution to detect historical demographic expansion of the major lineages. Expansion times (T) were estimated using the equation T (Rogers, 1995), where u is the substitution rate for the entire locus length and represented as 0.036 s/s/MY (Li et al., 2012), and τ is the mode of the mismatch distribution. Finally, the genetic distance within different geographic regions was determined using pairwise Φ_{st} -values, and analysis of molecular variance (AMOVA) was used for defining the divergence between each geographic group by using 1,000 permutations in Arlequin (Excoffier and Lischer, 2010).

Divergence Patterns within the Korean Populations

We also used Arlequin to calculate pairwise Φ_{st} and to conduct MANTEL tests with 10,000 permutations to estimate the correlation significance by using geographic distances calculated using four different hypothesis (Figure 7) based on dispersal mechanisms: (1) via freshwater and saline habitats (freshwater stream distance + shoreline distance), (2) via freshwater streams only (freshwater stream distance), (3) via current and historical freshwater streams [freshwater stream distance + Paleostream distance of the Last Glacial Maximum (LGM)] (Yoo et al., 2016), and (4) via land or freshwater streams (direct linear distance). Hypotheses 1, 2, and 3 assume that individuals migrate via aquatic habitats. Hypothesis 1 assumes some salinity resistance and allows for dispersal along the seashore, whereas hypothesis 2 does not allow for salinity resistance. Comparison of these results might help in determining whether the seashore is a barrier. Hypothesis 3 considers the historical impact of paleostreams in the current phylogeographic pattern. Hypothesis 4 assumes that individuals could disperse across land, with mountain ranges being ineffective barriers. In addition, a specific test was performed using pairwise Φ_{st} -values and GPS data to reveal historical barriers by using software BARRIER 2.2 (Manni et al., 2004).

In all, 92 groups of sites composed of 301 individuals from the Korean Peninsula were used for the MANTEL test. The samples from island sites were excluded because the island had no linkages with water streams or shorelines to compare the hypotheses. Negative Φ_{st} - values were set to zero. Geographic distances were measured using Google maps and Geographic Distance Matrix Generator 1.23 (Ersts, 2012).

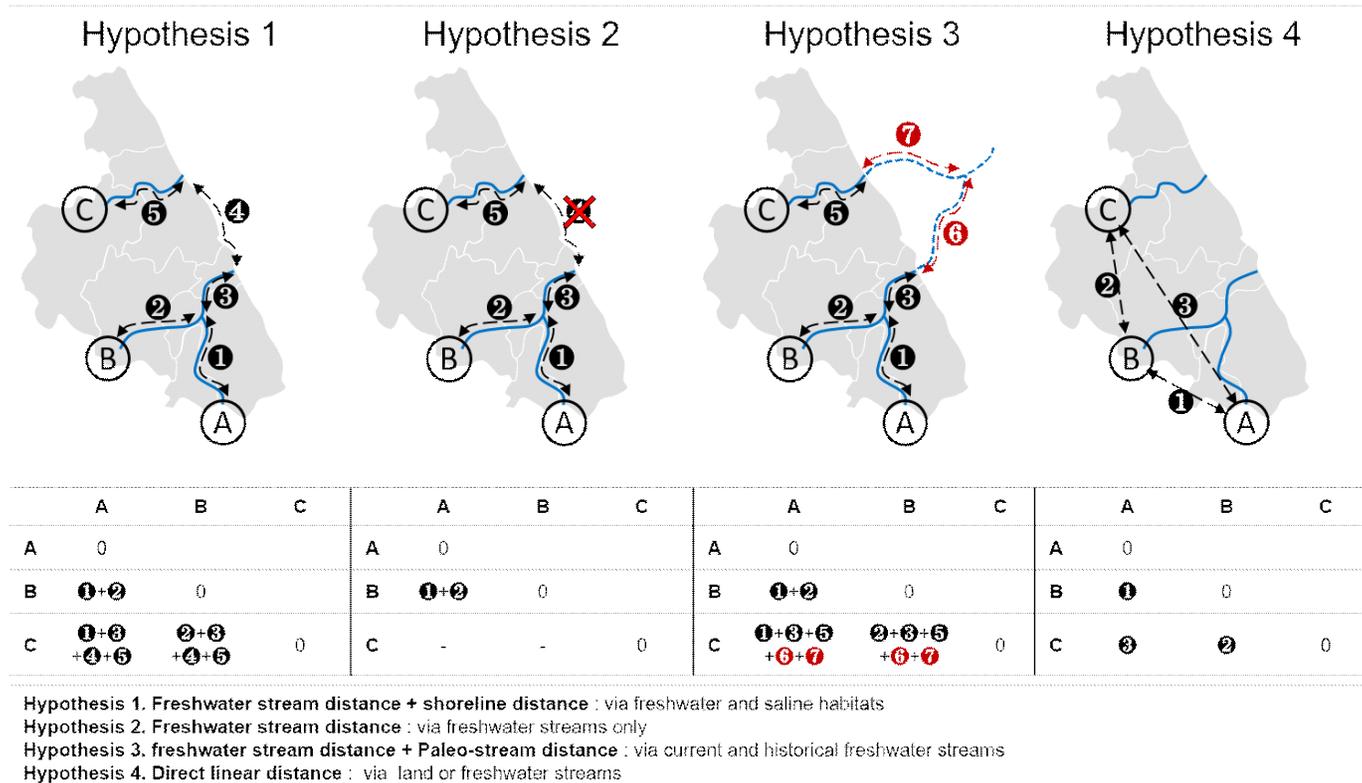


Figure 7. Four alternative methods to measure the distance between populations. Solid blue lines are modern stream routes, and dotted blue lines are paleostream routes. The measured distances are marked with double-headed arrows.

2-3. Results

Dispersal history estimates

We divided the regional populations and grouped them as A1 and A2 (Korea); CN (China); and B1, B2, and B3 (Japan) by using AMOVA. Long-term interruption of gene flow was revealed among the subdivided lineages, with evidence of high Φ_{st} values (Table 4). The highest Φ_{st} value (0.93953, $P < 0.001$) for the “[A1, A2, CN]; [B1]; [B2]; and [B3]” groups suggested that the populations from Korea and China and each population from the three Japanese groups were nearly isolated. In addition, the highest Φ_{ct} (0.84981, $P < 0.001$) for the “[A1, CN]; [A2]; [B1]; [B2]; and [B3]” groups implied that a potential geographic barrier existed between region A2 (part of Gyeongsang Province) and region A1 (rest of South Korea) and CN.

Neutrality tests were performed using Tajima’s D and Fu’s F_S to estimate recent population demographics. Tajima’s D value was significant ($P < 0.01$) only for lineage A1 (-2.35722; Table 5). Conversely, Fu’s F_S values were significant ($P < 0.02$) for sublineages A1 and A2, and both showed negative values. Negative values of Tajima’s D and Fu’s F_S indicate population expansion after bottleneck or purifying selection (Table 5). Mismatch distribution plot for all lineages exhibited unimodal shape (Figure 8), revealing that the sublineages of Korean wrinkled frogs had undergone sudden population expansion. The P values of the sum of squared deviation and Harpending’s raggedness index of both sublineages A1 and A2 were not significant (Table 5) and suggested goodness-of-fit supporting population expansion because of mismatch distribution. Next, we calculated that the mean times of population expansion (T) were 57,717 years ago (YA; late Pleistocene of Quaternary, estimated range

= 3,657–241,530 YA) in sublineage A1 and 46,346 YA (late Pleistocene of Quaternary, estimated range = 14,868–199,001 YA) in sublineage A2.

All the association tests between genetic and geographic distances showed significant correlation ($P < 0.001$) and positive correlation coefficient value ($r > 0$) (Table 6). The higher the value of correlation coefficient, the stronger is the relation between genetic and geographic distances. Our analysis revealed the highest r value for hypothesis 2 by using the distances via freshwater stream that had no linkage with shorelines ($r = 0.593$, $P = 0.000$). Second, hypothesis 3 assuming dispersion via freshwater paleostreams revealed slightly lower correlation ($r = 0.438$, $P = 0.000$). Both correlation coefficient values of hypothesis 1 obtained using distances of freshwater streams from shoreline ($r = 0.026$, $P = 0.000$) and hypothesis 4 obtained using direct linear distances ($r = 0.182$, $P = 0.000$) were low (under 0.3), indicating weak correlations. That is, the dispersal via freshwater streams had medium correlation with genetic distance, but the possibility of movement through the shoreline or downstream area was low.

We also tested geographic barriers (Figure 10). Genetic barriers were detected between the sampling sites of Yeongdong (HYB), Gimcheon (CCA), Seongju (CSA), Goryeong (CGA), Changnyeong (SCA), Gyeongju (CKB), and Pohang (CPA) and those of Yeongdong (HYD), Muju (NMA), Hapcheon (SPA, SPB), Miryang (SMA), and Gyeongju (CKA). This result clustered central–northern Gyeongsang Province and separated the rest of South Korea. One barrier was assigned for the isolation of an area surrounding the Sobaek and Palgong Mountain Ranges (wide part of the northern Gyeongsang Province), and a second barrier was formed by the Sudo (©) and Biseul (Ⓓ) ridge lines. Most Korean wrinkled frog habitats showed allopatry with the

Sobaek Mountain Range as a barrier. Sympatric sites were detected on the peripheral regions of the barrier: Donghae (KDB), Yeongdong (HYA, HYE), Gimcheon (CCF, CCG), Yangsan (SYA), Miryang (SMA), and Muju (NMA). However, one site, Yangju (GYA), showed sympatry at the region with the distribution of sublineage A1.

Table 4. Results of hierarchical analysis of molecular variance (AMOVA).

Grouping option	Φ_{ST}	Φ_{SC}	Φ_{CT}	Among groups	Among populations within groups	Within populations
1group [A1, A2, CN, B1, B2, B3]	0.78313*					
2group [A1, A2, CN];[B1, B2, B3]	0.92746*	0.71970*	0.74121*	74.12%	18.62%	7.25%
3group [A1, A2];[CN];[B1, B2, B3]	0.91809*	0.72031*	0.70714*	70.71%	21.09%	8.19%
3group [A1, A2, CN];[B1, B2];[B3]	0.93371*	0.70692*	0.77383*	77.38%	15.99%	6.63%
4group [A1, A2, CN];[B1];[B2];[B3]	0.93953*	0.69040*	0.80469*	80.47%	13.48%	6.05%
4group [A1, CN];[A2];[B1, B2];[B3]	0.88473*	0.35675*	0.82080*	82.08%	6.39%	11.53%
4group [A1];[A2];[CN];[B1, B2, B3]	0.87875*	0.42116*	0.79053*	79.05%	8.82%	12.13%
5group [A1, CN];[A2];[B1];[B2];[B3]	0.88812*	0.25507*	0.84981*	84.98%	3.83%	11.19%
6group [A1];[A2];[CN];[B1];[B2];[B3]	0.88505*	0.25755*	0.84518*	84.52%	3.99%	11.49%

* $P < 0.001$

Table 5. Comparisons of pairs of each lineage by using Φ_{ST} , mismatch distribution exhibited using sum of squared deviation (SSD) and Harpending's raggedness index values, and neutrality test performed using Tajima's D and Fu's F_S .

Lineage	Tajima's D	Fu's F_S	SSD	Harpending's Raggedness index
A1	-2.35722 $P=0.00000$	-24.32092 $P=0.00050$	0.00998 $P=0.83800$	0.00687 $P=0.98300$
A2	-1.26759 $P=0.08180$	-22.34176 $P=0.00010$	0.00813 $P=0.51000$	0.00736 $P=0.74200$
B	0.69173 $P=0.80990$	8.39330 $P=0.99750$	3.4E+38 $P=0.00000$	0.00000 $P=1.00000$

Table 6. The correlation between genetic and geographic distances obtained using the four hypotheses was estimated using Mantel values.

	H1	H2	H3	H4
<i>Z</i>	3539006.7522	38208.2692	323916.7871	358011.8215
<i>r</i>	0.2592	0.5931	0.4383	0.1822
<i>P</i>	0.0000	0.0000	0.0000	0.0000

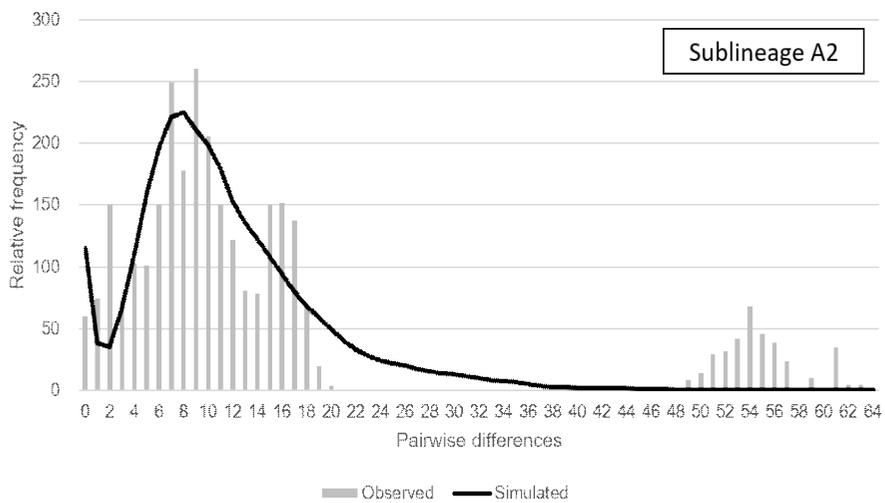
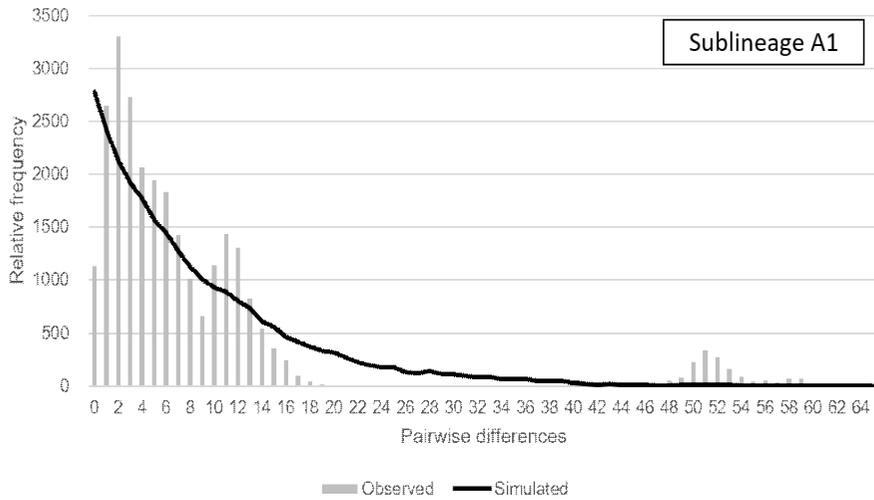


Figure 8. Mismatched distributions of the three lineages of genus *Glandirana*. Goodness of fit of the observed and simulated mismatch distributions was tested using the sum of squared deviation (SSD) and Harpending's raggedness index.

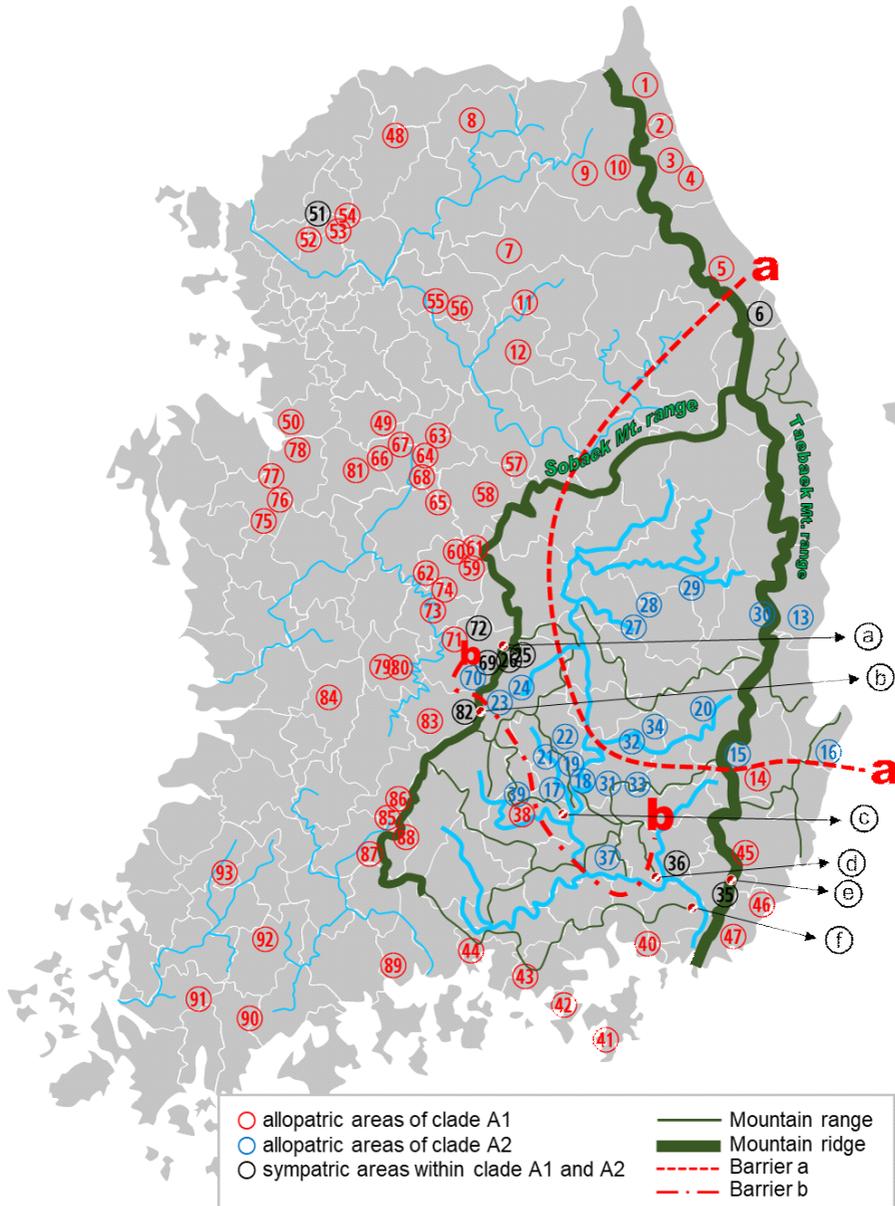


Figure 9. Sampling sites are marked by three types colors in accordance with sublineages A1 (Red), A2 (Blue), and sympatric sites (Black) of Korean wrinkled frogs. Several mountains and mountain ranges (green lines) are overlaid on the map, and possible barriers are marked with dotted red lines.

2-4. Discussion

Dispersed via independent freshwater streams

This study focused on the historical dispersal of Korean wrinkled frogs by comparing hypotheses to estimate how they had diverged. According to the results of the four hypothesis tests for dispersal, genetic distances of wrinkled frogs in South Korea revealed significant positive correlation with the hypothesis that they had dispersed via independent freshwater streams from the LGM (hypothesis 3, $r = 0.438$) to present (hypothesis 2, $r = 0.593$). These hypotheses indicated that the wrinkled frogs in South Korea migrated along independent freshwater streams that were possibly interconnected with paleostreams in the LGM. These results also support the hypothetical scenario of Sekiya et al. (2010) who suggested that a land bridge existed between the Korean Peninsula and Japanese Archipelago during the ice age and the paleo-Nakdong stream might have extended to the Korea Straits at that time. Otherwise, the low level of correlations of dispersal through geographic proximity (hypothesis 4, $r = 0.182$) or coasts, including brackish water zones (hypothesis 1, $r = 0.259$), inferred that land or sea water may play a role in hindrance to disperse.

Genetic and geographic barriers

Results of AMOVA and barrier test showed that wrinkled frogs in South Korea diverged into two clusters, and the divergence was affected by geographic barriers. The Sobaek Mountain Range (mean: 500 m) and ridges of the southern part of the Korean Peninsula were assumed as geographic barriers because wrinkled frogs in South Korea were reported to prefer sites located between latitude 83.7m and 434m

(mean: 259.1m) (Song and Lee, 2009). The Korean Peninsula features west-tilting topology with the Taebaek Mountain Range, which was estimated to have formed during 7–20 MYA during the Miocene (Kim et al., 2008; Lee, 1987) of Neogene. The Taebaek Mountain Range extends from north to south in the eastern part, and, because of this topology, most of the rivers in South Korea flow westward. However, a few rivers flowing southward were affected by the Sobaek Mountain Range originating from the Taebaek Mountain and flowed toward the southwest. The Sobaek Mountain Range separates Gyeongsang Province (Nakdong River watershed region) from the rest of the Korean Peninsula (Chu et al., 2017; Kwon et al., 2016). Thus, we suggested that the Sobaek Mountain Range is a major barrier, and Sudo and Biseul ridge lines (Figure 10 ㉔ and ㉕, respectively) are the southern barriers.

Sympatric areas of sublineages A1 and A2 were revealed at low-latitude regions of the Sobaek Mountain Range (Muju, Yeongdong, and Gimcheon) and strike-slip movement regions of the Yangsan fault (Yangsan, Miryang). Expected factors affecting the formation of sympatric areas were mountain passes (Figure 9 ㉖: Chupungryeong, ㉗: Deoksanjae) and Yangsan fault (Figure 9 ㉘) because the Chupungryeong Mountain pass is the lowest spot of the Sobaek Mountain Range (altitude, 221m) and the Yangsan fault had continued to slip until the Holocene (Kyung, 2009). Low mountain range latitude and tectonic movements might have facilitated the voluntary or passive migration of wrinkled frogs. Thus, lineage A2 was isolated by a geographic barrier—Sobaek mountain ranges—and lineage A1 was distributed in the rest of South Korea.

Historical dispersal reconstruction

Mountain range formation has close causality with freshwater stream formation in the historic process of weathering and erosion. Thus, the Sobaek Mountain Range might have affected the different dispersal histories of the two sublineages of wrinkled frogs in South Korea since it separates a southward waterway (Nakdong River watershed region) from the other regions. As expected, geographic barriers—Taebaek and Sobaek Mountain Ranges, including Sudo and Biseul ridge lines—were formed during 7~20 MYA (Miocene) (Kim et al., 2008; Lee, 1987). After the rise of mountain ranges, the present form of freshwater streams was revealed between the late Mindel glacial [450 thousand years ago (KYA)] and last glacial (10~30 KYA) of Pleistocene, following the general classification of river terrace (Park and Gong, 2001). Wrinkled frogs in South Korea expanded around the same period, the late Pleistocene. The estimated expansion time ranges were 3.6~242 KYA for Lineage A1 and 14.9~199 KYA for Lineage A2. The recently reported impact cratering in Hapcheon, southern Gyeongsang Province, at the end of late Pleistocene (Lim et al., 2020) could have caused the bottleneck-driven divergence in South Korean wrinkled frog populations because of sudden climate change. Conversely, the interconnection of freshwater streams caused by the landization of the Yellow Sea and lowering of the sea level during the LGM (16~31 KYA) (Yoo et al., 2016) could have affected species dispersal. Since glaciation did not affect most regions of South Korea (Park and Gong, 2001) and Southern Japan, the thermal environment of these areas might not have affected the dispersal of amphibians. The continental lineage of ancestral wrinkled frogs could have migrated to southern Japan through freshwater paleo streams within the land bridge between the Korean Peninsula and Japan (Sekiya et al., 2010).

The existence of sympatric areas was also affected by geological characters and evolutionary history. First, low latitude regions of the Sobaek Mountain Range (Muju, Yeongdong, and Gimcheon) formed mountain basins having humid and windy weather. These geographic topologies and climate ecologically linked both the sides of the Sobaek Mountain Range, Yeongdong and Gimcheon (Bae, 2003). Second, tectonic movements in areas (Yangsan, Miryang) of Yangsan fault could have affected the passive migration of wrinkled frog populations. Yangsan fault started to move after 45 MYA of Eocene, and dextral displacements occurred between 14 and 42 MYA (Chang et al., 1990; Choi and Park, 1986; Reedman and Um, 1975). Yangsan fault continued strike-slip until the most recent event in 1314 cal. YA of Holocene (A.D. 536) (Kyung, 2009). Third, a sympatric area far from the expected barrier regions (Yangju) led to the inclusion of one individual (unique haplotype 107) in sublineage A2 of the seven Yangju individuals. Haplotype 107 formed a small monophyletic taxon with haplotypes from Uiseong (Hap 25), Pohang (Hap 99, 149), Daegu (Hap 104), Changnyeong (Hap 159), and Yeongcheon (Hap 104, 136). This was possibly caused by human activities such as freshwater fish farming or fishing practices. We speculate that population genetic analysis to estimate gene flows at these sympatric areas by using early developed microsatellite markers (Kim et al., 2016) could yield interesting results.

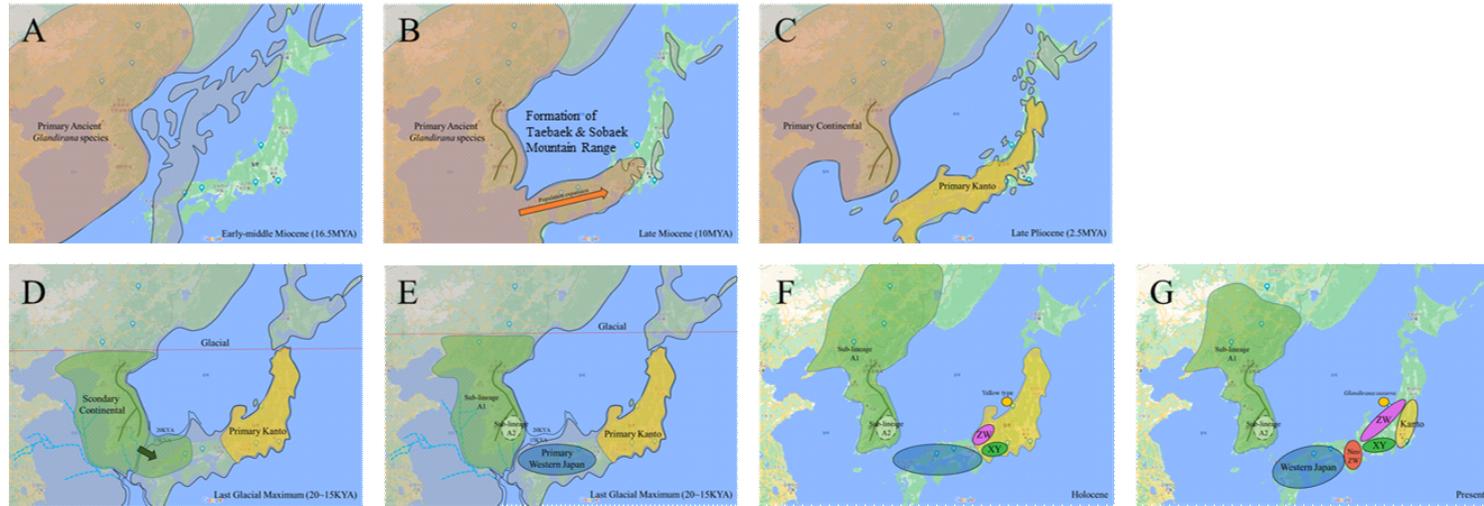


Figure 11. Paleogeographic maps of the era from Miocene to Pliocene (map A~C) were redrawn following a previous study of Noda and Goto (2004). Shape of the Yellow Sea coast line is incomplete owing to limited data availability. Maps of the Last Glacial Maximum era to present (map D~G) were referred to Yoo et al. (2016), Yang et al. (2014), Yoo et al. (2003), Xu et al. (1997) and Min (1993). The hypothetical reconstruction of phylogeographic history was modified from the results of this study and those of Sekiya et al. (2010).

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Appendix 1. A list of specimens used in this study, including their collection information.

No.	Site Codes	N. Specimens	GPS		Province	City, County	Streams	Paleo-Streams
			Latitude	Longitude				
1	KGA	7	38.369919	128.431519	Gangwon	Goseong	Buk creek	unknown
2	KCB	4	38.179428	128.526261		Sokcho	Cheongcho creek	unknown
3	KYA	4	38.053356	128.607133		Yangyang	Yangyangnamdae creek	unknown
4	KYB	3	37.995464	128.679414			Gwangjung creek	unknown
5	KKA	5	37.671486	128.839828		Gangneung	Gangneung-namdae creek	unknown
6	KDB	6	37.510189	129.046417		Donghae	Jeon creek	unknown
7	KHA	7	37.698067	127.955475		Hongcheon		
8	KFA	3	38.123083	127.761800		Hwacheon		
9	KIB	1	38.034103	128.311417		Inje	Han river	Paleo-Yellow Sea
10	KIC	6	37.987186	128.109542				
11	KEA	7	37.479594	127.958581		Hoengseong		
12	KWB	5	37.290447	127.946725		Wonju		

(Continued)

No.	Site Codes	N. Specimens	GPS		Province	City, County	Streams	Paleo-Streams
			Latitude	Longitude				
13	CYA	7	36.376158	129.253128	Northern Gyeongsang	Yeongdeok	Osib creek	unknown
14	CKA	4	35.769111	129.164950		Gyeongju	Hyeongsan river	unknown
15	CKB	3	35.865139	129.090575				unknown
16	CPA	7	35.897364	129.523383		Pohang	Janggi creek	unknown
17	CGA	1	35.778072	128.263869				
18	CGB	2	35.802806	128.294692		Goryeong		
19	CGC	3	35.812614	128.289028				
20	CWB	6	36.093533	128.883986		Yeongcheon		
21	CSA	5	35.844186	128.261017				
22	CSB	1	35.879167	128.283333		Seongju		
23	CCA	2	36.031811	128.023881				
24	CCB	2	36.060361	128.049528		Gimcheon		
25	CCF	1	36.170472	128.057750				
26	CCG	3	36.176389	128.004917			Nakdong river	Paleo-Nakdong
27	CEA	1	36.432819	128.548122				
28	CEB	1	36.452406	128.550639		Uiseong		
29	CAA	2	36.493444	128.824444		Andong		
30	COB	8	36.390397	129.140044		Cheongsong		
31	DGA	3	35.777186	128.455353				
32	DZB	2	35.918486	128.578375		Daegu Metropolitan		
33	DJA	1	35.754544	128.570039				
34	DYA	1	35.992917	128.683639				

(Continued)

No.	Site Codes	N. Specimens	GPS		Province	City, County	Streams	Paleo-Streams	
			Latitude	Longitude					
35	SYA	8	35.310139	129.067944		Yangsan			
36	SMA	4	35.436694	128.851278		Miryang			
37	SCA	1	35.458961	128.559406		Changnyeong	Nakdong river		
38	SPA	1	35.571556	128.087139					
39	SPB	2	35.661361	128.052944	Southern Gyeongsang	Hapcheon		Paleo-Nakdong	
40	SJA	1	35.142489	128.780250		Changwon	Daejang creek		
41	SKA	3	34.781694	128.599833		Geoje	Yulpo creek		
42	STA	1	34.919000	128.376683		Tongyeong	Gwangdo creek		
43	SGA	5	35.037892	128.187769		Goseong			
44	SHA	1	35.067361	127.918139		Sacheon	Sacheon river	Paleo-Seomjin	
45	UUA	7	35.488278	129.117906	Ulsan Metropolitan	Taehwa river	unknown		
46	BJA	1	35.319270	129.250600	Busan Metropolitan	Jwagwang creek	unknown		
47	BHA	2	35.178428	129.142044		Suyeong river	unknown		
48	GFA	1	38.050400	127.306500		Pocheon	Imjin river		
49	GAA	7	36.961111	127.360517		Anseong			
50	GPA	5	36.950067	126.967039		Pyeongtaek	Asan lake		
51	GYA	7	37.696844	126.981567		Yangju			
52	GGA	1	37.676472	126.966361	Gyeonggi	Goyang			
53	GEA	1	37.719850	127.027560					
54	GEB	6	37.726680	127.028240		Uijeongbu			
55	GOA	3	37.549881	127.570922		Yangpyeong			
56	GOB	3	37.522389	127.618167			Han river	Paleo-Yellow Sea	
57	HCA	7	36.905236	127.960006		Chungju			
58	HKA	6	36.668289	127.849372		Goesan			
59	HBB	2	36.538322	127.840661					
60	HBC	2	36.549681	127.826731	Northern Chungcheong				
61	HBD	2	36.548410	127.846650		Boeun			
62	HBH	1	36.482389	127.604194				Geum river	
63	HEA	5	36.960169	127.612014				Han river	
64	HEB	1	36.883033	127.562553			Eumseong	Geum river	

(Continued)

No.	Site Codes	N. Specimens	GPS		Province	City, County	Streams	Paleo-Streams
			Latitude	Longitude				
65	HZA	7	36.728164	127.620711		Jeungpyeong		
66	HGB	2	36.898125	127.354244				
67	HGD	1	36.929822	127.406678		Jincheon		
68	HGG	3	36.857856	127.567089				
69	HYA	3	36.159694	127.985306	Northern Chungcheong		Geum river	
70	HYB	1	36.091764	127.935167		Yeongdong		
71	HYD	1	36.216683	127.879447				
72	HYE	2	36.243667	127.932800				
73	HOB	1	36.342028	127.689306				
74	HOC	2	36.372780	127.622950		Okcheon		Paleo-Yellow Sea
75	OYC	4	36.574158	126.850528		Yesan	Sapgyo lake	
76	OYD	3	36.679878	126.955969				
77	OAA	4	36.726900	126.909544	Southern Chungcheong	Asan	Asan lake	
78	OAB	3	36.886308	127.020528				
79	OGA	2	36.017472	127.516944		Geumsan		
80	OGB	5	36.017364	127.517578				
81	OCA	7	36.837333	127.306439		Cheonan	Geum river	
82	NMA	6	35.956239	127.869950		Muju		
83	NMB	1	35.836158	127.673417				
84	NWA	2	35.976272	127.204928	Northern Jeolla	Wanju	Mangyeong river	
85	NJB	1	35.483514	127.538506		Jangsu	Seomjin river	Paleo-Seomjin
86	NJC	6	35.504014	127.536164				
87	NNC	2	35.378256	127.506122				
88	NND	5	35.481822	127.581597		Namwon	Nakdong river	Paleo-Nakdong
89	JGA	1	34.969917	127.632333		Gwangyang	Gwangyangseo creek	Paleo-Seomjin
90	JAA	2	34.739028	126.957417	Southern Jeolla	Jangheung	Tamjin river	
91	JYA	4	34.769917	126.740617		Yeongam		Paleo-Yellow Sea
92	JHA	1	35.065053	126.981997		Hwasun	Yeongsan river	
93	JJA	1	35.297719	126.779850		Jangseong		

(Continued)

No.	Site Codes	N. Specimens	GPS		Province	City, County	Streams	Paleo-Streams
			Latitude	Longitude				
94	JPN	5	<i>Glandirana rugosa</i> : unknown specific site, Northern Japan					
95	JPW	6	<i>Glandirana rugosa</i> : unknown specific site, Western Japan					
96	JPO	2	<i>Glandirana rugosa</i> : Higasi-gu, Okayama City, Okayama Prefecture, Japan					
97	LC536284	1	<i>Glandirana rugosa</i> : Shizuoka City, Shizuoka Prefecture, Japan					
98	LC536283	1	<i>Glandirana rugosa</i> : Nagaoka City, Niigata Prefecture, Japan					
99	LC536282	1	<i>Glandirana rugosa</i> : Kamogawa City, Chiba Prefecture, Japan					
100	LC536281	1	<i>Glandirana rugosa</i> : Higashihiroshima City, Hiroshima Prefecture, Japan					
101	CNL	2	<i>Glandirana emeljanovi</i> : Huanren County, Liaoning Province, China					
102	KU641020	1	<i>Glandirana emeljanovi</i> : Fusong County, Baishan City, Jilin Province, China					
103	TTT	5	<i>Glandirana tientaiensis</i> : Tiantai County, Taizhou City, Zhejiang Province, China					
104	KJ941041	1	<i>Glandirana tientaiensis</i> : unknown specific site,					
105	KF771342	1	<i>Glandirana tientaiensis</i> : Ninghai County, Zhejiang Province, China					
106	NC002805	1	<i>Pelophylax nigromaculata</i> : Ushita, Hiroshima City, Hiroshima Prefecture, Japan					
107	NC024748	1	<i>Sylvirana guentheri</i> : Mount Huang, Anhui Province, China					
108	NC028521	1	<i>Rana huanrensis</i> : Huanren County, Benxi City, Liaoning province, China					

Appendix 2. Primers used in this study.

Amplified region	Primer	Sequence	Reference
cytochrome <i>b</i>	981F	5'-CAT CGA CCT TCC AGC CCC ATC AAA CAT-3'	Min et al, 2004
	981R	5'-TGT TCT ACT GGT TGG CCT CCA ATT CA-3'	Min et al, 2004
d-loop	F17	5'-CCA TAC TTC TCC TAC AAA GAC-3'	custom product
	R1	5'-TAG CGG GGG TAC GAT AGG GT-3'	custom product
12S:tRNA-val:16S	12L20	5'-AAA GTA HAG CAC TGA ARA CGC DAA G-3'	custom product
	12SR	5'-TTT CAT GTT TCC TTG CGG TAC-3'	Wang et al., 2000

국 문 초 록

한국 옴개구리(*Glandirana emeljanovi*)의 분류학적 고찰 및 계통지리

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서울대학교 대학원

수의학과

수의생리학(수의생화학) 전공

옴개구리(wrinkled frog)는 옴개구리속(Genus *Glandirana*) 무미양서류로써 이에 속하는 다섯 종, *G. emeljanovi*, *G. rugosa*, *G. tientaiensis*, *G. minima*, *G. susurra*가 분류되어 있다. 한국에는 옴개구리 종 하나만 서식하고 있지만, 이 종은 *G. rugosa* 혹은 *G. emeljanovi*로 혼용되어 기재되어 있다. 원기재 기록에 의하면 *G. rugosa*는 일본에 서식하는 옴개구리를 총모식표본(syntype)으로 삼아 발표하였고, *G. emeljanovi*는 중국 흑룡강성 일면포역에서 발견한 옴개구리를 완모식표본(holotype)으로 삼아 발표하였다. 선행연구에 따르면 한국 옴개구리는 지역에 따라 두 개 아계통군(sub-lineage)을 형성하며, 일본 옴개구리 *G. rugosa*와 유전적 차이가 있고, 중국 옴개구리와는 단계통군(monophyletic taxon)을 형성한다고 알려져 있다. 그러나 한국 개체군을 분석하면서 일본 개체와만 비교하거나, 또는 한국과 중국 개체를 비교하는 데 그쳐 한국 옴개구리의 정확한 계통분류학적 관계를 추정하는 것에 어려움이 있었다. 따라서 본 연구는 옴개구리의 분류학적 쟁점을 해결하고자 한국, 중국, 일본 옴개구리 개체군 시료를 모두 이용하여 계통유전학 분석기법을 이용하여 통합적으로 비교·분석하였다. 또한, 한국 옴개구리의 계통지리적 분화 및 진화역사 추론을 시도하였다.

이를 위해, 미토콘드리아 DNA 유전자 5개(Cytochrome *b*, 817bp; D-loop, 765~774bp; 12S rRNA, 823~828bp; tRNA-val, 69~71bp; 16S rRNA, 62bp)의 염기서열을 분석하였다. 본 연구에 사용된 옴개구리 유전자 시료는 총 324개체로부터 수집하였으며, 남한 93개 지역 304개체, 중국 2개 지역 7개체, 일본 3개 지역 13개체의 유전자 시료를 직접 수집하였다. 이와 더불어 *G. emeljanovi*의 동모식표본으로써 중국 길림성 개체(KU641020)와 일본 내에서 지역적으로 뚜렷한 그룹으로 나뉘는 지역별 4개체의 염기서열 정보(LC536281~LC536284)를 미국 국립생물정보센터(NCBI) GenBank에서 얻어 분석에 사용하였다. 아울러 *G. tientaiensis*와 더불어 Raninae아과에 함께 속하는 *Rana huanrensis*, *Sylvirana guentheri*, *Pelophylax nigromaculata*를 outgroup으로 사용하였다.

총 2,630개 염기서열을 얻어 한국, 중국, 일본 옴개구리 개체군을 통합적으로 비교한 결과, 한국과 중국 개체군은 모두 일본 개체군과 유전적으로 뚜렷하게 구분되었다. 또한, 한국과 중국 옴개구리는 함께 단계통군을 형성하는 것을 확인하였고, 총 198개 하플로타입(Haplotype)을 발견하였다. 그 중 하플로타입 9는 대표적인 하플로타입으로서 중국 지린성(동모식표본, KU641020) 및 랴오닝성과 남한 21곳에서만 관찰되는 것으로 파악되었다. 본 연구결과와 기존 연구들을 종합적으로 고찰해 볼 때, 한국 옴개구리는 중국 옴개구리와 함께 학명 *G. emeljanovi*에 속하는 동일종으로 취급하는 것이 타당하리라 사료된다. 한편 일본 *G. rugosa*는 중국 *G. tientaiensis*와 함께 측계통군(paraphyletic taxon)을 형성하는 것을 확인하였다.

한국 옴개구리 개체군을 분석한 결과, 한국 옴개구리는 두 개의 아계통군, A1과 A2로 나뉘는 것을 확인하였다. 아계통군 A1은 경상도 중북부 지역, 아계통군 A2는 그 외 대부분의 내륙 지역에 분포하며 경상도 남부 일부 지역에서 공서지역이 확인되었다. 또한, Barrier test를 시행하여 두 아계통군이 이소성을 형성하는 지역 사이에 유전적 장벽이 존재함을 확인하였고 이는 소백산맥과 남부의 지맥이 지리적 장벽을 형성하여 영

향을 미친 것으로 사료된다. 두 아계통군이 분화한 원인을 알아보기 위해 확산경로를 추정하는 네 가지 가설을 수립하고 MANTEL test를 실시하여 각 가설을 비교하였다. 그 결과, 독립된 각 민물수계 내에서 확산한다는 가설(가설2)과 마지막 최대 빙하기(LGM, last glacial maximum) 동안 연장 형성된 고대 민물수계를 통해 확산했다는 가설(가설3)에서 유의미한 상관관계를 확인하였다. 연안 및 기수지역을 통해 이동할 수 있다는 가설(가설1)과 직선거리로 가까운 지역으로 확산한다는 가설(가설4)에서는 낮은 상관관계를 확인하였다. 개체군 확장 시점은 아계통군 A1이 약 46,000년 전, 아계통군 A2가 약 58,000년 전인 것으로 계산되었다. 따라서 두 아계통군 모두 제4기(Quaternary) 후기 홍적세(Late Pleistocene)에 개체군 확장이 시작된 것으로 추정되었다.

위 연구결과와 선행된 한반도의 지사학적 연구를 종합하여 한국 옴개구리의 진화역사 추론을 시도했다. 한국 옴개구리는 한반도 남부를 동북~서남 방향으로 가로지르는 소백산맥과 경상남도 중부지역을 나누는 수도지맥, 비슬지맥을 지리적 장벽으로 삼아 현재 두 아계통군으로 나뉜다. 중신세(Miocene) 동안에 형성된 산맥들은 한반도 민물수계 형성에 직접적인 영향을 미쳤으며, 민물수계는 홍적세(Pleistocene)를 거치면서 수계형태가 안정된 것으로 알려져 있다. 특히 소백산맥으로 분리된 낙동강 수계의 고립은 독립된 민물수계 내 확산에 의존하는 한국 옴개구리가 두 아계통군을 분화시키는 역할을 했을 가능성이 크다고 사료된다. 또한, 홍적세(Pleistocene) 동안 서해, 남해, 대마도 인근 동해 지역에서 반복적으로 형성된 육지와 민물수계는 중국을 비롯한 한반도의 *G. emeljanovi*와 일본의 *G. rugosa* 사이의 종분화 뿐만 아니라 각 종 내 아계통군 형성에도 밀접한 영향을 미친 것으로 판단된다.

결론적으로 본 연구결과 한국 옴개구리는 두 개의 아계통군이 존재하며 유전적으로 일본 *G. rugosa*와 뚜렷하게 다른 종으로 분화했음을 확인하였고, 중국 *G. emeljanovi*와 단계통군을 형성함을 확인하였다. 또한, 한국 옴개구리가 독립된 민물수계 내 확산을 통해 진화해왔으며 후

기 홍적세의 기후 및 해수면 변동으로 인해 지리적으로 분리된 두 개의 아계통군으로 분화되었음을 추정하였다. 따라서 본 연구에서 한국 움개구리 학명은 *G. emeljanovi*로 사용함이 더 합리적임을 제시하였고, 한국 *G. emeljanovi*에서 2개의 아계통군과 일본 *G. rugosa*에서 3개의 아계통군을 확인하였으며, 이들의 확산과 분화과정을 추론함으로써 진화역사에 대한 기초자료를 제공한 데 의의가 크다고 판단한다. 그러나 연구결과 추풍령 지역과 양산단층대 지역에서 한국 *G. emeljanovi*의 두 아계통군이 모두 서식하는 공서지역이 발견되었고, 이 지역에 대한 추가적인 관찰과 연구를 제안한다.

주요어 : 동아시아, 양서류, 움개구리, 계통지리학, 분류학, 제4기, 홍적세, 소백산맥

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한국양서·파충류학회

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지인 : 한지숙, 김태극, 조병희, 이은정

항상 묵묵히 기다려주시고 지지해주시는 부모님, 감사하고 사랑합니다.

A Poem by Tecumseh

So live your life that the fear of death

죽음이 두렵지 않은 인생을 살아라

Trouble no one about their religion

남의 종교를 욕하지 말고,

Respect others in their view and demand that they respect yours

모두의 의견을 존중하며 너 또한 그들에게 존중을 받아라

Love your life, perfect your life, beautify all things in your life

너의 인생을 사랑하고, 완벽하게 만들고, 아름답게 하여라

Prepare a noble death song for the day when you go over the great divide

결단의 순간이 오는 날을 위해, 죽음을 늘 준비하라

**Always give a word or a sign of salute when meeting or passing a friend,
even a stranger when in a lonely place**

친구를 만날 때나 떠나보낼 때, 경의가 담긴 인사를 해주어라

심지어 그가 외로운 이방인일지라도

Show respect to all people and grovel to none

세상 모두를 존중할지언정 절대 굴복하지 마라

When you arise in the morning give thanks for the food and for the joy of living

아침에 일어날 때 양식과 삶의 즐거움에 감사하라

If you see no reason for giving thanks, the fault lies only in yourself

감사할 이유를 알지 못한다면, 그 잘못은 오로지 너에게만 있을 것이다

Abuse no one and no thing

그 누구도 그 무엇도 악용하지 마라

For abuse turns the wise ones to fools and robs the spirit of it's vision

악용함은 현자에게서도 통찰력을 빼앗아가 결국 그를 멍청이로 만든다

**When it comes your time to die be not like those whoes hearts are filled
with the fear of death**

죽음의 순간이 다가온다면 남들처럼 죽음의 공포를 느끼지 마라

**So that when their time comes, they weep and pray for a little more time
to live their lives over again in a different way**

남들처럼 엎드려 구걸하며, 조금 더 살게 해달라며 추하게 굴지 마라

Sing your death song and die like a hero going home

죽음을 환영하여라 집으로 돌아가는 영웅처럼.