Master's Thesis of Veterinary Biomedical Sciences

Population genetic structure and phylogeography of the unique Asian Plethodontid, *Karsenia koreana*

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Abstract

The Korean crevice salamander (*Karsenia koreana*) is a unique plethodontid salamander species that lives only in South Korea. Despite its enigmatic phylogenetic and biogeographic history, population genetics and evolutionary studies on this species are limited. In this study, I investigated population genetic structure, genetic diversity, and evolutionary historical dispersal of the species across South Korean habitats. I collected samples from 204 individuals across 11 geographical populations, and 14 microsatellite markers were newly developed for this study. Various levels of expected heterozygosity (0.457–0.769) and significant genetic differentiation were identified among populations, with low levels of gene flow. Strong indication of "isolation by distance" were also observed. In particular, the distinct population genetic structure showed a unilateral northward dispersal route. Since the complex mountain ranges of the Korean Peninsula served as glacial refugia at the southern tip of the Northeast Asian region, not having been covered by ice sheets, results of this study could be well interpreted as an example of "refugia within refugia" theory. Because of insufficient gene flow and genetic diversity of the species, it is assumed that the populations are susceptible to genetic drift and environmental fluctuations. In conclusion, I propose a practical management unit system composed of seven groups for conservation of the species. Additional studies utilizing different types of genetic markers for future genetic monitoring and a newly-found northern population are needed.

Keyword : *Karsenia koreana*, Asian Plethodontid, phylogeography, population genetic structure, genetic diversity, glacial refugia
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1. Introduction

1.1. Study Background

Phylogeography is a study of biogeography taking advantage of phylogenetics (Avise, 2000; Juan *et al.*, 2000). It has been widely applied to investigate the dispersal pathways and diversification processes over a long-term period for a particular species or higher taxon (Avise, 2000; Juan *et al.*, 2000). Generally, the contemporary biodiversity and distribution have been shaped by recent historical dispersal and differentiation processes, especially along with isolation caused by geological events and climate change (Hewitt, 2011; Gillespie and Roderick, 2014). Due to Pleistocene ice sheets, considerable terrestrial wildlife became locally extinct or migrated southward to occupy the southern refugial sites (Hewitt, 2000). After ice sheet retraction, this wildlife recolonized northern areas or remained in refugia and diversified (Hewitt, 2000; Gómez and Lunt, 2007; Abellán and Svenning, 2014). Therefore, southern refugial regions generally display higher levels of biodiversity than their northern counterparts (Hewitt, 1996, 2000).

Against this backdrop, the Korean Peninsula is a flourishing southern refugium. The southern tip of the Far East, was not covered by ice sheets during the Quaternary ice ages but was indirectly affected by them (Chung *et al.*, 2017). Furthermore, the complex Baekdudaegan mountain range, along the Korean Peninsula has provided diverse climatic refugial habitats for local ecosystems (Chung *et al.*, 2018), resulting in high biodiversity and endemism, even within small geographical ranges. Genetic studies on two salamander genera in South Korea, *Hynobius* and *Onychodactylus*, have indicated highly differentiated phylogeographic structures and reliable evidence of cryptic diversity (Baek *et al.*, 2011; Suk *et al.*, 2018) in southern regions of South Korea in particular. Studies have also indicated that the southern refugia supplied for groups that migrated southward have been suggested by evolutionary

genetic connections between northern and southern populations (Baek *et al.*, 2011; Suk *et al.*, 2018).

Disagreements have arisen on the biogeographic and phylogenetic history of plethodontid salamanders, which is the most diverse family of order Caudata (Wiens *et al.*, 2006; Kozak *et al.*, 2009; Zhang and Wake, 2009; Vieites *et al.*, 2011; Shen *et al.*, 2016). Complicating this controversy is the discovery of Korean crevice salamander, *Karsenia koreana*, the only plethodontid in Asia (Min *et al.*, 2005; Vieites *et al.*, 2007, 2011; Pyron and Wiens, 2011; Shen *et al.*, 2016; Park *et al.*, 2019). This factor has suggested that Plethodontidae, mostly native to Europe and Americas (AmphibiaWeb, 2020), was once distributed in Asia (Song *et al.*, 2017; Wake, 2017). Since its first record in South Korea (Min *et al.*, 2005), attempts to unravel its phylogenetic position have been conducted to infer how this species came to be dispersed in Asia (Wake, 2013, 2017). The current hypothesis is that this species is a descendant of the western North American lineage that migrated to Eurasia via the Bering land bridge around 65 Ma (Vieites *et al.*, 2007; Shen *et al.*, 2016). However, no follow-up studies have focused on the phylogeography of *K. koreana*.

1.2. Study Species

Out of 754 salamander species in Order Caudata, Plethodontidae is the largest family with 487 species. The Korean crevice salamander, *Karsenia koreana*, is the only member inhabiting Asia, which is also the only species of its genus (AmphibiaWeb, 2020). In terms of South Korean salamander species, on the other hand, five of the six are endemic (Lee and Park, 2016), including *K. koreana*. The species has never been found elsewhere (AmphibiaWeb, 2020). However, according to Borzée *et al.* (2019), its geographical distribution across South Korea is relatively wide, even though its distribution pattern is narrow along mountain chains. As a member of the Family Plethodontidae, the salamander is lungless, entirely

terrestrial, but dependent on water, similar to other family members (Lee and Park, 2016; AmphibiaWeb, 2020). Its common name, “Korean crevice salamander”, was given because it prefers habitats under rock slides and/or mossy limestone (Min *et al.*, 2005; Jung *et al.*, 2019).

The snout–vent length (SVL) is on average 42 mm long and the total length, including the tail, is approximately double or slightly more than doubles its SVL (AmphibiaWeb, 2020). In comparison with other Korean salamanders, it has a small body but is more nimble and self-regenerates after tail self-amputation (Lee and Park, 2016). Commonly, the dorsal body is a dark brown color, with a dorsal stripe from the snout to the tail end, ranging from pale yellow to reddish brown (Figure 1). Its ventral side is brighter than the dorsal, with numerous white blotches. It has a well-defined head and relatively short limbs and digits (Figure 1).

Although this species has been extensively studied in terms of morphology (Buckley *et al.*, 2010; Sever *et al.*, 2016), cytogenetics (Sessions *et al.*, 2008), and ecology (Moon and Park, 2016; Do *et al.*, 2017; Song *et al.*, 2017; Jung *et al.*, 2019; Lee, 2019), its behavioral and evolutionary characteristics remain unclear. Particularly, the population genetics of this aberrant plethodontid requires study, as it has been 15 years since it was first described (Min *et al.*, 2005) and almost 50 years since the first specimen was collected (Nishikawa, 2009).

Figure 1. The photo of *Karsenia koreana* from its dorsal side. The reddish brown stripe appears from the snout to the tip of tail.

1.3. Purpose of Research

Previous studies showed that plethodontid salamanders were considerably differentiated ("species complex"; e.g. Moritz *et al.*, 1992; Tilley and Mahoney, 1996; Bingham *et al.*, 2018; Kuchta *et al.*, 2018) at both the mitochondrial and nuclear DNA, even over small geographical ranges (e.g. Verrell and Tilley, 1992; Highton, 1995; García-París *et al.*, 2000; Velo-Antõn *et al.*, 2013). In this context, 14 novel microsatellite markers were developed to identify fine-scale and recent population genetic structure (Hewitt, 2004; Teacher *et al.*, 2009) and to reveal evolutionary pathways of *Karsenia koreana* after its arrival in Eurasia. Specifically, the research objectives included the investigation of 1)genetic diversity and genetics differentiation of the species, and of each population and 2)population dispersal patterns imprinted in DNA.

2. Materials and Methods

2.1. Sample Collection

Karsenia koreana sampling covered all geographical regions and habitats known until the date (Figure 2, Table 1). When individuals were found, a tip of tail was clipped and immediately stored in 70 % EtOH for DNA extraction. Animals were then immediately released to its original location. In total, 204 individuals were collected from 11 geographical populations. Sample collection was conducted under permit from local government offices according to the Wildlife Protection and Management Act of the Korean Ministry of Environment and guidelines, and also guidelines on ethical animal experimentation from the Seoul National University Institutional Animal Care and Use Committee.

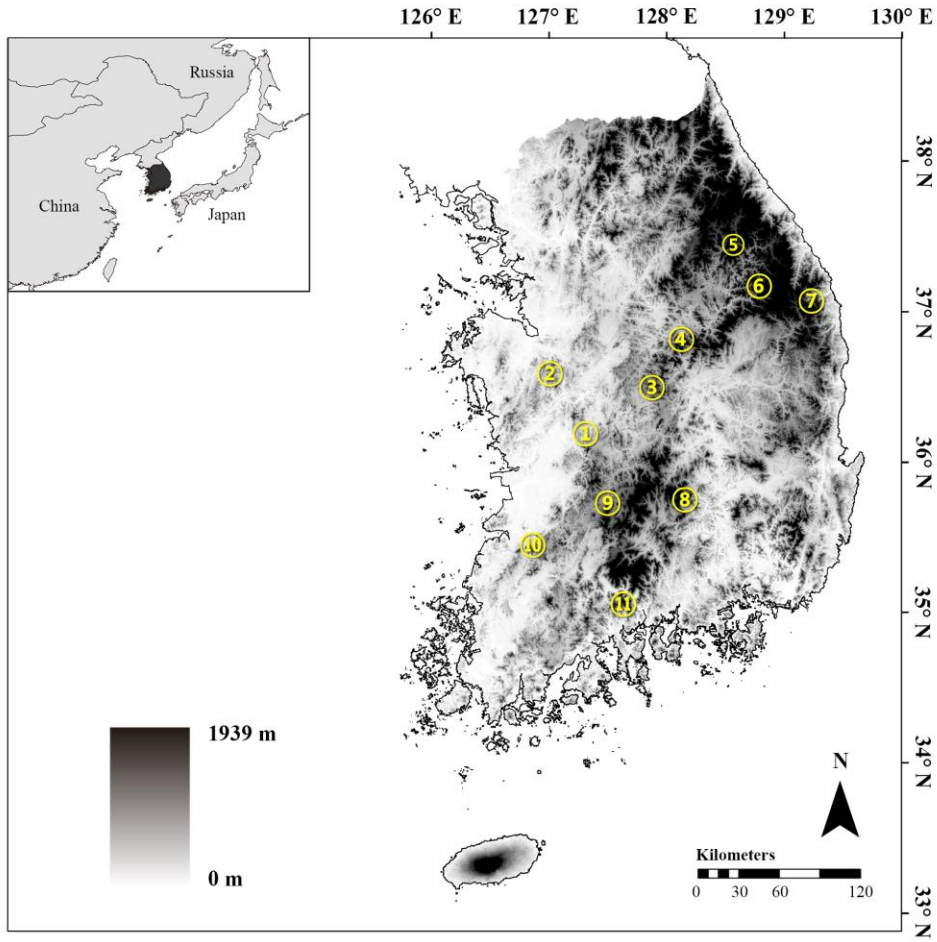

Figure 2. The location of 11 sites throughout the Korean Peninsula where the *Karsenia koreana* sample collection was conducted in this study. The foundational DEM (Digital Elevation Model) file was freely shared from http://www.biz-gis.com. Each number designated each site, as following: 1: DaeJeon (DJ), 2: GongJu (GJ), 3: BoEun (BE), 4: JeCheon (JC), 5: PyeongChang (PC), 6: JeongSeon (JS), 7: SamCheock (SC), 8: HapCheon (HC), 9: JinAn (JA), 10: JeongEup (JE), and 11: GwangYang (GY). See Table 1 for more detailed information of sampling sites.

Table 1. Detailed sampling sites information of geographic population of *Karsenia koreana* throughout South Korea in the study. Sample sizes of microsatellite analyses were given along with geographic information.

Location	Code	Longitude	Latitude	Altitude (m)	Sample size
DaeJeon	DJ	36.18826	127.3337	277	22
GongJu	GJ	36.35361	127.2201	253	9
BoEun	BE	36.52549	127.8582	328	25
JeCheon	JC	36.86138	128.0549	370	20
PyeongChang	PC	37.47148	128.5436	802	24
JeongSeon	JS	37.20680	128.7156	691	18
SamCheok	SC	37.09339	129.1771	310	6
HapCheon	HC	35.80649	128.0963	673	20
JinAn	JA	35.76491	127.4747	424	25
JeongEup	JE	35.48768	126.8939	272	16
GwangYang	GY	35.11225	127.6029	778	19
Total					204

2.2. Laboratory Protocols

Genomic DNA was extracted according to the manufacturer's instructions using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). After extraction, DNA quantity and quality checks were performed on the Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA). DNA samples were then equally diluted to 10–20 ng/μL and stored at −20℃.

For microsatellite marker development, the whole genomic DNA was sequenced on the MiSeq platform (Illumina, San Diego, USA) of Macrogen Inc (Seoul, South Korea) using the pair−end sequencing mode. Adaptor sequences in extracted reads were removed after sequencing. Repetitive DNA regions were identified using RepeatModeler (Smit *et al.*, 2014; http://www.repeatmasker.org/), and reads containing simple sequence repeats were annotated by SSR Finder (Stieneke and Eujayl, 2007; ftp://ftp.gramene.org/pub/gramene/archives/software/scripts/). In total, 52 candidate markers (32 tetra−, 10 tri−, 10 dinucleotide) were selected and tested for polymorphic amplification using eight individuals (two individuals from four geographical populations). Markers passing these tests were chosen to genotype all 204 samples.

PCR amplification was performed in a 20 μL mixture comprising 10X PCR buffer (with 2 mM MgCl2) (2 μL), 2.5 mM dNTPs (1.6 μL), 10 μM forward and reverse primers (0.5 μL each), i−StarTaq polymerase (iNtRON Biotechnology, Seongnam, South Korea) (0.2 μL, 1 unit) and genomic DNA (1 μL). The TaKaRa PCR Thermal Cycler Dice® Gradient (Takara bio) was used. PCR conditions are outlined in Table 2. Genotyping was performed on ABI 3730xl instrument at NICEM Inc (Seoul, South Korea). GeneMapper 3.7 (Themo Fisher Scientific, Waltham, MA, USA) was used for quality checking and peak detection in genotyped samples.

Table 2. Touch-down PCR condition for *Karsenia koreana* microsatellites in the study.

Cycle	Step	Temperature	Time
1	Denaturation	94 ℃	5 min
20	Denaturation	94 ℃	20 sec
	Annealing	60-50 ℃ (-0.5 ℃ / cycle)	20 sec
	Extension	72 ℃	20 sec
20	Denaturation	94 ℃	20 sec
	Annealing	50 ℃	20 sec
	Extension	72 ℃	20 sec
1	Extension	72 ℃	7 min

2.3. Data Analyses

The presence of large allelic dropout, null alleles, and scoring errors was inspected using Micro-Checker 2.2.3 (van Oosterhout *et al.*, 2004). Hardy-Weinberg equilibrium and potential linkage disequilibrium between markers were checked using Fisher's exact test (10,000 dememorization, 100 batches, and 5,000 iterations per batch) by Genepop 4.7.2 (Rousset, 2008). GenAlEx 6.503 (Peakall and Smouse, 2006) was used to compute the number of alleles (N), the number of effective alleles (N_a), observed and expected heterozygosity (H_O and H_E), and the fixation index (F_{IS}) for respective loci and populations. Pairwise relatedness between samples, to exclude duplicate individuals, was calculated in GenAlEx 6.503 (Peakall and Smouse, 2006). No duplicates were identified according to Queller-Goodnight's (Queller and Goodnight, 1989) and Lynch-Ritland's methods (Lynch and Ritland, 1999).

FSTAT 2.9.4 (Goudet, 1995, 2003) and Arlequin 3.5.2.2 (Excoffier *et al.*, 2005) were used to calculate pairwise F_{ST} and R_{ST} with 1,000 permutations, respectively. To identify population genetic structure, several different analyses were used. First, AMOVA in Arlequin 3.5.2.2 (Excoffier *et al.*, 2005) was used with 1,000 permutations to investigate genetic divergence among populations. GenAlEx 6.503 (Peakall and Smouse, 2006) was used to assess "Isolation by distance (IBD)," positive correlations between geographical distance and Slatkin's linearized F_{ST} (F_{ST} / (1-F_{ST}); Rousset, 1997), and covariance-standardized Principal Coordinate Analysis (PCoA) to visualize the distribution of individual genetic characteristics among populations. Genetic barriers, reflecting geographical locations, against gene flow between populations were analyzed by BARRIER 2.2 (Manni *et al.*, 2004) following Monmonier's maximum difference algorithm (Monmonier, 1973) with each 1,000 bootstrapped Nei's chord distance (D_A; Nei, 1987) matrices, generated using MSA 4.05 (Dieringer and Schlötterer, 2003), or F_{ST} matrices, generated by R package FinePop (Kitada *et al.*, 2017). Last, STURUCTURE

(Pritchard *et al.*, 2000) analysis was conducted from K =1 to K = 11 (number of geographic populations) with 10 iterations of 100,000 MCMC (10,000 burn-in) to identify distinct genetic clusters and the degree of distinctness throughout the population genetic structure. The optimal K for this was detected by STRUCTURE HARVESTER (Earl and Vonholdt, 2012) according to Evanno *et al.*, (2005).

For advanced analyses, historical demography and evolutionary history were investigated using several different approaches. Garza-Williamson index (M ratio; Garza and Williamson, 2001) was estimated utilizing AGARst 3.3 (Harley, 2001) to check whether any population had values significantly <0.68, suggesting a substantial population decline. We also investigated the possibility of historical bottleneck in each population using BOTTLENECK 1.2.02 (Cornuet and Luikart, 1997) with 1,000 iterations at IAM, SMM, and TPM (70% SMM, 30% variance). Significant deviations in mode shifts from a typical allele frequency distribution (L-shaped) of a population were considered as serious historical bottlenecks. Population evolutionary history was inferred from >100 pre-set scenarios in DIYABC 2.1.0 (Cornuet *et al.*, 2014). Scenarios were compared in a tournament fashion. To lessen the computational load, seven distinct genetic clusters were defined to group genetically close populations, following results from prior analyses. Posterior probabilities after >100,000 simulations per scenario were compared by the direct approach and logistic regression to decide the most probable scenario. In case of discrepancies between direct and logistic approach comparisons, the confidence of high-ranked scenarios was supplementarily appraised to be discriminated. For the last two scenarios, 1,000,000 simulations per scenario were performed to calculate effective populations sizes and divergence dates.

3. Results

3.1. Marker Characteristics and Genetic Diversity

Of the 52 candidate microsatellite markers, two di−, two tri, and 10 tetra−nucleotide markers were selected based on successful amplification and adequate polymorphism among populations. Large allelic dropout was not observed, but marker stuttering (K1040) was indicated for the whole population. The presence of null alleles was also indicated in all markers according to Brookfield 1 estimation (Brookfield, 1996) in the Micro−Checker result. However, in considering that null alleles were suspected in only one to two populations per locus, and no specific populations had null alleles for all loci, inbreeding or substructure within populations were unlikely. To check the potential impact of null allele−loci on population genetic analyses, analyses were repeated excluding markers (see Oromi *et al.*, 2019) with null allele frequencies >low−frequency zone (K1039 and K1040; see Dakin and Avise, 2004) in total and those having null alleles in two or more populations (K1011 and K1040). Thus, all these three markers (K1011, K1039 and K1040) were retained for downstream analyses since no difference (with or without these markers) was observed between analyses. Linkage disequilibrium between loci was not detected for all available pairs.

The overall genetic diversity from 14 microsatellite loci was moderately high (Table 3). Specifically, tetra−nucleotide loci indicated a higher level of genetic diversity, H_E of 0.841−0.921, whereas di− and tri−nucleotide loci indicated a lower level of genetic diversity, H_E of 0.677−0.874, and higher F_{IS} values, suggesting the presence of null allele presence (Table 3). The level of genetic diversity was modestly different among populations, yet the genetic diversities of [PC], [JS], and [SC] were particularly low (Table 4).

Table 3. Summary statistics of newly developed 14 microsatellite markers in the study.

Locus	Repeat motif	Allele range	N_a	N_e	H_O	H_E	uH_E	F_{IS}
K1004	TCTT	142 ~ 250	24	11.319	0.770	0.912	0.914	0.156
K1005	GAAA	203 ~ 259	15	9.901	0.799	0.899	0.901	0.111
K1006	CTTT	127 ~ 195	17	8.205	0.770	0.878	0.880	0.124
K1008	TAGA	159 ~ 227	18	10.451	0.824	0.904	0.907	0.089
K1011	GAAA	156 ~ 232	17	6.291	0.683	0.841	0.843	0.187
K1012	AGAA	230 ~ 290	16	10.359	0.755	0.903	0.906	0.164
K1018	TCTA	170 ~ 278	26	8.271	0.632	0.879	0.881	0.281
K1019	AAGA	119 ~ 227	17	8.746	0.598	0.886	0.888	0.325
K1020	CTAT	147 ~ 243	25	12.594	0.735	0.921	0.923	0.201
K1021	ATCC	162 ~ 222	16	7.658	0.667	0.869	0.872	0.233
K1039	TAC	114 ~ 159	13	4.168	0.309	0.760	0.762	0.594
K1040	TTA	153 ~ 207	16	3.098	0.328	0.677	0.679	0.515
K1049	AC	134 ~ 190	24	4.704	0.539	0.787	0.789	0.315
K1051	TG	146 ~ 178	17	7.909	0.534	0.874	0.876	0.388

Data comprises repeat motif, allele range, N_a (the number of alleles), N_e (the effective number of alleles), H_O (observed heterozygosity), H_E (expected heterozygosity), uH_E (unbiased expected heterozygosity) and F_{IS} (fixation index) of each microsatellite marker.

Table 4. Summary statistics of 11 *Karsenia koreana* geographic populations throughout South Korea revealed by 14 microsatellite loci.

Population	N	N_a	N_e	H_O	H_E	uH_E	F_{IS}
DJ	22	9.857 (1.199)	6.119 (0.758)	0.727 (0.059)	0.769 (0.054)	0.787 (0.055)	0.050 (0.037)
GJ	9	7.286 (0.759)	5.358 (0.627)	0.730 (0.060)	0.758 (0.043)	0.803 (0.045)	0.043 (0.057)
BE	25	8.643 (0.708)	5.159 (0.619)	0.680 (0.059)	0.728 (0.055)	0.743 (0.056)	0.078 (0.034)
JC	20	7.571 (1.026)	4.318 (0.691)	0.671 (0.048)	0.683 (0.052)	0.700 0.054)	0.002 (0.031)
PC	24	3.929 (0.559)	2.462 (0.372)	0.449 (0.082)	0.457 (0.079)	0.467 (0.080)	0.041 (0.048)
JS	18	5.357 (0.617)	2.720 (0.407)	0.480 (0.066)	0.510 (0.068)	0.525 (0.070)	0.038 (0.038)
SC	6	3.929 (0.385)	2.732 (0.332)	0.476 (0.067)	0.551 (0.060)	0.601 (0.065)	0.124 (0.085)
HC	20	8.643 (0.905)	5.002 (0.616)	0.654 (0.058)	0.723 (0.055)	0.742 (0.057)	0.086 (0.041)
JA	25	10.071 (1.112)	5.639 (0.677)	0.697 (0.069)	0.747 (0.059)	0.762 (0.061)	0.059 (0.054)
JE	16	7.143 (1.079)	4.345 (0.741)	0.647 (0.082)	0.629 (0.076)	0.650 (0.078)	−0.040 (0.048)
GY	19	8.000 (0.646)	5.120 (0.518)	0.744 (0.045)	0.768 (0.033)	0.788 (0.034)	0.029 (0.040)

Data shows N (samples size), N_a (the number of alleles), N_e (the effective number of alleles), H_O (observed heterozygosity), H_E (expected heterozygosity), uH_E (unbiased expected heterozygosity) and F_{IS} (fixation index) of each geographic population.

3.2. Historical Demography and Population Structure

Every population had an *M*−ratio of >0.68, the critical value of significant population decline (Table 5). Mode−shift of all populations from BOTTLENECK analysis had a typical L−shaped allele frequency distribution, meaning historical bottleneck features were not observed in any populations (Table 5).

Table 5. Historical demographic indices of *Karsenia koreana* geographic populations analyzed by 14 microsatellite loci.

Population	Garza−Williamson index (*M*−ratio)	BOTTLENECK (mode−shift indicator)
DJ	0.796	L−shaped
GJ	0.812	L−shaped
BE	0.768	L−shaped
JC	0.752	L−shaped
PC	0.893	L−shaped
JS	0.798	L−shaped
SC	0.704	L−shaped
HC	0.723	L−shaped
JA	0.716	L−shaped
JE	0.877	L−shaped
GY	0.804	L−shaped

Data shows Garza−Williamson index (*M*−ratio) and BOTTLENECK (mode−shift indicator), two different indices of historical demographic fluctuation, analyzed by allele frequencies of 14 microsatellite loci. Every population indicates *M*−ratio of more than 0.68 and typical L−shaped allele frequency distribution, implying a lack of detectable effective population size reduction or genetic bottleneck of *Karsenia koreana* geographic populations throughout South Korea.

Pairwise F_{ST} and R_{ST} values were high, signifying high genetic differentiation among populations. As pairwise R_{ST} values were much higher than F_{ST} counterparts in general, it was presumed that population genetic differentiation was caused by geographical isolation of populations rather than by genetic drift. [JA], [JE], and [GY] especially displayed high genetic differentiation distinct to other populations (Table 6). A significant positive correlation between geographical distance and genetic distance, that is, "Isolation by distance," was also identified (Figure 3) among populations. For example, spatially proximate populations, such as [DJ], [GJ], and [BE] group or [PC], [JS], and [SC] group, showed substantially low genetic distances. AMOVA result using each F_{ST} and R_{ST} suggested significant value of among-population variations, implying putative population genetic structure (Table 7).

Table 6. Pairwise F_{ST} and R_{ST} among 11 *Karsenia koreana* populations analyzed by 14 microsatellites.

	DJ	GJ	BE	JC	PC	JS	SC	HC	JA	JE	GY
DJ		0.160	0.261	0.086	0.544	0.501	0.414	0.123	0.733	0.825	0.436
GJ	0.077**		0.389	0.226	0.701	0.640	0.532	0.193	0.692	0.841	0.491
BE	0.130	0.153		0.120	0.343	0.310	0.202	0.259	0.715	0.828	0.535
JC	0.122**	0.179	0.075		0.444	0.408	0.317	0.164	0.735	0.830	0.502
PC	0.283	0.334	0.172	0.221		0.000^{NS}	0.150	0.486	0.761	0.904	0.768
JS	0.242	0.292	0.135	0.190	0.073		0.060*	0.440	0.721	0.877	0.728
SC	0.197	0.227*	0.076**	0.154	0.120	0.048^{NS}		0.334	0.651	0.851	0.661
HC	0.096	0.129	0.161	0.185	0.334	0.290	0.238**		0.664	0.743	0.276
JA	0.182	0.179	0.178	0.218	0.334	0.301	0.240	0.193		0.399	0.682
JE	0.225	0.239	0.259	0.273	0.417	0.377	0.329	0.227	0.192		0.818
GY	0.155	0.152	0.158	0.193	0.334	0.287	0.216	0.178	0.178	0.228	

R_{ST} values are shown above the diagonal and F_{ST} values below diagonal. All estimated values are highly significant ($p < 0.001$) unless the values are indicated by ** ($p < 0.01$), * ($p < 0.05$) and 'NS'.

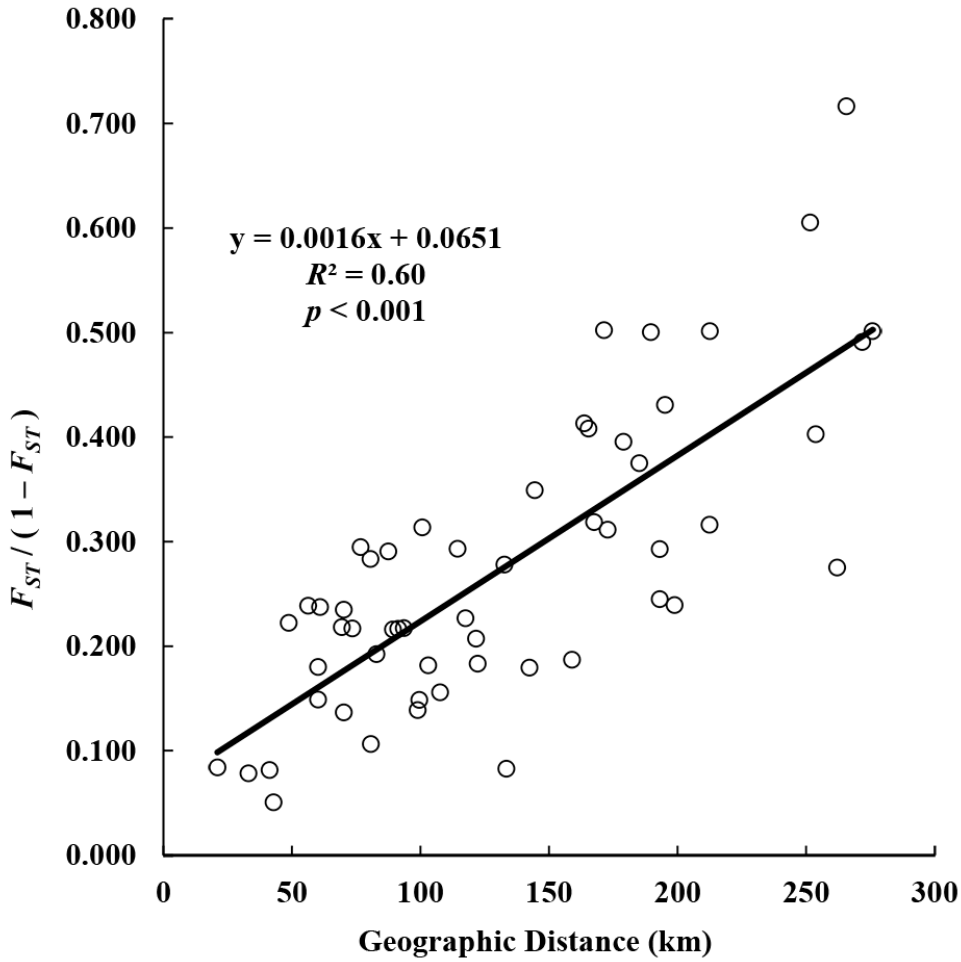

Figure 3. A plot of 'Isolation by distance' among *Karsenia koreana* geographic populations estimated by 14 microsatellite loci. X and Y axis indicate geographic distance (km) and Slatkin' s linearized F_{ST} (F_{ST} / (1 − F_{ST})), respectively.

Table 7. AMOVA results estimated by 14 microsatellite loci computed by (a) F_{ST} and (b) R_{ST}.

(a)

Source of variation	d.f.	Sum of squares	Variance components	% variation	Fixation index	p−value
Among populations	10	529.319	1.31081	21.43	0.21425	< 0.001
Within populations	397	1908.478	4.80725	78.57		
Total	407	2437.797	6.11806			

(b)

Source of variation	d.f.	Sum of squares	Variance components	% variation	Fixation index	p−value
Among populations	10	970326.626	2600.01428	62.25	0.62254	< 0.001
Within populations	397	625851.516	1576.45218	37.75		
Total	407	1596178.142	4176.46646			

From PCoA, four subdivided groups, namely, [PC + JS + SC], [BE + JC], [DJ + GJ + HC + GY], and [JA + JE], were detected among populations with a little overlap between groups (Figure 4). Populations within these subdivisions were largely located in spatial proximity to each other (Figure 4). The use of the Evanno method (Evanno *et al.*, 2005) to detect population genetic structure by STRUCTURE analysis was unsuccessful in determining the optimal K value for genetically distinct clusters in Bayesian structure analysis. Still, the pattern of population genetic structure was similar to the result of PCoA, looking at [JA] and [JE] together that were markedly distinct from other populations (Figure 5).

Gene flow barriers, estimated by bootstrapped D_A, also indicated that [JA] and [JE] were significantly isolated from other populations, whereas no significant gene flow barriers were found among [BE], [JC], [PC], [JS], and [SC] (Figure 6a). Gene flow barriers, estimated by bootstrapped F_{ST}, did not detect obvious barriers among populations; instead, populations were divided by barriers almost equally likely (Figure 6b).

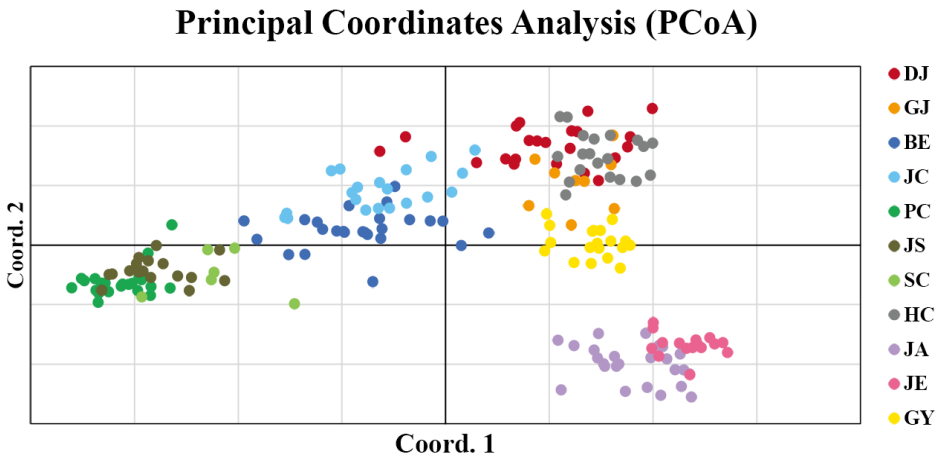

Figure 4. Covariance-standardized PCoA plot estimated by microsatellite genetic distance values presenting genetic differentiation among *Karsenia koreana* geographic populations. The first coordinate (Coord. 1) and the second coordinate (Coord. 2) each explain 14% and 5.79 % of the whole variation.

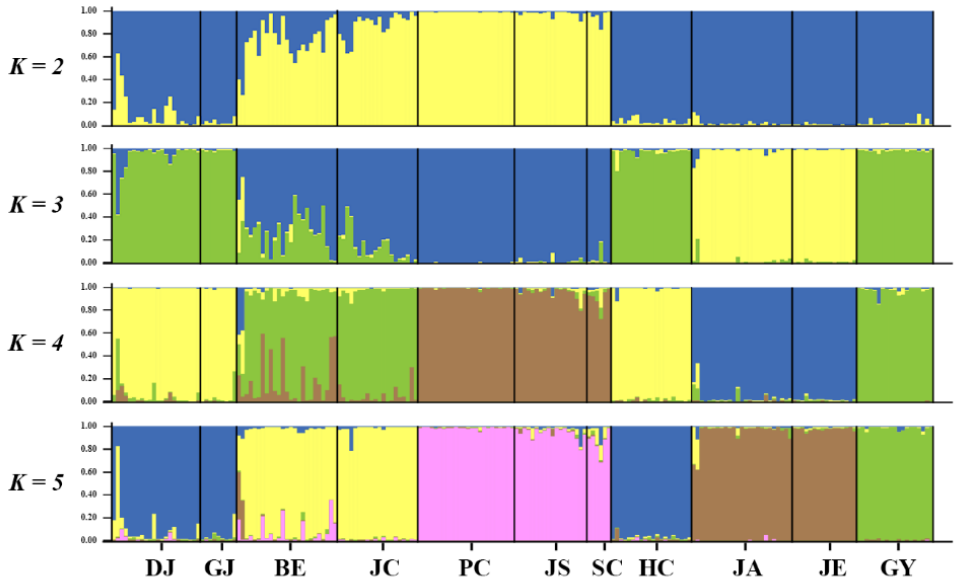

Figure 5. STRUCTURE bar plots among *Karsenia koreana* geographic populations estimated by Bayesian method based on the 14 microsatellite loci. Genetic structuring of four continuous plots from K = 2 to 5 were shown in parallel because the program could not discriminate the optimal number of genetic clusters.

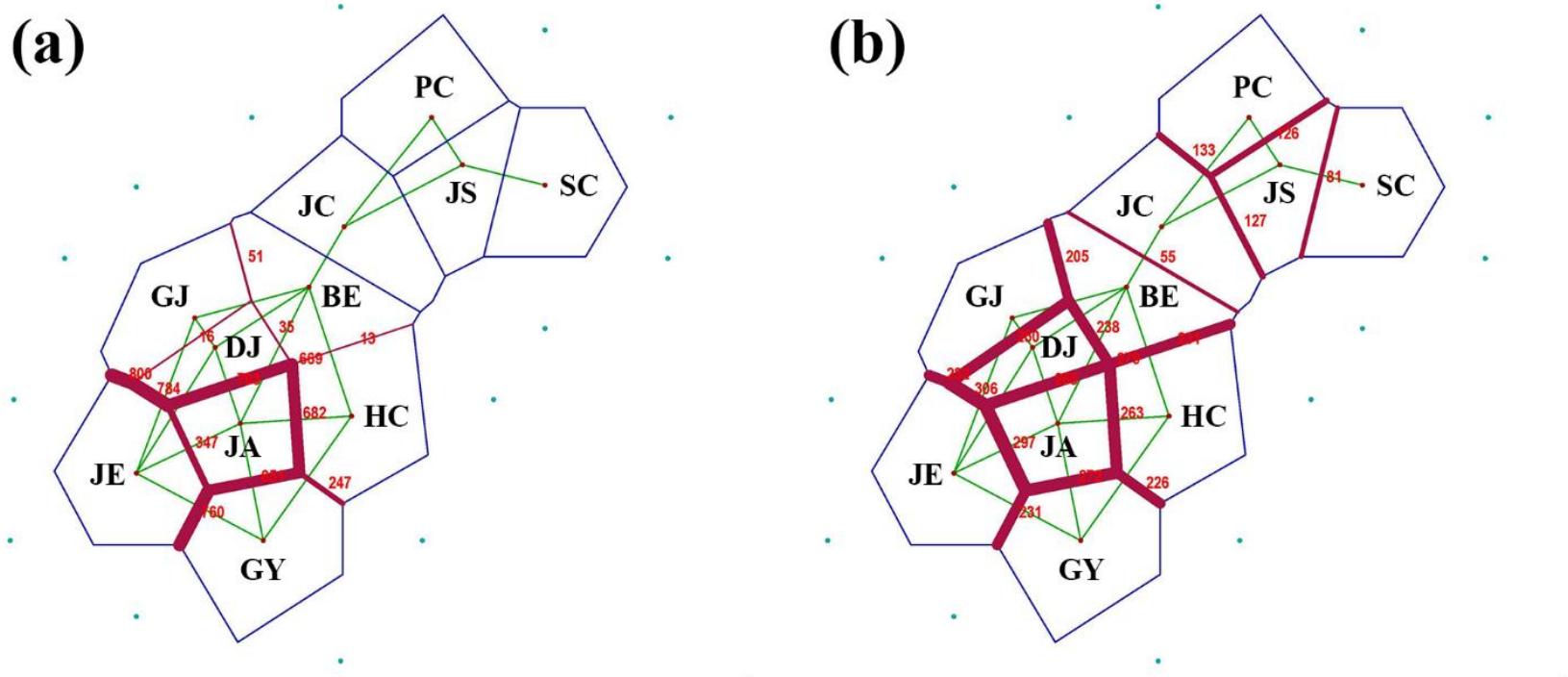

Figure 6. BARRIER results for *Karsenia koreana* geographic populations computed by (a) bootstrapped D_A, and (b) bootstrapped F_{ST}. The significance levels by bootstrapping are indicated by the thickness of red-lines, with thicker being more significant. (a) The most noticeable genetic barrier was recovered between group [JA + JE] and the other groups. (b) The genetic barriers are inferred nearly equally likely compared to (a).

Two evolutionary dispersal scenarios were recognized, most likely based on Bayesian posterior probability (Figure 7). The first scenario (Figure 7) showed that [PC+JS+SC], [JA], and [GY] were divided from an ancestral population approximately 5,380 generations ago, and [JE] was divided from [JA] approximately 2,950 generations ago. [BE+JC], [DJ+GJ], and [HC] were separated from the northern mountainous population: [PC+JS+SC]. The second scenario (Figure 7) indicated that [JA] and [DJ+GJ] were divided from [GY] approximately 13,200 generations ago, and [JE] was separated from [JA] around 5,710 generations ago. In the [GY] lineage, [BE+JC] and [HC] were divided approximately 2,090 years ago, and the northern mountainous population, [PC+JS+SC], was separated from [BE+JC] approximately 263 generations ago. Posterior probability comparison results (Figure 7) could not discriminate between the first and second scenarios, but the confidence test (Table 8) demonstrated that the second scenario was more reasonable. Estimated parameters, including effective population size and measures of central tendency, are summarized in Table 9.

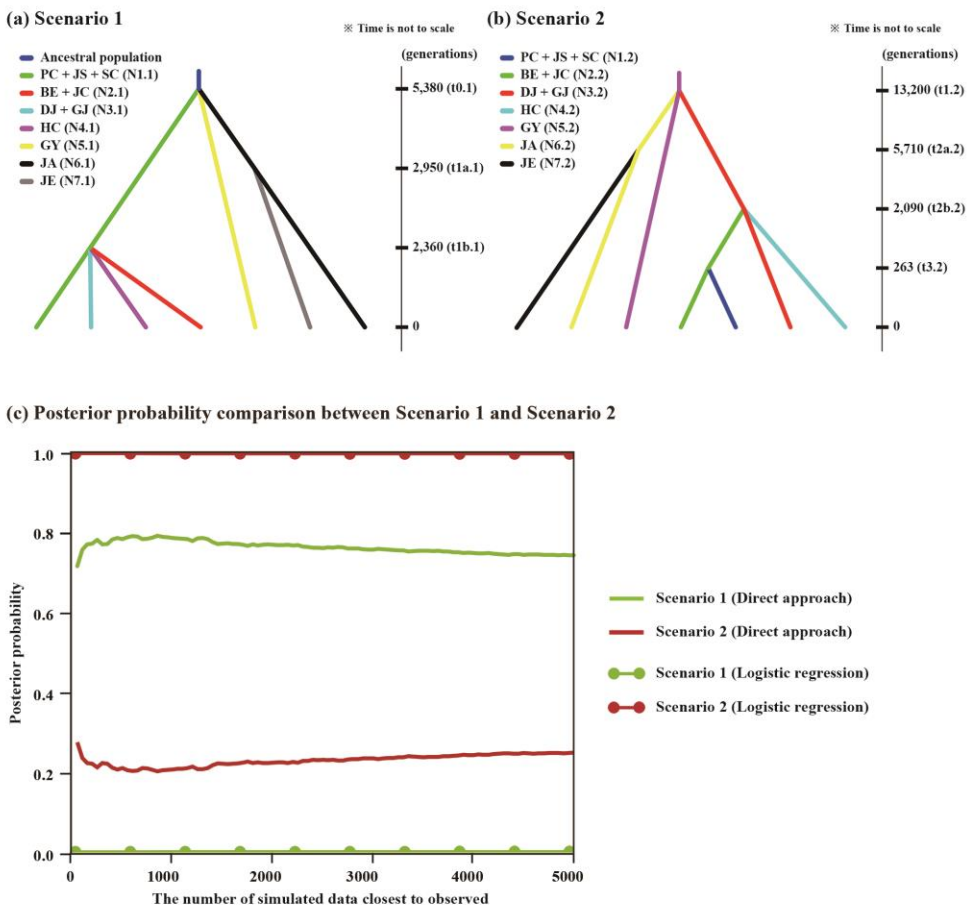

Figure 7. The results of DIYABC analyzed by 14 microsatellites using Approximate Bayesian Computation (ABC) to infer the most probable evolutionary divergence pattern among geographic populations. (a) The pattern of Scenario 1. (b) The pattern of Scenario 2. The time points of each divergence are presented on the right axis in generations, which is not corresponding to the actual time scale. (c) The result of posterior probability comparison between two scenarios. A direct counting of closest simulated datasets to the observed dataset and a weighted logistic regression for the probability of each scenario were performed to compare the deviations of simulated datasets from the observed summary statistics. Scenario 2 was chosen to be the most likely one. See Table 8 for the result of confidence evaluation between Scenario 1 and 2.

Table 8. The result of confidence evaluation of Scenario 1 and 2 of DIYABC based on microsatellites.

		Pseudo-observed data sets simulated with	
		Scenario 1	Scenario 2
Direct approach	Scenario 1	328	60
	Scenario 2	672	940
Logistic regression	Scenario 1	327	18
	Scenario 2	673	982

Numerical values in each cell stands for the count of times the scenario on its left, among 1,000 simulated datasets based on Scenario 1 or Scenario 2, has the highest posterior probability. The numerical values were calculated by the direct count (Direct approach) or the logistic regression estimate (Logistic regression) of the chosen scenario.

Table 9. Estimated parameters and their measures of central tendency among seven *Karsenia koreana* clusters in two selected scenarios of DIYABC. N = effective population size, t = estimated coalescent time in the number of generations, q = quantiles for estimated mean value. Numerical code of N and t of each scenario follows Figure 7.

(a) Scenario 1

Parameter	Mean	Median	Mode	q025	q050	q250	q750	q950	q975
N1.1	2.00e+3	1.67e+3	1.55e+3	5.89e+2	7.03e+2	1.19e+3	2.36e+3	4.47e+3	5.77e+3
N2.1	6.98e+3	7.08e+3	7.20e+3	3.68e+3	4.22e+3	5.98e+3	8.11e+3	9.39e+3	9.66e+3
N3.1	8.74e+3	8.93e+3	9.28e+3	6.48e+3	7.03e+3	8.33e+3	9.36e+3	9.83e+3	9.91e+3
N4.1	5.46e+3	5.36e+3	5.13e+3	2.14e+3	2.57e+3	4.13e+3	6.69e+3	8.84e+3	9.37e+3
N5.1	5.03e+3	4.91e+3	5.10e+3	1.86e+3	2.26e+3	3.72e+3	6.16e+3	8.52e+3	9.15e+3
N6.1	7.48e+3	7.62e+3	7.76e+3	4.40e+3	5.00e+3	6.68e+3	8.44e+3	9.50e+3	9.72e+3
N7.1	4.40e+3	4.14e+3	3.34e+3	1.42e+3	1.75e+3	3.03e+3	5.50e+3	8.11e+3	8.87e+3
t0.1	1.04e+4	5.38e+3	2.53e+3	1.02e+3	1.33e+3	2.94e+3	1.11e+4	3.90e+4	5.59e+4
t1a.1	4.24e+3	2.95e+3	1.65e+3	6.41e+2	8.47e+2	1.78e+3	4.98e+3	1.16e+4	1.61e+4
t1b.1	2.73e+3	2.36e+3	2.18e+3	7.83e+2	9.62e+2	1.66e+3	3.33e+3	5.60e+3	6.70e+3

(b) Scenario 2

Parameter	Mean	Median	Mode	q025	q050	q250	q750	q950	q975
N1.2	1.71e+3	1.29e+3	1.15e+3	3.86e+2	4.67e+2	8.53e+2	1.98e+2	4.62e+2	6.37e+2
N2.2	6.05e+3	6.05e+3	5.73e+3	2.77e+3	3.29e+3	4.87e+3	7.21e+3	8.98e+3	9.45e+3
N3.2	8.07e+3	8.24e+3	8.38e+3	5.35e+3	5.95e+3	7.45e+3	8.87e+3	9.63e+3	9.81e+3
N4.2	5.29e+3	5.18e+3	5.03e+3	2.08e+3	2.48e+3	3.94e+3	6.46e+3	8.65e+3	9.29e+3
N5.2	4.92e+3	4.78e+3	4.66e+3	1.94e+3	2.34e+3	3.70e+3	5.95e+3	8.19e+3	8.97e+3
N6.2	6.88e+3	6.97e+3	7.36e+3	3.60e+3	4.19e+3	5.94e+3	7.91e+3	9.30e+3	9.64e+3
N7.2	4.04e+3	3.74e+3	2.95e+3	1.22e+3	1.51e+3	2.67e+3	5.07e+3	7.89e+3	8.78e+3
t1.2	1.56e+4	1.32e+4	1.07e+4	5.15e+3	6.05e+3	9.78e+3	1.82e+4	3.22e+4	4.28e+4
t2a.2	5.72e+3	5.71e+3	5.42e+3	1.90e+3	2.40e+3	4.31e+3	7.14e+3	9.14e+3	9.51e+3
t2b.2	2.63e+3	2.09e+3	1.19e+3	5.39e+2	6.89e+2	1.34e+3	3.31e+3	6.82e+3	8.07e+3
t3.2	3.34e+2	2.63e+2	1.92e+2	6.08e+1	8.24e+1	1.68e+2	4.00e+2	8.03e+2	1.06e+3

4. Discussion

4.1. Population Genetic Diversity

Microsatellites heterozygosity (H_O = 0.449–0.744) was similar to or higher than other related salamanders, including other plethodontids (e.g. H_O = 0.189–0.420; *Plethodon cinereus* in Cameron *et al.*, 2017) or Northeast Asian salamanders (e.g. H_O = 0.28–0.61; Japanese *Onychodactylus* spp. in Yoshikawa and Nagata, 2017). From my study populations, the northern mountainous populations, [PC], [JS], and [SC], exhibited the lowest heterozygosity. This result was opposite to mitochondrial diversity reported by Jung (2020) that indicated lower nucleotide diversity than other relevant salamanders and higher genetic diversities of [PC], [JS], and [SC] when compared with [BE] or [HC].

However, by comparing my study with Jung (2020), population-level genetic structure patterns as analyzed by microsatellites and mitochondrial DNA, respectively, were analogous overall. The genetic and geographical distances showed significant positive correlations for both genetic markers, suggesting geographical location strongly influenced gene flow. Considering that this species has low mobility and high philopatry, preventing long-range migration, these patterns of isolation by distance appear reasonable. Additionally, [PC], [JS], and [SC] appeared to have high levels of gene flow with each other from both microsatellite and mitochondrial DNA results. This could be attributed to the proximity among these populations and favorable mountainous habitat, allowing the species to move relatively freely between them.

A disagreement between two kinds of genetic markers that [DJ+GJ] and [HC] were grouped together in the microsatellite results but were separated in the mitochondrial DNA results was also found. Yet, such incongruity was not unusual for different inheritance modes and evolutionary time scales applied to the two

kinds of genetic markers. Several theories have sought to clarify this incongruity, those are, selective sweeps, sex-biased dispersal, recent secondary contact, incomplete lineage sorting, or coincidental homoplasy until the date (Moritz, 1994; Mead *et al.*, 2001; Estoup *et al.*, 2002; Zeisset and Beebee, 2008; Karl *et al.*, 2012; Toews and Brelsford, 2012).

Moreover, as *Karsenia koreana* is endangered, the effective population size analyzed by each genetic marker would be different (Moritz, 1994; Cook *et al.*, 2007), lowered in mitochondrial DNA. Therefore, random genetic drift more than likely affected mitochondrial DNA, potentially leading to differences in genetic diversity and structure patterns of the two markers.

4.2. Phylogeography

According to Hewitt (Hewitt, 1996, 2000), during the Pleistocene glacial periods, animal distribution was contracted to "southern refugia", where genetic diversity was retained, and many of the species population became extinct. Later, when the glaciers retreated during interglacial periods, many animals have recolonized northern regions. Populations first arriving in these regions may have dominated and impeded the settlement of following populations, experiencing founder effects at the same time. If favorable habitat resources were abundant in "southern refugial" areas, animal species may have remained and increased population sizes, improving genetic diversity. Thus, they may have adapted to these refugia rather than returning to northern areas. This "refugia within refugia" concept was proposed by Gómez and Lunt (2007) to indicate that multiple refugia along complex mountain ranges could have been shaped within a big, single refugium. The "refugia within refugia" theory was empirically corroborated by several studies (e.g. Avise *et al.*, 1998; Alexandrino *et al.*, 2000, 2002) including plethodontids (Rovito, 2010; Shafer *et al.*, 2010) and animals in East Asia (Zhang, 2002; Matsui *et al.*, 2008a, 2008b; Malyarchuk *et al.*, 2013; Honda *et al.*, 2019).

These data also fit well in this line of explanation. Salamander populations are distributed along South Korean mountain ranges and display discrete genetic structure. Southern populations have shown higher levels of genetic differentiation when compared with other populations; northern populations have exhibited lower levels of genetic diversity, and evolutionary dispersal patterns suggested a northward expansion. When the data are considered all together, the Korean peninsula appears to function as a large refugium containing multiple small refugia in mountainous ranges. The Korean Peninsula and the Baekdudaegan mountain chain, which harbored most of habitats in this study, are also considered glacial refugia (Zhang *et al.*, 2008; Kim *et al.*, 2013; Chung *et al.*, 2016, 2017, 2018; Lee *et al.*, 2018), similar to other peninsulas (i.e. Balkan Peninsula, Iberian Peninsula, Italy, Anatolia, and Florida) and mountainous terrains therein around the northern hemisphere (Zamudio and Savage, 2003; Martínez-Solano *et al.*, 2006; Giokas *et al.*, 2011; Pabijan *et al.*, 2015; Iannella *et al.*, 2018).

In previous studies, it was hypothesized that the ancestral plethodontid of *Karsenia koreana* crossed the Pacific ocean from western North America to East Asia via Bering land bridge around 65 million years ago (Vieites *et al.*, 2007; Shen *et al.*, 2016). Since the species was not found in any other places of Asia until the date, only a small subset of species may have survived in the Korean Peninsula during the long history. After the settlement in South Korea, when Pleistocene ice sheets were developing in northern regions, the species may have contracted southward. Eventually, some *K. koreana* populations could be trapped in favorable mountain pockets, meaning that the populations settled and became genetically differentiated at each habitat, whereas other populations recolonized norther regions after ice sheets retreated. Combining data from this study with Jung (2020) demonstrates a one-way northward dispersal from the southernmost population, [GY], along mountain range terrains of the Korean Peninsula, except few cases. Yet, further studies using other types of genetic markers (i.e. nuclear genes or SNP markers) and recently discovered GoSeong

population in the Demilitarized Zone are required to corroborate this explanation.

4.3. Implications for Conservation

Karsenia koreana is currently listed on the IUCN red list as "Least Concern" (IUCN SSC Amphibian Specialist Group, 2019). This conservation status reflected the widespread distribution of the species across South Korea. Still, as demonstrated here and Borzée *et al.* (2019), the species distribution is restricted mostly along mountain chain terrains and is affected by microhabitat characteristics (Jung *et al.*, 2019). Furthermore, I demonstrated that historical gene flow among populations barely existed. This may have been due to fragmented distribution and presumed small population sizes (Jung, 2020), decreasing genetic diversity in the past.

The designation of "Management Unit" (MU) is especially primary to conserve current populations and their standing genetic diversity (Moritz, 1994). MUs are independent populations within a species shaped by limited gene flow with other populations (Moritz, 1994). In considering the MU definition based on genetic and geographical information, seven MUs should be used for *K. koreana*; [PC+JS+SC], [BE+JC], [DJ+GJ], [JA], [JE], [HC], and [GY], which are based on combined data from this and Jung (2020). This proposal may have significant implications for species conservation.

Since the Korean War, South Korea has rapidly developed at the expense of nature preservation (Lee and Miller-Rushing, 2014). This situation will become exacerbated in the near future with increasing population and developmental demands. Additionally, ongoing climate change exerts negative impacts on species survival in the long term (Araújo *et al.*, 2006; Jenkins *et al.*, 2010; Borzée *et al.*, 2019), bearing in mind this species prefers shady, cool, and moist shelters (Jung *et al.*, 2019), and is sensitive to natural perturbations (Welsh and Droege, 2001). By accounting for these factors, special conservation measures specific to this species must

be based on the MUs suggested here. Consistent population monitoring, wide-ranging field surveys, and further comprehensive studies on ecological characteristics, especially reproduction, will be indispensable for conservation planning.

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아시아 유일 미주도롱뇽인 이끼도롱뇽의 집단유전적 구조와 계통지리 연구

국문 초록

이끼도롱뇽(*Karsenia koreana*)은 미주도롱뇽과 (Plethodontidae) 유미양서류 중 아시아에 서식하는 유일한 종이며 현재까지 전세계에서 남한에만 서식하는 것으로 알려져 있다. 이 종의 진화에 관한 지식이 학술적으로 매우 중요함에도 불구하고 이 종의 계통유전 및 생물지리적 역사는 여전히 불분명한 채로 남아있으며, 이끼도롱뇽 집단의 유전적 구조 혹은 진화 역사에 대한 연구는 지금까지 거의 수행되어오지 못했다. 특히 유전적 다양성 평가 및 보전유전학적 측면의 연구는 전무한 상태이다. 이에 본 연구는 이끼도롱뇽의 집단유전적 구조, 유전적 다양성, 그리고 남한 내에서의 진화적 분산 경로를 밝히고, 보전을 위한 관리단위를 설정하고자 수행되었다. 본 연구를 위하여 남한의 11개 지리적 개체군에서 총 204 개체의 시료를 수집하였고 14개의 새로운 미소부수체 표지자(microsatellite marker)를 개발하여 집단유전학적 분석에 사용하였다. 그 결과, 집단 간에 비교적 다양한 수준의 이형접합자 빈도(0.457 ~ 0.769)가 존재하고, 유전적 교류 수준이 낮음에 따라 집단 간 유전적 분화가 유의미하게 이루어져 있음이 드러났다. 또한 '지리적 거리에 따른 유전적 격리(Isolation by distance)' 의 현상도 확연하게 나타났다. 특히, 집단 간 유전적 구조가 뚜렷하였으며, 북쪽을 향하는 일방향적 분산 경로를 보였다. 이 연구 결과는 '남방 피난처(Southern refugia)' 이론과 '피난처 내의 피난처(Refugia within refugia)' 이론의 관점에서 적합하게 설명할 수 있다. 한반도는 동북아시아의 남쪽 끝에 위치하여 빙하가 존재하지 않았고, 반도 내 복잡한 산맥 지형은 빙하기의 피난처 및 소피난처로 기능하였을 것으로 추정된다. 이끼도롱뇽의 유전적 다양성과 집단 간의 유전적 교류 정도가 그리 높지 않음을 고려할 때, 이 종과 그 집단들은 유전적 부동 및 환경적 변동에 매우 취약할 것으로 예상된다. 아울러, 이끼도롱뇽의 보전을 위해 7개 그룹으로 이루어진 관리단위(management units)를 제시하였으며, 추후 연구에서는 다른 종류의 유전적 표지자들과 새롭게 발견된 북쪽 집단을 연구범위에

추가할 것을 제안하였다.