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Population genetics of two endemic loach species, *Iksookimia hugowolfeldi* and *I. longicorpa* (Cypriniformes, Cobitidae), illuminate taxonomy, paleo-drainages, and river capture in the southern Korean Peninsula

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Abstract

Pleistocene climatic fluctuations reshaped the East Asian coastline, facilitating the formation of extensive paleo-river networks that influenced freshwater biodiversity. Here, we examine how the formation of ancient river systems has influenced the population genetic structures of two sister species of freshwater loaches, *Iksookimia hugowolfeldi* and *I. longicorpa*, found in the southern Korean Peninsula. We conducted a multi-locus genetic analysis of an extensive sample of 208 individuals from 21 populations across their entire distribution range. According to a phylogenetic tree constructed using nuclear DNA, the two sister species diverged approximately 5.5 million years ago. However, there was a discrepancy between the taxonomic identification of *I. hugowolfeldi* populations and the phylogenetic result. A wrong species boundary between the two sister species previously assumed and a genetic signature of headwater river capture were found to explain the discrepancy. An overall radiative diversification of multiple allopatric genetic clusters in the sister species highlights the significant influence of geographic barriers in today's isolated river systems. Simulations of ancient river connections, shaped by fluctuating sea levels, compellingly illustrate how localized reconnections through these historic networks during the ice ages have significantly shaped the population genetic structures of these sister species. The present study identifies the important role of paleo-river systems in facilitating the geographic connectivity of *Iksookimia*, which provides useful insights into the broader phylogeography of other riverine organisms in the Korean Peninsula and East Asia.

Key Words

Climate and oceanographic change, freshwater fish, geological event, phylogeography, speciation

Introduction

Geological events strongly influence the distribution and genetics of aquatic organisms by fragmenting or merging populations within the distributional range of a species (Cisne 1974; Winemiller 1992; Knouft and Page 2003; Netto et al. 2012). In particular, catastrophic geological changes and climatic fluctuations have significantly influenced the evolution of aquatic organisms worldwide (Chamberlin 1909; Hoorn et al. 2010; Davis et al. 2013; Dumont 2022). In the case of freshwater fish, historical dispersal among different rivers is inevitably constrained by sea waters, often leading to geographical splits of species or populations corresponding to different river drainages (Unmack 2001; De Bruyn et al. 2013; Jeon and Suk 2023; Yatsuyanagi et al. 2024; Yun et al. 2024). Historical changes in river

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connectivity, driven by climate-induced sea-level fluctuation, can be considered to have fundamental involvement in shaping the phylogeography of freshwater fish.

The coastal rivers of East Asia provide a key test bed for studying freshwater fish divergence, shaped by historical river connectivity and geological changes. (Tada 1994; Maruyama et al. 1997; Cukur et al. 2011; Kwan et al. 2014; Ho et al. 2015; Jeon and Suk 2023). A recent study (Sun et al. 2025) presented an example suggesting that the paleo-drainage system of the Yellow Sea had a significant impact on the distribution of freshwater fish. A study of the mitochondrial phylogenetic tree of cobitid fish revealed that the endangered *Cobitis choii*, previously thought to be restricted to Korea's Miho Stream, is also found in Hebei, China (Sun et al. 2025). This finding suggests that past river connections during an ice age played a significant role in shaping the distribution and evolution of fish in the region.

The Korean Peninsula and its rivers are surrounded by the extremely shallow Yellow Sea, while the South Sea of Korea is southerly connected to another shallow sea, the East China Sea. This unique geographic and topographic setting of the Korean peninsula would have placed the river drainages under incessant changes over geologic time. Consequently, the historical development and modifications of regional river systems often serve as key explanations for the current distribution and evolution of freshwater fish species on the Korean Peninsula and in neighboring East Asian countries (Lindberg 1972; Nishimura 1974; Yoon et al. 2018; Jeon and Suk 2023).

The Korean fish genus *Iksookimia*, which belongs to the freshwater loach family Cobitidae (Kim 1997), has experienced historical patterns of speciation influenced by various dispersal barriers. These barriers include the likely formation of mountain ranges inland during the Neogene period of the Cenozoic era, as well as changes in the intervening seas between river basins along the peninsula's coastline since the Pleistocene epoch (Kwan et al. 2015; Kwan et al. 2018). There are currently six valid species within this genus: *Iksookimia koreensis*, *I. pumila*, *I. pacifica*, *I. yongdokensis*, *I. hugowolfeldi*, and *I. longicorpa* (Kim 1997, 2009). They are geographically isolated and allopatrically distributed in different drainage systems (Suppl. material 1). In the present study, we conducted a population genetic analysis of two sister species of cobitids: *I. hugowolfeldi* and *I. longicorpa* (Fig. 1). These species inhabit the tributaries of large rivers, isolated independent streams that directly flow to sea without connecting to main rivers, and islands on the southern Korean Peninsula. *Iksookimia hugowolfeldi* inhabits rivers and streams flowing into southwestern regions of the Korean Peninsula, including the Yeongsan River (which includes the type locality for this species), the Tamjin River, several adjacent small streams that independently discharge into the sea, and nearby offshore islands (Park and Kim 1996; Kim 1997; Choi 2003; Park et al. 2018; Kim et al. 2020a, b). In contrast, *I. longicorpa* is found in rivers and streams flowing into southeastern regions of the Korean Peninsula, including the Seomjin River (which includes the type locality), Nakdong River, and nearby independent small streams and offshore islands (Kim 2009; Seo et al. 2019).

These two sister species have a complex taxonomic history. They were initially both classified as *Cobitis taenia* (Uchida 1939) but later were reclassified as the distinct species *Cobitis longicorpus* by Kim et al. (1976). Nalbant (1993) subsequently established a new genus, *Iksookimia*, separating it from the genus *Cobitis*, with *Cobitis longicorpus* reclassified as *Iksookimia longicorpus*. A distinguishing morphological feature of the genus *Iksookimia* is the absence of four rows of dark stripes and spots, referred to as Gambetta zones, along the dorsolateral body, which are characteristic of the genus *Cobitis*. Instead, *Iksookimia* has thin vertical stripes (Nalbant 1993). *Iksookimia longicorpus* remained the accepted scientific name for four years (Nalbant 1993; Park and Kim 1996) until it was revised to *Iksookimia longicorpa* by Kim and Park (1997). This revised name has since been used as the official name by subsequent researchers (Kim 1997; Park and Kim 2000; Kim and Ko 2005; Yun et al. 2021a). In the same paper in which Nalbant (1993) established the genus *Iksookimia*, he identified *Cobitis longicorpus*, found in the Yeongsan River in the southwestern part of the Korean Peninsula, as a new species based on morphological differences in vertical stripes, the lamina circularis, and body length (Fig. 1), naming it *Iksookimia hugowolfeldi* (Nalbant 1993; Kim 2009). Populations of *Iksookimia* inhabiting the Tamjin River and nearby small streams, which are geographically close to the Yeongsan River, were subsequently identified as *I. hugowolfeldi* (Park and Kim 1996; Kim 1997; Kim et al. 2003; Park et al. 2018).

Iksookimia hugowolfeldi and *I. longicorpa* have recently been reported to be the most recently diverged sister species within the genus (Kwan et al. 2018). Previous studies of these two species have investigated their morphological classification (Kim et al. 1991; Park 1991; Kim et al. 2003), preferred habitats and prey (Kim et al. 2003; Kim and Ko 2005; Park 2016; Park et al. 2018), egg production and early life histories (Ko et al. 2009; Ko and Bang 2016), and phylogeny (Park et al. 1991; Šlechtová et al. 2008; Perdices et al. 2016; Kwan et al. 2017; Kwan et al. 2018). In addition, previous genetic research on the two sister species based on allozyme electrophoresis (Park et al. 1991) and molecular phylogenetic analysis has revealed phylogenetic discrepancies between mitochondrial and nuclear genes (Park 1991; Kim 2000; Šlechtová et al. 2008; Perdices et al. 2016; Kwan et al. 2018).

Despite this body of knowledge, no studies to date have investigated the geographic boundaries between these two putative sister species or potential intraspecific genetic structuring. In addition, previous studies have been limited in their geographical scope and sampling size, with no prior sampling campaign covering the entire geographic range of the species. Field researchers have also reported that populations in several habitats, including Tamjin River, Goheung Peninsula, Namhae Island, and Geoje Island, cannot be clearly classified based on the

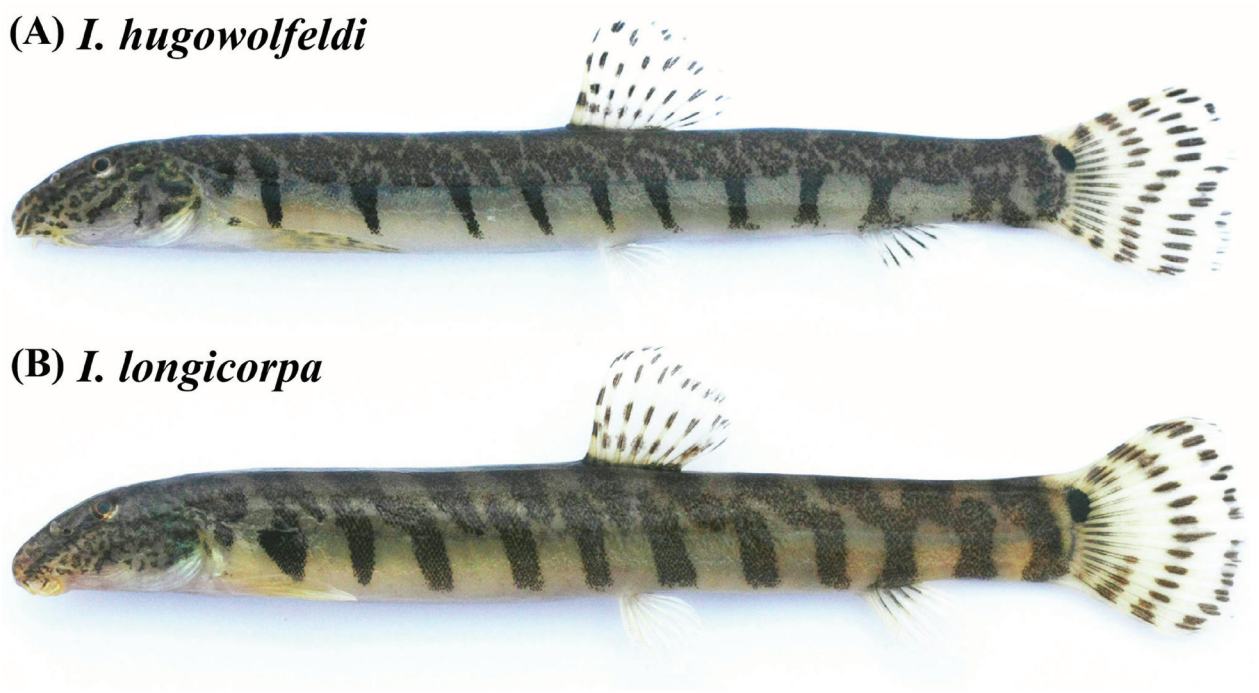


Figure 1. Photographs of representative specimens of the two *Iksookimia* species investigated in this study. **A.** *I. hugowolfeldi* from the Yeongsan River, South Korea; **B.** *I. longicorpa* from the Seomjin River, South Korea.

currently used identification key based on the appearance of vertical stripes and body length (unpublished results).

Barriers such as isolated river systems composed of major and minor independent streams, gaps in terrestrial and marine habitats, and past river reconnections through paleo-river systems caused by lower sea levels during ice ages may have influenced the population genetic structures of *I. hugowolfeldi* and *I. longicorpa*. It would be useful to determine which barriers in particular have influenced these sister species, and how they have done so, in order to understand the evolutionary relationships between abiotic factors and the two species in general. Furthermore, this research would also provide valuable insights into the general evolutionary processes affecting other riverine organisms within the region.

In line with this, we conducted population genetics and phylogenetics experiments using multi-locus genetic data from the mitochondrial cytochrome *b* (Cyt *b*) gene and three nuclear genes sequenced from 208 fish across 21 populations within the known range of *I. hugowolfeldi* and *I. longicorpa*. Using the obtained multi-locus genetic data, we assessed patterns of phylogenetic relatedness and intraspecific genetic structure within and between the two species.

Materials and methods

Sample collection

We attempted to collect as many *I. hugowolfeldi* and *I. longicorpa* individuals from as wide an area as possible to assess patterns in the population genetic structure between main-channel rivers, smaller streams and rivers, and offshore islands. The smaller streams and rivers targeted directly in the present study flow into the sea without connecting to the main-channel rivers. In this study, we followed Korean administrative standards, which categorize rivers as larger than streams based on total length and width (Kwater 2007). We sampled 208 fish samples, consisting of 108 *I. hugowolfeldi* individuals from 11 sampling sites (populations) and 100 *I. longicorpa* individuals from 10 sampling sites (Fig. 2, Table 1). We use the term "population" to refer to sampling localities for simplicity, and genetic clusters were inferred later based on the results of this study. Suppl. material 2 presents the voucher numbers assigned to the samples in the Molecular Ecology Laboratory of the Division of EcoScience at Ewha Womans University, South Korea. We collected the fish samples from 2015 to 2017 using a kick net (mesh size: 4 mm × 4 mm) in rivers and small streams in the southern part of the Republic of Korea. All captured individuals were humanely euthanized using tricaine methane sulphonate (MS-222) in the field. The right pectoral and pelvic fins were then dissected and placed in a 1.5 ml EP tube with 95% alcohol for transport to the laboratory and subsequent storage before the genetic experiments. This study was approved by the Institutional Animal Care and Use Committee (IACUC) at Ewha Womans University (permit no. 14-086, 15-104).

DNA sequencing and genotyping

Genomic DNA was extracted from the tissue of each fin using a DNeasy Tissue Kit (QIAGEN, Hilden, Germany).

Table 1. Sampling locations for the two *Iksookimia* species analyzed in this study. Under the "Drainage system" column, abbreviations with uppercase, two-letter codes represent the names of relatively large, main-channel rivers, while lowercase, two-letter codes represent smaller, inland streams that independently flow to the ocean without connection to other major rivers. Fish from each sampling locality were interpreted as forming a local subpopulation. Pop., population; N, number of samples.

Taxon	Pop. ID	N	Locality (county, province)	Drainage system	GPS
Iksookimia hugowolfeldi	IH01	10	Hwangnyong-myeon, Jangseong-gun, Jeollanam-do	**Yeongsan River (YS)**	35°19'25.9"N, 126°44'18.7"E
	IH02	10	Nam-myeon, Damyang-gun, Jeollanam-do	**Yeongsan River (YS)**	35°10'38.1"N, 127°01'45.8"E
	IH03	10	Cheongpung-myeon, Hwasun-gun, Jeollanam-do	**Jiseok Stream, Yeongsan River (YS)**	34°51'54.7"N, 126°58'25.7"E
	IH04	8	Busan-myeon, Jangheung-gun, Jeollanam-do	**Tamjin River (TJ)**	34°43'36.1"N, 126°55'44.7"E
	IH05	10	Gangjin-eup, Gangjin-gun, Jeollanam-do	**Gangjin Stream (gj)**	34°38'24.3"N, 126°44'26.4"E
	IH06	10	Gogun-myeon, Jindo-gun, Jeollanam-do	**Hyangdong Stream (hd)**, (Jin Island)	34°26'54.9"N, 126°20'58.3"E
	IH07	10	Yongsan-myeon, Jangheung-gun, Jeollanam-do	**Namsang Stream (ns)**	34°37'07.5"N, 126°55'22.5"E
	IH08	10	Geumsan-myeon, Goheung-gun, Jeollanam-do	**Eojeon Stream (ej)**, (Geogeum Island)	34°26'20.6"N, 127°09'24.5"E
	IH09	10	Beolgyo-eup, Boseong-gun, Jeollanam-do	**Childong Stream (cd)**	34°50'19.1"N, 127°18'00.8"E
	IH10	10	Seo-myeon, Suncheon-si, Jeollanam-do	**Suncheondong Stream (sc)**	35°01'30.2"N, 127°30'07.6"E
	IH11	10	Hwayang-myeon, Yeosu-si, Jeollanam-do	**Hwayang Stream (hy)**	34°40'17.6"N, 127°35'38.6"E
Iksookimia longicorpa	IL01	10	Bokheung-myeon, Sunchang-gun, Jeollabuk-do	**Churyeong Stream, Seomjin River (SJ)**	35°26'12.2"N, 126°56'43.7"E
	IL02	10	Sikjeong-dong, Namwon-si, Jeollabuk-do	**Yo Stream, Seomjin River (SJ)**	35°27'23.4"N, 127°26'06.9"E
	IL03	10	Nam-myeon, Hwasun-gun, Jeollanam-do	**Boseong River, Seomjin River (SJ)**	35°00'28.6"N, 127°06'25.3"E
	IL04	10	Bonggang-myeon, Gwangyang-si, Jeollanam-do	**Gwangyangseo Stream (gy)**	35°00'36.0"N, 127°35'13.4"E
	IL05	10	Samdong-myeon, Namhae-gun, Gyeongsangnam-do	**Hwa Stream (h)**, (Namhae Island)	34°47'39.0"N, 128°01'24.1"E
	IL06	10	Jeongdong-myeon, Sacheon-si, Gyeongsangnam-do	**Sacheon River (sc)**	35°02'00.1"N, 128°09'45.5"E
	IL07	10	Dongbu-myeon, Geoje-si, Gyeongsangnam-do	**Sanyang Stream (sy)**	34°48'48.1"N, 128°37'21.3"E
	IL08	10	Macheon-dong, Jinhae-gu, Changwon-si, Gyeongsangnam-do	**Daejang Stream (dj)**, (Geoje Island)	35°07'30.6"N, 128°46'53.9"E
	IL09	9	Yeongo-myeon, Goseong-gun, Gyeongsangnam-do	**Yeongcheon River, Nam River, Nakdong River (ND)**	35°06'37.6"N, 128°13'53.1"E
	IL10	11	Punggak-myeon, Cheongdo-gun, Gyeongsangbuk-do	**Miryang River, Nakdong River (ND)**	35°38'04.6"N, 128°37'29.0"E

The genetic loci used in this study were the mitochondrial Cyt *b* gene and three nuclear genes: the SH3 and PX domain containing three proteins (SH3PX3), early growth response protein 2b (EGR2B), and recombination activating gene 1 (RAG1). Using the primers presented in Suppl. material 3A, we amplified the four target loci following the PCR conditions listed in Suppl. material 3B. The amplified PCR products were purified using PCR purification kits (MGmed, South Korea) and QIAquick PCR Purification Kits (QIAGEN, Hilden, Germany). Purified PCR products were sequenced in both directions using an ABI 3730*xl* automatic sequencer (Applied Biosystems, Foster City, CA) at Cosmogentech (Seoul, South Korea). We used Geneious Prime v.2022.2 (Biomatters, Auckland, New Zealand) for gene alignment and editing after visually reviewing the quality of the sequences. Double peaks in chromatograms were treated as heterozygous sites for PHASE input. The DNA sequences were resolved using PHASE v.2.1.1 (Stephens et al. 2001; Stephens and Scheet 2005). We concatenated the three nuclear DNA (nDNA) sequences using Geneious Prime v.2022.2 (Biomatters, Auckland, New Zealand). The datasets generated during the current study were deposited under the GenBank accession numbers PP433046–PP434173 and PP438000–PP438315 at the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/).

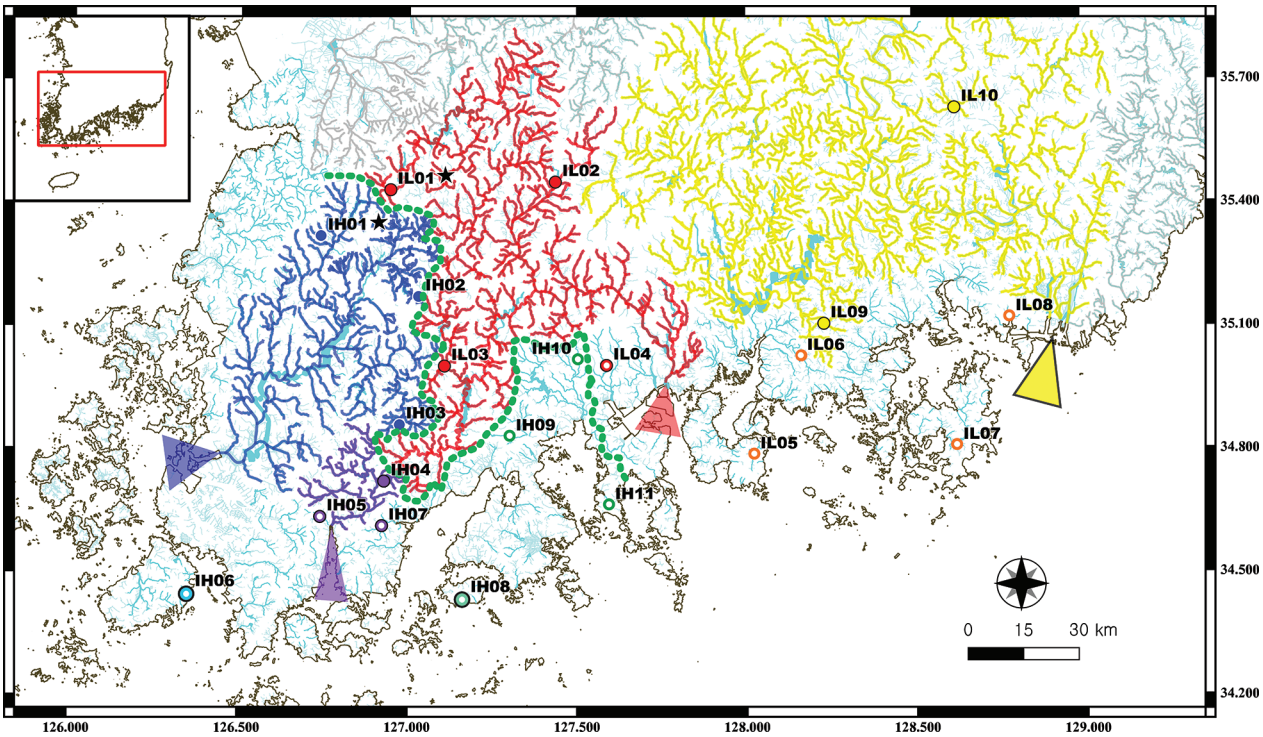


Figure 2. Map of the sampling sites in the study area on the Korean Peninsula (inset). Small dots representing sampling sites are colored to match the seven genetic clusters identified during DNA analysis. Hollow circles indicate sites in streams that flow directly to the ocean and are not connected with other major rivers. Blue line, Yeongsan River (YS); purple line, Tamjin River (TJ); red line, Seomjin River (SJ); yellow line, Nakdong River (ND); star, the type locality; sky blue line, all rivers and streams within the map's scope; gray line, the rivers where neither of the two *Iksookimia* species were recorded. The tip of each triangle points to the mouths of the four main river basins, which are color-coded to match, and the green dotted lines represent the presumed boundary between the habitats of the two focal sister species. The map was created using QGIS (Congedo 2021).

Data analysis

Genetic diversity indices were calculated using DnaSP v5 (Librado & Rozas, 2009) for all four loci and the concatenated nDNA sequence data for each population, while the average expected heterozygosity and observed heterozygosity were calculated using Arlequin v.3.5.2.2 (Excoffier and Lischer 2010). The diversity indices were subsequently visualized using Microsoft Excel.

To identify patterns in the intraspecific genetic structuring of the two focal species, three analyses were carried out. STRUCTURE analysis was conducted to investigate the patterns and agreement between clusters based on Hardy–Weinberg and linkage equilibrium loci and inferred groups (Hubisz et al. 2009). Using PGDspider (Lischer and Excoffier 2012), the nDNA data were converted to an input file utilizing STRUCTURE v.2.3.4 (Hubisz et al. 2009). The individual membership coefficient (q-value; percent ancestry for each K cluster) was computed under the following conditions: assumption of correlated allele frequencies and admixed populations, 10 iterative runs with a hypothetical number of populations (K) from 1 to 21, and a burn-in of 1×10^6 followed by 1×10^6 Markov chain Monte Carlo (MCMC) iteration for each cluster. The most likely number of clusters (K) was determined using ΔK, the rate of change in the estimated log-probabilities of the data, Pr ($X \mid K$), for each value of K (Evanno et al. 2005). These statistics were calculated using the STRUCTURE Harvester (Earl and VonHoldt 2012). To construct bar graphs for the estimated q-values, iterations were averaged for each K using CLUMPAK (https://clumpak.tau.ac.il/; (Kopelman et al. 2015). We visualized the CLUMPAK results using Distruct v.1.1 (Rosenberg 2004). We used only nDNA data for our STRUCTURE analysis because the mitochondrial sites were inappropriate, although mitochondrial DNA (mtDNA) can be used for haplotype network and phylogenetic tree analyses (Falush et al. 2003).

Phylogenetic analysis was conducted to infer the evolutionary history of the 21 sampled populations. The phylogenetic relationships were estimated using the maximum-likelihood (ML) method, while nodal support was estimated with 1000 bootstrap replicates using MEGA X (Kumar et al. 2018). We visualized the populations and clusters of *I. hugowolfeldi* on the resulting tree using cool colors (e.g., blue) and *I. longicorpa* using warm colors (e.g., red). Pairwise evolutionary distances were computed using the model described in Suppl. material 4A. Mitochondrial data were processed using the Tamura three-parameter model, and the concatenated nDNA was processed using the Hasegawa-Kishino-Yano model. Haplotype networks were calculated and graphed to establish the relationships among the 21 populations. The sequences for the outgroup species used in the analysis were downloaded from GenBank through the NCBI website (Suppl. material 5).

We also constructed coalescent-based species trees using *BEAST implemented in BEAST v.2.7.7 (Bouckaert et al. 2019). The best-fit sequence substitution models for the three nuclear genes were calculated using ModelFinder v.1.4.2 (Kalyaanamoorthy et al. 2017) based on the Bayesian information criterion (BIC) and the "models = BEAST2 (NUC)" setting. The substitution models are listed in Suppl. material 4B. We employed the fossil-calibrated divergence time between *I. koreensis* Kim, 1975, *I. hugowolfeldi*, and *I. longicorpa* of 9.28 (13.95–6.16) million years ago (MYA) from Kwan et al. (2018). To set 13.95 MYA and 6.16 MYA as the 95% upper bound and minimal age offset, respectively, a lognormal distribution with a mean of 1.137, a standard deviation of 0.55, and an offset of 6.16 was applied to the calibration point. Four independent runs of 100 million generations were performed, sampling parameters every 10,000 generations. We confirmed convergence and checked that the effective sample size (ESS) was over 200 using Tracer v.1.7.2 (Rambaut et al. 2018). These four runs were combined with LogCombiner v.2.7.7 in BEAST. The first 10% of the resulting trees were discarded, and the remaining trees were annotated using TreeAnnotator v.2.7.7 in BEAST. FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) was used to visualize the topology of the consensus tree generated with the divergence times.

We used DnaSP v.5 (Librado & Rozas, 2009) to convert the sequences for the four DNA loci, and the nDNA loci considered separately, into haplotype data. These haplotype data were used for analysis in PopART v.1.7 (http://popart.otago.ac.nz) (Leigh and Bryant 2015) and Arlequin v.3.5.2.2 (Excoffier and Lischer 2010). PopART v.1.7 was used to infer the TCS haplotype networks from the datasets. *Cobitis hankugensis* was included to check the effect of the introgression of the mtDNA and contrast it with the nDNA data. In addition, *I. koreensis* was added as a closely related outgroup because it is from the same genus as the focal taxa. These outgroup sequences were downloaded from GenBank and are presented in Suppl. material 5.

To analyze and compare specific geographical groups to the general pattern for the genetic groups, we calculated the genetic distances among these groups using the Kimura two-parameter model (Kumar et al. 2008) with MEGA X (Kumar et al. 2018). The pairwise distances were measured, and we used Student's *t*-tests on any interesting geographic pairs that diverged from the average pairwise genetic distances among all groups. For example, we used a Student's *t*-test to evaluate the statistical difference in the genetic distance between the type locality groups of *I. hugowolfeldi* in the Yeongsan River and *I. longicorpa* in the Seomjin River. To investigate genetic differentiation between populations, an analysis of molecular variance (AMOVA) was conducted, and pairwise genetic differentiation (F_{ST}, Wright's F_{ST}) was measured between the populations in Arlequin v.3.5.2.2 (Excoffier and Lischer 2010) using the haplotype data from DnaSP v5. The F_{ST} values were summarized by a non-metric multidimensional scaling (NMDS) plot (Kruskal 1964; Wickelmaier 2003), which we generated in Past version 4.13 (Hammer and Harper 2001; Paramasivam et al. 2017).

To identify the correlation between geography and genetic diversity, isolation-by-distance (IBD) analysis was conducted using Microsoft Excel. Two types of geographic distance were used: the GPS distance measured as the shortest straight-line distance between two sites along the surface of the Earth and the distance combining the length of the rivers or streams and the shortest distance between their mouths (i.e., the river–ocean distance). The unit of these two distances used kilometers (km). The GPS distance was measured in Microsoft Excel according to the formula of the shortest distance between two points in the spherical coordinate system. The river–ocean distance was estimated using a tool reflecting a spherical Earth in QGIS (QGIS.org, QGIS Geographic Information System. QGIS Association. http://www.qgis.org) (Graser 2016; Congedo 2021). To determine the correlation between pairwise genetic differentiation (F_{ST}) values and the geographic distance, Mantel and Pearson correlation tests were performed. The Mantel test was run in Arlequin v.3.5.2.2 (Excoffier and Lischer 2010), and the Pearson correlation test was conducted in Microsoft Excel.

All of the maps except that in Suppl. material 1 were created using QGIS (QGIS Geographic Information System, QGIS Association; available at: http://www.qgis.org). We downloaded the shapefile (SHP) for the rivers and streams used to build the map from the Water Environment Information System, a Korean geographic information agency (https://water.nier.go.kr/web/gisKrf?pMENU_NO=89). We downloaded the coastline SHP for South Korea from the National Geographic Information Institute of the Ministry of Land, Infrastructure, and Transport. Kwan et al. (2018) estimated that the divergence time between *I. hugowolfeldi* and *I. longicorpa* was 4.45–1.4 MYA. Therefore, we approximate that the divergence between the populations of these species occurred during the Pleistocene. We used QGIS to simulate changes in the topography on and around the Korean Peninsula caused by sea level fluctuations during the last Ice Age, approximately ~18,000 years before the present during the Pleistocene (Yoo and Park 2000; Grant et al. 2012). We also employed the plugin designed to simulate sea-level changes available in the GitHub repository (https://github.com/patrick-morrison/qgis_sea_level_tool.git). We downloaded the Grid file used to build the seabed and the study area from the General Bathymetric Charts of Oceans (GEBCO; Mayer et al. 2018; accessed at https://download.gebco.net) and the National Land Information Platform of the National Geographic Information Institute in Korea (https://map.ngii.go.kr/mn/mainPage.do). Finally, we downloaded the coastline SHP for Japan and China from HUMANITARIAN DATA EXCHANGE v1.78.1 PY3 (https://data.humdata.org/). Flow accumulation was analyzed by downloading PCRaster (Plugin ID: 2497), a software package from QGIS (https://jvdkwast.github.io/qgis-processing-pcraster/).

Results

We obtained 890 bp Cyt *b*, 667 bp EGR2B, 785 bp RAG1, and 613 bp SH3PX3 sequence alignments from 208 fish individuals. ML phylogenetic analysis revealed that the populations of the two nominal sister species *I. hugowolfeldi* and *I. longicorpa* were divided into multiple radiating groups, meaning that the taxa were para-/polyphyletic rather than two monophyletic groups as expected based on their current taxonomic classification. In particular, these two species' populations were grouped into five to eight distinct clusters. These clusters corresponded to several distinct geographical areas isolated by land barriers and the sea, including drainage basins (Figs 2–5, Tables 2, 3). We inferred the taxonomy and evolution of *I. hugowolfeldi* and *I. longicorpa* in relation to the geographic barriers in the study area, with both mtDNA and nDNA data indicating a pattern of radiative evolution across the southern part of the Korean Peninsula (Figs 4, 5, Suppl. material 7).

Table 2. Estimates of the divergence time for nodes and the corresponding 95% highest posterior density (HPD) for each divergence-time estimate. Nodes correspond to those on the species tree in Fig. 5A.

Node	Divergence Time (Million years ago)	
	Median	95%HPD
α	5.471	8.456–3.001
β	2.48	4.328–1.209
γ	1.36	2.371–0.653
δ	1.751	3.485–0.530
ε	0.63	1.11–0.28
* (calibration point)	9.79	13.9–6.16

Table 3. Composition of distinct genetic clusters inferred from nuclear DNA (nDNA) in STRUCTURE and employed to label samples in phylogenetic clusters and haplotype networks.

Cluster	Population ID	Drainage system
A-1	IH01–03	Yeongsan River
B	IH04, 05, 07	Tamjin River, Gangjin Stream, Namsang Stream
C	IH06	Hyangdong Stream
D	IH08	Eojeon Stream
E	IH09–11	Childong Stream, Suncheondong Stream, Hwayang Stream
A-2	IL01–04	Seomjin River, Gwangyangseo Stream
F	IL05–08	Hwa Stream, Sacheon River, Sanyang Stream, Daejang Stream
G	IL09, 10	Nakdong River

Around seven clusters were consistently inferred from the nDNA data using three methods: STRUCTURE assignment tests, haplotype network analysis, and phylogenetic analysis (Figs 3–5, Table 2, 3). The STRUCTURE results based on the three nuclear gene loci revealed that the 21 populations were divided into seven clusters (K = 7), as indicated by the highest peak for ΔK at seven (Fig. 3A, B). Mapping these clusters onto the sampling sites and the drainage systems they belonged to indicated that the genetic structure of the two sister species was closely related to their geographic location (Fig. 3C). These clusters suggest potential conservation units critical for preserving the genetic diversity shaped by Korea's unique geographic setting and paleo-drainage history. Fish populations located near each other or within the same watershed were more likely to belong to the same genetic cluster. In contrast, the genetic composition of populations that were geographically distant tended to differ significantly from one another (Fig. 3C, Table 3).

The haplotype network and phylogenetic tree analyses revealed a similar number of distinct clusters (Figs 4, 5, Suppl. material 7), though the demarcation of these clusters was subjective. To simplify our analyses and description, we tentatively considered the seven clusters identified in STRUCTURE to represent all of the divergent haplotype groups inferred from nDNA data. We labeled these clusters A–G (Figs 3, 4A). To facilitate comparison, we consistently employed the seven-cluster labeling scheme for nDNA to label the corresponding fish individuals in the phylogenetic tree based on nDNA (Fig. 5A, Suppl. material 7). This labeling was also used in the haplotype network and tree for the mitochondrial Cyt *b* gene (Figs 4B, 5B).

Genetic nDNA data indicated that each fish population belonged to one of the seven clusters. The sampled fish populations were distributed across various river drainage systems and offshore islands, leading to the formation of genetic groups that reflected their geographical locations. These genetic clusters exhibited a divergent pattern that appeared to be influenced by geographical isolation barriers such as seas, islands, river basins, and distance (Fig. 3C, Table 3). However, surprisingly, results that did not follow this pattern were observed for the Yeongsan and Seomjin Rivers, which are the type localities for *I. hugowolfeldi* and *I. longicorpa*, respectively. Because these are separate main rivers, there is an assumed species boundary between the two species (as indicated by the green dotted line in Fig. 2), with the mouths of the Yeongsan and Seomjin Rivers located in the western and southern regions of the Korean Peninsula, respectively (Fig. 2). This geographical separation suggests that, even during historical glacial periods, these rivers were probably not directly connected. However, our analysis revealed that the populations in these two rivers had only minor genetic differences. The inter-river populations were divided into two close clusters of haplotypes, with genetic distances only a few mutational steps apart (identified as A-1 and A-2 in Figs 3, 4A and Table 3). These clusters even shared some of the same haplotypes. The genetic distances between A-1 and A-2, which are subclusters of A, were significantly shorter than the average distance to the other 14 rivers or six clusters (i.e., B through G) (Table 4, Suppl. material 8). According to the nDNA data, the average pairwise genetic distance between the seven clusters was 0.008 (standard deviation [s.d.] = 0.001), while the genetic distance between subclusters A-1 and A-2 was

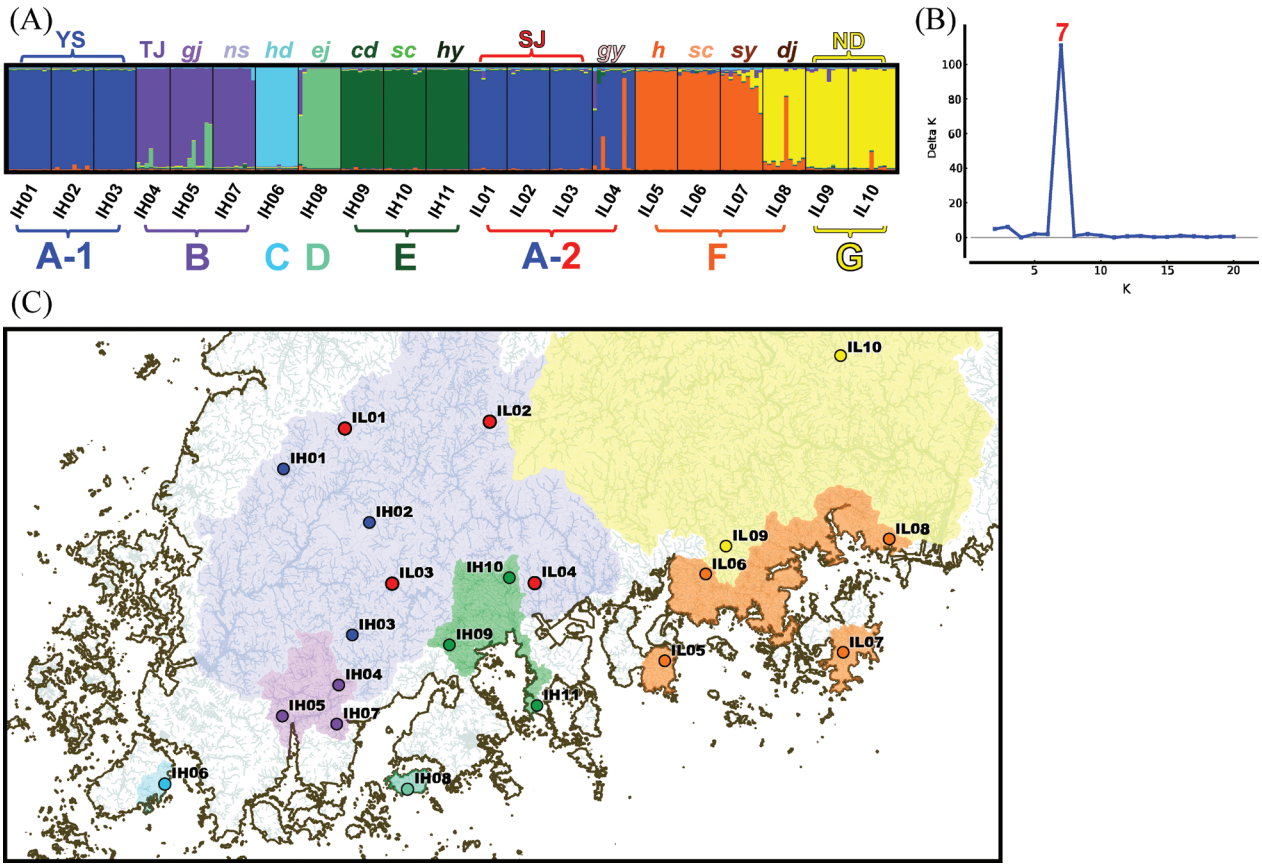


Figure 3. Results of genetic assignment analysis in STRUCTURE version 2.3.4 (Hubisz et al. 2009). **A.** STRUCTURE results for $K = 7$ genetically distinct clusters; **B.** the highest ΔK (= 7) obtained from the STRUCTURE harvester using the method reported by Evanno et al. (2005); and **C.** a map representing the estimated area of each genetic cluster from STRUCTURE. Vertical bars represent the probability of an individual being assigned to each cluster (individual membership coefficient, *q*-values; these also estimate percent ancestry), and clusters are assigned differently (see map and main text for additional details). Uppercase codes represent each river or stream site and correspond to those in Table 1. Uppercase, two-letter abbreviations represent the names of large rivers, while lowercase, two-letter abbreviations indicate offshore islands or short inland streams that are separate from the larger, main-channel rivers. The map was created using QGIS (Congedo 2021).

0.002, a difference that was significant in the corresponding Student's *t*-test ($p = 7.02 \times 10^{-12}$) (Table 4). Similarly, for the Cyt *b* gene, the average pairwise genetic distance between the seven clusters was 0.053 (s.d. = 0.021), while the distance between subclusters A-1 and A-2 was significantly shorter at 0.016 ($p = 1.02 \times 10^{-7}$) (Suppl. material 8). However, because subclusters A-1 and A-2 were assigned to the same cluster in the STRUCTURE analysis (Fig. 3A), we classified the two subclusters as part of a larger cluster (cluster A), despite the gene tree and haplotype network for Cyt *b* indicating a distinction between them (Figs 4B, 5B). The nDNA and Cyt *b* gene trees also indicated that the two sister species do not form mutually exclusive monophyletic groups (Fig. 5, Suppl. material 7). Representative fish images from the genetic clusters were shown in Fig. 5C as visual references. The pattern of dark stripes and spots varied among the clusters with a variable degree.

Analysis of the geographic distribution of the genetic variation also raised doubts about the nominal taxonomic status of *I. hugowolfeldi* and *I. longicorpa*. The results from the AMOVA indicated that the seven distinct genetic clusters represented more biologically meaningful units of variation than the traditional taxonomic distinction between the two species (Table 5). To conduct an AMOVA, three different grouping methods were utilized: the current taxonomy of the two species, the river drainage systems within the study area, which includes 16 isolated rivers and streams (Fig. 2, Table 1), and the seven genetic clusters inferred from nDNA analysis using STRUCTURE and supported by phylogenetic nDNA and mtDNA analysis (Table 3). These comparative analyses revealed significant differences in the among-group variation between the grouping methods. When individuals were grouped by species, the partitioning of among-group variation was lowest, with values of 9.6% for nDNA and 18.8% for Cyt *b*. In contrast, grouping individuals by the 16 river drainage systems resulted in the highest levels of among-group variation, with values of 58.4% for nDNA and 94.0% for Cyt *b*. Grouping individuals into the seven clusters yielded among-group variation values of 55.3% for nDNA and 79.1% for Cyt *b*, which were similar to the results obtained from the 16 drainage systems. The analysis revealed that the seven clusters accounted for most

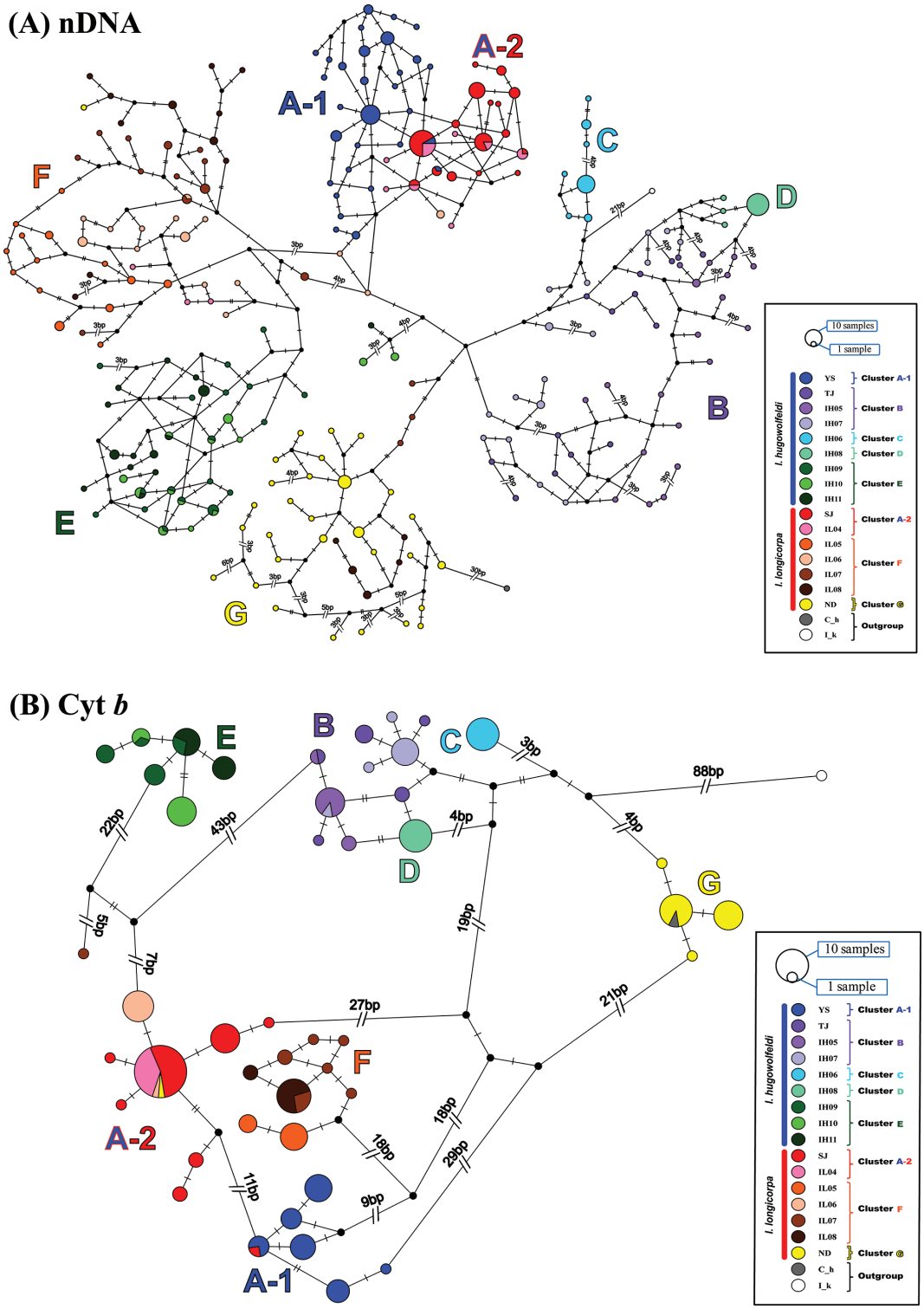


Figure 4. Haplotype network results. **A.** concatenated nuclear DNA data (nDNA) haplotype network; and **B.** mitochondrial Cyt *b* gene haplotype network. The colors represent each river or stream and correspond to the genetic cluster and drainage system colors in Fig. 3A. The size of the circles is proportional to the haplotype frequency. C_h, *Cobitis hankugensis*; I_k, *Iksookimia koreensis*.

of the genetic variance observed between the two sister species distributed across the different drainage systems. The lowest F_{ST} values were observed between the populations of the Yeongsan and Seomjin Rivers, indicating that even geographically adjacent drainage systems can exhibit low genetic differentiation. This pattern challenges the validity of the current taxonomic boundaries between *Iksookimia hugowolfeldi* and *I. longicorpa*, suggesting that drainage-based genetic structure may better reflect evolutionary history. Similarly, geographically adjacent pairs of main rivers and small tributary streams exhibited low F_{ST} values; for example, the F_{ST} between IH04 and IH07 was 0.022 for nDNA and 0.137 for Cyt *b*, while that between IL02 and IL04 was 0.101 for nDNA and 0.000

Table 4. Pairwise genetic distances for seven distinct genetic clusters of *Iksookimia* and Student's *t*-test results for concatenated nDNA data. **A.** Genetic distances between clusters based on the Kimura two-parameter model of sequence evolution; **B.** Student's *t*-test results. Genetic distances between the clusters indicated as subclusters A-1 and A-2 were used as the hypothesized mean during the *t*-test analysis.

Genetic distances between seven distinct genetic clusters							
	A	**B**	**C**	**D**	**E**	**F**	**G**
A	–						
B	0.00664	–					
C	0.00727	0.00547	–				
D	0.00676	0.00463	0.00359	–			
E	0.00770	0.00893	0.00981	0.00934	–		
F	0.00547	0.00730	0.00846	0.00793	0.00709	–	
G	0.00842	0.00993	0.00986	0.00953	0.01030	0.00787	–

Student's *t*-test results	
Parameter	**Result**
mean	0.00773
Variance	0.00000
standard deviation	0.00185
Observations	21
Hypothesized Mean	**0.00201**
t	14.15034
P(T<=*t*) *p*-value (one-tailed)	**0.00000**
t critical value (one-tailed)	1.72472

Table 5. Analysis of molecular variance (AMOVA) for the two *Iksookimia* species according to three grouping methods: nominal species ("Species"), 16 river drainage systems ("Drainage system"), and seven genetic clusters ("Clusters"). The genetic cluster grouping configuration was determined and justified based on our STRUCTURE, phylogenetic tree, and haplotype network results.

	nDNA (three concatenated nuclear DNA loci)			**Cyt *b***		
	Species (2)	**Drainage system (16)**	**Clusters (7)**	**Species (2)**	**Drainage system (16)**	**Clusters (7)**
Among groups (φ_{CT})	9.63	**58.44**	**55.29**	18.75	**93.97**	**79.11**
Among populations within groups (φ_{ST})	54.22	**4.43**	**10.24**	76.83	**1.33**	**16.65**
Within populations (φ_{ST})	36.15	**37.14**	**34.47**	4.41	**4.70**	**4.24**

for Cyt *b* (Suppl. material 11). In contrast, geographically isolated drainage systems acted as a source of diversification for the two species. This was supported by the relatively high pairwise F_{ST} values inferred from the nDNA and mtDNA markers between populations located in different rivers and streams (Suppl. material 10).

A significant relationship between the genetic differentiation of the populations and the geography of the drainage systems was also observed in the IBD analysis. The results of IBD tests for GPS distance and river–ocean distances are presented in Suppl. materials 12, 13. IBD was observed in both nDNA and Cyt *b* markers and across both distance measures, though the correlation coefficients were relatively low (Suppl. materials 12, 13). The Pearson's and Mantel's correlation coefficients between F_{ST} and the geographic distance were statistically significant for all cases ($p < 0.05$) except for nDNA analysis using the river–ocean distance (Mantel's $r = 0.158$; $p = 0.07$) (Suppl. material 13). Notably, GPS distance consistently yielded higher correlations than river–ocean distance; in particular, for nDNA, Mantel's r was 0.279 ($p = 0.004$), while it was 0.284 ($p < 0.001$) for Cyt *b*.

NMDS plots indicated that the current taxonomic classification does not accurately distinguish between the two sister species and their populations (Suppl. material 14). Notably, some *I. hugowolfeldi* populations were found to be genetically more similar to certain *I. longicorpa* populations than to other *I. hugowolfeldi* populations. NMDS analysis of nDNA revealed clusters of populations that were fairly evenly distributed, resembling the seven clusters, with a significant differentiation observed between sub-clusters A-1 and A-2 and between clusters F and G (Suppl. material 14A). This pattern may arise from the frequency-based approach employed for F_{ST}, which considers haplotype frequencies between populations without accounting for genetic distances. Conversely, the mtDNA results were partly inconsistent with those of nDNA, indicating a possible signature of past introgression of mtDNA (Suppl. material 14B).

Analysis of the genetic diversity among the 21 *Iksookimia* populations revealed several noteworthy patterns. First, there was no significant correlation between genetic diversity and either the species or stream size (Suppl. materials 15–17). Secondly, populations located

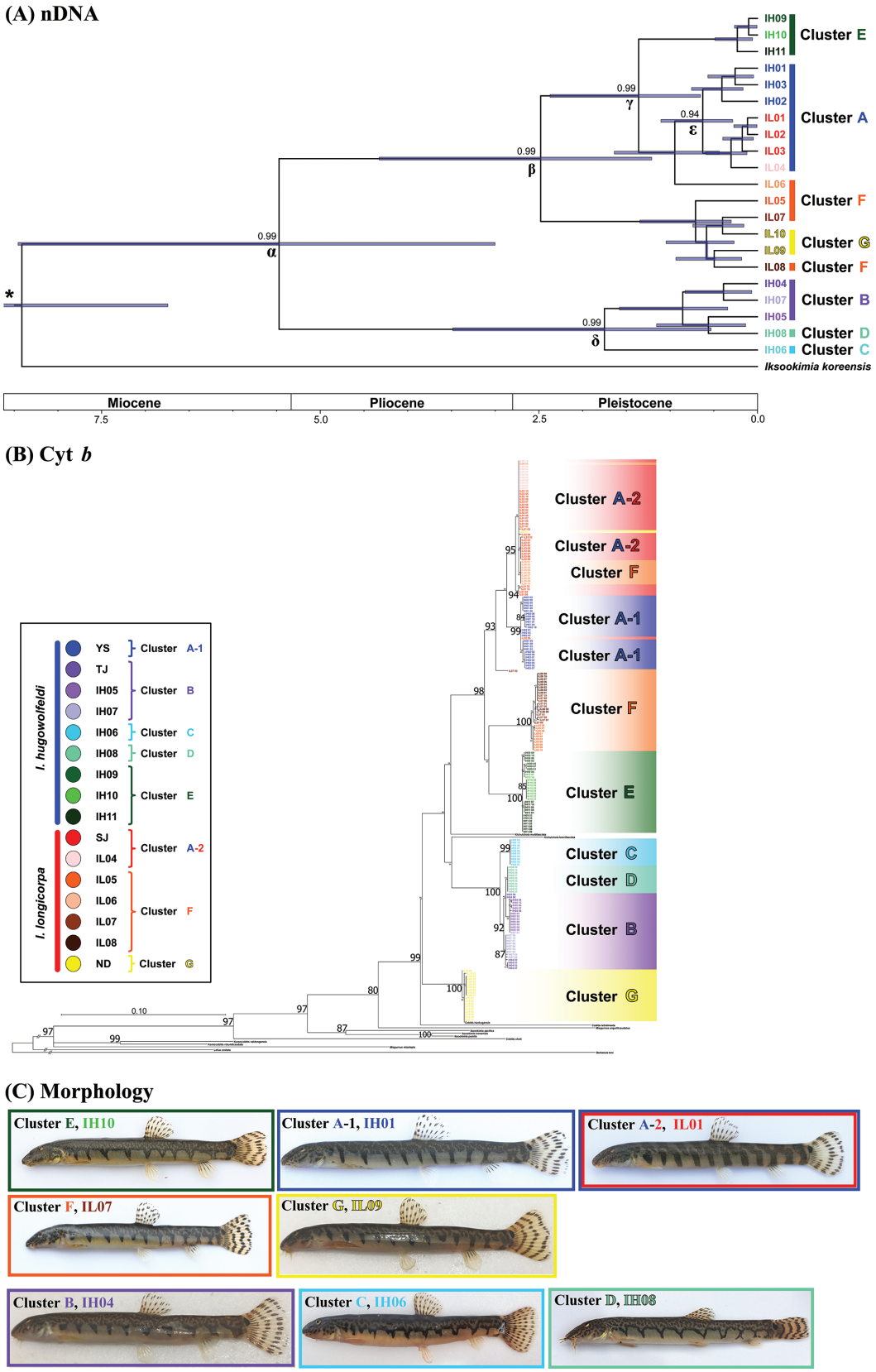


Figure 5. Rooted phylogeny of the study taxa. **A.** *BEAST time-calibrated concatenated species-tree based on three nuclear genes; **B.** maximum-likelihood phylogeny based on a mitochondrial Cyt *b* gene tree; **C.** representative photos of each cluster, with the border color of the photos indicating the color of each cluster. Clusters and sets of individuals in the trees are colored according to the seven genetic clusters identified in STRUCTURE (Fig. 3A), and black codes indicate outgroup species. For additional details, see the main text and color coding in Figs 3, 4.

on offshore islands generally exhibited lower genetic diversity, with the exception of population IL07, which is located on Geoje Island, the second-largest island in Korea. Despite being an island population, IL07 exhibited high genetic diversity. Thirdly, based on Cyt *b* data, IL09 had the highest nucleotide diversity, but its haplotype diversity was relatively low (Suppl. materials 15, 16). This unique characteristic was not observed in the nDNA data for IL09. The Cyt *b* phylogenetic tree indicated that, of the nine individuals in the IL09 population, eight contained mtDNA from *Cobitis hankugensis*, while one individual had mtDNA from *I. longicorpa*. Notably, in the IL10 population, all individuals possessed the Cyt *b* haplotype of *C. hankugensis*, providing an even more striking case of mitochondrial introgression. This strongly suggests ancient mitochondrial introgression from *C. hankugensis* to *I. longicorpa*. Therefore, employing mtDNA genes as genetic markers to study the phylogeny and population genetics of cobitid fish requires careful consideration and the concurrent use of nDNA.

To clarify the evolutionary relationships between the geographical populations and distinct genetic clusters, we examined phylogenetic trees using both nDNA and mtDNA data. A key finding from this phylogenetic analysis was that the seven genetic clusters formed distinct groups that were mostly mutually exclusive (Fig. 5), though there were a few exceptions. Another important finding was that, except for those lineages with taxonomic issues (specifically the Yeongsan River populations), the most deeply divergent lineages from the node of α descended to the two taxonomic species (*I. longicorpa* and *I. hugowolfeldi*) in the nDNA tree (Fig. 5A). However, as mentioned above, the Cyt *b* tree positioned the majority of cluster G at the outgroup location from the two taxonomic species due to the ancestral introgression of *Cobitis hankugensis* (Fig. 5B). The two lineages representing the two sister species of *Iksookimia* in the nDNA tree divided the fish populations and genetic clusters into two groups: clusters A, E, F, and G corresponding to *I. longicorpa* and clusters B, C, and D corresponding to *I. hugowolfeldi* (Fig. 5A). The results for the phylogenetic analysis thus aligned well with the STRUCTURE and haplotype network findings. According to the results from *BEAST, based on the three nuclear genes, the divergence of the 21 populations or seven clusters was estimated to have occurred during the Miocene and Pleistocene periods over approximately 7 million years (Fig. 5A, Table 2). The estimated divergence time between the Yeongsan River groups (IH01–IH03, subcluster A-1) and the Seomjin River groups (IL01–IL03, subcluster A-2), both of which belonged to cluster A, was 0.63 MYA. This time was significantly more recent compared to the estimated divergence times among the seven clusters, which ranged from 5.47 MYA to 0.56 MYA. Additionally, the estimated divergence time between two main lineages leading to the clusters B, C, and D and the other clusters was approximately 5.47 MYA, with a confidence interval of 8.46–3.00 MYA (95% HPD, node α; Fig. 5A and Table 2). This divergence occurred during the Miocene to Pliocene period, leading to the speciation of *I. longicorpa* and *I. hugowolfeldi*.

Discussion

The present study investigated the population genetics and phylogenetics of two closely related freshwater loach species, *I. hugowolfeldi* and *I. longicorpa*, found in the southern Korean Peninsula. Based on extensive population sampling and comparative genetic analysis, we made several unexpected discoveries. The most significant finding was that we identified discrepancies between the taxonomy and genetic identification of *I. hugowolfeldi* populations. In particular, populations IH01–03 in the Yeongsan River, which flows into the Yellow Sea, and populations IH09–11 from the independent Childong, Suncheondong, and Hwayang Streams, which flow into Yeoja Bay in the South Sea of Korea, were conclusively identified as *I. longicorpa* (Figs 2, 3). In contrast, *Iksookimia* sp., which was previously regarded as *I. hugowolfeldi*, was exclusively found in populations IH04–08 in the Tamjin River, Jin Island, Geogeum Island, and the two nearby independent streams Gangjin and Namsang (Figs 2, 3). As a result, the previously assumed allopatric distribution boundary between these two species requires revision; it should now be drawn at the southwestern tip of the Korean Peninsula.

Taxonomic interpretation and genetic relationships

Freshwater loaches in the *Iksookimia* genus from southern South Korea were initially classified as *Cobitis taenia* (Uchida 1939). However, subsequent taxonomic revisions led to significant changes in their classification. In 1976, these loaches in South Korea were reported to be the new species *C. longicorpus* (Kim et al. 1976). Nalbant (1993) subsequently proposed *Iksookimia* as a new genus and reclassified *C. longicorpus* from the Yeongsan River as a distinct species based on morphological differences, naming it *Iksookimia hugowolfeldi* (Nalbant 1993; Kim 2009). The *C. longicorpus* populations from other rivers were reclassified as *Iksookimia longicorpa*. Other studies have found that *I. hugowolfeldi* also inhabits the Tamjin River and several nearby independent small streams (Park and Kim 1996; Kim 1997; Kim et al. 2003; Park et al. 2018).

The taxonomic status of the genus *Iksookimia* is a topic of ongoing debate; however, it is widely recognized as an endemic genus in Korea by local ichthyologists and some international experts familiar with this group. Since Nalbant (1993) established *Iksookimia* as a distinct genus separate from *Cobitis*, species within this group

have consistently been referred to as *Iksookimia* (Nalbant 1993; Kim 2009; Kwan et al. 2018; Kim and Park 2020; Kim et al. 2020a). While some studies have questioned this classification, they often rely solely on mitochondrial data (Chen and Chen 2016) or a single nuclear gene (Šlechtová et al. 2008), which are insufficient for resolving the taxonomic status of *Iksookimia* in relation to *Cobitis*. The issue is particularly significant in the family Cobitidae, where mitochondrial introgression complicates genus-level taxonomic conclusions (Šlechtová et al. 2008; Kwan et al. 2019; Lee et al. 2022). To maintain consistency and avoid confusion, this study will use the name *Iksookimia* throughout. A more detailed discussion of this taxonomic issue is beyond the scope of this paper and deserves separate examination.

Iksookimia hugowolfeldi and *I. longicorpa* are currently classified based on various morphological characteristics, including body pigmentation, the lamina circularis, and diagnostic morphometric traits such as differences in standard length, body depth, and eye diameter (Nalbant 1993; Kim 2009). The lamina circularis is a bony plate located at the base of the pectoral fin in adult male Cobitidae fishes (Erkakan et al. 1998) and is one of the most important characteristics for classifying Cobitidae species (Nakajima and Suzawa 2016). The two sister species can typically be differentiated by their body pigmentation, with the first spot of *I. longicorpa* darker than the other spots, whereas *I. hugowolfeldi* lacks this prominent dark first spot (Fig. 1) (Nalbant 1993; Kim 1997). *Iksookimia hugowolfeldi* also exhibits a sharper tip spot pattern than *I. longicorpa* on the lateral (Fig. 1) (Nalbant 1993; Kim et al. 2005; Kim 2009). In addition, *I. longicorpa* has a significantly larger adult body size than *I. hugowolfeldi*, other species within the genus, and members of the sister genus *Cobitis* (Kim 2009). However, identifying *I. hugowolfeldi* and *I. longicorpa* in the field, particularly in the islands and coastal independent streams, has been difficult, leading researchers to classify them based on an assumed geographic species boundary (as marked by the green dotted line in Fig. 2).

Morphological features have historically played a crucial role in species classification (Gams and Bissett 2002; Will and Rubinoff 2004; Kruk et al. 2010). However, the traditional morphology-based approach to fish taxonomy faces significant challenges due to the presence of multiple morphs within a single species and cryptic species that closely resemble their sister species. This issue is particularly problematic among freshwater fish (Miner et al. 2005; Lara et al. 2010; Stevens 2016; Dick et al. 2018; Pereira et al. 2021).

Our genetic analysis highlights a significant discrepancy between the taxonomic classification and genetic identity of *I. hugowolfeldi* and *I. longicorpa*. This discordance is most evident in the Yeongsan (A-1) and Seomjin River (A-2) populations, the type localities of the two species (Figs 3–5). Despite their current classification as separate species, these populations exhibit minimal genetic differentiation, questioning the validity of their taxonomic distinction (Table 4, Suppl. material 8, Fig. 4). Their relatively recent divergence (Fig. 5, Table 2) further supports this ambiguity. STRUCTURE analysis ($K = 7$) and phylogenetic trees (nDNA and mtDNA) indicate that these species do not form monophyletic groups but instead exhibit intermixed lineages with para- and polyphyletic patterns (Figs 3, 5). This implies that current classification based on morphology and assumed species boundaries of *I. hugowolfeldi* and *I. longicorpa* (Fig. 2) can mislead our understanding of their evolutionary history. These findings emphasize the need for a revised taxonomic framework that integrates molecular evidence to better reflect the natural diversification of these loaches.

The nDNA phylogeny revealed two distinct branches. Although *I. longicorpa* and *I. hugowolfeldi* are currently recognized as distinct species based on morphological traits (Nalbant 1993), our results suggest that this taxonomic distinction is not fully supported by genetic evidence. The tree contains two deep branches split from node α, leading to the two sister species; one branch for *I. longicorpa* contains clusters A, E, F, and G, while the other branch for *Iksookimia* sp. contains clusters B, C, and D (see α in Fig. 5). The estimated divergence time for these two branches was 5.47 MYA (8.46–3.00 MYA, 95% HPD; Fig. 5A, Table 3), which is comparable to divergence times of about 3 to 5 MYA estimated for individual species within the genera *Iksookimia* and *Cobitis* from their respective sister species in Korea (see Table 1 and Fig. 2 in Kwan et al. 2018). The type locality of *Iksookimia hugowolfeldi* is the Yeongsan River (Nalbant 1993), which corresponds to cluster A in our phylogenetic tree. Since cluster A is genetically indistinguishable from populations currently assigned to *I. longicorpa*, we propose synonymizing *I. hugowolfeldi* in the Yeongsan River with *I. longicorpa* (Fig. 3). Conversely, clusters B, C, and D (populations IH04–08) represent a distinct lineage and cannot be confidently regarded as conspecific with the type material of *I. hugowolfeldi* (Figs 2–5, Table 1). Therefore, we recommend that these populations be described as a new species,*Iksookimia* sp., in future taxonomic revisions.

Using evidence-based taxonomy along with morphological comparison will be essential for accurately identifying and successfully conserving the two *Iksookimia* species and their unique phylogenetic lineages in the future. This approach will help ensure the protection of the distinct biodiversity that these species represent. The present genetic results also indicate that the degree of difference in morphological traits used to distinguish the two *Iksookimia* species may be influenced by environmental factors instead of reflecting genuine evolutionary differences. In addition, this study demonstrates that limited geographical sampling for molecular and morphological analyses may lead to the misclassification of closely related fish species. Therefore, morphological studies for species identification purposes should be thorough, with sufficient sampling across the entire geographical range of each species.

Biogeography

Historical biotic and abiotic influences can leave a biogeographical signature on living organisms in the form of patterns of genetic variation and isolation across geographical barriers (Barker et al. 2002; Gilbert et al. 2006; Jeon and Suk 2023). Based on the genetic data and results, we identified four major biogeographical and environmental factors influencing the genetic structure and divergence processes for the 21 populations: 1) the isolation of rivers inland and due to intervening saltwater habitats between river mouths, 2) separation of offshore islands from the mainland by marine barriers, 3) connection and disconnection between rivers via the paleo-river systems of East Asia, and 4) habitat overlap with other cobitid species. It is reasonable to conclude that these geographical barriers have similarly impacted the phylogeography of other freshwater fish species in the southern part of the Korean Peninsula. This study not only offers valuable insights into the population genetics of endemic loach species but also provides a model for understanding evolutionary processes in freshwater fish, particularly in regions shaped by the same palaeo-river systems. The ability of fish to move between habitats within the contemporary and paleo-river systems and the geographical distance between these habitats also likely affected gene flow and shaped the observed genetic patterns (Willis et al. 2010; Cooke et al. 2012; Jeon and Suk 2023). These factors explain why island populations of the two species are genetically similar to nearby mainland populations (Fig. 3C, Table 3, Suppl. material 11).

It is commonly observed among vertebrates that populations that are geographically closer will tend to have a smaller genetic distance (Selander and Johnson 1973; Cavalli-Sforza et al. 1994; Choi et al. 2014). Freshwater fish populations are excellent examples of this IBD because they are typically restricted to isolated rivers or streams (Durand et al. 2005; Kim et al. 2013). Numerous studies have reported that geographically distinct river drainage systems serve as dispersal barriers, resulting in the genetic isolation of freshwater fish (Radinger and Wolter 2015; Crookes and Shaw 2016; Radinger et al. 2017; Jeon et al. 2022; Jeon and Suk 2023). In this study, the genetic analysis revealed a significant isolating effect of the 16 river drainage systems in southern Korea. Individuals within the same genetically distinct clusters identified using STRUCTURE and phylogenetic analyses (Figs 2–5) were primarily found within the same main-channel river or other geographically proximate smaller rivers (Figs 3C, 6, 7). Furthermore, genetic and phylogenetic analyses identified seven genetic clusters, which were interpreted to be descendant lineages arising from radiative evolutionary divergence from a common ancestral lineage (Figs 2–5, Table 4, Suppl. material 7).

Notably, the genetic distance between pairs of genetic clusters correlated with the geographic location of the clusters. For example, the distance from the mouth of the river containing cluster F to the mouth of the Seomjin River, which contained cluster A, was shorter than the distance to the mouth of the Tamjin River, which harbored cluster B. Cluster F was consequently found to have a closer genetic relationship to cluster A than cluster B (Figs 2–5).

It is important to note that it is almost impossible for freshwater fish to disperse across the land or sea directly without utilizing drainage systems (Olden et al. 2011; Jeon and Suk 2023). Thus, ancient fish populations may have dispersed through paleo-drainage systems formed by the connection of several drainage systems (De Bruyn et al. 2013; Won et al. 2020; Jeon and Suk 2023). These past geographical factors may have influenced the genetic relationship among fish populations, in addition to contemporary isolation caused by the current river drainage systems on land. The coastlines of the Korean Peninsula have continuously changed since the Pleistocene due to the fluctuation of the sea level during ice ages (Dumont 2022) (Fig. 6). These changes have led to the repeated connection and disconnection of the southern Korean Peninsula, the Chinese continent, and the Japanese archipelago (Lee and Nam 2003; Won et al. 2020) (Suppl. material 20). The changes in habitable areas caused by sea level fluctuations have greatly affected the fauna of Northeast Asia, including the well-known historical migration of Japanese ancestors along with other mammals such as mammoths, ancient extinct rhinoceroses, and wild boars (Dobson 1994; Hammer et al. 2006; Choi et al. 2020; Ma et al. 2021). Notably, Sun et al. (2025) documented the presence of *Cobitis choii* in both the Miho Stream of Korea and the Luanhe River in China. These two river systems converge in the Yellow Sea, providing potential pathways for species dispersal. During the ice ages, lower sea levels caused most rivers and streams in southern Korea to extend in length and merge into the expansive paleo-Yellow River system (Park et al. 2000; Lee and Nam 2003; De Bruyn et al. 2013; Kwan et al. 2015; Won et al. 2020). Additionally, some rivers flowing south from Korea could have connected with those flowing north and west from the Kyushu region of Japan when the sea levels reached their lowest point. These historical changes may have facilitated the dispersal of freshwater fish to new habitats and the expansion of their ranges (Son and Song 1998; Lee and Nam 2003; Jeon et al. 2020; Taniguchi et al. 2021). The historical reorganization of river systems could explain the basic genetic patterns observed among the populations in the isolated rivers in this study.

An interesting example of this was observed in the Sachen River population IL06, which is located between the Seomjin and Nakdong Rivers (Fig. 2, Table 1). Although the sampling site on the Sacheon River was closer to the Nakdong River in terms of land distance than to any other river or stream, it is not currently connected to the Nakdong River. On the other hand, the Sacheon River estuary is closer to the Seomjin River estuary than the Nakdong River estuary (Figs 2, 7B, C). As expected, the genetic makeup of the IL06 population was found to be more

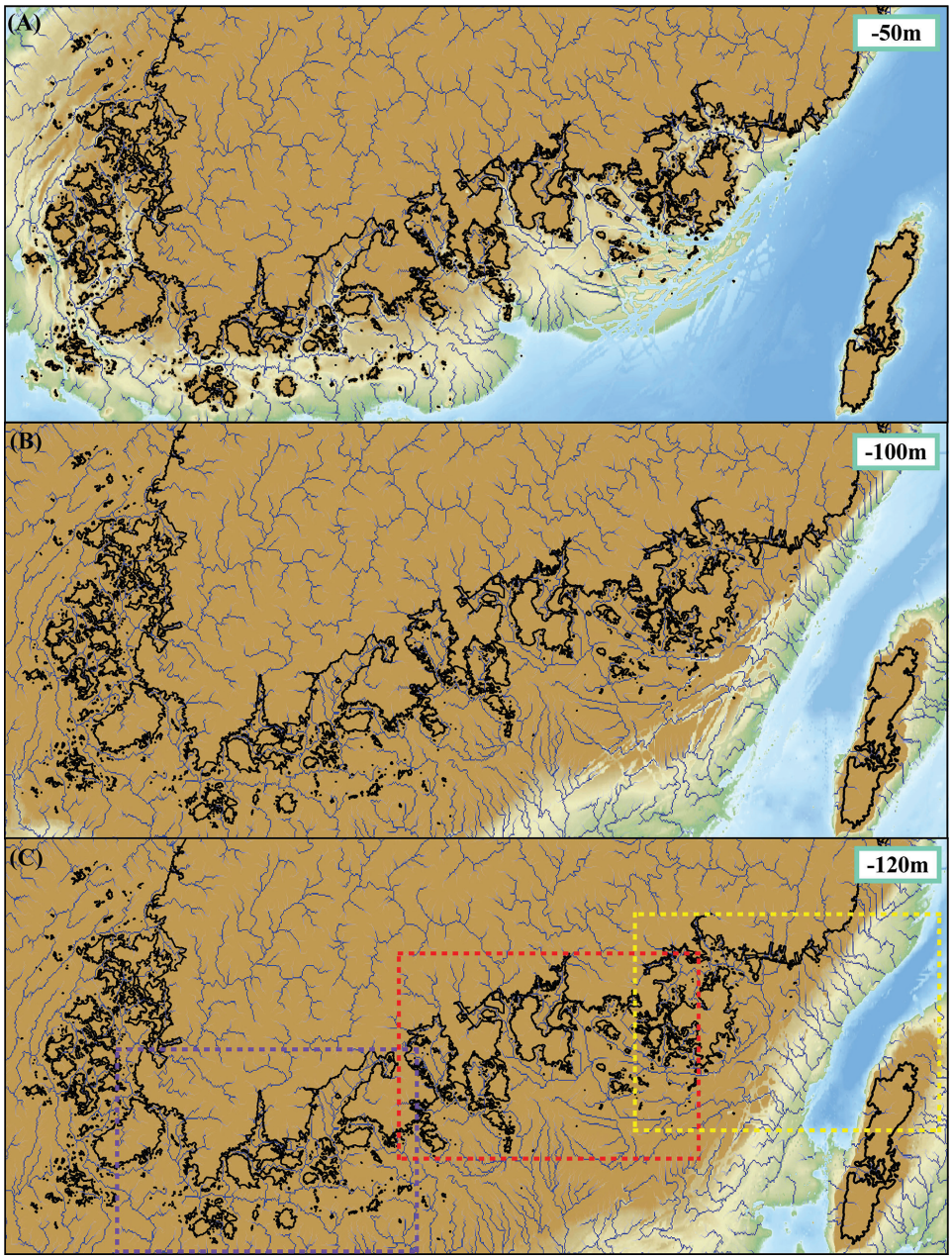


Figure 6. Results from simulations of changes in the coastline and paleo-drainage systems with sea level variation conducted in QGIS (Congedo 2021). The black line shows the current coastline of South Korea and Japan at multiple sea levels, including **A.** sea level 50 m lower (–50 m), **B.** 100 m lower (–100 m), and **C.** 120 m lower than present (–120 m). The areas within the purple, red, and yellow dotted lines are linked to the areas in Fig. 7. The grid file used to build the seabed was downloaded from GEBCO and can be accessed at https://download.gebco.net (Mayer et al. 2018).

similar to that of the Seomjin River than the Nakdong River (Figs 4, 5). Moreover, the river mouth for population IL06, which was in cluster F, was found to be closer to small rivers containing other populations in cluster F than to the Nakdong River (Figs 2–7).

One surprising discovery in this study was that the fish populations in the Yeongsan and Seomjin Rivers both belonged to cluster A despite being classified as different species (*I. hugowolfeldi* and *I. longicorpa*, respectively) (Figs 3–5). The reason for this is not entirely clear, but it is difficult to believe that paleo-drainage systems were responsible. The estuaries on these rivers are quite distant from each other, especially compared to other rivers (Fig. 2). For example, the Yeongsan River estuary is closer to the Tamjin River than the Seomjin River, yet the genetic distance from the Tamjin River population was much higher than that from the Seomjin River (Figs 2–5).

It is worth noting, however, that the headwaters of the Yeongsan (blue) and Seomjin (red) are located very close to each other in deep inland (Fig. 8). This led us to speculate that there might have been a river capture event in the past somewhere in the upper regions of these two rivers. During this event, freshwater fish could have spread from one river to the other without passing

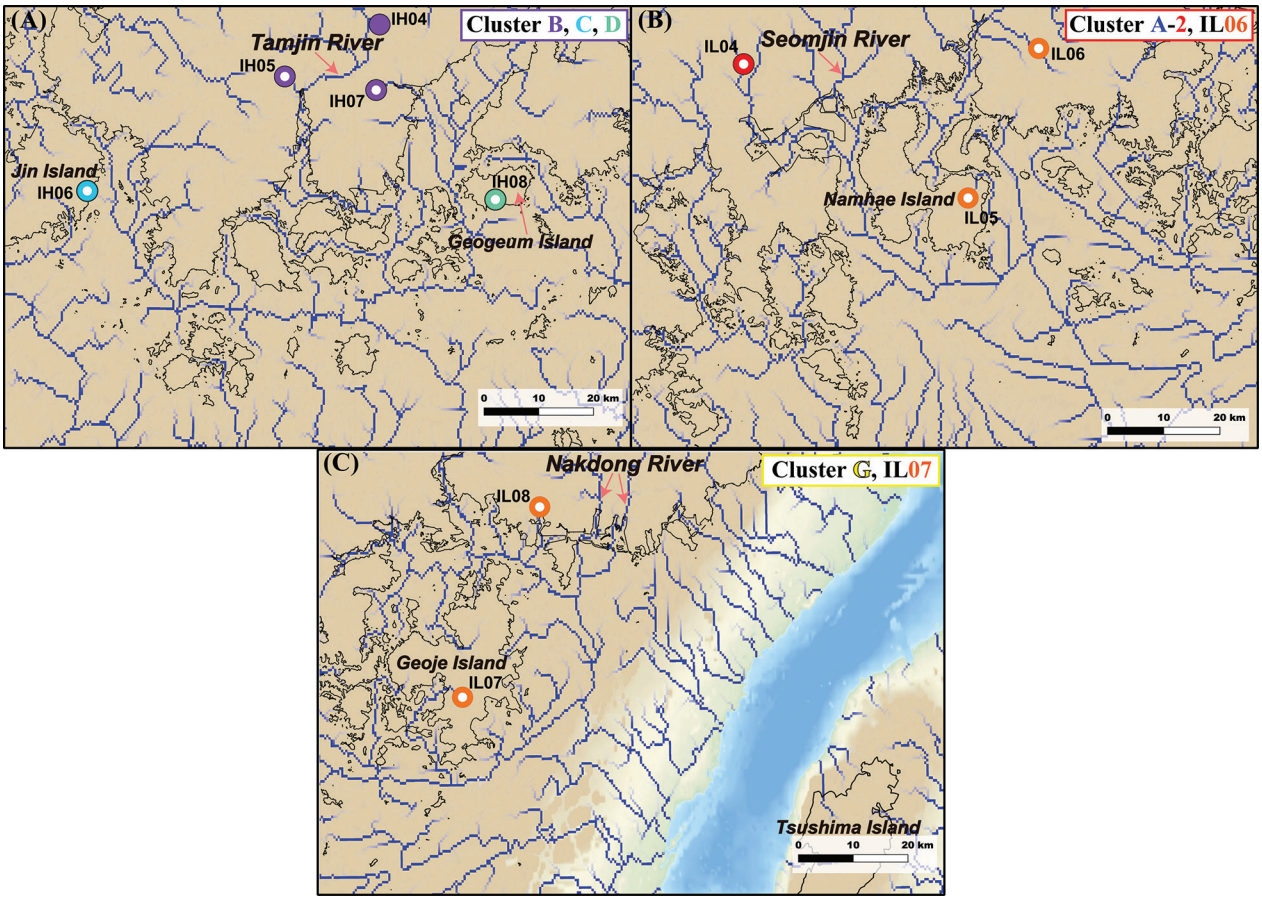


Figure 7. Results from simulations of major local regions when the sea level is 120 m lower than present, conducted in QGIS (Congedo 2021). **A.** area inside the purple dotted line in Fig. 6; **B.** area inside the red dotted line in Fig. 6; and **C.** area inside the yellow dotted line in Fig. 6.

through paleo-drainage systems during an ice age. River capture is a geological phenomenon that occurs when one river absorbs the tributaries of another due to a difference in altitude and erosion power between two adjacent rivers (Morisawa 1989; Bishop 1995; Aslan et al. 2005). When river capture occurs, the tributary being absorbed may introduce new gene pools or species (Albert et al. 2018; Waters et al. 2020). There are several areas located between the Yeongsan and Seomjin Rivers where the distance between the upper tributaries of the rivers is less than 2 km (Fig. 2). According to Kang (2004) and Son (2014), two areas between the Yeongsan River and the Seomjin River were identified as possible sites where river capture via geologic erosion was suspected (Fig. 8). Therefore, it is possible that the founding ancestors originated in the Seomjin River and spread through connected upper tributaries to the Yeongsan River, or the reverse could also be probable. However, considering the difference in altitude between the potential sites for river capture, it seems more likely that the flow was from the Seomjin River toward the Yeongsan River.

Typically, the genetic diversity of island-bound animals tends to be lower than that of mainland populations (Jones et al. 2004; White and Searle 2007; Wang et al. 2014). In line with this, our analysis indicated that the genetic diversity of the populations on islands was generally lower than those on the mainland, as seen in populations IH06, IH08, and IL05 (Suppl. materials 15–17). An exception to this, however, was observed for population IL07 on Geoje Island. Geoje Island, the second-largest island in Korea, is only around 740 m from the mainland at its closest point (Fig. 7C) (Son and Song 1998; Song and Jung 2015). Its proximity and large size likely explain the higher genetic diversity, which is consistent with findings that larger and more proximate islands tend to exhibit greater genetic diversity (Stuart et al. 2012; Wang et al. 2014; Portillo et al. 2019). Indeed, a previous study by Son and Song (1998) highlighted the diverse fish fauna on Geoje Island. The relatively large size of Geoje Island may have contributed to a larger effective population size, supporting a higher genetic diversity. The preservation of ancient phylogenetic lineages from various water sources through the reconnection of paleo-drainage systems is another plausible explanation (Kim et al. 2013). The southern Korean Peninsula was historically connected to the Chinese continent and the Japanese archipelago via land bridges formed during ice ages since the Pleistocene (Fig. 6, Suppl. material 20) (Maruyama et al. 1997). These land bridges created paleo-drainage systems, which are now submerged by seawater, and connected isolated rivers (Yoo et al. 2016). These historical reconnections may have

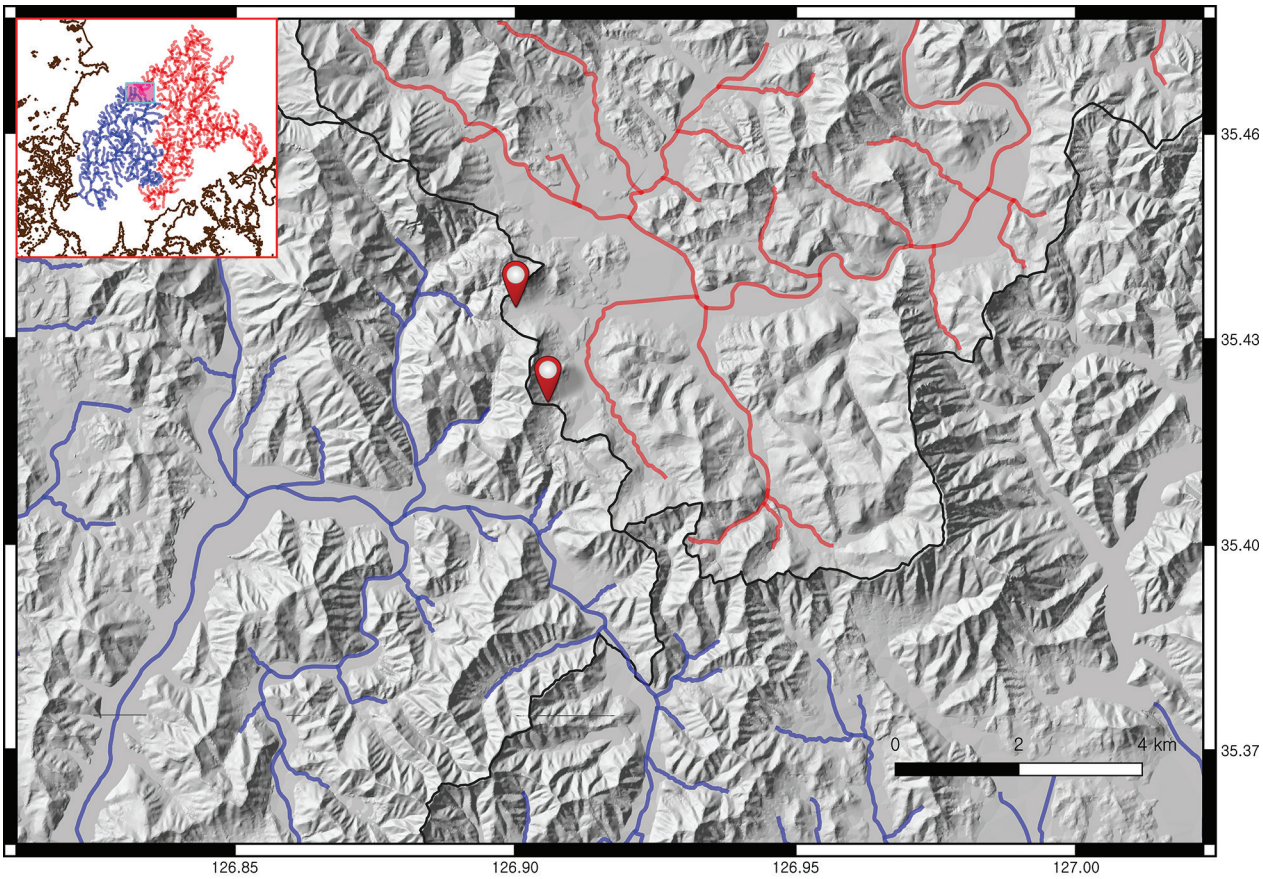


Figure 8. Candidate area for river capture. Location markers represent the possibilities suggested by Kang (2004). The map was created using QGIS (Congedo 2021). The black line shows the boundaries of three cities in Korea. Blue line, Yeongsan River (YS); Seomjin River (SJ). The Grid file used to build this map was downloaded from the National Land Information Platform of the National Geographic Information Institute in Korea and can be accessed at https://map.ngii.go.kr/mn/mainPage.do

influenced the current ichthyofaunal diversity and distribution in the southern Korean Peninsula (Kim 1997; Son and Song 1998; Taniguchi et al. 2021). Geoje Island may thus have been connected to the Korean Peninsula and its rivers to other mainland rivers during ice ages (Kim 1997; Maruyama et al. 1997; Son and Song 1998).

The overlap of *I. longicorpa* habitat with that of other cobitid fish, including *Cobitis hankugensis*, in the Nakdong River and other adjacent small rivers, appears to have influenced the sequences for population IL09. Of the nine individuals in the IL09 population, eight exhibited mitochondrial haplotypes identical to those of *C. hankugensis*, while the other individual had mitochondrial haplotypes from *I. longicorpa* (Fig. 5B). Consequently, population IL09 exhibited the highest nucleotide diversity (Suppl. material 15A, B), although its haplotype diversity was relatively low. The presence of *C. hankugensis* mtDNA in the eight IL09 individuals was attributed to mitochondrial introgression mediated by interspecific hybridization between *I. longicorpa* and *C. hankugensis* where their habitats overlap. In addition, individuals with the mitochondrial genes of *C. hankugensis* have been found among *I. longicorpa* from several sites along the Nakdong River in past studies (Saitoh et al. 2004; Kwan et al. 2019).

Patterns of introgression can often complicate the understanding of the evolutionary history of cobitid fishes in mitochondrial analyses, even more so than in analyses using nuclear DNA (Kwan et al. 2019; Lee et al. 2022; Dedukh et al. 2024). For example, despite the presence of *C. hankugensis* mtDNA in the IL09 population, no nuclear gene sequences from *C. hankugensis* were observed in the nDNA sequences of *I. longicorpa* in the present study (Fig. 5A), which is in accordance with the findings reported by Kwan et al. (2019). This can be explained by the unique hybridogenetic reproductive mechanism observed in the diploid–triploid hybrid complex between these species (see Fig. 2 in Kwan et al. 2019). Because most hybrids are female, with all males being infertile (Park et al. 2011; Yun et al. 2021b), hybrids produce offspring by mating with males of their parental species as sperm donors. This reproductive mechanism results in mito-nuclear mosaic genomes in varying combinations, with either the nuclear genome of *I. longicorpa*–mitochondrial genome of *C. hankugensis* or the nuclear genome of *C. hankugensis*–mitochondrial genome of *I. longicorpa* depending on the demographic and ecological features of the secondary contact location (see Figs 2, 4 in Kwan et al. 2019). Introgression events have only

been observed in mtDNA and not in nDNA (Saitoh et al. 2010; Kwan et al. 2019). In population IL10 from the Nakdong River, all of the *I. longicorpa* individuals had the mtDNA of *C. hankugensis* (Fig. 5B), but no *C. hankugensis* sequence was detected in the nuclear genes (Fig. 5A). This suggests that habitat overlap between the two species has affected the mitochondrial Cyt *b* gene of the two IL populations sampled in the Nakdong River. Given the potential for mitochondrial introgression through interspecific or intraspecific hybridization, we prioritized the results from concatenated nDNA over those from Cyt *b* when determining distinct genetic clusters. This allowed for a clearer understanding of the genetic data presented here.

Conclusion

This study investigated the population genetic structure and molecular phylogeny of two closely related freshwater loach species, *Iksookimia hugowolfeldi* and *I. longicorpa*, throughout their distribution range on the southern Korean Peninsula. Our findings revealed that the current taxonomy does not accurately reflect their natural classification. Specifically, we challenged the traditional classification that is based solely on morphological traits or hypothetical geographic boundaries between the two species. The nDNA phylogenetic tree indicated the presence of two main lineages among our fish samples, which diverged approximately 5.47 million years ago, suggesting that there are indeed two distinct species. However, the mismatch between the taxonomy and nDNA tree was evident in the samples of *I. hugowolfeldi* collected from the Yeongsan River, Childong Stream, Suncheondong Stream, and Hwayang Stream, indicating that these should be reclassified as *I. longicorpa*. Furthermore, the nDNA tree suggests that *Iksookimia* specimens from the Tamjin River, Jindo Island, and Geogeumdo Island should be classified as a new species, *Iksookimia* sp. Population genetic analyses conducted on the sampled 21 populations led to several key findings: (i) potential historical headwater river capture events between the Yeongsan and Seomjin Rivers, (ii) the identification of seven distinct genetic groups influenced by geographic isolation and various dispersal barriers, and (iii) a strong relationship between genetic connectivity and ancient river connection among the population. Our paleo-river reconstructions indicate that this connectivity was facilitated by the formation of paleo-river systems during a glacial period, which was caused by a drop in sea levels that connected terrestrial and island habitats. This study emphasizes the critical role of paleo-river systems in promoting geographic connectivity among freshwater fish. The findings provide valuable insights into the phylogeography and evolution of other riverine organisms in the Korean Peninsula and East Asia.

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Supplementary material 1

Distribution of all *Iksookimia* species (modified from Fig. 1 in Kwan et al. (2018))

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: tif
Explanation note: The blue and red areas indicate the presumed habitat of *I. hugowolfeldi* and *I. longicorpa*, respectively. The other four species were not included in this study. The thick green line indicates that mountains played an important role in the differentiation of the genus *Iksookimia*.
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Link: https://doi.org/10.3897/zse.101.152180.suppl1

Supplementary material 2

Voucher accession numbers for the fish samples used in this study

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: xlsx
Explanation note: These numbers were available from the Molecular Ecology Laboratory, Division of Eco-Science, Ewha Womans University. Pop., population; N, number of samples.
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Link: https://doi.org/10.3897/zse.101.152180.suppl2

Supplementary material 3

PCR parameters

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: xlsx
Explanation note: **A.** primers used in this study; and **B.** temperature and time information for PCR amplification. *: Touch-down PCR (1 °C/cycle).
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Link: https://doi.org/10.3897/zse.101.152180.suppl3

Supplementary material 4

Best-fit models

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: xlsx
Explanation note: A. model computing the pairwise evolutionary distances used in the phylogenetic analysis by MEGA X; and B. a substitution model used in the BEAST analysis.
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Link: https://doi.org/10.3897/zse.101.152180.suppl4

Supplementary material 5

Outgroup resources

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: xlsx
Explanation note: Outgroup sequences used to portray phylogeny and haplotype networks were downloaded from NCBI (National Center for Biotechnology Information) Gene Bank (https://www.ncbi.nlm.nih.gov/).
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Link: https://doi.org/10.3897/zse.101.152180.suppl5

Supplementary material 6

Haplotype network for each of the three nDNA genes

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: tif
Explanation note: **A.** EGR2B; **B.** RAG1; and **C.** SH3PX3. The colors represent each river or stream and correspond to the drainage system in Fig. 3A. The size of the circles is proportional to the haplotype frequency. C_h, *Cobitis hankugensis*; I_k, *Iksookimia koreensis*.
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Link: https://doi.org/10.3897/zse.101.152180.suppl6

Supplementary material 7

Rooted maximum-likelihood phylogeny based on concatenated nDNA from the study taxa

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: tif
Explanation note: Clusters and sets of individuals in the trees are colored according to the seven genetic clusters identified in STRUCTURE (Fig. 3A), and black codes indicate outgroup species. For additional details, see the main text and color coding in Figs 3, 4.
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Link: https://doi.org/10.3897/zse.101.152180.suppl7

Supplementary material 8

Genetic distances between seven clusters and Student's t-tests of Cyt *b* sequence data

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: xlsx
Explanation note: **A.** genetic distances between clusters based on the Kimura 2-parameter model; and **B.** Student's *t*-tests. The genetic distances between the subclusters A-1 and A-2 were used as the hypothesized mean.
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Link: https://doi.org/10.3897/zse.101.152180.suppl8

Supplementary material 9

Analysis of molecular variation (AMOVA) for the two *Iksookimia* species

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: xlsx
Explanation note: Analysis of molecular variation (AMOVA) for the two *Iksookimia* species according to three grouping methods: five groups, grouping clusters B, C, and D as one group; six groups, grouping clusters B, C, and D as one group and separate subclusters A-1 and A-2; and eight groups, with separate subclusters A-1 and A-2.
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Link: https://doi.org/10.3897/zse.101.152180.suppl9

Supplementary material 10

Pairwise F_{ST} results between populations

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: tif
Explanation note: **A.** nDNA; **B.** mitochondrial Cyt *b*; **C.** EGR2B; **D.** RAG1; and **E.** SH3PX3.
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Link: https://doi.org/10.3897/zse.101.152180.suppl10

Supplementary material 11

Pairwise F_{ST} results between populations

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: xlsx
Explanation note: after P-value, $\alpha < 0.05$; Bold, after Bonferroni correction, $\alpha < 0.000238$, **A.** nDNA; **B.** mitochondrial Cyt *b*.
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Link: https://doi.org/10.3897/zse.101.152180.suppl11

Supplementary material 12

IBD patterns with F_{ST} values

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: tif
Explanation note: **A.** F_{ST} values for nDNA and GPS distance; **B.** nDNA and river–ocean distance; **C.** F_{ST} values for mitochondrial Cyt *b* and GPS distance; and **D.** Cyt *b* and river–ocean distance.
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Link: https://doi.org/10.3897/zse.101.152180.suppl12

Supplementary material 13

Correlation coefficients for Suppl. material 12

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: xlsx
Explanation note: **A.** F_{ST} values for nDNA and GPS distance; **B.** nDNA and river–ocean distance; **C.** F_{ST} values for mitochondrial Cyt *b* and GPS distance; and **D.** Cyt *b* and river–ocean distance.
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Link: https://doi.org/10.3897/zse.101.152180.suppl13

Supplementary material 14

Bi-dimensional representation of genetic differentiation (F_{ST}) between the populations obtained via NMDS

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: tiff
Explanation note: Results are presented for **A.** concatenated nDNA and **B.** the mitochondrial Cyt *b* gene. Dots indicate sites from one of the four main rivers in this study, while triangles indicate sites from smaller tributary streams that flow directly into the ocean and are not connected with the major rivers. Points are colored to match the groupings in Figs 3, 4.
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Link: https://doi.org/10.3897/zse.101.152180.suppl14

Supplementary material 15

Genetic indices and heterozygosity

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: tif
Explanation note: The sampling sites of the Yeongsan River are in blue letters, the Tamjin River in purple, the Seomjin River in red, the Nakdong River in yellow, and the islands in bold. To the left of the vertical green dotted line is *I. hugowolfeldi*, and to the right is *I. longicorpa*. **A.** Nucleotide diversity per site; **B.** haplotype diversity; **C.** expected heterozygosity; and **D.** observed heterozygosity.
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Link: https://doi.org/10.3897/zse.101.152180.suppl15

Supplementary material 16

Genetic indices for each population and locus. nDNA, concatenated nDNA

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: xlsx
Explanation note: N, number of individuals; S, number of polymorphic sites; π, nucleotide diversity per site; h, number of haplotypes; H_d, haplotype diversity.
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Link: https://doi.org/10.3897/zse.101.152180.suppl16

Supplementary material 17

Heterozygosity

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: xlsx
Explanation note: **A.** expected heterozygosity (H_e); and **B.** observed heterozygosity (H_o).
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Link: https://doi.org/10.3897/zse.101.152180.suppl17

Supplementary material 18

Probability value for the Hardy–Weinberg equilibrium for each locus

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: xlsx
Explanation note: after Bonferroni correction, $\alpha < 0.000793$.
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Link: https://doi.org/10.3897/zse.101.152180.suppl8

Supplementary material 19

Lamina circularis for the two *Iksookimia* species (modified from Fig. 2 in Kim (2009)

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: tif
Explanation note: **A.** *I. hugowolfeldi* and **B.** *I. longicorpa*.
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Link: https://doi.org/10.3897/zse.101.152180.suppl19

Supplementary material 20

Results from simulations of changes in the coastline with sea level variation conducted in QGIS (Congedo 2021)

Authors: Da-Song Choi, Hyo-Jin Kim, Myeong-Hun Ko, Ra-Yeon Myung, Yong-Jin Won
Data type: tif
Explanation note: The black line shows the current coastline of South Korea, Japan, and China at multiple sea levels, including **A.** sea level 50 m lower (–50 m); **B.** 100 m lower (–100 m); and **C.** 120 m lower than present (–120 m). The grid file used to build the seabed was downloaded from GEBCO and can be accessed at https://download.gebco.net (Mayer et al. 2018).
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Link: https://doi.org/10.3897/zse.101.152180.suppl20