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Population Structure and Genetic Diversity of the Spotted Sleeper *Odontobutis interrupta* (Odontobutidae), a Fish Endemic to Korea

Kang-Rae Kim [1], Hee-kyu Choi [2], Taek Won Lee [2], Hyuk Je Lee [2] and Jeong-Nam Yu [1,*]

[1] Animal & Plant Research Department, Nakdonggang National Institute of Biological Resources, Sangju 37242, Republic of Korea; kimkangrae9586@gmail.com
[2] Molecular Ecology and Evolution Laboratory, Department of Biological Science, Sangji University, Wonju 26339, Republic of Korea; chk9987@naver.com (H.-k.C.); 2taek135@gmail.com (T.W.L.); lhjk622@gmail.com (H.J.L.)
* Correspondence: susia000@nnibr.re.kr

Abstract: The spotted sleeper, *Odontobutis interrupta*, is a fish species endemic to Korea and shows potential as an aquaculture species. Nevertheless, the population size of this species has declined significantly in recent years. To characterize the population structure and genetic diversity of *O. interrupta* in Korea, we analyzed four microsatellite loci in twelve populations from four major river systems. The provenance of the population was investigated to discern the origin of the translocated populations. The genetic diversity of the microsatellite ranged from 0.440 to 0.756, showing a high level of diversity similar to that of other freshwater fishes. However, mitochondrial DNA analysis exhibited low genetic diversity (H_d: 0.000–0.674, π: 0.00000–0.00159). The F_{ST} values of microsatellites and mitochondrial DNA ranged from 0.096 to 0.498 and −0.046 to 0.951, suggesting genetic admixture among populations. All populations exhibited an effective population size of <100; therefore, preservation efforts to prevent inbreeding depression would be required. The genetic structure could be divided into unique genotypes from the Seomjingang and Geumgang Rivers. However, genetic admixture was observed in all populations, rendering it impossible to distinguish them. Our findings provide fundamental but significant genetic insights pursuant to devising conservation strategies for *O. interrupta*.

Keywords: microsatellite; bottleneck; population structure; genetic diversity; *Odontobutis interrupta*

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1. Introduction

Current species extinction rates indicate a sixth mass extinction [1]. Most known species are deemed amenable to conservation efforts because of their high prevalence or economic value [2]. However, except for species of interest, most species are threatened or likely to become extinct [3]. Endemic species are confined to a specific geographic region, which can vary in size [4,5]. Notably, endemic species are more vulnerable to anthropogenic threats than non-native species because of their limited distribution range [3,6]. Their susceptibility to extinction can be attributed to the following five factors: (1) overexploitation by humans [7], (2) a decrease in population size [8], (3) low reproductive potential [9], (4) habitat damage caused by human activities [10], and (5) threats of extinction by invasive species [11]. Species affected by any of the aforementioned causes should be prioritized for conservation, and effective monitoring strategies should be implemented to manage them [5].

The Korean Peninsula induces genetic differences because, biogeographically, the uplift of mountain ranges hinders genetic flow [12]. Identifying these genetic differences is an important factor in conservation planning [4].

Genetic diversity is an essential factor in the conservation and management of conservation biology [9]. Genetic diversity is often associated with population survival and can,

therefore, influence the capacity of wild populations to adapt to rapidly changing environmental conditions [8,9]. Endemic species with restricted distribution tend to exhibit low genetic diversity [13]. Habitat damage owing to anthropogenic activities causes a decline in population size, ultimately resulting in low genetic diversity [14]. Diminished genetic diversity leads to a reduced capacity for evolution, ultimately increasing the likelihood of extinction [9].

The spotted sleeper, *Odontobutis interrupta*, is a fish species endemic to Korea and is found in the rivers draining into the West Sea, specifically in the north of the Geumgang River in the Korean Peninsula. This species inhabits the middle and lower reaches of slow-flowing rivers and is known for its carnivorous feeding behavior, consuming organisms such as aquatic insects, fish, and shrimp. It can grow up to 17 cm in length [15]. *Odontobutis potamophila* is closely related to *O. interrupta* and is a promising aquaculture species in China that can grow up to 11 cm [16]. Given its larger size than *O. potamophila*, *O. interrupta* could be more suitable for aquaculture. Breeding strategies are needed to conserve endemic species that show potential for aquaculture [15,16]. In addition, the numbers of *O. interrupta* are declining because of habitat loss or pollution owing to anthropogenic activities [17]. Although there is no official record, it is presumed that genetic admixture has occurred owing to artificial discharge between water systems for sustaining species diversity and resource generation. Damage to these habitats reduces population size, and genetic admixture can result in unexpected negative consequences [4]. Hence, it is imperative to conduct a genetic assessment to establish effective conservation strategies and genetic management units [4].

The Korean spotted sleeper is presumed to have been recently introduced into the Nakdonggang River water system and the Hyeongsangang River [18]. Introduced species are problematic for the structure and function of native ecosystems and can negatively affect species, particularly those occupying the same ecological niche [19–21]. *Odontobutis platycephala* is closely related to *O. interrupta* and occupies a similar ecological niche. Thus, the invasion of the Nakdonggang and Hyeongsangang Rivers by *O. interrupta* can potentially have an adverse impact on the native *O. platycephala*. Assessing the success of an invasive species can provide insights into the effect of the origin and genetic diversity of a population on its dissemination and establishment [22]. Since *O. interrupta* appears to be successfully established in the water system, from a conservation genetics perspective, assessing its origin and genetic diversity in the context of translocations is necessary.

Thus far, genetic research on *O. interrupta* has only centered around the characteristics of its mitochondrial genome; however, population genetics investigations, such as microsatellite analysis for genetic diversity, are yet to be conducted. Microsatellites are commonly analyzed in population genetics studies because of their high intraspecific polymorphism and codominance [23,24]. Mitochondrial cytochrome c oxidase I regions are typically used as barcoding regions and are useful for tracking translocation because they facilitate discrimination between species and populations within species [25–27]. In this study, *COI* was selected because it facilitates species identification of introduced populations and similar species. The integrated analysis of microsatellites and mitochondrial DNA (mtDNA) markers can significantly improve the investigation of genetic diversity and structure [28].

In this study, we analyzed the genetic diversity and structure of *O. interrupta*, a species endemic to Korea, and provided fundamental yet important insights into the provenance of the translocated population. In this study, we developed the first microsatellite loci to investigate the genetic diversity and population structure of *O. interrupta* in Korean rivers. The information from nuclear loci combined with mtDNA markers provides valuable genetic insights for population management in the development of conservation strategies for *O. interrupta*.

2. Materials and Methods

2.1. Sampling and DNA Extraction

Odontobutis interrupta (1985) is endemic to Korea; therefore, animal ethical approval was waived. Twelve populations of *O. interrupta* were sampled (location and latitude/longitude details are provided in Figure 1 and Table S1). Sampling was conducted in October 2019, and the fin tissues of the fish were collected and soaked in 99% ethanol. Genomic DNA was extracted using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The extracted genomic DNA was stored at −20 °C after dilution to 50 ng/µL to amplify *COI* and microsatellite loci.

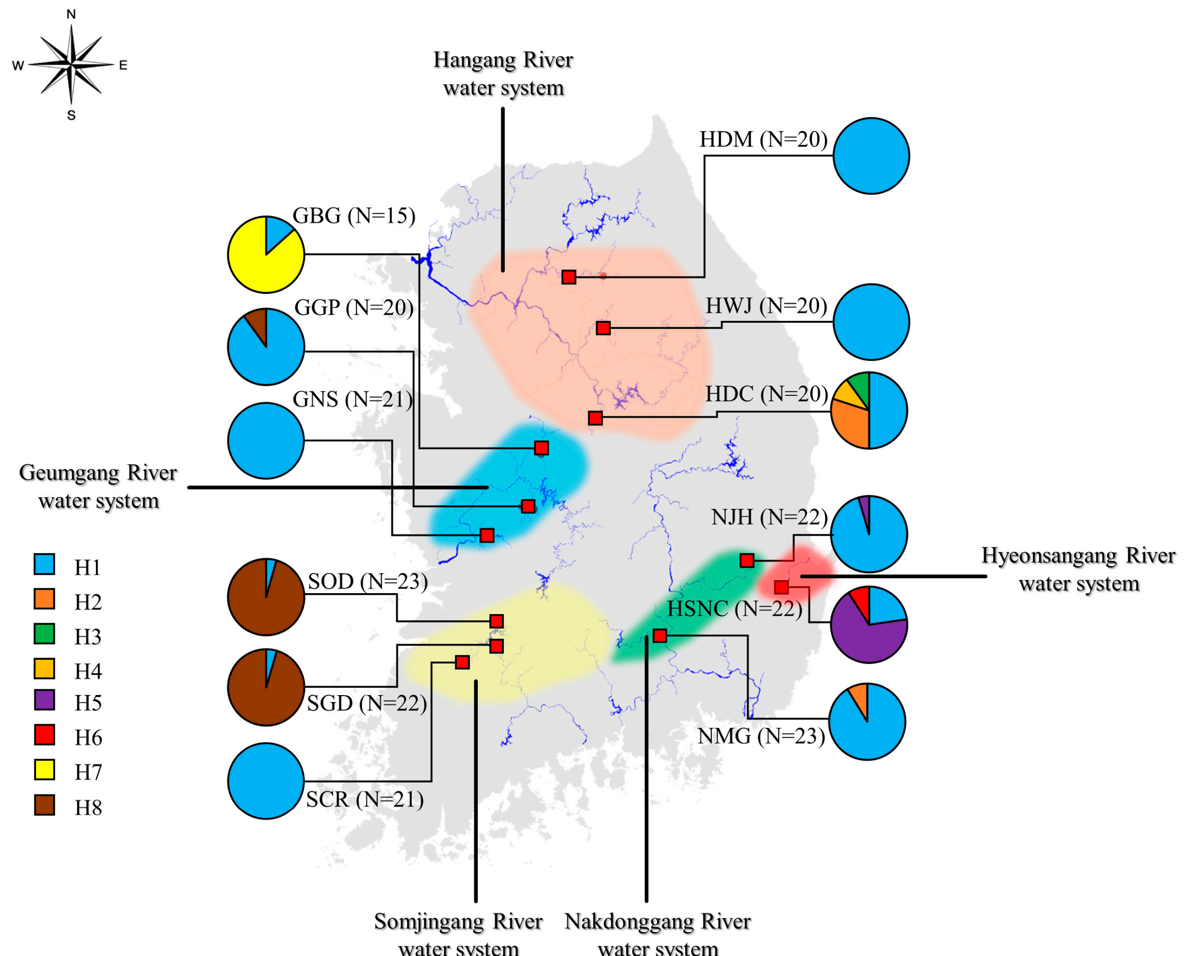

Figure 1. Sampling location of *Odontobutis interrupta* and haplotype distribution of mitochondrial DNA for the *COI* gene. Abbreviations for populations are given in Table S1.

2.2. Whole-Genome Sequencing and Microsatellite Screening

For whole-genome sequencing, the HDM01 (37°41′39″ N 127°40′06″ E) individual was used. Whole-genome sequencing was performed using a 150 bp paired-end library constructed by GnCBIO (GnCBio Inc., Daejeon, Republic of Korea) and sequenced using an Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA). Contigs assembled from SOAPdenovo2 were selected for di-, tri-, tetra-, penta-, and hexa-sequences with more than five repetitions using the MISA tool (http://misaweb.ipk-gatersleben.de/ (accessed on 1 June 2023)). Using Primer3 (https://github.com/primer3-org/primer3 (accessed on 1 June 2023)), we set the length of the appropriate primer to 20–24 bp, the size of the amplification product to 150–400 bp, and the melting temperature (Tm) value to 58–60 °C.

2.3. Microsatellite Genotyping and mtDNA Sequencing

One-hundred regions potentially containing microsatellite motifs were randomly selected across the sequenced genome to design primers for microsatellite amplification in a panel of twenty-five specimens from three sampled locations at distinct river basins. PCR was performed using performed using 10 ng genomic DNA, 0.5 units of Ex-Taq polymerase (TaKaRa, Kusatsu, Japan), 1× Ex-Taq buffer, 200 μM dNTP mixture (2.5 mM), 0.4 μM forward primer, 0.8 μM reverse primer, and 0.4 μM fluorescent label. The final volume of the reaction mixture was 20 μL, including the M13 (TGTAAAACGACGGCCAGT) primer labeled with the fluorescent dyes NED and PET. The PCR conditions were as follows, according to the method described by Schuelke [29]: pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 56–58 °C for 45 s, and extension at 72 °C for 45 s. After 30 repetitions, denaturation at 94 °C for 30 s, annealing at 53 °C for 45 s, and extension at 72 °C for 45 s were repeated another 8 times. The final extension was performed at 72 °C for 10 min, and the temperature was held constant at 4 °C. The amplified PCR products were separated using 2% agarose gel electrophoresis to confirm the presence or absence of a band and the size of the amplified fragment. Microsatellite PCR product fragments were prepared by mixing a GeneScan™ 500 ROX size standard ladder (Applied Biosystems, Foster City, CA, USA) and HiDi™ formamide and performing denaturation at 95 °C for 2 min, followed by termination at 4 °C. The allele sizes were determined using an ABI 3730xl DNA Analyzer (Applied Biosystems). Genotyping was performed using GeneMarker® 2.6.7 program (SoftGenetics, State College, PA, USA). The microsatellite markers were deposited at Genbank (OQ656882-OQ656885).

The primers for mtDNA were obtained from the study by Ward et al. [26] (FishF1: TCAACCAACCACAAAGACATTGGCAC, FishR1: TAGACTTCTGGGTGGCCAAAGAAT CA), and PCR was performed using a Mastercycler® pro gene amplifier. For PCR, an AccuPower® PCR Premix Kit (BIONEER Co., Daejeon, Republic of Korea) was used, and 1 μL of genomic DNA, 1 μL of each of the forward and reverse primers (1.0 mM), and 17 μL of tertiary distilled water were added. All components were mixed to a final volume of 20 μL. The PCR conditions were as follows: pre-denaturation at 95 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min and termination at 4 °C. The amplified *COI* fragments were sequenced using an ABI 3730xl DNA Analyzer (Applied Biosystems). The *COI* haplotype sequences identified in this study were deposited at Genbank (OR352370-OR352377) and compare with the already available *COI* sequences for *Odontobutis* species (*Odonbutis platycephala* OL674337, *Odonbutis potamophila* KF874495, *Odonbutis haifengensis* NC_036056, *Odonbutis yaluensis* NC_027160, *Odonbutis sinensis* NC_022818, *Odonbutis obscura* MW646297).

2.4. Microsatellite and mtDNA Genetic Diversity Analyses

MICRO-CHECKER software (Ver. 2.2.3) [30] was used to examine the presence or absence of scoring errors in the microsatellite loci. Genetic diversity was measured as the number of alleles (N_A), expected heterozygosity (H_E), and observed heterozygosity (H_O) using the CERVUS software (Ver. 3.0) [31]. The population inbreeding coefficient (F_{IS}) and Hardy–Weinberg equilibrium (HWE) analyses were performed using GENEPOP (Ver. 4.2) [32] and ARLEQUIN software (Ver. 3.5) [33]. Two methods were used to estimate bottlenecks: one involving the BOTTLENECK software (Ver. 1.2.02) [34], a program for estimating bottlenecks through heterozygous excess testing, and the infinite allele model (IAM) [35]. A two-phase model (TPM) and stepwise mutation model (SMM) [36] were used to estimate; TPM was performed with 10% variance and 90% SMM. In addition, each model had 10,000 iterations, and significance was verified using the Wilcoxon signed-rank test [37]. The effective population size (Ne) was determined using the linkage disequilibrium estimation from LDNe software [38].

MtDNA analysis was performed by aligning the *COI* sequences using the MEGA software [39]. Parameters of haplotype diversity and nucleotide diversity were determined

using DnaSP software (Ver. 5.0) [40]. A median-joining network analysis was performed using Network software (Ver. 10.2.0.0) [41] to create a haplotype network to determine the phylogenetic affinity between mtDNA sequences.

2.5. Population Genetic Structure Analysis

ARLEQUIN software (Ver. 3.05) [33] was used for the microsatellite and mtDNA data to analyze pairwise genetic differentiation, F_{ST}, between studied populations. For microsatellite data, a hierarchical analysis of molecular variance (AMOVA) based on the hydrographical structure of the studied locations was performed. In addition, groups of NJH, NMG, and HSNC were grouped as introduced populations and AMOVA analysis was performed. Using the microsatellite genotypes, the STRUCTURE software (Ver. 2.3) [42] applying the Markov Chain Monte Carlo (MCMC) Bayesian method was used to estimate the minimum number of genetically homogenous units (K) over sampled individuals. Runs for each possible K from 1 to 10 were repeated 10 times. Each run consisted of a burn-in period with 10,000 iterations and a run length after burn-in of 100,000 iterations, and a suitable default admixture model was applied to the mixture of water systems. The most likely value of K was selected following the methodology described by Evanno et al. using STRUCTURE HARVESTER [43,44]. A discriminant analysis of principal components (DAPC) of the microsatellite dataset was performed on the population using the R package ADEGENET (Ver. 2.1.3) [45].

3. Results

3.1. Microsatellite and Mitochondrial DNA Genetic Diversity

We obtained 4,075,232 reads through whole-genome sequencing, with a total read length of 1,083,799,779 bp. The assembly and microsatellite screening results are shown in Table S2. Only in four out of the one-hundred sequenced regions with microsatellite motifs did the designed primers result in amplification yielding reliable microsatellite variation across the three populations in the twenty-five specimens used for screening (Table S3). A total of 100 individuals were screened for the four markers; markers that were not amplified and those without polymorphisms in each population were excluded. The N_A ranged from 9 to 12, and the polymorphism information index was >0.5, indicating an appropriate marker. The HWE analysis did not yield significant results for any of the four loci; therefore, they were used for population analysis. Genotyping using the MICRO-CHECKER software showed no evidence of scoring errors or null alleles (Table S3).

The four loci were analyzed for genetic diversity indices in 12 populations (Table 1). The N_A ranged from 3.75 to 8.25, the H_O ranged from 0.440 to 0.756, and the expected heterozygosity ranged from 0.517 to 0.780. We found that the SCR, HDC, and NJH populations deviated from the HWE, whereas others followed the HWE. Inbreeding was observed solely in the SCR population, with significant values of F_{IS} ($p < 0.001$). The NA ranged from 3.75 at the NMG location to 8.25 at GGP, the H_O ranged from 0.440 to 0.756, and the expected heterozygosity ranged from 0.517 at SGD to 0.780 at HSNC. Despite the presumption that the HSNC population was translocated, it exhibited high genetic diversity (H_O = 0.732). The SCR and HDC populations exhibited low genetic diversity (H_O = 0.440, 0.467), albeit being a natural population (i.e., non-translocated).

Table 1. Genetic diversity based on four microsatellite loci and mitochondrial DNA.

ID	Water System	MtDNA/ Microsatellite	h	H_d	Nucleotide Diversity (π)	D	F	N_A	H_O	H_E	P_{HWE}	F_{IS}
SOD	Somjingang River	23/22	2	0.087	0.00033	−1.51496	−0.153	6.75	0.756	0.752	0.062	−0.044
SCR	Somjingang River	20/21	1	0.000	0.00000	-	-	3.00	0.440	0.570	0.013 *	0.257 ***
SGD	Somjingang River	22/24	2	0.091	0.00034	−1.51481	−0.112	4.75	0.500	0.517	0.270	0.051
HWJ	Hangang River	20/20	1	0.000	0.00000	-	-	5.00	0.726	0.697	0.740	−0.082
HDM	Hangang River	20/20	1	0.000	0.00000	-	-	5.00	0.600	0.642	0.149	0.078
HDC	Hangang River	20/23	4	0.674	0.00159	1.15776	−0.400	4.50	0.467	0.537	0.006 **	0.003
NJH	Nakdonggang River	22/17	2	0.091	0.00034	−1.51481	−0.122	5.25	0.635	0.726	0.027 *	0.005
NMG	Nakdonggang River	23/22	2	0.166	0.00031	−0.66215	−0.213	3.75	0.529	0.548	0.375	0.012
GGP	Geumgang River	20/23	2	0.189	0.00071	−0.76857	0.909	8.25	0.750	0.719	0.557	−0.045
GNS	Geumgang River	21/22	1	0.000	0.00000	-	-	6.50	0.727	0.703	0.609	−0.036
GBG	Geumgang River	15/23	2	0.248	0.00047	−0.39883	0.133	4.75	0.670	0.595	0.402	−0.110
HSNC	Hyeongsangang River	22/23	3	0.498	0.00155	1.13094	0.867	7.50	0.732	0.780	0.167	0.062

h: Number of haplotypes, H_d: Haplotype diversity, F: Fu's values, D: Tajima's values, N: Number of samples, N_A: Number of alleles, H_O: Observed heterozygosity, H_E: Expected heterozygosity, PIC: Polymorphic information content. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Analysis of the 531 bp-long *COI* partial sequences in the mtDNA region in the 12 populations revealed six variable regions, with two-hundred and forty-eight sequences exhibiting eight haplotypes (Table 2, Figure 1). The most common haplotype was H1, which was shared among between 1 and 21 individuals in all populations. The H8 haplotype was common among the SOD, SGD, and GGP populations, and the H8 haplotype in the GGP population accounted for a notable proportion (N = 2). The H3 and H4 haplotypes were observed only in the HDC population (H3: N = 2; H4: N = 2), and the H7 haplotype was observed only in the GBG population (N = 13). The NJH and NMG populations, which were presumed to be translocated populations, exhibited substantially high proportions of the H1 haplotype, whereas the HSNC population exhibited a high proportion of the H5 haplotype and shared a haplotype with the NJH population. The phylogenetic tree clustered *O. interrupta* as the first clade in all populations that were individually clustered (Figure 2). Interestingly, it was confirmed that *O. potamophila*, *O. haifengensis*, *O. yaluensis*, and *O. sinensis* were clustered in the same group and had the same sequence.

Table 2. Distribution of the eight haplotypes in twelve populations of *Odontobutis interrupta*.

	Seomjingang River Basin			Hangang River Basin			Geumgang River Basin			Nakdonggang River Basin		Hyeongsangang River
	SOD	SCR	SGD	HWJ	HDM	HDC	GGP	GNS	GBG	NJH *	NMG *	HSNC *
H1	1	20	1	20	20	10	18	21	2	21	21	5
H2						6					2	
H3						2						
H4						2						
H5										1		15
H6												2
H7									13			
H8	22		21				2					

*: Translocated populations.

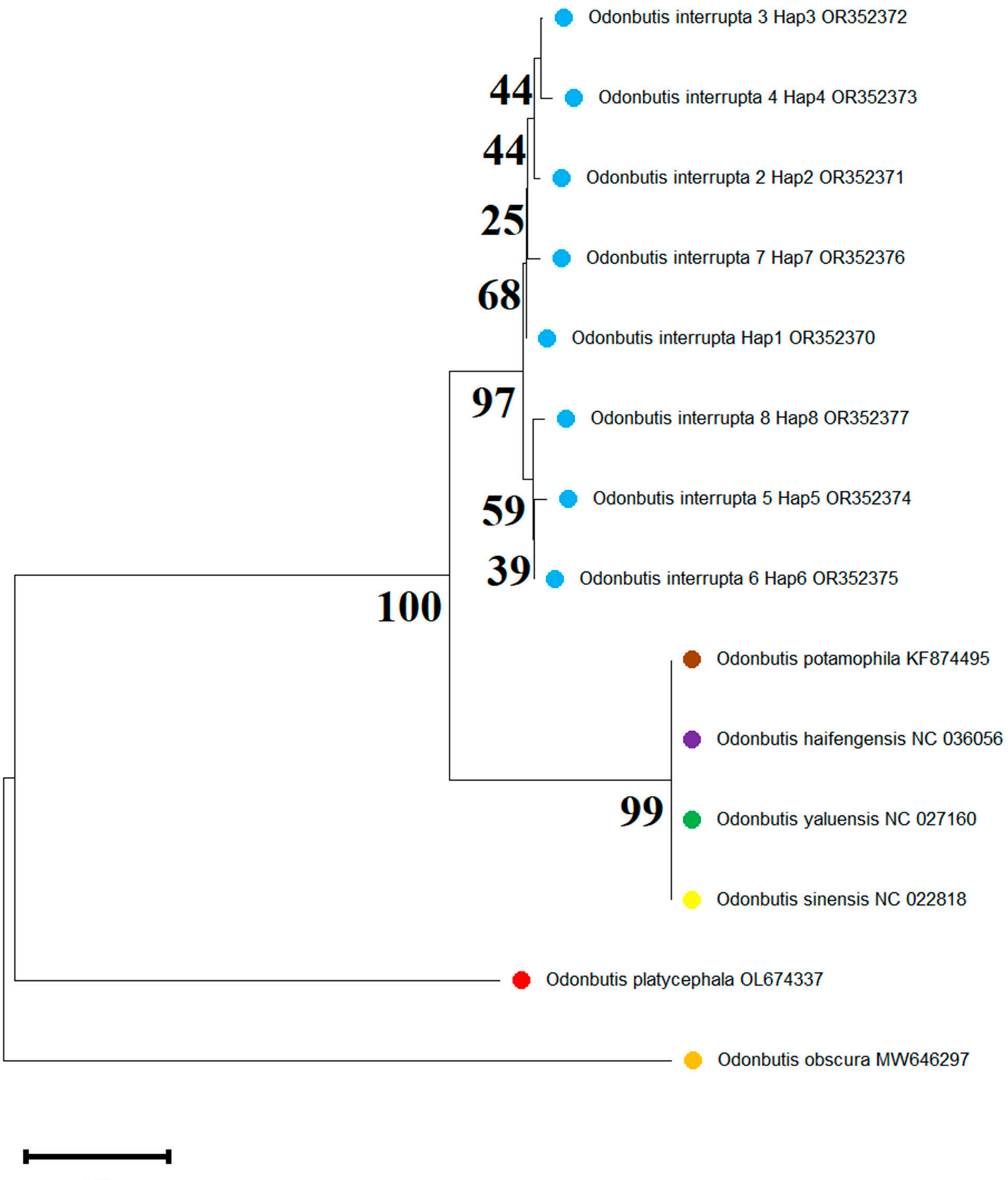

Figure 2. The phylogenetic tree was generated using the maximum likelihood method (MegaX software ver. 11.0.13). Colors in circles represent species.

Haplotype diversity (H_d) ranged from 0.000 to 0.674; the SCR, HWJ, HDM, and GNS populations exhibited the lowest scores, while the HDC population exhibited the highest scores (Table 1). In this study, the haplotype diversity of *COI* was found to be low. Nucleotide diversity (π) ranged from 0.00000 to 0.00159. The HSNC population, presumed to be a translocation population, exhibited higher H_d and π values than the other populations (H_d = 0.498, π = 0.00155).

3.2. Bottleneck Analysis

Using the infinite mutation model (IAM), we identified significant bottlenecks in the SOD, SCR, HWJ, NJH, and HSNC populations ($p < 0.05$). Using the TPM model, we identified a bottleneck in the HWJ population (Table 3). The SCR and NJH populations exhibited recent mode shifts, thereby showing evidence of a bottleneck.

Table 3. Summary statistics regarding the bottleneck signature, effective population size, and estimated M-ratio for populations at microsatellites.

Population ID	N	Wilcoxon Sign-Rank Test				Ne^	(95% CI)
		P_{IAM}	P_{TPM}	P_{SMM}	Mode-Shift		
SOD	22	0.031 *	0.844	0.906	L-shaped	82	(15–∞)
SCR	21	0.031 *	0.063	0.063	SHIFTED	3	(1–124)
SGD	24	0.906	1.000	1.000	L-shaped	4	(1–18)
HWJ	20	0.031 *	0.031 *	0.063	L-shaped	25	(6–∞)
HDM	20	0.156	0.563	0.906	L-shaped	23	(8–1646)
HDC	23	0.563	0.938	0.969	L-shaped	-	-
NJH	17	0.031 *	0.063	0.063	SHIFTED	9	(3–39)
NMG	22	0.094	0.844	0.844	L-shaped	10	(2–∞)
GGP	23	0.563	1.000	1.000	L-shaped	61	(20–∞)
GNS	22	0.063	0.844	0.906	L-shaped	36	(4–∞)
GBG	23	0.438	0.438	0.438	L-shaped	16	(6–105)

N: Numbers of Sample, N_e: Effective population size. P_{IAM}: *p*-value of bottleneck test using infinite allele mutation model, P_{TPM}: *p*-value of bottleneck test using two-phase mutation model (10% variance and 90% proportions of SSM), P_{SMM}: *p*-value of bottleneck test using the stepwise mutation model, Ne^: estimated effective population size by LDNe software, CI: confidence interval. * $p < 0.05$.

Among the 12 populations, the effective population size ranged from 3 to 82 individuals, except for the HSNC and HDC populations, for which estimation was not possible. The effective population size of the SOD population was 82; for the SCR population, it was 3, which was the smallest (Table 3). The effective population size of the NJH and NMG populations, which were presumed to be translocation populations, was substantially low, at 9 and 10, respectively, although their 95% CI was 3–39 and 2–infinity, respectively (Table 3). These results may be erroneous because of their small sample size. None of the populations exhibited a minimum effective population size of 100, which is necessary to prevent inbreeding depression.

3.3. Population Structure and Genetic Differentiation Analyses

The F_{ST} values for the mtDNA dataset were significant, with the highest divergence observed between the SOD and GNS populations (F_{ST} = 0.952, $p < 0.001$, Table 4). Despite being in the same water system, the SOD and SGD populations exhibited substantially higher F_{ST} values than the SCR population (F_{ST} = 0.951, $p < 0.001$; F_{ST} = 0.950, $p < 0.001$). NJH and NMG, presumed to be translocated populations, showed 0.000 F_{ST} values for NJH vs. SCR, HWJ, HDM, and GNS. No significant mtDNA differences were observed between the two populations NJH and NMG, and between these two populations and SCR, HWJ, HDM, and GNS. However, HSNC, which was presumed to be a translocated population, showed F_{ST} values of 0.595 or higher with all populations, which were significant ($p < 0.001$).

Table 4. Pairwise genetic differentiation and F_{ST} among populations according to microsatellite and mtDNA analysis.

	SOD	SCR	SGD	HWJ	HDM	HDC	NJH	NMG	GGP	GNS	GBG	HSNC
SOD	-	0.951 ***	0.000	0.951 ***	0.951 ***	0.806 ***	0.907 ***	0.915 ***	0.844 ***	0.952 ***	0.927 ***	0.739 ***
SCR	0.279 ***	-	0.950 ***	0.000	0.000	0.298 ***	0.000	0.037	0.053	0.000	0.876 ***	0.707 ***
SGD	0.107 ***	0.406 ***	-	0.950 ***	0.950 ***	0.801 ***	0.905 ***	0.913 ***	0.840 ***	0.951 ***	0.925 ***	0.734 ***
HWJ	0.135 ***	0.267 ***	0.273 ***	-	0.000	0.298 ***	0.000	0.037	0.053	0.000	0.876 ***	0.707 ***
HDM	0.246 ***	0.266 ***	0.419 ***	0.167 ***	-	0.298 ***	0.000	0.037	0.053	0.000	0.876 ***	0.707 ***
HDC	0.244 ***	0.380 ***	0.348 ***	0.160 ***	0.246 ***	-	0.267 ***	0.194 ***	0.237 ***	0.305 ***	0.608 ***	0.595 ***
NJH	0.120 ***	0.324 ***	0.315 ***	0.114 ***	0.246 ***	0.212 ***	-	0.022	0.007	0.000	0.781 ***	0.645 ***
NMG	0.172 ***	0.331 ***	0.348 ***	0.244 ***	0.287 ***	0.360 ***	0.231 ***	-	0.054	0.040	0.791 ***	0.683 ***
GGP	0.129 ***	0.332 ***	0.246 ***	0.075 ***	0.244 ***	0.096 ***	0.118 ***	0.277 ***	-	0.056	0.699 ***	0.595 ***
GNS	0.155 ***	0.291 ***	0.314 ***	0.066 ***	0.215 ***	0.201 ***	0.104 ***	0.257 ***	0.137 ***	-	0.880 ***	0.712 ***
GBG	0.318 ***	0.384 ***	0.498 ***	0.294 ***	0.337 ***	0.310 ***	0.318 ***	0.442 ***	0.318 ***	0.302 ***	-	0.750 ***
HSNC	0.125 ***	0.261 ***	0.323 ***	0.139 ***	0.133 ***	0.203 ***	0.083 ***	0.211 ***	0.143 ***	0.163 ***	0.250 ***	-

Pairwise genetic differentiation of mtDNA (above). F_{ST}: Pairwise genetic differentiation of microsatellite (below). *** $p < 0.001$.

The F_{ST} values for the microsatellite dataset were all significant, with the highest F_{ST} value between SGD and GBG (F_{ST} = 0.498). The SOD, SCR, and SGD populations in the Seomjingang River water system exhibited high F_{ST} values between SOD and SGD vs. SCR (F_{ST} = 0.279 and 0.406, respectively), similar to the mtDNA data. The NJH and NMG populations, presumed to be translocated populations, exhibited the lowest F_{ST} values between the NJH and GNS populations (F_{ST} = 0.104). The NMG population exhibited weak genetic differentiation, with the lowest F_{ST} values with SOD (F_{ST} = 0.172).

The median-joining network revealed that the H1 haplotype was present across all populations, suggesting a high degree of genetic admixture among said populations (Figure 3). The NJH and NMG populations were clustered with 21 samples as H1; in the case of NJH, haplotype H5 was shared with HSNC. In HSNC, the H5 haplotype occupied more than half (15/22) of the population, and there was a unique haplotype (H6). GBG was found in a high proportion in unique haplotype H7. The unique haplotype H8, which was observed in the SOD and SGD populations, was not observed in the SCR population, where only the H1 haplotype was present. Similar to the haplotype network, HDC, GGP, HSNC, SGD, and SOD were clustered separately.

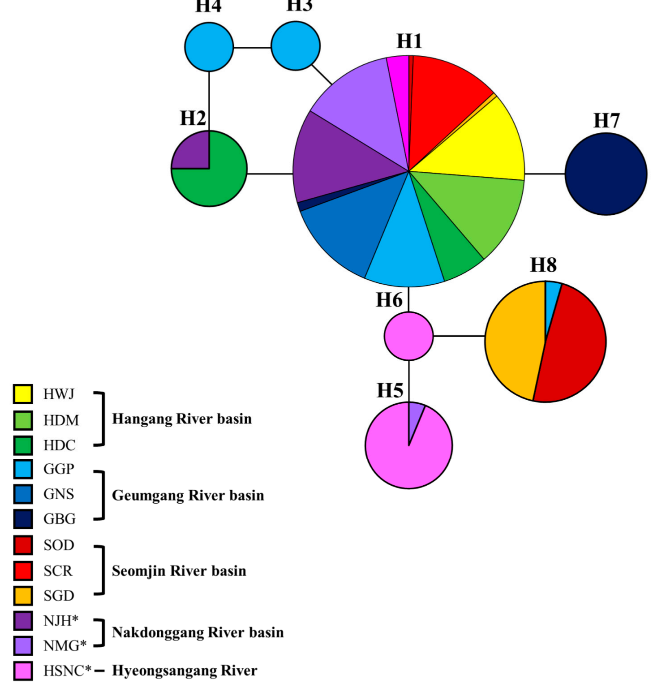

Figure 3. Haplotype networks from the median-joining networks. Colors in circles represent populations that share a haplotype. *: translocation population.

Bayesian clustering analysis maximized the Δ*K* value for population structure at *K* = 3 (Figure 4). At *K* = 3, the first group included SOD, SGD, and NMG; the second included SCR, HDC, and GBG. The third group included HWJ, HDM, NJH, GGP, GNS, and HSNC, but exhibited genetic admixture among various groups. In the case of NJH, NMG, and HSNC, genes from the three groups (Hangang River, Geumgang River, Somjingang River) were mixed. NJH was assigned to the Hangang River, NMG to the Seomjingang River, and HSNC were all mixed and assigned, suggesting that these presumed translocated populations exhibited distinct genetic origins. Results of non-model-based DAPC analysis showed similar trends to those of the STRUCTURE analysis (Figure 5).

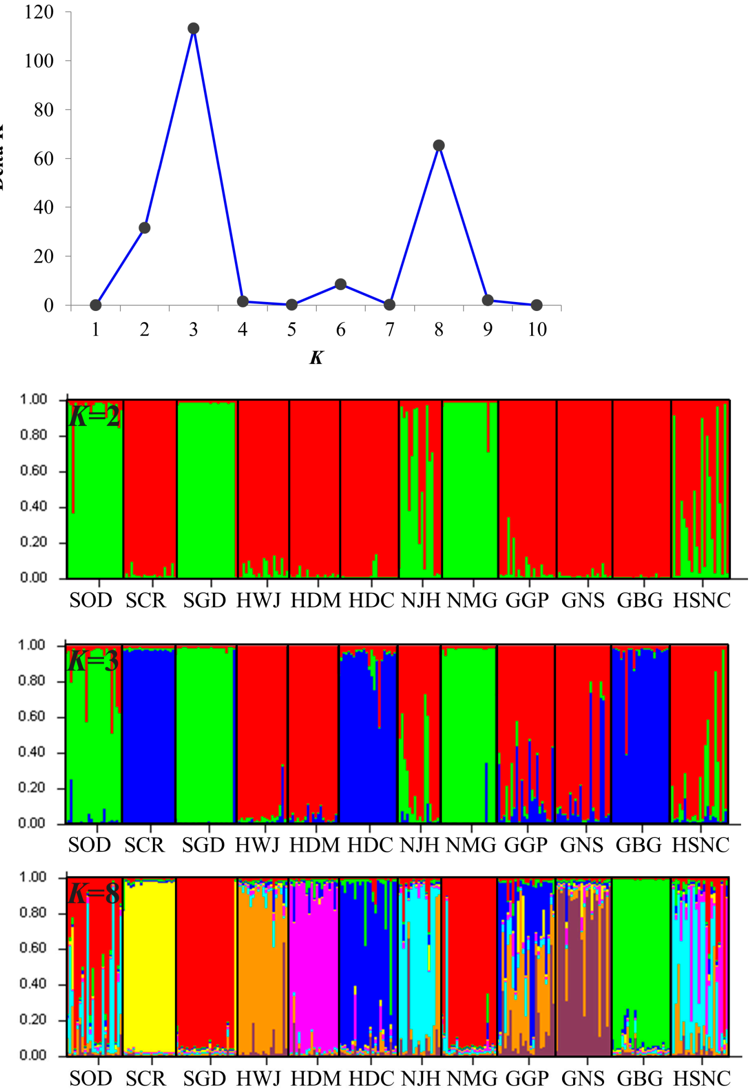

Figure 4. Population genetic structure of *O. interrupta*. Each individual is represented as a vertical bar partitioned into segments of different color according to the proportion of the genome belonging to each of the identified clusters (*K* = 2, 3, and 8) by STRUCTURE software.

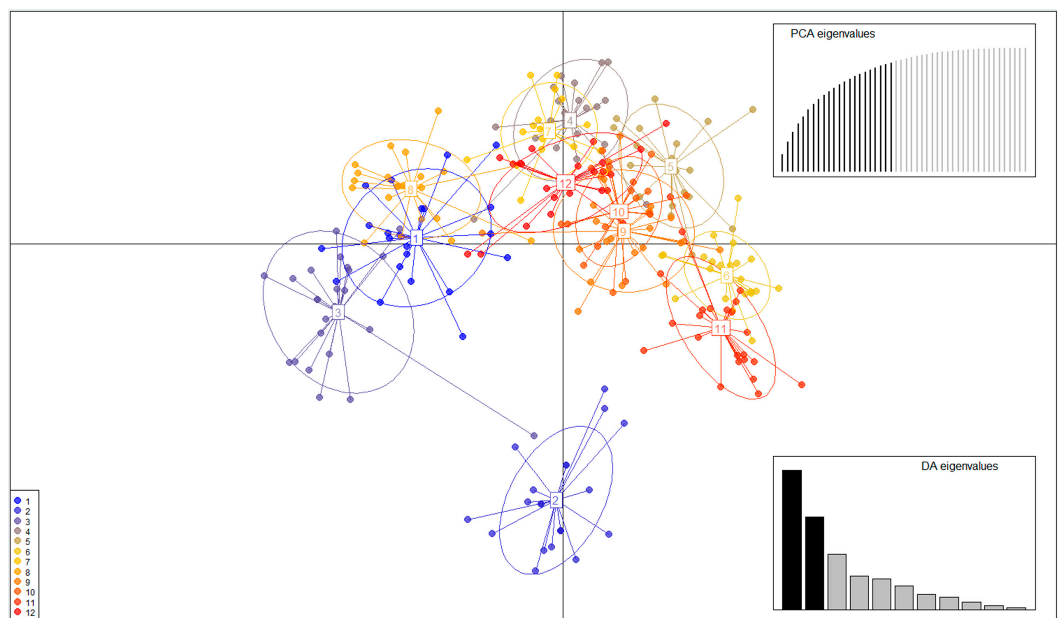

Figure 5. Scatterplots of the discriminant analysis of principal components (DAPC) for *Odontobutis interrupta*. The numbers shown on the plot are the population names (1: SOD, 2: SCR, 3: SGD, 4: HWJ, 5: HDM, 6: HDC, 7: NJH, 8: NMG, 9: GGP, 10: GNS, 11: GBG, 12: HSNC).

AMOVA for *O. interrupta* was performed on the 12 populations to determine their genetic structure (Table 5). AMOVA based on microsatellites indicated a larger proportion of genetic diversity among populations within river basins (21.97%) than among river basins (3.64%). AMOVA based on mtDNA and grouping locations according to shared haplotypes revealed an among-group variance of 63.05% and a within-population variance of 17.77%.

Table 5. Summary information for analysis of molecular variance for populations of *O. interrupta*.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variance	*F*-Statistics
Microsatellite markers (Four groups based on their distribution in water systems (SOD, SGD, SCR vs. HWJ, HDM, HDC vs. GBG, GNS, GGP vs. NJH, NMG, HSNC)					
Among groups	3	55.644	0.04485	3.64	F_{CT} = 0.036 *
Among populations within groups	8	100.979	0.27091	21.97	F_{SC} = 0.228 ***
Within populations	508	466.129	0.91758	74.40	F_{ST} = 0.256 ***
Total	519	622.752	1.23333	100.00	
Mitochondrial DNA (Four groups based on the distribution of shared haplotypes (SOD, SGD, GGP vs. GNS, SCR, HWJ, HDM, HDC, NJH, NMG, vs. GBG vs. HSNC)					
Among groups	3	71.3995	0.444	63.05	F_{CT} = 0.630 ***
Among populations within groups	8	23.727	0.135	19.18	F_{SC} = 0.519 ***
Within populations	236	29.527	0.125	17.77	F_{ST} = 0.822 ***
Total	247	124.649	0.704	100.00	

d.f.: degrees of freedom. * $p < 0.05$, *** $p < 0.001$. F_{ST} is based on standard permutation across the full dataset.

4. Discussion

4.1. Genetic Diversity and Population Bottleneck

Genetic diversity plays an essential role in an organism's ability to cope with changing environments and in the persistence of species during adaptation and evolution [9]. In this study, we observed relatively higher levels of genetic diversity in microsatellites, with H_O values ranging from 0.440 to 0.756, compared to mtDNA. Other freshwater fishes, such as *O. potamophila* and *Perccottus glenii*, also exhibit high genetic diversity [46,47]. As for the genetic diversity of the microsatellite and mtDNA datasets, the SGD population exhibited low levels. Conversely, the SOD population exhibited high microsatellite diversity but substantially low mtDNA H_d diversity. Similar patterns have also been reported for *Gymnocypris potanini* [48]. *Odontobutis interrupta* is a paternal care species wherein the male builds spawning grounds and guards the eggs [49]. A male-to-female ratio of 1:0.85 has been reported for fish belonging to the genus *Odontobutis* [50]. A bottleneck owing to population decline may be caused by the relatively smaller number of females relative to males, resulting in lower haplotype and nucleotide diversity in the mtDNA [51,52]. Since microsatellites are present in nuclear DNA, they are less likely to be affected by reduced genetic diversity with a female-biased sex ratio, unlike mtDNA, which represents maternal inheritance. This may be explained by the hypothesis that a decrease in mtDNA genetic diversity (owing to a reduction in females) has little effect on the nuclear marker microsatellite genetic diversity. However, additional studies are needed to clarify this. Moreover, the N_e of mtDNA is one-quarter of that of the nuclear genome; therefore, the genetic diversity of mtDNA is theoretically expected to be lower than that of nuclear DNA [53].

The NJH and NMG populations, presumed to be artificially introduced populations, exhibited moderate levels of genetic diversity. Tajima's D and Fu's FS values were insignificant but appeared as negative values. Negative neutrality test values may indicate rapid population expansion or recent bottlenecks [54]. Notably, the HSNC population (putatively translocated) exhibited relatively high genetic diversity of haplotypes and microsatellites (H_d = 0.498, N_A = 7.5, H_O = 0.732), suggesting the repeated introduction of the species from source populations of different genetic origins [54]. In general, microsatellite genetic diversity tends to be similar between donor and translocation populations [55]. Although they are identical translocation populations, NJH, NMG, and HSNC exhibited different levels of genetic diversity, indicating that they might have originated from distinct donor populations.

Inbreeding often reduces the survival and reproduction of a given population, i.e., inbreeding depression, ultimately resulting in the loss of genetic diversity and posing challenges to the preservation of a species [9,56]. In the SCR population, the HWE deviated, and the inbreeding coefficient (F_{IS}) was significant ($p < 0.001$), suggesting that inbreeding occurred. SOD, HWJ, GGP, GNS, and GBG exhibited negative F_{IS} values, albeit not significant ($p > 0.05$), suggesting an influx of outbred populations.

Because the SMM has few strictly followed loci, estimating the expected heterozygosity excess in the IAM is suitable for estimating current bottlenecks [33]. The most recent bottlenecked populations in the IAM were SOD, SCR, HWJ, NJH, and HSNC ($p < 0.05$). NMG, which is believed to be a translocated population, had no recently detected bottlenecks. Human activities, such as habitat destruction, reduce population sizes and create bottlenecks [9]. The previously encountered bottleneck could be attributed to human activity, but it could also be attributed to a simple sampling bias because of the low number of samples analyzed [37]. Thus, further studies are needed for more definitive evidence. Nonetheless, active conservation efforts are required since five populations in this study have suffered population size declines, and the recently detected populations also appear to be experiencing bottlenecks.

Species that commonly inbreed in nature experience inbreeding depression and reduced evolutionary capacity [57]. The N_e is essential for maintaining a species' adaptability to changing environments, that is, evolutionary potential [9]. Small population sizes can

accelerate the risk of local extinction, owing to genetic drift and inbreeding depression effects. In this study, except for the HDC and HSNC populations, whose N_e were difficult to measure, the remaining ten populations showed considerably low effective population sizes ($N_e < 100$). Frankham et al. suggested that N_e should be >100 to avoid inbreeding depression [58]. However, all populations have a median N_e of $\leq$100; therefore, they will likely suffer from inbreeding depression. In the case of the SCR population, it is estimated that inbreeding depression cannot be avoided because they are experiencing inbreeding. Except for the SCR population, the remaining 11 populations did not show significant F_{IS} values. However, inbreeding depression cannot be avoided because of low N_e values during subsequent inbreeding.

4.2. Genetic Structure and Gene Flow for Wild and Restoration Populations

In this study, *O. interrupta* inhabiting the Hangang, Seomjingang, and Geumgang Rivers, and the populations in the Nakdonggang and Hyeongsangang Rivers, which were presumed to have been introduced, were analyzed for their genetic structure and their origin was discerned. The twelve populations of *O. interrupta* showed significant genetic differences in the microsatellite and mtDNA datasets. The results of this study differed from previously reported results, indicating clear genetic differences between watersheds owing to geographic differences [12,59]. This might suggest the effects of human-mediated artificial translocations among genetically divergent populations from different river drainages in South Korea [60,61].

Freshwater fishes typically exhibit genetic differences depending on the geographical water system and little genetic differentiation within the same water system [12]. Interestingly, SCR exhibited significant genetic differences with SOD and SGD. The SOD, SCR, and SGD populations in the Seomjingang River water system were expected to exhibit low genetic differences due to gene exchange before the dam was constructed; however, a high level of genetic difference was observed. These distinct genetic differences are corroborated by the DAPC and STRUCTURE results. Solid genetic differentiation can be explained by non-natural processes, such as anthropogenic activities or fragmentation, despite being in the same water system. However, since fragmentation takes around 50 years for substantial genetic differentiation to occur, these results are likely attributed to translocation owing to anthropogenic activities.

Conversely, the populations of the Geumgang and Hangang Rivers were geographically separated and were expected to exhibit distinct genetic differences. Nonetheless, these populations, excluding GBG, showed little genetic difference. It is not known which populations of the Hangang and Geumgang Rivers first mixed, but considering the significant genetic differences between GBG vs. GGP and GNS, which occupy the same water system of the Geumgang River, an artificial genetic admixture is expected to have occurred. This was corroborated by the DAPC and STRUCTURE results. However, GBG also exhibited a mixed genotype with the Hangang River populations; therefore, we presume that genetic mixing of the Hangang River water system populations occurred.

The NJH, NMG, and HSNC populations are thought to have undergone genetic admixture with populations from various water systems. NJH shared an H1 haplotype in the mtDNA, but an H5 haplotype was also observed, suggesting that it was introduced from another population. Based on the microsatellite data, the genetic differences in SOD, GNS, and HWJ were the smallest. The DAPC and STRUCTURE results suggested that genes must have been introduced from the Seomjingang and Hangang River water systems. The NMG population shared the H2 haplotype of HDC, while microsatellite data showed the fewest genetic differences with SOD, suggesting introgression from both populations. However, it cannot be assumed that it was introduced, as the populations of SOD and HDC also appear to be genetically admixed; further research is needed to confirm the origin of this introgression. In the case of HSNC, the genetic differences in the microsatellites SOD, HWJ, NJH, and GGP were small, suggesting that genetic introgression has occurred in many groups; the results of DAPC and STRUCTURE analysis support this hypothesis.

However, the unique H6 haplotype in the HSNC population suggests translocation in populations other than those investigated in this study.

Genetic variation between the groups revealed different results for mtDNA and microsatellite markers. In mtDNA AMOVA, the genetic variation among groups showed significantly high differentiation (63.05%), but in microsatellites, it was only 25.09%. In contrast, the within-population variability was 17.77% for mtDNA and 74.24% for microsatellites. The low within-population variability of mtDNA may be attributed to rapid population expansion or slow evolution compared with microsatellites [62]. This trend was also observed in *Channa striata* and *G. potanini* [48,63]. The differences in genetic diversity between the mtDNA and microsatellite marker datasets can be explained in three ways. First, nuclear DNA microsatellites may reflect higher genetic diversity and recent bottlenecks than mtDNA [62]. *COI* genes inherited maternally have 2–3-fold lower effective population sizes than microsatellites inherited from both parents [63]. *Odontobutis interrupta* uses a reproductive method in which females select males when their fathers tend to spawn. When the sizes of females decrease, the nucleotide diversity of mtDNA, which is of maternal inheritance, is reduced. Thus, unlike the microsatellites inherited from both parents, mtDNA is more vulnerable to reduced genetic diversity. Second, the small sample size may have prevented the detection of genetic variants with unusual variabilities. Third, when considering the results of H1 haplotype structure, DAPC, population structure, and genotype mixture occurred in almost all water systems. This mixture may have resulted in minor differences in genetic variation between groups.

4.3. Conservation Implications

Genetics is an essential component of conservation strategies [9]. *Odontobutis interrupta* is an endemic species that only inhabits the Korean Peninsula; if it becomes extinct in this region, it will not be found anywhere in the world [63,64]. This study is the first to analyze the genetic diversity and structure of *O. interrupta* populations, which could aid in its conservation.

A species management unit is typically set up by grouping genetically similar populations to avoid genetic perturbations [65]. However, the Seomjingang, Hangang, and Geumgang River water system populations of *O. interrupta* are genetically admixed. Therefore, the SOD, SGD, and GBG populations, which exhibit genetic diversity unique to each water system, should not be introduced to other populations with divergent genetic structures. The SCR population requires protection owing to its low genetic divergence and effective population size. In addition, considering genetic differences, clarifying the origins of translocations, and increasing and sustaining the population size are necessary.

The HWJ, HDM, and HDC populations of the Han River water system represent mixed genotypes with the GGP and GNS populations of the Geumgang River. Therefore, care must be taken when establishing conservation strategies for these populations. In the case of populations in the Hangang and Geumgang River water systems, owing to their small effective population sizes and low genetic diversities, breeding programs should be implemented by selecting individuals unique to each water system [66].

Invasive species could disrupt native ecosystems and negatively affect species occupying similar ecological niches [19,21]. *Odontobutis interrupta* was translocated into the Nakdonggang and Hyeongsangang River water systems (NJH, NMG, HSNC) and can potentially compete with the native *O. platycephala* for spawning. In both species, males build spawning grounds to attract females during the spawning season. Therefore, *O. platycephala*, which initially occurs, may be eliminated through competition, and hybridization may occur, resulting in a decrease in the fertility of the native species [67]. In the case of the NJH and NMG populations, negative values appeared in the neutrality test, which was presumed to be due to their rapid expansion, because they did not show any recent bottlenecks. The rapid expansion of invasive species has the potential to pose a threat to native species [68]. Therefore, genetic and ecological monitoring of *O. interrupta* should be performed to assess their influence on native ecosystems. Future genetic studies of *O.*

interrupta and *O. platycephala* populations in the Nakdonggang and Hyeongsangang River water systems will help to conserve both species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15080913/s1, Table S1: Details of the sampling sites and number of Korean spotted sleeper *Odontobutis interrupta*; Table S2: Summary of microsatellite screening and raw data of sequencing in *Odontobutis interrupta*; Table S3: Details of microsatellite loci in *Odontobutis interrupta*.

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Institutional Review Board Statement: The study was conducted in accordance with the Nakdonggang National Institute of Biological Resources guidelines and regulations. In addition, all experiments were performed in accordance with ARRIVE-related guidelines and the regulations of the Nakdonggang National Institute of Biological Resources. Population samples were collected according to the guidelines of the Nakdonggang National Institute of Biological Resources.

Data Availability Statement: The microsatellite marker sequences were deposited in GenBank (OQ656882–OQ656885). The mitochondrial DNA sequences were deposited in GenBank (OR352370–OR352377).

Conflicts of Interest: There authors declare no conflict of interest.

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