

Development and Characterization of 10 Polymorphic Microsatellite Loci in the Korean Endemic Freshwater Fish *Iksookimia koreensis*, and Their Cross-species Amplification in the Endemic *I. longicorpa*

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ABSTRACT

The genus *Iksookimia* (Actinopterygii: Cypriniformes: Cobitidae) is a bottom-dwelling freshwater loaches, which are well-known as their endemism and high geographic variation. However, population genetic relationships among *Iksookimia* spp. have remained unclear due to a shortage of genetic markers that can be applied generally in the genus. Here, we developed high-resolving microsatellite markers using *I. koreensis* and *I. longicorpa* as representatives of *Iksookimia* species because of their wide distribution range and phylogenetic position. Ten of polymorphic microsatellite loci were isolated from *Iksookimia koreensis* and were successfully cross-amplified in *I. longicorpa*. The mean number of observed alleles per locus was about 10.4 (range, 2–17) for *I. koreensis* and about 13.2 (range, 2–24) for *I. longicorpa*. The loci, *IK03* and *IK08*, deviated from the Hardy-Weinberg equilibrium in *I. koreensis*, after applying the Bonferroni correction. The microsatellite markers obtained in the present study will be useful to evaluate population genetic structure and to establish conservation strategies for *I. koreensis* and related *Iksookimia* species.

Keywords: *Iksookimia*, loaches, marker, microsatellites, population genetics

INTRODUCTION

Iksookimia (Actinopterygii: Cypriniformes: Cobitidae) is a Korean endemic genus of bottom-dwelling freshwater loaches, and includes six allopatrically distributed species (Kim, 1997, 2009). One of these species, *Iksookimia koreensis*, is mostly distributed in the rivers and streams flowing into the Yellow Sea, while *I. longicorpa*, *I. hugowolfeldi*, and *I. yongdokensis* are observed in most streams flowing toward the south and the southeast and *I. pacifica* is distributed in the streams flowing into the East Sea (Kim, 1997, 2009). This particular species distribution suggests that the isolation of river systems might be closely related with *Iksookimia* speciation (Kim, 1997, 2009). Therefore, the phylogenetic relationships within *Iksookimia* should be investigated considering their population genetic structure among the river systems. Unfortunately, previous molecular

studies on this genus focused only on the taxonomic verification in the family Cobitidae (Šlechtová et al., 2008; Kim et al., 2013; Chen et al., 2015; Chen and Chen, 2016; Perdicies et al., 2016). Little is known about regarding population genetic relationships within *Iksookimia* (Yang et al., 1989; Kim et al., 2016) due to the limitation on the resolution of genetic markers to clearly detect genetic variation at the population scale. As a need for new high-resolving genetic markers for this genus has increased, a few of polymorphic microsatellite loci were characterized, but some of these markers failed to be amplified in the other *Iksookimia* species (Bang et al., 2009; Yu et al. 2014). Thus, in the present study, we newly developed additional microsatellite markers which showed high probability of being applied to *Iksookimia* species to identify population genetic relationships within the genus.

Iksookimia koreensis and *I. longicorpa* was chosen as

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the target species of this study based on the phylogenetic relationship within the genus. The genus *Iksookimia* were divided into at least two genetic lineages according to the previous phylogenetic studies based on mitochondrial genes while *I. pacifica* were shown as a basal species in the phylogenetic trees of nuclear genes (Šlechtová et al., 2008; Chen et al., 2015; Chen and Chen, 2016; Perdices et al., 2016). One of the lineages involved *I. koreensis*, *I. pumila*, and *I. pacifica*, and another lineage involved *I. longicorpa*, *I. hugowolfeldi*, and *I. yongdokensis*. Within each of the lineages, *I. koreensis* and *I. longicorpa* are more widely distributed than others, respectively (Kim, 1997, 2009). Interestingly, high geographic variations have been observed in the two *Iksookimia* species (Kim, 1981; Yang et al., 1989), sometimes leading to the delimitation of a new species. Indeed, *I. hugowolfeldi* and *I. yongdokensis* was proposed as a valid species from a subspecies of *I. longicorpa* (Nalbant, 1993; Kim and Park, 1997), and *I. pumila* was suggested as one of *I. koreensis* populations (Yang et al., 1989; Nalbant, 1993). For these reasons, we considered that genetic markers which are developed and characterized from *I. koreensis* and *I. longicorpa* would have a lot of potential for being applied to other *Iksookimia* species.

Candidate microsatellite markers were selected using *I. koreensis* following the steps described in a previous study that developed polymorphic markers for the spined loach *Cobitis lutheri* (Molecular Ecology Resources Primer Development Consortium et al., 2011), currently named *Cobitis nalbanti* (Vasil'eva et al., 2016). The polymorphism of can-

didate microsatellite markers was tested using 27 *I. koreensis* individuals obtained from Gangneung and Samcheok, South Korea. Genotyping of the *I. koreensis* individuals was performed by a model 4300 automatic sequencer (LI-COR, Lincoln, NE, USA) using PCR products obtained under the following conditions: 94°C for 5 min; followed by 30 cycles at 94°C for 30 sec, 50°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 7 min. PCR was performed in 10 µL containing 1 × PCR buffer, 0.2 mM dNTPs, 1 × bovine serum albumin, 5 pmol designed primers, 2 pmol IRD-700 labeled primers (LI-COR) and 0.05 U of nTaq-Tenuto DNA polymerase (Enzynomics, Daejeon, Korea), and 10–50 ng of template. Microsatellite loci were characterized by estimating the Hardy-Weinberg equilibrium (HWE) and gametic equilibrium between loci in GENEPOP 3.2 (Raymond and Rousset, 1995). Expected (H_E) and observed (H_O) heterozygosities were calculated in CERVUS 3.0 (Kalinowski et al., 2007) and significant levels were adjusted using Bonferroni correction for multiple testing (Rice, 1989). Additionally, the frequency of null alleles and the probability of heterozygote deficiency were estimated for some markers which showed significantly low HWE values using CERVUS and GENEPOP (Raymond and Rousset, 1995). The developed microsatellite loci from *I. koreensis* were also tested using *I. longicorpa*. Twenty-seven individuals of *I. longicorpa* collected from the Seomjin River were genotyped and characterized following the methods described above.

Table 1. Characterization of 10 microsatellite markers for *Iksookimia koreensis*

Locus	Primer sequence (5'-3')	Repeat motif	Accession No.	<i>k</i>	Range	H_O	H_E	HWE	F_{null}
<i>IK01</i>	F: M13F-TGAGAGGAGCAAAGTCAGCA R: M13R-CCAGATAAGGCCAGCAGAAG	(CA) ₃₀	KY500071	17	128–178	0.880	0.905	0.6623	+0.0004
<i>IK02</i>	F: M13F-TGTTTCGTTTCTCAGCCAGA R: M13R-CCTCCACACTTCCATCTCT	(CA) ₄ CG(CA) ₂₀	KY500072	10	157–177	0.704	0.815	0.0085	+0.0731
<i>IK03</i>	F: M13F-TTTGTTGTGGCTGACCTCTG R: M13R-CTCGCTGCACAAACACAAAT	(CA) ₁₈	KY500074	13	253–277	0.680	0.903	0.0047 ^a	+0.1361
<i>IK04</i>	F: M13F-CGGCAACACTTCAGGTCA R: M13R-CTTTTGTAAATGCCGCAAAT	(CA) ₁₀	KY500075	2	222–224	0.296	0.425	0.1646	+0.1692
<i>IK05</i>	F: M13F-CTACCATCTGGACCGCTTTC R: M13R-TGGTTACATCCGAACAATCC	(CA) ₁₅	KY500076	17	237–303	0.885	0.891	0.6876	–0.0058
<i>IK06</i>	F: M13F-AATGGCTGTTTATGCTGCT R: M13R-AATTTGAGGAGCCTGTGCGAA	(CA) ₂₄	KY500077	16	145–185	0.696	0.896	0.0066	+0.1246
<i>IK07</i>	F: M13F-AGCCTCGCTGTGTATTTGTG R: M13R-GAAACGCTGTCCAACGTAAA	(CG) ₆ (CA) ₂₀	KY500078	14	187–217	0.889	0.843	0.9829	–0.0369
<i>IK08</i>	F: M13F-ACCCATCTCACATAAACCTG R: M13R-ACAAGACACCAGAACAACCT	(CACG) ₇ (CA) ₁₃	KY500079	15	148–212	0.731	0.894	0.0010 ^a	+0.1004
<i>IK09</i>	F: M13F-AACCATCCTACTGCCAGGAA R: M13R-AAGCACAGAGGAGCCTGAAC	(CA) ₁₃	KY500080	2	149–151	0.481	0.484	1.0000	–0.0065
<i>IK10</i>	F: M13F-ACACGGCATCTCCTCAGAT R: M13R-TTTTTGTTGGTTGCTTTGTG	(CA) ₁₇	KY500081	4	164–170	0.444	0.708	0.0175	+0.2235

k, number of alleles; H_O , observed heterozygosity; H_E , expected heterozygosity; HWE, probability under the assumption of Hardy-Weinberg equilibrium; F_{null} , frequency of null alleles.

^a $p < 0.0050$; after Bonferroni correction.

Table 2. Characterization of 10 microsatellite markers for *Iksookimia longicorpa*

Locus	<i>k</i>	Range	<i>H_o</i>	<i>H_e</i>	HWE
<i>IK01</i>	15	140–192	0.9630	0.8810	0.5881
<i>IK02</i>	24	211–315	1.0000	0.9600	0.5793
<i>IK03</i>	16	269–329	0.7780	0.9160	0.0542
<i>IK04</i>	23	230–308	0.8460	0.9460	0.0224
<i>IK05</i>	12	195–237	0.7780	0.8860	0.0195
<i>IK06</i>	15	125–197	0.7780	0.9010	0.2757
<i>IK07</i>	15	197–245	0.8640	0.8820	0.8660
<i>IK08</i>	11	154–184	0.8000	0.8710	0.1850
<i>IK09</i>	11	149–182	0.8080	0.8000	0.3479
<i>IK10</i>	2	152–160	0.4810	0.5070	1.0000

k, number of alleles; *H_o*, observed heterozygosity; *H_e*, expected heterozygosity; HWE, probability under the assumption of Hardy-Weinberg equilibrium.

RESULTS AND DISCUSSION

Ten polymorphic microsatellite loci were developed and characterized for *I. koreensis* and were successfully amplified in *I. longicorpa* (Tables 1, 2). The mean number of alleles per locus (*k*) was about 10.4 (range, 2–17) for *I. koreensis* and 13.2 (range, 2–24) for *I. longicorpa*. Seven microsatellite loci except for *IK04*, *IK09*, and *IK10* were highly polymorphic in both species. However, *IK03* and *IK08* significantly deviated from HWE ($p < 0.0050$ after Bonferroni correction) in *I. koreensis*. This low HWE might be due to the presence of null allele(s) because these markers showed no heterozygote deficiency (*IK03*, $p = 0.0056$; *IK08*, $p = 0.0576$) but relatively high frequencies of null allele(s) (*IK03*, 0.1361; *IK08*, 0.1004). Significant gametic disequilibrium was not detected among loci ($p < 0.0050$ after Bonferroni correction). The successful amplification and characterization of the newly developed markers suggests that their high applicability for *Iksookimia* species in comparison with the previously developed markers. For example, 11 microsatellite loci were developed from *I. koreensis* in the previous study, but only 7 of them were successfully characterized in *I. longicorpa* (Yu et al., 2014).

The 10 newly developed markers presented here will be valuable for studying the population genetic structure of *I. koreensis* and related *Iksookimia* species, and thus for resolving the relationship between rivers' isolation and *Iksookimia* speciation. Furthermore, these markers will contribute to establish conservation strategies for the Korean endemic genus *Iksookimia*, considering its genetic diversity.

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