ASED http://e-ASED.org
Animal Systematics, Evolution and Diversity

Anim. Syst. Evol. Divers. Vol. 33, No. 2: 136-139, April 2017
https://doi.org/10.5635/ASED.2017.33.2.002

Short communication

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*

Ye-Seul Kwan[1], Hyo-Jin Kim[2], Bit-Na Lee[3], Yong-Jin Won[2,3,*]

[1]Nakdonggang National Institute of Biological Resources, Sangju 37242, Korea
[2]Interdisciplinary Program of Ecocreative, Ewha Womans University, Seoul 03760, Korea
[3]Division of EcoScience, Ewha Womans University, Seoul 03760, Korea

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; Perdices 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

*To whom correspondence should be addressed
Tel: 82-2-3277-4471, Fax: 82-2-3277-4514
E-mail: won@ewha.ac.kr

eISSN 2234-8190

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 candidate 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.	*k*	Range	H_O	H_E	HWE	F_{null}
IK01	F: M13F-TGAGAGGAGCAAAGTCAGCA R: M13R-CCAGATAAGGCCAGCAGAAG	$(CA)_{30}$	KY500071	17	128–178	0.880	0.905	0.6623	+0.0004
IK02	F: M13F-TGTTTCGTTTCTCAGCCAGA R: M13R-CCTCCCACACTTCCATCTCT	$(CA)_4CG(CA)_{20}$	KY500072	10	157–177	0.704	0.815	0.0085	+0.0731
IK03	F: M13F-TTTGTTGTGGCTGACCTCTG R: M13R-CTCGCTGCACAAACACAAAT	$(CA)_{18}$	KY500074	13	253–277	0.680	0.903	0.0047[a]	+0.1361
IK04	F: M13F-CGGCAACACTTCAGGTCA R: M13R-CTTTTGTAATGCCGCCAAAT	$(CA)_{10}$	KY500075	2	222–224	0.296	0.425	0.1646	+0.1692
IK05	F: M13F-CTACCATCTGGACCGCTTTC R: M13R-TGGTTACATCCGAACAATCC	$(CA)_{15}$	KY500076	17	237–303	0.885	0.891	0.6876	−0.0058
IK06	F: M13F-AATGGCTGGTTTATGCTGCT R: M13R-AATTTGAGGAGCCTGTCGAA	$(CA)_{24}$	KY500077	16	145–185	0.696	0.896	0.0066	+0.1246
IK07	F: M13F-AGCCTGCGTGTGTATTTGTG R: M13R-GAAACGCTGTCCAACGTAAA	$(CG)_6(CA)_{20}$	KY500078	14	187–217	0.889	0.843	0.9829	−0.0369
IK08	F: M13F-ACCCATCTCACATAAACCTG R: M13R-ACAAGACACCAGAACAACCT	$(CACG)_7(CA)_{13}$	KY500079	15	148–212	0.731	0.894	0.0010[a]	+0.1004
IK09	F: M13F-AACCATCCTACTGCCAGGAA R: M13R-AAGCACAGAGGAGCCTGAAC	$(CA)_{13}$	KY500080	2	149–151	0.481	0.484	1.0000	−0.0065
IK10	F: M13F-ACACGGCATCTCCTTCAGAT 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	*k*	Range	H_O	H_E	HWE
IK01	15	140–192	0.9630	0.8810	0.5881
IK02	24	211–315	1.0000	0.9600	0.5793
IK03	16	269–329	0.7780	0.9160	0.0542
IK04	23	230–308	0.8460	0.9460	0.0224
IK05	12	195–237	0.7780	0.8860	0.0195
IK06	15	125–197	0.7780	0.9010	0.2757
IK07	15	197–245	0.8640	0.8820	0.8660
IK08	11	154–184	0.8000	0.8710	0.1850
IK09	11	149–182	0.8080	0.8000	0.3479
IK10	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.

ACKNOWLEDGMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and future Planning (2015R1A2A2A01007117) and Korea Science and Engineering Foundation grant funded by the Korean government (MOST) (R01-2007-000-20955-0) to Y.-J. Won.

REFERENCES

Bang IC, Kim WJ, Lee IR, 2009. Characterization of polymorphic microsatellite loci in the endangered Miho spine loach (*Iksookimia choii*) and cross-species amplification within the Cobitidae family. Molecular Ecology Resources, 9:281-284. https://doi.org/10.1111/j.1755-0998.2008.02452.x

Chen Y, Chen Y, 2016. A new species of the genus *Cobitis* (Cypriniformes: Cobitidae) from the Northeast China. Zoological Systematics, 41:379-391. https://doi.org/10.11865/zs.201643

Chen Y, Sui X, Dekui H, Chen Y, 2015. Three new species of cobitid fish genus *Cobitis* (Teleostei, Cobitidae) from the River Pearl basin of China. Folia Zoologica, 64:1-16.

Kalinowski ST, Taper ML, Marshall TC, 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Molecular Ecology, 16:1099-1106. https://doi.org/10.1111/j.1365-294X.2007.03089.x

Kim D, Conway KW, Jeon HB, Kwon YS, Won YJ, 2013. High genetic diversity within the morphologically conservative dwarf loach, *Kichulchoia brevifasciata* (Teleostei: Cobitidae), an endangered freshwater fish from South Korea. Conservation Genetics, 14:757-769. https://doi.org/10.1007/s10592-013-0462-2

Kim D, Hirt MV, Simons AM, Won YJ, 2016. Small fishes crossed a large mountain range: quaternary stream capture events and freshwater fishes on both sides of the Taebaek Mountains. Integrative Zoology. https://doi.org/10.1111/1749-4877.12228

Kim IS, 1981. Geographic variation in the cobitid fish, *Cobitis longicorpus* Kim, choi and Nalbant. Chonbuk National University Basic Science Review, 4:123-128 (in Korean).

Kim IS, 1997. Illustrated encyclopedia of fauna and flora of Korea. Vol. 37. Freshwater fishes. Ministry of Education, Seoul, pp. 1-629 (in Korean).

Kim IS, 2009. A review of the spined loaches, family Cobitidae (Cypriniformes) in Korea. Korean Journal of Ichthyology, 21(Supplement):7-28.

Kim IS, Park JY, 1997. *Iksookimia yongdokensis*, a new cobitid species (Pisces, Cobitidae) from Korea with a key to the species of *Iksookimia*. Ichthyological Research, 44:249-256. https://doi.org/10.1007/BF02678704

Molecular Ecology Resources Primer Development Consortium, Agata K, Alasaad S, Almeida-Val VMF, Álvarez-Dios JA, Barbisan F, Beadell JS, Beltrán JF, Benítez M, Bino G, Bleay C, Bloor P, Bohlmann J, Booth W, Boscari E, Caccone A, Campos T, Carvalho BM, Climaco GT, Clobert J,

Congiu L, Cowger C, Dias G, Doadrio I, Farias IP, Ferrand N, Freitas PD, Fusco G, Galetti PM, Gallardo-Escárate C, Gaunt MW, Ocampo ZG, Gonçalves H, Gonzalez EG, Haye P, Honnay O, Hyseni C, Jacquemyn H, Jowers MJ, Kakezawa A, Kawaguchi E, Keeling CI, Kwan YS, La Spina M, Lee WO, Leśniewska M, Li Y, Liu H, Liu X, Lopes S, Martinez P, Meeus S, Murray BW, Nunes AG, Okedi LM, Ouma JO, Pardo BG, Parks R, Paula-Silva MN, Pedraza-Lara C, Perera OP, Pino-Querido A, Richard M, Rossini BC, Samarasekera NG, Sánchez A, Sanchez JA, Henrique C, dos Anjos Santos CH, Shinohara W, Soriguer RC, Sousa ACB, da Silva Sousa CF, Stevens VM, Tejedo M, Valenzulea-Bustamante M, Van de Vliet MS, Vandepitte K, Vera M, Wandeler P, Wang W, Won YJ, Yamashiro A, Yamashiro T, Zhu C, 2011. Permanent genetic resources added to Molecular Ecology Resources Database 1 December 2010−31 January 2011. Molecular Ecology Resources, 11:586-589. https://doi.org/10.1111/j.1755-0998.2011.03004.x

Nalbant TT, 1993. Some problems in the systematics of the genus *Cobitis* and its relatives (Pisces, Ostariophysi, Cobitidae). Review Roumain de Biologie Animals, 38:101-110.

Perdices A, Bohlen J, Šlechtová V, Doadrio I, 2016. Molecular evidence for multiple origins of the European spined loaches (Teleostei, Cobitidae). PLos One, 11:e0151228. https://doi.org/10.1371/journal.pone.0151228

Raymond M, Rousset F, 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity, 86:248-249. https://doi.org/10.1093/oxfordjournals.jhered.a111573

Rice WR, 1989. Analyzing tables of statistical tests. Evolution, 43:223-225.

Šlechtová V, Bohlen J, Perdices A, 2008. Molecular phylogeny of the freshwater fish family Cobitidae (Cypriniformes: Teleostei): delimitation of genera, mitochondrial introgression and evolution of sexual dimorphism. Molecular Phylogenetics and Evolution, 47:812-831. https://doi.org/10.1016/j.ympev.2007.12.018

Vasil'eva ED, Kim D, Vasil'ev VP, Ko MH, Won YJ, 2016. *Cobitis nalbanti*, a new species of spined loach from South Korea, and redescription of *Cobitis lutheri* (Teleostei: Cobitidae). Zootaxa, 4208:577-591. https://doi.org/10.11646/zootaxa.4208.6.5

Yang SY, Park BS, Kim JH, 1989. Systematic studies of the genus *Cobitis* (Pisces: Cobitidae) in Korea I. Geographic variations and classification of *Cobitis koreensis*. The Korean Journal of Zoology, 32:242-251.

Yu JN, Kim BJ, Yun YE, Kim HS, Bae CH, Kim S, Oh K, Lim CE, 2014. Characterization of 11 novel polymorphic microsatellite loci in the threatened Korean loach, *Iksookimia koreensis*, isolated using a next-generation sequencing method. Biochemical Systematics and Ecology, 56:132-137. https://doi.org/10.1016/j.bse.2014.05.016

Received February 22, 2017
Revised April 22, 2017
Accepted April 22, 2017