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Development of polymorphic microsatellite loci in *Odontobutis obscura* using Illumina paired-end sequencing and the test of cross-species amplification

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ABSTRACT. *Odontobutis obscura* is a bottom-dwelling freshwater fish native to East Asia. Its range encompasses southwest China, western Japan, and Geoje Island in South Korea. Despite its widespread range in China and Japan, only a small and spatially isolated population is found in South Korea. We developed a total of 23 novel and polymorphic

microsatellite loci of *O. obscura* using Illumina paired-end shotgun sequencing and characterized them using 80 Japanese and Korean samples. An extensive genetic polymorphism was detected at these 23 loci, with the observed number of alleles at a locus ranging from 2 to 15 and expected and observed heterozygosities ranging from 0 to 0.656 and 0 to 0.547, respectively. Korean *O. obscura* exhibited a much lower level of genetic variability than the Japanese population did, probably as a result of long-term isolation combined with historical bottlenecks. The Japanese and Korean populations showed a high level of genetic differentiation with F_{ST} = 0.700 and R_{ST} = 0.913. Many of our primer sets were successfully transferable to congeneric *O. interrupta* and *O. platycephala*, which exhibited even greater polymorphism than Korean *O. obscura*. In conclusion, our study showed that these 23 microsatellite markers are useful for understanding the conservation biology and population genetic structure of *O. obscura* and other congeneric species.

Key words: Microsatellites; Illumina; *Odontobutis obscura*; Odontobutidae; Cross-species amplification

INTRODUCTION

The dark sleeper, *Odontobutis obscura* Temminck & Schlegel (Odontobutidae: Perciformes) is a bottom-dwelling freshwater fish native to East Asia. Its range encompasses southwest China, western Japan, and Geoje Island, which is situated near the southern tip of South Korea (Iwata et al., 1985; Wu et al., 1993; Sakai et al., 1998). Despite its widespread range in China and Japan, the Korean population is small and spatially isolated (Chae, 1999). It is currently designated as endangered under the Protection Act of Wild Fauna and Flora by the Korean Ministry of Environment, and its natural habitats have been progressively damaged by anthropogenic modification and fragmentation, possibly leading to demographic decline. A comprehensive genetic study of *O. obscura*, including the Korean population, is critically needed, since such a scattered geographic structure may preserve important historical imprints of postglacial colonization and geological events throughout East Asia. In addition, information on genetic variability could provide a fundamental framework for the design of long-term conservation plans for the endangered Korean *O. obscura*. Previous genetic studies using allozymic and mitochondrial tools showed that Japanese *O. obscura* could be divided into four geographic groups: West Kyushu, West Seto, East Seto, and Sanin-Biwa-Ise (Sakai et al., 1998; Mukai and Nishida, 2003). Korean *O. obscura* showed relatively higher genetic affinity to East Seto than any other Japanese geographic groups in a genetic study based on 18 allozyme markers (Sakai et al., 2002).

Highly variable microsatellite loci may be a promising solution for quantifying the genetic diversity of the endangered and isolated Korean *O. obscura* and for assessing the spatial genetic structure among populations throughout East Asia. In the present study, a total of 23 novel and polymorphic microsatellite markers of *O. obscura* were developed and characterized using Illumina paired-end sequencing. Two basic tests were performed based on these microsatellites to determine 1) whether the isolated Korean population is characterized by low genetic variability and 2) whether the Korean population is differentiated from Japanese conspecific samples. In addition, cross-species amplification of these 23 loci was attempted in three other odontobutid species: *O. interrupta*, *O. platycephala*, and *Micropercops swinhonis*.

MATERIAL AND METHODS

Sampling

A total of 80 samples (42 Korean and 38 Japanese samples) of *O. obscura* were used for the genetic analyses. The Korean samples were collected from Sanyang River in Geoje Island, South Korea, in 2011 and 2015 with the official permission of a local environmental office of the Korean Ministry of Environment. According to the rules prescribed in the permission for the 2015 samples, tissues were obtained by removing a 1 x 1-mm fin-clip from the tip of each tail fin, and all individuals were returned to the collection site. The fin-clip tissues were immediately stored in 95% ethanol. A total of 38 Japanese samples were collected from the Tabe River, a tributary of the Koya River located in Shimonoseki, Yamaguchi Prefecture (Western Honshu), Japan, in 2012. The specimens of three related species stored in Yeungnam University, *O. interrupta* (N = 16; collected from Nakdong River, South Korea, in 2011), *O. platycephala* (N = 16; collected from Nakdong River, South Korea, in 2011) and *M. swinhonis* (N = 8; collected from Jilnal swamp, South Korea, in 2010) were used in the test of cross-species amplification. Our tissue sampling and population genetic analyses were approved by the Yeungnam University Institutional Animal Care and Use Committee (protocol #2015013). DNA for genotyping analysis was isolated using a Wizard Genomic DNA Purification Kit (Promega; Madison, WI, USA) following the manufacturer protocol for animal tissue with proteinase K.

Isolation of genomic microsatellites using Illumina paired-end sequencing

For genomic microsatellite isolation, total genomic DNA of a single individual (from the Korean population) was extracted with a DNeasy Blood and Tissue Kit following the manufacturer protocol (Qiagen, Dusseldorf, Germany). One microgram of genomic DNA was sheared in a hydrodynamic shearing system (Covaris, Woburn, MA, USA) to produce fragments of less than 800 bp, and the remaining overhangs were enzymatically removed. Following adenylation of 3'-ends of the fragments, the libraries were constructed using an Illumina Paired-End DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) following manufacturer protocols. The libraries constructed were electrophoresed on 1% agarose gel to extract fragments of approximately 500 bp in length. The purity of eluted fragments was checked using a Quant-iT™ dsDNA High Sensitivity Assay Kit (Invitrogen, Carlsbad, CA, USA) on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The final library preparation was subjected to pair-end sequencing with 100 bp read length in the HiSeq 2500 platform (Illumina). Raw image files were processed using Illumina Real-Time Analysis (RTA) for image analysis and base calling.

De novo genome assembly was performed using the de Brujin graph algorithm and the SOAPdenovo2 software package (v. 2.04) with the default settings, except for the *k*-mer value (Luo et al., 2012). Genome size was estimated based on the *k*-mer frequency spectrum, and a 17-mer assembly was finally chosen by adjusting various parameters. Repetitive DNA sequences, such as microsatellites, transposable elements, and *r*DNAs, were searched using RepeatMasker and RepeatModeler (http://www.repeatmasker.org/; Smith et al., 2014).

SSR Finder (ftp://ftp.gramene.org/pub/gramene/archives/software/scripts/; Stieneke and Eujayl, 2007) was used to identify reads containing di-, tri-, tetra-, penta-, and hexa-nucleotide microsatellite motifs. Reads were annotated as perfect microsatellite fragments if they contained tandem repeats of at least 12 bp in length. Once positive reads were detected,

primer pairs were designed using Primer3 (http://fokker.wi.mit.edu/primer3/; Untergasser et al., 2012) with default parameters. A total of 80 primer pairs exhibited di- and tetranucleotide repeat motifs and were synthesized for the assessment of amplification and polymorphism.

Microsatellite genotyping

Eighty candidate fragments containing 40 di- and 40 tetranucleotides were examined for amplification and polymorphism using 16 *O. obscura* individuals (8 Korean and 8 Japanese). PCR was performed in a 10-μL reaction mixture containing 10-25 ng genomic DNA extract, 5 pmol dNTP mix, 10 pmol each primer, 1X PCR buffer and 1.25 U *Taq* polymerase (Genetbio, Daejeon, South Korea) under the following thermal cycling conditions (GenePro, Bioer, Hangzhou, China): an initial denaturation at 94°C for 5 min followed by 30 cycles each of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and an extension at 72°C for 45 s, with a final extension at 72°C for 10 min. The amplified fragments were electrophoresed on 2% agarose gel and stained in 0.5 μg/mL ethidium bromide solution. The fragments were considered as confident marker loci if clear polymorphic bands were observed on the gel. The forward primers of confident marker loci were labeled with a fluorescent dye of 6-FAM, VIC, NED, or PET (Applied Biosystems, Foster City, CA, USA; Table 1). The fluorescence-labeled PCR products were fragmented on an ABI3730XL genetic analyzer following the standard protocol (Applied Biosystems) for Macrogen Inc. (Seoul, South Korea). Allele peaks were scored using GeneMapper 3.7 (Applied Biosystems) and Peak Scanner 1.0 (Applied Biosystems).

Microsatellite diversity

FSTAT 2.9.3.2 (Goudet, 2001) and Arlequin 3.5 (Excoffier and Lischer, 2010) were used to estimate various microsatellite diversity indices, including average number of alleles per locus, allelic richness, observed (H_O) and expected heterozygosities (H_E), and inbreeding coefficient (F_{IS}). Mean null allele frequencies per locus were examined by testing the significance using 10,000 bootstrap replicates in the FreeNA software (Chapuis and Estoup, 2007). MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004) was also used to check for genotyping accuracy by detecting the likelihood of large allele dropout or stuttering. The deviations from genotypic proportions expected under Hardy-Weinberg equilibrium (HWE) were tested for each locus with the exact test following Markov chain parameters, with 1000 batches and 10,000 iterations per batch (Guo and Thompson, 1992) implemented in GENEPOP 4.2 (Raymond and Rousset, 1995). The linkage disequilibrium between each pair of microsatellite loci was detected by Fisher exact tests under the Markov chain algorithm implemented in GENEPOP. The level of statistical significance for simultaneous multiple HWE tests was corrected using sequential Bonferroni adjustments (Rice, 1989). ML-Relate (Kalinowski et al., 2006) was employed to simulate the relatedness (unrelated, full-sibling, half-sibling, and parent-offspring) of all possible pairs of individuals within populations (95% confidence set with 10,000 randomizations). The genetic differentiation between the two populations of *O. obscura* was assessed based on pairwise-F_{ST} and -R_{ST} estimates in Arlequin and testing the significance by Fisher exact tests after 10,000 permutations. SPAGeDi 1.5 (Hardy and Vekemans, 2002) was used to examine the contribution of stepwise mutation to genetic differentiation between populations by testing whether the observed R_{ST} value was significantly greater than the permutated R_{ST} values (pR_{ST}; 20,000 permutations) calculated from the genotype data (Hardy et al., 2003).

Table 1. Details of 23 polymorphic microsatellite loci developed for *Odontobutis obscura*.

Locus	Motif	Allele size range (bp)	Primer (5'-3') forward (above) and reverse (below)	Ta (°C)	N_A	H_O	H_E	F_{null}	P_{HWE}	F_{IS}	Accession No.
Oob2	$(AC)_{23}$	106-166	ATTACGAGACTGTCCCAGACC	58	14	0.547	0.656	0.051	0.046	0.169	KU163394
			GACCAGACTCTGATGCTATTGG								
Oob3	$(AC)_{22}$	142-178	CAGGCGTCAAAAACATGC	58	12	0.367	0.388	0.000	0.183	0.054	KU163395
			GGTGATGGCAGGACCAAG								
Oob4	$(AC)_{22}$	101-166	GCTGGTTAATAGTGTACCACGG	58	9	0.457	0.613	0.090	0.088	0.256	KU163396
			CTGTGGGTCCCATCTGTC								
Oob6	$(AC)_{22}$	115-139	CAGAAAATCCTTCCCACTGC	58	6	0.382	0.374	0.001	0.258	-0.019	KU163397
			TCTGTTTCCTCACTCTGACTCC								
Oob7	$(AC)_{22}$	145-189	CTCATCAGTGTGATCATGGG	58	3	0.060	0.078	0.029	0.235	0.233	KU163398
			ACACTGTGCATGTGTACGTCTC								
Oob8	$(AC)_{22}$	143-182	GCTCCTGTCAAGATTGTACACC	58	5	0.289	0.330	0.024	0.596	0.122	KU163399
			TGTAACCTGGCTGGTTGTTC								
Oob9	$(AC)_{22}$	159-203	CACCTAGGCTTCTACAAAGAGC	58	13	0.342	0.377	0.004	0.148	0.093	KU163400
			CTGAGCGACTGAATAGCAGC								
Oob10	$(AC)_{22}$	89-138	GAGAGCCAATGACGAGAGAC	58	7	0.326	0.372	0.023	0.962	0.122	KU163401
			CTGCTGCTGGTGGTTATTG								
Oob11	$(AC)_{22}$	139-151	ACACAGACCTCAACAATCGC	58	5	0.368	0.361	0.001	0.991	-0.021	KU163402
			AGAACCAGGAATCGACACTAGC								
Oob13	$(AC)_{22}$	152-200	TGTAGCAGTACCGATCTGGG	58	8	0.368	0.401	0.018	0.467	0.082	KU163403
			CTCATCTGCATGTGTCTACGTG								
Oob16	$(AC)_{22}$	174-282	CACTGGTAACAATAGGCTGTGG	58	9	0.197	0.195	0.001	0.435	-0.012	KU163404
			GTGAGAGGTTACTGGAGTCCAAC								
Oob17	$(AC)_{22}$	139-206	AGATAGCAGTTCTGCCACTCAG	58	6	0.316	0.310	0.017	0.096	-0.020	KU163405
			GATGCAATAGTGATGCAGGG								
Oob18	$(AC)_{21}$	174-213	ACTATTGTCAGACCTGCTTCCC	58	15	0.452	0.519	0.031	0.196	0.127	KU163406
			AGACAGAGCTGAATGAGAGGAG								
Oob19	$(AC)_{21}$	107-144	CAGCTCAAACGCTATCCATC	58	3	0.013	0.039	0.050	0.039	0.660	KU163407
			CACTGGGAAGTAATGAGCAGAG								
Oob20	$(AC)_{21}$	143-180	GCTCGAGAATCCTCTCAGTATC	58	8	0.342	0.401	0.023	0.273	0.147	KU163408
			CTGTGAAGGCTGCTGTTCTC								
Oob23	$(AGAT)_{11}$	96-193	CCTCTCACTCACAGTTAGTCCAC	58	4	0.214	0.172	0.001	0.343	-0.243	KU163409
			GCTACAACTGATACCGATACCC								
Oob25	$(ATAG)_{11}$	142-180	CCAAATGGCTCATACCAGTC	58	6	0.313	0.318	0.000	0.799	0.016	KU163410
			CTGTGTATGGATGTGTGTGTGG								
Oob31	$(GATT)_{10}$	165-189	ACACTGCTAAAGGTGTGGGTAG	58	4	0.079	0.074	0.001	1.000	-0.060	KU163411
			ATGTGTCTGAGGTCCCTGATAG								
Oob32	$(TACA)_{10}$	100-150	TATGTCTATCGTGGCCTCTG	58	6	0.186	0.304	0.078	**0.002***	0.389	KU163412
			AGCTGCATGCTGTTGTCTG								
Oob33	$(TCAT)_{10}$	147-187	GCTGTAACATAGCTGCTGAACG	58	3	0.190	0.225	0.024	0.483	0.155	KU163413
			CTACTGGTGCCAATACAGCAC								
Oob35	$(TCTG)_{10}$	156-193	GTGAGAGAAACCTCGTGCATAC	58	5	0.049	0.048	0.000	1.000	-0.012	KU163414
			AGACTGTGTCAGAAGTGTGTCG								
Oob37	$(AAAT)_9$	173-197	GTCTGAGGCTTAATGTGACACC	58	9	0.434	0.411	0.001	0.684	-0.057	KU163415
			GGACCTTAATCCAGGTCAGAAC								
Oob38	$(AAAT)_9$	185-191	GCTTCACCGACCCTTTATAC	58	2	0	0	0.001	-	1.000	KU163416
			TTGGAGTTGACAGGTGACAG								

Data comprise locus name, repeat motif, allele size range, forward and reverse primer sequences, annealing temperature (Ta), number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities, frequency of null allele calculated using FreeNa (F_{null}), significance level of deviation from Hardy-Weinberg equilibrium (P_{HWE}), inbreeding coefficient (F_{IS}) and GenBank accession number. Microsatellite diversity indices were quantified using 80 Japanese and Korean samples. *P_{HWE} showing significance after Bonferroni correction was highlighted with bold.

RESULTS

Of the 80 primer sets tested, 52 were amplified reliably. Among these, 23 showed clear polymorphism and were labeled with fluorescent dyes. In the 80 Japanese and Korean *O. obscura* samples, extensive genetic polymorphism was detected at 23 loci, with the observed number of alleles at a locus ranging from 2 (*Oob38*) to 15 (*Oob18*) and expected and observed heterozygosities ranging from 0 (Oob38) to 0.656 (*Oob2*) and 0 (*Oob38*) to 0.547 (*Oob2*), respectively (Table 1). Although two alleles were detected in *Oob38*, each allele was completely fixed to either the Korean or Japanese population. A significant deviation from HWE was detected only at a single locus, *Oob32*, following Bonferroni correction (N = 23; $\alpha = 0.002$) for multiple testings (Table 1). The frequency of null alleles per locus was estimated to be less than 0.1 based on the FreeNA results. No evidence of amplification errors was found in the tests by MicroChecker. No signature of linkage disequilibrium was detected following the Bonferroni correction, indicating that there was no physical linkage among the loci examined in this study.

At the population level, Korean *O. obscura* exhibited a much lower level of genetic variability than the Japanese population did (Table 2). The Korean population contained only a single allele at 13 loci examined (mean number of alleles = 1.61), whereas polymorphism was found across 19 loci in the Japanese population (mean number of alleles = 5.70; Table 2). Maximum likelihood estimation of relatedness (ML-Relate) also demonstrated higher levels of family relationship (full-sibling, half-sibling, and parent-offspring) in the Korean population (34.38%) than in the Japanese population (11.10%). Our 23 loci showed a single-parent and parent-pair exclusion probability of more than 0.99, which allows reliable estimation of paternity in the Japanese population. A direct comparison of genetic variation solely based on several microsatellite loci can be problematic, since different levels of variation can be generated when different loci are used. In our study, however, many loci were used, and the Korean population consistently showed only a couple of alleles at all loci examined. Such severely reduced genetic variability in the Korean *O. obscura* may be attributable to geographic isolation for long periods of time combined with historical bottlenecks from its small population size. To confirm our results regarding genetic diversity, microsatellite loci isolated from congeneric species (Shen et al., 2015) could also be used.

The Japanese and Korean populations showed a high level of genetic differentiation, with $F_{ST} = 0.700$ and $R_{ST} = 0.913$. No allele was shared by the two populations at 16 of the 23 loci (69.5%). Stepwise mutations have likely contributed to the microsatellite differentiation between two populations, since the value of R_{ST} was significantly greater than the mean permuted R_{ST} value (SPAGeDi; $pR_{ST} = 0.719$; P = 0.001), indicating a long history of geographic isolation between two regions. Although strong differentiation was predicted between the two areas, these values are greater than generally expected from a comparison among intraspecific populations, suggesting the need for further taxonomic reevaluation of Korean *O. obscura*. However, the genetic differentiation quantified in our study is not likely to represent the exact level of historical isolation of Japanese and Korean *O. obscura*, since it was not based on extensive investigation of Japanese populations throughout their entire range. In the future, the microsatellites we identified could be used to investigate population genetic structure among Japanese and Korean populations, confirming the results obtained from allozymic and mitochondrial analyses (Sakai et al., 1998, 2002; Mukai and Nishida, 2003).

Eleven primer sets were successful in the amplification of *O. interrupta* samples (Table 2), although three of them (*Oob9*, *Oob11*, and *Oob16*) showed a significant deviation

from HWE following Bonferroni correction. *O. platycephala* samples showed reliable amplification at 19 primer sets (Table 2), and deviation from HWE was only detected at a single locus (*Oob7*). Only four loci were amplified in *M. swinhonis*, and no polymorphism was found (Table 2).

Table 2. Diversity indices of four odontobutid species, *Odontobutis obscura*, *O. interrupta*, *O. platycephala*, and *Micropercops swinhonis*, quantified using 23 microsatellites developed in this study.

Locus	*O. obscura*						*O. interrupta*			*O. platycephala*			*M. swinhonis*		
	Korea			Japan											
	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E
Oob2	3	0.357	0.466	13	0.737	0.845	-			14	1	0.915	-		
Oob3	2	0.024	0.024	10	0.711	0.752	-			-			-		
Oob4	2	0.31	0.441	7	0.605	0.781	-			11	0.875	0.891	-		
Oob6	1	0	0	5	0.763	0.749	-			10	0.938	0.901	-		
Oob7	2	0.119	0.155	1	0	0	3	0.438	0.524	7	0.563	0.806	1	0	0
Oob8	1	0	0	4	0.579	0.659	-			4	0.563	0.595	1	0	0
Oob9	1	0	0	12	0.684	0.754	10	0.563	0.821	2	0.063	0.063	-		
Oob10	2	0.048	0.047	5	0.605	0.696	11	0.813	0.831	5	0.688	0.611	-		
Oob11	1	0	0	5	0.737	0.722	10	0.6	0.834	-			-		
Oob13	1	0	0	8	0.737	0.802	4	0.438	0.609	11	0.813	0.905	-		
Oob16	1	0	0	8	0.395	0.391	9	0.5	0.815	19	1	0.956	1	0	0
Oob17	1	0	0	5	0.632	0.62	8	0.75	0.726	5	0.75	0.766	1	0	0
Oob18	2	0.167	0.155	15	0.737	0.883	2	0.063	0.063	11	0.875	0.871	-		
Oob19	1	0	0	2	0.026	0.077	1	0	0	1	0	0	-		
Oob20	1	0	0	7	0.684	0.801	-			1	0	0	-		
Oob23	3	0.429	0.346	1	0	0	1	0	0	1	0	0	-		
Oob25	3	0.048	0.047	3	0.579	0.59	-			1	0	0	-		
Oob31	1	0	0	3	0.158	0.149	-			1	0	0	-		
Oob32	1	0	0	5	0.371	0.605	-			-			-		
Oob33	2	0.381	0.45	1	0	0	-			1	0	0	-		
Oob35	3	0.071	0.07	2	0.026	0.026	2	0.063	0.063	7	0.625	0.688	-		
Oob37	1	0	0	8	0.658	0.638	-			-			-		
Oob38	1	0	0	1	0	0	-			2	0.063	0.063	-		

Data comprise number of alleles (N_A), observed (H_O), and expected heterozygosities (H_E).

Considering that these 23 microsatellite loci were developed from *O. obscura*, it is particularly intriguing that *O. interrupta* and *O. platycephala* showed much greater genetic variation in the loci that were successfully amplified than did Korean *O. obscura* (Table 2), supporting the extremely monomorphic nature of the Korean population. Genetic diversity has been recognized as a critical factor affecting a population's fitness and potential persistence (Frankham, 2005; Bouzat, 2010; Furlan et al., 2012). The genetic data in this study have fundamental implications for the conservation status of Korean *O. obscura*, and urgent management strategies for this population are needed to alleviate the further loss of genetic variation and the negative effects of inbreeding and genetic drift (see also Furlan et al., 2012). In the Japanese populations, our markers could be applied for the identification of lineages (geographic groups) or for tests of reproductive isolation. Taken together, our studies showed that 23 newly developed microsatellite markers are useful for understanding the conservation biology and population genetic structure of *O. obscura* and other congeneric species.

Conflicts of interest

The authors declare no conflict of interest.

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