Genetics and Molecular Research

Polymorphic microsatellite markers for the endangered fish, the slender shiner *Pseudopungtungia tenuicorpa* and cross-species amplification across five related species

K.S. Kim[1], S.J. Moon[2], S.H. Han[2], K.Y. Kim[3] and I.C. Bang[2]

[1]East Sea Environment Research Center, East Sea Research Institute, Korea Institute of Ocean Science & Technology, Uljin, Republic of Korea
[2]Department of Life Science & Biotechnology, Soonchunhyang University, Asan, Republic of Korea
[3]AquaGenTech Co., Ltd., Busan, Republic of Korea

Corresponding author: I.C. Bang
E-mail: incbang@sch.ac.kr

Genet. Mol. Res. 15 (3): gmr.15038496
Received January 26, 2016
Accepted March 18, 2016
Published September 2, 2016
DOI http://dx.doi.org/10.4238/gmr.15038496

ABSTRACT. The slender shiner *Pseudopungtungia tenuicorpa* (Cypriniformes; Cyprinidae; Gobioninae) is an endangered freshwater fish species endemic to Korea. The current strategies for its conservation involve the study of population genetic characters and identification of management units. These strategies require suitable molecular markers to study genetic diversity and genetic structure. Here, we developed nine polymorphic microsatellite markers for *P. tenuicorpa* for the first time by applying an enrichment method from a size-selected genomic library. The developed microsatellite markers produced a total of 101 alleles (average 11.2). The observed and expected heterozygosities

averaged 0.805 and 0.835, respectively. Among the nine identified markers, five markers showed successful amplification across five related Korean Gobioninae species. Thus, the microsatellite markers developed in this study will be useful to establish conservation strategies for both *P. tenuicorpa* and other related species.

Key words: *Pseudopungtungia tenuicorpa*; Microsatellite; Endangered species; Genetic diversity; Cross-species amplification; Conservation

INTRODUCTION

The slender shiner *Pseudopungtungia tenuicorpa* (Cypriniformes; Cyprinidae; Gobioninae) is a freshwater fish endemic to Korea. This species resides in gravel and rock in the upper and middle layers of rapid streams. It attracted much attention due to a recent report of its egg deposition in the spawning grounds of the Korean aucha perch *Coreoperca herzi* (Lee et al., 2014). *P. tenuicorpa* is strongly threatened by anthropogenic disturbances, including river channel development and gravel mining. It has been designated as an endangered species by the Korean Ministry of Environment according to Wildlife Protection and Management Act (ME, 2012) and the near threatened category in the Red Data Book in 2012 (NIBR, 2012).

The current strategies for conservation of endangered fish species involve the study of population genetic characters and identification of management units. However, no studies of *P. tenuicorpa* have been conducted. Thus, it is necessary to develop efficient molecular markers to analyze its genetic characteristics. Microsatellite markers are a popular genetic tool for studies of population genetics, because the simple sequence regions (SSRs) contained within the microsatellite markers usually have a high degree of polymorphism and are co-dominantly inherited (Liu and Cordes, 2004; Duran et al., 2009). Development of microsatellite markers generally involves much labor and high costs due to the construction of the enriched SSR library, which inevitably involves processes such as cloning, Sanger sequencing, and fluorescence primer design (Zane et al., 2002; Zalapa et al., 2012). However, many studies have demonstrated that cross-species amplification of already developed microsatellite markers may also enable the use in closely related species (e.g., Kang et al., 2012; Wang et al., 2015).

In this study, we developed microsatellite markers of *P. tenuicorpa* for the first time and applied them to study its population genetic diversity. Moreover, we tested their usefulness by cross-species amplification across five closely related fish species belonging to the subfamily Gobioninae.

MATERIAL AND METHODS

Specimen collection and DNA preparation

A total of 29 specimens of *P. tenuicorpa* were collected from the Dal stream, a tributary of the Han River after permission from Ministry of Environment, Korea. A piece of the pectoral fin from each specimen was preserved in absolute ethanol and kept at -20°C until genomic DNA (gDNA) extraction. gDNA was extracted with TNES-urea buffer using

the phenol-chloroform method (Asahida et al., 1996). The quantity and quality of gDNA was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and a 1.5% agarose gel.

Construction of microsatellite-enriched library

A microsatellite-enriched library with GT repetitive sequences was constructed as described by Hamilton et al. (1999) with slight modifications. Approximately 2 µg gDNA was digested with a restriction enzyme, *Alu*I. Fragments in the range of 200-600 bp were purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The purified fragments were ligated to the SNX adaptor and amplified by polymerase chain reaction (PCR). Thereafter, the PCR products were hybridized with a biotin-labeled $(GT)_{10}$ repeat oligo-nucleotide and captured on streptavidin-coated magnetic beads (Promega, Madison, WI, USA). After elution, the microsatellite-enriched fragments were again amplified by PCR and purified using the QIAquick Gel Extraction Kit (Qiagen).

Cloning, selection, and sequencing

The purified microsatellite-enriched fragments were ligated into the pUC18 vector (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) and transformed into XL1-Blue MRF strain cells (Stratagene, Inc., La Jolla, CA, USA). Positive clones were screened by colony PCR using $(GT)_{10}$ repeat oligo-nucleotides with universal M13 primers. Only the positive clones that produced multiple or smearing PCR bands were selected. Plasmid DNAs were purified from 236 identified positive colonies using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced using universal M13 primers on an ABI 3730*xl* DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Primer design and genotyping

Primers were designed using the Primer3 software (Rozen and Skaletsky, 2000) based on flanking regions. Microsatellite markers were amplified in a 20-µL volume containing approximately 50 ng gDNA and 0.5 µM each primer with *AccuPower*® PCR Premix (Bioneer, Seoul, Korea). The PCR conditions were as follows: 10 min at 94°C, followed by 34 cycles of 45 s at 94°C, 45 s at the annealing temperature optimal for each primer pair (see Table 1), 30 s at 72°C, and a final extension at 72°C for 5 min. The PCR products were subsequently separated on 1.5% agarose gels. For their fluorescent detection, primers were labeled with fluorescent dyes, 6-FAM, HEX, and NED (Applied Biosystems) at the 5' end of the forward primer. Polymorphisms of developed microsatellite markers were screened using an ABI 3730*xl* DNA Analyzer. Allele sizes of the PCR products were assessed with reference to a molecular size marker (GeneScan™ 400 HD; Applied Biosystems). Fluorescent DNA fragments were analyzed using the Peak Scanner™ 1.0 software (Applied Biosystems).

Cross-species amplification

Cross-species amplification tests were performed with gDNA of three selected specimens inhabiting the River *Pseudopungtungia nigra* (N = 6), *Gobiobotia naktongensis* (N

= 9), *Gobiobotia macrocephala* (N = 6), *Gobiobotia brevibarba* (N = 6), and *Microphysogobio koreensis* (N = 6). The PCR and genotyping conditions were the same as described earlier.

Table 1. Characteristics of nine microsatellite loci identified from *Pseudopungtungia tenuicorpa*.

Locus	Motif repeats	Primers (5'→3')	Ta (°C)	N_A	H_O	H_E	PIC
PTms105	$(GT)_{22}$	F: TCAAAGTGCTCATCTTCCAGG R: CAGAGTTGGAGACAGTTGGTTCA	58	19	0.897	0.940	0.918
PTms119	$(CA)_{22}$	F: AAAGGGTGGGACAACTCCAT R: CCTGATAGGAGGAAGTGCCC	60	16	0.931	0.901	0.876
PTms120	$(GT)_7$…$(GT)_2$ $(GC)_2(GT)_7$	F: TCCACTGATTCGGTTCTATCG R: TTTACTGCCCCCACTGTTTG	58	5	0.586	0.668	0.601
PTms138	$(CA)_{15}$	F: CCTCGTGTGGTAAGGGGAAG R: CGCCACTGAGGTTTTCTTCA	58	15	0.862	0.903	0.878
PTms150	$(CA)_{16}$…$(CA)_3$ $TA(CA)_{15}$	F: CTCCCCACATCTCTGGCAT R: TCCTCACTTTCTGTTCCTGCAT	58	13	0.897	0.919	0.895
PTms155	$(CA)_8$	F: CACCTCACCATCTCACGAGC R: GCCTGGCGTTGTCAGTTAAA	58	5	0.759	0.744	0.686
PTms177	$(CA)_{11}$	F: GACCTCAGCATGGACCACAG R: TCAGTTCTATATGTGTATGATGCCG	58	8	0.759	0.831	0.791
PTms185	$(GT)_{19}$	F: GCACCACCTCGAAAACGATA R: TGAATCAGGACATGCACACG	58	15	0.862	0.927	0.904
PTms239	$(CA)_{13}$	F: GAGGTCAGGAGGAGGACGAG R: CCTGAGAGGAAATGGGTGTG	58	5	0.690	0.682	0.606

Ta = optimized annealing temperature; N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; PIC = polymorphic information content.

Data analysis

MICRO-CHECKER 2.2.3 was used to test for evidence of null alleles, large allelic dropout, and genotyping errors (Van Oosterhout et al., 2004). Allele frequency, observed heterozygosity (H_O), expected heterozygosity (H_E), and the polymorphic information content (PIC) were calculated using CERVUS 3.0.7 (Marshall et al., 1998). Deviations from the Hardy-Weinberg equilibrium (HWE) for each locus and all pairwise combinations of loci were tested for linkage disequilibrium (LD) using GENEPOP on the web (Rousset, 2008).

RESULTS AND DISCUSSION

A $(GT)_n$ enriched-genome library for *P. tenuicorpa* was constructed, and 236 positive colonies were successfully screed from a total of 480 white colonies using a colony PCR method. The sequencing of the 236 positive colonies showed that 68 colonies (28.8%) contained SSRs with at least seven repeats. This result indicated a similar efficiency as that in a previous study of *Rhinogobio ventralis* (29.5%; Shao et al., 2015). Among the 68 colonies that contained SSR sequences, only 37 colonies had long and unique flaking regions suitable for primer design. Among these 37 primer pairs, 24 primer pairs only amplified the expected PCR products. Of these, nine loci were finally proven to have polymorphisms with a clear signal. Their sequences were deposited to National Center for Biotechnology Information under GenBank accession Nos. (JX179147-JX179152, JX179154-JX179156).

Conventional methods for microsatellite marker development are labor intensive, time consuming, and have low microsatellite isolation efficiency (Zane et al., 2002). In this study, we developed microsatellite markers for *P. tenuicorpa* from a partial genomic library, by using the enrichment method, which is faster and cheaper than that by using conventional methods.

A total of 101 alleles were found among the nine polymorphic microsatellite markers. The number of alleles per locus ranged from 5 (PTms120, PTms155, and PTms239) to 19

(PTms105) with an average of 11.2 alleles (Table 1). Moreover, all microsatellite markers developed in this study were highly informative (PIC > 0.60), and null allele, HWE, and LD values were not statistically significant. The microsatellite markers showed a high degree of variability indicating that they may have a great potential as a tool for future population genetic studies (DeWoody and Avise, 2000; Sethy et al., 2006).

H_O and H_E ranged from 0.586 to 0.931 (average 0.805) and from 0.668 to 0.940 (average 0.835), respectively (Table 1). Gene diversity (average H_E) is a good parameter to measure genetic variation and to assess genetic diversity within a population (Serrano et al., 2009; Sun et al., 2015). A wild population of *P. tenuicorpa* in the Dal stream showed a higher average H_E than those by *R. ventralis* (average 0.627) (Shao et al., 2015) and *G. brevibarba* (average 0.759) (Kim et al., 2014). These results suggest that the decline in wild populations of *P. tenuicorpa* is not the result of a decrease in genetic diversity but of human activities (Escalona et al., 2009). In addition, our results indicated that the genetic diversity of *P. tenuicorpa* was not significantly lower than that observed in other closely related species. A limitation of this study was that the analysis of the genetic diversity index included only one wild population of *P. tenuicorpa*. Therefore, further studies will be necessary to fully characterize the related conservation indices, such as evidence for a genetic bottleneck and effective population size, by including multiple, diverse populations.

Cross-species amplifications of the nine polymorphic loci were conducted across five closely related fish species (*P. nigra*, *G. naktongensis*, *G. macrocephala*, *G. brevibarba*, and *M. koreensis*), all belonging to the subfamily Gobioninae. As shown in Table 2, the PTms177 locus produced no PCR products for any species, whereas the PTms119, PTms155, and PTms239 loci were less effective due to lower amplification rates and/or monomorphic alleles. However, the other five microsatellite markers produced successful PCR products in all five species. The PTms105 locus, in particular, successfully produced PCR products for all individuals of the five species and displayed notable polymorphisms.

Table 2. Cross-species amplification in endangered freshwater fishes of the subfamily Gobioninae in Korea.

Locus	*Pseudopungtungia nigra* (N = 6)			*Gobiobotia naktongensis* (N = 9)			*Gobiobotia macrocephala* (N = 6)			*Gobiobotia brevibarba* (N = 6)			*Microphysogobio koreensis* (N = 6)		
	N	N_A	Size range (bp)	*N*	N_A	Size range (bp)	*N*	N_A	Size range (bp)	*N*	N_A	Size range (bp)	*N*	N_A	Size range (bp)
PTms105	6	8	190-236	9	7	164-186	6	11	192-276	6	10	194-264	6	3	166-172
PTms119	1	1	178	4	3	166-176	0	–		0	–		2	2	148-170
PTms120	0	–		3	1	152	0	–		0	–		6	3	252-270
PTms138	6	5	122-134	8	6	122-134	0	–		5	3	132-136	4	4	110-160
PTms150	6	9	196-226	0	–		6	9	178-196	6	7	178-212	6	9	188-246
PTms155	0	–		5	2	210-230	0	–		1	1	138	1	2	126-164
PTms177	0	–		0	–		0	–		0	–		0	–	
PTms185	6	2	92-96	0	–		0	–		0	–		2	2	208-232
PTms239	2	2	352-358	0	–		6	2	352-360	1	2	352-360	1	2	352-360

N = number of individuals; *N* = number of successfully genotyped individuals; N_A = number of alleles, (–) = no or nonspecific amplification.

In conclusion, we developed nine microsatellite markers for *P. tenuicorpa* for the first time. They exhibited high polymorphisms, no null alleles, and no significant deviations from HWE or evidence for LD. Therefore, our microsatellite markers will be useful for establishing conservation strategies for *P. tenuicorpa*. Moreover, of the nine identified microsatellite

markers, five showed polymorphisms with potential usefulness for comparative genetic studies across other closely related species belonging to the Gobioninae.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the Soonchunhyang University Research Fund.

REFERENCES

Asahida T, Kobayashi T, Saitoh K and Nakayama I (1996). Tissue preservation and total DNA extraction from fish stored at ambient temperature using buffers containing high concentration of urea. *Fish. Sci. Japan* 62: 727-730.

DeWoody JA and Avise JC (2000). Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. *J. Fish Biol.* 56: 461-473. http://dx.doi.org/10.1111/j.1095-8649.2000.tb00748.x

Duran C, Appleby N, Edwards D and Batley J (2009). Molecular genetic markers: discovery, applications, data storage and visualisation. *Curr. Bioinform.* 4: 16-27. http://dx.doi.org/10.2174/157489309787158198

Escalona T, Engstrom TN, Hernandez OE, Bock BC, et al. (2009). Population genetics of the endangered South American freshwater turtle, *Podocnemis unifilis*, inferred from microsatellite DNA data. *Conserv. Genet.* 10: 1683-1696. http://dx.doi.org/10.1007/s10592-008-9746-3

Hamilton MB, Pincus EL, Di Fiore A and Fleischer RC (1999). Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *Biotechniques* 27: 500-502, 504-507.

Kang JH, Park JY and Jo HS (2012). Rapid development of microsatellite markers with 454 pyrosequencing in a vulnerable fish, the mottled skate, *Raja pulchra. Int. J. Mol. Sci.* 13: 7199-7211. http://dx.doi.org/10.3390/ijms13067199

Kim KS, Lee HR, Park SY, Ko MH, et al. (2014). Development and characterization of polymorphic microsatellite marker for an endangered freshwater fish in Korea, *Gobiobotia brevibarba. Conserv. Genet. Resour.* 6: 91-93. http://dx.doi.org/10.1007/s12686-013-0012-8

Lee HH, Choi Y and Choi SH (2014). The best spawning timing in a day and the first spawning position of Korean endangered fish, *Pseudopungtungia nigra* (Teleostei: Cyprinidae). *Korean J. Ichthyol.* 26: 11-16.

Liu ZJ and Cordes JF (2004). DNA marker technologies and their applications in aquaculture genetics. *Aquaculture* 238: 1-37. http://dx.doi.org/10.1016/j.aquaculture.2004.05.027

Marshall TC, Slate J, Kruuk LE and Pemberton JM (1998). Statistical confidence for likelihood-based paternity inference in natural populations. *Mol. Ecol.* 7: 639-655. http://dx.doi.org/10.1046/j.1365-294x.1998.00374.x

ME (2012). Enforcement of Wildlife Protection and Management Act (Ordinance of the Ministry of Environment No. 633).

NIBR (2012). Korean red list of threatened species: mammals, birds, reptiles, amphibians, fishes and vascular plants. National Institute of Biological Resources, Incheon.

Rousset F (2008). genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Mol. Ecol. Resour.* 8: 103-106. http://dx.doi.org/10.1111/j.1471-8286.2007.01931.x

Rozen S and Skaletsky HJ (2000). Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics methods and protocols: methods in molecular biology (Krawetz SA and Misener S, eds.). Humana Press, Totowa, 365-386.

Serrano M, Calvo JH, Martínez M, Marcos-Carcavilla A, et al. (2009). Microsatellite based genetic diversity and population structure of the endangered Spanish Guadarrama goat breed. *BMC Genet.* 10: 61. http://dx.doi.org/10.1186/1471-2156-10-61

Sethy NK, Shokeen B, Edwards KJ and Bhatia S (2006). Development of microsatellite markers and analysis of intraspecific genetic variability in chickpea (*Cicer arietinum* L.). *Theor. Appl. Genet.* 112: 1416-1428. http://dx.doi.org/10.1007/s00122-006-0243-0

Shao K, Yan S, Xiong M, Li W, et al. (2015). Development of sixteen novel polymorphic microsatellite markers in *Rhinogobio ventralis* (Teleostei, Cyprinidae, Gobioninae). *Conserv. Genet. Resour.* 7: 225-227. http://dx.doi.org/10.1007/s12686-014-0341-2

Sun LF, Li J, Liang XF, Yi TL, et al. (2015). Microsatellite DNA markers and their correlation with growth traits in mandarin fish (*Siniperca chuatsi*). *Genet. Mol. Res.* 14: 19128-19135. http://dx.doi.org/10.4238/2015.December.29.22

Van Oosterhout C, Hutchinson WF, Wills DPM and Shipley P (2004). MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* 4: 535-538. http://dx.doi.org/10.1111/j.1471-8286.2004.00684.x

Wang K, Zhang S, Wang D, Xin M, et al. (2015). Development of 27 novel cross-species microsatellite markers for the endangered *Hucho bleekeri* using next-generation sequencing technology. *Conserv. Genet. Resour.* 7: 263-267. http://dx.doi.org/10.1007/s12686-014-0353-y

Zalapa JE, Cuevas H, Zhu H, Steffan S, et al. (2012). Using next-generation sequencing approaches to isolate simple sequence repeat (SSR) loci in the plant sciences. *Am. J. Bot.* 99: 193-208. http://dx.doi.org/10.3732/ajb.1100394

Zane L, Bargelloni L and Patarnello T (2002). Strategies for microsatellite isolation: a review. *Mol. Ecol.* 11: 1-16. http://dx.doi.org/10.1046/j.0962-1083.2001.01418.x