

Short Note

Development of Nine Markers and Characterization of the Microsatellite Loci in the Endangered *Gymnogobius isaza* (Gobiidae)

Kiwako S. Araki *, Takefumi Nakazawa, Atsushi Kawakita, Hiroshi Kudoh and Noboru Okuda

Center for Ecological Research, Kyoto University, Hirano 2-509-3, Otsu 520-2113, Japan;

E-Mails: take.nkzw@gmail.com (T.N.); kawakita@ecology.kyoto-u.ac.jp (A.K.);

kudoh@ecology.kyoto-u.ac.jp (H.K.); nokuda@ecology.kyoto-u.ac.jp (N.O.)

* Author to whom correspondence should be addressed; E-Mail: akiwako@ecology.kyoto-u.ac.jp;
Tel.: +81-77-549-8214; Fax: +81-77-549-8201.

Received: 5 April 2012; in revised form: 28 April 2012 / Accepted: 7 May 2012 /

Published: 11 May 2012

Abstract: *Gymnogobius isaza* is a freshwater goby endemic to Lake Biwa, Japan. They experienced a drastic demographic bottleneck in the 1950s and 1980s and slightly recovered thereafter, but the population size is still very small. To reveal dynamics of genetic diversity of *G. isaza*, we developed nine microsatellite markers based on the sequence data of a related goby *Chaenogobius annularis*. Nine SSR (Simple Sequence Repeats) markers were successfully amplified for raw and formalin-fixed fish samples. The number of alleles and expected heterozygosities ranged from one to 10 and from 0.06 to 0.84, respectively, for the current samples, while one to 12 and 0.09 to 0.83 for historical samples. The markers described here will be useful for investigating the genetic diversity and gene flow and for conservation of *G. isaza*.

Keywords: bottleneck; formalin-fixed samples; *Gymnogobius isaza*; Lake Biwa; microsatellite

1. Introduction

Gymnogobius isaza (Gobiidae) is a freshwater goby endemic to Lake Biwa, Japan. This goby is somewhat unusual in that it has been adapted to a pelagic habitat with its strong swimming ability, whereas most other gobiid fish are benthic. In addition, *G. isaza* plays important roles in the lake ecosystem as a keystone predator coupling pelagic and benthic food webs by diel vertical

migration [1]; Briones *et al.*, unpublished data and also as a socioeconomically valuable fishery target. Fishery data [2] show that the population suddenly collapsed (<1%) in the 1950s and 1980s. The population decline in the 1980s is probably attributable to combined effects of various ecosystem disturbances including lakeshore habitat degradation [3], explosion of introduced piscivores [4,5], and warming-induced hypoxia in the deep water [6]. Although the population recovered slightly thereafter in the late 1990s, it is still relatively low and thus this goby is now categorized as “critically endangered (CR)” in the Red Data Book of Japan [7]. We expect that the genetic diversity of *G. isaza* has been substantially reduced due to the demographic bottleneck. This can exert strong negative impacts on individual fitness, population dynamics, and future extinction risk of the fish [8,9], and thus be crucial for its species conservation and resource management. At present, however, nothing is known about the genetic consequences of the demographic bottleneck because no information is available for its genetic components. Fortunately, specimens of *G. isaza* have been archived since 1962 by the Center for Ecological Research, Kyoto University, where the specimens were initially fixed in 10% formalin and subsequently preserved in 70% ethanol [10,11]. In this study, in order to reveal changes in genetic diversity from current and archived *G. isaza* samples, we developed microsatellite markers for *G. isaza* from sequences of related species.

2. Results and Discussion

With 15 primer pairs, nine loci were successfully amplified from both raw and formalin-fixed samples (Table 1). The number of alleles per locus ranged from one to 10 for 18 frozen current samples and one to 12 for 32 formalin-fixed historical samples (Table 2). For the current samples, the values of observed and expected heterozygosities ranged from 0.06 to 0.78 and 0.06 to 0.84, respectively, while 0.00 to 0.72 and 0.09 to 0.83 for the historical samples. Linkage disequilibria between pairs of loci and deviations from the Hardy-Weinberg equilibrium were also tested. *Isaz5* significantly deviated from the Hardy-Weinberg equilibrium, suggesting null alleles in this locus. No significant linkage disequilibria were detected.

Table 1. Characteristics of nine microsatellite loci in *Gymnogobius isaza*. Shown for each primer pair are the forward (F) and reverse (R) sequence, repeat type, size range of the fragment, labeling dye, optimal annealing temperature (T_a) and accession number.

Locus	Primer Sequence (5'~3')	Repeat	Size Range (bp)	Labeling dye	T_a (°C)	Accession No.
Isaz1	F: CTGAAGGTCAGAGGTCAGAGGTCA R: GGGAAAGACATGAGGCAAAA	(GT) ₄ GAGGGGCGTGG CTAAAGCTA(GT) ₄	225–252	PET	58	AB703105
Isaz2	F: GGAGAGGAGAAAGGGTTTGG R: CAGGCTCCTTACCTCCAGTG	(AG) ₁₀	238–224	6-FAM	58	AB703106
Isaz4	F: CCATCACTCTCGCTCTGTT R: AGAGACAACCTGCCTTCAGA	(CT) ₃ TT(CTCTCGTT) ₂ (CT) ₃ TT(CT) ₃	180	NED	58	AB703107
Isaz5	F: ATGAGGATGTCACAAGTGGAGCAG R: CTGGGTGAATGTAGGGCAGT	(GA) ₁₈	168	6-FAM	58	AB703108

Table 1. Cont.

Locus	Primer Sequence (5'~3')	Repeat	Size Range (bp)	Labeling dye	T _a (°C)	Accession No.
Isaz9	F: ATGGACAAGTCGGAAACTCG R: AAAGTTTCTAAAGACCAACAC	(AC) ₁₃	150–165	VIC	56	AB703109
Isaz10	F: GGTTCGTCCCACTTTGCCTGT R: GTGAAAGGTTGTGCATGTGG	(CA) ₄ AA(CA) ₂ TA(CA) ₂	144–151	NED	56	AB703110
Isaz12	F: CTTGGCATGAAATTGGCTTT R: CCTGCTTTCTATTCCCAGTTGTAA	(GA) ₁₄	323–333	VIC	56	AB703111
Isaz14	F: ATCTGTATCGCCTCCTTTCTCC R: GCGGTTCAAAGCCCCGGTCTGT	(CT) ₁₁	125–162	VIC	58	AB703112
Isaz15	F: CATAGCACCGCCTAGTGTGA R: ACTCCACGGACGAATACTG	(CA) ₈	178–198	NED	56	AB703113

Table 2. Results of nine microsatellite primers in two populations of frozen current and formalin-fixed historical samples of *Gymnogobi isaza*.

Locus	Frozen Current (2010, N = 18)			Formalin-Fixed Historical (1983–2002, N = 32)		
	A	H _o	H _e	A	H _o	H _e
Isaz1	8	0.56	0.75	8	0.72	0.77
Isaz2	2	0.06	0.06	3	0.09	0.09
Isaz4	1	n.s.	n.s.	1	n.s.	n.s.
Isaz5	1	n.s.	n.s.	4	0.00	0.62
Isaz9	10	0.33	0.29	8	0.59	0.74
Isaz10	2	0.78	0.84	4	0.22	0.47
Isaz12	6	0.72	0.76	7	0.56	0.80
Isaz14	7	0.72	0.83	9	0.63	0.83
Isaz15	7	0.50	0.61	12	0.22	0.56

N: number of individuals; A: number of alleles; H_o: observed heterozygosity; H_e: expected heterozygosity; n.s.: not stated because of A = 1.

3. Experimental Section

We downloaded a data source of 15 DNA sequences containing microsatellite repeats (Accession No. AB557719 to AB557733) of *Chaenogobius annularis* (Gobiidae) from DDBJ (DNA Data Bank of Japan) [12]. Primer pairs were designed using Primer 3 (v.0.4.0) [13]. Simultaneously, to design primers at more conservative regions, homologous sequences in *Danio rerio* were searched by using BLASTN system in NIBB [14] and *C. annularis* sequences as queries. Conservative regions were identified by visual inspection, and additional primers were designed accordingly.

Total genomic DNA was extracted from fins by incubating with 600 µL of 1 × STE buffer, 60 µL of 10% SDS and 300 µg proteinase K at 55 °C for three days and then 300 µg proteinase K were added every 24 h. The sample was then extracted by Tris/phenol, phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol, in this order, before ethanol precipitation and washing. Although we used the above protocol to extract DNA for both frozen and formalin-fixed samples, our preliminary examination indicated that a simplified method with 3 h proteinase K incubation and without Tris/phenol and phenol/chloroform/isoamyl alcohol treatments sufficient for frozen (flesh) samples. PCR was performed with 5 ng of extracted DNA in final volume, 10 µL of 1 × Ex Taq HS buffer (TaKaRa, Otsu, Japan) and 10 µM of both forward and reverse primers. According to the manufacturer's recommendation, amplification was done with initial denaturation of 3 min at 94 °C, followed by 45 cycles of 30 s at 94 °C, 30 s at annealing temp (56 or 58 °C), and 1 min at 72 °C, with a final extension for 5 min at 72 °C. The PCR product size was measured using an ABI PRISM 3130 Genetic Analyser with GeneMapper analysis software (Applied Biosystems, Carlsbad, California, USA). Nine primer pairs successfully yielded PCR products. To confirm that the amplified products contain the target microsatellite regions, these fragments were ligated to the pGEM-T easy vector in accordance with the manufacturer's instruction (Promega, Madison, Wisconsin, USA). Recombinant clones were identified by using blue/white screening on Luria-Bertani agar plates containing ampicillin, X-Gal and IPTG. Insert-positive clones were amplified using M13 primers and sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA). Complete sequences of all of nine loci including target microsatellite regions were determined as published in DDBJ (Accession No. AB703105 to AB703113).

To confirm effectiveness of designed primers for investigating genetic diversity, 18 frozen (current) and 32 formalin-fixed (historical) samples were used for the examination of polymorphisms for all 15 loci. Frozen samples were collected from the northwest shore of the Lake Biwa in 2010 and kept in −40 °C freezer. Formalin-fixed samples were caught in 2002 and fixed in formalin immediately. Polymerase chain reactions (PCRs) were performed with the standard protocol of the QIAGEN Multiplex PCR Kit (QIAGEN), in a final volume of 10 µL, which contained 0.2 µM of each multiplexed primer, 5 µL of Multiplex PCR Master Mix and 5 ng of extracted DNA. Forward primers were labeled with the fluorochrom 6-FAM or VIC, NED or PET (Applied Biosystems, USA). The amplification profiles included initial denaturation at 95 °C for 15 min; followed by 30 (40 for formalin-fixed samples) cycles of 30 s at 94 °C, 1.5 min at appropriate annealing temperature, and 1 min at 72 °C; then final extension at 60 °C for 30 min. The primer pairs of *Isaz9*, *10*, *12*, and *15* were simultaneously amplified at 56 °C annealing temperature and *Isaz1*, *2*, *4*, *5* and *14* at 58 °C (see Table 1 for details). The observed and expected heterozygosities were calculated by using GENEPOP version 4.0.10 [15].

4. Conclusion

Developed SSR (Simple Sequence Repeats) markers described here will be used for both formalin-fixed and fresh frozen samples and useful for investigating genetic diversity and population dynamics in *G. isaza*. These markers developed for *G. isaza* are also potentially useful for assessing genetic diversity and gene flow of other *Gymnogobius* species.

Acknowledgement

The authors thank Y. Sakai for collecting fish samples. This study was supported by Global COE program (A06) of Kyoto University; a research fellowship of the JSPS for Young Scientists (201100907 to K.S.A. and 2103033 to T.N.); a Grant-in-Aid, MEXT, Japan (Special Research on Priority Areas, 21024005 to H.K.), JSPS Grants-in-Aid (20370009 and 23657019 to N.O.); the Environment Research and Technology Development Fund (S-9) of the Ministry of the Environment, Japan (to N.O.).

References

1. Ishikawa, T.; Narita, T.; Urabe, J. Long-term changes in the abundance of *Jesogammarus annandalei* (Tattersall) in Lake Biwa. *Limnol. Oceanogr.* **2004**, *49*, 1840–1847.
2. Shiga Statistics and Information Office. *Annual Report of the Agriculture, Forestry and Fisheries Statistics of Shiga*; Shiga Statistics and Information Office: Shiga Prefecture, Japan, 1962–2011.
3. Nakanishi, M.; Sekino, T. Recent drastic changes in Lake Biwa biocommunities, with attention to exploitation of the littoral zone. *GeoJournal* **1996**, *40*, 63–67.
4. Nakai, K. Recent, Faunal Changes in Lake Biwa, with Particular Reference to the Bass Fishing Boom in Japan. In *Ancient Lakes: Their Cultural and Biological Diversity*; Kenobi Production: Ghent, Belgium, 1999; pp. 227–241.
5. Nakazawa, T.; Ishida, N.; Kato, M.; Yamamura, N. Larger body size with higher predation rate. *Ecol. Freshw. Fish* **2007**, *16*, 362–372.
6. Kumagai, M. Lake Biwa in the context of world lake problem. *Verh. Internat. Verein. Limnol.* **2008**, *30*, 1–15.
7. Ministry of Environment of Japan. *Red Data Book of Japan*; Ministry of the Environment of Japan: Tokyo, Japan, 2007.
8. Charlesworth, D.; Charlesworth, B. Inbreeding depression and its evolutionary consequences. *Annu. Rev. Ecol. Evol. Syst.* **1987**, *18*, 237–268.
9. Falconer, D.S.; Mackay, T.F.C. *Introduction to Quantitative Genetics*, 3rd ed.; CABI: Wallingford, CT, USA, 1996.
10. Ogawa, N.O.; Koitabashi, T.; Oda, H.; Nakamura, T.; Ohkouchi, N.; Wada, E. Fluctuations of nitrogen isotope ratio of gobiid fish (Isaza) specimens and sediments in Lake Biwa, Japan, during the 20th century. *Limnol. Oceanogr.* **2001**, *46*, 1228–1236.
11. Nakazawa, T.; Sakai, Y.; Hsieh, C.H.; Koitabashi, T.; Tayasu, I.; Yamamura, N.; Okuda, N. Is the relationship between body-size and trophic niche position time-invariant in a predatory fish? First stable isotope evidence. *PLoS One* **2010**, *5*, doi:10.1371/journal.pone.0009120.
12. Hirase, S.; Kanno, M.; Kijima, A. Isolation and characterization of 15 microsatellite DNA loci for assessing population structure of the intertidal goby, *Chaenogobius annularis*. *Mol. Ecol. Resour.* **2010**, *10*, 1106–1108.
13. Rozen, S.; Skaletsky, H. Primer3 on the WWW for General Users and for Biologist Programmers. In *Bioinformatics Methods and Protocols: Methods in Molecular Biology*; Humana Press: Totowa, NJ, USA, 2000; pp. 365–386.

14. National Center for Biotechnology Information. Available online: <http://www.ncbi.nlm.nih.gov/guide/> (accessed on 20 November 2011).
15. Rousset, F. GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Mol. Ecol. Resour.* **2008**, *8*, 103–106.

© 2012 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (<http://creativecommons.org/licenses/by/3.0/>).