

Development of Microsatellite Markers for the Two Giant Salamander Species (*Andrias japonicus* and *A. davidianus*)

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Abstract: The Japanese giant salamander (*Andrias japonicus*) is a near threatened species endemic to western Japan and is strictly protected by law. However, available information regarding the genetic diversity and genetic structure in this species, essential for its effective conservation, has been limited. We developed four microsatellite markers from *A. japonicus* and characterized these markers for two populations of this species, as well as for some captive Chinese giant salamanders (*A. davidianus*) of unknown original locality or localities. These markers, showing expected heterozygosities of 0.00–0.50 in the former and 0.63–0.89 in the latter, will be useful

in documenting population genetic properties for each of the two species.

Key words: *Andrias japonicus*; *Andrias davidianus*; Microsatellite; Genetic diversity; Conservation

INTRODUCTION

The Japanese giant salamander, *Andrias japonicus*, is endemic to the western half of mainland Japan including western Honshu and Kyushu, and possibly Shikoku. This species is currently assigned to the Red List category, “Vulnerable” and “Near Threatened”, by the National Government of Japan (Matsui, 2000) and IUCN (2011), respectively, because many populations of the species have supposedly been declining due to recent artificial destruction of their habitats. Thus, *A. japonicus* is now strictly protected by Japan’s National laws, which prohibit killing or harming it. Such law-regulations have also been preventing studies on biological aspects of the species due to rather complicated procedure to get its handling permission from the Government (Matsui and Hayashi, 1992). Thanks to recent methodological progress, a few molecular genetic studies have been conducted for *A. japonicus* (Matsui et al., 2007, 2008) using tissue samples obtained by non-invasive methods. Nevertheless, information regarding genetic diversity and genetic structure among and within the Giant salamander populations is still limited, despite apparent importance of such information in planning effective conservation procedure. Moreover, *A. davidianus*, the other extant species of the genus native to continental China, has recently established nonnative breeding colonies in west-central Honshu, where genetic introgression from the species to *A. japonicus* through hybridization is concerned.

Application of polymorphic microsatellite markers is known to be effective in elucidating various genetic parameters of a given organismal population or population assemblage. Therefore, we have isolated four microsatellite markers from *A. japonicus* and characterized them for the species, as well as for *A. davidianus* as below.

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MATERIALS AND METHODS

Microsatellite markers were developed using the improved technique for isolating codominant compound microsatellite markers of Lian and Hogetsu (2002) and Lian et al. (2006). An adaptor-ligated, restricted DNA library for *A. japonicus* was constructed according to the following procedure: The whole genomic DNA was first extracted from fresh tail-fin tissues of an *A. japonicus* from Ota-gawa River System of Hiroshima Prefecture (kept in The Hiroshima City Asa Zoological Park) using standard phenol–chloroform extraction procedures (Hillis et al., 1996). The extracted DNA was digested with the blunt-end restriction enzymes *EcoRV* and *SspI*, and restriction fragments were then ligated with a specific blunt adaptor (consisting of the 48-mer: 5'-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGCCCGGGCTGGT-3' and an 8-mer with the 3'-end capped with an amino residue: 5'-ACCAGCCC-NH₂-3') using the Takara DNA ligation kit. Fragments were amplified by PCR from the *EcoRV* and *SspI* DNA libraries using compound SSR primers (AC)₆(AG)₅, (TC)₆(AC)₅ and an adaptor primer (5'-CTATAGGGCACGCGTGGT-3'). The amplified fragments, ranging from 300 to 800 bp, were then separated on a 1.5% LO3 agarose gel (Takara) and purified using the QIAquick Gel Extraction Kit (Qiagen). The purified DNA fragments were cloned using the QIAGEN PCR Cloning plus Kit (Qiagen)

following the manufacturer's instructions. The cloned fragments were amplified using the M13 forward and reverse primers from the plasmid DNA. Amplified fragments were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). For each fragment containing a compound SSR sequence at one end, a specific primer was designed from the sequence flanking the compound SSR using OLIGO 6 Primer Analysis Software (National Biosciences). Polymerase chain reaction (PCR) amplifications were performed following the standard protocol of the Qiagen Multiplex PCR Kit (Qiagen) in a final volume of 6 µl, which contained 5 ng of extracted DNA, 3 µl of 2x Multiplex PCR Master Mix, and 0.2 µM of each multiplexed primer. Compound SSR primers [(AC)₆(AG)₅ or (TC)₆(AC)₅] were labeled with fluorochromes FAM and VIC (Applied Biosystems), respectively. Polymerase chain reaction amplifications were performed with the GeneAmp PCR System 2700 thermal cycler (Applied Biosystems) using the following conditions: initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing for each of the designed specific primers at the temperatures shown in Table 1 for 1 min 30 s, extension at 72°C for 1 min, and final extension at 60°C for 30 min. The size of the PCR products was measured using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and GENESCAN analysis software (Applied Biosystems).

TABLE 1. Characteristics of four compound microsatellite loci for *Andrias japonicus* and *A. davidianus*.

Locus	Repeat motif	Primer sequences (5'-3')	T _a (°C)	Size range (bp)	Accession no.
AJ034	(TC) ₆ (AC) ₇	CCTCACTGCTTAAATAGTTA TCTCTCTCTCTCACACACACAC	57	203–223	AB679189
AJ037	(TC) ₆ (AC) ₁₅	CCATTTTCATTTGAGATATG TCTCTCTCTCTCACACACACAC	57	101–117	AB679190
AJ044	(TC) ₆ (AC) ₁₉	TGACGTGCTAGTAAATTAT TCTCTCTCTCTCACACACACAC	59	126–156	AB679191
AJ118	(AC) ₆ (AG) ₁₃	AAAGATGGACCACACAGAT ACACACACACACAGAGAGAGAG	59	55–99	AB679192

T_a, annealing temperature of primer pair.

Polymorphism of the markers was evaluated for two populations of *A. japonicus*: Pop. 1 ($n=15$) of Iga-shi (formerly Aoyama-cho), Mie Prefecture; and Pop. 2 ($n=10$) of Kitahiroshima-cho, Hiroshima Prefecture. In addition, efficiency of the markers in *A. davidianus* obtained from zoos and aquariums was also examined (15 individuals from unknown locality or localities). Deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were tested using GENPOP on the web (version 4.0.10; Raymond and Rousset, 1995). The frequency of null alleles was calculated using Micro-Checker version 2.2.3 (Oosterhout et al. 2004).

RESULTS AND DISCUSSION

Four polymorphic loci were identified that showed a clear, strong single band for each allele (Table 1). In *A. japonicus*, the number of alleles per locus ranged from 1–3 with an average of two (Table 2). The observed and expected heterozygosities (H_O and H_E) ranged from 0.00–0.80 ($\bar{x}=0.20$) and from 0.00–0.50 (0.12) in Pop. 1, and from 0.00–0.20 (0.08) and from 0.00–0.19 (0.07) in Pop. 2, respectively. In Pop. 1, AJ118 locus showed significant deviation from HWE with heterozygote excess. There was no evidence for significant linkage disequilibrium between these loci ($P>0.05$) and for the presence of null alleles. In *A. davidianus*, those four loci were also ampli-

fied, and the number of alleles per locus ranged from 7–12 with an average of nine (Table 2). The observed and expected heterozygosities (H_O and H_E) ranged from 0.40–0.60 ($\bar{x}=0.53$) and from 0.63–0.89 (0.74), respectively. Significant deviations from HWE at two loci (AJ034 and AJ044; $P<0.05$) with heterozygote deficient, and significant linkage disequilibrium between AJ034 and AJ044 loci ($P<0.05$) were observed. We found no evidence for the presence of null alleles.

The two populations of *A. japonicus* analyzed in this study possessed extremely low genetic variability, whereas *A. davidianus* was highly polymorphic. Although the *A. davidianus* sample used here was an assemblage of pet-traded individuals without any reliable information regarding original locality or localities, it is probable that these individuals actually represent several geographically distant populations. Results of our present study coincide with those of some previous studies on the giant salamanders by use of allozyme and mitochondrial DNA data (Matsui and Hayashi, 1992; Murphy et al., 2000; Matsui et al., 2008). Detailed genetic structures and variations within these two species are still unclear, and the microsatellite markers described here will be useful for future genetic and ecological studies on the *Andrias* species, and also for establishing effective management plan for conservation of their populations.

TABLE 2. Variability of four microsatellite loci in *Andrias japonicus* and *A. davidianus*. *A*: number of alleles per locus, H_O : observed heterozygosity, H_E : expected heterozygosity.

Locus	<i>A. japonicus</i>						<i>A. davidianus</i> ($n=15$)		
	Pop. 1 ($n=15$)			Pop. 2 ($n=10$)					
	<i>A</i>	H_O	H_E	<i>A</i>	H_O	H_E	<i>A</i>	H_O	H_E
AJ034	1	0.000	0.000	1	0.000	0.000	8	0.400*	0.629
AJ037	1	0.000	0.000	1	0.000	0.000	7	0.533	0.700
AJ044	1	0.000	0.000	3	0.200	0.185	9	0.600*	0.740
AJ118	2	0.800*	0.500	2	0.100	0.095	12	0.600	0.887
Average	1.25	0.200	0.125	1.75	0.075	0.070	9	0.533	0.739

* A significant deviation from Hardy–Weinberg equilibrium expectations ($P<0.05$).

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