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Isolation, Characterization and PCR Multiplexing of Microsatellite Loci for Western Sand Lance (*Ammodytes japonicus* Duncker and Mohr 1939)

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Abstract

Ten novel microsatellite markers were developed for the western sand lance, *Ammodytes japonicus*, which has decreased significantly in many fishing grounds because of overexploitation and deterioration of its habitat. The 10 markers were successfully amplified in two sets of multiplex polymerase chain reactions (PCR). A total of 63 individuals collected in two successive years from a single population were used to assess the characteristics of the 10 markers. The number of alleles per locus ranged from seven to 24 with the observed heterozygosity ranging from 0.397 to 0.921. None of the loci deviated significantly from the Hardy-Weinberg equilibrium, and there was no evidence of linkage disequilibrium between any loci-pairs. Almost all of these novel microsatellite markers were also confirmed to be successfully amplified, not only for the other regional *A. japonicus*, but also for the closely-related *Ammodytes heian* and *Ammodytes hexapterus.* These polymorphic microsatellite markers for multiplex PCR will largely improve the throughput of microsatellite DNA analysis, and contribute to the effective genetic monitoring of *A. japonicus* and other sand lances around Japan.

Discipline: Genetic resources
Additional key words: *Ammodytes heian*, *Ammodytes hexapterus*, cross-amplification, genetic monitoring, polymorphic microsatellite markers

Introduction

Western sand lance *Ammodytes japonicus*, identified as *A. personatus* before being systematically revised (Orr et al. 2015), are small marine fish distributed throughout the southern Sea of Okhotsk, along the coasts of Japan, and in the East China and Yellow Seas (Han et al. 2012, Orr et al. 2015). It is a very important commercial fish and also plays an important role in the ecosystem as a food source for larger, commercially important, carnivorous fishes (Uzaki et al. 2010). However, *A. japonicus* catches have decreased significantly in many fishing grounds because of overexploitation and habitat deterioration (Akimoto et al. 2002, Fujiwara 2008, Uzaki et al. 2010). For the effective management for *A. japonicus* and sympatric predatory fishes, genetic monitoring of the sand lance is a very important issue. Polymorphic microsatellite DNA markers are a useful tool for genetic monitoring. Furthermore, development of multiplex polymerase chain reactions (PCR) sets for microsatellite markers considerably improves the cost and throughput of microsatellite DNA analysis (Guichoux et al. 2011), contributing to effective genetic monitoring. However, there is no multiplex PCR set for microsatellite markers

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for the genus *Ammodytes*. Eleven microsatellite markers for the genus *Ammodytes* are already developed (Ren et al. 2009). It is also possible to apply these markers for *A. japonicus*, a previously identified southern lineage of *A. personatus* (Ren et al. 2015, Kim et al. 2017). However, these markers have various annealing temperatures, which is not ideal for multiplex PCR. Furthermore, six of these 11 microsatellite markers show high possibility of null allele presence (Ren et al. 2009). Of the remaining five microsatellite markers, only two markers are perfect repeat motif type, which is a desirable characteristic for analyses based on the stepwise mutation model (Guichoux et al. 2011). Because only two of the markers were ineffective for multiplex PCR and genetic monitoring, the development of novel microsatellite markers for *A. japonicus* is necessary for design of a microsatellite multiplex PCR. In the present study, novel polymorphic microsatellite markers for PCR multiplexing were developed for genetic monitoring of *A. japonicus* by using a next generation sequencer.

Materials and methods

Total genomic DNA was extracted from muscle tissue of one specimen of *A. japonicus* (82.4 mm in standard length: SL), randomly selected from fisheries catches off the coast of Hojo, Ehime, Japan (33.95 °N, 132.75 °E) in May 2014, using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The genomic DNA sample was outsourced to the Hokkaido System Science Co. Ltd. (Sapporo, Hokkaido, Japan) for sequencing in a Roche GS FLX+ 454 system. Pyrosequencing returned 108,967 reads totalling 63,289,150 base pairs (average read length = 580.81 bp ranging 24 to 1,106 bp) over a phred-equivalent quality score of 30. The resulting sequences were screened for potential microsatellite loci by MSATCOMMANDER 0.8.2 (Faircloth 2008). Among the 108,967 reads, 33,981 reads had putative microsatellite motifs of di- and tetranucleotides (Fig. 1). A total of 28 microsatellite loci were selected and checked for similarity of sequences to previously reported microsatellite loci for the genus *Ammodytes* (Ren et al. 2009) with the basic local alignment search tool BLAST+ v 2.2.29 (Zhang et al. 2000). Primer sets were designed for the 28 microsatellite loci using Primer3Plus (Rosen & Skaletsky 2000). A PIG-tail sequence (GTTTCTT) was added to the 5′ end of each reverse primer in order to enhance "A" addition (Brownstein et al. 1996). The tailed forward primer method described in Blacket et al. (2012) was also applied in PCR amplification. Tail primers were labeled at the 5′ end with FAM, HEX, NED and PET, following Blacket et al. (2012). Initially, PCR reactions were performed in simplex for each primer set in 10 µl volume including 1 µl of template DNA, 5 µl of HotStarTaq Plus Master Mix (Qiagen), 0.25 µl (4 mM) of tailed forward and tail primers and 0.5 µl (4 mM) of PIG-tailed reverse primer. The thermocycler profile consisted of 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 30 min. PCR performance of each primer set was checked using 2% agarose gel electrophoresis with amplicons for the same genomic DNA sample sequenced. For microsatellite loci showing clear PCR amplification, characteristics of each locus were evaluated using 31 individuals of *A. japonicus* (mean ± S.D. = 131.1 ± 6.4 mm in SL) caught off the coast of Hojo on April 1, 2014. PCRs were performed using the same protocol as described for simplex PCR above. Resulting PCR

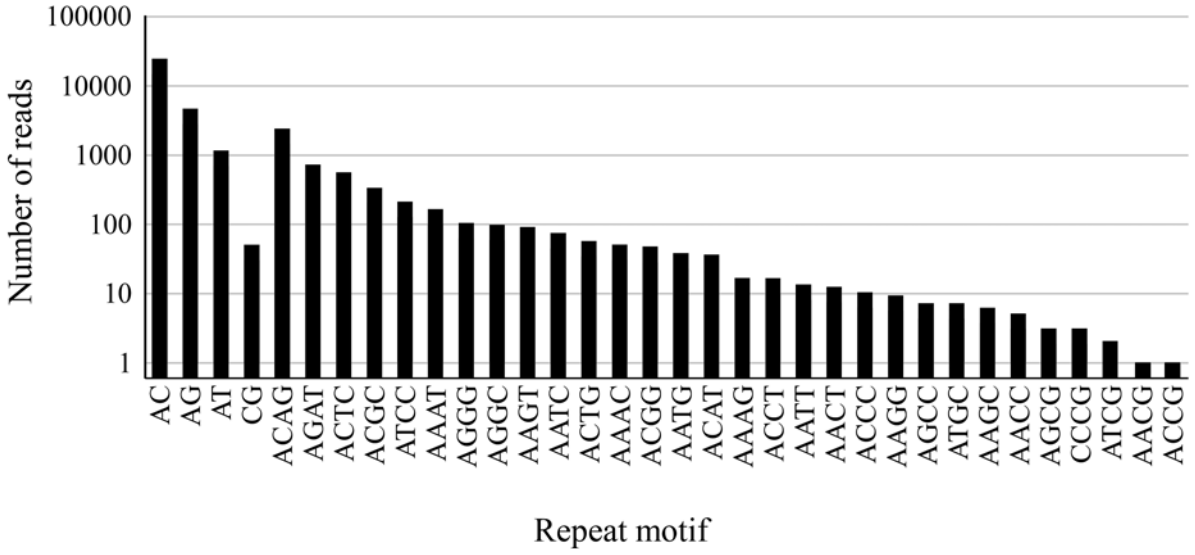

Fig. 1. Read numbers, including microsatellite motifs of di- and tetranucleotides

products were analyzed using ABI 3130xl Sequencer (Applied Biosystems, Life Technologies) with 600LIZ size standard (Applied Biosystems), and allele size was determined using Peak Scanner v2.0 (Applied Biosystems). Microsatellite markers are desirable for clear genotyping, in which alleles clearly distribute in periodic sizes expected from repeat motif size. Therefore, markers showing microvariants in which allele size deviates from the expected periodicity of repeat motif size were removed from further analyses. Microsatellite markers that did not show significant departure from Hardy-Weinberg equilibrium (HWE), any significant linkage disequilibrium (LD), and null allele presence were assigned to some multiplex reaction sets using Multiplex manager (Holleley & Geerts 2009). Performance of each multiplex set was evaluated using 32 individuals of *A. japonicus* (mean ± S.D. = 77.5 ± 4.7 mm in SL) caught off the coast of Hojo on June 9, 2015. Furthermore, to confirm the effectiveness of the developed microsatellite DNA markers for other regional specimens and closely-related species, 30 individuals of *A. japonicus* (mean ± S.D. = 164.8 ± 19.1 mm in SL), four individuals of *Ammodytes heian* (mean ± S.D. = 162.2 ± 21.2 mm in SL), and 10 individuals of *Ammodytes hexapterus* (mean ± S.D. = 151.6 ± 13.8 mm in SL) caught off the coast of Wakkanai, Hokkaido (45.58 °N, 142.25 °E) on June 1, 2015 were analyzed. Multiplex PCR products were amplified in 10 µl volume including 0.5 µl of template DNA, 5µl of Type-it Multiplex PCR Master Mix (Qiagen), 1 µl Q-solution (Qiagen), 0.05 µl (20 mM) of tailed forward and tail primers and 0.1 µl (20 mM) of PIG-tailed reverse primer. PCR was performed using the following touch-down PCR cycle: 95 °C for 5 min, and 35 cycles of 95 °C for 30 s, annealing for 30 s at progressively lowered temperatures from 68 to 59 °C by 1 °C every cycle, followed by 24 remaining cycles at 59 °C and 72 °C for 1 min, followed by a final extension at 72 °C for 30 min. Resulting PCR products were analyzed using an ABI 3130xl Sequencer (Applied Biosystems), same as described above.

Genepop v3.3 (Raymond & Rousset 1995, Rousset 2008) was used to estimate expected (H_E) and observed (H_O) heterozygosity and number of alleles (N_A). Cervus v3.0.7 (Kalinowsli et al. 2007) was used to estimate polymorphic information content (PIC) and probability of identity (P_{ID}). Departure from HWE of each locus, LD between loci and difference in allele distribution of each locus between two-year populations were examined in Genepop v3.3. Significance of HWE departure, LD and difference in allele distribution were determined using sequential Bonferroni correction of the significance level for multiple comparisons (Rice 1989). Significance of fixation index between regions was examined in GenAlEx 6.502 (Peakall & Smouse 2012). Presence of null alleles was checked with MICRO-CHECKER v2.2.3 (Van Oosterhout et al. 2004).

Results

BLAST analysis showed that the 28 microsatellite loci evaluated in this study did not match any previously reported microsatellite loci of the closely-related species *Ammodytes personatus* from the Yellow Sea, China (Ren et al. 2009). Of the 28 microsatellite loci, 12 were successfully amplified and were easily genotyped without confusion by microvariants (Table 1). In the 12 markers, there was no evidence of LD between any loci-pairs (Table 1). Two markers, *Amm019* and *030*, showed significant deviation from HWE and evidence of null alleles (Table 1). Therefore, these two markers were rejected from multiplex PCR sets. Two sets of 5-plexing were designed for the retained 10 markers (Table 1). In analyses for 32 *A. japonicus* collected in 2015, both 5-plex PCR successfully amplified all loci for all 32 specimens. It was also confirmed for specimens of the 2015 population that there was no significant HWE departure and LD, and no evidence of null allele in any of the 10 loci (Table 1). There was no significant difference in allele distribution of each locus between the 2014 population analyzed with simplex PCR and the 2015 population analyzed with multiplex PCR (Table 1). In summary, of 10 microsatellite loci in a total of 63 specimens collected in two successive years, the number of alleles per locus ranged from seven to 24, with the observed and expected heterozygosity and PIC ranging from 0.397 to 0.921, 0.467 to 0.937 and 0.447 to 0.925, respectively (Table 2).

The 10 microsatellite loci were successfully amplified for other regional specimens in multiplex PCR (Table 3), although significant departures from HWE and the presence of null allele were detected in *Amm008* (Table 2). There were significant differences in allele distribution in eight of the 10 loci between the two regions (Table 2). Significant genetic differentiations in *A. japonicus* between Ehime and Hokkaido were also supported by the test for fixation index (F_{st} = 0.043, P < 0.001).

It was confirmed that the 10 microsatellite loci were successfully amplified in multiplex PCR, with similar product size ranges for the closely-related species, *Ammodytes heian* (Table 3). For another closely-related species, *Ammodytes hexapterus*, almost all of the loci were also successfully amplified, but *Amm027* showed a low success rate of amplification. Product size ranges

Table 1. Characteristics of the twelve microsatellite loci for *Ammodytes japonicus*

Locus ID	GeneBank Accession No.	Repeat motif	Primer sequence (5′- 3′)	Tail sequence[a] and Fluorescent dye	T_A (°C)	2014 (n = 31)						Multiplex set	2015 (n = 32)						P_{GD}
						N_A	Allele Size (bp)	H_O	H_E	P_{HWE}	Null allele		N_A	Allele Size (bp)	H_O	H_E	P_{HWE}	Null allele	
Amm008	LC075605	$(ACAG)_{14}$	F: ACCTACACACAGTGAAAGGTACCTTGC	Tail B-VIC	59	14	271–339	0.871	0.909	0.527		1	14	271–339	0.844	0.866	0.259		0.674
			R: GCTCTTGGCTGATCTGCATGCAAC																
Amm016	LC075613	$(GT)_{10}$	F: GCATGTGCAATAGTGTGAATAAGTGTGG	Tail B-VIC	59	8	119–133	0.871	0.819	0.401		2	8	119–133	0.719	0.76	0.414		0.325
			R: TGAGCCATGGGTGTAGGCAGTG																
Amm017	LC075614	$(GT)_{15}$	F: TGTCCTCTCCATCTGCTCTGTGTCTC	Tail D-PET	59	18	147–209	0.806	0.937	0.128		1	20	147–209	0.906	0.933	0.553		0.302
			R: TACCATTGAGCATGAGCAGGCCG																
Amm019	LC075616	$(GT)_{15}$	F: TGCCTATGGTCTAATCATAGCAGTAAGG	Tail D-PET	59	21	171 – 219	0.567	0.949	＜0·001*	Detected	-	-	-	-	-	-	-	-
			R: GGCAGCAGCTTATTCATCTTGATTTCAC																
Amm021	LC075618	$(GT)_{15}$	F: GGAAACCCAATTTGCTGCCAACAGTC	Tail B-VIC	59	21	153–209	0.871	0.951	0.174		1	20	153–209	0.969	0.919	0.528		0.612
			R: CTCCTGTCCATAATGGCCTTGAAGACC																
Amm022	LC075619	$(AGAT)_{11}$	F: CAGCCATCACATGCTTCTGCTGC	Tail A-FAM	59	18	131–207	0.871	0.937	0.473		1	16	131–207	0.844	0.936	0.024		0.811
			R: GCTGCAGAAACAGATGATTGTCAAGAGG																
Amm024	LC075621	$(GGCT)_{12}$	F: CCCACGCCAAATGCAGCACATATC	Tail D-PET	59	16	142–218	0.839	0.911	0.410		2	17	142–218	0.875	0.922	0.761		0.515
			R: TGGAAGTTGCTGACTCACCTCTGC																
Amm025	LC075622	$(AAAC)_6$	F: AGGACTAGGAGGAATAAAGACCGAGGC	Tail C-NED	59	5	181–205	0.742	0.711	0.952		2	6	181–205	0.719	0.683	0.967		0.273
			R: TTTAATGCCGCAGTGGTGTGTTTGTC																
Amm027	LC075624	$(ATCC)_6$	F: TCGGAGAAATGGCTGGAGGC	Tail A-FAM	59	7	168–200	0.419	0.524	0.094		2	8	168–200	0.375	0.410	0.438		0.424
			R: TCGCTCACTTAGTCGCAGATTGAC																
Amm028	LC075625	$(AG)_9$	F: GGGAGTCACACAAGACACAGCCTTAAC	Tail B-VIC	59	7	195–215	0.742	0.69	0.501		2	8	195–215	0.688	0.719	0.933		0.518
			R: TTCATTGGGACCATCTTTCTCCCGC																
Amm029	LC075626	$(AG)_9$	F: TGTTTGATCTTTATGATGGTGGCAGAGC	Tail C-NED	59	8	187–205	0.645	0.608	0.477		1	4	187–205	0.563	0.589	0.237		0.812
			R: AACGTGACGCTATAGTGGCCGTC																
Amm030	LC075627	$(AG)_9$	F: GTCCCAGAGTGAGTCCATTGCTTCAC	Tail D-PET	59	10	215–243	0.419	0.809	＜0·001*	Detected	-	-	-	-	-	-	-	-
			R: GAGGGCGTTGACCTTAGACAACTGG																

T_A, annealing temperature; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; P_{HWE}, P values of Hardy-Weinberg equilibrium test; P_{GD}, P values of genic differentiation test between years.
a: ID and sequences of tails followed Blacket et al. (2012): Tail A (5′- 3′), GCCTCCCTCGCGCCA; Tail B, GCCTTGCCAGCCCGC; Tail C, CAGGACCAGGCTACCGTG; Tail D, CGGAGAGCCGAGAGGTG.
*: loci showed significant difference ($\alpha < 0.05$) after sequential Bonferroni correction for multiple tests.

were also similar in all loci for *A. hexapterus*, but only one allele was detected in *Amm025* and *Amm027* (Table 3).

Discussion

In the current study, 10 novel polymorphic microsatellite markers, which were successfully amplified in PCR multiplexing combined with the fluorescently labeled universal tail primer method (Blacket et al. 2012), were found. Multiplex PCR dramatically reduces the cost and effort of microsatellite DNA analysis (Guichoux et al. 2011) and fluorescence tail primer techniques contribute to reducing the cost of expensive fluorescence primers (Blacket et al. 2012). These improvements in efficiency in microsatellite DNA

Table 2. Comparison of characteristics of *A. japonicus* microsatellite DNA between Ehime and Hokkaido populations

Multiplex set	Locus ID	Ehime (n = 63)						Hokkaido (n = 30)								
		Allele Size (bp)	N_A	H_O	H_E	PIC	P_{ID}	Allele Size (bp)	N_A	H_O	H_E	PIC	P_{ID}	P_{HWE}	*Null allele*	P_{GD}
1	*Amm008*	271–339	16	0.857	0.889	0.870	0.025	259–343	17	0.714	0.920	0.895	0.017	<0.001*	Detected	<0.001*
	Amm017	147–209	24	0.857	0.936	0.924	0.010	147–233	20	0.967	0.934	0.913	0.012	0.008		0.717
	Amm021	153–209	24	0.921	0.937	0.925	0.009	157–229	26	0.933	0.964	0.945	0.005	0.587		0.004*
	Amm022	131– 207	19	0.857	0.934	0.922	0.010	127–247	19	1.000	0.934	0.913	0.012	0.987		<0.001*
	Amm029	187–205	8	0.603	0.596	0.514	0.245	187–211	9	0.633	0.730	0.674	0.123	0.240		<0.001*
2	*Amm016*	119–133	8	0.794	0.799	0.764	0.072	117–127	6	0.500	0.678	0.617	0.161	0.199		<0.001*
	Amm024	142– 218	19	0.871	0.933	0.904	0.015	142–226	16	1.000	0.922	0.896	0.017	0.886		0.245
	Amm025	181–205	7	0.730	0.701	0.642	0.147	185–193	3	0.333	0.315	0.278	0.508	0.378		<0.001*
	Amm027	168–200	8	0.397	0.467	0.447	0.304	168–204	10	0.767	0.794	0.756	0.073	0.352		<0.001*
	Amm028	195–215	9	0.714	0.705	0.657	0.133	203–215	7	0.500	0.719	0.657	0.136	0.090		<0.001*

N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphic information content; P_{ID}, probability of identity; P_{HWE}, *P*-values of Hardy-Weinberg equilibrium test; P_{GD}, *P*-values of genic differentiation test between populations

*: loci showed significant difference ($\alpha < 0.05$) after sequential Bonferroni correction for multiple tests.

Table 3. Success rates of multiplex polymerase chain reactions amplification for the *A. japonicus* of the different population at Hokkaido and in cross-amplifications for *A. heian* and *A. hexapterus* by using specimens collected off the coast of Wakkanai, Hokkaido.

Multiplex set	Locus ID	Hokkaido : *A. japonicus* (*n* = 30)		*A. heian* (*n* = 4)		*A. hexapterus* (*n* = 10)	
		Success rate of amplification (%)	Size range (bp)	Success rate of amplification (%)	Size range (bp)	Success rate of amplification (%)	Size range (bp)
1	*Amm008*	93	259–343	100	263–295	90	247–371
	Amm017	100	147–233	100	167–177	100	153–171
	Amm021	100	157–229	100	171–199	100	159–219
	Amm022	100	127–247	100	143–167	60	131–195
	Amm029	100	187–211	100	195–197	100	193–201
2	*Amm016*	100	117–127	100	119–123	100	121–125
	Amm024	87	142–226	100	134–214	90	146–174
	Amm025	100	185–193	100	185–193	100	177
	Amm027	100	168–204	100	164–216	40	172
	Amm028	100	203–215	75	211–213	100	209–211

analyses will greatly contribute to the conservation and management of *A. japonicus* by facilitating genetic monitoring.

These 10 novel markers were confirmed to have a stable successful rate of amplification for *A. japonicus* collected in plural years and regions, which were close to most southern and northern populations around Japan. These results strongly supported the effectiveness of these novel microsatellite markers, although locus *Amm008* should be used carefully because of the possibility of null allele presence in *Amm008* for the Hokkaido population of *A. japonicus*.

In this study, the novel 10 markers revealed significant genetic differentiation between Ehime and Hokkaido populations in *A. japonicus*. Genetic structures for *Ammodytes* around Japan have illustrated that there are two lineages based on the investigation for the mixture of *A. japonicus* and *A. heian*, which were recognized as southern and northern lineages of *A. personatus*, respectively (Ren et al. 2015, Kim et al. 2017), before revision of the systematics of the genus *Ammodytes* (Orr et al. 2015). This study provided the first suggestion of the presence of multiple genetic populations of *A. japonicus* around Japan. To understand the dynamics of fisheries resources of sand lances throughout Japan, further investigation into the separate genetic structures of *A. japonicus*, *A. heian*, and *A. hexapterus* is needed, and could be performed by analyzing the microsatellite DNA genotypes for a lot of regional samples throughout the Japanese coastal area. Almost all of the 10 novel microsatellite markers were confirmed to be successful in PCR amplification, not only in *A. japonicus*, but also in *A. heian* and *A. hexapterus*, except care should be taken in using *Amm025* and *Amm027* for *A. hexapterus*. The multiplex sets of 10 microsatellite DNA markers developed in this study will contribute to elucidate the genetic structure of sand lances in Japan.

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