GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Isolation and characterization of 19 highly polymorphic microsatellite markers in the devil stinger, *Inimicus japonicus* (Synanceiidae)

X.H. Dong, M.Y. Li, X.N. Tang, X.L. Zhang and L. Miao

College of Life Science and Biotechnology, Ningbo University, Ningbo, China

Corresponding author: M.Y. Li
E-mail: limingyun@nbip.net

Genet. Mol. Res. 10 (3): 1314-1319 (2011)
Received October 14, 2010
Accepted December 10, 2010
Published July 5, 2011
DOI 10.4238/vol10-3gmr1105

ABSTRACT. *Inimicus japonicus*, the devil stinger, has an extensive distribution along the coast of China, Japan and the Korean Peninsula. Nineteen highly polymorphic microsatellite markers were isolated and characterized in *I. japonicus*. Twenty-eight individuals from a wild population were tested for polymorphism using this set of polymorphic microsatellite markers. The number of alleles per locus ranged from 4 to 14. The ranges of observed and expected heterozygosity were 0.500-0.892 and 0.521-0.910, respectively. Significant deviations from Hardy-Weinberg equilibrium were detected at two loci. To the best of our knowledge, these were the first microsatellite loci characterized from the Synanceiidae; they can be used for estimating genetic diversity, population structure studies, parentage analysis, genetic linkage map construction, germplasm classification and identification, gene identification, quantitative trait loci mapping, and marker-assisted selection in breeding of *I. japonicus* and other species of this family.

Key words: *Inimicus japonicus*; Devil stinger; Microsatellite DNA; Genetic diversity

INTRODUCTION

The devil stinger, *Inimicus japonicus*, has an extensive distribution along the coastal areas of the Korea Peninsula, China and Japan. It is one of the species for which artificial reproduction and cultivation have been developed along the coastal areas of Japan to increase the harvest yield since the early 2000s, and it was considered to be a new species to be introduced into the aquaculture industry (Kadomura et al., 2006). *Inimicus japonicus* gradually became one of the commercially important fishes in Japan. In China, some hatcheries begin to produce the artificial seed, and artificial reproduction has promoted commercial cultivation of the devil stinger in recent years. However, the wild population of the devil stinger has declined rapidly because of excess fishing and habitat destruction. Information regarding its population structure and genetic diversity within and between populations, which could provide useful information for developing conservation and management plans, has not been previously reported. There is an urgent need for researchers to learn about the genetic diversity of the wild population, and to find some effective measures to conserve this species.

Microsatellites are one of the most powerful molecular markers for estimating population genetic parameters and performing detailed parentage and gene flow analyses because of the highly polymorphism content, co-dominance and bi-parentally inherited characteristics (Li et al., 2002). To the best of our knowledge, microsatellite markers in *I. japonicus*, even in the Synanceiidae, have never been developed and reported.

Here, 19 polymorphic microsatellite loci of *I. japonicus* were developed as a useful study tool for the estimation of genetic diversity and population genetic structure in wild and cultured populations. A total of 28 specimens from a wild population were captured along the coast in the East China Sea.

MATERIAL AND METHODS

High molecular weight DNA was extracted from muscle tissue using a phenol/chloroform method, followed by ethanol precipitation (Sambrook and Russell, 2001). An enriched genomic library of the target species was produced according to Glenn and Schable (2005) with slight modifications. The genomic DNA was digested with the restriction enzyme *Rsa*I (GE Healthcare), with fragments of approximately 300 to 1000 bp. The genomic DNA fragments were immediately ligated to SuperSNX linkers (forward: 5'GTTTAAGGCCTAGCTAGCAGA ATC, reverse: 5'pGATTCTGCTAGCTAGGCCTTAAACAAAA). The SuperSNX incorporated a GTTT "pig-tail" to facilitate non-template A addition by Taq DNA polymerase during polymerase chain reaction (PCR), which could be used for TA cloning (Glenn and Schable, 2005). The linker-ligated DNA was hybridized with three 3'-biotinylated tetranucleotides $(AC)_{12}$, $(AG)_{12}$ and $(AAG)_8$, which were captured with 600 µg streptavidin-coated magnetic beads (Streptavidin Magnesphere Paramagnetic Particles, Promega). The hybridization mixture was washed with 2X SSC, 0.1% SDS and 1X SSC, 0.1% SDS, respectively, twice at room temperature, and washed twice with 1X SSC, 0.1% SDS at 50°C. PCR recovery of enriched DNA was performed in a volume of 25 µL containing 50-100 ng of the captured DNA, 0.5 U rTaq polymerase (TaKaRa), 1X PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, and 0.4 µM Primer SuperSNX Forward. The PCR profile was as follows: initial denaturation at 95°C for

5 min and 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 10 min. The recovered fragments were cloned using the pGEM-T Easy kit (Promega) and transformed into *Escherichia coli* strain (DH10B). The transformed cells were grown at 37°C for 16 h on an LB agar plate containing ampicillin, X-gal and IPTG for blue/white selection. Insert-positive bacterial clones were amplified by colony-direct PCR using M13(-20) forward and M13 reverse primers, and 177 clones with inserts were sequenced on an ABI3100 sequencer (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Thirty-two microsatellite sequences were randomly selected for primer design using Primer 3 (Rozen and Skaletsky, 2000).

The PCR optimum annealing temperature of each set of primers was achieved in a Mastercycler gradient thermocycler (Eppendorf). PCR was performed in a volume of 25 μL containing 50-100 ng total DNA, 0.5 U rTaq polymerase (TaKaRa), 1X PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, and 0.4 μM of each primer (Table 1). The PCR profile was as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, a primer-specific annealing temperature for 30 s (Table 1) and 72°C for 30 s, with a final extension at 72°C for 10 min. The PCR products were checked by electrophoresis on 8% denaturing polyacrylamide gels, and visualized with silver staining.

Alleles of each locus were sized with QUANTITY ONE, version 4.4 (BioRad, Hercules, CA, USA), by referring to MAKER 1 (TIANGEN, Beijing). The observed heterozygosity (*Ho*) and the expected heterozygosity (*He*) were calculated using POPGENE 32 (version 1.32) (Yeh et al., 1999). Tests for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were conducted using GENEPOP 3.4 (Rousset, 2008). Sequential Bonferroni's correction was used to modify the P values, using multiple statistical comparisons (Rice, 1989).

RESULTS AND DISCUSSION

Of the 32 primer pairs examined, 19 showed polymorphisms in the sample population. The number of alleles varied from 4 to 14 per locus (Table 1). *Ho* and *He* ranged from 0.500 to 0.892 and from 0.521 to 0.910, respectively. Two loci (IJ004, IJ014) showed significant deviation from HWE (Table 1), which might have been due to the small sample size or the existence of null alleles. Two locus pairs (IJ003 and IJ010, IJ007 and IJ014) showed significant linkage disequilibrium ($P < 0.05$, adjusted sequential Bonferroni's correction), which might have resulted from null alleles, non-random sampling or physical linkage.

The 19 highly polymorphic microsatellite markers described here could be very useful for population studies on our target species *I. japonicus* and could constitute a set of molecular tools that can be very helpful in research programs aimed at the sustainable management and conservation of wild and cultured *I. japonicus*. They could also be used in the construction of genetic linkage maps and the analysis of QTLs for marker-assisted selection of this important species, *I. japonicus*. Besides, they may serve as efficient tools for the evaluation of the genetic diversity, ecology, conservation, and fishery management in other fishes of the Synanceiidae, as this was the first isolation and characterization of microsatellite markers in the Synanceiidae.

Table 1. Characterization of 19 highly polymorphic microsatellite markers in devil stinger, *Inimicus japonicus*.

Locus	Primer sequence (5'-3')	Repeat motif	*Ta* (°C)	A	Allele size	*Ho*	*He*	P	Accession No.
IJ001	F: TTGAGCTTTGGTTTGGACCGT R: ATGAGGACTTGACCCAGGATT	$(CT)_{12}(AC)_3$	55	7	176-194	0.535	0.521	0.410	HQ317683
IJ002	F: ATAACTTCGGATGGCTGAAC R: GGAAAATAAAATGGCTCTTG	$(CA)_{14}$	51	5	228-244	0.750	0.733	0.210	HQ317684
IJ003	F: TCTGGCTCATTGCTTTGCTG R: TTGTAGTCAGCGGCGGATGT	$(GA)_{25}$	57	11	224-254	0.785	0.868	0.017	HQ317685
IJ004	F: TATGGATATGATAGCGTAACC R: CTCGTGCTTTAGTCAGTGC	$(CA)_{20}$	53	14	172-194	0.500	0.910	0.000*	HQ317686
IJ005	F: ATCACTCCTCACAGTGCCTAT R: CTTTCTGTAGTTCTCCTCCAAT	$(GT)_{13}$	50	9	163-185	0.857	0.763	0.474	HQ317687
IJ006	F: CTGTTCCTCAGACAGACCTC R: GCTACTTAATTTATTTCTCAATCC	$(CA)_{12}$	50	7	210-232	0.678	0.742	0.016	HQ317688
IJ007	F: GTAGTGAGTCAGATGTTTCCA R: GCTAAACTCTATATTCCGCCT	$(CT)_{19}$	50	10	220-248	0.857	0.876	0.924	HQ317689
IJ008	F: CGCTGATCTGAGCCACAATAG R: ACTGAGCCCCGAGAACTATCC	$(CA)_{15}$	53	6	236-260	0.607	0.864	0.005	HQ317690
IJ009	F: TTGTCAACTTGGCATATTTTCA R: CCAGCTGTAATGTTTTGCAGTA	$(CA)_{12}\cdots(CA)_9$	55	7	200-234	0.750	0.772	0.039	HQ317691
IJ010	F: GAGACTCAGGCTAAACTGGA R: AAAGAGGCAAGCAGTGATTAG	$(AC)_{22}$	53	12	182-200	0.714	0.844	0.057	HQ317692
IJ011	F: AGCCTCCTCTGGGACTTTGT R: AGACGTTTGTGAACGACCTG	$(AC)_{13}$	55	5	205-221	0.642	0.727	0.417	HQ317693
IJ012	F: ATGCAGTCTTAGTCTTTATTTACC R: ACAGCAACATGGATTGTCTT	$(CA)_{13}\cdots(GA)_6$	52	8	212-230	0.750	0.785	0.639	HQ317694
IJ013	F: GGTCTTCTGGATGGCCGAAA R: GTGAGGAGCAGGGTCGGGTG	$(GT)_{10}$	58	9	180-218	0.892	0.821	0.803	HQ317695
IJ014	F: CCAACGAGTCTGTGATCTGC R: TTGTCTCCCTGTCTCCATTT	$(CA)_{13}$	53	7	195-217	0.500	0.808	0.001*	HQ317696
IJ015	F: TGCTTGACTGAATACCAAA R: TTAAAATTGTGACAAGTGGG	$(CA)_{16}$	55	12	174-196	0.785	0.885	0.012	HQ317697
IJ016	F: AAACGCAGCACAAGTGTCTCAC R: CCGCCTGTTCAAATGTTCATAG	$(GT)_{13}AT(GT)_4(GA)_5$	53	13	207-229	0.750	0.906	0.004	HQ317698

Continued on next page

Table 1. Continued.

Locus	Primer sequence (5'-3')	Repeat motif	*Ta* (°C)	A	Allele size	*Ho*	*He*	P	Accession No.
IJ017	F: TGAGGTGGTCAGTCTTTTGGC R: GAGGCTTCTGGGAGGCGTGT	$(CA)_{15}$	56	4	175-185	0.500	0.533	0.225	HQ317699
IJ018	F: AATAATGGAATAAAGGGAGCAG R: TGTGACCCAAATTCAGGAGA	$(AC)_{16}$	56	8	183-206	0.678	0.794	0.520	HQ317700
IJ019	F: CCGTTACGAAGGAACATACAA R: ACATGCAGACAAAATCAAGCA	$(CA)_{13}$	58	9	155-173	0.821	0.829	0.555	HQ317701

Ta = annealing temperature; A = number of alleles; *Ho* = observed heterozygosity; *He* = expected heterozygosity; P = probability that genotype proportions conform to Hardy-Weinberg equilibrium. *Statistically significant after Bonferroni's correction.

ACKNOWLEDGMENTS

Research supported by Changjiang Scholars and Innovation Team Development Program (#IRT0734) and the K.C. Wong Magna Fund in Ningbo University. We thank Cheng Jiong, Shi Yuhong, Li Changhong, and Cao Xiao for technical assistance.

REFERENCES

Glenn TC and Schable NA (2005). Isolating microsatellite DNA loci. *Methods Enzymol*. 395: 202-222.

Kadomura K, Nakashima T, Kurachi M, Yamaguchi K, et al. (2006). Production of reactive oxygen species (ROS) by devil stinger (*Inimicus japonicus*) during embryogenesis. *Fish Shellfish Immunol*. 21: 209-214.

Li YC, Korol AB, Fahima T, Beiles A, et al. (2002). Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. *Mol. Ecol.* 11: 2453-2465.

Rice WR (1989). Analyzing tables of statistical tests. *Evolution* 43: 223-225.

Rousset F (2008). Genepop'007: a complete re-implementation of the Genepop software for Windows and Linux. *Mol. Ecol. Res.* 8: 103-106.

Rozen S and Skaletsky H (2000). Primer 3 for General Users and Forbiologist Programmers. In: Bioinformatics Methods and Protocols: Methods in Molecular Biology (Krawetz S and Misener S, eds.). Humana Press, Totowa, 365-386.

Sambrook J and Russell DW (2001). Molecular Cloning: A Laboratory Manual. 3rd edn. In: Preparation and Analysis of Eukaryotic Genomic DNA. Cold Spring Harbor Laboratory Press, New York, 547-609.

Yeh FC, Yang R, Boyle TJ and Ye Z (1999). POPGENE 32, Microsoft Window-based Freeware for Population Genetic Analysis, Version 1.32. University of Alberta, Edmonton.

