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

Isolation and characterization of novel microsatellite markers from Siamese fighting fish (*Betta splendens*, Osphronemidae, Anabantoidei) and their transferability to related species, *B. smaragdina* and *B. imbellis*

V. Chailertrit[1,2,3], A. Swatdipong[4], S. Peyachoknagul[4,5], J. Salaenoi[6] and K. Srikulnath[1,4,5]

[1]Laboratory of Animal Cytogenetics and Comparative Genomics, Department of Genetics, Faculty of Science, Kasetsart University, Bangkok, Thailand
[2]Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand
[3]Center of Excellence on Agricultural Biotechnology, Bangkok, Thailand
[4]Department of Genetics, Faculty of Science, Kasetsart University, Bangkok, Thailand
[5]Center for Advanced Studies in Tropical Natural Resources, National Research University Kasetsart University, Bangkok, Thailand
[6]Department of Marine Science, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand

Corresponding author: K. Srikulnath
E-mail: kornsorn.s@ku.ac.th / ksrikulnath@yahoo.com

Genet. Mol. Res. 13 (3): 7157-7162 (2014)
Received March 28, 2013
Accepted September 30, 2013
Published September 5, 2014
DOI http://dx.doi.org/10.4238/2014.September.5.1

ABSTRACT. Ten novel microsatellite markers were developed and characterized from Siamese fighting fish (*Betta splendens*). Nine of

ten markers were polymorphic, exhibiting an allelic number (N_A) from 2 to 6 alleles per locus. The effective number of alleles (N_E) ranged from 1.60 to 3.08 (average of 2.30). The observed (H_O) and expected (H_E) heterozygosities ranged from 0.13 to 0.67 (average of 0.39) and 0.29 to 0.63 (average of 0.50), respectively. Linkage disequilibrium was not significantly detected for any pair of loci, and only two loci (BettaMS23 and BettaMS28) showed significant deviations from Hardy-Weinberg expectations. Of these, six loci could be amplified in genomic DNA of the closely related species *B. imbellis* and three loci in *B. smaragdina*. These microsatellite markers could be used as a tool to investigate genetic diversity and population structure, as well as breeding programs in hatcheries.

Key words: Microsatellite; Siamese fighting fish; *Betta splendens*; Heterozygosity; Transferability

INTRODUCTION

The Siamese fighting fish (*Betta splendens*, Anabantoidei) is an ornamental fish that widely inhabits northern and central Thailand, Malaysia, Cambodia, and Vietnam. Males behaviorally build bubble nests and care for developing eggs until they reach the larval stage. Beautiful body features of *Betta* species, such as color and scale pattern, and body shape and fin size, make them a flourishing species in markets worldwide. However, the local population of the Siamese fighting fish has been rapidly declining because of loss of its natural habitat and contamination from artificially bred fighting fish. This issue needs serious attention in the context of biodiversity and conservation, since all wild populations of *B. splendens* are now critically threatened (Vidthayanon, 2011). To delineate the management of population structures in the wild and in breeding programs in hatcheries, effective and reliable methods using molecular markers such as microsatellite markers are necessary. However, *Betta*-specific microsatellite markers have not yet been developed for population monitoring. In this study, we report ten novel *Betta splendens* microsatellites markers, most of which were successfully amplified in two closely related species, *B. smaragdina* and *B. imbellis*.

MATERIAL AND METHODS

Whole genomic DNA was isolated from tail muscle tissues of a single individual using a standard phenol-chloroform protocol as previously described (Srikulnath et al., 2010). Microsatellite markers were developed following the enriched library protocol (Zane et al., 2002) with slight modification. Total genomic DNA was digested with *Mse*I. The fragmented DNAs were ligated to specific adapters (5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGAC TCAT-3'), and then amplified by polymerase chain reaction (PCR) using the oligo-adaptor as primer. PCR products were hybridized to eight streptavidin-biotinylated oligo simple sequence repeat (SSR) complexes: $(CA)_{15}$, $(GA)_{15}$, $(GC)_{10}$, $(ACC)_{10}$, $(CCT)_{10}$, $(GAT)_{10}$, $(CAG)_{10}$, and $(GCT)_{10}$ with magnetic beads (Dynabeads® Myone™ Streptavidin C1, Invitrogen, Carlsbad, CA, USA). The enriched DNAs were molecularly cloned using pGEM®-T easy Vector

(Promega, USA) and transformed into competent *E. coli* DH5α cells (Promega). To confirm the presence of microsatellite sequences, the positive clones were subjected to dot-blot hybridization using the SSR probes as mentioned above and a North2South® Chemiluminescent Hybridization and Detection kit (Pierce, USA).

A set of 114 positive clones were randomly selected and sequenced. The nucleotide sequence of the DNA fragments was determined (1st Base DNA sequencing service, Seri Kembangan, Malaysia) and seventy clones were discarded because of inappropriate properties, such as extremely long or short repeat stretches or SSR located close to the cloning site. The clones with sufficient flanking region were recovered and used to design the specific primers using Primer3 (Rozen and Skaletsky, 2000). The candidate SSR primers were tested for their ability to produce a readable pattern with genomic DNA of fifteen individuals of *B. splendens* collected from a population in Nakhon Pathom, Thailand (13°45'N, 100°04'E). Twenty-five nanograms of genomic DNA were added to 20 µL 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5.0 µM specific primers, and 0.5 U *Taq* DNA polymerase (Invitrogen). PCR cycling conditions consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 50°/60°C for 30 s, and primer extension at 72°C for 1 min, and then post-cycling extension at 72°C for 5 min (Table 1).

The PCR products were subsequently detected on a 6% denaturing polyacrylamide gel (w/v) and visualized by silver staining as previously described (Benbouza et al., 2006). The sizes of allele were measured using the GelAnalyzer program (www.gelanalyzer.com/index.html). The expected DNA fragments were then extracted from silver-stained gels and cloned into pGEMT-Easy Vector System I (Promega), and their nucleotide sequencing was carried out as mentioned above. Nucleotide sequences of all loci were deposited in DDBJ (DNA Data Bank of Japan, http://www.ddbj.nig.ac.jp/index-e.html).

RESULTS AND DISCUSSION

Ten SSR primer pairs were successfully amplified from genomic DNA of fifteen individuals of *B. splendens* as seen from the high quality single-banded products of the expected sizes. However, only nine markers exhibited polymorphism. These markers were then subjected to determination of the allelic number (N_A), effective number of alleles (N_E), observed heterozygosity (H_O), expected heterozygosity (H_E), Hardy-Weinberg equilibrium (HWE), and linkage disequilibrium (LD) using the GENEPOP 4.0 program (Raymond and Rousset, 1995). Thirty-five alleles were obtained from all loci. N_A ranged from 2 to 6, and the N_E ranged from 1.60 to 3.08 (average of 2.30). In addition, H_O and H_E ranged from 0.13 to 0.67 (average of 0.39) and 0.29 to 0.63 (average of 0.50), respectively (Table 1). No evidence of significant LD was observed in any loci pair, and only two loci (BettaMS23 and BettaMS28) showed significant deviations from HWE.

Considering the specifications of behavioral and morphological characters, all nominal species of fighting fish in the genus *Betta* could be classified into 15 clades, one of which is the *Betta splendens* (bubble-nest building bettas) clade comprising *B. splendens*, *B. smaragdina*, *B. imbellis* and *B. stiktos* (Witte and Schmidt, 1992). *B. smaragdina* and *B. imbellis* are widely distributed in the northeast and south of continental Thailand, respectively, whereas *B. stiktos* inhabits Cambodia (Goldstein, 2001, Tan and Ng, 2005). Reports on molecular phylogenies of genus *Betta* suggest that *B. splendens* is more closely related to *B. imbellis* than to *B. smaragdina* (Ruber et al., 2004; Sriwattanarothai et al., 2010). These findings led us to predict that our SSR markers could be more transferable in *B. imbellis* than in *B. smaragdina*.

Table 1. Characteristics of ten novel microsatellite DNA loci isolated from *Betta splendens* and their transferability to related species, *B. smaragdina* and *B. imbellis*.

Locus	Primer sequence (5' to 3')	Repeat	Ta (°C)	*Betta splendens*							*Betta smaragdina*							*Betta imbellis*					
				Accession No.	Size range (bp)	N_A	N_E	H_O	H_E	P-HWE	Accession No.	Size range (bp)	N_A	N_E	H_O	H_E	P-HWE	Accession No.	Size range (bp)	N_A	N_E	H_O	H_E	P-HWE
BettaMS4	F: GTTTCATCAGGAGCAGCAGCATAA R: CTGTTTGATGGCCGACTTTT	$(GA)_n$	59	AB777403	259-315	5	2.78	0.47	0.54	0.167	AB777404	180-198	3	2.86	0.18	0.68	0.0016*	AB777405	162-220	4	3.17	0.62	0.72	0.0453
BettaMS5	F: GTTTCGTCACCTTCTGAGCAAACA R: AAATGCGCTGGGTAGACTTG	$(GA)_n$	59	AB777406	198-218	4	2.71	0.67	0.63	1	-	-	-	-	-	-	-	-	-	-	-	-	-
BettaMS8	F: CGTGAGCTGCAAAGAAAACA R: GCTGTTGCACATGAATCCAG	$(GA)_n$	57	AB777407	223	1	1	0	0	-	-	-	-	-	-	-	-	AB777408	223	1	1	0	0	-
BettaMS14	F: GCTGAGCTCTGAGGAACACC R: CAAGGTGTTGAGCGTTTCAG	$(CA)_n$	57	AB777409	227-250	2	1.6	0.33	0.29	1	AB777410	240-282	7	3.79	0.67	0.73	0.0008*	-	-	-	-	-	-
BettaMS15	F: ACTGTAACCGGGCTGTTCTG R: AACGCACCCAGAAACAAATC	$(GA)_n$	57	AB777411	216-225	3	2.02	0.33	0.48	0.1479	-	-	-	-	-	-	-	AB777412	227-294	6	4.09	0.36	0.78	0.0156
BettaMS17	F: AAGCAGGTCTTTCACCTCCA R: TCACCCTGCGTCTAAGTCAA	$(GA)_n$	61	AB777413	194-221	5	2.02	0.27	0.41	0.0863	-	-	-	-	-	-	-	AB777414	182-217	5	2.7	0.29	0.62	0.0035*
BettaMS23	F: GTTTGAGAAATGGGTTCTTCG R: TCACTACGCTGCCAAATCAG	$(CT)_n(CA)_n$	55	AB777415	277-296	3	1.63	0.13	0.35	0.0045*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BettaMS25	F: GTTTGGGTAAAACCCAACTCTGG R: AACGTCACGTGGAACAGATG	$(GT)_n$	55	AB777416	194-224	4	2.44	0.47	0.6	0.1032	-	-	-	-	-	-	-	AB777417	196-217	3	1.47	0.14	0.26	0.2172
BettaMS28	F: GTTTCTATTGCCTTAGGGCTCCA R: TGCTCCTGAGAGGACTATTGG	$(GA)_n$	57	AB777418	222-232	3	2.39	0.13	0.61	0.0001*	AB777419	212	1	1	0	0	-	AB777420	219-229	3	2.64	0.67	0.65	0.1179
BettaMS40	F: CAGTACATTTGACTGATCGCAGA R: CAGGATGCTTCCTTGGGTAA	$(GA)_n$	57	AB777421	136-165	6	3.08	0.67	0.62	0.3066	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Locus designation (Locus), primer sequences, repeat motif (Repeat), annealing temperature (Ta), accession number, size range of PCR products (Size range), allelic number (N_A), effective number of allele (N_E), observed heterozygosity (H_O), expected heterozygosity (H_E) and, P value of Hardy-Weinberg equilibrium (P-HWE). *Significant departures from Hardy-Weinberg equilibrium (HWE) ($P < 0.01$).

Cross-species amplification was then performed to determine the transferability of SSR markers. Fifteen individuals of each fighting fishes (*B. smaragdina* collected from a population in Ubon Ratchathani (15°15'N, 104°50'E) and *B. imbellis* collected from a population in Trang (7°33'N, 99°36'E) were used, and PCR was conducted as mentioned above. The results showed that three of ten microsatellite markers (BettaMS4, BettaMS14, and BettaMS28) were successfully amplified in *B. smaragdina*. However, only two markers exhibited polymorphism.

In total, ten alleles were obtained, with N_A ranging from 3 to 7, and N_E ranging from 2.86 to 3.79 (average of 3.33). H_O and H_E ranged from 0.18 to 0.67 (average of 0.42) and 0.68 to 0.73 (average of 0.71), respectively. Six of the ten SSR markers (BettaMS4, BettaMS8, BettaMS15, BettaMS17, BettaMS25, and BettaMS28) showed cross-species amplification to *B. imbellis*. Five of the six loci, being polymorphic markers, could provide 21 alleles in *B. imbellis*, with N_A ranging from 3 to 6, N_E ranging from 1.47 to 4.09 (average of 2.81). H_O and H_E were found to range from 0.14 to 0.67 (average of 0.42) and 0.26 to 0.78 (average of 0.61), respectively. Interestingly, two polymorphic markers (BettaMS4 and BettaMS28) could be amplified in the genomic DNA of all species. These results suggest that the flanking and microsatellite region of these loci may be highly conserved in the lineage of *Betta splendens* clade.

To the best of our knowledge, this is the first report on SSR marker transferability in the genus *Betta*. These markers could become a powerful tool for assessing genetic diversity and supporting conservation studies. However, several wild fighting fish populations are required to test the polymorphism of markers, and many species of *Betta* need to be deployed to confirm the transferability of SSR markers.

ACKNOWLEDGMENTS

Research supported by the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Office of Higher Education Commission, Ministry of Education. (AG-BIO/PERDO-CHE) and Center for Advanced Studies in Tropical Natural Resources, National Research University-Kasetsart University, Kasetsart University, Thailand (CASTNAR, NRU-KU, Thailand).

REFERENCES

Benbouza H, Jacquemin JM, Baudoin JP and Mergeai G (2006). Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. *Biotechnol. Agron. Soc. Environ.* 10: 77-81.

Goldstein RJ (2001). Bettas A complete Pet Owner's Manual. Barron's, New York.

Raymond M and Rousset F (1995). GENEPOP (version-1.2): population genetics software for exact tests and ecumenicism. *J. Hered.* 86: 248-249.

Rozen S and Skaletsky HJ (2000). Primer3: Bioinformatics Methods and Protocols. Humana Press. In: Methods in Molecular Biology (Krawetz S and Misener S, eds.) New Jersey, 365-386. Available at [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi]. Accessed July 15, 2013.

Ruber L, Britz R, Tan HH, Ng PK, et al. (2004). Evolution of mouthbrooding and life-history correlates in the fighting fish genus *Betta*. *Evolution* 58: 799-813.

Srikulnath K, Matsubara K, Uno Y, Thongpan A, et al. (2010). Genetic relationship of three butterfly lizard species (*Leiolepis reveesii rubritaeniata*, *Leiolepis belliana belliana*, *Leiolepis boehmei*, Agamidae, Squamata) inferred from nuclear gene sequence analysis. *Kasetsart J.* (*Nat. Sci.*) 44: 424-435.

Sriwattanarothai N, Steinke D, Ruenwongsa P, Hanner R, et al. (2010). Molecular and morphological evidence supports the species status of the Mahachai fighter *Betta* sp. Mahachai and reveals new species of Betta from Thailand. *J. Fish. Biol*. 77: 414-424.

Tan HH and Ng PKL (2005). The fighting fishes (Teleostei: Osphronemidae: genus *Betta*) of Singapore, Malaysia and Brunei. *Raffles Bull. Zool.* 13 (Suppl 13): 43-99.

Vidthayanon C (2011). *Betta splendens*. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.2. Available at [http://www.iucnredlist.org]. Accessed December 6, 2012.

Witte KE and Schmidt J (1992). *Betta* brownorum, a new species of anabantoids (Teleostei: Belontiidae) from northwestern Borneo, with a key to the genus. *Ichthyol. Explor. Freshw.* 2: 305-330.

Zane L, Bargelloni L and Patarnello T (2002). Strategies for microsatellite isolation: a review. *Mol. Ecol.* 11: 1-16.

