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TECHNICAL NOTE

Isolation and characterization of polymorphic microsatellite loci from a dinucleotide-enriched genomic library of obscure puffer (*Takifugu obscurus*) and cross-species amplification

Hongyu Ma · Songlin Chen · Xiaolin Liao · Tianjun Xu · Jiachun Ge

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Abstract Obscure puffer (*Takifugu obscurus*) is an anadromous fish species in China. Here, we reported 10 polymorphic microsatellite loci isolated from a dinucleotide-enriched genomic library of *T. obscurus*. The number of alleles, observed and expected heterozygosity per locus in 30 individuals ranged from four to 10, from 0.57 to 0.86 and from 0.68 to 0.90, respectively. Three loci significantly deviated from Hardy–Weinberg equilibrium after Bonferroni correction and no significant linkage disequilibrium between pairs of loci was found. Cross-species amplification of these microsatellite loci in additional three fish species was performed. These polymorphic microsatellite loci would be useful for investigating genetic population structure and construction of genetic linkage map in *T. obscurus*.

Keywords Obscure puffer · *Takifugu obscurus* · Microsatellite · Enriched genomic library

Obscure puffer (*Takifugu obscurus*) is an anadromous fish species, mainly inhabiting South of China. They usually grow in the sea before sexually mature, then return to fresh water to spawn, and they are famous for puffing behavior, powerful toxins in the internal organs, and edible muscle (Akira et al. 2005). Puffer fish are considered to be good model animals and the genome project of *T. rubripes* was completed in 2002 (Aparicio et al. 2002). The wild resource of *T. obscurus* has sharply decreased under the effect of overfishing and water pollution. Documents about *T. obscurus* mostly focused on the physiology (Yan et al. 2004) and mitochondrial DNA (Shao et al. 2006). There are no molecular markers such as microsatellite isolated and application in this species reported to date, in spite of many molecular markers including AFLP, SSR have been developed and used broadly in other fish species (Liu et al. 2003; Chen et al. 2007). Lack of enough polymorphic microsatellite markers has limited the development of genetic diversity, population structure, and molecular marker-assisted breeding in this species.

In the present study, we developed 10 polymorphic microsatellite loci isolated from a dinucleotide-enriched genomic library of *T. obscurus* and tested their applicability to additional three fish species including redfin puffer (*Takifugu rubripes*), tawny puffer (*Takifugu flavidus*) and yellowfin puffer (*Takifugu xanthopterus*).

A sample of 30 individuals of *T. obscurus* were selectively collected in Nanjing city, China. Genomic DNA was extracted from the fin tissue as described by Ma et al. (2007). A dinucleotide-enriched genomic library was constructed using the fast isolation by amplified fragment length polymorphism (AFLP) of sequences containing repeats (FIASCO) protocol (Zane et al. 2002), and the detailed procedure was described by Liao et al. (2007). In brief, the whole genomic DNA was digested with *Mse* enzyme (New England Biolabs) and ligated to the adapters (Oligo A: 5′ HO-TACTCAGGACTCAT-OH 3′ and Oligo B: 5′ HO-GACGATGAGT CCTGAG-OH 3′).

H. Ma · S. Chen (✉) · X. Liao · T. Xu
Key Lab for Sustainable Utilization of Marine Fisheries Resources, Ministry of Agriculture, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Nanjing Road 106, Qingdao 266071, China
e-mail: chensl@ysfri.ac.cn

H. Ma
College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China

J. Ge
Freshwater Fisheries Research Institute, Nanjing 210017, China

Bio-labelled probes $(GT)_{13}$ were used to hybridize with the recovered products of pre-amplification (the size ranged from 300 to 900 bp). Subsequently, the hybrids were captured by streptavidin-coated magnetic beads (Promega Corporation), and the final DNA fragments eluted from the magnetic beads were amplified using the corresponding primers. The amplification products were directly cloned into pMD-18T vector as described by Chen et al. (2004) and 60 recombinant clones were randomly selected for sequencing using ABI Prism 3730 automated DNA sequencer (PE Corporation), of which 55 clones containing microsatellite repeats. Finally, PCR primers were designed for 20 loci using the software Primer Premier 5.0, of which 10 loci showing polymorphic. These microsatellite sequences have been deposited in GenBank (EU391391–EU391400).

Polymerase chain reaction (PCR) were performed on a Peltier Thermal Cycler (PTC-200) in 25 μl total volume that included 0.4 μM each primer, 0.2 mM each dNTP, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.75 unit *Taq* polymerase, and approximately 100 ng template DNA under the following conditions: one cycle of denaturation at 94°C for 4 min; 30 cycles of 30 s at 94°C, 50 s at a primer-specific annealing temperature (Table 1), and 50 s at 72°C. As a final step, products were extended for 7 min at 72°C. The PCR products were separated on 6% denaturing polyacrylamide gel, and visualized by silver-staining. The size of alleles were estimated according to the pBR322/*Msp* marker (TianGen Biotech CO., LTD.). Genetic diversity indexes were calculated using POPGENE version 1.31 (Yeh et al. 1999) software. Significance values for all multiple tests were corrected by sequential Bonferroni procedure (Rice 1989).

Ten of the 20 tested microsatellite loci were polymorphic in 30 individuals of *T. obscurus* (Table 1). The number of alleles per locus ranged from 4 to 10, and observed and expected heterozygosity ranged from 0.57 to 0.86 and from 0.68 to 0.90, respectively. Three loci (Tob11, Tob61 and Tob107) showed significant departures from Hardy–Weinberg equilibrium after sequential Bonferroni correction ($P < 0.005$). Such deviations might be caused by the limited sample size used in our test or the presence of null alleles confirmed by MICRO-CHECKER version 2.2.3 software (Van Oosterhout et al. 2004), but no evidences for stuttering and allelic dropout were found in all loci. No significant genotypic linkage disequilibrium (LD) was found between all pairs of the 10 loci after Bonferroni correction ($P > 0.005$).

The 10 polymorphic microsatellite loci were also tested for cross-amplification in additional three fish species including redfin puffer (*T. rubripes*), tawny puffer (*T. flavidus*) and yellowfin puffer (*T. xanthopterus*)

Table 1 Characterization of 10 polymorphic microsatellite loci in obscure puffer (*T. obscurus*)

Locus	Repeat sequence	Primer sequences (5′-3′)	T_a (°C)	N_a (size range, bp)	H_O	H_E	*P*	Accession no.
Tob10	$(CA)_{190}$	ACCCACTCCGTCCTTCCT TCAACCGCCCTTCCAACT	59	5 (320–368)	0.83	0.78	0.4851	EU391391
Tob11	$(CA)_4$...$(CA)_3$...$(CA)_4$...$(CA)_4$ ...$(CA)_5$...$(CA)_{21}$	GCCATATTGACCACTCACC ACCACAGAATGTCCTGCTT	56	10 (214–270)	0.76	0.88	0.0000*	EU391392
Tob13	$(CA)_{20}$	AGTAGAACGCTCGGTCAG GTTTGTAATCATCAAAAGG	59	4 (304–338)	0.57	0.73	0.1940	EU391393
Tob25	$(TG)_{21}$	ACTCTTTCTCCAGCTCTTC TGCTTCCTTTGATTTGTAT	53	4 (244–260)	0.62	0.68	0.1086	EU391394
Tob53	$(GATG)_{11}(GACG)_2(GATG)_3$ $(GACG)_2$	CCTACATCTCACCCAGTG AGGAAGCAAGACAAATAAG	54	9 (218–270)	0.84	0.83	0.2280	EU391395
Tob55	$(AC)_{17}GC(AC)_3GC(AC)_3$	GCGCAGCTTGCACTGTAT TAGCCTCTTTAGTCTTGATGG	56	6 (170–202)	0.69	0.71	0.9082	EU391396
Tob61	$(TG)_{30}$	AGAGGCTCCTGGGGAATT CAGCCCTGTCTCACACAT	57	10 (142–196)	0.79	0.87	0.0018*	EU391397
Tob91	$(AC)_3$...$(AC)_7GC(AC)_5GC$ $(AC)_3$...$(AC)_3GC(AC)_2GC(AC)_4$	ATTACATCGACCAGAGCCT CACCTATACATCTTAGAATACCC	59	9 (130–214)	0.83	0.85	0.9712	EU391398
Tob107	$(CA)_{35}$	GACCAGTCTCACTCCCTCC TGTGGTAACGGCCATTTCT	57	10 (234–282)	0.83	0.90	0.0000*	EU391399
Tob108	$(GT)_{15}GA(GT)_5$...$(GT)_5$...$(GT)_5$ ...$(GT)_4$...$(GT)_4$...$(GT)_3$...$(GT)_4$	TCCATTACCACAGAATGTCCTGC GTGCCATATTGACCACTCACCTA	58	10 (170–268)	0.86	0.86	0.5061	EU391400

T_a, annealing temperature; N_a, observed number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; * indicated significant deviation from HWE after Bonferroni correction (adjusted *P*-value = 0.005)

Table 2 Cross-species amplification of 10 microsatellite loci in additional three fish species including redfin puffer (*T. rubripes*), tawny puffer (*T. flavidus*) and yellowfin puffer (*T. xanthopterus*)

Species	Locus									
	Tob10	Tob11	Tob13	Tob25	Tob53	Tob55	Tob61	Tob91	Tob107	Tob108
T. rubripes (8)	2	4	3	3	4	5	3	5	5	3
T. flavidus (7)	3	4	4	3	3	5	5	4	4	4
T. xanthopterus (8)	5	6	3	2	4	4	3	4	4	4

The number in each cell indicates the number of observed alleles; The numbers in parenthesis mean numbers of individuals analyzed

(Table 2). Eight or seven individuals of each species were tested and PCR was carried out under the conditions as described previously. The result showed high degree of polymorphism of these loci in the three fish species. All 10 loci were polymorphic in each fish species, and the number of alleles per locus ranged from two to six in the three fish species.

To the best of our knowledge, it is the first time to report polymorphic microsatellite markers in *T. obscurus* that will allow studies of the population structure and genetic diversity of *T. obscurus* in the future.

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