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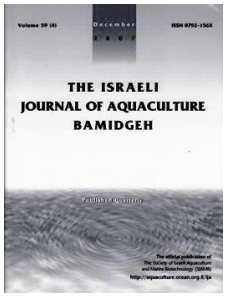

Microsatellite-Based Analysis of Genetic Diversity and Relationship of Artificial Hybrid Jiyan-1 Puffer and their Parents, *Takifugu flavidus* and *Takifugu rubripes*

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Keywords: Jiyan-1 puffer; *T. flavidus*; *T. rubripes*; genetic diversity; genetic relationship

Abstract

In this study, the genetic diversity and relationship of artificial hybrid Jiyan-1 puffer and their parents (*T. flavidus* ♀ and *T. rubripes* ♂) were evaluated using 15 microsatellite markers. The average number of alleles (*Na*), observed heterozygosity (*Ho*), and expected heterozygosity (*He)* of *T. flavidus* were higher than the average values of *T. rubripes*. Jiyan-1 puffer showed a relatively high level of genetic diversity, with an average allele number of 6.467 and mean observed and expected heterozygosity of 0.560 and 0.592, respectively. UPGMA cluster analysis indicated that Jiyan-1 puffer inherited more genetic information from female parents. This study indicates that the microsatellite markers will be useful for investigation of genetic background of puffer fish, as well as better conservation and sustainable utilization of puffer fish in aquaculture.

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Introduction

The genus *Takifugu* commonly known as puffer fish is mainly distributed in coastal waters of central and northern China, Japan, Korea, and Russia. They are high quality, edible fish and have been artificially cultivated for decades in Japan and China. Puffer fish contain the potent and deadly toxins tetrodotoxin and/or saxitoxin which can cause severe illness and death. Imports of puffer fish to the USA are authorized only after meticulous tests aimed at insuring that that they are free of lethal toxins and organs (FDA 1988). Consumers should eat puffer fish only from known safe sources.

Puffer fish aquaculture is a thriving and prosperous industry in China, producing more than 14,000 metric tons in 2013 (Department of Fisheries, 2014). Among the 22 species in the genus *Takifugu*, tiger puffer *Takifugu rubripes* and tawny puffer *Takifugu flavidus* are two representative aquaculture species in China. *T. rubripes* possesses the largest body size in *Takifugu* genus. Compared to *T. rubripes*, *T. flavidus* has smaller body size and slower growth rate, however its superior flavor brings in the highest market price of all aquaculture puffer species in China (Zhang et al., 2010). Jiyan-1 puffer, the F1 hybridof *T. flavidus* (♀) and *T. rubripes* (♂), were artificially reproduced and cultured in China in order to combine the desirable traits of *T. rubripes* and *T. flavidus*. The Jiyan-1 puffer exhibits obvious heterosis for growth performance, flavor, and stress tolerance (Gao et al., 2013; Fan et al., 2011) and has become a promising candidate for aquaculture, however information about the genetic background of Jiyan-1 puffer and their parents is limited.

Microsatellite markers, also known as simple sequence repeat (SSR) markers, have been widely employed in the detection of genetic diversity due to their co-dominance, high mutation rates, abundance throughout the genome and relative ease of scoring (Guichoux et al., 2011). In this study, Jiyan-1 puffer and their parents (*T. flavidus* and *T. rubripes*) were screened with 20 microsatellite markers in order to understand their genetic diversity and relationship. Sound information of genetic diversity provided by microsatellite loci will help in the design of conservation strategies for the genus *Takifugu*. Characterization of parental relationships and identification of hybrid using microsatellite markers will greatly accelerate the crossbreeding process in puffer aquaculture.

Materials and Methods

Sampling and DNA extraction. T. flavidus, *T. rubripes,* and Jiyan-1 puffer were provided by Tianjin Haisheng Aquatic Product Co., Ltd, Tianjin, China. Each population was sampled with 20 individuals. Total genomic DNA was extracted by a modified protocol according to Li et al. (2011). Concentration and quality of DNA were estimated using a Qubit 2.0 Fluorometer (Life Technologies, San Diego, USA) and agarose gel electrophoresis.

SSR markers screening. The potential microsatellite loci were searched from 1,730,788 bp genomic DNA of *T. rubripes* (AC156266) in Genebank using MISA software. About 35 loci were used for primer design using Primer Premier 3.0 (Premier Biosoft International), of which 20 pairs successfully amplified to yield PCR products of the expected size were labeled with a fluorescent dye of FAM at its 5’ end (Wuhan Genecreate Bioengineering Co., Ltd, Wuhan, China) for subsequent microsatellite amplification (Table 1).

Microsatellite amplification. Polymerase chain reaction (PCR) was performed in a 50 μL reaction volume containing 2 μL genomic DNA extraction product, 2 mM of each dNTP, 10 μM of each primer, 25 mM $MgSO_4$, 5 μL 10×KOD buffer, and 1 μL KOD-Plus. Thermal cycling was performed under the following conditions: an initial denaturation at 93°C for 3 min followed by 35 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and a final extension at 68°C for 8 min. PCR products amplified with fluorescent primers were genotyped on an ABI3730 genetic analyzer (Applied Biosystems) at Wuhan Genecreate Bioengineering Co., Ltd (Wuhan, China).

Table 1. Characteristics of the microsatellite markers

Locus	*Primer sequences (5'-3')*	*Repeat motif*	*Size range (bp)*
T1	F: TCTGAGTTGTGCATGGAAGC R: GCACAGAAGCGTCGACATAA	(CCCT)5	231
T2	F: CCTGTCACACCACAAAGGTG R: AAGTGGCGGGACTACACAAC	(GTGC)5	209
T3	F: TGAGCAAATTGTTTTCTGCG R: CGGATGATGTGAAGGTGACA	(CTG)5	215
T4	F: AAATGCCAGTGAAACCCTTG R: ACGCCGTCTGATTTGTTTTC	(GGA)5	268
T5	F: GTCAAGGGCAGGATCACAGT R: AGTTTGGCTGCCTCACATTC	(TG)6	211
T6	F: CCCCGTTAGACACAATGACC R: CGCACTCAGGAGATGACAAA	(GCT)10	264
T7	F: AGGAGTGCAGCACAGCTTTC R: GCATCATGGAGCAGAGTTGA	(GGC)6	242
T8	F: GTCTGTCAAATCATGCACGC R: AAACACGTTTTGCTCCCATC	(GT)11	228
T9	F: GCCTCCATAGTTCAAGCTGC R: AGATGCAGTTGATGTCGCTG	(CTC)5	225
T10	F: TGAAATTTGATGCACAGTGATG R: GACCAGCCCCATGCTAATAA	(TCT)5	253
T11	F: TTGCCTCACTGACAGGAAGTT R: ACAGTGAAGGATGGGAGGTG	(AAC)6	263
T12	F: AAACAGTTAACCCCGCATTG R: AGTGTGTGCACAACGGAGG	(GCA)5	180
T13	F: CCACTTCATCACGGTCGTC R: TTTAGGGCTGCCATTACCAC	(AC)6	275
T14	F: CTCTGCTGTCCTACGGGTGT R: GGAGTCGGTTTGCTGAGAAG	(GT)9	255
T15	F: AGAATCCGGAGGGCTACAAG R: TCAGGCTGACCACATAACCA	(GGC)6	209
T16	F: ATGCTGCTGGAGGAGAGCTA R: TCCTTTTTCTCCCTCCGTTT	(CCT)5	219
T17	F: TCCTCCCACACTTTGTCTCC R: GCTCTTTGGCCTCTTCCTCT	(GGA)8	276
T18	F: GCTGCAGGAGTCCAGAAAAC R: ATTTCAGGAGAGCGTGCCTA	(AGC)5	260
T19	F: CAAAGGTGGAGCAGAGGAAG R: ACCTGGCTTGAAATGGTTTG	(CAG)5	279
T20	F: GAAGACAGGAAACCACCCAA R: GGTAGACCAGCACCCGTAAA	(AGAT)18	246

Data analysis. To evaluate the genetic diversity of Jiyan-1 puffer and their parents (*T. flavidus* and *T. rubripes*), allele number (*Na*), effective allele number (*Ne*), observed heterozygosity (*Ho*), and expected heterozygosity (*He)* were estimated using GenAlEx 6.5. UPGMA cluster analysis based on genetic coefficient and performed using the software NTSYS.

Results

Five microsatellite loci, G3, G5, G7, G9 and G12 screened, were not polymorphic. The other 15 polymorphic microsatellite loci were used to evaluate the genetic diversity of Jiyan-1 puffer and their parents (see results in Table 2). These loci were highly polymorphic with sizes ranging from 209 bp to 279 bp. The allele numbers at Jiyan-1 puffer, *T. flavidus* and *T. rubripes* ranged from 2 to 12, 3 to 18 and 2 to 11, respectively. The effective allele number at the above three populations varied from 1.280 to 6.612, 1.581 to 12.308, and 1.051 to 5.926, respectively. Values of observed and expected heterozygosity of Jiyan-1 puffer ranged from 0.150 to 1.000 and 0.219 to 0.849, with an

average of 0.560 and 0.592, respectively. Both these average values were lower than those of *T. flavidus*, but higher than those of *T. rubripes*.
heterozygosity.

Table2. Genetic diversity analyses of Jiyan-1 puffer and their parents, *T. flavidus* and *T. rubripes*

Locus	Jiyan-1 puffer				*T. flavidus*				*T. rubripes*			
	Na	*Ne*	*Ho*	*He*	*Na*	*Ne*	*Ho*	*He*	*Na*	*Ne*	*Ho*	*He*
G1	2	1.280	0.250	0.219	3	1.831	0.600	0.454	2	1.051	0.050	0.049
G2	3	1.292	0.250	0.226	4	1.581	0.200	0.368	2	1.051	0.050	0.049
G4	5	1.613	0.450	0.380	7	2.100	0.500	0.524	6	2.041	0.650	0.510
G6	4	2.279	0.150	0.561	8	3.636	0.350	0.725	3	2.198	0.100	0.545
G8	8	1.970	0.400	0.493	10	2.556	0.700	0.609	2	1.471	0.000	0.320
G10	4	2.694	0.350	0.629	4	1.865	0.400	0.464	3	2.381	0.000	0.580
G11	7	2.778	0.350	0.640	6	4.145	0.500	0.759	4	1.865	0.200	0.464
G13	11	6.349	0.750	0.843	12	6.667	0.350	0.850	7	3.226	0.700	0.690
G14	9	3.419	0.700	0.708	15	10.127	0.800	0.901	2	1.980	0.800	0.495
G15	5	1.699	0.500	0.411	5	2.778	0.350	0.640	3	1.946	0.550	0.486
G16	4	2.807	0.850	0.644	6	3.419	0.850	0.708	2	1.980	0.600	0.495
G17	8	5.594	1.000	0.821	5	3.376	1.000	0.704	7	2.963	0.850	0.663
G18	12	4.762	0.900	0.790	18	12.308	1.000	0.919	6	2.817	0.550	0.645
G19	4	2.996	0.500	0.666	6	3.125	0.550	0.680	4	2.374	0.300	0.579
G20	11	6.612	1.000	0.849	15	9.091	0.950	0.890	11	5.926	0.900	0.831
Average over loci	6.467	3.210	0.560	0.592	8.267	4.574	0.607	0.680	4.267	2.351	0.420	0.493

Na: allele number, *Ne*: effective allele number, *Ho*: observed heterozygosity, *He*: expected

As shown in Fig.1, F1 hybrid Jiyan-1 puffer could be differentiated from their parents using microsatellite markers. Jiyan-1 puffer and *T. flavidus* run together into one branch, and *T. rubripes* go to one branch separately. The genetic coefficient between Jiyan-1 puffer and *T. flavidus* was larger than that between Jiyan-1 puffer and *T. rubripes*, suggesting that Jiyan-1 puffer inherited more genetic information from female parents.

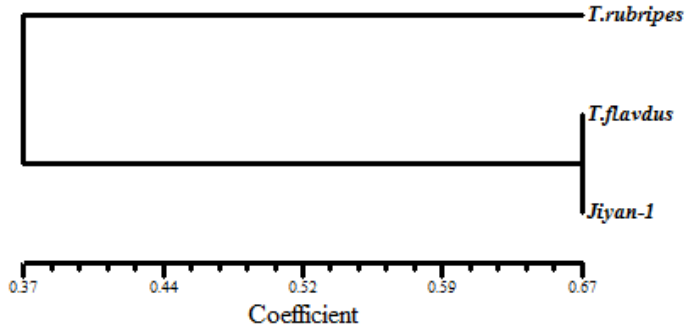

Fig. 1. Genetic relationship revealed by the microsatellite markers

Discussion

Microsatellites are useful tools for assaying genetic variation and provide efficient means to link phenotypic and genotypic variation (Varshney et al., 2005). All 15 loci used have relatively long repeating units and most of them are perfect repeats. Moreover, fluorescent detection with capillary electrophoresis was applied to genotype. The genetic diversity of Jiyan-1 puffer and their parents could be considered reliable. The number of alleles and heterozygosity are important indices for assessing population diversity at the genetic level (Leberg, 2002). In the present study, the average number of alleles, *Ho* and *He* of *T. flavidus* were relatively high compared to the average values of *T. rubripes*, suggesting that cultured *T. flavidus* population had greater breeding potential than cultured *T. rubripes* population. *T. flavidus* was artificially cultivated in China from 2001 (Shi et al., 2010) while *T. rubripes* was first cultured in the 1980s (Wan et al., 2013). Scientific selection and breeding measures should be adopted to maintain genetic diversity of cultured *T. rubripes*.

Interspecific hybridization is important to broaden the genetic base and create novel fish forms in breeding programs (Bartley et al., 2001). For puffer fish, natural hybrids occasionally appear (Lu et al., 2016); the artificial cross breeding started decades ago to combine the desirable traits of two parental species, often exhibiting superiority which never appeared in the parents (Yamashita, 1968). Successful artificial crosses were achieved between several cultured puffer species, such as *T. flavidus* and *T. rubripes* (Fan et al., 2011), *T. rubripes* and *T. niphobles* (Wang et al., 2011), *T. rubripes* and *T. porphyreus* (Wang et al., 2012), and *T. flavidus* and *T. obscures* (Zhang et al., 2015). It is important to avoid uncontrolled release of these artificially reared hybrids into the environment, as it will endanger native puffer species in regards to direct space and/or food competition, and also because of highly probable introgression into the genome of native species.

In this study, we found that F1 hybrid of *T. flavidus* and *T. rubripes* (Jiyan-1 puffer) had relative high genetic diversity. Caution is needed when introducing non-native species and their hybridization (Lin et al., 2015). The genetic relationship between Jiyan-1 puffer and their parents were not equal. The female parent transferred more genetic loci to Jiyan-1 puffer, which corresponded with previous study results for growth performance and flavor. The flavor of Jiyan-1 puffer is considered to be equivalent to *T. flavidus*, and distinctly better than *T. rubripes* (Gao et al., 2013).

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