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Short Communication

Isolation and characterization of microsatellite markers for the White Cloud Mountain minnow (*Tanichthys albonubes*) in wild and cultured populations

J. Zhao[1,2], D.-Y. Tseng[3], H.-D. Lin[4] and X.-T. Lin[1]

[1]Institute of Hydrobiology, Jinan University, Guangzhou, China
[2]Guangdong Provincial Key Laboratory for Healthy and Safe Aquaculture, Key Laboratory of Ecology and Environment Science in Guangdong Higher Education, School of Life Science, South China Normal University, Guangzhou, China
[3]Department of Biological Sciences and Technology, National University of Tainan, Tainan, Taiwan
[4]The Affiliated School of National Tainan First Senior High School, Tainan, Taiwan

Corresponding author: X.-T. Lin
E-mail: tlinxt@jnu.edu.cn

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ABSTRACT. We developed 12 microsatellite loci for the endangered minnow species, *Tanichthys albonubes*, using PCR-based isolation of microsatellite arrays. These new markers were tested in 26 individuals from a wild population collected from Guangzhou in China and 26 individuals from a cultured strain. The number of alleles ranged from two to nine and the expected heterozygosity from 0.177 to 0.853. The wild population had significantly higher allelic richness than the cultured strain, with a mean allelic richness of 5.52 (range = 3.69-8.64) and 3.13 (range = 1.99-5.73) for the wild population and the cultured

strain, respectively. No evidence of a recent bottleneck was detected in the wild population, but it was found in the cultured strain based on the BOTTLENECK test. These primers can be used to understand the demography and to examine genetic differences between the cultured *T. albonubes* strains and wild populations to help determine conservation and reintroduction strategies.

Key words: *Tanichthys albonubes*; Microsatellite marker; Bottleneck; Endangered species

The White Cloud Mountain minnow, *Tanichthys albonubes* Lin, is a small and popular ornamental cyprinid fish, originally found in Guangzhou, China. The wild populations of this species were thought to be extinct because there were no reports of this fish in the wild since 1980. It is a second-class state protected animal in China and classified as "extinct in nature" in the China Red Data Book (Yue and Chen, 1998) and China Species Red List, Vol. II (Wang and Xie, 2009). A wild population was discovered in mountain spring wells in the vicinity of Guangzhou (Liang et al., 2008), and a nature reserve was established for its protection. *Tanichthys albonubes* has characteristics similar to that of the model species *Danio rerio*, e.g., small in size, short generation time, laying eggs in batches, and transparent embryos, which render *T. albonubes* suitable for transgenic research.

Microsatellites are DNA markers that have proven to be highly polymorphic and co-dominant and have been used to address the genetic diversity and population structure of freshwater fishes (Crooijmans et al., 1997).

These markers have been widely used to determine genetic variation among wild and cultured populations of fish (Was and Wenne, 2002). They are also used to study population bottlenecks (Jarne and Lagoda, 1996) to facilitate specifically breeding programs (Jackson et al., 2003). The aim of this study was to isolate and characterize 12 novel microsatellite loci for the investigation of the genetic structure and bottleneck effect in wild and cultured populations of *T. albonubes*.

A microsatellite library was constructed using polymerase chain reaction (PCR)-based isolation of microsatellite arrays (PIMA) as proposed by Lunt et al. (1999). It takes advantage of the fact that the random amplified polymorphic DNA (RAPD) fragments contain microsatellite repeats more frequently than random genomic clones. The DNA was extracted from fin and/or muscle following the phenol-chloroform protocol. The RAPD-PCR amplifications were performed in a thermal cycler (Bio-Rad) with a reaction mixture (50 µL) containing 20-100 ng DNA, 0.2 mM of each dNTP, 2 mM $MgCl_2$, 0.5 U Taq polymerase (Promega), and 5 pmol RAPD primer. RAPD-PCR products were size-selected to preferentially obtain small fragments (500-1, 200 bp). Approximately 100 ng PCR product was ligated into a pGEM-T vector (Promega) according to manufacturer instructions, and the ligation mixture was transformed into competent *Escherichia coli* cells. Clones were screened using microsatellite-specific primers and two vector primers (Lunt et al., 1999). In positive clones, the repeat-specific and vector primers amplified DNA fragments containing microsatellites, whereas no amplification was found in negative clones. Plasmid DNA from positives was purified using the High-Speed Plasmid Mini kit (Geneaid). Both strands of the DNA insert were sequenced with an Applied Biosystems Model 377A automated sequencer (Applied Biosystems). Specific-

primer pairs were designed according to the nucleotide sequences upstream and downstream of the repetitive DNA using the Primer 3 software (Rozen and Skaletsky, 2000). Preliminary assessment of polymorphism was performed on a few individuals. Reactions were performed in a total volume of 15 µL containing 10 ng genomic DNA, 0.2 mM dNTPs, 2 mM $MgCl_2$, and 0.12 µM of each primer. PCRs were as follows: 94°C for 4 min followed by 40 cycles at 94°C for 30 s, 30 to 50 s at primer-specific annealing temperature (Table 1), 72°C for 45 s, and a final extension step at 72°C for 10 min. Electrophoresis was performed in denaturing 6% polyacrylamide gels using 10-bp ladder for molecular size standard (Invitrogen) to estimate allele sizes via ethidium bromide straining. A total of 52 *T. albonubes* specimens were collected from a wild population (GM) in Guangzhou, China, and a cultured population (CR) in Taiwan.

Genotype data files were interconverted for the various analytical software programs using CREATE (Coombs et al., 2008) to minimize errors. Arlequin version 3.11 (Excoffier et al., 2005) was used to estimate mean numbers of alleles, observed heterozygosity (H_O), expected heterozygosity (H_E), and Hardy-Weinberg equilibrium (HWE) status. Allelic richness and fixation index (F_{ST}) (Weir and Cockerham, 1984) were estimated using the FSTAT for Windows version 2.9.3 software (Goudet, 2002). Inbreeding coefficient (F_{IS}) (Weir and Cockerham, 1984) and the significance of these values were calculated for each population in GenePop Web Version 4.0.10 (Rousset, 2008). The allelic richness differences between wild and cultured populations were compared using the Wilcoxon signed-rank test (JMP 8, SAS Institute Inc.).

A recent expansion or bottleneck in each population was tested using the version 1.2.02 BOTTLENECK software (Piry et al., 1999), using a coalescent procedure to compare variation (excess or deficiency) in heterozygosity (Cornuet and Luikart, 1996). The observed allele frequency distribution was compared with that of a population in mutation-drift equilibrium assuming the SMM (stepwise mutation model; Kimura and Ohta, 1978) and TPM (two-phase model; Di Rienzo et al., 1994) with 70% SMM and 30% infinite allele model. The significance of the test was assessed with sign and Wilcoxon tests. Statistically, the first test offers low power, but the second test displays relatively high statistical power and can be applied with as few as four polymorphic loci and any number of individuals (Cornuet and Luikart, 1996).

Locus designation, GenBank accession number, repeat motif, PCR product size range, and number of alleles for the 12 microsatellite markers are listed in Table 1. The mean number of alleles was 3.16 and 5.83 in the CR and GM populations, respectively. Allelic richness was calculated to compare the number of alleles in samples of unequal size. The wild population had significantly higher allelic richness than the cultured strain, with an average allelic richness of 5.52 (range = 3.69-8.64) and 3.13 (range = 1.99-5.73) for the wild population and the cultured strain, respectively, which may be caused by hatchery selection and inbreeding (cf. Table 2). *F*-statistics showed significant population differentiation between the cultured strain and wild population (F_{ST} = 0.259; P = 0.000). The strong genetic differentiation observed in this study demonstrates that microsatellite markers can detect population structure in *T. albonubes* and will facilitate population genetic studies including assignment tests, pedigree analysis and mapping studies. Deviations from Hardy-Weinberg expectations of heterozygosity were seen in both wild and cultured populations (Table 2), both exhibiting significant departures ($P < 0.05$) from HWE for 7 and 5 loci, respectively. The paired-sample *t*-test of H_O and H_E implied that there was no significant difference between wild and cultured populations

Table 1. Primer sequence, GenBank accession No., repeat motif, size range, and number of alleles for 12 microsatellite loci of *Tanichthys albonubes*.

Locus	GenBank accession No.	Primer sequence (5' to 3')	Repeat motif	Size range (bp)	Total No. of alleles	Tm (°C)
MITA01	JF304956	F: TCTGAGCGGAGTCTCTGGAT R: AGGACCGCTGAGTCTCTTGA	$(CA)_{14}$	208-222	6	60
MITA02	JF304957	F: CGGCCCTGACTGACTTTAAT R: GGATTCTGGAAACTGGCAAC	$(CA)_4(CT)_{10}(CA)_{21}$	232-272	9	60
MITA03	JF304958	F: CAGTTCGTTCTTCCGTCCTC R: CTCAGCTGCTGCTTTTCCTT	$(CA)_8$	234-256	5	58
MITA04	JF304959	F: GTCCCCTTGTGTCTGTCC R: GAGCTCACAGTGTGCCAGTC	$(AC)_{10}$	220-246	5	54
MITA05	JF304960	F: GGCCCTGTCAGGAGTGATTA R: GGAAGTGTACATCGCAGCAA	$(AC)_9$	174-196	10	59
MITA06	JF304961	F: GCATCTCCCAGGTCCAACTA R: AAGGGCTGGGATTTCCTAAA	$(TG)_4$-$(TG)_7$	152-168	8	58
MITA07	JF304962	F: GCCACTGCGATATACTGCAAC R: CTGCTGATTCTGCTCTGTGC	$(TC)_2(CA)_6$	136-156	8	60
MITA08	JF304963	F: ATGCATTTCGGAGAGACGAG R: GAGCGAGACACACAGACAGC	$(CA)_{23}$	188-216	10	59
MITA09	JF304964	F: TGTCAGCTGTGTGTCGTCTG R: CCATCAGCTTTCATCCGTTT	$(CA)_{15}$	168-190	7	59
MITA10	JF304965	F: TGGACATGTCTCATCCAGTG R: TCAACACTGGACTGCCTAAAAA	$(TG)_8$-$(CA)_{10}$	254-308	9	59
MITA11	JF304966	F: TGGAGCCTGTGGTCATTACA R: GGCTTCCTCAATCAGTTTGC	$(AC)_{33}$	198-250	9	59
MITA12	JF304967	F: ACCCCCGAAGCTAATGAAAT R: ATAGAGGCCGGTCTGTTTT	$(CA)_{12}$	210-230	5	59

Tm = melting temperature.

Table 2. Variability in 12 microsatellite loci in two *Tanichthys albonubes* populations between wild (GM) and cultured (CR) populations.

Site	Measure of diversity	MITA01	MITA02	MITA03	MITA04	MITA05	MITA06	MITA07	MITA08	MITA09	MITA10	MITA11	MITA12	Average across loci
CR	A	2	4	2	2	3	5	6	5	2	3	2	2	3.167
N = 26	A_R	2.000	3.960	1.999	2.000	3.000	4.931	5.731	4.999	2.000	2.993	2.000	2.000	3.136
	H_O	0.615	0.250	0.192	0.153	0.730	1.000	0.961	0.208	0.923	0.375	0.846	0.680	0.578
	H_E	0.434	0.640	0.177	0.265	0.540	0.717	0.800	0.768	0.506	0.562	0.506	0.506	0.535
	P_{HW}	0.056	0.000	1.000	0.076	0.055	0.000	0.000	0.000	0.000	0.028	0.000	0.114	
	F_{IS}	-0.429	0.615	-0.087	0.425	-0.361	-0.404	-0.205	0.733	-0.852	0.339	-0.692	-0.351	-0.080
	P_{FIS}	0.057	0.000	1.000	0.077	0.059	0.000	0.000	0.000	0.000	0.026	0.001	0.114	
GM	A	6	4	5	4	9	8	7	7	4	5	7	4	5.833
N = 26	A_R	5.593	3.698	4.898	3.662	8.641	7.590	6.541	6.424	4.000	4.983	6.308	4.000	5.528
	H_O	0.346	0.269	0.193	0.307	0.730	0.961	0.666	0.826	0.684	0.461	0.807	0.615	0.572
	H_E	0.565	0.248	0.537	0.334	0.844	0.808	0.567	0.595	0.695	0.720	0.641	0.717	0.606
	P_{HW}	0.001	1.000	0.000	0.654	0.000	0.008	1.000	0.389	0.897	0.000	0.512	0.109	
	F_{IS}	0.393	-0.084	0.647	0.080	0.137	-0.194	-0.179	-0.400	0.017	0.364	-0.267	0.144	0.057
	P_{FIS}	0.000	1.000	0.000	0.653	0.000	0.013	1.000	0.379	0.896	0.000	0.493	0.110	

N = sample size; A = number of alleles; A_R = allelic richness; H_O = observed heterozygosity; H_E = expected heterozygosity; P_{HW} = P values of chi-square tests for Hardy-Weinberg equilibrium; F_{IS} = fixation index; P_{FIS} = P values of chi-square tests for inbreeding coefficient fixation index.

(P = 0.962 and 0.349, respectively). Additionally, the GM and CR populations exhibited significant differences in allelic richness (Wilcoxon signed-rank test, Z = 2.98702, P = 0.0028), where GM showed a significantly higher value for allelic richness than CR (Table 2). The mean score of GM (16.883) was higher than that of CR (8.1667), but there was no significant difference in overall heterozygosity. Similar results were presented by Norris et al. (1999), indicating that allelic diversity is a more sensitive measure of variability between wild and cultured populations compared to heterozygosity.

The bottleneck test with the SMM and TPM models showed deviations from mutation equilibrium for cultured population (CR) with sign and Wilcoxon tests (Table 3). No evidence of a recent bottleneck was detected in the wild population. The reduced genetic variability that we observed in the cultured strains is probably due to a low number of successful breeders during the establishment of these strains and is similar to a recent bottleneck effect in terms of impact on genetic variability (Allendorf, 1986).

Table 3. Bottleneck results for *Tanichthys albonubes* from cultured (CR) and wild (GM) populations, showing mode shift and heterozygote excess (significant P values in bold) from the sign test and the Wilcoxon signed-rank test.

Populations		Heterozygote excess P values	
		CR	GM
Sign test	SMM	**0.00747**	0.05962
	TPM	**0.00428**	0.16881
Wilcoxon test	SMM	**0.00122**	0.98291
	TPM	**0.00037**	0.95386
Mode shift		Shifted mode	Normal L-shaped distribution

SSM = stepwise mutation model; TPM = two-phase model (70% SMM and 30% infinite allele model). Values in boldface type are significant at P < 0.05.

This study showed that the 12 microsatellite loci are useful genetic markers to address the genetic difference between the wild and cultured populations, indicating a tendency towards a reduction in variability within the cultured *T. albonubes* strain and an increase in differentiation between the cultured *T. albonubes* strain and the wild population.

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