Development of transcript-associated microsatellite markers in *Ancherythoculter nigrocauda* and cross-amplification in *Culter alburnus*

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ABSTRACT. Twenty-eight polymorphic microsatellite markers were developed from the transcriptome of *Ancherythoculter nigrocauda*. These loci were used to characterize the genotypes of 48 individuals. The observed number of alleles per locus ranged from 5 to 11, with an average of 7.7. Expected and observed heterozygosities ranged from 0.437 to 0.978 and from 0.373 to 1.000, respectively. Four of these polymorphic microsatellite loci (HWB14, HWB18, HWB24, and HWB30) deviated significantly from the Hardy-Weinberg equilibrium after use of the sequential Bonferroni correction ($P < 0.05$). Twenty of the 28 loci could be successfully amplified in *Culter alburnus.* These novel markers will be useful for germplasm resource conservation and management of *A. nigrocauda* and *C. alburnus*.

Key words: *Ancherythoculter nigrocauda*; *Culter alburnus*; Polymorphism; Simple sequence repeat; Cross-amplification

INTRODUCTION

The fish species *Ancherythoculter nigrocauda*, which belongs to subfamily Culterinae, family Cyprinidae, and order Cypriniformes (Luo and Chen, 1998), is endemic to the upper reaches of the Yangtze River in China. Recently, its natural resources have been declining in China because of habitat fragmentation as a result of dam construction, long-term overfishing and water pollution (Xiong et al., 2006). Therefore, there is an urgent need for monitoring and preserving genetic diversity in this species. Microsatellite or simple sequence repeat (SSR) markers are widely used in assessing population genetic structures and genetic diversity because of their hyper-variability and abundance throughout most vertebrate genomes. Some anonymous SSRs have been developed previously for *A. nigrocauda* (Sun et al., 2014); however, SSRs derived from expressed sequence tags (EST-SSRs), which are associated with biological functions, are not so far available for this species. Compared to anonymous SSRs, EST-SSRs can provide more useful information for investigating genetic diversity, such as determining the ability to adapt to the environment (Reed and Frankham, 2003). In this study, we developed 28 EST-SSRs for *A. nigrocauda*, with the aim of providing new molecular tools to investigate genetic diversity in an *A. nigrocauda* population.

MATERIAL AND METHODS

A total of 11,211 putative microsatellites from the *A. nigrocauda* transcriptome were generated by direct high-throughput Illumina sequencing of a liver cDNA library (unpublished results). Of these, 36 microsatellite-containing sequences were randomly selected to design primers using PRIMER PREMIER 5.00 (Premier Biosoft International). Polymorphisms in all loci were initially tested in eight *A. nigrocauda* and eight *Culter alburnus* individuals. Subsequently, the polymorphic markers were used to investigate 48 *A. nigrocauda* individuals from Wu Lake in Wuhan, Hubei Province of China and 48 *C. alburnus* individuals from Danjiangkou, Hubei Province of China. Genomic DNA was extracted from ethanol-preserved fin tissues using the standard phenol-chloroform method. A 25-µL reaction mixture was used for PCR; this mixture contained 50-100 ng genomic DNA, 5 µM forward primer (5' modified with FAM, HEX, or ROX), 5 µM reverse primer, 10 mM dNTPs, 10X Taq buffer (15 mM $MgCl_2$), and 0.5 unit Taq polymerase (Takara). PCR amplifications were carried out with the following conditions: 95°C for 5 min; 35 cycles comprising 94°C for 30 s, the optimized annealing temperature (Tables 1 and 2) for 30 s, and 72°C for 30 s; and a final extension of 72°C for 7 min. The PCR products were genotyped on an ABI 3730 DNA sequencer (ABI) and analyzed using Genescan 3.7 (ABI).

The number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), exact test of Hardy-Weinberg equilibrium (HWE), and linkage disequilibrium were analyzed using ARLEQUIN version 3.0 (Excoffier et al., 2005).

RESULTS AND DISCUSSION

The 28 polymorphic loci identified 5 to 11 alleles per locus, with an average of 7.7, in a wild population (Table 1). H_E ranged from 0.437 to 0.978 and H_O from 0.373 to 1.000. Four of the polymorphic microsatellite loci (HWB14, HWB18, HWB24, and HWB30) showed significant deviations ($P < 0.05$) from HWE after use of the sequential Bonferroni correction. There was no evidence of linkage disequilibrium among the loci at the 5% significance level. These polymorphic

Table 1. Isolation and characterization of polymorphic microsatellite loci from *Ancherythoculter nigrocauda*.

Locus	Primer sequence (5'-3')	Repeat motif	Ta (°C)	N_A	Size range (bp)	H_E	H_O	GenBank accession No.
EHWB01	CATCACAACAGCAAAACAACT	CACATCCAATAATAGAAAGCG	$(GT)_7$	60	8	129-157	0.744	0.812	KM588228
EHWB02	TAAAGATGGAAAAGAAGAAA	CGATGTAAGATTGAGTGAGA	$(CT)_9$	50	5	301-329	0.437	0.373	KM588229
EHWB03	GACAGACTTATCGCAGGA	TGAACCCAGAAGTGAAAC	$(AC)_9$	60	5	239-251	0.598	0.479	KM588230
EHWB04	TTGTTCGTCTTTTCCTGTTTC	TGGTCACTGTCCAAGTCTGTT	$(TTC)_6$	48	6	242-286	0.500	0.687	KM588231
EHWB06	CTGATTCAAGCACTCAAAAAAACT	GAAAAACAGAACGAAAGCAACAC	$(AG)_6$	52	7	255-283	0.708	0.768	KM588232
EHWB07	GCATTGCCTGATTAGAGAGC	AGTGTTGACTGTGTTGGGGT	$(AC)_6$	52	9	237-293	0.958	0.730	KM588233
EHWB08	TTATTTTTATTGGTTCCCCT	CCATACACGATTATCTACGG	$(TG)_7$	52	8	258-320	0.791	0.697	KM588234
EHWB09	GACGGCATCCCCTTCACAAC	CGTAGAAAAGACGCCCCAGAG	$(CT)_6$	55	10	179-209	0.958	0.783	KM588235
EHWB11	GCAATAACAGGTTCAATCATA	CGCTCAGAGACATAGTAAAGA	$(CT)_6$	50	11	257-311	0.978	0.878	KM588236
EHWB12	CCATCAGCAGACTAAGCCAC	TATGTGTTCCCCAGTCCAAA	$(CA)_6$	55	6	276-294	0.534	0.574	KM588237
EHWB13	GTTGTATTTAGGATGTAAGCGAG	AACCTTTGAGTGTTTTATGTGTC	$(CA)_6$	52	7	256-288	0.793	0.792	KM588238
EHWB14	GCAAACAGATGACTGGAGC	AACTGAAATGGCAAAGAGC	$(TTCT)_5$	50	10	132-246	0.659	0.386*	KM588239
EHWB15	CTAAAAGGCATTGAAAACT	TGACTAAAGCCAACTGAAGA	$(ATT)_6$	56	5	114-126	0.826	0.972	KM588240
EHWB16	AAAAATAATACCAAGCGTGC	TCAGCCAATAGGAGAGAGG	$(TG)_7$	58	6	289-313	0.558	0.694	KM588241
EHWB17	GAGTGTTTGTTTCTGCTCTTGGC	ATCTGGCTGATGTGAACTGGGA	$(GA)_6$	55	8	232-278	0.811	0.971	KM588242
EHWB18	AAAGTCCCGCTCTGTTATCAT	TAGAAAACTGTGCTGTTCCTGT	$(GT)_9$	60	9	102-114	0.768	1.000*	KM588243
EHWB19	GGCACACGCACACATACAC	CAAAAGGAGATTTAGGAGGGGA	$(CT)_6$	54	10	198-296	0.556	0.692	KM588244
EHWB20	TAACGCTCTCTTGAATAAT	CTAAACAAACGAACTGAAA	$(AGC)_5$	50	8	245-256	0.583	0.583	KM588245
EHWB21	TGTCGGTGGGTTTAGTTT	CATTCATCCTCCATTTCC	$(TG)_7$	55	8	256-290	0.889	0.922	KM588246
EHWB22	GACTGTCCGCTCCTCAC	TACCAAGCACCCAAATG	$(AC)_6$	56	9	127-153	0.793	0.892	KM588247
EHWB23	TCGTGCTAAACATCTCCT	TTCTCGGTTGAACTATCC	$(TG)_7$	52	6	302-326	0.877	1.000	KM588248
EHWB24	AGGCTAAGATTGATTGACAG	GCATTTATGGGATGGTATT	$(CT)_8$	60	11	254-302	0.756	0.979*	KM588249
EHWB25	ACTCCAGATGCCTTGTTG	GTCTGCTTTAGCCCACTT	$(TCC)_8$	50	6	137-167	0.656	0.879	KM588250
EHWB26	ATCAGCAGCGATACTTCAA	GGCGGTAACATTTCTTCAT	$(AG)_7$	60	10	161-181	0.804	0.978	KM588251
EHWB27	TCCGTCTCACTGAAACAAGC	AGCCTCCACCCTGAACAA	$(AG)_6$	60	7	212-224	0.761	0.829	KM588252
EHWB28	CTACTTTGTCACAGCAGC	CATCAGAAAACCGTCAT	$(AT)_6$	50	7	131-183	0.875	0.912	KM588253
EHWB29	CCATACCGACCCGAAATAA	GTGCTGGATTGGGAAGAACG	$(GAG)_5$	52	9	268-298	0.821	0.871	KM588254
EHWB30	ATTGAAATGTTACCGACCAC	TCACAGAGCACCAGCAGA	$(TG)_7$	60	6	220-326	0.673	0.957*	KM588255

Ta = annealing temperature; N_A = number of alleles; H_E = expected heterozygosity; H_O = observed heterozygosity. *Significant deviation from the Hardy-Weinberg equilibrium after Bonferroni correction.

Table 2. Characterization of polymorphic microsatellite loci in the *Culter alburnus*.

Locus	Repeat motif	Ta (°C)	N_A	Size range (bp)	H_E	H_O	GenBank accession No.
EHWB01	$(GT)_7$	60	5	127-133	0.373	0.437	KM588228
EHWB03	$(AC)_9$	60	8	243-253	0.688	0.500	KM588230
EHWB04	$(TTC)_6$	48	8	240-280	0.505	0.680	KM588231
EHWB06	$(AG)_8$	52	7	253-281	0.705	0.761	KM588232
EHWB08	$(TG)_7$	52	9	256-322	0.790	0.691	KM588234
EHWB09	$(CT)_8$	55	10	179-205	0.954	0.783	KM588235
EHWB14	$(TTCT)_5$	50	11	130-240	0.768	0.708*	KM588239
EHWB17	$(GA)_6$	55	8	230-268	0.814	0.971*	KM588242
EHWB18	$(GT)_9$	60	8	102-116	0.730	0.958	KM588243
EHWB20	$(AGC)_5$	50	6	248-259	0.698	0.792	KM588245
EHWB22	$(AC)_6$	56	9	123-151	0.793	0.890	KM588247
EHWB24	$(CT)_8$	60	9	256-296	0.783	1.000	KM588249
EHWB26	$(AG)_7$	60	14	161-193	0.879	0.958	KM588251
EHWB27	$(AG)_6$	60	6	214-228	0.574	1.000	KM588252
EHWB30	$(TG)_7$	60	3	318-324	0.535	0.979	KM588255

Ta = annealing temperature; N_A = number of alleles; H_E = expected heterozygosity; H_O = observed heterozygosity. *Significant deviation from the Hardy-Weinberg equilibrium after Bonferroni correction.

microsatellite markers will therefore provide a powerful tool for investigating genetic diversity and population genetic structures in *A. nigrocauda*.

The 28 polymorphic markers were tested for cross-species amplification in *C. alburnus*, a closely related species to *A. nigrocauda*. Of these, 20 loci successfully amplified a product and 15 showed polymorphisms. The N_A per polymorphic locus ranged from 3 to 14 with an average of 8.1. H_E ranged from 0.373 to 0.954 and H_O from 0.437 to 1.000 (Table 2). Two of the polymorphic microsatellite loci (HWB14 and HWB17) showed significant deviations ($P < 0.05$) from the HWE after use of the sequential Bonferroni correction. There was no evidence of linkage disequilibrium among loci at the 5% significance level. Therefore, these cross-species microsatellite markers from *A. nigrocauda* are also potentially of value for conservation and management of germplasm resources in *C. alburnus*.

We identified strong heterozygosities for some microsatellite loci, such as HWBE18/HWBE23/HWBE24/HWBE27, which are therefore potentially suitable for genetic mapping in *A. nigrocauda* and *C. alburnus*.

In this study, we have described 28 polymorphic microsatellite loci in *A. nigrocauda* and showed that 15 of these markers can identify polymorphic loci in *C. alburnus* through cross-species amplification. These loci will be of value for investigating genetic variation and population structures in *A. nigrocauda* and *C. alburnus*, and will also prove helpful in the management of their fisheries and in the design of conservation strategies.

Conflicts of interest

The authors declare no conflict of interest.

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