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RESEARCH PAPER

Development of Polymorphic Microsatellite Markers from AFLP Products in the Xinjiang Arctic Grayling (*Thymallus arcticus grubei*) and a Test of Cross-Species Amplification

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Abstract

The population of Xinjiang arctic graylings (*Thymallus arcticus grubei*) has dramatically declined in China. A total of 22 polymorphic microsatellite loci were isolated and characterized from AFLP products in the Xinjiang arctic grayling. The number of alleles (Na) per locus varied from 3 to 13. Observed (H_O) and expected (H_E) heterozygosities ranged from 0.60 to 0.97, and from 0.58 to 0.89, respectively. These new microsatellite markers are a helpful tool for genetic analyses, stock management, and resource conservation efforts for *T. arcticus grubei*. According to the total number of alleles, the total effective number of alleles, and the total number of genotypes, the Fuhai population showed the highest diversity among all the populations while the Fuyun sample was the lowest. Five additional fish species: Red seam bream (*Pagrus major*), Lenok (*Brachymystax lenok*), Sea perch (*Lateolabrax japonicus*), Taimen (*Hucho taimen*), and Amur grayling (*Thymallus grubii*) were assessed for cross-species amplification. Three out of the five species showed at least two polymorphic loci. In addition, six loci were found to be polymorphic in at least one species.

Introduction

The Xinjiang arctic grayling, *Thymallus arcticus grubei*, one of the salmonid fishes, naturally occurs in the Xinjiang Irtysh River basin, China (Liu, Liu, & Wang, 2016). However, fisheries have reported heavily declined populations in China, due mainly to human activities, such as, but not limited to overfishing, coastal development, and environmental pollution (Liu *et al*., 2016). Thus, it's key to protect the natural resources of *T. arcticus grubei*. In the long run, a comprehensive understanding of the genetic diversity, population structure, and genetic differentiation of *T. arcticus grubei* is required to create management plans for conservation (Liu *et al*., 2016). Microsatellites, or simple sequences repeats (SSR), are widely distributed throughout the genomes of animals, plants, and microbes (Zeng *et al*., 2013; Zhao, Zhao, & Peng, 2014; Xin *et al*., 2016). Microsatellites are highly variable and most are thought to be selectively neutral. Therefore, microsatellites have become the marker of choice for gene studies and genome evolution for all taxa. Many studies have examined the genetics of salmonid fishes using microsatellite markers in recent years (Kordicheva*et al*., 2010; Khrustaleva, Volkov, Stocklitskaya, Mugue, & Zelenina, 2010; Afanasiev, Rubtsova, Shitova, Shaĭkhaev, & Zhivotovskii, 2011; Shaikhaev & Zhivotovsky, 2014; Rubtsova*et al*., 2016); however, many microsatellite sequences in GenBank for *T. arcticus grubei* are still unknown. We must screen for more polymorphic microsatellite markers in *T. arcticus grubei* in order to improve fine-scale population structure, stock management and enhancement, genetic linkage map construction, and molecular marker-assisted breeding. In this study, we isolated 22 polymorphic microsatellite markers derived

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from *T. arcticus grubei* to develop a batch of microsatellite markers and to assess the genetic variability of natural populations of *T. arcticus grubei*.

Materials and Methods

Fish Sampling

The Xinjiang arctic grayling samples were collected from Fuhai county (Fuh), Habahe county (Hab), and Fuyun county (Fuy) in Xinjiang Irtysh River basin, Aletai, China in August, 2015. Geographic locations and sample sizes are shown in Figure 1. The samples of *Pagrus major, Brachymystax lenok, Lateolabrax japonicus*, *Hucho taimen*, and *Thymallus grubii* were collected from Qingdao in the Shandong province, Linjiang in the Jilin province, Rizhao in the Shandong province, Tumen in the Jilin province, and Mohe in the Heilongjiang province in China, respectively.

DNA Extraction

DNA extraction was performed as described by Liu, Chen, and Li (2005a) and Liu, Chen, Li, Wang and Liu (2005b) with minor modifications. DNA was collected after brief centrifugation and was washed twice with 70% ethanol, air-dried, and redissolved in double distilled water. The concentrations were measured with a GENEQUANT Pro (Pharmacia Biotech Ltd, Cambridge, England) RNA/DNA spectrophotometer for absorption at 260 nm.

Microsatellite Sequence Screening

Procedures of AFLP analysis were based on Vos*et al*. (1995) with some modifications. About 120 ng of total DNA was digested with 5U of EcoRI and MseI in 1× NE buffer 2 at 37°C for 3 h. To generate the DNA template for subsequent PCR amplification, the digested DNA fragments were ligated with 2.5 pmol of EcoRI and 25 pmolMseI adapters in a reaction mixture containing 0.25 mg BSA, 5 pmol ATP, 0.05 U T4Dnase, and 10 × NE buffer 2 at 37°C for 8 h. The sequences for the EcoR I primer were 5’-GACTGCGTACCAATTC-3’, and for the Mse I primer, the sequences were 5’-GATGAGTCCTGAGTAA-3’. Co-dominant AFLP bands were hand selected, excised from the dried gel, and placed individually into tubes containing 0.25 ml of TE buffer as described by Liu, Liu, Li, and Li (2009) with minor modifications. The gel pieces were then incubated for 3 h at 57°C to elute the DNA fragments. Then, the incubation buffer was stored at 5°C before being used as a template for PCR re-amplification of the AFLP bands. The re-amplified PCR products were then checked with agarose gel (1.2%) electrophoresis and purified with a purification kit (Qiagen, Germany). The purified PCR products were ligated into T-vectors and then transformed to *E. coli* of DH5α for the next sequencing (Lunt, Hutchinson, & Carvalho, 1999; Yokota & Oishi, 1990). We sequenced 160 randomly selected clones of recombinant *E. coli* of DH5α. Microsatellite sequences were screened using Tandem Repeats Finder (version: 2.02) (Benson, 1999). Tandem Repeats Finder’s standardized identification of

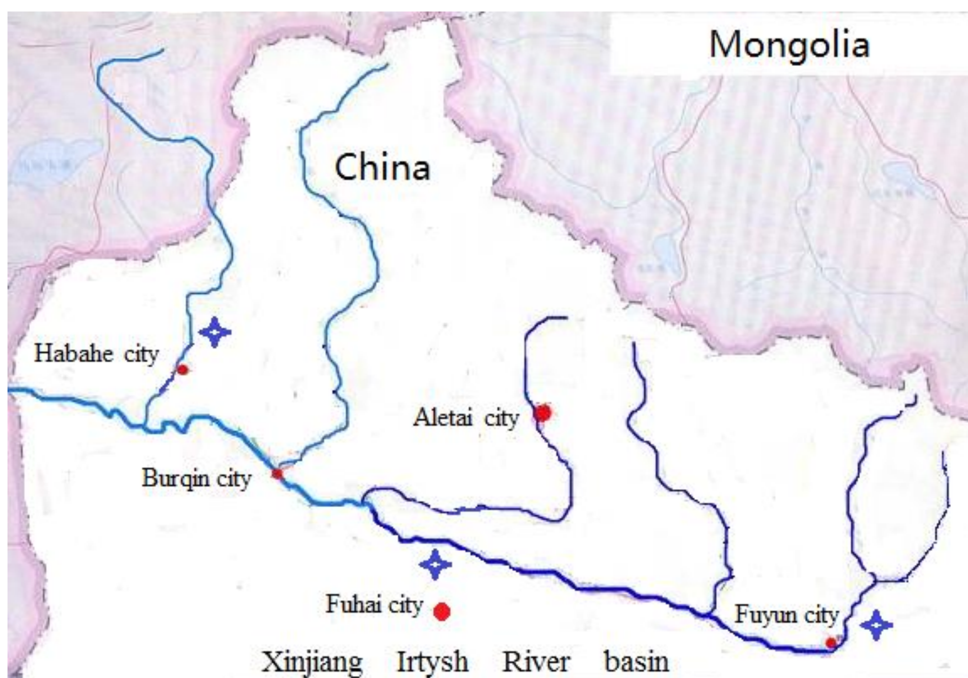

Figure 1. Geographic origin of Xinjiang arctic grayling (*Thymallus arcticus grubei*) samples. Fuh, Fuhai county (n=30); Hab, Habahe county (n=30); Fuy, Fuyun county (n=30).

microsatellites was 8 or more repeats for dinucleotide repeat sequences, 5 or more repeats for trinucleotide repeat sequences, and 4 or more repeats for tetranucleotide repeat sequences.

Microsatellite Amplification and Polymorphism Detection

Microsatellite amplification was performed as described by Liu, Guo, Hao, and Liu (2012). PCR was performed in a 25-µl reaction mixture composed of 10 pmol of each primer set, 100 µM of dNTPs, 10 mMTris–HCl (pH 8.3), 50 mMKCl, 2.0 mM $MgCl_2$, about 1.2 unit of Taq polymerase (Sangon Corp.), and approximately 150 ng of template DNA. PCR cycles were performed according to procedure described by Liu *et al*. (2012). Microsatellite polymorphism was detected using ABI 377 automated DNA sequencer.

Data Analysis

ARLEQUIN 3.0 (Excoffier, Laval, & Schneider, 2005) and POPGENE 1.3.1 (Yeh, Yang, & Boyle, 1999) were used to calculate observed (H_O) and expected (H_E) heterozygosities and linkage disequilibrium, respectively. Significance values for all tests were corrected following Sequential Bonferroni procedure (Rice, 1989). The Hardy–Weinberg equilibrium (HWE) at each locus was assessed by a test analogous to the Fisher's exact test using the Markov-chain method (the Markov-chain parameters used were: steps, 100,000; dememorization, 10,000).

Results

A total of 95 microsatellites were found of which 62 pairs of primers were designed and tested. A total of 22 microsatellite markers were found to be polymorphic among 30 individuals of *T. arcticus grubei* collected from the Xinjiang Irtysh River in China. The number of alleles per locus ranged from 3 (XJBJH02) to 13 (XJBJH17), and the observed and expected heterozygosity ranged from 0.60 to 0.97 and from 0.58 to 0.89, respectively (Table 1). All the loci conformed to Hardy-Weinberg equilibrium (HWE) was determined with the Markov-Chain method. Out of 231 possible pairwise comparisons between the 22 loci applied to *T. arcticus grubei*, none showed significant linkage disequilibrium. Considerable differences were found among Fuh, Hab, and Fuy populations in the number of alleles, effective number of alleles, and number of genotypes at all loci (Table 2). In regard to total number of alleles, total effective number of alleles, and total number of genotypes, the Fuh population showed the highest diversity among all the samples while the Fuy sample was the lowest. These new microsatellite markers are a helpful tool for genetic analyses of and resources conservation efforts for *T. arcticus grubei*. The method is also practically efficient in rapid screening of polymorphic microsatellite markers based on AFLP products from aquaculture resources.

Cross-species PCR amplification was tested on five other fishes using the same methodology like for *T. arcticus grubei*. All primer pairs were tested on 30 individuals from each of the five species: Red seambream (*Pagrus major*), Lenok (*Brachymystax lenok*), Sea perch (*Lateolabrax japonicus*), Taimen (*Hucho taimen*), and Amur grayling (*Thymallus grubii*). In traditional taxonomy, each represents five different species from distant species to interrelated species. Three out of the five species (*Brachymystax lenok*, *Hucho taimen*, and *Thymallus grubii*) showed at least two polymorphic loci. In addition, six loci were found to be polymorphic in at least one species. The results, summarized in Table 3, highlight the potential of some microsatellite markers of *T. arcticus grubei* being used in studies on other fish species. Eleven microsatellite loci have amplification products, and five are polymorphic in the Amur grayling. Thus, the Xinjiang arctic grayling may have a closer phylogenetic relationship with the Amur grayling and a more distant ancestry, as denoted by no polymorphic amplification, to Red seam bream and Sea perch.

Discussion

Vos *et al*. (1995) proved that the AFLP technique is a PCR-based DNA fingerprinting that can rapidly identify thousands of band polymorphisms associated with restriction sites and has been successfully applied to a wide range of organisms. The AFLP polymorphisms usually include microsatellites: indels (insertions and deletions) and SNPs (Liu *et al*., 2015; Bradeen & Simon, 1998). Therefore, polymorpic AFLP products contain many co-dominant bands caused by microsatellite sequences. Typically, microsatellite markers have been isolated from small insert size of genomic libraries of the species of interest, screening hundreds of clones through hybridization with repeat containing probes (Rassmann, Schlotterer, & Tautz, 1991). However, it requires southern hybridization, which is time-consuming. If sequenced randomly without hybridizing for microsatellite enrichment, the yield of microsatellite-containing sequences is low. We utilized a fast and effective protocol in this study for quick isolation of sequences containing microsatellite repeats from co-dominant AFLP bands of *T. arcticus grubei*. This method is very effective in amplifying large amounts of DNA in a suitable size for direct cloning and sequencing. Despite lacking library construction and hybridization screening, the method ended with a relative high microsatellite yield (Liu *et al*., 2007). We sequenced a total of 160 clones from co-dominant AFLP bands of *T. arcticus grubei*, and 95 microsatellite-containing sequences were obtained. Nearly 60% of clones contained microsatellites. However, only 22

Table 1. Characterization of twenty twopolymorphic microsatellite lociderived from*Thymallus arcticus grubei*

Locus	Repeat sequence	Primer sequences (5'-3')	Ta (°C)	Na (size range, bp)	H_O	H_E	*P*	Accession no.
XJBJH01	$(AC)_8$	F: GGTGAAAGAAAGAGGGAGGG R: AGCCCTCACCAAGATGAGAA	58	4 (230-310)	0.73	0.70	0.663	KY471332
XJBJH02	$(TAGA)_7$	F: CCTTTTATGCATTTCTGTGGG R: TTTGTGCACTTTCGTTCTGG	57	3 (130-180)	0.90	0.88	0.165	KY471333
XJBJH03	$(CA)_9$	F: TCACACATCCTGGCTGTCAT R: TCCCCAGATAGTCGTCAAGG	58	7(190-230)	0.67	0.58	0.231	KY471334
XJBJH04	$(GA)_7$	F: GATGACCAGCAGGGTCAAAT R: ATGACTCCTAGCTGTCCCCA	56	5(200-240)	0.67	0.64	0.102	KY471335
XJBJH05	$(GA)_{15}$	F: ATTACAGAAAACCCCCTCCG R: TCTCTCTCTCGCTCTCTGCC	59	9 (250-290)	0.60	0.59	0.376	KY471336
XJBJH06	$(CCAT)_6$	F: GCAAAGAAACCCTGCTTGAG R: TCCGCTTCCTCAGAAACAGT	57	4(130-180)	0.77	0.68	0.089	KY471337
XJBJH07	$(GT)_{12}$	F: GCAATGATTTTGCAGTGGTG R: ATCCCCTCTGGTGTGTCAAA	58	6 (110-150)	0.97	0.89	0.192	KY471338
XJBJH08	$(TATC)_7$	F: TCGGGATTGCACTAAGCTCT R: GCCAGCAAGGTTGATTGATT	57	6 (100-150)	0.90	0.86	0.410	KY471339
XJBJH09	$(ATAG)_{18}$	F: CTTTCGACCAATCACACCCT R: ACAAAGAAATTTCCCCCTCG	56	12 (130-170)	0.83	0.75	0.225	KY471340
XJBJH10	$(GT)_9$	F: TTTTGACCAGGACCCATAGC R: CTTACACCTGGCTGGTCACA	58	8(250-290)	0.87	0.82	0.921	KY471341
XJBJH11	$(AG)_{10}$	F: CCAGCTGAGAGAGGGAGAGA R: AGTTGTCCCAGGTTCAAACG	56	5 (220-270)	0.67	0.63	0.476	KY471342
XJBJH12	$(GA)_{12}$	F: GCCATAACCAGCCTCTCAAA R: ACTGCAGTCCCAGAGATGCT	56	6 (230-270)	0.87	0.82	0.202	KY471343
XJBJH13	$(GT)_{13}$	F: TCACATCTTTAGTTGTGCATGTG R: TGAGGACCAAGGGAACAATC	55	8 (140-180)	0.80	0.75	0.305	KY471344
XJBJH14	$(CA)_{10}$	F: CCCCAGGACAGTCATACCAC R: GTTCCCCTCCACTAAATGGC	58	7(110-160)	0.77	0.71	0.166	KY471345
XJBJH15	$(TTAC)_7$	F: AGCTCATGAAACATGGGACC R: CGGGAGAGAGACTAGCCCTT	57	4(160-190)	0.90	0.85	0.094	KY471346
XJBJH16	$(GAT)_{14}$	F: GCACATTTTGATGTATGCCC R: TGTGTTTTTGAAGCAGGCAC	56	5 (170-200)	0.87	0.79	0.361	KY471347
XJBJH17	$(TCAC)_9$	F: GAGTTTGAACCGATTTGGGA R: CCAAGAATGCTGGAGGTGAT	58	3(230-270)	0.87	0.83	0.136	KY471348
XJBJH18	$(TATC)_{23}$	F: TGCTCTGGTAGTAGGACCTGG R: CAGCCAACGCACATTTATCA	55	13(190-230)	0.80	0.74	0.239	KY471349
XJBJH19	$(AG)_{10}$	F: ATGCCTTTTTGCACCTCCTT R: AGAAATGTCCTCTCGGGGAT	57	5(250-310)	0.80	0.76	0.702	KY471350
XJBJH20	$(AG)_{11}$	F: CCTGGACACTCAGCATCTCA R: GCACGTTATTGGAGGGAAGA	58	7(150-190)	0.87	0.73	0.094	KY471351
XJBJH21	$(AG)_{13}$	F: ACTGTGGGAGAGGAAGGGAT R: AGTAGGCTCTGGCTGTCTGG	57	7(120-160)	0.83	0.75	0.296	KY471352
XJBJH22	$(CA)_{10}$	F: CCGTTGTGAAAGTGAACCCT R: TGGACCTGCATGTGTCTGAT	59	9(170-210)	0.70	0.62	0.179	KY471353

Ta is annealing temperature (°C); Na is number of alleles; H_O is observed heterozygosity; $H_E$is expected heterozygosity.

microsatellite sequences, approximately 23%, showed polymorphic loci. The other microsatellites had inappropriate flanking regions on one or both sides of the simple sequence repeats or contained only a few repeats and thus, had less potential for polymorphism. The Fuh population showed the highest diversity among all the populations based on the analysis using the microsatellite markers. This phenomenon might be a result of the Fuh population that was sampled from Fuhai county, located at the middle place of the Irtysh River. The geographic proximity increased the chance of gene flow between the Hab population and Fuy population.

Microsatellite loci have ancient origins and show considerable evolutionary conservation, suggesting that microsatellite primers developed for any single locus may often be useful across a wide range of taxa (Liu *et al*., 2009). For example, Liu *et al*. (2007) tested cross-species amplification of 68 existing microsatellite loci in 6 species of the Sparidae family: *Acanthopagrus butcheri*, *Sparus aurata, Pagrus auratus, Chrysophrys major, Pagellus bogaraveo, Pagellus erythrinus*, and one species of Bothidae, *Paralichthys olivaceus*. Of the 68 loci screened, sixteen were found to be polymorphic when tested in 20 individual black sea bream, or *Acanthopagrus schlegeli*. Cross-species amplification is

Table 2. Allelic variability attwenty two microsatellite loci in three different populationsof *Thymallus arcticus grubei*

Microsatellite Loci	Populations								
	Fuh			Hab			Fuy		
	A	a_e	G	A	a_e	G	A	a_e	G
XJBJH01	5	4.16	8	5	4.09	8	4	3.02	6
XJBJH02	5	4.13	8	4	3.88	6	3	2.23	5
XJBJH03	6	5.56	9	6	5.23	9	6	4.87	9
XJBJH04	6	4.15	9	5	3.70	7	5	3.51	7
XJBJH05	11	8.22	14	10	7.92	12	9	7.46	10
XJBJH06	4	2.92	6	4	2.92	4	4	2.77	4
XJBJH07	6	4.73	7	6	4.67	6	5	4.46	6
XJBJH08	8	5.33	10	7	4.65	9	7	4.29	9
XJBJH09	13	8. 90	14	13	8. 32	14	12	7. 73	12
XJBJH10	8	5.74	10	8	5.68	10	8	5.68	10
XJBJH11	7	5.16	5	5	3.76	5	4	3.04	4
XJBJH12	7	4.64	11	7	4.13	9	5	3.93	6
XJBJH13	9	5.34	13	8	5.20	12	8	5.02	11
XJBJH14	7	4.15	7	7	4.15	7	7	3.89	7
XJBJH15	5	3. 65	6	4	2.56	6	3	2.27	4
XJBJH16	6	3.34	8	6	3.10	8	6	2.195	6
XJBJH17	4	2.23	6	3	1.89	4	3	1.89	4
XJBJH18	13	8.97	15	13	8.25	15	10	7.13	12
XJBJH19	6	4.18	6	5	3.30	6	4	3.01	5
XJBJH20	8	6.55	10	8	6.64	10	8	6.39	10
XJBJH21	8	4.67	9	7	4.52	9	6	3.94	8
XJBJH22	9	7.29	12	9	7.06	12	8	6.49	10
Total	161	101.46	203	150	97.3	188	135	87.49	165

Number of alleles per locus (A), Effective number of alleles (a_e), Number of genotypes (G), are given for each population and locus.

Table 3. Cross-species amplification and PCR product size range of twenty two microsatellite loci from *T. arcticus grubei* in different fish species of the vertebrates including *Pagrus major, Brachymystax lenok, Lateolabrax japonicus*, *Hucho taime*, *Thymallus grubii*

Locus	*Pagrus major*	*Brachymystax lenok*	*Lateolabrax japonicus*	*Hucho taimen*	*Thymallus grubii*
XJBJH01	0	0	0	0	0
XJBJH02	0	0	0	1	0
XJBJH03	0	0	0	0	0
XJBJH04	0	1	0	0	0
XJBJH05	1*	1*	1*	3 (250-290)	1*
XJBJH06	0	0	1†	1†	0
XJBJH07	0	0	0	1	1
XJBJH08	0	2(100-130)	0	0	2(100-130)
XJBJH09	0	0	0	0	1
XJBJH10	0	2 (270-280)	0	3(270-290)	2 (270-280)
XJBJH11	0	0	0	0	0
XJBJH12	1†	1	1†	0	3 (230-270)
XJBJH13	0	1	0	0	0
XJBJH14	0	0	0	0	0
XJBJH15	0	0	0	0	5 (160-190)
XJBJH16	0	0	0	0	0
XJBJH17	0	0	0	1	1
XJBJH18	0	1	0	0	0
XJBJH19	0	2 (250-310)	0	3（250-300）	5 (250-310)
XJBJH20	0	0	0	0	1
XJBJH21	0	0	0	0	0
XJBJH22	0	0	0	0	1

The number in each cell indicates the number of observed alleles; "0" indicates no amplification or smear only; "*" indicates larger than expected size; "†" indicates smaller than expected size.

a practical method to extend the utilization of microsatellite markers. Although cross-species amplification is convenient, it may lead to low or incomplete amplification, since as little as a single dinucleotide mismatch between the primer and the target DNA sequences could lead to erroneous results (Zane, Bargelloni, & Patarnello, 2002). This problem can often be resolved by employing less stringent polymerase chain reaction (PCR) conditions such as lowering the annealing temperature or increasing the magnesium concentration in the reaction (Liu *et al*., 2007). In this study, three microsatellite loci show polymorphism in Taimen and Lenok. Other studies have reported that they have a polymorphic amplification product in closely related species in cross-species amplification of microsatellite markers (Dallimer, 2015; Delghandi*et al*., 2016).

In conclusion, the findings of this study prove the usefulness of co-dominant AFLP bands as a valuable source for the identification of microsatellite from the Xinjiang arctic grayling. The polymorphic microsatellite loci developed in this study for *T. arcticus grubei* should be used in the future studies of systematic and population genetics in this endangered species. Cross-species amplification on five other fishes indicates that some Xinjiang arctic grayling microsatellite loci are conservative in closely related species.

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