Online Resources

Isolation and characterization of microsatellite markers in a highland fish, *Pareuchiloglanis sinensis* (Siluriformes: Sisoridae) by next-generation sequencing

Weitao Chen[1,2] and Shunping He[1*]

[1] The Key Laboratory of Aquatic Biodiversity and Conservation of Chinese Academy of Sciences, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei, 430072, China

[2] Pearl River Fisheries Research Institute, CAFS, Guangzhou, Guangdong 510380, China

* For correspondence. E-mail: clad@ihb.ac.cn.

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Introduction

Pareuchiloglanis sinensis (Siluriformes: Sisoridae) is an endemic and highland fish species, which only detected in Jinsha River, Dadu River and Bailong River (Additional file 1: Figure S1) (Chu *et al*. 1999). In our study, 48 individuals were collected from Dadu River. Dadu River, the biggest tributary of Min River, has being heavily exploited primarily for hydroelectric power. As of March 2014, a total of 26 dams are completed, under construction or planned for the river, which poses a new threat to freshwater ecosystems and fish diversity in the Dadu River

(https://www.wilsoncenter.org/publication/interactive-mapping-chinas-dam-rush). To facilitate a better understanding of genetic diversity and population structure of *P. sinensis* for resource conservation, we have isolated and characterized 28 polymorphic microsatellites from *P. sinensis* since microsatellites are the markers of choice for a variety of population genetic study. Compared with traditional methods of simple sequence repeats (SSRs) marker development, next generation sequencing is more cost-efficient (Liu *et al*. 2017; Zheng *et al*. 2013). RNA-seq data was generated by Ma *et al*. (2016). In this study, we used unigenes assembled from RNA-Seq to develop polymorphic SSRs to understand population genetics of *P. sinensis* with by a Perl script known as MISA (http://pgrc.ipk-gatersleben.de/misa/misa.html).

Materials and methods

Sample collection

The methods involving animals in this study were conducted in accordance with the Laboratory Animal Management Principles of China. Forty eight individuals of *P. sinensis* were collected from Dadu River.

RNA-seq data

RNA-seq data was generated by Ma *et al*. (2016). FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) was used to control the reads quality. We trimmed the adapter sequence and sites lower quality reads (Phred score < 20) with Cutadapt (Martin 2011). These cleaned reads were assembled using Trinity (Haas *et al*. 2013) software with default parameters. Contigs longer than 200 bp were retained for further analysis. CD-HIT-EST program (Li and Godzik 2006) with an identity threshold of 95% was used to remove low-coverage artifacts or redundancies. The unigenes were used for further microsatellite marker detection.

EST-SSRs detection and primer development

Microsatellites within the assembly unigene were detected by a Perl script known as MISA (http://pgrc.ipk-gatersleben.de/misa/misa.html). The SSR loci were only considered to contain two to six nucleotides motifs with a minimum of 6,5,5,5 and 5 repeats, respectively. Mononucleotide repeats were excluded from the EST-SSRs search as their polymorphism is often difficult to interpret (Lopez *et al*. 2015).

The EST-SSR primers were designed using Primer 3.0 (Untergasser *et al*. 2012) under following criteria: (1) primers length ranging from 18 to 25 bases (optimum 20 bp); (2) PCR product size ranging from 100 to 300 bp; (3) melting temperature between 58°C and 63°C (optimum 60°C); (4) a GC content of 40%–60% (optimum 50%) .

DNA extraction, PCR conditions and amplification of SSRs

We dissected a small piece of white muscle tissue or fin was from the right side of the body of each specimen. All of the tissue samples were preserved in 95% ethanol. Total genomic DNA was extracted from the muscle tissue or fin by performing a standard salt extraction.

The polymerase chain reaction (PCR) amplication was carried out in 30 μl reaction mixture with approximately 100 ng of template DNA, 1μl of each primer (10 pmol), 3μl of 10× reaction buffer, 1.5μl of dNTPs (2.5 mM each), and 2.0 U of Taq DNA polymerase.

The PCR conditions for SSR included an initial denaturation step at 94°C for 5 min; followed by 30 cycles of denaturation at 94°C for 30s, annealing at 60°C for 40 seconds, and extension at 72°C for 30 seconds; followed by a final extension at 72°C for 10 min and storage at 4°C.

Amplification products were separated using 20% polyacrylamide gel. Some loci did not amplify in all samples although we adjusted the PCR conditions. These loci were

excluded from further testing. Besides, only those loci showed polymorphism were considered for genotyping analyses. Fluorescently labeled primers were further synthesized to ensure the accuracy of visualized lengths in polyacrylamide gel.

Genotyping

Forward primers (Additional file 2: Table S1) were labeled with FAM or HEX dye on the 5'end. The PCR reaction conditions were the same as described above. The amplified products were detected on an ABI 3130xl Genetic Analyzer, and scored using the GeneMapper software (Applied Biosystems)

Microsatellites data analysis

Important genetic parameters of polymorphic microsatellite loci such as polymorphism information content (PIC), the number of alleles (A), observed heterozygosity (H_O), expected heterozygosity (H_E) were calculated using POPGENE 1.32 (Quardokus, 2000). Possible deviations from Hardy–Weinberg equilibrium (HWE) were tested by Fisher's exact test with Bonferroni correction.

Result and discussion

In this study, 47989 unigenes generated using RNA-seq data were used to detect potential microsatellite loci. A total of 7832 sequences were identified containing 9471 SSRs. 1354 sequences containing more than 1 SSR (Table 1). There were 70 motifs obtained, of which the most frequent was AC/GT (428, 54.65%), followed by AG/CT (406, 16.43%), ATC/ATG (138, 5.02%), AGG/CTT (123, 3.99%), AAG/CTT (101, 4.35%) andGTA/CAT (88, 3.79%) (Additional file 2: Table S1). Detailed analysis showed that dinuleotide repeats were the most frequent (72.53%), followed by tri- (22.56%), tetra- nucleotide (4.56%) repeats. SSRs with nine tandem repeats 1980

(20.90%) were the most common, followed by eight tandem repeats 1333 (14.07 %) (Figure 1).

To test the applicability and polymorphisms of SSR markers, 120 primer pairs were chosen randomly and validated across 48 *P. sinensis* individuals. 86 of 120 (71.67%) were successfully amplified. 28 of the microsatellite loci showed polymorphism (Additional file 3: Table S2). Fluorescently labeled primers were further synthesized for these loci. The result showed that the number of alleles (N_A) for each locus ranged from 2 to 14 and the mean number of alleles per locus was 7. The observed heterozygosity (H_O) and expected heterozygosity (H_E) varied from 0.104 to 0.958 and from 0.157 to 0.844, with an average of 0.583 and 0.613, respectively (Additional file 3: Table S2). Twenty loci exhibited high polymorphism (PIC>0.5). Across all samples, 14 loci among 28 showed significant departures from Hardy-Weinberg equilibrium (HWE) (Additional file 3: Table S2).

Pareuchiloglanis sinensis is an endemic species with narrow distribution, which faced with threat from human disturbance and habitat destruction. Thus, it is crucial that the current resources of *P. sinensis* be protected. Microsatellite markers developed in our study serve as a useful tool for the conversation genetic study and population evaluation of *P. sinensis*.

Acknowledgments

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Table 1: Summary of SSRs identified in *P. sinensis* transcriptome unigenes.

Summary of SSRs identified in *P. sinensis* transcriptome unigenes.

Information	Number
Total number of sequences examined:	47989
Total size of examined sequences (bp):	37172544
Total number of identified SSRs:	9471
Number of SSR containing sequences:	7832
Number of sequences containing more than 1 SSR:	1354
Number of SSRs present in compound formation:	492

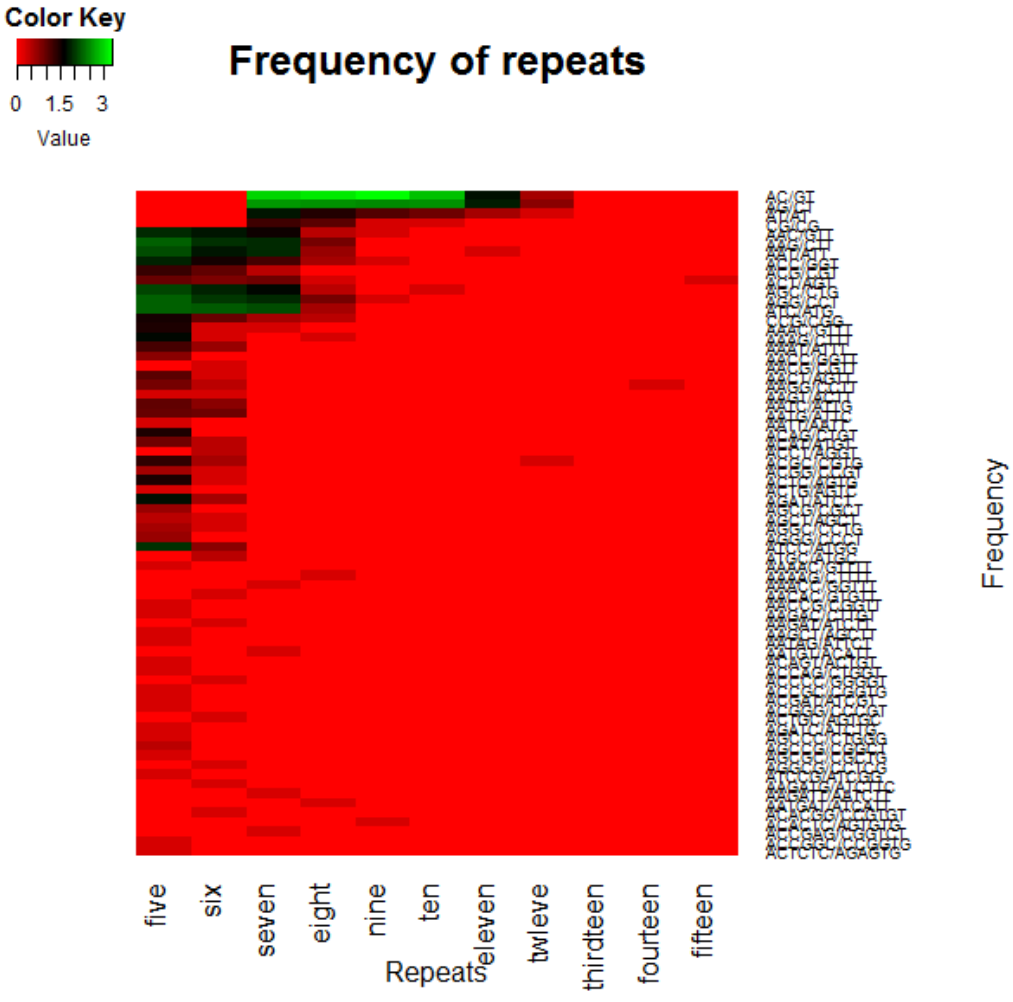
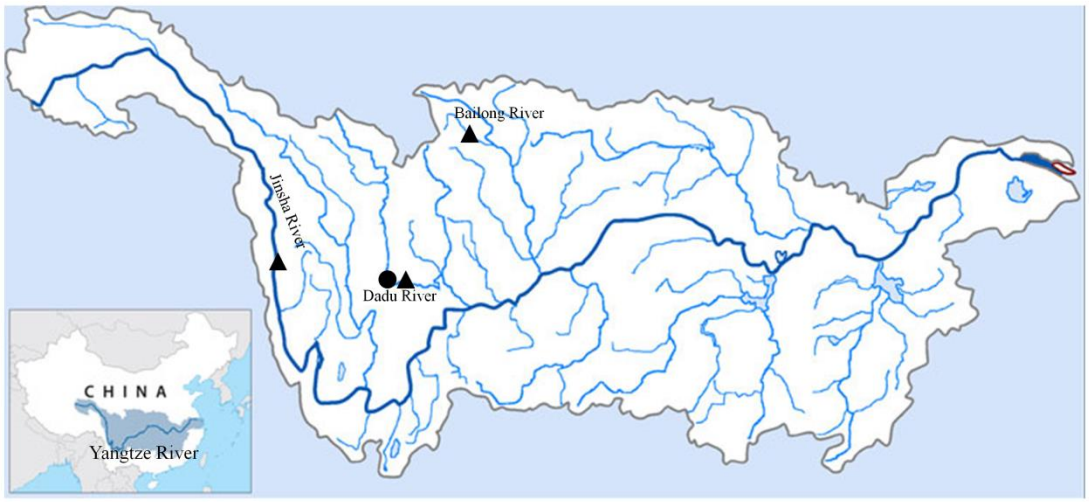


Figure 1: Heatmap of frequency of repeats identified by RNA-seq. Dinuleotide repeats were the most frequent (72.53%), followed by tri- (22.56%), tetra- nucleotide (4.56%) repeats. Simple sequence repeats (SSRs) with nine tandem repeats (20.90%) were the most common.

Supporting information



Additional file 1: Figure S1: Natural distribution (triangle) of *P. sinensis* and sample site (rectangle) in our study.

Table S2: Characterization of 28 transcriptome-derived microsatellites of *P. sinensis*. Primers redesigned from original sequence, N_A, number of alleles, *Ho*, observed heterozygosity; H_E, expected heterozygosity; P, probability of deviation from Hardy–Weinber equilibrium from heterozygote deficiency with significant values in bold.

Locus	Primer sequence (5'–3')	Repeat motif	Product size	Tm (℃)	N_A	H_o	H_E	P	PIC
BMK4	F:ACACACGCGTCTCTTCCTCT	(GT)7	285-289	60	3	0.479	0.559	0.526	0.455
	R:TGTGTGTCGGACCCTGAGACT								
BMK9	ACATGCTTTTTACAAGCCCC	(ATC)6	152-164	60	6	0.875	0.721	0.598	0.659
	GCCCCCAAAGAAGAAAGCTA								
BMK11	ACCGGAGTCTTTGGTCCTTT	(GTGA)5	272-316	60	9	0.729	0.745	**0.000**	0.698
	GAATTTGCCTCATTTCCCAA								
BMK26	AGCATATCGGAAAGTGCCTG	(AC)7	249-253	60	3	0.104	0.157	**0.006**	0.148
	TTTCTTCTCCGCCGTTAAAA								

BMK31	AGGCATCAAGCACATCAGTG AGGGAGATCTGGAGAGGGAG	(GT)8	236-264	60	8	0.458	0.511	**0.0207**	0.472
BMK43	ATGCAGAACACTCCCATTCC CATCAACGTGCTAATGTGCC	(GT)7	282-286	60	3	0.354	0.476	0.248	0.369
BMK46	ATGGTGAGTGCGCTACTGTG GGAAAGCAGCAAGCAGAAAA	(CA)8	104-114	60	6	0.729	0.713	0.644	0.654
BMK51	CAACAGCACGGTAGCTTCAA GTTGCTGAGCGGTCTCAGAT	(AT)8	118-134	60	7	0.708	0.734	**0.000**	0.684
BMK62	CACAGGTGTTCAGTCATCGG ATTAATCGTCCCCATTTCCC	(AC)7	262-300	60	11	0.688	0.774	0.066	0.738
BMK72	CATGTACAGGGGTTTGTGGG CAAATGCAAATGCAATCCAC	(TG)8	165-177	60	5	0.708	0.684	**0.000**	0.615
BMK74	CATGTCGATTCACAGTTCGG TACGCTCCTAACGTCTGCCT	(TTA)5	187-190	60	3	0.563	0.585	**0.001**	0.505

Table S2 (Continued)

Locus	Primer sequence (5'–3')	Repeat motif	Product size	Tm (℃)	N_A	H_o	H_E	P	PIC
BMK81	CGAACGTGATCTCGAACTGA TCTGCAGGTCCATTTAGCAA	(TGT)6	262-271	60	3	0.271	0.274	1.000	0.248
BMK82	CGAAGAACTCTGATAGCGGG TGTAGAAGAAATGGGGGCTG	(CA)7	293-339	60	14	0.708	0.735	1.000	0.706
BMK89	CGATGAAGGTGTTGGTGATG GAAGGGATGACGCGAATTTA	(GAT)6	293-308	60	4	0.479	0.575	0.245	0.500
BMK100	CTCAAAGAAACCTGAAGCCG TCTTGATGCGATACAAAGCG	(ACTA)5	240-256	60	4	0.188	0.192	0.080	0.179
BMK104	CTCCGCTCGTACACACTTCA TATGAACACACGCCCCAGTA	(CA)7	274-300	60	6	0.146	0.282	**0.000**	0.266

BMK118	CTGTATGGCTTGCGACAGAA GGAGGGTTGAAATGGGGTAT	(AG)8	255-287	60	11	0.750	0.797	**0.030**	0.762
BMK129	GACGAGAGCGAGAGAGAGGA GGAGAGAAAAGTTGGGGGAG	(AC)8	234-250	60	8	0.771	0.771		0.730
BMK132	GAGAAATGTGACACCTCGCA CTCTCTCTGTTCCGGTTTCG	(GT)8	274-300	60	13	0.958	0.844	**0.000**	0.816
BMK3	GAGCTGCTGGAAGAGTCACC TCGGAGCATTCCTTTCAGAT	(CT)8	287-317	60	13	0.833	0.884	0.944	0.862
BMK5	GAGCTTGGAGCAGAAAGCAG TCCCTCCTGAGCACTTGACT	(TTA)6	199-214	59	5	0.688	0.666	0.984	0.599
BMK6	GATGGCGTCTGAGTGTGAAT GTACATGCCGAACAACATGC	(AAG)6	112-124	59	4	0.500	0.451	0.808	0.394
BMK11	GCAAATGAAAAGCTGCGTAA CGGCACTGTAGGTCCTGTTT	(GT)9	174-194	59	8	0.771	0.658	**0.000**	0.591
BMK19	GCACTGCTACAACAGCGTTC TGTACCTGCCAACGTTCAAG	(TG)7	255-279	60	6	0.583	0.659	**0.000**	0.587

Table S2 (Continued)

Locus	Primer sequence (5'–3')	Repeat motif	Product size (bp)	Tm (℃)	N_A	H_o	H_E	P	PIC
BMK21	GCATCGATCATTTCACATGG TTTGACAGCTAAGGCAGGAAA	(TA)8	170-202	60	13	0.729	0.884	**0.000**	0.862
BMK28	GCTACACAGCCGAAGGAAAC GTCTTCTGTCTGGCTTTCGG	(AC)7	235-275	60	11	0.521	0.710	**0.000**	0.673
BMK36	GCTCGTTCGCTTTGCTTTAC TTTCCACTTTCTCGCAATCC	(TG)8	271-311	60	14	0.688	0.783	**0.030**	0.752
BMK38	GCTCTGTACAAGACCTCGCC TTCCCTGACTCGGATCAGTT	(AAC)5	117-120	60	2	0.333	0.333	1.000	0.275