ONLINE RESOURCES

Cross-species amplification of microsatellites in genera *Megalobrama* and *Parabramis*

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Introduction

Genus *Megalobrama* (subfamily: Cultrinae; family: Cyprinidea), is one of the most economically important species in Chinese freshwater polyculture system. According to several recent studies, based on morphological data, the *Megalobrama* genus includes four valid species: *M. amblycephala*, *M. pellegrini*, *M. terminalis* and *M. skolkovii* (Chen *et al.* 1998; Xu and Xiong 2008). Another economically important species, *P. pekinensis*, belongs to the sister genus *Parabramis* (Dai *et al.* 2005).

Microsatellites or simple sequence repeats (SSR) have been widely used as DNA markers in population genetic studies, parentage and kinship analyses, because of their high level of polymorphism and codominant Mendelian inheritance (O'Connell and Wright 1997). The drawback of microsatellite markers the need to develop a new set of loci for each targeted species because isolating markers costly and time-consuming (Moraes *et al.* 2012). As the flanking sequences of microsatellite markers are usually conserved in the same genus or family, the primers designed for one species can be tested for their ability to amplify homologous products in related species, a process known as 'cross-species amplification' or transferability (Barbara *et al.* 2007). Thus, cross-species amplification can be regarded as a kind of fast and effective method for developing microsatellite markers, and therefore is an important tool in population genetics. There have been several studies which isolated and characterized polymorphic microsatellite markers in *M. amblycephala* (Li *et al.* 2006, 2007; Li 2010; Gao *et al.* 2012). However, none of them has been tested on other *Megalobrama* genus species through cross-species amplification. Except for *M. pellegrini* which has one report about microsatellite development (Wang *et al.* 2012), there are no microsatellites reported in the other three species from *Megalobrama* and *Parabramis* genera as far as we know.

In this study, the microsatellite markers from *M. amblycephala* transcriptome database were chosen to test their utility for cross-species amplification. The obtained polymorphic microsatellites in this study could be directly applied in the genus *Megalobrama* and *Parabramis* for facilitating large-scale genetic studies on population structures, systematics and evolutionary history.

Materials and methods

Materials

The materials of our studied species were collected from their natural habitat in rivers and lakes of China over the period from July 2011 to November 2011. *M. amblycephala* and *P. pekinensis* were collected from Liangzi lake of Hubei province, *M. pellegrini* were from Longxi river of Sichuan province, *M. terminalis* were from Xijiang river and *M. skolkovii* were from Beijiang river, both these rivers being located in Guangdong province. All of the samples were preserved in 95% ethanol and stored at −20°C. Thirty samples of each species were chosen for DNA extraction.

Thirty microsatellite markers from *M. amblycephala* transcriptome database and characterized by Gao *et al.* (2012) were selected for cross-species amplification testing (table 1). These 30 microsatellites were chosen based on the number of repeat and the number of amplified alleles. Most loci have more than 10 repeats based on the transcriptome data of *M. amblycephala* and have more than three alleles in *M. amblycephala* tested population.

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Table 1. The primer sequences, annealing temperature and repeat motif of 30 microsatellite markers used in this study.

Locus	Primer sequence (5′–3′)		T_a (°C)	Repeat motif
Mam_EST7	F: GTTGAAAAGGGAGGGACT	R: TGGGGGACAAATAAAAGC	56	$(GT)_{10}$
Mam_EST9	F: GGGTTTGTCCATTACTGCC	R: TCCCTGGTCCGACTTTCC	49.5	$(CAT)_{11}$
Mam_EST11	F: ATGCCAGTCTGCCAACAA	R: TTCAATGATCGTCCGTCTT	58	$(TG)_{11}$
Mam_EST12	F: TCGTGCGAAGTAAACAAG	R: CAGGCAATAATAACAAAACC	54	$(TCTT)_{13}$
Mam_EST13	F: TCTTTCACAAACAAACCCTT	R: GGATTATCAAACGCGGACT	55.5	$(AC)_{14}$
Mam_EST22	F: TGCCTCGGTCTCACTCTG	R: AATCTCCTGGAACACTCTTTG	59	$(AC)_{11}$
Mam_EST24	F: ACTGAAGCCCTCAACCTC	R: TCACAGCAGACATCCAACT	58	$(GT)_{10}$
Mam_EST37	F: CACAAACCATAAACACAG	R: AATGCCCATAAAACACAC	54	$(TG)_8$
Mam_EST46	F: TAAAGGGAAATTCTGGT	R: AGTATAAGTTGAGTGGGTG	50.5	$(ATCT)_{25}$
Mam_EST61	F: TGGCAAATGAAGATGAAG	R: TTACAACGCACCACTGAC	52	$(CA)_{13}$
Mam_EST72	F: CTTTTCTTCTTTCCCCCT	R: CTTCCTCATTCCCTTGTT	52.7	$(CA)_{12}$
Mam_EST90	F: CTTACAGACTCCGACAGG	R: ATCCACGACTTCCAGAAC	61.4	$(AC)_{12}$
Mam_EST97	F: TCTCCTGGTGGGCTTGTCTG	R: CGTCAAAGGCTGGTTCTTCC	57.5	$(AC)_{14}$
Mam_EST98	F: TCATGCTTGAAGCGTGTTGC	R: CGCCTGCCATCCTAAGTGTT	57.5	$(AC)_{16}$
Mam_EST99	F: TTTCACCAGTGAGGAACTC	R: AGATTAGCAGGCGTTTCA	57	$(TG)_{13}$
Mam_EST100	F: GCGTATGAACGTCAGAGC	R: TGTTGGATTATTATGGGATG	54.6	$(GT)_{20}$
Mam_EST124	F: TACGAAGGTGAGCCAAGA	R: GAGATCAGAGCCTAGATAGAGC	57.1	$(AC)_{19}$
Mam_EST821	F: AGACGGAACAAACCCAGAG	R: TATTTGTGCCCGAGTGAA	53	$(CA)_{10}$
Mam_EST147	F: ACTGGATGACTTTAGTTAGGGTTA	R: CAGAAACGGCTTATCAGACC	57	$(TG)_{12}$
Mam_EST166	F: GGTACTGTTTGTGCTGGGC	R: CTGCTCACTCAACTTATTGTAGGTC	60	$(GT)_{16}$
Mam_EST851	F: ATTGGTCCAGTCTGTTGT	R: TGTATCTTGCACGCTCTA	54.5	$(AAGA)_{14}$
Mam_EST102	F: AGACGCCGTCAGGGAAAC	R: AACTCAAATCGCAATCAGC	54.6	$(AC)_{13}$
Mam_EST106	F: CCTCGCCATCTACAAGTG	R: CGTCCAAGCAGCAAAACA	60	$(CA)_{14}$
Mam_EST110	F: GCCTGACAGTCTTCTGC	R: GCTATCCGATTATCATTTAC	59	$(AC)_{13}$
Mam_EST116	F: CTATTTACAGTTTCATGCTTTCCTC	R: ATCCCGTCCGCCGCTTACT	62	$(AC)_{13}$
Mam_EST184	F: ACCGGTAGGCAACTTGAGTGTATTT	R: TGCCATTTCAAAGGCTTCTATCCTA	58	$(TA)_{10}$
Mam_EST195	F: GTGTAACGGTTATGAACGAGTG	R: TGGGAAAGGGAAAGTGTATG	57	$(CA)_{10}$
Mam_EST196	F: GAAGCAGGTGAACATCGTG	R: GGGTAGGTATAGTGTAGGGTGA	58	$(AC)_{10}$
Mam_EST197	F: GACAGCCTCGATTTACTCATCC	R: GAGCGTTTCACAGCCTTGC	60	$(GT)_{11}$
Mam_EST198	F: TTAGTGTCGCCCATTTGTG	R: ATGTGCCTTGGTTGCTTG	57	$(AT)_{10}$

DNA extraction and amplification

The genomic DNA was extracted from fin tissues using the classical phenol–chloroform method (Sambrook and Russel 2002). Concentration and quality of DNA were estimated using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, MA, USA) and agarose gel electrophoresis. Conditions for polymerase chain reaction (PCR) were optimized for each pair of primers. PCR amplifications were carried out in 10 μL volumes on a PTC-100 Thermocycler (MJ Research, MA, USA), the mixture containing 50 ng genomic DNA, 0.1 U of *Taq* DNA polymerase, 1 μL 5× *Taq* Buffer (Mg^{2+} plus), 0.25 μM for each primer, 200 μM dNTPs. The amplification condition was an initial denaturation step at 95°C for 3 min, 32 cycles at 95°C for 30 s of denaturation, a specific annealing temperature for 45 s (see table 1), 60 s of extension at 72°C, and a final cycle of 72°C for 10 min. Amplified fragments were size-fractionated on 8% nondenaturing polyacrylamide gels running at 160 W for about 150 min. Products were visualized by silver staining. The size of amplifications for each microsatellite was estimated by reference to a DNA ladder (pUC18 DNA/Mspl marker, Sangon, Shanghai, China).

The number of alleles per locus (N_a), observed heterozygosity (H_o) and expected heterozygosity (H_e) were calculated using Cervus 3.03 (Marshall *et al.* 1998). Tests for population-wide linkage disequilibrium (LD) between pairs of loci and deviations from Hardy–Weinberg equilibrium (HWE) were estimated using GenePop 4.0.10 (Raymond and Rousset 1995), and polymorphic information content (PIC) at each locus was estimated using Arlequin 3.11 (Excoffier *et al.* 2005). All *P* values were adjusted for multiple tests using the sequential Bonferroni method (Rice 1989).

Results and discussion

All the 30 chosen microsatellite markers generated bands in the target species. However, some had shadow bands and some amplified nonspecific bands. Consequently, based on the accuracy and polymorphism, only 13 markers were chosen for further analysis, most of which had clear target bands and showed high polymorphism.

The expected and observed heterozygosity per locus ranged from 0.267 to 0.892 (H_e) and 0.067 to 1.000 (H_o), respectively (table 2). The average levels of observed and expected heterozygosity were 0.433 (H_o) and 0.699 (H_e), respectively. Of the tests for departure from HWE, six loci (Mam_EST 97 in *M. skolkovii*, Mam_EST 24, 99, 110 in *M. pellegrini* and Mam_EST 147 in *M. pellegrini* and *M. amblycephala*) indicated a significant heterozygote deficiency (table 2). These results indicate that the majority

Table 2. The polymorphic information for the five studied fish species at 13 microsatellite loci.

Locus		Mam _EST9	Mam _EST12	Mam _EST13	Mam _EST22	Mam _EST24	Mam _EST46	Mam _EST72	Mam _EST97	Mam _EST99	Mam _EST100	Mam _EST110	Mam _EST147	Mam _EST851
M. amblycephala	R	200–220	190–250	210–240	150–176	167–187	270–396	282–320	183–201	172–180	194–210	200–230	160–180	270–300
	N_g	10	28	10	21	21	78	21	10	3	10	15	6	10
	N_a	4	7	4	6	6	12	6	4	2	4	5	3	4
	N_e	3.109	4.296	3.209	3.83	3.371	7.287	4.467	3.403	1.998	3.175	4.301	2.067	2.748
	H_o	0.5	0.833	0.767	0.808	0.7	0.733	1	0.533	0.367	0.533	0.609	1	0.7
	H_e	0.678	0.767	0.688	0.739	0.703	0.863	0.776	0.706	0.499	0.685	0.767	0.516	0.636
	PIC	0.6185	0.7312	0.6304	0.7048	0.6651	0.8486	0.7406	0.6536	0.3747	0.627	0.7318	0.3991*	0.5648
M. terminalis	R	220–240	190–220	200	142–170	171–189	290	282–298	185	148–176	184–214	196–220	160–180	270–300
	N_g	6	10	1	36	10	1	10	1	21	28	6	6	10
	N_a	3	4	1	8	4	1	4	1	6	7	3	3	4
	N_e	2.187	2.848	1	6.36	3.266	1	2.909	1	3.399	5.939	2.685	2.401	2.778
	H_o	0.4	0.467	0	0.967	0.552	0	0.643	0	0.593	0.786	0.593	0.423	0.6
	H_e	0.543	0.649	0	0.843	0.694	0	0.656	0	0.706	0.832	0.628	0.584	0.64
	PIC	0.4402	0.5857	–	0.8245	0.6348	–	0.5906	–	0.6644	0.8098	0.5542	0.5009	0.5789
M. skolkovii	R	200	190–240	226	146–184	167–197	278–370	278–320	183–201	166–182	194–215	200–230	160–200	280–290
	N_g	1	28	1	45	21	10	28	15	10	21	15	21	3
	N_a	1	7	1	9	6	4	7	5	4	6	5	6	2
	N_e	1	4.737	1	6.371	4.358	2.853	4.306	3.696	3.867	4.17	4.133	5.036	1.918
	H_o	0	0.733	0	0.931	0.633	0.667	0.633	0.067	0.69	0.571	0.586	0.759	0.448
	H_e	0	0.789	0	0.843	0.771	0.649	0.768	0.729	0.741	0.76	0.758	0.801	0.479
	PIC	–	0.75708	–	0.82427	0.73557*	0.60062	0.73077	0.68401*	0.69339*	0.71979	0.71598*	0.77318*	0.36408
M. pellegrini	R	224–230	190–230	226	140–170	167–199	290	278–298	183–215	154–166	194–214	210–230	160–200	280–300
	N_g	3	15	1	15	6	1	10	10	6	3	3	10	6
	N_a	2	5	1	5	3	1	4	4	3	2	2	4	3
	N_e	1.385	1.479	1	4.082	2.532	1	2.135	2.088	2.486	1.6	2	2.439	2.234
	H_o	0.267	0.367	0	1	1	0	0.7	0.467	0	0.5	1	1	0.448
	H_e	0.278	0.324	0	0.755	0.605	0	0.532	0.521	0.598	0.375	0.5	0.59	0.552
	PIC	0.2392	0.3116	–	0.7171	0.527	–	0.4223	0.4719	0.5169	0.3047	0.375	0.5039	0.4583
P. pekinensis	R	206–220	190–230	200–220	146–200	167–195	290	282–298	183–201	154–200	184–200	186–220	160–190	260–300
	N_g	6	15	10	66	36	1	10	10	45	21	10	10	15
	N_a	3	5	4	11	8	1	4	4	9	6	4	4	5
	N_e	2.699	4.216	3.403	9.231	6.228	1	2.641	3.422	5.573	5.341	3.388	3.745	3.571
	H_o	0.433	0.517	0.467	0.867	0.767	0	0.655	0.5	0.767	0.733	0.72	0.885	0.552
	H_e	0.629	0.763	0.706	0.892	0.839	0	0.621	0.708	0.821	0.813	0.705	0.733	0.72
	PIC	0.557	0.7224	0.6498	0.8819	0.8193	–	0.573	0.6514	0.7999	0.786	0.6477	0.6842	0.6712

R, allele size ranges; N_g, number of observed genotypes; N_a, number of alleles; N_e, effective alleles; H_e, expected; H_o, observed heterozygosity; PIC, polymorphic information content.
*Significant deviation from Hardy–Weinberg equilibrium (HWE) after Bonferroni correction ($P < 0.05$).

of these microsatellites will be useful in studying genetic diversity of these five species. Besides, loci Mam_EST 9, Mam_EST 13, Mam_EST 46 and Mam_EST 97 showed monomorphic or polymorphism in different species, which could be used as specific molecular markers for population or species identification.

It is concluded that the cross-species transferability of the microsatellite markers among genera *Megalobrama* and *Parabramis* species is very high and the 13 polymorphism microsatellite markers were developed for the studied species. These microsatellite markers would be useful in large-scale population genetic studies of species in the genus *Megalobrama* and *Parabramis*.

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