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RESEARCH ARTICLE

Identification of microsatellite loci in *Channa marulius* (Channidae: Perciformes) through cross species amplification

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Abstract

C. marulius, which belongs to the family Channidae, has high commercial value and medicinal importance, categorized under lower risk- near threatened status (LRnt). The present investigation identified seven polymorphic microsatellite loci namely, CA05, CHA5, CHA13, CHM91, CHM123, CHM151 and CHM 176 and twenty monomorphic loci from three different cross species of Channa. The observations showed that the submitted microsatellite sequence of *C. argus*, *C. maculate* and *C. striata* could be used to identify microsatellite loci in *C. marulius*. Only seven primers which amplified bands at the expected allele size regions were identified as polymorphic microsatellite loci.

INTRODUCTION

Murrels are considered potential candidate species for diversification of aquaculture, in all of them *Channa marulius* is most important due to consumer preference and high market price, also constitute a significant component of freshwater fishery in India. This is principally a freshwater riverine fish, occurs in sluggish or standing water, canals but frequently available in reservoirs, lakes, swamps and large marshy water areas. *C. marulius* has a worldwide distribution occurring in almost all states of India, Sri Lanka, Bangladesh, Pakistan, Burma and Thailand. In India, it is reported from the rivers of Indo- Gangetic plains and also in many parts of Peninsular India. Introduced range includes parts of Broward County and Florida in U.S.A where it is recognized as a potentially invasive species.

It is categorized to lower risk- near threatened status (LRnt) due to its declining number in wild (CAMP,1998). Understanding of population genetic structure of a species provides critical information for developing conservation and management strategies for natural fish populations as well as fishes having threatened status. The identification of molecular markers and documentation of genetic diversity of *Channa marulius* is, therefore, important considering that such information is valuable in breeding programmes, genetic stock identification and fishery management, besides to provide insight into evolutionary genetics of species (Chondar, 1999). Microsatellites are one of the most popular and versatile molecular markers for addressing questions in population genetics and evolution (Estoup and Angers, 1998). Due to their high level of polymorphism, relatively small size and rapid detection protocols, these markers are widely used in the related field of life sciences (Chistiakov et al., 2006). In compare to genomic library creation, Cross species amplification of Microsatellite loci is less time consuming, cost saving and requires no extra-effort which is essential for genetic conservation and management of stock who is already facing threatened and requires extra effort to conserve that population at its natural environment. The success of cross-species amplification across genera observed in this study agreed with the reports that microsatellite loci can be obtained by using primer sequences developed for related species (Zheng et al., 1995; Zardoya et al., 1996; Yue et al.,2002).

2. Materials and Methods

2.1 Primer designing- Sixty six primers (microsatellite flanking regions) designed from the online available microsatellite sequence of different cross species of genus Channa like *Channa argus*, *Channa maculate* and *Channa striata* (downloaded from NCBI site), through online available software PRIMER 3 (Rozen and Skaletsky, 2000).

2.2 Sample collection: Specimens of *Channa marulius* were collected through commercial catches from several rivers, belonging to different basins across its natural range of distribution. The blood was extracted through caudal puncture and fixed in 95% ethanol in ratio 1:5. These samples are collected different river basins from India like Ganga at Bijnore, U.P. (29˚ 23' N, 79˚11' E), Mahanadi at Cuttack (21º 58' N, 86º 07' E), Teesta at Teesta Barrage (26 º 45' 16.83"N; 88º 36' 02.83"E), Chambal at Kota, Rajasthan (25 º11'N, 75 º50' E), Sharda at Palia, U.P. (28 º22'N,80º 33' E) and Gomti at Sultanpur, U.P. (26˚ 16' N, 82˚ 4' E).

2.3 Isolation of genomic DNA from the Ethanol fixed blood: Total genomic DNA was extracted from the Ethanol fixed blood of *C. marulius* with proteinase–K and phenol chloroform method (Ruzzante et al., 1996), which removes proteins and other cellular components from the nucleic acids and pure genomic DNA was obtained.

2.4 Determination of quality and quantity of isolated DNA: 210mg agarose was dissolved in 3ml of 5X TAE and 27ml double distilled water. Agarose solution was heated to dissolve and 0.5µl of ethidium bromide was added before cooling down to approximately 50^{o}C. Solution was poured in casting plate with already adjusted gel comb. It was left for solidification at room temperature for 30 mins before loading the sample in the gel. Cold 0.5X TAE as gel running buffer was used. 8µl of DNA solution with bromophenol blue was loaded in the wells along with a known quantity of DNA in adjacent wells. It was run at 70V for 15 to 20 min and DNA band was observed with ultraviolet transilluminator.

2.5 Polymerase chain reaction (PCR): A total of 66 microsatellite primers were used to screen suitable primers for *C. marulius* species. In this study, PCR amplification was performed in a 25 µl reaction mixture, that included 1X PCR buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.01% gelatin), 0.2 mM of each dNTP, 2.0 mM of $MgCl_2$, 5 pmol of each primer, 1.5 U Taq DNA polymerase and 25–50 ng of template DNA. PCR (MJ PTC-200 thermal cycler) cycles were as follows (i) 1 cycle of denaturation at 94 °C for 5 min, (ii) 25 cycles of denaturation at 94 °C for 30 s, relevant annealing temperature for 30 s, elongation at 72 °C for 1 min, (iii) a final elongation of 1 cycle at 72 °C for 4 min and stored at 4 °C. The reaction mixture and PCR cyclic conditions have been shown in the Table 1, 2**.**

2.6 Seperation of Microsatellite Alleles using PAGE and silver staining for visual Scoring:

Amplified microsatellite loci were visualized via silver staining (silver staining kit, Amersham Biosciences, USA). Alleles were designated according to PCR product size, calculated relative to a molecular marker (pBR322 DNA/MspI digest) with Image master 1D Elite v3.01 (Amersham Biosciences, USA). A non-denaturing electrophoresis system has been found to provide the same resolution as that obtained with denaturing acrylamide gels and silver staining with the additional advantage of ease of use for analyzing large sample sizes (Wang et al., 2003). Moreover, Bovo et al. (1999) demonstrated that nondenaturing electrophoresis is not responsible for spurious or multiple bands in microsatellite analysis. The gels were run for 5 hours at 10V/cm at 4-6^{o}C.

2.7 Study of Microsatellite Band Pattern: Molecular weight of each microsatellite band was calculated comparing the distance run by the standard molecular weight bands through the BIOVIS-ID analysis software. Each individual was genotyped for; each of the microsatellite locus either as homo or heterozygote. Single band depicts monomorphic loci while a polymorphic locus is depicted by a double band on a gel.

3. Results and discussion

3.1 Identification of Microsatellite markers

In cross-species amplification of microsatellite primers sixty six primer pairs from three resource species were tested for the identification of homologous microsatellite loci in *Channa marulius* (Table 4).

Table 1: Composition of the reaction mixture per reaction

Components	Volume/reaction	Concentration/reaction
Water	17.25µl	
Buffer (10X)	2.5µl	1X
dNTPs	2µl	0.2mM
Primer	0.5µl	5 pmol
MgCl	0.5µl	2.0mM
Taq DNA polymerase	0.25µl	1.5U
DNA	2µl	50ng
Total	**25µl**	

Table 2: Details of the cycling conditions in a thermal cycler

	Conditions		**Cycles**
Steps	**Temperature**	**Time**	
Initial Denaturation	94◦C	5 min	1 cycle
Denaturation	94◦C	30 sec	25 cycles
Annealing	5-10◦C below Tm value	30 sec	
Extension	72◦C	1 min	
Final Extension	72◦C	4 min	1 cycle
Soak	4◦C		

Table 4. Microsatellite primers of related species tested for cross species amplification in *Channa marulius*.

S.No.	Donor Species	No. of Primers Pair tested	Loci/Primer	Gene bank Accession No.	References
1	*Channa argus*	**39**	CA01	GU253340	Gul et al.,(2010)
2			CA02	GU253341	
3			CA03	GU253342	
4			CA04	GU253343	
5			CA05	GU253344	
6			CA06	GU253346	
7			CA07	GU253347	
8			CA08	GU253348	
9			CA09	GU253349	
10			CA10	GU253350	
11			CHA2	GQ131289	
12			CHA3	GQ131290	
13			CHA4	GQ131291	
14			CHA5	GQ131292	
15			CHA7	GQ131294	
16			CHA8	GQ131295	
17			CHA9	GQ131296	
18			CHA10	GQ131297	
19			CHA12	GQ131299	
20			CHA13	GQ131300	
21			CarA118	HM030806	King T.L. and Johnson, R.L., 2010
22			CarC6	HM015825	
23			CarC7	HM015826	
24			CarC104	HM015827	
25			CarC110	HM015828	
26			CarC113	HM015829	

27			CarC116	HM015830	
28			CarD1	HM015831	
29			CarD3	HM015832	
30			CarD6	HM015833	
31			CarD108	HM015834	
32			CarD116	HM015835	
33			CarD119	HM015836	
34			CarD121	HM015837	
35			CarD126	HM015838	
36			CarD129	HM015839	
37			CarD133	HM015840	
38			CarD138	HM015841	
39			CarD139	HM015842	
40	*Channa striata*	**5**	CS1-H09	GU321682	Jamsari et al;(2009)
41			CS1-E12	GU321680	
42			CS1-C07	GU321678	
43			CS1-A11	GU321676	
44			CS1-A05	GU321675	
45	*Channa maculate*	**22**	CHM27	GU480032	Zhu, S. and Li, J., 2010
46			CHM32	GU480033	
47			CHM42	GU480035	
48			CHM46	GU480036	
49			CHM77	GU480038	
50			CHM80	GU480040	
51			CHM83	GU480041	
52			CHM91	GU480043	
53			CHM112	GU480047	
54			CHM113	GU480048	
55			CHM116	GU480049	

56		CHM123	GU480051
57		CHM124	GU480052
58		CHM126	GU480053
59		CHM139	GU480055
60		CHM145	GU480056
61		CHM189	GU480057
62		CHM151	GU480058
63		CHM157	GU480060
64		CHM165	GU480063
65		CHM172	GU480064
66		CHM176	GU480066
	Total Tested	**66**	

Cross priming tests revealed that seven loci were polymorphic while twenty were found to be monomorphic. Successful amplification was observed in *Channa marulius* with twenty seven primer pairs of related species i.e. twelve from *C. argus* (30.76%), five from *C. striata* (100%) and ten from *C. maculate* (45.45%) out of which three polymorphic microsatellite loci were found in C. argus, four in C. maculate and the remaining were monomorphic found in related resource species (Table 3). The optimum annealing temperature was determined through experimental standardization for each primer pair. The optimum annealing temperatures, to obtain scorable band pattern in *C. marulius* for all the seven primers vary within a range of 40-55^{o}C. (Table 5)

Table3: Cross species amplification of microsatellite loci of *C. argus, C. striata and C. maculate* in *C. marulius*.

	Test Species- *C. marulius*		
Resource Species	**Total no. of Primers tested**	**Amplified**	**Polymorphic**
C. argus	39	12	3
C. striata	5	5	
C. maculate	22	10	4

Table 5. Sequences, concentration and annealing temperature of selected microsatellite primers in *Channa marulius*

S.No.	Primers		Sequence (5'-------3')	Conc. (nmol)	Ta for each Primer
1	CA05	F	ACTAATCTCTGGTCGTCTCC	33.8	41
	CA05	R	ATGAATGATAGCCTCTGGTG	21.8	45
2	CHA5	F	CAGGGCAAAAGTGAGACTGG	16.9	49
	CHA5	R	GCTAGCCCCTGACATTATGC	19.8	48
3	CHA13	F	GGTTTCAAAGGTCGGGAGAG	17.9	49
	CHA13	R	GCTGTCCTGTCTGCTCCATT	34.5	48
4	CHM91	F	TAGGGAGGTCATTTGACTCAGG	24.2	49
	CHM91	R	CAGGGATACCAGTGTGTGAATG	23.6	48
5	CHM123	F	GATGGATTGCTCAGGTACAACC	17.1	52
	CHM123	R	GGTAGGCATACACACGCACA	17.4	51
6	CHM151	F	AGCTGTCAGGACTCCCAAGA	20.1	48
	CHM151	R	GCAGGGTGGTGTACCATAGG	29.2	47
7	CHM176	F	GGCCTAGTGTCACTGCAAGTC	29.2	47
	CHM176	R	GACCTCACTCATGGTTGCTC	29.5	46

3.2 Number of alleles and Repeat sequences in identified microsatellite loci

A total of seven polymorphic microsatellite loci were identified in *C. marulius*. The number of alleles at polymorphic locus CA05, CHA5, CHA13, CHM91, CHM123, CHM151 and CHM176 were four, two, six, six, four, four and five respectively (Table 6).

Table 6: Number of alleles and Repeat sequences in identified microsatellite loci

S.No.	Primers	No. of allele (Size-range)	resource-species	Test Species
1	CA05	4 (227-239)	(GT)n	(GT)n
2	CHA5	2 (111-123)	(GT)n	(GT)n
3	CHA13	6 (142-152)	(CA)n	(CA)n

4	CHM91	6 (182-206)	(GT)n	(GT)n
5	CHM123	4 (132-138)	(GT)n	(GT)n
6	CHM151	4 (125-145)	(GT)n	(GT)n
7	CHM176	5 (164-170)	(CA)n	(CA)n

Microsatellite loci are abundant and distributed throughout the eukaryotic genome and each locus is characterized by a conserved DNA sequence. These sequences consist of both unique DNA and a repetitive DNA motif. Many microsatellite loci, despite their high rates of repeat evolution, are quite conserved in their flanking regions and hence can persist unchanged over a long evolutionary time. High levels of conservation of such flanking sequences have been reported in a large variety of fishes (Scribner and Pearce, 2000).

The success of cross-species amplification of the primer pairs of the family Channidae in *C. marulius* shows the evidence of remarkable evolutionary conservation of microsatellite flanking sequences within the family. Similar results were also reported in fishes and other organisms (Watanabe et al., 2001; Sukmanoman et al., 2003; Revaldaves et al., 2005; Megleez et al., 2007). These data indicated that the cross species amplification might be locus specific (Watanabe et al., 2001).

4. Conclusion

The present study identified seven polymorphic loci namely CA05, CHA5, CHA13, CHM91, CHM123, CHM151 and CHM176 and twenty monomorphic loci from *C. argus*, *C. maculate* and *C. striata* respectively. The observations showed that the primer from these three resource species which belong to the same genus Channa could be used to identify microsatellite loci in *C. marulius*. Related Size range of the identified loci confirmed the presence of polymorphic microsatellite loci. Our results suggested that these identified microsatellites can be used for population genetic analysis and to assess or monitor genetic variation. The availability of conserved microsatellite markers is important for gene mapping, marker assisted selection and evolutionary studies.

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