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Molecular analysis of dinucleotide microsatellite in growth hormone gene of Asian seabass (*Lates calcarifer*) from Mumbai, India

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Abstract

In the present study, out of four alleles amplified from seabass (*Lates calcarifer*) genome inhabiting Mumbai water by PCR using growth hormone (GH) gene-specific primers, two DNA fragments (SGMS1, 233 bp and SGMS2, 239 bp) were eluted from gel, cloned using pTZ57R (2.886 kb) vector into *E. coli* DH5α, characterized by restriction endonuclease analysis and sequenced by automated DNA sequencer. After blasting and multiple alignment of the above sequences, SGMS1 showed 97% and SGMS2 93.3% homology with promoter region of GH gene containing microsatellite of Australian seabass and 94.6% homology between both the fragments. These sequences SGMS1 and SGMS2 were submitted to NCBI GenBank. On blasting, these sequences with gene databases, SGMS1 and SGMS2 showed partial homologies with *Seriola quinqueradiata* (26.9%, 12.9%), flounder (15.8%, 15.8%), *Oreochromis nilotica* (23%, 7.9%), *Oreochromis mossambicus* (23%, 7.9%) and *Danio rerio* (8.2%, 7.5%). Critical analysis showed the presence of microsatellite $(CA)_{16}$ and $(CA)_{19}$ repeats in fragments SGMS1 and SGMS2, respectively in seabass from Mumbai water in comparison to $(CA)_{14}$ repeats from the Australian seabass. Further, on sequence comparison, single nucleotide mismatches detected at their several positions in relation to seabass GH gene of Australia. These nucleotide variations detected in SGMS1 and SGMS2 in comparison to those of the Australian seabass may be due to mutations owing to environmental or habitat changes that seem to have definite potentials for development of genetic markers, which would be useful for identification and selection of superior germplasm with desirable commercial traits such as high growth rate.

Key words

Growth hormone gene, Nucleotide polymorphism, *Lates calcarifer*

Introduction

The Asian seabass (*Lates calcarifer)* locally known as Bhetki (in India) and Barramundi (in Australia), being euryhaline in nature, is a potential candidate fish for commercial aquaculture in freshwater, brackishwater and marine environment. Because of its delicious taste and nutritive value, this species is in high demand and fetches comparatively better price in South East Asian countries (Chou and Lee, 1997). To fulfill the gap of demand and supply, there in an urgent need to increase growth rate of *Lates calcerifer* using modern molecular and aquaculture innovations. In fact, growth hormone, a 22 KD single polypeptide protein, plays significant role in normal somatic growth, bone maturation and development of all vertebrates (Barianaga *et al.,* 1985). In teleosts too, this hormone is involved in osmoregulation (electrolyte balance) and several metabolic functions besides its growth-promoting activity (Björnsson, 1997; McCormick, 2001; Canosa *et al*., 2007).

Microsatellite loci consist of simple tendemly repeated sequences of 1 to 6 bp in length (Litt and Luty, 1989; Tautz, 1989). Because of the variations in number of repeat units, mini- and microsatellite may exhibit a high degree of length polymorphism which are abundant in fish genomes (Wright and Bentzen, 1994; O'Reilly and Wright, 1995). Microsatellites are useful in aquaculture and fisheries management specifically in selective

breeding, identification of superior germplasm and genomic mapping (Ward *et al*., 1994; Singh *et al*., 2011). Their abundance, polymorphic nature and amenability to amplification by polymerase chain reaction (PCR) make microsatellite as ideal markers for studies on forensic, quantitative and population genetics (Gill *et al.,* 1994; DeLeon *et al.,* 1997) making systems in natural populations and relatedness to broodstock individuals (Norris *et al*., 2000; Singh *et al*., 2011). Prolonged environmental and habitat changes in the ecology of aquatic organisms may also bring about molecular changes and mutation in their genome. Yue and Orban (2001) characterized the microsatellite in GH and insulin-like growth factor (IGF-2) of *Lates calcartifer* inhabiting Australian waters. Therefore, the present study was undertaken on nucleotide polymorphism in the alleles of growth hormone gene containing microsatellite of the seabass from Mumbai (India).

Materials and Methods

Fish samples and genomic DNA isolation : Out of forty *Lates calcarifer* collected from Mumbai, West Coast of India (Arabian Sea), twenty randomly selected fishes were utilized for DNA isolation from their fins. Genomic DNA was isolated using traditional phenol-chloroform method (Miller *et al.,* 1988). Purity, integrity and quantity of total genomic DNA were analyzed by 0.8% agarose gel electrophoresis (Bangalore Genei) and UV-spectrophotometer (Thermospectronic, UK). Forward and reverse primers (20 bas pairs) of the following sequences were designed and got synthesized from Qiagen Operon using published sequence of seabass GH gene in the promoter of GH gene (Yue *et al*., 2001).

Forward primer **5'** TCAAATCAGTTTGTGACACG **3'**

Reverse primer **5'** GTCTTGGCTCTGGATCAGTG **3'**

PCR amplification : PCR amplification of each DNA sample was performed using Thermal Cycler (Eppendorf, Germany) in 25 l volume containing 1.5 mM $MgCl_2$, 200 M each dNTP, 0.2 M of each primers, 1.0 U Taq polymerase (MBI Fermentas) and 30 ng template DNA. The cycling conditions for amplification were an initial denaturation at 94ºC for 2 min, annealing at 50ºC for 30 seconds and extension at 72ºC for 30 sec which were repeated for 34 cycles. Then final extension step at 72ºC was prolonged for 5 min. Amplified DNA fragments were electrophoresed both on 2% agarose gel as well as 5% polyacrylamide gel. The DNA bands of about 240 bp and 250 bp size were cloned after eluting from agarose gel by using Millipore Ultrafree®-DA (DNA extraction kit).

Cloning of PCR product and selection of recombinant clones : Cloning of PCR fragments, SGMS1 (240 bp) and SGMS2 (250 bp), were done after ligation with vector pTZ57R (2.886 kb) by using of Ins T/A cloneTM PCR product cloning kit of MBI, Fermentas, following manufacturer's instructions. The positive (white) clones were selected from X-gal/IPTG and ampicillin containing LB agar plates and grown individually in LB media containing ampicillin (40 µg ml^{-1}). After overnight culture, positive recombinants were confirmed by PCR using specific forward and reverse primers and recombinant plasmids were isolated from the positive clones following the method of Sambrook *et al.* (1988).

Restriction enzyme digestion of recombinant plasmids : Isolated recombinant plasmids (about 1g DNA) were digested with restriction enzymes including BamH I and Xba I (New England Biolabs) at 37ºC for 2 hr. The digested DNA was electrophoresed on 1% agarose gel using 1 X TAE buffer. The gels were visualized to ascertain the presence of insert (240 and 250 kb) under UV transilluminator and photographed using Gel Doc Bio Imaging system (SYNGENE).

Storage of recombinants : The positive recombinant clones selected out from the LB agar plates and grown overnight in 5 ml LB media (containing ampicillin) were aliquoted into 1.5 ml sterile microfuge tubes with 15% glycerol and stored at -20ºC as mother stock.

DNA sequencing : Sequencing of both recombinant plasmids having DNA fragments (SGMS1 and SGMS2) of seabass GH gene containing microsatellite was performed by ABI PRISM dye terminator cycle sequencing kit according to manufacture's recommendation using an automated DNA sequencer model 310 (Applied Biosystems).

Sequence analysis : On BLAST (Basic Local Alignment Search Tool) searching and after manual correction for ambiguous positions by comparing with electopherograms, SGMS1 and SGMS2 were found to be exactly 233 and 239 bp, respectively. To find homology, single nucleotide-nucleotide blasting was performed. Microsatellite repeats were analyzed using programme Tandem Repeats Finder (Version 2.02) and Microsatellite Analysis Server (MICAS). Then, sequence alignment of SGMS1, SGMS2 and DNA fragment of GH gene-containing microsatellite of *Lates calcarifer* (GenBank U16816; Yowe and Epping, 1995) was performed with the help of clustalw programme. These sequences SGMS1 (GenBank AY803731) and SGMS2 (GenBank AY803732) were submitted to NCBI GenBank.

Results and Discussion

The PCR-based amplification of *Lates calcarifer* DNA from Mumbai coast produced very explicit and repeatable pattern of four DNA bands of about 240, 250, 370 and 400 bp using GH gene containing microsatellite specific forward and reverse primers (Fig. 1).

Restriction endonuclease analysis of recombinant clones showed the presence of about 240 and 250 bp inserts after PCR

amplification. The size of these inserts in recombinant plasmids (3.116 Kb and 3.136 Kb) were further confirmed on double digestion with BamHI and XbaI (Fig. 2).

Further, these recombinant plasmids after sequencing showed and confirmed the size of insert SGMS1 as 233 bp and SGMS2 as 239 bp having microsatellite $(CA)_{16}$ repeats and $(CA)_{19}$ repeats, respectively (Table 1). In addition, very interesting single nucleotide polymorphisms in DNA fragments of GH gene containing microsatellite were also found after sequence comparison (Table 1, Fig.3).

In fishes, relationships and association between GH gene polymorphism and growth has been observed (Gross and Nilsson, 1995). Polymorphism has been found to be correlated with the longest uninterrupted run of repeat units within a microsatellite, and generally dinucleotide microsatellites are expected to be polymorphic if it is more than 10 repeats units without interruption (Weber, 1990). Accordingly, Weber (1990) classified microsatellites into three categories- perfect (uninterrupted run of repeats), imperfect (interrupted, with base substitution) and compound microsatellite (two or more runs of different repeat units). Imperfect and compound microsatellites, however, seem to be less polymorphic that might be expected from the length of uninterrupted repeats runs of perfect microsatellite (Jarne and Lagoda, 1996). In the present study, the presence of total four alleles (Fig. 2) and uninterrupted runs varying between $(CA)_{16}$ to $(CA)_{19}$ motifs for perfect dinucleotide microsatellite were found suggesting that polymorphism in DNA sequence of SGMS1(GenBank AY803731) and SGMS2 (GenBank AY803733) in comparison to $(CA)_{14}$ repeats in GH gene of *Lates calcarifer* of Australian origin (GenBank U16816) (Table 1). Similarly, the allelic variation has been reported within the growth hormone gene of the common bream (Gross *et al*., 1996).

Yue *et al*. (2001) characterized total four microsatellites in insulin-like growth factor-2 (IGF-2) and GH genes of Asian seabass by screening the DNA sequences described by Yowe and Epping (1995) (GenBank U16816). They found two microsatellites in IGF-2 gene both of which were located in second intron, namely prefect $(GA)_{13}$ and compound microsatellite $(CG)_4$ $(CA)_8$. The other two microsatellites identified in the GH gene were perfect one $(CA)_{14}$, located in promoter region between TATA and CAAT sites and an imperfect one $(TG)_3TTGT(TG)_2TTGT(TG)_2$ $T(TG)_2$ TTGT in second intron of GH

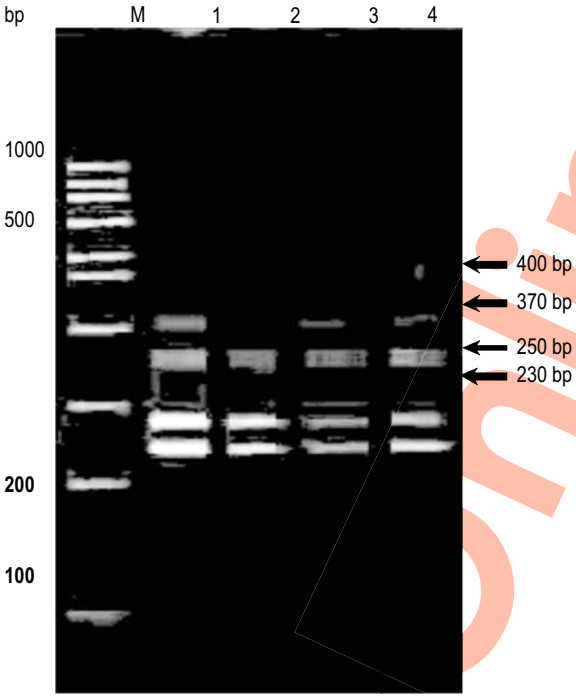

Fig. 1 : PCR amplification of four alleles (DNA fragments) of GH gene containing microsatellite of seabass genome from Mumbai (India). Lane M denotes DNA molecular weight marker 100 bp ladder. Lane 1, 2, 3 and 4 denote DNA fragments (alleles)

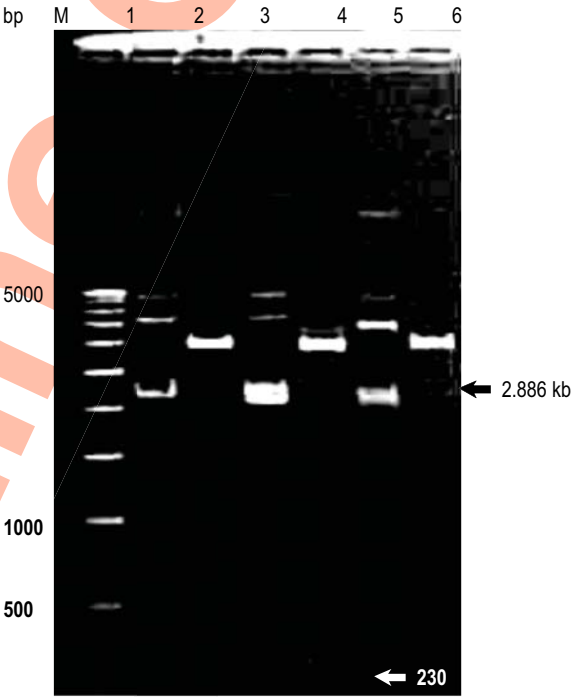

Fig. 2 : Restriction endonuclease analysis of r-plasmids after double digestion with BamHI and XbaI and confirmation of insert size. Lane M: DNA molecular weight marker, 500 bp ladder. Lane 1: Uncut recombinant plasmid (3.136 Kb) with insert 250 bp. Lane 2: Recombinant plasmid (3.136 Kb) releasing 250 bp insert. Lane 3: Uncut recombinant plasmids (3.116 Kb) with insert 230 bp. Lane 4: Recombinant plasmid (3.116 Kb) releasing 230 bp insert. Lane 5: Uncut non-recombinant plain plasmids (pTZ 57R, 2.886 kb). Lane 6: Linear plasmids (pTZ 57R, 2.886 kb)

gene. After analysis of DNA sequences of SGMS1 (GenBank AY803731) and SGMS2 (GenBank AY803732) of Indian *Lates caolcarifer,* it was found that dinucleotide perfect microsatellite $(CA)_{16}$ times repeats in the former sequence whereas $(CA)_{19}$ times repeats in the latter one, which is in good agreement with similar results published for Nile tilapia (Lee and Kocher, 1996) and Atlantic salmon (Slettan *et al*., 1993). In the present investigation after nucleotide sequence analysis, surprisingly extra two times (CA) repeats in sequence of SGMS1 and extra five times (CA) repeats in sequence of SGMS2 DNA fragments was observed in comparison to $(CA)_{14}$ repeats reported by Yue *et al*. (2001) for seabass from Australia (GenBank U16816). Such extra polymorphic microsatellites have also been reported in the common carp (Crooijmanas *et al*., 1997).

In fact, occurrence of polymorphism in GH gene of cattle and chicken has also been reported (Hoj *et al.,* 1993; Feng *et al.,* 1998). This polymorphism may be due to variation in microsatellite repeats in intron or promoter of GH gene. In salmonids and bleak fishes, polymorphism in GH gene appears to be located in introns *i.e.* non-coding region of gene (Park *et al*., 1995, Schlee *et al*., 1996). Almuly *et al.* (2000) have also reported presence of many mini- and microsatellite repeat sequences in non-coding region of gilthead seabream which result in extensive allelism due to different number of length repeats. In the present investigation, we have also found the occurrence of microsatellite variation in the promoter region of GH gene of Asian seabass which is in consonance with similar finding in *Lates calcarifer* of Australia (GenBank U16816) and pufferfish (Venkatesh and Brenner, 1997).

Further on sequence comparison, very interesting single nucleotide mismatches at several positions of both DNA fragments/alleles of GH gene containing microsatellite were found in comparison to *Lates calcarifer* of Australia (GenBank U16816). In DNA fragment SGMS1 at position 54, C was found instead of T, at position 64, G was found in place of A, and between position 111-114 additional $(CA)_2$ was present. Likewise in SGMS2, at position 54, C was found instead of T, at position 121, additional C was found at position 173, T was there in place of C, at position 178, 180, 188 and 189, T is there instead of A and between 111-120, additional $(CA)_5$ was found in comparison to DNA sequence of *Lates calcarifer* from Australia (GenBank No. U16816) (Table 1).

Similarly, on comparing the nucleotide sequence of SGMS1 and SGMS2 alleles of GH gene between themselves, the noticeable variations at the following positions were observed. At position 64, G was there instead of A, at position 149 and 173, C was found instead of T and at position 178, 180, 188 and 189, A was there in place of T in above sequences, respectively. Further at position 121, additional C and between position 115-120 additional $(CA)_3$ was found in case of SGMS2 (Table 1). These variations in nucleotide sequences of DNA fragments SGMS1 and SGMS2 of GH gene containing microsatellite may be due to substitution/insertion of bases, leading to mutation due to environment or habitat changes of Asian seabass of India in comparison to that of the Australian origin. The predisposition of genetic variation may be the result of mutation that occurs at protein or DNA level.

On blasting the SGMS1 and SGMS2 sequences with gene databases, partial homologies with *Seriola quinqueradiata* (26.9%, 12.9%), *Fundulus heteroclitus* (15.8%, 15.8%),

Table 1 : Multiple sequence alignment showing homologies among nucleotide sequence of SGMS1(AY803731), SGMS2(AY803732) of *Lates calcarifer*

Nucleotide position	Nucleotide in L. calcarifer (U16816)	Nucleotide in SGMS1 (AY803731)	Nucleotide in SGMS2 (AY803732)
54	T	C	C
64	A	G	A
82-120	$(CA)_{14}$	$(CA)_{16}$	$(CA)_{19}$
121	-	-	C
149	T	C	T
178	A	A	T
180	A	A	T
188	A	A	T
189	A	A	T

Table 2 : Homology between nucleotide sequence of SGMS1 with *Lates calcarifer*, *Seriola quinqueradiata*, *Oreochromis nilotica*, *Oreochromis mossambicus*, *Fundulus heteroclitus* and *Danio rerio*

Sequence name	GenBank. Acc. No	Sequence size (bp)	% Homology
SGMS2		239	100
Lates calcarifer	U16816	229	93.3
Fundulus heteroclitus	D29737	40	15.8
Seriola quinqueradiata	D50368	32	12.9
Tilapia nilotica	M97765	19	7.9
Oreochromis mossambicus	AF033805	19	7.9
Danio rerio.	AY286447	18	7.5

Table 3 : Homology between nucleotide sequence of SGMS2 with *Lates calcarifer, Seriola quinqueradiata, Oreochromis nilotica, Oreochromis mossambicus, Fundulus heteroclitus* and *Danio rerio*

Sequence name	GenBank. Acc. No	Sequence size (bp)	% Homology
SGMS2		239	100
Lates calcarifer	U16816	229	93.3
Fundulus heteroclitus	D29737	40	15.8
Seriola quinqueradiata	D50368	32	12.9
Tilapia nilotica	M97765	19	7.9
Oreochromis mossambicus	AF033805	19	7.9
Danio rerio.	AY286447	18	7.5

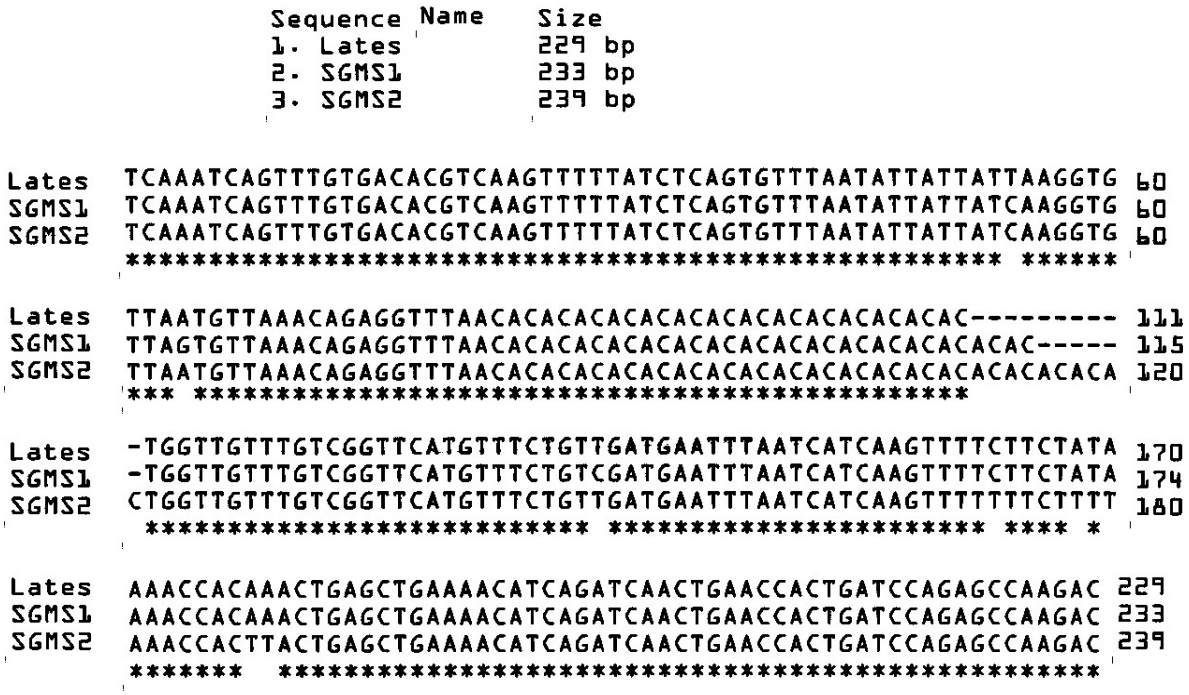

Fig. 3: Multiple sequence alignment showing homologies among nucleotide sequence of SGMS1 (AY803731), SGMS2 (AY803732) of *Lates calcarifer*

Oreochromis nilotica (23%, 7.9%), *Oreochromis mossambicus* (23%, 7.9%) and *Danio rerio* (8.2%, 7.5%), were also observed (Table 2, 3).

These findings indicated that sequence of two alleles of GH gene containing microsatellite of *Lates calcarifer* from Mumbai coast exhibited very less percentage homology with several above fishes except with seabass of Australian origin. In fact, similar results of no product amplification from genomic DNA of Asian arowana (*Scleropages formosus*) and *Tetraodon nigroviridis* using above primers have also been reported by Yue *et al*. (2001). Such molecular variations can be used as reference points for identification, isolation and manipulation of genes and characterization of animals carrying the transgenes (Mitra *et al.,* 1999).

The variations observed in microsatellite and individual nucleotide level in two alleles of seabass GH gene of Mumbai coast in comparison to those of Australian origin are useful after further validations in the development of genetic markers. These, in turn, shall have implications in characterization and identification of seabass biodiversity, selection of superior germplasm and their conservation from different environmental ecotypes having commercial traits like better growth rate and disease resistance to boost productivity of the seabass.

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