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Characterization and physical mapping of 18S and 5S ribosomal genes in Indian major carps (Pisces, Cyprinidae)

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ABSTRACT

Characterization of the major (18S) and minor (5S) ribosomal RNA genes were carried out in three commercially important Indian major carp (IMC) species, *viz. Catla catla*, *Labeo rohita* and *Cirrhinus mrigala* along with their physical localizations using dual colour fluorescence *in situ* hybridization. The diploid chromosome number in the above carps was confirmed to be 50 with inter-species karyo-morphological variations. The 18S rDNA signals were observed on 3 pair of chromosomes in *C. catla* and *L. rohita*, and two pairs in *C. mrigala*. The 5S rDNA signal was found on single pair of chromosome in all the species with variation in their position on chromosomes. The sequencing of 18S rDNA generated 1804, 1805 and 1805 bp long fragments, respectively, in *C. catla*, *L. rohita* and *C. mrigala* with more than 98% sequence identity among them. Similarly, sequencing of 5S rDNA generated 191 bp long fragments in the three species with 100% identity in coding region and 23.2% overall variability in non-transcribed spacer region. Thus, these molecular markers could be used as species-specific markers for taxonomic identification and might help in understanding the genetic diversity, genome organization and karyotype evolution of these species.

1. Introduction

During the last two decades, the role of genetics in conservation biology and ecology, in general, has greatly been emphasized. The assessment of genetic diversity of species in various populations, natural or captive, wild or domesticated, is now widespread. Potential molecular tools are being increasingly developed and used to estimate the extent and organization of genetic diversity, infer the causes of its spatio-temporal dynamics and to suggest strategies for conservation and wise use of genetic resources. In this era of dramatic human exploitation, consumption of natural biological resources and concomitant development of biotechnologies, the emerging field of conservation genetics can help to guide the necessary harmony between economic development and nature protection. Conservation genetics, hence, provides important tools for the assessment of biodiversity as per the Convention on Biological Diversity of the United Nations.

The four carp species, *viz.* catla (*Catla catla*), rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*) and kalbasu (*Labeo calbasu*) found in Indian waters, are grouped together as Indian major carps (IMCs). These IMCs are natural inhabitant of the freshwater river system of northern and central India along with the rivers of Pakistan, Bangladesh and Myanmar. Among these, catla, rohu and mrigal represent important fishery resources and also major contributor to aquaculture production in India and Bangladesh. Until recently, very limited attempts have been made to genetically characterize any of the major carp species. Further, no reports are available on existence of geographically distinct populations of the major carps species.

Some of the classical cytogenetic techniques, like karyomorphology, chromosomal distribution of heterochromatin and different staining techniques have been utilized earlier for characterization of Indian fish species, including IMCs (Lakra, 1996; Kushwaha et al., 2001; Nagpure et al., 2001). At National Bureau of Fish Genetic Resources (NBFGR), number of fish species, including major carps, have been cytogenetically investigated (Ponniah, 1997). Although, the diploid chromosome number in IMCs was reported to be 50 by number of workers, as such no molecular cytogenetic data are available for IMCs.

In higher eukaryotes, the moderately repetitive ribosomal RNA genes (rDNAs) are arranged in two different families: the nucleolus forming major (45S) and the non-nucleolus forming minor (5S) rDNAs. The major family is composed of the regions coding for 18S, 5.8S and 28S rRNA genes separated by internal transcribed spacers (ITS 1 and ITS 2) and surrounded by non transcribed spacer (NTS) sequences (Long and David, 1980; Pendas et al., 1993). The nucleolus organizer regions (NORs) contain 45S rDNA gene cluster, which has also been studied by means of $AgNO_3$ and CMA_3 staining. The minor family is composed of a highly conserved 120 bp long coding

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sequences separated by variable non transcribed spacer (NTS). In several fish species, chromosome location of the two rDNA families are usually different (Martinez et al., 1996; Fujiwara et al., 1998; Sajdak et al., 1998; Martins et al., 2000; Ferro et al., 2001), while in others species they are co-localized on the same chromosome (Moran et al., 1996; Inafuku et al., 2000; Fontana et al., 2003; Tigano et al., 2004).

The present study was envisaged to characterise 18S and 5S rRNA gene and physical mapping of these genes using dual colour fluorescence *in situ* hybridization (FISH) in three IMC species so that it can be used as species-specific molecular markers for taxonomic identification and to deduce phylogenetic relationship between these species.

2. Materials and methods

2.1. Specimen collection and chromosome preparations

Live specimens ($n = 8$ each) of three IMC species namely, *C. catla* (Hamilton), *L. rohita* (Hamilton) and *C. mrigala* (Bloch) were taken from the NBFGR farm for the present study. All the specimens were in juvenile stage and the sex was unidentifiable by visual examination. Metaphase chromosome spreads were prepared from anterior kidney cells using conventional hypotonic treatment, methanol-acetic acid fixation and flame-drying technique (Bertollo et al., 1978). For DNA isolation, blood samples were collected from the fish specimens.

2.2. Genomic DNA isolation, PCR amplification and sequencing of rDNA

Genomic DNA was extracted from whole blood using standard phenol-chloroform-isoamyl alcohol technique as described by Sambrook and Russel (2001). For amplification and sequencing of 18S rDNA, four sets of primers (F1: 5′-CGGCGCAAGACGGACGAA AGC-3′ & R1: 5′-ATGCTTTCGCTTTCGTCCGTCTTG-3′; F2: 5′-CGGCTACCACATCCAAGGAAGG-3′ & R2: 5′-ATGGGTAAT-TTGCGCGCCTGCTG-3′; F3: 5′-CGTGCGGTCGGCGTTCAACTTC-3′ & R3: 5′-CCTTGTTACGACTTTTACTTCCTC; and F4: 5′-CTCAAA-GATTAAGCCATGCAGGTC-3′ & R4: 5′-GACCTGTTATTCCTCCATCT CGCG-3′) were designed using conserved sequences of different teleost fishes. For amplification of the 5S rDNA, primer sequences (F: 5′-TACGCCCGATCTCGTCCGAT C-3′ & R: 5′-CAGGCTGGTATGGCCGTAAGC-3′) were taken from Moran et al. (1996).

PCR amplifications of 18S and 5S rDNAs were performed in 50-μl reaction volume containing 10× buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 10 pM of each primer, 1 U of *Taq* polymerase (Fermentas) and 50 ng genomic DNA. The PCR cycling conditions were: initial denaturation step at 94 °C for 4 min, followed by 34 cycles of denaturation at 94 °C for 1 min, annealing of primers at 55 °C for 30 s, primer extension at 72 °C for 1 min, followed by final extension at 72 °C for 7 min. Amplified products were visualized after electrophoresis on 1.5% agarose gel stained with ethidium bromide. All these amplified products were sequenced from both strands using custom services.

2.3. Probe labeling

The amplified 18S rDNA was labeled with Fluorescein 12-dUTP (Fermentas) by nick translation (http://www.riedlab.nci.nih.gov/publications/CGH%20Nick%20Translation.pdf), whereas the 5S rDNA was labeled with Biotin (Vector Labs, Burlingame, California) according to the manufacturer's instructions.

2.4. Dual colour FISH

Dual color FISH was performed to determine the simultaneous localization of 18S and 5S rDNA clusters using the protocol described by Winterfeld and Roser (2007), with minor modifications in post-hybridization washing at 45 °C for 5 min (Mani et al., 2010).

2.5. Fluorescence microscopy and image acquisition

Chromosome spreads were visualized and photographed with a fluorescence microscope (Leica, Germany) equipped with a computer assisted charge-coupled device camera. The DAPI, FITC and rhodamine fluorescences were detected using appropriate filter sets (DAPI: excitation 340/40 nm, emission 430/50 nm; FITC: excitation 480/20 nm, emission 510/30 nm and rhodamine: excitation 560/20 nm, emission 610 nm). Images were captured with appropriate filters and processed further using Leica CW4000 FISH software.

2.6. Sequence analysis

The 18S and 5S rDNAs sequences analyses with other fish species were done using BLASTn (Altschul et al., 1997). The comparative sequence analyses of 18S and 5S rDNAs among the subject species were done using 'MEGA5' software (Tamura et al., 2011).

3. Results

A distinct conservation of 50 diploid chromosomes (2n) was observed in the three undertaken IMC species with inter-species variations in their karyo-morphology. The localization of fluorescent signals on chromosomes of *C. catla*, *L. rohita* and *C. mrigala* with dual color FISH allowed visualization of four to six sites of 18S and two sites of 5S rDNAs (Fig. 1a–c). In *C. catla* and *L. rohita*, the 18S rDNA signals were found on 3 pairs of chromosomes, whereas in *C. mrigala* the signals were observed on 2 pairs of chromosomes. The 5S rDNA site was present on one pair of chromosome in all the three species.

The sequencing of 18S rDNA amplicon generated fragment size of 1804 bp in *C. catla*, 1805 bp in *L. rohita* and 1805 bp in *C. mrigala* (Table 1). In all the three species, single base differences in terms of substitutions or insertion-deletions were found in the nucleotide sequences. The 18S sequence of *C. catla* showed 99.3% identity with *L. rohita* and 98.3% identity with *C. mrigala*, whereas identity between *L. rohita* and *C. mrigala* was 98.5%. All these nucleotide sequences showed more than 92% overall identity with other fish species listed in NCBI database.

The total size, including both coding and NTS regions, of 5S rDNA amplicons were 191 bp in all the three IMC species (GenBank Accession Nos. GU967676, GU967677, GU967678). The nucleotide sequences of the 5S coding regions shared a highly conserved region (100% identity) of 120 bp among the three species, which contained three elements, namely Box A, Box C and intermediate element (IE), of the internal control regions (Fig. 2). T-rich (TTT) sequences were also identified at 3′ end of coding region and 5′ end of NTS region in these species.

NTS region of the minor rDNA family was identified to be 71 bp long in all the three species. NTS contained one TAAATA box like sequences in all the species under study. The comparative analyses of 5S NTS region among the three species showed 23.2% overall distance, whereas the distance between *C. catla* and *L. rohita* and *C. catla* and *C. mrigala* were 32.3 and 27.9%, respectively. The distance between *L. rohita* and *C. mrigala* was 9.3%. The comparative analyses of complete 5S sequence showed 10.2% distance between

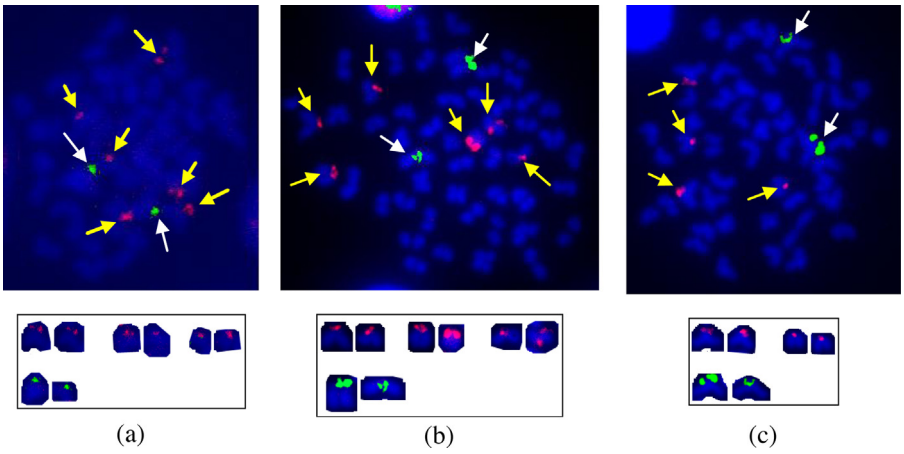

Fig. 1. Dual colour FISH on metaphase spread of: (a) *C. catla*, (b) *L. rohita*, and (c) *C. mrigala*. The micrographs were taken with triple band filter allowing the simultaneous visualization of DAPI stained chromosomes, the hybridization sites of 18S (red, indicated with yellow arrow) and the 5S (green, indicated with white arrow) rDNA probes. Pairs of homologous chromosomes bearing hybridization signals for 18S (red) and 5S (green) are shown in frame. Bar: 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Sequences details of 18S rDNA among the subject species.

Species	Sequence length (bp)	Base composition (%)				GC content (%)	NCBI Acc. No.
		A	T	G	C		
C. catla	1804	~22.1 (398)	~20.7 (373)	~30.3 (547)	~26.9 (486)	~57.2 (1033)	GU967673
L. rohita	1805	~22.3 (403)	~20.7 (374)	~30.3 (546)	~26.7 (482)	~57.0 (1028)	GU967674
C. mrigala	1805	~22.9 (414)	~20.8 (376)	~29.6 (534)	~26.7 (481)	~56.3 (1015)	GU967675

Note: Values presented in parenthesis are actual number of bases in the sequence.

C. catla and *L. rohita*, 9.6% between *C. catla* and *C. mrigala*, and 3.8% between *L. rohita* and *C. mrigala*, whereas overall distance was 7.9% among the IMC species. The 5S rDNA sequences of the IMC species showed more than 80% average similarity with other fishes listed in GenBank.

4. Discussion

The changes in chromosomal organization during evolution lead to intra- and inter-species as well as population variability. More exhaustive, integrated and complementary research

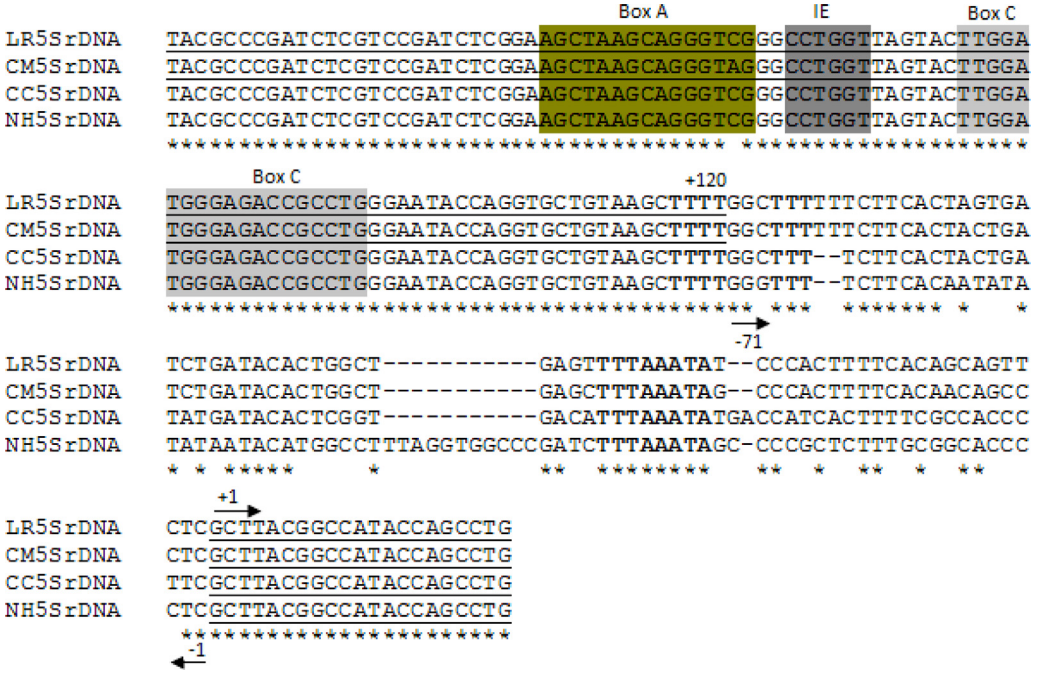

Fig. 2. Aligned nucleotide sequences of 5S rDNA in: *C. catla* (CC5SrDNA), *L. rohita* (LR5SrDNA) and *C. mrigala* (CM5SrDNA). The 5S rDNA sequence (GenBank with Accession No. GU568380) of *Neolissochilus hexagonolepis* (NH5SrDNA) was taken as out-group. The coding 5S rDNA sequences are underlined. Box A, Box C and IE sequences are indicated in shaded colors. TATA and TTT like elements are indicated in bold characters.

Table 2
Review on karyological studies in three IMC species.

Species	2n	Karyo-morphology				FN	Ag-NOR pairs	Reference
		m	sm	st	t			
C. catla	50	4	24	–	22	–	–	Khuda-Bukhsh and Manna (1976)
	50	8	16	26	–	–	–	Manna (1977)
	50	6	32	12	–	–	–	Majumdar and Ray Chaudhuri (1976)
	50	12	16	22	–	78	–	Zhang and Reddy (1991)
	50	12	16	22	–	88	–	Jana (1993)
	50	10	16	8	16	76	2[a]	Nagpure et al. (2001)
L. rohita	50	18	8	24	–	–	–	Manna and Prasad (1971)
	50	6	26	18	–	–	–	Majumdar and Ray Chaudhuri (1976)
	50	10	16	24	–	–	–	Gui et al. (1986)
	50	10	18	22	–	78	–	Zhang and Reddy (1991)
	50	–	18	22	–	–	–	Jana (1993)
	50	10	14	8	18	74	2[a]	Nagpure et al. (2001)
C. mrigala	50	6	8	–	36	–	–	Manna and Prasad (1971)
	50	6	26	–	18	–	–	Majumdar and Ray Chaudhuri (1976)
	50	12	18	–	20	–	–	Zhang and Reddy (1991)
	50	12	18	6	14	80	1[a]	Nagpure et al. (2001)

[a] Nucleolar organizing region (NOR) localized after silver nitrate impregnation.

approaches are needed to evaluate degrees of genetic exchange among species and populations, especially for the species having wide geographical distribution. In this respect, cytogenetic techniques have been used to characterize populations, species, genera and families, and many of them have proved to be efficient markers in identifying intra and inter-specific chromosome variations. The development of specific cytogenetic banding/staining techniques have facilitated accurate chromosome identification and permitted a better understanding of karyo-morphology. These findings have revealed relationships among species and the mechanisms involved in the evolutionary processes. Recently, the studies in chromosome structure and evolution were challenged with the introduction of new molecular cytogenetic techniques that has enabled taxonomic identification of species with the use of genes or specific genomic segments. This will eventually help in fisheries development through better management of genetic resources.

The results of the present study supported the conservedness of the diploid chromosome numbers (*i.e.* 2n = 50) in these cyprinid species. However, variation in karyo-morphology has been reported by many workers in these species, which is summarized in Table 2. It is evident from the frequency distribution (Klinkhardt et al., 1995) that 2n = 50 is by far the most common diploid chromosome number in cyprinids. The variation in karyo-morphology between species may be due to prevalence of non-robertsonian rearrangements (Kirpichnikov, 1981). In these IMC species, the karyo-morphological variation could be attributed to the presence of acrocentric chromosomes that suggest a substantial role of pericentric inversions during the course of evolutionary process.

The dual color FISH helped simultaneous chromosomal localization of the two rDNA families (18S and 5S) on the chromosomes of three IMC species and is being reported for the first time. In the earlier study, Nagpure et al. (2001) observed NOR signals on 2, 2 and 1 pair(s) of chromosomes, respectively, in *C. catla*, *L. rohita* and *C. mrigala* using silver nitrate staining that stains only transcriptionally active regions, whereas the FISH is able to detect 18S rDNA on 3, 3 and 2 pair of chromosomes in the present study. Thus, the molecular karyotyping using FISH technique helps precise characterization of these species. Further, the 18S rDNA probe has been considered as an important marker to evidence the karyotypic differentiation, which is not detected with conventional tools, in species considered karyotypically conserved and uniform (Almeida et al., 2010). The heteromorphism in signal intensity observed between homologous chromosomes may be caused by a variety of mechanisms, namely unequal crossing over, transposition, tandem amplification and other rearrangements involving homologous segments causing structural modifications in the NORs (Vicari et al., 2006; Sczepanski et al., 2010).

The 5S rDNA loci were observed on one of the largest submetacentric chromosome in all the species. There was no variation observed in number of 5S rDNA loci in these species, which suggests that no other sequences are included in a repeat, apart from its coding and NTS regions. Localization of 5S rDNA sites on single pair of chromosomes seems to be a general rule for cyprinid species. The 5S rDNA cluster has been observed on single chromosome pair in many fish species, namely *Salmo salar* (Pendas et al., 1994), *Anguilla anguilla* (Martinez et al., 1996), *Salvelinus fontinalis* (Phillips et al., 2002), but presence on several chromosomes has also been observed in *Oncorhynchus mykiss* (Moran et al., 1996), *Oncorhynchus masou*, *Hucho perryi*, *S. fontinalis* (Fujiwara et al., 1998), *Leporinus* (Martins and Galetti, 1999) and *Thymallus thymallus* (Jankun et al., 2003). Interestingly, Souza et al. (2008) observed two 5S rDNA loci on the same chromosome in *Salminus brasiliensis* that could be related to the two 5S rDNA NTS classes. The presence of two or more 5S rDNA clusters at the individual level may be attributed to compositional variations in the NTS region present due to insertions/deletions, mini-repeats and pseudo-genes (Martins and Wasko, 2004).

The 45S and 5S rDNA loci may be syntenic (Moran et al., 1996). The simultaneous detection of major and minor rDNAs in IMC species demonstrated unambiguously that 18S and 5S rDNAs are not syntenic. Martins and Galetti (2000) suggested that the localization of 5S and 45S rDNA loci on different chromosomes, as observed for the majority of the vertebrates, could permit them to evolve independently, since the divergent evolutionary tendencies may exist in a single genome and divergent functional dynamics of these sequences required physical distancing.

This study also analyzed 18S and 5S rDNA sequences in the IMC species and a close sequence identity of 18S rDNA was observed among them. These sequences showed maximum homology with other cyprinids (*e.g. Tor* species, *Cyprinus carpio*, *Carassius carassius*). Little variations were observed at about 32 sites in 18S rDNA sequences due to addition, deletion and substitution in these species. At the molecular level, it reflected sequence conservation of 18S. Several regions of 18S rRNA, such as those interacting with 5.8S, are the most conserved since these domains possibly cooperate during maturation of pre rRNA yielding 18S product (Kupriyanova, 2000).

The rDNA is one of the most conserved genes in eukaryotes and these sequences are very GC rich (Escobar et al., 2011; Lisowski et al., 2012). Conservation of rDNA sequence has been demonstrated in both linear sequences and secondary structures, which suggests that rRNA is under strong purifying selective pressure. Within vertebrates, the highest GC content in rDNA was found in mammals, birds and ray-finned fishes (like siluriformes, cypriniformes and clupeiformes). Further, the helices are GC richer than loops in the rDNA. The GC-rich RNA sequences form more stable secondary structures, than AU-rich, and can prevent the process of transcriptional backtracking and aid in the rate of recovery from the backtracks (Zamft et al., 2012). The multiples rDNA copies in the genomes evolve in a concerted manner, through unequal crossing over and/or gene conversion.

In most of the studies, the 5S rDNA has been characterized by presence of 120 bp long transcribing region highly conserved even between non-related species with variable NTS region owing to insertions-deletions, substitutions and pseudogenes frequently characterized in coregonid and tilapia fish (Sajdak et al., 1998; Martins et al., 2000). NTS regions are presumably free to vary because they are not under strong selective pressure, while most 5S rRNA gene mutations are selectively or nearly neutral only when they occur in a subcritical proportion (Cronn et al., 1996). PCR with primers designed from NTS regions have successfully been used for identification of the fish species (Ferreira et al., 2007; Mani et al., 2010; Tognoli et al., 2011). In fact, various classes of 5S rRNA gene (class I: 203 bp; class II: 340 bp; and class III: 477 bp; class IV: 188 bp; class V: 765 bp; class VI: 1.4 kb), characterized by difference in NTS sequence lengths and compositions, have been reported in fishes, which indicates that NTS region evolves rapidly. These classes may be present simultaneously or separately and may be the result of the influence of polyploidy on the organization and evolution of the multigene 5S rRNA family in fish (Martins et al., 2002; Qin et al., 2010; Rodrigues et al., 2012). The smallest NTS sizes of 5S rDNA described so far, in eukaryotes including fishes, are 62 bp (Martins and Wasko, 2004; Santos et al., 2006) and 56–67 bp (Fujiwara et al., 2009).

The 5S rRNA is transcribed alone by RNA polymerase III with the participation of a specific transcription factor IIIA (TFIIIA) and the three elements of internal control region, as also observed in the present study, *i.e.* Box A, Box C and IE, act as a promoter for transcription of the gene (Pieler et al., 1987). Box A is a general binding sequence for RNA polymerase III, and the IE and Box C are interaction sites for the TFIIIA. Another characteristic typically found in the presumed-functional 5S rRNA genes is a T-rich (TTTT) terminator region, as initially reported by Korn and Brown (1978) and also in present study. T-rich sequence was also identified at the 3′ end of 5S rDNA in *Oreochromis niloticus* (Martins et al., 2000), and 5′ end of NTS region in *Engystomops* spp. (Rodrigues et al., 2012) that act as a termination signal found in a variety of other genes transcribed by RNA polymerase III (Huang and Maraia, 2001). NTS region also contained control element TATA like motif sequences in all the three species. TATA box sequences were also reported in NTS region of *Tor* spp. (Singh et al., 2009; Mani et al., 2010) and of *Lebias fasciata* (Tigano et al., 2004) that might play important role in the regulation of gene expression. Campo et al. (2009) reported an additional TATA-like region in the NTS regions of the 5S rDNA sequences of the fishes *Merluccius merluccius*, *M. senegalensis* and *M. capensis*, and suggested that this TATA-box may serve as a 'backup'.

In the present study, variations in number of FISH signals and their position in the karyotype (submetacentric and subtelocentric) along with variation in nucleotide sequences of two ribosomal gene families have been observed in the three IMC species. These markers may be further useful for discrimination of stocks/populations of closely related species and their hybrids. The identification of four to six chromosomes carrying the rDNA loci may eventually help to understand the karyo-evolution of these species and further investigate the possibilities of the presence of transposable elements adjacent to rDNA region that serve point for transposition and integration of rDNA in to other part of the genome, enabling variation in number and position of rDNA loci.

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