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Nucleotide variation and physical mapping of ribosomal genes using FISH in genus *Tor* (Pisces, Cyprinidae)

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Abstract Molecular cytogenetic studies were carried out for localization of 18S and 5S ribosomal DNAs on chromosomes of three cyprinid fish species viz., *T. khudree*, *T. mussullah* and *T. mosal mahanadicus* using two color fluorescence in situ hybridization (FISH). All the species typically possessed 100 diploid chromosomes with minor variation in karyo-morphology. The 18S rDNA signals were observed on two pair of chromosomes in *T. khudree* and *T. mussullah*, and three pairs in *T. mosal mahanadicus*. The location of 18S signals also showed affinity to silver nitrate and chromomycin A_3 staining. Similarly, variation in localization of 5S rDNA among the three species has been detected with the presence of FISH signals on one pair of chromosome in *T. khudree* and *T. mussullah*, and on two pairs in *T. mosal mahanadicus*. These molecular markers could be used as species specific markers for taxonomic identification and can further add in understanding the dynamics of genome organization and karyotypic evolution of these species. The 18S rDNA region was sequenced that generated 1811, 1810 and 1776 bp long 18S sequence in *T. khudree*, *T. mussullah* and *T. mosal mahanadicus*, respectively. The 18S rDNA sequence showed 95–98% identity among the subject species. Similarly, 5S sequencing generated 203 bp long fragments in these species with 100% identity in coding and 9.63% variability in non-transcribed spacer regions. The nucleotide sequence variations could be used for understanding the genetic diversity and will add new informative characters in comparative genomics. These results, in general, would enhance the value and interpretation of ecological assessment data for conservation of *Tor* species.

Keywords 18S · 5S · FISH · rDNA · *Tor*

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Introduction

Owing to the difficulty in producing reliable chromosomal bands with conventional techniques, the moderately repetitive DNA sequences, such as ribosomal DNA (rDNA), have been extremely useful as chromosomal markers in fish cytogenetics [1]. In eukaryotes, genes of ribosomal RNA (rRNA) are organized in two distinct classes: the major rRNA gene cluster, which encodes 18S, 5.8S and 28S rRNAs, separated by internal transcribed spacer (ITS 1 and ITS 2) sequences, and the minor rRNA gene cluster, which encodes 5S. The major rRNA gene sequences are present in multiple copies and are found in the nucleolar organizing region (NOR) on the chromosome [2]. Another multi-copy minor rRNA gene is arranged in tandem arrays, which comprise a highly conserved 120 bp long coding sequences with a variable non-transcribed spacer (NTS) and has no role in the nucleolus formation [3, 4]. Molecular organization and cytogenetic mapping of ribosomal genes and other repetitive DNA sequences have contributed significantly to the characterization of biodiversity and the evolution of fish [5]. In many fish species,

chromosome location of the two rDNA families are different [6–9], though in some species they are co-localized on the same chromosome [10, 11].

The fishes of genus *Tor* (2n = 100) belong to the family cyprinidae, which is the most abundant and globally widespread family of the freshwater fishes, comprising of 220 genera and about 2,420 species [12]. Due to different anthropogenic activities, the ecological status of *Tor* species has been assigned as endangered, especially *T. khudree* [13] and *T. mussullah* [14]. The species *T. mosal mahanadicus*, reported from river Mahanadi only in the Deccan plateau [15] with the characteristic head length larger than the body depth, was described as *T. khudree mahanadicus* subspecies by [16]. It is also considered as endangered (www.fishingchimes.com/onmah.htm). Earlier methodologies adopted in identification of *Tor* species were based on several morphometric and meristic characters, which could not identify and characterize them accurately. This is fundamental problem for fisheries and stock management [17]. DNA markers are appropriate in order to obtain information about gene flow, allele frequencies and other parameters that are important in the population biology [18].

The karyomorphological analysis and conventional banding pattern have been utilized earlier for characterization of different species, populations and/or stocks of fishes including *Tor* [19, 20–23]. No molecular cytogenetic data are available for *T. khudree, T. mussullah* and *T. mosal mahanadicus*, except for other *Tor* species (*T. chelynoides, T. progeneius, T. putitora* and *T. tor*) reported from our lab [24]. The present study was aimed: (1) to simultaneous localization of 18S and 5S rDNAs on the metaphase chromosomes using dual color fluorescence in situ hybridization (FISH) and (2) to analyze 18S and 5S rDNA sequences of these *Tor* species. The study will lead to development of species-specific molecular markers for taxonomic characterization, and further guide to conservation of these species and to establish phylogenetic relationship among the species.

Materials and methods

Specimen collection

Live specimens of *Tor khudree* (n = 12), *T. mussullah* (n = 6) and *T. mosal mahanadicus* (n = 22) were collected from River Cauvery, Bangalore, Karnataka (India); TATA Power Company Reservoir, Lonavala, Maharashtra (India) and River Mahanadi, Sonepur, Orissa (India), respectively, with the help of local fishermen. All the specimens were in juvenile stage and the sex was unidentifiable by visual examination.

Chromosome preparations, Ag-NOR and CMA_3 staining

The metaphase chromosomes were prepared from anterior kidney cells of the live fish using conventional hypotonic treatment, methanol-acetic acid fixation and flame-drying technique [25]. NORs were stained with $AgNO_3$ according to the protocol described by [26] and the CMA_3 staining was performed according to [27]. For isolation of DNA, blood samples were collected from the fish specimens.

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 [28]. For amplification and sequencing of 18S rDNA in *T. khudree*, *T. mussullah* and *T. mosal mahanadicus*, four sets of primers (F1: 5′-CTCAAAGATTAAGCCATGCAGGTC-3′ and R1: 5′-ATGGGTAA TTTGCGCGCCTGCTG-3′; F2: 5′-CGGCTACCACATCCAAGGAAGG-3′ and R2: 5′-ATGCTTTTCGCTTTCGTCCGTCTTG-3′; F3: 5′-CGGCGCAAGACGGACGAAAGC-3′ and R3: 5′-GACCTGTTATTCCTCCATCTCGCG; F4: 5′-CGTGCGGTCGGCGTTCAA CTTC-3′ and R4: 5′-CCTTGTTACGACTTTTACTTCCTC-3′) were designed using conserved sequences of different teleost fishes. For amplification of the 5S rDNA, primers (F: 5′-TACGCCCGATCTCGTCCGATC-3′ and R: 5′-CAGGCTGGTATGGCGTAAGC-3′) were taken from Moran et al. [29].

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 pico moles 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 an ethidium bromide-stained 1.5% agarose gel. All these amplified products were sequenced from both strands using custom services.

Probe labeling and dual color FISH

Both direct and indirect labeling methods were used for probe construction. In direct 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 indirectly labeled with Biotin (Vector Labs,

Burlingame, California) according to the manufacturer's instructions.

Dual color FISH was performed to determine the co-localization of 18S major and 5S minor rDNA clusters using the protocol described by Winterfeld and Roser [30], with minor modifications in post-hybridization washing at 45°C for 5 min. In procedure, 2–3 days old metaphase slides were baked at 90°C for 1 h in Thermal cycler/Thermobrite. The multi chamber water bath was set at 37 and 75°C. Chemical aging of slide was done in 2× SSC at 37°C for 1 h followed by slide treatment with 0.005% pepsin at 37°C for 12 min, and then slide washing with phosphate buffered saline (PBS) for 5 min. The slide was then washed with 1% formaldehyde solution for 5 min and again washed with PBS for 5 min. The slides were then used for serial dehydration with 70, 90 and 100% ethanol for 2 min each at room temperature. 10 µl of hybridization mixture (50 ng of each probe, 50% formamide, 10% dextran sulphate, 2.5 µg salmon sperm DNA) was denatured at 95°C for 10 min and immediately chilled on ice for 5 min. The denatured probe was transferred at 37°C in incubator for 15–60 min for pre-hybridization. The slide was incubated in denaturation buffer (70% deionized formamide, 2× SSC, 0.1 mM EDTA, pH 7.0) for 3 min at 75°C and then washed with cold 2× SSC for 2 min, repeated this step twice. Serial dehydration of slide was again performed with 70, 90 and 100% chilled ethanol for 2 min each and the slide was dried at 42°C for 5 min. 10 µl mixture of denatured probes was applied on the slide. Metaphase spreads were hybridized with 18S and 5S rDNA probes simultaneously and the hybridization was carried out overnight at 37°C.

Before detection of the biotin labeled 5S rDNA probe signals, the slide was incubated in 1× blocking solution (Vector Labs) at 37°C for 30 min and the detection reagents were diluted in 1× blocking solution approximately 30 min before use to further reduce any non-specific binding. Detection of the biotin labeled probe was carried out with rhodamine avidin DCS 5 µg/ml (Vector Labs) followed by 2 rounds of signal amplification. After each step of amplification, slide was washed in 1× blocking solution. The preparations were then rinsed with 4× SSC/0.1% Tween 20 twice for 5 min and counter stained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted in Vectashield mounting medium (Vector Labs).

Fluorescence microscopy and image acquisition

Chromosome spreads were viewed and photographed with a fluorescence microscope (Leica, Germany) equipped with a computer assisted CCD camera. The DAPI, FITC and rhodamine fluorescence were detected using appropriate filter sets (DAPI filter cube: excitation 340/40 nm, emission 430/50 nm; FITC filter cube: excitation 480/20 nm, emission 510/30 nm and Rhodamine filter cube: excitation 560/20 nm, emission 610 nm). Blue, green, and red images were captured with appropriate filter for DAPI, FITC and rhodamine excitation and overlapped in Leica CW4000 FISH software. Analyzed numbers of metaphase plates for each species are given in Table 1.

Sequence analysis

The comparative sequence analyses of 18S rDNA among the species were done using 'BLAST 2 Sequences' tool [31]. The 18S and 5S rDNAs sequences were analyzed using BLASTN [32]. The sequence alignment of 5S rDNAs in the species was done using ClustalX [33].

Results

A discernible chromosomal conservation of 100 diploid chromosomes (2n) in the three *Tor* species was observed with inter-specific variations in their karyo-morphology. The karyotype of *T. khudree* was composed of 20 metacentric (m), 14 submetacentric (sm), 22 subtelocentric (st) and 44 telocentric (t) chromosomes. The karyotype formulae of *T. mussullah* and *T. mosal mahanadicus* were 22m + 24sm + 24st + 30t and 22m + 12sm + 22st + 44t chromosomes, respectively.

The conventional staining and two color FISH revealed an interesting chromosomal pattern both in the major and minor rDNA clusters in the three *Tor* species examined. In *T. khudree* and *T. mussullah*, the numbers of Ag-NOR signals were on two pairs of chromosomes, whereas in *T. mosal mahanadicus* the signals were on three pairs of chromosomes (Fig. 1a–c). Fluorescent signals were detected on two pair of chromosomes by CMA_3 staining on the metaphase spreads of *T. khudree* and *T. mussullah*, and three pairs on the metaphase spreads of *T. mosal mahanadicus* (Fig. 2a–c).

The localization of major and minor ribosomal genes on chromosomes of *T. khudree*, *T. mussullah* and *T. mosal mahanadicus* with dual color FISH allowed visualization of four to six sites of major and one to two sites of minor rDNAs. The major rDNA signals were detected on 5th sm

Table 1 Numbers of metaphase plates analyzed in *Tor* species

S. no.	Species studied	Metaphase plates		
		$AgNO_3$	CMA_3	FISH
1.	*T. khudree*	122	105	64
2.	*T. mussullah*	90	75	53
3.	*T. mosal mahanadicus*	152	133	68

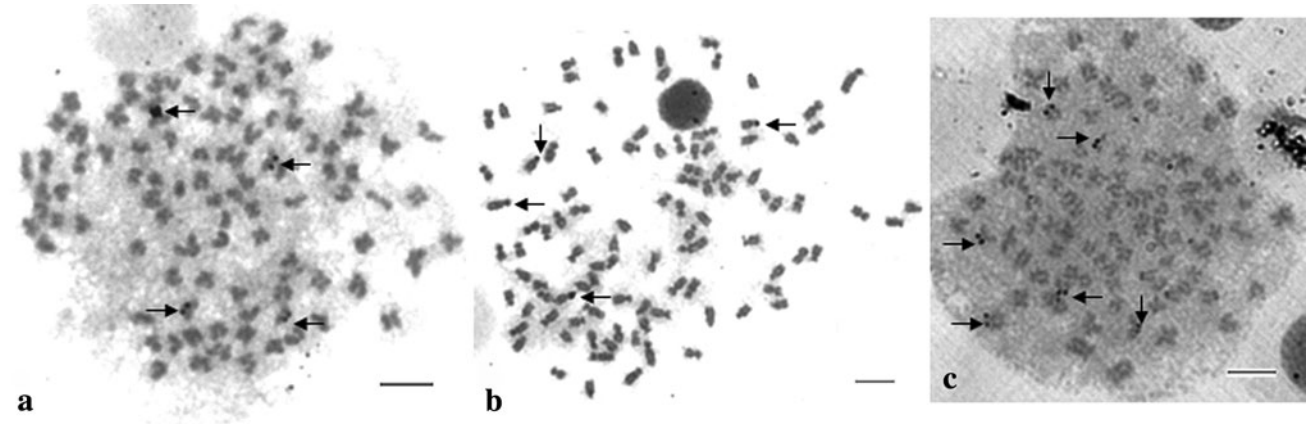

Fig. 1 Metaphase spreads showing Ag-NOR signals (*black arrows*) in **a** *T. khudree*, **b** *T. mussullah*, and **c** *T. mosal mahanadicus. Bar* 5 μm

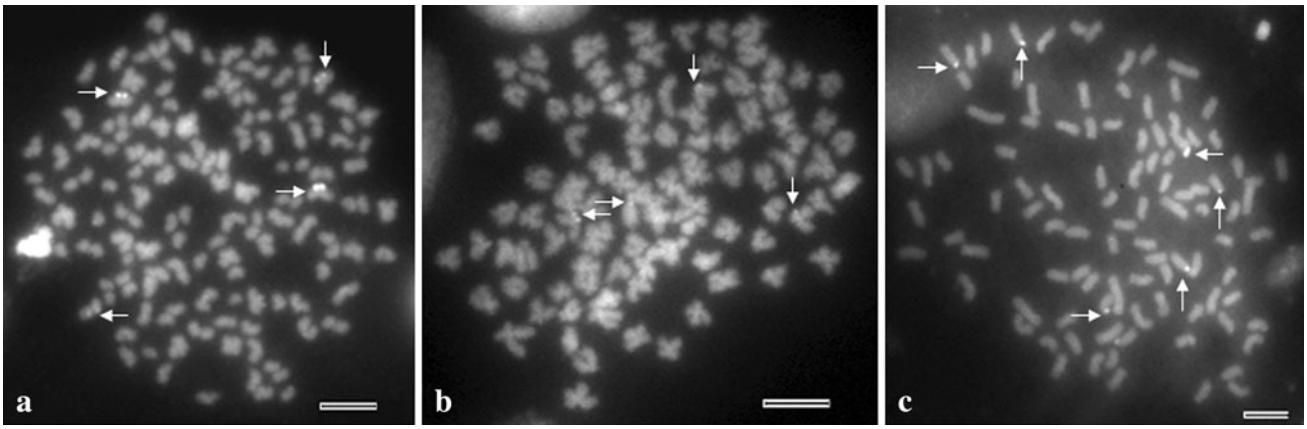

Fig. 2 Metaphase spreads showing CMA_3 signals (*white arrows*) in **a** *T. khudree*, **b** *T. mussullah*, and **c** *T. mosal mahanadicus. Bar* 5 μm

and 2nd st chromosomes in *T. khudree,* (Fig. 3a), 4th sm and 4th st chromosomes in *T. mussullah* (Fig. 3b) and 5th sm, 7th and 10th st chromosomes in *T. mosal mahanadicus* (Fig. 3c). Chromosomes that exhibited silver stained NORs and CMA_3 positive sites were identical in number and position, respectively. The minor rDNA sites were present on the p arms of the 1st sm chromosomes, close to the centromeric region in all the three species, while one additional 5S rDNA signal was localized on 9th st chromosomes in *T. mosal mahanadicus*. Locations of the 18S and 5S ribosomal genes on chromosome were presented in the ideogram (Fig. 4a–c).

The sequencing of 18S rDNA amplicon generated fragment size of 1811 bp in *T. khudree*, 1810 bp in *T. mussullah* and 1776 bp in *T. mosal mahanadicus* (NCBI Accession Nos. FJ598648, FJ233066 and GU249599) (Fig. 5a–c). In all the three species, single base differences in terms of substitutions or insertion-deletions were found in the nucleotide sequences. The partial 18S sequence of *T. khudree* showed 96% identity with *T. mussullah* and 98% identity with *T. mosal mahanadicus,* whereas such identity between *T. mussullah* and *T. mosal mahanadicus* was 95%. All these nucleotide sequences showed more than 91% identity with other fish species listed in NCBI database.

The size of 5S rDNA amplicon was 203 bp in all three fishes including NTS region (NCBI Accession Nos. GU249605, FJ233064 and GU249604). The nucleotide sequences of the 5S rDNA coding regions among the three species shared a highly conserved region (100% identity) of 120 bp long which contained three elements, namely Box A, Box C and IE, of the internal control regions (Fig. 6). Other characteristics of T-rich (TTTT) sequence were identified at the 3′ end of the 5S rRNA coding sequence of all the three *Tor* species. NTS region of the minor family was identified to be 83 bp long in all the three species. The comparative analyses of 5S rDNA sequences among the three species showed 9.63% variability in the NTS region. NTS also contained one TATA box like sequences in all the species. The coding sequences of minor rDNA family in *T. khudree*, *T. mussullah* and *T. mosal mahanadicus* showed an average sequence similarity of 96% with other fishes belonging to order Cypriniformes.

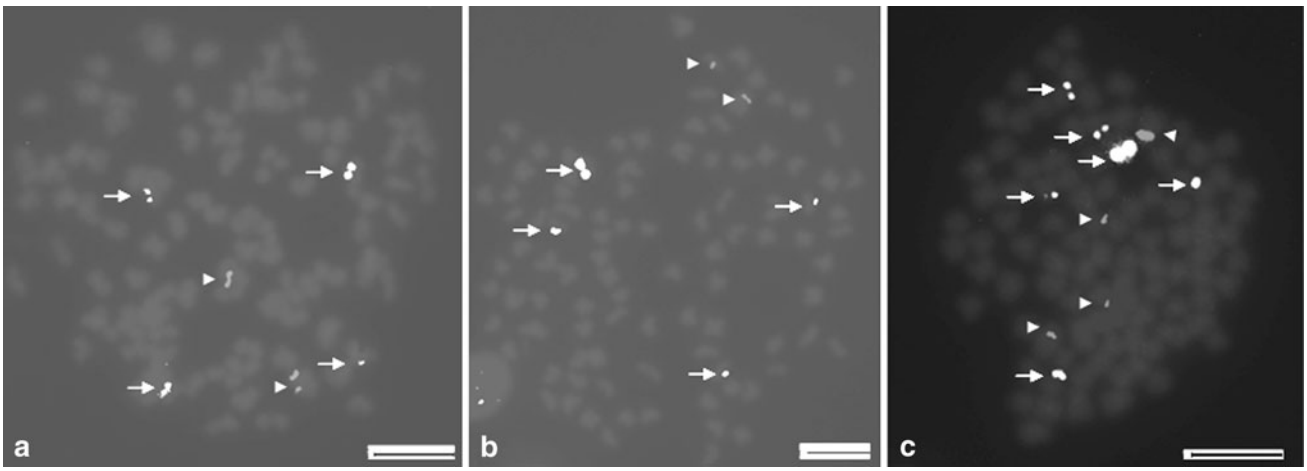

Fig. 3 Dual colour FISH on metaphase spreads of **a** *T. khudree,* **b** *T. mussullah*, and **c** *T. mosal mahanadicus.* 18S (*arrows*) and 5S (*arrow heads*) rDNA probes. *Bar* 5 μm

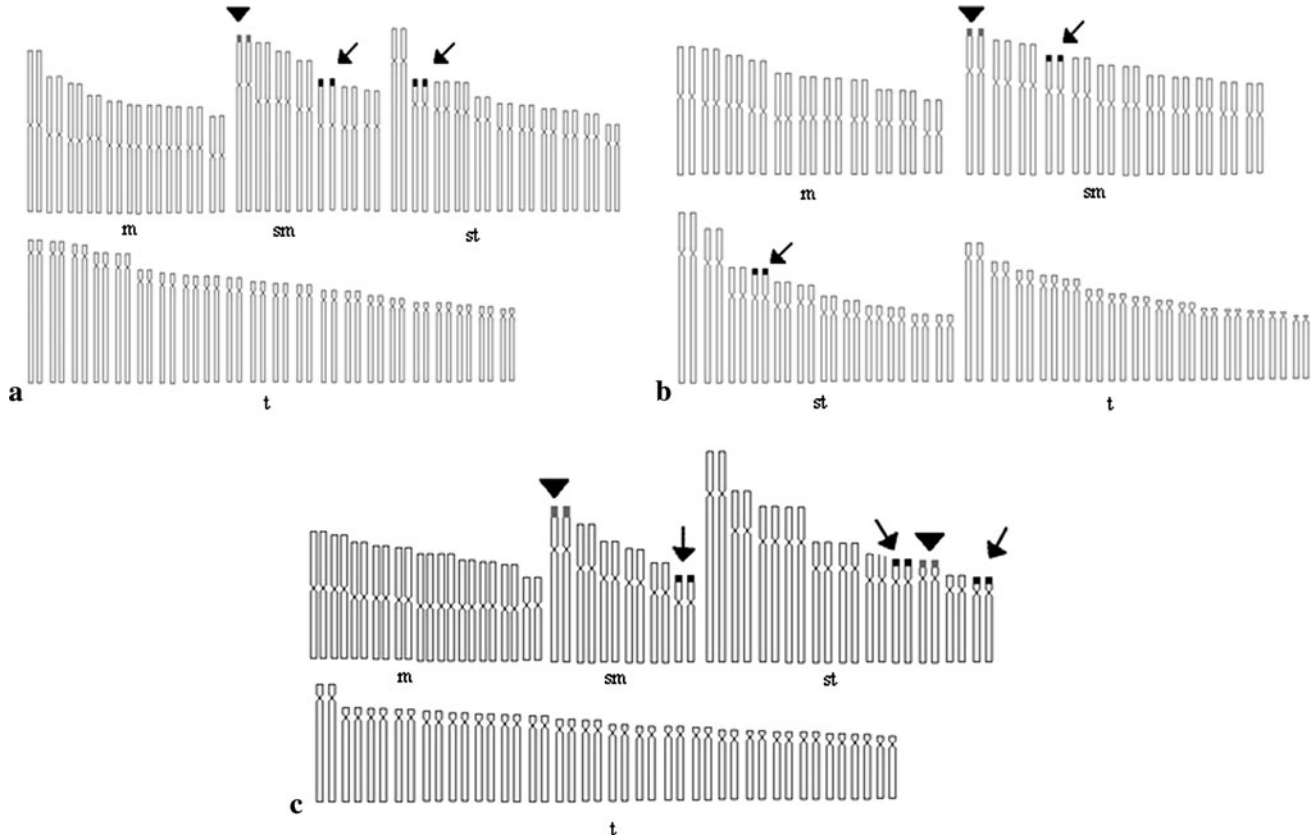

Fig. 4 Ideograms of **a** *T. khudree*, **b** *T. mussullah*, and **c** *T. mosal mahanadicus* showing 18S and 5S signals. *Arrow* indicates the 18S signals, whereas *arrow head* indicates 5S signals

Discussion

The species belonging to genus *Tor* are considered conservative in maintaining the same diploid chromosome numbers. A karyotype, with a diploid number of 100 chromosomes, consisting of 12–22 metacentric, 14–30 submetacentric, 6–44 subtelocentric and 26–60 telocentric chromosomes, is characteristic for the genus *Tor* [19, 20, 21, 23, 34, 35]. The occurrence of 100 diploid chromosome numbers in *Tor* species can be explained on the basis of polyploidization (tetraploidization) of a model diploid chromosome number of 50 [35], as also suggested by

Fig. 5 Nucleotide sequence of 18S rDNA in **a** *T. khudree*, **b** *T. mussullah*, and **c** *T. mosal mahanadicus*

a *Tor khudree*

CTCAAAGATTAAGCCATGCAGGTCTAAGTACACACGGCCGGTACAGTGAAAATGCGAATGGC
TCATTAAATCAGTTATGGTCCCTTTGATCGCTCCACCCGGTACTTGGATAACTGTGGCAATTCC
AGAGCTAATACATGCAAACGGGCGCCGACCTGCGCCCCCCCCCCGGGGGGGGTGCGGGGACGCG
TGCATTTATCAGATCCAAAACCCATCCGGGGTGTCGGGGCTCCGGCCTCGCCCCCCCGGTCCCT
TTGGTGACTCTAGATAACCTCGGGCCGATCGCGCGCCCTCCGCGGCGGCGACGATTCTTTCGA
ATGTCTGCCCTATCAACTTTCGATGGTACTTTAGGCGCCTACCATGGTGACCACGGGTAACGG
GGAATCAGGGTTCGATTCCGGAGAGGGAGCCTGAAAAACGGCTACCACATCCAAGGAAGGCA
GCAGGCGCGCAAATTACCCATTTCCGACTCGGAGAGGTAGTGACGAAAAATAACAATACAGG
TCTCTTTCGAGGCCCTGTAATTGGAATGAGCGTATCCTAAACCCATGGGCGAGGACCCATTGG
AGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGCT
GCAGTTAAAAAGCTCGTAGTTGGATCTCGGGAGTGGGCTGGCGGTCCGCCGCGAGGCGAGCC
ACCGCCTGTCCCGGACCCTGCCTCCCGGCGCCCCCCGGATGCCCTTAGCTGGGTGTCCGGTCA
CCCAAAGGGGCCCGGAGCGTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCCGCCCGTC
GCCGCTGAATACCGCAGCTAGGAATAATGGAATAGGACTCCGGTTCTATTTTGTGGGTTTCTG
GAACCCGGAGCCATGATTAAGAGGGACGGCCGGGGGCATTCGTATTGCGCCGCTAGAGGTGA
AATTCTTGGACCGGCGCAAGACGGACGAAAGCGAAAGCATTTGCCAAGAATGTTTTCATTAAT
CAAGAACGAAAGTCGGAGGTTCGAAGACGATCAGATACCGTCGTAGTTCCGACCGTAAACGA
TGCCGACCCGCGATCCGGCGGCGTTATTCCCATGACCCGCCGGGCAGCGTGCGGGAAACCAC
GAGTCTTTGGGTTCCGGGGGGAGTATGGTTGCAAAGCTGAAACTTAAAGGAATTGACGGAAG
GGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAACCTCACCCGGCC
CGGACACGGAAAGGATTGACAGATTGATAGCTCTTTCTCGATTCTGTGGGTGGTGGTGCATGG
CCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTCATTCCGATAACGAACGAGACTCCGGCTTGT
TAAATAGTTACGCGGCCCCGTGCGGTCGGCGTTCAACTTCTTAGAGGAACAAGTGGCGTTCAG
CCACGCGAGATGGAGGAATAACAGGTCTGTGATGCCCTTAGATGTCCGGGGCTGCACGCGCG
CCACAATGGGCGGATCAGCGTGTGTCTACCCTGCGCCGAGAGGCGCGGGTAACCCGCTGAAC
CCCGCTCGTGATCGGGACTGGGGATTGAAACTATTTCCCATCAACGAGGAATTCCCAGTAAGC
GCGGGTCATAAGCTCGCGTTGATTAAGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACC
GATTGGATGGTTTAGTGAGGTCCTCGGATCGGCCCCGCCGGGGCTCCTCGCGGGCCCTGGCGG
AGCGCCGAGAAGACGATCAAACTTGATCATCTAGAGGAAGTAAAAGTCGTAACAAGG

b *T. mussullah*

CTCAAAGATTAAGCCATGCAGGTCTAAGAACACCCCGCCGGTACAACGGAAATGCGAATGGC
TCATTAAATCAGTTATGGTCCCTTTGATCGCTCCACCCGGTACTTGGATAACTGTGGCAATTCC
AGAGCTAATACATGCAAACGGGCGCCGACCTGCGCCCCCCCCCCGGGGGGGGTGCGGGGACGCG
TGCTTTTATCATATCCAAAACCCCTCCGGGGTGTCGGGGCTCCGGCCTCCCCCCCCGGCCCTTT
TGGGGATTCAAAAAAACCTGGGGCCAATGGGCCCCCCCCCGCGGCGGCAAAAATTTTTTAAA
ATGTCCCCCCTATCATTTTTCGATGGTTTTTTAGGCCCCCACCGTGGTGACCCCGGATTAGGGG
AAATGGGGGTTCTATTCCGAAGAGGGACCCGAAAAAACGGCTACCACATCCAAGGAAGGCAG
CAGGCGCGCAAATTACCCATTTCCGACTCGGAGAGGTAGTGACGAAAAATAACAATACAGGT
CTCTTTCGAGGCCCTGTAATTGGAATGAGCGTATCCTAAACCCATGGGCGAGGACCCATTGGA
GGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGCTG
CAGTTAAAAAGCTCGTAGTTGGATCTCGGGAGTGGGCTGGCGGTCCGCCGCGAGGCGAGCCA
CCGCCTGTCCCGGACCCTGCCTCCCGGCGCCCCCCGGATGCCCTTAGCTGGGTGTCCGGTCAC
CCAAAGGGGCCCGGAGCGTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCCGCCCGTCG
CCGCTGAATACCGCAGCTAGGAATAATGGAATAGGACTCCGGTTCTATTTTGTGGGTTTCTGG
AACCCGGAGCCATGATTAAGAGGGACGGCCGGGGGCATTCGTATTGCGCCGCTAGAGGTGAA
ATTCTTGGACGGCGCAAGACGGACGAAAGCGAAAGCATTTGCCAAGAATGTTTTCATTAATCA
AGAACGAAAGTCGGAGGTTCGAAGACGATCAGATACCGTCGTAGTTCCGACCGTAAACGATG
CCGACCCGCGATCCGGCGGCGTTATTCCCATGACCCGCCGGGCAGCGTGCGGGAAACCACGA
GTCTTTGGGTTCCGGGGGGAGTATGGTTGCAAAGCTGAAACTTAAAGGAATTGACGGAAGGG
CACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAACCTCACCCGGCCCG
GACACGGAAAGGATTGACAGATTGATAGCTCTTTCTCGATTCTGTGGGTGGTGGTGCATGGCC
GTTCTTAGTTGGTGGAGCGATTTGTCTGGTTCATTCCGATAACGAACGAGACTCCGGCTTGTTA
AATAGTTACGCGGCCCCGTGCGGTCGGCGTTCAACTTCTTAGAGGAACAAGTGGCGTTCAGCC
ACGCGAGATGGAGCAATAACAGGTCTGTGATGCCCTTAGATGTCCGGGGCTGCACGCGCGCC
ACAATGGGCGGATCAGCGTGTGTCTACCCTGCGCCGAGAGGCGCGGGTAACCCGCTGAACCC
CGCTCGTGATCGGGACTGGGGATTGAAACTATTTCCCATCAACGAGGAATTCCCAGTAAGCGC
GGGTCATAAGCTCGCGTTGATTAAGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCG
ATTGGATGGTTTAGTGAGGTCCTCGGATCGGCCCCGCCGGGGCTCCTCGCGGGCCCTGGCGGA
GCGCCGAGAAGACGATCAAACTTGATCATCTAGAGGAAGTAAAAGTCGTAACAAGG

[36, 37] after observing 50 diploid chromosome numbers in about 70% of the studied cyprinids. In this study, dual color FISH exactly localized the two rDNA families (18S and 5S rDNA) on the chromosomes of three *Tor* species. This is the first report on simultaneous chromosomal localization of both rDNA families in *T. khudree, T. mussullah* and *T. mosal mahanadicus*. Similar study was also performed in other *Tor* species, i.e. *T. chelynoides, T. progeneius, T. putitora* and *T. tor* using 18S ribosomal genes. The specimens of *T. chelynoides*, *T. putitora* and *T. progeneius* showed six

Fig. 5 continued

c *T. mosal mahanadicus*

CGGGGCCCGGTACGTGTAACTGCGAATGGCTCATTAAATCAGTTATGGTCCCTTTGATCGCTC
CACCCGGTACTTGGATAACTGTGGCAATTCCAGAGCTAATACATGCAAACGGGCGCCGACCC
GCGCCCCCCCCCGGGGGGGTGCGGGGACGCGTGCATTTATCAGATCCAAAACCCATCCGGGGTG
TCGGGGCTCCGGCCTCGCCCCCGGTCCCTTTGGTGACTCTAGATAACCTCGGGCCGATCGCGC
GCCCTCCGCGGCGGCGACGATTCTTTCGAATGTCTGCCCTATCAACTTTCGATGGTACTTTAGG
CGCCTACCATGGTGACCACGGGTAACGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCCTGA
GAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATACCGACTCGGAG
AGGTAGTGACGAAAAATAACAATACAGGTCTCTTTCGAGGCCCTGTAATTGGAATGAGCGTAT
CCTAAACCCATGGGCGAGGACCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTC
CAGCTCCAATAGCGTATATTAAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATCTCGGGAGT
GGGCTGGCGGTCCGCCGCGAGGCGAGCCACCGCCTGTCCCGGACCCTGCCTCCCGGCGCCCCC
CGGATGCCCTTAGCTGGGTGTCCGGTCACCCAAAGGGGCCCGGAGCGTTTACTTTGAAAAAAT
TAGAGTGTTCAAAGCAGGCCGCCCGTCGCCGCTGAATACCGCAGCTAGGAATAATGGAATAG
GACTCCGGTTCTATTTTGTGGGTTTCTGGAACCCGGAGCCATGATTAAGAGGGACGGCCGGGG
GCATTCGTATTGCGCCGCTAGAGGTGAAATTCTTGGACCGGCGCAAGACGGACGAAAGCGAA
AGCATTTGCCAAGAATGTTTTCATTAATCAAGAACGAAAGTCGGAGGTTCGAAGACGATCAG
ATACCGTCGTAGTTCCGACCGTAAACGATGCCGACCCGCGATCCGGCGGCGTTATTCCCATGA
CCCGCCGGGCAGCGTGCGGGAAACCACGAGTCTTTGGGTTCCGGGGGGAGTATGGTTGCAAA
GCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTG
ACTCAACACGGGAAACCTCACCCGGCCCGGACACGGAAAGGATTGACAGATTGATAGCTCTT
TCTCGATTCTGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTCAT
TCCGATAACGAACGAGACTCCGGCTTGTTAAATAGTTACGCGGCCCCGTGCGGTCGGCGTTCA
ACTTCTTAGAGGAACAAGTGGCGTTCAGCCACGCGAGATGGAGGAATAACAGGTCTGTGATG
CCCTTAGATGTCCGGGGCTGCACGCGCGCCACAATGGGCGGATCAGCGTGTGTCTACCCTGCG
CCGAGAGGCGCGGGTAACCCGCTGAACCCCGCTCGTGATCGGGACTGGGGATTGAAACTATT
TCCCATCAACGAGGAATTCCCAGTAAGCGCGGGTCATAAGCTCGCGTTGATTAAGTCCCTGCC
CTTTGTACACACCGCCCGTCGCTACTACCGATTGGATGGTTTAGTGAGGTCCTCGGATCGGCC
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GAGGAAGTAAAAGTCGTAACAAGG

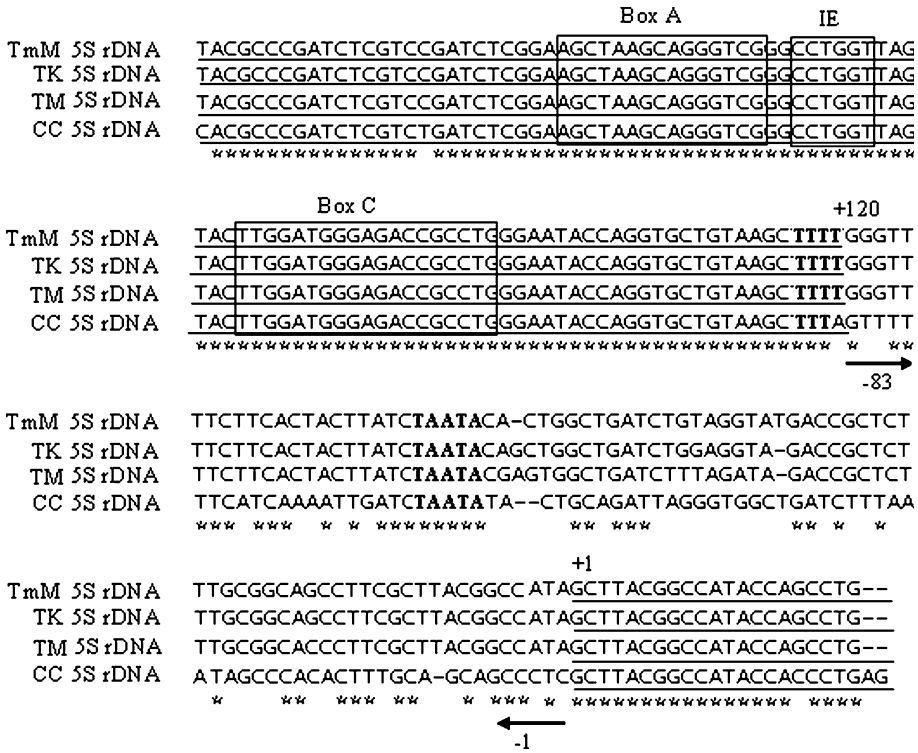

Fig. 6 Aligned nucleotide sequences of 5S rDNA in: *T. mosal mahanadicus* (5S rDNA TmM), *T. khudree* (5S rDNA TK), *T. mussullah* (5S rDNA TM) and *Cyprinus carpio* (5S rDNA CC). The 5S rDNA sequence of *C. carpio* was taken from GenBank Accession No. AB015590. The coding sequences are *underlined* with conserved Box A, Box C and IE element. TTTT and TATA like elements are indicated in *bold characters*

bright fluorescent signals of 18S rDNA, whereas *T. tor* exhibited ten such signals [24]. In these species of *Tor,* NORs signals were also characterized by silver and fluorescence (CMA_3) staining. The result of this technique encourages the molecular karyotyping in all organisms.

The 5S rDNA loci were observed on largest submetacentric chromosome in *T. khudree*, *T. mussullah* and

T. mosal mahanadicus. There was no variation observed in number and position of 5S rDNA loci in *T. khudree* and *T. mussullah*, while one additional 5S rDNA signal was examined on subtelocentric chromosomes in *T. mosal mahanadicus*. Singh et al. [24] reported localization of 5S rDNA sites on single pair of chromosomes of four *Tor* species. It may be a general rule for *Tor* species. Generally, the 5S rDNA clusters is located on a single chromosome pair in other fish species, i.e., *Salmo salar* [38], *Anguilla anguilla* [8], *Salvelinus fontinalis* [39], but this rDNA cistrons has also been present in several chromosomes in *Oncorhynchus mykiss* [29], *Oncorhynchus masou*, *Hucho perryi*, *S. fontinalis* [7], *Leporinus* [40] and *Thymallus thymallus* [41]. Besides, the 45S and 5S rDNA loci may be syntenic [29]. Martins and Wasko [42] suggested that the 5S rDNA clusters in fishes are most commonly located at interstitial chromosome site and this interstitial position is optimal for its organization in fishes, since it has been found in most species of several orders. Our findings in *Tor* species are in clear agreement with several studies in fishes that suggested common conservation pattern of 5S rDNA number and location found between closely related fish species [9, 43, 44]. The simultaneous detection of the rDNA in *Tor* species demonstrated unambiguously that 18S and 5S rDNA are not syntenic. In the present FISH study, polymorphism in the number and location of major rDNA loci was apparent and they are present on different chromosomes. Martins and Galetti [9] 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. Although, the 5S rDNA may be linked to the units of other tandemly repeating gene families such as histone genes [45], as reported in some crustaceans like *Artemia* spp. [46] and *Asellus aquaticus* [47], but it seems unlikely in the studied *Tor* species. The size of 5S rDNA unit suggests that no other sequences are included in a repeat, apart from its coding and NTS regions. The chromosomal location of the 5S rDNA clusters that observed in *T. mosal mahanadicus* is not a common feature in the genus *Tor*. This finding suggests 5S rDNA marker can be used in germplasm conservation.

The present study analyzed 18S and 5S rDNA sequences in three species of genus *Tor*. Highly close sequence identity of 18S rDNA was observed among *T. khudree, T. mussullah* and *T. mosal mahanadicus*. These sequences showed maximum homology with fishes (e.g. other *Tor* species, *Cyprinus carpio*, *Danio rerio*) and other organisms like *Xenopus* species, gastropods as well as humans (www.ncbi.nlm.nih.gov). Little variations were observed in 18S rDNA sequences due to addition, deletion and substitution in these *Tor* species. At the molecular level, this is reflected in the surprising sequence conservation and the universal secondary structure of certain regions of 28S and 18S rRNAs. Several regions of 18S and 28S rRNAs, such as those interacting with 5S and 5.8S rRNAs are the most conserved. Another reason for conservation is that these domains possibly cooperate during maturation of pre rRNA yielding 18S and 28S products [48].

Several authors reported ribosomal genes are GC rich and stain with fluorescence dye CMA_3 in many vertebrates including fish [49–51]. Richness of GC content (57%) was observed in these species of *Tor*. Moreover, high GC content is responsible for strong and stable secondary structure of ribosomal genes and also plays an important role in evolution of organisms. Tang and Tseng [52] reported a GC-rich sequence within the 5′ untranslated region of human basonuclin mRNA. The ability of this GC-rich sequence to form a large and stable secondary structure was suggested by experimental results from primer extension, RNAse resistance, and computer analysis of the sequence. To conclude, the regions coding for mature rRNAs are highly conserved among all organisms, which reflects conservation of the entire translation system.

The 5S rRNA is transcribed by RNA polymerase III and the three elements, i.e. Box A, Box C and IE, act as a promoter for expression of the gene [53]. A T-rich (TTTT) sequence was also identified at the 3′ end of 5S rDNA in *Oreochromis niloticus* [54] and *Xenopus* [55] that act as a termination signal found in a variety of other genes transcribed by RNA polymerase III [4, 56, 57].

Generally, the 5S rDNA coding sequence is highly conserved even between non-related species, the variation in the NTS owing to insertions-deletions, substitutions and pseudogenes have been frequently characterized in coregonid and tilapia fish [54, 58]. Similarly, the coding region of 5S rDNA showed 100% homology in three species of *Tor*. The slight differences were observed between NTS region of the subject species and those of other fish, indicating that this spacer region evolves rapidly. The reports has shown that the smallest size of NTS sequence of 5S rDNA, so far described in eukaryotes including fishes, is 62 bp [42, 44] and 56–67 bp [59]. The NTS region also contained TATA like motif sequences in all the three species. Moreover, 81 bp long NTS region of *T. chelynoides, T. progeneius, T. putitora* and *T. tor* also contained TATA like motif sequences [24]. This motif sequence has also been found in 5S rDNA region in *Lebias fasciata* that might play an important role in the regulation of gene expression [11]. Presumably, NTS regions are 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

[60]. This finding also suggests that NTS region may be useful in molecular discrimination of these closely related *Tor* species as observed in other related fish species [61–63].

The active process of genomic changes at different levels of chromosomal organization leads to a strong intra- and inter-specific as well as intra- and inter-population variability. This points the need for more extensive research; especially on the species with wide geographical distribution, possibly through integrated and complementary approaches to evaluate degrees of genetic exchange among species and populations. In this respect, intra-specific and intra-population cytogenetic polymorphism can have implications for stock identity. This will help in the fisheries management that is getting difficult due to over exploitation of fish stocks and different anthropogenic activities resulting into reduction of genetic resources and variations. Molecular cytogenetic based markers offer investigation of population structure and provide scientific data necessary to protect weaker populations and finally long term management of endangered fisheries resources. The FISH technique is now more suitable for major advances in the cytogenetics of fishes at the molecular level. Moderately repetitive ribosomal genes (rDNAs) were localized in these fish species and may be used as chromosome-, species- or population-specific probes for identification and conservation of *Tor* species. Similarly, these types of probes can be used as genome markers for examining inter-specific hybrids. Physical mapping of single copy genes, microsatellite loci and syntenic gene groups on chromosomes is now possible with FISH techniques and will be useful in isolating important quantitative trait loci (QTL) in fisheries science.

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 documented in the three *Tor* species. These markers may be useful for discrimination of stocks/populations of closely related *Tor* species and their hybrids. The identification of six to ten chromosomes carrying the rDNA loci in *Tor* species from India may eventually help to understand how the duplication occurred in these tetraploid species during evolution. Studies in other organisms [64–66] suggested the presence of transposable elements adjacent to rDNA serve point for their transposition and integration into other part of the genome. Therefore, the replicative as well as the non-replicative transposition may be the reason for increase in numbers and differences in the position of NORs in these species. Further, whole chromosome duplication followed by genomic rearrangements possibly have played role in development of tetraploidy and karyomorphological differentiation among the *Tor* species. However, a deeper insight into the polyploidy condition and the evolution of *Tor* species may probably be achieved through accurate nuclear DNA measurements and molecular genetic analysis.

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