Genetica (2009) 137:245–252
DOI 10.1007/s10709-009-9367-x

Chromosomal localization of 18S and 5S rDNA using FISH in the genus *Tor* (Pisces, Cyprinidae)

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Received: 30 August 2008 / Accepted: 24 April 2009 / Published online: 9 May 2009

Abstract Dual color fluorescence in situ hybridization (FISH) was performed to study the simultaneous chromosomal localization of 18S and 5S ribosomal genes in the genus *Tor* for the first time. The 18S and 5S rDNAs in four *Tor* species were amplified, sequenced and mapped on the metaphase chromosomes. The number and distribution of 18S and 5S rDNA clusters were examined on metaphase chromosome spreads using FISH. The specimens of *T. chelynoides*, *T. putitora* and *T. progeneius* showed six bright fluorescent signals of 18S rDNA and *T. tor* exhibited ten such signals. The 5S rDNA signals were present only on one pair of chromosomes in all the four *Tor* species. Ag-NORs were observed on two pairs of chromosomes in *T. chelynoides*, *T. putitora, T. progeneius* and four pairs in *T. tor*. Comparison of the observed 18S rDNA FISH signals and Ag-NORs strongly suggested a possible inactivation of NORs localized at the telomeres of a subtelocentric and telocentric chromosome pairs in all four species. The 5S rDNA contained an identical 120 bp long coding region and 81 bp long highly divergent non-transcribed spacers in all species examined. 18S and 5S rDNA sequencing and chromosomal localization can be a useful genetic marker in species identification as well as phylogenetic and evolutionary studies.

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

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Introduction

In higher eukaryotes, ribosomal RNA genes (rDNAs) are arranged in two different families, the nucleolus forming major rDNA (45S rDNA) family transcribed by RNA polymerase I and non-nucleolus forming minor rDNA (5S rDNA) family transcribed by RNA polymerase III. The major family is composed of clusters of multiple copies of tandemly repeated units that consist of a transcribed zone with coding regions 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 minor family is composed of multiple copies and arranged in tandem arrays which comprise a highly conserved 120 bp long coding sequences with a variable non transcribed spacer (NTS). In many 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 and Galetti 2000; Ferro et al. 2001) while in some 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).

Fishes of the genus *Tor* have been reported in various water bodies of India along with some other countries of the Asian subcontinent. They represent important fishery resources and attract anglers also due to its popularity as a game fish. Because of increased anthropogenic activities, the two main species namely *T. putitora* and *T. tor* are listed under the category of endangered species, facing the high risk of extinction in the wild, and, hence, conservation measures of these fishes are badly needed in the wild (Sarkar and Srivastava 2000). Some of the classical cytogenetic techniques like karyomorphology, chromosomal distribution of heterochromatin and different banding/

staining techniques have been utilized earlier for characterization of Indian fish species including *T. putitora* and *T. tor* (Khuda-Bukhsh and Nayak 1982; Kushwaha et al. 2001; Lakra 1996). But no molecular cytogenetic data are available for *T. chelynoides*, *T. progeneius*, *T. putitora* and *T. tor*. In the present study, with the aim to develop molecular markers for *Tor* species and to examine the relationship among them, the nucleotide sequences and chromosomal localization of 18S and 5S rDNAs were analyzed in these *Tor* species. In present study, we also examined the chromosomal distribution of nucleolar organizer regions (NORs) using silver nitrate and Chromomycin A_3 (CMA_3) staining and efforts have been made for the simultaneous chromosomal localization of the major (18S) and minor (5S) ribosomal RNA genes using dual color fluorescence in situ hybridization (FISH) in *T. chelynoides*, *T. progeneius*, *T. putitora*, and *T. tor*.

Materials and methods

Specimen collection, chromosome preparations, Ag-NOR and CMA_3 staining

Live specimens of the four *Tor* species, namely *T. chelynoides*, *T. putitora*, *T. progeneius* and *T. tor* were collected from the Alakhnanda River, near Garhwal, Uttarakhand; the Satluj River, near Bilaspur, Himachal Pradesh; the Jia-Bhoreli River, near Bhalukpong, Assam and the Tons River, near Rewa, Madhya Pradesh, India, respectively. 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). NORs were stained with $AgNO_3$ according to the protocol described by Howell and Black (1980) and CMA_3 staining was performed according to Sola et al. (1992). For DNA isolation, blood samples were collected from the fish specimens.

Isolation of genomic DNA and PCR amplification

The genomic DNA was extracted from the whole blood using the standard phenol-chloroform-isoamylalcohol method described by Sambrook and Russell (2001). For amplification of partial 18S rDNA in all the four species, primers were designed from the sequence of *Cyprinus carpio* (Accession No. AF133089) available in NCBI database and a standard PCR reaction was performed using 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 µM dNTPs mix, 10 pmoles of each primer (forward: 5′TTGGTGACTCTCGATAACCTC3′ and reverse: 5′CCT TGTTACGACTTTTACTT CCTC 3′), 2U Taq DNA polymerase (Fermentas) and 50 ng of genomic DNA in a final reaction volume of 50 µl. PCR cycling conditions were: initial denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, primer extension at 72°C for 2 min; with post-cycling extension at 72°C for 10 min. Amplification of 5S rDNA were carried out separately in 50 µl of PCR reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 µM dNTPs mix, 10 pmoles of each primer (forward: 5′TACGCCCGATCTCGTCCGATC3′ and reverse: 5′CAGGCTGGTATGG CGTAA GC3′) taken from Moran et al. (1996), 2U Taq DNA polymerase (Fermentas) and 50 ng of genomic DNA. Cycling conditions for amplification were: initial denaturation at 94°C for 4 min; followed by 35 cycles of denaturation at 94°C for 45 s, primer annealing at 57°C for 45 s and primer extension at 72°C for 1 min with post-cycling extension at 72°C for 7 min. Amplified products were run on 1.5% agarose gel stained with ethidium bromide.

Cloning and sequencing

Amplified products of partial 18S rDNA were purified by Qiagen PCR purification kit, according to manufacturer's instructions, ligated with pDrive T/A cloning vector (Qiagen) and incubated overnight at 16°C. The ligated products were transformed in *E. coli* DH5α competent cells (Invitrogen). Recombinant clones were screened with the help of blue/white colony selection and confirmed by restriction digestion. Amplification of 5S rDNA generated three bands of approximately 200, 400 and 600 bp in lengths. The 200 bp band was dissected from gel, purified by Qiagen gel extraction kit according to manufacturer's instructions and re-amplified with the same 5S primers. Cloned products of partial 18S rDNA were sequenced by primer walking and 5S rDNAs were sequenced directly using a custom service.

Probe labeling

The 18S rDNA probe was labeled with tetramethylrhodamine-5-2′-deoxyuridine 5′-triphosphate (Roche) by nick translation and the 5S rDNA probe was labeled with biotin (Vector Labs) according to manufacturer's instructions.

Dual color FISH

Dual color FISH was performed to determine the co-localization of the 18S major and 5S minor rDNA clusters. About 2 or 3 days aged chromosome preparations were baked at 90°C for 1 h followed by the FISH protocol described by Winterfeld and Roser (2007), with minor modifications in post-hybridization washing at 45°C. Before detection of the biotin labeled probe, slides were incubated in 1× blocking solution (Vector Labs) at 37°C

for 30 min and 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 fluorescein avidin DCS 5 μg/ml (Vector Labs) followed by 2 rounds of signal amplification. After each step of amplification, slides were washed in 1× blocking solution, while 18S rDNA probe detection do not required any antibody conjugate. The preparations were then rinsed 2 times each for 5 min with 4× SSC/0.1% Tween 20 and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted in Vectashield mounting medium (Vector Labs). The slides were examined under Leica fluorescence microscope (Leica) with triple band filter for simultaneous visualization of the three colors, i.e. DAPI, rhodamine, and fluorescein.

Results

PCR amplification of partial 18S rDNA generated a band of 1,561 bp in *T. chelynoides*, 1,560 bp in *T. putitora*, 1,561 bp in *T. progeneius* and 1,565 bp in *T. tor* (NCBI Accession No. EU835906, EU597006, FJ13815 and EU597007). In all the four species, small differences in terms of single base substitutions, deletions or insertions were found in the nucleotide sequences. Partial 18S rDNA sequence of *T. chelynoides* showed 99% identity to the sequence of *T. putitora* and 98% identity to the sequence of *T. progeneius* and *T. tor*, whereas such an identity between *T. tor* and *T. putitora* was 98%. *T. progeneius* has 98% identity to *T. puitora* and *T. tor*. All these sequences showed 96% identity on the average to the sequences of other fish species deposited in the NCBI database.

After sequencing, the length of single repeat of the minor rDNA family was found to be 201 bp long in all the four species (NCBI Accession No. EU835907, EU621853, EU621852 and FJ598657). The nucleotide sequences of the 5S rDNA coding region among the four *Tor* species shared a highly conserved gene (100% identity) of 120 bp long which contained three elements, namely Box A, Box C and I.E, of the internal control regions (Fig. 1) that functions as a promoter for the gene (Hallenberg et al. 1994). Non-transcribed spacer (NTS) region of the minor family was reported to be 81 bp long in all the four species. The comparative analyses of 5S rDNA sequences among the four species showed 25.92% variability in the NTS region. The NTS region contained TATA box like sequences in all the species but the number of these boxes were found to be three in *T. putitora* and two in *T. chelynoides*, *T. progeneius* and *T. tor* (Fig. 1). The coding sequences of minor rDNA family in *T. chelynoides*, *T. putitora*, *T. progeneius* and *T. tor* after searches using BLASTN program (Altschul et al. 1997) showed an average sequence similarity of 95.74% with other fishes belonging to order Cypriniformes,

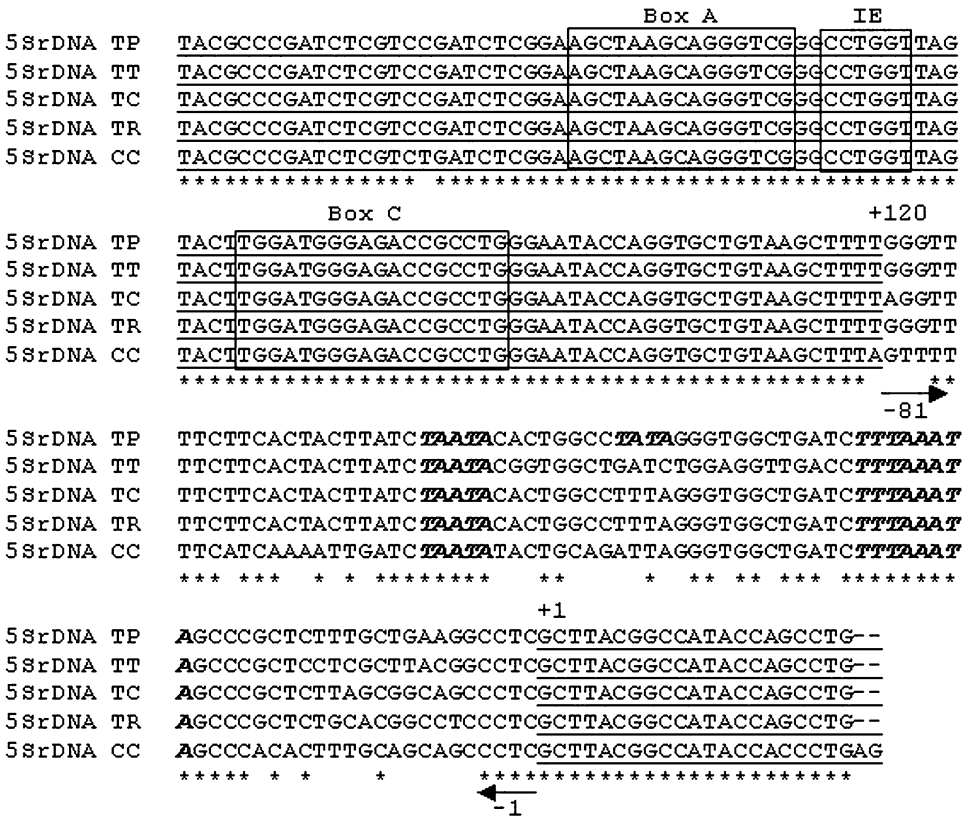

Fig. 1 Nucleotide sequences of 5S rDNA in *T. putitora* (5S rDNA TP), *T. tor* (5S rDNA TT), *T. chelynoides* (5S rDNA TC), *T. progeneius* (5S rDNA TR) and *Cyprinus carpio* (5S rDNA CC). The 5S rDNA sequence of *Cyprinus carpio* was taken from NCBI Gene bank with Accession No. AB015590. The coding sequences are *underlined* with conserved *Box A*, *Box C* and *I.E* element. TATA like elements are indicated in *bold* characters

Characiformes, Salmoniformes, Gadiformes, Perciformes and Tetradontiformes.

The karyotypes of *T. chelynoides* composed of 20 metacentric (M), 30 submetacentric (SM), 24 subtelocentric (ST) and 26 telocentric (T) chromosomes with a diploid chromosome number of 100. Similarly, the *T. tor* and *T. putitora* also possessed a diploid chromosome numbers of 100 but with different karyotypes (20M, 24SM, 24ST and 32T in *T. tor* and 12M, 22SM, 14ST and 52T chromosomes in *T. putitora*). The karyotype of *T. progeneius* contained 20M, 20SM, 20ST and 40T chromosomes with the same diploid chromosome number of 100. Conventional $AgNO_3$ staining and two color FISH revealed an interesting chromosomal pattern both in the major and minor rDNA clusters in the four *Tor* species examined. In *T. chelynoides*, *T. putitora* and *T. progeneius*, the number of Ag-NOR signals were on four (two pairs) chromosomes, whereas in *T. tor* signals were on eight chromosomes (four pairs) (Fig. 2a–d). Six fluorescent signals were detected by CMA_3 staining in the metaphase spreads of *T. chelynoides*, *T. putitora* and *T. progeneius*, whereas most of the metaphase spreads showed ten CMA_3 positive sites in *T. tor* (Fig. 3a–d). In present investigation with dual color FISH, six fluorescent signals of 18S rDNA were found in most of the metaphase spreads of *T. chelynoides* (on four STs and two Ts), *T. putitora* (on two Ms, two SMs and two Ts) and *T. progeneius* (on two Ms, two STs and two Ts). In *T. tor*, ten FISH signals (on six STs and four Ts), with heterogeneity in signal intensity, were observed (Fig. 4a–d). All the FISH signals of 18S rDNA were present on the telomeric region of the chromosomes. Comparison between profiles of FISH using 18S rDNA probe and Ag-NOR in

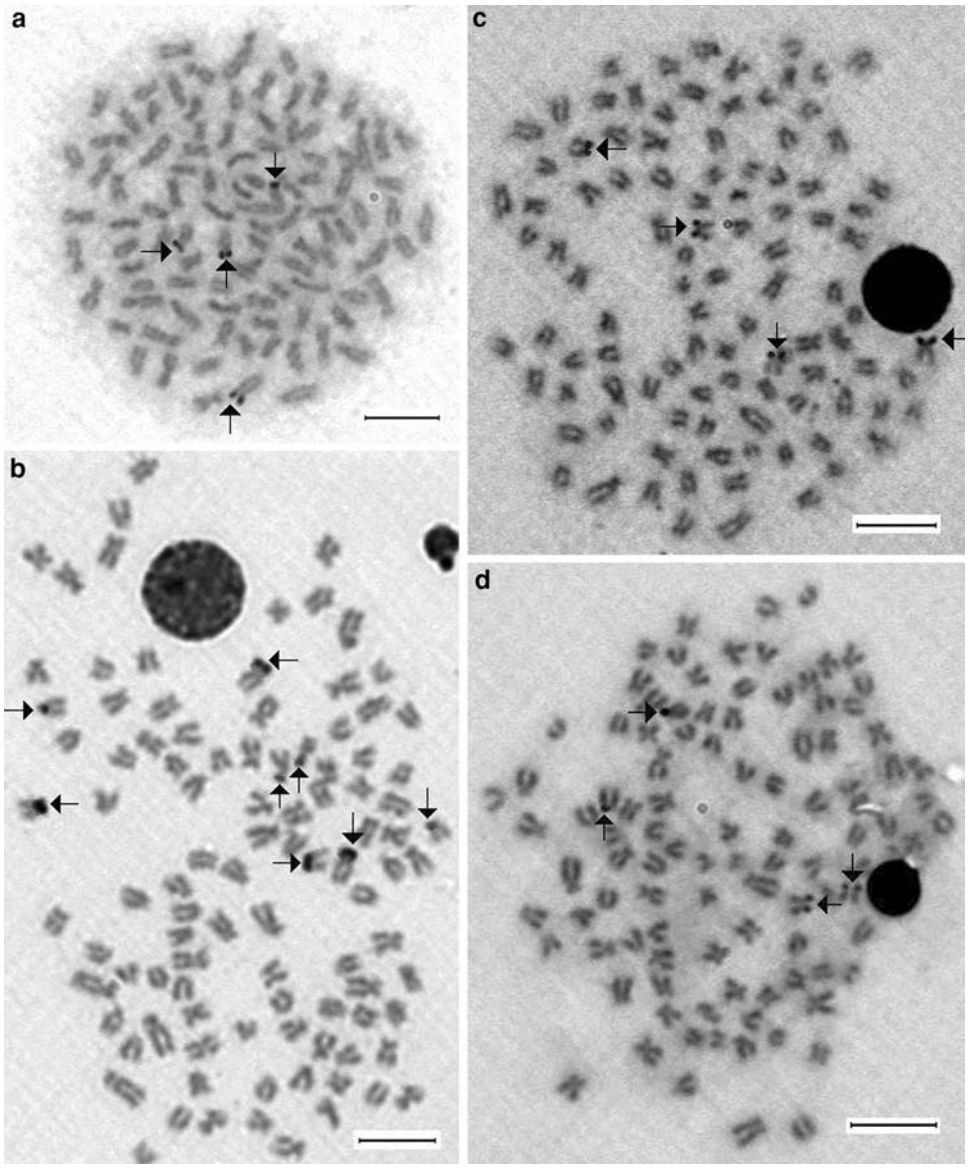

Fig. 2 Ag-NORs in metaphase spreads of **a** *T. putitora* **b** *T. tor* **c** *T. chelynoides* and **d** *T. progeneius*. *Black arrows* show Ag-NOR positive sites. *Bar* 5 μm

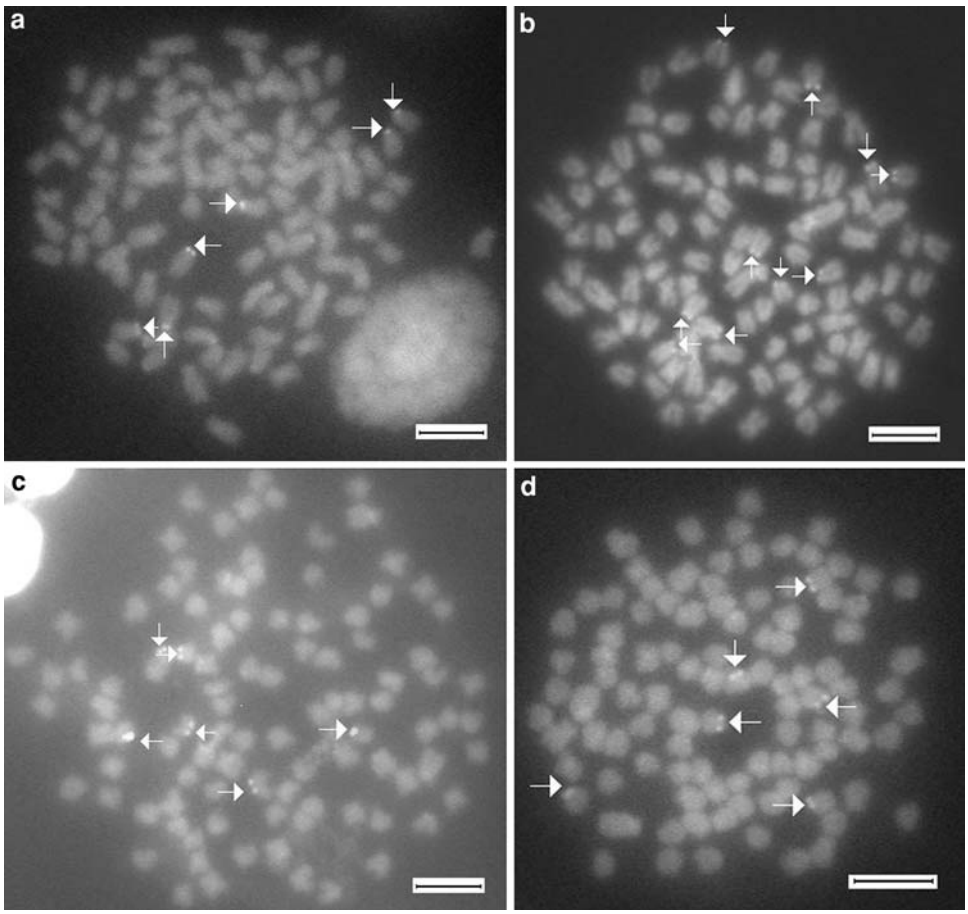

Fig. 3 CMA_3 staining of metaphase spreads of **a** *T. putitora* **b** *T. tor* **c** *T. chelynoides* and **d** *T. progeneius*. *White arrows* show CMA_3 positive sites. *Bar* 5 μm

T. chelynoides, T. putitora, T. progeneius and *T. tor* strongly suggested the possible inactivation of NORs localized at the telomeres of a ST and T pairs.

On the other hand, 5S rDNA loci were found only on single pair of chromosome in all the four species and the position was near to centromere of SMs in *T. tor*, *T. putitora* and *T. progeneius*, whereas in *T. chelynoides* it was present on telomeric region of STs (Fig. 4a–d).

Discussion

Molecular organization of 5S rDNA in cyprinid fishes is still not fully known. The coding region of 120 bp long 5S rDNA was highly conserved even in distinct taxa while the NTS region was highly variable and species specific. Moreover, it has been demonstrated that conserved sequences, which influence the transcription level of 5S rDNA in mammals, can also be located in the NTS region (Nederby-Nielson et al. 1993; Suzuki et al. 1996). Although little is known about the NTS sequences among fishes, a TATA like sequences has also been observed in NTS region of 5S rDNA in some other fishes (Pendas et al. 1994; Inafuku et al. 2000; Martins and Galetti 2000; Wasko et al. 2001; Tigano et al. 2004) that may involve in transcription of this gene. Comparison of the NTS region among selected fishes and four *Tor* species demonstrated the presence of 81 bp long NTS. Research work on several organisms has shown that the smallest length of NTS sequence of 5S rDNA so far described in eukaryotes, including fishes, is 62 bp (Martins and Wasko 2004; Santos et al. 2006).

In the present investigation, dual color FISH precisely localized the two rDNA families (45S and 5S rDNA) on the chromosomes of four *Tor* species. Results provided the accurate picture of positional and numerical variation in the rDNA location in all *Tor* species. This is the first report of simultaneous chromosomal localization of both rDNA families in *T. chelynoides*, *T. putitora*, *T. progeneius* and *T. tor*. No variation was found in the number and location of 5S rDNA loci in these species but location of 5S rDNA cluster was different in *T. chelynoides* as compared to *T. putitora*, *T. progeneius* and *T. tor*. Martins and Wasko (2004) 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

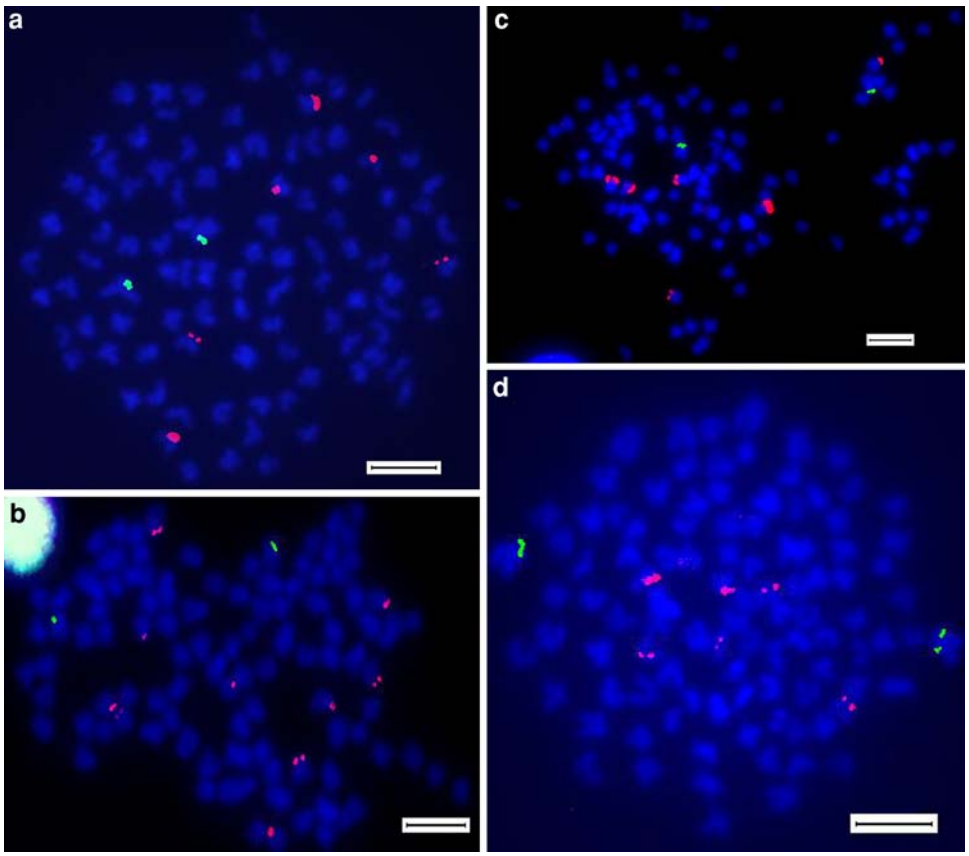

Fig. 4 Dual color FISH on metaphase spreads of **a** *T. putitora* **b** *T. tor* **c** *T. chelynoides* and **d** *T. progeneius*. The micrographs were taken with triple band filter allowing the simultaneous visualization of DAPI-stained chromosomes, the hybridization sites of 18S (*red*) and the 5S (*green*) rDNA probe. *Bar* 5 µm (color figure online)

with several studies in fishes that suggested common conservation pattern of 5S rDNA number and location found between closely related fish species (Martins and Galetti 2000; Gromicho et al. 2006; Santos et al. 2006). Even in the genus *Astyanax* (Characidae), which is reported for high rates of chromosome variations, 5S rDNA chromosome clusters are conserved among species (Almeida-Toledo et al. 2002; Mantovani et al. 2005).

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 (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.

We have observed extensive cytological variations in the number and location of major rDNA loci. Similarly, NOR polymorphism has also been reported in other species of the genus *Tor*, including *T. putitora* and *T. tor*, using Ag-NOR staining techniques (Kushwaha et al. 2001). The functional diversity/variation in the number of active NOR loci, as detected by FISH, among the four *Tor* species may possibly be explained by the altitudinal variations in their habitat, as Ferro et al. (2001), while analyzing the various populations of *Astynax scabripinnis* from different altitudes, observed higher number of active NORs in the specimens from lower altitude as compared to specimens from higher altitude. The *T. chelynoides*, *T. putitora* and *T. progeneius*, inhabitant of higher altitudes, contained two pairs of active NORs whereas *T. tor*, which inhabits lower altitudes, possessed four pair of active NORs. Polymorphisms related to transcriptional inactivation of NORs have long been described in many organisms, however, the precise etiology of such variations is not very clear. Guillén et al. (2004), while working with humans and chimpanzees, suggested rDNA elimination, DNA methylation and gene silencing due to position effects induced by heterochromatin (C-bands) and/or telomeres as three different mechanisms that produce inactivation of NORs. Cabrero and Camacho (2008) opinioned that the cryptic NORs might correspond to nascent NORs, i.e. a few rRNA gene copies moved to new locations, whereas the inactive rDNA loci might correspond to those being in the process of elimination. The abundance of silent and cryptic loci might be due to the transposition of a few rRNA genes to new chromosome locations, their amplification giving rise to new NORs, and the elimination of the old NORs.

Presence of major and minor rDNA clusters on the same chromosomes has been reported in some fishes (Pendas et al. 1994; Moran et al. 1996; Fontana et al. 2003) while

their localization on different pairs of chromosomes seems to be more common in vertebrates (Lucchini et al. 1993; Suzuki et al. 1996), including fishes (Martinez et al. 1996; Martins and Galetti 1999, 2001). In our study, we also found that both the major and minor rDNA clusters were present on different chromosome pairs. Unfortunately, little molecular and karyological information was available on the genus *Tor* for the characterization and elucidation of their evolutionary relationships. The data obtained in the present study indicated that nucleotide sequences of 18S rDNA and 5S rDNA and chromosomal localization of both rDNA families can serve as a suitable genetic marker for the evolutionary studies as well as the genetic identification of the related species of the genus *Tor*. Moreover, the use of ribosomal genes as chromosome markers has assumed importance for comparative genomic studies.

Acknowledgments The authors are thankful to the Department of Biotechnology, the Ministry of Science and Technology, Government of India for funding.

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