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Nucleotide Sequences and Chromosomal Localization of 45S and 5S rDNA in *Neolissochilus hexagonolepis* (Pisces, Cyprinidae), Using Dual-Color Fish

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Dual-color fluorescence *in situ* hybridization (FISH) was performed to study the simultaneous localization of major (45S) and minor (5S) family ribosomal RNA genes on chromosomes of *Neolissochilus hexagonolepis*. The partial 45S (18S, ITS 1, 5.8S, ITS 2 and 28S) and complete 5S (coding and NTS) rDNA units were amplified, sequenced, analyzed, and mapped on the metaphase chromosomes. The complete 18S, 5.8S and partial 28S rDNAs were 1849 bp, 157 bp and 1819 bp long, respectively. Internal transcribed spacers, namely ITS 1 (828 bp) and ITS 2 (359 bp), showed significant nucleotide variations from other fish species listed in NCBI database. The 5S rDNA contained an identical coding region of 120 bp and a highly divergent, non-transcribed 81-bp spacer. The specimens of *N. hexagonolepis* showed six bright fluorescent signals of 18S, while the 5S signals were present only on one pair of chromosomes. Subsequent analyses between conventional Ag-NORs and 18S rDNA FISH strongly suggested the possible inactivation of one pair of NORs that was localized at a telomeric position of a submetacentric chromosome. The sequencing and chromosomal localization of 45S and 5S rDNAs may serve as a useful genetic marker in taxonomic classification as well as in phylogenetic and evolutionary studies.

Key words: 18S, 5S, FISH, rDNA, chocolate mahseer

INTRODUCTION

In higher eukaryotes, ribosomal RNA genes (rDNAs) are arranged in two families, the nucleolus-forming major rDNA (45S) family transcribed by RNA polymerase I, and the non-nucleolus forming minor rDNA (5S) 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 of 18S, 5.8S, and 28S rRNA genes separated by internal transcribed spacers (ITS 1 and ITS 2) and surrounded by intergenic spacer (IGS). The IGS contains non transcribed spacer (NTS) sequences that is surrounded by 5' and 3' external transcribed spacers (ETS) in which 5' ETS is present at upstream of 18S rDNA and 3' ETS at downstream of 28S rDNA (Long and David 1980; Pendas et al., 1993). The minor family is composed of multiple copies arranged in tandem arrays, which comprise highly conserved, 120-bp coding sequences with a variable non-transcribed spacer (NTS). Each rRNA gene is organized into several divergent domains, also called expansion segments, interspersed between slowly evolving and, therefore, highly conserved cores (Hassouna et al., 1984). The evolution through insertion-deletion and/or substitution in the expansion domains and the cores of nuclear rRNA molecules (particularly 28S) make these genes useful for phylogenetic analyses. In many fish species, the chromosomal 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).

Neolissochilus hexagonolepis (McClelland), a freshwater fish of order: Cypriniformes and family: Cyprinidae commonly known as chocolate mahseer, have been reported from various water bodies in India along with some other

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countries of the Asian subcontinent, and represent important fishery resources (www.fishbase.org). It is an attractive sport fish having high economic value and aquaculture potential. In developing strategies for aquaculture and propagation assisted rehabilitation of chocolate mahseer fishery, there is a need to generate genetic information about the species. Currently, more than 20 species and subspecies are recognized in genus *Neolissochilus* (Eschmeyer et al., 2004; http://zipcodezoo.com/Animals/N/Neolissochilus_hexagonolepis/, version 2010) but there is still much confusion with regard to the taxonomy, systematics, and uniformity in identification of this genus. Because of increased anthropogenic activities, this species is listed under the category of endangered species facing the high risk of extinction in the wild and conservation measures for this fish is desperately needed in the wild (www.fishbase.org). Some of the classical cytogenetic techniques like karyomorphology, chromosome distribution of heterochromatin and different banding/staining techniques (C-, G-, NORs, CMA_3 etc.) have been utilized previously in the characterization of Indian fish species (Khuda-Bukhsh and Nayak, 1982; Kushwaha et al., 2001; Lakra, 1996; Sahoo et al., 2007), but no molecular cytogenetic data are available for *N. hexagonolepis*. Within the framework of the research on macroevolutionary processes in this previously uncharacterized species, we performed nucleotide sequence, simultaneous localization of 45S and 5S rDNAs by FISH, and characterization of genomic organization of 5S rRNA repeats by southern blot hybridization in the present study.

MATERIAL AND METHODS

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

Live specimens of *N. hexagonolepis* (n = 8) were collected from tributaries of Umiam Reservoir near Shillong in the Meghalaya state in India with the help of local fishermen. The specimens were at the juvenile stage, and the sex was unidentifiable by visual examination. Metaphase spreads were prepared from anterior kidney cells using conventional hypotonic treatment, acetic acid-methanol fixation and flame-drying technique (Bertollo et al., 1978). Nucleolar organizer regions (NORs) were stained with $AgNO_3$, according to the protocol described by Howell and Black (1980) and with chromomycin A_3 (CMA_3), according to Sola et al. (1992). Blood samples were collected from the specimens for DNA isolation.

Isolation of genomic DNA and PCR amplification

The genomic DNA was extracted from whole blood using standard phenol: chloroform: isoamylalcohol method, as described by Sambrook and Russell (2001). Primers for the amplification of 18S, ITS 1, 5.8S and ITS 2 were designed from the sequence of *Cyprinus carpio* (Accession No. AF133089) available in the NCBI database; primers for 28S rDNA were taken from Zardoya and Meyer (1996), and for 5S rDNA from Moran et al. (1996). The primers used in the present study are listed in Table 1. Standard PCR reactions were performed separately using 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM dNTPs mix, 10 pmols of each primer for all regions, 2U Taq DNA polymerase (Fermentas) and 50 ng of genomic DNA in a final reaction volume of 50 μl. The PCR cycling conditions were as follows: initial denaturation at 94°C for 4–5 min; followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 40–60 sec, primer extension at 72°C for 1–2 min; with final extension at 72°C for 10 min. Amplified products were run on 1.5% agarose gel stained with ethidium bromide.

Cloning and sequencing

Amplified products of 18S, ITS 1, and ITS 2 were purified using Qiagen PCR purification kit, according to manufacturer 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 length. The 200 bp band was sliced from the gel, purified by Qiagen gel extraction kit according to manufacturer instructions, and re-amplified with the same 5S primers. Cloned products of 18S, ITS 1, and ITS 2 were sequenced by primer walking, while 5.8S, 5S and domains of 28S were sequenced directly using a custom service. DNA sequencing was performed using the ABI Prism Big Dye Terminator version 3.0 or version 1.0 (nucleotides _280 to _1348 of the promoter) sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3100 machine. Sequences of 5S rDNA of *N. hexagonolepis* and other closely related species were aligned using ClustalW version 1.4 with default settings (Thompson et al., 1994). Determination of the nucleotide composition, i.e. G + C and A + T, of each fragment, was performed with the help of BioEdit version 7.0.5.

Probe labeling

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

Dual-color fluorescence in-situ hybridization

Dual -color FISH was performed to determine the localization of 18S major and 5S minor rDNA clusters. Two- or three-day-old

Table 1. List of primers used in the present study.

S. No.	Primer code	5'-3' sequence	Product size	Region
1	F1	AGCATTATGCTTGTCTCAAAGAT	1849 bp	18S complete
2	R1	CCTTGTTACGACTTTTACTTCCTC		
3	F2	TAGGTGAACCTGCGGAAGGATCATTA	828 bp	ITS1 complete
4	R2	CGAGTGATCCACCGCTAAGAGTTG		
5	F3	TACAACTCTTAGCGGTGGATCA	157 bp	5.8S complete
6	R3	AGCGACCCTCAGACAGGCGTGG		
7	F4	CACTTTGCGGCCCCGGGTTCCT	359 bp	ITS2 complete
8	R4	CCTCTTACCGGTTTCACGCCCT		
9	F5	CCCGCTGAATTTAAGCATATAAGTAAGCGG	397 bp	28S partial
10	R5	AACGGTTTCACGCCCTCTTGAACT		
11	F6	AAGTGGAGAAGGGTTCCATGTGA	500 bp	28S partial
12	R6	AGAGCCAATCCTTATCCCGAAGTT		
13	F7	CGGCGGGAGTAACTATGACTCTCTTAAGGT	410 bp	28S partial
14	R7	CCGCCCCAGCCAAACTCCCCA		
15	F8	TGAAATACCACTACTCTTATCGTT	671 bp	28S partial
16	R8	GGATTCTGACTTAGAGGCGTTCAG		
17	F9	TACGCCCGATCTCGTCCGATC	201 bp	Complete 5S coding & NTS
18	R9	CAGGCTGGTATGGCCGTAAGC		

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. Prior to detection of biotin-labeled probes, slides were incubated in 1X blocking solution (Vector Labs) at 37°C for 30 min and detection reagents were diluted in 1X blocking solution for approximately 30 min before use to further reduce any non-specific binding. Detection of biotin-labeled probes was carried out with fluorescein avidin DCS 5 μg/ml (Vector Labs) followed by two rounds of signal amplification. After each step of amplification, slides were washed in 1X blocking solution while 18S probe detection did not require any antibody conjugate. The preparations were then rinsed two times, each for 5 min with 4X SSC/0.1% Tween 20, and counterstained with DAPI and mounted on a Vectashield mounting medium (Vector Labs). Slides were examined under a fluorescence microscope (Leica) with triple band filter for simultaneous visualization of three colors, i.e. DAPI, rhodamine, and fluorescein.

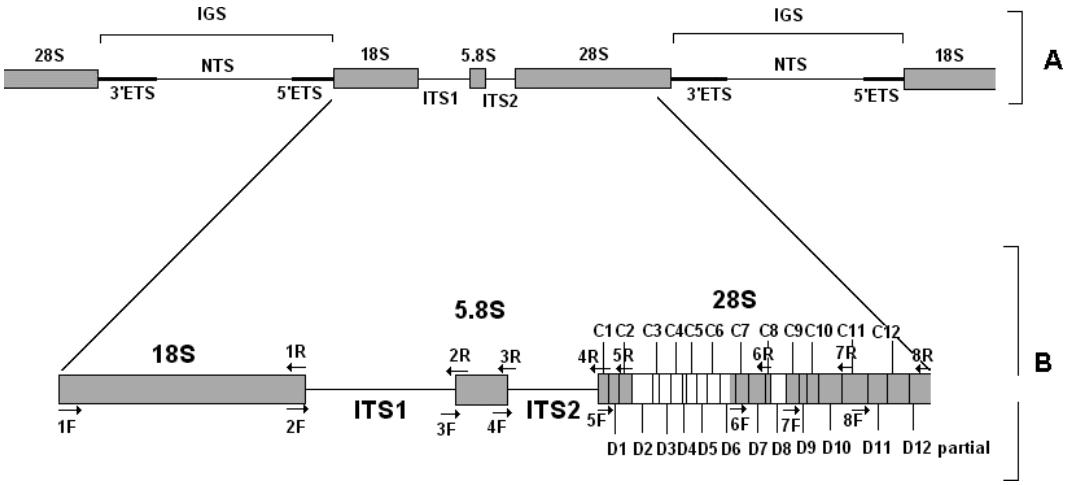

Fig. 1. Diagrammatic representation of 45S rDNA repeat unit. **(A)** shows the arrangement of 45S rDNA unit and **(B)** shows the regions that are sequenced in *N. hexagonolepis*. Primer annealing sites are also shown in the diagram. White blocks in 28S rRNA gene in part B shows the gaps or non-sequenced regions. This diagrammatic presentation is not to scale.

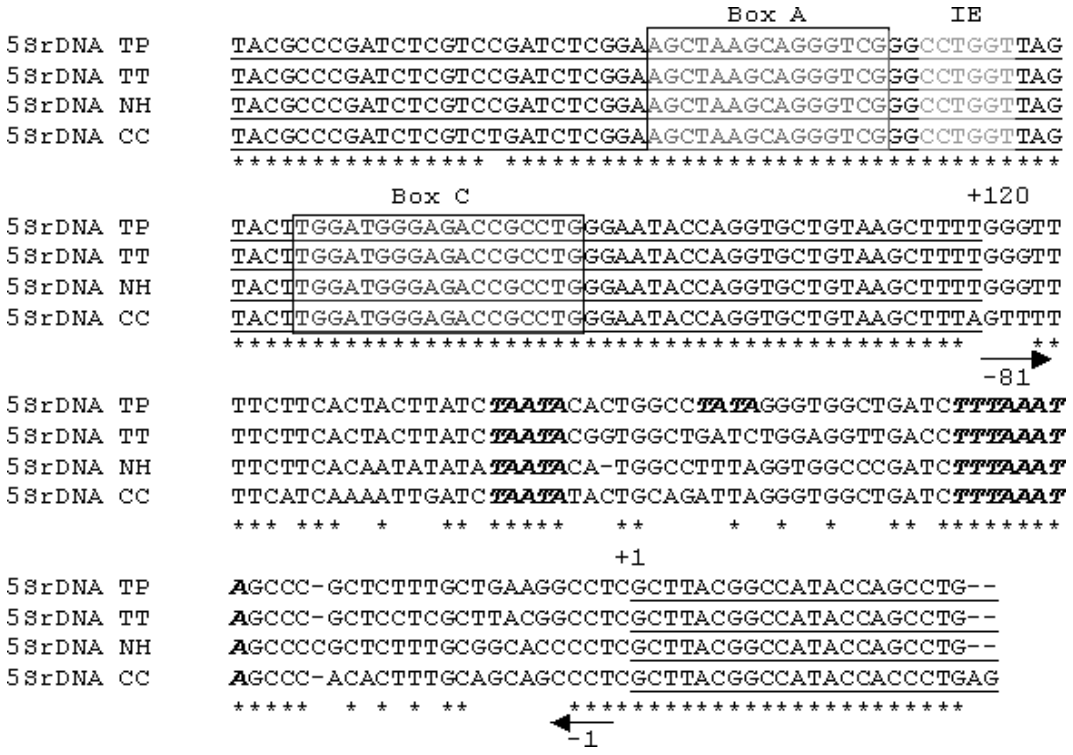

Fig. 2. Nucleotide sequences of 5S rDNA in *Tor putitora* (5S rDNA TP), *T. tor* (5S rDNA TT), *N. hexagonolepis* (5S rDNA NH) and *Cyprinus carpio* (5S rDNA CC). The 5S rDNA sequences of *T. putitora, T. tor,* and *C. carpio* were taken from NCBI Database with Accession Nos. EU621853, EU621852 and AF133089, respectively. The coding sequences are underlined. Box A and Box C sequences are indicated in red and blue colors, respectively, while IE are indicated in green color. TATA-like elements are indicated in bold italic characters.

Southern blot hybridization

The genomic organization of 5S rDNA was determined by Southern blot hybridization. Genomic DNA (10 μg) of *N. hexagonolepis* was partially digested with *Hin*dIII at 37°C for 30 min and complete digestion was performed overnight. Partially to completely digested DNA was subjected to electrophoresis in 1% agarose gel and transferred to a positively charged nylon membrane, as described by Sambrook and Russell (2001). The probe of the 5S rRNA gene of *N. hexagonolepis* was labeled with biotin, following the manufacturer's instructions (Vector Labs). Hybridization of filter immobilized DNA and post hybridization washes were performed according to Sambrook and Russell (2001). Hybridized DNA was detected by BCIP/NBT substrate kit (Vectors labs).

RESULTS

Nucleotide sequencing of both strands (forward and reverse) was performed in six individuals, and a single clone per specimen was analyzed. An 1849-bp 18S rDNA was identified in *N. hexagonolepis* (NCBI Accession GU568380), and the overall base composition of the 18S gene is characterized by the over-representation of G (29.80%) and under-representation of T (21.20%), whereas A (22.44%) and C (26.56%) distributions were median. Small differences in terms of single base substitutions, deletions or insertions were found in the nucleotide sequences when compared with other cyprinid sequences, and an average similarity of 96% to other fish species listed in NCBI database was observed. The ITS 1 was 828 bp long (NCBI Accession GU568380) and in terms of nucleotide composition, the T base (14.39%) was lowest followed by almost equal distributions of G (27.35%), A (28.78%) and C (29.49%). In this fish, ITS 1 is also characterized by the presence of a 36-bp stretch of AAAG repeats (9 times). The 5.8S rRNA gene is 157 bp (NCBI Accession GU568380) and GC rich (56.05%). The ITS 2 is a 359-bp (NCBI Accession FJ189513), GC-rich (66.85%) sequence, but showed an under-representation of A (13.09%). We also sequenced a 1819-bp partial 28S rRNA gene (NCBI Accessions FJ189518, FJ598649, FJ598650, FJ598651) that contained several conserved (C1, C2, C7, C9, C10, C11) and divergent (D1, partial D6, D7, D9, D10, D11, and partial D12) domains. The overall base composition was A-23.03%, C-25.07%, G-32.33%, and T-19.57%. The divergent domains of 28S are characterized by a slight bias against T and for high GC content. The arrangement of the major rRNA genes 18S, 5.8S and 28S, and spacers ITS 1 and ITS 2 with primer annealing sites are presented in Fig. 1.

After sequencing, the length of single repeat of a minor rDNA family was found to be 201 bp (NCBI Accession FJ598653) in *N. hexagonolepis*. The coding region of 5S shared a highly conserved gene of 120-bp sequence containing three elements, Box A, Box C, and IE, of the internal

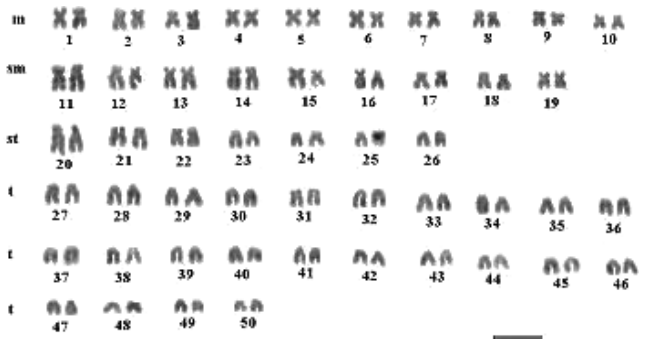

Fig. 3. Giemsa stained karyotype of *N. hexagonolepis* (m = metacentric, sm = submetacentric, st = subtelocentric and t = telocentric). Bar: 5 μm.

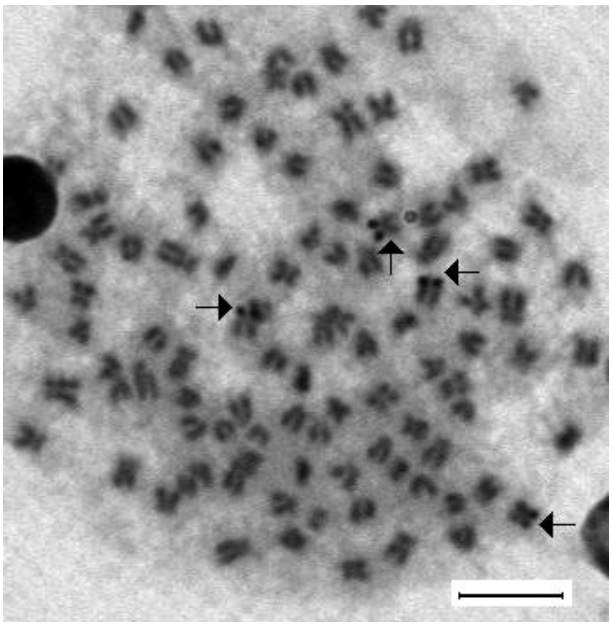

Fig. 4. Ag-NOR staining of metaphase spreads of *N. hexagonolepis*. Black arrows in micrograph show Ag-NOR positive sites. Bar: 5 μm.

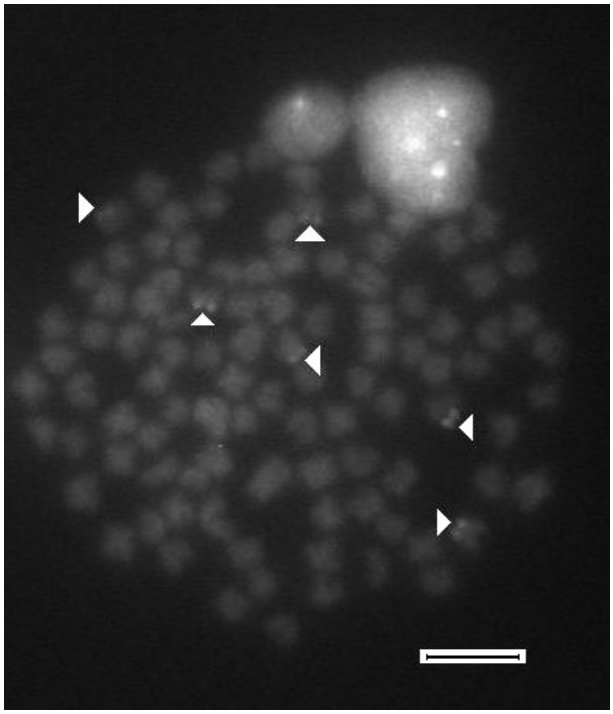

Fig. 5. Chromomycin A_3 (CMA_3) staining of metaphase spreads of *N. hexagonolepis*. White arrows heads in micrograph show CMA_3 positive sites. Bar: 5 μm.

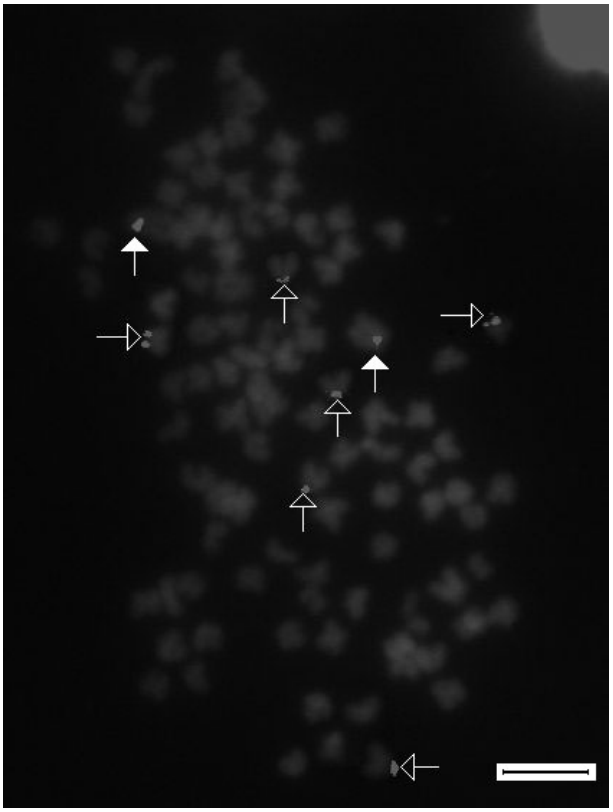

Fig. 6. Dual-color FISH on metaphase spread of *N. hexagonolepis*. The micrograph was taken with triple band filter allowing the simultaneous visualization of DAPI-stained chromosomes, the hybridization sites of 18S (red) and the 5S (green) rDNA probe. Bar: 5 μm.

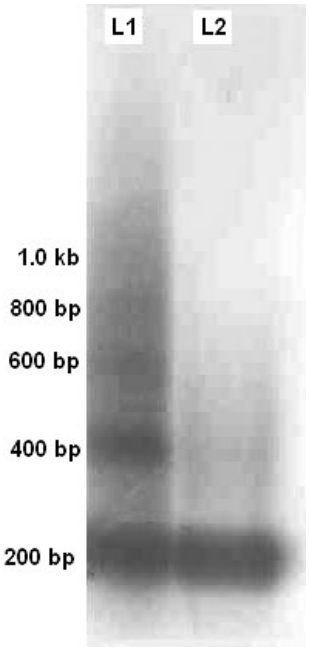

Fig. 7. Genomic organization of 5S rDNA in *N. hexagonolepis* determined by Southern blot hybridization. 10 μg of genomic DNA were partially digested (30 min) with 80 U of the restriction endonuclease *HindIII* in L1 and completely digested (overnight) in L2.

control regions (Fig. 2) that functions as a promoter for the gene (Hallenberg et al., 1994). Non-transcribed spacer (NTS) region of 5S was 81 bp, containing two TATA box-like sequences (Fig. 2). We also compared the nucleotide sequence of 5S rDNA of *N. hexagonolepis* with other closely related species, namely *Tor tor* (tor mahseer), *T. putitora* (golden mahseer), and *C. carpio.* The coding region of 5S showed 100% similarity but NTS showed 19.75% variability with other three species. The coding sequence of minor rDNA family in *N. hexagonolepis*, after searches using BLASTN program (Altschul et al., 1997), showed an average sequence similarity of 95.74% with other fishes belonging to orders Cypriniformes, Characiformes, Salmoniformes, Gadiformes, Perciformes and Tetradontiformes, while NTS showed extensive variations.

Ten giemsa (6%) stained plates per individual (~150 metaphase spreads per individuals) were analyzed for karyotyping. The karyotypes of *N. hexagonolepis* composed of 20 metacentric, 18 submetacentric, 14 subtelocentric, and 48 telocentric chromosomes with 100 diploid chromosome number (Fig. 3). Chromosome staining and FISH were performed in all the eight individuals studied. Ten plates in each individual (~150 metaphase spreads per individual) were analyzed to obtain the number and distribution of NORs and CMA_3. In order to establish the Ag-NOR- and CMA_3-positive chromosome correspondence among plates within individuals and among individuals, the ratio of the short arm (p) to the long arm (q) was used to classify the morphology of the chromosome (i.e. metacentric, submetacentric, subtelocentric and acrocentric) and subsequently the chromosomes were arranged according to their morphology and size, from largest to smallest. The numbers of Ag-NOR-positive signals were present on four (two pairs) chromosomes (Fig. 4). Six fluorescent signals were detected by CMA_3 staining in all the metaphase spreads of *N. hexagonolepis* (Fig. 5). In dual-color FISH, ten 18S and 5S probe hybridized plates per individuals were screened. With FISH, six fluorescent signals of 18S rDNA were found in the metaphase spread of *N. hexagonolepis* (four at telomeric region of metacentric chromosomes and two at telomeric region of submetacentric chromosomes) with heterogeneity in signal intensity (Fig. 6). In contrast, 5S rDNA loci were found to be present only on a single pair of chromosomes and the position was near the centromere (interstitial) of a submetacentric chromosome (Fig. 6). Genomic DNA of *N. hexagonolepis* was partially digested with *Hin*dIII, which only cleaves once in the 5S rRNA gene, and then hybridized with 5S rRNA gene probe to examine the organisation of the 5S rDNA. Analysis of band patterns of partially digested DNA showed a ladder of exact integers of 201 bp while complete digestion showed only one 201-bp band after southern blot detection (Fig. 7).

DISCUSSION

This is the first report that covers approximately 5.2 kb of nucleotide sequences collectively from single unit of major and minor rDNAs in *N. hexagonolepis*. As expected, 18S showed high degree of similarity with other fishes listed in the NCBI database whereas, both the spacers (ITS 1 and ITS 2) showed great variability when compared with closely related groups of fishes. The divergent domains of 28S

rDNA are GC rich. This base composition pattern with particularly large GC-rich stems reflects their potential ability to fold into secondary structures (Vawter and Brown, 1993). Divergent domains are commonly subjected to insertion and deletion events, coupled with substitution rates, that are at least two orders of magnitude higher than that for cores (Olsen and Woese, 1993) accounting for the large overall size variation of rRNA genes among eukaryotes. A clear bias toward nucleotide substitutions (especially transitions) rather than insertions or deletions is found in highly conserved core sequences (Olsen and Woese, 1993). However, the conserved core of the molecule has an almost equal distribution of the different types of nucleotides that is correlated with the presence not only of stem loop structures but also long single-stranded A-rich regions (Gutell et al., 1985), and which are thought to interact with other rRNA subunits and ribosomal proteins (Vawter and Brown, 1993).

The molecular organization of 5S rDNA in cyprinid fishes is still not fully known. The 120-bp coding region was found to be highly conserved, even in distinct taxa, while the NTS region was highly variable and species-specific. Moreover, it has previously 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 NTS sequences in fishes, TATA-like sequences have also been observed in the NTS region of 5S in some other fishes (Pendas et al., 1994; Inafuku et al., 2000; Martins and Galetti, 2000; Wasko et al., 2001; Tigano et al., 2004), which may be involved in the transcription of this gene. An AT-rich spacer region in *N. hexagonolepis* showed clear agreement with other fish species studied, such as Atlantic salmon and bitterling (Acheilognathinae) (Sajdak et al., 1998; Fujiwara et al., 2009). The coding region showed 100% similarity, while NTS showed 19.75% variability with *T. tor*, *T. putitora* and *C. carpio* that is consistent with our previously published data on four species of mahseer, namely *T. tor, T. putitora, T. chelynoides* and *T. progeneius* with 100% similarity in coding regions and 25.92% variability in NTS between species (Singh et al., 2009).

Comparison of the NTS region of *N. hexagonolepis* with that of other selected fishes studied here demonstrated the presence of an 81-bp NTS which have the optimum conserved sequences required for the organization and/or activity of 5S rDNA, but studies on a variety of organisms have shown even shorter 5S rDNA NTS sequences. Fujiwara et al. (2009) demonstrated a conserved 120-bp sequence of 5S rRNA gene and a short 56–67-bp NTS with two distinct portions, a conserved (5'-flanking portion at positions -1 to -38) and a variable part (3'-flanking portion), in six of ten species/subspecies examined from four genera: *Acheilognathus*, *Pseudoperilampus*, *Rhodeus*, and *Tanakia*. Martins and Wasko (2004) and Santos et al. (2006) reported a 62-bp NTS in fish. Organization of 5S, examined by southern blot hybridization, showed the presence of a single type of 5S repeat in *N. hexagonolepis*. Detection of a ladder of exact integers of 201 bp in partially digested DNA provides evidence that these 5S rDNA units were organized in repeats of tandem arrays.

A marked chromosomal conservation with the presence of 100 diploid chromosomes (2n) number in mahseer species, including *N. hexagonolepis*, has been observed in the present study and by others (Sahoo et al., 2007; Mani et al., 2009). Dual-color FISH precisely localized the two rDNA families (45S and 5S) on *N. hexagonolepis* chromosomes. This is the first report of simultaneous chromosomal localization of both rDNA families in the subject species. Martins and Wasko (2004) suggested that the 5S clusters are most commonly located at interstitial chromosome sites, and, as this has also been found in most species of several orders it appears that this interstitial position is optimal for its organization in fishes. Our findings in *N. hexagonolepis* showed clear agreement with our previously published data, as well as with those from several other studies in fishes that suggested common conservation pattern of 5S rDNA number and location in closely related fish species (Singh et al., 2009a, b; Martins and Galetti, 2000; Gromicho et al., 2006; Santose et al., 2006). Even in genus *Astyanax* (characidae), which is reported to exhibit high rates of chromosome variation, 5S rDNA chromosome clusters are conserved among species (Almedia-Toledo et al., 2002; Montavani et al., 2005). In the present FISH study, major and minor rDNA clusters were located on two different chromosomes. Martins and Galatti (2000) suggested that the localization of 5S and 45S rDNA loci on different chromosomes, as observed in majority of the vertebrates, might permit them to evolve independently, as divergent evolutionary tendencies may exist in a single genome and divergent functional dynamics of these sequences required physical distancing.

Six fluorescent signals with 18S rDNA probe in *N. hexagonolepis* strongly suggested possible inactivation of NORs localized at the telomeres of a submetacentric chromosome pair. 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 human and chimpanzees, suggested rDNA elimination, DNA methylation, and gene silencing, and 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) opined that 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 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.

Unfortunately, little molecular and karyological information was available on the species *N. hexagonolepis* for the characterization and elucidation of evolutionary relationships. The data obtained in the present study indicate that nucleotide sequences of 18S, 5.8S, 28S and 5S rDNAs, as well as of non transcribed and transcribed spacers and the chromosomal localization of both rDNA families can serve as suitable genetic markers for use in evolutionary studies and the genetic identification of related species of the genus *Neolissochilus*. Moreover, the use of ribosomal genes as chromosome markers was assumed to be of importance for comparative genomic studies.

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