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Physical mapping of rRNA gene in endangered fish *Osteobrama belangeri* (Valenciennes, 1844) (Family: Cyprinidae)

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Physical mapping of the 18S ribosomal RNA gene (rDNA) was carried out by fluorescent *in situ* hybridization (FISH) in the endangered freshwater fish *O. belangeri*. The specimens were collected from Imphal valley, Manipur, India and metaphase chromosome preparation was made using standard hypotonic treatment, methanol-acetic acid fixation and flame-drying technique followed by Giemsa, silver, CMA_3 staining for complete cytogenetic characterization of the species. The diploid chromosome number was found to be 50 and the karyotype composed of 6m+16sm+12st+16t (FN=72). One active rDNA site, located on short arm of 3rd submetacentric chromosome, was mapped by FISH and confirmed by silver and CMA_3 staining. The karyomorphology, chromosomal location of rDNA loci in this species and the utility of these cytogenetic markers have been discussed in the paper.

Keywords: Ag-NORs, Chromosome, CMA_3, FISH, *Osteobrama belangeri*

Genetic characterization of fish species particularly that of threatened or economically important species is useful for planning their conservation strategies for safeguarding biodiversity. The fish species, *Osteobrama belangeri* (Valenciennes, 1844; family: Cyprinidae; order: Cypriniformes), locally known as *pengba*, is a medium carp found in rivers and lakes of India (Manipur), Myanmar and China. It is moderate to highly vulnerable and categorized as 'Not Evaluated' by FAO (www.fishbase.org), as 'Extinct in wild' in the Conservation Assessment and Management Plan (CAMP) workshop conducted at National Bureau of Fish Genetic Research, Lucknow during 1997[1], as 'Threatened'[2] and as 'Endangered'[3,4]. In the past, *Osteobrama belangeri* formed a big fishery in Loktak lake but now this species has become rare or almost exterminated there because of the introduction of common carp. This species has not been investigated genetically so far and no information is available on the karyomorphology.

Cytogenetic markers have been considered as reliable tools for characterization of fish species as well as to screen putative hybrids[5]. Some of the classical cytogenetic markers have been utilized earlier for characterization of fish species and also have application in revealing phylogenetic relationship and resolving taxonomic ambiguity among related species by comparison of chromosome morphology and staining (like G-, NORs, CMA_3) pattern[6,7].

The physical maps of genes by fluorescence *in situ* hybridization (FISH) represent a potentially new source of chromosomal characters that may be cytogentically informative. So far, physical mapping has been focused mainly on highly repetitive DNA or multigene families because of the technical difficulties encountered for mapping low-copy genes[8]. Repetitive DNAs have been applied extensively as physical chromosome markers in comparative genome, chromosome evolution and characterization studies, identification of chromosome rearrangements and sex chromosomes and also in applied genetics[9]. Ribosomal DNA, the most frequently mapped repetitive gene, has emerged as a good marker for species characterization and population genetic studies.

Mapping of 18S rRNA gene on the chromosome of *O. belangeri* using FISH has been undertaken to determine the number and location of the rDNA loci in this species and to characterize the species

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cytogenetically using conventional karyotyping, staining methods, in order to generate cytogenetic data of *O. belangeri*. This may be the first report of chromosomal localization of 18S rRNA gene in *O. belangeri*.

Materials and Methods

Sample collection and chromosome preparation—Live specimens (12) of *Osteobrama belangeri* of juvenile stage were collected from Imphal valley, situated in North Eastern state of Manipur, India. The average wet weight and length of the specimens was 72.39 g (range 23-170 g) and 178.75 mm (range 125-265 mm), respectively. Metaphase chromosome spreads were prepared from anterior kidney cells using standard hypotonic treatment, acetic acid-methanol fixation and flame-drying technique[10]. The chromosomes were stained with 4% Giemsa in phosphate buffer (*p*H 6.8). Approximately, 250 chromosome complements were analyzed from the cells for establishing modal chromosome number and characteristic chromosome morphology. For karyotyping, chromosomes were grouped into metacentric (m), submetacentric (sm), subtelocentric (st) and telocentric (t) as per the classification proposed by Levan *et al*[11].

DNA extraction, PCR amplification and sequencing—The genomic DNA was extracted from whole blood using standard phenol: chloroform: isoamylalcohol method described by Sambrook *et al*[12]. For amplification of 18S rDNA in parts, a standard PCR reaction was performed using 10 m*M* Tris-HCl (*p*H 8.3), 50 m*M* KCl, 2 m*M* $MgCl_2$, 200 µ*M* dNTPs mix, 10 pmoles of each primer (forward1: 5′ CTCAAAGATTAAGCCATG CAGGTC 3′ and reverse1: 5′ ATGGGTAATTTGCGCGCCTGCTG 3′; forward2: 5′ CG GCTACCACATCCAAGGAAGG 3′ and reverse2: 5′ ATGCTTTCGCTTTCGTCCGTCTTG 3′; forward3: 5′ CGGCGCAAGACGGACGAAAG C 3′ and reverse3 5′ GACCTGT TATTCCTCCATCTCGCG 3′; forward4 5′ CGTGCGGTCGGCGTTCAACTTC 3′ and reverse4 5′ CCTTGTTACGACTTTTACTTCCTC3′), 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 final extension at 72°C for 10 min. Amplified products were run on 1.5% agarose gel stained with ethidium bromide and thereafter sequenced.

Probe labeling and FISH—The amplified 18S rDNA was labeled with fluorescein 12-dUTP (Fermentas) by nick translation for probe construction. Single colour FISH was performed to determine the localization of 18S major rDNA probe on the chromosomes. Two to three days aged chromosome preparations were baked at 90°C for 1 h followed by the FISH protocol described by Winterfeld and Roser[13], with minor modifications in post-hybridization washing at 45°C. The preparations were counterstained with DAPI and mounted in Vectashield mounting medium (Vector Labs). Slides were examined under fluorescence microscope (Leica) with double band filter for simultaneous visualization of the two colours, i.e. DAPI and fluorescein.

Chromosome staining—Nucleolar organizer regions (NORs) were studied by silver impregnation as well as CMA_3 staining. The Ag-NOR was performed according to the method of Howell and Black[14] with minor modifications. The chromosomes were stained by CMA_3 / DAPI staining as per the method described by Ueda *et al.*[15] with minor modifications. A particular band pattern was determined by studying a minimum of 20 metaphase spreads per specimen.

Results and Discussion

In *O. belangeri*, the diploid chromosome number was found to be 50 and based on the chromosome morphology, the karyotype formula for this species was derived as 6m+16sm+12st+16t (FN=72) (Fig. 1). So far, no published information seems to be available for this species, but in another species of

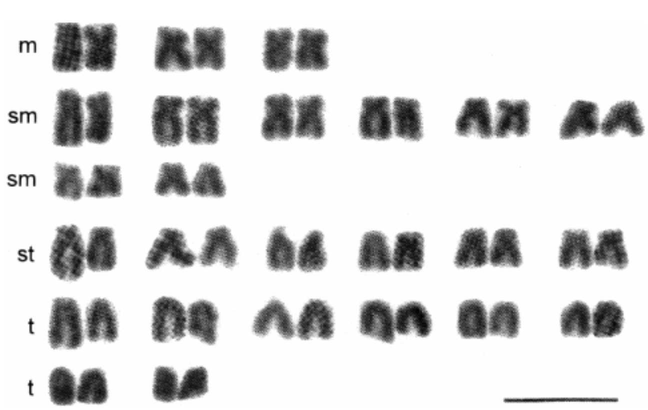

Fig. 1—Giemsa stained karyotype of *O. belangeri* (Bar = 5 µm)

this genus, i.e. *Osteobrama cotio cotio*, the 2n was reported to be 48[16,17] with karyotype formula as 18m+24sm+6st (FN=90)[18].

PCR amplification of 18S rDNA, in parts, produced bands of various sizes using different primers, which ranged from 416 to 565 bp. After sequencing, the length of single repeat of major 18S rDNA was found to be 1819 bp long in *O. belangeri* (NCBI GeneBank Accession No. FJ469676). The 18S sequence showed ~90% average similarity (103 BLAST hits) to other species listed in NCBI database.

In the present investigation, the FISH signals of 18S rDNA were clearly located in most of the metaphase spread of *O. belangeri* on short arms of one pair of submetacentric chromosome (Fig. 2a). The signals detected on the chromosomes were strong, indicating a high number of rDNA repeats at these loci. The chromosomal location of rDNA loci has a more substantial impact on the tempo of concerted evolution than the number of loci[19].

The nucleolar organizing regions (NORs), detected by silver impregnation, in *O. belangeri* were present in variable numbers on the chromosomes. In about 9% of the metaphase complements studied, Ag-NOR was found to be localized terminally on single chromosome, whereas in about 20% and 15% complements the signals were detected on 3 and 4 chromosomes, respectively. In about 56% of the metaphase spreads, it was present on both chromosomes (1 pair) of 3rd submetacentric chromosome on their short arm (Fig. 2b). Therefore,

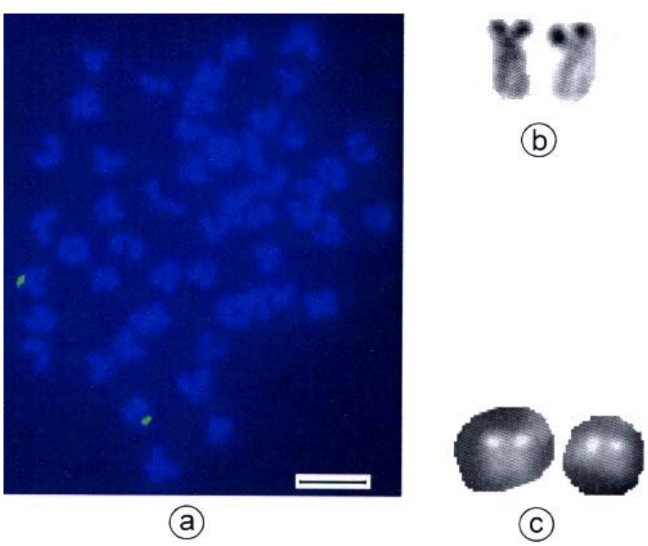

Fig. 2—a. Metaphase spread of *O. belangeri* showing FISH signals, b. chromosomes showing silver stained NORs, and c. chromosomes showing CMA_3 signals (Bar = 5 μm)

in the present study, the modal value was taken as the presence of one pair Ag-NOR signals on the standard metaphase complements, which was confirmed by FISH. The Ag-NORs are generally visualized in transcriptionally active site of rDNA and silver nitrate stains those NORs that are expressed themselves during the preceding interphase by binding to a complex of acidic proteins associated with nucleolus and nascent pre-RNA[20]. The variation in number of NOR is sometime observed when silver stains constitutive heterochromatin, in addition to heterochromatin associated with NORs[21]. In fishes, the presence of NOR on one pair of chromosome is a common feature, however, some species possessed NORs on more than one chromosomes. However, polymorphisms related to transcriptional inactivation of nucleolus organizer regions (NORs) have long been described in many organisms, however, the precise etiology of such variations is not very clear. The information on size, position and numbers of NORs are suitable for tracing intra-specific and inter-specific differences which may serve to demarcate and derive the taxonomic status of species in terms of karyo-evolution[22].

The NORs have been reported to contain GC-rich DNA in many vertebrates, including fish[23]. NOR-bearing chromosomes and GC-rich active regions were revealed through chromomycin A_3 (CMA_3) staining technique. Since silver staining generally demonstrates NORs, therefore, the CMA_3 staining has been suggested, not only for determining the number and localization of NORs, but also for pinpointing richness of GC content of transcriptionally active rRNA genes in several fish species[24-27]. The CMA_3 staining has also been useful in detecting the presence of several silent rDNA clusters[28]. The presence of CMA_3 signals on chromosomes was also variable in this species. In about 89% of the metaphase spreads, CMA_3 signals were present on two chromosomes (1 pair) while in 11% they were present on four chromosomes (2 pairs). Therefore, the standard number was reported as one pair that was present on short arm of 3rd submetacentric chromosome of *O. belangeri* in the present study (Fig. 2c).

The positions of 18S rDNA were similar to the Ag-NORs, CMA_3 and the FISH mapping. Earlier, Jankun *et al*.[26] reported a positive correlation between CMA_3 stained sites and active rRNA genes in some coregonid fish. Das and Khuda-Bukhsh[29] also found intimate association between the NOR-bearing chromosomes and CMA_3 stained GC-rich active rRNA genes in fishes. The co-localization of Ag-NOR, CMA_3 and FISH, in the present study, confirms the occurrence of single pair NOR in *O. belangeri* and the activeness of rDNA locus in this species. Such a co-localization has been found in many fish species[30,31].

Studies on ribosomal RNA gene activities have gained importance in a wide range of organisms, especially with respect to species/population characterization as well as phylogenetic and evolutionary relationships[32]. The localization of ribosomal DNA in several fishes including the representative of family cyprinidae has been reported[33-35]. Extensive variations in number and location of major rDNA loci have been found in the species studied till date; however, no specific pattern in distribution of major rDNA was observed. The data obtained in the present study indicated that nucleotide sequences of 18S rDNA and their chromosomal localization can serve as a suitable genetic marker for the evolutionary studies and genetic identification of the related species to aid in molecular taxonomy. The distribution and location of ribosomal DNA repeats have been employed for identification of stocks/species/populations. Further, these molecular markers have been utilized in conventional breeding, hybridization and genetic engineering programs to accelerate the genetic improvement through broadening of gene pool and marker assisted selection. The genome organization of several species and genetic exchange between populations have been studied using genomic DNA sequences, especially repetitive DNA, for future hybridization and genetic engineering programs[36]. The nucleotide sequence, distribution of ribosomal DNA and detailed karyological information for the endangered freshwater species, *O. belangeri* could be utilized for identification of different cytogenetic races, future hybridization and genetic engineering programme for conservation of this species.

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