Karyotype, Ag-NOR, CMA\textsubscript{3} and SEM studies in a fish (*Mystus tengara*, Bagridae) with indication of female heterogamety

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Somatic karyotypes in *M. tengara* contained 54 chromosomes, comprising 26 homomorphic pairs in both sexes and one pair of heteromorphic nature in female (one big submetacentric and one small subtelocentric chromosomes), while in males this pair was homomorphic (with two big sub-metacentric chromosomes). The Nucleolus Organizer Regions (NORs) were located at one arm of the suspected sex elements in both sexes, while another pair of metacentric chromosomes (No. 7) also showed Ag-positive arm. The CMA\textsubscript{3} technique revealed relatively bright fluorescing zones in the regions of chromosomes that showed Ag-positive staining, revealing thereby the predominance of GC-rich active sites of rRNA genes in NORs. SEM studies revealed clear heteromorphism to exist in the elements suspected as sex chromosomes in females.

Keywords: Chromomycin A\textsubscript{3}, Chromosome, Female heterogamety, Karyotype, *Mystus tengara*, Nuclear organizer region

Fish karyotypes are generally characterized by possession of a large number of small chromosomes and lack of morphologically distinguishable sex elements in all but a few species. Out of over 25,690 species of fish so far recorded taxonomically\textsuperscript{1,2}, some 2,500 odd species are now chromosomally known\textsuperscript{3,4-6}. Out of these, morphologically distinguishable sex elements have been recorded (mostly as heteromorphic pair in one sex, with corresponding homomorphic pair in the other) in slightly over one hundred species of fish belonging to some 50 families\textsuperscript{7}. Thus, the occurrence of morphologically detectable differences in chromosome structures and forms between sexes of any fish is of interest in view of the rarity of occurrence of morphologically distinguishable sex chromosomes and also for the difficulty in understanding the chromosomal sex determination mechanism in fish.

The somatic karyotypes of eight species of *Mystus* had earlier been studied from India\textsuperscript{4,6} but none excepting one species was reported to have the occurrence of any heteromorphic pair of chromosomes in any sex. Rishi and Rishi\textsuperscript{8} first reported that there was enough indication of female heterogamety in *Mystus tengara* through their karyotypic study with Giemsa-stained preparations.

However, the study was not repeated by others using any other technique to confirm or refute the occurrence of female heterogamety in this species. Therefore, an attempt has been made to study the karyotype in both sexes of *Mystus tengara*, using additional techniques like one-step silver nitrate and chromomycin A\textsubscript{3} (CMA\textsubscript{3}) for localization and confirmation of Nuclear Organizer Region (NOR) sites on metaphase chromosomes as well as the site for rDNA. Further, Scanning Electron Microscopy (SEM) has been attempted primarily to get enlarged view of the suspected sex elements (Z and W chromosomes in females and ZZ in males) in both sexes in order to confirm if any structural differences between them could be substantiated and also to reveal the exact location where silver was deposited on the metaphase chromosomes. To the best of our knowledge, the chromosomes of *M. tengara* have not been studied earlier by Ag-staining, CMA\textsubscript{3} or SEM techniques.

Materials and Methods

Eight specimens of male and six of female *Mystus tengara*, collected live locally, were intramuscularly injected with 0.03% colchicine @ 1ml/100g body weight and kept for 2.5 hr prior to sacrifice. The somatic chromosomes were prepared from their kidney cells by the flame-drying technique described elsewhere\textsuperscript{9} and their nomenclature adopted by following the method of Levan et al\textsuperscript{10}.
Some of the slides were routinely stained with Giemsa while some others were stained with silver nitrate by following the single-step method of Howell and Black\textsuperscript{11} for Ag-NOR locations; for localization of rDNA the CMA\textsubscript{3} technique, as suggested by Schweizer\textsuperscript{12}, was followed. For preparation of scanning electron microscopy the method of Sumner \textit{et al.}\textsuperscript{13} was followed.

Results

The Giemsa-stained typical diploid metaphase complements in both male (Fig. 1) and female (Fig. 5) contained 27 pairs of chromosomes. The chromosomes of male could be aligned into 27 homomorphic pairs comprising 7 pairs of metacentric (nos. 7,8, 15, 21, 22,24,26), 13 pairs of sub-metacentric (nos. 1-6, 9-14, and the suspected sex elements kept separately as no.27), 2 pairs of sub-telocentric (nos.17,19) and 5 pairs of acrocentric chromosomes (nos.16, 18, 20, 23, 25) (Fig. 2). However in female, the 54 chromosomes encountered in the female karyotype, one large metacentric Z and one small sub-telocentric chromosome suspected as W chromosomes, respectively (no. 27, Fig. 6).

In silver-stained metaphase preparations of both male (Fig. 3) and female (Fig. 7) complements, NORs could be observed in two pairs of chromosomes of both sexes; the small arm of largest sub-metacentric pair suspected as ZZ in male (no.27, Fig. 4) and also in one arm of another metacentric pair (no.7, Fig. 4) in the karyotype. Two pairs of NORs could also be localized in the female karyotype, one located at one arm of the medium-sized metacentric pair (no.7) while the other at the smaller arm of the heteromorphic chromosome pair consisting of one large sub-metacentric and one small sub-telocentric chromosomes, suspected as Z and W, respectively (no. 27, Fig. 8).

When the somatic complements in both male (Fig. 9) and female (Fig. 10) were subjected to SEM, the particular pair suspected as the sex elements appeared homomorphic in the male sex and heteromorphic in the female sex. When the sex elements (Z) (Figs 11,12 in male and Fig. 14 in female) and W (Fig. 13) were considerably enlarged, their gross difference in morphology and deposition of silver granules at the site of NORs (Figs 9-14 indicated by arrows) could be confirmed.

The CMA\textsubscript{3} preparations of metaphase complements in male (Fig. 15) and female (Fig. 16) showed greater fluorescence at regions which took up positive silver-staining depicting NOR-locations. Thus, the Ag-NOR locations denoted by silver-staining corroborated well with the CMA\textsubscript{3} staining, indicating thereby the active transcribing zones of NOR-bearing chromosomes to actually represent the GC-rich active sites for rRNA genes.

Discussion

The diploid number of 54 chromosomes and the occurrence of female heterogamety in this species as reported by Rishi & Rishi\textsuperscript{8} is confirmed in the present study through different techniques. However, there is some difference in the karyotypes observed by the different workers. While Rishi & Rishi\textsuperscript{8} reported 12 pairs of metacentric, 9 pairs of sub-metacentric and sub-telocentric and 5 pairs of acrocentric apart from a big sub-metacentric pair suspected as ZZ in male and a heteromorphic pair comprising one large sub-metacentric Z and one small acrocentric W reported in female, we observed a slightly different karyotype consisting of 7 pairs of metacentric, 12 pairs of sub-metacentric, 2 pairs of sub-telocentric, 5 pairs of acrocentric chromosomes apart from one pair of large sub-metacentric suspected as ZZ pair in male and one heteromorphic pair comprising a large sub-metacentric Z and a small sub-telocentric chromosome suspected as W chromosome. Such small variations in results may either reflect intra-specific variations naturally occurring in the materials studied in two geographically distant places of India, or else may partly represent observational errors on part of the observers in view of the small size of the chromosomes or their unsuitable disposition.

Interestingly, two pairs of chromosomes including the suspected sex chromosome pair showed localization of NORs as revealed from silver nitrate impregnation technique. The occurrence of regions taking silver (Ag) deposition has also shown positive fluorescence by CMA\textsubscript{3} technique depicting GC-rich region and confirming its role in ribosomal gene activity. Scanning electron microscopy conducted on silver treated preparation also confirmed the deposition of silver at regions depicted under light microscopy. The ultrastructure of the chromosomes appeared to be consisting of scaffold and chromatin
Figs 1-8 — Photomicrographs of metaphase complements of *M. tengara*: of male (Fig. 1) and female (Fig. 5) by ordinary Giemsa stain; Ag-stained diploid complements of male (Fig. 3) and female (Fig. 7); karyotypes, Figs. 2, 4, 6 and 8, prepared from Figs. 1, 3, 5 and 7, respectively. Bar=10 µm.
Figs 9-14 — Photographs of part of metaphase complements of M. tengara male (Fig. 9) and female (Fig. 10) under Scanning Electron Microscope; enlarged view of ZZ (Figs. 11, 12) and WZ (Figs. 13, 14), respectively.
Scale=60*3NM, 25KV, magnification 500X (Figs.9 and 10); 10*3NM, 25KV, magnification 3000X (Figs.11-14).

fibrillar structures. In earlier studies on mammalian chromosomes, the localization of silver deposition has been reported either on the sides of chromatin or in the fibrillar centers. Alternatively, staining of the dense fibrillar component has also been reported to occur in some cases. Therefore, the silver stainable material has been practically reported to be either located in a transcriptionally inactive part of the nucleolus organizer region, or can be less regularly located in the dense fibrillar component, the actual site of transcription. In metaphase chromosomes of M. tengara, the species under study, silver positive regions appear to be located in the fibrillar regions, rather than being located outside chromatin.

As mentioned earlier, the occurrence of morphologically detectable sex chromosome has been reported only in less than 0.5% of fishes karyotypically studied so far. The absence of heteromorphic sex pair in majority of fish species studied so far has been attributed to their "primitive" state, the sex chromosomes being very little differentiated from autosomes. Further, out of nearly 100 species showing morphologically detectable sex chromosomes, the most prevalent types of sex determination were XX female; XY male encountered in some 45 species, followed by some 30 species having ZZ male; ZW female while XO male; XX female and multiple sex chromosome mechanisms of X1X2X3X4 female; X1X2Y male have also been reported. However these suspected sex elements in majority of the species have not been verified by any banding studies. Park and Grimm could not find C-band and fluorescent staining technique to be of diagnostic value in differentiating ZZ pair in either the European (Anguilla anguilla) or American (Anguilla rostrata) eels.

In the primitive vertebrates, the chromosome morphology and behaviour of some sex-linked genes would indicate that the sex chromosome pair has remained largely homologous and sex determination is believed to be at genic rather than the chromosomal level. Differentiation of heteromorphic sex chromosomes probably resulted from inhibition of crossing over between the originally homologous pair, thus isolating the sex determining loci and stabilising differential accumulation of the two kinds of sex determining genes.

Morphological differentiation of Z and W or X and Y chromosomes could therefore imply that the initial homologue would possibly convert into heterologue through structural rearrangement.

In an earlier study, like in M. tengara, Ag-NOR was reported to be located in the suspected sex elements in a brackish water fish, Scatophagus argus.
showing male heterogamy. It is therefore of considerable academic interest if NORs are also found to be located on morphologically distinguishable sex elements reported in some other fishes, as this may point to the positive association of NORs and sex genes in fish. Thus, future studies in this direction may prove rewarding in gaining further knowledge in respect of presently unknown sex gene distribution in most fishes.

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