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Preponderance of GC-rich sites in silver-stained nucleolus organizing regions of *Rita rita* (Hamilton) and *Mystus gulio* (Hamilton) (Bagridae, Pisces), as revealed by chromomycin A_3-staining technique and scanning electron microscopic studies

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ABSTRACT. The karyotypes of two species of catfish, *Rita rita* (Hamilton) (2n = 54; 14m + 34sm + 6st; NF = 102) and *Mystus gulio* (Hamilton) (2n = 58; 30m + 12sm + 2st + 14t, NF = 100) were studied through Giemsa-, silver- and chromomycin A_3-staining techniques. The silver-stained karyotypes in both sexes of *R. rita* and *M. gulio* revealed that the nucleolus organizing regions were located terminally at the shorter arms (Tp) of one pair of submetacentric chromosomes, placed at positions Nos. 2 and 1, respectively, which was confirmed by scanning electron microscopy. Staining with a GC-specific fluorochrome, chromomycin A_3, produced bright fluorescence in the Ag-positive nucleolus organizer regions, suggesting thereby that nucleolus organizing regions actually included GC-rich sites of active r-RNA genes in metaphase chromo-

somes of these two bagrids. Further such studies are needed due to the extreme paucity of data on fish.

Key words: Ag-NORs, Chromomycin A_3, *Mystus gulio*, *Rita rita*, Scanning electron microscopy, Pisces

INTRODUCTION

Fish karyotypes are generally characterized by large numbers of small chromosomes. This discourages many researchers from pursuing fish karyotypic analysis, and therefore, karyological data on fish are available for only a small percentage (about 10%) of some 25,000 species taxonomically known so far (Ojima, 1985; Nelson, 1994; Klinkhardt et al., 1995; NBFGR, 1998; Arkhipchuk, 1999; Froese and Pauly, 2006).

Nucleolus organizer regions (NORs) are believed to be the chromosomal sites that synthesize 18S and 28S ribosomal RNA. These regions are usually detected through controlled silver-staining technique. As in other vertebrates, only specific chromosomes are involved in the formation of nucleoli in fish. These specific DNA templates are localized in certain specific regions of these specific chromosomes, known as nucleolar chromosomes. The composition of the NOR is not precisely known, but it is believed to contain nucleolar ribosomal DNA or rDNA and a surrounding matrix containing special proteins and other components essential for the synthesis of rRNA (Busch and Smetana, 1970). Although the sites of synthesis of some nucleoli are identified in prophase or metaphase chromosomes as secondary chromosome constriction(s) in fish, these sites of nucleolar synthesis are less defined regions and vary considerably in their distribution in different groups of fish, some being located at the terminal ends of the chromosomes, or they may occupy an intercalary position (Mayr et al., 1986; Takai and Ojima, 1986; Rab et al., 1991; Amemiya et al., 1992; Jenkin et al., 1992; Khuda Bukhsh and Tiwary, 1994; Martinez et al., 1996; Brassesco et al., 2004). Silver staining is a dependable method that can identify NORs. Chemically, the NORs have been reported to contain GC-rich DNA in many vertebrates, including fish (Gold and Zoch, 1990), but exceptions occur. Similarly, not every secondary constriction will necessarily be an NOR-bearing chromosome, and not all sites of rDNA necessarily appear as secondary constrictions. In some cases, NORs actually contained more condensed chromatin (Goessens, 1984). Since silver staining generally demonstrates NORs, additional techniques such as chromomycin A_3 (CMA_3) staining has been recommended, not only for determining the number and localization of NORs, but also for pinpointing GC-rich sites of transcriptionally active rRNA genes for synthesizing 18S or 28S rRNA in several fish species (Mayr et al., 1986; Jankun et al., 1998, 2003).

Recent advancements in understanding the internal organization of chromosomes have been possible through electron microscopic studies of chromosomes. The use of scanning electron microscopy (SEM) to study Ag-NOR-bearing chromosomes permits their observations at higher resolution than is possible by light microscopy. The site of silver staining has been investigated at very high resolution with electron microscopy by Angelier et al. (1982), who applied

Ag-NOR staining to spreads of transcriptional units from nucleoli and observed that silver was deposited exclusively on the transcribed part of rDNA and not on the untranscribed spacer regions. Schwarzacher et al. (1978) have clearly shown by SEM that the sites of Ag-NOR staining associated with chromosomes were not actually within the chromatin itself, but lie at the sides of chromatids. To our knowledge, similar studies have only been done on a limited number of fish species, and none from India. We decided to study the catfish, *Rita rita* and *Mystus gulio* (Bagridae).

MATERIAL AND METHODS

Eight live specimens each of both sexes of *R. rita* and five specimens each of both sexes of *M. gulio*, collected locally, were injected intramuscularly with 0.03% colchicine 1 mL/100 g body weight and maintained alive for 2.5 h prior to sacrifice. The somatic chromosomes were prepared from their kidney cells by the flame-drying technique (Khuda-Bukhsh, 1979) and their nomenclature adopted by following the method of Levan et al. (1964).

Some of the slides were routinely stained with Giemsa, while others were stained with silver nitrate, following the single-step method of Howell and Black (1980) for Ag-NOR locations; for localization of rDNA the CMA_3 technique (Schweizer, 1976) was followed. For SEM preparation, the method of Sumner et al. (1994) was used.

RESULTS

The Giemsa-stained karyotype of diploid metaphase complements of both male (Figure 1A) and female (Figure 1B) *R. rita* contained 27 pairs of chromosomes (NF = 102), comprising 7 pairs of metacentric (6, 7, 10-12, 21, 26), 17 pairs of sub-metacentric (1-5, 8, 13, 14, 17-20, 22-25, 27), and 3 pairs of sub-telocentric (9, 15, 16) chromosomes. The Giemsa-stained karyotype of typical diploid metaphase complements in male (Figure 1C) and female (Figure 1D) *M. gulio* contained 29 pairs of homomorphic chromosomes (NF = 100), comprising 15 pairs of metacentric (4, 7, 8, 9, 14-17, 21-27), 6 pairs of sub-metacentric (1-3, 5, 10, 12), 1 pair of sub-telocentric (29) and 7 pairs of telocentric (6, 11, 13, 18-20, 28; Figure 1A, B) chromosomes.

The NORs of both male and female *R. rita* were observed on the small arm of one pair of sub-metacentric chromosomes (Figure 1A, B). Similarly, NORs were observed on the small arm of one pair of sub-metacentric chromosomes (No. 1) in both sexes of *M. gulio* (Figure 1C, D).

When the silver-stained somatic complements in both male and female *R. rita* and *M. gulio* were studied with SEM, the ultra-structure of the chromosomal parts of NOR-bearing metaphase chromosomes was revealed and the exact location of silver deposition was confirmed (Figure 2A-F). Silver-positive regions were located in the fibrillar regions, rather than being located outside chromatin, unlike what is reported in some mammals (Schwarzacher et al., 1978; Angelier et al., 1982).

The CMA_3 preparations of metaphase complements in both male and female *R. rita* (Figure 3A, B) and *M. gulio* (Figure 3C, D) showed greater fluorescence at regions which took up positive silver-staining depicting NOR-locations and indicated the active transcribing zones of NOR-bearing chromosomes, which actually represented the GC-rich active sites for rRNA genes.

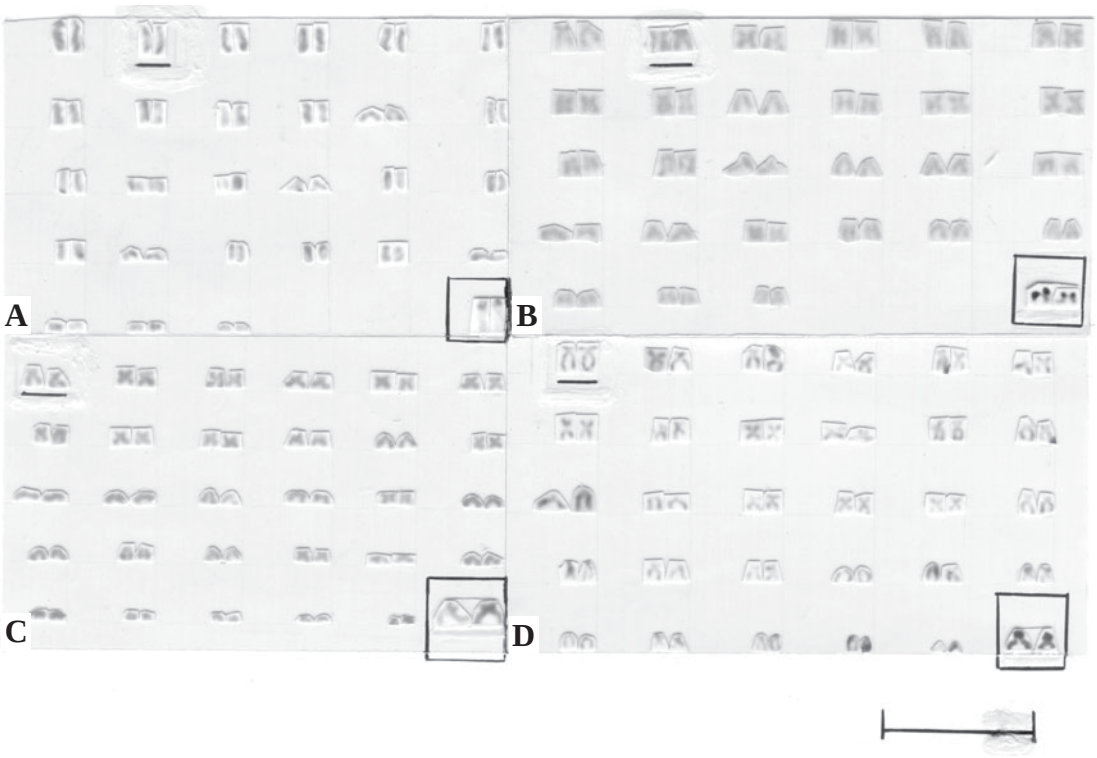

Figure 1. A. Giemsa-stained karyotype of metaphase complements of a *male Rita rita*. **B.** Giemsa-stained karyotype of metaphase complements of a female *R. rita*. The silver-stained NOR pair (No. 2) is shown in a box at the right hand corner of the karyotypes. **C.** Giemsa-stained karyotype of metaphase complements of a male *Mystus gulio*. **D.** Giemsa-stained karyotype of metaphase complements of a female *M. gulio*; the silver-stained NOR pair (No. 1) is shown in a box. Bar = 10 µm.

DISCUSSION

The somatic karyotypes of two species of *Rita* and eight species of *Mystus* from India have been published (Ojima, 1985; NBFGR, 1998; Das and Khuda-Bukhsh, 2003).

Our finding of one pair of NORs in *R. rita* and *M. gulio* agrees with the diploid number and NORs reported by Khuda-Bukhsh and Tiwary (1996). However, silver deposition in these two species had not been found either by SEM or by the additional method of CMA_3 staining in these earlier studies. SEM conducted on silver-treated preparations in these two species also demonstrated the deposition of silver at regions of metaphase chromosomes depicted under light microscopy. The ultrastructure of the chromosomes appeared to consist of scaffold and fibrillar chromatin structures. In earlier studies on mammalian chromosomes, silver deposition has been reported either on the sides of the chromatin (Schwarzacher et al., 1978) or in the fibrillar centers (Hernandez-Verdun et al., 1980; Fernandez-Gomez et al., 1983). Alternatively, staining of the dense fibrillar component has also been reported to occur in some cases (Ploton et al., 1984; Fakan and Hernandez-Verdun, 1986). Therefore, the silver-stainable material has been generally found either in a transcriptionally inactive part of the NOR, or less frequently in

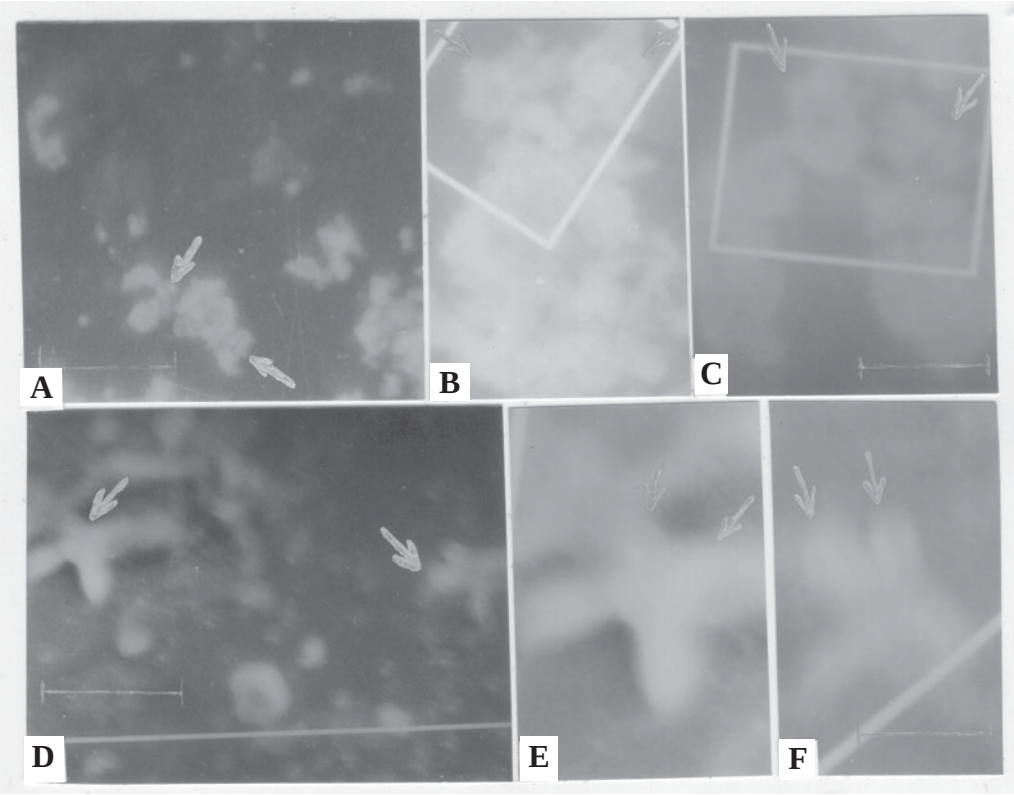

Figure 2. A. Scanning electron microscopic photographs of part of metaphase complements of *Rita rita*. Scale = 60,000 nm, 25 KV, magnification 500X. **B,C.** Enlarged view of NOR-bearing chromosomes of *R. rita*. Scale = 10,000 nm, 25 KV, magnification 3000X. **D.** Scanning electron microscopic photographs of part of metaphase complements of *Mystus gulio*, Scale = 60,000 nm, 25 KV, magnification 500X. **E,F.** Enlarged view of NOR-bearing chromosomes of *M. gulio*. Scale = 10,000 nm, 25 KV, magnification 3000X. NOR-bearing regions are indicated by arrows.

the dense fibrillar component, the actual site of transcription (Sumner, 1990). In metaphase chromosomes of *R. rita* and *M. gulio*, we found silver-positive regions in the fibrillar regions, rather than being located outside the chromatin. There was an intimate association between the NOR-bearing chromosomes and GC-rich active rRNA genes, revealed through CMA_3 in both *R. rita* and *M. gulio*. Earlier, Jankun et al. (2001) reported a positive correlation between CMA_3-stained sites and active rRNA genes (45S rRNA unit, which consists of a transcriptional unit that codes for 18S, 5.8S, 28S rRNA and intergenic spacer, IGS and minor 5S rRNA) in some coregonid fish. In that study, the regions with silver deposition had also shown fluorescence by CMA_3 technique, depicting a GC-rich region and thereby confirming its role in ribosomal gene activity. Recently, the same authors (Jankun et al., 2003) deployed a further improved method of PRINS/CMA_3 sequential staining to analyze the karyotypic correspondence between rRNA genes and NOR sites in Eurasian coregonid fish, confirming their earlier findings. We have now shown, by using CMA_3 staining, that GC-rich active rRNA genes are clustered at specific chromosome pairs in the two fish species, *R. rita* and *M gulio*. Further studies in this

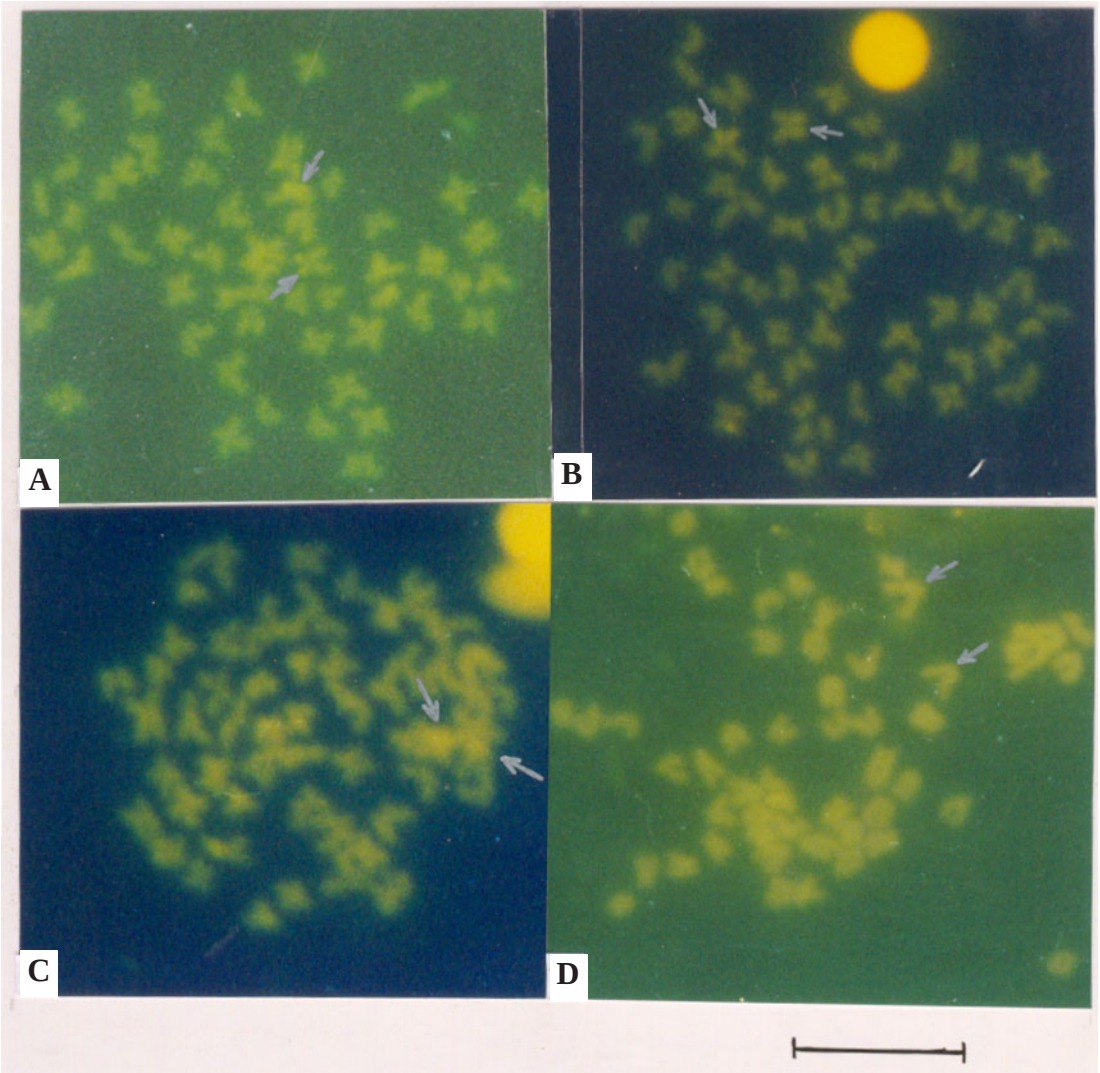

Figure 3. A. Photomicrograph of chromomycin A_3-stained diploid metaphase complement of a male *Rita rita*. **B.** Photomicrograph of chromomycin A_3-stained diploid metaphase complement of a female *R. rita*. **C.** Photomicrograph of chromomycin A_3-stained diploid metaphase complement of a male *Mystus gulio*. **D.** Photomicrograph of chromomycin A_3-stained diploid metaphase complement of a female *M. gulio*. Chromomycin A_3-fluorescent regions are indicated by arrows. Bar = 10 µm.

direction are needed to determine if the NOR sites of the metaphase chromosomes really include or overlap with active sites of rRNA genes rich in GC bases, or if there are other situations prevailing in other phylogenetically extant groups of fishes. Studies on ribosomal RNA gene activities have gained prominence in a broad range of animals and plants, especially in relation to species and population characterization and evolutionary relationships (Martins et al., 2000). Therefore, Ag-NOR bands, CMA_3-staining techniques and SEM studies need to be more widely used in fish; they may prove to be important cytotaxonomic markers to aid molecular taxonomy.

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