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Karyotypic diversity and evolution of seven mahseer species (Cyprinidae) from India

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Mahseer is a group of fish species that are well known as food and game fishes. The taxonomy of the mahseer species is confusing owing to the morphological variations and habitat adaptation. Detailed karyomorphological investigations have been carried out in seven species of mahseer, using karyotyping, Ag-NOR and fluorescent staining techniques. The basic diploid chromosome number (2n), in all mahseer species, was observed to be 100; however, the karyotype formula varied among the species, which were recorded as: 20m + 14sm + 22st + 44t (fundamental arm number, FN = 134) in *Tor khudree*; 22m + 24sm + 24st + 30t (FN = 146) in *Tor mussullah;* 12m + 22sm + 14st + 52t (FN = 134) in *Tor putitora*; 20m + 24sm + 24st + 32t (FN = 144) in *Tor tor*; 20m + 30sm + 24st + 26t (FN = 150) in *Tor chelynoides;* 20m + 20sm + 20st + 40t (FN = 140) in *Tor progeneius;* and 20m + 18sm + 14st + 48t (FN = 138) in *Neolissochilus hexagonolepis*. Silver staining of the chromosomes revealed the presence of multiple nucleolar organizer regions (NOR) in these mahseer species. The highest number of NORs was observed in *T. tor* (four pairs of chromosomes), whereas the other six species possessed Ag-NOR signals on only two pairs of chromosomes. Although chromomycin A_3 (CMA_3) staining induced bright fluorescence signals on same Ag-NORs sites, with CMA_3, one additional signal was observed on the p arm of subtelocentric chromosomes in *T. tor*, *T. chelynoides*, *T. progeneius* and *N. hexagonolepis*, which may indicate the presence of inactive NOR in these species. The information on cytogenetic profile of these mahseer species is discussed in the light of cytotaxonomic implications and understanding the karyoevolution of these fish species.

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Key words: Ag-NOR; chromomycin A_3; evolution; mahseer; *Neolissochilus*; *Tor.*

INTRODUCTION

Family Cyprinidae is the most abundant and globally widespread family of freshwater fishes, comprising 220 genera and *c*. 2420 species (Nelson, 2006). Among cyprinids, mahseer is the common name used for almost 50 species of freshwater fishes from

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genera *Tor*, *Neolissochilus* and *Naziritor*. These fishes are widely distributed along the Himalayan region of India, Pakistan, Bhutan, Bangladesh and Indonesia (Menon, 1992) and include some important game and food fish.

Differences of opinion prevail regarding the number of extant species in the genus *Tor* from India. Several authors have reported five species, namely, the golden or Himalayan mahseer *Tor putitora* (Hamilton), the Jungha mahseer *Tor progenies* (McClelland), the deep bodied or *Tor* mahseer *Tor tor* (Hamilton), the Deccan or Khudree mahseer *Tor khudree* (Sykes) and the humpback mahseer *Tor mussullah* (Sykes) (Nautiyal, 2006). Other mahseer species, namely the dark mahseer *Tor chelynoides* (McClelland), also known as *Puntius chelynoides*, and *Neolissochilus hexagonolepis* (McClelland), commonly known as chocolate mahseer, are also reported. *Neolissochilus hexagonlepis* has morphology similar to *Tor* species and the morphometric and meristic characters do not resolve its proper species identification. *Tor khudree, T. putitora* and *T. tor* are commercially important fishes, whereas *T. khudree, T. mussullah, T. putitora* and *T. tor* are considered as game fish. The ecological status of mahseer species has been recognised as endangered (Singh & Sharma, 1998; Menon, 1999; Sharma, 2003), especially *T. putitora* and *T. khudree* (Prasanna *et al*., 2000; Basavaraja & Hegde, 2004). Therefore, establishing the cytogenetic characterizations of these mahseer species is an urgent prerequisite for determining taxonomic ambiguities for future breeding and maintaining stocks of these populations in natural aquaculture resources.

Based on the cytogenetic studies on *Tor* species, all the *Tor* species have 100 diploid chromosomes (Froese & Pauly, 2009). Furthermore, the chromosomal analyses of different species, populations and stocks have revealed variation in karyomorphology (Khuda-Bukhsh, 1982; Lakra & Rishi, 1991; Lakra, 1996; Barat & Ponniah, 1998; Kushwaha *et al*., 2001). Among the cytogenetic markers, nucleolar organizer regions (NOR) have played an important role in the study of fish diversity. The localization of NORs has mainly been studied using silver nitrate ($AgNO_3$), mithramycin (MM) or chromomycin A_3 (CMA_3) staining (Amemiya & Gold, 1986; Phillips *et al*., 1989; Galetti & Rasch, 1993; Sola *et al*., 1997). CMA_3 stains brightly both active and inactive NORs by binding with GC-rich region of NORs. The Ag-NOR staining has been found to be useful to study interspecific variations in the species of genus *Tor* (Kushwaha *et al*., 2001).

The present investigation was designed to undertake detailed cytogenetic characterization and to explore the utility of karyomorphology and other cytogenetic markers like Ag and CMA_3 staining pattern in taxonomic, phylogenetic and systematic studies of mahseer species. This is the first attempt to study the karyomorphology in *T. chelynoides* and *T. progeneius* and Ag and CMA_3 staining in *T. chelynoides*, *T. progeneius* and *N. hexagonolepis*. In this way, a more detailed genetic differentiation and characterization of closely related mahseer species may contribute to improve conservation and restocking plans with considerable advantages for environmental protection of the species.

MATERIALS AND METHODS

Representative specimens of *T. khudree*, *T. mussullah*, *T. putitora*, *T. tor*, *T. chelynoides*, *T. progeneius* and *N. hexagonolepis* (Table I) were collected from the River Cauvery, Karnataka; from the TATA Electric Company reservoir, Lonavala, Maharastra; from the River

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Kosi, Manan, Almora, Uttarakhand; from the River Narmada near Hoshangabad, Madhya Pradesh; from the River Alaknanda, Garhwal, Uttarakhand; from the River Jia Bhoreli, near Bhalukpong, Assam and from tributaries of Umiam Reservoir, Shillong, Meghalaya, with the help of local fishermen (Fig. 1). All the specimens were in juvenile stage and the sex was unidentifiable by visual examination.

For chromosome preparations, the specimens were treated intramuscularly with 0·05% colchicine (1·0 ml 100 g^{-1} body mass) to arrest the chromosomes in metaphase stage and kept alive in a plastic bucket. After 2 hours, the specimens were killed, the kidney tissues were dissected out and further processed for chromosome preparations using hypotonic treatment–fixation (methanol:acetic acid)–flame drying technique. The chromosome slides were stained with 6% giemsa in phosphate buffer (pH 6·8). The karyotypes of chromosome complements were prepared from cells exhibiting the complete somatic chromosome number and characteristic chromosome morphology. Homologous chromosomes were paired based on their morphology and position of centromere. The chromosome pairs were arranged in decreasing order of morphology and size in the karyotype using Lieca CW4000Karyo software (www.leica.com). Averages of the paired chromosomes were taken for estimating the length of short arm (p), long arm (q), arm ratio, centromeric index and relative length (%). The arm ratio was used to classify the chromosomes as metacentric (m), submetacentric (sm), subtelocentric (st) and telocentric (t), as suggested by Levan *et al*. (1964). Ideograms were constructed according to p and q arm lengths. Diagrammatic representations of karyotype, *i.e.* karyogram, were constructed according to the centromeric index and relative lengths and evaluated for overall symmetry *v*. asymmetry in terms of centromere position and relative

FIG. 1. Geographical locations of mahseer sampling from India. A, Alaknanda River; C, Cauvery River; J, Jia Bhoreli River; K, Kosi River; L, Lonavala Reservoir; N, Narmada River; U, tributaries of Umiam Reservoir.

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TABLE I. Details of sample voucher number and fish body mass (mean and S.D.)

S. no.	Sample voucher number	Mean body mass (g)	Standard deviation	Name of species
1.	TKC01	211·95	123·48	*Tor khudree*
2.	TKC02			
3.	TKC03			
4.	TKC04			
5.	TKC05			
6.	TKC06			
7.	TKC07			
8.	TKC08			
9.	TKC09			
10.	TKC10			
11.	TKC11			
12.	TKC12			
13.	TML01	117·50	14·89	*Tor mussullah*
14.	TML02			
15.	TML03			
16.	TML04			
17.	TML05			
18.	TML06			
19.	TPK01	42·04	28·53	*Tor putitora*
20.	TPK02			
21.	TPK03			
22.	TPK04			
23.	TPK05			
24.	TPK06			
25.	TPK07			
26.	TPK08			
27.	TTN0...	361·00	81·94	*Tor tor*
28.	TTN02			
29.	TTN03			
30.	TTN04			
31.	TTN05			
32.	TTN06			
33.	TCA01	6·21	6·02	*Tor chelynoides*
34.	TCA02			
35.	TCA03			
36.	TCA04			
37.	TCA05			
38.	TCA06			
39.	TCA07			
40.	TCA08			
41.	TPRJ01	58·94	43·58	*Tor progeneius*
42.	TPRJ02			
43.	TPRJ03			
44.	TPRJ04			
45.	TPRJ05			
46.	TPRJ06			

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TABLE I. Continued

S. no.	Sample voucher number	Mean body mass (g)	Standard deviation	Name of species
49.	NHU01	35·14	34·43	*Neolissochilus hexagonolepis*
50.	NHU02			
51.	NHU03			
52.	NHU04			
53.	NHU05			
54.	NHU06			
55.	NHU07			
56.	NHU08			

size differences. Silver staining of NORs on chromosomes was performed according to the method of Howell & Black (1980). For CMA_3 staining, the method described by Ueda *et al*. (1987) was followed. A particular band pattern was determined for each species by studying a minimum of 25 metaphase spreads per specimen per species.

RESULTS

A marked chromosomal conservation with the presence of 100 diploid chromosomes (2n) number in the seven mahseer species has been observed in the present study. Furthermore, interspecific variations in their karyotype formula (KF) and fundamental arm number (FN) were established. The KF and FN in *T. khudree*, *T. mussullah*, *T. putitora*, *T. tor*, *T. chelynoides*, *T. progeneius* and *N. hexagonolepis* were derived as 20m + 14sm + 22st + 44t (FN = 134), 22m + 24sm + 24st + 30t (FN = 146), 12m + 22sm + 14st + 52t (FN = 134), 20m + 24sm + 24st + 32t (FN = 144), 20m + 30sm + 24st + 26t (FN = 150), 20m + 20sm + 20st + 40t (FN = 140) and 20m + 18sm + 14st + 48t (FN = 138), respectively [Fig. 2(a)–(g)].

The Ag-NOR of chromosomes revealed the presence of multiple NORs in all mahseer species with variation in numbers and positions. The NORs were present on two pairs of chromosomes in *T. khudree*, two pairs in *T. mussullah*, two pairs in *T. putitora*, four pairs in *T. tor*, two pairs in *T. chelynoides*, two pairs in *T. progeneius* and two pairs in *N. hexagonolepis* [Fig. 3(a)–(g)] that indicated the transcriptional activity of nucleolar organizing regions. In *T. khudree*, Ag-NORs were localized on the terminal portions of p arm of fifth sm, and second st chromosomes. In *T. mussullah* and *T. putitora*, p arm of fourth sm and fourth st chromosomes and second m and fourth sm chromosomes, respectively, exhibited terminal Ag-NORs. In *T. tor*, the terminal portion of p arm of third sm, sixth sm, fifth st and tenth t chromosomes exhibited silver impregnation. In *T. chelynoides*, the Ag-NORs were localized on the terminal portions of p arm of second and fourth metacentric chromosomes. In *T. progeneius*, p arms of eighth m and fifth sm chromosomes exhibited Ag-NOR signals, whereas in *N. hexagonolepis* Ag-NOR signals were observed on p arms of seventh sm and fifth st chromosomes.

The CMA_3 staining of metaphase complements in these mahseer species showed bright fluorescent signals at the regions [Fig. 3(a)–(g)] that were also stained with

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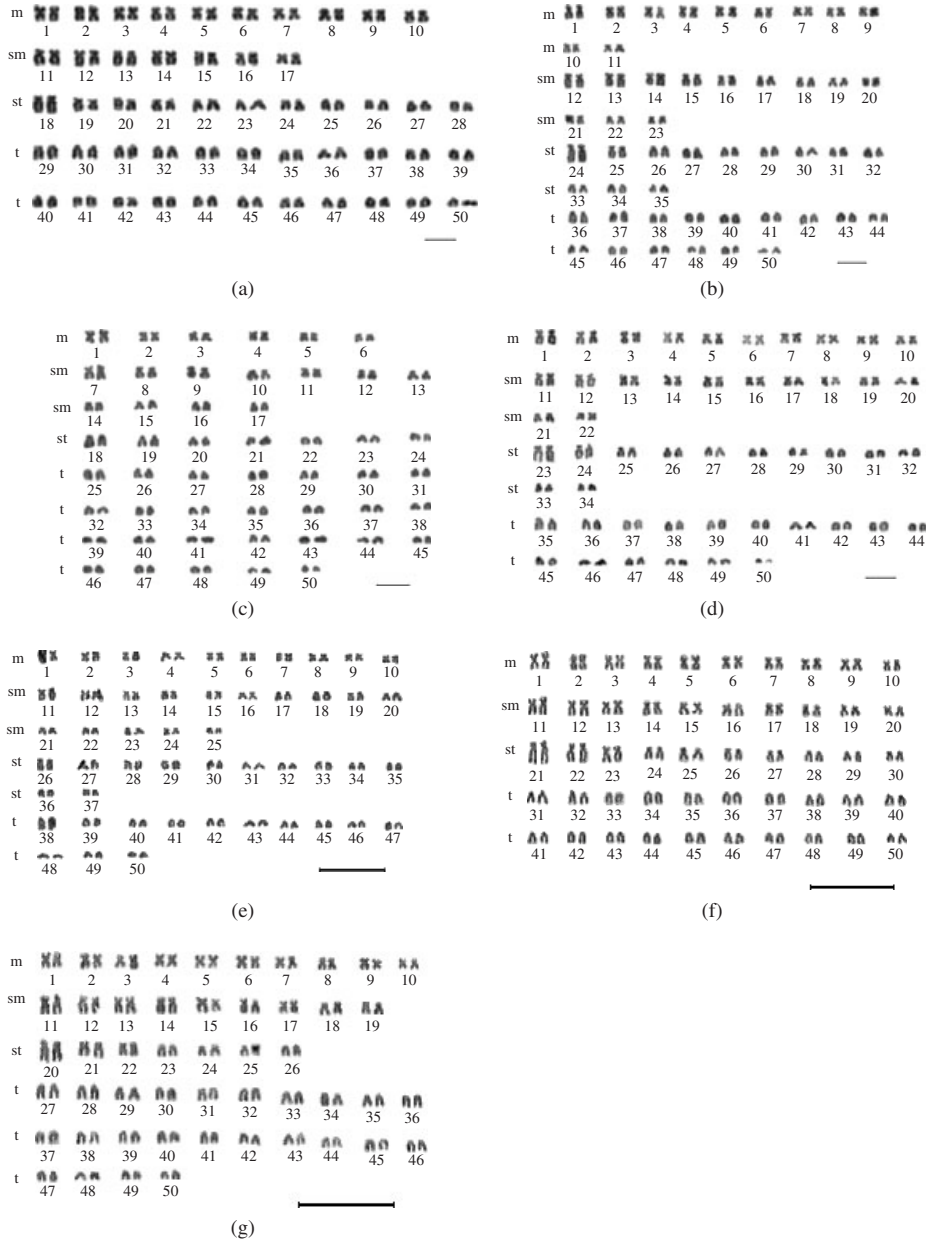

FIG. 2. Giemsa-stained karyotype of: (a) *Tor khudree*, (b) *Tor mussullah*, (c) *Tor putitora*, (d) *Tor tor*, (e) *Tor chelynoides*, (f) *Tor progeneius* and (g) *Neolissochilus hexagonolepis*. Bar, 5 μm.

$AgNO_3$, depicting the location of NORs and thus confirmed the position of active transcribing zones of NOR-bearing chromosomes. Furthermore, an additional CMA_3 signal was observed on p arm of tenth st chromosome in *T. tor*, second st in *T. chelynoides*, third st in *T. progeneius* and seventh st in *N. hexagonolepis* (Fig. 4).

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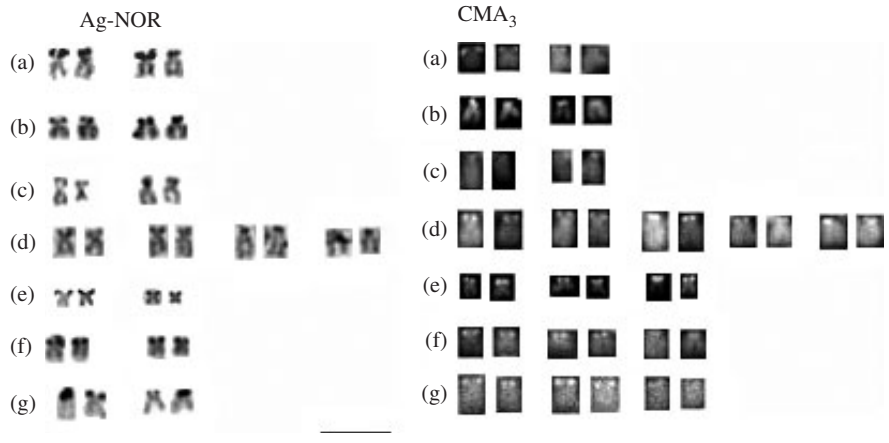

FIG. 3. Chromosome showing Ag-NOR and CMA_3 signals in: (a) *Tor khudree*, (b) *Tor mussullah*, (c) *Tor putitora*, (d) *Tor tor*, (e) *Tor chelynoides*, (f) *Tor progeneius* and (g) *Neolissochilus hexagonolepis*. Bar, 5 μm.

To compare the contribution of individual chromosome with the total haploid genome length, the relative length of chromosomes were estimated. The contribution of individual chromosome ranged from 2·53% (st) to 1·42% (t) in *T. khudree*, 3·44% (st) to 1·26% (t) in *T. mussullah,* 4·57% (st) to 1·04% (t) in *T. putitora*, 3·04% (t) to 1·19% (t) in *T. tor*, 3·89% (sm) to 1·19% (sm) in *T. chelynoides*, 3·73% (st) to 1·37% (sm) in *T. progeneius* and 3·54% (sm) to 1·18% (t) in *N. hexagonolepis*. Among the seven mahseer species, the karyogram of *T. tor* was found to be relatively symmetrical, whereas the *T. putitora* karyogram was positively skewed. NORs located on chromosomes in all the species of mahseer are shown in the ideogram.

DISCUSSION

The mahseer can be considered conservative in maintaining the same 100 diploid chromosome numbers that have been reported in all the mahseer species studied to date (Khuda-Bukhsh, 1980, 1982; Khuda-Bukhsh *et al*., 1986; Collares-Pereira, 1994; Lakra, 1996; Barat & Ponniah, 1998; Kushwaha *et al*., 2001; Sahoo *et al*., 2007). The presence of 100 diploid chromosomes has been regarded to be a plesiomorphic condition for the Cyprinidae family (Collares-Pereira, 1994). Several authors have considered mahseer species to be naturally polyploid in origin (Khuda-Bukhsh, 1982; Howes, 1991; Lakra, 1996). The presence of large 2n, as high as 446, has suggested the event of polyploidy in the Cyprinidae, which might have resulted due to complex evolutionary mechanisms (Yu & Yu, 1990).

To date, the cytogenetic investigations in the Cyprinidae have been reported for *c*. 410 species belonging to 152 genera (Froese & Pauly, 2009). The model diploid chromosome number in cyprinid fishes has been inferred as 50 (Yu *et al*., 1987; Rishi, 1989; Collares-Pereira, 1994), with the range from 34 to 446 (Froese & Pauly, 2009). In this family, there are seven genera, including *Tor* and *Neolissochilus* that possessed 100 diploid chromosome numbers. Assessment of the cytogenetic profile

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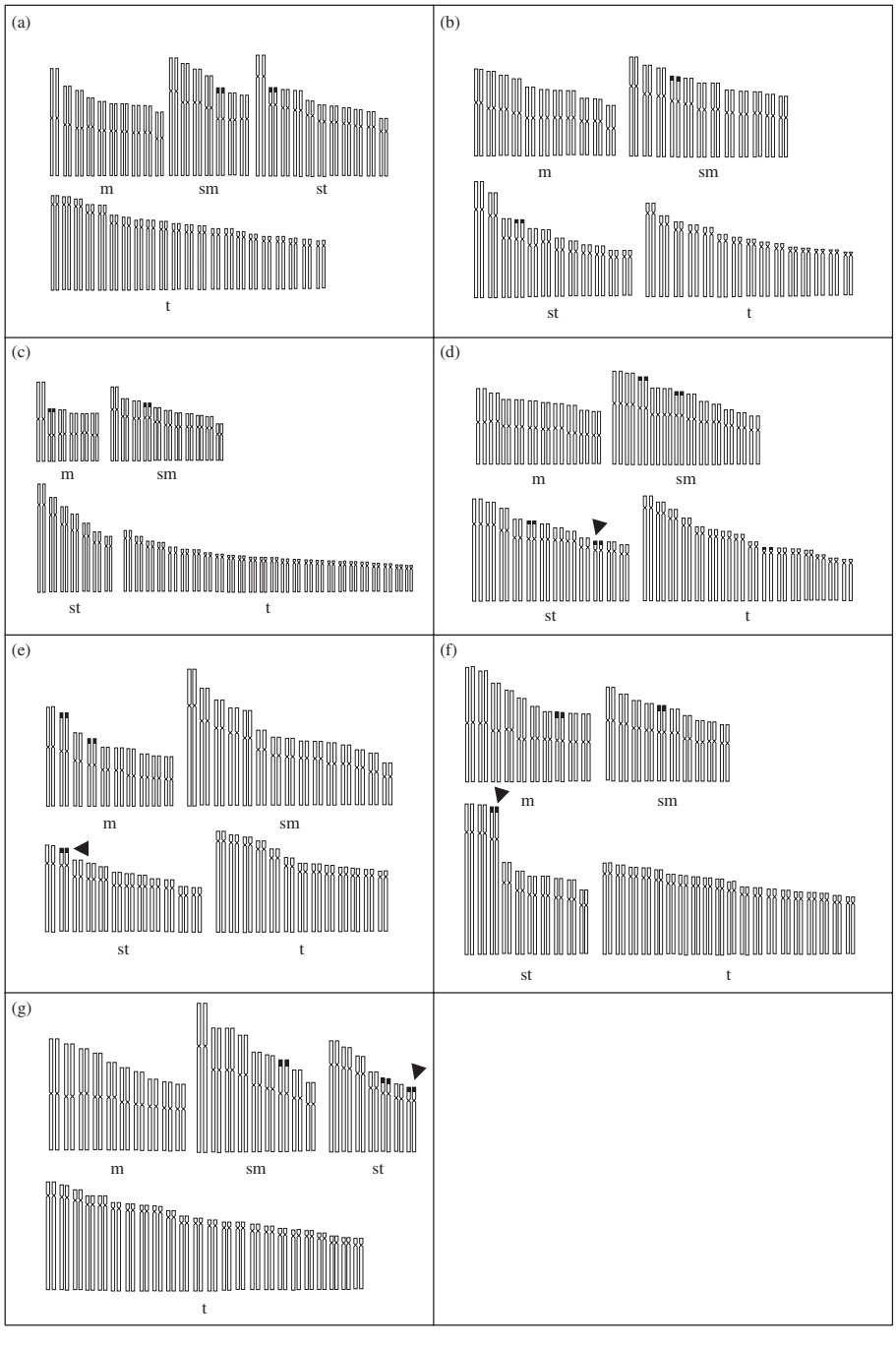

FIG. 4. Ideograms of: (a) *Tor khudree*, (b) *Tor mussullah*, (c) *Tor putitora,* (d) *Tor tor,* (e) *Tor chelynoides,* (f) *Tor progeneius* and (g) *Neolissochilus hexagonolepis* showing Ag-NORs and CMA_3 signals. Arrow heads indicate the additional CMA_3 signals. m, metacentric; sm, submetacentric; st, subtelocentric; t, telocentric.

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of the seven studied mahseer species revealed species-specific variation in chromosomal morphology and banding patterns. Comparison of the karyotypic formulae revealed deviations from the earlier reports for these species. For *T. mussullah*, the results, however, are in complete agreement with the earlier report (Kushwaha *et al.,* 2001).

It has been hypothesized that, during the phyletic evolution, the cyprinid fishes were differentiated into two large branches: Leuciscinae as one basic group and Barbinae as the other basic group. It was further suggested that Robertsonian translocation might have played a role in chromosome evolution of the Leuciscinae group, whereas polyploidization appears to have played a prominent role in the Barbinae group (Yu *et al*., 1987). The occurrence of 100 diploid chromosome numbers in mahseer species can be explained on the basis of polyploidization (tetraploidization) of a model diploid chromosome number of 50 (Khuda-Bukhsh *et al*., 1986), as also suggested by Manna (1983, 1984) after observing 50 diploid chromosome numbers in *c*. 70% of the studied cyprinids. Thus, the cyprinid ancestors were probably close to the barbin lineage where polyploidy had played a role in the karyoevolution of cyprinid fishes. Furthermore, it seems that rearrangements such as pericentric inversions (Sahoo *et al*., 2007) at different lengths of chromosomes have played an important role in the process of evolutionary divergence resulting in karyotypic diversification among the mahseer species. Similar karyotypic differences in the species of some fish genus have been attributed to pericentric rearrangements (LeGrande, 1981; Bertollo *et al*., 2004; Kavalco *et al*., 2005).

Karyotypic diversity analysis can also be complemented by the study of chromosomal localization of NOR signals. In fishes, the location of 45S rDNA (18S + 5·8S + 28S) has emerged as an important cytogenetic marker, with some groups having only one pair of NORs and others showing multiple. The majority of Indian freshwater fishes have been found to possess single NORs (John *et al*., 1993; Rishi & Mandhan, 1995; Tiwary & Khuda-Bukhsh, 1995; Kushwaha *et al.,* 2001; Nagpure *et al.,* 2001). Single pairs of NORs were also detected by $AgNO_3$ and GC-specific fluorochrome staining in the *Brycon* species (Almeida-Toledo *et al*., 1996; Margarido & Galetti, 1996) and in *Cyprinus carpio* L. (Anjum, 2005). In mahseer species, Ag-NORs were located on multiple chromosomes. The observation on number and position of NORs in *T. mussullah* was in complete agreement with Kushwaha *et al*. (2001), whereas in *T. khudree* the number of NORs was different, which may be due to population differences. Similar NOR number polymorphism was reported by Centofante *et al*. (2006) in *Oligosarcus hepsetus* (Cuvier) while working with Grande Stream (two pairs) and Santo Antonio Stream (three pairs) populations of the middle Paraiba do Sul River and by Barat *et al*. (1997) in Indian snowtrout, *Schizothorax richardsonii* (Gray), belonging to River Beas (one pair) and River Kosi (two pairs) populations. Comparative analysis of three Italian populations of European whitefish, *Coregonus lavaretus* (L.), showed four pairs of NORs in one population and five pairs in other two populations (Rossi & Gornung, 2005). In *T. putitora*, the position of NORs was in variation with the report of Barat & Ponniah (1998). In *T. tor*, *T. chelynoides*, *T. progeneius* and *N. hexagonolepis*, because no study has been done earlier, this may be the first report on localization of Ag-NORs.

GC-rich active regions of NORs are revealed by chromomycin A_3 (CMA_3) staining technique and, in general, a positive correlation was observed between $AgNO_3$ and CMA_3 stainings in these mahseer species, though an additional pair of positive CMA_3

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signals was detected in *T. tor*, *T. chelynoides*, *T. progeneius* and *N. hexagonolepis* which may indicate the presence of inactive NOR in these species. Interestingly, these additional signals were present on st (subtelocentric) chromosomes in all the four species. Jankun *et al*. (2001) also established a positive correlation between CMA_3-stained sites and active rRNA genes in their study in some coregonid fish. Das & Khuda-Bukhsh (2007) reported GC-rich sites in NOR of *Rita rita* (Hamilton) and *Mystus gulio* (Hamilton) using CMA_3 staining and scanning electron microscopy.

The presence of single NOR pair in fishes, in general, was considered to be plesiomorphic or a primitive condition and multiple NORs as apomorphic or derived condition (Gold & Amemiya, 1986). In view of this fact, the NOR signals, as detected by $AgNO_3$ and CMA_3, were present on only two pairs of chromosomes in *T. khudree, T. mussullah* and *T. putitora*; therefore, this character could be considered comparatively primitive one when compared with *T. chelynoides*, *T. progeneius* and *N. hexagonolepis* where NORs were present on three pairs and also with *T. tor* where signals were observed on five pairs of chromosomes. Furthermore, several studies in other organisms (Jakubczak *et al*., 1990, 1992; Eickbush *et al*., 1997) suggested that the presence of transposable elements adjacent to ribosomal genes can serve as point for transposition of rDNA regions and their integration into other part of the genome. Hence, the replicative as well as the non-replicative transposition may be the reason for increase in numbers and differences in the position of NORs in these mahseer species from the primitive ones during evolution.

The geographical distribution pattern shows *T. putitora* are predominantly distributed all along the Himalaya, whereas *T. chelynoides* is found in Himalaya as far east as Assam and in the Ganga River also. *Tor progeneius* and *N. hexagonolepis* are found in north-east Himalaya and eastern Himalaya and Assam region, respectively. *Tor tor* is distributed in sub-Himalayan regions within the Ganga and Narmada River systems. The *T. khudree* is found in Madhya Pradesh, Deccan and entire peninsular region. *T. mussullah* is found in the peninsular part of India, mainly in Rivers Krishna, Godavari and their tributaries (Talwar & Jhingran, 1991). The geographical distribution *T. putitora* and *T. chelynoides* are similar, whereas *T. mussullah* may be closer to *T. khudree*.

Detailed information related to the differences in chromosome morphology and the position as well as number of NORs in mahseer species suggested that these cytotaxonomic markers could be utilized in identification and characterization of these closely related species and their hybrids. Although the numeric chromosomal changes have not been observed in the evolution of mahseer species, the structural rearrangements might have played an important role in the speciation of this mahseer group. Moreover, the karyological data supplemented with technological progress in molecular cytogenetics will offer important evidences for the hierarchical classification and, possibly, for the recognition of phylogenetic relationships among these mahseer fish. Furthermore, a deeper insight into the polyploidy condition and their evolution will probably be achieved by both accurate nuclear DNA measurements and molecular genetic analysis.

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