

# Molecular cytogenetic analyses of *Epinephelus bruneus* and *Epinephelus moara* (Perciformes, Epinephelidae)

Guo Minglan<sup>1,2</sup>, Wang Shifeng<sup>3</sup>, Su Yongquan<sup>2</sup>, Zhou Yongcan<sup>3</sup>, Liu Min<sup>2</sup> and Wang Jun<sup>2</sup>

<sup>1</sup> Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, Guangdong, PR China

<sup>2</sup> College of Ocean and Earth Sciences, Xiamen University, Xiamen, Fujian, PR China

<sup>3</sup> Hainan Key Laboratory for Sustainable Utilization of Tropical Bioresource, Hainan University, Haikou, Hainan, PR China

## ABSTRACT

Genus *Epinephelus* (Perciformes, Epinephelidae), commonly known as groupers, are usually difficult in species identification for the lack and/or change of morphological specialization. In this study, molecular cytogenetic analyses were firstly performed to identify the closely related species *Epinephelus bruneus* and *E. moara* in this genus. The species-specific differences of both fish species showed in karyotype, chromosomal distribution of nucleolar organizer regions (NORs) and localization of 18S rDNA. The heterochromatin (interstitial C-bands) and distribution pattern of telomere (TTAGGG)<sub>n</sub> in *E. bruneus* revealed the chromosomal rearrangements and different karyotypic evolutionary characteristics compared to those in *E. moara*. The cytogenetic data suggested that the lineages of *E. bruneus* and *E. moara* were recently derived within the genus *Epinephelus*, and *E. moara* exhibited more plesiomorphic features than *E. bruneus*. All results confirmed that *E. moara*, which has long been considered a synonym of *E. bruneus*, is a distinct species in the family Epinephelidae. In addition, molecular cytogenetic analyses are useful in species differentiation and phylogenetic reconstruction in groupers.

Submitted 2 April 2014

Accepted 13 May 2014

Published 10 June 2014

Corresponding author

Wang Jun, junw@xmu.edu.cn

Academic editor

Linsheng Song

Additional Information and  
Declarations can be found on  
page 11

DOI 10.7717/peerj.412

© Copyright  
2014 Guo et al.

Distributed under  
Creative Commons CC-BY 4.0

**OPEN ACCESS**

**Subjects** Aquaculture, Fisheries and Fish Science, Evolutionary Studies, Genetics, Marine Biology, Taxonomy

**Keywords** Species-specific, Fish, Cytogenetics, Chromosomes, Taxonomy, Evolution

## INTRODUCTION

The family Epinephelidae comprises approximately 163 grouper species in 16 genera (*Craig, Sadovy de Mitcheson & Heemstra, 2011*). These species are of considerable economic value, especially in the coastal fisheries of tropical and subtropical areas (*Heemstra & Randall, 1993*). Taxonomic confusion in the Epinephelidae often occurs due to similarities of color patterns and ontogenetic changes in color (*Heemstra & Randall, 1993; Craig, Sadovy de Mitcheson & Heemstra, 2011*). *Epinephelus bruneus* (Bloch 1793) and *E. moara* (Temminck and Schiegel 1842) are two important aquaculture and commercial fish species. However, *E. moara* has long been considered a synonym of *E. bruneus* due to their similarities in coloration and overlapping in geographical distributions

(Heemstra & Randall, 1993; Craig, Sadovy de Mitcheson & Heemstra, 2011). Designation of correctly identified new species is important not only to the study of phylogenetic relationships, but also to the identification of fry and parent in grouper aquaculture. Based on morphological characteristics and molecular comparisons, *E. moara* has been suggested as a valid species (Guo et al., 2008; Guo et al., 2009; Liu et al., 2013). The interspecific differences between them were identified mainly based on the skeleton system as well as the meristic and morphometric characteristics (Guo et al., 2008). Gene differentiation (Guo et al., 2009) and mitogenome analyses (Liu et al., 2013) partially provided molecular information confirming their taxonomic status. Chromosomes are the carriers of genetic information, and chromosomal recombination plays a vital role in genetic diversity. Therefore, more other evidences are needed to support the hypothesis that *E. moara* is a valid species, such as molecular cytogenetic analyses.

Chromosomes are hereditary elements of the complete nuclear genome. Molecular cytogenetic studies on chromosomes constitute important approaches for characterizing species and reconstructing phylogenetic relationships (Galetti, Aguilar & Molina, 2000; Ocalewicz, Woznicki & Jankun, 2008; Cioffi, Martins & Bertollo, 2010; Ruiz-Herrera, Farre & Robinson, 2012). Karyological features indicate the evolutionary distance between species of different taxonomic categories (Dobigny et al., 2004). The nucleolar organizer regions (NORs) were particularly significant in chromosomal evolutionary analyses (Miller et al., 1976; Fujiwara et al., 1998). Heterochromatin corresponding to C-bandings is normally associated with rearrangements, quantitative variation, and formation of new karyotypes (Miklos & Gill, 1982; Rocco et al., 2002). Many taxonomic studies were based on the variations and polymorphism of the chromosomes containing major 18S rDNA (both active and non-active) (Cioffi, Martins & Bertollo, 2010; Britton-Davidian, Cazaux & Catalan, 2012), and minor 5S rDNA (Fujiwara et al., 1998; Mazzei et al., 2004) by fluorescent *in situ* hybridization (FISH). Location of telomeric sequence (TTAGGG)<sub>n</sub> provides direct evidence for cytotoxic studies and chromosomal evolution in fishes (Sola et al., 2003; Scacchetti et al., 2011). Therefore, molecular cytogenetic information has provided important contributions to the characterization of biodiversity and the evolution of ichthyofauna (Jesus et al., 2003; Vicari et al., 2008). The analyses of different methods above present a complete karyotypic picture for organisms.

The cytogenetic information provided by a variety of approaches will allow us to more fully explain the taxonomic and evolutionary statuses, and reveal the inherent differences of *E. bruneus* and *E. moara*. In this study, the karyotypic techniques, including Giemsa-staining, Ag-staining, C-banding and localization of 18S rDNA and telomere (TTAGGG)<sub>n</sub> by FISH, were used to investigate the molecular cytogenetic characteristics of *E. bruneus* and *E. moara*. Cytogenetic data provided a better definition of the specific epithet for these cryptic species. Molecular cytogenetic analyses were found to be applicable in differentiating between closely related species and reconstructing phylogenetic relationships in groupers.



**Figure 1** Specimen of adult *E. bruneus* and *E. moara*. Specimen of adult *E. bruneus* (Bloch, 1973) [standard length (LS), 550 mm] and *E. moara* (Temminck & Schiegel, 1842) (LS, 650 mm) (Xiamen, Fujian, China, ML Guo).

## MATERIALS AND METHODS

### Fish collection and identification

Twenty-five individuals of *E. bruneus* (standard length,  $L_S$ , 140–550 mm) and 24 individuals of *E. moara* ( $L_S$ , 188–650 mm) were collected alive from the coastal waters of Fujian, China, and reared in laboratory for one week before analyses. Specimen identification was based on external coloration (Fig. 1), skeleton and morphological characteristics established in previous studies (Guo *et al.*, 2008).

### Chromosome preparation, karyotyping and staining analyses

Fishes were injected with colchicine (3  $\mu\text{g/g}$  weight, Sigma) for 30 min. Mitotic chromosomes were obtained from cell suspensions of anterior kidney after the fishes were anesthetized with tricaine methanesulfonate (MS222, 100 mg/L, Sigma), using the conventional air-drying method (Ojima, Hitotsumachi & Makino, 1966). Chromosomes were stained using Giemsa and classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a) based on the arm ratios (Levan, Fredga & Sandberg, 1964). The nucleolar organizer regions (NORs) were visualized by Ag-staining (Howell & Black, 1980). Heterochromatin was identified by C-banding using barium hydroxide method (Sumner, 1972). After the acquisition of anterior kidney, tissue samples (mostly dorsal muscle) were collected and stored at  $-80^\circ\text{C}$ . All experiments were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee at Xiamen University.

### Chromosomal probes preparation

Genomic DNA of all specimens was extracted from muscle tissue using the phenol-chloroform method (Sambrook, Fritsch & Maniatis, 1989). 18S rDNA and 5S rDNA probes for chromosome hybridization were prepared as follows: The partial coding

region of 18S rDNA were amplified using the primers 18S rDNA-F (5'-GTAGTCA TATGCTTGTCTC-3') and 18S rDNA-R (5'-TCCGCAGGTTACCTACGGA-3') as described by *White et al. (1990)*. The coding region of 5S rDNA were obtained using the primers 5S rDNA-F (5'-TACGCCCGATCTCGTCCGATC-3') and 5S rDNA-R (5'-CAGGCTGGTATGGCCGTAAGC-3') indicated by *Martins & Galetti (1999)*. PCR reactions were performed as following: 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 54 °C (for 18S rDNA) or 62 °C (for 5S rDNA) for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The nucleotide sequences of 18S rDNA and 5S rDNA were obtained after cloning into the pMD-18T vector (Takara, Japan), and subjected to Blastn in NCBI database (<http://www.ncbi.nlm.nih.gov>). Telomere probes for chromosome hybridization were prepared as follows: telomeric repeat sequences (TTAGGG)<sub>n</sub> were amplified by PCR using (5'-TTAGGG-3')<sub>5</sub> and (5'-CCCTAA-3')<sub>5</sub> as primers (*Ijdo et al., 1991*). All probes were labeled with biotin-16-dUTP (Roche, Germany) by nick translation according to the manufacturer's instructions.

### Fluorescence *in situ* hybridization (FISH)

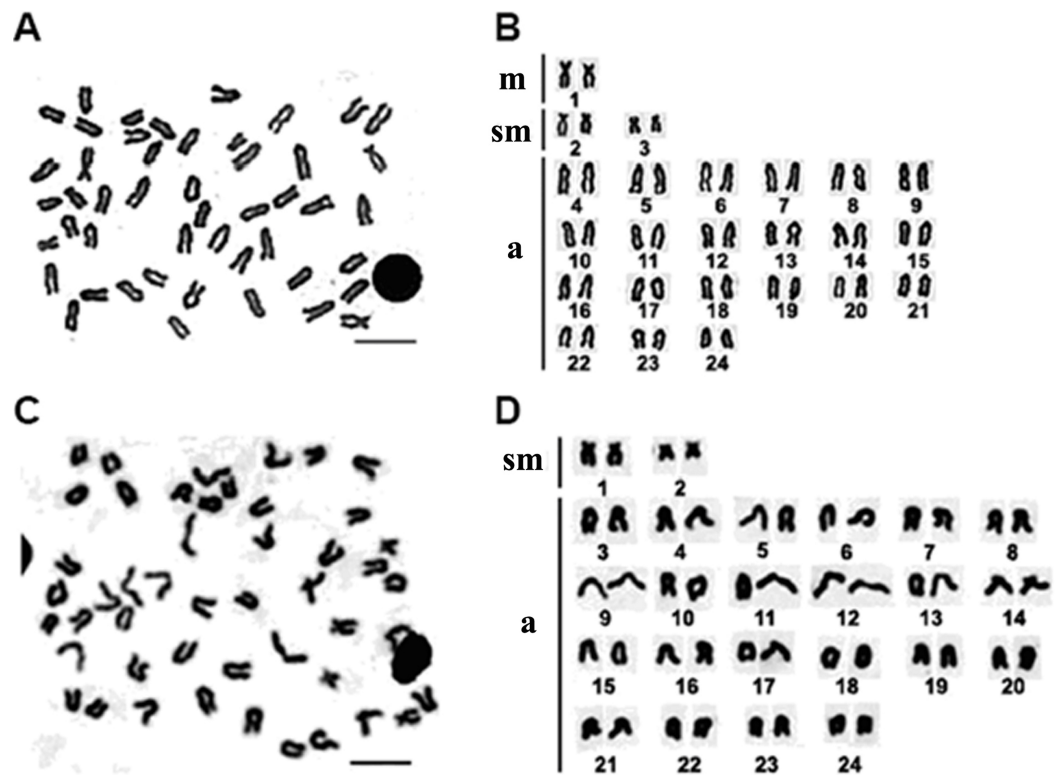
FISH and probe detection were conducted using methods as described previously (*Wang et al., 2010*). Briefly, avidin-fluorescein isothiocyanate (FITC) (Sigma, USA) was used for signal detection of probes 18S rDNA, 5S rDNA and telomere (TTAGGG)<sub>n</sub> based on the manufacturer's instruction. Chromosomes were counterstained with 1 µg/ml 4', 6'-diamidino-2-phenylindole (DAPI) (Roche, USA) in anti-fade solution of 70% glycerol, 2.5% DABCO [1,4-Diazabicyclo (2.2.2) octan], and 1× standard saline concentration (SSC) at pH 8.0. Hybridization signals were observed and analyzed under a fluorescence microscope Leica DM-400CCD.

## RESULTS

### Karyotypes and banding patterns

A total of 172 metaphases of *E. bruneus* and 156 metaphases of *E. moara* were analyzed to determine the karyotype structure. All specimens of *E. bruneus* and *E. moara* invariably showed the same diploid number of chromosomes,  $2n = 48$ . The karyotype formulas of *E. bruneus* and *E. moara* were  $2m + 4sm + 42a$ , giving a fundamental number (NF) equaled to 54 (*Figs. 2A and 2B*), and  $4sm + 44a$ ,  $NF = 52$  (*Figs. 2C and 2D*), respectively. Chromosomes pairs were numbered based on the relative length. The smallest chromosomes pairs No. 24 were submetacentric chromosomes (sm-3 for *E. bruneus* and sm-2 for *E. moara*, *Figs. 2B and 2D*). Chromosome pairs No. 9 in length were sm-2 for *E. bruneus* and sm-1 for *E. moara*. Chromosome pairs No. 2 were metacentric chromosomes m-1 for *E. bruneus*. Other chromosomes were acrocentric (a) chromosomes for both *E. bruneus* and *E. moara*.

Active NORs were identified on the terminal position of short arms or sub-centromere regions of those banded chromosomes. In *E. bruneus*, five actively transcribed NORs were located on the metacentric and submetacentric chromosomes (*Fig. 3A*). In *E. moara*, four Ag-NORs were found on the submetacentric chromosomes (*Fig. 3B*).

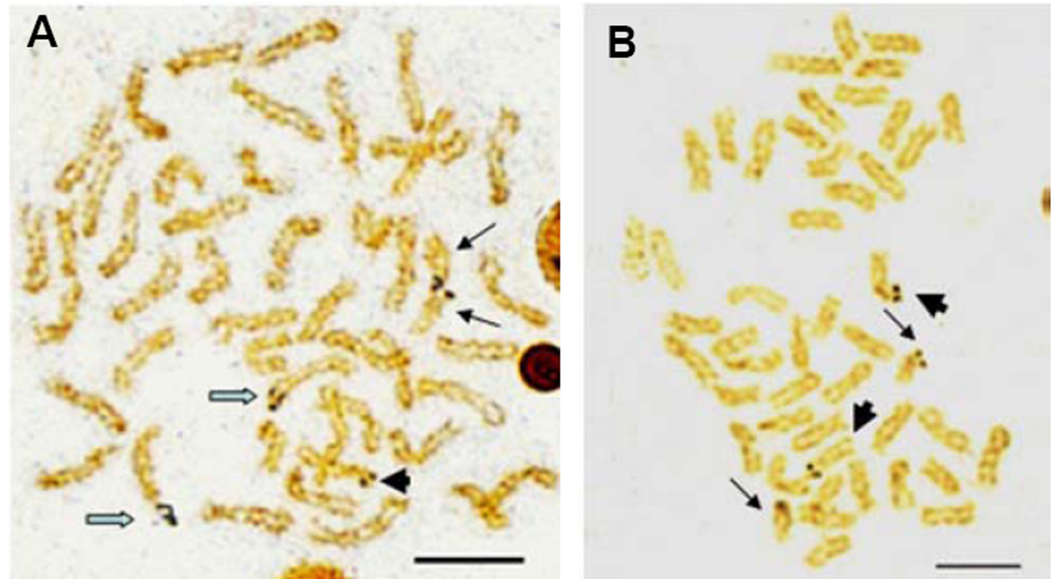


**Figure 2** Chromosome metaphase and corresponding karyotype of *E. bruneus* and *E. moara*. Chromosome metaphase (left, Giemsa staining) and corresponding karyotype (right) of *E. bruneus* (A and B) and *E. moara* (C and D). Scale bar = 5  $\mu$ m. a, acrocentric; m, metacentric; sm, submetacentric.

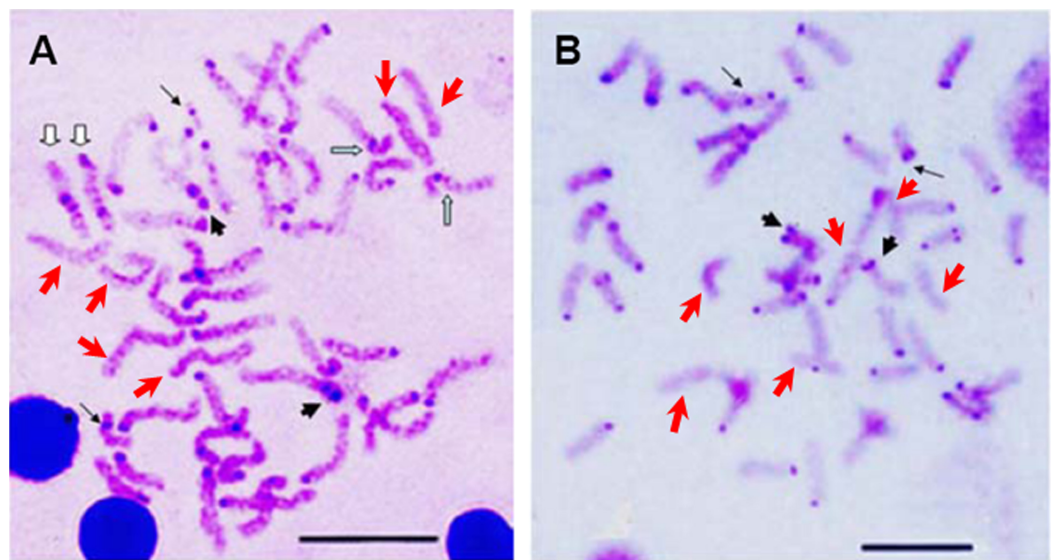
The constitutive heterochromatin was observed in the centromeric and/or pericentromeric region of most chromosomes for both *E. bruneus* and *E. moara*. And the biarmed chromosome pairs with positive Ag-NORs were coinciding with the positive heterochromatin C-bandings. While three pairs of acrocentric chromosomes were almost indiscernible in both fish species (Figs. 4A and 4B). However, the significant differences of heterochromatin were the heterochromatic blocks found in the interstitial region of the long arms of one pair of medium-sized acrocentric chromosome in *E. bruneus* (Fig. 4A).

### Sequences analyses

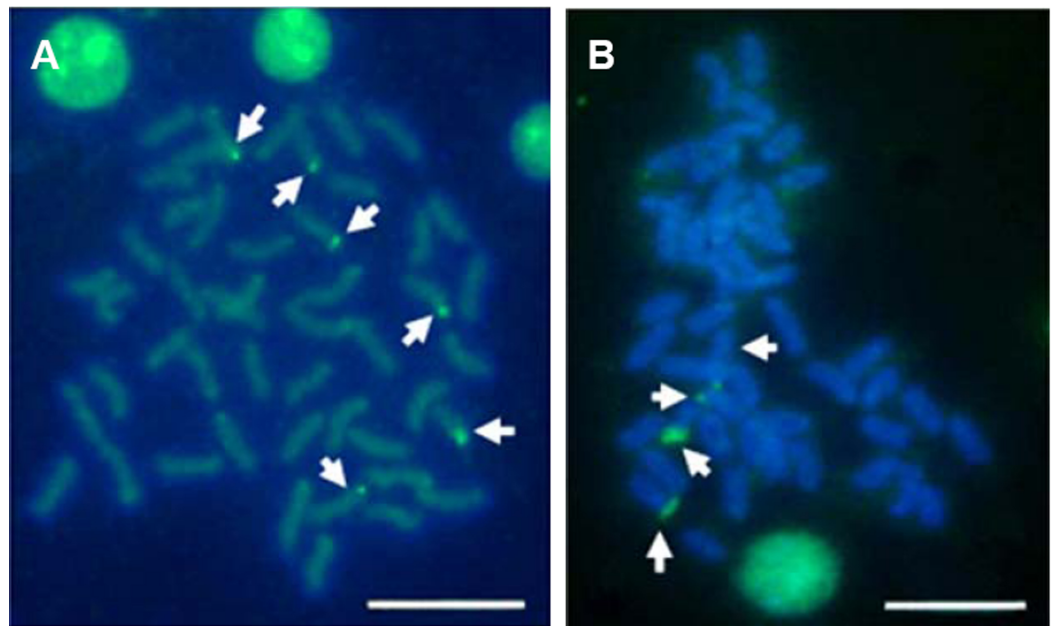
Sequences of 18S rDNA (GenBank accession nos. FJ176793 and FJ176794) and 5S rDNA (GenBank accession nos. FJ176796 and FJ176795) were amplified from genomic DNA of *E. bruneus* and *E. moara*. Sequence of 18S rDNA contained partial DNA of gene 18S rRNA. Partial DNA sequence of 5S rDNA included the encoding and non-transcribed spacer (NTS) region for both fish species. The determined sequences were highly conserved. The nucleotide similarities of partial 18S rDNA and 5S rDNA were 100% and 99.99%, respectively, for both fish species. The phylogenetic neighbor-joining (NJ) trees based on partial sequences of 18S rDNA and 5S rDNA strongly support the closed relationship of *E. moara* and *E. bruneus* (high bootstrap values of 92 and 100). And genera of the order



**Figure 3** Ag-NORs characteristics of *E. bruneus* and *E. moara* with silver staining. Ag-NORs characteristics of *E. bruneus* (A) and *E. moara* (B) with silver staining. Thick black arrows indicate the chromosomes No. 9 in length, thin black arrows represent the chromosomes No. 24 in length, and hollow arrows show the chromosomes No. 1 in length. Scale bar = 5  $\mu$ m.



**Figure 4** C-banding patterns of *E. bruneus* and *E. moara*. C-banding patterns of *E. bruneus* (A) and *E. moara* (B). Heterochromatic blocks were observed in the interstitial region of the long arms of acrocentric chromosome pair No. 12 in *E. bruneus* (white hollow arrows). Heterochromatin C-bands was consistent with the positive Ag-NORs sites on chromosome pair No. 2 in both fish species (thick black arrows). Heterochromatin C-bands were indiscernible for three pairs of chromosomes (Red arrows). Thin black arrows represent the chromosomes No. 24 in length. Scale bar = 5  $\mu$ m.



**Figure 5** Distribution of 18S rDNA on chromosomes of *E. bruneus* and *E. moara* by FISH. Distribution of 18S rDNA on chromosomes of *E. bruneus* (A) and *E. moara* (B) by FISH. White arrows indicate the banded chromosome, pairs No. 2, No. 9 and No. 24 in length, in both fish species. Scale bar = 5  $\mu$ m.

percomorpha were mostly reconstructed the phylogenetic relationship by partial sequences of 18S rDNA but not 5S rDNA (data not shown).

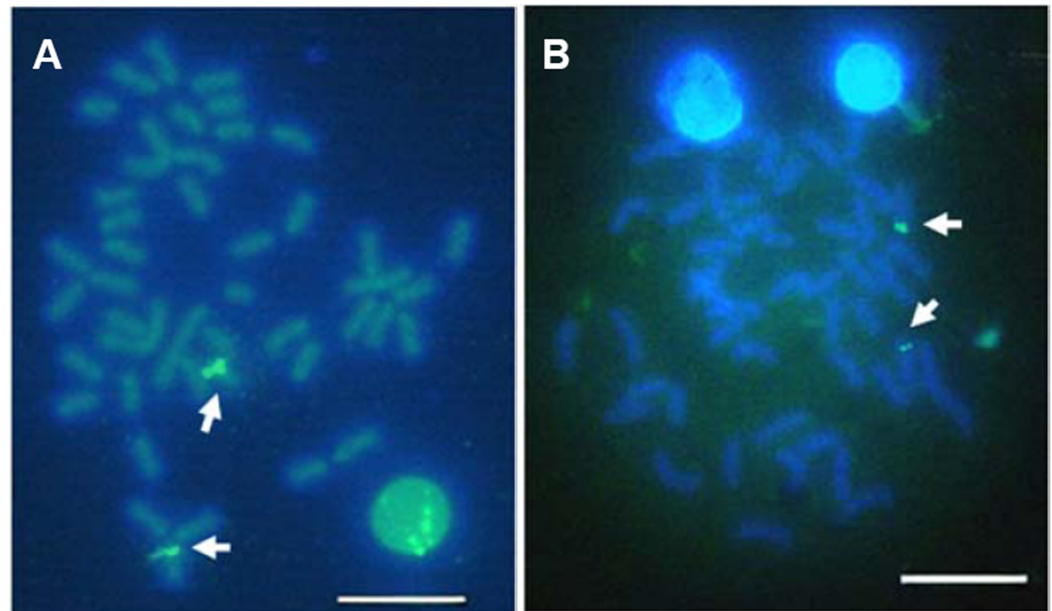
### FISH analyses

Multiple sites of 18S rDNA by FISH confirmed the data obtained by Ag-staining for NORs. In *E. bruneus*, six positive signals (both active and non-active) were identified, corresponding to metacentric (m) and submetacentric (sm) chromosomes (Fig. 5A). Four hybridization signals were observed on the short arms of submetacentric chromosomes in *E. moara* (Fig. 5B). 5S rDNA and 18S rDNA were found on different chromosomes. Two 5S rDNA sites were located on the arms of a medium-sized acrocentric chromosome pair in both *E. bruneus* and *E. moara* (Figs. 6A and 6B).

Telomeric repeats of (TTAGGG)<sub>n</sub> showed the typically telomeric signals on both telomeres and/or centromeric region of all chromosomes in *E. bruneus* and *E. moara*. No positive signal was detected at interstitial sites (Figs. 7A and 7B). Ten chromosome pairs of *E. bruneus* were significantly stronger than the signals of the others (Fig. 7A). However, *E. moara* were characterized by uniform telomeric signals in strength and size (Fig. 7B).

### DISCUSSION

Our previous study has distinguished *E. bruneus* and *E. moara* as two species based on their morphometric and skeletal characteristics (Guo *et al.*, 2008). The species-specific differences showed obviously on the bars of the body and stable skeleton characteristics of adult. And the pyloric caeca indicates their different feeding habits and digestive function, which means they could have different ecological niches. Mitogenome and molecular

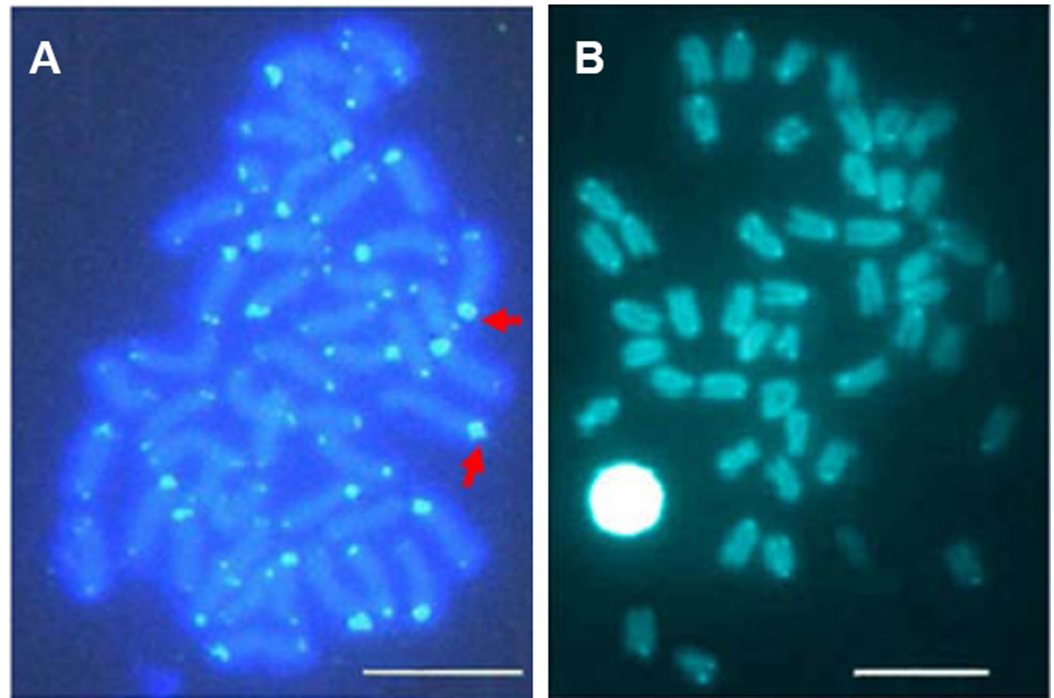


**Figure 6** Localization of 5S rDNA on chromosomes of *E. bruneus* and *E. moara* by FISH. Localization of 5S rDNA on chromosomes of *E. bruneus* (A) and *E. moara* (B) by FISH. White arrows indicate the two 5S rDNA clusters located on the arms of one of acrocentric chromosome pair. Scale bar = 5  $\mu$ m.

comparisons confirmed *E. moara* to be a valid species of the family Epinephelidae (Liu *et al.*, 2013). Further, we developed a molecular method to differentiate both fish species (Guo *et al.*, 2009). However, the cytogenetic backgrounds and evolutionary situation, which is very important to the cultivation and protection of fish resources, remains unclear for *E. bruneus* and *E. moara*. We here comprehensively analyzed the cytogenetic backgrounds, and reconstructed their phylogenetic relationships using molecular cytogenetic analyses.

For *E. bruneus* and *E. moara*, species-specific characteristics presented in karyotype, NORs, C-banding and telomere distribution patterns. Karyotype variation appears to parallel speciation events in many groups of vertebrates (Morescalchi *et al.*, 2007; Ruiz-Herrera, Farre & Robinson, 2012). Variations of NOR constituted a strong cytotaxonomic character in fishes (Fujiwara *et al.*, 1998; Galetti, Aguilar & Molina, 2000). Many species in genus *Epinephelus* showed the same karyotypic characteristics, such as karyotype formula and NORs (Wang *et al.*, 2012). However, karyotype formula and NORs were different between *E. bruneus* and *E. moara* (Table 1). Further, interstitial C-bandings were observed in *E. bruneus*, but not in *E. moara*. Similar interstitial heterochromatin was also found in *E. coioides* (Wang *et al.*, 2010) and *Diplectrum radiale* (de Aguilar & Galetti, 1997). The distribution patterns of (TTAGGG)<sub>n</sub> were different obviously between *E. bruneus* and *E. moara*. Cytogenetic differences were inter-specific, because *E. bruneus* and *E. moara* showed a similar geographical distribution (Heemstra & Randall, 1993; Guo *et al.*, 2009), and coupled with heterogeneously morphological characteristics (Guo *et al.*, 2008) and chromosomal structure.





**Figure 7** Distribution of telomeric (TTAGGG) $n$  sequence on chromosomes of *E. bruneus* and *E. moara* by FISH. Distribution of telomeric (TTAGGG) $n$  sequence on chromosomes of *E. bruneus* (A) and *E. moara* (B) with telomeric (TTAGGG) $n$  sequence using FISH. Red arrows indicate chromosomes with significantly stronger and larger telomeric signals than others in *E. bruneus*. Scale bar = 5  $\mu$ m.

The cytogenetic analyses suggested that the lineages of *E. bruneus* and *E. moara* recently derived within the genus *Epinephelus*. Both fish species share a uniform number of chromosomes to other species in the genus *Epinephelus* (Wang *et al.*, 2012). However, they contained more biarmed chromosomes such as metacentric and/or submetacentric chromosomes (Table 1). In fishes, 48 uni-armed chromosome types like acrocentric chromosomes represented the ancestral complement of diploid origin (Ohno, 1974; Vitturi *et al.*, 1991; Sola *et al.*, 2000). In addition, most species in genus *Epinephelus* showed a conserved, NOR-bearing chromosome pair No. 24 (Table 1), while *E. bruneus* and *E. moara* showed additional NORs on chromosome pairs. For most vertebrates, the presence of a single NOR pair seems to be an ancestral character state (Hsu & Pardue, 1975; Schmid, 1978; Galetti, Molina & Affonso, 2006). Both *E. bruneus* and *E. moara* show even more constitutive heterochromatin (related to chromosomal rearrangements or variation) than other species in *Epinephelus* (Sola *et al.*, 2000; Molina, Maia-Lima & Affonso, 2002; Phillips & Rab, 2001; Wang *et al.*, 2012).

Moreover, *E. moara* exhibited more plesiomorphic features than *E. bruneus*. The fundamental number (FN) of *E. bruneus* is larger than that of *E. moara*. Species with a larger FN are more derived in evolutionary terms (Martinez *et al.*, 1989; Ghigliotti *et al.*, 2007). Chromosomal rearrangements and genomic modifications were more obviously in *E. bruneus* compared to those in *E. moara*. Interstitial C-bandings appeared in *E. bruneus* imply the karyotypic rearrangement (Galetti, Aguilar & Molina, 2000),

**Table 1** Available cytogenetic data of the genus *Epinephelus*.

Species	2n	Karyotype formula	FN	NORs	C-banding	Reference
<i>E. adscensionis</i>	48	48a	48	SCR(24) <sup>a</sup> TR(2)	C(1-24)	(Molina, Maia-Lima & Affonso, 2002)
<i>E. akaara</i>	48	5st + 43a	48	/	/	(Wang et al., 2004)
<i>E. alexandrinus</i>	48	48a	48	SCR(24) <sup>a</sup>	NC(1-23), SCR(24) <sup>a</sup>	(Martinez et al., 1989)
<i>E. awoara</i>	48	48a	48	SCR(24)	NC(1-23), SCR(24) <sup>a</sup>	(Wang et al., 2012)
<b><i>E. bruneus</i></b>	<b>48</b>	<b>2m + 4sm + 42a</b>	<b>54</b>	<b>SCR(24, 9, 2)</b>	<b>NC (?)</b> <b>C (?)</b> <b>SCR(24,9,2)</b> <b>SA(2)</b> <b>IR (?)</b>	<b>Present study</b>
<i>E. caninus</i>	48	48a	48	SCR(24)	/	(Rodríguez-daga, Amores & Thode, 1993)
<i>E. coioides</i>	48	2sm + 46a	50	EA(24)	C(1-11, 13-24) SCR(5,12) EA(24)	(Wang et al., 2010)
<i>E. diacanthus</i>	48	2sm + 46a	50	/	/	(Natarajan & Subrahmanyam, 1974)
<i>E. fario</i>	48	4m + 6sm + 4st + 34a	62	/	/	(Zheng, Liu & Li, 2005)
<i>E. fasciatomaculosus</i>	48	48a	48	SCR(24)	/	(Li & Peng, 1994)
<i>E. fasciatus</i>	48	48a	48	SCR(24)	/	(Li & Peng, 1994)
<i>E. fuscoguttatus</i>	48	2sm + 46a	50	/	/	(Liao et al., 2006)
<i>E. guaza</i>	48	48a	48	SCR(24) <sup>a</sup>	NC(1-23) SCR(24) <sup>a</sup>	(Martinez et al., 1989)
<i>E. guttatus</i>	48	48a	48	/	/	(Medrano et al., 1988)
<i>E. lanceolatus</i>	48	4st + 44a	48	/	/	(Wang et al., 2003)
<i>E. malabaricus</i>	48	48a	48	SCR(24) <sup>a</sup> ? (5)	C(1-24) EA(24)	(Zou, Yu & Zhou, 2005)
<i>E. marginatus</i>	48	48a	48	SCR(24), TR(2)	C(1-24) SCR(24) <sup>a</sup> TR(2)	(Sola et al., 2000)
<i>E. merra</i>	48	4m + 6sm + 4st + 34a	62	/	/	(Zheng, Liu & Li, 2005)
<b><i>E. moara</i></b>	<b>48</b>	<b>4sm + 44a</b>	<b>52</b>	<b>SCR(24, 9)</b>	<b>NC(?)</b> <b>C(?)</b> <b>TR(?)</b>	<b>Present study</b>
<i>E. sexfasciatus</i>	48	2sm + 46a	50	/	/	(Chen et al., 1990)
<i>E. tauvina</i>	48	2sm + 46a	50	/	/	(Raghumath & Prasad, 1980)

**Notes.**

2n, diploid number; a, acrocentrics; C, centromeric; EA, nearly the entire arm; FN, fundamental number; IR, interstitial region; m, metacentrics; NC, almost indiscernible; NORs, nucleolar organizer regions; SA, short arm; sm, submetacentrics; st, subtelocentric; SCR, subcentromeric region; TR, telomeric region; /, not available; ?, not mentioned or measured; Numbers in parentheses, the number of chromosome pairs.

<sup>a</sup> Data estimated from illustrations and text in the respective papers.

robertsonian rearrangements and/or reciprocal translocations (Eler et al., 2007). Despite the conservation of (TTAGGG)<sub>n</sub> sequence and location, slight changes in the telomeric sequences have occurred during vertebrate evolution (Meyne et al., 1990). Uniform telomeric distribution in *E. moara* is similar to other species in *Epinephelus* (Table 1) (Sola et al., 2000; Wang et al., 2012). However, remarkably high repetitions of telomere sequences seem to exist on ten chromosome pairs with stronger signals in

*E. bruneus*, which appear to involve complex homologous or/and non-homologous recombination.

18S rDNA could be simultaneously applicable in the taxonomic and evolutionary analyses of groupers. The 5S rDNA seems to be unsuitable in the phylogenetic resolution, because the order percomorpha in the NJ trees were not recovered as monophylum. Distribution patterns of 5S rDNA of *E. bruneus* and *E. moara* are similar to other species, while that of 18S rDNA were different among species in genus *Epinephelus* (Sola et al., 2000; Wang et al., 2010; Wang et al., 2012). In addition, the different distributions of highly conserved 18S rDNA and telomere suggest the distinct genomes and evolutionary situation of the closely related species *E. bruneus* and *E. moara*.

In summary, many useful cytogenetic characteristics are available to distinguish *E. bruneus* from *E. moara*, such as karyotypes, NORs, C-banding, 18S rDNA and telomere (TTAGGG)<sub>n</sub> distribution patterns. Moreover, the lineage of *E. bruneus* and *E. moara* seems to be derived recently, and *E. moara* exhibits more plesiomorphic features than *E. bruneus*. Molecular cytogenetic analyses could be applicable in identification of closely related species and reconstruct their phylogenetic relationships in groupers.

## ACKNOWLEDGEMENTS

The authors wish to thank Miss Yan Cai, Dr. Marleen Perseke, and Dr. Li Zhang for providing language help and suggestions. We thank reviewers for their constructive comments.

## ADDITIONAL INFORMATION AND DECLARATIONS

### Funding

This work was supported by the grants from the Natural Science Foundation of China (40576064, 31001124 and 31060355) and the National Marine Public Welfare Research Project of China (No. 201205025). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### Grant Disclosures

The following grant information was disclosed by the authors:

Natural Science Foundation of China: 40576064, 31001124, 31260644, 31060355.

The National Marine Public Welfare Research Project of China: 201205025.

### Competing Interests

The authors declare there are no competing interests.

### Author Contributions

- Guo Minglan conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables.
- Wang Shifeng performed the experiments, prepared figures and/or tables.
- Su Yongquan analyzed the data, contributed reagents/materials/analysis tools.

- Zhou Yongcan contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Liu Min reviewed drafts of the paper.
- Wang Jun conceived and designed the experiments.

### Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

Institutional Animal Care and Use Committee (IACUC) of Xiamen University: 40576064.

### DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

GenBank: [FJ176793](#) and [FJ176794](#), [FJ176796](#) and [FJ176795](#).

## REFERENCES

- Britton-Davidian J, Cazaux B, Catalan J. 2012.** Chromosomal dynamics of nucleolar organizer regions (NORs) in the house mouse: micro-evolutionary insights. *Heredity* **108**:68–74 DOI [10.1038/hdy.2011.105](#).
- Chen Y, Rong S, Liu S, Zhang H, Pei M. 1990.** Analysis of the karyotype of *Epinephelus sexfasciatus*. *Journal of Zhanjiang Fisheries College* **2**:62–68 (in Chinese with English abstract).
- Cioffi MB, Martins C, Bertollo LAC. 2010.** Chromosome spreading of associated transposable elements and ribosomal DNA in the fish *Erythrinus erythrinus*. Implications for genome change and karyoevolution in fish. *BMC Evolutionary Biology* **10**:271 DOI [10.1186/1471-2148-10-271](#).
- Craig MT, Sadovy de Mitcheson YJ, Heemstra PC. 2011.** *Groupers of the world: a field and market guide*. Grahamstown: NISC (Pty) Ltd, 424.
- de Aguilar CT, Galetti PMJ. 1997.** Chromosomal studies in South Atlantic serranids (Pisces, Perciformes). *Cytobios* **89**:105–114.
- Dobigny G, Ducroz JF, Robinson TJ, Volobouev V. 2004.** Cytogenetics and cladistics. *Systematic Biology* **53**:470–484 DOI [10.1080/10635150490445698](#).
- Eler ES, Dergam JA, Venere PC, Paiva LC, Miranda GA, Oliveira AA. 2007.** The karyotypes of the thorny catfishes *Wertheimeria maculata* Steindachner, 1877 and *Hassar wilderi* Kindle, 1895 (Siluriformes: Doradidae) and their relevance in doradids chromosomal evolution. *Genetica* **130**:99–103 DOI [10.1007/s10709-006-0023-4](#).
- Fujiwara A, Abe S, Yamaha E, Yamazaki F, Yoshida MC. 1998.** Chromosomal localization and heterochromatin association of ribosomal RNA gene loci and silver-stained nucleolar organizer regions in salmonid fishes. *Chromosome Research* **6**:463–471 DOI [10.1023/A:1009200428369](#).
- Galetti PMJ, Aguilar CT, Molina WF. 2000.** An overview of marine fish cytogenetics. *Hydrobiologia* **420**:55–62 DOI [10.1023/A:1003977418900](#).
- Galetti PMJ, Molina WF, Affonso P. 2006.** Assessing genetic diversity of Brazilian reef fishes by chromosomal and DNA markers. *Genetica* **126**:161–177 DOI [10.1007/s10709-005-1446-z](#).

- Ghigliotti L, Mazzei F, Ozouf-Costaz C, Bonillo C, Williams R, Cheng CHC, Pisano E. 2007.** The two giant sister species of the Southern Ocean, *Dissostichus eleginoides* and *Dissostichus mawsoni*, differ in karyotype and chromosomal pattern of ribosomal RNA genes. *Polar Biology* **30**:625–634 DOI [10.1007/s00300-006-0222-6](https://doi.org/10.1007/s00300-006-0222-6).
- Guo M, Su Y, Chen X, Ding S, Wang J. 2008.** Comparative studies on morphology of *Epinephelus moara* and *E. bruneus*. *Acta Oceanologica Sinica* **30**:106–114.
- Guo M, Su Y, Zhang Z, Ding S, Wang J. 2009.** Differentiation of *Epinephelus moara* from *E. bruneus* by improved nest-tetra-primer-specific PCR. *Progress in Natural Science* **19**:1221–1226 DOI [10.1016/j.pnsc.2009.04.003](https://doi.org/10.1016/j.pnsc.2009.04.003).
- Heemstra PC, Randall JE. 1993.** FAO species catalogue: vol. 16. Groupers of the world (Family Serranidae, Subfamily Epinephelinae). In: *FAO Fisheries Synopsis*. Rome: FAO, 378.
- Howell WM, Black DA. 1980.** Controlled silver-staining nucleolus organizer regions with a protective colloidal developer: a 1-step method. *Experientia* **36**:1014–1015 DOI [10.1007/BF01953855](https://doi.org/10.1007/BF01953855).
- Hsu TC, Pardue ML. 1975.** Distribution of 18S + 28S ribosomal genes in mammalian genomes. *Chromosoma* **53**:25–36 DOI [10.1007/BF00329388](https://doi.org/10.1007/BF00329388).
- Ijdo JW, Wells RA, Baldini A, Reeders ST. 1991.** Improved telomere detection using a telomere repeat (TTAGGG)<sub>n</sub> generated by PCR. *Nucleic Acids Research* **19**:Article 4780 DOI [10.1093/nar/19.17.4780](https://doi.org/10.1093/nar/19.17.4780).
- Jesus CM, Galetti PMJ, Valentini SR, Moreira-Filho O. 2003.** Molecular and chromosomal location of two families of satellite DNA in *Prochilodus lineatus* (Pisces, Prochilodontidae), a species with B chromosomes. *Genetica* **118**:25–32 DOI [10.1023/A:1022986816648](https://doi.org/10.1023/A:1022986816648).
- Levan A, Fredga K, Sandberg AA. 1964.** Nomenclature for centromeric position on chromosomes. *Hereditas* **52**:201–220 DOI [10.1111/j.1601-5223.1964.tb01953.x](https://doi.org/10.1111/j.1601-5223.1964.tb01953.x).
- Li XQ, Peng YD. 1994.** Studies on karyotype of *Epinephelus fasciatus* and *Epinephelus fasciatus*. *Journal of Zhanjiang Fisheries College* **14**:22–26 (in Chinese with English abstract).
- Liao JQ, Yin SW, Chen GH, Huang H, Lei CG, Lou TT. 2006.** The karyotype of grouper *Epinephelus fuscoguttatus*. *Fisheries Science* **25**:567–569 (in Chinese with English abstract).
- Liu M, Li JL, Ding SX, Liu ZQ. 2013.** *Epinephelus moara*: a valid species of the family Epinephelidae (Pisces: Perciformes). *Journal of Fish Biology* **82**:1684–1699 DOI [10.1111/jfb.12112](https://doi.org/10.1111/jfb.12112).
- Martinez G, Thode G, Alvarez MC, Lopez JR. 1989.** C-banding and Ag-NOR reveal a certain heterogeneity among karyotypes of serranids (Perciformes). *Cytobios* **58**:53–60.
- Martins C, Galetti PMJ. 1999.** Chromosomal localization of 5S rDNA genes in Leporinus fish (Anostomidae, Characiformes). *Chromosome Research* **7**:363–367 DOI [10.1023/A:1009216030316](https://doi.org/10.1023/A:1009216030316).
- Mazzei F, Ghigliotti L, Bonillo C, Coutanceau JP, Ozouf-Costaz C, Pisano E. 2004.** Chromosomal patterns of major and 5S ribosomal DNA in six icefish species (Perciformes, Notothenioidei, Channichthyidae). *Polar Biology* **28**:47–55.
- Medrano L, Bernardi G, Couturier J, Dutrillaux B. 1988.** Chromosome banding and genome compartmentalization in fishes. *Chromosoma* **96**:178–183 DOI [10.1007/BF00331050](https://doi.org/10.1007/BF00331050).
- Meyne J, Baker RJ, Hobart HH, Hsu TC, Ryder OA, Ward OG, Wiley JE, Wursterhill DH, Yates TL, Moyzis RK. 1990.** Distribution of non-telomeric sites of the (TTAGGG)<sub>n</sub> Telmeric sequence in vertebrate chromosomes. *Chromosoma* **99**:3–10 DOI [10.1007/BF01737283](https://doi.org/10.1007/BF01737283).
- Miklos GLG, Gill AC. 1982.** Nucleotide sequences of highly repeated DNAs: compilation and comments. *Genetics Research* **39**:1–30 DOI [10.1017/S0016672300020711](https://doi.org/10.1017/S0016672300020711).

- Miller DA, Miller OJ, Dev VG, Tantravahi R, Croce CM. 1976.** Expression of human and suppression of mouse nucleolus organizer activity in mouse–human somatic cell hybrids. *Proceedings of the National Academy of Sciences of the United States of America* **73**:4531–4535 DOI [10.1073/pnas.73.12.4531](https://doi.org/10.1073/pnas.73.12.4531).
- Molina WF, Maia-Lima FA, Affonso P. 2002.** Divergence between karyotypical pattern and speciation events in Serranidae fish (Perciformes). *Caryologia* **55**:299–305 DOI [10.1080/00087114.2002.10797880](https://doi.org/10.1080/00087114.2002.10797880).
- Morescalchi MA, Liguori I, Rocco L, Stingo V. 2007.** Karyotypic characterization and genomic organization of the 5S rDNA in *Erpetoichthys calabaricus* (Osteichthyes, Polypteridae). *Genetica* **131**:209–216 DOI [10.1007/s10709-006-9119-0](https://doi.org/10.1007/s10709-006-9119-0).
- Natarajan R, Subrahmanyam K. 1974.** A karyotype study of some teleosts from Portonovo waters. *Proceedings of the Indian Academy of Sciences* **79**:173–196.
- Ocalewicz K, Woznicki P, Jankun M. 2008.** Mapping of rRNA genes and telomeric sequences in *Danube salmon* (Hucho hucho) chromosomes using primed *in situ* labeling technique (PRINS). *Genetica* **134**:199–203 DOI [10.1007/s10709-007-9225-7](https://doi.org/10.1007/s10709-007-9225-7).
- Ohno S. 1974.** Protochordata, cyclostomata and pisces. In: John B, ed. *Animal cytogenetics*. Berlin: Getrüder Borntaerger, 92.
- Ojima YS, Hitotsumachi S, Makino S. 1966.** Cytogenetic studies in lower vertebrates. *Proceedings of the Japan Academy* **42**:62–66.
- Phillips R, Rab P. 2001.** Chromosome evolution in the Salmonidae (Pisces): an update. *Biological Reviews of the Cambridge Philosophical Society* **76**:1–25 DOI [10.1017/S1464793100005613](https://doi.org/10.1017/S1464793100005613).
- Raghumath P, Prasad R. 1980.** Chromosomes of six marine percoids from the Indian Sea. *Indian Biology* **11**:9–12.
- Rocco L, Morescalchi MA, Costagliola D, Stingo V. 2002.** Karyotype and genome characterization in four cartilaginous fishes. *Gene* **295**:289–298 DOI [10.1016/S0378-1119\(02\)00730-8](https://doi.org/10.1016/S0378-1119(02)00730-8).
- Rodríguez-Daga R, Amores A, Thode G. 1993.** Karyotype and nucleolus organizer regions in *Epinephelus canius* (Pisces, Serranidae). *Caryologia* **46**:71–76 DOI [10.1080/00087114.1993.10797248](https://doi.org/10.1080/00087114.1993.10797248).
- Ruiz-Herrera A, Farre M, Robinson TJ. 2012.** Molecular cytogenetic and genomic insights into chromosomal evolution. *Heredity* **108**:28–36 DOI [10.1038/hdy.2011.102](https://doi.org/10.1038/hdy.2011.102).
- Sambrook J, Fritsch EF, Maniatis T. 1989.** Molecular cloning: a laboratory manual. In: *Cold spring harbor*, 2nd edition. New York: Cold Spring Harbor Laboratory Press, 165–209.
- Scacchetti PC, Pansonato-Alves JC, Utsunomia R, Oliveira C, Foresti F. 2011.** Karyotypic diversity in four species of the genus *Gymnotus Linnaeus*, 1758 (Teleostei, Gymnotiformes, Gymnotidae): physical mapping of ribosomal genes and telomeric sequences. *Comparative Cytogenetics* **5**:223–235 DOI [10.3897/compcytogen.v5i3.1375](https://doi.org/10.3897/compcytogen.v5i3.1375).
- Schmid M. 1978.** Chromosome banding in Amphibia? Constitutive heterochromatin and nucleolus organizer regions in Ranidae, Microhylidae and Rhacophoridae. *Chromosoma* **68**:131–148 DOI [10.1007/BF00287145](https://doi.org/10.1007/BF00287145).
- Sola L, De Innocentiis S, Gornung E, Papalia S, Rossi AR, Marino G, De Marco P, Cataudella S. 2000.** Cytogenetic analysis of *Epinephelus marginatus* (Pisces: Serranidae), with the chromosome localization of the 18S and 5S rRNA genes and of the (TTAGGG)(n) telomeric sequence. *Marine Biology* **137**:47–51 DOI [10.1007/s002270000334](https://doi.org/10.1007/s002270000334).
- Sola L, Gornung E, Naoi H, Gunji R, Sato C, Kawamura K, Arai R, Ueda T. 2003.** FISH-mapping of 18S ribosomal RNA genes and telomeric sequences in the Japanese bitterlings

- Rhodeus ocellatus kurumeus* and *Tanakia limbata* (Pisces, Cyprinidae) reveals significant cytogenetic differences in morphologically similar karyotypes. *Genetica* **119**:99–106 DOI [10.1023/A:1024446910161](https://doi.org/10.1023/A:1024446910161).
- Sumner AT. 1972.** A simple technique for demonstrating centromeric heterochromatin. *Experimental Cell Research* **75**:304–306 DOI [10.1016/0014-4827\(72\)90558-7](https://doi.org/10.1016/0014-4827(72)90558-7).
- Vicari MR, Artoni RF, Moreira-Filho O, Bertollo LAC. 2008.** Colocalization of repetitive DNAs and silencing of major rRNA genes. A case report of the fish *Astyanax janeiroensis*. *Cytogenetic and Genome Research* **122**:67–72 DOI [10.1159/000151318](https://doi.org/10.1159/000151318).
- Vitturi R, Catalano E, Loconte MR, Alessi AM, Amico FP, Colombera D. 1991.** Intra-populational and intra-individual mosaicisms of *Uranoscopus scaber* L. (Perciformes, Uranoscopidae). *Heredity* **67**:325–330 DOI [10.1038/hdy.1991.96](https://doi.org/10.1038/hdy.1991.96).
- Wang SF, Cai Y, Qin YX, Zhou YC, Su YQ, Wang J. 2012.** Characterization of yellow grouper *Epinephelus awoara* (Serranidae) karyotype by chromosome bandings and fluorescence *in situ* hybridization. *Journal of Fish Biology* **80**:866–875 DOI [10.1111/j.1095-8649.2012.03230.x](https://doi.org/10.1111/j.1095-8649.2012.03230.x).
- Wang S, Su Y, Ding S, Cai Y, Wang J. 2010.** Cytogenetic analysis of orange-spotted grouper, *Epinephelus coioides*, using chromosome banding and fluorescence *in situ* hybridization. *Hydrobiologia* **638**:1–10 DOI [10.1007/s10750-009-9980-9](https://doi.org/10.1007/s10750-009-9980-9).
- Wang DX, Su YQ, Wang SF, Qin YX, Guo F. 2003.** Study on the karyotype of *Promicrops lanceolatus*. *Journal of Oceanography in Taiwan Strait* **22**:465–469 (in Chinese with English abstract).
- Wang YX, Wang HD, Zhang HF, Liu-Fu YZ. 2004.** Karyotypes of *Epinephelus coioides* and *Epinephelus akaara*. *Journal of Zhanjiang Ocean University* **24**:4–8 (in Chinese with English abstract).
- White TJ, Bruns T, Lee S, Taylor J. 1990.** Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, eds. *PCR protocols: a guide to methods and applications*. New York: Academic Press, 315–322.
- Zheng L, Liu CW, Li CL. 2005.** Studies on the karyotype of 4 groupers. *Marine Science* **29**:51–55 (in Chinese with English abstract).
- Zou JX, Yu QX, Zhou F. 2005.** The karyotypes, C-bands patterns and Ag-NORs of *Epinephelus malabaricus*. *Journal of Fisheries of China* **29**:33–37 (in Chinese with English abstract).