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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 chromosomal karyotypes, nucleolar organizer regions (NORs) distribution pattern and location of 18S rDNA on chromosome. The heterochromatin (interstitial C-bands) and distribution pattern of telomere $(TTAGGG)_n$ 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 new species in the family Epinephelidae. In addition, molecular cytogenetic analyses are useful in species differentiation and phylogenetic reconstruction in groupers.

Keywords: cytogenetics, fish, chromosomes, taxonomy, species-specific, 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 (Craig, Sadovy de Mitcheson & Heemstra, 2011; Heemstra & Randall, 199[illegible] *Epinephelus bruneus* (Bloch 1793) and *E. moara* (Temminck & 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 (Craig, Sadovy de Mitcheson & Heemstra, 2011; Heemstra & Randall, 1993). 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 characters and molecular comparisons, *E. moara* has been suggested as a valid species (Guo et al., 2008, 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 characters (Guo et al., 2008). However, more other evidences are needed to support the hypothesis that *E. moara* is a valid species, such as molecular cytogenetic analysis.

Chromosomes are hereditary elements of the complete nuclear genome. Molecular cytogenetic studies on chromosomes constitute important approaches for characterizing species and reconstructing phylogenetic relationships (Coiffi, Martins & Bertollo, 2010; Galetti, Aguilar & Molina, 2000; Ocalewicz, Woznicki & Jankun, 2008; 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 analysis (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) (Britton-Davidian, Cazaux & Catalan, 2012; Cioffi, Martins & Bertollo, 2010), and minor 5S rDNA (Fujiwara et al., 1998; Mazzei et al., 2004) by fluorescent in situ hybridization (FISH). Location of telomeric sequence $(TTAGGG)_n$ provides direct evidence for cytotaxonomic 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).

In this study, the karyotypic techniques, including Giemsa-staining, Ag-staining, C-banding and localization of 18S rDNA and telomere $(TTAGGG)_n$ by FISH, were conducted to investigate the molecular cytogenetic characters of *E. bruneus* and *E. moara*. Molecular cytogenetic analyses were found to be applicable in differentiating between closely related species and reconstructing phylogenetic relationships in groupers.

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 analysis. Specimen identification was based on external coloration (Fig. 1), skeleton and morphological characters established in previous studies (Guo et al., 2008).

Chromosome preparation, karyptyping and staining analysis

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 anatomy of fishes for the acquisition of anterior kidney, muscle tissues and fishes were stored immediately at -80℃ until used for total DNA extraction (The research was approved by the National Natural Science Foundation of China, 40576064).

Chromosomal probes preparation

Genomic DNA of all specimens was extracted from muscle tissue using the phenol-chloroform method (Sambrook, Fritsch & Maniatis, 1989). 18S rDNA probes for chromosome hybridization were prepared as follows: primers used for 18S rDNA amplification were 18S rDNA-F (5'-GTAGTCATATGCTTGTCTC-3') and 18S rDNA-R (5'-TCCGCAGGTTCACCTACGGA-3') (White et al., 1990), and 5S rDNA-F (5'-TACGCCCGATCTCGTCCGATC-3') and 5S rDNA-R (5'- CAGGCTGGTATGGCCGTAAGC -3') (Martins & Galetti, 1999). PCR reactions were performed as following: 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 sec, 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 $(TTAGGG)_n$ were amplified by PCR using $(5'\text{-}TTAGGG\text{-}3')_5$ and $(5'\text{-}CCCTAA\text{-}3')_5$ 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, 5SrDNA and telomere $(TTAGGG)_n$ based on the manufacturer's instruction. Chromosomes were counterstained with 1 μg/ml 4', 6'-diamidino-2-phenylin-dole (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 karyotypes of *E. bruneus* and *E. moara* were 2M+4SM+42A, giving a fundamental number (NF) equaled to 54 (Fig. 2A and B), and 4SM+44A, NF=52 (Fig. 2C and D), 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*, Fig. 2B and D). 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 two-armed 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).

The constitutive heterochromatin was observed in the centromeric and/or pericentromeric region of most chromosomes for both *E. bruneus* and *E. moara*. And the two-armed 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 (Fig. 4A and B). 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 analysis

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

FISH analysis

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). Two ribosomal gene families are located on different chromosome pairs. Two 5S rDNA sites were located on the arms of a medium-sized acrocentric chromosome pair in both *E. bruneus* and *E. moara* (Fig. 6A and B).

Telomeric repeats of $(TTAGGG)_n$ 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 (Fig. 7A and B). 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* to be two species based on the morphometric and skeleton characters (Guo et al., 2008). The species-specific differences showed obviously on the bars of the body and stable skeleton characters 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 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 analysis.

For *E. bruneus* and *E. moara*, species-specific characters presented in karyotype, NORs, C-banding and telomere distribution patterns. Karyotype variation appears to parallel speciation events in many groups of vertebrates (Ruiz-Herrera, Farre & Robinson, 2012; Morescalchi et al., 2007). Variations of NOR constituted a strong cytotaxonomic character in fishes (Galetti, Aguilar & Molina, 2000; Fujiwara et al., 1998). Many species in genus *Epinephelus* showed the same karyotypic characters, 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, 1997). The distribution patterns of $(TTAGGG)_n$ 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, and coupled with heterogeneously morphological characters and chromosomal structure.

The cytogenetic analysis 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 two-armed 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

198 additional NORs on chromosome pairs. For most vertebrates, the presence of a single NOR pair
199 seems to be an ancestral character state (Galetti, Molina & Affonso, 2006; Hsu & Pardue, 1975;
200 Schmid, 1978). Both *E. bruneus* and *E. moara* show even more constitutive heterochromatin
201 (related to chromosomal rearrangements or variation) than other species in *Epinephelus* (Wang et
202 al., 2012; Sola et al., 2000; Molina et al., 2002; Phillips & Rab, 2001).

203 Moreover, *E. moara* exhibited more plesiomorphic features than *E. bruneus*. The
204 fundamental number (FN) of *E. bruneus* is larger than that of *E. moara*. Species with a larger FN
205 are more advanced in evolutionary terms (Martinez et al., 1989; Ghigliotti et al., 2007).
206 Chromosomal rearrangements and genomic modifications were more obviously in *E. bruneus*
207 compared to those in *E. moara*. Interstitial C-bandings appeared in *E. bruneus* imply the
208 karyotypic rearrangement (Galetti, Aguilar & Molina, 2000), robertsonian rearrangements and/or
209 reciprocal translocations (Eler et al., 2007). In despite of the conservation of (TTAGGG)n
210 sequence and location, slight changes in the telomeric sequences have occurred during vertebrate
211 evolution (Meyne et al., 1990). Uniform telomeric distribution in *E. moara* is similar to other
212 species in *Epinephelus* (Table 1) (Wang et al., 2012; Sola et al., 2000). However, remarkable
213 high repetitions of telomere sequence seems to exist on ten chromosome pairs with stronger
214 signals in *E. bruneus*, which appears to involve in complex homologous or/and non-homologous
215 recombination.

216 18S rDNA could be simultaneously applicable in the taxonomic and evolutionary analysis
217 of grouper. The 5S rDNA seems to be not suitable in the phylogenetic resolution, because the
218 order percomorpha in the NJ trees were not recovered as monophylum. Distribution patterns of
219 5S rDNA of *E. bruneus* and *E. moara* are similar to other species, while that of 18S rDNA were
220 different among species in genus *Epinephelus* (Wang et al., 2010, 2012; Sola et al., 2000). In
221 addition, the different distributions of highly conserved18S rDNA and telomere suggest the
222 distinct genomes and evolutionary situation of the closely related species *E. bruneus* and *E.*
223 *moara*.

224 In summary, many useful cytogenetic charater are available to distinguish *E. bruneus* from
225 *E. moara*, such as karyptypes, NORs, C-banding, 18S rDNA and telomere $(TTAGGG)_n$

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 analysis 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. This work was supported by the grants from the Natural Science Foundation of China (40576064, 31001124 and 31060355). We thank reviewers for their constructive comments.

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377

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

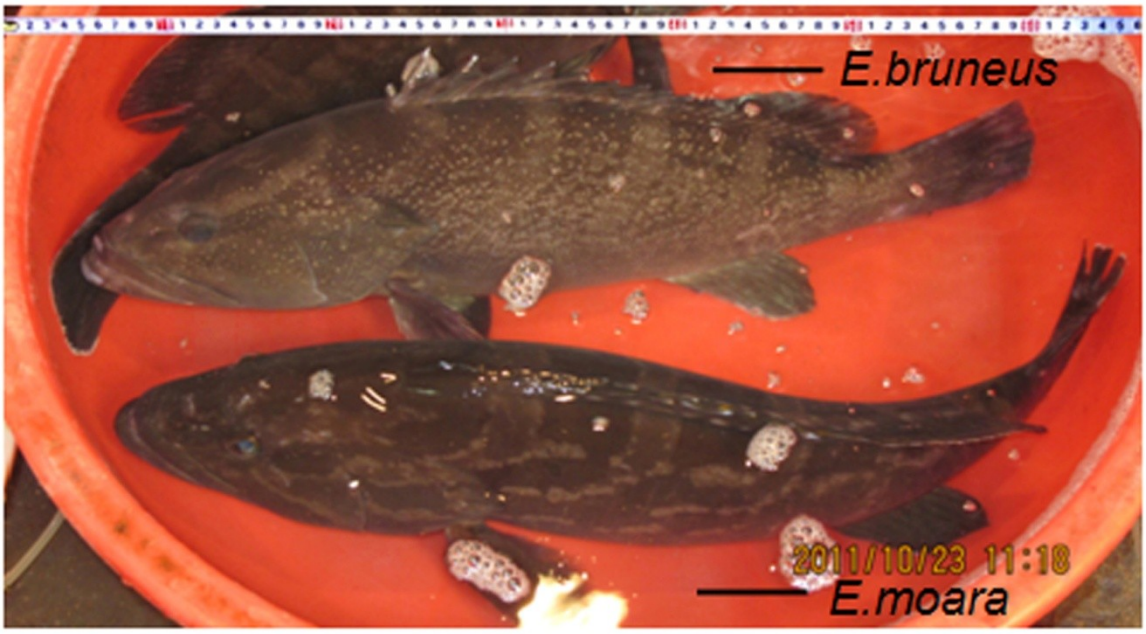

Figure 2 Chromosome metaphase (left, Giemsa staining) and corresponding karyotype (right) of *E. bruneus* (A and B) and *E. moara* (C and D). Scale bar=5 μm. A: acrocentric, M: metacentric, SM: submetacentric.

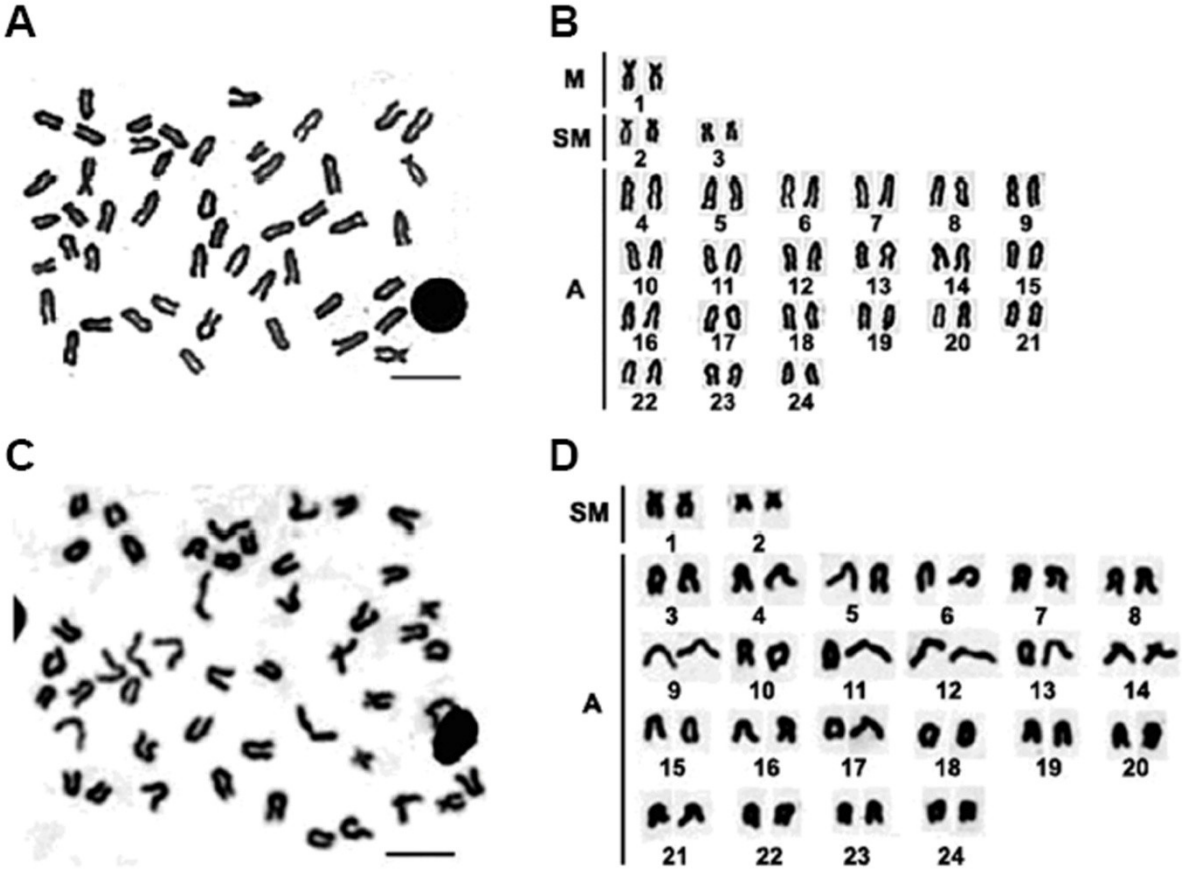

Figure 3 Ag-NORs characteristics of *E. bruneus* (A) and *E. moara* (B) with silver staining. Thick black arrows indicate the chromosomes No.9 in length, thin black arrows represent the chromosomes No.24 in length, and hollow arrows show the chromosomes No.1 in length. Scale bar=5 μm.

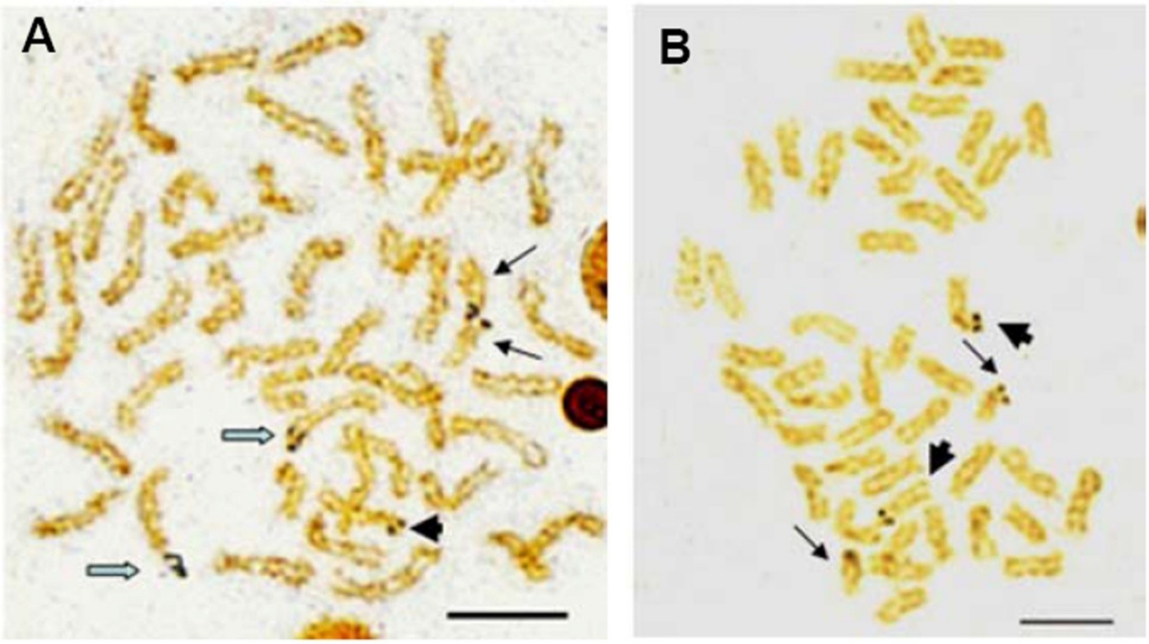

Figure 4 C-banding patterns of *E.bruneus* (A) and *E.moara* (B). Heterochromatic blocks were observed in the interstitial region of the long arms of acrocentric chromosome pair No.12 in *E. bruneus* (white hollow arrows). Heterochromatin C-bands was consistent with the positive Ag-NORs sites on chromosome pair No.2 in both fish species (Thick black arrows). Other arrow annotated with Fig.3. Scale bar=5μm.

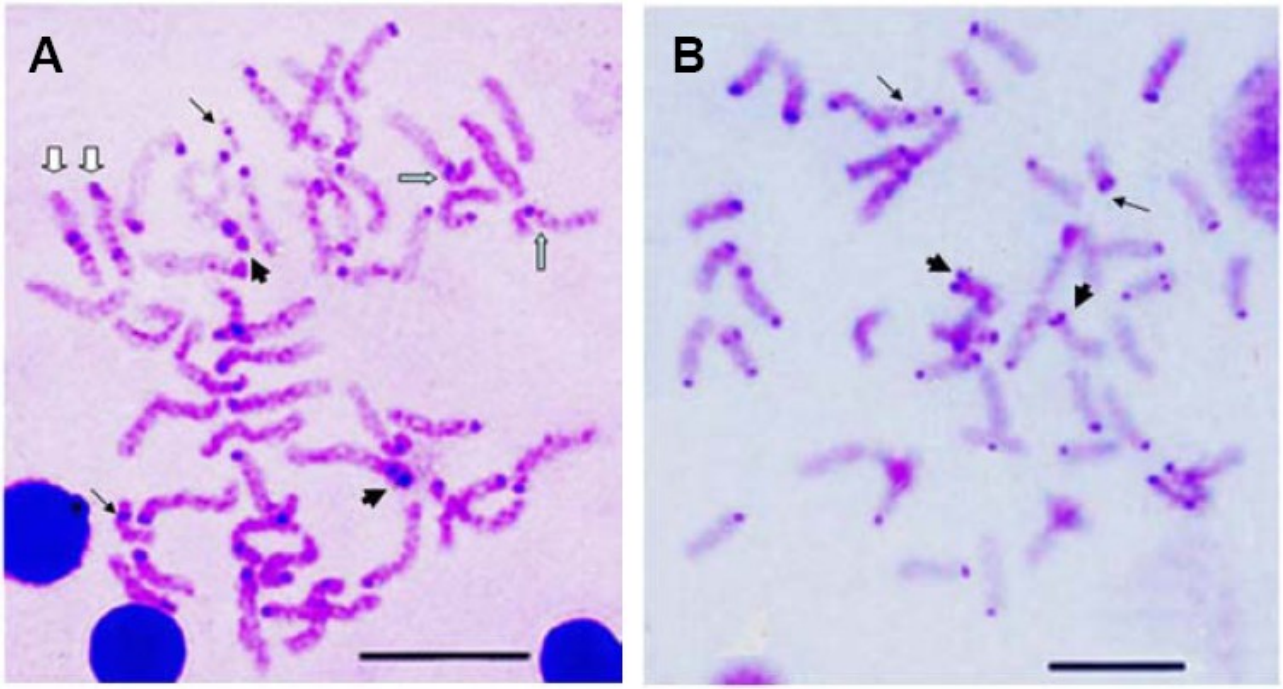

Figure 5 Distribution of 18S rNDA by FISH on chromosomes of *E. bruneus* (A) and *E. moara* (B). White arrows indicate the two-armed chromosome, pairs No.2, No.9 and No.24 in length, in both fish species. Scale bar=5μm.

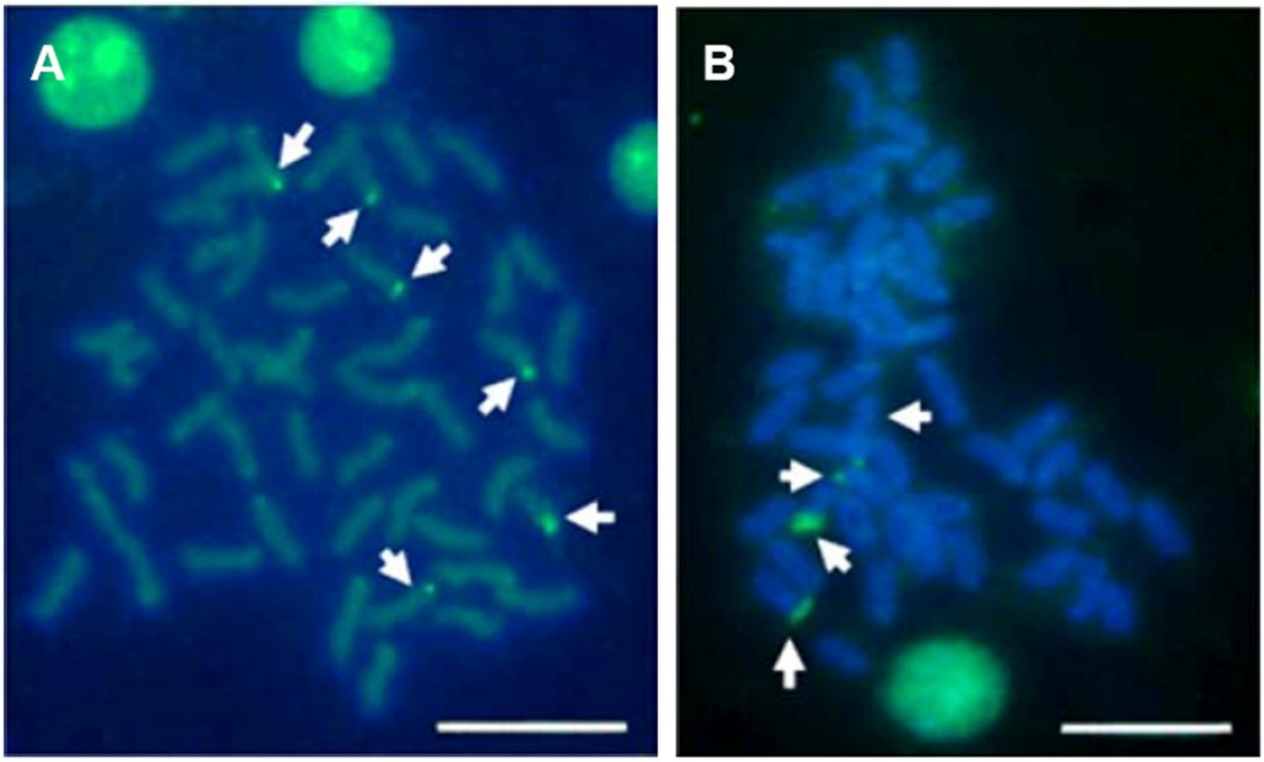

Figure 6 Metaphase plates of *E. bruneus* (A) and *E. moara* (B) with 5S rNDA by FISH. White arrows indicate the two 5S rDNA clusters located on the arms of one of acrocentric chromosome pair. Scale bar=5μm.

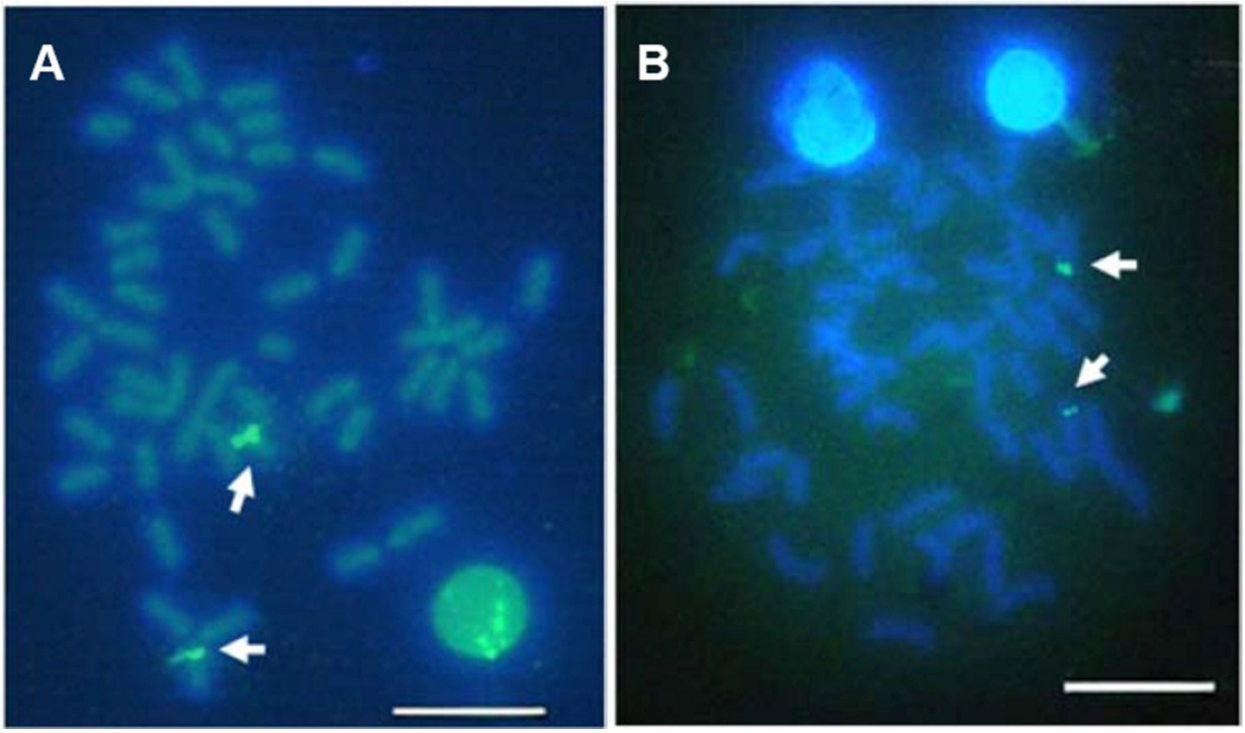

Figure 7 Metaphase chromosomes of *E. bruneus* (A) and *E. moara* (B) with telomeric (TTAGGG)n sequence using FISH. Red arrows indicate chromosomes with significantly stronger and larger telomeric signals than others in *E. bruneus*. Scale bar=5μm.

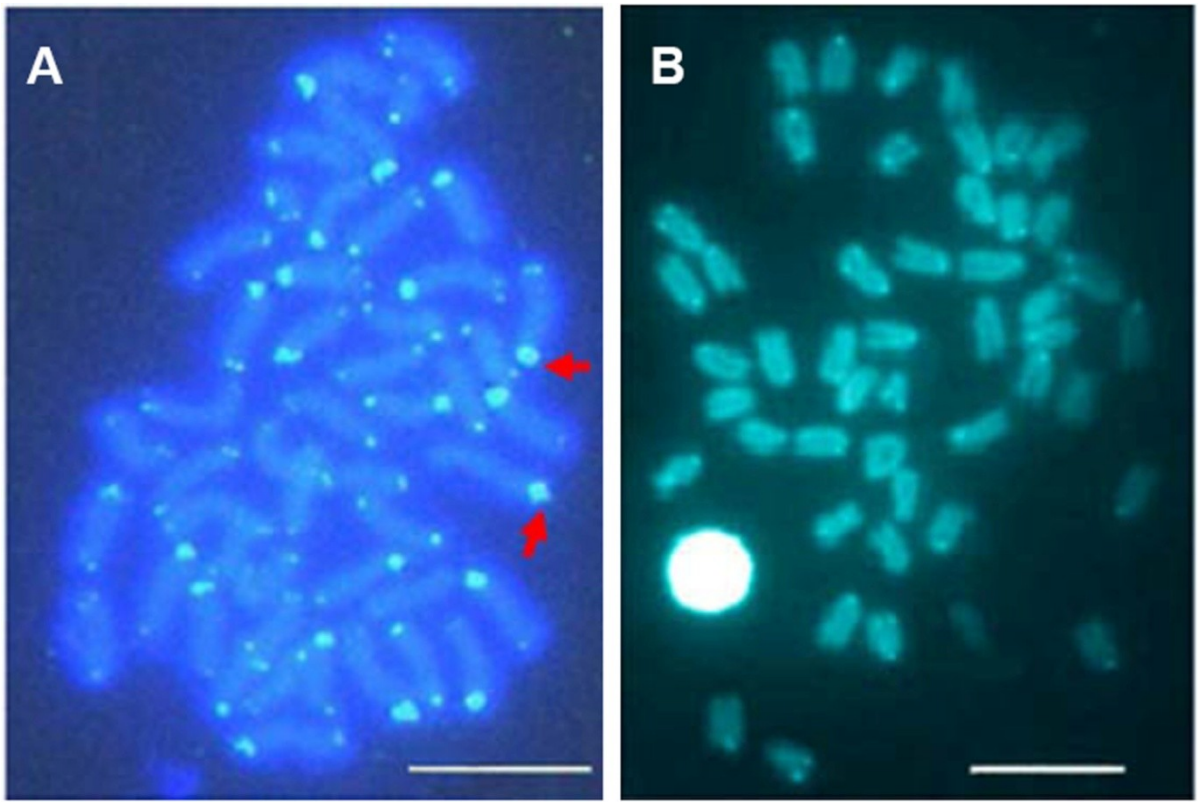

Table 1(on next page)

Table 1

Available cytogenetic data of the genus *Epinephelus*: diploid numbers (2 *n*), karyotype formula, fundamental number (FN), nucleolar organizer regions (NORs) and C-banding.

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

E. tauvina	48	2SM+46A	50	/	/	Raghumath & Prasad , 1980

2n, diploid number; A, acrocentrics; C, centromeric; EA, nearly the entire arm; FN, fundamental number; IR, interstitial region; M, metacentrics; NC, almost indiscemible; NORs, nucleolar organizer regions; SA, short arm; SM, submetacentrics; ST, subtelocentrics; SCR, subcentromeric region; TR, telomeric region; /, not available; ?, not mentioned or measured; Numbers in parentheses, the number of chromosome pairs; ,Data estimated from illustrations and text in the respective papers.