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Karyotype analysis and ribosomal gene localization of spotted knifejaw *Oplegnathus punctatus*

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ABSTRACT. The spotted knifejaw, *Oplegnathus punctatus*, is an important aquaculture fish species in China. To better understand the chromosomal microstructure and the karyotypic origin of this species, cytogenetic analysis was performed using Giemsa staining to identify metaphase chromosomes, C-banding to detect C-positive heterochromatin, silver staining to identify the nucleolus organizer regions (Ag-NORs), and fluorescence *in situ* hybridization (FISH) for physical mapping of the major (18S rDNA) and minor (5S rDNA) ribosomal genes. The species showed a karyotype of 2n = 48 for females, composed of 2 submetacentric and 46 telocentric chromosomes, with a fundamental number (FN) = 50, while the karyotype of males was 2n = 47, composed of 1 exclusive large metacentric, 2 submetacentric, and 44 telocentric chromosomes, with FN = 50. These karyotype results

suggest that *O. punctatus* might have an $X_1X_1X_2X_2/X_1X_2Y$ multiple sex chromosome system. C-positive heterochromatin was distributed in the centromeres of all chromosomal pairs and in the terminal portions of some chromosomes. A single pair of Ag-positive NORs was found to be localized at the terminal regions of the short arms of the subtelocentric chromosome pair, which was supported by FISH of 18S rDNA. After FISH, 5S rDNA were located on the interstitial regions of the smallest telocentric chromosome pair. This study was the first to identify the karyotype of this species and will facilitate further research on karyotype evolution in the order Perciformes.

Key words: *Oplegnathus punctatus*; Karyotype; 18S rDNA and 5S; rDNA Cytogenetic characteristics

INTRODUCTION

The spotted knifejaw, *Oplegnathus punctatus* (Perciformes, Oplegnathidae, Oplegnathus), is a widespread subtropical species in the Pacific Ocean along the coasts of Japan, Korea, and China (Meng et al., 1995), and is potentially one of the most important aquaculture fish species in China. Artificial breeding technology for this species has been studied in Japan since the 1970s. The available data on this species focus mainly on developmental biology, artificial propagation techniques, and taxonomy (Xiao et al., 2011). Studies have been carried out on interspecific hybridization between rock bream *Oplegnathus fasciatus* and *O. punctatus* by artificial insemination to investigate the reproductive system of the hybrids (Shimada et al., 2009), and on the relationship between the two species by mitochondrial DNA sequence characteristics (Xiao et al., 2011). However, few cytogenetic studies have been performed on this potential commercial fishery species.

As lower vertebrates, fishes present a broad range of sex-determining mechanisms, comprising six main types: XX/XY, ZZ/ZW, XX/XO, ZZ/ZO, $X_1X_1X_2X_2/X_1X_2Y$ (multiple sex chromosome systems), and an autosomal determination system (Xu et al., 2012). The guppy *Poecilia reticulata*, for example, has an XX/XY sex determination mechanism (Traut and Winking, 2001), and the half-smooth tongue sole *Cynoglossus semilaevis* has a ZZ/ZW mechanism (Jiang et al., 2014). However, fish with multiple sex chromosomes appear to be few. The first multiple $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system in fishes was reported in the Cyprinodontid *Megupsilon aporus* (Uyeno and Miller, 1971; Miller and Walters, 1972), with a karyotype of 48 in the female and 47 in the male. The male possesses an X_1X_2Y sex chromosome system, including an exclusive large metacentric chromosome considered to be an original Y chromosome. Subsequently, a trivalent observed in the first meiotic chromosomes during spermatogenesis, with the two X chromosomes attached end to end to the metacentric Y, provided clear evidence of the differentiation of multiple sex chromosome systems (Uyeno and Miller, 1972). Since then, multiple sex chromosome systems have been found in many fish species from various families (Uyeno and Miller, 1972) including the goby *Gobionellus shufeldti* (Pezold, 1984), Monodactylid fishes (Suzuki et al., 1988), the lutjanid fish *Lutjanus quinquelineatus* (Ueno and Takai, 2008), and the dolphinfish *Coryphaena equiselis* (Kitano and Peichel, 2012; Soares et al., 2014). These multiple sex chromosome systems are characterized by a large metacentric heteromorphic chromosome, probably resulting from the occurrence

of Robertsonian rearrangement between the original Y chromosome and an autosome. Males have one less chromosome than females do, but arm numbers are equal in both sexes (Xu et al., 2012). Recently, rock bream *O. fasciatus*, which is closely related to *O. punctatus,* was reported to have an $X_1X_1X_2X_2/X_1X_2Y$ mechanism (Xu et al., 2012). Therefore, it is meaningful to study the sex-determination mechanisms of *O. punctatus* and speculate on the chromosomal evolution of this family.

Chromosomal cytogenetic analysis in Perciformes has been performed thus far through conventional Giemsa-stained karyotyping, chromosome staining for C-bands, silver staining for nucleolus organizer regions (Ag-NORs), and fluorescence *in situ* hybridization (FISH) (Affonso and Galetti Jr, 2005; Molina et al., 2012; Paim et al., 2014). C-banding is the most widely used method for detecting heterochromatin distribution in fish chromosomes (Molina et al., 2012). NORs are chromosomal regions containing acidic proteins related to the transcription of major rDNA (Wang et al., 2009a). The number and location of NORs have been used to investigate the expression of major rDNA in fishes. Ag-NORs serve as excellent biological markers in some fish species (Galetti Jr et al., 2006). The major (45S rDNA) codes for the 18S, 5.8S, and 28S rRNAs and minor (5S rDNA) codes for the 5S rRNA. It has been demonstrated that these rRNA genes are highly conserved and are important landmarks for comparing the genomes of different species (Martins and Galetti Jr, 2001b; Wang et al., 2009a). These studies are broadly useful for characterizing karyotypes.

The present study provides the first data on the cytogenetic characteristics of *O. punctatus* by conventional and banding methods including Giemsa- and Ag-staining, C-banding, and FISH with major and minor rDNA probes. This study will contribute to our understanding of the karyotypic evolution of this artificially cultured species and provide new evidence concerning the sex-determining mechanisms in fish.

MATERIAL AND METHODS

Ethics statement

Spotted knifejaw *O. punctatus* samples were collected from local aquatic farms, and permission to collect samples was obtained from the local government of Yantai, Shandong, China. This study complied with and was approved by the Institutional Animal Care and Use Committee of the Ocean University of China.

Mitotic chromosome preparation

Healthy two-year-old spotted knifejaw *O. punctatus* specimens (10 females and 10 males) were obtained from Laizhou Mingbo Aquatic Co. Ltd., Yantai, China. Chromosome preparation was performed using the blood culture method as described by Wang et al. (2009b).

Karyotype analysis

Conventional karyotype analysis was performed by staining with 10% Giemsa in phosphate-buffered saline (PBS) for 20 min. Over 300 metaphase spreads per specimen were analyzed in both males and females to determine the diploid chromosome number and karyotype structure. Chromosome morphology was classified according to Levan et al. (1964).

Chromosome staining

Heterochromatin was detected by barium hydroxide C-banding according to Sumner (1972), with modifications. After handling at 65°C for 6 h, chromosome slides were denatured with saturated barium hydroxide at 60°C for 9-12 min, exposed to 0.4 M HCl for 11 min at room temperature, and renatured in 2X SSC at 60°C for 60 min. Next, the slides were stained in 10% Giemsa in PBS for 20 min. Chromosomes were also analyzed by $AgNO_3$ staining in order to visualize the NORs (Howell and Black, 1980). Then, 50% $AgNO_3$ and 2% gelatin (containing 1% formic acid) were mixed in a ratio of 2:1 and added to the stored preparations. A coverslip was added and the slides were incubated for 5-10 min at 60°C until they appeared golden brown. The slides were rinsed thoroughly in distilled water, dried, and observed under a Nikon microscope.

Probes

Major (18S rDNA) and minor (5S rDNA) ribosomal probes were isolated from the genome of *O. punctatus* by polymerase chain reaction (PCR). The 18S-28S rDNA probes were obtained with primers 18S F (5'-GTCGTAACAAGGTTTCCGTAG-3') and 18S R (5'-GATCAATGAGTCTGCAATTC-3') (Wang et al., 2009a). These probes were labeled with Digoxigenin-11-dUTP (Roche, Germany). The PCR was performed in a total volume of 25 μL containing 20 ng template DNA, 0.2 μM each primer, 200 μM dNTPs, 1 U Taq DNA polymerase (TaKaRa), and 1X PCR buffer. The amplification procedure was 94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s, followed by a final extension step of 72°C for 7 min. The 5S rDNA was amplified by the primers 5S F (5'-TACGCCCGATCTCGTCCGATC-3') and 5S R (5'-CAGGCTGGTATGGCCGTAACG-3') (Pendás et al., 1994), and the probe was synthesized following the same procedures. PCR products were verified by agarose gel electrophoresis and sequencing.

FISH

The FISH procedure and probe detection were performed according to previous studies (Wang et al., 2009a; Jiang et al., 2014), with minor modifications. Briefly, aged and formamide-denatured chromosome slides were hybridized with 20 μL rDNA probe mixture (containing digoxigenated probes, 10% dextran sulfate, 50% deionized formamide in 2X SSC) per slide at 37°C for 12-18 h in a dark, moist chamber. The negative control was performed without the rDNA probe mixture. After post-hybridization washing, chromosome slides were detected with Anti-Digoxigenin-Rhodamine (Roche) for 1 h at 37°C and counterstained with 4',6-diamidino-2-phenylindole (Vector) for 10 min at room temperature. Hybridization signals were photographed under a Leica microscope equipped with epifluorescence and a digital camera. Approximately 30 metaphases were analyzed for each individual.

RESULTS

Karyotype

The analysis of over 300 mitotic metaphases of *O. punctatus* in both females and

males by Giemsa staining showed that the diploid chromosome numbers were 48 for females and 47 for males (Figure 1). The female karyotype was composed of 2 submetacentric and 46 telocentric (FN = 50, Figure 1a) chromosomes, while the male karyotype consisted of 2 submetacentric, 44 telocentric, and 1 large metacentric chromosome without a homologous chromosome (FN = 50, Figure 1b). The unique and largest chromosome, which was easily recognized by its large size compared to the other chromosomes, was assigned as chromosome 1 in the male. The chromosomes of the female (Figure 1c) and male (Figure 1d) gradually decreased in size according to the average relative length. The relative length and type of the metaphase chromosomes are given in Table 1. The largest relative length of chromosomes in females was 5.86 ± 0.35, and the smallest was 2.24 ± 0.25. The relative length of the heteromorphic chromosome in males was 11.08 ± 0.39, which was the largest. The smallest relative length in males was 1.94 ± 0.32.

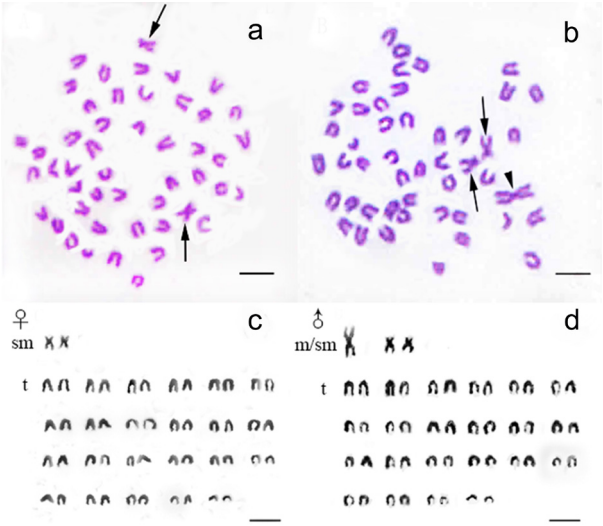

Figure 1. Metaphase chromosomes and karyotype of female (**a** and **c**, 2n = 48) and male (**b** and **d**, 2n = 47) *Oplegnathus punctatus* after Giemsa staining. Arrows indicate the submetacentric chromosome in the female (**a**) and male (**b**); the arrowhead indicates the large metacentric chromosome, which was found exclusively in males (**b**); m/sm: metacentric/submetacentric; t: telocentric. Bar = 5 μm.

C-banding

The distribution of heterochromatic regions on the chromosomes of *O. punctatus*, as detected by C-banding, is demonstrated in Figure 2. C-banding analysis revealed that heterochromatic bands occur in the centromeric regions of all chromosomes and the terminal portions of some chromosomes in both females (Figure 2a) and males (Figure 2b). In males, the heterochromatin of the large metacentric chromosome is distributed over the centromeric regions and two telomeres (Figure 2b). In addition, analysis of constituent heterochromatin clearly shows C-positive bands located on the short arm of the submetacentric chromosome pair in both sexes (Figure 2a and b).

Table 1. Relative length of metaphase chromosomes in *Oplegnathus punctatus*.

No. of chromosome	Female			Male		
	Relative length*	Arm ratio	Type	Relative length*	Arm ratio	Type
1	5.86 ± 0.35	1.98 ± 0.09	sm	11.08 ± 0.39	1.28 ± 0.10	m
2	5.23 ± 0.27	∞	t	5.44 ± 0.38	2.11 ± 0.13	sm
3	5.12 ± 0.18	∞	t	5.05 ± 0.30	∞	t
4	5.01 ± 0.20	∞	t	4.91 ± 0.24	∞	t
5	4.91 ± 0.15	∞	t	4.81 ± 0.15	∞	t
6	4.85 ± 0.17	∞	t	4.62 ± 0.29	∞	t
7	4.59 ± 0.08	∞	t	4.57 ± 0.16	∞	t
8	4.53 ± 0.10	∞	t	4.52 ± 0.12	∞	t
9	4.48 ± 0.15	∞	t	4.42 ± 0.08	∞	t
10	4.37 ± 0.09	∞	t	4.18 ± 0.13	∞	t
11	4.32 ± 0.07	∞	t	4.13 ± 0.09	∞	t
12	4.27 ± 0.23	∞	t	4.03 ± 0.10	∞	t
13	4.16 ± 0.17	∞	t	3.98 ± 0.13	∞	t
14	4.11 ± 0.15	∞	t	3.89 ± 0.11	∞	t
15	4.05 ± 0.10	∞	t	3.79 ± 0.08	∞	t
16	3.95 ± 0.16	∞	t	3.69 ± 0.12	∞	t
17	3.84 ± 0.22	∞	t	3.16 ± 0.15	∞	t
18	3.68 ± 0.20	∞	t	3.11 ± 0.22	∞	t
19	3.41 ± 0.08	∞	t	3.06 ± 0.17	∞	t
20	3.36 ± 0.17	∞	t	3.01 ± 0.23	∞	t
21	3.25 ± 0.10	∞	t	2.92 ± 0.16	∞	t
22	3.20 ± 0.09	∞	t	2.87 ± 0.09	∞	t
23	3.15 ± 0.18	∞	t	2.82 ± 0.12	∞	t
24	2.24 ± 0.25	∞	t	1.94 ± 0.32	∞	t

*Data are reported as means ± standard deviation; m = metacentric chromosome; sm = submetacentric chromosome; t = telocentric chromosome.

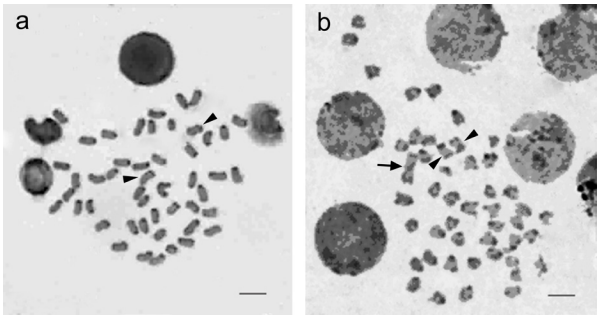

Figure 2. Metaphases of *Oplegnathus punctatus* in the female (**a**) and male (**b**) after C-banding. The arrow indicates the large metacentric chromosome in the male (**b**). Arrowheads indicate the submetacentric chromosome pair in the female (**a**) and male (**b**). Bar = 5 μm.

Ag-NORs staining

Silver nitrate staining of *O. punctatus*, which detected acidic proteins related to transcriptional activity, revealed a single pair of NORs in this species. Positive signals were located on the terminal regions of the short arms of the submetacentric chromosome pair in both the female (Figure 3a) and male (Figure 3b). Furthermore, the signal intensity of Ag-NORs observed on one chromosome was weaker than that on the other chromosome in females, whereas the Ag-NORs showed the same intensity in males.

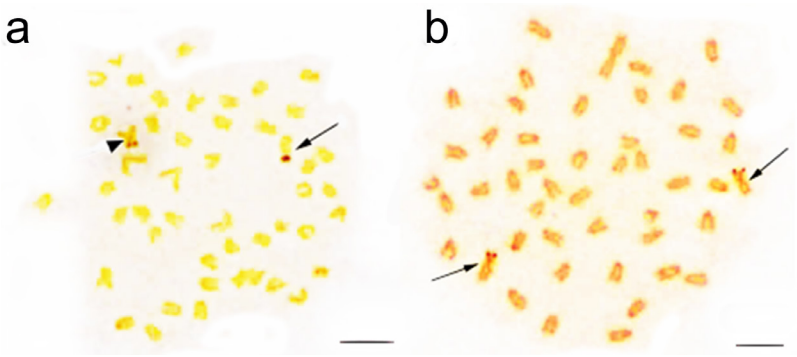

Figure 3. Silver-stained metaphase in female (**a**) and male (**b**) *Oplegnathus punctatus*. The arrowhead indicates the weaker AgNORs signal in the female (**a**). Arrows indicate AgNORs in the female (**a**) and male (**b**). Bar = 5 µm.

FISH with rDNA probes

The physical mapping of 18S rDNA and 5S rDNA sites showed that these genes were located in distinct chromosomal pairs in the karyotype of *O. punctatus*. There was no signal in the negative control. FISH with an 18S rDNA probe showed signals in the terminal region of the short arms of the submetacentric chromosome pair in both the female and male (Figure 4a and b). In female specimens, the hybridization signal intensity of 18S rDNA on one chromosome was weaker than that of the other chromosome. However, in male specimens, the hybridization signal intensity of 18S rDNA on both of the submetacentric chromosomes was similar. This pattern confirmed the previous results obtained by silver nitrate staining regarding the identification of AgNORs. No other inactive major ribosomal clusters were detected. When 5S rDNA probes were employed, a remarkable distribution pattern of 5S rDNA genes was observed in the interstitial region of the smallest telocentric chromosome pair (Figure 5a and b); it was not co-located with the distribution pattern of major rDNA. The signal strength on both of the telocentric chromosomes was nearly equal in the female and male.

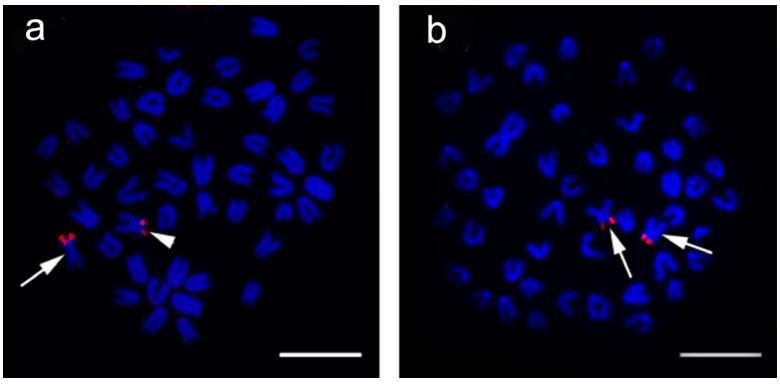

Figure 4. Metaphases of *Oplegnathus punctatus* after FISH with an 18S rDNA probe in the female (**a**) and male (**b**), revealing one NOR-bearing pair. The arrowhead indicates the weaker signal of 18S rDNA in the female (**a**). Arrows indicate the signals of 18S rDNA in the female (**a**) and male (**b**). Chromosomes are counterstained with 4',6-diamidino-2-phenylindole. Bar = 5 µm.

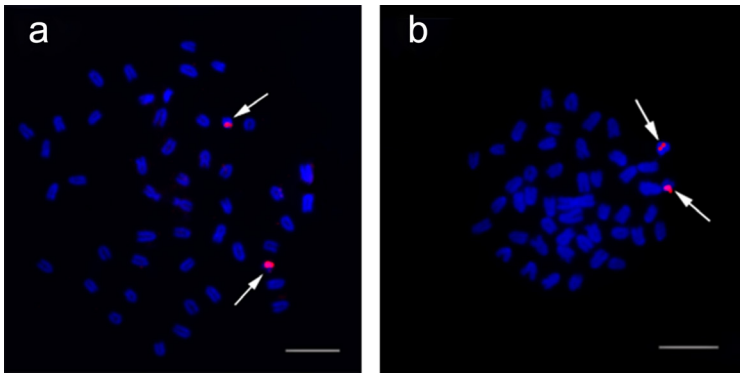

Figure 5. Metaphases of *Oplegnathus punctatus* after FISH with a 5S rDNA probe in the female (**a**) and male (**b**). Arrows denote the hybridization signals of 5S rDNA. Chromosomes are counterstained with 4',6-diamidino-2-phenylindole. Bar = 5 μm.

DISCUSSION

The present study indicates that the *O. punctatus* female chromosome number is 2sm + 46t (2n = 48, FN = 50), while the male chromosome number is 1m + 2sm + 44t (2n = 47, FN = 50), including a large metacentric chromosome without a homologous chromosome that seems to be the neo-Y chromosome. Chromosome numbers in the male and female would be identical if this species had the usual XY/ZW mechanism (Murofushi et al., 1980). Based on the chromosome complements, our preliminary speculation is that the karyotype of *O. punctatus* follows the $X_1X_1X_2X_2/X_1X_2Y$ sex determination mechanism as found in the Cyprinodontid *M. aporus* (Uyeno and Miller, 1971; Miller and Walters, 1972), filefish *Stephanolepis cirrhifer* (Murofushi et al., 1980), *L. quinquelineatu*s (Ueno and Takai, 2008), and *O. fasciatus* (Xu et al., 2012; Xu et al., 2013a,b). Female *O. fasciatus* have 44 autosomes and $X_1X_1X_2X_2$ sex chromosomes and male *O. fasciatus* have 44 autosomes and X_1X_2Y sex chromosomes, and it is difficult to morphologically identify X_1, X_2 from the autosomes. The large metacentric chromosome in the male is considered to be a Y chromosome derived from a Robertsonian chromosomal rearrangement between the original Y chromosome and an autosome (Suzuki et al., 1988; Ueno and Takai, 2008). However, it requires more cytogenetic methods such as meiotic during spermatogenesis (Ueno and Takai, 2008) to confirm the multiple sex determination of *O. punctatus*.

C-positive heterochromatin in Perciformes chromosomes is usually distributed over the centromeric/pericentromeric and/or NORs (Brum et al., 1995). In addition, heterochromatin blocks are situated in the telomeric regions in some chromosomes in the complement. Following this trend, *O. punctatus* is characterized by the distribution of C-bands close to the centromeres, telomeres, and the short arms of the submetacentric chromosome pair congruent with the NORs. The heterochromatin equivalent to NORs likely contributes to the occurrence of chromosomal rearrangement (Molina et al., 2012). The unique characteristics of the large metacentric chromosome in males, with heterochromatin distributed in the centromeric regions and two telomeres, confirm that the large chromosome is a Y sex chromosome.

In Perciformes, particularly in species with conserved karyotypes, the occurrence of a single interstitial NOR pair is the most frequently observed situation, and this NOR phenotype

is regarded as the basic and widespread pattern for various fish species (Aguilar, 1997; Affonso et al., 2001). Our study showed that a pair of AgNORs was located at the terminal position of the short arms on the submetacentric chromosomes in both female and male *O. punctatus*. The presence of only a single NORs pair in *O. punctatus* partially supports the idea that the presence of single NORs is a basic pattern in Perciformes, although NOR signals occupy the terminal regions on the short arms of chromosomes in this species. As expected, the distribution pattern of 18S rDNA clusters on the chromosomes in this species as determined by FISH was coincident with the results obtained by conventional silver nitrate staining (Ag-NORs). The location, number, and size of hybridization signals were nearly equivalent to those found by Ag-NORs in both females and males. In female *O. punctatus*, the different signal intensities of Ag-NORs and 18S rDNA on a submetacentric chromosome pair indicated that the major rDNA on the two chromosomes might have different transcriptional activity.

The chromosomal localization of minor rDNA genes (5S rDNA) has been described for many fish species. Among related fish species, the chromosome distribution of the 5S rRNA genes is highly conserved, occupying an interstitial position in the chromosomes. This distribution pattern could offer some advantage to its organization in fishes (Martins and Wasko, 2004). The 5S rDNA genes of *O. punctatus* were clustered in the interstitial position of the smallest telocentric chromosome pair in both the female and male, conforming to the conventional distribution pattern of 5S rDNA in most of the fish species studied thus far, which might also apply to vertebrates in general (Martins and Galetti Jr, 2001a; Sola et al., 2003). The distribution pattern of 5S rDNA in *O. punctatus* was not co-located with that of major rDNA, indicating that a non-system existed between both the ribosomal genes. This phenomenon seems to be the most common situation observed in fishes and suggests an independent evolution of major and minor genes under diverse selection pressures (Martins and Galetti Jr, 1999).

In conclusion, the present study provides the first data about the cytogenetic characteristics of *O. punctatus*. Our chromosomal analysis of a single pair of Ag-positive NOR and 18S rDNA in the pericentromeric region of 5S rDNA indicates that the karyotype of *O. punctatus* is a general state in fishes. The multiple sex chromosome system of *O. punctatus* seems to be an interesting phenomenon. However, in this study, cytogenetic methods such as meiotic during spermatogenesis were not performed to confirm the multiple sex chromosome system, so further investigation is needed.

Conflicts of interest

The authors declare no conflict of interest.

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