

Classical and molecular cytogenetics of *Belontia hasselti* (Perciformes: Osphronemidae): Insights into the ZZ/ZW sex chromosome system

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Abstract. Chaiyasan P, Mingkwan B, Jantarat S, Suwannapoom C, Cioffi MDB, Liehr T, Talumphai S, Tanomtong A, Supiwong W. 2021. Classical and molecular cytogenetics of *Belontia hasselti* (Perciformes: Osphronemidae): Insights into the ZZ/ZW sex chromosome system. *Biodiversitas* 22: 546-554. Karyotype of Java combtail fish, *Belontia hasselti*, from To Daeng peat swamp forest, Narathiwat Province, southern Thailand, was studied for the first time. Mitotic chromosome preparations were prepared directly from kidney cells from ten male and ten female fish. Conventional staining, NOR banding, and molecular cytogenetics with fluorescence in situ hybridization (FISH) using 5S and 18S rDNAs, as well as microsatellites d(CA)₁₅ and d(CAC)₁₀ as probes were applied. The diploid chromosome number (2n) was 48 and a female heterogametic sex chromosome system (ZZ/ZW) is suggested. The fundamental numbers (NF) were 48 and 49 in males and females, respectively. The karyotype of males comprised 48 telocentric chromosomes while the female ones were composed of one metacentric and 47 telocentric chromosomes. A single Ag-NOR-bearing chromosomal pair was identified. The NOR positions were characterized at the interstitial sub-centromeric region of pair 13, which coincided with signals of 18S rDNA and d(CAC)₁₀ probes. The 5S rDNA signals were located at interstitial sites of the largest telocentric pair. Microsatellite d(CA)₁₅ repeats were highly distributed throughout almost all entire chromosomes except for centromeric regions on some chromosome pairs, including sex chromosomes. The present study is a novel report for a ZZ/ZW sex chromosome system of this fish family in Thailand.

Keywords: Ag-NOR staining, *Belontia hasselti*, cytogenetics, fluorescence in situ hybridization (FISH), karyotype

INTRODUCTION

Java combtail fish, *Belontia hasselti* belongs to the family Osphronemidae, subfamily Belontiinae. This subfamily is represented only by genus *Belontia*, and two species *B. hasselti* and *B. signata*, are native from freshwater habitats in Southeast Asia, and Sri Lanka (Froese and Pauly 2014). Mostly the species occur in acidic freshwater biotopes with little water movement, particularly, in ancient forest peat swamps in which the water is stained dark-brown by humic acids and other chemicals released from decaying organic material (Kottelat 2013). In Thailand, *B. hasselti* is endemic in the To Daeng peat swamp forest.

Cytogenetic studies demonstrated a huge karyotypic diversity for the family Osphronemidae. The overall data showed that 2n ranges from 16 in *Sphaerichthys osphromenoides* to 48 chromosomes in several species as outlined in Table 1. Variations in karyotypic formula in the two species and their sub-populations result from differences in chromosome morphology.

Conventional staining technique has been used to determine chromosome number and karyotype composition. Structure, number, type, size, and morphology of a nucleolar organizer region (NOR) may be specific to populations, species, and subspecies. NOR-staining is frequently used to compare variations, as well as to identify and explain specifications. Molecular cytogenetic experiments have demonstrated that NORs are the chromosomal site of gene coding for 5.8S, 18S, and 28S rRNA, in humans and several mammalian species. NORs can be used as markers for evolutionary chromosome studies (Gornung 2013; Gálvez et al. 2018). Recently, molecular cytogenetic studies using fluorescence in situ hybridization (FISH) for mapping repetitive DNA sequences have provided important contributions to the characterization of biodiversity and the evolution of divergent fish groups (Cioffi et al. 2015). However, conventional cytogenetic and FISH techniques to investigate the chromosomal distribution of repetitive DNA sequences on *B. hasselti* have not yet been performed.

Accordingly, the present study is the first cytogenetic report on *B. hasselti* from Thailand, and is accomplished with both classical and molecular cytogenetics. The study moves forward our understanding of both the karyotype evolution mechanisms and speciation in the genus *Belontia*, and increases the knowledge available for implementation of polyploidy manipulation, hybridization, sex control, and other potential genetic improvements in the future.

MATERIALS AND METHODS

Sample collection

Ten males and ten females of *Belontia hasselti* were obtained from the To Daeng peat swamp forest, Narathiwat Province, Thailand. The fish were transferred to laboratory aquaria and kept under standard conditions for seven days before experimentations. The procedures followed ethical protocols, with anesthesia conducted by keeping samples in the freezer before euthanasia, as approved by the Institutional Animal Care and Use Committee of Khon Kaen University, based on the Ethics of Animal Experimentation of the National Research Council of Thailand ACUC-KKU-90/60.

Chromosome preparation, Giemsa's staining and Ag-NORs banding technique

Metaphase chromosomes were directly prepared *in vivo* as following Supiwong et al. (2012a, 2013b, 2015) and Kasiroek et al. (2017). Subsequently, chromosomes were stained with 20% Giemsa solution and 50 % silver nitrate for Ag-NOR banding (Supiwong et al. 2012b; Getlekha et al. 2017; Chaiyasan et al. 2018).

Chromosome checking

Twenty metaphases of each specimen were selected and photographed. The length of the short arm chromosome (Ls) and the long arm chromosome (Ll) were measured from 20 perfect metaphase plates of each sex, while the length of the total arm chromosome (LT) was calculated ($LT = Ls + Ll$). The relative length (RL), the centromeric index (CI), and standard deviation (SD) of RL and CI were estimated. The CI ($q/p + q$) between 0.50-0.59, 0.60-0.69, 0.70-0.89, and 0.90-1.00 are described as metacentric (m), submetacentric (sm), acrocentric (a), and telocentric (t) chromosomes, respectively. The fundamental number (NF) was obtained by assigning a value of 2 to the m, sm and a chromosomes and 1 to the t chromosome. All data were used in karyotyping and diagramming (Tanomtong et al. 2014; Choosangjaew et al. 2017).

Fluorescence in situ hybridization (FISH)

FISH was performed on metaphase chromosome spreads with specific probes for 5S and 18S rDNAs under highly stringent conditions (Rodrigues et al. 2012; Maneechot et al. 2016). Both rDNA probes were directly labeled with the Nick-translation Labeling Kit (Jena Bioscience, Jena, Germany), using the fluorescent labels Atto488 (18S rDNA) and Atto550 (5S rDNA), according to the manufacturer's manual (Supiwong et al. 2017).

The usage of microsatellites $d(CA)_{15}$, and $d(CAC)_{10}$ probes described by Cioffi et al. (2011) was followed with slight modifications. Sequences were directly labeled with Cy3 at 5' terminal during synthesis by Sigma (St. Louis, MO, USA). FISH was performed on mitotic chromosome spreads (Xu et al. 2017) under highly stringent conditions, as previously reported (Supiwong et al. 2017). The evaluation was carried out on an epifluorescence microscope Olympus BX50 (Olympus Corporation, Ishikawa, Japan).

RESULTS AND DISCUSSION

Diploid chromosome number (2n), fundamental number (NF) and karyotype of *Belontia hasselti*

The model diploid number of *B. hasselti* was $2n = 48$ chromosomes for both the sexes. The male karyotype was composed of 48 telocentric chromosomes (Figure 1.A-B), while females presented 47 telocentric and one large and unpaired metacentric chromosome (Figure 1.C-D), identified as the W chromosome. In addition to this, the female karyotype showed an exclusive telocentric chromosome, and the smallest chromosome was identified as the Z chromosome (Figure 1). Hence, a ZZ/ZW sex chromosomal heteromorphism was identified, with a large metacentric W-chromosome, which was similar in size to the first pair of karyotype complement (Figure 3). The NF were 48 and 49 in males and females, respectively (Figure 1, Table 1).

Chromosome marker of *Belontia hasselti*

The determination of a chromosome marker for this species was firstly obtained by Ag-NOR staining. The nucleolar organizer regions (NORs) were mapped to interstitial subcentromeric positions of the telocentric chromosome pair 13 (Figure 1 E-H).

Patterns of 5S and 18S rDNAs in *Belontia hasselti*

The 5S rDNA sequences were mapped at interstitial positions near the centromeres of the telocentric pair 1 (the first pair). The 18S rDNA probe showed coincident hybridization signals of NOR-carrying chromosomes, at the interstitial subcentromeric regions on the telocentric chromosome pair 13 (Figure 2.A-B).

Patterns of microsatellite $d(CA)_{15}$ and $d(CAC)_{10}$ repeats in *Belontia hasselti*

The mapping of microsatellite repeats on the chromosomes of *B. hasselti* showed that $d(CA)_{15}$ signals were observed on all chromosome pairs. These signals were distributed throughout the whole chromosomes except for centromeric regions of some pairs (pairs 2, 4, 8, 10, 15, and sex chromosomes in both sexes) (Figure 2.C-D). In turn, microsatellite $d(CAC)_{10}$ was highly accumulated at the interstitial subcentromeric region on chromosome pair 13, which corresponds to the NOR position and 18S rDNA patterns (Figure 2.E-F).

Table 1. Cytogenetics review in the family Osphronemidae

Species	2n	NF	Karyotype formula	Ag-NORs	Sex-chromosome	Reference
<i>Belontia hasselti</i>	48	48	48t	2	ZZ, M	Present study
	48	49	1m + 47t	2	ZW, F	Present study
<i>Betta splendens</i>	42	-	-	-	-	Arai (2011)
	42	45-56	12sm+14st/a+16a/t	-	-	Grazyna et al. (2008)
<i>Colisa chuna</i>	46	66	20m+26st/a	-	-	Grazyna et al. (2008)
<i>Colisa fasciata</i>	48	48	48a	-	-	Grazyna et al. (2008)
	48	68	20m+12st+16a	-	-	Grazyna et al. (2008)
	48	80-81	16m+16sm+15a(16a)	-	-	Grazyna et al. (2008)
<i>Colisa labiosa</i>	48	68	20m+10st+18a	-	-	Grazyna et al. (2008)
<i>Colisa lalia</i>	46	70	24msm+22a	-	-	Grazyna et al. (2008)
	45	-	26m/sm/st+19a	-	-	Grazyna et al. (2008)
	46	66	20m+8st+18a	-	-	Grazyna et al. (2008)
	16	30	14m+2a	-	-	Grazyna et al. (2008)
<i>Ctenops nobilis</i>	44	60	8m + 8sm + 28t	-	-	Arai (2011)
<i>Macropodus ocellatus</i>	46	54	4m + 4sm + 38t	-	-	Arai (2011)
	46	76	8m + 8sm + 14a + 16t	-	-	Arai (2011)
<i>Macropodus opercularis</i>	46	78	8m + 8sm + 16a + 14t	-	-	Arai (2011)
	46	72	4m + 10sm + 12a + 20t	-	-	Arai (2011)
	46	58	12m/sm + 34a/t	-	-	Arai (2011)
<i>Macropodus chinensis</i>	46	-	-	-	-	Grazyna et al. (2008)
<i>Macropodus concolor</i>	46	56	10m+2sm+22st+12a	-	-	Grazyna et al. (2008)
<i>Macropodus ocellatus</i>	46	64	8m+8sm+14st+16a	-	-	Grazyna et al. (2008)
<i>Macropodus opercularis</i>	46	58	12m/sm+34a	-	-	Grazyna et al. (2008)
	46	64	8m+8sm+16st+14a	-	-	Grazyna et al. (2008)
<i>Macropodus spechti</i>	46	80	10m + 2sm + 22a + 12t	-	-	Arai (2011)
<i>Osphronemus gorami</i>	48	50	2sm + 46t	-	-	Arai (2011)
<i>Parospromenus sumatranus</i>	46	46	46t	-	-	Arai (2011)
<i>Sphaerichthys osphromenoides</i>	16	30	14m/sm + 2t	-	-	Arai (2011)
	16	30	10m + 4sm + 2t	-	-	Arai (2011)
<i>Trichogaster chuna</i>	46	64	10m + 8sm + 28t	-	-	Arai (2011)
	46	80	20m + 8sm + 6a + 12t	-	-	Arai (2011)
	46	86	28m + 12sm + 6t	-	-	Arai (2011)
<i>Trichogaster fasciata</i>	48	86	16m + 16sm + 6a + 10t	2	-	Arai (2011)
	48	75	15m + 12sm/a+ 21t	-	ZW, F	Arai (2011)
	48	74	14m + 12sm/a + 22t	-	ZZ, M	Arai (2011)
	48	81	17m + 16sm + 15t	-	ZW, F	Arai (2011)
	48	80	16m + 16sm + 16t	-	ZZ, M	Arai (2011)
	48	83	15m + 16sm + 4a + 13t	6	ZW, F	Arai (2011)
	48	84	16m + 16sm + 4a + 12t	-	ZZ, M	Arai (2011)
	48	88	8m + 20sm + 12a + 8t	-	-	Arai (2011)
	46	76	18m + 12sm + 16t	-	-	Arai (2011)
<i>Trichogaster labiosa</i>	48	86	22m + 12sm + 4a + 12t	-	-	Arai (2011)
	48	78	12m + 6sm + 12a + 18t	-	-	Arai (2011)
	48	86	22m + 16sm + 10t	-	-	Arai (2011)
<i>Trichogaster lalius</i>	45	71	14m + 12sm/a + 19t	-	ZO, F	Arai (2011)
	46	72	14m + 12sm/a + 20t	-	ZZ, M	Arai (2011)
	46	66	14m + 6sm + 26t	-	XX, F	Arai (2011)
	45	65	14m + 6sm + 25t	-	XO, M	Arai (2011)
	46	82	14m + 10sm + 12a + 10t	-	-	Arai (2011)
	46	70	24m/sm + 22a/t	-	-	Arai (2011)
<i>Trichopodus leeri</i>	46	46	46t	-	-	Arai (2011)
	46	46	46t	-	-	Arai (2011)
<i>Trichopodus microlepis</i>	46	46	46t	-	-	Arai (2011)
	48	48	48t	-	-	Arai (2011)
<i>Trichopodus pectoralis</i>	46	46	46t	-	-	Arai (2011)
<i>Trichogaster sumatranus</i>	48	48	48st/a	-	-	Grazyna et al. (2008)
<i>Trichopodus trichopterus</i>	48	-	-	-	-	Arai (2011)
	46	46	46t	-	-	Arai (2011)
	46	46	46t	-	-	Arai (2011)
	46	46	46t	-	-	Arai (2011)
	46	46	46t	2	-	Supiwong et al. (2010)

Note: 2n: diploid chromosome number; NF: the fundamental number; m: metacentric; sm: submetacentric; a: acrocentric; t: telocentric; M: male; and F: female

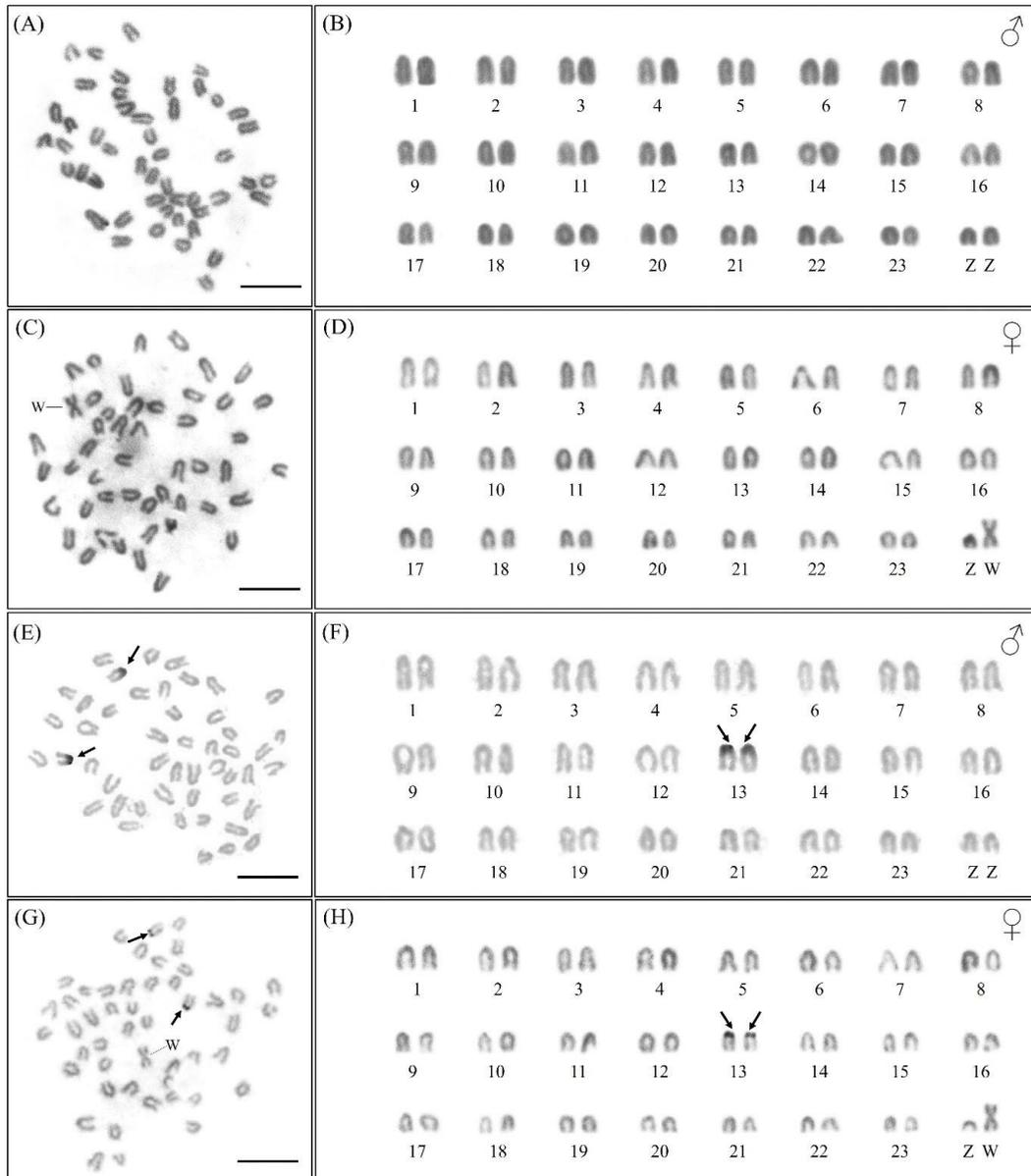


Figure 1. Metaphase chromosome plates and karyotypes of male (A-B and E-F) and female (C-D and G-H) Java combtail fish (*Belontia hasselti*), $2n=48$ by conventional straining (A-D) and Ag-NOR banding (E-H) technique. Scale bars indicate $5\mu\text{m}$. The arrows indicate nucleolar organizer regions/NOR

Idiograms of *Belontia hasselti* chromosomes

All previous results were summarized, and idiograms presenting shapes, sizes and probe signals on the chromosomes of *B. hasselti* are shown in Figure 3.

Discussion

The present study is the first one providing classical and molecular cytogenetics in the genus *Belontia*. The obtained results showed that the diploid chromosome number of *B. hasselti* was $2n=48$ for both sexes. It is the same as in some Osphronemidae species such as in *Osphronemus goramy*, *Trichogaster fasciata*, and *Trichogaster labiosa* (Grazyna et al. 2008; Arai 2011). However, it differs from most studied species in this family. The NFs were 48 and 49 in males and females, respectively (Figures 1-3). The

different Osphronemidae species underwent an extremely diversified karyotype evolution, considering the numerical and structural aspects of their complements, with NF that varied from 20 to 88 (Arai 2011). Each chromosome pattern allows its utilization in the identification of visible changes on the karyotype macrostructure of several families in the order of Perciformes (Nirchio et al. 2002; Gustavo and Molina 2005; Roesti et al. 2013; Molina et al. 2014; Almeida et al. 2017). Analyses performed to highlight the combined importance of the different chromosome rearrangements in the evolutionary modeling of their karyotypes, such as Robertsonian rearrangements or centric fission, fusion, and especially, pericentric inversions were important roles for chromosomal rearrangements during evolution of Perciformes fishes

(Ueno and Takai 2000; Jacobina et al. 2011). The occurrence of a large number of telocentric chromosomes in the karyotype is a common feature for Osphronemidae species, especially in the genus *Trichopodus*, and was also

observed in the present study. However, this fish group is characterized by the occurrence of a non-conservative karyotype, with 2n ranging from 16 to 48 chromosomes (Table 1.).

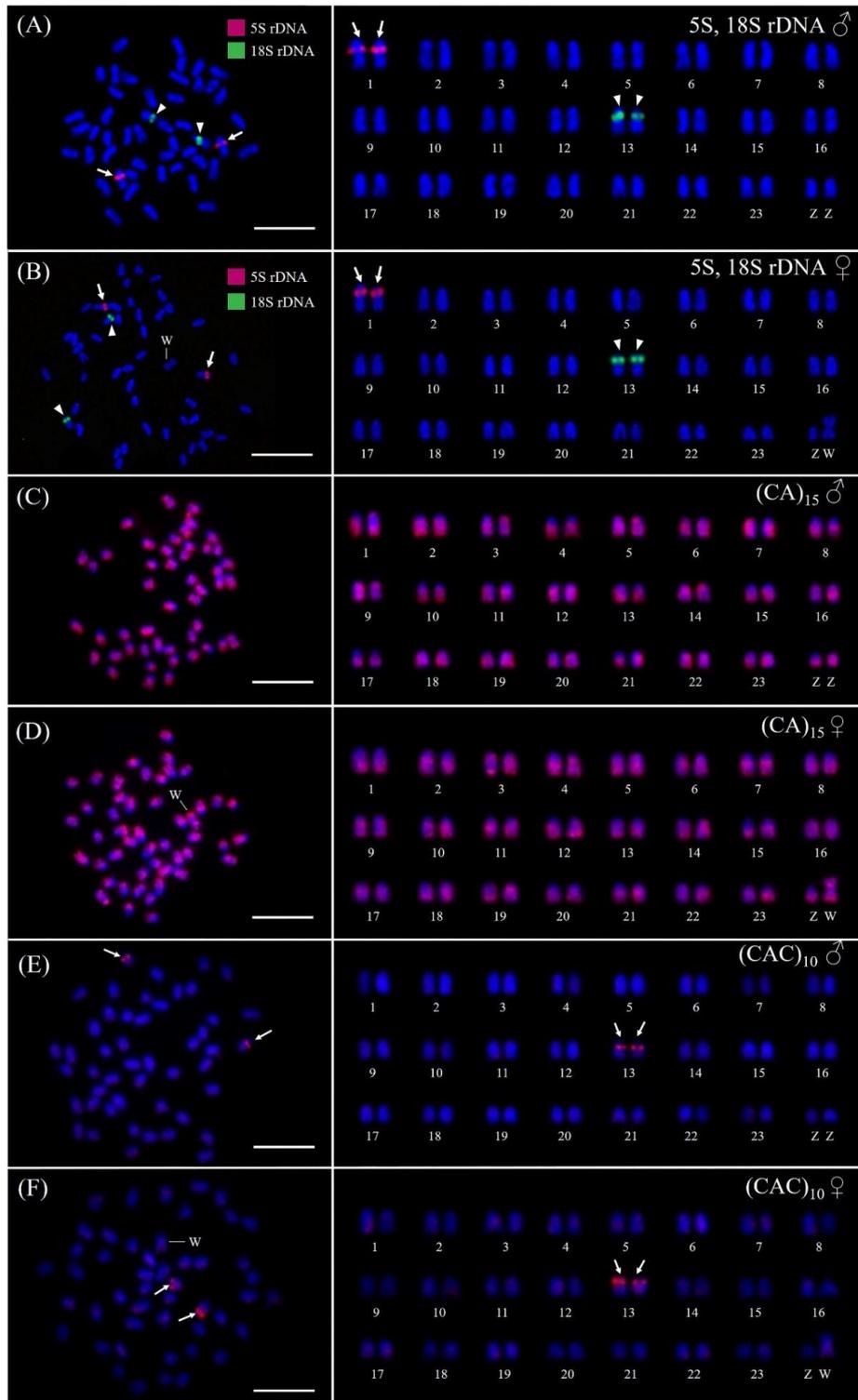


Figure 2. Karyotype of male (A, C, E) and female (B, D, F) Java combtail fish (*Belontia hasselti*), 2n=48 arranged from chromosomes after double-fluorescence in situ hybridization (FISH) with 5S rDNA (red) and 18S rDNA (green) probes (A-B), FISH with d(CA)₁₅ probe (C-D), FISH with d(CAC)₁₀ (E-F). Bars indicate 5 μm. The arrows indicate probe signals

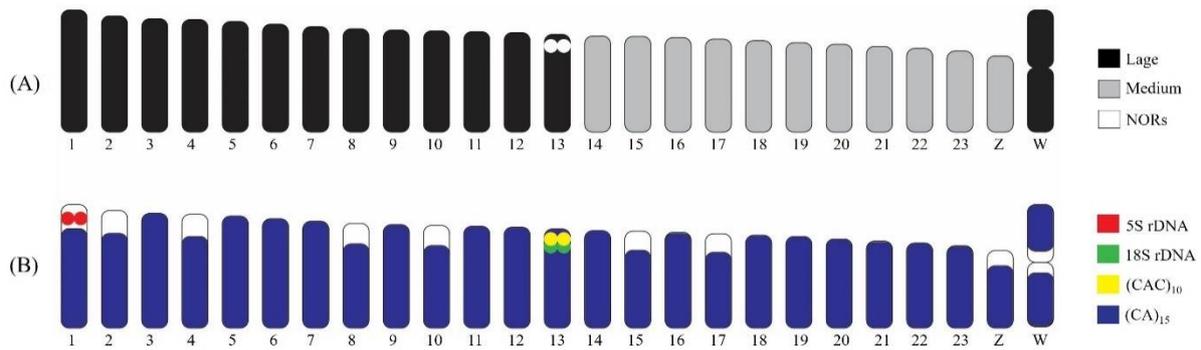


Figure 3. Idiograms of the Java combtail fish (*Belontia hasselti*), representing the haploid set ($n=23 + ZW$) by conventional staining, Ag-NOR banding (A) and FISH (B) techniques

Table 2. Means of the short arm length (Ls), long arm length (Ll) and total arm length of chromosomes (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI of 20 metaphase cells of the male and female Java combtail fish (*Belontia hasselti*), $2n=48$

Chro. pair	LS	LL	LT	RL \pm SD	CI \pm SD	Chro. Size	Chro. Type
1	0.000	1.437	1.437	0.052 \pm 0.003	1.000 \pm 0.000	Large	telocentric
2	0.000	1.368	1.368	0.050 \pm 0.003	1.000 \pm 0.000	Large	telocentric
3	0.000	1.334	1.334	0.048 \pm 0.002	1.000 \pm 0.000	Large	telocentric
4	0.000	1.324	1.324	0.048 \pm 0.002	1.000 \pm 0.000	Large	telocentric
5	0.000	1.301	1.301	0.047 \pm 0.002	1.000 \pm 0.000	Large	telocentric
6	0.000	1.270	1.270	0.046 \pm 0.002	1.000 \pm 0.000	Large	telocentric
7	0.000	1.241	1.241	0.045 \pm 0.001	1.000 \pm 0.000	Large	telocentric
8	0.000	1.216	1.216	0.044 \pm 0.001	1.000 \pm 0.000	Large	telocentric
9	0.000	1.201	1.201	0.044 \pm 0.001	1.000 \pm 0.000	Large	telocentric
10	0.000	1.192	1.192	0.043 \pm 0.001	1.000 \pm 0.000	Large	telocentric
11	0.000	1.182	1.182	0.043 \pm 0.001	1.000 \pm 0.000	Large	telocentric
12	0.000	1.171	1.171	0.043 \pm 0.001	1.000 \pm 0.000	Large	telocentric
13*	0.000	1.150	1.150	0.042 \pm 0.001	1.000 \pm 0.000	Medium	telocentric
14	0.000	1.130	1.130	0.041 \pm 0.001	1.000 \pm 0.000	Medium	telocentric
15	0.000	1.125	1.125	0.041 \pm 0.001	1.000 \pm 0.000	Medium	telocentric
16	0.000	1.112	1.112	0.040 \pm 0.001	1.000 \pm 0.000	Medium	telocentric
17	0.000	1.094	1.094	0.040 \pm 0.001	1.000 \pm 0.000	Medium	telocentric
18	0.000	1.076	1.076	0.039 \pm 0.001	1.000 \pm 0.000	Medium	telocentric
19	0.000	1.052	1.052	0.038 \pm 0.001	1.000 \pm 0.000	Medium	telocentric
20	0.000	1.033	1.033	0.038 \pm 0.001	1.000 \pm 0.000	Medium	telocentric
21	0.000	1.008	1.008	0.037 \pm 0.001	1.000 \pm 0.000	Medium	telocentric
22	0.000	0.985	0.985	0.036 \pm 0.001	1.000 \pm 0.000	Medium	telocentric
23	0.000	0.954	0.954	0.035 \pm 0.001	1.000 \pm 0.000	Medium	telocentric
Z	0.000	0.897	0.897	0.033 \pm 0.002	1.000 \pm 0.000	Medium	telocentric
W	0.680	0.757	1.437	0.052 \pm 0.018	0.528 \pm 0.118	Large	metacentric

Remarks: Chro.: Chromosome, *: NOR-bearing chromosome

In a comparative karyotypic analysis of males and females in *B. hasselti*, the presence of a female-specific large metacentric chromosome pointed towards the occurrence of a ZZ/ZW sex chromosome system, where the Z chromosome is represented by a small-sized telocentric. Inside the Osphronemidae family, two other species, named *T. fasciata* and *T. lalius* also presented differentiated sex chromosomes. *T. fasciata* has the ZZ/ZW sex chromosome system in some populations, whereas *T. lalius* present variations of sex chromosome systems as ZZ/ZO and XX/XO (Arai 2011). The absence of differentiated sex chromosomes can be considered as a plesiomorphic feature

(Vicari et al. 2008). Although most Neotropical fish species do not have differentiated sex chromosomes, some other kinds of sex chromosome systems have already described in some families. In certain groups, such as some species of Anostomidae and Triportheinae, only the ZZ/ZW system was identified, while in other groups, such as Belontiidae, Parodontidae and Erythrinidae, different sex chromosome systems were detected (Arai 2011; Cioffi et al. 2017). These results support the hypothesis of the differentiation of sex chromosomes in fishes having occurred independently in different groups (Almeida-Toledo et al. 2000). The presence of a sex chromosome system in the *B.*

hasselti reinforces the evidence of divergent karyotypic evolution in this group.

B. hasselti is characterized by a single pair of Ag-NORs, located at the interstitial subcentromeric regions of pair 13 in both the sexes. The single pair of NOR-bearing chromosomes is in accordance with *Trichopodus trichopterus*, but differs for the NOR location in telomeric region of the telocentric pair 2 (Supiwong et al. 2010). However, intraspecific NOR polymorphisms can be seen in *T. fasciata* in which one and three homologous chromosomes with NORs were reported (Sobita and Bhagirath 2007; Kushwaha et al. 2008). In fishes, the location of NORs in a terminal position, and close to the centromere, is also pondered to be a primitive feature. Single NORs are widespread in several fish taxa (Gornung 2013; Sochorová et al. 2018). Species with multiple NORs are not rare among fishes (Martinez et al. 2010). The presented results here indicated a plesiomorphic or a primitive condition, whereas multiple pairs of NORs were suggested to be an apomorphic or derived condition (Milhomem et al. 2013).

Our results revealed that the 5S rDNA and 18S rDNA sequences were present at interstitial subcentromeric regions of pairs 1 and 13, respectively. For comparison with other species in the same family, only one species, *T. trichopterus* has so far been studied. The present results differ, as the 5S rRNA gene is located at a proximal region on a pair of medium-sized chromosomes, whereas the 18S ribosomal gene is located at the telomeric region on long arms of the largest acrocentric pair (Pazza et al. 2009). Moreover, the 18S rDNA signals were located on chromosome pair 13, which is consistent with NORs regions. The location of NORs has been confirmed by FISH using rRNA or rDNA probes in fixed chromosomes of several vertebrates, including amphibians, humans, chimpanzees (Sluisa et al. 2012; Hirai 2020), and more recently, fishes (Rubert et al. 2011; Milhomem et al. 2013; Fernandes et al. 2019). In higher eukaryotes, the moderately repetitive ribosomal RNA genes (rDNAs) are arranged in two different families: the nucleolus forming major (45S), and the non-nucleolus forming minor (5S) rDNAs. The major family is composed of the regions coding for 18S, 5.8S and 28S rRNA genes, separated by internal transcribed spacers (ITS 1 and ITS 2), and surrounded by non-transcribed spacer (NTS) sequences (Singh et al. 2010). The nucleolar organizer regions contain 45S rDNA gene cluster, which has also been studied employing AgNO₃ and CMA₃ staining. The minor family is composed of highly conserved 120 bp long coding sequences separated by a variable non-transcribed spacer (NTS) (Da Silva et al. 2012). Martins and Galetti Jr. (2001) propose that the 5S rDNA interstitial position is optimal for its organization in fishes, since it has been found in most species of several orders. For these reasons, the conservation of the 5S rDNA distribution pattern may derive from the interstitial localization of these sites in the chromosomes.

The microsatellite repeats on the chromosomes of *B. hasselti* showed that d(CA)₁₅ signals are present on all chromosome pairs, throughout the whole chromosomes

except for centromeric regions of some pairs (pairs 2, 4, 8, 10, 15, and sex chromosome in both sexes), whereas, in most cases, they were found along the whole chromosomes. The patterns of d(CA)₁₅ in *B. hasselti* are similar to *Myxus* species (Supiwong et al. 2013a) and Thai pufferfish *Pao cochinchinensis* (Pissaparn et al. 2020). In turn, this pattern is inconsistent with other fish, for example, the *Hoplias malabaricus* Bloch 1794, d(CA)₁₅ probe signals provided a rich banding pattern in the subtelomeric region along most chromosome arms, while d(CA)₁₅ had only slight accumulation on the sex chromosomes (X and Y) (Cioffi et al. 2011). In the *Toxotes chatareus* Hamilton 1822, the d(CA)₁₅ repeats are abundantly distributed in all chromosomes, mostly in telomeric regions (Supiwong et al. 2017). From previous studies, it has been generally believed that microsatellites have specific zones as heterochromatin (telomeres, centromeres, and in the sex chromosomes) of fish genomes (Cioffi et al. 2011).

The pattern of d(CAC)₁₀ hybridized to the autosomes at the interstitial subcentromeric region on chromosome pair 13, showed interaction with NOR sites, and overlap with patterns of 18S rDNA in the present study. The pattern differs to that of d(CAC)₁₀ on *H. malabaricus*, producing a scattered distribution, and is thus more spread out along the autosomes. Furthermore, the microsatellite d(CAC)₁₀ was uniformly spread along the X chromosome, with just some weak signals in the heterochromatic areas and on the Y chromosome were slightly accumulated in the centromeric and/or telomeric heterochromatic regions (Cioffi et al. 2011). However, microsatellites have also been found in non-centromeric regions, many of them located either near or within genes (Rao et al. 2010), as found in the present study.

In summary, here we provided the first (molecular) cytogenetic study of the *B. hasselti*, proposing the probable occurrence of a ZZ/ZW sex chromosome system, that needs further confirmation. The results obtained here can be used to support further investigation of taxonomy and evolutionary relationship among the family Osphronemidae and others.

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