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Karyological analysis of the halfmoon triggerfish (*Sufflamen chrysopterum*) by the conventional and molecular cytogenetic techniques

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Abstract - This research aims to analyze the karyotype and other chromosomal characteristics of the halfmoon triggerfish (*Sufflamen chrysopterum*) from the Gulf of Thailand (Pacific Ocean). Chromosomes were prepared directly from kidney tissue of fish, and employed the conventional and the molecular cytogenetic analyses by 18S and 5S rDNA, together with CA_{15}, GA_{15}, and CAA_{10} microsatellites as probes. The results showed 2n=46 (46t; NF=46) in both male and female fish, and that the sex chromosomes could not be distinguished from both sexes. The C-positive heterochromatin was found in the centromeric regions of most chromosomes, and NORs localized single pairs which coincide with the location of the 18S rDNA probe on the telocentric chromosome No. 6. Moreover, the luminous signal of 5S rDNA probe was detected at the subcentromeric region of the same telocentric pair. The sign of $(CA)_{15}$, $(GA)_{15}$, and $(CAA)_{10}$ microsatellites is sparsely dispersed along all the chromosomes, especially the $(CA)_{15}$ sequences which indicates preferential accumulation at the telomeric and subcentromeric site of almost chromosome. Our study also provides additional data on the accumulation of repetitive sequences in the *S. chrysopterum*. Besides, the chromosomal organization of microsatellite DNAs among triggerfish species is also reviewed.

Keywords: Triggerfish, chromosome, repetitive sequences, fish cytogenetics

1. Introduction

The triggerfish (Balistidae) is a specious group with at least 40 species, representing the fish family that occur in the coral reef ecosystems, coral lagoons, and external reef slopes, where they play an important ecological role and are more frequently encountered by divers than by fishermen. They are widely distributed in tropical and temperate coastal regions, with a higher species' diversity occurring in the Atlantic, Indian and Pacific oceans (Schultz, 2004).

This family have eight genera have been extensively investigated in cytogenetic studies. Cytogenetic analysis has been indicating a model of conservative cytogenetic characteristics among triggerfishes, showing the highest acrocentric/telocentric chromosome. However, despite these suitable reports for chromosomal evolution studies, cytogenetic data are still confined to the conventional analyzes (Arai, 2011). The mapping of ribosomal sequences in the chromosomes of Atlantic species of the triggerfish was reported only one species (de Lima *et al*., 2011) and no molecular cytogenetic data are yet available for family Balistidae, limiting the understanding of its evolutionary chromosomal processes.

Thus, in order to investigate the evolutionary events associated with the chromosomal diversification in this group, the present study includes the chromosomal investigation, using conventional (Giemsa staining, Ag-NOR and C-banding) and molecular (*in situ* mapping of five different repetitive DNA classes) approaches in the halfmoon triggerfish (*Sufflamen chrysopterum*) from the Indo-Pacific region, that only two previous cytogenetic studies of the *Sufflamen* genus for example *S. chrysopterum* and *S. fraenatus* showing a same diploid chromosome number of 2n=46 (46a/t) (Arai & Nagaiwa, 1976; Takai and Ojima, 1987). This indicates that they are closely related. However, the results obtained from this study can provide cytogenetic information about *S. chrysopterum*, which is useful to support the taxonomy of the genus *Sufflamen*, and provides additional data on the repetitive sequences in among triggerfish species for its evolutionary chromosomal processes.

2. Materials and methods

2.1 Specimens, chromosomal preparations and banding

The halfmoon triggerfish, *Sufflamen chrysopterum* (5 males and 5 females) from the Gulf of Thailand (Pacific Ocean) were analyzed (Figure 1). The specimens were caught using hand-net. After capture, animals were placed in sealed plastic bags containing oxygen with clean water (seawater) and transported to the research station. The experiments followed ethical protocols and anesthesia with keeping the fish frozen before to sacrificing the animals to minimize suffering. The process was approved by the Ethics Committee of Khon Kaen University and by the RGJ Committee under licence no. U1-04484-2559. Mitotic chromosomes were obtained from cell suspensions of the anterior kidney, using the conventional air-drying method (Bertollo *et al*., 1978). The C-banding method was also employed to detect the distribution of the C-positive heterochromatin on the chromosomes (Sumner, 1972). The specimens were deposited in the fish collection of the Cytogenetic Laboratory, Department of Biology, Faculty of Science, Khon Kaen University.

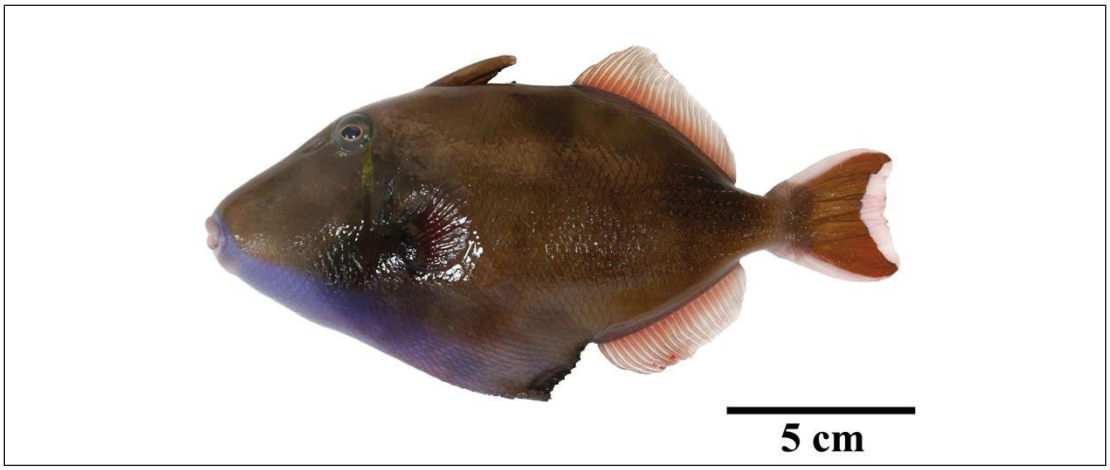

Figure 1. General characteristic of the halfmoon triggerfish (*Sufflamen chrysopterum*)

2.2 Chromosome probes and FISH experiments

Two tandemly-arrayed DNA sequences isolated from the genome of an Erythrinidae fish species, *Hoplias malabaricus*, were used as probes. The first probe contained a 5S rDNA repeat copy and included 120 base pairs (bp) of the 5S rRNA transcribed gene and 200 bp of the non-transcribed spacer (NTS) sequence (Martins *et al*., 2006). The second probe contained a 1,400 bp segment of the 18S rRNA gene obtained via PCR from nuclear DNA (Cioffi *et al*., 2009). The 5S and 18S rDNA probes were cloned into plasmid vectors and propagated in DH5α *Escherichia coli* competent cells (Invitrogen, San Diego, CA, USA).

The 18S and 5S rDNA probes were labeled with Spectrum Orange-dUTP and Spectrum Green-dUTP, respectively, using nick translation according to the manufacturer's recommendations (Roche, Mannheim, Germany).

The microsatellites $(CA)_{15}$, $(GA)_{15}$, and $(CAA)_{10}$ were used as probes and were synthesized according to previous work (Kubat *et al*., 2008). These sequences were directly labeled with Cy3 at the 5' terminus during synthesis by protocol of Sigma (St. Louis, MO, USA).

Fluorescence *in situ* hybridization (FISH) was performed under high stringency conditions on mitotic chromosome spreads (Pinkel *et al*., 1986). Metaphase chromosome slides were incubated with RNAse (40 µg/ml) for 1.5 h at 37 °C. After denaturation of the chromosomal DNA in 70% formamide/2x SSC, pH=7.0, at 70 °C for 4 min, the hybridization mixture (2.5 ng/µl probes, 2 µg/µl salmon sperm DNA, 50% deionized formamide, 10% dextran sulphate) was dropped on the slides, and the hybridization was performed overnight at 37 °C in a moist chamber containing 2x SSC. The first post-hybridization wash was performed with 2x SSC for 5 min at 65 °C, and a final wash was performed at room temperature in 1x SSC for 5 min. Finally, the slides were counterstained with DAPI and mounted in an antifade solution (Vectashield from Vector Laboratories).

2.3 Image processing

At least 30 metaphase spreads were analyzed to confirm the 2n, karyotype structure and FISH results. Images were captured using an Olympus BX50 microscope (Olympus Corporation, Ishikawa, Japan) with CoolSNAP camera and the images processed using Image Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD, USA). Chromosomes were classified as metacentric (m), submetacentric (sm), acrocentric (a) or telocentric (t) according to their relative length (Chaiyasut, 1989).

3. Results and discussion

3.1 Karyotype uniformity among *Sufflamen* species

This study aimed to clarify the cytogenetic analysis of the halfmoon triggerfish, *Sufflamen chrysopterum* from the Gulf of Thailand (Indo-Pacific). This represents the diploid chromosome number of *S. chrysopterum* is 2n = 46, with karyotypes predominantly formed by telocentric chromosomes in both males and females and a fundamental number (NF) is 42 (Figure 2 and Table 1). However, several species in the family Balistidae have been extensively investigated in cytogenetic studies (Table 2). The karyotype of *S. chrysopterum* and *S. fraenatus* showing a same diploid chromosome number of 2n=46 (46a/t) (Arai & Nagaiwa, 1976; Takai & Ojima, 1987; present study) this indicates that they are closely related. Besides, like all other species in the family Balistidae, no heteromorphic sizes of sex chromosomes were found (Arai & Nagaiwa, 1976; Kitayama & Ojima, 1984; Takai & Ojima, 1987,1988; Vitturi, *et al*., 1988,1992; Thode *et al*., 1994; Gustavo & Molina, 2004, 2005).

The karyotypes of the Sufflamen species exhibited normal structural patterns, including displaying 2n = 46 and having a high number of telocentric chromosomes like the common ancestors of marine fish. The chromosomal basis of the genomes of these species is the low variability of chromosomal rearrangements. These characteristics are present in among of the Balistid fish analyzed so far except *Balistoides* and *Pseudobalistes* genera (Takai and Ojima, 1988; Supiwong *et al*., 2013).

Table 1. Mean length of short arm chromosome (Ls), length of long arm chromosome (Ll), length of total arm chromosome (LT), relative length (RL), centromeric index (CI), and standard deviation (SD) of RL, CI from 20 metaphase cells of the male and female the halfmoon triggerfish, *Sufflamen chrysopterum*, 2*n*=46

Chromosome pair	Ls	Ll	LT	RL±SD	CI±SD	Chromosome type
1	0.00	3.69	3.69	0.069±0.010	1.000±0.000	telocentric
2	0.00	3.67	3.67	0.056±0.005	1.000±0.000	telocentric
3	0.00	3.40	3.40	0.052±0.005	1.000±0.000	telocentric
4	0.00	3.40	3.40	0.048±0.004	1.000±0.000	telocentric

Table 1. Mean length of short arm chromosome (Ls), length of long arm chromosome (Ll), length of total arm chromosome (LT), relative length (RL), centromeric index (CI), and standard deviation (SD) of RL, CI from 20 metaphase cells of the male and female the halfmoon triggerfish, *Sufflamen chrysopterum*, 2*n*=46 (cont.)

Chromosome pair	Ls	Ll	LT	RL±SD	CI±SD	Chromosome type
5	0.00	3.32	3.32	0.050±0.004	1.000±0.000	telocentric
6*	0.00	3.29	3.29	0.049±0.004	1.000±0.000	telocentric
7	0.00	3.26	3.26	0.052±0.005	1.000±0.000	telocentric
8	0.00	3.15	3.15	0.045±0.004	1.000±0.000	telocentric
9	0.00	3.12	3.12	0.043±0.004	1.000±0.000	telocentric
10	0.00	3.03	3.03	0.049±0.007	1.000±0.000	telocentric
11	0.00	2.98	2.98	0.045±0.006	1.000±0.000	telocentric
12	0.00	2.96	2.96	0.045±0.006	1.000±0.000	telocentric
13	0.00	2.94	2.94	0.042±0.005	1.000±0.000	telocentric
14	0.00	2.93	2.93	0.041±0.005	1.000±0.000	telocentric
15	0.00	2.86	2.86	0.037±0.004	1.000±0.000	telocentric
16	0.00	2.74	2.74	0.046±0.005	1.000±0.000	telocentric
17	0.00	2.53	2.53	0.042±0.005	1.000±0.000	telocentric
18	0.00	2.49	2.49	0.035±0.007	1.000±0.000	telocentric
19	0.00	2.41	2.41	0.036±0.007	1.000±0.000	telocentric
20	0.00	2.29	2.29	0.034±0.006	1.000±0.000	telocentric
21	0.00	1.89	1.89	0.031±0.007	1.000±0.000	telocentric
22	0.00	1.66	1.66	0.029±0.008	1.000±0.000	telocentric
23	0.00	1.49	1.49	0.024±0.007	1.000±0.000	telocentric

Remark: * NOR-bearing chromosome.

No cytologically distinguishable sex chromosomes were observed. It is possible that the change in the sex chromosomes of the fish depends on the onset of differentiation (Supiwong *et al*., 2012). Therefore, chromosomes containing sex-determination gene cannot be found by cytogenetic analyses. The origin and development of sex-chromosome has been reported on Neotropical fish in Brazil (Bertollo *et al*., 2004).

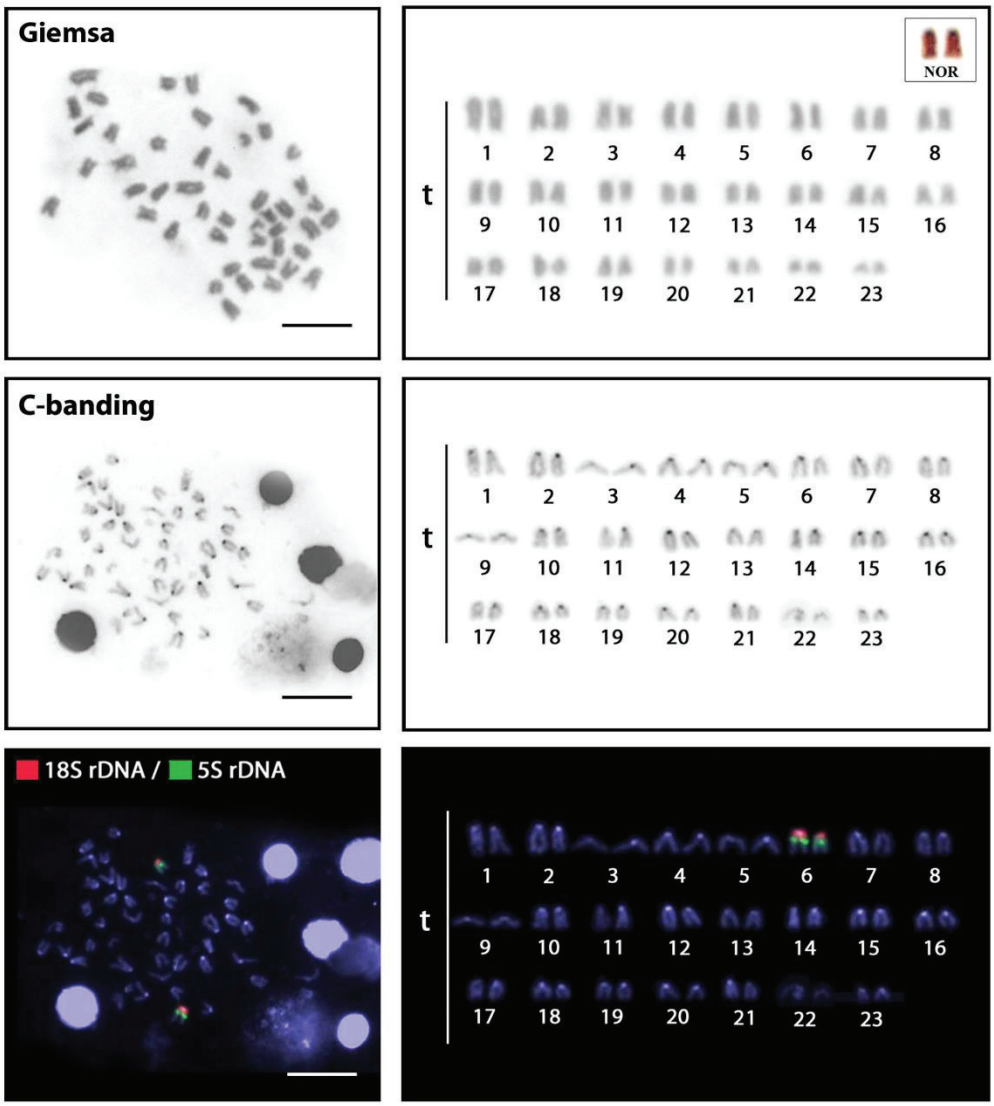

Figure 2. Metaphase and karyotypes of the *Sufflamen chrysopterum* arranged from conventionally Giemsa-stained, Ag-stained (showed in the box), C-banded and after fluorescence *in situ* hybridization with 18S rDNA and 5S rDNA probes. Bar = 5 μm.

3.2 Chromosome markers of *S. chrysopterum*

The C-positive heterochromatin in the chromosomes of *S. chrysopterum* was detected on the centromeric regions of most chromosomes (Figure 2). This recurring distribution pattern is similar to those reported for species of other *Balistidae* genera, such as *Melichthys* (Gustavo & Molina, 2005). In addition, the Ag-NOR sites of *S. chrysopterum* occupy the centromeric portion of the telocentric chromosome (pair 6) (Figure 4) that match the exclusive locations of an 18S rDNA site (Figure 2). Normally, the 18S rDNA gene are syntenic sequences of the 5S rDNA gene and we found that the 5S rDNA site appeared

on the same chromosome exclusively on the subcentromeric region (Figure 2). The syntenic organization of these genes is frequent and a probable apomorphic condition in Balistidae (Fontana *et al*., 2003; Penda ´s *et al*., 1993). Genes encoding ribosomal RNAs (rRNA) have been used as suited markers for chromosomal investigations. The rDNAs play an important role in protein synthesis, in which 45S rDNA encodes for 18S, 5.8S and 28S rRNAs, and the 5S rDNA encodes for 5S rRNA. All rDNAs are processed to form ribosome subunits in which 45S rDNAs are generated in the nucleolus (nucleolus organizer region), while 5S rRNAs are first synthesized in the nucleoplasm and which later enter the nucleolus to form a functional component of the large ribosomal subunit (Supiwong *et al*., 2014; Yano *et al*., 2017).

Only one pair of Ag-NOR/18S rDNA sites are useful chromosomal markers which are shared among the *Sufflamen* species. *S. chrysopterum* was found to have similar chromosome bearing nucleolar organizer regions as the *S. fraenatus* previous studies (Takai & Ojima, 1987) and also similar to what has been found in most species of the family Balistidae (Table 2). However, the mapping of ribosomal sequences in the chromosomes of Atlantic species of the triggerfish was reported only one species (de Lima *et al*., 2011) and no molecular cytogenetic data are yet available for family Balistidae. Beside, cytogenetic data in the *Sufflamen* genus is restricted to few species and it is still confined to the conventional analyzes (Arai, 2011).

3.3 Organization of repetitive DNAs in the chromosomes of *S. chrysopterum*

This is the first report in which the organizational patterns of microsatellite sequences in the heterochromatin of the *S. chrysopterum* were studied on the $(CA)_{15}$, $(GA)_{15}$, and $(CAA)_{10}$. Chromosomal mapping of all microsatellite repeats which indicated that scattered signal on the chromosomes differed from each other and without preferential accumulations in any of the chromosomal pairs (Figure 3). The $(CA)_{15}$, $(GA)_{15}$, and $(CAA)_{10}$ microsatellite sequences sparsely detected a weak signal in most chromosomes. However, some of those sequences are also conspicuous clusters in some chromosome pairs especially the (CA)15 sequence which is clearly exhibited on the telomeric and subcentromeric region of some pair (Figure 3). All the microsatellite markers were detected, and had the same patterns in both male and female.

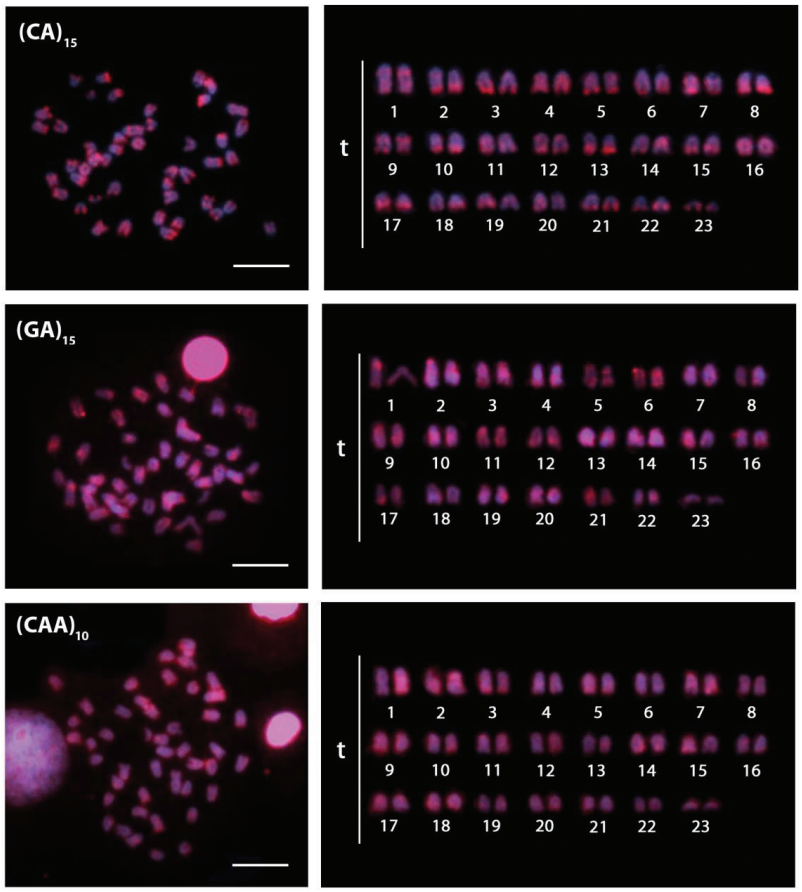

Figure 3. Chromosomal mapping of $(CA)_{15}$, $(GA)_{15}$, and $(CAA)_{10}$ microsatellites in the chromosomes of the *Sufflamen chrysopterum* by fluorescence *in situ* hybridization. Bar = 5 μm.

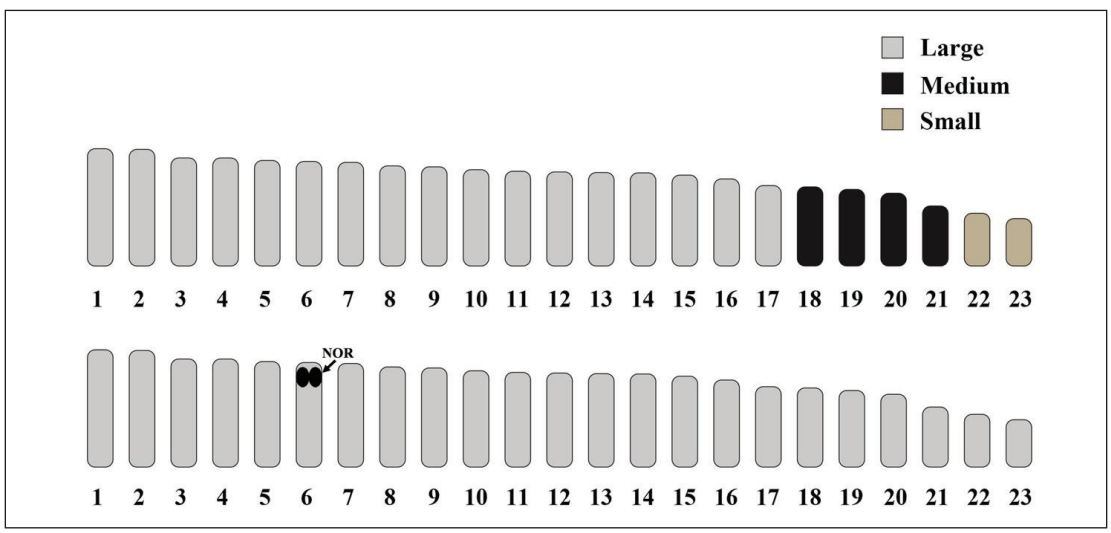

Figure 4. Standard idiogram of the *Sufflamen chrysopterum* arranged from conventionally Giemsa-stained and Ag-stained (arrow indicated NOR)

However, this data is useful for comparing the phylogenetic proximity of this triggerfish with might share the same distribution pattern of the microsatellite sequences. This points to independent evolutionary pathways constituting homoplastic chromosomal characters. The previous data indicated that the repetitive DNA has specific zones as heterochromatin (telomeres, centromeres, and in the sex chromosomes) of eukaryotic genomes (Cioffi *et al*., 2012). Rarely, molecular cytogenetic data from the Balistidae family are available, some reports indicated microsatellites have also been found in non-centromeric regions. Many of them were located either near or within genes (Rao *et al*., 2010). However, since these sequences are subject to high rates of change, might exhibit substantial evolutionary divergence in their distribution. (Cioffi *et al*., 2012). In fact, the organization of microsatellite sequences demonstrates particular arrangements that repetitive DNAs can be achieved in different species providing thus relevant information about the chromosomal structure in genomes of various taxa.

Table 2. Cytogenetic reviews of the family Balistidae (8 genera).

No.	Subfamily/Species	2*n*	NF	NORs	Formula	References
1	Balistapus undulates	42	42	2	42a/t	Takai and Ojima (1987)
2	Balistes capriscus	44	44	2	44t	Vitturi *et al.* (1988)
3	B.carolinensis	44	44	2	44t	Vitturi *et al.* (1992)
		44	44	2	44t	Thode *et al.* (1994)
4	B. vetula	44	44	2	44t	Gustavo and Molina (2005)
5	Balistoides conspicillus	44	44	2	44t	Takai and Ojima (1987)
		44	44	2	44t	Gustavo and Molina (2004)
6	B. viridescens	44	48	2	2m+2sm +40a/t	Takai and Ojima (1988)
		44	60	3	2m+14a +28t	Supiwong *et al.* (2013)
7	Melichthys niger	40	40	2	40t	Gustavo and Molina (2005)
		40	40	2	40a/t	de Lima *et al.* (2011)
8	M. vidua	40	40	2	40a/t	Kitayama and Ojima (1984)
9	Odonus niger	42	–	–	42a/t	Kitayama and Ojima (1984)
10	Pseudobalistes flavi-marginatus	44	–	–	2m+42a/t	Arai and Nagaiwa (1976)
11	Rhinecanthus aculeatus	44	44	2	44t	Arai and Nagaiwa (1976)
		44	44	2	44t	Kitayama and Ojima (1984)
12	R. echarpe	44	–	2	44a/t	Kitayama and Ojima (1984)
13	R. verrucosus	44	44	2	44t	Arai and Nagaiwa (1976)
14	Sufflamen chrysopterus	46	46	–	46a/t	Arai and Nagaiwa (1976)
15	S. fraenatus	46	46	2	46a/t	Takai and Ojima (1987)

Remarks: 2*n* = diploid chromosome number, NF = fundamental number (number of chromosome arm), m = metacentric, sm = submetacentric, a = acrocentric, t = telocentric chromosome, NORs = nucleolar organizer regions and – = not available.

3.4 Chromosome evolution of the family Balistidae

Diversification of karyotype patterns among saltwaterfish species has been caused by chromosomal rearrangements (Getlekha *et al*., 2018). The different Balistidae species underwent an extremely diversified karyotype evolution, considering the numerical and structural aspects of their complements, with diploid chromosome number varying from 2n=40 to 46, and marked differences in the NF that varied from 40 to 60, possibly due to the occurrence of pericentric inversions (Getlekha *et al*., 2018). Analyses performed highlight the combined importance of the different chromosome rearrangements in the evolutionary modelling of their karyotypes, such as centric fission, centric fusion, and especially, pericentric inversions (Gustavo & Molina, 2005; Getlekha *et al*., 2016a; 2016b; 2018). The family Balistidae has 2n values lower than 2n=48 with most of their representatives presenting acrocentric and telocentric chromosomes with the occurrence of concerted pericentric inversions or centric fusions in *Balistoides* and *Pseudobalistes* (Takai and Ojima, 1988; Arai & Nagaiwa, 1976).

However, we noticed that this karyotypic pattern was also observed in the present study in of *S. chrysopterum* (2n=46). The origin of the reduced diploid chromosome numbers in these species seems to be centric fissions but chromosomes lost in tandem, which seems to be common in other species of the family.

4. Conclusion

Mapping of repetitive DNA sequences of the halfmoon triggerfish, *Sufflamen chrysopterum* from the Gulf of Thailand (Indo-Pacific) has been generated, providing an important source of information for better understanding the involvement of repetitive DNA sequences in chromosomal organization. DNA sequence data combined with the chromosomal mapping of these repeated elements utilizing cytogenetic techniques can provide a clearer picture of the genome, which is not yet clearly defined, even if it has already been sequenced. This is the first record of the karyological studies of *S. chrysopterum* and the family Balistidae in Thailand.

Up to the present, there are only two reports that have *Sufflamen* species cytogenetically analyzed. Those species provide remarkable karyotype features for chromosomal and genetic conservatism discussion. Further studies of other species as well as additional information and molecular techniques for chromosome analyses are expected to clarify and explain the reasons behind the karyotype pattern and chromosome evolution in triggerfishes.

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