Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Kamika Sribenja, Alongklod Tanomtong, Nuntaporn Getlekha (2022). Chromosome Mapping of Repetitive DNAs in the Picasso Triggerfish (*Rhinecanthus aculeatus* (Linnaeus, 1758)) in Family Balistidae by Classical and Molecular Cytogenetic Techniques. *Caryologia* 75(3): 3-11. doi: 10.36253/caryologia-1731

Received: July 08, 2022

Accepted: November 19, 2022

Published: April 5, 2023

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Chromosome Mapping of Repetitive DNAs in the Picasso Triggerfish (*Rhinecanthus aculeatus* (Linnaeus, 1758)) in Family Balistidae by Classical and Molecular Cytogenetic Techniques

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Abstract. This work presents the cytogenetic analysis conducted on the Picasso triggerfish (*Rhinecanthus aculeatus* (Linnaeus, 1758)) from Thailand. Mitotic chromosomes were prepared from the anterior kidney. The cell suspensions were harvested by *in vivo* colchicine treatment. The present study includes the chromosomal investigation on *R. aculeatus*, using conventional (Giemsa staining, Ag-NOR and C-banding) and molecular approaches (*in situ* mapping of five different repetitive DNA classes including 18S rDNA, 5S rDNA, $(CA)_{15}$, $(GA)_{15}$ and $(CAA)_{10}$ as markers.) The results showed that *R. aculeatus* has karyotypes formed exclusively by telocentric chromosomes (44t; NF=44). The C-positive heterochromatic blocks are preferentially located in the centromeric and telomeric regions of some chromosomal pairs. The Ag-NOR sites occupy the interstitial position of the long arms of the largest telocentric pair (pair 1). The exclusive location of the major ribosomal sites in these pairs was confirmed by hybridization with 18S rDNA probes. However, the 5S rDNA genes are not located on 18S rDNA-bearing chromosomes, but instead located exclusively in the subcentromeric region of pair 4. The mapping of $(CA)_{15}$, $(GA)_{15}$ and $(CAA)_{10}$ microsatellites are sparsely dispersed along all the chromosomes. The karyotype formula of *R. aculeatus* is 2n (44) = 44t.

Keywords: Triggerfish, Chromosome, Repetitive sequences, Fish cytogenetics.

INTRODUCTION

The family Balistidae belongs to order Tetraodontiformes which includes the triggerfish of often brightly colored fish (Nelson et al., 2016). Around 40 species distributed in 12 genera are classified in this family (Allen et al., 2017). Triggerfish fishes are usually found in tropical and subtropical oceans throughout the world, with the greatest species richness in the Indo-Pacific.

The most abundance of species are found in relatively shallow, coastal habitats, especially at coral reefs (Allen et al., 2017). In the present, several species from this family are popular in the marine aquarium trade.

Out of 40 described species of Balistidae, only 15 species have been karyologically investigated: *Balistapus undulates*, *Sufflamen fraenatus* (Takai and Ojima, 1987), *Balistes capriscus* (Vitturi et al., 1988), *Balistes carolinensis* (Vitturi et al., 1992; Thode et al., 1994), *Balistes vetula* (Gustavo and Molina, 2005), *Balistoides conspicillus* (Takai and Ojima, 1987; Gustavo and Molina, 2004), *Balistoides viridescens* (Takai and Ojima, 1988), *Pseudobalistes flavimarginatus*, *Rhinecanthus verrucosus*, *Sufflamen chrysopterus* (Arai and Nagaiwa, 1976), *Rhinecanthus aculeatus* (Arai and Nagaiwa, 1976; Kitayama and Ojima, 1984), *Rhinecanthus echarpe* (Kitayama and Ojima, 1984), *Melichthys niger* (Gustavo and Molina, 2005) and *Melichthys vidua*, *Odonus niger* (Kitayam and Ojima, 1984). The members of the family Balistidae have 2n ranging from 40 to 46, and most species have the karyotype present as acrocentric and telocentric chromosomes except *B. viridescens* and *P. flavimarginatus*, which are comprised of metacentric and submetacentric chromosomes (Table 1).

The study aims to investigate the evolutionary events associated with the chromosomal diversification in the Picasso triggerfish (*Rhinecanthus aculeatus*). The chromosomal investigation was conducted by obtaining the standard karyotype and idiogram using conventional (Giemsa staining, Ag-NOR and C-banding) and molecular analyses to identify the chromosomal patterns and organization of five classes of repetitive DNAs [18S rDNA, 5S rDNA, $(CA)_{15}$, $(GA)_{15}$, and $(CAA)_{10}$]. Since there were only three previous cytogenetic studies of the genus *Rhinecanthus* showing a diploid chromosome number of 2n=44 (Arai and Nagaiwa, 1976; Kitayama and Ojima, 1984), the results obtained from this study will increase our basic knowledge of the cytogenetics of *R. aculeatus*, which could form the basis for future research and support taxonomy of genus *Rhinecanthus*.

MATERIAL AND METHODS

Specimens collected and conventional methods

Cytogenetic analyses were conducted on the Picasso triggerfish, *Rhinecanthus aculeatus* (4 males and 4 females) from Thailand Gulf (Figure 1). The specimens were caught using a hand-net, placed in sealed plastic bags containing oxygen and clean water and transported to the research station. The experiments followed ethical protocols and

Table 1. 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 flavimarginatus*	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.

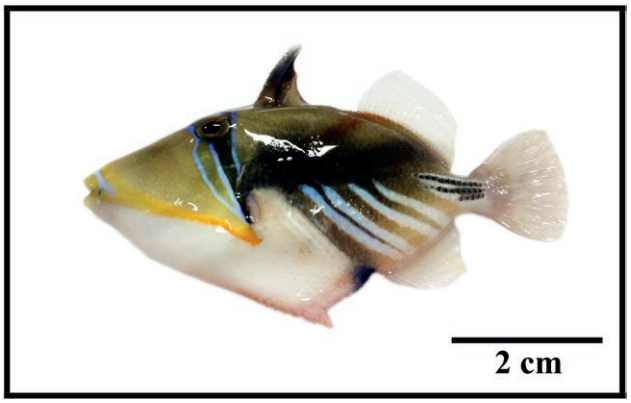

Figure 1. General characteristic of *Rhinecanthus aculeatus*, its respective collection sites in the Indian (Andaman Sea) and Pacific Oceans (Gulf of Thailand).

anesthesia with clove oil prior 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 no. PHD/K0081/2556. Mitotic chromosomes were obtained from cell suspensions of the anterior kidney, using the conventional air-drying method. The C-banding method was also employed to detect the distribution of C-positive heterochromatin and silver staining to detect the Ag-NOR location on chromosomes. The specimens were deposited in the fish collection of the Cytogenetic Laboratory, Department of Biology, Faculty of Science, Khon Kaen University.

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 and included 120 base pairs (bp) of the 5S rRNA transcribed gene and 200 bp of the non-transcribed spacer (NTS) sequence. The second probe contained a 1400 bp segment of the 18S rRNA gene obtained via PCR from the nuclear DNA. The 5S and 18S rDNA probes were cloned into plasmid vectors and propagated in DH5a *Escherichia coli* competent cells (Invitrogen, San Diego, CA, USA). The 5S and 18S rDNA probes were labeled with Spectrum Green-dUTP and Spectrum Orange-dUTP, respectively, using nick translation according to the manufacturer's recommendations (Roche, Mannheim, Germany).

The microsatellites $(CA)_{15}$, $(GA)_{15}$, and $(CAA)_{10}$ were synthesized. These sequences were directly labeled with Cy3 at the 5' terminus during synthesis by Sigma (St. Louis, MO, USA).

Fluorescence *in situ* hybridization (FISH) was performed under high stringency conditions (Yano et al., 2017). Metaphase chromosome slides were incubated with RNAse (40 µg/ml) for 1.5 h at 37°C. After the denaturation of the chromosomal DNA in 70% formamide/2x SSC at 70°C for 4 min, 20 µl of 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).

Image processing

Approximately 20 metaphase spreads were analyzed to confirm the diploid chromosome number, karyotype structure and FISH results. Images were captured using an Olympus BX50 microscope (Olympus Corporation, Ishikawa, Japan) with CoolSNAP and the Image Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD, USA). Chromosomes were classified according to their arm ratios as metacentric (m), submetacentric (sm), acrocentric (a) or telocentric (t).

RESULTS

The Picasso triggerfish (*Rhinecanthus aculeatus*) have 2n=44. Its karyotype is formed exclusively by telocentric chromosomes (44t) and a fundamental number (NF=44) (Figure 2). The C-positive heterochromatic blocks are preferentially located in the centromeric regions, with some pairs exhibiting blocks in the telomeric ones (Figure 2). The Ag-NOR sites are located in the interstitial region of the long arms of the largest telocentric pair (pair 1), the exclusive location of major ribosomal sites in these regions was confirmed by *in situ* hybridization with 18S rDNA probes (Figure 2). However, the 5S rDNA genes are not located on 18S rDNA-bearing chromosomes, but instead located exclusively in the subcentromeric region of pair 4, while the 18S rDNA sites are instead located on the interstitial position of the long arms of pair 1 (Figure 2).

The chromosomal mapping of all microsatellite sequences indicates a weak and dispersed distribution, without preferential accumulations in any of the chromosomal pairs (Figure 3). The $(CA)_{15}$ $(GA)_{15}$ and $(CAA)_{10}$

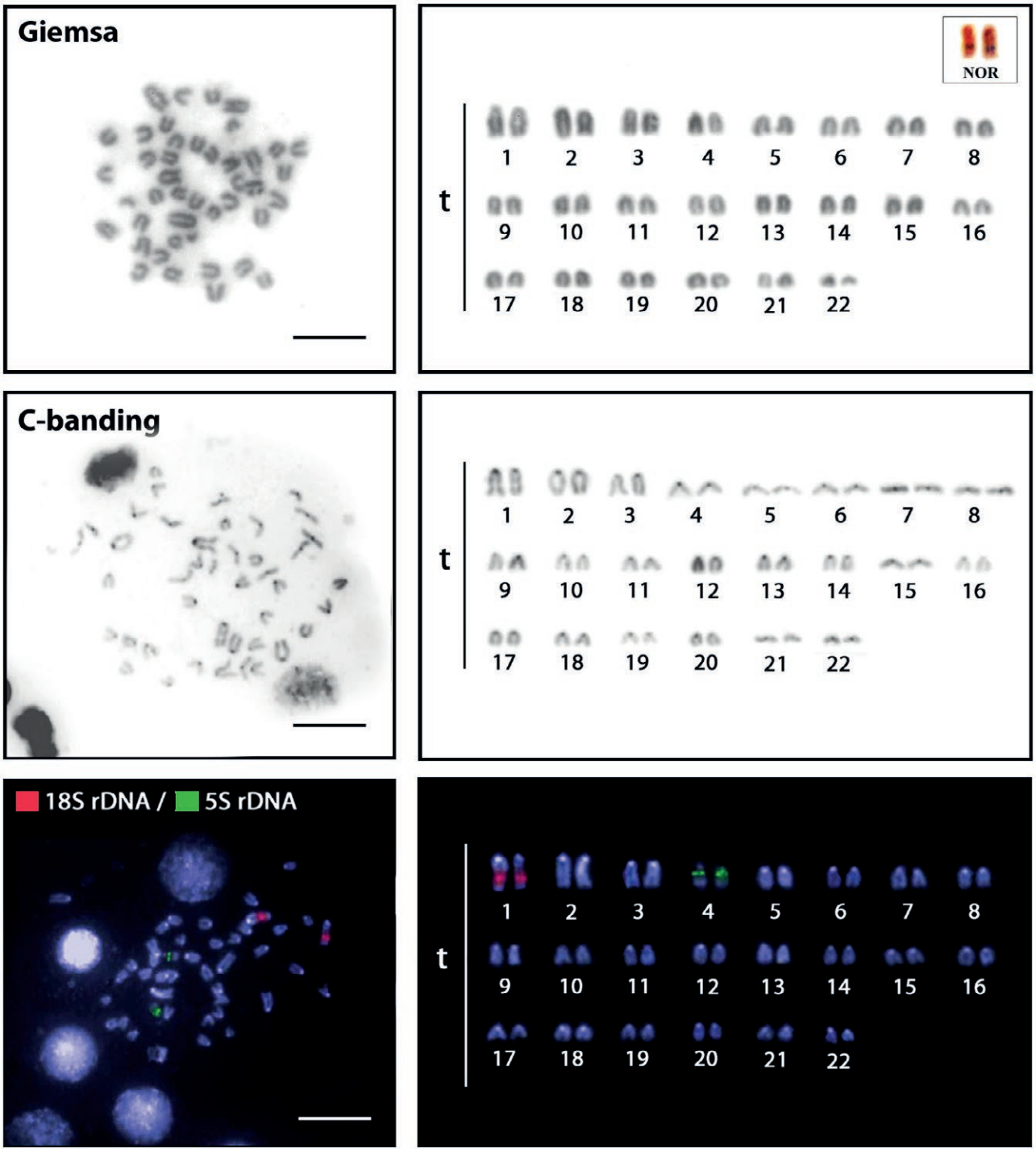

Figure 2. Mataphase and karyotypes of *Rhinecanthus aculeatus* arranged from conventionally Giemsa-stained, Ag-stained (highlighted in the boxes), C-banded and after fluorescence *in situ* hybridization with 5S and 18S rDNA probes. Bar 5 μm.

microsatellites are sparsely dispersed in most chromosomes though they can still form conspicuous clusters. They however exhibit less defined clusters in some chromosome pairs (Figure 3). These clusters occupy the centromeric regions of chromosomes but at rather low frequency. For all the chromosomal markers, no differential hybridization patterns were detected between males and females.

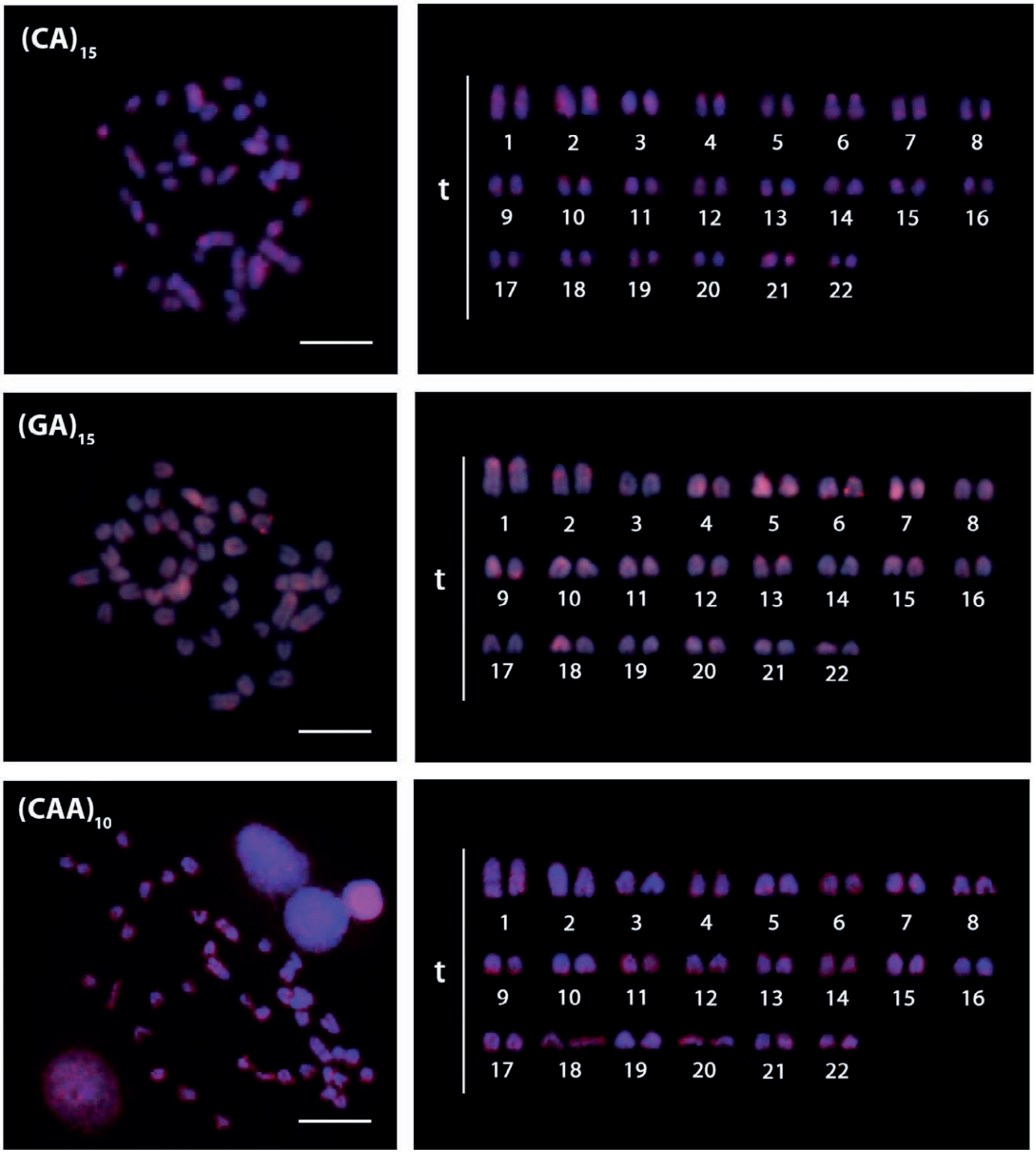

Figure 3. Chromosomal mapping of di- and tri-nucleotide microsatellites in the chromosomes of *Rhinecanthus aculeatus* by fluorescence *in situ* hybridization. The general distribution pattern of $(CA)_{15}$, $(GA)_{15}$ and $(CAA)_{10}$ microsatellites is mainly diffuse, with the occurrence of few conspicuous clusters in some centromeric and chromosome arm regions. Bar = 5 μm.

The idiogram of *R. aculeatus* represents gradually declining length of the chromosomes (Figure 4). The karyotype is notably attributed solely to telocentric chromosomes. The karyotype formula of *R. aculeatus* is as follow: 2n (44) = 44t

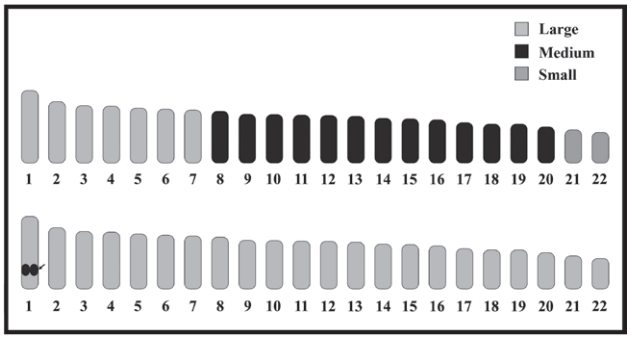

Figure 4. Standardized idiogram showing lengths and shapes of chromosomes of *Rhinecanthus aculeatus* (2*n*=44) by conventional staining and Ag-NOR banding techniques. The arrow indicates nucleolarnucleolar organizer regions.

DISCUSSIONS

Karyotype uniformity among Rhinecanthus *species*

Cytogenetic analyses were conducted on *Rhinecanthus aculeatus* from the Gulf of Thailand (Indo-Pacific). The 2n of *R. aculeatus* is 44 chromosomes in all specimens, with karyotypes predominantly formed by telocentric chromosomes (Figure 2, 3 and Table 2). However, considerable cytogenetic research has been conducted on a number of species in the family Balistidae (Table 1). The karyotype of *Rhinecanthus aculeatus* is 44t; this finding is consistent with that of other species in the genera *Rhinecanthus*, *Balistes*, and *Balistoides*, particularly *Balistes capriscus*, *B. carolinensis*, *B. vetula*, *Balistoides conspicillus*, *Rhinecanthus aculeatus*, *R. echarpe*, and *R. verrucosus*, which still have 2n=44t. It suggests that even after speciation, their karyotypes remain conserved. Although *B. viridescens* and *Pseudobalistes flavimarginatus* have the same number of chromosomes (2n=44) with above species but exhibit an asymmetrical karyotype due to both species are the high variability of chromosomal rearrangements and their higher adaptive divergence. Moreover, like all other species in the family Balistidae, in which the morphologically differentiated sex chromosome could not be observed (Arai and Nagaiwa, 1976; Kitayama and Ojima, 1984).

In addition, *Rhinecanthus* species exhibited karyotypes which are broadly similar in structural patterns, with all of them displaying 2n = 44 and a high number of telocentric chromosomes. These characteristics present in all of the *Rhinecanthus* species analyzed so far (Arai and Nagaiwa, 1976; Kitayama and Ojima, 1984; Montanari et al. 2016; present study).

Chromosome markers of R. aculeatus

The only one pair which bear Ag-NOR/18S rDNA sites are useful chromosomal markers shared among the *Rhinecanthus* species. The result here is also similar to the chromosome bearing nucleolar organizer region in previous studies (Table 1) except *Balistoides viridescens* that found tree NORs (Supiwong et al., 2013) this suggests that this event may be related to chromosomal change during evolution.

Furthermore, the interstitial region of the largest telocentric chromosome pair 1 of *R. aculeatus* showed clearly observable nucleolar organizer regions. This is quite consistent with the report by de Lima et al. (2011) on the karyotype of *Melichthys niger* in the same family. Their study reported the presence of a conspicuous secondary constriction in the interstitial position on the long arm of the chromosome pair No. 2 which was, corresponding to the nucleolar organizer regions, identified by Ag-NOR sites and by *in situ* hybridization with an 18S rDNA ribosomal probe.

Normally, most fishes have only one pair of NORs on chromosomes. Only some fishes have more than two NORs, which may be caused by the translocation between some parts of the chromosomes that have NOR and another chromosome (Sharma et al., 2002). The present study shows that the species analyzed presents NOR site on a single chromosome pair. This is considered a simple isomorphic condition in fish (Almeida-Toledo, 1985). Another peculiar cytogenetic aspect of Tetraodontiformes is the small quantity of heterochromatic regions, localized in telomeric or centromeric positions on most of the chromosome pairs (Supiwong et al., 2013).

Organization of repetitive DNAs in the chromosomes of R. aculeatus

The C-positive heterochromatins in the chromosomes of *R. aculeatus* are distributed in centromeric and telomeric positions in most of the chromosomes (Figure 2). This recurring distribution pattern is similar to those reported for species of other *Balistidae* genera, such as *Melichthys* (de Lima et al., 2011).

The 18S rDNA sites are equally located on the interstitial position of pair 1, whereas 5S rDNA sites occur in the subcentromeric region of pair 4. The non-syntenic organization of these genes is frequent and it could be a plesiomorphic condition in Balistidae.

This is the first report of the presence of microsatellite sequences in the heterochromatin of *R. aculeatus* which show recognizable organizational patterns.

Table 2. Mean length of short arm chromosome (Ls), length long arm chromosome (Ll), length 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 Picasso triggerfish (*Rhinecanthus aculeatus*), 2*n*=44.

Chromosome pair	Ls	Ll	LT	RL±SD	CI±SD	Chromosome type
1*	0.00	2.60	2.60	0.070±0.009	1.000±0.000	telocentric
2	0.00	2.20	2.20	0.059±0.004	1.000±0.000	telocentric
3	0.00	2.06	2.06	0.056±0.005	1.000±0.000	telocentric
4	0.00	2.04	2.04	0.055±0.004	1.000±0.000	telocentric
5	0.00	1.97	1.97	0.052±0.003	1.000±0.000	telocentric
6	0.00	1.93	1.93	0.051±0.003	1.000±0.000	telocentric
7	0.00	1.90	1.90	0.051±0.005	1.000±0.000	telocentric
8	0.00	1.86	1.86	0.049±0.003	1.000±0.000	telocentric
9	0.00	1.75	1.75	0.047±0.003	1.000±0.000	telocentric
10	0.00	1.74	1.74	0.047±0.004	1.000±0.000	telocentric
11	0.00	1.72	1.72	0.046±0.005	1.000±0.000	telocentric
12	0.00	1.71	1.71	0.045±0.003	1.000±0.000	telocentric
13	0.00	1.68	1.68	0.044±0.003	1.000±0.000	telocentric
14	0.00	1.61	1.61	0.043±0.003	1.000±0.000	telocentric
15	0.00	1.59	1.59	0.042±0.003	1.000±0.000	telocentric
16	0.00	1.55	1.55	0.041±0.003	1.000±0.000	telocentric
17	0.00	1.45	1.45	0.038±0.003	1.000±0.000	telocentric
18	0.00	1.40	1.40	0.036±0.005	1.000±0.000	telocentric
19	0.00	1.40	1.40	0.036±0.006	1.000±0.000	telocentric
20	0.00	1.30	1.30	0.034±0.006	1.000±0.000	telocentric
21	0.00	1.19	1.19	0.030±0.007	1.000±0.000	telocentric
22	0.00	1.10	1.10	0.028±0.007	1.000±0.000	telocentric

Remark: * NOR-bearing chromosome.

The $(CA)_{15}$ $(GA)_{15}$ and $(CAA)_{10}$ microsatellites present a weak and diffuse distribution on all chromosomes, but they also present a small number of conspicuous clusters characterized by intense signs in some parts of chromosomes (Figure 3). Thus, this data is useful for comparing the phylogenetic proximity of this genus that may share the same distribution pattern of the microsatellite sequences which points to independent evolutionary pathways, constituting homoplastic chromosomal characters. However, since these sequences are subject to high rates of change, their distribution may show marked evolutionary differentiation (Cioffi et al., 2011; Molina et al., 2014a; 2014b). In fact, the organization of microsatellite sequences demonstrates the particular arrangements that repetitive DNAs can be achieved in different species.

Chromosome evolution of the family Balistidae

Chromosomal rearrangements represent the main cause of karyotype diversification among several Perciformes species (Arai, 2011; Molina and Galetti, 2002). 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 fusion, and especially pericentric inversions (Getlekha et al., 2016a; 2016b).

The family Balistidae has 2n values lower than 2n=48 with most of their representatives presenting acrocentric and telocentric chromosomes. This karyotypic pattern was also observed in the present study in *R. aculeatus* (2n=44). The origin of the reduced diploid chromosome numbers in these species seems to be centric fissions but chromosome lost in tandem, which seems to be common in other species of the family (Arai and Nagaiwa 1976; Marques et al., 2016).

CONCLUSION

Based on the chromosome study of the Picasso triggerfish (*R. aculeatus*) using conventional analyses (Giemsa staining, Ag-NOR and C-banding) and molecular analysis (*in situ* mapping of five different repetitive DNA classes including 18S rDNA, 5S rDNA, $(CA)_{15}$, $(GA)_{15}$ and $(CAA)_{10}$ as markers), this research can verify diploid chromosome, fundamental number and distribution patterns of microsatellites on the chromosomes. The results show that *R. aculeatus* has 2n=44 with predominantly telocentric chromosome. The fundamental number (NF) was 44. The C-positive heterochromatic blocks are preferentially located in the centromeric and telomeric regions of some chromosomal pairs. The Ag-NORs sites were located on the interstitial region of long arms of the telocentric chromosome pair 1. The exclusive location of the major ribosomal sites in these pairs was confirmed by *in situ* hybridization with 18S rDNA probes. However, the 5S rDNA genes are not located on 18S rDNA-bearing chromosomes, but instead located exclusively in the subcentromeric region of pair 4. The mapping of $(CA)_{15}$, $(GA)_{15}$ and $(CAA)_{10}$ microsatellites are sparsely dispersed along all the chromosomes.

The idiogram of *R. aculeatus* represents gradually declining length of the chromosomes. The karyotype is notably attributed solely to telocentric chromosomes Like in common ancestor of marine fish, the high ability of distribution results in high gene flow and low chromosome evolution. The karyotype formula of *R. aculeatus* is 2n (44) = 44t.

Up to the present, there are 3 species of the Genus *Rhinecanthus* that were cytogenetically analyzed. *Rhinecanthus* species provides remarkable karyotype features for chromosomal and genetic conservatism discussion. Further studies of other species as well as additional information from molecular chromosome analyses are expected to explain the karyotype pattern and chromosome evolution in these fishes.

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