Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

Citation: Sribenja, K., & Getlekha, N. (2024). Mapping of five classes of repetitive DNAs and microsatellite repeats in the genome of the Rainbow Shark, *Epalzeorhynchos frenatum* (Fowler, 1934). *Caryologia* 77(2): 57-64. doi: 10.36253/caryologia-2596

Received: March 11, 2024

Accepted: September 09, 2024

Published: November 10, 2024

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.

Mapping of five classes of repetitive DNAs and microsatellite repeats in the genome of the Rainbow Shark, *Epalzeorhynchos frenatum* (Fowler, 1934)

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Abstract. The karyotype and chromosomal characteristics of *Epalzeorhynchos frenatum* (Fowler, 1934) were examined using different staining techniques (Conventional banding, C-banding and Ag-NORs banding) and the use of fluorescent *in situ* hybridization (FISH) to detect 18S, 5S rDNA sites and microsatellite sequences ($(CA)_{15}$, $(GA)_{15}$ and $(CGG)_{10}$ as markers). The result revealed karyotypes with 2n = 50 chromosomes (NF=86), consisting of 12 metacentric, 12 submetacentric, 12 acrocentric, and 14 telocentric chromosomes; there were no heteromorphic sex chromosomes. The NORs site was a noticeable proximal heterochromatic block on the short arm of pair No. 13, and C-positive heterochromatin was detected in the centromeric sections of the chromosomes as well as the telomeric regions of other pairs. On the short arm of pairs No. 13 and 15, the telomeric regions contained the 18S and 5S rDNA sites, respectively. Several chromosomes bearing these repetitive DNA sequences were shared, alongside with some exclusive chromosomal markers especially CGG rich segment (No. 13). This means that, as verified by two methods, it involves a syntenic condition for the 18S rDNA, NORs, and $(CGG)_{10}$ microsatellite probes.

Keywords: *Epalzeorhynchos frenatum*, Rainbow Shark, Chromosome, Repetitive sequences, Fish cytogenetics.

INTRODUCTION

Freshwater fish of the family Cyprinidae (Cypriniformes) are found in Thailand's main hydrographic basins and are distributed widely throughout Southeast Asia. One of this family's biggest subgroups is the subfamily Cyprininae that belong to the major large tribes such Labeonini, Poropuntini, and Smiliogastrini (Phimphan et al., 2020). In Thailand, freshwater fish from over 200 different species are utilized as ornaments. The family Cyprinidae is responsible for over half of Thailand's ornamental fish population. The most well-liked species include *Epalzeorhynchos frenatum* (Fowler, 1934), that

one of the several fish species that are important to Thailand's economy as ornamentals (Sermwatanakul, 2005).

Several cytogenetically examined of *E. frenatum*, exhibit conservative karyotypic diversification and are readily distinguished in terms of size, shape, and chromosomal number, indicating the existence of separate species (Bertollo et al., 1986, 2000). Numerous fish species have been subjected to molecular cytogenetic analysis employing repetitive DNAs, such as rDNA repeats, satellite DNAs, telomeric sequences, and other classes of microsatellite sequences. These investigations have shown how much potential there is to learn more about karyotype differentiation through the study of repetitive DNAs. These genomic elements have the ability to alter the molecular makeup of chromosomes and slow down the rate of recombination between them, two essential processes in chromosome differentiation.

This study presents a chromosomal characteristic analysis of *E. frenatum*, using different staining techniques (Conventional banding, C-banding, and Ag-NORs banding) and fluorescent *in situ* hybridization (FISH) with repetitive DNA probes (18S rDNA and 5S rDNA probs) and microsatellite sequences ($(CA)_{15}$, $(GA)_{15}$, and $(CGG)_{10}$ as markers). The distribution of repeated DNA sequences within the chromosomes serves as a key indicator of the comprehensive karyotype characterization that this approach provided.

MATERIAL AND METHODS

Specimens collected and conventional methods

Ten male and ten female of Rainbow Sharks (*Epalzeorhynchos frenatum* Fowler, 1934) from the Mae Klong River in the Ratchaburi area of Thailand were subjected to cytogenetic analysis (Figure 1). A hand net was used to collect the specimens, which were then brought to the study station in sealed plastic bags with oxygen and clean water inside. In order to reduce animal suffering, the trials adhered to ethical guidelines and used clove oil as anesthetic before slaughtering the animals. The process was approved by the Ethics Committee of Muban Chombueng Rajabhat University and by the RGJ Committee under no. U1-04484-2559. Mitotic chromosomes were extracted from anterior kidney cell suspensions by standard air-drying techniques. Additionally, the distribution of C-positive heterochromatin was found using the C-banding approach, and the Ag-NOR position on chromosomes was found using silver staining. The specimens were deposited in the fish collection of the Cytogenetic Laboratory, Department of Biology, Faculty of Science and Tecnology, Muban Chombueng Rajabhat University.

Figure 1. General characteristic of *Epalzeorhynchos frenatum* (Fowler, 1934).

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 $(CGG)_{10}$ 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 counter-

stained 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 Rainbow Shark (*Epalzeorhynchos frenatum*) has 2n=50. All four types of chromosomes (metacentric, submetacentric, acrocentric, and telocentric chromosome) make up its karyotype structure, which has a fundamental number (NF) of 86. The C-positive heterochromatic bands were observed in the centromeric and telomeric regions of many chromosomes. The Ag-NOR sites are located in the telomeric region of the biggest acrocentric pair's short arms (pair 13), the exclusive location of major ribosomal sites in these regions was confirmed by *in situ* hybridization with 18S rDNA probes. The 18S rDNA sites are found in the telomeric position of the short arms of pair 13, but the 5S rDNA genes are only found in the telomeric area of acrocentric pair 15 (Figure 2).

The chromosomal mapping of all microsatellite sequences indicates a different dispersed distribution. It was observed that the $(CA)_{15}$ microsatellite was primarily concentrated in the telomeric regions of each chromosome. Although the sequence $(GA)_{15}$ was broadly distributed on the chromosomes, it displayed weaker signals than the sequence $(CA)_{15}$. It also shows a dispersed distribution without preferential accumulations on the centromeric and telomeric regions in any of the chromosomal pairs. While the sequence $(CGG)_{10}$ showed hybridization signals on just one of the short arms of the largest acrocentric pair (pair 13), it looked to be weakly accumulated in several chromosomes (Figure 3), no differential hybridization patterns were detected between males and females.

The idiogram of *Epalzeorhynchos frenatum* represents gradually declining length of the chromosomes (Figure 4). The karyotype is asymetrical karyotype with metacentric, submetacentric, acrocentric and telocentric chromosomes. The karyotype formula of *Epalzeorhyn-*

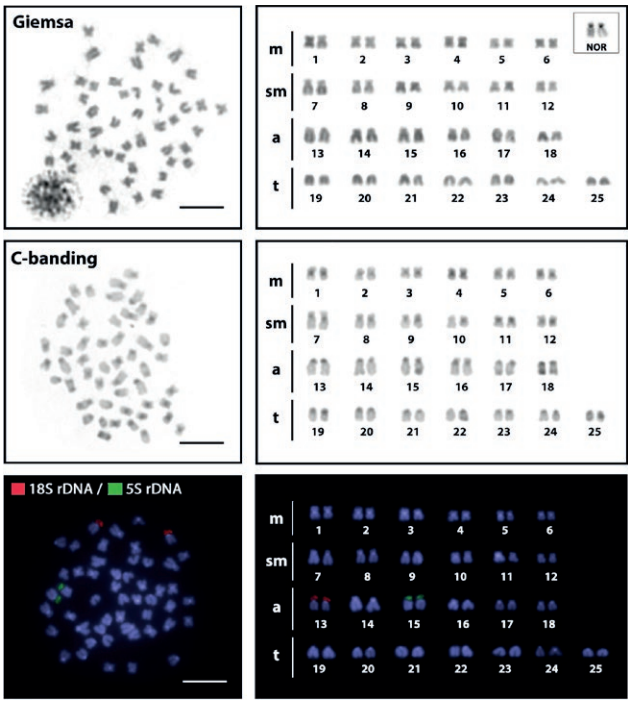

Figure 2. Mataphase and karyotypes of *Epalzeorhynchos frenatum* arranged from conventionally Giemsa-stained, Ag-stained (highlighted in the boxes), C-banded and after fluorescence *in situ* hybridization with an 18S rDNA probe. Bar 5 μm.

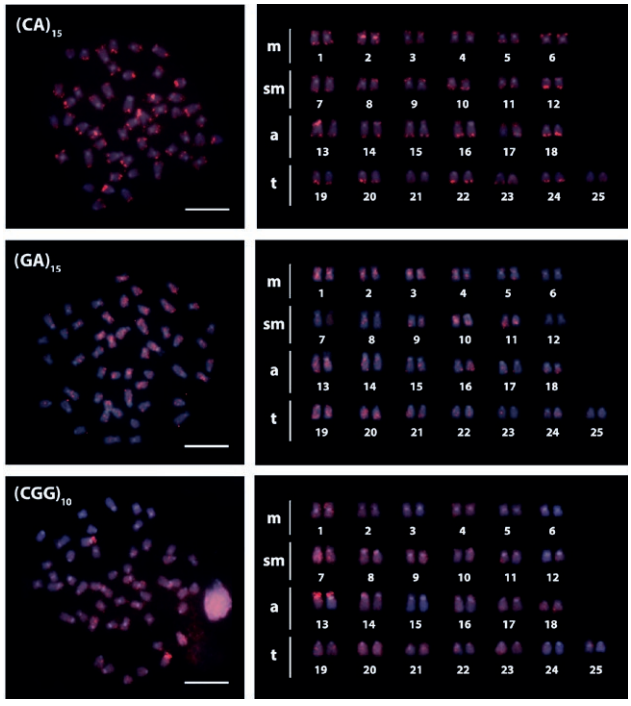

Figure 3. Chromosomal mapping of di- and tri-nucleotide microsatellites in the chromosomes of *Epalzeorhynchos frenatum* by fluorescence *in situ* hybridization. The general distribution pattern of $(CA)_{15}$, $(GA)_{15}$ and $(CGG)_{10}$ microsatellites as probe. Bar = 5 μm.

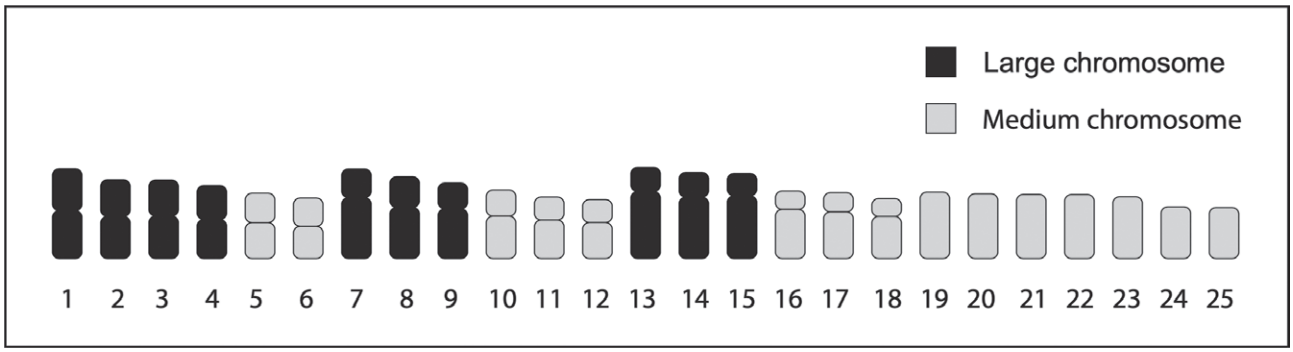

Figure 4. Standardized idiogram showing lengths and shapes of chromosomes of *Epalzeorhynchos frenatum* (2*n*=50) by conventional staining techniques.

chos frenatum is as follow: 2n (50) = L^m_8+L^{sm}_6+ L^a_6+ M^m_4+M^{sm}_6+ M^a_6+ M^t_{14}

DISCUSSIONS

Karyotype uniformity among Epalzeorhynchos *species*

Epalzeorhynchos frenatum from Mae Klong River, Ratchaburi province, Thailand, was subjected to cytogenetic analysis. All specimens of *E. frenatum* have 50 chromosomes, and their karyotypes are asymmetric (Figure 2, 3 and Table 2). On the other hand, a few species of the genus *Epalzeorhynchos* have been the subject of extensive cytogenetic study (Table 1). The karyotype of *E. frenatum*, *E. bicolor*, and *E. munensis* is 2n=50, which is in line with other species' findings. However, there is a difference in *E. frenatum* (2n = 48), as described by Magtoon and Donsakul (1993). It implies that their karyotypes continue to be conserved even after speciation. However, each species' greater adaptive divergence and substantial chromosomal rearrangement variability cause them to display an asymmetrical karyotype.

In addition, karyotypes with essentially comparable structural patterns were seen in *Epalzeorhynchos* species; majority of these showed the formation of asymmetric karyotypes and 2n = 50. All of the *Epalzeorhynchos* species that have been examined to date (Magtoon and Donsakul, 1993; Donsakul et al., 2012; Phimphan et al., 2020; current study) share these traits. Furthermore, these fishes have somewhat different karyotypes due to changes in NFs. These discrepancies might stem from population-level species-specific changes or from incorrectly identifying one species as another because of species complexity.

Additionally, similar to every other species in the genus *Epalzeorhynchos*, it was not possible to observe the physically distinct sex chromosome (Magtoon and Donsakul, 1993; Donsakul et al., 2012; Phimphan et al., 2020). Numerous species in this family exhibit the same phenomena (Arai, 2011).

Chromosome markers of E. frenatum

This is the first report on the distribution of C-positive heterochromatins at the centromeric and telomeric sites of the majority of the *E. frenatum* chromosomes (Figure 2). This distribution pattern resembles those of other fish that have had their C-banding documented (Cioffi, et al., 2009).

Table 1. Reviews of cytogenetic reports in the genus *Epalzeorhynchos*. (2n = diploid number, m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric and NORs = nucleolar organizer regions, NF = fundamental number, – = not available).

Species	2n	NF	Formula	NORs	Reference
E. bicolor	50	74	20m+4sm+2st+24a	-	Magtoon and Donsakul (1993)
E. munensis	50	84	22m+12sm+2st+14a	-	Donsakul et al. (2012)
E. frenatum	48	72	14m+10sm+8st+16a	-	Magtoon and Donsakul (1993)
	50	78	18m+10sm+10st+12a	2	Phimphan et al. (2020)
	50	86	12m+12sm+12a+14t	2	Present study

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 (*Epalzeorhynchos frenatum*), 2*n*=50.

Chromosome pair	Ls	Ll	LT	RL±SD	CI±SD	Chromosome type
1	0.672	0.769	1.441	0.051±0.005	0.528±0.051	metacentric
2	0.587	0.677	1.264	0.045±0.002	0.531±0.049	metacentric
3	0.577	0.683	1.260	0.044±0.003	0.543±0.034	metacentric
4	0.545	0.627	1.172	0.041±0.004	0.534±0.049	metacentric
5	0.472	0.577	1.049	0.037±0.005	0.546±0.057	metacentric
6	0.453	0.519	0.972	0.034±0.002	0.529±0.081	metacentric
7	0.460	0.978	1.438	0.051±0.004	0.680±0.047	submetacentric
8	0.492	0.822	1.314	0.046±0.004	0.633±0.056	submetacentric
9	0.415	0.799	1.214	0.044±0.003	0.648±0.040	submetacentric
10	0.420	0.681	1.102	0.039±0.002	0.614±0.028	submetacentric
11	0.370	0.618	0.988	0.035±0.003	0.621±0.038	submetacentric
12	0.368	0.579	0.947	0.033±0.003	0.612±0.043	submetacentric
13*	0.388	1.076	1.464	0.052±0.003	0.734±0.045	acrocentric
14	0.397	0.986	1.383	0.048±0.004	0.718±0.044	acrocentric
15	0.359	1.007	1.366	0.049±0.001	0.731±0.039	acrocentric
16	0.294	0.790	1.084	0.039±0.003	0.719±0.058	acrocentric
17	0.309	0.749	1.057	0.037±0.003	0.709±0.026	acrocentric
18	0.287	0.676	0.964	0.034±0.002	0.702±0.005	acrocentric
19	0.000	1.066	1.066	0.038±0.004	1.000±0.000	telocentric
20	0.000	1.040	1.040	0.037±0.002	1.000±0.000	telocentric
21	0.000	1.029	1.029	0.036±0.003	1.000±0.000	telocentric
22	0.000	1.027	1.027	0.036±0.002	1.000±0.000	telocentric
23	0.000	0.992	0.992	0.035±0.002	1.000±0.000	telocentric
24	0.000	0.831	0.831	0.029±0.002	1.000±0.000	telocentric
25	0.000	0.819	0.819	0.029±0.001	1.000±0.000	telocentric

Remark: * NOR-bearing chromosome.

Ag-NOR/18S rDNA sites are the sole pair of relevant chromosomal markers that all *E. frenatum* share. NORs are chromosomal landmarks made up of tandemly repeated ribosomal gene sequences (rRNA). Three genes that code for 18S, 5.8S, and 28S ribosomal RNA make up each unit in eukaryotes (Sharma et al., 2002). Since these chromosomal features are frequently species-specific, the number and location of rDNA clusters have been extensively exploited in systematics and phylogenetic reconstructions (Britton-Davidian et al., 2012). The outcome here is similar to that of earlier research on the chromosome-bearing nucleolar organizer area (Phimphan et al., 2020). This trait is shared by a variety of fish species and other vertebrates (Supiwong et al. 2012, 2013).

Additionally, there were discernible nucleolar organizer areas in the telomeric region of the short arms of *E. frenatum*'s biggest acrocentric chromosome pair 13. This aligns well with the Phimphan et al. (2020) research on the same species' karyotype. According to their research, there is a noticeable secondary constriction in the areas next to the telomere of chromosome pair 10 (submetacentric), which corresponds to the regions known as nucleolar organizer regions and is distinguished by Ag-NOR sites.

The majority of fishes typically only have one pair of NORs on their chromosomes; only a small number of fishes have more than two pairs, such as two pairs (*Cyclocheilos enoplos* (Bleeker, 1849): Magtoon and Arai, 1993), three pairs (*Cyclocheilichthys apogon* (Valenciennes, 1842): Chantapan, 2015) and four pairs (*Puntius denisonii* (Day, 1865), *P. semifasciolatus* (Günther, 1868): Nagpure et al., 2004; *P. filamentosus* (Valenciennes, 1844): Nagpure et al., 2003), which may be caused by translocation between certain regions of the chromosomes that have NOR and another chromosome (Sharma et al., 2002). The current analysis demonstrates that the studied species has a NOR site on a single pair of chromosomes. In fish, this is seen as a straightforward isomorphic requirement (Almeida-Toledo, 1985).

Organization of repetitive DNAs in the chromosomes of E. frenatum

This is the first report of the presence of repetitive DNAs on *E. frenatum*, the 5S rDNA sites are found in the telomeric area on the short arm of pair 15, the 18S rDNA sites are evenly distributed on the telomeric location on the short arm of pair 13. These genes frequently display a non-syntenic arrangement, which may indicate a plesiomorphic state in fish.

The identifiable organizational patterns seen in the heterochromatin of *E. frenatum*'s microsatellite sequences. On all chromosomes, the $(CA)_{15}$, $(GA)_{15}$, and $(CGG)_{10}$ microsatellites have a weak and diffuse distribution; yet, in certain regions of the chromosomes, they also exhibit a few prominent clusters with strong signals (Figure 3). It may appear from earlier and ongoing research that microsatellites make up every heterochromatin in fish genomes (Cioffi and Bertollo, 2012). Nevertheless, microsatellites have also been discovered in noncentromeric areas; a large number of these were found in or close to genes (Rao et al., 2010). This is consistent with the microsatellite $(CGG)_{10}$ pattern found in this investigation. As a result, this information is helpful for analyzing the phylogenetic proximity of this genus, which may have similar microsatellite sequence distribution patterns, indicating separate evolutionary routes that result in homoplastic chromosomal features. However, because to the rapid changes in these sequences, there could be a noticeable evolutionary divergence in their distribution (Cioffi et al., 2011; Molina et al., 2014a; 2014b). Actually, the way repeating DNAs are arranged in different species is demonstrated by the structure of microsatellite sequences.

Chromosome evolution of the genus Epalzeorhynchos

The primary factor causing karyotype diversification in several Cypriniformes species is chromosomal rearrangements. With diploid chromosome numbers ranging from 2n=48 to 50 in the tribes Labeonini and Smiliogastrini, while the tribe Poropuntiini is more conserved at 2n = 50 (Arai, 2011), the various Cyprinidae species underwent an extremely diversified karyotype evolution when considering the numerical and structural aspects of their complements. There were also notable differences in the NF, possibly as a result of the occurrence of pericentric inversions (Getlekha et al., 2018). The results of the analyses demonstrate the combined significance of the various chromosome rearrangements such as centric fission fusion and particularly pericentric inversions in the evolutionary modeling of their karyotypes (Getlekha et al., 2016a; 2016b).

The majority of members of the genus *Epalzeorhynchos* have asymmetrical karyotypes with metacentric submetacentric acrocentric and telocentric chromosomes, and nearly all of them have 2n = 50. In the current investigation, same karyotypic pattern was also seen in *E. frenatum* (2n=50). Pericentric inversions, which appear to be frequent in other freshwater fish species, particularly in the family Cyprinidae, are thought to be the source of the diploid chromosomal numbers in these species (Arai & Nagaiwa, 1976; Marques et al., 2016).

CONCLUSION

This research can verify diploid chromosome, fundamental number, and distribution patterns of microsatellites on the chromosomes based on the chromosome study of the Rainbow Shark (*Epalzeorhynchos frenatum*) using conventional analyses (Giemsa staining, Ag-NOR, and C-banding) and molecular analysis (*in situ* mapping of five classes of repetitive DNAs and microsatellite repeats, including 18S rDNA, 5S rDNA, $(CA)_{15}$, $(GA)_{15}$, and $(CGG)_{10}$ as markers). According to the data, *E. frenatum* has an asymmetric karyotype with 2n=50. 86 was the fundamental number (NF). The centromeric and telomeric regions of certain chromosomal pairs are where the C-positive heterochromatic blocks are more commonly found. The unique position of the major ribosomal sites in these pairs was confirmed by *in situ* hybridization with 18S rDNA probes, and the Ag-NORs sites were discovered on the telomeric region of the short arms of the largest acrocentric chromosome pair 13. Nevertheless, the 5S rDNA genes are only found in the telomeric region of the short arms of the acrocentric chromosome pair 15. Moreover, microsatellites $(CA)_{15}$, $(GA)_{15}$, and $(CGG)_{10}$ have been sparingly mapped throughout all chromosomes, with the CGG rich section being the exception (No. 13). This means that, as verified by two methods, it involves a syntenic condition for the 18S rDNA, NORs, and $(CGG)_{10}$ microsatellite probes.

Three species in the genus *Epalzeorhynchos* have undergone cytogenetically analyzed research thus far. Significant karyotype traits are provided by the *Epalzeorhynchos* species for the debate of chromosomal and genetic conservatism. It is anticipated that more research on different species and more data from molecular chromosomal analysis will clarify the chromosome evolution and karyotype pattern in these fishes. Gaining a rudimentary understanding of cytogenetics can help in the future development of potentially marketable species and stains. Research on karyotypes aids in examin-

ing the genetic makeup of aquatic animal species within each habitat, allowing for the precise identification of species relationships. This could potentially aid in future hybridization efforts aimed at improving strains (Sofy et al., 2008), managing chromosome sets in organism breeding (Na-Nakorn et al., 1980), and choosing brood stocks (Mengampan et al., 2004).

ACKNOWLEDGEMENTS

This work was supported by the Thailand Science Research and Innovation (Fundamental Fund; FF) and Toxic Substances in Livestock and Aquatic Animals Research Group, Khon Kaen University and Department of Biology, Faculty of Science and Technology, Muban Chombueng Rajabhat University and by the Brazilian agencies FAPESP and CNPq.

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