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Comparative chromosome mapping of repetitive DNA in four minnow fishes (Cyprinidae, Cypriniformes)

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Abstract. The present study focused on the repetitive DNA of the chromosome in four minnow fishes from the genera *Danio* Hamilton, 1822, *Devario* Heckel, 1843 and *Rasbora* Bleeker, 1859. Chromosomes were analysed using fluorescence *in situ* hybridization (FISH) with microsatellite probes including $(CA)_{15}$, $(CAC)_{10}$, $(CGG)_{10}$, $(GC)_{15}$ and $(TA)_{15}$ staining. All species retained the diploid chromosome number 2n = 50 in male and female. The microsatellite sequences were mapped in the chromosomes of *Danio albolineatus* (Blyth, 1860), *Devario regina* (Fowler, 1934), *Rasbora aurotaenia* Tirant, 1885 and *R. paviana* Tirant, 1885. In most cases, the microsatellite was dispersed in the chromosome with conspicuous markings in the telomeric region and the whole genome, which suggests that sequences contribute to chromosome structure and may have played a role in the relationship of this fish group. The comparative genome mapping data presented here provide novel information on the structure and organisation of the repetitive DNA region of the minnow's genome and contribute to a better understanding of the genomes of these minnows.

Keywords: Cyprinidae, Danioninae, FISH, microsatellite.

INTRODUCTION

The level of molecular cytogenetics plays an important role in precise characterisation of the structure of fish genomes (Cioffi and Bertollo 2012). The family Cyprinidae is the most abundant and globally widespread family of freshwater fish, comprising 3,000 species are one of the largest groups (Eschmeyer and Fong 2015). Thailand is one of the important areas for fish fauna in terms of both diversity and endemicity. The total number of fish spe-

cies in Thai waters is over 2,700 with about 2,000 marine and 720 freshwater species (Vidthayanon 2005). *Danio albolineatus* (Blyth, 1860), *Devario regina* (Fowler, 1934), *Rasbora aurotaenia* Tirant, 1885 and *R. paviana* Tirant, 1885 are four of the species of minnows, belonging to the family Cyprinidae (subfamily Danioninae-Danionini). They are tropical freshwater fish of minor commercial importance, which are native in Thailand. Their distributions include the Mekong, Chao Phraya, and Meklong Basins (Froese and Pauly 2012) and they can be easily found in large and small rivers, ponds, ditches, lakes, paddy field, and swamps. It rarely occurs in low oxygen waters (Brittan 1954; 1971; 1998). They could be used to assess if they were sensitive to change in environmental problems and aquatic pollution (Blazer 2002; Frame and Dickerson 2006; Raskovic et al. 2010; Yenchum 2010; Reddy and Rawat 2013). However, cytogenetic studies in minnows are quite scarce, in which only conventional technique reported to determine chromosome number and karyotype composition has been performed.

Up to the present time, cytogenetic studies on subfamily Danioninae (Cyprinidae, Osteichthyes) with 20 genera and about 335 species have been undertaken for only 21 species from three genera (*Danio*, *Devario* and *Rasbora*) as yet, only conventional cytogenetics have been applied to determine chromosome numbers and karyotype complements. Diploid chromosomes number (2n) varies between 48-50. Cytogenetic studies of the genera *Danio*, *Devario* and *Rasbora* have been reported in Table 1. From the previous reports, in most cases, more descriptions of karyotypes seem to be inconclusive when they are not used in combination with other methods to produce more accurate chromosome markers. More recently, molecular cytogenetics begun to be implemented in a finer-scale characterization of karyotype structures in certain Cyprinid taxa (Spoz et al. 2014; Saenjundaeng et al. 2018; Phimphan et al. 2020). More specifically, FISH-based repetitive DNA mapping, multiple DNA copies of repetitive DNAs are a large substantial portion of the genome of eukaryotes that can be generally classified into two main classes: tandem repeats, such as the multigene families and satellite DNAs; also, there are dispersed elements, such as transposons and retrotransposons, known as transposable elements (TEs) (Jurka et al. 2005). Repetitive DNA sequences display a high degree of polymorphism because of the variation in the number of repetitive units, which results from specific evolutionary dynamics. The taxonomic status by DNA genome as well as the phylogenetic relationships of Danioninae species is confirmed and well established based on previous study (Rüber et al., 2007; Tang et al., 2010).

Recently, molecular cytogenetic studies, using fluorescence *in situ* hybridization (FISH) for mapping of repetitive DNA sequences, have provided important contributions to the characterisation of biodiversity and evolution of divergent fish groups (Cioffi and Bertollo 2012). Moreover, some microsatellite repeats are species-specific characters amongst some fish groups (Cioffi et al. 2015). An important role of repetitive DNAs in genome evolution has been reported for different fish groups (Cioffi and Bertollo 2012; Cioffi et al. 2010, 2015; Moraes et al. 2017; 2019; Sassi et al. 2019; Terencio et al. 2013; Yano et al. 2014).

Thus, the present study is the report on chromosomal characteristics of FISH mapping of the microsatellites repeats in *Da. albolineatus*, *De. regina*, *R. aurotaenia* and *R. paviana* by using molecular cytogenetic protocols. The knowledge gained can provide cytogenetic information potentially useful for further study in this family.

MATHERIAL AND METHODS

Individuals from both sexes of the four minnows were collected for analyses from river basins in, Thailand (Table 2 and Fig. 1). The fishes were transferred to laboratory aquaria and kept under standard conditions for three days before the experiments. The procedures followed ethical protocols, as approved by the Ethics of Animal Experimentation of the National Research Council of Thailand U1-04498-2559.

Preparation of fish chromosomes was from kidney cells (Phimphan et al. 2020). The chromosomes were stained with Giemsa's Solution for 10 min. Metaphase figures were analysed according to the chromosome classification of Levan et al. (1964). Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) or acrocentric (a). The Fundamental number, NF (number of chromosome arms) is obtained by assigning a value of two (2) to metacentric and submetacentric chromosomes and one (1) to subtelocentric and acrocentric chromosomes.

FISH was performed under stringent conditions on metaphase chromosome spreads with microsatellite $(CA)_{15}$, $(CAC)_{10}$, $(CGG)_{10}$, $(GC)_{15}$ and $(TA)_{15}$ probes (Kubat et al. 2008; Liehr 2009) which were directly labelled with Cy3 at 5′terminal during synthesis by Sigma (St. Louis MO, USA). FISH, under stringent conditions on mitotic chromosome spreads (Pinkel et al. 1986), was carried out by previous protocols as reported by Supiwong et al. (2019) and Yano et al. (2017). The hybridzation signals were checked and analysed on an

Table 1. Reviews of cytogenetic reports in the genera *Danio*, *Devario* and *Rasbora*. (2n = diploid number, m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric, NORs = nucleolar organizer regions, NF = fundamental number, + = positive - = not available).

Genus/Species	2n	NF	Karyotype formula	FISH	Reference
Danio rerio	48	-	-	-	Post (1965)
	50	100	50m	-	Endo and Ingalls (1968)
	50	100	10m + 40sm	-	Fontana et al. (1970)
	50	100	10m + 12sm +28a	-	Rishi (1976)
	50	100	16m + 32sm + 2a	-	Schreeb et al. (1993)
	50	100	12m + 26sm + 12a	-	Pijnacker and Ferwerda (1995)
	50	100	4m + 16sm + 30a	-	Daga et al. (1996)
	50	100	4m + 16sm + 30a	-	Gornung et al. (1997)
	50	100	F: 7m + 7sm + 36a M: 6m + 8sm + 36a	-	Sharma et al. (1998)
	50	100	12m + 26sm + 12a	-	Ueda and Naoi (1999)
	50	100 100	4m + 30sm + 16a or* 4m + 20sm + 26a	-	Amores and Postlethwait (1999)
	50	100	4m + 16sm + 30a	+	Phillips and Reed (2000)
	50	100	4m + 16sm + 30a	+	Sola and Gornung (2001)
Da. Roseus	50	100	8m+34sm+8a	-	Kaewtip et al. (2021)
Da. albolineatus	50	99	10m+39sm+1a	-	Arai (2011)
	50	**100**	**8m + 14sm + 28a**	**+**	**Present study**
Devario laoensis	50	100	6m+10sm+34a	-	Aiumsumang et al. (2021) Kaewtip et al. (2021)
	50	96	6m+20sm+20a+4t	-	
De. aequipinnatus	50	96	6m+34sm+6st+4t		Sukham et al. (2013)
De. regina	50	100	6m+12sm+32a	-	Aiumsumang et al. (2021)
	50	**100**	**6m+12sm+32a**	**+**	**Present study**
Rasbora agilis	50	100	24m+26sm	-	Donsakul et al. (2009)
R. aurotaenia	50	92	14m+26sm+2a+8t	-	Seetapan and Moeikum (2004)
	50	98	8m+16sm+24a+2t	-	Aiumsumang et al. (2012)
	50	**98**	**8m+16sm+24a+2t**	**+**	**Present study**
R. borapetensis	50	88	24m+14sm+12t	-	Donsakul et al. (2005)
R. buchanani	50	100	30m+18sm+2a	-	Manna and Khuda-Bukhsh (1977)
R. caudimaculata	50	98	20m+26sm+2a+2t	-	Donsakul and Magtoon (2002)
R. daniconius	50	80	18m+6sm+6a+20t	-	Khuda-Bukhsh et al. (1979)
	50	92	32m+8sm+2a+8t	-	Donsakul et al. (2005)
R. dorsiocellata	50	92	18m+24sm+8t	-	Donsakul et al. (2009)
R. einthovenii	50	94	6m+30sm+8a+6t	-	Donsakul et al. (2005)
	50	100	16m+18sm+16a	-	Yeesaem et al. (2019)
R. heteromorpha	48	-	-	-	Post (1965)
	48	74	14m+10sm+2a+22t	-	Donsakul et al. (2005)
R. myersi	50	90	20m+14sm+6a+10t	-	Donsakul and Magtoon (2002)
R. paviei	50	100	10m+24sm+16a	-	Donsakul and Magtoon (2002)
R. paviana	50	98	8m+16sm+24a+2t	-	Aiumsumang et al. (2021)
	50	**98**	**8m+16sm+24a+2t**	**+**	**Present study**
R. retrodorsalis	50	88	26m+10sm+2a+12t	-	Donsakul and Magtoon (2002)
R. rubrodorsalis	50	82	16m+16sm+18t	-	Donsakul et al. (2009)
R. sumatrana	50	94	26m+16sm+2a+6t	-	Donsakul and Magtoon (1995)
R. trilineata	48	-	-	-	Post (1965)
	50	94	26m+16sm+2a+6t	-	Donsakul et al. (2005)

Table 2. Collection sites of the analyzed species show the sample number.

Species	Number of specimens in site sampling					
	Mae Khong Basin	Sirindhorn Peat Swamp Forest	Pasak Basin	Chi Basin	Chao Phaya Basin	Songkhram Basin
Danio albolineatus	-	-	10 ♀ 10 ♂	04 ♀ 05 ♂	05 ♀ 05 ♂	-
Devario regina	05 ♀ 06 ♂	06 ♀ 08 ♂	-	-	-	-
Rasbora aurotaenia	-	-	-	-	08 ♀ 07 ♂	05 ♀ 08 ♂
Rasbora paviana	05 ♀ 08 ♂	03 ♀ 04 ♂	05 ♀ 07 ♂	04 ♀ 05 ♂	-	-

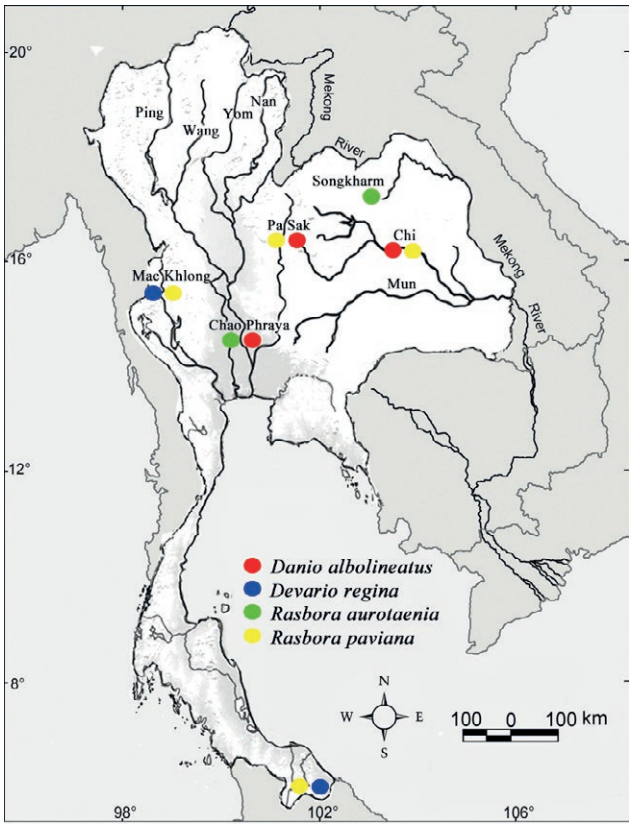

Figure 1. Map showing the collection sites of *Danio albolineatus* [red circles], *Devario regina* [blue circles], *Rasbora aurotaenia* [green circles] and *Rasbora paviana* [yellow circles] for studied herein.

epifluorescence microscope Olympus BX50 (Olympus Corporation, Ishikawa, Japan).

RESULTS

Diploid number and fundamental number of Da. albolineatus, De. regina, R. aurotaenia and R. paviana

The four minnow fishes have the same diploid number of 2n = 50. Although the minnow fishes analysed share the same 2n, there are differences in the fundamental number (NF) i.e. *Da. albolineatus* and *De. regina* NF = 100, while for the two *Rasbora,* NF = 98, The karyotype complements of *D. albolineatus* composed of 8m + 14sm + 28a, *D. regina* was m6+sm10+a34, while *R. aurotaenia* and *R. paviana* were 8m+16sm+24a+2t (Figs. 2A-D).

Patterns of the microsatellite repeat in the genomes of Da. albolineatus, De. regina, R. aurotaenia and R. paviana

The result of the mapping of the microsatellite repeats $(CA)_{15}$ show that hybridization signals are abundantly distributed on telomeric regions in all species (Figs. 3E-H), $(CAC)_{10}$ showing moderate abundance in *Da. albolineatus* and *R. paviana*, while *De. regina* and *R. aurotaenia* were not detected (Figs. 3I-L). The region hybridized $(CGG)_{10}$ of *Da. albolineatus* and *R. paviana* identified a partial genome, *De. regina* has a hybridization pattern throughout the genomes and *R. paviana* was not detected (Figs. 3M-P). $(GC)_{15}$ hybridized in *Da. albolineatus* and *R. paviana* as whole genomes, *Da. albolineatus* as telomeric regions and *R. paviana* was not detected (Figs. 3Q-T). The microsatellite $(TA)_{15}$ probe showed hybridization on the whole genomes of *Da. albolineatus* and *De. regina*, while two *Rabora* were not detected (Figs. 3U-X) (Table 3).

DISCUSSION

Diploid number of and fundamental number Da. albolineatus, De. regina, R. aurotaenia and R. paviana

Da. albolineatus had 2n = 50 which is in accordance with one single previous report (Arai 2011) and the same in other species in genus *Danio* (Post 1965; Endo and Ingalls 1968; Fontana et al. 1970; Rishi 1976; Schreeb et al. 1993; Pijnacker and Ferwerda 1995; Daga et al. 1996; Gornung et al. 1997; Sharma et al. 1998; Ueda and Naoi 1999; Amores and Postlethwait 1999; Phillips

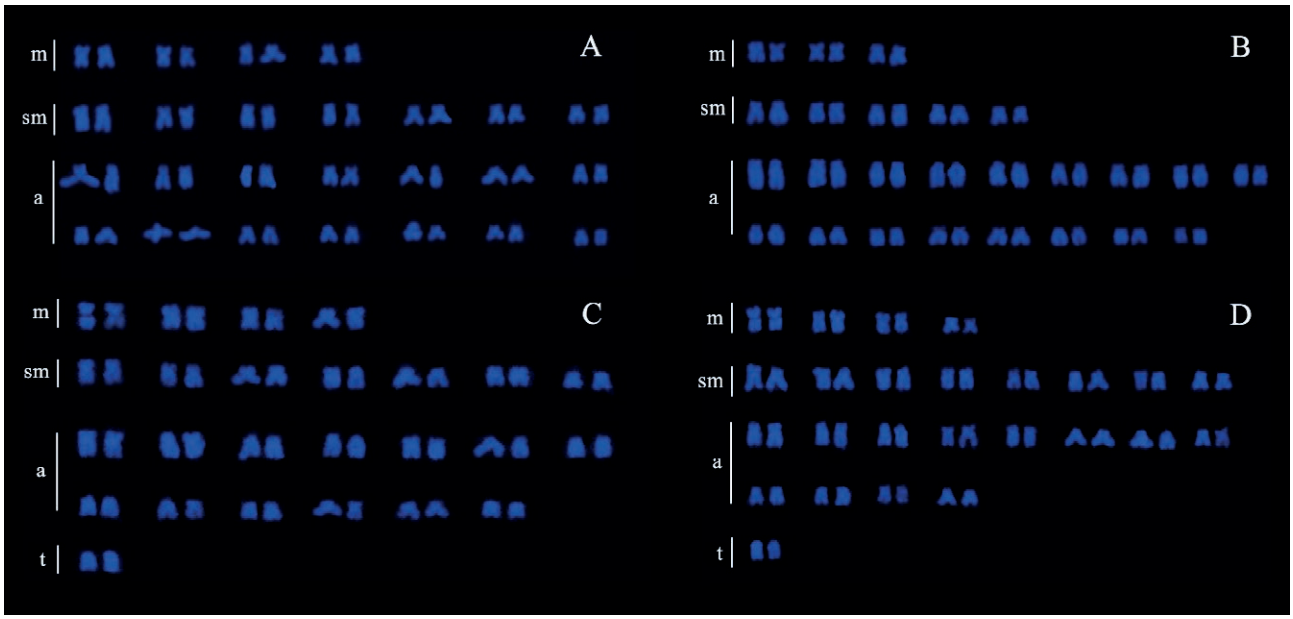

Figure 2. Karyotype of *Danio albolineatus* [A], *Devario regina* [B], *Rasbora aurotaenia* [C], *Rasbora paviana* [D], m = metacentric, sm = submetacentric, a = acrocentric and t = telocentric chromosomes.

Table 3. Molecular cytogenetic studies on four minnow fishes. [2n = diploid chromosome number, NF fundamental number (number of chromosome arm)].

Species	2n	NF	$(CA)_{15}$	$(CAC)_{10}$	$(CGG)_{10}$	$(GC)_{15}$	$(TA)_{15}$
Da. albolineatus	50	100	telomere	partial genome	partial genome	whole genome	whole genome
De. regina	50	100	telomere	not detected	whole genome	telomere	whole genome
R. aurotaenia	50	98	telomere	not detected	not detected	partial genome	not detected
R. paviana	50	98	telomere	partial genome	partial genome	whole genome	not detected

and Reed 2000; Sola and Gornung 2001), *Da. Roseus*: 2n=50 (Kaewtip et al. 2021) and *Da. albolineatus*: 2n=50 (Arai 2011). The 2n of *De. regina* is the same as that of *De. laoensis* Pellegrin & Fang, 1940 (Aiumsumang et al. 2021; Kaewtip et al. 2021) and *De. aequipinnatus* reported by Sukham et al. (2013). *R. aurotaenia* has 2n=50 according to Seetapan and Moeikum (2004) and Aiumsumang et al. (2012). The diploid chromosome number of *R. paviana* was 50, which is the same as that from the previous study by Aiumsumang et al. (2021). This 2n is considered the same as for the other species of subfamily Danioninae (*Danio*, *Devario* and *Rasbora*).

Patterns of microsatellite repeats in the genomes of Da. albolineatus, De. regina, R. aurotaenia and R. paviana

The chromosomal distribution of repetitive DNA elements revealed remarkable differences amongst the analysed species. In this study, the number and distribution of microsatellite sequence $(CA)_{15}$, $(CAC)_{10}$, $(CGG)_{10}$, $(GC)_{15}$ and $(TA)_{15}$ were not conserved among four analyzed species. The microsatellite sequence $(CA)_{15}$ was mapped on chromosomes of *Da. albolineatus*, *De. regina*, *R. aurotaenia* and *R. paviana*, and abundantly located and distributed in all chromosomes, usually in telomeric regions and a few chromosome pairs showed the strongest signal intensities, these not really being suited to serve as chromosomal markers. Microsatellites are usually located in the heterochromatic regions (telomeres/centromeres) of fish genomes, where a significant fraction of repetitive DNA is localized (Cioffi and Bertollo, 2012). Indeed, this distribution pattern is found in *Epalzeorhynchos frenatum* (Fowler, 1934), *Puntigrus partipentazona* (Fowler, 1934), *Scaphognathops bandanensis* Boonyaratpalin & Srirungroj, 1971 (Phimphan et al, 2020); *Catlocarpio siamensis* Boulenger, 1898 and *Probarbus jullieni* Sauvage, 1880 (Saenjundaeng et

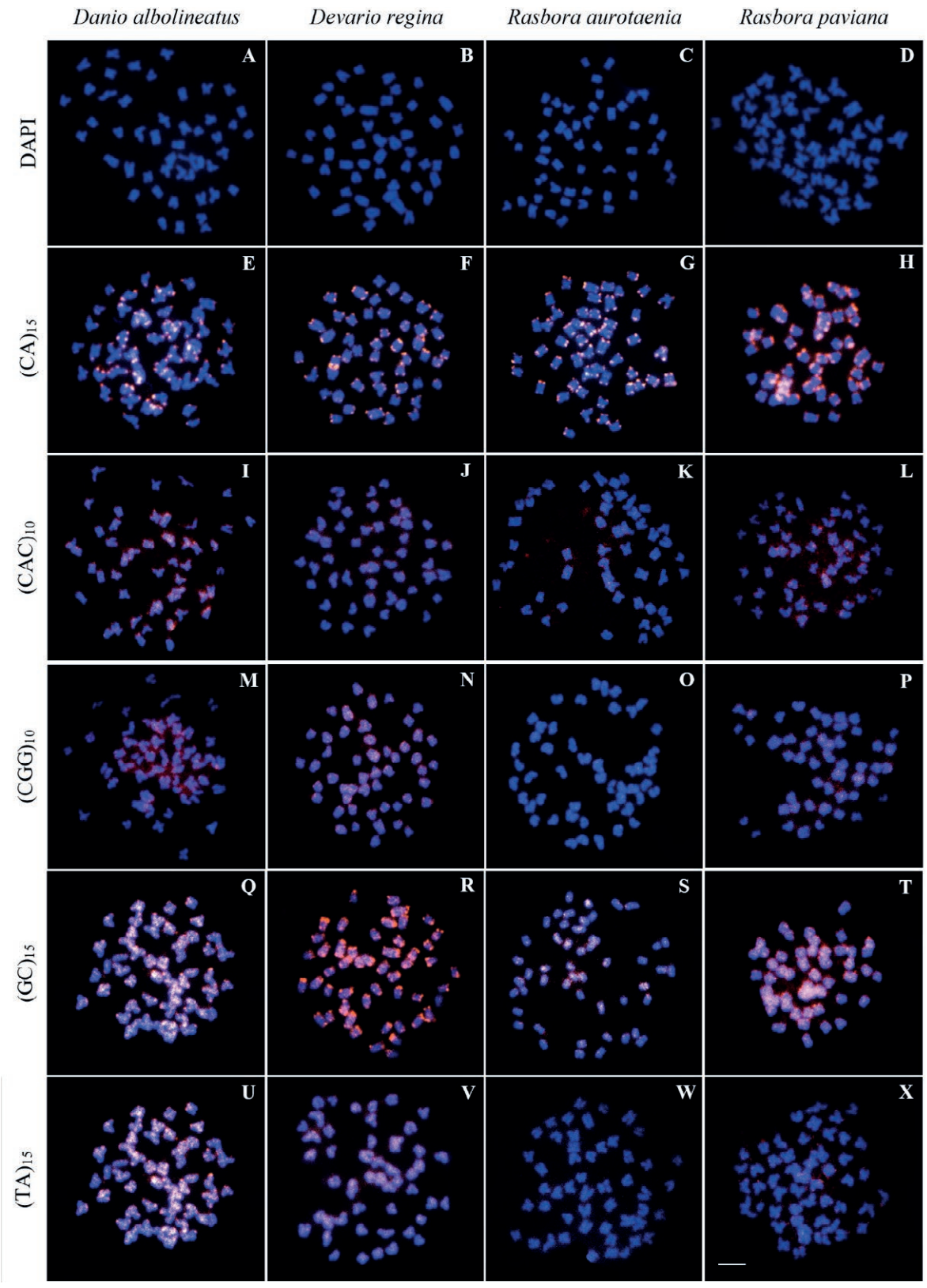

Figure 3. FISH using DAPI (A-D), $[CA]_{15}$ (E-H), $[CAC]_{10}$ (I-L), $[CGG]_{10}$ (M-P), $[GC]_{15}$ (Q-T), $[TA]_{15}$ (U-Z) of *Danio albolineatus*, *Devario regina*, *Rasbora aurotaenia* and *Rasbora paviana*, respectively. Scale bar 5 μm.

al. 2018); *Clarias* species (Maneechot et al. 2016) and *Channa micropeltes* (Cuvier, 1831) (Cioffi et al. 2015).This distribution pattern are differences known for *Channa gachua* (Hamilton, 1822), *C. lucius* (Cuvier, 1831), *C. striata* (Bloch, 1793) (Cioffi et al. 2015), *Hemibagrus wyckii* (Bleeker, 1858) (Supiwong et al. 2017) and *Monopterus albus* (Zuiew, 1793) (Supiwong et al. 2019). For the mapping of $(CAC)_{10}$ repeats in *Da. albolineatus* and *R. paviana,* partial genomes were identified, while *De. regina* and *R. aurotaenia* were not detected. In *Da. albolineatus*, *De. regina*, *R. aurotaenia* and *R. paviana,* most microsatellites $(CGG)_{10}$ and $(CG)_{15}$ were abundantly distributed in all chromosomes with the exception of $(CGG)_{10}$ in *R. aurotaenia,* this not being a signal probe. Nevertheless, comparative analyses among the species indicate that the microsatellites have also preferential zones of accumulation at telomeric heterochromatin. For *De. regina* the accumulation of microsatellites $(GC)_{15}$ in all chromosomal pair could point out a species–specific type of heterochromatin is similar to previous reports in *Channa micropeltes* (Cioffi et al. 2015). In *Da. albolineatus R. aurotaenia* and *R. paviana* the same microsatellites are not only located at the telomeric region of some chromosomes, but they are also found at the centromeres of the chromosome pair. $(TA)_{15}$ repeats in *Da. albolineatus* and *De. Regina,* displaying high accumulations at the whole genome, while not detected in the *Rasbora* group.

Repetitive DNA sequences display a high degree of polymorphism because of the variation in the number of repetitive units, which results from a specific evolutionary dynamic. Amongst these elements, microsatellites (or short tandem repeats) are the most polymorphic and consist of short sequences of one to six nucleotides repeated in tandem throughout the DNA (Tautz and Renz 1984). Due to their supposed neutral evolution, these molecular markers have been widely used in population genetics, to identify taxonomic limits and in hybridization and forensic studies (Goldstein and Pollock 1997; Filcek et al. 2005; Racey et al. 2007; McCusker et al. 2008) and can be used to spot genomic evolution as previously been reported for different fish groups (Cioffi et al. 2010; Cioffi and Bertollo 2012; Terencio et al. 2013; Yano et al. 2014; Cioffi et al. 2015; Moraes et al. 2017; 2019; Sassi et al. 2019).

The comparative study on four species showed that the diploid chromosome is the same, but the patterns of microsatellite repeat on chromosomes have differences amongst them. Thus, the molecular cytogenetic data may be a tool for classification of fish species where there is similar morphology, such as the stripe. Overall, it is believed that the microsatellites have specific zones of accumulation in genomes, preferentially in heterochromatic regions (Supiwong et al. 2014). In fact, microsatellites are located in the heterochromatic regions (telomeres, centromeres and in the sex chromosomes) of fish genomes (Cioffi and Bertollo 2012, including the present study). However, the distribution of microsatellites was not only restricted to heterochromatin, but also dispersed in euchromatic regions of the chromosomes (Getlekha et al. 2016). Additionally, such variability is also reinforced by the dynamism of repetitive elements in the genome, especially by the differential distribution and accumulation of rDNA sequences amongst chromosomes (Cioffi et al. 2015). Although not yet completely understood, this marked diversity is likely linked to the lifestyle of these fishes and to population fragmentation, as already identified for other fish species.

CONCLUSIONS

Our study is the first one to offer reliable chromosomal data for *Da. albolineatus*, *De. regina*, *R. aurotaenia* and *R. paviana* by molecular cytogenetic protocols, this study were useful tools in highlighting the remarkable chromosomal diversification which was characterised in the four minnow fishes. Besides, data from comparative genomic hybridization experiments also highlighted an advanced stage of repetitive DNA divergence, evidencing their evolutionary diversification.

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