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A comparative chromosome study on five Minnow fishes (Cyprinidae, Cypriniformes) in Thailand

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Abstract. The cytogenetic comparisons of five Minnow species from Thailand were presented here, i.e., *Devario regina*, *D. laoensis*, *Rasbora paviana*, *R. aurotaenia* and *Esomus metalicus*. The mitotic chromosomes were prepared directly from renal cells. Conventional staining and Ag-NOR banding techniques were applied to stain the chromosomes. The results revealed that all Minnow fishes studied possessed the same diploid chromosome number ($2n$) as 50 chromosomes. The fundamental numbers (NF) of *D. laoensis*, *D. regina*, *R. paviana*, *R. aurotaenia* and *E. metalicus* are 100, 100, 98, 98, and 98 respectively. Their karyotypes composing of metacentrics-submetacentrics-acrocentrics-telocentrics were as follows: 6-12-32-0 in *D. regina*, 6-10-34-0 in *D. laoensis*, 8-16-24-2 in *R. paviana*, 8-16-24-2 in *R. aurotaenia* and 8-10-30-2 in *E. metalicus*. The Ag-NOR banding technique provides the nucleolar organizer regions (NORs) at subtelomeric region of the short arm chromosome in the a submetacentric or acrocentric chromosomes that are located differently in the different chromosome pairs among species.

Keywords: karyotype, Minnow, fish chromosome, Cyprinid fishes, Minnow fishes.

INTRODUCTION

Devario laoensis, *D. regina*, *Esomus metalicus*, *Rasbora aurotaenia,* and *R. paviana* are some 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 distribu-

tions 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, Rawat 2013).

The current spurt in the fish cytogenetical studies has its origin in the standardization of newer techniques and the realization of an immense applied value of the cytogenetic data of fishes. The study on fish chromosomes has received considerable attention in recent years because of their importance in classification, evolution, heredity, systematic (Gold *et al*. 1990, Ueda *et al*. 2001, Barat *et al*. 2002, Barat and Sahoo 2007), fish breeding, rapid production of inbred lines including cytotaxonomy (Kirpichnikov 1981) and prove the ploidy status in some sturgeons (Zhou *et al*. 2013). The several methods namely, conventional staining, C-banding, Ag-NOR banding, and fluorescence *in situ* hybridization (FISH) have been used by ichthyologists for gathering of cytogenetic information of fish (Sola *et al*. 2000, Kavaco *et al*. 2005, Zhou *et al*. 2013), yet each of these methods provides a different aspect of the karyotype characteristics. For example, Ag-NOR staining shows the regions containing the actively transcribed ribosomal RNA genes (rDNA). NORs characterization can be a cytogenetic marker for cytotaxonomic studies and has been used for studying on phylogenetic relationships among the Cyprinids (Amemyia and Gold 1988, Gatetti Jr 1998, Almeida-Toledo *et al*, 2000). However, cytogenetic studies conducted on this group (*Devario*, *Esomus* and *Rasbora*) are quite scarce. There are some karyotype reports, including *Rasbora trilineata* and *R. heteromorpha*: 2*n*=48 (Post 1965), *R. buchanani*: 2*n*=50 (Manna and Khuda-Bukhsh 1977), *R. daniconius*: 2*n*=50 (Khuda-Bukhsh *et al*. 1979), *R. sumatrana*: 2*n*=50 (Donsakul and Magtoon 1995), *R. caudimaculata*, *R. myersi*, *R. paviei* and *R. retrodorsalis*: 2*n*=50 (Donsakul and Magtoon 2002), *R. aurotaenia*: 2*n*=50 (Seetapan and Moeikum 2004), *R. trilineata*, *R. heteromorpha, R. daniconius*, *R. borapetensis* and *R. einthovenii*: 2*n*=50 (Donsakul *et al*. 2005), *R. agilis*, *R. dorsicellata* and *R. rubrodorsalis*: 2*n*=50 (Donsakul *et al*. 2009), *E. metallicus*: 2*n*=50 (Neeratanaphan *et al*. 2017) and *R. einthovenii*: 2*n*=50 (Yeesaem *et al*. 2019) (Table 1). The studies on the karyotypes help to investigate the genetic structure of aquatic animal species in each habitat, thus it can determine what species are related to each other in an accurate manner. This may help to facilitate the hybridization between them in the future for strain improvement (Sofy *et al*. 2008).

In the present study, we conducted chromosomal analyses using conventional staining and Ag-NOR banding techniques. The examined karyotypes of five Minnow species from Thailand belonging to three different genera (*Devario*, *Esomus,* and *Rasbora*); *D. laoensis*, *D. regina* and *R. paviana* were reported chromosomes characterized for the first time. The obtained results will provide useful cytogenetic information for further studies on taxonomy and evolutionary relationship of fishes.

MATHERIAL AND METHODS

Chromosome preparation

Individuals from both sexes of five analyzed Minnows were collected from various river basins in Thailand (Table 1 and Fig. 1). The fishes were transferred to

Table 1. Collection sites of the analyzed species show the sample number.

Species	Number of specimens in site sampling								
	Mae Khong Basin	Sirindhorn Peat Swamp Forest	Ping Basin	Yom Basin	Pa-Sak Basin	Chi Basin	Chao Phraya Basin	Song-khram Basin	Remark with Fig. 1.
Devario regina	05 ♀ 06 ♂	06 ♀ 08 ♂	-	-	-	-	-	-	Site 1
D. laoensis	-	-	03 ♀ 05 ♂	-	-	-	-	-	Site 2
Rasbora paviana	05 ♀ 08 ♂	03 ♀ 04 ♂	-	-	05 ♀ 07 ♂	04 ♀ 05 ♂	-	-	Site 3
R. aurotaenia	-	-	-	-	-	-	08 ♀ 07 ♂	05 ♀ 08 ♂	Site 4
Esomus metalicus	-	-	-	04 ♀ 05 ♂	10 ♀ 10 ♂	-	-	-	Site 5

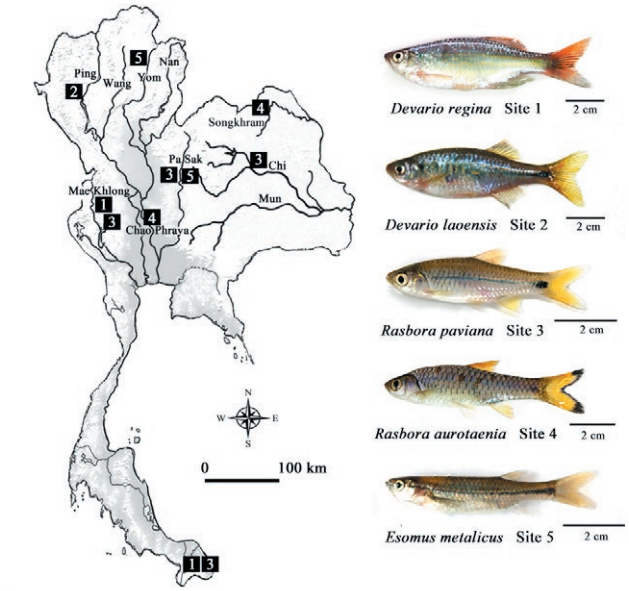

Figure 1. Collection sites of cyprinid fishes studied herein. 1=*Devario regina,* 2=*Devario laoensis*; 3=*Rasbora paviana*, 4=*Rasbora aurotaenia*, 5=*Esomus metalicus.*

laboratory aquaria and kept under standard conditions for three days before the experiments. Chromosomes were prepared *in vivo* as follows (Supiwong *et al.* 2014). The colchicine was injected into the fish's intramuscular and/or its abdominal cavity at a dose of 0.1 mL/100 g of body weight and then left for 1-2 hours. The kidney was cut into small pieces then squash mixed with 0.075 M KCl. After discarding all large piece tissues, 8 mL of cell sediments were transferred to a centrifuge tube and incubated for 30 minutes. The KCl was discarded from the supernatant after centrifugation at 1,200 rpm for 8 minutes. Cells were fixed in fresh cool Carnoy's fixative (3 methanol: 1 glacial acetic acid) allows to preserve the internal structure of the cells for better staining of the chromosomes (Pradeep *et al.* 2011) to which up to 8 mL were gradually added before being centrifuged again at 1,200 rpm for 8 minutes, at which time the supernatant was discarded. The fixation was repeated until the supernatant was clear and the pellet was mixed with 1 mL fixative. The mixture was dropped onto a clean and cold slide by micropipette followed by air-drying technique.

Chromosome staining

Conventional staining was carried out using 20% Giemsa's solution for 15 minutes (Phimphan *et al.* 2017). Ag-NOR banding was performed by adding 4 drops of 50% silver nitrate and 2% gelatin on slides (Howell and Black 1980). The slides were then sealed with cover glasses and incubated at 60°C for 5 minutes. After that, the slides were soaked in distilled water until the cover glasses were separated. Then, they were stained with 20% Giemsa's solution for 1 minute.

Chromosome check and Image processing

Twenty clearly observable metaphase cells with a well-spread chromosome of each male and female were selected. Images were captured under a light microscope Nikon ECLIPSE by a digital CCD camera (Nikon DS-Fi1). The chromosomes were classified based on the position of a centromere as metacentric (m), submetacentric (sm), acrocentric (a), telocentric (t) according to the arm ratios (Chaiyasut 1989).

RESULTS

Five minnow fishes were similar in the diploid number of 2*n*= 50, with the karyotype composed of m6+sm12+a32 in *D. regina*. The mean values calculated from twenty mitotic metaphases showed the relative length (RL) of chromosomes complement ranging from 0.041±0.010 to 0.033±0.004. The NOR was found on the short arm of chromosome pair 15 (Fig. 2A). The chromosome complements of *D. laoensis* consisting of m6+10sm+34a. The mean value of relative length ranged from 0.0.44±0.005 to 0.030±0.002. The NOR was presented on the short arms of chromosome pair 11 (Fig. 2B). Karyotype of *R. paviana* composes of 8m+16sm+24a+2t. The present investigation in this fish species revealed that the mean value of RL from 0.048±0.001 to 0.032±0.004. Ag-NOR banding result showed that NOR-bearing chromosomes locate at subtelomeric on the short arm of chromosome pair 9 (Fig. 2C). The karyotypic analysis result revealed that the chromosome complements of *R. aurotaenia* consisting of 8m+16sm+24a+2t. The parameters of all chromosomes were measured and it showed the mean value of RL from 0.0.054±0.003 to 0.033±0.002. The result of silver-staining exhibited the NORs show that it locates) at short arm of chromosome pair 23 (Fig. 2D). The karyotype of *E. metalicus* consisting of 8m+10sm+30a+2t. The mean value of RL from 0.0.51±0.001 to 0.025±0.002. The NOR was presented on the short arms of chromosome pair 7 (Fig. 2E).

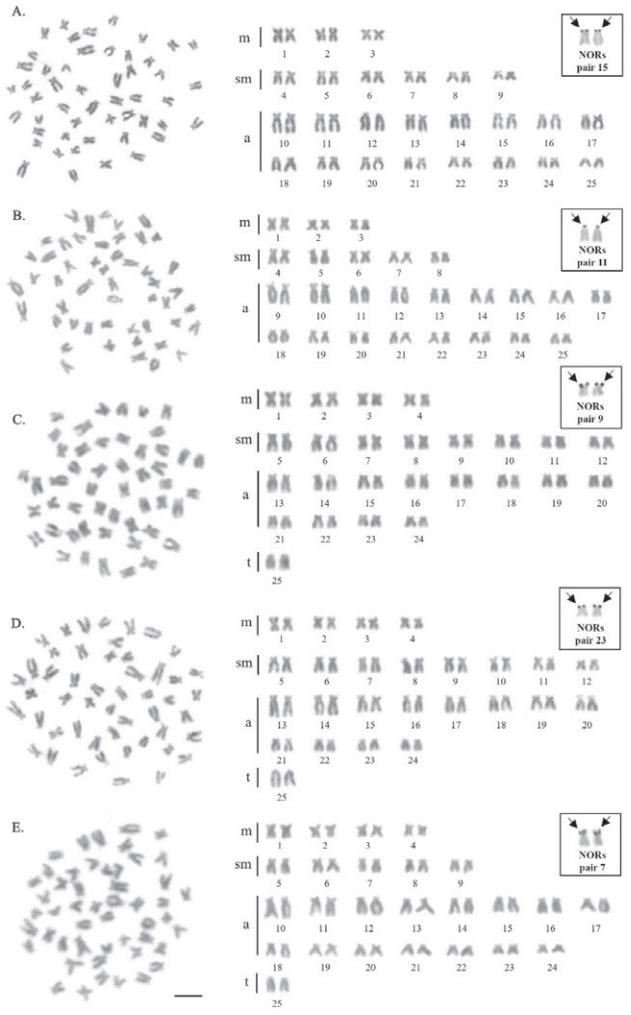

Figure 2. Metaphase chromosome plates and karyotypes of the *Devario regina* (A.), *D. laoensis* (B.), *Rasbora paviana* (C.), *R. aurotaenia* (D.) and *Esomus metalicus* (E.), by conventional staining. The arrows indicate NOR banding by Ag-NOR staining technique (inserted box). All species share the karyotype composed of 50 chromosomes. Scale bar indicates 5 μm.

DISCUSSION

The details of each metaphase chromosome spread and karyotype of five Minnow fishes, including *D. regina*, *D. laoensis*, *R. paviana*, *R. aurotaenia,* and *E. metalicus* are shown in Figure 2. The present study is the first report on the chromosomal characteristics of *D. laoensis*, *D. regina,* and *R. paviana* determined using conventional staining and Ag-NOR banding techniques. The diploid chromosome number of all species provided 50 chromosomes, which is shared by most of the cyprinid species previously analyzed (Post 1965, Manna and Khuda-Bukhsh 1977, Khuda-Bukhsh *et al.* 1979, Donsakul and Magtoon 1995, Donsakul and Magtoon 2002, Seetapan and Moeikum 2004, Donsakul *et al.* 2005, Donsakul *et al.* 2009, Neeratanaphan *et al.* 2017, Yeesaem *et al.* 2019) (Table 2). The NFs of *D. laoensis* and *D. regina* are 100 equally, while those of *R. paviana*, *R. aurotaenia,* and *E. metalicus* are equal to 98 in both sexes. To compare with previous studies, they are differences from Seetapan and Moeikum (2004) who reported the NF=92 in *R. aurotaenia* and Neeratanaphan *et al.* (2017) showed the NF of *E. metallicus* as 100. The differences in NF values are caused by the difference in the number of monoarm chromosomes. This phenomenon may be resulting from the intra-specific variation between populations of those species. This finding is in agreement with other species such as *R. daniconius* (Khuda-Bukhsh *et al.* 1979, Donsakul *et al.* 2005), *R. einthovenii* (Donsakul *et al.* 2005, Yeesaem *et al.* 2019), and *R. rebrodorsalis* (Donsakul and Magtoon 2002, Donsakul *et al.* 2009). The NF of these genera varied from 74 to 100 (Table 2). All species were analyzed herein display without morphologically differentiated sex chromosomes. This character is the same as in previous studies of this family (Arai 2011).

Although five Minnows analyzed herein have the same diploid number, there are differences in karyotype complements as follows (Fig. 2). *D. regina* has six metacentric (m) (pairs 1-3), 12 submetacentric (sm) (pairs 4-9) and 32 acrocentric (a) (pairs 10-25) chromosomes. The mean values were calculated from twenty mitotic metaphases showed the centromeric index (CI) of chromosome complements ranging from 0.548±0.004 to 0.808±0.005. The karyotype formula of *D. regina* could be deduced as $2n(50)$ = 6m+12sm+32a. *D. laoensis* has six metacentric (pairs 1-3), 10 submetacentric (pairs 4-8) and 34 acrocentric (pairs 9-25) chromosomes. The mean values of CI ranged from 0.553±0.005 to 0.798±0.002. The karyotype formula of this species is $2n(50)$ = 6m+10sm+34a. *R. paviana* consisted of eight metacentrics (pairs 1-4), 16 submetacentrisc (pairs 5-12), 24 acrocentrics (pairs 13-24) and two telocentrics (t) (pair 25). The mean values of CI ranged between 0.526±0.002 and 1.000±0.000. The proposed karyotype of this species was $2n(50)$ = 8m+16sm+24a+2t. *R. aurotaenia* shows eight metacentrics (pairs 1-4), 16 submetacentrics (pairs 5-12), 24 acrocentrics (pairs 13-24) and two telocentrics (pair 25) chromosomes. The mean values of CI in this species ranged from 0.569±0.003 to 1.000±0.000. The karyotype of this species was $2n(50)$ = 8m+16sm+24a+2t, which differs from the previous study by Seetapan and Moeikum (2004) that reported the karyotype of this species consisting of $2n(50)$ = 14m+26sm+2st+8a. In *E. metalicus*, the karyotype composed of eight metacentric (pairs

Table 2. Cytogenetic reported of the genera *Devario*, *Esomus* and *Rasbora*.

Species	2*n*	NF_1	NF_2	Karyotype formula	NOR	Reference
Devario laoensis	**50**	**100**	**66**	**6m+10sm+34a**	**2**	**Present study**
D. regina	**50**	**100**	**68**	**6m+12sm+32a**	**2**	**Present study**
Esomus metallicus	50	100	86	14m+22sm+14a	-	Neeratanaphan *et al.* (2017)
	50	**98**	**68**	**8m+10sm+30a+2t**	**2**	**Present study**
Rasbora agilis	50	100	100	24m+26sm	-	Donsakul *et al.* (2009)
R. aurotaenia	50	92	90	14m+26sm+2a+8t	-	Seetapan and Moeikum (2004)
	50	**98**	**74**	**8m+16sm+24a+2t**	**2**	**Present study**
R. borapetensis	50	88	88	24m+14sm+12t	-	Donsakul *et al.* (2005)
R. buchanani	50	100	96	30m+18sm+2a	-	Manna and Khuda-Bukhsh (1977)
R. caudimaculata	50	98	96	20m+26sm+2a+2t	-	Donsakul and Magtoon (2002)
R. daniconius	50	80	74	18m+6sm+6a+20t	-	Khuda-Bukhsh *et al.* (1979)
R. daniconius	50	92	90	32m+8sm+2a+8t	-	Donsakul *et al.* (2005)
R. dorsicellata	50	92	92	18m+24sm+8t	-	Donsakul *et al.* (2009)
R. einthovenii	50	94	86	6m+30sm+8a+6t	-	Donsakul *et al.* (2005)
	50	100	84	16m+18sm+16a	2	Yeesaem *et al.* (2019)
R. heteromorpha	48	-	-	-	-	Post (1965)
	48	74	72	14m+10sm+2a+22t	-	Donsakul *et al.* (2005)
R. myersi	50	90	84	20m+14sm+6a+10t	-	Donsakul and Magtoon (2002)
R. paviei	50	100	84	10m+24sm+16a	-	Donsakul and Magtoon (2002)
R. paviana	**50**	**98**	**74**	**8m+16sm+24a+2t**	**2**	**Present study**
R. retrodorsalis	50	88	86	26m+10sm+2a+12t	-	Donsakul and Magtoon (2002)
R. rubrodorsalis	50	82	82	16m+16sm+18t	-	Donsakul *et al.* (2009)
R. sumatrana	50	94	92	26m+16sm+2a+6t	-	Donsakul and Magtoon (1995)
R. trilineata	48	-	-	-	-	Post (1965)
R. trilineata	50	94	92	26m+16sm+2a+6t	-	Donsakul *et al.* (2005)

Abbreviations: diploid chromosome number (2*n*), fundamental number m, sm, a =2, t=1 (NF1), fundamental number m, sm, =2, a, t=1 (NF2), metacentric (m), submetacentric (sm), acrocentric (a), telocentric (t), Nucleolar Organizer Region (NOR).

1-4), 10 submetacentric (pairs 5-9), 30 acrocentric (pairs 10-24), and two telocentric (pair 25) chromosomes. The mean values of CI ranged between 0.558±0.003 and 1.000±0.000. The karyotype of *E. metalicus* showed 2*n*(50) = 8m+10sm+30a+2t. These results are inconsistent with previous cytogenetic data (Neeratanaphan *et al.* 2017). This fact suggests that some pericentric inversions have occurred in the karyotype differentiation of this species. Besides), the occurrence of chromosomal rearrangements has been considered a relatively common evolutionary mechanism inside the Cyprinidae family reviewed (Arai 2011). Family Cyprinidae are diploid chromosome ranges from 48–50 in the tribes Labeonini and Smiliogastrini while the tribe Poropuntiini and Danionini are more conserved as 2n = 50 (Phimphan *et al.* 2020).

Karyotype diversification processes in species are subjected to multiple factors, whether intrinsic (genomic or chromosomal particularities) or extrinsic (historic contingencies) factor. Among these, restricted gene flow between populations is an important factor for the fixation of karyotype changes. For example, after the occurrence of an inversion, it can be lost in the polymorphic state or, under the proper conditions, spread in the population until it is fixed. Inversions maintain areas of imbalance between alleles in loci within or influenced by these rearrangements, leading to an adaptive condition, primarily along environmental gradients. This could occur, particularly concerning possible historical expansion and adaptation to new environments for a review Hoffmann (2008). As mention above, the chromosomal study is very important and clearly exhibits the benefits.

Moreover, the karyological and NORs characteristics in cyprinid fishes were reported in some species.

The present study is the first report on the NOR phenotypes in five Minnow species studied. The single pair of NOR-bearing chromosomes were observed at subtelomeric regions on the short arm chromosomes in all species analyzed. However, there are differences in chromosome types and pair numbers as follows. The NORs were observed on acrocentric chromosome pair 15 in *D. regina* whereas those were found on acrocentric chromosome pair 11 in *D. laoensis*. In the genus *Rasbora*, the NORs located on the submetacentric chromosome pair 9 in *R. paviana* and distinct revealed on the acrocentric chromosome pair 23 in *R. aurotaenia*. For *E. metalicus*, NOR-baring chromosomes were found on the submetacentric chromosome pair 7 (Fig. 2). To compare with the same genus in previous report, *R. einthovenii* has single pair of NOR on chromosome pair 4 (Yeesaem *et al*. 2019). Moreover, the single pair of NOR bearing chromosomes can be observed in other cyprinids such as *Aspius aspius* (Ràb *et al*. 1990), *Osteochilus waandersi* (Magtoon and Arai 1993), *Barbonymus gonionotus* (Khuda-Bukhsh and Das 2007), *Puntioplites proctozysron* (Supiwong *et al*. 2012), *Puntius brevis* (Nitikulworawong and Khrueanet 2014). Also, the subtelomeric region of chromosome pair showed clearly observable NORs in most cyprinid fishes. However, NOR variation can be revealed in among populations of the same species as found in *Garra rufa*. This variation is caused by geographically isolated populations (Arzu and Ergene 2009). Normally, most fishes have only one pair of small 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). Our present study showed that the species analyzed had a NOR site on a single chromosome pair at a subtelomeric position. This is considered a simple condition in fish (Almeida-Toledo 1985).

In the present study, five Minnows belong to genera of which have closely related species. The obtained results have shown that this fish group shares the same 2*n*. However, there are differences in karyotype complements and NOR-bearing chromosome markers. These seem to be that cytogenetic methods can be used for the systematics of this fish family.

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