รายงานครั้งแรกทางพันธุศาสตร์เซลล์ของปลาซิวใบไผ่แถบขาว (*Danio albolineatus*)

Classical Cytogenetics of Pearl Danio, *Danio albolineatus* (Cyprinidae, Cypriniformes): First Karyomorphological Report

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บทคัดย่อ

วิเคราะห์ลักษณะทางพันธุศาสตร์เซลล์ของปลาซิวใบไผ่แถบขาว (*Danio albolineatus*) ด้วยเทคนิคการย้อมสีแบบดั้งเดิม การเตรียมโครโมโซมใช้วิธีการเตรียมโดยตรงจากเนื้อเยื่อไต ย้อมสีโครโมโซมแบบธรรมดาด้วยสีจิมซ่าและย้อมเทคนิคแถบสีแบบนอร์ (Ag-NOR banding) ผลการศึกษาโครโมโซมของปลาซิวใบไผ่แถบขาวทั้งเพศผู้และเพศเมียพบจำนวนโครโมโซมดิพพลอยด์เท่ากับ 50 แท่ง จำนวนโครโมโซมพื้นฐานเท่ากับ 100 นำมาการจัดเรียงแคริโอไทป์ประกอบด้วยโครโมโซมชนิดเมทาเซนทริก 8 แท่ง โครโมโซมชนิดซับเมทาเซนทริก 14 แท่ง และ โครโมโซมชนิดอะโครเซนทริก 28 แท่ง จำแนกตามขนาดของโครโมโซมประกอบด้วยโครโมโซมขนาดใหญ่ 18 แท่ง และโครโมโซมขนาดกลาง 32 แท่ง การศึกษาโครโมโมโซมของปลาซิวใบไผ่แถบขาวไม่พบความแตกต่างของโครโมโซมเพศ จากการย้อมแถบสีแบบนอร์ พบตำแหน่งนอร์บริเวณปลายแขนข้าวยาวของโครโมโซมคู่ที่ 5 ผลการศึกษาโครโมโซมของปลาซิวใบไผ่แถบขาวเขียนเป็นสูตรแคริโอไทป์ได้ ดังนี้ $2n$ (50) = $L^{m}_{4} + L^{sm}_{6} + L^{a}_{8} + M^{m}_{4} + M^{sm}_{8} + M^{a}_{20}$

ABSTRACT

A cytogenetic analysis was carried out on specimens of Pearl Danio (*Danio albolineatus*) by classical technique. Mitotic chromosome preparation was prepared directly from kidney cells follow by standard protocol and stained by conventional staining and Ag-NOR banding techniques. The result show that diploid number ($2n$) of this species is 50 and the fundamental number (NF) is 100 in both male and female. The karyotype consisted of eight metacentric, 14 submetacentric and 28 acrocentric chromosomes classifying as 18 large and 32 medium. No heteromorphic sex chromosome was observed in this species. Nucleolus organizer regions (NORs) appeared in the terminal position of the long arms of chromosome pair 5. The Karyotype formula of *D. albolineatus* is $2n$ (50) = $L^{m}_{4} + L^{sm}_{6} + L^{a}_{8} + M^{m}_{4} + M^{sm}_{8} + M^{a}_{20}$.

คำสำคัญ: แคริโอไทป์ โครโมโซมปลา ปลาซิวใบไผ่แถบขาว

Keywords: Karyotype, Fish chromosome, *Danio albolineatus*

INTRODUCTION

The pearl danio (*D. albolineatus*) belongs to the family Cyprinidae (Cypriniformes) which, with about 2,010 species, grouped in 210 genera, is the most speciose family among the freshwater fishes (Nelson, 2006). It is located within the *D. rerio* species group, but it can be considered as a separate species subgroup – the *D. albolineatus* species subgroup (McCluskey and Postlethwait, 2015). Within the *D. rerio* species group, the *D. albolineatus* species subgroup was found to be most distantly related to *D. rerio* (Sola and Gornung, 2001). The zebrafish (*D. rerio*) is a cyprinid species adopted in research as a model system for the analysis of vertebrate development (Quigley et al., 2004). *D. albolineatus* has seven dorsal soft rays (total) and 12-13 anal soft rays. Incomplete lateral line, without infraorbital process; with 6-7 branched dorsal fin rays and 13-14 branched anal fin rays. Two pairs of long barbels. Rostral barbels reaching to or slightly anterior to vertical through the middle of the orbit. Maxillary barbels exceed the origin of pectoral fin (Fang, 2000). This species has body pink with two light yellow-white (iridescent in life) stripes from below dorsal origin to caudal base (Kottelat, 2001) (Figure 1).

Studies of cytogenetics are important in an aquaculture context in the use of chromosome manipulation techniques, including induction of polyploidy, gynogenesis, androgenesis, and inter- or intra-species hybridization. For the family Cyprinidae, there were some cytogenetical studies reported about *Danio*. The first report on the chromosomes of *Danio* species dates back to the sixties, when Post (1964) proposed a diploid number (2*n*) of 48, based on the observation of meiotic cells. A few years later the chromosome were reported of *D. rerio* has 2*n*=50, karyotype several independent papers such as 50 bi-arm chromosomes (Endo and Ingalls, 1968), 10m (metacentric) + 40sm (submetacentric) (Fontana et al., 1970), 10m + 12sm + 28a (acrocentric) (Rishi, 1976), 16m + 32sm + 2a (Schreeb et al., 1993), 12m + 26sm + 12a (Pijnacker and Ferwerda, 1995), 4m + 16sm + 30a (Daga et al., 1996; Gornung et al., 1997), female: 7m + 7sm + 36a, male: 6m + 8sm + 36a (Sharma et al., 1998), 12m + 26sm + 12a (Ueda and Naoi, 1999), 4m + 30sm + 16a (Amores and Postlethwait, 1999) and 4m + 16sm + 30a (Phillips and Reed, 2000; Sola and Gornung, 2001).

This cytogenetic study, provide the very first report on chromosome standardization, including chromosome measurements of shape and size, karyotype formulation, idiograming and chromosomal characteristics of the nucleolar organizer regions (NORs) of *D. albolineatus*. Among the species of Cyprinidae, *D. albolineatus* is probably the one species that has been the examined from a cytogenetic point of view. This is why it might encourage future cytotaxonomic and cytogenetic studies on representatives of this family, as well as in teleosts, in general and could be applied to several studies, especially for their extinction protection.

MATERIAL AND METHODS

Biological Materials and Chromosome preparation

Individuals from both sexes of *D. albolineatus* analyzed were collected from various river basins in Thailand (Figure 1 and Table 1). The fish were transferred to laboratory aquaria and were kept under standard condition to the experiments. All the experiments followed ethical protocols, as approved by the Ethics of Animal Experimentation of the National

Research Council of Thailand U1-04498-2559 and anesthesia with clove oil was used prior to sacrificing the animals to minimize suffering. Mitotic chromosomes were obtained from cell suspensions of the anterior kidney, using the conventional air-drying method as follows (Supiwong et al., 2014). The colchicine was injected into the fish's intramuscular and/or its abdominal cavity at a dose of 0.05mL/100 g of body weight and then left for one hour. Kidney were 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 1,500 rpm for 8 minutes. Cells were fixed in fresh cool fixative (3 methanol: 1 glacial acetic acid) to which up to 7 mL were gradually added before being centrifuged again at 1,500 rpm for 10 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.

Giemsa's staining, Ag–NORs banding technique and karyotype

The chromosomes were conventionally stained with 20 % Giemsa's solution for 30 minutes (Phimphan et al., 2017). Ag-NOR banding technique was performed according to Howell and Black (1980) by applying four drops of 50% silver nitrate and 2% gelatin on slides. The slides were then sealed with cover glasses and incubated at 60ºC for 5 minutes. Next, the slides were soaked in distilled water until the cover glasses were separated and allow slide to air dry at room temperature. Approximately 30 metaphase spreads per specimen were analyzed to confirm the diploid chromosome number and karyotype structure. Metaphases were observed under the Nikon ECLIPSE microscope and photographed with digital CCD camera (Cannon EOS 500D). The chromosomes were measured and the centromeric index (CI) and relative length (RL) were estimated. The metaphase figures were analyzed according to the chromosome classification of Chaiyasut (1988). The centromeric index (CI) between 0.500-0.599, 0.600-0.699, 0.700-0.899, and 0.900-1.000 were described as metacentric (m), submetacentric (sm), acrocentric (a), and telocentric (t) chromosomes, respectively. The fundamental number (NF, number of chromosome arms) were obtained by assigning a value of two to metacentric, submetacentric and acrocentric chromosomes and one to telocentric chromosome. All parameters were used in karyotyping and idiograming.

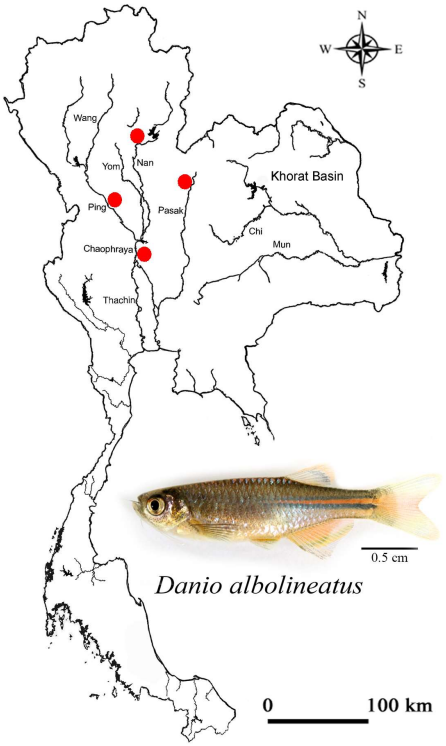

Figure 1. Collection sites of cyprinid fishes studied herein and general characteristics of the Pearl Danio (*D. albolineatus*).

Table 1. Collection sites of the analyzed *D. albolineatus* with the sample size.

Collection sites	*N*
– Nan Basin	(05 Female; 05 Male)
– Ping Basin	(07 Female; 08 Male)
– Pa Sak Basin	(10 Female; 10 Male)
– Chao Phraya Basin	(08 Female; 05 Male)

RESULTS

The present investigation revealed that the somatic chromosome number of *D. albolineatus* is 2*n*=50, the results of samples from different sources are the same. The respective fundamental number (NF) was 100 in both males and females. No cytologically distinguishable sex chromosomes were observed in this species analyzed. The chromosome types comprise two large metacentric, three large submetacentric, four large acrocentric, two medium metacentric, four medium submetacentric and ten medium acrocentric chromosomes (Figure 2A). The average lengths of each chromosome including short and long arm length, total length, relative length, and centromeric index were calculated and are presented in Table 2. The mean values calculated from twenty mitotic metaphases showed the relative length of chromosomes complement ranging from 0.043±0.01 to 0.029±0.001. Ag-NOR banding result showed clearly observable nucleolar organizer regions (NORs) were found appeared in the terminal position of the long arms chromosome pair 5 (Figure 2B). The standard idiogram of *D. albolineatus* showed the gradually decreasing length and size of the chromosomes from conventional staining and Ag-NOR banding techniques are shown in Figure 3.

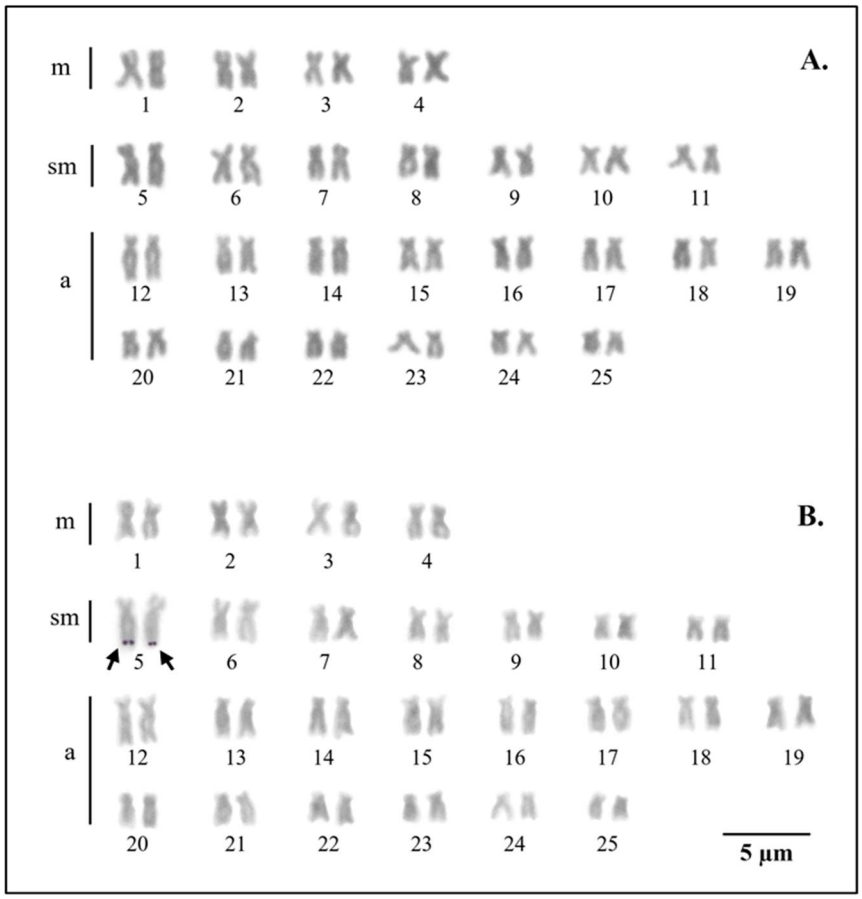

Figure 2. Karyotype of *D. albolineatus* from conventional staining (A.) and Ag-NOR banding techniques (B.), 2*n*=50, m=metacentric, sm=submetacentric and a=acrocentric chromosomes. The arrows indicate nucleolar organizer regions (NORs) on chromosome pair 5.

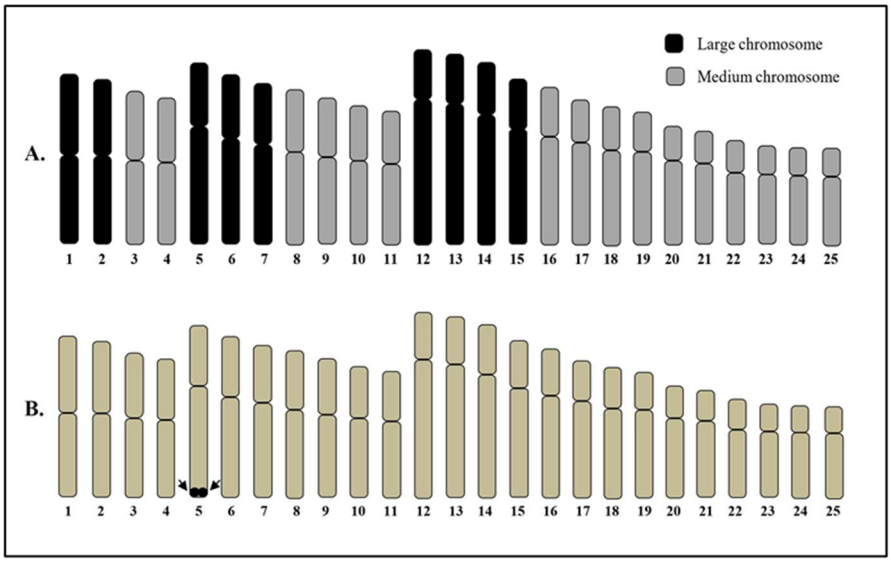

Figure 3. Idiogram of *D. albolineatus* from conventional staining (A.) and Ag-NOR banding techniques (B.) Arrows indicate NORs bearing.

Table 2. Karyomorphological details of D. albolineatus from 20 metaphases chromosome, 2n (diploid)=50.

Chro. pairs	Ls (μm)	Ll (μm)	LT (μm)	CI±SD	RL±SD	Chro. size	Chro. type
1	1.628	1.851	3.479	0.532±0.037	0.043±0.001	Large	metacentric
2	1.576	1.879	3.455	0.544±0.030	0.043±0.000	Large	metacentric
3	1.414	1.774	3.188	0.556±0.007	0.040±0.000	Medium	metacentric
4	1.302	1.694	2.996	0.565±0.022	0.037±0.001	Medium	metacentric
5	1.295	2.955	4.250	0.695±0.003	0.053±0.002	Large	submetacentric
6	1.196	2.496	3.692	0.676±0.025	0.046±0.000	Large	submetacentric
7	1.222	2.311	3.533	0.654±0.002	0.044±0.000	Large	submetacentric
8	1.150	2.089	3.239	0.645±0.006	0.040±0.001	Medium	submetacentric
9	1.049	1.940	2.990	0.649±0.013	0.037±0.000	Medium	submetacentric
10	0.988	1.645	2.634	0.625±0.012	0.033±0.001	Medium	submetacentric
11	0.963	1.603	2.566	0.625±0.044	0.032±0.001	Medium	submetacentric
12	1.035	3.356	4.391	0.764±0.009	0.055±0.003	Large	acrocentric
13	0.871	2.907	3.779	0.769±0.030	0.047±0.001	Large	acrocentric
14	0.887	2.746	3.632	0.756±0.010	0.045±0.001	Large	acrocentric
15	0.818	2.481	3.299	0.752±0.013	0.041±0.002	Large	acrocentric
16	0.845	2.367	3.212	0.737±0.014	0.040±0.002	Medium	acrocentric
17	0.852	2.433	3.284	0.741±0.006	0.041±0.000	Medium	acrocentric
18	0.784	2.227	3.010	0.740±0.004	0.037±0.001	Medium	acrocentric
19	0.775	2.286	3.061	0.747±0.016	0.038±0.001	Medium	acrocentric
20	0.788	2.002	2.789	0.718±0.010	0.035±0.001	Medium	acrocentric
21	0.708	2.202	2.910	0.757±0.007	0.036±0.000	Medium	acrocentric
22	0.685	2.143	2.828	0.758±0.013	0.035±0.001	Medium	acrocentric
23	0.684	1.924	2.608	0.738±0.004	0.032±0.000	Medium	acrocentric
24	0.709	1.756	2.465	0.712±0.004	0.031±0.000	Medium	acrocentric
25	0.655	1.706	2.361	0.723±0.017	0.029±0.001	Medium	acrocentric

Remarks: Ls=short arm chromosome, Ll=length of long arm chromosome, LT=length of total chromosomes, RL=relative length, CI=centromeric index, SD=standard deviation, Chro.=chromosome and *=NORs bearing chromosomes (satellite chromosome).

DISCUSSION

The karyotype and other chromosomal markers as revealed by Giemsa staining and Ag-NOR banding techniques were studied in *D. albolineatus*. This information is the first report for the following members of this species. Other species previously studied have a common karyotype consisting of 2*n* = 48-50 and NF=100 (all bi-arm chromosomes). The cytogenetic features reported here for the examined specimens of *D. albolineatus* revealed that the species has 2*n*=50 (all bi-arm chromosomes), which is shared by most of *Danio* species previously analyzed, such as chromosomes Post (1964); 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 and Reed (2000); and Sola and Gornung (2001) (Table 3). The karyotype in Figure 2 has been ordered numbering chromosomes according to their type and length, indicating the morphological classification based on the centromere position under the single

chromosome pair, as done by Chaiyasut (1988). The karyotype complements of *D. albolineatu* composed of 8m + 14sm + 28a chromosomes, there are a few differences in chromosome type for previous report (*D. rerio*) shown on Table 3.

The analysis of the NORs with the Ag-NOR banding technique detected a maximum of two Ag-positive signals in this species. In *D. albolineatus*, the Ag-positive signals are located along the long arm chromosome pair 5. The objective of the Ag-NOR band technique is to determine the nucleolar organizer region (NOR), which represents the location of genes (loci) that function in ribosome synthesis (Sharma et al., 2002). 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 we analyzed had a NOR site on a single chromosome pair in a subtelomeric position. This chromosome study has no heteromorphic pairs of chromosomes were observed in both sexes. It may be possible that the fish sex chromosomes are at the initiation of differentiation, and hence these chromosomes which contain the sex determination genes cannot be detected by cytogenetic analyses (Supiwong et al., 2013). Our results providing a rich source of information for comparative karyological analyses in *Danio* species and in related fish species.

Table 3. Karyological studies on *genus Danio.*

Species	2*n*	NF	Karyotype formula	References
Danio rerio	48	-	-	(Post, 1964)
	50	100	50m, sm, a	(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	(Sharma et al., 1998)
			M: 6m + 8sm + 36a	
	50	100	12m + 26sm + 12a	(Ueda and Naoi, 1999)
	50	100	4m + 30sm + 16a or*	(Amores and Postlethwait, 1999)
		100	4m + 20sm + 26a	
	50	100	4m + 16sm + 30a	(Phillips and Reed, 2000)
	50	100	4m + 16sm + 30a	9Sola and Gornung, 2001)
D. albolineatus	**50**	**100**	**8m + 14sm + 28a**	**This study**

Remarks: 2*n*=diploid chromosome, NF=fundamental number (arm of chromosome), m=metacentric, sm=submetacentric, a=acrocentric chromosomes and *****depending on the degree of chromosome condensation

CONCLUSION

This study reported the cytogenetics of *D. albolineatus*, obtained through classical techniques. The results showed that the diploid chromosome number was 2*n*=50, and the arm number was 100 in both male and female. No strange-sized chromosomes related to sex were observed. The analysis of nucleolus organizer regions (NORs) by silver staining has also revealed the existence of variable NORs, in addition to those on the appear terminal regions of the long arms of the chromosome pair 5. The karyotype formula of the species could be deduced as: $2n$ (50) = L^{m}_{4} + L^{sm}_{6} + L^{a}_{8} + M^{m}_{4} + M^{sm}_{8} + M^{a}_{20}.

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