Standardized Karyotype and Idiogram of White Eye Barb (*Cyclocheilichthys repasson*) (Cypriniformes, Cyprinidae) in Thailand by Conventional and Ag-NOR Staining Techniques

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ABSTRACT

The conventional and Ag-NOR banding techniques were performed in the white eye barb (*Cyclocheilichthys repasson*) from the Chao Phraya River at Ton Pho Subdictrict, Muang Singburi Dictrict, Sing Buri Province in the central of Thailand. The mitotic chromosome preparations were directly obtained from kidney tissues of eight males and eight females. The results showed that the diploid chromosome number of *C. repasson* was 2n=50, the fundamental number was 96 in both male and female. The karyotype of chromosomes consisted of six large metacentric, eight large submetacentric, two large acrocentric, six medium metacentric, 14 medium submetacentric, 10 medium acrocentric and four medium telocentric chromosomes. There are no irregularly sized chromosomes related to sex. The regions adjacent to the short arm near the telomere of metacentric chromosome pair 1, submetacentric chromosome pairs 9 and 12, and acrocentric chromosome pair 18 showed clearly observable nucleolar organizer regions (NORs). The karyotype formula for *C. repasson* is as follows:

$$2n \text{ (diploid) } 50 = L^{m}_{6} + L^{sm}_{8} + L^{a}_{2} + M^{m}_{6} + M^{sm}_{14} + M^{a}_{10} + M^{t}_{4} \text{ or } 12m + 22sm + 12a + 4t$$

Keywords: *Cyclocheilichthys repasson*, Chromosome, Ag-NOR staining

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Introduction

An important characteristic of nucleolar organizer regions (NORs) in fish is its' inter-and intraspecies polymorphism. NOR characterization can be a cytogenetic marker for cytotaxonomic studies and can even aid in constructing phylogenetic hypotheses (cytosystematics) for several fish groups [1-3]. Some fish groups present a simple NOR system characterized by ribosomal cistrons on only one chromosome pair, whereas others have a multiple NOR system which compose of cistrons dispersed over several chromosomes [2]. Family Cyprinidae is the most abundant and globally widespread family of freshwater fish, comprising 3000, livings and extinct species in about 370 genera [4]. There are many genera and species of the family Cyprinidae in South-East Asia, which the systematic positions are vague. Therefore, comparative karyology has become a useful tool in fish systematic studies [5-7].

The genus *Cyclocheilichthys* has eight species including *C. apogon*, *C. armatus*, *C. enoplos*, *C. furcatus*, *C. heteronema*, *C. lagleri*, *C. microlepis* and *C. repasson* [8]. The characteristic of the genus *Cyclocheilichthys* is the extension of dorsal scales to the head, dorsal scales reach the level of the orbit. Moreover, there are rows of parallel pores, which are well underlined by small black dots, clearly visible under the eye. The white eye barb (*C. repasson*) is very similar to *C. lagleri,* but clearly distinguished by the four barbels [8]. *C. repasson* wildly distribute in freshwater of the Mekong basin in Laos, Thailand, Cambodia and Vietnam; Chao Phraya basin and Sundaland. One of most common species of the genus in the Mekong. This species, like other small members of the genus, moves out into the flooded forest during the high-water season. Little is known about the precise timing of its movement. Its diet consists primarily of insects with some aquatic macrophytes.

Although chromosome numbers of most species in the *Cyclocheilichthys* have been described [7, 9-15]. Chromosome analyses of *Cyclocheilichthys* species have shown the diploid chromosome number of 2n=50 (Table 1). The information on diploid chromosome numbers (2n), fundamental number (NF), size and type of chromosomes which have been obtained from cytogenetic studies have aided in the understanding of evolutionary mechanisms in the groups investigated [9-10]. These studies are also important steps towards the establishment of genetic improvement techniques involved in chromosome manipulations and chromosome determinations, such as polyploidy induction, gynogenesis and androgenesis, sex control and inter- and intra-specific hybridizations [11-12]. These genetic techniques have been widely applied to improve farmed stocks in many aquaculture species in the world [14-16]. However, there was quite scarce of the study on chromosomal banding especially Ag-NOR banding in this genus.

Accordingly, the present study is the first cytogenetic study on *C. repasson* accomplished with the Ag-NOR staining technique. Our results provide new cytogenetic information for further study on taxonomy and evolutionary relationship. Moreover, we provide basic and useful information for the conservation, breeding and chromosome evolution study of this fish.

Materials and methods

Sample collection

The *C. repasson* (five males and five females) (Figure 1) samples were obtained from the Chao Phraya River at Ton Pho Subdictrict, Muang Singburi Dictrict, Sing Buri Province in the central part of Thailand. The fish were transferred to laboratory aquaria and kept under standard condition for seven days prior to the experiments. Species was identified morphologically using the keys and description of Rainboth [8].

Chromosome preparation

Chromosomes were directly prepared *in vivo* as follows by Supiwong *et al.* [17-18]. The 0.05% colchicine (1 mL per 100 g body weight) was injected into the intramuscular or abdominal cavity of fish and left for 1 h before sacrificing. The kidney tissues were cut into small pieces then squashed, mixed with hypotonic solution (0.075 M KCl) and incubated for 30 min. The cell suspension was centrifuged at 1200 rpm for 8 min and cells were fixed in fresh and cold Carnoy's fixative (3 methanol: 1 acetic acid) about 2–3 times. 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 glass slide by micropipette followed by an air-drying technique.

Chromosome staining

Conventional staining was performed using 20% Giemsa's solution for 30 min, and Ag-NOR banding [19] was carried out by adding four drops of 50% silver nitrate and two drops of 2% gelatin on a slide. The slides were then sealed with cover glasses and incubated at 60°C for 5 min. After that they were soaked in distilled water until the cover glasses were separated. The slides were stained with 20% Giemsa's solution for 1 min.

Karyotyping and idiograming

Twenty clearly observable cells with well spread chromosomes of each male and female were selected and photographed. The length of the short arm chromosome (Ls) and the long arm chromosome (Ll) were measured and the length of the total arm chromosome (LT, LT = Ls + Ll) was calculated. The relative length (RL), the centromeric index (CI), and standard deviation (SD) of RL and CI were estimated [20]. The CI (q/p +q) between 0.50 and 0.59, 0.60–0.69, 0.70–0.89, and 0.90–0.99 are described as metacentric, submetacentric, acrocentric and telocentric chromosomes, respectively. The fundamental number (NF, number of chromosome arms) is obtained by assigning a value of 2 for the metacentric, submetacentric and acrocentric chromosomes and 1 for the telocentric chromosome. All data were used in karyotyping and idiograming.

Results and discussion

Diploid chromosome number, fundamental number and karyotype

Karyological analysis of the *C. repasson* using kidney tissues revealed that the chromosome number is 2*n* (diploid)=50 in both males and females. This is the same chromosome number of the *Cyclocheilichthys* as reported in previous studies (Table 1) [7, 21-28]. Among subfamily Cyprininae in Thailand, the majority of species have chromosome 2n=50, which is an apparent modal diploid number. The diploid number, 2n=50 would probably be the

ancestral diploid number of the subfamily because the characteristic that frequently occurs in a group can be considered as ancestral [29]. The fundamental number (NF) of *C. repasson* is 96 in both male and female, which is different from the report of Donsakul *et al.* [24] who detected the NF was78. This may be caused by the interpopulation variation in this species. This phenomenon can be found in other *Cyclocheilichthys* [7, 21-24, 26, 28] and other cyprinids such as *Puntioplites proctozysron* [30-31]. The NF is specific to morphology can be found in the butterfly fishes. Arai and Inoue [33] and Supiwong *et al.* [32] revealed that the NFs in the family Chaetodontidae can classify in to two groups such as NF=48 and NF>48. These characters are specific to the number of anal fin spines. The NF=48 group has three anal fin spines whereas the NF>48 one has four anal fin spines. The *C. repasson*'s karyotype consisted of six large metacentric, eight large submetacentric, two large acrocentric, six medium metacentric, 14 medium submetacentric, 10 medium acrocentric and four medium telocentric chromosomes (Table 2 and Figures 2-3). The present study is the first analysis of the sizes of the chromosomes in this species. To consider only the types of the chromosomes, the obtained result showed that the types of chromosomes composed of 12 metacentric, 22 submetacentric, 12 acrocentric and four telocentric chromosomes. It is inconsistent with the report of Donsakul *et al*. [24], which revealed that the *C. repasson* has the following chromosomes: 12 metacentric, 16 submetacentric, six acrocentric and 16 telocentric chromosomes. In addition, it is different from report of Seetapan [26], who found that *C. repasson* 's karyotype comprised eight metacentric, 10 submetacentric, 12 acrocentric and 20 telocentric chromosomes. The reasons of these variations may be caused from the intraspecific variations among distinct populations, and/or the chromosome rearrangements during chromosomal evolution through the pericentric inversions. No cytologically distinguishable sex chromosome was observed, similar to other species of the genus *Cyclocheilichthys* [7, 21-28]. It may be possible that the fish's sex-chromosomes are at the initiation of differentiation. Therefore, we are unable to find out which of those chromosomes contain the sex-determination gene through cytogenetic studies [34]. The origin and development of sex-chromosomes have been reported on Neotropical fish in Brazil [35]. The chromosomes characteristics of other *Cyclocheilichthys* species (*C. apogon, C. enoplos, C. lagleri* and *C. repasson*) have been only reported in Thailand. Thus, the chromosome evolution of this group is still incomplete savvy.

***Chromosome marker of* C. repasson**

The present study provides the first cytogenetic report of *C. repasson*, accomplished using the Ag-NOR staining technique. The technique is to identify nucleolar organizer regions (NORs) representing the location of genes (loci) that have function in ribosome synthesis (18S and 28S ribosomal RNA). NORs produce numerous gene expressions and contain more nonhistone protein than other chromosome regions. Accordingly, the specific dark band is induced by the reduction of organic silver by these proteins that change silver to be dark [36]. *C. repasson* has four pairs of NORs, the regions adjacent to the telomere of the short arm of metacentric chromosome pair 1, submetacentric chromosome pairs 9 and 12, and acrocentric chromosome pair 18 showed clearly observable nucleolar organizer regions (Figures 3 and 4). *C. repasson* have multiple NORs compared to other species in the genus *Cyclocheilichthys* (e.g. *C. amatus*, *C. apogon*, *C. enoplos*, *C. lagleri*). From the results, this fish is considered to be advance characters of NORs because Gold and Amemiya [37] proposed that fish having multiple NORs is considered to be apomorphic or derived condition whereas single pair of NORs is considered to be plesiomorphic or a primitive condition. The important

chromosome marker of the *C. repasson* is the asymmetrical karyotype that was found for four types of chromosomes regarding the centromere position. The idiogram shows a continuous length gradation of chromosomes. The largest and smallest chromosomes show approximately two-fold size differences. The data of the chromosome measurement on mitotic metaphase cells are shown in Table 2. Idiogram by conventional staining and Ag-NOR banding is shown in Figure 4. Furthermore, the information of chromosome morphology and the position as well as number of NORs in *C. repasson* suggested that these cytotaxonomic markers could be utilized in contribute toward clarifying the karyotypic evolution and phylogenetic relationships in this group.

Acknowledgements

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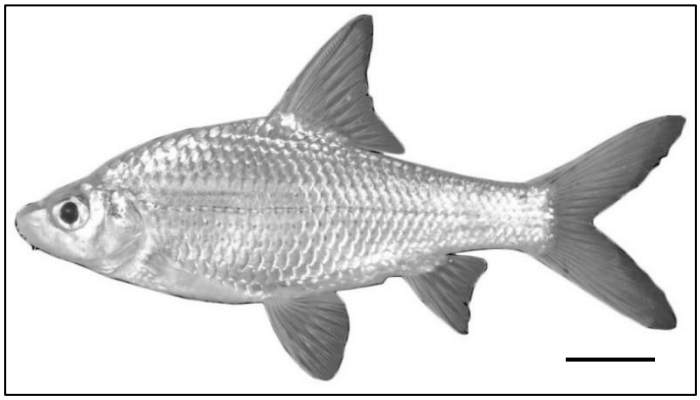

Figure 1 General characteristics of the white eye barb (*Cyclocheilichthys repasson*). Scale bar indicates 1cm.

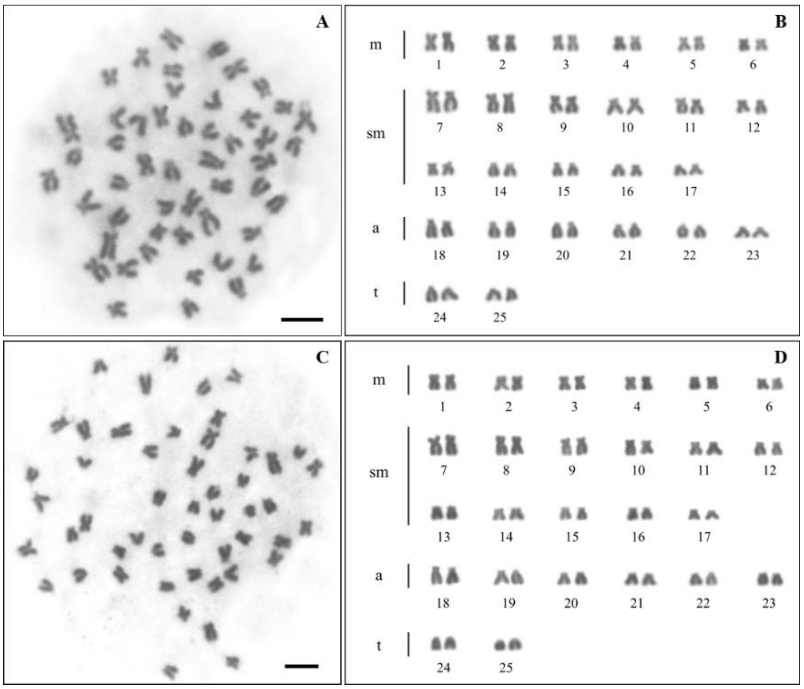

Figure 2 Metaphase chromosome plates and karyotypes of the male (A-B) and female (C-D) white eye barb (*Cyclocheilichthys repasson*) by conventional staining technique. Scale bars indicates 5 µm.

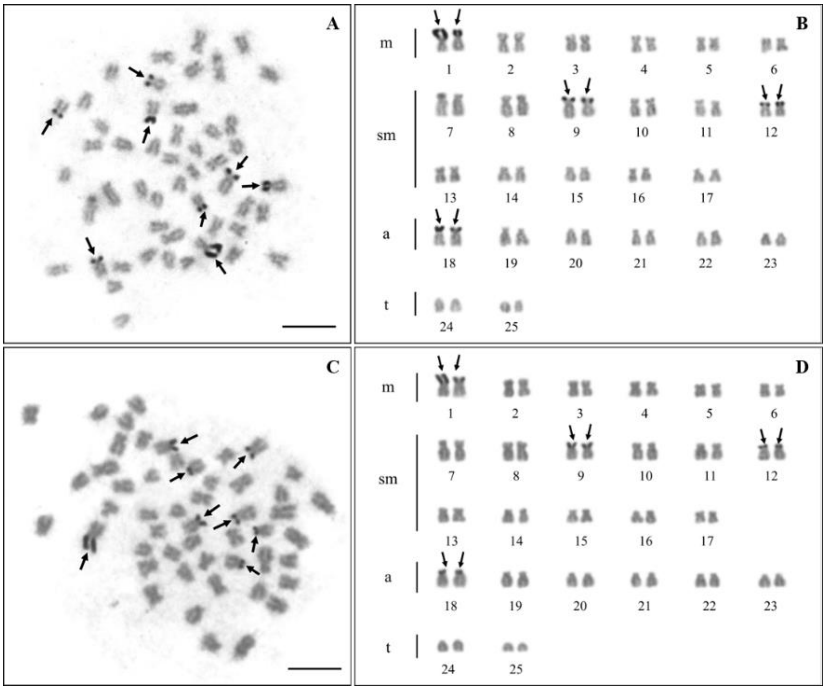

Figure 3 Metaphase chromosome plates and karyotypes of the male (A-B) and female (C-D) white eye barb (*Cyclocheilichthys repasson*) by Ag-NOR banding technique. Arrows indicate nucleolar organizer regions (NORs). Scale bars indicate 5 µm.

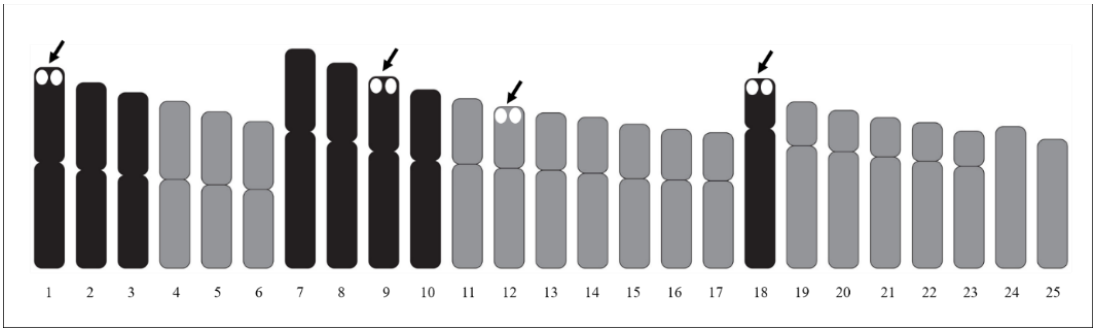

Figure 4 Idiogram of the white eye barb (*Cyclocheilichthys repasson*), representing the haploid set (n=25) by conventional staining and Ag-NOR banding techniques. Arrows indicates nucleolar organizer regions (NORs). (each color bar indicates the sizes of chromosomes, black = large (L) and gray = medium (M).

Table 1 Review previous studies on cytogenetics of the genus *Cyclocheilichthys* in Thailand.

Species	2*n*	NF	Karyotype formula	NOR	References
Cyclocheilichthys amatus	50	94	12m+18sm+14a+6t	S(TR)4	[27]
Cyclocheilichthys apogon	50	70	12m+8sm+6a+24t	–	[21]
	50	76	18m+8sm+4a+20t	–	[23]
	50	86	10m+16sm+10a+14t	2	[28]
Cyclocheilichthys enoplos	50	90	10m+30sm+4a+6t	4	[7]
	50	72	14m+8sm+10a+18t	–	[22]
Cyclocheilichthys lagleri	50	86	24m+12sm+2a+12t	–	[25]
Cyclocheilichthys repasson	50	-	8m+10sm+12a+20t	–	[26]
	50	78	12m+16sm+6a+16t	–	[24]
	50	**96**	**12m + 22sm + 12a + 4t**	**S(TR)8**	**Present study**

Remark: m =metacentric, sm = submetacentric, a = acrocentric, t = telocentric, S = short arm, TR = telomeric region, and –= not available.

Table 2 Means of the short arm length (Ls), long arm length (Ll) and total arm length of chromosomes (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI of 20 metaphase cells of the male and female white eye barb (*Cyclocheilichthys repasson)*, 2*n*=50.

Chromosome pairs	Ls (μm)	Ll (μm)	LT (μm)	RL ± SD	CI ± SD	Chromosome size	Chromosome type
1*	1.148	1.276	2.424	0.049±0.004	0.527±0.010	L	Metacentric
2	1.057	1.184	2.241	0.045±0.002	0.529±0.011	L	Metacentric
3	0.998	1.123	2.121	0.043±0.002	0.529±0.010	L	Metacentric
4	0.945	1.071	2.017	0.041±0.002	0.532±0.012	M	Metacentric
5	0.882	1.007	1.890	0.038±0.001	0.534±0.009	M	Metacentric
6	0.820	0.951	1.770	0.036±0.001	0.537±0.013	M	Metacentric
7	0.999	1.649	2.648	0.053±0.002	0.623±0.021	L	Submetacentric
8	0.948	1.530	2.478	0.050±0.003	0.617±0.018	L	Submetacentric
9*	0.903	1.411	2.315	0.047±0.002	0.610±0.013	L	Submetacentric
10	0.858	1.298	2.156	0.043±0.002	0.602±0.015	L	Submetacentric
11	0.791	1.257	2.048	0.041±0.002	0.615±0.021	M	Submetacentric
12*	0.743	1.208	1.951	0.039±0.001	0.620±0.019	M	Submetacentric
13	0.693	1.183	1.876	0.038±0.001	0.630±0.023	M	Submetacentric
14	0.677	1.146	1.824	0.037±0.001	0.629±0.018	M	Submetacentric
15	0.658	1.083	1.741	0.035±0.001	0.622±0.019	M	Submetacentric
16	0.614	1.063	1.678	0.034±0.002	0.634±0.018	M	Submetacentric
17	0.580	1.056	1.636	0.033±0.004	0.644±0.027	M	Submetacentric
18*	0.605	1.685	2.290	0.046±0.002	0.736±0.029	L	Acrocentric
19	0.531	1.478	2.010	0.041±0.001	0.736±0.023	M	Acrocentric
20	0.499	1.408	1.907	0.039±0.001	0.739±0.025	M	Acrocentric
21	0.475	1.344	1.818	0.037±0.001	0.740±0.022	M	Acrocentric
22	0.464	1.294	1.758	0.035±0.001	0.736±0.016	M	Acrocentric
23	0.426	1.231	1.657	0.034±0.001	0.742±0.019	M	Acrocentric
24	0.000	1.712	1.712	0.035±0.002	1.000±0.000	M	Telocentric
25	0.000	1.557	1.557	0.031±0.002	1.000±0.000	M	Telocentric

Remarks: * = NOR-bearing chromosomes, L = large, M = medium