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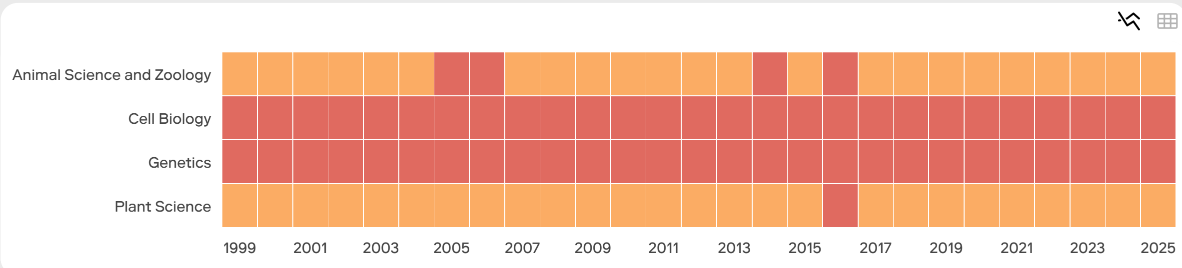

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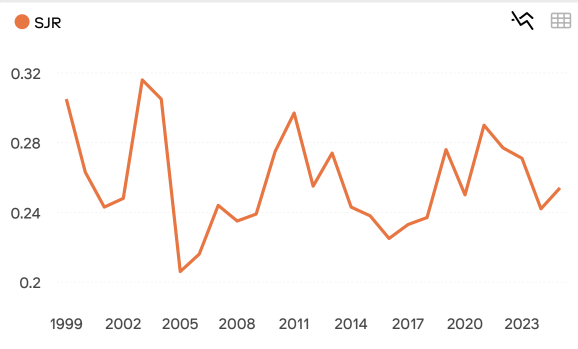

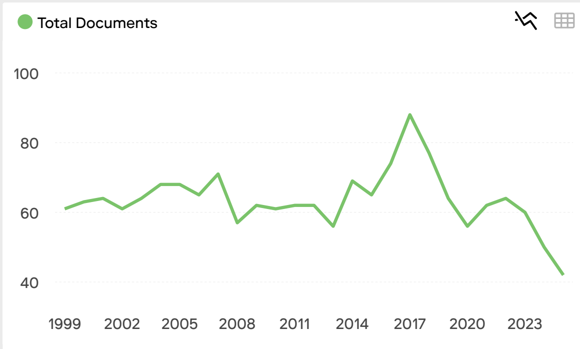

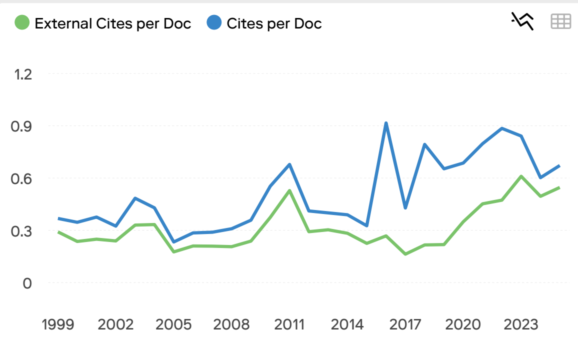

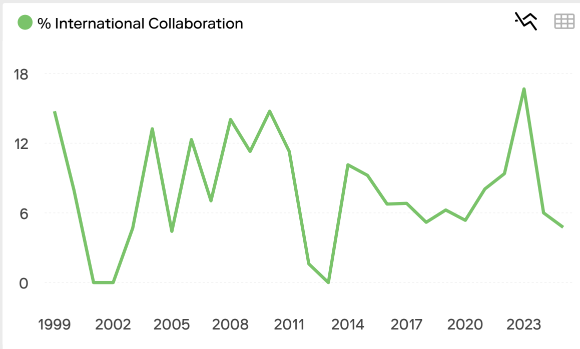

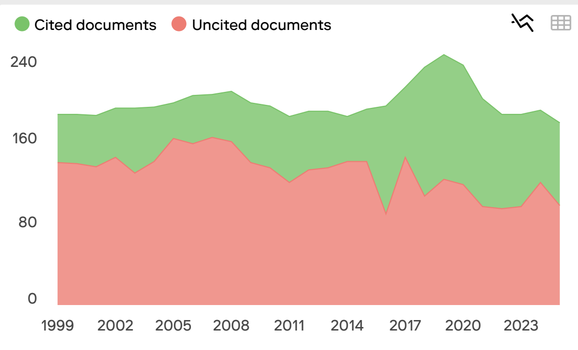

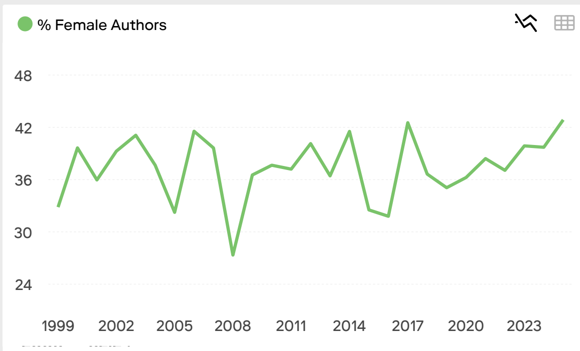

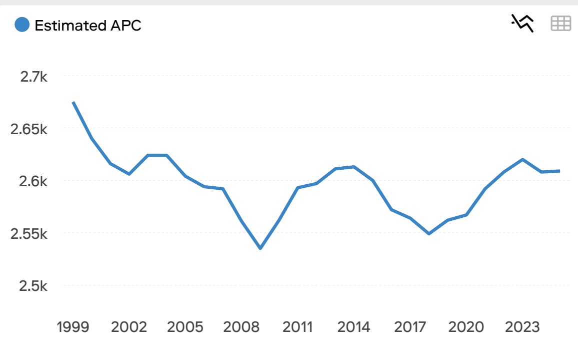

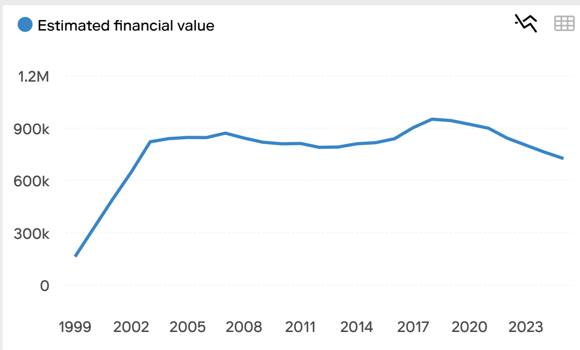

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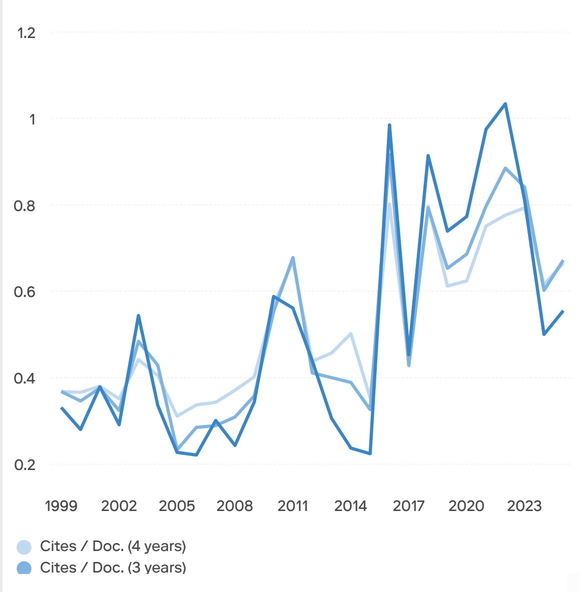

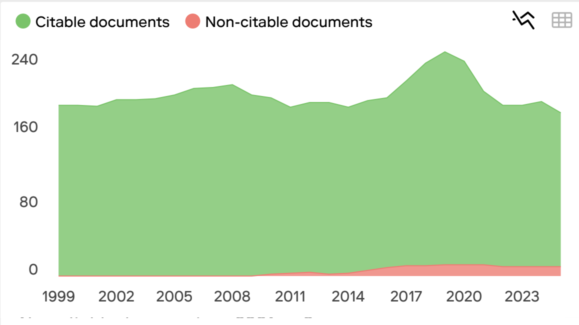

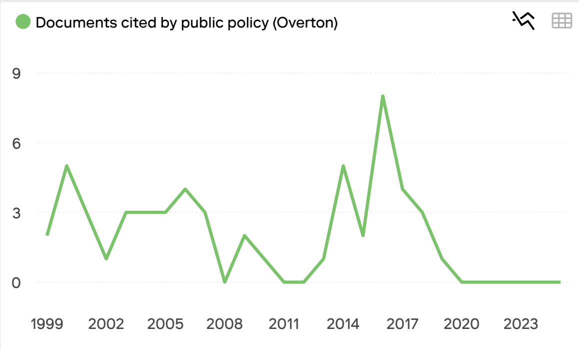

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Karyological investigation of three Boraras species (Danionidae, Rasborinae) from Thailand

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Manuscript Number:	CYTOLOGIA-D-25-00051R2
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Full Title:	Karyological investigation of three Boraras species (Danionidae, Rasborinae) from Thailand
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First Author:	Sumalee Phimphan
Order of Authors:	Sumalee Phimphan
	Surachest Aiumsumang
	Intira Komphan
	Natnapha Buking
Corresponding Author:	Sumalee Phimphan Phetchabun Rajabhat University Phetchabun, central THAILAND
Abstract:	Summary This research aimed to examine karyotypes of three Boraras species, including Boraras maculatus, B. naevus, and B. urophthalmoides from Thailand. Chromosome preparations for karyotype analysis were obtained from renal tissues. Conventional staining technique was applied to stain the chromosomes with Giemsa's solution. The results of this study revealed that all Boraras under study possessed the same diploid chromosome number of 50. Their karyotypes were as follows; Boraras maculatus had 3 metacentric (m) + 21 submetacentric (sm) + 1 acrocentric (a) pairs with the arm number (NF) of 100. B. naevus possessed 5m + 17sm + 3a pairs and NF=100, and B. urophthalmoides had 6m + 17sm + 2a pairs and NF=100.
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Regular Article

Karyological investigation of three *Boraras* species (Danionidae, Rasborinae) from Thailand

Surachest Aiumsumang[1], Intira Komphan[1],

Natnapha Buking[1], and Sumalee Phimphan[1*]

[1] Biology Program, Faculty of Science and Technology, Phetchabun Rajabhat University, Phetchabun 67000, Thailand

*Corresponding author, e-mail: sumalee.phi@pcru.ac.th

Numbers of tables (4), grayscale figures (2), and color figures (1)

Short title: Karyological investigation of three *Boraras*

Summary This research aimed to examine karyotypes of three *Boraras* species, including *Boraras maculatus*, *B. naevus*, and *B. urophthalmoides* from Thailand. Chromosome preparations for karyotype analysis were obtained from renal tissues. Conventional staining technique was applied to stain the chromosomes with Giemsa’ s solution. The results of this study revealed that all *Boraras* under study possessed the same diploid chromosome number of 50. Their karyotypes were as follows; *Boraras maculatus* had 3 metacentric (m) + 21 submetacentric (sm) + 1 acrocentric (a) pairs with the arm number (NF) of 100. *B. naevus* possessed 5m + 17sm + 3a pairs and NF=100, and *B. urophthalmoides* had 6m + 17sm + 2a pairs and NF=100.

Keywords Karyotype, *Boraras maculatus*, *Boraras naevus*, *Boraras urophthalmoides*

The genus name *Boraras* is an anagram of Rasbora, a reference to the inverse ratio between the fin rays of the ventral and caudal fins of these species. The *Boraras* genus is geographically restricted to Southeast Asia, primarily in the freshwater peat swamp habitats of islands like Borneo, Sumatra, and Malaysia, as well as parts of Thailand. In contrast, the larger *Rasbora* genus has a broader distribution across Southeast Asia and southern China, though specific species like the harlequin rasbora also have overlapping ranges with Boraras in some countries like Malaysia, Singapore, and Indonesia (Stout *et. al*., 2016). *Boraras* currently includes six species, most of which were formerly placed in *Rasbora* Bleeker: *B. brigittae* (Vogt, 1978), *B. maculatus* (Duncker, 1904), *B. merah* (Kottelat, 1991), *B. micros* (Kottelat and Vidthayanon, 1993), *B. urophthalmoides* (Kottelat, 1991) and *B. naevus* (Conway and Kottelat, 2011). Several phylogenetic investigations have recovered *Boraras* as amonophyletic group and suggest that the genus *Trigonopoma* Liao, Kullander and Fang (=*R. pauciperforata*-group of Kottelat and Vidthayanon, 1993) may represent the sister group to *Boraras* (Conway, 2005; Liao *et al*., 2010; Tang *et al*., 2010). Members of the cyprinid genus *Boraras* are small, brightly colored fishes that inhabit swamps and slow-flowing streams throughout much of Southeast Asia (Kottelat and Vidthayanon, 1993). Reaching maximum adult sizes less than 20 mm in standard length, all members of *Boraras* are considered miniature fishes (Weitzman and Vari, 1988) and exhibit a number of reductive characteristics, including the absence of the body lateral line, reduced cephalic lateral line system, low numbers of scales, branched fin rays, gill rakers and pharyngeal tooth rows (Kottelat and Vidthayanon, 1993), and the complete absence of a number of skeletal elements (Conway, 2005; Britz and Conway, 2009).

Karyotype studies have played a crucial role in understanding the evolutionary history and diversity of fish species. Indeed, fish are excellent models for cytogenetic studies because they exhibit diverse karyotypes, including diploid and polyploid genomes, in addition to sex chromosomes, which offer unique insights into chromosome structure and behavior (Cioffi and Bertollo, 2012). They are used to identify genetic disorders and can be helpful in understanding evolutionary relationships between species. Nowadays, several methods namely, conventional staining, C-banding, Ag-NOR banding and FISH (fluorescence *in situ* hybridization) have been used by ichthyologists for the gathering of cytogenetic information of fish (Sola *et al.*, 2000; Kavaco *et al.*, 2005), yet each of these methods provides a different aspect of the karyotype characteristics. Further, karyotypic information can throw light on phylogenetic relationship between different species and karyotype evolution in fish species . In view of the above, cytogenetic data for *Boraras* are scarce. In the present research, karyotype of *Boraras* species typically involves a diploid chromosome number of 2n=50, with no observed differences in sex chromosomes between males and females. The fundamental number (NF), which represents the total number of chromosome arms, varies. For example, *B. maculatus* has a NF of 92, while *B. urophthalmoides* has a NF of 94. The karyotype formula describes the number and types of chromosomes. For instance, *B. maculatus* has a karyotype formula of 2n (50) = 6m+15sm+2a+2t, while *B. urophthalmoides* is 2n (50) = 9m+13sm+1a+2t (Donsakul *et al.*, 2009).

In the present study, we explore the cytogenetic report of three *Boraras,* including *B. maculatus*, *B. naevus*, and *B. urophthalmoides* .This study is the first chromosome record of *B. naevus* .In the future, basic knowledge and cytogenetics of three *Boraras* species could be applied to several studies, especially for their cytotaxonomy.

Materials and methods

Preparation of chromosome spreads

B. maculatus, *B. naevus* and *B. urophthalmoides* individuals (5 males and 5 females) were collected from the Pa Sak River, Thailand (Figure 1). This research was conducted under animal use permission of scientific purposes code U1-05662-2559. The chromosomes were prepared *in vivo* (Aiumsumang *et al*., 2021), as follows, Colchicine was injected into the fish's intramuscular and/or its abdominal cavity and then left for 2 hours. Kidney, and/or gills were cut into small pieces then squash mixed with 0.075M KCl. After discarding all large pieces tissues, 8 ml of cell sediments were transferred to a centrifuge tube and incubated for 25-35 minutes. The KCl was discarded from the supernatant after centrifugation at 1,200 rpm for 8 minutes. Cells were fixed in fresh cool fixative (3 methanol: 1 glacial acetic acid) 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 using the conventional air-drying method. Conventional staining was done using 10% Giemsa's solution for 10 min (Rooney, 2001). Metaphases were photographed under Olympus Bx50 microscope (Olympus Corporation, Ishikawa, Japan). Chromosomes photographs of 100 metaphase spreads were taken from each species by conventional Giemsa's staining and the best 20 were used for karyological examination. 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 by software adobe Photoshop. The relative length (RL) and centromeric index (CI) were estimated. CI was also computed to classify the types of chromosomes according to Chaiyasut (1989). The fundamental numbers (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 data were used in karyotyping and idiograming.

Results and discussion

All studied species share the same diploid chromosome number as 2*n*=50 and fundamental number (NF)=100, but there are differences in karyotypes. The diploid number of *B. maculatus* and *B. urophthalmoides* were in concordance with previous cytogenetics data (Donsakul *et al.*, 2009), and similar to another species in *Rasbora* (Post, 1965; Khuda-Bukhsh, 1979; Donsakul and Magtoon, 1995; Manna and Khuda-Bukhsh, 1977; Donsakul and Magtoon, 2002; Seetapan and Moeikum, 2004; Donsakul *et al.*, 2005; Donsakul *et al.*, 2009; Yeesaem *et al.*, 2019; Aiumsumang *et al.*, 2021; 2022a; 2022b) .These species have the chromosome diploid number of 2*n*=50, which is the apparent modal diploid number of the *Rasbora* . Accordingly, it can be concluded that chromosome number in this genus is conserved. However, it differs from some species of previous report in the *R. heteromorpha* (Post, 1965; Donsakul *et al.*, 2005) and *R .trilineata* (Post, 1965) which was 2*n*=48.

The karyotypic analysis revealed that the chromosome complement of *B. maculatus* consisting of 3 metacentric (m) + 21 submetacentric (sm) + 1 acrocentric (a) chromosomes (Figure 2A). The mean values calculated from twenty mitotic metaphases showed the centromeric index of chromosome complements ranging from 0.581±0.094 to 0.710±0.064 (Table 2). The karyotype formula of *B. maculatus* could be deduced as : 2n(50) = 6m+42sm+2a. The karyotype of *B. urophthalmoides* was composed of 6m + 17sm + 2a chromosomes (Figure 2B). The mean values calculated from twenty mitotic metaphases showed the centromeric index of chromosome complements ranging from 0.528±0.028 to 0.755±0.029 (Table 3). The karyotype formula of *B.*

urophthalmoides could be deduced as : 2n(50) = 12m + 34sm + 4a. The type chromosomes and NF of two species, including *B. maculatus* and *B. urophthalmoides* herein differ from those previously reported (Donsakul *et al.*, 2009). These differences may be due to inter- populational variation in this species. This difference may be on account of inter-population variation emnating from pericentric inversions. Comparison of the present results and previous reports (Table 1). This is the first report on *B. naevus* showed 5m + 17sm + 3a chromosomes (Figure 2C). The present investigation in this fish species revealed that the mean value of centromeric index ranged from 0.543±0.043 to 0.702±0.003 (Table 4). The suggested karyotype of this species was 2n (50) = 10m + 34sm + 6a. The NFs of the genus *Boraras* range from 92 to 94 and karyotypes are composed of both mono- and bi-arms chromosome. Nirchio *et al*., (2014) proposed that species with high NF is advanced state or apomorphic character whereas one with low NF is a primitive state or plesiomorphic character. Thus, the *Boraras* seems to be advanced karyotype. The differences in chromosome numbers among the different taxa are not the result of hybridization in regions where there is geographic overlap between them. This chromosomal number is also seen in diploid members of other cyprinid lineages, in addition to diploid Danionidae fish. Indeed, 2n = 50 appears to be preserved in various Danionidae fish lineages. However, such conserved 2n is evidently associated with extensive intrachromosomal variations, which stress the role of structural rearrangements, such as pericentric inversions, chromatin additions/deletions, transpositions, and non-Robertsonian translocations as, e.g., demonstrated byin other cyprinoid lineage, chondrostomine species. (Khensuwan, et al., 2023).

The ideogram (Figure 4) shows a continuous length gradation of chromosomes. The size differences between the largest and smallest chromosomes show approximately two-fold. The data of the chromosome measurement on mitotic metaphase cells (from all specimens) are shown in Table 2, 3 and 4.

In summary, the present cytogenetic procedure results provide basic information of the karyotypic features in three yet understudied *Borarus* species. Each species can be clearly distinguished by cytogenetic analysis. These results also provide important data for systematics within this family. Further studies, other species comprising a high number of species in the *Borarus* should be studied additionally by both cytogenetic and molecular hybridization procedure techniques to provide a better understanding about the chromosomal evolution and to characterize biodiversity.

Author contributions

Surachest Aiumsumang, Intira Komphan, Natnapha Buking, and Sumalee Phimphan provided the samples and identified them. Surachest Aiumsumang planned the study. Surachest Aiumsumang, Intira Komphan, Natnapha Buking conducted cytogenetic studies. Sumalee Phimphan analyzed the data and wrote the manuscript. All authors approved the final manuscript.

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Table 1 Cytogenetic publications of the genus *Boraras*.

Species	2*n*	*n*	NF	Karyotype formula	References
Boraras maculatus	50	25	92	6m+15sm+2a+2t	Donsakul *et al.* (2009)
	50	25	100	3m+21sm+1a	This study
B. naevus	50	25	100	5m + 17sm + 3a	This study
B. urophthalmoides	50	25	94	9m+13sm+1a+2t	Donsakul *et al.* (2009)
	50	25	100	6m+17sm+2a	This study

Remarks :2n=diploid chromosome, 2=haploid chromosome NF=fundamental number m, sm, a =2, t=1, m=metacentric, sm=submetacentric, a =acrocentric and t=telocentric chromosome.

Table 2. Karyomorphological details of *B. maculatus* from 20 metaphases, *n* (haploid)=25.

Chromosome pairs	Ls (μm)	Ll (μm)	LT (μm)	CI±SD	RL±SD	Chromosome size	Chromosome type
1	0.953	1.353	2.306	0.587±0.033	0.057±0.005	Large	metacentric
2	0.722	1.351	2.072	0.652±0.035	0.051±0.004	Large	submetacentric
3	0.729	1.315	2.044	0.643±0.045	0.051±0.003	Large	submetacentric
4	0.639	1.249	1.888	0.662±0.100	0.047±0.003	Large	submetacentric
5	0.641	1.196	1.838	0.651±0.034	0.045±0.002	Large	submetacentric
6	0.608	1.189	1.798	0.661±0.053	0.044±0.002	Large	submetacentric
7	0.630	1.127	1.757	0.641±0.027	0.043±0.002	Large	submetacentric
8	0.580	1.140	1.720	0.663±0.044	0.042±0.002	Medium	submetacentric
9	0.588	1.095	1.683	0.651±0.044	0.042±0.003	Medium	submetacentric
10	0.616	1.043	1.659	0.629±0.038	0.041±0.002	Medium	submetacentric
11	0.556	1.049	1.605	0.654±0.027	0.040±0.002	Medium	submetacentric
12	0.521	1.071	1.591	0.703±0.064	0.039±0.002	Medium	acrocentric
13	0.597	0.972	1.569	0.620±0.025	0.039±0.002	Medium	submetacentric
14	0.499	1.063	1.562	0.681±0.094	0.039±0.005	Medium	metacentric
15	0.536	0.997	1.533	0.650±0.049	0.038±0.003	Medium	submetacentric
16	0.531	0.999	1.531	0.653±0.075	0.038±0.003	Medium	submetacentric
17	0.578	0.943	1.520	0.620±0.040	0.038±0.003	Medium	submetacentric
18	0.537	0.936	1.473	0.635±0.036	0.036±0.001	Medium	submetacentric
19	0.540	0.917	1.457	0.629±0.037	0.036±0.003	Medium	submetacentric
20	0.511	0.921	1.432	0.643±0.059	0.035±0.003	Medium	submetacentric
21	0.500	0.848	1.348	0.629±0.042	0.033±0.002	Medium	submetacentric
22	0.499	0.844	1.342	0.629±0.038	0.033±0.004	Medium	submetacentric
23	0.532	0.779	1.311	0.594±0.029	0.032±0.003	Medium	metacentric
24	0.502	0.787	1.289	0.611±0.049	0.032±0.002	Medium	submetacentric
25	0.446	0.701	1.147	0.611±0.080	0.028±0.002	Small	submetacentric

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.

Table 3. Karyomorphological details of *B. naevus* from 20 metaphases, *n* (haploid)=25.

Chromosome pairs	Ls (µm)	Ll (µm)	LT (µm)	CI±SD	RL±SD	Chromosome size	Chromosome type
1	0.697	1.276	1.973	0.647±0.006	0.054±0.001	Large	submetacentric
2	0.574	1.251	1.825	0.685±0.007	0.050±0.002	Large	submetacentric
3	0.600	1.160	1.760	0.659±0.011	0.048±0.001	Large	submetacentric
4	0.615	0.937	1.552	0.604±0.028	0.042±0.002	Large	submetacentric
5	0.612	0.956	1.568	0.610±0.012	0.043±0.001	Large	submetacentric
6	0.488	1.150	1.637	0.702±0.003	0.044±0.000	Large	acrocentric
7	0.491	0.957	1.448	0.701±0.008	0.039±0.002	Medium	acrocentric
8	0.560	0.940	1.500	0.627±0.028	0.041±0.000	Medium	submetacentric
9	0.727	0.864	1.591	0.543±0.043	0.043±0.001	Medium	metacentric
10	0.630	0.998	1.628	0.573±0.035	0.044±0.003	Medium	metacentric
11	0.538	0.929	1.467	0.634±0.027	0.040±0.001	Medium	submetacentric
12	0.471	1.054	1.524	0.701±0.015	0.041±0.002	Medium	acrocentric
13	0.583	0.805	1.388	0.580±0.006	0.038±0.000	Medium	metacentric
14	0.485	0.891	1.376	0.648±0.002	0.037±0.001	Medium	submetacentric
15	0.557	0.995	1.551	0.641±0.035	0.042±0.002	Medium	submetacentric
16	0.484	0.895	1.379	0.649±0.005	0.037±0.001	Medium	submetacentric
17	0.542	0.877	1.419	0.618±0.036	0.039±0.001	Medium	submetacentric
18	0.538	0.871	1.409	0.618±0.025	0.038±0.004	Medium	submetacentric
19	0.401	0.876	1.277	0.686±0.013	0.035±0.001	Medium	submetacentric
20	0.528	0.812	1.340	0.606±0.011	0.036±0.000	Medium	submetacentric
21	0.520	0.757	1.278	0.593±0.008	0.035±0.001	Medium	metacentric
22	0.546	0.854	1.400	0.610±0.004	0.038±0.002	Medium	submetacentric
23	0.377	0.819	1.196	0.585±0.008	0.032±0.001	Medium	metacentric
24	0.430	0.777	1.207	0.644±0.002	0.033±0.001	Medium	submetacentric
25	0.402	0.706	1.108	0.637±0.012	0.030±0.000	Medium	submetacentric

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.

Chromosome pairs	Ls (μm)	Ll (μm)	LT (μm)	CI±SD	RL±SD	Chromosome size	Chromosome type
1	0.715	1.331	2.046	0.651±0.033	0.054±0.004	Large	submetacentric
2	0.667	1.226	1.893	0.648±0.014	0.050±0.001	Large	submetacentric
3	0.595	1.199	1.794	0.668±0.017	0.047±0.002	Large	submetacentric
4	0.646	1.091	1.737	0.588±0.028	0.046±0.002	Large	metacentric
5	0.575	1.142	1.717	0.665±0.037	0.045±0.001	Large	submetacentric
6	0.669	1.034	1.702	0.608±0.034	0.045±0.006	Large	submetacentric
7	0.583	1.108	1.691	0.755±0.029	0.044±0.002	Large	acrocentric
8	0.567	1.081	1.647	0.656±0.028	0.043±0.003	Large	submetacentric
9	0.564	1.014	1.578	0.703±0.028	0.041±0.003	Medium	acrocentric
10	0.574	0.990	1.564	0.633±0.036	0.041±0.003	Medium	submetacentric
11	0.646	0.907	1.553	0.584±0.022	0.041±0.004	Medium	metacentric
12	0.555	0.949	1.504	0.631±0.025	0.039±0.001	Medium	submetacentric
13	0.574	0.926	1.500	0.617±0.033	0.039±0.001	Medium	submetacentric
14	0.553	0.948	1.501	0.632±0.020	0.039±0.001	Medium	submetacentric
15	0.590	0.894	1.484	0.592±0.030	0.039±0.001	Medium	metacentric
16	0.537	0.888	1.425	0.623±0.032	0.037±0.002	Medium	submetacentric
17	0.555	0.851	1.406	0.585±0.022	0.037±0.002	Medium	metacentric
18	0.547	0.853	1.400	0.609±0.015	0.037±0.002	Medium	submetacentric
19	0.549	0.851	1.401	0.607±0.016	0.037±0.002	Medium	submetacentric
20	0.519	0.844	1.363	0.619±0.029	0.036±0.003	Medium	submetacentric
21	0.474	0.845	1.319	0.641±0.054	0.035±0.002	Medium	submetacentric
22	0.508	0.742	1.249	0.594±0.041	0.033±0.002	Medium	submetacentric
23	0.479	0.767	1.246	0.596±0.022	0.033±0.002	Medium	metacentric
24	0.449	0.756	1.205	0.627±0.045	0.032±0.003	Medium	submetacentric
25	0.488	0.710	1.197	0.593±0.034	0.031±0.001	Medium	metacentric

Table 4. Karyomorphological details of *B. urophthalmoides* from 20 metaphases, *n* (haploid)=25.

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.

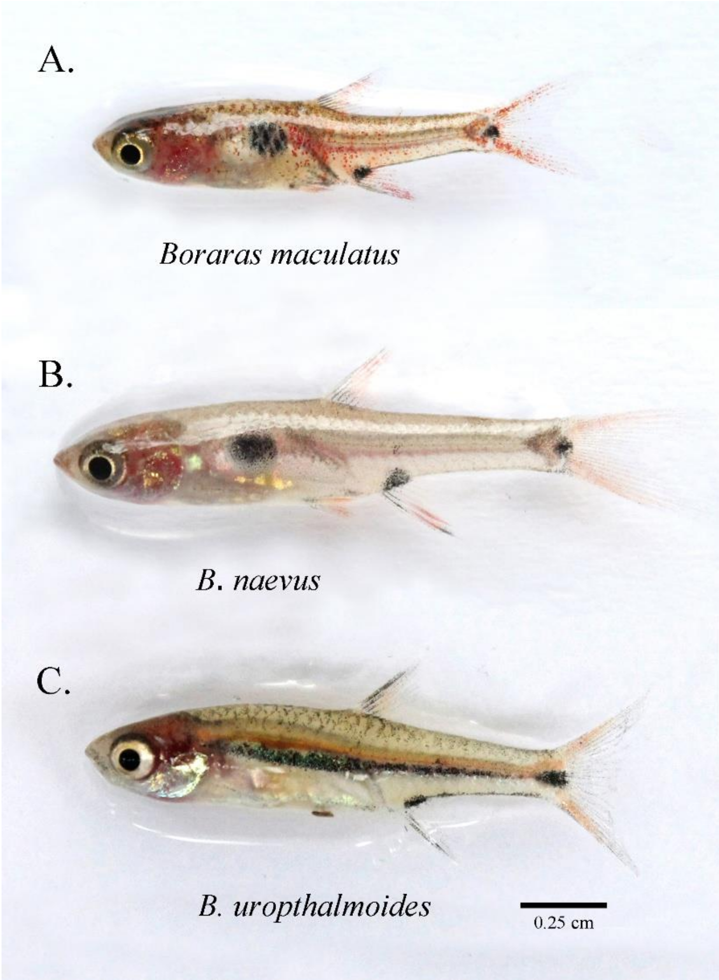

Fig. 1. General characteristics of *Boraras* (A.) *Boraras maculatus*, (B.) *B. naevus* and (C.) *B. urophthalmoides* from Thailand.

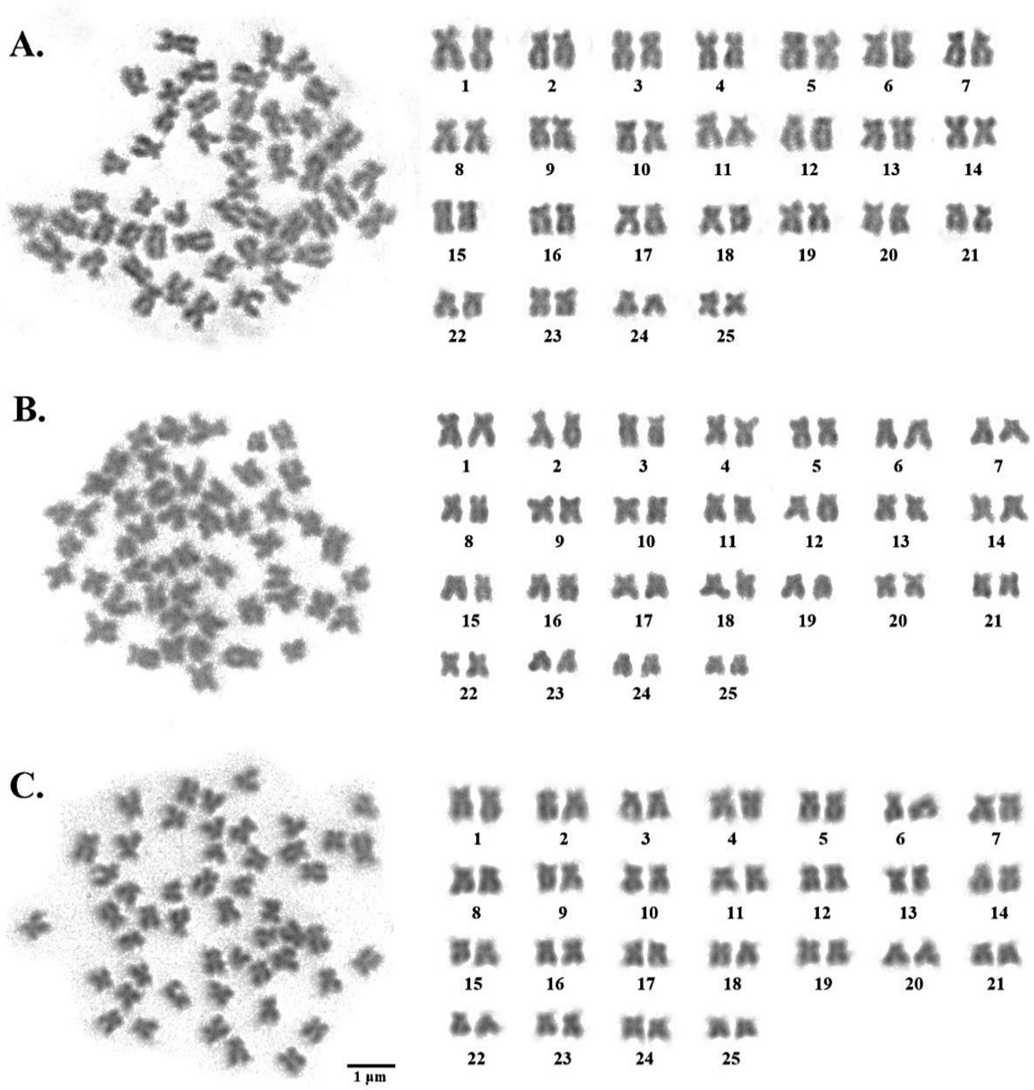

Fig. 2. Metaphase chromosome plates and karyotypes of *Boraras* (A.) *Boraras maculatus*, (B.) *B. naevus* and (C.) *B. urophthalmoides*, by conventional staining. All species share the karyotype composed of 50 chromosomes, *n*)haploid(=25.

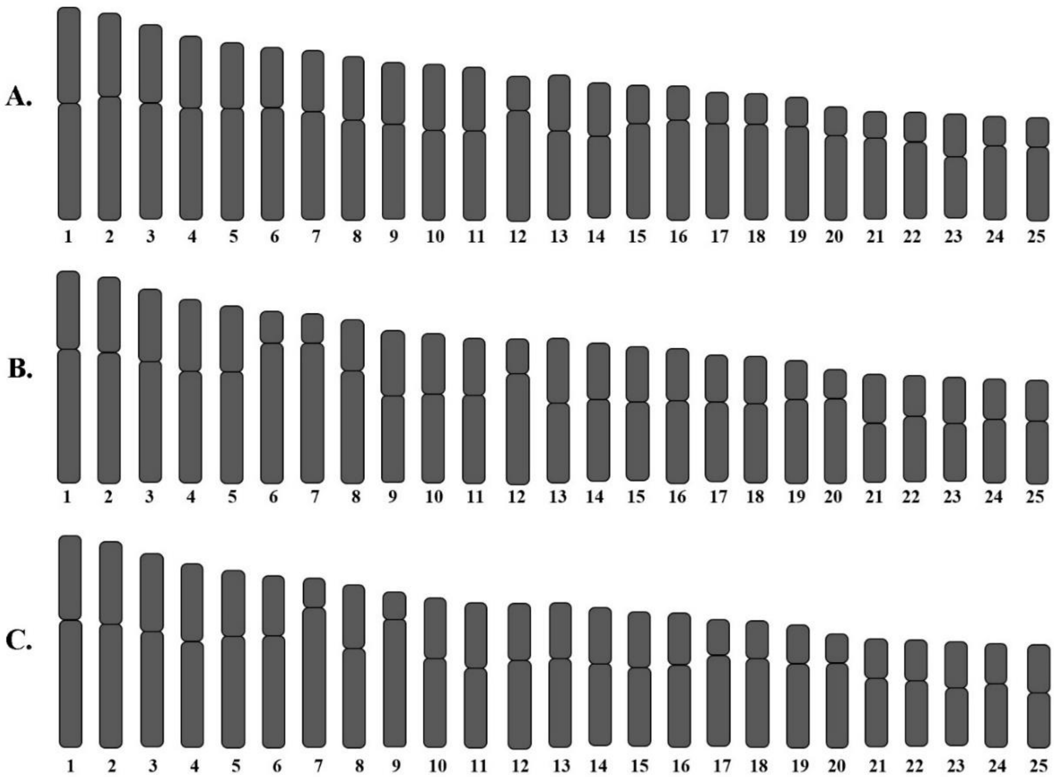

Fig. 3 Idiogram showing lengths and shape of chromosomes of *Borarus* (A.) *Boraras maculatus*, (B.) *B. naevus* and (C.) *B. urophthalmoides*, *n* (haploid)=25, by conventional staining technique.

Dear Editor and Reviewer:

Thank you very much for the valuable comments provided. The response for comment is as followed;

Reviewer 1 Major revision	**Authors' response to reviewers' comments**
1) The methodology used to perform the karyotype (Turpin and Lejeune, 1965) was developed for use with human chromosomes; it is not the methodology used by Levant et al. (1964) in the background work for the genus Boraras. For this reason, the results of this manuscript cannot be fully compared with the bibliographic references. The values used by both methodologies for the q/p ratio to create the karyotype are different, so it is likely that the karyotypes obtained in the manuscript are different from those in the references, solely due to the methodology used.	The author reviewed and compared the karyotype values again and found that they are consistent with the research of Chaiyasut, 1989
2) The karyotypes are shown in the manuscript sometimes for the haploid chromosome complement and sometimes for the diploid chromosome complement. Karyotypes are generally presented for the haploid chromosome complement, as shown in Tables 2, 3, and 4 of the manuscript. The way in which the karyotype is displayed should be standardized.	The author has added additional information (haploid chromosome) to Table 1, and added explanations for the figure 2 and tables 2, 3 and 4.
3) There are too many punctuation errors in the bibliographic citations, and the first reference has authorship errors and is incomplete. Therefore, it is suggested that the references be revised.	The MS has been revised as the reviewers' comments.
4) I reiterate that the discussion should be improved by relating the chromosome numbers obtained for Boraras to those of Rasbora, and their significance in phylogenetic relationships. Furthermore, there are references to hybridization in captivity and in natural populations among Danionidae fish. It should be discussed whether the differences between the chromosome numbers of the different taxa are the result of hybridization in regions where there is geographic overlap between them.	The MS has been revised as the reviewers' comments.