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RESEARCH ARTICLE

Cytotaxonomic Studies in Four Species of Genus *Puntius* (Hamilton, 1822) from Central India

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Abstract The cytotaxonomic analyses of four species of genus *Puntius*, viz. *P. chola*, *P. conchonius*, *P. sophore* and *P. ticto* from central India was carried out for the first time. The metaphase chromosomes were prepared from kidney and gill tissues. The diploid chromosome number and karyotype formula (KF) were found to be 50 & 2m + 4sm + 2st + 42t in *P. chola*; 50 & 14m + 28sm + 8st in *P. conchonius*; 48 & 4m + 2st + 42t in *P. sophore* and 50 & 14m + 24sm + 8st + 4t in *P. ticto*. Based on KF, the fundamental arm number were determined as 56, 92, 52 and 88, respectively, for these species. The karyomorphological features indicated that *P. conchonius* and *P. ticto* are closely related and the same holds true for *P. chola* and *P. sophore*. The chromosomes of all the four species exhibited constitutive heterochromatic blocks at their centromeric position, as detected by Cbanding technique. Variations in number of NORs were observed with presence of single pair of NORs in *P. chola* and *P. conchonius*, whereas in *P. sophore* and *P. ticto* multiple NORs were observed. Thus, based on the karyological features it can be hypothesized that *P. conchonius* and *P. ticto* may be in advanced stage of karyo-evolution.

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Introduction

The cyprinid fish genus *Puntius* comprises of more than 60 species found in India and new species are being discovered, especially from the North–Eastern and Southern parts of India [1, 2]. Many species of this genus are considered as weed fishes, while some of them are of ornamental value. Taxonomic ambiguities exist between many closely related *Puntius* species [2–4]. Further, these fish inhabits and breeds in common water-bodies; therefore, chances of inter-breeding and hybridization are higher. Cytotaxonomic studies are, therefore, required to document inter- as well as intra-specific variations and to resolve taxonomic ambiguities among the species.

The study on fish chromosome has received considerable attention because of their importance in classification, evolution and heredity [5, 6]. The cytogenetic techniques are considered as authentic tools for species characterization and have extensively been used to resolve taxonomic ambiguities in closely related species, identification of strains/cytotypes, genetic polymorphisms, sex determination, polyploidy etc. [7–9]. Comparison of chromosome number and structure among different species reveals phylogenetic relationship and throws light on their karyo-evolution and can also be helpful in planning conservation strategies for threatened fish species [10].

Four *Puntius* species, viz. *P. chola* (known as 'swamp barb'), *P. conchonius* (known as 'rosy barb'), *P. sophore* (known as 'pool barb') and *P. ticto* (known as 'two spot barb') collected from central India, were investigated to find out karyotypic variations and cytotaxonomic relationship among them. The nucleolar organizer regions (NORs) staining is considered to be one of the most commonly used technique for cytogenetic characterization of fish. The silver nitrate ($AgNO_3$) stained NORs detects only transcriptionally active sites [11], while GC specific fluorochrome chromomycin A_3 (CMA_3) stains both active and inactive NORs probably due to their high GC content [12]. C-banding is another useful technique used to study the localization of constitutive heterochromatic (CH) bands and its staining with fluorescent dye may further increase the resolution of bands.

As far as authors are aware, there is no information available on the CH bands and NORs in these four species of genus *Puntius*. Therefore, the present study was aimed to analyse the karyotypic characteristics, with particular reference to the variation in CH and NORs, and establish cytotaxonomic relationship among these species.

Materials and Methods

Live fish specimens of *P. chola* ($n = 6$), *P. conchonius* ($n = 10$) and *P. ticto* ($n = 5$) were collected from Pahuj river, Jhansi, Uttar Pradesh, while specimens of *P. sophore* ($n = 10$) were obtained from Ramsagar reservoir at Barauni, Datia, Madhya Pradesh with the help of local fishermen. Sharp pointed needle, like divider, and stainless steel ruler were used for recording body measurements [13]. Specimens were identified up to species level following taxonomic keys described by Jayaram [14], Talwar and Jhingran [15] and Srivastava [16]. The average total length and wet weight of *P. chola* specimens were 8.25 cm (range 7.3–9.0) and 6.35 g (range 5.4–7.2). In *P. conconius, P. sophore* and *P. ticto*, the total length and wet weight of specimens were 7.57 cm (range 6.3–10.0) & 6.16 g (range 4.4–13); 8.04 cm (range 6.9–9.0) & 6.58 g (range 5.0–10.2), and 6.45 cm (range 5.9–6.8) & 3.20 g (range 2.0–4.2), respectively. The specimens were at juvenile stage and the sex was unidentifiable by visual examination.

The chromosomes were obtained from kidney and gill cells following hypotonic (KCl) treatment, fixation (methanol-acetic acid) and air drying technique as described by Bertollo et al. [17] and the dried slides were stained with Giemsa. For karyotyping, the chromosomes were classified as per the method described by Levan et al. [18]. A total of 50 slides were prepared from each species and 6 good spreads from each slide were used for karyo-morphological analyses. Chromosome lengths were measured using 'MicroMeasure' (version 3.2) [19] computer software. C-banding was carried out according to Sumner [20], but the slides were stained with fluorescent propidium iodide according to Fontana et al. [21]. The method of Howell and Black [11] was used for silver staining of NORs, while fluorescent CMA_3 staining of NORs was done according to Sola et al. [22]. All the photographs were taken at 100 $\times$ magnification using fluorescent microscope and a total of 80 random spreads from each species were considered for determining a particular banding/staining pattern.

Results and Discussion

Metaphase Spreads and Karyotype

The metaphase complements and karyotype of *P. chola, P. conchonius, P. sophore* and *P. ticto* are shown in Fig. 1 and the chromosomal morphometric data are described in Table 1. In *P. chola, P. conchonius,* and *P. ticto*, the diploid chromosome number ($2n$) was found to be 50, whereas in *P. sophore* the $2n$ was recorded as 48. Further, variations in karyo-morphology were observed among the species. The specimens of *P. chola* possessed one metacentric pair (m), two submetacentric (sm) pairs, one subtelocentric (st) pair, and 21 telocentric (t) pairs of chromosomes and the karyotype formula (KF) was derived as 2m + 4sm + 2st + 42t with fundamental arm number (FN) of 56. In *P. conchonius*, the KF was derived as 14m + 28sm + 8st and FN as 92. In *P. sophore*, the karyotype composed of two pairs of metacentric, one pair of subtelocentric and 21 pairs of telocentric chromosomes with FN as 52. The karyotype of *P. ticto* specimens composed of seven pairs of metacentric, 12 pairs of submetacentric, four pairs of subtelocentric and two pairs of telocentric chromosomes with FN as 88.

Chromosome Morphometry and Index

The total length of the chromosomes (TL) in *P. chola* varied from 0.97 to 2.73 µm, whereas the centromere index (CI) and relative length (RL) ranged from 14.46 to 47.15 µm and from 2.55 to 7.17 %, respectively. In *P. conchonius* specimens, the TL varied from 1.63 to 2.97 µm, and the CI and RL from 17.08 to 44.61 µm and from 3.0 to 5.46 %, respectively. In *P. sophore,* the TL ranged from 0.84 to 2.68 µm, CI from 13.73 to 48.88 µm and RL from 2.15 to 6.87 %. In *P. ticto*, the TL varied from 1.55 to 3.64 µm and CI from 8.98 to 48.04 µm and RL from 2.70 to 6.35 %.

NOR Staining

The chromosome complements showing $AgNO_3$ and CMA_3 stained NORs in the *Puntius* species are presented

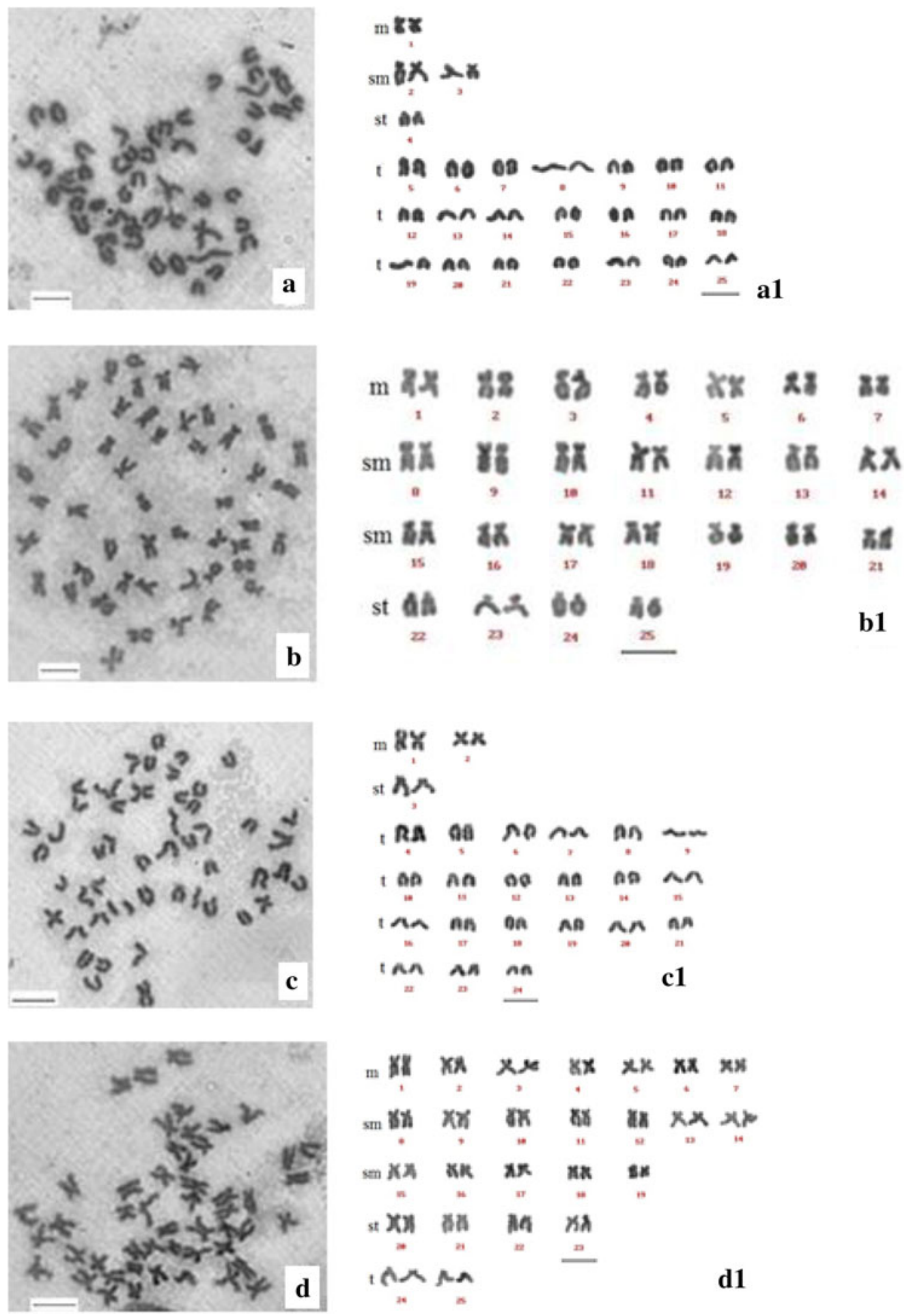

Fig. 1 Metaphase spread and karyotype of: *P. chola* (**a** and **a1**), *P. conchonius* (**b** and **b1**), *P. sophore* (**c** and **c1**) and *P. ticto* (**d** and **d1**). *Bar* = 5 μm

in Fig. 2. In *P. chola* and *P. conchonius,* single NOR pair was observed at the end of short arms of subtelocentric chromosome using both $AgNO_3$ and CMA_3 staining. In *P. sophore* specimens, however, NOR signals were found terminally on three pairs of telocentric chromosome. Similarly in *P. ticto*, NOR signals were detected at terminal/sub-terminal positions of two pairs of metacentric and one pair of submetacentric chromosomes.

Constitutive Heterochromatin

The C-banding technique revealed the localization of constitutive heterochromatic blocks and the C-banded metaphase complements are presented in Fig. 3. The CH blocks were detected on the chromosomes of all the species and were centromeric in position. No significant variation in the position and size of the bands were observed among these species.

Table 1 Chromosome morphometric data and chromosome types of *Puntius* sp

CPN	*P. chola*					*P. conchonius*					*P. sophore*					*P. ticto*				
	AR	TL	CI	RL (%)	CT	AR	TL	CI	RL (%)	CT	AR	TL	CI	RL (%)	CT	AR	TL	CI	RL (%)	CT
1	1.12	1.93	47.15	5.07	m	1.56	2.97	39.06	5.46	m	1.05	2.68	48.88	6.87	m	1.41	3.64	41.48	6.35	m
2	2.25	2.73	30.77	7.17	sm	1.43	2.73	41.02	5.02	m	1.2	1.87	45.45	4.79	m	1.52	2.90	39.65	5.06	m
3	2.92	2.08	25.48	5.46	sm	1.46	2.42	40.49	4.45	m	6.28	2.33	13.73	5.97	st	1.08	2.81	48.04	4.90	m
4	5.91	1.66	14.46	4.36	st	1.65	2.34	37.61	4.31	m	0	2.34	0	5.99	t	1.19	2.46	45.53	4.29	m
5	0	2.33	0	6.12	t	1.33	2.15	42.79	3.96	m	0	2.25	0	5.76	t	1.20	2.42	45.45	4.22	m
6	0	2.25	0	5.91	t	1.24	1.95	44.61	3.58	m	0	2.14	0	5.48	t	1.67	2.27	37.44	3.96	m
7	0	1.76	0	4.62	t	1.40	1.95	41.54	3.59	m	0	1.96	0	5.02	t	1.18	1.64	45.73	2.86	m
8	0	1.74	0	4.57	t	2.52	2.43	28.39	4.47	sm	0	1.93	0	4.95	t	1.96	2.61	33.72	4.55	sm
9	0	1.48	0	3.88	t	2.73	2.43	26.75	4.47	sm	0	1.61	0	4.13	t	2.17	2.60	31.54	4.53	sm
10	0	1.44	0	3.78	t	2.70	2.41	26.97	4.43	sm	0	1.61	0	4.13	t	2.60	2.52	27.78	4.39	sm
11	0	1.44	0	3.78	t	2.02	2.33	33.05	4.29	sm	0	1.59	0	4.07	t	2.64	2.51	27.49	4.37	sm
12	0	1.43	0	3.75	t	2.43	2.3	29.13	4.23	sm	0	1.56	0	3.99	t	2.57	2.50	28.00	4.36	sm
13	0	1.35	0	3.55	t	2.52	2.29	28.38	4.21	sm	0	1.52	0	3.89	t	1.90	2.44	34.43	4.25	sm
14	0	1.35	0	3.55	t	2.46	2.22	28.83	4.09	sm	0	1.52	0	3.89	t	1.84	2.33	35.19	4.06	sm
15	0	1.34	0	3.52	t	2.46	2.15	28.84	3.96	sm	0	1.41	0	3.61	t	2.04	2.16	32.87	3.77	sm
16	0	1.32	0	3.46	t	2.31	2.12	30.19	3.90	sm	0	1.38	0	3.54	t	2.17	2.03	31.53	3.54	sm
17	0	1.32	0	3.46	t	2.14	1.95	31.79	3.59	sm	0	1.32	0	3.38	t	2.09	1.98	32.32	3.45	sm
18	0	1.31	0	3.44	t	2.78	1.93	26.42	3.55	sm	0	1.32	0	3.38	t	2.43	1.75	29.14	3.05	sm
19	0	1.29	0	3.39	t	2.49	1.85	28.65	3.41	sm	0	1.31	0	3.36	t	1.92	1.55	34.19	2.70	sm
20	0	1.27	0	3.33	t	2.06	1.81	32.59	3.33	sm	0	1.30	0	3.33	t	5.82	2.32	14.65	4.05	st
21	0	1.19	0	3.13	t	1.96	1.63	33.74	3.00	sm	0	1.29	0	3.30	t	6.06	2.19	14.15	3.82	st
22	0	1.05	0	2.76	t	4.63	2.03	17.73	3.74	st	0	0.98	0	2.51	t	6.34	2.13	13.62	3.72	st
23	0	1.03	0	2.71	t	4.15	2.01	19.40	3.69	st	0	0.96	0	2.46	t	4.24	1.99	19.09	3.47	st
24	0	1.01	0	2.65	t	4.85	1.99	17.08	3.66	st	0	0.84	0	2.15	t	9.55	1.90	9.47	3.31	t
25	0	0.97	0	2.55	t	4.54	1.94	18.04	3.57	st	–	–	–	–	–	10.13	1.67	8.98	2.91	t

CPN Chromosome pair number, *AR* Arm ratio, *TL* Total length of chromosome, *CI* Centromeric index, *RL (%)* Relative length in percent; *CT* Chromosome type

The most commonly occurring 2*n* in fish family cyprinidae is 50 with the range from 34 to 446 [23, 24]. According to the studies performed by various workers on *Puntius* species in India, the $2n = 50$ seemed to be the modal number for the genus [25], with the range from 48 to 52 (www.fishbase.org/ version 06/2012). In the present study, the $2n = 50$ was found in *P. chola*, *P. conchonius* and *P. ticto*, whereas in *P. sophore* the same was found to be 48. The comparison of results of the present study with earlier reports has been presented in Table 2. The karyomorphology of all the species showed variation from the earlier reports, except of *P. sophore* in which the KF (4m + 2st + 42t) and FN (52) was in confirmation with the finding of Rishi and Rishi [26].

The presence of different populations, races and/or sub species arising from mutation, race improvement and hybridization with other indigenous species could be the possible explanation for differences in number and type of chromosomes reported in a species that is distributed in different aquatic ecosystems [27, 28]. Intra-specific variation in karyo-morphology have also been ascribed to ambiguities in classification due to border-line centromere positions caused by cell to cell variation in the extent of chromosome contraction, which is a general problem in the description of the relatively small chromosomes of cyprinids [29–31]. Differences in FN among closely related species corroborated the importance of pericentric inversions as the main mechanism of karyotypic evolution in several modern fish orders [32–36]. Different FN reported in *Labeo rohita* from China (i.e. 76), Thailand (80) and India (70) reflected local differentiation in the karyotype [37, 38]. The chromosomal morphometric data revealed that *P. ticto* possessed the longest chromosome (3.64 µm), while the smallest (0.84 µm) was observed in *P. sophore*. The maximum numbers of metacentric chromosomes (14) were found in *P. conchonius* and *P. ticto*, whereas the maximum numbers of telocentric chromosomes (42) were found in *P. chola* and *P. sophore*. No telocentric and

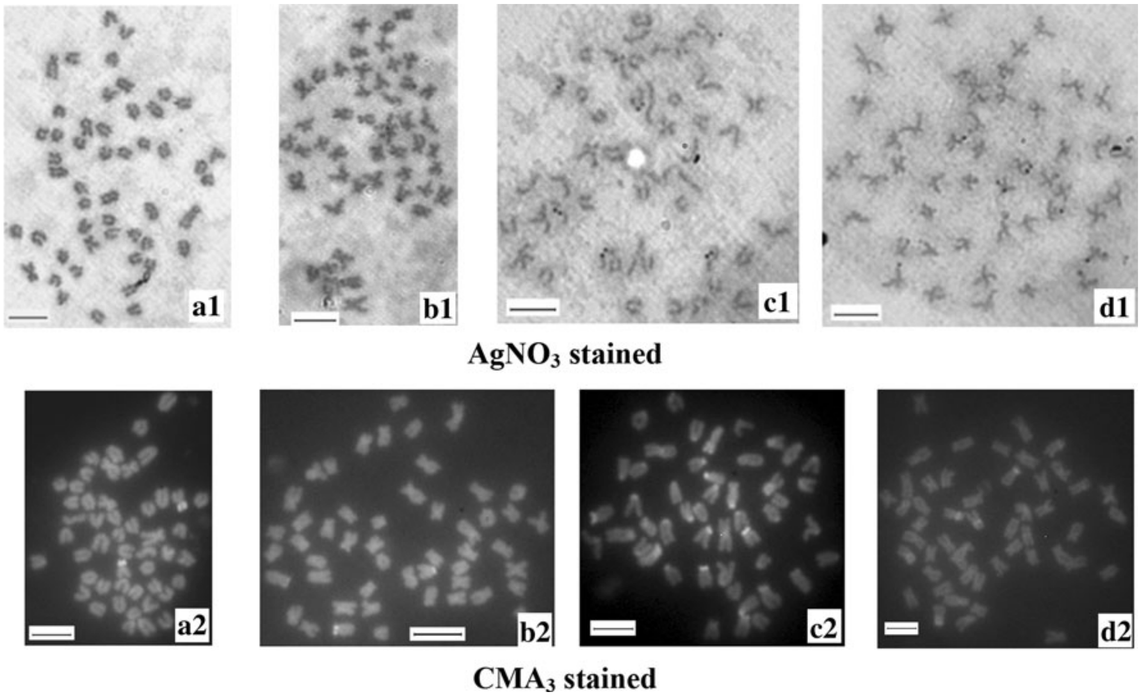

Fig. 2 Metaphase plates and chromosome pair(s) showing NOR regions detected by $AgNO_3$ and CMA_3, respectively, in: *P. chola* (**a1**, **a2**), *P. conchonius* (**b1**, **b2**), *P. sophore* (**c1**, **c2**) and *P. ticto* (**d1**, **d2**). *Bar* = 5 µm

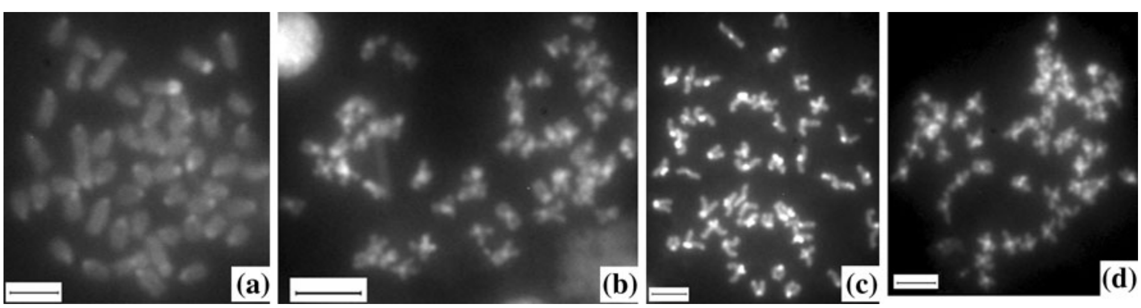

Fig. 3 Metaphase spreads showing constitutive heterochromatin regions, as detected by C-banding, in: *P. chola* (**a**), *P. conchonius* (**b**), *P. sophore* (**c**) and *P. ticto* (**d**). *Bar* = 5 µm

submetacentric chromosomes were observed in *P. conchonius* and *P. sophore*, respectively. The karyo-morphological features indicated *P. conchonius* and *P. ticto* to be closely related and the same holds true for *P. chola* and *P. sophore*. Similar closeness of *P. chola* with *P. sophore* and *P. conchonius* with *P. ticto* were also recorded on the basis of mitochondrial cytochrome b gene [39]. A phylogenetic study based on restriction fragment polymorphism analysis among different species of genus *Puntius* also indicated closeness of *P. chola* with *P. sophore* [4].

The banding studies can help in the precise mapping of genes onto the chromosomes and the evolutionary relationship between species can be inferred at a gross level by comparing banding patterns. The C-banding is very often species-specific and its distribution may vary considerably from species to species [40]. The characteristics of the C-bands, which aids to the identification of species, are their size, location on the chromosome and the position of the C-banded chromosome in the karyotype [41]. In the present study; however, the CH bands were found at the centromeric position of the chromosomes in all the species and no variation were observed with regards to the position and size/intensity of bands. Similar distribution pattern of heterochromatin throughout the chromosomes have been reported by several workers in many closely related species belonging to the same genus: *Notropis lutrensis* and *N. venustus* [42], *Schizodon borelli* and *S. isognathum* [43] and *Vimba vimba* and *V. elongata* [44]. Nuclear satellite

Table 2 Comparison of chromosomal morphology of *Puntius* species reported by different workers

Species	2n	KF	FN	Region	References
P. chola	50	2m + 2sm + 46t	54	Arunachal Pradesh	Sahoo et al. [55]
	50	2m + 4sm + 2st + 42t	56	Uttar Pradesh	Present study
P. conchonius	50	22m + 16sm + 12t	88	Kashmir	Ganai and Yousuf [25]
	50	16m + 24sm + 2st + 8t	90	Orissa	Khuda Bukhsh et al. [56].
	48	10m + 20sm + 10st + 8t	78	Jammu & Kashmir	Sharma and Agrawal [57]
	50	14m + 28sm + 8st	92	Uttar Pradesh	Present study
P. sophore	48	2m + 46t	50	Orissa	Biswal et al. [58].
	50	2m + 4sm + 44t	56	Tamil Nadu	Khuda Bukhsh et al. [52].
	48	4m + 2st + 42t	52	Haryana	Rishi and Rishi [26]
	48	4m + 4st + 40t	52	West Bengal	Manna and Prasad [59]
	48	4m + 2st + 42t	52	Madhya Pradesh	Present study
P. ticto	50	28m + 16sm + 6t	94	Arunachal Pradesh	Sahoo et al. [55].
	50	14m + 22sm + 6st + 8t	86	West Bengal	Manna and Prasad [60]
	50	14m + 24sm + 8st + 4t	88	Uttar Pradesh	Present study

DNA have one property in common, namely heterochromatinization, despite of their species specificity, and the apparent species specificity may be the result of natural selection for duplicated short polynucleotide segments. The centromeric heterochromatin is believed to confer protection and strength to the centromeric chromatin [45]. This condition may arise due to the Robertsonian fusions [46] or could have been formed by tandem duplication/pericentric inversion of heterochromatic DNA [47]. It is opined that karyotypic stability might reached after canalization to an optimal karyotypic configuration [48], which could be a reason for similarity in distribution of heterochromatin in these *Puntius* species.

The NORs are the chromosomal sites of genes that were presumably transcribed at preceding interphase and are important in view of their intimate relationship with protein synthesis [11, 49]. An important characteristic of NORs in fish is related to its inter- and/or intra-species polymorphism. NOR characteristics can be utilized as a marker for cytotaxonomic studies and can even aid in constructing phylogenetic hypotheses (cyto-systematics) for several fish groups [8]. In fish, presence of NORs on single pair of chromosome was considered to be plesiomorphic or primitive condition [50]. Single pair of NOR was observed at the end of the short arms of subtelocentric chromosomes (4th pair) in *P. chola* and at the end of the short arms of subtelocentric chromosome in *P. conchonius* with both CMA_3 and $AgNO_3$ staining, whereas multiple NORs were found in *P. sophore* and *P. ticto* that could be species-specific character. The information on size, position and number of NORs are suitable for tracing intra- and inter-specific differences and may serve to demarcate and derive the taxonomic status of species in terms of karyo-evolution [49, 51–53].

Comparative phylogenetic analyses have been employed to examine the evolutionary history of fish chromosome. The most parsimonious ancestral state for major actinopterygiian clades has been observed as 48 chromosomes [54]. Moreover, the presence of more number of telocentric chromosomes is also an ancestral condition. Based on the karyo-morphological features, *P. chola* and *P. sophore* may be considered as primitive species in the present study. On the other hand, *P. conchonius* and *P. ticto* may be considered as derived species or may be in the advanced stages of karyo-evolution due to presence of many numbers of bi-armed chromosomes. In the present study, *P. chola* satisfies the condition of being primitive species due to presence of single NOR. Surprisingly, *P. sophore* does not follow the condition of primitive species due to presence of multiple NORs, as proposed by Gold and Amemiya [50]. The species belonging to the genera *Puntius* have similarity in external phenotypic characters that lead to taxonomic uncertainty. Moreover, *P. chola* and *P. sophore* looks alike with similar morphometric and meristic characters, except the presence of one pair of maxillary barbells and an extra band on dorsal fin in *P. chola*. Similarly, *P. conchonius* greatly resembles with *P. ticto* based on morphology, except the presence of one black spot at anterior body in the later [14–16]. Further studies using molecular tools may add to reaffirm the cytotaxonomic and phylogenetic relationships in these species.

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