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Karyotypic analysis of Crucian carp, *Carassius carassius* (Linnaeus, 1758) from cold waters of Kashmir Himalayas

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Abstract. *Carassius carassius* (Linnaeus, 1758) is an exotic fish to Kashmir, locally known as “gang gad” and commonly called as “crucian carp”. It belongs to family Cyprinidae. The present study aimed to identify the chromosome number of the *Carassius carassius* and to optimize the colchicine concentrations (0.01%, 0.025%, and 0.05%) and hypotonic treatment timings (25, 35, and 45 minutes) for the chromosome preparation in *Carassius carassius* in order to obtain the highest number of clear and identifiable metaphasic chromosomal spreads. Data collected was analyzed and the means of each treatment was compared. The findings of the present study indicated that there was a significant influence of colchicine concentration, hypotonic timings as well as colchicine concentration × hypotonic timings ($P < 0.01$) on the number of metaphase chromosome spreads. Furthermore a significant ($P < 0.01$) strong positive correlation obtained between colchicine concentrations, hypotonic timings and the number of metaphase chromosome spreads. The findings of the present study recommends further research into chromosomal modification techniques such as fish polyploid production, gynogenesis, androgenesis, and inter or intra-species hybridization is needed to generate unique and good inbred lines in aquaculture.

Keywords: *Carassius carassius*, Colchicine, optimization, metaphase chromosome, kidney, hypotonic solution.

INTRODUCTION

The Cyprinidae (Teleostei: Cypriniformes) is a globally widespread and one of the richest and most vital family of fish (Al-Sabti, 1991; Kalbassi,

2008). It is a large family of freshwater fishes commonly called the carp family comprising of vast majority of bony fishes (Abdoli, 1999). The Crucian Carp (*Carassius carassius* Linnaeus, 1758) is a medium-sized fish in the Cyprinidae family and is a widespread European species. *Carassius carassius* is locally known as “gang gad” and more specifically known as “crucian carp” and was introduced into Dal Lake in Kashmir, India between 1956 and 1958 (Shafi, 2012). It is one of the most abundant cyprinids in Dal Lake Kashmir and it has adapted to a wide range of habitats to cope with abiotic environmental challenges such as low oxygen levels and water temperature variations (Holopainen and Hyvarinain, 1985).

The study of fish chromosomes has long been a popular topic of study due to its usefulness in fish population conservation, cytotaxonomy, phylogeny research and evolutionary studies Luca *et al.* (2010); Bazaz *et al.* (2022). The development of techniques such as karyotyping has made it possible to visualize undetected chromosomal anomalies such as small portions of chromosomes and translocations of tiny parts of chromosomes to one another Bazaz *et al.* (2022). Fish cytology may be beneficial in interpreting the evolution of higher vertebrates and the interrelationships among the numerous divisions of fishes, because they are a primordial chordate group. In terms of species characterisation, evolution and systematics, chromosomal studies have acquired a lot of traction in recent years (Barat *et al.*, 2002). One of the most critical parts of fish conservation is genetic characterisation. Using cytogenetic, biochemical-genetic and advanced molecular genetic approaches, this is conceivable. Cytogenetic characterisation is concerned with the study of chromosomes, which serve as genetic vehicles for a variety of genetic investigations, from the creation of gene maps and chromosome organisation models to the investigation of gene function and dysfunction. Various methods are now being used to study fish chromosomes, with improvements and modifications such as the pretreatment with colchicine and hypotonic solution, which causes the cells to enlarge and the chromosomes to separate (Luca *et al.*, 2010; Bazaz *et al.*, 2022).

There are a number of factors that may have contributed to the decline of fish species including habitat loss and degradation, displacement via competition with introduced species such as the polyploid biotype such as the goldfish (*Carassius auratus*) and the common carp (*Cyprinus carpio communis*). Moreover, all species of *Carassius*, including the crucian carp (*Carassius carassius*), Prussian carp (*Carassius gibelio*) and goldfish (*Carassius auratus*) are often confused due to their morphological similarity Knytl *et al.* (2021). Such confusion may

lead to inappropriate stocking of wrong species instead of intended support of a population of crucian carp with negative consequences.

Genetic contamination seems to be a very important but a hidden threat. Hybridization occurs and has been reported in various *carassius* species between such as *Carassius carassius* and *Carassius gibelio* (Prokes and Barus 1996). This type of hybridization was also confirmed using molecular (Wouters *et al.* 2012) and cytogenetic techniques (Knytl *et al.* 2013).

Hybrids between *Carassius carassius* and *Carassius auratus* (Hanfling *et al.* 2005; Smartt 2007) and intergeneric hybrids between *Carassius carassius* and *Cyprinus carpio* (Hanfling *et al.* 2005) has been observed. Moreover, molecular data suggest that these hybrids are able to reproduce and form relative generations by backcrossing (Hanfling *et al.* 2005, Wouters *et al.* 2012).

The cytogenetics of *Carassius carassius* is still inadequately understood. Interestingly, different diploid chromosome numbers $2n = 50$ Raicu *et al.* (1981), $2n = 98$ Manna, (1983), $2n = 100$ Knytl *et al.* (2021) and $2n = 104$ Chiarelli *et al.* (1969) has been reported. Similarly Knytl *et al.* 2021 reported the number of chromosomes in three *Carassius* species (*C. auratus*, *C. carassius* and *C. gibelio*) was uniform ($2=100$). The authors reported no differences in chromosomal morphology between male and female individuals. This findings confirmed homomorphic sex chromosomes at least in diploid biotypes of the genus *Carassius*.

Such an unclear condition encourages to present cytogenetic analyses of *Carassius carassius* with respect to hybridization processes and the related threats to indigenous species in Kashmir waters. Furthermore, the considerable karyotypic study on *Carassius carassius* has not been published in the Kashmir valley from decades. The cyto-taxonomic status of *Carassius carassius* with respect to the species hybridization and diploid/tetraploid status has remained unclear. In respect to its unclear distribution of possible diploid and/or tetraploid forms as well as hybridization process, the present study is an important contribution to the cytogenetics and cytotaxonomy of *Carassius carassius*. The present study deals with chromosomal characteristics of crucian carp (*Carassius carassius*) from the cold waters of Kashmir Himalayas.

MATERIALS AND METHODS

The research work was carried in the Division of Fish Genetics and Biotechnology, Faculty of Fisheries, SKUAST Kashmir, Rangil, Ganderbal, J&K, India.

Sample collection

A total of 45 specimens of *Carassius carassius* were collected from Dal Lake Srinagar, Kashmir and were transported live in an insulated box containing ice packs and equipped with aerators to the Fish Genetics and Biotechnology laboratory, faculty of fisheries, SKUAST-Kashmir. Fish were kept in 50L tubs equipped with continuous aeration. Identification of *Carassius carassius* was done following the diagnostic characters described by Kullander *et al.* (1999).

Chromosome preparation

Chromosome preparation for *Carassius carassius* species was done by the methods described by Killgerman and Bloom (1977), and Shao *et al.* (2010) with some modifications. For each treatment, five samples were used. Three different concentrations of freshly prepared colchicine (0.01%, 0.025% and 0.05%), were injected intramuscularly at 1ml/100g of body weight for 90 minutes to depress the mitotic division at the metaphase stage (Table 1). The fish were anesthetized using clove oil and were dissected. The anterior kidney was removed, homogenized and hypotonised simultaneously in 0.56% KCl for 25, 35 and 45 minutes at room temperature. Thereafter, the cell suspension was centrifuged for 10 min at 1300 rpm. Supernatant was removed and cells were fixed by chilled carnoy's fixative solution (methanol: glacial acetic acid) and left overnight, fixation process was repeated two-three times until the clear suspension was obtained with the fresh chilled fixative solution replaced every time. The suspension was centrifuged at 1300 rpm for 10 minutes before to each subsequent procedure in the formal treatment; the supernatant was

then removed, leaving one ml of the solution above the cell pellet in its place. The next solution was used to resuspend the cells. Slides were prepared by dropping method, three drops of the cell suspension on the pre-heated slide from about 40-50 cm height with slide slightly placed in an inclined position. The slides were stained using Giemsa stain (10%) distilled water and air-dried. Coverslips were placed on slides by mounting with D.P.X mountant to make them permanent and protect them from drying out.

Screening of slides

The slides were observed under the field illuminated trinocular microscope (Olympus CX-21) fitted with camera at 100X objective and screened for good metaphase chromosomal spreads.

Statistical analysis

The data obtained from the different treatments was tested statistically by applying TWO-WAY ANOVA (SPSS, version 20) to evaluate the significant differences between each treatment and their interactions. Differences among the means between the groups and within the groups were tested by "Duncan's multiple mean range test" $P < 0.01$.

RESULTS

The highest (Mean \pm SE) number of chromosomal spreads among the treatment groups were recorded in group T9 at 64.46 \pm 2.50 and the lowest (Mean \pm SE) number of chromosomal spreads were recorded in group T1 at 1.93 \pm 1.83 (Table 2). A significant ($P < 0.01$) positive correlation between colchicine concentration and the number of metaphasic chromosomal spreads at various hypotonic timings was observed as presented in Table 3 indicating that increase in colchicine concentration from 0.01% to 0.05%, the number of chromosomal spreads also increased (Table 3). Hypotonic treatment timings also estimated a significant ($P < 0.01$) positive correlation with number of metaphasic chromosomal spreads (Table 4). The modal diploid chromosome number of *C. carassius* was found to be $2n = 100$ (Figure 1). The chromosomes were classified into 20 metacentric, 36 sub-metacentric, 44 sub-telocentric and telocentric chromosomes 20 (M), 36 (SM), 44 (ST/T) (Figure 2).

Figure 3 depicts the frequency percentage of diploid chromosome number ranging from 94 to 102 per

Table 1. Experimental details.

Treatments	Colchicine concentration	Hypotonic treatments timings (mins)	No. of Samples/ treatment	No. of Slides / treatment
T1	0.01%	25	5	15
T2	0.01%	35	5	15
T3	0.01%	45	5	15
T4	0.025%	25	5	15
T5	0.025%	35	5	15
T6	0.025%	45	5	15
T7	0.05%	25	5	15
T8	0.05%	35	5	15
T9	0.05%	45	5	15

Table 2. Average number of metaphase chromosome spreads among various treatments.

Treatments	Number of metaphasic chromosomal spreads (Mean±SE)
T ₁	1.93±1.83
T ₂	3.6±2.84
T ₃	9.6±2.84
T ₄	26.13±2.55
T ₅	40.8±3.40
T ₆	47.86±2.32
T ₇	28.53±2.16
T ₈	50.93±1.79
T ₉	64.46±2.50

Table 3. Correlation between colchicine concentrations and number of metaphase chromosome spreads.

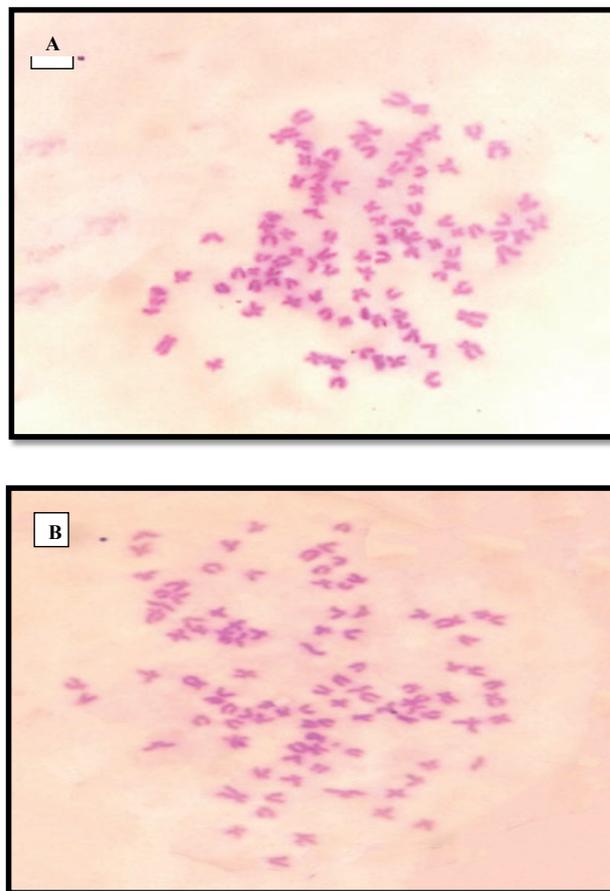
	Colchicine concentrations (25 minutes of hypotonic timings)	Colchicine concentrations (35 minutes of hypotonic timings)	Colchicine concentrations (45 minutes of hypotonic timings)
Metaphase Chromosome Spreads	0.890*	0.942*	0.969*

Table 4. Correlation between Colchicine concentrations and number of metaphase chromosome spreads.

	Hypotonic timings (0.01% Colchicine concentration)	Hypotonic timings (0.025% Colchicine concentration)	Hypotonic timings (0.05% Colchicine concentration)
Metaphase Chromosome Spreads	0.761*	0.939*	0.980*

* Significant at 0.01 level of significance.

metaphases. A total of 250.00 metaphasic chromosomal spreads studied from the cells of kidney tissues of *Carassius carassius* revealed that the modal diploid chromosome number of this species was found to be $2n = 100$ which is valid over 70% of metaphasic cells. Figure 4 depicts the number of metaphase chromosome spreads at different colchicine concentrations (0.01%, 0.025%, 0.05%) and varied hypotonic treatment timing (25, 35, 45 minutes) indicating an increasing pattern of number of chromosome spreads with an increase in colchicine concentration and hypotonic treatment timings.

**Figure 1.** (A&B): Metaphase chromosome spreads of *Carassius carassius* ($2n=100$) at 100x oil emulsion.

DISCUSSION

Chromosomes are best Karyotyped during somatic metaphase whereby one can study chromosomal number, size and morphology. To date, a variety of Karyotyping techniques have been developed to visualise chromosomes of fish at various developmental stages, including tissue cultures (Lomax *et al.*, 2000), squashing techniques (Armstrong and Jones 2003) and cell suspensions of tissues undergoing mitosis (Fan and Fox 1990; Henegariu *et al.*, 2001). Nonetheless, approaches aimed at the embryonic and larval stages of fish have struggled to achieve a consistent number of recognisable and widely distributed metaphase chromosomes, owing to differences in mitotic cell division rates among fish species (Shao *et al.*, 2010). Pretreatment with colchicine combined with various kayotyping techniques has led to a revolution in fish chromosomal studies (Roberts, 1964; Denton and Howell, 1969). Colchicine is a spindle poison used in traditional chro-

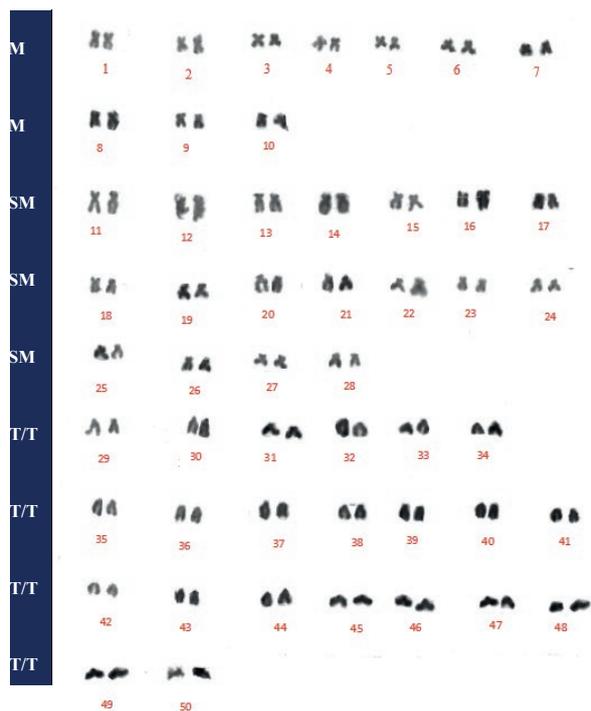


Figure 2. Chromosomal classification of diploid *Carassius carassius* 2n= 100. Metacentric (M)= 20, Sub-metacentric (SM)=36, Sub-telocentric/ Telocentric (ST/T)= 44.

mosomal preparation techniques to stop the cells from entering metaphase (Kligerman and Bloom, 1977). It is a naturally occurring alkaloid found in the *Colchicum autumnale* plant which binds to tubulin, preventing it from polymerizing, affecting microtubule dynamics and causing mitosis to be disrupted, generating a shift in the tubulin dimer, which is largely irreversible. When a tubulin dimer binds to colchicine the polymer becomes unstable and disassembles. (Schliwa 1986). Cells or larvae must be treated in a hypotonic solution after mitotic spindle inhibition to expand the nuclei and disperse the chromosomes over the slides (Moore and Best, 2001). Pretreatment with colchicine solution two hours before collection of the tissue in hypotonic sodium citrate or potassium chloride, followed by bringing the cells in suspension, fixation of cells in Cornoy’s fixative solution, smear on slides. and staining in various stains is now widely used for Karyotypic preparation. The staining techniques (e.g., aceto-orcein, haematoxylin, Giemsa, Wright and Leishman stains) or banding techniques (e.g., Q-banding, G-banding, R-banding, C-banding and high-resolution banding) are used to stain chromosomes for various purposes (Calado *et al.*, 2013; Moore and Best 2001).

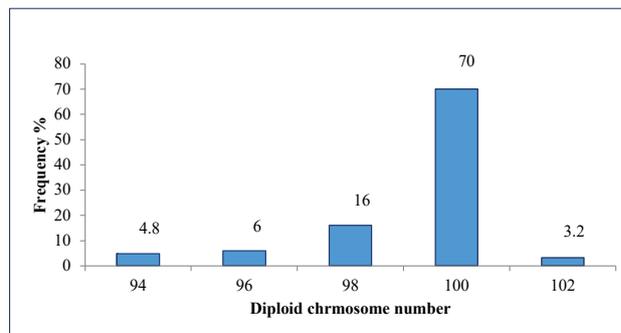


Figure 3. Frequency percentage of diploid Chromosome number of *Carassius carassius*.

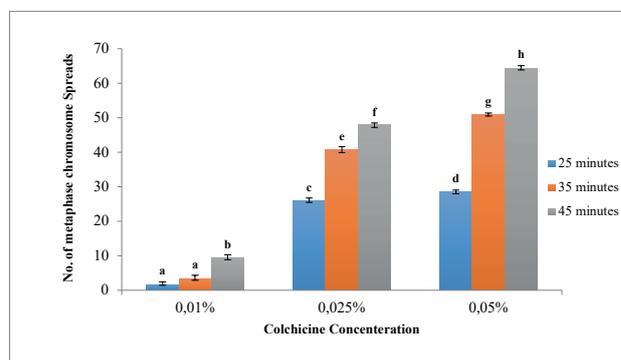


Figure 4. Graphical representation of number of metaphase chromosome spreads at different colchicine concentrations and varied hypotonic treatment timing. Data shown are mean ± SE with different letters are significantly different from each other (P≤ 0.01).

The choice of a right concentration and duration of exposure of colchicine is very important. This is because in sufficient amount of could fail to arrest the target cells amount could fail to arrest the target cells at metaphase stage (Rieder & Palazzo, 1992; Caperta *et al.*, 2006), however too high a concentrations or prolonged exposure, on the other hand, may lead to chromosomal condensation. As a result, the first stage in this technique was to optimize colchicine concentration to inhibit cell division in *Carassius carassius*. The optimum concentration of 0.05% for colchicine recorded in this study is similar to the findings of Karami *et al.* (2015), the authors reported that the optimum concentration of colchicine was observed at 0.05% among the 0.01%, 0.025% and 0.05% tested in African catfish (*Clarias gariepinus*) and the zebrafish (*Danio rerio*). Similarly, Okomoda *et al.* (2018) reported that juveniles of *Pangasianodon hypophthalmus* and *Clarias gariepinus* had better chromosome spreads at 0.05% colchicines concentration compared to 0.01% and 0.025%. This, however, contra-

dicts with the findings of Shao *et al.* (2010) and Pradeep *et al.* (2011) in chromosomal preparation of fish larvae and reported the best metaphase chromosomal spreads at colchicine concentrations of 0.02% and 0.01%, respectively. While, this may be a sufficient amount concentration to penetrate the vitelline membrane of the egg and the thin walls of the larvae, the present study shows this is not optimum for adult *Carassius carassius*.

It's vital to choose a suitable hypotonic solution to swell the mitotic cell nuclei to the point of bursting and spread out the chromosomes after mitotic spindle inhibition (Moore & Best, 2001). Choosing an improper hypotonic solution and incubation period may result in overlapping or significant loss of chromosomes (Baksi and Means, 1988). Hypotonic treatment allows the swelling of the cell, which facilitates cell disruption and the dispersion of chromosomes when the cell contents are spread on slides. Potassium chloride (KCl 0.075 M) is one of the most commonly used hypotonic solutions in chromosomal preparation protocols Bayat and Woznicki, (2006); Shao *et al.* (2010); Pradeep *et al.* (2011); Bazaz *et al.* (2022). The efficiency of potassium chloride (KCl 0.075M) over distilled water was demonstrated by Okomoda *et al.* (2018) reported that the number of clear metaphase chromosome spreads were significantly higher using the former than the latter. Similarly, Ida *et al.* (1978) reported that the use of potassium chloride showed the best chromosome spreads as compared to other two hypotonic solutions of sodium citrate and distilled water. In order to improve chromosome spreads, hypotonic treatment is essential. Hypotonic treatment induces swelling when cell contents are distributed on slides, facilitating cell disintegration and chromosomal dispersion. To procure a desirable number of clear chromosome spreads, the hypotonic solution should be modified according to the species of fish and/or larval age (Karami *et al.*, 2015). The current study found that hypotonic treatment time of 45 minutes was more effective than 25 and 35 minutes, however majority of the chromosomes were overlapped at 25 minutes of hypotonic treatment. In rainbow trout, chromosomal spreading was insufficient at 0.56% KCl for hypotonic treatment at a lower temperature Chourrout and Happe (1986). Furthermore, it was shown that the same concentration of KCl yielded superior results when the experiments were carried out at ambient temperature. However, 0.56% KCl for hypotonic treatment with an adequate time at room temperature provided good results in the current investigation.

Chromosome counts have long been used to characterize an organism or lineages most basic genetic

characteristics. Chromosome counts have been utilized as useful phylogenetic indicators (Guerra 2008) and their role in evolutionary processes has been discussed several times (Mayr 1982; Clark and Donoghue, 2018). Cyprinid fishes have long been the considerable cytogenetic research, with a focus on evolutionary issues (Taki *et al.*, 1977). The 2n number in the Cyprinidae family ranges from 44 to 100 (Arai, 1982). The most common diploid number in the Cyprinidae family is 50, which is regarded as the modal number in this family (Manna, 1984; Rishi, 1989). Cyprinid fishes have short chromosomes with centromere placements that range from median to nearly terminal, making it difficult to allocate some chromosomes to specific chromosomal groups as a result, making accurate identification of individual chromosomes nearly impossible (Rab and Collares-Pereira, 1995). The results of the current study revealed that *Carassius carassius* (crucian carp) possess 2n=100 chromosomes in its somatic cells. The results of the current study are in agreement with findings of Spoz *et al.* (2014); Knytl *et al.* (2013); Boron *et al.* (2010); Wang *et al.* (1995); Kasama and Kobayasi (1991); Mayr *et al.* (1986); Sofradzija *et al.* (1978); Hafez *et al.* (1978); and Kobayasi *et al.* (1970), as they all reported the same diploid chromosome count of 2n=100. However, Raicu *et al.* (1981) reported the diploid chromosome number 2n = 50 in Danube Delta of *Carassius carassius* individuals. In contrast to Raicu *et al.* (1981), Chiarelli *et al.* (1969) reported a diploid chromosomal number of 2n=104, which might most likely be attributed to preparation artifact.

CONCLUSION

The results obtained from the present study showed that 0.05% colchicine concentration resulted highest number of chromosomal spreads compared to other colchicine concentrations. KCl treatment for 45 minutes in *Carassius carassius* proved to be more effective than other treatment timings in terms of chromosome spread quality and highest number of metaphase chromosome spreads. A significant ($P < 0.01$) positive correlation between colchicine concentration and the number of metaphasic chromosomal spreads at various hypotonic timings indicating an increase in colchicine concentration from 0.01% to 0.05%, the number of chromosomal spreads also increased. The modal chromosome number of *Carassius carassius* species was found to be 2n = 100.

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