Optimization of the cytogenetic protocol for *Pangasianodon hypophthalmus* (Sauvage, 1878) and *Clarias gariepinus* (Burchell, 1822)

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To obtain well spread chromosomes, the cytogenetic protocol for *Pangasianodon hypophthalmus* and *Clarias gariepinus* were optimised. This includes, the colchicine concentration (0.01, 0.025, 0.05 %) / exposure duration (1, 3, and 5 h), hypotonic solution (distilled water or 0.075M KCl solution) /exposure duration (30min, 1h, and 2h), the time of cell suspension preparation (at hypotonic treatment or before slide preparation) and chromosome aging period (0, 3, and 7 days in Carnoy's fixative). In addition, the type (i.e. fin, gill or kidney) and the amount of tissue (10, 50, 100 or 150mg) were also investigated. Regardless of the species, the result obtained showed that well-spread chromosomes could be obtained using the following optimised protocol: Juveniles are injected with 0.05% colchicine (at $1mlkg^{-1}$) and allowed to swim for 3h. Then, 50mg of gill tissue is made into cell suspension in 0.075M KCl for 1h. The cell suspension is treated in Carnoy's fixative (changed 3times at 20min interval) and then aged for three days. Finally, chromosome slides are made and stained with 10% Giemsa for 1h.

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Abstract

To obtain well spread chromosomes, the cytogenetic protocol for *Pangasianodon hypophthalmus* and *Clarias gariepinus* were optimised. This includes, the colchicine concentration (0.01, 0.025, 0.05 %) / exposure duration (1, 3, and 5 h), hypotonic solution (distilled water or 0.075M KCl solution) /exposure duration (30min, 1h, and 2h), the time of cell suspension preparation (at hypotonic treatment or before slide preparation) and chromosome aging period (0, 3, and 7 days in Carnoy's fixative). In addition, the type (i.e. fin, gill or kidney) and the amount of tissue (10, 50, 100 or 150mg) were also investigated. Regardless of the species, the result obtained showed that well-spread chromosomes could be obtained using the following optimised protocol: Juveniles are injected with 0.05% colchicine (at 1mlkg^{-1}) and allowed to swim for 3h. Then, 50mg of gill tissue is made into cell suspension in 0.075M KCl for 1h. The cell suspension is treated in Carnoy's fixative (changed 3times at 20min interval) and then aged for three days. Finally, chromosome slides are made and stained with 10% Giemsa for 1h.

Keywords: African catfish, Asian catfish, Colchicine, Giemsa stain, metaphase.

INTRODUCTION

The first level of genome analysis of any organism involves karyotyping of mitotic chromosomes to determine the genome organization at the cytological level (Gormam, 1973). It is an essential tool in providing basic information on breeding programs such as inter-

specific/generic hybridization (Crego-Prieto et al., 2013), polyploidy induction (Christopher et al., 2010; Gilna et al., 2014; Thresher et al., 2014), and genetic improvement of commercial exploited or novel fish stocks targeted towards commercialization (Gui and (Zhu et al., 2012). An effective method of chromosome preparation is essential for cytogenetic research (Shao et al., 2010). Several techniques have been optimised to obtain well spread mitotic chromosomes in fish and this may differ for different species (e.g. as observed by Karami et al., 2010). These are usually in terms of chemical types, concentration, and duration of exposure. Firstly, the cells spindle fiber is arrested at metaphase by inoculation with a spindle poison (Rieder and Palazzo 1992; Silva et al., 2011). After which the cells are incubated in an appropriate hypotonic solution, to ensure swelling and bursting of the nuclei (Moore and Best 2001). This is then followed by fixation in Carnoy's fixative solution, preparation of a cell suspension, slide and finally staining (Moore and Best 2001; Wang et al., 2010; Calado et al., 2013).

Despite the ease of chromosome preparation from eggs and larvae of fish (Shao et al., 2010, Karami et al., 2010; Amar-Basulto et al., 2011; Botwright 2015), cytogenetic studies involving specific tissues of fingerlings, juvenile or adult fish may have some merits over the use of whole larvae or egg. Aside the possibility of having a large amount of tissue from which metaphase chromosome can be isolated; this method has a pride of place in the characterization of intergeneric distance crosses between different fish species. This is because of the need to match phenotypic characteristics of the progenies gotten with their equivalent ploidy levels because of the possible presence of ploidy polymorphism (see e.g. Liu et al., 2007; Zou et al., 2007; Zhao et al., 2015). However, due to similarities in egg or larvae of fishes, it is practically impossible to march phenotypic characters to different ploidy levels at this stage of development.

Chromosome preparation in post larvae fishes can be isolated from different tissues. These includes; fins (McPhail and Jones, 1966), gills (Yoo et al., 2017), scales (Ojima et al., 1972), kidney (Zhao et al., 2015; Huang et al., 2017) abdominal cavity fluid (Fan and Fox, 1990), gonads (Tan et al., 2004) to mention but a few. Despite successfully chromosomes isolation from these tissues, variations in the mitotic cell division rates could result in differences in the quality and quantity of the chromosome observed. More so, the quantity or amount of tissue used for this processes could affect the concentration of cell suspension and consequently the visibility of chromosome spread. However, to the knowledge of the researchers, there has been no report in

which the tissue amount was optimised. Hence, in addition to colchicine and hypotonic treatment, this study attempted to optimise different tissue type/amount as well as identify the appropriate time for making cell suspension and the effects of different aging time on metaphase chromosomes of two important catfishes namely; *Pangasianodon hypophthalmus* (Sauvage, 1878) and *Clarias gariepinus* (Burchell, 1822).

Materials and Methods

Juveniles of *P. hypophthalmus* and *C. gariepinus* (weighing between 10-50g) were obtained from the School of Fisheries and Aquaculture Science hatchery of the Universiti Malaysia Terengganu, in Malaysia. They were acclimatized for two weeks in rectangular fiberglass tanks and fed on a commercial diet (35% Crude Protein) till the experiment was conducted. The method of Liu et al. (2007) was used as the basis of protocol optimization in a stepwise manner. For each optimised procedure, five fish were used (per species) for each treatment. Firstly, colchicine concentration and the duration of inoculation were investigated. Juveniles were intramuscularly injected with freshly prepared colchicine solution at 0.01, 0.025, and 0.05% colchicine for 1, 3 and 5 h at $1mlkg^{-1}$ of the body weight of the juvenile. Using the selected concentration and duration of colchicine, the suitability of distilled water and KCl solution (0.075M) has a hypotonic solution was tested for 0.5, 1 and 2h. It is important to state that cytogenetic chemical treatment after colchicine treatment was done in a 1.5ml tube and a uniform volume of 600µl of these chemicals was adopted for comparative purposes. Following colchicine and hypotonic solution optimization, 10, 50, 100 or 150mg of fins, gill and kidney were extracted and followed by the procedure of Liu et al. (2007).

No alternative fixative, ratio or duration of exposure was considered in this study as freshly prepared Carnoy's solution (methanol-acetic acid at ratio 3:1) is commonly used with a unanimous exposure time of 20 mins with three changes (Fopp-Bayat and Woznicki 2006; Karami et al., 2010; Pradeep et al., 2011 etc.). However, the best time for making cell suspension was determined by initiating the process (i.e. chopping the tissue) during hypotonic treatment or prior to slide preparation (after fixation in Carnoy's solution). Before every subsequent step in the formal treatment, the suspension is centrifuged at 2500rcf for 10min, and then the supernatant is discarded leaving 1ml of the solution above the cell pellet. The cell is then re-suspended using the next solution. Also, the effect of aging was investigated on the quality of chromosome spread. The cell suspension or tissue was allowed to age for 0, 3 and 7days in the

Carnoy's solution followed by slide preparation. In all trial, slides were prepared by dropping method (two drops of the cell suspension on the slide at 1m height) and incubation in 10% Giemsa stain (prepared in 0.01M phosphate buffer at pH 7) for 1h. Similarly, the metaphase spreads in all the trials were microphotographed using a Nikon Eclipse 80i compound microscope, and the images processed using the NIS element Basic Research software (at 100× magnification).

In all the trial, the number of the well-spread chromosome was recorded except for the hypotonic treatment where the percentage of the complete / well-spread chromosome was computed. Prior to running analyses of data gotten in this study, normality, and homogeneity of data was tested (Tabachnick and Fidell 2001). For all treatment involving concentration/types vs duration of exposure (i.e. colchicine, and hypotonic solution respectively), a two-way Analysis of variance (ANOVA) was employed to evaluate the interactions. A similar analysis was done for tissue type vs amount (quantity) and for the time of cell suspension preparation vs aging period. All data analysis in this study was done using Mini tab 14 computer software.

Results

The result obtained shows that the juveniles injected with 0.05% colchicine (at $1mlkg^{-1}$) for 3h had better chromosome spread in both species when compared to other concentration and exposure times (Figure 1a and 2a). Treatment in KCL for 1hr (Figure 1b and 2b) and the use of 50mg of gill tissue sample (Figure 1c and 2c) also proved to be more effective in this study. Also, preparation of cell suspension before hypotonic treatment and ageing for 3days resulted in well spread chromosomes (Figure 1d and 2d). A sample of the metaphase chromosome produced in *Clarias gariepinus* and *Pangasianodon hypophthalmus* are presented in Figure 3.

Discussions

The choice of a right concentration and duration of exposure of colchicine is very important. This is because insufficient amount could fail to arrest the target cells at metaphase stage (Rieder and Palazzo 1992; Caperta et al., 2006), however too high a concentrations or prolonged exposure, on the other hand, may lead to chromosomal condensation (Wood et al., 2001). The optimum values recorded in this study for colchicine (i.e. 0.05% for 3h) is similar to the findings for optimization in post-larval stages of some other fish species (e.g. Liu et al., 2001; Botwright

2015; Zhao et al., 2015; Huang et al., 2017). This is, however, at variance with the finding of Shao et al., (2010) for eggs and larvae of Japanese flounder (*Paralichthys olivaceus*) and summer flounder (*Paralichthys dentatus*) as they reported best metaphase chromosomes with 0.02% for 1-2hours. While this may be a high enough concentration to penetrate the vitelline membrane of the egg and the tin walls of the larvae, the present study shows that this is not optimum for the post-larva of African and Asian catfishes. This is despite using intramuscular injection procedure which delivers the spindle poison directly to the fish bloodstream ensuring effective circulation to all tissues.

Subsequent upon mitotic spindle inhibition, it is pivotal to use an appropriate hypotonic solution to swell the nuclei of the mitotic cell to the point of bursting and spread out the chromosomes (Moore and Best 2001). Choosing an improper hypotonic solution and incubation period may result in overlapping or significant loss of chromosomes (Baksi and Means 1988). The efficacy of Potassium chloride (KCl 0.075 M) over distil water was demonstrated in this study. In both species, the number of clear metaphase chromosome spreads was significantly higher using the former than the latter. Karami et al., (2010) had earlier stated that using KCl caused extensive cell burst and chromosomal loss when compared to distil water treated larvae of *C. gariepinus*. However, chromosome loses in this study was observed in both hypotonic solutions when the tissue was incubated beyond 1hour while below this reference point, metaphase chromosomes were largely overlapping. The differences in observation of the two studies despite the similarity of the species may be linked to the different developmental stages of fish used.

The present study has for the very first time, shown that too much or too little of tissue amount could affect the quantity of identifiable metaphase chromosomes. The optimum amount as observed in this study was 50mg. While the scanty number of chromosome observed below 50mg could be explained by reduced cell concentration, the observation beyond the reference amount may be because of high cell/tissue concentration which resulted into darkening of the slide background. Hence, this may have covered some well spread metaphase chromosome and the spaces between chromatids, consequently making them unobservable. However, this may be remedied (hypothetically) by respectively concentrating or diluting the final volume of cell suspension prepared in the Carnoy's solution. The difference in the results from the different

tissue is largely connected with the mitotic cell division rates of these tissues as earlier stated. This assumption is similar to the findings of Shelton et al. (1997).

The observation of a higher number of well-spread metaphase chromosomes in the gills may be connected to the vulnerability of the gills to environmental imbalances (Moyle and Cech, 1996). This may imply that cell division in this organ could be more rapid to compensate damage from environmental influences. Wakahara (1972) had earlier demonstrated that the mitotic cell division rate of the ventral tail-fin epidermis of the larval African clawed frog (*Xenopus laevis*) is largely affected by changes in environmental factors. Therefore, future studies can be designed to understand the effect of environmental changes on the proliferation of the mitotic cell in different tissues of fish. However, the differences in mitotic division rates may also explain the differences in the response to colchicine and hypotonic solutions treatment by the two fishes understudied in this research.

The observation from this study suggests that making cell suspension during hypotonic treatment led to increased number of observable metaphase chromosome than initiating this process prior to slide preparation. The efficiency of the former is likely connected to the increased surface area of the dissociated cell to the different chemical treatments, hence making their effect more pronounced than the latter whose tissue was intact through the chemical treatment phase until slide preparation. Similarly, aging of the cell suspension or tissue for three days in the Carnoy's fixative produces better chromosome spread than preparing slide on the very day of cytogenetic treatment or seven days after. While the underlining principles responsible for this observation are not well understood, it was speculated that aging might have dissociation effect on the chromosomes. This is because appreciable percentages of the identifiable chromosome in the slide prepared on the very day of cytogenetic treatment were compacted and overlapping, while significant chromosome loss was characteristics of the observations after seven days of aging. However, these observations are lesser when cells were aged for just three days.

Conclusions

The metaphase chromosome produced in this study could be used for karyotype analysis. Although species-specific technicalities have been recommended to obtain well-spread metaphase chromosomes in different species (Karami et al., 2010), the method described in this

study, however, seem to give a satisfactory result for both species using similar protocol. The optimised protocol reported in this study may also be effective for cytogenetic studies involving other closely related catfish species (*Clarias macrocephalus*, *Heterobranchus longifilis* *Pangasius gigas* etc). This could be the focus of future researches.

Acknowledgements

The authors are indebted to the School of Fisheries and Aquaculture Science, Universiti Malaysia Terengganu, Malaysia for providing juveniles of *P. hypophthalmus* and *C. gariepinus* used in this study. We also acknowledge the help of some technical staffs of the PPSPA hatchery department and laboratory officers of AKUATROP during experimental trials of this study. This study is part of the first author's Ph. D research.

Compliance with Ethical Standards

We declare that no fund was received for the conduct of this research; hence, we have no conflict of interest what so ever (financial or otherwise). More so, all applicable guidelines for the care and use of animals were followed for this study (which includes international, national, and/or institutional guidelines).

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Figure 1(on next page)

Effects of different treatments on the number of clear and identifiable metaphase chromosome spreads in *Clarias gariepinus*.

(A) Colchicine concentration × duration of exposure interaction. (B) Tissues type × mass of tissue interaction. (C) Hypotonic treatment × duration of exposure. (D) Cell suspension preparation × aging time. Data shown are Mean±SE. Bars with different letters are significantly different from each other (P≤0.05).

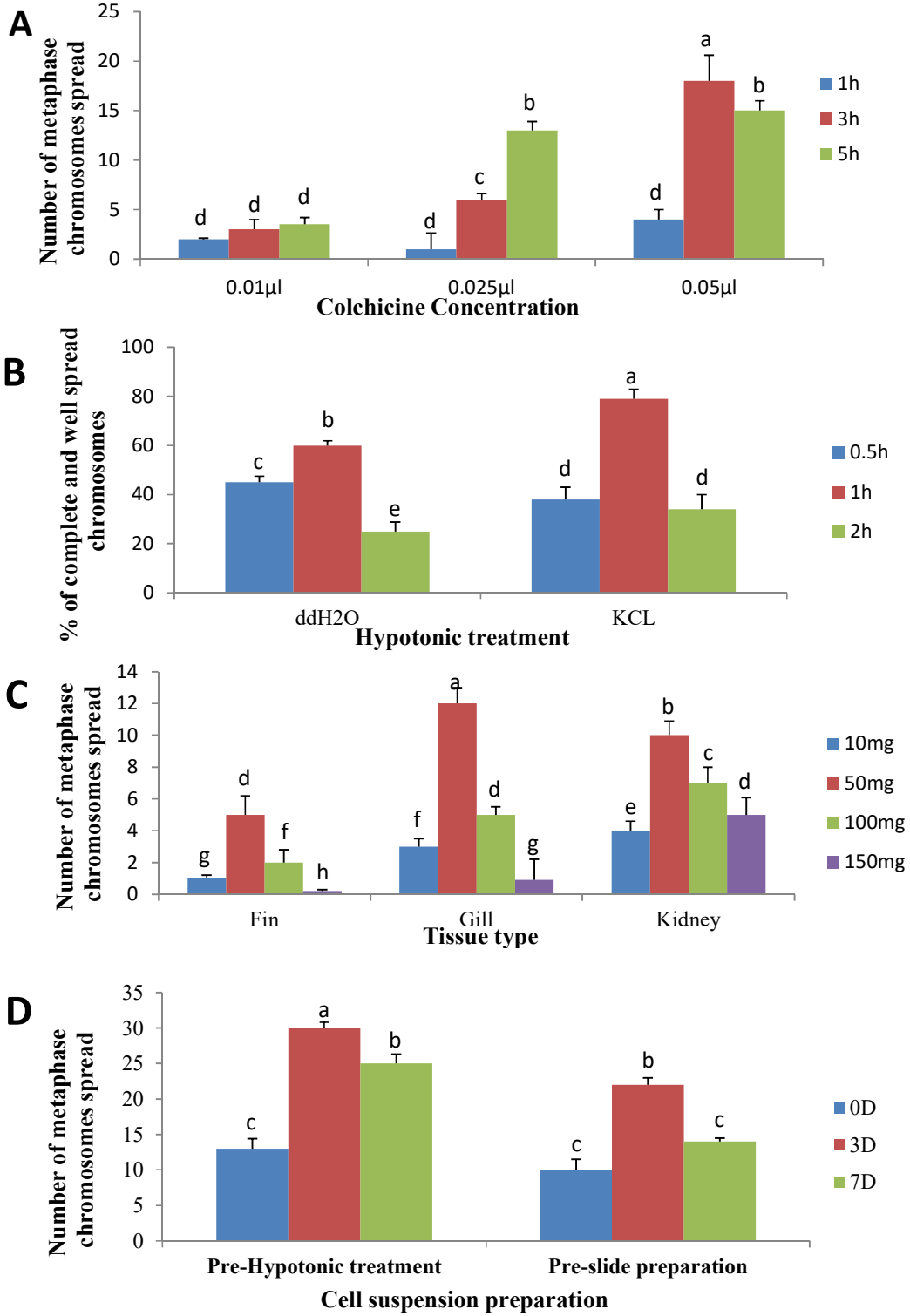

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Figure 2(on next page)

Effects of different treatments on the number of clear and identifiable metaphase chromosome spreads in *Pangasianodon hypophthalmus*.

(A) Colchicine concentration × duration of exposure interaction. (B) Tissues type × mass of tissue interaction. (C) Hypotonic treatment × duration of exposure. (D) Cell suspension preparation × aging time. Data shown are Mean±SE. Bars with different letters are significantly different from each other (P≤0.05).

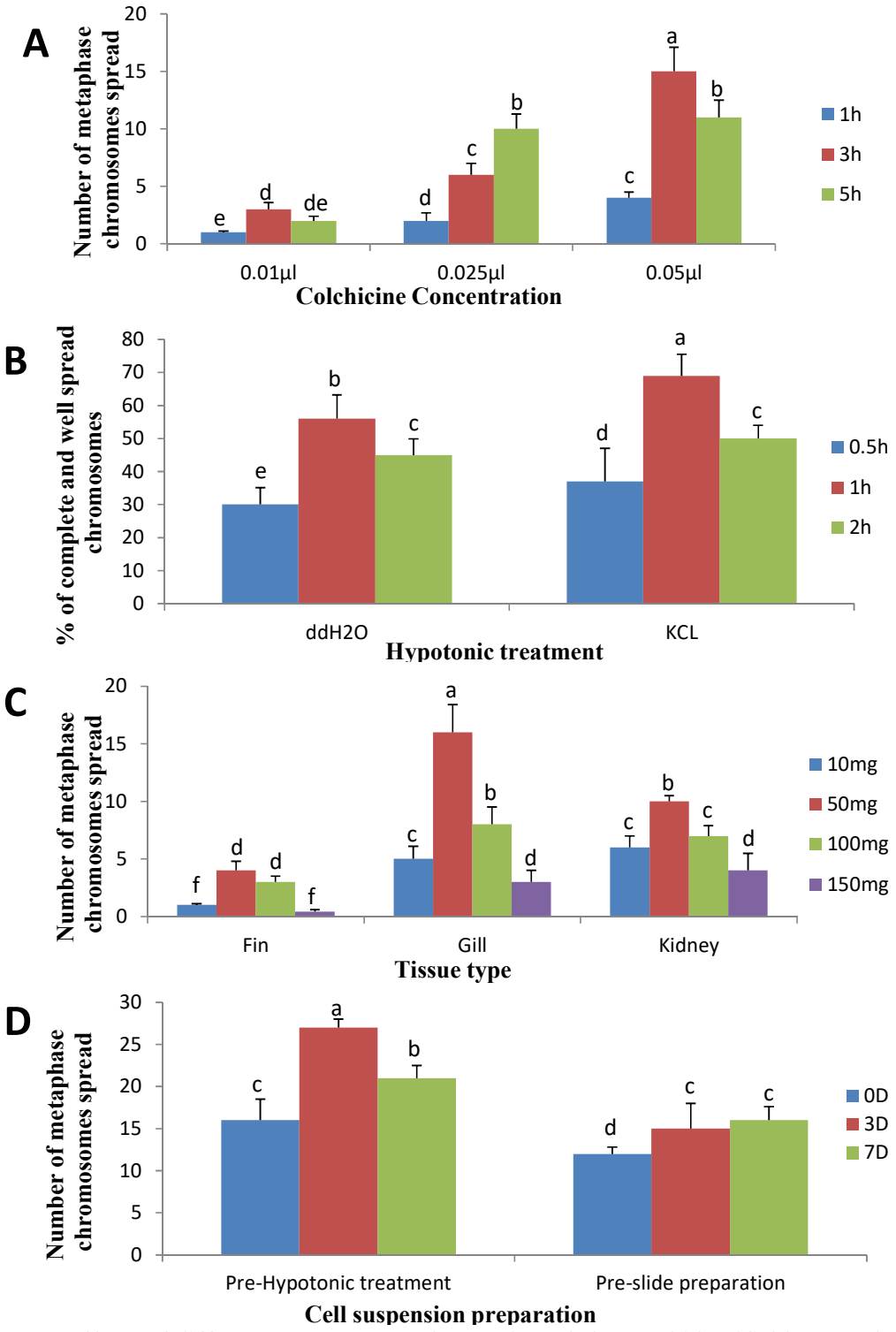

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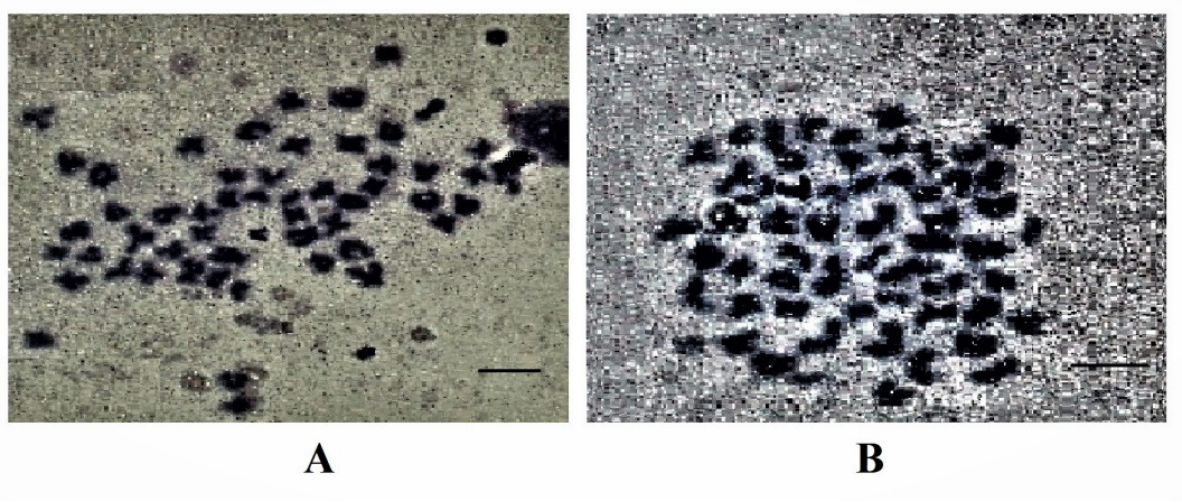
Figure 3(on next page)

Figure 3: Metaphase chromosome of (a) *Clarias gariepinus* (2n=56) and (b) *Pangasianodon hypophthalmus* (2n=60).

Bar =5µm.



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