Mutation Research 726 (2011) 209–214

Contents lists available at SciVerse ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Profenofos induced DNA damage in freshwater fish, *Channa punctatus* (Bloch) using alkaline single cell gel electrophoresis

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A R T I C L E   I N F O

Article history:
Received 18 March 2011
Received in revised form 6 August 2011
Accepted 19 September 2011
Available online 25 September 2011

Keywords:
Channa punctatus
Comet assay
DNA damage
Genotoxicity
Profenofos

A B S T R A C T

The aim of the present study was to evaluate the induced genotoxicity (DNA damage) due to organophosphate pesticide profenofos (PFF) in gill cells of freshwater fish *Channa punctatus* using single cell gel electrophoresis (SCGE)/Comet assay. The 96 h LC_{50} value of PFF (50% EC) was estimated for the fish species in a semistatic system and then three sub-lethal of LC_{50} concentrations *viz* the sub-lethal 1, sub-lethal 2 and sub-lethal 3 concentrations were determined as 0.58 ppb, 1.16 ppb and 1.74 ppb, respectively. The fish specimens were exposed to these concentrations of the pesticide and the gill tissue samplings were done on 24 h, 48 h, 72 h and 96 h post exposure for assessment of DNA damage in terms of percentage of DNA in comet tails. In general, a concentration dependent response was observed in the gill cells with induction of maximum DNA damage at the highest concentration of PFF. The results of the present investigation indicated that PFF could potentially induce genotoxic effect in fish, even in sub-lethal concentrations and SCGE as a sensitive and reliable tool for *in vivo* assessment of DNA damage caused by the genotoxic agents.

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1. Introduction

Pesticides of various categories are frequently used against a number of pests, in the field to increase the crop production, though these chemicals are highly toxic to other species in the environment [1,2]. Pesticide residues reach the aquatic environment and represent a risk for the non-target organisms and finally finding their way to the food chain threatening the ecological balance and the biodiversity of the nature [3,4]. These contaminators of surface waters have been well documented worldwide and constitute a major issue that gives rise to concerns at local, regional, national and global scales [5,6].

Profenofos (*O*-4-bromo-2-chlorophenyl-*O*-ethyl *S*-propyl phosphorothioate), is a broad-spectrum organophosphate pesticide used widely for agricultural and household purposes in India [1,7,8] and other countries *viz* Australia [9], China [10], Korea [11], Pakistan [12], and Egypt [13]. Profenofos (PFF) had been investigated to be highly toxic to different organisms including mammals [14], insects [15], and fish [12,16]. It also has been classified as moderately hazardous (toxicity class II) pesticide by WHO and it has a moderate order of acute toxicity following oral and dermal administration [17].

Genotoxicity and mutagenicity of pesticides for non-target organisms and their influence on ecosystems are of worldwide concern [18]. The genotoxic chemical becomes more dangerous when it possesses bio-accumulative properties and enters in the food chain of the ecosystem. Fishes as bio-indicators of pollutant effects are very sensitive to changes in their environment and play significant roles in assessing potential risk associated with contaminations of new chemicals in aquatic environment [19]. The pesticide, owing to its stability, contaminates the aquatic environment even at sub-lethal concentrations and tends to accumulate in tissues of aquatic organisms [20,21].

Fishes are being used as useful genetic models for the evaluation of pollution in aquatic ecosystems [22]. Ecotoxicological characteristics of air-breathing freshwater food fish *Channa punctatus* such as its wide distribution and availability throughout the year, easy maintenance in the aquaria/wet lab, and commercial importance make this species an excellent model for toxicity studies [21,23]. The effects of genotoxicity are reported to be several-folds on fitness traits like reproductive success; genetic patterns and subsequent population dynamics in fish have been highlighted during genotoxicity assessment experiments [24,25]. Since there is growing concern over the presence of genotoxins in the aquatic environment, it is important to develop methods for detection of genotoxic effects in aquatic organisms [26,27].

The single cell gel electrophoresis (SCGE) or Comet assay, detects DNA strand breaks and alkali labile sites by measuring the

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doi:10.1016/j.mrgentox.2011.09.011

migration of DNA from immobilized nuclear DNA [28] and useful technique for environmental contamination bio-monitoring [29]. This is a rapid and sensitive procedure to measure DNA lesions in any organ regardless of its mitotic activity. A number of studies have shown that SCGE or Comet assay is the most effective assay for detection of genotoxic effect under the field and laboratory conditions [21,23,30,31]. This assay has been widely applied to aquatic environment both on vertebrate and invertebrate organisms for the detection of the genetic/chromosomal damage after exposure to genotoxic agents [32–34]. An increase in global food demand has resulted in a significant increase in the use of pesticides in agriculture. This has caused great concern among health and environmental scientists, since some of these chemicals induce mutations (somatic as well as germ-line) in experimental systems in various *in vitro* and *in vivo* studies [35,36].

The information on DNA damaging nature of PFF in aquatic organisms is meager especially the data pertaining to the acute genotoxic effect in fish. Therefore the present study was undertaken to investigate the DNA damage due to PFF using Comet assay in gill cells of *C. punctatus* after *in vivo* exposure.

2. Materials and methods

2.1. Experimental fish specimens

The freshwater fish *C. punctatus* (Bloch) belongs to family: Channidae and order: Perciformes, was obtained from the local sources. The specimens were treated with 0.05% $KMnO_4$ solution to avoid any dermal infections. They were acclimated in the laboratory condition for 10 days before experimentation. They were kept in a large holding FRP tank of 500 liters capacity during the acclimatization. Length and weight of the fish ranged from 12.0 ± 3.0 cm and 23 ± 2.0 g, respectively. Fishes were fed on boiled chicken, eggs or poultry waste material daily.

2.2. Test chemical

The pesticide for the study, Technical-grade Profenofos (50% EC), CAS No. 41198-08-7 with product name CELCRON (manufactured by EXCEL Crop Care Ltd., Mumbai, India) was purchased from the local market. Low melting agarose (LMA), normal melting agarose (NMA), Triton X-100, disodium ethylene diamine tetra acetate (Na_2-EDTA), dimethyl sulfoxide (DMSO), Tris-(hydroxy methyl)-amino methane (Tris–HCl), ethidium bromide (EtBr) and all other common chemicals were obtained from Sigma.

2.3. Determination of sub lethal concentration

The acute toxicity bioassays to determine the LC_{50} – 96 h value of PFF were conducted in the semi-static system. A set of 10 acclimatized fish specimens was randomly exposed to each of the three PFF target concentrations. The LC_{50} – 96 h value of PFF was determined as 2.31 ppb for *C. punctatus* using SPSS software and was found to be 2.675 ppb by arithmetic method of Karber as described by Dede and Kaglo [37]. Based on the LC_{50} – 96 h value using SPSS software, the three test concentrations of PFF *viz* sub-lethal 1 (25% of LC_{50} = 0.58 ppb), sub-lethal 2 (50% of LC_{50} = 1.16 ppb) and sub-lethal 3 (75% of LC_{50} = 1.74 ppb) were used for the assessment of genotoxicity of the pesticide.

2.4. In vivo exposure experiment

The fish specimens were exposed to the three aforementioned test concentrations of PFF in a semi-static system for 96 h. The exposure was continued up to 96 h and tissue sampling was done at intervals of 24, 48, 72, and 96 h on each sampling day. The gill cells were collected and immediately processed for Comet assays.

2.5. Cell viability assay

Before running the SCGE, viability of cells was tested in gill tissue. The gill tissue was washed two times with chilled phosphate buffer saline (Ca^{2+} Mg^{2+} free) to remove blood cells and transferred to ice-cold homogenization buffer (1× Hanks' balanced salt solution, 20 mM EDTA, 10% dimethyl sulfoxide (DMSO), pH 7.0–7.5). The tissue was cut into small pieces using scissors and finally homogenized to obtain single-cell suspension. The cell suspension was centrifuged at 3000 rpm at 4 °C for 5 min and the cell pellet was finally suspended in chilled phosphate buffered saline for Comet assay. Viability of gill cells was evaluated by the Trypan blue exclusion test [38]. The tissue samples showing cell viability higher than 85% were further processed for SCGE/Comet assay.

2.6. SCGE/Comet assay

The alkaline SCGE/Comet assay was performed as a three-layer procedure [28] with slight modifications [39].

In brief, about 15 μl of cell suspension (approx. 20,000 cells) were mixed with 85 μl of 0.5% low melting-point agarose (LMPA) and layered on one end of a frosted glass slide pre-coated with a layer of 200 μl of 1% normal agarose. It was covered with a third layer of 100 μl LMPA, after solidification of the gel and then the slides were immersed in lysing solution (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, pH 10, with 10% DMSO and 1% Triton X-100, added fresh) overnight at 4 °C. The slides were placed side by side in a horizontal gel electrophoresis unit immersed in fresh cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2-EDTA and 0.2% DMSO, pH > 13.5), and left in solution for 20 min at 4 °C for the DNA unwinding and conversion of alkali-labile sites to single strand breaks. Alkaline electrophoresis was carried out using the same alkaline electrophoresis buffer for 20 min using 15 V (0.8 V/cm) and 300 mA at 4 °C. The slides were neutralized gently with 0.4 M Tris buffer at pH 7.5 and stained with 75 μl ethidium bromide (20 μg/ml). For positive control, the gill cells were treated with 100 μM H_2O_2 for 10 min at 4 °C. Comet images were analyzed using a comet image analyzer system (Komet-5.5, Kinetic Imaging) attached to the fluorescent microscope (Leica) equipped with appropriate filters. Images of 100 randomly selected cells (50 cells from each of two replicated slides) were scored randomly from each specimen. The parameter selected for quantification of DNA damage was percent tail DNA (i.e. % tail DNA = 100 − % head DNA) as determined by the software.

2.7. Statistical analysis

Statistical analysis was performed with the SPSS 10.1 computer program (SPSS Inc. Chicago, IL, USA). The results were expressed as mean ± SE. The one-way analysis of variance (ANOVA) was employed to compare the mean differences in % tail DNA damage. A *p* values less than 0.01 was considered statistically significant.

3. Results

3.1. Physicochemical properties of the test water

The temperature of test water varies from 18.2 to 24.6 °C and pH values ranged from 7.1 to 8.3. The dissolved oxygen (DO) ranged from 6.0 to 8.4 mg/l. The conductivity of the water ranged from 250 to 304 μM/cm while total hardness ranged from 166 to 185 mg/l during experiment period, respectively.

3.2. LC_{50} and application factor

Acute toxicity bioassays, of different lethal concentrations at different exposures are listed in Table 1. A dose dependent increase and time dependent decrease were observed in mortality rate from 24 to 96 h. The safe levels of PFF at 96 h LC_{50} value based and using with different "application factors (AF)" are listed in Table 2. The safe level values of PFF in *C. punctatus* under estimation varied from 2.31×10^{-1} to 2.31×10^{-5} ppb.

3.3. DNA damage

The DNA damages measured as percentage tail DNA in the gill tissues of the control and exposure groups are presented in Fig. 1, Table 3. The DNA strand breaks after exposure to PFF in fish *C. punctatus* are shown in Fig. 2. The fish specimens exposed to different concentrations of PFF exhibited significantly higher DNA damage ($p < 0.01$) in their tissue than the control samples. In general, the DNA damage was found to be concentration-dependent in gill tissues, with the highest damage at the sub-lethal 3 concentration, followed by sub-lethal 2 and sub-lethal 1. The highest DNA damage was observed on 96 h in gill cells (23.14%) at the highest concentration.

With respect to the effect of duration on DNA, a significant effect ($p < 0.01$) in gill cells was also observed in specimen exposed to various PFF concentrations. The lowest DNA damage was observed at 24 h followed by linear increase. The highest DNA damage was observed on 96 h in all treatment groups.

Table 1
Lethal concentrations of profenofos (ppb) (95% confidence intervals) depending on exposure time for *C. punctatus*.

Lethal concentration	Exposure Time (h)			
	24	48	72	96
LC_{10}	3.14 (2.03–3.74)	2.07 (1.12–2.64)	1.33 (0.57–1.86)	1.07 (0.52–1.49)
LC_{50}	4.59 (3.94–5.15)	3.53 (2.85–4.14)	2.74 (2.02–3.38)	2.31 (1.74–2.84)
LC_{90}	6.72 (5.85–9.31)	6.03 (4.97–9.45)	5.61 (4.37–9.95)	4.99 (3.90–8.04)

Table 2
Estimation of safe levels of profenofos at 96 h exposure duration.

Chemical	96 h LC_{50} (ppb)	Method	AF	Safe level
Profenofos	2.31	Sprague [80]	0.1	2.31×10^{-1}
		CWQC [81]	0.01	2.31×10^{-2}
		NAS/NAE [82]	0.1–0.00001	2.31×10^{-1}–2.31×10^{-5}
		IJC [83]	5% of 96 h LC_{50}	0.12

Table 3
Mean ± SE % tail DNA damage observed in the gill cells of *C. punctatus* due to exposure at different concentrations of profenofos.

Exposure time (h)	Exposure concentration				
	Control	Positive control	0.58 ppb	1.16 ppb	1.74 ppb
24	3.64 ± 0.32^{a1}	6.14 ± 0.66^{a23}	5.21 ± 0.30^{a2}	7.86 ± 0.34^{a34}	9.15 ± 0.34^{a4}
48	3.64 ± 0.32^{a1}	6.14 ± 0.66^{a2}	8.15 ± 0.48^{b23}	9.97 ± 0.50^{ab34}	11.27 ± 0.56^{a4}
72	3.64 ± 0.32^{a1}	6.14 ± 0.66^{a2}	9.45 ± 0.49^{b3}	11.68 ± 0.56^{bc3}	17.05 ± 1.02^{b4}
96	3.64 ± 0.32^{a1}	6.14 ± 0.66^{a2}	9.74 ± 0.40^{b3}	13.97 ± 0.60^{c4}	23.14 ± 0.84^{c5}

Note: Values with different alphabets differ significantly ($p < 0.01$) between durations within concentration. Values with different numeric superscripts differ significantly ($p < 0.01$) between concentrations within duration.

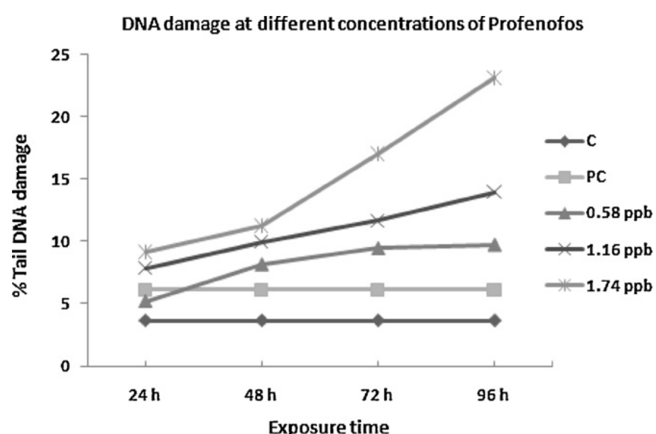

Fig. 1. DNA damage in gill cells of *C. punctatus* induced due to exposure at different concentrations of profenofos.

4. Discussion

In the recent years, several studies have been reported on the SCGE or Comet assay [40,41] to evaluate environmental genotoxic agents in different organisms, *viz* mammals [42,43], birds [44], reptiles [29,45], mollusk [46,47] and amphibians [47,48]. The various species of fishes (freshwater and marine) have been used for environmental biomonitoring *viz* Zebra mussel (*Dreissena polymorpha*) [49], *C. punctatus* [27,50,51], sea catfish [52], bullheads (*Ameiurus nebulosus*) [53] and carps (*Cyprinus carpio*) [54,55]. Fish and aquatic invertebrates can serve as excellent source of material for the study of genotoxic, mutagenic and carcinogenic potential of toxicants for environmental risk assessment [51,56].

Fishes are often used as sentinel organisms for ecotoxicological studies because they play a number of roles in the trophic web, accumulate toxic substances and respond to low concentration of mutagens [52,57]. Therefore, the importance of uses of fish as bio-indicators of the effects of pollution is increasing and can permit early detection of aquatic environmental problems [58,59].

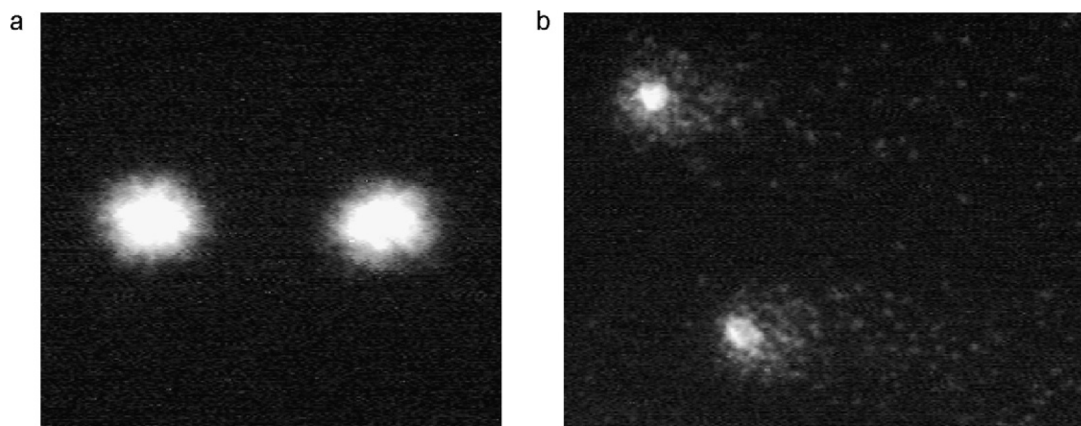

Fig. 2. Gill cells of *C. punctatus* showing: (a) control DNA and (b) DNA damaged after exposure to profenofos.

In the Comet assay many fish tissues can be extensively used, e.g., gill, brain, liver, and blood, however, as non-invasive method i.e. without killing the animal, is highly preferred [60,61].

The concurrent assessment of cytotoxicity is critically important for data interpretation. For gill cell viability test, a part of the cell suspension was used for Trypan blue exclusion test and another part for Comet assay. Tice et al. [62] were of view that an assessment of *in vivo* cytotoxicity on simple dye exclusion techniques (e.g., Trypan blue) is non-informative if used mincing or homogenization to provide single cells or nuclei and recommended the use of dual dye viability assay based on a combination of 5–6 caboxyfluorescein diacetate and ethidium bromide. However, in our experiments, we found that Trypan blue assay was quite useful in determination of cytotoxicity in gill.

Pesticides are used in a variety of combinations and the researchers are actively involved in carrying out the studies in exposed populations to assess the risk involved in occupational exposure. Because of the mutational basis of cancer occurrence, bio-monitoring of exposed animal populations to potential genotoxic and mutagenic compounds in polluted environments is a warning system with respect to human health protection. There are reports on positive genotoxic effects in populations exposed to pesticides [63,64]. PFF is known to bio-accumulate in the tissues of test organisms and the estimates of tissue concentrations may be more valuable for the assessment of situations in the natural environment [1,9]. Earlier study indicated that PFF is neurotoxic for inhibition of brain and gill AChE in the fish, *Oreochromis mossambicus* [1].

The 96 h LC_{50} value of 2.31 ppb for PFF (50% EC) as determined in the present *C. punctatus* indicated that PFF is highly toxic to the test fish. To the best of our knowledge, no literature is available on the acute toxicity of PFF to *C. punctatus*. The estimated safe levels of PFF in *C. punctatus*, as calculated by multiplying the LC_{50} with application factor (AF) recommended by different methods varied from 2.31×10^{-1} to 2.31×10^{-5}. However, the estimated safe levels cannot be guaranteed because of large variations found in different methods has resulted in controversy over its acceptability [23,65]. In this approach, some safe limits in extrapolation of laboratory studies to field are not always meaningful value and hence, it is difficult to decide an acceptable concentration based on the laboratory experiments that may be considered 'safe' in the environmental concentrations [23,66].

In general, gill cells showed significant DNA damage. It has been shown that the % tail DNA damage increases with dose and time. The higher DNA damage in gill cells could be justified because gill being appropriate organ that is directly and constantly exposed to the DNA damaging chemicals dissolved in the water [27]. The suitability of gill tissue for genotoxicity studies has also been demonstrated using shellfish, rainbow trout and gold fish [67–69]. Earlier investigations in various fish species indicated the higher sensitivity of the gill cells to DNA damage than the kidney, liver, erythrocytes, or lymphocyte cells [31,51,70,71].

In the present study the alkaline SCGE was favoured because pH > 13 is expected to maximize the expression of alkali-labile sites as single strand breaks. This is because the DNA denatured and unwound at pH values above 12 causes the disruption of hydrogen bonds between double-stranded DNA. At pH 12.6 or higher, alkali labile sites (apurinic sites) are quickly transformed to DNA strand breaks [72,73]. By weakening the *N*-glycosidic bond, the formation of depurinating DNA adducts can also lead to the formation of alkali-labile sites (after spontaneous depurination in alkaline conditions), which in turn can cause DNA strand breaks [34]. In this turn releases, fragments of broken DNA as well as relaxes the loops of super-coiled DNA near the sites of cleavage. During electrophoresis, the DNA fragments as well as relaxed DNA loops move towards the anode producing the tail of the comet. The DNA fragments tend to move freely during the electrophoresis, whereas the relaxed DNA loops are dragged out of the nuclear head. The tail length determines how far the DNA has migrated out of the cell. Since smaller DNA fragments move the farthest, the tail length is predominantly dictated by the size of the DNA fragments generated during the alkaline unwinding step of the Comet assay [74]. On the other hand, the percentage DNA in the tail (% tail DNA) is a popular and suitable parameter [70] which measures the percentage of DNA that has migrated from the head.

The biotransformation of xenobiotics often results in the production of reactive intermediates such as reactive oxygen species (ROS), which are highly toxic and can cause DNA breakage either directly (for H_2O_2 and OH^-) incomplete repair of the resulting oxidised DNA bases [34]. Although organisms are equipped with an antioxidant defence system to protect tissues against oxidative lesions, if the rate of ROS production exceeds the capacity of defence mechanisms, cellular and DNA lesions can occur [57,75,76]. Oxidative DNA damage generated by ROS, such as those inevitably arising from respiration, has been attributed for the high and variable DNA damage in the gill cells of *Mytilus edulis* [49,71].

The effect of acute concentrations of PFF noticed in this investigation suggested a serious concern towards its potential dangers to aquatic organisms. Our study further credence to other report where organophosphate pesticide PFF and other showed that PFF is highly toxic even at sub-lethal concentrations to the mosquito fish, *Gambusia affinis* [8] and evaluated their potential mutagenic effects in chromosomal aberration analysis in both somatic and germ cells of male mice [77] and has genotoxic and mutagenic effects of somatic chromosome in *Lathyrus sativus* L. [7] and other living organisms. Hence, use of PFF should be restricted in agricultural practices. *In vivo* genotoxicity of PFF to erythrocytes of Chinese native amphibian (*Rana spinosa*) Tadpoles showed that PFF was highly genotoxic pesticide [78]. The pesticide PFF on cultured human peripheral blood lymphocytes induced cytotoxicity and genotoxicity and showed poisoning effect in humans [79]. Genotoxicity and mutagenicity of pesticides for non-target organisms and their influence on ecosystems are of worldwide concern [18].

Thus the present study has indicated the genotoxic potential of PFF at acute concentrations, which has a serious concern towards its potential dangers to aquatic organisms and judicious and careful use in agricultural and non-agricultural practices. This biomarker has a broad perspective in aquatic toxicology, as fish gill cells are constantly being exposed to environmental pollutants.

5. Conclusions

Our results on *C. punctatus* clearly indicated that profenofos possesses genotoxic and mutagenic threats and also showed the Comet assay to be a potent tool for biomonitoring of genotoxicity in aquatic environment. It may be anticipated that site of contact genotoxicity and mutagenicity would occur *in vivo*. These findings may provide biomarker for PFF induced genotoxicity and mutagenicity that could be useful for investigating the impacts of acute toxic effects on the freshwater fish species. This system might be a useful tool for assessing the exposure of fish population to genotoxins and evaluate the mutagenic hazard in surface water; therefore, the Comet assay could be much more sensitive in ecogenotoxicological studies.

Funding

National Bureau of Fish Genetic Resources (Indian Council of Agricultural Research), Lucknow, India.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors wish to thank the Director, National Bureau of Fish Genetic Resources (ICAR) Lucknow, India and Head, Department of Zoology, University of Lucknow, Lucknow, UP, India for encouragement and providing the necessary laboratory facilities to conduct this study.

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