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Immune gene expression in cyprinid herpesvirus-2 (CyHV-2)–sensitized peripheral blood leukocytes (PBLs) co-cultured with CyHV-2-infected goldfish fin cell line

Sweta Das[1] · Arathi Dharmaratnam[1] · Charan Ravi[1] · Raj Kumar[1] · Thangaraj Raja Swaminathan[1]

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Abstract

Goldfish is one of the preferred ornamental fish which is highly susceptible to cyprinid herpesvirus-2 (CyHV-2) infection. The present study aimed to analyse immune gene expression in a co-culture of CyHV-2-sensitized goldfish peripheral blood leukocytes (PBLs) with CyHV-2-infected fantail goldfish fin cell lines (FtGF). Goldfish were sensitized with intraperitoneal $TCID_{50}$ dose ($10^{7.8\pm0.26}$/mL) of CyHV-2. After 2 weeks, PBLs were collected and co-cultured with CyHV-2-infected FtGF cells keeping both uninfected FtGF cells and PBL control groups. After 2 days of co-culture, WST-1 assay for cell proliferation was performed at 450 nm during the 2nd, 4th and 6th days of co-culture. The results showed a significant increase ($p < 0.05$) in cell density in CyHV-2-infected PBL and virus-infected FtGF cells during the 4th day post co-culture which confirmed effector cell generation. Expressions of few immune genes were checked taking RNA samples of CyHV-2-induced PBLs post co-culture with infected FtGF cells along with uninfected FtGF cells as control group at different time periods (2nd, 4th and 6th days) in triplicate. The results indicated increased expression of CD8α, IFNγ, b2m, MHC I, LMP 7, IL-10, IL-12 and GATA3 except Tapasin. From the above study, we concluded that goldfish showed both Th1- and Th2-mediated immune responses to CyHV-2. The current findings support the scope for further vaccine development against CyHV-2 for goldfish.

Keywords Goldfish · Cyprinid herpesvirus-2 · Peripheral blood leukocytes · Immune gene expression

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✉ Sweta Das
swetabt@gmail.com

Extended author information available on the last page of the article

Introduction

Goldfish (*Carassius auratus*) is one of the most common ornamental fish species cultured throughout the world due to their popularity as companion animals in aquariums and as an important laboratory animal (Groff et al. 1998). However, the species is highly susceptible to cyprinid herpesvirus-2 (CyHV-2) infection. CyHV-2 is a *Cyprinivirus* of the *Alloherpesviridae* family which causes goldfish herpesviral hematopoietic necrosis (HVHN) in goldfish (*C. auratus*), Prussian carp (*C. gibelio*) and silver crucian carp (*C. carassius*) (Goodwin et al. 2006). CyHV-2 outbreak in goldfish was first reported in Japan causing 100% mortality in all sizes of goldfish (Jung and Miyazaki 1995). Thereafter, the virus is being reported throughout the globe such as in the USA, China, Czech, the UK, Taiwan and Australia (Chang et al. 1999; Stephens et al. 2004; Goodwin et al. 2006; Jeffery et al. 2007; Goodwin et al. 2009; Danek et al. 2012; Xu et al. 2013). While a lot of studies on CyHV-2 infection in goldfish were conducted, however, there are only few reports on the host-pathogen interaction. Increased expressions of many immune-related genes were evident in crucian carp (*C. carassius*) to CyHV-2 infection such as intelectin, interleukin-11 (IL-11), purine nucleoside phosphorylase 5a (PNP5a), keratin8, myeloid-specific peroxidase (MPO), dual specificity phosphatase 1 (dusp1), MHC-I, IRF3 and MAPK7 (Xu et al. 2014, 2016; Lu et al. 2017).

Recently, the virus was reported in India by Sahoo et al. (2016) in goldfish. The virus culture is now possible with the development of fantail goldfish fin cell lines (FtGF) (Lu et al. 2018; Dharmaratnam et al. 2020). Further host-pathogen interaction studies will provide scope for disease management approaches. Understanding immune response to CyHV-2 will provide platform for generation of subsequent viral vaccines in goldfish. Therefore, the present work was carried out to check expression of some of the signature genes (CD8α, IFNγ, b2m, MHC I, LMP 7, IL-10, IL-12, GATA3 and Tapasin) for T-helper cell response (Fischer et al. 2013) to CyHV-2 in sensitized goldfish peripheral blood leukocytes (PBL) against CyHV-2-infected FtGF cells. Prior to the expression studies, a cell proliferation assay was conducted to confirm the presence of effector cells in leukocytes specifically generated against CyHV-2.

Materials and methods

Fish

Healthy goldfish weighing 20 g were procured from local commercial aquarium showing no apparent clinical signs of disease and maintained in 50-L tubs for acclimatization under optimal aeration. The fish were fed with commercially available feed at 3% of their body weight twice daily. One-third of water was replaced every 2 days to remove waste feed and faecal material.

Fish cell line

FtGF cell line was obtained from NBFGR cell line repository (Dharmaratnam et al. 2020) and cultured in Leibovitz's L-15 (L-15) medium (HiMedia, Nashik, India) with 5% foetal bovine serum (FBS) (Life Technologies, Paisley, UK) using a 25-cm^2 flask (Thermo Scientific, Roskilde, Denmark) at 28 °C. After being confluent, FtGF cells were sub-cultured to a new 25-cm^2 culture flask. Briefly, cells were washed with 1 mL phosphate-buffered saline (PBS)

(Life Technologies, Grand Island, NY) for two times and then treated with 0.25% trypsin-EDTA (Life Technologies, Grand Island, NY) in PBS. The cells were harvested by vigorous pipetting with 2 mL of fresh L-15 medium with 5% FBS. The cells were sub-cultured at a split ratio of 1:2. The flasks were observed daily under an inverted light microscope (Nikon, Melville, NY) and sub-cultured regularly upon being 80–90 % confluent. The cells were counted with a Neubauer haemocytometer before each experiment.

CyHV-2 sensitization

Ten healthy goldfish were challenged with an intraperitoneal injection at standardized $TCID_{50}$ dose of $10^{7.8\pm0.26}$/mL (Dharmaratnam et al. 2020). Control fish were injected with equal amount of PBS under the same condition.

Blood samples were collected after 2 weeks with anticoagulant and PCR was set with CyHV-2-specific primers CyHVpol-FOR (CCCAGCAACATGTGCGACGG) and CyHVpol-REV (CCGTARTGAGAGTTGGCGCA) (Jeffery et al. 2007). Briefly, DNA was extracted from blood drawn from the challenged fish using Qiagen DNeasy Kit (Qiagen, Austria) according to the manufacturer's instructions. Concentration and purity of the extracted DNA were checked by measuring OD at 260 and 280 nm using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, USA). One microgram of DNA was taken as template in 25 μL total reaction volume containing 10 pmole of each forward and reverse primers and 12.5 μL of 2X Taq DNA polymerase enzyme mix (Takara, Japan). The PCR conditions were set at 95 °C for 3 min followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min.

Collection of peripheral blood leukocytes (PBL)

After 2 weeks of challenge, blood was collected with anticoagulant and processed through density gradient centrifugation using HiSep (HiMedia, Nashik, India). Briefly, blood was collected aseptically from lateral line with anticoagulant after 2 weeks of intraperitoneal injection with CyHV-2. Then, the blood was diluted three times with PBS and overlaid on HiSep density gradient of 1.077 g/mL. After 30 min of centrifugation at 400 x g at room temperature without break, the white buffy coat was collected from the middle layer. The isolated leukocytes were then washed with PBS twice by centrifuging at 250 x g at room temperature and cultured in L-15 medium with 5% FBS. The cells were visualized under an inverted light microscope. Few cells were stained with 10% Giemsa solution and visualized under light microscope (Leica, Wetzlar, Germany). The cells were counted with a Neubauer haemocytometer. Antibacterial and antifungal agents (200 IU ml^{-1} penicillin, 200 μgml^{-1} streptomycin and 0·5 μgml^{-1} amphotericin B, Life Technologies, Grand Island, NY, and 1X gentamycin/ amphotericin B, Life Technologies, Grand Island, NY) were added to avoid contamination.

Effector cell proliferation assay

The FtGF cells were cultured in 96-well plate for overnight at a concentration of 2.76×10^5 cells/mL. Then, the cells were infected with at a standardized $TCID_{50}$ dose of $10^{7.8\pm0.26}$/mL (Dharmaratnam et al. 2020) along with PBS control. The cells were kept for half an hour and then washed with PBS. To inhibit further cell proliferation, the target cells were treated with

mitomycin C (10 μg/mL) (HiMedia, Nashik, India) for 20 min and washed with PBS. Freshly isolated PBLs, which consisted almost entirely of lymphocytes and monocytes, were added (200 μL) at a concentration of 5.03×10^5 cells/mL to both CyHV-2-infected and PBS control FtGF cells. Control leukocytes and a no-leukocyte control group containing only FtGF cells were also kept as negative controls. After 2 days of co-culture, cells were processed for cell proliferation using EZcount™ WST-1 Cell Assay Kit (HiMedia, Nashik, India). Cells were incubated with activated WST-1 reagent for 3 h as per manufacturer's instructions, and optical density (OD) was checked in a MULTISKAN GO (Thermo Scientific, USA) at 450nm at the 2nd, 4th and 6th days of co-culture. The average OD for triplicate samples for each time period was calculated and presented as mean ± SE (Fig. 1). Further, differences between the mean values were analysed using t-tests of infected and uninfected FtGF cells, with values $p < 0.05$ as significantly different.

Gene expression analysis

In another experiment, FtGF cells were seeded at a concentration of 1.04×10^6 cells/mL in eighteen 12.5-cm^2 flasks (Thermo Scientific, Roskilde, Denmark) and cultured overnight. Nine of the flasks were infected with CyHV-2 and 9 were treated with PBS (control). After 1 h, the cells were washed with PBS. Then, the cells were co-cultured with freshly isolated PBL at a concentration of 2.16×10^6 cells/mL. Prior to the collection of PBL (2 weeks before), 30 goldfish were challenged with CyHV-2 at standardized $TCID_{50}$ dose of $10^{7.8 \pm 0.26}$/mL. Cells were collected in 1 mL of RiboZol (Amresco, USA) after the 2nd, 4th and 6th days of co-culture from both infected and uninfected control groups in triplicate. RNA was isolated as per the manufacturer's instructions from the above collected cells, and cDNA were prepared using Verso cDNA Synthesis Kit (Thermo scientific, USA). Before cDNA preparation, RNA samples were treated with DNase I (Thermo scientific, USA) as per the instructions.

Primers were designed for ten genes (Table 1), and their respective annealing temperatures (Ta) were standardized with PCR using Qiagen DNeasy Kit (Qiagen, Austria). Quantitative PCR was performed using DyNAmo S SYBR Green qPCR Kit (Thermo Scientific, USA) in Aria MX Real-time PCR (Agilent, California, USA). Briefly, a total reaction mix of 10 μL was prepared using 5 μL of 2 X DyNAmo S SYBR Green mix, 0.3 μL of primer (5 pmole) mix and 2.4 μL of H_2O provided in the kit using 2 μL of previously prepared cDNA as template.

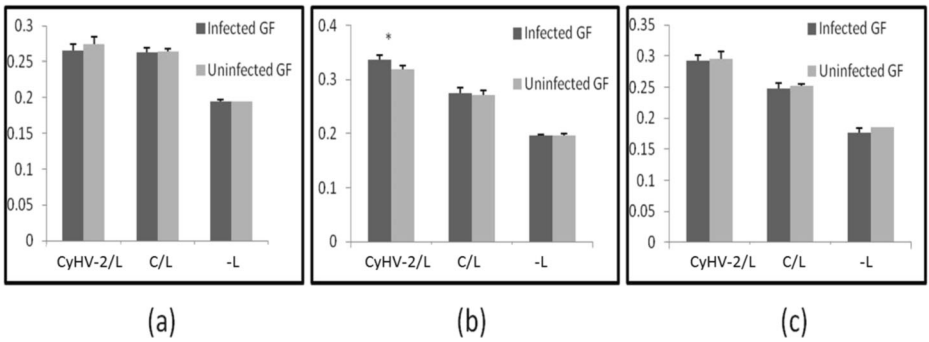

Fig. 1 Effector cell proliferation assay generated from PBL specifically raised against CyHV-2 co-cultured with infected goldfish fin cell line (FtGF) at the 2nd (**a**), 4th (**b**) and 6th (**c**) days of co-culture. CyHV-2/L leukocytes raised against CyHV-2, C/L control leukocytes, -L no leukocyte control (only infected and uninfected FtGF cells). * denotes significant difference ($p < 0.05$)

Table 1 List of primers used to study gene expression

Sl. No.	Primer name	Primers (5′ –> 3′)	Annealing temperature (Ta)	Product size (bp)
1	GATA3	ACAAGATGAACGGCCAGAAC CGTGGTCGTTTGACAGTTTG	59 °C	201
2	IL-10	TAAAGCCATGGGAGAGCTTG AGGGTGAAGTCCATTTGTGC	62 °C	198
3	IFNγ	GACTTCAGGATGGGCAACAT TGCTCAGGTTCCTCGAGATT	63 °C	171
4	CD8α	AGAGGTGGTACCGGTCGATT GCGCAGGTGTAGAAACCACT	63 °C	219
5	MHC I	AACGCCTCTGATGATGGTTC AAGCAAGATGGAGAGCAGGA	63 °C	119
6	b2m	GCCCTGTTCTGTGTGCTGTA GTCAGTTTGCTGCGTGTCAG	59 °C	219
7	IL-12	GCTTGTGGTGGATGTTGATG TCAGGTAGGAGCCCTCATTG	61 °C	203
8	LMP7	TGGGAGAGACTCCTGGCTAA CAGACGAGTGCCATTGTCAT	59 °C	201
9	Tapasin	CTGGACTGTGGCTTCTGGAT ATCAGAGAAGCGTTGCCTGT	57 °C	200
10	βActin	GTGATCACGGTTGACACAGG GGATCAGATTCTCCGCATTC	57–63 °C	155

The program consisted of pre-denaturation at 95 °C for 10 min and 45 cycles of amplification at 95 °C for 10 s, Ta (Table 1) for 10 s and 72° C for 20 sec. All the cycles were performed simultaneously with reference gene, β-actin in the same plate in triplicates. β-Actin was chosen as a reference gene after testing several other potential reference genes such as 18S ribosomal RNA, glyceraldehyde-3P-dehydrogenase and elongation factor 1 alpha (Dharmaratnam et al. 2021). In the present study too, the Cq values of β-actin gene did not change significantly ($P > 0.05$, ranged between 11 and 12). Quantitative PCR was verified by melt curve analysis at a temperature of 95 °C for 10 s, 65 °C for 1 min and 95 °C for 1 min. Blank was taken for each plate. Cq values were obtained using Agilent Aria Software set up 1.5, and n-fold differential expression was calculated using the comparative Cq method (Livak and Schmittgen 2001) by calculating the average of each Cq for the triplicate samples. Fold difference was calculated as $2^{-\Delta\Delta Cq}$. The control samples taken at each time periods were used as calibrators. The average fold expression for triplicate samples for each time period was calculated and presented as mean ± SE. Further, differences between the mean values were analysed using t-tests, with values $p < 0.05$ as significantly different. All values of n-fold differential expression were plotted in graph (Fig. 2).

Results

Effector cell proliferation assay

From the cell proliferation assay, a significant increase in cell number (in terms of optical density) was observed only during the 4th day of co-culture in CyHV-2-infected PBL with CyHV-2-infected FtGF cells (Fig. 1). No significant increase in cell density was observed in the rest of the time periods (2nd and 6th day) as well as in the control groups.

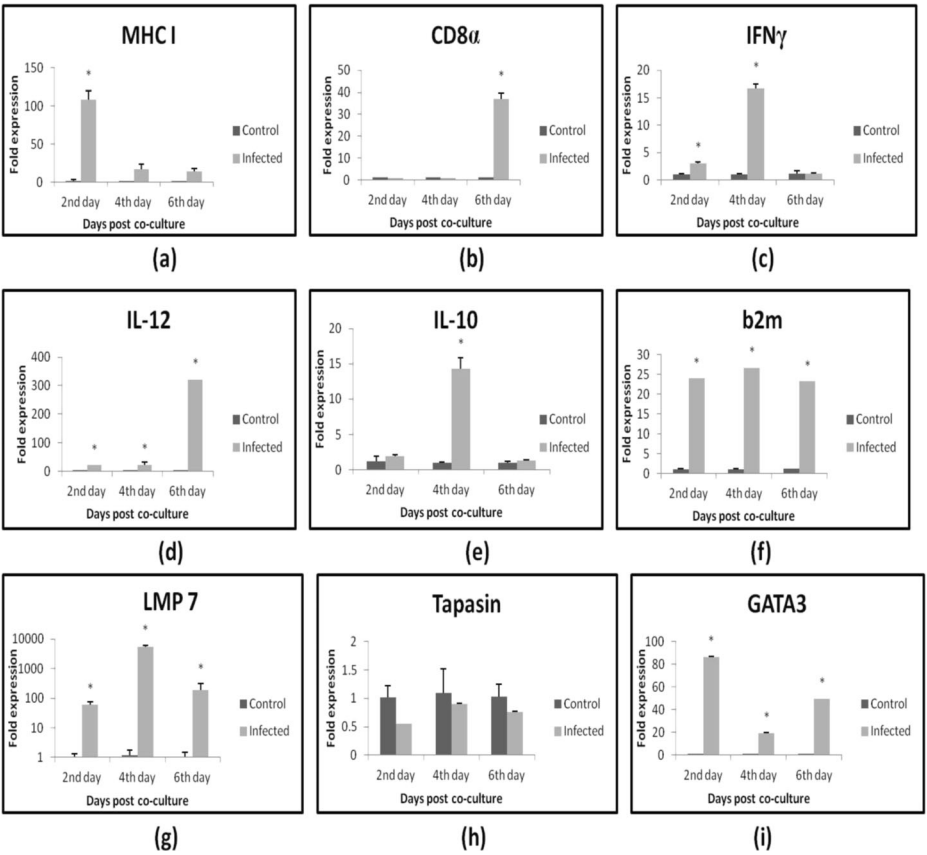

Fig. 2 Fold expression of MHC I (**a**), CD8α (**b**), IFNγ (**c**), IL-12 (**d**), IL-10 (**e**), b2m (**f**), LMP 7 (**g**), tapasin (**h**) and GATA3 (**i**) at different time periods (2nd, 4th and 6th days) post co-culture of CyHV-2-infected FtGF and PBL collected from CyHV-2-immunized goldfish. * denotes significant difference ($p < 0.05$)

Gene expression studies

Almost all the immune genes tested of cell-mediated immunity were up-regulated such as CD8α, GATA3, IFNγ, b2m, MHC I, LMP 7, IL-10 and IL-12 except for Tapasin (Fig. 2). The MHC I gene was up-regulated during the 2nd day post CyHV-2 infection and normalized thereafter. The CD8α gene was up-regulated only at the 6th day post viral infection. Increased expression of IL-10 was observed only in the 4th day of infection, whereas IFNγ was up-regulated during the 2nd day and continued until the 4th day of co-culture. However, GATA3, b2m, LMP7 and IL-12 up-regulated starting from the 2nd day until the 6th day post viral infection.

Discussion

In the present study, effector cell proliferation was observed only in CyHV-2-sensitized PBL at the 4th day post co-culture with infected FtGF cells. Since there was no difference in cell

proliferation in the control leukocytes in goldfish to CyHV-2-infected FtGF cells, the effector cells from goldfish can be regarded as viral antigen dependent. Leukocytes from crucian carp haematopoietic necrosis virus (CHNV)–infected ginbuna carp were also found to kill CHNV-infected target cells specifically (Somamoto et al. 2002). Further, majority of lymphoblast cells were observed at the 4th day post co-culture of responder primary lymphocytes from CHNV-infected fish with CHNV-infected stimulator cells (Somamoto et al. 2009).

In the expression study, MHC I up-regulated (up to 100-fold) at early 2nd day post co-culture, which signifies MHC I presentation of CyHV-2 in goldfish. In orange-spotted grouper (*Epinephelus coioides*) MHC I presentation was also evident to nodavirus infection along with increased expression of CD8α gene (Chang et al. 2011). The specific cytotoxicity studied in ginbuna crucian carp to CHNV showed MHC Class I restricted presentation (Somamoto et al. 2000, 2002) and overexpression of TCRβ and CD8α genes (Somamoto et al. 2006). Effector cells collected at 6, 12 and 24 days post co-culture in ginbuna crucian carp showed an increased expression of TCRβ and CD8α genes (Somamoto et al. 2009). Similarly, in the current study, increased expression (up to 37-fold) of CD8α was evident only at the 6th day post co-culture indicating activation of T cells by recognition of viral antigen. Increased expression of CD8α was also evident in Japanese flounder (*Paralichthys olivaceus*) (Byon et al. 2005, 2006) and rainbow trout (Fischer et al. 2006; Utke et al. 2007) to viral haemorrhagic septicaemia virus (VHSV). In the present study, IFNγ gene was up-regulated at the 2nd day and the highest expression was noticed at the 4th day post co-culture which was normalized at the 6th day. Though IFNγ is the main cytokine for innate cellular response, activated T cells also express IFNγ for the generation of memory $CD8^+$ cells (Mosmann and Coffman 1989; Perussia 1991; Sad et al. 1995). Previously, IFNγ was shown to induce antiviral activity in trout (Robertsen et al. 2006) and Atlantic salmon (*Salmo salar*) against infectious pancreatic necrosis virus (IPNV) and salmonid alphaviruses (SAV) (Sun et al. 2011). A mild increase in the expression of IL-12 at the 2nd and 4th days post co-culture in goldfish indicated regulated expression of IL-12. However, sudden increase (up to 300-fold) in expression level at the 6th day post co-culture coincides with CD8α expression which may be a sign of regulation of $CD8^+$ cell generation. Similar results were obtained in common carp where IL-12 and IFNαβ expression occurred concomitantly with CD8αβ up-regulation exclusively at the 4th day post viral infection (Forlenza et al. 2008). In the current study, β2m gene was up-regulated in all the studied time periods. Though β2m is believed to interact and stabilize the tertiary structure of MHC I, it also known to induce interleukin-6, -8 and -10 in several cell types, regulate the expression of hormone or growth factor and coordinate the interaction between cytokines and their receptors (Li et al. 2016). Previous studies showed an increased expression of β2m during infectious hematopoietic necrosis virus (IHNV) in rainbow trout (Hansen and La Patra 2002). The highest expression was noticed in LMP7 gene in all the studied time periods. Similar results were obtained in Nigerian spotted lungfish (*Protopterus dolloi*) to poly I:C (polyinosinic:polycytidylic acid) induction (Tacchi et al. 2013) and turbot (*Scophthalmus maximus*) to lymphocystis disease virus (LCDV) infection (Zhang et al. 2015). However, its exact role during the immune response to viral infection is yet to be explored. There was no effect on gene expression of tapasin in induced PBL indicating its least role during immune response of goldfish to CyHV-2 infection. However, reports on its induced expression were marked during viral infection in rainbow trout (Landis et al. 2006).

Besides Th1 response, up-regulation of IL10 and GATA3 indicated that Th2 response was also evident in goldfish to CyHV-2 infection which includes B cell activation and antibody production (Fischer et al. 2013). Previous reports on Atlantic salmon showed an increased

expression of IL10 gene to infectious pancreatic necrosis virus (IPNV) (Ingerslev et al. 2009). However, in grass carp (*Ctenopharyngodon idellus*) GATA3 expression was not marked during grass carp reovirus (GCRV) infection (Wang et al. 2013).

Conclusion

From the above study, we concluded that with the increased expression of these marker genes, goldfish utilizes highly-sophisticated T helper cell mediated immunity to CyHV-2 in terms of Th1 and Th2 response comparable to those of mammals. These results may help further to develop vaccines against CyHV-2 infection for goldfish.

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Data availability statement The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval All the experiments were conducted following the guidelines of the Institute Animal Ethics Committee.

Conflict of interest The authors declare that there is no conflict of interest.

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Affiliations

Sweta Das[1] · Arathi Dharmaratnam[1] · Charan Ravi[1] · Raj Kumar[1] · Thangaraj Raja Swaminathan[1]

[1] Peninsular and Marine Fish Genetic Resources Centre, ICAR-National Bureau of Fish Genetic Resources, CMFRI Campus, P.O. Number 1603, Kochi, Kerala 682018, India