Schizothorax richardsonii dsRNA dependent protein kinase has three double-stranded RNA binding motifs with a promoter lacking conserved kinase sequence

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Ankur Saxena
Directorate of Coldwater Fisheries Research

Kiran Belwal
Directorate of Coldwater Fisheries Research

Preeti Chaturvedi
ICAR -Directorate of Coldwater Fisheries Research Bhimtal

Amit Pande
Directorate of Coldwater Fisheries Research

amit.pande@icar.gov.in Corresponding Author
ORCiD: https://orcid.org/0000-0001-6794-2471

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Abstract

Background: Double stranded RNA (dsRNA) dependent protein kinase (PKR) is an interferon (IFN) stimulated antiviral protein. It inhibits protein synthesis by phosphorylation of the eukaryotic translation initiation factor 2-alpha (eIF2-α) by its serine-threonine kinase activity that prevents virus replication. This is the first report on the in silico analysis of PKR coding region and its promoter from a Coldwater fish of the Indian Himalayan region. Snow trout, Schizothorax richardsonii is an important Coldwater food fish. It is being over fished and therefore requires conservation. Being a vulnerable species it is listed under the red list of IUCN. Here we discuss the identification, cloning and sequencing of PKR coding region and its promoter.

Results: We have revealed the complete coding region of dsRNA dependent protein kinase (PKR) and its promoter from Schizothorax richardsonii, one of the several species of snow trout inhabiting sub-Himalayan fresh water bodies. An amplicon of 2884bp containing 5’ and 3’ untranslated regions (UTR) of 234 and 558 bases was obtained while the deduced open reading frame (ORF) of 2076 bases encoded a polypeptide of 691 amino-acids. Snow trout PKR protein contains three double stranded RNA binding motifs (dsRBM) at N terminal, besides possessing a serine/threonine protein kinase and a C terminal catalytic domain. Moreover, a stretch of 791 nucleotide bases was identified as the promoter upstream the ORF. The identified promoter has two interferon stimulated response elements (ISRE) besides the presence of core promoter elements. Moreover, the snow trout PKR promoter has a TATA box but lacks kinase conserved sequence (KCS) that is present in mammalian PKR promoters.

Conclusion: The promoter of snow trout was identified as a stretch of 791 nucleotide bases. It has two interferon-stimulated response elements (ISRE) besides core promoter elements. Intriguingly, unlike the mammalian PKR promoter, the snow trout PKR promoter has a TATA box but lacks the conserved kinase sequence (KCS) present in human counterpart. Keywords: Schizothorax richardsonii, Interferon Stimulated Genes, PKR protein, Antiviral state, Homology modeling, dsRNA binding protein kinase

Background
The protein kinase R (PKR) is an interferon-inducible dsRNA dependent protein kinase with antiviral properties. It is known to limit viral replication by blocking eIF2α mediated translation. PKR is a Serine/Threonine kinase that phosphorylates the α-subunit of eukaryotic translation initiation factor 2 (eIF-2α) which in turn leads to the inhibition of protein synthesis [1]. PKR is known to be expressed constitutively at moderate levels in cells besides being activated by viral dsRNA as well as ds RNA analog poly I:C. Viral infection leads to induction of interferon and PKR is induced as an effector protein against viral invasion [2]. Besides the antiviral activity, PKR protein also responds to various cellular stresses, regulates cell growth and also has a role in inflammatory processes, apoptosis, and metabolism [3]. PKR protein is composed of two major domains, one dsRNA binding domain (dsRBD) and a kinase domain. The dsRBD consists of two to three tandem dsRNA binding motifs (dsRBM) [3]. Virus-derived dsRNA binds to the dsRBD enabling homo-dimerization and auto-phosphorylation in a stretch of amino-acids known as activation segment. In the activation segment, residues Thr\textsuperscript{446} and Thr\textsuperscript{451} are steadily phosphorylated during the process of activation. Active PKR is known to bind and phosphorylate Ser\textsuperscript{51} residue of eIF–2α that results in the activation of the kinase domain [4, 5].

PKR has been identified from a number of fish like grass carp, rock beam, Japanese halibut and fresh water pufferfish [6,7, 8, 9]. In puffer fish Tetraodon nigroviridis, three PKR genes are known to be induced by poly I:C, one containing three double-stranded RNA binding domain (dsRBD) and two containing one dsRBD domain. On the contrary in zebra fish, one PKR has been described with three dsRBD. In Fugu, three-spined stickle back and fat head minnow two PKR genes with a putative dsRBD have been identified. Whereas one gene with two dsRBD has been reported in Japanese media. In Japanese flounder one, PKR gene with similar characteristic features like that of mammalian PKR has been described. It has been demonstrated that over expression of PKR increases the phosphorylation of eIF-2α and inhibits the replication of turbot (Scophtalmus maximus) rhabdovirus in embryonic cells [8]. Functional analysis of dsRNA binding motifs has been carried out in grass carp where the third dsRBM has been shown to enhance dsRNA binding [10]. Thus, the knowledge about fish PKR is quite intriguing which needs to be further unravelled and application of bioinformatic tools can prove
helpful in this direction. The knowledge on three dimensional structures of fish PKR is inadequate and a comparative structural and sequence analysis of the fish PKR with its mammalian orthologs thus needs in-depth exploration. This will help in understanding the fish immune mechanisms and will generate knowledge about different ways the immune system adopts to combat the pathogen attack. We have attempted to identify and characterize PKR and its promoter from an Indian Coldwater fish, snow trout. *Schizothorax richardsonii* one of the several species of snow trout, is an important conserved cyprinid fish of the mountainous riverine system. It is an economically important food and game fish facing a serious risk. Overfishing, pollution of aquatic bodies, damming of rivers and introduction of salmonids and other exotics have threatened its existence. Therefore, it has been listed as vulnerable species as per the IUCN red list of threatened species (2010). In order to identify and characterize PKR and its promoter from a Coldwater fish species inhabiting the foothills of Himalayas, we have identified, cloned and sequenced the PKR coding region and its promoter. Moreover, this is first report on the *in silico* analysis of PKR coding region and its promoter from a Coldwater fish, *Schizothorax richardsonii*.

**Results**

*Amplification of PKR gene*

The nucleotide sequence obtained by PCR was analyzed, and partial coding region of dsRNA dependent protein kinase of 900bp was submitted to GenBank (Accession No. KX447496). Two fragments of 1400bp and 800bp were obtained by 5’ and 3’ RACE analyzed on 1% agarose gel documented using GelDoc XR (BioRad), after sequencing and analysis of RACE PCR products, a full-length PKR nucleotide sequence of 2884 bases were obtained. Bioinformatic analysis of the obtained sequence revealed that it has an open reading frame (ORF) of 2076 nucleotides with a 5’ UTR of 234 nucleotides and a 3’ UTR of 558 nucleotides. The complete coding sequence was submitted to GenBank (Accession No. KX447496.1). 3’ UTR was predicted to have four instability motifs (ATTTA) and one polyadenylation signal (AATAAA) before PolyA tail (Fig 1).

**Amplification of PKR promoter region**

To identify the PKR promoter region, genome walking was carried out. PCR products were obtained
from the genomic libraries acquired by the complete digestion of chromosomal DNA with DraI, EcoRV, 
Nsbl, PvuII, Scal, SspI and Stul. Two amplicons of 1060 and 1700bp were obtained with Nsbl 
(Fermentas), an amplicon of 1687 bp, 845bp and 3761bp were obtained with PvuII, SspI, and Stul 
respectively. No amplicons could be visualized upon PCR from genomic libraries of DraI, EcoRV, and 
Scal. Nucleotide sequencing of the products and their analysis revealed a promoter sequence of 
791bases (Accession No. MF678851).

Analysis of PKR promoter

PKR promoter (Accession No.MF678851) was analyzed for the presence of core promoter elements, 
and interferon-stimulated response elements (ISRE). The analyses of the nucleotide sequence using 
YAPP and JASPAR revealed the presence of core promoter elements (Fig 2). In the predicted core 
promoter, a transcription start site (TSS) a TATA Box, 25–36 bases upstream the TSS and a 
downstream promoter element (DPE) 15–19 bases downstream the transcription start site were 
present. Moreover, two ISRE were also detected in the promoter, the first ISRE (ISRE1) is located 131– 
140 bases upstream the TSS while the second ISRE2, is present 98–106 bases upstream the TSS.

Analysis of PKR Protein

The snow trout PKR protein was predicted to be a polypeptide of 691 amino acids. The molecular 
weight of the protein was predicted to be 77.954 kDa with a predicted isoelectric point (pI) as 8.71. 
Domain analysis revealed the presence of two major domains a dsRNA binding domain and a Ser/Thr 
kinase domain (Fig 3). The dsRNA binding domain has three dsRNA binding motifs (dsRBM) while an 
ATP binding site “IGKGGFGRVFKARRKLEKKYFAVKI” between position 408–431 and a kinase active site 
“LIHRDLKPKNIMF” between positions 541–553 in the kinase domain. PKR also possesses a bipartite 
nuclear localization signal and four N–Glycosylation sites in the dsRBD.

Homology Modelling

The snow trout dsRBM1 has 41% identity with a first dsRBM domain of mouse PKR (PDB ID: 1x49 A) 
while dsRBM2 and dsRBM3 had 30% and 44% identity with a second dsRBM domain of mouse PKR 
(PDB ID: 1x49 A). The kinase domain had 44% identity with human PKR kinase domain (PDB ID: 3UIU).
Therefore it was selected as a template to develop a homology model. The dsRBM1 and dsRBM3 had a typical feature of mammalian dsRBMs as it has three β-sheets sandwiched between two α-helices (Fig 4). However, the dsRBM2 differs from the other two dsRBMs as it has two β-sheets sandwiched between two α-helices (Fig 4) whereas PKR kinase domain is formed of β-sheets at its N-terminal and α-helices at C-terminal (Fig 5). The N-lobe of the kinase domain consists of 5 stranded antiparallel β-sheets and one α-helix, which contains the ATP binding site. The C-lobe of the kinase domain consists of eight α-helices and two pairs of antiparallel β-sheets.

**Phylogenetic analysis**

Evolutionary analysis of PKR protein from various fish species was carried out using maximum likelihood method in MEGA7 to get a phylogenetic tree along with PKR from *Schizothorax richardsonii* (Fig 6). PKR of fish and mammalian species separated into two major clades one including the fish while the other includes the mammalian species. One of the fish species *Larimichthys crocea*, of *Sciaenidae* family separated itself from fish as well as mammalian species, forming a separate clade quite distant from both. PKR of *Schizothorax richardsonii* grouped itself among other members of *Cyprinidae* family. Moreover, the evolutionary analysis of fish and human RNA binding motifs (dsRBMs) revealed that the dsRBM from fish and human clustered into three separate clades. The first clade includes the dsRBM1 of fish along with dsRBM2 of a human while dsRBM2 of fish grouped itself with dsRBM1 of human (Fig 7). However, the third dsRBM of fish (dsRBM3) clustered itself into a separate clade. The fish species included are *Schizothorax richardsonii* (Sr), *Carassius auratus* (Ca) and *Ctenopharyngodon idella* (Ci).

**Discussion**

Initially, a partial sequence of snow trout PKR was amplified, and the complete sequence was deciphered by 5′ and 3′ RACE using the primers derived from the deduced partial sequence. The complete nucleotide sequence of PKR consists of 2884 bases with an open reading frame (ORF) of 2076 nucleotides. Further, the sequence contains a 5′ UTR of 234 nucleotides and a 3′ UTR of 558 nucleotides. The 5′ UTR of the coding sequence contains core promoter elements along with one interferon stimulated regulatory element (ISRE). PKR is interferon stimulated gene (ISG) having a role
in anti-proliferative activity against viruses while the presence of ISRE in the promoter region is an important prerequisite for interferon-dependent transcriptional induction [1, 11]. Nucleotide sequencing of the amplified promoter region of PKR gene revealed the presence of two ISREs. PKR promoter has the characteristic features of a core promoter as it has a transcription start site (TSS), a downstream promoter element (DPE), a TATA box beside two ISREs. Intriguingly, human and mouse PKR promoter regions are without TATA box and contain a unique kinase conserved sequence (KCS) which is a 15bp sequence [12]. However, KCS is absent in the PKR promoter of snow trout. KCS is a unique feature of TATA-less PKR promoter and responsible for the increased ISRE activity of the promoter. It has a role in both optimal basal expression and interferon-induced expression of PKR protein [11]. So, the absence of KCS in snow trout PKR promoter can be implicated to the presence of TATA box within its promoter which is a prerequisite for the basal expression and IFN induced expression of the gene.

Snow trout PKR coding sequence encodes a polypeptide of 691 amino acids with a molecular weight of 77.954 kDa and isoelectric point (pI) 8.71. The protein contains a bipartite nuclear localization signal (NLS) affirming that the snow trout PKR is a nuclear protein. In humans, PKR has been demonstrated to have both nuclear and cytoplasmic localization [13]. However, the bipartite nuclear localization signal in snow trout PKR suggesting its translocation into the nucleus. The role of the cytoplasmic form of PKR is well known to inhibit translation initiation factor, eIF2-α while the role of the nuclear form of PKR is not very evident. However, the role of the bipartite nuclear localization signal in nuclear signalling has been suggested [14]. It will be quite interesting to know whether knocking out the bipartite NLS of snow trout PKR retains its function besides localization in the cytoplasm.

The PKR protein possesses two domains, a dsRNA binding domain (dsRBD) and a kinase within it. The dsRBD consists of dsRNA binding motifs (dsRBMs) that help to bind the dsRNA while, the catalytic domain or the kinase domain is a serine/threonine kinase [3, 6]. So far, a complete homology model for PKR has not been reported, however, separate models for both PKR kinase domain and dsRNA binding domain (dsRBD) have been described [1,6,9]. Here we have attempted to predict the 3D
structure of the dsRNA domain and kinase domains using homology modelling. Snow trout PKR has three dsRNA binding motifs (dsRBMs) within the dsRBD, while the reference mouse model had only two. So, individual models were developed for each dsRBM and kinase domain. Snow trout dsRBM1, dsRBM2, and dsRBM3 were having a maximum identity with the first dsRBM of mouse PKR (PDB ID: 1x49A) thus used as a template for the homology modelling of the three motifs.

The dsRNA binding domain (dsRBD) consists of two or three dsRNA binding motifs (dsRBM) [6]. dsRBD of snow trout PKR has three tandem dsRBMs. Presence of three tandem dsRBMs in dsRNA binding domain has also been reported from PKRs of other fish [6, 9]. Two dsRBMs are reported from mammalian PKR while three tandem dsRBMs has been reported universally in fish [6, 9]. Three PKR genes are known to be induced by poly I:C in puffer fish one containing three double-stranded RNA binding domain (dsRBD) while two possess one dsRBD domain. However, one PKR has been described with three dsRBD in zebra fish. Two PKR genes with a putative dsRBD have been identified in fugu, three-spined stickle back and fat head minnow. Whereas Japanese media has one gene with two dsRBD, but the lone PKR gene of Japanese flounder has similar characteristic features like that of mammalian PKR [8]. The dsRBM1 of fish is closely related to dsRBM2 of a human while dsRBM2 of fish is more closely related to dsRBM1 of human, while the third dsRBM3 of fish is unique and closely related across fish species. The dsRBM1 and dsRBM3 had a typical feature of mammalian dsRBMs that is three β-sheets sandwiched between two α-helices. The dsRBM2 varies from the other two dsRBMs in the number of β-sheets as it has only two β-sheets sandwiched between two α-helices. However, the evolutionary analysis suggests that dsRBM1 and dsRBM2 are related to the two dsRBMs of human PKR while dsRBM3 is exclusive to fish and hence a unique feature to certain fish. Functional analysis of the three dsRBMs from grass carp revealed that the dsRBM1 and dsRBM2 are essential for the function of PKR while the third dsRBM3 enhances the function of PKR [10].

PKR kinase domain has the Serine/Threonine kinase activity that works either by auto-phosphorylation or phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF–2α) [1]. The kinase domain has an ATP binding site and a kinase active site. At the N-terminal of the kinase domain of PKR, an ATP binding site is present that contains a conserved glycine-rich stretch close to a lysine
residue [15]. The glycine stretch corresponds to amino acid position 408–417 that is present in the vicinity of Lys$^{431}$ in case of snow trout PKR. The Lys$^{431}$ in the kinase domain of snow trout PKR may be involved in ATP binding. The kinase domain of snow trout PKR retains the signature of kinase active site at 541–553. The kinase active site of the kinase domain of PKR requires a conserved aspartic acid residue for its catalytic activity[16] which acts as a proton acceptor and it corresponds to Lys$^{545}$ of snow trout PKR. It can be asserted that the functional features of the PKR kinase domain appear to be conserved through evolution. Thus the fish PKR has the same functional role as is evident for mammalian PKR.

The PKR kinase domain is formed of two lobes—an N-terminal lobes (N-Lobe) formed of β-sheets while the other C-terminal lobe (C-Lobe), formed of α-helices [17]. N-lobe of the kinase domain in human is formed of five antiparallel β-sheets and two α-helixs. One helix is at the top and the other one at the base whereas the helix at the base contains ATP binding site [17]. The N-Lobe of snow trout PKR kinase domain contains five antiparallel β-sheets and one helix at the base but lacks a helix at the top. ATP binding site or the functional site of the N-lobe is present in the α-helix at the base indicating that the snow trout PKR kinase domain retains the functional site. Furthermore, the C-lobe of human PKR kinase domain is composed of eight α-helices and two pairs of antiparallel β-sheets [17]. The C-Lobe of snow trout PKR kinase domain also contains the two pairs of antiparallel β-sheets with six α-helices. Although the kinase domain in snow trout lacks some structural features of human PKR kinase domain, but it retains the essential functional features of the PKR kinase domain.

The PKR protein of snow trout is a nuclear form of dsRNA-dependent protein kinase. The protein has one dsRNA binding domain at N terminal and a kinase domain at C terminal. The dsRNA binding domain has three dsRBMs, a characteristic feature of fish PKRs. Two dsRBMs of snow trout are similar to human dsRBMs while the third dsRBM may be unique to fish. The kinase domain of fish PKR structurally varies from the human PKR, lacking two α-helixs. However, it retains the functional features of the kinase domain, the ATP binding site, and the kinase active site.

Conclusions
Double-stranded RNA dependent protein kinase (PKR) an important molecule participates in the
innate immunity by recognizing dsRNA of viral origin. Snow trout PKR has two domains the dsRNA binding domain (dsRBD) and the kinase domain. The deduced nucleotide sequence of PKR coding region is 2884bp along with its UTRs. ORF of PKR is 2076bp and encodes a protein of 691 amino acids. Snow trout PKR protein contains three double-stranded RNA binding motifs (dsRBM) at N terminal besides possessing a serine/threonine protein kinase and a catalytic domain at C terminal. The promoter of snow trout was identified as a stretch of 791 nucleotide bases. It has two interferon-stimulated response elements (ISRE) besides the presence of core promoter elements. Intriguingly, unlike the mammalian PKR promoter, the snow trout PKR promoter has a TATA box but lacks the conserved kinase sequence (KCS) present in human PKR promoters.

Methods
Sample collection
Care and use of animals during the study was carried out as per the applicable international, national, and/or institutional guidelines (Institutional Animal Ethics Committee of ICAR-DCFR).
Snow trout (Schizothorax richardsonii) weighing nearly 8-10g were collected from Kalsa stream Chaafi, District Nainital, Kumaon region in Uttarakhand, India. The coordinates of the location were 29.37° N, 79.57° E with an altitude of 1238m above sea level. Fish were acclimatized in laboratory aquaria for a week before the commencement of the experiment. Aquaria were maintained at 24°C with adequate aeration and 12 hours of light. Periodic cleaning of the aquaria was done by siphoning off the debris besides changing three fourth volume of the water with fresh tap water.

Cloning and sequence analysis of PKR gene
Fish acclimatized to laboratory conditions for a week were injected intraperitoneally with poly (I:C) at a dose of 200µg per fish in a volume of 25µl [18] using a tuberculin syringe. Prior to dissection, the fish were anesthetized by dipping them in 0.05ml/l clove oil for 15-20 min [19]. Liver, spleen, and kidney were collected from treated fish after 72 hours of treatment. Total RNA was isolated from the collected tissues using Ribozol (Amresco) according to manufacturer’s recommendations. cDNA was prepared using total RNA as template and reverse transcriptase (RevertAid Reverse Transcriptase, Thermo) in a reaction volume of 20µl containing 5µg total RNA, 0.5µg random hexamers, 20pmol
dNTP mix, 20U RNase inhibitor and 200U of RevertAid Reverse Transcriptase. The reaction was incubated at 25ºC for 10min followed by 42ºC for an hour. Finally, the contents were heated at 70ºC for 10 min to stop the reaction. Primers were designed from the conserved region PKR coding sequences of Cyprinids after aligning the available sequences (Table 1).

Table 1: GenBank accession number of PKR mRNA from Cyprinids

| Fish                        | mRNA | Accession number |
|-----------------------------|------|-----------------|
| *Carassius auratus*, Gold fish | PKR  | JN091442        |
| *Ctenopharyngodon idella*, Grass carp | PKR  | JX511974        |
| *Cyprinus carpio*, Common carp | PKR  | JX516101        |

A fragment of approximately 900 base pairs was amplified, in a reaction volume of 25µl using 2.5µl of standard Taq Buffer, 10pmol of forward and reverse primers, 10pmol of dNTP mix, 1.5mM MgCl2, 1.25U of Taq DNA polymerase (NEB) and 2.5µl of cDNA template. PCR reactions were amplified by initial denaturation at 95ºC for 5min, followed by 35 cycles of denaturation at 95ºC for 45s, primer annealing at 60ºC for 1min, primer extension at 72ºC for 1min and a final extension of 10min at 72ºC. A PCR product of expected size (900 base pairs) was gel purified, cloned using InsTAclone PCR cloning kit (Thermo Scientific) and outsourced for nucleotide sequencing.

**Rapid Amplification of cDNA Ends - Polymerase chain reaction (RACE-PCR)**

Gene-specific primers for 5’ and 3’ RACE were designed (Table 2) using the partial nucleotide sequence of snow trout PKR coding region (Accession No. KX447496). Using SMARTer® RACE 5’/3’ Kit (Clontech) 5’ and 3’ ends of the PKR gene were amplified following the manufacturer’s recommendations. Cycling conditions included 35 cycles of denaturation at 94ºC for 30s, annealing at 68ºC for 1min and primer extension at 72ºC for 3 min. The two fragments 1400bp (5’ RACE) and 800bp (3’ RACE) obtained were cloned using InsTAclone PCR cloning kit (Thermo Scientific) and outsourced for nucleotide sequencing.

Table 2: List of primers their sequences and the gene amplified

| Primer ID | Gene Amplified          | Primer Sequence |
|-----------|-------------------------|-----------------|
| PKRmidFP-2 | PKR coding Sequence    | GAAGGAATACCCCGAAGGACAAGG |
| PKRmidRP-2 | PKR coding sequence    | CGTTTAGTCGGTTCCAGC |
| PKR 3RACE  | PKR 3’ RACE             | GATTACGCCAAGCTTTGGG |
| PKR S5RACE | PKR 5’ RACE             | GGATTGAGATTACGCCAAGCTTTGGG |
| PKR Ncol F1F | PKR Full length      | TCGCATT |
| PKR Xhol F2R | PKR Full length       | CATGCCATGGCTGTATCTCAGAGGAACTAT |
| PKR Pro GSP1 | PKR Promoter          | GATTACGCCAAGCTTTGGG |
| PKR Pro GSP2 | PKR Promoter          | GATTACGCCAAGCTTTGGG |
Nucleotide Sequence Analysis

The 3' UTR was analyzed while 5' UTR was analyzed for the presence of any regulating elements within it using YAPP (http://www.bioinformatics.org/yapp/cgi-bin/yapp.cgi) and JASPER [20].

Amplification and cloning of PKR promoter region

Genome walking was performed to identify the PKR promoter region. Briefly, genomic DNA was isolated from snow trout liver and kidney using Wizard® Genomic DNA purification kit (Promega). For genome walking, six genomic libraries were prepared by digesting genomic DNA with six different restriction enzymes namely DraI and Nsil (Fermentas), EcoRV (Takara), PvuII, Scal and SspI (NEB). Restriction digestion reaction was setup with 3μg genomic DNA and 10 units of respective restriction endonuclease. The reaction mix was incubated for 18 hours at 37°C for complete digestion. Digested DNA was analyzed by agarose gel electrophoresis on 0.6% agarose gel in 0.5X Tris Borate EDTA (TBE) buffer. The digested products were purified using phenol-chloroform-isoamyl alcohol (25:24:1).

Purified digested product (100ng) was ligated with universal adaptor 25μM using 3 units of T4 DNA ligase (Thermo Fischer) in a 10μl reaction volume containing 2x ligation buffer. The reaction mixture was incubated overnight at 16°C, heat inactivated at 70°C for 5 min and finally diluted with 70μl of nuclease-free water. Genomic libraries ligated with universal adaptor were used as template for primary PCR along with forward primer (AP1) a universal adaptor primer 1, along with a reverse primer (PKRproGSP-1) as gene-specific outer primer (Table 1). Secondary PCR was carried out using primary PCR product as template along with universal adaptor primer 2 (AP2) as forward primer and gene-specific inner primer (PKRproGSP-2) as reverse primer (Table 1). A master mix for seven reactions each of 50μl was prepared using 1X Long Amp Taq buffer, 10 pmol each forward and reverse primers, 300μM dNTPs and 5 units of Long Amp Hot Start Taq DNA polymerase (NEB). To the aliquoted master mix, 1μl template was added while the template was replaced with nuclease-free water in control reaction. Conditions used for amplification were 7 cycles of initial denaturation at 94°C for 30s and annealing at 72°C for 3min followed by 32 cycles of denaturation at 94°C for 30s, annealing 67°C for 3min and a final extension at 72°C for 6 min. After amplification, a 5μl aliquot was
analyzed on 1% agarose gel along with 1kb DNA molecular weight marker. Finally, the PCR products obtained were cloned using InstaClone PCR cloning kit (Thermo Scientific) for obtaining the nucleotide sequence. Analysis for the presence of regulatory elements within the promoter, online tools YAPP (http://www.bioinformatics.org/yapp/cgi-bin/yapp.cgi) and JASPER [20] were employed.

**PKR protein analysis**

The coding sequence of PKR was translated by Expert Protein Analysis System (EXPASY) translation tool [21]. Deduced protein sequence was analyzed for various structural and functional features using online prediction tools. The physicochemical properties of the protein were unravelling using ProtParam [22]. Domain analysis of the protein was carried out to reveal the functional features of the PKR protein. The protein domain features were predicted using PROSITE [23]. To interpret the N-glycosylation sites in the protein, NetNGlyc was used (http://www.cbs.dtu.dk/services/NetNGlyc/). The sub-cellular localization of the protein within the cell was predicted using WoLF PSORT II and the nuclear localization signal was detected by cNLS mapper [24-25].

**Phylogenetic analysis**

To carry out phylogenetic analysis of PKR protein from various fish and mammalian species, PKR protein sequences were retrieved from GenBank. The protein sequences were aligned using ClustalW in MEGA7 software [26]. Evolutionary history was inferred by using the maximum likelihood method based on the JTT matrix-based model [27]. Maximum parsimony method was applied for automatic heuristic search to obtain initial trees. Tree was drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 [26]. Moreover, the dsRNA binding motifs of fish *Schizothorax richardsonii* (Sr), *Carassius auratus* (Ca) and *Ctenopharyngodon idella* (Ci) were aligned using ClustalW along with two dsRNA binding motifs of human PKR protein for analyzing the evolutionary relationship.

**Homology Modelling**

To develop the homology model for PKR protein, a template model was required. To obtain such template, position-specific iterated BLAST (PSI-BLAST) was carried out. Stereo-chemical quality of the
models was validated by constructing Ramachandran Plot using PROCHECK [12]. Moreover the accuracy of the models was also validated in Verify_3D using SAVES (services.mbi.ucla.edu/SAVES/).

Abbreviations
Ca- *Carassius auratus*; C-Lobe- C-terminal lobe; Ci- *Ctenopharyngodon idella*; dsRNA- Double stranded RNA; dsRBM- RNA binding motifs; DPE- Downstream promoter element; eIF2-α- eukaryotic translation initiation factor 2-alpha; EXPASY- Expert Protein Analysis System; IFN- Interferon; ISRE- Interferon stimulated response elements; ISG- Interferon stimulated gene; KCS- Kinase conserved sequence; NLS- Nuclear localization signal; N-Lobe- N-terminal lobes; ORF- Open reading frame; PKR- Protein kinase; pl- Isoelectric point; PSI-BLAST- Position-specific iterated-Basic Local Alignment Search Tool; Sr- *Schizothorax richardsonii*; TBE- Tris Borate EDTA; TSS- Transcription start site; UTR- Untranslated regions

Declarations

**Ethics approval and consent to participate**

All applicable international, national, and/or institutional guidelines (Institutional Animal Ethics Committee of ICAR-DCFR) were followed for the care and use of animals during the study.

**Consent for publication**

Not Applicable

**Availability of data and materials**

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

**Competing interest**

The authors declare that they have no competing interest.

**ARRIVE Guidelines**

Not applicable as no *in vivo* studies were carried out.

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Authors Contribution

AP conceptualized the work. AP and AS finalized the Methodology. Formal analysis and investigation were done by AS, KB and PC. Writing and original draft were prepared by KB, AS and PC. Writing review and editing were performed by PC and AP. AP provided Resources and Funding acquisition. All authors have read and approved the manuscript.

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Figures
Diagrammatic representation of the full length coding sequence amplified for Snow trout PKR along with 5′ UTR and 3′ UTR analysis. The 5′ UTR analysis revealed presence of TATA box, transcription start site (TSS) and downstream promoter element (DPE) along with an interferon stimulated regulatory elements (ISRE). The 3′ UTR is having four instability motifs and a polyadenylation signal upstream the polyA tail.
Figure 2

Snow trout PKR promoter analysis, showing interferon stimulated response element (ISRE), TATA Box, Transcription start site (TSS) and downstream promoter element (DPE).
Figure 3

Domain Analysis of Snow trout PKR protein along with bipartite nuclear localization signal and N-Glycosylation sites in dsRNA binding motif.
Figure 4

Homology Model of dsRNA Binding Motifs (dsRBM) of snow trout PKR protein.
Figure 5

Homology model for kinase domain of snow trout PKR
Figure 6

Phylogenetic analysis of PKR with Maximum likelihood method in MEGA7.
Evolutionary analysis of dsRNA binding motifs (dsRBM) of fish and human. The fish species included are Schizothorax richardsonii (Sr), Carassius auratus (Ca) and Ctenopharyngodon idella (Ci).