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# Molecular cloning and *in silico* analysis of elongation factor 1A in *Schizothorax richardsonii*

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**Abstract**
In this manuscript, we report the cloning and *in silico* characterization of elongation factor 1A in a coldwater Trans-Himalayan cyprinid, *Schizothorax richardsonii.* The sequenced elongation factor 1A (*SrEF1A*) consisted of 1290 bp long partial ORF, which included a start codon (ATG) and 430 deduced amino acids. The *SrEF1A* sequence showed high homology (≥90%) with other teleosts and higher vertebrates. Phylogenetic analysis, multiple sequence alignment and prediction of conserved residues indicated a close evolutionary relationship among the cyprinids and conservation of *SrEF1A* protein across the vertebrate class. The deduced *SrEF1A* protein did not contain any signal peptide, but had two potential N-glycosylation motifs at 284$^{th}$ and 314$^{th}$ amino acid residue. Presence of many serine, threonine and tyrosine phosphorylation sites was also predicted, suggesting a potential post-translational regulation of the *SrEF1A* protein. *In silico* predictions of sub-cellular localization, function and protein-protein network illustrate the role of *SrEF1A* in the protein synthesis machinery of the cell. Finally, a reliable tertiary structure of *SrEF1A* protein was predicted with ten helixes and nineteen beta sheets. Ligand (GDP) binding sites in the tertiary structure were predicted at 15-22, 154, 156, 157 and 194-196 amino acid residues.

**Keywords:** Snow trout, elongation factor-1 alpha, house-keeping gene, reference gene, protein translation

## 1. Introduction
Elongation factor 1A (*EF1A*) is an important cellular protein involved in the decoding of mRNA codons to amino acids and thereby functional proteins [1]. It belongs to the family of G-protein and is one of the four subunits found in eukaryotic elongation protein complex [2, 3] In fact, *EF1A* is one of the most abundant protein found in the cytoplasm of a normal growing cell, accounting for 1-3% of total protein [4, 5]. The GTP bound form of *EF1A* delivers aminoacyl-t RNA to the A site of ribosome, and after the correct codon and anticodon pairing of mRNA and tRNA, a conformational change in *EF1A* results in hydrolysis of GTP to GDP and release of EF1A-GDP complex from A site. The GDP of *EF1A* is actively exchanged for GTP by *EF1B* resulting in active *EF1A* which participate in another round of elongation [1, 6]. *EF1A* is attributed with many other functional roles apart from protein syntheses such as interaction with cytoskeleton proteins or actin binding property and the ability to bundle F-actin [7], microtubule severing [8] and its stabilization [9]. The above functions suggest that *EF1A* plays a secondary role as a regulator of cytoskeletal rearrangements [8], but its biological significance is uncertain. Further, a large body of evidence supports the role of *EF1A* in nuclear export of t-RNA and shuttling of *EF1A* between nucleus and cytoplasm [1]. The *EF1A* is also known to direct misfolded proteins from ribosome to the proteasome [10]. Other functions include their role in apoptosis [11]; interaction with viral RNAs [12]; activator of protein kinases [13] and also altered expression of *EF1A* is implicated many kinds of tumors [14, 15]. Other non-canonical functions of *EF1A* are detailed elsewhere [1]. These diverse functions suggest that *EF1A* is a multifunctional protein with many physiological roles.
*EF1A* has been characterised in many fishes (as listed in Table 1) and is highly conserved across the vertebrate class (Table1), which implies a corresponding functional conservation. A number of isoforms of *EF1A* are reported in plants and animals. For example, five *EF1A* isoforms have been discovered in Senegalese sole with different spatial expression pattern, though their functional significance is not yet known [16]. On the other hand, *EF1A* has been widely used as a reference gene or internal control for normalising the mRNA expression data

[17, 18]. In the present study, we characterised 1290 bp of *EF1A* in *Schizothorax richardsonii*, in order to know the sequence of the transcript and protein, its phylogenetic relationship and protein structure. *S. richardsonii* is a coldwater cyprinid fish with wide distribution in the trans-Himalayan region. It forms an important component of subsistence fishery and is considered as a promising candidate species for aquaculture in Indian uplands.

## 2. Materials and methods

### 2.1. Amplification and cloning of *EF1A*

Total RNA was extracted from dorsal muscle tissue of *S. richardsonii* (weighing 15 g) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), as per manufacturers protocol. The extracted RNA was quantified using Take 3 system (Biotek Eon3 VT, USA). RNA quality was assessed in agarose gel electrophoresis using 1 µg of total RNA. First strand cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Thermo Fisher Scientific, MA, USA) and random hexamer, as per the manufacturer's instruction.

Gene-specific primers were designed based on the conserved regions identified by aligning available sequences of *EF1A* from closely related fishes of the cyprinid family, using Clustal omega online resource. Apt primers were chosen using Gene runner primer designing software (forward primer ATCAATCATGGGAAAGGAAAAG and reverse primer CCTCATGTCACGCACAGC). PCR was performed using the cDNA template, gene-specific primers, and Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA), as per manufacturers protocol. Amplified products were gel eluted, purified, ligated into pCR-4-TOPO vector and subsequently transformed into TOPO 10 cells (Invitrogen, Carlsbad, CA, USA). Positive colonies were screened and sequenced after plasmid extraction and confirmation through restriction digestion of plasmid.

### 2.2. *In silico* analysis of *SrEF1A*

The nucleotide sequence of *SrEF1A* was assembled in BioEdit version 7.0.5.2. The amino acid sequences were deduced using ExPASy (http://www.expasy.org/tools/). NetPhos 3.1 server was used to predict serine, threonine or tyrosine phosphorylation sites (http://www.cbs.dtu.dk/services/NetPhos/). N-glycosylation sites were predicted in the coding region using NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc /NetNGlyc1.0Server/). CELLO2GO web server was used for protein subcellular localization prediction (http://cello.life.nctu.edu.tw/cello2go/). Protein-protein interaction was predicted using STRING web server (https://string-db.org/). Multiple sequence alignments were performed using the ClustalW2.0 program (http://www.ebi.ac.uk/tools/clustalw2) in CLC Genomics Workbench v.7.5.2 (CLC Bio, Aarhus, Denmark). Evolutionarily conserved residues of deduced protein were predicted using Consurf server (http://consurf.tau.ac.il/2016/). Homology modeling was performed using Swiss model [19] and I-TASSER online server [20]. I-TASSER COFACTOR was used to deduce ligand binding sites. The protein models were visualised in UCSF Chimera. The predicted structures were validated using SAVES v5.0 (http://servicesn.mbi.ucla.edu/SAVES/), Rampage Ramachandran plot (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) and ProSA-web server (https://prosa.services.came.sbg.ac.at/ prosa.php) for checking overall model quality (Z score) and local model quality (residue wise energy plot). Phylogenetic tree was constructed using the Neighbour-Joining method based on the deduced amino acid sequences of *SrEF1A* and other reported species *EF1A* in MEGA 7 software. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Poisson correction method.

## 3. Results and Discussion

The PCR amplification resulted in an expected fragment of 1290 bp of *EF1A (*Fig.1), which was confirmed by sequencing. The nucleotide sequence of *SrEF1A* was deposited in NCBI Genbank under the accession number KU715836. The sequence result covered 5' start codon (ATG) but not 3' stop codon; thus the reported sequence is a partial cds. The deduced amino acid sequence of partial *SrEF1A* consisted of 430 amino acid residues. The full-length *EF1A* in other species is reported to be 462 in cyprinids, 461 in Xenopus, 461 in salmonids, 504 in elasmobranch, 462-463 in birds and 462 in mammals [16, 21] (number of residues as calculated from NCBI protein BLAST). The NCBI protein BLAST results further showed that >90% *SrEF1A* residues (Table 1) were similar across vertebrates, suggesting a resultant functional similarity. The phylogenetic tree constructed with 39 species *EF1A* including representatives of all vertebrate class resulted in three major clades (Fig. 2). The first clade included higher vertebrates, elasmobranchs and salmonids, whereas the second and third clade exclusively grouped the teleost *EF1A.* In the second clade, *S. richardsonii EF1A* was closely clustered with other cyprinids such as C*tenopharyngodon idella*, *Tor putitora*, *Cyprinus carpio, Carassius auratus* and *Danio rerio,* suggesting a common evolutionary linkage. Multiple sequence alignment of *SrEF1A* with selected other vertebrate *EF1A* (Fig. 3) and the presence of conserved residues across a larger space (Fig. 4) suggests evolutionary conservation of *EF1A* protein in vertebrates.

The Cello2GO predicted subcellular localization (Fig. 5) indicates that *SrEF1A* protein is mainly localised in cytoplasm (68.8%). This is in agreement with its major role in protein synthesis which occurs in cytoplasm, as observed by other in-vitro studies [22]. Likewise, the functional prediction of *SrEF1A* protein (Fig. 6) indicates multiple functions which include translation factor, GTPase and hydrolase activities; and RNA, nucleic acid and ion binding. These predicted functions correlate exactly with their major role in protein synthesis. It is known that *EF1A* binds to t-RNA in its GTP form and then hydrolysis of GTP ensures the fidelity of protein synthesis (GTP hydrolysis takes place only when correct pairing anticodon and codon is achieved); while Mg ion coordinates GDP+Pi state [23]. In any living system, protein-protein interaction is imperative to exert their proper function in vital processes such as cell growth, reproduction, motility, intracellular communication, and apoptosis [24]. As depicted in the interaction network diagram (Fig. 7), the major protein-protein interaction of *SrEF1A* included ribosomal protein L3, eukaryotic translation elongation factor 2b, eukaryotic translation elongation factor 1 gamma and other ribosomal protein, which are all involved in the protein synthesis machinery of eukaryotic cell [23, 6]. *SrEF1A* was also shown to interact with guanine nucleotide binding protein (G protein) and beta polypeptide 2-like 1 (GNB2L1), which

suggests their involvement in cellular processes other than protein synthesis such as signal transduction [1, 25]. All the predicted protein-protein interactions were apparently significant with high confidence scores (> 0.9).

*SrEF1A* did not contain any signal peptides (Fig. 8), but the presence of two glycosylation sites at 284th (NITT motif) and 314th (NVSV motif) residue was predicted by NetNGlyc 1.0 server (Fig. 9). Generally, proteins without signal peptides are implausible to be exposed to N-glycosylation and thus *SrEF1A* may not be glycosylated (*in vivo*) despite the presence of potential motifs [26]. An in-depth *in-vivo* study is required to ascertain the possibility or lack of *SrEF1A* glycosylation. Another important post-translational mechanism that regulates protein activity occurs through phosphorylation and dephosphorylation, employing kinases and phosphatases [27]. Many studies have demonstrated that *EF1A* is subjected to phosphorylation by various kinases such as protein kinase C, multipotential S6 kinase, cyclin dependent kinase, casein kinase II and TGF-ß receptor I which either up-regulates or down-regulates protein translation [28-30, 1]. In agreement, *SrEF1A* was predicted to contain many serine, threonine and tyrosine phosphorylation sites (Fig. 10), which suggests that *SrEF1A* activity might be regulated through post-translational phosphorylation.

The functional relevance of any protein at the molecular level is better understood through its three dimensional structure. When X-ray crystallography structure is unavailable, comparative homology modelling is commonly used to predict the structure of a query protein [20]. In this way, we also predicted the secondary and tertiary structure of *SrEF1A*. The predicted secondary structure of *SrEF1A* consisted 18.06 % helix, 35.81 % beta sheets and 45.58 % loop. The tertiary structure of *SrEF1A* (amino acid residue 5-430; Fig 11A & B) was built based on comparative homology modelling using 5lzs mammalian ribosomal elongation complex with aminoacyl-tRNA, eEF1A and didemnin B as a template. The structure consisted of 10 helixes and 19 beta sheets. Ramachandran plot (Fig.12) of the predicted structure showed that 96.5 % of the amino acid residues were within the favourable region, 3.3% in the allowed region and only a single residue was outside the allowed region. Further, ProSA overall model quality (Z score = -8.86; Fig.13A) suggested that the predicted structure was in confirmation with the experimentally determined protein structure available in PDB. Similarly, local model quality (Fig. 13B) suggested that absence of problematic or erroneous regions in the predicted structure as there were no positive energy values (thick green line indicates residue wise energy plot calculated based on smoothened average energy score over each-40 residues). Taken together, all these structure validation parameters suggest that the predicted protein structure is reliable and satisfactory. To illustrate, superimposition of *SrEF1A* structure (Fig.11 C) with yeast elongation factor complex *eEF1A* (1f60) resulted in a RMSD value of 0.34 A$^{o}$ indicating high similarity between the structures. In addition, the ligand (GDP) binding sites in the *SrEF1A* protein structure were predicted as follows: HIS-15, VAL-16, ASP-17, SER-18, GLY-19, LYS-20, SER-21, THR-22, LYS-154, ASP-156, SER-157, SER-194, GLY-195, TRP-196 and they are clearly depicted in Figure 14A & B). Molecular simulation and dynamic studies are further required to understand the conformation changes that occur between GTP and GDP form of *SrEF1A* [23].

**Table 1:** Similarity of *SrEF1A* with other teleost and higher vertebrate species

| Species | Similarity (%) | NCBI Accession |
|---|---|---|
| ***EF1A*** | | |
| *Schizothorax richardsonii* | 100 | AQV11481 |
| *Tor putitora* | 99 | AQV11477 |
| *Ctenopharyngodon idella* | 99 | BAO56800 |
| *Cyprinus carpio* | 98 | AAO49408 |
| *Danio rerio* | 97 | NP_571338 |
| *Carassius auratus* | 97 | BAB64567 |
| *Pimephales promelas* | 97 | AAT91089 |
| *Clarias batrachus* | 97 | BAO56800 |
| *Gobiocypris rarus* | 95 | ADI60310 |
| *Oryzias latipes* | 93 | XP_004074126 |
| *Salmo salar* | 92 | NP_001117101 |
| *Oncorhynchus mykiss* | 91 | NP_001117811 |
| *Fundulus heteroclitus* | 91 | XP_012707957 |
| *Oreochromis niloticus* | 92 | NP_001266576 |
| *Gadus morhua* | 90 | ABD62881 |
| *Sparus aurata* | 91 | AAD56406 |
| *Epinephelus coioides* | 91 | AOW69105 |
| *Xenopus tropicalis* | 92 | NP_989301 |
| *Gallus gallus* | 91 | NP_001308445 |
| *Mus musculus* | 90 | AAH04005 |
| *Bos taurus* | 90 | CAA34756 |
| *Homo sapiens* | 90 | BAD96702 |

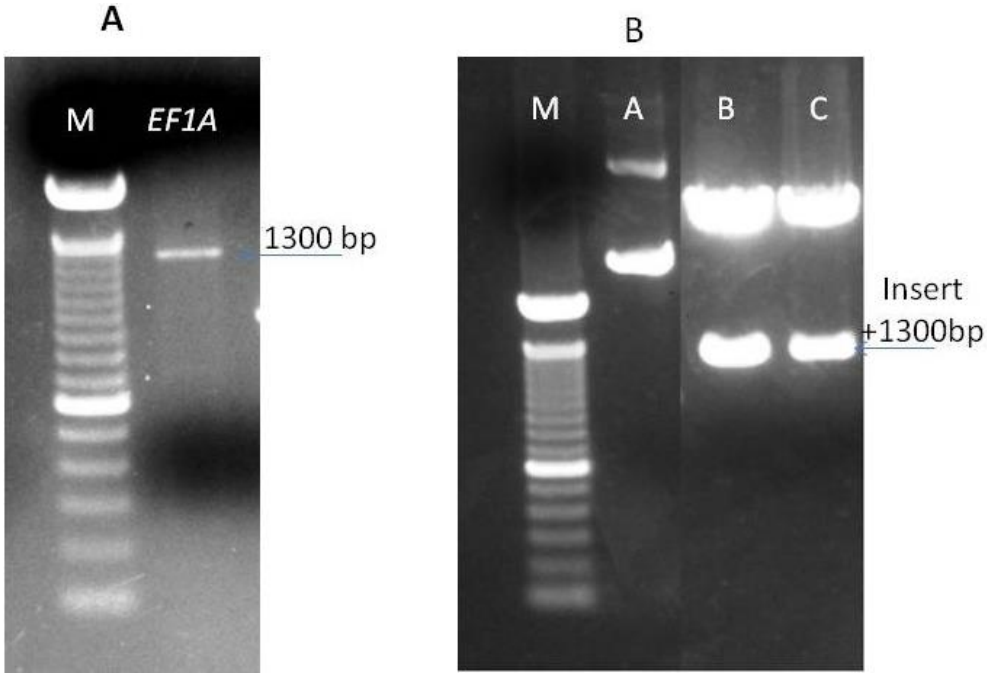



**Fig 1:** Agarose gel image of amplified and cloned *SrEF1A* fragment. (A) Amplified PCR product of *SrEF1A*; (B) Cloned pCR 4.0 TOPO plasmid showing a clear insert (*SrEF1A*) of expected size (+1300 bp) after restriction digestion. M: marker, A: uncut intact plasmid with insert; B & C: restriction enzyme digested plasmid containing *SrEF1A* insert.

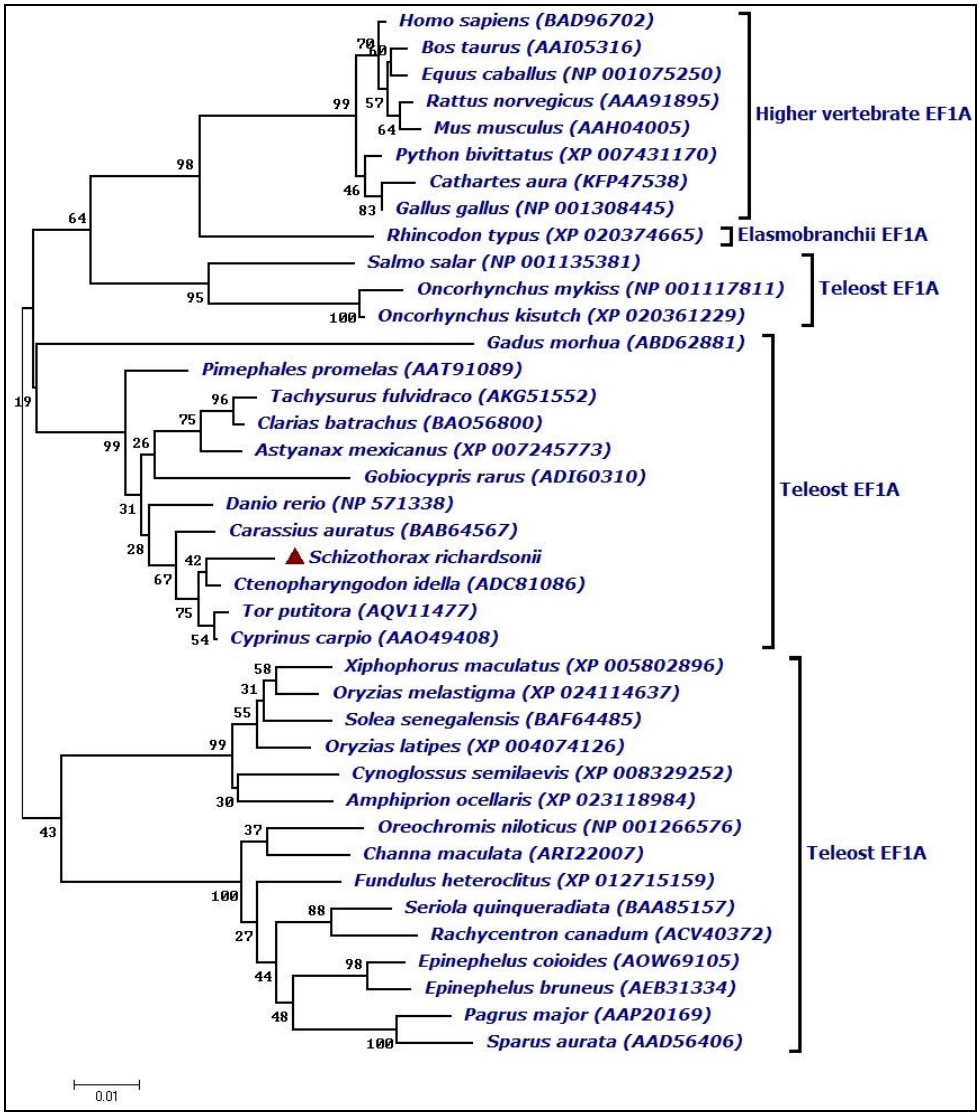



**Fig 2:** Phylogenetic tree constructed based on the deduced amino acid sequences of *SrEF1A* and other vertebrate *EF1A*, using MEGA 7 software. Numbers next to the branches indicate bootstrap values and scale bar represents evolutionary distance.

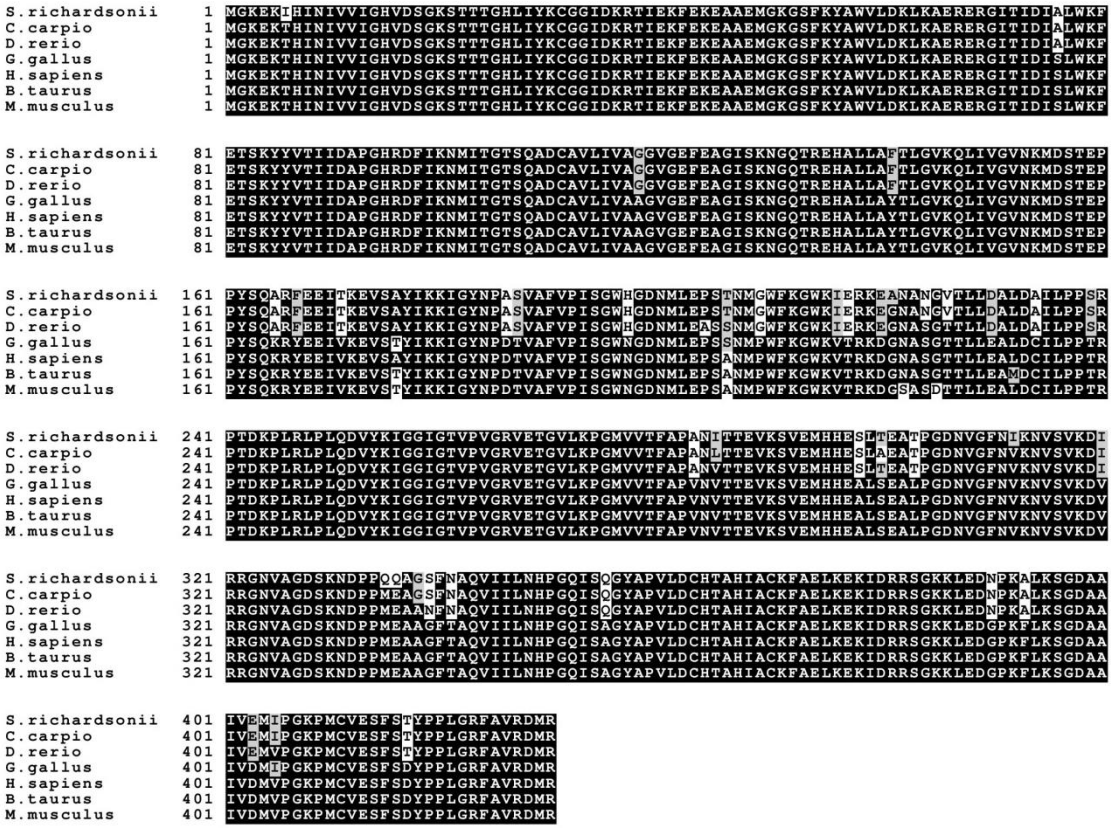



**Fig 3:** Multiple sequence alignment of *S. richardsonii EF1A* with other fishes and vertebrates. Protein sequence of *Cyprinus carpio* (AAO49408); *Danio rerio* (NP_571338); *Gallus gallus* (NP_001308445); *Homo sapiens* (BAD96702); *Bos tarus* (AAI05316); *Mus musculus* (AAH04005) were obtained from NCBI database.

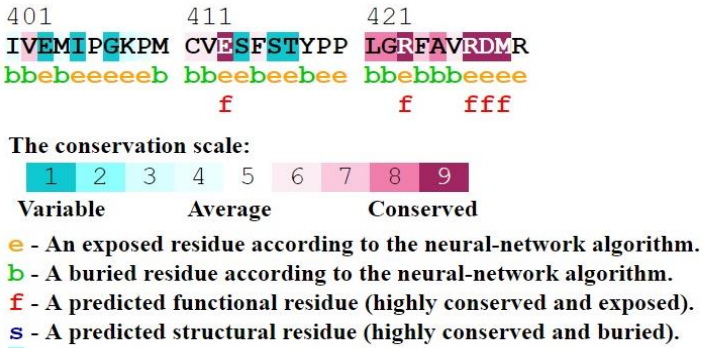



**Fig 4:** Evolutionary conservation of *SrEF1A* amino acid residues as construed by ConSurf

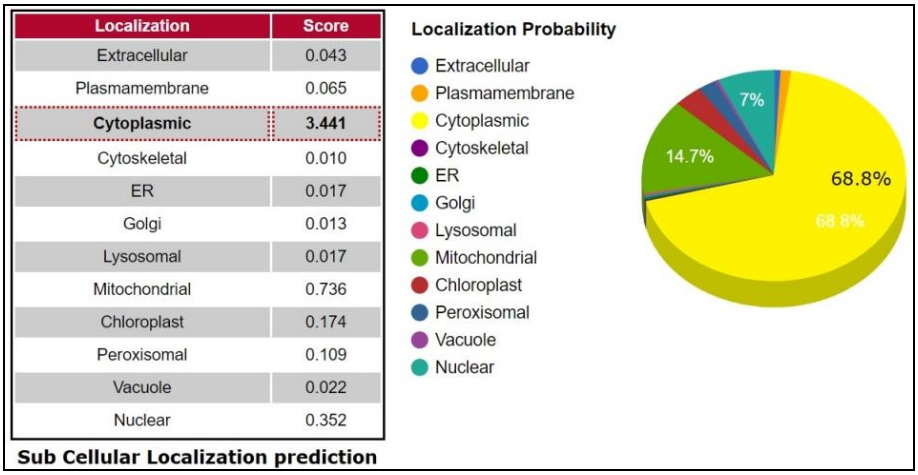



**Fig 5:** Subcellular localisation of *SrEF1A* as predicted by CELLO2GO

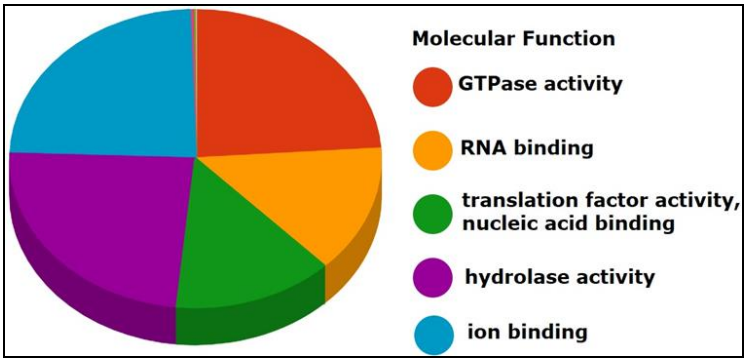
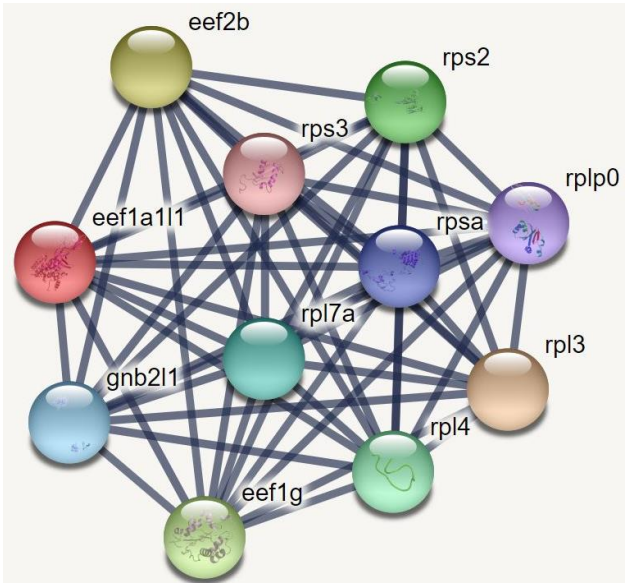



**Fig 6:** Molecular function of *SrEF1A* as predicted by CELLO2GO



| Predicted Functional partners: | | Score |
|---|---|---|
| rpl3 | *Ribosomal protein L3 (403 aa)* | 0.995 |
| eef2b | *Eukaryotic translation elongation factor 2b (858 aa)* | 0.995 |
| eef1g | *Eukaryotic translation elongation factor 1 gamma; Probably plays a role in anchoring the complex to other cellular components (486 aa)* | 0.994 |
| rps2 | *Ribosomal protein S2 (280 aa)* | 0.987 |
| rpl4 | *Ribosomal protein L4 (375 aa)* | 0.985 |
| rpl7a | *Ribosomal protein L7a (266 aa)* | 0.985 |
| gnb2l1 | *Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1; Involved in the recruitment, assembly and/or regulation of a variety of signalin...* | 0.982 |
| rpsa | *Ribosomal protein SA; Required for the assembly and/or stability of the 40S ribosomal subunit. Required for the processing of the 20S rRNA- precursor...* | 0.980 |
| rplp0 | *Ribosomal protein, large, P0; Ribosomal protein P0 is the functional equivalent of E.coli protein L10 (316 aa)* | 0.970 |
| rps3 | *Ribosomal protein S3 (245 aa)* | 0.965 |

**Fig 7:** Interaction network of *SrEF1A* protein showing different functional partners as predicted by STRING (with confidence scores)

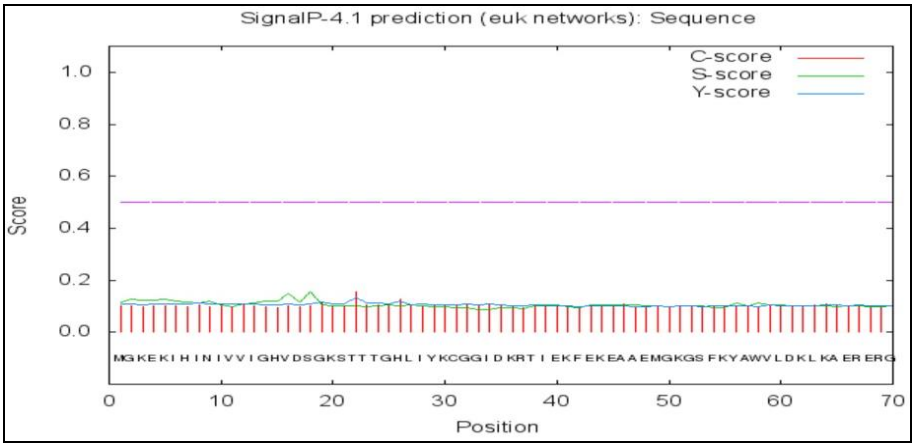



**Fig 8:** The deduced signal peptide of *SrEF1A* protein (SignalP-4.1)

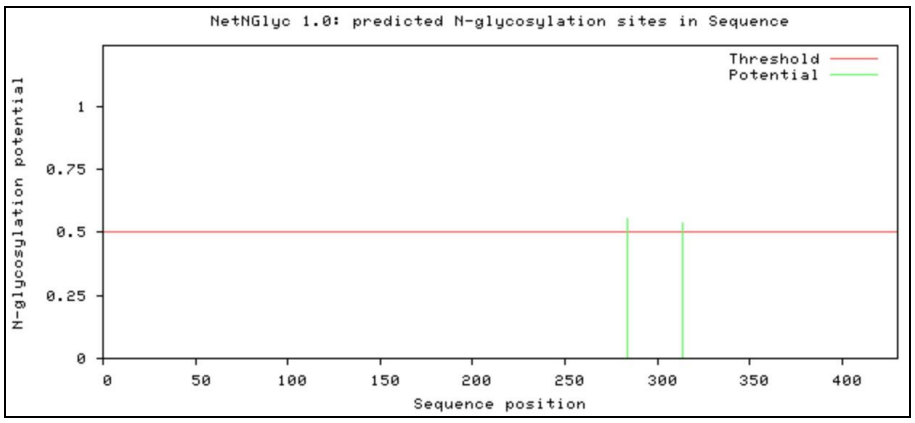



**Fig 9:** Potential N-glycosylation sites found in the *SrEF1A* protein

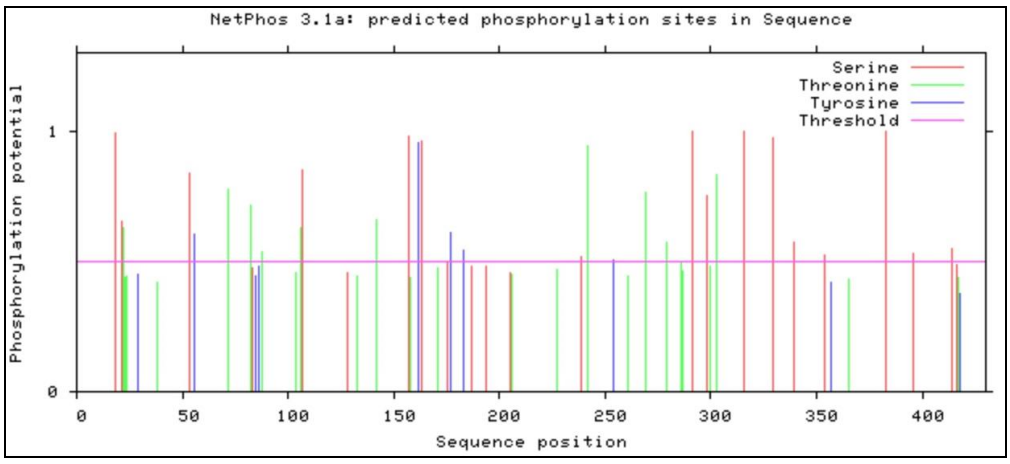



**Fig 10:** Potential serine, threonine, and tyrosine phosphorlyation sites found in the *SrEF1A* protein

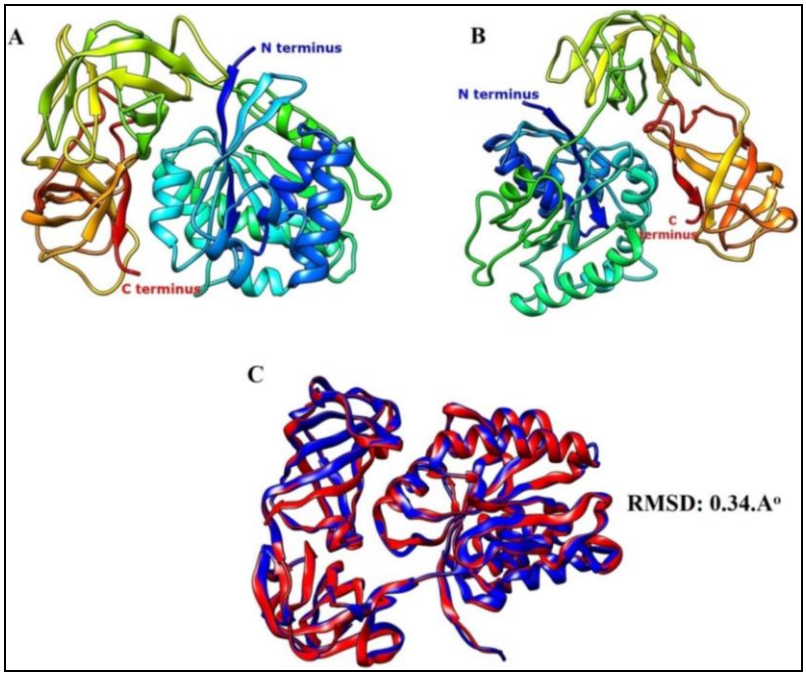



**Fig 11:** Predicted tertiary structure of *SrEF1A*. (A) front view; (B) back view; (C) superimposition of model *SrEF1A* structure with yeast elongation factor complex *EF1A* (1f60).

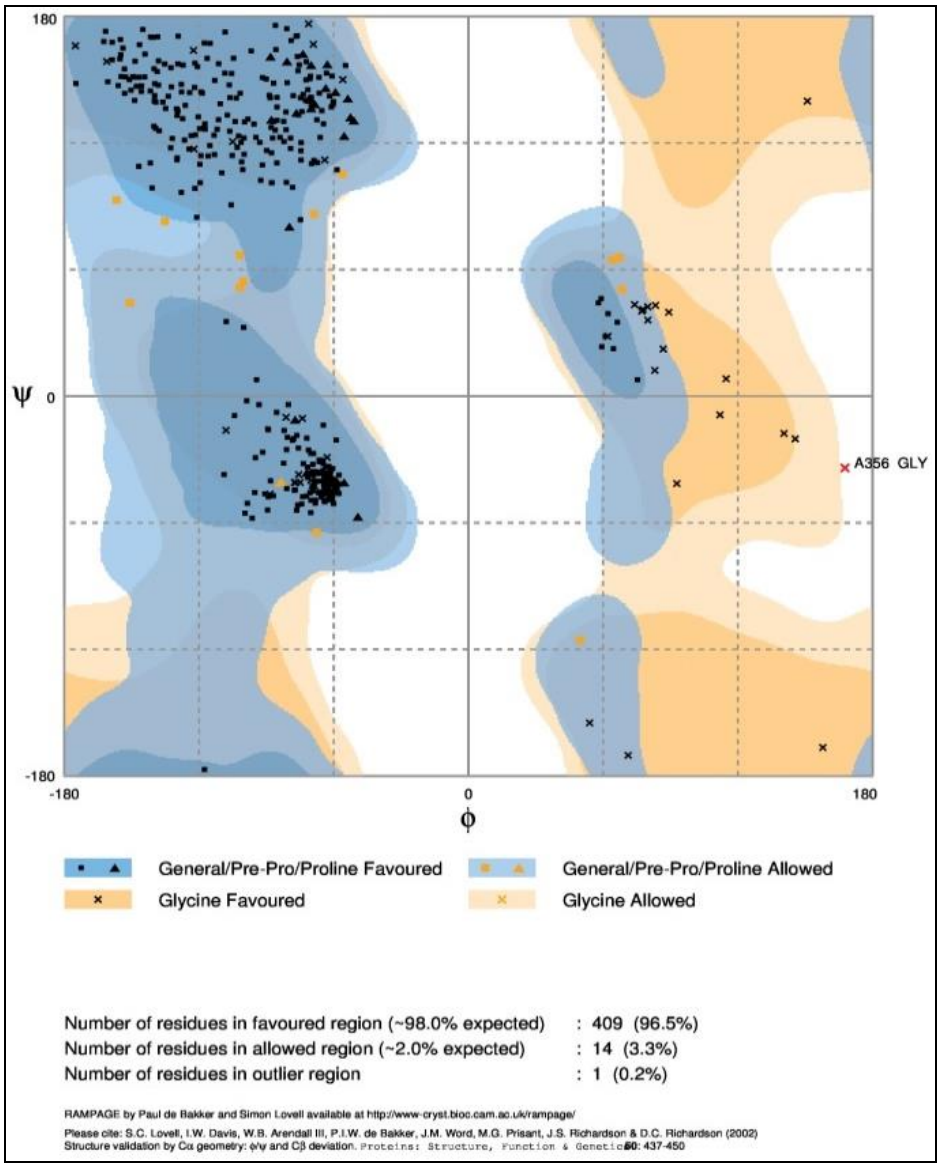



**Fig 12:** Ramachandran plot (Rampage) showing dihedral angles Psi and Phi of amino acid residues in the predicted tertiary structure of *SrEF1A.*

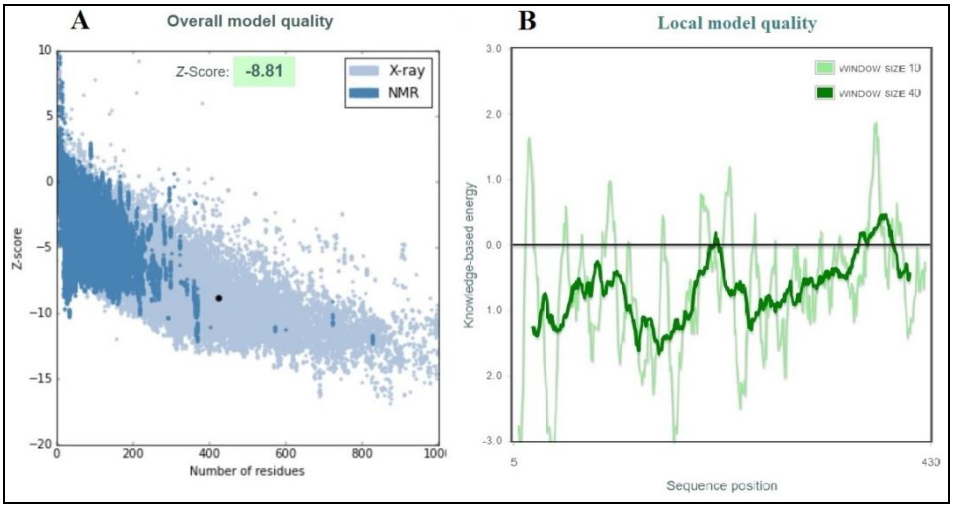



**Fig 13:** ProSA overall model quality/Z score plot (A) and ProSA local model quality/residue-wise energy plot (B) of the predicted tertiary structure of *SrEF1A*.

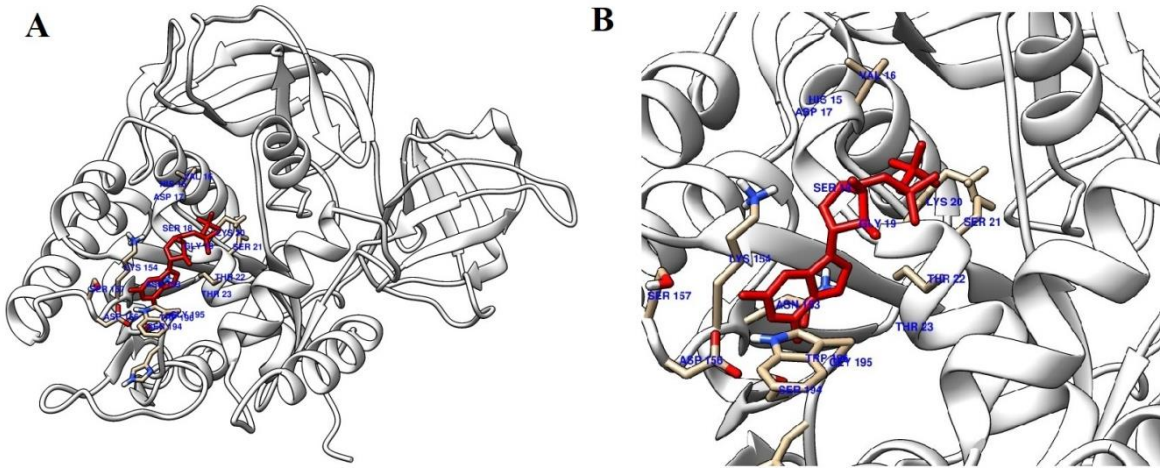



**Fig 14:** Ligand binding sites in the predicted *SrEF1A* tertiary structure (A); closer view of the ligand binding sites (B).

## 4. Conclusion

In conclusion, we characterized 1290 bp long coding region of Elongation factor 1A (*SrEF1A*) in a Himalayan cyprinid, *Schizothorax richardsonii*. Phylogenetic analysis showed that *SrEF1A* clustered along with other cyprinids, indicating their common ancestral root. Moreover, *SrEF1A* sequence showed high similarity (> 90%) with that of other vertebrates, suggesting its evolutionary conservation. The presence of potential glycosylation and phosphorylation sites in *SrEF1A* indicates the possibility of post-translational activation of the protein through phosphorylation. The predicted sub-cellular localization, function and protein-protein network coherently emphasizes the role of *SrEF1A* in the protein translation machinery of the cell. Besides, we also predicted a reliable tertiary structure of the *SrEF1A* protein along with its ligand binding sites. Further studies on full-length cloning, molecular docking, simulation and dynamics of *SrEF1A* will reveal more subtle information about this protein and help us get a deeper insight into its molecular function.

## 5. Acknowledgements

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