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ARTICLE

Identification of microRNAs from Atlantic salmon macrophages upon *Aeromonas salmonicida* infection

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Computational approach was used in to identify potent macrophage specific miRNAs involved in basic biological process of *Salmo salar*. Analysis of 1119 ESTs from macrophages of Atlantic salmon (*Salmo salar*) infected with *Aeromonas salmonicida* revealed expression of 3 miRNAs. Phylogenetic analysis of both the pre-miRNA sequence revealed its evolutionarily conserved nature among various species. Identified targets of the predicted miRNAs revealed the role of miRNA in pathogenesis, stress response and allosteric exchange of histones. Further detailed studies of these miRNAs will help in revealing its function in different biological process necessary for the action of macrophages upon pathogen infection.

Keywords: miRNA; *Salmo salar;* stress response; miRNA target; EST sequence; Phylogenetic

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Introduction

The fish Atlantic salmon (*Salmo salar*) belongs to the family Salmonidae with average size ranges from 28-30 inches (71-76 cm) long and weight 8-12 pounds (3.6-5.4 kg) after two years at sea. Wild populations of Atlantic salmon have declined worldwide due to various diseases that are caused by reduction in immune systems and the non-specific immunity in fish is developed by phagocytic cells such as macrophages and neutrophils [1]. Among the immune cells macrophages plays a key role in infectious disease of Atlantic salmon. The infectious disease salmonid rickettsial septicemia which kills millions of farm fish is caused by the intracellular bacterial pathogen *Piscirickettsia salmonis* [2] and recently 71 differentially expressed *Piscirickettsia salmonis* infected macrophage specific transcripts were reported [2]. Clinical sign of fish disease includes necrosis and inflammation furthermore intracellular activities are altered in infected macrophages [2]. Diseases in fish could be managed by improving necessary knowledge of the transcriptome and its regulation [3].

MicroRNAs (miRNAs) are short noncoding RNA molecules consisting of 21-25 nucleotides (nt) in length which are important regulator of gene expression in the cells [5]. They are mainly involved in RNA based regulation of gene expression and it is an evolutionarily conserved mechanism found in all species [4]. Expressed sequence tags (ESTs) analysis is a powerful tool in identification of conserved microRNAs from species where the whole

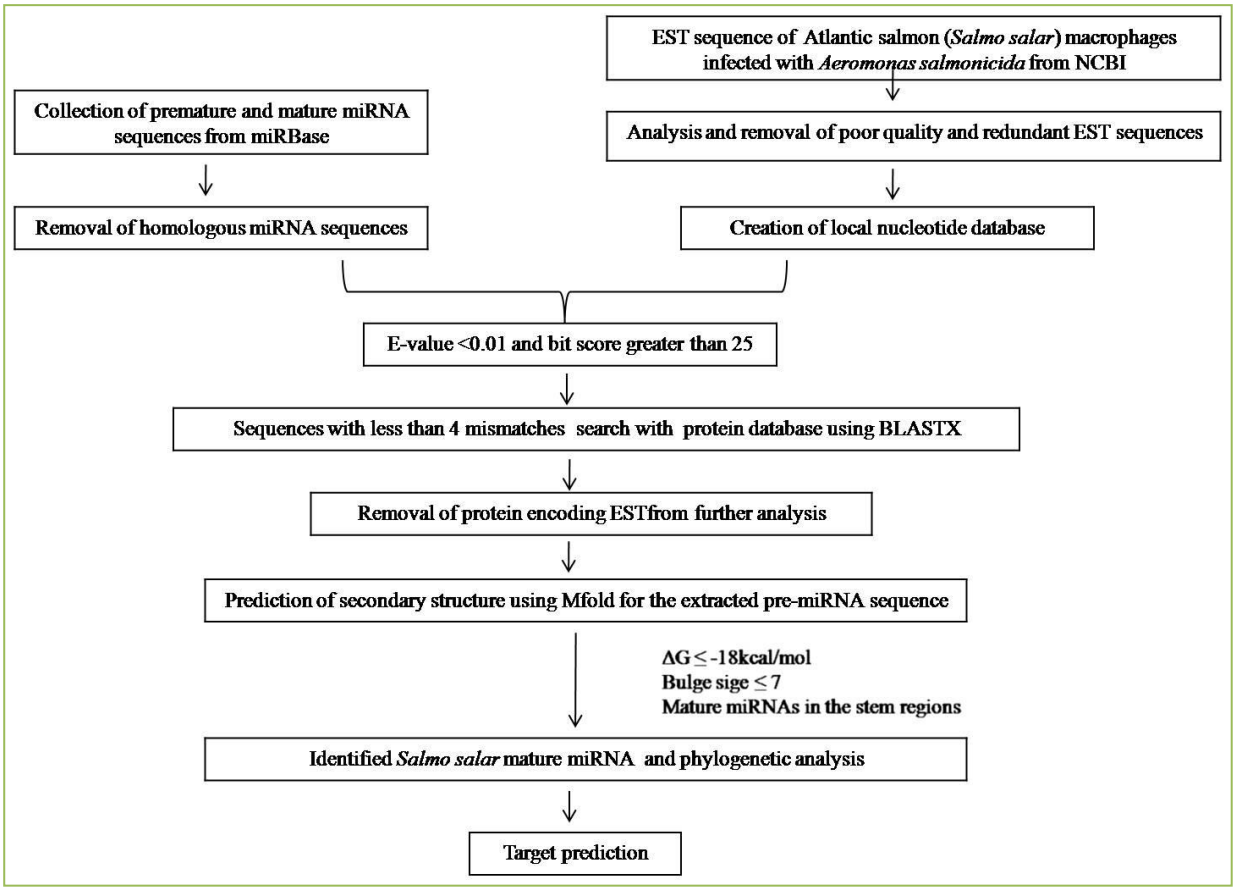

Fig 1. A schematic illustration of different steps involved in prediction of *Salmo salar* miRNA and their target prediction as described by Thirugnanasambantham *et al* 2013.

genome sequences is not available. In this novel study, we identified Atlantic salmon (*Salmo salar*) miRNAs from its ESTs resource using all miRNAs reported from different species and we reported 3 potent miRNAs from *Salmo salar* ESTs, using computational approach and analysed its target genes.

Materials and methods

1. Collection of reference miRNA and EST sequences

Macrophage specific ESTs resources from Atlantic salmon (*Salmo salar*) infected with *Aeromonas salmonicida* were used to identify miRNAs. ESTs sequences (1119, as on 2013) of *Salmo salar* macrophage were downloaded from NCBI EST database (http://www.ncbi.nlm.nih.gov) by providing the search name "Atlantic Salmon macrophage - Aeromonas". Local nucleotide database was created after eliminating redundant and poor quality sequences. The sequences of all pre-miRNA (21264, as on 2013) and mature miRNA (21623, as on 2013) were retrived from miRBase (http://www.mirbase.org/). The miRNAs after removal of homologous sequences were used for searching *Salmo salar* miRNAs.

2. Identification of precursor miRNA sequences

The pre-miRNAs retrieved from public database were used as reference material for search of its homolog in *Salmo salar* transcriptome at e-value threshold < 0.01 using BLAST + 2.2.22 program with all other parameters as default [6]. The entire candidate ESTs with homology towards the pre-miRNA sequences were saved as FASTA formats. Reference miRNA sequence was aligned against the corresponding singleton ESTs using Clustal W (Thompson et al. 1994) and the selected sequences with not more than 4 mismatches were validated for their non protein encoding phenomenon using BALST against protein database at NCBI using blastx with default parameter [7].

3. Structural validation of candidate pre-miRNAs and target prediction

The structural validation of pre-miRNAs were performed by analyzing the secondary structure created by Mfold version 3.2 (http://www.mfold.rna.albary.edu/). The precursor RNA sequence are considered to be a candidate miRNA precursor as described by Zhang et al. [8] and the parameters includes a) it could able to fold into an appropriate stem-loop hairpin

Table 1. Details of predicted *Salmo salar* miRNA

New *Salmo salar* miRNA	Source miRNA	Soruce organism	PL	MFE ΔG	MS	ME	SE	Strand	A+U %
Ssa-miR-21 -5p	hsa-miR-21 -5p	Homo sapiens	73	-37.7	UAGCUUAUCAGACUGGUGUUGA	21/22	DR695909	5’	59.09
Ssa-miR-22 3-3p	hsa-mir-22 3	Homo sapiens	121	-46.3	UGUCAGUUUGUCAAAUACCCCA	22/22	DR695353	3’	59.09
Ssa-miR-22 3-5p	hsa-mir-22 3	Homo sapiens	121	-46.3	AGUGUAUUUGACAAGCUGAGUU	21/22	DR695353	5’	63.64

PL = Pre-miRNA Length, MEF = Minimal Free Energy, MS = Mature sequence, ME = Match Extent, SE = Source EST

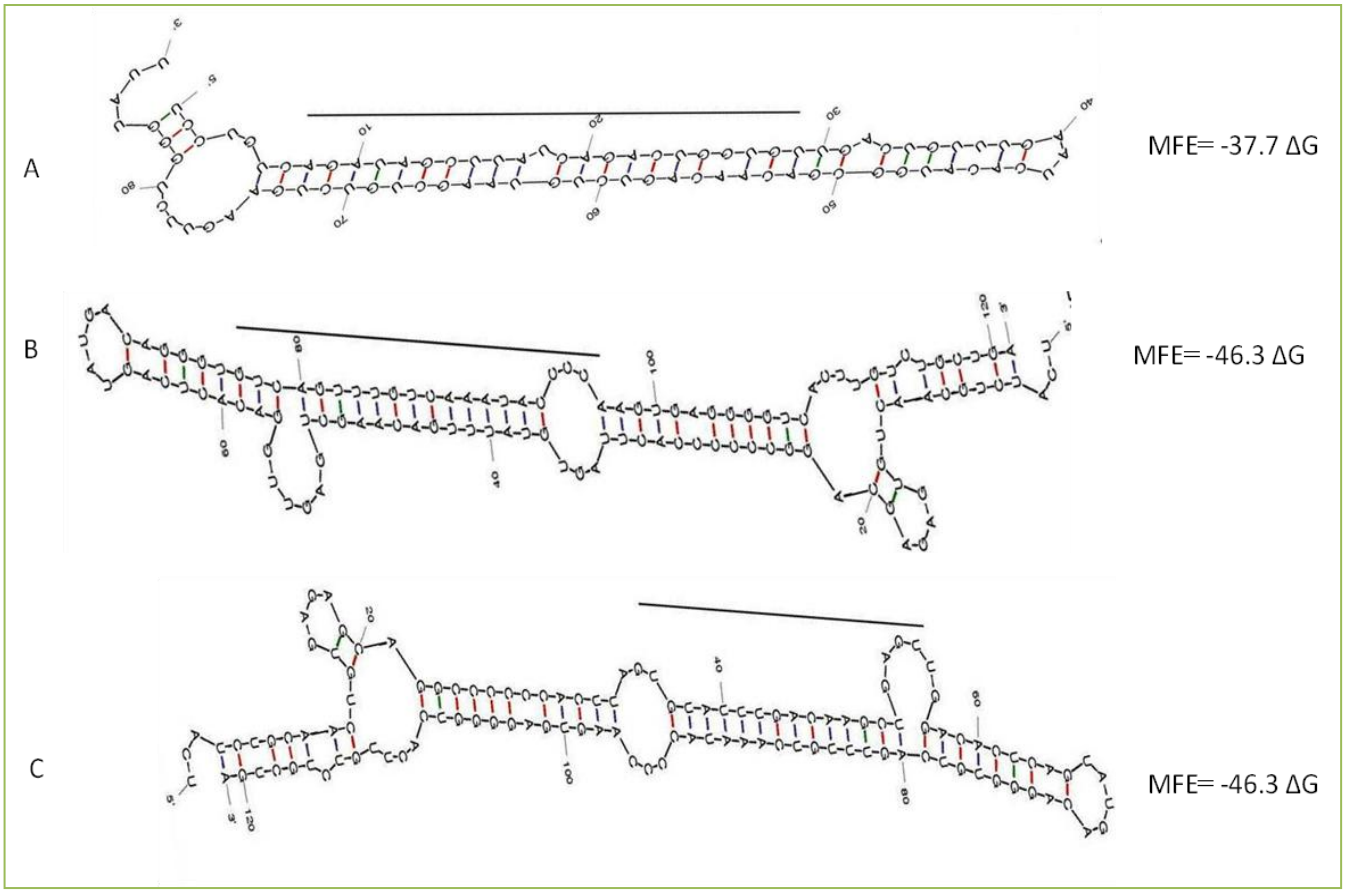

Fig 2. Hairpin structure of predicted *Salmo salar* miRNA. A. Ssa-miR-21-5p; B. Ssa-miR-223-3p; C. Ssa-miR-223-5p

secondary structure, b) mature miRNA sequence site in one arm of the hairpin structure, c) miRNAs should have less than seven mismatches with the opposite miRNA* sequence in the other arm, d) loop or break in miRNA sequences should not be noticed, e) predicted secondary structures must have higher negative energy MFEs (≤–18 kcal/mol), and 40-70% A + U contents. The structurally validated sequences were selected and subjected to RNA-hybrid, a miRNA target prediction tool (http://www. bibiserv.techfak.uni-bielefeld.de/rnahybrid/)

4. Nomenclature of predicted miRNAs and Phylogenetic analysis

The predicted miRNAs were named as per the pattern of miRBase [9]. The mature sequences were designated ‘miR’, and the precursor hairpins were labeled as ‘mir’ with the prefix ‘Sas’ for *Salmo salar*.

Phylogenic relationship of predicted *Salmo salar* miRNA was compared with their orthologues from other species to reveal the conservation. Predicted candidate miRNAs from *Salmo salar ESTs* were searched and corresponding precursor sequences of homolog miRNA’s were identified collected and aligned using Clustal W [10]. Phylogenetic analysis miRNA sequences were performed with MEGA5. The evolutionary distances were computed using the Maximum Composite Likelihood method [11] and are in the units of the number of base substitutions per site. Finally the

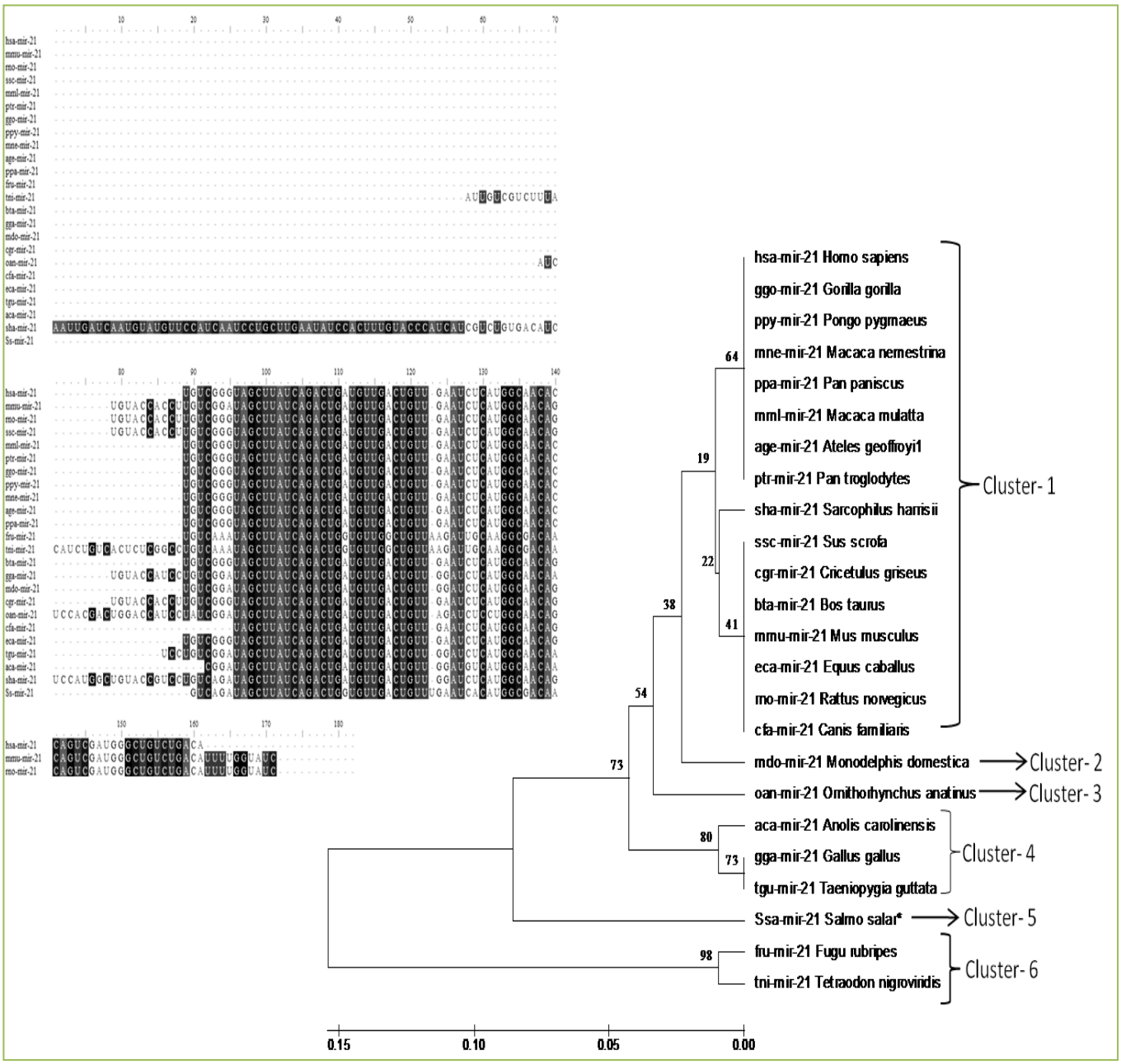

Fig 3. A. Multiple sequence alignment of Ssa-miR-21 with its related species showing conserved region. B. Phylogenetic tree showing evolutionary conservation of Ssa-miR-21.

evolutionary tree was constructed and analyses were conducted in MEGA5 [12].

Results

Transcriptomic resource in form of ESTs from *Salmo salar* macrophage was used for computational prediction miRNA as described in Fig. 1. As on 2013, 1119 *S.salar* macrophage specific EST sequences were retrieved from NCBI EST database and its corresponding miRNAs were predicted. From whole EST analysed sequences we have observed that only 54 sequences were recorded with less than four mismatches with the corresponding pre-miRNA orthologue. Further 20 non proteins encoding ESTs were identified using BLAST against protein database at NCBI using blastX. Finally three new *S.salar* miRNAs were reported by evaluation of secondary structure analysis. For the predicted miRNAs Table 1 provides details such as source sequences, length of precursor sequences and their minimum folding free energies and A+ U content.

Two miRNAs predicted from the *S. salar* EST resource were noticed in direct strand and the one was observed in indirect strand. The minimum free energy of the predicted

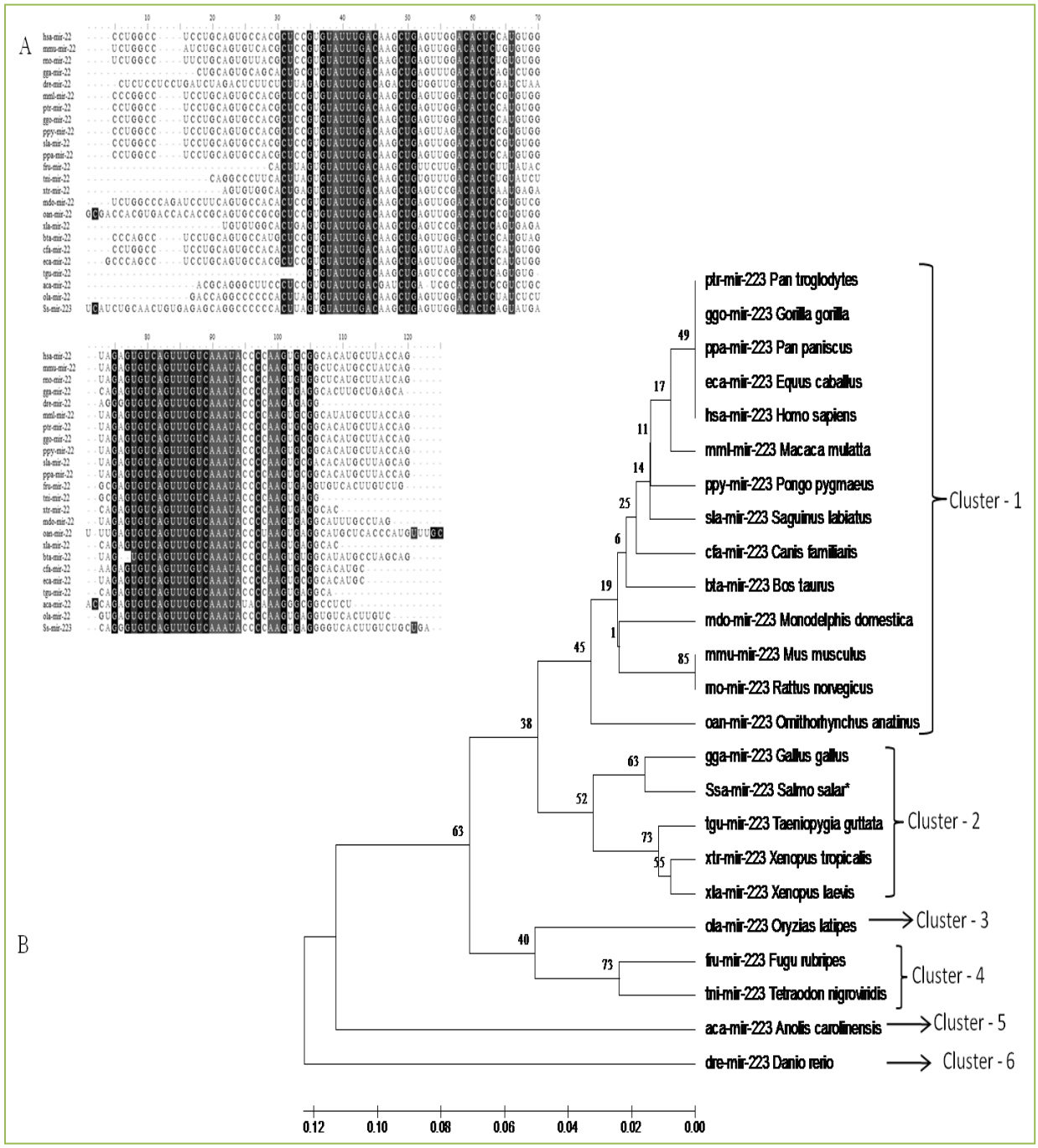

Fig 4. A. Multiple sequence alignment of Ssa-miR-223 with its related species showing conserved region. B. Phylogenetic tree showing evolutionary conservation of Ssa-miR-223.

miRNA ranged from -37.7 to -46.3 kcal/mol with an average of -43.43 kcal/mol. While considering the A+U percentage in the predicted miRNAs, it ranges from 59 % to 63% with an average value of 60.6% (Table 1). Among the miRNAs predicted Ssa-miR-223-3p sequence was 100% matched with their homologue in miRNA database. Analysis of RNA folding using Mfold revealed that, all the mature *S. salar* miRNAs were found in the stem portion of the hairpin structures are break or loopless inside the sequences (Fig. 2, Table 1) and containing less than 7 mismatches in the other arm.

The present study was the first report of miRNAs (miR-21-5p, miR-223-3p and miR-223-5p) from macrophages of *S.salar*. Phylogenetic analysis of the predicted miRNAs revealed that both the miRNA (Ssa-mir-21 and 223) were highly conserved, with more similarity among the species studied. Analysis of phylogeny of Ssa-miR-21 revealed its evolutionary conservation among from aquatic organism to human (Fig. 3). Based on our

Table 2. Targets of predicted *Salmo salar* miRNA

Sl. No	*Salmo salar* miRNA	Mfe energy	p-value	Target protein	NCBI Acc. No.	Function	Reference
1	SSa-miR-21-5p	-31.9 kcal/mol	0.157424	kelch-like 32 (Klhl32), transcript variant 2	NM_001033531	Inflammatory responses, Ubiquitination	27, 28
2	Ssa-miR-223-3p	-34.1 kcal/mol	0.004701	ASF1 anti-silencing function 1 homolog B	NM_024184	Histone Complexes	29
3	Ssa-miR-223-5p	-34.4 kcal/mol	0.025286	Signal peptide, CUB and EGF-like domain-containing protein 1	NM_022723	Develomental Process	30

Phylogenetic tree, we have observed 6 distinct clusters for the analysed miR-21, the cluster -1 was predominantly occupied with the mammals, which includes dog, horse, pig, rat, mouse, monkeys, chimpanzee, gorilla and human. The Brazilian opossum (*Monodelphis domestica*) and platypus (*Ornithorhynchus anatinus*) were observed as separate clusters as cluster-2 and cluster-3 respectively. The cluster-4 is recorded with species of reptiles and birds. While the Atlantic salmon was observed as separate cluster (Cluster-5) with more similarity to the cluster-6, which is occupied with the puffer fish. Similar to the miR-21, Phylogenetic analysis of miR-223 revealed 6 distinct clusters (Fig. 4). The cluster-1 was fully dominated with the members of mammalian species. The miRNA from Atlantic salmon reported in the present study was observed in cluster-2 and shared the position with birds and amphibians. The cluster-3 and cluster-4 was occupied with killer fish (*Oryzias latipes*) and puffer fish (*Fugu rubripes* and *Tetraodon nigroviridis*) respectively. The reptile lizard (*Anolis carolinensis*) and zebra fish (*Danio rerio*) was noticed as separate cluster named cluster-5 and cluster-6 respectively.

The results from our study on identification targets of the predicted miRNAs (Table 2). We have observed kelch-like 32 (Klhl32), transcript variant 2 as target of SSa-miR-21-5p, ASF1 anti-silencing function 1 homolog B as target of Ssa-miR-223-3p and Signal peptide, CUB and EGF-like domain-containing protein 1 as target of Ssa-miR-223-5p.

Discussion

Micro RNAs are involved in regulation of diverse and important processes but not limited to events such as B-cell differentiation [13], adipocyte differentiation [14], cardio genesis [15], insulin secretion [16], antiviral defense [17] and the development of cancer [18]. Computational approach is more economic and fast than other methods used for miRNA identification. Among various computational methods prospecting of miRNAs from EST resource directly reveals the expression of the identified miRNA. Limitations in miRNA research in the area of host–pathogen interactions has been eliminated by accumulating evidences from vertebrates, which suggests that miRNAs play major roles, including anti-pathogen responses targeting the microorganism directly or altering the expression of host genes that are beneficial to the microorganism [11, 19]. Our study revealed expression of three miRNAs in response to *Aeromonas salmonicida* infection. Though Reyes et al. [3] had reported 41 and 266 homologous and novel microRNAs, respectively using vertebrate miRNA dataset, they failed to predict the miRNA homologue which are absent in vertebrate. Bizuayehu et al. [20] has reported conserved and novel pre-miRNAs in a non-model vertebrate (*Atlantic halibut*) lacking substantial genomic resources using next generation sequencing technologies. Hackl et al. [21] extracted pre-miRNA from reference genomes, folded in silico to verify correct structures from Chinese hamster ovary (CHO) cell lines.

Our study shows the evolutionary conservation among the identified miRNA and conservation in miRNA sequence was observed among the other species too [22, 23]. Although, there are so many microRNAs prediction online softwares available to find out target genes, but each software may vary to predict the target gene and currently there is no specific software available for fish microRNAs target prediction. Here we used RNA hybrid computational method in which the available ESTs sequences of Atlantic salmon were used to derive target gene for the identified microRNAs. we have observed that kelch-like 32 (Klhl32) responsible for protein-protein interaction involved in multiple cellular processes, including cytoskeleton organization and dynamics, ion channel modulation, transcriptional regulation, and protein ubiquitination was targeted by the SSa-miR-21-5p. The kelch-like 32 proteins are substrate adapter proteins which mediate interaction between ubiquitin ligase and the substrates targeted for degradation [24]. Kelch like genes are conserved from *Drosophila melanogaster* to *Homo sapiens* [25] and plays key role in poxvirus virulence and host range through modulation of inflammatory responses [26]. Transcription factor Nrf2 (NF-E2-related factor-2) essentially involved in oxidative and electrophilic stress responses are tightly regulated at the protein level through proteasomal degradation by Kelch-like ECH-associated protein mediated ubiquitination [27]. Ssa-miR-223-3p targeted ASF1 anti-silencing function 1 homolog B involved in allosteric exchange of histones H3-H4 complexes [28]. Gene encoding Signal peptide, CUB and EGF-like domain-containing protein (Scube) expressed

in developing tissues, including gonads, the central nervous system, and limb buds during mouse embryogenesis, which implies their potential roles during development [29] was targeted by Ssa-miR-223-5p and are conserved from zebrafish to humans.

In conclusion we have identified three miRNAs named Ssa-miR-21-5p, 223-3p and 223-5p from Atlantic salmon macrophage specific EST resource expressed during *Aeromonas salmonicida* infection. From our knowledge this is the first report on microRNAs identification using computational approach in Atlantic salmon. Phylogenetic analysis of both the pre-miRNA sequence revealed its evolutionarly conservation among the different species. Identified targets of the predicted miRNAs revealed the role of miRNA in pathogenesis, stress response and allosteric exchange of histones. The above results reveals the identification of new microRNAs in relevance to fish diseases from Atlantic salmon, which can be used to better characterize the role of microRNAs to manage the biotic stress in fishes.

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References

1. Haugland GT, Jordal AEO, Wergeland HI. Characterization of Small, Mononuclear Blood Cells from Salmon Having High Phagocytic Capacity and Ability to Differentiate into Dendritic like Cells. PLoS ONE 2012; 7: e49260.
2. Rise ML, Jones SRM, Brown GD, von Schalburg KR, Davidson WS, Koop BF. Microarray analyses identify molecular biomarkers of Atlantic salmon macrophage and hematopoietic kidney response to Piscirickettsia salmonis infection. Physiol Genomics 2004; 20: 21–35.
3. Reyes D, Cepeda V, Gonz âlez R, Vidal R. Ssa miRNAs DB: Online repository of in silico predicted miRNAs in Salmo salar. Bioinformation 2012; 8: 284-286.
4. Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP. MicroRNAs in plants. Gene Dev 2002; 16: 1616–1626.
5. Bartel DP. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. Cell 2004; 116: 281–297.
6. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Bio 1990; 215: 403–410.
7. Altschul SF, Madden TL, Sch äffer AA, Zhang J, Zhang Z, Miller W. et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997; 25: 3389–3402.
8. Zhang B, Pan X, Anderson TA. Identification of 188 conserved maize microRNAs and their targets. FEBS Letter 2006; 580: 3753–3762.
9. Griffiths-Jones S, Bateman A, Marshall M, Khanna A, Eddy SR. Rfam: an RNA family database. Nucleic Acids Res 2003; 31: 439–441.
10. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. Nucleic Acids Res 1994; 22: 4673–4680.
11. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007; 24: 1596–1599.
12. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 2004; 101: 11030–11035.
13. Chen S, Zhang A, Blyn LB, Storz G. MicC, a second small-RNA regulator of Omp protein expression in Escherichia coli. J Bacteriol 2004; 186: 6689–6697.
14. Esau C, Kang XL, Peralta E, Hanson E, Marcusson EG, Ravichandran LV. et al. MicroRNA-143 regulates adipocyte differentiation. J Biol Chem 2004; 279: 52361–52365.
15. Zhao F, Xuan Z, Liu L, Zhang MQ. TRED: a transcriptional regulatory element database and a platform for in silico gene regulation studies. Nucleic Acids Res 2005; 33: D103–D107.
16. Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE. et al. A pancreatic islet-specific microRNA regulates insulin secretion. Nature 2004; 432: 226–230.
17. Lecellier C, Dunoyer P, Arar K, Lehmann-Che J, Eyquem S, Himber C. et al. A cellular microRNA mediates antiviral defense in human cells. Science 2005; 308: 557–560.
18. Martello G, Rosato A, Ferrari F, Manfrin A, Cordenonsi M, Dupont S. et al. A microRNAtargeting dicer for metastasis control. Cell 2010; 141: 1195–1207.
19. Xiao C, Rajewsky K. MicroRNA control in the immune system: basic principles. Cell 2009; 136: 26–36.
20. Bizuayehu TT, Fernandes JMO, Johansen SD, Babiak I. Characterization of Novel Precursor miRNAs Using Next Generation Sequencing and Prediction of miRNA Targets in Atlantic Halibut. PLoS ONE 2013; 8: e61378. doi:10.1371/journal.pone.0061378.
21. Hackl M, Jadhav V, Jakobi T, Rupp O, Brinkrolf K, Goesmann A. et al. Computational identification of microRNA gene loci and precursor microRNA sequences in CHO cell lines. J Biotechnol 2012; 158: 151–155.
22. Thirugnanasambantham K, Hairul-Islam VI, Saravanan S, Subasri S, Subastri A. Computational Approach for Identification of Anopheles gambiae miRNA involved in Modulation of Host Immune Response. Appl Biochem and Biotech 2013; 170: 281-291.

23. Mead EA, Tu Z. Cloning, characterization, and expression of microRNAs from the Asian malaria mosquito, Anopheles stephensi. BMC Genomics 2008; 9: 244.

24. Robinson DN, Cooley L. Drosophila kelch is an oligomeric ring canal actin organizer. The J Cell Biol 1997; 138: 799–810.

25. Adams JC, Kelso R, Cooley L. The kelch repeat superfamily of proteins: propellers of cell function. Trends Cell Biol 2000; 10: 17–24.

26. Pires de Miranda M, Reading PC, Tscharke DC, Brendan JM, Smith GL. The vaccinia virus kelch-like protein C2L affects calcium independent adhesion to the extracellular matrix and inflammation in a murine intradermal model. J Gen Virol 2003; 84: 2459–2471.

27. Suzuki T, Shibata T, Takaya K, Shiraishi K, Kohno T, Kunitoh H, et al. Regulatory Nexus of Synthesis and Degradation Deciphers Cellular Nrf2 Expression Levels. Mol Cell Biol 2013; 33: 2402-2412.

28. Zhang W, Tyl M, Ward R, Sobott F, Maman J, Murthy AS, et al. Structural plasticity of histones H3-H4 facilitates their allosteric exchange between RbAp48 and ASF1. Nature Struct Mol Biol 2013; 20: 29-35.

29. Grimmond S, Larder R, Van Hateren N, Siggers P, Morse S, Hacker T, Arkellet al. Expression of a novel mammalian epidermal growth factor-related gene during mouse neural development. Mech Dev 2001; 102: 209–211.