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Comparative analysis of shrimp (*Penaeus vannamei*) miRNAs expression profiles during WSSV infection under experimental conditions and in pond culture



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ABSTRACT

In recent years, the importance of viral and host microRNAs (miRNAs) in mediating viral replication and control of host cellular machinery, has been realised and increasing efforts have been taken in order to understand the interactions of miRNAs from host and pathogen during infection. However, all existing studies has thus far been conducted in controlled experimental conditions and the veracity of these data for field conditions are yet to be established. In this framework, small RNA sequencing was performed to identify the miRNAs involved in shrimp (*Penaeus vannamei*) immune responses under two different WSSV infection conditions of natural infection and experimentally challenged conditions. The expression profiles of miRNAs of shrimp infected with WSSV under two contrasting conditions were compared and as a result, 23365 known miRNAs and 481 novel miRNAs were identified. Amongst the most abundantly expressed miRNAs, the hypoxia related miR-210 and immune pathway related miR-29b were expressed only in infected shrimps of both conditions. miR-8-5p, having a functional role in modulation of chitin biosynthesis was exclusively represented in higher numbers in the WSSV -infected shrimps under natural conditions whilst four of the miRNAs (mja-miR-6493-5p, mja-miR-6492, mmu-miR-3968, tcf-miR-9b-5p) identified from shrimps collected from pond culture targeted chitinase, an important enzyme involved in growth and moulting in shrimps, indicating an interaction between WSSV infection and moult cycle under culture conditions. Some of the miRNAs (tca-miR-87b-3p, cte-miR-277a) and miRNAs belonging to class miR-9, miR-981 that were identified only in WSSV infected shrimps under experimental conditions, are known to respond against WSSV infection in shrimps. Moreover, the miRNA target prediction revealed several immune-related gene targets such as cathepsin, c-type lectin, haemocyanin and ubiquitin protein ligase were commonly identified under both the conditions. However, the miRNAs identified from challenge experiment had wide number of gene targets as compared to the miRNAs of natural infection. The shrimp miRNA mja-miR-6489-3p, was also found to target early virus gene wsv001 of WSSV. Our study, therefore, provides the comparative analysis of miRNA expression from shrimp during WSSV infection in two different conditions.

1. Introduction

Small non-coding RNAs, microRNAs (miRNAs) regulate the post transcription expression of messenger RNAs by sequence-specific base pairing and consequent silencing of the target mRNA which in turn controls many biological processes [1]. miRNAs were first identified in *Caenorhabditis elegans*, followed by identification in several other organisms including shrimp. The first report from shrimp, identified 35 miRNAs from *Penaeus japonicus* [2] and since numerous other miRNAs related to different functions have been subsequently identified from

several other shrimp species. For example, miRNAs linked with copper stress [3], innate immunity [4] or in response to bacterial infection [5] have been characterized for different penaeid species.

In shrimps, the majority of studies have shown implications of miRNAs in regulating virus-host interactions. Host miRNAs play an important role in the shrimp immune system [6] whereas viral miRNAs can help in virus replication [7] or impede host defence mechanisms, such as apoptosis of infected cells [8]. Ruan et al. [2] reported correlation between WSSV infection and expression of 22 miRNAs in *P. japonicus*. The miRNAs isolated from *P. japonicus* were shown to be

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involved in the regulation of apoptosis, phagocytosis and pro-phenoloxidase (proPO) system [4]. miR-S5, isolated from *P. japonicus*, was observed to be involved in WSSV infection by regulating haemocyte phagocytosis and apoptotic processes; furthermore, this miRNA conferred protection against *V. alginolyticus* infection by regulating proPO system, superoxide dismutase activity and phagocytosis. Shrimp mortality following WSSV infection was reduced by inhibition of miR-S5, indicating WSSV used the host's defence mechanisms for its replication. However, inhibition of miR-S5 increased mortality in shrimps infected with *V. alginolyticus*, indicating its protective role in anti-bacterial process [9]. Several other shrimp miRNAs have been identified as differentially expressed during WSSV infection and specifically target viral genes during infection. For example, in *P. japonicus* the up-regulation of miR-7 and its interaction with WSSV early gene wsv477 [6] and the up-regulation of miR-965 and its involvement in targeting the early infection gene wsv240 [10] have been reported. On the other hand, in *P. vannamei* miR-10a, which targeted viral genes *vp26*, *vp28*, and *wsv102*, was found to promote viral replication [11].

In spite of all the studies involving miRNA and host-pathogen interaction, information about shrimp miRNAs characterized against WSSV infection is still very limited. Moreover, all studies of shrimp host/virus miRNA interaction, to date, have been reported from controlled laboratory infection studies. In light of the acknowledged complexity of host pathogen interactions [12,13], it is essential that we corroborate observations reported from laboratory experiments with what happens during virus infectious outbreaks in pond culture. The present study represents a first attempt to compare *P. vannamei* miRNA expression in response to WSSV infection under the two different conditions of natural infection and challenge experiment.

2. Materials and methods

2.1. WSSV-infected shrimp from pond sampling

WSSV infected shrimps were collected from *P. vannamei* shrimp culture ponds located at Elavur village, of Tamil Nadu, India (13.4628 °N 80.1191 °E). To assess the viral load, gill tissues of infected shrimp samples were processed for DNA extraction (QIAamp DNA Mini Kit, QIAGEN). Healthy shrimps collected from the same location were used as uninfected control samples. All shrimp samples were tested for WSSV infection by nested PCR using WSSV gene specific primers (Table 1); animals tested positive at the nested PCR were deemed infected, while uninfected animals tested negative.

2.2. Laboratory WSSV challenge experiment

P. vannamei juvenile shrimps were intramuscularly injected with WSSV (WSSV virus isolate: WSSV_CIBA_003, GenBank accession no. MH883319) with 100 µl of 10⁻¹ dilution of viral stock containing 5.3 × 10⁷ µl⁻¹ of viral copies. The control shrimps were injected with PBS. The WSSV infected shrimps were sampled at different time points of infection ranging from 1.5 h to 56 h (n = 3) and tested for WSSV

Table 1
Primers used for WSSV nested PCR and qPCR.

S. no.	Primer sequence (5'-3')	Expected amplification size (bp)	PCR
1	F: GACAGAGATATGCACGCCAA R: ACCAGTGTTCGTATGGAG	643	nested PCR
2	F: GGTAGATTCTGGTATTAGG R: ACCTGGCGTAGTCTTGC	298	nested PCR
3	F: ATGTGTCCTTTGACAGCGAC R: GCCCTCCACGGGAGTGATGA		qPCR
4	[6FAM]AAGTCTGATGCACAGATGA [TAM]		qPCR probe

infection by nested PCR using WSSV gene specific primers (Table 1). Gill tissues from three each of WSSV- infected and control shrimps were analyzed for estimating the WSSV viral copies by qPCR using TaqMan Universal Master Mix II (ABI technologies, USA) using probe as shown in Table 1.

2.3. Library preparation and sequencing

The WSSV- infected shrimp gill tissues collected from shrimp ponds and from WSSV challenge experiments, along with respective uninfected control samples were used for preparation of four small RNA sequencing (SmRNA) libraries. SmRNA libraries were prepared with TruSeq[®] Small RNA Library Prep Kit (Illumina, San Diego, California, U.S.A.) using shrimp WSSV- infected and control shrimp total RNA (1.0 µg). Briefly, the 3' adapters were ligated to mi RNAs followed by ligation of 5' adapter. Adapter ligated fragments were reverse transcribed with Superscript III reverse transcriptase (Invitrogen, CA, USA) by priming with reverse transcription primers. The cDNA libraries were amplified by PCR amplification (15 cycles). Amplified libraries were analyzed using polyacrylamide gel. Library was size selected in the range of 140 bp - 160 bp by gel elution and precipitation in presence of glycogen, 3 M sodium acetate (Sigma, Saint Louis, Missouri, USA) and absolute ethanol. The pellet was re-suspended in nuclease free water (Invitrogen, CA, USA). Illumina-compatible sequencing library was quantified by Qubit fluorometer (Thermo Fisher Scientific, MA, USA) and its fragment size distribution was analyzed on Agilent 2100 Bioanalyzer. Illumina Nextseq Single-end sequencing (75x1) was used as sequencing platform.

2.4. Raw sequence data processing

The raw data of single end reads of length 75 bp was generated on Illumina platform in FASTQ format. *Clip_adapters.pl* script of miRDeep2 package [14], was used to trim 3' adapter sequences. The low quality and contaminated reads were removed based on the following criteria: i) low quality reads (< q30); ii) reads without 3' adapters; iii) reads without insert; iv) reads < 16 bp and > 40 bp and v) reads matching to other ncRNAs were all eliminated. The filtered reads were further aligned to Rfam database using bowtie [15] to eliminate other small RNA sequences (rRNA, tRNA, snRNA, snoRNA and piRNA). The unaligned sequences were considered for miRNA prediction.

2.5. miRNA identification

To identify conserved and novel miRNA and their regulation along with target identification in the dataset, a multiphase data analysis approach was used. In absence of *P. vannamei* miRNAs, the reads were aligned against all the animal mature miRNAs from miRBase v22 database [16]. Homology search by NCBI Blast was performed with word size set to 11, e-value 0.0001, alignment as ungapped and with percentage identity set to 100. The results were further filtered by retaining hits only with query coverage greater than 70%. For novel miRNA prediction, the reads were made unique by collapsing the reads using a perl script *collapse_reads_md.pl* from the miRdeep2 package [14]. Mireap v 0.2 (<https://sourceforge.net/projects/mireap/>) was used to predict novel miRNAs. Mireap uses a miRNA biogenesis model and combines it with small RNA position and depth to discover miRNAs.

2.6. Differential gene expression

To find out the differential expressed miRNAs between the control and infected groups, a count table was generated by aligning the clean reads to the known and novel predicted miRNAs. The count table was further analysed by DESeq [17], an R package, and counts were normalized by giving dispersion method as "blind" and sharing mode as "fit only". A negative binomial test was conducted to obtain the

expression values such as log2 fold change and p-value less than 0.05.

2.7. Target prediction

Differentially expressed known and novel mature miRNAs were considered for target prediction against a *P. vannamei* transcriptome. The target prediction algorithm, miRanda [18] was used to search and predict targets for miRNAs against published *P. vannamei* shrimp transcriptomes accession no.s (GCVY00000000, GDTK00000000, GDUV00000000, GETD00000000, GGKO00000000, GGUK00000000, GGUT00000000, HAAW00000000). miRanda was run with 'strict' option with other parameters set to default. The hits having an energy threshold less than -20 Kcal were considered for target prediction. The miRNA targets were categorized by gene ontology and KEGG pathway analysis using blast2GO software. The network between miRNAs and their respective targets were plotted using Cytoscape [19].

3. Results and discussion

3.1. WSSV challenge experiment

The WSSV infection was confirmed by nested PCR which revealed expected PCR product size of 643 bp using outer primer set and 298 bp using nested primers. The qPCR analysis showed a peak in WSSV infection containing 4.0×10^7 WSSV viral copies/mg gill tissues at 18 h post WSSV challenge time interval (data not shown). Hence, the gill samples collected from WSSV infected and control shrimps at 18 h were used for small RNA sequencing.

3.2. Raw data processing

Annotation using Rfam allowed the identification of various categories of small RNAs (e.g. rRNA, tRNA, snRNA, snoRNA), which were present in the libraries in a range from 2.73% to 6.07% and were subsequently excluded. Finally, 11982259 and 7594765 clean reads obtained from respective control shrimps and 10291747 and 11272341 clean reads from the WSSV infected shrimps of culture pond and from the challenge experiment were considered for further analysis (Table 2). The majority of reads had a length of 22 nts (Fig. 1).

3.3. miRNA identification

Using small RNA sequencing, miRNAs have been identified using different tissues of WSSV infected shrimp such as the lymphoid organs from *P. japonicus* [8], haemocytes from *P. monodon* [20], *P. japonicus* [2], cephalothorax from *F. chinensis* [21], hepatopancreas from *F. chinensis* [22] and *P. vannamei* [23].

The gill tissues of shrimps which is one of the primary site for WSSV replication has been used for identification of miRNAs against WSSV infection using microarray [7], and northern blotting [24]. However, to our knowledge, the use of small RNA sequencing of gill tissues of shrimp for identification of miRNA has not been reported so far. Hence, we chose to characterize miRNAs from gills in response to WSSV infection in *P. vannamei* in this study.

Table 2

Processed raw reads obtained from the four libraries of the control shrimp and WSSV infected shrimp collected from culture pond and from challenge experiment.

	Culture pond		Challenge experiment	
	Control shrimp	WSSV infected shrimp	Control shrimp	WSSV infected shrimp
Total number of raw reads	30910346	26070623	17620812	24502176
Reads within 16 and 40 bp	12318515	10907640	8085632	11616594
Percentage of reads eliminated by length filtering	60.15	58.17	54.12	52.59
Reads after Rfam filtering	11982259	10291747	7594765	11272341
Percentage of contaminated reads eliminated	2.73	5.65	6.07	2.96

The numbers of known miRNAs (11337) and novel miRNAs (144) were identified using miRBase v22.0, from the shrimps collected from pond. Whereas, 12028 known miRNAs and 337 novel miRNAs were identified from the shrimps of challenge experiment (Supplementary Table 1).

The miRBase v22.0 homology search of shrimp miRNAs resulted in identifying conserved known miRNAs from the control and WSSV infected shrimp collected from pond and challenge experiment having orthologs in other species. The top miRNAs that were identified in shrimps collected from culture pond and from the challenge experiment are shown in Fig. 2. The miRNAs (miR-100, miR-124, miR-92a, miR-184, miR-1 and miR-9) were expressed in high numbers in both control and infected shrimps collected from pond culture and WSSV challenge experiment.

High conservation of shrimp miRNAs with other species has been observed, suggesting similar functions of miRNAs across the species [3,4]. miRNAs miR-1, miR-7 and miR-34, are highly conserved in shrimp, fruit fly and humans and exert their biological role in similar pathways (i.e. apoptosis and MAP-kinase) in all these species [25]. It is worth noting that, in the challenge experiment, miR-1 and miR-7 were within the top eight most abundant miRNAs in the libraries (Fig. 2). miR-7 which was expressed in infected shrimp collected from pond is known to interact directly with WSV477, an early gene of WSSV and inhibits viral replication [25]. The top miRNAs identified in this study (miR-let7a, miR-184, miR-1 and miR-7) were reported to be up-regulated, while miR-9 was down-regulated against WSSV infection [25]. In addition Huang et al. [25] reported upregulation of miR-100, a well conserved miRNA across species, which is involved in innate immunity and apoptosis regulation in response to WSSV infection [26].

Amongst the abundantly expressed miRNAs, we observed miR-210 and miR-29b to be expressed only in infected shrimps in both pond culture and laboratory WSSV challenge experiments. The expression of these miRNAs only under infected conditions indicates the effect of WSSV on shrimp immune and physiological pathways. miR-210 is reported to be induced under hypoxic conditions [27]. Hypoxia is one of the stress factors which has been argued as pre-disposing a susceptibility of shrimp to WSSV infection [28]. The other abundantly expressed miRNA miR-29b has been reported to interact with NF- κ B and Wnt/ β -catenin pathways [29]. Interestingly, the shrimp NF- κ B pathway is activated by WSSV genes for regulation of viral gene expression [30] and Wnt/ β -catenin of shrimp is involved in regulating AMPs and the host response against bacterial and WSSV infection [31]. The role of shrimp miR-210 and miR-29b on immune functional pathways during WSSV infection remains to be further explored.

miRNA analysis revealed 4453 known miRNAs and six novel miRNAs in common between control and WSSV infected shrimp samples collected from culture pond (Fig. 3). From the challenge experiment, 5378 known miRNAs and 39 novel miRNAs were in common between control and WSSV infected shrimp samples (Fig. 4).

3.4. Differential gene expression

Five known miRNAs were differentially expressed in the shrimps collected from the culture pond, out of which four miRNAs (mja-miR-

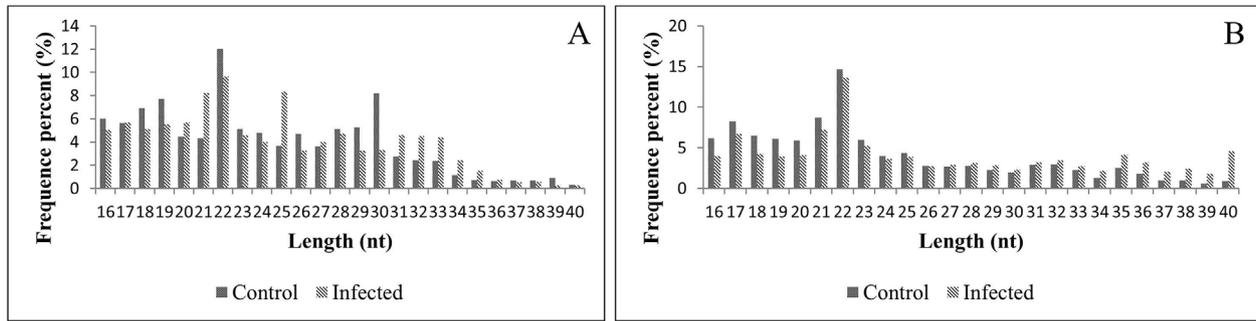


Fig. 1. Length (nt) distribution of *P vannamei* miRNAs in control and WSSV infected shrimp collected from (A) culture pond and (B) WSSV challenge experiment.

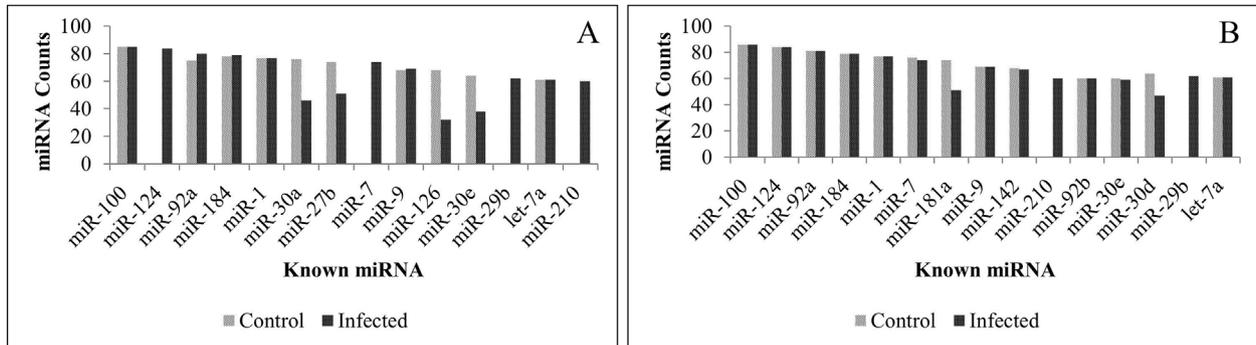


Fig. 2. The top miRNAs that were identified in the shrimp collected from (A) culture pond and from (B) the challenge experiment.

6494, gga-miR-1692, mja-miR-6492, mja-miR-6493–5p) were down-regulated, whereas only one miRNA was up-regulated (tca-miR-276–5p) (Table 3). Some of these miRNAs have been already identified from other shrimp, including miR-6494, miR-6492 and miR-6493 from *P. japonicus*, *P. monodon*, *F. chinensis* and *P. vannamei* in response to WSSV infection [20,22,23] and cold adaptation [32]. The down regulation of mja-miR-6492, that targets the gene ‘tyrosine decarboxylase-like’, is in agreement with the observations of Sun et al. [23] who found this miRNA to be down regulated in WSSV infected *P. vannamei*. miR-276–5p has been reported to be down regulated in WSSV infected *F. chinensis* and has been predicted to target a gene coding for a fatty acids binding protein [22]. This gene is known to play an important role in shrimp innate immunity against WSSV, although the exact mechanism is not known [33].

Three known miRNAs were differentially expressed in the shrimps collected from the WSSV- challenge experiment, out of which two miRNAs were up-regulated (tch-let-7a-5p and gmo-miR-100b-5p) and one miRNA (mja-miR-6489–3p) was down-regulated (Table 3). The let-7 miRNA, acting as tumour suppressor, was first discovered in *C. elegans*, and is highly conserved from nematodes to humans [34]. The dme-let-7-5p miRNA was shown to target Toll 2 gene in WSSV infected

P. vannamei [35]. Another differentially expressed miRNA, gmo-miR-100b-5p, a *Gadus morhua* ortholog, was upregulated and is reported to be highly expressed in the respiratory tree of sea cucumbers [36]. However, this miRNA has not been reported in crustaceans and its functional regulation requires further studies. The miR-6489–3p another miRNA identified from WSSV- challenge experiment, has been reported to target the *Ras* gene in *P. monodon* during ovarian development [37]. The Ras-regulated phagocytosis is involved in host pathogen interaction [38]. This miRNA, was shown to have serine/arginine repetitive matrix protein 1-like as predicted target [23]. Hence, these miRNAs require further in-depth studies to characterize their role in regulating WSSV pathogenesis through host genes.

Twelve miRNAs were identified to be exclusively present in WSSV-infected shrimps collected from culture pond (Table 4). Eight of these miRNAs belonged to the class miR-8-5p. This highly conserved miRNA class has been previously identified from WSSV-infected *P. monodon* [20] and *F. chinensis* [22]. Interestingly, this miRNA class is associated with the modulation of chitin biosynthesis in insects [39]. In arthropods, chitin metabolism is of great importance as it is the major constituent of the exoskeleton and it is replaced during ecdysis to increase body size of the organism. Interestingly, post-moult stages are

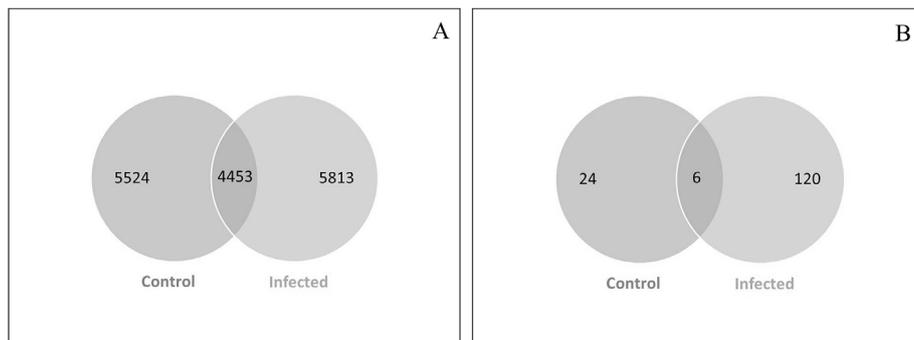


Fig. 3. Common miRNAs identified between the control and WSSV-infected shrimp samples collected from culture pond (A) known miRNAs (B) novel miRNAs.

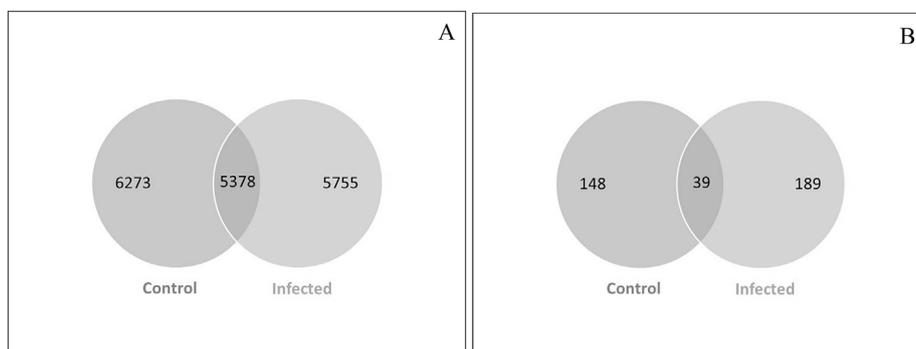


Fig. 4. Common miRNAs identified between the control and WSSV-infected shrimp samples collected from WSSV challenged experiment (A) known miRNAs (B) novel miRNAs.

Table 3
Fold change values of differentially expressed miRNAs identified from control and WSSV infected shrimp from culture pond and WSSV challenge experiment.

miRNA	Normalised expression value			
	control group	infected group	fold change value	log2fold change
Culture pond				
mja-miR-6494	284.58	1.14	0.00	-7.95
gga-miR-1692	7028.49	82.73	0.01	-6.40
mja-miR-6492	2133.08	53.23	0.02	-5.32
mja-miR-6493-5p	600.50	19.15	0.03	-4.97
tca-miR-276-5p	15.66	437.40	27.92	4.80
Challenge experiment				
tch-let-7a-5p	39.37	221.88	5.63	2.49
gmo-miR-100b-5p	222.09	1021.28	4.59	2.20
mja-miR-6489-3p	23971.92	5483.82	0.22	-2.12

Table 4
miRNAs expressed only in WSSV infected shrimp collected from culture pond and challenge experiment.

miRNA Culture pond	Normalised expression value
Culture pond	
mle-miR-745b-3p	3753.90
mle-miR-92a-3p	479.53
cqu-miR-8-5p	408.67
dvi-miR-8-5p	402.16
mmu-miR-3968	401.78
tca-miR-8-5p	398.33
dqu-miR-8-5p	397.56
bmo-miR-8-5p	394.88
dme-miR-8-5p	387.60
tcf-miR-8-5p	377.26
tcf-miR-9b-5p	373.05
aae-miR-8-5p	372.28
Challenge experiment	
hsa-miR-7975	1367.98
tca-miR-9e-5p	688.44
tca-miR-87b-3p	107.97
cte-miR-277a	72.31
mmu-miR-467g	51.50
tcf-miR-981	41.60

reported to be more susceptible to WSSV infection, since in this phase of the moult cycle the cuticle is softer, thinner and less developed in comparison to inter-moult and pre-moult stages [40].

Six miRNAs (hsa-miR-7975, tca-miR-9e-5p, tca-miR-87b-3p, cte-miR-277a, mmu-miR-467g and tcf-miR-981) were exclusively present in WSSV infected shrimps collected from WSSV challenge experiment

Table 5
miRNAs expressed only in control shrimp collected from WSSV challenge experiment.

miRNA	Normalised expression value
pca-miR-316-5p	466.39
ame-miR-316-5p	454.28
api-miR-316	388.66
bmo-miR-2779	361.40
aga-miR-281	245.31
oni-miR-7550	187.76
ipu-miR-7550	182.72
mse-miR-2779	171.61
tcf-miR-745	83.78
dpu-miR-745	80.76
mmu-miR-3082-5p	78.74
hme-miR-316	50.47
mse-miR-316	49.46
tca-miR-316-5p	46.43
hpo-miR-87b-3p	36.34
bmo-miR-316-5p	31.29
Novel-GETZ01063859	2488

(Table 4). The up-regulation of miRNA tca-miR-87b-3p in this study was similar to the results obtained by Sun et al. [23], who reported an up-regulation of this miRNA against WSSV infection in *P. vannamei*. The miRNA cte-miR-277a identified in this study was reported to target FAD-dependent oxidoreductase in WSSV infected *P. vannamei* [23]. Rajesh et al. [41], reported that the expression levels of FAD-dependent oxidoreductase changed following salinity stress in *P. monodon*, hence this suggests that this gene may also act as a stress induced gene during WSSV infection in shrimps. The miRNAs (tca-miR-9e-5p and tcf-miR-981) belong to the family of miRNAs miR-9 and miR-981 and are reported to be differentially expressed in WSSV infected *P. japonicus* [25]. Another miRNA expressed only in WSSV infected shrimp in this study, mmu-miR-467g has been recently reported to regulate osteoblastogenesis in mice [42]. However, the function of this miRNA is not known in shrimps and requires further in-depth studies.

Seventeen miRNAs were exclusively expressed in the control shrimps of challenge experiment (Table 5). Of these, miR-316-5p has been recently identified from WSSV infected *F. chinensis* [22] and is reported to target alpha-amylase gene. This gene has been recently reported to be differentially expressed in WSSV infected shrimps [43], whilst in the current study its expression was only regulated in control shrimp. Moreover, another miRNA (miR-281) found to be regulated in control shrimp in this study was reported to be up-regulated in response to dengue viral infection and facilitated viral replication [44]. miR-281 targets ecdysone receptor in the silkworm *Bombyx mori* [45], an essential gene for moulting and other growth parameters in crustaceans. Further, miR-281 and miR-2779 (both expressed in the uninfected group in this study) are reported to target fatty acid synthase [37], which is thought to be essential for WSSV morphogenesis [46]. miR-

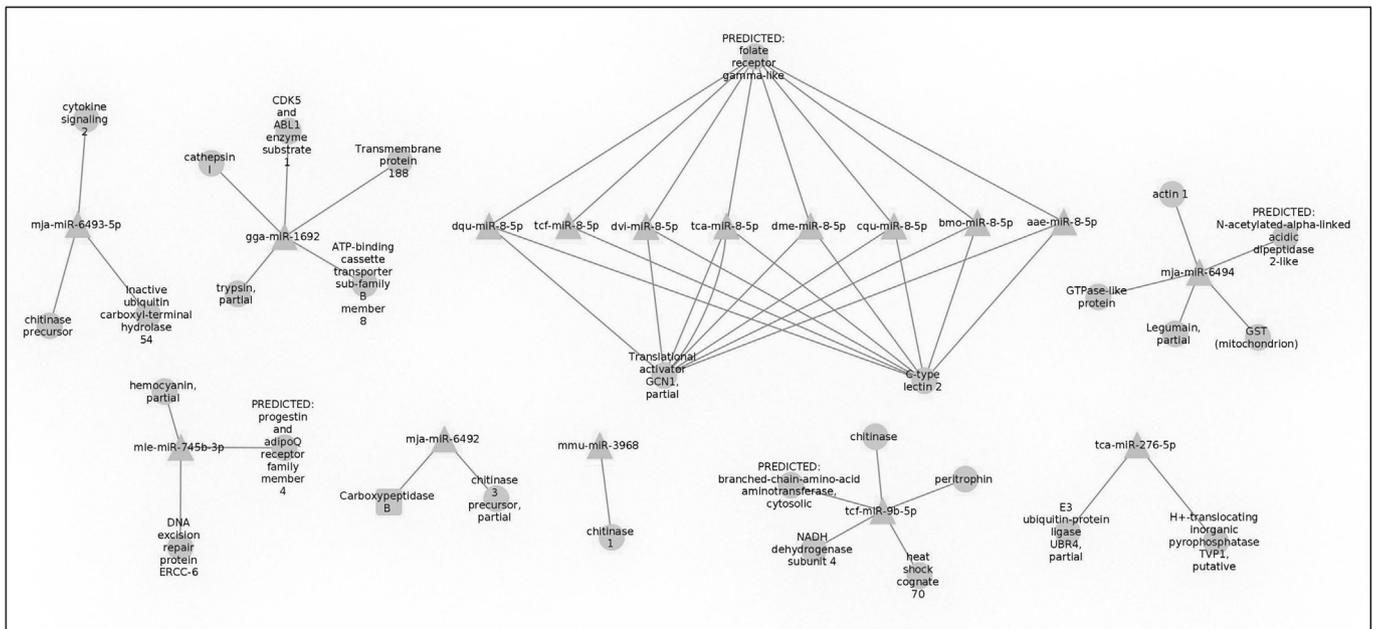


Fig. 5. The interactions between miRNAs and target genes of *P. vannamei* from culture pond.

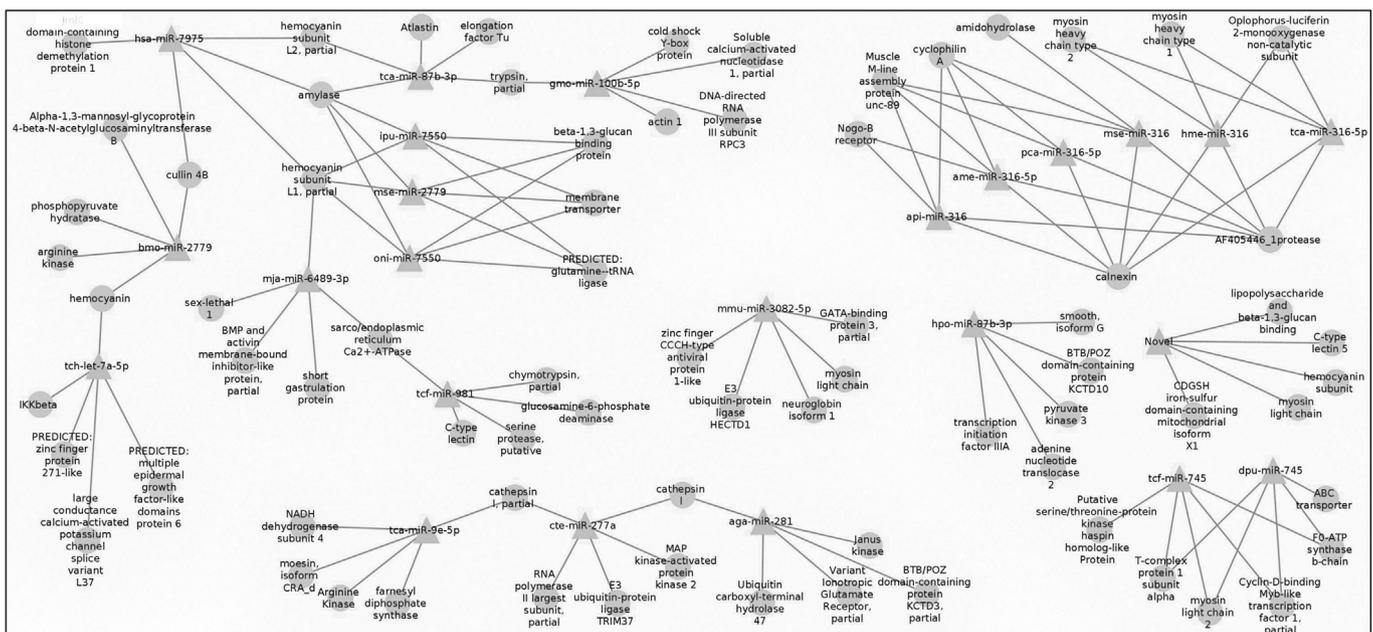


Fig. 6. The interactions between miRNAs and target genes of *P. vannamei* from WSSV challenge experiment.

2779 is predicted to target transporter genes [22]. Interestingly, the surface proteins such as glucose transporter 1 is reported to interact with WSSV envelope protein, VP53A [47]. Clearly, our data from uninfected shrimp in the current study contrast with the work of previous groups and this emphasizes the need for further and wider studies of the regulation of host miRNA expression in response to WSSV infection in both experimental and field conditions in order to corroborate and confirm our understanding of these important host/pathogen interactions.

3.5. miRNA target prediction

The target prediction of the miRNAs with miRanda from culture pond and WSSV challenge experiment identified 391 and 1411 number of mRNA targets respectively (Supplementary Table 2). The target

genes of miRNAs predicted by miRanda algorithm from the shrimps collected from culture pond are shown in Supplementary Table 3. The miRNAs belonging to the class miR-8-5p were predicted to target immune genes like C-type lectin 2, the pattern recognition molecules which interact with WSSV envelope proteins [48]. The miRNA gga-miR-1692 was found to target cathepsin, which is reported to be involved in the immune response against WSSV [49]. The cytokine signalling 2 was the target gene predicted in this study for mja-miR-6493-5p. The JAK/STAT pathway, a constituent of cytokine signalling pathway, is known to confer anti-viral immunity in shrimp [50]. Another immune gene, GTPase-activating protein was identified to be targeted by mja-miR-6494. GTPase-activating proteins are involved in regulation of WSSV replication. For example, Sritunyalucksana et al. [51] reported that PmRab7, a Ras-related protein identified in *P. monodon*, was involved in interaction with WSSV and is a receptor for

VP28 envelope protein of WSSV. Chitinase, an important enzyme in crustaceans involved in growth and moulting was another target identified in the shrimps infected under natural conditions. It was predicted to be targeted by mja-miR-6492, mja-miR-6493-5p, mmu-miR-3968 and tcf-miR-9b-5p miRNAs. Chitinase has been recently reported to be involved in regulation of humoral and cellular response against viral and bacterial pathogens in shrimp [52]. miRNA tcf-miR-9b-5p was found to target peritrophin, which is reported to bind to chitin and WSSV envelope proteins [53]. Hence, together with the expression of miR-8-5p, which is associated with the modulation of chitin biosynthesis and targets genes such as chitinase and peritrophin by the miRNAs identified from shrimps from natural infection, it is indicative that moult stages of shrimp during culture period influences WSSV infection. Our observation are in agreement with the earlier report by Corteel et al. [40] that shrimp have increased susceptibility to WSSV infection via immersion after moulting. The other immune genes, haemocyanin recently shown to possess antiviral peptides against WSSV [54] and heat shock cognate 70, an inhibitor of apoptosis induced by WSSV [55] were predicted as targets of miRNAs mle-miR-745b-3p and tcf-miR-9b-5p respectively in this study.

The target genes of miRNAs from the shrimps collected from WSSV challenge experiment are shown in Supplementary Table 4. Shrimp immune genes and genes associated with signalling pathway were also identified as miRNA targets from shrimp collected from WSSV challenge experiment. Some of the immune related genes targeted by miRNAs from WSSV challenge experiment and also identified under natural infection conditions included C-type lectin, (tcf-miR-981), cathepsin (aga-miR-281, tca-miR-9e-5p), haemocyanin (bmo-miR-2779). Targeting of Janus kinase by miRNA, aga-miR-281 indicates that miRNAs of shrimp also regulate the JAK-STAT and other signalling pathways by controlling the expression of their genes. Previous studies have shown miRNAs (miR-9041 and miR-9850) suppresses STAT expression resulting in increase in WSSV replication in *M. rosenbergii* [56]. β -1,3-glucan binding protein which has a functional role in stimulating cellular immune responses such as phagocytosis, melanization, encapsulation and coagulation in shrimps [57] was predicted to be targeted by the miRNAs ipu-miR-7550, mse-miR-2779 and oni-miR-7550.

Interestingly, the novel miRNA identified in this study (GETZ01063859) was predicted to target three major immune related genes of shrimp such as C-type lectin, hemocyanin and beta-1,3-glucan binding protein. This novel miRNA requires further characterization for its networking with multiple targets during WSSV infection. Only a few miRNAs from the laboratory-challenged and pond infected shrimp could be identified targeting viral genes (Supplementary Table 5). Four shrimp miRNAs specifically targeted viral genes, including lymphoid organ expressed yellow head virus receptor protein (tca-miR-9e-5p, dpu-miR-745, tcf-miR-745) and wsv001 (mja-miR-6489-3p). The envelope protein called “collagen-like protein” of WSSV is encoded by an early virus gene wsv001 and it is predicted to interact with integrin alpha, integrin beta and syndecan proteins of shrimp [58].

3.6. Gene ontology (GO) and KEGG analysis

miRNA target prediction which resulted in 370 and 1058 mRNAs targeted (with less than -20 Kcal energy threshold) from the shrimp culture pond (Supplementary Table 6) and WSSV challenge experiment (Supplementary Table 7) respectively were categorized into three classes of biological process (BP), molecular function (MF) and cellular component (CC). These putative target genes belonged to various functions, however, organic substance and cellular metabolic processes, ion binding and intracellular functions were the top functions identified under each category of biological process, molecular function and cellular component respectively. The KEGG pathway analysis revealed biosynthesis of antibiotics, oxidative phosphorylation and carbon fixation pathways in prokaryotes as the top three pathways for miRNAs targets identified from shrimps of culture pond (Supplementary

Table 8). Whereas, biosynthesis of antibiotics, glycolysis/gluconeogenesis and purine metabolism were the top three pathways for miRNA targets identified from shrimps of challenge experiment (Supplementary Table 9).

The interactions between miRNAs and target genes of *P. vannamei* for culture pond and from WSSV challenge experiment are shown in Figs. 5 and 6 respectively. The genes were predicted to be targeted by multiple miRNAs. The interactions between miRNAs and target genes of *P. vannamei* from culture pond revealed that in one of the interactions, C-type lectin 2, translational activator, folate receptor were targeted by multiple miRNAs belonging to miR-8-5p class. In shrimps, the C-type lectins, which are involved in immune response and in calcium dependent carbohydrate binding, have wide tissue distribution and act as pattern recognition molecules [59]. miR-8-5p is reported to be involved in host virus interaction in *Drosophila*, where it was observed that decreased levels of miR-8-5p supported *Drosophila* C virus replication [60]. The folate receptor is reported to act as co factor facilitating entry of marburg and ebola viruses [61]. It will be interesting to find if WSSV also uses folate receptors and if miR-8-5p levels in shrimps affects WSSV replication.

In case, of the interactions between miRNAs and target genes of *P. vannamei* from challenge experiments, one specific interaction showed the lectin chaperone calnexin which helps in maturation of viral glycoproteins [62], to be targeted by multiple miRNAs. Calnexin is reported to be differentially up-regulated at different time points post WSSV infection in shrimp [63]. Calnexin is involved in glycoprotein maturation in viruses [64], hence further study of the functional role of calnexin in WSSV maturation will add to the information pertaining to host virus interactions in shrimps. In conclusion, the present study has revealed that miRNAs are important regulators and have functional role in host-WSSV interactions and are expressed in a complex network during host immune response against viral infections in shrimps.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2019.07.057>.

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