Chapter IV

BLACK GILL DISEASE IN *L. VANNAMEI* BROODSTOCK

4.1. Introduction

The fungal infections are most severe problems in crustacean species in the marine environment. Black gill disease in the shrimps is caused mainly by a fungus belonging to the category ‘Fusarium’ and is commonly called as the ‘black gill Fusarium’. Black gill disease in the shrimp is described to be affected through a measure of causes for mortality due to damage of gills and the obstacle of gas interchange crosswise into the gill lamellae which leads to asphyxia (Lightner and Fontaine., 1975)

The black gill illness is identified firstly to create gill yellowing and progressively leads to black gill state which specifies melanisation of gills noticed to cause death (Khoa *et al.*, 2004; Khoa and Hatai, 2005). In general, the black gill state in the shrimp primarily creates generalized gill discoloration which increasingly grows into blackened gill condition and eventually leads to the death of affected individuals (Khoa *et al.*, 2005).

This disease is categorized by the occurrence of black spots and necrosis in the gills and eventually, the gills undergo necrosis and get collapsed at the advanced stages of disease development (Egusa and Udea, 1972). The black spots in the gills are recognized to be caused through an inflammatory reaction connecting initiation of prophenol oxydase that phenolic compound to melanin as well as the death of host (Bian and Egusa, 1981).

Fusarium is the reason for the reduction of osmolarity of hemolymph and concentration of Na+ and Cl- in the cry fish (Maestracci and Vey, 1989). The fungal enzymes remain identified to be complicated in the injury of cell membranes and in specific, act on shrimp carapace (Da Silva *et al.,* 2011). The black gill disease of pond-cultured kuruma prawn, *Penaeus japonicus*, was first identified in Japan (Egusa and Ueda, 1972).

The earlier findings have stated that the Fusarim species such as *Fusarium solani, F. monilifome* and *F. oxysporum* caused black gill diseases in *Penaeus japonicus* and brown shrimp *Penaeus californiensis* (Hose *et al.,* 1984; Rhoobunjongde *et al*., 1991; Souheil *et al.,* 1999; Khoa *et al.,* 2005). Ornate rock lobster, *Panulirus ornatus* is also affected by the black gill disease in culture farm (Nha *et al*., 2009). Shrimp hatchery and culturing has grown-up from a traditional, small scale business in Southeast Asia into a global industry than the other culture systems.

The fast development of shrimp culturing led to a commercial prosperity but, unfortunately, the outburst of several diseases has augmented the economic risks and reduced the development of this industry. In this context, Specific Pathogen Free (SPF) stain of Pacific white leg shrimp *Litopenaeus vannamei* was introduced in India in the year 2009 which revived the shrimp culture in India. Unluckily, the SPF shrimp was introduced in those regions where contaminations were prevalent.

The SPF status does not confirm that it is free from all the diseases. Therefore, the threat of shrimp diseases continues to remain. The practice of bio-

security measures like bird fencing, crab fencing, filtration and disinfection of water before pumping into the main pond helped to control the spread of the disease, particularly the carriers of the pathogens.

The implementation of Better Management Practices (BMP) at every stage also helped to control the spread of the disease. However, some unregistered hatcheries were supplied with the post larvae developed from domesticated SPF broodstock which led to deterioration of quality in the seed creating the stress in post larva stage, the stress is additionally intensified as a result of abiotic causes leading towards the fungal infections.

The diseases of *P. monodon* have similarly been recognized in *L. vannamei*, signifying the transfer of pathogens from *P. monodon* to *L. vannamei.* However, data on the disease outbreaks of the broodstock of *L. vannamei* have not been surveyed extensively in India. In the recent years, the shrimp hatcheries and aqua farms were severly affected by gross clinical symptoms of black gills. Black gill disease reported in *L. vannamei* are only very few and there is no systematic study in the hatchery mainly in the broodstock section. So the present work deals to study the black gill disease in *L. vannamei* broodstock from the hatcheries of Tamil Nadu.

4.2. Materials and Methods

4.2.1. Sample collection, gross observation and prevalence

The black gill disease affected shrimps (52-62 g) broodstock were collected from the Coastal Aquaculture Authority (CAA) approved hatcheries of Chennai coast, Tamil Nadu, India and were grossly observed. The estimation prevalence was

calculated by using the findings of Natividad and Lightner, 1992. The diseased shrimps were transported to the laboratory for further study.

4.2.2. Wet mount study

The normal and black gills were separated with a sterile blade. After separation of gills, wet mount slide was prepared separately for both normal and black gills and the samples were observed using the light microscope at different magnifications.

4.2.3. Histopathology

Davidson's fixative was injected into the heapatopancreases and muscle of live infected shrimp in order to avoid autolysis. After injection, the whole shrimp was immersed in the same fixative for 48 h before further processing. The black gill infected portions of gills were dissected out and immersed in Davidson's fixative for 48 h and transferred to 70% ethanol and processed by the method described by Bell and Lightner (1988). Tissues were dehydrated, embedded in paraffin wax and sectioned at 3µm using microtome (YORCO, YSI 115). Sections were stained with haematoxylin and eosin and subsequently were examined under a light microscope.

4.2.4. Microbiological investigation

The black gills were removed from the infected shrimps and were observed under a light microscope. The black gill infected shrimps were washed three times with sterile physiological saline (0.85% NaCl) and was plated on the Sabourad Dextrose Agar (SDA) plates supplemented with Amphotericin-B and Streptomycin sulphate (25µg/ml) to inhibit unwanted bacterial contamination. Further, the plates were incubated for 4 days at 25ºC on SDA slants for subsequent experiments. The

fungal strain was identified through Lacto phenol cotton blue mount and the morphology was observed under light microscopy in different magnification.

4.2.5. Microscopic observation of fungi

The fungi which were isolated from black gills on potato dextrose agar were taken on the clean slide and were stained with lactophenol cotton blue. The stained fungi were covered with cover slip and observed under a light microscope in different magnifications.

4.2.6. Scanning Electron Microscopy (SEM)

Black gill was dissected by using a sterile blade, the dehydrated and sputter-coated with gold and were examined using JEOL JSM-7401F scanning electron microscope at an accelerating voltage of 15 Kv GB low.

4.2.7. DNA extraction, 16S rRNA amplification, sequencing and Phylogenetic analysis

The bacterial genomic DNA was extracted according to the method adopted by Marmur (1961) and nearly a full length 16S rRNA sequence was amplified by using a universal bacterial primer 27F (5´-AGA GTTTGA TCC TGG CTC AG-3´) and 1492R (5´-GGT TAC CTT GTT ACG ACTT-3´). Polymerase chain reaction (PCR) was performed under the conditions of 35 cycles consisting of initial denaturation at 95º C for 5 min, denaturation at 95 ºC for 30 S, annealing at 55 ºC for 30 S and followed by a final extension of 5 min at 72 ºC. The 16S rRNA gene was sequenced in Macrogen Inc. Korea. The sequence similarity was analyzed using BLAST (Basic Local Alignment Search Tool) algorithm and the phylogenetic tree was constructed using the MEGA 6 software.

4.3. Results

4.3.1. Gross observation and prevalence

In gross sign, white and brown to black colour gill was observed. The percentage of gill affected broodstocks have gradually increased from December 2013 to March 2014 and then enhanced hastily to reach a peak of 2% during the month of March 2014 in a male shrimp. Whereas, the percentage of gill affected female broodstocks have gradually increased from October 2013 to March 2014 and enhanced hastily to reach a peak of 8.75% during the month of March 2014. The highest prevalence of 10.75% was observed in both the sex (male and female) during the month of March 2014 (Table 1). At this stage, the broodstocks were lethargic as well as an abnormal swimming behaviour and substantial numbers of dead broodstocks were observed. Initial stage of infected gills was slightly black in colour and finally it was converted to dark black colour (Fig. 1).

Table 1. Incidence (%) of Black gill disease in male and female

Months	Infected shrimps			Prevalence (%)		
	Total No	Male	Female	Total %	Male	Female
Oct -13	2	0	2	0.5	0	0.5
Nov-13	6	0	6	1.5	0	1.5
Dec-13	16	2	14	4.0	0.5	3.5
Jan -14	25	3	22	6.25	0.75	5.5
Feb -14	34	6	28	8.5	1.5	7.0
Mar-14	43	8	35	10.75	2.0	8.75
Total	**126**	**19**	**107**	**31.5**	**4.75**	**26.75**

4.3.2. Wet mount

The results of wet mount of normal gill were transparent with less debris. The infected shrimp black gill filaments were surrounded with macroconidias of *Fusarium solani*. Fungal hyphae have grown out from the gill filaments. The macroconidias were present in side of the central axis of gill and filaments (Fig. 2A,

B, C and D). Colony morphology of *Fusarium solini* with white cottony colony isolated from the black gill and reverse plate of *Fusarium solini* shows a yellowish pigmentation (Fig. 3 A and B).

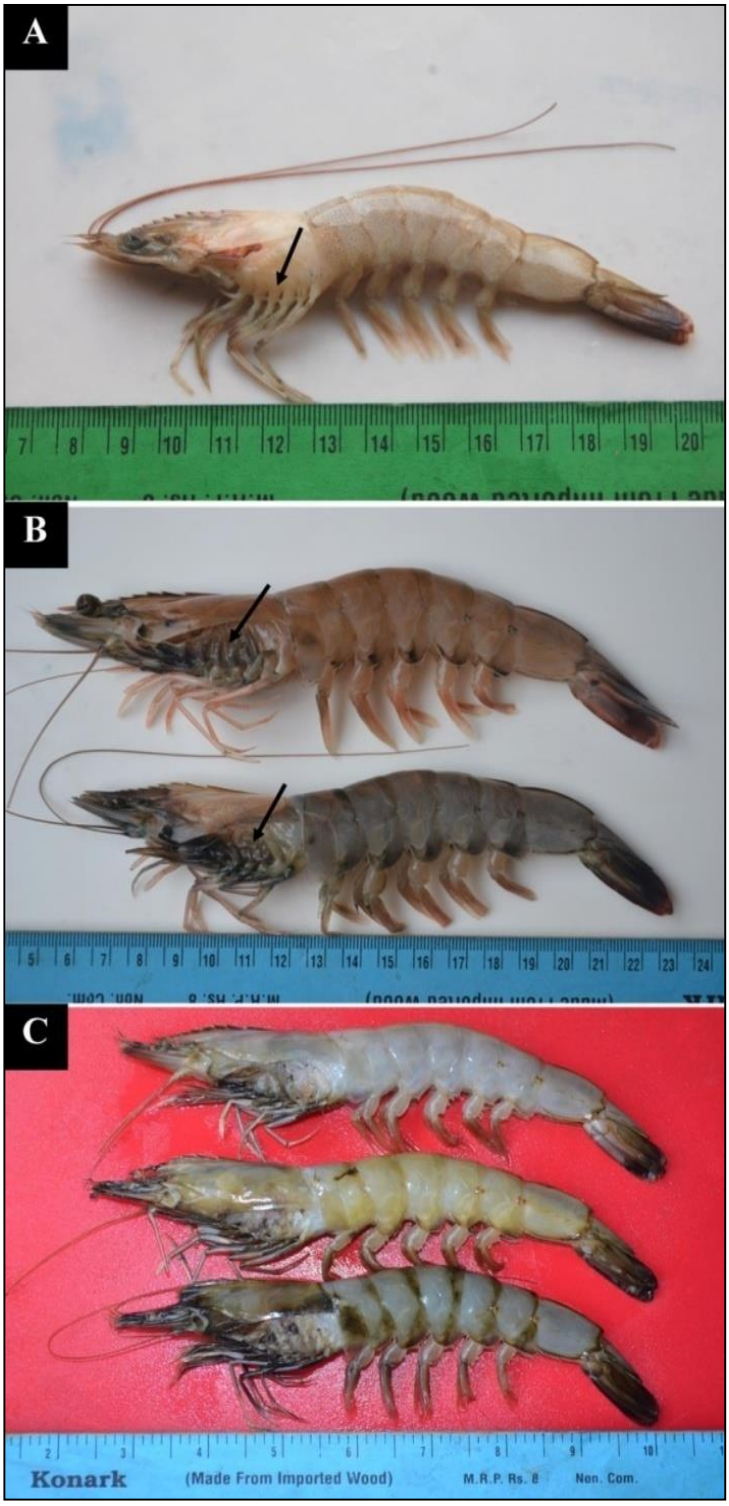

Figure1. Gross observation of normal shrimp with white gill (A), infected shrimps with black gill (B), Brown to black coloration of infected gill (C).

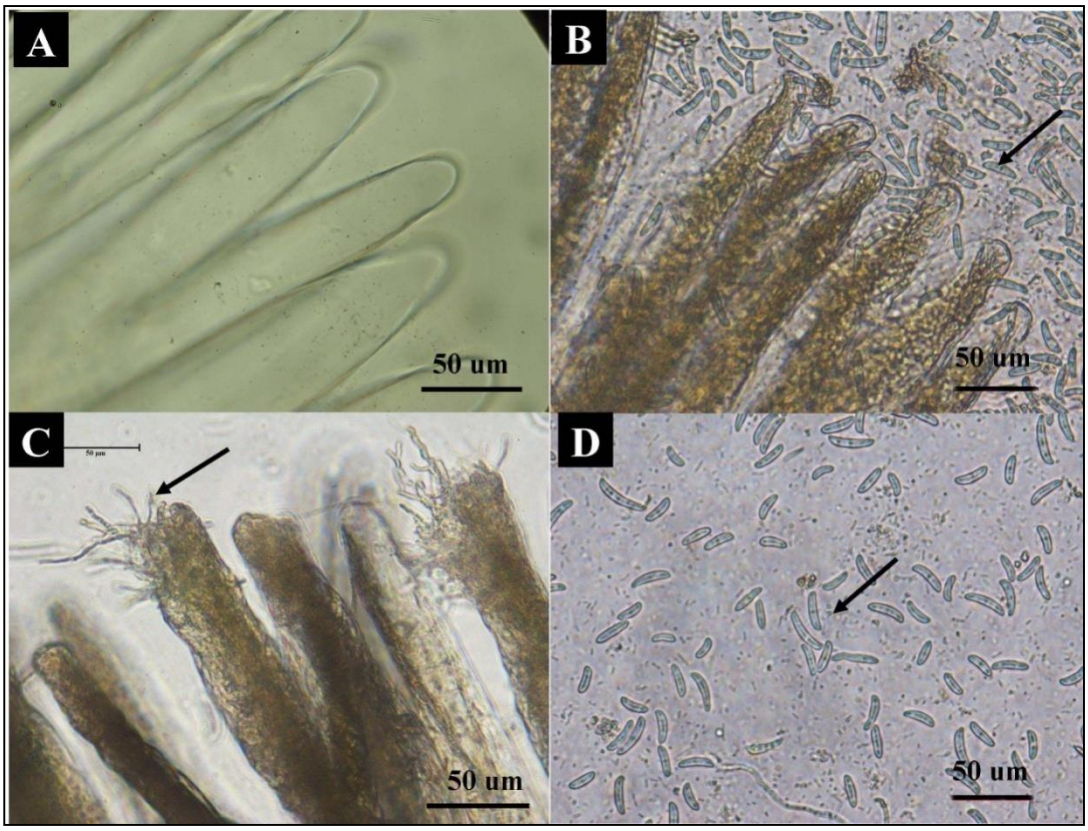

Figure 2. Wet mounts of the gill. Transparent normal gill lamellae (A), black gill surrounded with fungus (B), Fungal hyphae grow from black gill lamellae (C), macroconidias of *Fusarium* (D).

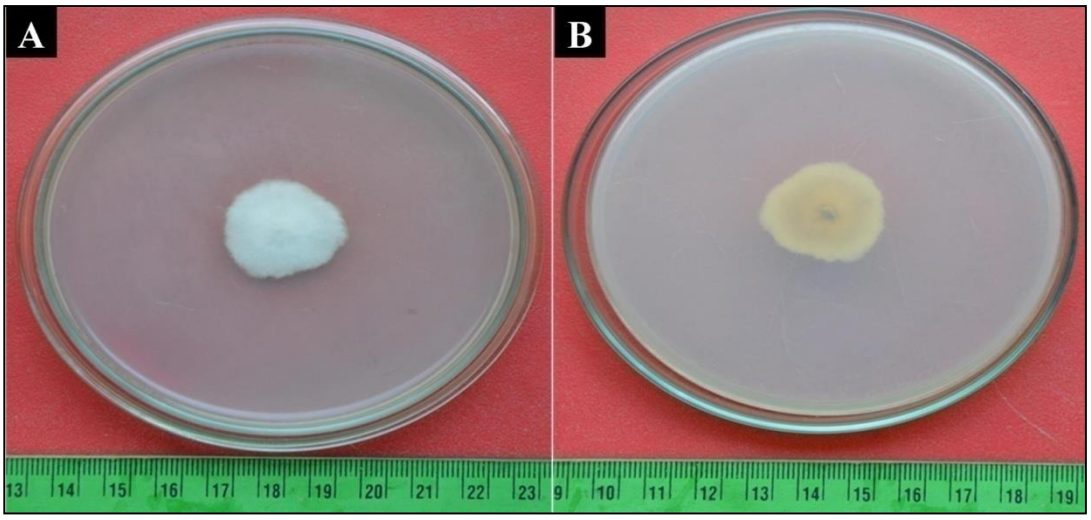

Figure 3. Colony morphology of *Fusarium solini*. White cottony colony isolated from black gill (A), reverse plate of *Fusarium solini* shows yellowish pigmentation (B).

4.3.3. Microscopic observation

Mycelium of *Fusarium solini* was dense and hyline. Hyphae were highly septate and an apical growth was also observed. Macroconidias ranged from 8-10 µm in length and were looking like sickle like curved shape. Macroconidias were septate by a 1-2 septa revealed by light and scanning electron microscope (Fig. 4A, B, C and D).

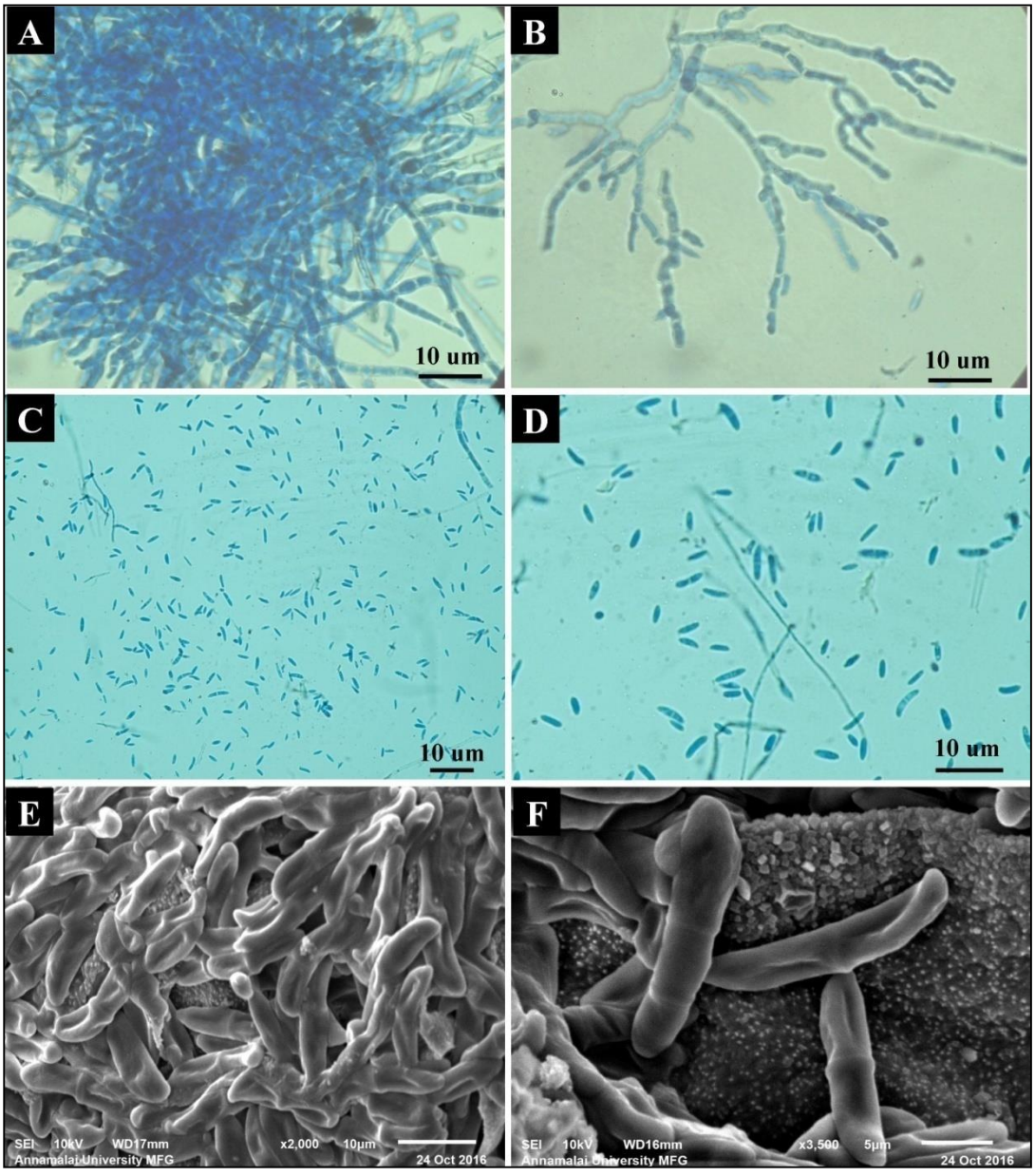

Figure 4: Light and scanning electron microscope. Dense mycelium (A), Septate hyphae (B), Macroconidia (C and D) SEM image of microconidia (E and F).

4.3.4. Histopathology

Normal gill was in a regular intact form without any microbial load. In a normal gill, primary filament and secondary filament were well arranged. Efferent vessel, afferent vessel and lacunae were of the integral form. Haemocytes were in circulating form in gill lamella. Secondary filament of black gill was in a necrotized state. Central axis was damaged. Spores of *Fusarium solani* were evident in gill filaments (Fig. 5).

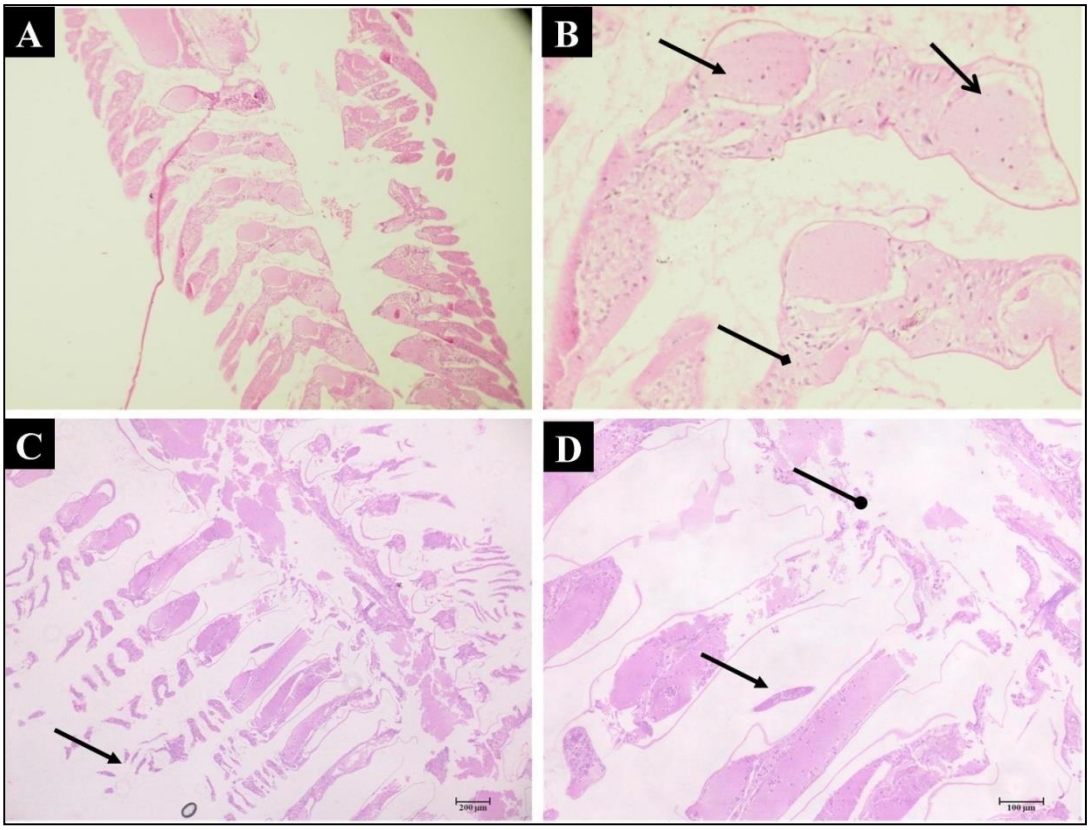

Figure 5. Histology of normal in black gill. Normal gill (A), afferent vessel and efferent vessel of the normal gill, hemocytes in circulating manner (B), Necrotized secondary gill filament (C), Degrades central axis of black gill with macroconidias (D).

4.3.5. Phylogenetic analysis

Based on the ITS gene sequencing, the pathogenic fungus was found to be *Fusarium solanii* (Genbank accession No.KU951245). In the analysis of basic local

alignment search tool, fungi which were isolated from black gills showing 84% similarity to *Fusarium solani* (KX349467.1) (Fig. 6). In the phylogenetic tree, it was clustered in the same clad of *Fusarium solani*.

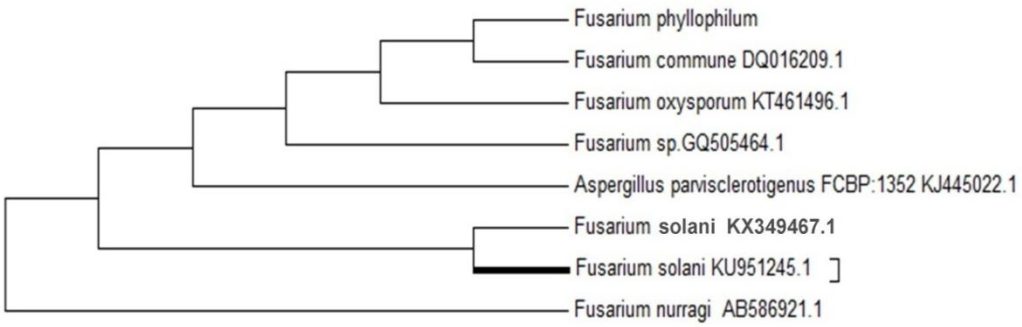

Figure 6. Phylogenetic tree of *Fusarium solani*

4.4. Discussion

L. vannamei is the main candidate species replaced by the Indian tiger shrimp in many countries. Among these countries, United States is the lone source of broodstock of *L. vannamei*. India is in the second place after China in shrimp production but various diseases pose a threat to shrimp industries of the world including of those in India. Hatcheries are one of the foremost departments in shrimp industries where larval production and its maintenance is the main work to provide healthy seeds to the farmers.

Black gill disease was first identified in Japanese Kuruma prawn, *Penaeus japonicus* by Ishikawa (1968). It is believed that Fusarium species was the causative agent for this black gill disease, later Fusarium species was measured as the most detrimental pathogen of Kuruma prawn in Japan.

In India, Ramaiah (2006) has reported that fusarium is the causative agent for Fusariosis and the black gill disease in penaeid shrimps and is identified to reason high mortality, whereas, in the current investigation, female shrimps show higher prevalence in all the 6 months compared to the male shrimps. In broodstock black gill disease, a total prevalence of 31.5% was recorded which is very low when compared to the farm level.

The Wet mount study revealed the fouling of fungi in the gill of shrimps. The present study also shows that the gill colour becomes light yellowish in the initial stage and gradually change into brown as well as red colours and finally turn into black in colour.

Brock and Lea Master (1992) stated that the Fusarium is reported to infect the shrimp by colonizing the cuticular wound and also infects the gills, walking legs, eye lens and body wall. The high stocking density of shrimp is well known to enrich the blowout of disease. But in the present study, Fusarium was found in the gills only. According to Soderhall *et al.* (1979) phenoloxidase of haemocytes is one of the factor which is involved in the formation of melanised lesions.

The present study also reveals that the melanin pigment was partially evident in the infected gill. The pathogenic fungi in shrimp is also known to secrete proteins and is involved in adhesion and invasion in host tissue; and play a significant part in devastation of cell membrane (Da Silva *et al*., 2011). The Wet mount study revealed the presence of fungal hyphae in the gill which grow out from the apex of gill lamellae.

The microconidias was present in high density in the gill of diseased shrimp which denotes severity of infection. Curved shape microconidias was easy to recognize because of its identical shape. The fungus was cottony in nature with undulated margin. Colony colour was white but the reverse plate shows brown colour.

The fungal colony changes the colour from white to slight pinkish which is an identical character of *Fusarium solani*. Gill has multiple functions in crustacean like osmotic regulation, acid-base balance, ammonia excretion and bioaccumulation of toxic metals (Henry *et al*., 2012). But in the present study, histological observations revealed the existence of macroconidias of *Fusarium solini* and gill filament necrosis which leads to block the respiration in shrimp.

Black gill disease in shrimp is described to be affected through a number of causes as well as it is possible noticed in mortality of diseased shrimps due to the damage of gills and through the obstacle of gas interchange crosswise , the gill lamellae leads to asphyxia (Lightner *et al.,* 1975). The black gill haemocytes are highly spread in the gill when compared to the normal gill which indicates inflammatory response.

Lacto phenol cotton blue staining of fungi revealed the dense septate hyphae and microconidia which was septate from the centre. An isolated fungus was identified by the internal transcribed spacer (ITS) gene sequencing method. Basic local alignment search tool results shows similarity with Fusarium species.

On the basis of the morphological and molecular identification fungus was recognized as *Fusarium solani* which is constantly reported by several investigators. The present study shows that the black gill disease is mostly ensued in the maturation tank bottom, committed on algal matt owing to the feeding of live broodstock. Furthermore, the broodstock are apprehended in more than six months under controlled condition for the production of highly healthy nauplii.

At the same time the female stocks are transferred from male tank for matting and the brood stock are handled several times for mating as well as spawning. Due to this the female broodstock become weak, inactive and easily infested. The brood stocks are maintained at 28°C for the production commitments. The result strongly suggest that the holding of broodstocks for more than six months should be avoided as well as the handling of broodstock should be minimised at the time of mating and spawning and strict feeding regimes for the entire cycles for the production of high health nauplii. Reza *et al*., (2014) have stated that the Black gill disease is a disease affected by the accumulation of melanocytes and can be identified in the gills. Black or brown pigments obtainable in gills are the melanin which gets accumulated at the inflamed areas of necrotic tissue. Black gills event usually happens after toxic exposure to inflammation-causing chemicals such as cadmium, copper, potassium permanganate, ozone, oil, acids, ammonia and nitric. Other caused factors are diseases like vibrios, mikoz infection, mycobacterium and ascorbic acid deficiency.

Karthikeyan and Gopalakrishnan (2014) suggested the infested shrimp's species which is considered to be unfit for human consumption. The poor water quality conditions are a budding problem of cultural pond. A study of Debansu *et*

al., (2015) revealed that in the year 2009 and 2010, black gill disease occurrence was frequently reported in the coastal ponds of Northern Odisha at the grow-out stage affecting shrimp production. No ample attention had been given to this disease, and its incidence, reason and effect in yields of shrimps in the coastal ponds of Northern Odisha, whereas some scattered work was found in other regions of India. Khoa *et al*., (2015) have reported the black gill diseased *Penaeus monodon* caused by *Fusarium incarnatum* from grow-out ponds in Vietnam.

The black gill diseased shrimps exhibited typical signs of black gill disease and mortalities around a month previous to harvest. Moreover, Selvakumar and Murugasen (2015) confirmed the black gill disease infection in shrimp affected by the fungus Aspergillus sp. Similarly, Naresh Kumar *et al.* (2015) have experimentally described the fungal spores and hyphae which were detected both in internal and external gill surface of infected shrimps. Fungal spore was round in shape and mature sporangium. In addition, histopathological observations clearly showed that the gill tissue was spoiled by *A. flavus.*