Fusarium moniliforme (Sheldon) Isolated from Gills of Kuruma Prawn
Penaeus japonicus (Bate) with Black Gill Disease

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Fusarium moniliforme was isolated from gill lesions of kuruma prawn *Penaeus japonicus*, with black gill disease at a private farm in Okinawa Prefecture in 1989. The colonies of the fungus cultured on upper surface of potato dextrose agar were floccose, creamy white, undersurface a lavender to violet, but did not grow on mycobiologic agar containing cycloheximide. The present report describes the first case of *F. moniliforme* infection in crustacea. An experimental infection using kuruma prawn was made by intramuscular injection with the conidia of *F. moniliforme* NJM 8995. For comparison, *Fusarium solani* NJM 8996 isolated also from a kuruma prawn with black gill disease in Okinawa Prefecture in 1989, was used as a reference. The clinical signs and pathological findings of the disease caused by the two species of the fungi were similar. Identification of the fungi isolated from the lesions was based principally upon the cultural characteristics. The use of a media lacking cycloheximide is recommended for the isolation of *F. moniliforme*.

Cultured kuruma prawns *Penaeus japonicus* suffering from black gill disease were sampled from a private farm in Okinawa Prefecture in 1989. This disease was originally observed by Egusa & Ueda¹⁾ and the causative agent was identified as *Fusarium solani* by Hatai *et al.*²⁾ Only one species of the genus *Fusarium*, *F. solani*, has so far been identified as the pathogen causing black gill disease in the kuruma prawn. *F. solani* is a comparatively well documented species with detailed examinations of its pathogenicity,³⁻⁹⁾ pathogenesis¹⁰⁾ and histopathology.¹¹⁻¹³⁾ The present study describes a case of *Fusarium* infection in a kuruma prawn caused by *Fusarium moniliforme*, which has never been reported from crustaceans as a pathogen.

The clinical signs of the diseased prawns showed many black spots on their gills which occasionally fused to a stone-like mass in advanced stages (Fig. 1). In the early stages, the gills appeared to show a slight change in colour from opaque white to a yellow or brown colour prior to black spots developing. Fresh mounts prepared from infected gills typically contained hyphae (Figs. 2, 3) and two kinds of conidia. The fungus was classified in the genus *Fusarium* from the shape of the characteristic macroconidia.

The conidia of *F. moniliforme* were intramuscularly inoculated to prove whether the isolated

fungus was capable of causing black gill disease and death in prawns. The histopathological examinations were also made on naturally and experimentally infected prawns.

Materials and Methods

Fungal Isolation and Identification

The moribund kuruma prawn *Penaeus japonicus* were obtained from a private farm in Okinawa Prefecture in April 1989. Isolation of fungus was made by incubating a small piece of gill from a diseased kuruma prawn onto Potato Dextrose Agar "Eiken" (PDA) containing 500 µg/ml each of penicillin G and streptomycin and also onto Mycobiologic agar "Difco" (MBA) at 25°C. A piece of an agar (PDA) block with mycelium was cut out and transferred onto PDA to obtain a pure culture. The isolated fungus was identified according to Booth¹⁴⁾ and Nelson.¹⁵⁾ Furthermore the potassium chloride (KCl) medium was used to observe typical long chains of microconidia.

Histopathology

Gills were fixed in 10% phosphate-buffered formalin solution, embedded in paraffin, sectioned and then stained with haematoxylin and eosin (H & E), periodic acid Schiff's stain (PAS) and Grocott, using routine histological methods.

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Artificial Infection

A strain (NJM 8995) of the present fungus *F. moniliforme* was used for an artificial infection. *Fusarium solani* NJM 8996, isolated from a kuruma prawn with black gill disease at a farm in Okinawa Prefecture in 1989, was used for comparison with *F. moniliforme*. Both strains were cultured on PDA at 25°C. After 2 weeks of incubation, 10 ml of sterile artificial seawater was added onto the plates to obtain the conidial suspension for both strains. The number of conidia in the final suspension was adjusted to approximately 800 and 80,000 conidia per ml of sterile artificial seawater for the both strains. Healthy cultured kuruma prawns of 15–28 gm in body weight and 12–15 cm in body length with no visible black spots in the gills, obtained from Kagoshima Prefecture, were used for the artificial infection. The experimental prawns were allotted into 5 groups of 20 prawns each, and they were injected intramuscularly at the site of the third somite of the abdomen with 0.1 ml of the conidial suspension mentioned above. Ten prawns were used as a control. The conidia injected prawns and control prawns were kept separately in plastic tanks containing 150 litre of seawater without sand at the bottom and maintaining in recirculation by flowing seawater outside the tanks. During the course of the experiment, all prawns were not fed. Mortality of the prawns, seawater temperature and salinity were recorded every day. Seawater temperatures varied between 19–22°C and salinity ranged from 24 to 32‰. Two prawns were randomly removed to observe the gill condition and the fungal infection under microscope at 1, 4, 7, and 14 days after inoculation. Attempts were made to isolate the inoculated fungus from their gills, and fix the gills in 10% phosphate-buffered formalin solution for the histopathological observations.

Effect of Cycloheximide and Temperature on the Growth

Each agar block with the mycelium at the edge of growing colonies of both strains, *F. moniliforme* NJM 8995 and *F. solani* NJM 8996, after 7 days of incubation were removed using a No. 2 cork borer approximately 5.5 mm in diameter and put onto the center of the PDA plates containing cycloheximide at concentrations of 0, 25, 100 and 400 ppm. These agar plates were incubated at 25°C. Further, agar blocks with mycelia of the two isolates were put onto the center of the PDA plates and incubated at various temperatures 5, 10, 15, 25, 30 and 37°C. For both experiments, the same procedures were repeated four times to measure the growing colonies in mm of diameter at 4, 7 and 10 days after inoculation.

Results

Fungal Isolation and Identification

A fungal species was isolated from the inoculated material (Fig. 4). The colonial size of the fungus grown on PDA at 25°C was approximately 35, 53 and 65 mm in diameter at 4, 7 and 10 days after inoculation, respectively. The fungus was classified to the group of the rapidly growing fungus. The colony on PDA was floccose, initially creamy white in colour, then darkened to a lavender colour as it grew, becoming violet in the center and lavender at the margin within 2 weeks. No growth was observed on MBA that contained cycloheximide. Cycloheximide at the concentration of 400 ppm in MBA was effectively able to inhibit growth of the fungus.

Fungal hyphae were hyaline and 2.5–6.0 µm in diameter. Conidiogenous cells with long monophialides were abundantly formed laterally on aerial mycelium or on sympodially branched conidiophores, and were hyaline, subulate 2.0–

Fig. 1. Gross appearance of the gills of a naturally infected kuruma prawn *Penaeus japonicus*. Note: necrotic tissue fused to a stone-like mass (arrow).

Fig. 2. *Fusarium moniliforme* in the gills of a naturally infected kuruma prawn showing hyphae growing out from the tip of gill filament, producing conidiophore and conidia. Fresh mount.

Fig. 3. The same lesion as Fig. 4. at high magnification.

Fig. 4. Colony on Potato Dextrose Agar (PDA) 4 days after inoculation.

Fig. 5. Six-celled macroconidia and one-celled microconidia of *F. moniliforme* producing by aerial hyphae growing out from the tip of gill filament. Fresh mount.

Fig. 6. Microconidial chains produced from a conidiophore on KCl medium 7 days after inoculation, Fresh mount.

Fig. 7. Histology of the macroconidia and hyphae within a gill lamella. Grocott.

Fig. 8. Histological section through a blackened gill branch showing the blockage of brachial blood vessels due to encapsulated fungal conidia and hyphae. Grocott.

Table 1. Comparison of morphological characteristics of the present isolate (NJM 8995) and *F. solani* isolated from kuruma prawn, *Penaeus japonicus* with black gill disease and *F. moniliforme* reported by several workers

Characteristics	Present strain, NJM 8995	<i>F. solani</i> * ¹	<i>F. moniliforme</i> * ²
Colony	White aerial mycelium grows rapidly	White aerial mycelium grows rapidly	White aerial mycelium grows rapidly
Conidiophores	Undersurface violet Long unbranched and branched monophialides	Undersurface brown Long unbranched and branched monophialides	Undersurface violet Long unbranched and branched monophialides
Macroconidia			
Length (μm)	26–50	30–45	25–60
No. of cells range (usually)	4– 6 (4)	4– 6 (4)	4– 8
Shape	Cigar	Canoe	Cigar
Microconidia			
Length (μm)	6–12	6–10	5–12
No. of cells range (usually)	1–2 (1)	1–3 (1)	1–2 (1)
Shape	Fusoid or clavate	Ovoid, oblong or slightly curved	Fusoid or clavate
Form	Cluster, long chains	Only cluster	Cluster, long chains
Clamydospore	Absent	Present	Absent
Hyphae			
Diameter (μm)	2.5– 6	3–4.5	—
Cycloheximide	Sensitive	Resistant	Sensitive

*¹ Egusa & Ueda (1972), and Hatai *et al.* (1978).

*² Booth (1977), Young (1978), Nelson (1983) and Frelief (1985).

4.0 μm in diameter. Macroconidia were present but only rarely and their appearance varied from slightly sickle- to cigar-shaped, 3–4 septa, rarely 5 septa and 26.0–50.0 μm in length (Fig. 5). Microconidia were abundant and variable on shape and size from ovoid to elliptical, 0–1 septa, rarely 2 septa, 6.0–20.0 μm in length, and were produced in chains mostly from a simple conidiophores (Fig. 6) and false heads on PDA and KCl medium but especially with longer chains on the KCl medium. Clamydospore was absent. Morphological characteristics of the isolate are listed in Table 1, comparing with those of *F. solani* described by Egusa & Ueda,¹⁾ Hatai,²⁾ and *F. moniliforme* described by Booth,¹⁴⁾ Nelson,¹⁵⁾ Young¹⁶⁾ and Frelief¹⁷⁾ On the basis of these characteristics, this culture was identified as *Fusarium moniliforme* of the section Liseola.^{14,15)}

Histopathology

Fungal hyphae and occasionally 1–2 celled microconidia and 4–6 celled macroconidia were observed in the collapsed gills (Fig. 7). Both types of conidia were also observed on the external hyphae which were growing out of the gill filament. Thrombosis caused by clotted haemocytes and hyphae, typically encapsulated

by melanized multiple layers of fusiform haemocytes and fibrocytes, were found in the haemocoel of the gill axes and branches (Fig. 8). Furthermore, the latter was surrounded by amorphous mass of substances caused by coagulative necrosis.

Experimental Infection

In the early stage of the infection, no fungal elements were observed under light microscope in the gills of the dead prawns and no growth of hyphae on the PDA also developed at 25°C. The inoculated fungus was able to reisolate from the gills from 4th day of post-inoculation. Prawns which died prior to this 4th day were not included in the mortality data.

As shown in the Fig. 9, the prawns injected with the lower concentration of the conidia (80 conidia/prawn) of *F. moniliforme* NJM 8995 and *F. solani* NJM 8996 first showed mortality 8–9 days after inoculation. The mortality rate of prawns infected with the *F. moniliforme* was slightly higher than that of the *F. solani*. However, the prawns injected with the large number of the conidia (8,000 conidia/prawn) of the both strains died 4 days after inoculation and all the prawns finally died within 2 weeks. The gills infected with both strains

showed the typical black gills condition variable from sparse brown spots to extensive black. On the contrary, no death occurred in the control prawns, no black spots were observed in the gills, and all prawns were free from the pathogenic fungus during the course of the experiment.

Effects of Cycloheximide and Temperature on the growth

The effects of cycloheximide on the growth of

F. moniliforme NJM 8995 and *F. solani* NJM 8996 are shown in Fig. 10. The *F. solani* was relatively resistant to cycloheximide. The growth of the *F. moniliforme* was completely inhibited by 400 ppm of cycloheximide.

Effects of the temperature on the growth of *F. moniliforme* NJM 8995 and *F. solani* NJM 8996 are given in Table 2. The optimum temperature for both fungi ranged from 25°C to 30°C with both strains growing slightly at 37°C, but zero growth was recorded at 5°C.

Discussion

Fusarium moniliforme distributes widely in tropical and subtropical regions as a pathogen in various hosts including rice, sugar cane, banana, cotton, maize, etc.¹⁴⁾ Despite the widespread distribution of *F. moniliforme*, a few cases of the infection with the fungus have been previously reported in man and animals. In man, cases of cutaneous infection,¹⁵⁾ systemic infection during treatment for malignant lymphoma¹⁶⁾ and the infection after allogenic marrow transplantation¹⁰⁾ have been reported. In all cases, the predisposed cause was based on the disturbance of the immune system. In animals, *F. moniliforme* infection has been reported only in a captive alligator *Alligator mississippiensis* as a fatal pulmonary infection.¹⁷⁾ In decapod crustacean, *Fusarium* infection except with *F. solani*, is limited in *F. tabacinum* which infected a crayfish, *Austropotamobius pallipes*.²⁰⁾ *F. moniliforme* infection in kuruma prawn is the first report as the mycotic disease of crustaceans.

Species identification of *Fusarium* is difficult

Fig. 9. Mortality rate of kuruma prawns intramuscularly injected with *F. moniliforme* NJM 8995 and *F. solani* NJM 8996 isolated from kuruma prawns at a farm of Okinawa Prefecture.

- 8,000 conidia of *F. moniliforme* NJM 8995
- 8,000 conidia of *F. solani* NJM 8996
- 80 conidia of *F. moniliforme* NJM 8995
- 80 conidia of *F. solani* NJM 8996
- ▲—▲ control.

Fig. 10. Growth rate of *F. moniliforme* NJM 8995 and *F. solani* NJM 8996 on PDA containing various amounts of cycloheximide.

Table 2. Effects of temperature on growth of 2 species of *Fusarium* isolated from kuruma prawn *Penaeus japonicus*

Temperature (°C)	Days after incubation					
	<i>F. moniliforme</i> NJM 8995			<i>F. solani</i> NJM 8996		
	4	7	10	4	7	10
5	NG	NG	NG	NG	NG	NG ^{*1}
10	NG	1.9	4.5	NG	0.2	0.4 ^{*2}
15	12.8	29.3	43.6	8.3	20.6	30.5
25	34.4	53.2	64.3	29.1	57.9	74.6
30	33.8	54.0	70.8	29.6	54.5	72.2
37	4.7	9.9	11.5	1.7	4.8	6.3

*1 NG: no growth.

*2 Average in diameter of colony (mm).

because of the rapid changes in their morphology and colonial colour.^{14,15} The most distinctive features of *F. moniliforme* from *F. solani* are the formation of microconidia in chains and the absence of clamydospore. Sometimes the formation of microconidial chains were difficult to observe, and in such a case, the fungus might be wrongly classified to either *F. oxysporum* or *F. solani*. Therefore, the presence or absence of microconidial chains is an important characteristic differentiating species of *Fusarium* Section Liseola. The KCl medium to induce the formation of microconidial chains has been used for the identification of *F. moniliforme* and the present isolated produced longer microconidial chains on the medium.

The artificial infection in this study showed the *F. moniliforme* can produce typical black gill condition in kuruma prawn similar to that caused by *F. solani*. The fungus was established as a pathogen of kuruma prawn from the results of the present experiments. The pathogenesis of *F. moniliforme* and the histopathological findings in the kuruma prawn were probably identical to that caused by *F. solani*. Based on the histological observations, failure of gas exchange in the gills was thought to be the main cause of death.

The fungus *F. moniliforme* can not grow on the standard Mycobiotic agar "Eiken" which is usually used to isolate *F. solani* in kuruma prawn. It was suggested that a media lacking cycloheximide should be used together with the standard medium when it was attempted to isolate the causative agent from the lesions showing black gill conditions.

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