Morphology and Molecular Phylogeny of *Fusarium solani* Isolated from Kuruma Prawn *Penaeus japonicus* with Black Gills

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ABSTRACT—Nine strains of fungi in the genus *Fusarium* were isolated from the lesions with black gills of cultured kuruma prawn *Penaeus japonicus* in Japan between 2000 and 2003. All strains showed same morphological characters. Two strains selected at random showed pathogenicity to kuruma prawns by intramuscular injection. From the precise morphological features of a representative isolate, it was identified as a member of the *Fusarium solani* complex. Phylogenetic analyses based on the sequences of its internal transcribed spacer region, including 5.8S ribosomal DNA and a partial 28S ribosomal DNA region, showed that all five strains tested were monophyletic. The present strains and the phytopathogenic *Fusarium solani* were clearly distinguished by the morphological and phylogenetical characteristics.

Key words: *Fusarium solani*, molecular phylogeny, *Penaeus japonicus*, black gill, morphology

Some species in the genus *Fusarium* such as *Fusarium solani* (Egusa and Ueda, 1972), *F. moniliforme* (Rhoobunjongde *et al.*, 1991) and *F. oxysporum* (Hassan *et al.*, 1999) have been isolated from kuruma prawns *Penaeus japonicus* with black gills. Among these species, *F. solani* has subsequently been reported as an important pathogen. *F. solani* has also been isolated from American lobsters *Homarus americanus* (Lightner and Fontain, 1975), brown shrimp *Penaeus californiensis* (Hose *et al.*, 1984), bonnethead sharks *Sphyrna tiburo* (Muhvich *et al.*, 1989), and captive scalloped hammerhead sharks *Sphyrna lewini* (Crow *et al.*, 1995). The pathogenicity of *F. solani* to prawns and the histopathology of the infected animals have been widely studied by fish pathologists (Hatai and Egusa 1978; Chinain and Vey, 1988; Bian and Egusa, 1981; Momoyama, 1987). Morphology and some biological characteristics of *F. solani* isolated from prawns have been reported first by Hatai and Egusa (1978). In addition, *F. solani* is also known as an important plant pathogen that causes root and fruit rot in *Cucurbita* spp.,

sudden dead syndrome in soybean, and wilt in *Ipomoea batata* (O'Donnell, 2000).

F. solani was originally proposed as a major species in the section Martiella in the genus *Fusarium* (Wollenweber, 1913). Later the section included five species, ten varieties, and four forms (Wollenweber and Reinking, 1935). Snyder and Hansen (1941) combined three species (*F. solani*, *F. martii* and *F. coeruleum*) into *F. solani*. This taxonomy, however, was not approved by Gerlach and Nirenberg (1982). Booth (1971) and Gerlach and Nirenberg (1982), included four and six species in the section Martiella, respectively, from conidiogenesis and shapes of conidia which are major criteria for the classification. The main species, *F. solani*, in the genus *Fusarium* has been later reported in the literatures as *formae speciales* (f. sp.), mating population (MP I – MP VII), or anamorph of *Nectria haematococca* due to its polytypic appearances (Matuo and Snyder, 1973). Because of its pathogenic importance, studies on the biological specification of *F. solani* species has also been developed (O'Donnell, 2000). Previous studies on the taxonomy of this complex fungal species have contributed valuable information on the limits of the specification and evolutionary relationships

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within the species, *F. solani* (Matuo and Snyder, 1973; VanEtten, 1978; Hawthorne *et al.*, 1992; Suga *et al.*, 2000; O'Donnell and Gray, 1995; O'Donnell, 2000). However, *F. solani* isolated from aquatic animals including crustaceans and fishes have never been studied in detail in previous reports.

This paper describes the pathogenicity to kuruma prawn, the morphology and phylogenetics of the strains isolated from kuruma prawns naturally infected with *F. solani*.

Materials and Methods

Fungal isolation

Kuruma prawns *Penaeus japonicus* (9 to 27 g in body weight) with *Fusarium* infection were obtained from different prawn farms in Kagoshima, Tokushima and Osaka Prefectures, Japan during 2000 to 2003. Small pieces of the gills were taken from the diseased prawns and observed under a biological microscope to determine whether fungal elements of *Fusarium* were present. A small piece of the gills with *Fusarium* was washed in sterile seawater (SW), and inoculated onto potatoes dextrose agar (PDA: Nissui Co. Ltd., Tokyo, Japan). A small amount of ampicillin and streptomycin, then, were spread onto the agar medium to prevent bacterial contamination. A single spore culture to obtain a pure culture was made 4 days after incubation of the

medium at 25°C.

Artificial infection

Two fungal strains, NJM 0001 and NJM 0180 isolated from cultured kuruma prawns in Kagoshima Pref., Japan in 2000 and 2001, respectively, were used for an artificial infection. The experiments were done using kuruma prawns (average 3.2 g in body weight) at Fisheries Research Institute, Tokushima, Japan. Fungal strains were cultured on PDA at 25°C for 10 days. The fungal conidia were harvested in sterile SW, and the number of conidia was measured using a

Table 1. *Fusarium solani* isolated from *Penaeus japonicus* used in this study

NJM*	Date	Prefecture in Japan	Farm
0001	—, 2000	Kagoshima	A
0178	Dec 30, 2001	Kagoshima	A
0180**	Dec 30, 2001	Kagoshima	A
0271	Jan 25, 2002	Tokushima	B
0272	Jan 25, 2002	Tokushima	B
0273	Jan 25, 2002	Tokushima	B
0377	Feb 25, 2003	Osaka	C
0378	Feb 25, 2003	Osaka	C
0379	Feb 25, 2003	Osaka	C

* Strain number of the Culture Collection in the Division of Fish Diseases (Nippon Veterinary and Animal Science University, Japan)

** Accession number: AY633744

Table 2. Comparative strains used for phylogenetic analysis*

NRRL	Accession number	Anamorph	Teleomorph	Mating population (MP) of <i>Nectria haematococca</i>
22101	AF178398	<i>Fusarium striatum</i>	<i>Nectria ipomoeae</i>	
22142	AF178411	<i>F. solani</i> f. sp. <i>cucurbitae</i>	<i>N. haematococca</i>	V
22153	AF178410	<i>F. solani</i> f. sp. <i>cucurbitae</i>	<i>N. haematococca</i>	I
22157	AF178421	<i>F. solani</i> f. sp. <i>mori</i>	<i>N. haematococca</i>	III
22158	AF178396	<i>Fusarium</i> sp.		
22161	AF178395	<i>F. solani</i> f. sp. <i>robiniae</i>	<i>N. haematococca</i>	VII
22163	AF178394	<i>F. solani</i> f. sp. <i>xanthoxyli</i>	<i>N. haematococca</i>	IV
22230	AF178420	<i>F. solani</i> f. sp. <i>mori</i>	<i>N. haematococca</i>	III
22275	AY320140	<i>Fusarium</i> sp.		
22277	AF178401	<i>F. solani</i> f. sp. <i>xanthoxyli</i>	<i>N. haematococca</i>	IV
22316	AF178423	<i>F. staphyleae</i>	<i>N. atrofusca</i>	
22387	AF178402	<i>Fusarium</i> sp.		
22389	AF178404	<i>Fusarium</i> sp.	<i>Nectria</i> sp.	
22395	AF178405	<i>Fusarium</i> sp.		
22400	AF178407	<i>F. solani</i> f. sp. <i>batatas</i>		
22402	AF178408	<i>F. solani</i> f. sp. <i>batatas</i>		
22412	AF178414	<i>Fusarium</i> sp.		
22436	AY381138	<i>Fusarium</i> sp.	<i>Neocosmospora africana</i>	
22570	AF178422	<i>F. solani</i> f. sp. <i>piperis</i>		
22574	AF178409	<i>Fusarium</i> sp.		
22586	AF178416	<i>F. solani</i> f. sp. <i>robiniae</i>		
22823	AY220215	<i>F. virguliforme</i>		
22825	AY220216	<i>F. virguliforme</i>		

* Cited from O'Donnell (2000) and Aoki *et al.* (2003).

NRRL: Strain number of Agricultural Research Service Culture Collection (National Center for Agricultural Utilization Research, Peoria, Illinois, USA).

Strains shown as bold were used for comparison with conidial sizes of *F. solani* NJM 0180.

hemocytometer and adjusted to 5×10^5 conidia/mL. In the experiments, 30 prawns for each isolate were intramuscularly injected with the conidial suspension of 0.1 mL. Prawns used in these experiments were maintained for 2 weeks in 100 L plastic tanks containing approximately 40 L of natural SW, 2 to 3 cm thick anthracite bottom bed, and running SW. Moribund and fresh dead prawns were sampled to observe clinical signs and fungal elements.

Fungal morphology

The fungal isolate, NJM 0180, was inoculated onto PDA and incubated at 25°C for 4 to 7 days in the dark. Morphology of fungal colony was recorded according to Kornerup and Wanscher (1978). Morphological characteristics of the isolate, which was inoculated onto SNA, after incubation on SNA (KH_2PO_4 1 g/L, KNO_3 1 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g/L, KCl 0.5 g/L, glucose 0.2 g/L, saccharose 0.2 g/L, agar 23 g, 1N NaOH 0.6 mL/L) at 25°C for 7–14 days in the dark were illustrated using the Adobe Illustrator™ version 9.0 (Adobe System Co., San Jose, California, USA). Thirty conidia selected randomly from the isolate were measured in their size and calculated in their average dimensions and their associated standard deviations (SD). The comparative isolates were obtained from National Institute of Agro-Biological Science (NIAS), Tsukuba, Japan.

Fungal molecular phylogeny

The ribosomal DNA (rDNA) template was extracted from fungal mycelia of nine isolates by vigorously mixing a small portion of mycelia with a 50:25:24:1 solution of distilled water:phenol:chloroform:isoamyl alcohol. The mixture was centrifuged for five min at $13,000 \times g$. One microliter (μL) of the supernatant was used as the template for polymerase chain reaction (PCR) along with a primer pair ITS5 and NL4 (White *et al.*, 1990; O'Donnell, 1992). The cycling conditions for the PCR were modified from Suga *et al.* (2000) as follows: initial denaturation at 95°C for 15 min; followed by 45 cycles of denaturation at 94°C for 20 s, annealing at 55 to 56°C for one min, elongation at 72°C for 50 s; and a final elongation at 72°C for ten min; and a 4°C soak. PCR products were purified and used for direct sequencing with six primers: ITS2, ITS3, ITS4, ITS5, NL1 and NL4 (White *et al.*, 1990). DNA sequences from both strands were read on an ABI PRISM 377 DNA sequencing system (Applied Biosystems, Foster City, CA, USA).

Sequence data were aligned using Clustal X programme version 1.8^{*1} and the GeneDoc programme version 2.6^{*2}. Phylogenetic analyses were performed from sequences by the neighbour-joining (NJ) and the most-parsimonious (MP) methods using PAUP version

4.0b8 (Sinauer Associates, Inc. Publishers, Sunderland, Massachusetts, USA). NJ trees were reconstructed with the HKY 85 model, and MP trees were reconstructed using the heuristic search option. Bootstrap values were evaluated for the best tree based on 1000 replicates. Fungi isolated from the infected prawns (Table 1) were sequenced, and sequence data for the other comparative strains (Table 2) were obtained by a BLAST search of GenBank^{*3}.

Results

Fungal isolation

Black spots due to formation of melanotic pigment were observed in the gills of the diseased prawns (Fig. 1). Septate mycelia extending from the tip of the gill filaments and their conidia were clearly observed under a microscope (Fig. 2). The fungi were isolated from the gills in all collected samples by inoculating a small piece

Fig. 1. A kuruma prawn *Penaeus japonicus* naturally infected with *Fusarium solani* showing melanization of the gill filaments (arrow). Scale bar = 2 cm.

Fig. 2. Gills with fungal mycelia. Note: Fungus producing conidia in and on the gills (arrows). Scale bar = 30 μm .

^{*1} <http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html>

^{*2} <http://www.psc.edu/biomed/genedoc/>

^{*3} <http://www.ncbi.nlm.nih.gov/BLAST/>

of gills onto PDA, and preliminary classified in the genus *Fusarium*. No fungi were isolated from the gills of five healthy prawns.

Artificial infection

Fungal hyphae and conidia were observed in the gills of moribund and freshly died prawns on 4 day after inoculation. The experimentally infected prawns showed similar black gills as compared with prawns naturally infected with the fungus. Accumulated mortalities after 2 weeks in the prawns inoculated with the isolates, NJM 0001 and NJM 0180, were 80% and 67%, respectively (Fig. 3). The injected fungus was re-isolated from the gill lesions using PDA. In contrast, 10% prawns died in the control group, but no fungi were found in the prawns.

Fig. 3. Accumulative mortality of *Penaeus japonicus* caused by *Fusarium solani* intramuscularly injected with ca. 5×10^4 conidia per prawn. Two strains of *F. solani*, NJM 0001 and NJM 0180, were used in the experiment.

Fungal morphology

Colony growth on PDA at 25°C in the dark was rapid with entire margin. On PDA, colony pigment on the reversed side of the plates was yellowish white to olive yellow or pale yellow, and turning brownish yellow in aged cultures. An odor was not perceptible from the cultures. Aerial conidiophores were long and unbranched or latterly branched, slightly narrow towards the apex, 37.5 to 85.0×2.50 to 3.25 μm , and lateral aerial conidiophores varied in length, and were usually long and branched. The aerial conidiophores bore mostly monophialidic conidiogenous cells, producing 0 to five-septate conidia; of which 0 to one-septate conidia (one to two-cell conidia) were abundant, and cohering in false head (Fig. 4a). These aerial conidia generally produced on the aerial mycelia at three to four day of post-inoculation on SNA, and they were predominantly oval, from ellipsoid to subcylindric. One-cell conidia on SNA in complete darkness were $9.89 \pm 2.03 \times 3.24 \pm 0.67$ μm , and two-cells conidia on SNA in complete dark-

Fig. 4. Microscopic morphology of *Fusarium solani* NJM 0180 isolated from an infected *Penaeus japonicus*. Scale bar = 25 μm

- a: Aerial conidiophore is long and unbranched, slightly narrow towards the apex, monophialidic, producing abundant 0 to one-septate conidia that cohere in a false head.
- b: Oval or ellipsoid one-cell conidia and subcylindric or slightly curved two-cells conidia.
- c: Unbranched aerial conidiophore bear three to four-septate conidia and are slightly curved with a short and blunt apical cell and slightly notched basal cell.
- d: A lateral branched aerial conidiophore producing one to four-septate conidia.
- e: Irregularly and verticillately branched sporodochial conidiophores, bearing monophialides and producing three and four-septate conidia. Conidia extend from the basal part and curve to the apex. The dorsal side is more curved than ventral side, and there is a blunt apical cell and slightly notched basal cell.
- f: A sporodochial conidiophore verticillately forming monophialides in the early stage of sporulation.
- g: A terminal chlamydospore from conidiophore is smooth-walled, globose.
- h: Intercalary chlamydospores in the hypha are smooth-walled, globose, and in a pair.

ness were $14.43 \pm 2.10 \times 3.60 \pm 0.56$ μm (Fig. 4b). The lateral aerial conidiophores contained two to five-septate conidia (Fig. 4c) and were subcylindric and regularly curved with a short and blunt apical cell, although they sometimes were slightly tapered towards the apex with very slightly notched basal cell (Fig. 4d). Three-septate conidia on SNA in complete darkness were 33.93 ± 5.02

$\times 4.39 \pm 0.43 \mu\text{m}$, four-septate conidia on SNA in complete darkness were $40.95 \pm 3.43 \times 4.98 \pm 0.17 \mu\text{m}$, and five-septate conidia on SNA in complete darkness were $45.68 \pm 3.78 \times 4.97 \pm 0.25 \mu\text{m}$. Sporodochial conidiophores were irregularly or verticillately branched and

bore monophialides mostly, producing three- to five-septate conidia, and rarely six- or seven-septate conidia (Fig. 4e and 4f). Sporodochial conidia were usually straighter and longer than those produced by aerial conidiophores, slightly curving from central dorsal area

Fig. 5. A neighbour-joining tree based on the ITS rDNA including the complete sequences of 5.8S rDNA regions and a partial sequence of the 28S rDNA from 27 of the in-group taxa within *Fusarium solani* complex. Note: The tree was rooted by an outgroup taxon *Fusarium staphyleae* NRRL 22316. All branches were supported with above 50% bootstrap values from 1000 replicates. Branches with 100% bootstrap support are shown with bold lines. Five strains from prawns were indicated in bold letters. Scale bar represents proportional branch distances.

toward the apex, with a blunt and sometimes pointed apical cell and indistinct notched basal cell (Fig. 4e and 4f). Chlamydospores were abundant, terminal or intercalary, smooth-walled, globose to subglobose, and occurred mostly in pairs. They were sometimes single and in short chains, 7.5 to 14 μm in diameter, and greenish in color. A terminal chlamydospore from conidiophore (Fig. 4g) and an intercalary chlamydospore in the hyphae (Fig. 4h) were smooth-walled, globose, and in pair. The mass of sporodochial conidia was greenish. Three-septate conidia on SNA in complete darkness were $48.14 \pm 5.83 \times 4.08 \pm 0.38$, 4-septate conidia on SNA in complete darkness were $51.83 \pm 4.42 \times 4.29 \pm 0.35$ μm , and five-septate conidia on SNA in complete darkness were $54.71 \pm 3.40 \times 4.66 \pm 0.32$ μm . Based on the morphology as mentioned above, the isolate NJM 0180 was identified as *Fusarium solani* Martius 1842, which is also known as *Fusisporium solani* or *Fusarium solani* var. *solani*.

Fungal molecular phylogeny

Because the rDNA sequences of the nine isolates of *F. solani* randomly selected were identical, five strains (NJM 0001, NJM 0180, NJM 0271, NJM 0272 and NJM 0273) among the nine isolates were randomly chosen for the phylogenetic analysis. In this study, a phylogenetic analysis on a total 27 in-group taxa within *F. solani* complex was performed, in which *F. staphyleae* strain NRRL 22316 (teleomorph = *Nectria atrofusca*) was used to root the tree as described by O'Donnell (2000). The sequence data sets included DNA fragments, each of which contained 5.8S rDNA gene, a partial 28rDNA gene, ITS regions and was 1,127 base pair in length without the PCR-amplified primers.

The NJ tree (Fig. 5) shows two major clades (clade 1 and clade 2). All clusters of the NJ tree corresponded to one of the biological species, but *F. solani* f. sp. *glycines* (NRRL 22823 and NRRL 22825) and *F. solani* f. sp. *phaseoli* (NRRL 22158 and NRRL 22275) were clustered in one group with a 100% bootstrap value. The strains from the prawn used in this study were included in clade 2 with a high bootstrap support (85%). Moreover, the strains were also clustered in a mono-

phylogenetic group with sister group of MP V (NRRL 22142) and the holothallic *Fusarium* sp. NRRL 22389. A high bootstrap value of 100% supported the grouping of the prawn strains. In addition, *F. solani* f. sp. *piperis* NRRL 22570 and *F. solani* f. sp. *cucurbitae* race 1 NRRL 22153 (MP I), and *Fusarium* sp. NRRL 22389 and *F. solani* f. sp. *cucurbitae* race 2 NRRL 22142 (MP V) were resolved in the two clusters with 93% and 52% bootstrap values, respectively.

Discussion

Morphology of the *F. solani* isolated from cultured kuruma prawns agreed well with systems provided by Booth (1971) and Gerlach and Nirenberg (1982). The species formed long aerial conidiophores and produced typical oval aerial conidia with one to two cells. And sporodochial conidiophores produced predominantly three-five-septate conidia that were slightly curved, short, and with a blunt apical and indistinctly pedicellate basal cell. These features are most distinguishing and diagnostic characteristics of the *F. solani*. Moreover, recent strains from prawns produced neither strong dark violet-blue pigment nor occasional polyphialides. The features differentiated them from *F. solani* var. *coeruleum*, which causes powdery rot of potatoes in storage (Booth, 1977).

Based on phylogenetic analysis, the *F. solani* isolated from prawns were cladistically sister of MP III, MP V and MP VII biological species. For this reason, conidial sizes of the prawn strain NJM 0180 were compared with four strains of the *Nectria haematococca* (Table 3). The sporodochial conidia of the strain NJM 0180 were longer and slightly more slender than the four strains of *Nectria haematococca*, biological species of the *F. solani* complex. Only the five-septate sporodochial conidia of strain NRRL 22157 (MP III) was similar to that of NJM 0180. However, strains NRRL 22161 and NRRL 22586 (MP VII) showed the differences in the lengths of the sporodochial conidia. In addition, phylogenetic studies of phytopathogenic fungus *F. solani* based on ITS rDNA have clearly showed that there was not a close association between

Table 3. Comparison of conidial sizes between *Fusarium solani* NJM 0180 and four strains of *F. solani* obtained from National Institute of Agro-Biological Science (NIAS)

Conidia formation	Number of cell	Strains				
		NJM 0180	NRRL 22142 (MP V)	NRRL 22157 (MP III)	NRRL 22161 (MP VII)	NRRL 22586 (MP VII)
Aerial	1	$10 \pm 2 \times 3.2 \pm 0.7^*$	$8.7 \pm 1.6 \times 4 \pm 0.4$	$9 \pm 2 \times 3.4 \pm 0.6$	$9.2 \pm 2 \times 3 \pm 0.6$	$9 \pm 2.2 \times 3.2 \pm 0.5$
	2	$14.5 \pm 2 \times 3.6 \pm 0.6$	$12.9 \pm 1.6 \times 4.5 \pm 0.4$	$16 \pm 2 \times 4 \pm 0.4$	$14.8 \pm 3 \times 4 \pm 0.5$	$14 \pm 2 \times 4 \pm 0.4$
Sporodochial	4	$48 \pm 6 \times 4 \pm 0.5$	$41 \pm 3.5 \times 4.7 \pm 0.3$	$38 \pm 3.5 \times 4.5 \pm 0.3$	$41 \pm 4 \times 4.4 \pm 0.3$	$34.3 \pm 6 \times 4.4 \pm 0.3$
	5	$52 \pm 4.5 \times 4.5 \pm 0.3$	$46 \pm 3 \times 5 \pm 0.2$	$40 \pm 5.3 \times 4.5 \pm 0.3$	$47 \pm 9 \times 4.7 \pm 1$	$38 \pm 2.2 \times 4.7 \pm 0.3$
	6	$55 \pm 3.4 \times 4.7 \pm 0.3$	$49 \pm 4.5 \times 5 \pm 0.2$	$55 \pm 6 \times 4.8 \pm 0.2$	$50 \pm 3.3 \times 5 \pm 0.2$	$42.3 \pm 3.1 \times 4.8 \pm 0.3$

* average length $\pm$ SD $\times$ average width $\pm$ SD (μm)

macroconidia types and phylogenetic relationships (Suga *et al.*, 2000).

Overall, the phylogenetic analysis using both NJ and MP methods in this study strongly supported that the strains from prawns formed a part of a monophyletic group. Although, molecular phylogeny is strong tool for resolving the taxonomy among *F. solani* complex, more strains and different gene partitions of the *F. solani* isolated from prawn and the other aquatic sources should be studied to determine its precise phylogeny.

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