海生甲殻類から分離した病原卵菌類のITS1領域の塩基配列による同定とアルテミア孵化幼生に対する病原性

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Molecular Identification of Marine Crustacean-pathogenic Peronosporomycetes Using DNA Sequences of ITS1 and their Pathogenicity for Nauplii of Brine Shrimps

Yasunori Muraosa[1*,2], Ayako Sano[3] and Kishio Hatai[2]

[1]*Division of Clinical Research, Medical Mycology Research Center, Chiba University, Chiba 260-8673, Japan*
[2]*Laboratory of Fish Diseases, Nippon Veterinary and Life Science University, Tokyo 180-8602, Japan*
[3]*Department of Animal Sciences, Faculty of Agriculture, University of the Ryukyus, Okinawa 903-0213, Japan*

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ABSTRACT—We isolated 27 strains of marine crustacean-pathogenic Peronosporomycetes from various infected marine crustaceans collected in Japan, Thailand, Vietnam and the Philippines, and identified them morphologically. The internal transcribed spacer (ITS) I region of the nuclear rRNA gene of the 27 strains and 12 reference strains of six peronosporomycete species isolated from marine crustaceans were sequenced. The phylogenic relationship inferred from the sequences corresponded well to the morphological identification, indicating that sequencing of the ITS1 region is a useful tool for identification of marine crustacean-pathogenic Peronosporomycetes. Nauplii of brine shrimp *Artemia salina* were experimentally challenged by bathing in zoospore suspensions (1×10^4 zoospores/mL, 25°C) with 18 strains belonging to nine species in four genera. The 48-h mortalities varied from 1.1% to 99.4% among the species, though all the strains were isolated from diseased animals.

Key words: Peronosporomycetes, *Lagenidium*, *Haliphthoros*, *Halocrusticida*, *Atkinsiella*, pathogenicity, *Artemia salina,* brine shrimp

The class Peronosporomycetes (formerly Oomycetes) are fungus-like organisms classified into the kingdom Stramenopile (Dick, 1990). They have been classified into the kingdom Fungi because their thalli morphologically resemble those of filamentous fungi; however, they are phylogenetically distinct from true Fungi. The class Peronosporomycetes includes many species that are pathogens of several commercially important plants and animals (Ristaino, 2002; Grooters, 2003; Prasertwitayakij *et al*., 2003; Phillips *et al*., 2008; Saylor *et al*., 2010). *Lagenidium, Haliphthoros, Halocrusticida, Atkinsiella* and *Halioticida* species have been isolated from marine crustaceans (Nakamura and Hatai, 1995b; Atami *et al*., 2009). These organisms cause problematic infections at marine crustacean hatcheries, leading to high mortality in larvae (Nilson *et al*., 1976; Roza and Hatai, 1999; Hatai *et al*., 2000).

The life cycle of Peronosporomycetes comprises both a sexual and an asexual stage. The sexual stage is characterized by the oogonium and antheridium, while the asexual stage is characterized by biflagellate zoospores (the infective unit) (Diéguez-Uribeondo *et al*., 2009). Peronosporomycetes has been identified based on the morphological characteristics of the asexual and sexual reproductive structures. Sexual reproduction has not been observed in any Peronosporomycetes isolated from marine crustaceans, and asexual reproduction often declines with repeated subculturing. Therefore, the morphological identification at the species level is difficult and requires experience and expertise. In recent years, sequencing and comparison of some genes have become important tools for the identification of Peronosporomycetes, and they facilitate morphology-based identification. Nuclear rRNA gene regions have been used for identification and phylogenetic analysis of the members of the class Peronosporomycetes. The small subunit rRNA gene (18S rDNA) and the large subunit of rRNA gene (28S rDNA) have been demonstrated to be useful in phylogenetic analyses of higher taxa (genera, families, orders or subclasses) within the class Peronosporomycetes (Dick *et al*., 1999; Riethmüller *et al*., 1999, 2002; Petersen and Rosendahl 2000; Sekimoto *et al*., 2007, 2008). Internal transcribed spacer (ITS) I-5.8S rDNA-ITS2 regions have been used to establish the phylogenetic relationships of freshwater peronosporomycete species belonging to the genera *Aphanomyces* and *Pythium* (Diéguez-Uribeondo *et al*., 2009; Schurko *et al*., 2003). In our previous study, we

* Corresponding author
E-mail: y.muraosa@faculty.chiba-u.jp

demonstrated that the D1/D2 region of 28S rDNA was a useful tool to analyze phylogenetic relationships and to identify marine invertebrate-pathogenic peronosporomycete species at the genus level (Muraosa *et al.*, 2009).

In this study, we isolated marine crustacean-pathogenic Peronosporomycetes from various marine crustaceans collected in Japan, Thailand, Vietnam and the Philippines. We identified them at the species level by morphological analyses and compared DNA sequences of the ITS1 regions of the nuclear rRNA gene among morphologically identified species, to estimate the potential use of the DNA sequences for molecular identification.

Each marine crustacean-pathogenic Peronosporomycetes has been isolated from different host species, suggesting that they have a wide host range. Furthermore, there are some reports showing that some Peronosporomycetes isolated from marine crustaceans are pathogenic for the nauplii of brine shrimp (Tharp and Bland, 1977; Overton and Bland, 1981; Yasunobu, 2001), which suggests that experimental challenges against nauplii of *Artemia salina* can be used for evaluation of general pathogenicity of Peronosporomycetes. In this study, we challenged nauplii of *A. salina* with several peronosporomycete species to evaluate the potential use of the experimental challenges against the nauplii.

Materials and Methods

Isolates

Single spore culture was performed to yield pure cultures. Eggs or larvae infected with Peronosporomycetes were directly inoculated on peptone–yeast extract–glucose–seawater (PYGS) agar plates composed of 0.125% peptone, 0.125% yeast extract, 0.3% glucose, 1.2% agar, and 37.6 g/L artificial seawater (Aqua-Ocean, Japan Pet Drugs). Powdered streptomycin sulfate and ampicillin were directly sprinkled on the surface of PYGS agar plates. After 3 days incubation at 20–25°C, agar blocks located at the edge of growing colonies were transferred onto fresh PYGS agar plates. All isolates were identified by observing asexual morphological characteristics according to methods described by Vishniac (1958), Bland and Amerson (1973), Hatai *et al*. (1980), Hatai and Lawhavinit (1988), Nakamura and Hatai (1995b), Nakamura *et al*. (1995), Hatai *et al*. (2000).

DNA sequencing and molecular comparison

The ITS regions of 27 isolates obtained in this study and 12 reference strains were sequenced as follows (Table 1). After 3 days incubation at 20–25°C in PYGS broth, young growing hyphae were washed three times with phosphate-buffered saline (PBS) and frozen at −85°C. Total genomic DNA was extracted using the DNAzol® reagent (Invitrogen) according to the manufacturer's instructions. ITS1-5.8S-ITS2 regions were amplified by PCR with the primers ITS5 and ITS4 (White *et al*., 1990). Each 50 μL PCR reaction mixture contained 2.5 ng genomic DNA, 10 μL 10× Ex Taq® Buffer (Takara Bio), 8 μL 2.5 mM dNTP Mixture (Takara Bio), 1 μM each primer, and 0.8 U Takara Ex Taq® (Takara Bio). PCR was performed using the Gene Amp® PCR System 9700 (Applied Biosystems) under the following conditions: 94°C for 1 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, with a final extension at 72°C for 7 min.

PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) and sequenced by the direct sequencing method using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Primers ITS1, ITS4, ITS5 (White *et al*., 1990), ITS2P (5′-gCAgCgTTCTTCATCgATgT-3′), and ITS3P (5′-ACATCgATgAAgAACgCTgC-3′) were used for the cycle sequencing. Sequences were assembled using the ATGC version 3.0 (Genetyx) and Genetyx®-win version 5.2 (Genetyx).

Similarity of sequences was analyzed using the Genetyx®-win version 5.2 (Genetyx). Sequence alignments were performed with the sequence of *Albugo candida* obtained from Genbank as the out-group species, using the Clustal X program (Thompson *et al*., 1997). Phylogenetic analyses were performed with the neighbor-joining (NJ) method using PAUP version 4.0b8 (Sinauer Associates). Bootstrap values were evaluated by 1000 replications.

Experimental infection against nauplii of the brine shrimp A. salina

Brine shrimp were experimentally challenged with 18 strains belonging to nine species in four genera: *Lagenidium callinectes* NJM 0531; *Lagenidium thermophilum* NJM 0485, NJM 0493, NJM 0534; *Haliphthoros milfordensis* NJM 0444, NJM0487, *Haliphthoros* sp. group1 NJM 0443; *Haliphthoros* sp. group 2 NJM 0440, NJM 0449, NJM 0535; *Halocrusticida panulirata* NJM 0441, NJM 0445, NJM 0465, NJM 0483; *Halocrusticida okinawaensis* NJM 0464; *Halocrusticida parasitica* NJM 0468, NJM 0533; and *Atkinsiella dubia* NJM 0634. Dehydrated brine shrimp cysts (Japan Pet Drugs) were incubated in artificial seawater (Aqua-Ocean) for 24 h at 30°C with appropriate aeration. Hatched nauplii were experimentally challenged to estimate the pathogenicity of the isolates. Five hundred nauplii were placed in 50 mL sterile artificial seawater containing 200 μg/mL streptomycin sulfate and ampicillin in a 100-mL glass beaker, to which 10^4 zoospores/mL were added for challenge. Nauplii were maintained without challenge as negative controls.

Table 1. Peronosporomycetes isolated from various marine crustaceans and reference strains were analyzed for ITS1 sequences in this study

Strain category	Species	Strains	Host	Host stage	Locality	GenBank Accession No.	References
Isolates	*Lagenidium* species						
	L. callinectes	NJM 0531*	Swimming crab, *Portunus trituberculatus*	egg	Hyogo, Japan	AB285488	
	L. thermophilum	NJM 0140	Black tiger shrimp, *Penaeus monodon*	larva	Vietnam, Vung Tau	AB285492	
	L. thermophilum	NJM 0144	Black tiger shrimp, *Penaeus monodon*	larva	Chachensao, Thailand	AB285493	
	L. thermophilum	NJM 0485*	Whiteleg shrimp, *Penaeus vannamei*	larva	Chachensao, Thailand	AB285494	
	L. thermophilum	NJM 0493*	Whiteleg shrimp, *Penaeus vannamei*	egg	Chachensao, Thailand	AB285495	
	L. thermophilum	NJM 0534*	Mangrove crab, *Scylla serrata*	egg	Philippines	AB285496	
	Haliphthoros species						
	H. milfordensis	NJM 0139	Greasyback shrimp, *Metapenaeus ensis*	larva	Hiroshima, Japan	AB285502	
	H. milfordensis	NJM 0262	Black tiger shrimp, *Penaeus monodon*	larva	Vietnam, Nha Trang	AB285503	
	H. milfordensis	NJM 0444*	Swimming crab, *Portunus trituberculatus*	larva	Yamaguchi, Japan	AB285504	
	H. milfordensis	NJM 0487*	Whiteleg shrimp, *Penaeus vannamei*	larva	Chachensao, Thailand	AB285505	
	H. sp. group1	NJM 0143	Black tiger shrimp, *Penaeus monodon*	egg	Chachensao, Thailand	AB285506	
	H. sp. group1	NJM 0443*	Kuruma prawn, *Penaeus japonicus*	larva	Mie, Japan	AB285507	
	H. sp. group2	NJM 0440*	Swimming crab, *Portunus trituberculatus*	larva	Fukuoka, Japan	AB285508	
	H. sp. group2	NJM 0449*	Greasyback shrimp, *Metapenaeus ensis*	egg	Hiroshima, Japan	AB285509	
	H. sp. group2	NJM 0535*	Mangrove crab, *Scylla serrata*	egg	Philippines	AB285510	
	Halocrusticida species						
	H. panulirata	NJM 0261	Kuruma prawn, *Penaeus japonicus*	larva	Shimane, Japan	AB285512	
	H. panulirata	NJM 0441*	Swimming crab, *Portunus trituberculatus*	larva	Fukuoka, Japan	AB285513	
	H. panulirata	NJM 0445*	Swimming crab, *Portunus trituberculatus*	larva	Hiroshima, Japan	AB285515	
	H. panulirata	NJM 0465*	Swimming crab, *Portunus trituberculatus*	larva	Hiroshima, Japan	AB285514	
	H. panulirata	NJM 0483*	Greasyback shrimp, *Metapenaeus ensis*	larva	Hiroshima, Japan	AB285516	
	H. okinawaensis	NJM 0141	Greasyback shrimp, *Metapenaeus ensis*	larva	Mie, Japan	AB285518	
	H. okinawaensis	NJM 0464*	Whiteleg shrimp, *Penaeus vannamei*	larva	Chachensao, Thailand	AB285519	
	H. parasitica	NJM 0468*	Swimming crab, *Portunus trituberculatus*	larva	Fukuoka, Japan	AB285520	
	H. parasitica	NJM 0533*	Swimming crab, *Portunus trituberculatus*	egg	Hiroshima, Japan	AB285521	
	Atkinsiella species						
	A. dubia	NJM 0132	Swimming crab, *Portunus trituberculatus*	egg	Okayama, Japan	AB285522	
	A. dubia	NJM 0532	Swimming crab, *Portunus trituberculatus*	egg	Hyogo, Japan	AB285523	
	A. dubia	NJM 0634*	Swimming crab, *Portunus trituberculatus*	egg	Hiroshima, Japan	AB285524	
Reference strains	*Lagenidium* species						
	L. callinectes	ATCC[a] 24973	Blue crab, *Callinectes sapidus*	egg	North Carolina, USA	AB285486	Bland and Amerson (1973)
	L. callinectes	NJM 8989	Swimming crab, *Portunus trituberculatus*	larva	Okayama, Japan	AB285487	
	L. thermophilum	**ATCC 200318**[b]	Mangrove crab, *Scylla serrata*	egg	Bali, Indonesia	AB285489	Nakamura *et al.* (1995)
	L. thermophilum	NJM[c] 0031	Black tiger shrimp, *Penaeus monodon*	larva	Chachensao, Thailand	AB285491	Muraosa *et al.* (2006)
	L. thermophilum	NJM 9831	Greasyback shrimp, *Metapenaeus ensis*	larva	Okayama, Japan	AB285490	
	L. myophilum	**ATCC 66280**[d]	Northern shrimp, *Pandalus borealis*	adult	Ishikawa, Japan	AB285498	Hatai and Lawhavinit (1988)
	L. myophilum	ATCC 200325[e]	Coonstripe shrimp, *Pandalus hypsinotus*	juvenile	Hokkaido, Japan	AB285500	Nakamura *et al.* (1994)
	L. myophilum	NJM 8403	Northern shrimp, *Pandalus borealis*	adult	Hokkaido, Japan	AB285497	
	L. myophilum	NJM 9131	Coonstripe shrimp, *Pandalus hypsinotus*	larva	Hokkaido, Japan	AB285499	
	Haliphthoros species						
	H. milfordensis	ATCC MYA-3264[f]	Black tiger shrimp, *Penaeus monodon*	larva	Nha Trang, Vietnam	AB285501	Chukanhom *et al.* (2003)
	Halocrusticida species						
	H. baliensis	**GSM**[g] **9703**	Mangrove crab, *Scylla serrata*	larva	Bali, Indonesia	AB285517	Hatai *et al.* (2000)
	H. panulirata	NJM 9832	Mangrove crab, *Scylla serrata*	egg	Bali, Indonesia	AB285511	

Strains shown in bold are the ex-type strains. [a]American Type Culture Collection, Manassas, VA, USA. [b]NJM 9338. [c]Culture collection in the Division of Fish Diseases, Nippon Veterinary and Life Science University, Musashino, Tokyo, Japan. [d]NJM 8601. [e]NJM 9331. [f]NJM 0131. [g]Culture collection in the Gondol Research Station for Coastal Fisheries, Singaraja, Bali, Indonesia. *Strains used for experimental infection of nauplii of the brine shrimp.

Dead nauplii on the bottom of the glass beakers were transferred into a Petri dish containing fresh, sterile artificial seawater after 24 and 48 h challenge, and counted to obtain cumulative mortality rates. To confirm the infection, some dead nauplii were incubated at 25°C for an additional 24 h and examined with a light microscope for the presence of broad hyphae without septa, which are the morphological characteristic of Peronosporomycetes.

Results

Identification and phylogenetic analysis

Twenty-two strains isolated from various crustaceans were morphologically assigned to seven known species belonging to four genera —*Lagenidium,*

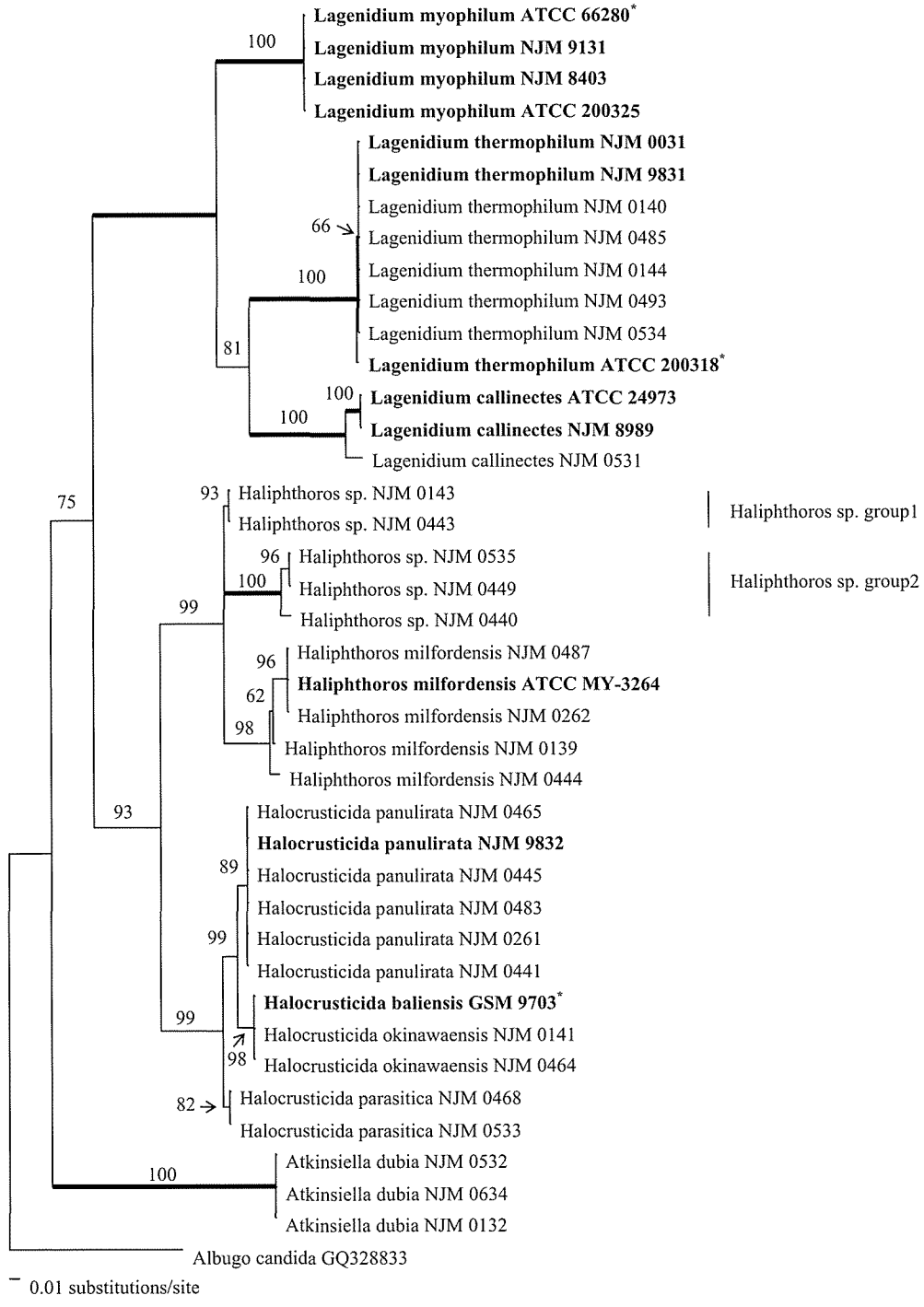

Fig. 1. NJ tree based on ITS1 from 39 of the in-group taxa. The tree was rooted by an outgroup taxon, *Albugo candida* GQ328833. All branches were supported by bootstrap values > 50% from 1000 replicates. Branches with 100% bootstrap values are shown with bold lines. Strains shown in bold are reference strains. *Ex-type strains.

Haliphthoros, Halocrusticida and *Atkinsiella*— and five strains were assigned to the genus *Haliphthoros* without specific identification (Table 1).

As DNA sequences of the ITS2 region were not obtained from four species, namely, *L. thermophilum*, *Halocrusticida okinawaensis, Halocrusticida baliensis,* and *At. dubia*, the sequences of ITS1 region only were used for sequence similarity and phylogenetic analyses, in which the sequences of primers were omitted. The sequence data obtained in this study were deposited in GenBank with accession numbers AB285486–AB285524 (Table 1). The phylogenetic tree inferred from ITS1 analysis is shown in Fig. 1.

In the genus *Lagenidium*, the similarity in ITS1 sequence clearly distinguished *L. callinectes, L. thermophilum* and *Lagenidium myophilum*; the intraspecific similarities were 96.8–100%, 96.8–100% and 100%, respectively, and the interspecies similarities between different species were 78.9–67.1%. In the genus *Haliphthoros*, the ITS1 sequence similarity clearly distinguished *Haliphthoros milfordensis* and two groups (*Haliphthoros* sp. group 1 and 2), the latter of which were not morphologically assigned to any known species; the intraspecific similarities were 97.3–100%, 100% and 97.0–100%, respectively, and the interspecies similarities were 80.6–83.2%. Each of the three groups including *Haliphthoros milfordensis* formed a discrete clade that was strongly supported by bootstrap values (98%, 93%, and 100%, respectively) as well. In the genus *Halocrusticida,* the ITS1 sequence similarity clearly distinguished *Halocrusticida panulirata* from *Halocrusticida parasitica*; intraspecific similarities were both 100%, and the interspecies similarity was 94.5%. The interspecies similarity between *Halocrusticida okinawaensis* and *Halocrusticida baliensis* was 100%. In *At. dubia*, the intraspecific similarity was 100%. The phylogenetic tree inferred from ITS1 analysis is shown in Fig. 1.

Experimental infection

Dead nauplii that sank on the bottom of the glass beaker were observed in all challenge experiments after 24 or 48 h (Table 2). However, broad hyphae without septa characterizing Peronosporomycetes were not observed by light microscopy at that time in the body of these nauplii. When dead nauplii after challenge were incubated for an additional 24 h, broad hyphae without septa were observed in the body cavity of nauplii (Fig. 2). The pathogenicity for nauplii differed greatly among species. *Halocrusticida parasitica* showed a highest cumulative mortality of above 98% at 48 h post-challenge, while the strains belonging to the genus *Lagenidium, Halocrusticida panulirata* and *Halocrusticida okinawaensis* showed moderate cumulative mortality of 8.1–41.8% at 48 h post-challenge. The strains belonging to genus *Haliphthoros* and *At. dubia* showed low cumulative mortality of below 5% at 48 h post-challenge. In control groups without challenge, the cumulative mortality rates remained lower than 1.0%.

Table 2. Pathogenicity of marine crustacean-pathogenic Peronosporomycetes in brine shrimp nauplii (n=500)

Species	Cumulative mortality rate (%)	
	24 h	48 h
Lagenidium species		
L. callinectes NJM 0531	6.1	8.1
L. thermophilum NJM 0485	0.8	13.4
L. thermophilum NJM 0493	5.2	15.6
L. thermophilum NJM 0534	23.2	25.5
Haliphthoros species		
H. milfordensis NJM 0444	0.1	2.7
H. milfordensis NJM 0487	0.0	2.2
H. sp. group 1 NJM 0443	0.0	2.0
H. sp. group 2 NJM 0440	0.0	3.7
H. sp. group 2 NJM 0449	0.6	4.9
H. sp. group 2 NJM 0535	1.4	2.1
Halocrusticida species		
H. panulirata NJM 0441	1.4	34.6
H. panulirata NJM 0445	5.9	25.0
H. panulirata NJM 0465	5.9	23.9
H. panulirata NJM 0483	5.5	41.8
H. parasitica NJM 0468	39.7	99.4
H. parasitica NJM 0533	79.9	98.7
H. okinawaensis NJM 0464	7.7	15.9
Atkinsiella species		
A. dubia NJM 0634	0.0	1.1
Controls	0.0–0.0	0.0–1.0

Discussion

The grouping and phylogenic tree inferred from the DNA sequences of ITS1 region corresponded well with the morphological identification, indicating that DNA sequencing and comparison of ITS1 can be a good tool for the identification of marine crustacean-pathogenic Peronosporomycetes.

Furthermore the DNA sequences provided taxonomically important information that was not obtained from morphological observation. The results of a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the ITS1 sequence similarity showed that *L. myophilum* shared 100% similarity with *Pythium flevoense* (AY59869, Lévesque and de Cock, 2004; AB507429, Miura *et al.*, 2010). Our previous phylogenetic study of marine invertebrate-pathogenic peronosporomycete species including *Halioticida noduliformans* based on the D1/D2 region of 28S rDNA showed that *L. myophilum* was included in a clade of the genus *Pythium* (Muraosa *et al.*, 2009). The results of phylogenetic studies using the *Cox 2* gene also were consistent

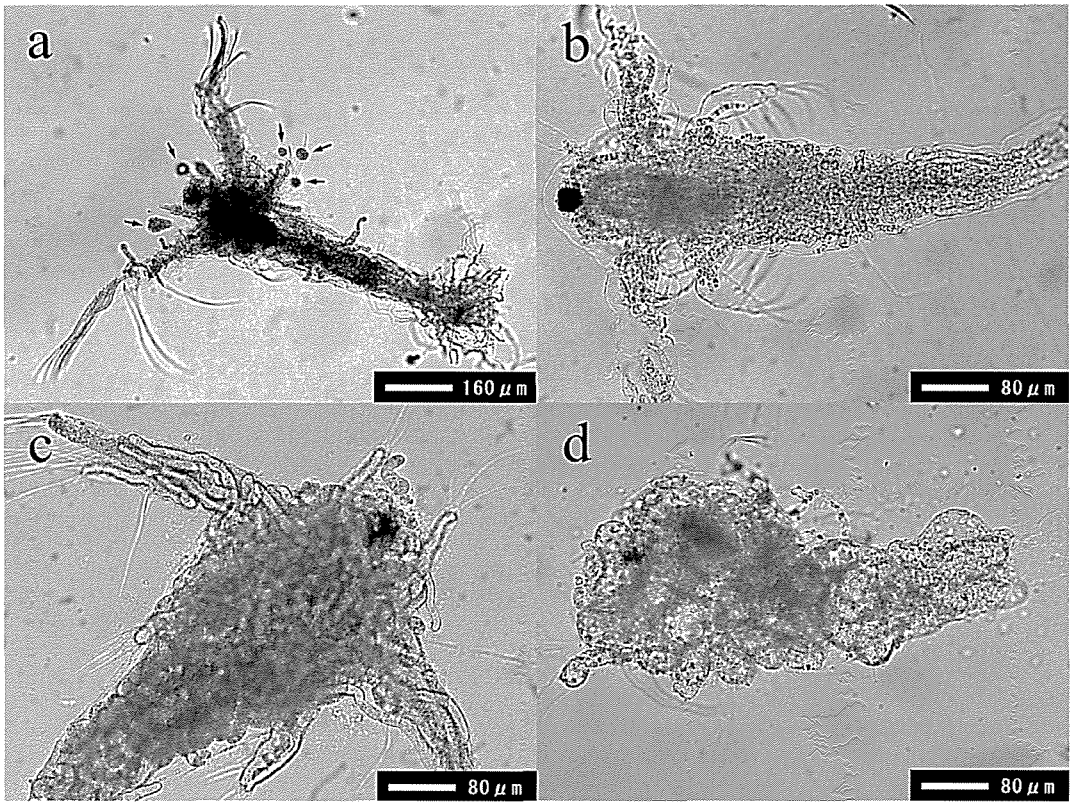

Fig. 2. Broad hyphae without septa in the body after additional 24 h incubation of dead nauplii. **a**. Nauplius infected with *L. thermophilum* NJM 0485. Vesicles formed on the surface of a nauplius (arrows). **b**. Nauplius challenged with Haliphthoros sp. group 2 NJM 0440. **c**. Nauplius challenged with *Halocrusticida parasitica* NJM 0468. **d**. Nauplius challenged with *At. dubia* NJM 0634.

with our results (Cook *et al*., 2001). It is highly probable that *L. myophilum* is synonymous with *P. flevoense*. Based on the DNA sequences, morphologically unidentified *Haliphthoros* spp. were divided into two clades: *Haliphthoros* sp. group 1 and *Haliphthoros* sp. group 2. *Haliphthoros* sp. group 1 and *Haliphthoros* sp. group 2 seemed to be two distinct species, the high intraspecific similarities within each group (100% and 97.0–100%, respectively) and the low interspecies similarities between the groups (80.6–81.6%) being considered. The 100% similarity in ITS1 sequence was shown between *Halocrusticida okinawaensis* and a reference ex-type strain of *Halocrusticida baliensis* GSM 9703. *Halocrusticida okinawaensis* and *Halocrusticida baliensis* were first reported as new species by Nakamura and Hatai (1995a) and by Hatai *et al*. (2000), respectively. Hatai *et al.* (2000) have stated that the yellowish colony pigmentation on PYGS agar only was the characteristic distinguishing *Halocrusticida baliensis* from *Halocrusticid okinawaensis* and the two species were similar to one other. Based on the molecular and morphological similarities, *Halocrusticida baliensis* may be a synonym of *Halocrusticida okinawaensis,* although further comparisons of their ex-type strains and analysis of some other genes are required to confirm their synonymity.

In the experimental challenges with different species and strains, contrary to our expectation, the cumulative mortality in the nauplii differed greatly between different peronosporomycete species, even though all the strains used had been isolated from infected eggs or larvae of marine crustaceans. The pathogenicity of marine crustacean-pathogenic peronosporomycete species may depend on host species, and the challenge experiments in nauplii of *A. salina* may not be used for evaluation of general pathogenicity of Peronosporomycetes isolated from marine crustaceans. Although the usefulness of the challenge experiments in nauplii of *A. salina* for evaluation of the pathogenicity of marine crustacean-pathogenic peronosporomycete species was not shown in the present study, and the strains belonging to *Haliphthoros* and *At. dubia* might be saprophytic, based on the low mortality (< 5%) in nauplii challenged with them.

Previous studies have investigated virulence factors of plant-pathogenic Peronosporomycetes including *Phytophthora infestans* (Morgan and Kamoun, 2007; Raffaele *et al*., 2010). In contrast, few reports are available on the virulence factors of marine crustacean-pathogenic Peronosporomycetes. For the species and strains showing clear pathogenicity for nauplii of *A. salina*, the experimental challenge to nauplii could be a useful model for further research on virulence factors

and for development of control measures against peronosporomycete infections in marine crustaceans.

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海生甲殻類から分離した病原卵菌類のITS1領域の塩基配列による同定とアルテミア孵化幼生に対する病原性

村長保憲・佐野文子・畑井喜司雄

海生甲殻類から分離した病原卵菌類27株について形態分類を行うとともに，対照とした卵菌類6種12株と合わせて，ITS1領域の塩基配列を比較した。その結果，塩基配列に基づく系統関係は形態学的分類に良く整合し，種の同定に有用であることが示された。また，9種18株の病原卵菌についてアルテミアのノウプリウス幼生に対する浸漬攻撃試験（1×10^4 zoospores/mL, 25°C）によって病原性の検討を行ったところ，死亡率は供試した卵菌類によって大きく異なった。

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感染実験からみた魚病細菌のサケ科魚類卵内感染機序

小原昌和・笠井久会・吉水　守

ニジマスおよびアマゴ卵を用いて *Flavobacterium psychrophilum*, *Renibacterium salmoninarum* および *Aeromonas salmonicida* の感染実験を行い，卵内感染機序を検討した。*F. psychrophilum* は卵の吸水時に卵門から侵入すると考えられた。*F. psychrophilum* 感染率は，汚染水吸水卵よりも卵表面汚染後に吸水させた卵で有意に高く，成立条件は 10^7 CFU/mL 以上であった。また，高濃度の *R. salmoninarum* で表面汚染した卵においても卵内感染がみられた。*F. psychrophilum* または *A. salmonicida* 汚染卵を吸水させたところ，*F. psychrophilum* は卵内侵入後に増殖したが，*A. salmonicida* は侵入後次第に消滅した。

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タイのテラピア養殖場から分離された *Aeromonas hydrophila* 多剤耐性株

N. Tipmongkolsilp・C. S. del Castillo・引間順一
T-S. Jung・近藤秀裕・廣野育生・青木　宙

タイのテラピア養殖場の感染魚より55株の *A. hydrophila* を分離し，11薬剤の最小発育阻止濃度を調べた。その結果，全ての分離株が1～8剤の組合せの耐性を示し，5剤以上の耐性菌が約半数を占めた。これらの薬剤耐性株中，1株からABPC，CP，SM, SMMXおよびTCの5剤に対して耐性を示す伝達性Rプラスミドを検出した。このプラスミドは，耐性遺伝子として *bla*OXA-35, *cat2*，*aadA1*，*sul1* および *tetA* を含んでいた。

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河川アユにおける *Edwardsiella ictaluri* 不顕性感染

E. S. Hassan・M. M. Mahmoud・河東康彦・永井崇裕
川口　修・飯田悦左・湯浅　啓・中井敏博

2008年から2010年にかけて，広島県下の1河川において *E. ictaluri* の保菌調査を実施した。アユからは本菌が高頻度で分離され，特に9月以降の保菌率は高く平均45.4%であった。アユ以外の魚種では1尾のギギから分離されたにすぎず，また菌の由来を探るべくおこなった放流アユ種苗からは本菌はまったく検出されなかった。一方，*E. ictaluri* の指標としてのファージが河川水から周年にわたって検出されたことから，本菌は河川環境に常在化し，それが河川アユへの感染源になると考えられた。

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在来マス及びアユに対する *Yersinia ruckeri* の病原性

坂井貴光・中易千早・伊東尚史・三輪　理
大迫典久・飯田貴次

ニジマス，イワナ，アマゴ及びヤマメの腹腔内に *Y. ruckeri* を 7.1×10^2 CFU/魚体重（g）接種して攻撃した。各魚種の累積死亡率は，100%，60%，30%，30%であり，すべての死亡魚がレッドマウス病の症状を示した。1.5×10^3～1.5×10^6 CFU/魚体重（g）で腹腔内接種したアユの累積死亡率は，0%～87%であった。また，浸漬攻撃でも高い累積死亡率が観察された。死亡したアユにサケ科魚類と同様のレッドマウス病の症状は見られず，眼球の突出や出血，腹水の貯留が観察された。

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ポルトガルで養殖ターボットに発生した *Streptococcus parauberis* 感染症

M. F. Ramos・J. F. Marques・J. V. Neves・T. Barandela
J. A. Sousa・A. Saraiva・P. N. Rodrigues

2004年の5月から8月にかけてポルトガル北部の1養殖場でターボット（体重90–1,020 g 水温12–14°C）に，眼球突出，背部や鰭基部の出血と浮腫，また病理組織学的には髄膜炎を特徴とする大量死亡が発生した。病魚の内臓諸器官からグラム陽性の α 溶血性球菌が分離され，それらは生化学的・血清学的性状および遺伝学的性状（16S rDNA を標的とした PCR）から *S. parauberis* に同定された。これはポルトガルにおける本菌感染症の初報告である。

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