Haliphthoros milfordensis isolated from eggs and larvae of mud crab (*Scylla tranquebarica*) in Sabah, Malaysia

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Abstract

Fungal infection occurred during mud crab spawning in a hatchery and caused almost 100% mortality in mud crab larvae after 5 days post-hatching. A fungus was isolated from eggs and larvae using PYGS agar and named IPMB 1603. During morphological identification, the strain IPMB 1603 was observed to produce a fragment inside the hyphae and the zoospores swam away from the tips of the discharge tube on several occasions, this closely resembling the genus *Haliphthoros*. Following comparisons made on the nucleotide sequence of the ITS1 region, the strain IPMB 1603 was identified belonging to a cluster of *Haliphthoros milfordensis* sharing 97-100% similarity. In this study, the strain IPMB 1603 was found to be an euryhaline fungus as the strain was able to grow on PYG agar containing NaCl. Strain IPMB 1603 only grew at certain salinity ranges, in which on PYGS agar contained seawater higher than 10 ppt. The optimum temperature range for fungal growth of strain IPMB 1603 was 30-35ºC. Different growth rates were demonstrated at pH ranging from 4 to 9, but optimum growth was observed at pH 6 to 8. This is the first record of *Haliphthoros milfordensis* infection in Malaysia.

Introduction

Mud crabs belonging to the genus *Scylla* spp. are known as an important fisheries species in Southeast Asia. The production of mud crab mainly relies on wild caught seed stock but currently market demand is higher than supply. Increasing global aquaculture production of mud crab is an alternative approach to support high market demand. Lately, China and Vietnam have become main contributors for crab production (Shelley, 2008). Consequently, disease outbreaks such as bacterial and fungal infections are the major threat in mud crab aquaculture where each outbreak has caused huge loss in production of mud crab (Jithendran et al., 2010). Previous studies have reported that marine oomycetes infections caused mass mortality in early life stages of mud crab (Lee et al., 2016). *Lagenedium* and *Haliphthoros* have been reported as important fungal pathogens in marine crustaceans and shellfish (Hatai, 2012). *Haliphthoros milfordensis* was reported as an endoparasite of eggs of the oyster, *Urosalpinx cinerea* (Vishniac, 1958). It was also isolated from juveniles of the American lobster, *Homarus americanus* (Fisher et al., 1975), adults of white shrimp, *Penaeus*

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setiferus (Tharp and Bland, 1977), and some marine algae (Fuller et al., 1964). In 2016, a fungal infection occurred in a hatchery during mud crab spawning and this caused almost 100% mortality in eggs and larvae. A strain IPMB 1603 was isolated from dead egg and larva. This paper describes the morphological identification, molecular analysis and biological characteristics of the isolated strain IPMB 1603.

Materials and Methods

Isolation

Mud crab, *S. tranquebarica* eggs and larvae were collected in a culture tank from a shrimp hatchery in Sabah, Malaysia. These eggs and larvae were examined using an Olympus CKX 41 microscope and selected for isolation if discharge tubes were seen. The selected egg and larvae were then inoculated onto PYGS agar (1.25g Bacto peptone, 1.25g Bacto yeast extract, 3g glucose, 12g agar and 35g/L artificial seawater). Streptomycin sulphate and ampicillin were added on top of the inoculated specimens to prevent bacteria growth. Whitish fungal colonies grew around the inoculated eggs and larvae after 5-7 days incubation. To yield a pure culture, the edge of a fungal colony was excised and inoculated onto fresh PYGS agar.

Morphological identification

A block from a fungal colony was cut and inoculated into PYGS broth, then incubated at 25°C for 3 days. White thread-like mycelia formed in the PYGS broth were rinsed three times with artificial seawater. Rinsed mycelia were transferred to new artificial seawater to induce zoospore production. After 24 h, the manner of zoospores production was observed using an Olympus CKX 41 microscope and identified according to methods of Sparrow (1960) and Karling (1981).

Phylogenetic analysis

An agar block of the isolated strain was cut and cultured in PYGS broth at 25ºC for 5 days. The mycelia formed was collected and washed three times with phosphate-buffered saline solution before being stored at -85ºC. Next, the mycelia were homogenised by Micro Smash ™ (Tomy Seiko Co., Ltd) with the addition of zirconia beads. Total genomic DNA was extracted using NucleoSpin Plant II (Macerey-Nagel GmbH & Co.) following the manufacturer's protocol. The ITS region of the isolated strain was amplified by PCR with the primes ITS4 and ITS5 (White et al., 1990). PCR analysis was performed using GoTaq DNA polymerase (Promega) with the following amplication conditions: 94°C for 1 min, followed by 30 cycles of 94°C for 30s, 55°C for 30s, 72°C for 1 min, and a final cycle of 72°C for 5 min. The resulting amplicon was purified using a NucleoSpin Gel and PCR Clean-up (Macerey-Nagel GmbH & Co.) according to manufacturer's protocol. The purified amplicon was sequenced using a BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific Inc.) and an automated DNA sequencer (ABI PRISM 3730 DNA analyser: Thermo Fisher Scientific), with both procedures performed by FASMAC Co. LTD., Japan. Finally, the resulting data was applied to generate a phylogenetic tree using the neighbour-joining method.

Effect of concentration of sodium chloride and potassium chloride on fungal growth

PYG agar (1.25g peptone, 1.25g yeast extract, 3g glucose, 12g agar) containing NaCl or KCl at different concentrations (0, 0.5, 1, 2, 3%) were prepared. Strain IPMB 1603 was cultured in advance on PYGS agar plate for 2 weeks to produce a large colony. Agar blocks were cut from the edge of the large colony using a No.

2 cork borer (5 mm diameter) and each was inoculated on a prepared PYG agar plate. Fungal growth was examined by measurement of the colony diameter at 3, 5, 7 and 10 days.

Effect of concentration of artificial seawater on fungal growth

The salinity of artificial seawater was prepared as 35g/L and adjusted to 30ppt. PYGS agar at different salinities (0, 5, 10, 15, 20 and 30ppt) were prepared by diluting with distilled water. An agar block was cut from theedge of a large colony cultured in advance and inoculated onto prepared agar plates. Fungal growth was determined as described above.

Effects of temperature on fungal growth

An agar block was removed from the edge of a large colony cultured in advance and inoculated onto PYGS agar plates, and then incubated at seven different temperatures (5, 10, 15, 20, 25, 30 and 35ºC). Fungal growth was determined as described above.

Effects of pH on fungal growth

The PYGS broth (without supplemented agar) were prepared and adjusted to pH (3, 4, 5, 6, 7, 8, 9, 10 and 11) by the addition of 1N solutions of HCl or NaOH. Each medium (5mL) was poured into the 6 well plate under sterile conditions and an agar block of strain IPMB 1603 was inoculated into each well, then incubated at 25°C for 10 days. The mycelial growth in each pH was evaluated by visual comparison of the number of mycelia grown around the agar block with control (pH 7). The results from pH adjustment were recorded as (-) no growth, (+) weak growth, (++) moderate growth and (+++) abundant growth.

Results

Isolation

Eggs and larvae were highly infected with fungus, reaching almost 100% mortality after 5 days post-hatching. Discharge tubes without vesicles were observed in the dead eggs and larvae. A few colonies were developed from the infected eggs and larvae and these colonies appeared to be the same species. Therefore, strain IPMB 1603 was selected randomly and used for this study.

Morphology

Strain IPMB 1603 grew as a whitish yellow colony on PYGS agar and the colony reached 4.8cm in diameter after incubation at 25ºC for 2 weeks. Vegetative hyphae were grown as branches with numerous vacuoles (Figure 1) in PYGS broth and measured 13.5-39.5μm in width. After being transferred to artificial seawater, the protoplasm within the hyphae was constricted and formed a fragment. The fragments developing within hyphae varied in size and shape, ranging from 13-23μm in diameter and 23-75μm in length (Figure 2). Individual zoospores divided in the zoosporangium and fragment and eventually released through tips of the discharge tube. Discharge tubes developing from each fragment were slightly curved and ranged from 131.5-603μm in length. The zoospores appeared arranged in rows inside the discharge tube before they released from the tips.

Phylogenic analysis

The nucleotide sequence containing the ITS1-5.8S-ITS2 region of rRNA gene for strain IPMB 1603 was analysed. The data obtained from the ITS1 region were used to construct a phylogenic tree of marine oomycetes, including the strain IPMB 1603 (Figure 3). The intraspecific similar-

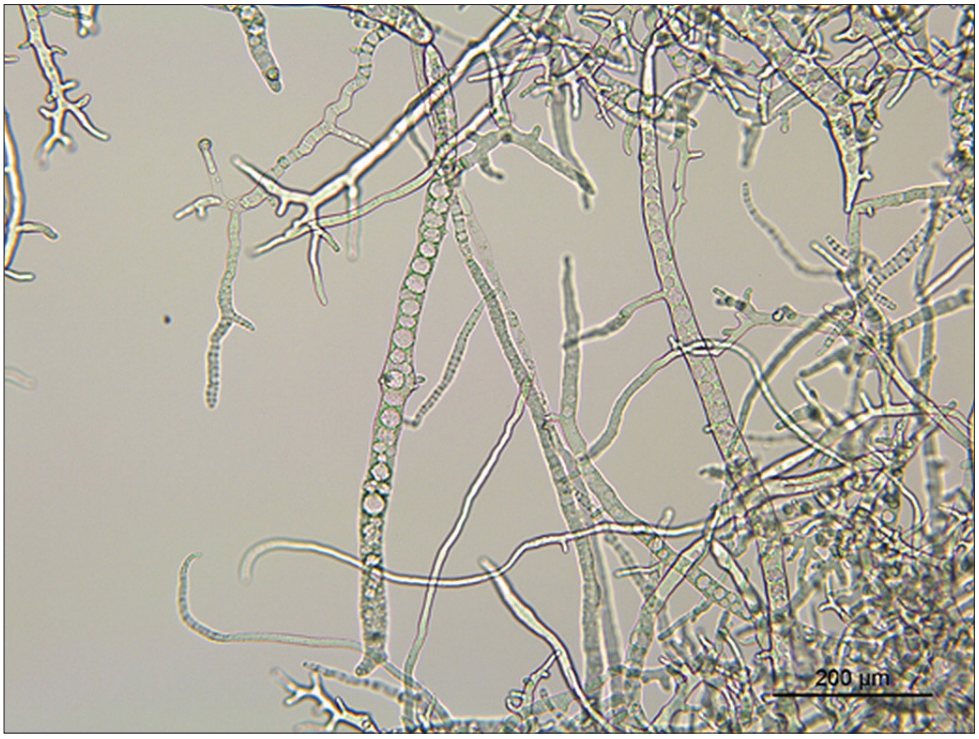

Figure 1. Vacuole production observed in the hyphae of the strain IPMB 1603 when incubated in PYGS broth at 25°C for 3 days.

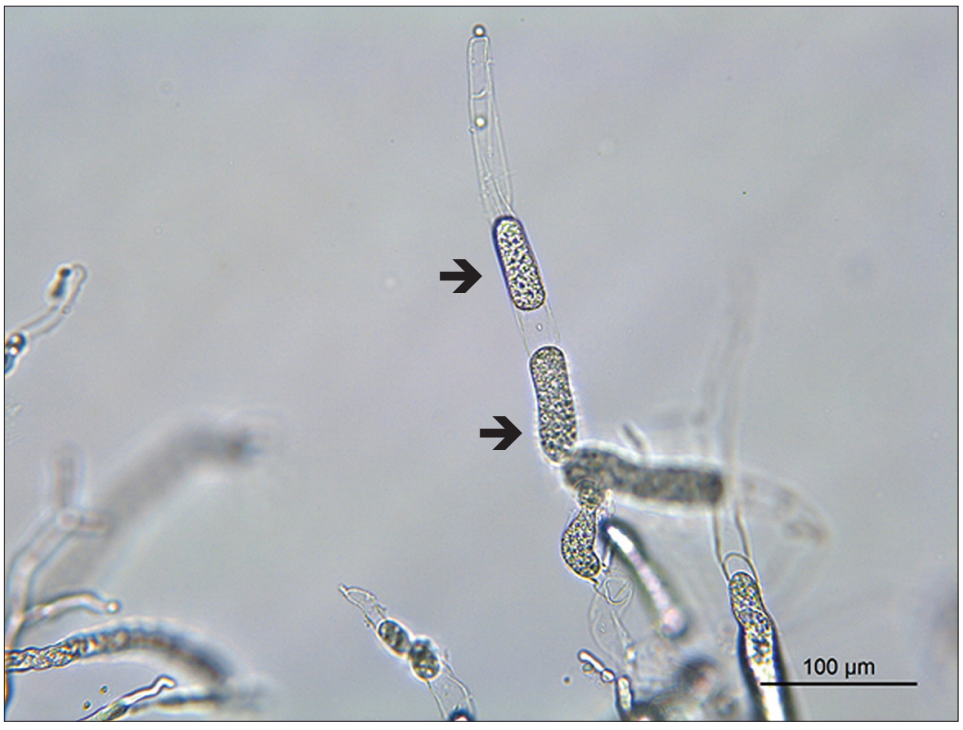

Figure 2. Fragment production (arrows) observed within the hyphae of the strain IPMB 1603 in the artificial seawater.

ity between strain IPMB 1603 and *Haliphthoros milfordensis* was 97–100%, indicating that the isolate (IPMB 1603) belongs in *H. milfordensis*. Subsequently, the sequence ID of *H. milfordensis* IPMB 1603 was registered in GenBank under accession number LC274624.

Effects of concentration of NaCl and KCl on fungal growth

Strain IPMB 1603 showed moderate growth on PYG agar supplemented with various concentrations of NaCl (Table 1) or KCl (Table 2) with PYGS agar (control) as a comparison. While, in contrast strain IPMB 1603 did not grow on PYG agar containing distilled water.

Effect of concentration of artificial seawater on fungal growth

Strain IPMB 1603 grew on PYGS agar at salinities of 10, 15, 20 and 30ppt (Table 3). The fungus did not grow at salinities less than 5 ppt.

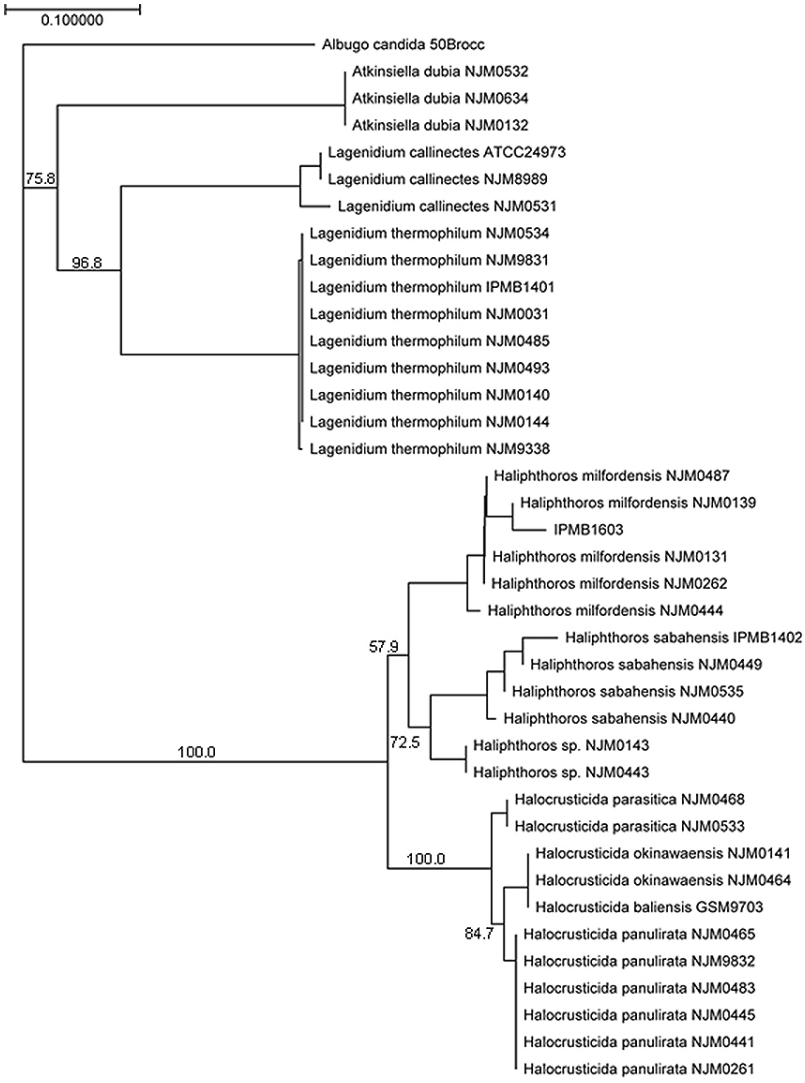

Figure 3. Phylogenetic tree of marine oomycetes, including the strain IPMB 1603, based on the nucleotide sequence of the ITS1 of rRNA gene region. The tree was rooted using Albugo candida 50 Brocc as the outgroup. The numbers indicate the occurrence of nodes during bootstrap analysis given as percentage of 1 000 reiterations.

Table 1. Effects of NaCl on hyphal growth of the strain IPMB 1603.

NaCl (%)	Days after incubation on PYG agar			
	3	**5**	**7**	**10**
0	-*	-	-	-
0.5	0.38**	0.78	1.18	1.65
1	0.80	1.65	2.25	3.08
2	0.95	1.88	2.68	3.68
3	0.43	1.08	1.75	2.63
PYGS agar	1.9	2.4	3.7	4.2

* no growth
** colony diameter in cm

Table 2. Effects of KCl on hyphal growth of the strain IPMB 1603.

KCl (%)	Days after incubation on PYG agar			
	3	**5**	**7**	**10**
0	-*	-	-	-
0.5	0.1**	0.1	0.1	0.1
1	0.35	0.85	1.33	2.03
2	0.53	1.33	2.08	2.68
3	0.43	1.28	1.7	2.53
PYGS agar	1.9	2.4	3.7	4.2

* no growth
** colony diameter in cm

Table 3. Effects of concentration of artificial seawater on hyphal growth of the strain IPMB 1603.

Salinity	Days after incubation on PYG agar			
	3	**5**	**7**	**10**
0	-*	-	-	-
5	-	-	-	-
10	0.6**	1.0	1.1	1.3
15	0.8	1.5	2.0	2.5
20	1.7	2.2	3.5	3.9
PYGS agar	1.9	2.4	3.7	4.2

* no growth
** colony diameter in cm

Effects of temperature on fungal growth

Strain IPMB 1603 grew rapid at 25 to 35ºC but slowly at 10ºC. There was only a slight increase in colony diameter at 15 and 20 ºC, whereas fungal growth was hardly observed at 5 ºC (Table 4).

Effects of pH on fungal growth

Strain IPMB 1603 grew well at pH 6 to 8 and moderate growth was observed at pH 4, 5 and 9. No mycelial growth was observed at pH 3, 10 and 11 (Table 5).

Table 4. Effects of temperature on the hyphal growth of strain IPMB 1603.

	Days after incubation on PYG agar			
Temperature (ºC)	**3**	**5**	**7**	**10**
5	-*	-	-	-
10	-	0.6	0.8	0.9
15	0.6**	0.8	1.1	1.1
20	1.3	1.7	2.7	3.6
25	1.9	2.4	3.7	4.2
30	2.8	4.3	5.5	7.2
35	2.9	4.5	5.5	7.4

* no growth
** colony diameter in cm

Table 5. Effects of pH on hyphal growth of the strain IPMB 1603.

	Days after incubation			
pH	**3**	**5**	**7**	**10**
3	-	-	-	-
4		+	+	+
5	+	+	++	++
6	++	++	++	++
7	++	++	+++	+++
8	++	++	++	++
9		+	+	+
10	-	-	-	-
11	-	-	-	-

Symbols: -: no growth, +, ++, +++ increasing amount of growth from weak to abundant.

Discussion

The genus *Haliphthoros* belongs to the family Haliphthraceae. At present, three species have been documented in the genus *Haliphthoros*: *H. milfordensis* Vishniac, *H. philippinensis* Hatai *et al*. and *H. sabahensis* Lee *et al*. *Haliphthoros* spp. differentiated themselves with other lower fungi based on their unique characteristics, in which fragment formation was found inside the hyphae. Strain IPMB 1603 was classified as a member of the genus *Haliphthoros* where the fragments were observed inside the hyphae. Zoospores of the strain IPMB 1603 swam away through the tips of the discharge tube, this feature is similar to that of *H. sabahensis* (Lee et al., 2017) and *H. milfordernsis* (Hatai, 1982) but differs from *H. philippinenesis*. Zoospores of *H. phillippinensis* are not only released from the tips of discharge tubes but also through the opening of fragment (Hatai et al., 1980). Recently, comparisons of DNA sequence have become important tools for pathogenic oomycetes identification (Muraosa et al., 2012). The ITS nucleotide sequence of the strain IPMB 1603 was compared with those of the other pathogenic *Haliphthoros* strains isolated from crustaceans. Based on the ITS1 nucleotide sequence, IPMB 1603 was classified to the same cluster as that of *H. milfordensis* and showed high similarity (97-100%). As a result, IPMB 1603 was identified as *H. milfordensis* based on the morphology and molecular similarities. The strain IPMB 1603 was characterised as an euryhaline fungus, where no growth was showed on NaCl free medium but fungal growth were observed on PYG agar containing NaCl. This result is similar with *H. philippinensis* and some strains of *H. milfordensis* but differed to that of *H. sabahensis* (Hatai et al., 1980; Nakamura and Hatai, 1995; Lee et al., 2017). *H. sabahensis* was concluded to be of obligatory marine because these fungi only grew on PYGS agar (Lee et al., 2017). This study demonstrated that strain IPMB 1603 shared similar growth environmental conditions with other fungi in the genus *Haliphthoros* in which fungal growth was observed only on PYGS agar at high salinities. The optimum temperature for strain IPMB 1603 was 30-35ºC, this result is similar with *H. philippinensis* (Hatai et al., 1980) and *H. sabahensis* (Lee et al., 2017) in which both species tolerated high temperatures. In contrast, *H. milfordensis* NJM 8977 appears to show optimum growth at a lower temperature range from 20-25ºC (Hatai et al., 1992). The present strain IPMB 1603, *H. philippinensis* and *H. sabahensis* were able to survive at temperatures more than 30ºC more likely because they were isolated from tropical countries. Therefore, it is believed that survival temperature of fungi highly depends on the host habitat. The strain IPMB 1603 was able to tolerate a range of pH 4 to 9, this finding was similar with *H. sabahensis*. However, other strains of *Haliphthoros* showed growth at high alkaline conditions up to pH 10 or 11. (Hatai, 1982; Chukanhom et al., 2003). In conclusion, we identified the strain IPMB 1603 as *Haliphthoros milfordensis* and studied the biological characteristics that will contribute to future investigations on prevention of fungal infection in mud crab aquaculture.

References

Fisher WS, Nilson EH and Shleser RS (1975). Effect of fungus *Haliphthoros milfordensis* on the juvenile stages of the American lobster, *Homarus americanus*. *Journal Invertebrate Pathology* **26**, 41-45.

Fuller MS, Fowles, BE and Mc Laguhlin D J (1964). Isolation and pure culture study of marine phycomycetes. *Mycologia* **56**, 745-756.

Chukanhom K, Borisutpeth P, Khoa LV and Hatai K (2003). *Haliphthoros milfordensis* isolated from black tiger prawn larvae (*Penaeus monodon*) in Vietnam. *Mycoscience* **44**(2), 123-127.

Hatai K (2012). Diseases of fish and shellfish caused by marine fungi in 'Biology of Marine Fungi', pp 16-52. Springer.

Hatai K, Bian BZ, Baticados MCL and Egusa S (1980). Studies on the fungal diseases in crustaceans. ii. *Haliphthoros philippinensis* sp. nov. isolated from cultivated larvae of the jumbo tiger prawn (*Penaeus monodon*). *Transaction Mycological Society of Japan* **21**, 47-55.

Hatai K (1982). On the fungus *Haliphthoros milfordensis* isolated from temporarily held abalone (*Haliotis sieboldii*). *Fish Pathology* **17**, 199-204.

Hatai K, Rhoobunjongde W and Wada S (1992). *Haliphthoros milfordensis* isolated from gills of juvenile kuruma prawn (*Penaeus japonicus*). *Transaction Mycological Society of Japan* **33**, 185-192.

Jithendran KP, Poornima M, Balasubramanian CP and Kulasekarapapandia S (2010). Diseases of mud crabs (*Scylla* spp.): An Overview. *Indian Journal of Fisheries* **57**(3), 55-63.

Karling JS (1981). Predominantly holocarpic and eucarpic simple biflagellate phycomycetes. J. Cramer, pp 225.

Lee YN, Hatai K and Kurata O (2016). First report of *Lagenidium thermophilum* isolated from eggs and larvae of mud crab *Scylla tranquebarica* in Sabah, Malaysia. *Bulletin of the European Association of Fish Pathologists* **36**(3), 111-117.

Lee YN, Hatai K and Kurata O (2017). *Haliphthoros sabahensis* sp. nov. isolated from mud crab *Scylla tranquebarica* eggs and larvae in Malaysia. *Fish Pathology* **52**(1), 31-37.

Muraosa Y, Sano A and Hatai K (2012). Molecular identification of marine crustacean-pathogenic peronosporomycetes using DNA sequences of ITS 1 and their pathogenicity for nauplii of brine shripms. *Fish Pathology* **47**(2), 41–48.

Nakamura K and Hatai K (1995). Three species of Lagenidiales isolated from the eggs and zoeae of the marine crab *Portunus pelagicus*. *Mycoscience* **36**, 87–95.

Shelley C (2008). Capture-based aquaculture of mud crabs (*Scylla* spp.) 'Capture-based aquaculture global overview'. *FAO Fisheries Technical Paper* **58**, 255–269.

Sparrow FK (1960) Aquatic phacomycetes. 2nd revised Edition. University of Michigan Press. Ann Arbor, Michigan, pp1187.

Tharp TP and Bland CE (1977). Biology and host range of *Haliphthoros milfordensis*. *Canadian Journal of Botany* **55**, 2936-2944.

Vishniac HS (1958). A new marine phycomycete. *Mycologia* **50**, 66-79.

White TJ, Bruns S, Lee S and Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications*.