Research Article

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Diversity of fungi isolated from carapace and gut of the marine crab *Portunus sanguinolentus* in northern waters of Taiwan

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Abstract: The fungal community associated with marine crabs is poorly known, except for the fungi causing diseases of marine animals of economic value. In this study we examined the diversity of fungi cultured from carapace and gut of the marine crab *Portunus sanguinolentus*, and the identification was based on nucleotide BLAST search results of the internal transcribed spacers of rDNA (ITS). A total of 256 fungal isolates representing 23 species were cultured from seven individuals of *P. sanguinolentus* including two unidentified species. The majority of the species belong to the Ascomycota, while three species of the Basidiomycota were isolated from the gut. Overall, *Candida tropicalis* (45.70 %, percentage occurrence), *Apiotrichum lignicola* (8.98 %) and *Rhodotorula* sp. (8.20 %) were the dominant fungi on the crab. The most dominant fungi on the carapace were *C. tropicalis* (66.95 %), *Emericellopsis maritima* (8.47 %), *A. lignicola* and *Purpureocillium lilacinum* (both 4.24 %). In the gut, *C. tropicalis* (27.54 %), *A. lignicola* (16.67 %), *Rhodotorula* sp. (15.22 %) and *Fusarium solani* (14.49 %) were dominant. The fungal diversity in the gut of *P. sanguinolentus* was higher than on the carapace according to the diversity indices. Although some of the isolated fungi were reported to be pathogenic, none were reported as pathogens of crabs, and no disease symptoms were noticed from the crab samples.

Keywords: Crustacea; Decapoda; epibiont/endobiont; marine fungi; Saccharomycetales

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1 Introduction

Compared to the diversity of fungi in the terrestrial environment, fungi are comparatively less known from the marine environment. Marine fungi have been mainly reported from coastal habitats, where organic substrata, such as wood, macroalgae, seagrasses and marsh grasses, are abundant for growth. Most of the 1857 marine fungi listed in marinefungi.org, are saprobic fungi of lignocellulosic materials belonging to diverse lineages (769 genera, 226 families, 88 orders, 22 classes, 7 phyla) (Jones et al. 2019), although fungi associated with marine animals were included (Pang et al. 2021).

Animal hosts of pathogenic marine fungi were mainly Chordata (100 species, 51.8 %), followed by the Arthropoda (68 species, 35.2 %) (Pang et al. 2021). Some fungi associated with the Crustacea were reported to be pathogenic, infecting a variety of shrimps and crabs (Pang et al. 2021). *Fusarium* species are the primary causes of black gill disease causing the gills of prawns and lobsters to form black spots eventually leading to mortality (Lightner and Fontaine 1975; Nha et al. 2009; Rhoobunjongde et al. 1991). *Exophiala cancerae* and *Fonsecaea brasiliensis* caused lethargic crab disease of the mangrove land crab *Ucides cordatus* in Brazil, infesting various internal organs of the crab leading to eventual death (Boeger et al. 2007; Vicente et al. 2012). *Trichomaris invadens* formed black crust on the carapace of the Alaska marine snow crab *Chionoecetes bairdi* and its hyphae invaded internal tissues following penetration of the carapace (Hibbits et al. 1981). Many external and internal tissues/organs were infected, including epidermis, subepidermal layers, muscles, blood vessels, hemopoietic tissue, gastrointestinal tract wall, wall of the heart, eyestalk, cephalothorax, retina and gill (Sparks 1982). Recently, Pang et al. (2019) and Shaumi et al. (2021) isolated 26 species of fungi from the marine hydrothermal vent crab *Xenograpsus testudinatus*, and species of *Aspergillus* were dominant (*A. clavatus*, *A. penicillioides*, *A. sydowii*, *A. terreus*, *Aspergillus* sp., *A. unguis*, *A. versicolor*). However, the possible roles of these fungi on this crab were unknown but assumed to be not pathogenic.

Portunus sanguinolentus or three-spot swimming crab belonging to the family Portunidae is one of the main catches in the crab fishery off northern marine waters of Taiwan (Hsueh et al. 2006). The last pair of legs is paddle-shaped which is characteristic of the Portunidae. This crab is mostly found in sandy to muddy bottoms of the sea and also has higher mobility than other crabs (Sumpton et al. 1989). Previously, eggs and zoeae of other portunid crabs *Portunus pelagicus* and *P. trituberculatus* were reported to be infected by three species of the oomycetous order Lagenidiales (Hamasaki and Hatai 1993; Nakamura and Hatai 1995), but no information is available on the mycota associated with this crab. In this study we examined the culturable diversity of fungi isolated from the carapace and gut of *Portunus sanguinolentus* based on nucleotide BLAST search results of the internal transcribed spacers of rDNA (ITS).

2 Materials and methods

2.1 Study site and sampling

Crabs (seven individuals) of *Portunus sanguinolentus* (Figure 1) were collected by a rectangular cage trap from the waters of northern Taiwan (121°85′E, 25°21′N) on 22 January 2020. The crabs were immediately put into sterile ziplock plastic bags on board, placed in a cool box during transportation to the laboratory, and kept at 4 °C before isolation (time from collection to isolation <12 h).

2.2 Isolation and identification of fungi

The crabs were initially rinsed with 0.1 % of Tween 80 (Sigma-Aldrich, Saint Louise, USA) in sterile natural seawater for 1 min and three times with sterile natural seawater. A flame-sterilized spatula was used to scrape the surface of the carapace and a pair of flame-sterilized scissors was used to dissect the gut. The resulted biofilm from the carapace and the content of the gut were separately suspended in 1 mL of sterile seawater. One hundred microliters of both suspensions were spread-plated (triplicate plates) onto two media: (1) marine agar (MA) (Himedia, Dindhori, Nashik, India), and (2) glucose-yeast extract-peptone seawater agar (GYPS; 1 % glucose [Bioshop, Burlinton, Canada], 1 % yeast extract [Oxoid, Basingstoke, UK], 1 % peptone [Oxoid, Basingstoke, UK], 15 g l^{-1} agar technical [Bioshop, Burlington, Canada] in natural seawater) supplemented with 0.5 g l^{-1} each of Penicillin G sodium salt (Bioshop, Burlington, Canada) and streptomycin sulfate (Bioshop, Burlington, Canada). The plates were incubated at 25 °C, and checked for fungal growth for up to one month. Fungi appeared on these plates were sub-cultured as pure cultures onto cornmeal agar (Himedia, Dindhori, Nashik, India) made with natural seawater (CMAS). These fungal isolates were grouped into colony morphotypes.

Figure 1: The marine crab *Portunus sanguinolentus* collected from northern waters of Taiwan.

2.3 Molecular analysis

Mycelia on CMAS were transferred to a mortar and pestle, and ground into fine powder in liquid nitrogen. Total genomic DNA were extracted using the DNeasy Plant DNA Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. ITS was amplified by polymerase chain reaction (PCR) using the primer sets ITS4 (5′-TCCTCCGCTTATTGATATGC-3′)/ ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White et al. 1990). PCR reactions were performed in 25 µl volumes containing 1 µl genomic DNA, 0.2 µM of each primer, 12.5 µl Gran Turismo PreMix (Ten Giga BioTech, Taiwan) and topped up with PCR water. The amplification cycle consisted of an initial denaturation step of 95 °C for 2 min, followed by 35 cycles of (a) denaturation (95 °C for 30 s), (b) annealing (54 °C for 30 s) and (c) elongation (72 °C for 30 s) and a final 10-min elongation step at 72 °C. The PCR products were analyzed by agarose gel electrophoresis (Bioman Scientific, New Taipei City, Taiwan), and sent to Genomics (New Taipei City, Taiwan) for sequencing with the same PCR primers. The sequences obtained were checked for ambiguity, assembled and submitted to the National Center for Biotechnology Information (NCBI) for a nucleotide BLAST search. The sequences generated in this study are deposited in GenBank and their accession numbers can be found in Supplementary Table S1.

2.4 Graphical and statistical analyses

A Krona Chart illustrating composition of fungal communities identified from the carapace and gut samples of *Portunus sanguinolentus* was prepared using the Krona Tools (https://github.com/marbl/Krona/wiki/KronaTools) (Ondov et al. 2011). The Shannon-Wiener (*H′*) (Shannon and Weaver 1949), species richness measured by the Margalef index (Margalef 1968), Pielou's evenness index (*J′*) (Pielou 1966) and Simpson's dominance index (*D*) (Simpson 1949) were carried out using the equation setting function in Microsoft Excel. FUNGuild analysis was performed at http://www.funguild.org/ (Nguyen et al. 2016).

3 Results

A total of 256 isolates of fungi were cultured from seven individuals of *Portunus sanguinolentus*: 118 from the

carapace and 138 from the gut. These isolates were grouped into colony morphotypes, and identified based on the BLASTn search results of the ITS rDNA in NCBI with the highest score, sequence coverage and similarity (Supplementary Table S1). These isolates represented 17 genera and 23 species with two unidentified species (Sordariomycetes sp., Sporormiaceae sp.), which belonged to the Ascomycota (20 species) and the Basidiomycota (three species) (Table 1, Figure 2). The three basidiomycetes were isolated from the gut. Eleven species were isolated from the carapace while 13 species were cultured from the gut. *Candida tropicalis* was the only common species isolated from the carapace and the gut.

Saccharomycetes (48 %, percentage occurrence), Sordariomycetes (23.8 %) and Microbotyomycetes (12 %) were the dominant classes on *P. sanguinolentus* (Figure 2). At the order level, Saccharomycetales (48 %), Hypocreales (16.6 %) and Sporidiobolales (12 %) constituted the highest percentage occurrence. Two yeast genera *Candida* (48 %) and *Rhodotorula* (12 %) were dominant on the crab while the other genera were below 9 %.

Differences in the diversity of fungi were observed between the carapace and the gut samples of *P. sanguinolentus* (Tables 1 and 2). A higher number of fungal isolates was cultured from the gut (138) than from the carapace (118). The gut samples (Shannon-Wiener Diversity Index = 1.91, Simpson's Index of Diversity = 0.84, Pielou's Evenness = 0.74, Margalef's = 4.03) had higher diversity, evenness and species richness indices than the carapace samples (1.42, 0.53, 0.59, 3.54, respectively; Table 2). The most dominant fungi of the carapace based on percentage occurrence were *C. tropicalis* (66.95 %), *Emericellopsis maritima* (8.47 %), *Apiotrichum lignicola* and *Purpureocillium lilacinum* (both 4.24 %). In the gut, the most dominant fungi were *C. tropicalis* (27.54 %), *A. lignicola* (16.67 %), *Rhodotorula* sp. (15.22 %) and *Fusarium solani* (14.49 %). Overall, *C. tropicalis* (45.70 %), *A. lignicola* (8.98 %) and *Rhodotorula* sp. (8.20 %) were the dominant fungi on the crab.

With the available data in the FUNGuild database for the fungal taxa isolated from *P. sanguinolentus*, a majority of taxa is reported to be pathotrophic (plant or human pathogens), especially in the gut samples Table 3. None of the fungi were reported as pathogens of crabs, and no disease symptoms were noticed in the crab samples.

Table 1: Fungal diversity on carapace and in gut of the marine crab *Portunus sanguinolentus*.

Taxa	Number (% occurrence) of isolates		Overall number (% occurrence) of isolates
	Carapace	Gut	
Acremonium egyptiacum (J.F.H. Beyma) W. Gams	3 (2.54)	–	3 (1.17)
Apiotrichum lignicola (Diddens) Yurkov *et* Boekhout	–	23 (16.67)	23 (8.98)
Candida parapsilosis (Ashford) Langeron *et* Talice	5 (4.24)	–	5 (1.95)
Candida tropicalis Berkhout	79 (66.94)	38 (27.54)	117 (45.70)
Cladosporium tenuissimum Cooke	–	3 (2.17)	3 (1.17)
Cyphellophora olivacea (W. Gams) Réblová *et* Unter.	1 (0.85)	–	1 (0.39)
Emericellopsis maritima Beliakova	10 (8.47)	–	10 (3.91)
Exophiala oligosperma Calandron *ex* de Hoog *et* Tintelnot	4 (3.39)	–	4 (1.56)
Fusarium solani Sacc.	–	20 (14.49)	20 (7.81)
Gibellulopsis nigrescens (Pethybr.) Zare, W. Gams *et* Summerb.	4 (3.39)	–	4 (1.56)
Hortaea werneckii (Horta) Nishim. *et* Miyaji	–	6 (4.35)	6 (2.34)
Parengyodontium album (Limber) C.C. Tsang, J.F.W. Chan, W.M. Pong, J.H.K. Chen, A.H.Y. Ngan, M. Cheung, C.K.C. Lai, D.N.C. Tsang, S.K.P. Lau *et* P.C.Y. Woo	–	1 (0.72)	1 (0.39)
Penicillium citrinum Thom	4 (3.39)	–	4 (1.56)
Penicillium griseofulvum Dierckx	2 (1.69)	–	2 (0.78)
Peroneutypa scoparia (Schwein.) Carmarán *et* A.I. Romero	–	2 (1.45)	2 (0.78)
Purpureocillium lilacinum (Thom) Luangsa-ard, Houbraken, Hywel-Jones *et* Samson	5 (4.24)	–	5 (1.95)
Rhodotorula mucilaginosa (A. Jörg.) F.C. Harrison	–	11 (7.97)	11 (4.30)
Rhodotorula sp.	–	21 (15.22)	21 (8.20)
Sordariomycetes sp.	–	3 (2.17)	3 (1.17)
Sporormiaceae sp.	1 (0.85)	–	1 (0.39)
Talaromyces sp.	–	6 (4.35)	6 (2.34)
Trichoderma atroviride Bissett	–	1 (0.72)	1 (0.39)
Trichoderma lixii (Pat.) P. Chaverri	–	3 (2.17)	3 (1.17)
Total no. of isolates (total abundance), *N*	118	138	256
Richness (total number of taxa in the community), *S*	11	13	23

4 Discussion

Fungal diversity associated with marine crabs is poorly understood while the available published reports were related to those causing diseases (Pang et al. 2021). In this study, a total 23 fungal species were cultured from the gut and carapace of seven individuals of *Portunus sanguinolentus*. Using the same isolation method, 12 species were cultured from the carapace of the marine crab *Xenograpsus testudinatus* (Shaumi et al. 2021). The fungal species common

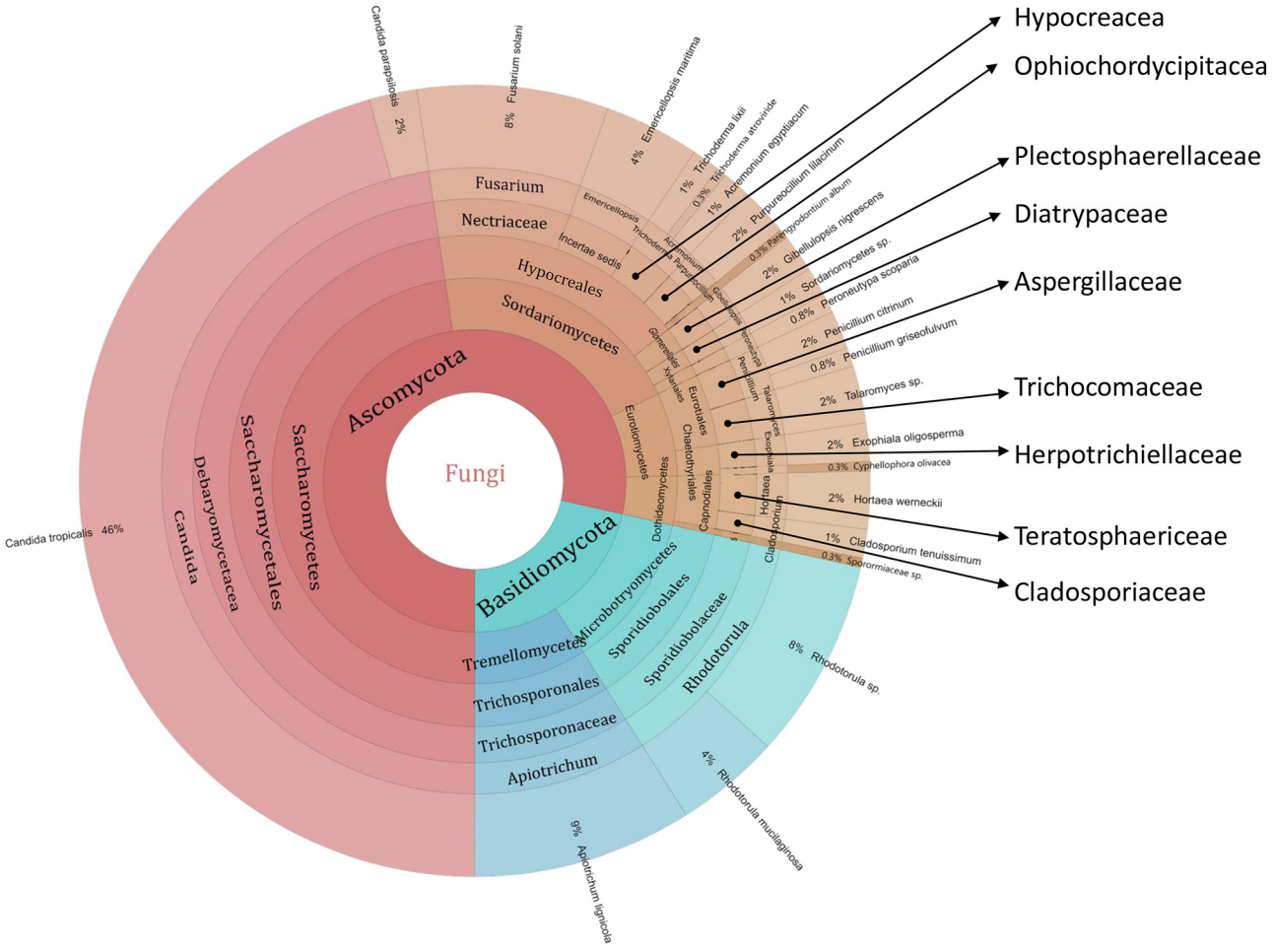

Figure 2: A Krona chart showing taxonomic classification of fungi isolated from the carapace and gut of the marine crab *Portunus sanguinolentus*.

to the two marine crabs include *Candida parapsilosis*, *Penicillium citrinum*, *Hortaea werneckii*, *Peroneutypa scoparia* and *Parengyodontium album*, although the latter three species were isolated from the gut in *P. sanguinolentus*. *Aspergillus* was the most speciose genus on *X. testudinatus*, but no *Aspergillus* species was isolated from *P. sanguinolentus*. Likewise, *Rhodotorula* spp. were abundant in the gut of *P. sanguinolentus*, but they were not found on the carapace of *X. testudinatus*. However, mycobiota in the gut of *X. testudinatus* was not examined (Pang et al. 2019; Shaumi et al. 2021). *Xenograpsus testudinatus* was collected from the hydrothermal vent area of Kueishan Island, Taiwan with the possibility of a higher water temperature and a low pH environment, and this may explain the discrepancy in the fungal diversities between the two crab species.

Table 2: Diversity indices of fungal community on carapace and in gut of the marine crab *Portunus sanguinolentus*.

Index	Carapace	Gut
Species richness (Margalef): $d = (S - 1)/\ln(N)$	3.54	4.03
Shannon-Wiener diversity index: $H' = -\Sigma[Pi \cdot \ln(Pi)]$	1.42	1.91
Pielou's evenness: $J' = H'/\ln(S)$	0.59	0.74
Simpson's index of diversity: $1 - D = 1 - \Sigma(Pi2)$	0.53	0.84

Yeasts, including *Candida tropicalis*, *C. parapsilosis*, *Rhodotorula mucilaginosa* and *Rhodotorula* sp. were dominant both on the carapace and in the gut of *P. sanguinolenus*. *Candida tropicalis* is widely distributed in tropical and subtropical marine environments (Silva et al. 2012; Yan et al. 2010). *Candida tropicalis* and *C. parapsilosis* were found to be pathogenic on the Okhotsk snailfish *Liparis ochotensis*, and attacked the gills, liver and kidney (Haridy et al. 2018). *Rhodotorula* is a common genus from subtropical marine environments (Ahearn et al. 1962; Jones et al. 2015). *Rhodotorula mucilaginosa* was one of the dominant fungi in the gut of *P. sanguinolentus*. *Rhodotorula mucilaginosa* was found to be one of the most commonly isolated yeast species in seawater, and can cause a range of human diseases (Wirth and Goldani 2012). This species was also isolated from skin lesions of sea lions (Alvarez-Perez et al. 2010). Although some

Table 3: Ecological Guild assignments for the fungi isolated from the carapace and gut of the marine crab *Portunus sanguinolentus* using FUNGuild database.

Taxon/Genus	Trophic mode	Guild	Notes
Apiotrichum Stautz	Saprotroph	Soil Saprotroph	From forest soil (Calduch et al. 2002)
Candida tropicalis Berkhout	Pathotroph Saprotroph Symbiotroph	Animal Pathogen-Endophyte- Undefined Saprotroph	Opportunistic human pathogen; common cause of candidemias (Trofa et al. 2008); host - Boraginaceae
Hortaea werneckii (Horta) Nishim. *et* Miyaji	Pathotroph	Null	Opportunistic human pathogen
Peroneutypa Berl.	Pathotroph	Plant Pathogen	Null
Purpureocillium Luangsa-ard, Hywel-Jones, Houbraken *et* Samson	Pathotroph	Fungal Parasite	Null
Rhodotorula mucilaginosa (A. Jörg.) F.C. Harrison	Pathotroph	Animal Pathogen	Likely opportunistic human pathogen (Irinyi et al. 2015)
Talaromyces C.R. Benj.	Saprotroph	Undefined Saprotroph	Null
Trichoderma atroviride Bissett	Symbiotroph	Endophyte	Endophyte detection method-culture (Busby et al. 2016)

of the isolated fungi were reported to be pathogenic, none were reported as pathogens of crabs and neither were disease symptoms noticed in the crab samples. Species of the Lagenidiales (Oomycota) were not isolated from *P. sanguinolentus* in this study, but they were isolated from eggs and larvae of *P. pelagicus* and *P. trituberculatus* (Hamasaki and Hatai 1993; Nakamura and Hatai 1995).

Fusarium solani is a cosmopolitan fungus with wide geographical and ecological distribution, and was the dominant filamentous fungus in the gut of *P. sanguinolentus*. *Fusarium solani* can cause diseases of marine animals including turtles and prawns (Pang et al. 2021). *Fusarium solani* causes sea turtle egg fusariosis, which kills embryos of turtles (Gleason et al. 2020).

As discussed above, a number of fungi isolated from *P. sanguinolentus* are potential pathogens of marine animals. Whether marine crabs are sinks of fungi with disease potential requires further study (Shaumi et al. 2021). However, the ecological role of most of the fungi on *P. sanguinolentus* is unknown. For example, *Apiotrichum lignicola* was originally described from wood pulp (Diddens 1934), and is not regarded as a marine species (Jones et al. 2019). *Apiotrichum* is classified as a saprotroph in FUNGuild.

Fungi in the gut of *P. sanguinolentus* were more diverse than on the carapace. The diet of *P. sanguinolentus* mainly includes crustaceans, fish remains and molluscs (Sukumaran and Neelakantan 1997). Whether these substrata support a higher fungal diversity or carry over exogenous fungi will require further study.

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