

Abundance of marine-derived fungi in nutrient-enriched *Kappaphycus* species

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Abstract

The occurrence of ice-ice disease is among the main issues affecting *Kappaphycus* production. The abiotic and biotic factors have major contributions to causing this disease. As biotic factors, marine-derived fungi (MDF) are known to be capable of causing the ice-ice disease. In this study, we determined the abundance of MDF from both healthy and ice-ice-infected nutrient-enriched *Kappaphycus alvarezii* and *K. striatus* in the seaweed farms of Tongehat, Sibutu, Tawi-Tawi, southern Philippines, following serial dilution procedure using malt extract agar. Results revealed that the abundance of MDF in ice-ice-infected nutrient-enriched *K. alvarezii* (3.04×10^4 CFU g⁻¹) had shown higher significance ($p < 0.05$) when compared with healthy nutrient-enriched *K. alvarezii* (3.32×10^3 CFU g⁻¹). Similarly, the abundance of MDF in ice-ice-infected nutrient-enriched *K. striatus* (9.31×10^3 CFU g⁻¹) also appeared to have higher significance ($p < 0.01$) than healthy nutrient-enriched *K. striatus* (6.10×10^2 CFU g⁻¹). In terms of seaweed species comparison, the abundance of MDF in healthy nutrient-enriched *K. alvarezii* had a higher significance ($p < 0.05$) than in healthy nutrient-enriched *K. striatus*, and the abundance of MDF in ice-ice-infected nutrient-enriched *K. alvarezii* also had higher significance ($p < 0.05$) than ice-ice-infected nutrient-enriched *K. striatus*. The great number of MDF in ice-ice-infected nutrient-enriched *Kappaphycus* suggests that MDF may be potential causative agents and have the capability of inducing ice-ice disease in farmed nutrient-enriched *Kappaphycus*.

Keywords: Abundance, Ice-ice disease, *Kappaphycus alvarezii*, *Kappaphycus striatus*, Marine-derived fungi

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1. Introduction

Marine-derived fungi (MDF) are a ubiquitous and diverse group of microorganisms (Jones & Pang, 2012; Simeões et al., 2013; Liu et al., 2015), known to be saprophytic, parasitic, and symbiotic organisms (Johnson & Sparrow, 1961; Kohlmeyer & Kohlmeyer, 1979). MDF are found mostly on seaweeds, mangroves, and sponges (Solis et al., 2010; Simões et al., 2015) and are grouped as true or obligate and facultative marine fungi (Johnson & Sparrow, 1961; Kohlmeyer & Kohlmeyer, 1979). These obligate and facultative marine fungi are well-known for sporulating the estuarine, marine environments, freshwater, and even those of terrestrial origin (Kohlmeyer & Volkmann-Kohlmeyer, 1991; Li & Wang, 2009). These

microorganisms are essential to soil components, for they serve as decomposers and symbioses of the so-called flora microorganisms (Jones & Pang, 2012; Simeões et al., 2013; Liu et al., 2015). MDF have evolved different techniques for their roles in nutrient transfer and chemical defense to increase their competitiveness for nutrient obtainment toward other microorganisms and to protect themselves from predation by animals (Bugni & Ireland, 2004; Taylor et al., 2007; Ding et al., 2011), and these microorganisms are potential sources of bioactive natural products (Shahidi & Rahman, 2018). However, some of these MDF can harm seaweeds. According to Solis et al. (2010), *K. alvarezii* and *K. striatus* infected by ice-ice disease have higher MDF, and these fungi were identified as *Aspergillus ochraceus*, *A. terreus*, and *Phoma* sp., which can show signs of



inducing ice-ice disease. This disease that occurs seasonally has been one of the common problems that could cause stress among farmed seaweeds (Hurtado & Agbayani, 2002; Solis et al., 2010; San, 2012; Tahiluddin & Terzi, 2021a; Tahiluddin & Terzi, 2021b).

Seaweed farmers in Sibutu, Tawi-Tawi, southern Philippines, practice the use of inorganic fertilizers (16-20-0 and 14-14-14 as N-P-K %) to combat ice-ice disease and improve the growth of *Kappaphycus* species (Tahiluddin, 2018; Sarri et al., 2021; Tahiluddin et al., 2021a; Tahiluddin et al., 2021b; Tahiluddin & Terzi, 2021a). This may influence the abundance of MDF. However, there are limited studies so far about the MDF found on farmed nutrient-enriched *Kappaphycus* species, and the variety and abundance of fungal microbes in saltwater environments have not yet been fully discovered (Richards et al., 2015). Thus, this study aimed to determine the abundance of MDF on nutrient-enriched *Kappaphycus* species (*K. alvarezii* and *K. striatus*) both from healthy and ice-ice-infected. This study could provide some information in understanding the role of MDF in the occurrence of ice-ice disease – a disease that affects the health of *Kappaphycus*, leading to a loss in the harvest.

2. Material and Methods

2.1. Study site

The collected samples were gathered from the seaweed farms of Brgy. Tongehat, Sibutu, Tawi-Tawi, southern

Philippines (Figure 1) and analyzed at the Microbiology Laboratory, College of Fisheries, Mindanao State University – Tawi-Tawi College of Technology and Oceanography (MSU-TCTO), Sanga-Sanga, Tawi-Tawi, southern Philippines for three (3) weeks from April 2-21, 2019.

2.2. Sample collection and transportation

A 30-day-old farmed *Kappaphycus* species (*K. alvarezii* and *K. striatus*), nutrient-enriched with ammonium phosphate (16-20-0 as N-P-K %), were randomly collected aseptically at seaweed farms of Tongehat, Sibutu, Tawi-Tawi, southern Philippines. About 5 grams for every 12 samples of mature thalli of cultivated *Kappaphycus* species were collected (3 mid-portion of healthy thalli and 3 ice-ice-infected thalli for *K. alvarezii*, 3 mid-portion of healthy thalli, and 3 ice-ice-infected thalli for *K. striatus*). Samples were placed in sealed sterile glass jars containing 225 ml of sterile seawater. These were placed in a styrofoam box with seawater ice to reduce fungal multiplication. These were transported and immediately placed in a freezer (0 °C). Then, on the following day, frozen samples were placed in a styrofoam box with seawater ice. These were transported to the Microbiology laboratory of College of Fisheries, MSU-TCTO, Sanga-Sanga, Bongao, Tawi-Tawi via boat and immediately kept in a freezer for 20 hours at 0 °C before analyzing.

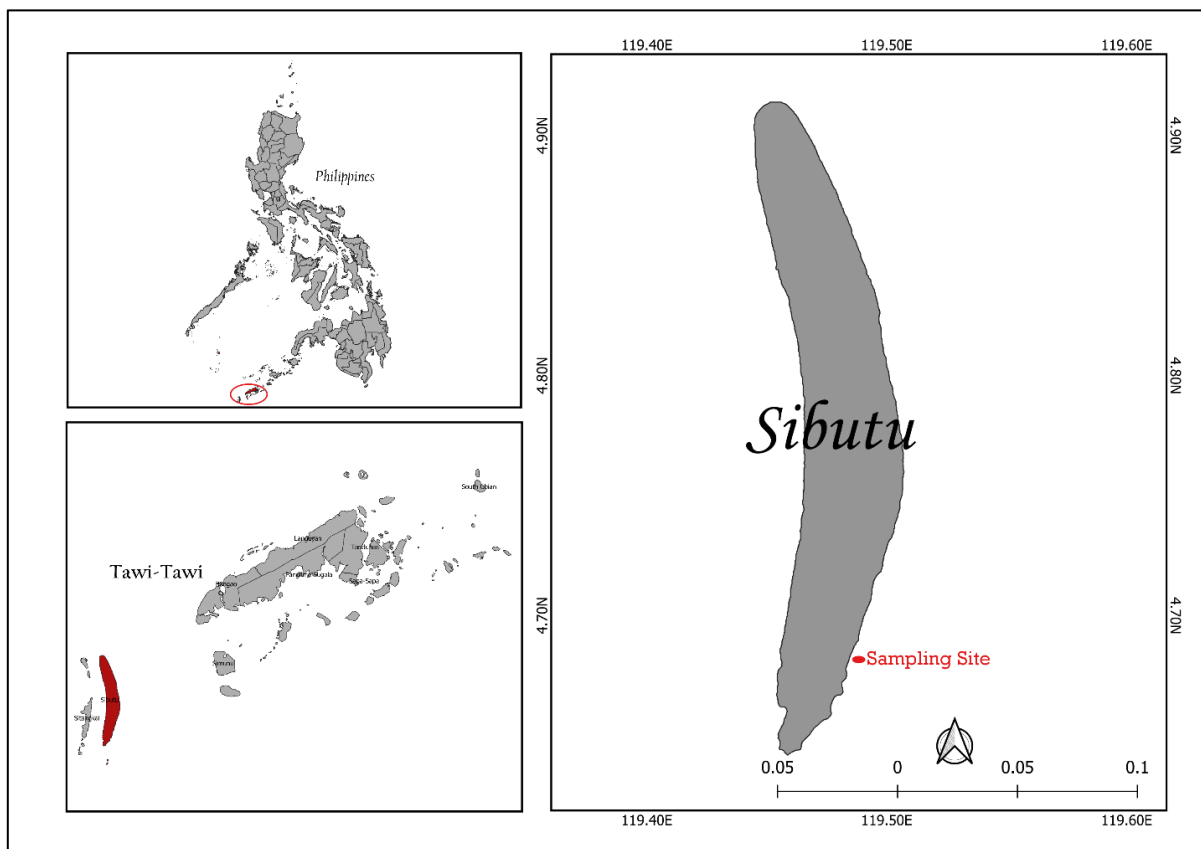


Figure 1. Map of the sampling site

2.3. Physico-chemical parameters of the farm water

Physico-chemical parameters of farm waters were measured by the time of collection, and these were temperature, pH, and salinity using a thermometer, pH meter (Smart Sensor), and refractometer (Atago Master), respectively. In addition, the water current was determined using an improvised drogue, and depth was measured using a calibrated rope.

2.4. Fungal analysis

The fungal analysis of seaweed samples was performed under sterile conditions. One (1) gram of sample was minced using a sterilized razor blade and forceps and placed in a test tube with 9 ml diluents for the vortexing procedure (2-3 minutes) (Tahiluddin et al., 2021a; Tahiluddin et al., 2021c). Samples were diluted up to 10^{-4} for healthy *Kappaphycus* species and 10^{-5} for ice-ice-infected *Kappaphycus* species. An aliquot of 0.1 ml was spread-plated in the media (malt extract agar). The samples were incubated for 2-3 days at room temperature in an inverted position (Solis et al., 2010). Colonies were manually counted, and the colony-forming units (CFU g^{-1}) were calculated using the formula below (Maturin & Peeler, 2001).

$$N = \frac{\sum C}{(n_1) + (0.1n_2) + (0.01n_3)d}$$

Where:

N : Number of colonies per g of sample,
 $\sum C$: Sum of all colonies on all plates counted,
 n_1 : Number of plates in first dilution counted
 n_2 : Number of plates in second dilution counted,
 n_3 : Number of plates in third dilution counted,
 d : Dilution from which the first counts were obtained.

2.5. Data analysis

A t -test was used to analyze the significant difference between samples used in the experiment using IBM SPSS version 20.

3. Results

The average CFUs of MDF in ice-ice-infected and healthy *Kappaphycus* species were 3.32×10^3 CFU g^{-1} for healthy *K. alvarezii*, 3.04×10^4 CFU g^{-1} for ice-ice-infected *K. alvarezii*, 6.10×10^2 CFU g^{-1} for healthy *K. striatus*, and 9.31×10^3 CFU g^{-1} for ice-ice-infected *K. striatus* (Table 1). A t -test had shown significant differences ($p < 0.05$) among *K. alvarezii* and *K. striatus*, both healthy and ice-ice-infected in terms of CFU g^{-1} (Figure 2). Abundance of MDF in ice-ice-infected *K. alvarezii* had a higher significance ($p < 0.05$) than in healthy *K. alvarezii*. Compared with ice-ice-infected *K. striatus*, the abundance of MDF had also shown higher significance ($p < 0.05$) than healthy *K. striatus*. In terms of species comparison, the abundance of MDF in healthy *K. alvarezii* revealed higher significance ($p < 0.05$) than in healthy *K. striatus*, and the

abundance of MDF in ice-ice-infected *K. alvarezii* had higher significance ($p < 0.05$) than in ice-ice-infected *K. striatus*.

Physico-chemical parameters like temperature, pH, salinity, and water current velocity of the two different sampling sites were more or less the same (Table 2). However, the depth of the sampling site of the *K. alvarezii* farm was deeper than the *K. striatus* farm. Thus, these physico-chemical parameters of the two different sampling sites are suitable for both *Kappaphycus* species, for they are away from pollution because of the absence of industry that discharges pollutants and domestic waste from urban dwellings (Table 2).

Table 1. Abundance (CFU g^{-1}) of MDF in *Kappaphycus* species

<i>Kappaphycus</i> species	CFU g^{-1}
Healthy <i>Kappaphycus alvarezii</i>	3.32×10^3
Ice-ice-infected <i>Kappaphycus alvarezii</i>	3.04×10^4
Healthy <i>Kappaphycus striatus</i>	6.10×10^2
Ice-ice-infected <i>Kappaphycus striatus</i>	9.31×10^3

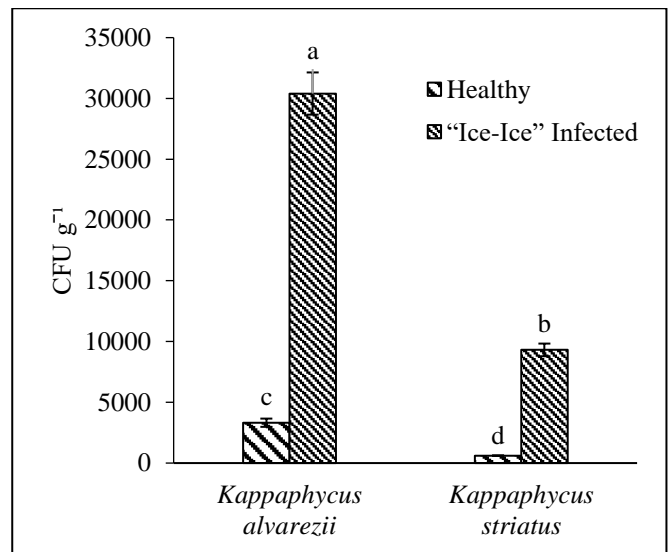


Figure 2. Abundance (CFU g^{-1}) of MDF in healthy and ice-ice infected nutrient-enriched *K. alvarezii* and *K. striatus*. Bars with different letters are significant different ($p < 0.05$). Error bars in SEM (standard error of mean), $n=3$.

Table 2. Physico-chemical parameters of the sampling sites (farms) in Tongehat, Sibutu, Tawi-Tawi, southern Philippines

Farm	Temperature (°C)	pH	Salinity (‰)	Depth (m)	Water current velocity (m s ⁻¹)
<i>Kappaphycus alvarezii</i>	30.5±0.29	8.16±0.01	33±0	4.95±0.03	0.11±0
<i>Kappaphycus striatus</i>	30.3±0.44	8.33±0.05	33±0	0.55±0.01	0.14±0.01

4. Discussion

The abundance of MDF was higher in ice-ice-infected *K. alvarezii* (3.04×10^4 CFU g⁻¹) than in healthy *K. alvarezii* (3.32×10^3 CFU g⁻¹). The presence of MDF in ice-ice infected *K. striatus* (9.31×10^3 CFU g⁻¹) was also higher than in healthy *K. striatus* (6.10×10^2 CFU g⁻¹). The diversity of fungal microbes was higher in ice-ice infected *Kappaphycus* species and had more strains of MDF than that of healthy *Kappaphycus* species (Solis et al., 2011). MDF in green algae *Flabellia petiolota* ranged from 4.8×10^2 CFU g⁻¹ to 1.3×10^3 CFU g⁻¹ and green *Charophytes* ranged from 0.02×10^4 CFU g⁻¹ to 1.2×10^4 CFU g⁻¹ (Gnavi et al., 2017; Kataržytė et al., 2017).

Fungi present in healthy seaweeds were lower, but when the disease occurred, the fungi in seaweeds increased in accordance with the decaying process (Miller & Jones, 1983). Pang et al. (2015) recorded that *K. alvarezii* had longer (290 mm) white and decayed parts than *K. striatus* (87 mm). This may be due to the ability of fungi that can penetrate through the tissue of some organisms like seaweeds, mangroves, and seagrass with their filamentous features and apical growth that may produce white rot and loss of wood even though these organisms have antimicrobial compounds (Valiela, 1979; Gonzalez-Farias & Mee, 1988; Mouzouras, 1989; Raghukumar et al., 1995).

Seaweed, like *K. striatus*, is capable of withstanding massive and uncertain conditions in intertidal zones where it is unprotected from fast-changing environmental factors. In contrast with *K. alvarezii*, it is often yielded by ice-ice disease; thus, *K. striatus* is frequently selected and used for research on field observation and cultivation since it has improved some defense mechanisms for survival in fast-changing environmental conditions in intertidal zones (Pang et al., 2015; Khoo et al., 2016).

Nevertheless, the abundance of MDF in *Kappaphycus* species may also be due to the location of the farm sites. *K. alvarezii* farm was located closer to the terrestrial environment (300 m - 400 m), while *K. striatus* was farther from the terrestrial environment (900 m - 1000 m). According to Jones (1994) and Richards et al. (2015), passive dispersal of spores and other propagules from terrestrial origin has evolved and influenced the presence of MDF in marine environments. Furthermore, some fungal diseases caused by Oomycetes and mitosporic fungi caused fungal diseases and high mortality rates in marine fish and abalones that comprise a group of the genera *Lagenidium*, *Haliphthoros*, *Halocrusticida*, *Halioticida*,

Atkinsiella, *Pythium*, *Fusarium*, *Ochroconis*, *Exophiala*, *Scytalidium*, *Plectosporium*, and *Acremonium* (Hatai, 2012).

According to Largo et al. (1999), whitening and bleaching that are frequently observed in the thalli of seaweeds during the induction of ice-ice infection have something to do with the hydrolytic enzymes. During infection, carrageenan-lysing bacteria were capable of entering the inner region of the seaweed thallus, which may produce epidermal deterioration, the decimation of the cell's pigment-containing plastids, and might have contributed to the primary bleaching of the infected parts. These may be the same as the mechanisms in inducing ice-ice disease caused by MDF which is known to have the ability to yield algal component-degrading enzymes and uses various algal components that might probably have contributed to the associated factors as causative agents that induced ice-ice disease in *Kappaphycus* species (Solis et al., 2010).

5. Conclusion

The abundance of MDF in both ice-ice-infected *Kappaphycus* species was 10-fold higher than those in healthy ones. The number of MDF in *K. alvarezii* was 10-100 times higher than in *K. striatus*. Hence, this study suggests that MDF may play an important role in ice-ice disease occurrence. Furthermore, *K. striatus* showed less susceptibility to MDF. This study could serve as baseline information for further studies in understanding the role of MDF, especially in ice-ice-infected *Kappaphycus*.

Conflict of interest

The authors declare that there is no conflict of interest.

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