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Fungal diversity and enzyme activity associated with sailfin sandfish egg masses in Korea

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ABSTRACT

The aggregation of decaying egg masses of sailfin sandfish along the mid-east coast of Korea is a major environmental problem with concurrent negative economic consequences. In an effort to ameliorate decaying egg masses, we investigated the diversity and community structure of fungi from egg masses and tested for their cellulase and protease activity. A total of 1108 strains were identified based on morphology and multigene analyses, and found to represent 184 fungal species. *Paradendryphiella salina* was the most dominant species, followed by *Penicillium crustosum* and *Penicillium aurantioviolaceum*. The fungal community displayed a significant degree of variation relative to both egg mass color and locality. Over 50% of species detected in this study exhibited both cellulase and protease activity. This study suggests that fungi play an important role in nutrient recycling at intertidal zones and thus may have potential industrial applications that can help resolve the environmental problems associated with egg mass aggregation.

1. Introduction

The sailfin sandfish, *Arctoscopus japonicus,* migrates between the mid-east coast of Korea, Hokkaido, and Alaska. Sailfin sandfish inhabit the sandy bottom of the deep sea (200–400 m) and migrate to shallow sea (2–10 m) near Korea during fall and winter (November to January) to spawn in seaweed beds (An et al., 2011). Egg color in this species varies from red, green, and yellow depending on diet (Morioka et al., 2005). Recently, climate change and human activity have led to whitening of the rocky shore in the east coast of Korea leading to a substantial decrease in the seaweed biomass that is used for spawning (Yang et al., 2009). Egg masses that lack this seaweed substrate tend to drift toward and accumulate on the shore. Aggregation of rotting egg masses has become a major environmental problem on the mid-east coast of Korea by contributing to the already serious problem of coastal landscape degradation. In addition, rotting egg masses have negatively impacted local economies due to the cost of treatment and removal of decaying egg masses.

Microbes are essential components of sustainable marine ecosystems. Many fungi have been isolated from various marine sources such as plants, animals, sediments, and wood (Bugni and Ireland, 2004; Jones and Pang, 2012) and play a major ecological role in nutrient recycling by decomposing substrates in various marine environments (Hyde et al., 1998). These fungi are found throughout the fungal tree of life and comprise an estimated 1500 species (Jones and Pang, 2012). *Aspergillus*, *Cladosporium*, *Fusarium*, and *Penicillium* are the dominant fungal genera found in most marine environments (Bugni and Ireland, 2004; Jones and Pang, 2012). However, few investigations have addressed the diversity of fungi from limited marine environments that included soil, plant, and animal specimens (Burgaud et al., 2009; Liu et al., 2010; Park et al., 2014) and no study has investigated fungi from fish egg masses.

To date, most fungi reported from marine environments have been identified based on morphological features; however, identification to the species level is difficult due to similar morphological features among genera and growth-dependent variation of morphological characteristics (Okuda, 1994; Visagie et al., 2014). To

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overcome these challenges, DNA-based methods, specifically those using the internal transcribed spacer (ITS) region as the primary fungal barcode gene, have significantly improved species identification (Schoch et al., 2012). Furthermore, protein-coding genes as a secondary marker have improved species and genus level resolution (Schoch et al., 2012). For example, *Aspergillus*, *Penicillium*, and *Trichoderma*, have been accurately identified primarily based on protein-coding genes (Samuels et al., 2006; Samson et al., 2014; Visagie et al., 2014).

Fungi from marine environments are known to produce secondary metabolites and enzymes (Bugni and Ireland, 2004; Rateb and Ebel, 2011; Bonugli-santos et al., 2015). *Aspergillus*, *Aureobasidium*, *Cerrena*, and *Penicillium* isolated from different substrates produce enzymes, such as alginase, cellulase, chitinase, ligninase, and protease (Park et al., 2014; Bonugli-santos et al., 2015; Park et al., 2016). Fungal enzymes have been used in five fields: biofuel and paper industries, food and beverages, animal feed, environmental applications, and pharmaceutical and cosmetic applications (Bonugli-santos et al., 2015). Several fungi including *Aspergillus*, *Cladosporium*, *Penicillium*, and *Trichoderma* are known to be capable of degrading and decolorizing environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs), synthetic dyes, and textile effluents. Fungi are known decomposers and the role of fungi in the decay process of substrates in marine environments is well reported for various enzymes (Hyde et al., 1998). Fungi are considered to be the key to recycling egg masses that cause environmental problems in marine environments.

The identification of fungal resources that may ameliorate the environmental problems caused by decaying egg masses of sailfin sandfish along the mid-east coast of Korea can be facilitated through describing the fungal diversity associated with egg masses. The primary objective of this study was to investigate the characteristics of fungi associated with sailfin sandfish egg masses by comparing the diversity and community structure of fungi across different locations and egg mass color variation. In addition, we examined enzyme activity associated with these fungi, including extracellular protease activity (gelatinase and caseinase) which degrades the proteins that are the main components of egg masses (Lee et al., 2005) as well as the activity of extracellular cellulase (endoglucanase and β-glucosidase) which degrades cellulose, the primary component of the seaweed inside and around egg masses (Park et al., 2016).

2. Materials and methods

2.1. Sampling

The green, red, and yellow colored egg mass of the sailfin sandfish were collected from five sites, Yeongok (YG), Jeong-am (JA), Sokcho (SC), Hwajinpo (HJ), and Chodo (CD), along the mid-east coast of Korea in January 2015, when these egg mass aggregations were most pronounced (Fig. 1). While multiple wet egg masses aggregated on CD beach, egg masses were dry in other areas. Seaweed was often found on eggs masses or on the beach with eggs. 250 g of each colored egg mass were collected in sterile bags and transported to the laboratory on ice. To remove surface debris and soil, each sample was washed with artificial sea water (ASW) containing 26.518 g L^{-1} NaCl, 2.447 g L^{-1} $MgCl_2$, 3.305 g L^{-1} $MgSO_4$, 1.141 g L^{-1} $CaCl_2$, 0.725 g L^{-1} KCl, 0.202 g L^{-1} $NaHCO_3$, 0.083 g L^{-1} NaBr (Huang et al., 2011). The components analysis of egg masses was conducted at the Korea Advanced Food Research Institute (KAFRI; Seoul, Korea).

To isolate fungi, the egg masses were cut to approximately 5 mm in length. 100 pieces per sample were placed on three different culture media plates: dichloran rose bengal chloramphenicol agar (DRBC; Difco, Sparks, MD, USA), glucose yeast extract agar (GYA; 1 g L^{-1} glucose, 0.1 g L^{-1} yeast extract, 0.5 g L^{-1} peptone, and 15 g L^{-1} agar), and Sabouraud dextrose agar (SDA; Difco, Sparks, MD, USA) supplemented with ASW. The plates were incubated at 25 °C for 7–15 d, then each strain was transferred to potato dextrose agar (PDA, Difco, Sparks, MD, USA) plate with ASW. The strains were maintained in 20% glycerol at −80 °C at the Seoul National University Fungus Collection (SFC) (Table S1).

2.2. DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted using the modified cetyltrimethylammonium bromide (CTAB) extraction protocol (Rogers and Bendich, 1994). The PCR amplifications of the ITS for the representative strains (one to three) from each morphological group were performed using the primers ITS1F and ITS4 (White et al., 1990). Actin (*act*) for *Cladosporium*, β-tubulin (*benA*) for *Penicillium*, calmodulin (*cam*) for *Aspergillus*, and translation elongation factor 1-α (*tef1*) for *Fusarium* and *Trichoderma* were amplified using the primers, ACT-512F and ACT-783R (Carbone and Kohn, 1999), Bt2a and Bt2b (Glass and Donaldson, 1995), CF1 and CF4 (Peterson et al., 2005) and EF1 and EF2 (O'Donnell et al., 1998), respectively. Each PCR was performed in a C1000 Thermal Cycler (Bio-Rad, Richmond, CA, USA) using previously described methods (Park et al., 2015). The PCR products were purified with the Expin™ PCR Purification Kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. DNA sequencing was performed at Macrogen (Seoul, Korea), using an ABI PRISM 3700 Genetic Analyzer (Life Technologies, Gaithersburg, MD, USA) with the indicated PCR primers. All the sequences were deposited in GenBank (Table S2).

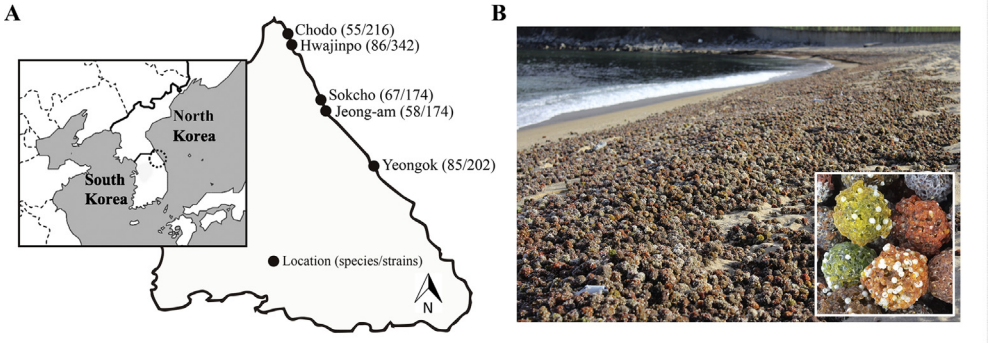

Fig. 1. Map showing the location of sampling sites along the mid-east coast of Korea (A) and aggregations of egg mass of the sailfin sandfish, *Arctoscopus japonicus*, on Chodo (CD) beach (B). Raw map was obtained from https://freevectormaps.com/and edited in Adobe Illustrator CS6 (Adobe Systems Inc., CA, USA).