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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).

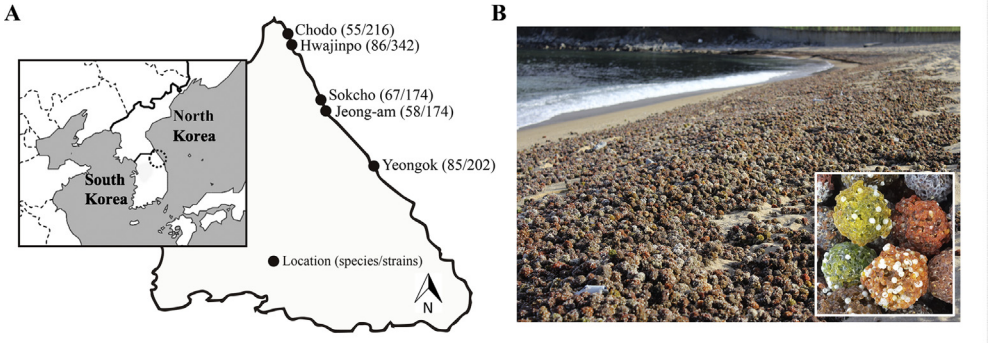

Fig. 1. Map showing the location of sampling sites along the mid-east coast of Korea (A) and aggregations of egg mass of the sailfin sandfish, *Arctoscopus japonicus*, on Chodo (CD) beach (B). Raw map was obtained from https://freevectormaps.com/and edited in Adobe Illustrator CS6 (Adobe Systems Inc.,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

2.3. Identification

Identification was performed in three steps. First fungal strains were grouped based on their cultural characteristics on PDA (color, margin, and shape of colony and diffusible pigment production) and micro-morphological characteristics (shape of conidiophores and spore). Representative strains (one to three) from each morphological group were chosen for molecular identification. Second, we identified all representative strains to the genus level by analyzing ITS sequences. Next, to identify strains to the species level, sequences from the ITS, *act*, *benA*, *cam*, and *tef1* loci were aligned separately (Table S3).

Sequences were assembled, proofread, and edited using MEGA5 software (Tamura et al., 2011). The sequences from each locus were aligned using MAFFT v7 (Katoh and Standley, 2013) and ambiguously aligned positions were adjusted manually. Maximum likelihood phylogenetic analyses were performed using RAxML (Stamatakis, 2006), with the GTR + G model of evolution and 1000 bootstrap replicates. Trees were visualized and edited using FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree).

2.4. Bioinformatic analysis

Bioinformatic analysis was conducted using QIIME version 1.8.0 (Caporaso et al., 2010) to calculate alpha diversity indices and community dissimilarities and R (R Core Team, 2014) to perform statistical tests and visualize data. Alpha diversity indices (Chao1 richness, Shannon's diversity, and Shannon's equitability) were calculated using QIIME with command *alpha_diversity.py*. Significance of difference was tested using one way ANOVA with Tukey's post hoc test, and P-values with multiple tests were corrected using the false discovery rate (FDR) of Benjamini and Hochberg (1995) using R. Constrained Analysis of Principal coordinates (CAP) analyses for CAP models were conducted to test the influence of factors (color of egg mass and locality) based on the Bray-Curtis and binary Jaccard distances using the phyloseq package (McMurdie and Holmes, 2013). CAP models were constrained to one factor and conditioned to the other factor [~Color of egg mass + Condition (locality), ~locality + Condition (Color of egg mass)]. Significance of the CAP model was calculated using permutational ANOVA with 999 permutations.

2.5. Enzyme assay

Extracellular cellulase (endoglucanase and β-glucosidase) and extracellular protease activity (gelatinase and caseinase) were assessed for each species using modified plate screening methods (Lee et al., 2015). The enzyme activity was determined by the formation of a clear zone surrounding the colony. Endoglucanase and β-glucosidase activity were assayed by growing the fungi on Mandels' medium (Lee et al., 2015) with 1% carboxymethylcellulose (Sigma-Aldrich, St. Louis, MO, USA) and 0.5% D-cellobiose (Sigma-Aldrich, St. Louis, MO, USA) as the primary carbon sources, respectively. The plates were flooded with Gram's iodine (Sigma-Aldrich, St. Louis, MO, USA) for 5 min (Sawant et al., 2015). Caseinase activity was assayed by growing the fungi for 5 days on yeast extract agar (Oxoid, UK) supplemented with 1.5% skim milk (Difco, Sparks, MD, USA) as the primary carbon source (Teng et al., 2001). Gelatinase activity was assayed by growing the fungi on glucose yeast extract peptone (GYP) agar medium (1 g L^{-1} glucose, 0.1 g L^{-1} yeast extract, 0.5 g L^{-1} peptone, 15 g L^{-1} agar, pH 6) supplemented with 0.4% gelatin (Difco, Sparks, MD, USA) (Maria et al., 2005) and then flooding the plates with saturated aqueous ammonium sulfate. Functional groups of fungal species were categorized based on enzyme activity. To compare differences in caseinase activity between representative fungal species, statistical significance was tested using ANOVA with Tukey's post hoc test.

3. Results

3.1. Identification of fungi isolated from the egg masses

A total of 1108 fungal strains were obtained from the egg masses at five sites in Korea. HJ (342) had the largest number of strains, followed by CD (216), YG (202), JA (174) and SC (174) (Fig. 1). On the basis of morphological comparisons and ITS sequences, 1108 strains were categorized into184 groups. Representative strains of each group were identified to the species level based on a genus-by-genus phylogenetic analyses using sequence of *act* for *Cladosporium*, *benA* for *Penicillium*, *ca* for *Aspergillus*, and *tef1* for *Fusarium* and *Trichoderma*, and ITS for the remaining genus (Fig. S1). Final identification is shown in an ITS phylogenetic tree (Fig. 2). These groups spanned three phyla, six classes, 15 orders, 27 families, 52 genera, and 184 species. 122 species were identified to the species level, whereas 62 species remained unidentified because of unclear morphology or phylogenetic relationships. These unidentified species were designated using confer (cf.) epithet. Among 1108 strains, 1096 (98.9%) belonged to Ascomycota, three (0.3%) to Basidiomycota, and nine (0.8%) to Zygomycota. The orders Eurotiales (39.8%, n = 441) and Pleosporales (33.1%, n = 337) were the dominant groups, followed by Hypocreales (11.8%, n = 131) and Capnodiales (7.5%, n = 83) (Fig. 3A). The dominant representative genera were *Penicillium* (36.6%) and *Paradendryphiella* (12.9%), followed by *Cladosporium* (7.4%), *Fusarium* (5.0%), *Epicoccum* (4.2%), *Trichoderma* (4.2%), and *Alternaria* (4.1%) (Fig. 3B). Dominant species were *Paradendryphiella salina* (n = 124), followed by *Penicillium crustosum* (n = 77), *Penicillium aurantioviolaceum* (n = 56), *Penicillium steckii* (n = 54), and *Cladosporium cladosporioides* (n = 51) (Table S1, Fig. 3C).

3.2. Diversity and community structure of fungi

Diversity indices of fungal communities were compared between egg mass colors and localities. Species richness, diversity, and evenness were not significantly different relative to egg mass color (Chao1: Corrected P = 0.314; Shannon: Corrected P = 0.312; Equitability: Corrected P = 0.243) (Table S4, Fig. S2A). There were, however, significant differences in species diversity and evenness depending on locality (Shannon: Corrected P = 0.002; Equitability: Corrected P < 0.001), although species richness was not significantly different (Chao1: Corrected P = 0.287) (Table S4, Fig. S2B). CD had lower diversity and evenness compared to the other regions.

Fungal community was compared between egg mass colors and between localities using CAP analysis. Fungal community were significantly different depending on egg mass color based on Bray-Curtis distance (P = 0.023, 7.5% explanatory power) (Fig. 4A). However, species presence in the community based on binary Jaccard distance was not significantly different (Fig. S3A), which means that community differences were probably due to differences in abundance of the dominant species. *Pa. salina* was the most dominant species of the three different egg mass colors (green; 10.3%, red; 14.3%, yellow; 8.6%) (Fig. 4C). The other dominant species, however, were different in relation to egg mass color. In green egg masses, *P. crustosum* (9.4%), *P. aurantioviolaceum* (6.7%), and *Penicillium rubens* (4.4%) were commonly isolated. In red egg masses, *P. steckii* (10.4%), *Penicillium* cf. *johnkrugii* (6.6%), and *C. cladosporioides* (6.3%) were common species. In yellow egg masses, *P. crustosum* (6.3%), *C. cladosporioides* (6.0%), and *Alternaria alternata* (4.1%) were commonly isolated (Fig. 4C).

Fungal community was significantly different depending on locality in both analyses based on a Bray-Curtis analysis (P = 0.004,

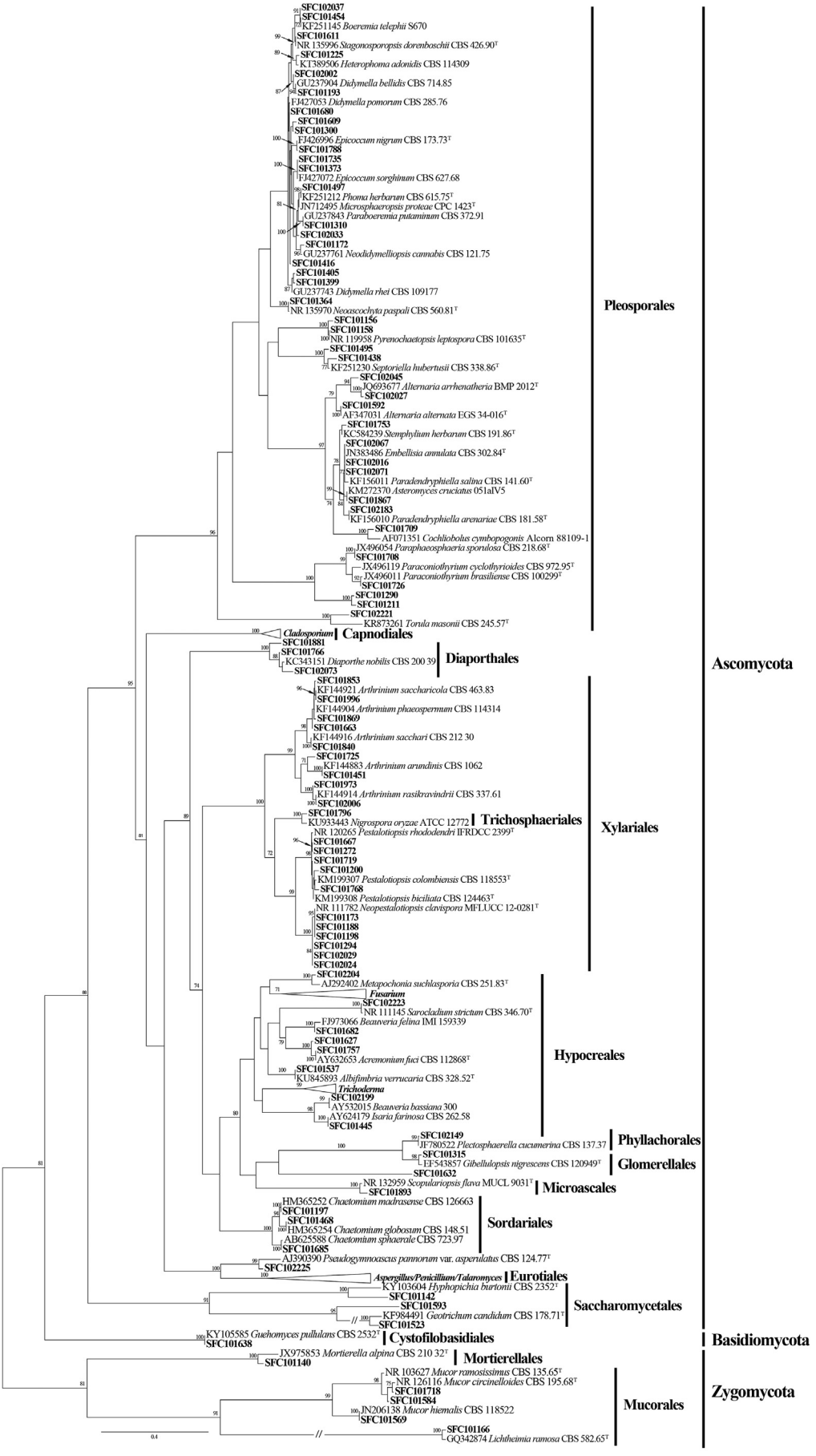

Fig. 2. Phylogenetic tree of fungi from egg masses of sailfin sandfish based on ML analysis of the ITS. Bootstrap scores are presented at the nodes if > 70. The scale bar indicates the number of nucleotide substitutions per site. "T" indicates ex-type strains. Representative strains of each species group based on morphological comparisons and ITS sequences are represented in bold font.

31.8% explanatory power) (Fig. 4B) and by binary Jaccard distance (P = 0.001, 21.9% explanatory power) (Fig. S3B). The fungal communities had different patterns in each locality. In particular, CD had a significantly different fungal community compared to the other regions that shared a high abundance of *P. salina* (Fig. 4D).

3.3. Enzyme activity of fungi

The major components of egg masses were crude protein (31.0–34.4%), crude fat (5.7–5.9%), ash content (8.9–11.4%), carbohydrate (0–0.2%), and water (49.8–53.0%) (Table S5).

The representative strains of each species were screened for extracellular enzyme activity of β-glucosidase, endoglucanase, caseinase, and gelatinase. *Aspergillus* and *Penicillium* (order: Eurotiales), and *Cladosporium* (Capnodiales), which were commonly isolated from egg masses, showed strong enzyme activity. *Acremonium* (Hypocreales) showed relatively strong β-glucosidase, endoglucanase, and gelatinase activity. *Asteromyces* and *Paradendryphiella* (Pleosporales) showed relatively strong β-glucosidase and endoglucanase activity (Fig. 5).

A total of 87 species showed β-glucosidase activity and 101 species showed endoglucanase activity. *Aspergillus candidus* (SFC102207) showed strong β-glucosidase activity, whereas *Penicillium nordicum* (SFC101546) and *Penicillium* cf. *polonicum* (SFC101499) showed strong endoglucanase activity.

67 species showed caseinase activity and 88 species showed gelatinase activity. *Penicillium oxalicum* (SFC101511) and *P.* cf. *johnkrugii* (SFC102200) showed strong caseinase activity, whereas *As. candidus* (SFC102207), *Beauveria felina* (SFC101682), and *Penicillium* cf. *concentricum* (SFC101540) showed strong gelatinase activity (Table S1).

Isolated fungi were categorized into six functional groups based on enzyme activity patterns to identify functional composition of fungal communities in the egg masses (Fig. 6). Group 1 comprised of fungi that exhibited both cellulase and proteases (caseinase and gelatinase) activity (*e.g. C. cladosporioides*). Group 2 consisted of fungi producing cellulase and caseinase (*e.g. P. crustosum*). Group 3 comprised of fungi showing cellulase and gelatinase activity (*e.g. Pa. salina*). Groups 4 and 5 consisted of fungi producing either only cellulase (Group 4) or proteases (Group 5) (*e.g. A. alternata* and *Epicoccum sorghinum*, respectively). Group 6 comprised of fungi showing no enzyme activity (*e.g. Neoascochyta paspali*). Composition of functional groups was similar respective to both egg mass color (Fig. 6A) and locality, except for CD (Fig. 6B). Generally, groups 1 and 2 comprised around 50% of the total community. In CD, however, group 3 was the most dominant functional group (Fig. 6B).

4. Discussion

To our knowledge, this is the first report focusing on fungi from egg masses of the sailfin sandfish. An abundant fungal diversity (a total of 1108 strains, 184 species, and 51 genera) was uncovered from this single substrate. 30 genera were previously reported fungi and 21 genera are new reports of fungi from marine environments. The relatively high diversity of fungi found in egg masses may be due to specific nutrient composition of egg mass, or improved identification based on multigene analysis including ITS and protein coding genes (compared to the identification using solely morphological features or ITS sequences) (Höller et al., 2000; Li and Wang, 2009). The majority of the fungi isolated belong to the phylum Ascomycota, whereas only a few Basidiomycota and Zygomycota taxa were isolated. This pattern is consistent with a previous study, where most of the approximately 1112 fungi from marine environments reported belonged to the Ascomycota (Jones et al., 2015). The fungal communities were primarily comprised of *Cladosporium*, *Fusarium*, *Paradendryphiella*, and *Penicillium* which is consistent with similar studies (Khudyakova et al., 2000; Cantrell et al., 2006; Bubnova, 2017).

Our results indicate that the fungal community was influenced by two environmental factors: egg mass color and locality. The abundance of common species was different depending on egg mass color, which suggests a preference among fungal species for egg mass color. The different patterns of microbial communities observed may depend on the color of the substrate. For example, the Hawaiian coral (*Montipora capitata*) and the cold-water coral (*Lophelia pertusa*) showed significantly different bacterial communities depending on color variation of coral species (Neulinger et al., 2008; Shore-Maggio et al., 2015). Differences in bacterial communities between white and red *L. pertusa* colonies are thought to be due to different bioactive substances produced by the coral colonies (Neulinger et al., 2008). Therefore, differences in color in sandfish eggs may be associated with different components of egg masses, an idea supported by a study that showed that the color of sandfish eggs was different depending on diet (Morioka et al., 2005). Although there was no difference among components of crude nutrients among the three different colors of egg mass in this study, small molecules in each of the colors of egg mass are likely to

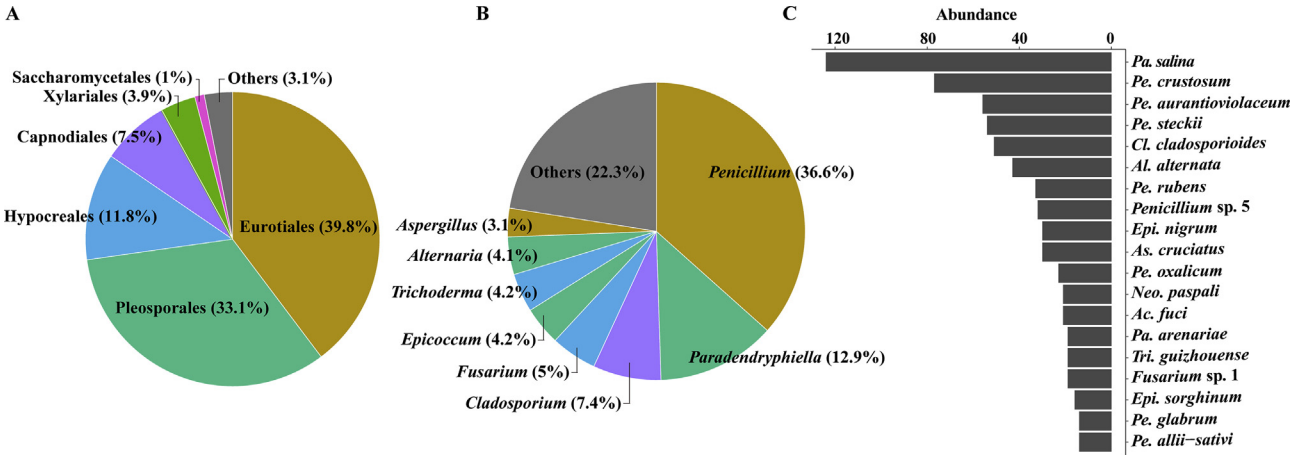

Fig. 3. Proportions of fungi isolated from egg masses at the order level (A), genus level (B), and species level (C).

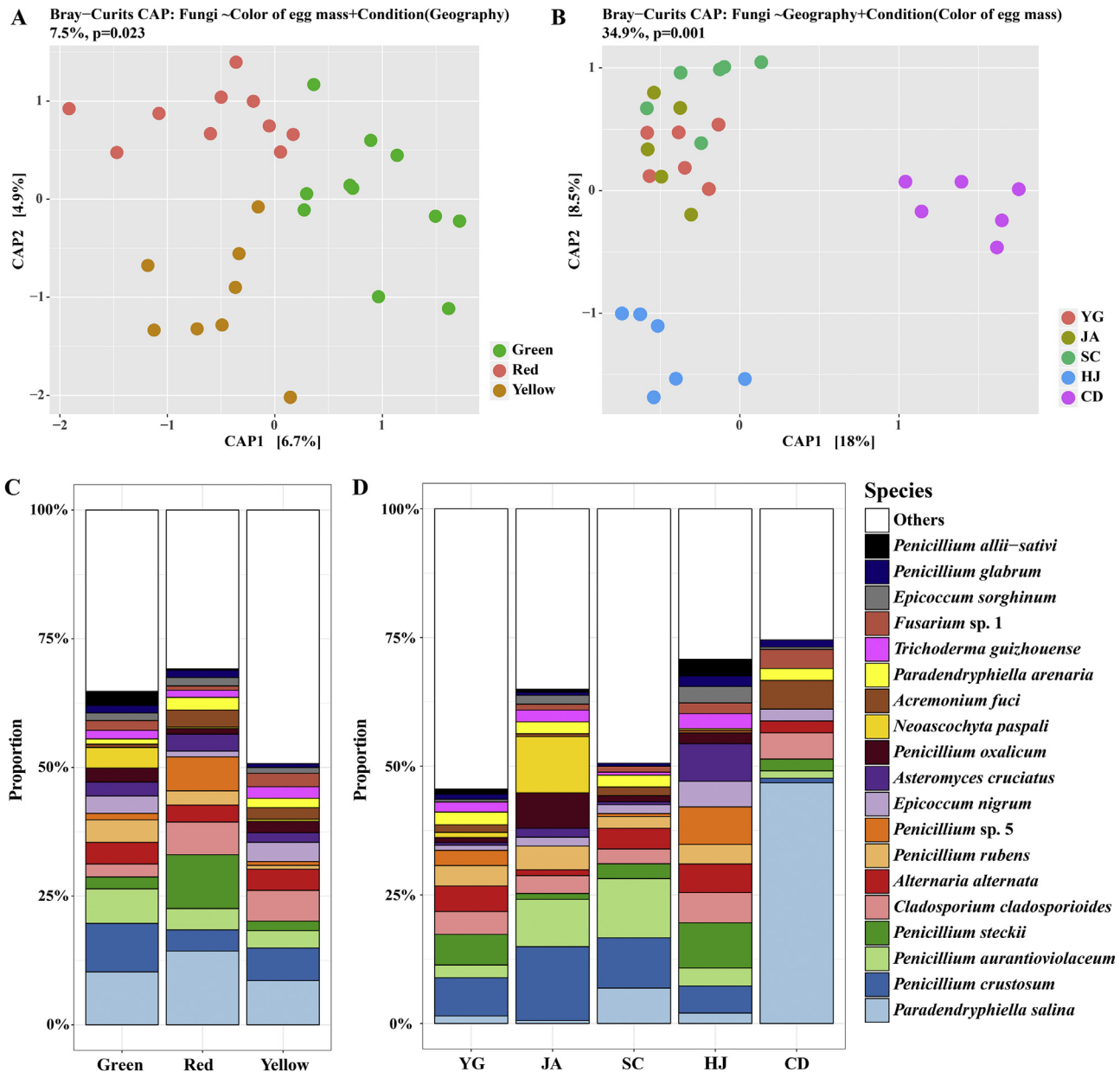

Fig. 4. The composition of fungal communities in different colors of egg masses and locality. Constrained Analysis of Principal coordinates (CAP) plots for fungal communities based on Bray-Curtis distance was constrained by color of egg mass and conditioned by locality (A), and constrained by locality and conditioned by color of egg mass (B). Significance of CAP models was evaluated using ANOVA with 999 permutations. Composition of major fungal species ($n \geq 10$) in different color of egg mass (C) and locality (D). Yeongok (YG), Jeong-am (JA), Sokcho (SC), Hwajinpo (HJ), and Chodo (CD) along the mid-east coast of Korea.

contribute to differences in fungal communities. Further studies are required to explain any possible relationship between egg mass components (i.e. nutrients) and fungal preference.

The structure of the fungal community was also different depending on locality. The large difference in the community structure at CD may be due to the condition of the egg mass of sailfin sandfish. The egg masses in CD were wet with seawater, while egg masses of the other regions were desiccated. Therefore, egg masses at CD were in the initial stage of decomposition, and presumably had recently washed ashore. It is likely that *Pa. salina* was involved in the initial stage of egg mass decomposition at CD, whereas dominant species in the other locations (*P. aurantioviolaceum, P. crustosum,* and *P. steckii*) were important in the middle and late stages of egg mass decomposition. It is well known that fungal communities exhibit distinct community structure during different stages of decomposition of substrates such as litter and wood (Frankland, 1998; Osono, 2007; van der Wal et al., 2013). Similarly, the distinct fungal community between wet and dry egg masses may be exhibiting different stages of the succession of fungal communities in the egg masses of sailfin sandfish.

Fungi isolated from marine environments are known to produce a variety of enzymes such as alginase, amylase, cellulase, chitinase, ligninase, protease, and xylanase (Bonugli-santos et al., 2015). Fungal species and their enzymes have been used in environmental applications such as de-colorization of synthetic dyes and degradation of PAHs, as well as various other industrial applications (Bonugli-santos et al., 2015). A relatively high proportion of fungi associated with egg masses exhibited endoglucanase, β-

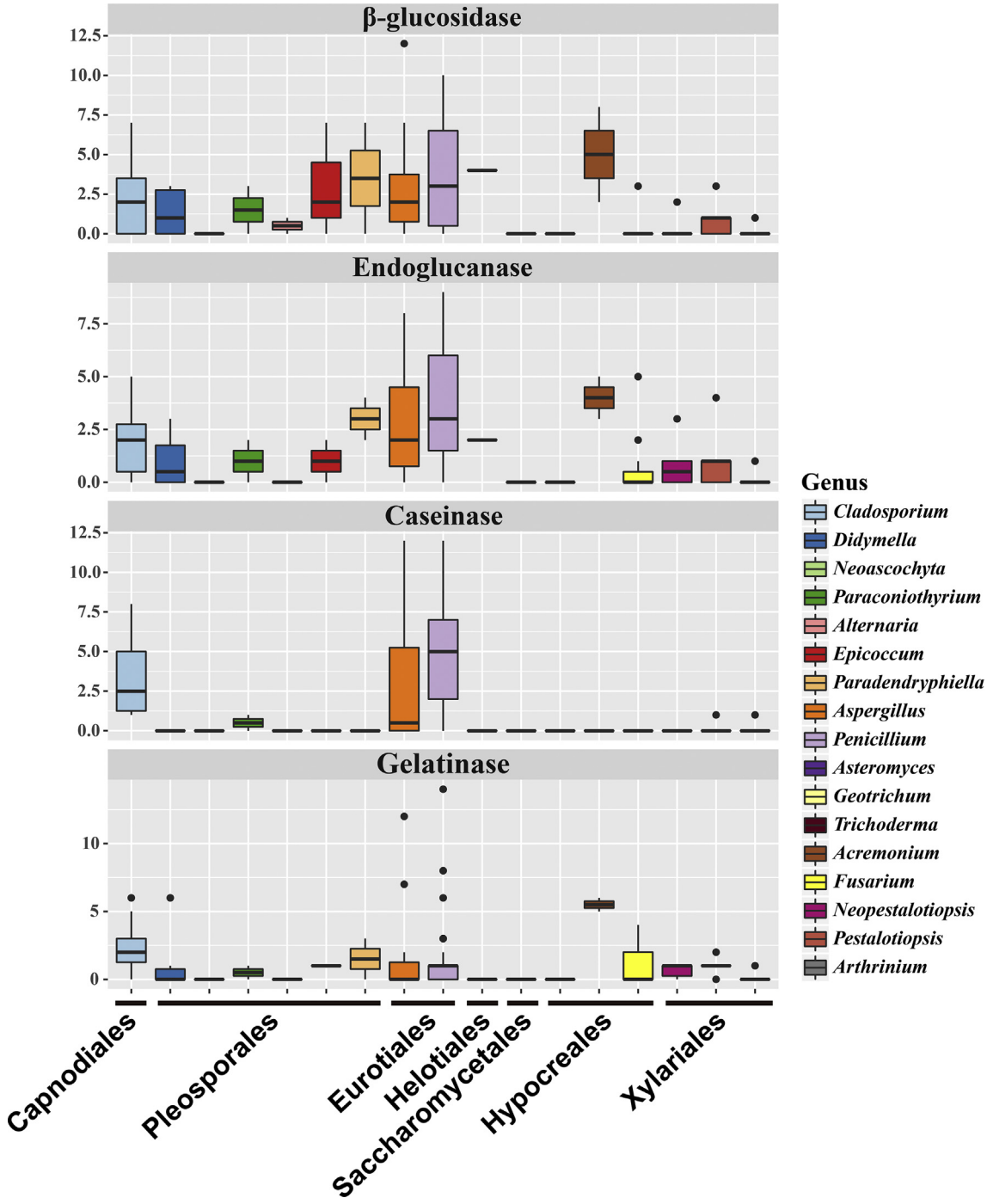

Fig. 5. Differences in enzyme activity among commonly isolated genera.

glucosidase, and/or gelatinase activity. In contrast, caseinase activity was observed in only a few genera, notably *Aspergillus*, *Cladosporium*, and *Penicillium*.

Fungi play an important role in marine nutrient recycling by decomposing substrates using cellulases such as β-glucosidase and endoglucanase (Bucher et al., 2004; Velmurugan and Lee, 2012). The genera *Aspergillus* and *Penicillium* showed relatively strong cellulase activity; (Velmurugan and Lee, 2012; Park et al., 2014). Although there is no cellulose in egg masses of sailfin sandfish, more than 50% of fungal species isolated from egg masses showed cellulase activity. The sailfin sandfish attach egg masses to seaweed during the winter season (Yang et al., 2009). When the eggs were collected from the beach in January 2015, seaweed was often found on eggs masses or on the beach with eggs. Thus, these cellulolytic fungi likely metabolize seaweed inside or around the egg masses rather than using the egg masses as a direct nutrient source.

Fungi isolated from various marine environments such as soft coral, sponge, deep-sea sediments have been reported to be producers of caseinase (Damare et al., 2006; Kamat et al., 2008). *Aspergillus* and *Penicillium* are known for their biotechnological potential in the production of proteases as well as other enzymes (Bonugli-santos et al., 2015). Previously, we showed that many

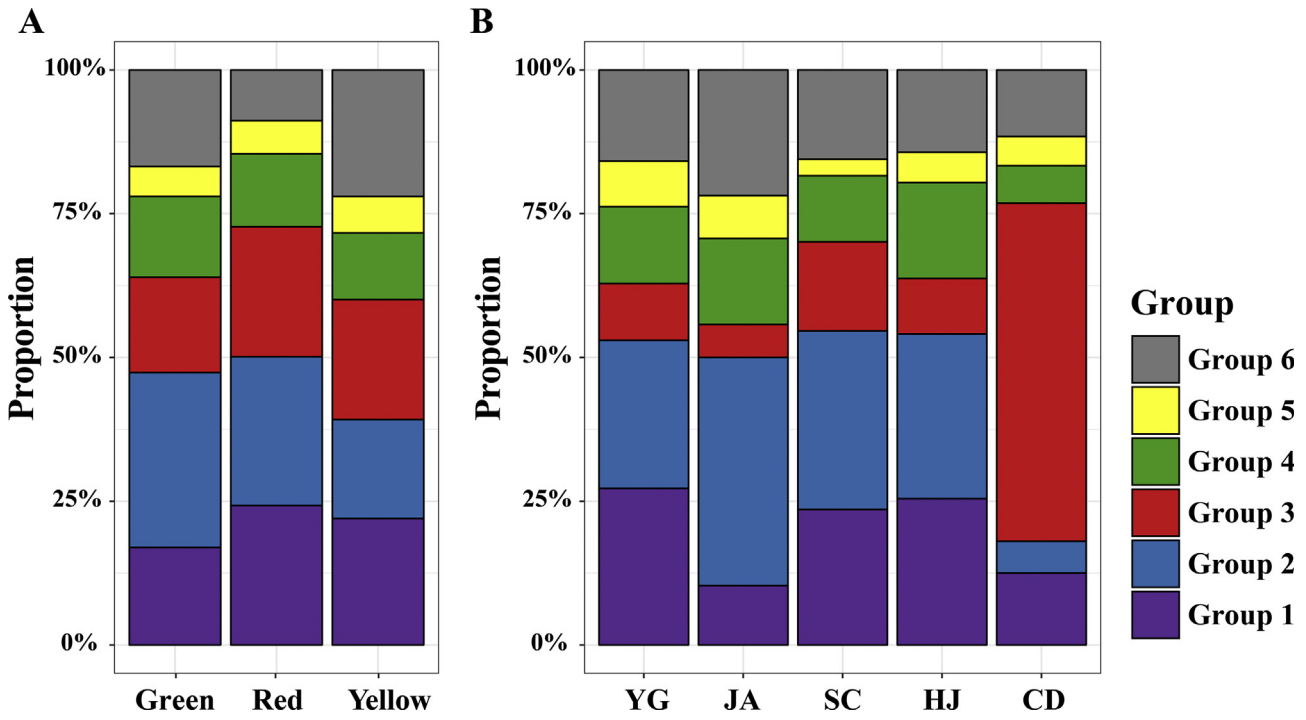

Fig. 6. Composition of functional groups based on the enzyme activities for egg mass colors (A) and localities (B). Group 1: all enzyme activity, Group 2: cellulase and caseinase, Group 3: cellulase and gelatinase, Group 4: cellulase, Group 5: protease, Group 6: no enzyme activity.

Penicillium species isolated from seaweed exhibited caseinase activity (Park et al., 2016). Similar results were detected in this study. Approximate 36% and 48% of the species examined in this study showed caseinase and gelatinase activity, respectively. While most studies of fungal gelatinase activity have focused on terrestrial fungi (Hutchison, 1990; Gopinath et al., 2005; Shukla, 2014), few studies have focused on the gelatinase activity of fungi from marine environments (Pisano et al., 1964; Pivkin, 2000). In this study, many genera, including *Aspergillus* and *Penicillium*, showed similar gelatinase activity. Although *A. candidus*, *B. felina*, and *Penicillium* cf. *janczewskii* were rarely isolated from egg masses, they exhibited relative strong gelatinase activity.

Over 50% of the species examined in this study showed cellulase and protease activity in all regions. Although community structure and species dominance were different depending on egg mass color and locality, functional groups based on enzyme activity had a similar composition across egg mass colors and across localities, with the notable exception of the CD location. A high proportion of functional groups 1 and 2 implies that caseinase plays an important role in the egg mass environment. In contrast to the other locations, functional group 3 was dominant in CD, which is likely related to egg mass condition. After fertilization, the egg envelope of fish gradually hardens (Nakano, 1956). The egg envelope, an egg-specific extracellular matrix, is composed of several types of N-linked glycoproteins, such as collagen, gelatin, and fibronectin (Lee et al., 2005). Therefore, functional group 3 may metabolize substrates such as gelatin from egg mass membranes and cellulase from seaweed on eggs masses. On the other hand, the species exhibiting caseinase activity in dry egg mass were dominant compared to species exhibiting gelatinase activity. The species showing caseinase activity may have used casein as a substrate from developing embryos or inside the egg chorion.

5. Conclusions

A morphological and multigene analysis showed that egg masses of sailfin sandfish harbored surprisingly high diversity of fungi with 51 genera and 184 species. Most isolated fungi had either cellulase and/or protease activity and are thought to play an important role in nutrient recycling in the decomposing egg masses of intertidal environments. This study improves our understanding of fungal ecology in coastal environments and identifies potential resources for various environmental problems including landscape degradation on the shore and the cost of treatment and removal of egg masses.

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Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.funeco.2018.03.004.

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