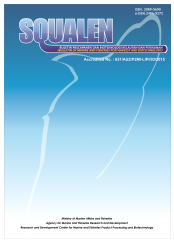

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FUNGAL COMMUNITY STRUCTURE OF MACROALGA *Ulva intestinalis* REVEALED BY MISEQ SEQUENCING

Agusman[1]* and Feng Danging[2]

[1]Research and Development Center for Marine and Fisheries Product Processing and Biotechnology
Jl. KS. Tubun Petamburan VI, Jakarta 10260 Indonesia

[2]College of Ocean and Earth Sciences, Xiamen University, Xiamen City, China

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Abstract

Since algae are well known as the host of fungi, the information about total fungal community associated with alga is needed to give the initial data that is useful for natural product discovery. The study on total fungal community associated with marine macroalga *U. intestinalis* from Xiamen-China was carried out using MiSeq, a powerful platform for microbial ecology studies. Metagenomics DNA was isolated from surfaces of *the U. intestinalis* thallus and rocks colonized by this alga and 18S ribosomal RNA region was sequenced using MiSeq Illumina to yield 81360 sequences from 162720 reads, which average read length is 245.61 bp. The sequences were assigned to operational taxonomic units (OTUs), where 101 genera represent 5 phyla, 17 classes and 64 families of fungal. *Ascomycota* was the highest abundance share of fungal community at phylum level. Meanwhile *Aspergillus*, *Blastobotrys*, *Alternaria, Knufia,* and *Fusarium* were some of dominant genera found associated with *U. intestinalis* which have been reported as the promising source of natural products. By using MiSeq platform, this study revealed the total community structure of algicolous fungi associated with *U. intestinalis*.

Keywords: fungi, community structure, MiSeq, *U. intestinalis*

1. Introduction

Algae have been well-known as host of abundant marine microorganism like bacteria and fungi (Godinho et al., 2013; Wichard et al., 2015), that may play an important rules in the ecosystem and bring the benefit for the growth of that algae (Burke, Thomas, Lewis, Steinberg, & Kjelleberg, 2011; Matsuo, Imagawa, Nishizawa, & Shizuri, 2014; Richards, Jones, Leonard, & Bass, 2012). Furthermore, the biochemical and physical processes which occur in the algal thallus surface are likely to play a role in structuring both qualitatively and quantitatively of the microbial community associated with algae (Egan et al., 2013; Maximilian et al., 1998; Tujula et al., 2010). Therefore the algal surface maybe associated with unique microorganism that might be prospective for new natural product discovery.

Algae are an important source for isolation of marine fungi, where more than 30% of all known marine fungi species associated with this plant (Gnavi et al., 2017). In addition, 27% of new natural products derived from marine fungi were isolated from macroalgae (Bugni & Ireland, 2004). Thus, algae are a reservoir of potential secondary fungi-derived metabolites. The data of fungal community structure is important to give an initial information that is beneficial for natural product discovery (Patantis, Rahmadara, Elfidasari, & Chasanah, 2013).

Several studies about fungi associated with algae have been conducted which most of them used culture dependent techniques (Gnavi et al., 2016; Loque et al., 2010; Godinho et al., 2013). The results showed that culture dependent technique only revealed less than 1% of fungal community (Handelsman, 2005). Other methods which have been used to study fungal community were Amplified Ribosomal DNA Restriction Analysis (Menezes et al., 2010) and PCR-denaturing gradient gel electrophoresis (DGGE) (Zuccaro et al.,

*Corresponding author.
E-mail: mr.agusman@gmail.com

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2008). Unfortunately, those traditional culture independent techniques were thought to only recover a small proportion of community in the environmental samples (Fu, Lv, & Chen, 2016; O'Brien, Parrent, Jackson, Moncalvo, & Vilgalys, 2005). In the other hand, high throughput sequencing platform has been using to explore the microbial diversity at an unprecedented scale of environmental samples (Logares et al., 2013; Ercolini, 2013).

The interaction of bacteria-algae (Egan et al., 2013) and the fungal community of *U. intestinalis ((*Godinho et al., 2013) have been studied using culture dependent method. Recently, the Illumina MiSeq is employed and recommended for analyzing microbial community of environmental samples. The advantages of this method are able to reveal the uncultured microorganism, time saving and low cost (Unno, 2015). Compared to other high throughput platforms, Illumina MiSeq gives more reads than Roche 454 (Werner, Zhou, Caporaso, Knight, & Angenent, 2011) and lower error rate results compared to Ion Torrent (Salipante et al., 2014). Therefore, the present study of fungal community associated with *U. intestinalis* of Xiamen origin was conducted using Illumina MiSeq. This study enabled us to understand the community structure of fungal associated with *U. intestinalis*.

2. Materials and Methods

2.1. Sample Collection

Samples of algal thallus of *U. intestinalis* (LU) and rocks colonized by algae (EU) were collected from Xiamen seaside (N 24°35'20.584'' – E 118°06'58.114'') on January 2016 (winter season) during low tide. EU samples were collected on area about 3x3 cm^2. The surfaces were flushed with sterile seawater and swabbed with sterile cottons. As many as 30 algal thallus were aseptically collected and put into sterile tubes. All samples were transported to laboratory and kept at -20°C. Prior swabbing, thallus were flashed with sterile seawater. The DNA were extracted and stored at -80°C for further experiment.

2.2. DNA Extraction and High-Throughput Sequencing of ITS

Total genomic DNA was extracted using Power Soil DNA isolation Kit (MO BIO Laboratories, Inc., CA., USA) and genomic DNA was used as template. The 18S ribosomal RNA genes were amplified using primers ITS1F (5¹-CTTGGTCATTTAGAGGAAGTAA-3¹) and 2043R (5¹-GCTGCGTTCTTC-ATCGATGC-3¹). PCR reactions were performed in triplicates of 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 ìM), 0.4 μL of FastPfu Polymerase, and 10 ng of DNA.

Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.), and quantified using QuantiFluor™-ST (Promega, U.S.). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on an MiSeq according to the standard protocols (Amato et al., 2013).

2.3. Sequencing and Analyzing of Fungal Community Structure Associated with *U. intestinalis*

Raw fastq files were demultiplexed and quality-filtered using QIIME (version 1.9.1) with the following criteria: (i) The 300 bp reads were truncated at any site receiving an average quality score <20 over a 50 bp sliding window, discarding the truncated reads that were shorter than 50bp. (ii) exact barcode matching, 2 nucleotide mismatch in primer matching, reads containing ambiguous characters were removed. (iii) only sequences that overlap longer than 10 bp were assembled according to their overlap sequence. Reads which could not be assembled were discarded.

Operational Units (OTUs) were clustered with 97% similarity cut off using UPARSE (version 7.1 http://drive5.com/uparse/) and chimeric sequences were identified and removed using UCHIME. The taxonomy of each ITS gene sequence was analyzed by RDP Classifier (http://rdp.cme.msu.edu/) against the ITS database (Unite 7) using a confidence threshold of 70% (Amato et al., 2013).

3. Results and Discussion

3.1. Sequence Analysis by MiSeq Sequencing

Total of 81,360 sequences from 162,729 reads with average length of 245.61 bp were generated by MiSeq sequencing. The sequences were assigned to 1066 operational taxonomic units (OTUs) after clustering at a 97% similarity level. The overlap of OTUs among EU and LU samples was calculated using a Venn diagram (Figure 1), as many as 265 of OTUs were overlaid and 536 OTUs specifically belong to LU and EU samples. Sequence information and the result of alpha diversity of fungal communities are shown in Table 1.

The rarefaction analysis was conducted to evaluate whether OTUs had been sufficiently recovered by MiSeq sequencing (Fu et al., 2016). Individual rarefaction curves of fungal samples showed a similar

pattern in reaching a saturation phase (Figure 2). It indicated that OTUs of both samples have been sufficiently recovered by MiSeq (Nam, Lee, & Lim, 2012).

3.2. Diversity of Fungal Community Associated with *U. intestinalis*

The richness and diversity of fungal community are represented in Table 1 . Sample EU had the higher fungal richness (ace=624.026 and chao=633.7568) compared to LU (ace=446.001 and chao = 445.1429). In addition, the rarefaction curves calculated at 97% level showed that the OTUs number of EU were higher than LU. Rarefaction curve is also a suitable tool for fungal diversity studies (Hughes et al., 2016). The results showed that the fungal richness based on rarefaction curve agreed with the alpha diversity analyses.

3.3. Composition of Fungal Communities Associated with *U. intestinalis*

The OTUs retrieved from both samples contained a large amount of unclassified fungi which was distributed in different phyla, classes and genus. The abundance of the unclassified species was defined as the percentages of the unclassified sequences. About 66.82%-72.83% at phylum level, 72.01 %-72.54 % at class level and 73.15 %-77.65 % at genus level were unable classified. These results are similar with study carried out by with Zheng, Wang, & Liu, (2014) which showed the advantages of using MiSeq to reveal the uncultured microorganism in environmental samples.

The unclassified species was omitted when calculating the abundances of the classified one (Zheng et al., 2014). Fungal communities from EU and LU samples were analyzed at phylum and class

Table 1. Sample information, fungal diversity and sequence abundance

Sample	Raw Sequences Information				Alpha Diversity				
	Sequence Number	Bases Number	Average Length	OUT Number	Shannon	Simpson	Ace	Chao	Coverage
LU	38923	9694875	2,490,783,085	443	436,815	0.054036	446,001	4,451,429	0.999741
EU	42437	10287956	2,424,289,182	622	4,493,706	0.057476	624,026	6,227,568	0.999808

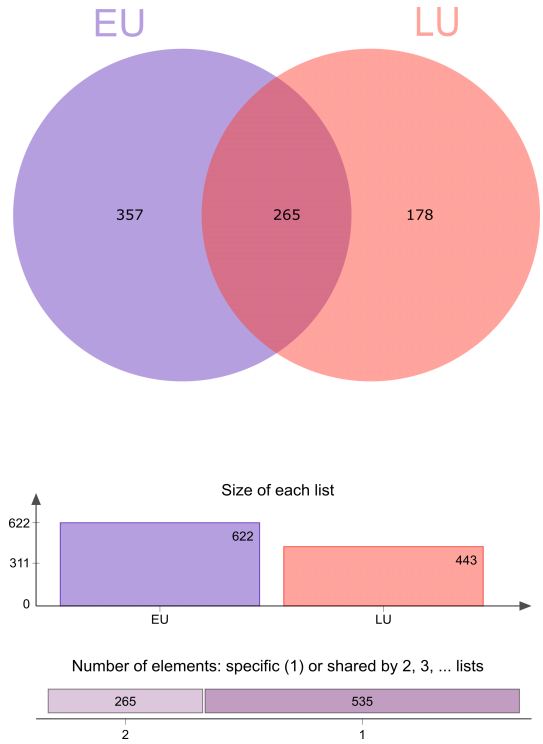

Figure 1. Venn diagram of OTUs generated from *U. intestinalis* thallus surface (LU) and rock surfaces colonized by *U. intestinalis* (EU).

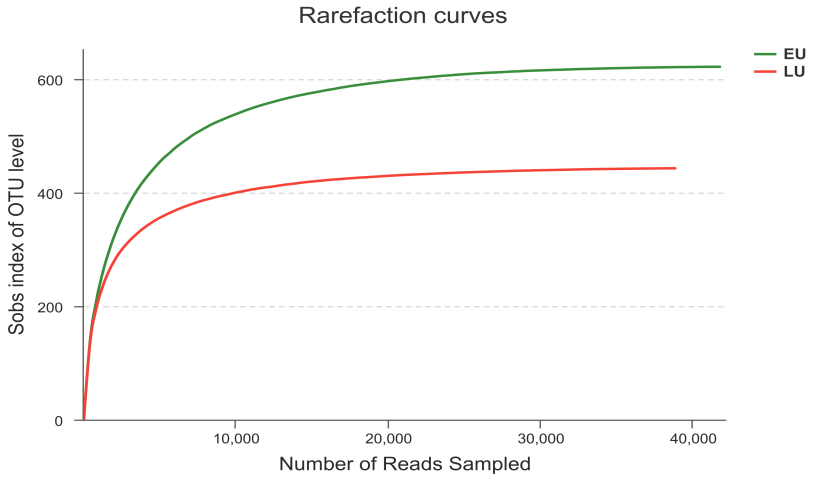

Figure 2. Rarefaction curves based on MiSeq sequencing of fungal community isolated from *U. intestinalis* thallus surface (LU) and rock surface colonized by *U. intestinalis* (EU).

level. The EU sample contained 4 phyla, 15 classes, 55 families and 77 genera, meanwhile the fungal community from LU sample contained 5 phyla, 15 classes, 45 families and 68 genera. Total of 5 phyla, 17 classes, 64 families and 101 genera of classified fungi were associated with *U. intestinalis.* Furthermore, there was an overlay taxon between classes, families and genera of EU and LU samples. The abundance and diversity of classified fungi in this study were higher than fungi associated with antarctic algae, which was only 21 genera revealed (Godinho et al., 2013). Furthermore alga *Fucus serratus* consist of 25 genera, which represented 2 phyla (Ascomycota and Zygomycota) (Zuccaro et al., 2008). Beside technique approach, the habitat condition may influence the community structure of marine microorganism (Allison & Martiny, 2008; Hou et al., 2017).

The community composition of fungi associated with *U. intestinalis* at phylum and class levels were summarized in Figure 3. The relative abundance was defined as classified sequence using Unite 7 database. As many as 11,882 reads of classified sequences was represented by 5 phyla. The dominant phyla and their abundances were similar among EU and LU samples. Ascomycota was the most dominant phylum, which representing about 78.95% - 83.25% of classified fungi associated with *U. intestinalis*. In addition, Basidiomycota were the second dominant phylum in EU and LU samples representing about 15.51% and 19.14 % of classified fungi, respectively. This results is agreed with other studies where Ascomycota were the most dominant fungi associated with antarctica algae (Godinho et al., 2013) and marine organism (algae, ascidian and sponge) of north coast of Sao Paulo, Brazil (Menezes et al., 2010).

Figure 3B presenting the population structure of fungi at class levels. The results showed that the main dominant classes and the abundance of fungi were varied among samples. Bottom of Form *Dothideomycetes* (35.83%) and *Eurotyomicetes* (27.98%) were the most dominant class in the EU and LU samples, respectively. There were two classes that unique in EU sample, *Ustilaginomycetes* and *Leotiomycetes,* while *Glomeromycetes* and *Wallemiomycetes* were only found in LU sample. However, 13 classes of fungi were found in both samples. *Dothideomycetes* was found at intertidal, primarily from mangrove habitats and mostly parasites or symbionts of sea grass or marine algae (Suetrong et al., 2009). *Eurotyomicetes* was also found associated with marine algae, ascidian and sponge of north coast of Sao Paulo (Menezes et al., 2010).

3.4. Shared Species Phylogenetic Tree of Fungal Communities Associated with *U. intestinalis*

The phylogenetic tree of fungal communities based on Bray-Curtis on the top 50 predominate bacteria at genus level was constructed. There were 12 classes associated with top 50 predominate fungi (Figure 4). Furthermore, the class of *Dothideomycetes* contributed the highest variant fungi in top 50 identified genus. The *Aspergillus* was found as the highest abundant genus in EU and LU samples, with total of 14 OTUs (4267 reads). Venn diagram also revealed that 33 and 24 genera were unique in EU and LU samples, respectively (Figure 5).

The top genera in EU sample were *Aspergillus* (10.84%), *Blastobotrys* (8.15%), *Stemphylium* (6.86%), *Alternaria* (5.49%), *Exsohilum* (5.06%). While

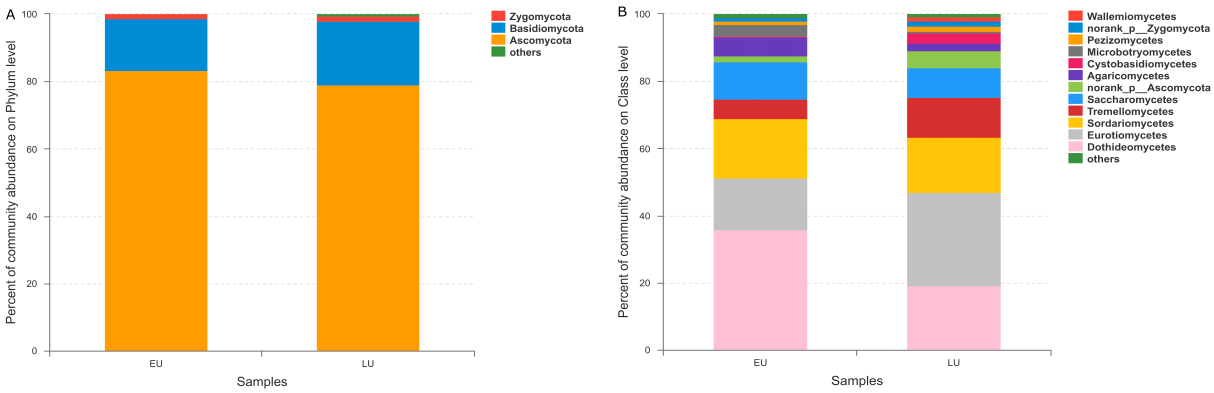

Figure 3. Community composition of fungal associated with *U. intestinalis* at A) phylum and B) class level revealed by MiSeq sequencing.

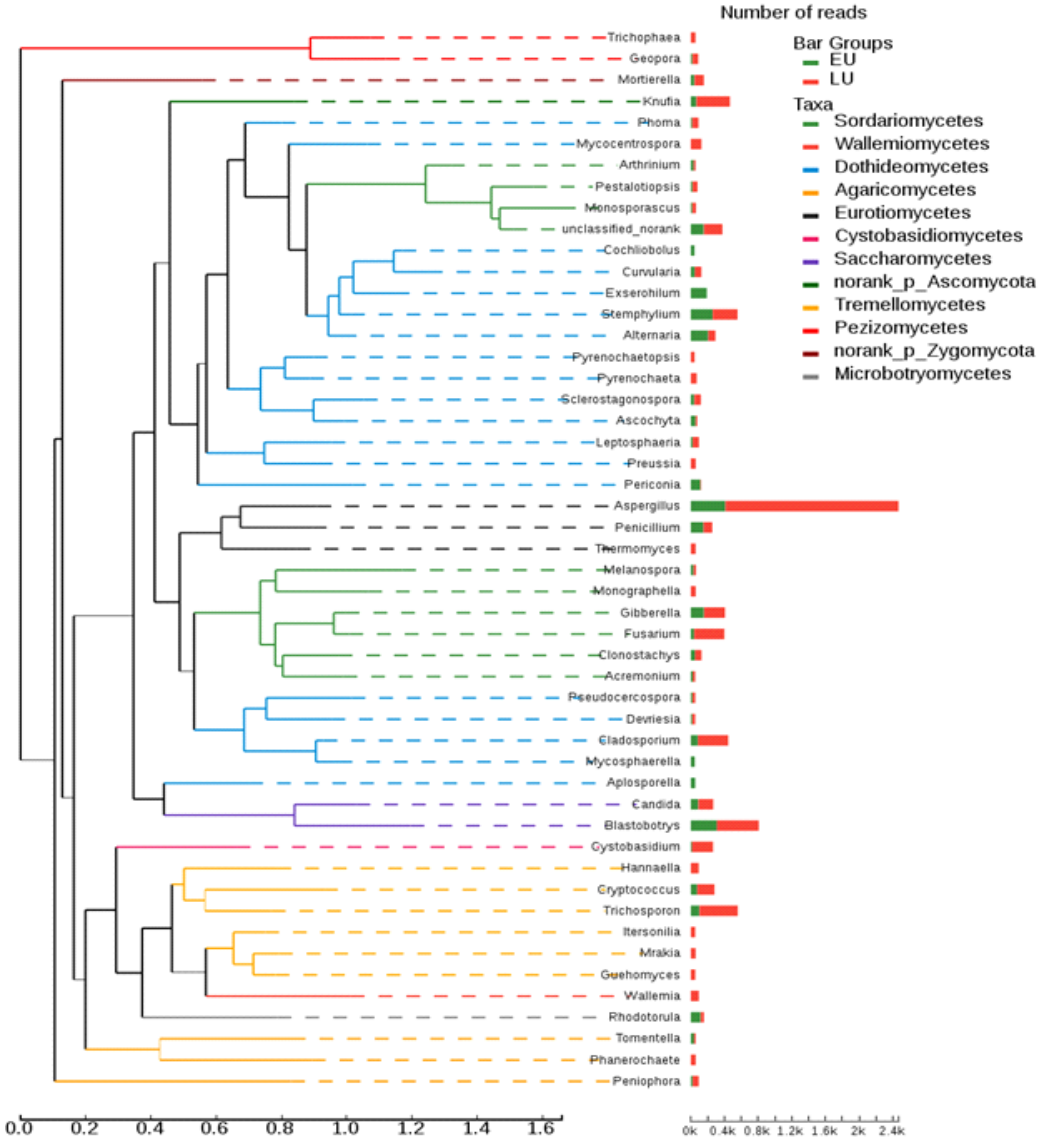

Figure 4. Phylogenetic three of fungi associated with *U. intestinalis* at genus level.

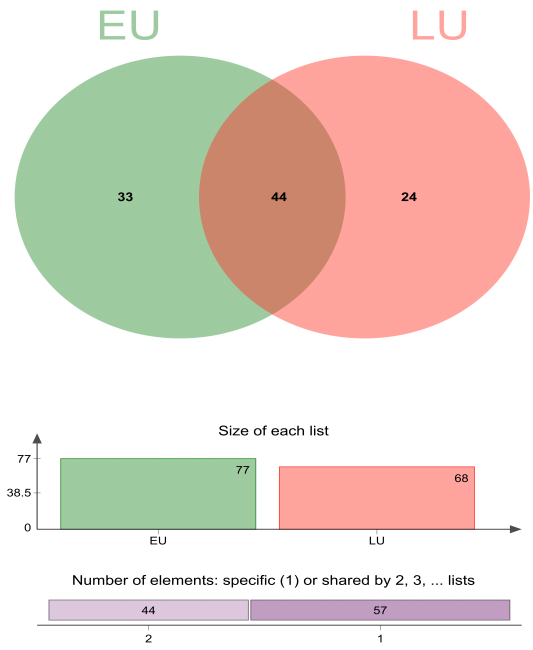

Figure 5. The Venn diagram of overlay genus of fungi generated from *U. intestinalis* surfaces (LU) and rock surfaces colonized by *U. intestinalis (EU).*

in LU were *Aspergillus* (25.40%), *Blastobotrys* (6.17%), *Trichosporon* (5.56%), *Knufia* (4.2%) and *Fusarium* (4.46%). Several fungi associated with *U. intestinalis* has been reported as sources of natural product. *Aspergilus,* the most abundant fungi associated with *U. intestinalis,* is well known as a good source of antibiotic. Natural products produced by marine origin *Aspergillus* such as stephacidin B and plinabulin showed antitumor activity (Lee et al., 2013). In addition, the crude extract of *Aspergillus terreus* had antibacterial activity against fish pathogen (Volkmann & Gorbushina, 2006).

Blastobotrys has been reported as a promising alternative yeast as donor cell which has thermo and osmotolerance ability (Flagfeldt, Siewers, Huang, & Nielsen, 2009). *Alternaria alternata* isolated from soft coral *Denderonephthya hemprichi* produced pyrophen and rubrofusarin has antimicrobial activity against *Candida albicans* (Shaaban, Shaaban, & Abdel-Aziz, 2012). The crude extract of *Knufia petricola* origin from green alga *Flabellia petiolata* showed antibacterial activities against *Burkholder metallica* and *Staphylococcus aureus* (Gnavi et al., 2016). Marine *Fusarium* isolated from Indian seawater showed the amylase activity (Lanka, Pydipally, & Latha, 2016). Neofusapyrone inhibiting the growth of *Aspergillus clavatus* while fusapyrone and deoxyfusapyrone inhibiting growth of *Pseudomonas aeruginosa* and *Aspergillus clavatus* were compound derived from marine *Fusarium* (Hiramatsu et al., 2006).

4. Conclusion

This research give an initial information about the community structures of fungi associated with *U. intestinalis*. As many as 101 genera represented 5 phyla, 17 classes and 64 families of fungal associated with *U. intestinalis*. Ascomycota (EU=78.95% and LU=83.25%) was the highest abundant phylum found in both samples. In addition, *Aspergillus* (EU=10.84% and LU=25.40%), was the most dominant genus in *U. intestinalis* thallus and rock surface colonized by *Ulva*. The dominant genera of fungi associated *with U. intestinalis* were reported as promising natural product producer. In summary, the large abundance of fungi associated with macroalgae give promising opportunity for marine natural compound sources.

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