Metagenomics Study of Fungi and Fungi-Like Organisms Associated With the Seagrass *Halophila Stipulacea* (Forssk.) Asch. From Al-Leith Mangroves, Saudi Arabia

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Abstract

This is the first metagenomics study of the fungal community associated with leaves of the seagrass *Halophila stipulacea*. Five leave samples were collected from the Al-Leith mangroves along the Red Sea coast of Saudi Arabia. Total DNA was extracted from 250 mg of each sample and the first 300 bp (contains D1-D2 variable regions) of the LSU rDNA amplicon was sequenced with the Illumina MiSeq (bTEFAP). A total of 928,626 reads were obtained from the five samples. The sequence reads belonged to the kingdoms: Metazoa (48.1% of the total reads), Viridiplantae (41.1%), Eukaryota (8.8%), Fungi (1.96%), Bacteria (0.09%), and Archaea (0.0001%). Fungi represented between 1.1% and 5.8% of the total reads in the five samples. A total of 18,279 reads (representing 1.96% of the total reads) were recorded from the 5 samples representing 296 molecular species (OTUs) that belong to 13 fungal phyla. At the phylum level, Basidiomycota dominated the community (37.2–51.6%) in three samples, while Neocallimastigomycota (37.5%) and Mucoromycota (42.1%) dominated the community in the fourth and the fifth sample respectively. High diversity of OTUs (28 molecular species) were recorded from the monokaryotic subkingdom with five unknown basal lineages that are not aligning with any known taxa. Total number of sequence reads of fungi-like organisms (Stramenopiles) from the five samples ranged between 0.16% of total reads in the fifth sample (AL-Hs05) to 2.9% in the first one (AL-Hs01). Majority of the fungi-like organisms reads (93.6%) belong to the phylum Oomycota, followed by Opisthokonts (Fungi/Metazoa group) representing 6.4% of fungi-like reads. Monokaryon phyla (i.e. Chytridiomycota, Mucoromycota and Neocallimastigomycota) and fungi-like organisms occupied a major portion of the sequences reads followed by Basidiomycota and Ascomycota. Our results support findings that the majority of fungi and fungi-like organisms' communities are so far unknown with seven deep branching lineages remain to be cultivated.

1. Introduction

Seagrasses are flowering plants inhabiting marine environments and have a worldwide distribution, with 50 species reported that cover about 60,000 km^2 (Logan 1992; Phang 2000; Raghukumar 2017). Seagrasses provide food, habitats, and breeding grounds to a variety of marine species. In addition, meadows of seagrass are important carbon sinks and sequester between 10 to 18% of the ocean's carbon reservoir for long-term storage (Pernice et al. 2016). Seagrasses prevent erosion by trapping and binding sediments and by their death, provide substantial amounts of nutrition to organisms living within the seagrass ecosystem, as well as to those in mangroves and corals and other ecosystems (Wilson 1998; Phang 2000; Raghukumar 2017). Primary productivity of seagrass meadows is among the highest of aquatic ecosystems (Duarte and Chiscano 1999). More than 50% of seagrass production enters the detrital food web (Duarte and Cebrian 1996). Seagrasses represent one of the most valuable ecosystems on Earth, with an estimated value of $ 2.8 10^6 /yr/km^2 (Costanza et al. 2014).

Fungi and fungi-like organisms (pathogens, mutualistic or saprobes) play a crucial role in functioning of the seagrass ecosystem. Both obligate and facultative marine fungi were reported from seagrasses (Cuomo et al 1985; Alva et al. 2002; Jones 2011; Jones et al. 2019). Considerable information is available on the occurrence of marine fungi on wood and other cellulosic material (Jones et al. 2019, 2020; Abdel-Wahab et al. 2020; Devadatha et al. 2021), but little is known of fungi associated with seagrasses (Raghukumar 2008; Sakayaroj 2010, 2012; Poli et al. 2020).

1.1. Marine fungi of seagrasses

Five marine fungi were reported from decaying leaves of *Zostera marina* L. namely *Alternaria* sp., *Corollospora maritima* Werdermann, *Lulworthia* sp., *Phoma* sp. and *Varicosporina ramulosa* Meyers et Kohlm. (Mounce and Diehl 1934; Kohlmeyer 1963a, 1966). Feldmann (1959) described the parasitic smut basidiomycete fungus, *Flamingomyces ruppiae* (Feldmann) R. Bauer, M. Lutz, Piątek, Vánky & Oberw. from rhizomes of *Ruppia maritima* L. from a salt lagoon in southern France. In addition, Kohlmeyer (1962) reported *Lulworthia* sp. from the same seagrass. Seven marine taxa reported from leaves of *Thalassia testudinum* Koning, namely *Corollospora lacera* Linder, C. *maritima, Lindra thalassiae* Orpurt, Meyers, Boral &

Simms, *Lulworthia* sp., *Paradendryphiella salina* (G.K. Sutherl.) Woudenb. & Crous, and *Varicosporina ramulosa* (Orpurt et al. 1964; Meyers and Kohlmeyer 1965; Meyers et al. 1965; Meyers 1969; Kohlmeyer and Kohlmeyer 1977). Kohlmeyer (1963b) reported *Halotthia posidoniae* Kohlmeyer and *Pontoporeia biturbinata* (Durieu & Montagne) Kohlm. on rhizomes of *Posidonia oceanica* (L.) Delile, and *Lulworthia* sp. on rhizome of *Syringodium filiforme* Kütz. Cuomo et al. (1985) recorded seven obligate marine fungi: *Corollospora maritima*, C. *intermedia, Halotthia posidoniae*, *Papulospora halima* Anastasiou, *Phoma* sp., *Pontoporeia biturbinata* and *Lulworthia* sp. from *Posidonia oceanica*.

Sterile mycelia forms dominated the fungal community of *Zostera marina* collected from Chesapeake Bay, USA (Newell 1981). Other reported fungi were *Acremonium* sp., *Cladosporium* sp., *Paradendryphiella salina*, *Lulworthia* sp., *Sigmoidea* sp., and *Varicosporina ramulosa*. Zoosporic fungi were completely absent (Newell 1981).

1.2. Endophytic fungi of seagrasses

Kuo (1984) and Kuo et al. (1990) examined *Zostera muelleri* Irmisch ex Asch. leaves and recorded fungal hyphae in the intercellular spaces and cell walls. Sathe and Raghukumar (1991) isolated species of the genera: *Acremonium*, *Chaetomium*, *Graphium*, *Humicola*, and *Penicillium* from surface sterilized, decomposing leaves of *Thalassia hemprichii* Asch. from the coral reef islands of the Lakshadweep in the Arabian Sea. While, Panno et al. (2011) isolated 88 fungal taxa (70 ascomycetes, 4 basidiomycetes and 14 unidentified fungi) all of which belong to terrestrial fungal genera with *Penicillium, Cladosporium* and *Acremonium* were the most abundant genera from four districts of a *Posidonia oceanica* meadow in the Mediterranean Sea. Wilson (1998) reported eleven endophytic fungi from three seagrasses: *Halodule bermudensis* Hartog, *Syringodium filiforme* and *Thalassia testudinum* collected from Bermuda. Alva et al. (2002) cultured 95 endophytic fungal isolates from three seagrasses: *T. testudinum*, *Zostera japonica* Asch. & Graebn and *Z. marina* collected from Hong Kong and the Philippines. Much lower diversity (only six endophytic fungi) were isolated from *Halophila ovalis* (R.Br.) Hook.f. collected from India. Thirteen endophytic fungal isolates were cultured from *T. testudinum* in Puerto Rico (Rodríguez 2008). Sakayaroj et al. (2010) cultured 42 endophytic fungal isolates from *Enhalus acoroides* (L.f.) Royle collected from Thailand. Molecular phylogenetic analyses based on ribosomal genes placed the isolated taxa in Ascomycota (98%) and Basidiomycota (2%). Ascomycota were represented by three major classes namely: Sordariomycetes (36%), Eurotiomycetes (33%) and Dothideomycetes (24%). In a recent study, Venkatachalam et al. (2015) cultured 305 endophytic fungal isolates representing 30 species from 10 seagrasses species from India. All isolated fungi belong to terrestrial genera with *Aspergillus*, *Paecilomyces* and *Penicillium* the most commonly encountered. Poli et al. (2020) listed 61 species from *Posidonia oceanica* including the new genus *Paralulworthia* A. Poli, E. Bovio, L. Ranieri, G.C. Varese & V. Prigione and two species belonging to the Lulworthiales: *P. gigaspora* A. Poli, E. Bovio, L. Ranieri, G.C. Varese & V. Prigione and *P. posidoniae* A. Poli, E. Bovio, L. Ranieri, G.C. Varese & V. Prigione. Thirty-seven fungi were reported for the first time from the Mediterranean Sea. Furthermore, by canonical analysis of principal coordinates (CAP) they demonstrated that the fungal communities on the three different marine hosts (*Posidonia oceanica*, *Flabellia petiolata* (Turra) Nizam. – green alga, and *Padina pavonica* (L.) Thivy – brown alga) were quite distinct.

A few obligate marine fungi were isolated as endophytes of seagrasses. Mata and Cebrian (2013) cultured 14 fungi from two seagrasses (*Halodule wrightii* and *Thalassia testudinum*) collected from north-central Gulf of Mexico including four obligate marine fungi namely: *Trichocladium alopallonellum* (Meyers & R.T. Moore) Kohlm. & Volkm.-Kohlm., *Halenospora varia* (Anastasiou) E.B.G. Jones, *Paradendryphiella arenariae* (Nicot) Woudenb. & Crous and *Lindra thalassiae* Orpurt, Meyers, Boral & Simms. Authors used light surface sterilization (0.5% bleach solution and sterile artificial sea water, 2 minutes each) and this might be adopted in future studies of endophytes of seagrasses.

1.3. Fungi-like organisms of seagrasses

Labyrinthula zosterae Porter & Muehlstein is the pathogen of the wasting disease that caused extensive, catastrophic losses of *Zostera marina* along the Atlantic coasts of North America and Europe in the 1930s (Tutin 1934; Short et al. 1986; Muehlstein et al. 1988; Sullivan et al. 2013). Several species of *Labyrinthula* were reported as pathogens from different species of seagrasses, however without causing serious losses (Durako and Kuss 1994; Steele et al. 2005; Leaño and

Damare 2012). *Aplanochytrium schizochytrops* (J.A. Quick) C.A. Leander & D. Porter was recorded from 80% of yellow to brown leaves of *Halodule wrightii* from Florida, USA (Quick 1974). Sathe and Raghukumar (1991) recorded straminipilan fungi from decaying seagrasses. They recorded *Aplanochytrium minutum* (S. W. Watson & Raper) C. A. Leander & D. Porter and *Thraustochytrium motivum* Goldstein from decomposing *Thalassia hemprichii* from the Lakshadweep Islands of the Arabian Sea.

Man in 't Veld et al. (2011) described *Phytophthora gemini* Man in 't Veld, K. Rosend., H. Brouwer & De Cock from decaying leaves and seeds of *Zostera marina* in Netherlands. Man in 't Veld et al. (2019) recorded seven taxa of oomycetes colonizing seeds and leaves of *Zostera marina* collected from Western Europe (Denmark, France, Germany, the Netherlands and Sweden) and the east coast of the USA. They described *Phytophthora chesapeakensis* Man in 't Veld & K. Rosendahl, and recorded the two known species *P. gemini* and *P. inundata* Brasier, Sánch. Hern. & S.A. Kirk, with three unidentified species of *Halophytophthora* and *Salisapilia sapeloensis* Hulvey, Nigrelli, Telle, Lamour & Thines. Ettinger and Eisen (2020) isolated two *Halophytophthora* species from leaves of *Zostera marina* collected from California, USA.

1.4. Microbial and fungal biomass in seagrasses

Blum et al. (1988) determined the total microbial biomass that ranged between 0.12 to 0.7% of detrital dry weight in four seagrasses namely: *Halodule wrightii*, *H. decipiens*, *Thalassia testudinum*, and *Syringodium filiformis*. While, Sathe and Raghukumar (1991) estimated a higher total fungal biomass of 3.4% in detritus of *Thalassia hemprichii* in the Lakshadweep islands of the Arabian Sea. However, that difference might be accounted for the methods that authors adopted.

1.5. The seagrass *Halophila stipulacea*

Halophila stipulacea (Hydrocharitaceae) is native to the tropical and subtropical waters of the Red Sea, Arabian Gulf and Indian Ocean (De la Torre-Castro and Ronnback 2004). *H. stipulacea* spread to the Mediterranean after the opening of the Suez Canal where it forms insulated, small populations across the basin (Gab-Alla 2001; El-Hady et al. 2012; Daisie 2015; Gisd 2015; Winters et al. 2020). In 2002, it was reported in the Caribbean Sea, where within less than two decades it spread to most of the Caribbean Island nations and reaching the South American continent. Unlike its invasion of Mediterranean, in the Caribbean *H. stipulacea* creates large, continuous populations in many areas. Reports from the Caribbean demonstrated the invasiveness of *H. stipulacea* by showing that it displaces local Caribbean seagrass species (Winters et al. 2020). Seagrasses play an important role in the ecology of various ecosystems and have been used in folk medicine for treatment of various diseases (De la Torre-Castro and Ronnback 2004). *H. stipulacea* was reported to have antioxidant and antibacterial activities (Rengasamy et al. 2012; Gumgumjee et al. 2018). Weidner et al. (2000) studied the phylogenetic diversity of the bacterial community associated with leaves of *H. stipulacea* in the northern Gulf of Elat, Red Sea using culture-independent method based on 16S rDNA clone library. In their study, the class Proteobacteria represented 62.6% of the clone sequences, while 7.1% of the sequences possibly belonged to the class Proteobacteria, but branched deeply from known subclasses. There is no previous report of fungi from *H. stipulacea* either at the morphological or metagenomics level.

1.6. Metagenomic study of microbes from seagrasses

Culture based methods uncover 1–5% of the total microbial diversity that exist in an environmental sample (Simões et al. 2013; Kennedy et al. 2020). While, culture-independent methods, e.g., metagenomics, provide wider microbial diversity as it circumvents culture-based biases (Cuadros-Orellana et al. 2013; Guo et al. 2015; Liu et al. 2015).

Fraser et al. (2018) studied the taxonomic and functional changes in the microbial communities of sediments from six seagrass meadows along gradients of salinity and phosphorus in Shark Bay, Australia. In their study, the dominant phylum was Proteobacteria representing 48–53% of sequences followed by Bacteroidetes (10–11%), Planctomycetes (6–9%), Firmicutes (5–6%), Actinobacteria (4.3–4.7%), and Cyanobacteria (3.6–5.9%). Previous surveys of fungi and fungi-like organisms from leaves and rhizomes of seagrasses have been based on culture-dependent techniques (e.g. Cuomo et al.

1985; Panno et al. 2011; Mata and Cebrian 2013; Man in 't Veld et al. 2019; Ettinger and Eisen 2020; Poli et al. 2020). Segovia et al. (2021) studied the epibiotic microeukaryotes on *Zostera marina* leaves, substrates, and planktonic microeukaryotes in ten meadows in the Northeast Pacific. They rinsed the surface of *Z. marina* leaves with sterile seawater, then swabbed the surface of the leaves and extracted total DNA from the swabs. They identified sixteen core microeukaryotes, including dinoflagellates, diatoms, and saprotrophic stramenopiles.

This study aims to document the fungal community from decaying leaves of the seagrass *Halophila stipulacea* using metagenomics study based on LSU rDNA.

2. Materials And Methods

2.1. Collection of samples and DNA extraction

On 7 April 2015, we collected five samples of decaying thalli of *Halophila stipulacea* (Fig. 1) randomly from distant locations from the intertidal zone of Al-Leith mangroves (20^{o} 02' 08' N 40^{o} 25' 66" E), 20 km south of Al-Leith city near Bin Saleh gas station, along the Red Sea coast, Saudi Arabia. *Avicennia marina* is the only mangrove tree occurring at this mangrove site. The forest floor was soft and muddy and the trees reached circa 6 meters (Abdel-Wahab et al. 2019). Decaying leaves of *Halophila stipulacea* thalli were washed in sterile seawater and homogenized in liquid nitrogen using a mortar and pestle, and 250 mg of the resulting homogenate was used for genomic DNA extraction using the UltraClean® Soil DNA Isolation Kit (MO BIO Laboratories Inc., USA) according to the manufacturer's instructions. DNA samples were quantified using a Nanodrop spectrophotometer (Spectrostar nano with optic, BMG, Germany). Total DNA samples were sent to MR DNA, Molecular Research LP, USA (www.mrdnalab.com) for metagenomics analyses.

2.2. Molecular methods

PCR, library construction, and Illumina MiSeq (bTEFAP) amplicon sequencing were carried out at MR DNA, Molecular Research LP, USA. All DNA samples were adjusted to 100 ng/µl, of which 1 µl was used for a 50 µl PCR reaction. The universal primers LROR (5'-ACCCGCTGAACTTAAGC-3') and LR22 (5'-CCTCACGGTACTTGTTCGCT-3') were used for amplifying the first 300 bp of LSU rDNA (Vilgalys and Hester 1990). HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was used for PCR under the following conditions: 94°C for 3 minutes followed by 32 cycles of 94°C for 30 seconds; 60°C for 40 seconds and 72°C for 1 minute; and a final elongation step at 72°C for 5 minutes. A secondary PCR was performed for FLX (Roche, Nutley, New Jersey) amplicon sequencing under the same condition by using designed special fusion primers with different tag sequences as: LinkerA-Tags-530F and LinkerB-1100R (Dowd et al. 2008a,b). After secondary PCR all amplicon products from different samples were mixed in equal volumes, and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA).

2.3. Sequence data analysis

The Q25 sequence data derived from the sequencing process was processed using a proprietary analysis pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX). Sequences are depleted of barcodes and primers then short sequences < 200bp were removed, sequences with ambiguous base calls removed, and sequences with homopolymer runs exceeding 6bp removed. Sequences are then denoised and Operational taxonomic units were defined clustering at 3% divergence (97% similarity) followed by removal of singleton sequences and chimeras (Dowd et al. 2008a, b; Edgar 2010; Capone et al. 2011). Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI (www.ncbi.nlm.nih.gov, DeSantis et al 2006, http://rdp.cme.msu.edu).

2.4. Phylogenetic trees

We placed core OTUs into phylogenetic trees to investigate their relationships and refine taxonomic assignments. OTUs were blasted in the GenBank and sequences with less than 95% similarity and unknowns were excluded from the analyses..

The most closely related sequences and the sequences of representative fungal species were aligned with our sequences by using CLUSTALX (Thompson et al. 1997). Ambiguously aligned regions were excluded from the alignment. Maximum-parsimony (MP) and maximum-likelihood (ML) phylogenetic analyses were carried out using MEGA X (Kumar et al. 2018). ML analysis (Felsenstein 1985) was performed using the Tamura–Nei model. Bayesian phylogenetic analysis was performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Five million generations were run in four chains with sampling every 100 generations, yielding 50,000 trees, of which the first 12,500 were discarded as "burn in." The phylogenetic trees were visualized using Njplot (Perrière and Gouy 1996) and edited using Adobe Illustrator CS6.

3. Results And Discussion

3.1. Microbial and fungal communities structure and diversity of the five metagnomic samples of *Halophila stipulacea*

A total of 928,626 reads were obtained from the 5 samples. The sequence reads belonged to the kingdoms: Metazoa (48.1% of the total reads), Viridiplantae (41.1%), Eukaryota (8.8%), Fungi (1.96%), Bacteria (0.09%), and Archaea (0.0001%). Fungi represented between 1.1% and 5.8% of the total reads in the five samples. A total of 18,279 reads (representing 1.96% of the total reads) were recorded from the 5 samples representing 296 molecular species (OTUs) that belong to 13 fungal phyla (Table 1, Fig. 2). Number of the recorded phyla from the five samples ranged between 10 in sample (AL-Hs04) and 13 in sample (AL-Hs03). At the phylum level, Basidiomycota dominated the community (37.2–51.6%) in three samples (AL-Hs01, AL-Hs02 and AL-Hs03), while Neocallimastigomycota (37.5%) and Mucoromycota (42.1%) dominated the community in the fourth sample (AL-Hs04) and the fifth sample (AL-Hs05) respectively. Other recorded major phyla were Ascomycota (3.1–21.2%), Chytridiomycota (14–24%) and Mortierellomycota (1.3–25.9%) (Fig. 1). Sixteen common genera (representing more than 1% of the total fungal reads) were reported from the five metagenomic libraries of *Halophila stipulacea*: *Mucor* (12.56%), *Mortierella* (11.58%), *Neocallimastix* "anaerobic" (11.32%), *Malassezia* "Yeast" (11.11%), *Entophlyctis* (3.31%), *Geranomyces* (2.07%), *Gonapodya* (2%), *Anaeromyces* (1.99%), *Clitocybula* (1.93%), *Cuphophyllus* (1.79%), *Coltricia* (1.76%), *Ascotaiwania* (1.6%), *Clavispora* "Yeast" (1.31%), *Allochytridium* (1.28%), *Pichia* "Yeast" (1.17%) and *Phellodon* (1.07%). The sixteen genera belonged to: Ascomycota (3 genera), Basidiomycota (5), Chytridiomycota (3), Monoblepharomycota (1), Mucoromycota (1) and Neocallimastigomycota (2). The three most common genera belonged to monokaryon phyla (Mucoromycota, Mortoerellomycota and Neocallimastigomycota). Monokaryotic phyla or lower fungi are dominating the fungal communities of the cellulosic materials in the sea (Jones et al. 2015, 2019, Devadatha et al. 2021). Although the diversity of marine Basidiomycota is low in culture-based methods, current and previous studies show high diversity of marine Basidiomycota in metagenomics studies (Simões et al. 2015).

Table 1 Sequence reads for fungi and fungi-like in the five studied samples

Sample	Total No. of raw reads	Fungi						Fungi-like			
		No. of reads	%	Monokaryon phyla (%)	Dikaryon phyla (%)	No. of genera	No. of OTUs	No. of reads	%	No. of genera	No. of OTUs
AL-Hs01	235,596	5,054	2.1	51.5	48.5	184	239	6,914	2.9	22	38
AL-Hs02	268,632	4,408	1.6	27.2	72.8	155	188	2,067	0.77	18	30
AL-Hs03	166,089	1,822	1.1	52.9	47.1	147	170	2,064	1.2	18	28
AL-Hs04	84,607	4,944	5.8	74.1	25.9	102	134	470	0.55	14	22
AL-Hs05	173,703	2,055	1.2	57.1	42.9	126	145	272	0.16	14	23

Table 2 Top 10 abundant fungal taxa recorded from the five metagenomic samples, figures are number of sequence reads:

Fungi	AL-Hs01	AL-Hs02	AL-Hs03	AL-Hs04	AL-Hs05	Total	%*
Mucor	180	268	145	826	857	2276	12.45
Neocallimastix	229	147	96	1506	92	2070	11.32
Mortierella	1286	275	236	245	24	2066	11.3
Malassezia	334	885	214	167	412	2012	11
Mortierella	1114	141	200	238	18	1711	9.36
Entophlyctis	32	15	13	541	5	606	3.32
Geranomyces	179	72	48	34	47	380	2.1
Anaeromyces	9	8	9	336	2	364	1.99
Clitocybula	7	8	4	332	2	353	1.93
Cuphophyllus	98	210	15	3	2	328	1.79

* Percentage of the total fungal reads in the five metagenomic samples.

3.2. Phylogenetic analyses of OTUs referred to Ascomycota

Ten OTUs grouped with genera in the order Saccharomycetales with moderate statistical support (58 ML/ 78 MP/ 84 BYPP), of which six OTUs have phylogenetic affinity with the genus *Pichia* E.C. Hansen, two OTUs with *Hanseniaspora* Zikes, one with *Yamadazyma* Billon-Grand, and one with *Cyberlindnera* Minter. Yeasts were frequently recorded from mangroves, with more than 138 species referred to Ascomycota and 75 taxa to Basidiomycota that were recorded from marine habitats (Jones et al. 2015). OUT-1001 is phylogenetically related to the *Aspergillus* clade in a highly supported clade (100 ML/ 100 MP/ 100 BYPP). Forty-seven species of *Aspergillus* were routinely isolated from various substrates in marine habitats (Jones et al. 2015, 2019). Two OTUs nested within *Hortaea werneckii* (Horta) Nishim. & Miyaji clade with

high statistical support (78 ML/ 94 MP/ 94 BYPP). *H. werneckii* was recorded from decaying wood and leaves of *Avicennia marina* (Forssk.) Vierh. from the Red Sea mangroves in Saudi Arabia (Abdel-Wahab et al. 2014; Hodhod et al. 2020). Two OTUs nested within the order Hypocreales that include one OTU related to *Fusarium* Link with high statistical support (100 ML/ 100 MP/ 100 BYPP) and one OUT grouped with *Trichoderma* Pers. in a highly supported clade (Fig. 3).

3.3. Phylogenetic analyses of OTUs referred to Basidiomycota

Six OTUs nested within the clade of the yeast genus *Malassezia* Baill. with high statistical support (92 ML/ 94 MP/ 100 BYPP). The genus *Malassezia* represented 11.11% of the total fungal reads in the current study. The sequences reads of *Malassezia* were frequently recorded in high percentages in previous metagenomics studies from marine habitats (e.g. Nagahama et al. 2011; Amend 2014). *Malassezia* species cause skin diseases in terrestrial and marine mammals (Guillot et al. 1998; Nakagaki et al. 2000; Pollock et al. 2000). One OUT formed a sister taxon to *Antrodiopsis oleracea* (R.W. Davidson & Lombard) Audet (Fig. 4).

3.4. Phylogenetic analyses of OTUs referred to monokaryotic subkingdom

High diversity of OTUs (23 molecular species) were recorded from the monokaryotic subkingdom with five unknown basal lineages that are not aligning with any known taxa. Six OTUs (AL-Hs-Mono-1) formed a basal clade to the phylum Entomophthoromycota, another 5 OTUs (AL-Hs-Mono-2) formed a basal clade to Kickxellomycota. The other three unknown clades (two OTUs each) formed a basal clade to other monokaryon phyla. Six OTUs clustered with known taxa in the phyla Chytridiomycota, Kickxellomycota and Mucoromycota (Fig. 5).

3.5. Phylogenetic analyses of OTUs referred to fungi-like organisms

Total number of sequence reads of fungi-like organisms from the five samples of *Halophila stipulacea* ranged between 0.16% of total reads in AL-Hs05 sample to 2.9% in AL-Hs01. Majority of the fungi-like organisms reads (93.6%) belong to the phylum Oomycota and represented by 18 genera followed by Opisthokonts (Fungi/Metazoa group) representing 6.4% of fungi-like reads and represented by 4 genera (Tables 1, 3). The most common genus was *Halioticida* Muraosa & Hatai and represented 5.5%-78% of the total fungi-like reads in the five studied samples followed by *Halophytophthora* H.H. Ho & S.C. Jong (3.2–62.9%) and *Enterobryus* Leidy (0.4–57.9%).

High diversity of OTUs (25 molecular species) were recorded from the fungi-like organisms in the kingdom Protista with two unknown basal clades. Twelve OTUs formed unknown basal clade (AL-Hs-FungL-1) to the families Crypticolaceae, Leptomitaceae, Myzocytiopsidaceae and Saprolegniaceae. Another six OTUs formed the second unknown clade (AL-Hs-FungL-2) that is a sister to the representatives of the family Pythiaceae. Two OTUs nested within *Halophytophthora* clade with moderate statistical support. One OUT formed a sister branch to the genus *Salisapilia* Hulvey, Nigrelli, Telle, Lamour & Thines with high statistical support (100 ML/ 100 MP/ 100 BYPP). One OTU formed a basal branch to the families Leptomitaceae and Saprolegniaceae. One OTU nested within Crypticolaceae closely related to the genera *Halocrusticida* K. Nakam. & Hatai and *Halodaphnea* M.W. Dick. The last OTU formed a basal branch to the families Haptoglossaceae and Eurychasmataceae (Fig. 6).

The genera *Halophytophthora* and *Salisapilia* were previously reported from the seagrass *Zostera marina*. Man in 't Veld et al. (2019) recorded three unknown species of *Halophytophthora* and *Salisapilia sapeloensis* from seeds and leaves of *Zostera marina* collected from Western Europe and the east coast of the USA. Also, Ettinger and Eisen (2020) isolated two *Halophytophthora* species from leaves of *Z. marina* collected from California, USA.

Table 3 Frequency of occurrence of the top ten fungi-like taxa recorded from the five						
metagenomic samples:						
Fungi/samples	AL-Hs01	AL-Hs02	AL-Hs03	AL-Hs04	AL-Hs05	Total
Oomycota, Chromista:						
Halioticida	77	63	78	8.1	5.5	70.3
Halophytophthora	3.89	11.7	7.65	12.9	66.2	7.7
Plasmodiophora	7.8	3.6	3	7.2	8.1	6.2
Halocrusticida	1.01	1.05	3.9	0.4	0	1.5
Saprolegnia	1.08	1.3	0.85	1.25	3.7	1.17
Haliphthoros	1	2	0	0.4	0.4	0.92
Lagenidium	0.11	1.05	0.85	6.8	2.5	0.73
Aphanomyces	1.01	0.05	0	0	0.7	0.6
Opisthosporidia:						
Sarcocystis	4.3	0.4	0.77	1.1	0	2.8
Protozoa:						
Enterobryus	0.49	13.25	2.5	57.9	5.6	5.49

4. Conclusion

Cuomo et al. (1985) recorded seven obligate marine fungi: *Corollospora maritima*, C. *intermedia, Halotthia posidoniae*, *Papulospora halima* Anastasiou, *Phoma* sp., *Pontoporeia biturbinata* (Dur. & Mont.) Kohlm., and *Lulworthia* sp. from *Posidonia oceanica*. On the contrary, of the previous results, Panno et al. (2011) isolated 88 fungal taxa (70 ascomycetes, 4 basidiomycetes and 14 unidentified fungi) all of which belong to terrestrial fungal genera from four districts of *Posidonia oceanica* meadows with *Penicillium, Cladosporium* and *Acremonium* the most abundant genera. This clearly demonstrate that the obtained results depends on the methods that authors adopt.

Newell and Fell (1980) and Newell (1981) concluded that fungi do not play a dominant role in the degradation of seagrasses under submerged condition, while fungi have an active role in the degradation of washed seagrasses in the intertidal zone. That is why documented mycobiota of seagrasses vary from one study to another. Samples that are freshly deposited to the wrack line in the intertidal zone have a microbial community that differs from those colonizing the heavily degraded seagrasses on the shore. Every method has its own bias. Endophytic isolation methods especially with heavy surface sterilization favor the isolation of marine-derived fungi (e.g. *Acremonium*, *Aspergillus*, *Cladosporium* etc.) (e.g. Sakayaroj et al. 2010), while direct examination of seagrasses produce obligate marine fungi (e.g. *Corollospora*, *Paradendryphiella*, *Lindra*, *Lulworthia* etc.) (e.g. Kohlmeyer and Kohlmeyer 1977; Cuomo et al. 1985). Fungi-like organisms, monokaryon phyla and Basidiomycota are well represented in the current metagenomics study with a wider range of taxa than those recorded by culture-based methods. However, we need to be careful when interpreting the metagenomics data as the primers and methodology might bias the obtained results.

In the current study, seven deep branching lineages that did not cluster with any known microbial taxa were recorded which clearly shows that a wide range of microbes are yet to be cultured from the sea grass *Halophila stipulacea.* In a previous

study of the bacterial community of *H. stipulacea* from Gulf of Elat, Red Sea based on 16S rDNA clone library (Weidner et al. 2000), they reported three unknown deep branching lineages that are not aligned with any known taxa. In summary, this study adds new knowledge of fungal diversity from decaying fronds of *H. stipulacea* and highlights a high diversity and abundance of fungi in the five samples investigated and previously not known. While the number of marine fungi documented (1,901: www.marinefungi.org), largely due to direct observation of substrates and culture-based studies is impressive, metagenomic studies and the development and analysis of sequence data will greatly add to our knowledge and lead to a better understanding of their physiological and biochemical role in the marine environment. Clearly the many new linages highlighted in this study require further investigation, especially their identity, host and function.

Declarations

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Author contribution

Conceptualization: Mohamed A. Abdel-Wahab and E.B.G. Jones. Methodology: Mohamed A. Abdel-Wahab, Ali H. Bahkali, Abdallah M. El-Gorban. Formal analysis and investigation: Mohamed A. Abdel-Wahab, Ali H. Bahkali, Abdallah M. El-Gorban and E.B. Gareth Jones. Resources: Mohamed A. Abdel-Wahab, Ali H. Bahkali, Abdallah M. El-Gorban and E.B. Gareth Jones. Writing—original draft preparation: Mohamed A. Abdel-Wahab, Ali H. Bahkali, Abdallah M. El-Gorban and E.B. Gareth Jones. Writing—review and editing: Mohamed A. Abdel-Wahab, Ali H. Bahkali, Abdallah M. El-Gorban and E.B. Gareth Jones. Funding acquisition: Ali H. Bahkali and E.B. Gareth Jones. All authors have read and agreed to the submitted version of the manuscript.

Conflict of interest

The authors declare no competing interests.

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Data availability

All data generated or analyzed in this study are included in this article. All alignments and trees from this study are available from TreeBASE (accession number 28249). The seventy sequences used to generate the four phylogenetic trees in this article are available from GenBank with accession numbers: MZ018046–MZ018115.

Reviewer access URL:
http://purl.org/phylo/treebase/phylows/study/TB2:S28249?x-access-code=f451aa07faaf8c8b2af9d541b361ff36&format=html

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Figures

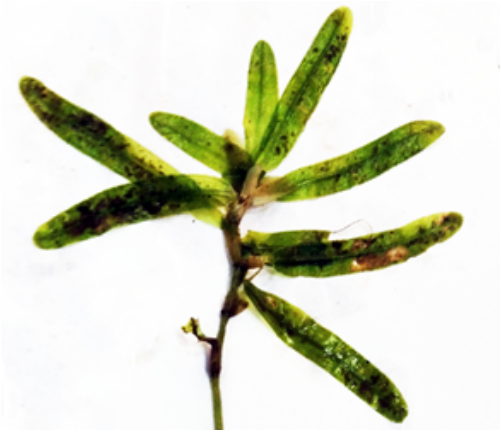

Figure 1

Decaying thallus of Halophila stipulacea.

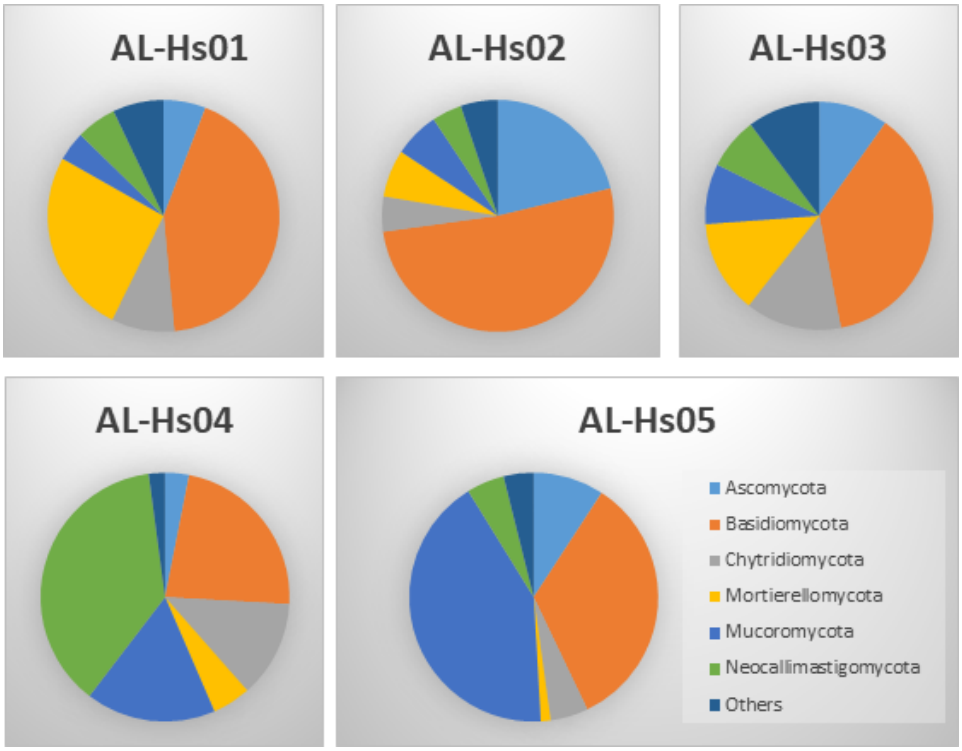

Figure 2

Percentages of the phyla recorded from the five metagenomics samples of Halophila stipulacea.

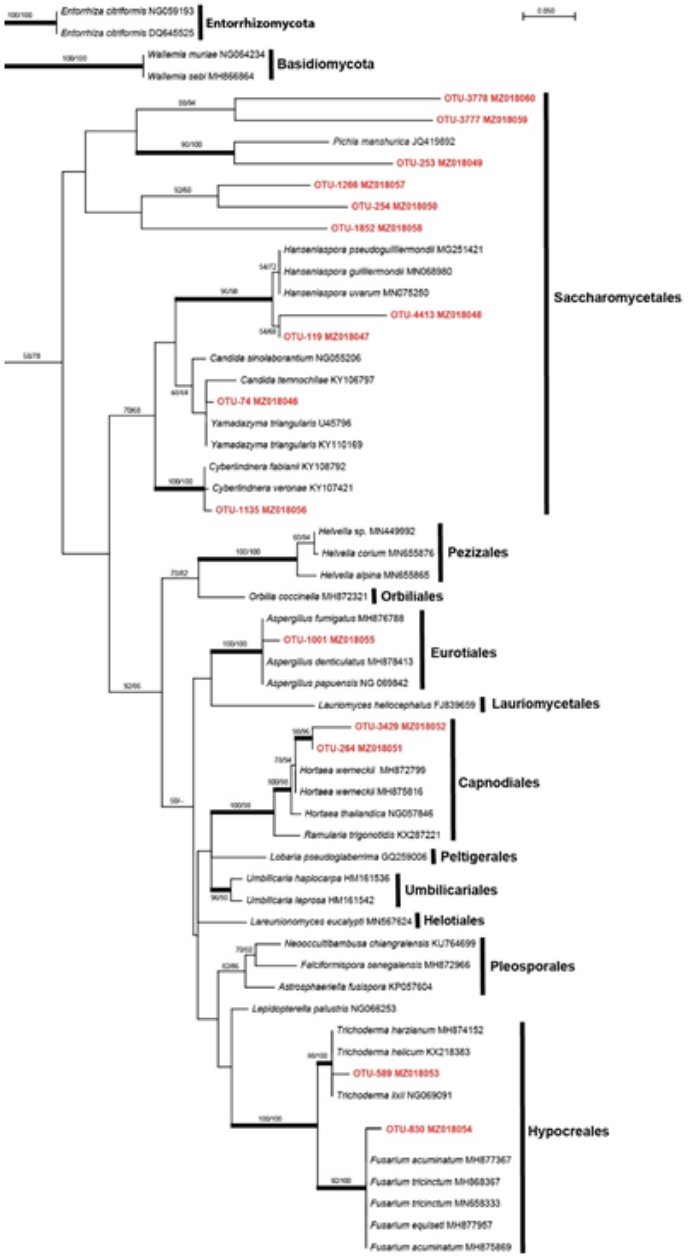

Figure 3

Maximum likelihood phylogenetic tree based on LSU rDNA of OTUs belong to Ascomycota and related sequences retrieved from the GenBank. The tree is rooted with representatives of Basidiomycota and Entorrhizomycota. Bootstrap support on the nodes represents ML and MP ≥50%. Branches with a BYPP of ≥95% are in bold. The sequences generated in this study are in red.

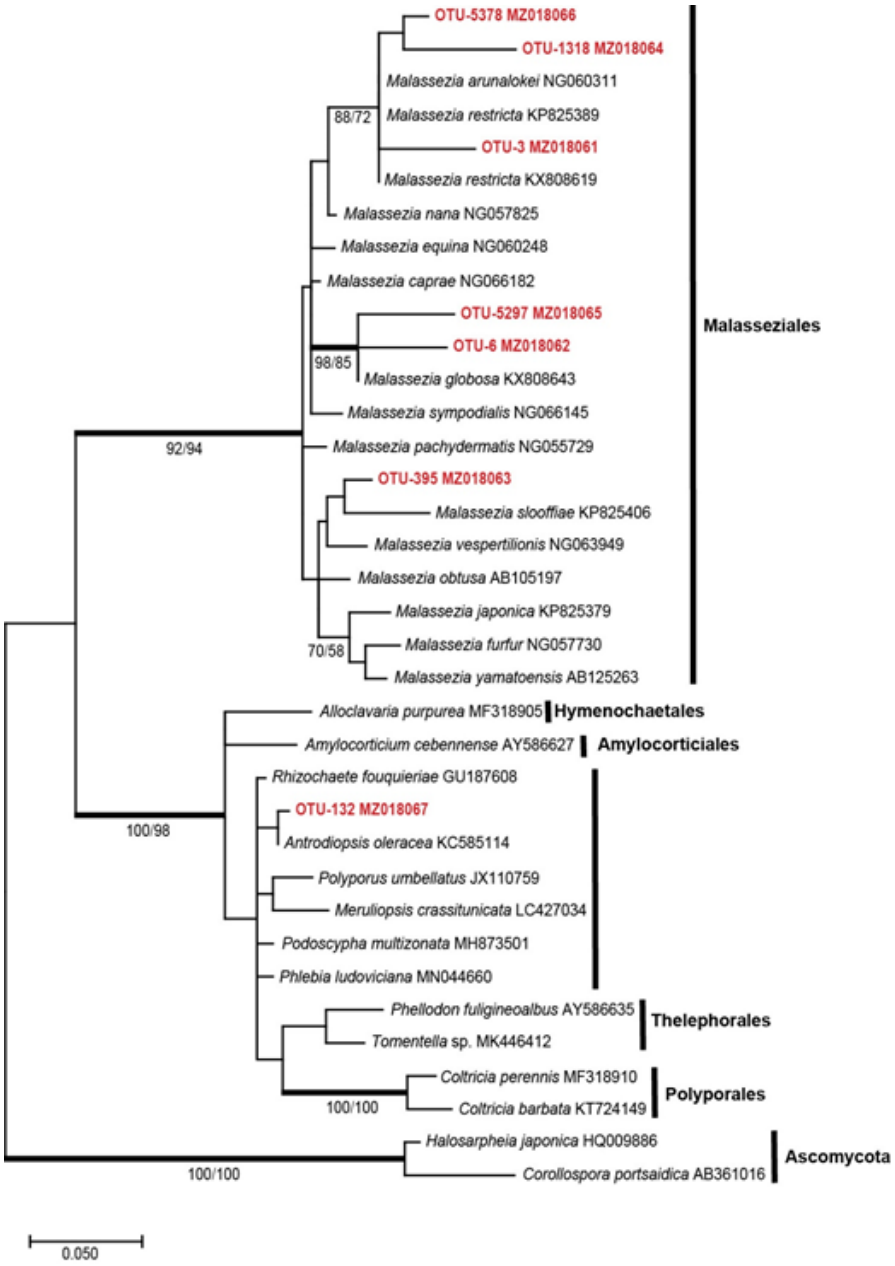

Figure 4

Maximum likelihood phylogenetic tree based on LSU rDNA of OTUs belong to Basidiomycota and related sequences retrieved from the GenBank. The tree is rooted with representatives of Ascomycota. Bootstrap support on the nodes represents ML and MP ≥50%. Branches with a BYPP of ≥95% are in bold. The sequences generated in this study are in red.

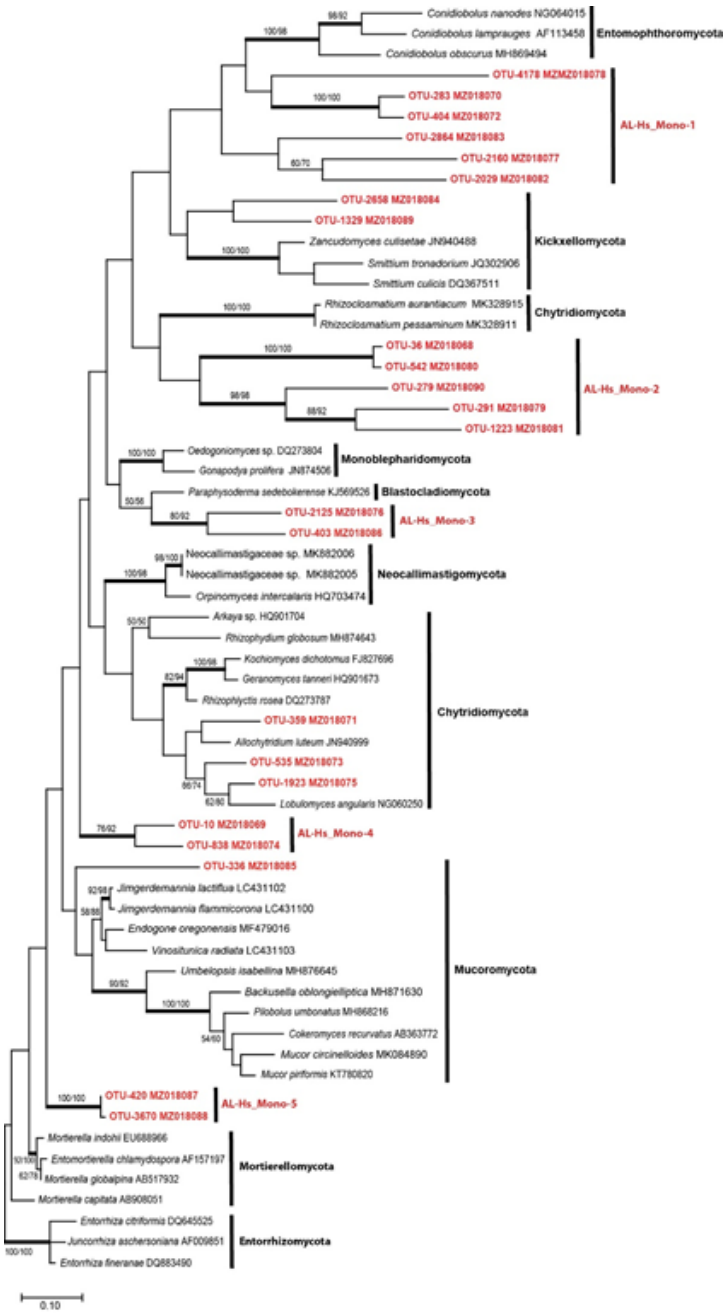

Figure 5

Maximum likelihood phylogenetic tree based on LSU rDNA of OTUs belong to monokaryon phyla and related sequences retrieved from the GenBank. The tree is rooted with representatives of Entorrhizomycota. Bootstrap support on the nodes represents ML and MP ≥50%. Branches with a BYPP of ≥95% are in bold. The sequences generated in this study are in red.

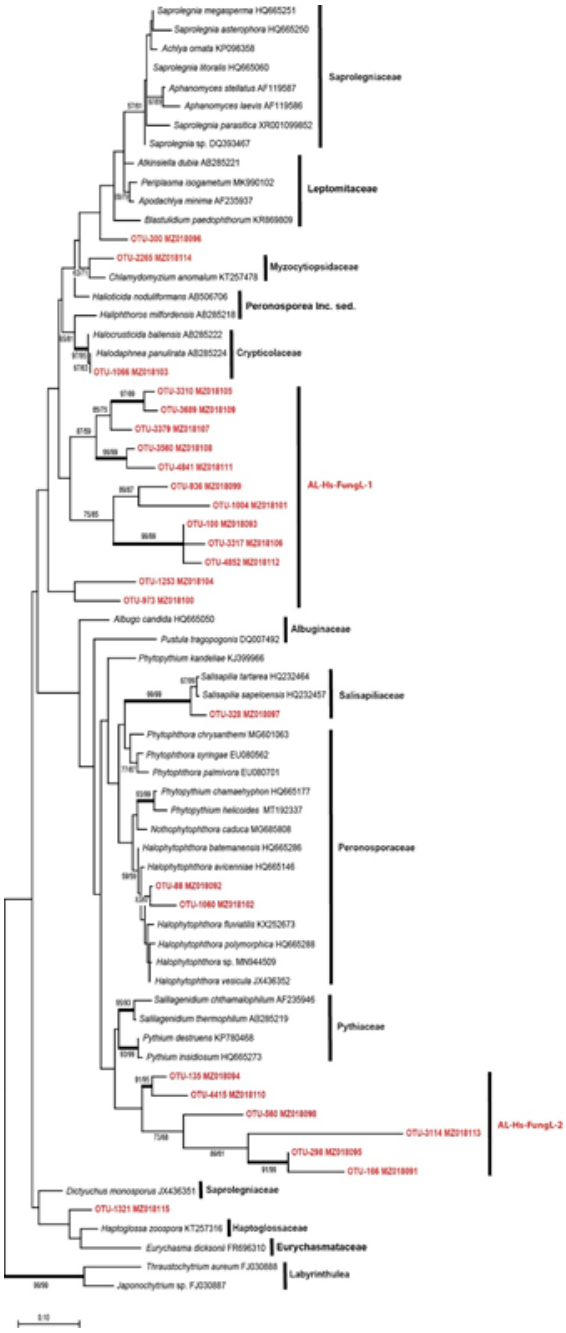

Figure 6

Maximum likelihood phylogenetic tree based on LSU rDNA of OTUs belong to fungi-like organisms and related sequences retrieved from the GenBank. The tree is rooted with representatives of Layrinthula. Bootstrap support on the nodes represents ML and MP ≥50%. Branches with a BYPP of ≥95% are in bold. The sequences generated in this study are in red.