

Fungal root symbionts of the seagrass *Posidonia oceanica* in the central Adriatic Sea revealed by microscopy, culturing and 454-pyrosequencing

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ABSTRACT: A significant part of the hitherto unexplored fungal diversity is hidden in the marine environment. At the same time, plant tissues host endophytic communities often dominated by yet undescribed fungal lineages. Here we focused on the Mediterranean endemic seagrass *Posidonia oceanica* and screened its root mycobionts at 8 localities in the Croatian central Adriatic Sea using (stereo-)microscopy, culturing from surface-sterilized root segments and 454-pyrosequencing. Our microscopic observations revealed that roots from all investigated localities possessed the typical dark septate endophytic association recently reported in this seagrass in the northwestern Mediterranean Sea. Both culturing and pyrosequencing detected very narrow fungal communities lacking typical terrestrial root endophytes. Similarly to the NW Mediterranean, these were dominated by an undescribed slow-growing mycobiont from the Pleosporales (1 operational taxonomic unit [OTU]: ca. 92% of 430 total isolates, and 3 molecular OTUs [MOTUs]: ca. 91% of 382 fungal sequences) and also comprised 2 yet undescribed mycobionts from the Lulworthiales (2 OTUs: ca. 8% of isolates, and 2 MOTUs: ca. 1.8% of sequences). Such a narrow, single-species dominated root mycobiont spectrum is unusual for photoautotrophic vascular plants and indicates a close symbiotic relationship between the dominating pleosporalean mycobiont and the dominant Mediterranean seagrass. Additionally, the apparent lack of typical terrestrial root endophytic fungi as well as their probable substitution by the pleosporalean mycobiont so far not known from other hosts or ecosystems implies relatively long specific coevolution of both marine organisms.

KEY WORDS: Seagrasses · Marine fungi · Root-associated fungi · Dark septate endophytes · Pleosporales · Lulworthiales · Mediterranean Sea

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INTRODUCTION

Fungi represent one of the major eukaryotic lineages as well as one of the most diverse organismal groups on our planet, equaling in number animals and exceeding plants (Blackwell 2011). It has been hypothesized that the global number of fungal species is at least 1.5 million, but probably as many as

3 million (Hawksworth 2012), and numbers up to 5.1 and 6 million have been proposed (O'Brien et al. 2005 and Taylor et al. 2014, respectively). Although some authors conclude that the hypothesized estimates of the global fungal diversity may be overestimated by 1.5- to 2.5-fold (Tedersoo et al. 2014), the few available fungal censuses suggest that there are only ca. 100–120 thousand accepted fungal species

(Hawksworth 2001, Kirk et al. 2008, Blackwell 2011), leaving the great majority of fungal species undescribed (Hawksworth & Rossman 1997). Many yet undescribed fungi dwell in the marine environment (Richards et al. 2012, 2015), and a recent analysis estimated that while there are some 500+ described obligate marine fungi, the true number might be more than 10 000 fungal taxa (Jones 2011).

Plant roots host an extensive and not yet fully explored diversity of fungal mutualists, endophytes, saprobes and parasites (Vandenkoornhuysen et al. 2002). In terrestrial ecosystems, roots of the majority of vascular plants are colonized by diverse spectra of mycorrhizal fungi; the resulting dual root–fungus organs are called mycorrhizae (Brundrett 2004). Mycorrhizae help their hosts with nutrient and water uptake as well as alleviation of other biotic and abiotic stresses (Brundrett 2009) and represent one of the most ancient plant symbioses which played a crucial role in the evolution of plant root anatomy and morphology (Selosse & Le Tacon 1998, Brundrett 2002). They have profound effects on plant productivity, nutrient cycling and ecosystem functioning and significantly influence the composition of plant communities (van der Heijden et al. 1998, Klironomos et al. 2000, Read & Perez-Moreno 2003).

In addition to mycorrhizal fungi, most, if not all, terrestrial plants host various root fungal endophytes which, by definition, grow asymptotically within living plant tissues and do not form mycorrhizal structures or cause any obvious disease symptoms (Wilson 1995, Rodriguez et al. 2009). In reality, however, many endophytes do influence their host fitness, either positively or negatively (Jumpponen & Trappe 1998, Newsham 2011, Mayerhofer et al. 2013, Lukešová et al. 2015). Terrestrial root endophyte communities are often dominated by the so-called dark septate endophytes (DSE) which produce melanized septate hyphae and intracellular microsclerotia (Jumpponen & Trappe 1998). In temperate and boreal forests, the spectrum of DSE is usually dominated by fungal symbionts (= mycobionts) from the *Phialocephala fortinii* s. l. — *Acephala applanata* species complex (Ascomycetes, Helotiales) (Grünig et al. 2008). In contrast, these helotialean mycobionts may be low in abundance or absent in (semi-)arid Northern Hemisphere grasslands whose root endophytic communities are usually dominated by pleosporalean species (Ascomycetes, Pleosporales) (Porras-Alfaro et al. 2008, Khidir et al. 2010, Knapp et al. 2012), in freshwater aquatic plants (e.g. Kohout et al. 2012, You et al. 2015) or in seagrasses (e.g. Panno et al. 2013, Vohník et al. 2016a).

Comparably less is known about the fungi colonizing marine plants, and this also holds true for seagrasses, the only vascular plants adapted to permanent life in the marine environment. On the one hand, their roots seem to be devoid of mycorrhizae (Nielsen et al. 1999). On the other hand, seagrasses may host diverse spectra of fungal root endophytes with practically unknown eco-physiological significance. These include typical terrestrial species, facultative marine fungi and obligate marine species, and some of them can be assigned as DSE (see Raghukumar 2017 and references therein).

The seagrass *Posidonia oceanica* (L.) Delile is endemic to the Mediterranean Sea where it forms large climax meadows dominating many sublittoral areas (Green & Short 2003). It is a long-lived species mostly relying on vegetative reproduction — its largest clones can spread over 15 km while being hundreds to thousands of years old (Arnaud-Haond et al. 2012). Rhizomes, roots and senescent leaf sheaths of *P. oceanica* form a characteristic peat-like seabed layer (matte) which may be several meters thick and thousands of years old (Hemminga & Duarte 2000). The matte is exceptionally resistant to microbiological decay, yet it comprises large amounts of nutrients in the organic form (Serrano et al. 2012). Higher plants cannot effectively utilize organically bound nutrients without the aid of symbiotic microorganisms such as bacteria or fungi (Smith & Read 2008), but to our knowledge, the role of fungi in the matte turnover and *P. oceanica* nutrient uptake has not yet been investigated.

The root mycoflora of *P. oceanica* has been studied by several authors with differing results: while Cuomo et al. (1985) and Torta et al. (2015) reported only 1 fungus (*Corollospora maritima* and '*Lulwoana* sp.', respectively), Vohník et al. (2016a) reported 3 (Pleosporales sp. MV-2012, Lulworthiales sp. MV-2012 and *Fuscoporia torulosa*) and Panno et al. (2013) reported 14 root-associated fungi. Additionally, an anatomically and morphologically unique root–fungus symbiosis has recently been discovered in this seagrass in the NW Mediterranean Sea (Vohník et al. 2015). Interestingly, this symbiosis was absent in the roots of the co-occurring seagrass *Cymodocea nodosa* and combined indirect evidence strongly suggested that it was formed by a single yet undescribed pleosporalean DSE mycobiont, the Pleosporales sp. MV-2012 (Vohník et al. 2015, 2016a). Intriguingly, another *P. oceanica* root mycobiont (the '*Lulwoana* sp.') has been recently proposed as DSE (Torta et al. 2015), but the reason for this assignment

remains unclear as no melanized hyphae or typical DSE melanized microsclerotia were reported by those authors, and the '*Lulwoana* sp.' is probably conspecific with the *Lulworthiales* sp. MV-2012 which does not form dark septate hyphae (Vohník et al. 2016a).

To resolve these incongruent results as well as to verify previous findings from the NW Mediterranean Sea (Vohník et al. 2015, 2016a), we focused on *P. oceanica* root mycobionts at 8 localities in the central Adriatic Sea off Croatia and searched for local-scale patterns of their root colonization, diversity and relative abundance, using stereomicroscopy, light microscopy, culturing (i.e. isolation of root mycobionts into pure culture followed by their molecular identification) as well as a culture-independent approach (tag-encoded 454-pyrosequencing, to our knowledge for the first time in a Mediterranean seagrass). We hypothesized that while the specific DSE root–fungus association previously reported from the NW Mediterranean (Vohník et al. 2015) would also occur in the central Adriatic, the combination of the finer local-scale approach, a different surface sterilization scheme before culturing and the culture-independent method would reveal more diverse root fungal spectra than those previously reported in *P. oceanica*.

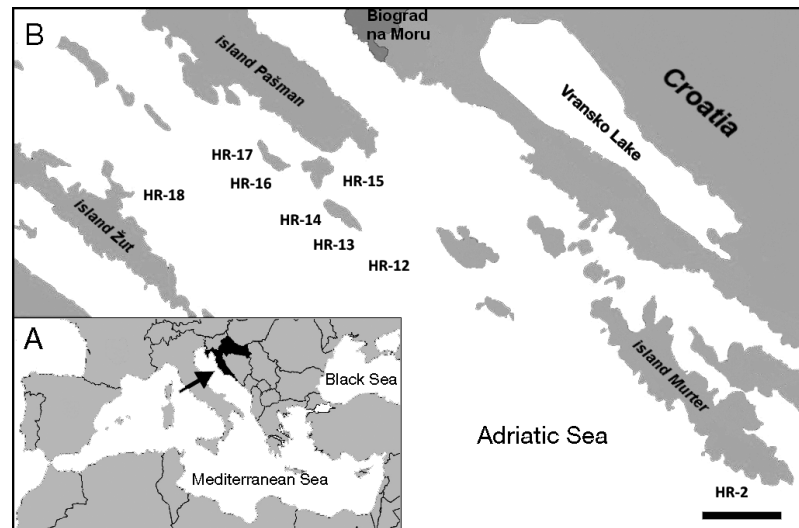


Fig. 1. (A) Study sites were located in the central part of the Adriatic Sea near Kornati National Park, ca. 45 km SE from Zadar (arrow), Croatia (black). (B) Detailed locations of the investigated sites in the Central Adriatic (numbered HR-2 and HR-12 to 18 in accordance with Table 1). Scale bar = 3 km

MATERIALS AND METHODS

Collection of root samples

Root samples of *Posidonia oceanica* were collected at different depths (8–21 m) by scuba diving at 8 localities in northern Dalmatia (central Adriatic Sea) some 45 km southeast of Zadar, Croatia, in September 2012 (Fig. 1, Table 1). In 1 case (site HR-2, see Table 1), we re-visited a locality sampled by Vohník et al. (2016a) for comparison of results. Roots from 5

Table 1. Sites investigated in this study with fungal culture morphotype diversity and abundance. Site numbering follows Fig. 1 and continues from Vohník et al. (2015, 2016a). Reference isolates and sequences are listed in Table 2. OTU: operational taxonomic unit

Site ID	Site name	GPS coordinates (°N, °E)	Morphotypes			Isolation success ratio (%)
			n	Frequency	Identity	
HR-2	Kukuljar	43.7596, 15.6341	2	91× Black 3× Yellow	Pleosporales sp. MV-2012 (OTU 1) Lulworthiales sp. MV-2012 (OTU 2)	58.8
HR-12	Obun	43.8500, 15.4553	2	41× Black 15× Yellow	Pleosporales sp. MV-2012 (OTU 1) Lulworthiales sp. MV-2012B (OTU 3)	35.0
HR-13	Gangaro II	43.8623, 15.4380	2	32× Black 3× Yellow	Pleosporales sp. MV-2012 (OTU 1) Lulworthiales sp. MV-2012B (OTU 3)	21.9
HR-14	Gangaro I	43.8639, 15.4341	1	45× Black	Pleosporales sp. MV-2012 (OTU 1)	28.1
HR-15	Veli Ošljak	43.8756, 15.4376	1	52× Black	Pleosporales sp. MV-2012 (OTU 1)	32.5
HR-16	Košara lighthouse	43.8822, 15.4022	1	44× Black	Pleosporales sp. MV-2012 (OTU 1)	27.5
HR-17	Košara	43.8838, 15.3994	2	56× Black 4× Yellow	Pleosporales sp. MV-2012 (OTU 1) Lulworthiales sp. MV-2012B (OTU 3)	37.5
HR-18	Veli Babuljaš	43.8741, 15.3528	2	36× Black 8× Yellow	Pleosporales sp. MV-2012 (OTU 1) Lulworthiales sp. MV-2012 (OTU 2)	27.5

different microsites (at least 10 m apart) were collected at each locality. These were pooled, inserted into 50 ml plastic beakers filled with seawater, delivered to the surface, stored in a fridge and processed for isolation of mycobionts in the evening of the respective collection day. Subsequently, the seawater was replaced with 70% ethanol, and the roots were transported to the laboratory of the Department of Mycorrhizal Symbioses, Institute of Botany, Czech Academy of Sciences, for microscopic observations and DNA isolation and amplification.

Microscopic observations

Fungal colonization of the roots was screened using an Olympus SZX12 stereomicroscope and an Olympus BX60 upright microscope equipped with differential interference contrast (DIC). We mainly screened root surfaces (stereomicroscope) and hand-made semi-thin longitudinal (ca. 3–5 mm in length) and transversal root sections (upright microscope). Photographs were taken with an Olympus DP70 camera at 400×, 600× and 1000× magnifications. The Olympus Deep Focus mode was employed when needed. Photographs were subsequently modified for clarity in Paint.NET (<https://www.getpaint.net/index.html>) as needed and assembled using the same software.

Isolation and identification of cultivable root endophytes

In our first study of the *P. oceanica* cultivable root mycobionts, we used root surface sterilization in 10% SAVO (common household bleach, Unilever; 100% SAVO contains 47 g kg⁻¹, i.e. 4.7% sodium hypochlorite = NaClO) for 30 s followed by rinsing the roots 2 times in sterile deionized water. This procedure is common in ericoid mycorrhiza research and is tuned for the very fine hair roots of Ericaceae where it typically yields hundreds of fungal isolates belonging to tens of fungal species (e.g. Bruzone et al. 2015, 2017). However, because it yielded a very narrow root mycobiont spectrum in the case of *P. oceanica* (Vohník et al. 2016a), we decided to change the surface sterilization scheme as follows. Healthy-looking turgid yellowish to brownish terminal fine roots (diam. ca. 1–2 mm) were surface-sterilized by 2 consecutive washes in 50% ethanol for 1 min followed by 2 washes in sterile tap water. The roots were then cut into ca. 3–5 mm segments and these were transferred to 4-compartment plastic Petri dishes (9 cm

diameter) containing potato dextrose agar (PDA; HiMedia) amended with Novobiocin sodium salt (50 mg l⁻¹; Sigma-Aldrich) to prevent growth of bacteria and sodium chloride (NaCl, 37 g l⁻¹) to adjust osmotic pressure to the average Mediterranean seawater salinity (3.7–3.8%). The isolation was done in a portable flow box to prevent air contaminants. There were 10 dishes each with 16 root pieces from each locality. The dishes were sealed with an air-permeable tape, kept at room temperature in the dark and observed for hyphal growth daily during the first 2 wk then once every 3–5 d. Hyphal colonies emerging from the surface-sterilized roots were observed with the SZX12 stereomicroscope and documented with the DP70 camera. The isolation experiment was terminated after ca. 3 mo; all obtained fungal colonies were conservatively grouped into morphotypes, and several representatives of each morphotype/locality combination were selected for DNA isolation.

Total DNA was extracted from representatives of all morphotypes (Table 1) using an Extract-N-Amp Plant Kit (Sigma-Aldrich) following the manufacturer's instructions. Partial large subunit (LSU) (28S) nuclear ribosomal (nr) DNA was amplified using the LR0R + LR5 primer pair (Vilgalys & Hester 1990). PCR and gel electrophoresis parameters were the same as in Vohník et al. (2012). PCR products were purified and sequenced in the MacroGen Europe Laboratory using the LR0R primer. The obtained sequences were screened in Finch TV v1.4.0 (http://jblseqdat.bioc.cam.ac.uk/gnmweb/download/soft/FinchTV_1.4/doc/) for possible machine errors and manually edited. The edited sequences were subsequently aligned in BioEdit v7.2.5 (Hall 1999) using ClustalW (Thompson et al. 1994), and the alignment was used as a matrix for a neighbor-joining (NJ) analysis in TOPALi v2.5 (Biomathematics & Statistics Scotland, www.topali.org). The threshold limit for grouping of sequences was set at 97%. Sequences within separate groups were further aligned to screen their heterogeneity, and the most divergent were subjected to BLAST searches (megablast/blastn algorithms) in GenBank (Altschul et al. 1997) (we preferred identified cultures with deposited vouchers and omitted sequences from environmental samples), and their taxonomic position was further roughly checked using Blast Tree View (NJ, maximum sequence difference = 0.75). As sequences of the major Black morphotype (operational taxonomic unit [OTU] 1) were identical (100/100 coverage/similarity) to those reported by Vohník et al. (2016a) (see Tables 1 & 2) we did not proceed with more precise phylogenetic analysis. To infer the phylogenetic

positions of the Lulworthiales spp. (OTUs 2 and 3), an LSU nrDNA sequence dataset was created using the matrix published by Azevedo et al. (2017) and the most similar sequences deposited in NCBI GenBank (see Fig. 3). The sequences were aligned using MAFFT6 (Katoh et al. 2009) and default settings. The aligned dataset had 460 positions from which 245 were variable and 187 were parsimony informative. A Bayesian phylogenetic analysis was conducted with MrBayes 3.0 (Ronquist & Huelsenbeck 2003) and 80 million replicates estimated together with a burn-in value (initial 20% of trees) in Tracer v1.5 (Rambaut & Drummond 2009). The substitution model (TN93+G+I) and other parameters were estimated in jModeltest 0.1.1 (Posada 2008). Members of Dothideomycetes (*Setosphaeria*, *Bimuria*, *Letendreaea*) were chosen as outgroups based on Azevedo et al. (2017).

Molecular detection of root endophytes by 454-pyrosequencing

Ca. 100 mg (fresh weight) of healthy-looking fine roots from each locality were cut into 5–10 mm segments, surface-sterilized for 30 s in 10% SAVO (as in Vohník et al. 2016a), washed twice in sterile water and kept in a freezer until used. Frozen roots were ground in liquid nitrogen using a mortar and pestle. DNA was extracted from homogenized root tissue using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions, with a 50 μ l elution volume of the AE buffer. The isolated DNA was 10 \times diluted with ddH₂O and used as a template for subsequent PCR reactions. From each DNA extract, 2 independent PCR reactions were run to avoid PCR bias. In the first step, the ITS region of the nrDNA was amplified using the fungal-specific primer combination ITS1F+ITS4 (White et al. 1990, Gardes & Bruns 1993). The PCR mix included 1 \times OmniTaq PCR Buffer (DNA Polymerase Technology), 0.2 mM of each of dNTPs, 0.4 μ M of each primer, 20 μ g of BSA (Thermo Scientific), 0.25 μ l of a mixture of 4% *Pfu* (2.5 U μ l⁻¹; Thermo Scientific) and 96% OmniTaq (10 U μ l⁻¹; DNA Polymerase Technology) DNA polymerases and 2 μ l of the template in a final volume of 25 μ l. Cycling conditions for the first PCR were 5 min at 94°C, followed by 38 cycles of 30 s at 94°C, 30 s at 55°C and 90 s at 72°C. The program was concluded by a final extension phase of 10 min at 72°C. The obtained PCR products originating from all 8 root extracts were then pooled, yielding a final volume of 400 μ l. Pooled samples were purified using a

QIAquick PCR Purification Kit (Qiagen), and the DNA was eluted into 20 μ l of ddH₂O. The purified PCR product (DNA concentration 70 ng μ l⁻¹) was used as a template for the second PCR reaction with tag-encoded 454-pyrosequencing primers ITS1+ITS4, also containing Titanium A or B adaptors (Roche). The mix for the second PCR reaction included 1 \times HF buffer with 1.5 mM MgCl₂ (Thermo Scientific), 0.2 mM of each of dNTPs, 0.1 μ M of each tagged primer, 1.5 μ l of DMSO PCR Reagent (Sigma-Aldrich), 1 U of Phusion High-Fidelity DNA polymerase (Thermo Scientific) and 1 μ l of the purified template in a final volume of 50 μ l. Cycling conditions for this PCR were 4 min at 94°C, followed by 10 cycles of 30 s at 94°C, 30 s at 55°C and 90 s at 72°C. The PCR product was then purified using AMPure Beads with NEBNext Sizing Buffer (New England BioLabs) and eluted to 10 μ l of 1 \times TE buffer. DNA concentration was quantified using Qubit dsDNA HS Assay Kit by Qubit 2.0 Fluorometer (Life Technologies), and the sample was equimolarly mixed with the other samples from the same 454-pyrosequencing plate. For exclusion of short reads, the mixed sample was separated by electrophoresis and the gel was purified using the Wizard SV Gel and PCR cleanup system (Promega), and subsequently purified once more by AMPure Beads and a QIAquick PCR Purification Kit as described above. The resulting DNA quantity was measured with a qPCR Kappa kit and used for emulsion PCR and subsequent sequencing on the GS Junior platform (Roche).

In total, pyrosequencing of the pooled *P. oceanica* root sample yielded 3144 raw sequences. All sequences with more than 1 error in the primer sequence were excluded from the dataset. The remaining flows were subjected to de-noising using Mothur 1.26.0 (Schloss et al. 2009) which yielded 702 sequences (such a high proportion of 454-pyrosequencing reads excluded because of quality-related issues is not an uncommon observation; see Lindahl et al. 2013). The obtained sequences were subsequently processed in the pipeline SEED 2.0.4 (Větrovský & Baldrián 2013). Sequences shorter than 380 bp were excluded and the data set was trimmed to the 380 bp sequence length. The obtained 550 sequences were then clustered to molecular OTUs (MOTUs) using UPARSE implementation in USEARCH ver. 8.1.1861 (Edgar 2013), with 97% similarity threshold. Chimeric sequences identified in this step (36) were deleted to prevent diversity overestimation. Also 19 global singletons were removed from the data set. The consensus sequences were constructed for each MOTU using MAFFT ver.

7.222 alignments (Kato et al. 2009), based on the most abundant nucleotide at each position. These consensus sequences were then checked for the closest hits using BLAST against the UNITE (Kõljalg et al. 2013) and GenBank databases.

RESULTS

Microscopic observations

While some parts of the terminal roots were free of any fungal colonization (Fig. 2A), most were often heavily colonized by melanized septate hyphae either on the root surface (Fig. 2B–H) or intracellularly (Fig. 2J–N). Hyphal colonization density differed especially on the root surface from no hyphae to single hyphae usually following sulci between rhizodermal cells (Fig. 2B,C) to 2-layered sheets (Fig. 2D,E) where the upper layer was typically formed by straight, relatively thick dark septate hyphae (Fig. 2D) and the lower layer by parenchymatous tissue formed by shorter thickened hyphae (Fig. 2E,F). The surface hyphae sometimes formed nearly geometrical patterns resembling various combinations of triangles and rectangles (Fig. 2G,H). In general, the intracellular colonization rarely occurred in the rhizodermis (Fig. 2J–L), very frequently in the hypodermis (Fig. 2M,N), rarely in the cortex-cell layer immediately below (Fig. 2N), and it was totally absent in the deeper cortex layers as well as in the vascular cylinder. It was represented either by loose hyphal hyaline to melanized loops (Fig. 2J–L) or microsclerotia typical for DSE fungi (Fig. 2M,N). The microsclerotia were either limited to the ‘entry cells’ (Fig. 2M; cf. Vohník et al. 2015) or dispersed through the whole hypodermis (Fig. 2N).

Morphologically unusual root hairs were infrequently observed in some root samples. Their cell wall seemed to be formed spirally (Fig. 2O) and their endings were infrequently rounded (Fig. 2P) but mostly finished by polymorphic swollen suction cup-like tips (Fig. 2R).

Isolation and identification of cultivable root endophytes

In total, we obtained 430 fungal colonies out of the 1280 surface sterilized root segments, i.e. on average 33.6% of the root segments yielded a fungal colony. This isolation success ratio varied among the investigated localities, being highest at the revisited locality

HR-2/Kukuljar (58.8%) and lowest at HR-13/Gangaro II (21.9%) (Table 1).

In agreement with Vohník et al. (2016a), the fungal colonies grouped into 2 morphotypes, Black and Yellow, which unmistakably differed in overall appearance and especially their growth rate, the former being significantly slower than the latter, often producing new colonies no earlier than after 1.5 mo from the beginning of the isolation procedure. With 397 isolates, the Black morphotype was by far the most frequent among the obtained cultivable spectrum (ca. 92% of the 430 total isolates), being recovered from all investigated localities and at the same time being the only morphotype recovered at 3 localities (Table 1).

Sequencing and subsequent analyses confirmed that the Black morphotype belonged to a single mycobiont (OTU 1) identical (100/100 sequence coverage/similarity) to *Pleosporales* sp. MV-2012 which dominates *P. oceanica* root mycobiont communities in the NW Mediterranean (Vohník et al. 2016a). That phylogenetic analysis placed this mycobiont as a putative new species in a new genus (Vohník et al. 2016a) within the recently established pleosporalean Aigialaceae family (Suetrong et al. 2009). The Yellow morphotype comprised 2 OTUs belonging to the Lulworthiales (Tables 1 & 2), OTU 2 being conspecific with Lulworthiales sp. MV-2012 which has been found as a mycobiont co-colonizing *P. oceanica* roots in the NW Mediterranean (Vohník et al. 2016a) (Fig. 3). OTU 3 most likely represents another yet undescribed species within the Lulworthiales; the closest published sequences identified by a BLASTn search belonged to *Lulworthia* sp. 107aIA (KM272360, 91%) and various other members of the Lulworthiales. The phylogenetic analysis placed OTU 3 as a well-defined lineage which clustered with *Kohlmeyeriella tubulata* (Fig. 3), a fungus known from European marine waters (Clipson et al. 2001). OTU 2 formed a well-defined lineage together with entries possessing identical or nearly identical (99.5%) sequences and originating from the Italian coast (polluted sea water, KU935711–2; Bovio et al. 2017) or *P. oceanica* roots from Croatia (KC736946; Vohník et al. 2016a). This lineage grouped with *Lulworthia* sp. 107aIA isolated from the Atlantic Ocean in Norway (KM272360; Rämä et al. 2014). Similarly to the previous study (Vohník et al. 2016a), practically no air contaminants occurred in the Petri dishes during the course of the isolation, suggesting that all the obtained mycobionts indeed originated from the surface-sterilized *P. oceanica* roots.

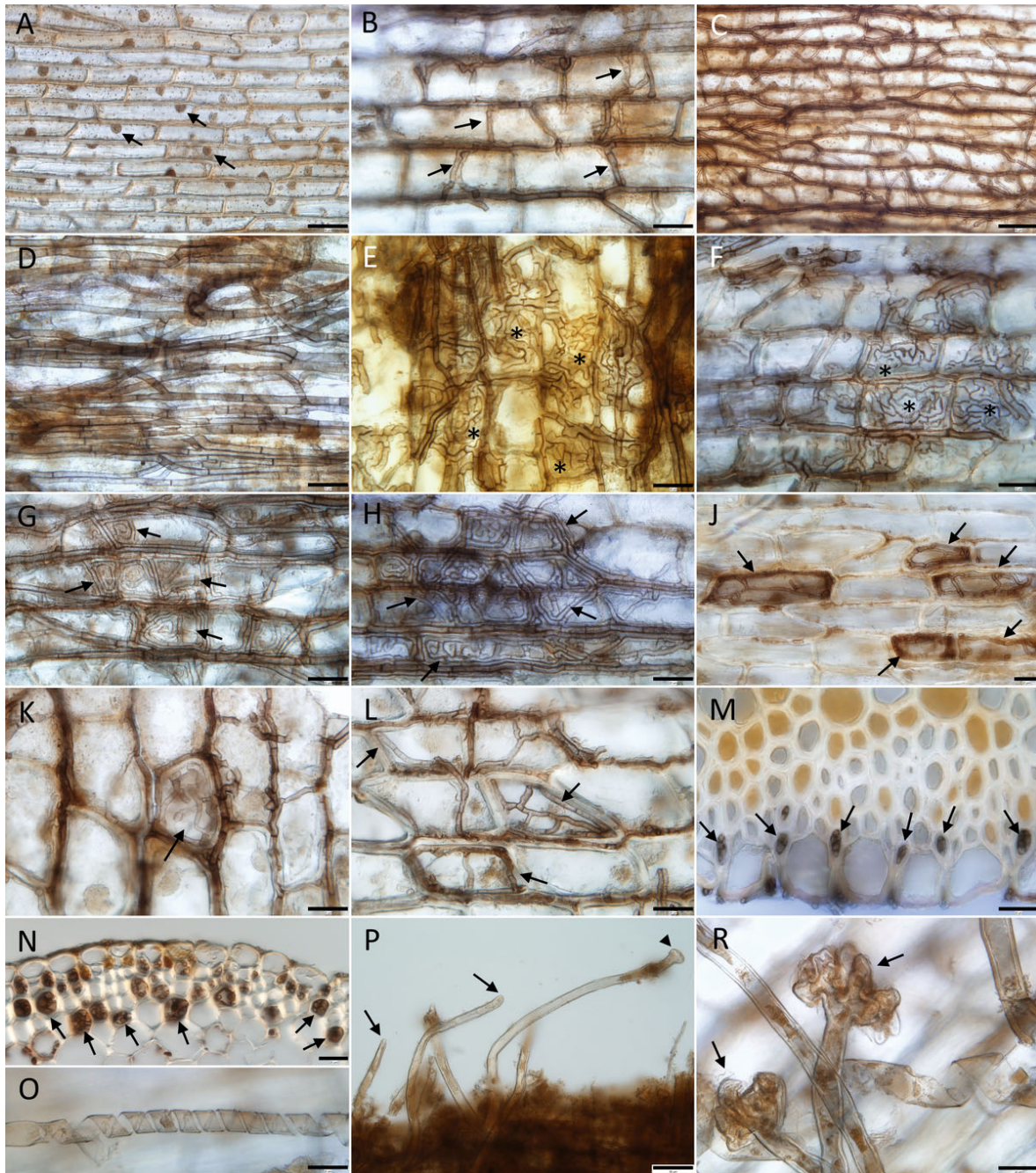


Fig. 2. Anatomical and morphological characteristics of root fungal colonization and root hairs in *Posidonia oceanica*. (A) Surface view of a root with no fungal colonization and with typical arrangement of the rhizodermal cells, most of them containing nuclei (arrows). (B) Single melanized septate hyphae (arrows) following sulci between the rhizodermal cells. (C) Macro view of melanized septate hyphae on the root surface. (D) Dense mycelial sheath formed by straight, relatively thick melanized septate hyphae. (E,F) Parenchymatous tissue formed by shortened and thickened hyphae (asterisks) on the root surface. (G,H) Melanized septate hyphae forming geometrical patterns resembling combinations of triangles and rectangles (arrows) on the root surface. (J–L) Intracellular septate hyaline to dark brown hyphae (arrows) in the rhizodermal cells of *P. oceanica*. (M) In the first phase of root colonization, the fungal intraradical mycelium seems to be limited to the 'entry cells' (see Vohník et al. 2015) just below the rhizodermis (arrows). (N) In later phases of root colonization, fully developed melanized intracellular microsclerotia (arrows) may occupy significant portions of the hypodermis as well as the cortex layers immediately below. (O) Spiral cell wall of the root hairs observed in some *P. oceanica* roots. (P) Rounded ends of the seagrass root hairs (arrows) eventually become swollen (arrowhead). (R) Most root hairs had polymorphic suction cup-like tips (arrows). Scale bars = 20 μm in all panels except A, C and P (50 μm)

Table 2. Identity of the culture morphotypes obtained in this study based on LSU nrDNA sequences and BLASTn searches in the NCBI database. Country codes —HR: Croatia; IN: India; IT: Italy; NO: Norway; PT: Portugal; TH: Thailand

Site ID	Morphotype/ OTU/reference isolate	Reference sequence acc. no.	Length	Closest GenBank match	Closest GenBank match Acc. no. Source, origin	Sequence coverage/ similarity (%)	Reference
HR-2	Black/OTU 1/M1	KY488006	538	Pleosporales sp. MV-2012 isolate P31 ^a	KC736945	100/100	Vohník et al. (2016a)
				<i>Rimora mangrovei</i> strain JK 5246A	GU301868	99/95	Schoch et al. (2009)
				<i>Aigiatus rhizophorae</i> strain BCC 33573	GU479781	99/94	Suetrong et al. (2009)
	Yellow/OTU 2/M2	KY488007	577	Lulworthiales sp. MV-2012 isolate P32	KC736946	100/100	Vohník et al. (2016a)
			Lulworthiales sp. MUT 1753	KU935712	100/99	Bovio et al. (2017)	
			<i>Lulworthia</i> sp. 107aIA	KM272360	99/99	Rämä et al. (2014)	
HR-12	Black/OTU 1/M3	KY488008	582	Pleosporales sp. MV-2012 isolate P31 ^a	KC736945	100/100	Vohník et al. (2016a)
				<i>Rimora mangrovei</i> strain JK 5246A	GU301868	100/95	Schoch et al. (2009)
				<i>Aigiatus rhizophorae</i> strain BCC 33573	GU479781	100/93	Suetrong et al. (2009)
	Yellow/OTU 3/M4	KY488009	837	<i>Lulworthia</i> sp. 107aIA	KM272360	99/91	Rämä et al. (2014)
			<i>Cumulospora varia</i> isolate GR78	EU848578	100/89	Pang et al. (unpubl.)	
			<i>Lulworthia</i> sp. isolate FCUL151007SP4	JN886842	100/89	Azevedo et al. (2017)	
HR-13	Black/OTU 1/M5	KY488010	622	Pleosporales sp. MV-2012 isolate P31 ^a	KC736945	100/100	Vohník et al. (2016a)
				<i>Rimora mangrovei</i> strain JK 5246A	GU301868	99/95	Schoch et al. (2009)
				<i>Rimora mangrovei</i> strain JK 5437B	GU479798	99/92	Suetrong et al. (2009)
	Yellow/OTU 3/M6	KY488011	850	<i>Lulworthia</i> sp. 107aIA	KM272360	99/91	Rämä et al. (2014)
			<i>Cumulospora varia</i> GR78	EU848578	100/90	Pang et al. (unpubl.)	
			<i>Lulworthia</i> sp. FCUL151007SP4	JN886842	100/89	Azevedo et al. (2017)	
HR-14	Black/OTU 1/M8	KY488012	806	Pleosporales sp. MV-2012 isolate P31 ^a	KC736945	100/100	Vohník et al. (2016a)
				<i>Rimora mangrovei</i> strain JK 5246A	GU301868	99/95	Schoch et al. (2009)
				<i>Rimora mangrovei</i> strain JK 5437B	GU479798	99/93	Suetrong et al. (2009)
	Black/OTU 1/M10	KY488013	672	Pleosporales sp. MV-2012 isolate P31 ^a	KC736945	100/100	Vohník et al. (2016a)
			<i>Rimora mangrovei</i> strain JK 5246A	GU301868	100/94	Schoch et al. (2009)	
			<i>Rimora mangrovei</i> strain JK 5437B	GU479798	100/92	Suetrong et al. (2009)	
HR-16	Black/OTU 1/M11	KY488014	799	Pleosporales sp. MV-2012 isolate P10	KC736941	100/100	Vohník et al. (2016a)
				<i>Rimora mangrovei</i> strain JK 5246A	GU301868	100/95	Schoch et al. (2009)
				<i>Rimora mangrovei</i> strain JK 5437B	GU479798	100/94	Suetrong et al. (2009)
	Black/OTU 1/M12	KY488015	505	Pleosporales sp. MV-2012 isolate P31 ^a	KC736945	100/100	Vohník et al. (2016a)
			<i>Rimora mangrovei</i> strain JK 5246A	GU301868	100/94	Schoch et al. (2009)	
			<i>Aigiatus rhizophorae</i> strain BCC 33573	GU479781	100/92	Suetrong et al. (2009)	
HR-17	Yellow/OTU 3/M13	KY488016	860	<i>Lulworthia</i> sp. 107aIA	KM272360	99/91	Rämä et al. (2014)
				<i>Cumulospora varia</i> GR78	EU848578	100/90	Pang et al. (unpubl.)
				<i>Lulworthia</i> sp. FCUL151007SP4	JN886842	100/89	Azevedo et al. (2017)
	Black/OTU 1/M14	KY488017	668	Pleosporales sp. MV-2012 isolate P31 ^a	KC736945	100/100	Vohník et al. (2016a)
			<i>Rimora mangrovei</i> strain JK 5246A	GU301868	100/94	Schoch et al. (2009)	
			<i>Rimora mangrovei</i> strain JK 5437B	GU479798	100/92	Suetrong et al. (2009)	
HR-18	Yellow/OTU 2/M15	KY488018	746	Lulworthiales sp. MV-2012 isolate P32 ^b	KC736946	100/100	Vohník et al. (2016a)
				Lulworthiales sp. MUT 263	KU935711	99/99	Bovio et al. (2017)
				<i>Lulworthia</i> sp. 107aIA	KM272360	100/99	Rämä et al. (2014)
				Pleosporales sp. MV-2012 from various locations in the NW Mediterranean Sea; see Vohník et al. 2016a)	KC736945	100/100	Vohník et al. (2016a)
			<i>Rimora mangrovei</i> strain JK 5246A	GU301868	100/94	Schoch et al. (2009)	
			<i>Rimora mangrovei</i> strain JK 5437B	GU479798	100/92	Suetrong et al. (2009)	
			Lulworthiales sp. MV-2012 isolate P32 ^b	KC736946	100/100	Vohník et al. (2016a)	
			Lulworthiales sp. MUT 263	KU935711	99/99	Bovio et al. (2017)	
			<i>Lulworthia</i> sp. 107aIA	KM272360	100/99	Rämä et al. (2014)	

^aSame score as KC736940–4 (all Pleosporales sp. MV-2012 from *P. oceanica* roots from various locations in the NW Mediterranean Sea; see Vohník et al. 2016a)

^bSame score as KC736939 (Lulworthiales sp. MV-2012 isolate P04 from a *P. oceanica* root from NE Spain)

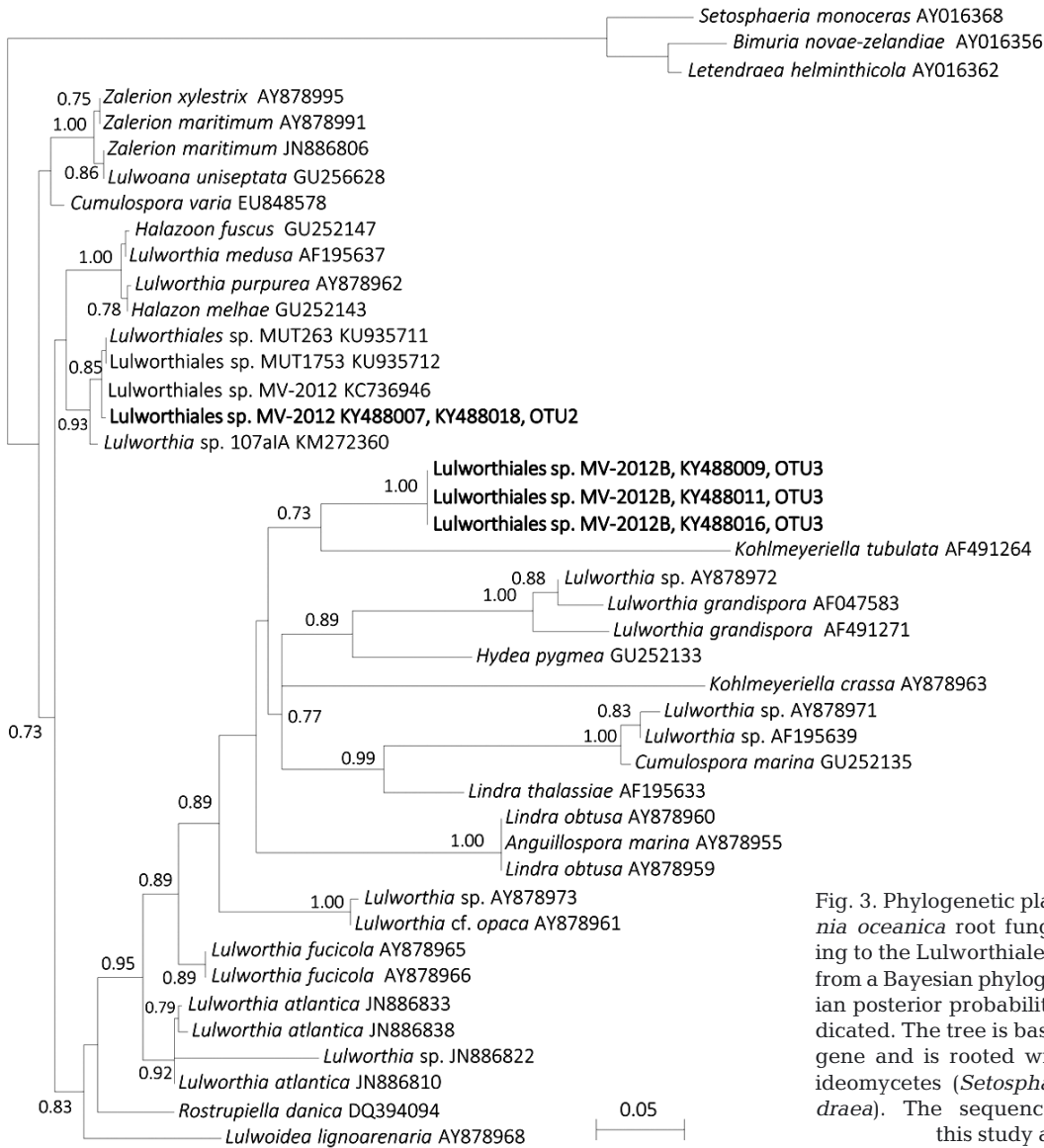


Fig. 3. Phylogenetic placement of the *Posidonia oceanica* root fungal associates belonging to the Lulworthiales. Topology is inferred from a Bayesian phylogenetic analysis. Bayesian posterior probability values ≥ 0.70 are indicated. The tree is based on the LSU nrDNA gene and is rooted with members of Dothideomycetes (*Setosphaeria*, *Bimuria*, *Letendreaa*). The sequences generated during this study are in bold

Molecular detection of root endophytes by 454-pyrosequencing

The obtained sequences were clustered into 11 MOTUs. Two MOTUs comprising 113 sequences represented the plant DNA. Out of the remaining 382 sequences, the 3 most frequent MOTUs (MOTU 1, 2 and 3) comprising 348 sequences matched with sequences derived from pure cultures of Pleosporales sp. MV-2012, i.e. OTU 1 from the cultivation part of this study. MOTUs 4, 5 and 7 matched with sequences of uncultured fungi from various soil samples and belonged to Ascomycota, Chytridiomycota and Basidiomycota, respectively (Table 3). MOTUs 6 and 9, representing 1.8% of the sequences, showed closest affinities to

Lulwoana sp. RP2 (KF719965; Torta et al. 2015). The infrequent MOTU 8 (0.8%) showed affinity to the terrestrial basidiomycetous saprobic fungus *Phlebiopsis gigantea*. A list of all detected fungal MOTUs is provided in Table 3. The counterpart of the third fungus identified from the cultures, i.e. Lulworthiales sp. MV-2012B (= OTU 3), was not detected in this analysis.

DISCUSSION

In agreement with our hypothesis, all sampled *Posidonia oceanica* individuals possessed the uniform root fungal colonization pattern previously reported in this seagrass at 10 other localities in the

Table 3. Fungal molecular operational taxonomic units (MOTUs) obtained in this study based on BLASTn searches in the UNITE and GenBank databases. Country codes — CO: Columbia; FR: France; IT: Italy; NL: The Netherlands

MOTU	No. of sequences (% frequency)	Taxonomic identity	Closest match (GenBank acc. no.)	Coverage/identity (%)	Source	Country	References
MOTU 1	292 (76.5)	Pleosporales sp. MV-2012	Pleosporales sp. MV-2012 isolate P15 (KC412712)	94/99	<i>Posidonion oceanica</i> roots	FR	Vohník et al. (2016a)
MOTU 2	31 (8.1)	Pleosporales sp. MV-2012	Pleosporales sp. MV-2012 isolate P15 (KC412712)	94/95	<i>P. oceanica</i> roots	FR	Vohník et al. (2016a)
MOTU 3	25 (6.5)	Pleosporales sp. MV-2012	Pleosporales sp. MV-2012 isolate P15 (KC412712)	93/98	<i>P. oceanica</i> roots	FR	Vohník et al. (2016a)
MOTU 4	13 (3.4)	Ascomycota	Uncultured soil fungus (HN1037646)	100/98	Agricultural soil	NL	Hannula et al. (2010)
MOTU 5	7 (1.8)	Chytridiomycota	Uncultured fungus (KC965614)	94/93	Soil	USA	Timling et al. (2014)
MOTU 6	5 (1.3)	<i>Lulwoana</i> sp. RP2	<i>Lulwoana</i> sp. RP2 (KF719965)	100/99	<i>P. oceanica</i> roots	IT	Torta et al. (2015)
MOTU 7	4 (1.1)	Basidiomycota	Uncultured fungus (EF434078)	96/85	Soil	USA	Taylor et al. (2007)
MOTU 8	3 (0.8)	<i>Phlebiopsis gigantea</i>	<i>Phlebiopsis gigantea</i> (KP067264)	100/100	Documentary material / indoor airborne	CO	Posado-Buitrago et al. (unpubl.)
MOTU 9	2 (0.5)	<i>Lulwoana</i> sp. RP2	<i>Lulwoana</i> sp. RP2 (KF719965)	83/96	<i>P. oceanica</i> roots	IT	Torta et al. (2015)

NW Mediterranean (Vohník et al. 2015). Thus, the currently known range of this specific symbiosis extends from the northeastern coast of Spain to the Mediterranean coast of France and to the southern coast of Croatia. It has yet to be investigated whether it also occurs along the coast of Italy, as it has not been reported in several recent studies from this region (e.g. Panno et al. 2013, Torta et al. 2015), as well as in the rest of the *P. oceanica* range in the Mediterranean Sea. The colonization pattern comprises sparse to dense mycelial sheaths and parenchymatous nets on the root surface which are characteristic for DSE associations (O'Dell et al. 1993, Fernando & Currah 1996, Vohník & Albrechtová 2011). Both the mycelial sheaths and nets resemble, at least to some extent, sparse hyphal mantles formed by some contact exploration ectomycorrhizal (EcM) fungi (Agerer 2001). On the other hand, the Hartig net, i.e. the EcM interface for the nutrient exchange between the plant and the fungus, is completely missing. Instead, the hypodermis is often filled with microsclerotia which are thought to be physiologically inactive towards the host plant and rather have a storage/dispersal function (Jumpponen & Trappe 1998). Thus, from the morphological point of view, this symbiosis can be viewed as endophytic and more specifically, due to the presence of melanized septate hyphae and melanized intracellular microsclerotia as well as its unique presence in *P. oceanica* roots, as a specific morphotype of DSE association.

In contrast to our hypothesis, both culture-dependent and -independent approaches revealed very narrow fungal spectra lacking typical terrestrial root endophytic fungi and aquatic hyphomycetes known to act as root endophytes (Selosse et al. 2008). On the other hand, the results of this study are in full agreement with previous findings from the NW Mediterranean (Vohník et al. 2016a) and confirm the dominance of a single slow-growing pleosporalean mycobiont, Pleosporales sp. MV-2012, among *P. oceanica* root mycobionts. This fungus belongs to the recently established Aigialaceae family (Ascomycetes, Pleosporales; Suetrong et al. 2009), and to our knowledge, it has not been reported from other hosts or ecosystems. Its closest relatives are probably non-biotrophic marine intertidal fungi known to colonize mangrove bark and wood in various tropical locations (e.g. *Aigialus grandis*, *Rimora mangrovei*) or colonizers of dead bamboo and palm tissues reported from SE Asia and Australia (e.g. *Fissuroma aggre-*

gata) (Suetrong et al. 2009, Liu et al. 2011). However, to our knowledge, no Aigialaceae member has been reported as a biotrophic associate of seagrass roots. Intriguingly, Pleosporales sp. MV-2012 was absent in the *P. oceanica* root mycobiont spectra recently reported from several sites in Italy (Panno et al. 2013, Torta et al. 2015), which may reflect its actual low abundance to absence at those sites or perhaps the methodological approaches employed in these studies, i.e. no surface sterilization prior to mycobiont isolation and too short a cultivation period, respectively (for further discussion, see Vohník et al. 2016a).

A small part (ca. 8% of the isolates and 1.8% of the sequences) of the mycobiont spectra reported here was represented by 2 members of the order Lulworthiales which comprises common marine fungi derived from terrestrial counterparts (Jones 2000, Kohlmeyer et al. 2000) but also fungi infrequently occurring in terrestrial saline and acidic soils (Hujšlová et al. 2010). In general, lulworthioid fungi are considered to be non-biotrophic saprobes ubiquitous in lignocellulosic substrates (Raghukumar 2017) and although a few are also known as seagrass associates, e.g. *Lindra thalassiae* on the seagrass *Thalassia*, *Lulworthia halima* on the seagrass *Zostera marina* (Kohlmeyer et al. 2000) and Lulworthiales sp. MV-2012 on *P. oceanica* (Vohník et al. 2016a, this study), the possible biotrophic mode of their interactions with the host remains dubious. Intriguingly, Torta et al. (2015) reported a lulworthioid mycobiont associated with *P. oceanica* roots and claimed that their paper was 'the first report of *Lulwoana* sp. as DSE in roots of *P. oceanica*' (p. 505). However, the rationale for these claims remains unclear, as the '*Lulwoana* sp.' taxonomical identification relied solely on 99–100% similarity of its ITS nrDNA with the GenBank entry KC145432 (representing Lulworthiales sp. MV-2012 isolated from surface-sterilized *P. oceanica* roots; Vohník et al. 2016a), and no dark septate mycelium was reported by Torta et al. (2015) in the screened *P. oceanica* roots.

In contrast to our hypothesis, pyrosequencing revealed only a few fungi not previously reported from *P. oceanica* roots. These comprised either mycobionts which could not be reasonably identified (MOTUs 4, 5 and 7) or typically terrestrial saprobic basidiomycetes (MOTU 8) with unknown roles in the marine environment/seagrass roots.

The narrow and single-species dominated spectrum of *P. oceanica* root mycobionts reported here is rare in photoautotrophic plants and rather evokes mycorrhizal specificity typically occurring in non-photosynthetic mycoheterotrophic plants (e.g. Taylor

et al. 2002, Bidartondo & Bruns 2005) or some orchids whose seed germination depends on associating with a suitable mycorrhizal partner (e.g. Irwin et al. 2007, Graham & Dearnaley 2012). In contrast, root endophytes are usually highly diverse as exemplified by temperate and boreal conifers (e.g. Holdenrieder & Sieber 1992, Grünig et al. 2002), grasses and understory plants (e.g. Khidir et al. 2010, Bruzone et al. 2015), freshwater aquatic plants (e.g. Kohout et al. 2012, You et al. 2015) and mangroves (e.g. Ananda & Sridhar 2002, Xing & Guo 2011). However, there are too few studies targeting seagrass root endophytes to make any solid general conclusion with respect to their diversity. Nevertheless, the dominance of the pleosporalean endophyte in the *P. oceanica* roots as reported here and in Vohník et al. (2016a) resembles, at least to some extent, the dominance of pleosporalean endophytes in the roots of the dominant desert grass *Bouteloua gracilis* as reported by Porras-Alfaro et al. (2008) and Khidir et al. (2010), even though these apparently have no close taxonomic relationship with the dominant seagrass mycobiont.

Next to nothing is known about the factors which influence root fungal colonization in *P. oceanica* as well as the diversity and distribution of its root mycobionts. Torta et al. (2015) reported that fungal colonization in this seagrass was higher in the roots anchored on rocks than on matte and sand. Hypothetically, the respective fungi could facilitate the seagrass nutrient uptake of minerals, similarly to the so-called rock-eating fungi (Landeweert et al. 2001, Rosenstock 2009). However, this has yet to be experimentally tested and such testing primarily depends on successful axenic cultivation of the slow-growing and difficult-to-cultivate Pleosporales sp. MV-2012 (cf. Vohník et al. 2016b). In this study, the fungal recovery (i.e. the number of obtained fungal isolates) apparently differed among the sampled localities, e.g. the HR-2/Kukuljar locality yielded nearly 3 times more pleosporalean isolates than the HR-13/Gangaro II locality while the number of the lulworthioid isolates was exactly the same (Table 1). Unfortunately, the reasons for these differences remain unknown. In general, fungal recovery may be significantly influenced by many factors, including the methodology used. For example, in this study, the re-visited site (HR-2/Kukuljar) yielded in total 94 isolates from 160 root segments surface-sterilized with ethanol, in contrast to Vohník et al. (2016a) where only 25 isolates were recovered from 80 root segments surface-sterilized with sodium hypochlorite, which nearly doubles the isolation success ratio (58.8 vs. 31.3%, respectively).

While screening *P. oceanica* root fungal colonization, we noticed the presence of morphologically unusual root hairs resembling the adhesive root hairs recently reported in seedlings of the same seagrass in Italy (Badalamenti et al. 2015). It has been suggested that they play a significant role in anchoring the seedlings to rocky substrates, with anchorage strength values up to 5.23 N (Badalamenti et al. 2015). This seems to be the first record of their presence in *P. oceanica* adult plants where their role is less clear. In our case, the root hairs were very scarce, possibly representing rudiments from the seedling stage (provided the sampled plants were of generative origin). Nevertheless, they may help the adults with anchoring in the substrate, similarly to the seedlings.

CONCLUSIONS

In agreement with previous reports from the NW Mediterranean Sea (Vohník et al. 2015, 2016a), all screened *Posidonia oceanica* individuals possessed the typical DSE root colonization, and their root-associated fungal communities were dominated by a single mycobiont, Pleosporales sp. MV-2012, as confirmed by both culturing and 454-pyrosequencing. Practically all detected *P. oceanica* endophytes represent yet undescribed pleosporalean and lulworthioid species, highlighting the *P. oceanica* root mycobiome as an interesting source of novel marine fungi. Although we have no solid proof that the specific fungal association reported by us in *P. oceanica* roots is beneficial to the host plant or is even mycorrhizal, its regular occurrence in visually healthy specimens possessing healthy-looking turgescerent roots along a significant part of the Mediterranean coast suggests an ecologically important biotrophic relationship which may influence the fitness of both symbiotic partners.

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