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Basidiomycota isolated from the Mediterranean Sea – Phylogeny and putative ecological roles

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ABSTRACT

Basidiomycota are an ecologically and taxonomically diverse fungal phylum, colonizing all terrestrial ecosystems, with 30,000 described species. By contrast, in marine habitats Basidiomycota are under-represented compared to Ascomycota. Recently, we investigated the marine mycobiota mainly in the Mediterranean Sea, confirming the scarcity of Basidiomycota. However, a low rank taxonomic identification based on morphological features, proved impossible, since most of the strains remained sterile in axenic culture. Nevertheless, considering the great potential and biotechnological value of Basidiomycota, it would be useful to define their precise taxonomic placement. To this end, 34 marine Basidiomycota isolated from different marine substrates underwent molecular analyses and 123 newly generated sequences were obtained and deposited in GenBank.

Sequencing of the Internal Transcribed Spacer (nrITS) regions allowed us to affiliate the 34 strains with six classes, but a lower taxonomic identification was reached with a multi-locus phylogenetic analysis.

1. Introduction

The marine environment, commonly divided into coastal regions (influenced by land), pelagic and deep-sea habitats (recognised as extreme), is home to a huge variety of microorganisms, among which fungi are often dominant (Richards et al., 2012). Marine fungi are classified as obligate if they grow and reproduce exclusively in the sea, or facultative (terrestrial species able to grow and reproduce in marine environments). Those fungi whose obligate or facultative marine nature is undefined are called marine-derived. Marine fungi have been retrieved worldwide from a broad range of biotic and abiotic substrates such as algae, sponges, corals, sediments etc. (Jones and Pang, 2012; Raghukumar, 2017). Living as mutualists (ecto- and endosymbionts), parasites, pathogens and saprobes, these organisms play an important role as primary decomposers, thus contributing to nutrient recycling (Richards et al., 2012; Raghukumar, 2017). Even though the total number of marine fungi has been estimated to exceed 10,000 taxa, a recent update on accepted classification described only 1112 species, mostly affiliated to Ascomycota (Jones et al., 2015). Basidiomycota are under-represented, with only 74 species (12 obligate and 62 facultative) contributing to marine fungal diversity (Jones and Pang, 2012; Jones et al., 2015; Raghukumar, 2017).

Basidiomycota living in marine habitats are an ecologically and taxonomically diverse group morphologically categorised as filamentous species, able to grow on several substrates such as seagrasses and mangrove wood, and single-celled yeasts, found in association with algae, seagrasses and dead animals or free-floating in the sea. In addition, some fungi (e.g. Cystobasidiales) can exhibit true dimorphism (Jones and Pang, 2012). Most of the Basidiomycota retrieved from the above mentioned unique environments belong to the following classes: Agaricomycetes (e.g. *Nia vibrissa* and *Schizophyllum commune, Grammothele fuligo, Peniophora* sp.), Microbotryomycetes (e.g. *Rhodosporidium diobovantum* and *R. babjeave*), Tremellomycetes (e.g. *Cryptococcus* spp.), Ustilaginomycetes (e.g. *Pseudozyma aphidis*), and Wallemiomycetes (e.g. *Wallemia sebi*) (Jones et al., 2015). However, a significant number of taxa remain undescribed or identified only at genus level. This is mainly due to the following reasons: (i) several isolates in axenic culture do not develop the reproductive structures necessary for morphological identification (sterile mycelia) (Panno et al., 2013; Bovio et al., 2017; Gnavi et al., 2017); (ii) molecular approaches relying on the internal transcribed spacers (nrITS), small or large ribosomal subunits (nrSSU and nrLSU) are not always exhaustive

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(Hibbett et al., 2007, 2014; Binder et al., 2013; Jancic et al., 2015).

Among marine fungi, the understudied Basidiomycota may be an untapped source of potentially novel enzymes and bioactive compounds due to the extreme environmental conditions they adapt to (high salinity and pressure, pH, oxidative stress, low temperature, chemicals and metals) (Bodke et al., 2012; Dalmaso et al., 2015). For instance, the production of ligninolytic enzymes, such as peroxidases and laccases, is well documented in the sponge derived strain *Peniophora* sp. CBMAI 1063 (Bonugli-Santos et al., 2012, 2016), in the mangrove associated *Phlebia* sp. MG-60 (Luo et al., 2005; Raghukumar et al., 2008) and in *Flavodon flavus* isolated from the seagrass *Thalassia hemprichii* (Mtui and Nakamura, 2008). This could signal the great potential of salt-tolerant Basidiomycota in treating coloured industrial effluents and in degrading aromatic recalcitrant pollutants (Bonugli-Santos et al., 2012).

Recently, the cultivable mycobiota associated with macro-algae, seagrasses, sponges and a crude oil contaminated site were investigated in the Mediterranean Sea and in the Atlantic Ocean, confirming the scarcity of Basidiomycota in comparison to their terrestrial counterpart and to the most dominant Ascomycota (Panno et al., 2013; Garzoli et al., 2015; Bovio et al., 2017; Gnavi et al., 2017). In this paper, by means of a combined multi-locus phylogenetic analysis, the authors provide a better phylogenetic placement of 34 Basidiomycota (Panno et al., 2013; Bovio et al., 2017, 2018; Gnavi et al., 2017, 2018), thus increasing our understanding of fungal diversity in the marine environment.

2. Materials and methods

2.1. Fungal isolates and DNA extraction

Fungal isolates used were previously retrieved from different substrates in the Mediterranean Sea and in the Atlantic Ocean (Table 1) and are preserved at the *Mycotheca Universitatis Taurinensis* (MUT). The organisms were originally isolated on Corn Meal Agar SeaWater (CMASW; SW, 3.4% w/v Sea Salt mix – Sigma-Aldrich, Saint Louis, USA – in ddH_2O) medium.

Genomic DNA was extracted from about 100 mg of mycelium, which was carefully scraped from Malt Extract Agar (MEA) plates, transferred to a 2 mL Eppendorf tubes and disrupted in a MM400 tissue lyzer (Retsch GmbH, Haan, Germany). A NucleoSpin kit (Macherey Nagel GmbH, Duren, DE, USA) was used and extraction proceeded according to the manufacturer's instructions. The quality and quantity of DNA samples were measured spectrophotometrically with Infinite 200 PRO NanoQuant (TECAN, Switzerland). DNA was stored at −20 °C.

2.2. PCR amplification and data assembling

Depending on the order/family of affiliation and on the availability of sequences in GenBank, specific markers were amplified in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) (Hibbett et al., 2007).

The nrITS rDNA region was amplified for all strains using the universal primers ITS1/ITS4 (White et al., 1990). Partial nrLSU rDNA was amplified for Polyporales, Psathyrellaceae and Schizophyllaceae using the universal primers LROR/LR7 (Vilgalys and Hester, 1990); partial nrSSU rDNA for Ustilaginaceae, Cistobasidiomycetes, Microbotriomycetes and Holtermanniales using primers PNS1/NS41 (Hibbett, 1996); D1/D2 region of nrLSU rDNA for Ustilaginaceae, Cistobasidiomycetes, Microbotriomycetes and Holtermanniales using primers NL1/NL4 (Kurtzman and Robnett, 1998). The thermocycler was programmed as previously described (Kurtzman and Robnett, 1998; Gnavi et al., 2017).

For Wallemiaceae, partial sequences of the protein coding genes RNA polymerase II subunits *rpb1* and *rpb2* were obtained by using primers RPB1WF/RPB1WR and RPB2WF/RPB2WR, respectively (Nguyen et al., 2015); RPB1-Af/RPB1-Cr were used to amplify *rpb1* in Polyporales and Psathyrellaceae (Matheny, 2005; Carlson et al., 2014). Translation elongation factor *ef-1α* was amplified by using primers EFdf/EF1-2218R (Matheny et al., 2007) for Cistobasidiomycetes, Microbotriomycetes, Schizophyllaceae, Holtermanniales, Psathyrellaceae and Polyporales. Finally, partial sequences of the pre-rRNA processing protein encoding gene *tsr1* were amplified for the Wallemiaceae with the specific primer pair TSR1WF/TSR1WR (Nguyen et al., 2015). PCR parameters for *rpb1* were: initial denaturation at 95 °C for 3 min; 36 cycles at 95 °C for 30 s, 50 °C for 1 min and 72 °C for 1 min; final extension of 8 min at 72 °C. A touchdown PCR protocol was set for *ef-1α* gene: initial denaturation at 94 °C for 2 min; 9 cycles at 94 °C for 40 s, 60 °C for 40 s (minus 1 °C per cycle), 72 °C for 2 min; 36 cycles at 94 °C for 45 s, annealing at 53 °C for 1 min 30 s, extension at 72 °C for 2 min; final extension at 72 °C for 10 min. The PCR profile for *rpb1W*, *rpb2W* and *tsr1W* was as described in Nguyern at al. (2015).

Reaction mixture consisted of 20 ng genomic DNA, 10× PCR Buffer (15 mM $MgCl_2$,500 mM KCl, 100 mM Tris-HCl, pH 8.3), 200 μM each dNTP, 1 μM each primer, 2.5 U Taq DNA Polymerase (Qiagen, Chatsworth, CA, USA), in 50 μL final volume. For problematic cases, additional $MgCl_2$ and/or 2.5% DMSO facilitated the reaction.

Amplicons were visualized on a 1.5% agarose gel stained with 5 mL 100 mL^{-1} ethidium bromide and a GelPilot 1 kb plus DNA Ladder was used; PCR products were purified and sequenced at Macrogen Europe Laboratory (Amsterdam, The Netherlands). The resulting ABI chromatograms were processed and assembled to obtain consensus sequences using Sequencer 5.0 (GeneCodes, Ann Arbor, Michigan, USA http://www.genecodes.com). Newly generated sequences were deposited in GenBank (Table 1).

2.3. Sequence alignment and phylogenetic analysis

Eleven datasets were assembled on the basis of BLASTn results and of recent phylogenetic studies that included allied taxa, as follows: Psathyrellaceae (Nagy et al., 2013; Orstadius et al., 2015), Peniophoraceae (Hallenberg et al., 1996), Ustilaginaceae (Wang et al., 2015a), Sporidiobolaceae (Wang et al., 2015b), Schizophyllaceae (Siqueira et al., 2016), Polyporales (Phleboid clade and *Trametes*) (Justo and Hibbett, 2011; Binder et al., 2013; Miettinen et al., 2016), Holtermanniales (Wuczkowski et al., 2011; Liu et al., 2015) and Wallemiales (Jancic et al., 2015) (Table 2; Table S1-S9). Sequences were retrieved from GenBank.

Alignments for each gene were generated using MUSCLE (default conditions for gap openings and gap extension penalties), implemented in MEGA v. 7.0 (Molecular Evolutionary Genetics Analysis), visually inspected and trimmed by TrimAl v. 1.2 (http://trimal.cgenomics.org) to delimit and discard ambiguously aligned regions. Since preliminary analyses suggested no incongruence among single-loci phylogenetic trees, alignments were concatenated into a single data matrix with SequenceMatrix v. 1.8 (Vaidya et al., 2011). The appropriate evolutionary model under the Akaike Information Criterion (AIC) was determined for each partition with jModelTest 2 (Darriba et al., 2012). Phylogenetic inferences were calculated using two approaches. First, Bayesian Inference (BI) was performed with MrBayes 3.2.2 (Ronquist et al., 2012) under GTR + I + G evolutionary model (best model). The alignment was run for 10 million generations with two independent runs each containing four Markov Chains Monte Carlo (MCMC) and sampling every 1000 iterations. The first 2500 trees were discarded as "burn-in" (25%). Using the Sumt function of MrBayes a consensus tree was generated and Bayesian posterior probabilities (BPP) were