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Early-diverging wood-decaying fungi detected using three complementary sampling methods ☆

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ABSTRACT

Wood-decaying fungi are essential components of degradation systems in forest ecosystems. However, their species diversity and ecological features are largely unknown. Three methods are commonly used to investigate fungal diversity: fruiting body collection, culturing, and environmental DNA analysis. Because no single method fully characterises fungal diversity, complementary approaches using two or more methods are required. However, few studies have compared the different methods and determined the best way to characterise fungal diversity. To this end, we investigated wood-decomposing Dacrymycetes (Agaricomycotina, Basidiomycota) using a complementary approach combining fruiting body collection, culturing, and environmental DNA analysis, thereby offering an effective approach for investigating the diversity of saprotrophic mushrooms. Fruiting body collection, culturing, and environmental DNA analysis detected 11, 10, and 16 operational taxonomic units (OTUs; 25 OTUs in total) and identified three, seven, and seven novel lineages, respectively. The three methods were complementary to each other to detect greater Dacrymycetes diversity. The culturing and environmental DNA analysis identified three early-diverging lineages that were not identified in the fruiting body collection suggesting that diverse lineages lacking observable fruiting bodies remain undiscovered. Such lineages may be important to understand Dacrymycetes evolution. To detect early branches of Dacrymycetes more efficiently, we recommend a combined approach consisting of a primary environmental DNA survey to detect novel lineages and a secondary culture survey to isolate their living mycelia. This approach would be helpful for identifying otherwise-undetectable lineages, and could thus uncover missing links that are important for understanding the evolution of mushroom-forming fungi.
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1. Introduction

Wood-decaying fungi are the primary decomposers in forest ecosystems, and are mainly mushrooms belonging to the Basidiomycota or Ascomycota (Deacon, 2006). Mushrooms are one of the most conspicuous and studied groups in the Kingdom Fungi; however, the species diversity of macroscopic fungi, including wood-decaying mushrooms, remains largely unknown (Mueller et al., 2007; Hibbett et al., 2011).

Three methods are used to detect mushroom diversity in the field: fruiting body collection, culturing, and environmental DNA analysis. Fruiting body collection is the most traditional and common method used to survey mushroom diversity (Watling, 1995; Straatsma et al., 2001). Mushroom specimens and sometimes cultures can be obtained from collected fruiting bodies. However, this approach depends on the visual confirmation of sporocarps and misses the strains that exist as microscopic mycelia without forming any visible fruiting body (Horton and Bruns, 2001; Moore et al., 2008). Even if a fungus has sporocarps large enough to be found, they could be undetected because fruiting body formation is seasonal and ephemeral.

The production of sporocarps is not a prerequisite for the other two methods; culturing (Rayner and Todd, 1979; Stenlid et al., 2008) and environmental DNA analysis (Johannesson and Stenlid, 1999; O'Brien et al., 2005; Lynch and Thorn, 2006; Porter et al., 2008a; Kubartová et al., 2012). Using culturing methods, fungi growing in decaying wood or fallen leaves can be grown in their mycelial form on agar plates. However, many mycorrhizal or parasitic fungi cannot be isolated using standard culturing methods (e.g., Allen et al., 2003). Even if they are potentially culturable, strains for which the culture conditions are unsuitable may be missed. Among the three methods, environmental DNA analysis has been shown to detect the highest diversity of operational

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taxonomic units (OTUs; Arnold et al., 2007; Lindner et al., 2011). This method has allowed the identification of novel strains, including deep-branching lineages of fungi such as Cryptomycota or Archaeorhizomycetes (van Hannen et al., 1999; Porter et al., 2008a; Lara et al., 2010; Jones et al., 2011; Rosling et al., 2011). However, this new technique has sometimes failed to detect the OTUs obtained in simultaneous fruiting body collection or culturing (Arnold et al., 2007; Porter et al., 2008b; Higgins et al., 2011; Langarica-Fuentes et al., 2014). This inconsistency might be because of differences in the sampling densities among the methods (Allmér et al., 2006; Lindner et al., 2011) or biases caused during DNA extraction or PCR (Martin-Laurent et al., 2001; Tedersoo et al., 2010; Lindahl et al., 2013). Environmental DNA analysis produces DNA or RNA sequences a few hundred bases long, and cannot provide additional biological data that the fruiting body and culture provide. As reviewed above, each method has advantages and disadvantages for investigating fungal diversity.

Complementary approaches using two or three of these methods have been conducted. Using such approaches, researchers have accumulated basic information to develop effective strategies to survey mushroom diversity (Johannesson and Stenlid, 1999; Allmér et al., 2006; Porter et al., 2008b; Lindner et al., 2011; Fischer et al., 2012; Hiscox et al., 2015). However, few studies have compared all three methods (Allmér et al., 2006; Lindner et al., 2011). Thus, the best way to determine total macro-fungal diversity has not been adequately discussed.

The goal of this study was to detect the diversity of wood-decaying fungi in a forest ecosystem. To achieve this goal, the class Dacrymycetes, a group of wood-decomposers in the mushroom-forming Agaricomycotina (Basidiomycota), was selected as a model group. This class is a monophyletic group containing approximately 110 species of brown-rot fungi, and is appropriate for studying the ecology and evolution of wood-decaying basidiomycetes (Shirouzu et al., 2013, 2014). There were three reasons for selecting this group: (1) they have visible fruiting bodies where most of their species diversity has been obtained from sporocarp studies (Kobayasi, 1939a,b; McNabb, 1973; Reid, 1974; Shirouzu et al., 2009b); (2) they are culturable on agar plates, and their remarkable yellow- to orange-coloured colonies are easily recognised in selective isolations; and (3) many reference DNA sequences are available to facilitate their molecular identification (Weiß and Oberwinkler, 2001; Shirouzu et al., 2009b, 2013). In this study, we surveyed the diversity of wood-decomposing Dacrymycetes using a complementary approach combining fruiting body collection, culturing, and environmental DNA analysis, and detected several new phylogenetic lineages.

2. Materials and methods

2.1. Study site and sample collection

A 40 × 40 m plot was established in a *Pinus densiflora* forest (36°15′N, 140°05′E; alt. 100 m) at Mt. Tsukuba, Ibaraki, Japan. Fruiting bodies of Dacrymycetes and fallen branches of *P. densiflora* were collected monthly from May 2013 to April 2014 except for February 2014, when the site was covered by heavy snow. All visible fruiting bodies of Dacrymycetes on *P. densiflora* branches (1–5 cm diam.) were collected during a 1-h collection at the plot. The fruiting bodies were morphologically observed under a light microscope for species identification (Shirouzu et al., 2009b). Cultures were obtained by multispore isolation from the fruiting bodies and were stored in sealed vials containing 0.1% cornmeal agar (0.2% CMA; Nissui, Tokyo, Japan) + 1.25% malt agar (2.5% MA; Nissui) medium (0.2% CMA 8.5 g, 2.5% MA 22.5 g, yeast extract 1 g, distilled water 1 l). Four fallen *P. densiflora* branches (1–5 cm diam.) in each of three decomposition stages (II, III, and IV; Berg and McClaugherty, 2003) were collected for culturing and DNA extraction.

2.2. Culture isolation from decaying branches

A high-throughput dilution-to-extinction technique (Collado et al., 2007) was modified and used to isolate fungi from the branches. Collected branches were debarked and washed with a brush in running tap water. A 10-g segment of wood was cut from the washed branch and pulverised with 500 ml distilled water using a blender (7011HS, Waring Commercial, Torrington, CT, USA) for 1 min at the "high" setting. Using an electric sieve shaker (M-3T, Tsutsui Scientific Instruments Co., Ltd., Tokyo, Japan), the pulverised wood was passed through four sieves (500-μm, 300-μm, 212-μm, and 106-μm mesh sizes) with running distilled water, and the particles that aggregated in the 106 μm sieve were collected. Particles from two branches at the same decomposition stage were mixed, and 1 mg composite was transferred to a 50-ml centrifuge tube. For particle washing, 20 ml distilled water was added to the tube. After centrifugation at 2200*g* for 3 min with a tabletop centrifuge (Model 4000, KUBOTA, Tokyo, Japan), the supernatant was removed from the tube and 20 ml fresh distilled water was added. This particle washing was repeated 10 times. The washed particles were diluted with a 1% CMC (carboxymethyl cellulose, No. 1190, Daicel FineChem Ltd., Tokyo, Japan) solution to a concentration of 2 particles/50 μl. A 50-μl aliquot of the CMC solution, including wood particles, was dispensed into each well containing 500 μl malt agar medium (2.5% MA 45 g, yeast extract 1 g, chloramphenicol 10 mg, distilled water 1 l) of a 48-well microplate. Two microplates were prepared for each particle composite (12 plates/sampling event). The microplates were sealed with parafilm and incubated at 25 °C (12 h light/12 h dark cycle) for 1 month. During incubation, the plates were observed every week under a stereomicroscope, and the colonies with characteristics of Dacrymycetes (white or yellow to orange in colour, velvety or wet in texture) were isolated and preserved in sealed vials containing 0.1% CMA + 1.25% MA medium. The isolated cultures are available from NBRC (NITE Biological Resource Center, Chiba, Japan).

2.3. DNA extraction from cultures and fruiting bodies, PCR, and sequencing

Genomic DNA was extracted from the cultured mycelia and the collected fruiting bodies with PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Primers D1 (Peterson, 2000) and NL4 (O'Donnell, 1993) were used to amplify the large subunit (LSU) ribosomal RNA (rRNA) gene D1–D2 region. Each PCR reaction mixture (10 μl) contained 1 μl genomic DNA, 5 μl EmeraldAmp PCR Master Mix (Takara Bio Inc., Shiga, Japan), 0.25 μl each primer (10 μM), and 3.5 μl distilled water. Cycling parameters were one cycle of 3 min at 94 °C; followed by 35 cycles of 30 s at 94 °C, 30 s at 51 °C, 1 min at 72 °C; and a final cycle of 5 min at 72 °C. The resulting PCR products were purified using an Illustra ExoProStar kit (GE Healthcare UK Limited, Buckinghamshire, UK) and were directly sequenced using a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's instructions. Capillary electrophoresis and data collection were performed on a 3130xl Genetic Analyzer (Applied Biosystems). Small subunit (SSU) rRNA genes and internal transcribed spacer (ITS) regions, including the 5.8S rRNA genes of the samples believed to be novel lineages, were additionally amplified with NS1/NS8 (White et al., 1990) and ITS1F (Gardes and Bruns, 1993)/ITS4 primers (White et al., 1990). The obtained PCR products were purified and sequenced as described above.

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