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
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 Cryptomycoporta or Archaeoarchaemycetales (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 (Weiβ 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 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 pulvérised 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 pulvérised 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 2200g 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. 1150, 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/sample event). The microplates were sealed with paraffilm 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) 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 N51/NSB (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.
2.4. DNA extraction from decaying wood and cloning

For DNA extraction from decaying branches, 2 mg pulverised and washed wood particles from each branch was placed in a 2-ml microtube with zirconia beads and ground with a Tissuelyser (Qiagen, Valencia, CA, USA) at 27 Hz for 5 min. The DNA was extracted from the wood powder and purified with a Genomic DNA Extraction Kit (Plant, RBC Bioscience, New Taipei City, Taiwan) and was purified again using a MagExtractor (Toyobo, Osaka, Japan). The LSU was selected as the target sequence because a large body of sequence data is available for this region, and it is suitable for phylogenetic analyses of Dacrymycetes (Shirouzu et al., 2013). The extracted DNA was used as the PCR template and amplified using the primers ITS2D (GTAGGACTACCGCTGAACCTAGGC/ NL4 (O'Donnell, 1993) in the same PCR mixtures as those described above. The primer ITS2D was designed based on a conserved region in the ITS2 regions of Dacrymycetes. To avoid producing chimeric sequences during the multi-template PCR, the cycling parameters were set at high annealing temperatures, and the rate of temperature change was set at 1 °C/s (Saitoh and Chen, 2008; Stevens et al., 2013). The PCR cycling parameters were as follows: one cycle of 3 min at 94 °C, six cycles of 30 s at 94 °C, 30 s at 67 °C → 62 °C, 1 min 30 s at 72 °C, 24 cycles of 30 s at 94 °C, 30 s at 62 °C, 30 s at 72 °C, and a final cycle of 5 min at 72 °C. To reduce the formation of heteroduplex cloning artefacts, 1 μl PCR product was purified with ExoProStar and used as a template for an additional amplification (one cycle of 3 min at 94 °C, three cycles of 1 min at 94 °C, 30 s at 62 °C, 2 min at 72 °C, and one cycle of 5 min at 72 °C) (Thompson et al., 2002). The final PCR product was purified with a HiYield Gel PCR DNA Fragment Extraction Kit (RBC Bioscience) and was used for DNA cloning with the pGEM-T Easy Vector System (Promega, Madison, WI, USA). Ten colonies of recombinant competent cells (XL10-Gold, Agilent Technologies, Santa Clara, CA, USA) were picked for each sample, amplified with the vectorspecific primers (T7/SP6), and sequenced. The obtained sequences were analysed with the Bellerophon program to detect and remove chimeric sequences (Huber et al., 2004).

2.5. Statistical analysis

The OTUs were defined on the basis of 97% or 99% (Lynch and Thorn, 2006; Porter et al., 2008b) sequence similarity of the LSU region. The sequences were clustered using BLASTclust (http://toolkit.tuebingen.mpg.de/blastclust) with a threshold setting of 70% coverage (default) and identities of 97% or 99%. Sample-based rarefaction of OTU curves were extrapolated using EstimateS 9 (Colwell, 2013) based on the presence/absence of OTUs obtained using each method at each sampling event. Chao 2 was used to estimate asymptotic richness. Analysis of variance (ANOVA) was performed to determine the factors affecting numbers of detected OTUs using R (R Core Team, 2014) with the car package (“Anova” function; Fox and Weisberg, 2011) through a generalised linear model (GLM) with a Poisson distribution (“glm” function). The numbers of OTUs were used as response variables, and the sampling method (fruiting body, culturing, and environmental DNA analysis), season of sampling event (spring [Mar.–May.], summer [Jun.–Aug.], autumn [Sep.–Nov.], and winter [Dec.–Feb.]) and decomposition stage (I, II, III, and IV) of the wood were used as the explanatory variables. To evaluate the factors affecting OTU compositions, a permutational multivariate analysis of variance (PERMANOVA) was performed using R with Jaccard distances from the MASS package (“adonis” function; n permutations = 5000; Venables and Ripley, 2002). In the data set for PERMANOVA, the occurrence of each OTU was entered as a binary value of 0 (absence) or 1 (presence).

2.6. Phylogenetic analysis

As well as the sequences obtained in this study, available sequences of Dacrymycetes and other Agaricomycotina species were downloaded from the DNA Data Bank of Japan (DDBJ) and were used to assemble datasets for the phylogenetic analyses. Multiple sequence alignments were generated with MAFFT 7 (mafft.cbrc.jp/alignment/software; Katoh and Standley, 2013). Poorly aligned regions of the alignments were removed for analysis. Molecular phylogenetic analyses of the LSU sequences were performed with RAxML 8.1.15 (Stamatakis, 2014) under a GTRGAMMA model. The maximum likelihood bootstrap proportions (MLBs) and trees were obtained by simultaneously running rapid bootstrap analyses of 1000 pseudoreplicates followed by a search for the most likely tree. Based on the generated phylogenetic tree, novel lineages were defined as OTUs that did not form clades with the previously known Dacrymycetes sequences, and that differed from other OTUs with a threshold of 97% similarity. We did not use the threshold of 99% similarity for this definition because many paraphyletic groups were made. To confirm the phylogenetic positions of the newly discovered lineages based on fruiting bodies and cultures, we conducted a multi-marker analysis using the sequences of LSU, SSU, and ITS regions. The ITS and multi-marker dataset was partitioned to allow different parameters for each gene region and was analysed using RAxML as described above. All aligned data were uploaded to TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:516449).

3. Results

The fruiting body collection, culturing, and environmental DNA analysis yielded 49 specimens, 18 isolates, and 53 clones of Dacrymycetes (Table S1). With a threshold of 97% (99%) sequence similarity, fruiting body collection, culturing, and environmental DNA analysis detected 11 (20), 10 (13), and 16 (24) OTUs, respectively (25 OTUs [46 OTUs] in total; Fig. 1). At 97% similarity, the rarefaction curves of environmental DNA and culture continued to increase, but that of the fruiting body was saturated at approximately 10 sampling events (Fig. 2). At 99% similarity, all curves were estimated to increase. The ANOVA results indicated that the sampling method and stage of decomposition significantly (Pr < 0.001) affected the numbers of OTUs (Table 1). The PERMANOVA result showed that the sampling method significantly (Pr < 0.001) affected the compositions of the OTUs (Table 2). A phylogenetic tree was constructed from all LSU sequences obtained using the three methods (Fig. 3). Three, seven, and seven of the OTUs detected by fruiting body, culturing, and environmental DNA analysis, respectively, were defined as novel lineages (A to M among 13 total OTUs; Fig. 3). Three single- and one multi-marker phylogenetic trees were constructed from the sequences of the LSU, SSU, and ITS regions derived from the collected spores or isolated cultures (Fig. 4). Three newly discovered lineages (1, L, and M) represented the early branches of the Dacrymycetes clade (Figs. 3, 4, and S1).

4. Discussion

4.1. A complementary approach for investigating saprotrophic mushrooms

At the threshold of 97% (99%) sequence similarity, 11 (10) OTUs were redundantly detected by two or three methods. However, 14 (36) OTUs were identified only using a specific method; the same trend was reported in previous studies (Fig. 1; Allmér et al., 2006; Lindner et al., 2011). The type of method affected both the
numbers and the composition of OTUs (Tables 1 and 2). These results confirm that mushroom diversity in a forest ecosystem cannot be fully described by any single method; therefore, a combined approach using two or more methods is recommended for detecting diverse fungal lineages.

Comparing the rarefaction curves of the fruiting body between the thresholds of 97% and 99% similarities, the former was saturated at approximately 10 sampling events, but the latter tended to increase (Fig. 2). If the number of sampling events increases, sporocarp collection is expected to identify new OTUs that are closely related to the strains already obtained. In contrast, the rarefaction curves for the environmental DNA and culture tended to increase, even at 97% similarity. These two methods are expected to detect a divergent array of Dacrymycetes, different from those detected through fruiting body collection.

Fruiting body collection detected the smallest number (three) of new lineages (Fig. 3). This result is reasonable because the reference sequences of Dacrymycetes had been obtained from the collected fruiting bodies (Weiß and Oberwinkler, 2001; Shirouzu et al., 2009b). Sporocarp collection is inexpensive, and provides mushroom specimens that are useful for species identifications and additional experiments, for example, chemical analyses. Therefore, this method could be the first choice for mushroom surveys, especially in poorly investigated areas. Although this approach detected three novel lineages in this study (Fig. 3), the rarefaction curves revealed that fruiting body collection has a limited capacity to reveal new strains at a well-investigated site.
Fig. 3. Phylogenetic tree of Dacrymycetes estimated with RAxML using previously known and newly obtained LSU sequences. Thick branches indicate MLBP > 80%. A–M are newly discovered lineages. Black triangles, white circles, and grey squares indicate sequences obtained using fruiting body, culture, and environmental DNA, respectively.
Fig. 3 (continued)
(Fig. 2). This limitation might arise from the fundamental shortcoming of this approach, which does not detect fungi that lack visible fruiting bodies (Horton and Bruns, 2001; Moore et al., 2008). We consider this disadvantage to be a common problem associated with studies of mushroom-forming fungi that have been investigated mostly based on collected sporocarps.

Comparable to the environmental DNA analysis, culturing detected seven new lineages (Fig. 3). Traditional isolation methods that involve incubating pieces of natural substrata on agar plates require special techniques to pick individual mycelia or spores from miscellaneous colonies growing from mixed plates (Guo et al., 2000; Shirouzu et al., 2009a). Moreover, fast-growing filamentous fungi, such as Trichoderma spp. or Mucor spp., often quickly cover the surfaces of agar plates before isolators can detect slow-growing species. This problem was solved using the high-throughput dilution-to-extinction method that, when modified in this study, succeeded in isolating slow-growing Dacrymycetes (Table S1; Collado et al., 2007). In this method, substrata such as soils or decaying wood are pulverised to fine particles (ideally, one particle includes one fungus) and are then diluted and dispensed into microplate wells to separate each colony. This method relieves the biases from technical differences among the isolators and avoids overestimates of fast-growing fungi.

A fundamental shortcoming of culturing is that the detected species are limited to strains that grow in the given conditions, so that species not culturable using standard methods are easily
overlooked. This problem might be partially avoided by adjusting the culture conditions to be more suitable for growth of difficult to culture organisms. For example, del Campo et al. (2013) successfully cultured eukaryotic protists by mimicking the nutritional conditions in their original habitats. Ecological or physiological information from previously non-culturable fungi might provide clues to establish suitable growth conditions in laboratories.

The environmental DNA analysis detected the most OTUs (16; Fig. 1). This result was also reported in a previous study investigating fungal communities in Picea abies logs (Lindner et al., 2011). These data demonstrated the high performance of environmental DNA analyses for detecting diverse fungal OTUs from environmental samples. The environmental DNA analysis, however, did not obtain sequences of Calocera cornea, Dacrymyces variisporus, and Dacryopinax sphenocarpa that were identified in simultaneous fruiting body collection or culturing (Fig. 3). This incongruity might come from differences in the sampling densities, which are difficult to keep consistent among the different methods, such as fungal fruiting bodies and fallen branches (Allmèr et al., 2006; Lindner et al., 2011). DNA extractions and PCR have the potential to generate biases in detecting microbial OTUs from environmental samples (Martin-Laurent et al., 2001; Tedersoo et al., 2010; Lindahl et al., 2013). During PCR, single mismatches between the primer and template could bias the sequence amplifications (Ihrmark et al., 2012). The primer ITS2D was designed based on a conserved region of the ITS2 sequences of Dacrymycetes; however, the primer has one or two nucleotide mismatches with the sequences of some dactylymeceteous species. Although the primer is not specific for Dacrymycetes and does not necessarily amplify the sequences of all dactylymeceteous strains, it functioned sufficiently to detect diverse lineages from the Dacrymycetes phylogeny (Fig. 3). PCR with two or more primer sets corresponding to intragroup polymorphisms may address the problem of primer mismatches.

Porter et al. (2008b) reported that sporocarp collection primarily recovered fungi with large distinct fruiting bodies, and environmental DNA analysis detected taxa with inconspicuous sporocarps. The latter technique identified novel deep-branches in the fungal phylogeny and provided incentives to collect further biological information about these newly discovered lineages (Jones et al., 2011; Rosling et al., 2011). In this study, environmental DNA analysis and culturing also showed the potential for detecting novel strains, including unknown early-diverging lineages, which may rarely or never produce visible fruiting bodies (Figs. 2 and 3). As an effective approach to study these hidden strains, we recommend a combined approach consisting of a primary survey with environmental DNA analysis to detect novel fungal lineages and identify their micro-habitats, followed by a secondary survey with culturing to isolate their living mycelia. The use of a next-generation sequencer would increase the detection efficiency in the primary survey. This combined approach is applicable for various saprotrophic fungi, and could extend our knowledge of fungal diversity.

4.2. Missing early branches in macro-fungal phylogeny

Dacrymycetes species produce fruiting bodies of various sizes and shapes, ranging from 0.5-mm pustulate to 10-cm dendroid sporocarps (Kobayasi, 1939a,b; McNabb, 1973; Reid, 1974; Shirouzu et al., 2009b). A phylogenetic analysis of Dacrymycetes showed that its early-diverging lineages produce inconspicuous small or thin fruiting bodies (Shirouzu et al., 2013). In this study, the novel lineages I, L, and M, which branched at early evolutionary stages, were detected by environmental DNA analysis and culturing. However, their sporocarps were not detected by fruiting body collection (Figs. 3 and 4). Among them, the lineage L was placed in a sister position in relation to the Dacrymycetales clade (Fig. 4), suggesting that it might be an independent group described as a new family or order. These intriguing lineages, which help us to understand the evolutionary history of Dacrymycetes, might rarely or never produce visible sporocarps, and could be overlooked by traditional collections based on direct observation.

In the other two higher taxa of mushroom-forming Agaricomycotina, Agaricomycetes and Tremellomycetes, the species lacking conspicuous sporocarps were estimated to diverge near the roots of their phylogenetic trees (Hibbett and Binder, 2002; Hibbett, 2004; Millanes et al., 2011). Our knowledge of the Agaricomycetes, which include common mushrooms (major decomposers and ectomycorrhizal symbionts) might be biased with an overrepresentation of lineages with observable fruiting bodies. Most studies on the diversity and phylogeny of Agaricomycetes have been based on fruiting body collection (e.g., Binder and Hibbett, 2006; Hibbett, 2006); thus, significant numbers of mushroom lineages might be missed if they do not produce visible sporocarps. The complementary approach using environmental DNA analysis and culturing could reveal the hidden diversity of not only Dacrymycetes but also other saprotrophic mushrooms.

5. Conclusions

The three methods tested in this study were complementary to each other for the detection of a diverse array of Dacrymycetes. Among these methods, environmental DNA analysis and culturing detected new strains, including early-diverging lineages that were not found by fruiting body collections. This result suggests that invisible lineages lacking distinct fruiting bodies maybe present in the substrata as mycelia, and that these are the key strains for discussing the evolution of Dacrymycetes. Previous studies focusing on the diversity and phylogeny of macro-fungi have largely depended on fruiting body collection, which may overlook fungal strains without observable sporocarps.

As an effective approach to investigate the invisible diversity of saprotrophic mushrooms, we recommend a combined approach consisting of a primary survey with environmental DNA analysis to search for novel lineages, and a secondary survey with culturing to isolate their living mycelia. Isolated cultures of newly discovered fungal lineages are needed to acquire additional biological information, such as genome sequences and various physiological and ecological data. Surveys of macro-fungal lineages lacking conspicuous fruiting bodies might uncover missing links, which could increase our understanding of the diversity and evolution of mushroom-forming fungi.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.01.015.

References
