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Ectomycorrhizal fungi identification in single and pooled root samples: terminal restriction fragment length polymorphism (TRFLP) and morphotyping compared

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Abstract

We describe here the results of a study conducted to evaluate a terminal restriction fragment length polymorphism (TRFLP) approach targeting rRNA genes for determination of ectomycorrhizal (ECM) communities colonizing the roots of loblolly pine (*Pinus taeda* L.). Root tips separated from soil cores were classified according to morphological characteristics and DNA was then extracted from each group of morphotyped tips. Labeled primers were used to generate terminal restriction fragments (TRF) for molecular fingerprinting of root colonizing fungi and to determine how well TRFLP could be used to discriminate between ectomycorrhizal types. Morphotypes generally correlated well with specific TRFs and sequence analysis confirmed that TRFs could be used to discriminate among fungal types. Sequence analysis indicated that important ECM fungi including Russulaceae, Thelephorales, and Tricholomataceae could be fingerprinted with TRFLP. In addition, a fixed proportion of the DNA extracted from each morphotype from the same core was used in a pooling experiment used to assess whether previously identified fungal species types could be distinguished from one another within reconstructed communities. Since some morphotypes share TRFs, dual analysis of ITS1 and ITS2 was necessary for accurate fingerprinting of fungal types. Approximately, 5.0 ± 0.3 phylotypes were detected per core with TRFLP-corrected morphotyping as compared to 4.0 ± 0.4 phylotypes using TRFLP on pooled community samples. TRFLP made on experimental sporocarp communities suggested that reduced ECM richness with TRFLP may be partly caused by differences in the amount of DNA available for PCR and primer bias. Nonetheless, TRFLP on pooled morphotypes accounted for more than 93% of colonized root tips. The method can be used to facilitate analysis of mycorrhizal communities using root tips collected from soil cores.
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Keywords: Community structure; Ectomycorrhiza; Morphotyping; *Pinus taeda*; Terminal restriction fragment length polymorphism

1. Introduction

The fungi forming ectomycorrhizal symbioses number over 5000 species (Molina et al., 2002) spanning all the phyla of true fungi. Analysis of natural ectomycorrhizal (ECM) communities has traditionally been a labor-intensive, highly-skilled process with heavy reliance on gross morphological characterization of the ECM root tips. Depending on the rigor of the classification protocol, it is possible to incorrectly assign dissimilar genetic entities into a morphotype when analyzing individual tips. This results in the need for multiple samples to be analyzed per morphotype to allow for mathematical subdivision of the group into genetic types. Because of the high spatial variability of naturally occurring ectomycorrhizas, a large number of samples are needed to effectively address questions concerning community structure, with many hundreds of individual tips to be analyzed per sample. The high labor requirement remains an obstacle to extensive research efforts, even with the addition of molecular verification of identified morphotypes using restriction fragment length polymorphisms (RFLP) which allows researchers to avoid in-depth microscopic study of root tips (Horton and Bruns, 2001). A rapid method for whole

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community analysis would allow researchers to reasonably expand research projects on ECM ecology to the level of replication necessary to overcome these sampling difficulties.

A number of molecular methods are currently in use for characterization of microbial communities; these include restriction fragment length polymorphism analysis (RFLP, Giovannoni et al., 1990), denaturing gradient gel electrophoresis (DGGE, Muyzer et al., 1993), terminal restriction fragment length polymorphism (TRFLP, Liu et al., 1997) and length heterogeneity PCR (LH-PCR, Suzuki et al., 1998). The most promising in terms of ease of use and resolution appears to be TRFLP (Liu et al., 1997). Since, first publication in 1997, TRFLP has been shown to be a highly effective method for analysis of microbial communities, to be relatively stable to variability in PCR conditions (Blackwood et al., 2003), and may detect a higher number of phylotypes than DGGE assays (Marsh et al., 1998; Moeseneder et al., 1999). In TRFLP analyses, fluorescent label unique to each PCR primer in a reaction allows for detection of the terminal fragments of restriction digested PCR products. These terminal restriction fragments (TRFs) contain the labeled primer and extend to the first instance of a restriction site for the enzyme used (Liu et al., 1997). Using this method, environmental samples can be rapidly analyzed providing extensive data on the community as defined by the specificity range of the primers. For comparison of microbial communities, TRFLP provides a relatively complete, culture-independent analysis.

TRFLP targeting rRNA genes has effectively been used to characterize fungal communities in soil including mycorrhizal communities (Edwards et al., 2004; Edel-Hermann et al., 2004; Vandenkoornhuyse et al., 2003; Dickie et al., 2002; Klamer et al., 2002). DNA profiles developed using TRFLP have been used to assess overall changes to fungal community structure in soil under elevated CO_2 (Klamer et al., 2002) and with organic matter amendment (Edel-Hermann et al., 2004). Other researchers have employed TRFLP as a molecular fingerprinting method for the identification of specific fungal taxa (Edwards et al., 2004; Dickie et al., 2002). This approach has been used to determine the vertical distribution of ECM in soil (Dickie et al., 2002) and to determine the effect of fertilization on ECM communities (Edwards et al., 2004). The use of TRFLP as a fingerprinting technique may provide researchers with a specific molecular approach for characterization of ECM communities that overcomes difficulties attendant with morphotyping. However, no one to date has examined how accurately TRFLP fingerprinting predicts ECM species richness of colonized root tips as compared to morphotyping or the resolution of the resulting analysis. In effect, are detected TRFs an accurate estimate of species-type richness or do some TRFs reflect closely-related fungal species or genera? Since, soil is a heterogeneous environment that is expected to contain fungal species with strategies ranging from mutualistic to saprophytic, efforts to assess ECM richness from soil hyphae can be obscured by the presence of additional non-ECM TRFLP phylotypes (Dickie et al., 2002), making accurate identification problematic. In addition, seasonally rare or cryptic species can be missed even when large numbers of samples are collected (Taylor, 2002).

In the current study, we compared traditional morphotyping techniques with molecular approaches for determining ECM richness of root tips separated from soil cores. Our purpose was to determine how well a TRFLP fingerprinting technique could estimate ECM richness compared to morphotyping and the specificity of TRFLP phylotypes at the taxonomic level. Our approach was to correlate morphotype, TRFLP fingerprinting and sequence information to develop a relatively accurate portrait of ECM richness in a small subsample of cores from a natural system. We then applied this information to analysis of root tip communities from those same cores.

2. Materials and methods

2.1. Site description and soil sampling

The study site is an 8-year-old loblolly pine (*Pinus taeda* L.) genetics plantation located in Scotland County, North Carolina, USA that is adjacent to the USDA Forest Service/North Carolina State University Southeastern Tree Research and Education Site (SETRES). Soils at the site are excessively-drained sandy loams (>90% sand), with a total water holding capacity of 12–14 cm in a 2 m soil profile. The site receives annual precipitation of approximately 120 cm with temperatures that average 26°/9 °C in the summer and winter, respectively. Between November 1993 and January 1994, seedlings of five open-pollinated loblolly pine families from the North and South Carolina Atlantic Coastal Plain (ACP) and five drought-hardy Texas families (TX) were field planted on the site, which had previously been occupied by an existing 10-year-old loblolly pine stand that was removed prior to planting. The site is currently surrounded on three sides by an 18-year-old loblolly pine stand and on the fourth side by a 40–60-year-old longleaf pine (*Pinus palustris*) stand, suggesting the presence of diverse sources of local ECM inocula. A total of 120 soil cores were collected from the site between 13 October and 22 November 2002 to a depth of 20 cm with a metal coring device measuring 15 cm in diameter (3.5 dm^3). Soil cores were collected from both fertilized and non-fertilized family plots. Each soil core was sieved at the site to separate pine roots and organic material from soil using a 0.5-cm mesh screen. Root, rhizosphere soil and organic material retained by the sieve constituted a sample, which was subsequently bagged and stored at 4 °C until processed in the laboratory.

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