DNA Sequence Identification of Tree Root Tips and Their Mutualist Fungi Using Landscape-Harvested Tissues

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Abstract

Plant-fungal mycorrhizal symbioses play an important role in the health of forest trees and the functioning of forest communities and ecosystems. Mycorrhizal fungi are often identified through DNA sequencing but different fungi can associate with different tree species so it can be important to know the tree species of the tree-fungal partnership as well. I refined a technique for the identification of tree root tips and their mycorrhizal fungal symbionts using PCR and DNA sequence analysis. Fungal ribosomal DNA and plant chloroplast DNA were amplified from extracts of single mycorrhizal root tips from a number of known tree species. Both members of each plant/fungal pair were identified using the BLAST program in the GenBank database. Also, different tree species were distinguishable by amplicon length on an agarose gel. The technique presented here is simple and reliable and has been used to identify plants and fungi at the genus or species level.

Introduction

Eastern hemlock (*Tsuga canadensis*) is an ecologically important conifer native to the forests of the Eastern United States. Late in the process of forest succession, this cool-climate species typically forms large, dense, persistent stands that are important because of their ability to retain rainwater, reduce evapotranspiration and nutrient loss, and

modulate ground humidity and temperature (Ellison et al. 2005). Eastern hemlock forests also shelter important birds and mammals (Tingley et al. 2002, Lishawa et al. 2007).

Mycorrhizal fungi are an important component in the health and survival of eastern hemlock, as they are for many forest trees (Amaranthus and Perry 1994). Mycorrhizal fungi form a symbiotic relationship with hemlock roots, providing nutrients such as nitrogen to the tree in exchange for sugar, which fungi cannot produce for themselves. The fungi also assist trees with water uptake (Rousseau et al. 1994), and protect roots from pathogenic fungi (Marx 1969). Different tree species associate with different suites of mycorrhizal fungal species and may overlap more or less in the species of mycorrhizal fungi with which they associate (Perry et al. 1990).

Severe hemlock population decline precipitated by attack from the hemlock woolly adelgid (*Adelges tsugae*) and climate warming combined with a predicted northward invasion of oak-hickory forests (Iverson and Prasad 2002) may mean that future hemlocks will be somewhat isolated and associated with a mycorrhizal community different from that found in climax hemlock stands. My broader study examines the effect of isolation on eastern hemlock trees within predominantly oak forests. Since some mycorrhizal fungal species are host-specific and the mycorrhizal community can have an effect the health of a tree, for instance by altering the nitrogen level in leaf tissue (Baxter and Dighton 2001), the isolation of hemlock trees in predominantly oak-hickory forests may have important implications for their health and survival.

I am examining differences in the ectomycorrhizal fungal symbionts found on eastern hemlock roots along a continuum from relatively dense groves of hemlocks to relatively isolated hemlock individuals within a predominantly oak forest. Mycorrhizal

fungal species are often determined by selecting root tips from soil cores and examining them under a microscope for fungal morphotypes (Allen 1991). Although this can lead to an accurate identification of the fungal species and is still used for preliminary screening of samples, it can be unreliable for some hard to identify fungi as well as in instances where more than one fungus has infected a root. Identification of mycorrhizal fungi through DNA sequencing is now widespread and relatively easy (Horton and Bruns 2001). Ribosomal DNA is used for PCR amplification and sequencing because it is composed of highly conserved regions (the 18S, 5.8S and 28S subunits) ideal for universal primer annealing, and two heterogeneous spacer regions (ITS 1 and ITS 2) that can be utilized to distinguish species (White et al. 1990; figure 1). Primers have been designed that anneal well only to fungal ribosomal DNA; this is important when working with root tips, since inadvertent amplification of plant rDNA may make fungal sequencing difficult (Gardes and Bruns 1993).

When different tree species (e.g., hemlock and oak) are growing in close proximity as in my study, uncertainty about the identity of a mycorrhizal root tip necessitates using DNA sequencing for identification. A common way to identify plants, which is generally reliable at the genus level, utilizes sequencing of the intergenic spacers and introns of plant chloroplast DNA that code for transfer RNA (Taberlet et al. 1991). Taberlet et al. (2007) have designed a primer pair that amplifies the small (10-140 bp) variable P6 loop of the plant chloroplast trnL (UAA) intron. The primer sites are highly conserved among angiosperms and gymnosperms, and since the region amplified is short, the primers work well with degraded DNA. Both of these features make the primers ideal

for my study; first, oak and hemlock belong to these two major groups, and second, root tips may have died by the time samples are taken.

The specific aim of the present study was to develop a reliable molecular technique for identifying the fungal and plant symbionts of single root tips, using primers for fungal ribosomal DNA and plant chloroplast DNA. This was achieved using single root tips of known, potted specimens representing the various tree species found in my outdoor study plots. I also wanted to differentiate between hemlock DNA and DNA from representatives of neighboring trees by amplicon length on an agarose gel. This would reduce the time and cost associated with sequencing both the fungal and tree DNA from each root tip.

Materials and Methods

Tissue samples Root samples of seven potted tree seedlings with known identity (*Tsuga canadensis* (Eastern hemlock); *Quercus velutina* (black oak); *Quercus rubra* (red oak); *Quercus alba* (white oak); *Quercus prinus* (chestnut oak); *Betula lenta* (black birch); and *Fagus grandifolia* (American beech) were collected and washed under tap water to remove all visible soil. Roots were transferred to Petri dishes and examined with a stereomicroscope. Individual root tips, some of which showed fungal infection, were excised from the root mass, dried in sterilized microcentrifuge tubes within a desiccation chamber, and frozen at -20° C for later use. Root tip masses were between approximately 0.25 and 1 mg.

DNA Extraction DNA from the root tips was extracted with a DNeasy® Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocols. Samples were homogenized in the lysis buffer with a plastic micropestle in 1.5 ml plastic centrifuge

tubes. Extracts were eluted from the spin columns with 50 ul nuclease free water and dilutions of the extracts (5x) were analyzed on a photospectrometer for DNA concentration.

PCR Two separate PCR reactions were run for each root tip extract, one with the fungal ribosomal DNA primers and the other with the plant chloroplast DNA primers. The forward (ITS 1F; fungal specific) and reverse (ITS 4) primers for amplification of the variable length and sequence ITS A and ITS B regions of ribosomal DNA were designed by Gardes and Bruns (1993) and White et al. (1990) respectively. The forward (g) and reverse (h) primers for amplification of plant chloroplast DNA were developed by Taberlet et al. (2008). This primer pair amplifies the variable length and sequence P6 loop of the chloroplast *trn*L (UAA) intron. Sequences and primer locations on their target DNA are shown in figure 1. Each 20 ul PCR reaction was performed with 10.0 ul of Go Taq Green Master Mix (Promega Corporation, Madison, WI), 8 pmol of each of the forward and reverse primers, 3 ng DNA, and a variable amount of nuclease-free water (depending on DNA concentration in the extract) to complete the volume. The DNA component in the negative controls was replaced with nuclease-free water. Reactions were performed in the thermocycler as follows: an initial denaturation for 5 min at 94°C was followed by 50 cycles of: 30 s at 94° C for denaturation, 30 s at 55° C for annealing and 30 s at 72° C for elongation. A final elongation step of 7 min was performed and reactions were held at 4° C until use, or storage at -20° C. Amplified DNA was run on 2% agarose gels containing ethidium bromide, and visualized with UV light.

PCR Product Purification and Sequencing PCR product was purified with the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA). Multiple bands from gel-

fractionated PCR product were individually excised and purified with the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA). Purified DNA was eluted from spin columns with 30 ul nuclease free water. DNA concentration was determined at 5x dilution with a spectrophotometer and product was prepared for sequencing by adding 80 ng of DNA, 10 pmol of the appropriate forward or reverse primer and nuclease free water to a final volume of 12 ul per reaction tube. Sequencing was performed by GENEWIZ, Inc. (South Plainfield, NJ). Returned sequences were compared with known sequences in the NCBI database using the BLAST program.

Results

A preliminary test of the plant chloroplast DNA primers on eastern hemlock, red oak and chestnut oak root tips revealed successful amplification of the plant DNA. Furthermore, the hemlock bands are seen to have migrated farther on the gel than either of the oak bands (figure 2). The seven known tree species were successfully amplified (figure 3, *left*) and accurately identified to at least the genus level using the BLAST program on the NCBI database. *Trn*L sequences containing the region amplified by the g and h primers were available in GenBank for eastern hemlock, red oak, black birch and American beech, which I identified to the species level with a 100% sequence match. White oak, black oak and chestnut oak did not have a *trn*L sequence in GenBank, but I was still able to match their sequences with other listed oak species with 97-100% accuracy (figure 4, *top*). The same root tip extracts were successfully amplified with the ribosomal DNA primers to reveal the fungal associates of each root tip (figure 3, *right*). Fungal species were identified with a with a 93-100% accuracy (figure 4, *bottom*).

Gels of the chloroplast PCR product revealed amplicons of differing length, in order from shortest to longest: eastern hemlock; the oaks; black birch; and American beech (figure 3, *left*). Alignments of the g and h primers with the returned GenBank sequences revealed that eastern hemlock has an amplicon length of 84 base pairs; white oak and chestnut oak have an amplicon length of 93 base pairs; red oak and black oak have an amplicon length of 94 base pairs, black birch has an amplicon length of 100 base pairs and American beech has an amplicon length of 104 base pairs.

Discussion

Uncertainty about the species identity of tree roots associated with mycorrhizal fungal symbionts from mixed forests necessitates using DNA sequencing to identify both the fungal and plant partners of any particular association. I successfully amplified and sequenced both plant and fungal DNA from root tips of known origin with unknown fungal associates. For the purposes of my larger study examining the mycorrhizal community associated with eastern hemlock trees in a mixed forest, I can use this technique not only to identify mycorrhizal fungi, but also to be sure that the root tips I examine belong to my target species, eastern hemlock.

The fungal identification results show that ribosomal DNA amplification and sequencing are acceptable for my study. However, for sequencing multiple fungi associated with a single root tip, gels must be run long enough to adequately separate bands, so that DNA from adjacent bands is not mixed during band excision from the gel. This may have happened, for instance in fungal lane 2 (figure 3, *right*), where clearly distinct bands have both been identified as *Hebeloma*.

A salutary result revealed by sequence analysis is that of all the species in my study plots whose roots may be found in a collected soil core, eastern hemlock has the shortest g-h primer amplicon length. Furthermore, its DNA can be distinguished from the other tree species by its lowest position on a 2% gel. This method can be made more reliable by adjusting gel conditions to accentuate differences in migration distance between hemlock DNA and DNA from the other tree species in my plots. This way, a simple check of chloroplast DNA PCR products on a gel could save considerable time and expense over individually sequencing the samples.

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Citations

Allen, M. 1991. *The Ecology of Mycorrhizae*. Cambridge University Press, Cambridge, UK.

Amaranthus M and Perry, D. 1994. The functioning of ectomycorrhizal fungi in the field: linkages in space and time. *Plant and Soil* 159:133-140.

Baxter J and Dighton, J. 2001. Ectomycorrhizal Diversity Alters Growth and Nutrient Acquisition of Grey Birch (Betula populifolia) Seedlings in Host--Symbiont Culture. *New Phytologist* 152(1):139-149.

Ellison A, Bank M, Clinton B. et al. 2005. Loss of foundation species: consequences for the structure and dynamics of forested ecosystems. *Fronteirs in Ecology and the* Environment 3(9):479-486.

Gardes M and Bruns, T. 1993. ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2:113-118.

Horton T and Bruns, T. 2001. The molecular revolution in ectomycorrhizal ecology: peeking into the black box. *Molecular Ecology* 10:1855-1871.

Iverson L and Prasad, A. 2002. Potential redistribution of tree species habitat under five climate change scenarios in the eastern US. *Forest Ecology and Management* 155:205-222.

Lishawa S, Bergdahl D and Costa, S. 2007. Winter conditions in eastern hemlock and mixed-hardwood deer wintering areas of Vermont. *Canadian Journal of Forest Research* 37(3):697-703.

Marx, D. 1969. The influence of ectotrophic ectomycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to pathogenic fungi and soil bacteria. *Phytopathology* 59:153-163.

Perry D, Borchers J, Borchers S and Amaranthus, M. 1990. Species migrations and ecosystem stability during climate change: the belowground connection. *Conservation Biology* 4:266-274.

Rousseau J, Sylvia D and Fox, A. 1994. Contribution of ectomycorrhiza to the potential nutrient-absorbing surface of pine. *New Phytologist* 128:639-644.

Taberlet P, Coissac E, Pompanon F, Gielly L, Miquel C, Valentini A, Vermat T, Corthier G, Brochmann C and Willerslev, E. 2007. Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. *Nucleic Acids Research* 35:e14 1-8.

Taberlet P, Gielly L, Pautou G and Bouvet, J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17:1105-1109.

Tingley M, Orwig D, Field R and Motzkin, G. 2002. Avian response to removal of a forest dominant: consequences of hemlock woolly adelgid infestations. *Journal of Biogeography* 29:1505–1516.

White T, Bruns T, Lee S and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a Guide to Methods and Applications* (eds Innis MA, Gelfand DH, Sninsky JJ, White TJ), pp. 315.322. Academic Press, London, UK.

Figures

Name	5'- 3' Primer Sequence	Primer Annealing Site	Region Amplified	Source	Comments
ITS 1-F	CTTGGTCATTTAGAGGAAGTAA	18S subunit of rRNA, fungi	ITS region of rDNA; 600-900 bp	Gardes and Bruns 1993	Fungal specific; will amplify some plant DNA.
ITS 4	TCCTCCGCTTATTGATATGC	28S subunit of rRNA	ITS region of rDNA; 600-900 bp	White et al. 1990	Universal primer.
g	GGGCAATCCTGAGCCAA	chloroplast DNA: trnL intron	P6 loop of trnL intron; 10-143 bp	Taberlet et al. 2007	Both primer sites highly conserved
h	CCATTGAGTCTCTGCACCTATC	chloroplast DNA: trnL intron	P6 loop of trnL intron; 10-143 bp	Taberlet et al. 2007	among angiosperms and gymnosperms.

a

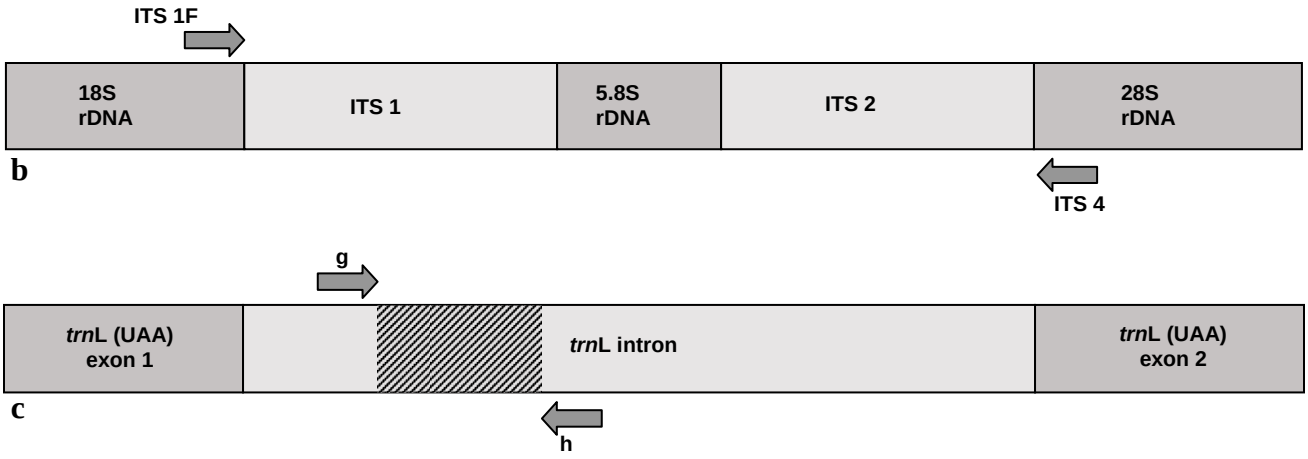


Figure 1. **a**) Primers used in this study. **b**) Position of the primers ITS 1F and ITS 4 on the ribosomal DNA. **c**) Position of the primers *g* and *h* on the intron of the chloroplast *trn*L (UAA) gene. The variable P6 loop amplified with primers g and h is indicated by hatching.

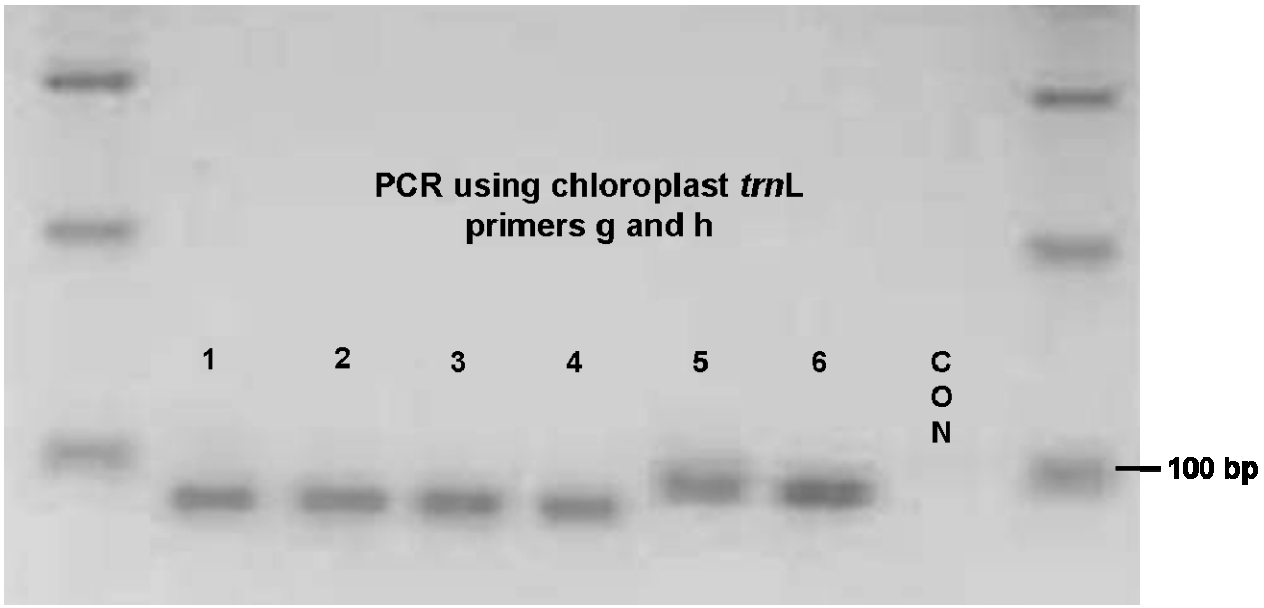
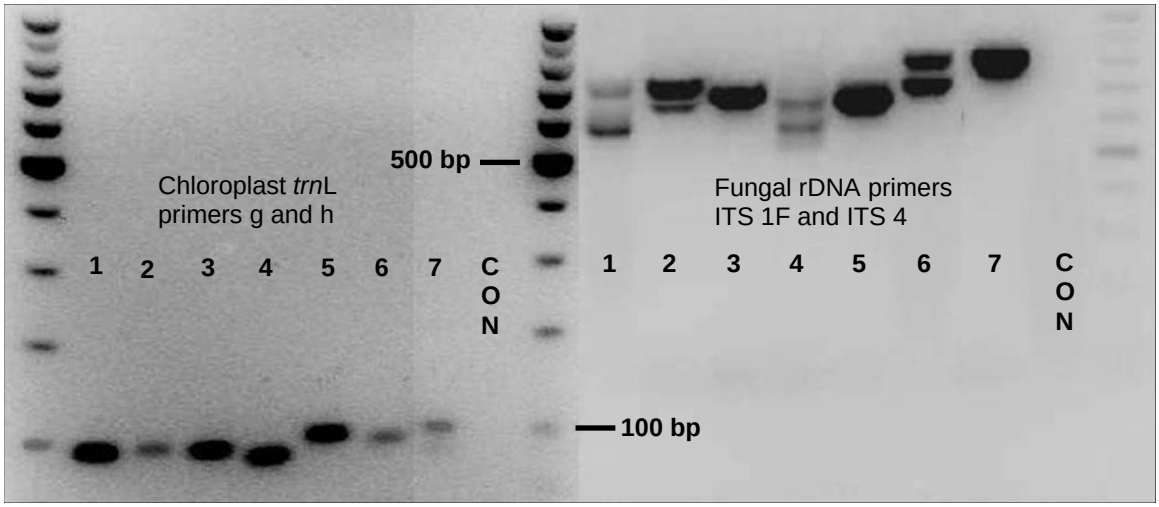


Figure 2. Agarose gel (2%) of the cDNA PCR product of four different hemlock root tips, a red oak and a black oak root tip. Note that the hemlock bands have migrated noticeably further than the oak bands.



Plant Component of Root Tip

1 *Quercus velutina* (black oak; 94 bp)

2 *Quercus rubra* (red oak; 94 bp)

3 *Quercus alba* (white oak; 93 bp)
4 *Tsuga canadensis* (e. hemlock; 84 bp)

5 *Betula lenta* (Black birch; 104 bp)
6 *Quercus prinus* (chestnut oak; 93 bp)

7 *Fagus grandifolia* (Am. beech; 100 bp)

Fungal Component of Root Tip

1 *Pleurotus ostreatus*
Cortinarius sp.
2 *Hebeloma sp.*
Hebeloma sp.
3 *Thelephora terrestris*
4 *Frankia sp.*
Cortinarius sp.
5 *Peziza sp.*
6 *Strobilurus trullisatus*
Laccaria sp.
7 *Strobilurus trullisatus*

Figure 3. *Left*: Gel of PCR products from root tips of seven tree species using chloroplast primers g and h. Amplicon length is in parentheses, after the common name. *Right*: Gel of PCR products from the same root tips, using rDNA primers ITS 1F and ITS 4. Multiple bands for a single lane are listed in the same order as they appear on the gel. Note that upper band 4 (*Frankia*) is a nitrogen fixing bacterium.

Known Tree Species	GenBank Tree Match	GenBank #	Accur.
Quercus velutina *	*Quercus sp.*	FJ480448.1	100%
Quercus rubra	*Quercus rubra*	X75707.1	100%
Quercus alba *	*Quercus pubescens*	DQ423789.1	100%
Tsuga canadensis	*Tsuga canadensis*	EF395421.1	100%
Betula lenta	*Betula lenta*	FJ012057.1	100%
Quercus prinus *	*Quercus mongolica*	AB107713.1	97%
Fagus grandifolia	*Fagus grandifolia*	AB066497.1	100%

Known Tree Species	GenBank Fungal Match	GenBank #	Accur.
Quercus velutina	*Pleurotus ostreatus*	EU520193.1	100%
	Cortinarius sp.	FJ553462.1	97%
Quercus rubra	*Hebeloma sp.*	EF564170.1	95%
Quercus alba	*Thelephora terrestris*	EU427330.1	99%
Tsuga canadensis	*Frankia sp.*	CP000820.1	100%
	Heliotales sp.	FJ197203	93%
Betula lenta	*Pezizales*	DQ469743.1	97%
Quercus prinus	*Strobilurus trullisatus*	DQ097370.1	98%
	Laccaria sp.	AJ534899.1	98%
Fagus grandifolia	*Strobilurus trullisatus*	DQ097370.1	99%

Figure 4. *Top*: List of seven known potted tree species and their matches in GenBank, based on PCR amplification and analysis. **Quercus velutina*, *Quercus alba* and *Quercus prinus* are not listed in Genbank, so the closest species match is given. *Bottom*: List of the same tree species (same root tips) with their identified fungal associates.