Genetic Diversity of *Pinus* species in New York: a baseline study for fungal endophytes assemblage analysis

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Abstract

Understanding how fungal endophyte communities differ in abundance, diversity, taxonomic composition, and host affinity over the geographic ranges of their hosts is key to understanding the ecology and evolutionary context of endophyte–pine associations. This study was undertaken to investigate the samples being characterize to make certain that any observed differences along Urban-Rural gradient were due to locality and not due to miss identification of pine species. The choloplast intergenic trnH-psbA spacer gene was used to characterize the pine samples. Results show that the white pine tree specimens located on the Rose Hill campus of Fordham University and Central Park are *Pinus strobus* and *Pinus wallichiana* respectively, even though both appeared to be morphologically white pine (*Pinus strobus*). Sequence analysis performed on samples which morphologically identified as red pines on the Rose Hill campus of Fordham University and in Central park were identified to be *Pinus nigra* (black pine). Our finding emphasis the importance of the genetic identification of samples utilized in any studies across an Urban-Rural gradient.

Key words: White pine, red pine, endophyte, DNA barcoding, intergenic spacer

Introduction

The study of endophytes has become great deal of interest among researchers in the recent years. Endophytes are microorganisms inhabiting plant organs that at some time in their

life, can colonize internal plant tissues without causing apparent harm to the host (Petrini, 1991). They produce a wide range of compounds useful for plants for their growth, protection to adverse environmental conditions, and herbivory. Endophytes also benefit host plants by preventing pathogenic organisms from colonizing them. Extensive colonization of the plant tissue by endophytes creates a "barrier effect", where the local endophytes outcompete and prevent pathogenic organisms from taking hold. Endophytes may also produce chemicals which inhibit the growth of competitors, including pathogenic organisms (Taghavi S *et al*., 2009). The endophytic population varies among plants, species, climatic conditions and host locality (Chareprasert S *et al.*, 2006). Resolving the importance of locality and host identity is important for understanding fundamental aspects of endophyte population.

Pines are conifer trees in the genus *Pinus*, in the family *Pinaceae*. Pines are gymnosperms. The genus is divided into three subgenera, based on cone, seed and leaf characters: *Pinus* subg. *Pinus*, the yellow, or hard pine group, generally with harder wood and two or three needles per fascicle. *Pinus* subg. *Ducampopinus*, the foxtail or pinyon group *Pinus* subg. *Strobus*, the white, or soft pine group, generally with softer wood and five needles per fascicle (Burton V. B, 2004). Their distribution is broad and continuous in the Northern hemisphere, where they form extensive forests, and rather scattered south of the equator. The pines are one of the world's most important resources of timber. Their wood is considered of high value for lumber, furniture, pulp for paper and special uses, with several well-known species as prime producers, including *Pinus strobus* and *Pinus resinosa*. In addition, pine helps in sustaining global biodiversity, and many species are considered excellent for afforestation and as ornamentals (Farjon A. 1984).

DNA barcoding (Hebert *et al.* 2003) is an increasingly attractive tool for species identification in terms of accuracy, speed, cost and functionality. It is currently receiving much attention as a complementary tool to morphology and biogeography for obtaining higher rates of species identification (Von Cra¨utlein *et al.,* 2011). Chloroplast intergenic trnH-psbA spacer has recently become a popular tool in plant molecular phylogenetic studies at low taxonomic level and is the region suitable for DNA barcoding studies (Armenise L, 2012).

Our long term goal is to investigate the distribution of endophytes in pine species at different environment gradient ie., urban-rural gradient in New York. But the morphological and molecular data indicate that pines have a complex evolutionary history and does possess genetic differentiation at the regional, species and family levels. So our immediate objective is to make sure that we are going to study endophytes on the same species of pine throughout urban-rural gradient. So the present study was undertaken to verify the genetic identity of pine species at different location in New York before beginning analysis of their resident endophytes.

Materials and Methods

Plant material, DNA isolation and molecular analyses:

Needles from one red pine and white pine were obtained from Fordham University's Rose Hill campus in Bronx, NY and Central Park in Manhattan. Morphologically white pine has blueish green, flexible needles that are in bundles of 3 to 5. The cones are long and thin. The trees grow up to 80 feet tall. Whereas the red pine has dark green, brittle needles that are in bundles of 2. The cones are short and broad. the tree grows up to 90 feet tall.

DNA extractions were performed with the DNeasy Plant Minikit (QIAGEN), following the manufacturer's instructions. Uniform PCR procedures were performed for all samples and

barcoding loci. Thermocycling conditions were as follows: 94 C for 3 min, followed by 50 cycles of 94 C for 40 s, 58 C for 30 s and 72 C for 40 s, with a final extension step of 5 min at 72 C. Primer pair for the investigated barcoding region are shown in Table 1 . PCR products were cleaned QIAquick PCR Purification kit and eluted in 30 µl of elution buffer. Standardized aliquots were then submitted to Genewiz (http://www.genewiz.com) for sequencing.

Sequence editing, alignment and assembly:

Sequences were aligned with ApE (http://biologylabs.utah.edu/jorgensen/wayned/ape/) under default parameters; alignments were visualized and checked with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). And a BLAST search was performed to identify the trees.

Table 1. Primer pair used to differentiate between pine species

Name	5' to 3' sequence	Exp Size (bp)	Source
trnH-psbA F inter	GCATGGTGGATTCACAATCC	~600	Primer to differentiate between conifer species (Armenise et
trnH-psbA R inter	GTTATGCATGAACGTAATGCTC		

Results

Each of the four samples barcoding loci was successfully amplified using standard primer pair and PCR protocol (Fig. 1). We obtained trnH-psbA DNA sequences from every single individual analyzed. BLAST searches performed on GenBank displayed multiple congeneric hits. Highest hits (100% identity) of the trnH-psbA sequence of white pine (Rose Hill campus) was with *Pinus strobus*, a eastern white pine belong to subgenus *Strobus* and native species of eastern North

America. Whereas the sequence of white pine (Central Park) displayed 100% identity with a Himalayan white pine (*Pinus wallichiana*), a native species of Himalayan region. On the other hand, the trnH-psbA sequences of red pine (Rose Hill and Central Park) displayed 100% identity with a black pine (*Pinus nigra*) sequence, a native species of southern and eastern Mediterranean Europe, but not with *Pinus resinosa*.

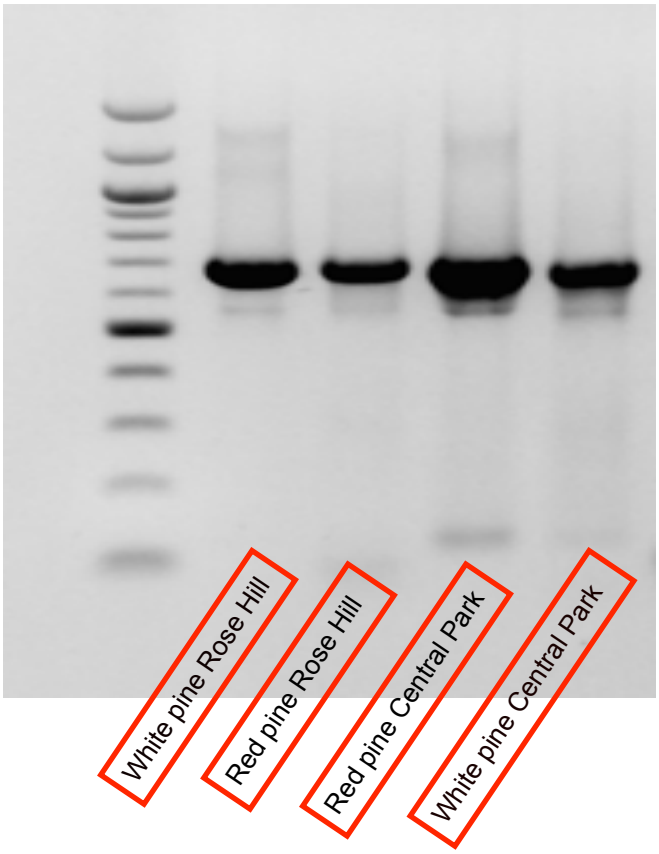

Figure 1. PCR results from amplification using primers for regions of the gene trnH-psbA

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Strobus          GCTGTTGAATCTATTTCAATAGGCGGATAATACTCTGATGGATTGTTATCGTGTATTGCT 60
Sample-1         GCTGTTGAATCTATTTCAATAGGCGGATAATACTCTGATGGATTGTTATCGTGTATTGCT 60
                 ************************************************************

Strobus          TAATTGAAGCATACCAAGCCTTTCAATAAAATGAAAGGCTTGGTATGCTTCAATTGTTTG 120
Sample-1         TAATTGAAGCATACCAAGCCTTTCAATAAAATGAAAGGCTTGGTATGCTTCAATTGTTTG 120
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Strobus          TTGTTATTCTTCATACTTCTTCCCCATTCCATCAATAATGGATATGTGCAGTTCCCCTGC 180
Sample-1         TTGTTATTCTTCATACTTCTTCCCCATTCCATCAATAATGGATATGTGCAGTTCCCCTGC 180
                 ************************************************************

Strobus          ATCCAGCAGGAATTGAACCCGCGAGTTCGCCAATTATGAGTTGGGCGCTTTAACCATTCA 240
Sample-1         ATCCAGCAGGAATTGAACCCGCGAGTTCGCCAATTATGAGTTGGGCGCTTTAACCATTCA 240
                 ************************************************************

Strobus          GCCATGGATGCTGGATAAAGATCATCAACATACTCATTCTATAATATGAGTATAGACTCA 300
Sample-1         GCCATGGATGCTGGATAAAGATCATCAACATACTCATTCTATAATATGAGTATAGACTCA 300
                 ************************************************************

Strobus          GATCTAAAATGGGTTAGTTCGGGATCGGGACCCATTTACATTCTTTCTCTCATAATTGAT 360
Sample-1         GATCTAAAATGGGTTAGTTCGGGATCGGGACCCATTTACATTCTTTCTCTCATAATTGAT 360
                 ************************************************************

Strobus          TCATGTCAAATATTATCAATAGACATTCAAATAACATTTCATTTTTATAATAAGCCGAAC 420
Sample-1         TCATGTCAAATATTATCAATAGACATTCAAATAACATTTCATTTTTATAATAAGCCGAAC 420
                 ************************************************************

Strobus          AACTTGTTCGAGAGTTGGGAGTTAGTCATCGATCTTGCTTTGATCCCCTATCAGAGTCAC 480
Sample-1         AACTTGTTCGAGAGTTGGGAGTTAGTCATCGATCTTGCTTTGATCCCCTATCAGAGTCAC 480
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Strobus          CAACCCACATACGAACAAACGTTAGCTCGTTTTTTCTTCT 520
Sample-1         CAACCCACATACGAACAAACGTTAGCTCGTTTTTTCTTCT 520
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Fig 2. Aligned sequences of Rose Hill white pine and *Pinus strobus* in Clustal Omega

The alignment for white pine of Rose Hill revealed similarity with the eastern white pine, *Pinus strobus* which is native to eastern North America (Fig 2).

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Wallichiana     TACGTCCGCCCCGGAAAAACAAGCCAATTTATCTATCTACAGTCATTTCTCCCAAGAAGA 60
Sample_4        TACGTCCGCCCCGG-AAAACAAGCCAATTTATCTATCTACAGTCATTTCTCCCAAGAAGA 59
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Wallichiana     AAAAACGAGCTAACGTTTGTTCGTATGTGGGTCGGTGACTCTGATAGGGGATCAAAGCAA 120
Sample_4        AAAAACGAGCTAACGTTTGTTCGTATGTGGGTCGGTGACTCTGATAGGGGATCAAAGCAA 119
                ************************************************************

Wallichiana     GATCGATGACTAACTCCCAACTCTCGAACAAGTTGTTCGGCTTATTATCAAAATGAAATG 180
Sample_4        GATCGATGACTAACTCCCAACTCTCGAACAAGTTGTTCGGCTTATTATCAAAATGAAATG 179
                ************************************************************

Wallichiana     TTTTTTGAATGTCTATTGATAATATTTGACATGAATCAAGTATGAGAGAAAGAATGTAAA 240
Sample_4        TTTTTTGAATGTCTATTGATAATATTTGACATGAATCAAGTATGAGAGAAAGAATGTAAA 239
                ************************************************************

Wallichiana     TGGGTCCCGATCCCGAACTAACCCATTTTAGATCTGAGTCTATACTCATATTATAGAATG 300
Sample_4        TGGGTCCCGATCCCGAACTAACCCATTTTAGATCTGAGTCTATACTCATATTATAGAATG 299
                ************************************************************

Wallichiana     AGTATGTTGATGATCTTTATCCAGCATCCATGGCTGAATGGTTAAAGCGCCCAACTCATA 360
Sample_4        AGTATGTTGATGATCTTTATCCAGCATCCATGGCTGAATGGTTAAAGCGCCCAACTCATA 359
                ************************************************************

Wallichiana     ATTGGCGAACTCGCGGGTTCAATTCCTGCTGGATGCAGGGGAACTGCACATATCCATTCC 420
Sample_4        ATTGGCGAACTCGCGGGTTCAATTCCTGCTGGATGCAGGGGAACTGCACATATCCATTCC 419
                ************************************************************

Wallichiana     ATTATTGATGGAATGGGGGAAGAAGTATGAAGAATAACAACAAACAATTGAAGCATACCA 480
Sample_4        ATTATTGATGGAATGGGGGAAGAAGTATGAAGAATAACAACAAACAATTGAAGCATACCA 479
                ************************************************************

Wallichiana     AGCCTTTCATTTTATTGAAAGGCTTGGTATGCTTCAATTAAGCAATACACGATAACAATC 540
Sample_4        AGCCTTTCATTTTATTGAAAGGCTTGGTATGCTTCAATTAAGCAATACACGATAACAATC 539
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Fig 3. Aligned sequences of Central Park white pine and *Pinus wallichiana* in Clustal Omega

Alignment of white pine of Central Park did not match eastern white pine (*Pinus strobus*), but the sequence did match *Pinus wallichiana* (Himalayan white pine), which is native to Himalayan region (Fig.3).

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nigra           AGGTGGATAATACTCTGATGGATTGTTATCGTGTATTGCTTAATTGAAGCATACCAAGCC 60
Sample_2        AGGTGGATAATACTCTGATGGATTGTTATCGTGTATTGCTTAATTGAAGCATACCAAGCC 60
                ************************************************************

nigra           TTTCATTCATTTTATTGAAAGGCTTGGTATGCTTCAATTGTATTGTTTGGTGTTATTCTT 120
Sample_2        TTTCATTCATTTTATTGAAAGGCTTGGTATGCTTCAATTGTATTGTTTGGTGTTATTCTT 120
                ************************************************************

nigra           CATACTTCCTTCTTCCCATTCCATCAATAATGGATATGTGCAGTTCCCCTGCATCCAGCA 180
Sample_2        CATACTTCCTTCTTCCCATTCCATCAATAATGGATATGTGCAGTTCCCCTGCATCCAGCA 180
                ************************************************************

nigra           GGAATTGAACCCGCGAGTTCGCCAATTATGAGTTGGGCGCTTTAACCATTCAGCCATGGA 240
Sample_2        GGAATTGAACCCGCGAGTTCGCCAATTATGAGTTGGGCGCTTTAACCATTCAGCCATGGA 240
                ************************************************************

nigra           TGCTGGATAAAGATCATCAACATATTCATTCTATAATATGAGTATAGACTCAGATCTAAA 300
Sample_2        TGCTGGATAAAGATCATCAACATATTCATTCTATAATATGAGTATAGACTCAGATCTAAA 300
                ************************************************************

nigra           ATTGGGTAGTTCGGGATTGGGACCCATTTACATTCTTTCTCTCATACTTGATTCATGTCA 360
Sample_2        ATTGGGTAGTTCGGGATTGGGACCCATTTACATTCTTTCTCTCATACTTGATTCATGTCA 360
                ************************************************************

nigra           AATATTCTCAATAGACATTCAAATAACATTTCATTTCATTGAATTAATTTGATAATAAGC 420
Sample_2        AATATTCTCAATAGACATTCAAATAACATTTCATTTCATTGAATTAATTTGATAATAAGC 420
                ************************************************************

nigra           CATACAACTTGTTCGAGAGTTGAGAGTTAGTCATCAATCTTGCTTTGATCCCCTATCAGA 480
Sample_2        CATACAACTTGTTCGAGAGTTGAGAGTTAGTCATCAATCTTGCTTTGATCCCCTATCAGA 480
                ************************************************************

nigra           GGTCACCGACGACCCACATAAGAACAAACGTTAGCTCATTTTTTATTCTTGGAAGAAATG 540
Sample_2        GGTCACCGACGACCCACATAAGAACAAACGTTAGCTCATTTTTTATTCTTGGAAGAAATG 540
                ************************************************************

nigra           ACTGTAGATAGATAAATTGGTT 562
Sample_2        ACTGTAGATAGATAAATTGGTT 562
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Fig 4. Aligned sequences of Rose Hill and Central Park red pine and *Pinus nigra* in Clustal Omega

The red pines of Rose Hill and Central Park did not match with *Pinus resinosa* a native species, as anticipated. But the sequences did match to *Pinus nigra* (black pine), which is native to southern and eastern Mediterranean Europe (Fig. 4).

Discussion

The present study detected considerably genetic differentiation between samples located in the Rose Hill and Central Park. The present barcoding results demonstrate that pine samples from Rose Hill campus of Fordham University and Central Park, Manhattan are different species, even though they look morphologically similar to white and red pines. The study of genetic diversity will helps in discrimination and identification of species which are morphologically similar but genetically different (cryptic species). Ideally, future study would include pine species identification from a geographic region along with endophyte population analysis in urban-rural gradient.

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