Analyses of ribosomal DNA internal transcribed spacer sequences from Juglans nigra and leaf-associated fungi in Zoar Valley, NY

by

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Submitted in Partial Fulfillment of the Requirements

for the Degree of

Masters of Science

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Abstract: A genetic analysis of samples of *Juglans nigra* (Black Walnut) from three different locations in Zoar Valley, New York, was conducted. Nuclear ribosomal DNA (nrDNA) intragenic spacer regions (ITS1, ITS2) were PCR amplified along with the 5.8S ribosomal RNA (rRNA) gene. Consensus sequence alignment of *J. nigra* DNA samples from Zoar Valley showed sequence variation (both base additions and substitutions) between samples. It is likely that at least some of the base substitutions in the consensus sequence are not an artifact of the method, and are different from the published sequence for *J. nigra*. This indicates the method has potential for examining within species variation for different populations of *J. nigra*.

A survey of fungi associated with the phyllosphere of two elm species native to Zoar Valley, NY, *Ulmus americana* and *Ulmus rubra*, was conducted on samples recovered from Zoar Valley, NY. Fungi were identified by sequencing cloned DNA of PCR amplified ribosomal DNA (rDNA) extracted from leaf tissue. Probable endophytes were identified (*Phoma, Coprinellus*), but the majority of fungi detected (*Cryptococcus, Ampelomyces, Colletotrichum*) were most likely parasites. Multiple genera of fungi were detected in single leaf tissue samples.

Introduction: DNA was isolated and sequenced from leaf samples collected in old growth forest in Zoar Valley, in hopes of forming a preliminary colonial history of *Juglans nigra* in Zoar Valley, and detection of specific hereditary variations in sequence within populations. This combined thesis was a byproduct of the universal eukaryotic ribosomal DNA primers (ITS1 and ITS4) used in this study. The original intention was to isolate and PCR replicate plant DNA only for further downstream application. As research methods progressed to DNA sequencing, it became apparent after the completion of numerous sequencing reactions, that fungal DNA was being PCR amplified and cloned along with plant DNA. Fungi from either within the leaves or upon their surface was being DNA extracted along with the leaves themselves, and was present in the DNA extracts to be PCR amplified.

Interestingly, only fungal DNA was extracted and PCR amplified from the leaves of *Ulmus americana* (American elm) and *Ulmus rubra* (Slippery elm), while no plant sequences were obtained for the elms. Conversely, no fungal DNA was sequenced from the *Juglans nigra* (Black walnut) leaf samples. PCR reactions performed on elm-leaf DNA extracts favored amplification of fungal DNA, rather than elm DNA. It was decided to use the fungal sequences we obtained in a second project.

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Title: Identification and comparison of ribosomal DNA internal transcribed spacer sequences (rDNA ITS) isolated from *Juglans nigra* leaf tissue collected from Zoar Valley, NY

Background: The genomes of all living organisms contain certain genes responsible for fundamental biochemical functions. These genes can be sequenced, aligned, and analyzed to study phylogenetic relationships, even among morphologically indistinguishable but otherwise distinct species (Hillis and Moritz 1990). This level of genetic variability is central to population biology because the amount of variability directly influences the evolutionary potential for a species or populations (Shaal and Learn 1988).

Nuclear ribosomal DNA (nrDNA) is a strong tool because it is ubiquitous in all organisms. Also, it is in relatively high copy number to chromosomal DNA, making it more accessible. Ribosomal DNA has been used to systemically evaluate and construct genetic histories, known as phylogenies, through many taxonomically diverse groups of plants, fungi, and animals. The nrDNA can be viewed as units, the 18S, 5.8S, and 26S ribosomal genes, and the two internal transcribed spacers, ITS1 and ITS2, located between the 18S and 26S coding regions. The ribosomal genes have a much slower rate of sequence change than do the internal transcribed spacer regions (Suh et al. 1993).

Ribosomal DNA has been very useful in the study of plant evolutionary biology. The DNA conserved sequences that code for the 18S, 5.8S, and 26S ribosomal subunits have provided information on phylogenetic relationships among the species within a genus, and have also illuminated higher level relationships (Shaal and Learn 1988). The

intergenic spacer (ITS) regions of ribosomal DNA are highly variable, with variation occurring within populations and in individuals of the same population (Shaal and Learn 1988).

The internal transcribed spacers (ITS1 and ITS2) and 5.8S rRNA gene of nuclear ribosomal DNA were sequenced and analyzed to determine genetic heredity in the angiosperm species *Juglans nigra*. The ITS1-5.8S-ITS2 stretch of eukaryotic nrDNA (each <300bp) can be readily amplified by PCR and sequenced using universal primers (Baldwin et al 1995) (see Figure 1). The ITS region is known to undergo rapid concerted evolution (Linder et al. 2000). Differences in ITS sequence between species can mostly be attributed to point mutations acquired over evolutionary time (Baldwin et al 1995). The ITS sequences have proven to be useful determining taxonomic relationships among many species of angiosperms, and species can be readily distinguished through sequence variation (Jobes and Thien 1997, Baldwin et al 1995).

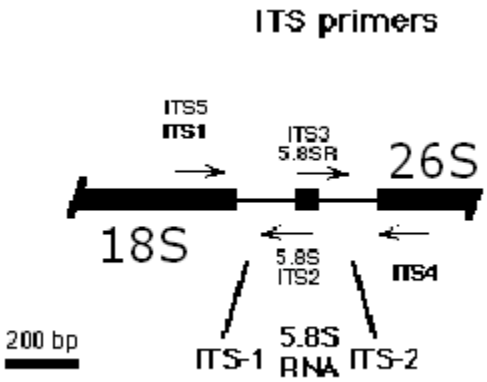


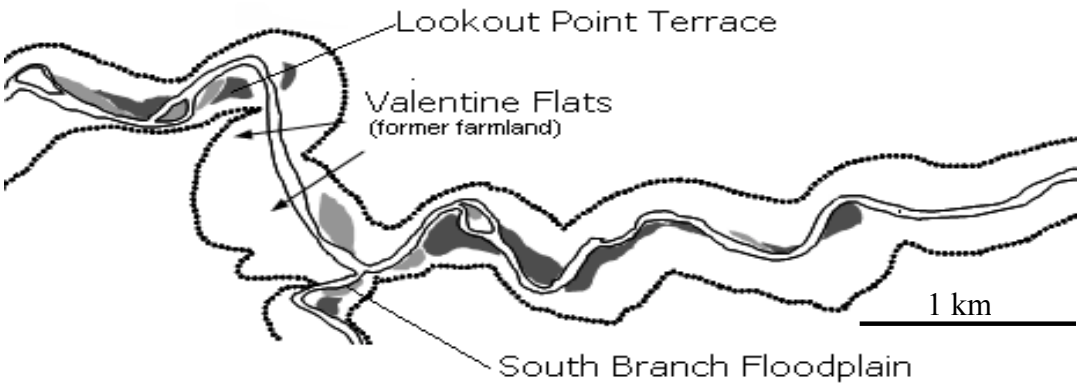
Figure 1. Ribosomal DNA amplified in this study with primer sites noted. Note ITS primer locations are found at the ends of each the highly conserved ribosomal genes

Previous studies (Gonzalez-Lamothe et al. 2002, Potter et al. 2001) have successfully utilized ribosomal DNA sequence, specifically the ITS1 and ITS2 spacer regions, to answer systematic questions and determine phylogenetic heredity. For example the 18S, 28S ribosomal genes, as well as the ITS1 – 5.8S- ITS2 region of the fungus *Spilocaea oleagina* (responsible for peacock leaf spot disease in olive trees) was sequenced to determine its identity. The phylogenetic classification of *S. oleagina* was previously undecided among mycologists. It was determined from nrDNA sequence data that *S. oleagina* belongs to the class Dothideomycetes, and is an anamorphic phase of a yet unidentified *Venturia* species (Gonzalez-Lamothe et al. 2002). In a plant example, ITS spacer sequences from the plant *Arabidopsis suecica* were PCR-amplified, cloned and sequenced, confirming that this allopolyploid species contains two distinct types of ITS sequences, one from *A. thaliana* and the other from *C. arenosa*, confirming they are the putative parents of *A. suecica* (O'Kane et al. 1996). In another landmark study, Paradox, a hybrid walnut cultivar [*J. regia* and *J. californica*] important to the California walnut industry, was analyzed for gene flow from other North American walnut species

[*J. major, J. hindsii* and *J nigra*]. ITS data showed that among various walnut industry sources of the Paradox strain, there was considerable genetic contribution from all North American species in at least some of these samples (Potter et al. 2001).

New Yok State's Zoar Valley was chosen for sampling due to its intact riparian ecosystem and healthy distribution of *J. nigra* (Black Walnut). The woodlands of Zoar Valley are highly varied and diverse, and collectively meet all objective criteria for eastern old growth forest (Diggins and Kershner 2005). Zoar Valley is the most intact forest-gorge landscape in the western New York region, having the largest area of virgin and secondary old growth forest, and contiguous climax forest. For these reasons and others, Zoar Valley has been studied scientifically and has become the subject of multiple conservation efforts.

A.



B.

Figure 2: Location of Zoar Valley in Western New York (A) and sample study areas within (B).

Juglans nigra [Black walnut, a dicotyledonous tree species of the family *Juglandaceae*] is an important tree economically, for both its edible nut and in its use in commercial wood production (Stanford et al. 2000). Black walnut was chosen as the focus of this study because it is well established inside Zoar Valley, both in old growth stands and in younger forest. Minimal *J. nigra* population structure exists outside Zoar Valley due to heavy logging (Diggins and Kershner 2005). This made *J. nigra* an ideal species to analyze for genetic variation within an isolated population since it is safe to assume minimal gene flow between *J. nigra* populations inside of Zoar Valley with populations outside.

Within Zoar Valley, three sites were strategically selected for sampling. The first two sites, South Branch Floodplain and Lookout Point Terrace, lie within the undisturbed forested valley, and are separated geographically by about 1 kilometer. The third site, Valentine's Flats Plantation, lies along the western rim of Zoar Valley within a kilometer of South Branch Floodplain and Lookout Point Terrace and consists of Black Walnut trees planted by the State of New York in the late 1960s.

Genetic analysis of *J. nigra* between Zoar Valley's old growth stands, developing forests, and Valentine Flats Plantation was performed using gene sequence alignment. Using ITS sequence analysis enabled the comparison of multi-aged, spatially distant tree stands throughout Zoar Valley. This knowledge was applied to determine a preliminary colonial history of the *J. nigra* population in Zoar Valley, expose genetic identity or variation inside of each distinct population, and link trees in young forest with their parent trees in old growth if possible. In all, some insight into the population dynamics of this species in Zoar Valley was provided, yielding a glimpse into the history, heredity, and future implications for these species.

Methods: Samples were obtained from Zoar Valley on October 4th, 2005. Samples were collected from three distinct areas of Zoar Valley, Lookout Point Terrace, South Branch Floodplain, and Valentine Flats Plantation. Samples of *J. nigra* leaf tissue were first identified morphologically, then collected and stored in deep freeze at -80 C° until DNA extraction was performed.

DNA extraction: Total DNA was extracted using a revised CTAB method of Doyle and Doyle (1987).

PCR: Extracted DNA was amplified with ITS specific primers. Forward primer used was **ITS1** [TCCGTAGGTGAACCTGCGG] and the reverse primer used was **ITS4** [TCCTCCGCTTATTGATATGC] of White et al. (1990). PCR performed on PTC-200 DNA engine.

Cloning: Purified PCR products were cloned using the TOPO TA Cloning Kit from Invitrogen (cat# K4530-20) or StrataClone PCR cloning kit from Stratagene (cat#240205)

Restriction Digest: A restriction digest using EcoR1 was used to screen clones before sequencing.

Figure 3: EcoR1 digest revealing restriction fragments of approximately 700bp, indicative of the PCR product generated using the universal primers ITS1 and ITS4 of White et al. (1990)

DNA Sequencing: Sequencing reactions were carried out on purified cloning extracts using Beckman Coulter Sequencing Kit, sequenced using primers M13 (-20, -47) Forward and M13 Reverse, with sites provided in the cloning vector.

Alignment: Sequences were aligned to determine identity or variation in ITS region of each species using computer programs. NCBI nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) was used to identify sequenced DNA and

ClustalW (http://www.align.genome.jp/sit-bin/clustalw) was used to construct a multiple alignment of the sequences.

Results and Discussion: Identity of the sequenced DNA was assessed by sequence comparison within NCBI Genbank.

Table 1. List of single run DNA sequences from trees morphologically identified as *J. nigra*

Sample # and primer set used (Forward or Reverse)	Collection Site within Zoar Valley	Closest match in NCBI Nucleotide Blast Search
1 (Forward)*	South Branch Floodplain	*Juglans nigra*
1 (Reverse)*	South Branch Floodplain	*Juglans nigra*
1 (Forward) (-47)**	South Branch Floodplain	*Juglans nigra*
3 (Forward)*	Lookout Point Terrace	*Juglans major*
3 (Reverse)*	Lookout Point Terrace	*Juglans nigra*
5 (Forward) (-47)**	Valentines Flat Plantation	*Juglans microcarpa*
5 (Reverse)*	Valentines Flat Plantation	*Juglans nigra*
11 (Forward)*	Lookout Point Terrace	*Juglans major*
11 (Reverse)*	Lookout Point Terrace	*Juglans nigra*
15 (Forward)*	South Branch Floodplain	*Juglans nigra*
15 (Reverse)*	South Branch Floodplain	*Juglans nigra*

* Forward or reverse primer supplied by Invitrogen cloning kit (cat# K4530-20)

** -47 sequencing primer supplied by Beckman Coulter Sequencing Kit

Sequence Identification of Single Run Forward and Reverse Reactions: Results from the Genbank sequence identity comparison (Table 1) showed that all single run sequence samples belonged to the *Juglans* genera. Samples #3, 5, and 11 showed variation within

the same sample, identifying better with other *Juglans sp*. than *J. nigra* when sequenced using the forward primer. However when sequenced with the reverse primer, all were most identical to *J. nigra* sequences in NCBI Genbank.

A total of five plasmids, from five different trees, were successfully sequenced with the forward and reverse primers. The inconsistent species identity observed when comparing the forward and reverse sequence of samples #3, 5, and 11 cannot be interpreted as variation within the same sample, since both the forward and reverse sequencing reactions were run from plasmid DNA extracted from the same clone.

It has been shown that reforestation by planting within a species' native range is an example of human mediated gene transport, and if trees in off-site plantations cross with those in native populations, diversity may increase in the next generation, although with negative consequences for local adaptation (Ledig 1992). The possible transport of non-native genes occurred with the planting of Valentine's Flats Plantation, and this may have further increased any genetic diversity in the adjacent J. nigra populations in Zoar Valley. This explanation, again, however unlikely (due to the relatively short time period between the planting of Valentine's flats and the amount of genetic variation observed) could account for the variation observed in the Valentines Flats and Lookout Point samples. *Juglans* species are renowned for their ability to form hybrids (Potter et al. 2001).

Referring to Table 1, if *J. microcarpa* (5 –Forward (-47)) or *J. major* (3 –Forward and 11 –Forward), or perhaps a hybrid of these two species was introduced to Zoar

Valley in the past, then it is possible that these genes were introduced into the Zoar Valley *J. nigra* population. If this happened, hybridized walnut trees were then created. They might be morphologically identical to the native *J. nigra* population, however revealing their true identity only in DNA analysis. (*J. microcarpa* and *J. major* are native to the southwestern U.S.). This possibility of ITS hybrids allows for the discrepancy observed between the forward and reverse primers. For further study of this observed variation, we must construct consensus sequences from all sequencing reactions for each sample, and then compare these consensus sequences with others in the sample set and with those published in NCBI GENBANK. The consensus sequences help to rule out error as a cause of variation.

Assembling and Analyzing the Consensus Sequence: Consensus sequences were constructed from the multiple sequence alignment of all single runs for each sample. These consensus sequences were constructed to represent the most highly observed sequence configuration, after alignment and comparison, for all sequenced plasmids. The consensus sequence was then subjected to an NCBI BLAST assay and the results were as follows:

Table 2. Tree DNA samples with the number of sequencing runs and successful consensus sequence constructed

Tree DNA Sample	# of Sequencing Runs	Consensus sequence constructed
1	3	yes
3	2	Yes
5	2	Yes

11	2	Yes
15	2	No

Consensus sequence nucleotides are listed either in capital letters (“A”) or lowercase letters bracketed by parenthesis (“(a/t)”). Capital letters stand for undisputed consensus nucleotides, while lowercase letters are found where there is an inconsistency between the single run sequences, with both nucleotides listed.

Table 3. Consensus Sequence and NCBI Blast best match of sample 1, with highest identity segment of overlap from Clustalw multiple alignment shown below

Sample 1 Consensus Sequence (799 bp)
CAACTTCGCCCTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCG ATACCTGCCCAGCATGCTAACGACCTGTGAACATGTAATAATAACC TTCTGGGTGGGGGTGTAATGCCCCCTCCCAGAAAACGG(t/g)GGGAG GG(c/g)CAACGTTGAGATTGGCCCACTGCTC(c/t)TCGGTGTG(g/t)GGT TGGGTCGATCCTCTCGTTCCCT(t/c)CCCGATCG(a/g)ACAATGAACCC CCGGCGCGGTCTGCGCCAAGG(a/g)ACTTAAAACAAGGAGTAACCA CGGGCGCCCCCGG(a/g/t)AAACGGTGTGCGTGTCGTTGGTGACGTCTT TACCATGATACATAACGACTCTCGGCAACGGATATCTCGTGCTCTC GCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTG CAGAATCCCGCGAATCATCGAGTCTTTGAACGCAAGTTGCGCCCGA AGCCATTCGGCCGAGGGCCACGTC(t/c)TGGCCTGGGGTGTCACGCAT CGTTGCCCCAACCCCAAACACTTCTTACGCTGTGCGGGGTGCGGGG AAGACGCCACGACAATCGGTGGTTGAGAAACCCTCGTGACCCGTCG TGTGTCGCCCGTCGCTGTGAAGGTGCTCCTCGACCCTATTGTGTCGT TCTTGCGACTCTACCATCGCGACCCCAGGTCAGGCGGGATTACCCGC TGAATTTAAGCATATCAATAAGCGGAGGAAAGGGCGATTCGCGGCC GCTAAATTCAATTCAGCCCTATAGTGAGTCGTATTACAATTCACTGG CGTA
NCBI BLAST Best Match: Juglans nigra isolate 836 18S ribosomal RNA gene **E Value:** 0.0

Identitiy: 95%

Figure 4. Segment of consensus sequence from Sample 1 multiple alignment of single runs (from Appendix)

```
1 Reverse            TTAAAACAAGGAGTAACCACGGG-CGCCCCCGGAAAACGGTGTGCGTGTC-GTTG 168
1                    TTAAA-CAAGGAGTAACCACGGG-CGCCCC--GGAAACGGTGTGCGTGTC-GTTG 290
1For                 GCCCGTCGCTGTGAAGGTGCTCCTCGACCCTATTGTGTCGTTCTTGCGACTGTAC 88
                           *   * * *    *    ** ***         **    * * * **

1 reverse       GTGACGTCTTTACCATGATACATAACGACTCTCGGCAACGGATATCTCG-GCTCTCGCAT 227
1               GTGACGTCTTTACCATGATACATAACGACTCTCGGCAACGGATATCTCGTGCTCTCGCAT 350
1For            CTGACGTCTTTACCATGAGACATAACGACTCTCGGCAACGGATATCTCG-GCTCTCGCAT 147
                 ***************** ***************************** **********

1 reverse       CGATGA 233
1               CGATGA 356
1For            CGATGA 153
                ******
```

The sample 1 consensus sequence is 95% (512/538 bp) identical to *J. nigra*. Three sequences were used to construct this consensus sequence, two forward and one reverse (Figure 4). This sequence was then assessed for matches in a BLAST assay (Figure 5).

Figure 5. Sample 1 NCBI BLAST result (partial)

```
S1      252  TTAAAACAAGGAGTAACCACGGGCGCCCCCGGAGAAACGGTGTGCGTGTCGTTGGTGACG  311
             || ||||||||||||||||||||| |||| | ||||||||||||||||||||||||||||
Gen     242  TT-AAACAAGGAGTAACCACGGGCG-CCCC-G-GAAACGGTGTGCGTGTCGTTGGTGACG  297

S1      312  TCTTTACCATGATACATAACGACTCTCGGCAACGGATATCTCGTGCTCTCGCATCGATGA  371
             |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Gen     298  TCTTTACCATGATACATAACGACTCTCGGCAACGGATATCTCG-GCTCTCGCATCGATGA  356
```

The segment of the consensus sequence used in the BLAST assay shown in Figure 5 has also been highlighted in Table 3. Figure 5 shows five distinct differences between the consensus sequence and the GENBANK closest match. The differences

observed are all base additions to the consensus sequence. None of the single base additions are conserved in all three of the single run sequences, making it unlikely that this is real sequence variation (Figure 4). These extra bases may have been erroneously added to the sample DNA sequence during the editing of the sequence chromatograms, and therefore are a likely byproduct of this protocol.

Table 4. Consensus Sequence and NCBI Blast best match for sample 3, with highest identity segment of overlap from ClustalW multiple alignment shown below

Sample 3 Consensus Sequence (808 bp)
GTGAACCTGCGGAAGGATCATTGTCGATACCTGCCCAGCAG AACGACCTGTGAACATGTAATAACCTTCTGGGTGGGGGTGT AATGCCCCCTCCCAAAAAACGGTTGGGAGGGCACGTTGAGA TTTGCCCACTGCTCCTCGTGTGTGGTTGGTCGATCCTCTCGTT CCCTTCCCGATCGAACAACGAACCCCGGCGCGGTCTGCGCCA (a/g)GGAACTTAA(a/c)CA(a/g)GGAGGTAACCACGGGGCGCCCC CGGGAAACGGGTGGGCGGTGTCGGTTGG(g/t)GACGTCTTTAC CAAGATACATAACGACTCTCGGCAACGGATATCTCGGCTCTC GCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGGT(g/t) GAATTGCAGAATCCCGCGAATCATCGAGTCTTTGAACGCAAG GTTGCGCCCGAAGCCATTCGGCCGAGGGCACGCCTGCCTGGG TGTCACGCATCGTTGCCCCAACCCCAAACACTTCTTACGCTGT GC(g/c)(g/c)GGTGCGGG(a/g)(a/g)A(g/a)(a/t)ACATTGGCCTCCCGT GCGCTTTTGCTCGCGGTTAGCCTAAAAGTGAGTCCTAGGCGAC GAGCGCCACGACAATCGGTGGTTGAGAAACCCTCGTGACCCG TCGTGTGTCGCCCGTCGCTGTGAAGGTGCTCCTCGACCCTATT GTGTCGTTCTTGCGACTCTACCATCGCGACCCCAGGTCAGGCG GGATTACCCGCTGAATTTAAGCA TATCAATAAGCGGAGGAAA GGGCGAATTCGTTTAAACAATGCAG
NCBI Best Match: Juglans nigra isolate 836 18S ribosomal RNA gene **E Value:** 0.0 **Identitiy:** 95%

Figure 6. Segment of consensus sequence from Sample 3 multiple alignment of single runs

```
3 Reverse       AAGCCATTCGGCCGAGGGCACGCCTGCCTGGGTGTCACGCATCGTTGCCCCAACCCCAAA 276
3               AAGCCATTCGGCCGAGGGCACGCCTGCCTGGGTGTCACGCATCGTTGCCCCAACCCCAAA 469
                **********************************************************

3 Reverse       CACTTCTTACGCTGTGC 293
3               CACTTCTTACGCTGTGC 486
                *****************
```

The sample 3 consensus sequence is 95% (717/748 bp) identical to *J. nigra*. Two sequences were used to construct this consensus sequence, one forward and one reverse (Figure 6). This sequence was then assessed for matches in a BLAST assay (Figure 7).

Figure 7. Sample 3 NCBI BLAST result (partial)

```
S3      AAGCCATTCGGCCGAGGGCACGCCTGCCTGGGTGTCACGCATCGTTGCCCCA  477
        |||||||||||||||||||||| |||||||||||||||||||||||||||||
Gen     AAGCCATTCGGCCGAGGGCACGTCTGCCTGGGTGTCACGCATCGTTGCCCCA  473

S3      ACCCCAAACACTTCTTACGCTGTGC 502
        |||||||||||||||||||||||||
Gen     ACCCCAAACACTTCTTACGCTGTGC 498
```

The segment of the consensus sequence used in the BLAST assay shown in Figure 7 has also been highlighted in Table 4. Figure 7 shows one distinct difference between the consensus sequence and the GENBANK closest match. The variant observed in this segment is found in both the forward and reverse runs of this sequence, and is found in an otherwise highly conserved stretch of DNA sequence between the consensus sequence and the Genbank entry (Firgure 6). This single nucleotide difference has the possibility of being a true variation. More sequences must be processed for sample 3 to determine if this is in fact real.

Table 5. Consensus Sequence and NCBI Blast best match for sample 5, with highest identity segment of overlap from Clustalw multiple alignment shown below

Sample 5 Consensus Sequence (833 bp)
TAGCGCACGTGGAATTGTAATACGACTCACTATAGGGTTCGAATTGAAT TTAGCGGCCGC GAATTCGCCCTTTCCTCCGCTTATTGATATGCTTAAATT CAGCGGGTAATCCCGCCTGAC CTGGGGTCGCGATGGTAGAGTCGCAAG AACGACACAATAGGGTCGAGGAGCACCTTCACA GCGACGGGCGACACA CGACGGGTCACGAGGGTTTCTCAACCACCGATTGTCGTGGCGCTC GTCG CCTAGGACTCACTTTTAGGCTAACCGCGAGCACAAGCGCACGGGAGGCC AATGTCT TCCCCGCACCCCGCACAGCGTAAGAAGTGTTTGGGGTTGGGG CAACGATGCGTGACACCC AGGCAGACGTGCCCTCGGCCGAATGGCTTCG GGCGCAACTTGCGTTCAAAGACTCGATGATTCGCGGGGATT(c/t)(/t/c) TGC a/g) ATTCA(a/c)(a/c)(a/c) C(a/c)A(g/a)GTATCGCATTTCGCTACGTTCTTCATC GATGCGAGAGCCGAAATATCCGTTGCCGAGAGTCG(g/t) TATGTATCATGG TAAAGACGTCACCAACGACACGCACACCGTTTCCGGGGCGCCCG(t/a)GGT (g/t) ACTCC(c/t)TGGGTAAGTTCCTTGGCGCAGACCGCGC(c/g)GGGGTTCAT TGTTCGATCGGGAAGGGAACGAGAGGATCGACCACCACACACGAGGGGC AGGGGGCAAATCTCAACGTGC(c/t) TGGGA(a/g)GGGGCC(t/g)GTAC(a/c)CC CCAGGAA(g/a)GGTATTATTACATGTTC(a/c) CAGGGTCGGTCT
NCBI Best Match: Juglans nigra isolate 834 18S ribosomal RNA gene **E Value:** 0.0 **Identitiy:** 96%

Figure 8. Segment of consensus sequence from Sample 5 multiple alignment of single runs

```
5 Reverse   CGAAATATCCGTTGCCGAGAGTCGGTATGTATCATGGTAAAGACGTCACCAA 125
5           CGAGATATCCGTTGCCGAGAGTCGTTATGTATCATGGTAAAGACGTCACCAA 538
            *** ******************** **************************


5 Reverse   CGACACGCACACCGTTTCCGGGGCGCCCGA 155
5           CGACACGCACACCGTTTCCGGGGCGCCCGT 568
            ******************************
```

The sample 5 consensus sequence is 96% (604/627 bp) identical to *J. nigra*. Two sequences were used to construct this consensus sequence, one forward and one reverse (Figure 8). This sequence was then assessed for matches in a BLAST assay (Figure 9).

Figure 9. Sample 5 NCBI BLAST result (partial)

```
S5      497  CGAAATATCCGTTGCCGAGAGTCGGTTATGTATCATGGTAAAGACGTCACCAACGACACG  556
             ||| ||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Gen     340  CGAGATATCCGTTGCCGAGAGTCG-TTATGTATCATGGTAAAGACGTCACCAACGACACG  282

S5      557  CACACCGTTTCCGGGGCGCCCGT
             |||||||||||||||||||||||
Gen     281  CACACCGTTTCCGGGGCGCCCGT
```

The segment of the consensus sequence used in the BLAST assay shown in Figure 9 has also been highlighted in Table 5. Figure 9 shows two distinct differences between the consensus sequence and the GENBANK closest match. One difference observed was a base addition to the consensus sequence. This single base addition was not conserved in both of the single run sequences, making it unlikely that this is real sequence variation (Figure 8). The other difference is a substitution of an "A" for a "G". This substitution is observed in the reverse run but not in the forward, and therefore is likely an editing mistake or an incorrect call by the sequencer.

Table 6. Consensus Sequence and NCBI Blast best match for sample 11, with highest identity segment of overlap from Clustalw multiple alignment shown below

Sample 11 Consensus Sequence (796 bp)
TCCTCTGTTTAAACCAATTCGCCCTTTCCTCCAGCTTATTGATATGCTTAAATTCA GCGG GTAATCCCGCCTGACCTGGGGTCGCGATGGTAGAGTCGCAAGAACGACAC AATAGGGTCG AGGAGCACCTTCACAGCGACGGGCGACACACGACGGGNTCACGA GGGTTTCTCAACCACC GATTGTCGTGGCGCTCGTCGCCTAGGACTCACTTTTAGGC TAACCGCGAGCAAAAGCGCACGGGAGGCCAATGTCTTCCCCGCACCCCGCACAGC GTAAGAAGTGTT(t/g) GGGGTTGGGGC(a/g) ACGATGCGTGACACCCAGGCAGACGT GCCCTCGGCCGGAATGGCTTCGGGCGCAACTTGC GTTCAAAGACTC(g/t) TATGAT

TCGCGGGATTCTGCAATTCACACCAAGTATCGCATTT(t/c)(c/g)GCCTACGTTCTTCA
TCGATGCGAGAGCCGAGATATCCGTTGCCGAGAGTCGTTATGTATCATGGTAAAG
ACGT(c/t)ACCAACGACACGCACACCGTTTCCGGGGCGCCCGTGGTTACTCCTTGTTT
AAGTTCCTTGGCGCA(g/a)ACC(g/c)(g/c)(g/c)CCGGGGTTCATTGTTC(g/c)ATCGGGAA
GGGAACGA(c/g)A(a/g)GATC(g/c)ACCA(a/c)CCACACACGAGGAGCAGTGGGCAAAT
CTCAACGTGCCCTCCCAACCGTTGCTGGGCAGGTATCGACAATGATCCTTCCGCAG
GTTCACCTACGGGAAGGCGAATTGCGG CCGCATATTCAATTGCC

NCBI Best Match: Juglans nigra isolate 836 18S ribosomal RNA gene
E Value: 0.0

Identitiy: 96%

Figure 10. Segment of consensus sequence from Sample 11 multiple alignment of single runs

```
11 Reverse      TGCAATTCACACCAAGTATCGCATTTCG-- 161
11              TGCAATTCACACCAAGTATCGCATTTTCGC 419
                *************************

11 Reverse      CTACGTTCTTCATCGATGCGAGAGCCGAGATATCCGTTGCCGAGAGTCGTT-ATGTATCA 220
11              CTACGTTCTTCATCGATGCNAGAGCCGAGATATCCGTTGCCGAGAGTCGTTTATGTATCA 479
                ****************** ****************************** ********

11 Reverse      TGGTAAAGACGTCACCAACGACACGCA 247
11              TGGTAAAGACGTTACCAACGACACGCA 516
                *********** **************
```

The sample 11 consensus sequence is 96% (629/653 bp) identical to *J. nigra*. Two sequences were used to construct this consensus sequence, one forward and one reverse (Figure 10). The consensus sequence was then assessed for matches in a BLAST assay (Figure 11).

Figure 11. Sample 11 NCBI BLAST result (partial) (from Appendix)

```
S11     394  TGCAATTCACACCAAGTATCGCATTTTCCGGCCTACGTTCTTCATCGATGCGAGAGCCGA  453
             ||||||||||||||||||||||||||| | | | ||||||||||||||||||||||||||||
Gen     393  TGCAATTCACACCAAGTATCGCATTT-C-G-C-TACGTTCTTCATCGATGCGAGAGCCGA  338

S11     454  GATATCCGTTGCCGAGAGTCGTTTATGTATCATGGTAAAGACGTCTACCAACGACACGCA  513
             ||||||||||||||||||||||| |||||||||||||||||||| ||||||||||||||
Gen     337  GATATCCGTTGCCGAGAGTCGTT-ATGTATCATGGTAAAGACGTC-ACCAACGACACGCA  280
```

The segment of the consensus sequence used in the BLAST assay shown in Figure 11 has also been highlighted in Table 6. Figure 11 shows six distinct differences between the consensus sequence and the GENBANK closest match. All these differences can be attributed to the editing process and subsequent construction of the consensus sequence. They are all extra base additions (Figure 11) and are due to having mismatched bases between the two single run sequences (forward and reverse), and having to include both possibilities in the consensus. This problem may have been avoided had there been at least one more single run sequence, since there would be a greater number of sequences to draw the consensus from, thereby avoiding the problem of having to include two possible bases in the sequence. In the example shown in Figure 10 the **11 (reverse)** sequence is identical to the Genbank acquired sequence shown in Figure 11. It is therefore the **11 (Forward)** sequence that appears to have been inaccurate in this instance.

Table 7. Consensus Sequence and NCBI Blast best match for sample 15

Sample 15 Consensus Sequence
No significant similarity is observed in the multiple alignment of the Forward and reverse reactions of sample 15 (from Appendix)

The reason for the lack of similarity of sample 15 is not completely understood. It is likely that it is a combination of poor sequence quality to begin with, as well as editing error. Looking at the NCBI BLAST of both the forward and reverse reactions for sample 15 Appendix 2), it is apparent that this sample did not have the high sequence

resolution of the other samples. Contamination of the plasmid or during the sequencing of sample 15 may have also played a part.

Consensus Sequence Multiple Alignment

Figure 12. Partial multiple alignment for all sample consensus sequences with J. nigra sequence from genbank included (from Appendix) [Additions (red)] [Substitutions (blue)] [Deletions (green)]

```
11                 G-TGTCG-TTGGTAGACGTCTTTACCATGATACATAAACGACTCTCGGCAACGGATATCT 300
J.nigra GENBANK    G-TGTCG-TTGGT-GACGTCTTTACCATGATACATAA-CGACTCTCGGCAACGGATATCT 338
3                  GGTGTCGGTTGGGTGACGTCTTTACCAAGATACATAA-CGACTCTCGGCAACGGATATCT 322
1                  G-TGTCG-TTGGT-GACGTCTTTACCATGATACATAAC-GACTCTCGGCAACGGATATCT 83
5                  G-TGTCG-TTGGT-GACGTCTTTACCATGATACATAACCGACTCTCGGCAACGGATATTT 274
                   * ***** ****  ************* *********  ****************** *

11                 CG-GCTCTCGCATCGATGAAGAACGTAGGCCGGAAAATGCGATACTTGGTG------TGA 353
J.nigra GENBANK    CG-GCTCTCGCATCGATGAAGAACGTAGC----GAAATGCGATACTTGGTG------TGA 387
3                  CG-GCTCTCGCATCGATGAAGAACGTAGC---GAAAATGCGATACTTGGTGGTG---TGA 375
1                  CGTGCTCTCGCATCGATGAAGAACGTAGC----GAAATGCGATACTTGGTG------TGA 133
5                  CG-GCTCTCGCATCGATGAAGAACGTAGC----GAAATGCGATACTCTGTGGTGTGTTGA 329
                   ** ************************      ************  ***      ***

11                 AT-TGCAGAA----TCCCGCGAATCATAACGAGTCTTTGAACGCAAG-TTGCGCCCGAAG 407
J.nigra GENBANK    AT-TGCAGAA----TCCCGCGAATCAT--CGAGTCTTTGAACGCAAG-TTGCGCCCGAAG 439
3                  AT-TGCAGAA----TCCCGCGAATCAT--CGAGTCTTTGAACGCAAGGTTGCGCCCGAAG 428
1                  AT-TGCAGAA----TCCCGCGAATCAT--CGAGTCTTTGAACGCAAG-TTGCGCCCGAAG 185
5                  ATCTGCAGAAGAATCCCCGCGAATCAT--CGAGTCTTTGAACGCAAG-TTGCGCCCGAAG 386
                   ** *******     ************  ****************** ************

11                 CCATTCCGGCCGAGGGC-ACGTCT---GCCTGGG-TGTCACGCATCGTCTGCCCCAACCC 462
J.nigra GENBANK    CCATTC-GGCCGAGGGC-ACGTCT---GCCTGGG-TGTCACGCATCGT-TGCCCCAACCC 492
3                  CCATTC-GGCCGAGGGC-ACGCCT---GCCTGGG-TGTCACGCATCGT-TGCCCCAACCC 481
1                  CCATTC-GGCCGAGGGCCACGTCTCTGGCCTGGGGTGTCACGCATCGT-TGCCCCAACCC 243
5                  CCATTC-GGCCGAGGGC-ACGTCT---GCCTGGG-TGTCACGCATCGT-TGCCCCAACCC 439
                   ****** ********** *** **   ******* ************* ***********
```

Figure 12 depicts the multiple alignment of the sample consensus sequences and *J. nigra* sequence taken from Genbank. Overall, the alignment pattern is comprised of short spans of high identity sequence of varying length, interrupted intermittently by sequence variation in one or more of the sample sequences, in the form of a nucleotide base addition, substitution, or deletion.

The majority of the sequence discrepancy is in the form of additions. As mentioned earlier, the likely culprit for these additions is human error during the

sequence editing process, or sequencer error. This theory is supported in that most of these additions were only observed in one of the sample sequences at any certain point throughout the sequence. Looking at Figure 12, when additions did happen in more than one sample at the same time in that sequence, they were sometimes conserved. It is those base additions that are conserved in more than one sequence that hold the best chances of actually being sample variation, and should be further studied.

Nucleotide substitutions were less frequent in the multiple alignment. They can also be byproducts of the sequence editing process. Several substitutions were visible in the first and second lines of Figure 12. These divergences of sequence may be indicative of true variation since they were conserved in both the forward and reverse reactions that went into the consensus sequence, and therefore should be examined further in future studies.

Only one deletion was observed. Located in the first line of Figure 12, sample 1, it appears that the nucleotide base had been shifted to the left somehow, since the missing nucleotide was listed as an addition in the adjacent space.

Comparison by location within Zoar Valley: All samples sequenced were positively identified as being of the species Juglans nigra. No clear discrepancy or pattern of genetic variance was able to be discerned between sample locations.

Conclusion: The primary goals of this study- to sequence multiple J. nigra subjects within 3 distinct populations inside Zoar Valley, and identify these sequences utilizing examples published in Genbank- were accomplished.

The use of a consensus sequence is of the utmost importance when sequencing DNA because each single run sequencing run can yield slightly different results. An example of this phenomenon was observed in this study, using a consensus sequence allowed for more accurate DNA sequencing. In the initial NCBI BLAST of the single run sequences (Table 1), one sequence was most identical to *J. microcarpa*, 5 (Forward -47), and two were most identical to J. major 3 (Forward) and 11 (Forward), instead of all the sequences being most identical to *J. nigra* as assumed they would be. The results of the consensus sequence analysis however showed that although one of the single run sequences may have been more identical to another *Juglans* species, when these single runs were used to construct a consensus sequence, that sequence was most identical to the *J. nigra* sequences in a BLAST assay. All the consensus sequences were matched with *J. nigra* sequences that have been submitted to and published Genbank.

While these results are very descriptive of the rDNA ITS regions of the sampled trees, the number of single run sequences used to construct the consensus sequences was minimal, with three sequences for sample 1 and two for the remaining samples. An increased number of sequencing and identification trials should be conducted before placing any of the sampled trees definitively under the classification of *J. nigra*, and will help determine which, if any, sequence variations observed in this study are real.

The results fell short of answering questions in key areas, such as determining a preliminary colonial history of *J. nigra* in Zoar Valley, and identification of specific

inherited variations in sequence within populations. While variation was observed, the results remain inconclusive because the total number of sequences was low, therefore further analysis is needed.

Many samples were lost during their processing from leaf tissue to DNA sequences, particularly during the methods of PCR, cloning, and sequencing. Perhaps if more samples had been successfully sequenced, the results of this study would provide more powerful evidence for the possible variation observed in the sample sequences from this study.

Future Research Implications: Three persistently problematic methods were: the consistent production of PCR products, efficiency in manufacture of positive (containing PCR product insert) clones, and reliable sequencing reactions.

PCR product was not consistently produced from DNA extracts. Many samples were contaminated, resulting in the amplification of fungal DNA instead of plant DNA, because the ITS primers used are universal for eukaryotes. Therefore mixes of plant and fungi DNA resulted in competition for the primers, reducing the yield of the desired plant DNA PCR product. Increased vigilance and focus on detail during collection of samples seemed to prevent this problem. Also, it is theorized that many failed reactions were the result of the ITS1 and ITS4 primers themselves. These primers are fairly short, and had there been any variation between the primer sequence and target DNA sequence, steric hinderance may have prevented the reliable annealing of the primers.

During cloning, the primary problem experienced was in generation of clones that contained a complete insert. This was believed to be due to free nucleotides present in the PCR product that were being preferentially inserted into the plasmid, rendering

plenty of clones, but that when subjected to EcoR1 digest, showed no insert. PCR cleanup kits were tried and did not rectify the problem. Another possible cause of the problem was contamination of the cloning kit chemicals. Two cloning kits were used (see Methods), and fresh supplies were purchased, but the problem remained.

Sequencing problems were in two parts: reliably generating sequence, and generating full sequences. Generation of sequences required careful quantification of the concentration of plasmid DNA and primers, and many reactions likely failed early on during this research due to incorrect quantification of DNA concentrations. As to generating relatively lengthy sequences, it seemed that low resolution of sequencing reaction products by the sequencer may have, in part with slightly miscalculated reagents, contributed to the generation of short fragments. It is recommended for the future that smaller PCR fragments be generated. Use of the ITS2 and ITS3 primers (White et al. 1990), located on either end of the 5.8S ribosomal gene, along with the ITS1 and ITS4 primers, would produce such shorter PCR products.

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Title: A molecular identification of fungal ribosomal DNA isolated from *Ulmus americana* and *Ulmus rubra* leaf tissue samples from Zoar Valley, NY using ribosomal DNA internal transcribed spacer sequences (rDNA ITS)

Background:

The entire, living plant leaf is known as the phyllosphere, and it includes the surface and interior. The plant leaf surface is a complex terrestrial habitat containing a wide variety of microorganisms including bacteria, filamentious fungi, and yeast (Carroll et al. 1977, Levetin & Dorsey 2006). Fungal endophytes are fungi that inhabit the tissue of plants without causing visible disease symptoms (Schulz & Boyle 2005). This is contrasted with the term "fungal epiphyte", which refers to organisms living on the outside of the plant (Wilson 1995). Phylloshpere fungi are those that found on the surface, and within living leaves (Petrini 1991).

In woody perennials, fungal endophytes are thought to protect the plants in which they live by one or more mechanisms (antibiosis, mycoparasitism, induced resistance, competitive exclusion), and are thought to develop from environmental and background inoculum, and are not transferred from generation to generation (Johnson & Whitney 1989; Crozier *et al*. 2006). Some endophytic fungi have been shown to effectively antagonize herbivores and pathogenic fungi (Carroll, 1988; Clay, 1988). Defensive mutualisms involving the protection of host plants by animals, primarily ants, are well known but may be far less common than defensive mutualisms with fungi (Clay 1988).

New Yok State's Zoar Valley was chosen for sampling due to its intact riparian ecosystem healthy distribution of the two elm species of interest. The woodlands of Zoar

Valley are highly varied and diverse, and collectively meet all objective criteria for eastern old growth forest (Diggins and Kershner 2005). No known previous analysis of leaf-associated fungi has been conducted in these woodlands on any native tree species. This study represents a preliminary survey of the phyllospheric fungi associated with the native elm species of Zoar Valley, from several distinct woodland areas within. Little is known concerning the common fungal species inhabiting the phyllosphere of non-economically important eastern woodland broadleaf tree species such as elms. A previous paper from Levetin and Dorsey (2006) analyzed the contribution of *Ulmus americana* leaf surface fungi to the air spora. Samples taken from leaves were cultured and identified morphologically and then compared with cultures grown from spores collected from the air.

Elms, in particular *U. americana* (American elm), have been an unmistakable landmark of culture in the North American continent. Elms are highly valued shade trees, once found lining city blocks from coast to coast (Hubbes 1999). Elm trees still thrive in Zoar Valley today, although their populations have been decimated elsewhere due to Dutch Elm Disease, a catastrophic infection caused by the fungus *Ophiostoma ulmi*, which usually results in death of the tree.

In 2003 the American Phytopathological society called for an increase in funding for research of many plant associated microbes, stating the importance of genetic screening and identification of plant-associated pathogenic microbes in disease prevention. Included in its list of "high priority species" for study were *Ophiostoma novo-ulmi*, as well as *Candidatus phytoplasma ulmi*, a phytoplasma that causes Elm Yellows. Analysis of phyllospheric communities may help identify endophytic

associations in elm trees in Zoar Valley, shedding light on the existence of certain colonizing fungi that help to prevent pathogenicity of other microbes.

Numerous studies analyzing temperate and tropical trees have showed that endophytes represent an important and quantifiable component of fungal biodiversity (Levetin and Dorsey 2006, Clay 1988, Arnold et al. 2001).

Methods: Samples were obtained from Zoar Valley over fall of 2005 and spring of 2006. Samples were collected from 5 distinct areas of Zoar Valley, Lookout Point Terrace, South Branch Floodplain, Burchfield Terrace, Skinny Dip Terrace, and Skinny Dip Streamside. Samples of *U. Americana* leaf tissue were first positively identified by analysis of leaf structures and bark, then collected and stored in deep freeze at -80 C° until DNA extraction was performed.

DNA extraction: Total DNA was extracted using a revised CTAB method of Doyle and Doyle (1987).

PCR: Extracted DNA is amplified with ITS specific primers. Primers used were ITS1 and ITS5 (forward) and ITS4 (reverse) of White et al. (1990). PCR performed on PTC-200 DNA engine.

Cloning: Purified PCR products were cloned using the TOPO TA Cloning Kit from Invitrogen (cat# K4530-20) or StrataClone PCR cloning kit from Stratagene (cat#240205)

Restriction Digest: A restriction digest using EcoR1 was used to screen clones before sequencing.

Sequencing: Sequencing reactions were carried out on purified cloning extracts using Beckman Coulter Sequencing Kit, sequenced using primers M13 (-20, -47) Forward and M13 Reverse, with sites provided in the cloning vector. Other sequences were from purified plasmid generated in this lab and sequenced at the Ohio State University Plant Microbe genomics facility using the same technique. Sequencing reactions were conducted at Ohio State University on a 3730 DNA analyzer from Applied Biosystems, Inc.

Alignment: Sequences are aligned to determine identity or variation in ITS region of each species using computer programs, such as NCBI nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and ClustalW (http://www.align.genome.jp/sit-bin/clustalw)

Results and Discussion:

Ten fungal samples were recovered from *Ulmus Americana* and 15 from *Ulmus rubra* leaves. Multiple fungal species were recovered from the same leaf sample in several cases. Sample 43 yielded seven different species of fungus.

Table 1. Identification of fungi using PCR amplified DNA extracted from elm leaves within Zoar Valley

Tree species fungal sample was isolated from	**Sample # and ITS ribosomal primer set used to sequence**	Collection Site within Zoar Valley	Closest match in NCBI Nucleotide Blast Search And **% Identity**
U. americana	**9 -Reverse**	South Branch Floodplain	Phlebia radiata **90%**
U. americana	**12 -Forward**	Lookout Point Terrace	Phoma sp. **90%**

U. americana	**12 -Reverse**	Lookout Point Terrace	Phoma sp. **90%**
U. americana	**63 -Forward**	South Branch Floodplain	Coprinellus sp. **97%**
U. rubra	**50 -Forward**	Skinny Dip Terrace	Uncultured leaf litter fungus **84%**
U. americana	**55 -Forward**	Skinny Dip Streamside	Coprinellus sp. **82%**
U. americana	**31D -Forward**	Burchfield terrace	Coprinellus sp. **96%**
U. americana	**31D -Reverse**	Burchfield terrace	Coprinellus sp. **96%**
. *U. americana*	**31E -Forward**	Burchfield terrace	Coprinellus sp. **99%**
U. rubra	**43i #4 -Forward**	Skinny Dip Terrace	Colletotrichum truncatum **98%**
U. rubra	**43i #5 -Forward**	Skinny Dip Terrace	Colletotrichum truncatum **99%**
U. rubra	**43s #2 -Forward**	Skinny Dip Terrace	Cryptococcus sp. **96%**
U. rubra	**43s #4 -Forward**	Skinny Dip Terrace	Phaeosphaeria sp. **97%**
U. rubra	**43s #5 -Forward**	Skinny Dip Terrace	Ampelomyces sp. **97%**
U. rubra	**43s #6 -Forward**	Skinny Dip Terrace	Gyoerffyella sp. **94%**
U. rubra	**43i#4 -Reverse**		Colletotrichum truncatum **96%**
U. rubra	**43i -Forward**	Skinny Dip Terrace	Didymella bryoniae **97%**
U. rubra	**43ii -Foreward**	Skinny Dip Terrace	Phoma glomerata **100%**
U. rubra	**43s#2 -Reverse**	Skinny Dip Terrace	Cryptococcus sp. **95%**
U. rubra	**43s#4 -Reverse**	Skinny Dip Terrace	Colletotrichum truncatum **98%**
U. rubra	**43s#6 -Reverse**	Skinny Dip Terrace	Colletotrichum truncatum **90%**
U. rubra	**45#1 -Forward**	Skinny Dip Terrace	Ampelomyces humuli

			95%
U. rubra	**45#2 -Forward**	Skinny Dip Terrace	Phoma sp. **97%**
U. americana	**53#1 -Forward**	Skinny Dip Streamside	Uncultured ectomycorrhiza **91%**
U. americana	**57#1 -Forward**	Skinny Dip Streamside	Uncultured glomeraceous **97%**

Table 2. Lineage of identified fungi and percentage variance with closest match in Genbank (Table 1)

Taxonomic Lineage of Closest Genabank Taxa

Genus	Phylum, Order, Family	% Difference between sample and closest match	# of Unique ITS sequences
Phlebia	*Basidiomycota; Polyporales; Meruliaceae*	4%	1
Phoma	*Ascomycota; Pleosporales; -*	0%	3
Coprinellus	*Basidiomycota; Agaricales; Psathyrellaceae*	3%	3
Colletotrichum	*Ascomycota; Phyllachorales; Phyllachoraceae*	1%	1
Cryptococcus	*Basidiomycota; Filobasidiales; Filobasidiaceae*	4%	2
Phaeosphaeria	*Ascomycota; Pleosporales; Phaeosphaeriaceae*	3%	1
Ampelomyces	*Ascomycota;* incertae sedis	3%	2
Gyoerffyella	*Ascomycota;* incertae sedis	6%	1
Didymella	*Ascomycota; Pleosporales;* Incertae sedis	3%	1

Classification based on the 9th edition of the Dictionary of Fungi: http://www.indexfungorum.org/Names/fundic.asp

Background on genetically identified genera (Listed in order of highest to lowest # unique sequences identified per genera):

***Phoma*:** In this study, we recovered three unique sequences identified from three different leaf samples (Table 1 and Table 2). Samples containing *Phoma* were from Lookout Point Terrace and Skinny Dip Terrace. *Phoma* is a large genus of anamorphic fungi in the form class Coelomycetes that is characterized by conidia formation in a

pycnidium, and their conidia are believed to be dispersed throughout a tree via a rain splash mechanism. *Phoma* species are documented endophytes, and have been frequently found in a wide array of plants species, including several types of cacti in Arizona, beech and giant dogwood in Japan, as well as American elm (Suryanarayanan et al. 2005, Osono and Mori 2003, Osono et al. 2004, Levetin and Dorsey 2006). *Phoma* species likely exist as endophytes in the trees sampled.

Coprinellus: Three unique sequences were detected in 3 different leaf tissue samples (Table 1 and Table 2). *Coprinellus* was found to be well distributed throughout the sampled areas of this study, with samples from South Branch Floodplain, Skinny Dip Streamside, and Birchfield Terrace (Figure 1). Species of this Basidiomycete genus have shown to be common endophytes in the stems and pods of agriculturally grown *Theobroma cacao*, the tree whose fermented and dried beans are used to produce chocolate (Crozier et al. (2006). *Coprinellus* has also shown promise as an agricultural agent in Chinese cabbage, effective for suppressing soil-borne pathogens, presenting new possibilities for biological control of vegetable diseases (Nakasaki et al. 2007). It is probable that *Coprinellus* exists in *Ulmus sp*. of Zoar Valley as an endophyte, but further studies would be needed to confirm.

Cryptococcus: Two unique samples of *Cryptococcus* were found in one geographic area, Skinny Dip Terrace. One species, *C. neoformans*, is a pathogenic yeast, and the most common fungal cause of meningitis in patients with AIDS (Litvinseva et al. 2007). This yeast opportunistically infects humans and other animals. *Cryptococcus* species have

been associated with soil, animal droppings, and other organic materials. Endophytic or pyllospheric colonization is unlikely. Presence of Cryptococcus in leaf samples is probably from an animal source.

Ampelomyces: Two unique sequences identified as *Ampelomyces* were detected in two samples from Skinny Dip Terrace. This genus includes many species of mycoparasitic fungi that cause powdery mildews. The typical *Ampelomyces* fungus infects and forms pycnidia inside of fungal hyphae of other fungi. Cells of this parasite therefore grow inside of the host, causing pathogenesis and death (Rotem et al. 1999). *Ampelomyces* species have been closely associated with apple shoots and aerial parts of 13 other flowering plant species. *Ampelomyces* has been shown to play an integral role in "bud bursting" of certain plants, such as apple trees in Holland (Szentivanyi and Kiss 2003). The presence of *Ampelomyces* in *Ulmus* samples likely indicates infection of a separate host fungus present in the leaf sample.

Colletotrichum*:* One unique sequence identified as *Colletotrichum* was isolated from Skinny Dip Terrace. Species of this genus have been observed to exist in the phyllosphere of giant dogwood trees in Japan (Osono et al. 2004) *Colletotrichum* species have caused increasing numbers of opportunistic human infections in recent years, usually in the immunocompromised HIV and transplant patients. The genus *Colletotrichum* is one of the most important genera of plant pathogens because of the diverse variety of economically important plants it colonizes (Cano et al. 2004). *Colletotrichum* species cause economically significant diseases of plants (generally

known as anthracnoses) that affect cereals and grasses, legumes, vegetables, and perennial crops, including fruit trees. *Colletotrichum* likely existed as a pathogen in the *Ulmus sp*. samples from Zoar Valley.

***Phlebia*:** *Phlebia* was found to exist in one sample from South Branch Floodplain. It is classified within class Basidiomycota, which is known for its abilities to degrade lignin. It has been found that proteosomal degradation upon nitrogen and carbon starvation is possibly involved in the regulation of ligninolytic activities in these wood decaying fungi (Staszczak 2007). These fungi are currently the subject of numerous microbial ecology studies for their lignin degrading abilities. *Phlebia* is not known for endophytic relationships and may be associated with soil contamination of specimens.

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Phaeosphaeria: One sequence identified as *Phaeosphaeria* was isolated from Skinny Dip Terrace. Many fungi placed taxonomically in the genus *Phaeosphaeria* were once found in the genus *Leptosphaeria*, and morphological distinctions of classification between these genera are often blurry. Some characteristics of *Phaeosphaeria* are production of ascospores with a distinguishing perispore, and the induction of Stagonospora leaf blotch diseases in cereals (Ueng et al. 2003). Phaeosphaeria exhibit pathogenesis of certain plants, and it is possible that it was existing parasitically with elm samples.

Gyoerffyella: One *Gyoerffyella* sequence was isolated from Skinny Dip Terrace. This genus of fungi is hypothesized to be a colonizer of the leaf phyllosphere. Species of this hyphomycete genus have been observed in rainwater collected after draining from forest

canopies in British Columbia (Gonczol and Revay 2006). Not much information is available on the species of this genus, although they are found extensively throughout North America and Europe. It is probable that *Gyoerffyella* colonized the phyllosphere of elm samples from Zoar Valley as an epiphyte, evidenced by its documented presence in rainwater collections .

Didymella: One *Didymella* sequence was detected in a sample from Skinny Dip Terrace. Species of this genus, such as *D. bryoniae*, are known plant pathogens. Plants affected by *Didymella* infection are wide ranging and include many economically important species, such as wheat, watermelon, pumpkin, cucumber and squash. One associated disease is Gummy Stem Blight. Appearance of spots on the leaves, petioles and stems are a typical sign of infection which usually become pale brown or gray in color. Gummy exudates may occur from cracks, especially in watermelon and pumpkin. Severe infection often results in death of the plant (Ferreira and Boley 1992). Didymella therefore may have existed as a pathogen in *Ulmus* species.

Skinny Dip Terrace showed the greatest variety of genera in our samples, with 7 of 9 total genera identified in this location. This resulted because sample 43, from Skinny Dip Terrace, was sequenced 12 times. 7 genera of fungi were detected in sample 43 alone. Most of the fungi implicated as pathogenic were taken from this sample.

Phoma and *Coprinellus*, the most likely of all genera detected to exist as endophytes, were relatively well dispersed in sampling areas, identified in 2 and 3 out of

five sample sites respectively. This finding supports the idea that these fungi are endophytes within elm species, since they were present in multiple samples and areas.

Conclusion:

This study represents a preliminary survey of phyllosphere fungi associated with *U. americana* and *U. rubra* in Zoar Valley. Genera detected were wide ranging and likely occupy various roles in their association with the trees they inhabit.

Future research should utilize a method of extraction to distinguish between endophytes and epiphytes, such as washing the leaf surface, then separately identifying fungi found in the wash from those isolated in leaf tissue. This will improve the understanding of how a fungus is associated with the plant, and enable the researcher to better hypothesize a plant-fungus relationship scenario.

Another improvement would be to increase the sample size. This would not only yield more genera of leaf-associated fungi for all locations, but would better characterize the phyllospheric ecosystems of *Ulmus sp.* within each location. This information could be vital in diagnosis and prevention of plant pathogens in the future.

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Appendix 1: Methods

DNA Extraction:

Johnston Lab CTAB DNA Extraction Protocol (Reference: Doyle and Doyle, 1987; and Cullings 1992)
Revised December 11th, 2006

1. Final preparation of CTAB buffer. Must use within 5-7 days. Add polyvinylpyrrolidone (PVP) and B-mercaptoethanol in 0.04 and 0.005 volumes respectively. Stir gently to dissolve.

CTAB Buffer	PVP	B-merc
0.5 ml	0.02g	2.5 ul
5 ml	0.2g	25 ul

2. Weigh out 40 mg of frozen plant tissue. Avoid using tissue samples that are discolored or show obvious freeze-thaw damage.
3. Grind tissue with glass pestle in liquid nitrogen cooled mortar
    a. Pestles must be acid-washed and rinsed well with DI water before reuse.
    b. Nitrogen gas will condense into liquid in the mortar. Allow sample to dry of liquid nitrogen, before adding CTAB, by removing the mortar from its base and placing on lab bench for several minutes.
4. Place freshly ground tissue in sterile microfuge tubes and add 500 ul of CTAB buffer.
5. Invert tube 5-10 times and incubate samples at 55 degrees C overnight.
6. Add 500 ul of 24:1 Chloroform: Iso Amyl Alcohol and mix well by shaking tubes.
7. Centrifuge 10 minutes at 13,000 RPM.
    a. Following centrifugation should have 3 layers in tube: top: aqueous phase, middle: protein debri, and bottom: chloroform.
    b. Aqueous phase contains DNA, pipette off quickly into a fresh microfuge tube.
8. Estimate the volume of the collected aqueous phase.
9. Add 0.1 volumes of cold 7.5 M ammonium acetate and 0.6 volumes of cold isopropanol (using combined volumes of aqueous layer + ammonium acetate). Ammonium acetate and isopropanol should be kept in the -20 freezer just prior to use.
10. Mix well. Place in -20 degrees C freezer for one hour to overnight.
11. Centrifuge for 3 minutes at 13,000 RPM
12. DNA pellet should be visible. Pour or pipette off supernatant, careful not to lose pellet. Pellet may vary in color from light brown to creamy white.
13. Add 700 ul of cold 70% ethanol and mix. Centrifuge at 13,000 RPM for one minute.
14. Pour off liquid. Add 700 ul of cold 95% ethanol and mix. Centrifuge one minute.
15. Carefully pour of liquid, being sure not to lose pellet.
16. Dry pellet by inverting on Kim-wipe for an hour or until dry.
17. Re-suspend sample in 100 ul of TE Buffer. Place in freezer -20 degrees C to store.

Stock Solutions:

CTAB Buffer: for 1 liter
100 ml of 1 M Tris, pH 8.0
280 ml of 5 M NaCl
40 ml of 0.5 M EDTA
20 g of CTAB (Cetyltrimethyl ammonium bromide)

1 M Tris, pH 8.0: 1 liter
121.1 g Tris
700 ml mqH2O
Dissolve Tris, bring volume to 900 ml
pH to 8.0 with concentrated HCl (~50 ml)
Bring to 1 liter

0.5 M EDTA, pH 8.0: 1 liter
186.12 g of EDTA
750 ml mqH2O
Add approximately 20 g of NaOH pellets until EDTA dissolves (~pH 8.0)

5 M NaCl: 1 liter
292.2 g of NaCl
700 ml of mqH2O
Dissolve and bring volume to 1 L

TE Buffer: 1 liter

10 mM	10 ml of 1 M Tris, pH 8.0
1 mM	2 ml of 0.5 M EDTA

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Generating PCR Fragments

Johnston Lab – PCR protocol for nrDNA Internal Transcribed Spacer (ITS) sequence amplification using primers ITS1 and ITS4 of White et al, 1990.

Plant DNA extracted and stored -20 degrees C in TE buffer until PCR set-up

Following Ingredient list is for a 50ul PCR reaction

Ingredient	**Conc. Used**	**Volume Used**
Sterile water	-	34.25ul
Immobuffer	10X	5ul
MgCl2	50mM	2.5ul
ITS1	25X	2ul
ITS4	25x	2ul
dNTPs	40mM	1.25ul
Immolase (Taq)	5 Units/ul	0.5ul
DNA	1/100 dil.	2.5ul
TOTAL		**50ul**

PCR cycle "IT" program:

1. 95 C for 7 min.
2. 94 C for 20s
3. 54.9 C for 30s
4. 72 C for 30s
5. Repeat 2-4 40 times
6. 72 C for 5 min.

Gels run on Agarose, 1.5% in TAE buffer

Cloning of fresh PCR product:

TOPO TA Cloning Kit for Sequencing (Invitrogen cat. No. K4530-20)
Recombinant colonies are analyzed with EcoRI restriction digest and selected for sequencing. Requires fresh PCR product with A-overhangs for the Topoisomerase-1 enzyme to efficiently ligate the PCR product. Non-recombinant colonies are selected through life cycle termination protein integrated into the vector, only activated during ligation of the vector without an insert.

StrataClone PCR cloning kit from Stratagene (Stratagene cat. No. 240206)

Uses the Topoisomerase-1 enzyme therefore requires fresh PCR product with A-overhangs. Cre recombinase gene in activated in vectors that ligate without the insert, therefore clones must be plated on X-gal/ampicillin plates (50ug/ml), and blue/white selection for recombinant clones.

Plasmid Isolation:

Plasmids are isolated from the cloned cells using the alkaline plasmid screen. Plasmid DNA is isolated from an overnight culture of Luria Broth plus 50ug/ml of Ampicillin. The plasmid DNA was eluted into sterile water instead of TE buffer in preparation for sequencing.

1. Inoculate using a flame sterilized needle, 5ml of LB broth with antibiotics in a sterile culture tube with a single recombinant colony. For quick screening, grow cells in 1.5 ml eppendorf tube. (plasmid must be high copy #)
2. Grow cells in 37˚C incubator overnight with shaking (200rpm)
3. For culture tubes, spin down at 3,000 rpm for 5 minutes, for eppendorf tubes 1 minute. Pour off the supernatant.
4. Resuspend cells in 150ul of P1 (15 mM Tris pH8, 10mM EDTA) + 10ug/ml RNAse A.
5. Add 150ul of P2 (0.2N NaOH 1%SDS). Mix genly by inverting 3 times.
6. Add 150ul of P3 (3M KOAc pH 5.5). Mix by gently inverting tube 5-10 times.
7. Remove white precipitate by centrifugation at 12,000 rpm for 10 minutes.
8. Carefully transfer the supernatant to a fresh 1.5ml eppendorf tube.
9. Add an equal volume of cold phenol:chloroform:isoamyl alcohol (25:24:1). Mix well, and centrifuge for 10 minutes.
10. Remove the aqueous phase (top) to a new 1.5ml eppendorf tube. This phase contains plasmid DNA.
11. Add two volumes of 95% ethanol. Incubate for 30 minutes at -20˚C.
12. Spin in the microcentrifuge for 10 minutes at 12,000 rpm. Decant the supernatant and wash the pellet once with cold 70% ethanol.
13. Air dry the pellet by inverting the tube over a Kimwipe. Resuspend in 30ul of TE buffer and store at -20˚C. The plasmid DNA is now ready for downstream applications like EcoR1 digestion and sequencing.

EcoR1 digestion of cloned DNA

Per single reaction:

Water	6.8ul
EcoR1 buffer	1ul
EcoR1	0.2ul
Miniprep	2ul

Combine in tube, being sure to keep enzyme at -20˚C while out of freezer, and add the enzyme to the tube last. Next incubate for two hours at 37˚C.

*For double digests (2 enzymes at once) use 0.2ul of each enzyme and 6.6ul of sterile water.

Sequencing:

Beckman Coulter sequencing protocol were followed in use of GenomeLab Dye Terminator Cycle sequencing with Quick Start Kit. Following the generation of nested DNA fragments, precipitation, and pellet formation, the pellets were left dry overnight until sequencing on the next morning. This was to avoid freeze-thaw complications regarding the sample loading solution (SLS).

Appendix 2: Sequence Data Comparison with Nucleotide Analysis Via Genbank

The following sequences are those of the Zoar Valley *J. nigra* samples specified in the title, underscored by their closest match, as determined through utilization the NCBI BLAST tool (see Methods). Sample #1 Forward has been labeled **{ }** with the intention of using it as a key to interpreting the BLAST data.

SAMPLE #1 Forward {SAMPLE}

> gi|18028823|gb|AF338492.1|AF338492 **{ACCESSION #}** Juglans nigra isolate 836 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence **{PUBLISHED SEQUENCE IDENTITY}**
Length=750

Score = 1026 bits (555), Expect = 0.0
Identities = 604/631 (95%), Gaps = 11/631 (1%)
Strand=Plus/Minus

***{for the purpose of this paper, the only identity value was utilized.Query represents the research derived sample DNA, while Sbjct (Subject) represents the Genbank published DNA sequence. Dots in the Sbjct represent identical nucleotide match with the Query}**

```
Query  82
CCGCTTATTGATATGCTTAAATTCAGCGGGTAATCCCGCCTGACCTGGGGTCGCGATGGT  141
Sbjct  749
............................................................  690

Query  142
AGAGTCGCAAGAACGACACAATAGGGTCGAGGAGCACCTTCACAGCGACGGGCGACACAC  201
Sbjct  689
............................................................  630

Query  202
GACGGGTCACGAGGGTTTCTCAACCACCGATTGTCGTGGCGCTCGTCGCCTAGGACTCAC  261
Sbjct  629
............................................................  570

Query  262
TTTTAGGCTAACCGCGAGCAAAAGCGCACGGGAGGCCAATGTCTTCCCCGCACCCCGCAC  321
Sbjct  569
............................................................  510

Query  322
AGCGTAAGAAGTGTTTGGGGTTGGGGCAACGATGCGTGACACCCAGGCAGACGTGCCCTC  381
Sbjct  509
............................................................  450
```

```
Query  382
GGCCGAATGGCTTCGGGCGCAACTTGCGTTCAAAGACTCGATGATTCGCGGGATTCTGCA  441
Sbjct  449
.............................................................  390

Query  442
ATTCACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGATATCCG  501
Sbjct  389
.............................................................  330

Query  502
TTGCCGAGAGTCGTTATGTATCATGGTAAAGACGTCACCAACGACACGCACACCGTTTTC  561
Sbjct  329  ............................................................-
.  271

Query  562
CGGGGGCGCCCGTGGTTACTCCTTGTTTTAAGTNCCCTTGGCGCAGACCGCNNCNGNGGT  621
Sbjct  270
.....-.....................-....T..-.............GC.G.-...  215

Query  622
NCATTNGTNCGATCGGGGANNGGANCGAGANGATCGACCCAACCCCACACCGAAGANCAG  681
Sbjct  214
T....-..T.........A.G-...A.....G.........-....A.....-..G..G...  159

Query  682
TNGGCCAATCTCAACGNTGCCCCTCCCCACC  712
Sbjct  158
.G...A.....-.....-....A...  130
```

SAMPLE #1 Reverse

> gi|18028823|gb|AF338492.1|AF338492 Juglans nigra isolate 836 18S ribosomal RNA gene, partial sequence;
internal transcribed spacer 1, 5.8S ribosomal RNA gene
and internal transcribed spacer 2, complete sequence; and
26S ribosomal RNA gene, partial sequence
Length=750

```
 Score =  780 bits (422),  Expect = 0.0
 Identities = 448/461 (97%), Gaps = 8/461 (1%)
 Strand=Plus/Plus

Query  76
TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATACCTGCCCAGCATNTAACGACCT  135
Sbjct  16   ..............................................G--
........  73

Query  136
GTGAACATGTAATAATAACCTTCTGGGTGGGGGTGTAATGCCCCCTCCCAGAAAACGGTT  195
Sbjct  74
.............................................................  133

Query  196
GGGAGGGCACGTTGAGATTTGCCCACTGCTCCTCGTGTGTGGTTGGTCGATCCTCTCGTT  255
```

```
Sbjct  134
.....................................................  193

Query  256
CCCTTCCCGATCGAACAATGAACCCCGGCGCGGTCTGCGCCAAGGAACTTAAACAAGGAG  315
Sbjct  194
.....................................................  253

Query  316
TAACCACGGGCGCCCCGGAAACGGTGTGCGTGTCGTTGGTGACGTCTTTACCATGATACA  375
Sbjct  254
.....................................................  313

Query  376
TAACGACTCTCGGCAACGGATATCTNNNNGCTCTCGCATCGATGAAGAACGTAGCGAAAT  435
Sbjct  314
.......................CG--.............................  371

Query  436
GCGATACTTGGTGTGAATTGCAGAATCCCGCGAATCATCGAGTCTTTGAACGCAAGTTGC  495
Sbjct  372
.....................................................  431

Query  496  GCCCGAAGCCATTNCGGCCGANNGNCACGTNCTGNCCTGGG  536
Sbjct  432  ............-.......GG.-.....-...-......  468
```

SAMPLE #5 Foreward

```
>  gi|18028818|gb|AF338487.1|AF338487  Juglans microcarpa isolate 108
18S ribosomal RNA gene, partial
sequence; internal transcribed spacer 1, 5.8S ribosomal RNA
gene and internal transcribed spacer 2, complete sequence;
and 26S ribosomal RNA gene, partial sequence
Length=735

 Score = 1094 bits (592),  Expect = 0.0
 Identities = 690/749 (92%), Gaps = 21/749 (2%)
 Strand=Plus/Minus

Query  90
CCGCTTATTGATATGCTTAAATTCAGCGGGTAATCCCGCCTGACCTGGGGTCGCGATGGT  149
Sbjct  734
.....................................................  675

Query  150
AGAGTCGCAAGAACGACACAATAGGGTCGAGGAGCACCTTCACAGCGACGGGCGACACAC  209
Sbjct  674
.....................................................  615

Query  210
GACGGGTCACGAGGGTTTCTCAACCACCGATTGTCGTGGCGCTCGTCGCCTAGGACTCAC  269
Sbjct  614
.....................................................  555
```

```
Query  270
TTTTAGGCTAACCGCGAGCACAAGCGCACGGGAGGCCAATGTCTTCCCCGCACCCCGCAC  329
Sbjct  554
.....................M.....................................  495

Query  330
AGCGTAAGAAGTGTTTGGGGTTGGGGCAACGATGCGTGACACCCAGGCAGACGTGCCCTC  389
Sbjct  494
.............................................................  435

Query  390
GGCCGAATGGCTTCGGGCGCAACTTGCGTTCAAAGACTCGATGATTCGCGGGATTCTGCA  449
Sbjct  434
.............................................................  375

Query  450
ATTCNACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGATATCC  509
Sbjct  374  ....-
.....................................................  316

Query  510
GTTGCCGAGAGTCGTTATGTATCATGGTAAAGACGTCACCAACGACACGCACACCGTTTC  569
Sbjct  315
.............................................................  256

Query  570
CNGGGCGCCCGTGGTTACTCCTNGTNTAAGTNCCTTGGCGCAGACCGCGCNNNNNTTCAT  629
Sbjct  255
.G...................T..T.....T.................CGGGG.....  196

Query  630
TGTTCGATCNGGANGGGAACGAGAGGATCGACCA-CCACACACNANGN-CA-TGGGNCAA  686
Sbjct  195
.........G...A.....................A........G.G.AG..G....-...  137

Query  687
NNTTTCAACGTGCCTTCCNAAC-GTTTTTTGGGNANGGGNCNT-AC-CCCNNCNNCCNAN  743
Sbjct  136
A-.C..........C...C...C..........-.G...G.A.T..A...C-.AC..-.G  81

Query  744
AAAGGTTATNATAACATGTTCCCANGGTCGGTCTGCCGGGGCAANGTNTNNACCAATGAN  803
Sbjct  80   ..-......T..T.........A..-.....T.....T...-..G-..A.CG..-
.....-  27

Query  804  TCCTTCCCNCANGNTTCNCCCTACGGAAA  832
Sbjct  26   .......G-..G.-...A..-........  1
```

SAMPLE #5 Reverse

> gi|18028823|gb|AF338492.1|AF338492 Juglans nigra isolate 836 18S ribosomal RNA gene, partial sequence;
internal transcribed spacer 1, 5.8S ribosomal RNA gene
and internal transcribed spacer 2, complete sequence; and
26S ribosomal RNA gene, partial sequence

```
Length=750

 Score =  507 bits (274),  Expect = 3e-140
 Identities = 323/349 (92%), Gaps = 7/349 (2%)
 Strand=Plus/Plus

Query  145
CGACCTGTGAACATGTANTNATACCCTTCCTGGGTGGGGGTGTACCTGCCCCCTCCCAGA  204
Sbjct  68
..................A.A...A.....-.............A-.............  125

Query  205
AAACGGTTGGGAGGGCACGTTGAGATTTGCCCCCTGCCCCTCGTGTGTGGTCGGTCGATC  264
Sbjct  126
.................................A....T............T........  185

Query  265
CTCTCGTTCCCTTCCCGATCGAACNATGANCCCCGGCGCGGTCTGCGCCAAGGAACTTAC  324
Sbjct  186
.........................A....A.............................A  245

Query  325
CCNGGGAGTNACCTCGGGCGCCCCGGAAACGGTGTGCGTGTCGTTGGTGACGTCTTTACC  384
Sbjct  246
A.AA.....A...A.............................................  305

Query  385
ATGATACATACCGACTCTCGGCANCGGATATTTCGGCTCTCGCATCGATGAAGAACGTAG  444
Sbjct  306
..........A............A.......C............................  365

Query  445  CGAAATGCGATACCTTGGTGTGAATCGCAAGAATCCCCGCGNAATTCAT  493
Sbjct  366  ............-...........T...-.......-...-...-...  409
```

SAMPLE #11 Forward

> gi|18028815|gb|AF338484.1|AF338484 Juglans major isolate 870 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence

```
Length=747

 Score =  870 bits (471),  Expect = 0.0
 Identities = 480/484 (99%), Gaps = 1/484 (0%)
 Strand=Plus/Plus

Query  45
CCGTAGGTGAACCTGCGGAAGGATCATTGTCGATACCTGCCCAGCAGAACGACCTGTGAA  104
Sbjct  19
..........................................................  78

Query  105
CATGTAATAACCTTCTGGGTGGGGGTGTAATGCCCCCTCCCAAAAAACGGTTGGGAGGGC  164
```

```
Sbjct  79
.....................................................  138

Query  165
ACGTTGAGATTTGCCCACTGCTCCTCGTGTGTGGTTGGTCGATCCTCTCGTTCCCTTCCC  224
Sbjct  139
.....................................A.................  198

Query  225
GATCGAACAATGAACCCCGGCGCGGTCTGCGCCAAGGAACTTAAACAAGGAGTAACCACG  284
Sbjct  199
.....................................................  258

Query  285
GGCGCCCCGGAAACGGTGTGCGTGTCGTTGGTGACGTCTTTACCATGATACATAACGACT  344
Sbjct  259
.....................................................  318

Query  345
CTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTG  404
Sbjct  319
.....................................................  378

Query  405
GTGTGAATTGCAGAATCCCGCGAATCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCC  464
Sbjct  379
.....................................................  438

Query  465
ATTCCGGCCGAGGGCACGTCTGCCTGGGTGTCACGCATCGTCGCCCCAACCCCCAACACT  524
Sbjct  439  ....-
.....................................T...........A......  497

Query  525  TCTT  528
Sbjct  498  ....  501
```

SAMPLE #11 Reverse

gi|18028823|gb|AF338492.1|AF338492 Juglans nigra isolate 836 18S ribosomal RNA gene, partial sequence;
internal transcribed spacer 1, 5.8S ribosomal RNA gene
and internal transcribed spacer 2, complete sequence; and
26S ribosomal RNA gene, partial sequence
Length=750

```
 Score =  968 bits (524),  Expect = 0.0
 Identities = 537/545 (98%), Gaps = 3/545 (0%)
 Strand=Plus/Minus

Query  72
TTATTGATATGCTTAAATTCAGCGGGTAATCCCGCCTGACCTGGGGTCGCGATGGTAGAG  131
Sbjct  745
.....................................................  686
```

```
Query  132
TCGCAAGAACGACACAATAGGGTCGAGGAGCACCTTCACAGCGACGGGCGACACACGACG  191
Sbjct  685
............................................................  626

Query  192
GGTCACGAGGGTTTCTCAACCACCGATTGTCGTGGCGCTCGTCGCCTAGGACTCACTTTT  251
Sbjct  625
............................................................  566

Query  252
AGGCTAACCGCGAGCAAAAGCGCACGGGAGGCCAATGTCTTCCCCGCACCCCGCACAGCG  311
Sbjct  565
............................................................  506

Query  312
TAAGAAGTGTTTGGGGTTGGGGCAACGATGCGTGACACCCAGGCAGACGTGCCCTCGGCC  371
Sbjct  505
............................................................  446

Query  372
GAATGGCTTCGGGCGCAACTTGCGTTCAAAGACTCGATGATTCGCGGGATTCTGCAATTC  431
Sbjct  445
............................................................  386

Query  432
ACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGATATCCGTTGC  491
Sbjct  385
............................................................  326

Query  492
CGAGAGTCGTTATGTATCATGGTAAAGACGTCACCAACGACACGCACACCGTTTCCGGGG  551
Sbjct  325
............................................................  266

Query  552
CGCCCGTGGTTACTCCTNGTTTAAGTTNCTNGGCGCAGACCGCGCCGNG-TN-ATTGT-C  608
Sbjct  265
.................T.........C..T................G.G.TC.....T.  206

Query  609  GATCG  613
Sbjct  205  .....  201
```

SAMPLE #11 Reverse II

> gi|18028823|gb|AF338492.1|AF338492 Juglans nigra isolate 836 18S ribosomal RNA gene, partial sequence;
internal transcribed spacer 1, 5.8S ribosomal RNA gene
and internal transcribed spacer 2, complete sequence; and
26S ribosomal RNA gene, partial sequence
Length=750

```
 Score =  926 bits (501),  Expect = 0.0
 Identities = 537/559 (96%), Gaps = 7/559 (1%)
 Strand=Plus/Minus
```

```
Query  70
GCTTATTGATATGCTTAAATTCAGCGGGTAATCCCGCCTGACCTGGGGTCGCGATGGTAG  129
Sbjct  747
.............................................................  688

Query  130
AGTCGCAAGAACGACACAATAGGGTCGAGGAGCACCTTCACAGCGACGGGCGACACACGA  189
Sbjct  687
.............................................................  628

Query  190
CGGGNTCACGAGGGTTTCTCAACCACCGATTGTCGTGGCGCTCGTCGCCTAGGACTCACT  249
Sbjct  627
....-........................................................  569

Query  250
TTTAGGCTAACCGCGAGCAAAAGCGCACGGGAGGCCAATGTCTTCCCCGCACCCCNNACA  309
Sbjct  568
.............................................................GC...  509

Query  310
GCGTAAGAAGTGTTTGGGGTTGGGGCAACGATGCGTGACACCCAGGCAGACGTGCCCTCG  369
Sbjct  508
.............................................................  449

Query  370
GCCGAATGGCTTCGGGCGCAACTTGCGTTCAAAGACTCTNATGATTCGCGGGATTCTGCA  429
Sbjct  448
.....................................-G.....................  390

Query  430
ATTCACACCAAGTATCGCATTTTCGCCTACGTTCTTCATCGATGCNAGAGCCGAGATATC  489
Sbjct  389
...................-.....-..................G..............  332

Query  490
CGTTGCCGAGAGTCGTTTATGTATCATGGTAAAGACGTTACCAACGACACGCACACCGTT  549
Sbjct  331
..............-......................C.....................  273

Query  550
TCCGGGNNGCCCGTGGTTACTCCTTGTTTAAGTTCCTTNNCGCANACC-CGCCCNNGGTT  608
Sbjct  272
......GC.............................GG....G...G....GG-....  214

Query  609  CATTGTNCCATCGGNAAGG  627
Sbjct  213  ......T.G.....G....  195
```

SAMPLE #3 Forward

> gi|18028815|gb|AF338484.1|AF338484 Juglans major isolate 870 18S ribosomal RNA gene, partial sequence;
internal transcribed spacer 1, 5.8S ribosomal RNA gene
and internal transcribed spacer 2, complete sequence; and

```
26S ribosomal RNA gene, partial sequence
Length=747

 Score =  850 bits (460),  Expect = 0.0
 Identities = 479/491 (97%), Gaps = 1/491 (0%)
 Strand=Plus/Plus

Query  2
AACCNGCGGAAGGATCATTGTCGATACCTGCCCAGCAGAACGACCTGTGAACATGTAATA  61
Sbjct  28
....T.....................................................  87

Query  62
ACCTTCTGGGTGGGGGTGTAATGCCCCCTCCCAAAAAACGGTTGGGAGGGCACGTTGAGA  121
Sbjct  88
.............................................................  147

Query  122
TTTGCCCACTGCTCCTCGTGTGTGGTTGGTCGATCCTCTCGTTCCCTTCCCGATCGAACA  181
Sbjct  148
.............................A...........................  207

Query  182
ACGAACCCCGGCGCGGTCTGCGCCAAGGAACTTAAACAAGGAGTAACCACGGGCGCCCCG  241
Sbjct  208
.T...........................................................  267

Query  242
GAAACGGTGTGCGTGTCGTTGGTGACGTCTTTACCAAGATACATAACGACTCTCGGCAAC  301
Sbjct  268
.....................................T.......................  327

Query  302
GGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATT  361
Sbjct  328
.............................................................  387

Query  362
GCAGAATCCCGCGAATCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCCG  421
Sbjct  388
.............................................................  447

Query  422
AGGGCACGCCTGCCTGGGTGTCACGCATCGTTGCCCCAANNCCAAACACTTCTTACGCNG  481
Sbjct  448
........T.............................CC..............T..T.  507

Query  482  TNCNCGGGTGC  492
Sbjct  508  .G.G-......  517
```

SAMPLE #3 Reverse

> gi|18028823|gb|AF338492.1|AF338492 Juglans nigra isolate 836 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene

and internal transcribed spacer 2, complete sequence; and
26S ribosomal RNA gene, partial sequence
Length=750

 Score = 824 bits (446), Expect = 0.0
 Identities = 481/498 (96%), Gaps = 9/498 (1%)
 Strand=Plus/Minus

```
Query  31
CCGCTTATTGATATGCTTAAATTCAGCGGGTAATCCCGCCTGACCTGGGGTCGCGATGGT  90
Sbjct  749
............................................................  690

Query  91
AGAGTCGCAAGAACGACACAATAGGGTCGAGGAGCACCTTCACAGCGACGGGCGACACAC  150
Sbjct  689
............................................................  630

Query  151
GACGGGTCACGAGGGTTTCTCAACCACCGATTGTCGTGGCGCTCGTCGCCTAGGACTCAC  210
Sbjct  629
............................................................  570

Query  211
TTTTAGGCTAACCGCGAGCAAAAGCGCACGGGAGGCCAATGTCTTCCCCGCACCCCGCAC  270
Sbjct  569
............................................................  510

Query  271
AGCGTAAGAAGTGTTTGGGGTTGGGGCAACGATGCGTGACACCCAGGCAGGCGTGCCCTC  330
Sbjct  509
...................................................A........  450

Query  331
GGCCGAATGGCTTCGGGCGCAACCTTGCGTTCAAAGACTCGATGATTCGCGGGATTCTGC  390
Sbjct  449
....................-.....................................  391

Query  391
AANNCNACCACCAAGTATCGCATTTTCGCTACGTTCTTNATCGATGCGAGAGCCGAGATA  450
Sbjct  390
..TT.-.-.............-............C.....................  334

Query  451
TCCGTTGCCGAGAGTCGTTATGTATCTTGGTAAAGACGTCCCCAANCGACACCGCCCACC  510
Sbjct  333
.........................A.............A....-.......-..A....  276

Query  511  CGTTTCNCNNGGGCGCCC  528
Sbjct  275  -......-.G-........  261
```

SAMPLE #6 Forward

> gi|17065874|emb|AJ251683.1|BAL251683 Betula alba 18S rRNA gene, 5.8S rRNA gene, 25S rRNA gene, internal transcribed spacer 1 (ITS1) and internal transcribed spacer

```
2 (ITS2)
Length=686

 Score =  756 bits (409),  Expect = 0.0
 Identities = 451/471 (95%), Gaps = 9/471 (1%)
 Strand=Plus/Minus

Query  79
GCTTAAATTCAGCGGGTAGTCCAGCCTGACCTGGGGTCGCGTTGGAAGCGTCGCTGGCGC  138
Sbjct  686
.....................C..............................A....  627

Query  139
GACACAGCAGGGTCAAAGAGCACACGATGAGCGACGCGGCACGCACGACGGGACACGAGG  198
Sbjct  626
................G.....................................  567

Query  199
GTTTGTCAACCACCGATTGTCGTGGCGCGCGTCGCCGAGGACTCGCTTTTGGGCCAACCG  258
Sbjct  566
.....A.........................................A.........  507

Query  259
CATGCATGAGCTCACGGGAGGCCAATTTCTGCCCCACAGGCCCCCTCGTCCCTTTGCAAG  318
Sbjct  506
.............................................................  447

Query  319
GAGATGGGGTTGGGGGCAACGATGCGTGACACCCAGGCAGACGTGCCCTCGGCCAGGTGG  378
Sbjct  446
.............................................................  387

Query  379
CTNCGGGCGCAACTTGCGTTCAAAGACTNCGATGATTCGCGGGATTCTGCAATTCACACC  438
Sbjct  386
..T.........................-..............................  328

Query  439
AAGTATCCGCATTTNCGCTACGTTNCTTCATCGATGCGAGAGCCGAGATATCCGTTTGGC  498
Sbjct  327
......-.......-.........-.............................-..-..  273

Query  499
CGAGAGNNGTGGTGGGTTCTAGACAAGATTCCGCCTCCCGCACGGCACACC  549
Sbjct  272
......TC..TA.-.................-.A.........-.......  225
```

15 Forward

> gb|AF338492.1|AF338492 Juglans nigra isolate 836 18S ribosomal RNA gene, partial sequence;
internal transcribed spacer 1, 5.8S ribosomal RNA gene
and internal transcribed spacer 2, complete sequence; and

```
26S ribosomal RNA gene, partial sequence
Length=750

 Score =  617 bits (334),  Expect = 1e-173
 Identities = 510/581 (87%), Gaps = 67/581 (11%)
 Strand=Plus/Minus

Query  59   CCGCTTATTGATATGCTTAAATTCAGCGGGTAATCCCGCCTGACCTGGGGTCGCGATGGT  118
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  749  CCGCTTATTGATATGCTTAAATTCAGCGGGTAATCCCGCCTGACCTGGGGTCGCGATGGT  690

Query  119  AGAGTCGCAAGAACGACACAATAGGGTCGAGGAGACAACCTTCAACAGACGAACGGGACG  178
            |||||||||||||||||||||||||||||||| || |||||| ||| ||| |||| ||
Sbjct  689  AGAGTCGCAAGAACGACACAATAGGGTCGAGGAG-CA-CCTTCA-CAG-CGA-CGGG-CG  636

Query  179  ACAAACACGACGGGTCAACGAGGGTTTCTCAAACCACCGATTGTCGTGGCGACTCGTCGC  238
            |||  |||||||||||| |||||||||||||| |||||||||||||||||| ||||||||
Sbjct  635  ACA--CACGACGGGTCA-CGAGGGTTTCTCAA-CCACCGATTGTCGTGGCG-CTCGTCGC  581

Query  239  CTAGGAACTCACTTTTAGGACTAAACCGCGAGACAAAAAGACGACAACGGGGAAGGGCCA  298
            |||||| |||||||||||| |||| ||||||| ||||| | || || |||| | || |||
Sbjct  580  CTAGGA-CTCACTTTTAGG-CTAA-CCGCGAG-CAAAA-G-CG-CA-CGGG-A-GG-CCA  532

Query  299  ATTGGTTCTTTCCCCGCAACCCCCGCAACAAGACGGTAAAGAAGTGTTTTgggggggttg  358
            || | | ||| ||||||| |||| ||| || | || ||| ||||||||| ||||   |||
Sbjct  531  AT-G-T-CTT-CCCCGCA-CCCC-GCA-CA-G-CG-TAA-GAAGTGTTT-GGGG---TTG  487

Query  359  gggggCAAACGAATGCCGTGACACCCAGGCAGAACGTGCCCTCGGCCGAAATGGCTTCGG  418
            |||  ||| ||| ||| ||||||||||||||| ||||||||||||||| |||||||||
Sbjct  486  GGG--CAA-CGA-TGC-GTGACACCCAGGCAGA-CGTGCCCTCGGCCGAA-TGGCTTCGG  434

Query  419  GCGCAACTTGACGTTCAAAGANCTCGAATGAATTCGTCGGGAT-CTGCAATTCACACCCA  477
            |||||||||| |||||||||| ||||| ||| |||| |||||| |||||||||||||| |
Sbjct  433  GCGCAACTTG-CGTTCAAAGA-CTCGA-TGA-TTCG-CGGGATTCTGCAATTCACACC-A  380

Query  478  AGTATTCGCATTTCGCTACGTTCTTCATTCGATGCGAGAGC-GAAGATATTCCCGTTGCC  536
            ||||| |||||||||||||||||||| |||||||||||| || ||||| || ||||||
Sbjct  379  AGTAT-CGCATTTCGCTACGTTCTTCAT-CGATGCGAGAGCCGA-GATAT-CC-GTTGCC  325

Query  537  GAGAGTCGTTAATGCATCATGGTAAAGAACGTCACCCAACGAACACGCCACCACCGTTTC  596
            |||||||||||| || |||||||||||| ||||||| ||||| ||||| || ||||||||
Sbjct  324  GAGAGTCGTTA-TGTATCATGGTAAAGA-CGTCACC-AACGA-CACGC-AC-ACCGTTTC  271

Query  597  CCGGGCGCC-GTGGGTAACTCCTTGGTTAAGT-CCT-GGCG  634
            | ||||||| |||| || ||||||| |||||| ||| ||||
Sbjct  270  CGGGGCGCCCGTGGTTA-CTCCTTGTTTAAGTTCCTTGGCG  231
```

15 Reverse

```
>  gb|AF338492.1|AF338492  Juglans nigra isolate 836 18S ribosomal RNA gene,
partial sequence;
internal transcribed spacer 1, 5.8S ribosomal RNA gene
and internal transcribed spacer 2, complete sequence; and
26S ribosomal RNA gene, partial sequence
Length=750

 Score =  558 bits (302),  Expect = 6e-156
 Identities = 371/399 (92%), Gaps = 25/399 (6%)
 Strand=Plus/Plus

Query  51   TTTCCGTAGGTGAACCTGCGGAAGGATC-TTGTCGATACCTGCCCAGACAGAACGACCTG  109
```

```
            |||||||||||||||||||||||||| |||||||||||||||| ||||||||||||
Sbjct  16   TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATACCTGCCCAG-CAGAACGACCTG  74

Query  110  TGAAC-TGTAATAATAAACCTTCTGGGTGGGGGTGTAATGCCCCCTCCCAGAAAACGGTT  168
            |||||  ||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  75   TGAACATGTAATAAT-AACCTTCTGGGTGGGGGTGTAATGCCCCCTCCCAGAAAACGGTT  133

Query  169  GGGAGAGCACGTTGAGATTTGCCCACTGCTCCTCGTGTGTGGTTGGTCGATCCTCTCGTT  228
            |||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  134  GGGAGGGCACGTTGAGATTTGCCCACTGCTCCTCGTGTGTGGTTGGTCGATCCTCTCGTT  193

Query  229  CCCTCCCCGATCGAAACA-TGAAACCCCGGCGACGGTCTGCGACCAAGGAACCTTAAAAC  287
            ||||  |||||||||| || ||||  |||||||| ||||||||| ||||||||| ||||| |
Sbjct  194  CCCTTCCCGATCGAA-CAATGAA-CCCCGGCG-CGGTCTGCG-CCAAGGAAC-TTAAA-C  247

Query  288  AAGGAGTAAACCAACGGGACGACCCCGGAAAACGGTGTGCGTGTCGTTGGTGAACGTCTT  347
            |||||||||  |||  ||||  ||  |||||||||  ||||||||||||||||||||| ||||||
Sbjct  248  AAGGAGTAA-CCA-CGGG-CG-CCCCGGAAA-CGGTGTGCGTGTCGTTGGTGA-CGTCTT  301

Query  348  TACCATGATGCATAAACGAACTCTCGGGCAACGGATATCTCGGCTCTCGCATCGATGAAG  407
            |||||||||  |||||  |||  |||||||  ||||||||||||||||||||||||||||||
Sbjct  302  TACCATGATACATAA-CGA-CTCTCGG-CAACGGATATCTCGGCTCTCGCATCGATGAAG  358

Query  408  AAACCGTAGCGAAATGCGATAACCTTGGTGTGAAT-GCA  445
            || | ||||||||||||||| | |||||||||| |||
Sbjct  359  AA-C-GTAGCGAAATGCGATA-C-TTGGTGTGAATTGCA  393
```

Appendix 3: Consensus sequence multiple alignment with *J. nigra* sequence acquired from Genbank

```
11              ---------------------------------------GGCAATTG--AATATGCGGCC 19
j               AAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATACCTGCCC 60
3               ----------------------GTGAACCTGCGGAAGGATCATTGTCGATACCTGCCC 36
1               ------------------------------------------------------------
5               ------------------------------------------------------------


11              -GCAATTCGCCTT-----CCCGTAG--GTGAACCTGCGGAAGGATCATTGTCGATACCTG 71
j               AGCAGAACGACCTGTGAACATGTAATAATAACCTTCTGGGTGGGGGTGTAATGCCCCCTC 120
3               AGCAGAACGACCTGTGAACATGTAA---TAACCTTCTGGGTGGGGGTGTAATGCCCCCTC 93
1               ------------------------------------------------------------
5               -----------------AGACCGACCCTGGTGAACATGTAATAATACCTCTTCCTGGTG 42


11              CCCAGCAACGGTTGGGAGGGCACGTTGAGATTTGCCCACTGCTCCTCGTGTGTGGGTTGG 131
j               CCAGAAAACGGTTGGGAGGGCACGTTGAGATTTGCCCACTGCTCCTCGTGTGTGG-TTGG 179
3               CCAAAAAACGGTTGGGAGGGCACGTTGAGATTTGCCCACTGCTCCTCGTGTGTGG-TTGG 152
1               ------------------------------------------------------------
5               TGGGGGGTGTACCAGGCCCCCTTCCCAAGGCACGTTGAGATTTGCCCCCTGCCCCTCGTG 102


11              TGCGATCCTTCGTCGTTCCCTTCCCGATGCGAACAATGAACCCCGGGCGCGCGGTTCTGC 191
j               T-CGATCCTC--TCGTTCCCTTCCCGAT-CGAACAATGAACCCCGG---CGCGGT-CTGC 231
3               T-CGATCCTC--TCGTTCCCTTCCCGAT-CGAACAACGAACCCCGG---CGCGGT-CTGC 204
1               ------------------------------------------------------------
5               TGTGGTGGTCGATCCTCTCGTTCCCTTCCCGATCGAACAATGAACCCCCGGCGCGGTCTG 162


11              GCCAAGG-AACTTAAACAAGGAG---TAACCACGGG-CGCCCC---GGAAACGG-TGTGC 242
j               GCCAAGG-AACTTAAACAAGGAG---TAACCACGGG-CGCCCC---GGAAACGG-TGTGC 282
```

```
3               GCCAAGGGAACTTAAACCAAGGGAGGTAACCACGGGGCGCCCCCG-GGAAACGGGTGGGC 263
1               ----------------------------ACGGG-CGCCCCCGGAGAAACGG-TGTGC 27
5               CGCCAAGGAACTTACCCAAGGGAGTACACCTACGGG-CGCCCC---GGAAACGG-TGTGC 217
                                        ***** ******    ******* ** **

11              G-TGTCG-TTGGTAGACGTCTTTACCATGATACATAAACGACTCTCGGCAACGGATATCT 300
j               G-TGTCG-TTGGT-GACGTCTTTACCATGATACATAA-CGACTCTCGGCAACGGATATCT 338
3               GGTGTCGGTTGGGTGACGTCTTTACCAAGATACATAA-CGACTCTCGGCAACGGATATCT 322
1               G-TGTCG-TTGGT-GACGTCTTTACCATGATACATAAC-GACTCTCGGCAACGGATATCT 83
5               G-TGTCG-TTGGT-GACGTCTTTACCATGATACATAACCGACTCTCGGCAACGGATATTT 274
                * ***** ****  ************* *********  ****************** *

11              CG-GCTCTCGCATCGATGAAGAACGTAGGCCGGAAAATGCGATACTTGGTG------TGA 353
j               CG-GCTCTCGCATCGATGAAGAACGTAGC----GAAATGCGATACTTGGTG------TGA 387
3               CG-GCTCTCGCATCGATGAAGAACGTAGC---GAAAATGCGATACTTGGTGGTG---TGA 375
1               CGTGCTCTCGCATCGATGAAGAACGTAGC----GAAATGCGATACTTGGTG------TGA 133
5               CG-GCTCTCGCATCGATGAAGAACGTAGC----GAAATGCGATACTCTGTGGTGTGTTGA 329
                ** ************************      ************  ***      ***

11              AT-TGCAGAA----TCCCGCGAATCATAACGAGTCTTTGAACGCAAG-TTGCGCCCGAAG 407
j               AT-TGCAGAA----TCCCGCGAATCAT--CGAGTCTTTGAACGCAAG-TTGCGCCCGAAG 439
3               AT-TGCAGAA----TCCCGCGAATCAT--CGAGTCTTTGAACGCAAGGTTGCGCCCGAAG 428
1               AT-TGCAGAA----TCCCGCGAATCAT--CGAGTCTTTGAACGCAAG-TTGCGCCCGAAG 185
5               ATCTGCAGAAGAATCCCCGCGAATCAT--CGAGTCTTTGAACGCAAG-TTGCGCCCGAAG 386
                ** *******     ************  ****************** ************

11              CCATTCCGGCCGAGGGC-ACGTCT---GCCTGGG-TGTCACGCATCGTCTGCCCCAACCC 462
j               CCATTC-GGCCGAGGGC-ACGTCT---GCCTGGG-TGTCACGCATCGT-TGCCCCAACCC 492
3               CCATTC-GGCCGAGGGC-ACGCCT---GCCTGGG-TGTCACGCATCGT-TGCCCCAACCC 481
1               CCATTC-GGCCGAGGGCCACGTCTCTGGCCTGGGGTGTCACGCATCGT-TGCCCCAACCC 243
5               CCATTC-GGCCGAGGGC-ACGTCT---GCCTGGG-TGTCACGCATCGT-TGCCCCAACCC 439
                ****** ********** *** **   ******* ************ ************

11              CCAAACACTTCTTAC---------------------------------------------- 477
j               C-AAACACTTCTTACGCTGTGCGGGGTGCGGGGAAGACATTGGCCTCCCGTGCGCTTTTG 551
3               C-AAACACTTCTTACGCTGTGCGCGCGGTGCGGGAGAGAGAATACATTGGCCTCCCGTGC 540
1               C-AAACACTTCTTACGCTGTGCGGGGTGCGGGGAAGAC---------------------- 280
5               C-AAACACTTCTTACGCTGTGCGGGGTGCGGGGAAGACATTGGCCTCCCGTGCGCTTGTG 498
                * *************

11              ------------------------------------------------------------
j               CTCGCGGTTAGCCTAAAAGTGAGTCCTAGGCGACGAGCGCCACGACAATCGGTGGTTGAG 611
3               GCTTTTGCTCGCGGTTAGCCTAAAAGTGAGTCCTAGGCGACGAGCGCCACGACAATCGGT 600
1               ----------------------------------------------GCCACGACAATCGGT 295
5               CTCGCGGTTAGCCTAAAAGTGAGTCCTAGGCGACG-------AGCGCCACGACAATCGGT 551


11              ------------------------------------------------------------
j               AAACCCTCGTGACCCGTCGTGTGTCGCCCGTCGCTGTGAAGGTGCTCCTCGACCCTATTG 671
3               GGTTGAGAAACCCTCGTGACCCGTCGTGTGTCGCCCGTCGCTGTGAAGGTGCTCCTCGAC 660
1               GGTTGAGAAACCCTCGTGACCCGTCGTGTGTCGCCCGTCGCTGTGAAGGTGCTCCTCGAC 355
5               GGTTGAGAAACCCTCGTGACCCGTCGTGTGTCGCCCGTCGCTGTGAAGGTGCTCCTCGAC 611


11              ------------------------------------------------------------
j               TGTCGTTCTTGCGACTCTACCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAATTT 731
3               CCTATTGTGTCGTTCTTGCGACTCTACCATCGCGACCCCAGGTCAGGCGGGATTACCCGC 720
1               CCTATTGTGTCGTTCTTGCGACTCTACCATCGCGACCCCAGGTCAGGCGGGATTACCCGC 415
5               CCTATTGTGTCGTTCTTGCGACTCTACCATCGCGACCCCAGGTCAGGCGGGATTACCCGC 671


11              ------------------------------------------------------------
j               AAGCATATCAATAAGCGGG---------------------------------------- 750
```

```
3               TGAATTTAAGCATATCAATAAGCGGAGGAAAGGGCGAATTCGTTTAAACAATGCAG---- 776
1               TGAATTTAAGCATATCAATAAGCGGAGGAAAGGGCGA-TTCGCGGCCGCTAAATTCAATT 474
5               TGAATTTAAGCATATCAATAAGCGGAGGAAAGGGCGAATTCGCGGCCGCTAAATTCAATT 731


11              ----------------------------------------
j               ----------------------------------------
3               ----------------------------------------
1               C-AGCCCTATAGTGAGTCGTATTACAATTC-ACTGGCGTA- 512
5               CGAACCCTATAGTGAGTCGTATTACAATTCCACGTGCGCTA 772
```

Appendix 4: Sample 1 consensus sequence and BLAST result

```
reverse         ------------------------------------------------------------
1               CAACTTCGCCCTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATACCTGCCCAGCA 60
1For            ------------------------------------------------------------


reverse         ------------------------------------------------------------
1               TGCTAACGACCTGTGAACATGTAATAATAACCTTCTGGGTGGGGGTGTAATGCCCCCTCC 120
1For            ------------------------------------------------------------


reverse         --------GGTGGGGAGGGGCAACGTTGAGATTGGCCCACTGCTCTTCGGTGTGGGGTTG 52
1               CAGAAAACGGTTGGGAGGGC--ACGTTGAGATTTGCCCACTGCTCCTCG-TGTGTGGTTG 177
1For            ------------------------------------------------------------


reverse         GGTCGATCCTCTCGT--CCTCCCCGATCGGACAATGAACCCCCGGCGCGGTCTGCGCCAA 110
1               G-TCGATCCTCTCGTTCCCTTCCCGATCGAACAATGAACCCC-GGCGCGGTCTGCGCCAA 235
1For            ------------------------------TTGAGAAACCCCGTCGTGACCC-CGTCGT 28
                                               *** *** * ** *  *  ** *


reverse         GGGACTTAAAACAAGGAGTAACCACGGG-CGCCCCCGGAAAACGGTGTGCGTGTC-GTTG 168
1               GGAACTTAAA-CAAGGAGTAACCACGGG-CGCCCC--GGAAACGGTGTGCGTGTC-GTTG 290
1For            GTGTCGCCCGTCGCTGTGAAGGTGCTCCTCGACCCTATTGTGTCGTTCTTGCGACTGTAC 88
                *   *      *   * * *    *    ** ***         **    * * * **


reverse         GTGACGTCTTTACCATGATACATAACGACTCTCGGCAACGGATATCTCG-GCTCTCGCAT 227
1               GTGACGTCTTTACCATGATACATAACGACTCTCGGCAACGGATATCTCGTGCTCTCGCAT 350
1For            CTGACGTCTTTACCATGAGACATAACGACTCTCGGCAACGGATATCTCG-GCTCTCGCAT 147
                 ***************** ***************************** **********


reverse         CGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGCGAATCATC 287
1               CGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGCGAATCATC 410
1For            CGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGCGAATCATC 207
                ************************************************************


reverse         GAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCCGAGGGC-ACGTCT--GCCTGG 344
1               GAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCCGAGGGCCACGTCCTGGCCTGG 470
1For            GAGTCTTTGTACGCAAGTTGCGCCCGAAGCCAA--AAACGAGGGC-ACGTCT--GCCTGG 262
                ********* **********************      ******* *****   ******


reverse         G-TGTCACGCATCGTTGCCCCAACCCCAAACACTTCTTACGCTGTGCGGGGTGCGGGGAA 403
1               GGTGTCACGCATCGTTGC---------------------------------------- 488
1For            G-TGTCACGCATCGTTGCCCCAACCCCAAACACTTCTTACCGTCTGTGGCGGGGGTTGGC 321
                * ****************


reverse         GACATTGGCCTCCCGTGCGCTTTTGCTCGCGGTTAGCCTAAAAGTGAGTCCTAGGCGACG 463
1               ------------------------------------------------------------
1For            GGGGGAAAGACCAATTG------------------------------------------- 338
```

```
reverse         AGCGCCACGACAATCGGTGGTTGAGAAACCCTCGTGACCCGTCGTGTGTCGCCCGTCGCT 523
1               ------------------------------------------------------------
1For            ------------------------------------------------------------


reverse         GTGAAGGTGCTCCTCGACCCTATTGTGTCGTTCTTGCGACTCTACCATCGCGACCCCAGG 583
1               ------------------------------------------------------------
1For            ------------------------------------------------------------


reverse         TCAGGCGGGATTACCCGCTGAATTTAAGCATATCAATAAGCGGAGGAAAGGGCGATTCGC 643
1               ------------------------------------------------------------
1For            ------------------------------------------------------------


reverse         GGCCGCTAAATTCAATTCAGCCCTATAGTGAGTCGTATTACAATTCACTGGCGTA 698
1               -------------------------------------------------------
1For            -------------------------------------------------------
```

>Sample 1 consensus

CAACTTCGCCCTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATACCTG
CCCAGCATGCTAACGACCTGTGAACATGTAATAATAACCTTCTGGGTGGGGG
TGTAATGCCCCCTCCCAGAAAACGG(t/g)GGGAGGG(c/g)CAACGTTGAGATTG
GCCCACTGCTC(c/t)TCGGTGTG(g/t)GGTTGGGTCGATCCTCTCGTTCCCT(t/c)CC
CGATCG(a/g)ACAATGAACCCCCGGCGCGGTCTGCGCCAAGG(a/g)ACTTAAAA
CAAGGAGTAACCACGGGCGCCCCCGG(a/g)AAACGGTGTGCGTGTCGTTGGTG
ACGTCTTTACCATGATACATAACGACTCTCGGCAACGGATATCTCGTGCTCTC
GCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATC
CCGCGAATCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCCG
AGGGCCACGTC(t/c)TGGCCTGGGGTGTCACGCATCGTTGCCCCAACCCCAAAC
ACTTCTTACGCTGTGCGGGGTGCGGGGAAGACGCCACGACAATCGGTGGTTG
AGAAACCCTCGTGACCCGTCGTGTGTCGCCCGTCGCTGTGAAGGTGCTCCTCG
ACCCTATTGTGTCGTTCTTGCGACTCTACCATCGCGACCCCAGGTCAGGCGGG
ATTACCCGCTGAATTTAAGCATATCAATAAGCGGAGGAAAGGGCGATTCGCG
GCCGCTAAATTCAATTCAGCCCTATAGTGAGTCGTATTACAATTCACTGGCGT
A

>Sample 1 consensus Fasta

CAACTTCGCCCTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATACCTG
CCCAGCATGCTAACGACCTGTGAACATGTAATAATAACCTTCTGGGTGGGGG
TGTAATGCCCCCTCCCAGAAAACGGtgGGGAGGGcgCAACGTTGAGATTGGCC
CACTGCTCctTCGGTGTGgtGGTTGGGTCGATCCTCTCGTTCCCTtcCCCGATCGag
ACAATGAACCCCCGGCGCGGTCTGCGCCAAGGagACTTAAAACAAGGAGTAA
CCACGGGCGCCCCCGGagAAACGGTGTGCGTGTCGTTGGTGACGTCTTTACCA
TGATACATAACGACTCTCGGCAACGGATATCTCGTGCTCTCGCATCGATGAA

GAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGCGAATCAT
CGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTCGGCCGAGGGCCACGTCt
cTGGCCTGGGGTGTCACGCATCGTTGCCCCAACCCCAAACACTTCTTACGCTG
TGCGGGGTGCGGGGAAGACGCCACGACAATCGGTGGTTGAGAAACCCTCGTG
ACCCGTCGTGTGTCGCCCGTCGCTGTGAAGGTGCTCCTCGACCCTATTGTGTC
GTTCTTGCGACTCTACCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGA
ATTTAAGCATATCAATAAGCGGAGGAAAGGGCGATTCGCGGCCGCTAAATTC
AATTCAGCCCTATAGTGAGTCGTATTACAATTCACTGGCGTA

Closest Genbank match

> gb|AF338492.1|AF338492 Juglans nigra isolate 836 18S ribosomal RNA gene, partial sequence;
internal transcribed spacer 1, 5.8S ribosomal RNA gene
and internal transcribed spacer 2, complete sequence; and
26S ribosomal RNA gene, partial sequence
Length=750

Sort alignments for this subject sequence by:

E value Score

Percent identity

Query start position

Subject start position

```
 Score =  828 bits (448),  Expect = 0.0
 Identities = 512/538 (95%), Gaps = 24/538 (4%)
 Strand=Plus/Plus

Query  12   TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATACCTGCCCAGCATGCTAACGACC  71
            |||||||||||||||||||||||||||||||||||||||||||||||||||| |  |||||||
Sbjct  16   TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGATACCTGCCCAGCA-G--AACGACC  72

Query  72   TGTGAACATGTAATAATAACCTTCTGGGTGGGGGTGTAATGCCCCCTCCCAGAAAACGGT  131
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73   TGTGAACATGTAATAATAACCTTCTGGGTGGGGGTGTAATGCCCCCTCCCAGAAAACGGT  132

Query  132  GGGGAGGGCGCAACGTTGAGATTGGCCCACTGCTCCTTCGGTGTGGTGGTTGGGTCGATC  191
             |||| || || |||||||||||| |||||||||||| || |||| |||||| ||||||||
Sbjct  133  TGGGA-GG-GC-ACGTTGAGATTTGCCCACTGCTCC-TC-GTGT-GTGGTT-GGTCGATC  185

Query  192  CTCTCGTTCCCTTCCCCGATCGAGACAATGAACCCCCGGCGCGGTCTGCGCCAAGGAGAC  251
            |||||||||||||| ||||||||| |||||||| |||||||||||||||||||||||| ||
Sbjct  186  CTCTCGTTCCCTT-CCCGATCGA-ACAATGAA-CCCCGGCGCGGTCTGCGCCAAGGA-AC  241

Query  252  TTAAAACAAGGAGTAACCACGGGCGCCCCCGGAGAAACGGTGTGCGTGTCGTTGGTGACG  311
            || |||||||||||||||||||||| |||| | |||||||||||||||||||||||||||
Sbjct  242  TT-AAACAAGGAGTAACCACGGGCG-CCCC-G-GAAACGGTGTGCGTGTCGTTGGTGACG  297

Query  312  TCTTTACCATGATACATAACGACTCTCGGCAACGGATATCTCGTGCTCTCGCATCGATGA  371
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  298  TCTTTACCATGATACATAACGACTCTCGGCAACGGATATCTCG-GCTCTCGCATCGATGA  356

Query  372  AGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGCGAATCATCGAGTCT  431
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357  AGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGCGAATCATCGAGTCT  416

Query  432  TTGAACGCAAGTTGCGCCCGAAGCCATTCGGCCGAGGGCCACGTCTCTGGCCTGGGGTGT  491
            |||||||||||||||||||||||||||||||||||| |||||| | |||||| |||
Sbjct  417  TTGAACGCAAGTTGCGCCCGAAGCCATTCGGCCGAGGGC-ACGTCT--G-CCTGGG-TGT  471
```

```
Query  492  CACGCATCGTTGCCCCAACCCCAAACACTTCTTACGCTGTGCGGGGTGCGGGGAAGAC  549
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  472  CACGCATCGTTGCCCCAACCCCAAACACTTCTTACGCTGTGCGGGGTGCGGGGAAGAC  529


 Score =  298 bits (161),  Expect = 3e-77
 Identities = 161/161 (100%), Gaps = 0/161 (0%)
 Strand=Plus/Plus

Query  549  CGCCACGACAATCGGTGGTTGAGAAACCCTCGTGACCCGTCGTGTGTCGCCCGTCGCTGT  608
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  589  CGCCACGACAATCGGTGGTTGAGAAACCCTCGTGACCCGTCGTGTGTCGCCCGTCGCTGT  648

Query  609  GAAGGTGCTCCTCGACCCTATTGTGTCGTTCTTGCGACTCTACCATCGCGACCCCAGGTC  668
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  649  GAAGGTGCTCCTCGACCCTATTGTGTCGTTCTTGCGACTCTACCATCGCGACCCCAGGTC  708

Query  669  AGGCGGGATTACCCGCTGAATTTAAGCATATCAATAAGCGG  709
           |||||||||||||||||||||||||||||||||||||||||
Sbjct  709  AGGCGGGATTACCCGCTGAATTTAAGCATATCAATAAGCGG  749
```

Appendix 5: Sample 3 consensus sequence and BLAST result

```
Reverse         ------------------------------------------------------------
3               GTGAACCTGCGGAAGGATCATTGTCGATACCTGCCCAGCAGAACGACCTGTGAACATGTA 60


Reverse         ------------------------------------------------------------
3               ATAACCTTCTGGGTGGGGGTGTAATGCCCCCTCCCAAAAAACGGTTGGGAGGGCACGTTG 120


Reverse         ------------------------------------------------------------
3               AGATTTGCCCACTGCTCCTCGTGTGTGGTTGGTCGATCCTCTCGTTCCCTTCCCGATCGA 180


Reverse         ------------------------GCCAGGGAACTTAACCAGGGAGGTAACCACGGGGCG 36
3               ACAACGAACCCCGGCGCGGTCTGCGCCAAGGAACTTAAACAAGGAG-TAACCACGGG-CG 238
                                        **** ********* ** **** ********** **

Reverse         CCCCCGGGAAACGGGTGGGCGGTGTCGGTTGGGGACGTCTTTACCAAGATACATAACGAC 96
3               CCCC--GGAAACGG-TGTGCG-TGTCG-TTGGTGACGTCTTTACCAAGATACATAACGAC 293
                ****  ******** ** *** ***** **** **************************

Reverse         TCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAAATGCGATACT 156
3               TCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAA-TGCGATACT 352
                ************************************************* *********

Reverse         TGGTGGTGGAATTGCAGAATCCCGCGAATCATCGAGTCTTTGAACGCAAGGTTGCGCCCG 216
3               TGGTG--TGAATTGCAGAATCCCGCGAATCATCGAGTCTTTGAACGCAAG-TTGCGCCCG 409
                *****   ****************************************** *********

Reverse         AAGCCATTCGGCCGAGGGCACGCCTGCCTGGGTGTCACGCATCGTTGCCCCAACCCCAAA 276
3               AAGCCATTCGGCCGAGGGCACGCCTGCCTGGGTGTCACGCATCGTTGCCCCAACCCCAAA 469
                ************************************************************
```

```
Reverse         CACTTCTTACGCTGTGCGGGGTGCGGGGAAGACATTGGCCTCCCGTGCGCTTTTGCTCGC 336
3               CACTTCTTACGCTGTGCCCGGTGCGGGAGAAT--------------------------- 501
                ****************  ********  *

Reverse         GGTTAGCCTAAAAGTGAGTCCTAGGCGACGAGCGCCACGACAATCGGTGGTTGAGAAACC 396
3               ------------------------------------------------------------

Reverse         CTCGTGACCCGTCGTGTGTCGCCCGTCGCTGTGAAGGTGCTCCTCGACCCTATTGTGTCG 456
3               ------------------------------------------------------------

Reverse         TTCTTGCGACTCTACCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAATTTAAGCA 516
3               ------------------------------------------------------------

Reverse         TATCAATAAGCGGAGGAAAGGGCGAATTCGTTTAAACAATGCAG 560
3               -------------------------------------------
```

Sample 3 consensus sequence for Thesis

GTGAACCTGCGGAAGGATCATTGTCGATACCTGCCCAGCAG
AACGACCTGTGAACATGTAATAACCTTCTGGGTGGGGGTGT
AATGCCCCCTCCCAAAAAACGGTTGGGAGGGCACGTTGAGA
TTTGCCCACTGCTCCTCGTGTGTGGTTGGTCGATCCTCTCGTT
CCCTTCCCGATCGAACAACGAACCCCGGCGCGGTCTGCGCCA
(a/g)GGAACTTAA(a/c)CA(a/g)GGAGGTAACCACGGGGCGCCCC
CGGGAAACGGGTGGGCGGTGTCGGTTGG(g/t)GACGTCTTTAC
CAAGATACATAACGACTCTCGGCAACGGATATCTCGGCTCTC
GCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGGT(g/t)
GAATTGCAGAATCCCGCGAATCATCGAGTCTTTGAACGCAAG
GTTGCGCCCGAAGCCATTCGGCCGAGGGCACGCCTGCCTGGG
TGTCACGCATCGTTGCCCCAACCCCAAACACTTCTTACGCTGT
GC(g/c)(g/c)GGTGCGGG(a/g)(a/g)A(g/a)(a/t)ACATTGGCCTCCCGT
GCGCTTTTGCTCGCGGTTAGCCTAAAAGTGAGTCCTAGGCGAC
GAGCGCCACGACAATCGGTGGTTGAGAAACCCTCGTGACCCG
TCGTGTGTCGCCCGTCGCTGTGAAGGTGCTCCTCGACCCTATT
GTGTCGTTCTTGCGACTCTACCATCGCGACCCCAGGTCAGGCG
GGATTACCCGCTGAATTTAAGCA TATCAATAAGCGGAGGAAA
GGGCGAATTCGTTTAAACAATGCAG

Fasta:

>sample 3 consensus
GTGAACCTGCGGAAGGATCATTGTCGATACCTGCCCAGCAGAACGACCTGTGAACATGTA
ATAACCTTCTGGGTGGGGGGTGTAATGCCCCCTCCCAAAAAACGGTTGGGAGGGCACGTTG
AGATTTGCCCACTGCTCCTCGTGTGTGGTTGGTCGATCCTCTCGTTCCCTTCCCGATCGAACA
ACGAACCCCGGCGCGGTCTGCGCCAagGGAACTTAAacCAagGGAGGTAACCACGGGGCGCCC
CCGGGAAACGGGTGGGCGGTGTCGGTTGGgt GACGTCTTTACCAAGATACATAACGAC
TCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAAATGCGATACT
TGGTGGTgtGAATTGCAGAATCCCGCGAATCATCGAGTCTTTGAACGCAAGGTTGCGCCCG
AAGCCATTCGGCCGAGGGCACGCCTGCCTGGGTGTCACGCATCGTTGCCCCAACCCCAAAC
ACTTCTTACGCTGTGCgcgcGGTGCGGGagagAgaatACATTGGCCTCCCGTGCGCTTTTGCTCGC
GGTTAGCCTAAAAGTGAGTCCTAGGCGACGAGCGCCACGACAATCGGTGGTTGAGAAACC
CTCGTGACCCGTCGTGTGTCGCCCGTCGCTGTGAAGGTGCTCCTCGACCCTATTGTGTCG

TTCTTGCGACTCTACCATCGCGACCCCAGGTCAGGCGGGATTACCCGCTGAATTTAAGCA
TATCAATAAGCGGAGGAAAGGGCGAATTCGTTTAAACAATGCAG

Closest Genbank Match

>gb|AF338487.1|AF338487 Juglans microcarpa isolate 108 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence
Length=735

```
 Score = 1190 bits (644),  Expect = 0.0
 Identities = 717/748 (95%), Gaps = 26/748 (3%)
 Strand=Plus/Plus

Query  1    GTGAACCTGCGGAAGGATCATTGTCGATACCTGCCCAGCAGAACGACCTGTGAACATG--  58
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10   GTGAACCTGCGGAAGGATCATTGTCGATACCTGCCCAGCAGAACGACCTGTGAACATGTA  69

Query  59   -TAATAACCTTCTGGGTGGGGGTGTAATGCCCCCTCCCAAAAAACGGTTGGGAGGGCACG  117
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  70   ATAATAACCTTCTGGGTGGGGGTGTAATGCCCCCTCCCAAAAAACGGTTGGGAGGGCACG  129

Query  118  TTGAGATTTGCCCACTGCTCCTCGTGTGTGGTTGGTCGATCCTCTCGTTCCCTTCCCGAT  177
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130  TTGAGATTTGCCCACTGCTCCTCGTGTGTGGTTGGTCGATCCTCTCGTTCCCTTCCCGAT  189

Query  178  CGAACAACGAACCCCGGCGCGGTCTGCGCCAAGGGAACTTAAACCAAGGGAGGTAACCAC  237
            ||||||| |||||||||||||||||||| |||||||||| ||| ||| ||||||||
Sbjct  190  CGAACAATGAACCCCGGCGCGGTCTGCGCCAA-GGAACTTAAA-CAA-GGA-GTAACCAC  245

Query  238  GGGGCGCCCCCGGGAAACGGGTGGGCGGTGTCGGTTGGGTGACGTCTTTACCAAGATACA  297
             ||||| |||| |||||| |||| || ||||| ||| ||||||||||||||| ||||||
Sbjct  246  -GGGCG-CCCC-GGAAAC-GGTGTGC-GTGTC-GTT-GGTGACGTCTTTACCATGATACA  298

Query  298  TAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAAATG  357
            |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  299  TAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCG-AAATG  357

Query  358  CGATACTTGGTGGTGTGAATTGCAGAATCCCGCGAATCATCGAGTCTTTGAACGCAAGGT  417
            |||||| |  ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  358  CGATAC-T--TGGTGTGAATTGCAGAATCCCGCGAATCATCGAGTCTTTGAACGCAA-GT  413

Query  418  TGCGCCCGAAGCCATTCGGCCGAGGGCACGCCTGCCTGGGTGTCACGCATCGTTGCCCCA  477
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  414  TGCGCCCGAAGCCATTCGGCCGAGGGCACGTCTGCCTGGGTGTCACGCATCGTTGCCCCA  473

Query  478  ACCCCAAACACTTCTTACGCTGTGCGCGCGGTGCGGGAGAGAGAATACATTGGCCTCCCG  537
            |||||||||||||||||||||||| | |||||||| || ||   ||||||||||||||
Sbjct  474  ACCCCAAACACTTCTTACGCTGTGCG-G-GGTGCGGG-GA-AG---ACATTGGCCTCCCG  526

Query  538  TGCGCTTTTGCTCGCGGTTAGCCTAAAAGTGAGTCCTAGGCGACGAGCGCCACGACAATC  597
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  527  TGCGCTTKTGCTCGCGGTTAGCCTAAAAGTGAGTCCTAGGCGACGAGCGCCACGACAATC  586

Query  598  GGTGGTTGAGAAACCCTCGTGACCCGTCGTGTGTCGCCCGTCGCTGTGAAGGTGCTCCTC  657
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  587  GGTGGTTGAGAAACCCTCGTGACCCGTCGTGTGTCGCCCGTCGCTGTGAAGGTGCTCCTC  646

Query  658  GACCCTATTGTGTCGTTCTTGCGACTCTACCATCGCGACCCCAGGTCAGGCGGGATTACC  717
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
Sbjct  647  GACCCTATTGTGTCGTTCTTGCGACTCTACCATCGCGACCCCAGGTCAGGCGGGATTACC  706

Query  718  CGCTGAATTTAAGCATATCAATAAGCGG  745
            ||||||||||||||||||||||||||||
Sbjct  707  CGCTGAATTTAAGCATATCAATAAGCGG  734
```

Appendix 6: Sample 5 consensus sequence and BLAST result

```
reverse         ------------------------------------------------------------
5               TAGCGCACGTGGAATTGTAATACGACTCACTATAGGGTTCGAATTGAATTTAGCGGCCGC 60


reverse         ------------------------------------------------------------
5               GAATTCGCCCTTTCCTCCGCTTATTGATATGCTTAAATTCAGCGGGTAATCCCGCCTGAC 120


reverse         ------------------------------------------------------------
5               CTGGGGTCGCGATGGTAGAGTCGCAAGAACGACACAATAGGGTCGAGGAGCACCTTCACA 180


reverse         ------------------------------------------------------------
5               GCGACGGGCGACACACGACGGGTCACGAGGGTTTCTCAACCACCGATTGTCGTGGCGCTC 240


reverse         ------------------------------------------------------------
5               GTCGCCTAGGACTCACTTTTAGGCTAACCGCGAGCACAAGCGCACGGGAGGCCAATGTCT 300


reverse         ------------------------------------------------------------
5               TCCCCGCACCCCGCACAGCGTAAGAAGTGTTTGGGGTTGGGGCAACGATGCGTGACACCC 360


reverse         ----------------------------------------------------GATGA 5
5               AGGCAGACGTGCCCTCGGCCGAATGGCTTCGGGCGCAACTTGCGTTCAAAGACTCGATGA 420
                                                                   *****

reverse         TTCGCGGGGATTCTTGCGATTCACACCAAGGTATCGCATTTCGCTACGTTCTTCATCGAT 65
5               TTCGCGGGA--TTCTGCAATTCNACACCAAGTATCGCATTTCGCTACGTTCTTCATCGAT 478
                ********   *  *** ****    * * ******************************

reverse         GCGAGAGCCGAAATATCCGTTGCCGAGAGTCGGTATGTATCATGGTAAAGACGTCACCAA 125
5               GCGAGAGCCGAGATATCCGTTGCCGAGAGTCGTTATGTATCATGGTAAAGACGTCACCAA 538
                ********** ******************* **************************

reverse         CGACACGCACACCGTTTCCGGGGCGCCCGAGGTGACTCCCTGGGTAAGTTCCTTGGCGCA 185
5               CGACACGCACACCGTTTCCGGGGCGCCCGTGGTTACTCCTTGT-TAAGTNCCTTGGCGCA 597
                **************************** *** ***** **  ***** **********

reverse         GACCGCGCCGGGGGTTCATTGTTCGATCGGGAAGGGAACGAGAGGATCGACCGACCACACA 245
5               GACCGCGCGGGGGGTTCATTGTTCGATCGGGAAGGGAACGAGAGGATCGACC-ACCACACA 656
                ******** ****************************************** ********

reverse         CGAGGGGCAGGGGGCAAATCTCAACGTGCCCTCCCAACCGTTTTCTGGGAGGGGGCAGGT 305
5               CAAGGG-CATGGGC--AATTTCAACGTGCCTTCCCAAC-GTTTTTTGGGAAGGGGCCT-T 711
                * **** ** ***   *** ********** ******* ***** ***** *****   *

reverse         ACACCCCCACCCAGGAAGGGTATTATTACATGTTCACAGG-TCGTTCTAAATGGGTCAGG 364
5               ACCCCCCCAACCA--AAAGGTTATATAACATGTTCCCAGGGTCGGTCTGCCGGGGCAAGG 769
                ** ****** ***  ** ***  *** ******** **** *** ***    ***  ***
```

```
     ****      **  *** ** *
```

Sample 5 consensus sequence

TAGCGCACGTGGAATTGTAATACGACTCACTATAGGGTTCGAATTGAATTTAGCGGCCGC
GAATTCGCCCTTTCCTCCGCTTATTGATATGCTTAAATTCAGCGGGTAATCCCGCCTGAC
CTGGGGTCGCGATGGTAGAGTCGCAAGAACGACACAATAGGGTCGAGGAGCACCTTCACA
GCGACGGGCGACACACGACGGGTCACGAGGGTTTCTCAACCACCGATTGTCGTGGCGCTC
GTCGCCTAGGACTCACTTTTAGGCTAACCGCGAGCACAAGCGCACGGGAGGCCAATGTCT
TCCCCCGCACCCCCGCACAGCGTAAGAAGTGTTTGGGGTTGGGGCAACGATGCGTGACACCC
AGGCAGACGTGCCCTCGGCCGAATGGCTTCGGGCGCAACTTGCGTTCAAAGACTCGATGATTCGCGGGGAT
T(c/t)(/t/c) TGC(a/g) ATTCA(a/c)(a/c)(a/c) C(a/c)A(g/a)
GTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAAATATCCGTTGCCGAGAGTCG(g/t)
TATGTATCATGGTAAAGACGTCACCAACGACACGCACACCGTTTCCGGGGCGCCCG(t/a)GGT(g/t)
ACTCC(c/t)TGGGTAAGTTCCTTGGCGCAGACCGCGC(c/g)GGGGTTCATTGTTCGATCGGGAAGGGAACGAGA
GGATCGACCACCACACACGAGGGGCAGGGGGCAAATCTCAACGTGC(c/t)
TGGGA(a/g)GGGGCC(t/g)GTAC(a/c)CCCCCA(c/a) CCAGGAA(g/a)GGTATTATTACATGTTC(a/c)
CAGGGTCGGTCT

>sample 5 consensus Fasta
TAGCGCACGTGGAATTGTAATACGACTCACTATAGGGTTCGAATTGAATTTAGCGGCCGC
GAATTCGCCCTTTCCTCCGCTTATTGATATGCTTAAATTCAGCGGGTAATCCCGCCTGAC
CTGGGGTCGCGATGGTAGAGTCGCAAGAACGACACAATAGGGTCGAGGAGCACCTTCACA
GCGACGGGCGACACACGACGGGTCACGAGGGTTTCTCAACCACCGATTGTCGTGGCGCTC
GTCGCCTAGGACTCACTTTTAGGCTAACCGCGAGCACAAGCGCACGGGAGGCCAATGTCT
TCCCCCGCACCCCCGCACAGCGTAAGAAGTGTTTGGGGTTGGGGCAACGATGCGTGACACCC
AGGCAGACGTGCCCTCGGCCGAATGGCTTCGGGCGCAACTTGCGTTCAAAGACTCGATGATTCGCGGGGAT
TcttcTGCagATTCAacacacCacAgaGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAAATATCCGTT
GCCGAGAGTCGgtTATGTATCATGGTAAAGACGTCACCAACGACACGCACACCGTTTCCGGGGCGCCCGtaGG
TgtACTCCctTGGGTAAGTTCCTTGGCGCAGACCGCGCcgGGGGTTCATTGTTCGATCGGGAAGGGAACGAGA
GGATCGACCACCACACACGAGGGGCAGGGGGCAAATCTCAACGTGCctTGGGAagGGGGCCtgGTACacCCCC
CAcaCCAGGAAgaGGTATTATTACATGTTCacCAGGGTCGGTCT

Closest Genbank Match

```
>   gb|AF338491.1|AF338491  Juglans nigra isolate 834 18S ribosomal RNA gene,
partial sequence;
internal transcribed spacer 1, 5.8S ribosomal RNA gene
and internal transcribed spacer 2, complete sequence; and
26S ribosomal RNA gene, partial sequence
Length=750

 Score = 1018 bits (551),  Expect = 0.0
 Identities = 604/627 (96%), Gaps = 17/627 (2%)
 Strand=Plus/Minus

Query  77   CCGCTTATTGATATGCTTAAATTCAGCGGGTAATCCCGCCTGACCTGGGGTCGCGATGGT  136
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  749  CCGCTTATTGATATGCTTAAATTCAGCGGGTAATCCCGCCTGACCTGGGGTCGCGATGGT  690

Query  137  AGAGTCGCAAGAACGACACAATAGGGTCGAGGAGCACCTTCACAGCGACGGGCGACACAC  196
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  689  AGAGTCGCAAGAACGACACAATAGGGTCGAGGAGCACCTTCACAGCGACGGGCGACACAC  630
```

```
Query  197  GACGGGTCACGAGGGTTTCTCAACCACCGATTGTCGTGGCGCTCGTCGCCTAGGACTCAC  256
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  629  GACGGGTCACGAGGGTTTCTCAACCACCGATTGTCGTGGCGCTCGTCGCCTAGGACTCAC  570

Query  257  TTTTAGGCTAACCGCGAGCACAAGCGCACGGGAGGCCAATGTCTTCCCCGCACCCCGCAC  316
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  569  TTTTAGGCTAACCGCGAGCAMAAGCGCACGGGAGGCCAATGTCTTCCCCGCACCCCGCAC  510

Query  317  AGCGTAAGAAGTGTTTGGGGTTGGGGCAACGATGCGTGACACCCAGGCAGACGTGCCCTC  376
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  509  AGCGTAAGAAGTGTTTGGGGTTGGGGCAACGATGCGTGACACCCAGGCAGACGTGCCCTC  450

Query  377  GGCCGAATGGCTTCGGGCGCAACTTGCGTTCAAAGACTCGATGATTCGCGGGGATTCTTC  436
            |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  449  GGCCGAATGGCTTCGGGCGCAACTTGCGTTCAAAGACTCGATGATTCGCGGG-ATTCT--  393

Query  437  TGCAGATTCAACACACCACAGAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGC  496
             ||| ||||| ||| | | ||  ||||||||||||||||||||||||||||||||||||
Sbjct  392  -GCA-ATTCA-CAC-C-A-AG--TATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGC  341

Query  497  CGAAATATCCGTTGCCGAGAGTCGGTTATGTATCATGGTAAAGACGTCACCAACGACACG  556
            ||| ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  340  CGAGATATCCGTTGCCGAGAGTCG-TTATGTATCATGGTAAAGACGTCACCAACGACACG  282

Query  557  CACACCGTTTCCGGGGCGCCCGTAGGTGTACTCCCTTGGGTAAGTTCCTTGGCGCAGACC  616
            |||||||||||||||||||||| ||| |||||| |||  ||||||||||||||||||||
Sbjct  281  CACACCGTTTCCGGGGCGCCCGT-GGT-TACTCC-TTGTTTAAGTTCCTTGGCGCAGACC  225

Query  617  GCGCCGGGGGTTCATTGTTCGATCGGGAAGGGAACGAGAGGATCGACCA-CCACACACGA  675
            ||||||||| ||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  224  GCGCCGGGG-TTCATTGTTCGATCGGGAAGGGAACGAGAGGATCGACCAACCACACACGA  166

Query  676  GGGGCAGGGGGCAAATCTCAACGTGCC  702
            || |||| ||||||||||||||||||
Sbjct  165  GGAGCAGTGGGCAAATCTCAACGTGCC  139
```

Appendix 7: Sample 11 consensus sequence and BLAST result

```
Reverse         ------------------------------------------------------------
11              TCCTCTGTTTAAACCAATTCGCCCTTTCCTCCAGCTTATTGATATGCTTAAATTCAGCGG 60


Reverse         ------------------------------------------------------------
11              GTAATCCCGCCTGACCTGGGGTCGCGATGGTAGAGTCGCAAGAACGACACAATAGGGTCG 120


Reverse         ------------------------------------------------------------
11              AGGAGCACCTTCACAGCGACGGGCGACACACGACGGGNTCACGAGGGTTTCTCAACCACC 180


Reverse         ------------------------------------------------------------
11              GATTGTCGTGGCGCTCGTCGCCTAGGACTCACTTTTAGGCTAACCGCGAGCAAAAGCGCA 240


Reverse         ----------------TCCCCGCACCCCGCACAGCGTAAGAAGTGTTGGGGGTTGGGGCG 44
11              CGGGAGGCCAATGTCTTCCCCGCACCCCGCACAGCGTAAGAAGTGTTTGGGGTTGGGGCA 300
                              ****************************** ***********


Reverse         ACGATGCGTGACACCCAGGCAGACGTGCCCTCGGCCGGAATGGCTTCGGGCGCAACTTGC 104
11              ACGATGCGTGACACCCAGGCAGACGTGCCCTCGGCCG-AATGGCTTCGGGCGCAACTTGC 359
                ************************************ ********************


Reverse         GTTCAAAGACTCG-ATGATTCGCGGGATTCTGCAATTCACACCAAGTATCGCATTTCG-- 161
11              GTTCAAAGACTCTTATGATTCGCGGGATTCTGCAATTCACACCAAGTATCGCATTTTCGC 419
                ************  ****************************************


Reverse         CTACGTTCTTCATCGATGCGAGAGCCGAGATATCCGTTGCCGAGAGTCGTT-ATGTATCA 220
11              CTACGTTCTTCATCGATGCNAGAGCCGAGATATCCGTTGCCGAGAGTCGTTTATGTATCA 479
                ****************** ****************************** ********


Reverse         TGGTAAAGACGTCACCAACGACACGCACACCGTTTCCGGGGCGCCCGTGGTTACTCCTTG 280
11              TGGTAAAGACGTTACCAACGACACGCACACCGTTTCCGGGGCGCCCGTGGTTACTCCTTG 539
                ************ ************************************************


Reverse         TTTAAGTTCCTTGGCGCAGACCGCGCCGGGGTTCATTGTTCGATCGGGAAGGGAACGAGA 340
11              TTTAAGTTCCTTGGCGCAAACCCGCCCGGGGTTCATTGTTCCATCGGGAAGG-----ACA 594
                ****************** ***   *************** **********     * *


Reverse         GGATCGACCAACCACACACGAGGAGCAGTGGGCAAATCTCAACGTGCCCTCCCAACCGTT 400
11              AGATCCACCACCC---------------------------------------------- 607
                 **** **** **


Reverse         TTTTGGGAGGGGGCATTACACCCCCACCCAGAAGGTTATTACATGTTCACAGGTCGTTCT 460
11              ------------------------------------------------------------


Reverse         GCTGGGCAGGTATCGACAATGATCCTTCCGCAGGTTCACCTACGGGAAGGCGAATTGCGG 520
11              ------------------------------------------------------------


Reverse         CCGCATATTCAATTGCC 537
11              -----------------
```

Sample 11 consensus for thesis:

TCCTCTGTTTAAACCAATTCGCCCTTTCCTCCAGCTTATTGATATGCTTAAATTCAGCGG
GTAATCCCGCCTGACCTGGGGTCGCGATGGTAGAGTCGCAAGAACGACACAATAGGGTCG
AGGAGCACCTTCACAGCGACGGGCGACACACGACGGGNTCACGAGGGTTTCTCAACCACC
GATTGTCGTGGCGCTCGTCGCCTAGGACTCACTTTTAGGCTAACCGCGAGCAAAAGCGCACGGGAGGCCAATGTCTTCC
CCGCACCCCGCACAGCGTAAGAAGTGTT(t/g) GGGGTTGGGGC(a/g)
ACGATGCGTGACACCCAGGCAGACGTGCCCTCGGCCGGAATGGCTTCGGGCGCAACTTGC GTTCAAAGACTC(g/t)
TATGATTCGCGGGATTCTGCAATTCACACCAAGTATCGCATTT(t/c)(c/g)GC
CTACGTTCTTCATCGATGCGAGAGCCGAGATATCCGTTGCCGAGAGTCGTTtATGTATCATGGTAAAGACGT(c/t)ACCAA
CGACACGCACACCGTTTCCGGGGCGCCCGTGGTTACTCCTTGTTTAAGTTCCTTGGCGCA(g/a) ACC(g/c)(g/c)(g/c)
CCGGGGTTCATTGTTC(g/c) ATCGGGAAGGGAACGA(c/g)A(a/g) GATC(g/c)ACCA(a/c)
CCACACACGAGGAGCAGTGGGCAAATCTCAACGTGCCCTCCCAACCGTT
GCTGGGCAGGTATCGACAATGATCCTTCCGCAGGTTCACCTACGGGAAGGCGAATTGCGG CCGCATATTCAATTGCC

>Sample 11 Fasta
TCCTCTGTTTAAACCAATTCGCCCTTTCCTCCAGCTTATTGATATGCTTAAATTCAGCGG
GTAATCCCGCCTGACCTGGGGTCGCGATGGTAGAGTCGCAAGAACGACACAATAGGGTCG
AGGAGCACCTTCACAGCGACGGGCGACACACGACGGGNTCACGAGGGTTTCTCAACCACC
GATTGTCGTGGCGCTCGTCGCCTAGGACTCACTTTTAGGCTAACCGCGAGCAAAAGCGCACGGGAGGCCAATGTCTTCC
CCGCACCCCGCACAGCGTAAGAAGTGTTtg
GGGGTTGGGGCagACGATGCGTGACACCCAGGCAGACGTGCCCTCGGCCGGAATGGCTTCGGGCGCAACTTGCGTTCAA
AGACTCgtTATGATTCGCGGGATTCTGCAATTCACACCAAGTATCGCATTTtccgGCCTACGTTCTTCATCGATGCGAGAGC
CGAGATATCCGTTGCCGAGAGTCGTTtATGTATCATGGTAAAGACGTctACCAACGACACGCACACCGTTTCCGGGGCGC
CCGTGGTTACTCCTTGTTTAAGTTCCTTGGCGCAgaACCgcgcgcCCGGGGTTCATTGTTCgcATCGGGAAGGGAACGAcgAag
GATCgcACCAac CCACACACGAGGAGCAGTGGGCAAATCTCAACGTGCCCTCCCAACCGTT
GCTGGGCAGGTATCGACAATGATCCTTCCGCAGGTTCACCTACGGGAAGGCGAATTGCGG CCGCATATTCAATTGCC

Closest Genbank Match:

> gb|AF338492.1|AF338492 Juglans nigra isolate 836 18S ribosomal RNA gene, partial sequence;
internal transcribed spacer 1, 5.8S ribosomal RNA gene
and internal transcribed spacer 2, complete sequence; and
26S ribosomal RNA gene, partial sequence
Length=750

Sort alignments for this subject sequence by:

E value Score Percent identity

Query start position Subject start position

```
 Score = 1051 bits (569),  Expect = 0.0
 Identities = 629/653 (96%), Gaps = 23/653 (3%)
 Strand=Plus/Minus

Query  34   GCTTATTGATATGCTTAAATTCAGCGGGTAATCCCGCCTGACCTGGGGTCGCGATGGTAG  93
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  747  GCTTATTGATATGCTTAAATTCAGCGGGTAATCCCGCCTGACCTGGGGTCGCGATGGTAG  688

Query  94   AGTCGCAAGAACGACACAATAGGGTCGAGGAGCACCTTCACAGCGACGGGCGACACACGA  153
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  687  AGTCGCAAGAACGACACAATAGGGTCGAGGAGCACCTTCACAGCGACGGGCGACACACGA  628

Query  154  CGGGNTCACGAGGGTTTCTCAACCACCGATTGTCGTGGCGCTCGTCGCCTAGGACTCACT  213
            |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  627  CGGG-TCACGAGGGTTTCTCAACCACCGATTGTCGTGGCGCTCGTCGCCTAGGACTCACT  569

Query  214  TTTAGGCTAACCGCGAGCAAAAGCGCACGGGAGGCCAATGTCTTCCCCGCACCCCGCACA  273
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  568  TTTAGGCTAACCGCGAGCAAAAGCGCACGGGAGGCCAATGTCTTCCCCGCACCCCGCACA  509

Query  274  GCGTAAGAAGTGTTTGGGGGTTGGGGCAGACGATGCGTGACACCCAGGCAGACGTGCCCT  333
            |||||||||||||||||| |||||||| ||||||||||||||||||||||||||||||||
```

```
Sbjct  508  GCGTAAGAAGTGTTTGGGG-TTGGGGCA-ACGATGCGTGACACCCAGGCAGACGTGCCCT  451

Query  334  CGGCCGGAATGGCTTCGGGCGCAACTTGCGTTCAAAGACTCGTTATGATTCGCGGGATTC  393
            |||||| |||||||||||||||||||||||||||||||||  ||||||||||||||||
Sbjct  450  CGGCCG-AATGGCTTCGGGCGCAACTTGCGTTCAAAGACTCG--ATGATTCGCGGGATTC  394

Query  394  TGCAATTCACACCAAGTATCGCATTTTCCGGCCTACGTTCTTCATCGATGCGAGAGCCGA  453
            |||||||||||||||||||||||| | | | ||||||||||||||||||||||||||||
Sbjct  393  TGCAATTCACACCAAGTATCGCATTT-C-G-C-TACGTTCTTCATCGATGCGAGAGCCGA  338

Query  454  GATATCCGTTGCCGAGAGTCGTTTATGTATCATGGTAAAGACGTCTACCAACGACACGCA  513
            |||||||||||||||||||||| |||||||||||||||||||| ||||||||||||||
Sbjct  337  GATATCCGTTGCCGAGAGTCGTT-ATGTATCATGGTAAAGACGTC-ACCAACGACACGCA  280

Query  514  CACCGTTTCCGGGGCGCCCGTGGTTACTCCTTGTTTAAGTTCCTTGGCGCAGAACCGCGC  573
            |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  279  CACCGTTTCCGGGGCGCCCGTGGTTACTCCTTGTTTAAGTTCCTTGGCGCAGA-CCGCGC  221

Query  574  GCCCGGGGTTCATTGTTCGCATCGGGAAGGGAACGACGAAGGATCGCACCAACCCACACA  633
             |  |||||||||||||| |||||||||||||||| || |||||| ||||||| |||||
Sbjct  220  -C--GGGGTTCATTGTTCG-ATCGGGAAGGGAACGA-GA-GGATCG-ACCAACC-ACACA  169

Query  634  CGAGGAGCAGTGGGCAAATCTCAACGTGCCCTCCCAACCGTTG-CTGGGCAGG  685
            |||||||||||||||||||||||||||||||||||||||||||  ||||| |||
Sbjct  168  CGAGGAGCAGTGGGCAAATCTCAACGTGCCCTCCCAACCGTTTTCTGGG-AGG  117


 Score = 86.1 bits (46),  Expect = 2e-13
 Identities = 46/46 (100%), Gaps = 0/46 (0%)
 Strand=Plus/Minus

Query  675  TGCTGGGCAGGTATCGACAATGATCCTTCCGCAGGTTCACCTACGG  720
            ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64   TGCTGGGCAGGTATCGACAATGATCCTTCCGCAGGTTCACCTACGG  19
```

Appendix 8: Sample 15 consensus sequence and BLAST result

```
reverse         ------------------TGCATTCACACCAAGGTTATCGC--ATTTCGCTACGGTTTCT 40
15              ACTATATCATTACCATAGGGCAATTGAATATAGACGGCCGCGAATTTCGCCCTTTCCTCC 60
                                  *** *   *   **     ***  *******       **

reverse         --TCATCGATGCG---AGAGCCGA--GATATCCGTTGCCCGA---GAGTTCGTTTATGCA 90
15              GCTTATTGATATGCTTAAATTCAGCGGGTAATC-CCGCCTGACCTGGGGTCGCGATGGTA 119
                  * ** ***  *   * *  *    * **  *   *** **   * * ***     * *

reverse         TCATGGTAAAGACGTT-CACCAACGACACGCACACCGTTTTCCGGGGTCGTCCCGT-TGG 148
15              GAGTCGCAAGAACGACACAATAGGGTCGAGGAGACAACCTTCAACAGACGAACGGGACGA 179
                   * * **  ***   **  *  * *  * * **    ***    * **  * *   *

reverse         TTTACTCCTTGTTTTAA----GGTTCCTTGG-TCGCAGACCGTCGCCGGGGTTTCATGTT 203
15              CAAACACGACGGGTCAACGAGGGTTTCTCAAACCACCGATTGTCGTGGCGACT-----CG 234
                   ** *   *  * **    **** **     * * **  ****  * *  *

reverse         TCGATCGGGGAGGGAACGAGAGGATCGACCAACCACACACGAGGA--GCAGTGGGCAAAT 261
15              TCGCCTAGGAACTCACTTTTAGGACTAAACCGCGAGACAAAAAGACGACAACGGGGAAGG 294
                ***    ** *   *     ****   * *  * * ***  * **   **  *** **

reverse         CTCAACGTGCTCTCCCAAC-CGTTTTCTGGGAGGGGGCATTACACCCCCACCCAGAAGGT 320
15              GCCAATTGGTTCTTTCCCCGCAACCCCCGCAACAAGACGGTAAAGAAGTGTTTTGGGGGG 354
                  ***   * ***  *  * *    * *  *   * *  ** *          *  **

reverse         TTATTATTACAGTTCACAGGTCGTTCTGTCTGGGCAG---GTATC----GACAAGATC-C 372
15              GTTGGGGGGCAAACGA-ATGCCGTGACACCCAGGCAGAACGTGCCCTCGGCCGAAATGGC 413
                 *       **    * * * ***     *  *****   **  *    * * * **  *

reverse         TTC---CGCAGGTTCACCTACGGAAAGGGCGTA---ATTCGTTTAAACCTGCAGGACTAG 426
15              TTCGGGCGCAACTTGACGTTCAAAGANCTCGAATGAATTCGTCGGGATCTGCAATTCACA 473
                ***   ****  ** ** * *  * *   ** *   ******    * *****   *

reverse         TCCCTTTAGTGAGGGTTAA---------------------------------------- 445
15              CCCAAGTATTCGCATTTCGCTACGTTCTTCATTCGATGCGAGAGCGAAGATATTCCCGTT 533
                 **   ** *     **

reverse         ------------------------------------------------------------
15              GCCGAGAGTCGTTAATGCATCATGGTAAAGAACGTCACCCAACGAACACGCCACCACCGT 593


reverse         -------------------------------------------
15              TTCCCGGGCGCCGTGGGTAACTCCTTGGTTAAGTCCTGGCGAC 636
```

No significant similarity