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DNA Barcoding and Phylogenetic Characterization of Mushrooms with in the Adirondack Park

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Abstract

In recent years, fungal identification has risen in popularity. It is often difficult to identify mushrooms using the morphologically-based identification system, due to extreme similarities between species. There are also notable differences in species at a mature stage versus a young fruiting body. The internal transcribed spacer (ITS) of nuclear DNA has been used for over 15 years as a focus when analyzing fungal diversity. Only recently has ITS been deemed as the official marker used in fungal DNA barcoding. The Adirondack Park is a region consisting of 6 million acres of “forever wild” terrain. This hosts over 3000 lakes and ponds and 30,000 miles of streams and rivers in the heart of its boreal forests. Mushroom sampling was performed within this region. The DNA was isolated, amplified, and sequenced, using ITS6 and ITS8 primers. Ultimately, all DNA sequences were uploaded into an international DNA database.

Keywords: Mushroom, DNA Barcode, Internal Transcribed Spacer, Adirondack Park, ITS primers

1. Introduction

The Adirondack Park, New York state, USA (Figure 1) hosts some of the world's most spectacular flora and fauna, abundant producers and consumers of energy. The nutrient cycle, however, would not be complete without a very important group of organisms. The decomposers recycle nutrients back into the environment, disabling the accumulation of waste. Fungi play a very heavy role in the decomposition world. Mushrooms do not contain chlorophyll like a plant and are unable to produce their own energy; so like an animal, they must receive nutrients from an external source (Waggoner, 1995). These single to multicellular organisms also create symbiotic relationships with the forest's flora, creating mycorrhizal associations necessary for plant survival.

The Fungi commonly referred to as "mushrooms" are much more than just caps and stems. Whether it is from the ground or on a dying tree, the complexity of the Fungi lies past our peripheral vision and into the depths of their substrate. This network is known as the mycelium. The mycelium is the vegetative body of the organism, consisting of a network of filament like branches known as hyphae (Figure 2) (Lepp, 2012).

It is nearly impossible to identify the genus of a fungus by its mycelium, which is where the mushroom truly becomes of interest. The mushroom itself is the fruiting body of the mycelium. Like the apple of a tree, it lasts for only a short period of time, where the mycelium remains alive throughout the year. Mushrooms have become of popular interest to humans because of their nutritional value. Wild mushrooms, like morels and chanterelles are a delicacy all over the nation.

Proper identification is necessary to ensure consumption is not lethal. Fungi identification keys allow mushroom foragers to decipher a delicious or toxic specimen.

There is still much room for error using a dichotomous key as a method of identification, being that mushrooms of different species bear extreme similarities. DNA analysis rarely shows a false result (Newmaster et al 2013). DNA from mushrooms can be extracted, amplified and sequenced in order to compare genetic codes of specimens with those that have already been entered into the National Center for Biotechnology Information (NCBI). Specimens entered into this database were not from the Adirondack Park, so this allowed for the observation of genetic differences found between species within the Park and those from around the world.

By using both dichotomous keys and the ribosomal internal transcribed spacer DNA region, it is possible to create an Adirondack DNA survey of species found within the Park. With the information gathered, it may then be possible to follow the evolutionary path of these organisms through phylogenetic trees.

2. Methods and Materials

DNA extraction and amplification were performed using a SIGMA Extract-and-Amp Plant PCR kit, ITS8 forward and ITS6 reverse primers, (Forward ITS8 primer: 5'-AGT CGT AAC AAG GTT TCC GTA GGT G-3' and Reverse ITS6 primer: 5'-TTC CCG CTT CAC TCG CAG T-3'). For the DNA extraction, mushroom tissue was used in the place of the plant leaf disk. A 0.5 to 0.7 cm disk of mushroom tissue was obtained.

DNA (100-200 ng) was amplified using the following conditions: 95°C for 3 minutes followed by

35 repeating cycles of 95°C for 30 seconds, 51°C for 30 seconds and 72°C for 62 seconds. A final step of 72°C for 3 minutes was performed. The samples were visualized in a 1.25% agarose gel (stained with ethidium bromide) and viewed using a gel documentation system. DNA bands were excised from the gel and purified using the QIAquick Gel Extraction kit (Qiagen). The samples were sequenced using the Clemson University Genomics Institute's sequencing services.

Species identification was achieved using dichotomous keys (Figure 3) and through a BLASTn DNA sequence identification program. Thirty five mushroom sequences were uploaded to the National Center for Biotechnology Information (NCBI) DNA database, with each sequence being assigned an accession number (Table 1)

3. Results

After the surveillance of the Adirondack Park, a total of 56 samples were collected. Of the 56 mushrooms found, all 56 of them have been identified using dichotomous keys (Figure 3). Further verification through genetic sequencing has been done on 35 of these mushrooms (Table 1). This left 21 mushrooms that were only identified through use of the dichotomous keys (Table 2). A sampling of the mushrooms identified using dichotomous keys are pictured in in Figure 4. The sampling of mushrooms included multiples of the following genera: *Amanita* (4), *Boletus* (3), *Ganoderma* (2), *Hericium* (2), *Lactarius* (3), and *Trametes* (2) (Tables 1 and 2). The four DNA samples from the genus *Amanita* were compared through a CLUSTAL DNA sequence alignment software program (Figure 5). The sequences demonstrated DNA differences within these four species of *Amanita*. This was further illustrated within the phylogenetic tree of these

four samples (Figure 6). However, our survey also included several identical species having slightly different DNA sequences (Figure 7).

4. Conclusions

As in many research projects, we were not able to successfully isolate DNA from all of the samples. We did, however, identify 35 mushroom samples through DNA barcoding. These were the first published mushroom samples within the Adirondack Park to be barcoded. Samples were initially identified using dichotomous keys and verified with DNA barcoding. The barcoding indicated that, even with the same species, there were slight differences in the DNA sequences (Figures 7 and 8). For example, eight different *Piptoporus betulinus* sequences collected worldwide were compared through sequence alignment (not shown) and phylogenetic tree analysis. Even though they were from the same species, phylogenetic analysis reveals their relatedness (Figure 8). If each of these samples were not barcoded, then these differences would not be verified by dichotomous keys alone.

The use of molecular techniques has certainly increased data regarding mushrooms, indicating an increase of diversity (Blackwell, 2011). Collecting mushrooms from a variety of locations is important for many reasons: the need to identify specimens, revisions of taxonomies, assessment of place in nature, biological control, and pharmacology (Blackwell, 2011). Mushrooms have the potential to be antimicrobial compounds (Alves et al., 2015). Mushrooms have outstanding nutritional value (Heleno et al., 2010). There are researched cases of ~100 medicinal functions of mushrooms and fungi (Chang & Wasser, 2012; Hobbs, 1995)

Hopefully, by entering the DNA sequences into the NCBI database, this will make people more aware of the types of mushrooms that can be found within the Adirondack Park. This can raise

awareness to people foraging, that the physiological identification of a mushroom is not always correct, and that it is beneficial to be 100% certain of a species prior to consumption. Future plans include further surveying, identification (through dichotomous keys) and DNA sequencing of mushroom samples located in the Adirondack Park.

References:

Alves, M., Ferreia, I.F.R., Dias, J., Teixeira, V., Martins, A., and Pintado, M. (2012). A review on antimicrobial activity of mushroom (*Basidiomycetes*) extracts and isolated compounds. *Planta Medica,* 78:16, 1707-1718.

Blackwell, M. (2011). The Fungi: 1, 2, 3 ---5.1 million Species? *American Journal of Botany* 98(3): 426-438

Bryn Dentynger, Simona Magaritescu, Jean-Marc Moncalvo. "Rapid and reliable high throughput methods of DNA extraction for use in barcoding and molecular systematics of mushrooms." Molecular Ecology (2010): 628-633.

Chang, S.T. and Wasser, S.P. (2012). The role of culinary-medicinal mushrooms on human welfare with a pyramid model for human health. *International Journal of Medicinal Mushrooms* 14:2, 95-134.

DEC. Comprehensive Wildlife Conservation Strategy for New York. 2012. <http:// www.dec.ny.gov/docs/wildlife_pdf/lkchamplaintxt.pdf>.

Eva Bellemain, Tor Carlsen, Christian Brochmann, Eric Coissan, Pierre Taberlet and Havard Kauserud. "ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases." BMC Microbiology (2010).

Heleno, S.A., Barros, L., Soursa, M.J., Martins, A. and Ferreira, I.C.F.R. (2010). Tocopherols composition of Portuguese wild mushrooms with antioxidant capacity. *Food Chemistry* 119:4, 1443-1450.

Hobbs, C. (1995). Medicinal Mushrooms. Santa Cruz; Botanica Press.

Lepp, Heino. The Mycelium. 26 June 2012. 8 October 2012 <http://www.anbg.gov.au/fungi/mycelium.html>.

Newmaster, S.G., Grguric, M., Shanmughanandhan, D., Ramalingam, S. and Ragupathy,S.(2013). DNA barcoding detects contamination and substitution in North American herbal products. BMC Medicine, Article retrieved on February 4, 2016 from http://www.biomedcentral.com/1741-7015/11/222

Waggoner, Ben. Fungi. 1995. Retrieved February 11, 2016: http://www.ucmp.berkeley.edu/people/bmw/bmw.html.

Figure 1. Map of New York state indicating placement of Adirondack State Park.

www.pic2fly.com

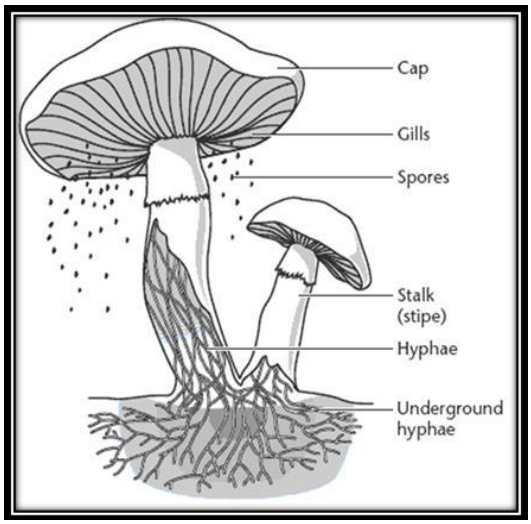

Figure 2. Diagram of typical mushroom with labeled parts. Image from

https://gcps.desire2learn.com/d2l/lor/viewer/viewFile.d2lfile/6605/8414/Parts%20of%20a%20Fungus.png

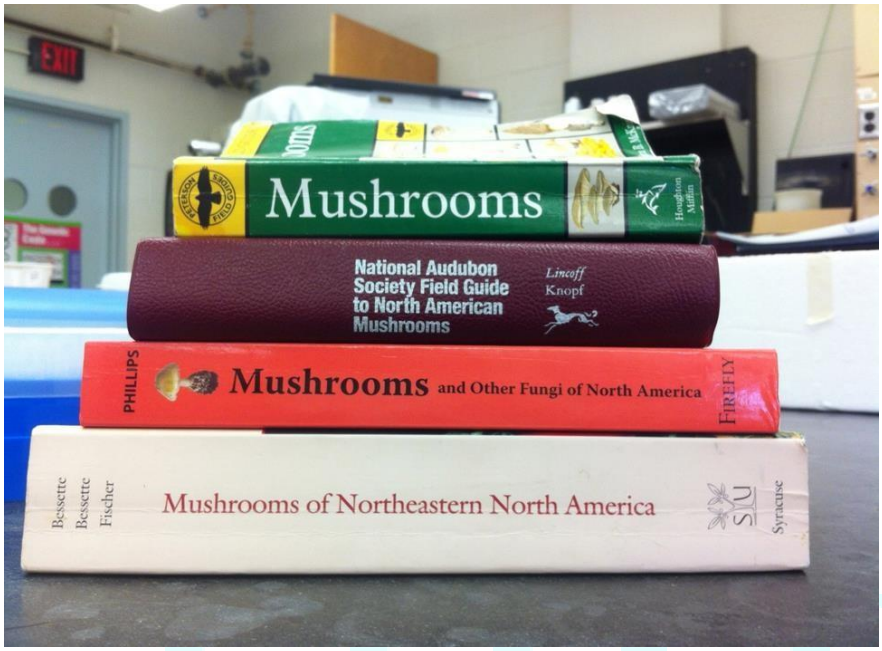

Figure 3. 1 2 3 4 Above are the dichotomous key guidebooks 5 6 that were used to identify mushroom samples.

7 8 9 10 11 12

14 15 23 17 18 20

21 22 24 25 26 27

13

19

16

Figure 4. Photograph samplings of Adirondack Park mushrooms.1.*Clavulina cinera 2. Mutinus caninus 3. Fomitopsis pinicola 4. Caprinoid sp.5. Fomes formentarius 6. Lycoperdon perlatum 7. Schizophyllum commune 8. Cerrena unicolor 9. Boletus edulis 10. Polyporus squamosus 11. Amanita citrina 12. Amanita muscaria 13. Lactarius fragilis 14. Amanita virosa 15. Hygrophoropsis aurantiaca 16. Amanita flavoconia 17. Boletus subvelutipes 18. Russula emetica 19. Artomyces pyxidatus 20. Entiloma sinuatum 21. Ganoderma aplanatum 22.*

Ganoderma tsugae 23. Oxyporous populinus 24. Lycoperdon perlatum 25. Omphalina epichisium

26. Cantharellus cibarius 27. Hericium erinaceus

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A.brunnescens       TTTTTATATACACACGGTTGTATGTCTATAGAATGAGA-TGTAGGCTTATTTAAAGCCAT
A.flavoconia        TTTTTTGACATACACGTTTGAATGTCTATAGAATGAAAATGTAGGCTT-TTGTCAGCCTT
A.virosa            TTTTATTACACAC-TAGTTGCATGTTTATAGAATGATGATTTG---------------AT
A.muscaria          TGTTCAGGTGTCTATGATTTTCTTTACATACA-TGAACACTTGTTGTA--CAGAATGTGA
* **           **   * *  *** * ***     *
A.brunnescens       TAAATGATAA-AGTACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
A.flavoconia        TAAATGATAA-AATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
A.virosa            TAAAT-ATAA-AATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
A.muscaria          TAAAAAATAGTAATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
****  ***  * ********************************************
A.brunnescens       GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
A.flavoconia        GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
A.virosa            GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
A.muscaria          GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
************************************************************
A.brunnescens       CGCATCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAAAGTATC
A.flavoconia        CGCATCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAAA-TATC
A.virosa            CGCACCTTGCACTCCTTGGCATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAA--CATC
A.muscaria          CGCATCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAAATTCTC
**** ***** ******** ********************************    **
A.brunnescens       TCAAAAGCTCTTATGTGTTATGCATATGGAACTTTTGGACAATTGGGAGTTGCCGGTCAC
A.flavoconia        TCAAAAAG-CTTGTGCTTTTTTGGCACAGGAGTTTTGGACA-TTGGGAGTTGCCGGCTGC
A.virosa            TCAAGA---CCTGTCTGTTTTTGATA----GGTATTGGATTTTTGGGGGTTGCAGGCTGT
A.muscaria          TCAAAG-----CATACACT-TGAGTGTG--TGTTTTGGATT-GTGGGAGTGTCTGCTGGC
****         *    * *           * *****    **** **  * *
A.brunnescens       TG-ATACAAGTGGTGGGCTCTTCTGAAAAGCATTAGTT-----GAGGAG----CTTTGCA
A.flavoconia        TGGATAACAGTGGTGGGCTCTTCTGAAAAGCATTAGTT-----GAGGAG----CTTTGCA
A.virosa            TT----CAGATAGCTTGCTCTCCTTGAATGTATTAGTG-----GAGAAAGAGCCATTGAA
A.muscaria          TT-----TATGAGCCAGCTCTCCTGAAAGACATTAGCTTTGGGGGGGGAGGTGCCAAGTCA
*           *   ***** **  **   *****       * * *     *     *
A.brunnescens       CT---------CTATTGGTGTGATAGA---------CTATCTATGCCAGGAGATGCATTA
A.flavoconia        CT---------CTATTGGTGTGATAGA---------CTATCTATGCCAGGAGACGCTTCA
A.virosa            CT---------CCATTGGTGTGATAAAA-------TCTATCAATGCCAGGAGCCATGTTA
A.muscaria          CTTCTGCCTTTCCATTGGTGTGATAGATGAATTAACTTATCTACGCCAGGAAAGCAGGCT
**         * ************ *          **** * *******
A.brunnescens       TTG-CCTCTGCTCTCTAACA----------GTCCTTAT----TGGACA---AGATGACGA
A.flavoconia        TGATCCTCTGCCATCTAACC----------GTCTTTAT----AAGACA---ATATGATAA
A.virosa            GTTCTCTCTGCTGTCTAACCCCTAACAGTTGTCTGTAAAAAATGGACA---ACTTGACCA
A.muscaria          GCAGGTGATGCACTGTGATCTCT--CTCTGCTCTCTAA----TTGACATTTGTCTGAT-A
***  * * *            **  **       ****      ***  *
A.brunnescens      ACTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTT
A.flavoconia       ACTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTT
A.virosa           ACTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTT
A.muscaria         ACTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTT
                   ************************************
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Figure 5. CLUSTAL W Multiple Sequence Alignment for the genius *Amanita*, * means the DNA sequence is identical. Sequences were analyzed using the CLUSTALW alignment program: http://www.genome.jp/tools/clustalw/

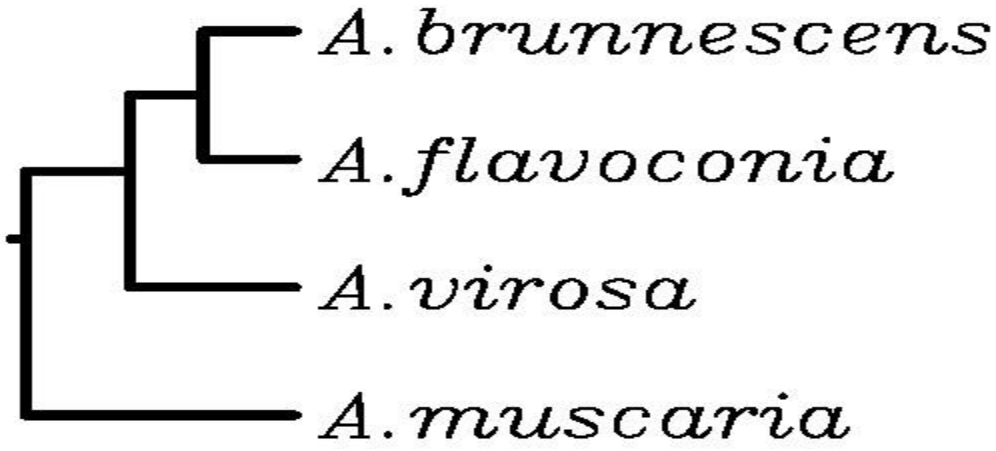

Figure 6. Rooted phylogenetic tree with branch length (UPGMA) from four species of the genus *Amanita* collected within the Adirondack Park. http://www.genome.jp/tools/clustalw/

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H.aurantiaca1        AAAGAGCCTATGTTTTTCTACACACCCAATTGTATGTCTATAGAATGTCTTTTTACAATT
H.aurantiaca2        AAAGAGCGTATGTTTTTCTACACACCCAATTGTATGTCTATAGAATGTCTTTTTACGATT
******* ********************************************** ***
L.griseus1           GGACTTTGGAGGCCTCTGCTGGCGTCTCTTGCCAGCTCCTCTCAAATGAATTAGCGGGGT
L.griseus2           GGACTTTGGAGGCCTTTGCTGGCGTCTCTTGCCAGCTCCTCTCAAATGAATTAGCGGGGT
*************** ******************************************

R.crustosa1          ACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCTCTAGTAACTGCG
R.crustosa2          ACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCG
********************************************** ************
```

Figure 7. Within the dichotomous key survey of the mushrooms, there were several samples that had the same genus and species, such as *Hygrophoropsis aurantiaca,* as shown above. Further analysis indicated sequence differences (highlighted in yellow). These include *H.aurantiaca*1 and 2 (Accession numbers KM373254 and KM37352); *L.griseus* 1 and 2 (Accession numbers KM373252 and KM373268) and *R.crustosa* 1 and 2 (Accession numbers KM373243 and KM373269). * means the DNA sequences are identical. Sequences were analyzed using the CLUSTALW alignment program: http://www.genome.jp/tools/clustalw/

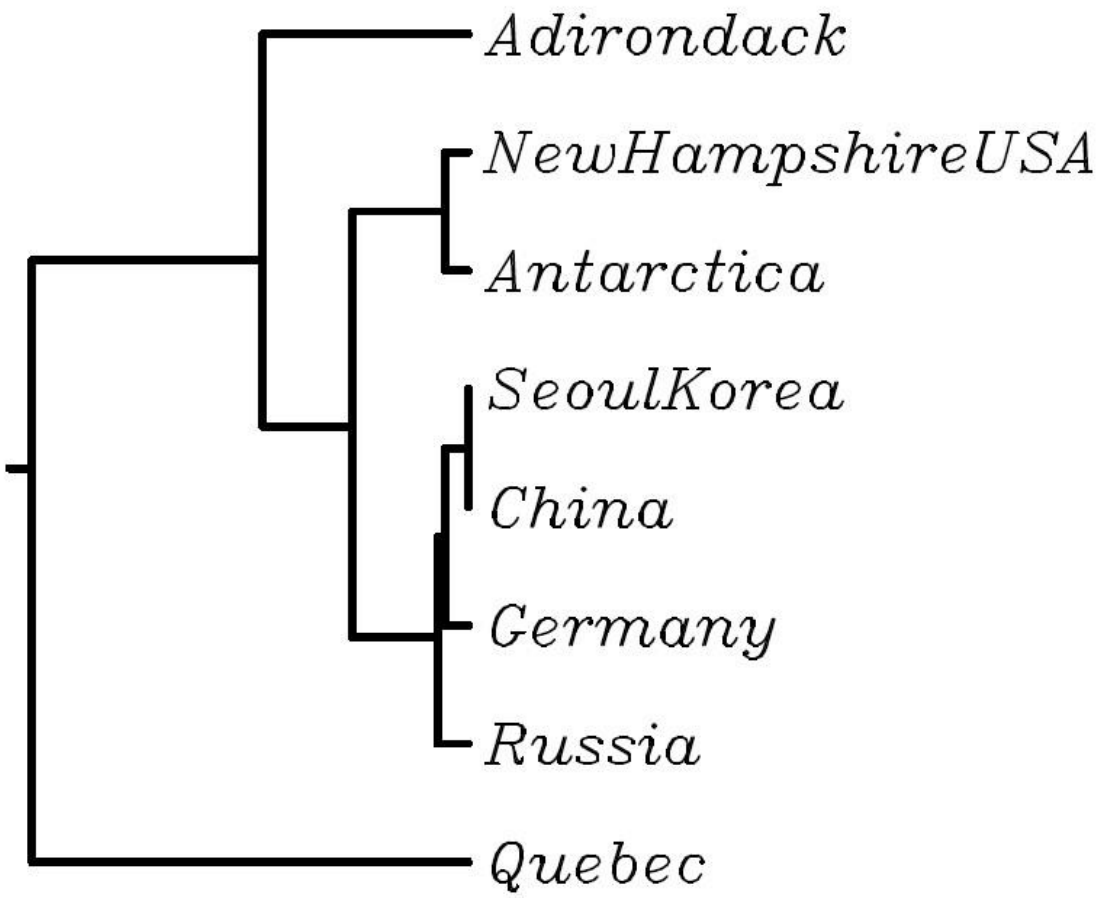

Figure 8. Predicted rooted phylogenetic tree of 8 different *Piptoporus betulinus* samples used for DNA barcoding. This tree was generated to show evolutionary relatedness. The accession numbers from the NCBI data base are: Adirondack KM373245; New Hampshire KC585371; Antarctica KM822755; Seoul Korea DQ491423; China KP171215; Germany FJ820597; Russia JX507733; Quebec KM248915. The phylogenetic tree was generated using http://www.genome.jp/tools/clustalw/.

Table 1. Mushrooms Identified by Dichotomous Key and DNA Barcoding	
Species	**Accession # NCBI Data base**

Agaricus bisporus	KM373263
Amanita brunnescens	KM373257
Amanita flavoconia	KM373250
Amanita muscaria	KM373246
Amanita virosa	KM373251
Artomyces pyxidatus	KM373237
Cerrena unicolor	KM373256
Clavulina cinerea	KM373238
Coprinopsis lagopides	KM373258
Curvibasidium cygneicollum	KM373247
Daedaleopsis confragosa (1)	KM373248
Daedaleopsis confragosa (2)	KM373255
Fomitopsis pinicola	KM373236
Gymnopus confluens	KM373266
Gymnopus dryophilus	KM373242
Hericium erinaceus	KM373260
Hygrophoropsis aurantiaca (1)	KM373254
Hygrophoropsis aurantiaca (2)	KM373264
Lactifluus griseus (1)	KM373252
Lactifluus griseus (2)	KM373268
Lactarius lignyotus	KM373270
Lactarius tabidus	KM373241
Lycoperdon pyriforme	KM373261
Megacollybia rodmanii	KM373244
Morganella subincarnata	KM373265
Mutinus caninus	KM373259
Oxyporus populinus	KM373249
Piptoporus betulinus	KM373245
Pluteus hongoi	KM373267
Royoporus badius	KM373240
Russula crustosa (1)	KM373243
Russula crustosa (2)	KM373269
Russula emetica	KM373253
Schizophyllum commune	KM373262

Trametes gibbosa	KM373239

Table 2. Mushrooms Identified by Dichotomous Key Only

Ganoderma tsugae	*Lactarius camphoratus*
Lycoperdon perlatum	*Trametes versicolor*
Fomes fomentarius	*Entiloma lividium*
Russula vinacea	*Polyporus squamous*
Omphalina epichysium	*Calvatia gigantica*
Amanita citrina	*Phaeolus schweinitzii*
Ganoderma applanatum	*Cantharellus cibarius*
Craterellus tubaeformis Hericium coralloides Morchella esculenta Boletinellus merulioides	
Boletus edulis	*Caprinoid sp.*
Boletus subvelutipes	