Classification, biogeography, and phylogeny of Northern Hemisphere *Lentinellus* species

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UNIVERSITY HONORS PROGRAM

SENIOR PROJECT - APPROVAL

Name: Laura McGhee

College: Arts & Sciences
Department: Microbiology

Faculty Mentor: Dr. Karen Hughes

PROJECT TITLE: Classification, biogeography, and phylogeny of Northern Hemisphere lentineellus species

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: Karen Hughes, Faculty Mentor

Date: May 7, 1999

Comments (Optional):
Classification, biogeography and phylogeny of Northern Hemisphere

*Lentinellus* species

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**Introduction**

*Lentinellus* is a large genus of Basidiomycete fungi consisting of approximately 15 known species. In the temperate forests of Eurasia and North America, there are at least six species, *L. ursinus*, *L. vulpinus*, *L. angustifolius*, *L. omphalodes*, *L. montanus*, and *L. micheneri* but these species are poorly delineated based on morphology. Their range and distribution is also unknown.

The purpose of these studies was to develop diagnostic molecular characters that delineate species, to determine the geographical distribution of each species, to determine if gene exchange is occurring between geographically separate populations within species and to develop a phylogeny based on molecular characters. Species delineated by molecular methods were compared with the morphological species and biological species to determine the cohesiveness of these concepts. There seems to be a second biological species sheltered under *Lentinellus omphalodes* and this unnamed taxon was also examined.

During these studies, a type I intron was identified within the ribosomal 18S gene. A type I intron is a mobile, self-replicating piece of DNA. The intron is transcribed but not translated. The length of this intron is about 350 base pairs long. The presence or absence of this intron was determined for all species and collections and the phylogenetic relationships of the intron and *Lentinellus* species (based on the ribosomal ITS sequence) were compared.

**History of the Genus**

Many of the species currently in *Lentinellus* were originally placed in *Agaricus* (Fries 1821), then later, segregated these species into the genus *Lentinus* (Fries 1863). Karsten established *Lentinellus* over 100 years ago with the species *L. cochleatus*, *L. friabilis*, *L. omphalodes*, *L. umbellatus* with type specimens from France (Karsten 1879). Into this genus, he placed taxa previously included in *Lentinus*, which were originally placed in the genus *Agaricus*.

At the same time as Karsten proposed *Lentinellus*, he also proposed *Hemicybe* that included some other taxa from *Lentinus* (Karsten 1879).

*Lentinellus ursinus* was discovered in 1821 by Fries and was first called *Agaricus ursinus* (Fries 1821). In 1825, it was moved to *Lentinus* and renamed *Lentinus ursinus* by Fries (Fries 1825). Karsten moved the species to *Hemicybe* and renamed it *Hemicybe ursina* in 1879 (Karsten 1879). According to Miller, in 1915, Murrill moved the species to *Panellus* and renamed it *Panellus ursinus* (Miller 1997). According to Miller and Stewart (Miller 1971), the species was transferred to *Lentinellus* in 1926 and renamed *Lentinellus ursinus* by Kuhner.
Lentinellus ursinus has also been called Lentinellus castoreus in 1946 (Romagnesi 1946). Miller and Stewart decided in 1971 that Lentinellus castoreus was the European version of the North American Lentinellus ursinus (Miller 1971). Fries had added the species to Lentinus in 1838 as Lentinus castoreus (Fries 1836-1838). Karsten moved Lentinus castoreus in 1879 to Hemicybe and renamed Hemicybe castorea (Karsten 1879). According to Miller, (Miller 1997), the species was placed in Lentinellus under the new name of Lentinellus castoreus in 1936 by Konrad and Maublanc.

According to Miller and Stewart, (Miller 1971), Lentinellus ursinus has also been called Lentinus anastomosans in 1938 by Rick. Lentinellus ursinus has also been labeled Lentinus hepatotrichus by Berkeley in 1860 (Berkeley 1860). In 1880, Kalchbrenner placed Lentinellus ursinus in Lentinus hyracinus (Kalchbrenner 1879-1880). Romagnesi christened the species as Lentinellus pusio in 1965 (Romagnesi 1965).

Lentinellus angustifolius was originally placed in Lentinus by Romell in 1901 as Lentinus angustifolius (Romell 1901). In 1952 Singer transferred the species to Lentinellus as Lentinellus angustifolius.

In 1803 Lentinellus vulpinus was classified as Agaricus vulpinus by Sow (Sow 1803). In 1821 the species was reconfirmed by Sow as being Agaricus (Sow 1821). According to Miller, in 1836, it was moved to Lentinus under the name Lentinus vulpinus (Miller 1997). It was transferred to Hemicybe by Karsten in 1879 (Karsten 1879). According to Miller, (Miller 1997), Murrill moved the species to Panellus in 1915. In 1934, the species was moved to Lentinellus by Kuhner and Maire (Kuhner 1934). Stalpers does not recognize the move to Panellus as being legitimate (Stalpers 1996).

Other names for Lentinellus vulpinus according to Miller were Lentinus auricula given in 1861 by Fries (Fries 1863), Lentinus hygrophanus given by Harz in 1889 and Lentinus tomentellus given by Karsten in 1887 (Miller 1997). The species was also classified as Hemicybe tomentella by Karsten in Hemicybe in 1889 (Miller 1997).

Lentinellus omphalodes was established by Karsten in 1879 (Karsten 1879). Before the establishment of this genus it was called Lentinus omphalodes by Fries since 1863 (Fries 1863).

Lentinellus cochleatus has also been called Agaricus cornucopioides (Bolton 1788), Agaricus cochleatus (Fries 1821), Lentinus cochleatus (Fries 1836-1838), according to Stalpers Lentinus umbellatus by Peck in 1876 (Stalpers 1996), Clavicorona dryophila (Maas 1976), Lentinellus umbellatus, and Lentinellus cornucopioides by Murrill (Miller 1997). It was finally transferred to L. cochleatus in 1971 by Miller and Stewart (Miller 1971). Miller and Stewart do not recognize the transfer to C. dryophila as legitimate (Miller 1971).

Lentinellus micheneri has also been known as Agaricus dentatus (Fries 1821) and Lentinus omphalodes (Fries 1863) before it was established as a separate species (Miller 1971).
According to Stalpers, *Lentinellus flabelliformis* has also been called *Claudopus subargillaceus* by Kauffm, *Lentinus scoticus* by Berkeley and Br., *Lentinus bisus* by Quel., and *Lentinus americanus* by Peck (Stalpers 1996).

*Lentinellus montanus* is a new species that was discovered and named by O.K. Miller in 1965 (Miller 1965).

**Methods and Materials**

**Collections**

Collections used in this study are given in Figure 1

**DNA Extraction/Preparation**

Cultures were maintained on Potato Dextrose, PD, agar slants at 4°C until ready for use. A portion of the culture was removed and half of the removed portion was put into liquid Potato Dextrose Media and half was transferred to a new Potato Dextrose agar slant tube. Both of these were incubated at 27°C for three weeks. At the end of three weeks the slant tube was returned to cold room storage if it had not become contaminated. The liquid culture was drained and the tissue was pressed to remove as much media as possible. The tissue was weighed, and 0.3-0.4g of tissue was removed from the culture tissue for DNA extraction. Carlson-Lysis buffer (Carlson et al 1991) and β-mercaptaethanol were heated to 74°C. The fungal tissue was ground with sterile sand and the hot Carlson-Lysis buffer and incubated at 74°C for one hour with inversion every 10 minutes. Cell debris and sand were sedimented by centrifugation for 10 minutes at 10,000 rpm. The supernatant was removed and chloroform added to precipitate proteins and polysaccharides while leaving the DNA in suspension. After shaking, the sample was centrifuged to separate the DNA from the proteins and polysaccharides. The top level was removed, being careful not to remove polysaccharides with it. Isopropanol was added to precipitate the DNA and the sample was incubated at room temperature for 30 minutes. The DNA was pelleted at the bottom of the tube by 10 minutes of centrifugation. DNA was washed off the sides with ice-cold ethanol to remove the isopropanol. The sample was centrifuged for 10 minutes to pellet the rest of the DNA. The pellet was dried and resuspended in TE buffer. The DNA was ready for further analysis.

**PCR Amplification**

DNA extracted from cultured tissues was used as a substrate for Polymerase Chain Reaction (PCR) amplification of the Internal Transcribed Spacer Region (ITS) between the 18 S ribosomal subunit gene and the large ribosomal subunit gene. This area is divergent enough to compare species within the same genus. The standard PCR reaction contained the following ingredients at the specified amounts:

- ddH20: 27.75μl
- 10X MgCl2 Free Buffer: 5μl
- MgCl2 25mM: 6μl
- dNTP mix 10μM each: 8μl
ITS4 primer 1µl
ITS5 primer 1µl
Taq polymerase 0.25µl
DNA 1µl

The thermocycler used was an Ericomp Single Block™ System in the Easy Cycler™ Series. Cycle times: Heat at 94°C for 3 minutes. Thirty-five cycles of one minute at 94°C, one minute at 52°C, and one minute at 72°C. Three minutes at 72°C. Store at 4°C when finished. PCR products were electrophoresed on a 1.5% agarose gel in TBE buffer to determine if amplification occurred.

When the DNA had been frozen for long periods of time, it would not amplify under standard conditions. To overcome this problem, Eppendorf made a Taq Enhancer that enables Taq polymerase to remain attached to the DNA strands for longer periods of time. With the addition of 20% Taq Enhancer, the PCR reaction proceeded and amplification occurred. The reaction mix for the PCR reaction with the Taq Enhancer was as follows:

- ddH20 20.75µl
- 10X Buffer with 15 mM MgCl₂ 5µl
- MgCl₂ 25 mM 3µl
- dNTP mix 10µM each 8µl
- Taq Enhancer heated to 65°C 10µl
- ITS4 primer 1µl
- ITS5 primer 1µl
- Taq polymerase 0.25µl
- DNA 1µl

PCR products were electrophoresed on a 1.5% agarose gel in TBE buffer to determine if amplification occurred.

The primers used throughout the standard PCR reactions are ITS-4 and ITS-5 primer. ITS-4 primer is a reverse primer that runs from the large ribosomal subunit gene into the internally transcribed spacer region. ITS-5 primer is a forward primer that starts in the 18S ribosomal subunit gene and runs to the large ribosomal subunit gene. (Diagram of ITS area is given in Figure 2. The sequence of the ITS-4 primer is TCCTCCGCTTATGATATGC (White et al 1990). The sequence of the ITS-5 primer is GGAAGTAAAAGTCGTAACAAGG (White et al 1990). These primers are also used for the sequencing of this region.

**PCR Amplification of part of the 18S ribosomal DNA.**

In the survey of the collections of *Lentinellus*, a portion of the 18S ribosomal RNA gene was PCR amplified to determine if the Group I Intron was present. Amplification of part of the 18S ribosomal RNA gene was accomplished using the primers SR1c and NS6. SR1c is a forward primer of the sequence, AGCAGCCGCGGTAA, (Hibbett 1992), while NS6 is the reverse primer that has a sequence of GCATCACAGACCTGTATTGCCTC, (White et al 1990).
The thermocycler used was an Ericomp Single Block™ System in the Easy Cycler™ Series. Cycle times: Heat at 94°C for 4 minutes. Thirty cycles of thirty seconds at 94°C, thirty seconds at 60°C, and two minutes at 72°C. Three minutes at 72°C. Store at 4°C when finished.

PCR reaction mix:

- ddH2O 30.75μl
- 10X MgCl2 Free Buffer 5μl
- MgCl2 8μl
- dNTP mix 10μM each 3μl
- SR1c primer 1μl
- NS6 primer 1μl
- Taq polymerase 0.25μl
- DNA 1μl

Reaction mixes were electrophoresed on a 1.5% agarose gel in TBE buffer to determine if amplification occurred. Hae III – digested Phi X was used as a molecular weight marker.

RFLP Analysis

ITS sequences of exemplars of 10 species of *Lentinellus* were examined to identify sequence differences that were diagnostic of each species. Restriction enzymes that recognized these differences were identified using the ‘map’ program in GCG and ‘Rebase’ (http://www.neb.com/rebase/rebase.html) to determine if the enzymes were commercially available. The PCR Products were digested according to manufacturer’s directions as follows.

Restriction Digest mix:

- ddH2O 4μl
- 10X Buffer 1μl
- DNA 4μl
- Enzyme 1μl

Samples were incubated at optimal digestion temperatures for each enzyme. The sample mixes were electrophoresed on 1.5% agarose gel in TBE buffer to determine the length of the restriction fragments.

Purification of the PCR Product and Sequencing

Four 50 μl PCR products were combined and electrophoresed on a 1.5% Nuseive GTG low melting temperature agarose gel with TAE buffer, and ethidium bromide. This separated the strands based on size. The dominant band at ~700 base pairs was excised with a sterile scalpel and placed in a microcentrifuge tube and heated to 70°C until all the agarose is melted. Using Quiagen’s Wizard Purification protocol, the PCR product is separated from the agarose and suspended in 70°C water so that it can be sequenced. The PCR product is amplified again with dideoxynucleotides using the ITS-4 and ITS-5 primers. The machine used in the Biology Sequencing Service Facility at the University of Tennessee is an ABI automated sequencer. The
ABI automated sequencer produces electropherograms of the amount of color tags that appear at each position.

Aligning Sequences

The sequences using the ITS-5 primer and the ITS-4 primer were automatically compared to each other using the ‘gap’ sub-program of the GCG program. The sequences were manually corrected based on the electropherograms from the automated sequencer. The ITS1-5.8S-ITS2 DNA sequences of multiple isolates were compared using ‘pileup’ and ‘lineup’ sub-programs in GCG (Genetics Cooperative Group). ‘Lineup’ incorporated multiple sequences while ‘Pileup’ did an initial alignment. The initial alignment was manually corrected using SeqLab in the GCG program.

Estimating a Phylogeny

The pileup file, **.msf, was adapted to work within the PHYLP program. Phylogenies were estimated using Neighbor-Joining and the strength of the branches was examined by Bootstrapping. The Neighbor-Joining program uses a Jukes-Cantor distance matrix to determine evolutionary relationships. Bootstrapping does 100 random replacements to evaluate the strength of the branches. For example, if one base replacement changes the whole topology of the tree, the branch supporting that area of the tree is very weak.

Results

Phylogeny of Lentinellus species based on sequences of the ribosomal ITS region

Aligned ITS sequences are given in Fig.3 for exemplars of each of the Lentinellus species in this study. Phylogenetic analyses (Figures 4-6) show that *L. vulpinus* and *L. cochleatus* form a single clade, differing from each other by 72 base pairs. The two collections of *L. ursinus* formed a second distinct clade. Two collections of *L. angustifolius* from the U.S. Southeast, formed a third clade and had identical sequences. *L. omphalodes* (Mating group IX), *L. montanus* and *L. micheneri* form a closely related group but another specimen identified morphologically as *L. omphalodes* (collection 9981) formed a separate clade. This collection also did not mate with *L. omphalodes* (R. H. Petersen, Pers. Com.) and is probably a new species.

Neighbor-Joining and Parsimony analyses are two different ways to group isolates. Neighbor-Joining analysis groups according to overall similarity, not according to evolutionary relationships. This method is acceptable because generally isolates that are the most similar to each other are usually the most closely related. Parsimony analysis groups according to evolutionary relationships. According to the phylogenetic trees generated with Parsimony and Neighbor-Joining analyses, the overall topology of the trees were very similar. The only difference came from the placement of collection 9981. In the Neighbor-Joining tree 9981 was in the same clade with collection 8452 from Mexico. In the Parsimony tree, collections 9981 and 8452 are not in the same clade (Fig 5 and 6). It is not known at the present time if these are the same biological species according to mating studies.
Restriction Length Fragment Polymorphisms (RFLP)

Specific restriction enzymes were identified that separated the different species of Lentinellus. Eco RI separated L. ursinus from all other Lentinellus species in this study, Figures 3 and 7, however, within L. ursinus, there were five collections that did not show the typical L. ursinus Eco RI RFLP pattern, Figure 1. Comparison of L. ursinus collections 2210 and 9986 showed that the loss of a restriction site was due to a single base pair substitution in which 9986 lost an adenosine base in the recognition site of the enzyme.

Eco RI separated L. ursinus from all other Lentinellus species in this study, Figures 3 and 7, however, within L. ursinus, there were five collections that did not show the typical L. ursinus Eco RI RFLP pattern, Figure 1. Comparison of L. ursinus collections 2210 and 9986 showed that the loss of a restriction site was due to a single base pair substitution in which 9986 lost an adenosine base in the recognition site of the enzyme.

TaqI separates L. angustifolius and L. vulpinus from all other Lentinellus species, however, these two species are not phylogenetically related and this similarity apparently represents convergent evolution, Figure 3. Other restriction enzymes were used to try to distinguish between species but these were not species-specific (Hpa II, Hinf I, Cla I and Rsa I).

Group I Intron

Results of PCR amplifications to determine the presence or absence of a group I intron are given in Figure 1. The Group I Intron in the 18S ribosomal DNA (Diagram of Group I Intron Figure 8) seems to have a geographic and species distribution. The Group I Intron occurs most frequently in the Southern United States around the Appalachian Mountains as can be seen in Figures 9-16. It occurs uniformly in L. omphalodes (Mating group IX), L. micheneri and L. omphalodes (Mating group VIII). It appears to be variable in L. angustifolius. Thus far the Group I Intron has not been found in L. ursinus. The phylogeny of the Group I Intron is similar to the phylogeny of the ITS region of the isolates containing the Group I Intron as can be seen in Figure 17.

Analysis of Placements

Lentinellus is closely related to the genera, Clavicorona and Panellus. At different times in history, there has been much debate about whether some Lentinellus species belong to these genera. The placement of L. vulpinus into the genus Panellus was not justified as shown by the alignment between 7996 and a Panellus isolate. The comparison between a Panellus isolate and L. vulpinus isolate can be seen in Figure 18. There is little similarity between the isolates. This data supports the stand taken by Stalpers that this species belongs in Lentinellus. The placement of L. cochleatus in the Clavicorona genus was not justified as shown by the alignment of 9985 and a Clavicorona isolate. The comparison between the Clavicorona and L. cochleatus can be seen in Figure 19. There is little similarity between these isolates. This data supports placement of this species in Lentinellus by Miller and Stewart.

Conclusions

Phylogenetic trees generated by neighbor-joining and parsimony analysis correspond well to the mating study data. Mating groups correspond to clades identified by phylogenetic analysis (Fig. 1 and Fig. 5). Branch lengths suggest that L. vulpinus and L ursinus are well separated from the remainder of the Lentinellus species and from each other. L. omphalodes
(Mating group IX), *L. montanus* and *L. micheneri* are closely related but still form separate clades and thus the separation of the latter two from *L. omphalodes* is justified. An unexpected finding was the separation of *L. omphalodes* (Mating group VIII) into a distinct clade associated with *L. sp.* from Mexico. This suggests that morphology was conserved or was convergent but that these are indeed separate species.

The biogeography of many of the species was previously unknown. The *L. ursinus* clade groups the two isolates from NC and SC. The mating study data indicated that the biological species was definitely cosmopolitan in its range. Samples from Russia, Sweden, Mexico and the United States confirmed this wide geographic distribution. The *L. angustifolius* clade groups two isolates that are very similar in sequence and are able to mate with each other. The isolates come from different areas within the southeast. The mating study data indicated that the biological species was cosmopolitan in its range. Collections from Russia, Austria, Costa Rica, Australia, and the United States confirmed this wide geographic distribution. The *L. cochleatus-L. vulpinus* clade occupies a boreal forest climate from MN and Austria. The *L. montanus-L. omphalodes* clade occupies a northern United States distribution. *L. montanus* according to Miller has a geographical range from Montana to Washington to Oregon (Miller, 1965). In the *Lentinellus omphalodes* complex (*L. omphalodes* mating group IX, *L. micheneri, L. montanus* and *L. omphalodes* Mating group VII), clades corresponding to the following geographic areas; boreal forests of North America and Europe, the TN/NC area, and global Northern Hemisphere. *Lentinellus omphalodes* inhabits a northern boreal forest climate with a short growing season and cool summers. The collections from Finland, Russia, Sweden and Alaska confirmed the northern boreal forest distribution of *L. omphalodes*. *Lentinellus micheneri* inhabits a southern Appalachian climate with a longer growing season and mild winters. Mating study data indicated that the TN/NC *L. omphalodes* were one biological species. The remaining clade is a cosmopolitan group containing isolates from Mexico, Austria, and Washington and may be comprised of more than one species.

The intercontinental distribution of *L. omphalodes* mating group IX has several possible explanations: 1) There is intercontinental gene flow by spores or by human-mediated transport of wood; 2) There is no significant intercontinental gene flow and the current intercontinental distribution represents an ancient connection between the continents. The most recent connection was via a North Atlantic land bridge in the Tertiary Period (Graham 1993); 3) The constipated duck theory states that birds and other animals carry the spores and tissue to other continents in their feces and on their bodies. The biogeographical disjunct between the species, *L. omphalodes, L. micheneri, and L. montanus*, is similar to the disjunct seen in *Flammulina* (Petersen and Hughes, Pers. Com.) and *Pleurotus* (Vilgalys and Sun 1994).

Collection 9981 is an unknown species at present. Morphological examinations need to be conducted to determine if it is a known species.

*L. vulpinus* and *L. cochleatus* are in the same clade. Based on sequence data alone, they are probably not the same species. The percent difference between the *L. vulpinus* and *L. cochleatus* isolates is 10.9%. Normally, disjunct populations of the same species have a percent difference of about 1%. To determine if this assumption is correct, mating studies need to be conducted to see if these two species inter-mate.
L. ursinus from NC and SC were sequenced. The sequence differences suggest some divergence in the Appalachian area. This does agree with other studies suggesting a high level of diversity in this region (Currie and Paquin 1987).

Diagnostic Molecular Characters to Delineate Species

There are few distinguishing morphological characters that can be used to characterize each of the different species of Lentinellus and these characters vary significantly with the age of the mushroom (Miller 1997). Restriction Fragment Length Polymorphisms can be used as molecular tools to help distinguish species. For example, L. ursinus can be distinguished uniquely by the presence of two Eco RI sites (if there are two sites present, the species is L. ursinus), yet some L. ursinus isolates do not have the second Eco RI site and will be missed by this diagnostic character. Taq I separates L. angustifolius, and L. vulpinus from all the other species. The sites that Taq I recognizes are not the same in these two species however, and there are difficulties with similar sized fragments that are produced upon digestion. By sequence comparison, two enzymes have tentatively been identified to distinguish species when used in combination, Mbo II and Sph I. Future studies will test these to see if they are reliable.

Group I Intron

The intron was probably vertically transferred. The phylogenetic relationship of species with the intron indicates that the intron was either lost or gained in an ancestor of present day species (Figure 20). If the intron had been horizontally transferred, there would have been no phylogenetic signal and the placement of the intron would be random, however, that is not the case. Species with the intron are phylogenetically related. Within Lentinellus, the loss or gain of the intron was apparently due to a single event that then evolved. The comparison of the intron tree vs. the ITS tree shows that there is some similarity between the two trees (Figure 17), however there are some major differences. The two trees are not entirely congruent. There are several explanations for these differences in the intron tree and the ITS tree. The two genes used for comparison may have evolved independently of each other and at different rates. The other explanation is that there is another mechanism at work here that is unknown at the present. As far as the geographical distribution of the intron, the intron could have been spread the same way that the organism was spread.

Further evidence for an ancient intron insertional event can be found by examining members of the family Auriscalpiaceae, including Lentinellus, Clavicorona and Auriscalpium, all of which have this intron. Comparison of a phylogenetic tree based on ribosomal 18S sequences with a phylogeny based on intron sequences shows concordance between these trees and indicates that this is an old element in this family (Ed Lickey, Pers. Com.).

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References


Figures
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Diagram of ITS region

ITS region PCR product ~700bp

18S ribosomal RNA gene → 5.8S ribosomal RNA gene → Large ribosomal subunit gene

Figure 2
Figure 3

MSF File with Eco RI sites Highlighted in Blue with red lettering of the recognition site.
MSF file with Taq I sites Highlighted in Yellow with Purple lettering of the recognition site.

9985 L. cochleatus
7966 L. vulpinus
9986 L. ursinus
2210 L. ursinus
8685 L. angustifolius
4101 L. angustifolius
9978 L. omphalodes
4243 L. omphalodes
6701 L. omphalodes
1159 L. montanus
5702 L. omphalodes
9159 L. micheneri
8452 L. sp. nov. 1
9981 L. sp. nov. 2
4027 L. sp. nov. 3

9985 L. cochleatus
7966 L. vulpinus
9986 L. ursinus
2210 L. ursinus
8685 L. angustifolius
4101 L. angustifolius
9978 L. omphalodes
4243 L. omphalodes
6701 L. omphalodes
1159 L. montanus
5702 L. omphalodes
9159 L. micheneri
8452 L. sp. nov. 1
9981 L. sp. nov. 2
4027 L. sp. nov. 3

9986 L. omphalodes
4243 L. omphalodes
6701 L. omphalodes
1159 L. montanus
5702 L. omphalodes
9159 L. micheneri
8452 L. sp. nov. 1
9981 L. sp. nov. 2
4027 L. sp. nov. 3

9985 L. omphalodes
9978 L. omphalodes
4243 L. omphalodes
6701 L. omphalodes
1159 L. montanus
5702 L. omphalodes
9159 L. micheneri
8452 L. sp. nov. 1
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5702 L. omphalodes
9159 L. micheneri
8452 L. sp. nov. 1
9981 L. sp. nov. 2
4027 L. sp. nov. 3

TGCGGAAGGA  
332 TCGA

Eco RI site == GAATTC

Taq I site == TCGA
Figure 4: Lentinellus Phylogeny

- **Russia**: 9978
- **Sweden**: 4243
- **Alaska**: 6701
- **Austria**: 9985
- **Minnesota**: 7966
- **South Carolina**: 9986
- **North Carolina**: 2210
- **Louisiana**: 8685
- **North Carolina**: 4101
- **Australia**: 4027
- **Mexico**: 8452
- **Austria**: 9981
- **Montana**: OKM
- **Washington**: 5702
- **Tennessee**: 9159

**Species**:
- **L. omphalodes**
- **L. cochleatus**
- **L. vulpinus**
- **L. ursinus**
- **L. angustifolius**
- **Species novum 1**
- **Species novum 2**
- **L. montanus**
- **Species novum 3**
- **L. micheneri**
Lentinellus Phylogeny
Parsimony Analysis

Species novum 1
Species novum 2
Species novum 3
Species novum 4

Figure 5
Figure 6

Neighbor-Joining Tree

L. cochleatus

L. omphalodes

L. micheneri

L. montanus

L. vulpinus

L. ursinus

L. angustifolius

New species

New Taxon

9985
7966
2210

9986

8685

4101

4027
Pattern 1

Pattern 2

Eco RI Banding Pattern

- Pattern 1 is *L. ursinus*
- Pattern 2 is any species
Diagram of 18S intron

SR1c       NS6

Intron Location (if present)

Figure 8
Introns within *Lentinellus vulpinus*

Figure 10

- No Group I Intron
- Group I Intron
- Heterozygous
Introns within *Lentinellus ursinus*

Figure 11

- No Group I Intron
- Group I Intron
- Heterozygous
Introns within *Lentinellus angustifolius*  

**Figure 12**

- **No Group I Intron**
- **Group I Intron**
- **Heterozygous**
Introns within *Lentinellus montanus*
Introns within *Lentinellus omphalodes* (IX)

Figure 14
Introns within *Lentinellus omphalodes* (VII) *L. micheneri*

**Figure 15**

- ● No Group I Intron
- ○ Group I Intron
- ● Heterozygous
Introns within *Lentinellus omphalodes* (VIII) New Taxon

Figure 16

- ● No Group I Intron
- ○ Group I Intron
- ● Heterozygous
Figure 17

18S Intron Tree

L. omphalodes

dshlomph

L. montanus

okm

L. micheneri

L. angustifolius

8685

L. sp. nov. 3

ITS Tree

4101

9159

6701

4243

9159

6701

okm

4101

8685
Figure 18
Panellus and Lentinellus
Percent Similarity: 65.719
7966 Lentinellus x 2675 Panellus

Lentinellus  1 ATAAAAGTGTAACAGTGTTTCCGTAGTGACCTGCGGAAGGATCATTA 50
  1 .......................................................... 38
  51 TCGAAAAACAAGGCGGCTAGGGCTGCTGCTGCCCTCCCTCAGGGGGGG 100
  31 TTGAATAACGCTTTTGGGAGGGTGGAGCTGGCTCTTT.............TCG 66
  101 GGGCATGTCACGCCGCGTGATCTCTTCACCCCTGACCTCTCTG 150
  67 AGCGATGTGCTCGCTACAAATGTTAAATCTCTACCTGACCTCTG... 113
  151 CGTGGTTTCTCGGCTTGGAGGCTCCCCTTCCTTCATACAC 200
  114 ........TTTGAGTGTCCTGGAAGAGCCACGTGTTATACATATTCAA 157
  201 CACCTTTTGATCTTTACGAGATTACATCGATAAAAGCATCTACTAA 250
  158 AGTTACGAAATGCTCTTGAGGCTTTATAA............AGTTAATAA 194
  251 CAACCTTCTAACACAGGATATCTCTTTCGAGGCTCCCCTCCCTACCA 300
  195 CAACCTTTCAACACGGGATCTCTTTCGAGGCTCCCCTCCCTACCA 244
  301 GAAATGCAGTAAAGTGTGAATGCGAGAAATCAGTAATCGATCTCGAATCT 350
  245 GAAATGCAGTAAAGTGTGAATGCGAGAAATCAGTAATCGATCTCGAATCT 294
  351 TTGAACGACCTTGGACACCTTTTGGTATTCGCGGAGGGTACCGCTGAG 400
  295 TTGAACGACCTTGGACACCTTTTGGTATTCGCGGAGGGTACCGCTGAG 244
  401 TGTCGTGAAATCTCTCAACCCCGCCGCTCTTTCGAGGCTGAGGCTGATT 450
  345 TGTCATTAACATATATACAC.............................362
  451 GGACCTTGGAGGCTTGGCAGACCCCGATTGGCTGCTGGGCTCTCTCT 500
  363 ........TTGAGGCTTTCTCTAGGCCTTGGATTTGGAGGGCTTGTCTTCC 409
  501 GCGCGTGTGCGGTACGGCTCCTCTCTCCTAAGGATTAGGAGCCCTCTGCG 550
  410 TTCAGGTGATTTGCTGTCCCTTTAATGCTTAATTGTGTGGCTTTGACAGT 459
  551 GGCCTCGGGTGATTAAGTTGCTTAGCCTGCGGCTAGCCTCTCTCGGGGGG 600
  460 CGTCGTGTGATAAAATTACACTACAC...........GTATTGACTGCA 499
  601 CCCCCTCTCAACCGCTCGCGAGGACACCTCTTCAATCGAAACTTGACCTCA 650
  500 CCTGCTTTAATACCAACCGGCTAGTTTGGACATTTGACCTCAATGCTAGGA 549
  651 GATCGAACGGCGACT...664
  550 CTACCGCGCTGAACCTAA 566
**FIGURE 19**

**CLAVICORONA AND LENTINELLUS**

PERCENT SIMILARITY: 67.097

4242 Clavicorona X 9985 Lentinellus

Clavicorona  
1 ........................ TAGGTGAA.CTGCGGAAG..ACATTATCGAAAAA 31

Lentinellus  
1 CGTAACAAAGGTTCGAGGACCTCGGAAGGATCATTAGCTGAAA 50

| 32 GCTTTCCGGTTTCTGCTGCT 75 |
| 51 AAAGGCCGTTGTGTTGCTGGGCCTGCAAGGCTATGCTGA 100 |
| 76 CACCGATTTTCACCTCCTACACACCCATGTGCACCTCTCCGGTGTTTGT 125 |
| 101 CGCCCATGCTGCCATCTCTCACACCCCCCTGTGCACTCTCTGGTGTTTG 150 |
| 126 CCTCTTTTACCAGGGGAAACGCGGCTTTTTCTACACACTCTTTTGTATG 175 |
| 151 TGGCTTTGCTCTCCGGCCTGCTATATACATATACAC..CTGTATG 198 |
| 176 CTTNAGAATGCTATTTGCTGCATACACAGCAATCAAAATTTTCAAC 225 |
| 199 CTTCCAGATGTCAAAATCGGCTATATAAAGCATCTATAACACTTTCAAC 246 |
| 226 AACCGATCTTTGCTCTCAGATGACAGTAAGAAGGCGAATGCGATA 275 |
| 247 AACCGATCTTTGCTCTCCGATCAGTGAAGAAGGCGAATGCGATA 296 |
| 276 AGTAATGTGAAATGCGAGATCTGATCATGAAATCTTGAGAAGAGCACC 325 |
| 297 AGTAATGTGAAATGCGAGATCTGATCATGAAATCTTGAGAAGAGCACC 346 |
| 326 TTGCGCTCTTTGNTCTTCGAGGAAACGCGCTTTTTGAGATGTGCTGC 375 |
| 347 TTGACACCCCTT.GGTATCCTGAGG..GTACCGCTCTGCTGAT..GTGCGT 392 |
| 376 AAATTCTCAGGACCTGGGTTTCTGAGATGTTGGAATGCTAC 425 |
| 393 AAATTCTCAACCCCACCCCTTTTGGGA..GGGCGATTTGGGATTGAC 440 |
| 426 TTGGAGTCTTTTGGCGGGTNTTTTACTAAATGCATCTGGCTTCTCTTAAATG 475 |
| 441 TGGAGGCTTTTGGCATGACACCCCCTTTTGGCCTGGTGTTTGATGCGC 490 |
| 476 TTAGTANGACCTCTTATTGGGAANAACCTCGGTGGTGGAAATATATTATCTACC 525 |
| 491 TCTCTCAAAGGCAATTAGCCCGGACCTTTTGGCCTGGTGTTTGATGATAAACT 540 |
| 526 CGCTCGTCTGTTCTGCTATATCCACTGTGGTATGAAACCTGCTTCTAAACCTGC 575 |
| 541 ATCTACGCCATGGGTGCTTTGCTGGAACCTCGGTGATGGGCCTTGATGACTTCGTCAC 588 |
| 576 TCCACGGGAANAATTTNAAATTTAGCTGAGAACCCTGACCCCTACGGCGGT 625 |
| 589 TCGTGAGGGGAACTTTTATATCGGACTACAGGCTTGAC 634 |

626 ACCNCTAAATNANANA 640

| : |

635 TACCCGCTGAA.... 645
Figure 20

Neighbor-Joining Tree

L. cochleatus

L. omphalodes

L. micheneri { L. montanus

L. vulpinus

L. ursinus

L. angustifolius

New species

New Taxon
Figure 21

*L. vulpinus* and *L. cochleatus*

PERCENT SIMILARITY: 91.339

*L. vulpinus* 7966 X *L. cochleatus* 9985

```
1  CGTAACAGGTGGTTCCGTAGTGAACTGCGGAAGGATCATTA 50
1  CGTAACAGGTGGTTCCGTAGTGAACTGCGGAAGGATCATTA 42
51  TCGAAACAAGAGCCCGGCTAGCTCGTGCCGCCCTTCGGGGGG 100
43  TCGTAACAAAGAGCCGGTGGTTCTGTGCTTCGCCCTCCCT..GGGG 89
101 GGGCATGTGCACGCACGCGGCTGCATCTTCACACCCCTTGACCTCTG 150
90  AGGCATGTGCACGCACGCGGCTGCATCTTCACACCCCTTGACCTCTG 139
151 CGTGGAAGTCGCCCTTGAGCCCTCTGAGGCTTCTGACCCGGGCTTCCTACACA 200
140 CGTGGAAGTCGCCCTTGAGCCCTCTGAGGCTTCTGACCCGGGCTTCCTACACA 189
201 CACCTTTGTATGTCCTTCAGAAATGTCACACCCGTCAAAAGACATCTAATA 250
190 CACCTTTGTATGTCCTTCAGAAATGTCACACCCGTCAAAAGACATCTAATA 235
251 CAACCTTCACACAGGCATCTCCTTGCTCAGTAGATAAGAAGGACGCCAGC 300
236 CAACCTTCACACAGGCATCTCCTTGCTCAGTAGATAAGAAGGACGCCAGC 285
301 GAAATGCATAGTAAGTGAATTCGAGATTCAGTGAATCATGAACTAGCT 350
286 GAAATGCATAGTAAGTGAATTCGAGATTCAGTGAATCATGAACTAGCT 335
351 TTGAAAGCACCTTGCACCCCCCTTGATTTACGAGGGGTACTGTCTGTGAG 400
336 TTGAAAGCACCTTGCACCCCCCTTGATTTACGAGGGGTACTGTCTGTGAG 385
401 TGTCAATTTCCAAACCGTCCTTCGCTTCTTCAGACCCGCTTCTGAGGAGTGG 450
386 TGTCAATTTCCAAACCGTCCTTCGCTTCTTCAGACCCGCTTCTGAGGAGTGG 435
451 GAGCTTTGAGGCTTCTTGAGCCTCCGCTTTCCGCTCTCCGCTCTCCTGGGG 500
436 GAGCTTTGAGGCTTCTTGAGCCTCCGCTTTCCGCTCTCCGCTCTCCTGGGG 478
501 GCAGATGGTGATCAGATCAGATGCTTAGCTCCTCTCAAGGACATTACGACAGACCCTCTC 550
479 .......GTTGGGATCAGGCCTTCTCAAGGACATTACGACAGACCCTCTC 521
551 GCAGCTGGTGATGAAATTTGCTACGCCCTGGCTTCTGAGCTTCCTGAGG 599
522 GCAGCTGGTGATGAAATTTGCTACGCCCTGGCTTCTGAGCTTCCTGAGG 571
600 ACCCGCTTCCAAACCGTCCTGAGGAGGACACCTTCATCGAAACATGACCTC 649
572 ACTTGGCTTCCAAACCGTCTCGTGAGGAGGACACTTTTATCGAAACATGACCTC 621
650 AGATCAGGCAGGACT........... 664
622 AGATCAGGCAGGACT........... 645
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