MOLECULAR SYSTEMATICS OF PLICARIA AND PEZIZA (PEZIZACEAE: ASCOMYCETES): TAXONOMIC IMPLICATIONS AND PATTERN OF ADAPTATION TO POSTFIRE HABITATS

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Molecular Systematics of *Plicaria* and *Peziza* (Pezizaceae: Ascomycetes): Taxonomic Implications and Pattern of Adaptation to Postfire Habitats

BY

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Abstract

Morphological similarity between *Plicaria*, which has spherical spores, and *Peziza*, which has elliptical spores, has lead to a great deal of disagreement over the recognition of *Plicaria* as a valid genus. The first objective of this study was to determine if *Plicaria* represented a phylogenetic grouping of taxa distinct from *Peziza*. Both taxa also show similarity in having adapted to occur in burned habitats following fires. The second component of this study was to compare the pattern of adaptation to burned habitats in *Plicaria* and *Peziza*.

To determine the phylogenetic relationships of *Plicaria* to *Peziza* and selected members of Pezizaceae, DNA sequences were obtained from the Small Subunit (SSU) gene, 3' Internal Transcribed Spacer region (ITS-1) and Large Subunit (LSU) gene of the nuclear-encoded ribosomal DNA.

Parsimony analysis of nucleotide characters showed that *Plicaria* and elliptical-spored *Peziza* species with a similar suite of morphological characters (*Plicaria*-like-*Peziza*) formed a closely-related monophyletic group distinct from other *Peziza* species. This makes *Peziza* paraphyletic, which can be dealt with by allowing *Peziza* to subsume *Plicaria*, merging *Plicaria*-like-*Peziza* species with *Plicaria*, or assigning members of the *Plicaria*-like-*Peziza* group to a separate genus.

Mapping of burn association character on the inferred phylogeny revealed that several monophyletic groups have evolved

in association with postfire sites. All taxa that branch basally within the *Plicaria/Plicaria*-like-*Peziza* clade are postfire species. This suggests that the ancestral condition is association with burns. A similar relationship was found within the *Peziza* clade but only a small number of taxa were sampled from this group.

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Introduction

1.1 General Introduction

A resurgence of interest in fungal evolution has been inspired by the combination of two unlikely partners, phylogenetic systematics and molecular genetics (Blackwell, 1994). The study of phylogenetics started in 1950 when Willi Hennig published "Grundzüge einer Theorie der Phylogenetischen Systematik." This method attempts to reconstruct genealogical relationships among groups of taxa and has allowed researchers to develop classifications that reflect phylogenetic relationships (Wiley, 1981). This system of empirical character analysis proved to be a powerful tool but the number of morphological characters limited its usefulness (Zambino and Szabo, 1993). Many authors also felt that homoplasies were being introduced into character analyses when authors mistakenly identified analogous characters as being derived from a recent common ancestor (Smith, 1994).

Advances in molecular genetics have allowed systematists to increase the number of characters which can be compared among taxa. This started in 1987 when Mullis and Faloona published a paper describing a method which allowed for enzyme-mediated amplification of DNA. This technique, called the Polymerase Chain Reaction (PCR), allowed non-molecular biologists to utilize techniques such as DNA

sequencing. The introduction of nucleotide sequencing allowed for the generation of large numbers of characters that could be analyzed using phylogenetic methodology. Large data sets of molecular characters could be used to generate phylogenetic trees which could be tested statistically (Taylor et al.,1993).

Nucleic acid sequencing has become the technique of choice for mycologists who are interested in resolving systematic questions. This method allows the researcher to select a gene or region with the appropriate degree of variation. Such flexibility has allowed authors, such as Sogin et al. (1989) and Van de Peer et al. (1993), to create a multikingdom phylogeny based on nuclear Small Subunit Ribosomal RNA (SSU rRNA) sequences. Similarly, Internal Transcribed Spacer ribosomal DNA (ITS rDNA) sequences have been used to differentiate at the population, species and generic level (Kim and Janson, 1994; O'Gorman et al., 1994). Molecular sequence data have also allowed for the incorporation of anamorph and teleomorph stages into phylogenetic studies, a task that is difficult, or impossible utilizing only morphological data. This has been especially important to mycologists, who have been able to infer anamorph-teleomorph (asexual-sexual) connections (Egger and Sigler, 1993) as well as the phylogenetic relationships which exist among mitotic and meiotic taxa (Lobuglio et al., 1993; Lobuglio et al., 1994). Finally,

sequence data have allowed researchers to resolve systematic questions when morphological characters are in conflict or missing (Berbee and Taylor, 1992; Spatafora and Blackwell, 1994).

The purpose of this study was three-fold: 1)to assess the phylogenetic and taxonomic validity of the genus *Plicaria* Fuckel emend. Boudier within the Pezizaceae 2)to assess the phylogenetic relationship of *Plicaria* to a group of morphologically similar *Peziza* L. species, and 3)to examine the evolution of the ecological association with postfire habitats in *Plicaria* and *Peziza*. This study focuses on the phylogenetic analysis of nucleotide characters obtained from the Small Subunit (SSU) gene, Internal Transcribed Spacer region (ITS) and Large Subunit (LSU) gene of the nuclear-encoded ribosomal DNA.

1.2 History and Classification of *Peziza* and *Plicaria*

The family Pezizaceae, which is a member of the order Pezizales, has historically included most of the larger, cupulate or sessile, operculate discomycetes (Seaver, 1928; Le Gal, 1947; Korf, 1954; Kimbrough, 1970). According to Kimbrough (1970), members of this family are described as having *Oedocephalum* Preuss or *Chromelosporium* Corda conidial stages, amyloid asci, and smooth or ornamented spores. Traditionally, the Pezizaceae has included the four genera *Peziza*, *Plicaria*, *Sarcosphaera* Auerswald and *Pachyella*

Boudier. However, additional genera included in the family at one time or another are *Iodophanus* Korf, *Thecotheus* Boudier, *Boudiera* Cooke, *Sphaerosoma* Klotzsch and others. One of the major reasons for disagreement is that the decisions as to which characters should be used to delineate a family is highly subjective. Also, as pointed out by Dissing (1974), the characters that are used to distinguish *Plicaria* from *Boudiera* (Pezizaceae), *Sphaerosoma* Klotzsch (Pyronemataceae) and *Sphaerozoma* (Pezizaceae) are not at all clear.

Typification and nomenclature of genera found in this family are also problematic. A considerable degree of controversy has surrounded the largest genus found in the Pezizaceae, *Peziza*, and its distinction from the closely-related genus, *Plicaria*. *Peziza* has been described as possessing cup-shaped to flattened, sessile or very short stalked, apothecia with thin receptacles and brittle-flesh, ascus tips that turn blue in iodine, and large elliptical spores which are either smooth or ornamented (Dennis, 1981). This description is similar for *Plicaria* that is described as possessing dark cup-shaped to flattened apothecia, ascus tips that turns blue in iodine, and spherical ascospores that are either smooth or ornamented (Dennis, 1981).

Present day controversy over *Peziza* had its beginnings in 1719 when Dillenius (cited in Rifai, 1968) introduced the name *Peziza*. Since that time, this genus, like many

classical genera, has undergone numerous amendments and interpretations. In 1754 Linnaeus adopted the name *Peziza* in "Species Plantarum" and "Genera Plantarum", after which the name *Peziza* has been found in the fungal and botanical literature. *Plicaria* was first introduced by Fuckel in 1870 (cited in Korf, 1960) when he transferred several elliptical and spherical spored taxa to the genus *Plicaria*. In 1885, Boudier restricted *Plicaria* to spherical-spored taxa. In 1907, in his book "Histoire et Classification des Discomycètes d'Europe," Boudier used the name *Aleuria* (Fr.) Gill to describe taxa that had smooth eguttulate spores and the name *Galactinia* Cooke for elliptical-spored taxa which possessed both spore ornamentation and guttules. For the next 80 years, numerous classification schemes were developed by authors such as Rehm (1887-1896), who used Plicaria *sensu* Fuckel, and Seaver (1928). However, in 1953, Le Gal published "Les Discomycetes de Madagascar" which marked a turning point for the nomenclature of the two genera when she suggested that the genus name *Galactinia* should include *Aleuria*. Under *Galactinia*, she included two sections: guttulisporae and eguttulisporae.

Authors such as Dennis (1960), Batra and Batra (1963) and Moser (1963) argued that *Plicaria* is validly separated from *Peziza* based upon shape of the ascospores. However, in 1960, Korf compiled a detailed history of the genus *Plicaria* in which he argued that the spherical ascospore character

was not sufficient to warrant a generic distinction and merged *Plicaria* with *Peziza*. This argument has been followed by a number of authors such as Le Gal (1963), Korf(1960), Denison (1963) and Berthet (1964). However, Dennis (1960,1968), Batra (1961), Rifai (1968), Eckblad (1968), Dissing and Korf (1980), Dissing and Pfister (1981), Egger (1987) and Moravec and Spooner (1988) all suggested that *Plicaria* may be a valid genus, based upon a suite of characters in addition to spore shape. Dissing and Pfister (1981) argued that the *Plicaria* genus concept is valid because members of this genus represent a natural grouping of species that is united by a suite of characters that include dark fruiting body pigments, paraphyses which adhere to one another, *Chromelosporium* anamorphs, a carbonicolous habitat, and an excipulum that contains globose and hyphal elements. However, all of these characters are found in elliptical-spored *Peziza* species. Classification of *Peziza* and *Plicaria* was further complicated by Egger (1987). He compared the ability of *Plicaria* and *Peziza* to oxidize tyrosinase substrates. Results showed that all *Plicaria* species and three *Peziza* species had a positive reaction, whereas most *Peziza* species had a negative reaction. Egger also noted that the three tyrosinase positive *Peziza* species (*Peziza atrovinosa* Cook and Gerard, *Peziza ostracoderma* Korf and *Peziza vacinii* (Velen.) Svrček) shared several morphological characters such as *Chromelosporium* anamorphs

similar to *Plicaria*. From this information, Egger suggested that it may be necessary to expand the *Plicaria* genus concept to include several elliptical-spored species. Moravec and Spooner (1988) added more support to this theory by pointing out that a number of morphological characters, such as brown spore colour, seemed to link several *Peziza* species which had *Chromelosporium* anamorphs, to *Plicaria*.

Adequate monographs of *Peziza* and *Plicaria* do not exist, therefore several different circumscriptions had to be followed. In this study, I used *Plicaria trachycarpa* (Curr.) Boudier, *Plicaria endocarpoides* (Berk.) Rifai, *Peziza petersii* Berkley and Curtis, *Peziza praetervisa* Bres., *Peziza violacea* Persoon, *Peziza repanda* Persoon and *Peziza vesiculosa* Bulliard in the sense of Rifai (1968). However, Rifai (1968) mistakenly referred to *Peziza praetervisa* by the name *Peziza tenacella* Phill.. *Peziza tenacella* is very similar to *Peziza praetervisa* but has smooth spores and enlarged paraphyses (Egger, 1987). Svrček (1976, 1977) claimed that the correct name for *Peziza violacea* is *Peziza lobulata* (Velen.) Svrček) and that the correct name for *Peziza praetervisa* is *Peziza subviolacea* Svrček. I used the concept of *Plicaria carbonaria* (Fuckel) Fuckel as described by Maas Geesteranus (1967) under the name *Peziza anthracina* Cooke. I also used the following concepts: *Peziza arvernensis* Boudier, *Peziza atrovinosa*, *Peziza badia* Persoon ex. Mérat., *Peziza cerea* Sowerby ex.

Mérat., *Peziza echinospora* Kasst., *Peziza ostracoderma*, *Peziza varia* (Hedwig) Fries, *Sarcosphaera crassa* (Santi ex. Steudel) Pouza (all sensu Dennis, 1981), *Plicaria acanthodictya* Dissing and Hauerbach (Dissing, 1974), *Kimbropezia campestris* Korf and Zhuang (Korf and Zhuang, 1991), *Peziza phyllogena* Cooke (Pfister, 1987), *Peziza quelepidotia* Korf and O'Donnell (Korf, 1973) *Peziza vacinii* (Svrček, 1977), *Glischroderma* sp. Fuckel (Korf, 1994) and *Pachyella clypeata* (Schw.) Le Gal (Pfister, 1973). Finally, I am using *Peziza* cfr. *linteicola* Phill. and Plowr. to describe a *Peziza* species that has brownish apothecia with a yellow hymenium and eguttulate punctate-roughened spores which are 13 X 9 µm. This species appears to be very similar to *Peziza linteicola* (Arroyo and Calonge, 1989; Eckblad, 1968).

1.3 Why Postfire Fungi Occur After Burns

Ascomycetes belonging to the order Pezizales commonly inhabit burned forest areas but usually only within three years of the fire (Petersen, 1985). This habitat, which is ephemeral and constantly shifting, would be expected to place special constraints upon the ability of these fungi to adapt and speciate. This is particularity important to *Plicaria* species which are normally found on postfire sites and to a lesser degree *Peziza* species which can occupy either burned, disturbed, undisturbed sites or a combination

of sites.

To explain why postfire fungi such as *Peziza* and *Plicaria* occur in such a transient habitat, a number of theories have been developed. First, it has been suggested that some postfire species may be reacting to the heat or chemical changes produced by burning, which releases them from mycostasis (Petersen, 1970b; Wicklow and Zak, 1979). Petersen (1970a) carried out germination studies on several postfire species. Results showed that *Plicaria endocarpoides*, *Peziza echinospora* and *Peziza praetervisa* germinate readily in water but heating may increase germination percentages. Secondly, species which inhabit burned sites may tolerate organic byproducts that are created during the burning process (Petersen, 1970a; Widden and Parkinson,1975). A study carried out by Egger (1986) suggests that *Plicaria endocarpoides*, *Plicaria trachycarpa* and several *Peziza* species produce a number of different degradative enzymes that include phenol oxidase enzymes. These enzymes have been implicated in the breakdown of lignins and detoxification of phenolic compounds, which are associated with burns. Thirdly, after burning, the heat from the fire may sterilize the soil or greatly reduce naturally occurring miroflora and fauna thereby reducing competition, allowing some of the less competitive species to flourish (Wicklow and Hirschfield, 1979). This is supported by competition studies that suggest that many post-fire

Plicaria and *Peziza* species are poor competitors (Egger, unpublished data). Sagara (1992) further added support to this theory when he found *Peziza ostracoderma* growing on soil that had been chemically sterilized using $CaCN_2$. This chemical treatment is similar to burning in that it generates heat and produces ash. Fourthly, it has been suggested that these fungi may have adapted to the physicochemical environment which is created by burning (El-Abyad and Webster 1968; Petersen, 1970[a]). Studies have shown that many *Peziza* and *Plicaria* species are normally found growing on burned soils that have elevated pH levels (Petersen, 1970[a]). However, by artificially increasing soil pH by liming, postfire species such as *Peziza praetervisa* (Petersen, 1970[a]) and *Peziza echinospora* (Sagara, 1992) have been stimulated to fruit on unburned soil.

Many *Peziza* and *Plicaria* species tend to be associated with disturbance. These disturbances can take many forms, such as burning of soil, soil compaction, excavations, upturned earth, river banks and others (Petersen, 1985). Studies have been carried out to determine the edaphic factor or factors which are of particular significance to these disturbance mediated species. In several studies, numerous factors have been examined such as minerals nutrients, soil pH, organic matter, surrounding tree and shrub species, conductivity and others (Petersen 1967; 1970[a]; 1970[b]; 1985). However, pH and organic matter content

appear to be the only two variables that can be correlated with the occurrence of most species (Petersen, 1985). These components may be of particular importance to the occurrence of post-fire species after a burn because the surface soil pH usually increases and the organic content decreases (Petersen, 1970"). Other studies have also found a link between artificially elevating soil pH by liming and the occurrence of postfire species on unburned soil (Petersen, 1970"; Turnau et al., 1991; Sagara, 1993).

Members of *Peziza* and *Plicaria* are restricted to burned sites to different degrees. There is a continuous progression from burned to unburned site inhabiting species. With this in mind, many authors have found it useful to establish groups based on the ability of these fungi to occupy burned, unburned or both types of habitats (Moser, 1949; Eberts, 1958; Petersen, 1970"). The first group includes species that are found exclusively on burned sites. Members from this group that were compared in this study include *Plicaria carbonaria*, *Plicaria trachycarpa*, *Peziza echinospora*, *Peziza petersii*, *Peziza violacea* (Petersen, 1970"), *Plicaria acanthodictya* (Dissing 1974) and *Peziza vacinii* (Moravec and Spooner, 1988). The second group includes those species which under natural conditions occur on burned sites but may also occur on unburned sites if the soil has been altered by some artificial means such as liming. Several examples of this group that were used in

this study include *Plicaria endocarpoides* and *Peziza praetervisa* (Petersen, 1970[a]). Collectively, these two groups make up the "obligate burn site species" (Petersen, 1970[a]). A third group, called the "facultative burned site species" include those fungi which occur on either burned or unburned sites. Facultative burned site species that were used in this study include *Peziza varia*, *Peziza repanda*, *Peziza arvernensis* and *Peziza vesiculosa* (Petersen, 1970[a]). *Peziza ostracoderma* is placed in the facultative postfire group even though I have found no record of it occurring on this habitat. The reasons for doing so are two fold, 1)the apothecia of *Peziza ostracoderma* are mostly found on sterilized soil, which shares a number of characteristics with burned soil and 2)Hennebert and Korf (1975) have found *Chromelosporium* anamorphs growing on burned soil that they have identified as belonging to *Peziza ostracoderma*. A fourth group of fungi which were examined in this study included the non-burned site species. These are distinguished from the other groups by the fact that they have never been found on burned sites but have been found on disturbed sites. These include *Peziza atrovinosa* (Moravec and Spooner, 1988), *Peziza badia* (Petersen, 1985), *Peziza quelepidotia* (Korf, 1973) *Kimbropezia campestris* (Korf and Zhuang 1991) and *Peziza cerea* (Rifai, 1968).

1.4 Ecological role of *Plicaria* and *Peziza*

Most *Peziza* and *Plicaria* species are found only in forested regions and this has lead to hypotheses regarding the role that these fungi play in forest ecology. Both genera are probably representatives of the wood and woody root decomposer community (Egger and Paden, 1986; Egger, 1986). Wood and woody root decomposers produce a wide range of enzymes such as cellulases and phenol oxidases that are capable of degrading lignin and are considered to be indicative of a more generalized mode of substrate hydrolysis (Egger, 1986).

The forest fungal community before a fire consists of species that are specialists in that they produce a limited number of extracelluar hydrolases. However, after a fire the fungal community shifts to species that are capable of producing a broad range of enzymes and a more generalized form of substrate hydrolysis (Wicklow and Hirschfield, 1979; Egger, 1986). These generalists have evolved life history strategies such as fast growth for rapidly colonizing habitats, short reproductive cycles and homothallic mating that allows them to exploit this short lived, constantly shifting habitat (El-Abyad and Webster, 1968; Egger, 1986).

1.5 Nuclear Ribosomal DNA

Ribosomal DNA (rDNA) has become the molecule of choice for many fungal molecular systematic studies. This region became popular when systematic studies were based on

ribosomal RNA sequencing. The RNA template was originally used because it was conservative and large amounts could be extracted (Bruns et al., 1991). However, with the development of PCR, rDNA sequencing replaced rRNA sequencing because it was faster, more accurate and not subject to post transcriptional editing (Bruns et al., 1991). The popularity of this region is partly due to the presence of highly conserved regions which contain interspersed variable elements. The nature of rDNA thus gives researchers the flexibility to analyze a wide range of phylogenetic relationships (Förster et al., 1990; Zambino and Szabo, 1993). It also allows for the design of primers which amplify across variable elements but are anchored in conserved regions (Bruns et al., 1991). Ribosomal DNA is also found in high copy numbers and very little material is needed for amplifying and sequencing. As well, large numbers of rDNA sequences have been generated and stored in data bases such as Genbank. This allows researchers to draw quickly upon the data of others for comparison and analysis.

The organization of the nuclear ribosomal DNA tandem repeat unit is shown in Figure 1 and consists of 3 RNA structural components used in the construction of ribosomes. These components consist of the 18S subunit, which is also called the small subunit (SSU rDNA), the 5.8S subunit and the 28S subunit, which is also known as the large subunit (LSU rDNA). Flanking the 5.8S subunit are two internal

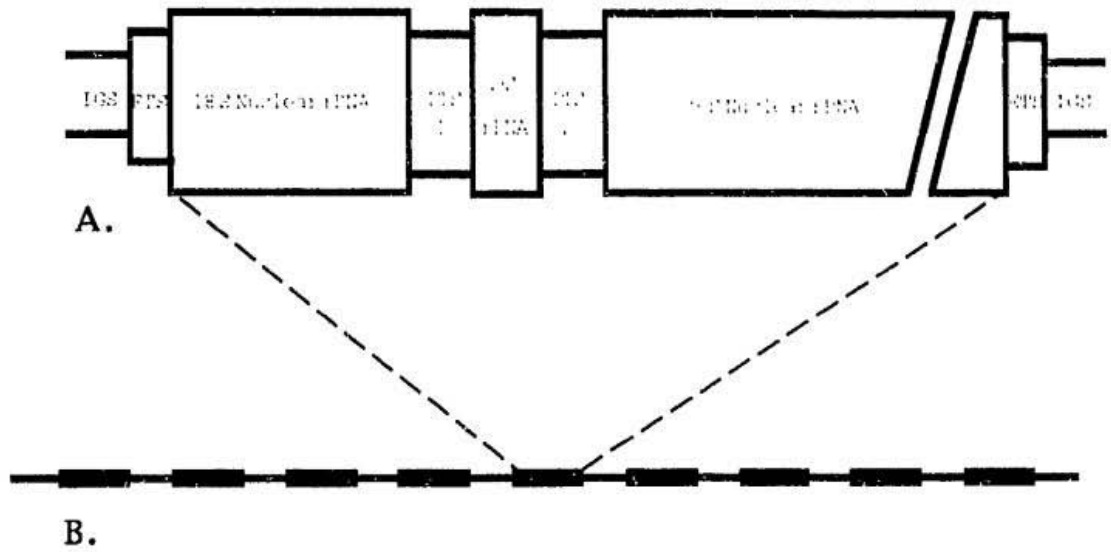


Figure 1. (A) Schematic diagram of a single 18S-28S repeat unit, ITS = internal transcribed spacer region, ETS = external transcribed spacer and IGS = intergenic spacer. (B) Schematic representation of a tandem array of rDNA repeats.

transcribed spacer regions which are called ITS1 and ITS2. These two components are transcribed, but are spliced out and degraded before ribosome construction. A second region which is transcribed but spliced out and degraded is called the external transcribed spacer. The first spacer is attached to the 5' end of the SSU gene and the second spacer is found on the 3' end of the LSU gene. The three structural components, the two ITS regions and the two ETS regions represent a transcriptional unit and is separated from the preceding and following transcriptional unit by the non-transcribed intergenic spacer (IGS) also known as the non-transcribed spacer (Singer and Berg, 1991; Rocheford, 1994).

The regions which are most commonly used in phylogenetic analysis are the SSU, ITS1 and LSU. The nuclear small subunit has mostly been used to resolve at or above the order level (Förster et al., 1990; Berbee and Taylor, 1992; Spatafora and Blackwell, 1994). The 5' end of the large subunit which includes two variable regions, divergent domain 1 (D1) and divergent domain 2 (D2), are more variable than the SSU and has been used to assess the phylogenetic relationships at the genus and species level. This region has been used to create phylogenies between closely related genera, (Peterson, 1993) species (Guadet et al., 1989; Sherriff et al., 1994) and intra-species level (O'Donnell, 1992; Moncalvo et al., 1993). Analyses of ITS-1 and ITS-2 have demonstrated the highest degree of variation. Studies

have used these two regions to determine relationships among closely related genera (Carbone and Kohn, 1993), species (Lee and Taylor, 1992) and populations (Fritz et al., 1994). Recently a number of studies have combined data from the LSU (D1 and D2) and ITS regions to determined phylogenetic relationships within a single genus (Sherriff et al. 1994) and between closely related genera (Peterson, 1993).

1.6 Polymerase Chain Reaction and DNA Sequencing

In 1987, PCR presented itself as an alternative way of amplifying DNA or RNA concealed within large chains of polynucleotides. This was less laborious and not as error prone as the more traditional technique of cloning. This technique is an *in vitro* nucleic acid synthesis process which uses DNA polymerase and oligonucleotide primers that flank the region which is to be amplified. The purpose of PCR is to duplicate the nucleotide sequence of each strand between the two annealed primers. This is accomplished by repeating a three step procedure of denaturation, primer annealing and primer extension for a predetermined number of times (Figure 2). First, a crude extract of DNA is heated so that the double stranded duplex denatures, remaining free in solution. Secondly, the temperature is lowered so that site specific primers can anneal to opposite strands flanking the region which is to be amplified. Finally, the temperature is raised to 72°C which is optimal for the 5'→3' extension of

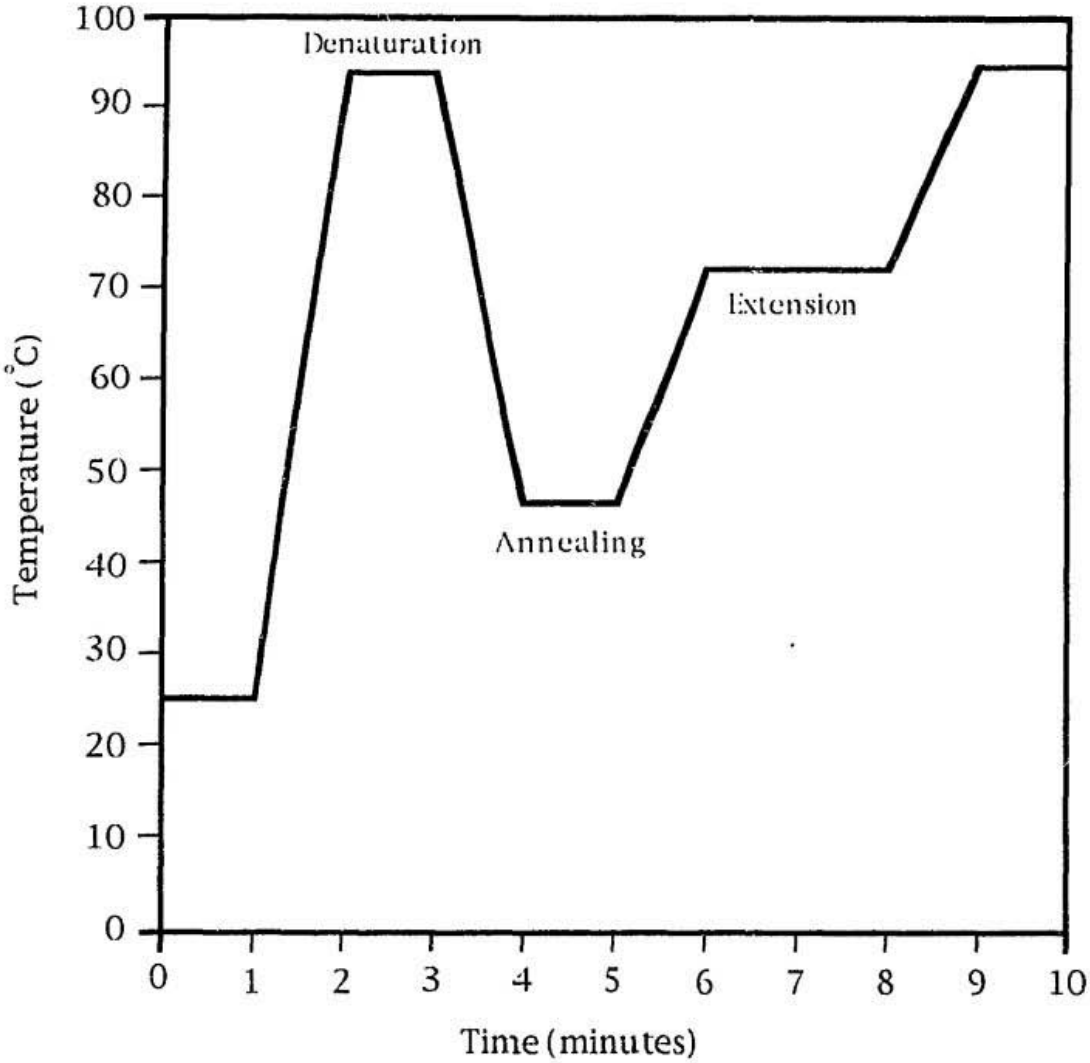


Figure 2. Temperatures and time during which denaturation, annealing and extension occur during the Polymerase Chain Reaction (PCR). Zero to eight minutes equals 1 cycle (based on Perkins-Elmer Cetus)

the primer-template duplex by a heat stable *Taq* DNA polymerase (Oste, 1988). This set of three steps is usually carried out between 25 to 40 times. After 25 cycles, one double stranded template can theoretically generate approximately 1 million copies of DNA (Lee and Taylor, 1990) because each newly synthesized strand can act as a template for the following cycle.

Just as PCR has changed the way many molecular systematists conduct research, the development of automated DNA sequencing has also changed the way research is carried out. DNA sequencing started in 1977 when two different techniques for obtaining DNA sequences were developed for the determination of the individual base constituents of DNA. The first method developed by Sanger et al. (1977) is an enzymatic method that utilizes DNA polymerase to make copies of the template DNA and randomly inserts radio-labelled chain terminating dideoxynucleotides. The second group, Maxam and Gilbert (1977), developed a base-specific chemical reaction method that allowed for base determination. Both methods have been used extensively but automated sequencing is quickly becoming the method of choice for those who can afford the initial purchase price of an automated sequencer. Automated DNA sequencing, like the Sanger et al. (1977) method, is an enzymatic method and works by randomly incorporating four distinguishable fluorescent tagged dideoxynucleotide terminators into a

synthesis reaction which is catalyzed by *Taq* DNA polymerase. DNA labelled with fluorescent tagged dideoxynucleotide are then electrophoresised through a polyacrylamide gel where a laser beam excites the fluorescent molecules causing them to emit light. This is detected by a photomultiplier tube which transfers the signal to a computer where the information is analyzed and stored (Prober et al., 1987). Automated sequencing has several advantages over manual sequencing. First, automated sequencing requires less time and is less laborious. Secondly, automated sequencing is non-radioactive and does not require delays for exposure and development of autoradiographs. Thirdly, manual sequencing requires four lanes (one for each nucleotide) while automated sequencing only requires one lane, which allows more samples to be run on a single gel. Finally, the data collection is automatic. To collect manual sequencing data requires a great deal of skill to interpret and record information from autoradiographs.

1.7 Study objectives

Current phenetic classification schemes developed for *Plicaria*, *Peziza* and members of Pezizaceae based on morphological similarity/dissimilarity are problematic. The purpose of this study is to utilize DNA sequence characters from the SSU, ITS-1 and LSU rDNA regions to establish a rigorous cladistic taxonomy that reflects the phylogenetic

relationships among *Plicaria*, *Peziza* and select members of Pezizaceae. A phylogenetic classification that only accommodates groups which have a single evolutionary origin, monophyly, is preferable to one that permits multiple or heterogeneous origins, polyphyly (Minkoff, 1983).

The first hypothesis being tested is that *Plicaria*, as currently delineated, is a monophyletic group. If it is not, *Plicaria* could be either paraphyletic, a group containing an ancestral species and some of its descendants, or polyphyletic. In either case, *Plicaria* would not be a valid genus, in a cladistic sense.

The second hypothesis being tested is that *Plicaria* and elliptical-spored *Peziza* species with a similar suite of morphological characters, inherited these characters-states from an immediate common ancestor, synapomorphy. If this is the case then these taxa should be accommodated in a single genus. Alternatively, the suite of morphological characters common to the spherical- and elliptical-spored taxa may be a set of shared primitive character-states, symplesiomorphy, or a case of convergent evolution, homoplasy.

The final component of this study is to compare how members of *Plicaria* and *Peziza* have evolved in association with burned habitats. Although *Plicaria* species are normally found on burned sites, *Peziza* is found on a variety of substrates including burned and disturbed sites. If postfire taxa form monophyletic groups, then this would suggest that

there has been adaptation and speciation in this ephemeral habitat over relatively long periods of time. If *Plicaria* is paraphyletic this would suggest that the adaptation to occur on burned sites has evolved multiple times independently. Phylogenetic analysis may also help resolve other questions concerning with relationships among species on burned and unburned sites, such as whether species on burned sites are recently derived from species on unburned sites, or vice versa.

Materials and Methods

2.1 Isolates Studied and DNA Extractions

The isolates chosen for this study represent several species of *Plicaria* and *Peziza* as well as several members of the family Pezizaceae as listed in Table 1. Isolates were cultured on E-strain agar (Egger and Fortin, 1990) for approximately 4 days at room temperature. Next, plugs were taken from around the growing colony edge using a glass pipet, freeze dried and stored at -5°C until required for DNA extraction.

DNA extraction was carried out on approximately 25 mg of freeze dried hyphal material or air dried apothecia, for those isolates for which cultures did not exist. Tissue was ground in a 1.5 mL microcentrifuge tube using liquid nitrogen and a 1.5 mL microcentifuge tube plastic pestle. Nucleic acids were extracted following a modification of the miniprep protocol of Zolan and Pukkila (1986). This consisted of incubating ground tissue at 60°C for 45 minutes in 700 µL extraction buffer (700 mM NaCl, 50 mM Tris pH 8, 10 mM EDTA, 1% CTAB, 0.2% mercaptoethanol). Cell debris and proteins were removed by emulsifying in chloroform:isoamyl alcohol (24:1), centrifuging for 10 minutes at 13000 rpm and transferring the aqueous phase to a new microcentrifuge tube. Nucleic acids were precipitated with isopropanol and resuspended in TE-8 (10 mM Tris-HCl pH 8, 0.1 mM EDTA). The

Table 1: Species, isolates, substrate and location of *Peziza*, *Plicaria* and select members of Pezizaceae.

Species	Collection number	Substrate	Location[a]	Source of isolate[b]	Determiner
Peziza taxa					
Peziza arvernensis Boudier	SA-455	litter/wood debris	Alberta Can	Abbott 455	S.P. Abbott
P. atrovinosa Cook & Gerard	2094	exposed soil	Maine USA	DAOM 199606	R.P. Korf
P. badia Persoon ex Mérat.	001	exposed soil	Nfld. Can	Egger 001	K.N. Egger
P. badia	002	exposed soil	Nfld. Can	Egger 002	K.N. Egger
P. cerea Sowerby ex Mérat.	2113	undisturbed soil	Quebec Can	DAOM 199736	K.N. Egger
P. echinospora Kasst.	TV-154	burned ground	Norway	Vrålstad 93/154	T. Vrålstad
P. cfr. *linteicola* Phil. and Plowr.	SA-093	debris on soil	Alberta Can	Abbott SA-093	K.N. Egger
P. ostracoderma Korf	2098	peat vermiculite	Quebec Can	DAOM 199608	K.N. Egger
P. ostracoderma	2138	peat vermiculite	Quebec Can	DAOM 199751	K.N. Egger
P. ostracoderma	A0-1471	steam sterilized potting soil	Quebec Can	DAOM 174176	S.J. Hughes
P. ostracoderma	A0-1525	roots of container grown pine	Alberta Can	DAOM 195061	R. Danielson
P. petersii Berkley & Curtis	A0-1079	burned soil	B.C. Can	DAOM 195796	K.N. Egger
P. pratervisa Bres.	305	burned litter	Ontario Can	DAOM 195809	K.N. Egger
P. pratervisa	882	charcoal and ash	B.C. Can	DAOM 195816	K.N. Egger
P. pratervisa	996	charcoal and ash	B.C. Can	DAOM 195829	K.N. Egger
P. quelepidotia Korf & O'Donn.	KD-2205	on "Jiffy-7 Pellet"	Michigan USA	O'Donnell 22205	R.F. Korf
P. repanda Persoon	353	burned litter	Ontario Can	DAOM 195813	K.N. Egger
P. vacinii (Velen.) Svrček	NW-6752	burned soil	Oregon USA	Weber 6752	N.S. Weber
P. varia (Hedwig) Fries	A0-2160	wallboard of house	B.C. Can	Egger A0-2160	J.W. Paden
P. vesiculosa Bulliard	SA-540	horse dung	Alberta Can	Abbott SA-093	S.P. Abbott
P. violacea Pers.	965	burned litter	B.C. Can	DAOM 195822	K.N. Egger
P. violacea	1074	burned soil and litter	B.C. Can	DAOM 199673	K.N. Egger
P. violacea	1113	burned soil and litter	B.C. Can	DAOM 199678	K.N. Egger
P. violacea	2040	wood ash	B.C. Can	DAOM 199561	K.N. Egger
Plicaria taxa					
Plicaria acanthodictya Diss.& Hauerbach	C-530	wood ash	Denmark	C 530	H. Dissing
P. acanthodictya	TV-181	burned ground	Norway	Vrålstad 93/181	C. Holm and T. Vrålstad
P. carbonaria (Fckl.) Fuckel	C-009	wood ash	Denmark	C	P. Rabenborg
P. endocarpoides (Berk.) Rifai	887	burned litter	B.C. Can	DAOM 195819	K.N. Egger
P. endocarpoides	985	burned litter	B.C. Can	DAOM 199089	K.N. Egger
P. endocarpoides	1076	burned soil	B.C. Can	DAOM 199675	K.N. Egger

Table 1
Continued

Species	Collection number	Substrate	Location[1]	Source of isolate[2]	Determiner
P. trachycarpa var. *ferruginea* (Curr.) Boud.	999	charcoal and ash	B.C. Can	DAOM 195830	K.N. Egger
P. trachycarpa var. *ferruginea*	2177	burned soil	Nfld. Can	Egger 2177	K.N. Egger
P. trachycarpa var. *ferruginea*	A0-1746	peat/vermiculite potting mix	B.C. Can	DAOM 210023	K.N. Egger
P. trachycarpa var. *ferruginea*	CU-005	charcoal and ash	Costa Rica	CUP-IN 22	S.C. Gruff
P. trachycarpa var. muricata Grelet	281	burned litter	Ontario Can	DAOM 199631	K.N. Egger
Taxa other than *Peziza* and *Plicaria*					
Glischroderma sp.	CUP-651	on leaves	New York USA	CUP-62651	R.P. Korf
Kimbropezia campestris Korf & Zhuang	CUP-2761	on soil	Canary Islands	CUP-MM-2761	R.P. Korf & Zhuang
Pachyella clypeata (Schw.) Le Gal	SA-363	rotted wood	Alberta Can	Abbott 363	S.P. Abbott
Sarcosphaera crassa (Santi ex Steudel) Pouza	2178	on soil	Colarado USA	Egger 2178	K.N. Egger
S. crassa	SA-289	on soil in needle litter	Alberta Can	Abbott 289	S.P. Abbott

[1] Location abbreviations: Can, Canada; Nfld. Newfoundland; B.C. British Columbia.
[2] Cultures or specimens which were obtained from culture collection or individuals: DAOM, Canadian Collection of Fungus Cultures, Ottawa, Canada; CUP and MM, Cornell University Plant Pathology Herbarium; C, Botanical Museum and Herbarium, University of Copenhagen, Copenhagen, Denmark; Dr. K.N. Egger, Department of Biology, Memorial University of Newfoundland, St. John's, Canada; Mr. S.P. Abbott, University of Alberta Microfungus Collection and Herbarium, Edmonton, Canada; Dr. N. S. Weber, Department of Forest Science, Oregon State University, Corvallis, USA; Ms. Trudé Vrålstad, Department of Biology, University of Oslo, Oslo, Norway; Dr. K. O'Donnell, Microbial Properies Research, National Center for Agriculture Utilization Research, ARS, USDA, Peoria, USA.

emulsification, precipitation and resuspension steps were repeated a second time to increase the purity of the DNA. Finally, DNA was resuspended in 50 μL of TE-8 and stored at -20°C until required for amplification.

2.2 Amplification of DNA

Three regions of nuclear ribosomal DNA were targeted for amplification and sequencing. The first region includes ITS1, a highly variable region which extends from the 3 end of the small subunit through the 5 internal transcribed spacer and anchors in the 5.8S subunit. To amplify this region, primers ITS9mun and ITS10mun were used (Figure 3). This primer set also amplified a portion of the 3' end of the SSU (SSU1) that is part of the highly conservative SSU gene. A second component of the SSU (SSU2) was amplified by primers NS11-12mun. The third region that contains the 5' end of the large subunit is slightly more conservative and contains divergent domains D1 and D2. This region was too long to sequence using a single primer set so two sets of overlapping primers were designed. The first primer set which amplified the 5' end of this region was NL5mun and NL6mun and the second primer set which amplified the 3' end of this region was NL7mun and NL8mun (Figure 3).

For PCR amplification 1 μL of undiluted genomic DNA was used as a template. Amplifications were carried out in 3, 100 μL reactions containing PCR buffer (50 mM KCl, 10 mM

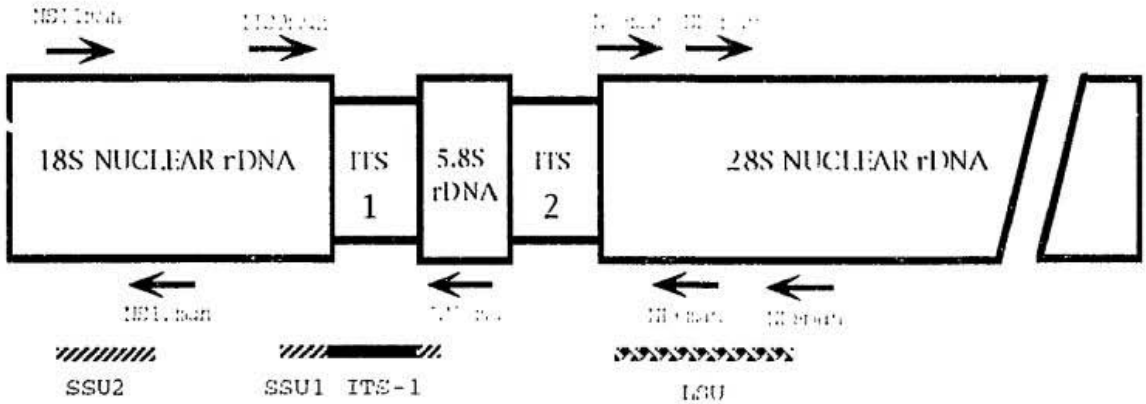


Figure 3. Schematic diagram of 18S-28S repeat unit showing PCR primer location. SSU2 designates the region flanked by primers NS11-12, SSU1 designates the 3' end of the SSU amplified using primers ITS9-10, ITS-1 designates the 5' ITS region amplified using primers ITS9-10 and LSU designates the region flanked by primers NL5-8. Highly conserved regions are indicated by ////, moderately conserved regions are indicated by ~~~ and highly variable regions are indicated by ▬ .

Tris-HCl (pH 9 at 25°C), 1.5 mM MgCl2, 0.01% gelatin (w/v), 0.1% Triton X-100), 50 mM each of dGTP, dATP, dTTP and dCTP (Pharmacia corp.), 0.4 μM of each primer (as listed in Table 2) and 2 units of *Taq* DNA polymerase (Promega corporation). PCR amplifications were carried out in a Perkin-Elmer Cetus DNA thermocycler with cycle parameters of 94°C for 1 minute, 46°C for 60 seconds and 72°C for 120 seconds initially and increasing by 1 second per cycle, with a 1 minute ramp time. Initial denaturation was carried out at 94°C for 2 minutes and a final extension step of 5 minute. With each set of PCR reactions, a DNA-free, negative control was also included to ensure that contamination did not occur while setting up the reaction. After thermocycling, 5 μL of amplified product was mixed with 3 μL of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 25% Ficoll), loaded onto a 1.0% agarose gel and electrophoresesed in 0.5X TBE buffer (45 mM Tris-borate, 45 mM boric acid, 1 mM EDTA pH 8) at 90v for 45 minutes. Gels were stained in a tank of 0.5 μg/mL Ethidium Bromide for 30 minutes and DNA bands were visualized on a UV-transilluminator (300nm). Gels were examined to ensure that only one PCR product band occurred per lane and that no bands were present in the control lane. After initial inspection gels were photographed.

Table 2. Oligonucleotide PCR and sequencing primers.

Primer	Primer Sequence	Region Amplified
ITS9mun	TGTACACACCGCCCGTCG	ITS1 (sense strand)
ITS10mun	GCTGCGTTCTTCATCGAT	ITS1 (antisense strand)
NL5mun	GCATATCAATAAGCGGAGGA	LSU D1 (sense strand)
NL6mun	CAAGTGCTTCCCTTTCAACA	LSU D1 (antisense strand)
NL7mun	TTGGGAATGCAGCTCTAAATG	LSU D2 (sense strand)
NL8mun	TTGGTCGGTGTTTCAAGACG	LSU D2 (antisense strand)

2.3 PCR Product Purification

PCR products were purified by the use of Wizard Magic PCR miniprep (Promega Corporation) technique following the manufacturer's procedure. This involved adding 100 μL of Magic PCR miniprep buffer (5 mM KCl, 1 mM Tris-HCl(pH 9 at 25°C), 150 μM MgCl2, 0.001% gelatin (w/v), 0.01% Triton X-100) to PCR product in a 5 mL polystyrene tube and vortexing. One millilitre of Magic PCR miniprep resin (6M Guanidine Thiocyanate) was then added to the mixture and vortexed 3 times over a one minute time period. The mixture was pipetted into a 3 cc syringe with a Magic PCR miniprep column attached to the luer lock. The syringe plunger was inserted and the solution pushed through the minicolumn. The syringe was then filled with 2 mL of 80% isopropanol and the syringe plunger was used to push the isopropanol through the minicolumn. Next, the minicolumn was removed, inserted into a 1.5 mL microcentrifuge tube and centrifuged at 13,000 rpm for 30 seconds. Following this, the minicolumn was placed into a new microcentrifuge tube, 50 μL of dH_20 or TE-8 was added, it was allowed to stand for 1 minute and centrifuged at 13,000 rpm for 30 seconds. Following PCR purification, concentrations were determined by spectrophotometry at 260 nm and stored at 4°C until ready for sequencing.

2.4 DNA Sequencing

Sequencing reactions for automated DNA sequencing were performed using 0.5 μg of double stranded PCR product, 3.2 pmol of primer and 7.0 μL of reaction premix (Applied Biosystems Inc.). Final volumes were adjusted to 19.2 μL with dH_2O. The sequencing reactions were then placed in a thermocycler and subjected to cycle sequencing parameters of 98°C for 1s, 50°C for 15s and 60°C for 2 min 30s for 25 cycles.

After cycle sequencing, excess primers and unincorporated nucleotides were removed by spin chromatography. This involved equilibrating G 50 fine sephadex (Pharmacia) in dH_2O, loading 2 mL into a spin column (5prime→3prime) and spinning for 1 minute at 1500 rpm to remove excess water. The sequencing reaction mixture was then loaded onto the top of the sephadex column, spun at 1500 rpm for 2 minutes so that the incorporated DNA will be eluted out the bottom of the column. The eluted DNA was then dried in a speed-vac and resuspended in 5 μL of 4.0 μM deionized formamide:50 mM EDTA at pH 8.0, heated to 90°C for 2 minutes, snap cooled on ice and loaded on a polyacrylamide gel (6% acrylamide, 8.3 M urea, 1X TBE) attached to an ABI 373A automatic DNA sequencer (Applied Biosystems). The gel was subjected to electrophoresis at 30 watts, 40-50°C for 8 hours.

2.5 Data Entry and Analysis

DNA sequences were generated in the form of dye intensity chromatographs, collected and analyzed with the use of the ABI 373A software package ver. 1.0.2. Consensus sequences were determined, for each isolate, by proof reading analyzed data for complementary strands. Other discrepancies were resolved by comparing multiple sequence chromatographs from different isolates of the same species.

Preliminary DNA alignments were determined using the default settings for both the automatic DNA sequencer software package SeqEd ver. 2.0 and CLUSTAL V. Final alignments were optimized by hand. Two data matrices were used for analysis, 1)aligned data for *Plicaria*, *Peziza* and select members of Pezizaceae were combined for SSU1, SSU2 and LSU 2)aligned data for *Plicaria* and members of *Plicaria*-like-*Peziza* were combined for ITS-1 and LSU. Both were analyzed using the maximum parsimony program PAUP 3.1q on a Macintosh IIcx computer. Missing or ambiguous characters or regions were not include in the analysis and transversions:transitions were weighted 2:1. Parsimony trees were constructed using heuristic search utilizing the tree bisection-recognition branch swapping algorithm for the SSU1, SSU2 and LSU data set, due to the large number of taxa analyzed, and the branch and bound search algorithm for the smaller ITS-1 and LSU data set. Bootstrap analyses were performed on both data sets, using the same search options

as described above to determine the confidence levels of the inferred phylogenies. Bootstrap indices (Felsenstein, 1985) were calculated using 100 replicates for the SSU1, SSU2 and LSU data set, due to the large number of taxa analyzed, and 1000 replicates for the smaller ITS-1 and LSU data set using PAUP 3.1q. This method attempts to approximate the underlying sampling distribution by resampling from the data set by randomly replacing assigned characters until a new data set is generated that is of the same size as the original. This new data set is then used to construct a new phylogeny. By repeating this procedure a large number of times a confidence limit is generated which compares how well the distribution of the original character set approximates a distribution of an infinitely large character set (Sanderson, 1989).

A modification of the jackknife technique (Lanyon, 1985: Spatafora and Blackwell, 1994) was also performed on the ITS-1 and LSU data to determine how select taxa were influencing the phylogeny (ie. importance of character state frequencies). This technique involves excluding taxa and reanalyzing the data using bootstrap analysis to determine the stability of branches.

2.6 Scanning Electron Microscopy (SEM)

The Hitachi S570 scanning electron microscope was used to view ascospore size, shape and ornamentation of

representatives of *Plicaria* and *Plicaria*-like-*Peziza* taxa. A small portion of apothecia tissue was placed in distilled water on a glass slide and macerated with a scalpel using a dissecting scope. Afterwards broken asci fragments and spores were transferred to an aluminum stub and allowed to air dried for 10 minutes. Dried tissue was gold coated with the use of a Edwards Vacuum S150A Sputter Coater. The stubs were then inspected with the use of the Hitachi 570 Scanning Electron microscope. Photographs were taken of spores which appeared to represent the most common shape, size and ornamentation with the use of Polaroid 665 black and white film

Results

3.1 Sequence Alignment for *Plicaria*, *Peziza* and selected members of Pezizaceae Utilizing SSU2, SSU1 and LSU Regions

The first region, SSU1 and ITS-1, was amplified using primers ITS9mun-ITS10mun which yielded a PCR product that was approximately 400 base pairs (bp) in length. This initial PCR product, which was used as a template in double stranded sequencing, reliably generated 360 bp of sequence data. Attempts to align all 22 taxa for the entire region, using the alignment programs SeqEd and CLUSTAL V, were unsuccessful due to the high degree of sequence divergence for the ITS-1 region. This was resolved by eliminating ITS-1 data from the analysis. Aligned data for SSU1 reveals that this region is highly conserved among all 22 taxa. Of 122 positions, 20 sites were variable (16%)(Figure 4).

The second region that was used in this analysis included a portion of the LSU gene. This segment of the LSU gene was 615 bp long and was originally amplified using primers NL5mun-NL8mun. However, initial automated sequencing data showed that ambiguities in base calling increased to an unacceptable level beyond 450 bp. To deal with this problem, two internal primers were designed. This allowed for the entire 615 bp segment to be amplified using two over-lapping sets of primers, NL5mun-NL6mun and NL7mun-NL8mun. Primers NL5mun-NL6mun amplified the first 360 bp and NL7mun-

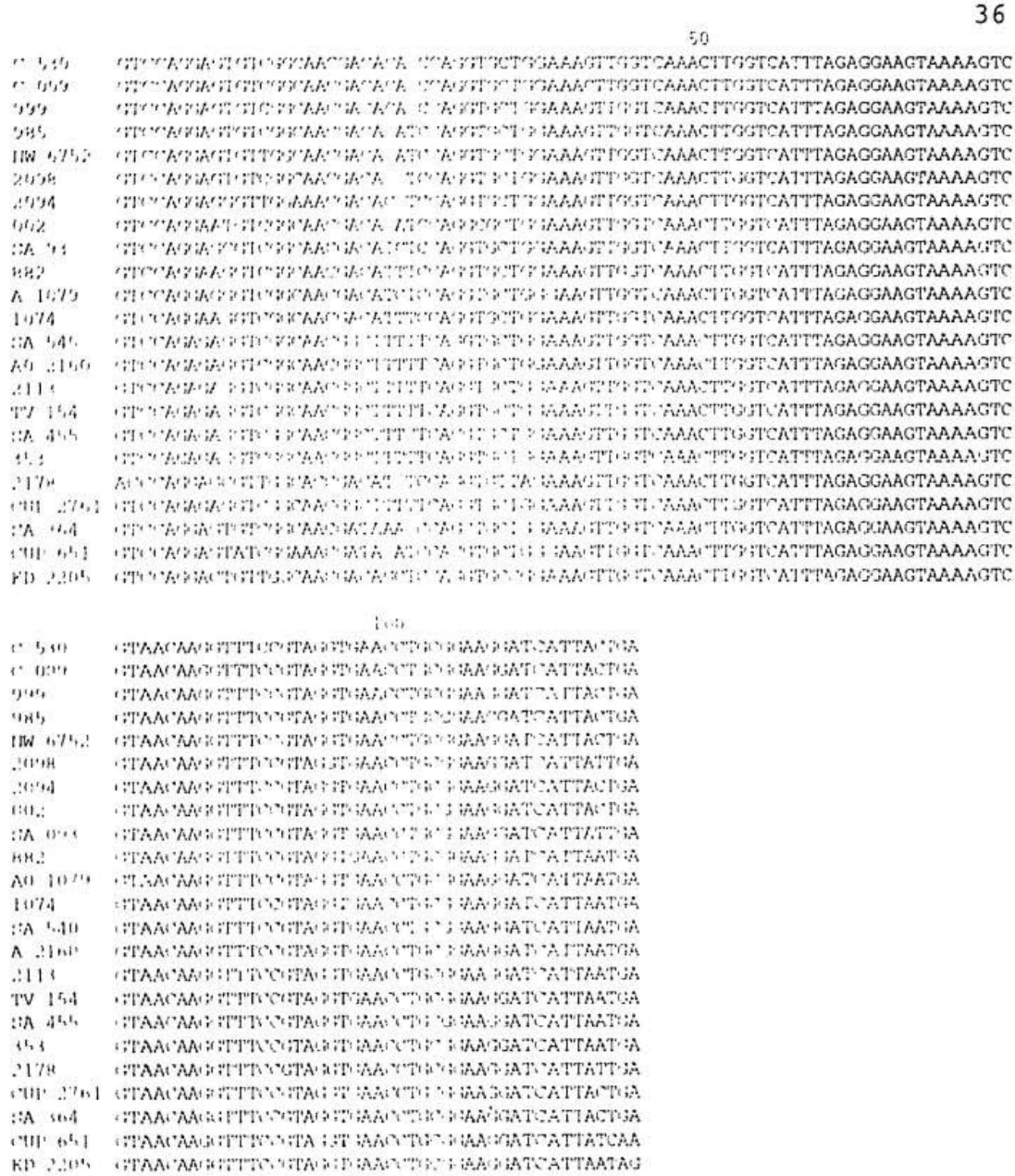


Figure 4. Aligned sequences for all 23 taxa for SSU1. Isolate reference numbers follow the same coding as found in table 1. Base positions are indicated every 50 bp. Gaps are represented by dashes.

NL8mun overlapped NL5mun-NL6mun by 150 bp and amplified the last 375 bp of the NL5mun-NL8mun segment. Combined sequence data yielded a total of 537 bp of which 138 sites were variable (26%) (Figure 5).

The final region which was used in the phylogenetic analysis of *Plicaria*, *Peziza* and select members of Pezizaceae was the SSU2 region. This segment was amplified using primers NS11-12 and produced a fragment which was 465 bp. Sequence data yielded a total of 423 bp of which 74 sites were variable (17%)(Figure 6).

It should be noted that *Peziza* cfr. *linteicola* was not included in the SSU1, SSU2 and LSU analysis because I was unsuccessful at obtaining a reliable sequence for the NS11-12 region. Numerous extractions, amplifications and sequencings attempts were tried but the sequence data that were generated were of very poor quality or contained many ambiguous sites. Comparison of the sequence data suggests that two different NS11-12 fragments are being sequenced concurrently. My inability to remove this contaminant, even after trying different DNA extractions, suggests that a fungal contaminant must be present in the apothecia of this specimen of *Peziza* cfr. *linteicola*

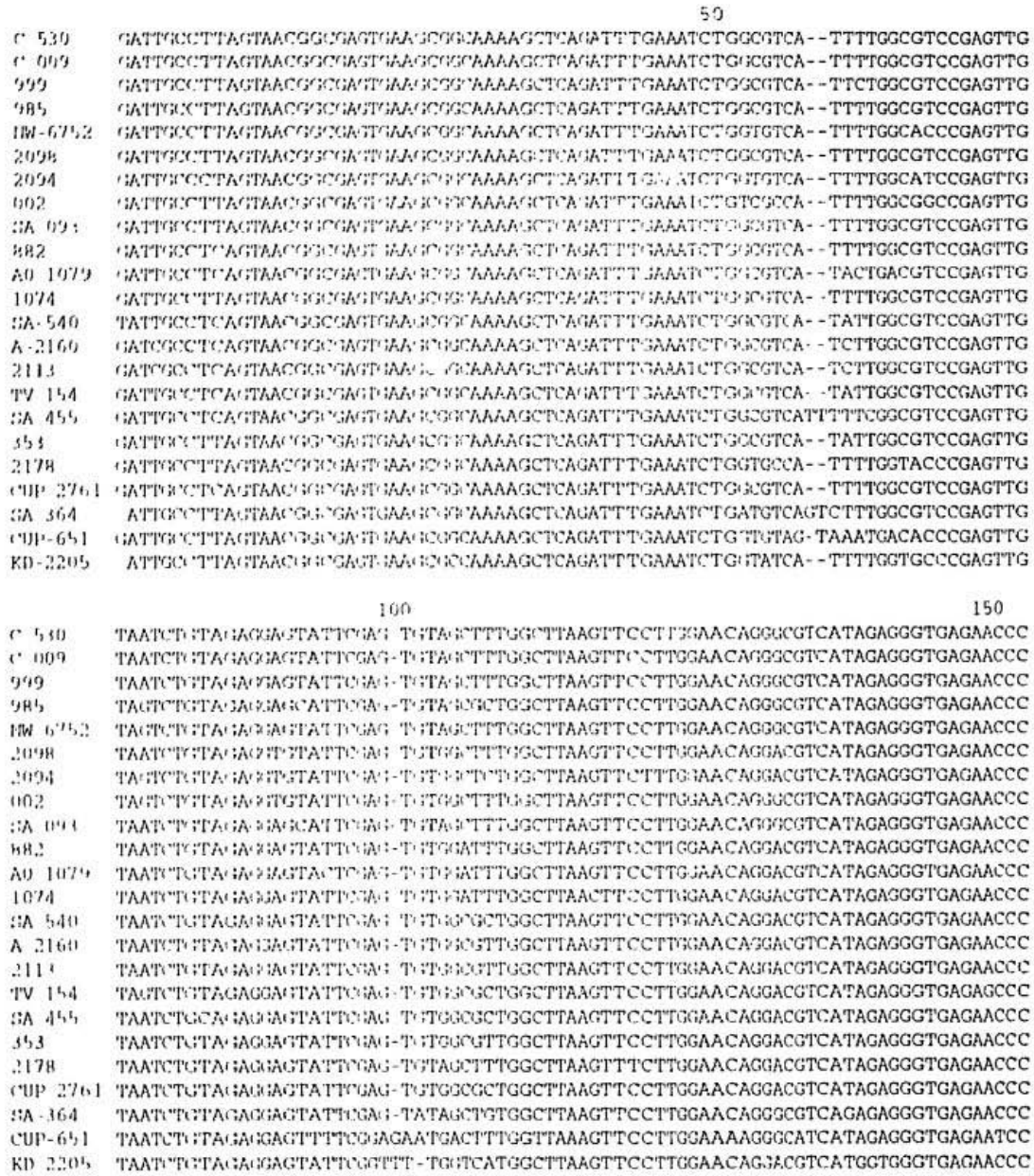


Figure 5. Aligned sequences for all 22 taxa containing a portion of the LSU rDNA repeat. Isolate reference numbers follow the same coding as found in table 1. Base positions are indicated every 50 bp. Gaps are represented by dashes.

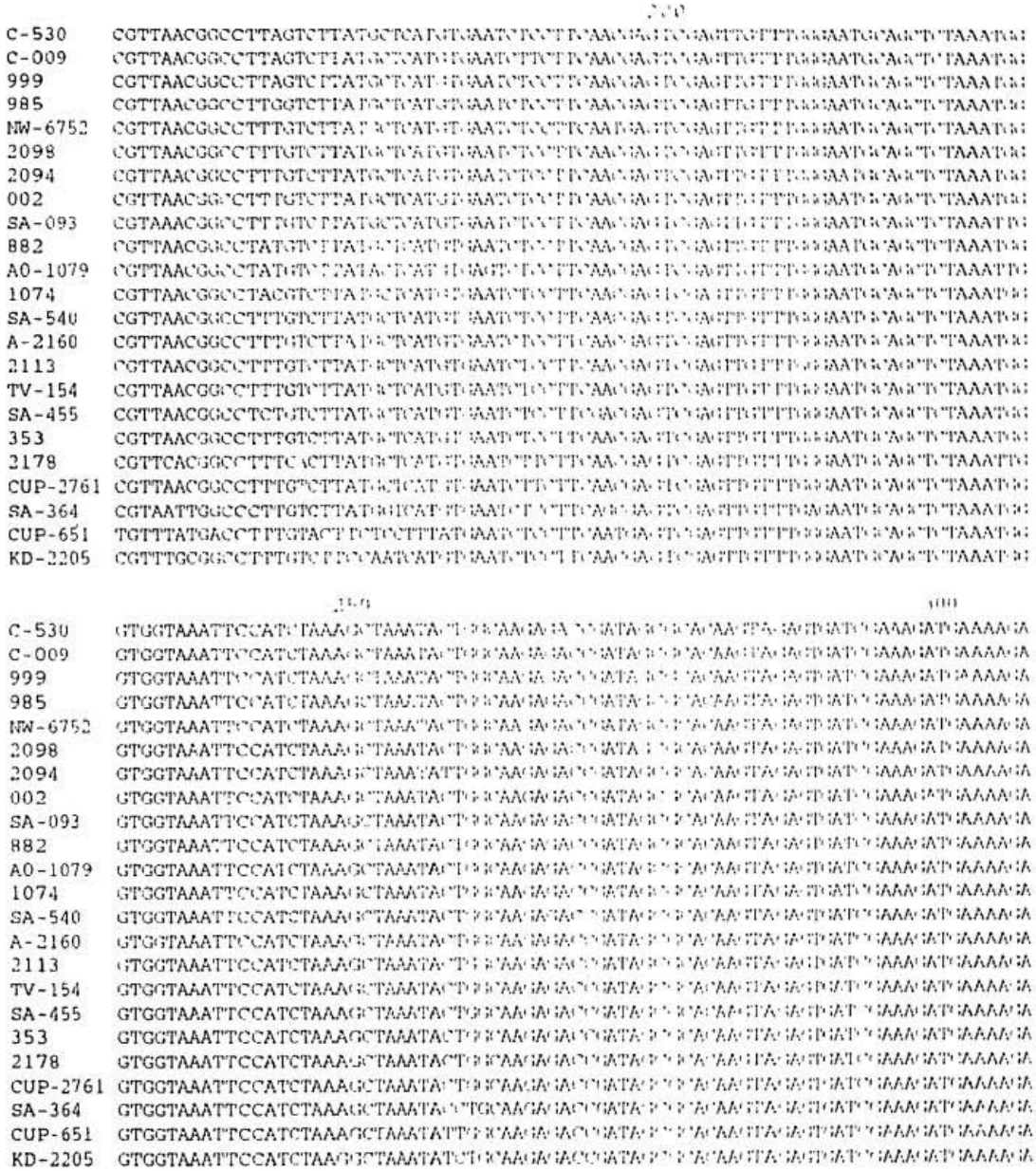


Figure 5 continued

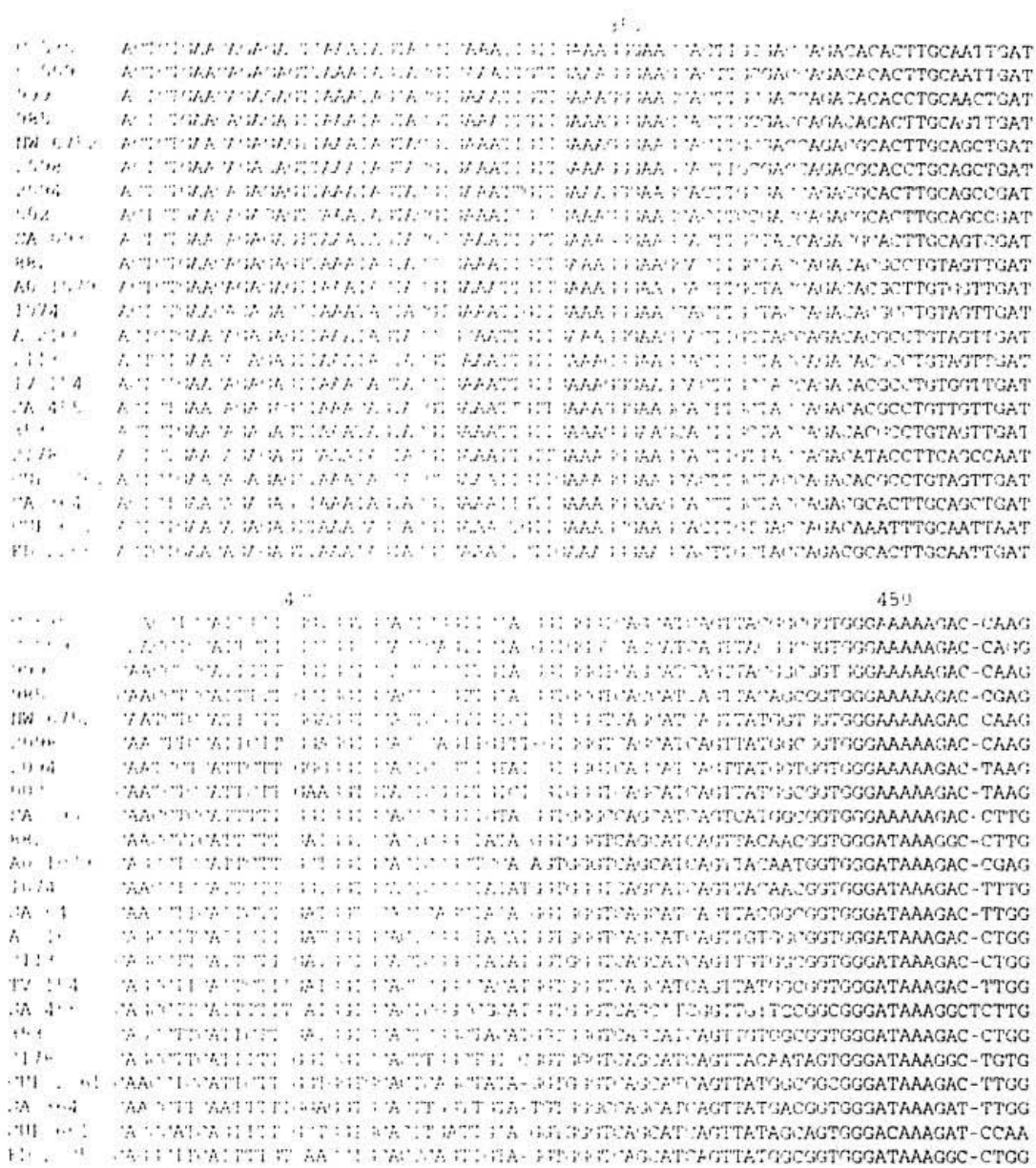


Figure 5 continued

Figure 5 continued

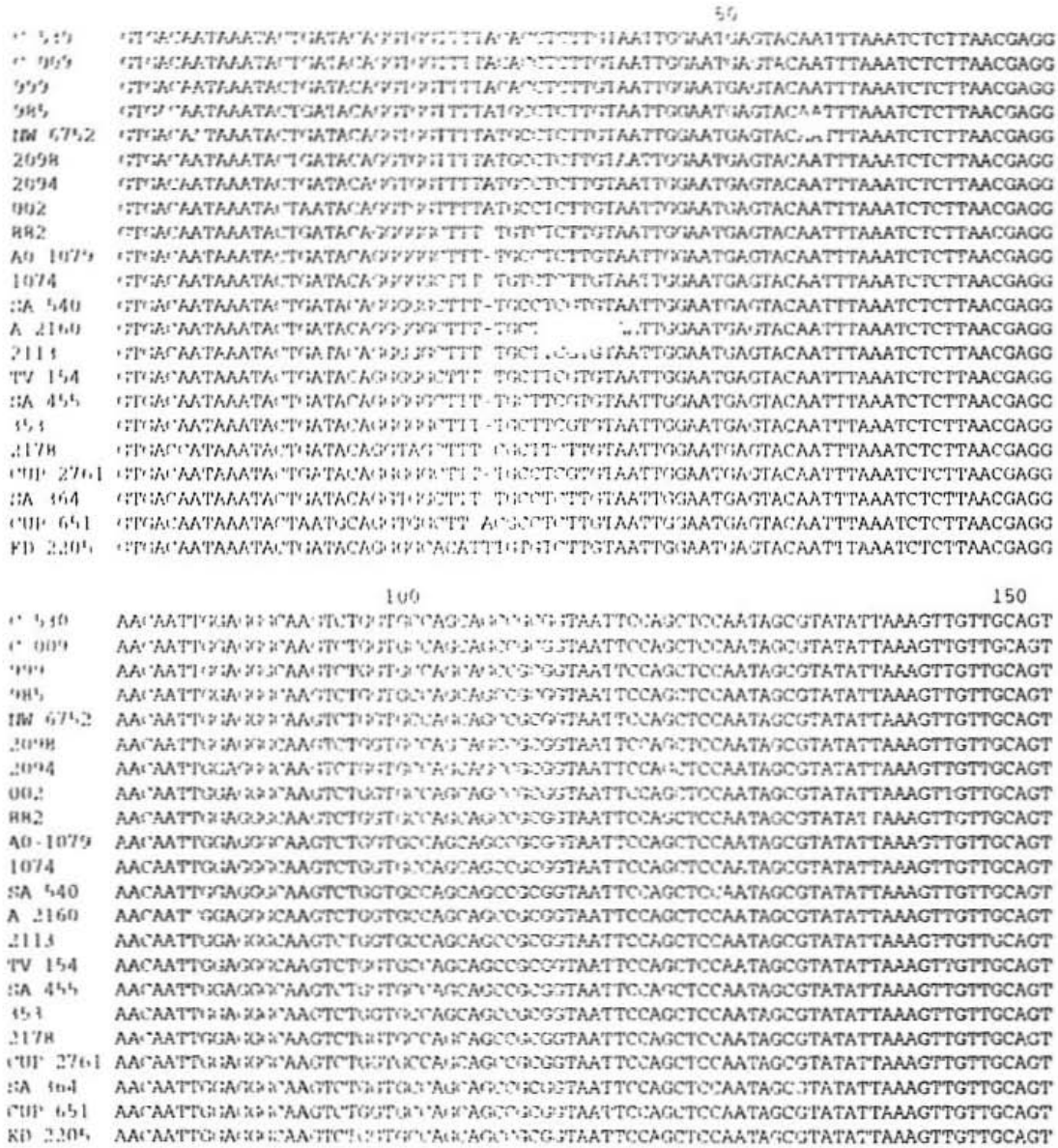


Figure 6. Aligned sequences for 22 taxa containing a portion of the SSU2 rDNA repeat. Isolate reference numbers follow the same coding as found in table 1. Base positions are indicated every 50 bp. Gaps are represented by dashes.

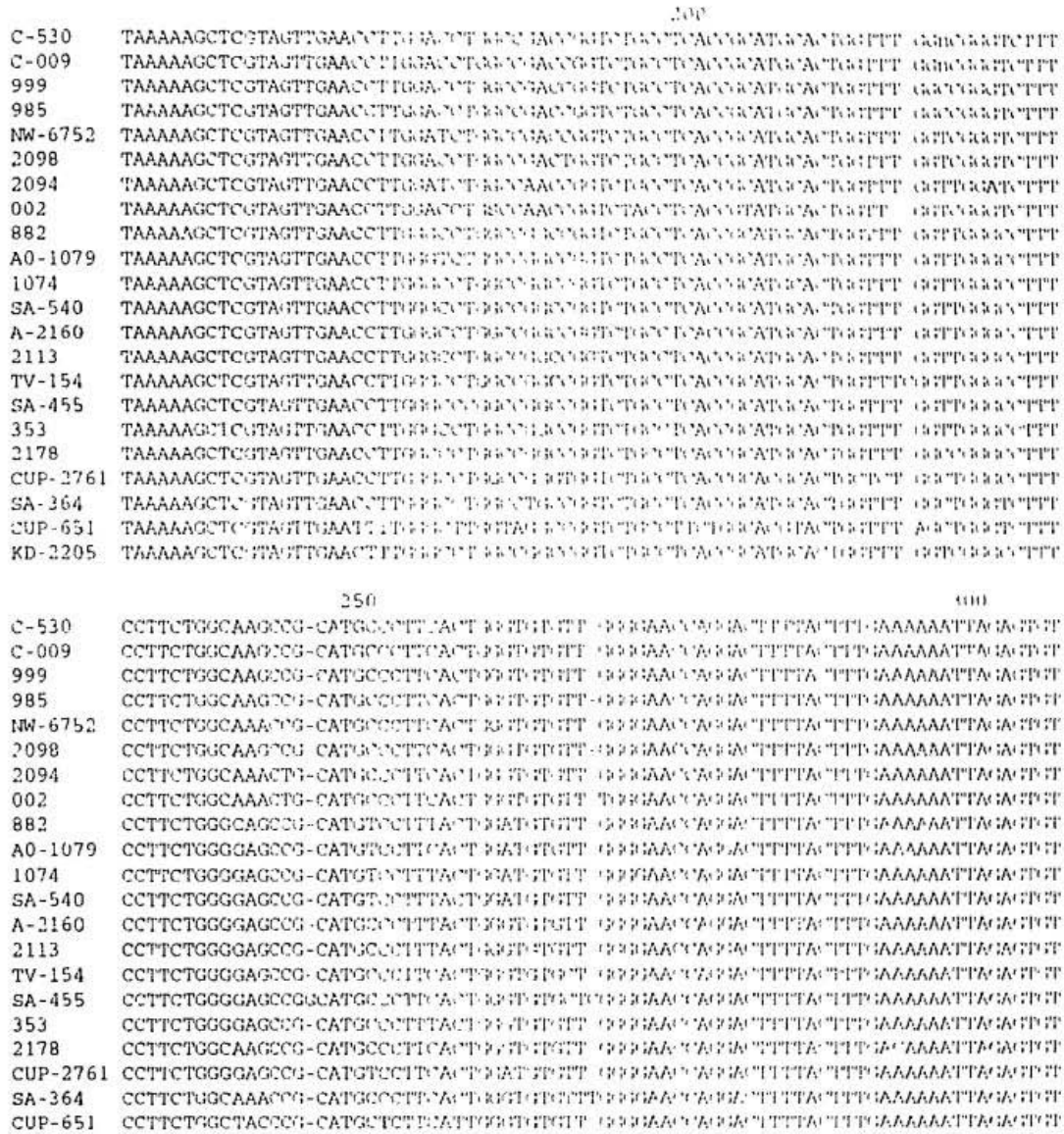


Figure 6 continued

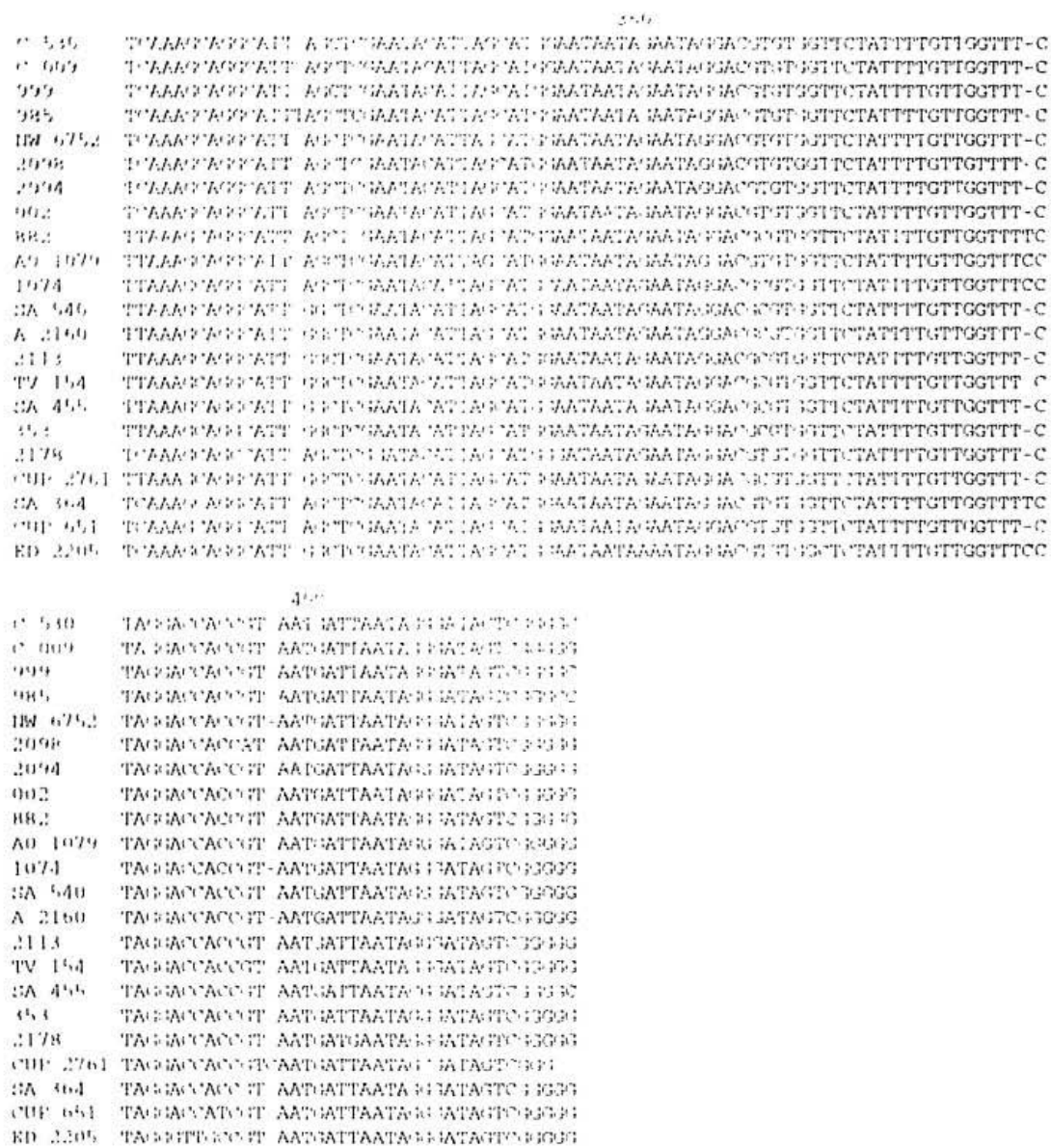


Figure 6 continued

3.2 Phylogenetic Analysis of *Plicaria*, *Peziza* and selected members of Pezizaceae Utilizing SSU1, SSU2 and LSU

The combined SSU1, SSU2 and LSU data set was used for phylogenetic analysis. The 22 taxon analysis consisted of 1074 positions of which 232 sites were variable (22%).

Based on sequence divergence and morphological differences, *Peziza quelepidotia* was chosen as the outgroup. The rDNA sequence of *Peziza quelepidotia* is the most divergent of all taxa compared in this study, and a number of divergent characters suggests that it cannot be accommodated within the *Peziza*, as presently conceived. The issue of proper taxonomic placement of *Peziza quelepidotia* will be addressed later.

Parsimony analysis, using the heuristic search option of PAUP 3.1q with transversion:transition weighting of 2:1, produced three equally parsimonious trees of 595 steps. A representative of the three most parsimonious trees is shown in Figure 7. Identical results were obtained from separate heuristic analyses utilizing general, stepwise addition and branch swapping algorithms. As well, the same tree topology was found when analysis parameters were changed to compare all sites, variant sites only or phylogenetically informative sites only. Transition/transversion weighting of 1:1 gave the same topology as 2:1 weighting, but support for the *Plicaria/Plicaria*-like-*Peziza* and group 1 *Peziza* nodes were generally lower than those found with a 2:1 weighting.

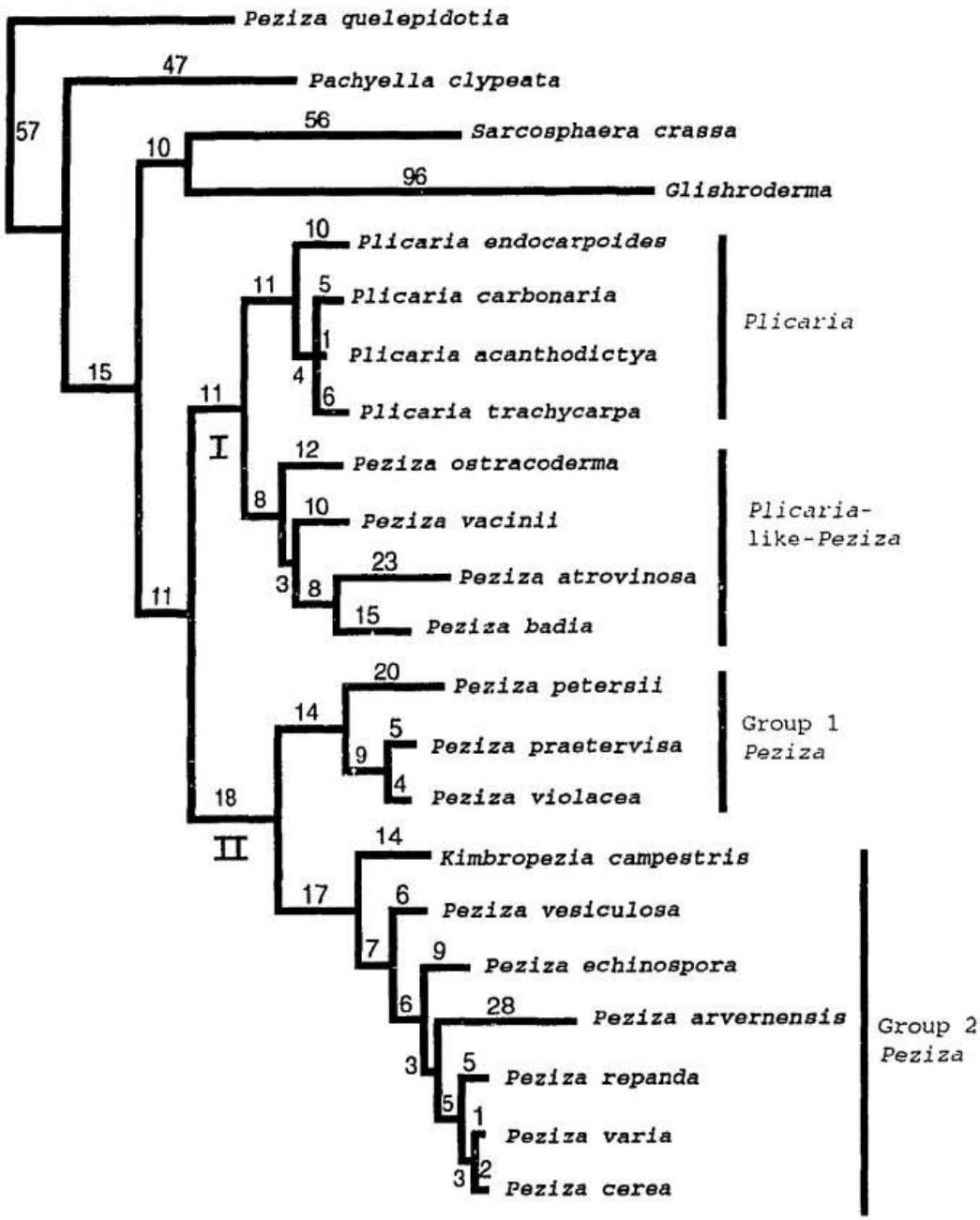


Figure 7. One of 3 trees most parsimonious trees generated of 595 steps inferring the phylogenetic relationship of *Plicaria* to *Plicaria*-like-*Peziza*, *Peziza* and selected members of Pezizaceae. Maximum parsimony analysis was performed on the SSU1, SSU2 and LSU data set. The numbers above the branches indicate the number of nucleotide character changes per node. The cladogram was generated using the heuristic option of PAUP 3.1q.

This suggests that the number of steps supporting these clades are low and that more branch support comes from transversions. This is partly due to the use of the highly conservative SSU regions which limit the number of intergroup substitutions. Support for several groups increased when 2:1 weighting was used. A comparison of other transitions/transversion weights of 3:1, 10:1 and 0:1 gave a similar level of support for the cladogram as 2:1.

Trees generated from the phylogenetic analysis divide the taxa into two large monophyletic groups, which have been designated clade I and clade II (Figure 7). Clade I contains all *Plicaria* species and members of *Plicaria*-like-*Peziza*, which include *Peziza vacinii*, *Peziza ostracoderma*, *Peziza badia* and *Peziza atrovinosa*. The second major clade, clade II, contains group 1 *Peziza* species that include *Peziza violacea*, *Peziza praetervisa* and *Peziza petersii*, and group 2 *Peziza* species that include *Peziza varia*, *Peziza cerea*, *Peziza vesiculosa*, *Peziza echinospora*, *Peziza repanda* and *Kimbropezia campestris*.

The basal branch of clade I is well supported with a bootstrap value of 90% (Figure 8). Clade I contains the *Plicaria* subclade that has bootstrap support of 95% and the *Plicaria*-like-*Peziza* subclade which was not as well supported, as indicated by the 83% bootstrap value. These data also show that the relationships within the *Plicaria* and the *Plicaria*-like-*Peziza* clusters are not well resolved.

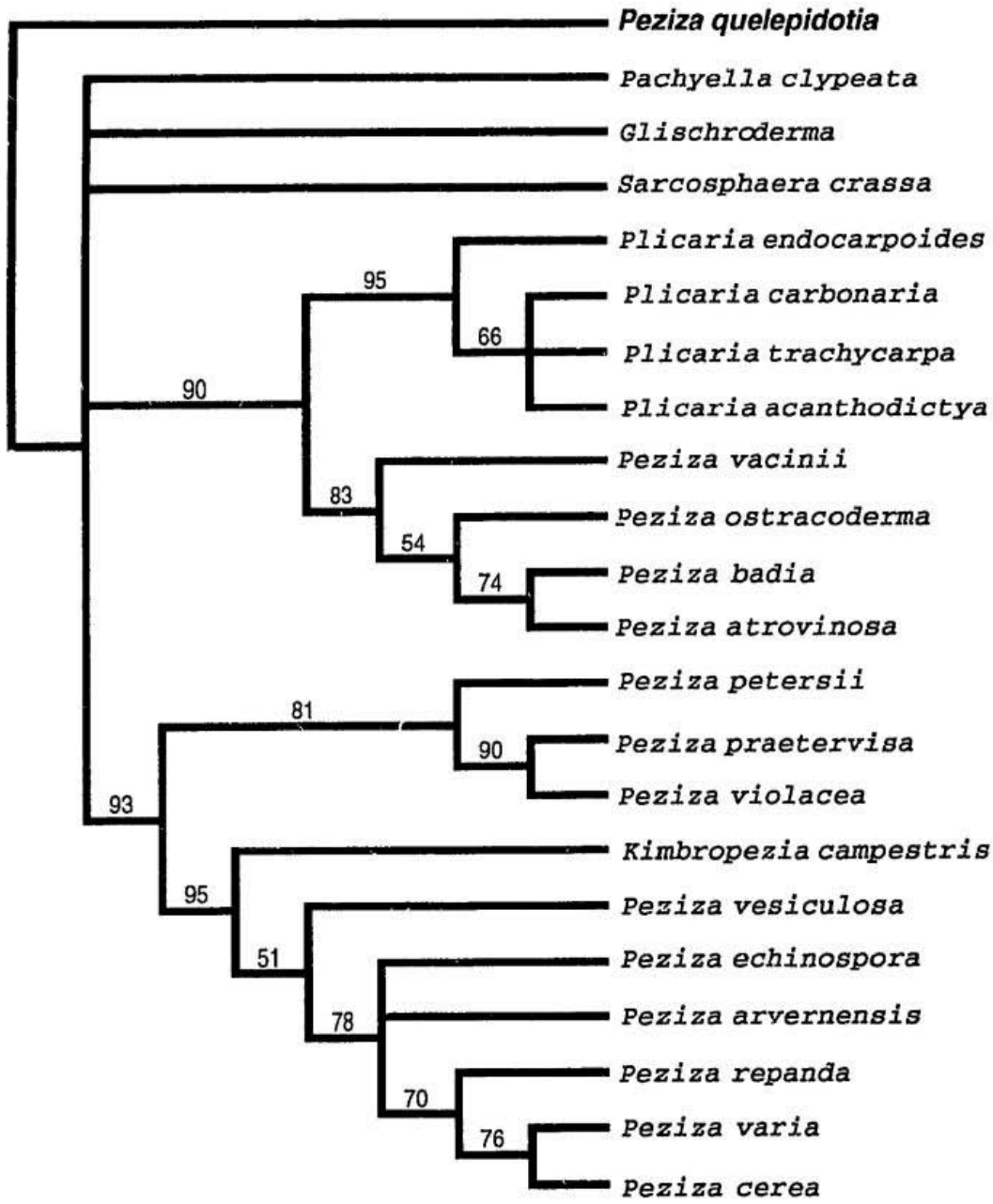


Figure 8. Bootstrap parsimony analysis for 22 taxa utilizing SSU1, SSU2 and LSU data set for *Plicaria*, *Plicaria*-like-*Peziza* and selected members of Pezizaceae. The numbers above the branches indicates percentage of support >50% for each node determined from 100 bootstrap replicates. The cladogram was generated using the bootstrap analysis heuristic option of PAUP 3.1q.

This is a result of the low level of intragroup sequence variation for the highly conserved SSU2 and SSU1 regions. Clade I is made up of two closely related monophyletic groups that have diverged from smooth-spored *Peziza* species. The occurrence of *Peziza* taxa within this clade makes *Peziza* paraphyletic.

Clade II, which mostly includes *Peziza* taxa, is well supported as indicated by a bootstrap value of 93%. This clade contains two monophyletic groups, the group 1 *Peziza* taxa and the group 2 *Peziza* taxa. The group 2 *Peziza* cluster is very well supported as shown by a bootstrap value of 95% but the group 1 *Peziza* cluster is less well supported as indicated by a 81% bootstrap. The close relationship between these two groups suggests they have evolved from a recent common ancestor.

Within the group 1 *Peziza* clade, *Peziza petersii* forms a basal branch to the well supported *Peziza praetervisa* and *Peziza violacea* node. The most basal branch found within the group 2 *Peziza* clade is defined by *K. campestris* the only taxon within clade II which is not a member of *Peziza*. Other taxa found within this group include *Peziza cerea, Peziza varia, Peziza repanda, Peziza arvernensis* and *Peziza vesiculosa* but the species relationships within this cluster are poorly resolved as indicated by the low bootstrap values placed on many of the branches.

All members of Pezizaceae other than *Plicaria* and

Peziza could not be resolved using this data set (Figure 8). The cladogram reveals that *Sarcosphaera crassa* and *Glischroderma* sp. cluster together on very long branches and form a sister group to the members of *Plicaria* and *Peziza*. However, as indicated by bootstrap analysis these branches collapse to form a polytomy with *Pachyella clypeata*.

3.3 Sequence Alignment Among *Plicaria* and *Plicaria*-like-*Peziza* Species for ITS-1 and LSU Regions

As indicated by the parsimony analysis of SSU1, SSU2 and LSU sequence, the *Plicaria*/*Plicaria*-like-*Peziza* clade is monophyletic and distinct from other *Peziza* taxa. However, the phylogenetic relationship of taxa found within the *Plicaria* and *Plicaria*-like-*Peziza* clades are not well resolved. In an attempt to resolve intragroup relationships, analysis of the more variable ITS-1 and LSU regions was performed on members of *Plicaria* and *Plicaria*-like-*Peziza*.

Initial alignments of the ITS-1 region for *Plicaria* and *Plicaria*-like-*Peziza* taxa were performed using the alignment program SeqEd and optimized by hand after alignment. Three regions, which spanned positions 85-96, 109-128 and 152-212, were omitted from the analysis because they were considered to be too variable to be aligned with confidence (Figure 9). With the three variable regions omitted, the ITS-1 region consisted of 131 positions of which 45 were variable (34%). In addition to the ITS-1 region, this analysis also

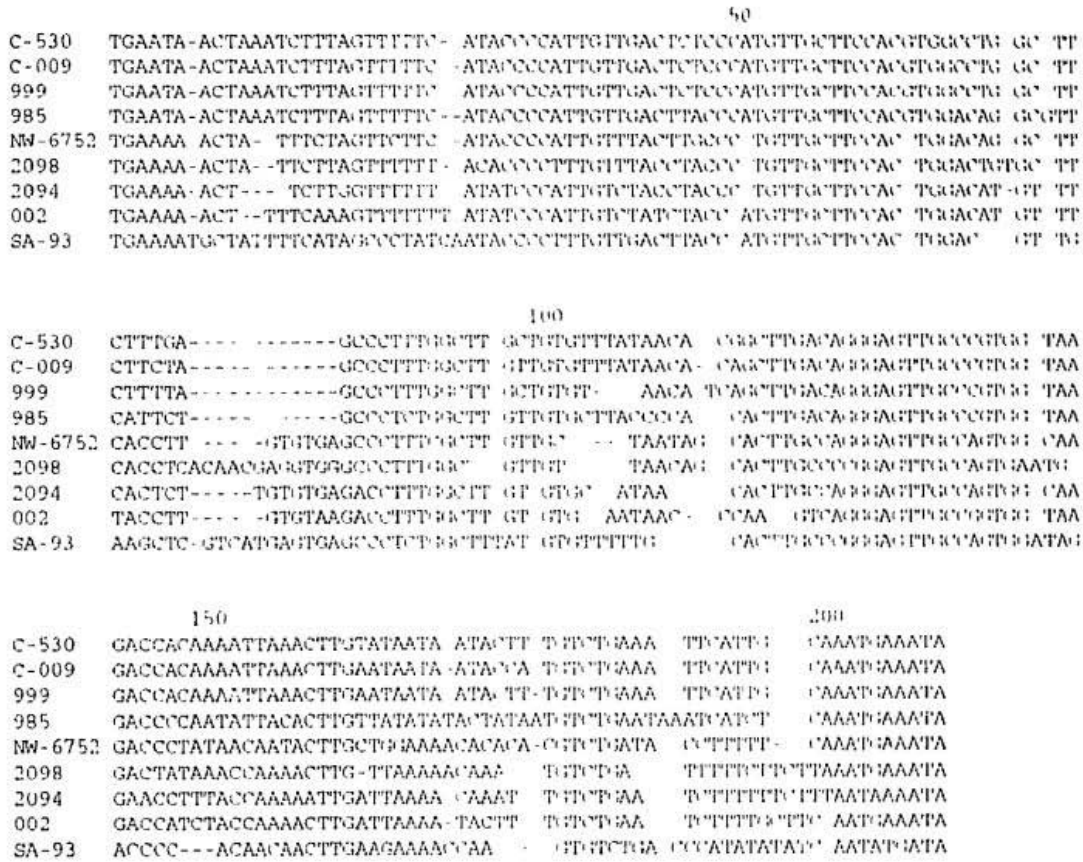


Figure 9. Aligned *Plicaria* and *Plicaria*-like-*Peziza* sequences for ITS-1 region of the rDNA repeat. Isolate reference numbers follow the same coding as found in table 1. Base positions are indicated every 50 bp. Gaps are represented by dashes.

contained the LSU region. The LSU sequence data yielded a total of 530 bp of which 65 sites were variable (12%). The combined ITS-1 and LSU ribosomal DNA data set included 9 taxa and consisted of 661 positions of which 110 sites were variable.

3.4 Phylogenetic Relationships of *Plicaria* to *Plicaria*-like-*Peziza*

Peziza cfr. *linteicola* was chosen as the outgroup for the ITS-1 and LSU analysis for several reasons, even though it was not included in the complete SSU1, SSU2 and LSU analysis. First, preliminary parsimony analysis including *Peziza* cfr. *linteicola* data for all 22 taxa suggests that this taxon forms a basal branch to either the *Peziza* or *Plicaria/Plicaria*-like-*Peziza* clades, and appears to possess intermediate characters of both clades. Second, *Peziza* cfr. *linteicola* was the only taxon that could be aligned with members of the *Plicaria/Plicaria*-like-*Peziza* clade throughout most of the ITS-1 region. However, it still possesses sequence elements that are common to members of the *Peziza* clade.

Parsimony analysis, using the branch and bound algorithm of PAUP 3.1q with a transition/transversion weight of 2:1, produced three equally parsimonious trees of 171 steps. An example of 1 of the 3 most parsimonious trees is found in Figure 10. Identical results were obtained from

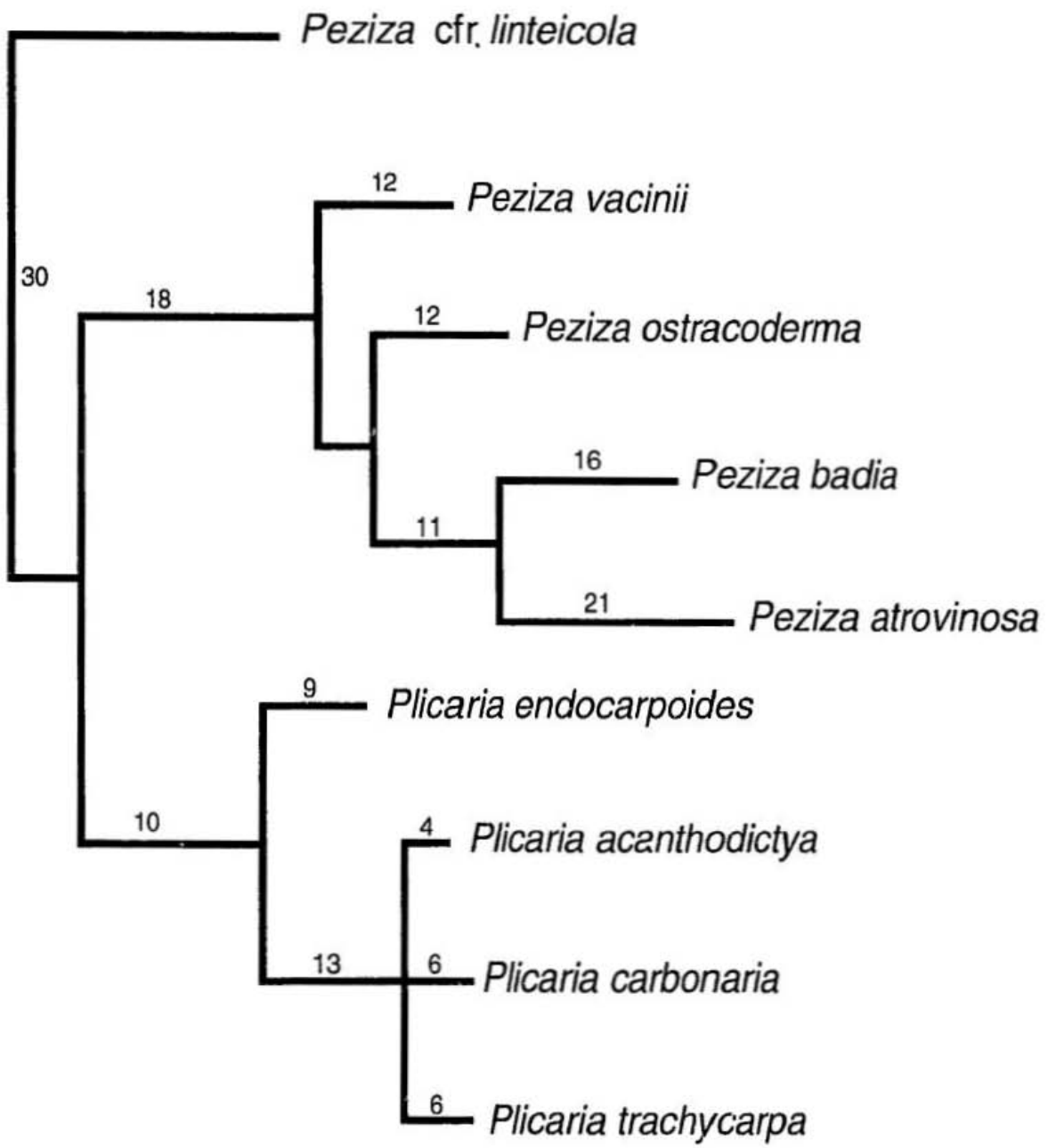


Figure 10. One of 3 most parsimonious trees of 173 steps inferring the phylogenetic relationship of *Plicaria* to *Plicaria*-like-*Peziza*. Maximum parsimony analysis was performed on combined nucleotide characters utilizing ITS-1 and LSU rDNA data sets. The numbers above the branches indicate the number of nucleotide character changes per node. The cladogram was generated using the branch and bound option of PAUP 3.1q.

separate branch and bound analyses utilizing different sequence addition strategies. Similarly, a number of different transition/transversions weights were also compared to determine the effect that different character weights had on tree topology and node support. A transition/transversion weighting of 1:1 gave the same topology as 2:1 weighting, but support for the *Plicaria*-like-*Peziza* node was reduced. This suggests that the *Plicaria* and *Plicaria*-like-*Peziza* clusters represent two groups of closely related taxa which have a similar number of intragroup substitutions as compared to intergroup substitutions but there are more transversions occurring between *Plicaria* and *Plicaria*-like-*Peziza* than within each cluster. A comparison of other transitions/transversion weightings of 3:1, 10:1 and 0:1 gave a similar level of support for the cladogram as did 2:1 weighting.

The cladogram generated from parsimony analysis using the ITS-1 and LSU regions divides the taxa into two monophyletic groups (Figure 10) the same as the parsimony analysis using SSU1, SSU2 and LSU (Figure 7). The largest difference between the two analyses is that the ITS-1 and LSU region shows greater resolution within the *Plicaria* and *Plicaria*-like-*Peziza* clades, as indicated by bootstrap analysis (Figure 11).

The *Plicaria* grouping is strongly supported as indicated by a bootstrap confidence level of 100%. Within

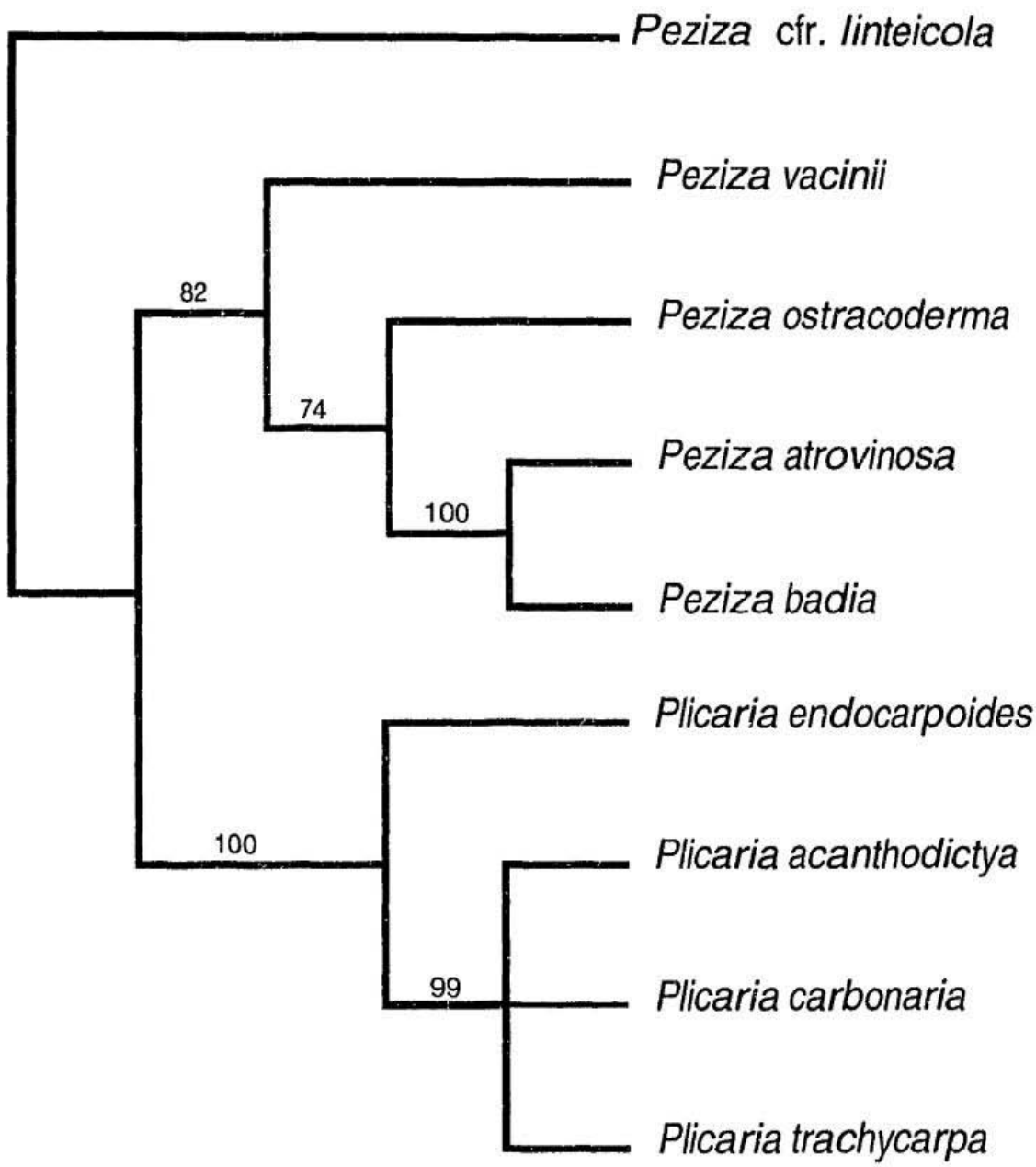


Figure 11. Bootstrap p[illegible]ar. lysis for *Plicaria* and *Plicaria*-like-*Peziza* [illegible]lizing combined nucleotide cha[illegible]ers from ITS-[illegible] [illegible]rDNA data sets. The numbers abo[illegible] branches indica[illegible]s percentage of support >50% for each [illegible] determined from 1000 bootstrap replicates. The clad[illegible] was generated using the bootstrap analysis branch and [illegible] option of PAUP 3.1q.

this group, smooth-spored *Plicaria endocarpoides* forms a sister group to the ornamented-spored *Plicaria acanthodictya*, *Plicaria carbonaria* and *Plicaria trachycarpa* trichotomy. The ornamented-spored *Plicaria* node received a high degree of support as indicated by the 99% bootstrap value, however, there was still not enough information to resolve the relationship between the three ornamented-spored *Plicaria* species.

The *Plicaria*-like-*Peziza* clade includes *Peziza vacinii*, *Peziza ostracoderma*, *Peziza atrovinosa* and *Peziza badia* is moderately well supported, as indicated by a 82% bootstrap value. The most basal branch in this group is defined by *Peziza vacinii*. Within this clade, *Peziza badia* and *Peziza* cluster together with 100% bootstrap support. *Peziza ostracoderma* forms a basal branch to the two species, however, this branch is not as well supported.

In order to assess the stability of the branches within the *Plicaria*-like-*Peziza* cluster a modified jackknife was performed on the data set. This was accomplished by leaving out taxa at random, perfcrming a parsimony analysis and determining the level of branch support using bootstrap analysis. The results of these analyses revealed that by removing *Peziza vacinii* the support for the *Plicaria*-like-*Peziza* group increased to 97% (Figure 12). Removal of other taxa but inclusion of *Peziza vacinii* always reduced the level of support for the *Plicaria*-like-*Peziza* group.

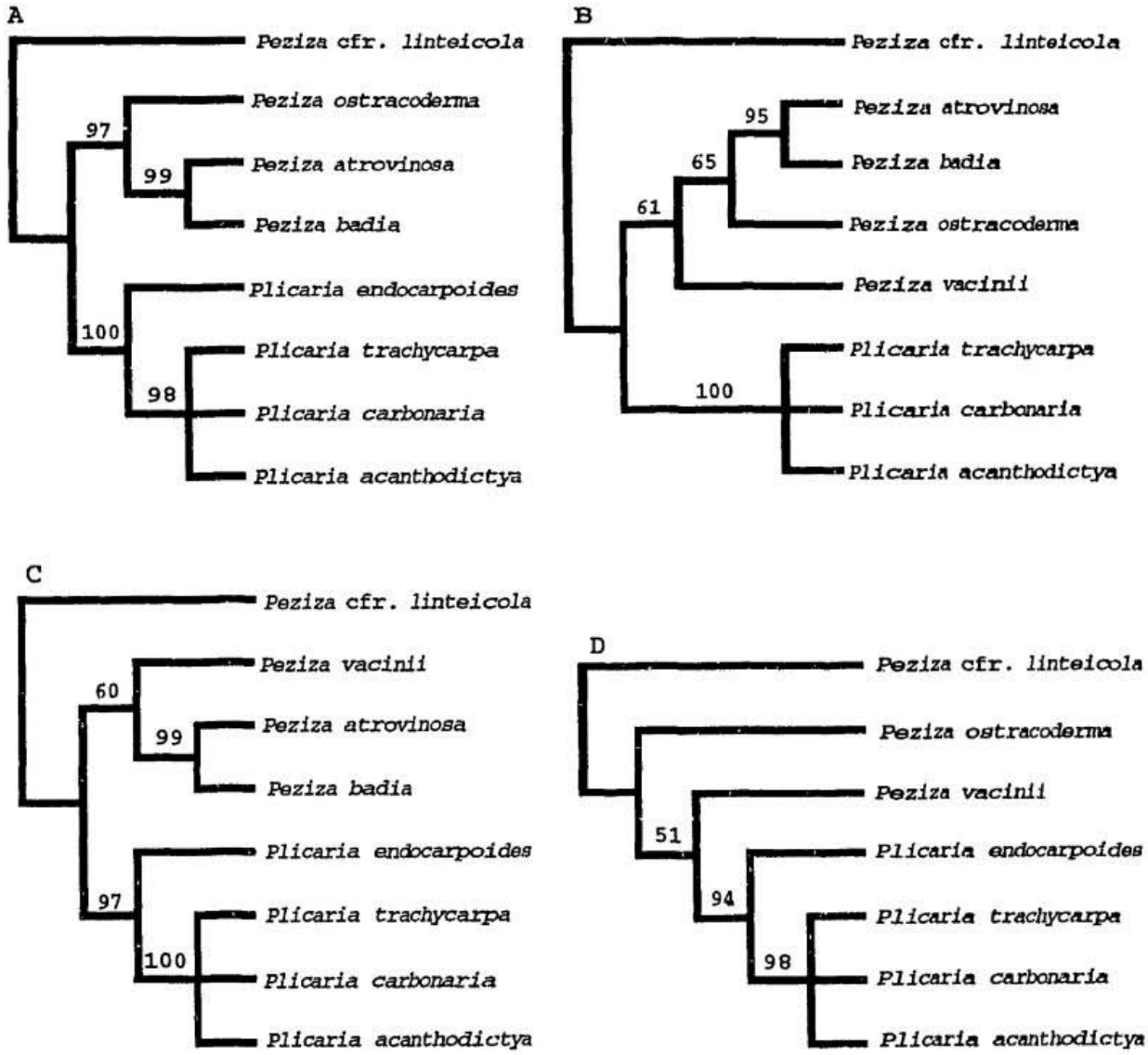


Figure 12. Bootstrap parsimony analysis for *Plicaria* and *Plicaria*-like-*Peziza* taxa utilizing combined nucleotide characters from ITS-1 and LSU rDNA data sets for 4 different samplings. A) *Peziza vacinii* omitted, B) *Plicaria endocarpoides* omitted, C) *Peziza ostracoderma* omitted and D) *Peziza atrovinosa* and *Peziza badia* omitted. The numbers above the branches indicates percentage of support >50% for each node determined from 1000 bootstrap replicates. The cladogram was generated using bootstrap analysis branch and bound option of PAUP 3.1q.

3.5 Intraspecific and Interspecific Variation Within *Plicaria*

In order to assess the degree of sequence variation that occurs within and between *Plicaria* species, ITS sequences were compared. Initial alignments of the ITS-1 region was performed using the alignment program SeqEd and then optimized by hand. Aligned sequence data for *Plicaria* isolates show that the ITS-1 region spanned 206 bp (Figure 13). Elimination of small subunit sequences and addition of ITS-1 sequences provided an adequate, but not excessive, degree of variation among taxa (Kohn, 1992).

A comparison of intraspecific variation within *Plicaria* revealed that *Plicaria trachycarpa* was the only taxon that displayed sequence variation among isolates. However, most of this variation could be attributed to a single region that has undergone an insertion/deletion event (indel). An exception was *Plicaria trachycarpa* var *muricata*, that showed a 3.5% sequence divergence from *Plicaria trachycarpa* but differed from *Plicaria carbonaria* by a single base. Examination of this indel region revealed that most of the variation appears to be in the number of AT repeats present. Tautz et al. (1989) suggests that this type of variation may arise as a result of polymerase slippage during DNA replication. Sequence comparisons show that the number of AT repeats varied starting at position 100 (Figure 13). *Plicaria trachycarpa* isolate 2177 has four,

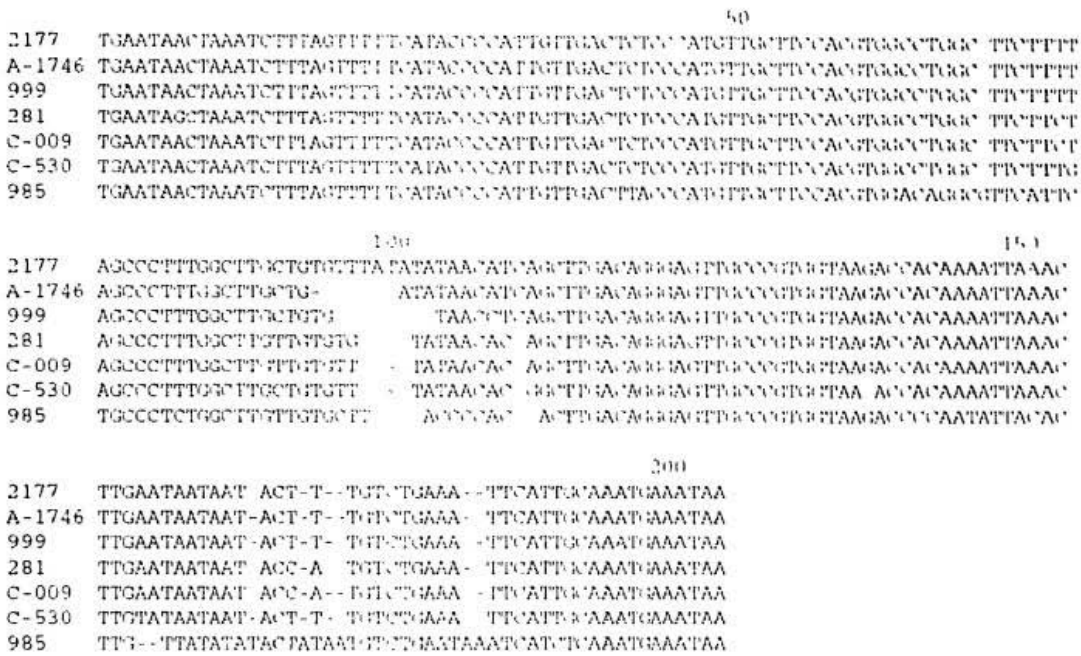


Figure 13. Aligned *Plicaria* sequences for ITS-1 region of the rDNA repeat. Isolate reference numbers follow the same coding as found in table 1. Base positions are indicated every 50 bp. Gaps are represented by dashes.

isolate A-1746 has two and isolate 999 has only one AT. Similarly, both *Plicaria* carbonaria and *Plicaria acanthodictya* appear to have two repeats while *Plicaria endocarpoides* does not have any. Although the number of repeats differs between taxa, these regions tend to display a high degree of homoplasy which restricts their usefulness in determining phylogenetic relationships.

A comparison of interspecific variation showed that there was a 23% sequence divergence rate among all *Plicaria* taxa for the ITS-1 region, but *Plicaria endocarpoides* is responsible for 13% of the total. A comparison of percent sequence divergence among ornamented-spored *Plicaria* taxa revealed that between *Plicaria trachycarpa* and *Plicaria carbonaria*, and between *Plicaria carbonaria* and *Plicaria acanthodictya* 3.5% sequence divergence was found but only 2.9% occurred between *Plicaria trachycarpa* and *Plicaria acanthodictya*.

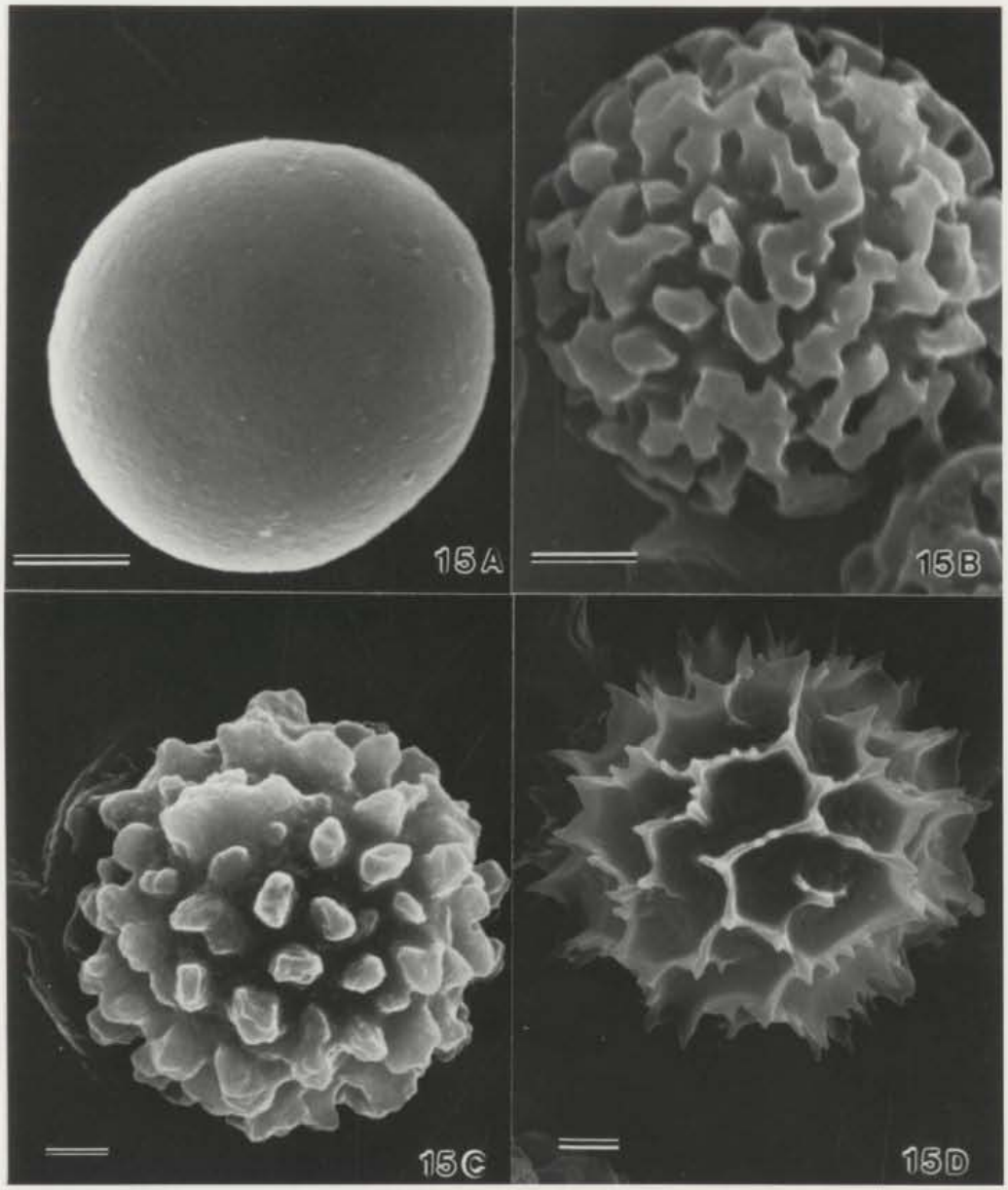
3.6 Spore Morphology Comparisons within *Plicaria* and *Plicaria*-like-*Peziza*

Plicaria endocarpoides, *Plicaria trachycarpa*, *Plicaria acanthodictya* and *Plicaria carbonaria* are differentiated primarily by spore morphology. *Plicaria endocarpoides* has smooth spores, but spore ornamentation among the other three taxa are less easily distinguish using light microscopy. However, scanning electron microscope (SEM) micrographs

taken of *Plicaria* taxa show that four different spore ornamentation patterns could be readily distinguished. This is consistent with the existence of four distinct species. It should be pointed out, however, that spore measurements determined using SEM are slightly smaller than those recorded in the literature. The likely reason is that spores examined using SEM were air dried and this resulted in shrinkage, while most measurements are done on spores in a liquid media such as water which causes the ascospores to swell and become turgid.

The SEM micrograph (Figure 15a) confirms that *Plicaria endocarpoides* spores are completely smooth and do not have any small warts or ridges which were unresolveable using the light microscope. The SEM comparisons reveal that the spore ornamentation patterns for the three rough-spored *Plicaria* taxa are different for each species. *Plicaria trachycarpa* has spores which are 8.0 µm in diameter excluding ornamentation. The ornamentation consists of broad flattened ridges, 1.0 µm long and joined together in a partial reticulum (Figure 15b). *Plicaria carbonaria*, is 11.2 µm in diameter excluding ornamentation. The ornamentation consists of 1.8 µm long coarse blunt warts that occasionally coalesce to form ridges (Figure 15c). It should also be pointed out that *Plicaria trachycarpa* var. *muricata*, which differed from *Plicaria carbonaria* by a single base, also had a similar spore ornamentation and size *as Plicaria*

Figure 15 Scanning electron micrographs of ascospores of four *Plicaria* species. A) *Plicaria endocarpoides*, B) *Plicaria trachycarpa*, C) *Plicaria carbonaria* and D) *Plicaria acanthodictya* Scale bar equals 2 µm.


15A
15B
15C
15D

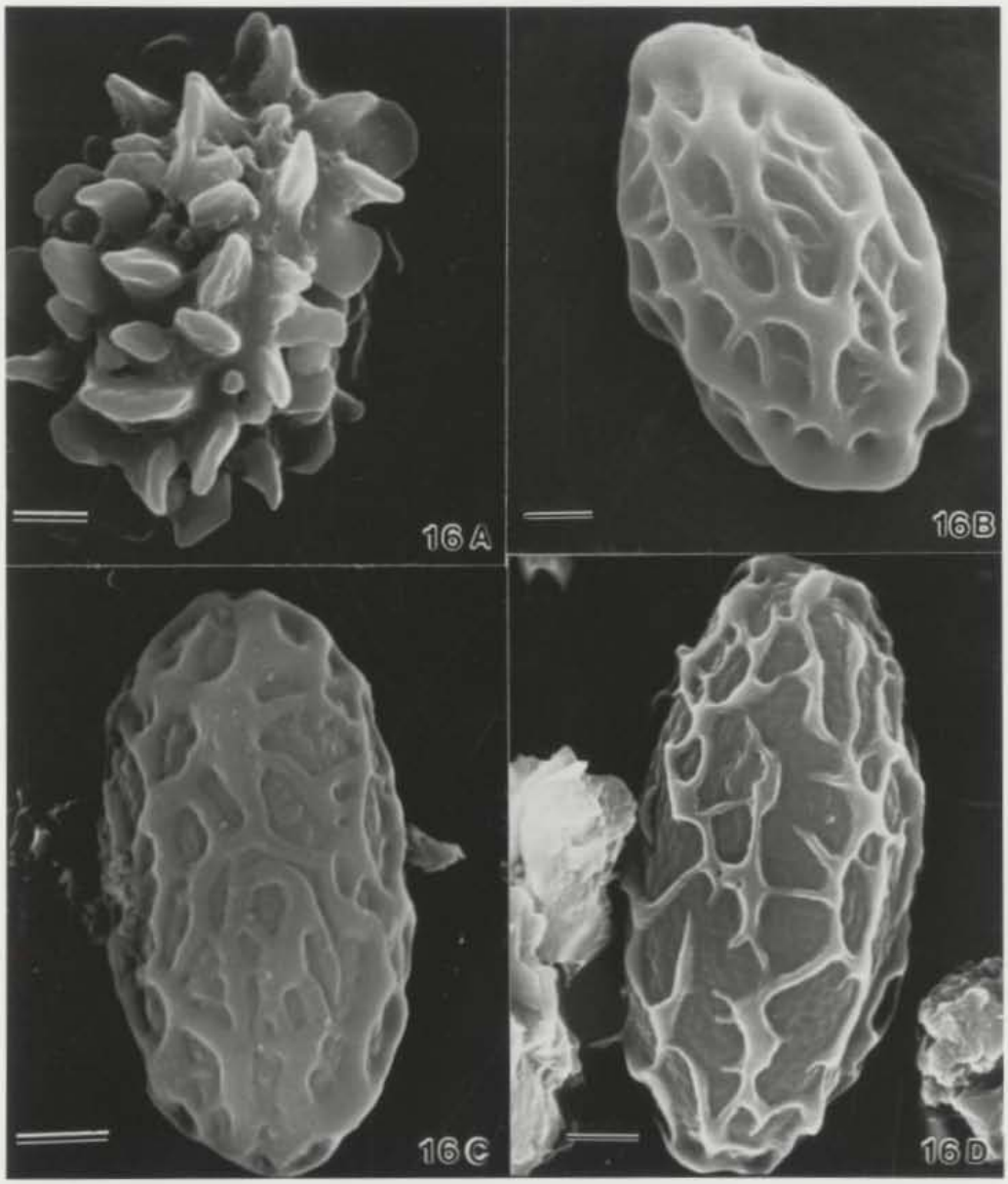
carbonaria. *Plicaria acanthodictya* is 12.2 µm in diameter excluding ornamentation. The ornamentation consists of thin spiny ridges that are 1.8 µm long and form a reticulum (Figure 15d).

Ascospore ornamentation patterns are not as important for the determination of species differences within *Plicaria*-like-*Peziza* as *Plicaria*. *Peziza vacinii*, *Peziza ostracoderma*, *Peziza atrovinosa* and *Peziza badia* can be differentiated based on apothecia size, colour and habitat as well as spore morphology. However, there is some disagreement on the degree of similarity of ascospore ornamentation between *Peziza atrovinosa* and *Peziza ostracoderma* based upon light microscopy.

Scanning electron micrographs of *Plicaria*-like-*Peziza* taxa show that four different spore ornamentation patterns could be distinguished. However, the similarity among *Peziza atrovinosa*, *Peziza ostracoderma* and *Peziza badia* indicates why these spore ornamentation patterns can be difficult to discern using compound light microscopy.

Ascospore examination using SEM shows that *Peziza vacinii* is the only species found within *Plicaria*-like-*Peziza* clade that does not possess a reticulum. *Peziza vacinii* has elliptical spores that are 11.4 µm x 6.8 µm excluding ornamentation (Figure 16a). Ornamentation consists of thick isolated ridges as well as warts which are up to 1.8 µm high. The ridges appear to be rounded at the top

Figure 16 Scanning electron micrographs of ascospores of four members of *Plicaria*-like-*Peziza*. A)*Peziza vacinii*, B)*Peziza atrovinosa*, C)*Peziza ostracoderma* and D)*Peziza badia*. Scale bar equals 2 µm.


16 A
16B
16 C
16 D

while the warts are pointed or blunt. This ornamentation pattern is most similar to that of *Plicaria carbonaria*.

The SEM comparisons among the three reticulate-spored *Plicaria*-like-*Peziza* species reveal that ornamentation patterns are very similar, but slight differences can be seen. *Peziza atrovinosa* has spores which are 12.0 µm x 5.6 µm excluding ornamentation (Figure 16b). The reticulation consists of broad flat ridges that are raised 1.2 µm and occasionally extend up to 2 µm. *Peziza ostracoderma* has truncated spores that are 11.0 µm x 5.1 µm excluding ornamentation (Figure 16c). The reticulation consists of broad flat ridges that are raised 0.5 µm. *Peziza badia* has spores that are 14.8 µm x 6.6 µm excluding ornamentation (Figure 16d). The ornamentation consists of thin ridges which are 0.7 µm high and form a reticulum .

3.7 Pattern of Adaptation to Burned Habitats

The cladograms were used to examine the evolution of the ecological association with burned habitats. This occurs in three states: 1)obligately associated with burns, 2)facultatively associated with burns, and 3)not associated with burns. This study was interested in determining whether this character was monophyletic or paraphyletic, and if the polarity of the character state change could be determined from the cladogram.

The taxa sampled in this study occur on several

different types of substrate. As shown in Figure 17, obligate postfire species include all *Plicaria* species, all group 1 *Peziza* species, *Peziza vacinii* of the *Plicaria*-like-*Peziza* group and *Peziza echinospora* of the group 2 *Peziza* species. Many of the species that are associated in a facultative manner with burned sites are found in Group 2 *Peziza* such as *Peziza varia*, *Peziza vesiculosa*, *Peziza repanda* as well as *Peziza ostracoderma* of *Plicaria*-like-*Peziza* clade. The taxa which do not occur on burns and that mostly inhabit disturbed areas include *Peziza atrovinosa*, *Peziza badia* of the *Plicaria*-like-*Peziza* group and *Peziza cerea*, and *K. campestris* of the group 2 *Peziza* cluster.

The cladogram suggests that *Plicaria* taxa form a well supported monophyletic group of obligate postfire species. This indicates that all members of this lineage occur on burned sites. This pattern of adaptation to burned habitats is also found within the group 1 *Peziza* clade, that also form a monophyletic group.

Obligate postfire species form basal branches in both the *Plicaria*/*Plicaria*-like-*Peziza* and Peziza clades. This suggests that members of *Plicaria* and *Peziza* have evolved within a common lineage where the ancestral condition was to occur on postfire sites. However, the taxa sampling for the *Peziza* clade is not as complete as that of the *Plicaria*/*Plicaria*-like-*Peziza* clade and these results should be interpreted in that light.

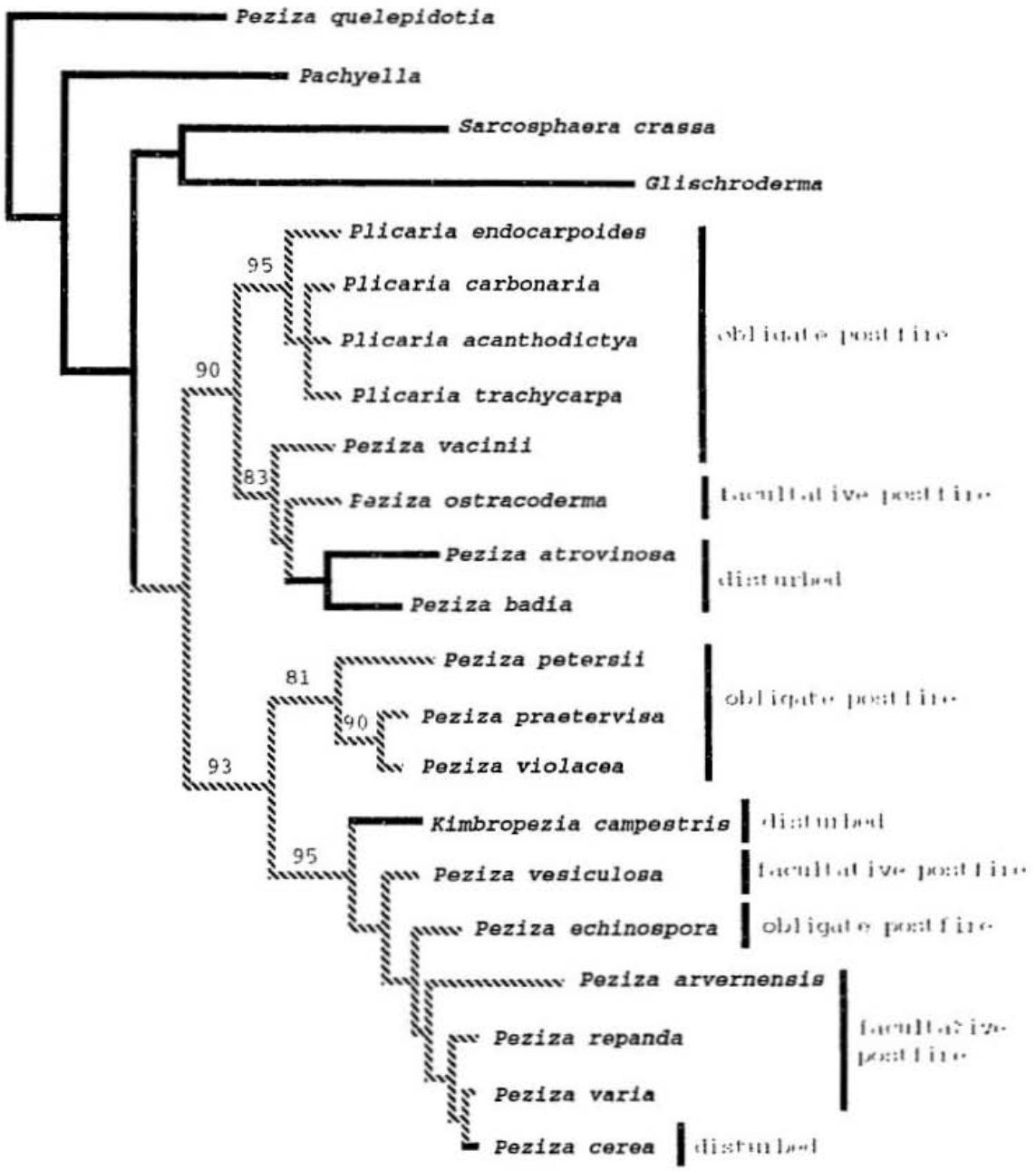


Figure 17. A strict consensus tree inferring adaptation to occur on postfire or disturbed habitats in *Plicaria*, *Plicaria* like -*Peziza* and other members of *Peziza*. Maximum parsimony analysis was performed on combined nucleotide characters from the SSU1, SSU2 and LSU. Branches showing lineages which have evolved to occur on fire are indicted by cross-hatches (≈). The number above branches indicates percentage of support for each node determined from 100 bootstrap replicates (>80%).

Similarly, the relationships of the facultative postfire species of group 2 *Peziza* do not contradict the results found within *Plicaria* or group 1 *Peziza*. However the relationships within this clade are not well resolved as shown by lower levels of bootstrap support. This limits what can be deduced from the cladogram regarding the adaptation to burned habitats.

Discussion

4.1 Phylogenetic relationship of *Plicaria* to *Peziza*

The phylogenetic relationship of *Plicaria* to other members of Pezizaceae was inferred from molecular characters using the SSU1, SSU2 and LSU data set. Initially, a portion of the 3' end of the ITS-1 region was also included in this analysis but this region was eliminated due to unreliability of the alignment. The difficulty experienced in obtaining alignments between *Plicaria* and *Peziza* suggests either that this is an ancient lineage which has undergone considerable divergence or that the lineage is young but evolving very rapidly.

Plicaria was originally delineated by Fuckel in 1870 when he transferred several *Peziza* species as well as four spherical-spored taxa to this genus (as cited in Rifai, 1968; Korf, 1960). Boudier (1885) was the first author to restrict *Plicaria* to spherical-spored taxa. He used the name *Galactinia* to refer to elliptical-spored taxa which possessed both spore ornamentation and guttules, while *Aleuria* was used for elliptical smooth-spored eguttulate taxa and *Peziza* was used for another genus.

As shown from the phylogeny inferred from SSU1, SSU2 and LSU, *Plicaria* forms a monophyletic group, but it is very closely related to the elliptical-spored members of the *Plicaria*-like-*Peziza* cluster. Together they form a well

supported clade. Although *Plicaria* can be delineated to comprise only round-spored taxa, such a structure would make both *Peziza* and *Galactinia* paraphyletic, since guttulate members of the *Plicaria*-like-*Peziza* lineage are separate from *Peziza praetervisa* and *Peziza petersii*, that Boudier included in *Galactinia*.

However, Boudier was partially correct in his hypothesis of the importance of guttules in the ascospores. The two clusters within the group 1 and group 2 *Peziza* clade can be distinguished from one another based on this character, with the exception of *Peziza violacea*. Boudier assigned *Peziza violacea* to *Aleuria* due to the absence of guttules but the cladogram places this taxon in the group 1 clade. The group 1 *Peziza* cluster includes *Peziza violacea*, *Peziza praetervisa* and *Peziza petersii*, but this cluster is only moderately supported due to difficulty in resolving the placement of *Peziza petersii*. This is in contrast to the *Peziza praetervisa* and *Peziza violacea* node, that is well supported, indicating that these two taxa are phylogenetically closely related. However, the inclusion of *Peziza praetervisa* and *Peziza violacea* in the same clade does not agree with morphological data. *Peziza praetervisa* has very finely warted ascospores that contain guttules whereas *Peziza violacea* has smooth spores which are eguttulate. This would suggest that *Peziza violacea* is more closely related to members of the group 2 *Peziza*, which also

do not possess spore guttules, than to *Peziza praetervisa*. However, Egger (unpublished data) has observed two tiny bipolar guttule-like structures in immature spores of *Peziza violacea* that are not present at maturity. This may indicate that guttules in this species become reduced during development. The presence of guttules should be interpreted with caution because thier occurrence may be due to convergence. *Peziza praetervisa* and *Peziza violacea* are also similar in that they both produce a violaceous hymenial pigment. *Peziza petersii* has warted, guttulate spores like *Peziza praetervisa* but possesses hymenial pigments that are reddish or vinaceous rather than violaceous.

The group 2 *Peziza* species clustered together with a high level of support. This clade, for the most part, corresponds to Boudier's *Aleuria* and is consistent with morphological data: these taxa have smooth ascospores which do not contain guttules. One of the taxa found in the group 2 *Peziza* clade was *Kimbropezia campestris*. This species was assigned to a monotypic genus based on the presence of a cyanophilic and congo-red staining lens-shaped disk within the ascus operculum (Korf and Zhuang, 1991). These authors felt that this species was sufficiently different from *Peziza* to warrant a separate genus. However, they compared sections of the apothecial excipulum of *K. campestris* to *Peziza cerea*, *Peziza varia* and *Peziza micropus* and found that all taxa showed four distinct tissue layers. The

similarity in excipulum morphology agrees with the phylogeny generated in this study. There is a high degree of support for including *K. campestris* as a member of this group.

4.2 Taxonomic Implications for *Plicaria* and *Peziza*

Results from the inferred phylogeny suggest that the round-spored *Plicaria* and spherical-spored *Plicaria*-like-*Peziza* taxa form a monophyletic group of very closely-related taxa that are distinct from *Peziza*. This indicates that the separation of *Plicaria* from *Peziza*, based on the round ascospore character, is not justified, an argument previously advanced by authors such as Korf (1960), Le Gal (1963), Denison (1963) and Berthet(1964). However, the *Plicaria* and *Plicaria*-like-*Peziza* taxa each have evolved into two separate lineages as defined by the *Plicaria* and *Plicaria*-like-*Peziza* clades. This supports the argument of authors such as Dennis (1960,1968), Batra (1961), Rifai (1968), Eckblad (1968), Dissing and Korf (1980) and Dissing and Pfister (1981).

The inclusion of *Plicaria* and *Plicaria*-like-*Peziza* within a clade separate from other members of *Peziza* makes *Peziza* paraphyletic. There are three way to alleviate the problem of *Peziza* being paraphyletic, each with different consequences.

If ascospore shape is used to separate *Plicaria* from *Plicaria*-like-*Peziza* and *Peziza*, members of *Plicaria*-like-

Peziza would have to be accommodated under a different genus. One suggestion would be to use *Galactinia*, but this genus concept would have to be emended. Originally, *Galactinia* referred to rough guttulate spored taxa but this definition would make *Galactinia* paraphyletic because two of the members of group 2 *Peziza* within the *Peziza* clade possess rough guttulate spores. It would be difficult to separate *Galactinia* from other members of *Peziza* based on morphological characters.

If ascospore shape is not used to separate the two genera, then *Plicaria* could be merged with *Peziza*, as suggested by Korf (1960). This alternative, however, is particularly unattractive because it does not accurately portray the degree of divergence which has occurred between the *Peziza* and *Plicaria/Plicaria*-like-*Peziza* clades. This would also leave *Peziza* intact as a diverse and unwieldy genus with over 100 species. A second option is to modify the generic concept of *Plicaria* to include members of *Plicaria*-like-*Peziza*. This option appears to be the best alternative because it allows for the separation of a natural grouping of species that have diverged from *Peziza*. It also recognizes the very close phylogenetic relationship which exists among *Plicaria* and *Plicaria*-like-*Peziza*. This is further corroborated by similarities in morphological characters among the two groups. The problem associated with this alternative is similar to that of *Galactinia*. There are

no definitive morphological characters which could be used to distinguish the two genera. Egger (1986) pointed out that *Plicaria ostracoderma* and most *Plicaria* species possess a *Chromelosporium* anamorph, while *Peziza* species have an *Oedocephalum* type anamorph. However, anamorphs have not been found for *Peziza vacinii*, *Peziza atrovinosa* and *Peziza badia*. It is also possible that they have lost their anamorph state. If *Chromelosporium* anamorphs could be found for these taxa, another character could be used to distinguished elliptical *Plicaria* taxa from other members of *Peziza*.

Another way of defining *Plicaria* would be to use a suite of characters, similar to the set used by Dissing and Pfister (1981), to separate *Plicaria* from *Peziza*. Although monographing and describing *Plicaria* to include both spherical and elliptical-spored taxa goes beyond the scope of this thesis, several characters which could be used in establishing a suite of morphological characters including: dark coloured apothecial pigment, paraphyses embedded in a granular matrix, guttules, ascospore walls turning brown at maturity, and a *Chromelosporium* anamorph.

4.3 Relationship of *Plicaria* and *Peziza* to other Genera Within Pezizaceae

Sarcosphaera crassa, which has been viewed by some as a partially hypogeous *Peziza*, has remained separate from

Peziza in the phylogenetic analysis. This genus has been distinguished from *Peziza* based on apothecia morphology, which is deeply cupulate and wholely immersed in the soil (Korf, 1973) but even these characters do not produce well defined generic boundaries. For example, Rifai (1968) pointed out that the young apothecia of *Peziza ammophila* are subterranean, but it is not included under *Sarcosphaera* because it differs considerably based on anatomical features. The results of this study were not capable of resolving the relationship between *Peziza* and *Sarcosphaera*. Morphological data shows some degree of similarity between *Sarcosphaera crassa*, that has minutely warted biguttulate spores and a violaceous hymenium, and members of the group 1 *Peziza* cluster.

Bruns et al. (1989) compared morphological and molecular divergence rates between the epigeous basidiomycete *Suillus* sp. and hypogeous *Rhizopogon* sp. They found that the morphological divergence rate was much greater than the molecular divergence rate. The difference in the two rates was attributed to an acceleration in morphological divergence due to an increase in selective pressures on a small number of developmental genes that were being selected as a result of the change in growth form from epigenous to hypogeous. However, I see no evidence for rapid morphological divergence between *Peziza* and *S. crassa*. *Sarcosphaera* is strongly differentiated based on molecular

and morphological characters.

The genus *Pachyella* was described by Boudier (1907) but Seaver (1928), Le Gal (1963) and Eckblad (1968) synonymized this genus with *Peziza*. Pfister (1973) suggested that *Pachyella* and *Peziza* show a high degree of similarity, however *Pachyella* differs from *Peziza* based on the presence of hyphoid hairs embedded in a gelatinous tissue as well as occurring on water soaked wood. The inferred phylogeny was unable to resolve the relationship between *Pachyella* and *Peziza* but the number of steps which separate the two genera suggests that they are not members of the same genus.

Glischroderma sp. resembles the *Chromelosporium* anamorph state of *Plicaria* and was suspected to be an anamorph of some *Peziza* species (Korf, 1994). However, the SSU1, SSU2 and LSU phylogeny revealed that *Glischroderma* does not appear to be closely related to *Plicaria* or *Peziza* but, like *S. crassa* and *Pachyella* clypeata, the branch which defines this taxon is not well resolved.

The taxon chosen as the outgroup of the parsimony analysis, *Peziza quelepidotia*, is considerably different from other members of *Peziza* based on molecular and morphological characters. This taxon was originally described as *Lepidotia hispida* by Boudier (1907). However, Korf (1973) stated that this genus could not be satisfactorily separated from *Peziza* because the asci blued at the tip (amyloid), and placed the two genera in synonymy,

reassigning *L. hispida* as *Peziza quelepidotia*. However, morphological differences which exist between *Peziza quelepidotia* and other members of *Peziza* suggest that *Peziza quelepidotia* belongs to a distinct genus. First, *Peziza quelepidotia* possesses an undescribed anamorph type which has not been found in any other *Peziza* species. Second, the apothecia of *Peziza quelepidotia* is yellow-green in colour and possesses a stipe, characters which are rarely found in *Peziza* (Korf, 1973). Based on morphology and sequence divergence, *Peziza quelepidotia* is not a member of *Peziza* and should be resigned to *Lepidotia*.

4.4 Relatedness of *Plicaria* taxa

The *Plicaria* group was well supported in both the expanded and restricted data analyses and this suggests that these taxa, which possess round ascospores, evolved within a single lineage. The most basal branch in this cluster contains *Plicaria endocarpoides* which is the only member which has smooth spores. This character state appears to be derived because it is the only member of this clade, which includes *Plicaria* and *Plicaria*-like-*Peziza*, that does not possess spore ornamentation. This is particularly important because a number of authors, such as Boudier (1907), have used this character to define taxonomic groups. In this case, *Plicaria endocarpoides* is closely related to the other members of *Plicaria* which are heavily ornamented. Based on

this information, a hypothesis can be formulated that the loss of spore ornamentation, as found in *Plicaria endocarpoides*, may be easier to achieve than the development of spore ornamentation in a species that previously did not possess this character.

The terminal node within the *Plicaria* cluster consisted of the rough-spored *Plicaria* species trichotomy which is well supported by the ITS-1 and LSU analysis. These two regions, however, were incapable of resolving the relationships that exist within this tricnotomy because all three species are closely related. This is evident by the lower percentage of sequence divergence among the rough-spored taxa for the ITS-1 region. Sequence divergence of 3.5% was found between *Plicaria carbonaria* and *Plicaria trachycarpa* as well as between *Plicaria carbonaria* and *Plicaria acanthodictya*. A slightly lower divergence of 2.9% was found between *Plicaria acanthodictya* and *Plicaria trachycarpa*. These values are comparable to the 3.5% ITS-1 sequence variation that Gardes et al. (1991) found between the closely related species of *Laccaria bicolour* and *L. laccata*. However, for the ITS-1 region, the percentage of sequence divergence found within a species and between closely related species overlaps. Anderson and Stasovski (1992) found 0.5% variation among closely related intersterility groups of *Armillaria*, while Lee and Taylor (1992) demonstrated that between 2.4-4.2% variation occurred

within *Phytophthora citrophthora*.

Confusion exists regarding the number of rough-spored taxa of *Plicaria*. A number of authors have argued that the differences between *Plicaria carbonaria* and *Plicaria trachycarpa* are not sufficient to warrant separate species status and have merged the two under *Plicaria trachycarpa*. In 1936, Grelet (cited in Maas Geesteranus, 1967) redefined *Plicaria carbonaria* as *Plicaria trachycarpa* var. *muricata*. Thind and Sethi (1957) later stated that members of *Plicaria trachycarpa*, which had finer warts than *Plicaria trachycarpa* var. *muricata*, should be referred to as *Plicaria trachycarpa* var. *ferruginea*. Waraitch (1977) added further confusion to this classification scheme when he suggested that *Plicaria carbonaria* possesses ascospores which have larger warts than *Plicaria trachycarpa* but, other than this character, both taxa are identical based on colour and anatomical characters. This analysis suggests that *Plicaria trachycarpa*, *Plicaria carbonaria* and *Plicaria acanthodictya* are distinct taxa that can be differentiated based upon molecular characters and ascospore morphology.

4.5 Relatedness of *Plicaria*-like-*Peziza* taxa

Egger (1986) was the first to suggest that the genus concept of *Plicaria* may have to be modified to include several elliptical-spored *Peziza* taxa with *Plicaria*-like characters. Moravec and Spooner (1988) went further in

suggesting that *Peziza vacinii* has several morphological characters in common with *Plicaria*, and that *Peziza vacinii* and *Plicaria carbonaria*, which have similar spore ornamentation, brown spore colour, dark apothecia and occur on burned habitats, differ only in spore shape. Although they correctly pointed out the similarity in morphological characters, their hypothesis regarding the relationship of *P. vacinii* to *P. carbonaria* is not supported by this analysis.

The molecular phylogeny also suggests that *Peziza vacinii* possesses characters which are symplesiomorphic with *Plicaria* and *Plicaria*-like-*Peziza*. This ambiguity is demonstrated by poor resolution of the branch that defines *Peziza vacinii*, as well as the results from the modified jack-knife analysis which showed that the support for the *Plicaria*-like-*Peziza* clade increased when *Peziza vacinii* was removed from the analysis. *Peziza vacinii* has elliptical spores similar to the members of the *Plicaria*-like-*Peziza* group, but it is the only member of this group which possesses warted spores, similar to *Plicaria carbonaria*, instead of reticulate spores. Although the data set with *Peziza vacinii* removed should be interpreted with caution, there is a trend in spore morphology between *Peziza atrovinosa*, *Peziza badia* and *Peziza ostracoderma*. *Peziza badia* and *Peziza atrovinosa* occur as sister species and share a common reticulate ascospore morphology. *Peziza*

ostracoderma occurs as a basal branch to these two sister species and has a similar spore reticulation pattern but differs in size, colour and habitat (Moravec and Spooner, 1988).

Two members of the *Plicaria*-like-*Peziza* group, *Peziza atrovinosa* and *Peziza ostracoderma*, have commonly been misidentified. Hennebert and Korf (1975) suggested that *Peziza atrovinosa*, which has much larger apothecia, brown spore walls and possess ascospores with broad irregular reticulations, is clearly distinguishable from *Peziza ostracoderma*. Moravec and Spooner (1988) are in agreement with Hennebert and Korf (1975) regarding the size of apothecia and spore colouration differences which occur between *Peziza atrovinosa* and *Peziza ostracoderma*. However, unlike Hennebert and Korf, they suggest that ascospore size and ornamentation of both species is very similar. A comparison of spore morphology determined using SEM agrees with Moravec and Spooner (1988). The size and spore ornamentation patterns are very similar and not as distinct as Hennebert and Korf (1975) suggest. This similarity in spore morphology may also explain why authors such as Dennis (1960, 1968) and Moser (1963) have misidentified *Peziza ostracoderma* as *Peziza atrovinosa*.

4.6 Adaptation and Speciation on Burned and Disturbed Sites

Mapping of the burn site association character on the cladogram reveals several interesting points. First, there are several monophyletic groups that share an association with burned habitats. These include the *Plicaria* and group 1 *Peziza* clades where all members of both clades are obligate burn site species. Second, the results from this study suggest that the ancestral condition for members of *Peziza* and *Plicaria* is association with burn habitats. All *Plicaria* species are obligately associated with burned sites. Similarly, the most basal branch within the *Plicaria*-like-*Peziza* clade, *Peziza vacinii*, is also an obligate burn species. These observations suggest that the ancestral condition for this clade was association with burns. The terminal taxa *Peziza atrovinosa* and *Peziza badia* are found in unburnt, but often disturbed, habitats. *Peziza ostracoderma*, which branches between *Peziza vacinii* and *Peziza atrovinosa/badia*, is facultatively associated with burns. It is mostly found on sterilized soil, but this is a substrate on which the obligate postfire species *Plicaria trachycarpa* and *Plicaria carbonaria* are occasionally found. This suggests that disturbed site species may have evolved from postfire ancestors via a transitional facultative stage.

The group 1 and group 2 *Peziza* clade does not contradict this hypothesis. The group 1 *Peziza* clade forms a

monophyletic group of obligately fire associated species, similar to the *Plicaria* clade. This clade also branches basally, which supports the hypothesis that the ancestral condition for this clade was burn association. The group 2 *Peziza* clade contains a mixture of obligate and facultative postfire species. The basal branch within this clade is a species which is not known to be associated with fire sites, *K. campestris*. However, this is a very long branch and this does not preclude the ancestral condition being association with fire sites. The remaining taxa in this clade are either obligately associated with burn sites (e.g. *P. echinospora*) or occasionally found on burned sites.

One of the more interesting facultative burned site associations is demonstrated by *Peziza vesiculosa*. This species is mostly found on the dung of herbivores and occasionally occurs on postfire sites. This relationship was pointed out by Wicklow (1975) who suggested that coprophilous fungi are sometimes capable of expanding their niche to include postfire sites. This may be due to similarities found between the two substrates such as the elimination of competitors and elevation in heat to allow ascospores to germinate. Our data supports his argument that the two substrates are similar in their ecological characteristics.

Conclusion

The phylogenies inferred from analysis of SSU1, SSU2, LSU and ITS-1 reveal that members of the *Plicaria* and *Plicaria*-like-*Peziza* clades form a closely related monophyletic group which is evolutionarily distinct from most *Peziza* species. This makes *Peziza* paraphyletic, a problem which can be dealt with in several ways. Because *Plicaria* forms a clade separate from *Plicaria*-like-*Peziza*, the *Plicaria* genus concept based on spherical spores could be maintained. This, however, would require a separate genus name for *Plicaria*-like-*Peziza* taxa, such as a *Galactinia*, but the generic concept based upon the presence of guttules would have to be modified because this character is also found in group 2 *Peziza*. Another option is to include members of the *Plicaria*-like-*Peziza* group in *Plicaria*. This alternative would involve emending the *Plicaria* genus concept.

The latter alternative appears to agree most closely with the molecular and morphological data. *Plicaria* is phylogenetically closely related to *Plicaria*-like-*Peziza* species. This option also agrees with many authors who have argued that the two groups are similar based on morphological characters.

If members of *Plicaria*-like-*Peziza* are removed from *Peziza*, a new *Peziza* genus concept would have to be

developed to include species found within group 1 and group 2 *Peziza*.

One taxon found within the group 2 *Peziza* clade is *Kimbropezia campestris*. The presence of a different genus within the *Peziza* clade makes *Peziza* paraphyletic. However, this taxon shares many other morphological characters with the members of the group 2 *Peziza* clade but was assigned to a monotypic genus based on a unique staining lens shape disk within the asci operculum. These data suggest this character is not sufficient to delineate a separate genus and supports the merging of *K. campestris* into the genus *Peziza*. After consulting with Dr. Richard Korf, it was suggested that this taxon be renamed *Peziza kimbropezia* because the species name *campestris* is already in use by another member of Peziza. This amendment will be done in publication.

The outgroup which was used in this study, *Peziza quelepidotia*, was originally called *Lepidotia hispida* by Boudier. It was, however, merged with *Peziza* based on the similarity of the bluing of the asci tip character. However, the data obtained in this study shows that this taxon does not belong to *Peziza*. This was also supported by several morphological characters which are found in *Peziza quelepidotia* but never or rarely found in *Peziza*. These data support the transfer of *Peziza quelepidotia* back to its previously described genus *Lepidotia* as *Lepidotia hispida* (Quel) Boud. This emendation will be done in publication.

The results from mapping of the burn association character on the cladogram show that several monophyletic groups have evolved in association with postfire sites. This suggests that even though the postfire sites are ephemeral and constantly shifting, it is stable enough for fungi to evolve and speciate in association with this habitat. There is also evidence that species on burned sites may give rise to lineages that are no longer obligately associated with burns, probably via a facultative intermediate. This study had insufficient data to determine the ancestral condition for all *Peziza*, however, it did set an important reference point for future studies on the evolution of fire association in Pezizales.

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