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Title: SYSTEMATICS OF PACIFIC NORTHWESTERN SPECIES OF THE GENUS *GYMNOMYCES* INFERRED FROM NUCLEAR RIBOSOMAL DNA INTERNAL TRANSCRIBED SPACER SEQUENCES

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Gymnomyces is an ectomycorrhizal basidiomycete genus in the family Russulaceae estimated to contain less than 50 species worldwide, with 23 species known to occur in the Pacific Northwest. *Gymnomyces* species are common in Pacific Northwest forests that include *Pseudotsuga, Abies, Tsuga,* and *Quercus.* They produce sequestrate (truffle-like) sporocarps with ornamented amyloid spores, which are a common food resource for a suite of mycophagous, both small and large mammals. *Gymnomyces* is one of five sequestrate genera (*Macowanites, Gymnomyces, Cystangium, Arcangeliella,* and *Zelleromyces*) long recognized as being more closely related to Russulaceae than to other sequestrate fungi as judged from similarities in spore ornamentation, tissue anatomy, and sporocarp morphology. The russuloid clade, where *Gymnomyces* resides, exhibits a great diversity of sporocarp morphologies. The hymenomycetous forms include sequestrate, resupinate, pileate, and coralloid, with smooth, toothed, lamellate, or poroid sporocarps forms. This clade is complex because it lacks an obvious morphological synapomorphy uniting all of the unique sporocarp forms. In this study I investigated infrageneric relationships in *Gymnomyces* to test current taxonomic hypotheses and species concepts. Through phylogenetic analyses of internal transcribed spacer nuclear ribosomal DNA sequences I found that *Gymnomyces* does not form a monophyletic group, and the ITS region clearly suggests at least six unique lineages of *Gymnomyces.* Because the ITS data is

consistent with the morphological autapomorphies of both *G. ellipsosporus* and *G. gilkeyae*, their removal from the genus *Gymnomyces* is suggested.

SYSTEMATICS OF PACIFIC NORTHWESTERN SPECIES OF THE GENUS *GYMNOMYCES* INFERRED FROM NUCLEAR RIBOSOMAL DNA INTERNAL TRANSCRIBED SPACER SEQUENCES

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CONTRIBUTION OF AUTHORS

Dr. Michael A. Castellano participated in the design of this study, editing of the manuscript, and provided funding for the project. Dr. James M. Trappe provided consultation and information regarding aspects of the project and participated in the editing of all drafts of the manuscript. Dr. Efren Cázares provided insightful comments and suggestions and was involved with the editing of the manuscript. Dr. Joseph W. Spatafora participated at all stages of the study including design, data collection and analyses, and editing.

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CHAPTER 1

INTRODUCTION

Gymnomyces is an ectomycorrhizal basidiomycete genus in the family Russulaceae with the highest species diversity known to occur in the Pacific Northwest. The season determines what particular species of *Gymnomyces* will be fruiting at one time; however, this group of fungi can be found throughout the year in the Northern Hemisphere and Australasia. *Gymnomyces* occurs in forest types that include *Pseudotsuga, Abies, Tsuga, Eucalyptus,* and *Quercus* spp. The sporocarps form underground and can commonly be found in the top 10 cm of the soil in the Pacific Northwest (PNW) (Luoma *et al.*, 1991). Sporocarps resemble minute potatoes and in cross section show lacunose chambers lined with hymenia. *Gymnomyces* species produce ornamented, amyloid spores typical of the Russulaceae family.

CLASSIFICATION

A major challenge of evolutionary biology is to understand the origin and diversification of biological form. Fungi reflect this challenge with their anatomical simplicity and sparse fossil record. Homobasidiomycetes include the mushroom-forming fungi and related taxa. Over 13,000 species of homobasidiomycetes have been described (Hibbet *et al.*, 1997), including approximately 1,600 sequestrate species (Castellano, unpublished).

Traditional classifications of fungi during the 19th century were based solely on macromorphology, especially that of the spore-bearing structure. Sequestrate fungi, puffballs, bird's nest fungi, earthstars, and stinkhorns were placed in the artificial grouping Gasteromycetes (Miller and Miller, 1988). Gasteromycetes do not forcibly discharge their spores. *Gastero-*, meaning "stomach," denotes where the spores are produced, internally rather than externally as in epigeous fungi (Arora, 1986). All fungi that produce spores on an exposed hymenophore were grouped in the class Hymenomycetes, which was composed of two orders: Agaricales, for gilled mushrooms, and Aphyllophorales, for polypores, toothed fungi, coral fungi, and resupinate, crust-like forms (Miller and Miller, 1988; Miller, 1988; Hibbett *et al.*, 1997).

Since the 19th century, anatomical studies by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) in conjunction with fungal ecology studies unequivocally showed that the "Gasteromycetes" is an artificial class (Donk, 1964; Miller, 1998; Hibbett *et al.*, 1997). Current molecular data suggest that sequestrate fungi have arisen independently many times in both the Ascomycota and the Basidiomycota (Bruns *et al.*, 1989; Vilglays *et al.*, 1993; Hibbett *et al.*, 1997; O'Donnell *et al.*, 1997). Many sequestrate genera are now well known to be derived from various lineages of epigeous mushrooms, e.g., *Gastrosuillus* from *Suillus*, *Podaxis* from *Coprinus, Pyrenogaster* from *Geastrum, Gymnomyces* from *Russula,* and *Hydnangium* from *Laccaria* (Bruns *et al.*, 1992; Vilgalys *et al,* 1993; Miller *et al.,* 2001; Pine and Mueller, 1993).

Vilgalys *et al.* stated (1993) "for taxonomy to reflect evolutionary relationships, genera need to be based on monophyly. Non-monophyletic taxa should be discarded in favor of generic concepts which reflect descent from a common ancestor." With the dissolution of Gasteromycetes many sequestrate fungi have since been arranged in clades that more accurately reflect molecular phylogenetic hypotheses and a natural classification. For example the Bolete clade includes *Hymenogaster, Gastrosuillus, Rhizopogon, Truncocolumella,* and

Scleroderma, and the Gomphoid-phalloid clade includes *Gautieria* and *Hysterangium* (Hibbett and Thorn, 2001).

In Hibbett and Thorn's (2001) relatively recent review of phylogenetic studies in homobasidiomycetes they provided a preliminary phylogenetic outline for the group at large. The Russuloid clade, where *Gymnomyces* resides, exhibits a great diversity of fruiting body morphologies. The hymenomycetous forms include resupinate (*Byssoporia*), pileate (*Russula*), or coralloid (*Clavicorona*) with smooth, toothed, lamellate, or poroid hymenophores as well as sequestrate forms. This clade is complex because it lacks an obvious morphological synapomorphy (a derived character state that is shared by two or more taxa and is postulated to have evolved from a common ancestor uniting all of the unique morphological forms (Hibbett and Thorn, 2001; Miller *et al.*, 2001). Many members of the russuloid clade have spores with amyloid ornamentation and gloeoplerous (includes laticiferous and oleiferous) cystidia or hyphae; however, the group is highly variable.

In conclusion, the concept of russuloid fungi has changed dramatically over the last decade through the application of molecular tools, and it remains a significant challenge to identify morphological synapomorphies that unite the group.

TAXONOMY

Many species of *Gymnomyces* have gone through several nomenclatural changes over the past century. *Gymnomyces* is one of five sequestrate genera (*Macowanites, Gymnomyces, Cystangium, Arcangeliella,* and *Zelleromyces*) long recognized as being more closely related to *Russula* or *Lactarius* than to other sequestrate fungi because of similarities in spore ornamentation, tissue anatomy, and sporocarp morphology (Lebel, 1998; Lebel and Trappe, 2000).

The sporocarps of *Gymnomyces* spp. are spherical to irregular and 1-5 cm in diameter (Singer and Smith, 1960). They lack a stipe but sometimes possess a reduced columella. The peridium is often delicate and easily worn off, sometimes absent, and generally difficult to observe in herbarium specimens. The peridiopellis is composed of a trichodermium (surface hyphae unequal in length, may be several cells long and project more or less vertically from the underlying context; the terminal cells may be cystidoid), a turf (the surface hyphae and/or cystidia are more or less arranged like blades of grass in a lawn, generally as single elongated cells), or of undifferentiated (peridiopellis which in not distinct from the peridial context) hyphae as defined by Lebel (1998). The gleba is loculate. The hymenophoral trama is usually either heteromerous with nests of sphaerocysts or with isolated inflated cells (Singer and Smith, 1960) but not always (Lebel and Trappe, 2000). The symmetric statismosporic spores of *Gymnomyces* vary between species in shape, size, and ornamentation. Spore shape ranges from globose to ellipsoid, 7-20(23) x 6-15(16) μm in size, and ornamentation from echinulate to reticulate 0.25-3.5 x 0.3-2.5 μm. The spore ornamentation exhibits differing degrees of amyloidity: in some species it's only spotted with amylacious material, while others have completely amyloid ornamentation (Singer and Smith, 1960). Although less documented, the spore walls tend to be hyaline to yellow-brown in Melzer's reagent with the exception of *Gymnomyces rolfalexii* and *Gymnomyces setigerus*, which exhibit amyloidity at the base of the spore (Singer and Smith, 1960).

Massee and Rodway described *Gymnomyces pallidus* and *Gymnomyces seminudus* in 1898. As was common at that time, they did not designate a type species for the genus. They found *Gymnomyces* to differ from *Octaviania* by the absence of a peridium in *Gymnomyces* and from *Gautieria* because of the hyaline, globose spores. In 1931, Clements and Shear designated *G. pallidus* as lectotype for the genus.

In 1924 Václav Melzer first employed the use of an iodine solution, today known as Melzer's reagent, for studying spore ornamentation in the order

Russulales. Melzer's accomplishment was unrecognized by many taxonomists working with sequestrate Russulales until the late 1940's. This produced considerable confusion in the establishment of generic and family relationships within this group of fungi. Using Melzer's reagent, Malençon (1931) demonstrated the spore ornamentation of a number of sequestrate Russulales to be amyloid, thereby providing strong evidence of the relationship between these fungi and epigeous Russulales.

In 1935 Cunningham transferred *G. pallidus* to *Octaviania* without knowledge of the amyloid reaction of the spore ornamentation of *Gymnomyces*. In 1960, Singer and Smith reinstated the genus *Gymnomyces* with *G. pallidus* as the type species after studying a Rodway collection of this species (not the type); however, it was only later when holotype material was studied by Smith (1962) that the decision to resurrect the genus was confirmed. The lack of sphaerocyst nests in the hymenophoral mediostratum was the character used to separate the genus *Martellia sensu* Singer and A.H. Smith (Singer and Smith, 1960) from *Gymnomyces*. Locating sphaerocysts requires careful scrutiny because they vary in size and distribution within a single sporocarp (Lebel and Trappe, 1988). Although Singer and Smith (1960) noted the sphaerocysts in the hymenophoral trama of the type specimen of *Martellia*, they considered them rare and usually absent in the genus.

Pegler and Young (1979) divided the sequestrate genera of Russulales into two groups based on spore development: one group included the genera producing ballistosporic basidiospores and the other included the genera producing statismosporic basidiospores. They believed the former clustered within the family Russulaceae and the latter to represent a family of its own, the Elasmomycetaceae.

Beaton *et al.* (1984) developed a key based on the anatomy of the hymenophoral trama and spore ornamentation as the main characters to separate the genera. Miller (1988) studied the ultrastructure of basidiospore development and recorded variation in both spore symmetry and morphology, concluding that

delimiting families and genera in the Russulales based on spore symmetry vs. asymmetry, as Pegler and Young (1979) proposed, was artificial.

Zhang and Yu (1990) also questioned generic concepts when unable to confidently separate Chinese genera they believed referable to the *Gymnomyces* group. Four collections were studied yielding two new species of gasteroid Russulales (*Gymnomyces lactifer* and *Martellia ramispina*); however, Zhang and Yu were still left with questions. After reviewing the taxonomic literature on the three sequestrate russuloid fungi, they agreed with Beaton *et al.* (1984) that spore ornamentation is a strong character and can be used to separate *Zelleromyces* (reticulate spores) from *Gymnomyces* and *Martellia* (spiny or warty spores). *Zelleromyces* also differs from the above two in the presence of a latex (observable in fresh specimens) that distinguishes its relationship with its epigeous counter-part *Lactarius* (Singer and Smith, 1960). However, Zhang and Yu (1990) were hesitant to place importance on the laticiferous hyphae that produce the latex seen in *Zelleromyces* and *Lactarius*, and disregarded this character.

They also believed the anatomy of the hymenophoral trama to be informative at the generic level and stated that *Gymnomyces* is distinguishable from the other two genera by having "true" nests of sphaerocysts as opposed to infrequent scattered inflated cells in the trama, or nests of sphaerocysts in other tissue layers. The character they used to separate *Zelleromyces* from *Gymnomyces* is reticulate ornamentation vs. spinose ornamentation. Species currently placed in *Gymnomyces* may possess varying degrees of a reticulum; spore ornamentation clearly varies greatly and cannot be used alone to separate genera.

Lebel and Trappe (2000) completed a comprehensive study of a large number of species that earlier authors (Singer and Smith, 1960) have placed in *Gymnomyces*, *Martellia*, and *Cystangium* from Australia, New Zealand, and the Pacific Northwestern USA. They found sphaerocysts present in the hymenophoral trama of the holotype of *M. mistiformis*. Sphaerocysts in the hymenophoral trama was the one character Singer and Smith (1960) used to distinguish *Gymnomyces* from *Martellia*, so the two genera could no longer be separated on that basis.

Singer and Smith (1960) distinguished *Cystangium* from *Martellia* and *Gymnomyces* by the reduced or percurrent columella and a peridiopellis composed of an epithelium of 3-5 tiers of inflated cells. Upon reviewing the generic concept of *Cystangium,* Lebel and Trappe (2000) reported varying size columella across the many collections studied, thus challenging Singer and Smith's definition of *Cystangium.* Trappe *et al.* (2002) published nomenclatural revisions on the sequestrate russuloid genera that moved some species of *Martellia* into *Gymnomyces* and others into *Cystangium* thus eliminating the genus *Martellia* (see Lebel and Trappe (2000) for emended generic concepts of the sequestrate russuloid fungi). The new emended generic description of *Gymnomyces* includes 23 species known to occur in the Pacific Northwestern United States (Singer and Smith 1960, Singer and Smith 1963). *Cystangium* now includes all sequestrate russuloid fungi with an epithelium of 3-5 tiers of inflated cells and the columella is no longer a defining character (Trappe *et al.,* 2002).

Recently, Calonge and Martin (2000) obtained DNA sequences of the ITS region of representative species of *Gymnomyces, Martellia,* and *Zelleromyces*, and from those suggested that the family Elasmomycetaceae be synonymized with Russulaceae. *Gymnomyces* and *Martellia* could be considered sequestrate forms of *Russula,* and *Zelleromyces* could represent a sequestrate form of *Lactarius.*

The objective of this study was to test selected current morphological species of the genus *Gymnomyces* through phylogenetic analyses of nuclear ITS rDNA sequence data. Preliminary investigations focused on type collections of ten PNW species of *Gymnomyces* formerly known as *Martellia* (Singer and Smith, 1960); however, they yielded little to no molecular information due to poor preservation: other collections of each species were sampled to supplement the types.

RIBOSOMAL DNA

Ribosomal DNA (rDNA) is frequently used in fungal systematic studies (Bruns *et al.*, 1991; Hibbett and Thorn, 2001). rDNA is composed of a tandem repeat of three rRNA genes: the 18S (nuclear small rDNA), the 5.8S, and the 28S (nuclear large rDNA), respectively. The 5.8S is sandwiched between two non-coding internal transcribed spacer regions (ITS), ITS 1 and ITS2, and an external transcribed spacer (ETS) is positioned at the 5' end of the transcribed RNA (Palumbi, 1989; Bruns *et al.*, 1991). The multiple copies of the repeat unit evolve in relative unison via concerted evolution and therefore tend to behave as a single copy gene (Zimmer *et al.*, 1980; Bruns *et al.*, 1991).

rDNA is used extensively in fungal systematics for the following reasons: 1) it presents universally conserved regions that serve as ideal primer sites (Bruns *et al.*, 1991; Horton and Bruns, 2001); 2) it is easily amplified through polymerase chain reaction (PCR) (Mullis and Falona, 1987; Baldwin *et al.*, 1995); 3) it is a multicopy gene that acts as a single-copy, and the tandem repeat configuration forms a mosaic pattern of conserved and variable regions which makes rDNA attractive for taxonomic investigation at many levels (Bruns *et al.*, 1991; Hibbett, 1995; Moncalvo *et al.*, 1995, Dowling *et al.* 1996); 4) and finally, a large sequence database with a great deal of rDNA sequence data is in constant construction, allowing individuals to download and add strength and depth to their analyses.

Taylor *et al.* (1990) cautioned that different fungal taxa exhibit varying levels of sequence diversity in a given region and that no unique region can be used to identify all fungal species or address phylogenetic relationships among all fungi. Moreover, certain regions will prove to be more useful for certain taxa.

ITS REGION

The ITS region generally refers to the ITS-1 region, the 5.8S subunit, and the ITS-2 region. In fungi, the ITS region is generally between 650 and 900 base pairs in size (Horton and Bruns, 2001). Although the ITS-1 and ITS-2 regions are part of the transcriptional unit of rDNA and are transcribed, they are not actually incorporated into the mature ribosome (Baldwin *et al.*, 1995). As a result these two spacer regions experience more nucleotide substitutions and insertion/deletion events (indels) (Horton and Bruns, 2001). This creates great variability in the ITS region, making it ideal for addressing systematic questions at lower taxonomic levels (inter- and intrageneric). The inherent variability within the ITS region made it a logical choice for assessment of relationships within *Gymnomyces* at the subgeneric level (Gonzales *et al.*, 1985; Vilgalys and Sun, 1994; Lee and Taylor, 1992). In addition, the ITS region has proven to be very successful for others working with Russuloid genera (Miller and Buyck, 2002).

Indels are dealt with in sequence alignments by inserting gaps. Gaps must be inserted into sequence alignments to attain the best possible positional homology of characters; however, the insertion of gaps is often problematic, because their placement can often be ambiguous when aligning sequences, and many placements could potentially provide equivocal results (Baldwin *et al.* 1995; Hillis *et al.*, 1996). In addition, any two sequences could be aligned perfectly if enough gaps are introduced (Hillis *et al.*, 1996).

Because a universal treatment of gaps is nonexistent, a variety of methods for recoding gaps for phylogenetic analysis can be found in fungal as well as plant literature. The character state "gap" is sometimes treated as a fifth (Wheeler and Gladstein, 1994) or binary (presence/absence) character, twenty-first amino acid, scored as missing, or completely excluded from the analysis (Swofford, 1998; Wojciechowski *et al.*, 1993). The process responsible for base substitution, insertion and deletion; however, are evolutionarily and mechanistically distinct, and the proper treatment of gaps is not clearly recognized (Swofford *et al.*, 1998). It

has been shown that the use of different methods of treating gaps influences the resulting phylogenetic hypotheses; however, a uniformly applied method of treating gaps is lacking in sequence-based phylogenetic studies and thus no other choices exist (Hillis *et al.*, 1996).

SEQUESTRATE FUNGAL ECOLOGY AND THE FOOD WEB CONNECTION

Nearly all known sequestrate species are thought to be ectomycorrhizal (ECM) (Trappe and Maser, 1977; Maser and Maser, 1978), forming a symbiosis with the roots of selected woody plant species. Sequestrate fungi as defined here include hypogeous (produced below-ground), true (ascomycetes) and false (basidiomycete) truffles, and secotioid fungi (basidiomycetes in which the pileus does not expand and the lamellae are convoluted and anastamosed) (Lebel, 1998). Kendrick (1992) coined the term sequestrate to refer to fungi fitting any of the categories listed above. An ECM fungus is one that forms mycorrhizae; the hyphae grow only intercellularly, most (with the exception of arbutoid mycorrhizae) never penetrating the cell walls of the host plant.

In addition to the importance of *Gymnomyces* as an ectomycorrhizal genus in the forests of the PNW, their sporocarps are a major food resource for a suite of mycophagous both small and large mammals (Fogel and Trappe, 1978; Maser *et al.*, 1978; Maser *et al.*, 1985; Carey, 1992; Colgan *et al.*, 1997; Cázares *et al.*, 1999; Colgan *et al.*, 1999). In tropical, boreal, subalpine, and temperate forests, such as those of the PNW, the sporocarps consumed by these mammals are formed by ECM fungi associated with species of the Pinaceae, Fagaceae, Betulaceae, Myrtaceae, Salicaceae, and other families (Molina *et al.*, 1992; Colgan *et al.*, 1999).

Elevation and habitat influence the diversity of sequestrate fungi eaten by animals and the degree to which animals rely on them as dietary items (Ure and

Maser, 1982; Maser and Maser, 1988; North *et al.,* 1997). Sequestrate fungus production depends on temperature and moisture conditions (Fogel, 1976); as a result, species number and biomass of sequestrate fungi vary greatly seasonally and yearly (Luoma, 1991; Luoma *et al.,* 1991).

Perhaps the most noted forest mycophagist is the northern flying squirrel (*Glaucomys sabrinus*). Abundant in the old growth Douglas-fir (*Pseudotsuga menziesii*) forests of the PNW, northern flying squirrels are the most important prey of the northern spotted owl (*Strix occidentalis caurina*) across most of its range, north of Douglas County, Oregon (Wells-Gosling, 1985; Zabel *et al.*, 1995; Colgan *et al.*, 1997). Northern flying squirrels feed primarily on sequestrate fungi and lichens. Although they are nocturnal and nest in the cavities of trees and snags (Wells-Gosling, 1985), their food preferences dictate that much time is spent on the ground foraging for sequestrate fungi. The northern flying squirrels' preference for sequestrate fungi makes them vulnerable to predation by spotted owls (and other predators) as they forage on the ground.

Maser *et al.* (1985) showed that as much as 90 percent of the material in the digestive tract of *G. sabrinus* was fungal, including lichens. Through investigation of the interactions among squirrels, mycorrhizal fungi and coniferous forests in Oregon, Maser and Maser (1988) found that of 26 genera of sequestrate fungi identified from the stomachs of 118 squirrels *Rhizopogon* was the dominant genus in all squirrel diets, followed by species of *Gautieria.*

Many studies on small mammal feeding preferences of fungal sporocarps suggest species of *Rhizopogon, Gautieria,* and *Hysterangium* are the most common sequestrate genera consumed (Hayes *et al.*, 1995; Colgan *et al.*, 1997; Colgan *et al.*, 1999; Cázares *et al.*, 1999). Russuloid sporocarps are also reported to be consumed (Cázares *et al.*, 1999); however, little is mentioned about *Gymnomyces*, because it cannot be differentiated from other genera of Russulaceae from spores alone (Castellano *et al.,* 1989). Hence we do not know how important *Gymnomyces* is in the diet of many small mammal species.

JUSTIFICATION OF THE STUDY

The Northwest Forest Plan (NFP) was created to coordinate management direction for the lands administered by the USDA Forest Service and the USDI Bureau of Land Management and to adopt complementary approaches by other federal agencies within the range of the northern spotted owl. The management of these public lands must meet dual needs: the need for forest habitat and the need for forest products. The NFP covers 24 million acres of federally managed land in Washington, Oregon, and northern California.

In a larger ecological perspective there is a new interest to understand habitat and distribution requirements of many fungi as a result of the NFP. The NFP has prompted intense research on a multitude of organisms, including fungi, lichens, vascular plants, amphibians, mammals, bryophytes, mollusks, and arthropods, related to old growth forests within the range of the northern spotted owl. The Forest Ecosystem Management and Assessment Team (FEMAT), an interagency, interdisciplinary team of expert scientists, economists, sociologists and others was created to develop management alternatives for Pacific Northwest forests that balance the economic and social needs of society with forest conservation (USDA Forest Service and USDI Bureau of Land Management. 1994a).

The Record of Decision document (USDA Forest Service and USDI Bureau of Land Management. 1994b) is the culmination of management strategies and ideas generated by FEMAT and further crafted by the president's cabinet after hearing comments from the public. The Secretary of Agriculture and the Secretary of the interior jointly amended the planning documents of nineteen National Forests and seven Bureau of Land Management Districts, which marked the first time in history that two of the largest Federal land management agencies have developed and adopted a common approach to managing the lands they administer throughout an entire ecological region.

The genus *Gymnomyces* (as *Martellia* and *Gymnomyces*) is among the many taxa of concern listed in the ROD document. In the original document ten sequestrate Russuloid species were listed in total, and of the twenty-three species of *Gymnomyces* thought to occur in the PNW, five were listed (3 as *Martellia*). Due to a high degree of uncertainty regarding the biological distribution of fungi amendments to the survey and manage ROD document were established and published in November of 2000 (USDA Forest Service and USDI Bureau of Land Management, 2000). The new criteria defined left the status of most fungi either unchanged, or changed to allow slightly increased management. The only major change that occurred in the sequestrate Russuloid species was the transfer of several samples listed as *Gymnomyces sp. nov.* to be mostly *G. abietis*. A phylogenetic approach was deemed necessary to resolve taxonomic uncertainties and to generate a more accurate estimate of *Gymnomyces* species occurring in the PNW. A better understanding of the distribution and habitat requirements of sequestrate fungi such as *Gymnomyces* spp. will contribute towards more informed forest management decisions.

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CHAPTER 2

INVESTIGATION OF INFRAGENERIC RELATIONSHIPS AMONG PACIFIC NORTHWESTERN SPECIES OF THE GENUS *GYMNOMYCES* BASED ON nrDNA INTERNAL TRANSCRIBED SPACER SEQUENCES

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ABSTRACT

Gymnomyces is an ectomycorrhizal basidiomycete genus in the family Russulaceae with less than 50 species currently known worldwide, and with 23 species documented in the Pacific Northwest. *Gymnomyces* species are common in Pacific Northwest forests that include *Pseudotsuga, Abies, Tsuga, Picea, Pinus* and *Quercus* spp. Maximum parsimony analyses of 137 nuclear ribosomal DNA internal transcribed spacer (ITS) sequences, including representatives from 50 collections of *Gymnomyces* and 87 *Russula* species, were conducted to investigate intrageneric relationships within *Gymnomyces* and examine *Gymnomyces* relationship to the closely related epigeous genus *Russula.* ITS data support at least six distinct *Gymnomyces* lineages, and reject the monophyly of *Gymnomyces.* The ITS data are consistent with the morphological autapomorphies of two "morphological species," *G. ellipsosporus* and *G. gilkeyae*, and their removal from *Gymnomyces.* The remaining clades of *Gymnomyces* are not characterized by any noted morphological synapomorphy and numerous morphospecies are found in more than one clade. The unavoidable and critical issue that arises when contemplating the reclassification of *Gymnomyces* is the reclassification of *Russula* and *Lactarius.* Miller *et al.* (2001) generated data indicating that *Russula* is not monophyletic and that *Lactarius* is nested within *Russula.* Before approaching the reclassification of *Gymnomyces* the broader issue of how to deal with *Russula* and *Lactarius* needs addressed. Therefore, at this time no nomenclatural changes are suggested due to the uncertainty of the future of *Russula* and *Lactarius.*

INTRODUCTION

Gymnomyces is an ectomycorrhizal basidiomycete genus in the family Russulaceae with less than 50 species currently known worldwide, and with 23 species documented in the Pacific Northwest. *Gymnomyces* species are common in

Pacific Northwest forests that include *Pseudotsuga, Abies, Tsuga, Picea, Pinus* and *Quercus* spp. They produce sequestrate (truffle-like) sporocarps with ornamented, amyloid spores. The sporocarps of *Gymnomyces* sp. are a food resource for a suite of mycophagous small and large mammals, including the northern flying squirrel (Fogel and Trappe, 1978; Maser *et al.*, 1978; Maser *et al.*, 1985; Carey *et al.*, 1992; Colgan *et al.*, 1997; Cázares *et al.*, 1999; Colgan *et al.*, 1999). Abundant in the old-growth Douglas-fir (*Pseudotsuga menziesii*) forests of the Pacific Northwest, northern flying squirrels are potentially the most important prey of the northern spotted owl (*Strix occidentalis caurina*), a threatened species that has been an icon in forest policy for preservation of old growth forests of the Pacific Northwest in the last decade (USDA Forest Service and USDI Bureau of Land Management, 1994a).

The Northwest Forest Plan (NFP) was created to coordinate management direction for the lands administered by the USDA Forest Service and the USDI Bureau of Land Management as well as to adopt complementary approaches by other federal agencies within the range of the northern spotted owl. The NFP prompted intense research on a multitude of organisms, including fungi, related to old growth forests within the range of the northern spotted owl. The Forest Ecosystem Management and Assessment Team (FEMAT), an interagency, interdisciplinary team of expert scientists, economists, sociologists and others was created to develop management alternatives for Pacific Northwest forests that balanced the economic and social needs of society with forest conservation (USDA Forest Service and USDI Bureau of Land Management, 1994a). The Record of Decision document (USDA Forest Service and USDI Bureau of Land Management, 1994b) is the culmination of management strategies and ideas generated by FEMAT and further crafted by the president's cabinet after hearing comments from the public. In addition, the ROD document is an interesting historical landmark in that it was the first time in history that the two largest Federal land management agencies (National Forests and Bureau of Land Management Districts) have

developed and adopted a common approach to managing the lands they administer throughout an entire ecological region.

The genus *Gymnomyces* (as *Martellia* and *Gymnomyces*) is among the many taxa of concern listed in the ROD document. Originally the document contained ten sequestrate Russuloid species including five species of *Gymnomyces* (3 as *Martellia*). Given the high degree of uncertainty regarding the biological distribution of fungi, amendments to the survey and manage ROD document were established and published in November of 2000 (USDA Forest Service and USDI Bureau of Land Management, 2000). The new criteria defined left the status of most fungi either unchanged, or changed to allow slightly increased management. No significant changes occurred in the sequestrate Russuloid species. *Gymnomyces* is one of five sequestrate genera (*Macowanites, Gymnomyces, Cystangium, Arcangeliella,* and *Zelleromyces*) long recognized as being more closely related to Russulaceae than to other sequestrate fungi as judged from similarities in spore ornamentation, tissue anatomy, and sporocarp morphology (Lebel, 1998; Lebel and Trappe, 2000). Our current understanding of the taxonomy of *Gymnomyces* is based predominantly on the work by Singer and Smith (1960) on "Asterogastraceous fungi," with the description of the vast majority of the North American species, and a second recent generic type studies by Lebel and Trappe (2000). Prior to Lebel and Trappe's (2000) publication, two additional sequestrate genera, *Martellia* Mattirolo and *Elasmomyces* Cavara, were recognized (Singer and Smith, 1960).

Singer and Smith (1960) separated the 7 genera by "critical characters:" presence or absence of 1) a stipe-columella, 2) sphaerocysts in the glebal trama, and 3) latex production (based on the species known at that time) (Trappe *et al.*, 2002). In reviewing type specimens for sequestrate Russulaceae, Lebel and Trappe (2000) found that Singer and Smith's (1960) "critical characters" used for separating genera were often ambiguous and essentially inadequate, therefore proposed the use of alternative characters to circumscribe genera: this led to

merging of some genera and a revision of generic boundaries within the Russulaceae.

Lebel and Trappe (2000) found sphaerocysts in the glebal trama of the holotype specimens of both *Martellia mistiformis* and *Elasmomyces mattirolianus*. Sphaerocysts in the glebal trama was the single character Singer and Smith (1960) used to distinguish *Gymnomyces* from *Martellia* and *Macowanites* from *Elasmomyces*. Subsequently, because the genera could no longer be separated on that basis, *Martellia* was synonimyzed with *Gymnomyces* and *Elasmomyces* with *Macowanites*. Lebel and Trappe (2000) suggested the structure of the peridiopellis as a more appropriate character to delimit generic boundaries than those previously used by Singer and Smith (1960). Lebel and Trappe (2000) stated that the structure of the peridiopellis is a consistent character within a species and can be examined in both mature and immature sporocarps. With the peridiopellis as the distinguishing character, the majority of *Martellia* species were reclassified into *Gymnomyces* and the remainder into *Cystangium*. Species of *Elasmomyces* were reassigned to either *Cystangium* or *Macowanites* (Lebel and Trappe, 2000).

One problem identified in the current study is the peridiopellis often sloughs off. This makes its use as an informative character somewhat unreliable on dried specimens. To complicate matters further, some sequestrate fungi within the family Russulaceae lack a peridium even when fresh. In fact the genus *Gymnomyces* was originally described as having a peridium: "haud distinctum nullum," or "barely distinct to absent" (Masse, 1898).

Despite the magnitude of the aforementioned contributions towards understanding the systematics of *Gymnomyces*, questions remain: Do current species concepts of *Gymnomyces* allow us to accurately differentiate species? How many species of *Gymnomyces* exist? Is *Gymnomyces* by current concepts monophyletic? This study has begun to unravel taxonomic uncertainties within the genus *Gymnomyces*, and suggests fewer species exist in the PNW than current literature suggests.

MATERIAL AND METHODS

Fungal specimens

Type specimens of various *Gymnomyces* species were obtained from The New York Botanical Garden (NY), The University of Michigan Fungus Collection (MICH), and from the Oregon State University Herbarium (OSC). Some collections were unavailable for loan due to the small amount of existing material; however, amplification of the following holotype collections was attempted: *G. brunnescens* (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano, *G. californicus* (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano, *G. cinnamomeus* Singer and A.H. Sm., *G. cremeus* (Zeller and C.W. Dodge) Trappe, T. Lebel and Castellano, *G. ellipsosporus* (Zeller) Trappe, T. Lebel and Castellano, *G. fallax* (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano, *G. ferruginascens* Singer and A.H. Sm., *G. fulvisporus* (A.H. Sm.) Trappe, T. Lebel and Castellano, *G. gilkeyae* (Zeller and C.W. Dodge) Trappe, T. Lebel and Castellano, *G. mistiformis* (Mattir.) T. Lebel and Trappe, *G. monticola* (Harkn.) Trappe, T. Lebel and Castellano, *G. occidentalis* (Harkn.) Trappe, T. Lebel and Castellano, *G. rolfalexii* Trappe, T. Lebel and Castellano, *G. subfulvus* (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano, *G. abietis* Trappe and Castellano, *G. nondistincta* Trappe and Castellano.

No type specimens amplified with a strong signal, so more recent *Gymnomyces* specimens were obtained from the Mycological Collection of the Oregon State University Herbarium (OSC). This herbarium contains the largest collection of sequestrate fungi from North America. Collections of all 23 "morphological species;" known to occur in the Pacific Northwest were unattainable. Of these, 17 were available and successfully sampled.

Approximately 200 representatives from 200 collections of *Gymnomyces* were critically examined. All collections either had a species name on the packet

or a "?" indicating either an undescribed species, or a species that proved difficult to identify.

In this study, samples were initially attempted to be keyed out using available keys by Singer and Smith (1960) and Trappe (unpublished), to confirm or reject the name on the packet. This proved to be extremely difficult when dealing with species other than *G. ellipsosporus* and *G. gilkeyae* because frequently a specimen exhibited features of several uniquely defined "species." When this situation arose samples were critically examined to see if they fit within the defined species concept of the given name. Subsequently the names on the packets were either confirmed or rejected and labeled with a new name.

Most of the first approximation identifications were by Dr. James M. Trappe who used available keys by Singer and Smith (1960), and the confirmations or amendments were by the author through the use of current keys and literature on the genus *Gymnomyces*. Identifications believed to be incorrect were annotated and labeled as such. There were few occurrences where the first approximation was thought to be incorrect; therefore, the vast majority of names presented in all tables and figure 2.1 represent *Gymnomyces* spp. concepts *sensu* Trappe with agreement by the author.

Fifty collections, believed to represent the 17 of the 23 putative species of *Gymnomyces* in the Pacific Northwest, were selected for ITS rDNA analyses. Analyses were conducted, ITS rDNA sequences obtained and further combined with the *Russula* data set of Miller & Buyck (2002), which included an additional 87 ITS rDNA *Russula* sequences and two distantly related outgroup taxa. Miller and Buyck (2002) chose *Albatrellus flettii* and *Gloeocystidiellum aculeatum* as the outgroup after evaluating a number of different taxa because they provided the most stable topology. Therefore they were maintained in the current study to retain consistency. When possible, multiple collections of each species were sampled (14 taxa had multiple collections sampled). See table 2.1 for a list of taxa included in the analyses.

DNA extraction

DNA was extracted from dried herbarium specimens. A modified CTAB method (Gardes & Bruns, 1993) was used to prepare crude DNA extracts. Samples were ground in micro centrifuge tubes with reusable pellet pestles (previously autoclaved) attached to a Mater Mechanic cordless drill (Fisher Scientific, Pittsburgh, PA). After maceration, 600 µL 65° 2x CTAB buffer was added to the ground tissue and the solution vortexed. The buffer-suspended samples were then frozen and thawed four times by passing the tubes between a dry-ice-ethanol bath and 65° C water bath. After the final thaw, the tissue was incubated at 65 C° for 1 hr. Two chloroform: indol-acetic acid (24:1) extractions were conducted after the incubation. DNA was purified from the aqueous phase of the second chloroform extraction with the GeneClean III kit (Bio 101 Inc., Vista, CA) following manufacturer's recommendations.

Polymerase Chain Reaction (PCR)

The internal transcribed spacer (ITS) region of the nrDNA was amplified via polymerase chain reaction (PCR) (Mullis and Fallona, 1987; Saiki et *al.*, 1988) using primer set ITS 4 and ITS 5 (White *et. al.*, 1990). Reaction mixtures were made to a total volume of 50 µL containing double-distilled H2O, 1 µL DNA template, 4.0 µL of each 10 µM of primer, 25 µL of buffer E (MasterAmp 2x PCR PreMixes: 100 mM Tris-Hcl (PH 8.3, 22 C), 100 mM KCl, 400 µM each dNTP, 5 µM MgCl2, and 4x MasterAmp Enhancer), .5 µL of 5 U/µL *Taq* polymerase. The DNA was amplified with a MJ Research Programmable Thermal Controller (PTC)-100 thermal cycler (Watertown, Massachusetts). Thermal cycling parameters for amplification consisted of one initial cycle with denaturation at 95 C for 3 min. The following conditions were performed for 34 cycles: denaturation at 95 C for 1

min., annealing at 53 C for 30 s, and extension at 72 C for 1 min. The final cycle was set with an extension at 72 C for 2 min followed by enzyme denaturation for 30 min at 4 C.

The primers ITS 4 and ITS 5 did not amplify the ITS nrDNA region of many herbarium collections. Unsuccessful amplification is usually caused by degraded DNA, which is common in older herbarium specimens. For these cases two additional primers, ITS 2 and ITS 3 (White *et. al.*, 1990), were employed to split the approximately 650-800 bp region into two segments. ITS 5 was coupled with ITS 2 to amplify the ITS1 region, and ITS 3 with ITS 4 to amplify the ITS2 region. PCR reactions and reaction parameters were performed as mentioned above.

DNA purification and sequencing

PCR products were electrophoresed on 1% agarose gels (Gibco-BRL ultra PURE, Life Technologies) in 5 μL aliquots, stained with ethidium bromide and visualized by use of a transiluminator. A hundred bp low DNA mass ladder (Gibco-BRL, Life Technologies) was used to estimate PCR product size. PCR products were purified with QIAquick PCR Purification Kit (QIAGEN, Inc., Valencia, CA) according the manufacturer's protocol. The ITS-1, ITS-2, and 5.8S subunits were sequenced with combinations of the primers ITS 5, ITS 2, ITS 3, and ITS 4 on an ABI 377 automated sequencer at either the Central Services Laboratory at the Center for Gene Research and Biotechnology at Oregon State University, or on an ABI 373XL at the Spatafora Mycology Laboratory at Oregon State University.

Table 2.1. GenBank number, voucher, and herbarium location of species from which the internal transcribed spacer (ITS) regions and 5.8S subunit were sequenced.

Species	GenBank Accession Number	Voucher Number[1]	Herbarium[2]
G. abietis Trappe and Castellano	AY239347	T 16022	OSC
G. abietis Trappe and Castellano	AY239348	T 16017	OSC
G. brunnescens (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano	AY239326	OSC 64226	OSC
G. brunnescens (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano	AY239327	OSC 51046	OSC
G. brunnescens (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano	AY239328	OSC 39986	OSC
G. californicus (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano	AY239308	T 16027	OSC
G. californicus (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano	AY239312	OSC 56055	OSC
G. californicus (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano	AY239318	OSC 41312	OSC
G. cinnamomeus Singer and A.H. Sm.	AY239338	T 19930	OSC
G. compactus Singer and A.H. Sm.	AY239342	T 13565	OSC
G. compactus Singer and A.H. Sm.	AY239303	T 13171	OSC
G. ellipsosporus (Zeller) Trappe, T. Lebel and Castellano	AY239304	OSC 61608	OSC
G. ellipsosporus (Zeller) Trappe, T. Lebel and Castellano	AY239305	OSC 60157	OSC
G. ellipsosporus (Zeller) Trappe, T. Lebel and Castellano	AY239306	OSC 58973	OSC
G. fallax (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano	AY239330	T 19916	OSC
G. fallax (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano	AY239329	OSC 44426	OSC
G. foetens (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano	AY239316	OSC 49110	OSC
G. fragrans (A.H. Sm.) Trappe, T. Lebel and Castellano	AY239331	PNW 5607	OSC
G. fragrans (A.H. Sm.) Trappe, T. Lebel and Castellano	AY239332	OSC 38887	OSC
G. gilkeyae (Zeller and C.W. Dodge) Trappe, T. Lebel and Castellano	AY239345	OSC 13548	OSC
G. gilkeyae (Zeller and C.W. Dodge) Trappe, T. Lebel and Castellano	AY239346	T 2572	OSC

Table 2.1. *(Continued).*

Species	GenBank Accession Number	Voucher Number[1]	Herbarium[2]
G. mistiformis (Mattir.) T. Lebel and Trappe	AY245519	MM 1653	MM
G. abietis (Harkn.) Trappe, T. Lebel and Castellano	AY239313	OSC 56167	OSC
G. abietis (Harkn.) Trappe, T. Lebel and Castellano	AY239314	OSC 55997	OSC
G. abietis (Harkn.) Trappe, T. Lebel and Castellano	AY239315	OSC 58479	OSC
G. nondistincta Trappe and Castellano	AY239350	T 17838	OSC
G. rolfalexii Trappe, T. Lebel and Castellano	AY239333	OSC 50307	OSC
G. rolfalexii Trappe, T. Lebel and Castellano	AY239335	T 14997	OSC
G. rolfalexii Trappe, T. Lebel and Castellano	AY239334	OSC 64229	OSC
G. setigerus (Zeller) Trappe, T. Lebel and Castellano	AY239317	OSC 29622	OSC
G. subalpinus (A.H. Sm.) Trappe, T. Lebel and Castellano	AY239309	OSC 56196	OSC
G. subalpinus (A.H. Sm.) Trappe, T. Lebel and Castellano	AY239311	OSC 56182	OSC
G. subalpinus (A.H. Sm.) Trappe, T. Lebel and Castellano	AY239310	OSC 49213	OSC
G. subfulvus (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano	AY239319	T14995	OSC
G. subfulvus (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano	AY239321	T 14998	OSC
G. subfulvus (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano	AY239320	T 16404	OSC
G. subfulvus (Singer and A.H. Sm.) Trappe, T. Lebel and Castellano	AY239322	OSC 39985	OSC
G. subochraceus (A.H. Sm.) Trappe, T. Lebel and Castellano	AY239323	OSC 19416	OSC
G. subochraceus (A.H. Sm.) Trappe, T. Lebel and Castellano	AY239324	T 16403	OSC
G. subochraceus (A.H. Sm.) Trappe, T. Lebel and Castellano	AY239325	T 13386	OSC
G. subochraceus (A.H. Sm.) Trappe, T. Lebel and Castellano	AY239349	T 7753	OSC
Gymnomyces sp.	AY239341	T 17893	OSC
Gymnomyces sp.	AY239302	T 8032	OSC

Table 2.1. *(Continued).*

Species	GenBank Accession Number	Voucher Number[1]	Herbarium[2]
Gymnomyces sp.	AY239339	T 23819	OSC
Gymnomyces sp.	AY239337	T 13003	OSC
Gymnomyces sp.	AY239338	T 19930	OSC
Gymnomyces sp.	AY239343	T 17205	OSC

[1,2] OSC: Mycological Collection of the Oregon State University Herbarium; T: James M. Trappe; MM: Amer Montecchi and Oreste Mattirolo, personal collection, Sardinia, Italy.

Phylogenetic analysis

DNA sequences were assembled and edited on a Power Macintosh G4 by use of SeqEd Ver. 1.0.3 (Applied Biosystems, Inc., 1992), manually aligned with color font, and combined with a preexisting data set from Miller & Buyck (2002, TreeBase matrix, S682 and M1067).

An alignment of 819 nucleotide bases representing the ITS-1, ITS-2, and 5.8S subunit was analyzed for three different exclusion sets (exsets) that represented three different indels and exclusion treatments. The three exsets were as follows: 1) **most conservative**: all areas of ambiguous alignment were excluded and gaps treated as missing data (**SSU**: 24; **ITS1**: 60-67, 81, 91-99, 111-114, 123-130, 142-145, 166-167, 175-190, 193-195, 206-213, 221-222, 226-229; **5.8S**: 236-240, 250-261, 275-276, 290-355; **ITS2**: 461-487; **LSU**: 488-492, 497-502, 509, 516-520, 526-539, 544, 566, 574, 610, 633-647, 665-667, 672-735, 742-753, 804-819); 2) **medium conservative**: areas of ambiguous alignment deemed salvageable were kept while those without any character information were excluded and remaining gaps treated as missing data (**ITS1**: 94-98, 112-114, 142-145; **5.8S**: 290-355; **ITS2**: 474-487; **LSU**: 633-647, 672-712, 741-746, 804-805, 816-818); 3) **least conservative**: all areas of ambiguous alignment included and remaining gaps treated as missing data (**5.8S**: 290-355).

Maximum parsimony analyses were performed with PAUP* 4.0 b10 (Swofford, 2002). *Gloeocystidiellum aculeatum* and *Albatrellus flettii* were chosen as outgroup taxa for phylogenetic analyses to remain consistent with TreeBase Matrix M1067 of Miller & Buyck (2002). A dense sampling of terminal clades prevented finding all most parsimonious tress. Therefore, a two-step heuristic search as detailed below was employed (Olmstead and Palmer, 1993; Hibbett and Donoghue, 1995). First, 1000 heuristic searches were conducted with random sequence addition and TBR (Tree Bisection-reconnection) branch swapping algorithms, saving no more than 2 trees per replication and with MULTRE turned off. Second, trees from step one were used as starting trees and another heuristic

search saving a maximum of 5,000 trees with TBR and MULTRE in effect was conducted. To measure relative support for resulting clades, 10,000 bootstrap replications with the fast-bootstrap option (Felsenstein, 1985), were performed on phylogenetically informative characters (329 included and 490 excluded).

To test alternative phylogenetic hypotheses for *Gymnomyces* spp., i.e. monophyly of *Gymnomyces*, constraint topologies were constructed in PAUP* 4.0b10 (Swofford, 2002). Constraint topologies forced the monophyly of *Gymnomyces* spp., but left all other nodes of the tree as unresolved. These topologies were used as starting trees in maximum parsimony analyses; search options were as described above. The most parsimonious trees recovered from the constraint analyses were statistically compared to the trees recovered from the maximum parsimony analyses with the Templeton WSR test and the Kishino-Hasegawa test, both implemented in PAUP* 4.0b10. (Swofford, 2002).

RESULTS

The ITS rDNA data set contained 137 ingroup taxa and two outgroup taxa. The ingroup taxa consisted of sequences of 50 *Gymnomyces* collections and 87 *Russula* collections. The outgroup consisted of sequences of *Gloeocystidiellum aculeatum* and *Albatrellus flettii*. ITS rDNA template amplified for these taxa were on average 750 base pairs long.

No major differences in tree topology were observed from the three exclusion sets; therefore, exset two was arbitrarily chosen for the remainder of the analysis. Bootstrap values varied slightly among the different exsets, but remained essentially unchanged and provided mid to high support for the nodes that did not collapse into a polytomy.

Results from the three analyses are summarized in table 2.2. Three hundred and twenty-nine characters were parsimony informative out of 819. Analyses yielded 5000 most parsimonious trees of 1990 steps each with a consistency index

(CI) of 0.2719, retention index (RI) of 0.6799, and rescaled consistency index (RC) of 0.1854.

Trees inferred from constraint analyses were significantly longer than most parsimonious trees (2027 steps long, CI of 0.2669, RI of 0.6707, and RC of 0.1790), thus the monophyly of *Gymnomyces* was rejected ($p < 0.0001$). The strict consensus tree for exset two is presented in figure 2.1. Topology of the strict consensus tree shows six distinct *Gymnomyces* clades dispersed among *Russula* taxa, which will be arbitrarily referred to as clade 1 through clade 6 (see figure 2.1). **Clade 1** contains three taxa (two of which fit no current species descriptions): *G. compactus*, and is the sistergroup to subsection *Viridantinae* (for further reference to sections/subsections in *Russula* see Miller and Buyck, 2002) in the strict consensus tree; however, this evolutionary relationship is not supported by bootstrap analyses. *G. compactus* and one undescribed taxon remain closely related with strong internal bootstrap support while the other undescribed taxon mixes among species of *Russula*. **Clade 2** consists of what was thought to be at least six different morphological species: *G. fulvisporus, G. californicus, G. subalpinus, G. abietis, G. foetens,* and *G. setigerus.* It is the sistergroup to the *Russula* section *Polychromae* and subsection *Laricinae* and has high bootstrap support. **Clade 3** consists of what was believed to be one morphological species: *Gymnomyces abietis.* This clade is the sistergroup to *Russula* subsection *Integroidinae*, and has very strong bootstrap support. **Clade 4** is characterized by what was thought to be two morphological species: *G. ellipsosporus* and *G. compactus*, and is the sistergroup to *Russula* clade O (see Miller and Buyck, 2002, Fig.2), which includes *Russula aurata, R. romellii, R. roseipes,* and *R. turci.* Clade 4 also exhibits strong bootstrap support. **Clade 5** consists of what was thought to be two morphological species: *G. gilkeyae* and one undescribed taxon is the sistergroup to *Russula* subsection *Sardoninae,* and has strong bootstrap support. **Clade 6** is comprised of what was thought to be at least eleven different morphological species: *G. californicus, G. cinnamomeus, G. subfulvus, G. subochraceus, G. fragrans, G. rofalexii, G. brunnescens, G. fallax, G. fulvisporus,*

Table 2.2. Results from maximum parsimony analysis of three indels and exclusion treatments.

Indel coding		Most Parsimonious trees					
Analysis Treatment[1]	Gaps as missing	Number of Characters[2]	Number	Length	CI	RI	RC
1	yes	222	5000	1,427	0.2516	0.6620	0.1670
2	yes	329	5000	1,990	0.2719	0.6799	0.1854
3	yes	381	5000	2,332	0.2723	0.6794	0.1850

[1] Different treatment of indels and exclusion sets (see text for further discussion):

1 = Most conservative: all areas of ambiguous alignment were excluded and gaps treated as missing data.

2 = Medium conservative: areas of ambiguous alignment deemed salvageable were kept while those without any character information were excluded, and remaining gaps treated as missing data.

3 = Least conservative: all areas of ambiguous alignment included and remaining gaps treated as missing data.

[2] Number of parsimony informative characters included in the analysis

G. nondistincta, and *G. mistiformis,* and is the sistergroup to the *Russula* subsection *Foetentinae* of Miller and Buyck (2002), and has relatively high bootstrap support (see figure 2.1).

DISCUSSION

The current classification of Gymnomyces

The russuloid clade (Hibbett and Thorn, 2001), where *Gymnomyces* resides, exhibits diverse sporocarp morphologies. The hymenomycetous forms include sequestrate, resupinate, pileate, and coralloid, with smooth, toothed, lamellate, or poroid sporocarps forms. This clade is complex because it lacks an obvious morphological synapomorphy diagnostic of the inclusive taxa, and appears to be ecologically variable as well (Hibbett and Thorn, 2001). The russuloid clade includes ectomycorrhizal, pathogenic, saprotrophic, and possibly lichenized species (Hibbett and Thorn, 2001).

Sequestrate fungi in general pose a great challenge for taxonomists due to their morphologically reduced sporocarps that include a limited number of taxonomically informative characters. In addition, because sequestrate fungi develop more slowly than epigeous fungi, multiple developmental stages may exist in a single collection (Molina and Trappe, 1994). Due to limited techniques at the time, it is possible that Singer and Smith (1960) interpreted ontogenetic variation within a species as different species, thus creating multiple species descriptions for a single species.

Grubisha *et al.* (2002) illustrate a case where many of the sectional relationships within *Rhizopogon* Fr., as Smith originally defined them, are well supported (sections *Amylogon* and *Villosuli*); however, many of the lower taxonomic groupings (subsections, series, and stirps) are polyphyletic. A similar

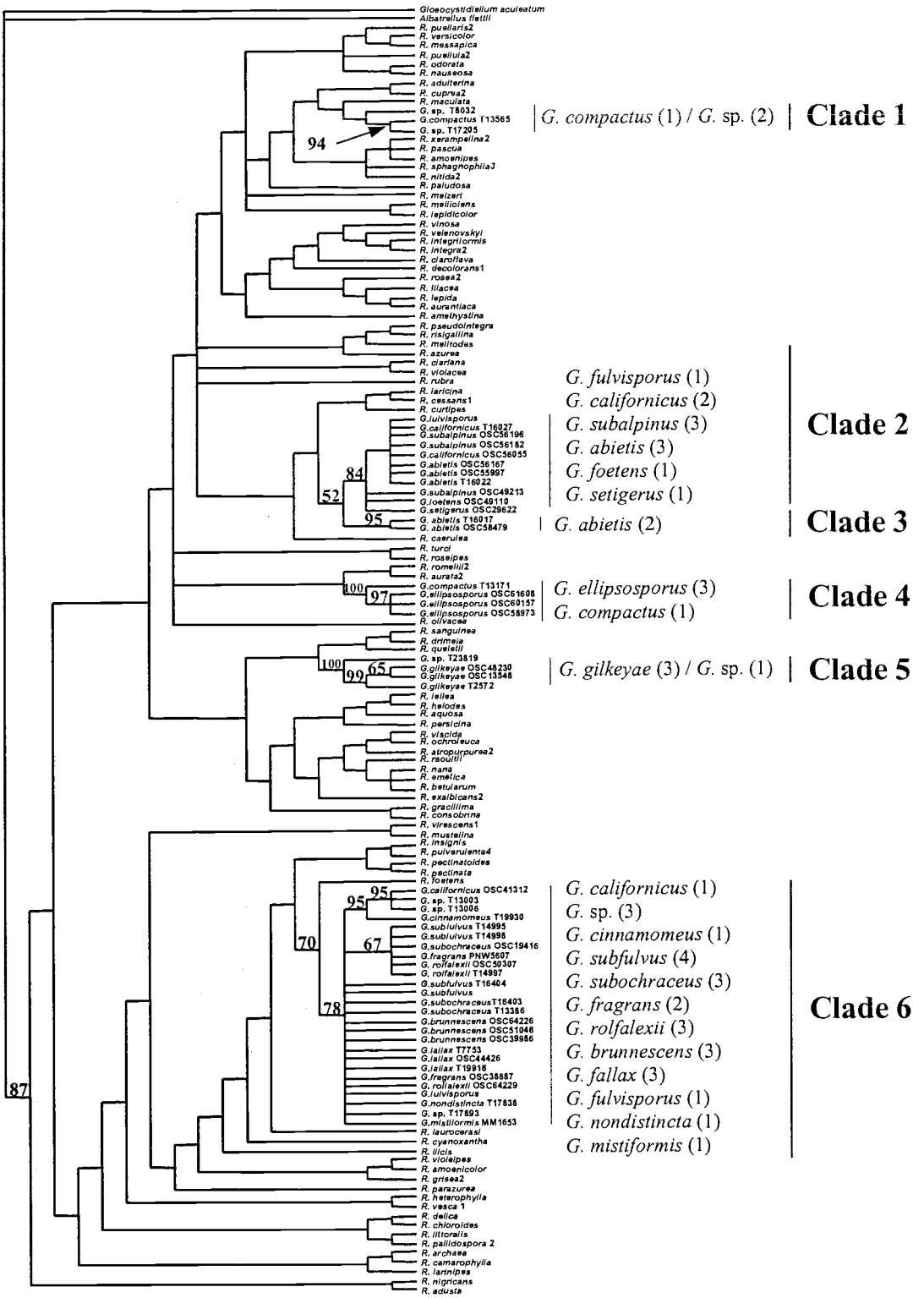

Figure 2.1. Strict consensus cladogram of 5000 equally most parsimonious (MP) trees of 1990 steps based on ITS nrDNA sequences using exset 2 (see table 2.2). Bootstrap values above 50% are indicated at the respective internode. CI=0.2669, RI=0.6706, RC=0.1790. ( ) indicate # of samples.

problem may exist in *Gymnomyces*. Under current taxonomic classification 23 species occur in the Pacific Northwest (Singer and Smith, 1960; Trappe *et al.*, 2002); however, results of this study suggest that fewer species may exist than current literature suggests. ITS data support at least six distinct *Gymnomyces* lineages, and reject the monophyly of *Gymnomyces*.

Examination of infrageneric relationships in Gymnomyces

Of the 18 morphological species included in these analyses (17 from the Pacific Northwest, plus the type species for the former genus *Martellia* from Italy), mostly defined by Singer and Smith (1960), ITS data are consistent only with *Gymnomyces ellipsosporus* and *G. gilkeyae*, which are clades 4 and 5, respectively. The remaining clades of *Gymnomyces* are not characterized by an obvious morphological synapomorphy and numerous morphospecies are found in more than one clade. Clade 4 includes 3 *G. ellipsosporus* specimens and one specimen identified as *G. compactus*, and is strongly supported with a high bootstrap value of 100. *Gymnomyces ellipsosporus* has a unique spore morphology that can be easily distinguished from other taxa. Its spores of are relatively large (15-20 (23) x 12-15 (16) μm) compared to other *Gymnomyces* spores (<15 x 13 μm) (James Trappe, personal communication) and broadly ellipsoidal in shape. Its spore ornamentation consists of irregular warts, short ridges, and spines (1.8-2.5 (3) x 0.4-1μm tall) (Singer and Smith, 1960). *Gymnomyces compactus* is also noted by Singer and Smith (1960) as having broadly ellipsoid spores (10-14 x 9-12 μm tall); however, the spores are believed to be significantly smaller. This species was identified as *Hydnangium compactum* by Zeller. According to Dodge and Zeller (1936) the "co-type" of *H. compactum* Harkn. has spores 5-6 μm in diameter and has a thick peridium of jellified hyphae (Singer and Smith, 1960).

Clade 5, including 3 *G. gilkeyae* specimens and one undescribed taxon, is strongly supported with a bootstrap value of 100. *Gymnomyces gilkeyae* possesses a distinct spore morphology and can be accurately distinguished from other taxa. Spores of *G. gilkeyae* are noticeably larger (12-16 x 12-15 μm) than the typical *Gymnomyces* spore size. Ornamentation consists of long prominent simple to forked spines, or truncated cones (3.4-5μm in length) (Singer and Smith, 1960), and the spores often have a prominent sterigmal appendage.

Clade 1, including one "morphological species (*G. compactus*)" and two other taxa, is not supported by bootstrap analyses; however, two taxa, *G. compactus* and *Gymnomyces* sp. T17205, remain in a single clade with high bootstrap support of 94 (see figure 2.1). The third and lone taxon mixes among *Russula* species (shown in the bootstrap tree). The morphological species "*G. compactus*" appears in two clades, clade 1 and clade 4 (see table 2.3 to see the distribution of "morphospecies" across clades). *Gymnomyces compactus*, as stated before, is described by Singer and Smith (1960) as having broadly ellipsoid spores which corresponds to the spore description of *G. ellipsosporus* and to most other *Gymnomyces*; however, the two taxa differ in spore size: *G. compactus* spores are in the range of 10-14 x 9-12 μm and *G. ellipsosporus* are in the range of 15-20 (23) x 12-15 (16) μm. The warted spore ornamentation of *G. compactus sensu* Singer and Smith (1960) is noted to be about 1 μm high compared to the prominent single to forked spines and truncated cones (3.5-5 x 1-2.5 μm) of *G. gilkeyae sensu* Singer and Smith (1960). Both taxa have a cellular subhymenium according to Singer and Smith (1960); however, *G. compactus sensu* is noted to have scattered sphaerocysts near the subhymenium. The character descriptions of the two taxa appear different when looking at spore size and ornamentation; however, this may be an artifact of the age of the two specimens.

Clade 2 consists of what was believed to be at least 6 different morphological species and has high bootstrap support of 84 (see figure 2.1). The dominant "morphological species" that appear in this clade are *G. abietis* (3), *G.*

subalpinus (3 species), and *G. californicus* (2 species). Singer and Smith (1960) did not list definitive characters of *G. subalpinus* other than lack of sphaerocysts in the peridium (distinguishing it from an Australian species called *Zelleromyces australiensis*), and almost globose spores (distinguishing it from *Zelleromyces alveolatus*).

Clade 3 includes only one "morphological species," *G. abietis* (figure 2.1). Trappe and Castellano (2000) described what they thought to be a new species (*G. abietis*) associated with *Abies* in the Pacific Northwest. They noted that this "new taxon" fit the description of *G. monticola* (Harkn.) Singer and A.H. Sm.; however, Singer and Smith (1960) list Smith #60297 from Idaho as the only material they examined of *G. monticola,* which does not match Harkness's original description of the holotype. Upon examination of Harkness #13, the holotype of *Octaviania monticola,* Trappe and Castellano (2000) observed differences in glebal color and spore shape thus determining that what Singer and Smith (1960) were calling "*G. monticola"* was the same taxon as *G. abietis*. The main differences described between *G. abietis* and *G. monticola sensu* Harkn. include spore shape, size, ornamentation, and cell size of the subhymenium (Trappe and Castellano, 2000).

Clade 6, composed of what was thought to be at least 11 morphological species, has relatively high bootstrap support (see figure 2.1). Not surprisingly, this clade consists of a species complex that equally befuddles both expert and amateur taxonomists. The dominant morphological species appearing in clade 1 are *G. brunnescens* (3 specimens), G. *subfulvus* (4 specimens), *G. subochraceus* (3 specimens), *G. fallax* (3 specimens), and *G. rolfalexii* (3). Singer and Smith (1960) distinguished *G. brunnescens* through its possession of a "turf of dermatocystidia," and "amyloid spores with isolated to grouped but unconnected elements of ornamentation with only moderate height."

Gymnomyces subfulvus was described by Singer and Smith (1960) to be distinctive through color reactions in potassium hydroxide (KOH), and a unique turf epicutis. *Gymnomyces subfulvus* was said to possess similar features to *G. fallax* but lacked hymenial cystidia and exhibited a paler gleba and paler spores in

Table 2.3. List of *Gymnomyces* species and the respective clades they appear in figure 2.1.

Species	Clade 1	Clade 2	Clade 3	Clade 4	Clade 5	Clade 6
G. abietis		X	X			
G. brunnescens						X
G. californicus		X				X
G. cinnamomeus						X
G. compactus	X			X		
G. ellipsosporus				X		
G. fallax						X
G. foetens		X				
G. fragrans						X
G. fulvisporus		X				X
G. gilkeyae					X	
G. mistiformis						X
G. nondistincta						X
G. rolfalexii						X
G. setigerus		X				
G. subalpinus		X				
G. subfulvus						X
G. subochraceus						X

KOH (Singer and Smith, 1960). Cystidia are often times difficult to locate and often times hard to differentiate from basidioles; color in general is a subjective character, and is often correlated with spore maturity.

Singer and Smith (1963) distinguished G. *subochraceus* by its relatively small spores 7-9 (10) μm, absence of sphaerocyts in the peridium, enlargement of cells in the subhymenium, and lack of odor when fresh. Spore size is also somewhat correlated with spore maturity so a small pale spore may be an immature rendition of a darker larger spore when fresh. Lebel and Trappe (2000) noted that the distribution of sphaerocysts can vary even within a single sporocarp, so much study is required in those taxa that do not possess distinctive features. Singer and Smith (1960) noted that enlarged cells in the subhymenium might represent a developmental stage of the fungus; however, they thought this is highly unlikely. Odor, like color, is a very subjective feature and is often correlated with sporocarp maturity, especially with sequestrate fungi that develop aromas at maturity to attract small mammal mycophagists. Many characters used in the past to delineate species boundaries in *Gymnomyces* seem to be highly variable, and potentially dependent on the developmental stage. The descriptions of some of the morphological species may represent ontogenetic variation within a species.

Clade 6 also contained a *Gymnomyces* specimen *from* Italy and not known from the Pacific Northwest. *Gymnomyces mistiformis* was formerly the type species for the genus *Martellia*, which was recently collapsed into the genera *Gymnomyces* and *Cystangium* (Trappe *et al.*, 2002). It was inserted into the analysis to see with what clade it shared the closest affinity, which resulted in clade 6. I did not succeed in obtaining a sample of the type species of *Gymnomyces* (*Gymnomyces pallidus*) from Tasmania. I planned to test if these two types grouped together or separately, to support or reject the recent merging of many *Martellia sp.* into the genus *Gymnomyces.*

While our work was not bolstered by phylogenetic data from type specimens, phylogenetic data was successfully collected from the largest pool of sequestrate fungal specimens available for this group in North America. In a

similar vein, it is likely that a large percentage of the diversity of North American *Gymnomyces* spp. existing among "collected specimens" was sampled.

The ITS region clearly suggests at least six unique lineages of *Gymnomyces*. Because the ITS data are consistent with the morphological autapomorphies of both *G. ellipsosporus* and *G. gilkeyae*, their removal from the genus *Gymnomyces* is suggested. In the future the expansion of the data set to include representatives of all Russuloid sequestrate fungi would be invaluable, both to investigate further placements of the remaining sequestrate fungi relative to *Gymnomyces,* and to increase our knowledge of the Russuloid clade in general.

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CHAPTER 3

CONCLUSIONS

In this study, ribosomal DNA sequences from the ITS1, 5.8S, and ITS2 regions were used to address questions of monophyly of the genus *Gymnomyces* and intrageneric boundaries within the genus *Gymnomyces*. Phylogenetic analyses supported the inference that the *Gymnomyces sensu* Lebel and Trappe is not monophyletic, suggesting at least six unique lineages or clades. Moreover, fewer species may exist than have been proposed.

Many of the characters used to delineate species boundaries in *Gymnomyces* such as presence or absence and location of sphaerocysts, enlargement of cells in the subhymenium, spore size, and sporocarp odor when fresh appear to vary considerably within a species and may depend on the developmental stage of the fungus. One possible explanation is that ontogenetic variation within a species has been interpreted as multiple species that are subsequently not supported by a phylogenetic criterion.

Maximum parsimony analyses yielded $\geq$5000 most parsimonious trees of 1990 steps with a consistency index (CI) of 0.2719, retention index (RI) of 0.6799, and rescaled consistency index (RC) of 0.1854. Trees yielded from constraint analyses were significantly longer than most parsimonious trees (2027 steps long, CI of 0.2723, RI of 0.6794, and RC of 0.1850), so the monophyly of *Gymnomyces* is rejected ($p < 0.0001$). The strict consensus tree is presented in figure 2.1. Topology of the strict consensus tree shows six distinct *Gymnomyces* clades dispersed among *Russula* taxa.

Clade 1 is not supported by bootstrap analyses; however, 2 out of 3 taxa have a high degree of support (bootstrap of 94 %). Two of the 3 taxa were undescribed *Gymnomyces* specimens with no readily apparent defining characters, and the third taxa was identified as *G. compactus*, which appears again in clade 4.

Two of the six clades (clade 2 and clade 6) are characterized by including 6 to 11 "morphospecies," respectively. Morphological synapomorphies uniting the individual clades are not obvious, nor are morphological characters to separate clade 2 and clade 6. Clade 3 resembles clade 2 and clade 6 in that there does not seem to be a discrete morphological synapomorphy uniting the clade.

Two of the six clades (clade 4 and clade 5) are characterized by distinct spore morphology. Because the ITS data are consistent with the morphological autapomorphies of both *G. ellipsosporus* and *G. gilkeyae*, their removal from the genus may be appropriate after further investigation.

While direct sequencing of a selected gene region, such as the ITS, within a particular fungal genome is a powerful tool for assessing potential genetic diversity, the data must be interpreted with caution. Different levels of information about fungal evolution will be obtained by study of different DNA molecules and genes so it may be premature to make taxonomic recommendations on a 1-gene study. This study can be strengthened by analyzing additional genes and preferably more taxa, which are unquestionably necessary to further circumscribe species boundaries. It would be most useful to use a gene that can address interspecific variation between closely related species, such as the β-tubulin or the IGS.

The unavoidable and critical issue that arises when contemplating the reclassification of *Gymnomyces* is the reclassification of *Russula* and *Lactarius*. Miller *et al.* (2001) generated data indicating that *Russula* is not monophyletic and that *Lactarius* is nested within *Russula*. This study included Russuloid sequestrate genera; *Gymnomyces* was shown to be nested within *Russula* along with *Cystangium* and *Macowanites*, while *Zelleromyces* and *Arcangeliella* were phylogenetically positioned within *Lactarius*. Before approaching the reclassification of *Gymnomyces* the broader picture including *Russula* and *Lactarius* needs to be approached.

Expansion of the data set to include representatives of all russuloid sequestrate fungi would be invaluable, both to investigate further placements of the remaining sequestrate fungi relative to *Gymnomyces* and increase our knowledge of

the russuloid clade in general. In this case it would be appropriate to use the large subunit rRNA, because it has proven useful for analyzing relationships at the familial and generic levels (Hibbett and Vilgalys, 1993; Moncalvo *et al.*, 1995). In conclusion, a multi-gene phylogeny with representative taxa throughout the sequestrate and epigeous Russulales would be ideal to further investigate inter- and intra-generic relationships among this important group of ectomycorrhizal fungi.

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