The Pennsylvania State University

The Graduate School

College of Agricultural Sciences

SYSTEMATICS, BIOGEOGRAPHY AND CONTROL OF ARTILLERY FUNGI (*SPHAEROBOLUS* SPP.)

A Thesis in

Plant Pathology

by

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Submitted in Partial Fulfillment
of the Requirements
for Degree of

Doctor of Philosophy

August, 2004

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ABSTRACT

Sphaerobolus is a basidiomycete with dual lignicolous and coprophilous ecology that is often found on landscape mulch. Mycologists have studied its spore dispersal mechanism, phototropism, and its possible control using biocontrol agents and different types of mulch. Virtually no research has been done, however, on the systematics, population structure, and chemical control of artillery fungi. Therefore, the goal was to elucidate the systematics of *Sphaerobolus* species using both molecular and morphological data, to obtain information about the biogeography of different species in the genus, and to investigate the effectiveness of various fungicides as candidates for control.

Phylogenetic analyses of artillery fungi (*Sphaerobolus* spp.) isolates (n=26) were conducted to identify species boundaries in the genus *Sphaerobolus*. Multiple gene genealogies inferred from maximum likelihood, Bayesian and maximum-parsimony analyses of sequence data from individual loci (mtSSU, ITS, EF 1-□, and LSU) and a combined dataset (mtSSU, ITS, EF 1-□) concordantly indicate the existence of three deeply divergent lineages in the genus *Sphaerobolus*, each representing a phylogenetic species. These three phylogenetic species correspond to two known species: *Sphaerobolus iowensis* (n=12) and *Sphaerobolus stellatus* (n=10), and a newly discovered species, provisionally named *Sphaerobolus ingoldii* prov. sp. nov. (n=4). This latter species is going to be described in a separate publication. Suprageneric phylogenetic analyses of the mtSSU and LSU datasets containing representatives of related genera of the gomphoid-phalloid clade of homobasidiomycetes suggested the monophyly of the genus *Sphaerobolus* and that *S. ingoldii* prov. sp. nov. likely is more closely related to *S. stellatus* than to *S. iowensis*.

Macro- and micro-morphological analyses of colony and basidiocarp characters confirmed that these three phylogenetic species correspond to two known species: *S. iowensis* and *S. stellatus*, and *Sphaerobolus ingoldii* prov. sp. nov. Species can be distinguished based on colony morphology and growth rate on agar media, and the composition and morphology of cells within the gleba.

Despite the considerable amount of DNA polymorhism found in all species, nested clade analyses of *S. iowensis* and *S. stellatus* indicated little phylogeographic structure in either species. A very likely explanation is the extensive dispersal, due to the dual coprophilous and lignicolous ecology of *Sphaerobolus* species, that provides many possible dispersal scenarios over great geographic distances. However, statistically significant genotype-geography associations were detected in one clade of each species, suggesting restricted gene flow due to isolation by distance for some isolates.

The inhibitory effect of 14 fungicides, 13 of which were known to be effective against the basidiomycete *Agaricus*, was tested at two concentrations (5 and 20 ppm) on the *in vitro* growth of three species of artillery fungi: *S. iowensis*, *S. stellatus*, and *Sphaerobolus ingoldii* prov. sp. nov. Opus®, Topsin M 70WP®, triphenyltin acetate, Difolatan®, and Terraguard 50W® were the most effective inhibitors of growth for all three *Sphaerobolus* species; reduction in growth was directly related to fungicide concentration. Five fungicides showed varying results, depending on fungal species and fungicide concentration. Four fungicides showed no significant inhibition on these artillery fungi. Although this preliminary study provided interesting results, potential fungicides must be tested in the field and registered by E.P.A. before recommendations can be made to homeowners who want to minimize adverse effects of artillery fungi.

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ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude and appreciation to the members of my doctoral research committee: David Geiser, Donald Davis, Blair Hedges, Daniel Royse, and Elwin Stewart for all their support and encouragement, and for providing a perfect environment for my academic development. I am particularly indebted to my co-advisers, David Geiser and Donald Davis, for their guidance and inspiration, for providing freedom in my research, and for their criticism that allowed me to improve as a scientist. My special thanks go to Daniel Royse for welcoming me in his lab when I first came to Penn State as a visiting scholar supported by a joint scholarship of the Hungarian Fulbright Commission and the Soros Foundation.

I want to thank the Pennsylvania Department of Agriculture, the Mycological Society of America, the Deep Hypha, the College of Agricultural Sciences and the Department of Plant Pathology of PSU for providing financial support for my research and for attending professional meetings. I am grateful for the following persons for providing cultures or herbarium specimens: Elizabeth Brantley, Richard Hanlin, David Hibbett, Kentaro Hosaka, David McLaughlin, Jean Lodge, Amy Rossman, Greg Thorn, Lori Trummer, Kazumasa Yokoyama, and the USDA Forest Products Laboratory. I also thank Barrie Overton and Elwin Stewart for their help in the micro-morphological investigations. I am grateful for Rytas Vilgalys and François Lutzoni for their hospitality and guidance during my collecting trip at Duke University.

My sincere thanks go to Gordon Blood, Constance Kossan, and the graduate student clinicians at the Penn State Speech and Hearing Clinic whose help played a major part in my personal development at Penn State.

I was very fortunate to work in a lab, where I was surrounded by friends: David Geiser, Ameena Nalim, Ning Zhang, María del Mar Jiménez-Gasco, Carla Garzón and Jean Juba were all much more than mere colleagues. They were ready to help in any situation, making it easier to withstand life's challenges in a foreign country.

I dedicate my work to my family. First of all, I thank my wife, Johanna Geml, for her never-ending love, support, inspiration and patience, and for putting my best interest a priority when making important decisions in our lives. I could not imagine having a better partner beside me. I also thank our daughter, Maya Geml, for filling my days with joy. My great appreciation and gratitude go to my parents, József Geml and the late Emilia Ilosvai, who provided a caring and intellectually challenging environment, and made it possible for me to attend the best available academic institutions throughout my education. They awoke and encouraged my interest toward Nature, and more specifically to Biology, and helped me to find a career that I love. I also thank my sister, Ágnes Geml, and numerous friends in Hungary and other parts of the world; they all enriched my life in many different ways, for which I am very grateful.

Chapter 1

Introduction

Since the first description of *Sphaerobolus* in 1729 by Micheli under the name *Carpobolus*, many mycologists have been fascinated by this unique fungus capable of ejecting a 1 mm diameter glebal mass over 6 m into the air toward the strongest light source in its environment (Buller 1933). The early work of Walker and Anderson (1925) and Walker (1927) on the cytology of fruiting bodies during development in several isolates was fundamental to the current knowledge of this fungus. Since then, most researchers have focused on the mechanism, physiology, cytology of gleba dispersal and mycelial growth (Alasoadura 1963, Buller 1933, Dykstra 1982, Fletcher and Cooke 1984, Ingold 1972, Walker 1927, Walker and Anderson 1925). More recently, attention has been given to potential control measures inhibiting the growth of the fungus (Brantley *et al*. 2001a, Brantley *et al*. 2001b). These latter studies were necessitated by the fact, that artillery fungi has become a source of distress to homeowners, landscape mulch producers and insurance companies due to the strong adhesion of the discharged gleba to artificial surfaces (e.g. house sidings, cars, and windows) (Lehman 1985).

Virtually no research has been done on the molecular systematics, population structure, and fungicide control of artillery fungi. The goal of this research was to elucidate the molecular evolution and systematics of *Sphaerobolus* species, and to investigate the effectiveness of various control strategies. A better understanding of the phylogenetic relationships among *Sphaerobolus* species, the description of concordant morphological and biochemical (e.g. resistance to fungicides, if any) characteristics will provide a systematic framework of the genus

that can serve as a base for future research, including but not limited to applied control studies, population genetics etc.

Classification of *Sphaerobolus*

Fig. 1-1. *Sphaerobolus stellatus* (photo by the author). The bar represents 1 mm.

Based on morphology, the genus *Sphaerobolus* has been classified as a member of the class gasteromycetes along with other fungi having passive spore discharge, including bird's nest fungi (*Cyathus, Crucibulum* etc.) puffballs (*Lycoperdon, Langermannia* etc.), earth balls (*Scleroderma* etc.), stinkhorns (*Phallus, Mutinus, Pseudocolus* etc.), and earth stars (*Geastrum* etc.). Within this class, authors have placed artillery fungi (Fig. 1-1) into different families and orders: Sphaerobolaceae in Nidulariales (Ulloa and Hanlin 2000); Sphaerobolaceae in Sclerodermatales (Hawksworth *et al.* 1996). However, molecular phylogenetic analyses of Hibbett *et al.* (1997) revealed that the Gasteromycetes represents a polyphyletic, artificial taxon that does not represent a true evolutionary group within the Basidiomycota. Based on molecular data, the genus *Sphaerobolus* is best placed in the gomphoid-phalloid clade with the following genera as closest relatives: *Geastrum, Phallus, Pseudocolus, Ramaria, Clavariadelphus, Gomphus, Gautieria* etc. (Hibbett *et al.* 1997, Moncalvo *et al.* 2002). These results seem to be incorporated in the most recent classification by Kirk *et al.* (2001), who placed *Sphaerobolus* in the family Geastraceae, order Phallales, class Basidiomycetes, phylum Basidiomycota.

The Greek origin of the name *Sphaerobolus* ("sphere thrower") is related to the ability of these fungi to shoot their gleba (mass of asexual and sexual spores) a considerable distance. The genus contains two currently recognized species, *S. stellatus* (Tode) Pers. and *S. iowensis* Walker (Hawksworth *et al.* 1996), that are distinguished by micromophological characteristics detailed later in this chapter. However, several other names appear in culture collections, including *S. bombardioides, S. carpobolus, S. corii, S. crustacus, S. epigaeus, S. minimus, S. minutissimus, S. rubidus, S. sparsus,* and *S. tubulosus*. The origin of these binomials cannot be found in the literature, and thus cannot be considered scientifically valid.

Ecology of artillery fungi

Sphaerobolus is a cosmopolitan genus. It has been reported from Alaska, British Columbia, most parts of the continental U.S. (particularly the Northeast), Europe (from Greece to Iceland), Asia (including Japan), Australia, New Zealand, Africa, and Latin America etc. (Aplin 1961, Dring 1964, Halgrimsson *et al.* 1992, Herrera and Perezsilva 1987, Ingold 1972, McGray pers. comm. 2002, McKenzie and Foggo 1989, Zervakis *et al.* 1998).

Artillery fungi produce spherical, whitish or orange colored basidiocarps, 1-2 mm in diameter, in which a single spore mass (gleba or peridiole) is formed. The gleba contains numerous spores of two types, uninucleate, thick-walled basidiospores and thin-walled, asexual, dikaryotic gemmae. The two spore types play different roles in the dual survival mechanism as a lignicolous and as a coprophilous fungus, that greatly increases the chance of survival of the species. Basidiospores are stimulated to germinate when exposed to proteolytic enzymes, such as pepsin, and to relatively high temperatures, such as those found in mammal herbivores (Dykstra

1982). This mechanism is elicited in the event that the gleba lands on a surface that is consumed by an herbivore. If no herbivore ingests the glebal mass, the gemmae can germinate on wood or other plant debris. A further back-up system to improve survival is the long term viability of the ejected spore mass, inferred from the fact that gleba have been reported to germinate after at least 11 years of storage (Walker 1927). Once germination occurs, colonization of the substrate begins. The characteristic bleaching associated with this white rotting fungus can be attributed to the digestion of cellulose, hemicellulose and lignin present within the substrate. Temperatures between 20 and 25 C are ideal for the fungi to grow vegetatively, but temperatures between 10 and 20 C are considered ideal for stimulating the fungi to produce reproductive structures (Alasoadura 1963). For this reason, artillery fungi are categorized as a cool season fungus. After colonizing the substrate for some time the fungi produce phototropic fruiting bodies. At maturity these basidiocarps orient themselves towards the brightest light source and forcibly discharge their gleba into air (Nawaz 1967). Similar active/passive mechanisms for dispersing spore masses can be observed in other dung-inhabiting fungi as well, including *Ascobolus* (Pezizomycetes) and *Podospora* (Sordariomycetes) of the Ascomycota, and *Pilobolus* (Mucorales) of the Zygomycota. This feature is likely an adaptation to the coprophilous lifestyle, and evolved multiple times independently during evolution. *Nidularia* and *Cyathus* in the Nidulariales (Basidiomycota) also have a similar dispersal mechanism and habitat, and was the basis at one time for *Sphaerobolus* being incorrectly classified in the Nidulariales.

Basidiocarp morphology, gleba discharge and practical concerns

The first signs of basidiocarp development in the mycelium consist of globular knots of binucleate hyphae. As development advances, variably sized hyphae with protoplasmic content can be observed. In *S. stellatus* some of the hyphae at the center of these knots have clavately enlarged ends that are the beginnings of the first basidia (Walker 1927). These primary basidia become young basidium centers around which new basidia are formed, eventually producing four (occasionally eight) basidiospores. The basidia entirely break down and disappear as soon as the spores are mature, making room for the enlargement and maturation of other basidia. In *S. iowensis*, the development of primary basidia is followed by the formation of the characteristic cavities or chambers in which the spores are produced (Walker 1927).

In a nearly mature basidiocarp the spore-bearing regions and the enlarged sterile hyphae and gemmae intermingled in the gleba can be clearly distinguished. The sterile hyphae undergo disintegration and become broken down by the time the glebal mass is discharged. The gemmae are more or less scattered through the gleba, but are more abundant toward the periphery. They are somewhat elongated, basidium-like cells in form with two or more nuclei (Walker 1927).

The mature basidiocarps of artillery fungi comprises a peridium (the “cup”) and a gleba. Just prior to opening they are spherical in shape and more or less imbedded in the surrounding mycelium and substrate. When mature the peridial layers of the basidiocarps break open on top in a stellate manner revealing the spherical glebal mass on the inside.

The peridium consists of a three-layered endoperidium and three-layered exoperidium. Beside the presence of glebal chambers as mentioned above, *S. iowensis* is also distinguished from *S. stellatus* based on the absence of a gelatinous layer in the outer peridium (Walker 1927).

Parenchyma-like cells join the inner and outer peridial layers at six or more points. Gleba release occurs as a result of an increase in osmotic pressure due to the conversion of glycogen into maltose and glucose. The concentration of these simple sugars increases in the inner and outer peridial layers at maturity, and the resulting hydration of these tissues causes tension to develop in the two layers (Fletcher and Cooke 1984, Walker and Anderson 1925). Simultaneously, the fruiting body orients itself towards the brightest light source up to five hours before sporulation (Lehman 1985). The fruiting body is better able to reorient itself if it is within a 60-degree angle of the brightest light source (Nawaz 1967). Prior to sporulation, the hyphae in the glebal region begin to decompose and collect at the bottom of the inner peridial layer, coating the gleba (Dykstra 1982, Walker 1927). This amorphous cellular debris is responsible for the adhesive nature of the gleba. The mechanism of ejection is attributed to tension at the points adjoining the inner and outer peridial layers. The spore mass is expelled when the endoperidial layer suddenly inverts. This action can propel the gleba up to 2 meters high and over 6 meters distance (Buller 1933).

Due to this discharge mechanism, and to the fact that artillery fungi are commonly found on landscape mulch, property owners recently have expressed concern about finding an abundance of brown-black expelled gleba on surfaces such as windows, cars, and structural sidings (Akina 2000, Brantley *et al*. 2001a, Brantley *et al*. 2001b, Lehman 1985). This occurrence is damaging because the gleba are extremely difficult to remove when dry, and they permanently stain surfaces (Akina 2000, Brantley *et al*. 2001a, Brantley *et al*. 2001b). Mulch producers, distributors, and landscape maintenance firms have been asked by customers to pay for the repainting of siding or vehicles, or replace siding stained by the fungus. Lawsuits and numerous insurance claims have been filed as a result of damage to property (Akina 2000).

Control of growth and sporulation of the artillery fungus

Artillery fungi are only some of many microorganisms present in landscape mulch. A vast population of bacteria and other fungi play integral roles in the decomposition of wood products, including various mushrooms, slime molds, bird's nest fungi etc. (Agrios 1997). Wood and bark mulches contain a variety of carbohydrates, from simple sugars through cellulose, hemicellulose, and lignin. Therefore, there is a fungal succession that occurs within the decomposing mulch, with one set of fungi decomposing the simple sugars and a different set of fungi decomposing the more complex carbohydrates. White-rotting fungi, such as *Sphaerobolus*, are capable of degrading the lignin, and have been observed to colonize somewhat later in the decomposition cycle (Agrios 1997).

Since microorganisms can inhibit the growth of other microorganisms colonizing the same substrate, a possible solution to the artillery fungi problem is the use of microorganisms to limit the growth and/or sporulation of *Sphaerobolus* through competition, antagonism and/or parasitism. For this purpose Brantley *et al.* (2001a) successfully used *Trichoderma* and *Bacillus* species *in vitro* as biological control agents against artillery fungi. Numerous other microorganisms with biological control potential have been reported to be present in several composted waste products, e.g. spent mushroom substrate (SMS) that is readily available throughout Pennsylvania (Cronin *et al.* 1996, Hoitink and Grebus 1994, Yohalem *et al.* 1996). Furthermore, Brantley *et al.* (2001b) observed that substrates with high N content generally do not encourage the growth and sporulation of *Sphaerobolus*. Therefore, besides being able to support a rich microbial community, the SMS with its high N content is a good candidate for biological control experiments aimed at reducing sporulation of artillery fungi.

An alternative control method is prevention, i.e. the use of certain types of landscape mulch that do not encourage growth and sporulation of artillery fungi. For this reason Brantley *et al*. (2001b) investigated twenty-five different types of landscape mulch to determine which mulches supported or inhibited artillery fungi. Their research showed that although the fungus was able to grow on most types of mulch, there were substantial variations in sporulation between the different types. In their experiments *Sphaerobolus* did not grow or sporulate well on pine bark, Atlantic cedar and cypress. Brantley *et al*. (2001b) observed that sporulation on bark mulches was significantly less than on wood, and even in mixed mulches, artillery fungi usually grew on the wood pieces rather than on the bark. Although mulch products with the highest C:N ratio produced low numbers of gleba, Brantley *et al*. (2001b) concluded that the C:N ratio is not useful indicator by itself to estimate the ability of mulch products to support colonization and sporulation by artillery fungi.

Recently there has been an increasing popular demand by homeowners for fungicides effective against artillery fungi (Davis pers. comm. 2002). However, no research has been published on this control measure of *Sphaerobolus*. Therefore, one of the focus areas of my research was to evaluate selected fungicides as candidates for control.

The use of various genes for molecular phylogenetic studies of fungi

Knowing the evolution and systematics of *Sphaerobolus* species is crucial for understanding the biology, geographical distribution, ecology of these taxa. This information then can subsequently be used for practical investigations (e.g. developing control measures).

Separation of the *Sphaerobolus* genus from other genera is simple. However, the correct identification of the species is much more difficult. Taxonomists in general often have to consider subjective, variable traits that are often influenced by environmental conditions. Phylogenetic assesment based on DNA offers several advantages over traditional morphological methods. For example: (1) the number of informative characters that can be obtained from DNA sequences is much greater than those from morphological analyses, (2) the character states are very objective, and generally do not depend on the personal opinion of the evaluator (given that subjectivity is avoided in making aligments), (3) DNA sequences of genes present in all living organisms (e.g. rDNA) make it possible to compare datasets among very different, distantly related taxa (from bacteria to human), therefore providing a tremendous help in assembling the Tree of Life.

The ribosomal RNA genes

The vast majority of molecular systematic studies in fungi have utilized sequence variation found in ribosomal RNA genes (rDNA), that can be used to determine relationships between various fungal taxa (for example, see Bruns *et al.* 1991, Hillis and Dixon 1991, Hopple and Vilgalys 1999, Lutzoni and Vilgalys 1995, Moncalvo *et al.* 1995, Pine *et al.* 1999). The reasons for the systematic versatility of rDNA include the numerous rates of evolution among different regions of rDNA (both among and within genes), and the presence of many copies of most rDNA sequences per genome (Hillis and Dixon 1991). These features facilitate the analysis of rDNA either by sequencing or by restriction enzyme methodologies, and make it well-suited to infer phylogenetic history across a very broad spectrum of taxa, from studies among the basal

lineages of life to relationships among closely related species and populations (Hillis and Dixon 1991). Sequence analyses of rDNA is extremely useful in fungi, that rarely, if ever, produce sporocarps, which are the structures traditionally used in taxonomic determinations in higher fungi (Hinkle *et al.* 1994).

The nuclear ribosomal RNA genes (rDNA) of fungi exist on a single chromosome as a multiple-copy gene family comprised of highly similar DNA sequences, from 8 to 12 kb, arranged in a tandem repeat. Each repeat has coding regions for one major transcript spanning the genes encoding the 16-18S (Small Subunit, SSU), 5.8S, and 25-28S (Large Subunit, LSU) rRNAs (which are transcribed by RNA polymerase I as a single transcriptional unit), punctuated by one or more non-tanscribed intergenic spacer (IGS) regions. In the Basidiomycota, each repeat also has a separately transcribed coding region for 5S RNA whose position and direction of transcription may vary among groups (Page and Holmes 1998, Palumbi 1996, Geiser pers. comm. 2002). The most conserved regions are generally the coding regions (LSU and SSU regions) being most suitable for analysis of distantly related groups. The 5S and the non-coding regions (ITS and IGS) are much less conserved, and therefore, can be used to elucidate differences between closely related taxa at the species or infraspecies level (Page and Holmes 1998, Palumbi 1996).

The mitochondrial ribosomal RNA genes (mt rDNA) – unlike the nuclear rDNA – are not arranged as tandem repeat, rather as single genes in a mitochondrial genome. However, since numerous mitochondria exist within a cell, they are present in many copies within each cell. An additional difference is that mt rDNA contains the 12S mtSSU, and 16S mtLSU genes, and does not contain the ITS and IGS regions.

Bruns and Szaro (1992) observed that the mtSSU gene evolves 16 times faster than the nuclear SSU gene, but is less variable than the nuclear ITS regions. Therefore, the mtSSU gene, by combining the advantages of nSSU and ITS regions (i.e. sufficient variability with relatively easily alignable regions), represents a good source of phylogenetic information, and is believed to have the potential to fill the gaps between the resolution levels of nSSU and ITS (Hong *et al.* 2002). However, multiple insertions, introns and hypervariable regions have been observed in this gene in some species, that can cause difficulties in sequence alignments (Hibbett pers. comm. 2002, Hibbett and Donoghue 1995). For example, in *Sphaerobolus*, at least three types of independent insertions have been observed with different insertion sites, length, and sequence composition. Furthermore, these insertions are polymorphic for presence and absence even in closely related isolates (Geml unpubl. data).

Despite their popularity as phylogenetic tools, rDNA arrays do not always evolve in a simple manner. In particular, when rDNA sequences are compared within and between species, there is often a high degree of sequence similarity within species, yet a great deal of divergence between them. This unusual evolutionary pattern is due to concerted evolution, through the mechanisms of unequal crossing-over and gene conversion that transfer DNA sequences between genes so that they evolve together (Page and Holmes 1998). In addition to this limitation in shallow phylogenetic analyses, several studies using rDNA genes in the Basidiomycota have shown that basal branches that connect relatively distantly related genera or family-level clades are not well resolved (Hibbett *et al.* 1997, Moncalvo *et al.* 2002), which indicates that their use is sometimes limited at higher taxonomic levels as well.

The translation elongation factor 1-α (EF 1-α) gene

The EF 1-α gene is a nuclear gene encoding a protein that is involved in the translation of mRNA to protein. Elongation factor complexes 1 and 2 both consist of several proteins with specific roles in translation. The EF 1-α functions in the transport of an aminoacyl-tRNA to the ribosome, and its amino acid sequence is highly conserved among taxa, even those from different kingdoms. However, the intron positions tend to vary greatly, making it difficult to predict their arrangements in newly investigated taxa (Palumbi 1996). The EF 1-α gene has been used for phylogenetic studies for revealing both deep and shallow phylogenies. While mainly the exons' nucleotide and protein sequences have been used for investigating deep divergences, e.g. in Oomycetes (van't Klooster *et al.* 2000), and in inter-kingdom analyses (Baldauf 1999, Roger *et al.* 1999); the much more variable introns are useful for studying molecular evolution within closely related organisms, e.g. in Mucorales (Zygomycota) (O'Donnell *et al.* 2001), and in the ascomycete genus *Fusarium* (Baayen *et al.* 2001, O'Donnell *et al.* 1998). A growing number of studies indicates the utility of EF 1-α nucleotide or protein sequences for molecular phylogenetics. O'Donnell *et al.* (1998) and O'Donnell *et al.* (2001) reported, that of the loci sequenced in their study (EF 1-α gene, SSU and LSU rDNA) EF 1-α provided the most phylogenetic information. Within the EF 1-α gene, 95% of the phylogenetic signal is derived from intron sequences that appear to be under relaxed evolutionary constraints as inferred from substitutional patterns (O'Donnell *et al.* 1998). In the EF 1-α genes of higher fungi the expected intron sequence is between 200-400 bp that is distributed among one to multiple intron positions. In combination with the nearly 400 3rd positions in the exons, the EF 1-α gene provides access to numerous sites evolving presumably at or near neutral evolutionary rates and are a rich source of

characters for shallow phylogenetics (Rehner pers. comm. 2002). Although paralogous EF 1-α genes, capable of generating misleading phylogenetic signals, have been reported in the zygomycete *Mucor racemosus* (Linz *et al*. 1986), they did not interfere with the phylogenetic analyses in other fungal species (O'Donnell *et al*. 1998, O'Donnell *et al*. 2001).

The phylogenetic species concept using multiple gene genealogies

Mayden (1997) characterized the existing species concepts as either theoretical or operational. While several operational species concepts have been recognized, there is only one theoretical species concept, the Evolutionary Species Concept (ESC). ESC defines species as *"...a single lineage of ancestor-dependent populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate"* (Wiley 1978). Operational species concepts are also aimed at distinguishing evolutionary lineages, but they differ in having recognition criteria, therefore they can be used to diagnose or recognize species in practice. The most common operational species concepts are the Morphological Species Concept (MSC, defined as a group of individuals that have some reliable morphological characters distinguishing them from all other species), the Biological Species Concept (BSC, defined as a set of actually or potentially interbreeding organisms), and the Phylogenetic Species Concept (PSC, defined as is the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent) (Cracraft 1983).

The currently dominant species concept is the MSC that is based on morphological descriptions. However, MSC often fails to recognize biological and/or phylogenetic species. Good examples of this are the morphological species *Armillaria mellea* and *Fusarium solani* that

both consist of several biological and numerous phylogenetic species (Anderson and Stasovski 1992, Anderson and Ulrich 1979, Geiser pers. comm. 2002, O'Donnell 2000). On the other hand, there still are "good" morphological species that have the same boundaries regardless of the recognition criteria (MSC, BSC, PSC), as in the case of *Fusarium avenaceum* (Nalim pers. comm. 2002). However, these cases of concordance are rather rare, and in recent years researchers have revealed numerous cryptic species when investigating single morphological species (for example, see Cruse *et al*. 2002, Geiser *et al*. 1998, O'Donnell *et al*. 1998, Shen *et al*. 2002). Concordance in gene genealogies determines the limit of the species as follows (Taylor *et al*. 2000): "*Where the different gene trees are concordant they have the same tree topology due to fixation of formerly polymorphic loci following genetic isolation; these concordant branches connect species. Conflict among the gene trees is likely to be due to recombination among individuals within a species, and the transitions from concordance to conflict determines the limits of species*."

The recognition of phylogenetic species can lead to the observation of variation in morphological and/or biological characteristics that otherwise would be much more difficult to detect. For example, an *a posteriori* investigation revealed variation - correlating with the phylogenetic species - in the presence and size of sclerotia and variation in mycotoxin production of *Aspergillus flavus* (Geiser *et al*. 2001). Furthermore, distinguishing separate phylogenetic species showing different pathogenicity patterns within pathogenic organisms can help to develop more efficient strategies against these diseases both in plant pathology and in medical research (Taylor *et al*. 1999).

The review of intraspecific phylogenetic methods used in this study

Evolutionary relationships above and below the species level are different in nature. The relationship is hierarchical between species due to the reproductive isolation and population fission over relatively long time that led to fixation of different alleles and to non-overlapping gene pools. By contrast, relationship between individuals of the same species is not hierarchical, because of sexual reproduction, smaller number of recent mutations and recombination (Posada and Crandall 2001). The traditional methods developed to estimate interspecific relationships (e.g. minimum evolution, maximum parsimony, and maximum likelihood) often give poor resolution or inadequately portray genealogical relationships due to assumptions that are often invalid at the population level. For example, these methods assume that ancestral haplotypes are no longer in the population, however coalescent theory predicts that ancestral haplotypes are usually the most frequent sequences sampled in a population (Clement *et al*. 2000, Posada and Crandall 2001, Templeton 1998). Additionally, traditional methods assume no recombination and require relatively large numbers of variable characters, neither of which is usually a characteristic of population studies. Furthermore, evolutionary processes commonly acting at the population level, such as recombination of genes and hybridization between lineages, and homoplasy generate reticulate relationships within the population. Traditional methods, based on primary bifurcating trees, do not allow such reticulations (Posada and Crandall 2001).

Therefore, network representations of the population level genealogical information have been developed (Templeton *et al*. 1992, Templeton 1998). Networks allow the incorporation of multifurcating nodes, the presence of ancestral haplotypes as internal nodes, therefore give more accurate genealogical information associated with population level divergences. The common

phylogeographic approach is to overlay a haplotype network on the geographical sampling area and infer explanations through visual inspection. However, this approach lacks statistical support. Nested clade analysis (NCA) (Templeton 1998) allows for statistical testing of the H_0 of no association between the haplotype and the geographic location. In NCA, a haplotype tree is used to define a series of nested clades that are used in the evolutionary analysis of the geographic distribution of genetic variation (Templeton 1998). The nested clade information, sample size and geographical location for each clade are used to calculate clade dispersion (D_c) and clade displacement (D_n), and to test them for significance at the 5% level using permutation technique (Posada *et al*. 2000). Clade dispersion (D_c) is the average distance of all individuals in clade X from the geographical center of that clade, while clade displacement (D_n) is the average distance of individuals in clade X from the geographical center of clades of the next highest nesting level. Where significant D_c and/or D_n values are detected, a set of criteria is used to discriminate between the effects of contemporary (e.g. gene flow) and historical (e.g. allopatric fragmentation, range expansion) processes (Posada *et al*. 2000, Templeton 1998).

Research objectives

The objectives of my study were as follows:

1. Reveal the evolutionary history of *Sphaerobolus* based on multiple gene genealogies.

2. Determine species boundaries in *Sphaerobolus* based on genealogical concordance phylogenetic species recognition following Taylor *et al*. (2000).

3. Detect morphological differences *a posteriori* between determined phylogenetic species.

4. Characterize biogeography of *Sphaerobolus* species using nested clade analysis approach.

5. Determine influence of selected fungicides on *in vitro* growth of artillery fungi.

Chapter2

Molecular evolution of *Sphaerobolus* based on multiple gene genealogies

Abstract

Phylogenetic analyses of 26 artillery fungus (*Sphaerobolus* sp.) isolates were conducted to identify species boundaries in the genus *Sphaerobolus*. Multiple gene genealogies inferred from maximum likelihood, Bayesian and maximum-parsimony analyses of sequence data from individual loci (mtSSU, ITS, EF 1-α, and LSU) and a combined dataset (mtSSU, ITS, EF 1-α) concordantly indicate the existence of three deeply divergent lineages in the genus *Sphaerobolus*, each representing a phylogenetic species. These three phylogenetic species correspond to two known species: *S. iowensis* and *S. stellatus*, and a newly discovered species, named *Spaerobolus ingoldii* prov. sp. nov.. Suprageneric phylogenetic analyses of the mtSSU and LSU datasets containing representatives of related genera of the gomphoid-phalloid clade of Homobasidiomycetes suggested the monophyly of the genus *Sphaerobolus* and that *S. ingoldii* prov. sp. nov. likely is more closely related to *S. stellatus* than to *S. iowensis*.

Introduction

Since the first documentation of *Sphaerobolus* nearly 300 years ago under the name *Carpobolus* (Micheli 1729), many mycologists have been fascinated by these unique fungi. The first species in the genus was named *S. stellatus* (Tode) Pers. Walker (1927) later described a

second species (*S. iowensis*) and a new variety (*S. stellatus var. giganteus*). These fungi are extremely common in temperate climates, encountered most commonly on wood mulches used in landscaping. Several researchers have studied the growth and unique spore dispersal mechanism of artillery fungi (Alasoadura 1963, Buller 1933, Dykstra 1982, Fletcher and Cooke 1984, Ingold 1972, Walker 1927, Walker and Anderson 1925).

Based on morphology, the genus *Sphaerobolus* historically has been classified as a member of the class Gasteromycetes along with other fungi having passive spore discharge, including bird's nest fungi, puffballs, earth balls, stinkhorns, and earth stars. Within Gasteromycetes, authors have placed artillery fungi into different families and orders, including Sphaerobolaceae in Nidulariales (Ulloa and Hanlin 2000) and Sphaerobolaceae in Sclerodermatales (Hawksworth *et al.* 1996). However, molecular phylogenetic analyses of Hibbett *et al.* (1997) revealed Gasteromycetes to be a polyphyletic taxon that does not represent a true evolutionary group within the Basidiomycota. Sequences of nuclear and mitochondrial genes placed the genus *Sphaerobolus* in the gomphoid-phalloid clade with an extremely morphologically diverse set of genera as closest relatives: *Geastrum, Phallus, Pseudocolus, Ramaria, Clavariadelphus, Gomphus, Gautieria* etc. (Hibbett *et al.* 1997, Moncalvo *et al.* 2002). These results are incorporated in the most recent classification by Kirk *et al.* (2001), who placed *Sphaerobolus* in the family Geastraceae, order Phallales, class Basidiomycetes, phylum Basidiomycota.

The genus contains two currently recognized species, *S. stellatus* (Tode) Pers. and *S. iowensis* Walker (Hawksworth *et al.* 1996), that are distinguished by micromorphological characteristics (Walker 1927). Early gleba development in young basidiocarps of *S. stellatus* is marked by the formation of globular knots of binucleate hyphae. These later become centers

around which new basidia are formed, eventually producing four, occasionally eight, basidiospores per basidium. The basidia entirely decompose and disappear by the time the spores are mature, making room for the enlargement and maturation of other basidia. In *S. iowensis*, the development of young basidia is followed by the formation of characteristic cavities or chambers in which the spores are produced (Walker 1927). In addition, the peridium consists of a three-layered endoperidium and three-layered exoperidium in *S. stellatus*, whereas in *S. iowensis* a gelatinous layer in the outer peridium is absent (Walker 1927).

Since no molecular systematic work had been published investigating species limits in this genus, our goal was to elucidate the molecular phylogenetics of *Sphaerobolus* species to provide better understanding of the biology of artillery fungi. For this purpose we generated nucleotide sequences of three ribosomal DNA regions and one protein coding gene region: mitochondrial ribosomal RNA small subunit (mtSSU), internal transcribed spacer regions of the nuclear ribosomal gene repeat (ITS), the nuclear large ribosomal RNA subunit (LSU), and translation elongation factor 1-□. (EF 1-□). These regions have been successfully used in several studies to reveal both deep and shallow phylogenies in fungi (Baayen *et al.* 2001, Bruns *et al.* 1991, Hillis and Dixon 1991, Hopple and Vilgalys 1999, Lutzoni and Vilgalys 1995, Moncalvo *et al.* 1995, O'Donnell *et al.* 1998, O'Donnell *et al.* 2001, Pine *et al.* 1999) and are well suited for determining phylogenetic species boundaries using genealogical concordance as outlined by Taylor *et al.* (2000).

Materials and methods

Isolates and DNA extraction

Isolates of artillery fungi included in this study either come from culture collections or were isolated from gleba isolated in the field or received from homeowners (Table 2-1). In order to isolate pure cultures from gleba, the gleba were agitated in 20% bleach solution for 3 min, washed with distilled water, air-dried, sprayed with 70% ethanol, and finally air-dried again on filter paper under aseptic conditions (Davis pers. comm. 2001). The surface-sterilized gleba were placed on oatmeal agar (OA) and were sealed with Parafilm® (American National Can, Chicago, IL) to prevent dehydration. Growth medium was made using 30 g DIFCO® oatmeal agar (Becton Dickinson Microbiology Systems, Sparks, MD) per 1 liter of media that was autoclaved and poured in 100 x 15 mm dishes. Although the 30 g per liter is half of the oatmeal agar concentration suggested by the manufacturer, according to my observations it supports growth just as well, and it is much easier to work with when compared to the 60 g per liter formula. The harvested and ground mycelia were used to isolate DNA using the DNeasy Plant Mini Kit (QIAGEN, Inc., Valencia, CA) according to the manufacturer's protocol. The prepared DNA was used as template for PCR amplification.

Table 2-1. Code, geographic origin and GenBank accession numbers for generated sequences of *Sphaerobolus* isolates investigated in the phylogenetic study.

Species	Isolate code	Geographic origin	GenBank accession number			
			mtSSU	ITS	EF 1-α	LSU
Sphaerobolus ingoldii prov. sp. nov.	T-800	Kellogg Biological Station Long Term Ecological Research, Michigan	AY654739	AY654737	AY654734	AF139975*
	9597	Inst. for Fermentation, Otsu, Japan	AY488022	AY487971	AY487996	AY439013
	SS19	Atlanta, Georgia	AY488015	AY487965	AY487990	AY439012
	SS42	Hershey, Pennsylvania	AY654740	AY654738	AY654735	-
Sphaerobolus iowensis Walker	ATCC 52850	East Lansing, Michigan	AY488008	AY487958	AY487984	AY439014
	SS1	Indiana	AY488000	AY487950	AY487976	-
	SS2	Elizabethtown, Pennsylvania	AY488001	AY487951	AY487977	-
	SS4	Langhorne, Pennsylvania	AY488003	AY487953	AY487979	-
	SS5	State College, Pennsylvania	AY488004	AY487954	AY487980	-
	SS9	Chapel Hill, North Carolina	AY488006	AY487956	AY487982	AY439010
	SS16	Olney, Maryland	AY488012	AY487962	AY487988	-
	SS18	Olney, Maryland	AY488014	AY487964	AY487989	-
	SS20	Olney, Maryland	AY488016	AY487966	AY487991	-
	SS21	Galion, Ohio	AY488017	AY487967	AY487992	-
	SS22	Ithaca, New York	AY488018	AY487968	AY487993	-
	SS23	Medina, Ohio	AY488019	AY487969	AY487994	-

Sphaerobolus stellatus (Tode)Pers.	ATCC 18339	Maryland	AY488007	AY487957	AY487983	AY439011
	CBS 321.32	The Netherlands	AY488026	AY487975	AY487999	-
	DSH 96-015	Great Brook State Park, Massachusetts	AY488009	AY487959	AY487985	-
	MIN 864513	Elm Creek Nature Reserve, Minnesota	AY488025	AY487974	AY487998	-
	SS3	State College, Pennsylvania	AY488002	AY487952	AY487978	-
	SS7	West Mifflin, Pennsylvania	AY488005	AY487955	AY487981	-
	SS13	Erie, Pennsylvania	AY488010	AY487960	AY487986	-
	SS14	Lucinda, Pennsylvania	AY488011	AY487961	AY487987	-
	SS25	Newton Centre, Massachusetts	AY488021	AY487970	AY487995	-
	SS28	Anchorage, Alaska	AY488024	AY487973	AY487997	-

* Submitted to Genbank by R.G. Thorn on 1 Apr 1999 under the name *Sphaerobolus stellatus*

PCR amplification and DNA sequencing

Portions of the mtSSU, LSU, EF 1-α, and the entire ITS region were PCR amplified in reaction mixtures containing 37 μl PCR water, 5 μl 10x PCR buffer (0.5M KCl, 0.1M Tris HCl pH 8.3, 0.025M $MgCl_2$), 5 μl 10x dNTPs (2mM of each dNTP), 0.1 μl AmpliTaq® DNA polymerase (Perkin-Elmer, Foster City, CA), 1 μl of 10μM forward primer and reverse primer for the region of interest, and 1 μl template DNA (100-fold dilution of original DNA solution extracted). PCR reactions were performed in a 96-well thermocycler (PTC-100 Programmable Thermal Controller, MJ Research, Inc.) using the following temperature program for all ribosomal DNA regions: 94 C/5 min; 34 cycles of 94 C/1 min, 53 C/1 min, 72 C/1 min; and 72 C/5 min. For the single copy gene EF 1-α a "touchdown" PCR setting were used with an annealing temperature of 65 C in the first cycle, then successively reduced by 1 C per cycle to 56 C, after which the annealing temperature will be maintained at 56 C for the remaining 30-36 cycles (Stephen Rehner pers. comm.). The following primers were used for amplification: ITS5 and ITS4 for ITS (White *et al*. 1990), NL1 and NL4 for LSU (O'Donnell 1996), MS1 and MS2 for mtSSU (White *et al*. 1990), and EF1-983F (GCY CCY GGH CAY CGT GAY TTY AT) and EF1-1567R (ACH GTR CCR ATA CCA CCR ATCTT) for EF 1-α (Stephen Rehner pers. comm.). Amplification products were electrophoresed in a 3.0% agarose gel and stained with ethidium bromide for visualization of the bands. Primers for amplification of genes to be used in my study are shown in Table 5 and Fig. 15. PCR products will be purified directly from reactions using the QIAquick® PCR Purification Kit (QIAGEN, Inc., Valencia, CA).

Purified amplification products were sequenced via the dideoxy chain-termination method (Sanger *et al*. 1977) using the Applied Biosystems (ABI) BigDye® v. 3.0 terminator kit and an ABI 377 automated DNA sequencer (Perkin-Elmer, Foster City, CA). Each sample was sequenced in both directions with the same primers used for PCR. The only exception is the 1567R primer of the EF 1-α gene that was replaced by the 1567Ra primer (ACH GTR CCR ATA CCA CC) for better results (Stephen Rehner pers. comm. 2002).

Phylogenetic analyses

Sequence data obtained for both strands of each locus were edited and assembled for each isolate using Sequencher 3.1 (Gene Codes, Ann Arbor, MI). Sequence alignments were made by Clustal X (Thompson *et al*. 1997). Analyses were conducted using maximum-likelihood (ML) and maximum-parsimony (MP) methods in PAUP* 4b10 (Swofford 2002), and Bayesian analysis in Mr.Bayes 3.0 (Huelsenbeck and Ronquist 2001). Since these methods follow different theories and algorithms, congruent features found in all three types of analyses were considered meaningful.

In the first set of analyses, i.e. the model-based approach, ambiguously aligned regions were excluded, consisting of the following positions: 208-259, 299-309, 324-330, 338-342, 470-480 in the suprageneric mtSSU, 205-255, 296-307 in the intrageneric mtSSU, 57-63, 79-85, 169-206, 227-236, 270-279, 492-497 in the ITS, and 204-260, 410-462 in the EF 1-α alignments. Interestingly, regions that could not be aligned unambiguously between the three different phylogenetic species were highly similar and sometimes identical within lineages, indicating the existence of fixed differences. The outcomes of the model-based analyses depend on the

evolutionary models used. Since systematic analyses should avoid *a priori* assumptions about evolutionary processes in their methods (Mindell and Thacker 1996), I conducted preliminary data analyses, and applied *a posteriori* corrections (e.g. choosing evolutionary model) to maximize objectivity in the analyses. Different evolutionary models with varying values of base frequencies, substitution types, α parameter of the Γ-distribution of variable sites, and proportion of invariable sites, among other parameters, were compared via the likelihood ratio test for each locus using PAUP* and Modeltest 3.06 (Posada and Crandall 1998) to determine the best-fit evolutionary model for both ML and Bayesian analyses. I tested 56 models – comparing the likelihood scores of trees constructed by the NJ method - starting with the simplest model (Jukes-Cantor model, number of substitution types=1, equal base frequencies, equal rate of evolution for variable sites, proportion of invariable sites=0) and progressing toward the most complex model (General Time-Reversible model, number of substitution types=6, estimated base frequencies, estimated rate matrix, rate of evolution for variable sites following a Γ-distribution with an estimated α parameter, estimated proportion of invariable sites). The likelihood scores corresponding to the different models were compared by the likelihood ratio test and the *P*-value was determined (Page and Holmes 1998). The likelihood ratio test statistic was calculated as twice the difference between the log likelihood scores of the two models contrasted. When the model representing the null hypothesis is a special case of the alternate model (as was the case in the models compared), this statistic fits a chi-square (χ^2) distribution with a number of degrees of freedom equal to the number of parameters that freely vary between the two models (Posada and Crandall 1998). ML analyses were carried out with the heuristic search option using the "tree bisection and reconnection" (TBR) algorithm with 100 random sequence additions to find the overall optimum instead of local optima. To test the statistical reliability of the generated trees

and test the stability of clades, the bootstrap test (Felsenstein 1985) was used with "full heuristic search" and 100 replicates. In Bayesian phylogenetic analyses, 100,000 generations were run in four chains. The chains were sampled every 100th generation. When the likelihood scores of trees sampled approached similar values, they were considered to have converged. In each run, only trees after this convergence point were included in computing the consensus tree.

In the MP analyses previously excluded ambiguous regions were included after being recoded with program INAASE 2.3b (Lutzoni *et al*. 2000). The code and step matrices obtained were attached to the appropriate alignments. Heuristic searches were carried out with the "tree bisection and reconnection" (TBR) algorithm with 100 random addition sequences to find the overall optimum instead of local optima. The bootstrap test was used with 500 replicates.

Phylogenetic trees obtained by analyzing each locus were compared to detect clades that are supported by every tree. Phylogenetic species were recognized based on the criteria of Taylor *et al*. (2000), i.e. by determining the transition points between concordant and conflicting information derived from different gene genealogies. Gene genealogies should be congruent between phylogenetic species, where gene trees should represent organismal trees. In contrast, gene genealogies should be incongruent within species as the result of recombination of unlinked loci by independent assortment of chromosomes or by crossing over during sexual reproduction.

Results

Intrageneric phylogenetic analyses

The intrageneric mtSSU, ITS, EF 1-α, and the combined datasets consisted of 583, 769, 628, and 1980 characters, respectively, including gaps. Of these 63, 78, 110, and 251 ambiguous positions were excluded in the intrageneric analyses. Three types of long (>1kb), independent insertions, each with a unique insertion site, length and nucleotide sequence, were detected in the mtSSU region of some of the isolates. These insertions were excluded from the alignment. The Hasegawa-Kishino-Yano model (Hasegawa *et al.* 1985) with no proportion of invariable sites (I=0) and equal variation rates for all sites (HKY) was selected by hierarchical likelihood ratio tests as the best-fit evolutionary model for the mtSSU and ITS datasets. The base frequencies for the mtSSU and ITS alignments were freqA = 0.3304, freqC = 0.1610, freqG = 0.2410, freqT = 0.2676, and freqA = 0.2617, freqC = 0.2256, freqG = 0.2012, freqT = 0.3115, respectively. The transition/transversion ratios were ti/tv = 1.4824 and ti/tv = 1.8035. The Tamura-Nei model (Tamura and Nei 1993) with no proportion of invariable sites (I=0) and estimated α-parameter of Γ-distribution (TN +G) was selected as the best-fit evolutionary model for the EF 1-α dataset. The base frequencies were freqA = 0.2349, freqC = 0.2869, freqG = 0.2492, freqT = 0.2290. The substitution rate matrix was as follows: R(a) [A-C] = 1.0000, R(b) [A-G] = 2.7043, R(c) [A-T] = 1.0000, R(d) [C-G] = 1.0000, R(e) [C-T] = 11.3650, R(f) [G-T] = 1.0000. The among-site rate variation was characterized by variable sites following a Γ-distribution with an estimated α-parameter = 0.3767. Since the HKY model was determined earlier as best-fit model for two of

the three loci, that model was chosen for ML and Bayesian analyses of the combined dataset as well.

Likelihood values converged after about 16000, 20000, 19000, and 16000 generations in Bayesian analysis of the mtSSU, ITS, EF 1-α, and combined datasets, respectively. The consensus trees were computed from 840, 800, 810, and 840 trees, after discarding the first 161, 201, 191, and 161 trees as "burn in". The ML phylograms and the Bayesian consensus trees generated using the models described above had similar topologies at each locus. The ML trees of individual loci and the combined dataset are shown in Fig.s. 2-1, 2-3, 2-5, and 2-7, respectively, with bootstrap (ML) and posterior probability (Bayesian) values of the supported branches. After including the character matrices of the ambiguous regions recoded by INAASE, the final alignments of mtSSU, ITS, and EF 1-α for MP analyses consisted of 522, 697, 520, and 1739 characters, respectively. One of the 11 (mtSSU), 46 (ITS), 85 (EF 1-α), and 18 (combined) most parsimonious trees are shown with bootstrap values in Fig.s. 2-2, 2-4, 2-6, and 2-8, respectively.

In all analyses, *Sphaerobolus* isolates formed three deeply divergent, highly supported clades. One of the clades corresponds to *S. stellatus*, as indicated by the presence of two isolates previously identified as *S. stellatus* from different culture collections: ATCC 18339, CBS 321.32. Isolates in the second clade are considered *S. iowensis*, as this group included a specimen previously identified as *S. iowensis* (ATCC 52850). The third clade has no former taxonomic connection and represents a newly discovered taxon, described in this paper as *Sphaerobolus ingoldii* prov. sp. nov.. The species-level clades received 100% support in all analyses, and numerous subgroups receiving varying levels of support were found in *S. iowensis* and in *S. stellatus*. Characteristic fixed DNA polymorphisms used in the description of *S. ingoldii* prov.

sp. nov. were determined based on the sequence alignments used in the phylogenetic analyses. Only those characters present in all *S. ingoldii* prov. sp. nov. isolates and none other *Sphaerobolus* isolates were included. These fixed DNA polymorphisms are indicated with capital and italicized letters (nucleotides) or italicized numbers (gaps) in the species description.

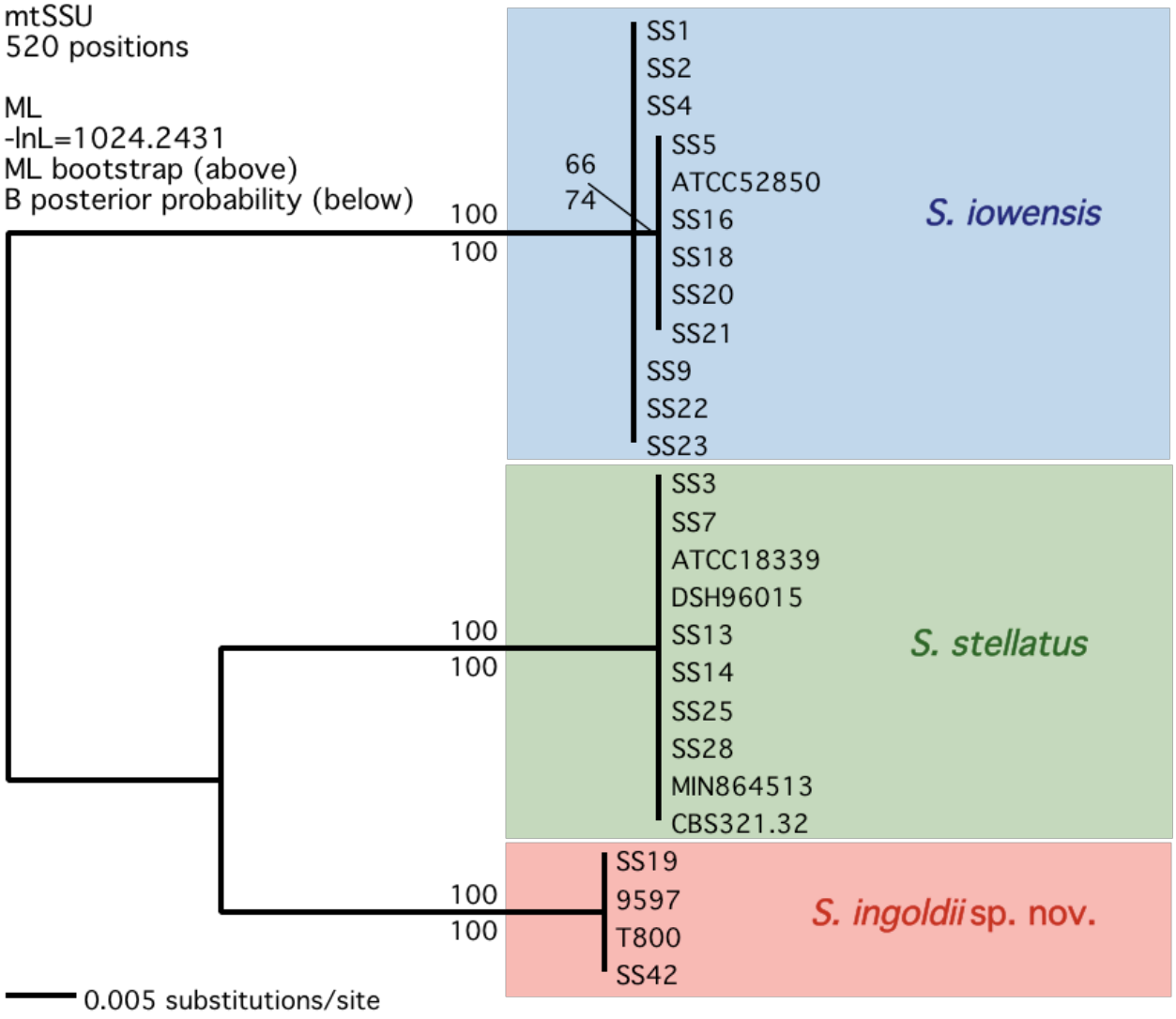

Fig. 2-1. Phylogram generated by ML analyses of the intrageneric mtSSU dataset. The tree is midoint rooted. Bootstrap and posterior probability values are indicated above and below the branches, respectively.

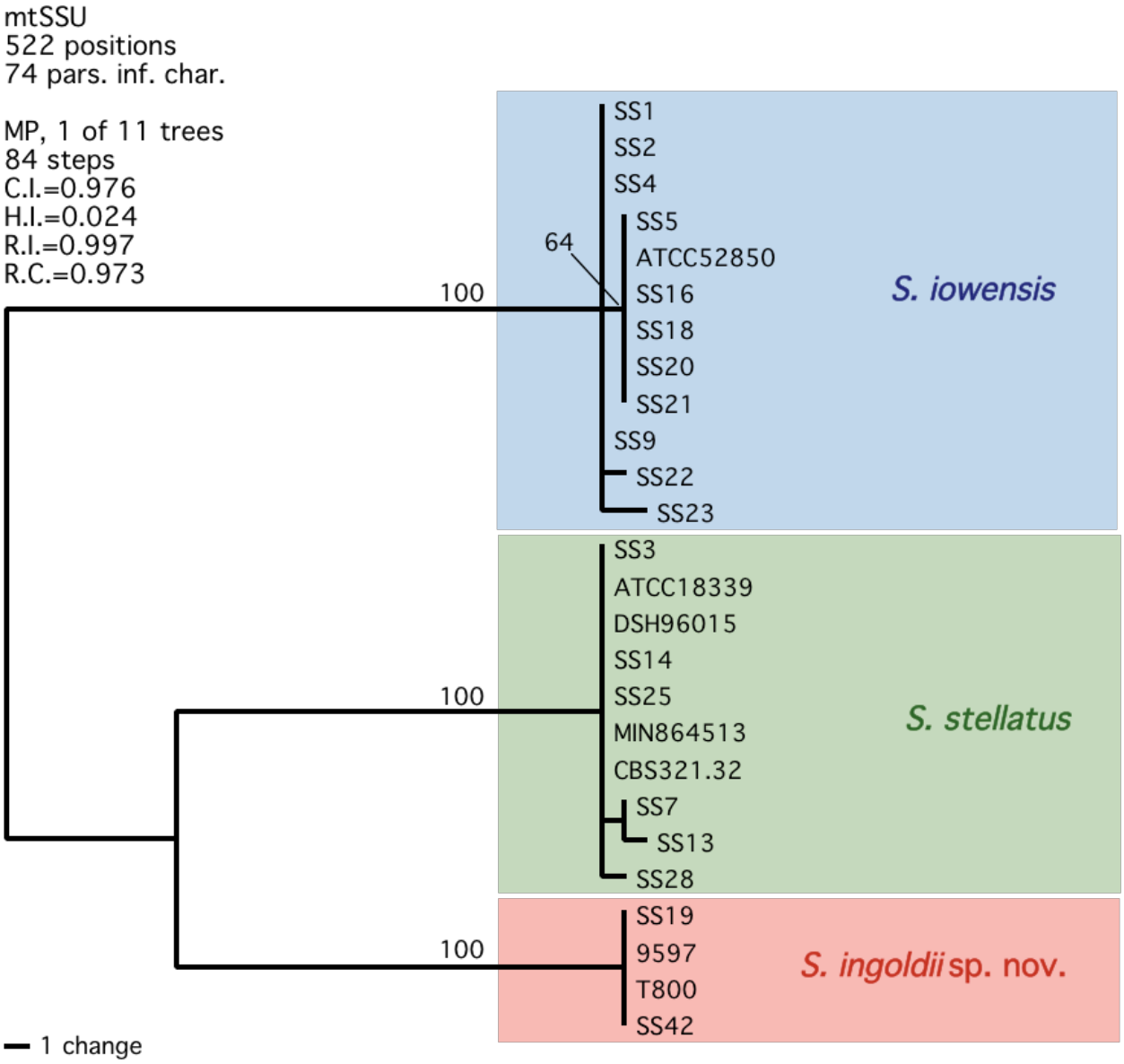

Fig. 2-2. Phylogram generated by MP analyses of the intrageneric mtSSU dataset. The tree is midoint rooted. Bootstrap values are indicated above the branches.

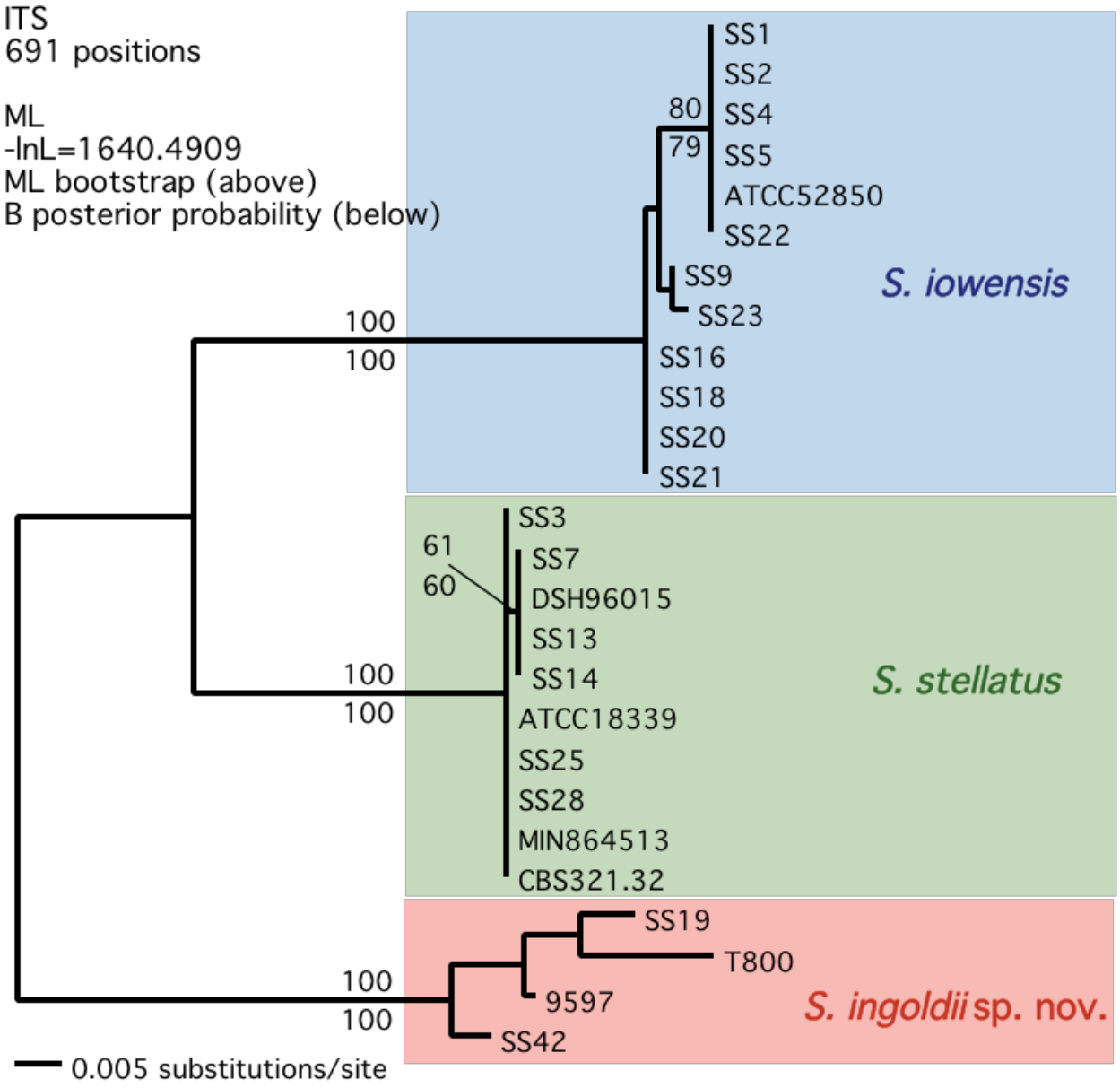

Fig. 2-3. Phylogram generated by ML analyses of the ITS dataset. The tree is midoint rooted. Bootstrap and posterior probability values are indicated above and below the branches, respectively.

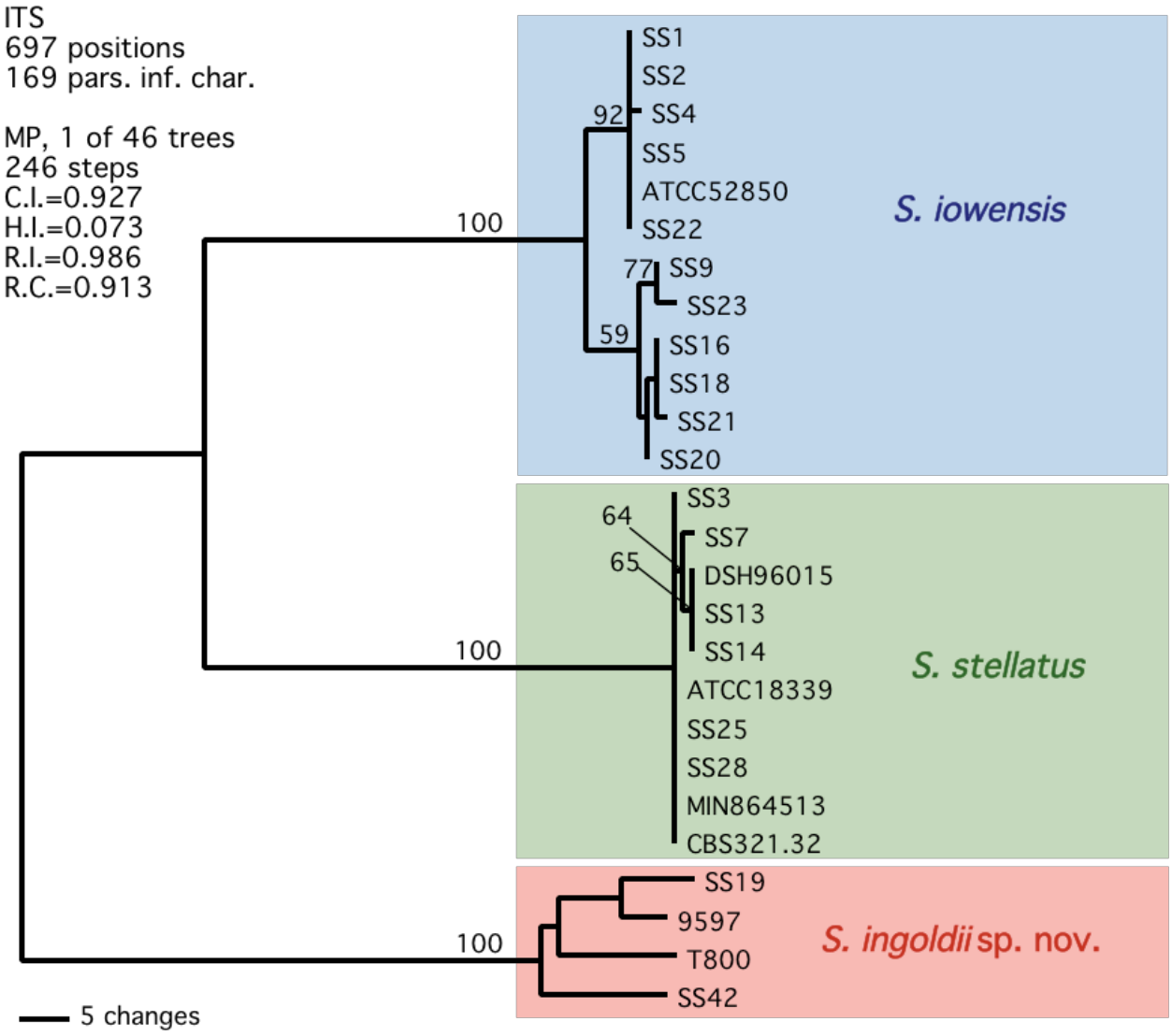

Fig. 2-4. Phylogram generated by MP analyses of the ITS dataset. The tree is midoint rooted. Bootstrap values are indicated above the branches.

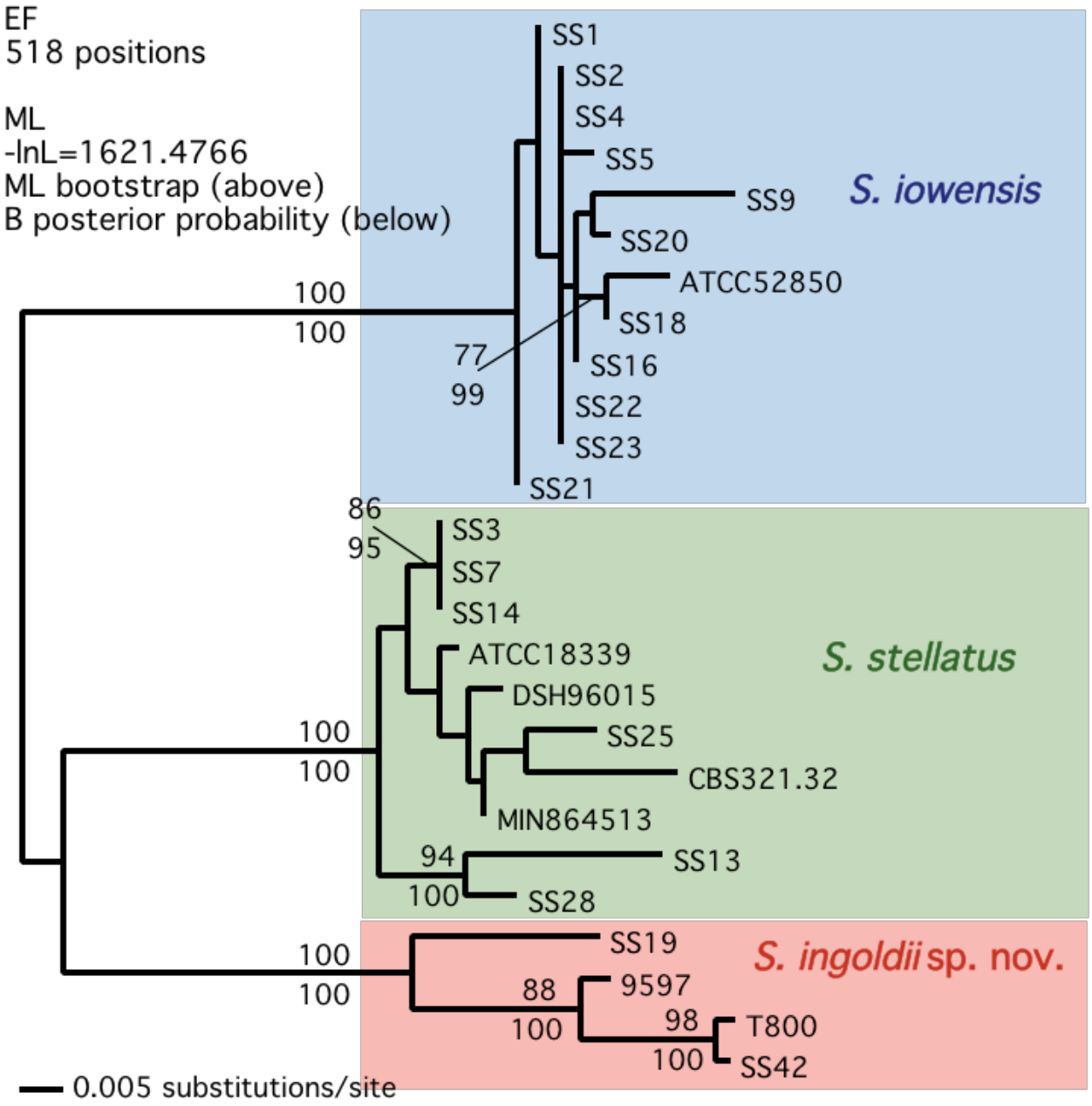

Fig. 2-5. Phylogram generated by ML analyses of the EF dataset. The tree is midoint rooted. Bootstrap and posterior probability values are indicated above and below the branches, respectively.

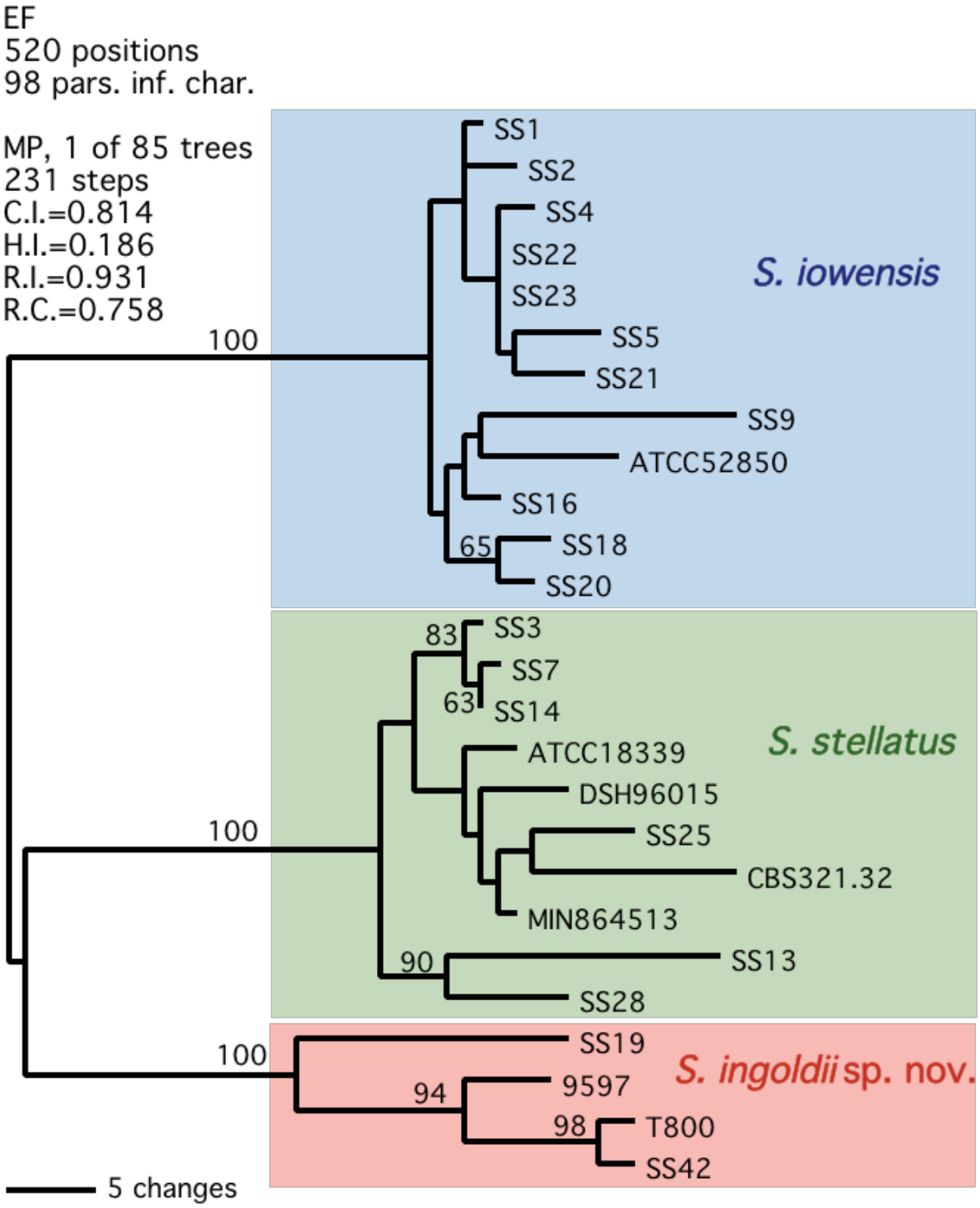

Fig. 2-6. Phylogram generated by MP analyses of the EF dataset. The tree is midoint rooted. Bootstrap values are indicated above the branches.

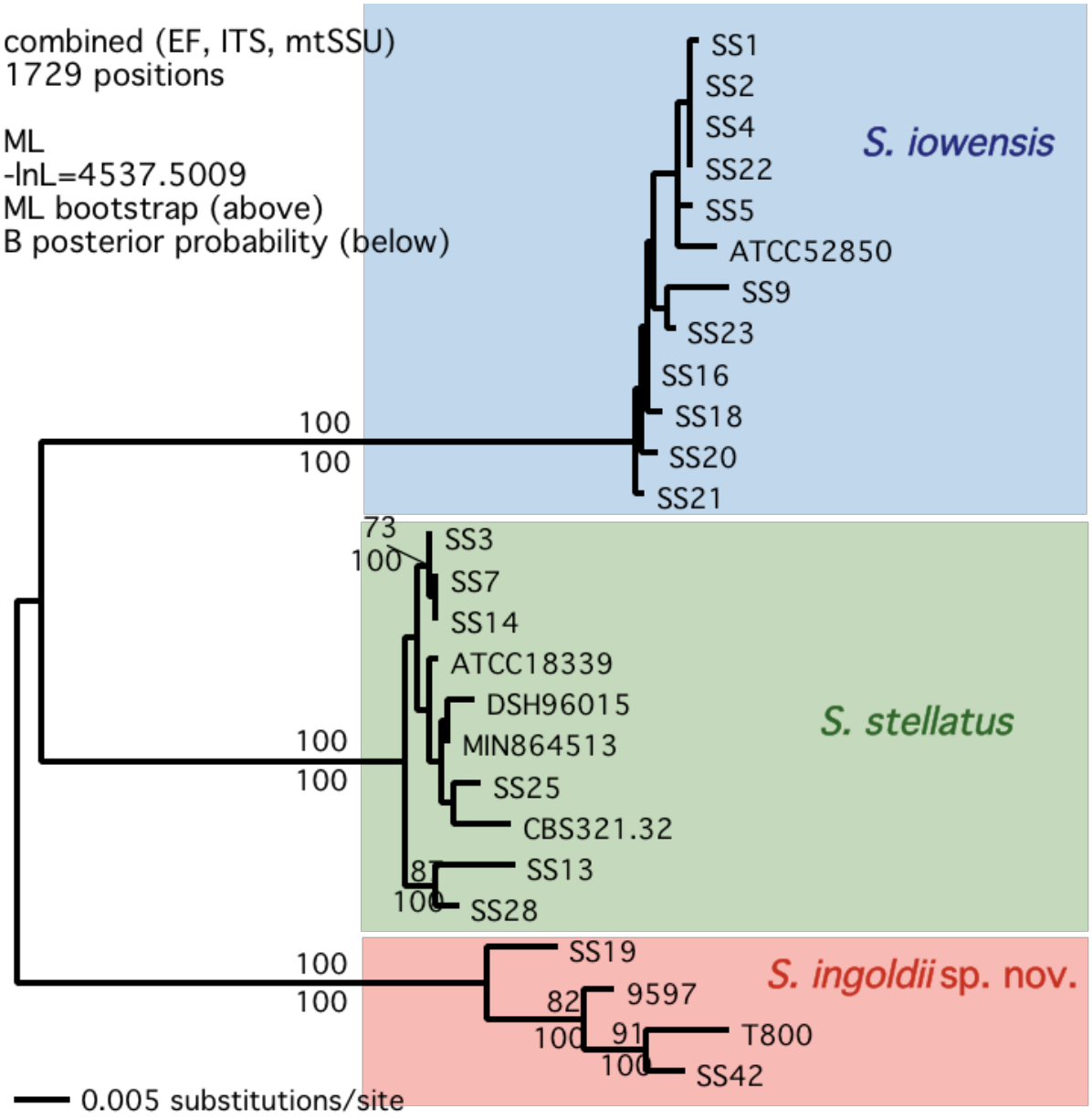

Fig. 2-7. Phylogram generated by ML analyses of the combined (mtSSU, ITS, EF) dataset. The tree is midoint rooted. Bootstrap and posterior probability values are indicated above and below the branches, respectively.

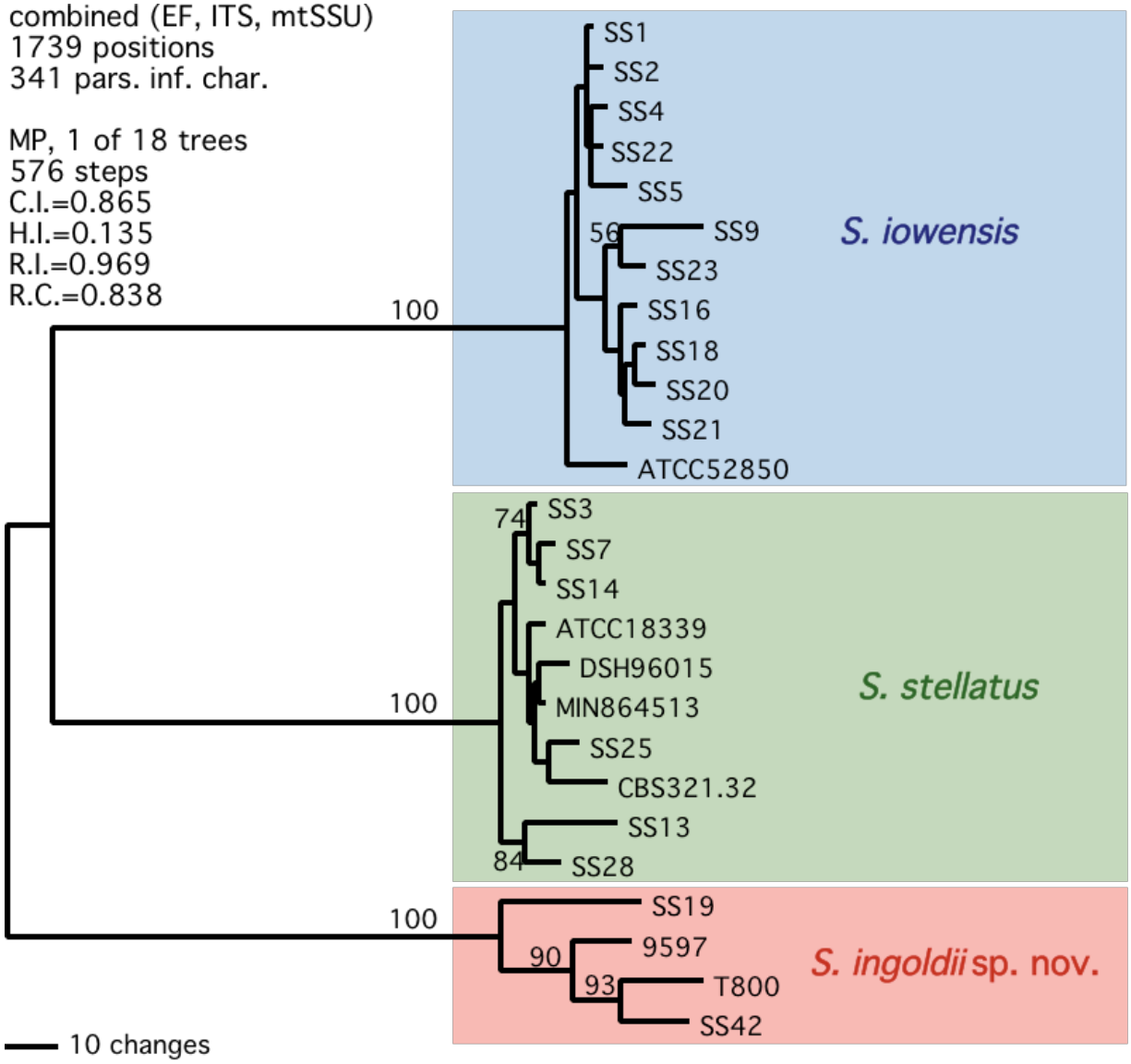

Fig. 2-8. Phylogram generated by MP analyses of the combined (mtSSU, ITS, EF) dataset. The tree is midoint rooted. Bootstrap values are indicated above the branches.

Suprageneric phylogenetic analyses

The mtSSU and LSU alignments of *Sphaerobolus* and related genera consisted of 601 and 577 characters, respectively, including gaps. 86 ambiguous positions were excluded from the mtSSU dataset, while the entire LSU region aligned well. The Hasegawa-Kishino-Yano model with no proportion of invariable sites (I=0) and estimated □-parameter of □-distribution (HKY +G) and the Tamura-Nei model with equal base frequencies, no proportion of invariable sites (I=0) and estimated □-parameter of □-distribution (TNef +G) were selected as the best-fit evolutionary models for the mtSSU and LSU datasets, respectively. Likelihood values of Bayesian analysis converged after about 9100 and 10200 generations in mtSSU and LSU datasets, respectively, and the consensus tree was computed from 910 and 899 trees after discarding the first 91 and 102 trees as "burn in". After including the character matrix of the ambiguous regions recoded by INAASE and ARC, the final alignment of mtSSU for MP analyses consisted of 543 characters. MP bootstrap cladograms for both datasets are shown in Fig. 4. ML, MP and Bayesian trees showed all *Sphaerobolus* isolates forming a monophyletic group, with strong bootstrap and posterior probability values in the mtSSU (88% [ML], 84% [MP], 100% [Bayesian]). However, in the LSU data, the monophyly of all *Sphaerobolus* isolates received weak support in all three analyses, the MP analysis giving the highest value of 60%. Within the genus *Sphaerobolus*, all of the three phylogenetic species received 100% support (ML, MP, and Bayesian) in the mtSSU analyses, while clades in the LSU tree, representing *S. iowensis*, *S. stellatus* and the undescribed taxon, were supported by bootstrap and posterior probability values of 88%, 99%, and 96% (ML), all 99%(MP) and all 100% (Bayesian),

respectively. Furthermore, *S. stellatus* and *S. ingoldii* prov. sp. nov. formed a subgroup with strong and moderate support in the mtSSU (83% [ML], 93% [MP], 99% [Bayesian]) and LSU (<50% [ML], 60% [MP], 81% [Bayesian]) trees.

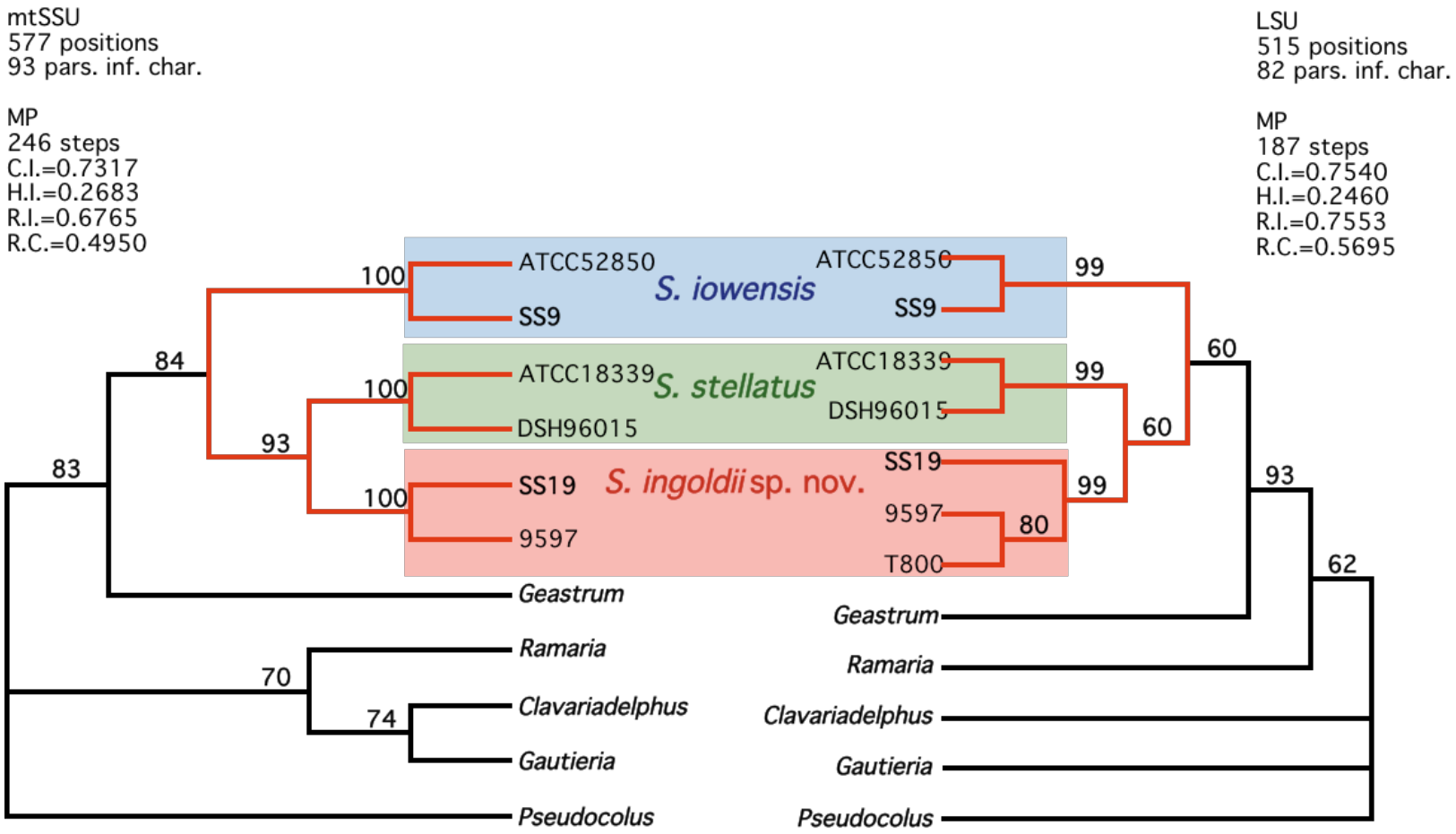

Fig. 2-9. Bootstrap cladograms of *Sphaerobolus* and related genera based on maximum parsimony analysis of the mtSSU and LSU rDNA datasets. The trees are rooted with *Pseudocolus* as outgroup. Bootstrap values are indicated above the branches.

Discussion

Data from gene genealogies from each locus, and the polymorphism and divergence analyses concordantly suggested three deeply divergent lineages corresponding to the taxa *S. iowensis*, *S. stellatus* and a newly discovered, undescribed species (see Chapter 3 for detailed discussion on morphology).

Suprageneric phylogenetic analyses of the mtSSU and LSU datasets containing representatives of related genera of the gomphoid-phalloid clade of Homobasidiomycetes indicated that the genus *Sphaerobolus* is monophyletic, and suggested that *S. ingoldii* prov. sp. nov. is more closely related to *S. stellatus* than to *S. iowensis* (Fig. 2-9). Levels of sequence divergence among *Sphaerobolus* species are similar to what is observed within morphologically diverse genera of basidiomycetes. Despite the small number of known species, values of pairwise HKY85 distances found in the first 900 bp in the 5' of the LSU of *Sphaerobolus* (5.35%, 5.38%, 8.12% between *S. stellatus* and *S. iowensis*, *S. iowensis* and *S. ingoldii* prov. sp. nov., and *S. stellatus* vs. *S. ingoldii* prov. sp. nov., respectively) are comparable to the greatest infrageneric distance values in many other homobasidiomycete genera (e.g., *Pleurotus purpureus* vs. *P. djamor* 10.62%, *Agaricus arvensis* vs. *A. bisporus* 5.72%, *Coprinopsis lagopides* vs. *Coprinopsis friesii* 10.79%; data generated by Moncalvo *et al.* 2002). While species pairs having such HKY85 distances are usually very distinct morphologically, the macromorphological differences are much less expressed in *Sphaerobolus*.

Some regions of the EF 1-α and ITS sequences were difficult to align between species, another indication of relatively deep divergence, whereas closely related species of fungi tend to have easily alignable EF 1-α and ITS sequences (Geiser *et al.* 2001, Geml and Royse 2002,

Geml *et al*. 2004, O'Donnell *et al*. 1998, Peintner *et al*. 2003). There may be a large number of unsampled taxa, extinct or extant, that would fill these phylogenetic gaps. In addition, no obvious sign of increased mutation rate was observed as unusually long branches in any of the genes examined in suprageneric phylogenetic analyses, confirming the ancient split between the lineages as the most likely explanation for the divergences observed between them.

The phylogenetic relationship of *S. ingoldii* prov. sp. nov. to the two other species is not clear. Results of suprageneric phylogenetic analyses suggest that *S. ingoldii* prov. sp. nov. likely is more closely related to *S. stellatus* than to *S. iowensis*, despite the fact that nucleotide differences between *S. iowensis* and *S. ingoldii* prov. sp. nov. were sometimes smaller than those between *S. stellatus* and *S. ingoldii* prov. sp. nov. Increasing the number of sampled and analyzed isolates in all species should clarify this question.

Chapter 3

Differences in basidiocarp morphology and colony characters associated with phylogenetic species of *Sphaerobolus*

Abstract

Despite mycologists' interest in its unique spore dispersal mechanism, systematic studies of the genus *Sphaerobolus* have received little attention. In our previous work, multiple gene genealogies indicated the existence of three deeply divergent lineages in the genus *Sphaerobolus*, each representing a phylogenetic species. Macro- and micro-morphological analyses of colony and fruiting body characters confirmed that these three phylogenetic species correspond to two known species: *S. iowensis* and *S. stellatus*, and *Sphaerobolus ingoldii* prov. sp. nov. Species can be distinguished based on colony morphology and growth rate on agar media, and the composition and morphology of cells within the gleba.

Introduction

Sphaerobolus was first documented nearly 300 years ago under the name *Carpobolus* (Micheli 1729). Since then researchers have studied its growth and reproduction (Walker and Anderson 1925, Walker 1927, Buller 1933, Alasoadura 1963, Ingold and Peach 1970, Ingold 1971, Ingold 1972, Dykstra 1982, Fletcher and Cooke 1984). Its common name "artillery

fungus" comes from its capability of ejecting a 1-mm diameter gleba up to 6 m toward the brightest light in its environment (Walker 1927, Buller 1933). In recent years, artillery fungi have become a source of distress to homeowners, landscape mulch producers and insurance companies due to the strong adhesion of the discharged gleba to artificial surfaces including house siding, cars, and windows (Lehman 1985, Brantley *et al*. 2001a, Brantley *et al*. 2001b).

Based on morphology, the genus *Sphaerobolus* has been classified as a member of the class Gasteromycetes along with other fungi having passive spore discharge, including bird's nest fungi (*Cyathus, Crucibulum* etc.), puffballs (*Lycoperdon, Langermannia* etc.), earth balls (*Scleroderma* etc.), stinkhorns (*Phallus, Mutinus, Pseudocolus* etc.), and earth stars (*Geastrum* etc.). Within this class, authors have placed artillery fungi into family Sphaerobolaceae in the following different orders: Nidulariales (Ulloa and Hanlin 2000); Sclerodermatales (Hawksworth *et al*. 1996). Based on molecular data, the genus *Sphaerobolus* is best placed in the gomphoid-phalloid clade with the following genera as closest relatives: *Geastrum, Phallus, Pseudocolus, Ramaria, Clavariadelphus, Gomphus, Gautieria* etc. (Hibbett *et al*. 1997, Moncalvo *et al*. 2002). These results are incorporated in the classification of Kirk *et al*. (2001), who placed *Sphaerobolus* in the family Geastraceae, order Phallales, class Basidiomycetes, phylum Basidiomycota.

Based on what is known about *S. stellatus*, the most widely studied of the three species, artillery fungi produce spherical, whitish or orange colored basidiocarps (1-2 mm in diameter), in which a single spore mass (gleba or peridiole) is formed. The gleba contains numerous spores of two types, uninucleate, thick-walled basidiospores and thin-walled, elongated gemmae (asexual spores) with two or more nuclei (Walker 1927, Ingold 1972). These two types play different roles in the dual survival mechanism as a lignicolous and as a coprophilous fungus, that greatly

increases the chance of survival of the fungus. Basidiospores are stimulated to germinate when exposed to proteolytic enzymes (e.g. pepsin) and relatively high temperatures (body temperature of mammal herbivores) (Dykstra 1982). This mechanism is elicited in the event that the gleba lands on a surface (e.g. a blade of grass) that is consumed by an herbivore. If no herbivore ingests the glebal mass, the gemmae can germinate on wood or other plant debris. A further back-up system to improve survival is the long term viability of the ejected spore mass, inferred from the fact that gleba have been reported to germinate after at least 11 years of storage (Walker 1927). Once germination occurs, colonization of the substrate begins. The characteristic bleaching associated with this white rotting fungus can be attributed to the digestion of cellulose, hemicellulose and lignin present within the substrate. Temperatures between 20 and 25 C are ideal for the fungus to grow vegetatively, but temperatures between 10 and 20 C are considered ideal for stimulating the fungus to produce reproductive structures (Alasoadura 1963). For this reason, artillery fungi are categorized as a cool season fungus. After colonizing the substrate for some time the fungus produces phototropic fruiting bodies. At maturity these fruiting bodies orient themselves towards the brightest light source and shoot their spore mass (gleba) into air (Nawaz 1967). Similar active/passive mechanisms for dispersing spore masses can be observed in other dung-inhabiting fungi as well, including Pezizomycetes (e.g. *Ascobolus*) and Sordariomycetes (e.g. *Podospora*) of the Ascomycota, and Mucorales of the Zygomycota (e.g. *Pilobolus*). This feature is likely an adaptation to the coprophilous lifestyle, and evolved multiple times independently during evolution.

The Greek origin of the name *Sphaerobolus* ("sphere thrower") is related to the ability of these fungi to propel their gleba a considerable distance. The genus contains two currently recognized species, *S. stellatus* (Tode) Pers. and *S. iowensis* Walker (Hawksworth *et al.* 1996),

that are distinguished by the micromophological characteristics detailed below. However, several other names of unknown origin exist in herbaria, including *S. bombardioides, S. carpobolus, S. corii, S. crustaceus, S. epigaeus, S. minimus, S. minutissimus, S. rubidus, S. sparsus,* and *S. tubulosus*. The origin of these binomials cannot be found in the literature, and thus cannot be considered scientifically valid. In the developing gleba of the young basidiocarps of *S. stellatus* some of the binucleate hyphae at the center of the globular knots have clavately enlarged ends; these are the beginnings of the first basidia (Walker 1927). These primary basidia become young basidium centers around which new basidia are formed, eventually producing four (occasionally eight) basidiospores. The basidia entirely break down and disappear as soon as the spores are mature, making room for the enlargement and maturation of other basidia. In *S. iowensis*, the development of primary basidia is followed by the formation of the characteristic cavities or chambers in which the basidiospores are produced (Walker 1927). In both species, in a nearly mature basidiocarp the sterile hyphae undergo disintegration and become broken down by the time the glebal mass is discharged.

Sphaerobolus is a cosmopolitan genus. It has been reported from Alaska, British Columbia, most parts of the continental U.S. (it is particularly abundant in the Northeast), Europe (from Greece to Iceland), Asia (including Japan), Australia, New Zealand, Africa, and Latin America (Aplin 1961, Dring 1964, Ingold 1972, Herrera and Perezsilva 1987, McKenzie and Foggo 1989, Halgrimsson *et al*. 1992, Zervakis *et al*. 1998, McGray pers. comm. 2002).

Materials and methods

Isolates and growing methods

Two isolates were selected from each phylogenetic species determined by the phylogenetic analyses for all morphological analyses (*S. ingoldii* prov. sp. nov.: T800, SS19; *S. iowensis*: SS5, ATCC 52850; *S. stellatus*: SS8, SS13, see details in Chapter 2). For basidiocarp production, isolates were grown on oatmeal agar (OA) (see Chapter 2) and wheat straw agar (WSA) that was made by placing approximately 20 g of dry, chopped, previously sterilized straw in 100 x 15 mm Petri plate and by pouring DIFCO® water agar (12 g agar per liter) on top of the straw. WSA was included in the fruiting experiments because preliminary studies showed that while *S. stellatus* and *S. ingoldii* prov. sp. nov. readily produce fruiting bodies on OA, all of the *S. iowensis* isolates consistently failed to fruit on this medium. Based on the findings of Flegler (1984) fruiting *S. iowensis* on wheat straw-based media, we adopted the wheat straw, although made the culture medium according to a different method. The cultures were inoculated with a colonized piece of agar and incubated in growth chambers at 25 C with an initial dark period of 3 weeks followed by 6 weeks of fluorescent lighting and lower temperature (16-18 C) in order to provide optimal fruiting conditions, a slight modification of methods described by Flegler (1984) and Ingold (1972). High (95-100%) relative humidity (RH) was maintained during the entire period. We used three replicates per treatment that were arranged according to a completely randomized design.

The growth rate experiments were conducted with the same set of isolates used for the fruiting experiments. Six replicates per isolate were inoculated with a colonized piece of agar in

the center, and were incubated in growth chamber. In addition, to OA and WSA, which were used in the fruiting experiments as well, DIFCO® potato-dextrose agar (PDA) was also used to analyze growth rates at 25 °C and 95-100% RH. The replicates were arranged according to a completely randomized design.

Collection and analysis of data

For morphological analyses, well-developed, unopened basidiocarps were sampled for microscopic analyses. Thin (10-20 μm) sections were sliced from the selected fruiting bodies using a cryotome (Model CTD International Harris Cryostat, International Equipment Company) with TBS Tissue Freezing Medium® (Triangle Biomedical Sciences, Durham, NC). Microscope slides were prepared with lactic acid and were examined using a Nikon® compound microscope to detect micro-morphological differences, including those described by Walker (1927) as distinctive characteristics between *S. stellatus* (Tode) Pers. and *S. iowensis* Walker (detailed in Chapter 1). Additonally, five mature basidiocarps were sampled from each culture for direct three dimensional examination using a dissection scope. The diameters of fifty expelled gleba attached to the Petri dish lid from each phylogenetic species (two isolates per species combined) were measured to detect any size differences. Five expelled gleba from each culture were also chosen for investigation using a Nikon® compound microscope to measure the sizes of basidiospores and to detect the presence of gemmae. Photographs were taken by a SONY® 3CCD Color Video Camera. The gleba diameter and basidiospore size data were analyzed using one-way analysis of variance (ANOVA) using Minitab 13 (Minitab Inc.). Where the null-hypothesis was rejected (i.e. the means are not all the same), the Fisher's *LSD* (Least Significant

Difference) multiple range test (Ott 1993) was used to detect significant differences by testing each mean to each mean.

Growth rate was recorded weekly by measuring two diameters of the colony at right angles to each other. The data were analyzed using one-way analysis of variance (ANOVA) using Minitab 13 (Minitab Inc.) to obtain information about the influence of genotype on growth. Where the null-hypothesis was rejected (i.e. the means were not all the same), the Fisher's *LSD* multiple range test was used to detect significant differences by testing each mean to each mean. Additionally, the cultures were examined macroscopically to detect and record any unique morphological characters.

Results

Basidiocarp and basidiospore characteristics

During the fruiting experiments, twenty-five gleba per isolate (fifty gleba per species) were measured. The only exception was *S. iowensis*, for which only one plate of ATCC 52850 isolate fruited, producing only eight basidiocarps. All gleba analyzed were produced on WSA, because of a lack of fruiting body production in *S. iowensis* on OA. Although *S. ingoldii* prov. sp. nov. and *S. stellatus* fruited abundantly on OA as well, *S. iowensis* failed to produce basidiocarps on OA, despite our repeated efforts. Therefore, this medium was excluded from the gleba and basidiospore morphology evaluation. Singificantly smaller ($P<0.001$) gleba were produced by *S. ingoldii* prov. sp. nov. (0.977±0.198 mm) than produced by the other two species

(*S. iowensis*: 1.549±0.156, *S. stellatus*: 1.495±0.289). Microscopical examinations revealed the existence of basidial chambers (Fig. 3-1), a distinct character used in the species description by Walker (1927), in the immature gleba of *S. iowensis*. Neither of the other species exhibited this trait (Fig. 3-2). We found significant differences in the lengths and widths of basidiospores produced (both $P<0.001$). The largest spores were produced by *S. ingoldii* prov. sp. nov. (8.780±0.761 × 5.874±0.583 μm), followed by *S. iowensis* (7.215±0.510 × 4.917±0.217 μm) and *S. stellatus* (7.266±0.498 × 4.571±0.288 μm). Basidiospores and gemmae of the species are shown on Fig.s 3-3, 3-4 and 3-5.

Growth rate differences

Growth rates of the three species on OA and WSA were significantly different (both $P<0.001$), but not on PDA ($P=0.113$). *Sphaerobolus ingoldii* prov. sp. nov. showed the fastest growth on both OA and WSA, with a daily linear growth of 2.095±0.036 mm and 2.089±0.029 mm, respectively. The growth rate of *S. iowensis* was 1.310±0.241 mm (OA) and 1.221±0.081 mm (WSA), while *S. stellatus* grew at a daily rate of 1.381±0.356 mm (OA) and 1.213±0.288 mm (WSA). Growth rates on PDA for *S. ingoldii* prov. sp. nov., *S. iowensis* and *S. stellatus* were 1.345±0.425 mm, 1.025±0.131 mm and 1.101±0.484 mm, respectively (Table 2). Examples of colony morphology of the three species on OA are shown in Fig.ures 3-6, 3-7, 3-8.

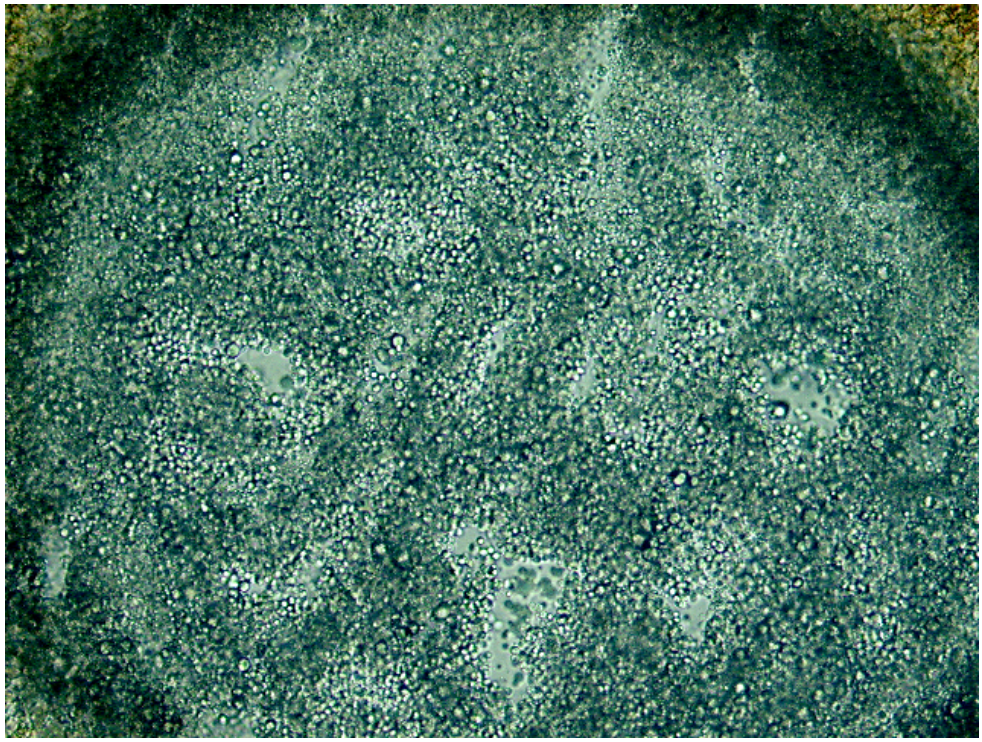

Fig. 3-1. Cross section of immature basidiocarp of *S. iowensis* showing the basidial chambers in the gleba.

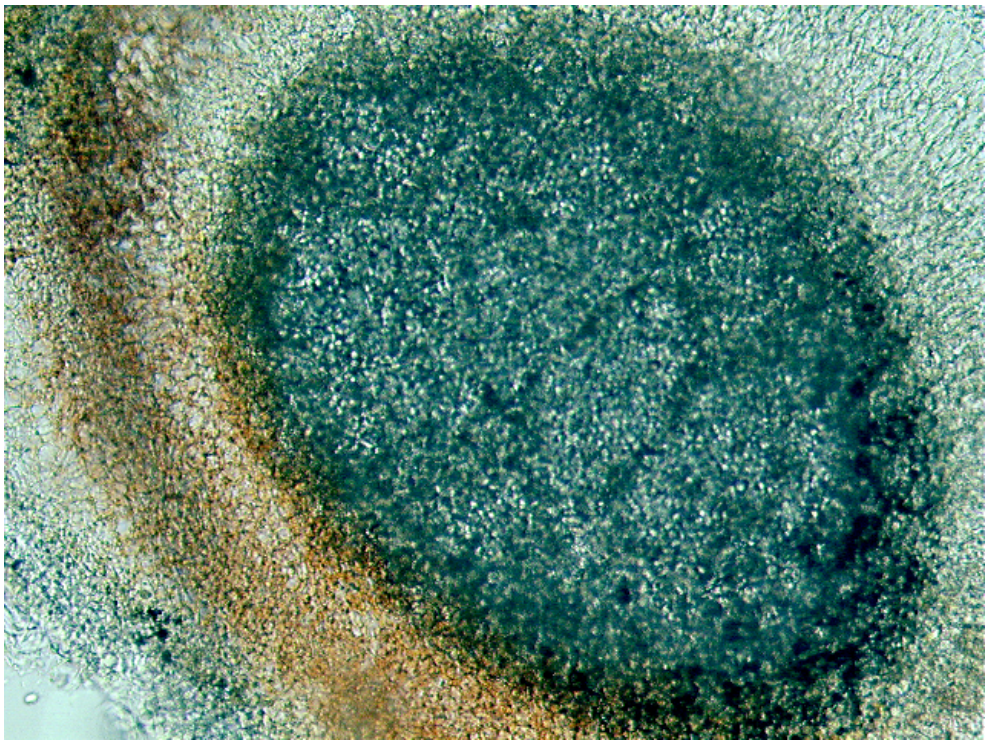

Fig. 3-2. Cross section of immature basidiocarp of *S. ingoldii* prov. sp. nov. showing the absence of basidial chambers in the gleba.

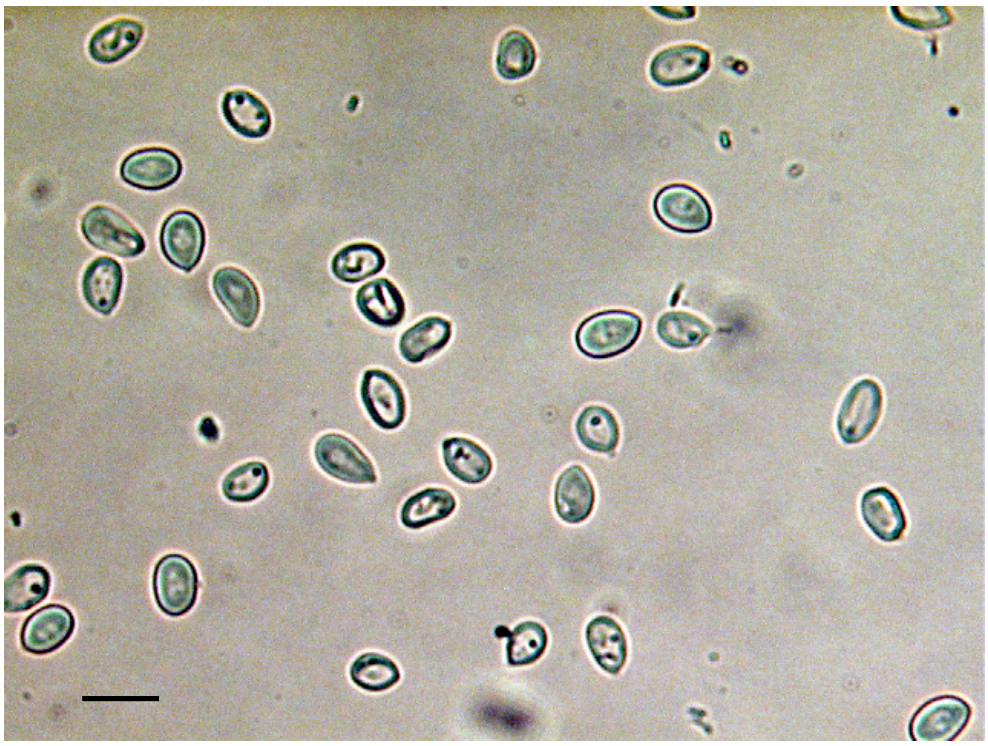

Fig. 3-3. Basidiospores of *S. ingoldii* prov. sp. nov. (T-800). The bar represents 10 μm.

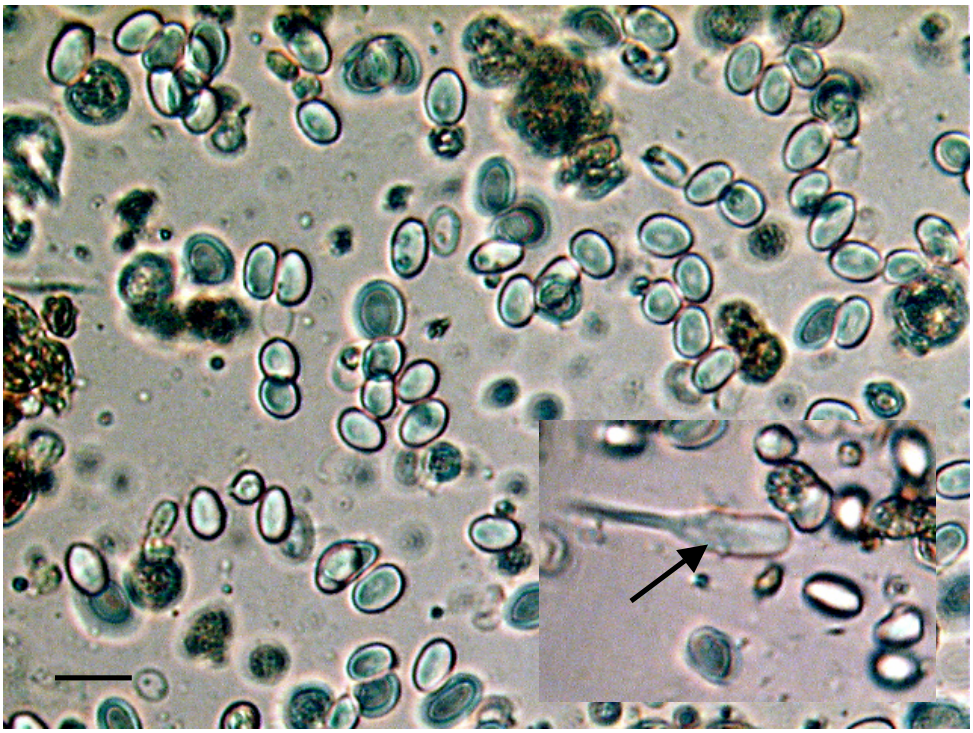

Fig. 3-4. Basidiospores and germinating gemmae (marked by an arrow in the inset) of *S. iowensis* (ATCC 52850). The bar represents 10 μm.

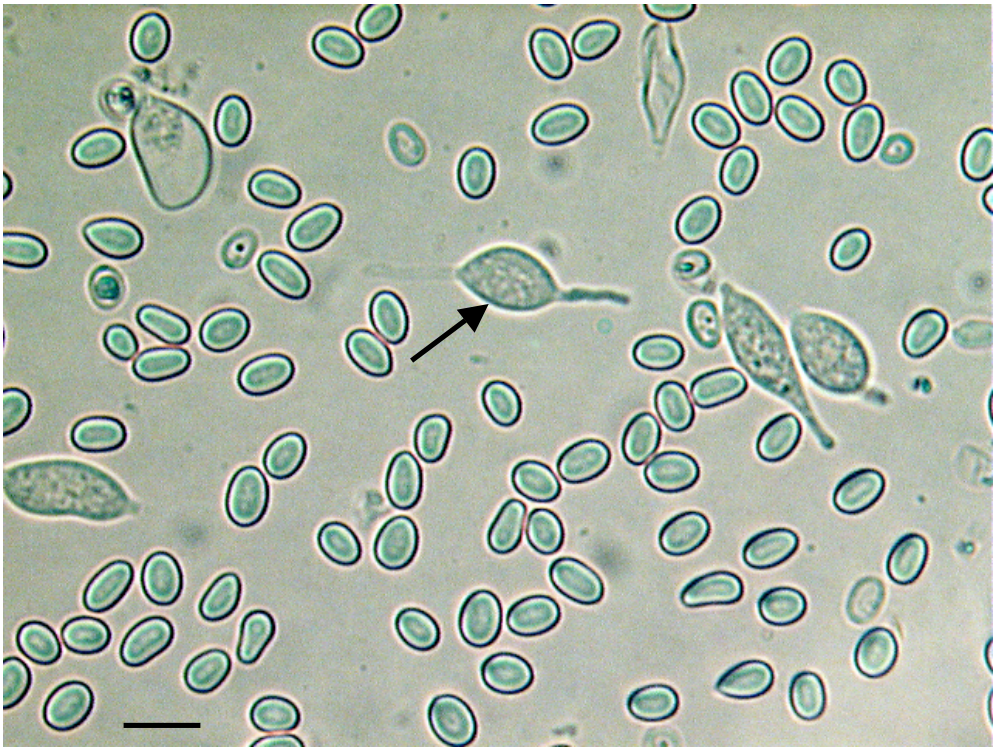

Fig. 3-5. Basidiospores and germinating gemmae (marked by an arrow) of *S. stellatus* (SS13). The bar represents 10 □m.

Fig. 3-6. Colony morphology of *S. ingoldii* prov. sp. nov. (SS19 and T-800) on OA.

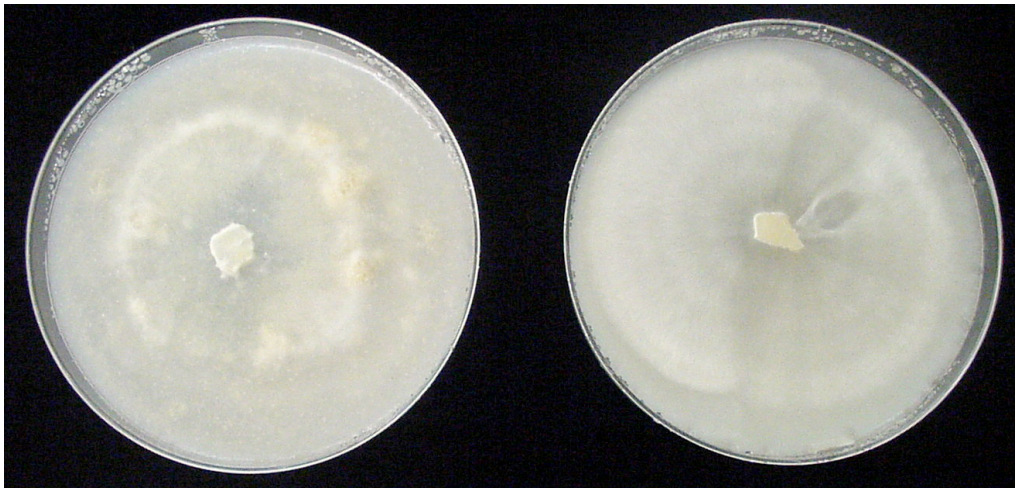

Fig. 3-7. Colony morphology of *S. iowensis* (SS5 and ATCC 52850) on OA.

Fig. 3-8. Colony morphology of *S. stellatus* (SS8 and SS13) on OA.

Table 3-1. Morphological characteristics of *Sphaerobolus* species. Mean value ± standard deviation is given for each character. Different letters in parentheses following the values indicate significant differences.

Character		***Sphaerobolus ingoldii* prov. sp. nov.**	***Sphaerobolus iowensis* Walker**	***Sphaerobolus stellatus* (Tode)Pers.**	***P*-value (ANOVA)**
Glebal structure	Basidial chambers	absent	present	absent	
	Gemmae	absent	present	present	
Gleba diameter (mm)	Sample size	N=50	N=8	N=50	
	Mean ± st. dev.	0.977±0.198 (a)	1.549±0.156 (b)	1.495±0.289 (b)	<0.001
Basidiospore length (μm)	Sample size	N=50	N=50	N=50	
	Mean ± st. dev.	8.780±0.761 (a)	7.215±0.510 (b)	7.266±0.498 (b)	<0.001
Basidiospore width (μm)	Sample size	N=50	N=50	N=50	
	Mean ± st. dev.	5.874±0.583 (a)	4.917±0.217 (b)	4.571±0.288 (b)	<0.001
Growth rate on OA (mm/day)	Sample size	N=12	N=12	N=12	
	Mean ± st. dev.	2.095±0.036 (a)	1.310±0.241 (b)	1.381±0.356 (b)	<0.001
Growth rate on WSA (mm/day)	Sample size	N=12	N=12	N=12	
	Mean ± st. dev.	2.089±0.029 (a)	1.221±0.081 (b)	1.213±0.288 (b)	<0.001
Growth rate on PDA (mm/day)	Sample size	N=12	N=12	N=12	
	Mean ± st. dev.	1.345±0.425 (a)	1.025±0.131 (a)	1.101±0.484 (a)	0.113 (N.S.)

Discussion

Morphological investigations, revealing several distinctive macro- and micro-morphological differences, confirmed the existence of the three species detected earlier in the phylogenetic analyses. *Sphaerobolus ingoldii* prov. sp. nov. differs from *S. iowensis* and *S. stellatus* in having smaller fruiting bodies and gleba, slightly larger basidiospores, no gemmae, faster mycelial growth rates on OA and WSA, and often cord-like mycelium on OA and WSA. We found very little difference in the measured morphological characters (none of which proved to be statistically significant) between *S. iowensis* and *S. stellatus*, in agreement with Walker (1927). However, the presence of basidial chambers in the premature gleba of *S. iowensis*, a distinct character used in the species description by Walker (1927), and the molecular data concordantly indicated the validity of these two species.

S. ingoldii prov. sp. nov. was found to produce only basidiospores, but no gemmae. Gemmae are assumed to play a crucial role in the lignicolous lifestyle of *Sphaerobolus*, because basidiospores were found not to germinate on wood (Ingold 1972). Since most of the isolates of *S. ingoldii* prov. sp. nov. were found on wood, this raises the question whether the basidiospores of *S. ingoldii* prov. sp. nov. in fact require the presence of proteolytic enzymes and high body temperature to stimulate germination as had been reported in *S. stellatus* (Dykstra 1982), or are able to germinate on wood and other plant debris directly after discharge. It is possible that the *in vitro* fruiting conditions were inappropriate for stimulating gemmae production, although gemmae were produced *in vitro* in *S. iowensis* and *S. stellatus*. Future investigations on the ecology of the genus should address this question, and study *S. iowensis* as well, since little is

known of the ecology of this latter species other than what is thought to be similar to *S. stellatus* based morphological comparisons.

Chapter 4

Biogeography of *Sphaerobolus* species in North America

Abstract

The genetic variation and biogeographic structure present within species of *Sphaerobolus* from different geographic areas were examined. Despite the considerable amount of DNA polymorhism found in all species, nested clade analyses of *S. iowensis* and *S. stellatus* indicated little phylogeographic structure in either species. The null hypothesis of no geographical association of haplotypes could not be rejected in most clades, suggesting either panmixia and/or extensive dispersal or inadequate geographical sampling; possibilities that cannot be distinguished with this method. Based on what is known about the ecology of the artillery fungi, a very likely explanation is the extensive dispersal, due to the dual coprophilous and lignicolous ecology of *Sphaerobolus* species, that provides many possible dispersal scenarios over great geographic distances. However, statistically significant genotype-geography associations were detected in one clade of each species, suggesting restricted gene flow due to isolation by distance for some isolates.

Introduction

Sphaerobolus is a cosmopolitan genus, containing two known and one newly described species: *S. iowensis* Walker, *S. stellatus* (Tode) Pers., and *S. ingoldii* prov. sp. nov., respectively (Hawksworth *et al.* 1996, and see Chapters 2 and 3 in this work). The distinct identities of these species have been confirmed in our previous work using both molecular and morphological data. Based on our current knowledge, only *S. stellatus* can be regarded as truely cosmopolitan having been reported from Alaska, British Columbia, most parts of the continental U.S. (it is particularly abundant in the Northeast), Europe (from Greece to Iceland), Asia (including Japan), Australia, New Zealand, Africa, and Latin America (Aplin 1961, Dring 1964, Ingold 1972, Herrera and Perezsilva 1987, McKenzie and Foggo 1989, Halgrimsson *et al.* 1992, Zervakis *et al.* 1998, McGray pers. comm. 2002). The other formerly described species, *S. iowensis*, is known only from North America, and previously was found in only two localities: Hunters, Iowa (type locality) and East Lansing, Michigan (Walker 1927, Flegler 1984). Although, *S. ingoldii* prov. sp. nov. has been found in various places, including the Kellogg Biological Station Long Term Ecological Research site, Michigan (type locality); Atlanta, Georgia; Sandusky, Ohio; Hershey, Pennsylvania and Osaka, Japan, its distribution in other areas of the world is unknown.

The artillery fungi are well-known for their unique spore dispersal mechanism, in which their fruiting bodies eject their adhesive gleba up to several meters. The gleba usually contain two types of spores, the sexual basidiospores and the asexual gemmae, that play different roles in the dual survival mechanism as a lignicolous and as a coprophilous fungus, greatly increasing the chance of survival (*S. ingoldii* prov. sp. nov. is likely an exception, since only basidiospores have been observed). Basidiospores are stimulated to germinate when exposed to proteolytic enzymes

(e.g. pepsin) and relatively high temperatures (body temperature of mammal herbivores) (Dykstra 1982). This mechanism is elicited in the event that the gleba lands on a surface (e.g. a blade of grass) that is consumed by an herbivore, and the basidiospores germinate in the dung. If no herbivore ingests the glebal mass, the gemmae can germinate on wood or other plant debris. A further characteristic to improve survival and dispersal is the long term viability of the ejected spore mass, inferred from the fact that gleba have been reported to germinate after at least 11 years of storage (Walker 1927).

This dual ecology is well suited not only for survival, but for dispersal as well. Since the artillery fungi recently have become a source of distress to homeowners, landscape mulch producers and insurance companies due to the strong adhesion of the discharged gleba to artificial surfaces (e.g. house sidings, cars, and windows) (Lehman 1985), recent works focused on the potential control measures inhibiting the growth of the fungus (Brantley *et al*. 2001a, Brantley *et al*. 2001b). The effectiveness of these and future control methods likely are influenced by the genetic diversity of artillery fungi communities to which the measures are appplied. Therefore, our goal was to evaluate the genetic variation present in *Sphaerobolus* samples from different geographic areas, the role of dispersal as a shaping force in the history of these populations, and to reveal any biogeographic structure within species. For this purpose, we evaluated intrspecific DNA polymorphism and conducted Nested Clade Analysis that had been used with a wide range of organisms for making inferences about both ongoing gene flow and historical processes that may have influenced population structure (e.g., Carbone and Kohn 2001, Masta *et al*. 2003, Schultheis *et al*. 2002).

Materials and methods

Isolates and DNA extraction

Eighty isolates were sampled in various parts of the U.S. or received from collections. Isolates were grown on oatmeal agar (OA) inoculated with a collected and surface sterilized gleba (as discussed in Chapter 2). Plates were incubated at room temperature. The DNA extraction, the PCR amplification and sequencing of the ITS region of nuclear rDNA were performed as described in Chapter 2.

Population phylogenetic analyses of artillery fungus species based on DNA sequences

The isolates obtained from the sampled communities were sorted based on their sequence data into phylogenetic species determined in Chapter 2. The overall frequency and distribution of the three phylogenetic species were determined. Biogeographical patterns linked to the different phylogenetic species were investigated. For this purpose, Nested Clade Analysis (NCA) (Templeton 1998) was used for statistical testing of the H_0 of no association between the haplotype and the geographic location. Maximum parsimony haplotype networks were generated by TCS v.1.13 (Clement *et al*. 2000). These haplotype trees were used to define a series of nested clades that in turn were used to perform random, two-way, contingency permutation analysis to detect any association between geographic distribution and genetic variation (Templeton 1998). The nested clade information, sample size for each haplotype, and

geographical location of each clade (latitude and longitude coordinates) were entered into the software package GeoDis v.2.0 (Posada *et al*. 2000). GeoDis was used to calculate clade dispersion (D_c) and clade displacement (D_n), and to test them for significance at the 5% level using permutation technique with 1000 resampling replicates (Posada *et al*. 2000). Clade dispersion (D_c) was calculated as the average distance of all individuals in clade X from the geographical center of that clade, while clade displacement (D_n) was the average distance of individuals in clade X from the geographical center of clades of the next highest nesting level. Where significant D_c and/or D_n values were detected, a set of criteria were used to discriminate between the effects of contemporary (e.g. gene flow) and historical (e.g. allopatric fragmentation, range expansion) processes (Posada *et al*. 2000, Templeton 1998).

Measures of intrspecific DNA polymorphism

The number of polymorphic sites and their distribution among species was determined for ITS sequence data generated ITS. Within species, nucleotide variability was measured using θ, the proportion of polymorphic sites in a sample (Watterson, 1975), and π, the average number of nucleotide differences among sequences in a sample (Nei and Li, 1979). Tajima's *D* (Tajima, 1989) and Fu and Li's *D** and *F** (Fu and Li, 1993) test statistics were calculated to test for departures from the neutral theory of molecular evolution (Kimura, 1983). The neutral theory predicts that θ and π (Tajima's *D*) or *h* and *S* (the number of singleton mutations and the number of segregating sites, compared by Fu and Li's statistics) should estimate the same parameter, $4N_e\mu$, under the neutral model. Because these tests assume that populations are in both mutation-drift and migration-drift equilibrium, significant values may indicate that the populations

experienced past population growth, were previously subdivided or that sequences are not evolving in a neutral manner. Measures of variation and the tests for neutrality were performed with the computer program DnaSP v. 3.51 (Rozas and Rozas, 1999).

Results

Nested Clade Analyses

Of the 80 isolates included in the analyses, 48 were *S. iowensis*, 27 *S. stellatus*, and 5 *S. ingoldii* prov. sp. nov. according to the ITS-based identification method. The geographic distribution of the samples is shown in Figure 4-1. Since only 5 representatives of *S. ingoldii* prov. sp. nov. were available, only *S. iowensis* and *S. stellatus* were included in the Nested Clade Analyses.

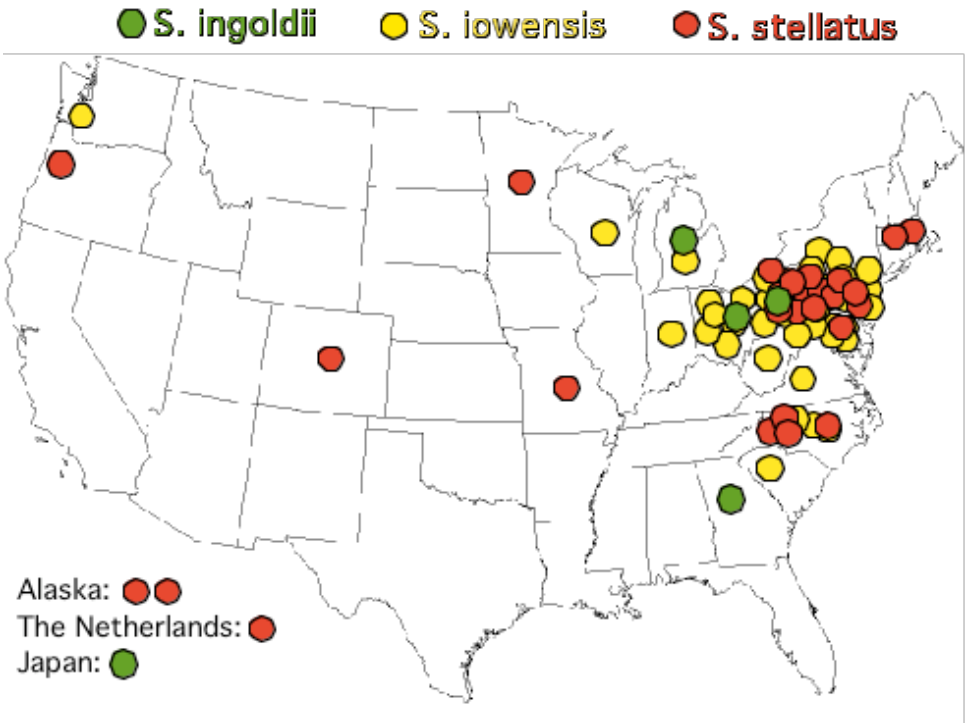

Fig. 4-1. The geographic distribution of *Sphaerobolus* isolates sampled.

The nested haplotype network of *S. iowensis* comprised of 35 zero-step clades (haplotypes), 28 one-step clades, 15 two-step clades, 6 three-step clades, 3 four-step clades, and the entire network (Fig. 4-2). The missing intermediate haplotypes (represented by black dots in the Figure) were retained during the nesting procedure for consistency in nesting (Crandall 1996). Haplotype "0" was by far the most numerous and was found in 9 individual samples from diverse locations, therefore it was considered the ancestral haplotype in the sample. Clade 4-1, showing the widest continuous distribution, contains the ancestral haplotype. The null hypothesis of no association between genotype and geographic origin could not be rejected at P=0.05 level in none of the clades, except in Clade 4-3 (tip), where significantly small clade distance (D_c) and nested clade distance (D_n) values were observed, P=0.007 and P=0.006, respectively. Based on the inference key (steps: 1. Yes; 2. Yes; 3. No; 4. No) of Templeton (1998), the significant

statistical association between haplotype and geography is due to restricted gene flow with isolation by distance. The inference key steps were the followings:

1. Are there any significant values for D_c, D_n, or I-T (interior to tip clades) within the clade? **Yes** (significant D_c values).
2. Are the D_c values for tip or some (but not all) interior clades significantly small or is the I-T D_c distance significantly large? **Yes** (significantly small D_c values).
3. Are any D_n and/or I-T D_n values significantly reversed from the D_c values, and/or do one or more tip clades show significantly large D_n values or interior clades significantly small D_n values or I-T significantly small D_n with corresponding D_c values being nonsignificant? **No**.
4. Do the clades (or two or more subsets of them) with restricted geographical distributions have ranges that are completely or mostly nonoverlapping with the other clades in the nested group (particularly interiors), and does the pattern of restricted ranges represent a break or reversal from lower level trends within the nested series (applicable to higher-level clades only)? **No**.

The distribution and frequency of *S. iowensis* haplotypes are shown in Table 4-1.

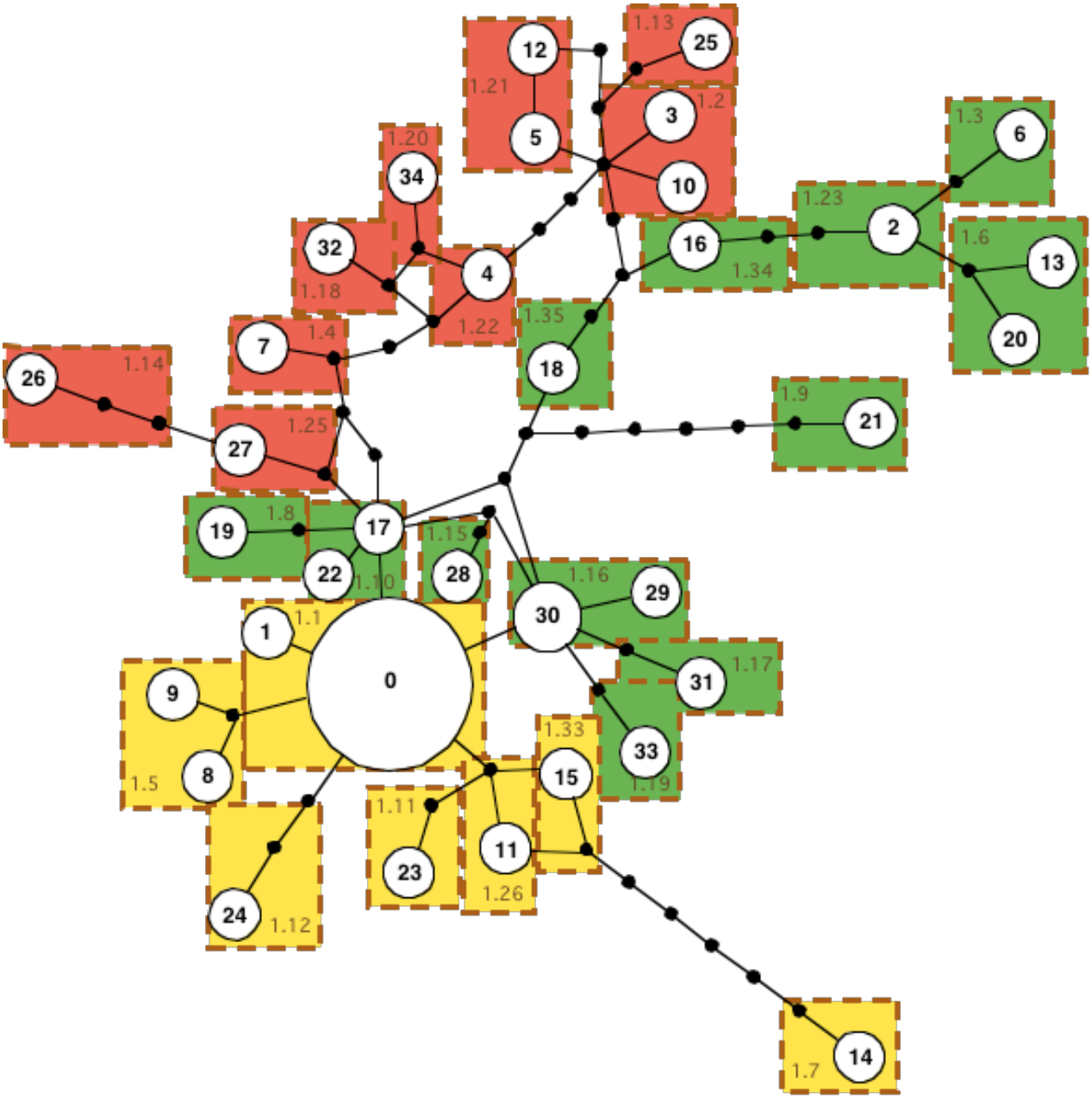

Fig. 4-2. ITS haplotype network of *S. iowensis* showing sampled haplotypes (white circles), unsampled, inferred haplotypes (black circles), 1-step clades (numbered rectangles) and highest-level nesting (4-step) clades (colored groups). Size of circles refers to the frequency of haplotypes.

Table 4-1. Geographic distribution, frequency, and GenBank accession numbers for sequences of *S. iowensis* haplotypes.

Haplotype	Isolate code	Geographic origin	GenBank accession number
0	ATCC 52850	East Lansing, Michigan	AY487958
	SS1	Indiana	
	SS2	Elizabethtown, Pennsylvania	
	SS5	State College, Pennsylvania	
	SS22	Ithaca, New York	
	SS37	Elderton, Pennsylvania	
	SS75	State College, Pennsylvania	
	SS100	Blythewood, South Carolina	
	SS113	Berkeley Springs, West Virginia	
1	SS4	Langhorne, Pennsylvania	AY487953
2	SS9	Chapel Hill, North Carolina	AY487956
3	SS16	Olney, Maryland	AY487962
	SS17	Olney, Maryland	
	SS18	Olney, Maryland	
4	SS20	Olney, Maryland	AY650228
5	SS21	Galion, Ohio	AY487967
6	SS23	Medina, Ohio	AY487969
7	SS110	Durham, North Carolina	AY650229
8	SS111	Durham, North Carolina	AY650230
9	SS116	Absecon, New Jersey	AY650231
10	SS34	Centralia, WA	AY650232
11	SS35	Branchville, NJ	AY650233
12	SS38-2	Blairsville, Pennsylvania	AY650234
13	SS39-1	Elderton, Pennsylvania	AY650235
14	SS43	Ridgway, Pennsylvania	AY650236
15	SS47-1	Hayward, Wisconsin	AY650237
16	SS48	Ebensburg, Pennsylvania	AY650238
17	SS49	Kresgeville, Pennsylvania	AY650239

18	SS51	Indiana, Pennsylvania	AY650240
19	SS56	Indiana, Pennsylvania	AY650241
20	SS57	State College, Pennsylvania	AY650242
21	SS65-2	Clyde, Pennsylvania	AY650243
22	SS69	Indiana, Pennsylvania	AY650244
23	SS73	Newport, Pennsylvania	AY650245
24	SS80-1	Hancock co., West Virginia	AY650246
25	SS82	Imperial, Pennsylvania	AY650247
26	SS83-1	Steubenville, Ohio	AY650248
27	SS83-3	Steubenville, Ohio	AY650249
28	SS84	Moon, Pennsylvania	AY650250
29	SS86-2	Belmont, Ohio	AY650251
30	SS89-1	Plum Creek, Pennsylvania	AY650252
	SS94	Cleveland, Ohio	
31	SS89	Plum Creek, Pennsylvania	AY650253
32	SS90	Altoona, Pennsylvania	AY650254
33	SS96	Morris, New Jersey	AY650255
34	SS99	Lambsburg, Virginia	AY650256

The nested cladogram of *S. stellatus* comprised of 12 zero-step clades (haplotypes), 9 one-step clades, 4 two-step clades, and the entire cladogram (Fig. 4-3). The missing intermediate haplotypes were treated in the same manner detailed earlier. Similarly, haplotype "0" was the most numerous and was found in 10 individual samples and was considered the ancestral haplotype in the sample. Clade 2-1, showing the widest overall distribution, contains the ancestral haplotype. As in *S. iowensis*, the null hypothesis of no association between genotype and geographic origin could not be rejected at P=0.05 level in none of the clades, except in Clade 1-2 (tip), where significantly small clade distance (D_c) and nested clade distance (D_n) vaues were observed, P=0.032 and P=0.014, respectively. Based on the inference key (steps: 1. Yes; 2. Yes;

3. No; 4. No) detailed earlier, the significant statistical association between haplotype and geography is due to restricted gene flow with isolation by distance, similar to that observed in *S. iowensis*. The distribution and frequency of *S. stellatus* haplotypes are shown in Table 4-2.

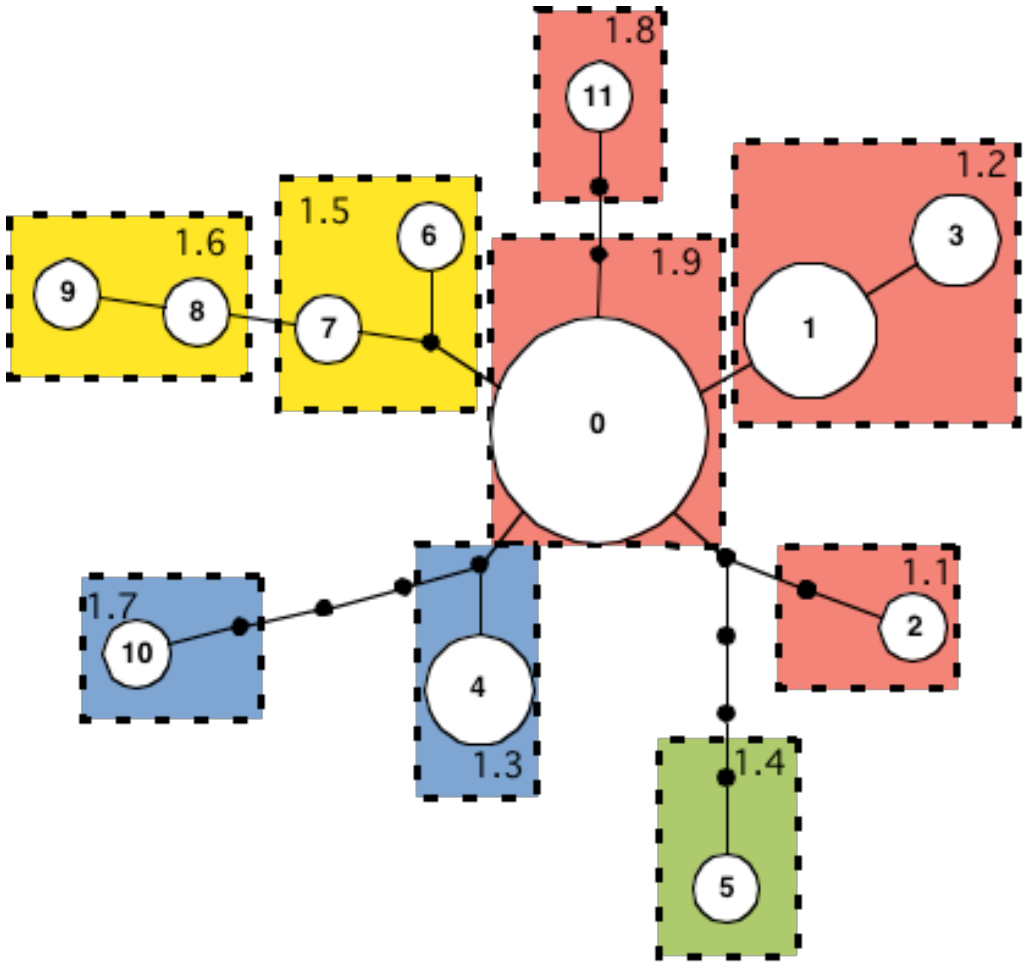

Fig. 4-3. ITS haplotype network of *S. stellatus* showing sampled haplotypes (white circles), unsampled, inferred haplotypes (black circles), 1-step clades (numbered rectangles) and highest-level nesting (2-step) clades (colored groups). Size of circles refers to the frequency of haplotypes.

Table 4-2. The distribution and frequency of *S. stellatus* haplotypes.

Haplotype	Isolate code	Geographic origin	GenBank accession number
0	ATCC 18339	Maryland	AY487957
	CBS 321.32	The Netherlands	
	MIN 864513	Elm Creek Nature Reserve, Minnesota	
	SS3	State College, Pennsylvania	
	SS25	Newton Centre, Massachusetts	
	SS28	Anchorage, Alaska	
	SS41	White River National Forest, Colorado	
	SS72	Shrewsbury, Pennsylvania	
	SS95	Poplar Bluff, Missouri	
	SS115	Harrisburg, Pennsylvania	
1	DSH 96-015	Great Brook State Park, Massachusetts	AY487959
	SS13	Erie, Pennsylvania	
	SS14	Lucinda, Pennsylvania	
	SS92	Kennett Square, Pennsylvania	
2	SS88	Martinsburg, Pennsylvania	AY650257
3	SS62	Holidaysburg, Pennsylvania	AY650258
	SS66	Cresson, Pennsylvania	
4	SS46	Mifflintown, Pennsylvania	AY650260
	SS63	Duncansville, Pennsylvania	
	SS65	Clyde, Pennsylvania	
5	SS59	Corvallis, Oregon	AY650259
6	SS7	West Mifflin, Pennsylvania	AY487955
7	SS112-1	Winston-Salem, North Carolina	AY650261
8	SS112-2	Winston-Salem, North Carolina	AY650262
9	SS112-3	Winston-Salem, North Carolina	AY650263
10	SS107	Durham, North Carolina	AY650264
11	SS27	Anchorage, Alaska	AY487972

DNA polymorphism

ITS sequences of *Sphaerobolus* species varied between 694-711 bp in length, excluding gaps (Table 4-3). There were 26, 28, and 12 segregating sites in the *S. ingoldii* prov. sp. nov., *S. iowensis* and *S. stellatus* intraspecific datasets, respectively. The highest values of the average number of nucleotide differences (θ) and the proportion of polymorphic sites (π) per site were observed in *S. ingoldii* prov. sp. nov., followed by *S. iowensis* and *S. stellatus*. Neither Tajima's *D* nor Fu and Li's *D** and *F** differed significantly from zero in any species, as expected under the neutral theory and in a population in equilibrium. However, near-significant ($0.1>P>0.05$) Fu and Li's *D** and *F** values and Tajima's *D* values were observed in *S. iowensis* and *S. stellatus*, respectively. Significantly negative values would indicate the possibility of non-neutral evolution of this locus or recent growth in population size.

Table 4-3. Nucleotide polymorphism in the ITS region of *S. ingoldii* prov. sp. nov., S. *iowensis,* and *S. stellatus*. Values of the average number of nucleotide differences ($\hat{\theta}$) and the proportion of polymorphic sites (θ) are given per site with variance estimates in parentheses. Tajima's *D*, Fu and Li's *D** and *F** statistics were calculated to test departure from neutrality.

Locus/ species	No. of sequences	Total sites	Segregating sites	$\hat{\theta}$ (per site)	θ (per site)	Tajima's D	Fu and Li's *D**	Fu and Li's *F**
S. ingoldii	5	694	26	0.01729 (1.3×10^{-5})	0.01798 (1.2×10^{-5})	-0.28656	-0.28656	-0.30826
S. iowensis	48	679	28	0.00522 (3×10^{-7})	0.00934 (3×10^{-6})	-1.47107	-2.08707[††]	-2.22311[††]
S. stellatus	27	711	12	0.00218 (3×10^{-7})	0.00438 (1.6×10^{-6})	-1.67678[††]	-1.25562	-1.61781

[†]Significant, $P<0.05$ (none of the test statistics)
[††]Not significant, but $0.1>P>0.05$

Discussion

While considerable amount of DNA polymorhism was found in all species, haplotype networks, in agreement with gene genealogies detailed in the phylogenetic part, indicated little phylogeographic structure in both species. In numerous instances, isolates from the same geographic region appeared in divergent portions of the networks, while isolates from geographically distant localities often clustered together. Geographic distribution of the highest-level nesting clades of *S. iowensis* and *S. stellatus* are shown in Fig. 4-4 and Fig. 4-5, respectively. The null hypothesis of no geographical association of haplotypes could not be rejected in most clades, suggesting either panmixia and/or extensive dispersal or inadequate geographical sampling, possibilities that cannot be distinguished with this method. However, it is worth noting that the dual coprophilous and lignicolous ecology of *Sphaerobolus* species provides many possible dispersal scenarios over great geographic distances. In ancient times animal migration might have been a considerable way of dispersal, occasionally supplemented with the natural movements of rotten wood and other plant debris, for example during the floodings of rivers. However, in modern times human activities likely play the major role in the dispersal. Such activities include the transportation of commercial wood products and livestock between distant areas.

The statistically significant results obtained in one clade of each species suggests restricted gene flow due to isolation by distance for some isolates. Because restricted gene flow implies only limited movement by individuals during any given generation, it takes time for a newly arisen haplotype to spread geographically. When a mutation first occurs, the resulting new

haplotype is found only in its area of origin, and it often remains within the geographical range of its ancestors for many generations under the isolation-by-distance model. As the ancestral haplotype is older than its mutational descendents, it usually has a wider geographic distribution under this model. This means that there is a strong tendency under restricted gene flow for tip clades to have a geographical range smaller and often nested within the range of the clades immediately interior to them. In addition, because the ancestral haplotype is expected to be most frequent near its site of origin, most mutational derivates will also occur in that area. This means that the geographic centers of all the clades nested together should be close, therefore, the clade distances and nested clade distances should show similar patterns under restricted gene flow. Interestingly, even though most clades of both *S. iowensis* and *S. stellatus* did not show statistically significant genotype-geography association, the clade distance and nested clade distance values were in fact similar in most cases, supporting the hypothesis of restricted gene flow among at least some of the isolates.

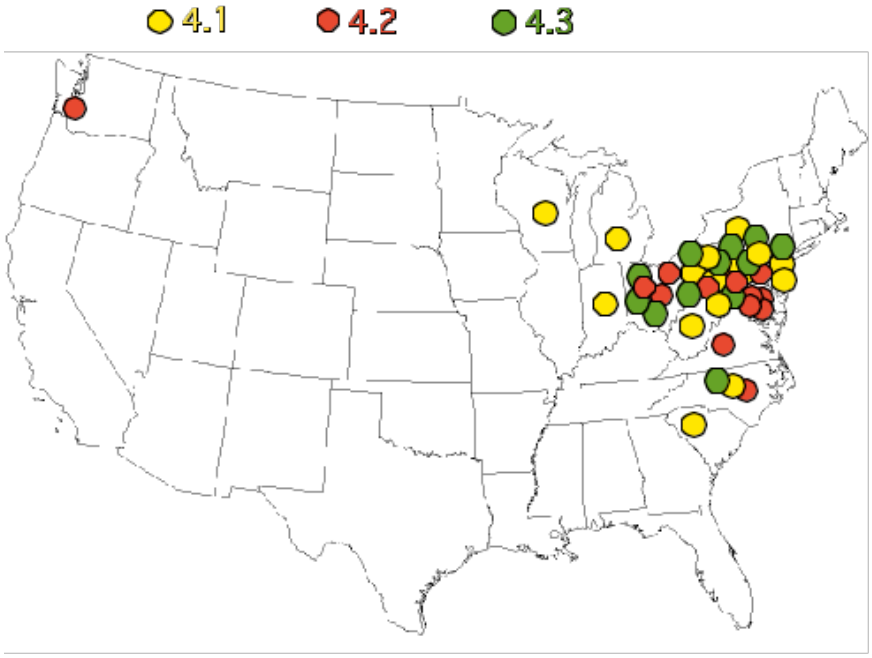

Fig. 4-4. Geographical distribution of the highest-level nesting clades of *S. iowensis*

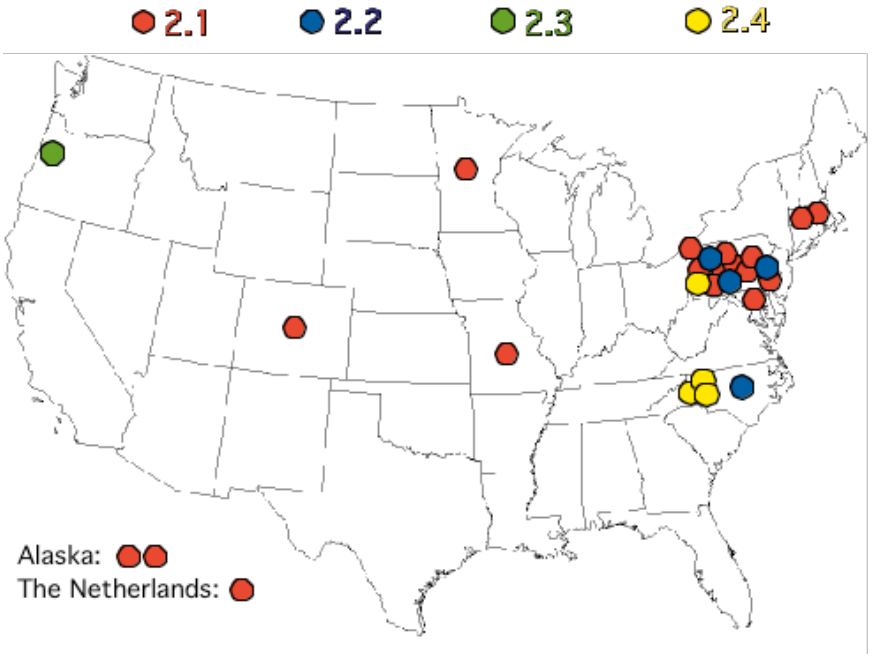

Fig. 4-5. Geographical distribution of the highest-level nesting clades of *S. stellatus*.

Chapter 5

Differences in the influence of selected fungicides on *in vitro* growth of artillery fungi (*Sphaerobolus* spp.)

Abstract

In our work reported herein we tested the inhibitory effect of various fungicides on the *in vitro* growth of representatives of all known artillery fungus species, namely *S. ingoldii* prov. sp. nov., *S. iowensis*, and *S. stellatus*. Fungicides were tested at two concentrations: 5 and 20 ppm. Opus®, Topsin M 70WP®, triphenyltin acetate, Difolatan®, and Terraguard 50W® were the most effective inhibitors of growth for all three *Sphaerobolus* species; reduction in growth was directly related to fungicide concentration. Five fungicides showed varying results, depending on fungal species and fungicide concentration. Four fungicides showed no significant inhibition on these artillery fungi. Although this preliminary study provided interesting results, potential fungicides must be tested in the field and registered by E.P.A. before recommendations can be made to homeowners who want to minimize adverse effects of artillery fungi.

Introduction

Artillery fungi are white-rotting wood-decay basidiomycetes, that are often found on rotten wood, for example decomposing landscape mulch. The genus *Sphaerobolus* contains three

described species: *S. iowensis* Walker, *S. stellatus* (Tode) Pers., and *S. ingoldii* prov. sp. nov. (Hawksworth *et al.* 1996, Geml *et al.* 2003, and see Chapters 2 and 3 in this work). Artillery fungi produce spherical, whitish or orange colored basidiocarps (1-2 mm in diameter), in which a single spore mass (gleba or peridiole) is formed. These fruiting bodies are phototropic and at maturity they orient themselves towards the brightest light source and shoot their gleba into air (Nawaz 1967). Prior to ejection, the hyphae in the glebal region begin to decompose and coat the gleba (Dykstra 1982, Walker 1927). This amorphous cellular debris is responsible for the adhesive nature of the gleba. The spore mass is expelled when the inner layer of the fruiting body suddenly inverts. This action can propel the gleba up to 2 meters high and over 6 meters distance (Bullers 1933). Temperatures between 20 and 25 C are ideal for the fungus to grow vegetatively, but temperatures between 10 and 20 C are considered ideal for stimulating the fungus to produce reproductive structures (Alasoadura 1963). For this reason, artillery fungi are categorized as a cool season fungus.

Due to the above-mentioned discharge mechanism, and to the fact that artillery fungi are commonly found on landscape mulch, property owners recently have expressed concern about finding an abundance of brown-black spherical masses (expelled gleba) on surfaces such as windows, cars, and structural sidings (Akina 2000, Brantley *et al.* 2001a, Brantley *et al.* 2001b, Lehman 1985). This occurrence is damaging because the gleba are extremely difficult to remove when dry, and they permanently stain surfaces (Akina 2000, Brantley *et al.* 2001a, Brantley *et al.* 2001b). Mulch producers, distributors, and landscape maintenance firms have been asked by customers to pay for the repainting of siding or vehicles, or replace siding stained by the fungus. Lawsuits and numerous insurance claims have been filed as a result of damage to property (Akina 2000).

As a possible solution, Brantley *et al*. (2001a) successfully used *Trichoderma* and *Bacillus* species *in vitro* as biological control agents against artillery fungi. Numerous other microorganisms with biological control potential have been reported to be present in several composted waste products, e.g. spent mushroom substrate (SMS) that is readily available throughout Pennsylvania (Cronin *et al*. 1996, Hoitink and Grebus 1994, Yohalem *et al*. 1996).

An alternative control method is prevention, i.e. the use of certain types of landscape mulch that do not encourage growth and sporulation of artillery fungi. Brantley *et al*. (2001b) reported great variations in sporulation between the different types of mulch, specifying pine bark, Atlantic cedar and cypress as substrates that did not encourage sporulation. Brantley *et al*. (2001b) also observed that sporulation on bark mulches was significantly less than on wood, and even in mixed mulches, artillery fungi usually grew on the wood pieces rather than on the bark. Although mulch products with the highest C:N ratio produced low numbers of gleba, Brantley *et al*. (2001b) concluded that the C:N ratio is not useful indicator by itself to estimate the ability of mulch products to support colonization and sporulation by artillery fungi.

Although the above-mentioned control strategies showed promising results, recently, there has been an increasing popular demand by homeowners for fungicides effective against artillery fungi (Davis pers. comm. 2002). In addition, earlier papers have been published only on the control of *S. stellatus*, while no report exists in the literature on the potential control methods for *S. iowensis* and *S. ingoldii* prov. sp. nov.. Since no research has been published on chemical control of *Sphaerobolus*, our goal was to evaluate the effect of selected fungicides on the in vitro growth of all known species of *Sphaerobolus*.

Materials and methods

Isolates and experimental design

Two isolates were selected from each phylogenetic species determined by the phylogenetic analyses for all morphological analyses (*S. ingoldii* prov. sp. nov.: T800, SS19; *S. iowensis*: SS5, ATCC 52850; *S. stellatus*: SS8, SS13, see details in Chapter 2). Fourteen fungicides, thirteen of which previously found to be effective against basidiomycetes (Royse pers. comm. 2002), were selected for the experiment (Table 5-1). Isolates were grown in Petri plates, on DIFCO® potato-dextrose agar (PDA) (Becton Dickinson Microbiology Systems, Sparks, MD) containing 5 ppm and 20 ppm of one of fourteen fungicides or no fungicide at all (control). The 20 ppm concentration has been found sufficient to control growth in *Agaricus*, a basidiomycete (Royse pers. comm. 2002). Each plate was inoculated with a small piece of agar plug (4 mm in diameter) colonized by the fungus, then sealed with Parafilm® (American National Can, Chicago, IL) in order to prevent dehydration. Three replicates were used for each treatment, arranged according to completely randomized design. Plates were incubated in growth chambers for 21 days at 25 °C, 95-100% relative humidity, and in complete darkness, providing ideal environmental conditions for the fungus to grow.

Collection and analysis of data

Colony diameters were recorded after 21 days by measuring two diameters of the colony at right angles to each other. The measured values of the two isolates were combined for each species. In the preliminary analyses the data were analyzed using two-way analysis of variance (ANOVA) for the whole dataset, using "species" as factor 1, and "fungicide treatment" as factor 2. Since significant *P*-values were obtained not only for both main effects, but for the interaction as well, therefore, this method was not used any further. The explanation is that in any given two-way ANOVA table, where rows represent the variables of factor 1 and columns the variables of factor 2, in absence of interaction in the model, it makes sense to look for overall row effects since they describe the differences between row levels regardless of the column level. Similarly, for column effects. If interaction is present in the model, it is not appropriate to talk about simple row effects because the row effects are column specific. Therefore, I analyzed the data in two separate analyses, both by one-way ANOVA: (1) comparing growth rate values of a certain isolate for all treatments (including the fungicide-free control) to obtain information about the influence of treatments on growth; (2) comparing growth rate values of a certain treatment for all isolates to obtain information about the influence of genotype on growth (and perhaps resistance levels). In analysis 1, after rejecting the null-hypothesis (colony diameter means are not all the same), two independent methods, the Dunnett's comparison and Fisher's Least Significant Difference (*LSD*) multiple range test (Ott 1993), were used to detect significant differences between the fungicide treatments and the control with no fungicide. A family error rate (the probability of making one or more type I errors for the entire set of comparisons) were used in both methods to control the rate of type I error. Results were presented as a set of confidence intervals for the difference between the mean of the control level and the other factor level means. I used the intervals to determine whether the means were different. When an

interval did not contain zero, there was a statistically significant difference between the corresponding means. In analysis 2, Fisher's *LSD* test was used to detect significant differences between species for any given treatment. Since neither species was assigned as control in agreement with the nature of the question to be answered, the Dunnett's comparison could not be used.

Table 5-1. Common and chemical names, and concentration of active ingredients (% A.I.) of fungicides tested in this study.

Name	Chemical name	% A.I.
Euparen M® (tolylfluanid)	*N*-dichlorofluoromethylthio-*N*',*N*'-dimethyl-*N*-*p*-tolylsulfamide	99
Demosan® (chloroneb)	1,4-dichloro-2,5-dimethoxybenzene	65
Fuberidazole	2-(2'-furyl)benzimidazole	99
Triphenyltin acetate	triphenyltin acetate	96
Terraguard 50W® (triflumizole)	[[4-chloro-2(trifluoromethyl)phenyl]imino]-2-Propoxy-ethyl-1H-imidazole	50
Glyodin	2-heptadecyl-2-imidazoline acetate	98
Difolatan® (captafol)	*N*-(1,1,2,2-tetrachloroethylthio)cyclohex-4-ene-1,2-dicarboximide	99
Karathane® (dinocap)	2,4-dinitro-6-octylphenyl crotonates	99
Folpet	*N*-(trichloromethylthio)phthalimide	99
Dazomet	3,5-dimethyl-1,3,5-thiadiazinane-2-thione	98
Ferbam	iron(3+) dimethyldithiocarbamate	98
Opus® (epoxiconazole/naphta)	(2*RS*,3*SR*)-1-[3-(2-chlorophenyl)-2,3-epoxy-2-(4-fluorophenyl)propyl]-1*H*-1,2,4-triazole	28
Bravo Zn® (chlorothalonil/zinc oxide)	2,4,5,6-tetrachloroisophthalonitrile	40.4
Topsin M 70WP® (thiophanate-methyl)	dimethyl 4,4'-(*o*-phenylene)bis(3-thioallophanate)	70

Results

Comparing fungicide treatments to the control

As expected, one-way ANOVA of the fungicide treatments showed highly significant *P*-values ($P<0.001$) in every species. When conducting Dunnett's comparison and Fisher's *LSD* multiple range test, many treatments differed significantly from the no-fungicide control in each species. Out of the fourteen fungicide, four four fungicides (triphenyltin acetate, Opus®, Bravo Zn®, and Topsin M 70WP®) significantly ($P<0.05$) inhibited growth of all three species of the artillery fungus and at both concentrations (Table 5-2). Interestingly, Bravo Zn® only moderately (yet, significantly) inhibited all three fungus species, with no significant difference between growth at the two concentrations. Moderate growth inhibition was observed in all species using 5 ppm triphenyltin acetate and Topsin M 70WP®; treatment efficacy greatly increased to almost full inhibition at 20 ppm in all three species. Opus® significantly inhibited growth at both 5 and 20 ppm, although the 5 ppm treatment resulted in less growth inhibition in all three fungus species.

Among fungicides exhibiting a wide range of inhibition, 5 ppm of Difolatan® showed virtually no inhibition of growth, while 20 ppm greatly inhibited growth in all three species of the artillery fungus. Similar results were obtained for glyodin and Terraguard 50W®, where all of the 20 ppm and some of the 5 ppm treatments resulted in significant growth inhibition, although the difference between the growth values for the two concentrations was not as great as with Difolatan®. The Euparen M®, Demosan®, and fuberidazole fungicides gave various results, showing moderate growth inhibition in every treatment, with only a few effects being

significant at 20 ppm and/or 5 ppm. Four of the 14 fungicides (Karathane®, folpet, dazomet, and ferbam) did not significantly inhibit growth of any artillery fungus species at either concentration, as compared to the control.

Comparing the growth rate of different species within fungicide treatments

The one-way ANOVA of the growth rates of different species of the artillery fungus showed highly variable *P*-values (from $P<0.001$ to $P=0.729$) when compared within the fungicide treatments (including the no-fungicide control). An example of the difference among colony growths at the different treatment is shown in Fig.ure 5-1. In those cases where growth rates significantly differed among species, we were able to identify which fungus species differed significantly from the rest using Fisher's *LSD* multiple range test. Since the growth of all species did not differ from each other in the control ($P=0.729$), we were able to compare the values among species directly without normalizing the data for each species relative to control values.

Among species, the 5 ppm Euparen M® treatment resulted in significantly less growth of *Sphaerobolus ingoldii* prov. sp. nov. than *S. iowensis* and *S. stellatus*, which in turn did not differ significantly from each other. In the 20 ppm triphenyltin acetate treatment, *S. iowensis* showed significantly less growth rate than *Sphaerobolus ingoldii* prov. sp. nov., while *S. stellatus* did not differ significantly from either species. Within the 20 ppm Terraguard 50W®, *S. iowensis* and *S. stellatus* did not differ significantly from each other, however, both species grew significantly less than *Sphaerobolus ingoldii* prov. sp. nov. *Sphaerobolus stellatus* showed the smallest growth rate on 20 ppm glyodin with value that was statistically different from both *Sphaerobolus*

ingoldii prov. sp. nov. and *S. iowensis*. Similarly, the growth of *S. stellatus* was significantly less than that of *S. iowensis* and *Sphaerobolus ingoldii* prov. sp. nov. in 20 ppm folpet treatment, although it was not statistically different from the *S. stellatus* control.

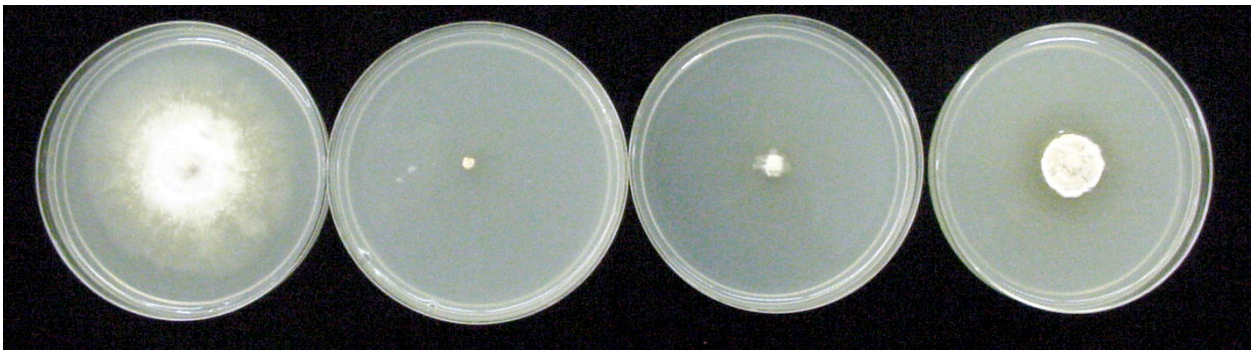

Fig. 5-1. Colony diameters of *Sphaerobolus ingoldii* prov. sp. nov. (T-800) showing different growth rates on PDA containing different fungicide treatments after 3 weeks at 25 C. The first plate is the no-fungicide control.

Interestingly, although ferbam was not significantly different from the control in the earlier analyses, the growth rates differed significantly among species at both 5 and 20 ppm. In both cases, *S. iowensis* and *Sphaerobolus ingoldii* prov. sp. nov. grew at the fastest rate, with no significant difference, while *S. stellatus* grew significantly slower. The fungicide Opus® was highly effective in all three species, yet it was somewhat less effective in *Sphaerobolus ingoldii* prov. sp. nov., showing significant difference from *S. iowensis* and *S. stellatus* both at 5 and 20 ppm. In contrast, Bravo Zn® proved to be most effective against *Sphaerobolus ingoldii* prov. sp. nov., that showed significantly slower growth rate than *S. iowensis*, while *S. stellatus* grew at a statistically similar rate between the other two species. Topsin M 70WP® was effective against all three species, showing greater inhibition at 20 than at 5 ppm. Although a significant difference was observed in *Sphaerobolus ingoldii* prov. sp. nov. compared to the other two species, this was likely an artifact of the extremely small standard deviation values in both *S. iowensis* and *S. stellatus*.

Table 5-2. Influence of selected fungicides on *in vitro* growth of *Sphaerobolus* species. Mean value ± standard deviation for colony diameter after 21 days of growth at 25 °C are given for each treatment. The cultures were inoculated with colonized agar plugs 4 mm in diameter, therefore 4 mm colony diameter refers to no growth.

Fungicide		**Colony diameter (mm) of species**			**ANOVA *P*-value**
Trade name (common name)	**Concentration (ppm)**	***Sphaerobolus ingoldii* prov. sp. nov.**	***Sphaerobolus iowensis***	***Sphaerobolus stellatus***	**Species vs. species**
Euparen M® (tolylfluanid)	5	33.92 ± 21.16^{x}*a*	56.83 ± 8.64*b*	49.92 ± 22.61*b*	0.015^{z}
	20	40.67 ± 21.47	59.67 ± 9.07	42.83 ± 28.29	0.069
Demosan® (chloroneb)	5	39.25 ± 25.09^{x}	55.67 ± 10.34	41.33 ± 27.49	0.162
	20	33.08 ± 24.39^{x}	43.83 ± 6.91^{xy}	32.50 ± 23.90	0.312
Fuberidazole	5	47.42 ± 32.09	57.58 ± 7.82	39.92 ± 33.06	0.287
	20	34.58 ± 29.86^{x}	47.25 ± 12.51^{x}	37.58 ± 30.65	0.461
Triphenyltin acetate	5	14.25 ± 7.35^{xy}	14.25 ± 3.08^{xy}	11.50 ± 4.76^{xy}	0.360
	20	9.25 ± 1.96^{xy}*a*	6.42 ± 0.90^{xy}*b*	8.00 ± 2.86^{xy}*ab*	0.008^{z}
Terraguard 50W® (triflumizole)	5	38.50 ± 20.40^{x}	47.50 ± 4.76^{x}	40.08 ± 29.93	0.543
	20	29.92 ± 2.50^{xy}*a*	10.75 ± 2.42^{xy}*b*	10.00 ± 6.55^{xy}*b*	<0.001^{z}
Glyodin	5	47.25 ± 14.96	47.75 ± 6.33^{x}	33.42 ± 26.13	0.096
	20	36.92 ± 11.60^{x}*a*	29.42 ± 8.84^{xy}*a*	18.83 ± 13.86^{xy}*b*	0.002^{z}
Difolatan® (captafol)	5	60.75 ± 17.79	56.67 ± 11.15	47.25 ± 27.91	0.257
	20	9.25 ± 2.26^{xy}	8.59 ± 2.11^{xy}	8.25 ± 3.28^{xy}	0.636
Karathane® (dinocap)	5	56.33 ± 16.71	56.83 ± 12.66	44.42 ± 29.54	0.272
	20	45.67 ± 13.88	55.17 ± 6.65	45.42 ± 25.13	0.291
Folpet	5	46.42 ± 16.05	61.33 ± 16.47	45.17 ± 25.73	0.102
	20	56.67 ± 14.39*a*	60.75 ± 16.60*a*	40.50 ± 25.73*b*	0.026^{z}

Dazomet	5	62.25 ± 15.39	51.42 ± 13.24	43.58 ± 28.54	0.091
	20	42.08 ± 23.54	58.17 ± 8.63	44.25 ± 22.07	0.101
Ferbam	5	58.42 ± 12.92*a*	56.50 ± 7.50*a*	37.42 ± 31.81*b*	0.029[z]
	20	52.42 ± 15.05*a*	53.08 ± 7.15*a*	33.58 ± 28.18*b*	0.025[z]
Opus® (epoxiconazole/ naphta)	5	14.92 ± 4.19[xy]*a*	4.42 ± 0.79[xy]*b*	4.83 ± 1.95[xy]*b*	<0.001[z]
	20	7.33 ± 3.17[xy]*a*	4.00 ± 0.00[xy]*b*	4.00 ± 0.00[xy]*b*	<0.001[z]
Bravo Zn® (chlorothalonil/ zinc oxide)	5	16.83 ± 3.38[xy]	31.08 ± 10.76[xy]	28.08 ± 24.67[x]	0.078
	20	15.33 ± 7.34[xy]*a*	37.58 ± 18.07[xy]*b*	30.00 ± 27.19[x]*ab*	0.025[z]
Topsin M 70WP® (thiophanate-methyl)	5	24.25 ± 10.52[xy]	30.08 ± 7.93[xy]	20.08 ± 18.82[xy]	0.194
	20	4.25 ± 0.45[xy]*a*	4.00 ± 0.00[xy]*b*	4.00 ± 0.00[xy]*b*	0.036[z]
Control (no fungicide)		53.08 ± 27.61	58.33 ± 8.467	51.00 ± 27.93	0.729
ANOVA *P*-value	**Fungicide vs. control**	*P*<0.001	*P*<0.001	*P*<0.001	

[x]The result is significantly different (*P*<0.05) from the control based on Fisher's *LSD* test (within species comparison).
[y]The result is significantly different (*P*<0.05) from the control based on Dunnett's comparison (within species comparison).
[z]Italicized small letters indicate significant differences (*P*<0.05) among the species for any given treatment based on Fisher's *LSD* test (within treatment comparison). The same letter indicates that the difference is not significant.

Discussion

Our results show significant differences among fungicides in growth inhibition of various *Sphaerobolus* species. Almost one-third of the fungicides tested showed no significant inhibition of the artillery fungus, despite their effectiveness against *Agaricus*, another basidiomycete. This emphasizes the importance of *a priori* testing of any fungicide before field application. In addition to the type of fungicide, significant differences were often observed between the two concentrations of a certain fungicide. Additionally, several fungicides expressed different extent of growth inhibition among species. For example, Terraguard 50W® was only moderately effective in *Sphaerobolus ingoldii* prov. sp. nov., but showed great inhibition on *S. iowensis* and *S. stellatus*. Since results detailed in the previous chapter revealed that these latter two species were more abundant than *Sphaerobolus ingoldii* prov. sp. nov., the Terraguard 50W® might provide sufficient control in most areas of the U.S. Glyodin showed similar differences in efficacy among species, but was not as effective as Terraguard 50W®. In contrast, Bravo Zn® was particularly effective against *Sphaerobolus ingoldii* prov. sp. nov., showing high growth inhibition, but was only moderately inhibitory against *S. iowensis* and *S. stellatus*. In some cases, despite the significant differences found among the species, some fungicides proved to be highly effective in all species (e.g. triphenyltin acetate, Opus®, Topsin M 70 WP®).

The results reported herein demonstrate the inhibitory effect of various fungicides as tools for chemical control for all three species of the artillery fungus: *Sphaerobolus ingoldii* prov. sp. nov., *S. iowensis*, and *S. stellatus*. Based on these initial results, Opus®, Topsin M 70WP®, triphenyltin acetate, Difolatan®, and Terraguard 50W® in 20 ppm concentration show

promise for further field testing against all *Sphaerobolus* species. However, these preliminary experiments used *a priori* application of fungicides in the growing media under *in vitro* conditions. At this time, none of the fungicides tested are registered by E.P.A. for use against mulch fungi. Of the fungicides tested in this study only Difolatan® has been used – beside a wide range of agricultural crops – in the lumber and timber industries to reduce losses from wood rot fungi in logs and wood products. However, this fungicide is not registered in the U.S., according to our knowledge. Further studies should include *in vivo* experiments determine whether the results presented here will be consistent in field trials.

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József Geml
Curriculum Vitae

Education:

Ph.D. in **Plant Pathology** and **Molecular Evolutionary Genetics**, The Pennsylvania State University, University Park, PA (2001-2004)
Ongoing **Ph.D.** in **Mycology**, Corvinus University of Budapest, Budapest, Hungary (1998-2004)
BSc./MSc., University of Agricultural Sciences in Gödöllõ, Gödöllõ, Hungary (1991-1996)

Professional Experience:

Asst. Manager, Korona Spawn Plant, National Korona Mushroom Union, Hungary (1996-2001)

Scholarships:

Visiting Fulbright-Soros Researcher, Penn State University, University Park, PA (2000-2001)
Exchange Student Scholarship, Purdue University, West Lafayette, IN (1995)
Visiting Student Scholarship, Zagazig University, Zagazig, Egypt (1996)
Visiting Student Scholarship, Universität von Bonn, Bonn, Germany (1993)

Awards:

MSA Graduate Fellowship, The Mycological Society of America (2004)
Howard E. Bigelow Mentor Student Travel Award, The Mycological Society of America (2004)
Graduate Research Prize, The Mycological Society of America (2003)
Graduate Student Competitive Grant, College of Agricultural Sciences, Penn State Univ. (2003)
James M. Trappe Mentor Student Travel Award, The Mycological Society of America (2003)
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Selected publications:

Geml, J., Davis, D.D., Geiser, D.M. 2004.Systematics of the genus *Sphaerobolus* based on molecular and morphological data, with the description of *Sphaerobolus ingoldii* sp. nov. *Mycologia* (in review).

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