DISSERTATION

COLORADO CYTOSPORA CANKER COMPLEX ON *POPULUS TREMULOIDES* MICHX.

Submitted by

Jeff B. Kepley

Department of Biology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2009

COLORADO STATE UNIVERSITY

January 16, 2009

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JEFF B. KEPLEY ENTITLED COLORADO CYTOSPORA CANKER COMPLEX ON *POPULUS TREMULOIDES* MICHX. BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Dr. William R. Jacobi

Dr. Elizabeth A. H. Pilon-Smits

Dr. David A. Steingraeber

Adviser Dr. F. Brent Reeves

Department Head Dr. Daniel R. Bush,

ABSTRACT OF DISSERTATION

COLORADO CYTOSPORA CANKER COMPLEX ON *POPULUS TREMULOIDES* MICHX.

Cytospora canker is a serious fungal disease affecting aspen in natural and commercial forests as well as urban sites. In Colorado the causal organism responsible for this canker disease is typically reported to be *Cytospora chrysosperma* (Pers.) Fr. However, a thorough understanding of the species of *Cytospora* attacking aspen in Colorado is lacking. Fungal identification has been based upon morphological characteristics of fruiting/vegetative structures despite the plasticity known to occur in such diagnostic features. Examinations of cankers on aspen stems in Colorado revealed a morphologically distinct *Cytospora*-like fungus that frequently co-occurs with *C. chrysosperma.* This fungus is a new species and is closely associated with and superficially resembles *C. chrysosperma.* Based on these findings Cytospora canker on aspen in Colorado is a complex of fungi, contrary to what is typically reported in the literature. Isoenzyme analysis was employed as an initial step to determine the genetic/biochemical differences that occur among and between *C. chrysosperma* and the new non-*C. chrysosperma* isolates. Of the twelve enzyme systems initially screened only three, viz., alpha esterase, amylase, and glucose-6-phosphate dehydrogenase, provided good resolution for all isolates. Following cluster analysis, two major clades well-delineated the two taxa. Phylogenetic analyses of ITS1-5.8S-ITS2 rDNA and EF-1α

sequences produced phylogenetic trees in which non-*C. chrysosperma* isolates formed a monophyletic clade (with strong bootstrap support and high posterior probability) within a *Cytospora* spp. phylogeny. Based on these results the non-*C. chrysosperma* isolates from aspen in Colorado are considered a new *Cytospora* species. External morphological features of the ascostromata and conidiomata (natural specimens) as well as histological sections of the new *Cytospora* sp. reveal conceptacles and conceptacle-like tissues which gives fruit bodies a unique target-like appearance. Cultures are darkly pigmented and display robust (large diameter) bead-like hyphae; many hyphal tips from young cultures lyse. Pycnidia produced *in vitro* do not enclose a multi-lobed locular structure; rather they have indentations/pockets with conidiophores lining these invaginations as well as pycnidial surfaces. In addition to the *Cytospora* anamorph a *Phialocephala*-like synanamorph is produced by some isolates. Descriptions of the new *Cytospora* species and *C. chrysosperma*, occurring on aspen in Colorado, are provided.

Jeff B. Kepley
Department of Biology
Colorado State University
Fort Collins, CO 80523
Spring 2009

ACKNOWLEDGEMENTS

I would first like to offer thanks to my graduate committee (Dr. F. Brent Reeves, Dr. David A. Steingraeber, Dr. Elizabeth A. H. Pilon-Smits and Dr. William R. Jacobi) for their support during the course of my studies, research, and writing of this dissertation. I must say it was a long and arduous process. I am fortunate you were patient and that you gave me the opportunity to see it through to fruition. Moreover, I would like to extend a very special thank you to Dr. Pilon-Smits who filled in, at very short notice, for Dr. Paul Kugrens after his passing.

I particularly want to express my sincere gratitude to Dr. Brent Reeves. Unfortunately, words cannot fully convey the degree of respect and admiration I have for you. You have been a wonderful mentor and a very good friend. Your vast knowledge in biology and practical approach to problem solving was/is a tremendous resource. However, your expertise in mycology cannot be overlooked or overstated. I feel honored to have had the opportunity to work with you, and the fact I was your final graduate student is very special to me. Thank you for your countless hours of help and support. Not only was your knowledge invaluable, but your candor and sense of humor made some difficult periods survivable. I have learned much from you regarding science, as well as life in general.

Special thanks to Dr. Amy Wangeline, my office mate and confidant during the course of my program. You are a wonderful person and friend, and I feel very fortunate

to have met you. Your positive attitude and spirit is infectious. I would also like to acknowledge Dr. Tracy Halward; you were a wonderful lab coordinator and helped so much as I made the difficult transition into student teaching. Additionally, I am particularly indebted to Dr. Gerard Adams at Michigan State University for his much needed input and advice. I feel very fortunate that you were willing to share with me your expertise concerning *Cytospora*.

Lastly, but by no means the least, I want to offer my most heartfelt gratefulness to Cynthia Benton, my significant other. You have been a true soul mate and friend for the time we have been together. The road I have followed during the course of this endeavor has been rough at times, but you have always been there to lend support and encouragement. Without your help I am not sure it would have been possible.

This dissertation is dedicated to my late father, and my mother and Dr. F. Brent Reeves. My father instilled in me the value of education and making it a continual part of life. My mother showed me the values of hard work and doing your best regardless of the task. Brent Reeves once told me "you can't run away from stress."

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF TABLES

Chapter 2

Chapter 3

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

LIST OF FIGURES (continued)

CHAPTER 3

LIST OF FIGURES (continued)

APPENDIX III

LIST OF FIGURES (continued)

INTRODUCTION/OVERVIEW OF DISSERTATION

The purpose of this introduction/overview is to briefly outline what is contained and/or addressed in each of the chapters and appendices of this dissertation. By no means are the summaries all inclusive; rather they emphasize some of the key components of each section.

Chapter 1 Literature Review

Chapter 1 consists of two main sections. The first provides an overview of Cytospora canker and begins with a discussion of disease distribution and woody plant hosts. This is followed by aspects of disease ecology, epidemiology, and etiology-included are factors that contribute to host predisposition as well as components of host penetration, infection and pathogenesis; perspectives pertaining to an endophytic habit for *Cytospora* spp. also are explored. Lastly, elements of fruit body formation including features of spore dissemination and germination are discussed. The second section of Chapter 1 provides an historical review and the taxonomy of *Cytospora* spp. and related teleomorphs, viz., *Valsa*, *Leucostoma*, *Valsella,* and *Valseutypella*. Species numbers, and those occurring on *Populus* spp. specifically, are addressed. Some of the problems that have made species identification difficult, i.e., morphological variation resulting from differences in host/host tissues and environment, and cultural, strain, and spore size variation, are outlined. Chapter 1 concludes with a summation of nine monographic works (in English) pertaining to *Cytospora* spp. and related teleomorphs.

Chapter 2 Differentiation of *Cytospora* isolates on *Populus tremuloides* based on isoenzyme analysis

Chapter 2 begins with a description of isoenzymes followed by discussions regarding their importance as a research tool, and prior biochemical and molecular studies involving *Cytospora* spp. and associated teleomorphs. Following a brief discussion defining the Colorado Cytospora canker complex on aspen (a fungal complex involving *C. chrysosperma* and a *Cytospora*-like fungus), and its implications, the remainder of Chapter 2 focuses on the chapters' crux, i.e., a study using isoenzymes to detect biochemical/genetic differences among and between isolates of the two taxa (*C. chrysosperma* and non-*C. chrysosperma*) comprising the Cytospora canker complex on aspen in Colorado. In this study 12 enzyme systems were initially screened. However, only three, viz., alpha esterase, amylase, and glucose-6-phosphate dehydrogenase, provided good resolution for all isolates. Following cluster analysis, two major clades well-delineated the two taxa. In cases where enzyme systems did not produce resolvable bands for all isolates, viz., acid phosphatase, alkaline phosphatase, peroxidase, and polyphenol oxidase, activity that did occur also clearly separated *C. chrysosperma* and non-*C. chrysosperma* isolates. It was uncertain, however, whether the genetic differences for the two taxa on aspen in Colorado, as detected by isoenzymes, constitute separate species. Therefore, a follow-up study employing DNA sequence comparisons was conducted.

Chapter 3 Molecular phylogeny and morphology of the fungi associated with the Cytospora canker complex on *Populus tremuloides* Michx. in Colorado

Chapter 3 begins with discussions pertaining to the importance of aspen, problems associated with identifying *Cytospora* spp., and the Colorado Cytospora canker complex on aspen. Following this, molecular methods for inferring fungal phylogeny

and fungal identification among Diaporthalean taxa are briefly addressed. The remainder of Chapter 3 involves molecular phylogenetic and morphological studies pertaining to the two taxa (*C. chrysosperma* and non-*C. chrysosperma*) comprising the Cytospora canker complex on aspen in Colorado. Phylogenetic analyses of ITS1-5.8S-ITS2 rDNA and EF-1α sequences produced phylogenetic trees in which non-*C. chrysosperma* isolates formed a monophyletic clade (with strong bootstrap support and high posterior probability) within a *Cytospora* spp. phylogeny. Based on these results the non-*C. chrysosperma* isolates from aspen in Colorado are considered a new *Cytospora* species. External morphological features of the ascostromata and conidiomata (natural specimens) as well as histological sections of the new *Cytospora* sp. reveal conceptacles and conceptacle-like tissues which gives fruit bodies a unique target-like appearance. Cultures are darkly pigmented and display robust (large diameter) bead-like hyphae; many hyphal tips from young cultures lyse. Pycnidia produced *in vitro* do not enclose a multi-lobed locular structure; rather they have indentations/pockets with conidiophores lining these invaginations as well as pycnidial surfaces. In addition to the *Cytospora* anamorph a *Phialocephala*-like synanamorph is produced by some isolates. Descriptions of the new *Cytospora* species and *C. chrysosperma*, occurring on aspen in Colorado, are provided.

Appendices

Appendices I, II, IV, and V pertain to materials and methods that complement those discussed, or those which are referred to, in Chapters 2 and 3 of this dissertation. Appendix III (a supplement to the primary investigative studies in Chapters 2 and 3) consists of five preliminary studies. The principal conclusions from each of those studies are as follows: Study 1: enzyme (amylase) mobility during electrophoretic runs is not affected by the volume of protein extract loaded in sample wells; Study 2: the expression

of polyphenol oxidase and peroxidase isoenzymes for *C. chrysosperma* and non-*C. chrysosperma* isolates is related to age of cultures, i.e., increasing enzyme expression occurs with increasing age of cultures; Study 3: incompatible mycelial interactions between isolates of *C. chrysosperma* and those of non-*C. chrysosperma* are reflective of the genetic differences occurring between the two taxa; Study 4: volume (depth) of medium (modified Leonian's, potato dextrose, and oatmeal agar) in Petri dishes has a profound effect on fungal morphology, e.g., smaller volumes (10 and 15 ml) of medium per dish results in more lobate culture margins of *C. chrysosperma* and non-*C. chrysosperma* isolates compared to isolates grown in dishes containing larger volumes (25 and 30 ml). Moreover, depth of media, as opposed to media alone, can affect the quality of fungal growth; Study 5: *C. chrysosperma* and non-*C. chrysosperma* isolates from aspen in Colorado are not pathogenic on field-grown aspen and cottonwood (*Populus* sp.) trees (the only fungal isolate exhibiting canker expansion was a *Cytospora* sp. collected from cottonwood).

CHAPTER 1 LITERATURE REVIEW

Overview of Cytospora canker

Distribution and hosts. Cytospora canker is a world-wide canker disease that affects branches and stems of numerous woody plant species (Agrios, 1997) as well as roots in certain species (Kohlmeyer & Kohlmeyer, 1971; Ross, 1976). According to Grove (1935), more than 60 genera of hardwood and conifer trees are affected. Agrios (1997) states that more than 70 species of shrubs, fruit trees, forest and shade trees are attacked. Adams et al. (2006) state that the canker disease occurs on over 85 woody host species; this estimate agrees with Farr et al. (1989) and Sinclair et al. (1987). Cytospora canker probably affects more tree species than any other canker disease (Agrios, 1997).

In Colorado, Hinds (1964) found Cytospora canker in 97% of the native aspen (*Populus tremuloides* Michx.) stands that he sampled. A number of urban tree species, in addition to aspen, are attacked in the Rocky Mountain region (Kepley & Jacobi, 2000). These include cottonwoods (*Populus* spp.), Lombardy and other poplars (*Populus* spp.), apples (*Malus* spp.), cherries (*Prunus* spp.), peaches (*Prunus* spp.), plums (*Prunus* spp.), birches (*Betula* spp.), willows (*Salix* spp.), honeylocust (*Gleditsia triacanthos* L. var. *inermis*), mountain ashes (*Sorbus* spp.), silver maple (*Acer saccharinum* L.), spruces (*Picea* spp.) and other conifers, and Siberian elm (*Ulmus pumila* L.) (Jacobi, 1994). Additionally, the disease occurs on green ash (*Fraxinus pennsylvanica* Marsh.) and alder (*Alnus* spp.) trees (Kepley & Jacobi, 2000).

Ecology, epidemiology, and etiology

Predisposing factors and host penetration. Cytospora canker is caused by various species of the anamorphic fungus *Cytospora* Ehrenb. Because the *Cytospora* anamorph is most commonly found, the disease is generally referred to as Cytospora canker (Agrios, 1997). *Cytospora* fungi mostly are opportunistic pathogens and parasitize hosts that are stressed; poor vigor leads to increased susceptibility (Hubert, 1920; Leonian, 1921; Schoenweiss, 1967; Hinds, 1985; Sinclair et al., 1987; Biggs, 1989; Adams et al., 2006). Such stresses include tree species planted outside their native range or "off-site", fire, drought, freezing, winter injury, shade weakened twigs inside canopies, crowding, insects, nematode infection of roots, defoliation or other injury due to fungi, dwarf mistletoe infections, sunscald, herbicides, salt spray, nutrient deficiencies, soil compaction, soils with high clay content, or soils with thin upper horizons or calcareous horizons, severe pruning, and mechanical injury (Long, 1918; Hubert, 1920; Leonian, 1921; Povah, 1921; Schreiner, 1931b; Willison, 1933; Willison, 1937; Zentmeyer, 1941; Wright, 1942; Waterman, 1955; Wright, 1957; Helton & Konicek, 1961d; Jorgensen & Cafley, 1961; Lukezic et al., 1965; Gross, 1967; Luepschen & Rohrbach, 1969; Scharpf & Bynum, 1975; Bertrand et al., 1976a; Schoeneweiss, 1981; Dhanvantari, 1982; English, 1982; Pinon, 1984; Smiley et al., 1986; Biggs, 1989; Jacobi, 1994; Burks et al., 1998; Adams et al., 2005). Trees with root damage appear especially prone to infection (Jacobi, 1994).

Observations by Christensen (1940), however, led to his questioning the degree of parasitism inflicted by *C. chrysosperma* (Pers.) Fr.; he felt the organism often times is not responsible for the injury with which it is associated and believed this genus to be one of the most common fungi forming fruit bodies on the bark of recently dead aspen.

Schreiner (1931b) stated that of the 135 described species of *Valsa* Fr.(anamorph *Cytospora* spp.) the majority were saprophytic in nature. Helton and Konicek (1961d) found that isolates obtained from stone fruit stem sections which were seriously diseased could be saprophytic rather than pathogenic. Likewise, an extensive series of inoculations (Willison, 1936) on the peach variety *P. persica* L. Batsch 'Elberta' suggested that *Valsa leucostoma* (Pers.) Fr. (anamorph *C. leucostoma* (Pers.) Sacc.) was either non-pathogenic or an exceptionally weak wound parasite, even though it had been isolated from internal tissues of cankers and spores from fruiting bodies on *P. persica*. A study by Christensen and Hodson (1954), in which various species of forest and plantation trees were wounded or banded to induce senescence, led to their concluding that it is extremely difficult for a person to tell, by means of ordinary external examination, whether a tree is declining, dying, or has recently died. They felt there was no adequate measure of vigor in a forest tree, so evaluations as to whether a fungal organism was a primary invader or invading trees that were already dying was difficult to discern. Spielman (1983) states it is often difficult to determine the primary cause of death based on the fact that *Cytospora* spp. can exist as saprophytes yet twig dieback is a main symptom of pathogenicity in woody plants. The occurrence of *C. variostromatica* G.C. Adams & M. J. Wingf. on *Populus deltoides* Bartr. ex Marsh. in South Africa was thought to be the result opportunistic colonization of senescent tissues since pathogenicity studies revealed it was not virulent in comparison to South African isolates of *Valsa nivea* (Hoffm.) Fr. and *V. sordida* Nitschke (anamorph *C. chrysosperma*) (Adams et al., 2006). They speculated a "host jump" from *Eucalyptus* might explain its presence on *Populus*.

Hildebrand (1947) believed that when injured or dead bark tissue was present and allowed saprophytic establishment, *V. cincta* (Fr.) Fr. (anamorph *C. cincta* Sacc.) and *V. leucostoma* could become pathogenic and invade healthy tissues of peach trees. Wounds (mechanical, natural etc.) or dead bark are necessary for infection as *Cytospora* species are not capable of invading healthy, intact bark (Long, 1918; Hubert, 1920; Leonian, 1921; Schreiner, 1931b; Willison, 1933; Forsberg, 1941; Wright, 1942; Hildebrand, 1947; Helton & Moisey, 1955; Helton & Konicek, 1961d; Jorgensen & Cafley, 1961; Lukezic et al., 1965; Scharpf & Bynum, 1975; Luepschen et al., 1979; Kamiri & Laemmlen 1981a; Dhanvantari, 1982; Hinds, 1985; Walla & Crowe, 1986; Ramaley et al., 1987; Biggs, 1989, 1990). Additionally, wounds are susceptible to infection for only a certain period of time which is partly based upon the time frame at which host response mechanisms initiate and develop. McIntyre et al. (1996) showed that on aspen, wound susceptibility and resistance to infection depended on water status of the host and time of year. During the spring this period was at least 2 to 6 days for stressed trees and approximately 48 hours for nonstressed trees. Kamiri and Laemmlen (1981a) found that on drought-stressed Colorado blue spruce (*P. pungens* Engelm.) the period of canker development following inoculation was shorter in comparison to that of non drought-stressed trees.

Endophytic possibilities. Recently, Alonso et al. (2005) used isolates of *C. eucalypticola* Van der Westh., obtained from cankered twig tissue on *Eucalyptus globulus* Labillardiere and *E. grandis* W. Hill ex Maiden, and demonstrated they could penetrate epidermal tissue without wounds in *E. globulus* and *E. grandis*; however, in these experiments no post inoculation symptoms of infection were observed. Such

outcomes might be attributed to the fact that young, actively growing trees were inoculated. Under such conditions the defense system of the host may have prevented pathogenesis from occurring since stress, such as drought, slows host resistance mechanisms such as periderm production (Puritch & Mullick, 1975), rate of lignification, and reaction zone size (Biggs et al., 1983). Additionally, it is possible the fungus never penetrated intact tissue which is a well documented limitation regarding this genus; rather, the fungus potentially may have entered through natural openings such as lenticels and leaf scars, or via minute undetected wounds in the epidermis. On the other hand, Schoeneweiss (1983) discovered that *C. kunzei* Sacc. var. *piceae* Waterman could be found colonizing asymptomatic drought-stressed and non drought-stressed Colorado blue spruce bark tissue at a point some distance from the wounding and inoculation site. In the case of the drought stressed trees, necrotic cankers formed in bark tissue around the inoculation site. Such studies do point out, however, the potential for *Cytospora* spp. to reside within healthy appearing host tissue in an innocuous/inconspicuous state, i.e., latent organisms surviving as asymptomatic endophytic infections, and possibly remain so until stress would allow the host to become susceptible to parasitism or saprobic fungal lifestyles.

A number of studies have addressed the potential endophytic nature of *Cytospora* spp. Both *C. chrysosperma* and *C. eucalypticola,* as well as other *Cytospora* spp., have been isolated from symptomatic and healthy tissues in *Eucalyptus* spp. (Bettuccii & Saravay, 1993; Fisher et al., 1993, Bettucci & Alonso, 1997; Bettucci et al., 1997; Bettucci et al., 1999). Because *Cytospora* spp. and *C. chrysosperma* have been found inhabiting healthy xylem and complete stems (Bettucci & Saravay, 1993), and

seedling stems more frequently than symptomatic stems (Bettucci et al., 1997), such organisms probably possess a latent, i.e., metabolically inactive, phase in their life cycle whereby latent invasions result from fungal propagules distributed widely but sparsely in functional sapwood; such propagules do not develop overtly due to the high water content present in the sapstream (Boddy & Griffith, 1989). If during this latent phase conditions such as dead and/or dying tissues become available, the organisms may then undergo saprotrophic expansion. A study by Bettucci et al. (1999) seems to suggest this. They examined healthy and symptomatic tissues and found *C. chrysosperma* colonizing both. Because severe drought had occurred when trees were initially transferred to the field and potentially induced bark cracking, they speculated saprotrophic expansion occurred on the dead and dying tissue.

Hosts other than *Eucalyptus* spp. have been examined to determine if *Cytospora* spp. are part of endophytic floras. Santamaria and Diez (2005) isolated *C. chrysosperma* from *Populus tremula* L. in both healthy, and dead and dying tissues while Hutchison (1999) recovered *Leucostoma niveum* (Hoffm.) Höhn. (anamorph *C. nivea* (Hoffm.) Sacc.) and *Valsa sordida* from healthy stems of *P. tremuloides.* Chapela (1989) also isolated *C. chrysosperma* from healthy *P. tremuloides* tissue. However, in his study fungal development within the wood was induced by drying stem and branch sections. Chapela concluded that *C. chrysosperma* was a xylotropic endophyte which grew into secondary xylem as drying of the wood took place. He made the distinction between this type of endophyte and the "typical" endophyte used and defined by earlier scientists as any fungus inhabiting the internal environment of a living plant. The main difference illustrated by Chapela is that xylotropic endophytes grow into secondary xylem upon

drying of the wood. Chapela's work somewhat replicates the earlier examination into this phenomenon conducted by Kern (1940). Kern's study showed that healthy appearing stem sections of *P. tremuloides,* and cuttings of *P. alba* L. and *Salix* sp. which had been surface disinfested, coated with a thin layer of hot paraffin, and then incubated developed fruit bodies of *C. chrysosperma.* Additionally, a living branch section of *Sorbus aucuparia* L. which failed to develop pycnidia after 30 days in the laboratory supported the formation of fruit bodies of *C. chrysosperma* after the bark was frozen sufficiently to kill the plant tissue. Furthermore, Boddy and Griffith (1989) isolated *C. ambiens* (Nitschke) Sacc. from healthy wood and bark, as well as dead bark, from attached twigs of oak. *C. ambiens* also was found colonizing healthy sapwood of detached oak twigs which had been subjected to drying. They believed that the drying of the tissue allowed saprotrophic expansion by the fungus. Similar to the hypothesis posed by Christensen, Boddy and Griffith thought it possible that disease symptoms associated with some endophytes might merely be due to their ability to rapidly colonize dying tissue as opposed to pathogenic behavior.

Adams et al. (2006) used morphology and DNA sequence homology of the internal transcribed spacer region of the nuclear DNA operon, i.e., ITS ribosomal DNA (ITS-rDNA), and found similarities existed between *Cytospora* isolates from non-native trees in South Africa and isolates from Australia, Europe and America. They believed such similarities pointed towards the import into South Africa of these pathogens as endophytes residing within their respective hosts.

Despite the preceding studies which point towards the potential for a latent phase of *Cytospora* spp. to reside within healthy, asymptomatic host tissue, along with the

potential for saprophytic and/or parasitic behavior, a study by McIntyre et al. (1996) that used *C. chrysosperma* and *P. tremuloides* concluded otherwise. In their study with *C. chrysosperma* and aspen in Colorado, the fungus only was isolated from the outer most bark surface and never from within healthy bark tissue. Moreover, Endert-Kirkpatrick (1986) could not isolate *C. cincta* from the wood of apparently healthy peach trees leading her to conclude that *Cytospora* might be a primary pathogen.

Infection and pathogenesis. Once inside the host *Cytospora* fungi can colonize aggressively (Biggs, 1989) typically killing the cambium and invading adjacent host tissues (Schreiner, 1931b; Biggs et al., 1983). Such colonization of host tissue occurs via ramification of hyphae through dead bark tissue within and between walls of partially disintegrated and crushed host cells. Schreiner (1931b) found mycelium present in vessels, fibers, occasionally wood parenchyma, and between pith ray cells. On small stems, pith tissue also was colonized. Biggs (1983) found rapid colonization in inoculated hybrid *Populus* (*P. maximowiczii* Henry X *P. trichocarpa* Torr. & Gray) stems resulted from mechanical ramification of dense wedges of large-diameter hyphae with tissues behind the wedge colonized inter- and intra-cellularly by small-diameter hyphae. Production of extracellular enzymes and toxins has been thought to aid *Cytospora* spp. during this ramification process (Schreiner, 1931b; Hildebrand, 1947; Gairola & Powell, 1971; Rozsnyay & Barna, 1974; Svircev, et al., 1991). Pluim et al. (1994) suggested that enzymatic degradation was not a major part of pathogenesis on *Amelanchier alnifolia* (Nutt.) Nutt. by *C. leucostoma* because of the lack of maceration of diseased tissues and no resultant correlation between oxalic acid production and pH. They state that in other fungi, such as *Sclerotium rolfsii* Sacc., oxalic acid acts synergistically with

polygalacturonase via chelation of calcium from host tissue and lowering pH to levels where tissue macerating enzymes can function in hydrolyzing pectins in the middle lamella. However, they did observe oxalic acid secretion by numerous isolates and suggested that sequestration of calcium by oxalic acid was an important part of pathogenesis.

Hampson and Sinclair (1973) found that during infection of peach trees by *C. leucostoma*, wilting and defoliation of non-girdled branches occurred. Inoculations done during the period of active tree growth resulted in acute and chronic wilt of peach tree branches. However, they attributed the wilt to a host response in which gum present in tissues adjacent to cankers either plugged vessels and tracheids directly or made xylary fluid more viscous affecting lateral movement of fluid among functional tracheids and vessels. Inoculations of Elberta peach seedlings with *C. cincta* by Banko and Helton (1974) showed the fungus penetrated the xylem rapidly and proceeded to colonize deeper into tissue via xylem ray cells with gum forming in vessels and tracheids at or just beyond the canker margin. However, Helton and Randall (1975) observed gum appearing not only in tissues next to infection sites of inoculated Italian prune (*P. domestica)* trees, but elements well beyond and generally above the infection site. Additionally, *Cytospora* spp. have been shown to be capable of penetrating wound gum during pathogenesis of peach by means of appressoria and penetration threads (Willison, 1932; Hildebrand, 1947) or possibly via gum dissolution (Banko & Helton, 1974).

Symptoms. Depending on the exact mode of entry, host species affected, and the stage of disease development, symptoms can vary. On stone fruit trees the disease is characterized by premature leaf senescence, twig and branch dieback, discoloration and

flagging of foliage, and extensive perennial cankers on the trunk, branches, and scaffold limbs (Helton & Konicek, 1961d; Gross, 1967; Luepschen & Rohrbach, 1969; Jones & Luepschen, 1971; Hampson & Sinclair, 1973; Tekauz & Patrick, 1974). Gum exudation is common as well (Willison, 1933; Hildebrand, 1947; Helton & Konicek, 1961d; Lukezic et al, 1965; Gross, 1967; Hampson & Sinclair, 1973; Helton & Randall, 1975; Sinclair et al., 1987). With poplars and willows, infected inner bark may be yellow, brown, reddish-brown, gray or black, and becomes watery and odorous as it deteriorates, while wood underneath the cankered bark is stained brown (Long, 1918; Walla & Conway, 1986). On conifers such as spruce, bark which has been killed internally is brown to reddish-brown and infiltrated with resin. Beneath this area, the sapwood is scarcely discolored despite killing of tissue and colonization by the pathogen. Large amounts of clear, amber resin commonly exudes from the canker edges (Wright, 1957; Scharpf & Bynum, 1975; Sinclair et al., 1987). Typically on *Picea* spp., the disease starts on lower branches resulting in premature death followed by lateral and upward spread (Jorgensen & Cafley, 1961; Kamiri & Laemmlen, 1981; Walla & Crowe, 1986; Sinclair et al., 1987). On true firs (*Abies* spp.) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco top-killing and dieback of upper branches is more typical, especially on larger trees (Wright, 1957; Scharpf & Bynum, 1975). Rogers and Noskowiak (1976) however, observed brown sapwood stain of ponderosa pine (*P. ponderosa* Laws.) logs in storage at a lumber mill in Washington State. The fungus isolated from stained areas was a species of *Cytospora* and sections associated with the brown-stained wood showed the fungus to be mostly confined to rays and longitudinal wood elements at ray crossings. Epithelial

resin duct cells and parenchymatous ray elements were well lysed by the fungus and wood tracheids were entered via pits with subsequent brown staining of their walls.

Fruit body formation. Fruiting structures may be found in association with cankered areas. Adams et al. (2005) do not accept *Cytospora* spp. as being either purely stromatic or pycnidial. However, they commonly are stromatic in nature.

Pycnidia or conidiomatal stromata typically form shortly after death of bark tissue and on deciduous trees appear as small, erumpent, pimple-like swellings beneath the bark (Walla & Conway, 1986; Sinclair et al., 1987). On spruce, however, such structures are not visible on the bark surface and are observed by superficial cuts in cankered bark (Walla & Crowe, 1986) or removal of the loose outermost bark scales (Sinclair et al., 1987).

Generally the teleomorph also is composed of stromatic tissue, i.e., ascostroma, and normally forms after maturation of the anamorph. As with pycnidia or conidiomatal stromata, ascostromata also may be erumpent (Wehmeyer, 1926; Marsden, 1948; Kern, 1955; Barr, 1978; Spielman, 1985). Barakat et al. (1995) observed that ascostromatal formation in *L. cinctum* (Fr.) Höhn. (anamorph *C. cincta*) on sweet cherry in orchards only occurred on infected branches that had been dead for 2 to 3 years which is in agreement with Bertrand and English (1976a) for *V. leucostoma* on *Prunus* spp; however, Adams et al. (1990) found perithecia of *L. persoonii* (Nitschke) Höhn. (anamorph *C. leucostoma*) to be present on **living** scaffold branches of peach trees in Michigan orchards. Similarly, Pluim et al. (1994) found the perfect stage of *C. leucostoma* to predominate at a site where disease incidence was low and orchard maintenance of *Amelanchier alnifolia* in Alberta, Canada was high, thus reducing the presence of older

wood. They speculated that, based on the extreme northern location of the site, environmental conditions or differences in length of day might trigger expression of genes controlling the sexual stage. Kamiri & Laemmlen (1981b) found that in nature perithecial numbers and ascospore viability was small in comparison to the numbers of pycnidia and conidia produced by *V. kunzei* on Colorado blue spruce. They believed this suggested that the teleomorphic stage may develop several years after a branch first becomes infected. Because teleomorphs were found most often in the spring or late fall on low growing branches or those protected from drying, Spielman (1983) suggested their formation was favored by high humidity and low temperatures. Adams et al. (1998) observed formation of ascocarps of *L. persoonii* and *L. cinctum* on dead peach branches which had been placed on the ground two years earlier in Michigan orchards.

Factors related to spore dispersal, spore germination, and their role in the infection process. Depending on the fungal species, perithecia may or may not occur in the same stromata as the pycnidia (Wehmeyer, 1926, Kern, 1955; Spielman; Walla & Conway, 1986; Sinclair et al., 1987). Generally the two states are more often found intermingled or inhabiting closely associated areas on the same branch; sometimes the teleomorph may occupy lower protected parts of the host (Spielman, 1983).

Conidiospores exude from pycnidia in a mucilaginous, polysaccharide matrix and appear as long, coiled threads called spore horns, spore tendrils, or cirrhi (Hildebrand, 1947; Spielman, 1983; Sinclair et al., 1987; Biggs, 1989). Such tendrils form under normal moisture conditions, but if atypical moisture is present, and spores are not washed away, globular, sticky masses of spores form around pycnidial openings which eventually dry down to produce hard, hemispherical masses (Hubert, 1920; Povah, 1921; Schreiner,

1931b). Helton and Konicek (1961d) state that the tendrils form during dry weather and as globular masses when conditions are wet. Cirrhi have been described to occur in a variety of different colors such as amber, red, dark red to reddish brown, reddish-orange to orange-brown, orange, salmon-orange, yellow to pale yellow, flesh, cream, white to off-white and pallid depending on the host and/or species of *Cytospora* or the literature consulted (Long, 1918; Hubert, 1920; Schreiner, 1931b; Christensen, 1940; Forsberg, 1941; Wright, 1942; Hildebrand, 1947; Kern, 1955; Waterman, 1955; Helton & Konicek, 1961d; Gross, 1967; Spielman, 1983; Walla & Conway, 1986; Walla & Crowe, 1986; Proffer & Jones, 1989; Pluim et al., 1994; Wier, 2000, Adams et al., 2006). Adams et al., (2006) report that for *Cytospora* spp. occurring on *Eucalyptus*, cirrhus color remains uniform within species as long as environmental conditions and age of cultures are similar.

Ascospores may similarly ooze from perithecia, but the matrix is less gelatinous and less abundant than that produced by pycnidia (Christensen, 1940); however, Kamiri and Laemmlen (1981a) report that both conidia and ascospores exude from fruit bodies on Colorado blue spruce in indistinguishable tendrils upon wetting. Furthermore, and in contrast to conidiospores, ascospores have been observed to be forcibly discharged (Saito et al., 1972; Bertrand & English, 1976a).

Although conidia and ascospores have the same general allantoid shape, dimensions are quite variable, and ascospores typically are larger (Schreiner, 1931b; Christensen, 1940; Kern, 1955; Sutton, 1980; Spielman, 1985; Adams et al., 2005). However, Adams et al. (2005) found that two species of *Cytospora* (*C. valsoidea*, and the anamorph of *Valsa myrtagena*) on *Eucalyptus* produced straight conidia in nature,

whereas in culture they were mostly allantoid. From the foregoing information it would seem that variability in color of cirrhi and shape of spores can occur based upon the environmental conditions or cirrhus age as well as whether cultures or natural specimens are being examined.

Cytospora fungi are prolific and have the potential to produce large amounts of inoculum. The number of spores produced by a pycnidium has been shown to vary and may range from 10,000 to more than 580,000,000 (Hildebrand, 1947; Christensen, 1940; Tekauz, 1972). Whether conidia or ascospores cause the initial infection is ambiguous at this time. Probably either one could initiate infection.

Biggs (1989) states that conidia initiate most new infections of Leucostoma canker on peach. However, work conducted by Adams et al. (1990) suggested that ascospores might be the effective propagules for infection of peach by *L. persoonii.* This hypothesis was based on the number of vegetative compatibility groups (v-c groups) which were found to occur in close proximity to one another within a single orchard. A later study by Wang et al. (1998) further pointed towards ascospores as potential inoculum sources for *Leucostoma* species. This finding was supported by the amount of genetic variation which was detected via colony morphology, small nuclear rDNA size polymorphisms, number of maternal lines observed based upon mitochondrial DNA restriction fragment length polymorphisms, and subsequent incompatibility reactions within those lines. An inoculation study by Wier et al. (2000) demonstrated conidia of *C. rhizophorae* Kohlm. & E. Kohlm. to be effective sources of inoculum for infection of red mangrove (*Rhizophora mangle* L.). Based on the limited (to single) v-c groups found within cankers, trees, and adjacent trees, Proffer and Hart (1988) speculated that conidia

were involved in the short-range dissemination of *L. kunzei*. Despite the tendency for a single or a few v-c groups to predominate in a localized area, isolates from trees at different sites or from trees not adjacent to each other showed more variety regarding v-c groups; such findings point towards ascospores as a dissemination mode for the fungus in addition to conidia. Kamiri and Laemmlen (1981a, b) suggested that ascospores were the infective inoculum for infection of Colorado blue spruce by *Valsa kunzei* Nitschke (anamorph *C. kunzei* Sacc.) whereas Schoeneweiss (1983) believed that conidia could serve as primary inoculum for the same fungal/host species combination. Waterman (1955) was able to obtain positive results during inoculation studies using both mature pycnidia and mycelium from agar cultures of *V. kunzei* var. *piceae*, and *Picea* spp. and *Pseudotsuga menziesii* (Mirb.) Franco as hosts. Whether use of mature pycnidia is evidence for conidia being infective propagules is somewhat ambiguous. In experiments conducted using white (*P. glauca* (Moench) Voss) and Norway (*P. abies* (L.) Karst) spruce, Jorgensen and Cafley (1961) concluded that both conidiospores and ascospores of *V. kunzei* var. *piceae* Waterman were infective. Leonian (1921) was able to induce canker formation on inoculated field grown apple trees using both conidia and ascospores of *V. leucostoma*. Lastly, although Bertrand and English (1976a) found conidia to be 10 to over 4,000 times more common than ascospores for *V. leucostoma* on French prune (*P. domestica* L. 'French') and President plum (*P. domestica* L. 'President') in California orchards, both spore types were capable of inducing cankers in inoculated French prune trees.

Release and dispersal of spores from fruiting structures is responsive to both moisture and temperature (Hubert, 1920; Schreiner, 1931b; Christensen, 1940; Forsberg,

1941; Wright, 1942,; Hildebrand, 1947; Leyendecker, 1952; Filer, 1967; Luepschen & Rohrbach, 1969; Saito et al., 1972; Bertrand & English, 1976a; Kamiri & Laemmlen 1981b; Spielman, 1983; Barakat et al., 1995). Splashing and wind-blown rain have been found to be the main modes of dissemination of conidia for *C. leucostoma* (Leupschen & Rohrbach, 1969; Bertrand & English, 1976a) with dispersion distances as great as 77 meters being recorded (Bertrand & English, 1976a). Likewise, ascospores could potentially be either forcibly discharged, or splashed up into air currents, resulting in long distance dispersal (Kamiri & Laemmlen 1981b).

Barakat et al. (1995) found that conidia produced by *L. cincta* on sweet cherry *(Prunus avium* L.) in Washington State were released most rapidly when free water was present. This occurred in less than 6 hours when pycnidia were incubated at 20 C. In contrast, it took more than 48 hours for sporulation to occur at a relative humidity of 100%. Conidia were observed to be released over a wide temperature range (2 to 28 C). They concluded that both conidia and ascospores were a source of inoculum for infection at lower temperatures as long as sufficient moisture was present. Furthermore, they reported that conidia in dried cirrhi could remain viable for up to 30 days under orchard conditions thereby increasing the likelihood of infection. Viable conidiospores have been found to be present throughout the year in numerous locals and for varied host/fungal species combination (Filer, 1967; Luepschen & Rohrbach, 1969; Bertrand & English, 1976a; Kamiri and Laemmlen, 1981b). Spielman (1983) speculated that the mucilaginous matrix which surrounds the conidia protects them from desiccating since viable spores can be found within completely dry tendrils. Luepschen and Rohrbach (1969) observed that from February through June, release of conidia was responsive to

relative humidity whereas during the rest of the year temperature was the main factor. Presence of ascospores, at least for some host/species combinations, appears to occur more in the spring-time than during other periods of the year (Bertrand & English, 1976a; Kamiri & Laemmlen 1981b). Spielman (1983) notes that the ability of fruit bodies to produce viable spores does not seem to be affected by periods of mild freeze and that fruit body formation may occur during midwinter thaws; production of viable ascospores and conidia also can take place whenever temperatures are above freezing. In addition to splashing and wind-blown rain, dissemination of spores also can occur via insects, birds, wind, and contaminated pruning tools (Long, 1918; Schreiner, 1931a; Forsberg, 1941; Hildebrand, 1947; Leyendecker, 1952; Jorgensen & Cafley, 1961; Leatherman et al., 1986; Walla & Conway, 1986; Walla & Crowe, 1986; Sinclair et al., 1987; Tattar & Wier, 2002).

Species of *Cytospora* have a wide range of temperatures at which spores will geminate, but temperatures around 27 C are the optimum in a number of studies (Rohrbach & Luepschen, 1968; Kamiri & Laemmlen, 1981b, Barakat et al., 1995). Barakat et al. (1995) demonstrated that by increasing the incubation period from 12 to 24 hours to 48 to 72 hours, germination of conidia of *L. cincta* could occur at lower temperatures such as those observed for ascospores. In both cases sufficient moisture is required. *In vitro* studies by Rohrbach and Luepschen (1968) indicated that conidiospores of *C. leucostoma* would not germinate when temperatures were below 4 C. Additionally, they believed that temperatures of 38 C for several days, as well as those below -12 C, would limit infections under natural conditions due to reduced viability. *In*

vitro studies also showed that spore germination would only occur only in a saturated atmosphere or free water, and with a source of carbon present.

Historical perspective and taxonomy

Historical review: *Cytospora* and *Valsa*. An excellent detailed synopsis of the taxonomic history of *Valsa* and *Cytospora* is given by Spielman (1983) followed by a more condensed version by the same author in 1985. By no means does the following summary of her works include all the mycologists and their contributions covered within her body of work; rather my summary highlights what I feel are some of the more important aspects.

The genus *Cytospora* was described by Ehrenberg (1818) and included several segregates from *Naemaspora* Pers. as well as four of Ehrenberg's new species. Fries (1823) adopted the genus although he misspelled it as *Cytispora* and as such this name was recognized and used throughout much of the early mycological literature. However, *Cytispora* is viewed as an orthographic variant by numerous authorities and most works following the third volume of Saccardo's Sylloge Fungorum (1884) have used Ehrenberg's original spelling (Spielman, 1980). Donk (1964) chose *C. chrysosperma* (Pers.) Fr. as the type specimen from amongst several species which he considered to be "the most eligible species."

Fries was the first to notice resemblances between *Cytospora* and *Valsa* and believed *Cytospora* was merely a developmental irregularity of *Valsa*. He erected *Valsa* as a genus in 1849, yet *Valsa* had been earlier named by Adanson (1763). Fries, though, did not include Adanson's species in his circumscription and instead included them in the genus *Diatrype* Fr. *Valsa* Adanson was later rejected and *Valsa* Fr. was conserved in order to preserve the modern generic concepts of *Valsa* Fr. and *Diatrype* Fr. (Adams et

al., 2005). Tulasne and Tulasne (1863) first hypothesized that *Valsa* and *Cytospora* were two forms of the same organism. Soon following, *Cytospora* species were described and named in association with known *Valsa* species although many of these newly described anamorphs were illegitimate because they had been given names in earlier works.

Following Fries' works *Valsa*, as a genus, was in a state of flux. Some mycologists were conservative while others such as Saccardo were more progressive. Initially, such conservatism led to many diverse (heterogeneous) groups within *Valsa*, but over time progressive taxonomic schemes led to segregation and the creation of separate genera from the *Valsa* complex. Such segregation yielded a number of smaller, but more homogeneous taxa. Additionally, Saccardo's system helped in the development of more natural genera as use of spore types could be associated with other pertinent features. Under such a system *Valsa* Fr. was emended by Saccardo (1875, 1882) and was limited to species having perithecia immersed in a stroma with beaks which converged, and ascospores which were hyaline and allantoid. During this period, i.e., second half of the nineteenth century, the number of species included in *Cytospora* grew; however, the incongruity associated with *Valsa* during this same time was much greater than that observed with *Cytospora*.

Von Höhnel (1906-1928a,b) was the first mycologist to scrupulously examine species of *Valsa* and *Cytospora*. In his studies of type specimens the number of species numbers decreased; many were found to belong to different genera while others were reduced to synonymy. Lectotypification of *Valsa* Fr. by *V. ambiens* (Pers.:Fr.) Fr. occurred with von Höhnel (1917).

Von Höhnel (1914b, 1923) also is noted for determining that anamorphs of *Valsa* consisted of various locular forms and comprised four, and perhaps five, anamorphic genera based on these locular as well as their ostiolar arrangements. Among them were *Cytophoma* Höhn., *Cytospora*, *Torsellia* Fr., *Lamyella* Fr., and *Leucocytospora* (Höhn.) Höhn. However, speculation surrounding *Leucocytospora* and whether von Höhnel had erected it as a genus or infrageneric taxon was addressed by Petrak (1921) and Sutton (1980). Spielman (1983) states that although she could find valid existence for *Leucocytospora* at the sectional rank, i.e., *Leucocytospora* (Höhn.) Petr., she was unable to determine whether it had ever been genuinely elevated to the level of genus. In recent years, *Cytophoma*, *Cytospora*, *Torsellia*, *Lamyella*, and *Leucocytospora* have been grouped within a single genus, namely *Cytospora*.

Lastly, von Höhnel (1918) was able to show the ordinal separation of members of the Diaporthales from the Diatrypales based upon characteristics of the centrum. This distinction was later confirmed by Wehmeyer (1926) and Nannfeldt (1932) during their work with pyrenomycetous fungi, and allowed for further species reduction by Nannfeldt (Adams et al., 2005). Studies by Grove (1932, 1935) on British species of *Cytospora* furnished descriptions of the known species of *Cytospora* while Gutner's work (1935) pertained to *Cytospora* spp. of the Soviet Union. However, these compilations unfortunately did not critically examine or evaluate species of *Cytospora*; type specimens were rarely cited, and in the case of Gutner, material used for descriptions is not specified. Gilman et al. (1957) provided a useful investigative study, but their examination of *Valsa* is limited to specimens collected only in Iowa. Kobayashi's work

(1970) is another compilation but it too is limited in that it deals with species from Japan only (Adams et al., 2005).

The monograph by Urban (1958) showed the relationship between von Höhnel's locular types of the anamorph and teleomorphic sections within *Valsa*. A study by Gvritishvili (1982) treated von Höhnel's anamorphic types as infrageneric rankings of subgenera. In his scheme von Höhnel's sections of *Cytospora* are grouped into three subgenera: sections *Cytospora* and *Cytophoma* (Höhn.) Gvrit., in subgenus *Cytospora*, sections *Leucocytospora* (Höhn.) Gvrit. and *Cytosporopsis* (Höhn.) Gvrit. in subgenus *Leucocytospora* Höhn., and sections *Torsellia* (Fr.) Gvrit. and *Lamyella* (Fr.) Gvrit. in subgenus *Torsellia* (Fr.) Gvrit.

Historical review: *Leucostoma, Valsella,* and *Valseutypella*. While Spielman provided insight into *Cytospora* and *Valsa,* Adams et al. (2005) present a well summarized taxonomic history of the other teleomorphs associated with *Cytospora* spp. Again my summary does not include all studies and mycologists covered by Adams et al., but once more emphasizes some of the more important aspects.

As part of his published work (1867 to 1870) with Pyrenomycetes, Nitschke considered *Leucostoma* as a subgenus of *Valsa*; however, von Höhnel raised it to generic rank in 1917. Nevertheless, studies by Gilman et al. (1957), Munk (1957), Gvritishvili (1982), and Vasilyeva (1988, 1994) questioned the generic separation of *Valsa* from *Leucostoma*, *Valsella,* and *Valseutypella*. They were uncertain if adequate distinctness was present to allow separation of these genera from *Valsa* at a generic level. Petrak (1919, 1969), Hubbes (1960a), and Vasilyeva (1988, 1994) supported the view that they were merely variations among species in *Valsa* and as such were either subgenera or

species with no further infrageneric rank. In contrast, other published works, e.g., Defago's (1942), Urban's (1957, 1958), and Barr's (1978, 1990), consider the genera to be distinctly separate.

The generic separation of *Leucostoma* and *Valsa* has traditionally and primarily been based upon the presence/absence of a darkly pigmented zone of fungal tissue - the conceptacle - delimiting the ascostroma. In the case of *Leucostoma* this tissue is typically present whereas in *Valsa* it is generally absent. Vasilyeva (1988, 1994) made note that in the Diatrypales, which has many commonalities with the Diaporthales, the conceptacle as a taxonomic character is considered pertinent only at the species rank. For this reason she felt fungi within the Diaporthales should be treated in the same manner and as such suggested *Leucostoma* be placed at the subgeneric level. By doing so this would emphasize the importance of the conceptacle as a taxonomic criterion in other genera. The importance of the conceptacle for taxonomic purposes at the rank of subgenus was also demonstrated by Gvritishvili (1982) as *Leucocytospora* (teleomorph *Leucostoma*) was viewed as a subgenus of *Cytospora*.

Use of the conceptacle as a criterion for classification appears to be a debatable subject however. For example, in particular collections of *Leucostoma auerswaldii* (Nitschke) Höhn. and *Leucostoma curreyi* (Nitschke) Défago, the conceptacle may be absent or the black basal zones not completely developed. Spielman (1983) also notes that the occurrence of partial or complete black boundaries may occasionally be observed in collections "conclusively identified" as species of *Valsa* based on other taxonomic informative traits. Additionally, she states similar occurrences with *Leucostoma* spp. where pertinent features are present except for the presence of a well-developed

conceptacle. In Vasilyeva's (1994) work such inconsistencies point toward confusion when interpreting her dichotomous key as *L. curreyi* is removed in relation to the presence of the black zone yet *L. auerswaldii* is retained. Other genera can be found that display this irregularity as well. In specimens where the anamorphs *Leucocytospora* and their respective teleomorphs, i.e., *Leucostoma* spp., occur on the same branch, it is not uncommon to find the black boundary absent in the anamorphs yet present in the teleomorphs. Additionally, in some anamorphs of *Leucostoma*, such as *Leucocytospora niveum* (Hoffm.:Fr.) Höhn., the conceptacle is almost always present although in other anamorphic species its presence is variable.

Petrak (1919) and Barr (1978) recognized *Valsella* to be a *Leucostoma* with asci containing more than eight ascospores. However, Petrak (1919, 1969) and Muller and von Arx (1973) failed to accept this trait as an adequate criterion to demarcate species of *Valsella* from *Leucostoma* species. Petrak believed *Valsella polyspora* Nitschke and *Valsella adhaerens* Fuckel to be forms of *Valsa auerswaldii* (subgenus *Leucostoma*) producing more than eight ascospores per ascus. Similarly, he considered *Valsella salicis* Fuckel, *Valsella fertilis* (Nitschke) Sacc., and *Valsella nigro-annulata* Fuckel merely polysporous forms of *Valsa translucens* (De Not.) Ces. & De Not. (subgenus *Leucostoma*). Despite such viewpoints, Vasilyeva (1994) continued to recognize *Valsella* at the taxonomic rank of genus.

The genus *Valseutypella* was erected by von Hohnel (1918a) and contained a single species, i.e., *V. tristicha* (De Not.) Höhn. Additional species, namely *V. multicollis* Checa, G. Moreno & M.E. Barr and *V. khandalensis* Vaidya, were described in 1986 and 1981 respectively. For two of the species, viz., *V. tristicha* and *V. multicollis*, *Cytospora*

anamorphs were described by Hubbes (1960b) and Checa and Martinez (1989) respectively. Vasilyeva (1994), however, reclassified *V. tristicha* as *Valsa tristicha* (De Not.) Lar. N. Vasilyeva. Such placement was based on Vasilyeva's de-emphasis on stromatic tissues being valid as generic characteristics; as such, differences in stroma became valuable at the species level, i.e., a species variable character, similar to the situation regarding the conceptacle in *Leucostoma*. Additionally, Vasilyeva had no intention of restricting the species of *Valseutypella* transferred to that of *V. tristicha* only; however, she apparently was unable to obtain herbarium specimens of the other species (G. C. Adams, pers. comm.).

Species numbers, *Cytospora* species present on *Populus*, and teleomorphic and anamorphic classifications. The number of *Cytospora* species causing disease is uncertain; Adams et al. (2002, 2006) cite works in which the number is approximately 500 and 541, respectively. According to Sutton (1980) as many as 400 species have been described, usually on the basis of host association. From 1882 to 1931 Saccardo described 334 species (Adams et al., 2005). In contrast to the extensive species numbers listed prior, Spielman (1983, 1985) accepted only six on woody angiosperms in North America. Use of physiological specialization, viz., host substrate rather than distinct morphological differences, for species determination has likely contributed to the extensive number of described species. Historically, much of the literature, however, has dealt with but a few select *Cytospora* species, i.e., *C. chrysosperma*, *C. cincta*, *C. leucostoma*, and *C. kunzei*. Presently there is general agreement for the existence of approximately 100 *Cytospora* species (Donk, 1964; Rossman et al., 1987; Kirk et al., 2001; Rossman et al., 2007).

Generally speaking, the bulk of the literature points toward *C. chrysosperma* as the causal organism which produces cankers on *Populus*. Other references, however, indicate a greater number of species are involved. For example, Farr et al. (1989) list six species of *Cytospora* occurring on *Populus*, namely *C. chrysosperma*, *C. leucosperma* (Pers.:Fr.) Fr., *C. leucostoma*, *C. nivea, C. sacculus* (Schwein.:Fr.) Gvrit., and *C. translucens* Sacc. Spielman (1983, 1985) states that three may be present- *C. chrysosperma, C. fugax* (Bull.:Fr.) Fr. and *C. sacculus.* The United States Department of Agriculture-Agricultural Research Service, (http://www.ars.usda.gov/main/site_main.htm?modecode=12-75-39-00), lists five occurring on *P. tremuloides*, i.e., *C. chrysosperma*, *C. leucosperma*, *C. leucostoma*, *C. nivea*, and *C. translucens*.

The causal organism responsible for Cytospora canker in Colorado and other regions of the Great Plains is typically reported to be *C. chrysosperma* (Hinds, 1964; Hinds & Krebill, 1975, Hinds, 1985; Walla & Conway, 1986; Leatherman et al., 1986; Kepley & Jacobi, 2000). Additionally, a number of studies have utilized *C. chrysosperma* isolated from aspen in Colorado as a fungal organism in investigative studies (Guyon et al., 1996; McIntyre et al., 1996; Kepley & Jacobi, 2000) leaving me to question how thorough the examination of morphological characteristics was. Since the identification of *Cytospora* spp. is difficult, and the fact that numerous species are reported to occur on *Populus* spp., I also must speculate whether *C. chrysosperma* was, indeed, the correct organism. This will be further addressed below as part of the discussion concerning host range and pathogenicity studies.

Many of the *Cytospora* species have not been associated with a teleomorph. However, when applicable, some incongruity exists regarding the names of the teleomorph depending on the literature source that is consulted. Barr (1978, 1990) lists *Valsa* Fr., *Leucostoma* (Nitschke) Höhn., *Valsella* Fuckel, and *Valseutypella* Höhn. In addition to the prior genera, another reference (Farr et al., 1989) includes *Eutypella* (Nitschke) Sacc. and *Naemaspora* Pers. as teleomorphs. However, the most current classification (Eriksson, 2006), which contains all accepted genera and higher rankings above the generic level in phylum Ascomycota, has *Valsa, Leucostoma, Valsella,* and *Valseutypella* as teleomorphs of *Cytospora* spp. This scheme is in general agreement with Kirk et al. (2001), Castlebury et al. (2002), Lee et al. (2004) and Rossman et al. (2007). The study conducted by Castlebury et al. determined the phylogenetic relationships of various genera in the Diaporthales by means of analyses of large subunit nuclear ribosomal DNA (nrDNA) sequences. Their work provided better resolution regarding families, i.e., at least six distinct lineages (four families and two complexes) were found to occur within the order, as well as a taxonomic framework for placing asexual (anamorphic) diaporthalean fungi. Molecular analyses placed *Valsa, Leucostoma,* and *Valsella* within Valsaceae and was supported by 100% bootstrap values. However, *Valseutypella* was not addressed during the course of the study. Kirk et al. (2001) accept the family Valsaceae Tul. & Tul. to accommodate the genera *Valsa, Leucostoma, Valsella,* and *Valseutypella.* Lee et al. (2004) used sequence data obtained from analyses of the large subunit nrDNA amplicon to show that isolates of *Valsa ambiens* (Pers.:Fr.) Fr., *L. niveum*, and *Valsella salicis* Fuckel grouped together with 94% bootstrap support within the Valsaceae. As with the study conducted by Castlebury et al.

(2002) *Valseutypella* was not examined. Rossman et al. (2007) did a follow-up study of Rossman et al. (2006) whereby three additional lineages, based on recent phylogenetic data, have been added to the Diaporthales; nine families now comprise the order. Once again *Valsa, Leucostoma,* and *Valsella* are placed within the Valsaceae and recognized as being closely related; however, *Valseutypella* is not addressed in the work.

Adams et al. (2005) conducted an extensive study of *Cytospora* spp. and their associated teleomorphs collected from species of *Eucalyptus* over a broad geographic range. Based on morphological examinations and phylogenetic sequence analysis of the internal transcribed spacer regions of the nuclear ribosomal DNA operon (ITS-rDNA), in which isolates from *Eucalyptus* and those from other hosts were compared for homology, they concluded that *Leucostoma, Valsella* and *Valseutypella* are very closely related to *Valsa*. Excluding *Valseutypella* this would be in agreement with Adams et al. (2002) and Castlebury et al. (2002) and Rossman et al., (2007). All teleomorphic genera based on Adams et al. (2005) are included within family Valsaceae. This study will be discussed in more detail with regards to pertinent monographic works in English which appears later in this text.

Per Eriksson (2006) *Leucostoma, Valsa, Valsella,* and *Valseutypella* are classified as pyrenomycetous (having perithecia) Ascomycetes, subclass Sordariomycetidae O. E. Erikss. & Winka, and belong to the order Diaporthales Nannf. *Valseutypella* is a member of the Gnomoniacaea G. Winter family while *Valsa, Leucostoma,* and *Valsella* are placed in Valsaceae Tul. & C. Tul. Kirk et al. (2001) state Valsaceae contains 293 species, and *Leucostoma, Valsa, Valsella,* and *Valseutypella* are comprised of 10, 60, 2, and 2 species, respectively. The placement of *Valseutypella* in the Gnomoniacaea is in agreement with

Barr (1978); however, in a more recent scheme (Barr, 1990), she places *Valseutypella* within the Valsaceae.

Using the Saccardo system for anamorphic fungi, *Cytospora* is classified as a coelomycetous deuteromycete belonging to the order Sphaeropsidales (having pycnidial-like fruit bodies), family Sphaeropsidaceae (Talbot, 1971). Sutton (1980), however, used a developmental system in which conidial ontogeny was a principal basis for classification of coelomycetes. According to this system *Cytospora* is placed in the class Blastodeuteromycetes (because of blastic origin of conidia), subclass Enteroblastomycetidae (because the origin of the conidium wall is enterothallic), order Phialidales (because of the phialidic ontogeny of the conidiospore), and suborder Phialostromatineae (because of the occurrence of stromatic conidiomata).

Problems associated with species identification of *Cytospora* and associated teleomorphs. For critical morphological studies, one must rely on the formation of the essential diagnostic features that are characteristic of a species and that occur on living plants in nature (Adams et al., 2005). *Cytospora* species have been described based on morphological studies of fruiting bodies from type specimens, other herbarium specimens, and fresh collections. Herbarium specimens from the inoculation of host tissues have been used as type material as well (Adams et al., 2005). Species identification of *Cytospora* has proved to be problematic, however. Adams et al. (2005) state that "accurately identifying a species of *Cytospora* based on morphological features is recognized as a problem that is perhaps insurmountable." Spielman (1983, 1985) states that "due to the difficulty in characterizing the anamorph of *Valsa ambiens* subsp. *ambiens* (Pers.:Fr.) Fr., if teleomorphs are absent the delimitation of *V. ambiens* subsp.

ambiens from other species of *Valsa* is nearly impossible." According to Helton and Konicek (1961), the complex nature of the Cytospora canker problem of stone fruits was apparent in the literature as seen by the "taxonomic confusion" which included synonymy, poor descriptions of environmental conditions, and questionable conclusions; many times there were "outright contradictions of valid conclusions." Regarding *C. chrysosperma* and *Valsa sordida* on *Populus* spp., Christensen (1940) thought that establishing the limits of fungal variation on the host species could lead towards making "some order out of the taxonomic chaos" surrounding these fungi. Concerning the Sphaeriales, Wehmeyer (1926) states the taxonomic literature is "riddled with inaccuracy and confusion" and that "any herbarium will show inaccurately determined exsiccati and immature or half-decayed material" upon which "many a species has been described from such an impossible remnant." He suggested many taxonomists found it easier to describe a specimen as a new species rather than to identify it. Leonian (1921) believed that identifying *Cytospora* to species was difficult due to the many morphological similar forms classified within the genus. It is apparent that rather than proposing new species, current taxa should be carefully reassessed (Spielman, 1983). Furthermore, Adams et al. (2005) make note that problems arise when interpreting the historic and current literature regarding descriptions of species of *Cytospora* as most are not clear as to the major features of infrageneric sections, e.g., locular, ostiolar, and chamber arrangement within stromata of conidiomata. For this reason re-evaluation of type specimens is often needed in order to correctly identify *Cytospora* spp.

Despite the dilemmas species of *Cytospora* have typically been described on the basis of the host, and on morphological features which have been shown to be plastic

(Adams et al., 2002). Spielman (1983) states, that for taxa within *Valsa*, "nearly every morphological character" has the potential for some degree of modification. Such variation, as well as a poor understanding of host ranges, has hindered species delimitation (Spielman, 1985). However, Adams et al. (2005) believe that for *Cytospora* and *Cytospora*-like pathogens of *Eucalyptus*, characters other than morphology and pathogenicity are a necessity to delineate species. They suggest that host preference does not provide a certifiable trait for the genus and the morphology of anamorphs in nature vary a great deal from those forming in culture. Numerous factors which contribute to this variation have made species identification for *Cytospora* difficult. Such factors include differences in hosts and host tissues, environment, culture, and strains. Additionally, variation in spore size and a limited number of host range studies to date, compound the problems associated with species delimitation.

Variation based on host/host tissue. On a single host species, the variability in *Cytospora* fruiting bodies has been shown to make species determinations difficult (Waterman, 1955; Spielman, 1983, 1985). Spielman (1983) believed that the development of the stroma was one of the most variable characteristics; when the natural substrate was absent stromatic fruiting structures could be atypical (Spielman, 1985). Within different collections of the same taxon both ecto- and endo-stroma could range from being very scarce to quite abundant. Thickness of bark and size of branches, i.e., large, thick-barked branches as opposed to small, thin-barked twigs, could have a profound effect on the internal and external structure of the stroma in a collection from a single tree on a given date as well. Spielman (1983) also found that, depending on the stage of development, the fruit bodies of a single taxon could display even more

variation. For example, collections from one date might exhibit fruiting structures with reduced ecto- and endo-stroma, no apparent discs, and thin-walled locules while those from a collection one month later could show profuse ecto-stroma, prominent discs, and thick-walled locules. Additionally, locular size and complexity can be variable. Spielman (1983) examined anamorphic stromata from 11 different hosts from the same local in British Columbia seven months after a light ground fire. All hosts were injured relatively uniformly by the fire and the locular development (as well as amounts of stroma) of *C. chrysosperma* varied widely based upon the host species which was infected. Spielman felt this was an example of the developmental possibilities, as seen by this morphological variation, for a given fungal species based upon differences in host substrates. Similarly, Adams et al. (2002) state that locular morphology can be affected based on the physical and physiological properties of the bark and cambium of the host which could make a given species of *Cytospora* appear dissimilar on different hosts. Adams et al. (2005) also found that locular morphology varied for specimens of *C. eucalypticola* from Australia based upon how deep into the bark fruit body formation occurred. Where development took place deep in the bark conidiomata were unilocular cytosporoid as opposed to rosette cytosporoid when formation occurred near the bark surface. Moreover, for certain *Cytospora* species, e.g., *C. variostromatica*, great variability in the form of locules (unilocular, cytosporoid, and lamyelloid) could be present on one specimen, while at other times such variability occurred only among collections from different geographical locations.

Adams et al. (2005) concur with Spielman (1983) in their observations that the presence and absence of the ecto-stromatic disc within *Cytospora* collections is highly

variable; not only within a collection but within a species as well. It is not uncommon to find conidiomatal stroma lacking a disc adjacent to one with a thick convex disc. As the stroma ages the appearance of the disc also may change from being white in young stromata to becoming dark as the fruit body ages and weathers. Therefore, when numerous collections are compared, variation in disc color can be the result of differences in specimen age at the time collections were made. Disc shape also can be variable based on how erumpent the fungal tissue is through the bark surface. Neck (the portion of the ostiole below the disc and bark) length of the ostioles is another highly variable characteristic within a species based upon the variability occurring in bark thickness of the host.

While working with *C. kunzei* from numerous coniferous hosts, Waterman (1955) found that the size of the stromata and the size and number of the pycnidial locules in a stroma varied widely on any one host. When similar ranges occurring on other hosts were compared, the differences were too small to be worthy of species delimitation. Kern (1955) thought that because stromatic parts could be variable based on the bark anatomy of the host, biological criteria were needed to go along with morphological data for species distinctions. He states that a well-known fungus in an atypical form on a new host could potentially be described as a new species. While growing the plum strain of *Valsa leucostoma* on apple branches, Leonian (1921) found the fungus lost its white stromatic hyphae around the ostioles. Additionally, the stromata became more embedded in the bark rather than adhering to the epidermis.

Variation based on environment. Stromatic fruiting structures can be variable and aberrant under different environmental conditions (Spielman, 1985). Leonian (1924)

believed the same fungus growing under the influence of one group of environmental factors may show the characteristics of one specific organism yet, when subjected to another set of environmental factors, it may produce different morphological features. For this reason he felt it was "highly essential" for the physiological relations of fungi to be investigated in order to properly classify them. Leonian (1921) discovered that while the apple strain of *V. leucostoma* did not develop a white disk out-of-doors in the New Mexico climate, such a disk was formed on sterilized apple twigs in culture tubes where moisture conditions were controlled. The stromatic tissues also expanded more forcefully and pushed through the epidermis in test tubes. Under natural climatic conditions in New Mexico, i.e., out-of-doors, apple branches were not ruptured by the enlarging stroma. Wehmeyer (1924) found that *V. kunzei* grown under axenic conditions in culture tubes on *Thuja plicata* Don. twigs produced abnormal amounts of superficial mycelium due to the high moisture content present in the air of the tubes. While growing on sterilized poplar twigs in culture tubes, Schreiner (1931) noticed there was often no clear differentiation into ecto- and ento-stromatic tissues, but under natural conditions there was typically a distinct grayish ectostroma and a darker but more diffuse entostroma. Christensen (1940) observed that if excess moisture were present on sterilized twigs of poplar, pycnidia were often produced on the bark surface by *C. chrysosperma* rather than within the bark. While working with *C. kunzei*, Waterman (1955) found that on sterilized twigs in culture flasks with high humidity, pycnidia were more stromatic and variable than those found in nature. As with host tissue differences leading to morphological variation, Kern (1955) also states that different external conditions can lead to irregularity in stromatic tissues. Adams et al. (2005) observed that

simple pycnidia were produced in culture for many *Cytospora* spp. which in nature produced cytosporoid conidiomata. Furthermore, species in sections *Lamyella* and *Torsellia* which produce multiple independent locules, viz., locule walls are separate, form rosette conidiomata where chambers have shared walls in culture. In the study conducted by Adams et al. (2006) they found environmental (or host) influences could produce "morphological forms" which can result in a species complex. Based on phylogenetic analyses of ITS-rDNA sequences, rarely reported *Cytospora* species (with unknown sexual states) grouped with the common species *C. chrysosperma*, and formed a clade regarded as the *Valsa sordida* species complex. They surmised that the rarely reported species were probably just an example of the population diversity, and that the morphological extremes exhibited in fruit bodies of the common species were due to environmental and/or host effects. Additionally, they believed that the *V. malicola*/*V. germanica* species complex, based on ITS sequence homology, represented two species, i.e., *V. malicola* Z. Urb. (anamorph *C. schulzeri* Sacc. & P. Syd.) and *V. germanica* Nitschke (anamorph *C. germanica* Sacc.) which were morphologically variable when present on different hosts. On the other hand, it is possible this species complex is representative of a species undergoing divergence. For example, on *Malus*, multiple ostioles form a ring within the disc of a conidioma whereas on *Populus* and *Salix* the conidioma typically displays one central ostiole (within a whitish disc). More thorough morphological studies using type specimens representative of species clustered within a species complex should provide a better idea of the plasticity which can occur with certain characters (Adams et al., 2006).

Cultural variation. The anamorph can be produced in culture from conidia or ascospores, but cultural appearance is quite variable. When the natural substrate and environment is absent, stromatic structures associated with fruit bodies may be atypical (Spielman, 1985). Christensen (1940) found that pycnidial structure, rate of growth on agar, and general cultural characteristics of collections of *Cytospora* from non-*Populus* hosts and collections of *C. chrysosperma* from *Populus* species could not be distinguished. He also found that cultures of some isolates of *C. chrysosperma* were almost identical with those of some isolates of *V. sordida*. Moreover, different isolates of *C. chrysosperma* were more variable than when comparing isolates of *C. chrysosperma* as a group with those of *V. sordida* as a group.

In a series of studies, Helton and Konicek (1961a, b, c) showed that carbon and nitrogen sources, as well as temperature, affected colony characteristics. They suggested the cultural conditions would have considerable bearing on whether or not an isolate could be related to isolates used by other workers. For comparative cultural studies of fungi, Helton and Konicek suggested that standardized laboratory environmental conditions are needed. In an earlier study, Helton and Moisey (1955) found that cultural characteristics were different upon reisolation from an inoculated host if the original host and the inoculated host were different. For example, isolates obtained from Italian prune showed more erratic and rapid growth following reisolation from artificially infected Myrobalan plum. Spielman (1983) observed extreme variability in the appearance of cultures of *Valsa* on eight different agar media. No specific traits, such as color and mycelial growth, were unique to a particular taxon, i.e., *V. ambiens* subsp. *ambiens* (from maple and non-maple hosts), *V. ambiens* subsp. *leucostomoides* (from four maple

species), and *V. ceratosperma* (from maple and non-maple hosts). Additionally, no sectional distinction between *Valsa* (*V. ambiens* subsp. *ambiens* and *V. ambiens* subsp. *leucostomoides*) and *Monostichae* (*V. ceratosperma*) could be made. However, Spielman did observe some correlation between the production of a diffusible purple pigment on some media, viz., potato dextrose agar (PDA), malt extract agar (MEA), oatmeal agar (OMA), and mycological agar-low pH (MYC), and morphological groupings. For example, production of the pigment occurred significantly more frequent in isolates of *V. ambiens* subsp. *leucostomoides* and *V. ceratosperma* from red maple than in *V. ambiens* subsp. *ambiens* or *V. ceratosperma* from non-maple hosts.

Despite the variation, use of cultural characteristics for distinguishing species, e.g., temperature optima for hyphal growth, colony margin and color, size of pycnidia etc., has been used in the past (Schreiner, 1931; Willison, 1936; Hildebrand, 1947; Waterman, 1955; Bertrand & English, 1976b; Dhanvantari, 1982; Proffer & Jones, 1989; Adams et al., 1989; Surve-Iyer et al., 1995; Adams et al., 1998) and continues to be used with some success (Adams et al., 2002). In the 2002 study by Adams et al. differences in cultural characteristics, along with multi-locus isoenzyme analysis and inferences based on phylogenetic analysis of ITS-rDNA, supported the recognition of a new species of *Cytospora* and its associated teleomorph. Adams et al. (2005) also obtained distinguishing *in vitro* information on 18 *Cytospora* species based on growth rates at 37 C, 32 C, and on 2 ppm cyclohexamide.

However, Adams et al. (2006) state that "culture morphology is not sufficient to identify or describe **any** *Cytospora* species" with no workable system available for identification of *Cytospora* species in culture, or on inoculated host tissues *in vitro*

(Adams et al., 2005). This is due to the fact that the present identification system hinges on essential morphological characteristics forming on bark tissues of trees in nature. Examination of many species of *Cytospora* revealed that conidiomatal diameter *in vitro* was not like that found in nature and varied with the medium used. Therefore, use of this feature, as well as conidiomatal density, was not reliable and reproducible to the degree necessary for species separation (Adams et al., 2005). They conclude that "clarifying morphological species concepts in *Cytospora*, constructing a molecular phylogeny, and describing the species in culture" will provide a more workable identification system.

Anamorph/teleomorph relationship. Normally, accurate identification of the anamorph, particularly for certain species, is dependent upon the teleomorph whose morphology is considerably more complex (Adams et al., 1995; Adams et al., 2002). Unfortunately, formation of the teleomorph can be rare in nature and on many economically important hosts they may rarely occur even when numerous cankers are formed (Adams et al., 2002). Teleomorphs also typically do not form on inoculated hosts (Adams et al., 2005).

Although both conidia and ascospores of a particular holomorph can incite infection and produce cankers *in situ* (Leonian, 1921; Jorgensen & Cafley, 1961; Bertrand & English, 1976), cultural studies, i.e., *in vitro*, definitively linking anamorph to teleomorph are limited. Spielman (1985) believed that conclusive evidence for the development of one state in culture, i.e., development of either the anamorph or teleomorph derived from a single spore of the other, was not available. In the same study she also points out that Défago (1942) found production of the teleomorph to be an uncommon and generally non-repeatable event in culture. Wehmeyer (1926) suggested

that while production of the perfect stage in artificial nutrient culture is difficult with pyrenomycetous fungi, more positive results are achieved when the natural substratum is used.

Leonian (1921) was able to induce formation of perithecia for a strain of *Valsa leucostoma* when a favorable nutrient medium was provided. He found that addition of 2 to 10% sucrose or sodium chloride to oatmeal agar stimulated formation of perithecia, whereas additions of more than 2% sucrose to cornmeal agar delayed or inhibited their formation. Leonian also discovered that ascospore isolations of *V. leucostoma* placed on steamed apple twigs produced both the imperfect and perfect stages. However, conidia collected from these pycnidia placed onto another group of steamed apple twigs produced only pycnidia while ascospores produced both pycnidia and perithecia. Leonian (1923) felt that two very similar strains of *V. leucostoma* were present. He presumed the only difference was that in one strain the ascigerous stage of the life cycles had been lost. As such, one strain gave rise to both the imperfect and perfect stages of the fungus regardless of the type of inoculum, i.e., mycelium, conidia, or ascospores. The second strain was incapable of producing perithecia regardless of the environmental conditions. Using the strain capable of producing both reproductive phases Leonian demonstrated that via environmental modifications, e.g., food concentrations (increase/decrease), types of nitrates (ammonium, calcium, sodium, and potassium), and sugars (maltose, glucose, levulose, and cane sugar) etc., production or suppression of pycnidia and perithecia could be manipulated. Leonian concluded that variations regarding control of reproduction were due to these environmental modifications rather than the intrinsic nature of the fungus strictly, i.e., heterothallism vs homothallism. Additionally, Wehmeyer (1926)

observed perithecial formation on sterilized twigs of *Thuja occidentalis* L. inoculated with mycelium from a culture obtained from a single ascus of *Valsa kunzei* (originally isolated from perithecia on twigs of *T. occidentalis*). Single ascus and single spore cultures obtained from these perithecia produced only the perfect stage when *T. occidentalis* twigs were again inoculated but this time placed at 0 to 3 C for *ca.* four months; inoculations made on 6% oat agar produced pycnidia only. Wehmeyer judged that separate sexual strains of the fungus were not needed for formation of perithecia due to the fact that perithecia and subsequent ascospores resulted from single spore inoculations. Rather, he believed it was due to differences in substratum since pycnidia formed solely on agar while perithecia formed only on twigs. Adams et al. (2005) speculated that such an occurrence was potentially the result of secondary homothallism in which a single ascospore could contain nuclei of two mating types. Schreiner (1931) was unable to induce formation of perithecia for *V. sordida* and *V. nivea* via single ascospore cultures on a number of different media including natural substratum.

Christensen (1940) could not find any previous work showing *V. sordida* had been obtained from its anamorphic state, *C. chrysosperma*. He wondered if the Cytosporas commonly found on ornamental poplars were identical with the *Valsa* and *Cytospora* on aspen or whether two or more fungi were present. Christensen had encountered the perfect stage frequently on aspen only. He speculated this was the result of several possible factors including *C. chrysosperma* and *V. sordida* being identical, and the environmental conditions necessary for perithecial formation being encountered less often on some poplar species and varieties; different strains were involved and those common to aspen produced perithecia more commonly; the fungi were taxonomically

distinct despite the similarity in their pycnidia. In an attempt to solve this problem Christensen inoculated sterile aspen bark with isolates of *C. chrysosperma* and *V. sordida* followed by various post inoculation treatments such as alternate freezing and thawing, alternate drying and moistening, ultraviolet light exposure, and long term storage, i.e., over two years. Regardless of the treatment no perithecia formed.

As with the anamorph, proper identification of the teleomorph is dependent upon distinctive morphological characters; however, such traits also can be quite variable (Leonian, 1921; Wehmeyer, 1926; Kern, 1955; Spielman, 1983, 1985; Adams et al., 2002, 2005). Among these variable features are the number, size, and orientation/arrangement of perithecia within ascostromata, the presence, abundance, shape, thickness and color of discs, the number and arrangement of ostioles in the disc, and the presence and abundance of ecto- and endo-stromatic tissues. Additionally, Adams et al. (2005) state that the "variable character of the ascostromata that most hinders determinations is the presence of the conceptacle in a species." Its absence is more typical among anamorphs but variability for both states, i.e., imperfect and perfect, ranges from being absent to pale brown to prominent and black-all within a single collection. As with the anamorph all these features can vary within a single species or collection of a single species depending on bark thickness and host species, stage of development of the fruit bodies on a single tree, or external conditions. Barr (1990) states that due to the vegetative nature of stromatic tissues, considerable developmental variation, i.e., amount and extent, within a taxon is possible. Therefore, such features are useful, but only in concurrence with more stable ones like those of asci and ascospores.

Regarding *Valsa*, Schreiner (1931) thought that characteristics separating described species were often negligible, and where such differences occurred it likely could result from a single species being under the influence of different environmental conditions. As such one would need to know the limits of variation within a given species when interpreting specimens. Similarly, Wehmeyer (1926) believed that the variation exhibited by the stromatic Sphaeriales as a group was fairly restricted, but at the species level they were widely variable. He suggested there is an "all-too-great tendency to separate species and genera without a sufficient conception of what the variation within a given species or genus might be under different conditions." New genera, he deemed, were too frequently erected for every slight variation from the type species. Adams et al. (2005) also point out that there are times when the morphology of the teleomorph is not taxonomically useful at the species level. This point is illustrated in their work with *V. ceratosperma* (Tode) Maire and its anamorph *C. sacculus* in which many specimens have the same necessary morphological characteristics for the species, i.e., small asci and ascospores, and small, numerous upright perithecia etc., but their sequence homology as compared to the specimens used as DNA sources (*V. ceratosperma sensu stricto*) is not closely shared. Thus, the present descriptions of *V. ceratosperma* comprise several different fungi which exist in separate lineages. For this reason species concepts were narrowed to most closely match those of the neotype specimen and the original species concept of Fries (1823). This was represented by the fungus commonly present on *Quercus* sp.

Strain variation. Helton and Konicek (1961a) believed the confusion and contradiction in the literature concerning *Cytospora* was partially due to comparisons of

work involving the teleomorphic state with those involving the anamorphic state. However, they suggested that most isolates were different physiological strains of whatever species they fell within (Helton & Konicek, 1961c). They speculated such strain/form variability within a distinct species could be greater than that between strains associated with related *Cytospora* species (Helton & Konicek, 1961d).

Variation in spore size. In addition to the preceding factors which have made species identification for *Cytospora* difficult, variation in spore size within one species can vary also (Kern, 1955). Christensen (1940) found that conidial sizes among what were considered to be different *Cytospora* species fell within a fairly narrow range. He believed that spore size alone was not a sufficient character to separate species, or that perhaps some species were invalid. Christensen, however, did find that ascospore size between specimens of *Valsa sordida* varied considerably, and suggested that if spore length were to be used as a criterion in taxonomic studies with *Valsa* spp., such variability should be considered. Gilman et al. (1957) concluded that distinctions between fungal species such as those observed in *Valsa* were small, and the decision as to which characteristics would be used for determinations were too subjective. To them, spore size was of less significance than its shape, i.e., the ratio of its length to diameter. For this reason other characteristics were of greater importance such as features of the stroma, disc development and color, and the arrangement of ostiolar necks. Spielman (1985) believed that some morphological characters, i.e., conidial dimensions, were negligible among species and as such made species delimitation difficult, particularly those within a single section. For this reason she did not consider conidial dimension as a species characteristic. For the teleomorph, ascospores of a single species could vary by

as much as 100%. It also was not uncommon to find asci containing less than eight spores in which case they tended to be larger than normal; some asci were found to hold both small and large spores.

The monographic work conducted by Kern (1955) examined European and American specimens and specifically addressed the use of spore size as a taxonomic criterion within the genus *Leucostoma*. Results from the study revealed the inadequacy of using morphological characteristics for delimitation at the species level, even in the presence of the teleomorph. Morphological differences that occurred in nature were small, indistinct, and overlapped, pointing out the existence of intermediate or related forms. Kern believed that a single species could be described as two separate species if one were to focus on the extreme ends of variation in spore size within a species.

Wehmeyer (1926) though, considered that spores provided "one of the most nearly constant and dependable characters" at the taxonomists' disposal with spore size being of use primarily as a specific character. He believed that because variations in spore characteristics were a species-related phenomenon and not due to environmental conditions, they could more easily resolve species variation than nearly any other character. However, he did suggest that other characters should be used in conjunction with spore characteristics as taxonomic criteria.

Adams et al. (2005) state conidial width is a distinctive characteristic, with differences of 1μm diameter versus 1.5μm diameter being significant. Therefore, accurate measurements could be used for species separation. However, they think that inaccurate measurements occur for many descriptions in the literature with type material commonly recorded as wider than what current measurements reveal. Adams et al. also

found ascospore width to vary little within a species, but among species it was distinctive and rare and thus useful as a basis for species differentiation.

Host range and pathogenicity studies. With species identification of *Cytospora* problematic due to a poor understanding of host ranges and the morphological variation that occurs with fruiting and vegetative structures as well as spore size, limited works investigating host range and pathogenicity have been conducted (Schreiner, 1931b; Wright, 1942; Sproston & Scott, 1954; Waterman, 1955; Helton & Moisey, 1955; Helton, 1961; Helton & Konicek, 1961d; Spielman, 1983; Proffer & Hart, 1994; Kepley & Jacobi, 2000). Many species of *Cytospora* attack a diverse group of woody plant species and other *Cytospora* spp. are host specific (Farr et al., 1989). The study conducted by Kepley and Jacobi (2000) examined but a few host tree species and associated fungal isolates in Northern Colorado. Although some of the isolates from this study were host specific it only suggested the possibility of different strains or species of *Cytospora.* Later work conducted by Jacobi & Adams (unpublished data) found that the morphological species *C. fugax* which was used in the experiments consisted of two genetically discrete fungi. In the experiments *C. fugax* isolates were pathogenic only on the *Salix* sp. from which they were isolated. Additionally, *C. sacculus* isolates obtained from *Ulmus pumila* L. were pathogenic on *U. pumila*, as well as *Populus deltoides* Bartr. ex Marsh. in one experiment. As was the case with *C. fugax*, *C. sacculus* was composed of two genetically distinct fungi as inferred by sequence differences in ITS-rDNA.

The preceding examples suggest that results of host range/preference studies need to be carefully examined. Adams et al. (2005) state that regardless of the care taken in the designing of host preference studies with *Cytospora* spp., problems can arise due to

the "dependence on morphological characteristics to identify species." Because numerous *Cytospora* spp. exist, and their host range is quite broad, as well as the belief that host range does not provide a verifiable characteristic for species delineation (Adams et al, 2005), it is apparent additional methodology to genetically identify *Cytospora* species should be utilized. Such methodology must be sensitive enough to sort out the overlap, even from one locale, which appears to occur between morphological species concepts and ranges of biological species (Adams et al., 2005). Despite the numerous difficulties associated with the use of morphological criteria, *Cytospora* species continue to be separated with macro and microscopic characters such as stromatic tissues of the pycnidium, locular size, shape, and arrangement, conidiogenesis, and spore characteristics.

Some pertinent monographic works in English. European plant material was used for much of the early taxonomic work with species of *Valsa, Leucostoma, Valsella*, and *Cytospora*. Nearly 200 species in *Valsa* and more than 50 in *Cytospora* were established for North American specimens on hardwood hosts based on works which used European plant material.

At present the most comprehensive taxonomic keys in English are Spielman's (1983, 1985) and Adams et al. (2005, 2006). Spielman's key only deals with North American species of *Valsa* and *Cytospora* on hardwoods. Adams et al. (2005) primarily address species on *Eucalyptus* worldwide while Adams et al. (2006) examines species from a diverse group of woody plants worldwide. Other less comprehensive works have also examined *Cytospora* and associated teleomorphs.

Barr (1978). As part of her assessment of the Diaporthales, Barr examined *Valsa*, *Leucostoma*, *Valsella*, and *Valseutypella*; however, her coverage of *Cytospora* is only briefly touched upon, in comparison to those of Spielman and Adams et al., and primarily addresses generic concepts. The extent of her treatment of the anamorphs is not of great detail stating that stromata are similar to those of the perithecial stromata, i.e., conidial locules are formed in stromata. Such locules may be uni-or multi-loculate. Additionally, conidiogenous cells are phialidic and produce one celled, hyaline, allantoid or cylindroid conidia. Actual conidial dimensions are given on three occasions only.

Barr's concept of the Diaporthales is based upon Luttrell's (1951) work for the family Diaporthaceae. Members of the Diaporthales have a centrum comprised of pseudoparenchymatous tissue during early stages of development. Paraphysis-like cells, **not true paraphyses**, may occur in the centrum, but usually are absent in mature perithecia. Such features are characteristic of Barr's Subclass Parenchemycetidae within the Euascomycetes. Asci of the Diaporthales are typically elliptic or broadly oblong, and for many genera become deliquescent near the base at maturity, thus floating free in the centrum. Barr states that the refractive apical ring of the ascus is chitinoid and nonamyloid, and "apparently", nonfunctional. The peridium, i.e., external wall tissues of the perithecium, is organized in a textura epidermoidea pattern. Elongate beaks are usually formed and periphyses line the canals of ostioles. Hyphae in the beak surfaces are organized in textura porrecta, forming elongated cells which are oriented vertically.

For this work Barr utilized the absence or presence and type of stromatic tissues, perithecial and beak positions in relation to the substrate, and ascospore shape, septation, and wall structure, i.e., firm or thin-walled, as the primary variable characteristics for

taxonomic evaluation. In this scheme *Valseutypella* is placed within the suborder Gnomoniineae, family Gnomoniaceae, subfamily Mamianioideae, and tribe Endothieae. *Valsa*, *Leucostoma*, and *Valsella* are all members of suborder Gnomoniineae, family Valsaceae, and subfamily Valsoideae; however, *Valsa* is placed within tribe Valseae while *Leucostoma* and *Valsella* are associated with tribe Diaportheae.

Barr separates *Valseutypella* from other Diaporthaceous members of the group, viz., *Leucostoma*, *Valsa*, and *Valsella*, based upon the presence of a stroma composed of sclerotial thick-walled cells, upright perithecia in which the beaks are central to eccentric and separately erumpent, i.e., they do not converge through a stromatic disc, and asci which are usually four spored. The other members of the group are marked by the presence of horizontal or oblique perithecia with beaks which are oblique or lateral and converge through a disc. *Valsa* is distinctive in that it lacks a dark marginal zone of compact tissues, defined as a black conceptacle, which delimits the stromata. Barr recognizes two sections within *Valsa,* i.e., Valsa and Monostichae, which are separated based on the number, size, and arrangement of perithecia, and the amount of development of the ento-stroma and ecto-stromatic discs. *Leucostoma* and *Valsella* are distinguished from one another based on the number of ascospores present within asci; *Leucostoma* typically contains eight or less, hyaline allantoid spores while *Valsella* has polysporous asci.

Barr (1990). In a more recent monograph, Barr reforms her earlier concepts of tribes. *Leucostoma*, *Valsa*, *Valsella*, and *Valseutypella* are grouped within the Valsaceae as members of class Hymenoascomycete, subclass Parenchymycetidae, order Diaporthales. Barr separates subclasses based upon centrum characteristics,

absence/presence and type of hamathecial tissues, and peridium structure. Parenchymycetidae is characterized by the thin-walled pseudoparenchymatous tissues of the centrum which break up during asci maturation allowing asci to fill this space. In some species short chains of cells or paraphyses can develop within the centrum and such sterile cells may become deliquescent as asci mature, or persist through asci maturation. Tissues of the peridium are typically comprised of a few rows of pseudoparenchymatous cells. Within the Diaporthales, members have ascomata which are normally immersed and erumpent, hemibiotrophic, biotrophic, or at times saprobic lifestyles, absence of paraphyses at maturity, and asci which become loose at the base and fill the centrum. Valsaceae is separated from other families within the Diaporthales based on the presence of allantoid ascospores.

Per Barr, *Valseutypella* has dark pseudoparenchymatous stromatic tissues, whereas *Leucostoma*, *Valsa*, and *Valsella* have prosenchymatous stromatic tissues with ascomata in a valsoid configuration and beaks which are convergent and erumpent through ectostromatic discs. *Valsa* is delimited by the presence of poorly developed ento-stromatic tissues and well developed ecto-stromatic discs while *Leucostoma* and *Valsella* have well developed ento-and ecto-stromatic tissues. As with her prior study of 1978, *Leucostoma* and *Valsella* are separated on the basis of spore numbers per asci, i.e., *Leucostoma* is octosporous whereas *Valsella* is polysporous. The description of Valsaceae per Tulasne and Tulasne (1861) states that the stroma may at times be delimited by a blackened marginal zone; however, for the descriptions of the prior genera Barr makes no note as to the presence or absence of such a zone for any of these genera. It appears that use of the conceptacle for taxonomic purposes is considered to be of no

relevance in this monograph. Coverage of *Cytospora* within this study is even less than that in the 1978 monograph.

Within her study pertaining to the Diaporthales Barr (1990) makes note of some other works which have examined and classified fungi within this group. Wehmeyer (1975) recognized three families within the Diaporthales, namely Gnomoniaceae, Diaporthaceae, and Valsaceae. His scheme separates Valsaceae based on the presence of allantoid ascospores with Gnomoniaceae and Diaporthaceae each containing genera with non-allantoid spores; however, members of Diaporthaceae are stromatic as opposed to non-stromatic forms in the Gnomoniaceae. O. Eriksson and Hawksworth (1988) accepted Barr's (1978) families plus an additional one, viz., Melogrammataceae; however, their study published in 1990 accepted only Melaconidiaceae and Valsaceae which is in agreement with Cannon (1988). Barr (1990) disagrees with Cannon's view that centrum details were not well known and therefore their use for taxonomic purposes may not be significant.

Gilman et al. (1957). The monograph by Gilman et al. addressed *Valsa* and *Valsella* but did not accept the generic separation of *Leucostoma* from *Valsa*. They state that the uniqueness in stromatic characteristics referred to by Wehmeyer were present, but not to the extent to imply generic distinction. As such, all taxa comprising *Valsa* and *Leucostoma* were considered members of *Valsa* (family Diaporthaceae, section Valseae); however, only specimens collected from Iowa were studied. In their work, 22 species on 21 host genera are described, but a considerable number have been reduced to synonymy. Additionally, anamorphic descriptions are barely addressed; conidial sizes for only some of the species are given.

Kern (1955). Although Kern's work examines *Leucostoma* primarily, he does address *Valsa* and *Valsella* stating that he is in agreement with von Höhnel in that all three should be raised to the rank of genera. *Valsa* is unique due to the absence of a black basal zone (conceptacle) which surrounds fructifications. *Leucostoma* and *Valsella* differ as *Leucostoma* has asci with 4 or 8 spores while asci in *Valsella* contain many spores. Kern recognizes two groups of species in *Leucostoma* with one having a white or whitish stromatic disc and the other marked by the presence of a grayish brown to dark brown disk. Additionally, species are delimited in his scheme by conceptacle shape and thickness, the range in ascospore size and host which is affected. Morphological features of the anamorphic states are examined in somewhat more detail than Barr (1978, 1990) and Gilman et al. (1957) with the structure, i.e., irregularly folded, chambered, simple, radiate, of conidial stromata (pycnidial cavity) noted, and disc color, number of ostioles, conceptacle thickness, and conidial sizes addressed.

Wehmeyer (1926). Wehmeyer's monograph examined the works of Lindau (1897) and von Höhnel (1918), and suggested a new arrangement for the allantoid spored Ascomycetes. Within his scheme Allantosphaeriaceae included the diatrypaceous forms (asci with long persistent stalks with formation of a definitive hymenial layer of asci) while Diaporthaceae comprised diaporthaceous forms (asci with short and temporary water soluble stalks leading to free asci within the perithecium). *Valsa*, *Leucostoma*, and *Valsella* were placed within the Diaporthaceae and tribe Valseae. As was the case with Barr's work (1978, 1990), generic concepts are the main focus in Wehmeyer's study.

Valsa is distinguished based on the formation of a conical ecto-stromatic disc which is erumpent, with perithecia lying below the ecto-stroma in the bark cortex. No

well differentiated area of ento-stroma is present; ento-stromatic tissue may exist to a slight degree around the perithecia only, leaving perithecia embedded in primarily unchanged bark cortex. Additionally, no dark marginal zone (conceptacle) is present. In *Leucostoma*, delimitation of the stromatic area by the conceptacle occurs early on during stroma development therefore limiting its size. The position, i.e., depth, of the conceptacle tissue varies with species, being positioned just beneath the periderm in some versus deep penetration into the bark in others. Perithecia are surrounded by well developed and strongly differentiated ento-stromatic tissues. *Valsella* differs from *Leucostoma* primarily on the basis of polyspored asci in *Valsella*. Wehmeyer discusses *Cytospora* in some depth in this study. He states that the chambers, i.e., locules, within which conidia are produced, are numerous and often coalesce to form a labyrinthiform structure. Conidia are small, one celled, non-pigmented and allantoid in shape. For *Valsa*, the thin-walled labyrinthiform locules of the anamorph are embedded in the cortex with sparse ento-stroma surrounding them at maturity. The anamorphs for *Leucostoma* and *Valsella* also have locules which are numerous or labyrinthine, but stromatic development is more extensive. Additionally, a conceptacle surrounds the stroma; however, at times the stromatic tissues become so blackened that the boundary may be masked.

With regards to the Sphaeriales as a whole, Wehmeyer discusses stromatic tissues in considerable detail. He believed the stroma depicted one of the best examples of the morphological variation which could occur within a given species (or genus). He concluded that differences associated with this tissue resulted in the many transitional forms found amongst "so-called species." Wehmeyer defines stroma as an accumulation

of vegetative/sterile mycelium, i.e., its origin is not the result of a sexual stimulus. This mass of differentiated mycelium may be loosely compacted, a mixture of hyphae and host tissue, or occur as a solid mass of pseudoparenchymatic or pseudoprosenchymatic fungal tissue. An outer distinct layer may or may not delimit the stroma. Differentiation of the stroma can result in two types of tissue, viz., ecto- and ento-stroma. Wehmeyer's uses the terms in relation to differences in structure and position rather than function since in some genera, such as *Leucostoma*, the conidial locules arise within the ento-stromatic tissue rather than the ecto-stroma which is more typical for fungi in this group. Ecto-stroma normally consists of fungal tissue only and can be located on the bark surface or, as in the case of *Cytospora* and its associated teleomorphs, beneath or within the periderm. Ento-stroma is located within the cortex or wood tissue of the host and has elements of both fungal and host tissue. Depending on the fungal species these tissues may be differentiated from one another quite sharply or in others not clearly defined. Also, there are cases where the terminology becomes rather loose such as with species of *Leucostoma* in which the mature ento-stroma only has but a few remnants of disintegrated bark cells.

Regarding the conceptacle which surrounds the ento-stroma in many genera of the stromatic Sphaeriales, Wehmeyer believed this blackened tissue served to protect the internal stromatic tissue from the nutritive absorptive hyphae in the surrounding bark cells of the host; ento-stromatic hyphae in this delimited area would bind bark cells and serve as protective and nutritive tissue for developing perithecia. Additionally, Wehmeyer states that in species where a disc, i.e., that portion of the stroma which erupts through the periderm or epidermis, is present it may be comprised of either ecto- or ento-

stromatic tissue. If the disc is composed of ecto-stroma it may appear as a very definitive conical stroma, or irregular and cushion-like and so intergrown with ento-stroma in the superficial bark layers that differentiation of the two tissues is hard to discern.

Spielman (1983, 1985) and Adams et al. (2005) also discuss stroma in some depth as well. Spielman states that reproductive structures are always associated with stroma - a sterile mass of fungal cells. A correlation between cell type and its location is evident in regards to stromatic tissue. More shallow areas like discs, or regions just beneath the bark, are composed of tightly packed cells with thick, pigmented (brown to olive-gray) walls. The contents of these cells are non-staining in cotton blue and their shape varies from hyphal-like to angular to globose near the disc surface. Cells of the disc region break up leaving an amorphous area of variable color which is responsible for the furfuraceous quality of this area. As per Wehmeyer (1926), Spielman refers to this tissue as ecto-stroma, and this tissue is the location of locular formation in anamorphs. Stroma in deeper regions of the reproductive structure is comprised of non-pigmented, thin-walled hyphal cells in a loose arrangement, along with host cells scattered throughout. Contents of the hyphal cells will stain with reagents such as cotton blue. Stroma in this region is called ento-stroma, again with reference to Wehmeyer (1926), and is the site of perithecial formation. Spielman reiterates Wehmeyer's (1926) comments regarding delineation of the two stromatic tissues, i.e., at times it may be distinct (an abrupt transition) whereas other times a margin is hard to locate (a gradual transition).

Adams et al. (2005) make reference to Spielman and Wehmeyer regarding their definitions of stromatic tissues which were designated according to hyphal tissue types and position within the bark. In contrast, Adams et al. choose to define ento-stroma as

any stromatic tissues, irrespective of the cell types, cell qualities etc. involved, which surround perithecia or pycnidia. Tissues external to this would be considered ecto-stroma. They think that using the nature of the cells (such as when cells begin to break down and become amorphous and furfuraceous, as described in Spielman's ecto-stromatic regions of the disc) as the defining feature is confusing. They point out that these qualities are common for discs of leucostomoid and polysporous leucostomoid *Valsa* species and their associated anamorphs. However, these tissues have more resemblance to the ento-stromatic tissue which surrounds the perithecia. Adams et al. are in agreement with Spielman in that tissues of the ascostroma in species of *Valsa* are pseudostromatic and therefore contain fungal cells interspersed with host cells.

According to Adams et al. (2005), the extent and profusion of stromatic tissues are important taxonomic characteristics for species delimitation, particularly for the region below the disc down to the perithecial globes. In some cases ecto-stroma may surround ostiolar necks for part of their length while at other times ento-stroma may encircle necks for their whole length, or envelop only a portion or the entire perithecial globes.

Careful examination of the literature reveals that the interpretation of stromatic tissue is somewhat subjective and as such has a large impact on fungal descriptions. With this in mind it seems the taxonomy for morphologically similar taxa is complicated by different perceptions or the use of different taxonomic parameters.

Spielman (1983, 1985). The monographic works by Spielman reevaluated the *Valsa* and *Cytospora* species present on hardwoods in North America based on morphological studies of type specimens, herbarium specimens, and fresh collections.

These works resulted in a reduction in the number of accepted *Cytospora* species, viz., only six are recognized, with some species being identified from a wide variety of hosts. Although her teleomorphic studies deal only with *Valsa*, she does make note (1983) as to it being closely related to *Leucostoma* and *Valsella* and that mycologists such as Munk and Gilman do not accept them as separate genera. Her separation of *Valsa* (1983, 1985) from the other two genera is based on the absence of a black, saucer-shaped zone in the well developed ento-stroma below perithecia, as well as a lack of chalky-white discs.

Teleomorph/anamorph association. Spielman based all connections between the teleomorph and the anamorph on the following criteria: (1) close association between teleomorph and anamorph; (2) consistent association in collections from different localities; (3) both states existing in the same stroma; and (4) similar tissue types occurring in the stromata of both states. She thought these criteria necessary because of a lack of evidence that a single spore of one state could give rise to the other state, and that the teleomorph could not be produced in culture. Additionally, the uniformity which occurred in conidial dimensions, the variability displayed in cultures of the anamorph, and irregularities that occurred in stromatic fruiting structures in the absence of natural substrate and environment contributed to the importance of these criteria as well.

Teleomorph morphology. The teleomorph consists of a fruiting structure (ascoma) that is erumpent through the periderm, that is pseudostromatic, and that contains a group of immersed perithecia. Beaks of the perithecia converge and emerge through a region of stroma called the disc which is externally visible. A region of internal stromatic tissue, defined by Wehmeyer (1926) as ento-stroma, is composed of loose hyphae and host cells; in the disc, and just beneath the bark, the stroma is

constructed strictly of fungal tissue consisting of compacted, thick-walled cells and are termed ecto-stroma (Wehmeyer, 1926). The development of this stromatic tissue occurs via the development and differentiation of immersed mycelium. The perithecial centrum is Diaporthaceous and follows Barr's (1978) classification scheme whereby asci are detached and have no associated paraphyses at maturity. Each ascus has a refractive, non-amyloid, apical ring and typically contains eight hyaline, allantoid ascospores.

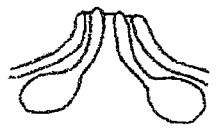
Anamorph morphology. Stromatic fruiting structures consist of pycnidia immersed in ectostroma. Each pycnidium opens to the outside by means of one to several ostioles. Cavities of the pycnidia are lined with phialidic conidiophores that produce hyaline, allantoid conidia.

***Valsa* sections.** Spielman's classification system is less complex than that of Gvritishvili's with no groupings into subgenera. Under her system species of *Valsa* are separated into three morphological sections: *Valsa*, *Monostichae* Nits., and *Cypri* Urban. (Figure 1.1). Section *Valsa* usually produces less than 20 perithecia per ascoma and is distinguished by large perithecia, asci, and ascospores. Perithecia are often in a circular arrangement and lie in the wood outside the disc. The beaks of perithecia are inserted laterally and do not fill the disc. Generally the ecto-stroma is well developed and the ento-stroma is sparse. Members of section *Monostichae* have small asci, ascospores, and perithecia and usually more than 15 perithecia per ascoma. Perithecia are grouped beneath the stromatic disc in somewhat of an upright position with the closely packed beaks normally filling the disc. The ecto-stroma is sparse while the ento-stroma is well developed. Section *Cypri* has fused perithecial beaks which form a characteristic "disc."

The large, upright to lateral perithecia occur in irregular clusters. Perithecia usually number less than 20 per ascoma and contain large asci and ascospores.

***Cytospora* sections.** According to Spielman, *Cytospora* has four morphological sections: *Cytospora*, *Torsellia* (Fr.) Gvrit., *Cytophoma* (Höhn.), and *Cytosporopsis* (Höhn.) Gvrit. (Figure 1.2). Section *Cytospora* is comprised of those species having one intricate, branched pycnidial locule per conidioma. This locule is composed of a continuous mass of ecto-stromatic tissue within which lies numerous interconnected, radially to irregularly arranged chambers. Section *Torsellia* is comprised of species with more than one locule per conidioma. Each locule is a bag-like structure with its own wall. Individual locules join one another only near the center and this convergence forms the ostiolar canal(s). The locules are easily separated despite the fact they are usually surrounded by ento-stromatic tissue. Section *Cytophoma* is distinguished by a single globose locule per conidioma. Just beneath the bark this locule has a distinctive ecto-stromatic collar encircling the ostiolar region. Section *Cytosporopsis* also has a single locule per conidioma, but differs in the fact that it is shaped like a flattened doughnut and has a circular ostiolar canal with a plug in the center.

Teleomorph/anamorph relationship. Within Spielman's system there is generally a one-to-one correlation between the teleomorphic and anamorphic sections: *Valsa* section *Valsa* pairs with *Cytospora*, *Valsa* section *Monostichae* with *Torsellia*, and *Valsa* section *Cypri* with *Cytophoma.* However, *V. melanodiscus* Otth, (and its associated anamorph which is in section *Cytosporopsis*) has features found in sections

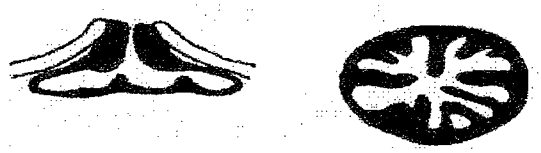


Valsa section *Valsa*

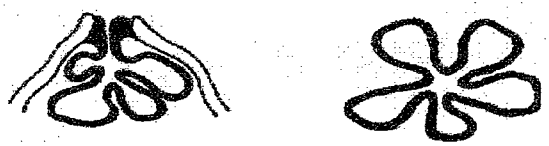
Valsa section *Monostichae*

Valsa section *Cypri*

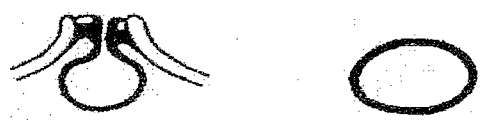
Figure 1.1. Diagrams illustrating the teleomorphic structures of the three sections of *Valsa* (based on Spielman, 1983 & 1985).



Cytospora section *Cytospora*



Cytospora section *Torsellia*



Cytospora section *Cytophoma*

Cytospora section *Cytosporopsis*

Figure 1.2. Diagrams illustrating the anamorphic structures of the four morphological sections of *Cytospora* (based on Spielman, 1983 & 1985). Longitudinal section on the left, and cross-section of the lower part of the conidioma on the right.

Valsa and *Monostichae*, but is placed in *Monostichae* (with ambivalence) due to ascospore size and ento-stromatic development. Urban's (1958) monograph placed *V. melanodiscus* in section *Valsa*, but he suggested it perhaps should be in a section of its own, and Spielman is inclined to agree. Furthermore, Spielman raises section *Leucocytospora*, which contains anamorphs of *Leucostoma* and *Valsella*, to the rank of genus. She also synonomizes *Lamyella* and *Torsellia*, i.e., section *Torsellia*; Gvritishvili's separation was based on differences in the number of ostioles which Spielman found to be a variable feature and not taxonomically pertinent at the generic or sectional level. Spielman makes no groupings into subgenera and therefore the anamorphs of *Valsa* species are separated among sections *Cytospora*, *Torsellia*, *Cytophoma*, and *Cytosporopsis.*

Adams et al., 2005. Within this monograph species of *Cytospora*, and *Cytospora*-like fungi, occurring primarily on *Eucalyptus* spp. world wide are addressed. Via morphological examination of type specimens, herbarium specimens, and various *in vitro* methods, the comprehensive study emends descriptions of several *Cytospora* spp. (with two species synonymized) to include locule forms in conidiomatal stromata, delimits a number of new species of *Cytospora* and *Valsa* on *Eucalyptus* via morphological characteristics of anamorphs and teleomorphs, provides corresponding cultural characteristics for the fungi, and constructs a phylogeny using ITS-rDNA sequence data.

For the phylogenetic analysis thirty-three unique ITS-rDNA sequences obtained from 62 *Cytospora* and three *Cytospora*-like isolates from *Eucalyptus* were compared with those of *Cytospora* species from different hosts. Additionally, fungal morphology

on natural substrates and in culture was studied to provide unique features for potential correlation with lineages derived from the analyses. For the collections examined, *Cytospora* on *Eucalyptus* showed considerable morphological and genetic variability with as many as 15 putative lineages revealed. Moreover, most of these lineages were found to be unrelated to those of commonly known *Cytospora* species on hardwoods and conifers in the Northern Hemisphere. Furthermore, *Valsa ceratosperma* on *Eucalyptus*, as well as other hosts (*Quercus*, *Malus*), fits the description of *Valsa ceratosperma* sensu stricto but consists of several distinct lineages. It was therefore necessary to choose one which most closely correlated to the original species concept.

Anamorphic morphology (conidiomatal forms). Morphological examination of specimens from *Eucalyptus* showed a wide range in variability with characteristics typical of the *Cytospora* infrageneric sections *Cytospora*, *Torsellia*, *Lamyella*, and *Leucocytospora*; most were found to be representative of members of section *Lamyella*. Some *Cytospora* species were found with a combination of locule types typical of several sections while other species displayed characteristics that were distinct and not associated with recognized sections. Adams et al. believe that the use of subgenera and sections complicates and reduces the understanding of the taxonomy for this group of fungi. Therefore, based on the phylogenetic analyses and morphological studies, they reject Spielman's (1985) and Gvritishvili's (1982) work in which infrageneric rankings are based upon morphological features such as locule types (sections *Cytophoma*, *Torsellia*, *Lamyella*, and *Leucocytospora*), and propose new descriptive terminology for locule forms which are more explicit and allow for easier distinction between locule forms/arrangements in stromatal tissues (Figure 1.3). The new descriptive terms for

conidiomata of *Cytospora* which have been substituted for characteristics common to former subgenera and sections are as follows:

cytophomoid: Conidiomata containing an undivided locule and wing-like (in longitudinal median cross section) or ring-like ecto-stroma around the ostiole.

unilocular: Conidiomata having an undivided locule without a wing- or ring-like ecto-stroma encircling the ostiole. A disc may or may not be present.

cyclocytosporoid: Conidiomata comprised of an undivided toroid (doughnut-shaped) locule and ostiolar tissue which forms a central column.

torsellioid: Conidiomata having multiple independent, i.e., separate walls, locules sharing one ostiole.

lamyelloid: Conidiomata containing multiple independent locules with multiple ostioles.

cytosporoid: Conidiomata having a single locule which is subdivided into several chambers by invaginations (locule walls are shared). Two distinct forms can occur with this type of conidiomata, i.e., **rosette cytosporoid** in which there are typically 3 to 7 regular, radially arranged locules, and **labyrinthine cytosporoid** where invaginations are complex, labyrinthine and irregular other than at the margin. Additionally, it is difficult to precisely count the number of locules and conidia and conidiophores tend to be larger than the rosette type. This is the type of conidiomata formed by the type species of *Cytospora* (*C. chrysosperma*).

leucocytosporoid: Conidiomata having the same characteristics as cytosporoid conidiomata except a dark conceptacle is present which delimits the stroma. Two

distinct forms can occur with this type of conidiomata, i.e., **rosette leucocytosporoid** in which there are typically 3 to 7 regular, radially arranged locules, and **labyrinthine leucocytosporoid** where invaginations are complex, labyrinthine and irregular other than at the margin.

leucotorsellioid: Conidiomata comprised of the torsellioid form, but with a dark conceptacle delimiting the stroma. This form is a combination of former sections *Torsellia* and *Leucocytospora.*

Distinguishing some of the locule types of *Cytospora* species can be extremely difficult, particularly when trying to determine if locule walls are independent or shared. Often times locules are crowded and compressed which alters their shape and orientation. Such deformation can make independent locules appear as a single locule divided by invaginations forming numerous chambers that share common walls. Adams et al. state that even with the most common conidioma type, i.e., a rosette shape formed when a single locule is divided into approximately six chambers that are radially arranged, interpretations as to whether a thick shared wall or a double wall exists are quite difficult. Such determinations must be made before one can place a species of *Cytospora* into the correct morphological (locule form) group, e.g., cytosporoid vs. torsellioid.

Teleomorphic morphology (ascostromatal forms). Adams et al. work is consistent with an earlier study (Adams et al., 2002) which suggested *Leucostoma* and *Valsella* were synonyms of *Valsa*. Additional data obtained from the 2005 monograph supports the hypothesis that *Valseutypella* should be included as a synonym of *Valsa* as well. *Leucostoma* and *Valseutypella* are considered collections of *Valsa* with distinct

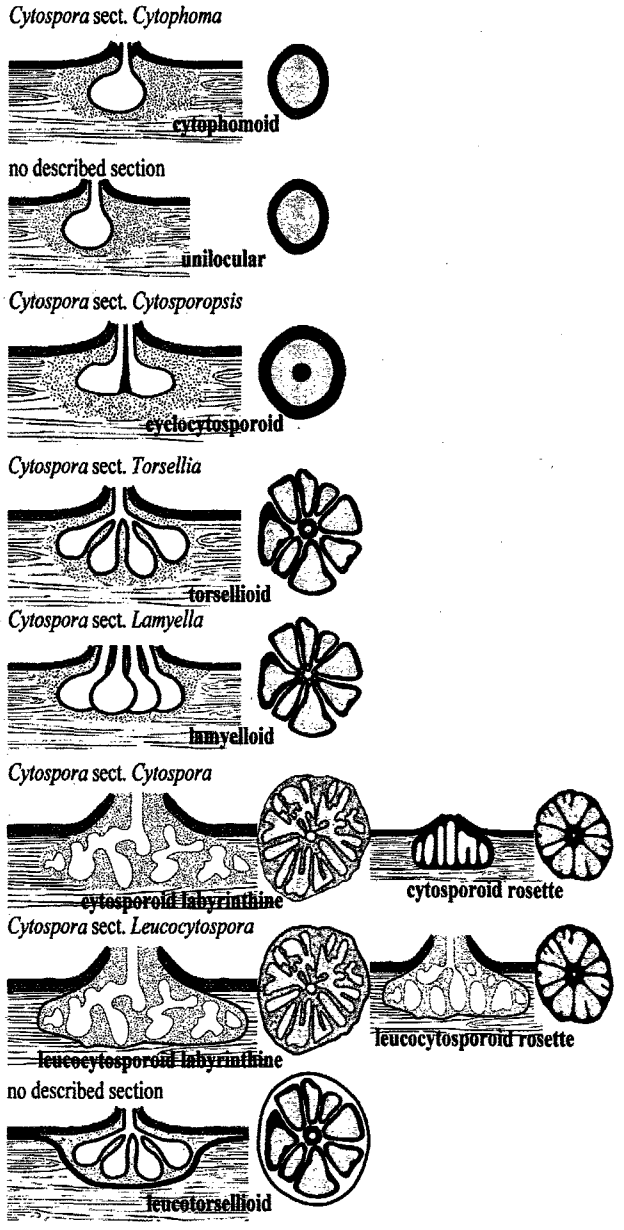


Figure 1.3. Diagrams of longitudinal and cross-sections of conidiomata illustrating the locule forms that are the basis of the former infrageneric sections of *Cytospora.* New descriptive terms, which appear in bold, are used for features of the former sections (based on Adams et al.. 2005).

ecto- and ento-stromatic teleomorphic morphologies while *Valsella* is considered to be variant populations of *Valsa* which produce multi-spored asci.

Similar to the anamorph, Adams et al. suggest that the established teleomorphic sections and subgenera are artificial, and fail to show natural or phylogenetic relationships. However, as with the infrageneric ranks of the conidiomatal forms, the sectional and subgeneric ascostromatal forms are of practical use in describing the morphology of species. Once again new descriptive terminology has been substituted for the unique morphological characteristics of the ascostromatal tissues which distinguish the former sections and subgenera (Figures 1.4 and 1.5). Such terminology, listed below, should allow for the inclusion of additional *Valsa* species with novel combinations of morphological traits and provides better clarity and understanding of the complex ascostromatal forms of *Valsa* species.

euvalsoid: Having ascocarps with or without stroma and not delimited by a conceptacle.

leucostomoid: Having ascostromata which are delimited by a conceptacle.

polysporous leucostomoid: Having ascostromata which are delimited by a conceptacle and asci that are polysporous.

valseutypelloid: Having ascostromata comprised of perithecia which are surrounded by well-developed stromatic tissues composed of pseudoparenchymatous tissues.

circinateous: Ascostromata containing only a few large perithecia with large ascospores (approximately 16 to 35μm long). Perithecia are laterally inclined and arranged circinately.

monostichous: Ascostromata having numerous small perithecia with small ascospores approximately 6 to 12μm in length. Perithecia are upright to inclined and crowded.

cyprious: Ascostromata containing perithecial ostioles which are fused.

Teleomorph/anamorph relationship (ascostromatal forms associated with conidiomatal locule types). Results from the Adams et al. (2005) study are not in agreement with Spielman's (1985) work which states there is a one to one correspondence (except for *Valsa melanodiscus*), based upon morphological differences, between the teleomorphic sections of *Valsa* and the sections of *Cytospora*. Adams et al. believe that the proposed sections for *Cytospora* do not correlate with specific holomorph (those based on teleomorphic morphology) sections. Such conclusions were supported by the fact that some anamorphs did not have the characteristic traits of established sections, i.e., their form was not assignable within the current sections, they were unique and differed from those of the assigned section, or their morphology comprised a range that overlapped a number of anamorphic sections. As such, the proposed relationships, which Adams et al. believe are useful only in a broad sense, are shown in Figure 1.6.

Adams et al., 2006. This monographic study examines *Cytospora* species from a diverse group of woody plants worldwide based on ITS-rDNA sequence data and morphological comparisons. Thirty-three *Cytospora* isolates from South Africa (collected from diseased trees, dead branches, and living trees) were compared with sequences from isolates (comprising 118 taxa) from various areas of the world,

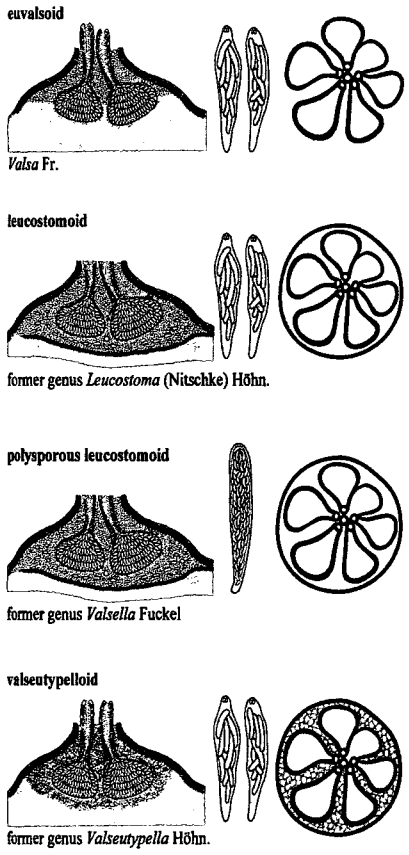


Figure 1.4. Diagrams of asci and longitudinal and cross-sections of ascostromata illustrating the ascostromatal forms of *Valsa* that are the basis of the former subgeneric divisions in *Valsa*. New descriptive terms, which appear in bold, are used for features of the former subgeneric divisions (based on Adams et al., 2005).

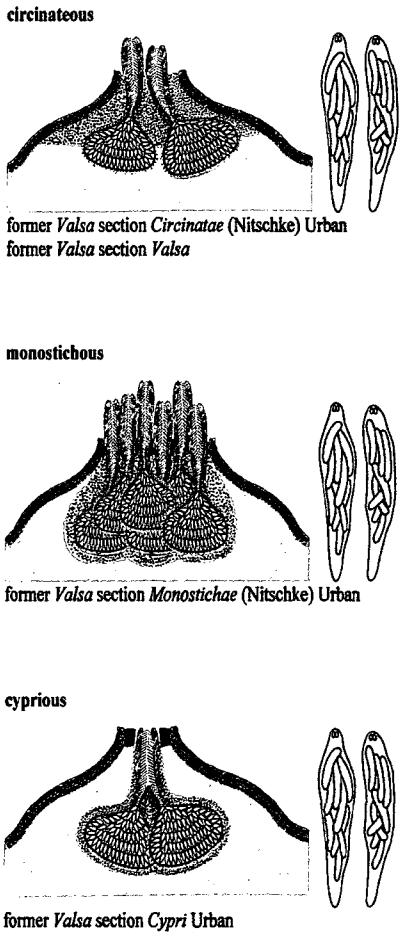


Figure 1.5. Diagrams of asci and longitudinal sections of ascostromata illustrating the ascostromatal forms of *Valsa* that are the basis of the former infrageneric sections in *Valsa*. New descriptive terms, which appear in bold, are used for features of the former sections (based on Adams et al., 2005).

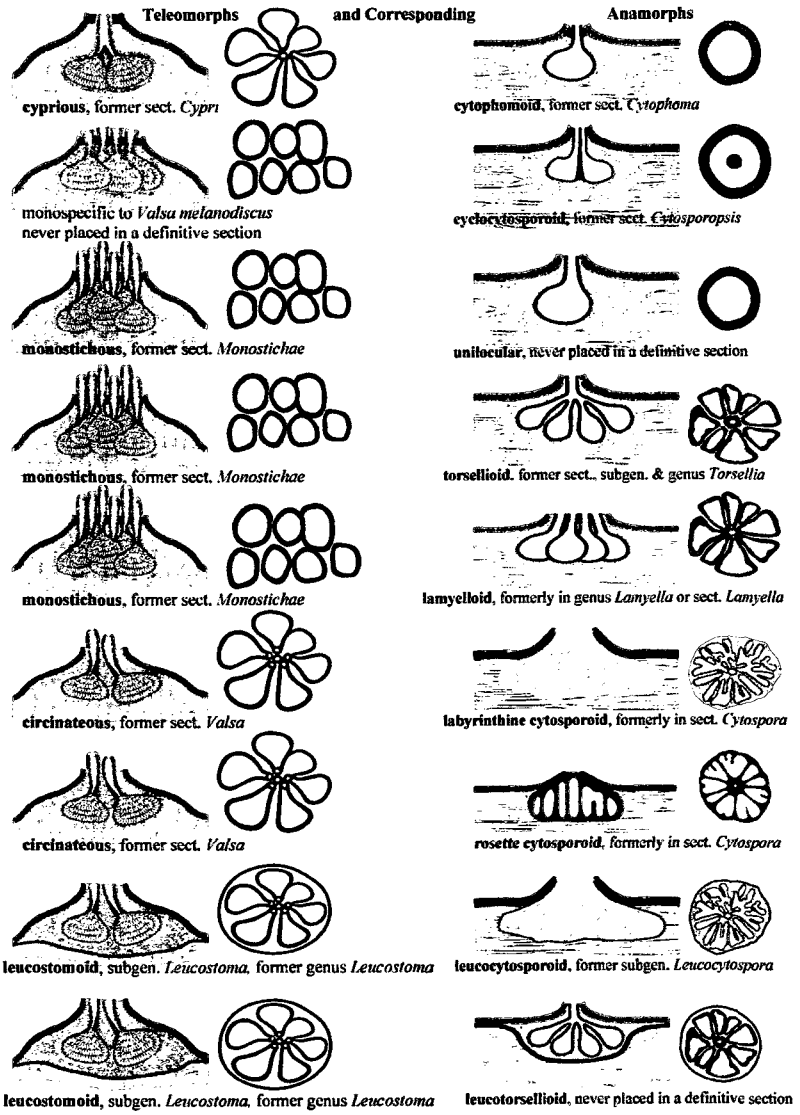


Figure 1.6. Diagrams of longitudinal and cross-sections of ascostromata and conidiomata illustrating the previously hypothesized correspondence between teleomorphic (former holomorph sections of *Valsa*) and anamorphic (former sections of *Cytospora*) morphologies. New descriptive terms, which appear in bold, are used for teleomorphic and anamorphic (locule forms) stromatic features (based on Adams et al., 2005).

geographic regions, and culture collections. Many of these sequences were used in the prior study by Adams et al. (2005). Species identification was based on morphological characteristics of the teleomorph on natural substrates, but when absent, species (and putative species) identification was based upon DNA homology with cultures obtained from sexual fruit bodies.

Phylogenetic analyses using 25 unique sequences obtained from the South African isolates, and sequence data collected from the 118 taxa from other parts of the world, showed South African isolates to reside within several clades which represented as many as 14 species and lineages. Of the 14 *Cytospora* species, nine are well known while three are recently described. Additionally, some of the South African isolates comprised distinct lineages representing unresolved taxa or putative species.

The taxonomic groupings, i.e., clades, within the phylogenetic trees were considered to be species complexes for both the South African isolates and many of the well known Northern Hemisphere species. Based on ITS sequence data reference isolates of commonly known species commonly cluster into statistically well-supported clades with reference isolates of less familiar taxa. Such species complexes were named for the most well known species within the clade. Several of the species complexes which were resolved in the study are discussed below.

***Valsa nivea* species complex (anamorph *C. nivea*).** Although species of *Populus* were the anticipated hosts, the first report of *V. nivea* occurring on *Malus* was made by Adams et al. However, based on sequence data, *Valsella melostoma* (Fr.) Sacc., which is often present on *Malus*, occurred in the same species complex. Additionally, the two species are morphologically similar; the only difference is that *V. melostoma* is

polysporous. Data from the study led Adams et al. to conclude that *V. melostoma* is a multispored variant of *V. nivea*; this concurs with their previous study (Adams et al., 2005).

***V. cypri* species complex (anamorph *C. pruinosa*).** In this two-part (a and b) complex, South Africa isolates are temporarily regarded as *V. cypri* despite the fact they differ significantly in ITS sequence homology from those of European and North American reference isolates. Moreover, South African specimens are morphologically different and do not form the ring-/wing-like collar of ecto-stromatic tissue which surrounds the ostiole (or it is difficult to discriminate). The inferred relationship between South African isolates from *Olea europaea* L. is considered to be that of a sister species to *V. cypri*, rather than a population component of the *Fraxinus* and *Syringa* isolates found in the Northern Hemisphere. The isolates may denote a closely related species that has yet to be described.

***V. kunzei* species complex (anamorph *C. kunzei*) *V. kunzei* var. *piceae* (anamorph *C. kunzei* var. *piceae*).** On *Pinus radiata* D. Don a South African specimen was found that was morphologically most similar to *V. kunzei* var. *piceae*, yet DNA sequence homology revealed it was closely related to the reference strains *V. pini* (Alb. and Schwein.) Fr. (anamorph *C. pini* Desm.) and *V. kunzei* var. *piceae* (anamorph *C. kunzei* var. *piceae*) from the Northern Hemisphere. Based on the phylogenetic analyses Adams et al. believe that *V. kunzei* and *V. pini* are potentially morphological variants of a single species; this would be the first report of a *Valsa* or *Cytospora* species occurring on *P. radiata*. However, there is the chance that the ITS sequence cannot effectively resolve two closely related species and the potential synonymy needs to be further investigated.

***V. malicola/V. germanica* species complex pro parte; *V. malicola* (anamorph *C. schulzeri*) and *V. malicola/V. germanica* species complex pro parte; *V. germanica* (anamorph *C. germanica*).** Phylogenetic analyses grouped a South African strain from *Populus* with North American collections of *V. malicola* from *Salix* and South African collections of *V. malicola* from *Malus*. On *Malus* the two strains formed a ring of multiple ostioles within the disc which is a characteristic exhibited by *C. schulzeri*. Strains from *Populus* and *Salix* produced only one central ostiole within a whitish disc which is a trait common to *C. germanica*. Based on these data, Adams et al. concluded that *V. malicola* and *V. germanica* depict a species complex which is morphologically variable depending on the host on which they are present. Furthermore, these results are in disagreement with Spielman's (1983, 1985) morphological studies in which she synonymized *V. germanica* and *V. sordida*, and *V. malicola* and *V. ambiens* respectively.

***V. sordida* species complex (anamorph *C. chrysosperma*).** This clade is composed of *Cytospora* species from the Northern Hemisphere with no known sexual state, i.e., *C. hariotti* Briard from *Populus*, *C. minuta* Thüm. from *Fraxinus*, *C. tritici* Punith. from *Triticum*, and *C. eutypelloides* Sacc. from *Prunus*, as well as *V. sordida* (with its *C. chrysosperma* anamorph) from *Salix* and *Populus* in South Africa and the Northern Hemisphere. This unusual grouping could be the result of the inability of the ITS sequence to separate the distinct species. However, Adams et al. believe the separate species depict the morphological variability and population diversity occurring within *V. sordida*. They also note that the host range for *V. sordida* is inclusive of members of the Salicaceae, but with the information inferred from the phylogenetic analyses the range could become more comprehensive by including *Ulmus*, *Triticum*, *Fraxinus*, and *Prunus*.

Because *Cytospora* species occurring in South Africa are known to be present mainly on exotic tree species, and the species show ITS sequence homology with *Cytospora* species occurring on the same hosts in the Northern Hemisphere, the authors suggest their introduction into South Africa occurred with seed or propagation stock where they resided as endophytes. The threat of movement of *Cytospora* species from non-native tree species to indigenous species is a concern and examples of pathogen movement already have been noted in South Africa, e.g., *Valsa nivea* moving from *Populus* to *Malus*.

A most recent comprehensive analysis of fungal phylogeny (Spatafora et al., 2006) strongly supports the monophyly of the Diaporthales within the Sordariomycetidae, with *Leucostoma*, *Valsa*, and *Valsella* as closest relatives based on a four-gene phylogeny, viz., nSSU and nLSU rDNA (nuclear large and nuclear small subunit ribosomal DNA), TEF (translation elongation factor) and RBP2 (second largest subunit of RNA polymerase II) (Zhang et al., 2006). Based upon multiple gene analysis (nSSU and nLSU rDNA, TEF1, and RBP2), another study (Hibbett et al., 2007) also concludes the order Diaporthales to be a natural group within the class Sordariomycetes. Bootstrap support values for maximum parsimony, weighted parsimony, and maximum likelihood analyses were 95, 94, and 77 percent, respectively.

Conclusions. Based on sequence analysis, *Cytospora* species constitute a phylogenetically natural group. *Cytospora* (and its morphological variants) is the anamorph of *Valsa* (and members of the Diaporthales). I agree with the conclusion of Adams et al. (2005) that *Leucostoma*, *Valsa*, *Valsella*, and *Valseutypella* all should be reduced to synonymy in the genus *Valsa* based on morphological studies and the close homology of the sequence analyses of both the anamorphs and teleomorphs.

CHAPTER 1 LITERATURE CITED

Adams, G., Hammar, S., and Proffer, T. 1990. Vegetative compatibility in *Leucostoma persoonii*. Phytopathology 80:287-291.

Adams, G. C., Roux, J., and Wingfield, M. J. 2006. *Cytospora* species (*Ascomycota, Diaporthales, Valsaceae*): introduced and native pathogens of trees in South Africa. Australasian Plant Pathol. 35:521-548.

Adams, G. C., Surve-Iyer, R. S., and Iezzoni, A. F. 2002. Ribosomal DNA sequence divergence and group I introns within the *Leucostoma* species *L. cinctum, L. persoonii,* and *L. parapersoonii* sp. nov., ascomycetes that cause Cytospora canker of fruit trees. Mycologia 94:947-967.

Adams, G. C., Wingfield, M. J., Common, R., and Roux, J. 2005. Phylogenetic relationships and morphology of *Cytospora* species and related teleomorphs *(Ascomycota, Diaporthales, Valsaceae)* from *Eucalyptus*. Stud. Mycol. 52:1-144.

Adanson, M. 1763. Familles des Plantes 2. Vincent, Paris, FR.

Agrios, G. N. 1997. Plant Pathology. Academic Press, San Diego, CA. 635 p.

Alexopoulos, C. J., Mims, C. W., and Blackwell, M. 1996. Introductory Mycology. John Wiley and Sons, New York, NY. 869 p.

Alonso, A., Lupo, S., and Bettucci, L. 2005. Pathogenicity evaluation of *Cytospora eucalypticola* isolated from *Eucalyptus* spp. cankers in Uruguay. Fitopathol. Bras. 30:289-291.

Banko, T. J., and Helton, A. W. 1974. *Cytospora* induced changes in stems of *Prunus persica*. Phytopathology 64:899-901.

Barr, M. E. 1978. The Diaporthales in North America. Mycol. Mem. 7:1-232.

Barr, M. E. 1990. Prodromus to non-lichenized pyrenomycetous members of the class Hymenoascomycetes. Mycotaxon 39:43-184.

Bertrand, P. F., and English, H. 1976a. Release and dispersal of conidia and ascospores of *Valsa leucostoma*. Phytopathology 66:987-991.

Bertrand, P. F., and English, H. 1976b. Virulence and seasonal activity of *Cytospora leucostoma* and *C. cincta* in French prune trees in California. Plant Dis. Rep. 60:106-110.

Bertrand, P. F., English, H., Uriu, K., and Schick, F. J. 1976a. Late season water deficits and development of Cytospora canker in French prune. Phytopathology 66:1318-1320.

Bertrand, P. F., English, H., and Carlson, R. M. 1976b. Relation of soil physical and fertility properties to the occurrence of Cytospora canker in French prune orchards. Phytopathology 66:1321-1324.

Bettucci, L., and Alonso, R. 1997. A comparative study of fungal populations in healthy and symptomatic twigs of *Eucalyptus grandis* in Uruguay. Mycol. Res. 101:1060-1064.

Bettucci, L., and Saravay, M. 1993. Endophytic fungi of *Eucalyptus globulus*: a preliminary study. Mycol. Res. 97:679-682.

Bettucci, L., Alonso, R., and Fernandez, L. M. 1997. A comparative study of fungal populations in healthy and symptomatic twigs and seedlings of *Eucalyptus globulus* in Uruguay. Sydowia 49:109-117.

Bettucci, L., Alonso, R., and Tiscornia, S. 1999. Endophytic mycobiota of healthy twigs and the assemblage of species associated with twig lesions of *Eucalyptus globulus* and *E. grandis* in Uruguay. Mycol. Res. 103:468-472.

Biggs, A. R. 1989. Integrated approach to controlling Leucostoma canker of peach in Ontario. Plant Dis. 73:869-874.

Biggs, A. R. 1990. Managing wound-associated diseases by understanding wound healing in the bark of woody plants. J. Arbor. 16:108-112.

Biggs, A. R., Davis, D. D., and Merrill, W. 1983. Histopathology of cankers on *Populus* caused by *Cytospora chrysosperma*. Can. J. Bot. 61:268-272.

Bloomberg, W. J., and Farris, S. H. 1963. Cytospora canker of poplars: bark wounding in relation to canker development. Can. J. Bot. 41:303-310.

Boddy, L., and Griffith, G. S. 1989. Role of endophytes and latent invasion in the development of decay communities in sapwood of angiospermous trees. Sydowia 41:41-73.

Burks, S., Jacobi, W. R., and McIntyre, G. A. 1988. Cytospora canker development on aspen in response to nitrogen fertilization. J. Arbor. 24:28-34.

Cannon, P. F. 1988. Proposal to merge the Phyllachorales with the Diaporthales, with a new family structure. Syst. Ascomyc. 7:23-43.

Castlebury, L. A., Rossman, A. Y., Jaklitsch, W. J., and Vasilyeva, L. N. 2002. A preliminary overview of the Diaporthales based on large subunit nuclear ribosomal DNA sequences. Mycologia 94:1017-1031.

Chapela, I. H. 1989. Fungi in healthy stems and branches of American beech and aspen: a comparative study. New Phytol. 113:65-75.

Checa, J., and Martinez, A. T. 1989. Description of the anamorph of *Valseutypella multicollis* in culture. Mycotaxon 36:43-45.

Checa, J., Moreno, G., and Barr, M. E. 1986. *Valseutypella multicollis* sp. nov. Mycotaxon 25:523-526.

Christensen, C. M. 1940. Studies on the biology of *Valsa sordida* and *Cytospora chrysosperma.* Phytopathology 30:459-475.

Christensen, C. M., and Hodson, A. C. 1954. Artificially induced senescence of forest trees. J. Forestry 52:126-129.

Défago, G. 1942. Seconde contribution à la connaissance des Valsées von Höhnel. Phytopathol. Z. 14:103-147.

Dhanvantari, B. N. 1982. Relative importance of *Leucostoma cincta* and *L. persoonii* in perennial canker of peach in southwestern Ontario. Can. J. Plant Pathol. 4:221-225.

Donk, M. A. 1964. Nomina conservanda proposita I. Proposals in fungi Deuteromycetes. Regnum Veg. 34:7-15.

Ehrenberg, C. G. 1818. Sylvae Mycologicae Berolinenses. Formis Theophili Bruschcke, Berlin, GM.

Endert-Kirkpatrick, E. 1986. Biology and ecology of *Cytospora cincta* Sacc. on peach (*Prunus persica* (L.) Batsch) (bacterial canker, stone fruit decline, *Pseudomonas syringae*). Ph.D diss. North Carolina State University. Raleigh, NC. 98 p. [Abstract].

English, H. 1982. Effect of ring and pin nematodes on the development of bacterial canker and Cytospora canker in young French prune trees. Plant Dis. 66:114-116.

Eriksson, O. E., (ed.). 2006. Outline of ascomycota-2006. Myconet 12:1-82.

Eriksson, O., and Hawksworth, D. L. 1988. Outline of the Ascomycetes. Syst. Ascomyc. 7:119-315.

Eriksson, O., and Hawksworth, D. L. 1990. Outline of the Ascomycetes-1990. Syst. Ascomyc. 8:119-318.

Farr, D. F., Bills, G. F., Chamuris, G .P., and Rossman, A. Y. 1989. Fungi on Plants and Plant Products in the United States. APS Press, St. Paul, MN. 1252 p.

Filer, T. H. Jr. 1967. Pathogenicity of *Cytospora*, *Phomopsis*, and *Hypomyces* on *Populus deltoides*. Phytopathology 57:978-980.

Fisher, P. J., Petrini, O., and Sutton, B. C. 1993. A comparative study of fungal endophytes in leaves, xylem and bark *Eucalyptus* in Australia and England. Sydowia 45:338-345.

Forsberg, J. L. 1941. New fungus disease caused by *Cytospora* threatens Chinese elm trees of Colorado. Colo. Farm Bull. 3:3-4.

Fries, E. M. 1823. Systema Mycologicum. Vol. 2(2). Berling, Lundae, GM.

Fries, E. M. 1849. Summa Vegetabilium Scandinaviae. Vol. 2. Typographia Academica, Leipzig, GM.

Gairola, C., and Powell, D. 1971. Extracellular enzymes and pathogenesis by peach Cytosporas. Phytopathol. Z. 72:305-314.

Gilman, J. C., Tiffany, L. H., and Lewis, R. M. 1957. Iowa Ascomycetes II. Diaporthaceae: Valsaceae. Iowa State Coll. J. Sci. 31:623-647.

Gross, H. L. 1967. Cytospora canker of black cherry. Plant Dis. Rep. 51:941-944.

Grove, W. B. 1923. The British species of *Cytospora*. Bull. Misc. Inform. R. Bot. Gard. 1922, No. 1. 1-30.

Grove, W. B. 1935. British Stem and Leaf-Fungi (Coelomycetes). Vol. 1. Cambridge University Press, Cambridge, MA. 488 p.

Gutner, L. S. 1935. Contributions to a monograph of the genus *Cytospora*. Trudy Bot. Inst. Akad. Nauk SSSR, Ser. 2, Sporov. Rast. 2:411-484.

Guyon, J. C., Jacobi, W. R., and McIntyre, G. A. 1996. Effects of environmental stress on the development of Cytospora canker of aspen. Plant Dis. 80:1320-1326.

Gvritishvili, M. N. 1982. The fungal genus *Cytospora* in the USSR. Izdatelstve Sabchota Sakarstvelo, Tbilici, RS. 214 p.

Hampson, M. C., and Sinclair, W. A. 1973. Xylem dysfunction in peach caused by *Cytospora leucostoma*. Phytopathology 63:676-681.

Helton, A. W. 1961. First year effects of 10 selected *Cytospora* isolates on 20 fruit and forest tree species and varieties. Plant Dis. Rep. 45:500-504.

Helton, A. W., and Konicek, D. E. 1961a. An optimum environment for the culturing of *Cytospora* isolates from stone fruits. I. Temperature. Mycopathol. Mycol. Appl. 16:2-26.

Helton, A. W., and Konicek, D. E. 1961b. An optimum environment for the culturing of *Cytospora* isolates from stone fruits. II. Carbon sources. Mycopathol. Mycol. Appl. 16:27-34.

Helton, A. W., and Konicek, D. E. 1961c. An optimum environment for the culturing of *Cytospora* isolates from stone fruits. III. Nitrogen sources. Mycopathol. Mycol. Appl. 16:125-132.

Helton, A. W., and Konicek, D. E. 1961d. Effects of selected *Cytospora* isolates from stone fruits on certain stone fruit varieties. Phytopathology 51:152-157.

Helton, A. W., and Moisey, J. A. 1955. *Cytospora* damage in Idaho prune orchards. Plant Dis. Rep. 39:931-943.

Helton, A. W., and Randall, H. 1975. Cambial gummosis in *Prunus domestica* infected with *Cytospora cincta.* Plant Dis. Rep. 59:340-344.

Hibbett, D. S., Binder, M., Bischoff, J. F., Blackwell, M., Cannon, P. F., Eriksson, O. E., Huhndorf, S., James, T., Kirk, P. M., Lücking, R., Lumbsch, H. T., Lutzoni, F., Matheny, P. B., McLaughlin, D. J., Powell, M. J., Redhead, S., Schoch, C. L., Spatafora, J. W., Stalpers, J. A., Vilgalys, R., Aime, M. C., Aptroot, A., Bauer, R., Begerow, D., Benny, G. L., Castlebury, L. A., Crous, P. W., Dai, Y-C., Gams, W., Geiser, D. M., Griffith, G. W., Gueidan, C., Hawksworth, D. L., Hestmark, G., Hosaka, K., Humber, R. A., Hyde, K. D., Ironside, J. E., Kõljalg, U., Kurtzman, C. P., Larsson, K-H., Lichtwardt, R., Longcore, J., Miadlikowska, J., Miller, A., Moncalvo, J-M., Mozley-Standridge, S., Oberwinkler, F., Parmasto, E., Reeb, V., Rogers, J. D., Roux, C., Ryvarden, L., Sampaio, J. P., Schüßler, A., Sugiyama, J., Thorn, R. G., Tibell, L., Untereiner, W. A., Walker, C., Wang, Z., Weir, A., Weiss, M., White, M. M., Winka, K., Yao, Y-J., and Zhang, N. 2007. A higher-level phylogenetic classification of the Fungi. Mycol. Res. 111:509-547.

Hildebrand, E. M. 1947. Perennial peach canker and the canker complex in New York with methods of control. Cornell Univ. Agric. Stn. Mem. 276:1-61.

Hinds, T. E. 1964. Distribution of aspen cankers in Colorado. Plant Dis. Rep. 48:610-614.

Hinds, T. E. 1985. Diseases. Pages 87-106 in DeByle, N. V., and Winokur, R. P. (eds.), Aspen: Ecology and Management in the Western United States. U.S. Dept. Agric., For. Serv., Rocky Mt. For. Range Exp. Stn., Gen. Tech. Rep. RM-119, Ft.Collins, CO.

Hinds, T. E., and Krebill, R. G. 1975. Wounds and canker diseases on western aspen. U.S. Dept. Agric., For. Serv., For. Pest Leaflet 152. 9 p.

Höhnel, F. von. 1906. Fragmente zur Mykologie 73. Über *Valsa melanodiscus* Otth. Sitzungsber. Kaiserl. Akad. Wiss., Math.-Naturwiss. Kl., Abt. 1, 115:660.

Höhnel, F. von. 1910. Mykologische Fragmente 536. *Torsellia sacculus* (Schw.) Fr. Sitzungsber. Kaiserl. Akad. Wiss., Math.-Naturwiss. Kl., Abt. 1, 119:13-14.

Höhnel, F. von. 1914a. Fragmente zur Mykologie 863. Über *Dendrophoma pruinosa* (Fr.) Sacc. Sitzungsber. Kaiserl. Akad. Wiss., Math.-Naturwiss. Kl., Abt. 1, 123:84-86.

Höhnel, F. von. 1914b. Beiträege zur Mykologie VIII. Über *Plectophoma umbelliferarum* v. Höhn., nebst Bemerkungen zu Diedickes Bearbeitung der Sphaeropsidales. Z. Gaerungsphysiol. 4:207-223.

Höhnel, F. von. 1917. System der Diaportheen. Ber. Deutsch. Bot. Ges. 35:631-638.

Höhnel, F. von. 1918a. Über die allantoidsporigen Sphaeriaceen. Ann. Mycol. 16:127-132.

Höhnel, F. von. 1918b. Mykologische Fragmente 264. Über die gattung *Valsa* Fries sensu Nitschke. Ann. Mycol. 16:134-135.

Höhnel, F. von. 1919. Zu meinem System der Diaportheen. Ann. Mycol. 17:131.

Höhnel, F. von. 1923. System der Fungi Imperfecti Fuckel. Mykologische Untersuchungen und Berichte 1:301-369.

Höhnel, F. von. 1927. Über die Europaischen Valséen und *Cytospora*-Arten auf *Cornus*. Mitt. Bot. Lab. TH Wien 4:69-73.

Höhnel, F. von. 1928a. Über die *Cytospora melanodiscus* (Otth.) Höhn. Mitt. Bot. Lab. TH Wien 5:16-18.

Höhnel, F. von. 1928b. Über die *Cytospora*-Arten auf *Acer*. Mitt. Bot. Lab. TH Wien 5:70-75.

Hubbes, M. 1960a. Systematisches und physiologische Untersuchungen an Valseén auf Weiden. Phytopathol. Z. 39:65-93.

Hubbes, M. 1960b. Untersuchungen über die Valsaceengatung *Valseutypella* v.H. Phytopathol. Z. 39:389-400.

Hubert, E. E. 1920. Observations on *Cytospora chrysosperma* in the Northwest. Phytopathology 10:442-447.

Hutchison, L. J. 1999. Wood-inhabiting microfungi isolated from *Populus tremuloides* from Alberta and northeastern British Columbia. Can. J. Bot. 77:898-905.

Jacobi, W. R. 1994. Cytospora canker. Service in Action Bulletin. Colorado State University Cooperative Extension. No. 2.937, Ft.Collins, CO. 1 p.

Jones, A. C., and Luepschen, N. S. 1971. Peach canker in Colorado. Colorado Agric. Exp. Stn. Prog. Rep. 62:1-2, Ft. Collins, CO.

Jorgensen, E. and Cafley, J. D. 1961. Branch and stem cankers of white and Norway spruces in Ontario. For. Chron. 37:394-400.

Kamiri, L. K., and Laemmlen, F. F. 1981a. Effect of drought stress and wounding on Cytospora canker development on Colorado blue spruce. J. Arbor. 7:113-116.

Kamiri, L. K., and Laemmlen, F. F. 1981b. Epidemiology of cytospora canker caused in Colorado blue spruce by *Valsa kunzei.* Phytopathology 71:941-947.

Kepley, J. B., and Jacobi, W. R. 2000. Pathogenicity of *Cytospora* species on six hardwood species. J. Arbor. 26:326-332.

Kern, H. 1955. Taxonomic studies in the genus *Leucostoma.* Mich. Acad. Sci. Arts Lett. 40:9-22.

Kirk, P. M., Cannon, P. F., David, J.C., and Stalpers, J. A. 2001. Ainsworth and Bisby's Dictionary of the Fungi. 9th ed. CAB International, Wallingford, UK. 655 p.

Kobayashi, T. 1970. Taxonomic studies of Japanese Diaporthaceae with special reference to their life histories. Gov. For. Res. Exp. Stn. Bull. No. 226, JA.

Kohlmeyer, J., and Kohlmeyer, E. 1971. Marine fungi from tropical America and Africa. Mycologia 63:831-861.

Leatherman, D. A., McNulty, H. E., Schomaker, M. E., and Lynch, D. L. 1986. Cankers. Pages 19-21 in Aspen: A Guide to Common Problems in Colorado. Colorado State University Cooperative Extension, Colorado State Forest Service. No. 117-0886, Ft. Collins, CO.

Lee, S., Groenewald, J. Z., and Crous, P. W. 2004. Phylogenetic reassessment of the coelomycete genus *Harknessia* and its teleomorph *Wuestneia* (Diaporthales), and the introduction of *Apoharknessia* gen. nov. Stud. Mycol. 50:235-252.

Leonian, L. H. 1921. Studies on the *Valsa* apple canker in New Mexico. Phytopathology 11:236-243.

Leonian, L. H. 1923. The physiology of perithecial and pycnidial formation in *Valsa leucostoma.* Phytopathology 13:257-272.

Leonian, L. H. 1924. A study of factors promoting pycnidium-formation in some Sphaeropsidales. Am. J. Bot. 11:19-50.

Leyendecker, P. J. Jr. 1952. Cytospora canker of apple in New Mexico. Plant Dis. Rep. 36:276-277.

Lindau, G. 1897. Pyrenomycetineae. Pages 321-491 in Engler, A., and Prantl, K. (eds.), Die Natürlichen Pflanzenfamilien, Vol. 1. Wilhelm Engelmann, Leipzig, GM.

Long, W. H. 1918. An undescribed canker of poplars and willows caused by *Cytospora chrysosperma.* J. Agr. Res. 13:331-343.

Luepschen, N. S., and Rohrbach, K. G. 1969. Cytospora canker of peach trees: spore availability and wound susceptibility. Plant Dis. Rep. 53:869-872.

Luepschen, N. S., Hetherington, J. E., Stahl, F. J., and Mowrer, K. E. 1979. Cytospora canker of peach trees in Colorado: survey of incidence, canker location and apparent infection courts. Plant Dis. Rep. 63:685-687.

Lukezic, F. L., DeVay, J. E., and English, H. 1965. Comparative physiology and pathogenicity of *Leucostoma persoonii* and *Rhodosticta quercina.* Phytopathology 55:511-518.

Luttrell, E. S. 1951. Taxonomy of the Pyrenomycetes. Univ. Missouri Stud. Sci. Ser. 24(3):1-120.

Madar, Z., Leisrael, K. K., Solel, Z., and Kimchi, M. 2004. First report of Cytospora canker caused by *Cytospora chrysosperma* on white poplar in Israel. Plant Dis. 88:220 [Abstract].

Marsden, D. H. 1948. A *Valsa* associated with cytospora canker of spruce. Phytopathology 38:307-308.

McIntyre, G. A., Jacobi, W. R., and Ramaley, A. W. 1996. Factors affecting Cytospora canker occurrence on aspen. J. Arbor. 22:229-233.

Müller, E., and Arx, J. A. von. 1973. Pyrenomycetes: Meliolales, Coronophorales, Sphaeriales. Pages 87-132 in Ainsworth, G. C., Sparrow, F. K., and Sussman, A. S. (eds.), The Fungi, Vol. IVA. Academic Press, San Diego, CA.

Munk, A. 1957. Danish Pyrenomycetes. A preliminary flora. Dansk Bot. Ark. 17:1-149.

Nannfeldt, J. A. 1932. Studien über die Morphologie und Systematik der nicht-lichenisierten inoperculaten Discomyceten. Nova Acta Regiae Soc. Sci. Uppsal., Ser. IV, 8(2):1-368.

Nitschke, T. 1867-1870. Pyrenomycetes Germanici: die Kernpilze Deutschlands. Eduard Trewendt, Breslau, GM. 320 p.

Petrak, F. von. 1919. Mycologische Notizer. I. Ann. Mycol. 17:59-100.

Petrak, F. von. 1921. Über die Gattungen *Leucostoma* (Nit.) v. Höhn. und *Leucocytospora* v. Höhn. Ann. Mycol. 19:128.

Petrak, F. von. 1969. Ergebnisse einer Revision der Grundtypen verschiedener Gattung der Ascomyceten und Fungi imperfecti. Sydowia 23:265-272.

Pinon, J. D. 1984. Management of diseases of poplars. Eur. J. For. Pathol. 14:415-425.

Pluim, R. A., Tewari, J. P., and Knowles, N. R. 1994. Strain variation and oxalic acid production by *Cytospora leucostoma* isolated from Saskatoon (*Amelanchier alnifolia*). Plant Dis. 78:551-557.

Povah, A. H. W. 1921. An attack of poplar canker following fire injury. Phytopathology 11:157-165.

Proffer, T. J., and Hart, J. H. 1988. Vegetative compatibility groups in *Leucocytospora kunzei*. Phytopathology 78:256-260.

Proffer, T. J., and Hart, J. H. 1994. Wound inoculations of Colorado blue spruce with isolates of *Leucostoma* (*Cytospora*) *kunzei* and other *Cytospora* species. J. Arbor. 20:215-221.

Proffer, T. J., and Jones, A. L. 1989. A new canker disease of apple caused by *Leucostoma cincta* and other fungi associated with cankers on apple in Michigan. Plant Dis. 73:508-514.

Puritch, T. J. and Bir Mullick, D. 1975. Effect of water stress on the rate of nonsuberized impervious tissue formation following wounding in *Abies grandis*. J. Exp. Bot. 26:903-910.

Ramaley, A. W., McIntyre, G. A., and Jacobi, W. R. 1987. Factors affecting infection of aspen by *Cytospora chrysosperma*. Phytopathology 77:1726 [Abstract].

Rogers, J. D., and Noskowiak, A. F. 1976. Brown sapwood stain of ponderosa pine caused by *Cytospora* sp.: cultural and histological aspects. Phytopathology 66:25-27.

Rohrbach, K. G., and Luepschen, N. S. 1968. Environmental and nutritional factors affecting pycnidiospore germination of *Cytospora leucostoma*. Phytopathology 58:1134-1138.

Ross, W. D. 1976. Fungi associated with root diseases of aspen in Wyoming. Can. J. Bot. 54:734-744.

Rossman, A. Y., Castlebury, L A.., and Samuels, G. J. 2006. Phylogeny and biodiversity of the Hypocreales and Diaporthales. Eighth International Mycological Conference, Cairns, AS, Book 2:337 [Abstract].

Rossman, A. Y., Farr, D. F., Castlebury, L. A. 2007. A review of the phylogeny and biology of the Diaporthales. Mycoscience 48:135-144.

Rossman, A. Y., Palm, M. E., and Spielman, L. J. 1987. A Literature Guide for the Identification of Plant Pathogenic Fungi. APS Press, St. Paul, MN. 252 p.

Rozsnyay, S. D., and Barna, B. 1974. Apoplexy of apricots. Acta Phytopath. Acad. Sci. Hung. 9:301-310.

Saccardo, P. A. 1875. Conspectus generum pyrenomycetum italicorum additis speciebus fungorum Venetorum novis vel criticis, systmate carpologico dispositorum. Atti Accad. Veneto-Trentino-Istriana Padua, Ser. 3, 4:77-100.

Saccardo, P. A. 1882. Sylloge Fungorum Omnium Hucusque Cognitorum. Vol. 1. Typis Seminarii, Pavia, IT.

Saccardo, P. A. 1884. Sylloge Fungorum Omnium Hucusque Cognitorum. Vol. 3. Typis Seminarii, Pavia, IT.

Saito, I., Tumuru, O., and Takakuwa, M. 1972. Ascospore dispersal in *Valsa ceratosperma,* the causal fungus of Japanese apple canker. Ann. Phytopathol. Soc. Jap. 38:367-374 [English abstract].

Santamaria, O., and Diez, J. J.: 2005 Fungi in leaves, twigs and stem bark of *Populus tremula* from northern Spain. Forest Pathol. 35:95-104.

Scharpf, R. F., and Bynum, H. H. 1975. Cytospora canker of true firs. Forest Pest Leaflet. U.S. Dept. Agric., For. Serv. No. 146. 5 p.

Schmidle, A. 1954. The *Cytospora* disease of poplar and the conditions for its occurrence. Rev. Appl. Mycol. 33:388-389 [English abstract].

Schoenweiss, D. F. 1967 Susceptibility of weakened cottonwood stems to fungi associated with blackstem. Plant Dis. Rep. 51:933-935.

Schoenweiss, D. F. 1981. The role of environmental stress in diseases of woody plants. Plant Dis. 65:308-314.

Schoenweiss, D. F. 1983. Drought predisposition to Cytospora canker in blue spruce. Plant Dis. 67:383-385.

Schreiner, E. J. 1931a. The role of disease in the growing of poplar. J. Forestry 29:79-82.

Schreiner, E. J. 1931b. Two Species of *Valsa* Causing Disease in *Populus*. Am. J. Bot. 18:1-29.

Sinclair, W. A., Lyon, H. H., and Johnson, W. T. 1987. Diseases of Trees and Shrubs. Comstock Publishing Associates, Cornell University Press, Ithaca, NY. 575 p.

Sorensen, I, and Traquair, J. A. 1991. Electrophoresis of *Cytospora* isolates from different hosts. Can. J. Plant Pathol. 13:285 [Abstract].

Spatafora, J. W., Hughes, K. W., and Blackwell, M. 2006. A phylogeny for kingdom fungi-deep hypha issue. Mycologia 98:829-1105.

Spielman, L. J. 1980. *Cytospora* vs. *Cytispora*: which is correct? Mycotaxon 10:473-478.

Spielman, L. J. 1983. Taxonomy and biology of *Valsa* species on hardwoods in North America, with special reference to species on maples. Ph.D diss. Cornell University. Ithaca, NY. 175 p.

Spielman, L. J. 1985. A monograph of *Valsa* on hardwoods in North America. Can. J. Bot. 63:1355-1378.

Sproston, T., and Scott, W. W. 1954. *Valsa leucostoma*, the cause of decay and discoloration in tapped sugar maples. Phytopathology 44:12-13.

Surve, R., and Adams, G. 1988. Differentiating *Leucostoma persoonii* from *L. cincta* by isozyme polymorphisms. Phytopathology 78:1518 [Abstract].

Surve, R. S., and Adams, G. C. 1990. Phenetic groups within *Leucostoma persoonii* and *L. cincta* based on allozyme analysis. Newsletter-Mycological Society of America 41:40 [Abstract].

Surve-Iyer, R. S., and Adams, G. C. 1991. Genetic comparison of ribosomal DNA of *Leucostoma* species. Newsletter-Mycological Society of America 42:35 [Abstract].

Surve-Iyer, R. S., Adams, G. C., Iezzoni, A. F., and Jones, A. L. 1995 Isozyme detection and variation in *Leucostoma* species from *Prunus* and *Malus*. Mycologia 87:471-482.

Sutton, B. C. 1980. The Coelomycetes: Fungi Imperfecti with Pycnidia, Acervuli and Stromata. Commonwealth Mycological Institute, Kew, Surrey, EN. 696 p.

Svircev, A. M., Biggs, A. R., and Miles, N. W. 1991. Isolation and partial purification of phytotoxins from liquid cultures of *Leucostoma cincta* and *Leucostoma persoonii.* Can. J. Bot. 69:1998-2003.

Talbot, P. H. B. 1971. Principles of Fungal Taxonomy. The Macmillan Press, London. 274 p.

Tattar, T. A., and Wier, A. M. 2002. Proposed etiology of *Cytospora rhizophorae* canker of *Rhizophora mangle* in southwestern Puerto Rico. Caribbean J. Sci. 38:156-158.

Tekauz, A. 1972. The role of leaf scar and pruning cut infections in the etiology and epidemiology of peach canker caused by *Leucostoma* species. Ph.D. diss. University of Toronto. 161 p.

Tekauz, A., and Patrick, Z. A. 1974. The role of twig infections on the incidence of perennial canker of peach. Phytopathology 64:683-688.

Treshow, M. J., Scholes, F., and Gardner, W. S. 1960. Pathogenic variation in *Cytospora rubescens* isolates on stone fruit varieties. Phytopathology 50:86 [Abstract].

Tulasne, L. R., and Tulasne, C. 1861. Selecta Fungorum Carpologia, Erysiphei Vol. 1. The Imperial Press, Paris, FR.

Tulasne, L. R., and Tulasne, C. 1863. Selecta Fungorum Carpologia, Xylariei, Valsei, Sphaeriei Vol. 2. The Imperial Press, Paris, FR.

Urban, Z. 1957. Vorlaufige Mitteilung der Ergebnisse einer Revision der Gattungen *Valsa* and *Valsella.* Preslia 29:394-395.

Urban, Z. 1958. Revise československých zástupků rodů *Valsa, Leucostoma* a *Valsella.* Rozpr. Česk. Akad. Véd. 68:1-101.

Vaidya, J. G. 1981. *Neotrotteria mangiferae* sp. nov. and *Valseutypella khandalensis* sp. nov.-New records from India. Curr. Sci. 50(20):901-902.

Vasilyeva, L. N. 1988. A new treatment of the family Valsaceae. Syst. Ascomyc. 7:13-21.

Vasilyeva, L. N. 1994. Pyrenomycetes of the Russian Far East, 2. Valsaceae. Institute of Biology and Pedology, Russian Academy of Sciences, Far East Department, Valdivostok, RS.

Walla, J. A., and Conway, K. E. 1986. Cytospora canker of cottonwoods and willows. Pages 48-49 in Riffle, J. W., and Peterson, G.W. (Technical coordinators), Diseases of Trees in the Great Plains. U.S. Dept. Agric., For. Serv., Rocky Mt. For. Range Exp. Stn., Gen. Tech. Rep. RM-129, Ft.Collins, CO.

Walla, J. A., and Crowe, F. J. 1986. Cytospora canker of spruce. Pages 132-133 in Riffle, J. W., and Peterson, G.W. (Technical coordinators), Diseases of Trees in the Great Plains. U.S. Dept. Agric., For. Serv., Rocky Mt. For. Range Exp. Stn., Gen. Tech. Rep. RM-129, Ft.Collins, CO.

Wang, D., Iezzoni, A., and Adams, G. 1998. Genetic heterogeneity of *Leucostoma* species in Michigan peach orchards. Phytopathology 88:376-381.

Waterman, A. M. 1955. The relation of *Valsa kunzei* to cankers on conifers. Phytopathology 45:686-692.

Wehmeyer, L. E. 1924. The perfect stage of the Valsaceae in culture and the hypothesis of sexual strains in this group. Mich. Acad. Sci. Arts Lett. 4:395-412.

Wehmeyer, L. E. 1926. A biologic and phylogenetic study of the stromatic Sphaeriales. Am. J. Bot. 13:575-645.

Wier, A. M., Tattar, T. A., and Klekowski, E. J. 2000. Disease of red mangrove (*Rhizophora mangle*) in southwest Puerto Rico caused by *Cytospora rhizophorae*. Biotropica 32:299-306.

Willison, R. S. 1932. Wound-gum in peaches and grapes. Its relation to the invasion of fungus wound-parasites. Sci. Agric. 12:484-505.

Willison, R. S. 1933. Peach canker investigations I. Some notes on incidence, contributing factors and control measures. Sci. Agr. 14:32-47.

Willison, R. S. 1936. Peach canker investigations II. Infection studies. Can. J. Res. 14:27-44.

Willison, R. S. 1937. Peach canker investigations III. Further notes on incidence, contributing factors, and related phenomena. Can. J. Res. 15:324-339.

Wood, F. A., and French, D. W. 1965. Hypoxylon canker of aspen: seasonal development of cankers and ascospore ejection in winter. Phytopathology 55:771-774.

Wright, E. 1942. *Cytospora abietis*, the cause of canker of true firs in California and Nevada. J. Agr. Research 65:143-153.

Wright, E. 1957. Cytospora canker of rocky mountain Douglas-fir. Plant Dis. Rep. 41:811-813.

Wysong, D. S., and Dickens, L. E. 1962. Variation in virulence of *Valsa leucostoma.* Plant Dis. Rep. 46:274-276.

Zentmyer, G. A. 1941. Cytospora canker of Italian cypress. Phytopathology 31:896-906.

Zhang, N., Castlebury, L. A., Miller, A. N., Huhndorf, S. M., Schoch, C. L., Seifert, K. A., Rossman, A. Y., Rogers, J. D., Kohlmeyer, J., Volkmann-Kohlmeyer, B., and Sung, G. 2006. An overview of the systematics of the Sordariomycetes based on a four-gene phylogeny. Mycologia 98:1076-1087.

CHAPTER 2

DIFFERENTIATION OF *CYTOSPORA* ISOLATES ON *POPULUS TREMULOIDES* BASED ON ISOENZYME ANALYSIS

Abstract

The causal organism responsible for Cytospora canker in Colorado and other regions of the Great Plains is typically reported to be *C. chrysosperma*. Fungal identification has been based upon morphological characteristics of fruiting/vegetative structures despite the plasticity known to occur in such diagnostic features. Examinations of cankers on aspen stems in Colorado revealed a morphologically distinct *Cytospora*-like fungus that frequently co-occurs with *C. chrysosperma*. This fungus was identified as a putative labyrinthine/rosette leucocytosporoid *Cytospora* species (referred to as non-*C. chrysosperma*) and is closely associated with and superficially resembles *C. chrysosperma* (a labyrinthine cytosporoid *Cytospora* sp.). Based on these findings it appears that the Cytospora canker on aspen in Colorado is a complex of fungi. Isoenzyme analysis was employed as an initial step to determine the genetic/biochemical differences that occur among and between *C. chrysosperma* and non-*C. chrysosperma* isolates. Of the twelve enzyme systems initially screened only three, viz., alpha esterase, amylase, and glucose-6-phosphate dehydrogenase, provided good resolution for all isolates. Following cluster analysis, two major clades well-delineated the two taxa. In cases where enzyme systems did not produce resolvable bands for all isolates, viz., acid

phosphatase, alkaline phosphatase, peroxidase, and polyphenol oxidase, activity that did occur also clearly separated *C. chrysosperma* and non-*C. chrysosperma* isolates.

Introduction

During examinations of cankers on aspen stems I found that along with *C. chrysosperma*, a morphologically distinct *Cytospora*-like fungus is frequently present. Based on morphological characteristics I identified this fungus as a putative labyrinthine/rosette leucocytosporoid *Cytospora* species as per the work of Adams et al. (2005). This fungus is closely associated with and superficially resembles *C. chrysosperma* (a labyrinthine cytosporoid *Cytospora* sp. per Adams et al., 2005), and, no doubt, the two have been confused. Based on these findings it appears that the Cytospora canker on aspen in Colorado is a complex of fungi. This contradicts what is typically reported in the literature. Because isoenzyme analysis can resolve taxonomic disputes it was utilized to assess the genetic/biochemical differences between the two taxa.

Isoenzymes: their description, importance, and use

Isoenzymes are different molecular forms of an enzyme having similar or identical enzymatic properties, i.e., they catalyze the same reaction, and may occur in either a single individual or in different members of the same species (Brewer, 1970; Micales et al., 1992; Bonde et al., 1993; Manchenko, 2003). These different molecular forms arise from slight amino acid sequence differences, and different isoenzymes can be visualized via gel or cellulose acetate electrophoresis. Because the amino acid content of the enzyme molecule is dependent on the sequence of nucleotides in the DNA, isoenzyme variation provides a means of assessing genetic variability (Micales et al., 1992). Isoenzymes with amino acid compositions that result in different net electrostatic charges, or in large differences in the shape of the enzyme, can be differentiated due to

differences in rates of migration during electrophoresis (Micales et al., 1986; Weeden, 1989; Micales et al., 1992; Bonde et al., 1993). Such charge differences can be the result of a mutation where a substituted amino acid changes the net charge on the protein (Brewer, 1970). Unfortunately, since many amino acids are neutral or because a mutation may result in the substitution of an amino acid carrying the same charge, it is believed that differentiation via electrophoresis represents only about a third of all the possible isoenzymes which may be present in a genetic system (Micales et al, 1986).

There are three main causes for the formation of multiple molecular forms of enzymes: 1) the presence of more than one gene locus coding for the enzyme; 2) the presence of more than one allele at a single locus coding for the enzyme (termed allozymes); 3) posttranslational modifications of the enzyme polypeptides which result in the formation of nongenetic or secondary isoenzymes (Micales et al., 1986; Micales et al., 1992; Bonde et al., 1993; Manchenko, 2003).

Isoenzymes are frequently employed as a research tool based on their simple methodology, efficiency, and low cost (Bonde et al., 1993). Manchenko (2003), when referring to isoenzyme use in general, i.e., those not specific to fungi or fungal-like organisms, believes that "enzyme electrophoresis remains to be the most simple and powerful tool for separation and identification of second-level structural gene products." He further elaborates, stating that despite the advent and advances in DNA technologies and DNA markers, gel electrophoresis and zymograms provide technically simple and fast methods whereby isoenzymes utilized as gene markers allow one to study genetic polymorphisms at numerous nuclear gene loci quickly, relatively easily, and cheaply. Additionally, inferences regarding genotypic interpretation of the variation in isoenzyme

banding patterns (electrophoretic phenotypes) can typically be made directly from the patterns themselves based on the enzymes highly conserved subunit structure and their codominant expression (as allozymes). Manchenko (2003) also states that as genic markers, isoenzymes do not require quantitative measurements; therefore quantitative standardization of the same staining solutions is not necessary for comparing band patterns.

Although electrophoretic banding patterns are often predictable because they are dependent on the genetic and nuclear condition of the organism, Micales et al. (1992) state that it may not be necessary to genetically interpret banding patterns to allow separation of fungal taxa; rather, simple band-counting measures can be used for calculating genetic similarities and distances. Likewise, Wendel and Weeden (1989), when discussing plant isoenzymes, state that for some types of studies the relative migration and number of bands observed between samples may provide sufficient information.

Molecular methodology tends to be used more frequently than isoenzyme analysis for examining fungal, and fungal-like, taxonomic issues. However, isoenzymes, either alone or in combination with other techniques continue to be used with success (Laday et al., 2000; Ramos et al., 2000; Smith & Sivasithamparam, 2000; Chen & Swart, 2001; Zervakis et al., 2001; Neeraja et al., 2002; Aly et al., 2003; Cacciola et al., 2003; Lebot et al., 2003; Nishizawa et al., 2003; Silveira et al., 2003; Arana-Cuenca et al., 2004; Balali & Kowsari, 2004; Duarte et al., 2004; Horvath & Vargas, 2004; Mohammadi et al., 2004; Cacciola et al., 2005; Dokmetzian et al., 2005; Leslie et al., 2005; Cacciola et al., 2006a;

Cacciola et al., 2006b; Bakonyi et al., 2006; Mwenje et al., 2006; Luo et al., 2007; Mahmoud et al., 2007).

Isoenzyme analysis is most frequently used to address taxonomic problems, particularly when morphological characteristics overlap or are plastic within a genus or species (Micales et al., 1986; Micales et al., 1992); additionally, isoenzymes can be used to reconstruct phylogenetic relationships between related species (Manchenko, 2003) or solve phytopathological problems, i.e., pathogen detection and identification (Bonde et al., 1993). Although their applications have been tested at many different taxonomic levels, analysis is usually most successful at distinguishing species (Micales et al., 1992).

Dokmetzian et al. (2005) state that "using the traditional morphological classification system for the genus *Ascobolus* Pers. has led many times to doubts when trying to establish clear limits between related or supposedly related species that exhibit high morphological similarities." In a study of the *Ceratocystis coerulescens* (Münch) B. K. Bakshi complex, Harrington et al. (1996) felt that isoenzymes were useful for delimiting species, and prior to describing new taxa or addressing species synonymy based on morphology, chose to employ isoenzyme analysis. Leslie et al. (2005) used isoenzymes as one of several methodologies for sorting out the confusion associated with the *Gibberella fujikuroi* (Sawada) Wollenw. species complex from sorghum and millet. They believed that five species of *Fusarium* were often confounded and most likely identified as the single species *F. moniliforme* J. Sheld. Such confusion has numerous negative implications, one being *Fusarium* spp. produce mycotoxins which are harmful to both humans and domesticated animals. Given the fact that not all species produce all

toxins, correct identification of the organism present is critical. Bonde et al. (1993) point out that isoenzymes provide a technique for identifying *Colletotrichum* species which attack strawberry. Identification on morphology alone is very difficult, but the given organisms are easily identified by isoenzyme profiles. They further state the rapid identification of pathogens via electrophoretic enzyme patterns can play an important role in plant disease quarantine regulation. Isoenzyme analysis was used by Micales et al. (1987) to verify that *Endothia eugeniae* (Nutman & F. M. Roberts) J. Reid & C. Booth was the same organism as *Cryphonectria cubensis* (Bruner) Hodges. *C. cubensis* is a canker producing fungus of *Eucalyptus* spp. while *E. eugeniae* is considered to be an opportunistic pathogen of clove. Ramifications of the species synonymy were large; clove, which is ubiquitous, could now be considered as an inoculum source for eucalyptus canker in tropical and subtropical areas throughout the world. Based on the foregoing examples it seems that isoenzymes can be included as but one tool used during a comprehensive examination of any taxonomical and/or phytopathological problem.

Prior biochemical and molecular studies with *Cytospora* spp. and associated teleomorphs

Adams et al. (2005) state that "accurately identifying a species of *Cytospora* based on morphological features is recognized as a problem that is perhaps insurmountable." Other techniques used for species identification, e.g., cultural characteristics, have problems, thus additional methodology employing biochemical and/or molecular techniques have become a necessity for species delimitation within this genus.

Although isoenzyme analysis has been used extensively for fungi, relatively little work on *Cytospora* spp. and associated teleomorphs has been conducted. A series of

studies utilizing isoenzymes was begun as a means for investigating *Leucostoma cinctum* and *L. persoonii* when the teleomorphs were absent (Surve & Adams, 1988; Surve & Adams, 1990; Surve-Iyer et al., 1995). Such an analysis was needed since the teleomorph, which provides the necessary morphological characteristics for anamorphic separation, is rarely found, and because the criteria necessary for identifying the two anamorphs in culture are highly variable.

Surve and Adams (1988) were able to differentiate several isolates of *L. cinctum* from isolates of *L. persoonii* from Michigan (and elsewhere) using cellulose acetate and starch gel electrophoresis and seven isoenzymatic systems (aconitase (ACON), general esterases (EST), hexokinase (HK), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), phosphoglucodehydrogenase (PGD), and phosphoglucoisomerase (PGI)). However, some electromorphs of *L. cinctum* were similar to those of *L. persoonii.*

A later study by Surve and Adams (1990) included isolates of *L. cinctum* and *L. persoonii* from six *Prunus* spp. and *Malus pumila* Mill. in North America, as well as isolates from the anamorph, viz., *Leucocytospora.* Cellulose acetate gel electrophoresis showed the presence of polymorphisms at 10 enzyme loci (glucose-6-phosphate dehydrogenase (G-6-PD), glutamate dehydrogenase (GDH), HK, IDH, malic enzyme (ME), mannitol dehydrogenase (MADH), PGD, PGI, and phosphoglucomutase (PGM)). Isolates of *Leucocytospora* and *L. persoonii* having cultural characteristics which were alike formed two distinct phenetic (genetically different) groups based on isoenzyme patterns. The first group, which was associated with the *L. persoonii* teleomorph, was widespread in occurrence while the second occurred in the same orchards in Michigan as

the first group but was not linked with a known teleomorph. Three phenetic groups, based on isoenzymatic patterns, were observed for isolates of *L. cinctum*. The group on *Malus* was distinct while those on *Prunus* formed two related groups. Teleomorphs having identical morphological characteristics of the fruit body were associated with each of the three groups. However, cultural features and temperatures for optimum growth differed among the three groups. The two phenetic groups for *L. persoonii* were distinct from those of *L. cinctum*.

Surve-Iyer et al. (1995) followed up the studies of Surve and Adams (1988 & 1990) using isoenzyme methodology to continue the investigation of *L. cinctum* and *L. persoonii*. Via cellulose acetate gel electrophoresis and eight isoenzymatic systems (G-6-PD, glucose-phosphate isomerase (GPI), GDH, IDH, ME, MADH, 6-phosphogluconate dehydrogenase (PGDH), and PGM) 31 alleles were resolved for 56 isolates obtained from anamorphic and teleomorphic stroma on seven *Prunus* spp. and varieties and *Malus domestica* Borkh. These isolates were of widespread geographic origin; they were collected in Michigan as well as other locals in North America and Canada. Electrophoretic banding patterns distinctly separated *L. persoonii* from *L. cinctum* while cluster analysis revealed the presence of three phenetic groups (PG1, PG2, and PG3) within the *L. persoonii* clade and three (PG4, PG5, and PG6) within the *L. cinctum* clade.

The PG1 *L. persoonii* phenetic group was widespread (Sierra foothills of California, North Carolina, Oregon, and Ontario) and occurred on various *Prunus* spp., and was unique in culture, typically forming lobate colony margins and having small (0.5-1.0 mm diameter) pycnidia. The remaining two phenetic groups, PG2 and PG3, were essentially identical to each other, and the isolates produced colonies with entire

margins and large (1.0-3.0 mm diameter) pycnidia. PG2 occurred on peach and native black cherry in Michigan; PG3 was found on nectarine in California's hot inner valley west of Fresno. Within the *L .cinctum* phenetic groups, PG4 and PG5 clustered into two similar groups and were found on various *Prunus* spp. while isolates of PG6 were found primarily on *M. domestica.* For the two *Leucostoma* taxa isoenzyme polymorphism was greater in *L. cinctum*, specifically in the phenetic groups PG4 and PG5, which also displayed the greatest amount of variation in colony characteristics. No isoenzyme variation occurred within PG6 which had distinct electrophoretic patterns.

Isolates of the three phenetic groups of *L. persoonii* (PG1, PG2, and PG3) were generally all olivaceous in color. Isolates of the phenetic groups within *L. cinctum* (PG4, PG5, and PG6) produced colonies with entire margins and large pycnidia. PG4 and PG5 were generally buff, vinaceous buff, hazel, umber or smoke grey in color while PG6 was unique, releasing a reddish brown pigment, producing reddish brown hues, and colony colors ranging from primrose to hazel, honey or buff. Isolates of the three phenetic groups of *L. persoonii* showed good growth at 33 C while those of *L. cinctum* would not continue growth at this temperature.

Surve-Iyer and Adams (1991) had previously compared the phenetic groups of *Leucostoma* via restriction fragment length polymorphism (RFLP) of the nuclear ribosomal RNA genes (rDNA), viz., the nuclear small rDNA (18S rDNA), the internal transcribed spacer regions (ITS) and about half of the nuclear large rDNA (28S rDNA). Four restriction enzyme patterns were able to distinguish isolates of *L. cinctum* and *L. persoonii* and four enzyme patterns could separate populations within *L. persoonii.* Differentiation by the latter restriction enzymes correlated with some of the isoenzyme

groups resolved during a prior study (Surve & Adams, 1990). However, differences between *L. cinctum* isolates from *Prunus* spp. and *Malus* were minor in comparison to results of the isoenzyme study; nevertheless, the 18S rDNA region revealed a size difference within *L. cinctum* isolates from *Malus* and *Prunus* spp.

A study by Adams et al. (2002) reexamined the phenetic groups revealed during prior works (Surve & Adams, 1990; Surve-Iyer & Adams, 1991; Surve-Iyer et al., 1995) via phylogenetic analysis of the internal transcribed spacers (ITS 1 and ITS 2) and 5.8S gene of the rDNA operon, and RFLP analysis of the ITS1-5.8S-ITS2 region, the 5′ end of the nuclear large subunit (LSU) and the nuclear small subunit (SSU) of the rDNA operon. They make note that the work of Wang et al. (1998) was not fully supportive of the isoenzyme analysis conducted by Surve-Iyer et al. (1995) and revealed considerable genetic variation within the *L. persoonii* phenetic group PG1 based upon the number of vegetative compatibility groups and maternal mitochondrial lines which were found to occur in a restricted locale, i.e., three orchards in southwest Michigan; Surve-Iyer et al. showed that isolates **within each** of the phenetic groups PG1, PG2, and PG3 had identical zymograms; such zymograms were indicative of a lack of genetic variation for that particular phenetic group. This was considered unusual for PG1 which was widespread in origin, and diverse in regards to its host range where it occurred on a variety of *Prunus* spp.

Ribosomal DNA sequence analysis by Adams et al. (2002), in which *L. persoonii* and *L. cinctum* isolates were compared with 44 different *Cytospora* strains, separated the former *L. persoonii* clade, i.e., *L. persoonii* sensu lato (comprised of isolates with identical teleomorphic morphological characteristics) into two distinct clades; isolates of

the former PG1 phenetic group, which were unique in having lobate colony margins, were now referred to as the *L. persoonii* sensu stricto group while those of PG2 and PG3, which were identical in ITS-rDNA sequence, were separated from *L. persoonii* sensu stricto as a new species, viz., *L. parapersoonii*. Additionally, the analysis separated the isolates of *L. cinctum* (phenetic groups PG4, PG5, and PG6), also with morphologically inseparable teleomorphic features, into a third, well-resolved group referred to as the *L. cinctum* group.

Based on these data, separation of PG1 from PG2 and PG3 agreed with the distinction resolved by the isoenzyme analysis carried out by Surve-Iyer et al. (1995). Furthermore, the phylogenetic clustering of isolates of PG1 and isolates of PG2 and PG3 concurred with their cultural separation as noted by Surve-Iyer et al. (1995), i.e., PG1 isolates had lobate colony margins whereas those of PG2 and PG3 had entire margins. Additionally, isolates in phenetic groups PG2 and PG3 exhibited little to no variation in sequence and RFLP analysis or cultural characteristics. This was in contrast to isoenzyme analysis which detected variation between PG2 and PG3. On the other hand, *L. persoonii* sensu stricto isolates (formerly PG1) showed much greater variation in sequence and RFLP analysis as compared to results of isoenzyme analysis. Phylogenetic clustering of isolates within the *L. cinctum* group did not correlate with the work of Surve-Iyer et al. (1995), where isoenzyme electrophoretic patterns and cultural characteristics for PG4 and PG5 were different from those of PG6. However, the RFLP analysis showed all PG4 and PG5 isolates to be unique; both contained two large insertions, i.e., length mutations comprised of 415 and 309 nucleotides respectively, in the small subunit of the nuclear rDNA; these insertions were identified as Group 1

introns. It is apparent, based on these results, that studies based on DNA sequences are not always in agreement with those based on isoenzymes.

Studies by other scientists also have utilized isoenzymes or total proteins although no follow-up work pertaining to molecular methodology was conducted. Sorensen and Traquair (1991) observed significant enzyme differences between *Cytospora* isolates using cellulose acetate electrophoresis and the PGI, PGM, and alcohol dehydrogenase (ADH) isoenzymatic systems. Such differences, in some instances, were correlated with host preference and differences in cultural characteristics, i.e., growth rate, colony morphology, and production of pycnidia. Spielman (1983) was able to distinguish species of *Leucostoma* from those of *Valsa* based on comparisons of soluble protein patterns.

Cytospora canker complex occurring on *P. tremuloides* in Colorado

Generally speaking, the bulk of the literature points toward *C. chrysosperma* as the causal organism which produces cankers on *Populus*. Other references, however, indicate a greater number of species are involved (Spielman, 1983 & 1985; Farr et al., 1989). The United States Department of Agriculture-Agricultural Research Service, (http://www.ars.usda.gov/main/site_main.htm?modecode=12-75-39-00), lists five occurring on *P. tremuloides*, i.e., *C. chrysosperma*, *C. leucosperma*, *C. leucostoma*, *C. nivea* and *C. translucens*. The causal organism responsible for Cytospora canker in Colorado and other regions of the Great Plains is typically reported to be *C. chrysosperma* (Hinds, 1964; Hinds & Krebill, 1975, Hinds, 1985; Walla & Conway, 1986; Leatherman et al., 1986; Kepley & Jacobi, 2000) and a number of studies have utilized *C. chrysosperma* isolated from aspen in Colorado as a fungal organism in investigative studies (Guyon et al., 1996; McIntyre et al., 1996; Kepley & Jacobi, 2000).

Because a number of species are reported to occur on aspen, and the fact that species identification of *Cytospora* is extremely difficult, I suspect that *C. chrysosperma* often was not the actual organism used in such studies.

Given the difficulties encountered in determining the nature of this Cytospora canker complex it would seem that prior research with aspen and *Cytospora* could be misleading. Without critical examination of the fructificatons one could easily overlook the fact that two distinctly different fungi are present. *Cytospora* fungi are typically examined via a squash mount of a pycnidium or conidioma, and identified by the presence of hyaline, aseptate conidia which are allantoid in shape. If one is satisfied with this methodology then the potential exists for these fungi to be used interchangeably in host range, pathogenicity, or other studies involving aspen. With reference to the Leucostoma canker complex occurring on *Prunus* spp. and *Malus domestica*, Surve-Iyer et al. (1995) state that correctly identifying field isolates from diseased tissue would contribute to the understanding of the biology and epidemiology of the disease; I believe the same is true for the species complex occurring on aspen in Colorado.

Study objectives

Because Cytospora canker on aspen in Colorado appears to be a complex of fungi the goal of this study was to determine if isoenzyme analysis could detect any biochemical/genetic differences among and between *C. chrysosperma* and non-*C. chrysosperma* isolates from aspen in Colorado. DNA sequence analysis would be used in follow-up studies for better resolution of the species complex at the conclusion of the isoenzyme study.

Materials And Methods

Sample collection and culture isolation. Location and collection dates of isolates of *C. chrysosperma*, putative labyrinthine/rosette leucocytosporoid *Cytospora* sp., and putative leucostomoid *Valsa* sp. used in this study are provided in Table 2.1. To isolate the fungi, surfaces of conidiomata or ascostromata on the specimen were initially washed several times with sterile water. Following this a single drop of sterile water was carefully placed over their surface and allowed to soak into the tissue for a few minutes. Once the conidioma was hydrated, slight pressure was exerted via a sterile inoculating needle causing spores to exude. Spores were then collected with a sterile pipette and dispersed on the surface of a variety of media for the initial isolations, viz., yeast-malt agar(YMA), malt extract agar (MEA), and modified Leonian's agar (see Appendix I for formulations). After sealing with wax-film, Petri dishes (90 mm) were stored on the shelf at room temperature and examined for spore germination. Following germination hyphal tip transfers were made and plated on modified Leonian's agar. Cultures were then put onto a shelf at room temperature for approximately three weeks at which time all isolates were transferred to slants containing potato dextrose agar (PDA-see Appendix I for formulation) and allowed to grow at room temperature. Slants were then sealed with wax-film and placed in the refrigerator at 5 C for long term storage.

Culture preparation. All isolates for isoenzyme analysis were initially subcultured from PDA slants under storage at 5 C. Plugs of inoculum approximately $4mm^2$ were plated on 90 mm Petri dishes containing PDA and cultures allowed to grow for approximately two weeks at 25 C. Isolates for protein extraction were grown in 250 ml flasks containing 20 ml of potato dextrose broth (PDB-see Appendix I for formulation) following inoculation with $4mm^2$ mycelial plugs taken from the margins of

Table 2.1. Isolate code, locality, and collection date of isolates used for isoenzyme analysis

Species	Isolate code	Locality	Date collected
C. chrysosperma	C1	Bayfarm Fort Collins, CO	September 1, 2002
C. chrysosperma	C2	Bayfarm Fort Collins, CO	September 1, 2002
C. chrysosperma	C3	Bayfarm Fort Collins, CO	September 1, 2002
C. chrysosperma	C5	Norwich Ct. Fort Collins, CO	September 4, 2002
C. chrysosperma	C7	Trenton Way Fort Collins, CO	October 16, 2002
C. chrysosperma	C8	Trenton Way Fort Collins, CO	October 16, 2002
C. chrysosperma	C9	Trenton Way Fort Collins, CO	October 16, 2002
C. chrysosperma	C13	River Oak Dr. Fort Collins, CO	October 7, 2002
C. chrysosperma	C14	Poudre Canyon CO (east of Cameron Pass)	August 20, 2004
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	1	Bayfarm Fort Collins, CO	September 1, 2002
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	2	Bayfarm Fort Collins, CO	September 1, 2002
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	3	Bayfarm Fort Collins, CO	September 1, 2002
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	4	Norwich Ct. Fort Collins, CO	September 4, 2002
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	5	Norwich Ct. Fort Collins, CO	September 4, 2002
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	6	Hinsdale Dr. Fort Collins, CO	August 30, 2002
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	7	Roaring Creek Trail Poudre Canyon CO	October 6, 2002
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	8	Roaring Creek Trail Poudre Canyon CO	October 6, 2002
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	10	Trenton Way Fort Collins, CO	October 16, 2002

Table 2.1. (continued) Isolate code, locality, and collection date of isolates used for isoenzyme analysis

Species	Isolate code	Locality	Date collected
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	11	Trenton Way Fort Collins, CO	October 16, 2002
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	12	Trenton Way Fort Collins, CO	October 16, 2002
Putative labyrinthine/rosette cytosporoid *Cytospora* sp.	13	Lynda Lane Fort Collins, CO	June 6, 2003
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	14	Lynda Lane Fort Collins, CO	June 6, 2003
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	15	Lynda Lane Fort Collins, CO	June 6, 2003
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	16	Pingree Park CO	June 25, 2004
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	17	Pingree Park CO	June 25, 2004
Putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.	20	Poudre Canyon CO (east of Cameron Pass)	August 20, 2004
Putative leucostomoid *Valsa* sp.	T1	Poudre Canyon CO (east of Cameron Pass)	August 20, 2004

cultures. For incubation, flasks were placed in a growth chamber in the dark at 25 C under static conditions.

Extraction, electrophoresis and staining. Protein samples were prepared from 10 day old cultures with mycelial mats harvested from the broth via vacuum filtration onto filter paper (Wattman grade no. 2). Following vacuum filtration mycelia were placed in a frozen mortar and put into the freezer. Once frozen the mycelia were ground with a frozen pestle in 0.8 ml of extraction buffer (see Appendix II for formulation), and 0.1 g each of polyvinylpyrrolidone (PVP) and alumina. Ground mycelium was then centrifuged in a tabletop centrifuge at 10,000 *g* for five minutes and the supernatant, containing the crude protein extract, collected and frozen for all isolates until electrophoresis was conducted.

Twelve enzymes (acid phosphatase (ACP), alkaline phosphatase (ALP), alanine dehydrogenase (ALADH), alcohol dehydrogenase (ADH), alpha esterase (α-EST), amylase (AMY), glucose-6-phosphate dehydrogenase (G-6-PD), glutamate dehydrogenase (GDH), malate dehydrogenase (MDH), peroxidase (PER), polyphenol oxidase (PPO), and superoxide dismutase (SOD)) were initially screened using one tank buffer system (see Appendix II for formulations). Only those enzyme systems which provided good resolution for **all** isolates were chosen for analysis.

Mycelial proteins were electrophoresed in a vertical slab gel apparatus with a 1.5 mm thick 8% polyacrylamide separating gel, a 8% cap and well-forming gel, and a 4.5% stacking gel (see appendix II for formulations of stock solutions and protocols for gel construction). Cold (4 to 5 C) buffer was added to the gel apparatus and electrophoresis tank after the addition of three drops of bromophenol blue which was used as a dye

marker to denote the migration front. Following this the tank was placed in a shallow tray and surrounded with ice to maintain running temperatures at approximately 4 to 5 C. Gels were subjected to a constant 200 v and 40 to 50 mA for approximately 1.25 to 1.5 hours after which slab gels were removed, rinsed with double distilled water and incubated with appropriate staining mixtures for the activity staining of the various enzyme systems (see Appendix II for stain formulations and procedures). After staining, gels were rinsed with double distilled water, fixed in 7% acetic acid, and photographed by placement on an illuminated light box. Electrophoretic runs were conducted a minimum of three times for each isolate and enzyme assay. However, where staining activity was either too high or low the volume of protein extract used during electrophoresis was adjusted accordingly. The amount of protein extract added did not appear to affect enzyme mobility, and was further examined in a preliminary study shown in Appendix III.

Data analysis. For each enzyme system and isolate, the relative mobility/position (R_f value) of the enzyme reaction, i.e., a stained band, was calculated as a percentage in reference to the dye marker by the formula- $R_f = (d/f) \times 100$ where d is equal to the migration distance of the band and f is the distance traveled by the bromophenol front. The relative staining intensities of bands was disregarded. Genetic interpretation of the banding patterns, viz., allelic frequencies, numbers of gene loci, genetic diversity etc., was not determined in this study as simple band counting measures, and subsequent calculations from these, provided adequate resolution for separation of the fungal taxa. Additionally, a definitive genetic basis for the patterns via progeny studies was not possible as formation of the teleomorphic state in culture could not be induced.

The NTSYSpc2.2 program (Rohlf, 2005) was used for data analysis. The data were organized into a binary matrix where bands were recorded as 0 if absent or 1 if present and each isolate assigned to a phenotype class based on the banding pattern. A similarity matrix in which pairwise comparisons are made between all isolates was then calculated using Jaccard's Coefficient via the SIMQUAL subprogram; this coefficient was chosen as it excludes consideration of negative matches (absence of an allele) and is therefore more appropriate for genetic studies of haploid organisms (Surve-Iyer et al., 1995). The similarity matrix was then analyzed via the unweighted pair-group method with arithmetic averages (UPGMA) using the SAHN subprogram to produce a phenogram. The efficiency, i.e., the goodness of fit of the UPGMA cluster analysis to the similarity matrix, was evaluated by computing cophenetic values and testing the cophenetic correlation using the COPH and MYXCOMP subprograms. Lastly, the tree subprogram was utilized to display a phenetic tree based on the cluster analysis.

Results

Only enzyme systems which showed clear and reproducible banding patterns for **all** isolates were included in the analyses. These systems were α-EST, AMY, and G-6-PD. For these three systems 17 distinct bands, i.e., those with different R_f values, were scored and analyzed. Out of the nine other systems, ALADH, ADH, GDH, MDH, and SOD failed to show reproducible enzyme activity. The remaining four systems (ACP, ALP, PER, and PPO) provided unambiguous and repeatable resolution, but not for all isolates. ACP and ALP exhibited excellent resolution for most **putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. and putative leucostomoid *Valsa* sp. (further designated as non-*C. chrysosperma* isolates)**; however, only a few *C. chrysosperma* isolates displayed strong activity. For PER and PPO, activity was

consistently stronger with isolates of *C. chrysosperma*, but expression of these enzymes appears to be a function of isolate age, particularly those of non-*C. chrysosperma*. A study regarding this occurrence is discussed in Appendix III. In all cases, however, resolvable isoenzymatic patterns for ACP, ALP, PER, and PPO are clearly unique for isolates of *C. chrysosperma* as compared to those of non-*C. chrysosperma* isolates from aspen (data not shown). Additionally, *Cytospora* sp. isolates obtained from *Populus alba* L. and cottonwood (*Populus* sp.), which were initially examined during the isoenzymatic studies, revealed banding patterns which were unique in comparison to those of *C. chrysosperma* and non-*C. chrysosperma* isolates from aspen; furthermore, the patterns displayed by the isolate from *P. alba* were distinct from those of the isolate from cottonwood (data not shown).

A total of 11 electromorphs were identified for the three isoenzymatic systems analyzed (Figures 2.1, 2.2, and 2.3). The α-EST system (Figure 2.2) exhibited the greatest variation, producing six electromorphs. More variation was seen with non-*C. chrysosperma* isolates where five electromorphs were observed (lanes 1-5). For *C. chrysosperma* isolates, only one electromorph was detected for the α-EST system (lane 6). The number of bands for each electromorph varied from five (lanes 1, 4, and 6) to six (lanes 2, 3, and 5). The AMY isoenzymatic system (Figure 2.1) produced three electromorphs; again non-*C. chrysosperma* isolates revealed greater variation forming two electromorphs (lanes 1 and 2) while those of *C. chrysosperma* once more had a single electromorph (lane 3). Band numbers per electromorph were either single (lanes 1 and 2) or three (lane 3). The least amount of variation was seen with the G-6-PD system (Figure 2.3) where a single electromorph each was produced for both non-*C.*

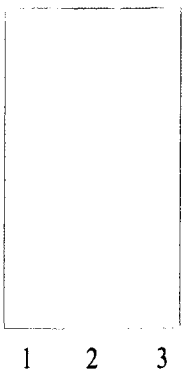


Figure 2.1. Electromorphs for the amylase isoenzymatic system after electrophoresis in polyacrylamide gels containing 0.05% starch. Lanes 1 and 2 represent isolates of putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. and putative leucostomoid *Valsa* sp. Lane 3 represents isolates of *C. chrysosperma.*

Figure 2.2. Electromorphs for alpha-esterase. Lanes 1-5 denote isolates of putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. and putative leucostomoid *Valsa* sp. Lane 6 denotes *C. chrysosperma* isolates.

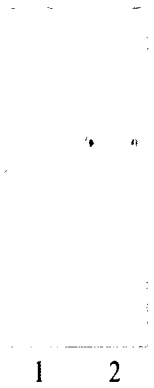


Figure 2.3. Electromorphs for the glucose-6-phosphate dehydrogenase isoenzymatic system. Lane 1 typifies isolates of putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. and putative leucostomoid *Valsa* sp. while those of *C. chrysosperma* are shown in Lane 2.

chrysosperma and *C. chrysosperma* isolates; each electromorph produced one band.

The phenogram (Figure 2.4) obtained from the cluster analysis based on the similarity matrix using the UPGMA method shows that isolates of *C. chrysosperma* and those of putative labyrinthine/rosette leucocytosporoid *Cytospora* species and leucostomoid *Valsa* sp. are well separated from one another, i.e., they form two distinct clades and share a low enzymic similarity of 15%. *C. chrysosperma* isolates comprise the first clade and show the maximum similarity of 100%; the non-*C. chrysosperma* isolates form the second clade which exhibits two subgroups associated by a similarity index of approximately 47%. The first subgroup forms two clusters united by a similarity index of about 59%. The first cluster is comprised of isolates 4, 5, 7, 8, 10, 11, 13, 14, and 15 which are linked to isolates 1, 2, 3, 6, 12. Within the first cluster a subcluster is formed with isolates associated by a similarity index of 79%. Isolates within this subcluster form three groups of terminal taxa, viz., 4 and 5; 7 and 8; and 10, 11, 13, 14, and 15; the similarity indices for isolates within each of these three groups is 100%. The second cluster within the first subgroup corresponds to isolates 1, 2, 3, 6 and 12 and show a similarity of 100%. Isolates 16, 17, 20, and T1, which form the second subgroup, represent an internal branch and form a separate cluster with 100% similarity. A very good fit between the phenetic tree and the original data was indicated by a high cophenetic correlation value of 0.993 (Surve-Iyer et al., 1995; Kaufman, 1996; Castrillo & Brooks, 1998; Ramos et al., 2000; Duarte et al., 2004).

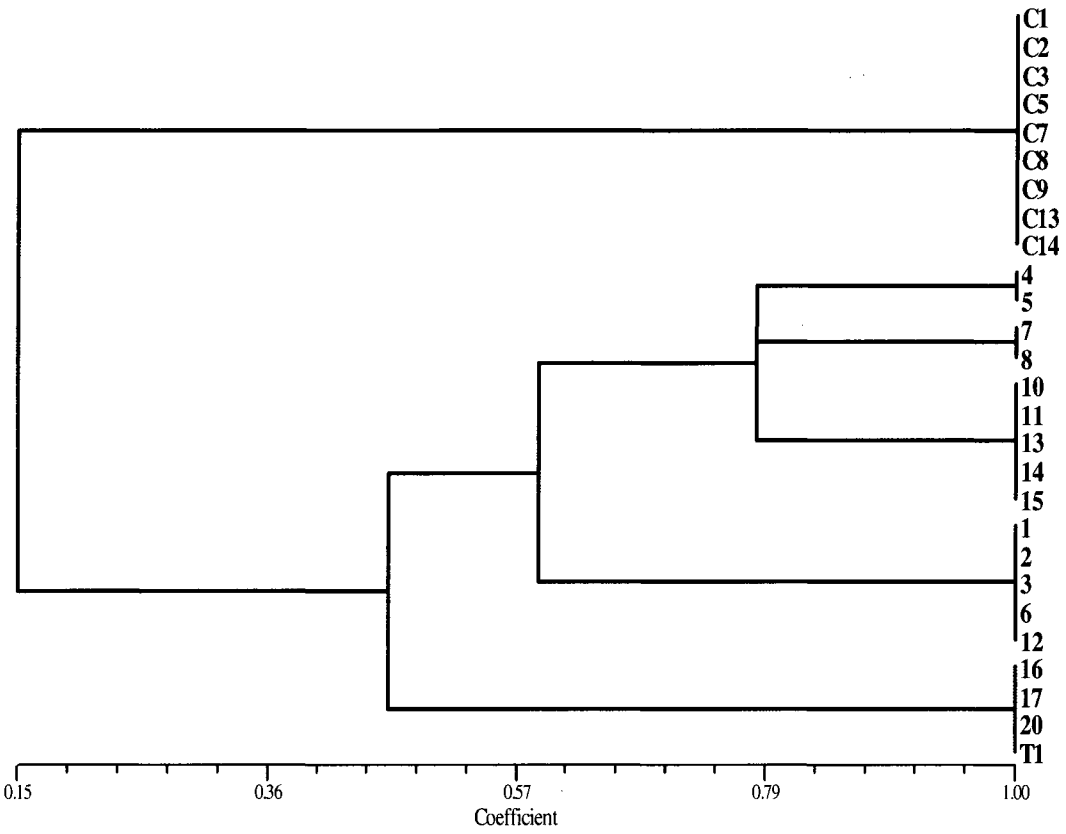


Figure 2.4. Phenogram representing genetic similarity based on Jaccard's coefficient among nine *C. chrysosperma* isolates and 18 putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. and leucostomoid *Valsa* sp. isolates. Similarity coefficients were based on 11 electromorphs generated from three isoenzymatic systems (AMY, α-EST, G-6-PD). Cluster analysis was performed via the unweighted pair-group method with arithmetic averages (UPGMA) using the NTSYSpc2.2 program. Numbered letters at the ends of branches correspond to isolates in Table 2.1.

Discussion

The use of polyacrylamide gels for electrophoresis of isoenzymes provided a very useful preliminary tool for revealing the apparent biochemical/genetic differences between *C. chrysosperma* and non-*C. chrysosperma* isolates from aspen in Colorado. Furthermore, the simplicity, low cost, short run times, and consistent results aided in the utility of isoenzymes as a diagnostic tool. Three enzyme systems (α-EST, AMY, and G-6-PD) displayed strong activity for all isolates and allowed a reliable analysis of the results; following cluster analysis two major clades which well delineated the two taxa were observed. However, even in cases where enzyme systems did not produce resolvable bands for all isolates, viz., ACP, ALP, PER, and PPO, activity that did occur also clearly separated *C. chrysosperma* and non-*C. chrysosperma* isolates. Likewise, with isolates from cottonwood and *P. alba*, banding patterns were unique when the given enzyme system tested proved successful. These findings suggest a canker complex is present for *Populus* spp. in general.

The α-EST system displayed the greatest amount of variation and total numbers of bands. This was to be expected, however, as esterases are considered nonspecific isoenzymes, and may encompass several classes of enzymes or be the products of several genetic loci. Additionally, their activities may be revealed via various artificial substrates (Brewer, 1970; Wendel & Weeden, 1989; Manchenko, 2003). Due to these facts, it is recommended that a study include regulatory enzymes involved in energy metabolism, e.g., G-6-PD, as they typically display less variation than nonregulatory enzymes, e.g., esterases and phosphatases, and thus provide an excellent measure of relatedness or ability to distinguish species (Bonde et al., 1993). Despite these shortcomings esterases have, and continue to be, used in studies involving fungal and fungal-like organisms

(Marlatt et al., 1996; Gottlieb et al., 1998; Boshoff et al., 1999; Ramos et al., 2000; Chen & Swart, 2001; Zervakis et al., 2001; Neeraja et al., 2002; Borges Da Silveira et al., 2003; Cacciola et al., 2003; Duarte et al., 2004; Mohammadi et al., 2004; Dokmetzian et al., 2005; Cacciola et al., 2006); in some instances they may be the sole isoenzyme analyzed (Aly et al., 2003; Nishizawa et al., 2003) or one of only a few (Abd-Elaah, 1998; Bruhn et al., 1998; Castrillo et al., 1998; Dorrance et al., 1999; Pane et al., 2005; Luo et al., 2007; Mahmoud et al., 2007).

All *C. chrysosperma* isolates from aspen displayed the same banding pattern for α-EST, whereas non-*C. chrysosperma* isolates showed considerable variation. Since nonregulatory enzymes are known to detect an overly high level of intraspecific variation (Bonde et al., 1993) it was expected that *C. chrysosperma* isolates also would show considerable variation. Whether this is reflective of the small sample set, or is truly indicative of a trend that non-*C. chrysosperma* isolates have greater intraspecific variation than *C. chrysosperma* from aspen at the local level in Colorado, needs to be investigated further.

The five α-EST electromorphs resolved for non-*C. chrysosperma* isolates (vs. two and one for AMY and G-6-PD respectively) would likely have a strong effect on the cluster analysis due to the limited number of enzyme systems examined and electromorphs revealed, and therefore potentially result in more subgroups and clustering within those subgroups. My initial hunch was that isolates collected from aspen occurring in natural areas in the Poudre Canyon perhaps would show a closer relationship with one another than with isolates collected from aspen in urban areas. Yet, isolates 7 and 8 clustered with those taken from aspen from various urban locations. However, the

geographic source of the aspen from urban areas is unknown because almost all are purchased from nurseries, garden centers etc.; therefore, relationships based on host/isolate local is misleading and must be viewed with discretion. With more isolates collected from a wider geographic area, a clearer picture of esterase variation within and between the two taxa might be revealed. Nonetheless, it is not uncommon for isolates of the same species collected from different geographic regions to have the same esterase banding patterns (Abd-Elaah, 1998; Dorrance et al., 1999; Ramos et al., 2000; Neeraja et al., 2002; Nishizawa et al., 2003; Mahmoud, 2007).

The system which produced the next greatest number of electromorphs for non-*C. chrysosperma* isolates from aspen was ACP where four were observed; however, it was not included in the analysis due to poor resolution which occurred with *C. chrysosperma* isolates. Perhaps any potential variation (or the lack of) within *C. chrysosperma* isolates was not detectable under the conditions used during this study. A similar situation occurred with the ALP isoenzymatic system. Whether both systems failed to efficiently resolve *C. chrysosperma* isolates or possibly represents putative character differences between the two taxa cannot be concluded at this time.

Dokmetzian et al. (2005) state that isoenzyme markers such as esterases and phosphatases are efficient for examining intraspecific variation, and when interspecific variability is to be investigated those with limited intraspecific variation are suggested. Regardless of whether regulatory or nonregulatory isoenzymatic systems are utilized, it seems that the more systems analyzed the more conclusive the data would be. However, some studies have delimited taxa based on a single enzyme system. Nishizawa et al., (2003) used esterase zymograms to delineate isolates of *Flammulina velutipes* (Curt.:Fr.)

Sing.; Aly et al. (2003) also employed the EST system to discriminate between species of *Fusarium*. In another study, Graham et al. (1998) were able to utilize glucose-6-phosphate isomerase (GPI) to clearly separate three species of *Phytophthora* while Erselius et al. (2000) used GPI to examine population changes of *Phytophthora infestans* (Mont.) de Bary; in the latter case it was an easy and rapid method for surveying because of clonal reproduction in the population and banding patterns within the population had previously been characterized. Smith and Sivasithamparam (2000) resolved the species identities of Australian *Ganoderma* isolates by means of G-6-PD only. Although a limited number of isolates and a relatively small number of isoenzymatic systems were examined in the present study, *C. chrysosperma* and non-*C. chrysosperma* isolates still could be distinguished easily; the α-EST, AMY, and G-6-PD isoenzymatic systems provided strong statistical support for their separation. In addition, the aim of the study was to determine if isoenzyme analysis could detect any biochemical/genetic differences between the two taxa and therefore support other phenotypic and genotypic variations, e.g., morphological/cultural features and DNA sequence analysis discussed in Chapter 3, and vegetative incompatibility reactions discussed in Appendix III. Therefore, a population study examining inter- and intraspecific variation was not the intent.

Because isozyme analysis detects only main differences in enzyme structure (Bonde et al., 1993), molecular methodology, e.g. RFLP, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and ribosomal RNA gene (rDNA) sequencing, often has been used alone or in conjunction with isoenzyme analysis for comparative purposes or increased resolution (Surve-Iyer & Adams, 1991; Faris-Mokaiesh et al., 1996; Castrillo et al., 1998; Wang et al., 1998; Stanosz et al., 1999;

Witthuhn et al., 2000; Zeller et al., 2000; Zervakis et al., 2001; Adams et al., 2002; Lebot et al., 2003; Leslie et al., 2005; Mwenje et al., 2006). Bonde et al. (1993) note that techniques such as RAPDs and RFLPs are more sensitive and thus capable of detecting very small changes in the genome of an organism. Such sensitivity allows for detection of single nucleotide changes (Castrillo et al., 1998) or other length mutations resulting from insertions, deletions and substitutions (Lebot et al., 2003). Regardless of the system employed, there is normally some level of correlation or agreement between the isoenzyme data and those resulting from the other methodology (Surve-Iyer & Adams, 1991; Faris-Mokaiesh et al., 1996; Stanosz et al., 1999; Witthuhn, et al., 2000; Zervakis, 2001; Adams et al., 2002; Leslie et al., 2005; Mwenjeet al., 2006); however, the increased resolution can show incongruence with isoenzyme data and reveal variation not detected by isoenzymes (Castrillo et al., 1998; Wang et al., 1998; Zeller et al., 2000; Adams et al., 2002; Lebot et al., 2003), fail to show variation as depicted by isoenzyme data (Adams et al., 2002), or cluster isolates previously separated by isoenzymes (Adams et al., 2002). Witthuhn et al. (2000) state that while isozyme data may not infer the true phylogeny regarding species involved in the *Ceratocystis coerulescens* (Münch) B. K. Bakshi complex, they are useful for delimiting morphological similar, host specialized species in the complex. For the preceding reasons a follow-up study (Chapter 3) was conducted employing DNA sequence analysis to provide better resolution regarding the Cytospora canker complex on aspen in Colorado.

As a preliminary tool, isoenzyme analysis is quite useful at separating *C. chrysosperma* isolates from those of putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. and putative leucostomoid *Valsa* sp. Despite the fact that only a limited

number of enzyme systems provided resolution for all isolates, the methodology was simple, fast, and cost efficient; also the results were repeatable and conclusive and separated the taxa into two well resolved clades. However, any variation, particularly within a taxon, must be viewed with caution due to the limited geographic area sampled and the small number of isolates collected.

An additional study including a larger geographic area with more representative isolates from those locals, as well as employing more regulatory and nonregulatory isoenzymatic systems, would lead to a better understanding of genetic diversity occurring within and between the two taxa. Furthermore, expanding the host range to include more species of *Populus* could provide insight regarding a canker complex occurring on *Populus* in Colorado. Whether the genetic differences for the two taxa on aspen detected by isoenzymes in this study constitute separate species is uncertain. A better clarification is expected to be found through DNA sequence comparisons.

CHAPTER 2 LITERATURE CITED

Abd-Elaah, G. A. 1998. The occurrence of fungi along the Red Sea coast and variability among isolates of *Fusarium* as revealed by isozyme analysis. J. Basic Microbiol. 38:303-311.

Adams, G. C., Surve-Iyer, R. S., and Iezzoni, A. F. 2002. Ribosomal DNA sequence divergence and group I introns within the *Leucostoma* species *L. cinctum, L. persoonii,* and *L. parapersoonii* sp. nov., ascomycetes that cause Cytospora canker of fruit trees. Mycologia 94:947-967.

Aly, I. N., Abdel-Sattar, M. A., Abd-Elsalam, K. A., Khalil, M. S., and Verreet, J. A., 2003. Comparison of multi-locus enzyme and protein gel electrophoresis in the discrimination of five *Fusarium* species from Egyptian cottons. Afr. J. Biotechnol. 2:206-210.

Arana-Cuenca, A., Roda, A., Téllez, A., Loera, O., Carbajo, J. M., Terrón, M. C., and González, A. E. 2004. Comparative analysis of laccase-isozymes patterns of several related *Polyporaceae* species under different culture conditions. J. Basic Microbiol. 44:79-87.

Bakonyi, J., Nagy, Z. Á., and Érsek, T. 2006. First report of *Phytophthora citricola* on false cypress in Hungary. Plant Dis. 90:1358 [Abstract].

Balali, G. R., and Kowsari, M. 2004. Pectic zymograms variation and pathogenicity of *Rhizoctonia solani* AG-4 to bean (*Phaseolus vulgaris*) isolates in Isfahn, Iran. Mycopathologia 158:377-384.

Bonde, M. R., Micales, J. A., and Peterson, G. L. 1993. The use of isozyme analysis for identification of plant-pathogenic fungi. Plant Dis. 77:961-968.

Borges Da Silveira, R. M., Saidman, B. O., and Wright, J. E. 2003. *Polyporus s. str.* in southern South America: isoenzyme analysis. Mycol. Res. 107:597-608.

Boshoff, W. H. P., Swart, W.J., and Pretorius, Z.A. 1999. Isozyme characterization of *Fusarium graminearum* isolates associated with head blight of irrigated wheat in South Africa. S. Afr. J. Bot. 65:281-286.

Brewer, G. J. 1970. An Introduction to Isozyme Techniques. Academic Press, New York, NY. 186 p.

Bruhn, J. N., Johnson, T. E., Karr, A. L., Wetteroff, J. J. Jr., and Leininger, T. D. 1998. Identification of *Armillaria* field isolates using isozymes and mycelial growth characteristics. Mycopathologia 142:89-96.

Cacciola, S. O., Cooke, D. E. L. Raudino, F., and Magnano di San Lio, G. 2006a. First report of brown rot and wilt of fennel caused by *Phytophthora megasperma* in Italy. Plant Dis. 90:110 [Abstract].

Cacciola, S. O., Pennisi, A. M., Agosteo, G. E., and Magnano di San Lio, G. 2003. First report of *Phytophthora palmivora* on *Grevillea* spp. in Italy. Plant Dis. 87:1006 [Abstract].

Cacciola, S. O., Spiea, D., Cooke, D. E. L., Raudino, F., and Magnano di San Lio, G. 2006b. Wilt and collapse of *Cuphea ignea* caused by *Phytophthora tropicalis* in Italy. Plant Dis. 90:680 [Abstract].

Castrillo, L. A., and Brooks, W. M. 1998. Differentiation of *Beauveria bassiana* isolates from the darkling beetle, *Alphitobius diaperinus*, using isozyme and RAPD analyses. J. Invertebr. Pathol. 72:190-196.

Chen, W., and Swart, W. J. 2001. Genetic variation among *Fusarium oxysporum* isolates associated with root rot of *Amaranthus hybridus* in South Africa. Plant Dis. 85:1076-1080.

Dokmetzian, D. A., Ranalli, M. E., and Saidman, B. O. 2005. Isozyme analysis of twelve species of the genus *Ascobolus*. Mycotaxon 92:295-309.

Dorrance, A. E., Miller, O. K., and Warren, H. L. 1999. Comparison of *Stenocarpella maydis* isolates for isozyme and cultural characteristics. Plant Dis. 83:675-680.

Duarte, F. L., Pais, C., Spencer-Martins, I., and Leão, C. 2004. Isoenzyme patterns: a valuable tool for the differentiation of *Zygosaccharomyces* species and detection of misidentified isolates. System. Appl. Microbiol. 27:436-442.

Erselius, L. J., Vega-Sánchez, M. E., and Forbes, G. A. 2000. Stability in population of *Phytophthora infestans* attacking tomato in Ecuador demonstrated by cellulose acetate assessment of glucose-6-phophate isomerase. Plant Dis. 84:325-327.

Faris-Mokaiesh, S., Boccara, M., Denis, J. B., Derrien, A., and Spire, D. 1996. Differentiation of the "*Ascochyta* complex" fungi of pea by biochemical and molecular markers. Curr. Genet. 29:182-190.

Gottlieb, A. M., Saidman, B. O., and Wright, J. E. 1998. Isoenzymes of *Ganoderma* species from southern South America. Mycol. Res. 102:415-426.

Graham, J. H., Timmer, L. W., Drouillard, D. L., and Peever, T. L. 1998. Characterization of *Phytophthora* spp. causing outbreaks of citrus brown rot in Florida. Phytopathology 88:724-729.

Harrington, T. C., Steimel, J. P., Wingfield, M. J., and Kile, G. A. 1996. Isozyme variation and species delimitation in the *Ceratocystis coerulescens* complex. Mycologia 88:104-113.

Horvath, B. J., and Vargas, J. M. Jr. 2004. Genetic variation among *Colletotrichum graminicola* isolates from four hosts using isozyme analysis. Plant Dis. 88:402-406.

Láday, M., Bagi, F., Mesterházy, Á., and Szécsi, Á. 2000. Isozyme evidence for two groups of *Fusarium graminearum*. Mycol. Res. 104:788-793.

Lebot, V., Herail, C., Gunua, T., Pardales, J., Prana, M., Thongjiem, M., and Viet, N. 2003. Isozyme and RAPD variation among *Phytophthora colocasiae* isolates from South-east Asia and the Pacific. Plant Pathol. 52:303-313.

Leslie, J. F., Zeller, K. A., Lamprecht, S. C., Rheeder, J. P., and Marasas, F. O. 2005. Toxicity, pathogenicity, and genetic differentiation of five species of *Fusarium* from sorghum and millet. Phytopathology 95:275-283.

Luo, J.-L., Bao, K., Nie, M., Zhang, W. Q., Xiao, M., and Li, B. 2007. Cladistic and phenetic analyses of relationships among *Fusarium* spp. in Dongtan wetland by morphology and isozymes. Biochem. Syst. Ecol. 35:410-420.

Mahmoud, Y. A. G., Gaafar, R. M., and Mubarak, H. M. 2007. Genetic diversity among Nile Delta isolates of *Rhizoctonia solani* Kühn based on pathogenicity, compatibility, isozyme analysis and total protein pattern. Turk. J. Bot. 31:19-29.

Manchenko, G. W. 2003. Handbook of Detection of Enzymes on Electrophoretic Gels. CRC Press, Boca Raton, FL. 553 p.

Marlatt, M. L., Correll, J. C., and Kaufmann, P. 1996. Two genetically distinct populations of *Fusarium oxysporum* f. sp. *lycopersici* race 3 in the United States. Plant Dis. 80:1336-1342.

Micales, J. A., Bonde, M. R., and Peterson, G. L. 1986. The use of isozyme analysis in fungal taxonomy and genetics. Mycotaxon 28:405-449.

Micales, J. A., Bonde, M. R., and Peterson, G. L. 1992. Isozyme analysis in fungal taxonomy and molecular genetics. Pages 57-79 in Arora, D. K., Elander, R. P., and Mukerji, K. G. (eds.), Handbook of Applied Mycology Volume 4: Fungal Biotechnology. Marcel Dekker, New York. 1114 p.

Micales, J. A., Stipes, R. H., and Bonde, M. R. 1987. On the conspecificity of the *Endothia eugeniae* and *Cryphonectria cubensis*. Mycologia 79:707-720.

Mohammadi, M., Aminipour, M., and Banihashemi, Z. 2004. Isozyme analysis and soluble mycelial protein pattern in Iranian isolates of several formae speciales of *Fusarium oxysporum*. J. Phytopathol. 152:267-276.

Mwenje, E., Wingfield, B. D., Coetzee, M. P. A., Nemato, H., and Wingfield, M. J. 2006. *Armillaria* species on tea in Kenya identified using isozyme and DNA sequence comparisons. Plant Pathol. 55:343-350.

Neeraja, C. N., Shenoy, V. V., Reddy, C. S., and Sarma, N. P. 2002. Isozyme polymorphism and virulence of Indian isolates of the rice sheath blight fungus. Mycopathologia 156:101-108.

Nishizawa, K., Amano, Y., Nozaki, K., Hosokawa, N., Shiroishi, M., and Kanda, T. 2003. Some characteristics of three groups in *Flammulina velutipes* classified by analysis of esterase isozymes. Mycoscience 44:19-23.

Pane, A., Cacciola, S. O., Adornetto, M., Proietto Russo, G., Badalà, F., and Magnano di San Lio , G. 2005. Root and basal stem rot of Scotch broom caused by *Phytophthora citricola* and *P. drechsleri* in Italy. Plant Dis. 89:1131 [Abstract].

Ramos, A. M., Ranalli, M. E., Forchiassin, F., and Saidman, B. O. 2000. Isozyme analysis of different species of the genus *Saccobolus* (Ascomycetes, Pezizales). Mycotaxon 74:447-462.

Rohlf, F. J. 2005. NTSYSpc: numerical taxonomy and multivariate analysis system. Version 2.2. Exeter Software. Setauket, NY.

Smith, B. J., and Sivasithamparam, K. 2000. Isozymes of *Ganoderma* species from Australia. Mycol. Res. 104:952-961.

Sorensen, I., and Traquair, J. A. 1991. Electrophoresis of *Cytospora* isolates from different hosts. Can. J. Plant Pathol. 13:285 [Abstract].

Spielman, L. J. 1983. Taxonomy and biology of *Valsa* species on hardwoods in North America, with special reference to species on maples. Ph.D diss. Cornell University. Ithaca, NY. 175 p.

Stanosz, G. R., Swart, W. J., and Smith, D. R. 1999. RAPD marker and isozyme characterization of *Sphaeropsis sapinea* from diverse coniferous hosts and locations. Mycol. Res. 103:1193-1202.

Surve, R., and Adams, G. 1988. Differentiating *Leucostoma persoonii* from *L. cincta* by isozyme polymorphisms. Phytopathology 78:1518 [Abstract].

Surve, R., and Adams, G. C. 1990. Phenetic groups within *Leucostoma persoonii* and *L. cincta* based on allozyme analysis. Newsletter-Mycological Society of America. 41:40 [Abstract].

Surve-Iyer, R. S., and Adams, G. C. 1991. Genetic comparison of ribosomal DNA of *Leucostoma* species. Newsletter-Mycological Society of America. 42:35 [Abstract].

Surve-Iyer, R. S., Adams, G. C., Iezzoni, A. F., and Jones, A. L. 1995. Isozyme detection and variation in *Leucostoma* species from *Prunus* and *Malus*. Mycologia 87:471-482.

Wang, D., Iezzoni, A., and Adams, G. 1998. Genetic heterogeneity of *Leucostoma* species in Michigan peach orchards. Phytopathology 88:376-381.

Weeden, N. F. 1989. Genetics of plant isozymes. Pages 46-72 in Soltis, D. E., and Soltis, P. S. (eds.), Isozymes in Plant Biology. Dioscorides Press, Portland, OR. 268 p.

Wendel, J. F., and Weeden, N. F. 1989. Visualization and interpretation of plant isozymes. Pages 5-45 in Soltis, D. E., and Soltis, P. S. (eds.), Isozymes in Plant Biology. Dioscorides Press, Portland, OR. 268 p.

Witthuhn, C. R., Harrington, T. C., Steimel, J. P., Wingfield, B. D., and Wingfield, M. J. 2000. Comparison of isozymes, rDNA spacer regions and *MAT*-2 DNA sequences as phylogenetic characters in the analysis of the *Ceratocystis coerulescens* complex. Mycologia 92:447-452.

Zeller, K. A., Jurgenson, J. E., El-Assiuty, E. M., and Leslie, J. F. 2000. Isozyme and amplified fragment length polymorphisms from *Cephalosporium maydis* in Egypt. Phytoparasitica 28:121-130.

Zervakis, G. I., Venturella, G., and Papadopoulou, K. 2001. Genetic polymorphism and taxonomic infrastructure of the *Pleurotus eryngii* species-complex as determined by RAPD analysis, isozyme profiles and ecomorphological characters. Microbiology 147:3183-3194.

CHAPTER 3

MOLECULAR PHYLOGENY AND MORPHOLOGY OF THE FUNGI ASSOCIATED WITH THE CYTOSPORA CANKER COMPLEX ON *POPULUS TREMULOIDES* MICHX. IN COLORADO

Abstract

With a wide distribution, and aesthetic, recreational and wood fiber qualities, aspen are a valued forest tree species. *Valsa sordida* (anamorph *Cytospora chrysosperma*) is typically reported as the causal organism for Cytospora canker occurring on aspen in Colorado and the Great Plains region. However, a thorough understanding of the species of *Cytospora* attacking aspen in Colorado is lacking. Fungal identification has been based upon morphological characteristics of fruiting/vegetative structures despite the plasticity known to occur in such diagnostic features. A new fungus that is closely associated with and superficially resembles *V. sordida* was discovered on stems of aspen in Colorado. Both morphological and molecular data (phylogenetic analyses of ITS1-5.8S-ITS2 rDNA and EF-1α sequences) suggest that the fungus belongs in *Cytospora* but is distinct from *V. sordida*. This fungus is illustrated and described as a new species, *Valsa notabilistromatica*. Diagnostic features are prominent, darkly pigmented conceptacles delimiting ascostromata, and inner zones of conceptacle-like (stromatic) tissues that surround prominent white to grayish-white discs. In addition to the *Cytospora* anamorph a *Phialocephala*-like synanamorph is produced by some isolates *in vitro*. The description of *V. sordida* occurring on aspen in Colorado also is provided.

Introduction

Cytospora canker is a world-wide canker disease that affects branches, stems, and roots of woody plants (Kohlmeyer & Kohlmeyer, 1971; Ross, 1976; Agrios, 1997). Estimates as to the number of woody host species found with the disease vary, but 85 or more have been cited by some authors (Sinclair et al., 1987; Farr et al., 1989; Adams et al., 2006).

In Colorado, Hinds (1964) found Cytospora canker in 97% of the native aspen stands which he sampled. Presently an aspen die-off/die-back, referred to as both "Sudden Aspen Decline" (SAD) or "Sudden Aspen Mortality" (SAM) that was first noted about 2002, is affecting thousands of acres of aspen throughout the western United States. In Colorado, results of aerial surveys in 2006 indicated that as many as 140,000 acres were impacted, with as much as 10 percent of the aspen affected in some areas (Bartos & Sheppard, 2006). Mortality due to this "decline" indicates that lateral roots also might be affected which would hinder vegetative regeneration via root suckering. Death of mature trees can be rapid (a year or two) and is believed to often begin at epicenters followed by radial spread throughout an aspen stand. A preliminary study (Worrall et al., 2008) in southwestern Colorado concludes that agents such as *Encoelia pruinosa* (Ellis & Everh.) Tork. & Eckblad and *Ganoderma applanatum* (Pers.) Pat., which typically kill mature trees in aspen stands, are unimportant in the present mortality. Rather, a group of secondary agents are thought to be involved. Among them, Cytospora canker ("usually caused by *Valsa sordida* Nitschke" anamorph *Cytospora chrysosperma* (Pers.) Fr.) is often present and is believed to play a major role in mortality in some instances.

Sinclair et al. (1987) state that *C. chrysosperma* is inconsequential in natural forests, but can cause devastating losses in nursery seedbeds, storage, newly established forest plantations, and landscape or shelterbelt plantings of *Populus* spp. A study by Jacobi et al. (1998) concluded that aspen sprout mortality following harvesting in Colorado resulted from drought and/or drought coupled with root flooding induced stress followed by infection by canker fungi, viz., *C. chrysosperma* and *Dothiora polyspora* Shear & R.W. Davidson. It is apparent that regardless of the predisposing and inciting factors associated with SAD/SAM and other cases of aspen mortality, species of *Cytospora* are often involved in disease of aspen in natural or commercial forests in the Western United States; in many instances *C. chrysosperma* is believed to be the specific agent (Hinds, 1964; Krebill, 1972; Hinds & Krebill, 1975; Ross, 1976; Juzwik et al., 1978; Walters et al., 1982; Jacobi et al., 1998). Additionally, aspen residing in urban forests in the Rocky Mountain region are often found with the disease (Kepley & Jacobi, 2000).

Aspen: Its importance in natural and urban forests

The importance of aspen cannot be overstated as it is the most widely distributed native tree species in North America (Harlow et al., 1996). Knight (2001) states "no other tree in the Rocky Mountain region is more highly valued for its amenities than aspen." In the western United States aspen is most plentiful in Colorado and Utah (Bartos & Sheppard, 2006), and some natural forests in Colorado and Utah have 15-35% aspen cover (Gruell & Loope, 1974). Approximately 10,000 acres of aspen forests are located on landscapes of Colorado State Parks (Kurzel, 2006). Next to riparian ecosystems, aspen ecosystems have the greatest diversity of plants and animals in the Interior West and serve to protect watersheds in addition to their aesthetic, recreation, and

wood fiber values (Campbell, 2006). Commercial use of aspen has increased in the central Rocky Mountains, and in Colorado and Utah, aspen comprises more than 25% of the commercial forests (Jones, 1985). However, aspen also is commonly planted as a landscape tree in urban sites because of its fast growth, striking white bark, and brilliant fall color.

Problems associated with species identification of *Cytospora*

A thorough understanding of the species of *Cytospora* occurring on aspen and other *Populus* spp. in Colorado and other regions of the Great Plains, as well as other areas of the United States, is lacking. Some sources list several species on aspen (Spielman, 1983 & 1985; Farr et al, 1989); one (United States Department of Agriculture-Agriculture Research Service @ http://www.ars.usda.gov/main/site_main.htm?modecode= 12-75-39-00) records five on aspen alone. The causal organism responsible for Cytospora canker in Colorado is typically reported to be *C. chrysosperma* (Hinds, 1964; Hinds & Krebill, 1975, Hinds, 1985; Walla & Conway, 1986; Leatherman et al., 1986; Kepley & Jacobi, 2000). Moreover, a number of investigative studies have employed *C. chrysosperma* isolated from aspen in Colorado (Guyon et al., 1996; McIntyre et al., 1996; Kepley & Jacobi, 2000). Fungal identification has most likely been based on morphological characteristics, but it is possible host association was the criterion for species recognition in some instances.

Use of morphological characters, e.g., stromatic tissues of the pycnidium, locular size, shape, and arrangement, conidiogenesis, and spore characteristics, for accurate identification of *Cytospora* spp. is quite problematic (Adams et al., 2005) given the plasticity known to occur with diagnostic features (Adams et al., 2002). Differences in

hosts and host tissues, as well as the environment, may alter some of these features and lead to variation. Adams et al. (2006) found environmental (or host) influences could produce "morphological forms" and, as a result, be recognized species complexes. Spielman (1983) believed that for taxa within *Valsa*, most morphological characters could be modified to some degree. Such variation, as well as a poor understanding of host ranges, has hindered species delimitation (Spielman, 1985). Furthermore, because the current identification system relies on key morphological characteristics forming on bark tissues of woody plants in nature, no workable system exists for identification of *Cytospora* spp. *in vitro* or on inoculated host tissues (Adams et al., 2005).

Cytospora canker complex occurring on *P. tremuloides* in Colorado

My examinations of cankers on aspen stems have revealed that along with *C. chrysosperma*, a morphologically distinct *Cytospora*-like fungus is frequently present. Based on morphological characteristics this fungus has been identified as a labyrinthine/rosette leucocytosporoid *Cytospora* morphotype as per the work of Adams et al. (2005). It typically co-occurs with and superficially resembles *C. chrysosperma* (a labyrinthine cytosporoid *Cytospora* sp. per Adams et al., 2005). Isoenzyme analyses and vegetative compatibility studies, as part of Chapter 2 and Appendix III respectively of this dissertation, demonstrate that isolates of the *Cytospora*-like fungus (**non-*C. chrysosperma* isolates**) are genetically distinct and easily separated from those of *C. chrysosperma*. It is almost certain that the two taxa have been confused, and Cytospora canker on aspen in Colorado is most likely a complex of fungi, contradicting what is typically reported in the literature. Even though isoenzyme analyses and vegetative compatibility reactions show genetic differences do exist between the two taxa, it cannot be concluded that such differences are indicative of a new *Cytospora* species.

Molecular methods for inferring fungal phylogeny and fungal identification

With approximately 100 species of *Cytospora* described (Donk, 1964; Rossman et al., 1987; Kirk et al., 2001; Rossman et al., 2007), and as many as five reported to occur on aspen, as well as the view that host range is not a definitive characteristic for species delineation (Adams et al, 2005), it is apparent more precise methodology to identify *Cytospora* species must be utilized. Additionally, new experimental techniques are necessary given the likelihood of overlap (even from one locale) between morphological species concepts and the areas encompassed by biological species (Adams et al., 2005).

Within the Kingdom Fungi molecular phylogenetic analysis has a history of only 15-25 years (Shenoy et al., 2007). However, methods employing comparisons of DNA sequence data have continued to improve immensely during that period and are now becoming the standard, especially when classical (traditional) morphological comparisons, and/or host affiliations, prove difficult. In reference to differentiation of *Cytospora* species, their *Valsa* teleomorphs, and *Cytospora*-like pathogens of *Eucalyptus* spp., Adams et al. (2005) consider DNA sequences to be valuable since they provide numerous characters for determining relationships among isolates and species of these fungi. Moreover, such an approach allows phylogenetic hypotheses to be statistically tested. Anamorph and teleomorph morphological data, and other types of phenotypic character data, e.g., culture morphology, growth rates, substrate utilization, pigment production, pathogenicity etc., may correlate with molecular phylogenetic studies and thereby provide more robust identification of fungal and fungal-like organisms; on the other hand, molecular phylogenies may show no congruence with such data resulting in disputes of taxonomic schemes. In either case, insight is gained as to what might be taxonomically informative characters.

Clarifying the taxonomic relationships between the Colorado *Cytospora*-like fungus and *C. chrysosperma*, as well as other *Cytospora* spp. and related teleomorphs, has numerous implications. If the *Cytospora*-like fungus is indeed a species of *Cytospora*, then its impact on aspen and other woody plant species must be evaluated, given the endophytic and pathogenic nature as well as broad host range of the genus. Additionally, sorting out species complexes and properly identifying disease causing fungal organisms are critical in designing control strategies (Stewart et al., 2006; Farr et al., 2001), plant disease quarantine regulation and breeding disease resistance (Bonde et al., 1993; Adams et al., 2002; Baayen et al., 2002; O'Donnell et al., 2004; Adams et al., 2005; Cortinas et al., 2006), understanding the biology and epidemiology of the disease(s) (Surve-Iyer et al., 1995; Uddin et al., 1998; Adams et al., 2005), and easing communication among plant pathologists, mycologists, and quarantine specialists (O'Donnell et al., 2004). Rossman et al. (2006) state that "accurately defining genera and species in such economically significant orders of fungi such as the Diaporthales and Hypocreales is the first step in controlling the diseases they cause or using them in biological control." Winka (2000) believed that the natural classifications provided by phylogenetic analyses helped in understanding evolutionary changes and allowed predictions to be made "regarding the biology, ecology, and physiology of organisms based on what is known about their close relatives."

The number of molecular phylogenetic studies involving fungi is indeed vast; the same can be said for the Sordariomycetes within which *Cytospora* teleomorphs are placed. Likewise, works determining relationships among *Cytospora* species (and related teleomorphs), other Diaporthalean taxa, as well as closely related orders, are quite

numerous. As with other groups of fungi, a number of loci have been investigated within the Diaporthales. One DNA region that has been targeted for genetic analysis is the sequence encoding the nuclear ribosomal gene repeat (rDNA). Each ribosomal DNA repeat contains a cluster of genes, viz., the small subunit (SSU=16S-18S) gene, the 5.8S gene, and the large subunit (LSU=26S-28S) gene. Between the SSU and the 5.8S gene, and the 5.8S gene and the LSU gene are noncoding spacer elements, i.e., the internal transcribed spacer regions ITS1 and ITS 2. The rDNA gene repeat is transcribed as a single unit followed by processing which removes the spacer regions (White et al., 1990; Palumbi, 1996; Piercey-Normore & Egger, 2001).

The choice of which region of the rDNA operon to examine depends on the phylogenetic level of the study, i.e., ordinal, familial, generic etc. To resolve higher level phylogenies (ordinal/familial), more conserved regions such as those encoding the ribosomal RNA genes (SSU, 5.8S, LSU) are typically utilized while the ITS regions, due to their faster rate of evolution, are often used to assess species level relationships (White et al., 1990; Talbot, 2001) The ITS regions (often in combination with other genic regions) have been used extensively in phylogenetic analyses of Diaporthalean taxa (Rehner & Uecker, 1994; Uddin et al.,1998; Farr et al., 1999; Farr et al., 2002a; Farr et al., 2002b; Castlebury et al., 2003; Roux et al., 2003; Gregory et al., 2004; Gryzenhout et al., 2004; Lee et al., 2004; Myberg et al., 2004; Rossman et al., 2004; Sogonov et al., 2005; Gryzenhout et al., 2006a; Gryzenhout et al., 2006b; Mejia et al., 2006; Moročko, 2006; Nakabonge et al., 2006, Sogonov et al., 2006; Green & Castlebury, 2007; Mejia et al., 2008). Several studies have focused on *Cytospora* species and associated teleomorphs (Adams et al., 2002; Adams et al., 2005; Adams et al., 2006). Because of

the multicopy nature of the nuclear rDNA array, ITS regions of fungal organisms are relatively easy to amplify from small, dilute, or highly degraded DNA samples (Sánchez-Ballesteros et al., 2000).

Kullnig-Gradinger et al. (2002) state that "the use of phylogenies based on single gene sequences is now generally discredited." They emphasize this is especially true where the ITS1 and/or ITS2 regions are employed due to paralogous copies which have been shown to occur in some fungi. For this reason they suggest using combined sequence analysis of ITS1 and ITS2 with various single copy genes. It is evident many scientists have adopted this philosophy as the number of genome regions examined per molecular phylogenetic study have increased in the last decade. Such an approach allows researchers to be more confident in their phylogenetic placement and identification of fungal taxa. Taylor et al. (2000) use the terminology Genealogical Concordance Phylogenetic Species Recognition (GCPSR) to describe fungal species recognition based on the concordance of multiple gene genealogies. They stress that phylogenetic analysis of single genes allows individuals to be placed into monophyletic groups, but does not provide the clarity necessary for species separation. Unfortunately, the GCPSR cannot recognize all evolutionary species, but Taylor et al. believe it is superior to species recognition based upon morphology and mating.

Another gene which has been examined either alone, or in combination with other genic regions, for phylogenetic analysis of Diaporthalean taxa is elongation factor-1 alpha (EF-1α) (Zhang & Blackwell, 2002; Niekerk et al., 2004; Castlebury, 2005; Schilder et al., 2005; Castlebury & Mengistu, 2006; Rensburg et al., 2006; Sogonov et al., 2007). Also, a database of EF-1α sequences from *Cytospora* spp. and related

teleomorphs is continuing to enlarge (Adams, pers. comm.). EF-1α is a protein which directs the GTP-dependent binding of aminoacyl-tRNA to the open acceptor site on the ribosome during the elongation cycle of translation in the cytoplasm of all eukaryotic cells (Linz et al., 1986). Because of its ubiquitous distribution and high degree of conservation, eukaryotic EF-1α often is employed to examine evolutionary questions (Keeling & Inagaki, 2004), particularly those involving deep-level phylogenies (Baldauf & Doolittle, 1997). Nevertheless, EF-1α can still be "a rich source of characters informative to low level phylogenetics" (Rehner, 2001). Another desirable feature is its copy number. Depending on the literature read, sampled taxa have displayed single or low copy numbers (Geiser et al., 2004; Baldauf & Doolittle, 1997; Baldauf, 1999; Rehner, 2001). To date no paralogs have been found in Sordarialean fungi (Rehner, pers. comm.) and Spatafora (pers. comm.) states that on "rare occasions paralogs have been amplified with the standard EF primers," although Spatafora has not experienced any such problems with the fungal taxa he has examined. No information/research regarding possible paralogs of EF-1α and *Cytospora* spp. is currently available.

Study objectives

Because Cytospora canker on aspen in Colorado appears to be a complex of fungi, and no known genetic or morphological analyses of these fungi have been conducted, the primary goals of the present study were: 1) to employ molecular phylogenetic analyses to determine if non-*C. chrysosperma* isolates from aspen in Colorado are members of the genus *Cytospora*, and, if so, do they constitute a new species; 2) to assess the phylogenetic relationships of non-*C. chrysosperma* isolates with other *Cytospora* spp. and related teleomorphs; 3) to compare cultural and morphological characteristics of non-*C. chrysosperma* isolates with those of *C. chrysosperma*. The ITS regions of the

nuclear rDNA array were chosen for fungal identification and inferring phylogenies as they are relatively simple to amplify and are good for species level examinations. Furthermore, because multi-gene phylogenies provide more robust assessments of taxonomic affiliations and/or fungal identification, particularly when single copy genes are incorporated, sequence comparisons of the EF-1α locus were included in the analyses.

Materials And Methods

Isolates studied. Specific information pertaining to the collection and culturing of isolates from aspen in Colorado are stated in the materials and methods section of Chapter 2 of this dissertation. Representative isolates included in sequence analyses were those collected by the author, viz., C 14 (*C. chrysosperma*) and 3, 16, and T1 (non-*C. chrysosperma*), in addition to those obtained from other sources (Table 3.1).

Culture preparation. All isolates for sequence analyses were initially subcultured from PDA slants under storage at 5 C. Plugs of inoculum approximately $4mm^2$ were plated on 90 mm Petri dishes containing potato dextrose agar (PDA-see Appendix IV for formulation) and cultures allowed to grow for approximately two weeks at 25 C. Isolates for DNA extraction were grown in 250 ml flasks containing 20 ml of potato dextrose broth (PDB-see Appendix IV for formulation) following inoculation with $2mm^2$ mycelial plugs taken from the margins of cultures. For incubation, flasks were grown under ambient light and temperature conditions.

DNA extractions. Mycelia for genomic DNA extractions were obtained from one week old cultures. Approximately $1cm^2$ of fungal tissue was aseptically removed from a colony margin and placed in a sterile microcentrifuge tube. Following the addition of 750 µl 0.1 M $MgCl_2$, and brief centrifugation in a tabletop centrifuge at 13,000 g, the resulting wash was removed with a sterile pipette. The fungal tissue was

Table 3.1. Hosts, geographic origin, and GenBank accession numbers/culture numbers of species/isolates used for phylogenetic studies

Species/isolate	Origin	Host	Accession/ culture no.
Valsa abietis	Switzerland	*Abies alba*	AY347336[a]
V. ambiens	Switzerland	*Taxus baccata*	AY347330
V. ambiens subsp. *ambiens*	New Jersey	*Acer rubrum*	AY347339
V. ambiens subsp. *leucostomoides*	New York	*Acer rubrum*	AY347345
V. auerswaldii	USSR		AY347337
*V. brevispora*1	Venezuela	*Eucalyptus. grandis*	AF192321
*V. brevispora*2	Congo	*E. grandis* x *tereticornis*	AF192315
*V. ceratosperma*1	Michigan	*Quercus alba*	AY347334
*V. ceratosperma*2	Switzerland	*Taxus baccata*	AY347333
V. cf. *ceratosperma*1	Michigan	*Rhus typhus*	AF192324
V. cf. *ceratosperma*2	Japan	*Malus pumila*	AF192326
*V. cinereostroma*1	Chile	*E. globulus*	AY347377
*V. cinereostroma*2	South Africa	*Mangifera indica*	AF260267
V. coenobitica	Netherlands	*Betula verrucosa*	CBS283.74[b]
*V. cincta*1	Michigan	*Prunus armeniaca*	AF191169
*V. cincta*2	Michigan	*Malus domestica*	AF191170
V. curreyi	Switzerland	*Larix* sp.	AF191172
*V. cypri*1	South Africa	*Olea europaea* var. *africana*	DQ243790
*V. cypri*2	South Africa	*Olea europaea* fruit	DQ243789
*V. cypri*3	Switzerland	*Syringa* sp.	DQ243801
*V. diatrypoides*1	Alaska	*Alnus incana*	GA[c]
*V. diatrypoides*2	Colorado	*Alnus incana*	GA
*V. diatrypoides*3	Alaska	*Alnus incana*	GA
V. eucalypti	California	*Sequoia sempivirens*	AY347340
*V. eugeniae*1	Malaysia	*Eugenia aquea*	AY347348
*V. eugeniae*2	Indonesia	*Eugenia* sp.	AY347343
*V. eugeniae*3	Australia	*Tibouchina heteromalla*	AY347364
*V. eugeniae*4	Indonesia	*Eugenia* sp.	AY347344
*V. eugeniae*5	Indonesia	*Eugenia* sp.	AY347342
*V. eugeniae*6	Tanzania	*Anarcardium occidentale*	IMI057979[d]
*V. fabianae*1	Australia	*E. globulus*	AY347359
*V. fabianae*2	South Africa	*E. grandis*	AF260265
*V. fabianae*3	South Africa	*E. dunnii*	AY347360
*V. friesii*1	Germany	*Abies alba*	AY347318
*V. friesii*2	Switzerland	*Abies alba*	AY347328

Table 3.1 (continued) Hosts, geographic origin, and GenBank accession numbers/culture numbers of species/isolates used for phylogenetic studies

Species/isolate	Origin	Host	Accession/ culture no.
V. germanica	Switzerland		AY347325
V. japonica	Japan	*Prunus persica*	AF191185
*V. kunzei*1	Canada	*Pinus contorta*	ATCC20502[e]
*V. kunzei*2	Michigan	*Pinus strobus*	GA
*V. kunzei*3	South Africa	*Pinus radiata*	DQ243791
V. kunzei var. *piceae*1	Michigan	*Picea pungens*	AY347320
V. kunzei var. *piceae*2	Michigan	*Picea glauca*	AY347320
*V. leucostoma*1	Florida	Unknown twig	GA
*V. leucostoma*2	North Carolina	*Prunus persica*	AF191178
*V. leucostoma*3	South Africa	*Prunus persica*	AF191180
*V. leucostoma*4	Michigan	*Chaenomeles japonica*	AF191179
*V. leucostoma*5	Michigan	*Prunus serotina*	AF191177
*V. leucostoma*6	Switzerland	*Prunus domestica* subsp. *institutia*	AF191175
*V. leucostoma*7	West Virginia	*Betula alleghaniensis*	ATCC74091
V. mali	Japan	*Malus domestica*	AF191186
*V. malicola*1	South Africa	*Malus domestica*	DQ243792
*V. malicola*2	Michigan	*Malus domestica*	DQ243802
V. massarianae	Switzerland	*Sorbus aucuparia*	AY347338
*V. melanodiscus*1	Colorado	*Alnus incana*	GA
*V. melanodiscus*2	Alaska	*Alnus incana*	GA
*V. melanodiscus*3	Colorado	*Alnus incana*	GA
*V. melanodiscus*4	Alaska	*Alnus incana*	GA
*V. melanodiscus*5	Colorado	*Alnus incana*	GA
*V. melanodiscus*6	Alaska	*Alnus incana*	GA
V. myrtagena	Hawaii	*Tibouchina urvilleana*	AY347363
*V. nivea*1	South Africa	*Malus domestica*	DQ243796
*V. nivea*2	Switzerland	*Populus nigra*	AF191174
*V. nivea*3	Oregon	*Alnus incana*	GA
V. parapersoonii	Michigan	*Prunus persica*	AF191181
V. pini	Switzerland	*Pinus sylvestris*	AY347332
V. platani	Switzerland	*Platanus acerifolia*	CBS198.42
V. pustulata	Switzerland	*Fagus sylvestris*	CBS202.42
V. salicina	Switzerland	*Salix* sp.	AY347323
*V. sordida*1	Colorado	*Salix* sp.	AY347321
*V. sordida*2	Wyoming	*Salix* sp.	GA
*V. sordida*3	South Africa	*Salix* sp.	AY347324
*V. sordida*4	South Africa	*Populus nigra* var. *italica*	DQ243797

Table 3.1 (continued) Hosts, geographic origin, and GenBank accession numbers/culture numbers of species/isolates used for phylogenetic studies

Species/isolate	Origin	Host	Accession/ culture no.
V. subclypeata	Netherlands	*Rhododendron ponticum*	AY347331
V. translucens	Switzerland	*Salix* sp.	AF191182
V. viridistroma	USA	*Cercis canadensis*	CBS249.54
*V. weiriana*1	British Columbia	*Pseudotsuga menziesii*	AF192551
*V. weiriana*2	British Columbia	*Chamaecyparis* sp.	AF192550
Valsa sp.1	Michigan	*Pinus sylvestris*	GA
Valsa sp.2	North Carolina	*Tsuga canadensis*	GA
Valsa sp.3	Wisconsin	*Pinus strobes*	GA
Cytospora abyssinica	Ethiopia	*E. globulus*	AY347354
C. acaciae	Spain	*Ceratonia siliqua* fruit	DQ243804
C. austromontana	Australia	*E. pauciflora*	AY347362
C. aff. *austromontana*	South Africa	*E. grandis*	AY347379
C. berkeleyi	California	*E. globulus*	AY347349
C. carbonacea	Germany	*Ulmus campestris*	DQ243805
C. cedri	Italy		AF192311
C. decorticans	Netherlands	*Fagus sylvatica*	CBS116.21
C. diatrypelloidea	Australia	*E. globulus*	AY347368
*C. disciformis*1	Australia	*E. globulus*	AY347357
*C. disciformis*2	Uruguay	*E. grandis*	AY347374
C. eriobotryae	India	*Eriobotrya japonica*	AY347327
*C. eucalyptina*1	Mexico	*E. grandis*	AF192317
*C. eucalyptina*2	Colombia	*E. grandis*	AY347375
C. eutypelloides	England	*Prunus armeniaca*	DQ243806
C. hariotii	Netherlands	*Populus* sp.	DQ243807
C. magnoliae	Louisiana	*Magnolia* sp.	IMI259790
C. minuta		*Fraxinus americana*	DQ243808
C. nitschkia	Ethiopia	*E. globulus*	AY347356
C. mougeotii	Norway	*Picea abies*	AY347329
C. rhizophorae		*Rhizophora mangle*	DQ996040
C. rhodophila	Germany	*Rosa* sp.	DQ243809
C. ribis	Netherlands	*Ribes rubrum*	DQ243810
C. sacchari	India	*Saccharum officinarum*	DQ243811
C. tritici	Germany	*Triticum aestivum*	DQ243812
C. valsoidea	Indonesia	*E. urophylla*	AF192312
*C. variostromatica*1	Australia	*E. globulus*	AY347366
*C. variostromatica*2	South Africa	*E. grandis*	AF260263
*C. variostromatica*3	South Africa	*E. camaldulensis*	AF260264
Cytospora sp.		*Acacia nilotica*	CMW5255[f]

Table 3.1 (continued) Hosts, geographic origin, and GenBank accession numbers/culture numbers of species/isolates used for phylogenetic studies

Species/isolate	Origin	Host	Accession/ culture no.
Galder Mich	Michigan	*Alnus incana* var. *rugosa*	GA
Worrall 7b	Wyoming	*Alnus incana*	GA
C14 (*C. chrysosperma*)	Colorado	*Populus tremuloides*	JK[g]
3	Colorado	*Populus tremuloides*	JK
16	Colorado	*Populus tremuloides*	JK
T1	Colorado	*Populus tremuloides*	JK
95-11	Colorado	*Fraxinus pennsylvanica*	GA
Diaporthe vaccinii	Massachusetts	*Oxycoccus macrocarpos*	AY952141
Phomopsis viticola		*Vitis vinifera*	GA
Valsella melostoma	Michigan	*Malus domestica*	AF191184
Valseutypella mollicollis	Spain	*Quercus ilex* subsp. *rotundifolia*	DQ243803

[a] GenBank sequences
[b] Culture numbers with prefix CBS are of the culture collection of the Centraalbureau voor Schimmelculture, Ultrecht, The Netherlands.
[c] Culture numbers designated GA are of the culture collection of Gerard Adams, at Michigan State University, East Lansing, Michigan.
[d] Culture numbers with the prefix IMI are of the culture collection of the International Mycological Institute, CABI Bioscience, Egham, Surrey, UK.
[e] Culture numbers with the prefix ATTC are of the American Type Culture Collection, Manassas,Virginia.
[f] Culture numbers with the prefix CMW are of the culture collection of M. J. Wingfield at the Tree Protection Cooperative Program, University of Pretoria, Pretoria, South Africa.
[g] Culture numbers designated JK are of the author of this dissertation.

again centrifuged at 13,000 g for one minute and the cells lysed by adding 300 µl of yeast cell lysis solution (MasterPure™ Yeast DNA Purification Kit, EPICENTRE® Biotechnologies, Madison, WI). After incubation of approximately three minutes the fungal tissue was ground with a sterile plastic pestle, incubated in a rotary shaker for 15 minutes at 65 C, and placed in ice for five minutes. Once removed from the ice, cellular debris was pelleted via the addition of 150 µl of MPC protein precipitation reagent (MasterPure™ Yeast DNA Purification Kit, EPICENTRE® Biotechnologies,) and centrifugation at 13,000 g for 10 minutes. Following centrifugation the supernatant was removed and transferred to a sterile microcentrifuge tube. DNA then was precipitated by adding 500 µl of 91% isopropyl alcohol, placement on ice for 10 minutes, and centrifugation for 10 minutes at 13,000 g. The resulting supernatant was removed and the DNA pellet washed with 0.5 ml 70% ethanol followed by brief centrifuging at 13,000 g. Any remaining ethanol was removed and the DNA suspended in 50 µl of TE buffer (MasterPure™ Yeast DNA Purification Kit, EPICENTRE® Biotechnologies) and stored at -20 C until DNA was quantified, and polymerase chain reaction (PCR) amplification studies were conducted. Quantification of DNA in samples was determined both after DNA extractions and PCR amplifications/product purifications by ultraviolet light (UV) absorbance using a tabletop spectrophotometer. DNA yields were calculated on the basis of UV absorbance X dilution and purity of the samples estimated by the ratio of UV absorbance at A_{260}/A_{280}.

PCR amplification, gel electrophoresis, and sequencing. For PCR studies DNA template concentrations were diluted with sterile double distilled water as needed to provide a final concentration of ca. 200 ng/µl. The primer pairs for amplification of the

ITS1, 5.8S, ITS2 region were ITS1 F (forward primer) and ITS4 R (reverse primer) (White et al., 1990). EF1 F and EF2 R (Geiser et al., 2004), EF1-526 F and EF1-1567 R (Rehner, 2001), and EF1-728 F and EF1-986 R (Carbone & Kohn, 1999) were used for amplification of the EF-1α gene. In an effort to achieve optimum PCR results the FailSafe™ PCR PreMix Selection Kit (EPICENTRE® Biotechnologies) was used to determine which PreMix (buffered salt solution, all four deoxynucleotide triphosphates, varying amounts of $MgCl_2$ and FailSafe PCR Enhancer (with betaine)) would be best for the DNA template/primer pair combinations. Once the optimal PreMix was determined a slightly modified protocol of the FailSafe™ PCR System with PreMix choice was employed (see Appendix IV for amplification reactions and PCR cycle conditions).

Amplified samples were fractionated by electrophoresis on 1% agarose gels buffered in sodium boric acid (SB) (see Appendix IV for formulations and gel construction protocols). SB was chosen as a buffer based on its ability to overcome heat generation occurring in Tris-acetic acid-disodium EDTA (TAE) and Tris-boric acid-disodium EDTA (TBE) buffers. Such heat can cause sample diffusion, convection, denaturation, and poor gel integrity and limits the ability to run gels at high voltage (Brody & Kern, 2004). Two microliters of ethidium bromide at 10 μg/ml was included in the gels to allow visualization of the DNA fragments with a UV transilluminator. For each sample well, 10 μl of PCR product was mixed with 2 μl of Blue/Orange 6X loading dye (Promega Corporation, Madison, WI) and 10 μl of this mixture loaded. The first well in all gels was loaded with a PCR marker (Promega Corporation, Madison, WI) mixed with the loading dye (1 μl dye plus 5 μl PCR marker with 6 μl loaded) for

comparison with DNA sample fragments. The gel was run for approximately 20 minutes under a constant current of 100 v for PCR products of the ITS regions.

For electrophoresis of EF-1α amplification products two protocols were employed. The first was identical to that used for the ITS regions; for the second, odd numbered lanes were loaded and electrophoresed at 75 v until the bromophenol blue component of the dye marker migrated ca. 10 mm into the gel. At this point electrophoresis was stopped and even numbered lanes were loaded and electrophoresis resumed. Electrophoresis was conducted until major bands in staggered sets of samples were well separated. Such an approach is suggested by Rehner (2001) in order to isolate desired PCR products since several of the EF-1α PCR primers are used as sequencing primers. Upon completion of electrophoresis gels were placed in the refrigerator at 4 C to firm up the gel texture followed by rapid band excision with a sterile scalpel. Gel slices containing the bands were placed in sterile microcentrifuge tubes and stored at -20 C.

Following amplification and gel electrophoresis, any purification of PCR products prior to DNA sequencing was done using the QIAquick Gel Extraction Kit (QIAGEN Sciences, Inc., Germantown, MD) according to the manufacturer's instructions when a microcentrifuge is used for gel extraction. Both purified (exposed to UV light) and unpurified PCR products were submitted to Macrogen USA, Inc., (Rockville, MD) for sequencing. PCR products were sequenced in both directions using the same primer pairs that were used in the amplification reactions. PCR products and primers were supplied at concentrations of approximately 50 ng/μl and 5 pM/μl respectively. Sequencing

reactions were achieved using BigDye® Terminator cycling conditions with nucleotide sequences determined with a 3730xl automated DNA sequencer.

Data analysis

Sequence alignment. Automatic alignment of the ITS1-5.8S-ITS2 rDNA and EF-1α sequences was performed in Clustal X 1.81 (Thompson et al., 1994; Thompson et al., 1997) followed by refinement via direct examination. To determine how insertions/deletions (indels) and gaps introduced for alignment purposes would be treated in final analyses, several coding schemes were briefly examined. Simple indel coding (SIC) (Simmons & Ochoterena, 2000) and modified complex indel coding (MCIC) (Müller, 2006; Simmons et al., 2007) were automatically implemented using SeqState (Müller, 2005) version 1.4 (available at http://systevol.nees.uni-bonn.de/software/SeqState); coding gaps as 5th states and treating gaps as missing data was achieved using PAUP* 4.0 Macintosh beta version 10 (Swofford, 2003).

Preliminary molecular phylogenetic characterization. To infer phylogenetic relationships of non-*C. chrysosperma* isolates to previously described (and putative) *Cytospora* spp. and related teleomorphs, several methods for comparing sequences were examined prior to final analyses. One hundred and two (101 ingroup and 1 outgroup) and 56 (55 ingroup and 1 outgroup) taxa were examined for the ITS1-5.8S-ITS2 rDNA and EF-1α sequence data sets, respectively. Distance analyses were calculated using PAUP with the neighbor-joining (NJ) algorithm and HKY85 (Hasegawa et a l., 1985) parameters in effect. Maximum parsimony analyses (MP) were performed with PAUP using heuristic search with tree-bisection-reconnection (TBR) branch-swapping algorithm, MulTrees option (saving of all optimal trees) in effect, and zero length branches collapsed. All characters were of the unordered type and had equal weight.

When applicable, the tree with the best -ln likelihood ranking was selected from among the equally most parsimonious trees (MPT), using the Kishino-Hasegawa (KH) test (Kishino & Hasegawa, 1989). Additionally, parsimony ratchet analyses (Nixon, 1999), employing PRAP version 2.0 (Wall et al., 2008) (available at http://systevol.nees.uni-bonn.de/software/PRAP2), were investigated. The PRAP program generates command files for PAUP which allow parsimony ratchet searches to be conducted. The parsimony ratchet has been shown to be effective in breaking "islands" of suboptimal trees which typically are quite close in topology and length to those of the shortest trees (Nixon, 1999). Ten random addition cycles of 200 ratchet iterations each were used. Each iteration involved two rounds of TBR branch-swapping, one on a randomly re-weighted data set (25% of the positions), and the other on the original matrix saving one shortest tree. For each of the sequence comparison procedures listed previously, strict consensus trees were compiled using PAUP when the tree with the best -ln likelihood score was unable to be determined. Support for phylogenetic groupings was determined by bootstrapping (Felsenstein, 1985) on 100 replicate data sets for all methods except where gaps were treated as missing characters. *Diaporthe vaccinii* Shear and *Phomopsis viticola* (Sacc.) Sacc. served as the outgroup for the ITS1-5.8S-ITS2 rDNA and EF-1α data sets respectively.

Final molecular phylogenetic characterization. For the final phylogenetic analyses both MP and Bayesian analyses were conducted for the two sequence data sets. For each analyses and data set, the number of taxa examined and outgroups used were the same as those listed previously. MP was performed using heuristic search with TBR branch-swapping algorithm, MulTrees option in effect, and zero length branches

collapsed. All characters were of the unordered type and had equal weight. Gaps were treated as missing data rather than being coded. For each data set the equally MPT with the greatest -ln likelihood ranking was selected via the KH test and drawn using FigTree version 1.1.2 available at http://tree.bio.ed.ac.uk/software/figtree/. To estimate the relative support for branches a 50% majority-rule consensus tree was employed. For each data set 2000 heuristic searches were performed by bootstrapping. Support values for branches on the consensus tree were placed onto the MPT.

Bayesian analyses were performed with MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2003; Ronquist & Huelsenbeck, 2005) for Macintosh using the GTR+I+G and TVM+I+G models for the ITS1-5.8S-ITS2 rDNA and EF-1α sequence data sets, respectively. The Akaike information criterion (AIC), as implemented by ModelTest version 3.7macX (Posada & Crandall, 1998), selected these models as the best-fitting model for their particular data set. Likelihood settings from the models, as estimated by ModelTest, were specified as starting values for Bayesian analyses. Gaps were treated as missing characters rather than being coded. A Markov chain Monte Carlo (MCMC) analysis with Metropolis coupling was run (Huelsenbeck et al., 2001). For calculation of the posterior probability distributions of branches on the 50% majority-rule consensus tree one million searches sampling every 100^{th} cycle were conducted for the EF-1α sequence data set; two independent Bayesian analyses were performed to check for convergence with four chains running simultaneously and the initial 2,500 cycles discarded as burn-in. For the ITS1-5.8S-ITS2 rDNA sequence data set, protocols were identical other than the use of two million searches and discarding of 5,000 initial cycles as burn-in. Stationarity of MCMC sampling and the appropriate burn-in values were

assessed with Tracer version 1.4 (Rambaut & Drummond, 2003) available at http://tree.bio.ed.ac.uk/software/tracer/. Consensus trees for both data sets were displayed using FigTree with posterior probability distributions for the branches placed onto the trees.

Morphological studies. For examination of fruiting bodies on bark, i.e., natural state conidiomata and ascostromata were excised from aspen tissues by cutting deep enough into the bark tissues to remove entire fruiting bodies. For examination of fruiting bodies from cultural specimens, mature pycnidia, i.e., those oozing spore masses, were cut out with a portion of the agar medium (modified Leonian's medium-20 ml per Petri dish; see Appendix V for formulation). These cultures were seven weeks old and grown at 25 C in the dark. Protocols for fixing, embedding, and sectioning followed a modified version of Adams et al. (2005) (see Appendix V for formulations and procedures). Measurements of characteristic structures from fruiting bodies on bark were derived from 20 observations when possible. Distilled water and several aqueous stains, viz., phloxine-KOH, lactophenol-cotton blue, and Melzer's solution (see Appendix V for formulations) were used as mounting media for observing general morphological characteristics, e.g., stromatal tissues, perithecial wall tissues, conidiogenous cells, asci, spores etc. For sections, staining was conducted using toluidine blue (see Appendix V for formulation).

Cultural characteristics were determined in triplicate from isolates (C 1, C 14, 3, 16, 20, and T1) grown in 90 mm Petri plates (wrapped with wax-film) containing 20 ml modified Leonian's agar under 12 hours continuous light and 12 hours continuous darkness at 25 C for 28 days. Colors were determined according to Rayner (1970).

Measurements of pycnidia, conidia, and hyphae were based on 20 observations each per isolate. Distilled water, and the aqueous stains listed prior, were again used as mounting media for microscopic examinations.

Results

PCR, electrophoresis and sequence alignment. PCR products of approximately 600 base pairs (bp) (ITS1-5.8S-ITS2 rDNA) and 300 bp (EF-1α) were amplified from all isolates. Isolates of *C. chrysosperma* required a different PCR PreMix, viz. D, than non-*C. chrysosperma* isolates (PreMix E) for optimum PCR results. For the ITS1-5.8S-ITS2 rDNA region the best results were achieved using thermal cycler Program 3 (Appendix IV) with an annealing temperature of 53 C. Amplification of the EF-1α gene for all isolates was achieved only using the EF1-728 F and EF1-986 R (Carbone & Kohn, 1999) primer pair and Touchdown PCR (Rehner, 2001) that started with an annealing temperature of 60 C and was reduced by 1 C for each cycle until 50 C was reached (thermal cycler Program 2- Appendix IV). No difference was observed in the two electrophoretic protocols used for the EF-1α amplification products. Sequencing results were positive using both purified and unpurified PCR products.

Initial BLAST searches (Altschul et al., 1990) of the GenBank database with the ITS1-5.8S-ITS2 rDNA and EF-1α sequences of non-*C. chrysosperma* isolates showed homology with *Cytospora* spp. and related teleomorphs. Therefore, many of the sequences used to infer phylogenetic relationships in the present study were the same as those used by Adams et. al (2005; 2006); additional sequences were obtained from the database of Gerard Adams (pers. comm.).

Molecular phylogenetic characterization

Preliminary analyses. Several different methods for treatment of indels and gaps, and for phylogenetic inference, were examined preliminarily to determine which would be employed in the final analyses. In all cases, regardless of the technique used for coding indels and gaps, or for comparing sequences, strict consensus and equally MPT with the best -ln scores using the KH test showed non-*C. chrysosperma* isolates from aspen in Colorado to be a distinct clade- along with one isolate from *Alnus incana* var. *rugosa* from Michigan (data not shown). Although bootstrap analysis was done for all but one of the inference methods, replicates were limited to 100 for each analysis. However, in all instances confidence levels for the clade with non-*C. chrysosperma* isolates ranged from 90 to 100%. Furthermore, for the EF-1α sequence data set, regardless of the gap treatment or inference method used, the group always nested within a large clade of *V. leucostoma* and *V. nivea* isolates; support values, however, were somewhat weak (71-85%). Interrelationships were less resolved using the ITS1-5.8S-ITS2 rDNA sequence data set, but non-*C. chrysosperma* isolates were typically a sister group of a large monophyletic group comprised of, among others, *V. leucostoma* and *V. nivea* isolates. Isolates which had been identified as *C. chrysosperma* (using morphological characteristics) from aspen in Colorado always grouped amongst a clade comprised, in part, of sequences of *Valsa sordida* (data not shown).

The primary differences associated with the various methodologies were in relation to the location of clades along the backbone/spine of the phylogenetic trees (data not shown). Various topologies were observed; however, in general the major clades consisted of the same taxa for each method. For these reasons it was concluded that gaps would be treated as missing data in the final MP and Bayesian analyses.

Final analyses

Maximum parsimony and EF-1α sequence data set. Alignment of the EF-1α sequence data set consisted of 56 taxa and included 208 parsimony-informative characters. Following heuristic searches in PAUP, 43 equally MPT of 1199 steps were retained (consistency index (CI) = 0.422; retention index (RI) = 0.724; re-scaled consistency index (RC) = 0.306). The tree displayed in Figure 3.1 had the greatest -ln likelihood score of 5667.821 as determined by the KH test. The names to the right of the thickened vertical bars are those of described species occurring on aspen. Non-*C. chrysosperma* isolates (3, 16 and T1) from aspen in Colorado (along with an isolate from *A. incana* var. *rugosa* from Michigan = Galder_Mich) form a monophyletic clade with strong bootstrap support (100%). This group is nested in a monophyletic cluster comprised of *V. leucostoma* and *V. nivea* isolates; an isolate of *V. cypri* is a sister species of this larger clade but with very low support (<50%). Based upon hypothesized common ancestry, non-*C. chrysosperma* isolates appear to be more closely related to *V. leucostoma* isolates than to those of *V. nivea* (81%). The sister group relationship between the *V. nivea* group and *V. leucostoma*/non-*C. chrysosperma* isolates has relatively poor support (71%). The isolate (C 14) from aspen in Colorado, identified as *C. chrysosperma* via morphological characteristics, groups robustly (100%) with *V. sordida* isolates.

Maximum parsimony and ITS1-5.8S-ITS2 rDNA sequence data set. Two hundred and twenty two parsimony-informative characters were included in ITS1-5.8S-ITS2 rDNA data set (102 taxa) following alignment. Heuristic searches in PAUP gave 5,340 equally MPT each of 1432 steps and CI of 0.518, RI of 0.759, and a RC of 0.393. Figure 3.2 shows the tree which had the best -ln likelihood ranking of 7901.33 using the

KH test. Names to the right of the thickened vertical bars are described species occurring on aspen. Non-*C. chrysosperma* isolates (and the Galder_Mich isolate) form a monophyletic clade (bootstrap =97%) which is concordant with the EF-1α gene tree (Figure 3.1). However, unlike in the EF-1α gene tree, isolates of this group are not nested within a clade comprised of *V. leucostoma* and *V. nivea* isolates. Rather, they fall outside a large clade comprised of the *V. leucostoma* and *V. nivea* groups, and a *V. parapersoonii* isolate. Based on the proximity of the two clades, i.e., recent common ancestry, it appears they are closely related. The bootstrap confidence level for this large clade, however, is less than 50%. Another described species occurring on aspen, viz., *V. translucens* resides within the *V. nivea* group but the bootstrap value for the cluster is low (<50%). As was the case in the EF-1α gene tree, the isolate (C 14) identified from aspen as *C. chrysosperma*, groups with *V. sordida* isolates (bootstrap =93%). Similar to the *V. leucostoma* group, the large clade within which the *V. sordida* and C 14 isolates reside, i.e., the *V. sordida* group, is relatively taxon-rich.

Some conflicts do exist regarding the topologies resolved from parsimony analyses of the EF-1α and ITS1-5.8S-ITS2 rDNA sequence data sets (Figures 3.1, 3.2). The primary differences are in the locations of clades along the backbone/spine of the respective trees. The inferred relationship of *V. diatrypoides* and *V. melanodiscus* isolates appears much closer in the EF-1α gene tree. Locations of these two clades in the tree constructed from the ITS1-5.8S-ITS2 rDNA sequence data are quite distant with the *V. leucostoma*, non-*C. chrysosperma*, and *V. nivea* isolate groups (as well as some additional taxa) separating them. Bootstrap support values for the two clades are still very robust at 98 and 100% respectively. The Worrall 7b isolate, which grouped with *V.*

diatrypoides in the EF-1α gene tree appears to be closely related, i.e., a sister taxon, to a clade comprised of *V. cypri* isolates based on the ITS1-5.8S-ITS2 rDNA sequence data, but confidence levels are less than 50%. Additionally, *V. cypri* isolates are more distant to those comprising the *V. leucostoma*/non-*C. chrysosperma*/*V. nivea* clade being separated by a cluster consisting of *V. cincta*, *V. japonica*, and *V. curreyi* isolates in the ITS1-5.8S-ITS2 rDNA sequence data set.

Bayesian analyses and EF-1α sequence data set. A 50% majority-rule consensus tree (Figure 3.3) with a mean tree length of 7.5995 and estimated marginal likelihood (arithmetic mean) of -5373.20 was produced by Bayesian analyses of the EF-1α sequence data set (56 taxa). The names to the right of the thickened vertical bars are those of described species occurring on aspen. Convergence diagnostics, i.e., average standard deviation (SD) of split frequencies, potential scale reduction factor (PSRF), were indicative that the two independent Bayesian analyses had reached convergence and thus a good sample from the posterior probability distribution had occurred. Analyses of the two runs using the Tracer program showed sample correlation was low (Effective Sample Sizes (ESSs) were high) and thus the posterior probability distribution was well represented. Moreover, raw trace plots indicated convergence had been reached.

The consensus tree (Figure 3.3) shows non-*C. chrysosperma* isolates (and the Galder_Mich isolate) form a monophyletic clade with a posterior probability of 1.00. Like in the EF-1α gene tree produced by MP analysis (Figure 3.1) this group is nested within a clade comprised of *V. leucostoma* and *V. nivea* isolates but with much stronger support, i.e., 1.00 posterior probability versus 71% bootstrap confidence. The *V.*

Figure 3.1. Phylogenetic tree based on parsimony analysis of EF-1α sequences of *Cytospora* species and related teleomorphs. The displayed tree, chosen from one of the equally most parsimonious trees (MPT), had the best –ln likelihood score of the MPT (-ln likelihood =5667.821, CI =0.422, RI =0.724, RC =0.306, length =1199 steps). Branch lengths correspond to inferred genetic distances with the scale bar equivalent to 10 nucleotide substitutions. The numbers at the nodes represent bootstrap support values based on 2000 resamplings (values of 50% and higher are shown). The tree was rooted to *Phomopsis viticola*.

Figure 3.2. Phylogenetic tree based on parsimony analysis of ITS1-5.8S-ITS2 rDNA sequences of *Cytospora* species and related teleomorphs. The displayed tree, chosen from one of the equally most parsimonious trees (MPT), had the best –ln likelihood score of the MPT (-ln likelihood =7901.33, CI =0.518, RI =0.759, RC =0.393, length =1432 steps). Branch lengths correspond to inferred genetic distances with the scale bar equivalent to 10 nucleotide substitutions. The numbers at the nodes represent bootstrap support values based on 2000 resamplings (values of 50% and higher are shown). The tree was rooted to *Diaporthe vaccinii*.

leucostoma/non-*C. chrysosperma*/*V. nivea* clade occupies a position at the top of the Bayesian consensus tree (as opposed to *V. eugeniae* in the MP tree) while the *V. cypri* isolate is located near the root of the tree. The sister relationship between *V. leucostoma* and non-*C. chrysosperma* isolates is highly supported (posterior probability =1.00) in the Bayesian analyses in comparison to that inferred by MP analysis (bootstrap =81%). Isolate C 14 once more clusters robustly with *V. sordida* isolates (posterior probability =1.00). The large clade within which C 14 and *V. sordida* isolates cluster has a posterior probability of 1.00 but interrelationships are partly unresolved as indicated by the polytomy. In MP analysis, taxa within this large clade are separated into several distinct clusters.

The large clade (.71 posterior probability) in the basal portion of the Bayesian consensus tree also displays considerable polytomy. When comparing this portion of the tree to that resolved by MP analysis several inconsistencies are present. Although bootstrap support values are weak (<50%) parsimony analysis shows *V. melanodiscus* isolates (and *C. eriobotryae* and *V.* cf. *ceratosperma*) to be more closely related to isolates of *V. diatrypoides* (and Worrall 7b) than to *V. kunzei* and *V. pini* isolates. Support for the *V. diatrypoides*/Worrall 7b and *V. kunzei*/*V. pini* relationship in the Bayesian analyses is, however, low (posterior probability =.62) as well.

Bayesian analyses and ITS1-5.8S-ITS2 rDNA sequence data set. Analyses of the ITS1-5.8S-ITS2 rDNA data set (102 taxa) produced a 50% majority-rule consensus tree with a mean tree length of 13.0379 and estimated marginal likelihood (arithmetic mean) of -7298.08 (Figure 3.4). Names to the right of the thickened vertical bars are described species occurring on aspen. A cladogram (Figure 3.5) also was drawn to

display the posterior probability distributions for the branches due to the short branch lengths in the phylogram. Convergence diagnostics indicated the two independent analyses had converged and analyses of trace files (generated by the Bayesian MCMC runs) with Tracer again showed low sample correlation and that convergence had been reached.

Branching order in the consensus tree (Figure 3.4) is poorly resolved with a preponderance of polytomy. Despite this fact some of the finer relationships within clades are better supported as compared to the parsimony analysis of the ITS1-5.8S-ITS2 rDNA data set (Figure 3.2). Furthermore, support for some of the lager, i.e., more comprehensive clades, improves as well.

Non-*C. chrysosperma* and Galder_Mich isolates comprise a monophyletic group with a posterior probability of .98. In agreement with MP analysis the group is not nested within the larger clade consisting of the *V. leucostoma* and *V. nivea* groups (and a *V. parapersoonii* isolate). Because of the polytomy a more defined location for the clade(s) cannot be hypothesized. Bayesian support for the *V. leucostoma* group is .94 compared to a very low bootstrap support of <50% in MP analysis. The *V. translucens* isolate is clustered amongst isolates of the *V. nivea* group but the support is again low (posterior probability =.53; bootstrap =<50%). *Valseutypella mollicollis* shows a sister relationship to a *V. nivea* isolate in the Bayesian analysis unlike in the parsimony analysis where it clusters within a nearby clade (separated by *V. diatrypoides* isolates). Isolate C 14 clusters with good probability (.95) within the same taxon-rich clade as was observed in parsimony analysis, but its relationship to isolates of *V. sordida* cannot be resolved. The *V. diatrypoides* and *V. melanodiscus* clades are strongly supported with posterior probabilities of .97 and .98 respectively, and Worrall 7b again has a poorly supported (.65) sister relationship with *V. cypri* isolates.

Figure 3.3. Phylogenetic tree of *Cytospora* species and related teleomorphs. The 50% majority rule consensus tree was generated from Bayesian analysis of EF-1α sequences using one million searches sampling every 100th cycle and the initial 2,500 cycles discarded as burn-in. Branch lengths correspond to inferred genetic distances with the scale bar equivalent to 0.1 nucleotide substitutions per site. Numbers at the nodes are posterior probability values greater than 0.50.

Figure 3.4. Phylogenetic tree of *Cytospora* species and related teleomorphs. The 50% majority rule consensus tree was generated from Bayesian analysis of ITS1-5.8S-ITS2 rDNA sequences using two million searches sampling every 100th cycle and the initial 5,000 cycles discarded as burn-in. Branch lengths correspond to inferred genetic distances with the scale bar equivalent to 0.1 nucleotide substitutions per site. Numbers at the nodes are posterior probability values greater than 0.50.

Figure 3.5. Cladogram of *Cytospora* species and related teleomorphs. The 50% majority rule consensus tree was generated from Bayesian analysis of ITS1-5.8S-ITS2 rDNA sequences using two million searches sampling every 100^{th} cycle and the initial 5,000 cycles discarded as burn-in. Numbers at the nodes are posterior probability values greater than 0.50.

Morphological characterization
Ascostromata

Putative leucostomoid *Valsa* species. The teleomorph, i.e., putative leucostomoid *Valsa* sp. (per Adams et al., 2005), associated with non-*C. chrysosperma* specimens produces typically ovoid (occasionally circular) ascostromata (2.0-3.0 x 1.2-1.8 mm) which are immersed in the host bark tissue and become erumpent (Figure 3.6). Ascostromata are interspersed amongst fruit bodies of the anamorph and may occur in the same stroma (Figure 3.6). A prominent olive-black to black conceptacle delimits the stroma (leucostomoid ascostromata) (Figure 3.6).

Ascostromatal discs (0.4-0.55 (-0.65) mm diameter) are prominent, snowy-white to grayish-white, furfuraceous, i.e., powdery or flaky, circular to ovoid, nearly flat, (Figure 3.6) and composed of loosely arranged *textura angularis* and *textura intricata.* Ostioles ((45-) 60-100 (-120) μm diameter) are olive-black to black, and level with or slightly above the disc surface (Figure 3.6). The ostioles emerge through the disc (Figure 3.6) and may occur scattered, in rows, or in a circular arrangement on the disc surface; as few as two or as many as 10 may be visible. An ovoid to circular zone of olive-black to black conceptacle-like tissue, i.e., thick-walled, darkly pigmented *textura angularis* and *textura intricata*, surrounds the disc becoming less prominent with increasing depth. This zone can be variable among specimens, or between ascostromata on the same specimen, ranging from well developed to somewhat obscure (Figure 3.6). When well developed, the conceptacle-like tissue gives the fruit body a distinct target-like appearance.

Perithecia are circinate and laterally inclined, typically globose ((0.25-) 0.3-0.40 (-0.50) mm diameter) (Figure 3.6), and olive-black to black; numbers per ascostroma vary from 6-15. Walls of perithecia are comprised of *textura*

epidermoidea and surrounded by well developed orangish, cinnamon, olive-gray to creamish-white entostroma consisting of *textura angularis* and *textura intricata* (Figure 3.6). Asci float freely in the centrum and are clavate to obclavate ((33-) 37-43 x 8-11 µm), 8-spored, and have a non-amyloid apical ring (Figure 3.10). Ascospores ((7.5-) 8.0-9.5 x 1.5-2.0 µm) are biseriate, allantoid, thin-walled, hyaline, and aseptate. Ascospores ooze from teleomorphic structures in salmon-colored masses.

Valsa sordida. *V sordida* and putative leucostomoid *Valsa* sp occurring on aspen in Colorado are easily distinguished from one another. Most apparent are the absence of conceptacles (euvalsoid ascostromata per Adams et al., 2005) delimiting the ascostromata and the lack of ovoid/circular conceptacle-like tissues surrounding the discs in *V. sordida* specimens (Figure 3.7). Additionally, discs of *V. sordida* are not snowy-white in color and never have ostioles emerging through them (Figure 3.7).

V. sordida ascostromata are circular to ovoid (2.0-3.0 x 1.5-2.0 mm), immersed in the host bark tissue and become erumpent. Ascostromata are interspersed amongst fruit bodies of the anamorph (*C. chrysosperma*) and never occur in the same stroma.

Ascostromatal discs ((0.25-) 0.3-0.4 (-0.55) mm diameter) are prominent (sometimes obscured by perithecial beaks), tan, gray, olive-gray to olive-black, furfuraceous, circular to ovoid, nearly flat, and composed of amorphous material (Figure 3.12). Ostioles ((-65) 80-120 (-145) µm diameter) are olive-black to black, circinately arranged around the margin of the disc, and level with or slightly above the disc surface (Figure 3.7). Ostiolar beaks are often swollen and may be fused with adjacent beaks.

Perithecia are typically globose (0.35-0.50 mm diameter), olive-black to black, circinate, and laterally inclined; numbers per ascostroma vary from 8-15 (Figure 3.7).

Walls of perithecia are comprised of *textura epidermoidea* and have poorly developed cinnamon, brown, olive-green to olive-black entostroma around them. The absence of well developed entostroma (Figure 3.7) is another key morphological feature which can be used to delineate *V. sordida* from putative leucostomoid *Valsa* sp. Asci are clavate to obclavate ((33-) 42-47 (-53) x 6-10 μm), 8-spored, have a non-amyloid apical ring, and float freely in the centrum. Ascospores (8.0-11.5 x 1-2 μm) are biseriate, allantoid, thin-walled, hyaline, with no septa and ooze from teleomorphic structures in milky-white to yellowish streams.

Conidiomata

Putative labyrinthine/rosette leucocytosporoid *Cytospora* species. Non-*C. chrysosperma* (putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. per Adams et al., 2005) conidiomata are often interspersed amongst conidiomata of *C. chrysosperma* (Figure 3.8); they also may occur in the same stroma as putative leucostomoid *Valsa* sp. Conidiomata are ovoid to circular (1.5-2.5 x 1.0-1.5 mm) immersed in the host bark tissue and become erumpent (Figure 3.8). A prominent olive-black to black conceptacle delimits the stroma (leucocytosporoid conidiomata) (Figure 3.8).

Conidiomatal discs (0.25-0.40 mm diameter) are prominent, white to grayish-white, furfuraceous, circular to ovoid, nearly flat, (Figure 3.8) and composed of thin-walled *textura angularis* and amorphous material. Ostioles (75-150 (-170) μm diameter) are olive-gray, olive-black to black, and level with or slightly above the disc surface (Figure 3.8); numbers per conidioma vary from 1 to 3. An ovoid to circular zone of olive-black to black conceptacle-like tissue surrounds the disc, and tends to extend deeper into the fruit body in comparison to similar tissues found in ascostromata.

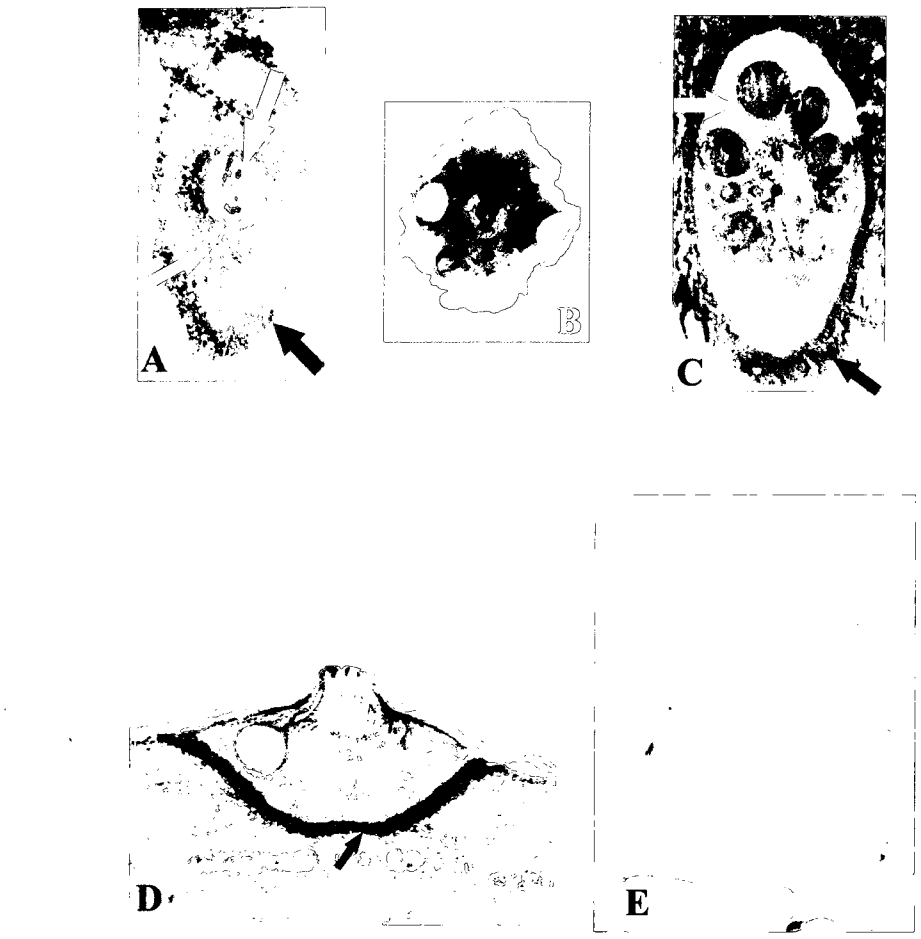
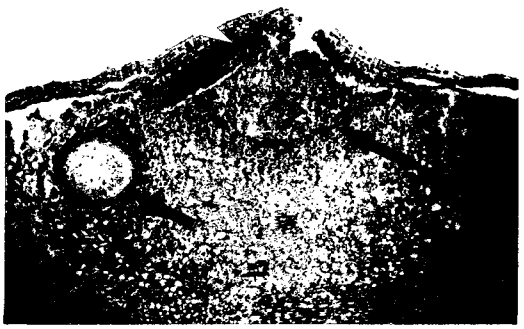


Figure 3.6. Ascostromata of putative leucostomoid *Valsa* sp. A. Erumpent ascostroma with white arrow pointing to prominent ovoid snowy-white disc and emerging black ostioles. Yellow arrow points to obscure black conceptacle-like tissue and black arrow the black conceptacle. B. Ascostroma lifted out showing anamorph in same stroma as teleomorph- red arrow points to a perithecium and green arrow to locular chambers of the anamorph. C. Horizontal cross section showing circinate perithecia surrounded by well developed cinnamon to creamish-white entostroma- red arrow points to a black globoid perithecium and black arrow the conceptacle. D. Vertical section with white arrow indicating ostiole emerging through disc; yellow arrow points to black conceptacle-like tissue surrounding the disc; red arrow points to a laterally inclined perithecium surrounded by entostroma; black arrow indicates conceptacle. E. Clavate asci (with ascospores) floating freely in perithecial centrum. Magnification (**approximate**): A-D = 20X; E = 300X.



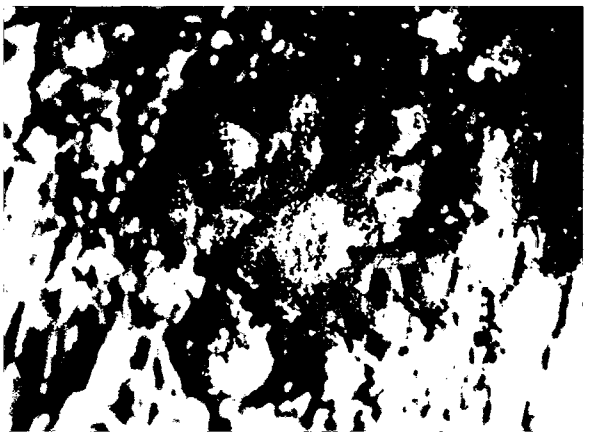


Figure 3.7. Ascostromata of *Valsa sordida*. Top picture: Vertical section with white arrow pointing to ostiole at margin of disc; brown arrow indicates stromatic tissue below disc; red arrow points to globose perithecium lacking well developed entostroma around it-note lack of conceptacle delimiting stroma. Bottom picture: Circinate laterally inclined perithecia with ostioles at margin of prominent tan to gray circular disc. Magnification (**approximate**): Top, bottom = 30X.

As seen with the teleomorph, the conceptacle-like tissue can be variable in terms of development, and when well developed conidioma take on a distinctive target-like appearance (Figure 3.8).

Conidiomatal stromata are rosette to labyrinthine, i.e., composed of multi-chambered locules that are subdivided by invaginations into regular and irregular radial chambers (lined with a layer of conidiophores) that share common walls (100 x 300 μm diameter) (Figure 3.8). Tissues surrounding the locules are well developed, cinnamon, olive-gray to creamish-white in color, and consist of *textura angularis* and *textura intricata* (Figure 3.8). Conidiophores are hyaline and branched, and made up of basal cells ((2.0-) 3.0-4.5 x 1.5-3.0 μm) subtending phialides (6.0-10.0 x 1.0-1.5 μm), or occasionally stalk cells (4.5-5.0 x 1.0 μm) (Figure 3.9). Long gelatinous hyphae are present between conidiophores forming a continuous gelatinous matrix. Conidiogenous cells are enteroblastic phialidic, cylindrical and taper to apices with collarettes. Like ascospores, conidia are hyaline, eguttulate, allantoid, and aseptate; they are, however, somewhat shorter in length (3.0-6.0 x 1.0 μm) (Figure 3.9). Conidia ooze from conidiomata in salmon-colored masses.

Cytospora chrysosperma. As with the teleomorphs, distinguishing between the anamorphs, i.e., *Cytospora chrysosperma* and putative labyrinthine/rosette leucocytosporoid *Cytospora* sp., occurring on aspen in Colorado is relatively easy. Absence of conceptacles delimiting the conidiomata (cytosporoid conidiomata per Adams et al., 2005) and the lack of ovoid/circular conceptacle-like tissues surrounding the discs in *C. chrysosperma* are the most diagnostic macroscopic features (Figure 3.10).

Conidiomata are ovoid to circular (1.0-1.7 x 0.5-1.0 mm) immersed in the host bark tissue and become erumpent (Figure 3.10)

Conidiomatal discs (0.30-0.55 (-0.60) mm diameter) are prominent, gray, olive-gray to olive-black, furfuraceous, circular to ovoid, nearly flat, (Figure 3.10) and composed of thin-walled *textura angularis* and amorphous material. Ostioles (60-135 (-150) μm diameter) are olivaceous-black to black, level with or slightly above the disc surface and occur singularly for each conidioma (Figure 3.10).

Conidiomatal stromata are labyrinthine with complex multi-chambered locules subdivided into numerous irregular chambers (lined with a layer of conidiophores) sharing common walls (100 x 250 μm diameter) (Figure 3.10). Tissues surrounding the locules are somewhat better developed than those surrounding the perithecia of the teleomorph, viz., *V. sordida*, and are comprised of gray, olive-green to olive-black *textura angularis* and *textura intricata*. Conidiophores are hyaline and branched, and made up of basal cells (2.0-4.0 x 2.0-3.5 μm) subtending phialides (6.0-9.0 x 1.0-2.0 μm), or occasionally stalk cells (3.5-6.0 x 1.0 μm); a continuous gelatinous matrix is formed by long gelatinous hyphae arising between conidiophores. Conidiogenous cells are enteroblastic phialidic, cylindrical and taper to apices with collarettes. Conidia are similar to spores of the teleomorph, i.e., hyaline, eguttulate, allantoid, and aseptate, but shorter (3.0-5.0 (-6.0) x 1.0 μm); they ooze from conidiomata in milky-white to yellowish streams.

Conidiomata *in vitro*. *In vitro* differences between mature pycnidia of *C. chrysosperma* isolates and those of putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. were most easily observed with fixed, embedded, and sectioned specimens.

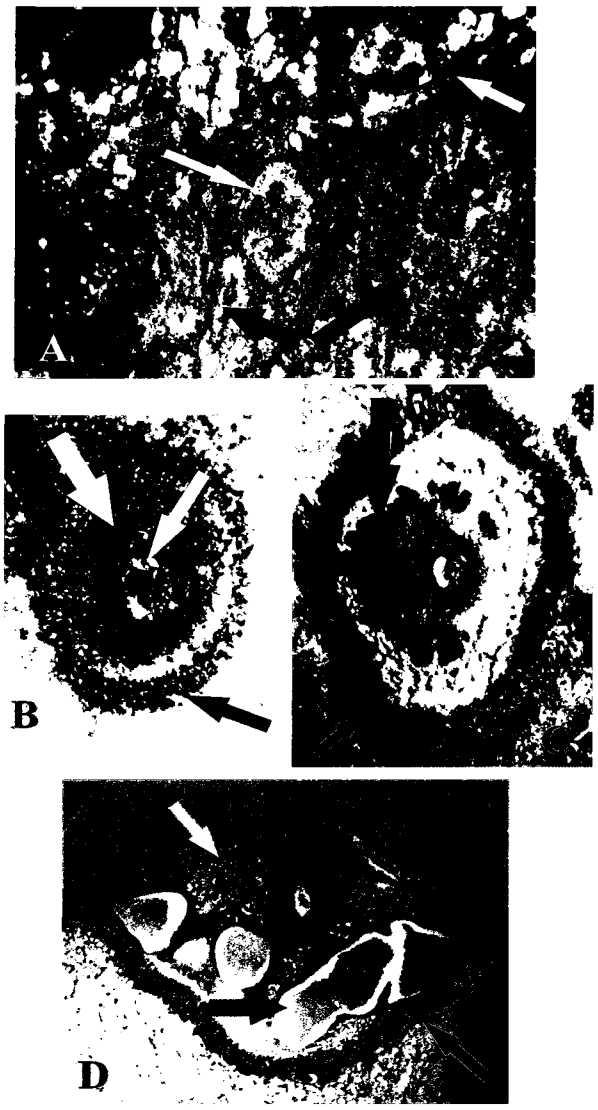


Figure 3.8. Conidiomata of putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. A. Interspersed conidiomata- white arrows point to those of putative *Cytospora* sp. and red arrows to those of *C. chrysosperma*. B. Erumpent conidioma with yellow arrow pointing to well developed black conceptacle-like tissue, white arrow to a prominent circular grayish-white disc with two emerging black ostioles, and black arrow to the black conceptacle. C. Horizontal cross section with green arrow indicating rosette multi-chambered locules surrounded by well developed creamish-white to olive-gray stromatic tissues; black arrow indicates the conceptacle. D. Vertical section with yellow arrow indicating conceptacle-like tissue surrounding the disc; green arrow points to rosette multi-chambered locules sharing common walls surrounded by well developed stromatic tissues; black arrow indicates the conceptacle delimiting the stroma. Magnification (**approximate**): A = 10X; B, C = 25X; D = 30X.

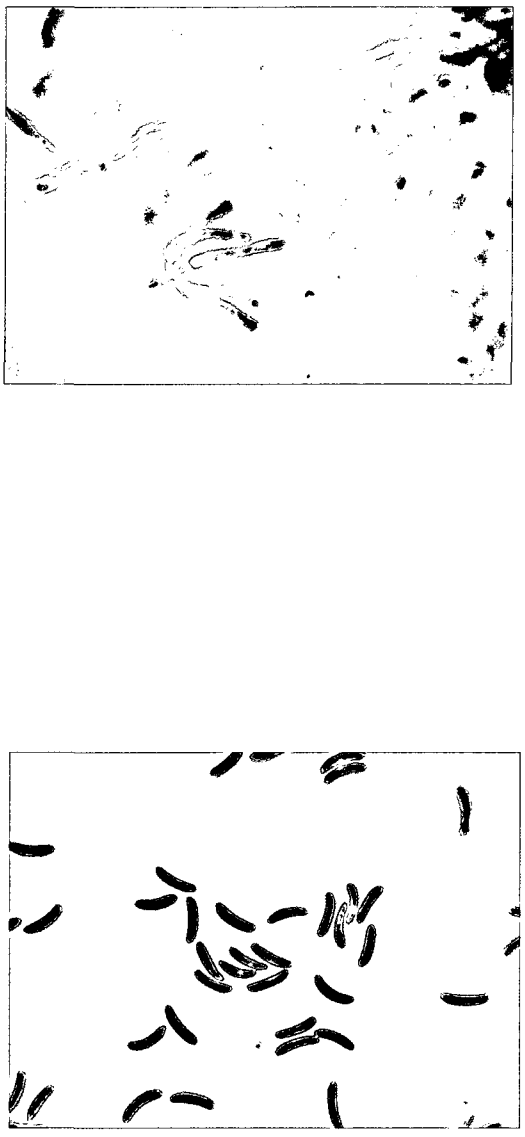


Figure 3.9. Conidiogenous cells and spores of putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. Top picture: Left-most arrow points to a basal cell subtending branching phialidic conidiophores; right-most arrow points to a spore at apex of phialide. Bottom picture: Hyaline, eguttulate, allantoid, and aseptate conidia. Magnification: Top, bottom = 1000X.

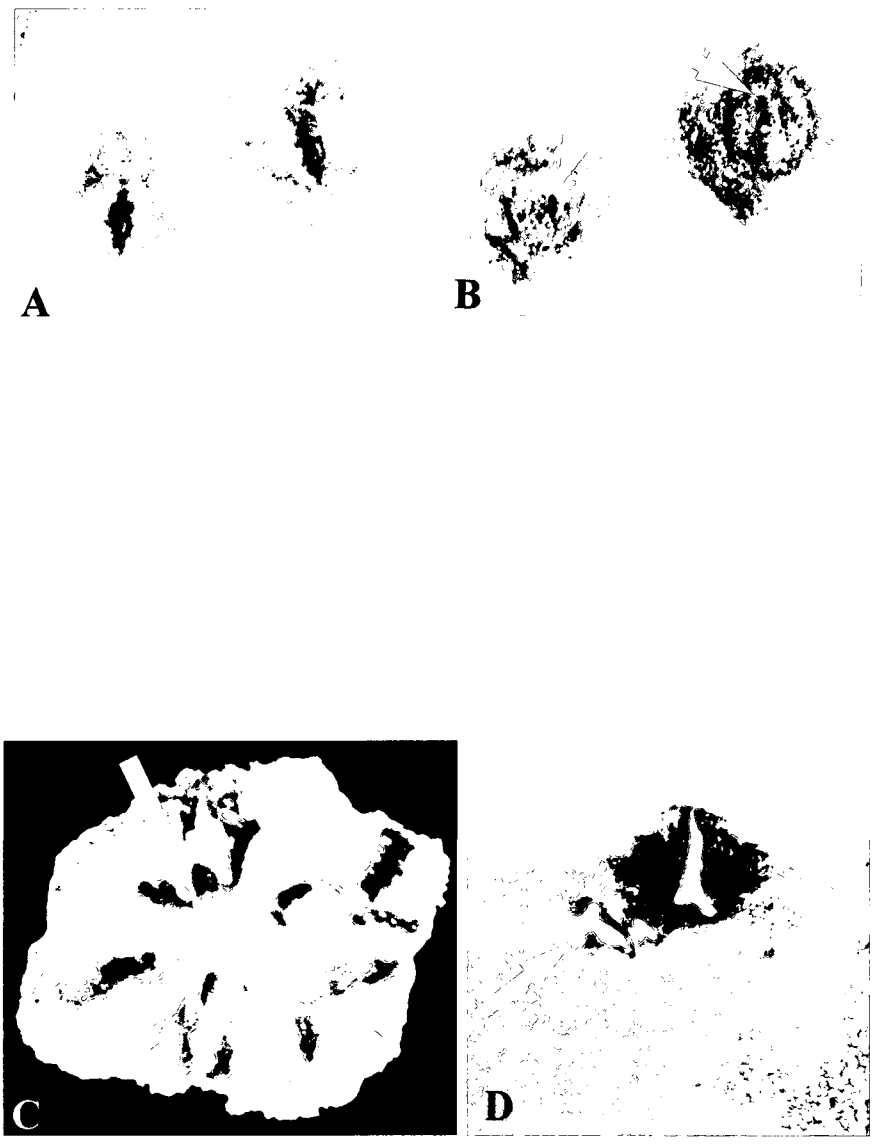


Figure 3.10. Conidiomata of *Cytospora chrysosperma*. A. Erumpent circular conidiomata- note lack of conceptacles and conceptacle-like tissues. B. Conidiomata with white arrows pointing to prominent gray, olive-gray to olive-black circular discs each with single black ostiole. C. Conidioma lifted out with green arrow pointing to labyrinthine multi-chambered locules surrounded by gray to olive-green stromatic tissues. D. Longitudinal cross section with green arrow pointing to labyrinthine multi-chambered locules sharing common walls surrounded by dark stromatic tissues (stroma of *C. chrysosperma* is better developed than entostroma of *V. sordida*); note lack of conceptacle and conceptacle-like tissue. Magnification (**approximate**): A, D = 15X; B = 30X; C = 40X.

Pycnidia produced by isolates of *C. chrysosperma* have a complex structure and resemble fruiting bodies formed on bark (natural state). The complex structure is comprised of multi-lobed locular chambers enclosed within the pycnidium (Figure 3.11). A layer of conidiophores, interspersed with long gelatinous hyphal cells, line the walls of the locular structure (Figure 3.11). In contrast, pycnidia produced by non-*C. chrysosperma* isolates are much simpler and less organized than those formed by *C. chrysosperma* isolates in culture, as well as fruiting bodies of putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. occurring naturally on bark. The simple pycnidial structure does not have an enclosed multi-lobed locular structure; rather it has invaginations, i.e., indentations/pockets, with conidiophores lining these invaginations as well as the surface of the pycnidium (Figure 3.11).

Cultural characteristics. Cultures of *C. chrysosperma* isolates (C 1 and C 14), were distinctly different than those of non-*C. chrysosperma* isolates (3, 16, 20, T1) when grown in 90 mm Petri plates containing 20 ml modified Leonian's agar under 12 hours continuous light and 12 hours continuous darkness at 25 C. *Cytospora chrysosperma* cultures were much lighter in color and zonated, viz., buff to honey-color with olivaceous concentric rings. The reverse were honey-colored with a small inner olivaceous-black zone. Aerial hyphae were dense and quite tall. Numerous spherical shaped, darkly pigmented (greenish-black) pycnidia, some covered with white, buff to honey-colored hyphae, formed primarily in the zonated regions. Spores masses were pale-luteous, saffron to orange-colored. Conidia were 3.0-4.5 x 1.0 μm with oil droplets (1-2) occasionally observed.

Cultures of non-*C. chrysosperma* isolates tended to be much darker in color ranging from olivaceous (3) to olivaceous-black (16, 20, T1) (Figure 3.12). The reverse were the same as the afore mentioned colors for the respective isolates. Hyphae were generally appressed and typically grew down into the agar. Pycnidia were darkly pigmented (greenish-black) and often covered with white, smoke-grey to olivaceous-grey hyphae. Isolates 16, 20, and T1 produced pycnidia that grew more upright/vertical forming beaks/necks with minimal exudate (milky-white); spore masses produced by isolate 3 were more numerous and ranged from milky-white, pale-luteous to orange. Conidia were 3.5-6.0 x 1.0 μm with 1-2 oil droplets frequently observed.

No differences were observed in the size of pycnidia amongst the isolates. However, examination of hyphal tips collected from five day old cultures and mounted in water showed hyphae produced by *C. chrysosperma* isolates to be different in size as well as morphology. Hyphae of *C. chrysosperma* isolates were 1.5-2.5 μm in diameter and uniformly straight; non-*C. chrysosperma* isolates had considerably larger hyphae (4.0-5.5 μm diameter) that was bead-like/wavy in appearance. Additionally, a bursting of hyphal tips was observed in young cultures (ca. seven days old) of non-*C. chrysosperma* isolates whereas no lysing occurred with isolates of *C. chrysosperma.*

Young cultures, i.e., approximately 7-10 days old, of non-*C. chrysosperma* isolates (16 and 20) were found to produce a *Phialocephala*-like anamorph (Figure 3.12). These synanamorphs were located in older regions of cultures just beginning to form pigmentation and where hyphae were aggregated into ball-like clusters. *Phialocephala*-like structures are comprised of darkly pigmented mononematous conidiophores arising off the main hyphae (Figure 3.12). Conidiophores are variable in length (short to quite

long), and in numbers of septa (three or more), and often times subtended by basal cells. Branching of conidiophores is initiated at or near septa with branching being variable, i.e., dichotomous to three or more, and occurring in a series (Figure 3.12). Conidiogenous cells are phialidic, cylindrical (5.0-12.0 x 2.0-3.0 μm) and taper to apices. Conidia are hyaline, allantoid, and aseptate.

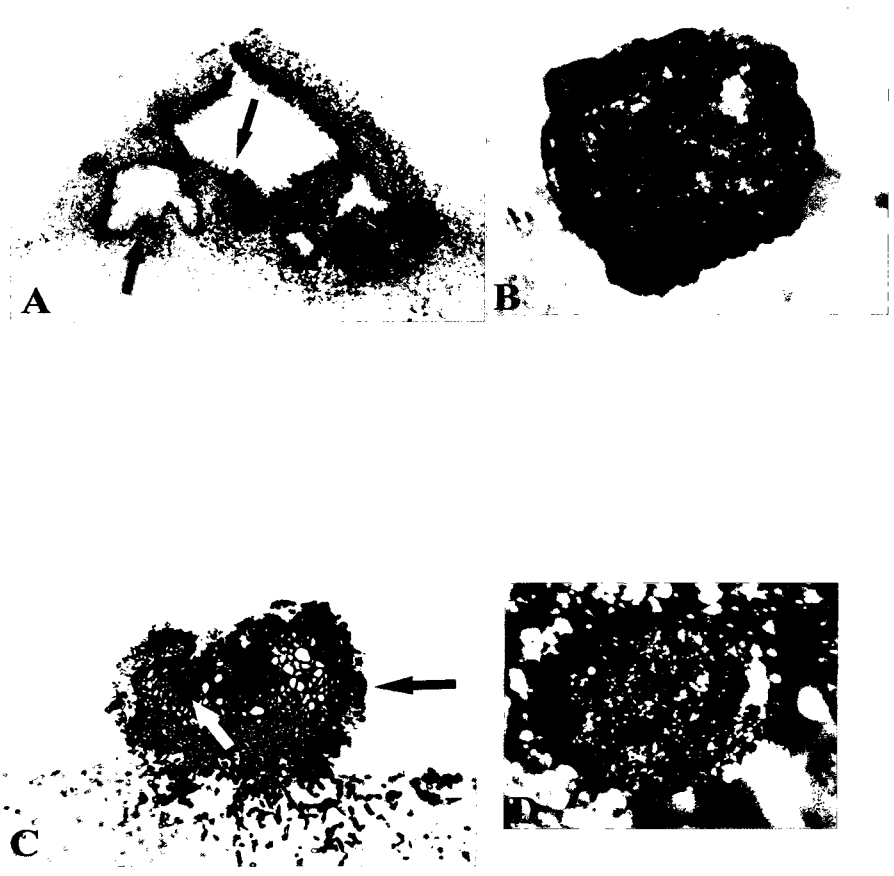


Figure 3.11. Conidiomata *in vitro*. A. Vertical section of pycnidium of *Cytospora chrysosperma* isolate C 14 with red arrow pointing to multi-lobed locular chambers and black arrow to the layer of conidiophores, interspersed with long gelatinous hyphal cells, lining the walls of the locular structure. B. Vertical section through pycnidium of *C. chrysosperma* isolate C 14 showing the complex labyrinthine locular structure. C. Longitudinal cross section of pycnidium of putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. isolate 3 with yellow arrow pointing to invaginations in the pycnidium which are lined with conidiophores; the black arrow points to conidiophores lining the surface of the pycnidium. D. Horizontal cross section through pycnidium of putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. isolate 3 showing the simple pycnidial structure which does not have an enclosed multi-lobed locular structure. Magnification (**approximate**): A = 75X; B-D = 50X.

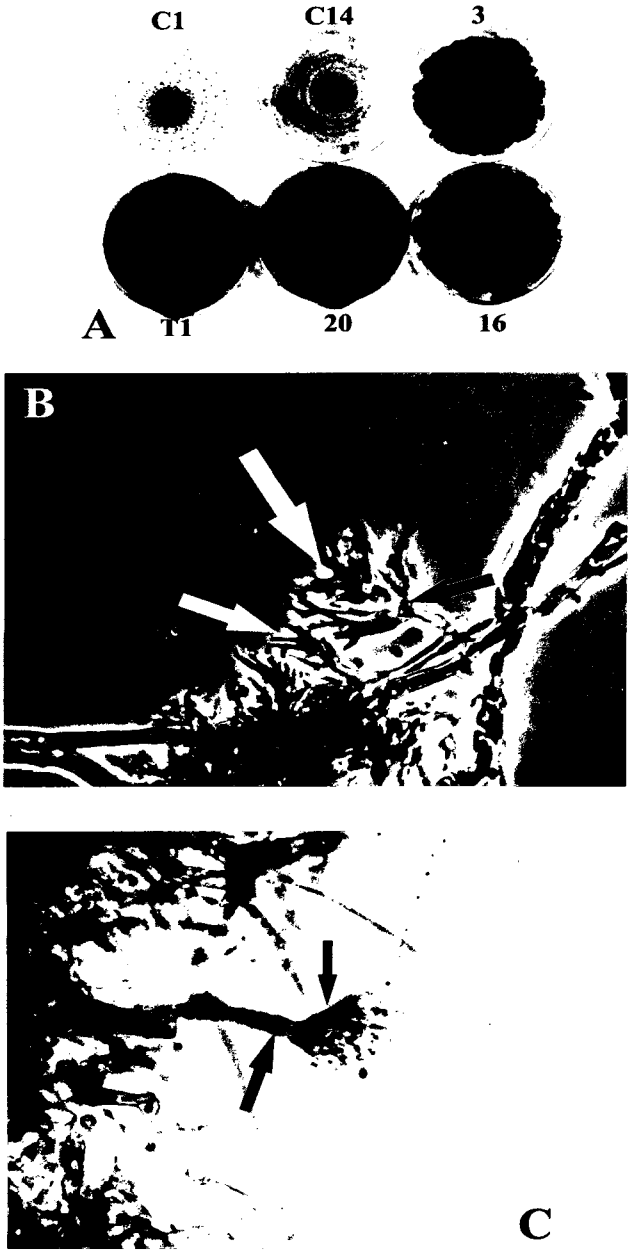


Figure 3.12. Cultural characteristics. A. Cultures of *C. chrysosperma* isolates (C 1 and C 14) are lighter in color and zonated (pycnidia forming in the zonated regions) vs. those of non-*C. chrysosperma* isolates (3, 16, 20, T1). B. *Phialocephala*-like synanamorph produced by non-*C. chrysosperma* isolates 16 and 20. Black arrow indicates the point where dichotomous branching of conidiophores begins; yellow arrows point to clusters of phialidic conidiogenous cells of two conidiogenous apparatuses. C. *Phialocephala*-like synanamorph produced by non-*C. chrysosperma* isolates 16 and 20. Black arrows point to septa and yellow arrow to phialidic conidiogenous cells of conidiogenous apparatus. Magnification (**approximate**): B, C = 250X.

Discussion

One of the main objectives of the present study was to use molecular techniques to sort out the Cytospora canker "species complex" occurring on aspen in Colorado. I was hopeful that answers for the following questions would be found: 1) are non-*C. chrysosperma* isolates members of the genus *Cytospora*?; 2) if so, do they represent a new species?; 3) how do non-*C. chrysosperma* isolates fit within a *Cytospora* spp. (and related teleomorphs) phylogeny? Based upon phylograms generated by the phylogenetic analyses of EF-1α and ITS1-5.8S-ITS2 rDNA sequence data sets the questions have been answered, albeit not without some incongruity. Firstly, for the isolates and geographic areas examined, non-*C. chrysosperma* isolates associated with the Cytospora canker complex on aspen in Colorado should be considered members of the genus *Cytospora* based upon their firm placement within a *Cytospora* spp. phylogeny. Furthermore, the clustering of isolates identified as *C. chrysosperma* with those of *V. sordida* isolates supports the literature with respect to *C. chrysosperma* as a causative agent of Cytospora canker on aspen in Colorado. Secondly, in all analyses (preliminary and final) non-*C. chrysosperma* isolates were strongly supported as a monophyletic clade and therefore it can be concluded they constitute a new (phylogenetic) species. Answering the third question, i.e., resolving interrelationships, unfortunately is somewhat problematic based upon inconsistencies and polytomies occurring in the phylograms.

In the final analyses the greatest resolution of interrelationships was achieved with MP analysis of the EF-1α sequence data set. Nevertheless, in preliminary analyses, regardless of the gap coding or inference method used, EF-1α gene trees were all generally well-resolved other than the clade comprised of *V. melanodiscus* isolates and the clade representing the root of the tree lineage. Furthermore, a close relationship

between non-*C. chrysosperma* and *V. leucostoma* isolates was observed in all instances with bootstrap values ranging from 73 to 100%. The primary inconsistency among trees generated using various methodologies involved a shifting of major clades along the tree backbone.

Bayesian analyses, on the other hand, generated an EF-1α gene tree with considerable polytomy. In spite of this, some interior branches displayed strong posterior probabilities as opposed to the weak bootstrap support from parsimony analysis. Of particular interest is the non-*C. chrysosperma*/*V. leucostoma* relationship (posterior probability =1.0 vs. bootstrap =81%) and the well supported nesting of non-*C. chrysosperma* isolates within a *V. leucostoma*/*V. nivea* clade (posterior probability =1.0 vs. bootstrap =71%). The Bayesian method and posterior probabilities have been criticized (Simmons et. al, 2004; Kolackowski & Thornton, 2007; Jones, 2008). Simmons et. al believe the Bayesian method overestimates support and that support values should not be regarded as probabilities for correctly resolved clades. Kolackowski/Thornton and Jones' primary objections regard the use of priors, i.e., prior assumptions about model parameters, which can greatly affect posterior probabilities. With this in mind, Simmons et al. (2004) advise use of the "conservative" bootstrap approach for estimating branch support which is known to underestimate clade probability (Hall, 2008).

Areas for concern regarding the final analyses of the ITS1-5.8S-ITS2 rDNA sequences are mainly associated with the large number of equally MPT and low bootstrap values (for larger clades) generated with MP analysis, and the large amount of polytomy seen with Bayesian analyses. The recovery of a large number of MPT can be a function

of the number of taxa analyzed, i.e., increasing terminal units increases number of trees generated (Felsenstein, 1978), the result of homoplasy (Sanderson & Donoghue, 1989; Farr et al., 2002a), or "very real polytomies in the tree" (Hall, 2008). Farr et al. believed the great number of MPT they encountered using parsimony analysis and ITS sequence data were the result of a large amount of homoplasy distributed across *Phomopsis* as a whole.

Examination of indices measuring the fit of data to tree topology, e.g., CI and RI, are often used to evaluate homoplasy levels. However, CI is highly correlated with the number of taxa analyzed and has been shown to decrease as the number of taxa analyzed increase (Sanderson & Donoghue, 1989). Naylor and Kraus (1995) cite studies which have shown RI are less sensitive to both numbers of taxa and characters in a data set (Archie, 1989), as well as not being influenced by uninformative characters (Farris, 1989). As such, RI may be better indicators of levels of homoplasy. Examination of CI and RI and equally MPT generated for the EF-1α and ITS1-5.8S-ITS2 rDNA sequence data sets in the final analyses seems to contradict some of these findings, however. For example, although the number of MPT are many times less for the smaller (56 taxa) EF-1α data set as compared to the larger (102 taxa) ITS1-5.8S-ITS2 rDNA data set, viz., 43 vs. 5340 respectively, CI values indicate more homoplasy to be present in the EF-1α set (0.422 vs. 0.518); moreover, RI were very similar (0.724 vs. 0.759). Use of different gap coding methods, e.g., SIC and MCIC, and MP analyses of the ITS1-5.8S-ITS2 rDNA sequence data in the preliminary analyses did result in considerably less MPT (SIC =2877 MPT, MCIC =747 MPT), yet CI and RI were approximately the same for each

method (CI ~0.51, RI ~0.75) and similar to the previously stated indices values in the final analyses.

Despite the prevalence of polytomy in the phylogram generated by Bayesian analyses of the ITS1-5.8S-ITS2 rDNA sequence data set, many of the clades recovered are the same as those generated with MP analysis. Additionally, some interrelationships are better supported compared to weak confidence levels observed for interior branches in the parsimony tree. For example, the taxon-rich *V. leucostoma* group has a bootstrap support of <50% using parsimony analysis, but displays a Bayesian posterior probability of .94. Again this must be viewed with some caution based upon the scrutiny associated with the Bayesian method and posterior probabilities. An anomaly which occurred in both the MP and Bayesian analyses is the separation of *V. friesii* isolates. Given both isolates were collected from the same host species in Europe, viz., *Abies alba*, I would not expect their inferred relationship to be so distant. Therefore, it is possible the database documentation is incorrect or the validity of the sequence is suspect.

One facet which draws attention regarding the Bayesian tree and the ITS1-5.8S-ITS2 rDNA sequence data set, is the relatively short branch lengths throughout the tree. Such lengths indicate somewhat minimal divergence has occurred between ingroup taxa. It could be questioned whether the correct model of evolution, as assessed by ModelTest, was chosen for the data. Although not referred to in the materials and methods section, Bayesian analyses for the ITS1-5.8S-ITS2 rDNA sequences were conducted whereby the MrBayes program was allowed to estimate model parameters. The Bayesian tree generated by these analyses displayed the same overall polytomy and branch lengths as the one generated when likelihood settings estimated by ModelTest were used as starting

values. Generally speaking, the greatest difference was in relation to posterior probability values, i.e., some increased and others decreased slightly.

Comparison of parsimony and Bayesian ITS1-5.8S-ITS2 rDNA trees from the current study to those from prior phylogenetic studies with *Cytospora* spp. and related teleomorphs (Adams et al.; 2002; Adams et. al., 2005; Adams et al., 2006) reveals considerable overlap. By and large phylograms generated from all analyses are very similar in regards to the clades resolved and their overall topology. The main differences pertain to a shift of some of the clades along the tree backbone and variation in bootstrap values. Bayesian ITS1-5.8S-ITS2 rDNA trees from the prior studies do show considerably less polytomy when compared to the one from the present study. Moreover, these trees display deeper internal branching. However, the lack of deep internal branching in the current tree would seem to be the result of polytomy since branches whose support are less than 50% are collapsed. For the most part, clades recovered amongst all the studies are similar.

The placement of the non *C .chrysosperma* isolates within the *Cytospora* spp. phylogeny is suggested by a number of morphological characteristics. Most notably are erumpent fruit bodies composed of stromatic tissues surrounding locular chambers, the thread-like conidiophores, and conidiogenous cells that produce hyaline, allantoid, and aseptate conidia. The presence of the anamorph within the same stromatic tissues as the teleomorph is somewhat unusual, however. Adams et al. (2005) state this occurs in only a few species such as *V. cincta* and *V. massarianae*. Based on the phylograms from the phylogenetic analyses, non-*C. chrysosperma* isolates are closely related to these taxa and cluster between the two clades.

In culture, non-*C. chrysosperma* isolates are easily separated from those of *C. chrysosperma*. Cultural variation among non-*C. chrysosperma* isolates, however, is negligible although isolate 3 produced more lobate growth, was less darkly pigmented, and did not produce pycnidia with beaks. Nonetheless, morphological characteristics of the specimen are within the "normal range" for putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. It is interesting that isoenzymatic studies reported in Chapter 2 of this dissertation show that isolate 3 is in a different subgroup than isolates 16, 20, and T1, although genetic similarity (based on Jaccard's coefficient) is 47%. Unfortunately, this group of isolates was not examined in the vegetative incompatibility study discussed in Appendix III. Phylogenetically, isolate 3 shows a closer relationship with an isolate from *Alnus incana* var. *rugosa* from Michigan, based on EF-1α sequence data analyses. Obviously, further genetic, molecular and morphological studies should be conducted for this phylogenetic cluster of taxa. The occurrence of two synanamorphs, albeit in culture, was quite unexpected. To the best of my knowledge only two other studies (Hildebrand, 1947; Helton & Konicek, 1961) have reported such findings. Helton and Konicek described "naked conidiophores" arising from dichotomous branching of hyphal tips in isolates from stone fruit trees. Hildebrand reported a similar occurrence with isolates of *V. leucostoma* from peach trees. Whether this occurs in the field is not known. Based on the trees generated from phylogenetic analyses, non-*C. chrysosperma* isolates are distinct but closely related to *V. leucostoma* isolates.

With time, methods for identifying fungi and assessing their phylogeny will continue to improve, and databases will continue to enlarge. Both will improve resolution among fungal taxa. Some of the methods I used in the present study were

based on prior studies with *Cytospora* species. Although I could continue to fine tune my methodology, or explore other methods, I strongly believe (based on the reasonably congruent analyses) non-*C. chrysosperma* isolates from aspen in Colorado represent a new *Cytospora* species. I think it is important to remember that phylogenetic analyses allow only inferences and hypotheses to be formulated. Hall (2008) summarizes this quite succinctly stating that "the right tree doesn't exist" and "all methods implicitly acknowledge that the trees produced are only a subset of the possible trees that are consistent with the data."

The importance of a recognizing new *Cytospora* species cannot be understated. Given *Cytospora* spp. endophytic and pathogenic nature and broad host range, future studies are necessitated in order to determine the biology, ecology, and physiology of these organisms.

Formal Description of New Species

Valsa notabilistromatica Kepley & Reeves, sp. nov.

Anamorph. ***Cytospora notabilistromatica*** Kepley & Reeves, sp. nov.

Etymology: "*notabilistromatica*" refers to the notable inner zone of conceptacle-like (stromatic) tissue surrounding the disc of ascostromata and conidiomata.

Ascostromata in cortice immersa, erumpentia, ovoidea vel circularia 2.0-3.0 x 1.2-1.8 mm, peritheciis 6-15 in entostromate aurantiaca, cinnamomea, olivacea-grisea, vel cramum-alba circinatim dispositis, e cellulis texturam angularum et intricatum farmans, conceptaculo prominentes olivacea-nigra vel nigra. Disci prominentes, niveus vel grisei-albi, paene plani, circulares vel ovoidei 0.4-0.55 (-0.65) mm diametro, furfuracei, e cellulis texturam angularum et intricatum farmans, ostiolis 2-10 lateraliter vel recte insertis praediti, conceptaculi similis circuli olivaceus-nigra vel nigra circumdatus. Perithecia olivacea vel olivacea-nigra, globosa, (0.25-) 0.3-0.40 (-0.50) mm diametro, parietibus e textura epidermoidea compositis praedita. Asci liberi, clavati vel obclaveti (33-) 37-43 x 8-11 µm, apparatu apicali non amyloideo, 8-spori. Ascosporae biseriatae, allantoideae, tenuitunicatae, hyalinae (massiter ad salmonea), unicae, (7.5-) 8.0-9.5 x 1.5-2.0.

Anamorpha plerumque in teleomorphas interspersa, interdum in stromate sed teleomorphas. Stromata conidiomatica in cortice immersa erumpentia, ovoidea vel circularia 1.5-2.5 x 1.0-1.5 mm, conceptaculo prominentes olivacea-nigra vel nigra. Disci prominentes, albi vel grisei-albi, paene plani, circulares vel ovoidei 0.25-0.40 mm diametro, furfuracei, e materia amorphica compositi et cellulis texturam angularum farmans, ostiolis 1-3 praediti, conceptaculi similis circuli olivaceus-nigra vel nigra circumdatus. Ostiola olivacea-grisea, olivacea-nigra vel nigra, 75-150 (-170) µm diametro, paene plana vel aliqua supra superficiem disci. Loculi ad typum complexum multi-locellatum pertinentes, introrsum per plicas in cavernulis irregulares radiatim dispositas parietibus communalibus praeditas partiti, 100 x 300 µm diametro, stromate cinnamomeus, olivaceus-griseus vel crami-albus e *textura angularis* et *intricata* circumdatus. Conidiophora in matrice continua gelatinosa inclusae, hyalina et ramosa 6.0-10.0 x 1.0-1.5 µm (phialides includentia), cellularum basalium orientibus (2.0-) 3.0-4.5 x 1.5-3.0 µm. Cellulae conidiogenae enteroblastice phialidicae, cylindricae, ad apicem contractae, collaretta minuta. Conidia hyalina (massiter ad salmonea), eguttulata, allantoidea, unica 3.0-5.0 x 1.0 µm.

Ascostromata immersed in bark, erumpent, ovoid to circular 2.0-3.0 x 1.2-1.8 mm, leucostomoid circinateous, 6-15 perithecia arranged circinately in well developed orangish, cinnamon, olive-gray to creamish-white entostroma composed of cells forming *textura angularis* and *intricata*, conceptacles prominent, olive-black to black. *Discs* prominent, snowy-white to grayish-white, nearly flat, circular to ovoid 0.4-0.55 (-0.65) mm diam, furfuraceous, composed of cells forming a *textura angularis* and *intricata*, 2-10 laterally to vertically inserted ostioles, surrounded by an ovoid to circular zone of olive-black to black conceptacle-like tissue. *Ostioles* olive-black to black (45-) 60-100 (-120) µm diam, nearly level to slightly above disc surfaces. *Perithecia* olive-black to

black, globose (0.25-) 0.3-0.40 (-0.50) mm diam, inclined, walls of *textura epidermoidea*. *Asci* free, clavate to obclavate (33-) 37-43 x 8-11 µm, apical apparatus non-amyloid, 8-spored. *Ascospores* biseriate, allantoid, thin-walled, hyaline (salmon-colored in mass), and aseptate (7.5-) 8.0-9.5 x 1.5-2.0.

Anamorph usually interspersed amongst teleomorphs but may occur in the same stromata as the teleomorphs. *Conidiomatal stromata* immersed in bark, erumpent, labyrinthine to rosette leucocytosporoid, ovoid to circular 1.5-2.5 x 1.0-1.5 mm, conceptacles prominent, olive-black to black. *Discs* prominent, white to grayish-white, nearly flat, circular to ovoid 0.25-0.40 mm diam, furfuraceous, composed of amorphous material and cells forming a *textura angularis*, 1-3 ostioles, surrounded by an ovoid to circular zone of olive-black to black conceptacle-like tissue. *Ostioles* olive-gray, olive-black to black, 75-150 (-170) µm diam, nearly level to slightly above disc surfaces. *Locules* multi-chambered, subdivided by invaginations into regular to irregular radially arranged chambers sharing common walls, 100 x 300 µm diam, surrounded with well-developed cinnamon, olive-gray to creamish-white stromata of *textura angularis* and *textura intricata*. *Conidiophores* hyaline and branched 6.0-10.0 x 1.0-1.5 µm, inclusive of phialides, arise from basal cells (2.0-) 3.0-4.5 x 1.5-3.0 µm, embedded in a continuous gelatinous matrix. *Conidiogenous cells* enteroblastic phialidic, cylindrical, tapering to the apices, minute collarettes. *Conidia* hyaline (salmon-colored in mass), eguttulate, allantoid, aseptate 3.0-6.0 x 1.0 µm.

Phialocephala-like anamorph: On modified Leonian's agar (20 ml/90 mm Petri dish) at 25 C with 12 hours continuous light and 12 hours continuous darkness syanamorphs are located in older regions of young cultures (7-10 d old) where pigmentation is forming and hyphae are aggregated into ball-like clusters. *Conidiophores* arising from main hyphae are mononematous, darkly pigmented, variable in length (short to quite long) and numbers of septa (three or more), often subtended by basal cells, branching variable (dichotomous to three or more), occurs in series, initiated at or near septa. *Conidiogenous cells* phialidic, cylindrical (5.0-12.0 x 2.0-3.0 µm), taper to apices. *Conidia* hyaline, allantoid, aseptate.

Cultures: Colony growth on modified Leonian's agar (20 ml/90 mm Petri dish) after 28 d at 25 C with 12 hours continuous light and 12 hours continuous darkness is olivaceous-black (top and reverse). Hyphae are generally appressed and grow down into the agar. Pycnidia are greenish-black, often covered with white, smoke-grey to olivaceous-grey hyphae, and typically form vertically-oriented beaks/necks. Exuded cirrhi are milky-white.

Hosts: *Populus tremuloides* Michx.

Known distribution: Colorado, USA

Holotype: USA, COLORADO: Upper Poudre Canyon east of Cameron Pass on dead bole of *P. tremuloides* in grove, 20 Aug 2004, collectors J. Kepley and F. B. Reeves.

Paratype: USA, COLORADO: Pingree Park on dead bole of *P. tremuloides*, 25 May 2004, collectors J. Kepley and F. B. Reeves.

CHAPTER 3 LITERATURE CITED

Adams, G. C., Roux, J., and Wingfield, M. J. 2006. *Cytospora* species (*Ascomycota, Diaporthales, Valsaceae*): introduced and native pathogens of trees in South Africa. Australasian Plant Pathol. 35:521-548.

Adams, G. C., Surve-Iyer, R. S., and Iezzoni, A. F. 2002. Ribosomal DNA sequence divergence and group I introns within the *Leucostoma* species *L. cinctum, L. persoonii,* and *L. parapersoonii* sp. nov., ascomycetes that cause Cytospora canker of fruit trees. Mycologia 94:947-967.

Adams, G. C., Wingfield, M. J., Common, R., and Roux, J. 2005. Phylogenetic relationships and morphology of *Cytospora* species and related teleomorphs (*Ascomycota, Diaporthales, Valsaceae)* from *Eucalyptus*. Stud. Mycol. 52:1-144.

Agrios, G. N. 1997. Plant Pathology. Academic Press, San Diego, CA. 635 p.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Evol. 215:403-410.

Archie, J. W. 1989. Homoplasy excess ratios: new indices for measuring levels of homoplasy in phylogenetic systematics and a critique of the consistency index. Syst. Zool. 38:253-269.

Baayen, R. P., Bonants, P. J. M., Verkley, G., Carroll, G. C., Aa, H. A. van der, Weerdt, M. de, Brouwershaven, I. R. van, Schutte, G. C., Maccheroni, W., Glienke de Blanco, C., and Azevedo, J. L. 2002. Nonpathogenic isolates of the citrus black spot fungus, *Guignardia citricarpa*, identified as a cosmopolitan endophyte of woody plants, *G. mangiferae* (*Phyllosticta capitalensis*). Phytopathology 92:464-477.

Baldauf, S. L. 1999. A search for origins of animals and fungi: comparing and combining molecular data. Am. Nat. 154:S178-S188.

Baldauf, S. L., and Doolittle, W. F. 1997. Origin and evolution of the slime molds (Mycetoza). Proc Natl. Acad. Sci. 94:12007-12012.

Bartos, D. L., and Shepperd, W. D. 2006. Draft of notes from the aspen "die-off summit." 19 p.
http://www.aspensite.org/pdf/Die-off/Aspen-Summit-Summary.pdf.

Bonde, M. R., Micales, J. A., and Peterson, G. L. 1993. The use of isozyme analysis for identification of plant-pathogenic fungi. Plant Dis. 77:961-968.

Brody, J. R., and Kern, S. E. 2004. Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. BioTechniques 36:214-216.

Campbell, R. 2006. Restoring forest health and properly functioning conditions in aspen ecosystems. In presentation outlines from the aspen "die-off summit." http://www.aspensite.org/die-off_summitt.html.

Carbone, I., and Kohn, L. M. 1999. A method for designing primer sets for speciation studies in filamentous Ascomycetes. Mycologia 91:553-556.

Castlebury, L. A. 2005. The *Diaporthe vaccinii* complex of fruit pathogens. Inoculum 56:12 [Abstract].

Castlebury, L. A., and Mengistu, A. 2006. Phylogenetic distinction of *Diaporthe/Phomopsis* isolates from soybeans. Inoculum 57:13 [Abstract].

Castlebury, L. A., Rossman, A. Y., and Farr, D. F. 2003. Phylogenetic placement of the anamorph genus *Sirococcus*. Inoculum 54:15 [Abstract].

Cortinas, M.-N., Crous, P. W., Wingfield, B. D., and Wingfield, M. J. 2006. Multi-gene phylogenies and phenotypic characters distinguish two species within the *Colletogloeopsis zuluensis* complex associated with *Eucalyptus* stem cankers. Stud. Mycol. 55:133-146.

Donk, M. A. 1964. Nomina conservanda proposita I. Proposals in fungi Deuteromycetes. Regnum Veg. 34:7-15.

Farr, D. F., Bills, G. F., Chamuris, G .P., and Rossman, A. Y. 1989. Fungi on Plants and Plant Products in the United States. APS Press, St. Paul, MN. 1252 p.

Farr, D. F., Castlebury, L. A., and Pardo-Schultheiss, R. A. 1999. *Phomopsis amygdali* causes peach shoot blight of cultivated peach trees in the southeastern United States. Mycologia 91:1008-1015.

Farr, D. F., Castlebury, L. A., and Rossman, A. Y. 2002a. Morphological and molecular characterization of *Phomopsis vaccinii* and additional isolates of *Phomopsis* from blueberry and cranberry in the eastern United States. Mycologia 94:494-504.

Farr, D. E., Castlebury, L. A., Rossman, A. Y., and Erincik, O. 2001. *Greeneria uvicola*, cause of bitter rot of grapes, belongs in the Diaporthales. Sydowia 53:185-199.

Farr, D. F., Castlebury, L. A., Rossman, A. Y., and Putman, M. L. 2002b. A new species of *Phomopsis* causing twig dieback of *Vaccinium vitis-idaea* (lingonberry). Mycol. Res. 106:745-752.

Farris, J. S. 1989. The retention index and the rescaled consistency index. Cladistics 5:417-419.

Felsenstein, J. 1978. The number of evolutionary trees. Syst. Zool. 27:27-33.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783-791.

Geiser, D. M., Jiménez-Gasco, M. del Mar, Kang, S., Makalowska, I., Veeraraghavan, N., Ward, T. J., Zhang, N., Kuldau, G. A., and O'Donnell, K. 2004. FUSARIUM-ID v. 1.0: a DNA sequence database for identifying *Fusarium*. Eur. J. Plant Pathol. 110:473-479.

Green, S., and Castlebury, L. A. 2007. Connection of *Gnomonia intermedia* to *Discula betulina* and its relationship to other taxa in the Gnomoniaceae. Mycol. Res. 111:62-69.

Gregory, N. F., Mulrooney, R. P., Rossman, A. Y., and Castlebury, L. A. 2004. Anthracnose caused by *Discula fraxinea* on the new host Chinese fringetree and white ash in Delaware. Plant Dis. 88:427 [Abstract].

Gruell, G. E., and Loope, L. L. 1974. Relationships among aspen, fire, and ungulate browsing in Jackson Hole, Wyoming. U.S. Dept. Agric., For. Serv., Intermt. Reg., Ogden, UT. 33 p.

Gryzenhout, M., Myburg, H., Merwe, N. A. van der, Wingfield, B. D., and Wingfield, M. J. 2004. *Chrysoporthe*, a new genus to accommodate *Cryphonectria cubensis*. Stud. Mycol. 50:119-142.

Gryzenhout, M., Wingfield, B. D., and Wingfield, M. J. 2006a. New taxonomic concepts for the important forest pathogen *Cryphonectria parasitica* and related fungi. FEMS Microbiol. Lett. 258:161-172.

Gryzenhout, M., Myburg, H., Wingfield, B. D., and Wingfield, M. J. 2006b. Cryphonectriaceae (Diaporthales), a new family including *Cryphonectria*, *Chrysoporthe*, *Endothia* and allied genera. Mycologia 98:239-249.

Guyon, J. C., Jacobi, W. R., and McIntyre, G. A. 1996. Effects of environmental stress on the development of Cytospora canker of aspen. Plant Dis. 80:1320-1326.

Hall, B. G. 2008. Phylogenetic Trees Made Easy- A How To Manual. 3rd edition. Sinauer Associates, Inc. Sunderland, MA. 233 p.

Harlow, W. M., Harrar, E. S., Hardin, J. W., and White, F. M. 1996. Textbook of Dendrology. McGraw-Hill, New York, NY. 534 p.

Hasegawa, M., Kishino, H., and Yano, T. 1985. Dating the human-ape split by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22:160-174.

Helton, A. W., and Konicek, D. E. 1961. Effects of selected *Cytospora* isolates from stone fruits on certain stone fruit varieties. Phytopathology 51:152-157.

Hildebrand, E. M. 1947. Perennial peach canker and the canker complex in New York with methods of control. Cornell Univ. Agric. Stn. Mem. 276:1-61.

Hinds, T. E. 1964. Distribution of aspen cankers in Colorado. Plant Dis. Rep. 48:610-614.

Hinds, T. E. 1985. Diseases. Pages 87-106 in DeByle, N. V. and Winokur, R. P. (eds.), Aspen: Ecology and Management in the Western United States. U.S. Dept. Agric., For. Serv., Rocky Mt. For. Range Exp. Stn., Gen. Tech. Rep. RM-119, Ft.Collins, CO.

Hinds, T. E., and Krebill, R. G. 1975. Wounds and canker diseases on western aspen. U.S. Dept. Agric., For. Serv., For. Pest Leaflet 152. 9 p.

Huelsenbeck, J. P., and Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17:754-755.

Huelsenbeck, J. P., Ronquist, F., Nielsen, R., and Bollback, J. P. 2001 Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294:2310-2314.

Jacobi, W. R., Kelly, E. F., Troendle, C. A., Anqwin, P. A., and Wettstein, C. A. 1998. Environmental conditions and aspen regeneration failure. U.S. Dept. Agric., For. Serv., Rocky Mt. Reg., Renewable Resources For. Health Management, Tech. Rep. R2-60, Golden, CO. 24 p.

Jones, G. 2008. On the reliability of Bayesian posterior clade probabilities in phylogenetic analysis. Available from Nature Precedings. http://hdl.handle.net/10101/npre2008.1494.1

Jones, J. R. 1985. Distribution. Pages 9-10 in DeByle, N. V., and Winokur, R. P. (eds.), Aspen: Its Ecology and Management in the Western United States. U.S. Dept. Agric., For. Serv., Rocky Mt. For. Range Exp. Stn., Gen. Tech. Rep. RM-119, Ft. Collins, CO.

Juzwik, J., Nishijima, T., and Hinds, T. E. 1978. Survey of aspen cankers in Colorado. Plant Dis. Rep. 62:906-910.

Keeling, P. J., and Inagaki, Y. 2004. A class of eukaryotic GTPase with a punctuate distribution suggesting multiple functional replacements of translation elongation factor 1 α. Proc. Natl. Acad. Sci. 101:15380-15385.

Kepley, J. B., and Jacobi, W. R. 2000. Pathogenicity of *Cytospora* species on six hardwood species. J. Arbor. 26:326-332.

Kirk, P. M., Cannon, P. F., David, J.C., and Stalpers, J. A. 2001. Ainsworth and Bisby's Dictionary of the Fungi. 9th ed. CAB International, Wallingford, UK. 655 p.

Kishino, H., and Hasegawa, M. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order of the Hominoidea. J. Mol. Evol. 29:70-179.

Knight, D. H. 2001. Summary: aspen decline in the West? U.S. Dept. Agric., For. Serv. Proceedings, Rocky Mt. Res. Stn.-P-18.

Kohlmeyer, J., and Kohlmeyer, E. 1971. Marine fungi from tropical America and Africa. Mycologia 63:831-861.

Kolaczkowski, B., and Thornton, J. W. 2007. Effects of branch length uncertainty on Bayesian posterior probabilities for phylogenetic inference. Mol. Biol. Evol. 24:2108-2118.

Krebill, R. G. 1972. Mortality of aspen on the Gros Ventre elk winter range. U.S. Dept. Agric., For. Serv., Intermt. For. Range Exp. Stn., Res. Pap. INT-129, Ogden, UT. 16 p.

Kullnig-Gradinger, C. M., Szakacs, G., and Kubicek, C. P. 2002. Phylogeny and evolution of the genus *Trichoderma*: a multigene approach. Mycol. Res. 106:757-767.

Kurzel, B. P. 2006. Colorado State Parks. In presentation outlines from the aspen "die-off summit."
http://www.aspensite.org/die-off_summitt.html.

Leatherman, D. A., McNulty, H. E., Schomaker, M. E., and Lynch, D. L. 1986. Cankers. Pages 19-21 in Aspen: A Guide to Common Problems in Colorado. Colorado State University Cooperative Extension, Colorado State Forest Service. No. 117-0886, Ft. Collins, CO.

Lee, S., Groenewald, J. Z., and Crous, P. W. 2004. Phylogenetic reassessment of the coelomycete genus *Harknessia* and its teleomorph *Wuestneia* (Diaporthales), and the introduction of *Apoharknessia* gen. nov. Stud. Mycol. 50:235-252.

Linz, J. E., Katayama, C., and Sypherd, P. S. 1986. Three genes for the elongation factor EF-1α in *Mucor racemosus*. Mol. Cell. Biol. 6:593-600.

McIntyre, G. A., Jacobi, W. R., and Ramaley, A. W. 1996. Factors affecting Cytospora canker occurrence on aspen. J. Arbor. 22:229-233.

Mejia, L. C., Castlebury, L. A., Rossman, A. Y., and White, J. F. Jr. 2006. Clarification of the nomenclature and relationships of the genera *Cryptosporella*, *Ophiovalsa* and *Winterella* (Gnomoniaceae). Inoculum 57:29 [Abstract].

Mejia, L. C., Castlebury, L. A., Rossman, A. Y., Sogonov, M. V., and White, J. F. 2008. Phylogenetic placement and taxonomic review of the genus *Cryptosporella* and its synonyms *Ophiovalsa* and *Winterella* (Gnomoniaceae, Diaporthales). Mycol. Res. 112:23-35.

Moročko, I. 2006. Characterization of the strawberry pathogen *Gnomonia fragariae*, and biocontrol possibilities. Ph.D. diss. Swedish University of Agricultural Sciences. Uppsala, SW. 41 p.

Müller, K. F. 2005. SeqState- primer design and sequence statistics for phylogenetic DNA data sets. Appl. Bioinformatics 4:65-69.

Müller, K. F. 2006. Incorporating information from length-mutational events into phylogenetic analysis. Mol. Phylogenet. Evol. 38:667-676.

Myburg, H., Gryzenhout, M., Wingfield, B. D., Stipes, R. J., and Wingfield, M. J. 2004. Phylogenetic relationships of *Cryphonectria* and *Endothia* species, based on DNA sequence data and morphology. Mycologia 96:990-1001.

Nakabonge, G., Roux, J., Gryzenhout, M., and Wingfield, M. J. 2006. Distribution of Chrysoporthe canker pathogens on *Eucalyptus* and *Syzygium* spp. in eastern and southern Africa. Plant Dis. 90:734-740.

Naylor, G., and Kraus, F. 1995. The relationship between *s* and *m* and the retention index. Syst. Biol. 44:559-562.

Niekerk, J. M. van, Groenewald, J. Z., Verkley, G. J., Fourie, P. H., Wingfield, M. J., and Crous, P. W. 2004. Systematic reappraisal of *Coniella* and *Pilidiella*, with specific reference to species occurring on *Eucalyptus* and *Vitis* in South Africa. Mycol. Res. 108:283-303.

Nixon, K. C. 1999. The parsimony ratchet, a new method for rapid parsimony analysis. Cladistics. 15:407-414.

O'Donnell, K., Ward, T. J., Geiser, D. M., Corby Kistler, H., and Aoki, T. 2004. Genealogical concordance between mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genet. Biol. 41:600-623.

Palumbi, S. R. 1996. Nucleic acids II: the polymerase chain reaction. Pages 205-247 in Hillis, D. H., Moritz, C., and Mable, B. K. (eds.), Molecular Systematics. Sinauer Associates, Sunderland, MA.

Piercey-Normore, M. D., and Egger, K. N. 2001. Identification and genotypic characterization of filamentous fungi. Pages 1-21 in Talbot, N. (ed.). Molecular and Cellular Biology of Filamentous Fungi: a Practical Approach. Oxford University Press, Oxford, UK.

Posada, D., and Crandall, K. A. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817-818.

Rambaut, A., and Drummond, A. 2003. Tracer MCMC trace analysis tool. University of Oxford. Oxford, UK.

Rayner, R. W. 1970. A mycological colour chart. Commonwealth Mycological Institute, Kew, UK. 34 p.

Rehner, S. 2001. Primers for elongation factor 1-α (EF1-α). 4 p. http://ocid.nacse.org/research/deephyphae/EF1primer.pdf.

Rehner, S. A., and Uecker, F. A. 1994. Nuclear ribosomal internal transcribed spacer phylogeny and host diversity in the coelomycete *Phomopsis*. Can. J. Bot. 72:1666-1674.

Rensburg, J. C. J. van, Lamprecht, S. C., Groenewald, J. Z., Castlebury, L. A., and Crous, P. W. 2006. Characterisation of *Phomopsis* spp. associated with die-back of rooibos (*Aspalathus linearis*) in South Africa. Stud. Mycol. 55:65-74.

Ronquist, F., and Huelsenbeck, J. P. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574.

Ross, W. D. 1976. Fungi associated with root diseases of aspen in Wyoming. Can. J. Bot. 54:734-744.

Rossman, A. Y, Castlebury, L. A., and Putnam, M. L. 2004. First report of ash anthracnose caused by *Discula fraxinea* in Oregon. Plant Dis. 88:222 [Abstract].

Rossman, A. Y, Castlebury, L. A., and Samuels, G. J. 2006. Phylogeny and biodiversity of the Hypocreales and Diaporthales. Eighth International Mycological Conference, Cairns, AS, Book 2:337 [Abstract].

Rossman, A. Y., Farr, D. F., Castlebury, L. A. 2007. A review of the phylogeny and biology of the Diaporthales. Mycoscience 48:135-144.

Rossman, A. Y., Palm, M. E., and Spielman, L. J. 1987. A Literature Guide for the Identification of Plant Pathogenic Fungi. APS Press, St. Paul, MN. 252 p.

Roux, J., Myburg, H, Wingfield, B. D., and Wingfield, M. J. 2003. Biological and phylogenetic analyses suggest that two *Cryphonectria* spp. cause cankers of *Eucalyptus* in Africa. Plant Dis. 87:1329-1332.

Sánchez-Ballesteros, J., González, V., Salazar, O., Acero, J., Portal, M. A., Julián, M., and Rubio, V. 2000. Phylogenetic study of *Hypoxylon* and related genera based on ribosomal ITS sequences. Mycologia 92:964-977.

Sanderson, M. J., and Donoghue, M. J. 1989. Patterns of variation in levels of homoplasy. Evolution 43:1781-1795.

Schilder, A. M. C., Erincik, O., Castlebury, L. A., Rossman, A. Y., and Ellis, M. A. 2005. Characterization of *Phomopsis* spp. infecting grapevines in the Great Lakes region of North America. Plant Dis. 89:755-762.

Shenoy, B. D., Jeewon, R., and Hyde, K. D. 2007. Impact of DNA sequence-data on the taxonomy of anamorphic fungi. Fungal Divers. 26:1-54.

Simmons, M. P., and Ochoterena, H. 2000. Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol. 49:369-381.

Simmons, M. P., Müller, K., and Norton, A. P. 2007. The relative performance of indel-coding methods in simulations. Mol. Phylogenet. Evol. 44:724-740.

Simmons, M. P., Pickett, K. M., and Miya, M. 2004. How meaningful are Bayesian support values? Mol. Biol. Evol. 21:188-199.

Sinclair, W. A., Lyon, H. H., and Johnson, W. T. 1987. Diseases of Trees and Shrubs. Comstock Publishing Associates, Cornell University Press, Ithaca, NY. 575 p.

Sogonov, M. V., Castlebury, L. A., Mejia, L. C., Rossman, A. Y., and White, J. F. 2006. The Gnomoniaceae on the Juglandaceae. Eighth International Mycological Conference, Cairns, AS, Book 1:20 [Abstract].

Sogonov, M. V., Castlebury, L. A., Rossman, A. Y., and White, J. F. Jr. 2005. Towards monophyletic genera in the Gnomoniaceae. Inoculum 56:54 [Abstract].

Sogonov, M. V., Castlebury, L. A., Rossman, A. Y., and White, J. F. 2007. The type species of *Apiognomonia*, *A. veneta*, with its *Discula* anamorph is distinct from *A. errabunda*. Mycol. Res. 111:693-709.

Spielman, L. J. 1983. Taxonomy and biology of *Valsa* species on hardwoods in North America, with special reference to species on maples. Ph.D diss. Cornell University. Ithaca, NY. 175 p.

Spielman, L. J. 1985. A monograph of *Valsa* on hardwoods in North America. Can. J. Bot. 63:1355-1378.

Stewart, J. E., Kim, M.-S., James, R. L., Kasten Dumroese, R., and Klopfenstein, N. B. 2006. Molecular characterization of *Fusarium oxysporum* and *Fusarium commune* isolates from a conifer nursery. Phytopathology 96:1124-1133.

Surve-Iyer, R. S., Adams, G. C., Iezzoni, A. F., and Jones, A. L. 1995 Isozyme detection and variation in *Leucostoma* species from *Prunus* and *Malus*. Mycologia 87:471-482.

Swofford, D. L. 2003. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Inc. Sunderland, MA.

Taylor, J. W., Jacobson, D. J., Kroken, S., Kasuga, T., Geiser, D. M., Hibbett, D. S., and Fisher, M. C. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genet. Biol. 31:21-32.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876-4882.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.

Uddin, W., Stevenson, K. L., Pardo-Schultheiss, R. A., and Rehner, S. A. 1998. Pathogenic and molecular characterization of three *Phomopsis* isolates from peach, plum, and Asian pear. Plant Dis. 82:732-737.

Wall, P. K., Leebens-Mack, J., Müller, K. F., Field, D., Altman, N. S., and Pamphilis, C. W. de. 2008. PlantTribes: A gene and gene family resource for comparative genomics in plants. Nucleic Acids Res. 36:D970-D976.

Walla, J. A., and Conway, K. E. 1986. Cytospora canker of cottonwoods and willows. Pages 48-49 in Riffle, J. W. and Peterson, G.W. (Technical coordinators), Diseases of Trees in the Great Plains. U.S. Dept. Agric., For. Serv., Rocky Mt. For. Range Exp. Stn., Gen. Tech. Rep. RM-129, Ft. Collins, CO.

Walters, J. W., Hinds, T. E., Johnson, D. W., and Beatty, J. 1982. Effects of partial cutting on diseases, mortality, and regeneration of Rocky Mountain aspen stands. U.S. Dept. Agric., For. Serv., Rocky Mt. For. Range Exp. Stn., Res. Pap. RM-240, Ft. Collins, CO. 12 p.

White, T. J., Bruns, T., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J. (eds.), PCR Protocols: a Guide to Methods and Applications. Academic Press, San Diego, CA.

Winka, K. 2000. Phylogenetic relationships within the Ascomycota based on 18S rDNA sequences. Ph.D diss. Umeå, University. Umeå, SW. 91 p.

Worrall, J. J., Egeland, L., Eager, T., Mask, R. A., Johnson, E. W., Kemp, P. A., and Shepperd, W. D. 2008. Rapid mortality of *Populus tremuloides* in southwestern Colorado, USA. Forest Ecol. Manage. 255:686-696.

Zhang, N., and Blackwell, M. 2002. Population structure of dogwood anthracnose fungus. Phytopathology 92:1276-1283.

Appendix I

Media for isolation, maintenance, isoenzyme studies, and long-term storage

Yeast-malt agar

3 g yeast extract
3 g malt extract
10 g dextrose
5 g peptone
20 g agar
1 L distilled water
30 mg streptomycin and 30 mg penicillin added after cooling to approximately 50 C.

Malt extract agar

20 g malt extract
20 g dextrose
1 g peptone
15 g agar
1 L distilled water
30 mg streptomycin and 30 mg penicillin added after cooling to approximately 50 C.

Modified Leonian's medium

6.25 g maltose
6.25 g malt extract
1.25 g KH_2PO_4
0.625 g $MgSO_4 \cdot 7H_2O$
0.625 g peptone
15 g agar
1 L distilled water
30 mg streptomycin and 30 mg penicillin added after cooling to approximately 50 C.

Potato dextrose agar

24 g potato dextrose broth (Difco Laboratories, Detroit, MI)
15 g agar
1 L distilled water

Potato dextrose broth

24 g potato dextrose broth (Difco Laboratories, Detroit, MI)
1 L distilled water

Media used for vegetative compatibility/incompatibility, cultural, and pathogenicity studies

Modified Leonian's medium

6.25 g maltose
6.25 g malt extract
1.25 g KH_2PO_4
0.625 g $MgSO_4 \cdot 7H_2O$
0.625 g peptone
15 g agar
1 L distilled water

Potato dextrose agar

24 g potato dextrose broth (Difco Laboratories, Detroit, MI)
15 g agar
1 L distilled water

Oatmeal agar (per Gooding & Lucas, 1959; used in vegetative compatibility and media volume studies)

75 g Quaker white oats added to 600 ml distilled water and ground in standard blender for 5 minutes.
After blending the mixture is heated to 45-55 C in a water bath and added to 20 grams of agar previously melted in 400 ml of distilled water in a microwave.
300 ml aliquots of the resulting mixture are then autoclaved for 90 minutes.

Oatmeal agar (per Adams et al., 2005; used in study to stimulate sexual fruiting)

60 g Quaker white oats added to 1000 ml distilled water and autoclaved.
Autoclaved mixture is homogenized in a standard blender for ca. 3 minutes.
After blending the mixture is filtered through 4 layers of cheesecloth.
Following filtering 120 g sucrose and 20 g agar are added and the mixture microwaved.
Once the ingredients go into solution distilled water is added to bring the volume up to 1 L.

Appendix II

Materials used for isoenzyme studies

Extraction buffer- 0.1 M Tris buffer, pH 8.2

3.03 g Tris base (2-amino-2-hydroxymethyl-1,3-propanediol)
75 g 30% sucrose
0.25 g 0.1% cysteine·HCl
0.25 g 0.1% ascorbic acid
Double distilled water to make 250 ml
pH adjusted to 8.2 at room temperature with concentrated HCl.

Tank buffer- 0.065 M Tris-borate buffer (5X stock solution)

Dilute 1:4 with cold double distilled water for final buffer (for 0.065 M solution).
39.35 g Tris base
5.46 g boric acid (H_3BO_3)
Double distilled water to make 1 L
Cool to 4 to 5 C overnight and adjust pH to 9.35.

Tris-citrate gel buffer system, pH 9.0 (stock solutions)

Stock solution 2a: Tris-citrate, 1.5 M Tris, 0.14 M citrate
18.15 g Tris base
14 ml 1 M citric acid
250 µl TEMED (N,N,N′,N′-tetramethylethylenediamine)
Double distilled water to make a final volume of 100 ml

Stock solution 3a: Tris-citrate, 0.3 M Tris, (Well and Cap gel buffer)
10 ml stock solution 2a
95 µl TEMED
Double distilled water to make a final volume of 30 ml

Stock solution 5a: 32% acrylamide
9.5 g acrylamide
0.24 g Bis (N, N′-methylenebisacrylamide)
Double distilled water to make a final volume of 30 ml

Stock solution 6a: Ammonium persulfate catalyst
Must be made fresh daily
0.04 g ammonium persulfate
10 ml double distilled water

Vertical slab polyacrylamide gel construction (one 1.5 mm thick mini-gel)

Layer 1: 8% separating gel

2.5 ml stock solution 2a
2.5 ml stock solution 5a
2.5 ml double distilled water
2.5 ml stock solution 6a

Solution is added to within 3 to 4 mm of the bottom of the well comb. A thin layer of double distilled water is placed over the gel to provide an even layer and aid in polymerization. After polymerization the water cap is removed.

Layer 2: 4.5% stacking gel

250 µl stock solution 2a
142 µl stock solution 5a
110 µl double distilled water
500 µl stock solution 6a

Solution is added to the bottom of the well comb and capped with a thin layer of double distilled water. After polymerization the water cap is removed.

Layer 3: 8% well-forming gel

500 µl stock solution 3a
500 µl stock solution 5a
1000 µl stock solution 6a

Solution is added and the well comb is immediately inserted. After polymerization the comb is carefully removed and individual wells are carefully washed out with double distilled water.

Protein extract carefully added to each well.

Layer 4: 8% cap gel

200 µl stock solution 3a
200 µl stock solution 5a
200 µl double distilled water
200 µl stock solution 6a

Protein extract is carefully covered and the wells filled and allowed to polymerize prior to beginning electrophoresis.

Enzyme stain formulations and procedures

Each name is followed by the enzyme code (EC) number recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology in 1992.

Acid phosphatase (ACP) E.C. 3.1.3.2

Preincubate gel in 50 ml of 0.1 M sodium-acetate buffer, pH 5.0, for approximately 0.75 hours.

Sodium-acetate buffer

1.16 ml glacial acetic acid
2.72 g sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$)
Double distilled water to make 200 ml

Stain in

50 mg α-naphthyl acid phosphate, sodium salt
50 mg Fast Garnet GBC salt
50 ml 0.1 M sodium-acetate buffer, pH 5.0
Incubate in the dark at 37 C

Alkaline phosphatase (ALP) E.C. 3.1.3.1

Preincubate gel in 50 ml of 0.05 M Tris-HCl buffer, pH 8.5, for approximately 1.25 hours.

Tris-HCl buffer

1.21 g Tris base
Double distilled water to make 200 ml
Adjust pH to 8.5 at room temperature with concentrated HCl (and as needed when preparing stains).

Stain in

50 mg α-naphthyl acid phosphate, sodium salt
20 mg $MgCl_2$
50 mg Fast Blue RR salt
50 ml 0.05 M Tris-HCl buffer, pH 8.5
Incubate in the dark at 37 C

Alanine dehydrogenase (ALADH) E.C. 1.4.1.1

0.1 M phosphate buffer, pH 7.0

To approximately 10 ml 0.2 M monobasic sodium phosphate add about 15 ml 0.2 M dibasic sodium phosphate. Dilute the 25 ml solution to 50 ml with double distilled water.

Alanine dehydrogenase (ALADH) (cont.)

Stain in

10 mg NAD
50 mg DL-α-alanine
10 mg MTT
2 mg PMS
50 ml 0.1 M phosphate buffer, pH 7.0
Incubate in the dark at 37 C

Alcohol dehydrogenase (ADH) E.C. 1.1.1.1

50 mM Tris-HCl buffer, pH 8.0

1.21 g Tris base
Double distilled water to make 200 ml
Adjust pH to 8.0 at room temperature with concentrated HCl (and as needed when preparing stains).

Stain in

10 mg NAD
1 ml ethanol
10 mg MTT
2 mg PMS
50 ml 50 mM Tris-HCl, pH 8.0
Incubate in the dark at 37 C

alpha-esterase (α-EST) E.C. 3.1.1.1

Prepare buffer and final staining solution about 10 minutes before use.

0.2 M phosphate buffer, pH 6.0

To approximately 43 ml 0.2 M monobasic sodium phosphate add about 7 to 10 ml 0.2 M dibasic sodium phosphate until pH is 6.0.

Stain in

Dissolve 50 mg Fast Blue RR salt in 50 ml 0.2 M phosphate buffer, pH 6.0.
Dissolve 10 mg of α-naphthyl acetate in 1 ml acetone. Add 1 ml double distilled water and stir. Add 1 ml of this solution to the Fast Blue RR/phosphate buffer solution.
Add 5 ml n-propanol to the Fast Blue RR/phosphate buffer stain solution.
Incubate in the dark

Amylase (AMY) E.C. 3.2.1.1 and 3.2.1.2

The polyacrylamide gel is prepared with 0.05% starch in the separating (8%) gel via a 0.2% starch solution ("starch 2a"), which is used in place of the stock 2a solution during gel construction (layers 2, 3, and 4 follow the standard protocols for gel building).

"Starch 2a" stock solution

Dissolve 0.04 g soluble starch in 20 ml double distilled water by gently heating in the microwave until the solution just begins to boil. Add 3.63 g Tris base, 2.8 ml 1 M citric acid, and 50 µl TEMED.

Following electrophoresis, incubate gel in 50 ml 0.2 M phosphate buffer, pH 7.0, for approximately 3 hours at 37 C to allow amylase to digest starch.

0.2 M phosphate buffer, pH 7.0

To approximately 20 ml 0.2 M monobasic sodium phosphate add about 30 ml 0.2 M dibasic sodium phosphate until pH is 7.0.

1% IKI_2 stock solution for staining

Dissolve 1 g I_2 and 1 g IK into double distilled water to make a final volume of 100 ml.

Final staining solution (0.05% IKI_2)

Dissolve 5 ml 1% IKI_2 stock solution in 100 ml double distilled water.

Incubate in light at room temperature.

Glucose-6-phosphate dehydrogenase (G-6-PD) E.C. 1.1.1.49

50 mM Tris-HCl buffer, pH 8.0

1.21 g Tris base

Double distilled water to make 200 ml

Adjust pH to 8.0 at room temperature with concentrated HCl (and as needed when preparing stains).

Stain in

5 mg NADP

50 mg $MgCl_2$

50 mg glucose-6-phosphate, Na_2-salt

10 mg MTT

2 mg PMS

50 ml 50 mM Tris-HCl, pH 8.0

Incubate in the dark at 37 C

Glutamate dehydrogenase (GDH) E.C. 1.4.1.2

50 mM Tris-HCl buffer, pH 8.0

1.21 g Tris base
Double distilled water to make 200 ml
Adjust pH to 8.0 at room temperature with concentrated HCl (and as needed when preparing stains).

Stain in

5 mg NADP
50 mg $CaCl_2$
50 mg L-glutamate, sodium salt
10 mg MTT
2 mg PMS
50 ml 50 mM Tris-HCl, pH 8.0
Incubate in the dark at 37 C

Malate dehydrogenase (MDH) E.C. 1.1.1.37

50 mM Tris-HCl buffer, pH 7.0

1.21 g Tris base
Double distilled water to make 200 ml
Adjust pH to 7.0 at room temperature with concentrated HCl (and as needed when preparing stains).

Stain in

5 mg NAD
50 mg $MgCl_2$
200 mg malic acid, monosodium salt
10 mg MTT
2 mg PMS
50 ml 50 mM Tris-HCl, pH 7.0
Incubate in the dark at 37 C

Peroxidase (PER) E.C. 1.11.1.7

50 mM sodium-acetate buffer, pH 5.0

0.6 ml glacial acetic acid
1.36 g sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$)
Double distilled water to make 200 ml

Stain in

50 mg $CaCl_2$
0.25 ml 3% hydrogen peroxide
50 ml 50 mM sodium-acetate buffer, pH 5.0
Dissolve 25 mg of 3-amino-9-ethylcarbazole in 2 ml of N,N-dimethylformamide. Add this solution to the sodium-acetate/ $CaCl_2$/hydrogen peroxide solution.
Incubate in the dark at room temperature.

Polyphenol oxidase (PPO) E.C. 1.10.3.1

0.2 M phosphate buffer, pH 6.8

To approximately 26 ml 0.2 M monobasic sodium phosphate add about 25 ml 0.2 M dibasic sodium phosphate until pH is 6.8.

1 M catechol stock solution

0.55 g catechol
Double distilled water to make 50 ml

Stain in

25 mg 0.10% *p*-phenylene diamine
5 ml 1 M catechol stock solution
50 ml 0.2 M phosphate buffer, pH 6.8
Incubate in the dark at room temperature.

Superoxide dismutase (SOD) E.C. 1.15.1.1

50 mM Tris-HCl buffer, pH 8.5

1.21 g Tris base
Double distilled water to make 200 ml
Adjust pH to 8.5 at room temperature with concentrated HCl (and as needed when preparing stains).

Stain in

15 mg $MgCl_2 \cdot 6H_2O$
10 mg MTT
6 mg PMS
80 ml 50 mM Tris-HCl buffer, pH 8.5
Incubate in the light at room temperature.

Appendix III

Introduction

As a supplement to the primary investigative studies in Chapters 2 and 3, five additional studies were conducted for the following reasons: (Study 1) to determine if the migration distance of isoenzymes during polyacrylamide gel electrophoresis were affected by the volume of protein loaded in sample wells; (Study 2) to examine how culture age affects the expression of peroxidase (PER) and polyphenol oxidase (PPO) isoenzymes; (Study 3) to conclude whether *Cytospora chrysosperma* isolates were vegetatively compatible/incompatible with putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. isolates; (Study 4) to determine if the depth of volume and/or types of media in Petri dishes affect the cultural characteristics of *C. chrysosperma* and putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. isolates and to see if sexual fruiting could be stimulated in culture; (Study 5) to find out if *C. chrysosperma* and putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. isolates differ in their pathogenicity on field-grown aspen and cottonwood trees.

Initial isolation, cultivation, and storage of fungal isolates used in these studies are outlined in Chapter 2. All isolates, except for the *Cytospora* sp. from cottonwood used in Study 5, are listed in Table 2.1. All isolates were subcultured from PDA slants under storage at 5 C. For Studies 1 and 2, culture preparation, protein extraction, electrophoresis, and staining protocols also are stated in Chapter 2.

Study 1: Isoenzyme Migration And Polyacrylamide Gel Electrophoresis: Does Sample Volume Affect Migration Distance?

Introduction

As discussed in Chapter 2, electrophoretic runs were conducted a minimum of three times for each isolate and enzyme assay. Where staining activity was either too high or low the volume of protein extract used during electrophoresis was adjusted accordingly. Despite the consistency between electrophoretic runs for a given assay, a preliminary experiment was conducted to determine if the amount (volume) of protein extract loaded in sample wells would affect enzyme mobility.

Materials And Methods

Only one isolate and enzyme assay, viz., C 5 (*C. chrysosperma*) and amylase, were examined in this study. A total of six sample volumes were tested. Protein volume per sample well was increased from 5 µl to 30 µl in increments of 5 µl. Lanes 1, 2, 9, and 10 were not utilized to avoid any potential migration differences that might occur near gel edges.

Results And Discussion

The migration distance of amylase isoenzymes for C 5 at each volume loaded was nearly identical (Figure A III.1). The 5 µl sample was only slightly slower in migration than the other five volumes. Although only one isolate and one enzyme system and electrophoretic run was examined, the conclusion lends evidence that the range of standard volumes of protein ordinarily loaded on gels does not affect isoenzyme mobility. All electrophoretic runs in Chapter 2 were conducted a minimum of three times for each isolate and enzyme assay with results highly consistent among runs. Additionally, in

Study 2, migration of isoenzymes with equivalent protein concentrations did not differ in migration from samples where proteins were not quantified. The only difference observed among samples was band intensity.

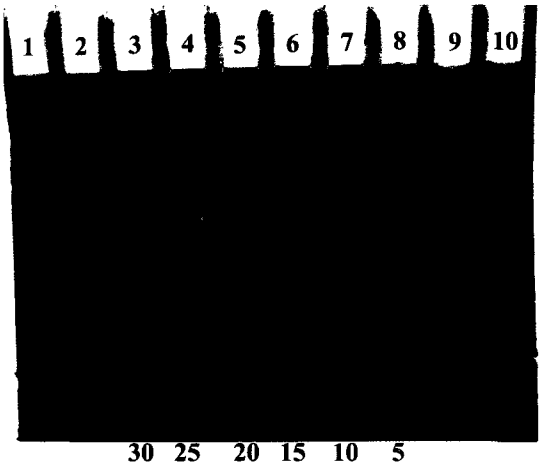


Figure A III.1. Enzyme mobility for isolate C 5 (*C. chrysosperma)* and the amylase isoenzymatic system following electrophoresis. Volumes (microliters) of protein extract loaded into wells 3 to 8 are listed below the diagram. Lanes 1, 2, 9, and 10 were not loaded.

Study 2: Polyphenol Oxidase And Peroxidase Expression As A Function Of Culture Age

Introduction

Polyphenol oxidases (PPO) and peroxidases (PER) are groups of ubiquitous oxidative enzymes found in a number of organisms including bacteria, fungi, plants, invertebrates, and vertebrates (Manchenko, 2003).

PPO enzymes contain copper and catalyze the oxidation of certain phenolic substances to quinones in the presence of molecular oxygen (Astarci, 2003); these quinone derivatives are then spontaneously polymerized (Shi et al., 2001). There are three major types of phenol oxidases, i.e., tyrosinase, catechol oxidase, and laccase, distinguishable by their substrate specificity (ability to oxidize different phenolic compounds) and response to inhibitors (Leathman & Stahmann, 1981; Astarci, 2003). Mayer (1987) believed that inhibitor studies alone could not prove that an enzyme was involved in a particular physiological process.

Phenol oxidase production has been observed in different groups of fungi including Basidiomycetes, Ascomycetes, and Deuteromycetes (Bell & Wheeler, 1986; Gianfreda et al., 1999). Fungi may secrete phenols and/or phenol oxidases directly as a part of pathogenesis or as defense mechanisms, viz., toxins, inhibitors, tissue degradation, toxin/inhibitor degradation etc. (Marbach et al., 1985; Geiger et al., 1986; Mayer, 1987; Rigling et al., 1989; Griffin, 1994; Arana-Cuenca et al., 2004). Additionally, phenols and phenol oxidases are associated with the production of dark-brown to black pigments called melanins (Bell & Wheeler, 1986). Langfelder et al. (2003) define melanins as "macromolecules formed by oxidative polymerization of phenolic and indolic compounds" with the resulting pigments usually black or brown, although numerous

other colors have been observed. These polymers are considered to be either "wall-bound", "extra-cellular", i.e., synthesized completely apart from cell walls, or heterogeneous melanins formed after secretion of phenols, or phenol oxidases, into culture media or the natural environment (Bell & Wheeler, 1986). Typically melanins are found in the cell walls of spores, hyphae (vegetative and reproductive), and appressoria. Possible functions of melanins include protection (from free radicals, environmental stress, and microbial attack/competition), as well as increased structural rigidity, pathogenicity, and virulence (Bell & Wheeler, 1986; Hamilton & Holdom, 1999; Jacobson, 2000; Butler et al., 2001; Gomez & Nosanchuk, 2003; Langfelder et al., 2003; Nosanchuk & Casadevall, 2003; Mrnka et al., 2006). The specific types of melanin found in various fungal species, and the precursors needed for their synthesis, are somewhat speculative (Bell & Wheeler, 1986; Griffin, 1994).

Peroxidase is an oxidant-degrading enzyme produced by fungi when challenged by oxidant stress exerted by the host, environment, and normal fungal metabolism (Chauhan & Calderone, 2004). Hydrogen peroxide (H_2O_2) is but one of many oxidant wastes, and may serve as a proton accepting substrate for peroxidase which then may catalyze a number of oxidative reactions (Manchenko, 2003). Furthermore, many fungi, particularly Basidiomycetes, secrete lignin and manganese peroxidase enzymes which are involved in lignin degradation (Hatakka, 1994; Hammel, 1997; Kamitsuji et al., 2004). Griffin (1994) believes oxidation of γ-glutaminyl-4-hydroxybenzene (GHB) can occur via peroxidase to produce a quinone that polymerizes nonenzymatically to form melanin.

Although isoenzymes have been used to differentiate *Cytospora* spp. (Surve & Adams, 1988; Surve & Adams, 1990; Surve-Iyer et al., 1995; Sorensen & Traquair,

1991) the use of PPO and PER have not been involved in any study that I am aware of. However, they have been, and continue to be, important assays used in many microbial and plant studies (Weber & Stahmann, 1964; Clutterbuck, 1972; Blakeman et al., 1976; Arinze & Smith, 1982; Salomé et al., 1983; Marbach et al., 1985; Geiger et al., 1986; Rigling et al, 1989; Goodin et al., 1993; Mathur & Vyas, 1996; Bending & Read, 1997; Hammel, 1997; Bestwick et al., 1998; Gramss et al., 1998; Ray & Hammerschmidt, 1998; Timonen & Sen, 1998; Hiromi et al., 1999; Sakharov & Ardila, 1999; Conesa et al., 2000; Cowan et al., 2000; González et al., 2000; Andersone & Ievinsh, 2002; Sen et al., 2002; Arana-Cuenca et al., 2004; Campos et al., 2004; Kamitsuji et al., 2004; Rudrappa et al., 2005; Zheng et al., 2005; Melo et al., 2006; Mrnka et al, 2006; Colak et al., 2007).

Due to the inconsistent results obtained with PPO and PER enzyme assays as part of the investigative study in Chapter 2, a follow up study was conducted to further explore their incongruity. Because various phenol oxidases and peroxidases are associated with the melanin formation in some fungi, and given that pigmentation with *C. chrysosperma* and putative labyrinthine/rosette *Cytospora* sp. (non-*C. chrysosperma*) isolates occurs in older cultures, it was hypothesized that enzyme expression may be related to culture age.

Materials And Methods

Isolates C1 (*C. chrysosperma*) and 3 (non-*C. chrysosperma*) were examined in this study. Protein extraction for each isolate was done when cultures were 7, 14, 21, and 25 days old. Proteins were quantified for all extractions following the Bradford microassay procedure (Bio-Rad Laboratories, Inc.) to ensure equivalent amounts of

protein were loaded for each sample prior to electrophoresis. By doing so, band intensity could be correlated with relative protein concentration.

Results And Discussion

Based on the results of this study the expression of PPO and PER isoenzymes for *C. chrysosperma* and non-*C. chrysosperma* isolates is related to the age of cultures. For peroxidase and polyphenol oxidase isoenzymes, expression (based on band intensities) of enzymes is well correlated with culture age for isolate 3 (Figures A III.2 & A III.3). At 7 days (lane 1) no bands are present. Enzyme expression is first noted in 14 day old cultures (lane 2) with increasing expression at 21 and 25 days (lanes 3 and 4 respectively). For polyphenol oxidase and isolate 3 (Figure A III.3), two bands are well resolved with the best resolution occurring with 21 day old cultures. Band resolution for isolate 3 and the peroxidase isoenzyme system (Figure A III.2) is not as discrete. It appears that two distinct bands are present, but at days 21 and 25 a thick, intensely stained region occurs above the slower migrating band making interpretation difficult.

The same "general" trends seen with isolate 3 also are displayed with isolate C 1 and the two isoenzyme systems (Figures A III.2 & A III.3). However, some differences can be observed. Most notable is that band intensity is not as strong, or as distinct, i.e., sharp, for C 1. For both isoenzyme systems two bands appear to be present for C 1. The slower migrating band is more distinct while the faster one is less clear due to a broader or longer stained region. Therefore, more than one band in this region cannot be discerned. With the PPO isoenzymatic system (Figure A III.3) no enzyme expression occurs in 7 day old cultures (lane 5). Enzyme expression is first noted at day 14 (lane 6), but only for the faster migrating band. Expression of the slower migrating band first occurs in 21 day old cultures (lane 7); however, staining intensity for the faster migrating

band is less than that observed at day 14. With 25 day old cultures (lane 8), both bands are the most intensely stained and distinct. For the PER isoenzymatic system and C 1 (Figure A III.2) enzyme expression for both bands occurs with 7 day old cultures (lane 5) with increasing intensity and resolution over time. As with the PPO system, the faster migrating band is more faint at 21 days (lane 7) vs. 14 days (lane 6), and staining intensity and band clarity is the greatest in 25 day old cultures (lane 8).

Although only two isolates were tested it can be concluded that under the conditions of the present study, viz., 7, 14, 21, and 25 day old cultures grown in 20 ml of PDB in the dark at 25 C under static conditions, expression of PPO and PER isoenzymes for *C. chrysosperma* and non-*C. chrysosperma* isolates generally increases with increasing age of cultures. The notable exception was for isolate C 1 where band intensity, and thus protein concentration, was less for the faster migrating band in 21 day old cultures as compared to 14 day old cultures. This is potentially an anomaly, but other works (Leatham & Stahmann, 1981; Arana-Cuenca et al., 2004) have shown separate peaks in polyphenol oxidase activity during time-course studies. Additionally, band clarity tended to increase with time as well. However for isolate 3 and the PER system, a broad, intensely stained region was observed above the slower migrating band at 21 and 25 days; isolate C 1 tended to have a similar region related to the faster migrating band for both isoenzymatic systems. Arana-Cuenca et al. (2004) make note that "different levels of glycosylation have been reported in laccases (by Giardina et al., 1999) which might be responsible for differences in band thickness in native gels."

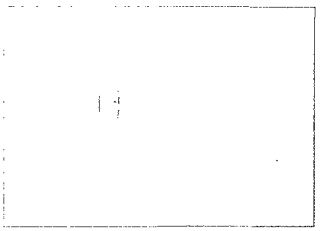


Fig. A III.2. Time study for the peroxidase isoenzymatic system after electrophoresis in polyacrylamide gels. Lanes 1-4 represent cultures of isolate 3 (putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.) grown in PD broth in the dark at 25 C for 7, 14, 21, and 25 days respectively. Lanes 5-8 are from cultures of C 1 (*C. chrysosperma*) grown under the same conditions.

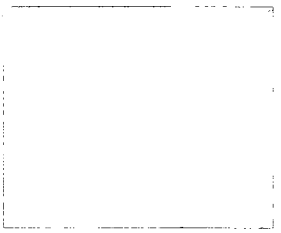


Fig. A III.3. Time study for the polyphenol oxidase isoenzymatic system. Lanes 1-4 represent cultures of isolate 3 (putative labyrinthine/rosette leucocytosporoid *Cytospora* sp.) grown in PD broth in the dark at 25 C for 7, 14, 21, and 25 days respectively. Lanes 5-8 are from cultures of C 1 (*C. chrysosperma*) grown under the same conditions.

For the present study the only variable examined was age of culture. Other studies (Leatham & Stahmann, 1981; Marbach et al., 1985; Astarci, 2003; Arana-Cuenca et al., 2004; Kamitsuji et al., 2004) have examined various media, nitrogen and carbon sources, inducers etc. and their effects on the activities of polyphenol oxidases and peroxidases. Astarci (2003) found that maximum polyphenol oxidase activities for cultures of *Thermomyces lanuginosus* Tsikl. were achieved at pH 8.0; yeast extract, as a source of nitrogen, as well as additions of copper and gallic acid all increased PPO production. It is possible that in *C. chrysosperma* and non-*C. chrysosperma* isolates, activity of these isoenzymes could be increased by methods other than culture age. The variation in expression of isoenzyme activity with age of culture emphasizes the importance of conducting studies on fungal cultures of equivalent age and physiological state when using isoenzymes in comparative studies.

Literature Cited

Andersone, U., and Ievinish, G. 2002. Changes of morphogenic competence in mature *Pinus sylvestris* L. buds *in vitro*. Ann. Bot. 90:293-298

Arana-Cuenca, A., Roda, A., Téllez, A., Loera, O., Carbajo, J. M., Terrón, M. C., and González, A. E. 2004. Comparative analysis of laccase-isozymes patterns of several related *Polyporaceae* species under different culture conditions. J. Basic Microbiol. 44:79-87.

Arinze, A. E., and Smith, I. M. 1982. Distribution of polygalacturonase, total phenolic substances, polyphenol oxidase and peroxidase in rot zones in sweet potato. Plant Pathol. 31:119-122.

Astarci, E. 2003. Production and biochemical characterization of polyphenol oxidase from *Thermomyces lanuginosus*. MS thesis. Middle East Technical University. Ankara, TU. 89 p.

Bell, A. A., and Wheeler, M. H. 1986. Biosynthesis and functions of fungal melanins. Ann. Rev. Phytopathol. 24:411-451.

Bending, G. D., and Read, D. J. 1997. Lignin and soluble phenolic degradation by ectomycorrhizal and ericoid fungi. Mycol. Res. 101:1348-1354.

Bestwick, C. S., Brown, I. R., and Mansfield, J. W. 1998. Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce. Plant Physiol. 118:1067-1078.

Blakeman, J. P., Mokahel, M. A., and Hadley, G. 1976. Effect of mycorrhizal infection on respiration and activity of some oxidase enzymes of orchid protocorms. New Phytol. 77:697-704.

Butler, M. J., Day, A.W., Henson, J. M., and Money, N. P. 2001. Pathogenic properties of fungal melanins. Mycologia 93:1-8.

Campos, Â. D., Ferreira, A. G., Hampe, M. M. V., Antunes, I. F., Brancão, N., Silveira, E. P. da, Osóreo, V. A., and Augustin, E. 2004. Peroxidase and polyphenol oxidase activity in bean anthracnose resistance. Pesq. Agropec. Bras. 39:637-643 [Abstract].

Chauhan, N., and Calderone, R. A. 2004. Adaptation to oxidative, nutrient, and pH stress by human pathogenic fungi. Pages 129-167 in San-Blas, G., and Calderone, R. A. (eds.), Pathogenic Fungi: Host Interactions and Emerging Strategies for Control. Caister Academic Press, Wymondham, EN. 469 p.

Clutterbuck, A. J. 1972. Absence of laccase from yellow-spored mutants of *Aspergillus nidulans*. J. Gen. Microbiol. 70:423-435.

Colak, A., Sahin, E., Yildirim, M., and Sesli, E. 2007. Polyphenol oxidase potentials of three wild mushroom species harvested from Liser High Plateau, Trabzon. Food Chem. 103:1426-1433.

Conesa, A., Hondel van den, C. A., Punt, P. 2000. Studies on the production of fungal peroxidases in *Aspergillus niger*. Appl. Environ. Microbiol. 66:3016-3023.

Cowan, M. M., Horst, E. A., Luengpailin, S., and Doyle, R. J. 2000. Inhibitory effects of plant polyphenoloxidase on colonization of *Streptococcus sorbrinus* 6715. Antimicrob. Agents Chemother. 44:2578-2580.

Geiger, J.-P., Nicole, M., Nandris, D., and Rio, B. 1986. Root rot diseases of *Hevea brasiliensis* I. Physiological and biochemical aspects of host aggression. Eur. J. For. Pathol. 16:22-37.

Gianfreda, L., Xu, F., and Bollag, J.-M. 1999. Laccases: a useful group of oxidoreductive enzymes. Bioremediation J. 3:1-25.

Gomez, B. L., and Nosanchuk, J. D. 2003. Melanin and fungi. Curr. Opin. Infect. Dis. 16: 91-96.

González, E. M., Ancos , B. de, and Cano, M. P. 2000. Partial characterization of peroxidase and polyphenol oxidase activities in blackberry fruits. J. Agric. Chem. 48:5459-5464.

Goodin, M. M., Biggs, A. R., and Castle, A. M. 1993. Changes in levels and isozymes of peroxidase in wounded peach bark. Fruit Var. J. 47:185-192.

Gramss, G., Günther, T., and Fritsche, W. 1998. Spot tests for oxidative enzymes in ectomycorrhizal, wood-, and litter decaying fungi. Mycol. Res. 102:67-72.

Griffin, D. H. 1994. Fungal Physiology. Wiley-Liss, New York, NY. 458 p.

Hamilton, A. J., and Holdom, M. D. 1999. Antioxidant systems in the pathogenic fungi of man and their role in virulence. Med. Mycol. 37:375-389.

Hammel, K. E. 1997. Fungal degradation of lignin. Pages 33-45 in Cadisch, G., and Giller, K. E. (eds.), Driven by Nature: Plant Litter Quality and Decomposition. Wallingford Publishers, Oxon, UK. 409 p.

Hatakka, A. 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Micrbiol. Rev. 13:125-135.

Hiromi, T., Shuji, I., and Akio, E. 1999. Hydroxyl radical generation by an extracellular low-molecular-weight substance and phenol oxidase activity during wood degradation by the white-rot basidiomycete *Trametes versicolor*. J. Biotechnol. 75:57-70.

Jacobson, E. S. 2000. Pathogenic roles for fungal melanins. Clin. Microbiol. Rev. 13:708-717.

Kamitsuji, H., Honda, Y., Watanabe, T., and Kuwahara, M. 2004. Production and induction of manganese peroxidase isozymes in a white-rot fungus *Pleurotus ostreatus*. Appl. Microbiol. Biotechnol. 65:287-294.

Langfelder, K., Streibel, M., Jahn, B., Haase, G., and Brakhage, A. A. 2003. Biosynthesis of fungal melanins and their importance for human pathogenic fungi. Fungal Genet. Biol. 38:143-158.

Leatham, G. F., and Stahmann, M. A. 1981. Studies on the laccase of *Lentinus edodes*: specificity, localization, and association with the development of fruiting bodies. J. Gen. Microbiol. 125:147-157.

Manchenko, G. W. 2003. Handbook of Detection of Enzymes on Electrophoretic Gels. CRC Press, Boca Raton, FL. 553 p.

Marbach, I., Harel, E., and Mayer, A. M. 1985. Pectin, a second inducer for laccase production by *Botrytis cinerea*. Phytochemistry 24:2559-2561.

Mathur, N., and Vyas, A. 1996. Biochemical changes in *Ziziphus xylopyrus* by VA mycorrhizae. Bot. Bull. Acad. Sin. 37:209-212.

Mayer, A. M. 1987. Polyphenol oxidases in plants-recent progress. Phytochemistry 26:11-20.

Melo, G. A., Shimizu, M. M., and Mazzafera, P. 2006. Polyphenoloxidase activity in coffee leaves and its role in resistance against the coffee leaf miner and coffee leaf rust. Phytochemistry 67:277-285.

Mrnka, L., Kulhánková, A., Vosátka, M., and Koukol, O. 2006. Competition of *Scleroconidioma sphagnicola* with fungi decomposing spruce litter needles. Can. J. Bot. 84:469-476.

Nichols-Orians, C. 1991. Differential effects of condensed and hydrolyzable tannin on polyphenol oxidase activity of attine symbiotic fungus. J. Chem. Ecol. 17:1811-1819.

Nosanchuk, J. D., and Casadevall, A. 2003. The contribution of melanin to microbial pathogenesis. Cell. Microbiol. 4:203-223.

Ray, H., and Hammerschmidt, R. 1998. Responses of potato tuber to infection by *Fusarium sambucinum*. Physiol. Mol. Plant Pathol. 53:81-92.

Rigling, D., Heiniger, U., and Hohl, H. R. 1989. Reduction of laccase activity in dsRNA-containing hypovirulent strains of *Cryphonectria* (*Endothia*) *parasitica*. Phytopathology 79:219-223.

Rudrappa, T., Neelwarne, B., Kumar, V., Lakshmanan, V., Venkataramareddy, S. R., and Aswathanarayana, R. G. 2005. Peroxidase production from hairy root cultures of red beet (*Beta vulgaris*). Electron. J. Biotechnol. 8:185-196.

Sakharov, I. Y., and Ardila, G. B. 1999. Variations of peroxidase activity in cocoa (*Theobroma cacao* L.) beans during their ripening, fermentation and drying. Food Chem. 65:51-54.

Salomé, M., Pais, S., and Barroso, J. 1983. Localization of polyphenol oxidases during the establishment of *Ophrys lutea* endomycorrhizas. New Phytol. 95:219-222.

Sen, S., Das, A. K., Pal, S., Das, S., and Viswavidyalaya, K. 2002. Peroxidase, polyphenoloxidase, total phenol and protein content in leaf tissues of *Colocasia esculenta* var. *antiquorum* and their relationship to *Phytophthora* leaf blight disease. J. Veg. Crop Prod. 8:83-89.

Shi, C., Dai, Y., Xia, B., Xu, X., Xie, Y., and Liu, Q. 2001. The purification and special properties of poyphenol oxidase I from *Nicotiana tabacum*. Plant Mol. Biol. Rep. 19:381a-381h.

Sorensen, I., and Traquair, J.A. 1991. Electrophoresis of *Cytospora* isolates from different hosts. Can. J. Plant Pathol. 13:285 [Abstract].

Surve, R., and Adams, G. 1988. Differentiating *Leucostoma persoonii* from *L. cincta* by isozyme polymorphisms. Phytopathology 78:1518 [Abstract].

Surve, R., and Adams, G. C. 1990. Phenetic groups within *Leucostoma persoonii* and *L. cincta* based on allozyme analysis. Newsletter-Mycological Society of America 41:40 [Abstract].

Surve-Iyer, R. S., Adams, G. C., Iezzoni, A. F., and Jones, A. L. 1995. Isozyme detection and variation in *Leucostoma* species from *Prunus* and *Malus*. Mycologia 87:471-482.

Timonen, S., and Sen, R. 1998. Heterogeneity of fungal and plant enzyme expression in intact Scots pine-*Suillus bovinus* and *Paxillus involutus* mycorrhizospheres developed in natural forest humus. New Phytol. 138:355-366.

Weber, D. J., and Stahmann, M. A. 1964. *Ceratocystis* infection in sweet potato: its effect on proteins, isozymes, and acquired immunity. Science 146:929-931.

Zheng, H. Z., Cui, C. L., Zhang, Y. T., Wang, D., Jing, Y., and Kim, K. Y. 2005. Active changes of lignification-related enzymes in pepper response to *Glomus intraradices* and/or *Phytophthora capsici*. J. Zhejiang Univ. Sci. B 6:778-786.

Study 3: Vegetative Compatibility Among *Cytospora chrysosperma* And Putative Labyrinthine/Rosette Leucocytosporoid *Cytospora* sp. Isolates

Introduction

A common observation on aspen (*Populus tremuloides* Michx.) cankers caused by *Cytospora* are dark lines ("zone lines") delimiting adjacent areas of infection (Figures A III.4 & A III.5). Fungal isolations from adjacent areas often result in cultures that are genetically different or even different species. Li (1981) states that isolates of *Phellinus weirii* (Murrill) Gilb. from separate infection centers develop darkly pigmented zone lines at their interface in culture, or in *P. weirii* colonized wood in response to antagonists. Works cited by Li (Nelson, 1964 & 1975) suggest zone lines exclude antagonistic microorganisms and thus improve the survival of *P. weirii* in colonized wood. Li (1981) concluded that polyphenol oxidase and peroxidase activities were higher in zone lines (vs. adjacent mycelium), and antagonistic fungal and bacterial organisms stimulated such an increase in activity. As a result, this stimulation could lead to the possible formation of melanin or melanin-like pigments. Unlike the zone lines resulting from woody tissue colonization by *P. weirii*, the lines of demarcation associated with Cytospora canker on aspen are situated in tissues of the inner and outer bark.

Alexopoulos et al. (1996) define vegetative (somatic) incompatibility as "a genetic system that restricts the fusion of mycelia of the same species that have different controlling genes- a form of heterogenic incompatibility." They cite Leslie (1993), who suggests that during fungal vegetative growth, this incompatibility system inhibits nuclei and other components of the cytoplasm from being transferred. Even though hyphae may fuse, rapid compartmentation and death of the hyphal fusion cell and surrounding cells usually follows to destroy the heterokaryon (when genetically different nuclei are in the

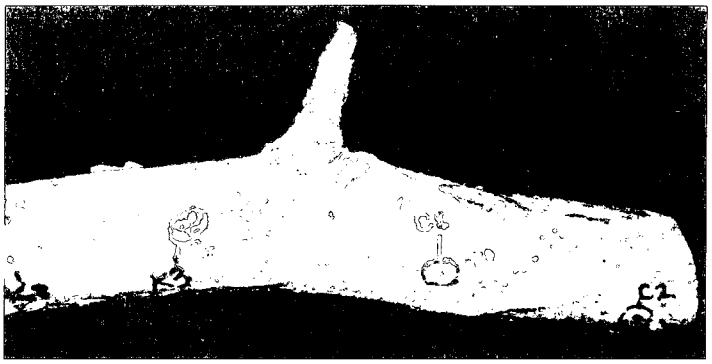


Figure A III.4. Aspen stem from which C 1, C 2, and C 3 isolates (*C. chrysosperma*), and isolates 1, 2, and 3 (non *C. chrysosperma*) were obtained from. White arrows point to lines of demarcation which delimit adjadent cankers.

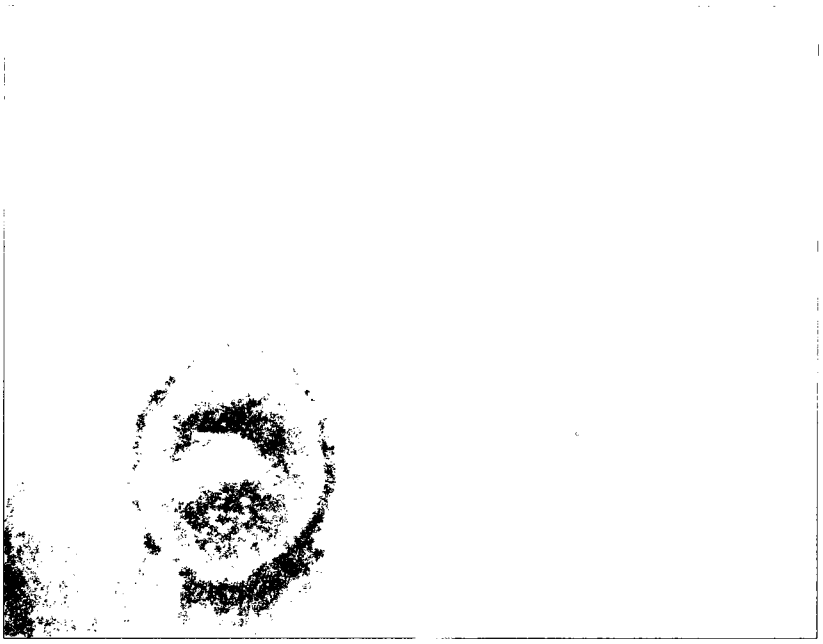


Figure A III.5. Non *C. chrysosperma* conidiomata occurring within a distinct cankered region on an aspen branch. Note partial formation of the three fruiting bodies along the line of demarcation at the top of the picture.

same mycelium or cell), or it becomes highly unstable (Glass et al., 2000; Glass & Kaneko, 2003; Kaneko et al., 2006). Thus, vegetative incompatibility (also known as nonself identification) can operate as a defense mechanism by reducing the spread of suppressive/infectious cytoplasmic genetic elements, such as viruses and plasmids, in natural fungal populations (Caten, 1972; Debets et al., 1994; Cortesi et al., 2001; Smith et al., 2006).

Griffin (1994) believes that hyphal fusions (anastomoses) generally do not serve a sexual purpose. He cites research (Raper, 1983; Glass & Kuldau, 1992) which refers to two kinds of genetic systems which affect heterokaryosis. One is concerned with mating-types (heteroallelic compatibility) and promotes outcrossing via identification of genetically different individuals. Under this system, formation of a sexually functional interaction requires that the two individuals have different alleles at the *mat* (mating type) loci. The other type of genetic system affecting heterokaryosis is vegetative compatibility (homoallelic compatibility), which functions in identification of individuals that are genetically similar and thereby limits heterokaryosis and genetic recombination via the parasexual cycle. For a vegetative compatible reaction to occur the two individuals must have identical alleles at the *het* (heterokaryon incompatibility) loci (also called *vic* for vegetative incompatibility loci). However, he points out that Glass & Kuldau (1992) believe allelic and nonallelic systems operate in controlling vegetative compatibility. With the allelic system only individuals with identical genotypes at all *het* loci are compatible, whereas in nonallelic vegetative incompatibility, as displayed by *Podospora anserina* (Rabenh.), interaction between specific alleles occurs at two separate *het* loci. Among Ascomycetes heterokaryon formation in vegetative mycelia is usually

controlled by *het* genes. However, in *Neurospora crassa* Shear & B.O. Dodge the *mat* locus also functions as a *het* locus. Moreover, expression of heterokaryon incompatibility typically occurs during vegetative growth, and as such enables heterothallic (outbreeding) individuals with many allelic *het* differences to still take part in sexual reproduction (Glass & Kaneko, 2003).

Based on the preceding information it is clear that vegetative compatibility/incompatibility is a complex process. The genetic determinants of vegetative compatibility have been examined in relatively few ascomycetous species. Probably the most intensively studied to date are *Cryphonectria parasitica* (Murrill) M.E. Barr, *P. anserina*, *N. crassa*, and *Aspergillus nidulans* (Eidam) Vuill. The complexity of the system is evident given the fact that for these fungi, 6, 9, 11, and 8 different *het* loci respectively, have been genetically identified by various researchers (Glass & Saupe, 2002). Moreover, Kaneko et al. (2006) provided definitive molecular and genetic proof that an allelic *het* locus functions in a nonallelic manner in *N. crassa*. Smith et al. (2006) used antibiotic/fungicidal forcing markers and found that in *C. parasitica*, heteroallelism at any of *vic*1, 2, 3, 6, or 7 loci was correlated with heterokaryon incompatibility, viz., slow growth or aberrant morphology, except at *vic*4 where barrage formation (demarcation or zone lines) occurred but not heterokaryon incompatibility, i.e., putative heterokaryotic sectors grew out from contact zones with no barrages in all pairings where there was no difference at any of the six loci; however, where a difference occurred only at *vic*4, putative heterokaryotic sectors were visible even though a barrage zone had formed. Such findings suggest heterokaryon incompatibility is a component of the incompatibility system, and although barrage formation may occur, it may not eliminate

the spread of infectious elements. Likewise, Micali and Smith (2003) suggested that in *N. crassa*, barrages and heterokaryon incompatibility could be considered "subsets" of vegetative incompatibility with barrage formation under complex genetic control that could be independent of heterokaryon incompatibility and mating type.

Micali and Smith (2003) define barrage as "a line or zone of demarcation that may develop at the interface where genetically different fungi meet." They state the barrage test is one of three methods used to place fungal strains into distinct vegetative incompatibility groups. Heterokaryon incompatibility and partial diploid analysis are the other two. The simplest of these is the barrage test where different fungal isolates are paired on agar media, allowed to grow out, and observed for barrage formation at the region of mycelial interaction. Micali and Smith (2003) found at least three types of barrages were formed in *N. crassa*- a dark line, a clear zone of two distinct types, and a raised aggregate of hyphae.

Barrage reactions have been used in a number of studies involving *Cytospora* spp. and associated teleomorphs (Proffer & Hart, 1988; Proffer & Jones, 1989; Adams et al., 1990; Wang et al., 1998). Adams et al. (1990) examined various media for use in vegetative compatibility studies and found that clarified oatmeal agar provided the best results. However, isolates grew with uniformly radial margins, merged, and exhibited antagonism on all media containing oatmeal. Vegetatively incompatible pairings formed dark brown or black barrage zones in 7-16 days when incubated in the dark at 22-26 C; increasing the incubation period to 30-35 days resulted in pycnidia forming along the zone line.

Because pairwise tests of mycelial compatibility can be used to obtain a general idea of genetic similarity (Molina et al., 2005) a vegetative compatibility test using *C. chrysosperma* and putative labyrinthine/rosette *Cytospora* sp. (non-*C. chrysosperma*) isolates was conducted. Evidence of non compatibility between the two taxa would provide another character for delineation and further support isoenzyme, morphological, cultural, and DNA sequence differences.

Materials And Methods

Isolates C 1, C 2, C 3 (*C. chrysosperma*) and 1, 2, and 3 (non-*C. chrysosperma*) were examined in this study. Each of the isolates were collected from separate fruiting bodies from distinct cankered regions occurring on the same stem. For isolates C 1 and 3, and C 3 and 2, the discrete cankered areas abutted one another. Pairwise tests were done using 5 mm diameter plugs of mycelium placed on the surface of oatmeal agar (Gooding & Lucas, 1959) contained in 60 X 15 mm Petri dishes. Plugs were placed directly across from one another at the farthest edges of the dish. Each isolate was paired with itself and the six other isolates with three replicates for each paired comparison. All plates were wrapped with wax-film and placed in the dark (other than short periods for examination) in a growth chamber at 25 C. After 21 days incubation all cultures were removed from the dark and assessed for the presence/absence of a barrage reaction at the zone of contact between mycelial outgrowths. Isolates forming a reaction were considered to be vegetatively incompatible.

Results And Discussion

Oatmeal agar (Gooding & Lucas, 1959) provides an excellent medium for assessment of vegetative compatibility reactions among *C. chrysosperma* and non-*C. chrysosperma* isolates. The barrage phenomenon observed *in vivo* can be replicated *in*

vitro in *Cytospora* isolates. Incompatible reactions are easy to observe between incompatible isolates (Figure A III.6) where darkly pigmented barrage zones are formed at areas of mycelial interactions. Adams et al. (1990) found clarified oatmeal agar provided the best results, and it is possible barrage zones would have been even more pronounced with its use. Cultures also were grown in the dark per Adams et al. (1990) to try and avoid pigment formation which can mask barrage zones. Although cultures still became pigmented, barrage zones were not masked. All parings between *C. chrysosperma* and non-*C. chrysosperma* isolates resulted in the formation of zone lines. Pycnidial formation often occurred along the area of demarcation (Figures A III.6, A III.7 & A III.8). Absence of barrage zones was observed whenever *C. chrysosperma* isolates were paired with each other or themselves (Figure A III.9); the same results occurred for non-*C. chrysosperma* isolates (Figure A III.9).

Because vegetative compatibility tests are simple, cheap, and relatively quick, yet provide a general idea of genetic similarity/dissimilarity they were employed here to examine if genetic differences occurred between *C. chrysosperma* and non-*C. chrysosperma* isolates. Based on the results of barrage formation between the two taxa it can be concluded genetic differences do exist. Such results support isoenzyme analysis in addition to other character differences such as culture and morphology. It must be noted, however, that compatible isolates are not always genetically identical as Molina et al. (2005) observed with use of RAPD markers. Additionally, vegetative compatibility tests can reveal more genetic variation (Wang et al., 1998) than can isoenzyme analyses (Surve-Iyer et al., 1995).

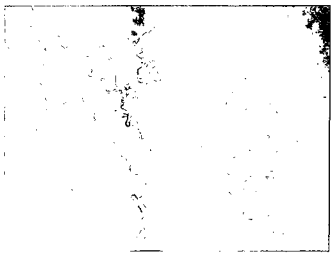


Figure A III.6. Vegetative incompatibility as noted by darkly pigmented barrage zone in center of picture. Isolate 3 (non *C. chrysosperma*) is to the left of the zone line and isolate C 3 (*C. chrysosperma*) is to the right. Note pycnidium of *C. chrysosperma* forming adjacent to the zone line.

Figure A III.7. Vegetative incompatibility reaction between isolate 2 (non *C. chrysosperma*) on the left and C 2 (*C. chrysosperma*) on the right.

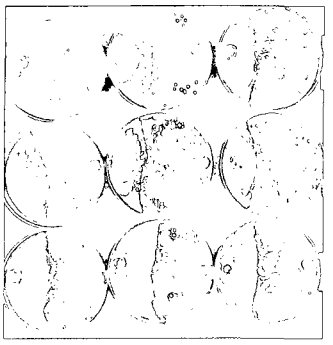


Figure A III.8. Vegetative incompatibility reactions between non *C. chrysosperma* isolates (numbers 1, 2, & 3) and *C. chrysosperma* isolates (C 1, C 2, & C 3). For each dish a non *C. chrysosperma* isolate is to the left of the barrage zone and a *C. chrysosperma* isolate to the right. Top row (left to right): 1 & C 1, 1 & C 2, 1 & C 3; Middle row (left to right): 2 & C 1, 2 & C 2, 2 & C 3; Bottom row (left to right): 3 & C 1, 3 & C 2, 3 & C 3.

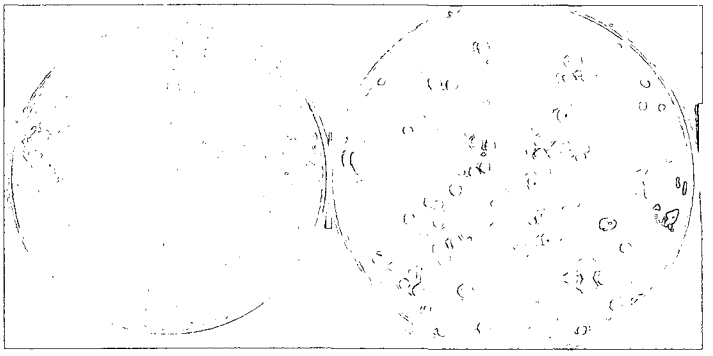


Figure A III.9. Examples of isolates which display vegetative compatibility. This is noted by the absence of a barrage zone between the isolates. Dish on the left contains non *C. chrysosperma* isolates 2 and 1 (left and right side respectively). Dish on the right contains *C. chrysosperma* isolates C 2 and C 3 (left and right side respectively).

Literature Cited

Adams, G., Hammar, S., and Proffer, T. 1990. Vegetative compatibility in *Leucostoma persoonii*. Phytopathology 80:287-291.

Alexopoulos, C. J., Mims, C. W., and Blackwell, M. 1996. Introductory Mycology. John Wiley and Sons, New York, NY. 869 p.

Caten, C. E. 1972. Vegetative incompatibility and cytoplasmic infection in fungi. J. Gen. Microbiol. 72:221-229.

Cortesi, P., McCulloch, C. E., Song, H. Lin, H., and Milgroom, M. C. 2001. Genetic control of horizontal virus transmission in the chestnut blight fungus *Cryphonectria parasitica*. Genetics 159:107-118.

Debets, F., Yang, X., and Griffiths, A. J. 1994. Vegetative incompatibility in *Neurospora*: its effect on horizontal transfer of mitochondrial plasmids and senescence in natural populations. Curr. Genet. 23:113-119.

Glass, N. L., and Kuldau, G. A. 1992. Mating type and vegetative incompatibility in filamentous ascomycetes. Ann. Rev. Phytopathol. 30:201-224.

Glass, N. L., and Saupe, S. J. 2002. Vegetative incompatibility in filamentous fungi. Pages 109-133 in Osiewacz, H. D. (ed.), Molecular Biology of Fungal Development. Marcel Dekker, NY. 607 p.

Glass, N. L., Jacobson, D. J., and Shiu, P. K. T. 2000. The genetics of hyphal fusion and vegetative incompatibility in filamentous Ascomycete fungi. Ann. Rev. Genet. 34:165-186.

Gooding, G. V., and Lucas, G. B. 1959. Factors influencing sporangial formation and zoospore activity in *Phytophthora parasitica* var. *nicotianae*. Phytopathology 49:277-281.

Griffin, D. H. 1994. Fungal Physiology. Wiley-Liss, New York, NY. 458 p.

Kaneko, I., Dementhon, K., Xiang, Q., and Glass, N. L. 2006. Nonallelic interactions between *het-c* and a polymorphic locus, *pin-c*, are essential for nonself recognition and programmed cell death in *Neurospora crassa*. Genetics 172:1545-1555.

Leslie, J. 1993. Fungal vegetative incompatibility. Ann. Rev. Phytopathol. 31:127-151.

Li, G. Y. 1981. Phenoloxidase and peroxidase activities in zone lines of *Phellinus weirii*. Mycologia 73:811-821.

Micali, C. O., and Smith, M. L. 2003. On the independence of barrage formation and heterokaryon incompatibility in *Neurospora crassa*. Fungal Genet. Biol. 38:209-219.

Molina, M. C., DePriest, P. T., and Lawrey, J. D. 2005. Genetic variation in the widespread lichenicolous fungus *Marchandiomyces corallinus*. Mycologia 97:454-463.

Nelson, E. E. 1964. Some probable relationships of soil fungi and zone lines to survival of *Poria weirii* in buried wood blocks. Phytopathology 54:120-121.

Nelson, E. E. 1975. Survival of *Poria weirii* in wood buried in urea-amended forest soil. Phytopathology 65:501-502.

Proffer, T. J., and Hart, J, H. 1988. Vegetative compatibility groups in *Leucocytospora kunzei*. Phytopathology 78:256-260.

Proffer, T. J., and Jones, A. L. 1989. A new canker disease of apple caused by *Leucostoma cincta* and other fungi associated with cankers on apple in Michigan. Plant Dis. 73:508-514.

Raper, C. A. 1983. Controls for development and differentiation of the dikaryon in Basidiomycetes. Pages 195-238 in Bennett, J. W., and Ciegler, A. (eds.), Secondary Metabolism and Differentiation in Fungi. Marcel Dekker, NY. 504 p.

Smith, M. L., Gibbs, C. C., and Milgroom, M. G. 2006. Heterokaryon incompatibility function of barrage-associatiated vegetative incompatibility genes (*vic*) in *Cryphonectria parasitica*. Mycologia 98:43-50.

Surve-Iyer, R. S., Adams, G. C., Iezzoni, A. F., and Jones, A. L. 1995. Isozyme detection and variation in *Leucostoma* species from *Prunus* and *Malus*. Mycologia 87:471-482.

Wang, D., Iezzoni, A., and Adams, G. 1998. Genetic heterogeneity of *Leucostoma* species in Michigan peach orchards. Phytopathology 88:376-381.

Study 4: Effects Of Depth Of Agar And Illumination On *Cytospora chrysosperma* And Putative Labyrinthine/Rosette Leucocytosporoid *Cytospora* sp. Cultures

Introduction

It is well documented that cultural variation can occur among species and strains of *Cytospora* and associated teleomorphs (Christensen, 1940; Helton & Moisey, 1955; Helton & Konicek, 1961a, b, c d; Spielman, 1983, 1985; Adams et al., 2006). Adams et al. (2006) state that "culture morphology is not sufficient to identify or describe **any** *Cytospora* species" with no workable system available for identification of *Cytospora* species *in vitro* or on inoculated host tissues (Adams et al., 2005).

In a series of studies, Helton and Konicek (1961a, b, c) showed that carbon and nitrogen sources, as well as temperature, affected colony characteristics. They suggested the cultural conditions would have considerable bearing on whether or not an isolate could be related to isolates used by other workers. For comparative cultural studies of fungi, Helton and Konicek suggested that standardized laboratory environmental conditions are needed. Surve-Iyer et al. (1995) emphasized that the criteria used for identification may not be defined in a given study, or, when stated, they may contradict those of other comparative studies.

An important component of studies I typically do not find stated within the methodology is the volume of media contained in Petri dishes used for fungal inoculation. Whether media is poured and "eyeballed" by the investigator for an approximate equal amount per dish, or the volume per plate is actually a measured quantity is not clear. However, I have found that this is a pertinent but overlooked criterion. A study by Okuda et al. (2000) examined several variables pertaining to *Penicillium* and *Aspergillus* cultivation, including medium ingredients, types of Petri

dishes, volume of media, method of inoculation, and incubation conditions, viz., temperature, air exchange, duration, and sporadic light exposure. Their results showed that volume of media and air exchange (dishes wrapped with two polyethylene bags vs. unwrapped dishes) affected colony appearance and growth to the greatest extent.

Adams et al. (2006) state that *Cytospora* spp. which produce colonies with highly lobate margins, e.g., *C. leucostoma*, also display poor growth on weak media, especially powdered commercial preparations of PDA. On clarified oatmeal agar, however, growth is nearly uniform which masks this distinct character trait. Additionally, Leonian (1921) found that for *Valsa leucostoma*, additions of 2-12% sugar to oatmeal agar stimulated the production of perithecia. However, Adams et al. (2005) were unsuccessful using such methodology.

Because cultural characteristics are used when describing new taxa, and the volumes (depth) of media in Petri dishes are typically not stated, a study was undertaken to see if the volume and/or type of media used would affect the cultural characteristics of *C. chrysosperma* and putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. (non-*C. chrysosperma*) isolates. Also, an attempt to stimulate sexual fruiting in culture was examined.

Materials And Methods

Four types of media were used in this study, i.e., modified Leonian's, PDA, and two formulations of oatmeal agar. With all media, plugs of mycelium 3 mm^2 were plated in the center of 90 mm diameter Petri dishes which were then wrapped with wax-film and placed under standardized growing conditions in growth chambers or diffused light. Isolates C 14 (*C. chrysosperma*) and 3 (non-*C. chrysosperma*) were examined in all depth of volume studies. For the study using modified Leonian's agar each isolate was grown

in both continuous light (CL) and continuous darkness (CD) at 25 C in dishes containing 10, 15, 20, 25, and 30 ml of media; three replications for each treatment were conducted. All cultures were examined at 9, 15, and 21 days. The study involving PDA followed the same protocols except cultures were observed at 9, 16, 26, and 37 days. Media for the oatmeal agar study used the formulation of Gooding and Lucas (1959). Volumes per dish and replications were the same as the previous two studies. However, cultures were grown at 25 C in CD only and inspected at 13, 21, and 30 days. Stimulation of sexual fruiting was attempted with isolates C 1, C 14, 3, 16, 20, and T 1. The media used was that of Adams et al. (2005) with 30 ml used for each Petri dish. All cultures were grown in CD at 25 C with three replicates for each isolate. Additionally, four cultures of T 1 were placed in diffused light at room temperature. After 90 days all cultures were examined for perithecial formation.

Results And Discussion

Results from the study indicate that the cultural morphology of *C. chrysosperma* and non-*C. chrysosperma* isolates is greatly dependent upon the type of medium used, the depth (volume) of the medium in 90 mm Petri plates, and whether cultures are grown under CL or CD (other than short periods for examination) at 25 C.

On modified Leonian's agar, C 14 displayed the greatest non-uniformity, i.e., lobate growth, in colony margins at the lesser (10 and 15 ml) volumes under both CL and CD (Figures A III.10, A III.11, & A III.12). A trend of increasing marginal uniformity with increasing depth resulted in the cultures showing more typical equal radial growth. Cultures of C 14 grown under CD tended to be more darkly pigmented as the volume of medium per dish increased (Figures A III.10, A III.11, & A III.12). This was not as pronounced for cultures grown under CL, but under both CL and CD cultures became

more darkly pigmented as they aged. When grown under the same conditions isolate 3 showed similar lobate growth at lesser (10 and 15 ml) volumes, but it was more pronounced for cultures grown under CD (Figures A III.10, A III.11, & A III.12). As with C 14 there was a trend of increasing marginal uniformity as media volume increased. Pigmentation also increased with cultural age under CL and CD. Unlike C 14, however, pigmentation for isolate 3 was darker at all volumes under CL light with the darkest pigmentation occurring, somewhat, at the lesser (15 and 20 ml) volumes; cultures growing under CD displayed similar uniformity of pigmentation at all volumes except at 30 ml where pigmentation was very light (Figures A III.10, A III.11, & A III.12).

Grown on PDA, cultures of C 14 exhibited lobate margins at the lesser (10 and 15 ml) volumes in 90 mm Petri dishes under CL and CD (Figures A III.13, A III.14, A III.15, & A III.16). As with cultures grown on modified Leonian's agar, there was a trend of increasing marginal uniformity with increasing volume of medium per dish. Marginal non-uniformity, particularly for 10 ml cultures grown under CD, was still very noticeable in the oldest cultures (37 days). This probably resulted from the coloration of the agar, i.e., under CD agar color became dark brown to black over time vs. yellow under CL. As before, pigmentation increased as cultures aged, and those grown in CD had the darkest pigmentation. However, unlike C 14 cultures grown on modified Leonian's agar, those grown on PDA revealed the darkest pigmentation at the lesser (10 and 15 ml) volumes of medium per dish (Figures A III.13, A III.14, A III.15, & A III.16). Pigmentation for cultures grown under CL showed no obvious trends, but at 16 days those with 30 ml of PDA per dish exhibited the least pigmentation. However, at 26 and 37 days, pigmentation was mostly the same at all volumes. Unlike what was observed on

modified Leonian's agar, the lobate growth habit for cultures of isolate 3 on PDA was very pronounced at various volumes under both CL and CD. The trend of increased marginal non-uniformity with decreasing volumes of media per dish was once more observed (Figures A III.13, A III.14, A III.15 & A III.16). Cultures were least uniform at volumes of 10, 15, and 20 ml per plate. The lobate growth habit was still very distinct after 37 days as compared to cultures grown on modified Leonian's agar. Pigmentation of cultures yet again increased over time under both CL and CD with those grown at the lesser (10, 15, and 20 ml) volumes being the most darkly pigmented. This was most pronounced for cultures grown under CL, particularly at 10 and 15 ml of PDA per dish.

Because cultures were not grown under CL in the oatmeal agar study, no conclusions can be drawn regarding its effect on cultural morphology for the two isolates. However, trends were still apparent when cultures were grown under CD. The lobate growth habit was not nearly as pronounced on oatmeal agar as on the other two media. However, C 14 exhibited slight lobate margins at 10 and 15 ml volumes per dish while the trait was more apparent at the same volumes for isolate 3 (Figure A III.18). Pigmentation once more increased over time for both isolates. Depth of media had a dramatic impact on pigmentation in isolate 3 as the darkest pigmentation occurred at volumes of 10 and 15 ml per dish (Figures A III.17 & A III.18). Although the effect was not as pronounced in C 14, pigmentation also was darkest at the lesser volumes. At 21 days cultures grown at 10 and 15 ml per dish had the darkest pigmentation (Figure A III.17); at 30 days 10, 15, and 20 ml volumes resulted in the darkest pigmentation (Figure A III.18).

Use of oatmeal agar to stimulate sexual fruit body formation failed for all isolates, both in CD and diffused light. However, isolates 3, 16, 20, and T 1 (non-*C. chrysosperma*) produced abundant pycnidia while C 1 and C 14 (*C. chrysosperma*) failed to produce any. Adams et al. (2005) also were unsuccessful using this protocol with *Cytospora* spp., and therefore it is possible that the isolates used in the present study are heterothallic, or require some other stimulus to reproduce sexually in culture.

A number of important conclusions can be drawn from this study. As was observed by Okuda et al. (2000), volume (depth) of medium in Petri dishes can have a profound affect on fungal morphology. In the case of *C. chrysosperma* and non-*C. chrysosperma* isolates this was exhibited most prominently in culture margins, i.e., as the volume of medium per dish decreased, the margins became more lobate. This generally occurred under conditions of both CL and CD. The relation between pigmentation and depth of media is less clear, however. No easily explained patterns were observed. It is possible that interactions between the isolate, the type and volume of media used, and illumination (or lack of) could have occurred which would make interpretations difficult.

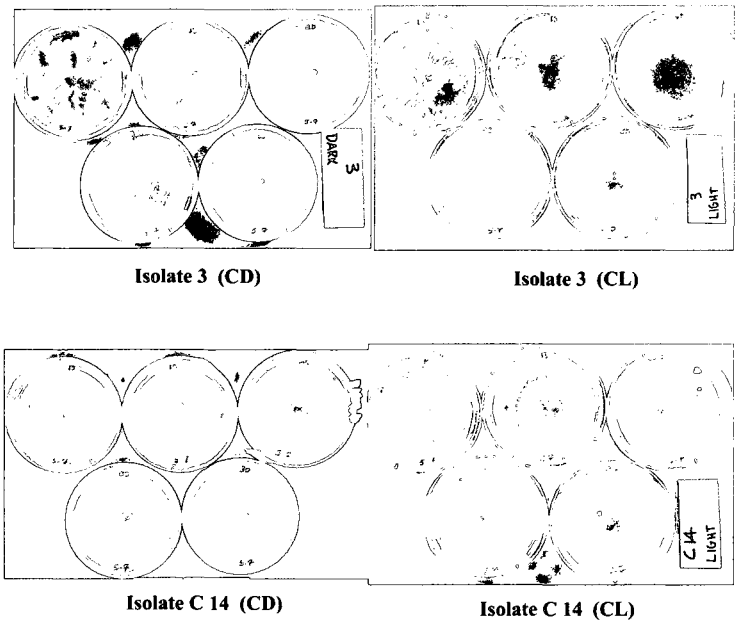
I believe this study points out some of the complexities involved in culturing fungi, and that it is imperative to standardize laboratory protocols so accurate comparative studies can be conducted. This was strongly emphasized by Helton and Konicek (1961a, b, c) who proposed that cultural conditions would have considerable bearing on whether or not an isolate could be related to isolates used by other workers. Unfortunately one facet of methodology often overlooked by researchers is a simple statement of how much medium was used for growing an isolate. Based on the results of

this study such inconsistencies could dramatically affect comparative studies leading to confusion, misconstrued results etc.

Another aim of this study was to determine if *C. chrysosperma* and non-*C. chrysosperma* isolates which produce colonies with highly lobate margins would display weak growth on powdered commercial preparations of PDA, or produce uniform growth on oatmeal agar. Isolate 3 (non-*C. chrysosperma*) and C 14 (*C. chrysosperma*) were utilized in these studies based on their lobate growth habit. Both isolates generally displayed more uniform and better overall growth when the volume of PDA in Petri dishes increased. Therefore it appears that depth of media, as opposed to media alone, can affect quality of growth. Similarly, the same isolates grown on oatmeal agar produced less uniform growth at lesser volumes of medium per dish. Despite the lack of production of cultures with "highly" lobate margins, and the use of non-clarified oatmeal agar, it can be concluded that the lobate growth habit failed to be completely masked. Perhaps with the use of clarified oatmeal agar uniform growth would have resulted at all volumes. Adams et al. (2005) reported that isolates with extremely lobate margins would produce nearly uniform growth on clarified oatmeal agar that masks the trait. Whether the slightly non-uniform growth observed in the present study is equivalent to "nearly uniform growth" is subjective. Unfortunately the use of non-clarified oatmeal is a drawback, but the point remains that the volume of media used in Petri dishes is a very important component of standardized laboratory protocol.

Another conclusion that can be drawn from this study is how easily these two taxa can be separated on the basis of cultural morphology- providing standardized methodology is used. There have been some slight cultural variations noted among

isolates within their respective taxon, but isoenzyme analyses did not correlate with these variations. This illustrates the importance of incorporating other methodologies, e.g., DNA sequence, RAPD, RFLP, when doing comparative studies. Such methodologies are more sensitive and as such will allow for a better clarification of such variations.



Isolate 3 (CD) **Isolate 3 (CL)**

Isolate C 14 (CD) **Isolate C 14 (CL)**

Figure A III.10. Isolate 3 (non *C. chrysosperma*) and isolate C 14 (*C. chrysosperma*) grown under CL and CD on modified Leonian's agar using five different volumes at 25 C for 9 days. For each isolate and CL or CD treatment the five volumes for each plate are: Top row- left to right: 10, 15, & 20 ml; Bottom row- left to right: 25 & 30 ml.

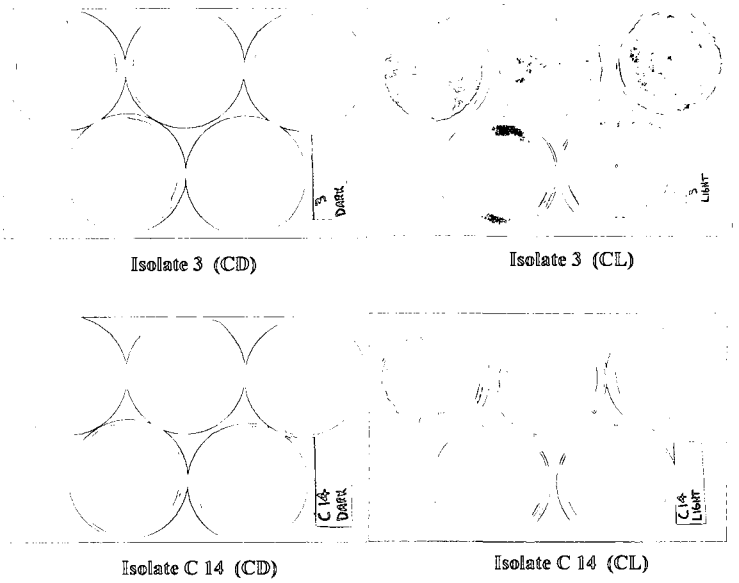


Figure A III.11. Isolate 3 (non *C. chrysosperma*) and isolate C 14 (*C. chrysosperma*) grown under CL and CD on modified Leonian's agar using five different volumes at 25 C for 15 days. For each isolate and CL or CD treatment the five volumes for each plate are: Top row- left to right: 10, 15, & 20 ml; Bottom row- left to right: 25 & 30 ml.

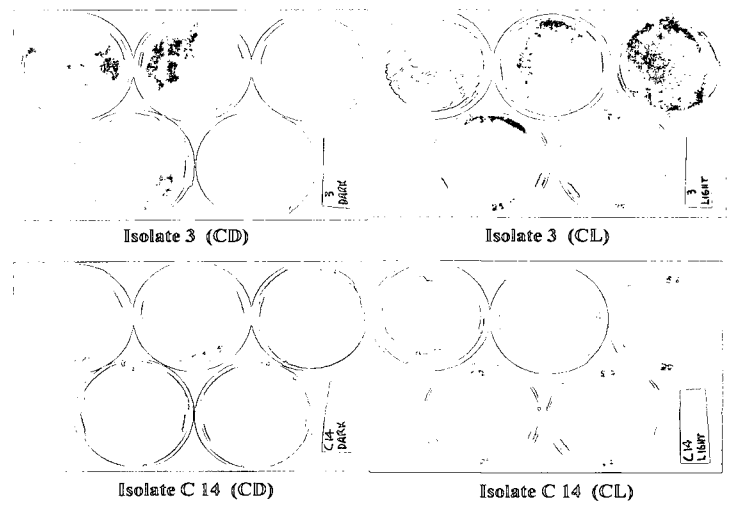


Figure A III.12. Isolate 3 (non *C. chrysosperma*) and isolate C 14 (*C. chrysosperma*) grown under CL and CD on modified Leonian's agar using five different volumes at 25 C for 21 days. For each isolate and CL or CD treatment the five volumes for each plate are: Top row- left to right: 10, 15, & 20 ml; Bottom row- left to right: 25 & 30 ml.

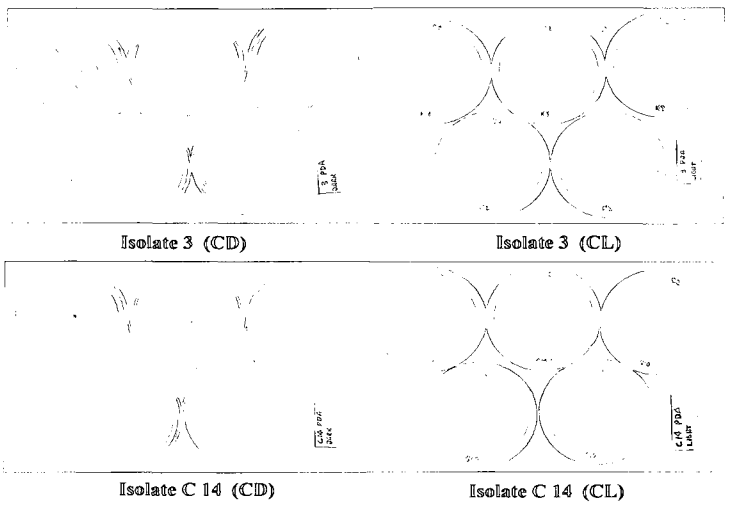


Figure A III.13. Isolate 3 (non *C. chrysosperma*) and isolate C 14 (*C. chrysosperma*) grown under CL and CD on PDA using five different volumes at 25 C for 9 days. For each isolate and CL or CD treatment the five volumes for each plate are: Top row- left to right: 10, 15, & 20 ml; Bottom row- left to right: 25 & 30 ml.

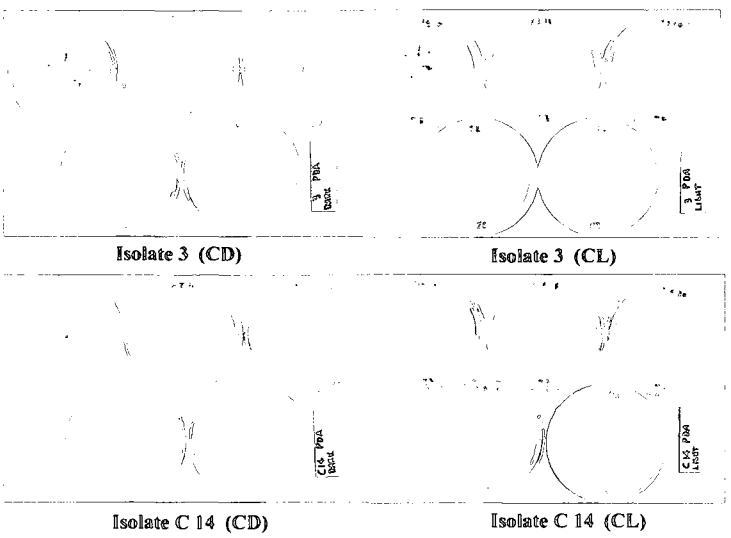


Figure A III.14. Isolate 3 (non *C. chrysosperma*) and isolate C 14 (*C. chrysosperma*) grown under CL and CD on PDA using five different volumes at 25 C for 16 days. For each isolate and CL or CD treatment the five volumes for each plate are: Top row- left to right: 10, 15, & 20 ml; Bottom row- left to right: 25 & 30 ml.

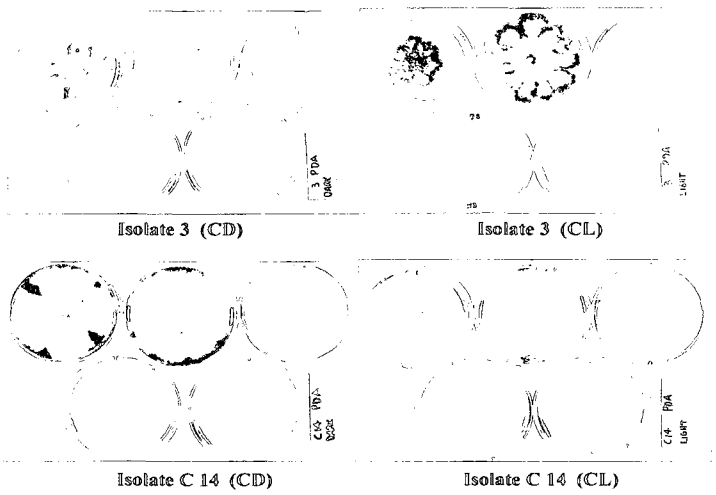


Figure A III.15. Isolate 3 (non *C. chrysosperma*) and isolate C 14 (*C. chrysosperma*) grown under CL and CD on PDA using five different volumes at 25 C for 26 days. For each isolate and CL or CD treatment the five volumes for each plate are: Top row- left to right: 10, 15, & 20 ml; Bottom row- left to right: 25 & 30 ml.

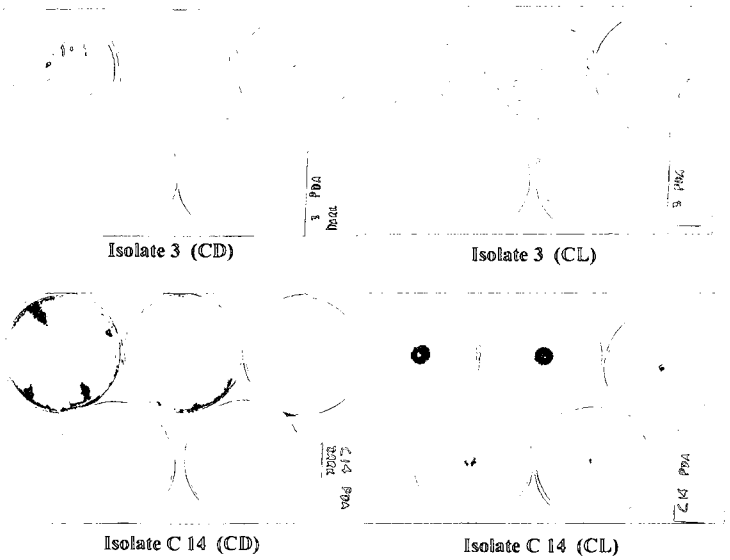


Figure A III.16. Isolate 3 (non *C. chrysosperma*) and isolate C 14 (*C. chrysosperma*) grown under CL and CD on PDA using five different volumes at 25 C for 37 days. For each isolate and CL or CD treatment the five volumes for each plate are: Top row- left to right: 10, 15, & 20 ml; Bottom row- left to right: 25 & 30 ml.

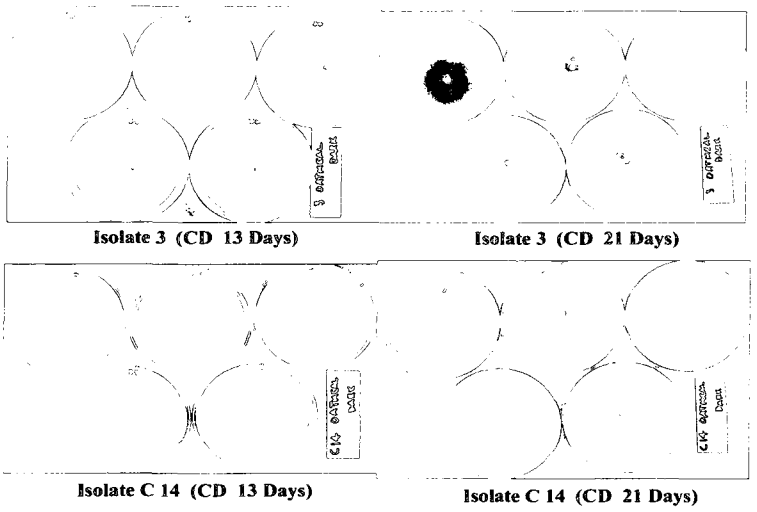


Figure A III. 17. Isolate 3 (non *C. chrysosperma*) and isolate C 14 (*C. chrysosperma*) grown under CD on oatmeal agar using five different volumes at 25 C for 13 and 21 days. For each isolate and treatment the five volumes for each plate are: Top row- left to right: 10, 15, & 20 ml; Bottom row- left to right: 25 & 30 ml.

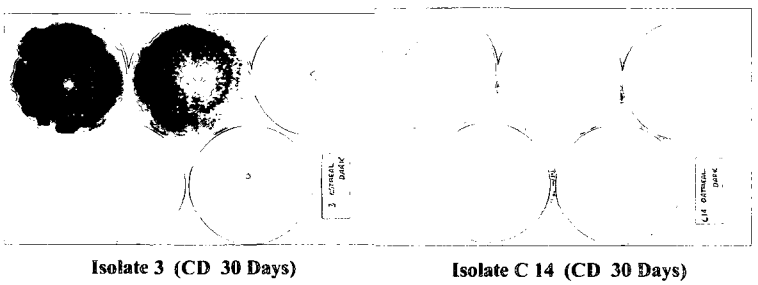


Figure A III. 18. Isolate 3 (non *C. chrysosperma*) and isolate C 14 (*C. chrysosperma*) grown under CD on oatmeal agar using five different volumes at 25 C for 30 days. For each isolate and treatment the five volumes for each plate are: Top row- left to right: 10, 15, & 20 ml; Bottom row- left to right: 25 & 30 ml.

Literature Cited

Adams, G. C., Roux, J., and Wingfield, M. J. 2006. *Cytospora* species (*Ascomycota, Diaporthales, Valsaceae*): introduced and native pathogens of trees in South Africa. Australasian Plant Pathol. 35:521-548.

Adams, G. C., Wingfield, M. J., Common, R., and Roux, J. 2005. Phylogenetic relationships and morphology of *Cytospora* species and related teleomorphs (*Ascomycota, Diaporthales, Valsaceae)* from *Eucalyptus*. Stud. Mycol. 52:1-144.

Christensen, C. M. 1940. Studies on the biology of *Valsa sordida* and *Cytospora chrysosperma.* Phytopathology 30:459-475.

Gooding, G. V., and Lucas, G. B. 1959. Factors influencing sporangial formation and zoospore activity in *Phytophthora parasitica* var. *nicotianae*. Phytopathology 49:277-281.

Helton, A. W., and Konicek, D. E. 1961a. An optimum environment for the culturing of *Cytospora* isolates from stone fruits. I. Temperature. Mycopathol. Mycol. Appl. 16:2-26.

Helton, A. W., and Konicek, D. E. 1961b. An optimum environment for the culturing of *Cytospora* isolates from stone fruits. II. Carbon sources. Mycopathol. Mycol. Appl. 16:27-34.

Helton, A. W., and Konicek, D. E. 1961c. An optimum environment for the culturing of *Cytospora* isolates from stone fruits. III. Nitrogen sources. Mycopathol. Mycol. Appl. 16:125-132.

Helton, A. W., and Konicek, D. E. 1961d. Effects of selected *Cytospora* isolates from stone fruits on certain stone fruit varieties. Phytopathology 51:152-157.

Helton, A. W., and Moisey, J. A. 1955. *Cytospora* damage in Idaho prune orchards. Plant Dis. Rep. 39:931-943.

Leonian, L. H. 1921. Studies on the *Valsa* apple canker in New Mexico. Phytopathology 11:236-243.

Okuda, T., Klich, M. A., Seifert, K. A., and Ando, K. 2000. Media and incubation effects on morphological characteristics of *Penicillium* and *Aspergillus*. Pages 83-99 in Samson, R. A., and Pitt, J. I. (eds.), Integration of Modern Taxonomic Methods for *Penicillium* and *Aspergillus* Classification. Hardwood Academic Publishers, Amsterdam, NL. 510 p.

Spielman, L. J. 1983. Taxonomy and biology of *Valsa* species on hardwoods in North America, with special reference to species on maples. Ph.D diss. Cornell University. Ithaca, NY. 175 p.

Spielman, L. J. 1985. A monograph of *Valsa* on hardwoods in North America. Can. J. Bot. 63:1355-1378.

Surve-Iyer, R. S., Adams, G. C., Iezzoni, A. F., and Jones, A. L. 1995 Isozyme detection and variation in *Leucostoma* species from *Prunus* and *Malus*. Mycologia 87:471-482.

Study 5: Pathogenicity Of *Cytospora chrysosperma* And Putative Labyrinthine/Rosette Leucocytosporoid *Cytospora* sp. Isolates On Field-Grown Aspen And Cottonwood

The objective of this test was to determine if the fungi comprising the Cytospora canker complex differ in their pathogenicity on field-grown aspen (*Populus tremuloides* Michx.) and cottonwood (*Populus* sp.) trees.

Materials and Methods

Plant material and isolates. Three aspen and one cottonwood tree located at the ARDEC research facility northeast of the Colorado State University campus were used in the experiments. A total of nine isolates were tested; two *C. chrysosperma* (C1, C8) and six putative labyrinthine/rosette leucocytosporoid *Cytospora* sp. (non-*C. chrysosperma* isolates- 1, 2, 5, 7, 11, 14) from aspen and one *Cytospora* sp. from cottonwood. All but one of the nine isolates were collected from urban landscapes in Ft. Collins, CO. The additional isolate was collected in the Poudre Canyon Northwest of Ft. Collins, CO. All isolates were grown in 90 mm diameter Petri dishes containing PDA at 25 C in the dark for one week prior to inoculation.

Inoculations. On May 19, 2004 three branches of the same approximate size, viz., approximately 0.75 inches diameter at the branch collar, from each tree were inoculated. Each of the three branches on each tree was inoculated with three fungal isolates and one control. The surface of each branch was surface disinfested with 70% ethanol and allowed to dry before wounding. Branches were wounded by removing a bark disk to the xylem with a 7 mm diameter sterilized leather punch. Each branch received four wounds. Placement was such that each consecutive wound was located 90° clockwise around the branch from the preceding one. A 6 mm diameter plug of mycelium from an actively growing colony was placed into each of the three wounds

with the mycelium facing the wound. The fourth wound received a 6 mm diameter plug of sterile agar as a control. Wounds were inoculated one at a time and wrapped with wax-film starting with the wound closest to the main stem of the tree and proceeding towards the branch tip. Wax-film was removed two weeks after inoculation and wounds inspected for canker development. An additional observation was made one week later.

Results And Discussion. The only fungal isolate which exhibited canker expansion was the *Cytospora* sp. collected from cottonwood. For this isolate, expanding cankers were produced on cottonwood as well as aspen branches. The lack of pathogenicity for the *C. chrysosperma* and non-*C. chrysosperma* isolates is speculative. *Cytospora* fungi are considered to be mostly opportunistic pathogens and generally attack hosts that are stressed and of poor vigor (Long, 1918; Hubert, 1920; Schreiner, 1931; Schoenweiss, 1967; Sinclair et al., 1987; Biggs, 1989). Drought stress has been shown to be a prominent predisposing factor where *Cytospora* spp. are involved (Bloomberg & Farris, 1963; Bertrand et al., 1976; Kamiri & Laemmlen, 1981; Biggs et al., 1983; Schoenweiss, 1983; Biggs, 1986; Guyon et al., 1996; McIntyre et al., 1996), and a critical region, i.e., threshold level, of drought stress has been noted to occur. When this threshold level is reached, or exceeded, cankers produced are larger and the time period wounds are susceptible to infection is increased (Bertrand et al., 1976; Schoenweiss, 1983; Guyon et al., 1996; McIntyre et al., 1996). Although these trees were not monitored as to their water potential status it is assumed they were not drought stressed given they were in a turf area receiving irrigation plus any additional precipitation. Therefore they may not have been in a predisposed state. Visually they appeared to be in vigorous condition, but this is subjective. Isoenzyme patterns of the *Cytospora* sp.

obtained from cottonwood were unique leading me to believe that it is a different species or strain. Kepley (1998) showed in a greenhouse inoculation study that well-watered trees could become infected by certain *Cytospora* sp. isolates. Based on this it is possible that the isolate from cottonwood was better able to overcome non-compromised host defense mechanisms resulting from adequate irrigation.

Post inoculation conditions during May were relatively warm to hot although specific temperature data were not recorded. Different *Cytospora* species are known to have different optimal temperatures for pathogenicity and virulence as well as *in vitro* growth and conidial germination. Schreiner (1931) observed that clones of *V. sordida* displayed most rapid growth on poplar decoction agar at 25 C although growth occurred at temperatures as low as 4 C and as high as 35 C. In contrast, clones of *V. nivea* displayed a very narrow temperature range predominantly growing only at 25 C. Schreiner believed temperature would likely influence the pathogenicity of *V. nivea.* Therefore it is possible that temperatures following inoculation exceeded the optima for *C. chrysosperma* and non-*C. chrysosperma* isolates as compared to those required by the *Cytospora* sp. from cottonwood. Furthermore, Wood and French (1965) showed that temperatures of healthy aspen bark on the south side of the tree in February and April could exceed that of ambient air temperatures by as much as 8 C which further could have altered the specific conditions needed by these fungi.

The time of year in which inoculations are conducted has been shown to have an effect on the outcome of studies for various host/*Cytospora* spp. combinations (Schreiner, 1931b; Willison, 1936; Zentmeyer, 1941; Helton & Moisey, 1955; Helton & Konicek, 1961; Filer, 1967; Bertrand & English, 1976; Dhanvantari, 1982; McIntyre, 1996).

Adams et al. (2006) state that in climates with long, cold winters autumn (September-October) inoculations provide maximum canker expansion which is measured in the spring. Inoculations in the present study were made on May 9, and I suspect host defense mechanisms were greater at this time than they would have been in the autumn due to dormancy factors.

Given the fact that stress, particularly drought, is a predisposing factor for infection it is surprising that many pathogenicity and virulence studies, for both trees growing in the field and potted trees in a greenhouse, have (prior to inoculation) either used visual assessment as to tree status, made no mention as to tree status, used vigorously growing/healthy trees, or have not monitored trees as to direct water potential or relative bark moisture when the technology was available (Long, 1918; Christensen, 1940; Zentmeyer, 1941; Wright, 1942; Hildebrand, 1947; Marsden, 1948; Schmidle, 1954; Helton, 1961; Wysong & Dickens, 1962; Lukezic et al., 1965; Gross, 1967; Bertrand & English, 1976; Kamiri & Laemmlen, 1981; Dhanvantari, 1982; Spielman, 1983; Proffer & Hart, 1994; Wier & Tattar, 2000; Madar et al., 2004; Alonso, 2005). Such factors could have a direct influence on the pathogenic and virulence potential of isolates used in a study or lead to misconstrued results when comparisons between isolates are made or the nature of a given isolate is assessed, i.e., is it a primary parasite or potentially a saprophyte or weak primary parasite. Additionally, evaluations of host resistance could also be misinterpreted.

Why the isolate from the cottonwood was pathogenic and the *C. chrysosperma* and non-*C. chrysosperma* isolates from aspen were not is difficult to speculate upon. Perhaps the cottonwood isolate is more virulent and able to incite disease when the hosts'

vigor is not reduced, or it has a different temperature optimum required for growth and pathogenicity. A number of studies have shown there can be a wide range in virulence in a collection of *Cytospora* isolates ranging from saprophytic or non-pathogenic to highly virulent (Willison, 1936; Hildebrand, 1947; Treshow et al., 1960; Helton, 1961; Helton & Konicek, 1961; Wysong & Dickens, 1962; Lukezic et al., 1965; Bertrand & English, 1976; Dhanvantari, 1982).

Adams et al. (2005) propose that pathogenicity may be a species-specific character that is especially difficult to determine. They illustrate this point with *V. sordida* inoculated on *Populus* which is highly virulent as opposed to *C. nivea* which is not. Both species are considered ubiquitous endophytes in *P. tremuloides.* In pathogenicity tests, *Valsa* or *Cytospora* isolates collected from fruit bodies on fallen branches may or may not be pathogenic. They conclude that in cases where they are not pathogenic they are merely saprophytes or low virulent endophytes capitalizing on the senescent tissue.

It is apparent that it is difficult to draw any definitive conclusions from this study due to the limited number of trees which were inoculated and the fact that tree water status was not monitored. Obviously controlled experiments utilizing more trees and more replications could potentially provide a better understanding regarding the pathogenicity and virulence of the fungi in this canker complex.

Literature Cited

Adams, G. C., Roux, J., and Wingfield, M. J. 2006. *Cytospora* species (*Ascomycota, Diaporthales, Valsaceae*): introduced and native pathogens of trees in South Africa. Australasian Plant Pathol. 35:521-548.

Alonso, A., Lupo, S., and Bettucci, L. 2005. Pathogenicity evaluation of *Cytospora eucalypticola* isolated from *Eucalyptus* spp. cankers in Uruguay. Fitopathol. Bras. 30:289-291.

Bertrand, P. F., and English, H. 1976. Virulence and seasonal activity of *Cytospora leucostoma* and *C. cincta* in French prune trees in California. Plant Dis. Rep. 60:106-110.

Bertrand, P. F., English, H., Uriu, K., and Schick, F. J. 1976. Late season water deficits and development of Cytospora canker in French prune. Phytopathology 66:1318-1320.

Biggs, A. R. 1986. Influence of irrigation on wound response in peach bark. Can. J. Plant Pathol. 8:405-408.

Biggs, A. R. 1989. Integrated approach to controlling Leucostoma canker of peach in Ontario. Plant Dis. 73:869-874.

Biggs, A. R., Davis, D. D., and Merrill, W. 1983. Histopathology of cankers on *Populus* caused by *Cytospora chrysosperma*. Can. J. Bot. 61:268-272.

Bloomberg, W. J., and Farris, S. H. 1963. Cytospora canker of poplars: bark wounding in relation to canker development. Can. J. Bot. 41:303-310.

Christensen, C. M. 1940. Studies on the biology of *Valsa sordida* and *Cytospora chrysosperma*. Phytopathology 30:459-475.

Dhanvantari, B. N. 1982. Relative importance of *Leucostoma cincta* and *L. persoonii* in perennial canker of peach in southwestern Ontario. Can. J. Plant Pathol. 4:221-225.

Filer, T. H. Jr. 1967. Pathogenicity of *Cytospora*, *Phomopsis*, and *Hypomyces* on *Populus deltoides*. Phytopathology 57:978-980.

Gross, H. L. 1967. Cytospora canker of black cherry. Plant Dis. Rep. 51:941-944.

Guyon, J. C., Jacobi, W. R., and McIntyre, G. A. 1996. Effects of environmental stress on the development of Cytospora canker of aspen. Plant Dis. 80:1320-1326.

Helton, A. W. 1961. First year effects of 10 selected *Cytospora* isolates on 20 fruit and forest tree species and varieties. Plant Dis. Rep. 45:500-504.

Helton, A. W., and Konicek, D. E. 1961. Effects of selected *Cytospora* isolates from stone fruits on certain stone fruit varieties. Phytopathology 51:152-157.

Helton, A. W., and Moisey, J. A. 1955. *Cytospora* damage in Idaho prune orchards. Plant Dis. Rep. 39:931-943.

Hildebrand, E. M. 1947. Perennial peach canker and the canker complex in New York with methods of control. Cornell Univ. Agric. Stn. Mem. 276:1-61.

Hubert, E. E. 1920. Observations on *Cytospora chrysosperma* in the Northwest. Phytopathology 10:442-447.

Kamiri, L. K., and Laemmlen, F. F. 1981. Effect of drought stress and wounding on Cytospora canker development on Colorado blue spruce. J. Arbor. 7:113-116.

Kepley, J. B. 1998. Pathogenicity of *Cytospora* species on six hardwoods. MS thesis. Colorado State University. Fort Collins, CO. 108 p.

Long, W. H. 1918. An undescribed canker of poplars and willows caused by *Cytospora chrysosperma.* J. Agr. Res. 13:331-343.

Lukezic, F. L., DeVay, J. E., and English, H. 1965. Comparative physiology and pathogenicity of *Leucostoma persoonii* and *Rhodosticta quercina.* Phytopathology 55:511-518.

Madar, Z., Leisrael, K. K., Solel, Z., Kimchi, M. 2004. First report of Cytospora canker caused by *Cytospora chrysosperma* on white poplar in Israel. Plant Dis. 88:220 [Abstract].

Marsden, D. H. 1948. A *Valsa* associated with Cytospora canker of spruce. Phytopathology 38:307-308.

McIntyre, G. A., Jacobi, W. R., and Ramaley, A. W. 1996. Factors affecting Cytospora canker occurrence on aspen. J. Arbor. 22:229-233.

Proffer, T. J., and Hart, J, H. 1994. Wound inoculations of Colorado blue spruce with isolates of *Leucostoma* (*Cytospora*) *kunzei* and other *Cytospora* species. J. Arbor. 20:215-221.

Schmidle, A. 1954. The *Cytospora* disease of poplar and the conditions for its occurrence. Rev. Appl. Mycol. 33:388-389 [English abstract].

Schoenweiss, D. F. 1967 Susceptibility of weakened cottonwood stems to fungi associated with blackstem. Plant Dis. Rep. 51:933-935.

Schoenweiss, D. F. 1983. Drought predisposition to Cytospora canker in blue spruce. Plant Dis. 67:383-385.

Schreiner, E. J. 1931. Two Species of *Valsa* Causing Disease in *Populus*. Am. J. Bot. 18:1-29.

Sinclair, W. A., Lyon, H. H., and Johnson, W. T. 1987. Diseases of Trees and Shrubs. Comstock Publishing Associates, Cornell University Press, Ithaca, NY. 575 p.

Spielman, L. J. 1983. Taxonomy and biology of *Valsa* species on hardwoods in North America, with special reference to species on maples. Ph.D diss. Cornell University. Ithaca, NY. 175 p.

Treshow, M. J., Scholes, F., and Gardner, W. S. 1960. Pathogenic variation in *Cytospora rubescens* isolates on stone fruit varieties. Phytopathology 50:86 [Abstract].

Wier, A. M., Tattar, T. A., and Klekowski, E. J. 2000. Disease of red mangrove (*Rhizophora mangle*) in Southwest Puerto Rico caused by *Cytospora rhizophorae*. Biotropica 32:299-306.

Willison, R. S. 1936. Peach canker investigations II. Infection studies. Can. J. Res. 14:27-44.

Wood, F. A., and French, D. W. 1965. Hypoxylon canker of aspen: seasonal development of cankers and ascospore ejection in winter. Phytopathology 55:771-774.

Wright, E. 1942. *Cytospora abietis*, the cause of canker of true firs in California and Nevada. J. Agr. Research 65:143-153.

Wysong, D. S., and Dickens, L. E. 1962. Variation in virulence of *Valsa leucostoma*. Plant Dis. Rep. 46:274-276.

Zentmyer, G. A. 1941. Cytospora canker of Italian cypress. Phytopathology 31:896-906.

Appendix IV

Media and materials used for DNA extraction and gel electrophoresis of polymerase chain reaction (PCR) products

Potato dextrose agar

24 g potato dextrose broth (Difco Laboratories, Detroit, MI)
15 g agar
1 L distilled water

Potato dextrose broth

24 g potato dextrose broth (Difco Laboratories, Detroit, MI)
1 L distilled water

10 mM sodium boric acid buffer (SB); pH 8.0 (20X stock solution)

Dilute 1:19 with double distilled water for final buffer (10 mM solution)
8 g sodium hydroxide
Double distilled water to make 1 L
Adjust pH to 8.0 with boric acid (H_3BO_3), which upon dilution to working strength (1X), yields a pH ~8.5.

1% agarose gel slab construction for electrophoresis of PCR products

Dilute 20X SB buffer to 1X.
1 g agarose added to 1X SB buffer. Bring volume up to 100 ml.
Microwave mixture until agarose goes into full solution.
After microwaving add 2 µl of ethidium bromide (EB-10 µg/ml) to the agarose solution.
Pour approximately 35-40 ml of the agarose/EB solution into the horizontal gel mold and insert well comb.
Allow gel to fully cool (at least 30 minutes) before removing well comb.
Place gel into electrophoretic apparatus and flood with 1X SB buffer until the sides and top of the gel are covered with buffer.
Carefully load wells with PCR product and loading dye mixtures. The first well is loaded with PCR marker and loading dye mixture.

PCR amplification reactions for ITS1-5.8S-ITS2 and EF-1α genomic regions

FailSafe Master Mix

Primers: ITS1 F and ITS4 R (White et al., 1990)

By using 0.25 µl of each undiluted primer per 25 µl amplification reaction the final concentration for each primer is 1 µM.

ITS1 F	0.25 µl
ITS4 R	0.25 µl
PCR Enzyme Mix	0.25 µl
Sterile distilled water	10.75 µl
Template DNA	1 µl
	12.5 µl total reaction volume

FailSafe PCR 2X PreMix D 12.5 µl

25 µl amplification reaction

Note: PreMix D was used in all amplification reactions containing sample DNA extracted from *C. chrysosperma* (C 1, C 14) and non-*C. chrysosperma* (3, 16, 20, T1) isolates.

FailSafe Master Mix

Primers: EF1 F and EF2 R (Geiser et al., 2004)

By using 0.2 µl of each undiluted primer per 25 µl amplification reaction the final concentration for each primer is ~1 µM.

EF1 F	0.2 µl
EF2 R	0.2 µl
PCR Enzyme Mix	0.2 µl
Sterile distilled water	11 µl
Template DNA	1 µl
	~12.5 µl total reaction volume

FailSafe PCR 2X PreMix D 12.5 µl

~25 µl amplification reaction

Note: PreMix D was used in all amplification reactions containing sample DNA extracted from *C. chrysosperma* (C 1, C 14) and non-*C. chrysosperma* (3, 16, 20, T1) isolates.

FailSafe Master Mix

Primers: EF1 526 F and EF1 1567 R (Rehner, 2001)

By using 0.1 µl of each undiluted primer per 25 µl amplification reaction the final concentration for each primer is ~1 µM.

EF1 526 F	0.1 µl
EF1 1567 R	0.1 µl
PCR Enzyme Mix	0.25 µl
Sterile distilled water	11 µl
Template DNA	1 µl
	~12.5 µl total reaction volume

FailSafe PCR 2X
PreMix D 12.5 µl

~25 µl amplification reaction

Note: PreMix D was used in all amplification reactions containing sample DNA extracted from *C. chrysosperma* (C 1, C 14) and non-*C. chrysosperma* (3, 16, 20, T1) isolates.

FailSafe Master Mix

Primers: EF1 728 F and EF1 986 R (Carbone & Kohn, 1999)

The primers were first diluted with sterile distilled water to get solutions with final concentrations of 25 µM. By using 1 µl of each 25 µM primer solution per 25 µl amplification reaction the final concentration for each primer is 1 µM.

EF1 728 F	1 µl
EF1 986 R	1 µl
PCR Enzyme Mix	0.25 µl
Sterile distilled water	9.25 µl
Template DNA	1 µl
	12.5 µl total reaction volume

FailSafe PCR 2X
PreMix D or E 12.5 µl

25 µl amplification reaction

Note: PreMix D was used in all amplification reactions of sample DNA extracted from *C. chrysosperma* (C 1, C 14) isolates. PreMix E was used for reactions associated with non-*C. chrysosperma* (3, 16, 20, T1) isolates.

Thermal cycler programs used for amplification of EF-1α and ITS1-5.8S-ITS2 genomic regions

Program 1: Touchdown PCR (Rehner, 2001)- employed for EF-1α amplification reactions when primers EF1-526 F and EF1-1567 R (Rehner, 2001) are used.

Minutes	Temperature (C)	
4	94 (denature)	
1	66 (anneal)	
1.5	72 (extension)	
1	94	↓
1	65	
1.5	72	↓
1	94	
1	64	↓
1.5	72	
1	94	↓
1	63	
1.5	72	↓
1	94	
1	62	↓
1.5	72	
1	94	↓
1	60	
1.5	72	↓
1	94	
1	59	↓
1.5	72	
1	94	↓
1	58	
1.5	72	↓
1	94	
1	57	↓
1.5	72	
1	94	↓
1	56	
1.5	72	

10 cycles

Following the first 10 cycles an additional 33 cycles of 1 minute at 94 C (denature), 1 minute at 56 C (anneal), and 1.5 minutes at 72 C (extension) each are run. Upon completion of these cycles a final extension period of 10 minutes at 72 C is conducted.

Program 2: Touchdown PCR (Rehner, 2001)- employed for EF-1α amplification reactions when primers EF1-526 F and EF1-1567 R (Rehner, 2001), and EF1-728 F and EF1-986 R (Carbone & Kohn, 1999) are used.

This program has the same overall protocol as Program 1 except the starting annealing temperature, which is 60 C, is reduced by 1 C for each cycle until 50C is reached. Following these 10 cycles, the remaining 33 cycles have annealing temperatures of 50 C. A final extension period of 10 minutes at 72 C concludes the program.

Program 3: Used for all ITS1-5.8S-ITS2 amplifications and EF-1α amplifications when primers EF1 F and EF2 R (Geiser et al., 2004) are used.

Minutes	Temperature (C)
4	94 (denature)
1	53 (anneal)
2	72 (extension)
1 Cycle	
1	94 (denature)
1	53 (anneal)
2	72 (extension)
34 cycles	
10	72 (final extension)

Appendix V

Materials used for the fixing, embedding, and sectioning of fruiting bodies from woody (natural state) and cultural specimens

Tissue hydrating solution- 2% potassium hydroxide (KOH)

2 g KOH
Distilled water to make 100 ml

0.1 M phosphate buffer, pH 7.4

To approximately 9.5 ml 0.2 M monobasic sodium phosphate add about 40.5 ml 0.2 M dibasic sodium phosphate. Dilute the 50 ml solution to 100 ml with distilled water.

Tissue fixating solution- 2% formaldehyde, 2% glutaraldehyde, 0.1 M phosphate buffer, pH 7.4

5.4 ml 37% formaldehyde
8 ml 25% glutaraldehyde
0.1 M phosphate buffer, pH 7.4, to make 100 ml

0.025 M phosphate buffer, pH 7.2

To approximately 7 ml 0.2 M monobasic sodium phosphate add about 18 ml 0.2 M dibasic sodium phosphate. Dilute the 25 ml solution to 100 ml with distilled water.

5% HCl solution

5 ml HCl
Distilled water to make 100 ml

Spurr's resin- low viscosity embedding medium

ERL (Vinylcyclohexene dioxide)	20 g
D.E.R.	12 g
(Diglycidyl ether of polypropylene glycol)	
NSA (Nonenyl succinic anhydride)	52 g
DMAE (Dimethylaminoethanol)	0.8 g

Preparation of medium:

The embedding medium should be freshly prepared. Weigh the components singly into a tared disposable plastic beaker with DMAE added last. Before adding DMAE gently mix the first three components (ERL, D.E.R., and NSA). After the addition of DMAE, thoroughly mix the complete medium by stirring.

1% toluidine blue in 1% borate stock solution for staining

1 g toluidine blue
1 g sodium borate
Distilled water to make 100 ml

Dissolve sodium borate in distilled water. Add toluidine blue and stir until dissolved. Filter the solution through a glass funnel.

Final staining solution (0.01% toluidine blue in 0.01% borate)

Dissolve 1 ml 1% toluidine blue/1% borate stock solution in 100 ml distilled water.

Procedures for the fixing, embedding, and sectioning of fruiting bodies from woody (natural state) and cultural specimens (modified version of Adams et al., 2005)

1) Hydrate the excised fruit body in a glass vial containing10 ml 2 % KOH (approximately 30 minutes).
   Note: Cultural samples do not require hydration.
2) Rinse specimen with distilled water.
3) After rinsing, fix the fruit body in 10 ml 2% formaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 24 hours.
4) Once fixed, decant the fixative and soak the sample in 5% HCl for ca. 3 hours.
5) After removal of the HCl solution, neutralize the specimen by washing in 0.025 M phosphate buffer, pH 7.2, for 1 hour.
6) Following removal of the phosphate buffer dehydrate the fruit body in a graded ethanol (ETOH) series, viz., 25%, 50%, 75%, 100%, 100%, 100%.
   Note: Decant the ETOH after each dehydration step.
7) After the final 100% ETOH dehydration step, transfer the specimen to a fresh vial containing ca. ¼ inch of 100% propylene oxide for further dehydration.
8) Begin to add Spurr's resin to the 100% propylene oxide in a drop-like manner beginning with ca. 5 drops. Mix the propylene oxide and resin until no lines are seen in the mixture (this is done each time more resin is added during the embedding process).
9) The slow introduction of Spurr's resin by drop-like additions is continued over an 8 to 12 hour period until the amount of resin is approximately 100%.
10) Remove the sample from the 100% resin mixture and place in a fresh vial containing 100% Spurr's. Leave the specimen in the resin for ca. 4 hours.
12) Transfer the fruit body to an embedding mold (film canister cap) filled with 100% resin-orient the specimen so the ostiole (s) faces upward. Let the mold sit for approximately 8 hours.
13) Cure the resin by placing the embedding mold in the oven at 70 C for ca. 16 hours.
14) Remove the mold from the oven and let sit at room temperature until the specimen is sectioned.
15) Specimen blocks are trimmed appropriately prior to sectioning.
16) An ultramicrotome with a glass knife is used to cut sections. Thickness of sections are approximately 1 μm.
17) Once cut, sections are slowly heated in distilled water with an alcohol lamp to allow them to unfold, stretch-out, and adhere to a microscope slide.
18) Following adhesion to the slide, sections are flooded with 0.01% toluidine blue in 0.01% borate and heated to ca. 100 C. Excess stain is rinsed off with distilled water. The slide is again heated and rinsed, and then dried.
19) After drying, a drop of immersion oil and a cover slip are placed over the stained section. The slide is heated as needed to remove any air bubbles.

Materials used for observing cultural characteristics, and general morphological features from woody (natural state) and cultural specimens

Modified Leonian's medium

6.25 g maltose
6.25 g malt extract
1.25 g KH_2PO_4
0.625 g $MgSO_4 \cdot 7H_2O$
0.625 g peptone
15 g agar
1 L distilled water

Phloxine-KOH (potassium hydroxide) staining solution

Note: each solution is kept in a separate bottle

95% ethyl alcohol
0.1% Phloxine (0.1 g in distilled water to make 100 ml)
4% KOH (4 g in distilled water to make 100 ml)

Fungal tissue is fixed/wetted by placing it in a drop of 95% ETOH on a microscope slide. After draining any excess alcohol, a drop of 0.1% phloxine is placed on the fungal tissue. Excess phloxine is then drained, followed by a drop of 4% KOH and the addition of a cover slip.

Amman's Lactophenol (plus Cotton Blue) staining solution

20 ml	Phenol
20 ml	Lactic acid
40 ml	Glycerin
0.05 g	Cotton Blue

Distilled water to make 100 ml

Fungal tissue is added to a drop of the staining solution on a microscope slide. A cover slip is placed over the tissue and the preparation is gently heated with an alcohol lamp.

Melzer's staining solution

100 g	Chloral hydrate
5 g	Potassium iodide
1.5 g	Iodine

Distilled water to make 100 ml

Literature Cited

Adams, G. C., Wingfield, M. J., Common, R., and Roux, J. 2005. Phylogenetic relationships and morphology of *Cytospora* species and related teleomorphs *(Ascomycota, Diaporthales, Valsaceae)* from *Eucalyptus*. Stud. Mycol. 52:1-144.

Carbone, I., and Kohn, L. M. 1999. A method for designing primer sets for speciation studies in filamentous Ascomycetes. Mycologia 91:553-556.

Geiser, D. M., Jiménez-Gasco, M. del Mar, Kang, S., Makalowska, I., Veeraraghavan, N., Ward, T. J., Zhang, N., Kuldau, G. A., and O'Donnell, K. 2004. FUSARIUM-ID v. 1.0: a DNA sequence database for identifying *Fusarium*. Eur. J. Plant Pathol. 110:473-479.

Rehner, S. 2001. Primers for elongation factor 1-α (EF1-α). 4 p. http://ocid.nacse.org/research/deephyphae/EF1primer.pdf.

White, T. J., Bruns, T., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J. (eds.), PCR Protocols: a Guide to Methods and Applications. Academic Press, San Diego, CA.