Systematics, efficacy and population dynamics of the biocontrol fungus, *Chondrostereum purpureum*

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We accept this dissertation as conforming
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ABSTRACT

Biological control shows great potential as a non-chemical alternative for forestry vegetation management. Current methods of deciduous weed control include mechanical and manual removal, combined with chemical herbicide application. Manual cutting is labour-intensive and ineffective due to the rapid re-sprouting of most deciduous species from cut stumps. The basidiomycete fungus *Chondrostereum purpureum* was identified as a promising candidate for development as a stump treatment to suppress re-sprouting. Phylogenetic relationships were estimated by comparison of chitin synthase gene fragments among *C. purpureum* and other fungi thought to be closely related, or of the same ecological niche. The removal of *C. purpureum* from the genus *Stereum* was supported by these analyses. This study provided an independent confirmation of evolutionary hypotheses based on ribosomal DNA sequences. PCR-based genetic markers were developed to confirm the identity and source of *C. purpureum* individuals in infected trees, wood samples and mycelial cultures, allowing hundreds of field trial samples to be assayed for *C. purpureum*. Field-inoculated *C. purpureum* was re-isolated and identified, satisfying Koch's postulates for plant pathogens. The extent of infection in different hosts by *C. purpureum* was related to the relative success of biocontrol in these treatments. A lower rate of *C. purpureum* infection of treated aspen stumps, as compared to Sitka alder, was correlated with less effective suppression of this species. The same diagnostic markers were also applied to assess the distribution of genetic variation among natural populations of *C. purpureum* and estimate the extent of gene flow and other evolutionary forces. Genetic variation within the species revealed little evidence of substructuring that could be attributed to evolutionary processes such as genetic drift or selection. Accordingly, no geographic or host specialization was evident in *C. purpureum* within B.C. Spore trapping experiments were designed to assess the persistence of individual genotypes of *C. purpureum* following a biocontrol application. The genotypes of *C. purpureum* isolated from the field site before the trial, and those isolated from spore traps, were compared with the released isolate. No increase in band sharing, which would be evidence for persistence of the genotype, was apparent among the post-trial *C. purpureum* cultures. The PCR primers used to identify and differentiate

C. purpureum amplified a number of polymorphic fragments, hypothesised to be repetitive DNA. These fragments were further characterized by comparison with published sequences and Southern hybridization. Based on sequence alignments, the repetitive DNA fragments amplified by the *C. purpureum* primers were hypothesised to be inactive retrotransposons, which is supported by the presence of methylation within the amplified fragments. Preliminary experiments showed that these primers can also be used to amplify polymorphic repetitive DNA from other basidiomycetes. The results of experiments summarized in this dissertation have provided an estimation of the evolutionary history of the genus by phylogenetic analysis, an assessment of the natural population structure of the species and an investigation of the dynamics of this fungus after field application. This research has expanded our understanding of fungal evolution, while concurrently supporting the development of a native fungus as a biological control agent for use in our forests.

Examiners:

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Dedication

to my Grandfather, Henry Becker

for his love, support, teaching and inspiration

Chapter 1. General Introduction

Integrated forest vegetation management

Forest values such as biodiversity, recreation and water quality are becoming of paramount importance to our society. At the same time, there is an increasing demand for forest products by the world's growing population. Future wood harvests will depend in part on the management of reforestation sites and the regeneration of natural forests. The integrated vegetation management (IVM) approach follows the idea of agricultural integrated pest management (IPM), by utilizing methods most suited to the ecology of a particular site to promote growth of crop species (Wagner 1993, McLean 1994). As more vegetation management tools become available as alternative treatments, more flexibility will exist for IVM that is tailored to a specific forest ecosystem.

The focus of most research in vegetation management for the past several decades has been the development of technology for the control of unwanted vegetation, or weeds (Wagner 1993). Many tall-growing hardwood tree species in British Columbia (B.C.) can be opportunistic and have faster initial growth than conifer species. In reforestation sites, these fast-growing species, which include *Acer macrophyllum* Pursh (bigleaf maple), *Alnus rubra* Bongard (red alder), and *Populus tremuloides* Michx. (trembling aspen), can out-compete conifer tree species, thereby reducing their survival and growth. The hardwood species are thus considered forest weeds in areas of conifer plantation or regeneration and efforts must be made to control their growth. The average annual harvest from the 51.2 million hectares of productive forests in B.C. from 1986-1990 was

about 77.5 million cubic meters and the estimated annual depletion due to forest weed species was 8.4 million cubic meters (McLean 1994). Early suppression of competing vegetation stimulates growth by channeling more resources into crop trees (Richardson 1993). Traditional methods of control of forest weed species include the use of chemical herbicides, mechanical and manual removal or damage, and the use of fire.

Tall-growing deciduous species are also problematic when growing in utility company rights-of-way (ROW) where trees can encroach upon power and telephone transmission lines and cause fires and power outages. An appropriate approach would promote the growth of low-growing shrubs requiring little maintenance. This would require a balance of suppression of certain fast-growing species and the management of low-growing species. The utility company B.C. Hydro maintains 65,000 km^2 of ROW in B.C., in which vegetation management is achieved primarily by mechanical methods in combination with chemical herbicide application. Manual cutting is labour intensive and is not effective in control of many hardwoods because of rapid and prolific resprouting from cut stumps or root suckers. The control of these species is currently accomplished by mechanical clearing followed by treatment with a chemical herbicide.

While the application of chemical herbicides is the most prevalent method of control of forest weeds in North America, the public demand for alternatives is high, with 71% of the public polled in B.C. opposed to the use of chemicals in forests (Wagner 1993). The most commonly used chemical herbicide, glyphosate, acts non-selectively on the foliage of crop species and weeds alike. Public perception of the danger of non-target effects of

herbicides on wildlife and humans has influenced policy makers who have limited the use of chemical herbicides in at least five Canadian provinces (Halleran 1990). The use of mechanical equipment and controlled fire are the primary alternatives where chemical methods are restricted. Other alternatives to the use of chemical herbicides in forestry applications have been explored with varied success (Wagner 1993). Negative public opinion about the dangers of chemical herbicides has created a need for non-chemical controls. Biological control, or the deliberate use of natural enemies to suppress the growth or reduce the population of a weed species, is a promising alternative.

Biological control

Biological control methods have been successfully used in agricultural settings and show great potential for the development of non-chemical alternatives for forestry applications (Wall *et al.* 1992, Templeton and Greaves 1984, Templeton *et al.* 1979, Jobidon 1991, TeBeest *et al.* 1992). Biological control is defined as the deliberate use of natural enemies of a species in order to suppress the growth or reduce the population of that species (Markin and Gardner 1993). The goal of plant pathology research has traditionally been disease prevention, but much of this research can be applied to the promotion or enhancement of disease in weeds. Examples of successful biological controls that have been developed are two products registered for agricultural use for major crops in the United States. The fungus *Phytophthora palmivora* Butler has been sold since 1981, under the trade name DeVine® for control of strangler vine (milkweed), *Morrenia odorata* Lindl., in Florida citrus groves. (Templeton *et al.* 1989). Another commercially available biological control is a formulation of *Colletotrichum*

gloeosporioides f.sp. *aeschynomene* R.J. Sm. & Fox, fungus sold under the name Collego® since 1982 to control northern jointvetch, *Aeschynomene virginica* (L.) Britton, Poggenb. and Sterns, in rice fields (Daniel *et al.* 1973, Templeton and Greaves 1984). Both products have been efficacious and have good grower acceptance (Templeton 1986, Templeton *et al.* 1989).

Mycoherbicide theory

The aforementioned biological controls are both examples of mycoherbicides, or indigenous fungi applied in an inundative manner to control native weeds (TeBeest and Templeton 1986, Auld 1990, Charudattan 2001). The theory behind this approach is that these indigenous fungi evolved with their hosts and are controlled by natural population checks, so application of these fungi in sufficient inoculum load at a critical time in their hosts' lifecycles might provide control of the weed species, followed by the pathogen's return to endemic levels. This method differs from the classical biological control strategy which uses the one-time release of exotic pathogens or insects to attack pests. Because many of the species considered to be forest weeds are native and serve a useful role in the ecosystem, the release of an alien pathogen is not being considered for forestry use (Wall *et al.* 1992). A second consideration arguing against the introduction of a foreign pathogen is that many of the species considered to be weeds in one context (*e.g.* ROW's) can have commercial value in plantations (*e.g.* Aspen groves). Other requirements for a biological control for use in forestry include: proven efficacy at levels similar to chemical controls, specificity to target hosts in a particular area of use, ease and practicality of production and application, and cost competitiveness (Van Dyke 1989).

The advantages for the use of a naturally occurring biological control agent also include biodegradation of applied materials, and greater public acceptance as compared to the use of chemical herbicides (Auld 1990, Wall *et al.* 1992, Markin and Gardner 1993).

Chondrostereum purpureum as a mycoherbicide for forest weeds

Most weeds are hosts to many natural pathogens, certain of which may show potential as biological control agents. Studies by Dutch and Canadian groups indicated that the basidiomycete fungus *Chondrostereum purpureum* (Pers. ex Fr.) Pouzar, was a promising candidate for use as a biological control of many deciduous forest weeds (Scheepens and Hoogerbrugge 1989, deJong *et al.* 1990, 1991, Wall 1986, 1990, 1991, 1994, 1996, Wall *et al.* 1992). Host plants are usually infected through fresh wounds, cut stumps or stem lesions. The fungus grows through the xylem tissues of the infected plant, causing cambial necrosis, decay, sapwood staining and sometimes death of the host (Rayner 1977, Wall 1986, 1991).

Chondrostereum purpureum morphology and taxonomy

Chondrostereum purpureum (syn. *Stereum purpureum* (Pers:Fr)Fr.) (Pouzar 1959) is a basidiomycete classified in the genus *Stereum* (Peck) in the early 19th century due to macromorphological similarity (stereoid fruitbodies). The species was then removed to *Chondrostereum* by Pouzar (1959) because of differences in other characteristics of its basidioma. Morphological differences supporting the distinction of *C. purpureum* include the monomitic hyphal system in basidiocarps with clamp connections on generative hyphae, whereas *Stereum* basidiocarps are dimitic and are without clamp

connections (Stalpers 1978). Other characteristic features distinguishing the genus are its inamyloid basidiospores and the presence of large sac-like vesicles (gloeocystidia) in the sub-hymenial zone (Nakasone 1990). Young *C. purpureum* basidioma are often light purple and resupinate, while mature basidiocarps are generally in a reflexed form, reaching 2 to 10 cm across, and becoming browner and darker over time. The upper surface of the basidiocarp is usually hairy and zoned and tawny to brown color while the hymenium-covered lower surface is purple to lilac in fresh specimens. Basidiospores are smooth, hyaline and inamyloid (Hawksworth *et al.* 1995), and are produced under conditions of high humidity (Butler and Jones 1949).

There is a lack of consensus in the taxonomic placement of *C. purpureum*. The species has been variously considered to be placed in the order Agaricales, family Schizophyllaceae (NCBI taxonomy ID: 58368) (Benson *et al.* 2000, Wheeler *et al.* 2000), order Polyporales, family Meruliaceae (Kirk *et al.* 2001), order Stereales, family Meruliaceae (Hawksworth *et al.* 1995, Ainsworth and Bisby 1971), order Meruliales, family Meruliaceae (Ginns and Lefebvre 1993), order Aphyllophorales ("*Stereum purpureum*" Kendrick 1992, Stalpers 1978), family Corticiaceae (Nakasone 1990, Alexopoulis and Mims 1979, Ainsworth *et al.* 1973), family Stereaceae (Arora 1986), and order Aphyllophorales, family Meruliaceae (Welden 1971).

Wood that is infected with a fungus like *C. purpureum* often does not show basidiocarps; hence the identity of the responsible fungus might only be ascertained using pure cultures derived from the wood (Stalpers 1978). Basidiomycetes in general do not form

basidiocarps in pure culture. As the taxonomy of these fungi is based on characters of the basidiocarp, identification using conventional keys would be impossible and microscopic characters and enzyme tests must be used (Stalpers 1978).

Life history and mating system

In the lifecycle of *C. purpureum*, basidiospores germinate on their substrate, normally a freshly wounded tree or shrub, and haploid homokaryotic hyphae grow through the woody tissue and extend into the xylem (Rayner and Boddy 1988). *Chondrostereum purpureum* has a heterothallic, tetrapolar mating system (Wall *et al.* 1996a) which ensures outcrossing (*Stereum* is homothallic). When homokaryotic mycelia with the same vegetative compatibility groups and differing mating types come into contact, they may anastomose, forming a dikaryon (n+n, containing two types of unfused haploid nuclei per cell). The dikaryotic mycelia may form a basidiocarp on the bark surface of the host. Within the basidiocarp, the basidia are formed on the hymenium, wherein karyogamy and meiosis occur, producing four haploid basidiospore progeny spores, each containing a single nucleus.

Ecological role of C. purpureum

The ecological role of *C. purpureum* is as a pioneer wound parasite, colonizing freshly wounded trees or shrubs. Although *C. purpureum* grows rapidly, its rate of wood decay is slow compared to other wood-destroying basidiomycetes (Rayner and Boddy 1988 p269). *Chondrostereum purpureum* has been characterized as having hemibiotrophic behavior, infecting living tissue, eventually killing it, then continuing to grow on the dead

tissue in competition with other saprotrophs. This confers upon the fungus the advantage of primary occupation of the food resource at the time of host death. Most other basidiomycete parasites are perthotrophs, meaning they kill host material in advance of penetration. In a similar manner, *C. purpureum* could exist as a latent infection that might become active when host susceptibility increases or compartmentalization is overcome (Spiers and Hopcroft 1988). Wall (1991) observed that *C. purpureum* could survive for at least six years in sucessfully compartmentalized infected wood. Thus living trees may act as a reservoir for *C. purpureum*, providing the inoculum for further infection.

Host susceptibility to infection by *C. purpureum* appears to increase in the spring and summer (Spiers *et al.* 1998, Wall 1991, Dumas *et al.* 1997). As *C. purpureum* produces basidiocarps during periods of high humidity, it has been noted (Wall 1991) that with the tendency to fruit at times when tree susceptibility is low, and the ability of healthy trees to compartmentalize infection, *C. purpureum* under normal conditions may be a threat only to trees which are compromised by stress. This has been further supported by de Jong *et al.* (1996) who found that *C. purpureum* basidiocarps on live trees were always associated with injury.

Pathogenicity and virulence

Besides wood decay, infection by *C. purpureum* can also produce symptoms of foliar discolouration or silvering, and in fruit trees it is the cause of silver-leaf disease. The impact of silver-leaf disease on orchard trees (Setliff and Wade 1973), and hardwood

stoolbed nurseries (Spiers 1985) has been reported widely. Silvering of the leaves is the optical result produced when the polygalacturonase toxin (EndoPG), produced by *C. purpureum* causes the palisade mesophyll cells to separate from the epidermis and from each other (Peace 1962). EndoPG is the only toxin produced by *C. purpureum* that has been investigated with respect to virulence (Miyairi *et al.* 1985).

It has been shown that *C. purpureum* collected from one host may readily infect a different host (Bishop 1979). Different hosts, however, vary in their susceptibility to infection by *C. purpureum*. The effectiveness of *C. purpureum* as a mycoherbicide has been evaluated for the control of *Acer rubrum* (red maple), *A. saccharum* (sugar maple), *A. macrophyllum* (bigleaf maple), *Alnus rubra* (red alder), *Betula alleghaniensis* (yellow birch), *B. papyrifera* (paper birch), *Fagus grandifolia* (beech), *Populus tremuloides* (trembling aspen), *Prunus pensylvanica* (pin cherry), and *P. serotina* (black cherry) (deJong *et al.* 1990, Wall 1986, 1990, 1991, 1994, 1996, Wall *et al.* 1992). *Chondrostereum purpureum* has been tested as a control for *P. serotina*, a forest weed in the Netherlands, and formulations of the fungus are being developed for commercial use as a mycoherbicide (Scheepens and Hoogerbrugge 1989, deJong 1992). Red alder (*Alnus rubra*) was particularly susceptible to *C. purpureum* infection, while bigleaf maple, aspen and conifers were highly resistant (Wall 1996, Dumas *et al.* 1997).

Succession and competitive forces

As a pioneer invader of fresh wounds, *C. purpureum* is usually replaced, upon weakening the host, by other more competitive saprobic fungi such as *Trametes versicolor* and

Schizophyllum commune. As a result of this succession, *C. purpureum* is not thought to persist at an increased level following the local inundation of stumps within a field site (Rayner 1977, Wall 1997). The local incidence of *C. purpureum* basidiocarps has been observed to increase in relation to the background levels following biocontrol application, then to drop to endemic levels within three or four years (Wall 1997).

Population structure and gene flow

Chondrostereum purpureum has been found on many trees and shrubs throughout the temperate zones of the world, on all continents except Antarctica (Chamuris 1988, Peace 1962). Isolates collected from across Canada were surveyed for differences in temperature response, virulence, and protein profiles. There was some variability among isolates indicated by these data, but no geographical or host specialization was detected (Ekramoddulah *et al.* 1993). Studies of the *C. purpureum* mating system confirmed that it is an outcrossing species which is capable of maintaining unrestricted gene flow provided there are no natural barriers to spore movement (Wall *et al.* 1996a). Besides dissemination by short-lived wind-borne basidiospores, movement of this fungus has probably also occurred through human activities such as the transport of forest and nursery products (Ekramoddullah *et al.* 1993).

Molecular markers for C. purpureum

Biochemical methods have allowed grouping of *C. purpureum* individuals by comparison of their total protein profiles and isozyme analysis using SDS-PAGE methodology (Ekramoddulah *et al.* 1993, Shamoun and Wall 1996). North American isolates of

C. purpureum were distinguished from isolates from other continents, including Europe and New Zealand, by comparison of rDNA restriction fragment length polymorphisms (RFLPs) (Ramsfield *et al.* 1996).

Generation of DNA fingerprints by random amplification of polymorphic DNA (RAPD) (Williams *et al.* 1990) has been successfully used to identify isolates of many fungal species (Khush *et al.* 1992, Huff *et al.* 1994, Nicholson and Rezanoor 1994). This method utilizes the polymerase chain reaction (PCR), in which the target DNA is thermally denatured and then one or two short oligonucleotide primers are allowed to anneal and become extended by a thermostable DNA polymerase, and the cycle is repeated, producing fragments which are amplified exponentially. After 35-45 cycles, there is sufficient DNA to observe directly by agarose gel electrophoresis. The RAPD reaction uses genomic DNA as template, and uses random sequence oligonucleotide primers usually selected singly to amplify small fragments of DNA. Changes in sequence within these fragments will alter the pattern of DNA which can be visualized by gel electrophoresis. This technique relies on the inherent genetic variation in populations accumulated during the course of evolution and has been successfully utilized in a wide variety of organisms including fungi, for a number of uses including DNA fingerprinting, population studies, and gene mapping (Foster *et al.* 1993, Peever and Milgroom 1993, Milgroom and Lipari 1995, McDermott *et al.* 1994, Kelly *et al.* 1994).

DNA fragments amplified using random primers can be further used to develop primer pairs that specifically amplify selected fragments called sequence characterized amplified

regions (SCAR) (Paran and Michelmore 1993). For this technique, selected DNA fragments amplified in RAPD reactions are cloned into bacterial plasmid vectors. The sequences of 5' and 3' termini of each fragment are then determined and used to design PCR primers. These SCAR primers are about twice as long as standard RAPD primers, and are therefore less sensitive to varying reaction conditions. It was hypothesised that a set of standard SCAR markers, which would be more stringent and reproducible than RAPD itself, could be developed using informative RAPD markers for *C. purpureum* and used for screening large populations.

Risk: non-target infection

The risk of infection to non-target trees in the Netherlands was assessed by field experiments, surveys, and simulations of basidiospore dispersal using a Gaussian plume model (deJong *et al.* 1990). Commercial fruit orchards are high risk areas due to pruning practices which can provide entry points for the fungus. The added infection caused by inundative application of *C. purpureum* was concluded to be of the same order of magnitude as that from sporulation of naturally occurring basidiocarps and therefore did not significantly add to current risks of infection from natural sources of inoculum. Based on these results, the Plant Protection Service of the Netherlands concluded that the risk of use of *C. purpureum* as a biological control in Dutch forests is acceptable when applied at least 500m away from fruit orchards (deJong *et al.* 1990).

Objectives

The intention of this dissertation was to contribute to the basic understanding of the biology of this fungus and provide a foundation for further studies in the ecology and genetics of *Chondrostereum purpureum.* The overall long term objective of this research project was to gain a fundamental understanding of the systematics of this fungus and of the evolutionary forces responsible for its population structure and dynamics.

Specific objectives of this project may be grouped into four areas of research: (1) phylogenetic analyses of *C. purpureum* and allies, (2) development of molecular markers for diagnosis and identification of *C. purpureum* in infected wood, (3) investigation of the population genetics of *C. purpureum* along with an assessment of the persistence of *C. purpureum* used as a biological control, and (4) characterization of repetitive DNA fragments in *C. purpureum.*

Chondrostereum purpureum has been classified as a member of various taxonomic groups, including orders Aphyllophorales, Agaricales, Stereales, Meruliales, and Polyporales and families Stereaceae, Meruliaceae, Corticiaceae, and Schizophyllaceae (Kirk *et al.* 2001, Hawksworth *et al.* 1995, Ginns and Lefebvre, 1993, Stalpers 1978, Nakasone 1990, Welden 1971, Benson *et al.* 2000, Wheeler *et al.* 2000). An objective of this dissertation was to test these hypothesized classifications, and estimate the evolutionary history and relatedness among members of these taxonomic groups, using sequence comparisons.

A diagnostic marker was required in order to confirm the identity of *C. purpureum* in infected trees, wood samples and mycelial cultures. Futhermore, a reliable and stringent fingerprinting marker system was needed to genetically characterize individuals of *C. purpureum.* One aim of this study was to apply genetic markers to monitor *C. purpureum* infection in field trial experimental plots. To satisfy Koch's postulates for plant pathogens, *C. purpureum* cultures would be recovered from treated stumps having symptoms of *C. purpureum* infection and tested to confirm their source. It was hypothesized that an assessment of the frequency of recovery of *C. purpureum* from treated stumps would be indicative of biocontrol efficacy. In order to test this hypothesis, a comparison of infection frequency among different treated hosts and *C. purpureum* strains were related to the relative success of biocontrol in those treatments.

A concurrent objective of this research was to apply diagnostic markers, designed to identify *C. purpureum* and differentiate individuals, to assess the distribution of genetic variation in natural populations of *C. purpureum* and estimate the extent of gene flow and other evolutionary forces that may be responsible for its distribution and genetic variability. It was necessary to determine what natural barriers exist that limit dispersal and establishment of particular *C. purpureum* genotypes. It was hypothesised that spore trapping experiments would allow an assessment of the persistence and environmental fate of individual genotypes of released isolates. Based on preliminary observations of the persistence of basidiocarps following application of *C. purpureum* as a biocontrol, local incidence of the genotype of an applied isolate was expected to increase after application, then return to previous levels over a few seasons. This hypothesis was tested

on the operational scale of field trials. The sampling of biogeographic diversity and analysis of gene flow in natural populations has applications in phytopathology, conservation biology and many other biological fields. An objective of this study of genetic variability and population structure of *C. purpureum* was to provide a framework for understanding the dynamics of plant pathogen species, which can be exploited for use in biocontrol strategies as well as for plant disease control.

A number of polymorphic fragments, hypothesized to be repetitive DNA, were amplified from genomic DNA of *C. purpureum* using SCAR primers. As a final objective of this dissertation, the marker DNA would be characterized to test this hypothesis, using Southern hybridization analyses and sequence comparison with published sequences.

Chapter 2. Evolutionary relationships of '*Aphyllophorales*' inferred by phylogenetic analysis of class II chitin synthase gene fragments

ABSTRACT

Chitin synthase-encoding partial genes were compared in order to reconstruct evolutionary relationships among wood-rotting species of *Basidiomycetes* which have previously been classified as *Aphyllophorales*. Polymorphic banding patterns of PCR products resulted which were due to variation in the occurrence and length of three introns found only in *Basidiomycetes* and usually conserved within genera. Fragments were sequenced and derived class II chitin synthase amino acid sequences were aligned with homologous published sequences. Parsimony, distance and maximum likelihood criteria were used to estimate their phylogeny. All three approaches resulted in evolutionary hypotheses that confirmed phylum and class level groupings and paired species of the same genus. Most current order and family level groups based on taxonomic placement using traditional morphological characters were not well supported. Within *Basidiomycetes*, the monophyly of several groups was identified and well-supported including the '*Agaricales*', '*Phlebia*', '*Boletales*' and '*Russulales*' clades. Within the '*Agaricales*' clade, *Collybia* formed a strongly-supported terminal clade with *Hypholoma* species. There was moderate support for *Phlebia* grouped with *Phanerochaete,* also suggested by their sequence similarity which was in the same range as that within genera. The grouping of *Stereum* species within the *Russulales* clade was moderately supported. The removal of *Chondrostereum purpureum* from the genus *Stereum* was supported. There was no support for *Schizophyllum* grouped within

Meruliales. This study provides a framework for future studies of fungal taxa using chitin synthase genes and independent confirmation of evolutionary hypotheses based on ribosomal DNA sequences.

INTRODUCTION

The *Aphyllophorales* (*sensu* Donk 1964) are non-gilled fungi [*Basidiomycota: Basidiomycetes*] capable of utilizing wood as a nutrient source by means of enzymatic digestion of wood cell walls. This cosmopolitan group is a major component of terrestrial ecosystems and has been the subject of many ecological and industrial studies (*e.g.* Rayner and Boddy 1986, Barr and Aust 1994). The taxonomy of wood-inhabiting members of Basidiomycetes is in a dynamic state especially at order and family level and there is no consensus of classification. The Friesian system based on macromorphological characters, while holding the advantage of allowing species to be identified based on field characters, has been recognized as unnatural. Donk (1964) included anatomical and biochemical characters to divide the Friesian families of *Aphyllophorales* into smaller families. While former members share common physiological characters in mode of nutrition, the *Aphyllophorales* is no longer considered a taxonomic group and has been recognized for some time as a paraphyletic assemblage of fungi with tough, non-gilled perennial basidiomata. Molecular analyses have recently indicated that gills have been repeatedly derived from non-gilled forms (Hibbett *et al.* 1997). Phylogenetic studies of subgroups once classed as *Aphyllophorales* have been greatly needed but have been hampered by the lack of informative morphological characters. Morphology of the hymenophore can be quite variable and it

is not uncommon for one fungus to express different growth forms. Many of these fungi are considered corticioid for their resemblance to bark as fruitbodies may be formed appressed or attached to the surface of the substratum. Biologically, these fungi decompose wood and can often cause tree diseases. Some are aggressive pathogens but a great majority are saprophytic, producing both brown and white rot types. The mode of wood decay has been an important taxonomic character but, like other traditional characters such as mating type, brown rot is now thought to have evolved repeatedly (Hibbett and Donaghue 2001). Phylogenetic hypotheses based on molecular characters will allow further insight into the evolution of important morphological characters in these fungi such as hymenial configuration, mating type, wood decay biochemistry and pathogenicity factors.

Studies of the evolution of ribosomal gene sequences have helped to resolve many questions in fungal systematics and have greatly contributed to our understanding of the evolution of higher taxa (Bruns *et al.* 1991, Bruns *et al.* 1993, Swann and Taylor 1993, 1995, Tehler *et al.* 2000, Binder and Hibbett 2002). Phylogenetic comparisons of other genes, having greater or lesser conservation, should provide independent evidence with which to evaluate these hypotheses. Slowly evolving (conserved) molecules have been used to resolve deep branches in fungal phylogenetic tree topology (Kohn 1992, Berbee and Taylor 1993). Chitin, the β 1-4 polymer of N-acetylglucosamine, is an important structural component of all fungal cell walls. Chitin is not found in plants or bacteria, making chitin synthesis an attractive target for the development of anti-fungal compounds. The genes responsible for encoding chitin-UDP acetyl-glucosaminyl

transferase or chitin synthase (CHS) (E.C. 2.4.1.16) are highly conserved in fungi (Bowen *et al.* 1992) and have been cloned and characterized in a number of taxonomically diverse species. The deduced amino acid sequences fall into four classes (I, II, III, IV), which correspond to homologous chitin synthase zymogens which most likely represent different functional groups (Bowen *et al.* 1992, Mehmann *et al.* 1994).

Several phylogenetic studies have been based on analyses of CHS genes (Bowen *et al.* 1992, Chua *et al.* 1994, Hintz 1999, Miyazaki *et al.* 1993), including a study of the evolution of ectomycorrhizal fungi (Mehmann *et al.* 1994). The resolution provided by analysis of this highly conserved gene suggested that CHS might also provide insight towards resolving the evolutionary history and relatedness of wood-rotting species of *Basidiomycetes*. In this study, I used parsimony, distance, and maximum likelihood criteria to estimate the phylogeny of the class II CHS subunit from fungal taxa representing eight families of *Basidiomycetes* along with published sequences that included taxa from three more basidiomycete families.

MATERIALS AND METHODS

The species examined in this study and specimen accession numbers are listed in Table 1, with taxonomic designations based on Ginns and Lefebvre (1993), and Hawksworth *et al.* (1995). Genomic DNA was isolated from freeze-dried fungal cultures using a CTAB (hexadecyltrimethylammonium bromide) extraction (Möller *et al.* 1992). Chitin synthase gene fragments were amplified from genomic DNA using degenerate 27-mer primers (Bowen *et al.* 1992) which were

Table 1. Collection numbers of fungi studied, with GenBank accession numbers for class II chitin synthase partial gene sequences. Taxonomic designations are as in Hawksworth *et al.* (1995) if not noted.

Species	Culture no. / GenBank no.	Order	Family
Chondrostereum purpureum (Pers.) Pouzar 1959	[a] 2090 / AY138385	*Meruliales*[b] *Stereales*[c]	*Meruliaceae*[c]
Collybia butyracea (Fr.) Staude 1857	OKM-7607 / AY138386	*Agaricales*	*Tricholomataceae*
Corticium floridense (M.J. Larsen & Nakasone) M.J. Larsen 1990	HHB-9663 / AY138387	*Hericiales*[b] *Stereales*[c]	*Vuilleminiaceae*[b] *Meruliaceae*[c]
Hypholoma fasciculare (Huds.) Quél. 1871	OKM-2932 / AY138388	*Agaricales*	*Strophariaceae*
Hypholoma subviride (Berk. & M.A. Curtis) Dennis 1961	FP-102544 / AY138389	*Agaricales*	*Strophariaceae*
Phanerochaete chrysosporium Burds. 1974	ME-446 / AY138390	*Meruliales*[b] *Stereales*[c]	*Phanerochaeteaceae*[b] *Meruliaceae*[c]
Phlebia centrifuga P. Karst. 1881	RLG-7588 / AY138391	*Stereales*	*Meruliaceae*
Resinicium bicolor (Alb. & Schwein.) Parmasto 1968	HHB-10108 / AY138392	*Meruliales*[b] *Stereales*[c]	*Meruliaceae*[bc]
Schizophyllum commune (L.) Fr. 1815	ATCC26889 / AY138393	*Meruliales*[b] *Schizophyllales*[c]	*Schizophyllaceae*
Stereum hirsutum (Willd.) Gray 1938	ATCC13240 / AY138394	*Stereales*	*Stereaceae*
Stereum sanguinolentum (Alb. & Schwein.) Fr. 1838	ATCC12233 / AY138395	*Stereales*	*Stereaceae*
Trametes versicolor (L.) C.G. Loyd 1921	ATCC44677 / AY138396	*Poriales*	*Coriolaceae*

[a] The isolate of *Chondrostereum* has been described by Ramsfield *et al.* (1996). All other cultures were obtained from the USDA Forest Products Laboratory (Madison, WI), except for those preceded by ATCC which were from the American Type Culture Collection (Manassas, VA).

[b] Ginns and Lefebvre 1993

[c] Hawksworth *et al.* 1995

designed to add *Hind* III and *Xho* I restriction sites to the 5' and 3' ends of the amplified fragment. Amplification was carried out in a Stratagene Robocycler using Pharmacia *Taq* polymerase and buffer following conditions of Bowen *et al.* (1992). The most prominent amplification products from each species were excised from a 1% low melting temperature agarose gel after electrophoretic separation, extracted using the Promega Wizard System and ligated to pGEM-T (Promega) cloning vector. Competent SURE *E. coli* cells (Stratagene) were transformed with the ligation mixture according to the TA Cloning System procedure. Sequencing was performed on purified DNA of putative transformants by cycle sequencing of double-stranded products using fluorescent dideoxy-terminators with an ABI 373A automated sequencer according to the manufacturer's instructions (Applied Biosystems). Sequence was determined for both strands of dsDNA and compared.

The amino acid sequences corresponding to the amplified DNA sequences were predicted using the Generunner computer program. Assignment to CHS homologue class and determination of coding regions were based on consensus with published sequences. DNA and amino acid sequences of class II chitin synthases from other species were obtained from SWISS PROT, GenBank, and PIR databases through the NCBI service BLAST (Altschul *et al.* 1990, Benson *et al.* 2000, Wheeler *et al.* 2000). Class II CHS sequences were aligned using ClustalX version 1.81 (Thompson *et al.* 1997). For protein sequences, the gap opening penalty was increased to 35.00 to make gaps less frequent and the extension penalty was set to 0.75 to make gaps shorter. The Gonnet protein weight matrix was used to determine the similarity of non-identical amino acids.

Phylogenetic analyses using character state (maximum parsimony) and distance (neighbor-joining) approaches were performed using the computer program PAUP version 4.0b4a for Macintosh (Swofford 2000). The heuristic search option and the following settings were used: the starting tree was obtained by stepwise addition, simple addition sequence, branch swapping was by tree-bisection-reconnection, steepest descent was not in effect, and initial 'MaxTrees' setting equaled 100. Branches were collapsed if maximum branch length was zero. 'Multrees' option was in effect, and topological constraints were not enforced. Relative robustness of derived phylogenetic tree branches was estimated by bootstrap resampling. Phylogenetic analyses using the maximum likelihood approach were performed using Tree-Puzzle 5.0 (Strimmer and von Haeseler 1996), using the JTT model of substitution of amino acids (Jones *et al.* 1992). The number of puzzling steps used was 10,000. Estimations of support to each internal branch of the derived tree were computed by likelihood mapping. When an outgroup was assigned, the class III CHS sequence from *Agaricus bisporus* (GenBank AY138384) was defined as the outgroup for the class II sequences from all taxa and the *Aspergillus nidulans* CHS class II sequence (M82941.1) was used as outgroup for sequence comparisons within the *Basidiomycota*.

RESULTS

Sequence analysis of PCR-amplified fragments

Amplification of genomic DNA of selected fungi (Table 1) with the degenerate CHS primers (Bowen *et al.* 1992) yielded polymorphic PCR products in the expected size range of approximately 550 to 800 bp. The most prominent products of each reaction

were cloned and DNA sequence determined. All sequences analysed in this study were deposited in GenBank (Benson *et al.* 2000, http://www.ncbi.nlm.nih.gov/Entrez) and their accession numbers are given in Table 1. Chitin synthase homologues were identified by multiple sequence alignment analysis. As previously observed (Bowen *et al.* 1992, Mehmann *et al.* 1994), class II CHS sequences were most often recovered and these were chosen for phylogenetic comparison. Alignment of *Basidiomycete* sequences required no gaps (Figure 1).

Comparison of intron presence and position

Published gene fragments and translated sequences were compared to identify intervening sequences of 48 to 88 bp in most of the class II CHS sequences from basidiomycetes (Figure 2). Exon/intron splice junction sequences followed a general pattern: GTNNNN.....C/TAG. When present, the number of introns varied among taxa but their positions in the class II gene fragment were conserved and are referred to as a, b and c. The corresponding three positions in the CHS class II DNA sequence were: a) 202, b) 257, and c) 560 (Figure 2). Three basidiomycetes had no introns within the class II CHS fragment: *Collybia butyraceae*, and the two *Hypholoma* species. Sequences with introns in all three positions were found in the majority of taxa, including *Phlebia, Phanerochaete, Trametes, Resinicium, Schizophyllum,* and *Laccaria*. Sequences with both a and b introns were found in *Chondrostereum, Cortinarius,* and the two bolete species, *Boletus* and *Xerocomus*. *Corticium* was the only taxon with a and c introns, and *Stereum sanguinolentum, Russula,* and *Lactarius* had b and c introns. Both species of

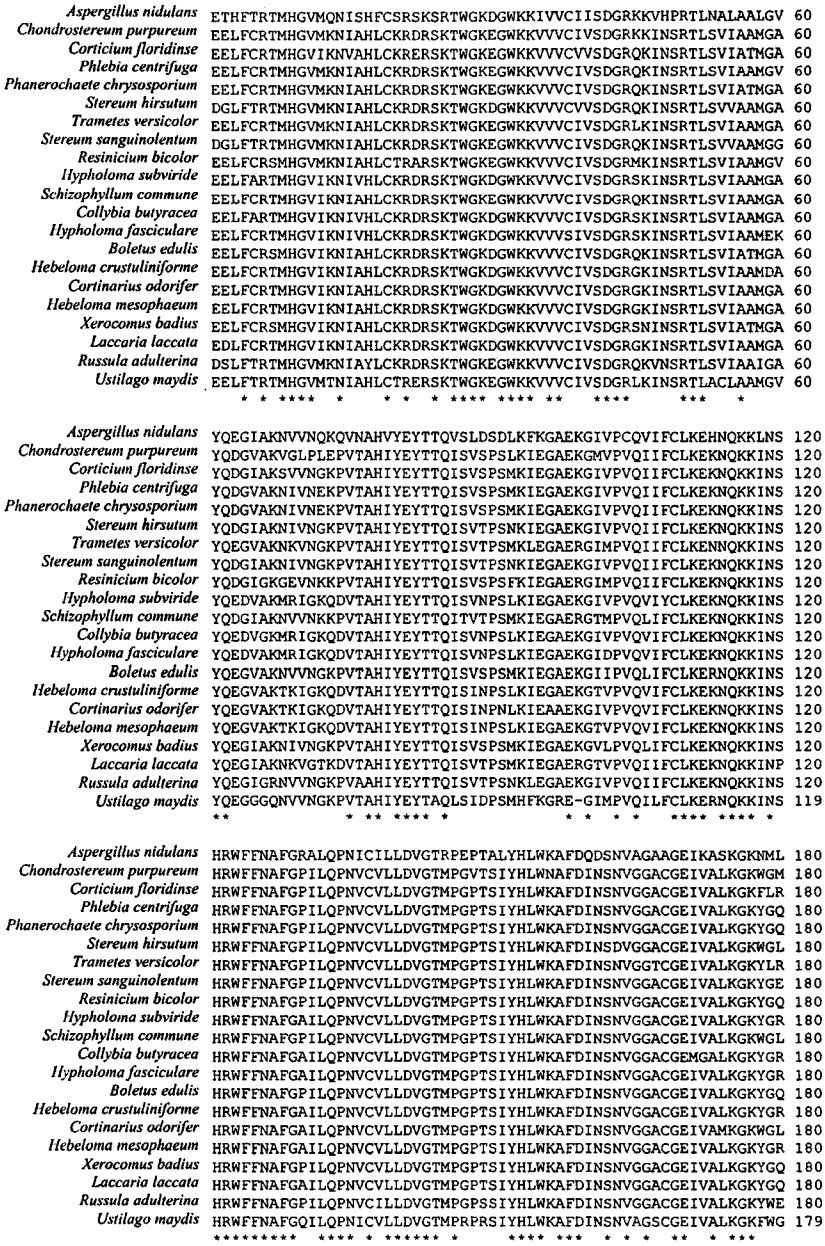

Figure 1. Alignment of *Basidiomycota* sequences. Multiple sequence alignment derived using Clustal X (Thompson *et al.* 1997) using deduced amino acid sequences of class II chitin synthase encoding gene fragments from basidiomycete taxa. The symbol '*' indicates fully conserved residues. Sequence accession numbers of class II chitin synthase-encoding sequences from *Basidiomycota* analyzed here are given in Table 1 (taxa from this study) and Figure 4 (published sequences).

Aspergillus nidulans	GLLNPLVAS	189
Chondrostereum purpureum	NLLNPLVAA	189
Corticium floridinse	NLLNPLVAA	189
Phlebia centrifuga	NLINPLVAA	189
Phanerochaete chrysosporium	NLINPLVAA	189
Stereum hirsutum	KLLNPLVAA	189
Trametes versicolor	NLINPLVAA	189
Stereum sanguinolentum	KLLNPLVAA	189
Resinicium bicolor	ALLNPLVAA	189
Hypholoma subviride	NLLNPLVAA	189
Schizophyllum commune	NLLNPLVAA	189
Collybia butyracea	NLLNPLVAA	189
Hypholoma fasciculare	NLLNPLVAA	189
Boletus edulis	YLLNPLVAA	189
Hebeloma crustuliniforme	NLINPLVAA	189
Cortinarius odorifer	NLLNPLVAA	189
Hebeloma mesophaeum	NLINPLVAA	189
Xerocomus badius	YLLNPLVAA	189
Laccaria laccata	NLINPLVAA	189
Russula adulterina	KLLNPLVAA	189
Ustilago maydis	ALLNPLVAA	188
	* *****	

Figure 1 (cont.). Alignment of *Basidiomycota* sequences.

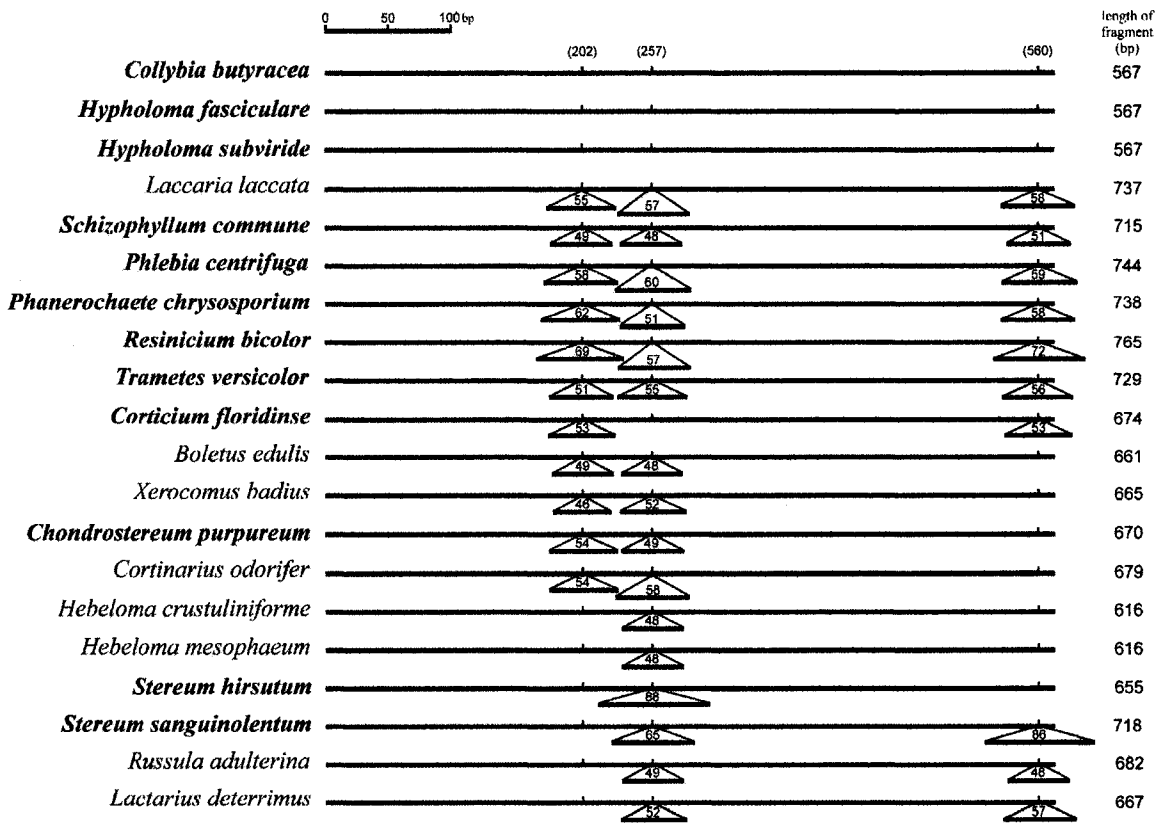

Figure 2. Structure of partial class II chitin synthase-encoding genes from Basidiomycetes. Taxa are shown within groups of similar intron presence. Triangles represent intron sequences with lengths of introns (bp) given within. Fragment lengths represent the products of PCR amplification of partial class II chitin synthase genes using degenerate primers (Bowen et al. 1992).

Hebeloma, and *S. hirsutum* had only the b intron. The presence and position of introns were usually conserved within terminal groups with the exception of the *Stereum* species, in which intron c was variably present (Figure 2).

Variability of nucleotide sequences

A similarity matrix was computed in PAUP using class II chitin synthase-encoding DNA sequences with intron sequences removed as well as the derived amino acid sequences (Figure 3). Identity between DNA sequences from the same genus ranged from 87 to 96%: *Stereum* were 87.1% identical, *Hypholoma* 94.4% and *Hebeloma* sequences (not shown) were 95.8% identical. DNA sequences from three other pairs of taxa considered to be in the same family or order were also within this range (88-91%) of similarity including members of *Agaricales*, *Hypholoma subviride* and *Collybia* (89.9%), *Hypholoma fasciculare* and *Collybia* (90.7%) and the boletes, *Boletus* and *Xerocomus* (88.5%) (not shown). Identity among derived amino acid sequences within genera ranged from 96.8% between *Stereum* sequences and 96.8% between *Hypholoma* (Figure 3), to 99.5% identity between *Hebeloma* sequences (not shown). There was 96.3% sequence identity between the two members of *Boletaceae* (not shown) and 98.9% identity between *Phlebia* and *Phanerochaete* sequences (Figure 3). Amino acid sequences from *Basidiomycota* were 65 to 69% identical to those of *Ascomycota* and 68 to 75% identical to those of *Zygomycota*. The amino acid sequence of the outgroup, *Agaricus* class III CHS, was 44 to 54% identical to the class II sequences (data not shown).

	C. purpureum	*C. butyracea*	*C. floridinse*	*H. fasiculare*	*H. subviride*	*P. chrysosporium*	*P. centrifuga*	*R. bicolor*	*S. commune*	*S. hirsutum*	*S. sanguinolentum*	*T. versicolor*	*A. nidulans*	*R. oligosporus*
Chondrostereum purpureum	-	86.8	89.4	86.8	87.8	92.1	92.1	88.4	89.9	88.4	88.9	88.4	65.6	72.5
Collybia butyracea	74.8	-	85.7	95.8	97.9	87.3	87.3	85.2	86.2	84.7	85.2	85.7	66.1	68.8
Corticium floridinse	77.1	75.8	-	85.7	86.8	93.7	92.6	89.4	92.1	90.5	90.5	90.5	66.7	73.5
Hypholoma fasciculare	73.0	90.7	75.5	-	96.8	87.3	87.8	85.2	86.8	84.1	85.2	86.2	65.6	68.8
Hypholoma subviride	72.7	89.9	74.1	94.4	-	88.4	88.4	85.2	87.3	85.7	86.2	86.8	66.7	69.3
Phanerochaete chrysosporium	75.1	79.9	83.8	78.7	77.8	-	98.9	91.5	92.6	91.5	93.1	93.1	67.2	74.6
Phlebia centrifuga	72.5	73.9	79.7	75.3	73.4	81.7	-	92.6	92.6	91.5	93.7	93.1	68.3	75.7
Resinicium bicolor	73.9	71.6	76.0	70.5	70.0	75.3	76.4	-	91.0	87.8	89.9	89.4	66.7	72.5
Schizophyllum commune	73.0	75.7	80.1	76.0	75.5	79.7	79.0	72.8	-	91.0	91.0	91.5	67.2	73.5
Stereum hirsutum	75.8	73.5	78.1	72.7	73.2	79.4	77.4	81.3	75.5	-	96.8	88.4	68.8	73.0
Stereum sanguinolentum	75.5	74.6	79.5	73.7	74.1	79.2	77.4	82.2	77.6	87.1	-	89.9	68.8	74.6
Trametes versicolor	75.3	76.7	81.7	76.5	75.8	84.5	80.6	73.5	79.4	76.7	75.1	-	66.7	73.0
Aspergillus nidulans P30584	62.3	63.8	63.5	63.5	64.6	64.9	63.8	64.4	63.3	66.1	66.7	63.1	-	66.1
Rhizopus oligosporus P30595	68.2	62.9	64.3	64.7	64.7	64.8	64.5	61.8	64.8	63.1	64.5	64.1	62.2	-

Figure 3. Similarity matrix of class II CHS sequences. Percent amino acid sequence identity is shown above the diagonal, and identity of DNA sequences with intron sequences removed is below. Similarity of sequences from basidiomycete taxa from this study to those from phyla *Ascomycota* and *Zygomycota* is shown using examples of *Aspergillus nidulans* and *Rhizopus oligosporus*, respectively.

Phylogenetic analyses of class II CHS sequences

The maximum parsimony approach

The derived class II CHS amino acid sequences were used in phylogenetic analyses. One thousand bootstrap resamplings were performed to evaluate support as a percentage of replicates that supported a given branch in a phylogenetic tree. Of the 199 total derived amino acid characters of equal weight from 43 taxa including outgroup (*Agaricus bisporus* CHS class III), 35 were constant, 36 were variable and uninformative and 128 were parsimony-informative (*i.e.* they contained variant states shared by two or more taxa) and these were used for analysis. The maximum parsimony approach produced nine trees which generally grouped major branches at phylum and class levels (Figure 4). A strongly supported (100 bootstrap replicates) terminal clade, *Mucor circinelloides* and *Rhizopus stolonifer*, was a sister group to the weakly supported (74) clade of other *Zygomycota* taxa. Within a moderately supported branch of *Ascomycota* taxa (86), a clade of nine species was strongly supported (99) which included *Aspergillus nidulans*, *A. niger*, *Phaeococcomyces exophialae, Exophiala jeanselmei, Xylohypha bantiana*, *E. dermatididis, Ajellomyces capsulatus*, *A. dermatitidis* and *Elaphomycetes muricatus*. The yeast species *Candida albicans* and *C. maltosa* were strongly supported (100), within a clade that included *Saccharomyces cerevisiae* (86). The branch composed of species of *Basidiomycota* was well-supported (98) with *Ustilago maydis* (*Ustilaginomycetes*) included, but the support of the clade of *Basidiomycete* taxa within this group was weaker (82). Within the *Basidiomycete* clade, seven members of *Agaricales* were grouped (91): two species of *Hypholoma*, *Collybia*, *Cortinarius*, two *Hebeloma* species, and *Laccaria*,

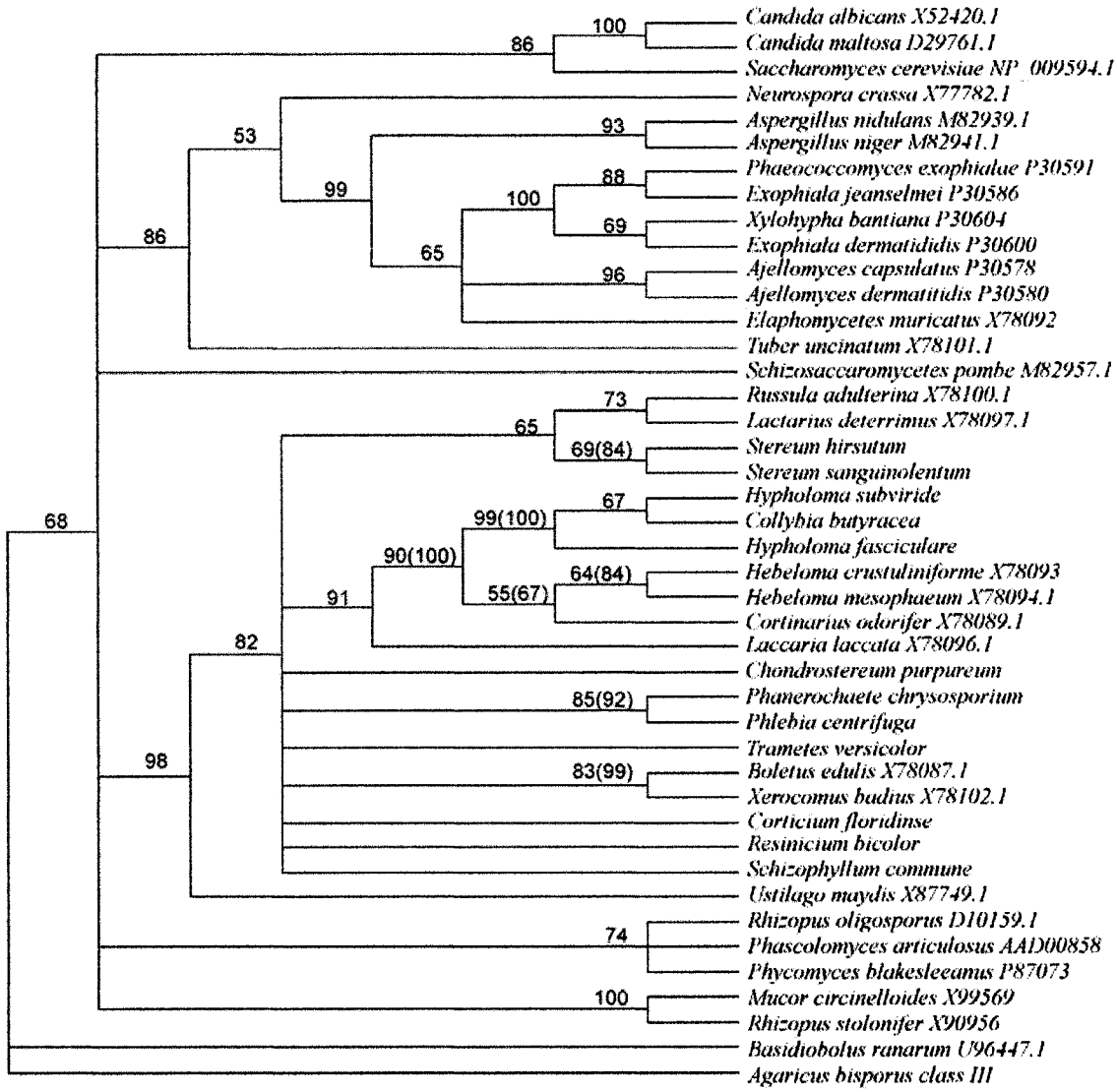

Figure 4. Maximum parsimony 50% majority-rule consensus tree.
A consensus of nine phylogenetic trees of equal length produced by comparison of derived amino acid sequence of the chitin synthase gene fragment encoding the class II zymogen using maximum parsimony criteria. Support of nodes as a percentage of 1000 bootstrap resamplings are given. Bootstrap values in brackets are the results of PAUP analysis of a smaller subset of taxa (Basidiomycota) which generally produced a similar tree topology as the all-inclusive analyses, but provided higher support values for terminal clades. Sequence accession numbers are given for published sequences.

and within this clade there was very strong support of the terminal clade containing the two *Hypholomas* and *Collybia* (99). There was weak-to-moderate support (Hillis and Bull 1993) for the pairing of the two *Russulales* (73), two species of *Stereum* (69), *Phlebia* with *Phanerochaete* (85), and two *Boletales* (83) (Figure 4).

In the subset of CHS class II sequences from 20 *Basidiomycota* taxa plus the outgroup *Aspergillus nidulans*, of 189 total derived amino acid characters of equal weight, 97 were constant, 52 were variable (parsimony-uninformative) and 40 were parsimony-informative and used for analysis. Using the heuristic search option with the optimality criteria of maximum parsimony, 28 trees with a score of 199 were retained. A bootstrap resampling of these data was performed with 1000 replicates using maximum parsimony optimality criteria. In the 50% majority consensus tree, a bootstrap value of 94 highly supported the branch that grouped *Hypholoma* species, *Collybia*, *Hebeloma* species, and *Cortinarius* (Figure 4). Within this group, the terminal clade containing *Hypholoma subviride*, *Collybia*, and *Hypholoma fasiculare* was strongly supported (99). There was moderate support (70) for *Laccaria* clustered basal to the *Agaricales* clade. The *Boletales* species *Xerocomus* and *Boletus* formed a well-supported clade with a bootstrap value of 93. Moderate support (89) was provided for the *Phlebia* with *Phanerochaete* grouping and weaker support (72) united the two *Stereum* species. The taxa *Chondrostereum*, *Resinicium*, *Trametes*, *Corticium*, and *Schizophyllum* remained unresolved in the consensus and formed a polytomy of sister groups to the *Agaricales*, *Boletales*, *Russulales-Stereum* and *Phlebia-Phanerochaete* groups (Figure 4).

Neighbor-joining

Neighbor-joining analysis of all sequence data grouped major branches of *Ascomycota* (99% of bootstraps) and *Basidiomycota* (100) and two groups of *Zygomycota*: *Rhizopus, Phascolomyces* and *Phycomyces* (96), and *Rhizomucor* with *Rhizopus* (100) (Figure 5). Within the *Basidiomycetes* (98) clade, monophyly of *Agaricales* taxa was strongly supported (98). This group contained well-supported subgroups of *Hypholoma* spp. (89) together with *Collybia* (99) and *Hebeloma* spp. (99) with *Cortinarius* (86). The two *Russulales* taxa (80) were grouped together with weak support (50) together with the two *Stereum* species (95). Other well-supported terminal nodes were the pairing of *Phlebia* with *Phanerochaete* (100) and the two *Boletales* (97) (Figure 5).

Maximum likelihood analyses

Within the larger set of 42 class II CHS sequences compared using the maximum likelihood approach, 27 of 199 amino acid residues (13.6%) were constant and the average distance over all taxa was 42%. Of 123,410 quartets analyzed, 6,037 (4.9%) were unresolved in the tree. The quartet puzzling tree grouped the major branches corresponding to taxonomic phyla with generally high support values, shown in brackets: *Zygomycota* (86), *Ascomycota* (96), and *Basidiomycota* (98) (Figure 6). There was very high support for internal nodes within each group. The zygomycetes *Rhizopus* and *Phascolomyces* comprised a highly supported (97) clade and there was very strong support (99) for the monophyly of the *Aspergillus* clade. Within the *Basidiomycota* clade, the subgroup corresponding to *Basidiomycetes* that excluded *Ustilago*

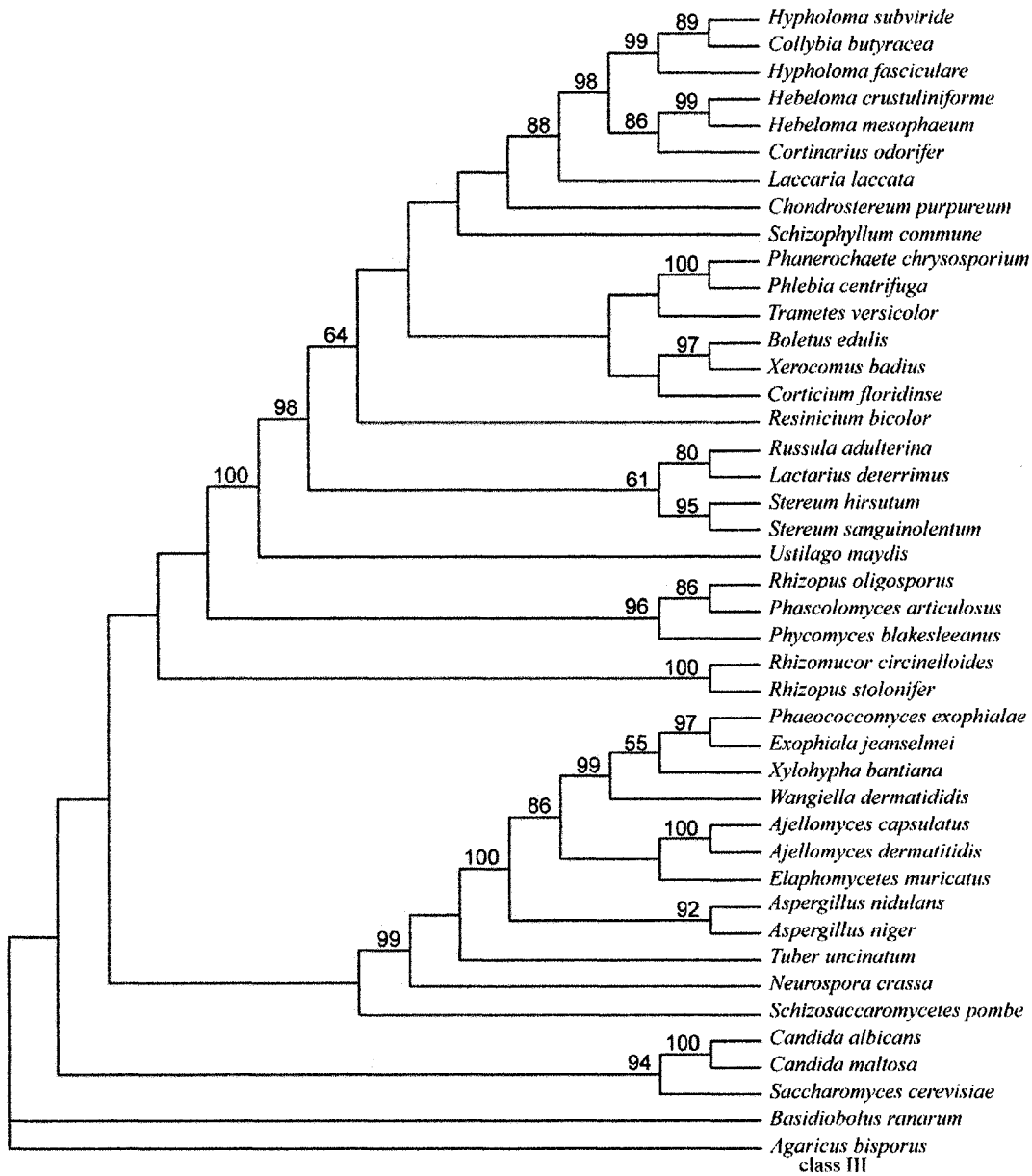

Figure 5. Neighbor joining tree produced using PAUP. Bootstrap resampling values indicate support for clades as a percentage of 1000 data resamplings.

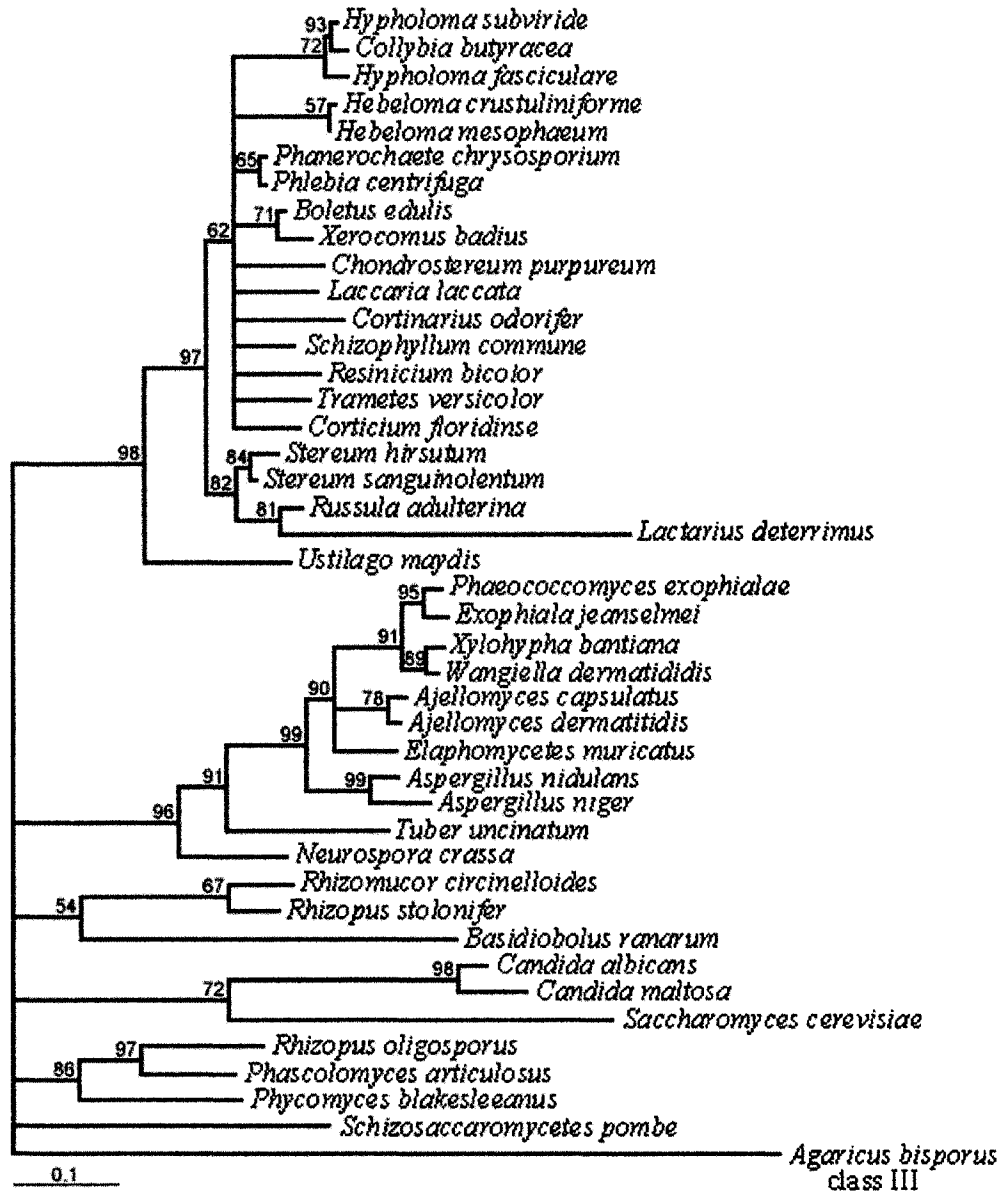

Figure 6. Maximum likelihood tree produced using Puzzle. Quartet puzzling values indicate support for clades as a percentage of resampling data 1000 times. Scale represents maximum likelihood branch length.

(*Ustilaginomycetes*) was strongly supported (97). Within this clade, there was moderately high support (82) of the clade that grouped the two *Stereum* taxa with the two members of *Russulaceae* (Figure 6). Maximum likelihood analysis using a subset of taxa (*Basidiomycota* plus *Aspergillus nidulans*) yielded similar tree topology but again gave higher support values for terminal nodes in this subset. The 'agaricales' subgroup was weakly (71) supported but contained the highly supported internal clade (96) of *Hypholoma* species and *Collybia*. Sister clades to the 'agaricales' were the bolete group which was strongly supported (97), *Phlebia* and *Phanerochaete* (72), and the *Stereum* species (57). The russula *Lactarius deterrimus* showed very long branch length in phylograms so was removed from some analyses to avoid the affects of long-branch attraction (Felsenstein 1978) or in case of non-homology. The remaining topologies were unchanged, however, upon removal of this taxon.

DISCUSSION

The analysis of chitin synthase genes has provided a useful tool for determining phylogenetic relationships in fungi (Bowen *et al.* 1992, Chua *et al.* 1994, Mehmann *et al.* 1994, Hintz 1999). The known chitin synthase gene family sequences are grouped into four classes (Mehmann *et al.* 1994), corresponding to chitin synthase enzyme homologues that appear to have differing functions in fungal chitin synthesis (Bulawa 1993). As previously observed, class II CHS sequences were most often recovered from PCR amplification of genomic DNA with degenerate primers (Bowen *et al.* 1992, Mehmann *et al.* 1994). Sequence alignment revealed highly conserved regions, which may represent functionally important regions such as binding sites of enzyme substrates.

Few sequence differences occurred within groups, while variation among groups was moderate to large. The relative divergence within the region of sequence studied ranged from 4-13% among DNAs from the same genus (which corresponded to a 0.5-3% difference in amino acid sequence) (Figure 3) to 38% divergence in DNA sequences from members of different taxonomic class. This resolution corresponded to that found within ITS sequences compared among similar groups of taxa (Boidin *et al.* 1998). Deviations from the expected range of variation among members of a single genus can give clues to reclassification of problematic groups. Sequences of *Stereum* species were more divergent than expected as DNA sequences were only 87% identical to one another, while *Hypholoma* species were 94.4% identical and *Collybia* sequence was 90% identitical to both *Hypholoma* species. Conversely, there was less divergence than expected between *Phlebia* and *Phanerochaete* (98.9% amino acid identity) (Figure 3), which may be indicative of problems in current taxonomic designations. It is likely that the third position (wobble bases) in codons have become randomized in protein coding sequences that have been diverged for a long time. Hence it was assumed that the 'signal-to-noise' ratio among distant taxa was improved by using derived protein sequences over DNA sequences.

Intervening sequences were present only in the CHS class II partial gene of taxa from *Basidiomycetes*. The intron splicing sites followed a general pattern (GTNNNN....C/TAG), but were somewhat different from the conserved sequence reported previously in filamentous fungal introns (GTANGT....C/TAG) (Gurr *et al.* 1987), reflecting the wide range of acceptable sequence for splice sites. Intron presence

and position were usually conserved in terminal clades, providing support for their members' shared evolutionary history. For example, the *Hebeloma* species both contained just the b intron, the two boletes had a and b introns and the two russulas had both the b and c introns. The only exception was *Stereum*, which varied in presence of the c intron (the *S. hirsutum* sequence had intron b and that of *S. sanguinolentum* had introns b and c). Since the positions of the three intron sites were conserved throughout disparate fungal taxa, the hypothesis of loss of introns from a common ancestor with all three introns in the class II CHS sequence is more likely than the gain of intron sites in descendants. A quick identification methodology could be developed which exploits the size polymorphisms in PCR-amplified CHS fragment patterns caused by the variation in presence and position of introns (Mehmann *et al.* 1994) (Figure 2).

Comparison of phylogenetic methods

The consistency of the various methods of reconstructing phylogenies based on CHS sequences depended on many factors including the topology and branch lengths of the true tree and the variability of the substitution rates. The maximum parsimony algorithm was used to search for the minimum number of genetic events (amino acid changes) required to transform a sequence to its nearest neighbor. The most parsimonious tree, or that which needed the fewest changes, was inferred from the set of aligned sequences. This led to many "equally most parsimonious trees" which made it difficult to justify the choice of a particular tree hypothesis. Other negative points were that the parsimony algorithm did not provide information on branch lengths, and was sensitive to codon bias and unequal rates of evolution (Swofford 2000). Information may have been lost as the

maximum parsimony method only uses informative sites that contain variant states that are shared by two or more taxa; thus this method may be less efficient than distance methods when the number of relevant sites is not large (Saitou and Nei 1987). Distance methods, like neighbor joining, convert sequence data into a matrix of pairwise distance values, then fit a tree to this matrix using cluster analysis. In this method, some loss of information occurs as sequences are only indirectly compared, but neighbor joining is considered to be appropriate for use with recently divergent sequences having high similarity scores as is the case among the highly conserved CHS sequences (Saitou and Nei 1987). In the maximum likelihood method, all sequences are considered separately at each site and the probability (log-likelihood) of the occurrence of a particular residue is calculated, given a hypothetical topology. This procedure is repeated for all possible topologies and that which shows the highest likelihood is chosen as the final tree. Unlike parsimony and distance methods, the maximum likelihood method uses all the information available in the sequences and seems to be robust and relatively insensitive to unequal rates of evolution or nucleotide bias (Strimmer and von Haeseler 1996).

Evolutionary hypotheses

Assuming that all of these approaches are equally valid, it can be expected that the different methods will show the same phylogenetic tree provided that the sequences have a strong phylogenetic relationship. The overall topology of derived trees from character state (maximum parsimony), distance (neighbor-joining) and maximum likelihood methods was indeed similar. All three approaches resulted in tree topology that confirmed phylum-level groupings (*Zygomycota, Ascomycota,* and *Basidiomycota*) and

paired species of the same genus, but generally provided weak or no support for most current order and family level groupings with a few exceptions. Several groups within the strongly-supported (98%) *Basidiomycetes* clade were identified that were supported by bootstrapping or maximum likelihood analyses and are referred to here as the 'agaricales', 'phlebia', 'bolete', and 'russuloid' clades. Generally, analyses using the subset of taxa (*Basidiomycota*) produced a similar tree topology to the all-inclusive analyses, but provided higher support values for terminal clades. These results provided independent molecular support for many well defined groups of fungi. Within the *Basidiomycetes*, eight major monophyletic groups have recently been proposed based on rDNA analysis: the polyporoid, euagarics, bolete, thelephoroid, russuloid, hymenochaetoid, canthareollid, and the gomphoid-phalloid clades (Hibbett and Thorn 2001).

The '*Agaricales*' clade was strongly-supported (98) as monophyletic group of taxa, and included *Hypholoma*, *Collybia*, *Hebeloma*, and *Cortinarius*. This grouping supports Ginns and Lefebvre's (1993) classification scheme over that of Hawksworth *et al.* (1995), in which *Hebeloma* and *Cortinarius* are placed in *Cortinariales*. I hypothesize that this group corresponds to the euagarics clade of Hibbett *et al.* (1997), which includes many *Agaricales* (gilled mushrooms) and former *Aphyllophorales*, as well as other 'non-agaric' fungi (puffballs and coral mushrooms). There was moderate support (80%) of *Laccaria* as an 'agaricales', which would support its current taxonomic designation (Hawksworth *et al.* 1995). As *Laccaria* and *Collybia* are both members of *Tricholomataceae*, I would have expected them to pair together and remain separate from the *Hypholoma* species

which are members of *Strophariaceae*. In this analysis however, *Collybia* is strongly supported (99% bootstrap value) as a member of a terminal clade together with *Hypholoma* species, suggesting that *Tricholomataceae* is not monophyletic or that the taxonomic designation of *Collybia* is not accurate.

The pairing of *Phlebia* with *Phanerochaete* was moderately supported (85% bootstrap, 72% maximum likelihood value, ML) by the different methods of analysis. Alternative taxonomic designations are given for *Phanerochaete*, in the order *Stereales* (Hawksworth *et al.* 1995) or *Meruliales* (Ginns and Lefebvre 1993) and *Phlebia* is referred to as a member of *Stereales* in the former scheme. In the absence of other information, I hypothesize that *Phanerochaete* be given the designation of Hawksworth *et al.* (1995) so as to group it with *Phlebia*. Hibbett and Thorn (2001) also placed *Phanerochaete* and *Phlebia* together in a monophyletic subgroup ('Phlebia clade') within their 'polyporoid' clade. The pairing of members of *Russulales* (81% ML) and of *Boletales* (97% ML), confirmed the current evolutionary hypotheses supporting these clades (Bruns *et al.* 1993). These groups are thought to be monophyletic based on rDNA analysis and comprise two ('bolete' and 'russuloid') of the proposed eight major clades of homobasidiomycetes (Hibbett and Donaghue 2001). The maximum likelihood and distance methods (neighbor-joining, NJ) grouped the two *Stereum* species together with the two russulas, (83% ML, 61% NJ). The grouping of *Stereum* in a monophyletic clade with russulas is supported by rDNA analyses (Hibbett and Donaghue 1995, Hibbett *et al.* 1997) which placed *Stereum* as well as other former *Aphyllophorales* in a 'russuloid' clade. The absence of intron 'a' from all taxa in this terminal clade also supports their

shared evolutionary history and close relationship. Support was provided for the removal of *Chondrostereum purpureum* from the genus *Stereum* (Pouzar 1959), as the lack of clustering of these taxa implied a more distant relationship. Further, this species did not group with any taxon considered *Stereales* and remained unresolved with respect to known groups. Similarly, *Schizophyllum* did not group with other taxa considered to be *Meruliales* (Ginns and Lefebvre 1993); hence its placement in *Schizophyllales* (Hawksworth *et al.* 1995) may be supported over that scheme. The family *Schizophyllaceae* was supported as a monophyletic group by Parmasto (1995) and was considered synonymous with *Meruliaceae*. The inclusion of more taxa from alternative groups should allow progress towards plausible phylogenetic hypotheses for both *Chondrostereum* and *Schizophyllum*. The taxonomic designation of *Corticium* as a member of *Stereales* (Hawksworth *et al.* 1995) was not supported in this study, as *C. floridinse* did not group with other taxa considered to be *Stereales*. An alternative placement in the order *Hericiales* (Ginns and Lefebvre 1993) can not be verified using available taxa. Recent phylogenetic analysis (Wu *et al.* 2001) has delineated a monophyletic group that may be classified as *Stereaceae*, and it is noted that "*Corticium* ... is not in this group, thus *Stereum* is not synonymous with Corticiaceae". Likewise, *Resinicium* did not cluster with any other taxon, so its alternative relationship with either *Stereales* or *Meruliales* was not resolved. The taxonomic groups *Aphyllophorales*, *Meruliales* as well as *Stereales* were not supported in this study. These groups appeared to be polyphyletic assemblages, which require further studies using more taxa in order to resolve their evolutionary histories with respect to one another.

It is often difficult to assess the state of fungal taxonomy as overviews of morphologically defined groups (monographs) are usually published separately (*e.g.* Gilbertson 1980, Gilbertson and Ryvarden 1986, Nakasone 1990, Ginns and Lefebvre 1993). Taxa for this study were mostly chosen from *Stereales* but their alternative taxonomic designations confused selection criteria. Supraspecific groupings (*e.g.* family, order) are contrived but the goal of their use should be to contribute some clarification to taxonomic groups that reflects their shared evolutionary history (Hibbett and Donoghue 1998). Phylogenetic trees aim at reconstructing the history of successive divergences which took place during the evolution between the considered sequences and their common ancestor. Recently, consideration has been given to replacing the current hierarchical (Linnaean) ranking system of taxonomy to one that reflects the evolutionary hypotheses represented in phylogenetic trees (Bryant and Cantino 2002).

These analyses placed known members of deeper branches (*Ascomycota*, *Basidiomycota*, *Basidiomycetes* and 'agaricales') with high confidence (>95% bootstrap) while phylogenetic resolution was low (<70%) in many terminal nodes. While none of the methods used here may guarantee the one tree true will be found, the hypothesis can be considered reliable when the widely different methods provide similar tree topologies. It was not possible using available data to resolve the order in which most species descended from a common ancestor, producing multifurcating nodes (polytomy) in the hypothetical phylogenetic tree. Smaller numbers of taxa and/or amount of sequence data will introduce sampling biases. Although sampling of fungal taxa was limited in this

study, the phylogenetic relationships inferred generally mirror other hypotheses of relationship, especially those based on molecular phylogenetic analyses.

Chapter 3. PCR-based genetic markers for detection and infection frequency analysis of the biocontrol fungus *Chondrostereum purpureum* on Sitka alder and trembling aspen. 1999. Biological Control 15: 71-80.

ABSTRACT

Diagnostic molecular genetic markers were developed to estimate the infection frequency following the application of the biocontrol fungus *Chondrostereum purpureum* on two target weed species in a field trial in British Columbia. Sitka alder (*Alnus sinuata*) and trembling aspen (*Populus tremuloides*) stump sections were sampled four months after treatment with the biocontrol fungus, chemical herbicide, or blank controls, and assayed for the presence of *C. purpureum*. A *Chondrostereum*-specific PCR primer pair, designed to amplify the intergenic region of ribosomal DNA (rDNA), allowed the detection of *C. purpureum* while a second sequence-characterized, amplified region (SCAR) marker was developed to identify the specific *C. purpureum* genotypes. Significantly, the biocontrol isolates were recovered only from stumps to which they were applied, suggesting that topical application of *C. purpureum* is highly target-specific. The absence of secondary infection on control stumps by biocontrol isolates of *C. purpureum* indicated that nontarget infection was absent. There was a significant difference in the infection frequencies of the two target weeds. Approximately 90% of biocontrol-treated Sitka alder stems and 40% of trembling aspen stems were successfully infected by *C. purpureum*. It is expected that this methodology will provide an early indication of success of biocontrol using *C. purpureum*.

INTRODUCTION

Productivity of conifer plantations is often reduced by opportunistic deciduous tree species which out-compete planted tree species for resources such as light, water, and nutrients. Suppression of competing vegetation can stimulate survival and growth of the conifers by channeling resources into the crop trees. Hardwood species are thus considered forest weeds in conifer reforestation sites and some form of selective vegetation management is required. These same hardwood species can also be a problem in utility rights-of-way, where tall-growing trees can interfere with power and telephone transmission lines, causing fires and power outages.

Traditional methods for control of forest weeds include mechanical and manual removal in conjunction with the use of chemical herbicides. Public concerns about nontarget effects have influenced policy makers to limit the use of chemical herbicides in at least five Canadian provinces and in several USDA Forest Service regions (Wagner 1993). Vegetation management that does not rely on chemical herbicide treatment alone is becoming the preferred strategy, and has created a demand for alternatives such as biological control. Mycoherbicides, which are indigenous fungi applied innundatively to control native weeds, provide one such alternative for forest vegetation management (Templeton *et al.* 1979, Wall *et al.* 1992, Wagner 1993). Studies by Canadian and Dutch researchers indicate that the hymenomycete fungus *Chondrostereum purpureum* (Pers. ex Fr.) Pouzar, is an excellent biological control candidate for many deciduous forest weeds (deJong *et al.* 1990, 1991, deJong 1992, Wall 1986, 1990, 1991, 1996, 1997, Wall *et al.* 1992). *Chondrostereum purpureum* has been used in the Netherlands to effectively

control American Black Cherry (*Prunus serotina* Ehrh.), an introduced forest weed (deJong 1992). This fungus is commonly found throughout the temperate zones and has a broad host range including most broad-leaved tree species (Wall 1996). Application of *C. purpureum* mycelium directly to cut surfaces of hardwood stems has been shown to reduce the number and viability of resprouts from numerous North American forest weed species (Wall 1990). Nontarget exposure is minimized as the fungus requires a wound through which to enter the host.

Prior to registration of *C. purpureum* as a biocontrol agent in North America, it is essential to establish the efficacy of this agent on different hosts and in different biogeographic regions. A national field trial was therefore established in the early summer of 1995 to compare the efficacy of *C. purpureum* treatments to that of chemical herbicide treatments in Atlantic Canada (Nova Scotia), central Canada (Ontario) and Pacific Canada (British Columbia) (Pitt *et al.* 1996). The Pacific region field trial was designed to test the efficacy of two different *C. purpureum* isolates on two target species, Sitka alder (*Alnus sinuata* (Regel) Rydb.) and trembling aspen (*Populus tremuloides* Michx.).

The causal relationship between the applied biocontrol agent and the development of disease symptoms in the host can best be confirmed by reisolation of the applied agent exclusively from symptomatic targets. To establish that the disease symptoms resulted from the specific isolate deployed, and not from colonization of the surface of the stump by other opportunistic pathogens, the infecting agent must be reisolated and identified.

This "release and recovery" survey requires the collection and identification of a large number of cultures isolated from wood samples. From a practical standpoint, *C. purpureum* in culture is difficult to distinguish from other wood decay fungi by traditional methods of identification based on morphological and biochemical tests (Stalpers 1978, Nakasone 1990). The polymerase chain reaction (PCR) can provide a relatively easy, quick, and reliable method for detection and identification of *C. purpureum* among a large number of isolates. My objective was therefore to develop a series of diagnostic PCR primers for the amplification of genetic markers that distinguish *C. purpureum* from other wood-rot fungi and to differentiate the different genotypes of this fungus. These genetic markers were also used to estimate the infection frequency following the biocontrol application of *C. purpureum* on two host species, Sitka alder and trembling aspen.

MATERIALS AND METHODS

Field trial establishment

Sitka alder and trembling aspen were selected as target species to test the field efficacy of cut stump treatments with two *C. purpureum* isolates: M0022 (Cp2139), isolated in British Columbia, and F0144 (JAM6), isolated in Ontario. The isolates were cultured and paste formulations of hyphae were prepared by two methods, referred to as the B.C. formulation (BCF) (Wall *et al.* 1996b), and the Ontario formulation (ONF) (Dumas *et al.*, 1997). The experimental layout of the field trials, developed by Pitt *et al.* (1996), was designed to assess the relative efficacy of the two *C. purpureum* isolates in combination with the two methods of formulation and also to compare the individual biocontrol

treatments with chemical herbicide treatment. A randomized complete block design was employed with five replicates of eight treatments. The four *C. purpureum* biocontrol treatments were comprised of the two isolates in combination with the B.C. and Ontario formulations. Biocontrol treatments using the B.C. formulation were designated BCF/F0144 and BCF/M0022. Biocontrol treatments using the Ontario formulation were designated ONF/F0144 and ONF/M0022. Control treatments consisted of both blank formulations, (BCF and ONF) which contained all the ingredients of the formulations except the active agent, as well as a brushed-only treatment (C). For biocontrol treatments and formulation controls, approximately 2-3 ml of formulation was applied from plastic squirt-bottles to cover each cut stump. Release® Silvicultural Herbicide (Dow Elanco, Indianapolis, IN), containing the active ingredient triclopyr ([3,5,6-trichloro-2-pyridinyl]-oxyacetic acid), was applied with a hand sprayer to simulate operational field application conditions (treatment R) (Pitt *et al.* 1999). Weed assessments were made prior to treatment, by gauging health, height, condition, and diameter at a 15-cm height for all stems within each sampling unit. Experimental treatments were applied on June 28, 1995, to the aspen trial, and the Sitka alder trial was treated on July 5, 1995. The weed species were manually brushed and the entire surface of the cut stumps was immediately treated with the formulated fungus, the formulation alone, or the chemical herbicide.

Infection frequency within the cut treatments was assessed four months after application. In the aspen trial site, six stumps were removed from the treated buffer zones from each experimental unit on October 24, 1995, 117 days after treatment. In the alder trial site,

two stumps were collected from each of three treated clumps in each treatment plot, on October 26, 1995, 112 days after treatment. A cross-sectional disk was cut at a depth of 2 cm below the treated surface of each of the control stumps while two disks, at 2 cm and 6 cm depths, were cut from each of the fungus-treated stumps to determine the extent of mycelial growth.

Fungal strains

A reference set of basidiomycetous fungal isolates were selected for the development of the species-specific marker (Table 2). The *C. purpureum* isolates represented a variety of geographic origins, including North America, New Zealand, Switzerland, Norway, and the Netherlands. Other hymenomycetes included in the reference set include *Trametes versicolor* and *Schizophyllum commune,* which are known to occupy a similar ecological niche as *C. purpureum* (Rayner and Boddy 1988). Examples of *Stereum*, *Resinicium*, and *Phanereochaete,* which have been grouped taxonomically with *Chondrostereum* (Hawksworth *et al.* 1995, Pouzar 1959), were also included. All fungal cultures were maintained on 1.5% malt extract (ME) agar (Difco).

Field recovery of fungi

Fungi from wood disks cut from the sampled field-trial stumps were cultured on a semi-selective medium. Four wood chips containing the cambium tissue were surface-sterilized for three minutes each in 95% ethanol and 15% bleach, rinsed with sterile water, and placed on 1.5% ME agar amended with benomyl (methyl 1-(butylcarbamoyl)-

Table 2. Species designation and geographical origin of Basidiomycete isolates used to screen the *Chondrostereum purpureum*-specific rDNA marker (APN1).

No.	Accession	Designation	Origin
1 [a]	2001	*Chondrostereum purpureum*	BC, CANADA
2	2139	*Chondrostereum purpureum*	BC, CANADA
3	NOF-663	*Chondrostereum purpureum*	AB, CANADA
4	NOF-671	*Chondrostereum purpureum*	AB, CANADA
5	2121	*Chondrostereum purpureum*	ON, CANADA
6	JAM-6	*Chondrostereum purpureum*	ON, CANADA
7	EA2	*Chondrostereum purpureum*	QC, CANADA
8	MB7	*Chondrostereum purpureum*	QC, CANADA
9	855	*Chondrostereum purpureum*	NB, CANADA
10	2096	*Chondrostereum purpureum*	NB, CANADA
11	CFMR-11	*Chondrostereum purpureum*	AK, USA
12	CFMR-12	*Chondrostereum purpureum*	AK, USA
13	CFMR-3	*Chondrostereum purpureum*	NY, USA
14	CFMR-8	*Chondrostereum purpureum*	VA, USA
15	ATCC64240	*Chondrostereum purpureum*	New Zealand
16	Swiss 3-6 #1	*Chondrostereum purpureum*	Switzerland
17	NOR-1	*Chondrostereum purpureum*	Norway
18	Cp2a2	*Chondrostereum purpureum*	Netherlands
19	ATCC64311	*Trametes versicolor*	WA, USA
20	FP-102357	*Trametes versicolor*	WI, USA
21	R-105	*Trametes versicolor*	NY, USA
22	ATCC26889	*Schizophyllum commune*	not available
23	FP-56473	*Schizophyllum commune*	PA, USA
24	FP-125035	*Schizophyllum commune*	NH, USA
25	ATCC34682	*Stereum hirsutum*	not available
26	ATCC13240	*Stereum hirsutum*	OR, USA
27	FP-133888	*Stereum hirsutum*	ID, USA
28	FP-105828	*Stereum hirsutum*	SD, USA
29	ATCC12233	*Stereum sanguinolentum*	ME, USA
30	ATCC44181	*Stereum sanguinolentum*	CANADA
31	FP-104730	*Stereum sanguinolentum*	CO, USA
32	FP-133911	*Stereum sanguinolentum*	ID, USA
33	HHB-10108	*Stereum sanguinolentum*	MN, USA
34	HHB-10731	*Resinicium bicolor*	VA, USA
35	ME-446	*Phanerochaete chrysosporium*	ME, USA
36	BKMF-1767	*Phanerochaete chrysosporium*	Kazachstan

[a] Isolates numbered 19, 22, 25, 26, 29 and 30 were obtained from the ATCC, Rockville MD, and those numbered 20, 21, 23, 24, 27, 28, 31, 32, 33, 34, 35 and 36 were obtained from the Forest Products Lab, Madison, WI.

2-benzimidazolecarbamate; Later Chemicals Ltd., Richmond, B.C.), neomycin sulfate (Sigma-Aldrich Corp., Oakville, ON), and streptomycin sulfate (Sigma-Aldrich Corp., Oakville, ON) (BNS medium) (Johnson 1995). Mycelium growing from the wood chips was transferred to 1.5% ME agar and visually checked to ensure that a pure culture was obtained. A 10-µl aliquot of guaiacol (Sigma-Aldrich Corp., Oakville, ON) test solution (5% w/v in ethanol) was dropped on actively growing mycelium in each plate culture and examined for the presence of a blue color indicative of laccase activity characteristic of *C. purpureum* (Stalpers 1978). Cultures with macroscopic mycelial features not found in standard cultures of *Chondrostereum* (Table 2, 1-18) were eliminated from further consideration. Those cultures that were assigned to the *C. purpureum* species based on morphology and the guaiacol test were stored in sterile distilled water at 4°C.

Preparation of fungal genomic DNA

The reference set of fungal isolates used for DNA marker development (Table 2) was grown at 20°C in 50 ml of 1.5% ME broth for two weeks. The mycelium was then harvested by centrifugation, washed twice with distilled water, and lyophilized for 48 h. Genomic DNA was extracted and purified using the method of Möller *et al.* (1992).

For genotype screening of the field-trial samples, 5-ml volumes of 1.5% ME broth were inoculated with recovered fungal isolates and incubated at 20°C for two weeks without shaking. Genomic DNA was extracted using a rapid "mini-prep" method from wet mycelium as described by Cenis (1992) using washed sterile sand and Teflon® pestles to grind mycelium with extraction buffer in microcentrifuge tubes.

Development of a species-specific rDNA marker for C. purpureum

Lambda DNA of an EMBL-3 genomic library clone, containing an entire rDNA cistron of *C. purpureum* single-spore isolate 2128u (Ramsfield *et al.* 1996), was digested with the endonucleases *Bgl* II and *Pst* I and the fragments were separated on an agarose gel. The 500 base pair *Bgl* II/*Pst* I fragment, located within the 3712 base-pair intergenic spacer (IGS) region between the sequences encoding the 5S and 18S RNA subunits (Figure 7), was purified from the gel using a Wizard PCR purification column (Promega Corp., Madison, WI), and subcloned into a *Bam* HI/*Pst* I digested pUC19 vector. The sequence of this fragment was determined by using the dideoxynucleotide sequencing approach (Sanger *et al.* 1977), with universal primers (Universal Forward/ M13 Reverse, Pharmacia Biotech Inc., Baie d'Urfe, PQ) and a T4 DNA Sequencing Kit (Pharmacia Biotech Inc., Baie d'Urfe, PQ). Design of oligonucleotide primers analogous to the termini of the cloned fragment was assisted by the use of the computer program OLIGO Version 3.4, to ensure minimal secondary structure and a melting point (Tm) greater than 60°C. Two primers, APN1-F, (5'-GCACGGAGAAGGAGAAGATTGGCT-3'), Tm=61.6 °C, and APN1-R, (5'-TTTCGGACTTTTGGGGCTCATTTCG-3'), Tm=64.7°C, were used to amplify the rDNA fragment. Amplification reactions contained 10mM Tris (pH 8.3), 50mM KCl, 2mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 200 µM each dNTP, 10 pmol each primer, 0.4 units of *Taq* polymerase (Pharmacia Biotech Inc., Baie d'Urfe, PQ) and ~10 ng DNA in a final volume of 20 µl. After overlaying the reaction mixture with mineral oil, amplifications were performed in a Perkin-Elmer Thermal Cycler (Perkin-Elmer Corp., Norwalk, CT). Genomic DNA from

reference fungi (Table 2), was amplified using APN1 primer pairs with 25 cycles of denaturation at 94°C for one minute, annealing at either 50°C, 60°C, or 70°C for 1.5 minutes, and extension at 72°C for two minutes. The amplification products were visualized on a 1% agarose/TAE gel run for 2 hours at 100 Volts and stained with ethidium bromide. As a positive control to check the quality of DNA, all non-*C. purpureum* DNA was also amplified using degenerate primers specific for chitin synthase (CHS) gene fragments (Bowen *et al.* 1992). Reactions were performed as above, using a 60°C annealing temperature.

Development of isolate-specific SCAR marker APM22D13 for C. purpureum

Genomic DNA of isolate M0022 served as a target for amplification of polymorphic DNA using the RAPD primer OPD13, 5'-GGGGTGACGA-3' (Operon Technologies Inc., Alameda, CA). For the RAPD reactions, the thermal cycler was programmed for 25 cycles of a 1-min denaturing step at 94°C, annealing at 37°C for 1.5 minutes, and extension at 72°C for 2 min. The resultant amplification products were purified using a PCR-purification kit (Promega Corp., Madison, WI), ligated with the pGEM-T plasmid vector DNA (Promega Corp., Madison, WI), and introduced into competent XL1-Blue *E.coli* cells. Plasmid DNA of selected clones were sequenced and primers were designed as described for APN1. The SCAR primer sets were screened using template DNA from a geographically diverse set of *C. purpureum* isolates. The primers designated APM22D13F, (5'-GGGGTGACGAGGACGACGGTG-3'), Tm=63.2 °C and APM22D13R, (5'-GGGGTGACGACATTATACTGCAGGTAGTAG-3'), Tm=61.2 °C were chosen for use in this application, based on the ability of patterns produced to

consistently differentiate the lead isolates from each other and from other *C. purpureum* isolates.

PCR analysis of field trial samples

Genomic DNA from field collected samples was PCR amplified using APN1 and APM22D13 primer pairs using 5 cycles of denaturation at 94°C for one minute, annealing at 55°C for 1.5 minutes, and extension at 72°C for two minutes followed by 25 cycles in which the annealing temperature was raised to 60°C. The amplification products were visualized on a 1% agarose/TAE gel run for 2 hours at 100 Volts and stained with ethidium bromide.

Statistical analysis

Infection-frequency data were grouped according to several variables, including the type of formulation (BCF vs. ONF), *C. purpureum* isolate (M0022 vs. F0144), and type of host weed species (Sitka alder vs. trembling aspen). Data sets were compared using the two-sample t-test assuming unequal variances and analysis of variance (ANOVA). Interactions between each of the four combinations of biocontrol treatment and either of the host weed species were also tested using analysis of variance.

RESULTS AND DISCUSSION

Development of a C. purpureum-specific marker

A key objective of this study was to develop a PCR-based species-specific molecular genetic marker to distinguish *C. purpureum* from other wood-rot fungi. In order to use

this marker as a diagnostic tool, it was necessary to establish that the primers would amplify the diagnostic bands from *C. purpureum* but not from other fungi, including those which commonly inhabit cut or wounded trees. The ribosomal DNA (rDNA) repeat, which has been found to be well suited for taxonomic and phylogenetic studies in fungi (Bruns *et al.* 1991), provided an appropriate target for the design of a diagnostic primer pair. Comparison of various regions of the rDNA repeat has allowed discrimination at the generic, specific, and intraspecific level (Hintz *et al.* 1989). The ribosomal RNA genes themselves are highly conserved in their primary nucleotide sequences. There have, however, been fewer evolutionary constraints on the intergenic portions of the rRNA repeat. Hence, greater variation has been incorporated into these regions. The large intergenic spacer (IGS), also known as the large nontranscribed spacer region (NTS-L), is located between the 5S and 18S ribosomal regions in hymenomycetes. In a recent study of genetic variability within *C. purpureum*, restriction polymorphisms in the large IGS region were analyzed from a world-wide set of isolates (Ramsfield *et al.* 1996). This region was very well conserved within the *C. purpureum* species and demonstrated little heterogeneity in restriction pattern. Because random mutations can accumulate in the IGS without conferring either positive or negative effects to the survival of the organism, the IGS region would be expected to be quite variable among genera of fungi, although the exact amount of relative sequence-divergence is unknown (Bruns *et al.* 1991). I hypothesized that primers designed to anneal to the *C. purpureum* IGS region would share sufficient homology with all *Chondrostereum* isolates to generate an amplification product but would not anneal with sufficient fidelity to foreign

DNAs to result in positive amplification. Such a selective primer pair could be used to differentiate *C. purpureum* from other fungi.

A 500 bp *Pst I/Bgl* II fragment of the *C. purpureum* rDNA intergenic region was subcloned (Figure 7), and the DNA sequence determined (data not shown). For the specific amplification of this fragment from genomic DNA, a pair of oligonucleotide primers (APN1F+R) was designed according to the terminal sequences of this fragment. To ensure a high degree of fidelity during PCR amplification, the oligonucleotide primers were designed to have a projected melting temperature of greater than 60 °C. This would permit annealing of the primers to complementary regions of the target DNA only at higher temperatures, resulting in amplification of the rDNA under very stringent conditions. The APN1 primers were used in PCR amplification of target DNA from a reference set of *C. purpureum* isolates and a series of other wood-rotting fungal genera (Table 2).

Three different annealing temperatures were employed to assess the effects of reduced stringency during PCR amplification. The PCR was first performed such that the annealing temperature of the reaction was 70°C, approximately 10°C higher than the projected Tm of the APN1 primers. Under these highly stringent conditions, a single intensly staining discrete band of 500 bp was amplified from *C. purpureum* template DNAs from all geographic regions represented (Figure 8a, lanes 1-18). There were no significant amplification products observed for the other fungi under these reaction

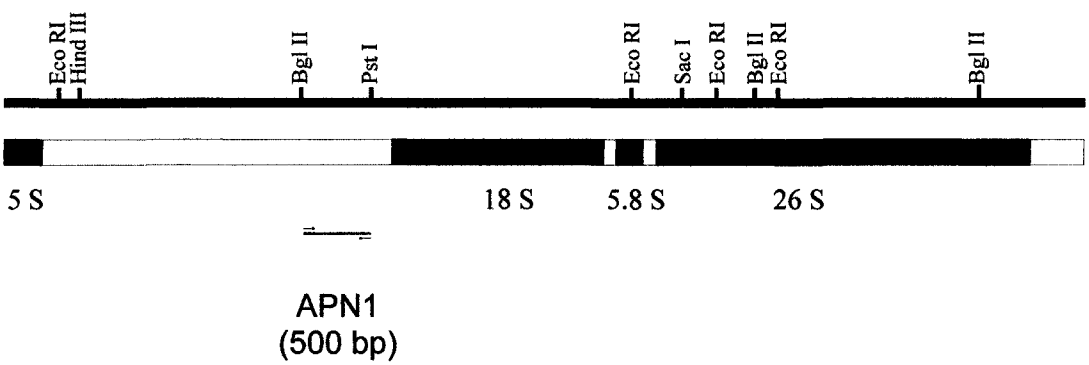

Figure 7. Restriction site map of *C. purpureum* ribosomal DNA

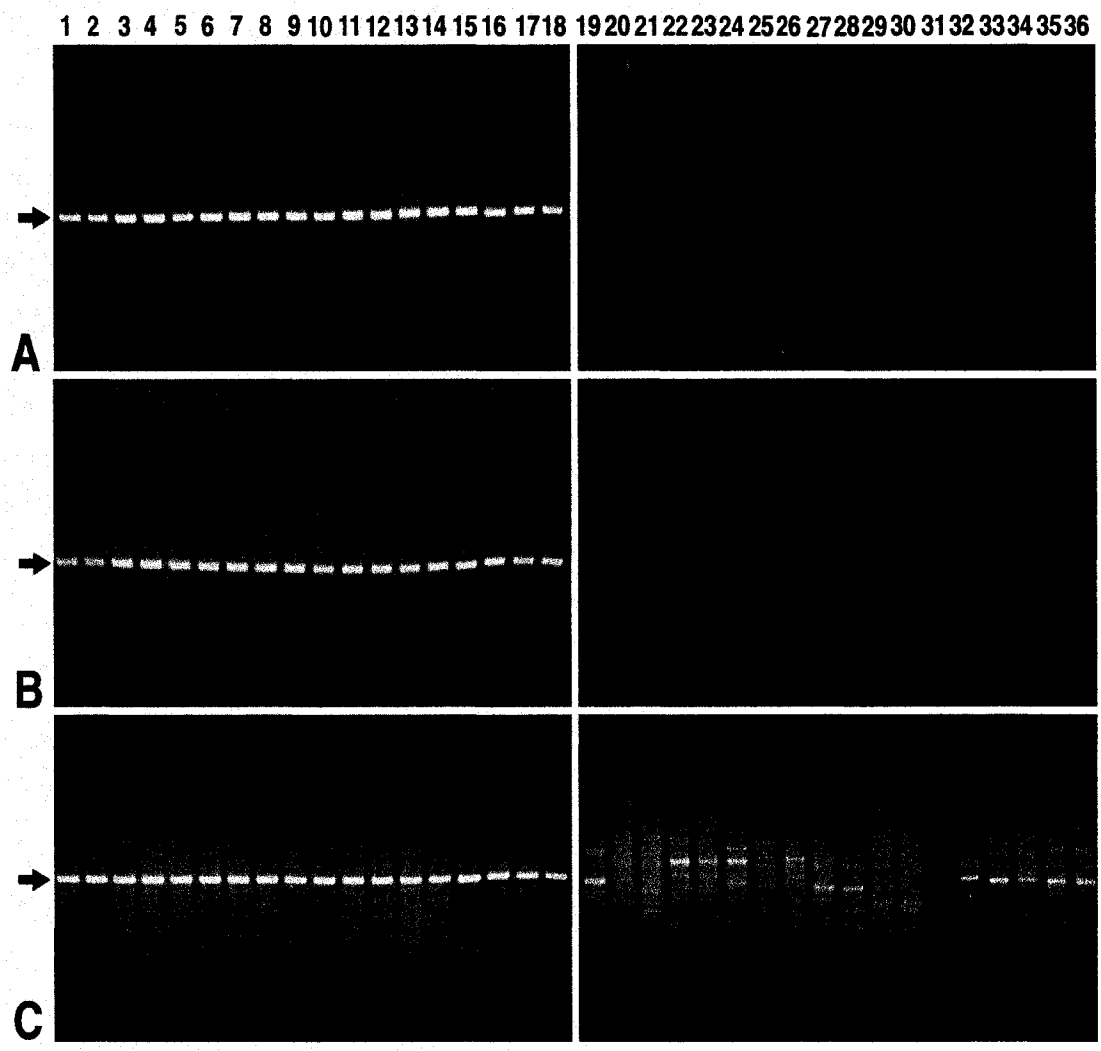

Figure 8. Evaluation of the discriminatory power of the APN1 marker. Amplification was performed using APN1 primers with template DNA extracted from the standard set of cultures (Table 1). Lanes are labeled with species designation and lane numbers correspond with the numbering of individual isolates in Table 1. Products of amplification of *C. purpureum* isolate DNAs are shown in lanes 1-18 and that of other fungal DNAs in lanes 19-36 in the three agarose gels shown after electrophoretic separation (a, b, and c). Amplification was performed under conditions of high stringency, using a 70°C annealing temperature (a), which yielded a 500 bp band from *C. purpureum* DNA exclusively (a, lanes 1-18). Lowering the stringency of the amplification reaction by using a 60°C annealing temperature (b), again produced a 500 bp band from *C. purpureum* DNA only. Very faint bands amplified from DNA of other fungi were visible, but were not comparable in intensity or size to the band amplified from *C. purpureum* DNA. Amplification was further performed at conditions of very low stringency, lowering the annealing temperature to 50 °C (~10°C < the Tm of the APN1 primers). These conditions again produced the single, strongly amplified band of 500 bp for the *C. purpureum* DNA set (c, 1-18). Non-specific amplification of *C. purpureum* DNA was additionally evidenced by smearing and faint bands of different sizes. Amplification of the DNA of other fungi (lanes 19-36), produced many-sized bands of a range of intensities but no single intense band of 500 bp was amplified from any one sample.

conditions (Figure 8a, lanes 19-36). The stringency was then reduced such that the annealing temperature was 60°C; approximating the calculated Tm of the APN1 primers (Figure 8b). These conditions again yielded a bright 500 bp band, uniformly amplified from all the *C. purpureum* DNAs (Figure 8b, lanes 1-18). Very faint bands of a range of sizes were visible upon amplification of DNA of other fungi, but no bands were comparable in intensity and size to the single 500 bp band amplified from the *C. purpureum* DNA (Figure 8b, lanes 19-36). The stringency of the PCR reactions was further reduced by dropping the annealing temperature to 50°C; approximately 10°C lower than the Tm of the APN1 primer. This low stringency again produced the bright band at 500 bp for the *C. purpureum* DNA set (Figure 8c, lanes 1-18) but DNA smearing and the presence of several faint bands indicated nonspecific amplification of *C. purpureum* DNAs at this low annealing temperature. Amplification of DNA from the other fungi (Figure 8c, lanes 19-36) resulted in many bands of a variety of sizes and staining intensities, but no samples amplified the diagnostic 500 bp band. It would appear that lowering the annealing temperature permitted a certain amount of mismatch between primers and the non-homologous target DNAs, resulting in amplification of a number of fragments of different sizes from the foreign DNAs. Whether these spurious bands represented foreign rDNA spacer or other anonymous sequence is not known. Since it is often difficult to distinguish between a failed PCR reaction and a null allele, it was fortuitous that amplification products were visible in the non-*C. purpureum* isolates at reduced stringency. Amplification at the lower temperature could be used as a positive control to verify that the target DNA was of adequate quality and concentration to be amplified. Test samples yielding negative amplification at 70°C or 60°C could thus be

re-amplified at 50°C for confirmation of the null allele. As a further quality control, the set of non-*C. purpureum* genomic DNA (Table 2,19-36) was subjected to PCR amplification using primers specific to CHS gene fragments. While only *C. purpureum* templates yielded a intensely staining 500bp fragment with the APN1 primers, template DNA of all of the non-*C. purpureum* reference isolates was amplified by the CHS primers (Figure 9). I concluded that PCR amplification at high stringency using the APN1 primers was extremely specific for the *C. purpureum* rDNA and could be considered to be diagnostic of this fungus.

Quality control during PCR screening

The effects of template DNA concentration and the limits of resolution were determined for the APN primer pair using a dilution series of *C. purpureum* (isolate M0022) DNA. The target DNA concentrations were measured by spectrophotometric methods and the APN fragment amplified under stringent conditions. Amplification was successful for concentrations ranging from 2 μg to less than 128 pg per reaction (Figure 10). Amplification using 128 pg of template DNA yielded a band of about half the intensity as that of the more concentrated DNA samples so this quantity should be considered the minimum necessary for unambiguous amplification using these primers. Because amplification was successful over such a wide range of DNA concentrations (>15,000 fold) it was not critical to quantify DNA extracted from field samples before amplification. This permitted the assay of field-collected samples following a rather crude DNA extraction. The nuclear rRNA genes are organized in a tandemly repeated unit present in 60-220 copies in a haploid fungal genome, so the sensitivity of detection

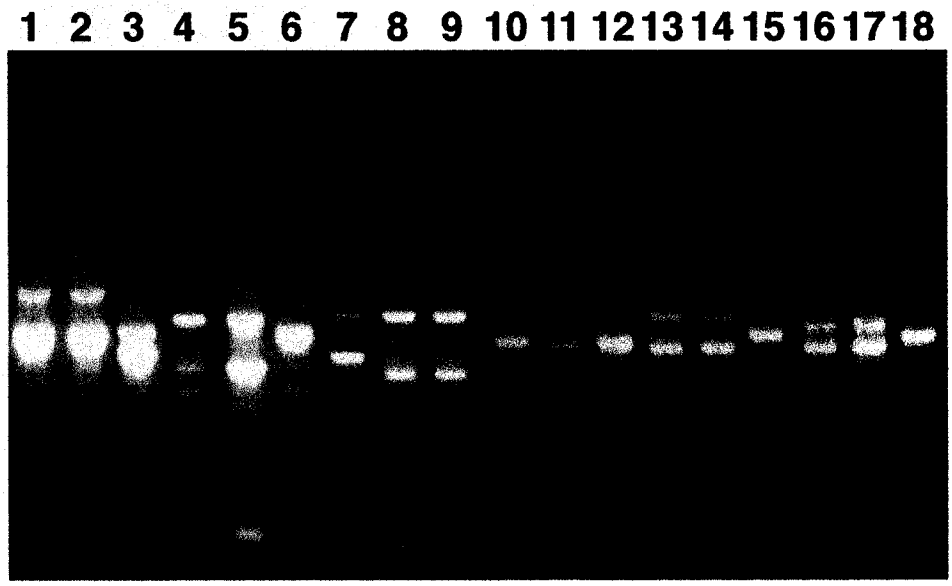

Figure 9. Amplification of target DNA with chitin synthase primers. To serve as a positive control for DNA quality, the reference set of genomic DNAs from non-*C. purpureum* fungi was subjected to amplification using primers specific to chitin synthase (Bowen *et al.,* 1992). Products are shown from the PCR of genomic DNA of isolates of *Trametes versicolor* in lane 1 (FP-102357), lane 2 (R-105) and lane 12 (ATCC64311); *Schizophyllum commune* in lane 3 (ATCC26889), lane 16 (FP-56473) and lane 17 (FP-124035); *Stereum hirsutum* in lane 4 (ATCC346682), lane 5 (ATCC13240), lane 8 (FP-133888) and lane 9 (FP-105828); *Resinicium bicolor* in lane 6 (HHB-10731) and lane 15 (HHB-10108); *Stereum sanguinolentum* in lane 7 (FP-104730), lane 11 (FP-133911), lane 13 (ATCC12233), and lane 14 (ATCC44181); and *Phanerochaete chrysosporium* in lane 10 (BKMF-1767), and lane 18 (ME-446).

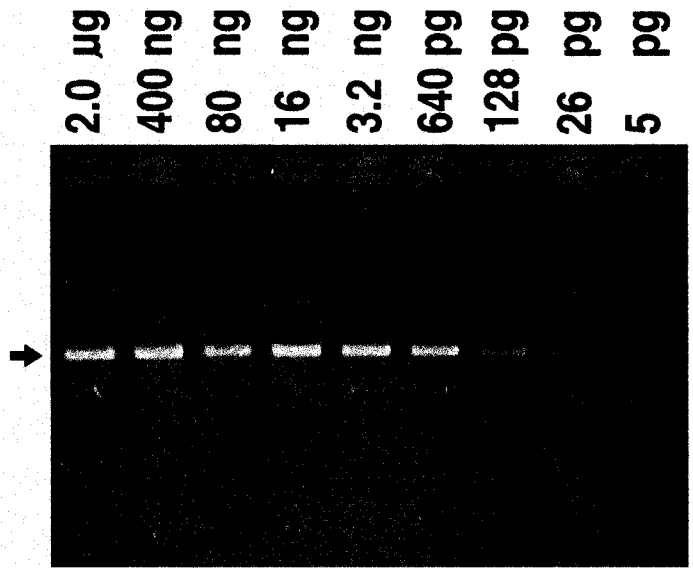

Figure 10. Limits of resolution of the APN1 primers. To determine the minimum concentration of target DNA required for successful amplification using the APN1 primer pair, genomic DNA of *C. purpureum* M0022 was serially diluted and subjected to PCR. Target DNA concentration ranged from 2 µg per reaction to 5 pg per reaction, as indicated. The 500 bp amplification product was amplified over a 15,000 fold range of DNA concentrations (2 µg - 128 pg).

would be expected to be much higher than that of single-copy genomic DNA. Despite the many copies of the rDNA repeat it appears that there is sufficient uniformity in the rDNA repeats within a genome to allow for unambiguous amplification of a single product from each *C. purpureum* individual under these conditions.

Development of a DNA fingerprinting marker

The APM22D13 SCAR primers were designed by cloning and sequencing a RAPD fragment produced by amplification of *C. purpureum* M0022 DNA with the OPD13 RAPD primer. Longer primers, which incorporated the 10 bases of the RAPD primer, were designed according to the DNA sequence of the cloned fragment termini. Primers designed in this manner usually produce a single band upon amplification of the original template DNA (McDermott *et al.* 1994). Unexpectedly, amplification of *C. purpureum* DNA using the APM22D13 primer pair consistently produced a series of discrete bands. The resultant fragment patterns vary to a sufficient degree to allow individual genotypes of *C. purpureum* to be identified (Figure 11). The unique banding patterns of the two *C. purpureum* isolates used in the field release (M0022, F0144) used in the field-release allowed them to be readily differentiated from one another and from other isolates of *C. purpureum* (Figures 11 and 12). Because the amplification was performed at a high annealing temperature, the results were highly reproducible.

While the APN1 marker was useful for identification to species level, a more discriminating marker was required to identify individual *C. purpureum* genotypes. This

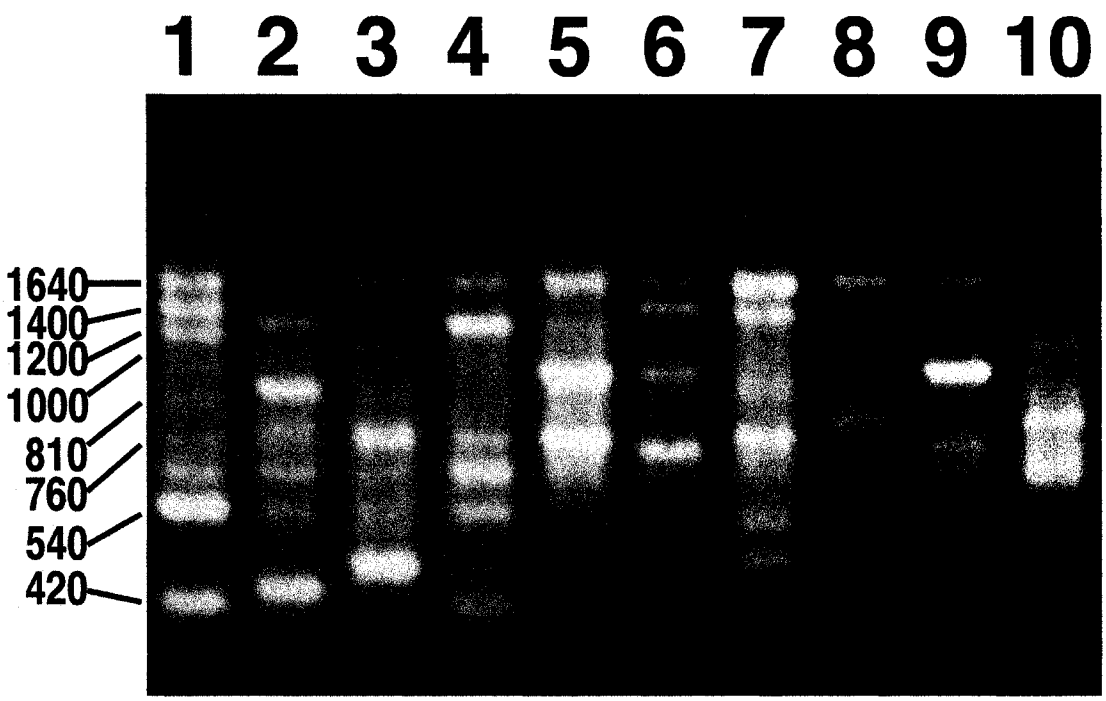

Figure 11. Banding patterns generated by isolate-specific PCR marker. To exemplify the discriminatory power of the fingerprinting marker, products of amplification of several *C. purpureum* DNAs using the SCAR primer pair APM22D13 are shown. *Chondrostereum* samples which have previously been described by Ramsfield *et al.* (1996) included lane 1, M0022 (=2139); lane 2, 2128; lane 3, 2121; lane 4, 2191; lane 5, 2193; lane 6, 2145; lane 7, JAM5; lane 8, F0144 (JAM6); lane 9, EA2; and in lane 10, NOR1.

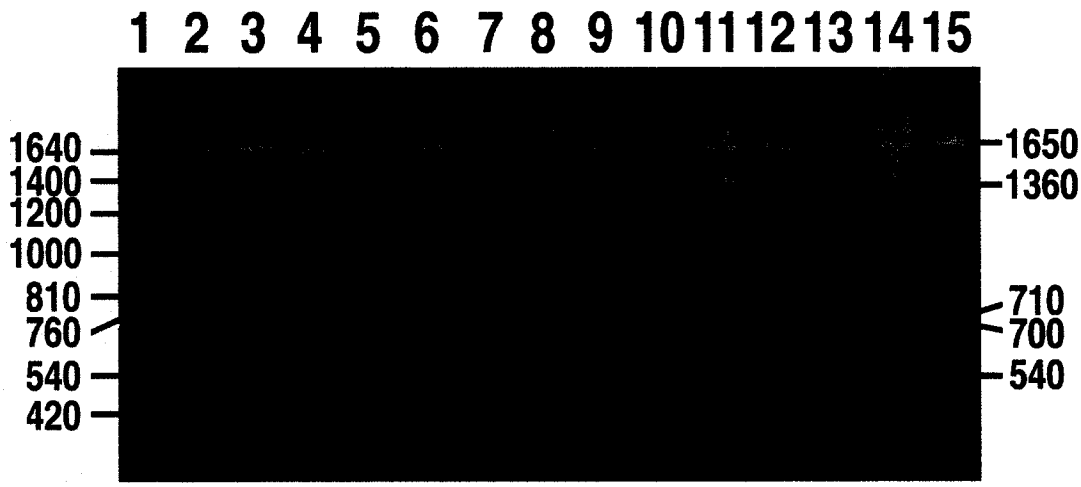

Figure 12. DNA fingerprinting of field-collected samples. An example of results of amplifications using SCAR primer pair APM22D13 with template DNA from several field trial samples are shown. DNA banding patterns indicative of biocontrol isolates M0022 are evident in lanes 1, 2, 4, 6, 7, 8, 9, 11, 12 and 14. The pattern indicating the F0144 isolate can be seen in lanes 3, 5, 10, 13 and 15. Approximate sizes of the most strongly amplified bands (in base pairs) are indicated, with bands seen in the M0022 amplification shown in the left of the photograph and F0144 bands shown on the right. A band of 540 base pairs was amplified by both *C. purpureum* isolates and may be homologous. The consistent, unambiguous banding patterns obtained illustrate the reproducibility of these markers applied to DNA samples isolated and cultured independently.

would permit the differentiation of a released *C. purpureum* isolate from other naturally-occurring *C. purpureum* following a field release. Randomly amplified polymorphic DNA (RAPD) markers have recently been used to study genetic variability within *C. purpureum* populations in Quebec and were shown to be useful for distinguishing *C. purpureum* individuals (Gosselin *et al.* 1996). There are, however, disadvantages to the use of RAPD markers. The low annealing temperature, due to the short length (10 bases) of the random primers, contributes to variability in the specificity and reproducibility of amplification. Amplification of RAPD fragments is sensitive to variations in quantity and purity of the target DNA, and the reaction can even be affected by the source of polymerase and type of thermocycler. This inconsistency led us to consider the development of SCAR markers for the amplifications of sequence-characterized regions of DNA (Paran and Michelmore 1993, McDermott *et al.* 1994). Whereas RAPD primers amplify anonymous fragments of DNA based on annealing of random-sequence primers, the longer SCAR primers, designed according to authentic sequence, could be used to amplify specific regions of the genome. The major advantage of SCAR primers is that due to the longer length of the primers, much higher annealing temperatures can be used, (55°C to 60°C compared with 35°C to 37°C). Amplification using the SCAR markers is thus significantly more stringent and is highly reproducible.

Epidemiological study in the field: Application of genetic markers to the analysis of infection

Having established that the rDNA-based molecular markers could be used to distinguish *C. purpureum* from other fungi, and that the APD13M22 marker could distinguish among

genetic individuals within populations of *C. purpureum*, I used these markers to survey the recovery of specific isolates following field release. Two field trial sites were established in British Columbia to compare the efficacy of *C. purpureum* with the use of chemical herbicides for the control of Sitka alder (Site 1) and trembling aspen (Site 2). As treatment efficacy can be dependent on the composition of the formulation materials, the isolates F0144 and M0022 were tested in two different formulations. To determine whether the adjuvants promoted infection of the stumps by local fungi, blank formulations without the biocontrol agent were also applied. Infection frequency of treated stumps was compared to stumps that were cut without any further treatment. Four months after application, destructive sampling of stumps was performed to estimate the extent of *C. purpureum* infection of the treated and control stumps. A total of 30 stumps were collected from each of the eight treatment regimens at the two field trial sites and the incidence of infection by the released isolates M0022 and F0144 was determined for each treatment (Table 3). Wood chips from the sampled stumps were plated on BNS medium which contained antibiotics to suppress bacterial growth and benomyl to select for most hymenomycetes (Worrall 1991). Recovered fungal isolates were further screened for the presence of *C. purpureum*-like fungi. DNA was extracted from all those cultures which had mycelium characteristic of *C. purpureum* (white, advancing zone of culture distantly spaced) (Stalpers 1978), and which gave a positive reaction with guaiacol, indicative of laccase activity. These DNAs were assayed using the APN1 primer pair for identification as *C. purpureum*. Where there were no amplification products visible, the reactions were repeated at a lower annealing temperature to verify that the lack of amplification was a consequence of a lack of primer homology and not

Table 3. Infection of treated stumps of Sitka alder (Site 1) and trembling aspen (site 2) by *C. purpureum*.

Treatment		Plot 1[a]					Plot 2					Plot 3					Plot 4					Plot 5				
Site 1: Sitka alder (Ripperto Creek)																										
ONF/ F0144	2cm	F[b]	0	F	F	0	F	F	0	F	F	F	F	F	F	F	F	F	F	0	0	F	F	F	0	F	F	0	F	L	F
ONF/ F0144	6cm	F	F	F	F	0	F	0	F	0	F	F	F	F	0	0	F	F	F	L	0	F	F	0	0	F	F	F	F	F	F
ONF/ M0022	2	M	M	M	M	M	M	M	0	M	M	M	0	M	M	0	M	M	M	0	M	M	M	M	M	0	M	M	M	M	M
ONF/ M0022	6	0	0	M	0	M	M	M	M	M	M	M	M	M	M	0	0	0	M	0	0	M	0	M	M	0	M	M	M	0	M
BCF/ F0144	2	F	F	F	F	F	0	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
BCF/ F0144	6	0	0	F	F	0	0	F	F	F	F	F	F	F	F	F	0	F	F	F	F	F	F	0	0	F	F	F	F	F	F
BCF/ M0022	2	M	M	M	M	0	0	M	M	M	M	M	M	M	M	M	M	M	M	M	M	0	M	M	M	M	M	M	M	M	0	M
BCF/ M0022	6	0	0	M	M	0	0	M	M	M	M	0	M	M	M	0	M	M	M	M	M	M	M	0	M	M	M	M	M	0	0	M
ONF alone[c]		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BCF alone[c]		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cut control[c]		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Triclopyr ®[c]		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Site 2: Trembling aspen (B.C. Mine Rd.)																										
ONF/ F0144	2cm	F	F	0	0	0	0	0	0	0	0	0	0	0	0	0	F	F	0	0	0	F	0	F	0	0	0	0	0	F	F
ONF/ F0144	6cm	0	0	0	0	0	0	0	0	0	0	0	0	0	F	F	F	F	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ONF/ M0022	2	M	0	M	M	0	M	M	M	0	M	0	M	M	M	0	M	0	0	0	0	0	0	M	0	0	0	0	M	0	0
ONF/ M0022	6	0	M	M	0	0	0	M	0	M	0	0	0	0	0	0	M	M	0	0	0	0	0	0	0	M	0	0	0	0	0
BCF/ F0144	2	0	F	0	0	F	0	0	0	0	0	0	0	0	0	F	0	F	F	0	0	0	0	F	F	0	F	F	0	0	0
BCF/ F0144	6	F	0	F	F	F	0	0	0	0	0	0	0	F	0	0	0	0	0	0	0	0	0	F	F	0	0	F	0	0	0	F
BCF/ M0022	2	M	M	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	M	0	0	0	0	0	0	0	0	0	M	0
BCF/ M0022	6	0	M	M	M	0	0	0	0	0	0	0	0	0	0	0	0	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ONF alone[c]		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BCF alone[c]		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cut control[c]		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Triclopyr ®[c]		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Six stumps were sampled from each of five experimental plots (1-5).

[b] The identity of the recovered fungus is indicated by an "F" (F0144), "M" (M0022), or "L" (local unknown isolate). A "0" is indicated where no *C. purpureum* was recovered.

[c] The controls, not treated with biocontrol, were sampled at a depth of 2 cm only. ONF = Ontario formulation; BCF = B.C. formulation.

simply a failed PCR reaction. To promote greater rigor in the experiment and reduce experimental or sampling error, DNA was extracted independently twice from each sample, and PCR amplifications repeated. The rDNA PCR screen was very effective at discriminating between *C. purpureum* and other hymenomycete fungi which were originally grouped together according to mycelial morphology and guaiacol reaction. Two cultures in the alder trial set were initially designated as *C. purpureum*, but did not amplify the APN1 marker, and thus were concluded to be some other hymenomycete (Table 3). APN1 marker verification eliminated four isolates from the aspen trial set, two of which were isolated from control stumps which would not be expected to harbor the released biocontrol fungus. Visual assessment in combination with the guaiacol test was highly accurate as a predictor of culture identification: 97.6 % of those recovered cultures were "APN1 positive". Since it was important to confirm that the specific isolates released (M0022 and F0144) were recovered from the targeted stumps, all those samples verified to be *C. purpureum* using the rDNA marker were further analyzed using the APM22D13 marker. A very low incidence of infection of cut stumps by endemic *C. purpureum* was recorded 4 months after site establishment. Of all the *C. purpureum* cultures tested, only two isolates, recovered from F0144-treated stumps in the Sitka alder trial, were genetically different from either M0022 or F0144, and likely represented local *C. purpureum* isolates (Table 3). Although only healthy trees were chosen for treatment, these stems may have already been infected with local *C. purpureum* prior to the field trial application. Alternatively, the stumps could have been co-infected by local *C. purpureum* spores landing on the freshly cut surface during biocontrol application.

Screening with APM22D13 indicated that the Ontario isolate (F0144) and the B.C. isolate (M0022) were recovered only from stumps to which they were applied (Table 3). There was no cross-contamination of nontarget stumps, suggesting that topical application with a paste formulation of *C. purpureum* is highly target-specific. No nontarget effects directly attributable to the release of *C. purpureum* were noted. Stumps not treated with the *C. purpureum* fungus were sampled in order to determine whether other treatments would promote infection by local isolates of *C. purpureum*. No *C. purpureum* was detected in any of those stumps when treated with blank formulations (BCF, ONF), herbicide (R), or left untreated (C) (Table 3). This could be due to a paucity of spores in the air at the time the stumps were cut. Fruit bodies generally form during the cooler and wetter times of year and the field trial was established in the driest part of the summer.

Approximately four months after treatment, mycelium of *C. purpureum* was detected at a depth of 6 cm in most infected stumps, indicating that the pathogen can grow at least 1.5 cm per month through Sitka alder and trembling aspen stumps during the hottest and driest months of the year. If infection at both 2 cm and 6 cm was taken as the criterion for pathogen establishment, the rate of alder infection (78 / 120 stems treated) was much greater than that of aspen (10 / 120 stems treated) (Table 3). There were as many (25) instances in each of the aspen and alder sites in which infection had proceeded to the 2 cm mark but the fungus was not recovered at 6 cm. This was initially interpreted as a slower growth pattern in some of the sampled stumps. There were, however, several instances where the fungus was recovered at a depth of 6 cm and not 2 cm. For example,

8 of the alder stems and 13 of the aspen stems demonstrated the presence of *C. purpureum* at the lower depth but not closer to the surface of the stump. The fact that several stumps were found to be infected at 6 cm yet *C. purpureum* was not recovered from the same stump at the 2-cm depth may indicate that *C. purpureum* grew in an irregular pattern down these stumps or may have lost viability closer to the application surface as resources became depleted. When infection frequency was calculated for either 2-cm or 6-cm depths, approximately 90% of the biocontrol-treated Sitka alder stems and 40% of the aspen stems were successfully infected. The average number of fungus-treated stumps found to be infected by biocontrol isolates of *C. purpureum* of six samples per plot ranged from 5.0 to 5.8 stumps in the alder trial and 1.4 to 3.2 stumps in the aspen trial (Table 4). There was a significant difference between the rates of infection of the two target weeds (Sitka alder vs. trembling aspen), but not in the infection rates of the two isolates applied (M0022 vs. F0144), or the type of formulation used (BCF vs. ONF).

CONCLUSION

The results of this study indicated that individuals of the released fungus can be reliably reisolated after biocontrol application and accurately identified using PCR-based methodology. Molecular markers were successfully designed and applied to confirm infection of treated stumps by *C. purpureum* in the two field trial sites. This approach allowed hundreds of field-trial samples to be quickly screened for *C. purpureum*

Table 4. Frequency of infection of treated stumps.

Treatment	Site 1: Sitka alder infection		Site 2: Trembling aspen infection	
	Average[a]	% Infection[b]	Average[a]	% Infection[b]
ONF[c] / F0144	5.0 ± 1.22	83.3 ± 20.3%	2.0 ± 1.41	33.3 ± 19.0%
ONF[c] / M0022	5.4 ± 0.55	90.0 ± 9.1%	3.2 ± 1.64	53.3 ± 27.3%
BCF / F0144	5.8 ± 0.45	96.7 ± 7.5%	3.0 ± 1.87	50.0 ± 31.2%
BCF / M0022	5.4 ± 0.89	90.0 ± 14.9%	1.4 ± 1.52	23.3 ± 25.3%
ONF[c] alone	0	0	0	0
BCF alone	0	0	0	0
Cut control	0	0	0	0
Triclopyr ®	0	0	0	0

[a] The average number of stumps infected with biocontrol isolates of *C. purpureum* at either 2 or 6 cm below their cut surfaces (± standard deviation), per six stump sampled in five treatment replications.

[b] The average number of stumps infected with biocontrol isolates of *C. purpureum*, expressed as the percentage of infected stumps per treatment replication (± standard deviation).

[c] ONF = Ontario formulation; BCF = B.C. formulation.

infection. In addition, this method enabled the fungal genotypes to be differentiated and identified. The lower rate of infection by *C. purpureum* on trembling aspen compared to Sitka alder is expected to result in less-effective suppression of the former species. Hence, if efficacy is correspondingly lower on aspen, this would indicate that the rate of *C. purpureum* infection must be improved before it can be expected to perform effectively as a biocontrol on that host. Improved infection of this host may be achievable through a change in application, formulation, or strain of *C. purpureum*. The infection data are being correlated with subsequent disease symptoms to establish causality and also to resprout-vigor to determine whether early *C. purpureum* establishment can be used to predict subsequent suppression of resprouts. This methodology might thus provide an early indication of success of biocontrol application of *C. purpureum*.

Chapter 4. *Chondrostereum purpureum* as a biological control agent in forest vegetation management. III. Infection survey of a national field trial. 1999. Canadian Journal of Forest Research 29: 859-865.

ABSTRACT

A nationally coordinated field trial was established in the summer of 1995 to assess the utility of *Chondrostereum purpureum* (Pers. ex Fr.) Pouzar as a biocontrol agent for several weedy deciduous species. To assess the proof of concept for this biocontrol, it was necessary to establish that disease symptoms (re-sprout suppression) resulted directly from the application of the specific biocontrol agent. In this study, molecular genetic markers were used to survey *C. purpureum* infection in cut stumps four months following application of the biocontrol agent in Ontario and New Brunswick. Genetic analysis of samples from the New Brunswick trials verified the infection of trembling aspen (*Populus tremuloides*) and red maple (*Acer rubrum*) with biocontrol isolates of *C. purpureum*, with no cross-contamination of treatment applications detected. Analyses of recovered fungal samples from the target weeds, trembling aspen and speckled alder (*Alnus rugosa*), in Ontario also confirmed success of biocontrol application and revealed some differences in the extent of *C. purpureum* infection. An average of 84% of biocontrol-treated speckled alder stems and 54% of trembling aspen stems were verified to have been infected by *C. purpureum*. It is hypothesized that, given a sufficient number of samples, this methodology will provide an early indication of the relative success of *C. purpureum* biocontrol.

INTRODUCTION

In managed forests, the establishment and growth of crop trees is often impeded by competing vegetation. Forestry vegetation management often relies on manual brushing combined with chemical herbicide treatment of forest weeds to stimulate growth by channeling resources into the crop trees. The desire for alternatives to chemical herbicides that can be integrated with present vegetation management practices has promoted research into biological control strategies for forestry use.

Researchers in the Netherlands have demonstrated the effectiveness of the basidiomycete fungus *Chondrostereum purpureum* (Pers. ex Fr.) Pouzar, as a biocontrol agent, or mycoherbicide, for the control of American black cherry (de Jong *et al.* 1990). In Canada, research and testing is in progress towards the development of indigenous *C. purpureum* as a biocontrol, with the aim of selectively minimizing the problematic resprouting of cut hardwood trees (Wall 1986, 1990, 1991, 1994, 1996, Wall *et al.* 1992, Jobidon 1998, Dumas *et al.* 1997). Commonly found throughout the temperate zones of the world, *C. purpureum* is a facultative saprophyte that has a broad host range with a preference for hardwood tree species (Wall 1996). Application of *C. purpureum* mycelium directly to the cut surfaces of hardwood stems has been shown to reduce the number and viability of resprouts in numerous weedy species (Wall *et al.* 1992). Deciduous species, which have traditionally been controlled by mechanical brushing followed by treatment with chemical herbicides, thus provide likely candidates for *C. purpureum* biocontrol testing.

Prior to registration of *C. purpureum* as a biocontrol in North America, it is necessary to establish effectiveness on different hosts and in different biogeographic regions. In a collaborative effort of the University of Victoria, Mycologic, Inc., the B.C. Ministry of Forests, and the Canadian Forest Service, a national field trial was established in the early summer of 1995. This trial was designed to test the biocontrol application of two different strains and formulations of *C. purpureum* on a total of four different target weeds under operational conditions in three regions of Canada. *Chondrostereum purpureum* biocontrol treatments were compared to conventionally used chemical herbicide treatment in Pacific Canada (British Columbia), central Canada (Ontario), and Atlantic Canada (New Brunswick) (Pitt *et al.* 1996). Trembling aspen (*Populus tremuloides*) was selected as a target species of national importance and was assessed in each region, while sitka alder (*Alnus viridis* ssp. *sinuata*), speckled alder (*Alnus rugosa*), and red maple (*Acer rubrum*) were species of regional importance in British Columbia, Ontario and New Brunswick, respectively. Two *C. purpureum* isolates were applied in combination with two methods of inoculum formulation.

In order to assess the efficacy of biological control, it was essential to establish that subsequent disease symptoms (re-sprout suppression) resulted directly from the application of the specific biocontrol agent. To establish that disease symptoms resulted from the specific isolate deployed, and not from the colonization of stump surfaces by other opportunistic pathogenic fungi, the infecting agent needed to be re-isolated and identified as the released isolate. Diagnostic genetic markers have recently been developed which can be used to distinguish *C. purpureum* from other wood rot fungi and

differentiate between different genetic individuals of this fungus (Becker *et al.* 1999a). In this study, these molecular genetic markers were used to survey and confirm *C. purpureum* infection in cut stumps four months following application of Eastern Canada field trial treatments.

MATERIALS AND METHODS

Field trial establishment

The experimental layout of the national field trials, developed by Pitt *et al.* (1996), was designed to investigate the relative resprout suppression of weed species resulting from different cut stump treatments. Trembling aspen and speckled alder in Ontario and trembling aspen and red maple in New Brunswick were selected as target weed species. All experimental treatments were applied in late June, 1995. Each trial consisted of five replicates of eight treatments as well as uncut controls. Target weeds were manually brushed and the entire surface of the cut stems was immediately treated with the formulated fungus, the formulation alone, or chemical herbicide. Two *C. purpureum* isolates were used in the national field trials, M0022 (= 2139) originally isolated in B.C., and F0144 (= JAM6), isolated in Ontario. Isolates were cultured separately and formulations of *C. purpureum* mycelia were prepared by two methods, and are referred to as the B.C. formulation (BCF) (Wall *et al.* 1996b) and the Ontario formulation (ONF) (Dumas *et al.* 1997), providing four combinations of biocontrol application. Biocontrol treatments using the B.C. formulation were designated BCF/ F0144, and BCF/ M0022, while *C. purpureum* treatments using the Ontario formulation were designated ONF/ F0144 and ONF/ M0022. Control treatments included both blank formulations, (BCF

and ONF) which contained all ingredients of the formulation except fungi, as well as a cut-only treatment (C). Release® Silvicultural Herbicide (Dow Elanco, Indianapolis, IN), containing the active ingredient triclopyr, was applied with a hand sprayer (R).

Field trial sampling protocols

Four months after treatment, six stumps were sampled from each field trial plot. Wood disks were cut at six cm below the cut surface of each stem section and chips, including the cambial layer, were placed on 3% malt extract agar medium supplemented with 500 PPM streptomycin sulfate, 100 PPM chlortetracycline, 100 PPM neomycin sulfate, and 5 PPM methyl benzamidazole carbamate phosphate (Pitt *et al.* 1996). The cultured fungal isolates were tested for laccase activity using a guaiacol drop test and were microscopically examined for morphological features characteristic of *C. purpureum* (Stalpers 1978).

One culture sample from each plot was selected from putative *C. purpureum* recovered from the trembling aspen and red maple samples taken from trials in New Brunswick. Three cultures per plot were selected from the Ontario trembling aspen and speckled alder trial samples. Samples from Ontario were coded prior to genetic analysis to ensure an impartial "blind" experiment and included several unrelated "quality control" *C. purpureum* samples (as a double-check of the molecular markers). Selected culture samples were stored in sterile distilled water in 1.5 ml tubes at 4°C after being mailed to the Biology Department at the University of Victoria.

Molecular genetic analysis

Fungal isolates from all field-collected samples that were determined to be similar to *C. purpureum* were cultured at 20 °C for two weeks in 5 ml volumes of 1.5% malt extract prior to DNA extraction (Cenis 1992).

Genomic DNA was amplified using the polymerase chain reaction (PCR) using an oligonucleotide primer pair, designated APN1F+R, designed to yield specific amplification of *C. purpureum* ribosomal DNA (rDNA) (Becker *et al.* 1999a). The individual genotype of the recovered isolates was determined with a second round of PCR using another set of primers designed from a sequence-characterised amplified region (SCAR). These primers, designated APM22D13F+R, were chosen for use in this application based on the ability of patterns produced to consistently differentiate the lead isolates from each other and from other *C. purpureum* isolates (Becker *et al.* 1999a).

Extracted DNA solutions were routinely diluted 100 fold in distilled water and a 10 µl aliquot was used in 20 µl total volume amplification reactions. Along with the template DNA solution, the PCR mixture was composed of 10mM Tris (pH 8.3), 50mM KCl, 2mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 200 µM each dNTP, 10 pmol each primer and 0.4 units of *Taq* polymerase (Pharmacia). Samples were amplified by 5 cycles of denaturation at 94°C for one minute, annealing at 55°C for 1.5 minutes, and extension at 72°C for two minutes followed by 25 cycles in which the annealing temperature was raised to 60°C. The PCR products were separated by electrophoresis on a 1% agarose/TAE gel and were visualized under UV light after staining with ethidium

bromide. To reduce experimental error, DNA was extracted independently twice from each sample, and the PCR reactions were repeated to verify results.

RESULTS AND DISCUSSION

Six field trial sites were established in British Columbia, Ontario and New Brunswick to test the efficacy of *C. purpureum* against chemical herbicides for the control of deciduous forest weeds. The B.C. trial results have been reported separately (Becker *et al.* 1999a) and I discuss the Ontario and New Brunswick trials in this report. In all trials, the *C. purpureum* isolates M0022 (2139) and F0144 (JAM6) were crossed with two different formulations (BCF and ONF) in order to determine whether there were any effects on survival or infection by the active biological agents as a result of the materials used to formulate the fungus. To determine whether the formulation adjuvants had intrinsic effects on stump resprouting,"blank" formulations, without the biocontrol agent *C. purpureum*, were also applied. Furthermore, the application of the blank formulation allowed the investigation of whether the formulations alone promoted infection by local fungi. Stumps that were cut without any further treatment provided an operational control for the treated plots. The control plots could also permit entry to local fungal spores, allowing an estimation of the local incidence of *C. purpureum* spores dispersed by natural inoculum at the time of application.

I have previously established that the PCR marker APN1 could be used to distinguish *C. purpureum* from other fungi and the APM22D13 marker could be used to distinguish among individuals within a *C. purpureum* population (Becker *et al.* 1999a). For this

study, *C. purpureum* infection was surveyed following biocontrol application on cut trembling aspen and red maple stems in New Brunswick and trembling aspen and speckled alder stems in Ontario. Four months after field application, destructive sampling of stumps was performed in order to estimate the extent of *C. purpureum* infection of the treated and control stumps. Five stumps per plot were (originally) collected for a total of thirty stumps from each of the eight treatment regimens at each field trial site. Stems were sampled at a six cm depth below the cut surface and wood chips containing the cambial layer were cultured on selective media. After preliminary screening of recovered cultures for laccase activity and *C. purpureum*-like morphology, molecular genetic analysis was used to confirm the presence and identity of *C. purpureum* in experimental stem samples. The cultured *C. purpureum* fell into one of three categories: (1) released biocontrol strain M0022, (2) released biocontrol strain F0144, or (3) naturally occurring wild types.

One culture sample from each plot was selected for DNA analysis from the trembling aspen and red maple trials in New Brunswick. Genetic analysis of samples from the New Brunswick trials provided verification that all recovered *C. purpureum* individuals were identical to those originally applied and were genetically uniform (Table 5). Two

Table 5. *C. purpureum* infection of trembling aspen and red maple in New Brunswick field trials.

	New Brunswick trembling aspen					New Brunswick red maple				
Treatment	Block1	2	3	4	5	Block1	2	3	4	5
ONF/ F0144	F [a]	F	F	F	0 [b]	F	F	F	0	0
ONF/M0022	M	M	M	M	M	0	0	M	0	M
BCF/ F0144	F	F	0	0	F	F	F	F	F	F
BCF/ M0022	0	M	M	M	0	M	M	M	0	0
ONF alone	0	0	0	+0 [c]	0	0	0	0	0	0
BCF alone	0	0	0	0	0	0	0	0	0	0
Cut control	0	0	0	0	0	0	0	0	0	0
Release®	0	0	+0	0	0	0	0	0	0	0

[a] "F" indicates the recovery of the *C. purpureum* isolate F0144, and M indicates the *C. purpureum* isolate M0022 from stump samples.

[b] "0" indicates that no *C. purpureum* was recovered from stump samples.

[c] "+0" indicates that a fungal culture was recovered having morphology like *C. purpureum*, but DNA test with *Chondrostereum*-specific marker (APN1) was negative.

samples that were both from control plots were originally classified as *C. purpureum* based on morphology but did not amplify the *C. purpureum*-specific marker, so were concluded to be some other hymenomycete. Thus, no verified *C. purpureum* was recovered from any of the control stump samples in the New Brunswick trials. Therefore, no incidence of local *C. purpureum* infection was recorded at the New Brunswick sites four months after field trial establishment. These results indicated that infection of the New Brunswick host weeds was generally successful and that there was no cross-contamination of *C. purpureum* applications during their preparation or field application.

Somewhat more extensive sampling was performed at the Ontario field release sites. Three culture samples were selected from each plot for DNA analysis. These samples, recovered from trembling aspen and speckled alder, were coded in a blind experiment to rigorously test the genotype determination methodology and to provide an impartial comparison of morphological analyses with DNA results. DNA was extracted twice from each culture and assayed using PCR with the APN1 primer pair which specifically amplifies rDNA from *C. purpureum*.

The APN1 marker was very effective at discriminating between *C. purpureum* and other hymenomycete fungi which were originally grouped together according to mycelial morphology (Tables 6 and 7). On speckled alder, an average of 79% of stumps from biocontrol-treated plots were estimated to be infected using morphological criteria. This

Table 6. *C. purpureum* infection of speckled alder in Ontario field trial.

	Morphological analysis[a]						DNA analysis[b]															
Treatment	Block						Block															
	1	2	3	4	5	%[c]	1			2			3			4			5			%[d]
ONF/F0144	2	5	5	5	5	73	--[e]	F[f]	F	F	F	F	F	F	F	F	F	F	--	F	F	87
ONF/M0022	4	5	5	6	5	83	M	M	M	M	--	--	M	M	M	M	M	M	M	M	M	87
BCF/F0144	6	5	6	5	5	90	--	F	*[g]	F	F	F	F	F	--	F	F	F	F	F	F	80
BCF/M0022	4	5	4	4	4	70	M	M	M	M	M	M	M	M	--	M	--	M	M	M	*	80
ONF alone	1	0	2	0	0	10	F[h]						L[i]	--					--			7
BCF alone	0	3	0	2	2	23				--	Q[j]	*				--	*		--	--		0
Cut control	2	1	2	1	0	20	--	--		--	Q		--	Q	*	--			--			0
Release ®	0	0	0	0	0	0																

[a] for morphological analyses, a total of 6 samples were taken per plot
[b] three samples per plot were subjected to DNA analyses
[c] percentage is of 30 total samples per treatment
[d] percentage is of 15 total samples per treatment
[e] "--" indicates a sample is not *C. purpureum* based on molecular analysis (APN1 negative)
[f] "F" indicates the infection of stump samples by *C. purpureum* biocontrol isolate F0144, while "M" indicates infection by *C. purpureum* isolate M0022.
[g] "*" denotes a missing sample
[h] an isolate having the same genotype as F0144 was recovered from a control stump treated with the ONF formulation alone
[i] "L" is a local *C. purpureum* isolate recovered from a control stump treated with ONF formulation alone
[j] "Q" signifies quality control samples

Table 7. *C. purpureum* infection of trembling aspen in Ontario field trial.

	Morphological analysis[a]						DNA analysis[b]															
Treatment	Block						Block															
	1	2	3	4	5	%[c]	1				2			3			4			5			%[d]
ONF/F0144	6	4	4	4	5	77	--[e]	--	--	--	--	--	F[f]	--	F	F	--	F	--	--	--	27
ONF/M0022	5	6	4	6	5	87	M	M	M	M	--	M	--	--	M	M	--	*[g]	M	M	--	60
BCF/F0144	3	6	5	4	5	77	F	F	--	--	F	--	F	F	*	F	--	*	F	F	F	60
BCF/M0022	5	6	5	4	6	87	M	M	--	M	--	--	--	M	M	--	M	M	M	M	M	67
ONF alone	0	3	3	5	4	50	--	--	--	--	--	--	--	--	*	--	*	*	--	--	*	0
BCF alone	1	4	4	3	4	53	--	Q[h]		--	--	*	--	--	--	--	--	--	--	--	--	0
Cut control	3		4	3	4	47	--	--	--				--	--	--	--	--	*	--	F[i]	*	7
Release ®	0	0	0	0	0	0																

[a] for morphological analyses, a total of 6 samples were taken per plot
[b] three samples per plot were subjected to DNA analyses
[c] percentage is of 30 total samples per treatment
[d] percentage is of 15 total samples per treatment
[e] "--" indicates a sample is not *C. purpureum* based on molecular analysis (APN1 negative)
[f] "F" indicates the infection of stump samples by *C. purpureum* biocontrol isolate F0144, while "M" indicates infection by *C. purpureum* isolate M0022
[g] "*" denotes a missing sample
[h] "Q" is a quality control sample
[i] an isolate having the same genotype as F0144 was recovered from a control stump treated with the ONF formulation alone

was in the same range as the 84% average infection estimated using DNA analysis (Table 6). On trembling aspen in Ontario, an average of 82% of stumps were estimated to be infected by *C. purpureum* using morphological analysis, yet an average of only 54% infection on aspen was confirmed using DNA tests, suggesting that morphological assessment alone could overestimate the infection frequency (Table 7).

The genetic markers were able to eliminate many false positives in the (non-herbicide treated) control stumps. Based on phenotypic criteria, *Chondrostereum*-like fungi were isolated from an average of 18% of cut control, ONF and BCF-treated speckled alder stumps and 50% of trembling aspen control stumps. Subsequent DNA analysis greatly reduced this estimate, as only one biocontrol-infected stump was verified in control plots in each of the Ontario field sites, representing a 2% average infection in cut control plots. Morphological assessment alone therefore does not provide an accurate estimation of *C. purpureum* infection.

As it was imperative to confirm that the specific biocontrol isolates released (M0022 and F0144) were recovered from the targeted stumps, those samples that were verified to be *C. purpureum* using the APN1 marker were further analyzed using the APM22D13 marker. With only two exceptions, the biocontrol isolates were recovered only from stumps to which they were applied in the Ontario trials (Tables 6 and 7). There was no cross-contamination of biocontrol-treated stumps. However, using the APM22D13 primers, an isolate which amplified the same banding pattern of DNA as F0144 was recovered from a BCF-treated plot in the speckled alder site and from a cut control plot in

the trembling aspen trial site. These isolates may have infected these control stumps during field trial application by contamination of equipment or applicators with the JAM6-containing paste formulation. Alternatively, they could be isolates local to these Ontario sites which are so closely related to the JAM6 isolate; they are indistinguishable by the marker employed.

Five of the coded samples from the Ontario data set produced patterns of bands that were unlike either biocontrol isolate upon amplification with the APM22D13 primers. Four of these isolates were later confirmed to be "quality control" *C. purpureum* samples that were completely unrelated to the biocontrol isolates. These samples had been coded and sent for DNA analysis along with trial samples to test the ability of the genetic screening to unambiguously type isolates. All the "QC" samples in the data set were unmasked by the APM22D13 primer pair (Tables 6 and 7). The other unknown *C. purpureum* isolate was recovered from a ONF formulation-alone control plot in the speckled alder trial, and represented a local *C. purpureum* isolate which may have infected the stump through a spore or spores landing on the freshly cut surface of the stump during the field trial application. Alternatively, this isolate may have already been infecting the stem prior to the field trial application, although only healthy-looking trees were chosen for treatment.

Based on samples that were verified to be biocontrol isolates using *C. purpureum*-specific molecular markers, the average infection of biocontrol treated speckled alder stems was 80% (BCF/ M0022 and BCF/ F0144) to 87% (ONF/ M0022 and ONF/ F0144) of treated stems. The trembling aspen trees appeared not to be as successfully infected

with *C. purpureum,* with a range of 27% (ONF/F0144) to 67% (BCF/ M0022) of stems estimated to be infected by biocontrol isolates. The variation in the degree of infection among treatments in the aspen trial implies that the capacity for improved infection may exist with selected *C. purpureum* strains and/or different application methodology.

CONCLUSIONS

Development and registration of *C. purpureum* mycelium as a safe and effective biocontrol agent requires an understanding of the dynamics of growth and interaction of the fungus with each weed host considered. It is fundamental to firmly establish that post-treatment effects on stump resprouting and mortality were directly attributable to infection of target weeds by biocontrol isolates of *C. purpureum.* In this study, molecular markers were successfully utilized to identify and differentiate recovered *C. purpureum* cultures four months after biocontrol field release, thus providing an accurate and easy-to-use tool for environmental monitoring. The application of both biocontrol isolates (M0022 and F0144) was very target specific, being for the most part limited to the stumps to which they were applied. No cross-contamination of biocontrol isolates (M0022 and F0144) was detected, suggesting that there were no errors in inoculum preparation or in field trial application of fungal treated plots. A low level of possible infection of control stumps by the released Ontario isolate F0144 was detected in 2% of brushed-alone stumps in Ontario trials. It may be significant that the B.C. isolate (M0022) was not found in the control plots. While precautions should be taken to avoid non-target infection in future applications, this small amount is not likely to result in further problems. A local isolate was found in a formulation-alone control plot in the

speckled alder trial site in Ontario, representing an average of 2% infection of control stumps in that trial. This rate of occurrence of "background" *C. purpureum* is consistent with previous studies of *C. purpureum* incidence in Western Canada (de Jong *et al.* 1996). No local *C. purpureum* isolates were found to be infecting stems from New Brunswick.

The difference in biocontrol infection frequency between the two weed tree hosts of the Ontario trial, 84% of specked alder and 54% of trembling aspen, is hypothesized to be predictive of the relative success/impact of biocontrol on speckled alder as a target species. Lower efficiency of infection may indicate that a particular host species may have sufficient natural defense mechanisms to inhibit the growth of *C. purpureum.* For improved biocontrol efficacy, the extent of infection by *C. purpureum* must be maximized for each potential weedy host species. With sufficient samples, it is expected that a survey of infection frequency using molecular markers could provide an early indicator for biocontrol treatment success.

Molecular genetic markers are indispensable diagnostic tools for biocontrol research and development. The *C. purpureum*-specific PCR primers will be further used to study the epidemiology and environmental fate of *C. purpureum* after biocontrol release. The application of the *C. purpureum*-specific DNA markers to national field trial samples in this study has demonstrated the value of genetic testing for monitoring biocontrol infection.

Chapter 5. Efficacy and persistence of *Chondrostereum purpureum* as a biocontrol for red alder.

INTRODUCTION

Environmental concerns have called for the reduction of chemical herbicide use in forests and have led to the support of concepts of integrated vegetation management, sustainable forestry and the maintenance of biodiversity. The use of chemical herbicides is at present the preferred approach for vegetation management in reforestation sites and in utility company rights-of way. Mechanical treatment alone is ineffective as the target tree species resprout vigourously without further treatment. There is therefore a need for effective alternative treatments such as indigenous plant pathogenic fungi formulated for use as biocontrol agents for weeds, known as mycoherbicides (Templeton *et al.* 1979). While biocontrol of forest weeds using indigenous mycoherbicides is designed to minimize risk, it is a new approach which should be carefully evaluated for all impacts.

The white-rot basidiomycete *Chondrostereum purpureum* (Pers.) Pouzar is found throughout the temperate zones of the world and has a broad-spectrum pathogenicity towards many hardwood species but is a facultative saprophyte. A primary invader of woody angiosperms, *C. purpureum* usually enters its host through a fresh wound, cut stump or stem lesion (Brooks and Moore 1926, Spiers and Hopcroft 1988, Rayner and Boddy 1988). The fungus grows through xylem tissues of the host plant, causing cambial necrosis, decay, sapwood staining and sometimes death of the host (Rayner 1977, Wall 1986, 1991). Infection by *C. purpureum* can also cause foliar discolouration or silvering,

and thus it is also known as silver-leaf disease of orchard fruit trees (Grosclaude *et al.* 1973, Setliff and Wade 1973, Bishop 1979).

In Canada, it has been demonstrated that application of *C. purpureum* mycelium to the cut surfaces of hardwood stems reduces the number and viability of resprouts (Wall 1990). The effectiveness of *C. purpureum* as a mycoherbicide has been evaluated for the control of *Acer rubrum* L. (red maple), *A. saccharum* Marsh. (sugar maple), *A. macrophyllum* Pursh (bigleaf maple), *Alnus rubra* Bong. (red alder), *Betula alleghaniensis* Britton (yellow birch), *B. papyrifera* Marsh. (paper birch), *Fagus grandifolia* Ehrb. (beech), *Populus tremuloides* Michx. (trembling aspen), *Prunus pensylvanica* Linn. f. (pin cherry), and *P. serotina* Ehrh. (black cherry) (de Jong *et al.* 1990, Dumas *et al.* 1997, Harper *et al.* 1999, Jobidon 1998, Pitt *et al.* 1996, Wall 1986, 1990, 1991, 1997, Wall *et al.* 1992, 1994). The *C. purpureum* biocontrol strategy involves covering the entire surface of freshly cut target stumps with mycelial fragments in a protective nutrient formulation.

Current understanding of the ecology and pathogenicity of *C. purpureum* is steadily growing. The fungus exists in all biogeoclimactic zones in North America, which precludes the risks associated with the introduction of a foreign species for biological control. A number of studies have examined the population structure of *C. purpureum* at regional to intercontinental scales, using different methods for differentiation of subgroups (Rayner and Boddy 1986 (mating interactions), (Ekramoddoullah *et al.* 1993 (SDS-protein profile), Ramsfield *et al.* 1996, 1999 (ribosomal DNA and mitochondrial

DNA), Gosselin *et al.* 1996, 1999 (RAPD), and Spiers *et al.* 2000 (morphology, pathogenicity, RAPD). The associated risk of moving isolates over large geographic areas and across biogeoclimatic zones was assessed with the conclusion that the *C. purpureum* population is panmictic and has no geographic or host specialization, Gosselin *et al.* 1999, Ramsfield *et al.* 1996, 1999).

The wide host range of *C. purpureum*, recorded on over one hundred hosts, is an advantage to a candidate biocontrol agent, but can be considered a hazard when assessing the associated risks of treatment. Studies have indicated a low risk of non-target infection associated with the application of *C. purpureum* mycelium (Becker *et al.* 1999a, 1999b), but secondary infection from spores originating from the biocontrol-treated stumps is of concern (Gosselin *et al.* 1999). Basidiocarps are usually produced by the fungus on infected stumps and trees and may persist for up to two years following biocontrol treatment (Wall 1997). When environmental conditions are favourable, basidiospores are released which may infect freshly wounded deciduous tree species. In Canada and in other countries, *C. purpureum* is commonly found in logged areas, woodpiles and pruned areas, and these are considered the most important inoculum sources for the phytopathogen.

A similar increase in spore load with a similar environmental impact is hypothesized to be associated with the use of *C. purpureum* as a stump treatment. An extensive study was performed in 1993 and 1994 of the natural occurrence of *C. purpureum* on Vancouver Island in relation to its use as a biocontrol agent, which found that the added

fructification due to biocontrol use was the same order of magnitude or less than naturally occurring levels (de Jong *et al.* 1996). A fate assessment of *C. purpureum*, concerned with the emission and transmission of spores, has been performed by modelling the dispersal part of the epidemiological process to simulate the dissemination of the disease (de Jong 1992, de Jong *et al.* 1990, 1991). Many factors influence or limit the spread of *C. purpureum* spores into the environment: weather conditions which influence the release, dispersal, deposition germination and survival of spores as well as the stress level and susceptibility of the hosts (Grosclaude *et al.* 1973, Wall 1991). Grosclaude *et al.* (1973), stressed the low effectiveness of *C. purpureum* to infect wounded trees. According to their observations, it is unlikely that one spore could establish infection in a wound; instead, infection of a susceptible *Prunus* tree required at least 22 spores to induce significant silverleaf symptoms and no infection occurred if too many spores germinated on a wound. However, while an exception to the usual results, infection of a fresh wound was successful from as few as two spores of *C. purpureum*. Spiers and Hopcroft (1988) showed that only a few spores are necessary and de Jong *et al.* (1990) reported that 10 spores on a wound were enough (20% probability) to cause infection.

The requirement of a fresh wound for infection allows *C. purpureum* to be applied specifically to host individuals. If the *C. purpureum* formulation is applied to only part of a stump, resprouting often occurs in the unaffected region of the stump. This dependence on a freshly wounded host is hypothesized to be the most important constraint limiting infection by *C. purpureum*. The application of *C. purpureum* mycelium to freshly cut stumps largely removes this constraint and hence efficacy of

biocontrol is then dependent upon other factors affecting colonization and competitive ability such as lateral rate of growth and production of phytotoxins. The production of *C. purpureum* basidiocarps has been estimated to result in 160 basidiospores per cubic meter at 500 meters from the point of emission, dropping to four basidiospores per cubic meter at 5000 meters (de Jong *et al.* 1990). It was hypothesized that indirect validation of spore dispersal models could be achieved by wounding susceptible trees all around a biocontrol site while basidiocarps are sporulating and relating occurrence of infection by the introduced individual (confirmed by genetic fingerprints) to spore concentrations predicted by dispersal models.

The following experimental trial was established to gain information on the biological impact, in terms of efficacy and environmental fate of *C. purpureum*, applied under nearly operational conditions as a formulation which could be developed as a commercial product. As part of this study, spore traps were established in order to assess the impact of the field trial on non-target host plants and the local *C. purpureum* population. Based on research by de Jong *et al.* (1990, 1991), spore traps were placed within areas in which a greater number of spores due to the field trial would be expected. Spore-trapping red alder trees were cut at various distances from the trial, when fructification on stumps within the trial was at a maximum. It was hypothesized that the biocontrol isolate, having vigorous growth characteristics, could persist around the treated area. The relative frequency of encountering a biocontrol isolate compared to a local isolate was compared in order to estimate the magnitude of effects of the trial compared to those due to the background levels of *C. purpureum* spores.

This field trial was established in a power line ROW, to examine the dynamics of this mycoherbicide pathosystem and to evaluate 1) the potential of a formulation of *C. purpureum* mycelium to inhibit red alder stump sprouting compared to manual brushing and chemical herbicide treatments, 2) the persistence of *C. purpureum* sporocarps, 3) the succession of other fungi on stumps, and 4) the persistence of the released *C. purpureum* genotype.

MATERIALS AND METHODS

Pre-trial population sampling

Prior to the establishment of the field trial, a collection of *C. purpureum* infected wood was taken from stumps which were also under the power lines and adjacent to the trial site. Wood chips containing cambium tissue were taken from immediately beneath *C. purpureum* basidiocarps and were surface sterilized, cultured, identified and stored as previously described (Becker *et al.* 1999a).

Site description and experimental design

The field trial site was established in a power line right-of-way 10 km west of the city of Duncan on Vancouver Island at latitude 48°49'N and longitude 123°50'W. The experimental area straddled a shallow stream and varied in slope and drainage. Buffer strips (3 meters wide) were cleared between plots during the early summer of 1994 and plots were established in September of 1994, a time of year considered suitable according to previous studies (Wall 1994). Each plot contained at least twenty healthy red alder

trees each with a diameter of 5 to 10 cm at base height. A total of 25 treatment plots were established in a complete randomized block design consisting of five blocks which were established based on location. Each block consisted of five treatment plots and each treatment plot had 20 tagged red alder trees that were cut and treated. Trees were cut by chainsaw and all stumps within a clump or coppice were similarly treated with one of: *C. purpureum* isolate 2139, glyphosate spray (12%), Carbopaste™ (12% glyphosate in a paste base material), or formulation alone. Control stumps were cut but left untreated.

Preparation of C. purpureum inoculum

Chondrostereum purpureum isolate 2139 was collected in southern Vancouver Island in June, 1994 from *Alnus rubra*. Inoculum was prepared from actively growing Petri dish cultures growing on 1.25% malt agar. Plugs, 5-8 mm in diameter, were removed aseptically from the advancing zone of the culture, macerated, and suspended in 100 ml aliquots of 1% malt extract broth in Ehrlenmeyer flasks. Flasks were placed on 100 r.p.m. rotary shakers at room temperature for two to five days and the contents then aseptically added to the dry formulation.

The dry formulation consisted of 375 g talc, 100 g kaolin, 12.5 g corn starch, 5 g pectin, 5 g monosodium glutamate, 1 g monopotassium phosphate and 1.5 g yeast extract (Wall 1996). Ingredients were thoroughly mixed, placed in autoclavable plastic bags and autoclaved at 15 p.s.i. for 20 minutes. After cooling, the bags were inoculated with the malt broth culture, controls (formulation only) receiving sterile malt broth only. After inoculation, the contents of each bag were agitated to distribute the mycelial fragments

and the bags were incubated at room temperature for 3-8 weeks. During incubation, bags were agitated at weekly intervals to maintain uniform growth. Immediately before inoculation in the field, the contents of each bag were mixed with 600 ml of of sterile 1% sucrose, 200 ml canola oil, 60 g finely powdered cellulose (Polyphila, LePages's Ltd., Brampton, ON) and the yolks from 2 grade A large eggs. The mixture was stirred vigorously to form a smooth paste, and applied to stumps in a plastic squeeze bottle.

Field treatments

Trees were felled with a chainsaw to leave 15 cm high stumps with horizontal surfaces. The diameter of each stump was recorded and inoculum applied within 30 minutes of felling. Inoculum was applied to the cambial region of each stump as a continuous band, at the rate of about 0.2 ml of liquid fomulation per cm of stump circumference (e.g. a 10 cm diameter stump would have received 0.2 x 10 π = 6.3 ml). Sterile control stumps (formulation only) were treated in the same manner using uninoculated formulation. Stumps treated with glyphosate were either sprayed to saturation with 12% spray or a thin bead of Carbopaste™ was placed on their perimeter (cambium).

Field trial assessment

Cut stumps in all the treatment plots were examined for resprouting and mortality during the mid summer of each of the succeeding two years. The number of living sprouts was recorded for each stump during the field examination and stump mortality was calculated based on the presence or absence of living sprouts on each of the 20 stumps within

treatment plots. The presence of basidiocarps of *C. purpureum* and other basidiomycetes on stumps was monitored continuously and was assessed 18 months after inoculation.

Sporetrap establishment and sampling

Untreated alder trees cut at a height of about three feet were established as spore traps in the fall of 1996 during a period of massive fruiting of *C. purpureum* within the field trial [established summer of 1994]. Approximately 100-200 trees (2-5 cm diameter) were cut in each of three spore-trap sites at distances of 50 meters, 700 meters and 1000 meters from the field site along the same right-of-way. Trees chosen as spore traps were healthy and vigorous and previously uncut, and were primarily from the edges of the ROW. During the following 12-24 months, spore traps were monitored and wood showing symptoms or signs of infection (staining, presence of basidiocarps) was cut and taken to the laboratory for culturing of fungi and genotype analysis.

DNA extraction and amplification

Mycelium for DNA extraction was grown in 5 ml volumes of 1.5% malt extract broth for approximately two weeks, collected by filtration, washed with water, frozen and freeze dried. Dry mycelium was ground with sterile sand with plastic pestles in 1.5 ml tubes and genomic DNA extracted according to Möller *et al.* (1992).

For genetic analyses, amplification reactions using the APD13 primers were performed as previously described (Becker *et al.* 1999a), using an annealing temperature of 60°C for 30 cycles. Reactions were each independently amplified twice, separated on 1.5%

agarose gels and visualized under ultraviolet light by ethidium bromide staining. Photographed gels were scored at least twice and banding patterns for each isolate were recorded as a binary code, with a '1' denoting the presence of a band, and a '0' its absence, at a particular locus defined as a band size. All DNA samples were also amplified using species-specific primers APN1 (Becker *et al.* 1999a) for verification of identity as *C. purpureum* and as a positive control for DNA quality (data not shown).

Analyses

A similarity index based on the proportion of bands shared by two individuals, was computed as twice the number of shared bands divided by the total number of bands scored in the two individuals (Nei and Li 1979). This was computed for each isolate in the pre- and post trial populations, compared with the released isolate.

The computer program PAUP Version 4.0b4a (Swofford 2000) was used to compute a distance matrix among all isolates. Isolates were compared using the Neighbor Joining algorithm (Saitou and Nei 1987) to test for subgrouping within and among the populations. Data were resampled by bootstrapping to test for reliability of clustering.

RESULTS

Trial assessment

Sprouting of cut stumps began during the spring of 1995 and had reached a maximum height of 50 cm by mid summer, the time of the first assessment. Significantly fewer living sprouts were found on stumps treated with *C. purpureum* or chemical herbicides

than found on the untreated controls (slash) (Table 8). Sprouting had occurred on many of the stumps treated with *C. purpureum*, but considerable sprout mortality was evident by mid-July. The number of living sprouts on stumps treated with *C. purpureum* was significantly less than on stumps in the 'slash' control but not the 'formulation control' plots.

Both the number of resprouts and the mortality of stumps differed significantly among treatments by 1995, one year after establishment of the trial (ANOVA, $p<0.05$) (Table 9). The overall *C. purpureum* treatment effects were not significantly different from that due to chemical herbicide treatment but were significantly different (Planned contrasts, $p<0.01$) from the overall untreated formulation and slash controls (Table 9). On the basis of individual treatments, *C. purpureum* plots were not different from either glyphosate or Carbopaste™ plots but were significantly different from formulation and slash controls (Table 9) as evident by 80% more reduction of resprouts and 20% more stump mortality (Table 8).

Field examination made in mid summer of 1996, the second growing season after field trial establishment, showed near-complete mortality of stumps treated with Carbopaste™ or *C. purpureum* and more than 95% mortality of liquid glyphosate-treated stumps (Table 8). In comparison with 1995, all the treatment plots exhibited less sprouting and more mortality of stumps (Table 8). The *C. purpureum* treatments in 1996 were not significantly different from chemical herbicides but were significantly different than

Table 8. Percent mortality and number of living sprouts on cut and treated *Alnus rubra* stumps.

Treatment	1995		1996	
	% Mortality	Sprouts per stump	% Mortality	Sprouts per stump
Slash control	65.0 b	4.45 a	86.0 ab	1.18 a
Formulation control	70.0 b	3.49 ab	72.0 b	0.37 b
C. purpureum 2139	92.0 a	0.45 c	100.0 a	0.00 b
Glyphosate	97.0 a	0.35 c	99.0 a	0.01 b
Carbopaste	100.0 a	0.01 c	100.0 a	0.00 b

Treatments with the same letter are not significantly different by Duncan's multiple range test at $P \leq 0.05$.

Table 9. Analysis of variance and planned contrasts (*P* values) of stump mortality and resprouting of *Alnus rubra* treated with *C. purpureum* and chemical herbicides

Source/contrast	1995		1996	
	Mortality	Living sprout #	Mortality	Living sprout #
Overall treatment	0.0010	0.0147	0.0737	0.0031
Cp vs. untreated	0.0022	0.0030	0.0163	0.0013
Cp vs. herbicides	0.0680	0.5965	0.8380	0.9809
Cp vs. glyphosate	0.5422	0.9424	0.9232	0.9461
Cp vs. carbopaste	0.3330	0.7509	1.0000	1.0000
Cp vs. formulation	0.0130	0.0378	0.0128	0.2198
Cp vs. slash	0.0032	0.0084	0.1868	0.0006

untreated formulation and slash controls (Table 9), which followed the trend seen by 1995.

Basidiocarp assessment

The fungus *C. purpureum* generally produces basidiocarps about 18 months after infection of red alder stumps. On the experimental site, the peak production of *C. purpureum* basidiocarps occurred in the spring of 1996 on 66% of the stumps treated with *C. purpureum*, about 19% of the stumps treated with chemical herbicides, and 43% of stumps in formulation and slash controls (Table 10). Basidiocarps of *Trametes versicolor* (L.) C.G. Loyd, *Schizophyllum commune* (L.) Fr., and other basidiomycetes were also observed on many of the stumps in all of the treatment plots (Table 10).

In a follow-up assessment of the trial in July 1997, basidiocarps of *C. purpureum* were observed on 23% of the stumps treated with the fungus. On the other treatments, occurrence of *C. purpureum* was less than 2%. No living sprouts were found on any stump bearing *C. purpureum* basidiocarps. Evidence of secondary colonization and advanced decay was apparent and basidiocarps of *T. versicolor*, *S. commune* and other basidiomycetes were recorded on many of the stumps.

Pre-trial population vs. spore traps

In the summer of 1997, eight isolates verified to be *C. purpureum* were recovered from 100 tagged alder stumps from the spore-trap site established 50 meters from the field trial. The spore-traps that were 700 meters from the field site yielded 34 verified isolates

Table 10. Percentage of *Alnus rubra* stumps in each treatment with basidiomycete fruitbodies.

Treatment	*Chondrostereum purpureum*	*Schizophyllum commune*	*Trametes versicolor*	Others
Slash control	42 bc	19 a	28 a	37 a
Formulation control	43 bc	17 a	13 ab	21 b
C. purpureum 2139	66 ab	13 a	18 ab	19 b
Glyphosate	15 c	3 a	6 b	11 b
Carbopaste	23 c	13 a	4 b	8 b

Treatment means with the same letter are not significantly different by Duncan's multiple range test at $P \leq 0.05$.

of *C. purpureum* from 200 spore traps. Sporocarps of *C. purpureum* were also noted on slash cut from spore-traps. At a distance of 1000 meters from the field site, only one isolate of *C. purpureum* was recovered from 200 spore-trap alder stems. Field-collected samples were amplified using SCAR fingerprinting markers (Becker *et al.* 1999a) and banding patterns (Figure 13) were compared. Relative similarity to the released *C. purpureum* isolate 2139 determined by band sharing was computed for each member of the pre-trial population and ranged from 12 to 64 % (Table 11). Members of the post-release population ranged from 12 to 64% similarity to the released isolate. There was no significant difference in average band sharing with the isolate 2139 among pre-trial (37% average) and spore-trap (38% average) populations (Table 11).

A distance matrix was computed which compared all isolates (data not shown). The phylogenetic tree produced using the distance-based neighbor joining algorithm did not cluster taxa of pre-and post- trial population sub-groups (data not shown). Bootstrap resampling of data (1000 times) resulted in a polytomy 'comb' with no grouping of any taxa. Likewise, when data from the released isolate (2139) were added to the analyses, no nodes were supported and there was no clustering among taxa. When the tree was rooted using the released isolate (2139), branch lengths from pre-and post- trial populations to the root were equal (data not shown).

DISCUSSION

Efficacy

Field trials can provide a proof of concept as well as an ecological experiment, which

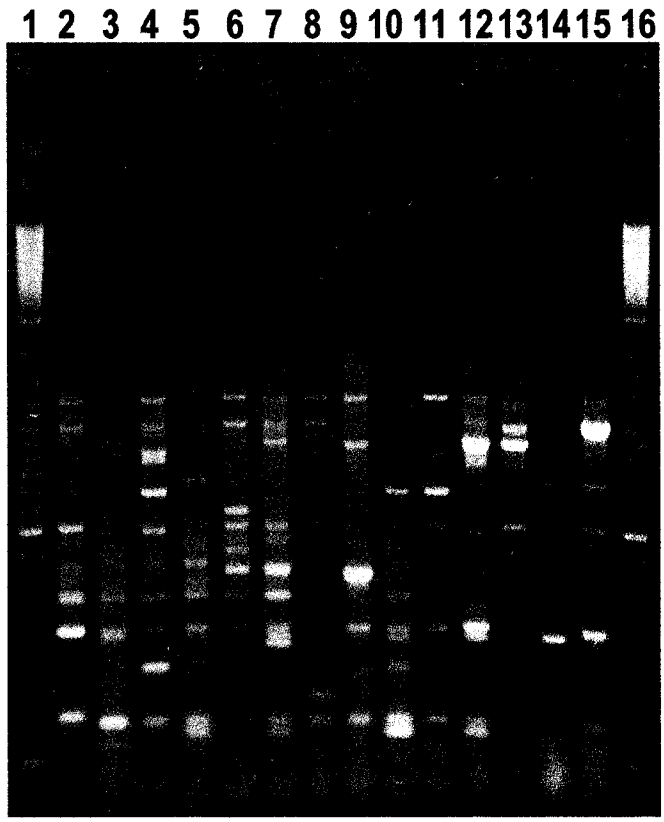

Figure 13. Banding patterns generated by isolate-specific PCR marker, from pre-trial and spore-trap collections of *C. purpureum*. Results of amplifications using SCAR primer pair APD13F+R with template DNA are shown for a selection of field samples. Biocontrol isolate 2139 is shown in lane 2, along with spore-trap samples ST056 (lane 3), ST062 (lane 4), ST083 (lane 5), ST105 (lane 6), ST114 (lane 7), ST122 (lane 8), ST156 (lane 9), ST157 (lane 10), ST167 (lane 11), and pre-trial samples F0167 (lane 12), F0168 (lane 13), F0170 (lane 14), F0172 (lane 15). Size marker (100 bp ladder, Pharmacia), which has an intensely staining 800 bp band, is shown in lanes 1 and 16.

Table 11. Similarity of *C. purpureum* isolate 2139 to isolates collected prior to and following field release, expressed as band sharing coefficents (Nei and Li 1979).

Pre-trial isolates		Spore-trap isolates		
Isolate	Similarity to 2139	Isolate	Distance from field trial (m)	Similarity to 2139
F0080	0.35	ST001	50	0.38
F0082	0.53	ST003	50	0.43
F0083	0.38	ST013	50	0.40
F0085	0.44	ST021	50	0.18
F0086	0.40	ST022	50	0.30
F0087	0.38	ST025	50	0.25
F0088	0.50	ST053	50	0.24
F0092	0.53	ST056	50	0.33
F0115	0.35	ST062	700	0.42
F0117	0.50	ST081	700	0.32
F0118	0.33	ST082	700	0.25
F0119	0.12	ST083	700	0.42
F0121	0.21	ST103	700	0.48
F0122	0.13	ST104	700	0.38
F0125	0.38	ST105	700	0.64
F0128	0.13	ST114	700	0.56
F0129	0.59	ST116	700	0.57
F0130	0.47	ST122	700	0.60
F0131	0.53	ST156	700	0.22
F0158	0.42	ST157	700	0.40
F0159	0.13	ST167	700	0.48
F0163	0.50	ST194	700	0.29
F0164	0.38	ST196	700	0.33
F0166	0.33	ST402	700	0.32
F0167	0.33	ST406	700	0.12
F0168	0.22	ST407	700	0.50
F0170	0.21	ST415	700	0.42
F0172	0.64	ST422	700	0.33
Mean	0.37179			0.37714
Median	0.38			0.38
Sum	10.41			10.56
Standard Deviation, s	0.14682			0.12803
Sample Variance, s^2	0.021556			0.016392

may allow us to disprove hypotheses and address questions posed by theoretical models, such as, "what are the biological impacts of mycoherbicide application? In this study, control of red alder by *C. purpureum* stump treatment was as effective as that obtained with glyphosate. There was no statistical difference between treatment of red alder with *C. purpureum* and treatment with glyphosate; both resulted in significant reductions in both the number of resprouts and the maximum height of resprouts. In spite of the high mortality in the untreated controls, the field applications resulted in statistically significant reduction in the amount and vigour of sprouting on red alder stumps treated with *C. purpureum* isolate 2139. Suppression of regrowth by *C. purpureum* was as effective as the liquid formulation of glyphosate and almost as effective as Carbopaste™. Plots treated with *C. purpureum* or with chemicals had little or no tree growth and would not require re-treatment for at least a decade. Most of the untreated control plots, however, had vigorous alder coppice growth which might need removal within five years to prevent their encroachment on power lines.

Generally, mycoherbicides will persist locally at an elevated level then return to endemic levels as the targeted weed population is depressed (Charudattan 1988). In the case of *C. purpureum*, the fungus is thought to persist as a saprophyte then return to endemic levels as its substrate (fresh wood) is depleted. As a primary invader of wounds, *C. purpureum* causes sapwood stain, decay and eventually host death (Rayner 1977, Rayner and Boddy 1986). Upon weakening the host, *C. purpureum* is replaced by other more aggressive saprobic fungi such as *Trametes versicolor* and *Schizophyllum commune* (Rayner and Boddy 1986, Wall 1997) which was observed in *C. purpureum* infected

stumps in this trial. These fungi may accomplish the death of the host and the rapid succession ensures *C. purpureum* will not persist at high levels following the inundation of an area for biocontrol treatment.

Biochemical features of *C. purpureum*, such as the silvering agent, endoPG (Miyairi *et al.* 1985) and the production of a unique group of sesquiterpene metabolites, sterpurenes (Xie *et al.* 1992, Strunz *et al.* 1997) have been characterized, but only endoPG has been linked to pathological symptoms. Virulence is dependent on the ability of the fungus to rapidly colonize its host, as well as phytotoxin production and effects. Hence the pathogenic mechanism of *C. purpureum* is likely dependent on many factors and has not yet been elucidated. Ekramoddoullah *et al.*(1993) found that most *C. purpureum* isolates varied slightly in virulence, and Wall *et al.* (1996) observed few significant differences in virulence of mono and dikaryotic isolates of *C. purpureum* on plant tissue cultures and rooted poplar cuttings.

While 2139, the isolate chosen for use as a biocontrol agent, was selected on the basis of virulence as expressed by canker size on red alder (Wall 1996), these selection criteria do not automatically correlate to virulence expressed as silver leaf symptoms, necrosis in plate disk assays or other virulence assays. Isolates selected for biocontrol use are not necessarily more "fit", but the concern has been expressed that these isolates will persist and cause an increase in the incidence of silverleaf disease in fruit trees.

If the isolate used in the field trial was more "fit" and was able to pass on proportionally more of its genotype than individuals in the local population, the population would become more similar to the biocontrol isolate. This effect would be similar to that seen due to genetic drift, population bottleneck or the founder effect, resulting in less diversity within the population and more diversity between populations. The genes or alleles that conferred a selective advantage would become more frequent in the population. Since selection is a stronger force for maintaining a particular allele than is gene flow, a positively selected gene will take over rapidly in the population. This could be detected as an increase in the similarity of the post-trial population to the released isolate, as compared to the similarity of the released isolate to the pre-trial (background) population. The field trial was therefore monitored over time to test the hypothesis that inundative biocontrol application would cause a local effect of "swamping" of the resident population by the applied isolate.

APD13 marker

Molecular genetic markers have provided tools for monitoring populations at different scales, from the fine scale of individual differentiation to across biogeoclimactic zones. The SCAR marker APD13 has shown its utility for highly reproducible characterization of individuals and has been utilized for monitoring the *C. purpureum* infection frequency within infected stumps in field trials (Becker *et al.* 1999a, 1999b). Polymorphism is detected as band presence versus absence and may be caused either by failure to prime a site in some individuals because of nucleotide sequence differences or by insertions or deletions in the fragment between two conserved primer sites. The presence or absence

of shared fragments can be used to estimate the relatedness of the DNA samples. These estimates of divergence do not depend on absolute counts of bands but on the proportion of bands that are shared by samples. Fortunately, the estimation of similarity using a comparison of band sharing is independent of the efficiency of PCR in amplifying all possible target fragments. If only a fraction of expected fragments are actually detected, providing this fraction is consistent for monomorphic and polymorphic sites, the estimation of the proportion of shared bands will remain valid (Clark and Lanigan 1993). Preliminary characterization of this marker has revealed its distribution throughout the genome in a repeated motif. No bands are unique to the released isolate but it has a characteristic pattern that can be easily identified.

Spore trap conclusions

Factors involved in persistence of an individual *C. purpureum* genotype include: the inoculum potential of the infected host(s), spore dispersal ability and the availability of fresh wounds to colonize, and the competitive ability of the fungus to establish itself in the host. The heterothallic tetrapolar mating system (Nakasone 1990) and presence of many mating types of *C. purpureum* (Wall *et al.* 1996a) also affects persistence of individuals, promoting outcrossing of haploid basidiospores derived through meiosis. Likewise, the absence of barriers to gene flow among populations of *C. purpureum* (Ramsfield *et al.* 1996, Gosselin *et al.* 1999) limits the possibility of fixation of alleles due to selection or stochastic effects (Wright 1951).

I was unable in this experiment to detect the genotype of the released isolate in spore traps following inundative application of this individual, nor was I able to detect any increase in similarity of the post-trial population to the released isolate that would have suggested its persistence. While these results do not rule out the possibility of the persistence or local adaptation of *C. purpureum* containing genes or alleles which confer a selective advantage, the outcrossing nature of the fungus and its capacity for gene flow counteract this risk, and allow the fungus to maintain a high level of genetic variability.

CONCLUSIONS

1) *Chondrostereum purpureum* inoculation is as effective as chemical herbicides Carbopaste™ and liquid glyphosate in causing cut stump mortality and in reducing resprouting.

2) Overall, more than 90% of stumps treated with *C. purpureum* died in the first year and 100% died in the second year.

3) In comparison with manual cutting, or slashing, *C. purpureum* caused 90% reduction in stump sprouting.

4) Naturally occuring *C. purpureum* spores colonized about 40% of untreated cut stumps and about 20% of stumps treated with chemical herbicides.

5) Amplification of the repeated marker DNA APD13 by the PCR is a rapid and highly reproducible means of detecting polymorphisms for strain identification and analysis of genetic distance. Markers were successfully used to identify and characterize *C. purpureum* genotypes in order to compare populations and monitor effects of mycoherbicide application.

6) There was no significant difference in average band sharing with the applied isolate among pre-trial (37% average) and spore-trap (38% average) populations. Persistence of the *C. purpureum* individual used in the field trial release was therefore undetectable.

Chapter 6. An inactive retrotransposon-like element and its occurrence in populations of *Chondrostereum purpureum* in British Columbia.

INTRODUCTION

The phytopathogenic basidiomycete fungus *Chondrostereum purpureum* has excellent potential as a biological control of North American deciduous weed species (Dumas *et al.* 1997, Jobidon 1998, Pitt *et al.* 1999, Wall 1990, 1991, 1994). As a component of the program to evaluate *C. purpureum* as a biological control agent, I have developed genetic markers to monitor the environmental fate of field-inoculated strains and to evaluate strain efficacy (Becker *et al.* 1999a, 1999b, Harper *et al.* 1999). This program required a molecular-based method of strain identification that permitted both species and strain identification, for any *C. purpureum* isolates collected in the field.

The development of these genetic markers is described in Becker *et al.* (1999a), and they are sensitive enough to differentiate genetic individuals of this fungus, allowing for their identification. The SCAR (sequence characterized amplified region) primers, referred to here as APD13F and APD13R, amplify a collection of variable-length fragments from target genomic DNA of a *C. purpureum* strain, providing a unique, reproducible polymorphic banding pattern for different individuals.

Preliminary sequence characterization of the genetic marker regions amplified using APD13F+R SCAR primers detected features suggestive of repetitive elements. There are three main categories of repetitive elements, namely microsatellites, minisatellites and

transposons. Microsatellites are very small repetitive sequences, typically 20-30 base pairs in length, containing tandem repetitions of 1 to 5 base pairs. Minisatellites have a larger, though still fairly short, basic sequence of 10 to 60 base pairs, which can be repeated for lengths of 0.1 to 30 kb, showing a high level of length polymorphism. Both micro- and minisatellite sequences are found in both prokaryotes and eukaryotes and are well described for the major taxa of the fungi (Wostemeyer and Kreibich 2002).

Transposons are genetic elements found in both prokaryote and eukaryote organisms. They are generally larger in size than other classes of repetitive elements (micro- and minisatellites) and contain recognized genes similar to those found in retroviruses. Their occurrence and features in bacteria, plants and animals have been well studied and, in more recent years, their existence in fungal species has been documented. They include two main classes, which distinguishes these elements based on their method of transposition. The class I elements transpose by the reverse transcription of an RNA intermediate with a reverse transcriptase, while class II elements transpose by the excision of DNA sequence from a donor site, followed by its re-integration elsewhere in the genome (Daboussi 1996, Kempken and Kuck 1998, Wostemeyer and Kreibich 2002). Although the detection of transposable elements in fungi has occurred more recently, when compared to other eukaryotes, fungal species have been found to exhibit a variety of both class I and II elements. The structural and functional features of transposable elements detected in fungi are described in Kempken and Kuck (1998), and in Wostemeyer and Kreibich (2002).

Transposable elements have been determined to influence gene expression, gene sequence and genetic recombination in other eukaryotic organisms, in addition to their ability to transpose within a host genome. Studies of the fungi have identified similar events related to the presence of transposable elements (Daboussi 1996, Kempken and Kuck 1998). The presence of transposable elements in fungal species has proven to be a useful feature of some economically important fungi. Elements have been used in population studies for the genetic fingerprinting of fungi pathogenic to humans (Debeaupuis *et al.* 1997, Franzot *et al.* 1998, Reiss *et al.* 1998) as well as plant pathogenic fungi (Daboussi 1996, Nitta *et al.* 1997, George *et al.* 1998, Becker *et al.* 1999a, Nakayashiki *et al.* 1999), using element sequences as molecular probes, or as the template for PCR-based genetic characterization. They have also been useful in studies of horizontal gene transfer (Daboussi 1996, Nakayashiki *et al.* 1999) and for transposon-based gene tagging systems that use insertional mutagenesis (Daboussi 1996, Kempken and Kuck 1998, Wostemeyer and Kreibich 2002).

In the present study, DNA sequence fragments amplified by APD13F+R primers have been subject to further analyses to determine their possible function and origin. As a concurrent objective, these genetic markers have been used to evaluate population structure of *C. purpureum* in different regions of British Columbia, Canada.

MATERIALS AND METHODS

Field collection of isolates

Chondrostereum purpureum isolates were collected from trees growing under B.C. Hydro power lines in Mission and Kemano B.C. Host trees had been operationally cut to prevent obstruction of power lines and had become infected by natural sources of *C. purpureum* inoculum. Wood chips were taken from directly beneath *C. purpureum* basidiocarps to obtain field isolates. These were cultured from surface-sterilized wood chips and were subsequently identified and stored as previously described (Becker *et al.* 1999a).

Culture conditions and genomic DNA extraction

For DNA extraction, fungal cultures were grown at 20 °C in 1.5% malt extract (Difco) broth for two weeks prior to harvest by filtration. Mycelium was then washed with water and lyophilized. Total genomic DNA was extracted from freeze-dried tissue according to Möller *et al.* (1992), and was resuspended in TE for Southern hybridizations, or diluted 1:200 in ddH_2O for PCR.

PCR amplification

PCR amplification was performed as previously described (Becker *et al.* 1999a). All DNA samples were amplified using species-specific primers APN1F+R (Becker *et al.* 1999a), for verification of isolate identity as *C. purpureum* and as a positive control for DNA quality. Amplification using APD13 SCAR primers was performed in 20 µl total reaction volumes and used an annealing temperature of 60 °C for 25 cycles in a Perkin

Elmer thermocycler. PCR products were separated by agarose gel electrophoresis followed by staining with ethidium bromide.

Population analyses

Computer-aided data analysis of the DNA fingerprints was performed using Molecular Analyst software (Bio-Rad). Unequivocally scorable and consistently reproducible amplified DNA fragments were transformed into binary character matrices (1 for presence, 0 for absence). A similarity coefficient for each pair of isolates was computed using the Dice coefficient which is defined as 2a/ 2a + u, where a is the number of positive matches and u is the number of nonmatches. These data were used to construct dendrograms using cluster analysis based on the unweighted pair group method with arithmetic means (UPGMA) (Sokal and Michener 1958), neighbor-joining (NJ) (Saitou and Nei 1987), and Ward (Ward 1963) algorithms.

Cloning of APD13 bands and sequence analysis

Genomic DNA of *C. purpureum* isolate 2139, previously described by Ramsfield *et al.* (1996), was amplified by APD13F+R primers (Becker *et al.* 1999a). The PCR products were cloned *en masse* into pGEM-T vector (Promega) using the T-vector cloning system. Positive clones were digested using appropriate restriction enzymes to remove the insert from the vector and determine its size by gel electrophoresis. The DNA inserts of selected clones were analyzed by cycle sequencing of double-stranded products in an ABI 373A automated sequencer according to manufacturer (Applied Biosystems). The sequence was determined for both strands of dsDNA and compared to obtain the final

sequence for each clone. Seven cloned *C. purpureum* sequences (D13-204, D13-304, D13-431, D13-542, D13-678, D13-1228, and D13-1419) were subject to further analysis. Searches in the BLASTN and BLASTX databases (Altschul *et al.* 1997) were done to identify homology to nucleotide and protein sequences, respectively, currently in the database (as of October, 2002).

Southern hybridization

To determine the distribution throughout the *C. purpureum* genome and copy number of the APD13 repetitive DNA, Southern blots of 1.125 µg *C. purpureum* genomic DNA digested with *Eco* RI, *Bam* HI and *Hae* III endonucleases (Pharmacia) were probed with ^{32}P-labeled APD13 F+R-amplified fragments. To detect the presence and visualize the extent of methylation of the fragments, genomic DNA was digested using *Hpa* II and *Msp* I endonucleases (Bio-Rad) and likewise probed with ^{32}P-labeled APD13 PCR fragments. The restriction enzymes *Hpa* II and *Msp* I are isoschizomers which recognize the 4 bp sequence C'CGG and when the external C in the sequence is methylated, neither can cleave. However, unlike *Hpa* II, *Msp* I can cleave the sequence when the internal C residue is methylated (Bio-Rad).

Genomic DNA of *C. purpureum* isolate 2139 was used as a template to produce the ^{32}P labeled probes. The PCR reactions were performed as previously described, except that the concentration of "cold" dCTP was reduced by 50% and 0.825 µM ^{32}P-dCTP (5µCi) was added to the reactions. Radiolabeled PCR products were purified using the Wizard PCR Prep Kit (Promega) and label incorporation was assessed by scintillation counting

prior to use. Southern blots were probed with labeled APD13 fragments according to the methods of Sambrook *et al.* (1989).

RESULTS AND DISCUSSION

Genetic variability and population structure of C. purpureum in B.C..

In order to examine the distribution of APD13 repetitive DNA within *C. purpureum* in B.C., isolates of the fungus were collected under power line rights-of-way from *Alnus*, *Populus* and *Salix* host trees in Mission, B.C. and from *Alnus rubra* hosts in Kemano, B.C. Putative *C. purpureum* cultures were verified using *C. purpureum*-specific markers (Becker *et al.* 1999a). Accession numbers of verified *C. purpureum* cultures, host tree identity, and the geographical origins of collections are listed in Table 12.

A variable number of polymorphic bands was produced upon amplification of total *C. purpureum* genomic DNA with APD13 primers under the stringent conditions of a high annealing temperature (60 °C) (Figure 14). Banding patterns were distinctive for each genetic individual tested, demonstrating that the APD13 SCAR is a very useful fingerprinting tool for this species. Gels were scanned and their patterns were input in the computer program Molecular Analyst v. 21 (Bio-Rad). Shared bands can represent overlapping alleles and can indicate movement or gene flow of individuals between geographic regions. A distance matrix was produced using the Dice coefficient, a measure of variability which depends on the number of shared bands and the total

Table 12. Host and geographic source of *Chondrostereum purpureum* isolates

Accession	Host	Source Location
F0301	*Alnus* sp.	Mission, B.C.
F0302	*Alnus* sp.	Mission
F0303	*Alnus* sp.	Mission
F0304	*Alnus* sp.	Mission
F0305	*Alnus* sp.	Mission
F0306	*Alnus* sp.	Mission
F0308	*Populus balsamifera* ssp. *trichocarpa*	Mission
F0309	*P. balsamifera* ssp. *trichocarpa*	Mission
F0310	*P. balsamifera* ssp. *trichocarpa*	Mission
F0311	*P. balsamifera* ssp. *trichocarpa*	Mission
F0312	*P. balsamifera* ssp. *trichocarpa*	Mission
F0315	*Alnus rubra*	Mission
F0345	*P. balsamifera* ssp. *trichocarpa*	Mission
F0346	*P. balsamifera* ssp. *trichocarpa*	Mission
F0347	*P. balsamifera* ssp. *trichocarpa*	Mission
F0348	*P. balsamifera* ssp. *trichocarpa*	Mission
F0349	*P. balsamifera* ssp. *trichocarpa*	Mission
F0351	*P. balsamifera* ssp. *trichocarpa*	Mission
F0353	*Alnus* sp.	Mission
F0354	*Alnus* sp.	Mission
F0355	*Salix* sp.	Mission
F0387	*Alnus rubra*	Kemano, B.C.
F0388	*Alnus rubra*	Kemano
F0389	*Alnus rubra*	Kemano
F0393	*Alnus rubra*	Kemano
F0395	*Alnus rubra*	Kemano
F0396	*Alnus rubra*	Kemano
F0397	*Alnus rubra*	Kemano
F0398	*Alnus rubra*	Kemano
F0399	*Alnus rubra*	Kemano
F0401	*Alnus rubra*	Kemano
F0402	*Alnus rubra*	Kemano
F0404	*Alnus rubra*	Kemano
F0405	*Alnus rubra*	Kemano
F0407	*Alnus rubra*	Kemano
F0408	*Alnus rubra*	Kemano
F0410	*Alnus rubra*	Kemano
F0411	*Alnus rubra*	Kemano
F0414	*Alnus rubra*	Kemano
F0415	*Alnus rubra*	Kemano
F0416	*Alnus rubra*	Kemano
F0407	*Alnus rubra*	Kemano
F0418	*Alnus rubra*	Kemano
F0419	*Alnus rubra*	Kemano
F0420	*Alnus rubra*	Kemano
F0421	*Alnus rubra*	Kemano

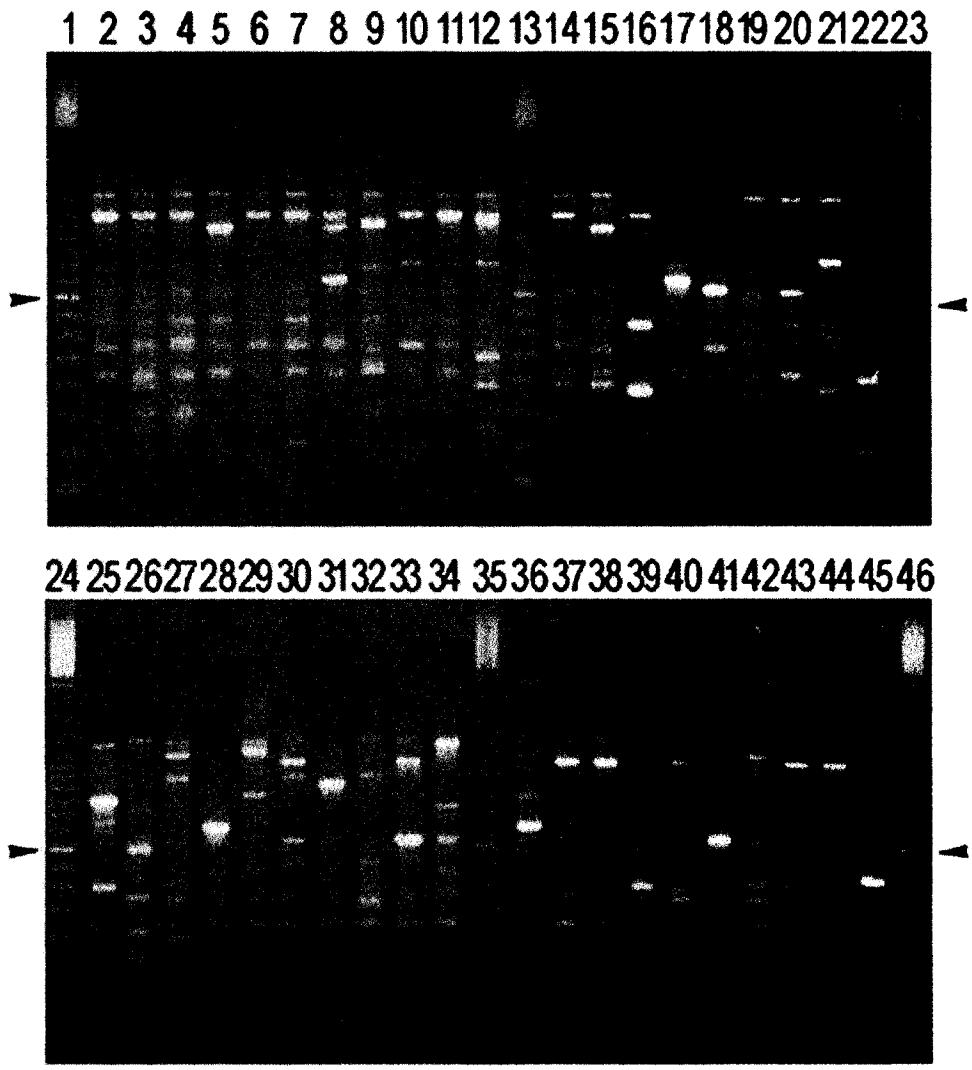

Figure 14. Amplification of *C. purpureum* isolates from Mission and Kemano, B.C., using APD13F+R primers. Isolates shown include F0301 (lane 2), F0302 (lane 3), F0303 (lane 4), F0304 (lane 5), F0305 (lane 6), F0306 (lane 7), F0308 (lane 8), F0309 (lane 9), F0310 (lane 10), F0311 (lane 11), F0312 (lane 12), F0315 (lane14), F0345 (lane 15), F0346 (lane 16), F0347 (lane 17), F0348 (lane 18), F0349 (lane 19), F0351 (lane 20), F0353 (lane 21), F0354 (lane 22), F0387 (lane 25), F0393 (lane 26), F0395 (lane 27), F0396 (lane 28), F0397 (lane 29), F0398 (lane 30), F0399 (lane 31), F0401 (lane 32), F0402 (lane 33), F0404 (lane 34), F0410 (lane 36), F0411 (lane 37), F0414 (lane 38), F0415 (lane 39), F0416 (lane 40), F0417 (lane 41), F0418 (lane 42), F0419 (lane 43), F0420 (lane 44), and F0421 (lane 45). Size markers (100 bp, Pharmacia) are shown in lanes 1, 13, 23, 24, 35, and 46, with the 800bp band indicated.

number of amplified bands for each pair of isolates and gives more weight to matching bands than Jaccard's coefficient (Bio-Rad).

Dendograms produced using different clustering algorithms were used to make inferences about the natural population structure of *C. purpureum.* The dendogram produced by the UPGMA algorithm showed no population substructuring, which might have indicated that isolates from the two regions did not freely interbreed (Figure 15). In addition, there was a lack of apparent clustering for host, or geographical origin. Similar results were obtained using NJ and Ward algorithms (not shown).

The minimal population substructuring suggests that there are no barriers to gene flow among populations of *C. purpureum* within B.C. There was likewise no evidence of selective pressures strong enough to result in clusters of genetically similar isolates. The lack of geographical or host specificity by *C. purpureum* in B.C. provides evidence that there would be a minimal risk to using one genetic individual for biocontrol applications in B.C., which is in agreement with previous risk analyses that employed different methodologies (de Jong *et al.* 1990, Ramsfield 1996, 1999, Gosselin *et al.* 1999).

Characteristics of the life history of *C. purpureum* promote gene flow among populations and this is supported by the results of my population analyses. Conidial spore production is absent in this species, which limits the clonal propagation of genotypes. The primary form of dispersal for this species is therefore the meiotically-produced basidiospore. This feature, combined with the tetrapolar, heterothallic mating system of *C. purpureum* will

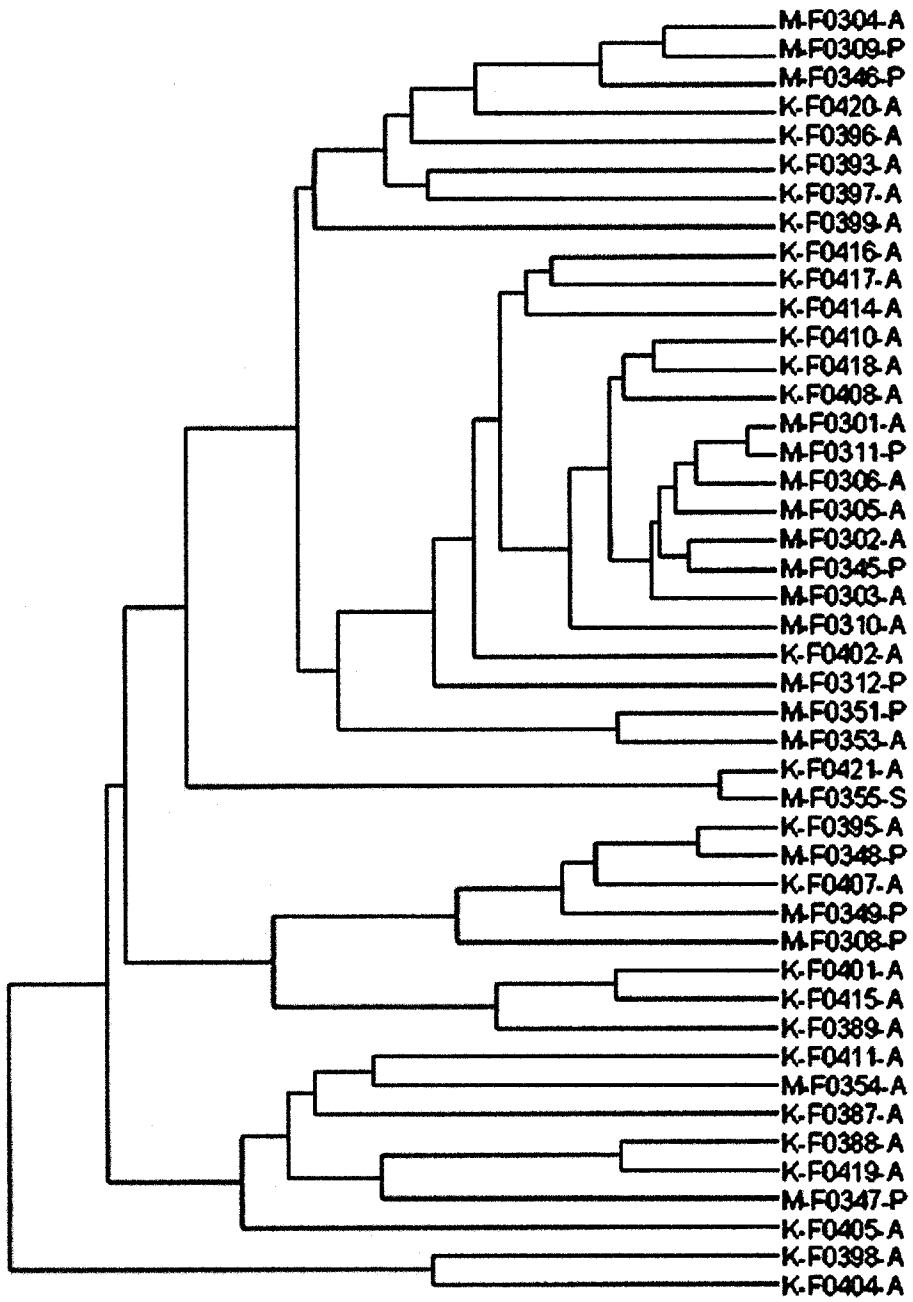

Figure 15. Dendogram representing *C. purpureum* from Kemano and Mission, B.C. Similarity coefficients (Dice's) were derived from comparisons of APD13 amplification patterns among all isolates and clustered using the UPGMA algorithm within Molecular Analyst software (BioRad). Accession numbers for *C. purpureum* are shown preceded by an M (Mission) or K (Kemano) to denote their source and followed by an A (*Alnus*), P (*Populus*), or S (*Salix*) to signify host tree from which they were collected.

promote gene flow among populations. The probable multi-allelic nature of the A and B mating type loci will likely result in a high level of compatibility for mated homokaryons from geographically disparate populations and thus promote outcrossing (Boidin 1986, Rayner and Boddy 1986, Stankis *et al.* 1992).

Analyses of repetitive DNA in C. purpureum

Distribution of repetitive DNA in C. purpureum genome

Genomic organization of the APD13 family in *C. purpureum* was analyzed by southern hybridization of genomic digests probed with a mixture of all fragments produced by amplification of *C. purpureum* using APD13F+R primers. Genomic DNA digested with *Eco* RI, *Bam* HI, and *Hae* III, and probed with APD13 fragments, revealed hybridization signal corresponding to a range of fragment sizes (Figure 16). Similar results were obtained by probing *C. purpureum* genomic DNA separately with each of the repetitive DNA fragments (not shown). Southern hybridization revealed a composite of a large number of restriction fragments of a similar range of size that hybridized to probes. Probe DNA hybridized at many loci of the *C. purpureum* genome, indicating that this repetitive DNA is present in a large number of copies and distributed throughout the genome. I can further assume that I observed only a small subset of all potential loci, since the use of my amplified sequences as probes only detected genomic DNA with sequences complementary fragments amplified by APD13F+R primers which were an appropriate length to allow for PCR amplification. This suggests that this repetitive DNA occurs more often than I can detect with the APD13 primers.

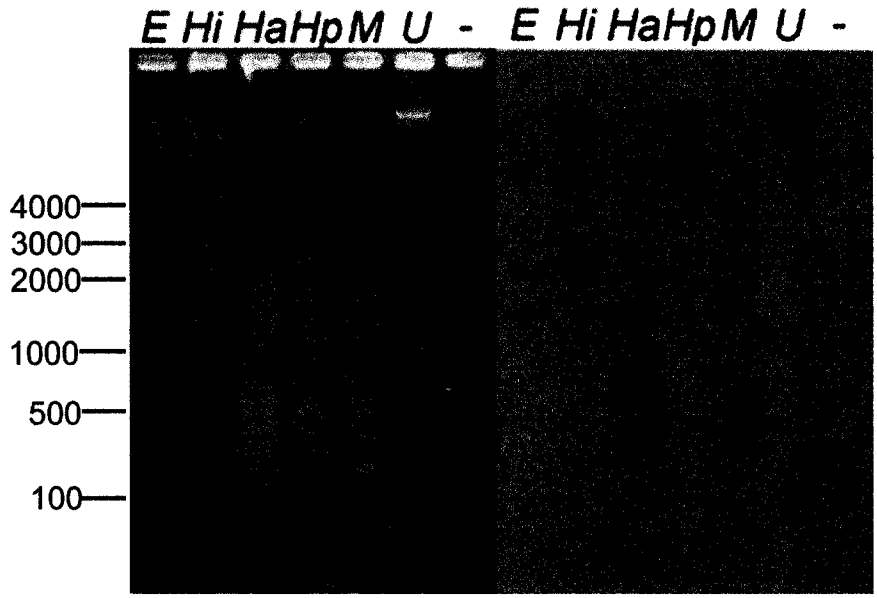

Figure 16. Distribution and methylation of repetitive DNA in *C. purpureum*. Agarose gel (a) showing 1.125 μg of *C. purpureum* total genomic DNA in each lane, digested with *Eco* RI (E), *Hin*d III (Hi), *Hae* III (Ha) *Hpa* II (Hp), and *Msp* I (M) restriction enzymes. Uncut genomic DNA (U) and a no-DNA control (-) lanes are shown. *Hpa* II and *Msp* I are isoschizomers which differ in recognition of methylated cytosine residues. The Southern blot (b) was probed with the radiolabeled amplification products of the PCR primer pair APD13F+R. Fragment lengths are indicated in base pairs.

Methylation of repetitive DNA in C. purpureum

In order to examine methylation within the repetitive DNA markers, total genomic DNA of *C. purpureum* was digested with isoschizomer restriction endonucleases that varied in recognition of methylated residues. Hybridization of labeled APD13 fragments to blots of the isoschizomer digests produced signal representing many copies of homologous fragments distributed throughout the *C. purpureum* genome (Figure 16). The size of fragments hybridizing to probe in the lane of *Msp* I-digested *C. purpureum* DNA was considerably smaller than that in the *Hpa* I digest. Likewise, faint bands that are visible in both digests, which are assumed to be analogous, are smaller in the *Msp* I lane. These results indicated that DNA amplified by these primers is methylated in the internal cytosine of the C'CGG recognition sequence of these enzymes. The methylation of this repetitive DNA is consistent with the hypothesis that this is non-transcribed or inactive sequence.

The methylation of repetitive DNA is a method by which these sequences can be rendered inactive by the host organism. Studies of fungi that have identified transposable elements found some of these sequences to be incomplete, or translationally inactive (Neuveglise *et al.* 1996, Bibbins *et al.* 1998, Chen *et al.* 1998). Elements may become inactive because of processes initiated by the host that silence these sequences. These include repeat-induced point mutations (RIP) and methylation-induced premeiotically (MIP). The former will result in C to G and G to A transitions, while the latter will result in methylation of cytosine residues in repeated sequences, which is a reversible process (Daboussi 1996, Kempken and Kuck 1998, Wostemeyer and Kreibich 2002). Both of

these processes are active before or during sexual recombination. Consequently, they are likely only active in those fungi like *C. purpureum* that exhibit meiosis, a rare event for some species of fungi (Daboussi 1996).

Processes that occur during vegetative growth may also inactivate expression of repeated sequences. Quelling appears to be a post-translational process that reduces the level of mature mRNA (Selker 1997, Wostemeyer and Kreibich 2002). Mutations that are detected in retrotransposons may be due to errors that occur during reverse transcription, for class I elements. Reverse transcription is considered to be error-prone and a major source of variability in a transposon population, possibly resulting in the formation of non-functional retroelements (Bhattacharyya *et al.* 1997).

Sequence analysis of repetitive DNA

Seven DNA fragments amplified from *C. purpureum* isolate 2139 by the APD13F+R primers were cloned and sequenced, and ranged in size from 204 bp to 1419 bp (Figure 17). These were named by their length (bp) and were deposited in GenBank with the following accession numbers: D13-204 (AF550613), D13-304 (AF550612), D13-431 (AF550614), D13-542 (AF550611), D13-678 (AF550617), D13-1228 (AF550616), and D13-1419 (AF550615) (Benson *et al.* 2000). Although the size of fragments varied greatly, alignment of DNA fragments revealed a highly conserved region of 123 bases adjacent to the return primer in all cloned sequences (Figure 17). The region adjacent to the forward primer was highly variable and not conserved among DNA fragments in this

```
d13-204         GGGGTGACGACATTATACTGCAGGTAGTAGGGTAACTACCCTACTGGGCTACATCCCGGC 60
d13-304         GGGGTGACGACATTATACTGCAGGTAGTAGGGTAACTACCCTACTGGGCTACTACCCGGC 60
d13-431         GGGGTGACGACATTATACTGCAGGTAGTAGGGTAACTACCCTACTGGGCTACATCCTGGC 60
d13-542         GGGGTGACGACATTATACTGCAGGTAGTAGGGTAACTACCCTACTGGGCTACATCCCGGC 60
d13-678         GGGGTGACGACATTATACTGCAGGTAGTAGGGCAGCTACCCTACAGGGCAAGTGCCAGCC 60
d13-1228        GGGGTGACGACATTATACTGCAGGTAGTAGGGTAACTACCCTACTGGGCTACATCCCGGC 60
d13-1419        GGGGTGACGACATTATACTGCAGGTAGTAGGGTAACTACCCTACTGGGCTACATCCCAGC 60
                ******************************** * ********* **** *   **   *

d13-204         CTGATGCATCACATCGTAGGGTAACTACCCAGATATCATGTATAATATTAGCACCACAAC 120
d13-304         TGGATGCATCAGGCCGTAGGGCAACTACCCAGATATCATGCTTAATCGTAGCAGCTATAG 120
d13-431         CTGATGCATCACATCGTAGGGTAACTACCCAGATATCATACATGATCATAGCACCCTAAG 120
d13-542         CTGATGCATCACATCGTAGGGTAACTACCCAGATATCATGTATAATATTAGCACCACAAC 120
d13-678         TGGATGCATGTTAGCGTAGGGCAACTACCCATATTTCATGTATCAGAGGAGCATCTACAG 120
d13-1228        CTGATGCATCACATCGTAGGGTAACTACCCAGATATCATGTATAATATTAGCACCACAAC 120
d13-1419        CTGATGCATCACATCGTAGGGTAACTACCCAGATATCATGTATAATATTAGCACCACAAC 120
                  *******     ******* ********* ** ****   * *    **** *   *

d13-204         CTGCAGAACAACCACAGGAATCGGATCCCCCGATCCCACCACGAACTAAAACCCCATCCC 180
d13-304         GTGAGCTTGCGTGTTCCGCTCACTCTCGTGCTCTTTCTTGCAACCCTCCTTTTATCTCCT 180
d13-431         GTGGGTTACACTCACACGGCGCTGCCTGTGTGCGCGCATTCTGAGTGCACCACTTATCTC 180
d13-542         CTGTTGAAGCAGGCTGGACGCAGATGGTACAAGACCTTCTGCAACATCATGAAGGAATTT 180
d13-678         GTGACATTAGGTCCAACCTCCCTACGCACCGGCGATTTTCCTGGATTTTTCTCGATCCCC 180
d13-1228        CTGCCGGCTCGACGCTGTGGAGGTGAGACCGGGAGCTGAGGTGGTGGTGGTTTGAAGGAG 180
d13-1419        CTGTTGAAGCAGGCTGGACGCAGATGGTATAAGACCTTCTGCAACATCATGAAGGAATTT 180
                 **

d13-204         CTTCACCGTCGTCCTCGTCACCCC---------------------------------- 204
d13-304         CTCATCCCCGACCATCTCATCCCACTTTTTGTTTGAAGCCATCACGCATCGCTTGTAGGT 240
d13-431         CCCTCTCCAGCGTAGCCTCCNANNGTACGCTATCGTTTTCTGGCTCGTTATCTTGATTCA 240
d13-542         GGGTTTACACGATCTGAGCACGATCATGCTGTATTTTTCCGATCCGATCCCTACATCATC 240
d13-678         CTAAATCCACTTCCTATACCGCGACACGACACAGCTACTAACGTATATACATAGCCACTC 240
d13-1228        GATTAGACGCTTGTGCCGAGCGGATTGTTTGTTGAGTTGAGTCCCGTGACTACCGTGACC 240
d13-1419        GGGTTTACACGATCTGAGCACGATCATGCTGTATTTTTCCGATCCGATCCCTACATCATC 240

d13-204         ---------------------------------------------------------- 
d13-304         GCTTCCACTTAACTGTAGTCGCGAGCCTTGCATACGGCCGATACACCGTCGTCCTCGTCA 300
d13-431         CCGTGTTAGTTTTCTCGTTATCAGATCTTTTCCATACCGACCTTCACTCGGTAAGTTTAT 300
d13-542         CTGTTCATTCACGTTGACGACATGACTATCATCACAGACAACGACATGGTCATGACACAG 300
d13-678         CGTTGTGAGACGAACTCTTAAGGGACTGTACTTCAGTGTACCTCCCTTGACGGCCAATAC 300
d13-1228        GTGAGACTCACTTACTGGGTTTGGGGATCAACCCCGGGATCGCGTCAGCCTCATTGTTTT 300
d13-1419        CTGTTCATTCACGTTGACGACATGACTATCATCACAAACAACGACATGGTCATGACACAG 300

d13-204         ---------------------------------------------------------- 
d13-304         CCCC------------------------------------------------------ 304
d13-431         AGCGATCGTTCCCCGCGCTATATGACTTACTCAATTCACACGCAGCATGAGCGAGCCCCA 360
d13-542         TTCAAGAACAAGATCAAAGCAAAGATCGAAGTCACCGACGCTGGTGAACTTCATTGGATG 360
d13-678         TGTCTCGTTGCAGAAGGGAAAGTCTTATCAAGATAATCCCCAATGATAACTGCGAATCCA 360
d13-1228        CAGCTTGCTTCCATTTTCGGCGGTGTTCTATCATGAGGAACTCATGCTTTTCTTGCCTCG 360
d13-1419        TTCAAGAACAAGATCAAAGCAAAGATCGAAGTCACCGACGCTGGTGAACTTCATTGGATG 360
```

Figure 17. Multiple sequence alignment of repetitive DNA fragments amplified using APD13F+R primers from *C. purpureum* genomic DNA. Sequences were aligned using ClustalX version 1.81, and are arranged here to show the return primer sequence (underlined) on the 5' end. Sequence complementary to the APD13F primer is underlined with a wavy line. The RAPD primer OPD13 (Operon), used in the design of the SCAR primer APD13R, is double-underlined. Residues that are identical in all repetitive DNA clones are marked with an asterix.

```
d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         CGTACGCTTTTCCAACACTTATACTACTGCCTACTATGCCTCGTCGTCGCCACCGTCGTC 420
d13-542         CTAGGTATCGAAGTTCGTCGAGTACGCTCGGCGCGTATCCTTCAGCTGTCTCAGAGAGCC 420
d13-678         ACGTGAGTTTCTTGGGCTCCGTAATTAGTTTTCATTTTCTCGAGTAGTACCACGGCCTTT 420
d13-1228        CCGTCTTTGTTCAATACTACCATGTCCTCACGAAAAAAAAGCGCCGGGGGGATGATGTCT 420
d13-1419        CTAGGTATCGAAGTTCGTCGAGTACGCTCGGCGCGTATCCTTCAGCTGTCTCAGAGAGCC 420

d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         CTCGTCACCCC------------------------------------------------ 431
d13-542         TATATCGACTCTATAATTGTCCGCTATGGTTTTAGCGACATCAAGCCCCTTTCTATTCCC 480
d13-678         TTACATATATACTGCCGAACGTTGCGGTCCACCCCCTTCTATCTTTCATCCTGGAGTGTA 480
d13-1228        TCCTCGAATATGACTTCACCGACGTAGGGGCCTCAGGGCAAAGCTCAGCGGCTGATGTTG 480
d13-1419        TATATCGACTCTATAATTGTCCGCTATGGTTTTAGCGACATCAAGCCCCTTTCTATTCCC 480

d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         ------------------------------------------------------------
d13-542         TTCGACCCTCATCTGCAGTTATCTGATACGCAGAGTCCTTCCACCGTCGTCCTCGTCACC 540
d13-678         CCAATCTTCCCCATTTCACGGACGAGTTCGGTATCGCCCAACACACAGATTATTCCGTCG 540
d13-1228        TAGTGGAGACCCTATCTCGCGACGGTCGACGCACAACCAAATCGATCTCGACAGTCCTTG 540
d13-1419        TTCGACCCTCATCTGCAGTTATCCGATACGCAGAGTCCTTCCACCGTCAACGAATCGCAC 540

d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         ------------------------------------------------------------
d13-542         CC-------------------------------------------------------- 542
d13-678         AACCACCTCGCTGGAAACGTTGCGCCGCTCAACCGTGCCCGTCTGTCAAAGACACTTCAG 600
d13-1228        CGAAGGAATCGGATGCTCTTCTCCCACCACCATCGATAAAACCCACCCCTGTACCCCCTC 600
d13-1419        TGATGCACAACCATCCTTACCGAGAAGCTCTCGGTGCCCTTCAATATCTCTCAGTTGCCA 600

d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         ------------------------------------------------------------
d13-542         ------------------------------------------------------------
d13-678         AACAACCACAGGAATCAGATCCCCCGATCCCACCACGAACCAAAACCCCATCCCCTTCAC 660
d13-1228        TAATCCCTTGCGCTGACGAGCCGTTGCTGGAGATCCCTTACGACAAAAAAGTCTATGATC 660
d13-1419        CTCGTCCTGACATAACCTACGCATTTCTCAATTGGCTCGTTTCATGCAAAACCCCGGTAT 660

d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         ------------------------------------------------------------
d13-542         ------------------------------------------------------------
d13-678         CGTCGTCCTCGTCACCCC------------------------------------------ 678
d13-1228        TTGCGGACTGGGTTACACACACGGATAGCTTTGCAATTCCATCTAAGGGGAAAAGGAGAA 720
d13-1419        CACACACTGGAACGCCCTTCGCCGTGTCTACGCCTATCTCAAAGGTACACGCGAACACTG 720

d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         ------------------------------------------------------------
d13-542         ------------------------------------------------------------
d13-678         ------------------------------------------------------------
d13-1228        AATATGCCTCATCAGTAAGTTTTATTTTTCTATGAAGAACACATGCCGCTACTTAATGAA 780
d13-1419        GCTCACCTATGGTTAAGCAGCCAACTGCTATCATCGGTACTCCGACGCCGACGGTATGTC 780
```

Figure 17 (cont.). Multiple sequence alignment of repetitive DNA fragments amplified using APD13F+R primers from *C. purpureum* genomic DNA.

```
d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         ------------------------------------------------------------
d13-542         ------------------------------------------------------------
d13-678         ------------------------------------------------------------
d13-1228        CTCTATGGATTTACAGGATCAGCCTCTCAAGGCTTGGACCCCATTTGCTTCCCAATACCT 840
d13-1419        CTCCGCTGATCGTCATGCCATCTCTGGCTATTGTTTCATGATCAACGGCGGTGCTGTATC 840

d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         ------------------------------------------------------------
d13-542         ------------------------------------------------------------
d13-678         ------------------------------------------------------------
d13-1228        CGACGAGTTTATTCGTCTCGAGGGGCGGGGATCCTTTGCCATGGCGGCATGCCCTTCCTG 900
d13-1419        TTGGTCTTCCAAGCGCCAGGAGATCGTTTCCCTTTCCACTACCGAAGCTGAGTATGTCGC 900

d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         ------------------------------------------------------------
d13-542         ------------------------------------------------------------
d13-678         ------------------------------------------------------------
d13-1228        CCATGAGGCTGCGGACCACGCCCCCCCAAANGCTGCGCTGCCGCGATTGCTTCCCACAGG 960
d13-1419        ACTCACTCACGCTAGCAAGGAAGCTATCTGGTTCGCAACTTCATCTCAGAAGTATTCGGA 960

d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         ------------------------------------------------------------
d13-542         ------------------------------------------------------------
d13-678         ------------------------------------------------------------
d13-1228        AGTTGTTTTGTGAAGGCTGCTTTGTTGAAAAGCACCACGAGCACCCATTGCACGTCACGG 1020
d13-1419        AAGATCTCAGACCCCATCCCCATTCATTCCGACAACCAGTCCGTATCGCATTGTCGAAAG 1020

d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         ------------------------------------------------------------
d13-542         ------------------------------------------------------------
d13-678         ------------------------------------------------------------
d13-1228        AGGTGAGTCGCAAAGCATTCCTTAATAGCCATTCTATCTAACGACCGAATAGAAGTGGAA 1080
d13-1419        ATGATCGCTTTCATGCACGCACCAAGCACATCGACATCCGATTCCATTTCATCCGCTATG 1080

d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         ------------------------------------------------------------
d13-542         ------------------------------------------------------------
d13-678         ------------------------------------------------------------
d13-1228        TGGGGTATGCTTCGAGCGCGTTCAACTGCACAGCCTGGGGCTTCGTGTTCAACTAGGGCA 1140
d13-1419        CCATCGAAGAAGGCAAGATCGTCCTCAAGTACTGCCCCACTAACGAGATGACCGCAGACA 1140

d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         ------------------------------------------------------------
d13-542         ------------------------------------------------------------
d13-678         ------------------------------------------------------------
d13-1228        TCCAACAGGGGTCGAGTGTCCCCGTCCAATCGAGCCCGGTGAGGTCACCCAGAAACGCTC 1200
d13-1419        TCTTTACCAAAGCTCTACCGTCTGTTAAGTCCAAGCATTTTGCTTCGTCGCTGGGACTCG 1200
```

Figure 17 (cont.). Multiple sequence alignment of repetitive DNA fragments amplified using APD13F+R primers from *C. purpureum* genomic DNA.

```
d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         ------------------------------------------------------------
d13-542         ------------------------------------------------------------
d13-678         ------------------------------------------------------------
d13-1228        AACCTTCACCGTCATCCTCGTCACCCC---------------------------------- 1227
d13-1419        CCAAGGCTTGAGGGGGAGTGTTAGATTCTCAGGCGTGACTACGCCACATCACATGCAAGC 1260

d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         ------------------------------------------------------------
d13-542         ------------------------------------------------------------
d13-678         ------------------------------------------------------------
d13-1228        ------------------------------------------------------------
d13-1419        TTTCCTTATCCATCTCTTACTGTACCACTACCACGCTGCCTCTTTCCCCTTCCGTATATC 1320

d13-204         ------------------------------------------------------------
d13-304         ------------------------------------------------------------
d13-431         ------------------------------------------------------------
d13-542         ------------------------------------------------------------
d13-678         ------------------------------------------------------------
d13-1228        ------------------------------------------------------------
d13-1419        TTTCCTCCTGTGCTTTACACCTATGCCTACCTTTTCCGTGCGTATCTGTCATTGTACTTA 1380

d13-204         ----------------------------------------
d13-304         ----------------------------------------
d13-431         ----------------------------------------
d13-542         ----------------------------------------
d13-678         ----------------------------------------
d13-1228        ----------------------------------------
d13-1419        TCTATTGTTCCAACGGTGCACCGTCGTCCTCGTCACCCC 1419
```

Figure 17 (cont.). Multiple sequence alignment of repetitive DNA fragments amplified using APD13F+R primers from *C. purpureum* genomic DNA.

family. The conservation of the sequence adjacent to the return primer might reflect selection against mutation in this region.

Of the seven cloned sequences studied, none were found to show nucleotide sequence similarity with any sequences in the BLASTN database (Wheeler *et al.* 2000). In searches of the BLASTX database, only clones D13-542 and D13-1419 were found to show protein sequence similarity to known sequences. A consensus sequence of these two clones was obtained and the subsequent search of the BLASTX database produced 498 significant alignments. The sequences identified were primarily retrotransposon, or retrotransposon-like sequences for genes in a variety of organisms (Table 13). The first five significant alignments to the D13-1419 sequence are shown in Figure 18.

The results of my sequence analysis indicate that some of the cloned sequences isolated from the *C. purpureum* genome represent incomplete portions of known retrotransposable elements (Table 13). The consensus sequence demonstrated homology with retrotransposon-like sequences and sequences that were identified as components of active retrotransposons. These included the *pol* region, where the domains for reverse transcriptase, RNase H, integrase and protease genes are typically found for the Class I retrotransposon elements (Kempken and Kuck 1998). The majority of significant alignments support homology of the consensus sequence with retrotransposon and retrotransposon-like sequences, providing good evidence for the identity of this repetitive *C. purpureum* sequence. Although these sequences are incomplete, they have been maintained in *C. purpureum*.

Table 13. Summary of BLASTX search results for consensus sequence of clones D13-542 and D13-1419, showing details of the first five significant alignments.

BLASTX search rank	Accession number	Sequence length (aa)	Sequence identity	Source organism
1	D83003	1338	Hypothetical protein of common tobacco retrotransposon Tto1	*Nicotiana tabacum*
2	NP_179010	1335	Putative retroelement *pol* polyprotein At2g13930	*Arabidopsis thaliana*
3	AAL75759	1005	Putative *pol* polyprotein	*Oryza sativa*
4	NP_192290	964	Putative retrotransposon protein At4g03810.1	*Arabidopsis thaliana*
5	NP_174900	1356	Putative polyprotein At1g37110.01	*Arabidopsis thaliana*

```
first     (964-996)LKQAPRQWYKKFESVMGQHGYKKTTSDHCVFAQ...(1005-1096)ILLLYVDDMLIVGRNVSRIN
second   (969-1001)LKQSPRQWNLRFDEFMRGIKYTRSAYDSCVYFK...(1010-1101)YLLLYVDDMLIASANKSEVN
third     (643-675)LKQSPRQWYKRFDLFMLSHGFKRSEFDSCVYIK.....(682-773)IYLLYVDDMLIAAKSKEQIT
fourth    (594-626)LKQASRSWNLRFNEAIKEFDFIRNEEEPCVYKK.....(634-725)FLVLYVDDILLLGNDIPLLQ
fifth    (992-1024)LKQSPRQWNKRFDRFMSSQQFIRSEHDACVYVK...(1033-1124)YLLLYVDDMLIAGASKAEIN
APD13      (42-74)LKQAGRRWYKTFCNIMKEFGFTRSEHDHAVFFR......(80-171)ILFIHVDDMTIITDNDMVMT
Reading frame                R1

SLKEQLSKFFAMKDLGPAKQILGMRIMRDREAKKLWLSQEKYIEKVLQRFNMEKTKAVSCPLANHFRLSTKQ...(1107-1133)
ELKQLLSREFEMKDLGDAKKILGMEISRDRDAGLLTLSQEGYVKKVLRSFQMDNAKPVSTPLGIHFKLKAAT...(1112-1138)
TLKKQLSSEFDMKDLGAAKKILGMEITRDRNSGLLFLSQQSYIKNVLQRFNMHDAKLVSIPIAPHFKLSVLQ...(784-810)
SVKTWLGSCFSMKDMGEAAYILGIRIYRDRLNKIIGLSQDTYIDKVLHRFNMHDSKKGFIPMSHGITLSKTQ...(736-762)
RVKEQLSTEFEMKDMGGASRILGIDIYRDRKGGVLKLSQEIYIRKVLDRFNMSGAKMTNAPVGAHFKLAAVR...(1132-1158)
QFKNKIKAKIEVTDAGELHWMLGIEVRRVRSARILQLSQRAYIDSIIVRYGFSDIKPLSIPFDPHLQLSDTQ...(181-207)
                             R1

MERIPYASAVGSLMYAMVCTRPDIAHA...(1122-1171)VGVVSRFLSNPGKEHWDAVKWILRYLRGTSKLCLCFGE...
MKIVPYANTIGSIMYSMIGTRPDLAYS...(1127-1176)LGVISRFMSKPLKDHWQAVKWVLRYMRGTEKKKLCFRK...
MSRVPYSSAVGSLMYAMVCSRPDLSHA.....(799-848)MSLVSRYMANPGKEHWKAVQWIFRYLRGTADACLKFGR...
MSKIPYASAIGSIMYAMLYTRPDVACA.....(751-800)LSMTSRYQSDPGESHWIVVRNIFKYLRRTKDKFLVYGG...
TDVVPYSSAVGSIMYAMLGTRPDLAYA...(1147-1196)ICLISRYMSKPGSMHWEAVKWVMRYLKGAQDLNLVFTK...
MHNHPYREALGALQYLSVATRPDITYA.....(208-245)ISQLARFMQNPGITHWNALRRVYAYLKGTREHWLTYG#...
               R3                                        R2

(1185-1237)AGDVDSRKSTSGYLINFSGGAVSWQSKLQKCVALSTTEAEFIAATEACKELIW...(1262-1294)
(1191-1243)GSNFDTRRSITGYVFTVGGNTISWKSKLQKVVAISSTEAEYMALTEAVKEALW...(1268-1300)
  (862-914)AADLDKRRSLTGYVFTISSCAMSWKATLQPVVAQSTTEAEYMAIAEACKESVW...(939-971)
  (815-867)QTDKDDFRSQSGFFFCLNGGAVSWKSTKQSTVADSTTEAEYIAASEAAKEVVW...(895-927)
(1211-1263)AADLDRRRSISGYVFTIGGNTVSWKASLQPVVAMSTTEAEYIALAEAAKEAMW...(1288-1320)
  (258-310)GMSSADRHAISGYCFMINGGAVSWSSKRQEIVSLSTTEAEYVALTHASKEAIW...(335-367)
                                  R2

IHLAKNASFHSRSKHIDVRYNWIRDVLEKKMLR
ITLAKNSVHHERTKHIDIRLHFIRDIICAGLIK
ICLTKDQMFHERTKHIDIKYHYVRDVVVQGRRI
IAQAKEPKSHQKSKHIQRRYHLIREIIDRGDVK
ICLSKNSVYHERTKHIDVRFNYIRDVVESGDVD
IALSKDDRFHARTKHIDIRFHFIRYAIEEGKIV
               R3
```

Figure 18. Multiple sequence alignment of the APD13 consensus sequence with homologous amino acid sequences. Alignments were prepared using Gene Runner version 3.00 (Hastings Software Inc.). The aligned sequences (Table 13), included the hypothetical protein of common tobacco retrotransposon Tto1 of *Nicotiana tabacum* (First), putative retroelement *pol* polyprotein At2g13930 of *Arabidopsis thaliana* (Second), putative *pol* polyprotein of *Oryza sativa* (Third), putative retrotransposon protein At4g03810.1 of *Arabidopsis thaliana* (Fourth), putative polyprotein At1g37110.01 of *Arabidopsis thaliana* (Fifth), with the APD13 consensus sequence of clones D13-542 and D13-1419, aligned in the first (R1), second (R2), and third (R3) reverse-complement reading frames. The number ranges preceding each set of aligned amino acid sequences indicate the portion of each individual sequence implicated in that reading frame alignment. Amino acid matches between the APD13 sequence and each of the five significant aligned sequences are shown in bold.

The sequences isolated from *C. purpureum* are incomplete and therefore translationally inactive. Although not all of the cloned sequences amplified by the SCAR primers showed homology with known retrotransposon sequences by BLAST analysis, they did exhibit a strong degree of homology with each other for a short conserved region of 123 bp. This provides evidence that this inactive retrotransposon-like sequence in *C. purpureum* does occur in multiple copies, in effect saturating the genome of this species, and that these sequences likely have a common origin. The conservation of the 123 base pair sequence among most cloned sequences is curious in that this region has no apparent coding function, based on the results of BLAST database searches. The reason for this short sequence being conserved is not apparent and requires further investigation.

Amplification of APD13 in other fungi

Preliminary results indicate that APD13F+R primers may be used to amplify polymorphic repetitive DNA fragments from genomic DNA of other fungi (Figure 19). Banding patterns of APD13 amplified fragments from basidomycete species *Stereum hirsutum, S. sanguinolentum, Schizophyllum commune* and *Trametes versicolor* were of a size range and frequency similar to those observed in *C. purpureum* and varied within the species examined. Further studies are required to evaluate the occurrence of this repetitive DNA in these and other fungi.

Summary

My population study demonstrates that the SCAR amplified regions do occur in all *C. purpureum* isolates examined from different geographical regions and include a range of

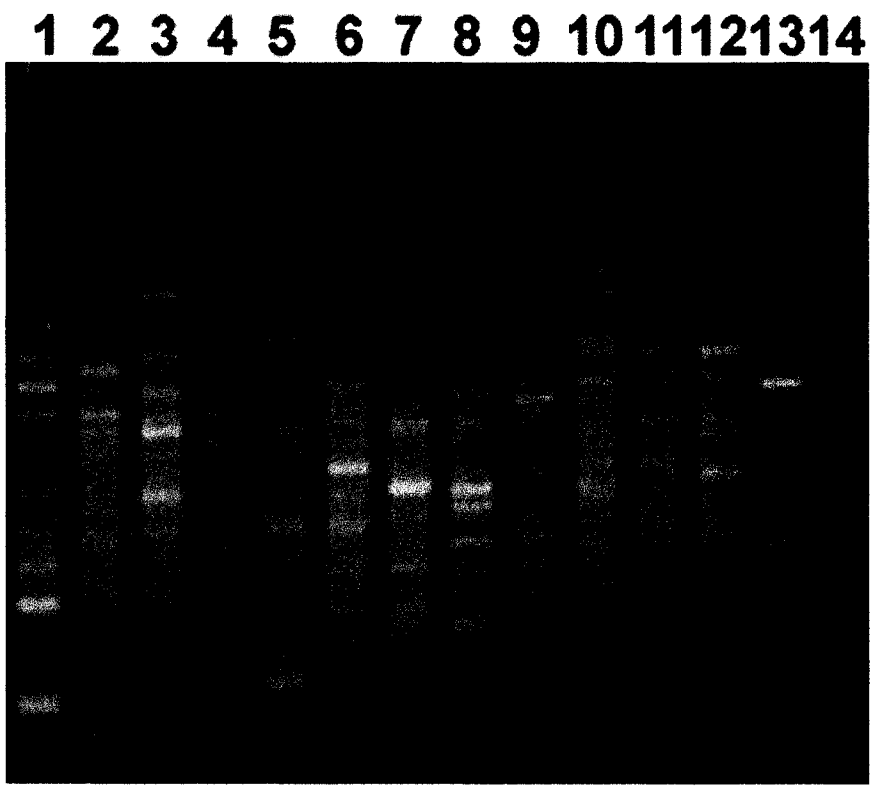

Figure 19. Amplification of repetitive DNA from genomic DNA of several Basidiomycete fungi, using primers APD13F+R. PCR products are shown from *C. purpureum* 2139 (lane 1), *Stereum hirsutum* ATCC34682 (lane 2), *S. hirsutum* ATCC13240 (lane 3), *Schizophyllum commune* LOO-14612-R (lane 3), *S. commune* FP-56473-T (lane 4), *S. commune* FP-125035-T (lane 5), *Stereum sanguinolentum* FP-104730-Sp (lane 6), *S. sanguinolentum* Fp-133911-Sp (lane 7), *S. sanguinolentum* Fp-102551-Sp (lane 8), *S. sanguinolentum* Fp-102357-Sp (lane 9), *S. sanguinolentum* FP-102485-Sp (lane 10), *Trametes versicolor* R-105 (lane 11), *T. versicolor* Fp-133255-Sp (lane 12), *T. versicolor* Fp-102485-Sp (lane 13), and no-DNA PCR control (lane 14). All cultures besides *C. purpureum* were obtained from the USDA Forest Products Laboratory (Madison, WI), except for those preceded by "ATCC", which were from the American Type Culture Collection (Manassas, VA).

fragment sizes. The variable length of the amplified fragments among many isolates also supports the incomplete nature of this element. Amplified fragments likely include both retrotransposon and non-coding regions of DNA. These sequences can serve as useful genetic markers for strain identification, due to their neutral character and their widespread occurrence among *C. purpureum* isolates. They are also useful in evaluating gene flow among populations of *C. purpureum* and allow the population structure of this species to be better understood. An accurate assessment of population structure will permit the safe use of this fungus as a bioherbicide agent. My results suggest furthermore, that the APD13 markers have potential for characterizing genotypes of other basidiomycete species, in addition to indicating the possible presence of retrotransposon-like sequences in these fungi.

Chapter 7. General Conclusions

Chondrostereum purpureum biology

This investigation of the biology of *Chondrostereum purpureum*, which included its phylogenetic relationships, ecology, pathogenicity and genetic structure, has expanded our understanding of fungal evolution while supporting the development of a native fungus as a practical biological control for use in our forests. Various aspects of the biology of *C. purpureum* were examined, providing an estimation of the evolutionary history of the genus using phylogenetic analysis, an assessment of the natural population structure of *C. purpureum* and an investigation of the dynamics of this fungus after field application. The results of experiments summarized in this dissertation have allowed a gain in our understanding of the systematics of *C. purpureum* and an estimation of the evolutionary forces which have shaped its population structure.

Phylogenetic relationships

Phylogenetic relationships were estimated among *C. purpureum* and other fungi thought to be closely related or sharing the same ecological niche. Evolutionary relationships among a group of fungi that had once been classified as 'Aphyllophorales' were inferred by phylogenetic analyses of class II chitin synthase gene fragments. The banding patterns of chitin synthase PCR products exhibited polymorphism, which resulted from variation in the occurrence and length of three introns found only in Basidiomycetes, and usually conserved within genera. Within Basidiomycetes, the monophyly of several

groups was identified and well-supported including the 'Agaricales', 'Phlebia', 'Boletales' and 'Russulales' clades. The removal of *Chondrostereum purpureum* from the genus *Stereum* was supported by these analyses, as was the grouping of *Stereum* species within the Russulales clade. This study provided an independent confirmation of evolutionary hypotheses based on ribosomal DNA sequences, as well as a framework for future studies of fungal taxa using chitin synthase genes.

Efficacy, strain and host interactions

Efforts to develop a biocontrol strategy to suppress vegetative re-sprouting of forest weeds were supported by the development of the *C. purpureum*-diagnostic marker and fingerprinting markers. Molecular markers were successfully applied to monitor infection by *C. purpureum* of treated stumps in field trial sites. Individuals of *C. purpureum* applied as biocontrol were re-isolated from symptomatic hosts and positively identified, satisfying Koch's postulates for plant pathogens. The PCR-based approach allowed hundreds of field trial samples to be quickly screened for *C. purpureum* infection. In addition, this method enabled genetic individuals of the fungus to be differentiated and identified. This allowed a comparison of infection frequency and colonization ability of different isolates of *C. purpureum* applied as a biocontrol to different weedy host species. The lower rate of infection by *C. purpureum* on aspen compared to Sitka alder was expected to result in less-effective suppression of this weedy species, which was indeed the result. This indicated that the rate of *C. purpureum* infection on aspen must be improved before it can be expected to perform effectively as a biocontrol on that host. While improved infection may be achievable through a change in

application, formulation or strain of *C. purpureum*, some weedy host species like aspen may be too resistant to *C. purpureum* infection to make their biocontrol practical. This methodology thus provided an early indication of success of *C. purpureum* biocontrol application.

Genetic characterization of C. purpureum

Molecular genetic markers have been used to examine the population genetic structure within many fungal species, allowing for the investigation of the ecological and evolutionary determinants of such structure. Markers which were developed to identify *C. purpureum* in samples of infected wood and differentiate individual genotypes were applied to a collection of *C. purpureum* sampled from two regions of British Columbia. Evidence of evolutionary effects due to biological factors of the fungus (*e.g.* dispersal ability, mating system, selection, competition, succession) was examined at this scale of genetic variation. The wind-borne dispersal of spores with no apparent barriers to gene flow, along with the outcrossing mating system, were hypothesized to contribute to the maintenance of a relatively high degree of heterozygosity within populations, while promoting homogenization of *C. purpureum* genotypes over their geographic range. Genetic variation revealed within *C. purpureum* supported this hypothesis and showed little evidence of substructuring that might have been due to evolutionary processes such as genetic drift or selection. No geographic or host specialization was revealed in *C. purpureum* within B.C., which corroborated the findings of minimal population substructuring encountered in previous studies.

Environmental fate and risk assessment

Risks associated with the biocontrol application of the fungus were assessed, and the usefulness of markers to identify and monitor strains of *C. purpureum* was demonstrated. PCR markers allowed a determination of the incidence of non-target infection within the experimental sites and the level of natural infection by local spores. The biocontrol treatment apparently did not lead to an added risk of infection of non-target trees. Information about the extent of genetic variation within the pathogen addressed concerns that proliferation and persistence of spores from a genotype used in weed biocontrol might affect resident populations. The dynamics of movement of particular alleles or combinations of alleles is dependent on, among other things, whether these alleles are selectively neutral or whether they confer a selective advantage to the fungal individual. The advantage of particular combinations of alleles was impossible to predict, but general hypotheses were made and tested in field trials. To assess the risk of the potential increased incidence of the released isolate following its mass inoculation, spore traps at various distances from the field sites were established and monitored. While no evidence for persistence of the release isolate was apparent from these tests, results of spore trapping experiments were not conclusive, *i.e.* absence of evidence was not evidence of absence. Further studies to test hypotheses of spore dispersal gradients could be performed within smaller distances from biocontrol applications to determine the resolution of detection of released isolates. Additional experiments in spore trapping could be performed using homokaryotic "bait" cultures of *C. purpureum* on Petri dishes (Vilgalys and Sun 1994).

Repetitive DNA

Repetitive DNA generated by PCR amplification using SCAR primers was cloned and sequenced, allowing for comparison with other sequences in the GenBank (BLAST). Two of the seven fragments for which sequence was determined showed homology to retrotransposon-like repetitive elements. It was hypothesized that these repetitive sequences are inactive retrotransposons, which is supported by the presence of CpG methylation within the amplified fragments. The fragments amplified by these primers are present in all individuals of *C. purpureum* tested, including isolates from world-wide sources. Preliminary experiments showed that repetitive DNA is amplified from genomic DNA of other basidiomycetes, including *Stereum hirsutum*, *S. sanguinolentum*, *Schizophyllum commune*, and *Trametes versicolor*. Directions for future research include investigations of the presence of the inactive retrotransposon-like element in other organisms, and of the utility of the repetitive DNA for differentiating individuals and revealing genetic variation appropriate for population studies.

Evolution and population dynamics of C. purpureum

There is a need for a better understanding of evolutionary influences on the population biology of plant pathogens and how movement of genes influences the geographical structure of populations. This should lead to a more complete understanding of the natural constraints on plant pathogens and how they can be exploited for use in integrated vegetation management strategies. Information about the population dynamics and evolution of plant pathogenic fungi can also be useful in devising strategies for disease control, as this information can indicate how fast novel genotypes (*e.g.* virulence genes)

might potentially spread between populations. Further work is required in this area so that a level of understanding of fungal population genetic structure can be obtained that is comparable to that in animals and plants.

Chondrostereum purpureum represents the first biocontrol agent for forestry applications in North America. The practical implications for this work include support for the production of guidelines for the safe use of *C. purpureum* as a biocontrol agent. This support was provided by this work in terms of an increased knowledge of the *C. purpureum* pathosystem as well as molecular genetic tools that have been developed to diagnose infection and monitor individuals of this fungus. Sampling of biogeographic diversity and analysis of gene flow in natural populations has applications in phytopathology, conservation biology and many other biological fields. This study of the systematics and population structure of *C. purpureum* has provided a framework for understanding the dynamics of this plant pathogen, which can be exploited for use in biocontrol strategies as well as for disease control. The methodologies employed in this work may be transferred to similar investigations of promising biocontrol candidates.

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