VARIATION IN THE MITOCHONDRIAL DNA OF *CHONDROSTEREUM PURPUREUM*

by

Tod Ramsfield

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Name: Tod Ramsfield

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Examining Committee:

Chair: Dr. N. Haunerland, Associate Professor

Dr. Z. Punja, Professor, Senior Supervisor
Department of Biological Sciences, S.F.U.

Dr. S. Shamoun, Research Scientist
Canadian Forest Service, Pacific Forestry Centre

Dr. W. Hintz, Assistant Professor
Department of Biology, U. Victoria

Dr. M. Smith, Professor
Department of Biological Sciences, S.F.U.

Dr. A. Levesque, Research Scientist
Agriculture and Agri-Food Canada
Public Examiner

Date Approved: DEC 9, 1997

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ABSTRACT

The mitochondrial DNA of the proposed biological control agent *Chondrostereum purpureum* was assessed as an indicator of total genetic variation in a sample population of isolates collected from North America, Europe and New Zealand. Extraction and purification of the mitochondrial genome allowed calculation of an approximate size of 67 kb. Restriction digestion of the total mitochondrial DNA from eight isolates of *C. purpureum* with the restriction endonuclease *Bam* HI and comparison of the resultant banding patterns by UPGMA analysis, indicated that isolates from British Columbia, Alberta, Finland, the Netherlands, and New Zealand had exactly the same restriction pattern. Two other isolates from British Columbia and Switzerland had different banding patterns and had similarity coefficients of 0.917 and 0.655, respectively. To study a larger sample population, a rapid polymerase chain reaction (PCR) assay was developed. PCR primers were designed to amplify two sequence-characterized amplified regions (SCARs). The first contained the NADH 4 gene and the second the ATPase VI and cytochrome b genes. Restriction digestion of the first SCAR with 24 restriction endonucleases failed to show any polymorphisms. Restriction digestion of the second SCAR with the endonuclease *Nsi* I was able to differentiate two mitochondrial haplotypes. A sample population of 84 isolates was screened and both haplotypes were found in all of the geographic regions surveyed. The calculated value of Nei's gene differentiation statistic, G_{ST}, was 0.0935; indicating that the diversity between sub-populations was not significantly different from the diversity within sub-populations. The low degree of variation in the total mitochondrial DNA, the inability of 24 restriction endonucleases to differentiate populations in the first SCAR, and the finding that two

mitochondrial haplotypes are distributed throughout the sample population suggest that the risk of introducing rare mitochondrial genes into new regions as part of biological control activities is low.

DEDICATION

I would like to dedicate this work to my grandfathers, Arthur Ramsfield and George Wellburn. I know they would be proud.

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TABLE OF CONTENTS

ABSTRACT iii

DEDICATION v

ACKNOWLEDGEMENTS vi

LIST OF TABLES ix

LIST OF FIGURES x

CHAPTER 1 - INTRODUCTION 1

1.1 VEGETATION MANAGEMENT 1
1.2 BIOLOGICAL CONTROL 3
1.3 *CHONDROSTEREUM PURPUREUM* - FUNGAL TAXONOMY AND CHARACTERISTICS. 6
1.4 *CHONDROSTEREUM PURPUREUM* AS THE CAUSAL AGENT OF SILVER LEAF DISEASE. 9
1.5 *CHONDROSTEREUM PURPUREUM* AS A BIOLOGICAL CONTROL AGENT. 11
1.5.1 Development *11*
1.5.2 Risk assessment *15*
1.5.2.1 Host range and spore dispersal 15
1.5.2.2 Genetic variation 16
1.5.2.3 Gene flow 18
1.6 FUNGAL MITOCHONDRIAL GENOMES 19
1.7 RESEARCH OBJECTIVES 24

CHAPTER 2 - MATERIALS AND METHODS 25

2.1 *CHONDROSTEREUM PURPUREUM* ISOLATION AND STORAGE 25
2.2 MITOCHONDRIAL DNA EXTRACTION 26
2.3 VARIATION IN THE TOTAL MITOCHONDRIAL DNA 28
2.4 CLONING OF MITOCHONDRIAL DNA 28
2.5 SEQUENCING OF FRAGMENTS, PCR PRIMER DESIGN AND AMPLIFICATION 29
2.6 SCREENING SCARS FOR POLYMORPHISMS 31
2.7 SAMPLE POPULATION SCREENING 32

CHAPTER 3 - RESULTS 34

3.1 ISOLATION OF MITOCHONDRIAL DNA, GENOME SIZE AND VARIATION 34
3.2 SCREENING MITOCHONDRIAL SCARS FOR POLYMORPHISMS 40
3.3 SCREENING OF SAMPLE POPULATION AND ASSIGNMENT OF HAPLOTYPES 43
3.4 MITOCHONDRIAL HAPLOTYPE AND RIBOSOMAL DNA HAPLOTYPE COMPARISON 47

CHAPTER 4 - DISCUSSION 49

4.1 TOTAL MITOCHONDRIAL DNA 50
4.2 MITOCHONDRIAL SCAR ANALYSIS 53
4.3 MITOCHONDRIAL INHERITANCE 56
4.4 MITOCHONDRIAL GENOME CONSERVATION AND EVOLUTIONARY IMPLICATIONS 58
4.5 CONCLUSIONS 65

CHAPTER 5 - FUTURE RESEARCH 67

5.1 MITOCHONDRIAL INHERITANCE 67
5.2 MATING SYSTEM 68
5.3 CONCLUSIONS 71

CHAPTER 6 - REFERENCES 73

APPENDIX 1 ..... **83**

1.1 DNA SEQUENCES READ IN THIS STUDY. 83

APPENDIX 2 ..... **85**

2.1 AMINO ACID SEQUENCE CONSENSUS OF SEQUENCES USED IN THIS STUDY. 85

LIST OF TABLES

TABLE 1. MITOCHONDRIAL SCAR PCR PRIMERS DEVELOPED IN THIS STUDY 30

TABLE 2. RESTRICTION ENDONUCLEASES AND CORRESPONDING RECOGNITION SEQUENCES USED TO DIGEST BOTH THE 3 KB AND 5 KB SCARS. 32

TABLE 3. SAMPLE POPULATION OF *CHONDROSTEREUM PURPUREUM* ISOLATE ORIGIN, HOST, YEAR COLLECTED, RIBOSOMAL AND MITOCHONDRIAL DNA TYPES. 43

TABLE 4. FREQUENCY DISTRIBUTION OF MITOCHONDRIAL HAPLOTYPES I AND II IN THE SAMPLE POPULATION STUDIED. 46

LIST OF FIGURES

FIGURE 1. CESIUM CHLORIDE GRADIENT SHOWING THE SEPARATION OF NUCLEAR AND MITOCHONDRIAL DNA. 36

FIGURE 2. RESTRICTION DIGEST OF DNA FROM TOP AND BOTTOM BANDS OF CESIUM CHLORIDE GRADIENT. 37

FIGURE 3. *BAM* HI DIGEST OF MITOCHONDRIAL DNA 38

FIGURE 4. DENDROGRAM BASED ON *BAM* HI DIGEST OF TOTAL MITOCHONDRIAL DNA GENERATED BY UPGMA ANALYSIS. 39

FIGURE 5. *NSI* I DIGEST OF ATPASE VI, CYTOCHROME B 5.1 KB SCAR 42

CHAPTER 1 - INTRODUCTION

1.1 Vegetation management

With ever-increasing demands for higher productivity being placed on a finite supply of forest land, vegetation management is becoming a major component of timber production. In regenerating forest plantations, competition from fast-growing, invasive hardwoods, such as red alder (*Alnus rubra* Bong.), sitka alder (*Alnus viridis* ssp. *sinuata* (Regel) Á. Löve & D. Löve), bigleaf maple (*Acer macrophyllum* Pursh.), paper birch (*Betula papyrifera* Marsh.), trembling aspen (*Populus tremuloides* Michx.), balsam poplar (*Populus balsamifera* L.) and black cottonwood (*Populus trichocarpa* Torr. & A. Gray) result in competition for light, water, nutrients, and space with the planted conifers. The result of this competition is often reduced growth of the regenerating conifers; therefore, the above-mentioned hardwoods are considered to be weeds (Biring et al. 1996).

Competing vegetation in a forestry environment is quite different from plant species which are considered to be weeds in an agricultural setting. Forest weeds display a high degree of variability in temporal, spatial and biological setting; they have various growth habits and competitive ability, and their ecological niches vary from bare ground to a complex of established species. The major difference between forest and agricultural weeds is that forest weeds are often perennial and native and they resprout vigorously from roots, rhizomes or cut stumps. Since most forest weeds are primary colonizers of disturbed sites, they do have important ecological roles, such as nitrification of soils, but in a forestry setting, this vegetation usually suppresses the commercial species, necessitating some degree of vegetation management (Watson and Wall, 1995).

Vegetation management is also implemented by utility companies in the maintenance of their rights-of-way. For example, in British Columbia, the Provincial power authority has 70,000 km of power lines located on 11,000 km of designated rights-of-way which must remain clear of vegetation to insure uninterrupted power service and to prevent electrical hazards (de Jong et al., 1996; Shrimpton et al., 1996).

Traditional weed control methods in forest regeneration sites and utility rights-of-way rely upon chemical herbicides and manual brushing. Both offer effective vegetation management, but also have undesirable side effects. Chemical herbicides, although effective, may result in damage to non-target vegetation and thier application is limited to non-riparian zones (Jobidon, 1991a). It is probable that restrictions on herbicide use will increase in Canada, since the majority of forestry operations occur on public land leased to timber companies, and non-timber values, such as wildlife, recreation and endangered species, as well as ecosystem degradation caused by herbicides, have increased public opposition to herbicide applications (Wagner, 1993; Wall et al., 1992). Herbicide use for forestry has already been restricted in Alberta and Saskatchewan and the probability is high that increased restriction will be placed on herbicide use in other regions of Canada (Campbell, 1990). Manual brushing offers an alternative to herbicide application, but often results in increased biomass due to vigorous resprouting of many hardwood species from below the cut surface, necessitating retreatment (Wall, 1994), and is labor-intensive and economically unfeasible (Wagner, 1993).

1.2 Biological control

Biological control in forest regeneration sites and utility rights-of-way as an alternative method to chemical herbicides and manual brushing is currently being investigated. Since restriction of herbicide use in forestry is likely, forest managers have ranked the development of alternate vegetation management strategies, including biological control, as the highest priority (Campbell, 1990). Biological control using plant pathogens has three distinct advantages over chemical herbicides: (1) they can be specific to the weed; (2) residue and toxicity problems would be reduced or eliminated; (3) there would be no accumulation of herbicide in soil and underground water (Wilson, 1969).

Biological control can take one of three strategies. Classical or inoculative biological control, the first strategy, assumes that the weed species have escaped their natural enemies, thus allowing the weeds to proliferate. Natural enemies are sought out in other regions and introduced into areas where they are absent and the weed is a problem, resulting in lower competitive ability of the weed and a subsequent reduction in the weed population (Mortensen, 1986). The goal of this control method is to establish a natural, self regulating balance between the host and pathogen (Hasan and Ayres, 1990). Rusts and other self dispersing agents which cause epidemics after their release, such as *Puccinia chondrillina* Bub. and Syd., which resulted in a 79% reduction of skeleton weed (*Chondrilla juncea* L.) in Australia, are examples of this biological control method. Before imported pathogens are released, however, they must undergo a thorough screening process to ensure host specificity and no effect on economically important crop species (Mortensen, 1986).

The second method of biological control, the inundative or bioherbicide approach, is accomplished through mass culturing of indigenous organisms as biocontrol agents, followed by application to the target weed. The use of indigenous fungi ensures that they will not normally persist in the environment at greater than endemic levels or spread from the treated site because natural controls are present to keep them in check (Wall et al., 1992). Inundative biological control agents have been registered for agricultural use in the United States. DeVine, using *Phytophthora palmivora* (Butler) Butler as the active ingredient for control of strangler (milkweed) vine (*Morrenia odorata* Lindl.) in Florida citrus groves, and Collego, a formulation of *Colletotrichum gloeosporioides* (Penz.) Sacc. f. sp. *aeschynomene* to control northern jointvetch (*Aeschynomene virginica* (L.) B.S.P. in rice and soybean fields, are the two successful examples of biological control of weeds (TeBeest and Templeton, 1985).

The third method of biological control is the augmentative, or integrated vegetation management approach, where natural populations of pests or pathogens of weeds are manipulated (Wall et al., 1992). Exploitation of allelopathy, the production of chemical compounds by one plant which cause other plants to be excluded from the environment around the plant, is an augmentative biological control strategy. The allelopathic effects of kikuyu grass (*Pennisetum clandestinum* Chiov.) were demonstrated in Taiwan, where significant reductions in the growth of weeds were observed without affecting the regeneration of Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook.). The allelopathic effect of straw mulches has also been shown in Quebec, which significantly reduced the growth of red raspberry shoots (*Rubus ideaus* L.) in a regenerating black spruce (*Picea mariana* (Mill.) B.S.P.) stand (Jobidon, 1991b). Another aspect of

integrated vegetation management is using biological control in combination with low doses of chemical herbicides, arthropod pests or manipulating species composition to achieve weed control (Hasan and Ayres, 1990).

The strategy for development of inundative biological control agents consists of three major stages: (1) discovery; (2) development and; (3) deployment. During the discovery stage, the weed of interest is identified and extensive field collections of diseased samples are made. Under laboratory conditions, the pathogens present are isolated from the weed and identified. Pure cultures of the pathogens are then applied to healthy and uninfected weeds under greenhouse conditions and the disease process is monitored. Once the symptoms are described, each pathogen is reisolated from the diseased tissue and identified. The identity of the pathogen isolated from the diseased plant must be same as that which the plant was initially inoculated with and result in the same symptoms to prove Koch's postulates, and thereby prove that the potential biological control agent is the causal agent of the disease. The development stage involves determination of conditions for optimum inoculum production, disease development, host range and evaluation of the efficacy of the pathogen as a potential biocontrol agent. During the deployment stage, mass production strategies for scale-up, formulation, regulatory, and marketing strategies for the biocontrol are developed (Watson and Wall, 1995). During these stages, the essential criteria of: (1) being able to produce abundant and durable inoculum in artificial culture; (2) genetic stability of the inoculum and specificity for the target weed; and (3) being able to infect and kill the weed under variable environmental conditions, are all assessed (Templeton, 1982; Sands et al., 1990). Failure of the potential biological control agent to meet any of these criteria will

result in the search for alternative pathogens.

Biological control is a realistic alternative to chemical herbicides in agro-forestry settings where vegetation management is required and is an important component of an integrated vegetation management approach to weed control. *Chondrostereum purpureum* (Pers.:Fr) Pouzar is a candidate biological control agent for forest weeds which is currently being developed for red alder (Shamoun et al., 1996; Wall, 1994), sitka alder (Harper et al., 1996), black cherry (Scheepens and Hoogerbrugg, 1988), and aspen (Dumas et al., 1997; Pitt et al., 1996).

1.3 *Chondrostereum purpureum* - Fungal taxonomy and characteristics.

Chondrostereum purpureum (syn. *Stereum purpureum* (Pers.:Fr) Fr.) (Pouzar, 1959) is a basidiomycete in the order Meruliales, family Meruliaceae (*in sensu* Ginns and Lefebvre, 1993). The fungus was initially classified in the genus *Stereum*, but has been segregated into the genus *Chondrostereum* because the fructifications are significantly different from those of *Stereum* (Ginns and Lefebvre, 1993; Pouzar, 1959). *Chondrostereum purpureum* basidiocarps are monomitic with clamp-bearing generative hyphae with thickened walls as opposed to those of *Stereum,* which are dimitic without clamp connections (Lentz, 1971; Reid, 1971). The presence of large sack-like vesicles in the area above the hymenium and the inamyloid basidiospores are also features distinctive to this genus (Reid, 1971).

The basidiocarp of *C. purpureum* is resupinate to reflexed, monomitic, with leptocystidia and the spores are hyaline, smooth and inamyloid (Hawksworth et al., 1995). Young basidiocarps are purplish in color, becoming dingy with age. The upper

surface is tawny to brown, frequently hairy and zoned, while the lower hymenial surface is purple to lilac in colour and covered with basidia. Mature basidiocarps are 2 to 8 cm across with a vesicular system of hyphae which nourish the hymenium. Basidiospores are produced rapidly under humid conditions (Butler and Jones, 1949).

Mycelium of *C. purpureum* in culture forms white mats, appressed to thin or slightly raised and arachnoid to downy at the point of inoculation, then becoming raised, moderately thick, woolly to cottony toward margins at 1 week. By 2 weeks, the mycelium is white to light buff, appressed to slightly raised, moderately thick to thick, felty to woolly throughout, with some isolates forming numerous, small, cottony, mycelial balls. At 6 weeks, the mycelium is white to ivory yellow, appressed to slightly raised, moderately thick to thick, firm, felty to floccose or tomentose with margins bayed, appressed, fimbriate. The odor of the culture is sweet and fruity at 1 week and absent at 2 and 6 weeks. Marginal hyphae are 6-7 μm in diameter, thin walled, nodose septate, sparsely branched with branches arising opposite clamps. Submerged hyphae are 1.5-6 μm in diameter, thin walled becoming thick walled at 6 weeks, simple septate with scattered clamps especially on wider hyphae, moderately to frequently branched. Aerial hyphae are similar to marginal hyphae except up to 9 μm in diameter and slightly thick walled. Some hyphae are similar to submerged hyphae and some fiber hyphae 1-2 μm in diameter which are thin walled at first, becoming thick walled or rough walled, simple septate, moderately to frequently branched and coated with resinous materials that disappear in 2% KOH. Some hyphae become conglutinate at 6 weeks. Cystidia are present on the mycelium and are clavate to fusiform, occasionally constricted or monilioid, often stalked, up to 15 μm in diameter, hyaline, thin walled to slightly thick

walled, terminal or occasionally lateral, simple septate at the base, smooth to heavily coated with resinous material that disappears in 2% KOH, are scattered to numerous in aerial mats and sometimes becoming thick walled with age to resemble chlamydospores (Nakasone, 1990; Ginns and Lefebvre, 1993).

The lifecycle is that of a typical basidiomycete. Basidiospores germinate on freshly wounded woody tissue and homokaryotic mycelium extends into the xylem (McHenry et al., 1996). On contact with homokaryotic mycelium of a compatible mating type, hyphal anastamosis occurs followed by bidirectional nuclear migration, resulting in a heterokaryon with clamp connections. A basidiocarp is formed on the bark of the host and karyogamy and meiosis occur in the basidium. Each nucleus then migrates into the sterigmata which differentiate to form basidiospores containing single nuclei (Ginns and Lefebvre, 1993). The mating system of *C. purpureum* is heterothallic and tetrapolar, which insures that populations are outcrossing (Ginns and Lefebvre, 1993; Gosselin et al., 1995; Rayner and Boddy, 1988; Wall et al., 1996).

The host species are angiosperms and rarely gymnosperm trees, wood, logs and branches and the fungus is distributed widely throughout North America (Ginns and Lefebvre, 1993) and temperate zones throughout the world (Ramsfield et al., 1996). *Chondrostereum purpureum* causes white rot (Nakasone, 1990) and is a primary wound invader, spreading rapidly after infection and fruiting within a year but with low combative ability, allowing it to be rapidly replaced by other fungi such as *Coriolus versicolor* L. ex Quél. (Rayner, 1977; Rayner, 1979). *Chondrostereum purpureum* has been found to infect balsam fir (*Abies balsamea* (L.) Mill.), but was not associated with advanced decay and the fungus could not be recovered in culture (Etheridge and Morin,

1963). It is also possible for *C. purpureum* to infect logs and waste wood as described by Fritz (1954) who observed *C. purpureum* infection of poplar logs in storage.

1.4 *Chondrostereum purpureum* as the causal agent of Silver leaf disease.

Silver leaf disease occurs on stone and pome fruit trees, including peaches, nectarines, apples, apricots, cherries and plums and is caused by *C. purpureum.* It is spread by wind borne basidiospores which enter the xylem through fresh wounds, or pruning cuts less than one month old, and initiate infection (Beever, 1970). Incidence of silver-leaf disease has been correlated with winter injury brought on by severe winters in both Washington State and Poland (Sprague and Hord, 1952; Borecki et al., 1978). During establishment and pathogenesis, the fungus is limited to the xylem tissues and obtains nutrients from the xylem sap (Beever, 1970). The silver leaf symptom is a result of translocation of a diffusable toxin produced by *C. purpureum* in the transpiration stream, which causes the upper epidermis to separate from the palisade cells, allowing the accumulation of air and the resultant silver sheen in the foliage (Butler and Jones, 1949). The exact nature of this translocatable toxin has not been elucidated, but proteases have been implicated because the only available nitrogen source within wood is from protein. Production of extracellular enzymes, such as polygalacturonase, result in wood degredation, making nitrogen available to the fungus (McHenry et al., 1996). Mortality of trees has also been reported without the characteristic silver-leaf symptom. Apple trees in Wisconsin were identified as killed by *C. purpureum* based on the morphology of basidiocarps located on cankers of the dead trees (Setliff and Wade, 1973).

Trees are susceptible to infection by *C. purpureum* at any time, but least susceptible in June, July and August because the soluble carbohydrate concentration, the major nutrient source available to the fungus, is at its lowest (Butler and Jones, 1949; Beever, 1970). Establishment of the pathogen in wounded tissue results in translocation of the toxin to the leaves, which turn silver, become bumpy, chlorotic and necrotic. Terminal growth of the affected branches is stunted and flowers or young fruit dehisce early (Williams and Cameron, 1956). Staining, due to mycelial growth, occurs in the central region of the xylem tissue and extends longitudinally up and down the stem into the roots much more rapidly than laterally (Butler and Jones, 1949). The pathogen may spread from the inoculation point and gradually take over the whole tree, resulting in mortality; or the tree may be able to produce a "gum barrier" which restricts *C. purpureum* growth and the tree may survive (Butler and Jones, 1949; Williams and Cameron, 1956). Basidiocarps form on cankered, dead tissue and basidiospores are released during wet weather, or under high relative humidity (RH > 90%) throughout the year (Spiers and Hopcroft, 1988). The production of basidiospores has been estimated to result in 160 basidiospores / m^3 at 500 meters from the point of emission, dropping to 4 basidiospores / m^3 at 5000 meters (deJong et al., 1990). It has been shown that spores of *C. purpureum* from basidiocarps produced on one host may readily infect a different host, but different hosts vary in their susceptibility to the disease (Bishop, 1979). This has been exemplified by Wall (1996) and Dumas et al. (1997), who found that red alder was highly susceptible to *C. purpureum*, while bigleaf maple, aspen and conifers were highly resistant.

Control of silver-leaf has been accomplished through the removal of infected material by pruning back to healthy wood during the summer when chances of reinfection are lowest. Exclusion of *C. purpureum* spores from freshly pruned branches can be accomplished through immediate treatment with grafting wax or fungicidal paints. Since *C. purpureum* can infect dead stumps and logs, all dead pruning materials should be removed from orchards and burned (Butler and Jones, 1949). Biological control of *C. purpureum* using *Trichoderma viride* Pers. ex Fr. spores applied with specially designed pruning shears, has been shown to inhibit *C. purpureum* from colonizing the cut branch (Grosclaude et al., 1973). *Trichoderma viride* in combination with triadimefon fungicide resulted in significant protection from infection of pruning wounds by *C. purpureum* in plum (Woodgate-Jones and Hunter, 1983). It was observed that pear trees infected with *C. purpureum* could recover by inserting *T. viride* colonized dowels, resulting in a 61% reduction in disease. The exact mode of action of *T. viride* is unknown, but since the propagules of *T. viride* and *C. purpureum* do not interact, it is hypothesized that some sort of antibiosis occurred as a result of translocated metabolites (Mukerji and Garg, 1988).

1.5 *Chondrostereum purpureum* as a biological control agent.

1.5.1 Development

The efficacy of *Chondrostereum purpureum* as a biological control agent for forestry has been thoroughly studied. In Canada, Wall (1986) studied the pathogenicity of *C. purpureum* to yellow birch (*Betula alleghaniensis* Britt.). Basidiocarps of *C. purpureum* were collected from recently killed birch saplings and it was determined that the fungus was viable at temperatures ranging from 5°C to 35°C, with an optimum of

25°C, and mortality occurred at 37°C after 7 days. Artificial inoculation of yellow birch resulted in rapid colonization of the cambial region and sapwood, with the pathogen spreading most rapidly longitudinally and slowly toward the center of the stem. No foliar symptoms or mortality were noted during the duration of the experiment, but the rapid colonization of the cambium and sapwood demonstrated that *C. purpureum* is a potential cause of cankers and premature mortality of deciduous trees (Wall, 1986). It is this rapid colonization ability which supports the potential use of *C. purpureum* as an inundative biological control agent (Wall, 1991).

The first reported use of *C. purpureum* as a biological control agent was in the Netherlands, where it was used to control American black cherry (*Prunus serotina* Erhr.), a species introduced from North America to improve the forest understory and soil structure. Due to it's ability to colonize open areas, black cherry competes with the newly planted forest trees and as a result is considered to be a weed. Biological control was tested as an alternative method for black cherry control, and inoculation of cut stumps with *C. purpureum* resulted in average mortality of 87%, which was significantly higher than the average mortality of 38% reported for the untreated controls (Scheepens and Hoogerbrugge, 1989).

Variation in susceptibility of different host species to *C. purpureum* infection was described by Wall (1990), who inoculated stumps of American beech (*Fagus grandifolia* Ehrh.), yellow birch, red maple (*Acer rubrum* L.), sugar maple (*Acer saccharum* Marsh.), trembling aspen (*Populus tremuloides* Michx.), paper birch (*Betula papyrifera* Marsh.) and pin cherry (*Prunus pensylvanica* L. f.). He observed that in all of the species, the proportion of stumps with living sprouts was less in the *C. purpureum* inoculated

treatments compared to control treatments. It was found that yellow birch was highly susceptible to infection, with greater than 100 basidiocarps produced on each treated stump and complete inhibition of resprouting. Trembling aspen showed the least evidence of infection and a negligible reduction in resprouting was observed. Variation in susceptibility of the different hosts to infection is likely due to variable levels of resistance to the fungus, as both the tree species studied and the fungus are endemic. The general reduction in resprouting, however, demonstrated that *C. purpureum* could be used as a tool in an integrated vegetation management program.

Recently, many experiments have been conducted to further test the efficacy of *C. purpureum* as a biological control agent. Red alder was treated and it was found that control of up to 80% was achieved (Wall, 1994). Shamoun et al. (1996) found that *C. purpureum* treatment in utility rights-of-way resulted in a level of control of red alder comparable to that of chemical herbicides. Sitka alder was also controlled very well by *C. purpureum*, resulting in 88% clump mortality which was not statistically different from triclopyr herbicide (Harper et al., 1996). However, resprouting of speckled alder (*Alnus rugosa* (Du Roi) Spreng) was not inhibited after treatment with *C. purpureum*, but the treated clumps were smaller than the untreated controls (Pitt et al., 1996). Treatment of trembling aspen and largetooth aspen (*Populus grandidentata* Michx.) resulted in significant reduction in stump resprouting and resprout mortality when compared to the untreated control, but herbicide treatment resulted in stump death (Dumas et al., 1997). The range of efficacy of *C. purpureum* can clearly be seen in these experiments. Red alder was highly susceptible to infection and control rates were not significantly different from herbicide application; while aspen regrowth, although reduced, was not eliminated

by *C. purpureum.*

Timing of application has also been found to affect efficacy of *C. purpureum* in some tree species. Wall (1991, 1994) found that host resistance was highest in the spring and decreased until mid summer at which time resistance began to rise. This appears to contradict the literature (Butler and Jones, 1949; Beever, 1970); however, a distinction must be made between inoculation success and subsequent invasion (Wall, 1991). In the earlier studies, natural inoculum (which is most prevalent in the damp fall and winter) served to initiate infection; while in the biological control application, the fungus was applied directly to a wound in a protective formulation, guaranteeing infection. Successful establishment is most likely related to seasonal carbohydrate levels which affect the ability of the tree to compartmentalize the fungus. In spring, when the metabolic activity of the tree is high, cambial activity is also high, allowing rapid compartmentalization of the fungus. However, when metabolic activity and the reserves are low, the tree is unable to compartmentalize the fungus, resulting in higher mortality. Seasonal effects were also studied by Dumas et al. (1997) in aspen, who found no significant difference between treatment time and the degree of pathogenesis. This indicates that not all tree species show strong seasonal effects, but it should be noted that aspen is highly resistant to *C. purpureum* (Wall, 1990), and the effect of season may be less important in resistant species.

Formulation technology can significantly increase the efficacy of biological control agents because, as living organisms, they are susceptible to environmental extremes and their growth environment. Protection from heat, desiccation, ultraviolet light and competing organisms is provided by the formulation that the biological control

agent is applied in (Wall et al., 1992). Fungal development is dependent upon water availability in the growth medium and environment, therefore protection against desiccation must be provided by the formulation. Protection from desiccation can be provided by encapsulation of the organism in clay or vermiculite, or water absorbing compounds such as sodium alginate, or by formulating the fungus as an invert emulsion so that water is trapped in oil. The addition of nutrients to the formulation, which increase fungal activity, also serves to increase the efficacy of the biological control agent by stimulating fungal growth (Boyette et al., 1996). Monitoring of the environmental conditions prior to application is advisable so that optimal temperature and humidity can be forecast before the biological control agent is applied (Hasan and Ayrers, 1990; Wall et al., 1992). Field trials have shown that *C. purpureum* is a viable alternative to chemical treatment to control stump resprouting in selected hardwoods. Optimization of this biological control agent through selection of isolates of the fungus that show high levels of pathogenesis, optimizing culture, formulation delivery and application techniques, as well as timing application to allow rapid establishment in the host, may result in increased bioherbice efficacy in species which are moderately resistant to the fungus (Wall, 1990).

1.5.2 Risk assessment

1.5.2.1 Host range and spore dispersal

Before the use of an inundative biological control agent is implemented in an area, the effects of the increased inoculum density above the natural inoculum level must be determined to quantify non-target risks. While modeling spore dispersal using the

Gaussian plume model, de Jong et al. (1990) calculated that a minimum separation of 500 meters between treated areas and susceptible non-target hosts was a safe distance to prevent secondary infection. Estimation of the increase in inoculum density after treatment in field studies have determined that the increase in *C. purpureum* fructification after biological control activities would be less than or equal to natural fructification, which is not considered significant (de Jong et al., 1996). Host range studies have shown that *C. purpureum* is not virulent on softwood trees and therefore poses no risk to the regenerating conifers (Wall, 1996). These studies have indicated that the risks of using *C. purpureum* as a biological control agent are low and do not exceed the risks associated with naturally occurring inoculum sources (Wall, 1997). The rapid replacement of *C. purpureum* by other competitive fungi, such as *C. versicolor*, ensures that it will not persist in the environment at endemic levels for an extended period of time (Rayner, 1977).

1.5.2.2 Genetic variation

Consideration of secondary risks to non-target vegetation must be coupled with an estimation of the risks associated with the possible introduction of new genetic material into the endemic population of the pathogen from the strains used in biological control activities to avoid the possible introduction of rare virulence alleles and the resultant general increase in the pathogen's virulence (Templeton et al., 1979). It is not possible to directly survey the variation at virulence loci of *C. purpureum* because they are uncharacterized. Therefore, other techniques must be used to assess the amount of genetic variation present in the population. Initial studies using protein profiles of a

Canadian sample population, indicated that no host or geographic races existed (Ekramoddoullah et al., 1993). API ZYM and isozyme analysis of a Canadian population also indicated a very low level of genetic variability (Shamoun and Wall, 1996). Variation in the DNA provides a better measure of genetic variation because the genotype, rather than phenotypic expression of a trait, is examined. Variation in the internal transcribed spacer region of the ribosomal DNA of a small sample population of *C. purpureum* by Shamoun et al. (1991) distinguished an isolate from New Zealand, suggesting the level of variation in the other regions studied (Canada and Europe) was low. A more extensive study using randomly amplified polymorphic DNA (RAPD) analysis on a Quebec population differentiated isolates, but no groupings could be correlated with host or geographic specialization (Gosselin et al., 1995). Recently, examination of levels of variation in the large non-transcribed spacer region of the ribosomal DNA of 107 isolates of *C. purpureum* identified three specific nuclear type patterns (Ramsfield et al., 1996). The first was distributed throughout the entire region surveyed, the second was detected only in North America, and the third was found only in Europe and New Zealand. The distribution of the two nuclear type patterns in North America indicated that gene flow was occurring across the continent. Each of these previous studies has indicated that the level of genetic variation of this fungus is limited, but since this fungus is in the process of being registered as a biological control agent, it is desirable to investigate the population structure using as many established marker systems as possible.

1.5.2.3 Gene flow

Coupled with an understanding of the amount of genetic variation, it is also important to determine if gene flow is acting to maintain genetic variability or homogeneity. Gene flow occurs by migration, gamete movement, the extinction and recolonization of populations or the movement of extranuclear DNA, such as mitochondrial DNA, plasmids and viruses. Gene flow within a species is the governing factor in determining the extent to which local populations are independent (Slatkin, 1985). The movement of one individual per generation between populations is all that is required to prevent differentiation between the two populations (McDermott and McDonald, 1993). Genotypic differentiation occurs through natural selection, allowing populations to adapt to their local environment, and mutation. Random genetic drift is an important mechanism of allele frequency change in small isolated populations. The evolutionary implications of gene flow are that it can result in the movement of single genes or genotypes throughout populations as a creative force, or it can be a limiting factor, restricting the ability of local populations to adapt (McDermott and McDonald, 1993). In this way, gene flow can constrain evolution by preventing adaptation or promote evolution by spreading genes throughout a species range (Slatkin, 1987).

Patterns of gene flow can be inferred from spatial distribution of alleles. An indirect assessment of gene flow can be made based on computing statistics from data and then relating the statistics to a particular model of gene flow. *F* statistics, developed by Wright (1951), are used to estimate gene flow. The inbreeding coefficient, *F*, has been partitioned into F_{IT}, the correlation coefficient between uniting gametes relative to the whole population, F_{IS}, the correlation coefficient of uniting gametes relative to alleles in a

particular subpopulation, F_{ST}, the correlation of two randomly chosen alleles in a subpopulation relative to alleles in the whole population. F statistics are valid assuming two alleles are present at a locus and there is an infinite number of sub populations. To overcome these limitations, Nei (1973) proposed the gene diversity index, G_{ST}, which can be used for multiple alleles and finite populations. The gene diversity index will be used in this study to investigate if diversity within sub populations is significantly different from diversity between sub populations.

Gene flow plays an important role in the natural populations of plant pathogenic fungi. The tools of population genetics allow investigations into the amount of gene flow in fungal populations. Use of molecular marker systems, which are able to assess genetic variation in populations, can be coupled with an analysis using population genetics to determine if populations are isolated or if gene flow is occurring between them. Completion of these studies before the application of a biological control agent is important because the population genetic studies will reveal whether or not gene flow is occurring. If gene flow is not occurring between populations, they are effectively isolated and biological control treatments must use isolates collected from each sub population as the biological control agent. If gene flow is occurring between populations, it has a homogenizing effect, indicating that the use of one particular isolate as a biological control agent is not restricted to specific regions.

1.6 Fungal mitochondrial genomes

The degree of variation in the mitochondrial genome of *C. purpureum* has not been researched and this marker will be used in this study to assess the amount of genetic

variation within the species and how much gene flow is occurring between sub populations. Gene flow is an important factor in mitochondrial genomes of fungi because it allows gene flow across otherwise isolated clonal mitochondrial DNA lines, making gene flow an important mechanism in maintaining mitochondrial variability (May and Taylor, 1988). Evolution of mitochondrial genomes in animal occurs at a rate 10 times faster than the nuclear genome; therefore, mitochondrial DNA may be a more sensitive measure of the level of variation between populations than some nuclear encoded markers (Contolini et al., 1992; Kistler et al., 1987; Taylor, 1986).

Mitochondrial genomes of fungi are unlike mitochondrial genomes in other organisms with respect to their variable size and mode of inheritance. Gene arrangement in fungal mitochondrial genomes is less organized compared to animal mitochondrial DNA, but is more organized than the mitochondrial DNA of plants (Clark-Walker, 1992). The size of fungal mitochondrial genomes has been shown to range from 17.6 kb in *Schizosaccharomyces pombe* Lindner (Zimmer et al., 1984) to 176 kb in *Agaricus bitorquis* (Quel.) Sacc. (Hintz et al., 1985) and the genome is typically circular, although linear exceptions do exist. The variability in mitochondrial genome size is due to A+T rich intergenic spacer regions, optional introns, and variation in gene copy number (Hudspeth, 1992; Pfeifer et al., 1992). In some fungal genera, species have been identified with three- to four-fold mtDNA size variation; while in others, mtDNA is much more uniform in size. Intraspecific mtDNA size variation has been observed in *Saccharomyces cerevisiae* Hansen, with variation from 68 to 85 kb due to differential intron presence (Clark-Walker, 1992). Races of *Ophiostoma ulmi* (Buisman) Nannf. vary in mitochondrial size from 74 - 78 kb, 65 - 69 kb and 50 - 59 kb (Bates et al., 1993;

Charter et al., 1996; Hintz et al., 1993; Jeng et al., 1991).

The typical fungal mitochondrial genome encodes eleven genes, representing the necessary subunits required during cellular respiration (cytochrome oxidase 1 - 3, apocytochrome b, NADH dehydrogenase 1 - 6, NADH dehydrogenase 4L), three genes coding the subunits of the ATP-synthetase complex (ATPase 6, 8 and 9), two genes coding ribosomal RNAs (small and large ribosomal RNAs) and multiple tRNAs (Paquin et al., 1997). The gene order in fungal mtDNA is highly variable and can even vary within a single species; however, levels of transcription are unaffected due to multiple promoters and continuous transcription. Different mitochondrial gene arrangements had no effect on growth rate of *Kluyveromyces lactis* (Dombrowski) van der Wal, providing experimental evidence that gene arrangement in fungi may be inconsequential (Clark-Walker, 1992).

Unlike the maternal inheritance and lack of recombination in mammalian mitochondrial genomes (Avise et al., 1987), basidiomycete mitochondrial genomes are not always inherited uniparentally and recombination has been shown to occur at the zone of interaction between two homokaryons (Fukuda et al., 1995; Matsumoto and Fukumasa-Nakai, 1993; 1996; Taylor, 1986). Although recombination of mitochondrial DNA has been shown, the recombined genome is typically limited to the zone of interaction and mitochondrial inheritance is paternal (ie. mitochondria of the parent homokaryon is retained in the heterokaryon). In basidiomycetes, hyphal anastomosis of two homokaryons is followed by bidirectional nuclear migration, resulting in the formation of a heterokaryon with identical pairs of nuclei throughout the mycelium (Hintz et al., 1988a). Under laboratory conditions, it was found that mating between *Armillaria*

bulbosa (Barla) Romagn. strains resulted in a mosiac of mitochondrial types throughout the heterokaryon (Smith et al., 1990); however, this appears to be an exception since matings of *Agaricus bitorquis* (Hintz et al., 1988a), *Coprinus cinereus* (Schaeffer : Fries) Gray C (May and Taylor, 1988), *Neurospora crassa* Shear et Dodge (Mannella et al., 1979), and *Lentinula edodes* (Berk.) Pegler (Fukuda et al., 1995) resulted in parental inheritance of the mitochondrial type, indicating an absence of mitochondrial migration after mating.

Mutations in mitochondrial genomes have been shown to cause phenotypic changes in fungi with defective mitochondria. Petite mutants of *S. cerevisiae* form at a rate of approximately 1% per generation and are induced by destabilization of the mitochondrial genome through excision of G+C clusters, which result in the petite phenotype (Clark-Walker, 1992). Senescence of the Ascomycete *Podospora anserina* (Cesati) Niessl is caused by rearrangement of the mitochondrial genome as a result of the amplification of senescence DNAs, which are initiated by inheritance of a cytoplasmically located factor (Jamet-Vierny et al., 1997). The *poky* mutations of *Neurospora crassa* are a result of a cytochrome deficiency and decreased ratios of the 19S to 25S mitochondrial ribosomal RNAs (Mannella et al., 1979). Hypovirulence in *Ophiostoma ulmi*, the causal agent of Dutch elm disease, and *Cryphonectria parasitica* (Murrill) Barr, the causal agent of chestnut blight, has been associated with mitochondrial mutations. In *O. ulmi*, a double stranded RNA (dsRNA) virus has been isolated from mitochondria (Rogers et al., 1987) which caused the induction of plasmid formation through recombination of long repeats in the mitochondrial large subunit rRNA, resulting in the hypovirulent phenotype (Abu-Amero et al., 1995). In the *C. parasitica* case, mutant mitochondria which have

respiratory deficiencies were transmitted to virulent strains by hyphal anastamosis and multiplied in the virulent host, resulting in the hypovirulent phenotype (Bell et al., 1996; Mahanti, et al., 1993).

Variation in mitochondrial DNA has been used in many studies to assess the level of intraspecific variation; the degree of variation reported to date is quite variable. Variation has been reported to be quite low in *Fusarium oxysporum* Schlect. and restriction fragment length polymorphisms (RFLP) patterns correlated with *formae speciales* (Kistler et al., 1987). Further study of *Fusarium oxysporum* f. sp. *melonis* (Leach & Currence) Snyd. & Hans. mitochondrial DNA indicated that each vegetative incompatability group (VCG) had a distinct mitochondrial type (Jacobsen and Gordon, 1990). Intraspecific variation was reported to be low compared to interspecific variation in *Sclerotinia* (Kohn et al., 1988), *Armillaria* (Smith and Anderson, 1989), *Phytophthora megasperma* Drechs. (Pm) (Förster et al., 1989) and *Agaricus* (Hintz et al., 1988b). Alternatively, highly variable mitochondrial genomes, and cluster analysis of RFLP patterns correlated to geographic groupings, have been reported in *Pleurotus ostreatus* (Jacq.:Fr.) P. Kumm. (Matsumoto and Fukumasa-Nakai, 1995), *Lentinula edodes* (Matsumoto and Fukumasa-Nakai, 1993; Fukuda et al., 1994), *Ophiostoma ulmi* (Bates et al., 1993; Hintz et al., 1993), and *Cryphonectria parasitica* (Milgroom and Lipari, 1993). The range of mitochondrial DNA variation reported for these species is due partly to differences in lifecycle and mode of propagation for each species. Some species exist as large clones and have clonal modes of propagation, while others are dispersed by sexually derived spores. As a result, the mitochondrial DNA would be expected to be more variable in outcrossing heterothallic species such as *C. purpureum* and less variable in

inbreeding homothallic species, such as *F. oxysporum*.

1.7 Research objectives

The overall objectives of this research study were to elucidate the population structure of *C. purpureum*, the level of gene flow, and to assess the risks of isolate movement during biological control activities through assessment of the variation in the mitochondrial DNA. The specific objectives of this study were threefold. The first was to extract mitochondrial DNA from *C. purpureum* and determine the size of the mitochondrial genome. Second, the variation in the total mitochondrial genome of a small sample population of *C. purpureum* which was collected from around the world was assayed. The third objective was to develop sequence characterized amplified region (SCAR) markers to further study the variation in a larger world wide sample population.

CHAPTER 2 - MATERIALS AND METHODS

2.1 *Chondrostereum purpureum* isolation and storage

Isolates of *C. purpureum* were obtained from naturally infected trees by one of the following methods: 1) stained cambial tissue was excised from an infected tree and in the laboratory wood chips were removed from the stained cambial tissue and surface sterilized by placing the infected wood chip in 95 % ethanol for 30 seconds, then transferring to 10 % bleach (Javex, Colgate-Palmolive Inc., Toronto) for 30 seconds and rinsing in sterile distilled water 3 times, followed by plating on 1.25 % malt agar (Difco, Detroit, MI) and incubation at 20°C in the dark until mycelium emerged; or 2) basidiocarps of *C. purpureum,* removed from infected trees, were surface sterilized using the same method as for the infected wood chips and a small piece was aseptically removed and plated on 1.25 % malt agar and incubated at 20°C in the dark; or 3) single spore isolates were collected by casting spores onto water agar by suspending a moist basidiocarp over sterile medium for 5 minutes, followed by incubation at 20°C in the dark for 24 hours and then excising germinated spores from the media before hyphal anastomosis with adjacent germinating spores occurred. In all cases, mycelium emerging from wood chips, basidiocarps or spores was transferred to fresh 1.25 % malt agar and incubated at 20°C in the dark to obtain pure cultures.

Isolates collected for this study originated in Canada, the United States, Europe and New Zealand and were collected from a variety of hardwood trees (Table 3). North American isolates were characterized as Western, Central or Eastern based on a 500 km border inland from the Pacific and Atlantic coasts. Isolates collected greater than 500 km from the coasts were considered to be central in origin. Total mitochondrial DNA was

isolated from 8 isolates of *C. purpureum* and 84 isolates were screened using a polymerase chain reaction (PCR) based assay. International isolates were gathered by researchers in the foreign countries surveyed using one of the described methods and then mailed to Dr. S.F. Shamoun of the Canadian Forest Service, Victoria, BC, Canada.

Long term storage of *C. purpureum* cultures was on 1.25% malt agar slants at 5°C, under sterile distilled water according to the method of Burdsall and Dorworth (1994), and in 15% glycerol and 5% dimethylsulfoxide submerged in liquid nitrogen.

2.2 Mitochondrial DNA extraction

To provide mycelial biomass material for DNA extraction, isolates were grown in 250 ml of 1.25% malt broth and shaken at 125 rpm at 22°C for 3 to 4 weeks with approximately a 10 hour photoperiod. Mycelium was then harvested by vacuum filtration, frozen at -86°C and freeze-dried.

Total DNA was extracted from 2 to 5 grams of freeze-dried mycelium using a modified phenol / chloroform extraction procedure (Ramsfield et al., 1996). Mycelium was ground to a fine powder, suspended in 15-25 ml of 50 mM ethylenediaminetetraacetic acid (EDTA) with 0.2% sodium dodecyl sulfate (SDS) and incubated at 65°C for 20 minutes. Cell debris was removed by centrifugation at 1000 x *g* for 10 minutes, the supernatant was transferred to a fresh tube and one-sixth volume of 3.0 M potassium acetate with 5.0 M acetic acid added. Following incubation on ice for 30 minutes, and centrifugation at 1000 x *g* to remove residual protein and other contaminants, an equal volume of isopropanol was added to the supernatant to precipitate the DNA. Whole cell DNA was then resuspended in 10 mM Tris (pH 7.5) with 10 mM

EDTA, extracted once with 10 mM Tris with 1 mM EDTA saturated phenol, and twice with chloroform : isoamyl alcohol (24:1). DNA was precipitated from the aqueous layer by the addition of 1/10 volume of 3 M sodium acetate and 2 volumes of 95% ethanol, followed by incubation on ice for 30 minutes. The precipitated DNA was pelleted by centrifugation and washed with cold 70% ethanol and finally resuspended in 3 ml TE buffer (10 mM Tris (pH 7.5); 10 mM EDTA).

The crude total DNA extract was further separated into mitochondrial and nuclear components by two rounds of ultracentrifugation based on modifications of the methods reported by Specht et al. (1983). Residual proteins and polysaccharides contaminating the DNA, which prevented the separation into the mitochondrial and nuclear components, were removed in the first round of ultracentrifugation. To the 3.0 ml of DNA containing solution, 4.0 g of cesium chloride and 400 µl of ethidium bromide (10 mg / ml) were added and the volume was brought to 5.0 ml with TE, giving a final concentration of 0.8 g / ml cesium chloride. The refractive index of the solution was measured to insure it was in the range of 1.3880 - 1.3900. Ultracentrifugation was performed in a Beckman model L8M ultracentrifuge using a 50.4ti rotor. Gradients were centrifuged at 48,000 rpm at 15°C for 15 hours. Purified total DNA was removed by side puncture with a hypodermic syringe and the DNA was extracted with cesium chloride-saturated isopropanol to remove the ethidium bromide and dialyzed in TE overnight to remove cesium chloride.

Separation of the total DNA into the nuclear and mitochondrial components was accomplished in the second round of ultracentrifugation. To 3.5 ml TE, 500 µl of DNA containing solution, 4.40 g cesium chloride (8.8 g / ml final) and bis benzimide (Hoesch 33258) to a final concentration of 200 µg / ml, was added. The refractive index was

adjusted to 1.3960 using cesium chloride or TE and the gradient was ultracentrifuged in a Vti65 rotor at 40,000 rpm at 20°C for 15 hours. Mitochondrial and nuclear DNAs were then removed from the gradient by side puncture with a hypodermic syringe and the DNA was extracted with cesium chloride-saturated isopropanol and dialyzed as before.

Verification of the nuclear and mitochondrial DNA fractions was performed by restriction digestion using the restriction endonuclease *Bam* HI, a restriction enzyme with a G-C rich six-base recognition sequence. Restriction digestion of the mitochondrial fraction gave a series of individual bands, while digestion of the nuclear DNA gave a smear of DNA fragments as visualized by ultraviolet light on an ethidium bromide stained 0.7% agarose gel.

2.3 Variation in the total mitochondrial DNA

Assessment of variation in the total mitochondrial DNA of eight isolates of *C. purpureum* and one isolate of *Stereum hirsutum* (Willd.:Fr.) S.F. Gray (collected in England) was determined by digesting the total mitochondrial DNA with the restriction endonuclease *Bam* HI, followed by electrophoresis through a 0.7% agarose gel and comparison of the resulting RFLP patterns. Bands were scored as present (1) or absent (0) and compared using Unweighted Pair-Group Method using Arithmetic means (UPGMA) analysis function of the Numerical Taxonomy and Multivariate Analysis System (NT-SYS) (Rohlf et al. 1982).

2.4 Cloning of mitochondrial DNA

Mitochondrial DNA of isolate 2088 was digested with the restriction

endonuclease *Bam* HI and ligated into the pUC-18 "Ready-to-go" vector (Pharmacia, Uppsala, Sweden) and transformed into *E. coli* by electroporation. This resulted in the cloning of fragments up to 9 kb in size. To avoid plasmids which contained two mitochondrial fragments ligated together, clones were further screened for their insert size and only the clones containing fragments that corresponded in size to the fragments observed in the total mitochondrial DNA were stored in 30% glycerol at -70°C.

2.5 Sequencing of fragments, PCR primer design and amplification

Isolation of mitochondrial DNA using cesium chloride gradients was time consuming and laborious. To screen a larger sample population, a rapid PCR based assay was developed. Two pUC-18 clones with inserts of 3 kb and another of 5.1 kb, were sequenced at the vector insertion points (Appendix 1). The first sequence was read by manual sequencing using the T7 sequencing kit (Pharmacia) and the second using an ABI 377 (Applied Biotechnology Incorporated) automated sequencer. The sequences were compared to sequences deposited in the Non-Redundant Amino Aacid (nr-aa) database using the Basic Local Alignment Search Tool (BLAST) to identify the genes the sequences encoded by degree of homology (Appendix 2).

Sequence data was used to design mitochondria-specific PCR primers to amplify the fragments from total DNA (total DNA extraction procedure provided in Ramsfield et al. 1996). Amplification of the first SCAR marker (3 kb), encoding the NADH 4 gene and unknown DNA, was accomplished using the primers CP12F and CP12R (Table 1). The second SCAR marker (5.1 kb), encoding the ATPase VI and cytochrome b genes, was amplified using the primers CP6000F and CP6000R (Table 1).

Table 1. Mitochondrial SCAR PCR primers developed in this study.

Primer	Sequence	Gene	Size
CP12F	5'-CAGTAAAGCGACTATCAAAGGGACGAGC-3'	unknown	3 kb
CP12R	5'-CTGATTACCTAAAGCTCATGCTGATTCAG-3'	NADH 4	3 kb
CP6000F	5'-TTACTTGGTTTTTAATTTCCC-3'	ATPase VI	5.1 kb
CP6000R	5'-GGGCAAATCTCCGCTAAATAT-3'	Cyt b	5.1 kb

Each PCR reaction for amplification of the 3 kb SCAR contained 160 ng of each primer, 200 mM each of dATP, dCTP, dGTP, dTTP, buffer (10 mM Tris (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin; and 0.1 % Triton X-100), 2.5 units *Taq* DNA polymerase (Pharmacia, Uppsala, Sweden) and 0.1 - 10 ng total DNA in a final volume of 50 µl. The mitochondrial DNA was amplified from a total DNA extraction using a step up amplification protocol, with 1 minute denaturing at 94°C, primer annealing for 1 minute at 50°C (step 1), 51°C (step 2), 52°C (step 3), 53°C (step 4), 54°C (step 4), 55°C (steps 5-30), and extension for 3 minutes at 72°C. Amplification of the 5.1 kb SCAR followed a similar protocol, with the same primer, dNTP and target DNA concentrations but 1/10 volume *TaqPlus Precision*™ buffer and 2.5 units of *TaqPlus Precision*™ (Stratagene, La Jolla, CA), a DNA polymerase specially formulated to amplify large fragments of DNA. The thermal cycling regime was then modified, with the number of cycles at 55°C primer annealing increased from 25 to 35 and 7 minute extension times at 72°C. All amplification reactions were conducted in a Perkin Elmer thermal cycler.

2.6 Screening SCARs for polymorphisms

Restriction digestion of the SCARs was used to find polymorphisms in amplified regions. DNA was amplified from isolates of *C. purpureum* with different geographic origins and ribosomal DNA types (Ramsfield et al. 1996) and then sub samples of five isolates, each with a different ribosomal DNA pattern (i.e. rDNA types I+I, II+II, III+III, I+II and I+III) and geographic origin were screened with restriction endonucleases. After amplification by PCR, 10 µl of the completed reaction was electrophoresed on a 0.7 % agarose gel to verify amplification. The remaining 40 µl was brought to 100 µl volume with sterile water, extracted once with 100 µl chloroform : isoamyl alcohol (24:1), centrifuged and the aqueous layer removed. To the aqueous layer, 10 µl 3M sodium acetate and 200 µl 95% ethanol were added. After incubation at -20°C for one hour, the precipitated DNA was collected by centrifugation at 14,000 rpm for 15 minutes in a 4°C microcentrifuge. The DNA was washed once with ice cold 70% ethanol, recentrifuged, air dried and resuspended in 34 µl sterile water. Restriction digestion was performed in a 10 µl volume, with 8.5 µl purified DNA, 1.0 µl of 10 X reaction buffer (or 7.5 µl DNA and 2.0 µl 10 X reaction buffer if the restriction enzyme used had an optimum 2 X final concentration of buffer), 0.5 µl restriction enzyme and allowed to incubate at 37°C for one hour. Restriction products were visualized by ultraviolet illumination of ethidium bromide stained 0.7% agarose gels. A total of 24 restriction endonucleases were screened to detect polymorphisms in the amplified fragments (Table 2).

Table 2. Restriction endonucleases and corresponding recognition sequences used to digest both the 3 kb and 5 kb SCARs.

Restriction enzyme	Recognition sequence
Alu I	5'-AG↓CT-3'
Ase I	5'-AT↓TAAT-3'
Ava II	5'-ATGCA↓T-3'
Bam HI	5'-G↓GATCC-3'
Bgl II	5'-A↓GATCT-3'
Bst EII	5'-G↓GTNACC-3'
Dde I	5'-C↓TNAG-3'
Dra I	5'-TTT↓AAA-3'
Eco RI	5'-G↓AATTC-3'
Eco RV	5'-GAT↓ATC-3'
Hae III	5'-GG↓CC-3'
*Hin*d III	5'-A↓AGCTT-3'
Nco I	5'-C↓GATGG-3'
Nde I	5'-CA↓TATG-3'
Nsi I	5'-ATGCA↓T-3'
Pst I	5'-CTGCA↓G-3'
Rsa I	5'-GT↓AC-3'
Sau 3AI	5'-↓GATC-3'
Sac I	5'-GAGCT↓C-3'
Sac II	5'-CCGC↓GG-3'
Spe I	5'-A↓CTAGT-3'
Ssp I	5'ATT↓ATT-3'
Taq I	5'-T↓CGA-3'
Xba I	5'-T↓CTAGA-3'

2.7 Sample population screening

The 5.1 kb SCAR was amplified from total DNA of the sample population and digested with the restriction endonuclease *Nsi* I following the protocols above. This enzyme was able to distinguish two mitochondrial haplotypes. Type I had one intact *Nsi* I recognition sequence, resulting in the 5.1 kb fragment being cut into 1375 bp and 3707 bp pieces. The recognition sequence was altered in the type II haplotype, therefore preventing cleavage into two pieces. To test if the failure of *Nsi* I to digest the DNA of

haplotype II was a result of an altered recognition sequence, and not inhibition by one of the reaction components, isolates of haplotype II were digested with other restriction enzymes to insure that the DNA was digestible. Geographic distribution of the haplotypes was compared using Nei's (1973) gene differentiation statistic, G_{ST}, to compare variation within the sub-populations to variation between the sub-populations screened.

CHAPTER 3 - RESULTS

3.1 Isolation of mitochondrial DNA, genome size and variation

Mitochondrial DNA was purified from the nuclear DNA of eight isolates of *Chondrostereum purpureum* and one isolate of *Stereum hirsutum* by ultracentrifugation (Fig. 1). Restriction digestion of the mitochondrial and nuclear components of the ultracentrifuged cesium chloride gradient verified that mitochondrial DNA was in the top band in the gradient because restriction digestion produced a limited number of fragments easily separated on an agarose gel, while a smear of DNA fragments from the bottom band represented nuclear DNA (Fig. 2). Purified mitochondrial DNA was then digested with the restriction endonuclease *Bam* HI, which cleaved the mitochondrial DNA into large fragments. Electrophoresis of the digested mtDNA allowed the mitochondrial genome size of *C. purpureum* to be estimated at 67 kb, while the mitochondrial genome of *S. hirsutum* was 125 kb (Fig. 3). This size determination was made by comparing the relative migration of the mitochondrial bands to the migration of the known band sizes of λ bacteriophage marker DNA digested with the restriction endonuclease *Hind* III.

The restriction patterns generated by *Bam* HI were compared using the UPGMA feature of the numerical taxonomy and multivariate analysis system (NT-SYS) and a dendrogram was constructed (Fig. 4). It was found that isolates from British Columbia, Alberta, Finland, the Netherlands, and New Zealand shared identical *Bam* HI banding patterns. Isolate 2088, from British Columbia, had one additional band of 2200 bp (Fig. 3, Lane 3) and had a similarity coefficient of 0.917. The location of one of the *Bam* HI sites was shifted in Swiss isolate 95026 so that the doublet formed at approximately 20 kb was absent and an additional band was present at approximately 10 kb, resulting in a

similarity coefficient of 0.655 (Fig. 3, Lane 4). The mitochondrial DNA of *S. hirsutum* was compared to the mitochondrial DNA of *C. purpureum* and it was found that some of the bands were shared, resulting in a similarity coefficient of 0.365 (Fig. 3, Lane 6). This assessment of the variation in the total mitochondrial genome resulted in the identification of three mitochondrial haplotypes among eight isolates screened. Haplotype distribution did not correlate to geographic origin but differences in the mitochondrial genome existed in geographically separated areas. The most common haplotype was found in all regions surveyed, indicating that it may represent an ancestral mitochondrial genome.

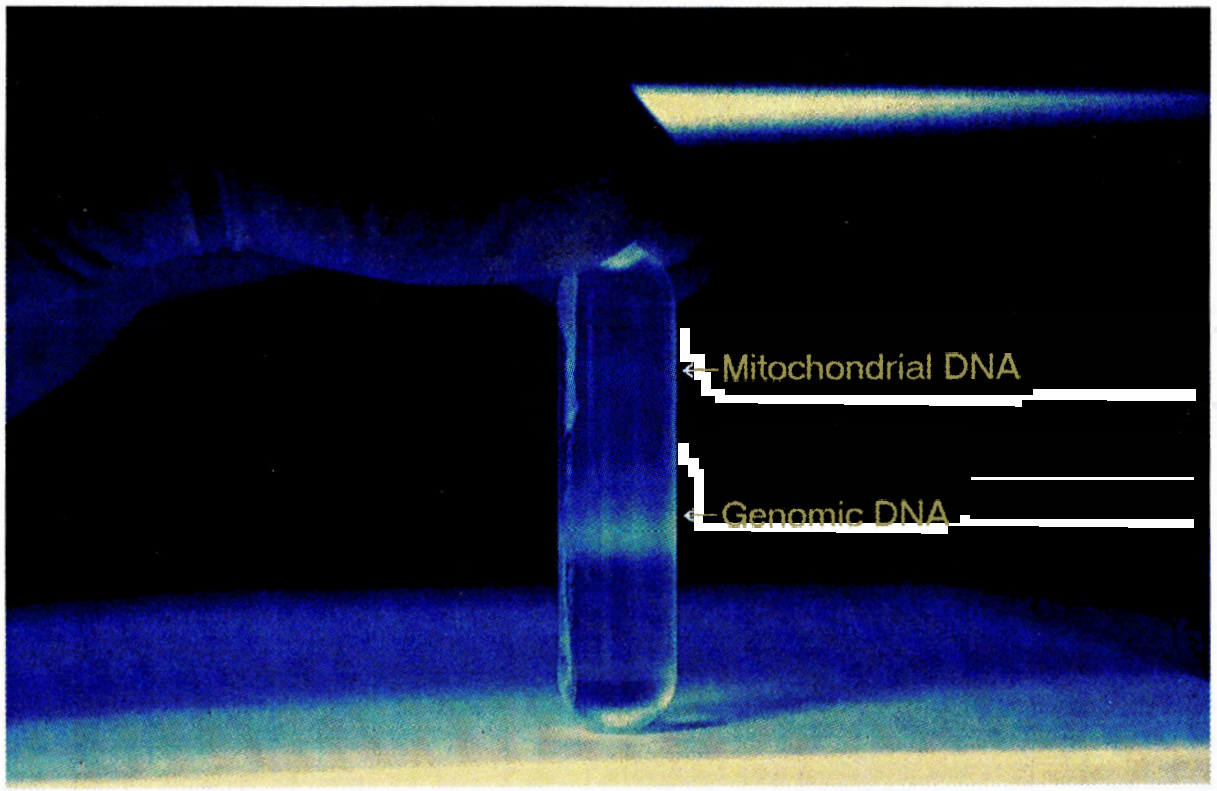


Figure 1. Cesium chloride gradient showing the separation of nuclear and mitochondrial DNA.

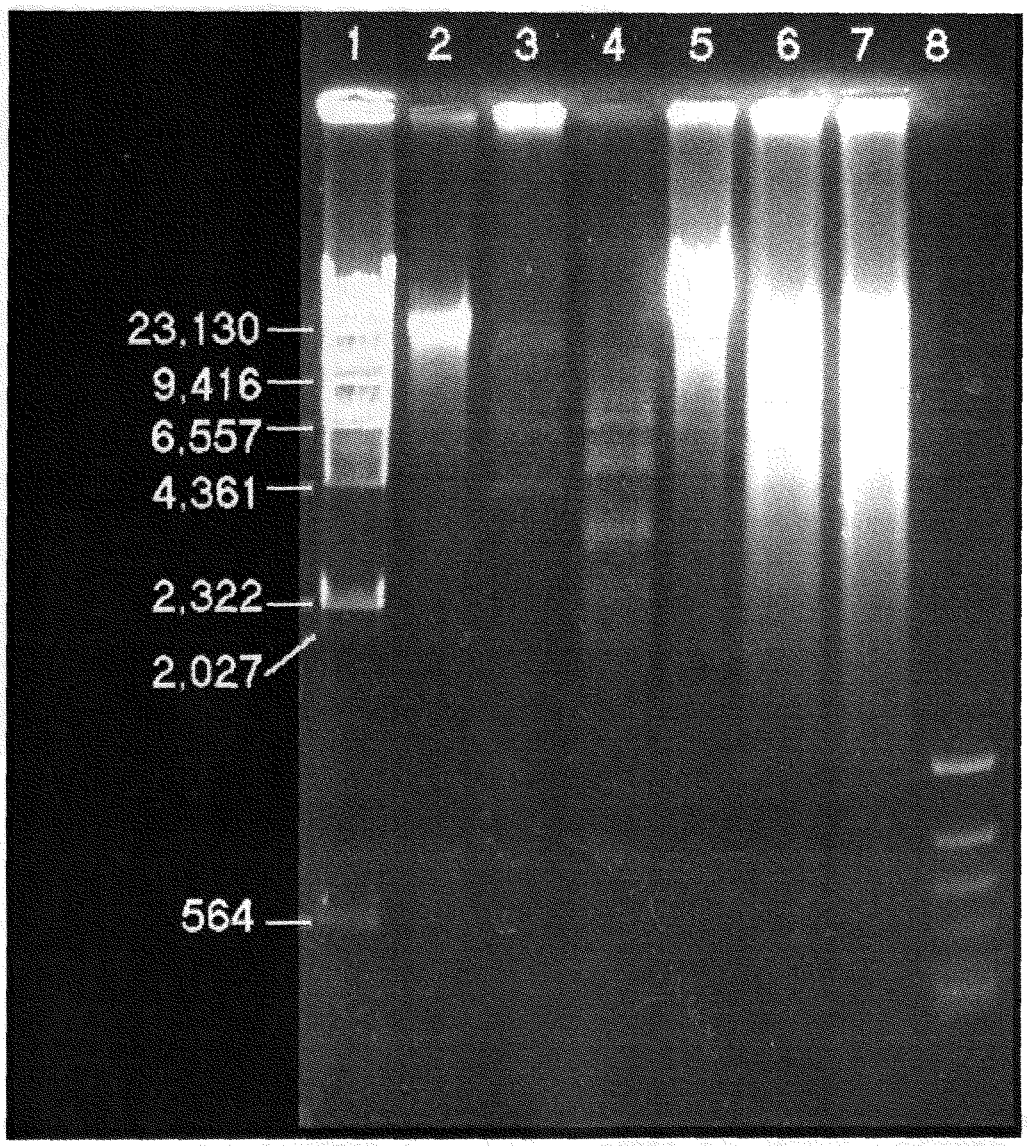
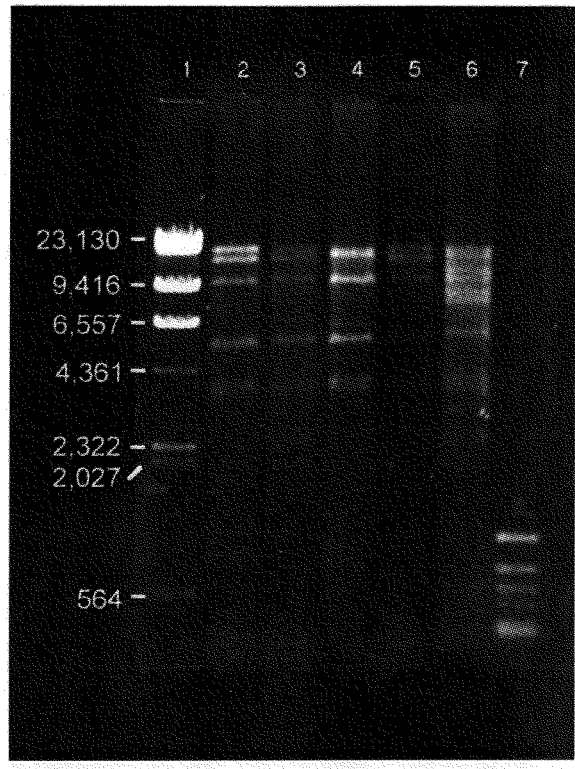


Figure 2. Restriction digest of DNA from top and bottom bands of cesium chloride gradient. Lane 1, λ *Hin*d III size marker. Lane 2, isolate 2064 P-25 mtDNA uncut. Lane 3, 2064 P-25 mtDNA *Eco* RI digest. Lane 4, 2064 P-25 mtDNA *Hin*d III digest. Lane 5, 2064 P-25 nDNA uncut. Lane 6, 2064 P-25 nDNA *Eco* RI digest. Lane 7, 2064 P-25 nDNA *Hin*d III digest. Lane 8, ϕX 174 / *Hin*c II size marker.

A.



B.

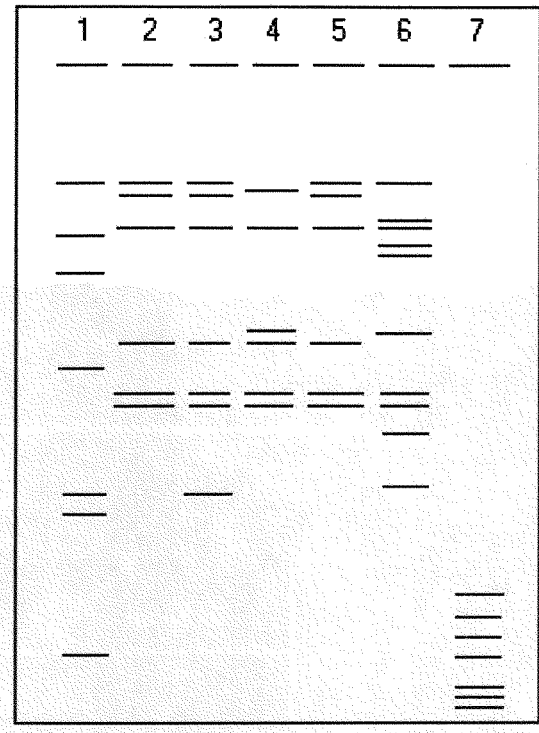


Figure 3. *Bam* HI digest of mitochondrial DNA. Lane 1, λ *Hind* III size marker. Lane 2, isolate 2202. Lane 3, isolate 2088. Lane 4, isolate 95026. Lane 5, isolate 94-179/3. Lane 6, *Stereum hirsutum*. Lane 7, ϕX 174 / *Hinc* II size marker. A. Actual gel, B. Schematic diagram.

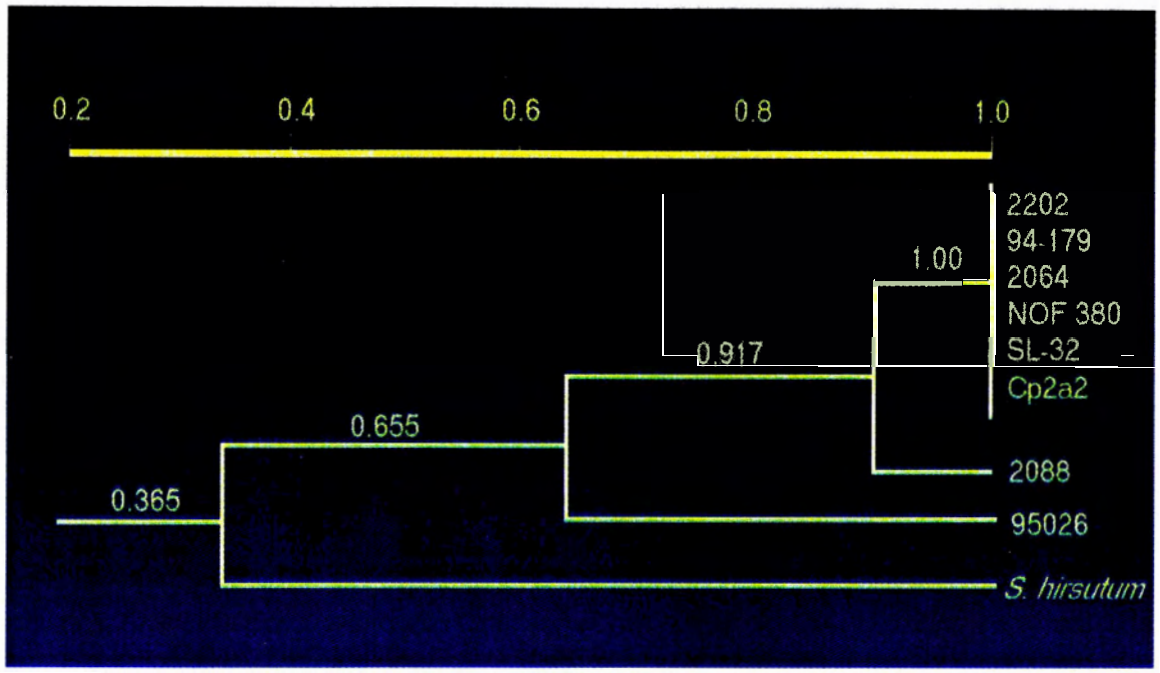


Figure 4. Dendrogram based on *Bam* HI digest of total mitochondrial DNA generated by UPGMA analysis. Isolates used were: 2202, British Columbia; 94-179, Finland; 2064, British Columbia; NOF-380, Alberta; SL-32, New Zealand; Cp2a2, Netherlands; 2088, British Columbia; and 95026, Switzerland. The outgroup was *Stereum hirsutum*, collected in England.

3.2 Screening mitochondrial SCARs for polymorphisms

Cloned mitochondrial DNA was sequenced and PCR primers were developed to amplify two fragments. The first SCAR contained the NADH 4 gene and the second contained the ATPase VI and cytochrome b genes (Appendix 2). Amplification and restriction digestion of these fragments to generate restriction fragment length polymorphisms (RFLPs) formed the basis of this assay. Mitochondrial DNA was amplified from total DNA extracted from each isolate screened using mitochondrial specific PCR primers.

Screening the 3.0 kb SCAR with 24 restriction enzymes (Table 2) failed to uncover any RFLPs. When digested with the restriction enzyme of interest, the result was always the same in the sub-sample tested: either the banding pattern generated was the same, or the restriction endonuclease was unable to cleave the fragment because the recognition sequence was not present. The inability of any of the restriction enzymes tested to produce polymorphisms in this fragment suggests that it is highly conserved. This was unexpected since the sequence of DNA at the 3' end of the fragment did not match any sequences in the database, suggesting it was non-coding spacer DNA (Appendix 2).

Screening the 5.1 SCAR with the same 24 restriction enzymes revealed that *Nsi* I was able to differentiate two mitochondrial haplotypes. The first, termed type I, had an intact *Nsi* I site, resulting in the 5.1 kb SCAR being cut into 1375 bp and 3707 bp fragments. The second haplotype, termed type II, had no *Nsi* I site, therefore the 5.1 kb SCAR remained uncut (Fig. 5). Sequencing the region where the *Nsi* I site was located and comparison with the sequence database indicated that the site was in the non-coding

spacer region between the two genes coded in this SCAR (Appendix 2). Since these genes were separated by a non-coding spacer region, it was surprising that no other restriction enzymes were able to differentiate these two haplotypes, providing more evidence for a conserved mitochondrial genome.

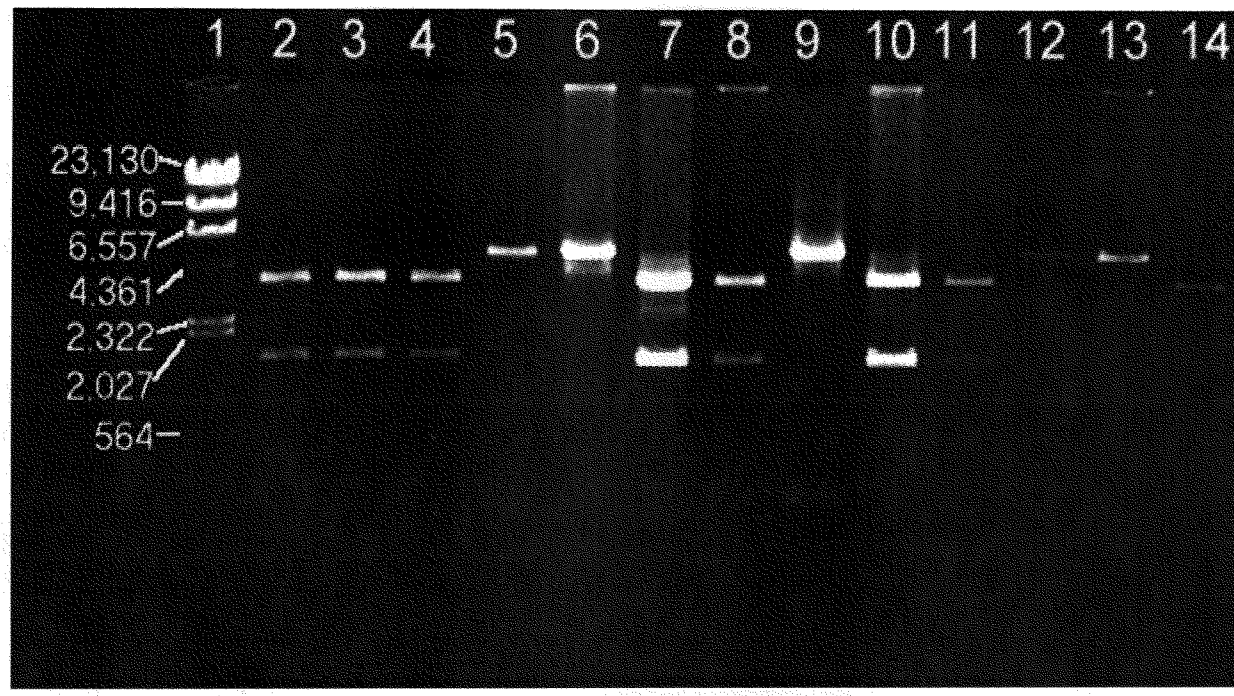


Figure 5. *Nsi* I digest of ATPase VI, cytochrome b 5.1 kb SCAR. Lane 1, λ *Hin*d III size marker. Mitochondrial haplotype I, lanes 2 (Nor-4), 3 (Nor-5), 4 (F889), 7 (95004), 8 (94-271/4), 10 (94-312/1), 11 (SL-8), 14 (SL-39); haplotype II, lanes 5 (Bath-1), 6 (95028), 9 (94-311/2), 12 (SL-26), 13 (SL-33). Isolates in parenthesis, refer to Table 3 for origin and host.

3.3 Screening of sample population and assignment of haplotypes

The 5.1 kb SCAR was amplified from a sample population of 84 isolates of *C. purpureum* collected in North America (n=44), Europe (n=22) and New Zealand (n=18) and was screened with the restriction endonuclease *Nsi* I. Mitochondrial haplotypes were then assigned as type I or II depending on the restriction pattern observed (Table 3). PCR amplification resulted in a 5.1 kb product in every case; there were no size polymorphisms present which would indicate gene duplication or large insertion / deletion events.

Table 3. Sample population of *Chondrostereum purpureum* isolate origin, host, year collected, ribosomal and mitochondrial DNA types.

Region	Isolate	City	Collection date	Host	rDNA[a]	mtDNA
New Zealand						
	SL-1	Aokautere	?	*Salix* sp. Clone 1184	I+III	I
	SL-2	Aokautere	?	*Salix* sp. Clone 1184	I+I	II
	SL-3	Aokautere	?	*Salix* sp. Clone 1184	I+III	I
	SL-4	Aokautere	?	*Salix* sp. Clone 1184	I+III	II
	SL-8	Aokautere	?	*Salix* sp. Clone 1040	I+I	I
	SL-10	Aokautere	?	*Salix* sp. Clone 1040	I+III	II
	SL-13	Aokautere	?	*Salix* sp. Clone 1040	III+III	I
	SL-14	Aokautere	?	*Salix* sp. Clone 1040	III+III	II
	SL-15	Aokautere	?	*Populus* sp.	I+III	I
	SL-17	Aokautere	?	*Alnus* sp.	III+III	I
	SL-33	Aokautere	?	Unknown	III+III	II
	SL-26	Palmerston North	?	*Prunus* sp.	I+III	II
	SL-29	Taranaki	?	*Malus* sp.	III+III	I
	SL-39	Taranaki	?	*Pyrus* sp.	I+III	I
	SL-30	Christchurch	?	*Malus* sp.	III+III	II
	SL-31	Christchurch	?	*Malus* sp.	III+III	I
	SL-35	Pohangia	?	Tree Lucerne	I+I	I
	atcc64240	Aokautere	1985	*Populus maximowicizii*	I+I	II
Norway						
	Nor-1	Gjovic	1996	*Betula* sp.	I+I	II

Region	Isolate	City	Collection date	Host	rDNA[a]	mtDNA
	Nor-2	Vennesla	1982	*Picea abies*	I+III	I
	Nor-3	Vennesla	1982	*Picea abies*	I+I	I
	Nor-4	Vennesla	1982	*Picea abies*	I+III	I
	Nor-5	Skoftestad	1960	*Populus tremula*	I+III	I
	Nor-6	Asker	1943	*Rubus idaeus*	III+III	I
Finland						
	94-179/3	Suonenjoki	1994	*Betula* sp.	I*	I
	94-271/4	Kikkonummi	1994	*Betula* sp.	I*	I
	94-286/3	Kikkonummi	1994	*Betula* sp.	III*	I
	94-311/2	Kikkonummi	1994	*Picea abies*	I*	II
	94-312/1	Kikkonummi	1994	*Picea abies*	I*	I
England						
	Bath-1	Bath	1995	Unknown	I+III	II
Netherlands						
	Cp2a1	Unknown	?	*Prunus serotina*	I+III	I
Germany						
	F889	Stuttgart	1989	*Fagus sylvatica*	I+I	I
Switzerland						
	Swiss 3-6#1	Zollikon	1994	*Prunus avium*	III+III	I
	Swiss 3-6#2	Zollikon	1994	*Prunus avium*	III+III	I
	95004	Zurich	1994	*Carpinus betulus*	III*	I
	95008	Zurich	1994	*Carpinus betulus*	I*	I
	95021	Uetliberg	1994	Unknown	III*	II
	95022	Uetliberg	1994	Unknown	III*	II
	95026	Bonstetten	1994	*Betula pubescens*	III*	II
	95028	Bonstetten	1994	*Betula pubescens*	III*	II
Eastern North America						
	CFMR-3	New York	1944	*Prunus japonica*	I+I	I
	CFMR-4	New Hampshire	1961	*Betula papyrifera*	I+II	I
	CFMR-8	Fairfax, VA	1932	*Fagus* sp.	I+I	I
	Cp92-16.1	Newfoundland	1992	*Betula papyrifera*	I+I	II
	Cp92-41.1	Newfoundland	1992	*Betula papyrifera*	I+I	I
	855	Fredericton, NB	1986	*Betula alleghaniensis*	I+II	I
	2102	Fredericton, NB	1989	*Betula alleghaniensis*	I+II	I
	2097	Fredericton, NB	1989	*Betula alleghaniensis*	I+I	I
	2110	Fredericton, NB	1989	*Betula alleghaniensis*	I+I	I
Central North America						

Region	Isolate	City	Collection date	Host	rDNA[a]	mtDNA
	DR-365	Michigan	1994	*Populus tremuloides*	I+II	I
	CFMR-1	Dog River, ON	1973	*Abies balsamera*	II+II	II
	CFMR-10	Ontario	1966	*Populus sp.*	I+I	II
	2121	Dryden, ON	1990	*Populus tremuloides*	II+II	I
	JAM-3	Thunder Bay, ON	1989	*Betula papyrifera*	I+II	I
	JAM-4	Thunder Bay, ON	1989	*Acer saccharinum*	I+I	I
	JAM-6	Blake Twp, ON	1989	*Betula papyrifera*	I+I	I
	MB7	Quebec	1992	Unknown	I+I	I
	EA2	Quebec	1992	Unknown	I+II	I
	NOF-380	Slave Lake, AB	1957	*Betula papyrifera*	I+II	II
	NOF-663	Edmonton, AB	1976	*Sorbus* sp.	II+II	I
	NOF-671	Edmonton, AB	1976	*Sorbus americana*	II+II	I
Western North America						
	CFMR-9	Alaska	1954	*Betula papyrifera*	II+II	II
	CFMR-11	Alaska	1990	*Populus* sp.	II+II	II
	CFMR-12	Alaska	1990	*Populus* sp.	II+II	II
	OR-1	Corvallis, OR	1995	*Alnus rubra*	II+II	I
	OR-3	Corvallis, OR	1995	*Alnus rubra*	II+II	I
	OR-4	Corvallis, OR	1995	*Alnus rubra*	II+II	I
	NOF-163	Cinema, BC	1949	Unknown	II+II	II
	2047	Port Renfrew, BC	1988	*Alnus rubra*	I+II	II
	2075	Genelle, BC	1988	*Populus tremuloides*	II+II	II
	2095	Genelle, BC	1989	*Alnus sinuata*	II+II	I
	2088	Saanichton, BC	1989	*Prunus persica*	II*	I
	2090	Saanichton, BC	1989	*Malus* sp.	II+II	II
	2091	Saanichton, BC	1989	*Malus* sp.	II+II	I
	2064	Mesachie Lk., BC	1988	*Acer macrophyllum*	I+II	I
	2065	Mesachie Lk., BC	1988	*Acer macrophyllum*	I+II	I
	2138	Mesachie Lk., BC	1993	*Alnus rubra*	II+II	II
	2140	Duncan, BC	1993	*Acer macrophyllum*	I+II	II
	2139	Cowichan, BC	1994	*Alnus rubra*	II+II	II
	2145	Cowichan, BC	1994	*Alnus rubra*	II+II	I
	2151	Cowichan, BC	1994	*Alnus rubra*	II+II	I
	2L8	Cowichan, BC	1995	*Alnus rubra*	I+II	I
	2101	Sooke, BC	1990	*Alnus rubra*	II+II	II
	2131	Sooke, BC	1992	*Alnus rubra*	I*	II

[a] rDNA type as presented in Ramsfield et al. (1996).
* Homokaryotic
mtDNA: Mitochondrial Type I - Cut, Type II - Uncut

When the data were analyzed on a geographic basis, both mitochondrial haplotypes were present in every region surveyed (Table 4). There was a near equal distribution of mitochondrial haplotypes in New Zealand (Table 4), while Europe and North America had twice the number of mitochondrial type I over type II (Table 4). Analysis of the North American population showed that mitochondrial type I was the dominant type in the East and as the sample moves west, the haplotype distributions became near equal (Table 4).

Table 4. Frequency distribution of mitochondrial haplotypes I and II in the sample population studied.

Sub-population	n	Type I	Type II
New Zealand	18	0.56	0.44
Europe	22	0.68	0.32
East North America	9	0.89	0.11
Central North America	12	0.75	0.25
West North America	23	0.48	0.52
Total	84	0.63	0.37

Calculation of Nei's (1973) gene differentiation statistic (G_{ST}) gave a value of 0.0935. Small values of G_{ST} indicate that the diversity between sub-populations is not significant compared to diversity within sub-populations (Brown, 1996). The distribution of mitochondrial haplotypes within each sub-population of the sample population therefore fits the same ratio as the total population.

3.4 Mitochondrial haplotype and ribosomal DNA haplotype comparison

As can be seen in Table 3, each member of the sample population was previously assigned a ribosomal (nuclear) DNA type by Ramsfield et al. (1996). In North America, nuclear type I was the major nuclear type on the east coast and nuclear type II was the dominant nuclear type on the west coast. The mitochondrial DNA types determined in this study mirror the distribution of the ribosomal DNA types, in that mitochondrial type I was the major mitochondrial type on the east coast and mitochondrial type II increased from 11% of the mitochondrial types on the east coast to 52% of the mitochondrial types on the west coast. This suggests that gene flow was occurring across the continent.

When the nuclear and mitochondrial types of each isolate were compared to measure the association between them using the Chi square test (χ^2=2.567), no significant relationship between the nuclear and mitochondrial types was found (p=0.633). This indicated that although the trend was the same for both marker systems, they were distributed independently of each other. This showed that nuclear type II is not linked to mitochondrial type II, but that they are inherited independently. This can be clearly seen in the data. In Western North America, isolates 2090 and 2091 were both collected in Saanichton, BC, on *Malus* sp., but 2090 has nDNA type II+II and mtDNA type II, while 2091 has nDNA type II+II and mtDNA type I. In Eastern North America, both Cp92-16.1 and Cp92-41.1 were collected in Newfoundland on *Betula papyrifera* but Cp92-16.1 has nDNA type I+I and mtDNA type II, while Cp92-41.1 has nDNA type I+I and mtDNA type I. This trend is also present in New Zealand, SL-30 and SL-31 were both collected in Christchurch on *Malus* sp., but SL-30 has nDNA type III+III and mtDNA type II, while SL-31 has nDNA type III+III and mtDNA type I. Clearly the nuclear and mitochondrial

markers are inherited independently of each other, which explains why although their distributions were similar, they were not correlated with each other on an isolate by isolate basis.

CHAPTER 4 - DISCUSSION

An assessment of the genetic variation in *Chondrostereum purpureum* is an important precursor to its widespread use as an inundative biological control agent to insure that rare virulence alleles are not introduced into local populations as a result of biological control applications. Also, it is required by the regulatory authorities to characterize and identify biocontrol agents prior to the registration and commercialization of biological control products (Anonymous, 1993). Variation at virulence loci cannot be quantified in *C. purpureum* as they are unknown; therefore, genetic variation had to be estimated using other marker systems. Studies on the genetic variation in *C. purpureum* based on protein profiles (Ekramoddoullah et al., 1993), API ZYM, isozyme patterns (Shamoun and Wall, 1996), ITS polymorphisms (Shamoun et al., 1991), and RAPD markers (Gosselin et al., 1995) have all indicated that no host or geographic specializations were present. The report of ribosomal DNA markers by Ramsfield et al. (1996) that were able to distinguish European and New Zealand isolates from North American isolates was the first to show geographic distinction between continents, but gene flow across North America was also observed. Characterization of variation in the mitochondrial genome has been used to assess genetic variation in other fungi, such as *Ophiostoma ulmi* (Bates et al., 1993; Charter et al., 1996; Hintz et al., 1993; Jeng et al., 1991) and species of *Armillaria* (Anderson et al., 1987; Guillaumin et al., 1996; Smith and Anderson, 1989; Smith et al., 1990) and is the marker which has been used in this study to assess the population structure of this fungus.

4.1 Total mitochondrial DNA

The mitochondrial genome of *C. purpureum* was found to be approximately 67 kb in size, over half the size of the 125 kb mitochondrial genome of *Stereum hirsutum* and well within the range of fungal genome sizes reported (Grossman and Hudspeth, 1985; Hudspeth, 1992). Until Pouzar (1959) reclassified *Chondrostereum*, *C. purpureum* and *S. hirsutum* were placed in the same genus and thought to be closely related. The huge difference in the mitochondrial genome size of these two closely related species is likely a result of introns and insertion / deletion events. This finding is not unusual, however, since both interspecific and intraspecific variation in mitochondrial genome sizes have been observed. The mitochondrial genome of *Agaricus bitorquis* was found to be nearly twice as large as *A. brunnescens* Peck (Hintz et al., 1988b), and the mitochondrial genomes of different races of *Ophiostoma ulmi* have been shown to range in size from approximately 55 kb to 70 kb (Bates et al., 1993; Charter et al., 1996; Hintz et al., 1993; Jeng et al 1991). The within-genus variation seen in fungal mitochondrial genomes indicates that they are quite plastic with respect to insertion / deletion events.

Restriction digestion of the total mitochondrial genome of eight isolates of *C. purpureum* revealed the presence of three mitochondrial haplotypes. The first haplotype was the most common, being shared by 6 isolates from British Columbia, Alberta, the Netherlands and New Zealand. The other two haplotypes were represented by the gain of a 2 kb band in a British Columbia isolate, and the movement of one of the *Bam* HI sites in a Swiss isolate. Movement of one of the *Bam* HI sites in the Swiss isolate is indicated by the presence of a single band of approximately 18 kb, rather than a doublet as in the other isolates and the presence of a doublet corresponding to 7.5 kb and 6.0 kb rather than a

single band as in the other isolates. The 2 kb band present in the British Columbia isolate is most likely not indicative of a 2 kb insertion into the mitochondrial genome, but rather the appearance of a *Bam* HI restriction site in one of the 20 kb fragments. It is very difficult to differentiate a 2 kb difference between bands of approximately 20 kb size on an agarose gel, thus preventing determination of the exact location of the new restriction site. The wide distribution of the most common haplotype suggests there is a high degree of conservation in the mitochondrial genome of *C. purpureum.* The worldwide distribution of the most common type indicates that there are no geographically isolated sub populations, as determined with this marker.

The low degree of variation indicated by restriction digestion of the total mitochondrial DNA of *C. purpureum* is characteristic of fungi that exist as large clones, such as *Armillaria*; in contrast to a highly outcrossing species like *C. purpureum*, which has been reported to have a large number of mating type alleles (Gosselin et al., 1995; Wall et al., 1996). However, *Armillaria* is not a good representative to compare with *C. purpureum*, since it is a root rot pathogen which is spread by mycelial growth and root contact, compared to meiotically derived basidiospores (Smith et al., 1990). The variation in the mitochondrial DNA of other basidiomycetes, such as *Pleurotus ostreatus* and *Lentinula edodes,* was also found to be very high. Analysis of *P. ostreatus* mitochondrial DNA variation resulted in the identification of 22 different mitochondrial haplotypes in 34 isolates (Matsumoto and Fukumasa-Nakai, 1995); 28 different mitochondrial haplotypes were observed in 51 isolates of *L. edodes* (Fukuda et al., 1994). In both cases, cluster analysis demonstrated that populations were geographically isolated. A high degree of mitochondrial DNA variation has also been observed in some

Ascomycetes. Diversity in *Cryphonectria parasitica* mitochondrial DNA was indicated by the presence of 24, 30 and 20 mitochondrial haplotypes in three different regions of New York and Vermont (Milgroom and Lipari, 1993). Hintz et al. (1993) found four different mitochondrial haplotypes in *Ophiostoma ulmi* collected in the city of Winnipeg, Canada.

The low amount of variation observed in the mitochondrial genome of *C. purpureum* is comparable to the oomycete *Phytophthora infestans*, in which 4 globally distributed mitochondrial haplotypes were observed (Carter et al., 1990). Low variation of mitochondrial haplotypes has also been correlated with *formae speciales* in *Fusarium oxysporum* (Kistler et al., 1987) and *Phytophthora megasperma* (Förster et al., 1989). Mitochondrial DNA haplotypes were found to be well correlated with vegetative compatibility groups in *Fusarium oxysporum* f. sp. *melonis* and it was suggested by the authors that mitochondrial haplotypes could be used to identify genetically isolated populations (Jacobson and Gordon, 1990). Since *Chondrostereum* has no host specializations (Ekramoddoullah et al., 1993; Gosselin et al., 1995; Shamoun et al., 1991; Shamoun and Wall, 1996), there are no *formae speciales* or vegetative compatability groups present to inhibit gene flow.

One explanation for the low amount of variation observed in the total mitochondrial genome of *C. purpureum* is that there is prevention of cytoplasmic exchange and a resultant inhibition of mitochondrial recombination during heterokaryon formation as observed in *Agaricus* by Hintz et al. (1988a). The most common mitochondrial haplotype observed in this study may be representative of an ancestral mitochondrial genome, as shown by its distribution throughout the sample population; the

other haplotypes identified in this study may be the result of mutations that have occurred in geographically separated areas. Thus, one ancestral mitochondrial type may have predominated in *Chondrostereum* and due to low recombination, even with sexual outcrossing, the mitochondrial DNA remained highly conserved. This hypothesis requires additional research, however.

4.2 Mitochondrial SCAR analysis

Screening of the mitochondrial SCARs with restriction enzymes suggested that the fragments selected were highly conserved. This was unexpected since both fragments were relatively large and were hypothesized to contain non-coding spacer DNA. SCARs with large sizes were chosen deliberately since the probability of detecting a mutation would be greater in a larger fragment of DNA than a smaller one. The disadvantage of this method is that variation can only be assessed at the restriction endonuclease recognition sites. A high degree of conservation was indicated by the failure of 24 enzymes to show polymorphisms in the 3 kb SCAR and the fact that only 1 of 24 enzymes produced polymorphisms in the 5.1 kb SCAR. As discussed earlier, mitochondrial genomes are subject to a high degree of insertion / deletion events and size polymorphisms are quite common; however, in all cases with both of these fragments, PCR amplification always produced uniform sized bands. This provides additional evidence for conservation, since both SCARs contained non-coding spacer regions and any mutation which could have occurred would not be selected against, therefore allowing the mutations to persist, which was not the case in this experiment.

Restriction digestion of the 5.1 kb SCAR with the endonuclease *Nsi* I resulted in the identification of two mitochondrial haplotypes. Both haplotypes were distributed throughout the sample population and a calculated value of 0.0935 for Nei's (1973) gene diversity statistic, G_{ST}, indicated that diversity between sub-populations was not significant compared with diversity within the sub-populations (Brown, 1996). This lack of variation between sub-populations is an indication that the risk of introducing new mitochondrial types into regions through biocontrol activities may be low, because both mitochondrial haplotypes were observed in every sub-population studied.

The observation of both mitochondrial SCAR haplotypes throughout the sample population suggests that long distance dispersal of the sexually derived basidiospores or movement of infected woody material has served to maintain both haplotypes. Allele frequencies can change as a result of random genetic drift because there is a larger number of gametes (basidiospores) produced than adults (heterokaryons) in the next generation; subsequently, only a small number of gametes result in adult formation. Over a large number of generations, allele frequencies may change until one allele becomes fixed. The effect of random genetic drift is minimized in outcrossing species by increasing the effective population size and therefore slowing fixation (Li, 1997). Because no sample population in this study showed fixation, the effective population size must be large and long distance dispersal may serve to preserve large populations. If the individual populations were reproductively isolated, fixation would have occurred for one of the alleles as a result of random genetic drift. The observation of both haplotypes in all sub-populations indicated that gene flow is occurring to prevent fixation at one haplotype.

Nuclear type distribution patterns in the ribosomal DNA study by Ramsfield et al. (1996) suggest that populations of *C. purpureum* from Europe and New Zealand do not intermix with North American populations and that a second ribosomal DNA type has arisen from a common ancestral pattern in both of these regions. Extrapolation of this finding to mitochondrial genome transmission suggests that mitochondrial haplotypes are not intermixing across these vast distances. The mitochondrial haplotypes observed may therefore each be ancestral, or by chance the insertion or deletion event that created or destroyed the *Nsi* I site has occurred in all regions surveyed. Another possible explanation is that the mutation occurred in one geographic location and then the fungus moved clonally, therefore distributing the mutant form over a large area. The independent inheritance of the nuclear and mitochondrial DNA markers observed, however, does not support the clonal hypothesis. If clonal propagation did occur, the nuclear and mitochondrial types would be expected to be correlated with each other on an isolate by isolate basis and they are not.

The data collected does not allow speculation as to the origin of the two mitochondrial haplotypes, but it does suggest that gene flow is occurring to maintain both haplotypes in each area. The distribution of mitochondrial haplotypes in North America is indicative of gene flow across the continent. The near equal distribution of haplotypes in the west decreased as the population moved eastward, but both haplotypes were present in all areas surveyed. As with the ribosomal DNA, it is impossible to determine if one haplotype is replacing the other or if one haplotype is ancestral and a mutation is occurring to cause conversion to the other haplotype. Long distance transport of nursery stock and infected woody products has been cited as a mode of dispersal which has

served to maintain gene flow and reduce genetic variation in this species (Ekramoddoullah et al., 1993). It should be noted that movement of nursery stock is in effect clonal propagation, because one genotype is moved across a large area without undergoing recombination; therefore, the resultant reduction in genetic variation may be a result of this clonal movement.

4.3 Mitochondrial inheritance

The independent mode of inheritance of the nuclear DNA and mitochondrial DNA observed, and also reported in *Agaricus* by Hintz et al. (1988a), can most likely be explained by the events that occur at hyphal anastomosis between two homokaryons. The basidiomycete lifecycle includes both homokaryotic and heterokaryotic phases, and in all cases, sexual reproduction must occur before basidiospores are produced. Recombination of nuclear genes occurs during meiosis preceeding the formation of basidiospores. Mitochondrial DNA does not undergo recombination before spore production, but it may occur in the junction region of two homokaryons after hyphal anastomosis. Pairing experiments in *Lentinula edodes* have been conducted to study the mechanism of mitochondrial inheritance. RFLP patterns of *L. edodes* mitochondrial DNA in newly established heterokaryons removed from the colony periphery on either side of the contact zone retained the mitochondrial RFLP pattern of the parent homokaryon, but at the junction zone, three mitochondrial RFLP patterns were found: each of the parent patterns and one representing a mixed pattern due to mitochondrial recombination (Matsumoto and Fukumasa-Naki, 1993). Nuclear DNA throughout the entire heterokaryon would represent both paternal types because of nuclear migration after hyphal anastomosis. The

observation of only two mitochondrial haplotypes in *C. purpureum* and highly conserved total mitochondrial genomes suggested that recombination of mitochondrial DNA was limited. No isolates of *C. purpureum* were detected which contained a combination of mitochondrial haplotypes, as would be indicated by the presence of both the cut and uncut haplotypes in the 5.1 kb amplification product. This provides evidence that in *C. purpureum*, mitochondrial DNA is inherited paternally and cytoplasmic exchange, resulting in bidirectional transmission of mitochondria in the heterokaryon, does not occur.

Independent inheritance of the ribosomal (nuclear) and mitochondrial DNA was indicated by the Chi square analysis, which found no significant relationship (χ^2=2.567, p=0.633) between the nuclear and mitochondrial DNA present in each individual isolate. It was found that isolates that were identical with respect to host, geographic origin and nuclear DNA type were different in their mitochondrial DNA types (Table 3). If the nuclear and mitochondrial DNA distributions were found to be perfectly correlated and linked, as was found in *Cochliobolus heterostrophus* (Drechs.) Drechs. (Garber and Yoder, 1984), it would indicate that two geographically distinct populations are present. Comparison of the nuclear and mitochondrial types of each isolate indicate that they are not linked, and thus clonal propagation (as would be indicated by a significant relationship between the nuclear and mitochondrial types) is not occurring and the populations are not geographically isolated because gene flow is occurring to maintain both haplotypes in each sub-population studied.

4.4 Mitochondrial genome conservation and evolutionary implications

The limited amount of variation present in the mitochondrial DNA of *C. purpureum* allows for some interesting speculation as to its evolutionary origins. The distribution of *C. purpureum* is not limited to specific regions or environments, since it is found in temperate regions throughout the world (Hawksworth et al., 1995), suggesting that it has had a long time to propagate itself. Human activity may be responsible for the introduction of *C. purpureum* to New Zealand, and evidence for this is provided by the ribosomal DNA distributions which are the same in Europe and New Zealand (Ramsfield et al., 1996). Human activity cannot be the sole mode of long distance dispersal of *C. purpureum,* however, because the fungus is found in both populated and isolated regions. Ribosomal DNA and mitochondrial DNA data in North America also demonstrate gene flow across the continent because each haplotype is near equally distributed in the center of the continent, while the coastal regions have a predominance of one of the haplotypes. The observation of gene flow across the continent suggests that *C. purpureum* has a high potential for outcrossing because the haplotypes are being independently distributed. It cannot be estimated how quickly gene flow is occurring, but based on the work of de Jong (1990), who estimated effective spore dispersal to be 500 m, it would take a relatively long period of time for the fungus to propagate itself over a large distance.

The evidence for an extended period of evolution in *C. purpureum* is not supported by the mitochondrial or ribosomal DNA (Ramsfield et al., 1996) data collected which show a low degree of genetic variation. The tetrapolar mating system of *C. purpureum* (Ginns and Lefebvre, 1993; Nakasone, 1990; Rayner and Boddy, 1986) ensures outcrossing, which results in frequent recombination and would theoretically

serve to increase the genetic variation (Gosselin et al., 1995; Wall et al., 1996). The ribosomal and mitochondrial DNA data collected suggest that *C. purpureum* may have recently gone through a population bottle neck, thereby reducing the genetic variation, followed by rapid distribution. As mentioned, it is unlikely that the dispersal of *C. purpureum* occurred quickly, therefore it is possible, based on the world wide distribution of the fungus, that *C. purpureum* has used a clonal method of propagation to disperse over large distances. Clonal propagation, however, is not supported by the independent inheritance of ribosomal and mitochondrial haplotypes observed in the dataset (Table 3). Clonal propagation is also not supported by the RAPD data collected by Gosselin et al. (1995), who were able to differentiate every isolate they screened using a combination of RAPD primer sets. These results indicate that genetic recombination must be occurring, to result in a reassortment of the genomic DNA at every mating.

The tetrapolar mating system ensures outcrossing by reducing the degree of self compatibility to 25%. Each haploid homokaryotic mycelium contains two independent mating type loci, *A* and *B*, each with one of many alleles. The *A* locus is responsible for clamp connection formation and the *B* locus is responsible for bidirectional nuclear migration and fusion of the clamp cell with the subterminal hyphal segment (Boidin, 1986). A compatible reaction occurs when homokaryons with different alleles at the *A* and *B* mating type loci anastamose, resulting in the formation of a heterokaryon. A heterokaryon with mating types *A1B1* and *A2B2* will generate spores with the mating types *A1B1*, *A1B2*, *A2B1* and *A2B2* in equal proportions due to random assortment at meiosis. The only compatible matings that can occur from the resultant homokaryons are between *A1B1* + *A2B2* and *A1B2* + *A2B1* because if the same alleles are present at either

the *A* or *B* loci, an incompatible reaction occurs. As the number of mating type alleles increase, the number of successful matings increases; spores produced by two different fruit bodies are usually fully intercompatible (Day, 1965).

The tetrapolar mating system of *C. purpureum* was studied by McLaughlin (1991), who observed four mating types produced by one isolate in the ratio of 1:5:5:1, which was not representative of the equal ratios expected due to random assortment. Fruiting was only observed in cultures containing clamp connections. It was observed that pairing of cultures with uncommon *A* and *B* alleles resulted in no reaction zone formation, as expected. Unexpectedly, matings with a common *A* allele also resulted in very little noticeable reaction at the confluence zone. Parings with a common *B* allele always resulted in barrage reactions of various intensity, as expected. Barrage reactions were only observed in 13 of 20 matings between isolates with common *A* and *B* alleles, and 2 of the reactions grew into each other without any reaction. Clamp connection formation was not uniform in all cases; some pairings resulted in asymmetric clamp connection formation, suggesting dominance of the host or donor *A* allele controls the frequency of clamp connection formation. These studies indicate that *C. purpureum* is generally heterothallic with a tetrapolar mating system, but it also indicates that exceptions may exist.

The diversity of mating type alleles present in *C. purpureum* was tested by Gosselin et al. (1995), who harvested single spore isolates from a heterokaryon, crossed them and identified the four mating types. The four mating types of five isolates were then crossed in all possible combinations and it was found that 100% of the crosses resulted in compatible mating reactions, as indicated by the presence of clamp

connections and fruit body production. No vegetative incompatability or antagonistic reactions occurred in any of the crosses, even between isolates collected from a 500 m^2 area. Gosselin et al. (1995) interpreted these findings to indicate a high degree of diversity in mating type alleles. Another plausible explanation is that *C. purpureum* does not have a true tetrapolar mating system. Gosselin et al. (1995) reported crossing four tester strains of five isolates for a total of 210 crosses. To obtain 210 crosses, every mating type was paired with another and itself. In a true tetrapolar mating system, self crosses will not result in the formation of a sexually compatible heterokaryon because mating type alleles are identical at both loci. For a compatible reaction to occur, mating type alleles must be different at both loci, resulting in a 25% self compatibility rate. Accordingly, of the 210 crosses made, 170 should have resulted in compatible mating, assuming that every isolate had a different mating type allele, and 40 should have failed. In order to select the four different tester strains originating from each heterokaryon, however, Gosselin et al. (1995) must have observed incompatibility reactions between single spore isolates with common *A* or *B* loci.

Mating experiments performed by Wall et al. (1996) with single spore isolates collected from a parent heterokaryons also indicated that *C. purpureum* has a tetrapolar mating system. Compatibility of isolates from wide ranging geographic origins was observed, indicating multiple alleles at the mating type loci. Wall et al. (1996), did find one exception; single spore isolates from one heterokaryotic parent were fully compatible with each other. This result was explained by Wall (1996) as the possibility that more than one genet was present in the family of single spore isolates, in which case compatible matings would be expected to be 100%.

In all three studies outlined, homothallic mating is a possible explanation for compatible matings between crosses with identical alleles at the *A* and *B* loci. Homothallism occurs when a single spore derived individual is able to sporulate without mating with another individual. Homothallism can take the form of true homothallism, in which all single spore isolates are fully compatible; secondary homothallism, in which two compatible nuclei are present in each spore; apomixis, in which the cells develop into spores without fertilization; or homokaryotic fruiting (Bayman and Collins, 1990). Genetic variation is reduced in homothallic species because meiosis results in recombination of identical nuclei fused during karyogamy (Bayman and Collins, 1989), therefore reducing the genetic variation in the population (Sherriff and Lucas, 1989). Homothallism offers two advantages: (a) the fungus does not require a mate and heterokaryon formation; self-fertility ensures that the fungus can regenerate itself quickly, and (b) a genotype well adapted to rapid colonization is maintained without regular recombination (Bayman and Collins, 1989).

Based on the number of published reports on the tetrapolar mating system of *C. purpureum* (Boidin, 1971; Gosselin et al., 1995; McLaughlin, 1991; Nakasone, 1990; Rayner and Boddy, 1988; Wall et al., 1996), it is unlikely that *C. purpureum* is completely homothallic. Spores of *C. purpureum* contain only one nucleus, therefore ruling out secondary homothallism (Boidin, 1971; Ginns and Lefebvre, 1993). Apomixis has been observed in *Stereum sanguinolentium* (Alb. & Schw. Ex Fr.) Fr., but *C. purpureum* has clamp connections on the heterokaryotic mycelium and only mycelium with clamp connections is capable of producing fruit bodies (McLaughlin, 1991). Clamp connections are not formed in species which demonstrate apomixis, therefore apomixis is

not a mode of homothallism in this species (Rayner and Turton, 1982). Monokaryotic fruiting is ruled out because only secondary mycelium (containing clamp connections and therefore two nuclei) can form fruit bodies. True homothallic mating, however, cannot be completly ruled out by the mating experiments previously outlined. It is apparent that some degree of self compatibility is present in all three studies, indicating that *C. purpureum* may be able to reproduce homothallicly. It is thought that homothallism results from mutations to heterothallic individuals which occur at mating type loci or the presence of an extra chromosome carrying the mating type locus, resulting in the loss of mating specificity (Bayman and Collins, 1990). As homothallism is thought to be a result of a mutation in a heterothallic species, this may indicate that some isolates of *C. purpureum* have mutated to become homothallic, while others have retained the heterothallic lifestyle. Heterothallism is one of the dividing features between *Chondrostereum* and *Stereum*, which contains two homothallic species, but perhaps some degree of true homothallism is retained by *Chondrostereum*. Experimental evidence has shown a high level of diversity in mating type alleles, suggesting that *C. purpureum* is capable of a high degree of outcrossing (Gosselin et al., 1995; Wall et al., 1996). Clearly, the mating system of *C. purpureum* needs further investigation. It appears that *C. purpureum* is basically heterothallic and tetrapolar, but the mysteries of it's mating system were outlined by McLaughlin (1991) who concluded in his study that "more questions were raised than answered about the incompatibility system of the sexuality of *C. purpureum*".

The tetrapolar mating system of *C. purpureum* may allow conservation of mitochondrial lines, and therefore clonal propagation of mitochondrial DNA, because

mitochondrial DNA is extranuclear and may not be subject to frequent recombination. No isolates in the sample population surveyed were found with more than one mitochondrial type, suggesting that mitochondrial recombination, if it occurs, is rare. If mitochondrial recombination is a rare event, the mitochondrial genome of *C. purpureum* is effectively a clonally reproducing entity, which explains why the amount of variation in the mitochondrial DNA is so low. The cytoplasmic location of the mitochondria, combined with an absence of cytoplasmic exchange, may allow conservation of the mitochondrial DNA while the nuclear genes are shuffled, resulting in genomic recombination. This could explain why Gosselin et al. (1995) found different RAPD patterns in every isolate while the data presented here indicates that the mitochondrial DNAs are highly conserved.

It is possible that the high degree of mitochondrial conservation is due to a mechanism which selects against mitochondrial mutation. The results of mitochondrial mutation are typically disadvantageous, resulting in hypovirulence in *Ophiostoma ulmi* (Abu-Amero et al., 1995; Rogers et al., 1987), and *Cryphonectria parasitica* (Bell et al., 1996; Mahanti et al., 1993), senescence in *Podospora anserina* (Jamet-Vierny et al., 1997; Silliker et al., 1996), and the petite mutants of *Saccharomyces cerevisae* (Clark-Walker, 1992). Protection against mitochondrial mutations has been observed in *Podospora anserina.* It was found that crosses of two homokaryotic isolates of *P. anserina* with the senescence phenotype resulted in the restoration of the healthy phenotype. This occurred by the elimination of the senescence mitochondrial type and the reestablishment of the wild-type mitochondria. The mechanism for this conversion is unknown, but either recombination of the mutant mitochondria by a repair process

occurred at sexual recombination, or wild-type mitochondria were transmitted and became the dominant type resulting in the elimination the mutant type (Silliker et al., 1996). The typically disadvantageous result of mitochondrial mutations, which result in mitochondrial dysfunction, and the importance of functional mitochondria for virulence, suggests that the low variation in the mitochondrial genome of *C. purpureum* may be the result of selection against any mutant mitochondrial phenotype.

4.5 Conclusions

Long distance gene flow has most likely served to preserve the two mitochondrial haplotypes observed in the 5.1 kb SCAR marker. The observation of the same RFLP haplotype in the total mitochondrial DNA over broad geographic distances may be representative of an ancestral mitochondrial type. The highly conserved SCAR markers, as indicated by the lack of length polymorphisms and the inability of 24 restriction endonucleases to differentiate the 3 kb SCAR and only 1 of 24 enzymes to identify two haplotypes present in the 5.1 kb SCAR, suggest that there is a low degree of variation in the mitochondrial DNA of *C. purpureum.* The mechanism of mitochondrial genome conservation is unknown, but it is possibly a result of inhibition of mitochondrial recombination by the absence of cytoplasmic exchange, or an aggressive repair mechanism that selects against mitochondrial recombination. Regardless of the method of conservation, it appears from this data that the mitochondrial genome is effectively clonally reproducing because mitochondrial recombination is rare. Taking into account the possibility of long distance gene flow and the highly conserved nature of the mitochondrial markers studied, the risk of introducing new mitochondrial genes into the

natural population of *C. purpureum* present in a treatment area is considered to be low. This data will also partially fulfill the requirements of the registration and commercialization of *C. purpureum* as a biological control agent for hardwood weed vegetation.

CHAPTER 5 - FUTURE RESEARCH

5.1 Mitochondrial inheritance

Further research is necessary to clarify the mode of mitochondrial inheritance in *C. purpureum.* From the data collected in this project, it is apparent that mitochondrial inheritance was independent of nuclear inheritance and that cytoplasmic exchange did not occur. It also appears that mitochondrial recombination was rare, resulting in clonal mitochondrial propagation, otherwise the amount of variation observed would be much higher, and more than one mitochondrial type may have been found in one isolate. Crossing two homokaryons of different mitochondrial types would allow mitochondrial inheritance to be investigated. Crossing the homokaryons would result in the formation of a heterokaryon, in which nuclei migrate bidirectionally, resulting in all cells containing a pair of nuclei.

Mitochondrial mixing may occur at the zone of hyphal anastamosis in the cells which have anastamosed to join the homokaryons. Careful microscopic monitoring of the events during formation of the heterokaryon may allow a subculture to be taken immediately, or shortly after, the formation of the heterokaryon. This subculture would be heterokaryotic and mitochondrial recombination could be monitored. Use of the PCR based SCAR marker developed in this project would allow both mitochondrial types to be recognized in the newly formed heterokaryon, but it could not be used to monitor recombination. It would be impossible to detect recombination using this method because if both mitochondrial types were present, PCR amplification would result in independent amplification of the SCAR and restriction digestion would show both mitochondrial types. PCR amplification and restriction digestion would fail to show

recombination because it is not limited to one mitochondrial genome; a mosaic of mitochondrial DNAs would be amplified. Mitochondrial recombination could be identified by extraction of total mitochondrial DNA, followed by restriction digestion. Comparison of the mitochondrial DNA from the original homokaryons with the mitochondrial DNA from the region of anastamosis would indicate recombination if the mitochondrial DNA from the anastamosis region was different from both parents and approximately the same size. Summation of the band sizes of a mosaic pattern composed of both parental mitochondrial types would be much larger than each mitochondrial DNA independently, but a recombined mitochondrial DNA would be approximately the same size as the parent mitochondrial DNAs. In this way mitochondrial recombination could be monitored. Continual subculture of the heterokaryon could be performed to observe the stability of the recombined genome. The low amount of variation observed in this experiment suggests that a conserving mechanism is in place, and subculturing may cause a recombined mitochondrial genome to be lost and the parental types reestablished.

5.2 Mating system

All the studies completed to date indicate that *C. purpureum* has a tetrapolar mating system which promotes outcrossing and recombination (Boidin, 1971; Gosselin et al., 1995; McLauglin, 1991; Nakasone, 1990; Rayner and Boddy, 1988; Wall et al., 1996). The mitochondrial and ribosomal DNAs, however, are highly conserved and the same types are spread over large geographic ranges. This high degree of conservation is more typical of an organism with a clonal mode of propagation such as homothallism. The widespread distribution of this fungus suggests that it has not rapidly colonized large

areas, but how has it remained so conserved at these markers? Conservation of the mitochondrial DNA is possible if cytoplasmic exchange is very limited and a mechanism is in place to prevent mitochondrial recombination. The ribosomal DNA may remain conserved because it is inherited as a single Mendelian marker.

The mating system of *C. purpureum* may provide a mechanism for genetic conservation. Experiments on the mating of *C. purpureum* have shown no incompatibility reactions between widely separated individuals, indicating that multiple alleles are present at the mating type loci and that the mating system is tetrapolar. Exceptions have been observed by McLaughlin (1991) and Wall et al. (1996) who both found single spore isolates of *C. purpureum* which were compatible with other single spore isolates of the same parent homokaryons containing the same mating type alleles. Wall et al. (1996) explained their findings as the potential existence of more than one genet in the single spore isolate collection from the parent heterokaryon. It may also represent homothallism, which would explain the low amount of genetic variation observed in the ribosomal and mitochondrial DNAs.

True homothallism, the ability of single spore isolates with the same alleles at the mating type loci to successfully mate, could be tested by pairing single spore isolates. Single spore isolates derived from a parent basidiocarp would be crossed in all possible combinations and the reactions at the zone where the homokaryotic mycelium meets would be monitored. This would allow the verification of the tetrapolar mating system by observing the resultant ratios of mating type alleles. If 100% of the crosses were inter-compatible, as determined by mycelial confluence, *C. purpureum* could be considered truly homothallic. The presence of barrage zones would indicate that isolates with the

same allele at the *A* or *B* locus are unable to cross, indicating the species is not homothallic. The experimental evidence presented (Gosselin, 1995; McLaughlin, 1991; Wall et al., 1996), indicates that some isolates may be homothallic and some may be heterothallic. It is therefore necessary to replicate this experiment with many different parent heterokaryons, so that many isolates are screened. Since the collection at the Pacific Forestry Centre contains isolates from many geographic origins, testing a diverse population will indicate if homothallism is present in one geographic location and heterothallism is present in another, or if both systems are present in many areas.

With advances in molecular biology, it is possible to fingerprint the mating types and monitor their progress after hyphal anasotomosis. Randomly amplified polymorphic DNA fingerprints could be assigned to each homokaryon, which would represent the nucleus of each mating type. After hyphal anastamosis, cultures taken from the zone of confluence and the perimeter, representing each parent, would have the following patterns: a) successful mating, DNA fingerprint from the zone of confluence and each parent would be a combination of both parental types as a result of bidirectional nuclear migration; or b) unsuccessful mating, zone of confluence represents both parental types, while each parental type is isolated in the perimeter cultures representing the failure of nuclear migration; or c) one way nuclear migration as represented by a combination of nuclear patterns at the zone of confluence and one parent having both nuclei and one parent having only the original. These mating experiments would help to clarify the exact role of homothallism in the *C. purpureum* lifecycle and would indicate what proportion of the isolates are able to reproduce homothallicaly.

Secondary homothallism is unlikely in *C. purpureum* because each spore contains one nucleus (Biodin, 1971; Ginns and Lefebvre, 1993). Apomixis is potentially ruled out because species with this form of homothallism do not form clamp connections before fruiting and *C. purpureum* does (McLaughlin, 1991). To positively rule out these forms of homothallism, a susceptible host of *C. purpureum* could be inoculated with mycelium derived from a single spore isolate and fruit body production on the infected host monitored. The isolate must be determined to be homokaryotic by the absence of clamp connections, and the experiment must occur under controlled conditions in a growth chamber to prevent any wild *C. purpureum* spores from germinating on the inoculated host which would result in the formation of a heterokaryon. Disease progress can be monitored because there is no difference in virulence between homokaryons and heterokaryons (Wall et al., 1996). Apomixis or secondary homothallism would be proven or refuted by monitoring for basidiocarp production. If the homokaryon was able to produce basidiocarps with viable basidiospores, secondary homothallism or apomixis would be demonstrated, providing another explanation for the high degree of genetic conservation.

5.3 Conclusions

Experiments designed to demonstrate inhibition of mitochondrial recombination or potential homothallism should be performed to identify possible reasons as to why *C. purpureum* has such a conserved mitochondrial genome. All data collected on the mating system of *C. purpureum* indicates that it is highly outcrossing, which is preferable for a

biological control agent. If *C. purpureum* does have a potential for homothallism, and therefore clonal propagation, it is possible that isolates used in biological control would persist in the environment for extended periods of time. It is likely that the mitochondrial genome has been maintained through prevention of recombination rather than homothallism because mitochondrial DNA is inherited cytoplasmically without recombination and independently of the nuclear DNA, but these experiments would help to clarify the situation.

CHAPTER 6 - REFERENCES

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APPENDIX 1

1.1 DNA sequences read in this study.

DNA presented in 5' to 3' direction. PCR primer locations bold face and underlined. *Nsi* I site of 5.1 kb middle sequence bold, italicized and underlined.

3 kb Forward Sequence:

AGACCTTTCTGTGCTACGCGCCCGGCCACCGCGCAGGCCCGTGAACTTCGAA
TCTTTCCTTTAAGCCACTAGAAGCAGTGAAGTTGCTAACGAAATGAAGCTAG
TAAGCTAGAGTTATAGGATCTGACTATCGGCGGGGTTATTTTTTATTTTTAAA
AAAGG**CAGTAAAGCGACTATCAAAGGGACGAGC**TCTAAGGAAACAGCTGA
GTTCTTCTAACCTACT

3 kb Reverse Sequence:

ATAAGCTTGGATTATCAAAAATTATTATGAATAGGTTTTTTTATAGCTTTTGCT
TTTAAAACTCCTTTATATCCTTTTGTAAT**CTGATTACCTAAAGCTCATGCTG**
ATTCAGTCTTAGCAGGTAGTATCATATTAGCAGCTACAATCTTAAAACTAGC
AGTTTATGGAGAATTAAGAGTGTTAATTAACTTTTTCTGATGCTACTTATTATT
TT

5.1 kb Forward Sequence:

CTACCACCNAAAAAAGNTTAATTTTATTTTTAATTTTATAATAATAATATGAA
GTATTAGATACCTGAATATATAACAGAATTTTCNTTTGTTAAAAAAATTATTT
TTTAATGTTAAATACTATTTTCNTTTCACCNTTAGAACAATTTGAAATTACNA
AT**TTACTTGGNTTTAATTTCCC**CNNATTGGGTTTTACAAATATAACTTTACT
NATTTTGGGATTATATTCNACTTTAAATTTAAAAATTGTTNTCCGGNTTCCCTT
TTTTGAGANNATGANTCCCCCTTAGTTCCCAATNAATGATCNATCNCCCCNTG

5.1 kb Middle sequence

CTTACAATTAGCCGCAGGATTAATTTAAGGCTAGAATATTAACATTAAGTTAG
TATTAAGGNTGAGCAGTAGGTAGCGCCTTTTTAACCCCTT***ATGCAT***CCTGTCC
TTATCTTTTTTATTATACCTTTTGCTTTAGTGATCCTACCCTTACTTACACCTTA
AAAAGTAGGAGCTGGAAATCCCAGCCTTATAAGGGACAAGATCTGAAAGGT
AAATGGGAATTAAGCTACCTTACAATAGCTCAGACTTTCCTGCGGTAAAAAG
CTAGATGTCTAACTAGTTTTAGTTAGAACAGCCGCAGCCGCAACAAAAAGGT
AATGAAAAAAAAATATATATATTGACACTTAGTATGTTACAATATTTTTTTAA
TAGTTTGAAAGACCTAATCCNAATTCTAACTTATTAAATTTAGGNGNAAAATC

CGCCCATTAANATTGGNCACTTTAAANCAAGGCTTCCCACCCTAANNGGGAG
GGAAGTAGGTCGAGGGGTCTAACTTAAGTTAAAAACGGAAAAACCATAACN
GGTTTAATTTGGNGTTCTGCTGGAGGGTTAATTAATATAGGTNTCTCGCCGCC

5.1 kb Reverse sequence

GGTTAAGAACGCTATTAACCAGATTTTCAATCAGTCACATTTTTCAATGAACT
TTCTTATATTAATGGCATT**GGGCAAATCTCCGCTAAATAT**ACTATCATTTAC
ATGATTGCATTCNACTGTGTTTTTTTAATAAACANTCNGGGGCTCCCGANTAT
GTNCCACCTCCNTATTTTGAAAAAATAAAAAAATNTTTCTAATCCGGGGNGT
CGGTAAACCAAATCCCTCCCCCNACNAAATTAAANCCAATTTTTGGAAATTA
TTTTTAATTNTTAACAAACCCCNTATGTTTTAAAGAANTNACCCTNNAANGGG
GGAAANT

APPENDIX 2

2.1 Amino acid sequence consensus of sequences used in this study.

3 kb Forward sequence: No amino acid matches in nr-aa database.

3 kb Reverse sequence: NADH 4

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                N                11                21                31                41                51                61
                1  LSLDLQKLLW    IGFFIAFAVK    TPLFPFHIWL    PEAHVEAPIA    GSIILAGIIL    KLAGYGILRL    LIPMF
1.  3kbr.pir       ISLDYQKLL*    IGFFIAFA FK   TPLYPF VI*L   PKAH ADSVLA   GSIILA AT IL   KLA VYGELRV   LINFF
2.  asterina.p     ----------    ---FIAF III K  MPIY GFHLWL   PKAHVEAPIA    GSMILA AILL    KLGGYG LLRT   IPLFY
3.  reclino.pi     -SQERQKLLW    LAFFI SFAIK   IPMVPFH VWL   PEAHVEAPTA    GSVLLAG VLL    KLGGYGILRF    SIPMF
4.  hansenul.p     LSIDLQSIIF    IGLIIGILVK    TPVFP VHTWL   PLVHAESPI S   GSIILAGII I    KLA IYAIIRL    -----
5.  tricticu.p     -----Q ILLW   IAFFASFAVK    VPMVP VHIWL   PEAHVEAPTA    GSVILAGILL     KLG TYGFLRF    SIPMF
6.  yarrow~1.p     LSLDLQ TIIW   LGTFMAMMVK    TPLFP IHEWL   PEEHSESPLA    GSMILAG LIL    KLA LYAMLRL    LL---
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1. *Chondrostereum purpureum*
2. *Asterina pectinifera*
3. *Reclinomonas americana*
4. *Hansenula wingei*
5. *Triticum aestivum*
6. *Yarrowia lipolytiva*

5.1 kb Forward sequence: ATPase VI

```
                N                11                21                31                41                51                61
                1  MLNTIITSPL    DQFEIR NLFG   FNAPLLGNSN    LNL
1.  5kbf2.pir      MLNTI FKSPL   EQFEI TNLLG   FNFP XLGFTN    ITL
2.  agaricus.p     MKTLF ILSPL   SQLEVT SLIG   LNAPILGHIN     LTL
3.  aspergil.p     -------SPL    DQFE VRDLLS   INANLLGNF H    LSL
4.  chondrus.p     ----IITSPL    EQFEI VTIFP   FSISGLNFS-     ---
5.  cocliobu.p     -------SPL    DQFEIRNLF S   IDTPLL ANMN    LSI
6.  hansenul.p     MTNIIVNSPL    DQF DIKVFIG   FVSPF IDLTN    INI
7.  saccharo.p     LLNTYITSPL    DQFEIRTLFG    LQSSF IDLSC    LNL
```

1. *Chondrostereum purpureum*
2. *Agaricus bitorquis*
3. *Aspergillus amstelodami*
4. *Chondrus crispus*
5. *Cochliobolus heterostrophus*
6. *Hansenula wingei*
7. *Saccharomyces cerevisiae*

5.1 kb Middle sequence: No amino acid matches in nr-aa database.

5.1 kb Reverse sequence: Cytochrome b

```
          N              11          21          31          41          51          61
          1  IVDWLWGGFS  VDNPTLNRFF  ALHFLLPFLI  LGAVLIHLIA  L
1.  5kbr.pir  ----------  VKNAINQIFN  QSHFSMNFLI  LM--------  -
2.  luc shor.p  ------GGFS  VDNPTLTRFF  ALHFLLPFLI  VGLTLV----  -
3.  myrmecia.p  ----------  VSNATLNRFY  SIHFILPFMI  LI--------  -
4.  p mega.pir  -VDWLWGGFA  VDNPTLNRFF  SLHFTFPFVI  VGAVLIHLIL  L
5.  s_cere s.p  IVSWLWGGFS  VSNPTIQRFF  ALHYLVPFII  AAMVIMHLMA  -
```

1. *Chondrostereum purpureum*
2. *Luscinia svecica*
3. *Myrmecia pilosula*
4. *Phytophthora megasperma*
5. *Saccharomyces cerevisiae*