Chapter 4

Sequencing of the ITS region, a β-tubulin Gene and an Extracellular Serine Protease Gene to Detect Genetic Variation in 25 Australian *Duddingtonia flagrans* Isolates

4.1 Introduction

In recent years, the nematode-trapping fungus *Duddingtonia flagrans* has been shown to have great potential for the control of gastrointestinal nematodes of livestock (Fernandez *et al.*, 1997; Knox and Faedo, 2001; Larsen *et al.*, 1998; Larsen *et al.*, 1997; Peloille, 1991; Sanyal, 2001). The use of this fungus in a biocontro situation requires its inundative application in livestock feed so it appears in faeces in sufficient quantity to substantially reduce larval survival and hence re-infection rates. This principle is well established in the aforementioned studies but before widespread application can occur it will be necessary to ensure no detrimental effects occur in environments where this biocontrol agent is applied.

One of the main requirements of a biological control agent is that it has little or no effect on non-target organisms. As nematophagous fungi do not show any particular nematode host specificity (Barron, 1977), there is the potential for increased numbers of an introduced biological control fungus to have detrimental effects on beneficial free-living soil nematodes. The most extensive study looking at the environmental impact of *D. flagrans* showed that numbers and diversity of soil nematodes and microarthropods were not affected by the release of *D. flagrans* and that it did not persist in the environment for greater than 24 weeks (Knox *et al.*, 2002). Despite these encouraging results, the inundative release of any biological control

agent must continue to be closely monitored for longer-term impacts. There is the possibility of a competitive effect with native fungi and over time displacement by a more biologically active isolate could occur.

There are examples where displacement of endemic organisms has occurred after the introduction of a biological control agent. A program which introduced the predacious snail *Euglandia rosea* to control the land snail *Achatina fulica,* resulted in the extinctions of some non-target, precinctive species of *Partula* land snails in the Pacific island of Moorea (Murray *et al.,* 1988). These *Partula* snails were not the intended target of the control program. Another biological control program which saw the release of the aphidophagous coccinellid, *Coccinella septempunctata,* to improve the biocontrol of the green peach aphid and the Colorado potato beetle which cause economic loss to potato crops, saw the displacement of two native coccinellids. These two species, *Hippodamia convergens* and *Coccinella transversoguttata,* are now very rare in areas they once dominated (Snyder and Clevenger, 2004).

Displacement has also been observed in closely related populations of the one species. Hughes *et al.* (2003) conducted a study in which two populations of the freshwater shrimp *Paratya australiensis* were mixed in the Brisbane River. The two populations were quite divergent genetically and seven years after the original translocation, the translocated population had caused the resident population to become extinct in the pool where the translocation took place and in several surrounding pools. There have also been studies investigating the dominant competitive effect hatchery salmon have over wild salmon (Rhodes and Quinn, 1998). In an aquarium experiment, it was shown that hatchery-reared salmon dominated naturally spawned salmon, even when the wild salmon were prior residents. They found the combined effects of

greater size and rearing experience of hatchery-produced salmon were sufficient to overcome a wild salmon's advantage of prior residence.

The identification of fungi using taxonomic keys of morphological features does not allow differentiation between isolates of the same species which may exhibit substantial biological differences as shown in Chapters 2 and 3. In contrast, modern molecular techniques offer the potential to monitor the release of a particular isolate provided an appropriate molecular signature can be found. Nuclear ribosomal DNA (rDNA), which encodes rRNAs, has been commonly applied in phylogenetic approaches for distinguishing kingdoms, phyla, classes or orders because of its highly conserved nature (Hwang and Kim, 1999). The spacer regions, intergenic spacer (IGS) and internal transcribed spacer (ITS), separating the rDNA coding regions, are often employed to resolve phylogenies in lower categorical levels among genera, species and populations because of their high variability (Hwang and Kim, 1999).

Studies of β-tubulin genes have been used to understand the evolution of trapping devices in nematophagous fungi and have been used extensively in phylogenetic studies of parasites and fungal species (Li *et al.*, 2005). For example, Keeling (2003) used β-tubulin and α-tubulin to determine that microsporidia is likely derived from a zygomycete-like ancestor. Einax and Voigt (2003) have used β-tubulin genes to study and confirm phylogenies of a broad range of fungal taxa and provided evidence that it is suited to the estimation of deep phylogenies and for the analysis of complex species groups. Hansen *et al.* (2005) used β-tubulin to analyse the evolutionary relationships of the fungi *Peziza* and *Pezizaceae*. The *tubA* β-tubulin gene has also been previously used in phylogenetic studies of several *D. flagrans* isolates from Europe,

the USA, India and Malaysia (Ahren *et al.*, 2004). No variation was found within these isolates.

Extracellular serine proteases have been implicated in the penetration and digestion of host tissues by many plant and animal parasitic fungi (Minglian *et al.*, 2004) and have been used to study phylogenies of the fungus *Trichophyton rubrum* (Jousson *et al.*, 2004). In addition, the PII protease gene has been used to study the genetic diversity within several isolates of *D. flagrans* from around the world (Ahren *et al.*, 2004).

In this study the ITS region, a β-tubulin gene and an extracellular serine protease gene have been sequenced with the aim of developing a technique for distinguishing between *D. flagrans* isolates and to further address the hypothesis that considerable diversity exists within the Australian *Duddingtonia* spp. complex and that it may be comprised of more than one species.

4.2. Material and Methods

4.2.1 Fungal Isolates

The 25 fungal isolates were obtained from original cultures derived from a field survey (Larsen *et al.*, 1994) and from further isolates maintained in the CSIRO culture collection. Subcultures were taken from parent stock and maintained on Potato Dextrose Agar (PDA) using the procedure outlined in Chapter 3.

4.2.2 DNA Extraction

Fungal mycelium scraped from a 5 day old culture was placed in a microcentrifuge tube containing 1 mL of 20 mM EDTA. The mycelium was then pelleted by spinning in a microcentrifuge for 30 sec at 14000 x g. Supernatant was removed using a pipette. One mL of 100% ethanol was then added to mycelium and the tube was placed on a vortex mixer for 5 sec to displace the mycelial pellet from the side of the tube. The mycelium was again pelleted by spinning for 30 sec at 14000 x g. Ethanol was removed using a pipette and the pellet was then completely dried under vacuum for 10 min. A small amount of sterile acid washed sand was then added to the pellet which was pulverised to a fine powder using a 1 mL plastic pipette tip that had been rounded-off using a blue flame. Three hundred µl of 50 mM EDTA pH 8.0, 0.2% sodium dodecylsulphate (SDS) was added to the ground mycelium. The tube was then incubated in a 65 °C water bath for 15 min and then spun for 10 min at 14000 x g. The supernatant was then removed to a new tube and the tube containing the pellet was discarded. 60 µl of chilled 5M potassium acetate pH 4.8 was then added to the tube and mixed. The solution was then incubated on ice for 1 hr. The chilled solution was then spun in a microcentrifuge at 14000 x g for 20 min. The supernatant was then removed to a fresh tube and the tube containing the pellet discarded. 300 µl of isopropanol was then added and the solution mixed by inverting the tube. The DNA was then pelleted by spinning in microcentrifuge at 14000 x g for 5 min. The supernatant was then removed and discarded and DNA pellet was washed using 0.5 mL 70% ethanol and spun for 5 min in a microfuge. Ethanol was then removed and the pellet was dried under a vacuum for 10 min. DNA was then resuspended for approx. 12 hr in 50 µl TE buffer and then stored at -4 °C for future use.

4.2.3 DNA Sequencing

The ITS region was amplified using the primers TW81 (GTTTCCGTAGGTGAACCTGC), and AB28 (ATATGCTTAAGTTCAGCGGGT) (Howlett *et al.*, 1992). The PCR was performed in a total volume of 25 µl containing 10-20 ng fungal DNA, 120 ng of each primer, 1X PCR reaction buffer (50 mM KCl; 10 mM Tris-HCl), 2 mM of DNTPs, 1 U *Taq* polymerase and 1.8 mM $MgCl_2$. Paraffin oil was added to prevent evaporation. Control reaction tubes lacked DNA. DNA was amplified using the following cycling parameters: [94 °C–2 min] + [94 °C – 10 sec, 50 °C –30 sec, 72 °C –1 min] x 35 + [72 °C –3 min]. The PCR products (2 µl) were analysed using electrophoresis on 0.8 % agarose gel in 1x TAE (40mM Tris-acetate, 1mM EDTA, pH 8.5) buffer. A λ *Hind* III DNA ladder was used as a size standard. The gels were stained using ethidium bromide (0.5µg/ml) and photographed using a UV transilluminator. PCR samples were prepared for sequencing using a Promega Wizard® PCR DNA purification kit and sent to the Biomolecular Research Facility at the University of Newcastle for sequencing.

The β-tubulin gene was amplified using the primers Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (ACCCTCAGTGTAGTGACCCTTGGC) (Faedo *et al.*, 2001). The PCR mix was as above and DNA was amplified using the following cycling parameters [94 °C–3 sec] + [94 °C – 10 sec, 55 °C –1 min, 72 °C –1 min] x 37 + [72 °C –10 min]. PCR products were analysed and prepared for sequencing as described above and sent to the Biomolecular Research Facility at the University of Newcastle for sequencing.

The extracellular serine protease gene was amplified using the primers Protease1 (GTATCCGCATTACCCACGAT) and Protease2 (CATAGGTCTTTCCGGCGATA) (Ahren *et al.*, 2004). The PCR mix was as described above and DNA was amplified using the following cycling parameters [94 °C–3 sec] + [94 °C – 10 sec, 55 °C –1 min, 72 °C –1 min] x 37 + [72 °C –10 min]. PCR samples were prepared for sequencing using a Millipore Montage™ (Billerica, MA) PCR purification kit and sent to the Biomolecular Research Facility at the University of Newcastle for sequencing.

The isolates that were sequenced for each gene are shown in Table 4.1.

4.2.4 Sequencing analysis

For analysing the polymorphisms in the ITS region, β- tubulin gene and protease gene, nucleotide alignments were constructed using the ClustalX or ClustalW (accurate) program (Thompson *et al.*, 1994). The parsimonious phylogenetic trees was calculated using the program DNAPars in the phylogenetic program PHYLIP (Felsenstein, 2004). Neighbour-joining distance trees and Maximum likelihood trees were constructed using the program PAUP 4.0 (Swofford, 2002), and TreeView (Page, 1996). Each of these trees was constructed using the closely related nematophagous fungus *Arthrobotrys musiformis* (Genbank no: U51948) as an outgroup.

Table 4.1 Isolates of *D. flagrans* that had the ITS1, 5.8S, ITS2 region, the B-tubulin gene *tubA* and an extracellular serine protease gene sequenced. X indicates sequencing.

Isolate	Gene region sequenced		
	ITS	B-tubulin	Protease
92/3197	X	-	-
92/4054	X	-	-
CP/1134	X	-	-
CP/1168	X	-	-
CP/1171	X	-	-
CP/1382	X	-	-
CP/1187	X	-	-
CP/1188	X	-	X
CP/1474	X	X	-
CP/1801	X	-	-
CP/870	X	-	-
Dfa	X	-	-
CP/1810	X	X	X
CP/1861	X	-	-
DH/DIXON	X	X	-
DH/MCOMP	X	-	-
CP845	X	-	-
DF/ 8	X	X	X
DF/3	X	X	X
DH/OSP	X	-	-
DH/SPB	X	-	-
DH/CHICK	X	X	X
DH SPA	X	-	-
CP/911	X	X	-
CP/912	X	X	X

4.3 Results

The ITS region of all 25 isolates of *D. flagrans* was amplified using the primer pair TW81/AB28 and sequenced. The size of the amplification product was approximately 600 bp. Direct comparison using the programs ClustalX or ClustalW (Thompson *et al.*, 1994) of the sequence information shows that the isolates fell into four groups (GP 1, GP 2, GP 3 and GP 4). The four ITS groupings are shown in Figure 4.1 and the nucleotide differences are indicated by orange stars. GP 1 consists of isolates CP 1188, CP 1471, DH DIXON, CP 1187, DFa, 92/ 870, CP 1810, CP 1134, DH MCOMP, CP 1801, CP 1168, 92 3197, CP 1382, CP 1171, CP 1861, 92/ 4054. GP 2 contains isolates CP 911, CP 912, DH SPB, DH SPA, DH OSP and DH CHICK. GP 3 is made up of DF 3 and DF 8 ad GP 4 consists solely of CP 845. In total there were 15 nucleotide differences and these are summarised in Table 4.2. GP 2 differs from GP 1 by 10 bp substitutions and an insertion of 1 bp. GP 3 differs from GP 1 by one transition mutation, which is in common with GP 2 and 1 transversion mutation. GP 2 differs from GP 3 by 9 bp substitutions and one insertion. GP 4, isolate CP 845, differs by one transitional point mutation to all the other isolates and also has 3 bp insertions before a string of A's which are also different to the other 24 isolates.

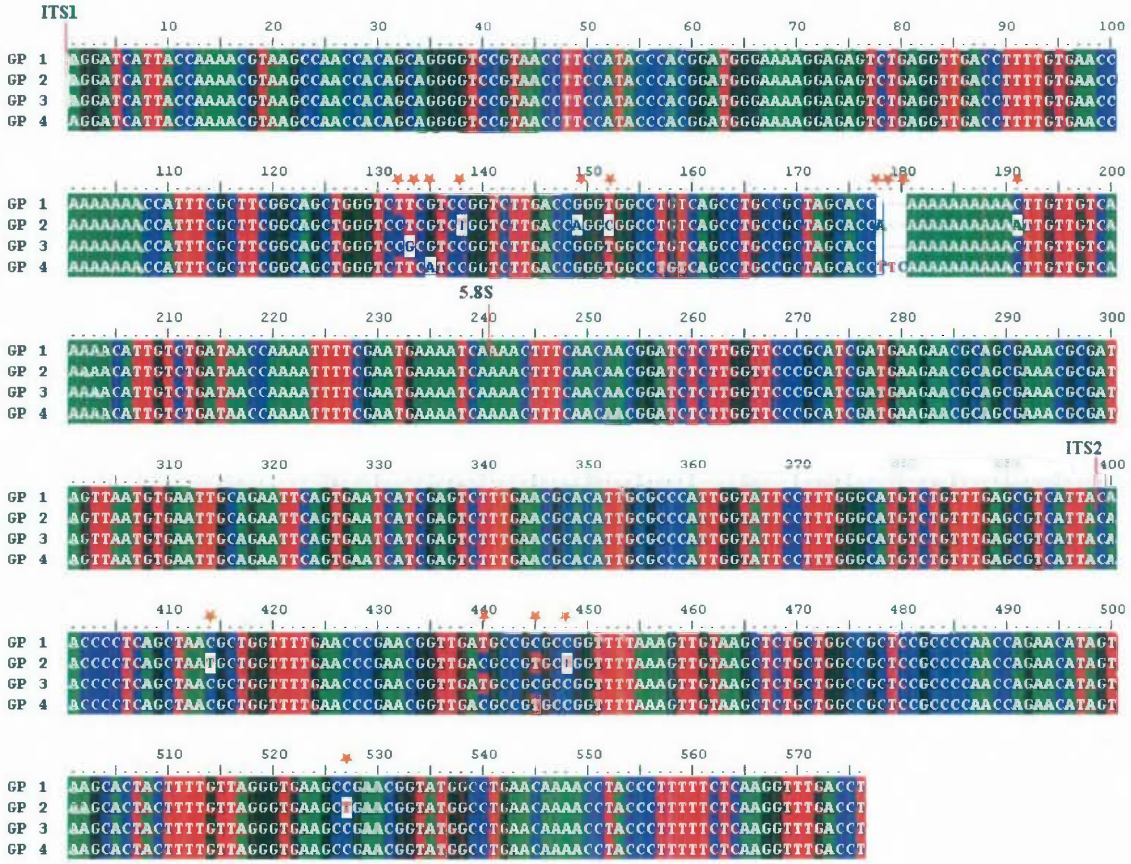

Figure 4.1 DNA sequence of the ITS region of *D. flagrans* where GP 1 is the sequence of CP 1188, CP 1471, DH DIXON, CP 1187, DFa, 92/ 870, CP 1810, CP 1134, DH MCOMP, CP 1801, CP 1168, 92 3197, CP 1382, CP 1171, CP 1861 and 92/4054 ; GP 2 is CP 911, CP 912, DH SPB, DH SPA, DH OSP and DH CHICK; GP 3 is DF 3 and DF 8; and GP 4 is CP 845. The ITS regions and the 5.8S region are indicated by red lines at their beginning. Orange stars highlight nucleotide differences.

Table 4.2 Nucleotide differences in the ITS region between 25 Australian Isolates of *D. flagrans*

Isolate	State	Source	Base Pair Number														
			132	133	135	138	149	152	178	179	180	191	414	440	445	448	527
92/3197	NSW	Sheep faeces	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
92/4054	Unknown	Unknown	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
CP/1134	WA	Sheep faeces	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
CP/1168	SA	Sheep faeces	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
CP/1171	SA	Sheep faeces	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
CP/1382	NSW	Sheep faeces	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
CP/1187	WA	Sheep faeces	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
CP/1188	NSW	Sheep faeces	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
CP/1474	Unknown	Unknown	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
CP/1801	WA	Unknown	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
CP/870	NSW	Unknown	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
Dfa	NSW	Soil	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
CP/1810	WA	Sheep faeces	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
CP/1861	Unknown	Sheep faeces	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
DH/DIXON	NSW	Soil	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
DH/MCOMP	NSW	Sheep faeces	T	T	G	C	G	T	-	-	-	C	C	T	C	C	C
CP845	SA	Sheep faeces	T	T	G	C	G	T	T	T	C	C	C	T	T	C	C
DF/ 8	QLD	Cattle faeces	C	G	G	C	C	T	-	-	-	C	C	T	C	C	C
DF/3	Unknown	Unknown	C	G	G	C	G	T	-	-	-	C	C	T	C	C	C
DH/OSP	NSW	Soil	C	T	G	T	A	C	A	-	-	A	T	C	T	T	T
DH/SPB	NSW	Soil	C	T	G	T	A	C	A	-	-	A	T	C	T	T	T
DH/CHICK	NSW	Soil	C	T	G	T	A	C	A	-	-	A	T	C	T	T	T
DH SPA	NSW	Soil	C	T	G	T	A	C	A	-	-	A	T	C	T	T	T
CP/911	NSW	Horse faeces	C	T	G	T	A	C	A	-	-	A	T	C	T	T	T
CP/912	NSW	Cattle faeces	C	T	G	T	A	C	A	-	-	A	T	C	T	T	T

The base pair number is the position in the sequence that a mutation is found. The State is the location where the isolates were originally collected and the Source is the medium from which they were collected. The white indicates GP 1, the yellow indicates GP 2, the orange indicates GP 3 and the green indicates GP 4.

Figure 4.2 shows the phylogenetic tree inferred from 1000 bootstrapped nucleotide sequence data. It uses distance matrices and the Jukes-Cantor model, which assumes equal base frequencies and equal mutation rates. Branch lengths are not displayed on the tree as proportional to genetic distance as they were created using PHYLIP's CONSENSE program and then drawn using Treeview. If this procedure is followed it is recommended to display PHYLIP consensus trees as rectangular cladograms (as presented) as the "Branch lengths" on trees obtained using PHYLIP's CONSENSE program are actually the frequency of the corresponding clade in the set of input trees (if the trees were bootstrap trees then these

correspond to bootstrap values) (Page 1996). See Table 4.3 for genetic distances between the 25 *D. flagrans* isolates and the out-group *A. musiformis.*

The groups in Figure 4.2 are separated into four groups as determined by the nucleotide sequence alignment. There is 73% support separating Gp 3 (isolates DF 3 and DF 8) into their own group, 100% support separating GP 2 (isolates CP 911, CP 912, DH SPB, DH SPA, DH OSP and DH CHICK) from the other isolates, 53% support separating GP 4 (CP 845) from the other isolates and 64% support clustering the GP 1 isolates together.

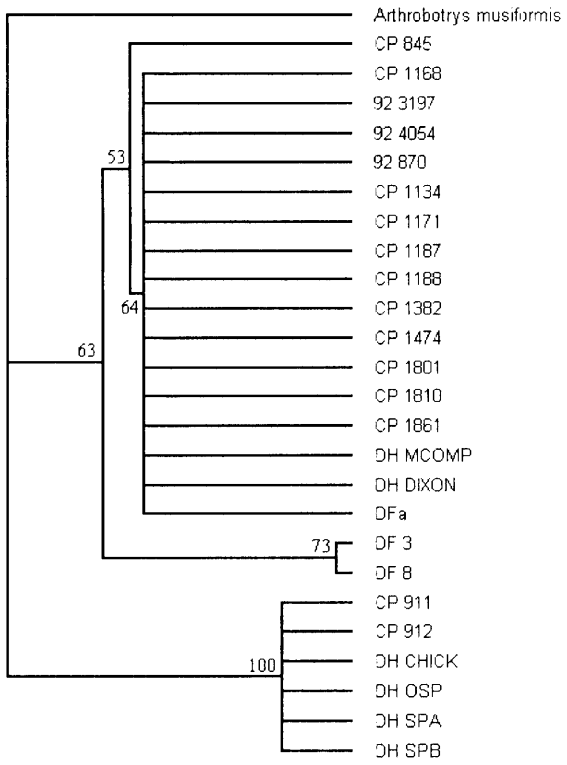

Figure 4.2 Consensus Neighbour-joining phylogenetic tree based on bootstrap replicates of the ITS region of 25 Australian Isolates of *D.flagrans* using the closely related fungus *A. musiformis* as an outgroup. Multiple sequence alignment was calculated using ClustalX (accurate) (Thompson *et al.*, 1994) and 1000 bootstrap replicates were calculated from this alignment using the phylogenetic analysis program PAUP 4.0 (Swofford, 2002). Distance matrices using the Jukes/Cantor distance method were calculated using PAUP 4.0 and the phylogenetic tree was constructed using TreeView (Page, 1996). The values on branches indicate the support percentage above 50%.

Figure 4.3 shows the phylogenetic tree displayed as a rectangular cladogram as suggested by Page (1996). The tree was generated using the Parsimony method. It assumes that "each site evolves independently, that different lineages evolve independently, that the probability of a base substitution at a given site is small over the lengths of time involved in a branch of the phylogeny, that the expected amounts of change in different branches of the phylogeny do not vary by so much that two changes in a high-rate branch are more probable than one change in a low-rate branch and that the expected amounts of change do not vary enough among sites that two changes in one site are more probable than one change in another" (Felsenstein, 2004). This tree separates the isolates into 4 clusters which again mimic the results of the sequence alignment. The groupings are different to that of the Neighbour-joining tree. Here there is only 29% support separating isolates DF3 and DF 8 from the other isolates. The remaining isolates form one group, but assemble into three clusters from the one group. There is 50% support placing CP 845 into its own cluster and very strong support (99%) separating the GP 2 isolates from CP 845.

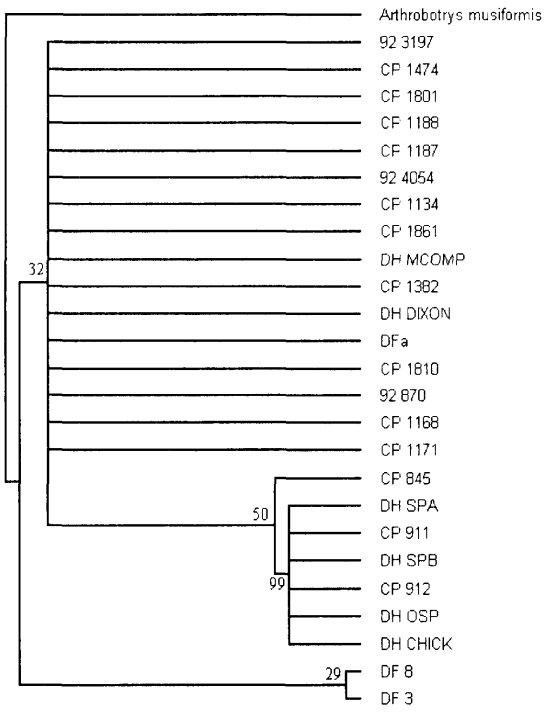

Figure 4.3 Consensus extended majority rule phylogenetic tree based on bootstrap replicates of the ITS region of 25 Australian isolates of *D.flagrans* using *A. musiformis* as an outgroup. Multiple sequence alignment was calculated using ClustalW (accurate) (Thompson *et al.*, 1994) and bootstrap replicates were calculated from this alignment using the Phylip bootstrapping program Seqboot with 1000 replicates. The Phylip program DNApars was used to carry out unrooted parsimony on DNA sequences. The phylogenetic tree was constructed using the Phylip Consense program. The values on branches indicate the support percentage the groupings achieve after 1000 replicates (Felsenstein, 2004).

Figure 4.4 shows the phylogenetic tree displayed as a rectangular cladogram as suggested by Page (1996). The tree was created using the maximum likelihood model which assumes that "the base substitution allows the expected frequencies of the four bases to be unequal, allows the expected frequencies of transitions and transversions to be unequal, and has several ways of allowing different rates of evolution at different sites" (Felsenstein, 2004). This tree is different again to the two other trees, however still forms clusters of the four ITS groups. Here only one group is formed with the clusters of the ITS group forming off this group. There is 100% support clustering the GP 3 isolates together, 100% support clustering the GP 2 isolates together and 63% support separating CP 845 from these 2 clusters.

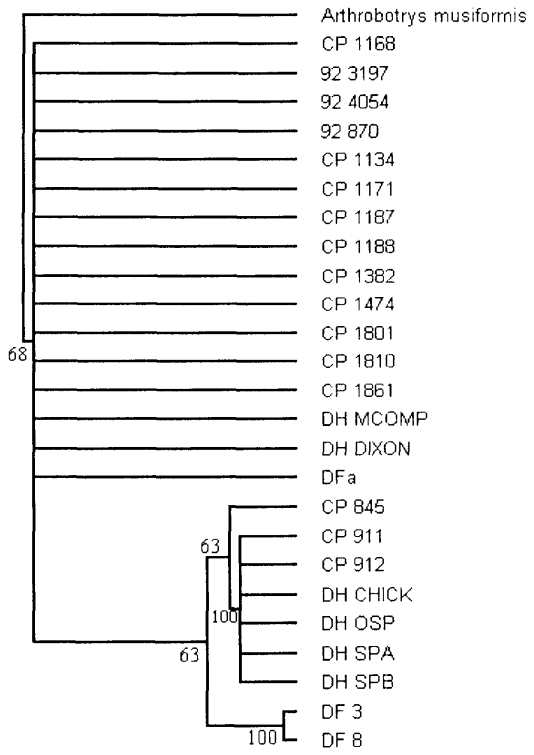

Figure 4.4 Consensus extended majority rule phylogenetic tree based on bootstrap replicates of the ITS region of 25 Australian Isolates of *D.flagrans* using *A. musiformis* as an outgroup. Multiple sequence alignment was calculated using ClustalX (accurate) (Thompson *et al.*, 1994) and 100 bootstrap replicates were calculated from this alignment using the phylogenetic analysis program PAUP 4.0 (Swofford, 2002). Maximum likelihood was calclated on the DNA sequences using PAUP 4.0 and the phylogenetic tree was constructed using TreeView (Page, 1996). The values on branches indicate the support percentage above 50%.

Table 4.3 shows the genetic distance between 25 isolates of *D. flagrans* and the closely related fungus *A. musiformis* calculated using the Phylip program dnadist (Felsenstein, 2004). The data reflects what has been demonstrated by the phylogenetic trees. All isolates of *D. flagrans* are most distantly related to *A. musiformis* (0.104- 0.115) and as expected closely related to each other (0.000- 0.018), however four clear clusters are again evident, placing the isolates in the groups detailed in table 4.2.

Table 4.3 Genetic distance between the ITS region of 25 Australian Isolates of *D. flagrans* and closely related fungus *A. musiformis*

	DH_SPB	DH_SPA	DH_OSP	DH_CHICK	CP_912	CP_911	DF_8	DF_3	CP_845	Dfa	DH_DIXON	DH_MCOMP	CP_1861	CP_1810	CP_1801	CP_1474	CP_1382	CP_1188	CP_1187	CP_1171	CP_1134	92_870	92_4054	CP_1168	92_3197	*A. musiformis*
A. musiformis	0.115	0.115	0.115	0.115	0.115	0.115	0.104	0.104	0.112	0.106	0.106	0.106	0.106	0.106	0.106	0.106	0.106	0.106	0.106	0.106	0.106	0.106	0.106	0.106	0.106	
92_3197	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
CP_1168	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000			
92_4054	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000				
92_870	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000					
CP_1134	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000						
CP_1171	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000							
CP_1187	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000								
CP_1188	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000									
CP_1382	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000										
CP_1474	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.005	0.000	0.000	0.000	0.000	0.000	0.000											
CP_1801	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.005	0.000	0.000	0.000	0.000	0.000												
CP_1810	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.005	0.000	0.000	0.000	0.000													
CP_1861	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.005	0.000	0.000	0.000														
DH_MCOMP	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.005	0.000	0.000															
DH_DIXON	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.005	0.000																
Dfa	0.018	0.018	0.018	0.018	0.018	0.018	0.003	0.003	0.009																	
CP_845	0.018	0.018	0.018	0.018	0.018	0.018	0.009	0.009																		
DF_3	0.018	0.018	0.018	0.018	0.018	0.018	0.000																			
DF_8	0.018	0.018	0.018	0.018	0.018	0.018																				
CP_911	0.000	0.000	0.000	0.000	0.000																					
CP_912	0.000	0.000	0.000	0.000																						
DH_CHICK	0.000	0.000	0.000																							
DH_OSP	0.000	0.000																								
DH_SPA	0.000																									
DH_SPB																										

Six isolates, two representatives ITS groups 1, 2 and 3, were sequenced in the case of the protease gene, and 8 isolates, two representatives from each of the ITS groups, and two others randomly chosen were sequenced for the β-tubulin gene. If variation was found in these isolates, the remaining isolates would then be sequenced. Figures 4.5 and 4.6 show the sequence of each of these genes. The size of the amplification product of the PII gene is approximately 180 bp. The sequence of the PII gene is identical to that of previously sequenced *D. flagrans* PII genes with accession nos. AY444709 TO AY444725 (Ahren *et al.*, 2004). The size of the amplification product of the *tubA* (β- tubulin) gene was approximately 440 bp. The sequence of this PCR product is identical to previously sequenced *D. flagrans tubA* genes with accession nos. AY444726 to AY444739. It was also identical to an undefined region of the β-tubulin gene of *Arthrobotrys pseudoclavata* with accession number AY773359 (Liu and Yang, 2004). In each case there was no variation between any of the isolates sequenced so sequencing of the remaining isolates was not undertaken.

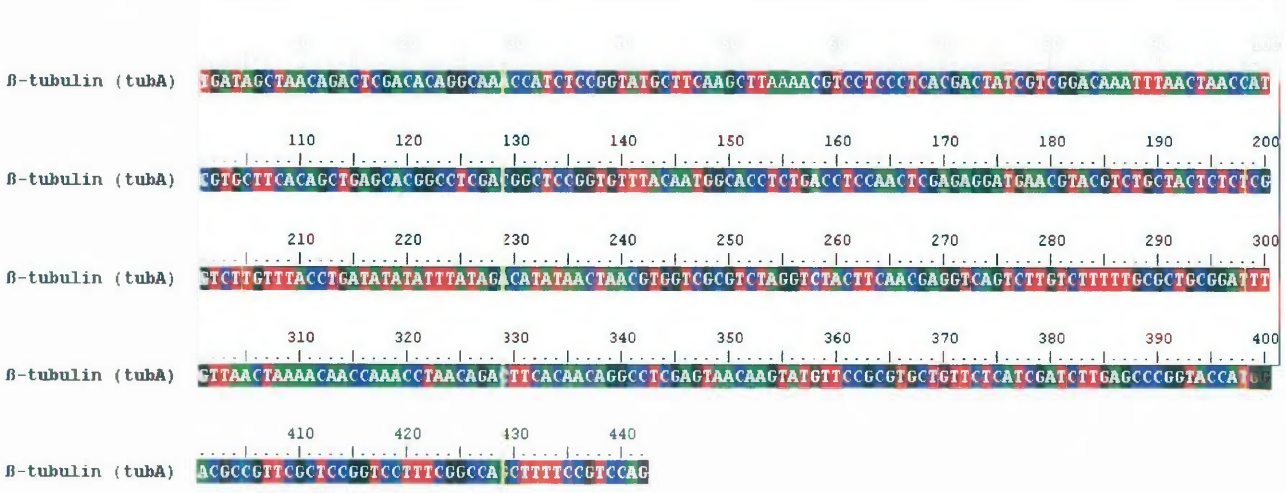

Figure 4.5 DNA sequence of the *tubA* gene for *D. flagrans* isolates CP 911, CP 912, DH CHICK, DH DIXON, CP 1810, CP 1474, DF 3 and DF 8

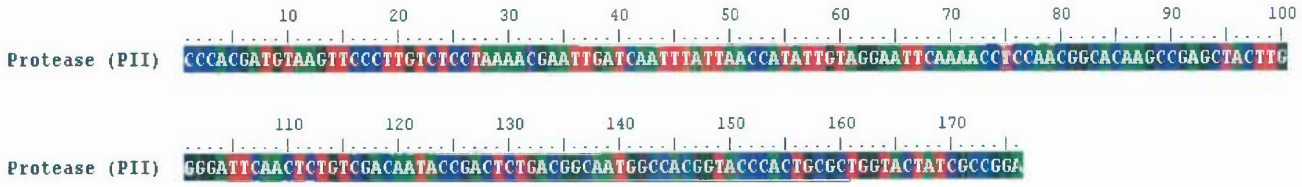

Figure 4.6 DNA sequence of the PII gene for *D. flagrans* isolates CP 1810, CP 1188, CP 912, DH CHICK, DF 3 and DF 8.

4.4 Discussion

Earlier chapters have described the biological differences between *D. flagrans* isolates in the CSIRO culture collection. The present chapter indicates that there are also molecular differences among these Australian isolates as a result of the ITS sequencing. The ITS1 and ITS2 sequencing produces four clear groups. Group 1 consists of isolates CP 1188, CP 1474, DH DIXON, CP 1187, DFa, 92/ 870, CP 1810, CP 1134, DH MCOMP, CP 1801, CP 1168, 92 3197, CP 1382, CP 1171, CP 1861 and 92/4054. Group 2 consists of CP 911, CP 912, DH SPB, DH SPA, DH OSP and DH CHICK, group 3 is DF 3 and DF 8 and group 4 consists of CP 845 alone. This has extended the work done by Faedo (1998) who also found four groups of isolates after sequencing the ITS1, 5.8S and ITS2 region of 15 of these 25 *D. flagrans* isolates. The additional 10 isolates sequenced in this work have added one extra isolate to group 3, four additional isolates to group 2 and five additional isolates to group 1. Group 4 still contains only CP 845.

The sequence variation of ITS regions has led to their use in phylogentic studies of many different organisms (Guarro *et al.*, 1999). The ITS regions are located between the 18S and 28S

rRNA genes and are separated by the 5.8 rRNA gene. They offer distict advantages over other molecular targets including increased sensitivity due to the exixtence of approximately 100 copies per genome (Henry *et al.*, 2000). Although the ITS regions are mainly used for identification to species level, it has ben used to determine the genetic diversity among clinical isolates of *Acremonium strictum*, which can cause mycetoma and other focal infections (Novicki *et al.*, 2003). ITS sequencing has also been used to differentiate between strains of *Pneumocystis carinii* that infect humans (Lu *et al.*, 1994; Lee *et al.*, 1998). *P. carinii* is a major cause of pneumonia in immunocompromised patients (Lu *et al.*, 1994). Variation was found between 15 strains of human *P. carinii* and four different sequence types were identified. They detetermined that because the majority of specimens examined contained only one type of *P. carinii* ITS sequence, that the nucleotide sequences of different copies of rRNA genes had homogenised and that each strain of *P. carinii* had a unique ITS sequence (Lu *et al.*, 1994). This nucleotide sequence homogenisation is known as concerted evolution (Hillis *et al.*, 1991) and has been been observed in almost all organisms whose rRNA gene sequences have been examined. It is quite likely that different types of ITS sequences represent different strains of *P. carinii* (Lu *et al.*, 1994).

The fact that CP 845 has a unique ITS1, ITS2 sequence could be very useful if this isolate was selected to be released as a biological control agent. As almost all organisms whose rRNA gene sequences have been examined have undergone concerted evolution (Hillis *et al.*, 1991), it is quite likely that different types of ITS sequences of *D. flagrans* represent different strains. It would enable this particular isolate to be easily monitored for persistence in the environment and would allow it to be distinguished from naturally occurring *D. flagrans* isolates (if any were present) to see if there was any kind of displacement occurring. From studies in Chapters

2 and 3, CP 845 was shown to have high trapping efficiency and a high rate of growth and spore production. Obviously further sampling would need to be done to see if CP 845 is found elsewhere and specific primers could be designed to check for the presence of the CP 845 sequence in other isolates of *D. flagrans* to determine if they shared its ITS1, ITS2 sequence.

There is support for these groupings in the phylogenetic trees. Three different methods were used to construct the phylogenetic trees: maximum likelihood, neighbour-joining and parsimony, and although there is a difference in the support values of the groupings all of these methods supported the separation of these isolates into four clusters.

Despite the differences noted above, no detectable differences were found in either the β-tubulin or the protease genes that were sequenced. It would not be unusual however, to see little variation within protein coding genes because of their highly conserved nature (Hwang and Kim, 1999). Previous work with these genes in *D. flagrans* isolates from Europe, North America and Asia, concluded that there is low genetic variation in *D. flagrans* because of the low level of variation in these nucleotide sequences (Ahren *et al* 2004). In the same study, looking at the same genes, significantly higher variation was found in the closely related fungus *A. oligospora.* These authors did however find a high level of variation in AFLP data, but concluded that because the AFLP data did not correlate with the geographical origins, the dispersal of *D. flagrans* is recent and ongoing. The findings here indicate that there are four clear groups of *D. flagrans* and our data essentially supports their findings that there is no geographical correlation between the origin of the isolates and the groupings. However, both the isolates in Group 3 isolates are believed to have been collected from northern Queensland (Knox, *pers comm.*) so there is the possibility that there is a unique genetic cluster of isolates in

this particular geographic area. There are however, only two isolates in this group, so the collection and analysis of additional isolates would need to be made to confirm this. There may also be other factors involved. Most of the isolates in Group 2 were collected from soil samples and all but one of the isolates collected from Group 1 were from sheep faeces so there may well be a correlation between substrate type and the nucleotide variation. Further studies also need to be done to confirm this.

From the work in Chapters 2 and 3, there does not appear to be any correlation between the four ITS groups and a given isolate's trapping efficiency or its growth performance on a particular substrate or at different temperatures. Of the four isolates that were identified as having significantly higher chlamydospore production and growth rates, three were from ITS GP 2 and the fourth was from ITS GP 1.

The main finding of this chapter was the separation of isolates into four clusters as a result of the ITS1, ITS2 sequencing. In particular, the unique sequence of isolate CP 845 has implications for the development of a commercial biological control agent and the ease of monitoring it once released. The following chapter will explore further techniques to distinguish amongst the *D. flagrans* isolates.

4.5 References

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