Chapter 5

Molecular Variation of 25 Australian Isolates of *Duddingtonia flagrans* as Determined by Random Amplified Polymorphic DNA Analysis and Sequence Tagged Microsatellite Profiling

5.1 Introduction

In assessing diversity of an organism, molecular markers are invaluable as they indicate the underlying genetic differences between individuals, populations, species or higher taxa (Bensch *et al.*, 1999). Non-molecular markers can have severe limitations. For instance, a trait in one group may not be present in other groups, making comparison between groups impossible. They may not reflect actual genetic variation due to the same genotype producing different phenotypes in different environments, or the same character state may be the result of similarity due to convergence rather than shared ancestry (Constantine, 2002). Earlier in this thesis, biological differences in growth, chlamydospore production and trapping efficiency of *D. flagrans* were examined under a range of environmental conditions and significant diversity was found between isolates within the Australian population. Studies of the ITS region have revealed significant differences between these Australian isolates whereas no differences were observed for a β-tubulin gene and an extracellular serine protease gene (see Chapter 4). Further differentiation between these isolates should be possible with recently developed methods for molecular characterization of fungi and other micro-organisms.

The technique of random amplified polymorphic DNA (RAPD) is a polymerase chain reaction (PCR) amplification of genomic DNA by a (usually) single short (5-15bp) random

oligonucleotide primer which produces complex patterns of anonymous polymorphic DNA fragments (Clark and Lanigan, 1994). Paired combinations of primers have also been used (Ruma *et al.*, 1996; Welsh and McClelland, 1991) with the resultant DNA fragments being separated on a gel and the bands scored as present/absent character states for each sample. There is no prior sequence information required but the technique detects a high level of variation and is relatively inexpensive, however, there can be problems with reproducibility (McDonald, 1997). RAPD is a low stringency technique, so any contaminating DNA or other substances will interfere with the banding pattern so genomic DNA must be purified. Also, different types of Taq polymerase and different thermocyclers may give varying results (McDonald, 1997). It is important to minimize these problems by developing reactions that reveal polymorphisms, consistently produce strong bands, produce uniform bands between replicate PCRs and are insensitive to DNA template concentrations (Stewart and Excoffier, 1996). RAPDs have been used to distinguish between 29 isolates of the nematophagous fungi *Paecilomyces lilacinus* (Tigano-Milani *et al.*, 1995), and between isolates of Race 2 of *Fusarium oxysporum* f. sp. *pisi*. They found that by examining patterns of several primers, fingerprinting of individual isolates was possible (Grajal-Martin *et al.*, 1993).

The use of RAPDs is becoming increasingly popular in population genetics (van de Zande and Bijlsma, 1995) and has been used in many phylogentic studies (Doherty *et al.*, 2003; Bailey *et al.*, 2004; Lourenco *et al.*, 2007; Jacobson and Hedrén, 2007), however one must be careful to avoid the problems of potentially co-migrating, non-homologous fragmants that have been identified by Adams and Reiseberg (1998) and Rieseberg (1996), and the difficulty in distinguishing between homozygotes and heterozygotes (van de Zande and Bijlsma, 1995). D. flagrans is a haploid so distinguishing between homozygotes and heterozygotes is not an issue

in the current study and non- homologous, co-migrating fragmaents will not be a problem as it was determined by Thormann *et al.* (1994) that homology is only less between species and genera, yet at an intraspecific level, all co-migrating bands are homologous.

Microsatellites, or simple sequence repeats (SSRs), are widely used for DNA fingerprinting. They are based on short (1-6 bp) tandem repeats of DNA sequences. These markers are highly variable due to differences in the number of repeat units. A major limitation of microsatellites is the time and cost required to isolate and characterise each locus when pre-existing DNA sequence is not available. Typically the process requires the construction and screening of a genomic library of size selected DNA fragments with SSR-specific probes. Once identified, the loci are amplified using specifically designed PCR primers and any variation can be identified by separating the products using either gel electrophoresis or capillary electrophoresis (Dupont, 2002). More recently, sequence tagged microsatellite profiling (STMP) has been developed which substantially reduces the cost of developing SSRs. This technique is based on anchored PCR, in which microsatellite amplification is achieved with one primer complementary to the flanking sequence and one that is specific to the repeat motif (Hayden *et al.*, 2004; Keiper *et al.*, 2006). Microsatellites have been used to distinguish between four races of *Fusarium oxysporum* f. sp. *ciceri*, an important fungal pathogen of chickpea (Assigbetse *et al.*, 1994). Sequence tagged microsatellite profiling has been used to develop SSRs for the barley scald fungus *Rhynchosporium secalis*, revealing a high level of polymorphism among the isolates (Keiper *et al.*, 2003).

The current study uses RAPD and STMP analysis to explore the molecular diversity between Australian *D. flagrans* isolates with the aim of generating unique fingerprints for each isolate.

5.2 Material and Methods

5.2.1 Fungal Isolates

The 25 fungal isolates were obtained from original cultures derived from a field survey conducted in 1991 (Larsen *et al.*, 1994) and subsequent additions to the CSIRO culture collection. Subcultures were taken from parent stock and maintained on Potato Dextrose Agar (PDA) using the procedure outlined in Chapter 2, section 2.2.1.

5.2.2 DNA Extraction

DNA was extracted from fungal mycelia by the procedure outlined in Chapter 4, section 4.2.2.

5.2.3 RAPD Analysis

25 isolates of *D. flagrans* were screened using a paired combination of the following primers: MYC1 (5'- GAGGAAGGTGGGGATGACGT -3'); 5SOR(5'- ATGGGAATACGACGTGCTGTAG -3'); and CN1 (5'- TACCCCCGCCCATATTCCAT -3') (Ruma *et al.*, 1996) (Faedo, 1998) the single primer 38a (5'- TGCGCCCTTC-3') (Sanyal and Mukhopadhyaya, 2002). Additionally, a RAPD kit containing twenty 10 mer primers was purchased from Operon Technologies ™ (Almeda, California, Kit OPB, Lot No: 073397). These are shown in Table 5.1.

Table 5.1 Primers used in RAPD analysis

Primer	5' to 3'
OPB-01	GTTTCGCTCC
OPB-02	TGATCCCTGG
OPB-03	CATCCCCCTG
OPB-04	GGACTGGAGT
OPB-05	TGCGCCCTTC
OPB-06	TGCTCTGCCC
OPB-07	GGTGACGCAG
OPB-08	GTCCACACGG
OPB-09	TGGGGGACTC
OPB-10	CTGCTGGGAC
OPB-11	GTAGACCCGT
OPB-12	CCTTGACGCA
OPB-13	TTCCCCCGCT
OPB-14	TCCGCTCTGG
OPB-15	GGAGGGTGTT
OPB-16	TTTGCCCGGA
OPB-17	AGGGAACGAG
OPB-18	CCACAGCAGT
OPB-19	ACCCCCGAAG
OPB-20	GGACCCTTAC

Primers MYC1, CN1, and 5SOR in paired combination, primer 38a and the primers listed in Table 5.1 were initially screened against two randomly selected *D. flagrans* isolates (CP 1382 and CP 911) to see if informative band patterns were present. Additionally, the primer pair combinations listed in Table 5.2 were similarly screened.

Table 5.2 Primer combinations screened against *D. flagrans*

Primer Combination		Primer Combination		Primer Combination		Primer Combination		Primer Combination	
Primer 1	Primer 2	Primer 1	Primer 2	Primer 1	Primer 2	Primer 1	Primer 2	Primer 1	Primer 2
OPB-01	OPB-02	OPB-02	OPB-08	OPB-03	OPB-07	OPB-12	OPB-03	OPB-18	OPB-08
OPB-01	OPB-03	OPB-04	OPB-02	OPB-05	OPB-02	OPB-12	OPB-06	OPB-18	OPB-09
OPB-01	OPB-04	OPB-04	OPB-03	OPB-05	OPB-03	OPB-12	OPB-07	OPB-18	OPB-10
OPB-01	OPB-05	OPB-04	OPB-05	OPB-05	OPB-06	OPB-12	OPB-08	OPB-18	OPB-11
OPB-01	OPB-06	OPB-04	OPB-06	OPB-05	OPB-07	OPB-12	OPB-09	OPB-18	OPB-13
OPB-01	OPB-07	OPB-04	OPB-07	OPB-05	OPB-08	OPB-12	OPB-10	OPB-18	OPB-14
OPB-01	OPB-08	OPB-04	OPB-08	OPB-05	OPB-09	OPB-12	OPB-11	OPB-18	OPB-15
OPB-01	OPB-09	OPB-04	OPB-09	OPB-05	OPB-10	OPB-12	OPB-13	OPB-18	OPB-16
OPB-01	OPB-10	OPB-04	OPB-10	OPB-05	OPB-11	OPB-12	OPB-14	OPB-18	OPB-17
OPB-01	OPB-11	OPB-04	OPB-11	OPB-05	OPB-12	OPB-12	OPB-15	OPB-18	OPB-19
OPB-01	OPB-12	OPB-04	OPB-12	OPB-05	OPB-13	OPB-12	OPB-16	OPB-18	OPB-20
OPB-01	OPB-13	OPB-04	OPB-13	OPB-05	OPB-14	OPB-12	OPB-17		
OPB-01	OPB-14	OPB-04	OPB-14	OPB-05	OPB-15	OPB-12	OPB-18		
OPB-01	OPB-15	OPB-04	OPB-15	OPB-05	OPB-16	OPB-12	OPB-19		
OPB-01	OPB-16	OPB-04	OPB-16	OPB-05	OPB-17	OPB-12	OPB-20		
OPB-01	OPB-17	OPB-04	OPB-17	OPB-05	OPB-18	OPB-18	OPB-02		
OPB-01	OPB-18	OPB-04	OPB-18	OPB-05	OPB-19	OPB-18	OPB-03		
OPB-01	OPB-19	OPB-04	OPB-19	OPB-05	OPB-20	OPB-18	OPB-06		
OPB-01	OPB-20	OPB-04	OPB-20	OPB-12	OPB-02	OPB-18	OPB-07		

Amplifications for primer 38a were performed in a 25 µl reaction with 200 µM dNTPs, I U TaqTi polymerase (Bioline), 1.5 mM $MgCl_2$, 1 x PCR reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$), 10 pmol primer, and 10-20 ng genomic DNA. The PCR cycling protocol was as follows: [94 °C –30 sec] + [94 °C –10 sec, 25 °C –1 min, 72 °C –1 min] x 40 + [72 °C –2 min]. Amplifications for primer combinations of MYC1, 5SOR and CN1 were performed in a final volume of 25 µl as described above, although using 10 pmol of each primer and 3.5mM $MgCl_2$. The cycling program was as above.

From the above single primers and primer pair combinations, the following produced informative band patterns and were screened against all 25 *D. flagrans* isolates: OPB-04; OPB-01/ OPB-04; OPB-01/ OPB-07; OPB-05/ OPB-012; OPB-12/ OPB-18. Amplifications were performed as for primer MYC1, CN1 and 5SOR.

All amplifications described above were performed in one of three thermocyclers: MJ Research PTC-200 DNA engine thermal cycler PCR (Geneworks); Hybaid PCR express thermal cycler (Integrated Sciences); and Eppendorf Mastercycler gradient Autorisierter thermocycler (Crown Scientific). All thermocyclers produced similar results.

Amplified DNA products were loaded onto 2 % NuSieve® (Cambrex) gels and electrophorised in 1 x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.5). The gels were stained using ethidium bromide (0.5μg/ml) and photographed using a UV transilluminator.

5.2.3.1 Optimization of RAPD

The greatest variable in RAPD PCR comes from $MgCl_2$ concentrations. For this reason, six different $MgCl_2$ concentrations were tested. These were 1.5 mM, 2.5 mM, 3.5 mM, 4.5 mM, 5.5 mM and 6.5 mM.

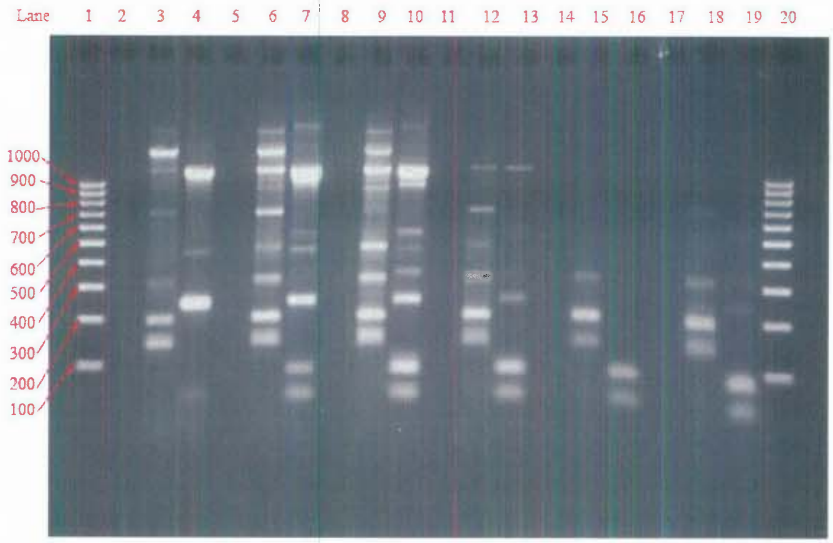

Figure 5.1 $MgCl_2$ trial.
Lane1: 100 bp ladder (BioRad®)
Lane2: -/control (No DNA). $MgCl_2$ 1.5 mM, Primer pair OPB-05/ OPB-10
Lane3: $MgCl_2$ 1.5 mM, Primer pair OPB-05/ OPB-10
Lane4: $MgCl_2$ 1.5 mM, Primer pair OPB-12/ OPB-18
Lane5: -/control (No DNA). $MgCl_2$ 2.5 mM, Primer pair OPB-05/ OPB-10
Lane6: $MgCl_2$ 2.5 mM, Primer pair OPB-05/ OPB-10
Lane7: $MgCl_2$ 2.5 mM, Primer pair OPB-12/ OPB-18
Lane8: -/control (No DNA). $MgCl_2$ 3.5 mM, Primer pair OPB-05/ OPB-10
Lane9: $MgCl_2$ 3.5 mM, Primer pair OPB-05/ OPB-10
Lane10: $MgCl_2$ 3.5 mM, Primer pair OPB-12/ OPB-10
Lane11: -/control (No DNA). $MgCl_2$ 4.5 mM, Primer pair OPB-05/ OPB-10
Lane12: $MgCl_2$ 4.5 mM, Primer pair OPB-05/ OPB-10
Lane13: $MgCl_2$ 4.5 mM, Primer pair OPB-12/ OPB-18.
Lane14: -/control (No DNA). $MgCl_2$ 5.5 mM, Primer pair OPB-05/ OPB-10.
Lane15: $MgCl_2$ 5.5 mM, Primer pair OPB-05/ OPB-10.
Lane16: $MgCl_2$ 5.5 mM, Primer pair OPB-12/ OPB-18.
Lane17: -/control (No DNA). $MgCl_2$ 6.5 mM, Primer pair OPB-05/ OPB-10.
Lane18: $MgCl_2$ 6.5 mM, Primer pair OPB-05/ OPB-10.
Lane19: $MgCl_2$ 6.5 mM, Primer pair OPB-12/ OPB-18.
Lane20: 100 bp ladder (BioRad®)

The clearest profile came from using an $MgCl_2$ concentration of 3.5 mM. A concentration of 2.5 mM also produced a clear banding pattern, however any less than 2.5 mM and any greater than 3.5 mM, the banding patterns produced fewer bands or the bands were less intense. A concentration of 3.5 mM was therefore chosen.

Three different sources of Taq polymerase were also tested. The hotstar taq (Qiagen Valencia, CA), TaqTi (Biotech), TaqF1 (Biotech), and GoTaq® (Promega, Madison, WI). Primer 38a produced reproducible, repeatable results using TaqTi, MYC1, CN1 and 5SOR using Taq F1 and the primers from Operon® produced the clearest results using GoTaq®. Three different thermocyclers were also used: MJ Research PTC-200 DNA engine thermal cycler PCR (Geneworks); Hybaid PCR express thermal cycler (Integrated Sciences); and Eppendorf Mastercycler gradient Autorisierter thermocycler (Crown Scientific). Results were identical and reproducible on all three thermocyclers.

Each RAPD PCR reaction was repeated at least twice, using different DNA preparations each time, to be sure of reproducibility.

5.2.3.2 Statistical Analysis

Gels were scored by distinguishing different bands and scoring each individual as having each band as present (1) or absent (0) across the 25 *D. flagrans* isolates. The data was bootstrapped with 1000 repetitions using Seqboot in the phylogeny inference program PHYLIP (Felsenstein, 2004). A distance matrix was then created from the bootstrapped data set using the Restdist program with a modified Nei/Li model in PHYLIP (Felsenstein, 2004). A dendogram was then generated using the neighbor-joining method in the program Neighbour in PHYLIP (Felsenstein, 2004). A data set that scored positive for all loci was created as an out group.

5.2.4 Sequence Tagged Microsatellite Analysis

5.2.4.1 SSR library construction

An attempt was made to develop microsatellite markers for four di-nucleotide compound repeat motifs using a modified sequence tagged microsatellite profiling procedure (Keiper *et al.*, 2006). Initially, a genomic DNA fraction was isolated by digesting 2 µg of DNA isolated from a single isolate (Isolate CP 1382). The genomic DNA was digested for 2 hours at 37°C with 40 U of the restriction endonuclease *Eco*R1 (New England Biolabs) in 200 µl of 1 x NEB buffer (New England Biolabs). 20 U of restriction enzyme was added initially, followed by another 20 U after 1 hr. The digested DNA was separated on a 1% agarose gel at 100 mV, and restriction fragments of less than 10 kB and greater than 200 bp in size were excised and purified using a QiaQuick Gel extraction kit (Qiagen).

Sequence tagged microsatellite libraries were constructed by digesting 200 ng of g DNA (3 ng/µl) for 2 hours at 37°C with 25 U of *Dpn*II (New England Biolabs) in 55 µl of 1x NE buffer *Dpn*II (100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM Bis Tris-HCl pH 6.0) (New England Biolabs). The reaction mixture was heated at 65°C for 20 min to inactivate the restriction endonuclease and a solution containing 1x NE buffer *Dpn*II, 3 mM dATP, 13.75 pmol of *Dpn*II adapter (Table 5.3) and 2.75 U T_4 ligase (New England Biolabs) was added to give a total volume of 82.5 µl. The mixture was then incubated for 3 hours at 37°C. PCR amplification of SSR sequences from 2µl of adapter-ligated DNA fragments was performed in a 20 µl reaction volume containing 0.2 mM dNTP, 1x PCR buffer (16mM $(NH_4)_2SO_2$, 0.01 % Tween 20, 100mM Tris-HCl pH 8.3), 1.5 mM $MgCl_2$, 5 pmol each of *Dpn*II adapter primer and a microsatellite anchoring primer (Table 5.3), and 1 U Taq Ti DNA polymerase (Bioline).

Amplification was performed for 32 cycles with the profile 60 s at 92°C, 60 s annealing (see below*), 60 s at 72°C. The annealing* temperature for the first cycle was 62°C, reducing by 1°C per cycle for the next seven cycles. PCR was completed with a final extension step of 10 min at 72°C. The reaction products were separated on a 2% agarose gel and fragments within the size range of 150-400 bp were excised and purified using a QiaQuick Gel purification column (Qiagen). Unfortunately the purification was inefficient. A loss of 90% of the product was determined through a trial on 150 bp products. The above procedure was then repeated, 'bulked up' by 7 x using only one di-nucleotide compound repeat motif (AC/AG) (Table 5.3). Fragments within the size range of 150-400 bp were again excised and purified using a QiaQuick Gel purification column (Qiagen). The purified SSR fragments were cloned into pGEM-T (Promega) and transformed into competent JM109 cells (Promega).

To minimise the number of DNA sequencing reactions required for SSR characterisation, plasmid clones were pre-screened by PCR using the D*pn*II adapter primer and the microsatellite anchoring primer (AC/AG) labeled at its 5' end with hexachloro-6-carboxylfluorescein (HEX). Single colonies were picked from LB-agar plates and were dipped into 10 µl of PCR mixture (0.2 mM dNTP, 1x PCR buffer, 1.5 mM $MgCl_2$, 5 pmol each of *Dpn*II adapter primer and a microsatellite anchoring primer) (Table 5.3), and 1 U Taq Ti DNA polymerase (Bioline) and amplified as outlined above. The colony PCR products were separated on an ABI PRISM® 377 DNA Sequencer to accurately determine the size of the cloned SSR fragment. Four plasmid clones contained uniquely sized SSR fragments, so were then prepared for sequencing using the CEQ® DTCS-Quick Start Kit and sequenced using the Beckman Coulter CEQ® 8000 Genetic analysis system (Figure 5.2). The size of the 4 clones were: 79 bp for clone 4; 62 bp for clone 9; 101 bp for clone 27; and 119 bp for clone 43.

Sequence tagged microsatellite (STM) primers specific to the DNA sequence flanking the repeat array were designed using the freeware primer design program Primer3 (Rozen and Skaletsky, 2000) (Table 5.3).

Table 5.3 Adapter and primer sequences used in the development of sequence-tagged microsatellites in *Duddingtonia flagrans*

Adapters	**Sequences (5' → 3')**
***Dpn* II adapter**	
sense-strand	GAGGATGAGTCCTGA
antisense-strand	GATCTCAGGACTCATC
Dpn II adapter primer	GGATGAGTCCTGAGAT
Microsatellite anchoring primers	
AC/AG	HEX-ACACACACACACACAGAGAGAG
AG/AT	HEX-AGAGAGAGAGAGAGATATATAT
AT/GC	HEX-ATATATATATATATGCGCGCGC
GC/AC	HEX-GCGCGCGCGCGCACACACAC
STM Primers	
4 tm-60	GATAAACCCGCCGAGGAC
9 tm-60	GTCAAGGAACCAGCACGAG
27 tm-60	AACCAAGCTGTCAGGAAACC
43 tm-60	TGGGCCGATAAATGACTTTC

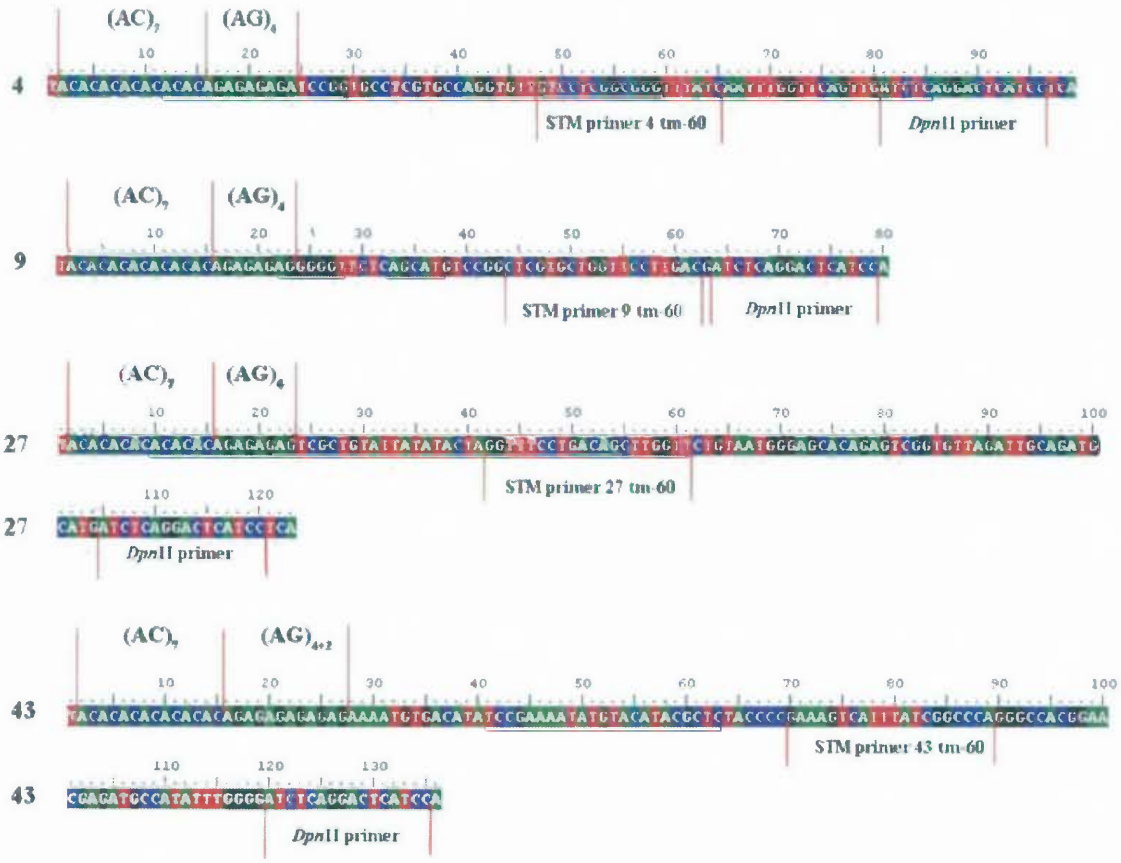

Figure 5.2 Sequence data of clones 4, 9, 27 and 43. Red lines show the AC/AG compound microsatellite and the *Dpn*II adapter primer for each sequence. The position of the STM primer designed using Primer3 (Rozen and Skaletsky, 2000) is also shown on each sequence. The primer sequence is shown in Table 5.3.

5.2.4.2 SSR Amplification

The amplification of sequence tagged microsatellites (STMs) from 10 ng of genomic DNA was performed using a 10 µl reaction mixture containing 5 µl Qiagen® Taq PCR master mix (Qiagen Inc. Scientific Products), 5 pmol each of STM and the microsatellite anchoring primer (AC/AG) labeled at its 5' end with hexachloro-6-carboxyylfluorescein (HEX) (Table 3). The Amplification process was as described for the SSR library construction, however was run for a total of 47 cycles. The PCR products were then separated on an ABI PRISM® 377 DNA Sequencer to detect polymorphisms.

5.2.4.3 Statistical Analysis

Microsatellite alleles were scored as present (1) or absent (0) across the 25 *D. flagrans* isolates. The data was bootstrapped with 1000 repetitions using Seqboot in the phylogeny inference program PHYLIP (Felsenstein, 2004). A distance matrix was then created from the bootstrapped data set using the Restdist program with a modified Nei/Li model in PHYLIP (Felsenstein, 2004). A dendogram was then generated using the neighbor-joining method in the program Neighbor in PHYLIP (Felsenstein, 2004). A data set was created that scored absent for all loci, and was used as an out group.

5.3 Results

5.3.1 RAPD Results

Of the 21 single primers and 90 primer pairs trialed, 9 in total gave informative band patterns that revealed differences between some isolates of *D. flagrans*. The other primers tested produced either too few bands for useful analysis or did not amplify any products.

The RAPD data was checked for monomorphic and polymorphic bands. A total of 79 individual band positions were generated, of which 33 were polymorphic. The polymorphic bands included those bands that were present in groups of individuals and those that were only present in one individual.

Table 5.5 summarises the RAPD results, showing the number of monomorphic and polymorphic loci for each primer/primer pair combination. The total number of loci amplified for each primer ranged from 6-13, and the number of polymorphic loci amplified ranged from 0-7. Depending on the primer or primer pair combination, 3-10 DNA fragments were amplified from a given template DNA. The discriminating power of the various primer/primer pair combinations varied greatly, with the number of genotypes defined ranging from 1-6 depending on the primer. Primer pair MYC1/CN1 had no discriminating power and primer pair OPB-12/OPB-18 could only define two genotypes, whereas primer 38a defined five genotypes and primer-pair OPB-01/OPB-04 was able to define six. The total number of genotypes defined from the composite RAPD data was 15 and these are highlighted in Table 5.5, where the polymorphic loci are shown for each primer/primer pair combination. Nine isolates: 92 4054, CP 1474, CP 1171, CP 1134, CP 1810, CP 1861, CP 1168, CP 1188 and CP 1801 make

up genotype 1. Isolates CP 911 and CP 912 make up genotype group 2 and isolates 92 870 and 92 3197 make up genotype 3. The remaining 12 genotypes all consist of one isolate each.

Figures 5.3-5.11 show the banding patterns of 25 *D. flagrans* isolates amplified with the 9 primer/primer pair combinations. The polymorphic bands are indicated by blue lines.

Figure 5.12 shows the Pattern of separation achieved from the RAPD data after 1000 bootstrap repetitions. There is strong support (92 %) clustering DH SPA, CP 912, CP 911, DH CHICK, DH OSP and DH SPB together. This cluster is further separated, grouping CP 912 and CP 911 together, and DH CHICK and DH OSP together. There is strong support (95 %) clustering DF 8, DF 3, DH DIXON, CP 845 and CP 1187 together. This is reflected in the genotyping results shown above even though the genotypes of these isolates are different. In the case of these five isolates they only differ from each other by 1-3 loci out of a total of 79 loci, so it would be expected that they would cluster together. The remaining isolates are grouped off the 95 % branch into three groups. The first group with 53 % support consists of two isolates, DH MCOMP and CP 1382. The second group with 59 % support consists of isolates DF a, CP 1861, CP 1168, CP 1188, CP 1171, CP 1134, CP 1474, 92 4054 and CP 1801. Isolates 92 870 and 92 3197 cluster together off this branch forming a third group with a support of 61%, again reflecting the genotypes shown in Table 5.5.

Table 5.4 Summary of RAPD analysis of *D. flagrans* displaying the number of loci amplified and the number of genotypes defined from each primer/primer pair combination analysed.

Primer/Primer pair combination	No. of genotypes defined	Range of fragments amplified	Total no. of loci amplified	No. of monomorphic loci amplified	No. of polymorphic loci amplified
38a	5	6-7	9	5	4
CN1/ 5SOR	3	9-10	11	9	2
MYC1/ 5SOR	3	9-10	13	6	7
MYC1/ CN1	1	9-9	9	9	0
OPB-04	4	3-5	7	2	5
OPB-01/ OPB-04	6	5-8	11	4	7
OPB-01/ OPB-07	3	5-6	7	4	3
OPB-05/ OPB-10	2	4-5	6	3	3
OPB-12/ OPB-18	4	4-6	6	4	2
Composite RAPD data	15	3-10	79	46	33

The number of monomorphic and polymorphic loci are also shown for each primer/primer pair combination.

Table 5.5 Polymorphic loci from RAPD data of 25 *D. flagrans isolates* for eight primer/primer pair combinations used.

Primer name	Genotype	OPB 05-10			MYC1/5SOR							OPB 01-04							OPB 01-07			OPB 04					38a				OPB 12-18		CN1/5SOR	
Isolate		1	2	3	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	1	2	3	4	5	1	2	3	4	1	2	1	2
DH SPA	4	1	0	0	1	0	0	1	1	1	0	0	0	0	0	0	1	1	0	1	0	1	0	1	1	0	1	0	1	0	1	1	0	1
DH SPB	5	1	0	0	0	1	0	1	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0	1	0	0	1	1	1	0	0	1
[illegible]	6	0	1	1	0	1	1	0	0	0	1	0	1	0	1	0	1	0	1	0	1	0	1	1	0	0	0	1	0	0	0	1	1	0
DHOSP	7	1	0	0	0	1	0	1	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	1	1	1	0	1
DH CHICK	8	1	0	0	0	1	0	1	1	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	1	1	1	1	0	1
[illegible]	9	0	1	1	0	1	1	0	0	0	1	0	1	0	1	0	1	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	1	0
DF 3	10	0	1	1	0	1	1	0	0	0	1	1	0	0	1	0	1	0	1	0	1	0	1	1	0	0	0	1	0	1	0	1	0	0
DF 8	11	0	1	1	0	1	1	0	0	0	1	1	0	0	1	0	1	0	1	0	1	0	1	1	0	0	0	1	0	1	0	0	0	1
Df a	12	0	1	1	0	1	1	0	0	0	1	1	0	0	1	0	1	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1
92 4054	1	0	1	1	0	1	1	0	0	0	1	1	0	0	1	0	1	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	0
92 870	3	0	1	1	0	1	1	0	0	0	1	1	0	1	1	0	1	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	0
92 3197	3	0	1	1	0	1	1	0	0	0	1	1	0	1	1	0	1	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	0
[illegible]	2	1	0	0	1	0	0	1	1	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	1	0	0	0	1	1	1	0	0	0
[illegible]	2	1	0	0	1	0	0	1	1	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	1	0	0	0	1	1	1	0	0	0
CP 1474	1	0	1	1	0	1	1	0	0	0	1	1	0	0	1	0	1	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	0
CP 1171	1	0	1	1	0	1	1	0	0	0	1	1	0	0	1	0	1	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	0
CP 1134	1	0	1	1	0	1	1	0	0	0	1	1	0	0	1	0	1	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	0
CP 1810	1	0	1	1	0	1	1	0	0	0	1	1	0	0	1	0	1	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	0
CP 1861	1	0	1	1	0	1	1	0	0	0	1	1	0	0	1	0	1	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	0
CP 1168	1	0	1	1	0	1	1	0	0	0	1	1	0	0	1	0	1	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	0
CP 1188	1	0	1	1	0	1	1	0	0	0	1	1	0	0	1	0	1	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	0
CP 1801	1	0	1	1	0	1	1	0	0	0	1	1	0	0	1	0	1	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	0
CP 1382	13	0	1	1	0	1	1	0	0	0	1	0	1	0	1	0	1	0	0	0	1	0	0	1	0	1	0	1	0	1	0	0	0	0
CP 845	14	0	1	1	0	1	1	0	0	0	1	1	0	0	1	0	1	0	1	0	1	0	1	1	0	0	0	1	0	1	0	1	0	0
CP 1187	15	0	1	1	0	1	1	0	0	0	1	1	0	1	1	0	1	0	0	0	1	0	0	1	0	1	0	1	0	1	0	0	0	0

Polymorphic loci are scored as 1 for band presence or 0 for band absence. Fifteen genotypes are shown, each one highlighted by a different colour.

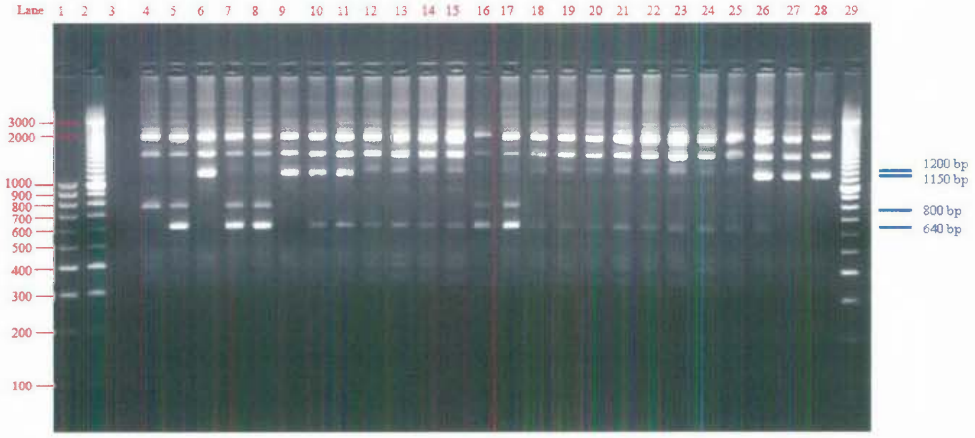

Figure 5.3 RAPD patterns of 25 isolates of *D. flagrans* obtained with primer 38a. Blue lines indicate the polymorphic bands differentiating the isolates.

Lane1: 100 bp ladder (BioRad®)	Lane9: DH MCOMP	Lane16: CP 912	Lane23: CP 1168
Lane2: 1000 bp ladder (	Lane10: DF 3	Lane17: CP 911	Lane24: CP 1188
Lane3: -/control (No DNA)	Lane11: DF 8	Lane18: CP 1474	Lane25: CP 1801
Lane4: DH SPA	Lane12: DFa	Lane19: CP 1171	Lane26: CP 1382
Lane5: DH SPB	Lane13: 92 4054	Lane20: CP 1134	Lane27: CP 845
Lane6: DH DIXON	Lane14: 92 870	Lane21: CP 1810	Lane28: CP 1187
Lane7: DH OSP	Lane15: 92 3197	Lane22: CP 1861	Lane29: 1000 bp ladder (BioRad®)
Lane8: DH CHICK			

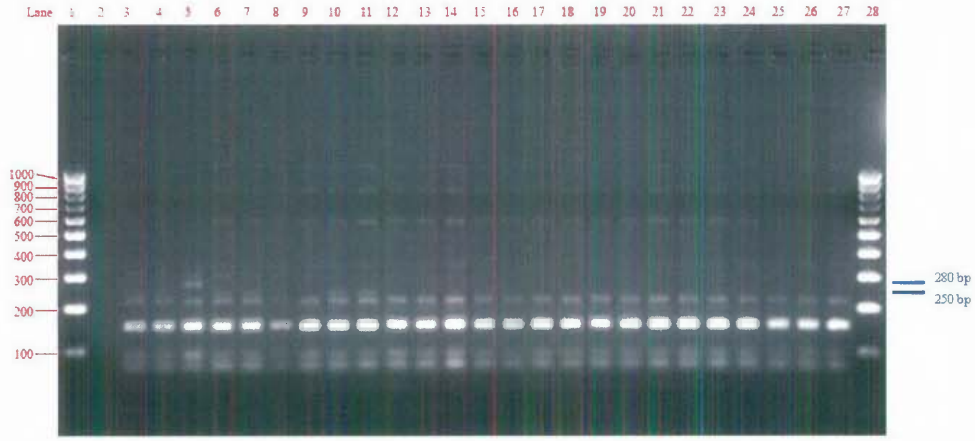

Figure 5.4 RAPD patterns of 25 isolates of *D. flagrans* obtained with primer CN1/5SOR. Blue lines indicate the polymorphic bands differentiating the isolates.

Lane1: 100 bp ladder (BioRad®)	Lane8: DH MCOMP	Lane15: CP 912	Lane22: CP 1168
Lane2: -/control (No DNA)	Lane9: DF 3	Lane16: CP 911	Lane23: CP 1188
Lane3: DH SPA	Lane10: DF 8	Lane17: CP 1474	Lane24: CP 1801
Lane4: DH SPB	Lane11: DFa	Lane18: CP 1171	Lane25: CP 1382
Lane5: DH DIXON	Lane12: 92 4054	Lane19: CP 1134	Lane26: CP 845
Lane6: DH OSP	Lane13: 92 870	Lane20: CP 1810	Lane27: CP 1187
Lane7: DH CHICK	Lane14: 92 3197	Lane21: CP 1861	Lane28: 100 bp ladder (BioRad®)

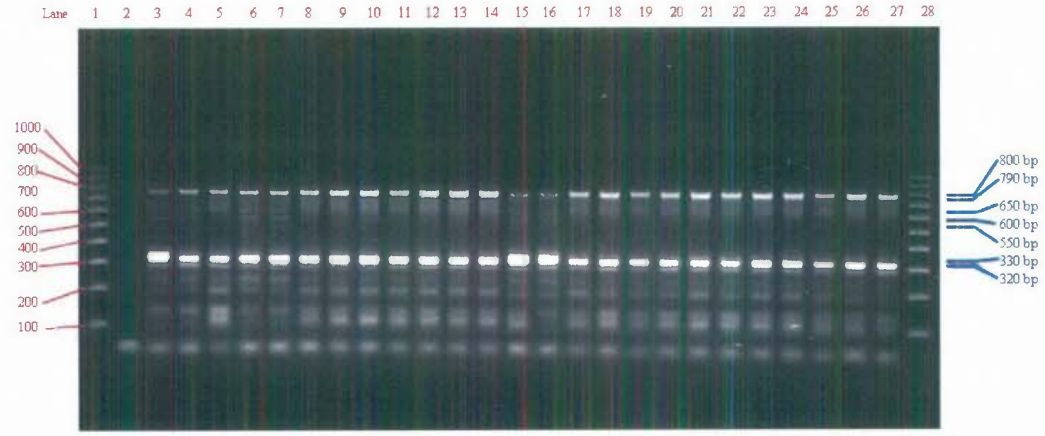

Figure 5.5 RAPD patterns of 25 isolates of *D. flagrans* obtained with primer MYC1/5SOR. Blue lines indicate the polymorphic bands differentiating the isolates.

Lane1: 100 bp ladder (BioRad®)	Lane8: DH MCOMP	Lane15: CP 912	Lane22: CP 1168
Lane2: -/control (No DNA)	Lane9: DF 3	Lane16: CP 911	Lane23: CP 1188
Lane3: DH SPA	Lane10: DF 8	Lane17: CP 1474	Lane24: CP 1801
Lane4: DH SPB	Lane11: DFa	Lane18: CP 1171	Lane25: CP 1382
Lane5: DH DIXON	Lane12: 92 4054	Lane19: CP 1134	Lane26: CP 845
Lane6: DH OSP	Lane13: 92 870	Lane20: CP 1810	Lane27: CP 1187
Lane7: DH CHICK	Lane14: 92 3197	Lane21: CP 1861	Lane28: 100 bp ladder (BioRad®)

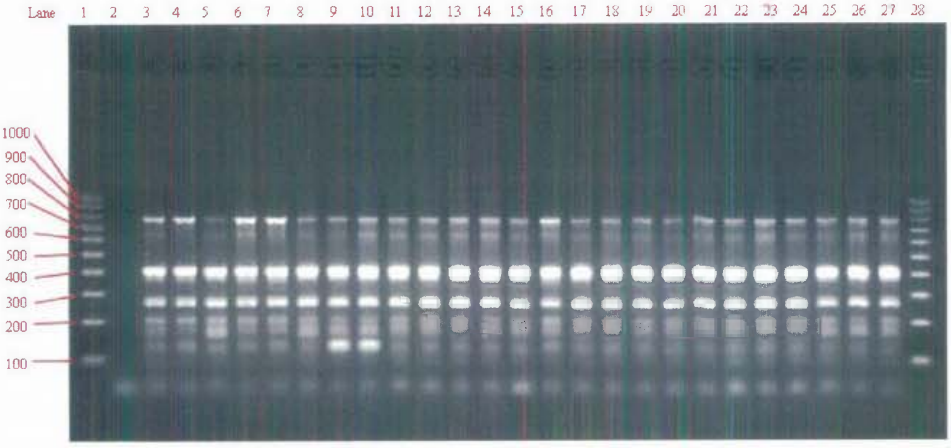

Figure 5.6 RAPD patterns of 25 isolates of *D. flagrans* obtained using primer pair MYC1/CN1.

Lane1: 100 bp ladder (BioRad®)	Lane8: DH MCOMP	Lane15: CP 912	Lane22: CP 1168
Lane2: -/control (No DNA)	Lane9: DF 3	Lane16: CP 911	Lane23: CP 1188
Lane3: DH SPA	Lane10: DF 8	Lane17: CP 1474	Lane24: CP 1801
Lane4: DH SPB	Lane11: DFa	Lane18: CP 1171	Lane25: CP 1382
Lane5: DH DIXON	Lane12: 92 4054	Lane19: CP 1134	Lane26: CP 845
Lane6: DH OSP	Lane13: 92 870	Lane20: CP 1810	Lane27: CP 1187
Lane7: DH CHICK	Lane14: 92 3197	Lane21: CP 1861	Lane28: 100 bp ladder (BioRad®)

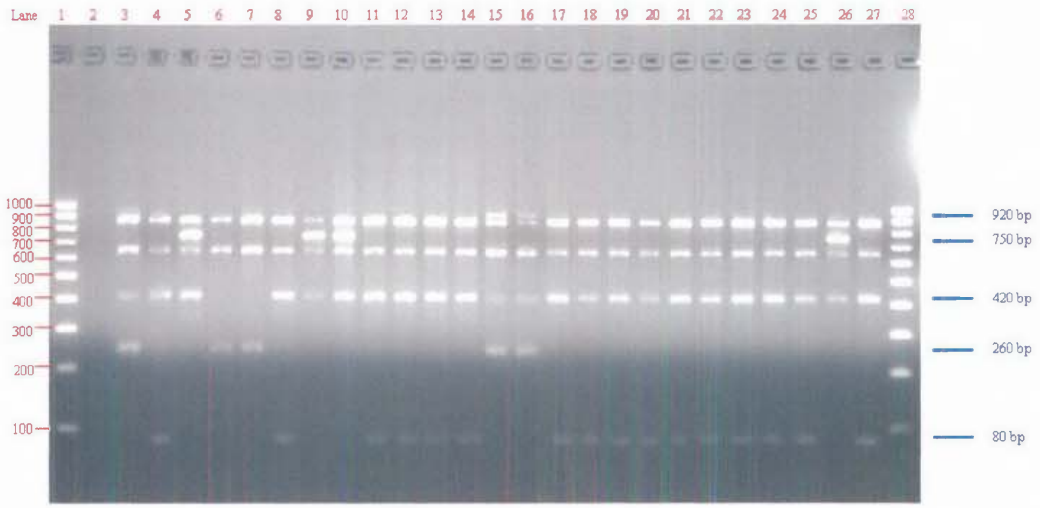

Figure 5.7 RAPD patterns of 25 isolates of *D. flagrans* obtained with primer OPB-04. Blue lines indicate the polymorphic bands differentiating the isolates.

Lane1: 100 bp ladder (BioRad®)	Lane8: DH MCOMP	Lane15: CP 912	Lane22: CP 1168
Lane2: -/control (No DNA)	Lane9: DF 3	Lane16: CP 911	Lane23: CP 1188
Lane3: DH SPA	Lane10: DF 8	Lane17: CP 1474	Lane24: CP 1801
Lane4: DH SPB	Lane11: DFa	Lane18: CP 1171	Lane25: CP 1382
Lane5: DH DIXON	Lane12: 92 4054	Lane19: CP 1134	Lane26: CP 845
Lane6: DH OSP	Lane13: 92 870	Lane20: CP 1810	Lane27: CP 1187
Lane7: DH CHICK	Lane14: 92 3197	Lane21: CP 1861	Lane28: 100 bp ladder (BioRad®)

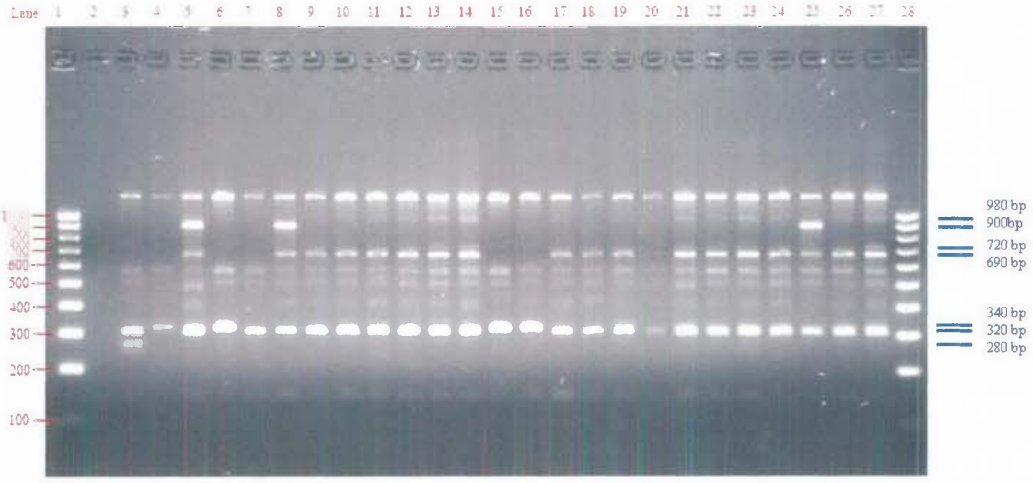

Figure 5.8 RAPD patterns of 25 isolates of *D. flagrans* obtained with primer OPB-01/OPB-04. Blue lines indicate the polymorphic bands differentiating the isolates.

Lane1: 100 bp ladder (BioRad®)	Lane8: DH MCOMP	Lane15: CP 912	Lane22: CP 1168
Lane2: -/control (No DNA)	Lane9: DF 3	Lane16: CP 911	Lane23: CP 1188
Lane3: DH SPA	Lane10: DF 8	Lane17: CP 1474	Lane24: CP 1801
Lane4: DH SPB	Lane11: DFa	Lane18: CP 1171	Lane25: CP 1382
Lane5: DH DIXON	Lane12: 92 4054	Lane19: CP 1134	Lane26: CP 845
Lane6: DH OSP	Lane13: 92 870	Lane20: CP 1810	Lane27: CP 1187
Lane7: DH CHICK	Lane14: 92 3197	Lane21: CP 1861	Lane28: 100 bp ladder (BioRad®)

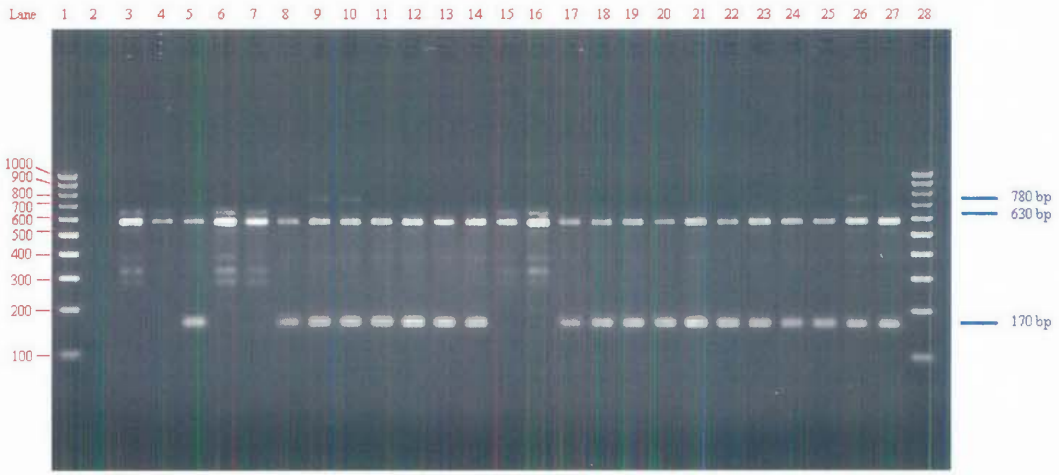

Figure 5.9 RAPD patterns of 25 isolates of *D. flagrans* obtained with primer OPB-01/OPB-07. Blue lines indicate the polymorphic bands differentiating the isolates.

Lane1: 100 bp ladder (BioRad®)	Lane8: DH MCOMP	Lane15: CP 912	Lane22: CP 1168
Lane2: -/control (No DNA)	Lane9: DF 3	Lane16: CP 911	Lane23: CP 1188
Lane3: DH SPA	Lane10: DF 8	Lane17: CP 1474	Lane24: CP 1801
Lane4: DH SPB	Lane11: DFa	Lane18: CP 1171	Lane25: CP 1382
Lane5: DH DIXON	Lane12: 92 4054	Lane19: CP 1134	Lane26: CP 845
Lane6: DH OSP	Lane13: 92 870	Lane20: CP 1810	Lane27: CP 1187
Lane7: DH CHICK	Lane14: 92 3197	Lane21: CP 1861	Lane28: 100 bp ladder (BioRad®)

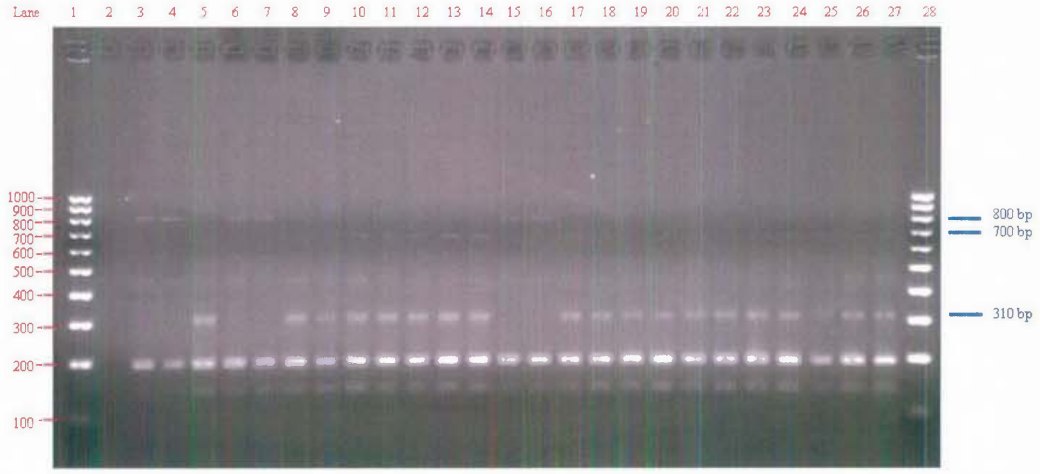

Figure 5.10 RAPD patterns of 25 isolates of *D. flagrans* obtained with primer OPB-05/OPB-10. Blue lines indicate the polymorphic bands differentiating the isolates.

Lane1: 100 bp ladder (BioRad®)	Lane8: DH MCOMP	Lane15: CP 912	Lane22: CP 1168
Lane2: -/control (No DNA)	Lane9: DF 3	Lane16: CP 911	Lane23: CP 1188
Lane3: DH SPA	Lane10: DF 8	Lane17: CP 1474	Lane24: CP 1801
Lane4: DH SPB	Lane11: DFa	Lane18: CP 1171	Lane25: CP 1382
Lane5: DH DIXON	Lane12: 92 4054	Lane19: CP 1134	Lane26: CP 845
Lane6: DH OSP	Lane13: 92 870	Lane20: CP 1810	Lane27: CP 1187
Lane7: DH CHICK	Lane14: 92 3197	Lane21: CP 1861	Lane28: 100 bp ladder (BioRad®)

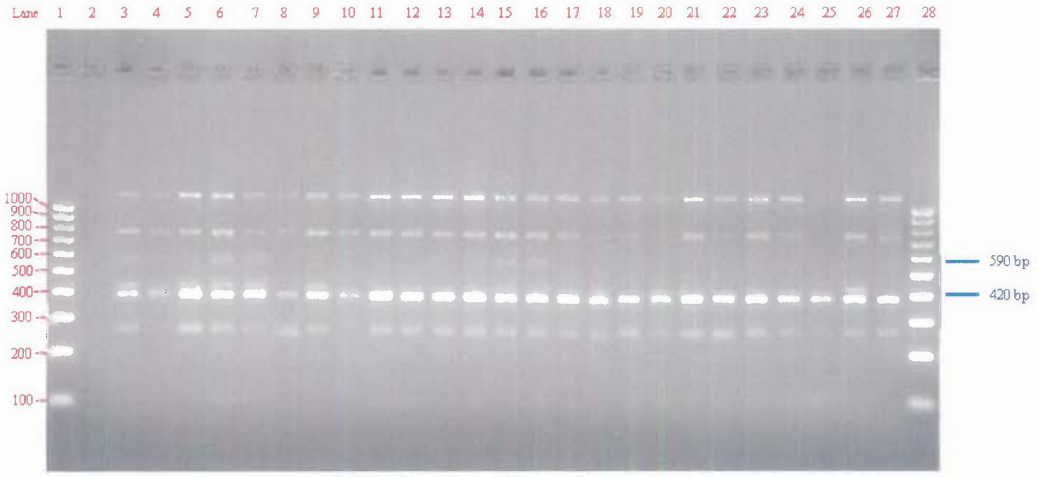

Figure 5.11 RAPD patterns of 25 isolates of *D. flagrans* obtained with primer OPB-12/OPB-18. Blue lines indicate the polymorphic bands differentiating the isolates.

Lane1: 100 bp ladder (BioRad®)	Lane8: DH MCOMP	Lane15: CP 912	Lane22: CP 1168
Lane2: -/control (No DNA)	Lane9: DF 3	Lane16: CP 911	Lane23: CP 1188
Lane3: DH SPA	Lane10: DF 8	Lane17: CP 1474	Lane24: CP 1801
Lane4: DH SPB	Lane11: DFa	Lane18: CP 1171	Lane25: CP 1382
Lane5: DH DIXON	Lane12: 92 4054	Lane19: CP 1134	Lane26: CP 845
Lane6: DH OSP	Lane13: 92 870	Lane20: CP 1810	Lane27: CP 1187
Lane7: DH CHICK	Lane14: 92 3197	Lane21: CP 1861	Lane28: 100 bp ladder (BioRad®)

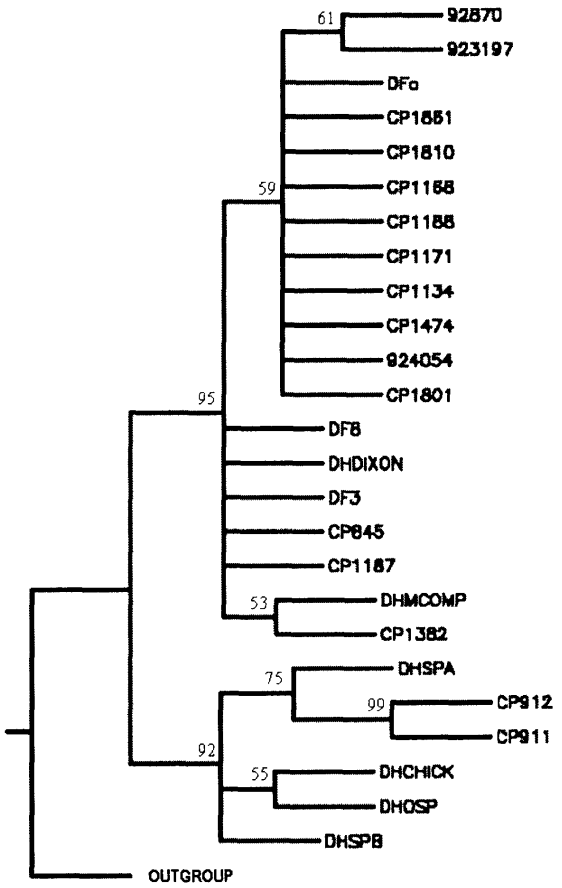

Figure 5.12 Neighbour-joining dendogram (Neighbour, PHYLIP) based on RAPD data using modified Nei/Li distance model (Restdist, PHYLIP) showing patterns of separation among 25 Australian isolates of *D. flagrans*. Values given on dendogram branches indicate the support percentage above 50 the clusters achieve after 1000 bootstrap iterations (Seqboot, PHYLIP). The outgroup was created to score positive for all loci (Felsenstein, 2004).

Table 5.6 shows the genetic distance between 25 isolates of *D. flagrans* and an outgroup, calculated using the Phylip program restdist (Felsenstein, 2004). The data reflects what has been demonstrated by Figure 5.12. All isolates of *D. flagrans* are most distantly related to the outgroup (0.161- 0.174) and as expected closely related to each other (0.000- 0.012).

Table 5.6 Genetic distance between 25 Australian Isolates of *D. flagrans* and an Outgroup based on RAPD data.

	CP_1187	CP_845	CP_1382	CP_1801	CP_1188	CP_1168	CP_1861	CP_1810	CP_1134	CP_1171	CP_1474	CP_911	CP_912	92_3197	92_870	92_4054	Dfa	DF_8	DF_3	DH_MCOMP	DH_CHICK	DH_OSP	DH_DIXON	DH_SPB	DH_SPA	Outgroup
Outgroup	0.168	0.169	0.172	0.172	0.172	0.172	0.168	0.169	0.168	0.172	0.172	0.172	0.172	0.172	0.172	0.172	0.168	0.173	0.173	0.171	0.170	0.170	0.174	0.161	0.171	
DH_SPA	0.012	0.012	0.011	0.011	0.011	0.011	0.012	0.011	0.012	0.011	0.011	0.002	0.002	0.011	0.011	0.011	0.012	0.012	0.012	0.011	0.004	0.005	0.012	0.009		
DH_SPB	0.008	0.009	0.009	0.009	0.009	0.009	0.008	0.008	0.008	0.009	0.009	0.007	0.007	0.009	0.009	0.009	0.008	0.010	0.010	0.010	0.006	0.004	0.011			
DH_DIXON	0.005	0.004	0.003	0.004	0.005	0.005	0.006	0.006	0.006	0.005	0.005	0.014	0.014	0.005	0.005	0.005	0.006	0.002	0.002	0.002	0.009	0.010				
DH_OSP	0.010	0.010	0.008	0.008	0.008	0.008	0.010	0.008	0.010	0.008	0.008	0.003	0.003	0.008	0.008	0.008	0.010	0.009	0.009	0.009	0.001					
DH_CHICK	0.009	0.009	0.007	0.007	0.007	0.007	0.009	0.007	0.009	0.007	0.007	0.004	0.004	0.007	0.007	0.007	0.009	0.008	0.008	0.008						
DH_MCOMP	0.004	0.004	0.000	0.003	0.002	0.002	0.005	0.003	0.005	0.002	0.002	0.012	0.012	0.002	0.002	0.002	0.005	0.003	0.003							
DF_3	0.002	0.002	0.002	0.001	0.002	0.002	0.004	0.004	0.004	0.002	0.002	0.011	0.011	0.002	0.002	0.002	0.004	0.004								
DF_8	0.002	0.002	0.002	0.001	0.002	0.002	0.004	0.004	0.004	0.002	0.002	0.011	0.011	0.002	0.002	0.002	0.004									
Dfa	0.001	0.002	0.004	0.002	0.002	0.002	0.000	0.004	0.000	0.002	0.002	0.011	0.011	0.002	0.002	0.002										
92_4054	0.003	0.004	0.002	0.001	0.000	0.000	0.002	0.001	0.002	0.000	0.000	0.010	0.010	0.000	0.000											
92_870	0.003	0.004	0.002	0.001	0.000	0.000	0.002	0.001	0.002	0.000	0.000	0.010	0.010	0.000												
92_3197	0.003	0.004	0.002	0.001	0.000	0.000	0.002	0.001	0.002	0.000	0.000	0.010	0.010													
CP_912	0.011	0.011	0.012	0.010	0.010	0.010	0.011	0.011	0.011	0.010	0.010	0.000														
CP_911	0.011	0.011	0.012	0.010	0.010	0.010	0.011	0.011	0.011	0.010	0.010															
CP_1474	0.003	0.004	0.002	0.001	0.000	0.000	0.002	0.001	0.002	0.000																
CP_1171	0.003	0.004	0.002	0.001	0.000	0.000	0.002	0.001	0.002																	
CP_1134	0.001	0.002	0.004	0.002	0.002	0.002	0.000	0.004																		
CP_1810	0.005	0.005	0.002	0.002	0.001	0.001	0.004																			
CP_1861	0.001	0.002	0.004	0.002	0.001	0.002																				
CP_1168	0.003	0.004	0.002	0.001	0.000																					
CP_1188	0.003	0.004	0.002	0.001																						
CP_1801	0.003	0.004	0.003																							
CP_1382	0.003	0.004																								
CP_845	0.001																									
CP_1187																										

5.3.2 STMP Results

From the SSR library, four clones were sequenced and four primers were designed using Primer3 (Rozen and Skaletsky, 2000). One of these primers revealed polymorphism between the 25 Australian *D. flagrans* isolates.

Figure 5.13 shows two SSR loci. The first is amplified by primer 4 Tm-60 and microsatellite anchoring primer AC/AG, producing a 79 bp product. There is no polymorphism between the isolates at this locus. The second locus is amplified by primer 9 Tm-60 and microsatellite anchoring primer AC/AG, producing a 62 bp product. Again, no polymorphism was revealed. These are close to the predicted size of the clones presented in Figure 5.2 which were 64 bp for clone 4 and 61 bp for clone 9. Any discrepancy can be accounted for by an error in reading the sizing of the products from the gels.

Figure 5.14 shows two SSR loci. The first is amplified by primer 27 Tm-60, producing an 81 bp product. There is no polymorphism between the isolates at this locus. The second locus is amplified by primer 43 Tm-60. Two bp products were produced by this primer, one at 79 bp and the other at 82 bp. This separates the isolates into two groups. Those isolates with an 82 bp product are DH SPA, DH SPB, DH OSP, DH CHICK, CP 911 and CP 912. The remaining isolates produce a product of 80 bp at this locus. The size of these products were larger than expected from figure 5.2. For the products generated from 27 Tm-60, a 60 bp product was expected, and for the products generated from 43 Tm-60, an 88 bp product was expected. Because of the consistent polymorpisms produced, the products are unlikely to be primer artefacts and so the size variation can only be explained by an error when the products were

sized from the gels. As the error is consistent for each product produced by each primer, the actual sizing error will not affect the microsatellite analysis as each allele is scored as present or absent and not by size.

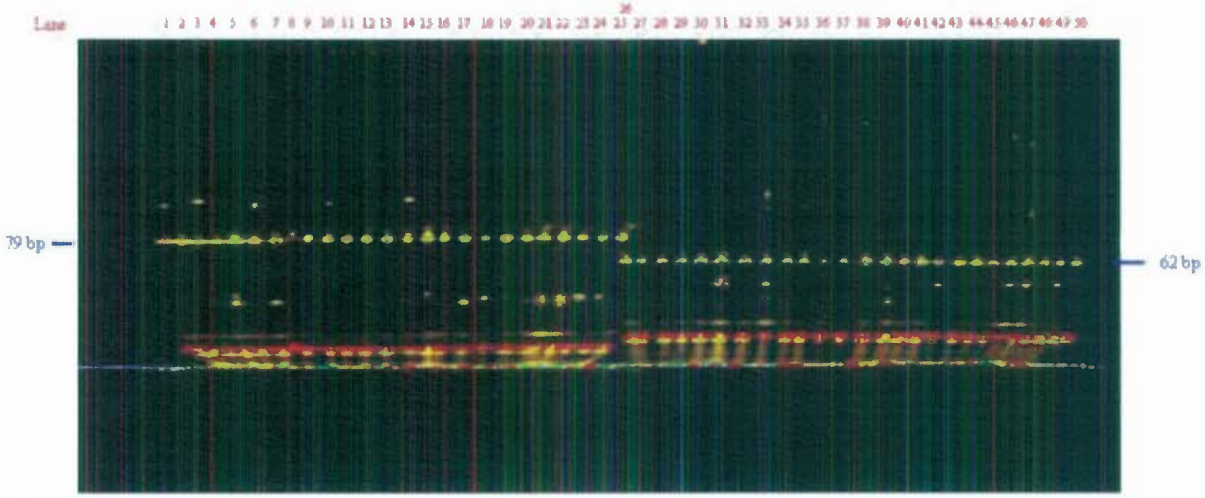

Figure 5.13 Two SSR loci with one allele showing no polymorphism between 25 Australian isolates of *D. flagrans*. Lanes 1-25 are derived from primer 4 Tm-60 and microsatellite anchoring primer AC/AG. Lanes 26-50 are derived from primer 9 Tm-60 and microsatellite anchoring primer AC/AG. Lanes 1-25 were run separately to lanes 26-50.

Lane 1	DH SPA	lane 26	DH SPA
Lane 2	DH SPB	lane 27	DH SPB
Lane 3	DH DIXON	lane 28	DH DIXON
Lane 4	DH OSP	lane 29	DH OSP
Lane 5	DH CHICK	lane 30	DH CHICK
Lane 6	DH MCOMP	lane 31	DH MCOMP
Lane 7	DF 3	lane 32	DF 3
Lane 8	DF 8	lane 33	DF 8
Lane 9	DFa	lane 34	DFa
Lane 10	92 4054	lane 35	92 4054
Lane 11	92 870	lane 36	92 870
Lane 12	92 3197	lane 37	92 3197
Lane 13	CP 912	lane 38	CP 912
Lane 14	CP 911	lane 39	CP 911
Lane 15	CP 1474	lane 40	CP 1474
Lane 16	CP 1171	lane 41	CP 1171
Lane 17	CP 1134	lane 42	CP 1134
Lane 18	CP 1810	lane 43	CP 1810
Lane 19	CP 1861	lane 44	CP 1861
Lane 20	CP 1168	lane 45	CP 1168
Lane 21	CP 1188	lane 46	CP 1188
Lane 22	CP 1801	lane 47	CP 1801
Lane 23	CP 1382	lane 48	CP 1382
Lane 24	CP 845	lane 49	CP 845
Lane 25	CP 1187	lane 50	CP 1187

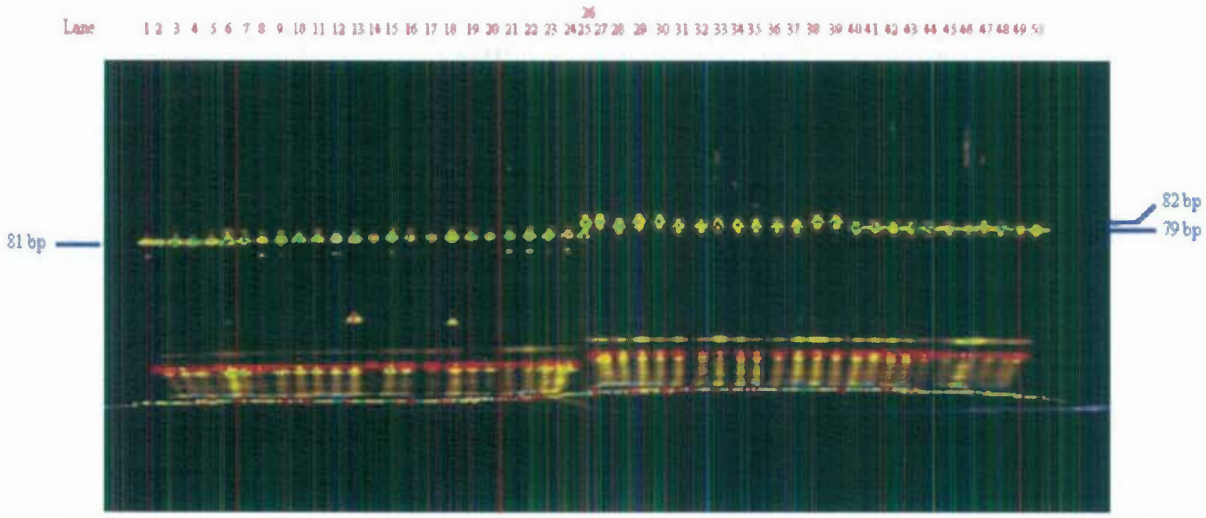

Figure 5.14 Two SSR loci with one allele showing polymorphism at one loci between 25 Australian isolates of *D. flagrans*. Lanes 1-25 are derived from primer 27 Tm-60 and microsatellite anchoring primer AC/AG. Lanes 26-50 are derived from primer 43 Tm-60 and microsatellite anchoring primer AC/AG. Lanes 1-25 were run separately to lanes 26-50.

Lane 1	DH SPA	lane 26	DH SPA
Lane 2	DH SPB	lane 27	DH SPB
Lane 3	DH DIXON	lane 28	DH DIXON
Lane 4	DH OSP	lane 29	DH OSP
Lane 5	DH CHICK	lane 30	DH CHICK
Lane 6	DH MCOMP	lane 31	DH MCOMP
Lane 7	DF 3	lane 32	DF 3
Lane 8	DF 8	lane 33	DF 8
Lane 9	DFa	lane 34	DFa
Lane 10	92 4054	lane 35	92 4054
Lane 11	92 870	lane 36	92 870
Lane 12	92 3197	lane 37	92 3197
Lane 13	CP 912	lane 38	CP 912
Lane 14	CP 911	lane 39	CP 911
Lane 15	CP 1474	lane 40	CP 1474
Lane 16	CP 1171	lane 41	CP 1171
Lane 17	CP 1134	lane 42	CP 1134
Lane 18	CP 1810	lane 43	CP 1810
Lane 19	CP 1861	lane 44	CP 1861
Lane 20	CP 1168	lane 45	CP 1168
Lane 21	CP 1188	lane 46	CP 1188
Lane 22	CP 1801	lane 47	CP 1801
Lane 23	CP 1382	lane 48	CP 1382
Lane 24	CP 845	lane 49	CP 845
Lane 25	CP 1187	lane 50	CP 1187

Table 5.6 shows the SSR alleles amplified by primers 4 TM- 60, 9 TM- 60, 27 TM- 60 and 43 TM- 60 for 25 Australian isolates of *D. flagrans.* The first three primers produce only one allele each and they are present in each of the isolates. Primer 43 TM-60 produces two alleles, clustering the isolates into two distinct groups. Isolates DH SPA, DH SPB, DH OSP, DH CHICK, CP 911 and CP 912 create one group, these are presented in yellow in the table. The remaining isolates make up the second group and they are presented in white.

Table 5.7 The presence or absence of SSR alleles from of 25 *D. flagrans isolates* amplified from four primers.

Isolate	Presence or absence of SSR alleles				
	4 Tm-60	9 Tm-60	27 Tm-60	43 Tm-60 allele 1	43 Tm-60 allele 2
DH SPA	1	1	1	1	0
DH SPB	1	1	1	1	0
DH DIXON	1	1	1	0	1
DH OSP	1	1	1	1	0
DH CHICK	1	1	1	1	0
DH MCOMP	1	1	1	0	1
DF 3	1	1	1	0	1
DF 8	1	1	1	0	1
DF a	1	1	1	0	1
92 4054	1	1	1	0	1
92 870	1	1	1	0	1
92 3197	1	1	1	0	1
CP 912	1	1	1	1	0
CP 911	1	1	1	1	0
CP 1474	1	1	1	0	1
CP 1171	1	1	1	0	1
CP 1134	1	1	1	0	1
CP 1810	1	1	1	0	1
CP 1861	1	1	1	0	1
CP 1168	1	1	1	0	1
CP 1188	1	1	1	0	1
CP 1801	1	1	1	0	1
CP 1382	1	1	1	0	1
CP 845	1	1	1	0	1
CP 1187	1	1	1	0	1

5.3.3 Combined RAPD, STM and Sequencing Results

Figure 5.15 shows the shows the pattern of separation achieved from the RAPD data, microsatellite data and ITS 1 and ITS 2 sequence data after 1000 bootstrap repetitions of each. 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. Page (1996) recommends displaying these particular trees as rectangular cladograms (as presented) as the branch lengths are actually 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). The tree is therefore a representation of the groupings and not drawn to scale. When the sequencing data, RAPD data and microsatellite is combined, three clusters are formed all within the one group. DF 3 and DF 8 make up one cluster with 67% support. DH OSP, DH SPB, DH CHICK, DH SPA, CP 911 and CP 912 make up a second cluster with 100 % support. The remaining isolates make up a third cluster.

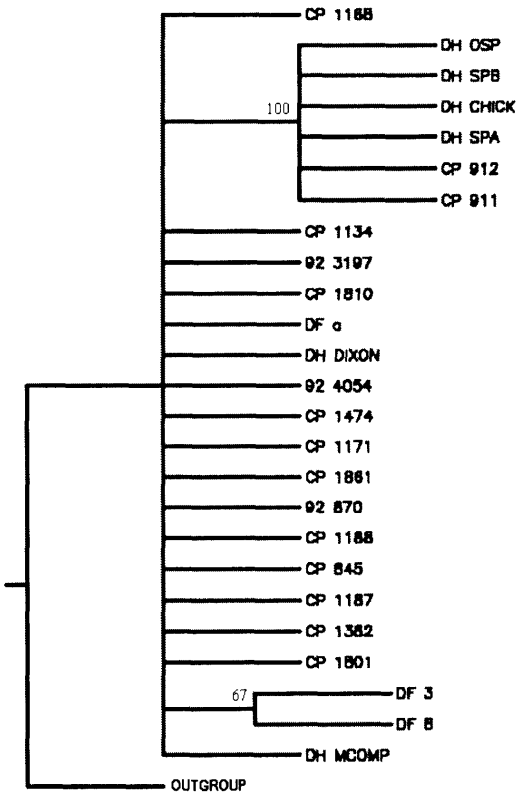

Figure 5.15 Neighbour-joining dendogram (Neighbour, PHYLIP) *flagrans* based on data from RAPD profiling, microsatellite analysis and ITS 1 and ITS 2 sequence data using modified Nei/Li distance model (Restdist, PHYLIP) showing patterns of separation among 25 Australian isolates of *D. flagrans*. Values given on dendogram branches indicate the support percentage above 50 that the clusters achieve after 1000 bootstrap repetitions (Seqboot, PHYLIP). The outgroup was created to score positive for all loci (Felsenstein, 2004).

5.4 Discussion

Random amplified polymorphic analysis and sequence tagged microsatellite profiling was used to determine differences between 25 Australian isolates of *D. flagrans*. Both techniques detected polymorphisms between the isolates, however they differed in the amount of variability detected. For both techniques, the polymorphism detected was the result of the presence or absence of a band. The RAPD data generated the most polymorphisms.

The method of identifying nematode trapping fungi at the species level using PCR-RFLP of the ITS region of the ribosomal RNA is described by (Persson *et al.*, 1996). They were able to distinguish between *D. flagrans, Hirsutella rhossiliensis, Nematoctonus leiosporus, Dactylaria gracilis, D. candida, Monacrosporium elegans, Arthrobotrys musiformis, A. robusta, A. conoides, A. oligospora* and *A. superba* and described it as a rapid and convenient method of distinguishing between species of nematophagous fungi. They were not however, able to distinguish between isolates of any one species. For biocontrol purposes a convenient method of identifying isolates is needed so a released isolate can be identified after re-isolation to evaluate population dynamics and survival in the environment.

The present study identified 15 genotypes among 25 Australian *D. flagrans* isolates. It has demonstrated that 12 of the 25 isolates can be individually identified with the use of a combination of eight primer/ primer pair combinations. The remaining 13 isolates fall into one of 3 groups (Table 5.5). There have been two previous studies using RAPDs to distinguish between isolates of *D. flagrans*. The first study looked at seven Australian isolates of *D. flagrans* (CP 911, CP 912, CP 1134, CP 1168, CP 1171, CP 1810 and 92 870), and used three

of the primer pair combinations used in this study. These were CN1/MYC1, CN1/5SOR and MYC1/5SOR (Faedo, 1998). No difference was observed between the isolates when primer pairs CN1/MYC1 and CN1/5SOR were used. The present results supported this observation as no difference was found between the 25 isolates in this study when primer pair CN1/MYC1 was used, and no difference between the seven isolates used in Faedo's study when primer pair CN1/5SOR was used. However three genotypes were identified using this primer among the total 25 isolates in the present study. Faedo (1998) identified two genotypes using primer pair MYC1/5SOR, CP 911 and CP 912 and the remaining 5 isolates. The present results again supported this finding, but also extended the information, with DH SPA joining CP 911 and CP 912 in one genotype, DH SPB, DH OSP and DH CHICK making a second genotype and the remaining 19 isolates making up a third. The second study compared two Indian isolates of *D. flagrans* (isolates S-2550 and S-2507) using primer 38a (Sanyal and Mukhopadhyaya, 2002). They amplified two different banding patterns and in the current study five different banding patterns were generated using this primer. Isolate S-2507 was identical to the banding patterns of 5 of the isolates in the current study (DH SPB, DH OSP, DH CHICK, CP 912 and CP 911), whereas isolate S-2550 had a unique profile.

The genetic diversity of a worldwide collection of 22 *D. flagrans* isolates was explored using amplified fragment length polymorphism (AFLP) analysis (Ahren *et al.*, 2004). There was significant variation found between the isolates, however there was no correlation between the genotypes identified and geographical origins of the isolates. From the RAPD study repeated here, there also does not appear to be any correlation between the groupings of the isolates in relation to their original location or to the substrate from which they were collected and their genetic variation (Chapter 4, Table 4.2).

There have been several studies which have successfully used RAPD analysis to distinguish between isolates of other fungal species. Tigano-Milani *et al.* (1995) found different RAPD fingerprints could be used to distinguish several isolates of *Paecilomyces lilacinus*. Their study involved 29 isolates of *P. lilacinus*. Grajal-Martin *et al.* (1993) used RAPD analysis to distinguish 28 isolates of *Fusarium oxysporum* f. sp. *pisi*. They found by examining patterns of several primers, fingerprinting of individual isolates was possible. Gunasekera *et al.* (2000) were able to differentiate among 15 isolates of *P. lilacinus* using RAPD-PCR, revealing sufficient diversity to allow tracking of strains released into the environment. They also tried to relate nematophagous activity to genetic clusters, however were unable to find a clear relationship. With further investigation into the development of RAPD profiles for *D. flagrans*, it is hopeful that further differentiation among *D. flagrans* isolates will be possible.

The phylogenetic tree based on the RAPD data (Figure 5.11) reflects the findings of the ITS sequencing from Chapter 4. The polymorphisms found through the RAPD analysis have identified approximately three clusters. Isolates DFa, CP 1861, CP 1810, CP 1168, CP 1188, CP 1171, CP 1134, CP 1474, 92 4054 and CP 1801 are consistently clustered together and make up the first group. 92 870 and 92 3197 are clustered together off this group. DH MCOMP and CP 1382 are also clustered together, but very closely to the first group. Isolates DF 8, DH DIXON, DF 3, CP 845 and CP 1187 make up the second cluster and isolates DH SPA, CP 911, CP 912, DH CHICK, DH OSP and DH SPB make up the third cluster. The only differences with the findings from Chapter 4 are that CP 845 is unique to all the other isolates based on the sequencing data, and DH DIXON and CP 1187 are found in cluster 1 rather than cluster 2 as shown in Figure 5.11.

This study was the first to look at microsatellite variation in *D. flagrans*. The STMP data revealed only one polymorphism, however analysis of more than four clones would undoubtedly produce more informative results. High levels of polymorphism have been revealed in studies with the 16 Australian isolates of the barley scald fungus, *Rhynchosporium secalis*. A total of 66 polymorphic SSRs exhibited a high level of polymorphism out of the 168 that were developed. Each isolate exhibited a unique genotype (Keiper *et al.*, 2006). Studies such as this one highlight the potential of using this technique to differentiate between fungal isolates, so further work needs to be done to generate more SSRs of *D. flagrans*, which have a high likelihood of adding to the information highlighting the diversity between *D. flagrans* isolates. From the microsatellite data generated, two distinct groups were formed, one consisting of six isolates, namely DH SPA, DH SPB, DH CHICK, DH OSP, CP 911 and CP912 and the other consisting of the remaining 19. This result supports the findings from the ITS sequencing data in Chapter 4 and RAPD data in this study, which both cluster these six isolates together.

This study has revealed a high level of diversity between *D. flagrans* isolates and a set RAPD primers has been developed that can be used to differentiate 12 out of 25 of the Australian *D. flagrans* isolates.

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