Chapter 6

General discussion

Due to the high levels of resistance to currently available anthelmintics, the need for alternative control methods for nematode parasites of livestock is ever increasing. *Duddingtonia flagrans* has in recent years been identified as a promising candidate for the biological control of gastrointestinal nematodes of livestock. The development of *D. flagrans* as a commercial biocontrol agent depends on identifying an isolate, or several isolates, that have a high efficacy against parasitic nematodes under a range of differing environmental conditions, are easily grown in mass culture, do not have a negative impact on non-target organisms, do not persist in the environment for extended periods after their release, and are easy to release. Whether or not one or several isolates should be used in the development of a commercial biocontrol agent depends on whether the effectiveness of particular *D. flagrans* isolates changes in different environmental conditions and whether there are genetic differences that could alter an endemic population of *D. flagrans*. If a range of isolates perform differently with variations in temperature, perhaps a selection of isolates would be better so the effectiveness is maximised over a wide temperature range. If the genetic differences are great, then perhaps an isolate already endemic to a particular area would be the best one to use, or an isolate that can be easily monitored after release, such as one in which a molecular signature can be identified.

In this thesis, 25 Australian isolates of *D. flagrans* were examined to determine and compare their trapping efficiency and their growth and chlamydospore production over a range of temperatures and on a range of substrates to identify an isolate, or isolates, which would be

most effective to use in a commercial production system. Genetic differences between the isolates were also investigated using DNA sequencing, RAPD and microsatellite techniques to further add to the information about the population biology and to develop molecular markers for monitoring the isolates if released as biological control agents.

A major question identified by Larsen *et al.* (1997) is how well a biological control candidate would perform under climatic conditions which differ from those prevailing where the fungus originated. There have been several studies which have looked at the preferred temperature of growth of biological control fungi (Bogus *et al.*, 2005; Fernandez *et al.*, 1999c; Grønvold *et al.*, 1996; Morgan *et al.*, 1997), although none of these identified whether there was a correlation between the optimum temperature for the growth of a particular isolate and the site where it originated. Chapter 2 addressed this question by assessing 25 isolates of *D. flagrans* to compare the differences in radial growth and chlamydospore production over five temperature regimes. There was no correlation between the optimum temperature for the growth and chlamydospore production of a particular isolate and the temperatures at the site from which they were originally collected. For example, DF 8 which was collected from North Queensland would be expected to grow well at high temperatures. This particular isolate however, had one of the lowest growth rates and lowest rates of spore production at the highest temperature tested when compared to the majority of other isolates.

Studies in Denmark (Grønvold *et al*, 1996) and India (Sanyal, 2000) have previously found *D. flagrans* to have the highest growth rates and highest chlamydospore production at temperatures ranging from 20-30 °C. This was supported by the present study where it was shown the optimum temperature for the growth of most the isolates was 27 °C. In addition,

four isolates, CP 911, DH OSP, DH SPB and CP 1187, were identified as having significantly higher chlamydospore production and growth rates than the other 21.

Laboratory tests are very useful for screening purposes in the selection of the best isolate to develop as a biological control product. Nematophagous fungi are subjected to the influence of different environmental factors and laboratory studies allow the testing of these different factors and their effects on fungal growth and ability to destroy the target organism. Temperature is considered one of the most important factors affecting fungal growth (Griffin, 1994), and many studies have been carried out which have looked at the effect of temperature in the laboratory when selecting isolates of various fungal species for further investigation (Larsen, 1991; Peloille, 1991; Grønvold *et al.*, 1996; Morgan *et al.*, 1997; and Fernandez *et al.*, 1999c). As mentioned previously, the ability to survive and grow well at a range of temperatures will be beneficial for any isolate deployed as a biological control agent as it will be required to be able to perform effectively in the varying temperature extremes seen throughout areas of Australia where livestock are produced. As laboratory studies are just a representation of environmental conditions that may be seen *in vivo,* it will now be important to conduct comparative studies of the four isolates with the best growth at varying temperatures (CP 911, DH OSP, DH SPB and CP 1187) in a field situation, preferably over a range of temperatures to see if similar results are observed. Fernandez *et al.* (1999d) found that a new isolate of *D. flagrans* (Troll A) which had a higher larval reducing capacity in the laboratory than a previously tested isolate (C13), also had a slightly higher, although not significantly so, nematode-destroying capacity than C13 when applied in a field study. The study of each isolate, however, was performed at different times of the year under different climate conditions which could affect the activity of the fungus against free-living larvae in the

faeces. The author stressed the need to study both isolates in the same environmental conditions to determine if the higher larval reducing capacity of Troll A in the laboratory could be translated in the field.

The fact that an isolate grows faster than others does not necessarily mean that it is a better predator. This lack of correlation between growth rate and trapping efficiency has been reported by Feder (1963), Fernandez *et al.* (1999c), and Heinz (1978) and has been demonstrated again in Chapter 3 of this thesis. All isolates were shown to reduce numbers of nematode larvae, which is in agreement with previous studies that show that *D. flagrans* has a high degree of trapping efficiency *in vitro* or when *D. flagrans* chlamydospores have been mixed with faeces against a broad range of nematode parasites from several livestock species (Fernandez *et al.*, 1999a; Fernandez *et al.*, 1999b; Grønvold *et al.*, 1999; Grønvold *et al.*, 1996; Henriksen S. A. *et al.*, 1997; Mendoza de Gives *et al.*, 1999; Sanyal, 2001). However, in the present study, those that had the highest growth rate and chlamydospore production were not significantly better predators than any of the other isolates except for isolates DF 3 and DF 8, which were the least effective predators. This is further supported by the fact that DF 8, one of the least effective predators at room temperature, had the highest rate of radial growth at 22 °C.

The predatory ability of nematophagous fungal isolates against free-living larvae of animal parasitic nematodes is also one of the most important factors to consider when selecting an isolate for use as a biological control agent. Laboratory studies using faecal cultures closely represent the *in vivo* environment where the fungal activity takes place, ie sheep faecal pellets or dung pats. These tests are fundamental in determining the number of spores needed to obtain a high effect on reduction of parasite free-living stages and to determine which isolates are

most effective in achieving that reduction. As mentioned above, several authors have demonstrated a large reduction in larval numbers when nematophagous fungi have been mixed with faeces containing nematode larvae (Sanyal, 2001; Henriksen S. A. *et al.*, 1997; Fernandez *et al.*, 1999a; Fernandez *et al.*, 1999b; and Grønvold *et al.*, 1999). The next stage in the development of a biological control agent is to test the efficacy of application in a field trial, as demonstrated by Fernandez *et al.* (1999d) and Knox and Faedo (2001), to determine if similar reductions are seen, and moreover, to determine if the selected isolates survive the rigours of gut passage through livestock.

Larsen *et al* (1991) developed *in vitro* procedures designed to mimic environmental stresses of the rumen and abomasal passage by fungi, which are important factors to consider in the development of a fungal biological control agent of livestock nematodes. These procedures were found to be a valuable screening procedure when the same fungi were then tested for gut passage *in vivo* (Waller *et al.*, 1994). Those fungi that survived *in vitro* stress selection also survived *in vivo*. Initially a large number of studies showed *Arthrobotrys* spp. to be very effective *in vitro* at trapping and destroying nematode larvae from several livestock species including sheep (Virat and Peloille, 1977), cattle (Pandey, 1973; Nansen *et al.*, 1986; Nansen *et al.*, 1988), horses and pigs (Nansen *et al.*, 1986; Nansen *et al.*, 1988). Further testing of *Arthrobotrys* spp. using the stress selection test developed by Larsen *et al* (1991) revealed six isolates that were viable, in addition to seven viable isolates of *D. flagrans*. Further assessment *in vivo* however, showed that *Arthrobotrys* spp. were not as able to survive passage through the gastrointestinal tract as *D. flagrans*, so *D. flagrans*, which also displayed excellent larval trapping ability, was determined to be the better candidate for development as a biological control agent (Larsen *et al.*, 1992). The use of animal derived isolates from the CSIRO culture

collection precludes the requirement for an *in vitro* stress selection step in the development of an Australian isolate for biological control purposes.

There have been other examples demonstrating the importance of laboratory screening studies in selecting biological control agents for potential commercial use. Johnsson *et al.* (1998) looked at the performance of the bacterial biocontrol agent *Pseudomonas chlororaphis*, strain MA 342, against a number of cereal seed-borne diseases in the field. The strain was selected from a number of bacterial strains because of a strong disease-controlling effect when applied to infected cereal grains in the laboratory. As a result it was tested in 105 field experiments under various climatic conditions, against several seed-borne diseases and during six growing seasons. The results supported the preliminary laboratory studies, finding that seed bacterisation with the strain MA 342 gives excellent control of several seed-borne cereal diseases in the field under normal cropping conditions. They further found that the effects were consistent not only over time, but under different climatic conditions. Pak *et al.* (1989) studied the effect of the inundative release in the field of four strains of the egg parasite *Trichogramma* (Hymenoptera, Trichogrammatidae) to control five lepidopterous species infesting brussel sprouts crops in the Netherlands. *Trichogramma* strains were selected on the basis of behavioural characteristics investigated in laboratory experiments such as parasitisation activity at low temperatures and host-species preference. There was found to be a translation of the observed behavioural characteristics and parasitism patterns seen in the laboratory with the results seen in the field, with high levels of parasitism seen from all the strains when host densities were high, however it was determined that a low host densities were a limiting factor for effective parasitism.

In general, laboratory screening studies are useful tools in the initial selection of any potential biological control agent. For nematophagous fungi, although it is unjustified to draw firm conclusions as to whether one fungus or another would be suited as a potential biological control agent against animal parasitic nematodes on laboratory tests alone, it has been shown that if faecal cultures are used in combination with tests which simulate potential environmetntal conditions and gastrointestinal tract passage, the laboratory assays are very good predictors of which fungal isolates would be likely to be efficient against reducing parasite larvae in the faecal environment and of surviving gut passage (Larsen, 1999).

The sequencing of the ITS1-5.8S-ITS2 region, the microsatellite analysis and the RAPD analysis have provided some of the most interesting results in this thesis. Chapter 4 extended a study by Faedo (1998) who sequenced the ITS region of 15 isolates of *D. flagrans*. Her findings were extended by the results presented. The sequencing of the ITS1-ITS2 region placed the isolates into 4 distinct groups. Group 1 consisted 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. Group 2 consists of the six isolates CP 911, CP 912, DH SPB, DH SPA, DH OSP and DH CHICK. Group 3 consists of the two isolates DF 3 and DF 8, and group 4 consists solely of isolate CP 845. There appears to be no correlation between these groups and trapping efficiency or growth rate on a particular substrate or at different temperatures. This is the first study which has looked at the possibility of the biological characteristics of *D. flagrans* being correlated with genetic diversity. There have been some attempts to relate a particular genetic group of *Paecilomyces lilacinus*, which is a successful antagonist of plant parasitic nematodes such as root-knot and cyst nematodes, to nematophagous ability (Gunasekera *et al.*, 2000; Tigano-Milani *et al.*, 1995a), however in both

these studies there was no clear relationship between genetic clusters and pathogenicity. There may however, be some correlation between the genetic clusters of *D. flagrans* and the site or substrate where the isolates were originally collected. The two isolates found in group 2, DF 3 and DF 8, are both thought to have come from the Townsville area (M.R Knox, pers. comm.). This suggests that maybe their genetic differences could be a result of geographic or climatic parameters. These two isolates have also consistently shown differences throughout the other aspects of this thesis. As discussed above, DF 3 and DF 8 were the least effective predators. DF 8 also has a significantly lower growth rate at higher temperatures than most of the other isolates. It also has a lower growth compared to the majority of isolates when grown on barley and millet.

All isolates collected from sheep faeces are found in group 1, except for CP 845 which is alone in group 4. There are, however, three isolates collected from compost in group 1 as well as several from unknown locations so further work would need to be undertaken to see if original substrate type was correlated with genetic diversity.

Population genetic studies have been carried out on the nematophagous fungus *Paecilomyces lilacinus* (Tigano-Milani *et al.*; 1995a Gunasekera *et al.*, 2000; Tigano Milani *et al.*, 1995b). Analysis of a world-wide collection of 15 isolates using RAPD and allozymes detected significant variation between the isolates and placed them into six genetic clusters. The authors demonstrated that some genetically related *P. lilacinus* exhibited widespread geographic distributions. Chapter 5 looked at the variation amongst the *D. flagrans* isolates using RAPD analysis. Fifteen genotypes were identified within three clusters and 12 isolates were able to be individually identified using a combination of eight primer/primer pair

combinations. The genetic clusters did not appear to be related to the geographic origin of the isolates. A study was also undertaken looking at the genetic variation between 12 isolates of the nematophagous fungi *Arthrobotrys oligospora* (Ahren *et al.*, 2004). The variation found within the protein coding genes *tubA* (β-tubulin), CMD1 (calmodulin), EF1α (translation elongation factor 1α) and PII (an extracellular serine protease), partly separated the isolates according to their geographic origin. Chapter 4 investigated the variation between *tubA* and PII of the 25 *D. flagrans* isolates. No differences were found between the isolates.

The microsatellite study in Chapter 5 separates the *D. flagrans* isolates into two groups. Microsatellites have previously been used to distinguish between strains of the barley scald fungus *Rhynchosporium secalis* (Keiper *et al.*, 2006), however the current study was the first to use microsatellite fingerprinting techniques to distinguish isolates of *D. flagrans*. The first microsatellite group combines groups 1, 3 and 4 of the ITS sequencing groups, and the second group is made up of the six isolates in group 2. This is quite exciting as it strongly supports the division of group 2 from the other isolates. These results are further enhanced by the RAPD analysis which shows these six isolates to again be separated from the other isolates. This very apparent separation of these six isolates from the others using three different molecular techniques raises the possibility that there may be a sub-species or even a separate species. Further work, both molecular and morphological, needs to be undertaken to investigate this suggestion. Bills *et al.* (1999) used the sequencing of the ITS1-5.8S-ITS2 region, RAPD analysis, microsatellite-primed PCR fingerprinting, morphological and biochemical techniques to reclassify *Zalerion arboricola*, a fungus which produces the metabolite pneumocandin which can be used in the treatment of systemic candidiasis, aspergillosis and pneumonia, to a new species, *Galarea lozoyensis*. It would be particularly interesting to see the generation of

more SSRs for *D. flagrans* since one of the four microsatellites developed revealed a polymorphism which is good evidence to suggest that further polymorphisms may be revealed in the generation of more SSRs. There particularly needs to be more work comparing international isolates of *D. flagrans* to Australian ones. Work by Ahren *et al.* (2004) has claimed there is little genetic diversity between isolates of *D. flagrans* after a study looking at 22 worldwide strains and suggested that a 'super-isolate' could be selected for world-wide release, despite finding significant differences between the isolates after AFLP analysis. No correlation was found between the genetic differentiation and the geographical origins of the isolates, however there was no investigation into whether a correlation may have been found between biological characteristics of the isolates. A more detailed investigation needs to be carried out to determine this.

The only recent morphological study looking at different isolates of *D. flagrans* was made by Skipp *et al.* (2002). They examined 13 isolates and compared general morphology, nematophagous habitat, mode of conidial development, and dimensions between their 13 isolates and published descriptions of type cultures of *D. flagrans* made by Duddington (1949) and Cooke (1969) and determined they were indicative of these isolates being one species. There is unlikely to be any big differences in morphology because of environmental adaptation to habitat and food source, however there were small variations in dimensions and spore size observed. It would be interesting to run a morphological study parallel to a genetic one to see whether observed variation between the isolates, which had initially been put down to within species variation, might actually be representative of more than one species or a sub-species where there is genetic variation to support this.

Contrary to the conclusions of Ahren *et al.* (2004), this study has highlighted the significant differences between Australian isolates of *D. flagrans.* Four isolates in particular: CP 911, DH OSP, DH SPB and CP 1187, have been identified as having superior growth rate and chlamydospore production, as well as growing well on a barley substrate and having a high trapping efficiency. Any one of these isolates could be potentially used in the development of a highly effective biological agent. Isolate CP 845 may also be a good choice for the development of a commercial product. It also has good growth and spore production and a high trapping efficacy, but it also has the advantage of having a unique ITS1-5.8S-ITS2 sequence which could be useful in monitoring its persistence in the environment after an inundative release as a biocontrol agent. The importance in preserving Australia's microbiological diversity is discussed by Davison *et al.* (1999). The ability to distinguish between isolates of *D. flagrans* and monitor an inundative release of a particular isolate will play a role in maintaining Australia's microbiological variety.

The biological differences observed between the isolates will most likely not have an effect on the use of a particular isolate in different climatic zones of Australia. As shown in this thesis, there are several isolates that have a very high performance at all temperatures. The main concern in the release of any one of these isolates is the possibility of the displacement of endemic fungal population by the introduction of a biocontrol species. Ahren *et al.* (2004) have discussed the use of a 'super-isolate' which could be widely applied, however this idea needs more thought. A detailed assessment of endemic *D. flagrans* populations should precede any importation and inundative release into non-endemic areas. This study has identified at least four genetically distinct Australian populations of *D. flagrans* and although there is no evidence to suggest recombination may occur with local isolates, displacement is still possible.

Intensive and long term application of an isolate may be potentially problematic, however if applied intensively for short terms at epidemiologically significant times and in conjunction with other worm control practices, there may be less chance of persistence in the environment and displacement of endemic strains of *D. flagrans*. We are now further along the path to having molecular fingerprints to be able to monitor the release of a *D. flagrans* isolate and an extension of the RAPD study and microsatellite work will hopefully generate enough variability between the isolates to be able to identify any of the studied *D. flagrans* isolates.

Recommendations and Future Research

For the development of a commercial biological control product using *D. flagrans* as the biological control agent, several main factors must be considered:

- Rapid and abundant growth and chlamydospore production so large quantities can easily be produced in mass culture for biological control delivery;
- High predatory ability for maximum effectiveness on release;
- Ease of monitoring on release as a biological control agent;
- No negative effects on non-target organisms or extended persistence in the environment after release.

This study addressed the first three of these points and highlighted five Australian *D. flagrans* isolates that could be recommended for further studies with the aim of developing them into a biological control agent. These isolates are CP 911, DH OSP, DH SPB, CP 1187 and CP 845. All these isolates had very high growth and chlamydospore production and excellent predatory

abilities. CP 845 also had a unique ITS-5.8S-ITS2 sequence compared to the other isolates tested which could potentially be used as a marker to monitor its persistence after release.

Further work based on these efforts is essential in order for the most effective biological control agent to be developed. Future studies could include:

- *In vitro* and *In vivo* testing to determine if the recommended isolates would pass through the gastrointestinal tract of an animal without losing viability;
- *In vivo* tests to determine if the recommended isolates have the same efficacy in the environment in relation to rapid growth in a faecal pat and high predatory ability;
- Further work developing RAPD and microsatellite markers so each of the recommended isolates can be monitored on release using a unique molecular signature;
- Intensive environmental impact studies to determine if the recommended isolates have a negative impact on non- target organisms- including the displacement of endemic fungal species;
- Further work comparing Australian isolates to global isolates of *D. flagrans* to gain greater knowledge of any possible differences or similarities and to further our understanding of their population biology.

6.1 References

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