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High throughput development of population genetic markers for the *Sirex noctilio* woodwasp and its nematode parasite, *Deladenus siricidicola*

By

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Declaration,

I, the undersigned, hereby declare that this thesis, submitted herewith for the degree of *Magister Scientiae* to the University of Pretoria, contains my own independent work and has not been submitted for any degree at any other University.

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Xolile Osmond Mnyamezeli Mlonyeni

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I dedicate this thesis to my late grandmother Nompucuko Muriel Mlonyeni and to my loving mother Kholiswa Olive Mlonyeni.

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Preface

Pine trees constitute a significant part of the forestry industry across the world, and specifically non-native plantations in the Southern Hemisphere. Across the Southern Hemisphere, *Sirex noctilio* is the most serious invasive insect pest of pine trees, causing devastating losses in some cases. In these situations, the nematode parasite, *Deladenus siricidicola*, is used as the primary biological control agent. Despite the potential value of population genetic data to understand critical aspects of the ecology of this invasive wasp and the biological control nematode, no such data currently exist. One of the reasons for this is the lack of molecular markers that can be efficiently applied to conduct these studies.

The molecular diversity of populations of the nematode *D. siricidicola* is a major focus of the thesis. Therefore, chapter one reviews the literature pertaining to the use of molecular tools to study various levels of nematode diversity, with a special focus on *Deladenus* spp. The limiting factor in resolving and characterizing nematode diversity is the narrow morphological difference that exists between and within the various taxonomic levels. The advent of molecular tools has helped to overcome this limitation. Its influence ranges from the ability to determine phylogenetic relationships of nematodes at the highest taxonomic classifications to understanding population diversity and structure. The review also considers the diversity of *Deladenus* spp. and populations, and the potential for molecular tools to better characterize this group.

Microsatellites are considered ideal markers for population diversity studies. These markers are co-dominant, characterized by a high degree of polymorphism, randomly distributed and frequently occur in the non-coding region of the genome. A shortcoming, however, is that they can be laborious to develop. In chapter two, next generation sequencing technology (454 pyrosequencing) was assessed as a tool to enhance the throughput of microsatellite discovery. In this chapter the efficacy of this tool is tested on three different organisms, including the fungus *Fusarium circinatum*, the insect *S. noctilio* and the nematode *D. siricidicola*.

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In chapter three and four the newly develop method of microsatellite discovery based on pyrosequencing of enriched libraries is used to develop markers to characterize genetic diversity of *D. siricidicola* and *S. noctilio*, respectively. For *D. siricidicola*, the markers were used to determine the population genetic variation between regions in the Southern Hemisphere. A comparison of genetic diversity between Southern Hemisphere and Canadian (Northern Hemisphere) sources of the nematode was also made. For *S. noctilio*, the diversity of the markers were tested and characterized on a selection of samples from Argentina, Australia and South Africa.

In the final chapter, the biological control system and the efficacy of *D. siricidicola* is critically reviewed. The review considers some of the well-studied aspects of the biology and handling of *D. siricidicola*. The review, however, mostly aims to engage with emerging issues relating to the ecology and evolution of the nematode populations that might impact its efficacy in biological control systems, including issues raised by the results presented in previous chapters of this thesis. These include the evolution of nematode virulence and wasp resistance, the nematode ability to adapt to different environments, and nematode compatibility with different fungal and wasp strains or populations.

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CHAPTER 1

Molecular tools and applications for understanding nematode diversity, with a special focus on *Deladenus siricidicola*

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1. Abstract

The Nematoda is the third most diverse phylum of the kingdom Animalia. It is thus ironic that approximately 90 % of the estimated number of species remains unknown. This is largely due to the predominant focus on economically significant species. Furthermore, early studies of nematode biodiversity relied exclusively on morphological characters for identification and systematics. The limiting factor associated with this as the primary tool is the relatively narrow morphological diversity available within and between taxa and the lack of universally coherent criteria to determine these differences. During the course of the past two decades, molecular tools have increasingly been used to address this gap. Beyond applications in higher-level nematode systematics, molecular tools have also revolutionized nematode species identification and population diversity studies. Such data is crucial to understand how and why nematode species inhabit certain ecosystems, explaining how evolutionary forces affect genetic diversity and also to understand the structure of populations. This knowledge can have important implications for the use of nematodes as biological control agents as well as for their management as invasive pests. A system where this is particularly relevant is in the biological control nematode *Deladenus siricidicola* used to control the invasive wasp, *Sirex noctilio*. This review seeks to highlight the critical importance of molecular tools in understanding nematode diversity.

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1.1 Introduction

The phylum Nematoda is the third most diverse phylum of the kingdom Animalia including an estimated 40 000 to 100 million species (Kampfer et al., 1998, Dorris et al., 1999, Coomans, 2000). The exceptionally large range of these, illustrates how little is really known about the diversity of nematodes. This phylum is sub-divided into two classes, Adenophorea (nematodes with causal glands) and Secernentea (nematodes with excretory system) (Maggenti, 1983, Kampfer et al., 1998, Coomans, 2000, Holterman et al., 2006). Adenophorea are largely marine, freshwater and soil nematodes including relatively few plant and animal parasitic nematodes. This class consists of eight orders namely the free-living Monochida, Enoplida, Chromadorida, Monhysterida, insect–parasitic Mermithida, vertebrate-parasitic Trichocephalida, and plant-parasitic Dorylaimida and Triplochida. The Chromadorida includes a diverse group of microbivorous nematodes and is suggested to be the paraphyletic ancestor of Secernentea (Blaxter et al., 1998, Dorris et al., 1999). The monophyletic class Secernentea occurs in terrestrial habitats and consists of free-living and parasitic nematodes. The nine orders in this class include the animal–parasitic Ascaridida, Spirurida, Oxyurida, Rhigonematida, Strongylida, predominantly plant–parasitic Tylenchida, Aphelenchida, free-living Diplogasterida and free–living and animal parasitic Rhabditida (Blaxter et al., 1998, Dorris et al., 1999).

Nematode classifications have largely been based on morphological characteristics such as the pharyngeal and buccal structures, reproductive systems, intestine, cuticle, lip and feeding structures, and sense organs. Other characteristics include ecology, biology, host–parasitic relationships (co-evolution) and life cycle (Lorenzen, 1983, Maggenti, 1983). The limitation with morphological characteristics is the relatively narrow morphological diversity available to distinguish between taxa (Hirschmann, 1983). This is compounded by bias error inherent in individual intuition, lack of coherent criteria to determine morphological differences, variable reliability and diagnostic importance of characters, and underestimated differences arising from host–induced effects for parasitic nematodes (Gibson, 1983). Most morphological descriptions are based on adult nematodes, which usually possess the largest number of characteristics, despite the fact that this represents a single stage in the nematode life cycle (Coomans, 2000).

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Nematode systematics using morphology is further complicated by the lack of informative fossil data. For these reasons, molecular tools have become widely used for the characterization of taxonomic diversity of and phylogenetic relationships between nematodes.

Knowledge of the genetic diversity of populations is crucial to better understanding population structure, the forces affecting diversity such as mutation, gene flow, genetic drift, selection and inbreeding. It can, for example, be important to understand the evolutionary adaptations that have occurred in specific populations of biological control agents. This makes it possible to develop effective management strategies and to understand introduction histories of pathogenic nematodes and much more. A variety of different techniques have been employed to characterize the diversity of nematode populations. These tools include both protein-based and various DNA based molecular markers.

The aim of this review is to consider the impact that molecular tools and associated applications have had on the understanding of nematode diversity. This includes taxonomic diversity and phylogenetic relationships from class down to species level. However, this review places the greatest emphasis on the use of molecular tools to distinguish species and to characterize population diversity. The review, furthermore, considers the phylogenetic placement of the genus *Deladenus*, which is an important focus of the thesis for which this document has been prepared.

1.2 Molecular tools used in nematode systematics

In recent years, molecular markers have become the most important tools used to study nematode classification. Marker selection in these studies is important and primarily based on the ability of a marker to enable measurable comparison of closely and distantly related species. This requires that the marker is consistent, reliable and provides an accurate resolution of characters. For an improved overall resolution for nematode classification, molecular markers and morphological characters are used congruently (Kampfer et al., 1998, De Ley and Blaxter., 2002).

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In eukaryotes, the ribosomal RNA (rRNA) molecule, a transcript of rDNA comprises of conserved subunits called small (SSU, 18S), 5.8S and large (LSU, 28S) subunits, and variable sequences called internal transcribed spacer (ITS) and intergenic spacer (IGS) regions (figure 1). In nematodes rRNA cistron is also present in multiple and largely identical copies and it is the marker of choice for classification studies (Dorris et al., 1999). The rRNA subunit/cistron is present in ± 55 directly repeated copies per nematode genome.

Sequence data from the SSU rDNA has been most widely used to reconstruct the phylogeny of phylum Nematoda (for example, see figure 2; Holterman et al., 2006). Blaxter et al. (1998) showed that animal parasitism originated independently a minimum of four times and that plant parasitism has arisen three times in the nematodes. For plant parasitic independent evolution, this occurred twice in Adenophorea (Dorylaimida and Triplonchida) and once in Secernentea (Tylenchida). Furthermore, the monophylectic class Secernentea emerged from ancestry Chromadoria within the paraphyletic class Adenophora. The limiting factor in this inferred phylogeny was that it was largely comprised of a relatively wide range of economically important species. Consequently, evolutionary relationships of many widespread taxa (e.g. marine nematodes) remained unincorporated and unresolved in these studies.

Recently, De Ley and Blaxter (2002) sought to reconstruct the phylogeny of phylum Nematoda by using morphological characters and SSU rDNA sequences. This study in particular sought to resolve the paraphyletic nature of Adenophorea with respect to Secernentea, and to test the hypothesis that parasitic nematodes emerged from ancestral free–living nematodes. The phylum Nematoda was consequently sub-divided into three classes, namely Chromadoria, Enoplia and Dorylaimia. Meldal et al. (2007) increased the number of sequences for marine nematodes and found that their results concurred with the proposed classification of De Ley and Blaxter (2002). However, both studies acknowledge the limited use of molecular sampling within “Adenophorea” resulting in less intensive analysis as compared to “Secernentea” species and, therefore, leading to a possible overemphasis on interpretation of morphological data. Although the results of Meldal et al. (2007) recovered the proposed classes, under Bayesian

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inferences, Chromadoria were never significantly supported, and only Enoplia and Dorylaimia were significantly supported (96% and 100%respectively).

In one of the most comprehensive phylogenetic studies, Holteman et al (2006) used an increased data set of 339 nematode taxa representing all major taxa, including scarce molecular data on the bacterivorous, fungivorous, carnivorous and omnivorous nematodes, to reconstruct the phylogeny of the entire phylum Nematoda. These authors showed a detailed relationship between Adenophorea and Secernentea. Their results suggested a revision of the phylum Nematoda to include 12 clades rather than the five proposed by Blaxter et al. (1998). Consistent with the findings of Blaxter et al. (1998), Holteman et al. (2006) also showed that Secernentea evolved from an ancestor within Adenophorea. Thus, the members of genus *Terocephalus* were suggested to be the closest living representatives of the common ancestor of Secernentea (figure 2). Despite the rather comprehensive phylogenetic studies in recent years, many gaps remain in the understanding of the phylogenetics and systematics of the Nematoda. These shortcomings relate to both sampling of the full diversity of the nematodes and adding more sequence data (multiple loci) to strengthen the inference and support for evolutionary relationships.

1.3 Molecular tools for species identification and population genetics

Protein-based isoenzyme markers represented the first non-morphological method used to detect polymorphisms among nematode species. Deoxyribonucleic acid (DNA) based tools have, however, superseded all other approaches as is true for all biological systems. The most commonly used of these tools include Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Microsatellites (SSR). In nematode studies, these tools have revolutionized the extent and sensitivity to which taxonomic, systematic and population genetics studies can be researched. However, the application of each of these approaches is strongly dependant on the particular research question being asked.

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1.3.1 *Isoenzymes*

Isoenzymes have been widely used to characterize inter- and intra-specific nematode diversity (Dickson et al., 1971, Esbenshade and Triantaphyllou, 1985, Fargette, 1987, Esbenshade and Triantaphyllou, 1990, Andrès et al., 2000, Andrès et al., 2001). Isoenzymes are specific non-regulatory enzymes that can be used as a variable molecular marker through electrophoresis and subsequently detection by an enzyme-specific staining method that reveals the isoenzyme profile. Two approaches are available to assess isoenzyme variation, namely polyacrylamide gel electrophoresis based on molecular weight (Hussey et al., 1972, Esbenshade and Triantaphyllou, 1985, Pais and Abrantes, 1989) and isoelectrofocusing electrophoresis (Andrès et al., 2000, 2001). Isoelectrofocusing electrophoresis has increased the resolution and possibility of detecting polymorphic enzymes, revealed by an increased number of bands observed.

In an early study using isoenzymes to distinguish nematode species, Hussey et al. (1972) compared the ability of soluble – proteins and eight isoenzyme profiles to distinguish between *Meloidogyne incognita* and *M. arenaria*. In that study, isoenzymes were more reliable markers than soluble-proteins to distinguish between the species, with significant variation also found between different isoenzymes. Esterase, malate dehydrogenase and α – glycerophosphate dehydrogenase were the most reliable markers amongst the isoenzymes tested. That study also showed that different host plants influenced isoenzyme production and activity, although some profiles were more stable (e.g. the esterase profiles of *M. incognita* populations) (Hussey et al. 1972).

Subsequent to the study of Hussey et al. (1972) other investigations have confirmed that esterase is a reliable isoenzyme to distinguish between *Meloidogyne* species, in particular the four major species, *Meloidogyne areanaria*, *M. incognita*, *M. javanica* and *M. hapla* (Esbenshade et al., 1985, Pais and Abrantes, 1989, Carneiro et al., 2004). Malate dehydrogenase was also reliable to distinguish *M. hapla* from the rest of the *Meloidogyne* species (Esbenshade et al., 1985, Pais and Abrantes, 1989). From these studies it was concluded

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that multi-enzyme profiles provide the most definitive characterization of *Meloidogyne* species. It was, however, clear that even multienzyme contribution is limited and largely supplied supportive criteria for species identification and characterization.

In a fairly recent study, Andrés et al. (2000) used seven isoenzymes to determine inter– and intraspecific diversity of 40 populations representing nine species of the root-lesion nematode *Pratylenchus*. They revealed that four isoenzymes were effective in differentiating *Pratylenchus* species and the outgroup *Radopholus similis* from these species. Phophoglucomutase differentiated seven species of *Pratylenchus*, while phophoglucose isomerase differentiated six species of *Pratylenchus* with two distinct groups detected within *R. similis* populations. The diagnostic applicability of phophoglucose isomerase, however, was poor due to the large number and concentration of bands in the upper half of the gel. Esterase showed low activity making the isoenzyme an unsuitable diagnostic character, despite the fact that it differentiated six *Pratylenchus* species and detected two defined groups within *R. similis* populations. Malate dehydrogenase effectively differentiated all *Pratylenchus* species from *R. similis*. Using cluster analysis, Andrés et al. (2000) showed that the banding patterns for the four isoenzymes were useful markers to differentiate between these species. They, however, concluded that these markers had limited applicability to determine population genetic variability among the populations of the *Pratylenchus* species studied.

Isoenzymes were used to determine inter– and intraspecific diversity of the species in the cereal cyst nematode *Heterodera avenae* complex (Andrés et al. 2001). The study showed that the isoenzymes used (esterase, malate dehydrogenase, phophoglucose isomerase, phophoglucomutase and super-oxide dismutase) could be used to detect, at varying degrees, the inter-specific variation among *H. avenae sensu stricto*, *H. latipons*, *H. filipjevi* and *H. mani* populations, which represented twenty geographically different origins. It also revealed intraspecific variation within the *H. avenae sensu stricto* populations. The results do concur with those of previous morphological identification studies and, while useful, remained supplementary to morphological characterization (Andrés et al. 2001).

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Although isoenzymes served as most important non-morphological markers initially used, they also had major limitations and this was recognized early. Hussey et al (1972) highlights some of the factors that could possibly influence enzyme variation, including (I) methods of collecting and storing nematodes, (II) the effect of nematode development, (III) protein extraction procedure, (IV) storage of protein extracts and (v) method of enzyme analysis. Other factors include the limited available tissue to conduct satisfactory protein analysis from individual nematodes due to their small size and insufficient number of detectable loci resulting in low levels of polymorphism (Nadler et al., 1995, Nasmith et al., 1996, Fullaondo et al., 1999, Chilton, 2004). These limitations necessitated the search for more stable, abundant and polymorphic markers to identify and characterize nematode diversity.

1.3.2 *Restriction Fragment Length Polymorphisms (RFLPs)*

Restriction fragment length polymorphism (RFLPs) studies involve restriction endonuclease digestion of homologous DNA loci that produce multiple banding patterns on an agarose gel due to the differences in size and electrophoretic ability of the resulting fragments. The restriction length variation can then be determined by Southern blot analysis that involves hybridization of complementary labeled DNA probes onto the target DNA. This provides a RFLP profile where band sizes and sequences can be determined. The polymorphisms in DNA fragments occur as a result of differences in restriction sites generated by nucleotide substitutions or rearrangement of DNA sequences, resulting in either a loss or gain of restriction sites (Botstein et al., 1980). RFLP markers are co-dominant, reproducible and relatively inexpensive to develop, but they are labor intensive, time consuming and prior knowledge of the genome sequence is required in order to produce these markers (Sunnuks, 2000).

Two types of DNA templates can be used to develop RFLP markers, namely the total DNA and specific DNA loci amplified through PCR. Total DNA RFLP's involve the digestion of either the nuclear genomic DNA (gDNA) or mitochondrial genome (mtDNA) (Hugall et al., 1994). PCR – RFLPs involve amplification of a specific fragment using sequence specific primers followed by

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digestion of the fragment by selected restriction endonucleases. This method does not require membrane hybridization and the need for labeled probes, which drastically reduces the time required to develop markers. Both total genomic DNA-RFLP and PCR–RFLP markers have been used to resolving questions of systematics (taxonomy and phylogeny) and population genetics of nematode (Nasmith et al., 1996, Subbotin et al., 1999, Maafi et al., 2003, Aikawa et al., 2006).

Several studies have used PCR – RFLP markers as to distinguish between and identify genera infecting a specific host (Nasmith et al., 1996, Iwahori et al., 2000, Waeyenberge et al., 2000, Amiri et al., 2002, Maafi et al., 2003, Zheng et al., 2003, Chilton, 2004, Han et al., 2004). These studies show how this technique can overcome some of difficulties of morphological identification. For example, it does not require a high level of expertise for accurate identification, the inability to distinguish morphologically similar yet pathologically variant species, selection of one sex over another for unequivocal species identification, the stability of the characters used and the amount of material required to perform a successful analysis. The commonly used target genes are the non-coding internal transcribed spacers (ITS-1 and ITS-2) and coding nuclear ribosomal genes (18S, 5.8S and 28S rRNA) of the ribosomal DNA array (rDNA). The ITS regions are typically more suitable for diagnostic purposes, given the higher level of conservation of the rRNA genes at or below species level (Cherry et al., 1997, Powers et al., 1997, Szalanski et al., 1997, Gasser et al., 1999, Subbotin et al., 2000, Bae et al., 2009).

One application of PCR-RFLPs has been to distinguish closely related species. For example, Nasmith et al. (1996) studied 15 strains of entomopathogenic nematodes from the Steinernematidae (*Steinernema feltiae* syn *bionis* (F), *S. glaseri*, and seven strains of *S. carpocapsae*) and Heterorhabditidae (four strains of *Heterorhabditis bacteriophora*) using PCR – RFLP analysis of ITS regions and 28S rRNA. The PCR – RFLP profiles were unique at an inter-specific level, except in the case of *S. feltiae* s (F) and *S. glaseri* glaseri (G) that showed no difference. In an application of the tool, one field isolate was shown to have the same profile as the *S. carpocapsae* strains. This confirmed the establishment of this species the year preceding this study, while a second field isolate had a profile unique to all the known strains. Similarly,

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Subbotin et al. (1999) conducted a comparative study using PCR – RFLP analysis and multivariate analysis of morphometric characters to distinguish seven species *Heterdera avenae* group *sensu lato* from twenty seven geographically distant populations. The study showed that both techniques gave similar results in distinguishing the species and populations within the *H. avenae* group, but PCR-RFLP is generally more efficient.

PCR-RFLPs have great value in screening isolates associated with plant disease material for the correct identification of associated species. Szalanski et al. (1997) used PCR – RFLP analysis of the ITS1 region to identify different cyst nematodes, that can have a devastating effect on their hosts. They found that the PCR – RFLP profiles of the globally distributed cyst causing species such as the pea cyst (*Heterodera goettingiana*), soybean cyst (*H. glycine*), sugar beet (*H. schachtii*) and clover cyst (*H. trifolii*), were unique and consistent, except for corn cyst (*H. zeae*). The study reports ITS1 heterogeneity affecting restriction site polymorphism within individual isolates of *H. zeae* samples. These variants have potential to be used as diagnostic markers for monitoring the distribution and spread of these populations, similar to what was reported by Chetty et al. (1997). Szalanski et al. (1997), however, also expressed caution regarding the use of the ITS region due to the possibility of comparing non-homologous ITS variants. Iwahori et al. (2000) and Zheng et al. (2003) also used PCR – RFLP analysis to distinguish between the morphologically similar *Bursaphelenchus xylophilus* and *B. mucronatus*, the former of which causes pine wilt disease and the latter that in non-pathogenic.

1.3.3 *Random Amplified Polymorphic DNA markers (RAPDs)*

Random Amplified Polymorphic DNA (RAPD) markers have been fairly widely used to characterize both intra-specific variation in nematodes in the same host or environment (Caswell-Chen et al., 1992, Fullaondo et al., 1999). Furthermore they have been used to assess inter-specific variation amongst individuals in populations of the same species or between different geographic populations (Nadler, 1996, Fullaondo et al., 1999, Randig et al., 2001, Carneiro et al., 2004, Syracuse et al., 2004, Vieira et al., 2007, Zhang et al., 2008, Devran et al., 2009). These markers are based on PCR amplification of random loci by short arbitrary

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oligonucleotides (10-mer) as primers. For each primer, the number of binding sites and varying amplicon lengths is dependent on loci containing complementary sequences. The detection of polymorphism is based on the presence or absence of an amplicon, placing RAPD's in the category known as dominant markers. The technique, however, has serious limitations such as an inability to detect heterozygosity, therefore, underestimating allelic and genotypic frequencies if the sample size is not sufficiently. There are also problems regarding reproducibility of the results. The advantage of the technique is that it enables markers to be scored with relative ease and without prior knowledge of the genome sequence.

Most of the studies using RAPDs have been focused on identification of important plant–parasitic nematodes such as cyst nematodes *Heterodera* spp., potato cyst nematodes *Globodera* spp. and root-knot nematodes *Meloidogyne* spp. As an example, Caswell-Chen et al. (1992) differentiated between *Heterodera schachtii* and *H. cruciferae*, nematodes that are sympatric and frequently occur in the same field in the same host. Eighteen RAPD primers were used to differentiate the species. All primers showed unique bands present in these species. Comparison of RAPD markers generated from single and multiple cysts originating from the same population showed a high level of reproducibility in this study. From these findings the authors conclude that RAPDs are suitable tools for nematode identification. More recent studies on root-knot nematodes in the genus *Meloidogyne* drew similar conclusions (Randig et al., 2001, Carneiro et al., 2004).

Caswell-Chen et al., (1992) analyzed *H. schachtii* population diversity using the same RAPD markers used to identify *H. schachtii* and *H. cruciferae*. They analyzed six geographically separated populations of *H. schachtii* using ten RAPD primers. Using extracted DNA from multiple cysts per population, the primers produced 78 RAPD markers that could be scored for similarity and difference between the populations. The cluster analysis revealed that one of the Imperial Valley populations (approximately 5 km apart from another) was distinct from the other five populations, while the two Clarksburg populations were closely related to the Lodi population although being approximately 34 km apart. These results raised caution regarding the assumption that there is a correlation between geographic proximity and genetic

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relatedness, and showed the value of RAPD markers to estimate population relatedness and the history of introductions.

An important application of RAPDs to intra-specific variation and introduction history has been on the pinewood nematode *Bursaphelenchus xylophilus*. Vieira et al. (2007) examined diversity in populations of this species from different geographic locations in Portugal (24 isolates) and considered the possible origin of the introduced population, either from Portugal via China or the USA. From 28 RAPD primers used on 27 isolates, excluding an outgroup *B. mucronatus* isolate from Germany, 471 bands could be scored. These markers showed a high level of genetic homogeneity amongst the 24 Portuguese isolates, while a cluster analysis showed that Portuguese isolates grouped most closely with isolates from China. These results strongly suggested a single introduction from China to Portugal, followed by dispersal within the country. Zhang et al. (2008) subsequently examined the geographic distribution of *B. xylophilus* in China and worldwide using D2/3 domain of the 28S rDNA, ITS region and RAPD markers and confirmed the findings of Vieira et al. (2007).

RAPD markers can be used to discover loci for species-specific primer development. For example, Fullaondo et al. (1999) developed species-specific primers from RAPD markers for the pathogenic potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*. They selected two candidate bands that distinguish the species, excised them from the agarose gel, re-amplified, cloned and sequenced these bands, and compared the sequences to the EMBL database. After ascertaining the unique nature of the sequences, two primer pairs were designed and amplified the region of interest and unambiguously identified their respective species. The species specificity of the primers was tested on sugar beet and cereal cyst nematodes *H. schachtii* and *H. avenae*, respectively. Although this technique is more time consuming than others, it is significantly more sensitive and reliable than RAPDs (Fullaondo et al., 1999, Dong et al., 2001).

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1.3.4 *Amplified Fragment Length Polymorphisms (AFLPs)*

Amplified Fragment Length Polymorphic (AFLP) markers have been used less frequently for molecular characterization of nematode genetic diversity than RAPDs and RFLPs, but they are much more powerful because the approach combines the strengths of RAPDs and RFLPs (Vos et al., 1995). AFLP markers are based on simultaneous digestion of genomic DNA with selective restriction enzymes (rare and frequent cutters) followed by ligation of adapters to the fragment ends. The adapters contain a core sequence, an enzyme-specific sequence and a selective extension nucleotide(s), which provide the priming sites to which complementary primers will anneal for a pre-selective amplification cycle. Following pre-selective amplification, the PCR products are amplified with more selective primers that reduce non-specific amplified fragments. These products can be visualized on a polyacrylamide gel under UV transilluminator that will show a banding profile that can be scored for the presence or absence of fragments. Like RAPDs, AFLP markers are dominant markers that are easy to assay and do not require prior knowledge of the genome, but are much more reproducible than RAPDs.

AFLPs have been used to characterize the genome-wide diversity and geographic structure (based on linkage disequilibrium analysis) of hermaphroditic nematode species. The model species, *Caenorhabditis elegans*, is a self-fertilizing hermaphrodite has been used in comparisons of populations of other hermaphroditic species. For example, Zauner et al. (2007) compared *Pistionchus pacificus* and *C. elegans* for the level of diversity and linkage disequilibrium amonst loci in their genomes. The study found that *P. pacificus* strains obtained from geographically isolated locations had strong levels of genome-wide linkage disequilibrium between loci, suggesting that *P. pacificus* has a predominantly self-fertilizing mode of reproduction. The AFLP analysis, together mtDNA analysis, showed that *P. pacificus* has a higher global genetic diversity than *C. elegans*. Similarly, Baïlle et al. (2008) found that *Oscheius tipulae*, a widely distributed soil hermaphroditic nematode, has a five-fold higher genetic diversity and more geographically differentiated populations than *C. elegans*. The higher diversity and greater genetic structure of both *P. pacificus* and *O. tipulae* were attributed to the

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low level of long-range migration of these species, as opposed to the more common human-caused long-range migration *C. elegans*.

Semblat et al. (1998) used AFLP markers to characterize populations of root-knot nematodes *Meloidogyne arenaria*, *M. incognita* and *M. javanica*. The study revealed that species *M. arenaria* and *M. javanica* were more closely related than they were to *M. incognita*, and that populations of *M. arenaria* were more variable compared to *M. incognita* and *M. javanica*. Furthermore, the AFLP data had high levels of homogeneity within each parthenogenetic root-knot nematode species and that no genetic correlation could be found with geographic origin of the populations within each species. These findings were similar to studies that used RAPD markers, although the AFLP markers showed a higher level of polymorphism between the root-knot nematode species and populations (Semblat et al., 1998 cited from Baum et al., 1994 and Castagnone – Sereno et al., 1994).

1.3.5 *Microsatellites or Simple Sequence Repeats (SSR's)*

In recent years, microsatellites have become the marker of choice for population genetic studies of nematodes (Hoekstra et al., 1997, Plantard and Porte, 2003, Plantard and Porte, 2004, Picard et al., 2004, Zhou et al., 2007, Wielgoss et al., 2007, Plantard et al., 2008, Wielgoss et al., 2008, Bai et al., 2009, Silvestre et al., 2009, Villate et al., 2009). Microsatellites are short tandem nucleotide repeats that consist of a range from 1 to 6 base pairs. These repeat regions typically evolve faster than the rest of the genome, due to strand slippage during replication (Tautz, 1989, Schlötterer and Tautz, 1992). They are randomly distributed and abundant in eukaryotic genomes, predominantly occurring in non-coding regions of a genome (Li et al., 2002). The unique flanking sequences enable specific primer design and locus-specific amplification of the microsatellite. These amplicons are then scored for allele size polymorphism between different isolates of a species. The repeatability of these neutral, co-dominant and hypervariable markers is what has made them the markers of choice in population studies in many organisms (Jarne and Lagoda, 1996, Provan et al., 2001, Chistiakov et al., 2006).

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Hoekstra et al. (1997) characterized the populations of *Haemonchus contortus*, a gastrointestinal parasitic nematode causing serious damage to goats and sheep, using 13 microsatellite markers. The microsatellite data revealed distinct population structures for the four nematode populations originating from Africa, Europe (two) and Asia used in this study. The Asian population and European populations were most closely related, possibly due to nematode mixture resulting from strong trade relations and the general practice of intensive husbandry in these regions, which could cause a reduction of genetic diversity. The divergence of the African population most likely reflects its historical and geographic isolation, as well as less intense husbandry (Hoekstra et al., 1997). These findings add important information to studies that attempt to understand the selection processes that lead to development of drug resistance in these nematodes.

Phytoparasitic nematodes are responsible for significant economic losses in a variety crops (Szalanski et al., 1997, Andrès et al., 2000, Plantard and Porte, 2003, Carneiro et al., 2004, Picard et al., 2004). Population diversity studies can contribute significantly to spread, resistance build-up to nematicides or breakdown of resistant varieties. As an example, Plantard and Porte (2003, 2004) characterized the population genetic structure of the sugar beet cyst nematode *Heterodera schachtii* using five polymorphic microsatellites to contribute to efforts to preserve resistant sugar beet varieties. A hierarchical sampling design on three spatial scales (region, field and plant) was followed, within three regions, each comprising of one to three fields that were two to 175 km apart. The findings revealed that high genetic variation within fields in a region (94.6 %), while low genetic variation was observed between fields in a region (3.75 %) and between regions (1.65 %). These results suggest that significant gene flow occurs between fields in a region and among regions. Plantard and Porte (2004) attribute such gene flow over long distances to the possible passive mode of cyst transport through human activities (soil transportation by farm machinery, sewage farms around sugar factories), water (irrigation, floods or drainage) and wind. Furthermore, they found *H. schachtii* to be a highly inbred species and suggest this to be due to the limited active dispersal of larvae (few

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centimeters or decimeters) from the same cyst, which favours the probability of (half) siblings mating.

Picard et al. (2004) investigated the dispersal abilities and population genetic structure of the potato cyst nematode *Globodera pallida*, in its native area in Peru, using eight polymorphic microsatellite markers described by Thiéry and Mugniéry (2000). The three geographic regions used were separated between 326 km to 832 km. Each region comprised of three fields with distances between the fields varying from three to 35 km. The results from this study suggested that *G. pallida* has a high level of gene flow between plants and between fields within a region, but that low gene flow occurs among regions. The long-distance gene flow within regions observed in the study is suggested to be due to the passive dispersal of cysts through anthropogenic or natural origin. *Globodera pallida* was also shown to be a highly inbred species, similar to that observed for *H. schachtii* (Plantard and Porte, 2004). The findings from both the Plantard and Porte (2004) and Picard et al. (2004) studies illustrate the value of microsatellite data to help develop an effective control programme by being able to track the production and dispersal of virulent nematodes, apply quarantine measures and consequently improve the management of resistant varieties.

Population genetic studies using microsatellites are very useful to characterize the introduction and dispersal histories of invasive pests, such as the pinewood nematode, *B. xylophilus*, causing pine wilt disease in Japan. Zhou et al. (2007) studied the population genetic structure of this nematode from infested *Pinus densiflora* and *P. thunbergii* trees using six polymorphic microsatellite markers. The microsatellite markers revealed a low degree of polymorphism in these populations, indicative of an introduced species that has been able to spread rapidly. The populations of the nematode in the geographically separated forests of Tanashi, Tsukuba and Chiba, however, appeared to have been derived from three different origins, with secondary gene flow evident between the latter two forests. Sub-populations (in trees) within each forest showed that the genetic difference between sub-populations is much greater than within subpopulations, which is most likely influenced by a founder effect and genetic drift within each tree. Furthermore, *B. xylophilus* genotypes showed an excess of homozygous allele pairs, which

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result from the Wahlund effect and possibly inbreeding caused by founder effects (Zhou et al., 2007). These findings illustrate how microsatellites can be applied to characterize pest diversity, infer route of pest introduction, factors influencing proliferation ability and determine nematode genetic structure.

1.3.6 *Mitochondrial DNA (mtDNA)*

Mitochondrial DNA (mtDNA) sequence data are used for both phylogenetic studies and population genetic studies on nematodes. Mitochondrial DNA normally has a higher evolutionary rate than nuclear DNA, a limited effect of recombination and is uniparentally inherited. The fact that it is haploid and has a smaller effective population size than the nuclear DNA, also means that it is more dramatically affected by demographic factors and ideal for investigation of genetic relatedness and variation amongst species and individuals within a population.

In an early study using mtDNA sequence data for nematode population genetic studies, Blouin et al. (1995) compared sequences of the region between the variable-length long non-coding region and 3' end of the NADH dehydrogenase 4 (ND4) gene between populations of Trichostrongylid nematodes that parasitize different ruminant species. The ruminants either had high or low dispersal abilities. They found that host dispersal ability primarily determines distribution of genetic variance within and between nematode populations. Near total genetic diversity within populations and high gene flow between populations were characteristic of high host dispersal abilities. In contrast, strongly structured populations resulted from restricted host dispersal ability. Furthermore, this study revealed that a high within population diversity was a general feature of trichostrongylids due to their large effective sizes within each population. These data were valuable for control strategies aimed at reducing the rate of anthelmintic resistance spread. In a later study, Blouin et al. (1999) used part of the 3' end of ND4 gene to show the importance of population size and geneflow on population genetic structure of these nematodes.

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Cytochrome oxidase mtDNA genes are widely used as sequence markers in the Animalia, with a part of the cytochrome oxidase I (CO I) gene being identified as the universal barcoding region for this kingdom (Hebert et al., 2003, Hebert et al., 2004, Elsasser et al., 2009, Derycke et al., 2010). It has also been used for population genetics in nematodes. For example, Hawdon et al. (2001) used this region to study the population genetic structure of the human hookworm *Necator americanus*. The results revealed how varying cultural practices create complex scenarios with sporadic occurrences of gene flow over short and long distances. Likewise, these practices result in fluctuating population sizes, which have a major bearing on the expected population genetic structures and thus make it difficult to determine generalized control strategies.

Mitochondrial DNA sequence data are also very useful to differentiate between related nematode species. For example, Blok et al. (2002), used the mtDNA region between cytochrome c oxidase II (CO II) and *I*RNA genes and the hypervariable 63 bp tandem repeat region to distinguish *Meloidogyne mayaguensis* from the *M. javanica*, *M. arenaria*, *M. incognita* and *M. hapla*. They found that the size polymorphism of CO II – *I*RNA region distinguished *M. mayaguensis* from the other species. This technique can be especially powerful when combined with other characteristics such as morphology or those relating to ecology.

Mitochondrial sequence data can be used to determine the centres of origin of species complexes or invasive species. Kanzaki and Futai (2002) used the mtDNA CO I gene to elucidate the phylogenetic relationships of species within the "*xylophilus"* group of *Bursaphelenchus* and the origin of the group. These results showed that the ancestral species (*B. abruptus*, *B. conicaudatus* and *B. fraudulatus*) originated from Eurasia where the preferred habitat was broad–leaved trees. It is hypothesized that through the process of distribution expansion, a new population emerged that preferred coniferous trees. Furthermore this population diverged, resulting in the closely related *B. mucronatus* and *B. xylophilus*. Plantard et al. (2008) used mtDNA cytochrome b to determine the origin of introduced Western Europe populations of *Globodera pallida* and found that the mtDNA data supported by microsatellite data showed

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with accuracy that these populations originate from southern Peru, the native range of *Globodera pallida*.

1.4 The genus *Deladenus*

Members of the genus *Deladenus* Thorne, 1941 (Nematoda: Neotylenchidae) have both free-living mycetophagous and/or insect parasitic life cycles (Chitambar, 1991). Each form is associated with spermatozoan and adult female functional dimorphism. In the free – living life cycle a mycetophagous female is fertilized by a small number of large amoeboid spermatozoa, whereas, a countless number of small amoeboid spermatozoa fertilize an entomophagous female (Bedding, 1967, 1972).

Classification within this genus has been based on at least thirty – eight morphological characteristics, with several species described from very small populations. Some species are morphologically or superficially similar yet reproductively isolated from each other. Differences between them were established using cross breeding experiments (Chitambar, 1991). Currently, twenty – three species are known in this genus namely *Deladenus durus* (*D. andrassy* and *D. paradurus* junior synonym), *D. obesus*, *D. indicus*, *D. saccatus*, *D. aridus* (*D. crassus* junior synonym), *D. norimbergensis*, *D. apopkaetus*, *D. ulani*, *D ipini*, *D. parvus*, *D. megacodylus*, *D. minimus*, *D. siricidicola*, *D. wilsoni*, *D. imperialis*, *D. proximus*, *D. rudyi*, *D. canii*, *D. nevexii* and special inquirenda *D. arboricolus* (Chitambar, 1991).

Only seven of the known species has a parasitic life cycle. These are, *D. siricidicola*, *D. wilsoni*, *D. imperialis*, *D. proximus*, *D.rudyi* and *D. canii* that are all parasitic on Siricid wasps, and occasionally beetles and parasitic wasps associated with Siricids. The host range of Siricids that each Deladenus spp. parasitizes varies. *Deladenus siricidicola* is known to parasitize *Sirex noctilio*, *S. juvencus*, *S. cyaneus*, *S. nitobei*, *Xeris spectrum*, an associated beetle *Serropalpus barbatus* (Bedding, 1972, Bedding and Akhurst, 1978). *Deladenus canii* and *D. imperialis* is known only to parasitize *S. cyaneus* and *S. imperialis*, respectively (Bedding, 1974, Bedding and Akhurst, 1978). *Deladenus rudyi* parasitizes *S. juvencus*, *S. cyaneus*, *Urocerus gigas*, *U. augur*, *U. japonicas*, *U. antennatus* and *X. spectrum* (Bedding, 1974, Bedding and Akhurst, 1978).

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Deladenus nevexii parasitizes *S. cyaneus*, *S. longicauda*, *U. albicornis*, *U. califonicus*, *X. spectrum* and *X. morrisoni* (Bedding, 1974, Bedding and Akhurst, 1978). *Deladenus proximus* parasitizes *S. nigricornis*, *S. abbottii* and *S. edwardsii* (Bedding, 1974, Bedding and Akhurst, 1978). *Deladenus wilsoni* parasitizes *S. juvencus*, *S. cyaneus* and *U. gigas* (Bedding and Akhurst, 1978).

All *Deladenus* spp. that parasitize Siricids feed on *Amylostereum* spp., the symbiotic fungal mutualists of Siricids, in their free-living or mycetophagous form. In their free – living cycle *D. wilsoni* feeds on both Siricid symbiotic fungi *Amylostereum areolatum* and *A. chailletii* Boidin while *D. siricidicola* specifically feeds only on *A. areolatum* and *D. canii*, *D. imperialis*, *D. rudyi*, *D. nevexii*, *D. proximus* only on *A. chailletii* Boidin (Bedding, 1974, Bedding and Akhurst, 1978). In this form, the ability to survive, reproduce and live independently is dependent on the availability of the fungal food source in the absence of the host. Generally the generation span of the free-living form is less (approximately two weeks) than the parasitic form, because the latter form is intrinsically connected to the rate of host larval development, which spans a year or more (Bedding, 1972).

A burst of research activity on the genus *Deladenus* followed the discovery that *D. siricidicola* infect the eggs of the woodwasp *Sirex noctilio* in New Zealand where this wasp was a serious pest (Bedding and Akhurst, 1974, Zondag, 1979). The wasp has since invaded most pine-growing areas of the Southern Hemisphere, leading to the widespread use of this nematode species as a biological control agent (Bedding and Iede, 2005). The bi-cyclic life cycle of this nematode makes it very suitable for use as a biological control agent. The free-living form where the nematodes feed on the fungal symbiont of *S. noctilio*, *A. areolatum*, is exploited to mass-rear the nematode in culture (Bedding and Akhurst, 1974). The nematodes are then inoculated into *S. noctilio*-infested trees where they continue to multiply in the free-living stage, feeding on the symbiotic fungus. Emerging juveniles are triggered to develop into the parasitic form by the micro-environmental conditions surrounding the *S. noctilio* larvae, namely increased CO_2 and low pH. This is followed by host penetration, development in the larvae, and eventual infestation of the developing eggs in the ovaries, which effectively sterilizes the female woodwasp (Bedding, 1967, Bedding, 1972, Bedding and Akhurst, 1978). The emerging females

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then spread the nematode when ovipositing their sterile, nematode-filled eggs into the trees that they infest.

Despite the success of *D. siricidicola* to control *S. noctilio* with *D. siricidicola* in some regions in the Southern Hemisphere, it has failed in others (Haugen and Underdown, 1990, Carnegie et al., 2005, Hurley et al., 2007). During the Green Triangle outbreak in Australia in the late 1980's, it was thought that a loss of virulence due to prolonged rearing in the free-living form was responsible. In other areas, more complex sets of factors might influence the success of the nematode, including drying of the wood, availability of sufficient food source (*A. areolatum*) to sustain large populations in the tree, fungi competing with *A. areolatum*, inoculation time and inoculation technique (Hurley et al., 2008). A hypothesis emerging from some of these studies is that incompatibility of the nematode strain and strains of the fungus and wasp might affect parasitism levels (Hurley et al. 2007). In this regard it is problematic that very little knowledge is available regarding the diversity of *D. siricidicola* populations.

Molecular tools have only rarely been used to characterize *Deladenus* spp. Holterman et al. 2009 included a *Deladenus* sp. in a phylogenetic study of the order Tylenchida based on SSU rDNA. The study showed that it is most closely related to Sphaerulariidae, grouping most closely to species of *Sphaerularia*, which are insect parasitic nematodes (overwintering gynes or bumblebee queens). At a population level, Bedding and Iede (2005) reported the use of RAPD markers to characterize Australian populations of *D. siricidicola*. They showed differential banding patterns between some populations of *D. siricidicola*, possibly potentially related to virulence levels, but no details regarding this study are available. Yu et al. (2009) used a combination of morphology, rDNA and cytochrome oxidase subunit 1 to confirm the identity and characterize *D. siricidicola* in Canada, where recent populations of the invasive woodwasp *S. noctilio* have been discovered. One of the reasons for the lack of studies on *Deladenus* diversity is the lack of reliable tools to study population diversity.

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1.5 Conclusions

As for many organisms, nematode systematics is yet to be fully resolved and the full extent of nematode biodiversity remains largely unexplored (Blaxter et al., 1998, Kampfer et al., 1998, Coomans, 2000, De Ley and Blaxter, 2002, Holterman et al. 2006, Meldal et al., 2007, van Megen et al. 2009). Given how widely this group of animals is distributed and its significant influence on plant, animal and agricultural well-being, a better understanding of their ecological relationships, habitats and genetic diversity is critical. Molecular tools have helped to overcome some of the limitations that hampered explorations of nematode diversity and a deeper understanding of their relationships. These tools have now provided a framework from which diversity can be explored and characterized on a much wider scale.

The rapid increase in the number of available nematode genome sequences is making phylogenomics more feasible at a phylum wide scale. Such studies promise to resolve many of the currently unresolved phylogenetics questions in coming years (Ragsdale and Baldwin, 2010). Genomics studies are also bound to reveal many of the genetic secrets to the ecological and evolutionary success of the Nematode. Such data will also be invaluable to reveal the underlying mechanisms of the unique life-styles of nematodes such as *D. siricidicola* with its mycetophagous and insect parasitic stage.

As is true for other microscopic organisms, nematode taxonomy has long been hampered by the fact that morphological characters have very low resolution and a very high level of expertise is needed to use these efficiently. A suite of molecular tools, including isoenzymes, RFLPs, RAPDs, AFLPs and microsatellites have been used to augment and substantially improve nematode classification. Sequence data, in particular of the ITS rDNA locus and mtDNA loci, are, however, by far the most reliable techniques to identify species. In recent years, this approach has become rapid and sufficiently affordable to ensure that it can be applied wide for species identification. Further developments such as species specific primers and PCR-RFLPs, based on information from sequence comparisons, can then be used as rapid and reliable identification tools.

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The same markers used for species identification have also been used in studies of the population diversity of various nematode species. Microsatellites have become the markers of choice for population genetics studies in nematodes and this is also true for most other organisms (Meimberg et al., 2010, Reusch et al., 2010, Torriani et al., 2010, Wright et al., 2010). Using data from these markers, the diversity within and between populations can be determined with sensitivity and reproducibly. These markers can be especially powerful when combined with information from mitochondrial DNA sequence data to investigate relatedness and phylogeographic patterns of species. Population genetic data have been used to determine routes of introduction, to characterize adapted populations in specific hosts or environments and to reveal the signatures of various evolutionary forces that affect nematode populations. Such information can be invaluable for developing effective management strategies against nematode pests, or where nematodes are used in biological control programs. For this reason, and because these markers are becoming more accessible and affordable to use, there is likely to be a substantial increase in their use in future.

Deladenus siricidicola is the most widely used and important biological control agent for *S. noctilio* in the Southern Hemisphere. The limited collection from which current populations in biological control programs was derived, as well as possible inbreeding following repeated culturing, suggests that the populations of the nematode in this region harbor little diversity. Nonetheless, the nematode is being applied across very different climatic and host groups. There are also indications that the nematode is adapted to specific strains of the wasp or fungus on which it feeds. Information about the diversity in these nematode populations is thus crucial in order to understand its ability to adapt and its response to the variation in the environment.

Undoubtedly, molecular tools have revolutionized the manner in which research is and can be conducted for taxonomy, systematics and population genetics of nematodes. Their impact is only just being fully felt and their ability to provide answers to complex questions in future is unprecedented. The future of nematode studies will largely rest on the optimal application of these tools.

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Figure 1. The ribosomal RNA (rRNA) cistron. Sizes are approximate and not to scale. Each cistron comprises the small subunit gene (SSU; also called 18S), internal transcribed spacer 1 (ITS 1), 5.8S gene, ITS 2 and large subunit gene (LSU or 28S). An external non-transcribed spacer (NTS) separates each transcribed cistron. The relative rate of sequence variation observed between taxa is shown schematically above the cistron and illustrate that the SSU and LSU sequences are the most conserved, followed by the ITS regions. The NTS region is the most variable in length and sequence. In addition to the gross variation between the segments of the rRNA cistron, the genes themselves comprise a mosaic of highly conserved and variable regions (Adapted from Dorris et al., 1999).

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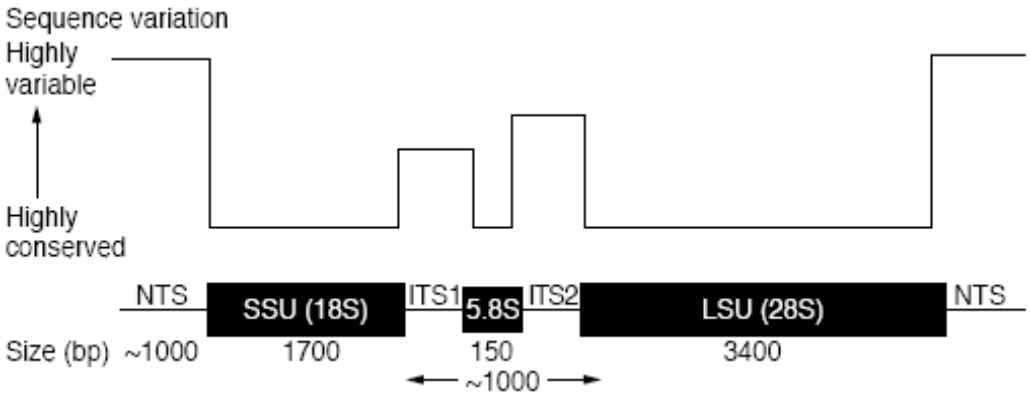

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Figure 2. Bayesian tree of the phylum Nematoda using the SSU rDNA. Alternating yellow and green backgrounds define the subdivision of the phylum Nematoda into 12 clades. Within each clade, nematode families have separate colors. Support values are indicated at the deep nodes: the first number (black) is the Bayesian posterior probability (PP) and the second number (orange) is the maximum parsimony (MP) bootstrap value. "-" indicates that the node was part of a polytomy in MP. Other nodes down to the family level are marked with a black asterisk if the support from the Bayesian inference (BI) tree is significant (PP ≥ 95) and an orange asterisk if the support from the MP tree is significant (bootstrap ≥ 65). Underlined family names are paraphyletic, family names marked with an asterisk are polyphyletic, and family names in italics are embedded in another family. The black and white bars indicate (sub- and infra-) orders as defined by De Ley and Blaxter (2002, 2004). Plant parasitic and fungivorous taxa are indicated by a pictogram and a purple (fungivores) or green (plant parasites) arrow or bar. The insert shows the most distal part of the tree in more detail (Adapted from Holterman et al. 2006).

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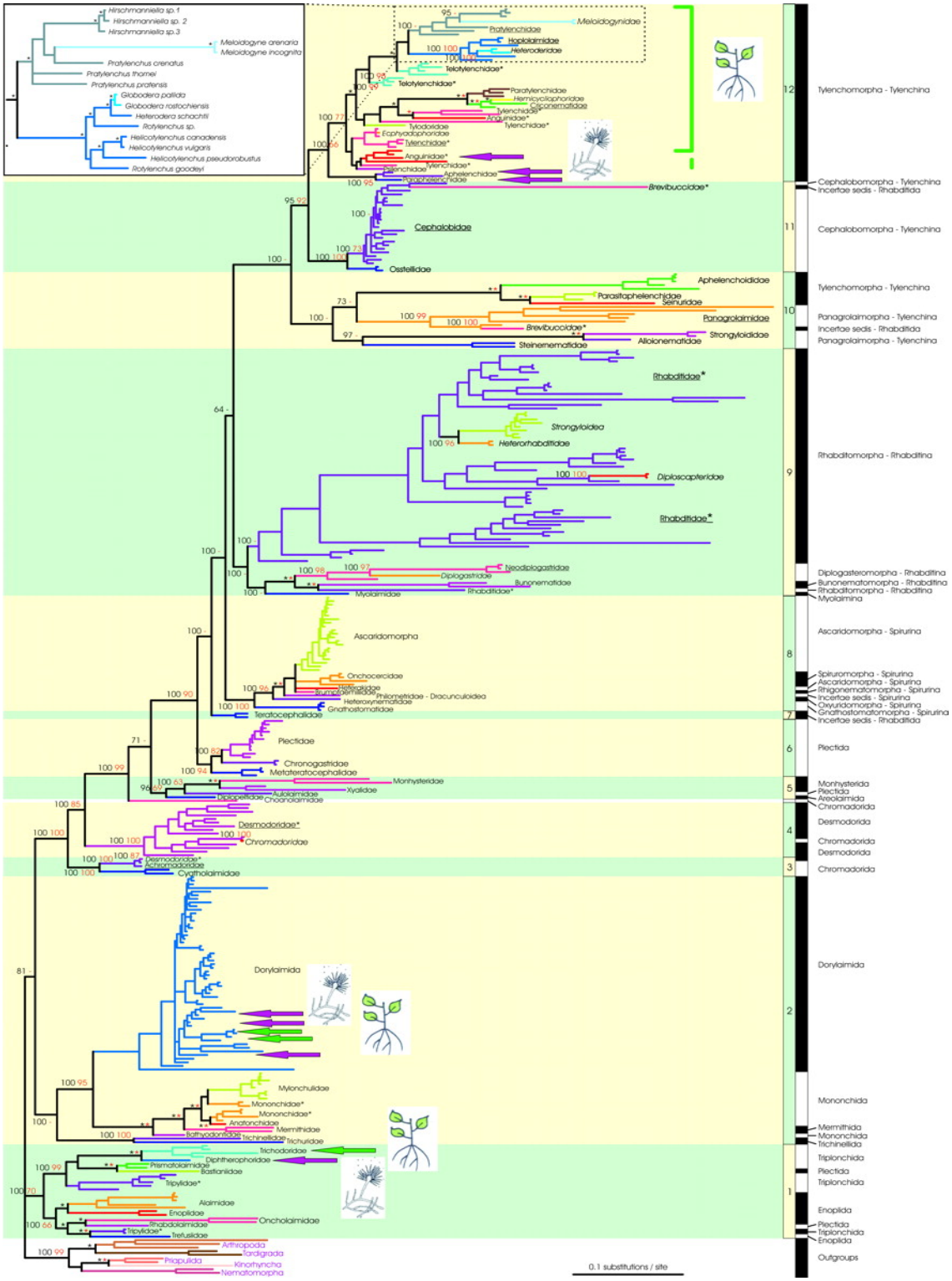

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CHAPTER 2

Microsatellite discovery by deep sequencing of enriched genomic libraries

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Reference: This chapter is also part of Mr Quentin C. Santana's PhD

My contribution to this paper: DNA isolation of *Deladenus siricidicola* and *Sirex noctilio*, sequencing, data analysis and contributing towards the writing of the paper.

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2. Abstract

Robust molecular markers such as microsatellites are important tools used to understand the dynamics of natural populations, but their identification and development are typically time consuming and labor intensive. The recent emergence of so-called next generation sequencing raised the question as to whether this new technology might be applied to microsatellite development. Following this view, we considered whether deep sequencing using the 454 Life Sciences/Roche GS-FLX genome sequencing system could lead to a rapid protocol to develop microsatellite primers as markers for genetic studies. For this purpose, genomic DNA was sourced from three unrelated organisms: a fungus (the pine pathogen *Fusarium circinatum),* an insect (the pine-damaging wasp *Sirex noctilio)* and the wasp's associated nematode parasite (*Deladenus siricidicola)*. Two methods, FIASCO (fast isolation by AFLP of sequences containing repeats) and ISSR-PCR (inter-simple sequence repeat PCR), were used to generate microsatellite-enriched DNA for the 454 libraries. From the resulting 1.2-1.7 megabases of DNA sequence data, we were able to identify 873 microsatellites that have sufficient flanking sequence available for primer design and potential amplification. This approach to microsatellite discovery was substantially more rapid, effective, and economical than other methods, and this study has shown that pyrosequencing provides an outstanding new technology that can be applied to this purpose.

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2.1 Introduction

Microsatellites or simple sequence repeats (SSR's) are DNA sequences that consist of tandem repeats of 1-6 nucleotides, found at varying frequencies in the genomes of just about every known organism and organelle (Chambers and MacAvoy, 2000). They belong to a class of highly mutable genomic sequences known as Variable Number of Tandem Repeat (VNTR) elements (Tautz and Renz, 1984, Buschiazzo and Gemmel, 2006) that show extensive levels of intraspecific polymorphisms in both eukaryotic (Weber, 1990, Tóth et al., 2000, Katti et al., 2001) and prokaryotic (Field and Wills, 1996, Gur-Arie et al., 2000) genomes. Because of their ease of use, co-dominance and high levels of polymorphism (Jarne and Lagoda, 1996) microsatellites have been particularly valuable in genome mapping, forensics, paternity testing, population genetics, conservation or management of biological resources and molecular typing of microbial strains (Jarne and Lagoda, 1996, Hennequin et al., 2001, Luikart et al., 2003, Lim et al., 2004).

Both the identification and development of microsatellite markers represent significant challenges. This is especially true in the case of organisms for which there are little or no sequence data and where the development of microsatellite markers requires the protracted steps of generating clone libraries and sequencing them (Queller et al., 1993, Jarne and Lagoda, 1996). For species with known genome sequences, in silico scanning of genome databases using bioinformatics tools can be used to identify microsatellites and to design primers targeting these regions (Tóth et al., 2000, Lim et al., 2004). However, genome sequences are available for relatively few eukaryotes and providing these is generally beyond the limited budgets of most research programs. Also, microsatellite loci can in some instances not be employed across distantly related species (Barbará et al., 2007) and they usually need to be identified and characterized de novo for each species, which can be a time intensive and expensive exercise. In general the success with which microsatellite markers are obtained and the size of clone libraries to be constructed are related to the frequency of occurrence of microsatellite sequences in the genome of interest (Zane et al., 2002, Selkoe and Toonen, 2006). However, the frequency of microsatellites observed in the genomes of plants, animals, fungi and

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prokaryotes has been reported to be significantly different (Tóth et al., 2000) and in some cases researchers have reported extreme difficulty in obtaining any microsatellite sequences (Dutech et al., 2007).

A number of methods are available to identify microsatellites (Dutech et al., 2007). Of these, the most commonly used methods employ targeted enrichment of DNA for microsatellites (Hamilton et al., 1999, Zane et al., 2002). One is known as inter simple sequence repeat PCR (ISSR-PCR) (Zietkiewicz et al., 1994). In this procedure, ISSR primers, which contain microsatellite motifs and three anchoring nucleotides at the 5' terminal end, are used to amplify regions of the genome that are thought to be abundant in microsatellites. The PCR products are cloned and subsequently sequenced to determine the presence of microsatellite sequences (van der Nest et al., 2000). More recently, DNA enrichment strategies involving hybridization with probes containing microsatellite sequences to genomic DNA fragments have been introduced (Zane et al., 2002). After exclusion of the non-hybridized DNA that presumably lacks repeat regions, the remaining microsatellite-rich fragments are cloned and sequenced to identify the microsatellite sequences. One of these approaches known as Fast Isolation by AFLP of Sequences Containing repeats (FIASCO) also uses amplified fragment length polymorphism (AFLP) (Vos et al., 1995) to aid in the enrichment process. Both the ISSR-PCR and FIASCO methods have been used widely in contemporary studies to isolate microsatellites from a wide variety of different eukaryotic species (Luque et al., 2002, Squirrell et al., 2003, Pfunder and Frey, 2006, Barnes et al., 2008).

Development of new microsatellite markers has been streamlined to some extent by optimizing the numerous steps in microsatellite identification and subsequent sequencing throughput, to make the process cheaper, more efficient and more successful (Zane et al., 2002, Selkoe and Toonen, 2006). However, using the currently available methods, certain factors–such as cloning efficiency, the necessity to sequence large numbers of cloned fragments and the need for a multitude of hybridization probes for enrichment–limit the success rate of microsatellite isolation. The recent appearance of next-generation sequencing such as Roche 454 genome

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sequencing (Margulies et al., 2005) which uses pyrosequening, raised the question whether this could facilitate more effective production of microsatellites.

2.2 Materials and Methods

In this study, the 454 Life Science/Roche GS-FLX genome sequencing system (Roche Applied Science, Penzburg, Germany) (Margulies et al., 2005) was used for the identification of microsatellite sequences, directly from microsatellite-enriched genomic DNA. To provide a broadly applicable test, we evaluated this method on three unrelated eukaryotes with little genome information available: *Fusarium circinatum* (a fungal ascomycete), *Sirex noctilio* (a hymenopteran insect), and *Deladenus siricidicola* (a tylenchid nematode).

2.2.1 *DNA extraction and microsatellite enrichment*

DNA was extract from *F. circinatum*, *S. noctilio* and *D. siricidicola* as previously described methods (Moller et al., 1992, Cortinas et al., 2006). The methods used to enrich genomic DNA for microsatellites were adopted from existing ISSR-PCR (Zietkiewicz et al., 1994) and FIASCO protocols (Zane et al., 2002, Cortinas et al., 2006) but without cloning of the microsatellite-containing DNA. ISSR-PCR enrichment was used for *F. circinatum*, the FIASCO enrichment method was used for *D. siricidicola* and both enrichment methods were used for *S. noctilio*. For the ISSR-PCR, the following primers were used: ISSR1 (5'-DDB$(CCA)_5$-3'), ISSR2 (5'-DHB$(CGA)_5$-3'), ISSR3 (5' YHY$(GT)_5$G-3'), ISSR4 (5'-HVH$(GTG)_5$-3'), ISSR5 (5'-NDB$(CA)_7$C-3'), ISSR6 (5'-NDV$(CT)_8$-3') and ISSR7 (5'-HBDB$(GACA)_4$-3'). For FIASCO the following probes were used: $(CAC)_7$, $(AAG)_7$, $(TCC)_7$, $(CA)_{10}$, $(CT)_{10}$, $(AG)_{10}$ and $(GACA)_5$.

2.2.2 *Pyrosequencing*

For each microsatellite-enriched genomic DNA pool, 5µg were analyzed on the Roche 454 GS-FLX platform at Inqaba Biotech (Pretoria, Gauteng, South Africa). For *F. circinatum* a single-lane sequencing run using portioned sections of the PicoTitrePlate™ was performed. Single runs were carried out for the *S. noctilio* and *D. siricidicola* DNA libraries, each using one section of

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the PicoTitrePlate™. Sample preparation and analytical processing such as base calling, were performed at Inqaba Biotech using the manufacture's protocol.

2.2.3 *Microsatellite discovery*

Sequence reads for *F. circinatum*, *S. noctilio* and *D. siricidicola* were assembled using the ContigExpress component of the Vector NTI software package (Invitrogen, Carlsbad, CA, USA). After sorting the assembled contigs and remaining single reads according to size, all contigs or single reads shorter than 100 base pairs (bp), were discarded from further study as they may complicate subsequent primer design.

All contigs or sequences longer than 100 bp were searched for microsatellites using the MSatFinder interface (http://www.genomics.ceh.ac.uk/msatfinder). For this purpose, perfect microsatellite repeats were identified using a regex-directed search engine. The minimum number of repeat motifs used to flag a sequence as containing a microsatellite was 12 repeats for mononucleotide motifs and 5 repeats for the remaining repeat classes (di-, tri-, tetra-, penta-, hexanucleotides). The search was performed for sequences only containing microsatellites such as mono- to hexanucleotide repeats, per the definition of a microsatellite region (Hennequin et al., 2001). Tab-delimited files were generated from the searches using the MSatFinder web interface, and converted to spreadsheet files for use in Microsoft Office Excel (Microsoft Corp., Redmond, WA, USA) for subsequent data analysis including sorting according to size, determination of repeat class numbers, and average repeat length, using the data filter command in Microsoft Office Excel.

Sequences were filtered according to the position of the microsatellite contained within the sequence data. This was done in Microsoft Excel as the results file from the MSatFinder contained the start and stop positions of the microsatellites. Since primers should be located in regions flanking the microsatellite sequences, those sequences starting within 20bp from the 5' terminal region or ending within 20bp of the 3' terminal region of the sequence were discarded. This step was performed to streamline future design of primers for amplification of microsatellite markers.

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To compare the sequence similarity between the microsatellites discovered in *S. noctilio* using ISSR-PCR versus FIASCO methods, the sequences that were amplifiable from each method (ISSR-PCR: 159; FIASCO: 296) were used in ContigExpress to construct contigs. Sequences that had > 95% sequence similarity including the microsatellite regions were assembled into a contig. Manual searching through these contigs was performed to determine if they contained shared sequences generated from the two methods, which allowed for an estimation of sequence similarity between the two methods.

To determine whether the technique displayed any bias towards a certain microsatellite repeat class, the distribution of microsatellites from genome sequences of taxa related to the studied organisms were downloaded from GenBank (http://www.ncbi.nlm.nih.gov). These organisms were the insect *Apis mellifera*, the nematode *Caenorhabditis elegans*, and the fungus *F. verticillioides*. Their sequences were searched for microsatellites using the MSatFinder web interface with the same selection criteria as that for *F. circinatum*, *S. noctilio* and *D. siricidicola*.

2.2.4 *Comparison between next generation sequencing and traditional cloning and sequencing*

The number of microsatellites generated following traditional cloning and sequencing was determined for *F. circinatum* to assess the efficiency of next generation sequencing. The pooled ISSR amplicons for this fungus were cloned using the pGEM®-T easy cloning kit (Promega, Madison, WI, USA). Cloned inserts (≥100 bp) were amplified from a total of 100 recombinant colonies using vector-specific primers and standard PCR conditions. PCR products were purified with the Invitek MSR® Spin PCRapace cleanup kit (Invitek. Berlin, Germany) and sequenced using Applied Biosystems' BigDye version 3.1 sequencing kit and ABI 3130xl Sequencer (Foster City, CA, USA). The resulting sequences were analyzed and filtered as described in the "Microsatellite discovery" section.

2.2.5 *Microsatellite primer design and testing for F. circinatum*

Following successful identification of microsatellites in *F. circinatum*, sequences were analyzed for primer design. In order to increase the possibility of targeting polymorphic loci, sequences

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were chosen that had a higher-than-average repeat number in comparison to the average repeat number for each microsatellite repeat class for *F. circinatum*. Primers were developed using the Primer3 web-based interface (primer3.sourceforge.net) (Rozen and Skaletsky, 2000), and then tested for reproducible amplification using standard PCR conditions with annealing temperatures altered according to primer sequence. Primer pairs were tested for their ability to amplify polymorphic bands by using them in PCR reactions that included eight *F. circinatum* isolates from different geographic regions. For this purpose, amplicons were separated and visualised using 12% polyacrylamide gel electrophoresis (PAGE) (Sambrook and Russell, 2001) and Syber Gold (Molecular Probes, Inc., Eugene, OR. USA). To confirm the identity of the microsatellites, amplicons were purified and sequenced as described in the previous paragraph.

2.3 Results

2.3.1 *Microsatellite enrichment and DNA pyrosequening*

To enrich genomic DNA for microsatellites, the ISSR-PCR and FIASCO methods were used. FIASCO enrichment using di- and tri-nucleotide repeat probes of the extracted *S. noctilio* and *D. siricidicola* genomic DNA, produced amplicons with a wide range of sizes, which appeared as smears following agarose gel electrophoresis. ISSR-PCR enrichment of the *F. circinatum* and *S. noctilio* DNAs using primers with di-, tri- and tetranucleotide repeats produced a defined number of amplicons that could be visualized as distinct bands on agarose gels. After pooling the enriched DNA for the respective individuals, it was subjected to Roche 454 GS-FLX pyrosequencing, which generated 1.67 megabases (Mb) of sequence for *F. circinatum* and 1.22Mb for *D. siricidicola* and 1.47Mb for *S. noctilio* (Table 1). These sequences represented large numbers of individual sequence reads, the majority of which could be assembled into contigs (Table 1).

2.3.2 *Microsatellite discovery and analysis*

Analysis of the assembled contigs and single reads (≥100bp) revealed that the fungal dataset contained the most (97%) microsatellite regions (Table 1). Only 41% of the sequences

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generated for the insect and nematode harbored microsatellites. However, a much higher proportion of the animal microsatellites were suitable for subsequent conversion to amplifiable markers. At least 70% and 45% of the nematode and insect microsatellites were situated towards the middle of contigs or sequence reads, while most of the fungal microsatellites (85%) were located within 20bp of the ends of a contig or sequence read. Overall, 495 of the *S. noctilio* sequences harbored microsatellite motifs that were flanked by regions suitable for designing primers that would allow their PCR amplification. For *D. siricidicola*, 296 such motifs were identified, while 231 were indentified for *F. circinatum*. For *S. noctilio*, more potentially amplifiable microsatellites were identified using FIASCO than ISSR-PCR enrichment (Table 1), and of these, only 10% shared high sequence similarity, suggesting that the two enrichment protocols targeted different microsatellite regions.

Analysis of the relative abundance of the various potentially amplifiable microsatellite classes revealed similar trends in the three species examined (Table 1 and Figure 1). In all cases the di- and trinucleotide repeat classes were much more abundant than the mono-, tetra-, penta- and hexanucleotide classes. In general, the average length of the microsatellite repeat motif also decreased with motif complexity (Figure 2). The only exception was for the nematode *D. siricidicola* that had, on average, more repeats in the tetra- and hexanucleotide motifs than in the di- and trinucleotide motifs. These results were comparable to those obtained from the analysis of complete genome sequences for related organisms (*A. mellifera*, *C. elegans*, and *F. verticillioides*) using similar filtering and microsatellite search criteria (Figures 1 and 2).

2.3.3 *Comparison between next generation sequencing and traditional cloning and sequencing*

To evaluate the efficiency of our new method for microsatellite discovery, we compared it to the traditional approach that involves cloning and Sanger sequencing. Of the 100 cloned inserts considered, all harbored microsatellites at their 3' and 5' prime ends. Sequence analysis using the same criteria as described in “Comparison between next-generation sequencing and traditional cloning and sequencing” (in “Materials and methods”) revealed that eight of the

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cloned inserts harbor microsatellite motifs that are potentially amplifiable. Of these four represented dinucleotide motifs and four represented trinucleotide repeats.

2.3.4 *Microsatellite markers for F. circinatum*

To determine whether the microsatellite discovery procedure described here allows for the identification of regions that can be converted to amplifiable microsatellite markers, we used the sequence information for *F. circinatum*. We designed 28 primer pairs to amplify microsatellite regions, 19 of which allowed amplification of single fragments in the expected size range. Their evaluation on a collection of *F. circinatum* isolates using PAGE rendered 13 primer pairs that yielded polymorphic amplicons for the isolates (Table 2). Sequencing of the amplicons (GenBank accession numbers: FJ436307-FJ436319) showed that all of the primer pairs amplified the targeted microsatellite loci.

2.4 Discussion

This study marks one of the first that employs Roche 454 technology for sequencing DNA libraries enriched for microsatellites. Our approach of deep sequencing of enriched nematode, insect and fungal genomic DNA facilitated the identification of large numbers of microsatellite-containing sequences. In fact, the number of sequences produced using this method resulted in the identification of more microsatellite sequences (by at least 1 order of magnitude) than what is usually generated in studies based on regular cloning technologies (Zane et al., 2002). This method substantially increased the throughput of microsatellite discovery by excluding the time-consuming steps of cloning and subsequent clone sequencing.

The frequency and length of the various microsatellite classes identified for the organisms included in this study closely resembled those of related taxa for which whole-genome sequence information is available (Figure 1). The microsatellite classes in *A. mellifera*, *C. elegans*, and *F. verticillioides*, have frequencies and corresponding repeat numbers that are not substantially different to those identified for *S. noctilio*, *D. siricidicola* and *F. circinatum*, respectively (Figures 1 and 2.). These relative abundance values are also comparable to those

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observed in a previous survey of eukaryotic genomes (Tóth et al., 2000). For example, trinucleotide motifs were found to be more abundant in fungi than animals, while dinucleotide motifs were more abundant in animals than in fungi (Tóth et al., 2000). *Sirex noctilio* was the only exception as we found no pentanucleotide microsatellite motifs (Figure 1) (Tóth et al., 2000), which could be a function of the insect's evolutionary history (Selkoe and Toonen, 2006). The new method presented here, therefore, is not biased for specific microsatellite repeat classes.

The difficulties associated with developing microsatellite markers for eukaryotes (Zane et al., 2002) and fungi in particular (Lim et al., 2004, Karaoglu et al., 2005, Dutech et al., 2007), are well documented. These difficulties mostly arise from the small genome size and/or the low genomic abundance of microsatellite loci (Lim et al., 2004, Karaoglu et al., 2005, Dutech et al., 2007). The fact that we did not encounter pentanucleotide microsatellites for *S. noctilio* (Figure 1) is probably a manifestation of the latter problem, where microsatellites are not only relatively rare, but certain classes of microsatellites also have limited distribution. Despite these potential limitations, the new technique allowed us to identify a large number of potentially amplifiable microsatellite motifs for the two animal and the fungal representatives (Table 1). For *S. noctilio* and *D. siricidicola* 16-29% of the sequenced contigs/singletons harbored potentially amplifiable microsatellite motifs, which is comparable to those reported in many animal microsatellite discovery studies (Plantard and Porte, 2003, Tsuchida et al., 2003, Kankare et al., 2004, Johnson et al., 2006). For the fungus, at least 12% of our contigs/sequence reads harbored microsatellites from which primers could be designed, which is within the range that has been reported previously for fungi (Lim et al., 2004, Karaoglu et al., 2005, Dutech et al., 2007). Our findings for *F. circinatum* are also consistent with those of previous studies, where no amplifiable microsatellites were found using Sanger sequencing of cloned ISSR amplicons (Britz et al., 2002) and FIASCO enriched (unpublished data) libraries. Therefore, the relatively low number of microsatellites identified for this fungus most likely reflects the limited representation of microsatellites in the genome of *F. circinatum*.

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The enrichment procedure employed in any microsatellite study is an important consideration. Our results clearly showed that ISSR-PCR targets significantly more microsatellites than FIASCO, although the latter method yielded more sequences that are potentially transformable to PCR markers (Table 1). This may be due to the fact that ISSR-PCR employs primers that contain microsatellite sequences, the application of which results in sequences containing microsatellites at their terminal ends. As such, these terminal microsatellites lack suitable flanking regions for primer design and thus, are not directly convertible to amplifiable markers. For this reason, we excluded all contigs and sequence reads with microsatellites located only in the terminal regions. Although considerable sequence information was lost in this way, large numbers of sequences–comparable to those identified using FIASCO–remained, from which primers could potentially be designed. We also performed direct comparisons of the two methods in terms of frequency distribution of microsatellite class and microsatellite length for the two *S. noctilio* genomic DNA libraries, enriched using the two methods. In this case, no significant differences (Figure 1) ($P>0.05$) were found. This is in contrast to anecdotal evidence (Dutech et al., 2007) suggesting that ISSR-PCR enrichment preferentially leads to the isolation of short microsatellites (Dutech et al., 2007). We therefore believe that either enrichment protocol can be used to characterize microsatellites for any organism of interest. However, we suggest that the more labor-intensive FIASCO method only be used in cases where specific microsatellite classes are needed, as enrichment with specific microsatellite probes will facilitate their quick identification. Where there is no need for the identification of a specific microsatellite class, ISSR-PCR represents an excellent enrichment option as numerous microsatellites and microsatellite classes can be identified rapidly. Furthermore, in cases where microsatellite loci are exceptionally rare, the ISSR-PCR based deep sequencing approach will be valuable. Even if microsatellites are not situated towards the middle of contigs, genome walking procedures could still be used to convert the terminal microsatellites to amplifiable markers.

Elimination of the construction of microsatellite-enriched clone libraries and substituting Sanger sequencing with pyrosequencing significantly enhances the cost-effectiveness of microsatellite discovery. For *F. circinatum*, a total of 231 amplifiable microsatellites were

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identified from the pyrosequencing data after one sequencing run using pooled ISSR-PCR products. In contrast, only eight amplifiable microsatellites were discovered following Sanger sequencing of 100 cloned ISSR-PCR fragments. To obtain the same number of microsatellites as generated from our pyrosequencing data, approximately 2800 additional clones would have to be sequenced. However, in reality, the number of clones will be substantially greater since our estimate assumes a 100% sequencing success and presence of usable microsatellite sequences in all clones. At the time of this study, the cost of generating the 1692 unique sequences that were obtained for *F. circinatum* through pyrosequencing would have been 62% greater if Sanger sequencing was employed. When only amplifiable microsatellites are considered, pyrosequencing resulted in a 276% cost reduction per microsatellite. As technology develops, the price of identifying new microsatellites with pyrosequencing will be further reduced, allowing for even cheaper development of microsatellites.

In order to show that the microsatellite regions that were sequenced were also useful for the development of polymorphic markers, we synthesized primers flanking 29 microsatellite regions in *F. circinatum*. Of these, 19 produced single amplicons, and their application on a set of only eight *F. circinatum* isolates showed that 13 primer pairs targeted polymorphic microsatellites loci (Table 2). Thus, in a space of 2 months, we were able to identify more PCR-based molecular markers than have been available previously for this species (Wikler and Gordon, 2000, Britz et al., 2002). What makes the technique described here particularly powerful is that while 29 microsatellite regions were chosen for analysis, a further 212 microsatellite regions are available for testing, without requiring any further cloning or sequencing. In addition, we were able to be selective regarding the regions used to develop primers. This is in contrast to previous studies with severe limitations in the amount of available sequence (Britz et al., 2002). Therefore, despite the fact that no sequence data are available for a specific species, our approach of microsatellite enrichment and deep sequencing genomic DNA libraries makes it possible to rapidly develop many of these powerful genetic markers.

The results of this study clearly demonstrate that sequencing essentially all enriched PCR amplification products for microsatellite regions using 454 genome sequencing technology

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represents a superior alternative to conventional screening of clone libraries using Sanger sequencing. This methodology for microsatellite discovery is much more robust than cloning and sequencing of individual fragments, which is substantially more time consuming and delivers few microsatellite markers from a large library. It is possible to generate high confidence sequence data that can be used to identify microsatellite regions in approximately one week. This technique also enables the sequencing of larger amounts of microsatellite-containing DNA that might have been lost due to inherent inefficiencies in the cloning of these fragments (Dutech et al., 2007). This considerable saving of time and effort makes the development of microsatellites a much more certain and reasonable exercise for most research groups.

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Table 1. Sequence data generated and microsatellites characterized from deep sequencing of libraries.

Species	Sequence data generated (Mb)	Sequence reads	Contigs and singletons	Sequences containing microsatellites [a]	Sequences with amplifiable microsatellite sequences[b]
F. circinatum (ISSR)	1.67	8644	1692	1644 (97%)	231 (14%)
S. noctilio (FIASCO)	1.47	7016	1840	463 (25%)	336 (18%)
S. noctilio (ISSR)			1013	512 (50%)	159 (15%)
D. siricidicola (FIASCO)	1.22	6388	1040	421 (40%)	296 (28%)

[a]Sequences that contain microsatellites that are larger than 100bp in size. (Percentage in parentheses show amount from original contig and single reads.)

[b]Microsatellites that are not within 20 bases of the terminal regions of the sequence. (Percentage in parentheses show the proportion of the amplifiable microsatellites relative to the total number of microsatellite-containing contigs/singletons.)

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Table 2. Polymorphic microsatellite PCR primers designed for *F. circinatum.*

Locus Name	GenBank accession number	Repeat sequence	Primer name	Primer sequence	Annealing Temperature (°C)
FCM-2	FJ436307	$(tttc)_6$	FCM-2A	CGGAAGCAATCAGGACATTT	50
			FCM-2B	GAGCATGATGTCTCTCGAAGC	
FCM-3	FJ436308	$(catgag)_6$	FCM-3A	CAGTATGATAAGGCACCCATGT	53
			FCM-3B	GACTGACCCCTTGCCCTTAT	
FCM-4	FJ436309	$(ttctt)_5$	FCM-4A	TGGTCCCGGCTCATTTACTA	50
			FCM-4B	AAAAGAAGACCCGCCTGATG	
FCM-6	FJ436310	$(gtgc)_7$	FCM-6A	GATGGAGATGAATGGGGAAA	50
			FCM-6B	GCCTCAGGTTGGTCTGGTTA	
FCM-7	FJ436311	$(aggaga)_5$	FCM-7A	ACGGCAGTGAAAAGAAGCAT	50
			FCM-7B	CAAGACCCTCTTGGCATCTC	
FCM-9	FJ436312	$(caacga)_8$	FCM-9A	CGACGACGACGACAACGAC	60
			FCM-9B	CTCCTCTTTGGCCCTCTTG	
FCM-16	FJ436313	$(catcca)_{18}$	FCM-16A	CGGATGAGAAAGCGAGAGAG	50
			FCM-16B	GGTGGATCACAGACCACAAA	
FCM-19	FJ436314	$(a)_{13}$	FCM-19A	GCGTCTTCCTCTGCCATTT	50
			FCM-19B	TAAGATTGAGGTTGTGCGGTTG	
FCM-20	FJ436315	$(actgt)_9$	FCM-20A	GCTGATCGAAGCCAATCG	50
			FCM-20B	TGACTACGCCAGAAGAGACG	
FCM-23	FJ436316	$(cactt)_8$	FCM-23A	GGAGGTTATTGTCCGTCTCAA	50
			FCM-23B	ACTGAGGTGTGCCAAGCTGT	
FCM-24	FJ436317	$(tgattg)_7$	FCM-24A	GACAGTTAGTCAGTCTTAGTCTCG	45
			FCM-24B	GTCTCTTGCAGTCACAATCAC	
FCM-25	FJ436318	$(tgtct)_6$	FCM-25A	TGATTCCTCCTGCCTCATTC	50
			FCM-25B	TAGGGCGATGTCTCTGGTTT	
FCM-26	FJ436319	$(a)_{12}$	FCM-26A	CAAACCGGCCAGAGACAG	45
			FCM-26B	TTCTCCAACCACCCTTGAAC	

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Figure 1. Distributions of microsatellite repeat classes for the organisms of interest. Percentages were calculated according to the total number of microsatellites for a respective class divided by the total microsatellites identified for that organism.

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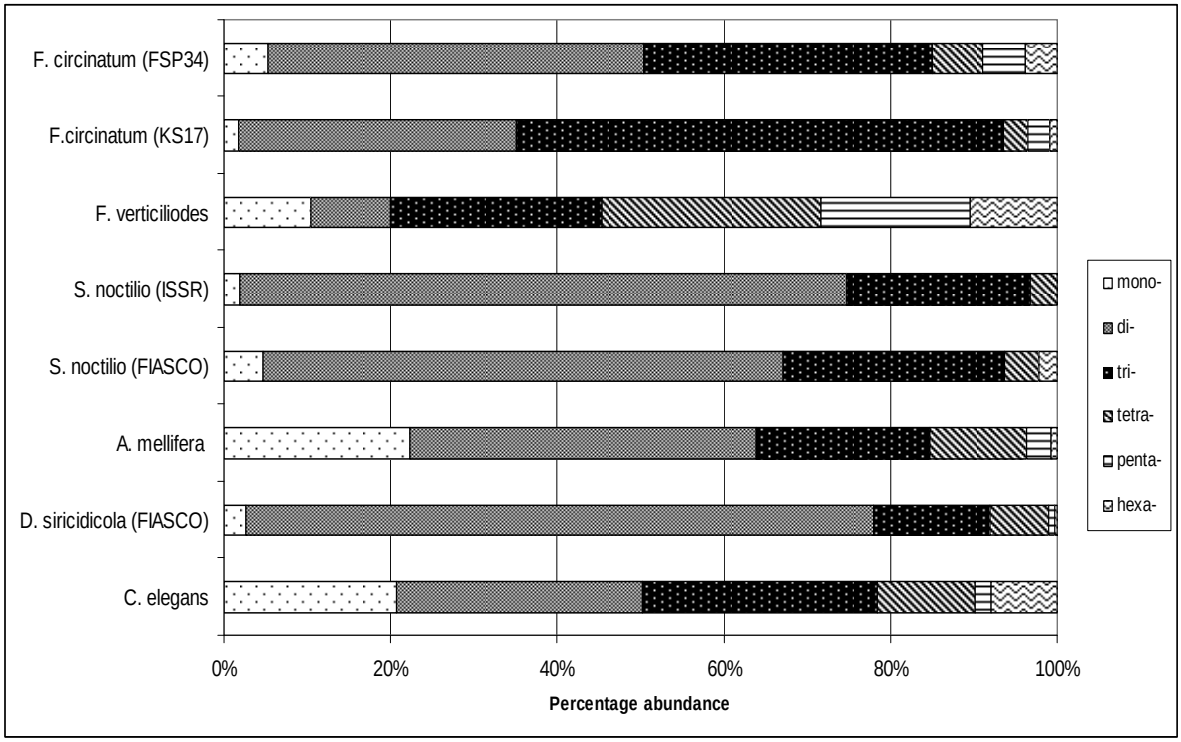

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Figure 2. Average repeat length of all microsatellites discovered for each repeat unit class. Repeats were measured as the total number of times a motif was repeated. The average was taken for all microsatellites identified in a specific repeat class.

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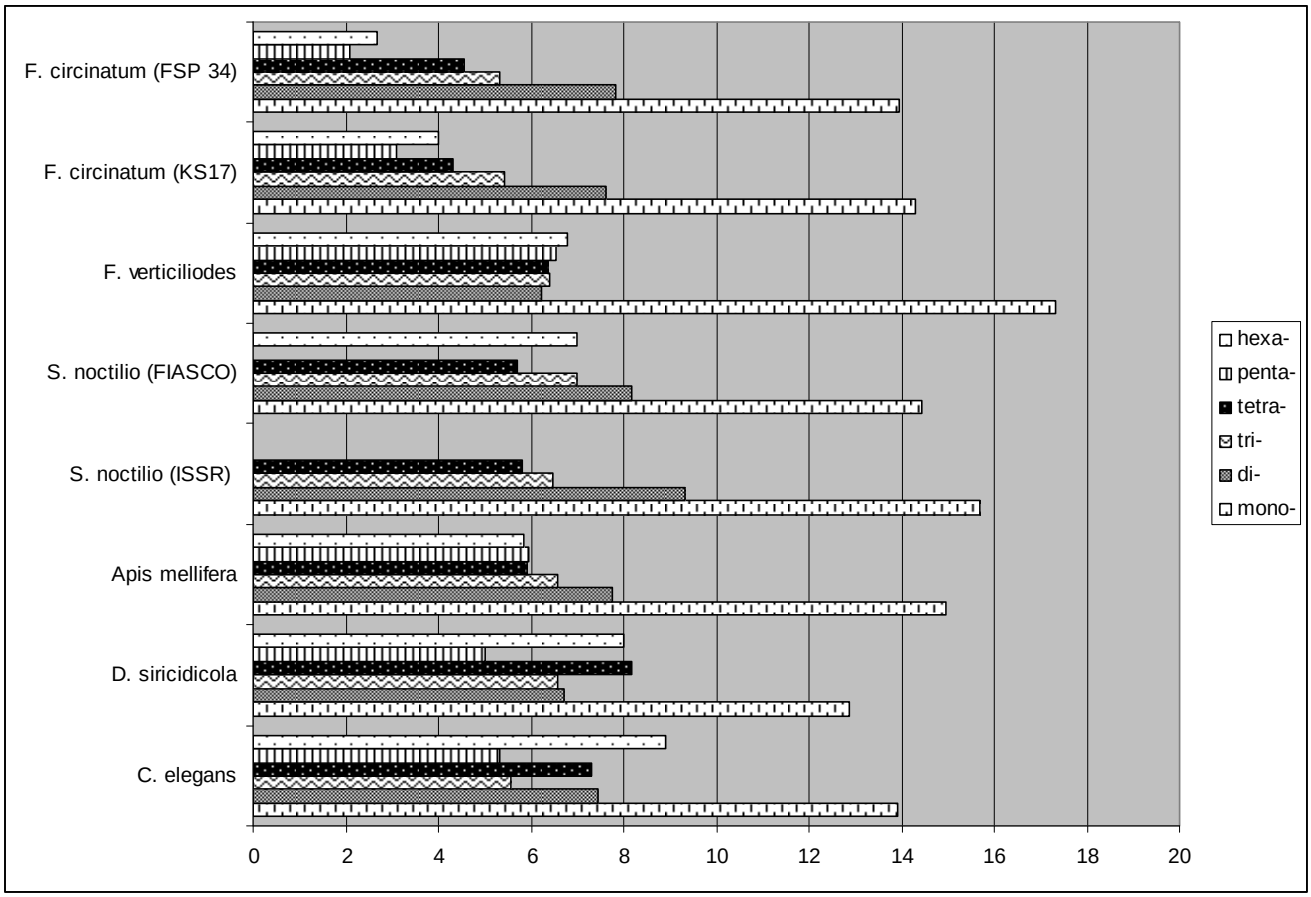

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CHAPTER 3

Extreme homozygosity in Southern Hemisphere populations of *Deladenus siricidicola*, a biological control agent of *Sirex noctilio*

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3. Abstract

The woodwasp *Sirex noctilio,* together with its mutualistic fungal symbiont *Amylostereum areolatum*, is the most damaging invasive pest of *Pinus* spp. in the Southern Hemisphere. The nematode *Deladenus siricidicola* parasitizes *S. noctilio* larvae and is the most effective biological control agent against this woodwasp. Nothing is known regarding the genetic diversity of *D. siricidicola,* even though such knowledge would be invaluable in improving sustainable biological control programs. The aim of this study was to develop microsatellite markers to study the genetic diversity of *D. siricidicola* populations. Microsatellite enrichment was performed using Fast Isolation by AFLP of Sequences Containing repeats (FIASCO) and fragments were then sequenced using 454 GS-FLX pyrosequencing. From the 1.2 megabases of sequence data, 166 microsatellite containing contigs were identified. Twenty-six primer pairs were designed using the web-based program Primer3 and screened for polymorphism in populations of the nematode from different sources in the Southern Hemisphere. Seventeen primers amplified microsatellite-containing loci of interest. No length polymorphism was present in any of the microsatellite repeats in these populations. Regions flanking the microsatellites also showed no polymorphism, except for one transition observed in an Argentinean strain for locus *Ds316*. Twelve of the loci showed polymorphism between the Southern Hemisphere and Canadian sources of *D. siricidicola*. The lack of diversity in Southern Hemisphere populations of *D. siricidicola* could affect the ability of this nematode to adapt to different environments and host types where it is used in biological control programs, and should thus be considered as a factor in future control strategies and research projects.

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3.1 Introduction

The nematode *Deladenus siricidicola* Bedding is the primary and most effective biological control agent against the invasive woodwasp *Sirex noctilio*. This wasp and its obligate fungal mutualist, *Amylostereum areolatum* Boidin, is one of the most serious pests of *Pinus* spp. in Southern Hemisphere forestry plantations. *Deladenus siricidicola* has a bi-cyclic life cycle (Bedding, 1967, Bedding, 1972, Bedding and Akhurst, 1978). During the free-living cycle, the nematode feeds on the basidiomycete fungus *A. areolatum* growing in the infested trees. In its parasitic cycle, the nematode penetrates and develops inside the wasp larvae. Eggs of the nematode are triggered to convert to the infective form by the high CO_2 and low pH conditions that surround the Sirex larvae (Bedding, 1967, Bedding, 1972, Bedding and Iede, 2005). Once wasp pupation begins, the fertilized female nematodes release juveniles that migrate and infest the reproductive organs, eventually entering the eggs and sterilizing the female *S. noctilio*.

Sirex noctilio is native to Eurasia and North Africa (Morgan, 1968, Spradbery and Kirk, 1978) and was accidentally introduced into the Southern Hemisphere via New Zealand in the early 1900's. Subsequent biological invasions were observed in Australia in 1952 (Neumann et al., 1987), Uruguay in 1980 (Maderni, 1998), Argentina in 1985 (Klasmer et al., 1998), Brazil in 1988 (Iede et al., 1998), South Africa in 1994 (Tribe, 1995), Chile in 2000 (Ahumada, 2002) and more recently the United States of America in 2005 (Hoebeke et al., 2005) and Canada (de Groot et al., 2006).

Deladenus siricidicola was discovered in 1962 in New Zealand, parasitizing *S. noctilio* (Zondag, 1969). An extensive research program was established in New Zealand and Australia to characterize the biology of the nematode, and to develop a biological control program. This program eventually resulted in the selection of a virulent Hungarian strain (Sopron strain) of *D. siricidicola* for use as a biocontrol agent (Bedding and Akhurst, 1974, Zondag, 1979). This strain was widely released in Sirex-infested plantations in Australia. In the late 1980's the Sopron strain was reported to have lost its virulence, presumably due to continuous mass rearing on the fungal symbiont (Haugen and Underdown, 1990, Bedding and Iede, 2005). A virulent strain

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of the nematode was then isolated from the Kamona forest (site of the original release of virulent Sopron strains) in Tasmania, and this Kamona strain was used to inoculate infested plantations in Australia (Bedding and Iede, 2005). The success of the program led to the distribution of the Kamona strain to other Sirex-infested countries in the Southern Hemisphere. A strain known as 'Encrusilada do sul' was isolated from the field-infested pines in Brazil and selected for biological control due to its higher level of virulence. The relationship between the Brazilian strain and Kamona has not been determined.

The rate of *D. siricidicola* parasitism of Sirex wasps in inoculated trees varies between 5 – 90 % in different regions of the Southern Hemisphere (Hurley et al., 2007). These authors hypothesized that a number of factors could affect this variation in parasitism by the nematode, including inoculation techniques, moisture content of the wood, loss of virulence of the nematode, incompatibility among the specific populations of *S. noctilio*, *A. areolatum* and *D. siricidicola* in that area, competition of saprophytic fungi with *A. areolatum*, and pine species affected. In a subsequent study, (Hurley et al., 2008) excluded inoculation techniques and loss of nematode virulence as factors affecting *D. siricidicola* parasitism in South Africa. While wood moisture content affected parasitism levels, this did not explain the breadth of the observed variation. This highlights the possible role of other factors that might affect nematode efficacy and that remain to be tested.

Population diversity studies on *D. siricidicola* and *S. noctilio* have yet to be conducted. The aim of this study was to characterize microsatellite containing markers specific for *D. siricidicola* that could be used in studies of population genetic variation. These markers are then applied to assess the diversity among strains from populations of *D. siricidicola* from Australia, Argentina, Brazil and South Africa as well as to determine the level of diversity and the historical relationship between these nematode strains. This diversity is also compared with that of strains of the nematode that have recently been discovered in Canada (Yu et al., 2009).

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3.2 Materials and Methods

3.2.1 *Nematode sources and harvesting*

Sources of *D. siricidicola* were from Argentina (two sources; Argentina1, Argentina2), Australia, Brazil and South Africa for the Southern Hemisphere, and Canada from the Northern Hemisphere (four sources; 181, 184, 484 and 1089). Nematode sources refer to cultures of the nematode made from an individual wasp in the different regions of origin. All nematode cultures are maintained by the Tree Protection Co-operative Program based in the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (http://www.fabinet.up.ac.za).

Nematodes were harvested from cultures grown in 500 ml Erlenmeyer flasks containing wheat, rice and *Amylostereum areolatum*, or on potato dextrose agar (PDA) plates onto which *A. areolatum* had been inoculated (Bedding and Akhurst, 1974). Nematodes were harvested from the flasks by soaking (15 min.) and washing with distilled H_20. The nematode suspension was poured through a sieve into 1L beakers and nematodes were allowed to settle for 20 min. For isolation from Petri dish culture plates, sterile distilled water (SABAX water, Adcock Ingram Ltd, Bryanston, RSA) was added to the plates to cover the cultures and left to stand for 20 min, while swirling four times during this period. The suspension was decanted into 50 ml beakers and nematodes were allowed to settle for 10 min. After settling, for both of the above techniques, excess water was discarded and nematodes were washed four times at intervals of 20 min in sterile distilled water, each time discarding excess water, prior to the subsequent wash. Following the last wash, 1 ml of sample was transferred to 1.5 ml Eppendorf tubes, centrifuged at 13 000 rpm for 3 min, the supernatant was discarded and the sediment was used for DNA extraction.

3.2.2 *DNA extraction*

Two DNA extraction methods were used. A phenol-chloroform DNA extraction method was used to extract DNA for microsatellite enrichment and discovery, from large numbers of

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harvested nematodes from flasks as described above (source Argentina1). The nucleic acids were precipitated by 0.1 volume of 2M NaAc (pH 5.6) and 1 ml of 100 % EtOH. The sample was left overnight and the DNA was obtained after centrifugation (Eppendorf Centrifuge 5417C), washing the pellet with 1 ml of 70 % EtOH, vacuum drying at 45°C for 5 min (Concentrator 5301), and resuspending with 100 µl of Sabax water. Five microlitres of RNase was added to the resuspended sample, followed by incubation on HB-2 heat blocks (Wealtec Corporation) at 37°C for 3 hours.

Pooled DNA extractions from all nematode sources were performed using PrepMan™ (Applied Biosystems, California, USA) in 20 µl, and using nematodes harvested from PDA culture plates as described above. Fifteen microlitres of PrepMan Ultra sample Preparation Reagent and a 2 mm-diameter sterile metal ball was added to each sample-containing Eppendorf tube. The samples were shaken at a frequency of 30 vibrations/s for 3 min using the Retsch® MM 301 (Retsch, Germany). The metal balls were then removed with a magnetic stick, and the samples vortexed for 15 s and incubated at 100 °C for 10 min. Following incubation, samples were centrifuged at 13 000 rpm for 3 min, after which the DNA containing supernatant was transferred into sterile 1.5 ml Eppendorf tubes. The concentration of the resulting DNA was determined using the ND – 1000 UV/Vis Spectrometer (NanoDrop Technologies, Wilmington, DE USA).

3.2.3 *Microsatellite enrichment and pyrosequencing*

The fast isolation by AFLP sequence containing repeats (FIASCO) method (Zane et al., 2002) with modifications (M-FIASCO) (Cortinas et al., 2006) was used for microsatellite enrichment of *D. siricidicola* source Argentina1. This was followed by pyrosequencing as described by (Santana et al., 2009).

3.2.4 *Primer design and amplification*

Sequences obtained from pyrosequencing were assembled into contigs using the ContigExpress component of Vector NTI Explorer software (Invitrogen, Carlsbad, CA, USA). From these contigs,

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microsatellite repeats were identified using the web-based program Simple Sequence Repeat Identification Tool (SSRIT) (Temnykh et al., 2001). The minimum number of repeats used in order to design primers for microsatellite containing sequences was five repeats for all repeat classes. Primers were designed using the PRIMER 3.0 software (Rozen and Skalestsky, 2000).

Primers were optimized and used on all *D. siricidicola* sources from harvested cultures (i.e. large numbers of nematodes pooled in each of the DNA isolation reactions). Polymerase Chain Reactions (PCR's) were performed using the thermal *i*Cycler (Bio-Rad). DNA from various populations was used as the template in a 25 µl reaction volume containing 10× PCR buffer (10x solution, 100 mM Tris-HCl, 500 mM KCl, pH 8.3) (Roche Diagnostics GmbH, Germany), 25mM $MgCl_2$ (Roche Diagnostics GmbH), 10 mM dNTPs, 10µM of each forward and reverse primer, 2.5 U of FastStart *Taq* DNA Polymerase (Roche Diagnostics GmbH) and sterile SABAX water was added to make up the total volume for PCR amplification. The thermocycler conditions were an initial denaturation step at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min and a final extension step of 72 °C for 10 min. PCR products were separated on 2 % agarose gels, stained with ethidium bromide (EtBr) and visualized under UV transilluminator imaging system (UVP, United Kingdom). A 100 base-pair (bp) molecular weight marker (Fermentas, O' Gene Ruler ™) was used for size estimates of PCR products.

The presence of *A. areolatum* DNA in *D. siricidicola* extracted DNA was determined using basidiomycete primers, P – 1 (5' TTG CAG ACG ACT TGA ATG G 3') (Hsiau, 1996) and 5S – 2B (5' CAC CGC ATC CCG TCT GAT CTG CG 3') (Coetzee et al., 2000). These primers targeting the IGS rDNA region were also tested on *A. areolatum* isolate F8 NCT (*A. areolatum* isolate from the field populations of *S. noctilio* in South Africa) using the same conditions as for *D. siricidicola* DNA.

For Southern Hemisphere sources of *D. siricidicola,* the microsatellite loci (excluding Ds 105, Ds 302, Ds 323, Ds 350 and 366) were also amplified from four single nematodes from all sources. For single nematode amplification, nematodes were transferred from *A. areolatum* cultures

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using 0.80 x 40 mm sterile needles (New Promex Corporation) into 96 well PCR microplates (Axygen Scientific, California) using a Stemmi 2000 microscope (Zeiss, Germany). Each well contained one nematode in a 25 µl of the PCR reagent mixture as described above. The thermocycler conditions for amplification were an initial denaturation step at 95 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60°C for 30 s, extension at 72 °C for 1 min and a final extension step of 72°C for 10 min. The PCR products were visualized as described above. In order to assess possible polymorphism, PCR products from single nematodes were separated by 15 % polyacrylamide gel electrophoresis (PAGE) (30 % acrylamide, 10x Tris-borate-EDTA buffer, 10 % APS and TEMED; 4h at 160V). Once the run was complete, the gels were placed into a Syber Gold (Molecular Probes, Inc., Eugene, OR, USA) solution for 30 min and then visualized under the UV transilluminator.

3.2.5 *DNA sequencing*

PCR products were purified using 6 % Sephadex G-50 columns with 50 – 150 µm bead size (Sigma Aldrich, Germany) following manufacturer's instructions, before sequencing. Purified PCR products were used as template DNA in cycle sequence reactions using the ABI BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems). The same primers used for PCR amplification were used to sequence both strands during cycle sequencing reactions. The thermocycler conditions were 25 cycles of denaturation at 96 °C for 10 s, annealing at 55 °C for 15 s and extension at 60 °C for 4 min using the thermal *i*Cycler (Bio-Rad). The products were purified as previously described and precipitated using the Concentrator 5301 (Eppendorf AG, Germany) at 45 °C for 30 min. Precipitated PCR products were run on an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, California, USA).

3.3 Results

Both harvesting methods yielded large numbers of nematodes from which DNA was extracted. DNA extraction from the flask-reared nematodes using the phenol – chloroform method was considerably more labor intensive and time consuming compared to DNA extraction performed using PrepMan™ (Applied Biosystems, California, USA) from PDA cultures.

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The outcomes of the FIASCO enrichment and pyrosequencing have previously been reported and compared with a similar application of these tools to the fungus *Fusarium circinatum* and *Sirex noctilio* by Santana et al. (2009). In summary, 1.2 Mb of sequence data were generated using this method, which was assembled into 397 contigs or fragments represented by single reads. Of these, 166 fragments contained microsatellites with dinucleotides, trinucleotides and tetranucleotides, comprising 93 %, 2 % and 5 % of the fragments, respectively.

A total of 26 primer pairs were designed to amplify microsatellite-containing loci, of which 17 could be optimized to amplify single fragments (Table 1). These fragments were confirmed to represent the loci of interest through sequencing and comparison with the original fragments. Amplifications were equally successful from DNA extracted from culture plates (including large numbers of nematodes per sample) and for amplifications from single nematodes. DNA of *A. areolatum* was detected in the extracted nematode DNA through amplification of the IGS rDNA region using the primers P-1 and 5S-2B. None of the primer pairs resulted in the amplification of fragments when DNA extracted from *A. areolatum* cultures was used in the reactions.

No length polymorphisms were identified in any of the 17 microsatellite containing loci using agarose and polyacrylamide gel electrophoresis for any of the five sources of *D. siricidicola* from the Southern Hemisphere. This was confirmed by sequencing 10 - 25 representative products for each of the 12 loci. These sequences also confirmed that there were no polymorphisms in regions flanking the microsatellites, except for one transition (T/C) observed for the strain Argentina2 for locus *Ds316*, compared to other sources of the nematode from the Southern Hemisphere. The sequence data for 17 primer pairs covered 3291 bp including 317 bp of microsatellite repeated sequences.

Comparison of PCR product sequences for the 17 selected loci between Southern Hemisphere and Canadian sources of the nematode (DNA isolated from culture plates) revealed polymorphisms in 12 of the loci (Table 1 and 2). These polymorphisms included microsatellite length variations, single nucleotide polymorphisms and insertions/deletions (indels) (Table 2).

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3.4 Discussion

This study is the first to consider the diversity among populations of *D. siricidicola* from different regions in the Southern Hemisphere. It was expected that there might be reduced diversity and a genetic linkage between these populations, given their shared history of introduction and the expected bottlenecks resulting from the rearing process. The extent of homozygosity was, however, unexpected. Among 3291 bp of sequence data, spanning 317 bp of microsatellite repeats, there was only one base pair variation between a source obtained from Argentina and all other sources of the nematode from the Southern Hemisphere. These results confirm that the Kamona strain has been distributed throughout the Southern Hemisphere, and from a single origin. In contrast, strains of *D. siricidicola* from Canada provided a firm basis for comparison and were clearly genetically distinct from all the Southern Hemisphere strains.

The analysis of the Southern Hemisphere populations of *D. siricidicola* using pooled DNA from cultures, confirmed by genotyping single nematodes from all populations, showed that a highly inbred population of the nematode exists throughout the Southern Hemisphere. This is likely the result of repeated genetic bottlenecks and inbreeding associated with the culturing and introduction of the nematode into new environments. When nematodes are reared, a circular plug of 13 mm in diameter containing between >100 – >2000 nematodes (Brett Hurley, unpublished data) is selected from a culture, which is then used to repeatedly produce subcultures. The plug is also likely to contain many nematode siblings, since female nematodes lay their eggs in groups. Such sub-culturing typically occurs bi-weekly, until the nematodes are transferred onto flasks for mass production or stored in liquid nitrogen (Bedding and Iede, 2005). When *D. siricidicola* is introduced into new regions for biological control programs, only a few cultures are typically introduced. These steps of multiplication, bulking and transfer to new environments, repeated in many laboratories during the course of the last two decades, will inevitably have resulted in genetic bottlenecks and inbreeding for the Kamona strain of *D. siricidicola*. Results of this study confirm this view.

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Founder effects and inbreeding have been shown to affect the population diversity of other nematodes in a similar fashion to *D. siricidicola*. For example, a study conducted on the pine wood nematode, *Bursaphelenchus xylophilus*, an introduced pathogen in Japan (Mamiya, 1987), showed a low degree of microsatellite polymorphism (relative genetic uniformity) in sub-populations (Zhou et al., 2007). This was attributed to a founder effect. Similarly, studies on the cyst nematodes *Heterodera schachtti* (Plantard and Porte, 2003, Plantard and Porte, 2004) and *Globodera pallida* (Picard et al., 2004) have shown that they are highly inbred species due to increased probability of interbreeding between siblings mating, which is influenced by their limited dispersal range. Larvae from a single cyst are siblings or half-siblings, depending on whether a single or several males fertilize a female. This mode of reproduction can significantly influence and reduce population diversity. The same situation appears to be true for *D. siricidicola* in the Southern Hemisphere.

Comparison of *D. siricidicola* sources from the Southern Hemisphere and Canada confirmed that each of these areas harbours a different strain of the nematode. *Deladenus siricidicola* was thought not to be present in North America, and was only recently discovered in that region (Bedding and Akhurst, 1978, Yu et al., 2009). Yu et al. (2009) confirmed the species identity using morphological characteristics and comparison with the Kamona strain based on DNA sequences. Using sequences for ribosomal DNA region and the cytochrome oxidase subunit 1 (COI), these authors also showed that there were sequence differences between North America *D. siricidicola* and the Kamona strain. These differences included seven substitutions and ten indels for the rDNA region, and two base pair differences for the COI. However, subsequent work has shown that a portion of the Canadian population is the same or similar to the Kamona strain (Isabel Leal, unpublished data). The microsatellite markers developed here will be useful to further characterize the differences between the Canadian and Kamona strain of *D. siricidicola*.

It is commonly accepted that *D. siricidicola* was accidentally introduced into Canada. Such an introduction would not be unusual, in view of the fact that the original discovery of the nematode in New Zealand during the early part of the 20th century resulted from its accidental

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introduction together with *S. noctilio* (Zondag, 1969). In tracing the origin of the *S. noctilio* invasion into the United States of America, (Nielsen et al., 2009) showed that *A. areolatum* found in North America was represented by two genotypes, which were different from the genotypes found in the Southern Hemisphere (Slippers et al., 2001, Slippers et al., 2002). These results suggest that the invasions of *S. noctilio* into the Southern Hemisphere and North America represent two separate events. In contrast, a recent study using multiple locus sequence data has suggested that one of the genotypes of *A. areolatum* in Canada is identical to that in the Southern Hemisphere (Bergeron et al., 2010). This would imply a shared origin of introduction of *S. noctilio* into North America and the Southern Hemisphere. Regardless of whether there is a link between the *S. noctilio* introductions in North America and the Southern Hemisphere, the introduction of *D. siricidicola* appears not to be connected to that of the fungal strains observed in the Southern Hemisphere. This provides an opportunity for North American (including Canada) and Southern Hemisphere countries to exchange nematode strains and thus to increase genetic diversity and potentially the efficacy of biological control programs in these regions.

The loci for which markers have been developed in this study might have broader application than simply microsatellite length variation in populations of *D. siricidicola*. A number of loci also contained SNPs and indels in the microsatellite flanking regions. These variations were in some cases fixed in the populations, for example between those of the Kamona strain from the Southern Hemisphere and strains from Canada. These markers could thus be used for rapid distinction between the strains using sequencing, real-time PCR probes or primers, or PCR-RFLP screens (Nasmith et al., 1996, Aikawa et al., 2006).

Next generation sequencing coupled to microsatellite enrichment protocols has provided important new opportunities for the effective development of microsatellite markers for population studies, as illustrated in this study. The use of pyrosequencing enabled the exclusion of the time–consuming cloning step, generated substantially more microsatellite containing sequences than would have been feasible with the traditional cloning and Sanger sequencing approaches, and substantially reduced the overall costs associated with development of

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microsatellites. This tool has recently been described in a series of studies, with slight modifications, and applied widely to various organisms. These include the venomous copperhead snake *Agkistrodon contortrix* (Castoe et al., 2010), the pine pathogenic fungus *Fusarium circinatum*, the pine-damaging woodwasp *Sirex noctilio* and its parasitic nematode *Deladenus siricidicola* (Santana et al., 2009), ancient DNA of the extinct heavy-footed moa *Pachyornis elephantopus* (Allentoft et al., 2009), and New Zealand's endangered blue duck *Hymenolaimus malacorhynchos* (Abdelkrim et al., 2009). Interestingly, in the latter two studies, microsatellites are known to occur in low frequency, and yet this tool enabled characterization of markers that are crucial in understanding past biodiversity and extinction processes.

The nematode harvesting method used in this study did not rid the *D. siricidicola* samples of all residual *A. areolatum* fragments, even after multiple washes. This meant that DNA extracts contained a mixture of fungal and nematode DNA, and therefore a possibility of inadvertently developing primers from fungal sequences. All primers were therefore tested for their ability to amplify pure *A. areolatum* DNA, but this did not occur. This is probably because the fungal DNA was in a much lower concentration compared to the nematode DNA. The primers can thus now be applied in situations in which even greater amounts of fungal DNA are present.

It is of substantial concern that Southern Hemisphere populations of *D. siricidicola* appear to be highly inbred, and also homozygous, at most of the loci tested. This lack of diversity is likely to significantly reduce the ability of the nematode to adapt and establish itself in different environments, on different *Pinus* spp. and on different populations of *S. noctilio* (Roderick and Navajas, 2003, Hufbauer and Roderick, 2005). The environments in which the nematode is applied for biological control purposes include a variety of climate types, from Mediterranean winter-rainfall, to continental summer-rainfall, cold temperate winter rainfall and subtropical environments. In addition, various *Pinus* spp. are involved, including *P. radiata, P. patula, P. elliottii, P. sylvestris, P. taeda,* and *P. caribaea, P. contorta var. latifolia* and *P. ponderosa*. It is also known that the fungus *A. areolatum*, which is an obligate food source for the nematode, and different wasp populations can differ genetically and phenotypically across regions (Hurley et al., 2007, Hurley et al., 2008). These authors also highlight the fact that there are other

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genera of fungi in trees where the nematode is applied, which also have the potential to reduce the efficacy of *D. siricidicola* as a biocontrol agent. These and probably other less obvious factors make it highly unlikely that a genetically homozygous population of *D. siricidicola* will be an effective biocontrol agent over the longer term. Thus, for an effective biological control program for the Southern Hemisphere, native and genetically diverse populations of *D. siricidicola* would need to be characterized and tested in different environments where biological control is required. Apart from native environments, New Zealand might also be a location from which diverse strains of *D. siricidicola* could be obtained, or observed. This region has had many introductions of the nematode spanning a long period of time. The Kamona strain of *D. siricidicola* has also not been deployed in New Zealand, and therefore the nematode populations in this country might be sufficiently genetically diverse to provide new sources of the biocontrol agent. The polymorphic markers produced in this study should be useful in future research requiring knowledge of the genetic diversity and structure of *D. siricidicola* populations.

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Table 1. Primer sequences, expected locus size per SSR primers, no. of SSR repeats, annealing temperature per primer of *Deladenus siricidicola.*

Locus	Primer name	Primer sequence (5' → 3')	Expected size	Repeats	Tm (°C)	Polymorphism (SH vs Can)
Ds 1	Ds 1F	CAATGTGCTGCGTCAATTTT	157 bp	$(GTA)_{10}$	56	Yes
	Ds 1R	ACCCAACGCGTAGTGATAGC			62	
Ds 7	Ds 7F	TTCAATTTTGTGTCTGGCAAA	249 bp	$(TG)_6$	55	No
	Ds 7R	AGCCACAAATCGCGACATA			58	
Ds 19	Ds 19F	CGTGACCGATTTCATTTTGC	192 bp	$(TCC)_5$	58	No
	Ds 19R	ACGACAGCGAAGAGAACGAT			60	
Ds 54	Ds 54F	CAGCCACAACAATTCACACC	155 bp	$(CA)_6$	60	Yes
	Ds 54R	GCACAAAAATCTCGCCTCAT			58	
Ds 83	Ds 83F	AGGCATAGAGCGAGTGGAAA	159 bp	$(GA)_5$	60	Yes
	Ds 83R	TCTCACGAACTTGTCCCTCA			59	
Ds 105	Ds 105F	TGGTAGCAATCGATCGAAAA	150 bp	$(AG)_9$	56	Yes
	Ds 105R	CGTGTCCACTTGTCCCTCTC			56	
Ds 201	Ds 201F	TGCATAGCTGGCGATAAATG	168 bp	$(TG)_7$	58	Yes
	Ds 201R	CGAGTCACGTACGCATTAGC			62	
Ds 302	Ds 302F	ATTGTTACGGTGTGGGCATT	215 bp	$(AC)_6$ $(CA)_5$	58	Yes
	Ds 302R	TGGATGTCCGTCTGTTGTGT			60	
Ds 308	Ds 308F	GCCTTTTCCTTAGCTGTTTGA	155 bp	$(TG)_6$	59	No
	Ds 308R	GTGGTCCTCCTCTCCCTTTT			62	
Ds 316	Ds 316F	TGCGGATATCTTCTCATTGTAA	382 bp	$(TC)_7$ $(TC)_5$	57	Yes
	Ds 316R	TCAAATGTTATGCGAAATTCTG			55	
Ds 318	Ds 318F	AGGGTACTCATGCCGAGGTT	127 bp	$(AC)_6$	63	No
	Ds 318R	ATGCGTATGTGCAGTTGTGT			58	
Ds 323	Ds 323F	TTTACCTGTTGGCTGTTACCG	165 bp	$(TG)_8$ $(TG)_6$	61	Yes
	Ds 323R	TGGGGTAAAAGTGGATTGGA			58	
Ds 325	Ds 325F	ACGCTTATGTGTGCCACTTG	298 bp	$(TG)_6$ $(GT)_8$ $(TG)_5$ $(CATA)_5$	60	Yes

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	Ds 325R	GGGTCTCTTGATGATGTTTCG			61	
Ds 350	Ds 350F	AGTCCTGAGTAACCTCCACCA	151 bp	$(GC)_5$	63	No
	Ds 350R	TACGGTACGCTGATGCTCAC			62	
Ds 366	Ds 366F	CGCTGCTGTACTGCTGTTTT	152 bp	$(GT)_5$	60	Yes
	Ds 366R	CACACAAATGCACACATGGA			58	
Ds 375	Ds 375F	GGCAGCTGAAATGATGACAA	188 bp	$(AC)_5$	58	Yes
	Ds 375R	ATCATCATCAGCAATATCCTCA			57	
Ds 388	Ds 388F	AAGTCAGCTGAAAGGCGAAG	228 bp	$(CA)_{10}$ $(AC)_4$	60	Yes
	Ds 388R	TGTGTGCATGAAAACGGAAC			58	

SH – Southern Hemisphere sources (Argentina1, Argentina2, Australia, Brazil & South Africa)

Can - Canadian sources (181, 184, 484, 1084)

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Table 2. Summary of polymorphisms observed per locus.

Primers	Strains	Types of polymorphism			No. of alleles
		SSRs	SNPs	Insertion	
Ds 1	SH	$(GTA)_{10}$	A/G	-	1
	Canadian	$(GTA)_{16}$		GTGGTAGT	1
Ds 54	SH	No variation	-	CACATACA	1
	Canadian	No variation		-	1
Ds 83	SH	No variation	G/T ; A/G	-	1
	Canadian	No variation		-	1
Ds 105	SH	$(AG)_9$	G/A	-	1
	Canadian	$(AG)_{13}$		-	1
Ds 201	SH	$(TG)_7$	G/A	T	1
	Canadian	$(TG)_4$		-	1
Ds 302	SH	No variation	-	-	1
	Canadian	No variation		T	1
Ds 316	SH	$(GA)_5$	-	-	1
	Canadian	$(GA)_7$		-	1
Ds 323	SH	$(TG)_8$	G/T	TTGT	1
	Canadian	$(TG)_7$		-	1
Ds 325	SH	$(GT)_8$	A/G ; A/T	-	1
	Canadian	$(GT)_9$		-	2
Ds 366	SH	$(GT)_5$	T/A	-	1
	Canadian	$(GT)_6$		-	1
Ds 375	SH	No variation	-	TGCACA	1
	Canadian	No variation		-	1
Ds 388	SH	$(GT)_4 (TG)_{10}$	C/T	-	1
	Canadian	$(GT)_5 (TG)_7$		-	1

SH – Southern Hemisphere sources (Argentina1, Argentina2, Australia, Brazil & South Africa)
Canadian - Canadian sources (181, 184, 484, 1084)

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CHAPTER 4

Development of polymorphic microsatellite markers for the invasive pest *Sirex noctilio* using pyrosequencing of SSR genomic enrichment libraries

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4. Abstract

Sirex noctilio is one of the most significant pests of *Pinus* spp. in areas of the Southern Hemisphere that it has invaded during the last century. Despite the long history of research on the pest, the genetic diversity of these invasive populations has not been studied. In this study, ten polymorphic microsatellite markers were characterized that could be used to study the *S. noctilio* population diversity. The diversity of these markers was estimated on 40 *S. noctilio* individuals from non-native populations in Argentina, Australia and South Africa (KwaZulu-Natal and Western Cape Provinces). The alleles varied between two to six per locus, with a total number of 35 alleles amongst the markers.

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4.1 Introduction

The woodwasp, *Sirex noctilio* Fabricius and its obligatory fungal symbiont, *Amylostereum areolatum*, is a devastating invasive pest complex of *Pinus* species in the Southern Hemisphere (Hurley et al., 2007). Female wasps seek out stressed or suppressed trees and introduce *A. areolatum* and a phytotoxic mucus during oviposition. Their combined action can result in tree death. In addition *A. areolatum* decays the wood in order to provide nourishment required for *S. noctilio* larvae development (Coutts and Dolezal, 1969, Bedding and Akhurst, 1974, Talbot, 1977). In regions of the Southern Hemisphere where *S. noctilio* populations became established, including Australasia, South America and South Africa, its damage has been devastating at times (Hurley et al., 2007). This is in contrast to its native range in Eurasia and North Africa where *S. noctilio* is regarded as a secondary pest (Talbot, 1977, Spradbery and Kirk, 1978). Recently, *S. noctilio* has been detected in the United States of America (Hoebeke et al., 2005) and Canada (de Groot et al., 2006). Despite a long history of intensive study of *S. noctilio*, especially in the Southern Hemisphere, nothing is known regarding the genetic diversity in its populations. The aim of this study was to develop polymorphic microsatellite markers for *S. noctilio* that can be used to characterize such population genetic diversity of this important pest.

4.2 Materials and Methods

4.2.1 *Sirex noctilio collection*

The isolates of *Sirex noctilio* were collected from *Sirex* infested logs from various locations in Argentina, Australia, Chile, South Africa, United States of America and Uruguay. Adult wasps that emerged were preserved in 95 % ethanol.

4.2.2 *DNA extraction*

Abdomen muscles of *Sirex noctilio* were used for extraction of genomic DNA. For the development of microsatellite markers an adult female *S. noctilio* was used. A phenol-

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chloroform DNA extraction method was used to extract DNA for microsatellite enrichment as described before (Mlonyeni et al., 2011 - chapter 3 in thesis).

4.2.3 *Microsatellite enrichment and pyrosequencing*

The fast isolation by AFLP sequence containing repeats (FIASCO) with modifications (M-FIASCO) (Cortinas et al., 2006), together with an Inter Simple Sequence Repeats (ISSR) method (Barnes et al., 2001) was used to enrich for microsatellites. The FIASCO probes used as described in Santana et al. (2009). Genomic DNA of *S. noctilio* was amplified using eight ISSR primers 5'DDB$(CCA)_5$, 5'DHB$(CGA)_5$, 5'HVH$(GTG)_5$, 5'NDV$(CT)_8$, 5'BDB$(ACA)_5$, 5'BV$(AT)_8$, 5'VH$(TG)_8$ and 5'HD$(GC)_8$. Fifty microlitres of PCR mixture containing 10× PCR buffer (10x solution, 100 mM Tris-HCl, 500 mM KCl, pH 8.3) (Roche Diagnostics GmbH, Germany), 25mM $MgCl_2$ (Roche Diagnostics GmbH), 10 mM dNTPs, 10 µM of each of the two ISSR primers used, 2.5 U of FastStart *Taq* DNA Polymerase (Roche Diagnostics GmbH) and sterile SABAX water added to make up the total volume. The thermocycler conditions were an initial denaturation step at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min and a final extension step of 72 °C for 10 min. The PCR products were separated on 1 % agarose gels, stained with ethidium bromide (EtBr) and visualized under UV transilluminator imaging system (UVP, United Kingdom).

PCR products were purified by adding 0.1 volume of 3M NaOAc and 5 volumes of 100 % EtOH per product, incubated on ice for 10 min and centrifuged at 13 000 rpm for 30 min. The supernatant was removed and 80 µl of 70 % EtOH was added followed by centrifugation at 13 000 rpm for 30 min. This step was repeated whereafter the pellet was vacuum dried at 45 °C for 30 min (Concentrator 5301). The samples were resuspended in SABAX water. Both FIASCO and ISSR enriched DNA containing microsatellite repeats were pooled and sequenced using pyrosequencing (Genome Sequencer FLX System™, Roche Diagnostics) as described in Santana et al. (2009).

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4.2.4 *Microsatellite discovery, primer design, amplification and DNA sequencing*

Sequences obtained from pyrosequencing were assembled into contigs using Vector NTI Explorer software (Invitrogen, Carlsbad, CA, USA), microsatellite repeats identified and primers designed using the web-based program Simple Sequence Repeat Identification Tool (SSRIT) (Temnykh et al., 2001) and PRIMER 3.0 software (Rozen and Skalestsky, 2000), respectively. Sixteen primers were optimized and tested for amplification on DNA from a total of ten *S. noctilio* individuals. These included a sample from Chile, the United States of America, Uruguay, two from Argentina and four from South Africa. The PCR was performed using the thermal *i*Cycler (Bio-Rad). DNA from each individual was used as the template in a 25 µl reaction volume containing 10× PCR buffer (10x solution, 100 mM Tris-HCl, 500 mM KCl, pH 8.3) (Roche Diagnostics GmbH, Germany), 25mM $MgCl_2$ (Roche Diagnostics GmbH), 10 mM dNTPs, 10µM of each forward and reverse primer, 2.5 U of FastStart *Taq* DNA Polymerase (Roche Diagnostics GmbH) and sterile SABAX water was added to make up the total volume. The thermocycler conditions included an initial denaturation step at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min and a final extension step of 72 °C for 10 min. PCR products were stained with GelRed™ (Biotium, Incoporation, USA) before being separated on 2 % agarose gels, and visualized under UV transilluminator imaging system (UVP, United Kingdom). The 100 base-pair (bp) molecular weight marker (Fermentas, O' Gene Ruler ™) was used for size estimates of PCR products. Selected PCR products were purified and sequenced as described in Chapter 3. The thermocycler conditions were 25 cycles of denaturation at 96 °C for 10 s, annealing at 58 °C for 15 s and extension at 60 °C for 4 min.

4.2.5 *Data analysis*

Following sequencing and agarose gel evaluation, four loci were found to be monomorphic for the amplified region of interest for all samples, while 12 were polymorphic in the *S. noctilio* samples. The forward primers of the polymorphic loci were fluorescently labeled (Applied Biosystems) (Table 1) for analysis using 40 additional isolates. Ten samples were used from

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each of the four regions namely Argentina, Australia, South Africa (Western Cape Province) and South Africa (KwaZulu Natal Province). The amplified PCR products were electrophoresed on an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems). The allele sizes were compared against a GeneScan LIZ – 500 size standard and determined using GENEMAPPER VERSION 3.0 (Applied Biosystems). Alleles of loci *Sn525* and *Sn189* could not be determined due to stutters/multiple peaks and thus were discarded from further analysis. A total of 35 alleles were observed across the remaining 10 loci (Table 1). The number of alleles per locus ranged from two to six.

For each population observed (H_o) and expected (H_e) heterozygosity (Levene, 1949), deviation from Hardy-Weinberg equilibrium and linkage disequilibrium (LD) were tested for all loci using the program POPGENE version 1.31 (Yeh et al., 1999).

4.3 Results and Discussion

The observed and expected heterozygosity respectively ranged from 0 to 0.5 and 0 to 0.6737 in the Argentina population, from 0 to 0.4 and 0 to 0.6797 in the Australian population (Table 2), from 0 to 0.5 and 0 to 0.742 in the Western Cape population from South Africa. The SA KwaZulu-Natal population lacked diversity compared to the other populations, which most likely reflects the recent bottleneck experienced by this population when it was introduced from the SA Western Cape population. The SA Western Cape population showed the highest number of loci in Hardy-Weinberg disequilibrium ($P<0.05$). The South Africa population (Table 2) consisting of the SA Western Cape and SA KwaZulu-Natal populations excludes males that were homozygous at all loci. No LD was detected between any loci, indicating random association of alleles in this tested population.

The level of gene diversity (Nei, 1973) was relatively low for all populations namely 0.3722 in Argentina, 0.2676 in Australia and 0.4637 in the SA Western Cape population, but it was the lowest for the SA KwaZulu-Natal (0.0815). Most alleles were shared between populations, with only 3 and 5 alleles being unique to the Argentina and SA Western Cape populations (Table 1), respectively. The limited population sampling make strong inferences about the history of *S.*

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noctilio introduction difficult, but it was noticeable that the Australian population shared all its alleles (n=22) with the other populations from the Southern Hemisphere. These data support the hypothesis that *S. noctilio* has spread between infested countries within the Southern Hemisphere (Slippers et al., 2002).

An interesting finding from this study was a number of male samples that were heterozygous at some loci. Siricids have a haplo-diploid mating system typical of Hymenoptera (Cook, 1993, Cook and Crozier, 1995). Males are thus expected to be most often haploid and females diploid. In certain cases, diploid males could increase in a population when diversity of sex determination loci is severely reduced (Hendrick et al., 2006). In this study, five out of 12 males were shown to be diploid based on a heterozygous locus. This is a fairly high number, suggesting that the genetic bottleneck during introduction might be affecting the male:female ratios in these populations.

The 10 polymorphic microsatellite markers developed for *S. noctilio* in this study will be very useful to study the population genetic diversity and structure of this important invasive pest of pine species. Such data reflect the routes and numbers of introdutions, as well as help to better understand the potential effects that bottlenecks have on population ecology.

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Table 1. Characteristics of 10 polymorphic microsatellite loci of *Sirex noctilio*. Prefix in italics before the forward primer sequence indicate flourescent dye identity.

Locus	Repeat motif	Primer sequences (5' - 3')	T_m (°C)	Range (bp)	N_A
Sn 90 [b,c]	$(GA)_8$	**F**: *FAM*-GGG TAG GAA AAG GGA TGT TCT C	60	102-109	4
		R: GTC TTT ATC GCT CGC TCG AC	60		
Sn 104	$(TTCG)_6$	**F**: *FAM*-TCT GAC ACC TAT TTT ACA CTG GAC A	60	174-178	2
		R: AAC GAG AAA AAC GCA TCG AC	60		
Sn 177 [b]	$(TG)_5$ $(TG)_9$	**F**: *VIC*-AGA ACG TGG TAC ATT GAA CAT TT	58	158-160	2
		R: TCC ATC CGT TGA TAC CTT ACT T	58		
Sn 185	$(AAG)_8$	**F**: *FAM*-CTA TGA AGC GAT GCC TCC TC	59	198-212	3
		R: GCT TCT TTG CTC CCT CTT CA	60		
Sn 231	$(TC)_5$ $(CT)_5$	**F:** *PET*-GAG CTC GAC AAC AGT GGA ATC	60	186-206	2
		R: ACA AGC GCG AAT GAA GAG AA	61		
Sn 350	$(CTA)_7$	**F**: *PET*-CGT CCA TCG AAC ACA TCA CT	60	174-177	2
		R: AAG GGT GAT CGG GGA AAT AG	60		
Sn A2 [c]	$(GA)_{15}$	**F**: *FAM*-TCG ACT TTA TCG TCG ACT GC	59	185-195	5
		R: CGT GTC GTG TCT TTG ACG TT	60		
Sn A3 [b,c]	$(GA)_{15}$	**F**: *NED*-TCG CTC GTG ACG TAA CAT GA	61	196-219	6
		R: GCT CTA GCG ACT CCC TGG TA	60		
Sn A7 [c]	$(AC)_5$ $(GA)_{10}$	**F**: *PET*-CGT TCA AAG ATG CGT GAG AA	60	197-203	3
		R: CTG TTG AGG ACT TGG GCA AT	60		
Sn B2 [c]	$(TG)_{10}$	**F**: *VIC*-TGA ATG AAC AAC ATG GCA CA	59	185-209	6
		R: TCA CCA GCC CTG AAC TAT CC	60		

T_m, melting temperature; N_a, number of alleles observed;

b, markers with alleles unique to Argentina; c, markers with alleles unique to South Africa (Western Cape Province)

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Table 2. Data analysis of the polymorphic microsatellite markers of *S. noctilio*.

	ARGENTINA			AUSTRALIA			SOUTH AFRICA		
Locus	H_o	H_e	P-value	H_o	H_e	P-value	H_o	H_e	P-value
Sn 90	0.5	0.3947	0.3545	0.1	0.1	1	0.31	0.579	0.055
Sn 104	0	0	-	0.1	0.1	1	0	0.517	0
Sn 177	0.4	0.5263	0. 4237	0	0	-	0	0	-
Sn 185	0.4	0.4421	0.7453	0.2	0.1895	0.808	0.15	0.465	0.021
Sn 231	0.4	0.4421	0.7453	0.222	0.4706	0.088	0.15	0.369	0.023
Sn 350	0.2	0.1895	0.8083	0.1	0.1	1	0.08	0.409	0.002
Sn A2	0.5	0.6737	0.4835	0.4	0.4421	0.745	0.62	0.766	0.602
Sn A3	0.3	0.5105	0.309	0.4	0.6421	0.228	0.39	0.705	0.005
Sn A7	0.13	0.125	1	0.1	0.1	1	0.08	0.471	0.002
Sn B2	0.5	0.6158	0. 9219	0.222	0.6797	0.02	0.08	0.496	0

H_o, observed heterozygosity; H_e, expected heterozygosity.

P-value for statistical significance of deviation from Hardy-Weinberg equilibrium (P<0.05)

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CHAPTER 5

Factors affecting the efficacy of *Deladenus siricidicola* in biological control systems

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5. Abstract

The nematode, *Deladenus siricidicola*, represents the cornerstone of *Sirex noctilio* biological control programs across the Southern Hemisphere. There is, however, significant variation in its efficacy in different regions. In this review, we consider emerging issues related to the biology and handling of the nematode that might influence its efficacy in biological control systems. Most practical aspects concerning the handling of *D. siricidicola* have been streamlined over the past half-century and these appear to be very efficient. However, large gaps remain in our knowledge about some key aspects of the biology of *D. siricidicola*. For example, very little is known regarding the evolution of virulence in the nematode populations, and the consequent evolution of resistance in *S. noctilio* populations. Furthermore, the levels of diversity in *D. siricidicola* and its ability to adapt to fungal, wasp and environmental variation are poorly understood. In this regard, new collections and storage of native populations of the *Deladenus* spp. are critical for the future research and management of this key biological control agent of *S. noctilio*.

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5.1 Introduction

Sirex noctilio was accidentally introduced into New Zealand around 1900 (Miller and Clark, 1935). The wasp soon became a serious pest in *Pinus radiata* plantations and during the 1940's and 1950's reached epidemic proportions (Rawling, 1955). The damage caused by the wasp sparked intensive studies on its biological control, initially focussed on parasitic wasp species (Hanson, 1939, Nuttall, 1989). During the course of this work, a nematode-infected female *S. noctilio* was discovered on the north island of New Zealand (Zondag, 1962). The nematode was thus naturally introduced together with *S. noctilio* into this region. It soon became clear that this nematode held promise as a biological control agent (Zondag, 1965, 1967, 1969). This stimulated the emergence of a research field that continues to today and that has made *D. siricidicola* one of the best studied entomopathogenic nematodes in any system.

Deladenus siricidicola has a bi-cyclic life cycle, including a mycetophagous or free-living and a parasitic cycle (Bedding, 1967). The two morphological forms associated with this unusual life history are so distinct that it might initially have been described in two families, the Neotylenchidae (where it is currently placed) and the Allantonematidae (Bedding, 1967, 1974). In the free-living cycle, the nematode feeds exclusively on *Amylostereum* spp., the fungal symbionts of Siricid wasps, and it reproduces oviparously (Bedding, 1967, 1972). In the parasitic cycle, female nematodes enter and develop in the haemocael of the Siricid larvae and reproduce ovoviviparously (Bedding, 1967, 1972). Nematode larvae produced by parasitic females are released inside the haemocael of Siricid larvae, and migrate towards and then infest the testes and developing eggs.

Deladenus siricidicola sterilizes the female of *Sirex noctilio*. The nematode does not affect oviposition and is consequently spread by the female wasps through infected eggs (Zondag, 1969, Bedding, 1972). Furthermore, the free-living cycle makes it possible to rear the nematode in large quantities in the laboratory and thus to be able to achieve mass releases in the field (Bedding, 1974, Bedding and Iede, 2005) (Figure 1).

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To date, seven *Deladenus* spp. have been described associated with the Siricid-*Amylostereum* symbiosis (Bedding, 1974). Several of these species also infest the parasitoids of Siricids and the beetle *Serropalpus barbatus* (Bedding, 1967, 1972, 1974). In addition, *D. siricidicola* is highly specific to Siricids and *A. areolatum*, including not infesting other hymenopteran parasitoids of Siricids (Bedding and Akhurst, 1974, 1978). These characters together have made *D. siricidicola* an ideal biological control tool. Initially it was introduced between plantations by moving infested logs (Zondag, 1969). Subsequently, artificial inoculation has become the preferred method of introduction into plantations, followed by natural spread by female wasps. This followed intensive work on artificial rearing of the nematode on *A. areolatum* cultures (Bedding and Akhurst, 1974, Bedding, 1979), and subsequent development of the method to inoculate it into cavities punched into tree stems together with a carrier gel solution (Bedding and Iede, 2005).

Today, *Deladenus siricidicola* is considered to be the cornerstone of ongoing biological control programs against *S. noctilio* in Australia, South America and South Africa. In New Zealand the nematode is no longer actively inoculated into trees, but it still contributes to the control of the wasp together with parasitic wasps (John Bain, personal communication). The methods to produce and inoculate *D. siricidicola* vary from region to region, but generally follows the basic principles described during the 1970's to 1990's in Australia (Figure 1).

Biological control using *D. siricidicola* is not equally successful in all regions of the world, with inoculation success varying from <5 - >99 % in different regions (Hurley et al., 2007). The factors affecting this variation in success have, however, not been widely studied. Hurley et al. (2008) attempted to identify a number of potential factors affecting *D. siricidicola* in summer rainfall regions of South Africa. The conclusions derived from this study were that a combination of factors including moisture in the wood, virulence, resistance, competing micro-organisms, and variation in the *A. areolatum* strain used for inoculation could contribute to inoculation success.

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Many excellent reviews treating the biology, history and use of *D. siricidicola* for the biological control of *S. noctilio* have been published. For example, Bedding (1979), Neumann et al. (1987) and Bedding and Iede (2005) reviewed the biology, development and application of *D. siricidicola* in field management systems. In a recent review, Hurley et al. (2007) considered programs in the Southern Hemisphere where *D. siricidicola* is being used for the control of *S. noctilio* and particularly focussed on the relative success of these programmes. The aim of this chapter is to consider emerging issues related to the biology and handling of the nematode that might influence its use in biological control systems.

5.2 Rearing, handling and storage

The success of *D. siricidicola* as a biological control agent requires effective methods to mass-rear, release and store the nematode. Developments in these areas (as described in Bedding and Iede 2005) have contributed substantially to the feasibility of using this nematode as a biological control agent effectively. This has also promoted its introduction into all Southern Hemisphere countries where *S. noctilio* is present. However, the use of *D. siricidicola* is not without challenges, and poor quality control in the rearing, handling and storing of *D. siricidicola* can drastically decrease *S. noctilio* control.

Contamination of the medium used to grow *A. areolatum*, the fungal food source for *D. siricidicola,* can result in greatly reduced numbers of nematodes produced. This is exacerbated by the fact that the nematode cultures take many weeks to mature. In worst cases, the majority of a rearing population can be lost in a short time (Brett Hurley, personal observation). The contaminants, including bacteria and mites, are often transferred by mites and can spread rapidly within a rearing facility. It is thus imperative to ensure that working conditions are as clean as possible. However, even under the sterile conditions, contamination is likely to occur during the mass rearing process and this needs to be considered when planning control programs.

The temperature of the nematodes during their transit to the field is a major factor influencing their survival. Nematodes are initially transported in water in sealed,

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breathable plastic bags and later suspended in a polyacrylamide gel before being inoculated into the trees, typically under cool conditions (<5 °C) (Bedding and Iede, 2005). Recent studies examining the survival of nematodes in water have confirmed that nematode survival decreases over time and as temperature increases (BP Hurley, unpublished data). For example, at 5 °C and 10 °C, over 80 % of the nematodes survived after 150 hours, whereas at 25 °C only 39 % survived after 30 hours, and at 30 °C only 7 % survived after 24 hours. Similarly, nematode survival in the polyacrylamide gel has also been shown to decrease with an increase in temperature (authors, unpublished data). The temperature of the gel is, however, less of a concern as the nematodes generally remain in the gel for only a short time before being inoculated into trees. In contrast, the nematodes can remain in the water-filled bags for numerous days. This increases the chance for them to be exposed to high temperatures and this can consequently greatly reduce the success of the inoculations.

A specially designed rebound hammer punch is used to inoculate trees with *D. siricidicola* (Bedding and Iede 2005). The hammers are designed to make clean holes in the wood without bending the tracheids, thus allowing entry of the nematodes into the wood. Nematode numbers introduced into trees are significantly reduced when blunt punches are used (Brett Hurley, unpublished data). Furthermore, the level of care with which contractors use the hammer punches to produce inoculation holes can also influence the quality of the inoculation site and thus nematode establishment.

Continuous rearing of the mycetophagous stage of *D. siricidicola* on *A. areolatum* over a number of years can result in a loss of virulence of the nematode (Bedding and Iede 2005). This can have serious negative consequences for biological control efforts, as was observed in the Green Triangle of Australia (Haugen, 1990, Haugen and Underdown, 1990, Haugen and Underdown, 1993). This problem can be largely solved by storing nematodes in liquid nitrogen outside of the inoculation season (Bedding and Iede 2005).

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5.3 Evolution of nematode virulence and wasp resistance

Variation and natural change in resistance of the *S. noctilio* populations to infection by *D. siricidicola*, and equally, changes in the virulence of *D. siricidicola* populations should be expected over time. Such variation and change are common patterns in biological interactions and they are thought to be linked to the evolution and maintenance of sexual reproduction in biological systems. The red queen hypothesis (van Valen, 1973) postulates that hosts and their parasites are in a continual "arms race" involving cycles of evolution of resistance (including tolerance) in the host and over-coming resistance (including higher levels of virulence) in the pathogen. The "trade-off" hypothesis between transmission and virulence predicts that parasites will evolve towards lower levels of virulence in situations where there is a restriction on spread linked to high levels of virulence (Alizon et al., 2009). This idea has been most intensively explored in human-pathogen interactions, but not in agricultural or forestry situations. It is especially relevant to some biological control systems, in particular classical biological control, such as the *S. noctilio-D. siricidicola* system, which relies on the natural dispersal of the parasite that is often linked to the dispersal of the host.

Virulence is defined here as the number of adults in a given *S. noctilio* population that are infested by *D. siricidicola* and are sterilized by it. In female wasps this only includes individuals with infested eggs. Usually all eggs in such females will be infested. The nematodes sometimes infest the females, but they do not enter the eggs (Bedding 1972, 1974, Yu et al., 2009). For the purposes of this discussion on nematode virulence, these female wasps are not sterile and are thus not included in counts of infected wasps in a population.

It is not known how effectively the *D. siricidicola* can spread if it does not infect the eggs. How this condition influences the fitness of the nematode populations is thus not known. This is an important question to answer, especially because the condition appears to be common in some regions. Bedding (1972, 1974) reported this condition in a number of populations. The latter studies also reported this to be the case for *D. imperialis* and *D. rudyi*. Yu et al. (2009) found that none of the 102

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nematode- infected *S. noctilio* females collected from various sites in Canada had infected eggs. Zondag (1969) noticed a small number of nematode infected *S. noctilio* females, where the eggs were not also infested. The possibility that the nematodes can spread without infecting the eggs is suggested by the fact that they can be found in the oviducts of the female wasp through which the eggs will pass through during oviposition. If there is no selective advantage to *D. siricidicola* spread via eggs and thus sterilizing the host over spreading without infecting the eggs, then it is hard to imagine why all populations would not exclusively spread outside the eggs. Furthermore, if this condition is genetically controlled, then cross-breeding between infective and non-infective strains of *D. siricidicola* should be vigorously avoided where Sirex control using this nematode is important. There is thus a critical need to better understand the mechanisms that underlie and drive the ability of the nematode to infect Siricid eggs. Futhermore, variation in this condition should be compared in the different regions of the world where *D. siricidicola* is being used for biological control.

Bedding (1974) noted that there is a direct correlation between time of release of juvenile nematodes by infective female wasps, and subsequent infection of the host eggs. He also speculated that the nematode forms that do not enter eggs have evolved in host species that are more solitary. This is in contrast to wasps that often infest the same trees that would allow the evolution of highly virulent, egg-infecting nematode forms, because non-infected hosts are likely to also oviposit in the same tree (Bedding 1972, 1974). Bedding and Iede (2005) report high levels of virulence even under very low levels of plantation infestation (>1 %) where attacks are very sparse. There are, however, no other data to make an evaluation of this hypothesis possible.

The infection of wasps by *D. siricidicola* is expected to have an effect on the population of *S. noctilio* even if the nematode does not infect the eggs. A number of researchers (Zondag 1969, Bedding and Iede, 2005, Corley and Villacide, 2005) have noted that infection by *D. siricidicola* leads to lowered fat reserves in the larvae and adults and that this affects their size and ability to fly. Their fitness would thus be

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negatively affected, in terms of numbers of eggs produced, dispersal distance and energy to oviposit. The effect of wasp infection without entering the eggs on the fitness of the *S. noctilio* population, however, cannot be quantified at present.

Zondag (1969) noticed that heavily infected wasps sometimes have no fat bodies remaining and they were often observed to die. He stated that '*The most important deviation from the normal pattern of the nematode infection is that the hosts can die when the immature hosts are heavily infected.*' Bedding (1972) disagreed with this view and concluded that heavy infestation by the nematode does not lead to the death of the larvae. This is an important question as it underpins a potential driving mechanism for the evolution towards lower virulence in *D. siricidicola*, which would emerge where inordinately high levels of virulence in the nematode increase mortality in the larvae. This could then also be affected by levels of artificial inoculation, where heavy inoculation might lead to high infection levels and high larval mortality. The consequence would be that nematodes that are less virulent and consequently kill less larvae would survive. In this regard, Bedding and Akhurst (1974) noticed that heavily inoculated logs (at 10cm intervals) produce smaller and fewer numbers of females, seemingly confirming this view.

Mechanisms of natural resistance in *S. noctilio* populations, other than the non-infection of eggs described above, might also exist. For example, Bedding (1972) noticed that nematodes, which infect and sterilize the Australian population of *S. noctilio*, do not do so in the Belgian population of *S. noctilio* where it originated. It is also obvious that any resistance that arises by mutation in a particular population of *S. noctilio* is likely to become fixed rapidly, especially under the strong selective pressure that would be emerge where nematode infection sterilizes all non-resistant individuals. The mechanisms that might drive such resistance are not understood and only open to speculation. One possibility might be that resistance to infestation by *D. siricidicola* involves co-ordination of the development of eggs and the nematodes. The nematodes must enter the egg at a very specific stage of development, or they will either stop egg development completely or fail to

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penetrate the eggs (Bedding, 1972). This is clearly an issue that needs urgent consideration.

Another source of variation in virulence in *D. siricidicola* might be by the loss of the ability to develop infective females due to continuous artificial rearing in the free-living cycle in the laboratory (Bedding, 1972). The continual selection of those individuals that develop into mycetophagous forms (as opposed to infective females) might lead to such a loss. This is thought to have been the cause of the collapse of the biological control system in Australia during the 1987-1990 outbreak in Victoria (Haugen and Underdown, 1993). The nematode that had previously consistently resulted in infection levels of above 95 % after inoculation, then infected less than 30 % of the wasp population after inoculation. To overcome this problem, a new and virulent nematode strain was re-isolated, which again brought parasitism levels after inoculation to >90 %. There is, however, not direct evidence that the loss of virulence was caused by this rearing process. Attempts to produce a non-infective strain, for research purposes, by ongoing studies to rear the nematode artificially currently spanning 6 years at the University of Pretoria, has failed to re-create this effect.

5.4 Introduction history and genetic diversity

In 1962, more than half a century after *S. noctilio* was first reported in New Zealand, Rudi Zondag noticed size changes in female and male reproductive organs of wasps from the Rotoehu forest (Zondag, 1962). Upon closer inspection, this was found to result in nematode infection. Subsequently, nematodes were also described from the parasitoid wasp *Rhyssa* spp. in New Zealand and from various Siricids and their parasitoids from England (Bedding 1967, 1968a , 1968b, 1972), including the description of the bicyclic life cycle of the species of *Deladenus*.

Subsequent to the discovery of the *D. siricidicola* and the realization of the obvious potential it has for biological control, extensive surveys were initiated by the CSIRO (Australia). Ultimately thousands of logs and tens of thousands of wasps from across Europe, North Africa, various sites in North America, Japan, Pakistan and India were

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collected and screened for nematodes (Bedding 1972, Bedding and Akhurst 1974, Bedding and Iede 2005). These collections represented various species of *Deladenus*, as well as strains of *D. siricidicola* that produced lower levels of infection, or smaller wasps. Bedding (1972) reports that '*…maintaining strains from many countries and already hundreds of millions of nematodes have been reared and distributed throughout many of the Sirex infested forests of Australia with encouraging results.*' After extensive screening of strains in the early 1970's, four strains from Corsica, Thasos, Sopron and New Zealand that gave high levels of infestation were selected for final trials. Of these, strain (198) from Sopron in Hungary was finally selected for wide scale application (Bedding and Iede, 2005).

The Sopron strain of *D. siricidicola* has been the predominant strain inoculated throughout Australia since the early 1970's. However, it is expected that other strains were dispersed on a limited scale in early years of development of the biological control program in that country. The nematode was not distributed in New Zealand and consequently its populations in that country are expected to still reflect original, accidental introductions of another strain(s). The 'Sopron' strain has subsequently also been used widely for inoculations in South America (Iede et al., 1998, Klasmer et al., 1998, Maderni, 1998, Ahumada, 2002, Bedding and Iede, 2005).

The Sopron strain of *D. siricidicola* has been reported to have lost its virulence in two cases, once in Australia in the late 1980's (Haugen and Underdown, 1993, Bedding and Iede, 2005) and once in Brazil (Bedding and Iede, 2005). This reported to be due to repeated culturing of the nematode for more than 15 years in the free-living form and consequently, its loss of ability to covert to the infective form, at least in culture (Bedding and Iede, 2005). No experimental data, however, exist to confirm that this was the reason for loss of virulence. Furthermore, such a loss of virulence could not been repeated in experiments in South Africa (Hurley et al., 2008) even though the nematodes in that study were only continually sub-cultured for about three years. The underlying reasons why *D. siricidicola* lost its virulence in Australia and Brazil remain unclear and this could be due to one or more of the factors discussed previously in this review.

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In Australia, the loss of virulence in the Sopron strain was resolved by re-isolating the nematode from the Kamona forest in Tasmania, where original releases of the Sopron strain had been made years before (Bedding and Iede, 2005). The culture was selected from a single male and female nematode (RA Bedding, personal communication). Subsequent inoculations resulted in high levels of virulence. This strain has subsequently been used extensively in the biological control program of *S. noctilio* in South Africa. In Brazil, nematode strains were also isolated from infested wasps to establish new colonies. A strain resulting from these isolations and known as 'Encruzilhado do Sol' (Southern Hybrid), is widely used today in South American countries.

One of the consequences of introductions of *D. siricidicola* into Australia, South America and South Africa is that is that there is a lack of genetic diversity in populations of the nematode. A recent study has shown that the nematode populations from across this region are homozygous for 17 microsatellite regions and 3291 bp of sequence data (Mlonyeni et al. 2011 – chapter 3 in thesis). This most likely result from a genetic bottleneck in the nematode population created during every round of sub-culturing. A selection of between >100 – >2000 nematodes is typically transferred between plates and this process is often repeated numerous times. Furthermore, inbreeding levels would also be expected to be high in this system and this would be expected to rapidly reduce heterozygosity. This lack of diversity can be a problem, because the nematode is used in a variety of environments, and in different populations of the wasp and fungus. Its selection for a specific environment is expected to be high due to the strong human selection during isolation, rearing and inoculation.

5.5 Interaction specificity – *Amylostereum* and *Sirex*

Specificity of *D. siricidicola* to specific wasp populations, and *vice versa*, has been observed. For example, Bedding (1972) and Bedding and Akhurst (1974) noticed that a strain of *D. siricidicola* from Japan never infects the eggs of *S. noctilio* females. This reflects a process that is influenced by the particular strain of the nematode. In contrast, a strain of the nematode from Belgium sterilizes Australian populations,

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but not Belgian populations of the wasp. In the latter case, it is the wasp population that clearly influences the effect. The drivers behind these apparently strain specific interactions are not clear. However, molecular tools not available when these observations were made should make it possible to better understand the relationships between different wasp and/or nematode populations, Irrespective of the driving forces behind host specificity in *D. siricidicola*, this factor clearly needs to be considered in *S. noctilio* management. This is especially because differences in wasp populations or invasions of new populations of the wasp can have far- reaching consequences on the efficacy of biological control programs. This is especially relevant given the lack of diversity in the nematode populations, discussed above.

The fungal strain that has been used to rear *D. siricidicola* in Australia (here referred to as the "nematode strain") is thought to have originated from early collections of wasps in Europe, possibly from *S. juvencus*. This strain has been shown to be distinct from the strain in the field in Australasia, South Africa and South America using VCG and molecular markers (Slippers et al., 2001, 2002, Nielsen et al., 2009). The "nematode strain" of the fungus is easily spread during the inoculation of the nematode, as harvesting of the nematodes from fungal cultures also contains many viable propagules of the fungus. This specific fungal strain is widely used across the South Hemisphere for mass rearing the nematode.

The difference between the "nematode strain" of the fungus and the strain of *A. areolatum* found in the field across the Southern Hemisphere is potentially important for biological control programs (Hurley et al., 2007). It has been observed that the nematode feeds and develops better on the "nematode strain" of the fungus than on other strains (RA Bedding, personal communication; Authors observations during mass rearing of the nematode). These preliminary observations could thus far not be quantified in experiments (BP Hurley, unpublished). Nor could the nematode be selectively bred to reproduce more effectively on the field strains collected from *S. noctilio* in South Africa, despite multiple generations over a two-year period (Hurley, unpublished). Given the importance of potentially lower fitness

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of *D. siricidicola* on fungal strains other than the "nematode strain", this question needs to be urgently addressed.

5.6 Variable environmental factors

For many years it was assumed that *S. noctilio* populations performed best in Mediterranean, winter rainfall regions similar to its most common distribution in Europe (Spradbery and Kirk, 1978). During the course of the last two decades the wasp has spread and prospered in winter and summer rainfall areas, in particular in Brazil and South Africa (Iede et al., 1998, Hurley et al., 2007). Projections based on current distribution also show that large parts of North and South America, Africa and Australasia would be suitable to future invasion by the wasp (Carnegie et al., 2006). From the South American and South African experiences in particular, it has become evident that the efficacy of the standard biological control programs developed in winter rainfall regions of Australasia, in particular with the nematode, will not be equally effective in all these regions.

Sirex noctilio populations are known to differ substantially in phenology in different climatic zones, which could affect the interaction with the nematode (Neumann and Minko, 1981, Carnegie et al., 2005, Hurley et al., 2007). In New Zealand, the Cape region of South Africa and south-eastern pine- growing regions of Australia (Victoria, New South Wales), *S. noctilio* emergence is from December to April (peaking in February), while it occurs between October to January in the summer rainfall regions of South Africa (peaking in November).

One of the major differences between summer and winter rainfall regions that affect *D. siricidicola* is the rate of change in wood moisture after infestation. In the winter rainfall regions the majority of the time that the nematodes are in the trees is during the wet season, while in the summer rainfall regions the nematodes are in the trees mainly during the dry season. In the summer rainfall area of South Africa, moisture content of the trees, especially in the upper sections of the trunks, often drops below 20 % (Hurley et al., 2007, 2008). It is not known what the threshold of moisture content is, below which the nematodes and / or the symbiotic fungus, *A.*

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areolatum, will survive, but lower moisture content has been linked to lower nematode parasitism (Hurley et al., 2008).

Recent studies have shown that some sap stain fungi compete strongly with *A. areolatum* for resources (authors, unpublished data). In particular, *Diplodia pinea*, a commonly occurring and important latent pathogen of pine in South Africa (Swart and Wingfield, 1991), grows faster than *A. areolatum*, especially in environments of lower moisture availability. Although *D. pinea* has not been found to overgrow *A. areolatum*, its faster growth enables it to capture more wood resources and thus to limit the growth of *A. areolatum*. Where the growth of *A. areolatum* is severely limited, this will influence the survival and reproduction of *D. siricidicola*. Thus, the composition of sap stain fungi, and conditions that favour the establishment and growth of these fungi in an area, is likely to influence the successful establishment of *D. siricidicola*, and consequently effective biological control.

Various other factors vary between regions and could possibly influence the establishment of *D. siricidicola*, but their effects have not been studied. For example, many different *Pinus* species are planted in the regions where *D. siricidicola* is applied for biological control. These include *P. radiata*, *P. patula*, *P. taeda*, *Pinus carribea*, *P. pondersae*, *P. elliottii*, *P. contorta* var. *latifolia*, and hybrids of some of these species and some could be more suitable as hosts for *S. noctilio*, *A. areolatum* or *D. siricidicola*. Besides these factors that might influence variation, the effects of interactions between the above mentioned factors are also unknown. It is, for example likely that factors such as moisture content in the trees and the effect of sap-stain fungi would be correlated. Many smaller affects working in an additive fashion, as opposed to a single dominant effect, could also result in major differences in parasitism in control programs in different regions.

5.7 Conclusions

The discovery in and description of the *D. siricidicola* in New Zealand and Australia represented a significant and exciting scientific breakthrough. The work that followed in this area during the subsequent decades led to the development of a

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biological control application that has saved potentially billions of dollars of damage to pine plantation industries across the Southern Hemisphere. Despite this success, there are regions where the nematode has not been successful or has failed completely. As *S. noctilio* continues to spread throughout previously unaffected areas, understanding the cause of this variation and improving the efficacy of biological control will be important. Information and interpretations provided in this review will hopefully clearly illustrate that much work remains in to understand the causes of variation in efficacy of *D. siricidicola* in various parts of the world. Such understanding should ultimately make it possible to predict problems that will result in ineffective control and to ultimately also avoid these effects.

This review has treated many factors that could potentially affect the usefulness of *D. siricidicola* as a biological control agent. These include factors such as handling of the nematode, environmental factors, variation in the wasp and nematode populations, competing fungi and others. It seems unlikely from current evidence that one major factor is responsible for the variation in nematode success as a biological control agent. Rather, an interaction between several of these factors, or a number of factors in concert will more likely combine to cause the overall effect of dramatically different parasitism results.

It is unfortunate that variation, both molecular and phenotypic, in the populations of *D. siricidicola* has not been considered previously. The potential variation in factors such as the ability of the nematode to adapt to variable environments and populations of the wasp are all of great relevance to control strategies. Furthermore, the mechanisms driving evolution of resistance and virulence in the *D. siricidicola* and *S. noctilio* populations should be high on the research agenda for the future. Recently developed molecular markers should also assist in this process. The potential to use rapidly developing novel approaches to DNA sequencing and thus to be able to analyse genetic and genomic factors underlying aspects such as virulence in *D. siricidicola* is great. Studies arising from the application of these technologies and others that have yet to emerge will surely improve our understanding of the biology of *D. siricidicola* and this will enable improved control.

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Perhaps the greatest factor hampering research on *D. siricidicola* currently is a lack of availability of natural variation in available *D. siricidicola* strains. During the height of the collection and research programmes on *D. siricidicola* supported by the Australian and UK governments in the 1970's, numerous strains of this nematode (and its relatives) were collected from across the Northern Hemisphere. Today none of those strains from Europe remain available for study and it is only the Kamona strain, and recently strains collected in Canada, that can be investigated. Without appropriate investment in this aspect of the research on *D. siricidicola*, it should be expected that failures in biological control programmes will occur in the future and especially as a result of resistance emerging in populations of *S. noctilio*.

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Figure 1. *Deladenus siricidicola* mass rearing strategy and bi-cyclic life cycle.

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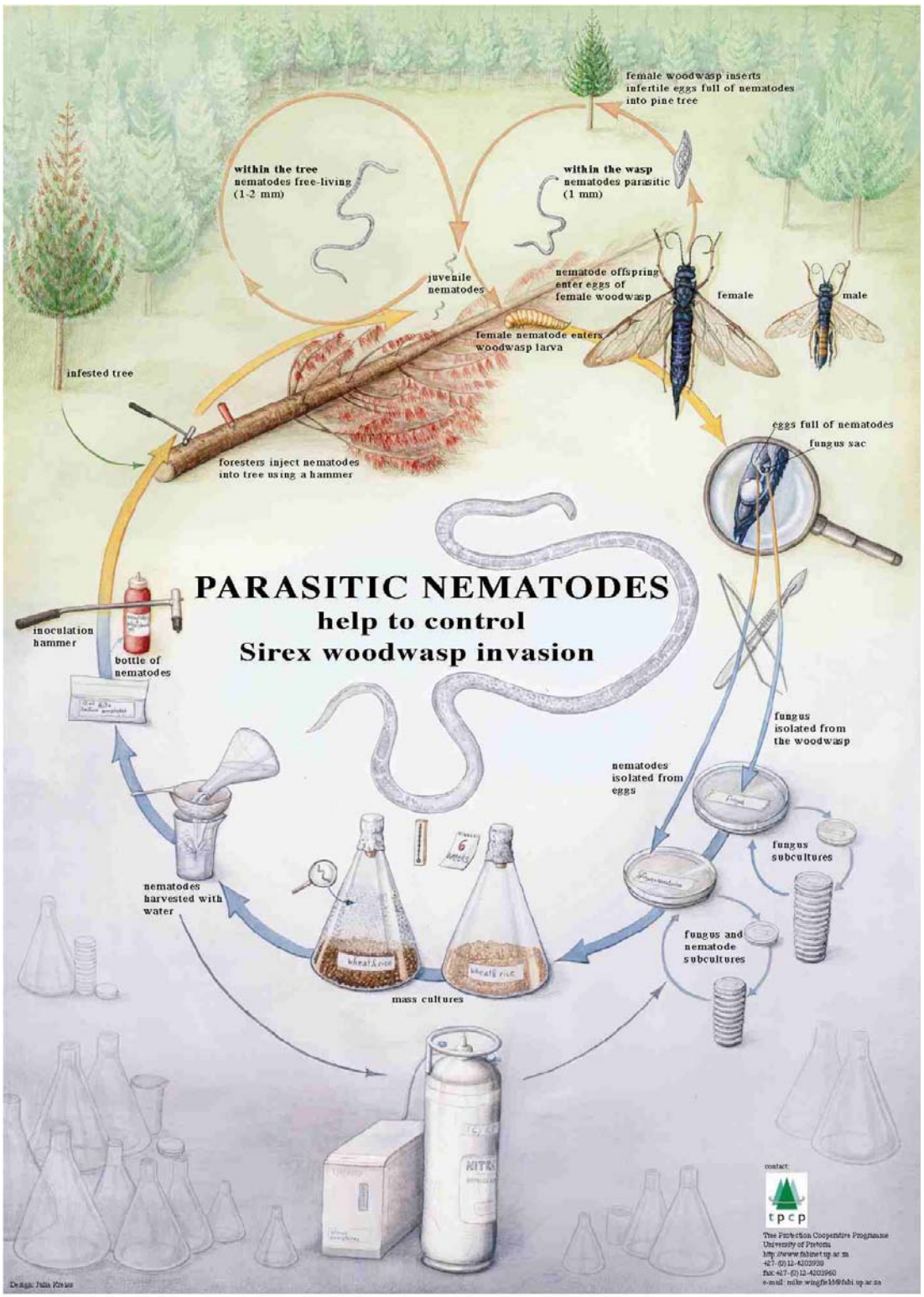

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Summary

Thesis title: High throughput development of population genetic markers for the *Sirex noctilio* woodwasp and its nematode parasite, *Deladenus siricidicola*

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Degree: MSc

Sirex noctilio is a major pest to *Pinus* spp. and threat to the Forestry industry in the Southern Hemisphere. The varying level of efficacy of its primary biological control agent, *Deladenus siricidicola*, is also of serious concern. There is a need to better understand the diversity of both the pest and its biological control agent, as one of the factors that could influence this biological control system. One of the short-comings is that molecular markers did not exist to study the population diversity of the nematode and wasp. As part of this study it was illustrated how next generation sequencing (454 pyrosequencing) can be used to enhance microsatellite marker discovery tools, and produce a more robust, rapid and economical tool for the development of these markers. The developed markers for *D. siricidicola* and *S. noctilio* proved to be highly efficient in characterizing the population diversity of both of these organisms.It was shown that the populations of *D. siricidicola* in the Southern Hemisphere is highly homozygous at all the loci developed, which likely resulted from human selection of favorable strains, genetic bottlenecks during transfer and subsequent inbreeding. This result likely suggested a reduced ability of the nematode to adapt to the diverse environmental conditions, different *Pinus* spp. and *S. noctilio* populations found in the various regions. The populations from Southern Hemisphere and Canadian sources were

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distinct at most loci and illustrate the unexplored potential of exploring diverse populations of *D. siricidicola* in order to screen for useful phenotypic variation. The microsatellite markers that were developed were also effectively used to characterize representative samples of *S. noctilio* in the Southern Hemisphere. The alleles are largely shared amongst countries, reflecting the shared history of introduction. It also shows a stepwise invasive process in South Africa, leading to a genetic bottleneck which might have a significant impact on the population ecology of the wasp, as it can influence its sex ratio. This thesis contributes critical tools and knowledge necessary to understand the population diversity of both *D. siricidicola* and *S. noctilio*. As argued in the final chapter, this information and the use of these tools in future is critical to understand aspects such as the evolution of virulence and population ecology of the organisms, which should be used to direct biological control programs.

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