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Population variation in traits of *Deladenus siricidicola* that could influence the biocontrol of *Sirex noctilio* in South Africa

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ABSTRACT

Deladenus siricidicola is the primary biocontrol agent of the invasive woodwasp, *Sirex noctilio*. The nematode is mass-reared in culture on the *S. noctilio* symbiotic fungus, *A. areolatum*, but can also be induced to convert into its infective form in culture. The aim of this study was to explore the level of variation among a collection of *D. siricidicola* strains to convert into the infective form in culture. In addition, we measured variation in growth of the nematode on a strain of *A. areolatum* that is commonly used for laboratory rearing. Both traits could influence laboratory rearing and efficiency in the field. The 22 strains from three countries showed significant variation in the frequency of conversion into the infective form in culture. Even genetically uniform strains from South Africa displayed a range of variation in this trait. There was also significant variation in the growth of *D. siricidicola* on *A. areolatum*. These results emphasise the importance of considering variation in traits in *D. siricidicola* that could influence a biological control program. They also provide tools that could be used to screen larger collections of *D. siricidicola* for variation in traits linked to virulence and reproduction.

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Biological control; population genetics; nematode-fungus interaction; parasitic nematode; woodwasp

1. Introduction

The nematode *Deladenus siricidicola* Bedding (Tylenchida: Neotylenchidae) is the primary biological control agent of *Sirex noctilio* Fabricius (Hymenoptera: Siricidae), an invasive woodwasp pest of pine trees in various parts of the world. It has bicyclic life cycle, where each cycle has a distinct morphological form and is independent of the other (Bedding 1967). In the mycetophagous phase of the life cycle, the nematode feeds exclusively on *Amylostereum areolatum* (Chaillet ex Fr.) Boidin (Russulales: Amylostereaceae), the fungal symbiont of *S. noctilio* (Zondag 1969; Bedding 1972). To enter the parasitic or infective phase of its life cycle, micro-environmental conditions (high concentration of CO_2 and low pH) surrounding the *S. noctilio* larvae are thought to trigger the development of female *D. siricidicola* juveniles into an infective form (Bedding 1993; Bedding and Iede 2005). Infective juveniles penetrate the *S. noctilio* larvae, feed and reproduce within the infected bodies, and eventually lead to the sterilization of female *S. noctilio* when nematode offspring invade the eggs. The infected female *S. noctilio* are then a natural vector of *D. siricidicola* to other trees (Bedding 1972; Bedding and Iede 2005).

The levels of parasitism of *S. noctilio* populations by *D. siricidicola*, defined as the percentage of infected wasps in a sampled population, has been shown to be variable across the Southern Hemisphere where it has been actively released (Hurley et al. 2007). Several studies have been undertaken to elucidate possible factors that may affect these parasitism levels (Haugen 1990; Haugen and Underdown 1993; Hurley et al. 2007; Villacide and Corley 2008). As an example, in the summer rainfall areas of South Africa, inoculation technique, nematode loss of virulence, incompatibility between *D. siricidicola* and *A.areolatum* populations were investigated and eliminated as primary factors responsible for the low (less than 10%) nematode parasitism observed in *S. noctilio* populations emerging from inoculated trees (Hurley et al. 2007; Hurley et al. 2008; Hurley et al. 2012). Wood moisture content was shown to have a weak effect on the parasitism levels. Thus, while low wood moisture was almost always correlated with low levels of parasitism, high wood moisture levels did not always result in high level of parasitism (Hurley et al. 2008). There are still factors influencing parasitism that are not understood and are thus outside the control of management programs.

A serious outbreak of *S. noctilio* in the Green Triangle of southwest Victoria and southeast South Australia occurred in 1987 (Haugen and Underdown 1990). It was suggested that this was due to a loss of virulence in *D. siricidicola* and consequently a severe drop in parasitism levels of *S. noctilio* populations was observed

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(Bedding and Iede 2005). Uninterrupted laboratory rearing of *D. siricidicola* over 20 years as part of the biological control program was suggested to have led to the loss of nematode virulence. It is thought that the maintenance of the mycetophagous life cycle through uninterrupted laboratory rearing could lead to a loss or reduced ability to convert into the infective form because of the absence of selection pressure to maintain this trait in culture. Furthermore, Slippers et al. (2012) hypothesized that evolution of virulence and resistance in this system could also be expected under natural conditions, because these are common outcomes of biological interactions.

No published study has been conducted on the variation of virulence in populations of *D. siricidicola,* neither those used in current biological control programs nor from native areas. This is presumably because it is difficult to measure nematode virulence in the *D. siricidicola*–*S. noctilio* system. An approach to achieve this goal has been to characterise the ability of the nematode to convert to the infective form in culture under conditions of high CO_2 and low pH as a proxy for virulence (Bedding 1993). While practitioners have applied this tool, there are no published reports or data where it has been used to systematically screen for variation in *D. siricidicola* populations. A standardized protocol has also not been published for this tool. Furthermore, it is also not clear how these *in vitro* tests correlate with field data. Such a tool and the information that it could provide regarding variation in *D. siricidicola* populations would have a profound influence on biological control programs.

Other than virulence, the variation that is seen in the ability of *D. siricidicola* to reproduce on different strains of *A. areolatum* can also have significant impacts on this biological control system (Morris et al. 2012; Morris et al. 2014; Caetano et al. 2016). Such variation can affect the efficiency of mass-producing the nematode in culture. It can also influence the reproduction of the nematode in trees, and consequently also potentially its efficiency at infecting *S. noctilio* populations (Morris et al. 2012). Yet this trait has not been studied in populations of the nematode in the Southern Hemisphere, where *D. siricidiciola* is used in biological control programs.

The aim of this study was to investigate the levels of variation in *D. siricidicola* traits that have relevance to its use in biological control programs. These include the ability of the nematodes to convert to the infective form in culture and their growth on a specific strain of *A. areolatum*. Both traits are likely to affect the virulence of the nematode in field populations. In order to do this, standardized culture-based tests for these traits were determined. These tools were then applied to a number of nematode strains that are used in biological control programs in the Southern Hemisphere, as well as to strains from "naturally occurring" populations in Canada. A subset of these strains was also tested in the field, to consider how the *in vitro* results might reflect virulence under natural conditions. To elucidate relationships between the strains we also characterized their genetic diversity using microsatellite markers.

2. Materials and methods

2.1. Nematode strains

Nematode strains in this study are defined as cultures that were obtained from a single female *S. noctilio*. Twenty-two nematode strains were used; these originated from South Africa, Chile and Canada, with seventeen, three and two strains available from each country, respectively (Table 1). The South African strains were isolated from wasps that were collected

Table 1. Strains of *Deladenus siricidicola* used in this study where a strain is defined as a culture from a single female *S. noctilio*.

Year isolated from wasp	Strain	Region	Locality	Tree species
2013	RSA24	KwaZulu-Natal	Epsom	*Pinus patula*
	RSA32	KwaZulu-Natal	Underberg	*Pinus patula*
	RSA47	KwaZulu-Natal	Underberg	*Pinus patula*
	RSA107	KwaZulu-Natal	Golboa	*Pinus patula*
	RSA410	Mpumalanga	Helvetia	*Pinus patula*
	RSA417	Mpumalanga	Helvetia	*Pinus patula*
	RSA419	Mpumalanga	Houtboschoek	*Pinus patula*
	RSA500	Mpumalanga	Sjonajona	*Pinus patula*
	RSA735	Western Cape	Witelsbos	*Pinus radiata*
	RSA751	Western Cape	Jonkersberg	*Pinus radiata*
	ChileBF1	Los Noglas	BASA	*Pinus radiata*
	ChileBF3	Santa Ines	FVSA	*Pinus radiata*
	ChileBF5	Las Trancas	FVSA	*Pinus radiata*
2012	RSAD9	KwaZulu-Natal	Underberg	*Pinus patula*
	RSALY24	KwaZulu-Natal	Comrie	*Pinus patula*
	RSALY39	KwaZulu-Natal	Epsom	*Pinus patula*
	RSALY65	KwaZulu-Natal	Demagtenburg	*Pinus patula*
	RSA767	Western Cape	Witfontein	*Pinus radiata*
	RSA808	Western Cape	Geelhoutvlei	*Pinus radiata*
2011	RSA747	Western Cape	Ruigtevlei	*Pinus radiata*
2010	Can280	Ontario	Goderich	*Pinus sylvestris*
	Can470	Ontario	Goderich	*Pinus sylvestris*

from the three major pine-growing areas namely, KwaZulu-Natal, Western Cape and Mpumalanga provinces. All nematode strains were reared in the laboratory at the Biocontrol Centre of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa. Thirteen of the nematode strains had been mass reared since 2013, six since 2012, one since 2011 and two since 2010. This study was conducted between 2014 and 2015.

2.2. Multilocus genotyping and genetic diversity of nematodes

Multilocus genotyping was performed using microsatellite markers to confirm the identity and relationship amongst the nematodes strains. Total DNA isolation from the 22 strains was obtained using PrepMan™ (Applied Biosystems, USA) as described by Mlonyeni et al. (2011). Twelve fluorescently labelled microsatellite markers were used (Table S1; Mlonyeni et al (2011). Polymerase Chain Reaction (PCR) amplifications were performed in a 25 μL reaction volume using a ProFlex PCR System (Applied Biosystems, USA). The PCR reaction and visualization was done according to Mlonyeni et al. (2011). The PCR conditions were an initial denaturation step of 95 °C for 2 min, followed by 35 cycles of (i) denaturation at 95 °C for 30 s, (ii) annealing at 60 °C for 30 s, (iii) extension at 72 °C for 2 min; and final extension at 72 °C for 45 min. The exceptions were the annealing temperatures for microsatellite markers Ds33, Ds38 and Ds323 which were 55 °C, 57 °C and 58 °C, respectively. Three μL of PCR products were mixed with 1.5 μL of GelRed™ (Biotium Inc., USA) and run on a 2% agarose gel and visualized using Gel Doc™ EZ Imager (Bio-Rad, USA). The PCR product size estimations were done using a 100 base pair molecular weight marker (Fermentas, USA). For each nematode strain, three panels were run, respectively, with eight, three and one microsatellite markers. The PCR products in a solution containing 14:1000 ratio of Liz-500 size standard and HiDi™ Formamide were run on a 3500xl Genetic Analyzer (Applied Biosystems, USA) at the DNA sequencing facility at the University of Pretoria.

The alleles were scored using GeneMapper® Software version 4.1 (Applied Biosystems, USA). Allele distribution between the countries from which *D. siricidicola* strains originated was determined using the web-based program Venn diagram generator (http://www.bioinformatics.lu/venn.php). Multilocus genotype profiles were manually deduced from the genotypes associated with the 12 microsatellite markers used. Genetic diversity of the strains was determined using the population genetic analysis program POPGENE version 1.31 (Yeh et al. 1999). To determine the relationship between the countries, a network was reconstructed using the microsatellite data based on a median-joining algorithm via the program Network version 5.0.0.0 (www.fluxus-engineering.com).

2.3. Nematode egg collection and culturing

For each nematode strain, only nematode eggs were harvested from cultures. This was achieved by adding 4 mL of distilled water to the cultures, swirling once every minute for 3 min and then transferring the water containing nematodes and their eggs into an excavator block (Australian Entomological Supplies Pty. Ltd., Australia). After 15 min and under a dissection microscope (Nikon SMZ645, USA), the nematodes were separated from their eggs by slightly agitating the excavator block and pipetting out the resuspended nematodes. In some instances small numbers of juvenile nematodes remained in the egg-containing water. Five hundred μL of distilled water was added to the excavator block containing nematode eggs and mixed thoroughly to ensure that eggs were resuspended. Three, one μL samples were taken and each counted using a nematode counting slide with 3 mm^2 grids under a dissection microscope to determine the number of eggs per drop of water. Before each one μL sample was taken, the nematode egg suspension was thoroughly mixed. Following enumeration, the total volume of water in the excavator block was adjusted so as to allow a drop of one μL to contain approximately 180 eggs. An *A. areolatum* isolate (CMW40871), a commercially sourced isolate from Ecogrow Environment Pty Ltd in Australia (henceforth referred to as Australia_E) that is commonly used for rearing *D. siricidicola*, and three one μL of nematode eggs were inoculated onto a 0.2% lactic acid-containing half-strength Potato Dextrose Agar (PDA) (Difco™ Potato Dextrose Agar, LOT 2347 278) (19.5 g l^{-1} potato dextrose extract, 17.5g l^{-1} agar) plate. Approximately 540 eggs were inoculated per plate by placing each egg drop equidistant from each other and half way between the fungal plug and the edge of the plate. In each case two independent experiments were conducted; the first had three replicates per nematode strain, while the second had five replicates.

2.4. Quantifying variation in development into infective or mycetophagous forms

An initial experiment was conducted to evaluate *D. siricidicola* conversion to the infective or mycetophagous forms under a range of different CO_2 levels (10%, 5% and 1%) at 23 °C in a controlled CO_2 incubator (Forma Direct Heat CO_2 incubator Class 100 HEPA; Thermo Electron Corporation, USA). Females associated with the mycetophagous and infective state (characteristic of the parasitic life cycle) have distinct morphological forms. Those of the infective form have

a pronounced stylet that is absent in the mycetophagous form (Bedding 1967).

Under normal ambient conditions all *D. siricidicola* would develop into the mycetophagous form in cultures with *A. areolatum* (Bedding and Iede 2005). The percentage of nematodes that developed into the infective form under low pH (0.2% lactic acid-containing half-strength Potato Dextrose Agar (PDA) plate) is reported here as the "conversion ratio" between infective and mycetophagous forms. To confirm reliability of the scoring technique in the conversion ratio experiment, replicates with high nematode density were scored twice. For the first experiment, 10 strains had a minimum of two replicates with high nematode density while the second experiment had 15 strains with a minimum of three replicates. Six strains were present in both experiments.

New numbers were assigned to all plates to hide the identity of the cultures to the person evaluating them. After 16 days of incubation at 23 °C, nematode cultures were flooded with 4 mL of distilled water, allowed to stand for approximately 20 min while swirling the cultures every five minutes and thereafter eluting the suspension into a glass Petri dish. This was repeated twice per strain to remove all nematodes. Thereafter, the Petri dish was allowed to stand for one hour at an angle of approximately 40° on the base or cover of a larger Petri dish so as to allow the nematodes to sink to the bottom. Excess water was discarded one or two mL of distilled water were added to the nematodes. This was mixed and one mL of the suspension was transferred onto a Hawksley slide with 3 mm^2 grids and thereafter filled by adding distilled water.

One hundred female nematodes were characterized per culture for numbers of infective or mycetophagous stage, performed under a compound light microscope (Nikon ECLIPSE 50*i*, USA) at 10X magnification.

2.5. Field inoculation trial

Nine *D. siricidicola* strains that represented the observed range of conversion rates from mycetophagus to the infective form were selected for a field trial. Those chosen were seven South African (RSA24, RSA32, RSA47, RSA107, RSA410, RSA500, RSA735) and two Canadian (Can280, Can470) strains. The South African strains were all isolated in 2013 and Canadian strains were isolated in 2010.

The field trial was conducted in compartment area D8 at the Monta Hills plantation in Graskop, Mpumalanga. Thirty *Pinus patula* trees were felled and inoculated following a standard procedure described by Hurley et al. (2008). The trees were inoculated on 10 March 2015. Each nematode strain was inoculated into three trees and three control trees were not inoculated with nematodes. In October and after six months, all trees were collected and each tree was cut into six logs. Each log was placed in an emergence drum and emerging wasps were collected as described by Hurley et al. (2008). The emergence drums were stored at the Linwood wasp emergence depot in KwaZulu-Natal. From 27 October to 15 December 2015, wasps emerging from drums were sent to the FABI Biocontrol Centre, University of Pretoria for dissections. Both male and female wasps were dissected with the lower abdomen and eggs examined for nematode infestation, respectively.

2.6. Nematode propagation assay

The variation in growth of six nematode strains (RSA47, RSA107, RSA500, RSALY65, Can280 and ChileBF3), that span the higher or lower range of conversion rates from mycetophagus to the infective form, was determined on a commonly used strain of *A. areolatum* (CMW40871). Strains RSA47, RSA107, RSA500 and Can280 were also used in the field trial. The strains were cultured on half strength PDA. Four cultures were scored for each strain. The fungus and nematode eggs were inoculated as described above and plates were incubated for 25 days at 23 °C.

Following incubation, harvesting of the nematodes from the plates followed a similar procedure as described above, however, only one mL of distilled water was added after eluting the excess water. For each culture, nematodes were thoroughly mixed and 100 μL of this mixture was transferred into three glass Petri dishes, each containing 500 μL of distilled water. From each of these, 100 μL of the solution was removed, after being well mixed, and transferred to a Hawksley slide that was filled with distilled water. The total number of nematodes was then counted at 10× magnification under a compound light microscope. The total number of nematodes per culture was calculated from the averages of three counts per plate.

2.7. Data analysis

A single measures intra-class correlation coefficient (ICC) was performed, using a Two-Way Mixed model and Absolute Agreement type with a 95% confidence interval (CI) that shows the lower and upper bound, to determine the reliability of the scoring technique. One-way Analysis of Variance (ANOVA) using Tukey HSD at $P < 0.05$ was used to determine statistical differences in the mean conversion ratio of nematodes to the infective form and the reproduction rate assay of nematode strains. Pearson's correlation coefficient was used to determine the relationship between field parasitism and *in vitro* conversion ratio of nematode strains. These analyses were performed using the statistical programme IBM® SPSS® Statistics version 23.

3. Results

3.1. Multilocus genotyping and genetic diversity of nematodes

A total of 30 alleles from the 12 markers (Table S1) were detected in this study, with strains from Canada, Chile and South Africa containing 13, 22 and 20, alleles each. Total allele distribution included five alleles shared between the countries, 12 exclusively shared between Chile and South Africa, three exclusively between Chile and Canada. Five alleles were unique to the Canadian strain, while the Chilean strain had two and South African strain had three unique alleles each. From the eight multilocus genotypes (Table 2), none were shared between the countries. Within South Africa four multilocus genotypes were found, one was dominant and shared in all regions, while one closely related multilocus genotype was shared between the Western Cape and KwaZulu-Natal strains and one was unique for KwaZulu-Natal strain (Table S2; Figure 1). The dominant multilocus genotype included 13 individuals, another consisted of two individuals and the rest comprised of an individual each (Figure 1). The multilocus genotype B and C, each differed from the dominant A at one locus, respectively. Multilocus genotype D differed from A at six loci, with five of these loci sharing the same genotypes as ChileBF1 (Table S2; Figure 1). Strains from Chile had the highest diversity, while Canada was the lowest (Table 2).

3.2. Quantifying variation in development into infective or mycetophagous forms

Under CO_2 conditions, the majority of the eggs did not develop while those that hatched had juveniles that appeared stressed (illustrated by coiling) (data not shown). Increased CO_2 was therefore not used to study variation in development into infective or mycetophagous forms.

Under ambient incubation at 23 °C for *D. siricidicola* in low pH cultures with *A. areolatum*, reliability of the scoring technique showed the intra-class correlation coefficient (ICC) was 0.986 with 95% CI (0.965, 0.994) for the first experiment using two replicates per strain. For the second experiment using three replicates per strain, the ICC was 0.988 with 95% CI (0.978, 0.993).

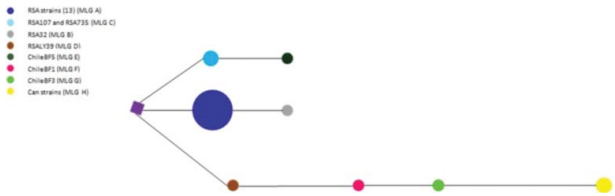

Figure 1. Microsatellite genotype network of *Deladenus siricidicola* strains. Different coloured nodes (circles) represent a haplotype (or MLG) with the size of the circles corresponding to the number of nematode strains in that haplotype. The purple block is the hypothetical median vector generated by Network version 5.0.0.0. Strains: RSA – South Africa, ChileBF – Chile and Can – Canada.

Deladenus siricidicola strains showed significant variability in conversion ratios to the infective form (Figure 2). Canadian and Chilean strains had a lower conversion ratio on average to the infective form compared with the South African strains, which ranged from having the highest and lowest ratios. For the South African strains, the conversion ratio into infective form was not related to the number of years for which the nematodes had been reared in culture (Figure S1). Furthermore, no correlation was observed between the level of conversion to the infective form (Figure 2) with the geographic origin of these South African strains (Table 1).

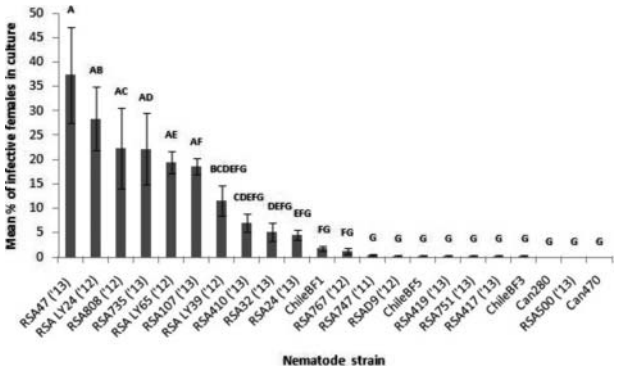

Figure 2. Female nematode infective form conversion ratio in culture. The bars represent the mean with error bars being standard errors. One-way ANOVA Tukey HSD at $P < 0.05$ was used to determine statistical difference between nematode strains. Different letters on the error bars per strain represent significant difference between strains.

Table 2. Genetic diversity of *Deladenus siricidicola* using 12 microsatellite markers.

Country	N	N_{all}	*I*	H_o	H_e	MLGs
South Africa	17	1.6667	0.1073	0.0343 (0.0303)	0.0500 (0.0713)	4
Chile	3	1.8333	0.5244	0.4167 (0.2887)	0.3657 (0.1796)	3
Canada	2	1.0833	0.0578	0.0833 (0.2887)	0.0417 (0.1443)	1

N – Sample size
N_{all} – Mean oberserved number of alleles
I – Shannon's diversity index [Lewontin (1972)]
H_o – Observed heterozygosity
H_e – Expected heterozygosity [Nei (1973)]
MLGs – Multilocus genotypes

3.3. Field inoculation trial

A total of 1915 wasps emerged from102 of the 180 collected logs, of which 189 (~10%) were female and 1727 (~90%) were male. Of these 666 wasps (187 females, 479 males) were dissected. The average parasitism level was 10.5%, of which 7.9% of the females and 17.1% males were parasitized. Wasps from control logs were not parasitized by nematodes. For analysis, a log from which less than five wasps emerged, as well as a treatment where five or more wasps emerged from less than three logs were excluded.

There was variation in wasp parasitism by nematode strains (Figure 3). The parasitism percentage in the field (Figure 3) and conversion ratio in the *in vitro* culture study (Figure 2) did not correlate statistically (Pearson's correlation coefficient = 0.381, $n = 7$, $p = 0.398$) (Figure S2). However, one strain (RSA735) scored relatively highly while others (RSA24, Can280, RSA500) were low in both the *in vitro* culture and field parasitism study.

3.4. Nematode propagation assay

The mean growth of nematode strain RSALY65 (2830 ± 336.7) was the highest and showed significant difference from strains RSA500 (840 ± 290.2), RSA47 (780 ± 202.6) and RSA107 (725 ± 161.3), which had the lower mean growth *Deladenus siricidicola strain* Can280 (1315 ± 301.9) and ChileBF3 (1500 ± 621.2) showed no significant difference in growth as compared with the South African strains. The growth of these nematodes on *A. areolatum* (CMW40871) showed an ability to survive and reproduce on this fungal strain (Figure 4).

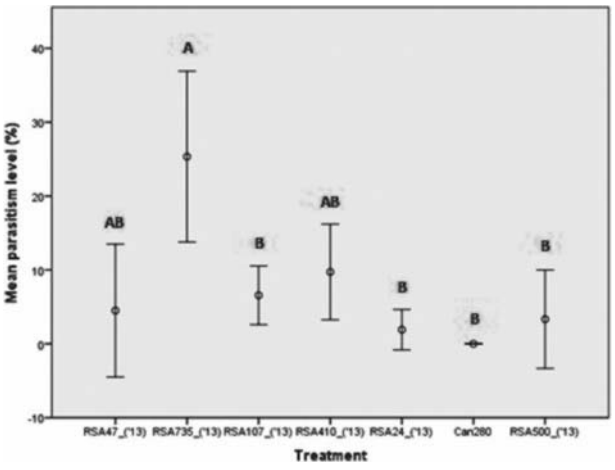

Figure 3. *Deladenus siricidicola* field parasitism of *Sirex noctilio*. The circle dots represent the mean and the error bars are standard errors. One-way ANOVA Tukey HSD at $P < 0.05$ was used to determine statistical difference between nematode sources. Different letters on the error bars per strain represent significant difference between strains.

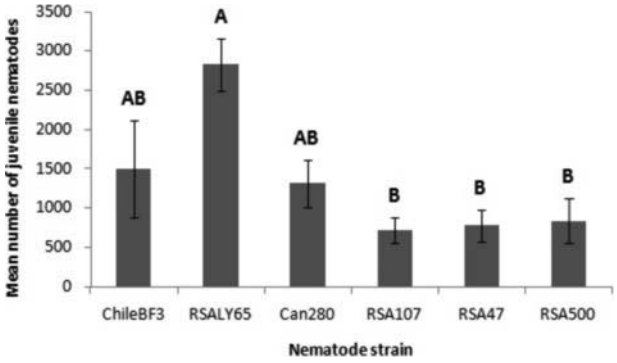

Figure 4. Mean number of *Deladenus siricidicola* juveniles produced for six selected strains, cultured on *Amylostereum areolatum* isolate CMW40871. The mean growth is represented by bars while the error bars are standard errors. One-way ANOVA Tukey HSD at $P < 0.05$ was used to determine statistical difference between nematode strains. Different letters on the error bars per strain represent significant difference between strains.

4. Discussion

The results of this study demonstrate variation in the ability of *D. siricidicola* strains commonly used in *S. noctilio* biological control programs to convert to the infective form. They also showed that there are differences in the growth of nematode strains on a commonly used strain of the fungus *A. areolatum* in culture. These traits varied even amongst strains of the nematode from populations where there was little genetic variation at neutral molecular markers. The observed variation in these traits demonstrates a need to consider such variation (also in other traits) in biological control programs.

A modified protocol was developed to screen for the ability of *D. siricidicola* strains to convert to the infective form in culture. This protocol was shown to provide repeatable results between experiments. Different approaches to screen for this trait in *D. siricidicola* have been reported previously (Bedding 1993; Bedding and Iede 2005). These included inducing nematode eggs to develop into the infective form when inoculated to PDA media with 0.2% lactic acid and placed in dark plastic bags with an approximately 20% CO_2 level or a 10% CO_2 level inside a desiccator (Bedding 1993; Bedding and Iede 2005). In this way, it was possible to distinguish between the highly parasitic Kamona strain, a defective strain reported to have lost its virulence and a hybrid of these strains (Bedding 1993). The preliminary experiments in the present study using levels of CO_2 between 1%–10% in an incubator, resulted in the death of the majority of the eggs or stressed, inactive (coiled) juveniles. Similarly, studies investigating the response of parasitic nematodes to CO_2, as a cue to host location, showed how the nematode *Haemonchus contortus* coiled when exposed to 1% CO_2, while *Ancylostoma caninum* and *Strongyloides stercoralis* were active at CO_2 levels below 5%

(Sciacca et al. 2002). Gaugler et al. (1980) attributed inactivity of *Steinernema carpocapsae* possibly due to anoxia when under conditions of constant elevated CO_2. We consequently excluded using increased CO_2 levels because the nematodes still developed into the infective form on a medium having a low pH level. The simplified approach that we have used successfully in this study should make it possible for practitioners of the biological control program to test *D. siricidicola* strains to convert into the infective form *in vitro* relatively easily.

Strains from the South African *D. siricidicola* population varied in the frequency with which females converted to the infective form. The variation was extreme – from 37% to 0%. Similarly, Bai et al. (2005) observed that 22 inbred lines of infective juveniles of the entomopathogenic nematode *Heterorhabditis bacteriophora* generated from a monoxenic culture of a founding population varied in parasitism (insect host mortality) under laboratory conditions. Clearly, some variation in traits linked to host infectivity can remain in populations of biological control organisms despite reduced population genetic diversity.

Results on this study using microsatellite markers showed that the South African population lacked variation, consistent with the common origin of the strains. The phenotypic variation in conversion to the infective form was consequently in contrast to the nematode genetic diversity. Most *D. siricidicola* strains with the highest and lowest ratios of conversion to the infective form had the same multilocus genotypes. This was with the exception of two strains (RSA735 and RSA107) that only differed at one marker (Ds38), as would be expected as they would both be related to original Kamona isolates imported into the country from a common source. In contrast, Bedding (2009) reported a link between random amplified polymorphic DNA (RAPD) markers and virulence in *D. siricidicola*. However, virulence is likely to be a complex trait (Semblat et al. 2000) and unlikely to be easily linked to single genetic markers (Aikawa et al. 2013). For example, Ding et al. (2016) identified 117 single nucleotide polymorphic markers (SNPs) linked to virulence in the pine wood nematode *Bursaphelenchus xylophilus* following genome and transcriptome sequencing of *B. xylophilus* virulent and avirulent strains. This follows the common application of ITS-RFLP marker (Iwahori et al. 1998; Aikawa et al. 2003, 2006), which was shown not to be reliable in distinguishing virulence of *B. xylophilus* strains (Aikawa et al. 2013). Nevertheless, the microsatellite markers used in the present study were helpful to confirm the identity and relations amongst the strains.

The two Canadian and two Chilean strains of *D. siricidicola* generally had lower conversion ratios to the infective form in culture than South African strains. The Canadian strains were isolated from invasive *S. noctilio* populations (i.e. not as part of a formal biological control program; Yu et al. 2009), while the Chilean strain were from a biological control program in that country (Beèche et al. 2012). The low *in vitro* conversion ratio to the infective form in the Canadian strains may not be surprising as *D. siricidicola* strains from North America are known to have low *S. noctilio* parasitism levels (Ryan et al. 2012; Zylstra and Mastro 2012; Kroll et al. 2013). However, the observed low parasitism is largely attributed to the presence of the non-sterilizing strain of *D. siricidicola* (Yu et al. 2009; Williams et al. 2012; Kroll et al. 2013). For Chilean strains the conversion ratio to the infective form is difficult to explain given the fact that these strains originate from a relatively successful biological control program (Beèche et al. 2012). It would be interesting to use the approach developed in the present study to screen a larger number of strains from Chile to help resolve this question.

The present study included strains that have continuously been subcultured every two weeks over a period of two years (approximately 52 generations). This an opportunity to infer whether such culturing might select for the *D. siricidicola* mycetophagous form, and lead to a loss of ability to convert to the infective form. Interestingly, the results showed no loss in the ability of the nematode to convert to the infective form, irrespective of the age of the culture. It is possible that two years is insufficient to result in a loss of virulence. Alternatively, loss of virulence in Australian cultures (Bedding 1993; Haugen and Underdown 1993) could have been a unique event. Unfortunately, there are no data available to shed more light on the exact nature of the change that was observed in the Australian cultures in the 1980's. Using tools such as the one described in the present study over longer time periods could help test this important hypothesis, which is potentially important for biological control programs.

Significant variation in the growth of *D. siricidicola* strains on a strain of *A. areolatum* that is commonly used in mass rearing was evident in this study. This is important because the successful mass production of *D. siricidicola* on *A. areolatum* cultures is fundamental to the augmentative biological control program for *S. noctilio* (Bedding and Akhurst 1974). Other recent studies have also highlighted the fact that the Kamona strain of *D. siricidicola*, which is the origin of the South African biocontrol strain and commonly used in the biological control program throughout the Southern Hemisphere, has variable growth when feeding on different *A. areolatum* strains (Morris et al. 2012; Morris et al. 2014). These authors showed that the commonly used Australia_E *A. areolatum* strain produced less *D. siricidicola* Kamona nematodes than *A. areolatum* strains associated with *S. noctilio* in the USA (Morris et al. 2014). In an earlier study, Hurley et al. (2012) found no significant difference in *D. siricidicola*

Kamona growth on the Australia_E and South African field *A. areolatum* strains. However, it is worth noting that many of these studies used different techniques, such as nematode production in Petri dishes and flasks, to assess the *D. siricidicola* growth It would be important to test the growth of *D. siricidicola* Kamona and other nematode strains on different *A. areolatum* strains using the technique described earlier (nematode production in Petri dishes), which is similar to that described by Morris et al. (2014). This would help to standardize the method used for testing *D. siricidicola* growth.

Despite the limitations, the *in vivo* (in field-grown trees) experiment applied in the present study suggest that the *in vitro* tests for conversion rate could be relevant to the field virulence of the nematode strains, and could be further considered for this purpose. Parasitism of *S. noctilio* by *D. siricidicola* within a tree is subject to influence by several factors such as wood moisture content, competition between *A. areolatum* and other fungi (Hurley et al. 2008; Hurley et al. 2012), and differential *A. areolatum* growth in different *Pinus* spp. or hybrids (Nahrung et al. 2016). Consequently, a perfect overlap between the *in vitro* and *in vivo* results should not be expected. However, even where results between *in vitro* and *in vivo* tests are partially correlated, as our results suggest might be the case, then these *in vitro* tools would become valuable. They could then be used to screen large numbers of nematode strains to select candidates to be tested in field to improve the impact of augmentative biocontrol efforts. This could help circumvent the challenge of costly and impractical large field trials. In addition, this *in vitro* screening tool could provide an opportunity to characterize local evolution of virulence within the *Sirex-Deladenus* system. Such knowledge could feed into transcriptomics studies aimed at understanding the genetic basis of this interaction.

The invasion pattern of the *S. noctilio* wasp populations is complex (Boissin et al. 2012). Consequently, the effect of the observed mismatch in diversity with *D. siricidicola* (Mlonyeni et al. 2011) and possible effect of wasp-nematode specificity (Bedding 1972) should be further explored to improve effectiveness of *S. noctilio* control. The phenotypic and genetic characters considered in this study can contribute to the process to develop effective and locally tailored biological control programs. The data also illustrate the extent of local variation that might exist to be exploited for this purpose, despite the limited genetic diversity usually associated with biocontrol populations.

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