Sirex biocontrol: cryptic nematode field strain prompts urgent review of program

Final report

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Sirex biocontrol: cryptic nematode field strain prompts urgent review of program

Prepared for

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by

Helen Nahrung[1], Katrin Fitza[2], Brett Hurley[2], Irene Barnes[2], Angus Carnegie[3], Firehiwot Eshetu[2] and Bernard Slippers[2],

[1]Forest Industries Research Centre, University of the Sunshine Coast, Queensland
[2]Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa
[3]NSW-Department of Primary Industries, Parramatta, NSW.

Forest & Wood Products Australia Limited
Level 11, 10-16 Queen St, Melbourne, Victoria, 3000
T +61 3 9927 3200 F +61 3 9927 3288
E info@fwpa.com.au
W www.fwpa.com.au

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Researcher/s:
Helen Nahrung
Forest Industries Research Centre
University of the Sunshine Coast
Sippy Downs, Queensland 4558

Bernard Slippers, Katrin Fitza, Brett Hurley, Irene Barnes, Firehiwot Eshetu
Forestry and Agricultural Biotechnology Institute (FABI),
University of Pretoria, South Africa

Angus Carnegie
NSW Department of Primary Industries
Parramatta, New South Wales 2150

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Forest & Wood Products Australia Limited
Level 11, 10-16 Queen St, Melbourne, Victoria, 3000
T +61 3 9927 3200 F +61 3 9927 3288
E info@fwpa.com.au
W www.fwpa.com.au

Executive Summary

1. *Pinus radiata* is Australia's most valuable softwood resource, and extremely susceptible to mortality from Sirex woodwasp, the most serious invasive softwood pest in the Southern Hemisphere. Australia pioneered the main Sirex management technique used globally: inoculation of infested trees with a bicyclic nematode (*Deladenus* (*Beddingia*) *siricidicola*; hereafter "nematode") that feeds on the Sirex fungus, parasitises its larvae, and sterilises emerging females, which then infest the next generation via oviposition of nematodes into trees with uninfected Sirex.
2. Research funded by the National Sirex Coordination Committee (NSCC) discovered a genetically distinct nematode strain ("Lineage D") predominating in Sirex populations in Australia, despite the introduction of hundreds of millions of commercial-strain nematodes ("Kamona") annually, at a program cost of >$0.5Mpa. This project sought to characterise the new strain to understand its biology, sterilising ability, interactions with commercial strain, and mechanisms behind its field dominance. Results will inform the need to review perceived failings in the current trap tree plot (TTP) management system to assess its contribution to the low recovery rates of commercial nematodes in the field.
3. Four hundred and sixty-eight nematode samples from different wasps were collected and shipped to the and Agricultural Biotechnology Institute (FABI) where they were genotyped. Fifty-eight samples were from resolved outbreak sites at Sunny Corner and Green Hills, and the remainder were from other sites throughout Sirex' distribution. Overall, inoculated trees yielded 77% Kamona, and uninoculated trees 17% Kamona. Nematodes were never detected in Queensland Sirex background populations, and all ten samples from inoculated trees there were Kamona.
4. All nematodes collected from Sunny Corner outbreak — where 16M Kamona were inoculated into NSTs prior to Sirex population crash — were Lineage D, and half of the samples collected from Green Hills immediately after the resolution of outbreak were Lineage D. All samples collected at Green Hills since then were Lineage D.
5. The number of Kamona inoculations between 2005 and 2019 did not show any relationship with Kamona recovery, and nor did the block or region level show any pattern with number of trees inoculated in the 2-3 years prior to sample collection for this study.
6. Reproductive rate trials on different fungal isolates suggest that a higher reproductive rate of Lineage D may contribute to its success in the field. Recovery of Lineage D from inoculated trees suggests that it may outcompete Kamona if already established prior to inoculation. Field inoculations in South Africa also suggested that background nematodes may reduce inoculation success, with very low recovery of inoculated Lineage D against a background of 90% field parasitism by Lineage B.
7. Lineage D was confirmed to be a sterilising strain, entering Sirex eggs at a similar rate to Kamona. From bins with a single strain and from which ≥10% of emergees were genotyped, bins with Kamona and Lineage D yielded similar parasitism rates.
8. When lineages B and D were paired in 'interaction trials', hybridized freely, but produced a greater than expected number of heterozygotes. There appears to be no mating barrier between the strains under these conditions. Genetic barries that are maintained in the field are thus not relating to mating or somatic incompatibility, but rather an ecological, postzygotic or operational reason.
9. There is a rich genetic diversity of *D. siricidicola* in field populations in Australia. The results of the recent studies suggest strongly that this diversity should be explored to

increase the efficiency of the biological control program, possibly through regular reisolation and introduction into the rearing populations, as is done elsewhere in the world. If nothing else, lab rearing can be improved by selecting faster reproducing strains on most common local strains of the fungus.

10. The value and role of the TTP program should be considered in light of the low persistence of Kamona in background populations, and low yield (number of parasitised females) produced in each plot. Its value as a monitoring tool and means of 'concentrating' Sirex is clear, but operations (e.g inoculation technique or timing) and the nematode strain used could be explored as areas that can be improved.
11. Kamona could be considered as a successful inundative biocontrol agent, as it directly impacts the generation against which it was released, while Lineage D appears to be established as a classical biocontrol agent, parasitising Sirex without further intervention.
12. The studies show the value of ongoing collections and evaluation of the biological control agents in the system. There is a rich set of optimized molecular and other tools available from the current study for strain identification, hybridization mapping and study of the biology of the nematode. The study raises hypotheses about genetic and ecological/operational mechanisms that maintain the unique lineages and diversity in the Australian populations.

Table of Contents

Executive Summary i
Introduction 1
Methodology 2
Nematode collections 2
Sunny Corner and Green Hills outbreak sites 3
Potential drivers of nematode recovery rates 4
Nematode "genotyping" using SSR 4
Nematode reproduction rate 5
Field inoculation trials 6
Sterilising ability 7
Nematode interaction trial 7
Results 9
Nematode "genotyping" 9
Sunny Corner and Green Hills outbreak sites 10
Comparison with previous studies 13
Potential drivers of nematode recovery rates 15
Nematode reproduction rate 18
Field inoculation trials 20
Sterilising ability 21
Nematode interaction trial 21
Inoculation trials and infection studies 23
Conclusions 25
Recommendations 26
References 27
Acknowledgements 29
Appendix 1: COI protocol to distinguish nematode lineages 30

Introduction

Pinus radiata comprises 74% of Australia's 1M ha of softwood plantations, a mill-door industry sector valued at >$1B per year (ABARES 2018). It is Australia's most valuable softwood resource, and extremely susceptible to mortality from Sirex woodwasp, the most serious invasive softwood pest in the Southern Hemisphere. Australia pioneered the main Sirex management technique used globally: inoculation of infested trees with a bicyclic nematode that feeds on Sirex symbiotic fungus, parasitises its larvae, and sterilises emerging females (Bedding 2009).

This nematode, *Deladenus (Beddingia) siricidicola*, is the primary Sirex management tool in its global invasive range (Fitza et al. 2019). It was discovered in Sirex first adventive range, New Zealand (Zondag 1962), and subsequently found in native Sirex populations in Europe (Bedding 2009). Three different European strains of this nematode (from Hungary, Corsica and Greece), and a strain from New Zealand, were released in Victoria following the establishment of Sirex in mainland Australia (Bedding 2009). However, ultimately the Hungarian strain (Sopron168 – originating from *Sirex juvencus*) was chosen for mass-rearing and release because of its lower impact on Sirex adult size (Bedding 2009). After several years of continuous laboratory rearing for the Sirex management program it appeared to lose its virulence, contributing to the devastating Green Triangle Sirex outbreak in the late 1980s that killed millions of trees (Carnegie & Bashford 2012). The original strain was re-isolated from a site in Tasmania (Kamona) and rearing practices were altered to ensure that continuous rearing did not impact control. The Kamona strain is now the mainstay of Sirex biocontrol in the Southern Hemisphere, and in Australia the nematode inoculation program costs the softwood industry around $0.5M per year (Cameron et al. 2018).

The nematode has two distinct life stages: a free-living form that feeds on Sirex symbiotic fungus and a parasitic form that enters Sirex larvae and reproduces, sterilising female wasps by invading developing eggs, and spreading to new trees during Sirex oviposition. The free-living form is strongly influenced by different strains of its food source, *Amylostereum areolatum* (Morris et al. 2012, 2014), and interactions with other biotic factors including bluestain fungus (Yousuf et al. 2014a), Ips bark beetles (Clarke et al. 2016) and pine taxa (Nahrung et al. 2016). Abiotic factors including temperature (Yousuf et al. 2014b), and moisture content (Hurley et al. 2008) also influence nematode performance. Its ability to convert to the parasitic form is also very variable and dependent on several factors including strain origin (Mlonyeni et al. 2018). For example, the North American strain was long considered non-sterilising, however a recent study found evidence for a sterilising strain in the field in North America (Bittner et al. 2019). Further, while nematode populations were initially considered to be virtually clonal in the introduced/invaded range (Mlonyeni et al. 2011), they have lately been found to comprise more complex diversity patterns including strain hybridisation (Fitza et al. 2019). From fungal strain incompatibility (Hurley et al. 2012; Morris et al. 2012, 2014) to inter- (Bittner et al. 2019) and intra- (Fitza 2020; Fitza et al. 2022) strain-specific interactions, multiple factors are recognised as potentially influencing biological control outcomes (Hurley et al. 2008; Slippers et al. 2012).

Recent research funded by NSCC discovered a genetically distinct nematode strain predominating in Sirex populations in Australia (Eshetu et al. 2019, 2020), despite the introduction of hundreds of millions of commercial-strain nematodes annually, at an annual program cost of >$0.5M. Given these costs and the serious risk that Sirex poses to the softwood industry – estimated at up to $60Mpa if uncontrolled (Carnegie & Bashford 2012) - we urgently needed to characterise the new strain to understand its biology, sterilising ability, interactions with the commercial strain (Kamona), and mechanisms behind its field dominance. We also need to review the current trap tree plot (TTP) management system (Harper 2017, Nahrung et al. 2020a) to assess its contribution to the low recovery rates of commercial nematodes in the field. The effectiveness of the TTP approach has been questioned over the last few years due to very low yields (Nahrung & Griffiths 2016, 2017; Harper 2017, 2018), the failure of nematodes to establish through the TTP process in Queensland plantations (Nahrung & Griffiths 2016, 2017), the susceptibility of TTPs to interference by Ips bark beetles (Yousuf et al. 2014), and the discovery of the different prevalent nematode strain in background Sirex populations (Eshetu et al. 2019, 2020).

The current inoculation strategy comprises two techniques: poisoning a block of trap trees to render them attractive to ovipositing female woodwasps, then felling and inoculating them; or felling naturally-struck trees within the forest and inoculating them. Anecdotally, the latter produces a greater yield of parasitised female Sirex, however, this may have reflected dispersal of the field strain, rather than success of the commercial strain (Nahrung et al. 2020b). This project seeks to quantify the extent of the distribution of the new nematode lineage, identify potential mechanisms behind its success, and to make important recommendations to industry for ongoing Sirex management based on these results.

Methodology

Nematode collections

Nematodes from parasitised females were collected by NSCC project partners in Tasmania, NSW, Victoria and South Australia during the Sirex 2019-21 flight seasons. Collections were made from inoculated and uninoculated trap trees (TTs) and naturally-struck trees (NSTs) in emergence facilities as part of normal Sirex operations. Samples comprising abdomens dissected from parasitised male and female wasps, abdominal contents, or nematodes extracted directly from trees, were sent to HFN where they were labelled, transferred to Eppendorf tubes where required, databased and shipped to South Africa. Queensland samples were from Sirex that emerged in 2018 from inoculated logs of the most recent TTPs undertaken there, and Tasmania's were from female wasps collected at SIRNOC-lure baited panel traps in the field.

Because the primary question in this study is about the success (or failure) of Kamona in an applied industry perspective, we sought simply to identify the dominant strain in samples as one of the four currently-recognised strains (Eshetu et al. 2020). "Genotyping*" was therefore conducted using primers designed specifically to amplify the *Deladenus* mtCOI gene (Morris et al., 2013). Polymerase chain reaction (PCR) was performed following the process in Fitza et al. (2019) and precipitated PCR products were sent for sequencing to a partner laboratory

(DNA Sequencing Facility, Faculty of Natural and Agricultural Science, University of Pretoria, South Africa) (http://seqserve.bi.up.ac.za//). mtCOI haplotypes (Lineage B (containing Kamona) and Lineage D (the novel lineage)) were allocated to nematode samples from inoculated and uninoculated trees (and traps) throughout Australia. SSR was performed on a subsample of nematode isolates to provide additional information where COI samples were ambiguous, appeared mixed, or where wasps containing both genotypes emerged from the same log.

* in this context & throughout, "genotyping" simply refers to allocation of COI haplotype, and "Kamona" is used to refer to Lineage B unless otherwise noted.

Sunny Corner and Green Hills outbreak sites

Nematode collections from a recent Sirex outbreak at Sunny Corner, Bathurst, NSW (see Nahrung et al. 2020b), where there had been "flooding" with Kamona strain nematodes and outbreaks had subsequently resolved (Figure 1), were genotyped to determine the nematode responsible.

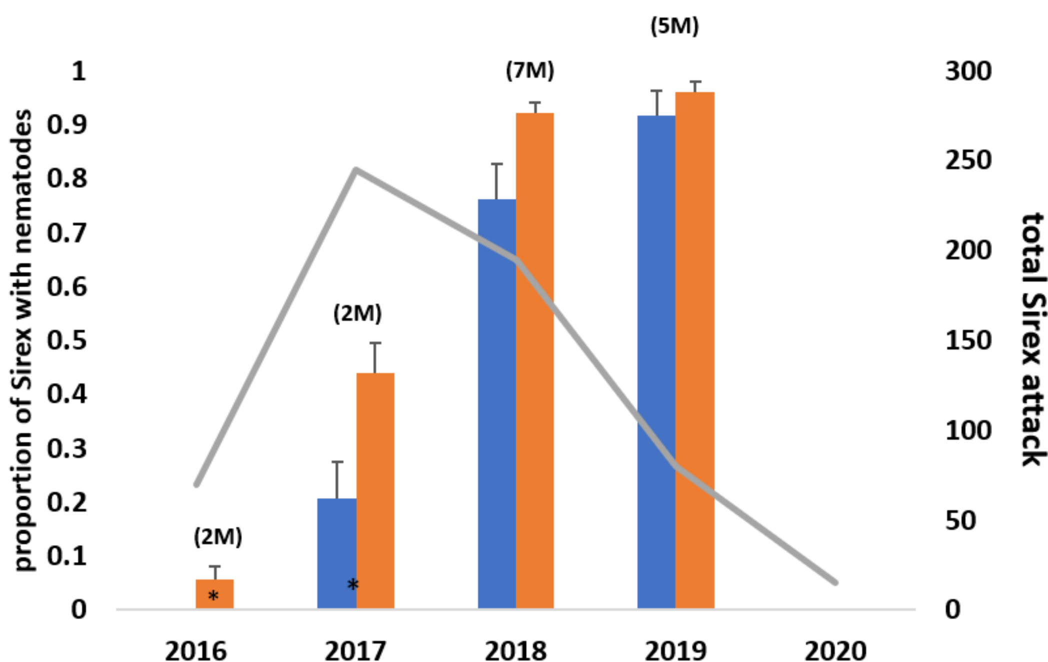

Figure 1: Background (uninoculated naturally-struck tree) parasitism rates (proportion + SE of wasps with nematodes) of female (blue) and male (orange) Sirex emerging in the rearing facility each season from Sunny Corner outbreak study compartments between 2016 and 2019 (x-axis is the year of inoculation). The grey line indicates the total number of new trees struck in each season, and the number above the bars is the approximate number of Kamona nematodes inoculated earlier that year (before the parasitism assessment for that same year). *genotyped as Lineage D (Eshetu unpubl.). Figure is from Nahrung et al. (2020a).

Nematodes from parasitised wasps that emerged in late 2019 and early 2020 (after the 2019 inoculation) and from 2021 from Sunny Corner, and a sample of nematodes extracted directly from trees in August 2020 following the crash of the Sirex population were genotyped (N=52). This information is important to understand the outbreak dynamics observed, specifically whether the "flooding" with Kamona contributed to the decline in Sirex attack observed over the study period, or whether Lineage D, already present in the area, was potentially responsible for the decline. Ten of these were analysed using five microsatellite markers (SSR) to check for possible presence of hybrids.

Six samples collected in 2012 from a resolved outbreak at Green Hills, Tumut (NSW) were also genotyped. The Green Hills Sirex outbreak ran for about six years between 2006 and 2012, with several million nematodes released up to 2011. The 2012 samples were all from wasps that emerged from naturally-struck, uninoculated trees.

Potential drivers of nematode recovery rates

Nematode recovery rates for Victorian samples were further explored to determine parasitism rates for each strain (Kamona/Lineage D) from drums, as well as potential relationships with numbers of emerging adults, and tree status (NST/TT). These analyses used a subset of Victorian emergence bin data from the last two years (Programs P2018-19-20 and P19-20-21 where in total 437 bins yielded 9207 Sirex; only data pertaining to those bins for which nematodes were sequenced from parasitised wasps are analysed here: N = 80 bins from which 1955 Sirex emerged, of which 162 were genotyped). Relative nematode recovery rates of the two lineages were also examined at the block and region level in relation to inoculation site histories to try to understand potential Lineage D displacement of Kamona strain. Using bins from which only one nematode strain was collected, and from which genotyped samples represented ≥10% of emerged adults, bin parasitism rates were compared between Lineage D and Kamona.

Nematode "genotyping" using SSR

Nematodes from parasitised females were collected by NSCC project partners in Tasmania, NSW, Victoria and South Australia during the Sirex 2019-21 flight seasons. Using the mitochondrial cytochrome oxidase subunit 1 (mtCOI) gene region the 458 samples were determined to be either one of the two currently recognised Lineages (Eshetu et al. 2020). In addition, five microsatellite markers previously designed (Fitza et al., 2016, Mlonyeni et al., 2011) were selected to further characterise the genotypes of 41 of these isolates. Samples for SSR analysis were chosen from each state (except Queensland) to compare COI results with SSR results. In particular, trees that had been inoculated with Kamona but yielded Lineage D, or where wasps with nematodes from both COI genotypes had emerged from the same bin, and from the Sunny Corner outbreak were selected for SSR analysis. Polymerase chain reaction (PCR) with the labelled microsatellite markers was performed following the process in Fitza et al. (2019) and the labelled PCR products were sent for Genescan analysis at a partner laboratory (DNA Sequencing Facility, Faculty of Natural and Agricultural Science, University of Pretoria, South Africa) (http://seqserve.bi.up.ac.za//). The program GeneMapper v.6 was used to determine the allele sizes of the various samples and markers.

Nematode reproduction rate

The reproduction rate of the mycetophagous phase of different nematode lineages (Kamona, Lineage B and Lineage D nematode strain) was performed on their food source, *Amylostereum areolatum*. Several studies have demonstrated differential performance linked with co-adaptation for different nematode-fungal isolate combinations (e.g. Morris et al. 2014; Mlonyeni et al. 2011). 1. The first step was to investigate the differential performance investigating media-fungal-nematode isolates. MEA and PDA was investigated, four fungal isolates and 2 Lineage D and 3 Lineage B nematode isolates. From that study the media and fungal isolate for use in subsequent trials was determined. To further investigate the difference in the reproductive rate between Lineage B and D, we selected and prepared *D. siricidicola* isolates (5 lineage B and 8 lineage D; Table 1) for further study of the interaction of the nematode and the Australian fungus CMW49995, NSW (N72). The fungal culture CMW49995 was established on 90 mm Petri dishes three days before adding the nematode eggs. Nematode eggs from parent cultures were harvested by flooding the plates with 4 mL of water and collecting the eggs into an excavated block . The eggs were washed with water five times to get rid of any fungal spores and then the number of eggs was counted three times. For each treatment plate approximately 500 eggs were placed opposite the fungal plug Petri dishes were incubated for 25 days at 23°C, then flooded with 5 mL distilled water and the number of live nematodes was counted as a measure of reproductive rate. Each treatment (nematode isolate) had three replicates, and was repeated with the same nematode and fungal isolates. Tables 1 and 2 outline the nematode and fungal isolates used in this trial, respectively. The experiment was conducted using two different culture media - Full strength Malt Extract Agar (MEA) and 1/3 strength Potato Dextrose Agar plus Agar (PDA) culture media in 90 mm diameter Petri dishes for each of the nematode-fungus combinations.

Analysis of Variance (ANOVA) was used to compare the interaction between nematode isolate and abundance. Square root transformation was used on the data to ensure constant variance and normality. As post-hoc analysis, the Tukey's Honestly Significant Difference (HSD) was run to compare pairwise treatment combinations. The analyses were run using R.

Table 1: Isolates of *Deladenus siricidicola* used in the reproductive rate study

Sample ID	State	District	Block name	Lineage	Details
V452	Victoria	Gippsland	Longford	D	Genotype #D2
V457	Victoria	Gippsland	Longford	D	Genotype #D4, control from Eshetu et al. (2020)
V450	Victoria	Gippsland	Longford	D	Main genotype #D1
N99	NSW	Bathurst	Sunny Corner	D	Genotype #D6
N102	NSW	NSW	Mt Lambia	D	Main genotype #D1
N104	NSW	Tumut	Green Hills N	D	Genotype #D7
S12	S. Australia	Comaum	Comaum	D	Main genotype #D1
S14	S. Australia	SA	Sandwood	D	Genotype #D8
Kamona				B	Kamona genotype
V176	Victoria	Shelley	Koeteng	B	Kamona genotype
V473	Victoria	Myrtleford	Merriang	B	Genotype #B1
V465	Victoria	Shelley	Koeteng	B	Genotype #B2
V484	Victoria	Benalla	Stanley	B	Genotype #B2

Table 2. Isolates of *Amylostereum areolatum* used in reproductive rate study.

Country	Region	CMW number	Isolate
Australia	South Australia	CMW53336	S21
	NSW	CMW49995	N72
	Old Ecogrow*	CMW40871	Old Ecogrow
South Africa	Commercial-RSA	CMW46043	SA14(2013)

*Ecogrow strain ~2000-2015 from Mt Gambier, not currently-used isolate (see Nahrung 2017).

Field inoculation trials

A field inoculation trial was established in a 21-yo *Pinus radiata* plantation (33˚ 54.4288 S 23˚26.6758 E) in the Western Cape, South Africa, in June 2020. Sirex-infested trees were identified based on characteristic external symptoms, felled and debranched. The trial consisted of five inoculation treatments (four nematode lineages and uninoculated control) with six trees per treatment (Table 3). Standard inoculation procedures were used (Bedding & Iede 2005, Hurley et al. 2008), with approximately 1M nematodes per tree applied in polyacrylamide gel.

Table 3: Nematode isolates used for field inoculation trial in South Africa

Lineage	Country	State	Region	Isolate
Lineage D	Australia	Victoria	Gippsland	V450
		NSW	Bathurst	N99
		South Australia	Penola	S3
South Africa	South Africa	Mpumalanga north	Blyde	SA922(2019)

Billets were collected from inoculated and control trees in the week of 19 October 2020, prior to wasp emergence. The trees were cut into 80cm-long billets that were examined for larval tunnels to confirm the presence of Sirex. Billets where tunnels were observed were prioritized for selection, however due to the limited number of trees / billets, other billets were also included. Billets that showed larval tunnels from other insects (e.g. cerambycids) were discarded. The number of billets selected therefore differed between trees, with some of the inoculated trees found to be unsuitable after cross-section. Selected billets were labelled and transported to the FABI Biocontrol Centre where they were placed into emergence cages.

Emerging wasps were collected between 01 Dec 2020 and 06 April 2021, dissected and the presence of nematodes in the eggs of females or abdomens of males determined. Dissected wasps were also examined for nematodes outside the eggs. When nematodes were found, eggs of the female, or the body of the male, were stored in 96 % EtOH. From each of the five inoculation treatments a subset of parasitized wasps was used to identify the nematode lineage. For this purpose, primers in the mtCOI region were used to determine whether the extracted nematodes were in Lineage D, i.e. isolates V450, N99 or S3 from Australia, or lineage B, i.e. the SA922 isolate that formed one of the treatments. Lineage B was the assumed strain of the nematodes already established at the inoculation site.

Sterilising ability

Live female Sirex from within Australia were shipped by NSCC partners to HFN for dissection (Jan and Feb 2021) to determine egg sterilisation rates (i.e. penetration of developing Sirex eggs prior to oviposition such that emerging Sirex females lay eggs containing mycetophagous nematodes, rather than healthy Sirex larvae). Assessment comprised dissection under a dissecting microscope (x100) where ovaries were removed from anaesthetized live females, rinsed with distilled water, and five eggs from the proximal ovary (closest to oviduct) of each female were transferred to a glass cavity block, washed to remove nematodes from the surface, and observed for nematodes visible through the egg chorion, or, if nematodes were not visible, eggs were dissected using two pairs of fine forceps and mounted micro-pins. Notes on the density of juvenile and adult nematodes in the abdominal cavity and any other relevant observations were recorded. Live nematodes recovered and ovaries of infected females were transferred to 96% EtOH in Eppendorf tubes, labelled, sealed with parafilm and databased for shipment to FABI for genotyping. 61 samples from live dissected females emerging from inoculated and uninoculated trees were genotyped, and subsequently examined for patterns and parasitism once lineages were identified. These samples were also included in the general population census analyses.

Nematode interaction trial

Due to the observations that nematodes from wasps from trees previously inoculated with Kamona (Lineage B) were often identified as Lineage D *D. siricidicola* using mtCOI, it was of great interest to determine the outcome of direct interaction between the two Lineages. For these trials two Lineage B nematode strains and four Lineage D strains were selected (Figure 1; Table 2). We used the same *Amylostereum* strain originating from NSW (CMW49995, N72) as in the reproduction trials on PDA media.

Table 4: Isolates of *Deladenus siricidicola* used in the competition/hybrydization study

Sample ID	State	District	Block name	Lineage	
Kamona				B	Kamona genotype
V484	Victoria	Benalla	Stanley	B	Genotype #B2
N99	NSW	Bathurst	Sunny Corner	D	Genotype #D6
S14	S. Australia	Penola	Sandwood	D	Genotype #D8
N102	NSW	NSW	Mt Lambia	D	Main genotype #D1
V457	Victoria	Gippsland	Longford	D	Genotype #D4, control from Eshetu et al. (2020)

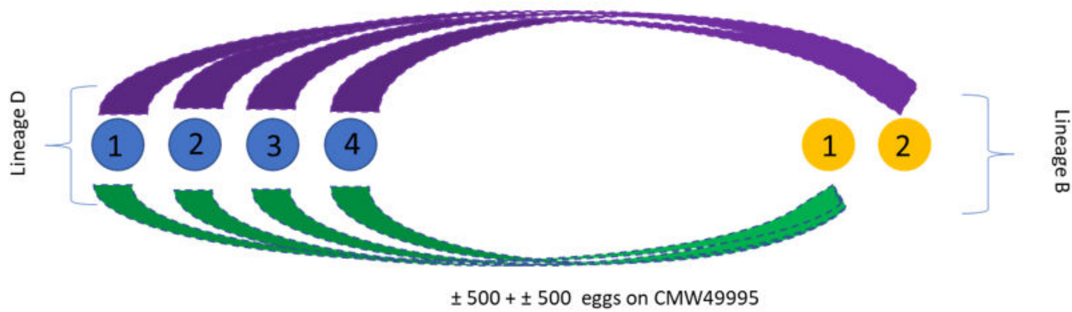

Figure 1: Schematic representation of the combinations/crosses conducted in the interaction trial.

The fungal culture CMW49995 was established on 9 mm Petri dishes three days before adding the nematode eggs. Nematode eggs from parent cultures were harvested by flooding the plates with 4 mL of water and collecting the eggs into an excavated block. The eggs were washed with water five times to get rid of any fungal spores and then the number of eggs were determined (3 counts). On each plate approximately 500 eggs from one Lineage D and approximately 500 eggs from one Lineage B were placed on a single plate (Fig. 1). This provided us in total with eight combinations. After 20 days eggs were collected and counted and approximately 500 eggs were placed on a plate. The F_1 nematode were collected and up to single nematodes were picked. The same was done for F_2. Single nematode extraction was performed as described in Fitza et al., (2022). A single nematode was placed in a solution of 5µl Proteinase K, 1µl PCR buffer without $MgCL_2$ and 9µl of double distilled water. After spinning down the samples shortly, the samples were placed for 10 min into -80 ºC freezer. Later, the samples were placed into the PCR machine for proteinase K activation 65 ºC for 60 min and deactivation 95 ºC for 15 min. Two microsatellite markers (Ds1, Ds323) previously designed (Fitza et al., 2016, Mlonyeni et al., 2011) were selected to characterise the genotypes of these isolates. Polymerase chain reaction (PCR) with the labelled microsatellite markers was performed following the process in Fitza et al. (2019) and the labelled PCR products were sent for Genescan analysis at a partner laboratory (DNA Sequencing Facility, Faculty of Natural and Agricultural Science, University of Pretoria, South Africa) (http://seqserve.bi.up.ac.za//). The program GeneMapper v.6 was used to determine the allele sizes of the various samples and markers. The proportion of F1 and F2 offspring that were homozygous for either parent or represented hybrids (heterozygous for alleles of each parent) were determined. The procedure was followed as described above for the second trial.

Results

Nematode “genotyping”

Inoculated trees: 219 wasps from 95 drums were genotyped from inoculated trees. Kamona was recovered from 77% of bins, although 9% of these also contained Lineage D; 23% of bins containing inoculated trees contained Lineage D alone, and 77% contained Kamona alone. Overall, 77% of wasps emerging from inoculated trees were Kamona. Prior to results in this and our previous trial (Eshetu unpubl.) the expectation was that nematodes recovered from inoculated trees would be Kamona.

Queensland never recovered nematodes from background populations (hence, nematodes from inoculated trees were Kamona, as expected) (Figure 2). ACT also had only Kamona recovered from inoculated trees.

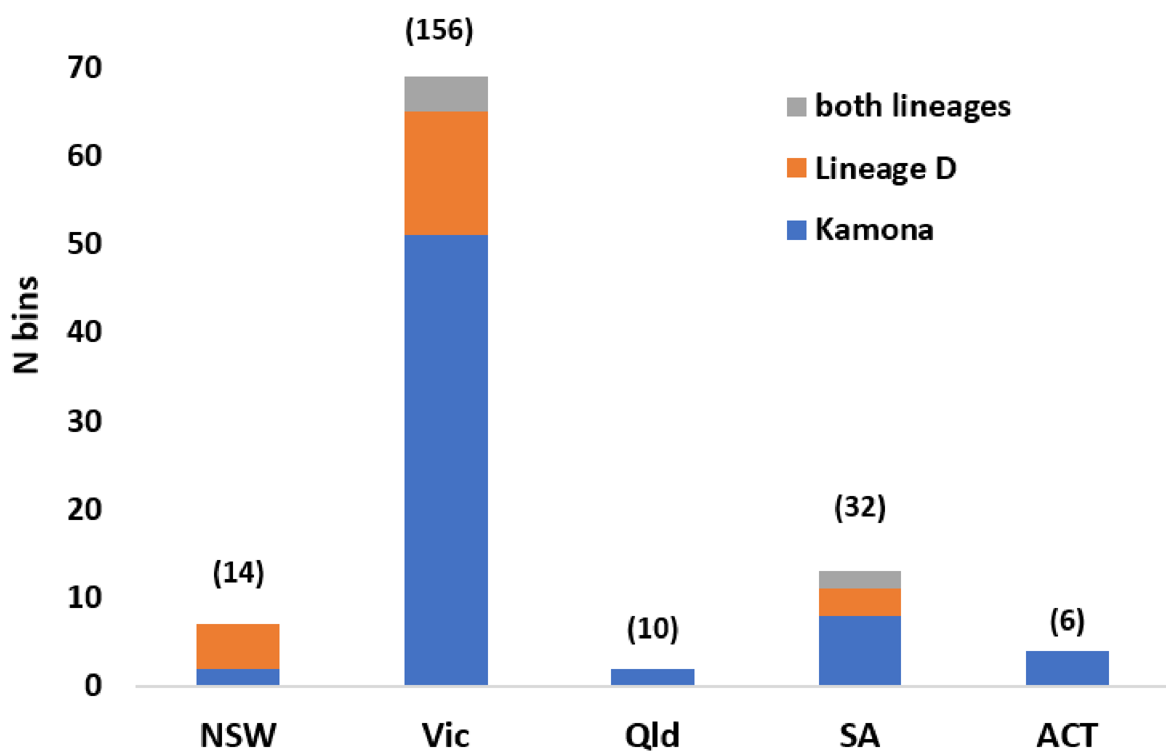

Figure 2: Number of bins containing inoculated trees from each state, which contained nematodes genotyped as Kamona (blue), Lineage D (orange), or both lineages (grey). Number above bars shows the number of wasps genotyped per state.

While it is clear that inoculation with Kamona does not guarantee its recovery, of the four bins containing mixed lineages for which billet data were provided, two represented single trees, suggesting that Kamona is able to develop in trees that already contain Lineage D at inoculation.

Uninoculated trees: For uninoculated trees, the collected nematodes represent those present in background populations, and the ability of Kamona to be transferred into the next generation of wasps. We genotyped 179 nematode samples from uninoculated trees (excluding Sunny Corner and Green Hills outbreak sites), of which 17% were Kamona. There were 70 bins containing uninoculated billets, of which 76% contained Lineage D only, 11%

contained Kamona only, and 13% contained both lineages (Figure 3). This was the first time that ACT was included, and the single background wasp contained Lineage B (not same genotype as Kamona – see SSR results). After no recovery of Kamona from South Australia in our previous study, Lineage B (not same genotype as Kamona – see SSR results) was recorded from 12 wasps in 3 bins here.

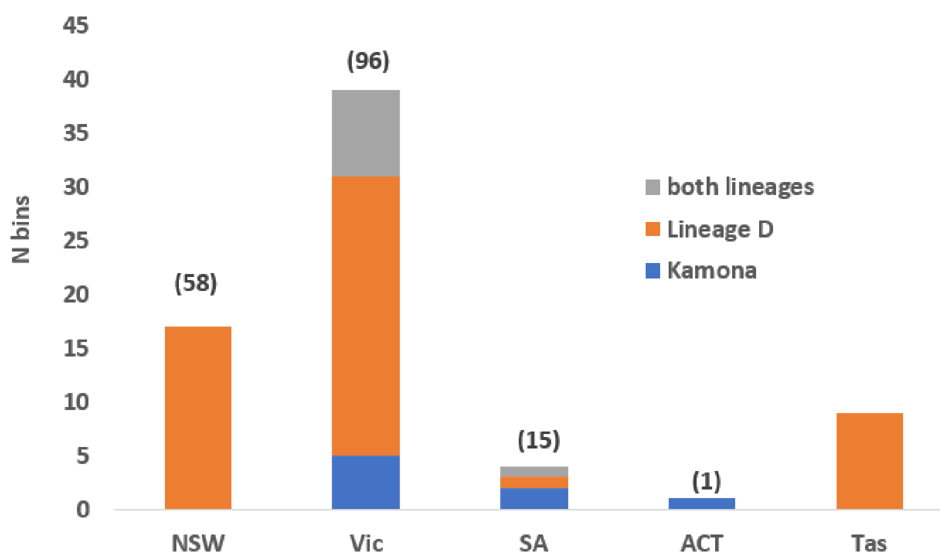

Figure 3: Number of bins containing uninoculated trees from each state, which contained nematodes genotyped as Kamona (blue), Lineage D (orange), or both lineages (grey). Number above bars shows the number of wasps genotyped per state.

Sunny Corner and Green Hills outbreak sites

Nematodes from 51 wasps from at least 15 trees across 7 Sunny Corner compartments were genotyped, along with one sample of nematodes extracted directly from a struck tree collected in 2020. Wasps from three inoculated trees were sampled, while the remainder were uninoculated naturally-struck trees representing background nematode populations following the inoculation of ~16M Kamona nematodes in the preceding two years (Figure 1).
Genotyping results revealed that nematodes from all 52 samples were Lineage D.

While it is good news is that the outbreak resolved quickly, and killed mostly only wildings and suppressed trees (Nahrung et al. 2020b), our failure to recover Kamona from the outbreak site suggests that inoculation played little part in control, and that Lineage D was the main contributor to the observed Sirex population crash. It may also suggest that the notion of superior performance of NSTs for inoculation, as well as previous results reporting background parasitism rate, may simply have reflected the success of Lineage D.

The limited sampling (six samples comprising three parasitised wasps each) from the resolved Green Hills Tumut outbreak in 2012 returned three samples as Kamona, two samples as Lineage D, and one as a probable mixed sample of both genotypes. Thus, both lineages were present in approximately equal numbers, and inoculation appears to have been successful, with Kamona established in background populations in the year following cessation of

inoculation. However, in subsequent years (4 NST samples taken in 2016; 24 in 2019/20) no more Kamona were detected at Green Hills, with all 29 wasps tested infected with Lineage D.

Forty-one *S. noctilio* samples were selected for further characterization of their infecting nematode isolates based on maternally inherited mtCOI sequences (Table 4). To further characterize the genotypes of those nematode samples, five microsatellite markers were applied. All alleles found were the same as those identified in Eshetu et al. (2020). Nine samples (all from Victoria) showed evidence of admixture/hybridisation that was not identified by COI, while four samples were identified by COI as containing a mixture of nematodes from both lineages. SSR marker 54 in all the samples had the same profile for Lineage B and D and therefore was not informative. Sample LH867 from Tasmania showed both Lineage B and D mtCOI profiles, indicating double infection, yet all its SSR markers were associated with Lineage D. In South Australia, three samples (S2, S33, S18) had one or two SSR markers with alleles linked to Lineage D, although the mtCOI results assigned them to Lineage B. Four of the Victorian samples showed SSR markers linked to Lineage B with the mtCOI grouping the samples to Lineage D. Sample V644 has the mtCOI profile of Lineage B while its SSR marker Ds38 had the allele profile of Lineage D. Of the three samples from Victoria that had both mtCOI profiles, two samples (V789, V800) showed allele profiles of Lineage D, whereas sample V950 shows allele profiles of Lineage B. Interestingly, the ACT samples showed no association with Lineage D; all these samples are from trees inoculated with Kamona, and from which the resultant nematodes were identical to Kamona at these markers. It is important to remember that the DNA used was not from single nematodes, but directly from wasps containing multiple nematodes. The mtCOI data indicates the maternally inherited Lineage, while the SSR markers can indicate both maternal and paternal inheritance. In samples with both mtCOI sequence profiles there has to be a mixed infection from both lineages. Not all these show SSR profiles of both lineages though, which might be a PCR artefact or indicating that one of the lineages dominate in the infection.

Table 4: Summary of nematode samples genotyped using mtCOI sequence and microsatellite markers. Blanks in the table indicate alleles that could not be scored. Samples were there is a double mtCOI profile present, or where there is a difference between the mtCOI and the SSR markers are highlighted. Green represents Lineage D and purple Lineage B. Mix refers to the conclusion that the sample contained nematodes of both lineages, admix represented crossing/hybridisation of lineages.

sample	State	Details	COI	DS1	DS33	DS38	DS54	DS105	SSR
N14	NSW	inoc TT, SC o’break	D	207/207	337/337	117/117	152/152	173/181	D
N15	NSW	inoc TT, SC o’break	D	207/207	337/349	114/169	152/152	173/181	D
N16	NSW	inoc TT, SC o’break	D	207/207		114/114	152/152	173/181	D
N9	NSW	uninoc TT, SC o’b	D	207/207	337/337	114/117	152/152	173/173	D
N20	NSW	uninoc NST, SC o’b	D	207/207	337/349	114/114	152/152	173/181	D
342,3	NSW	uninoc NST, SC o’b	D	207/207	349/349	114/114	152/152	181/181/	D
N23	NSW	nematodes from tree, SC o’b	D	207/207	349/349	114/114	152/152	181/181	D
339.2	NSW	uninoc NST, SC o’b	D	207/207		114/114	152/152	173/181	D
21-N-19	NSW	uninoc NST, SC o’b	D	207/207	349/349	117/117	152/152	173/181	D
N7	NSW	uninoc NST, SC o’b	D	207/207	337/349	114/114	152/152	181/181	D
T1	Tas	trapped female	D	207/207	349/349	114/117	143/152	173/173	D

T2	Tas	trapped female	D	198/207	349/349	114/117	152/152	173/181	D
BU862	Tas	trapped female	D	207/207		114/117	152/152	171/171	D
BC873	Tas	trapped female	D	207/207	349/349	114/117	152/152	167/167	D
LH867	Tas	trapped female	B/D	207/207	349/349	117/117	152/152	171/171	(D) mix
T4	Tas	trapped female	D	207/207	349/349	114/117	152/152	173/173	D
S2	SAus	uninoc NST	B		244/244	114/114	152/152		admix
S20	SAus	inoc NST	D		349/349	114/114	152/152	173/181	D
S33	SAus	uninoc TT	B		224/224	114/114	152/152	151/151	admix
S18	SAus	uninoc TT	B		349/349	114/114			admix
21-S-12	SAus	uninoc NST	D	207/207	349/349	114/114	152/152	167/173	D
S14	SAus	inoc NST	D	207/207		114/114	152/152	167/171	D
V661	Vic	inoc TT Benalla	D	159/159	349/349	114/114	152/152	173173	D
V660	Vic	inoc TT Myrtleford	D	155/155	244/244	114/114	152/152		admix
V751	Vic	inoc TT Ballarat	D	207/207	349/349	117/117	143/152		D
V763	Vic	inoc TT Benalla	D	155/207	244/349	114/117	143/152	151/173	admix
V593	Vic	inoc TT Myrtlford*	D	207/207	349/349	114/209	143/152	173/173	admix
V644	Vic	inoc TT Myrtlford*	B	155/155	244/244	114/114	143/152	151/151	admix
V685	Vic	uninoc NST Myrfd	D	155/155	244/244			151/173	admix
V670	Vic	uninoc TT Benalla	D	159/207	349/349			167/167	D
V674	Vic	uninoc NST Shelley	B	155/155		209/209		151/151	B
V780	Vic	uninoc NST Benalla	D	155/155	244/244	209/209	143/152	151/151	admix
V948Q	Vic	uninoc TT Gippslnd	D	216/216	349/349	114/117		167/173	D
V872Q	Vic	uninoc TT Central	D		349/349	114/114	143/152	173/173	D
V732	Vic	uninoc TT Ballarat	D	207/207	349/349	114/117	152/152	167/173	D
V789	Vic	inoc NST Benalla	B/D			114/114	152/152		(D) mix
V808	Vic	uninoc TT Benalla	B/D	207/207	349/349	114/117	152/152	167/173	(D) mix
V950	Vic	inoc TT Benalla	B/D	155/155	244/244	209/209	152/152	151/151	(Kam) mix
21-A-2	ACT	uninoc Parks	B	155/155	244/244	209/209	143/152	151/151	B
21-A-7	ACT	inoc Parks	B	155/155	244/244	209/209	152/152	151/151	Kam
21-A-4	ACT	inoc Parks	B	155/155	244/244	209/209	152/152	151/151	Kam
Kamona			B	155/155	244/244	209/209	152/152	151/151	Kam

The drivers of the high level of homozygosity in the populations of *D. siricidicola* in Australia, which is again highlighted be the SSR data collected here, remains to be resolved. The data presented in Table 4 suggest that lineage B (which is used in the biological control program) is more homozygous than lineage D. It is tempting to speculate that this is influenced by the mass rearing process, but this too remains to be demonstrated.

The SSR profiles using these five markers were combined with those from Eshetu et al. (2020) in a minimum spanning network (Figure 4) to visualise genetic distances between nematode samples from inoculated and uninoculated trees.

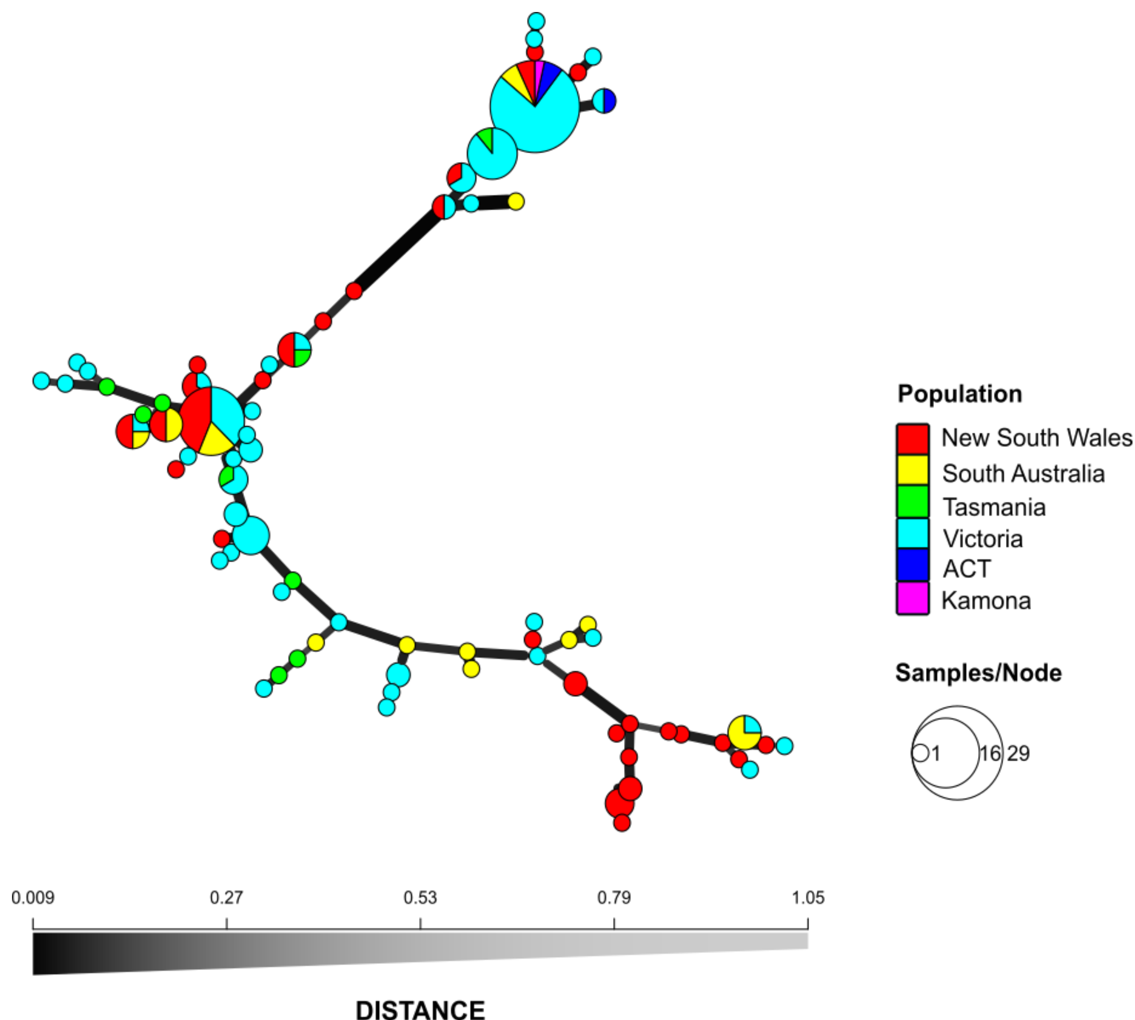

Figure 4: Representation of genetic similarities between nematode samples collected from throughout Australia and assessed using five microsatellite markers from this and our previous (Eshetu et al 2020) study.

Comparison with previous studies

No Kamona were recovered from background populations from Tasmania in this or our previous (Eshetu et al. 2020) study, except in one wasp where COI suggested a mixed sample. We previously found three Lineage B samples in NSW background populations (two with same SSR genotype as Kamona from the same tree; one with SSR genotype different from Kamona), and none in South Australia (Eshetu et al. 2020). Our current genotyping results confirm those of Eshetu et al. (2020) that Lineage D is widespread throughout Sirex range in Australia (with the exception of Queensland which has no background nematodes) and that it is dominant in background populations.

A genetic profiling study was undertaken on nematode populations in the mid-late 1990s using RFLPs, with the aim of ascertaining the extent of 'defective' nematodes in background populations (CSIRO 2000), identifying two-thirds of NSW nematodes as Kamona, where in contrast only three were recovered in our previous study (Eshetu et al. 2020), and none in the current study (Figure 5). A single NSW nematode sample used in Mlonyeni et al. (2011) and later by Fitza et al. (2019) was also genotyped as Kamona. No Kamona strain nematodes were recovered from background populations in Victoria in the earliest study, despite five years of

releases (Collett & Elms 2009). One-third of background nematodes were Kamona in samples from 2014-18 (Eshetu et al. 2020), but Victoria's background levels appear to have halved to 17% in the current study (Figure 6). South Australian samples contained hybrids between Lineage B and D (Table 4); eight other SA samples that were genotyped remain unassigned because inoculation status was not available.

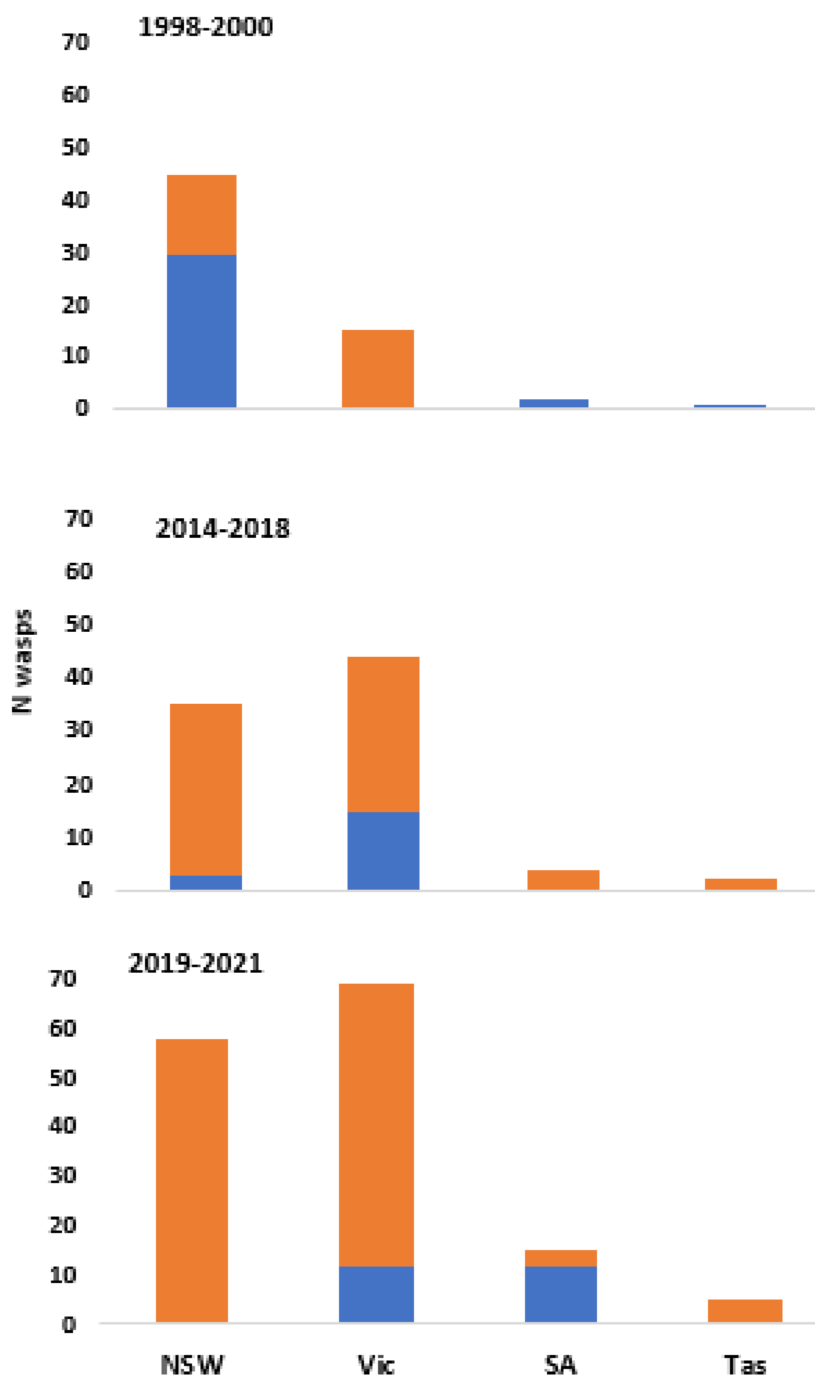

Figure 5: Results of genetic profiling of background nematodes from four states in 1998-2000 (using RFLP), 2014-2018 (using mtCOI and SSR), and in 2019-2021 (using mtCOI) Nematodes were classified as Kamona (blue) or not Kamona (orange).

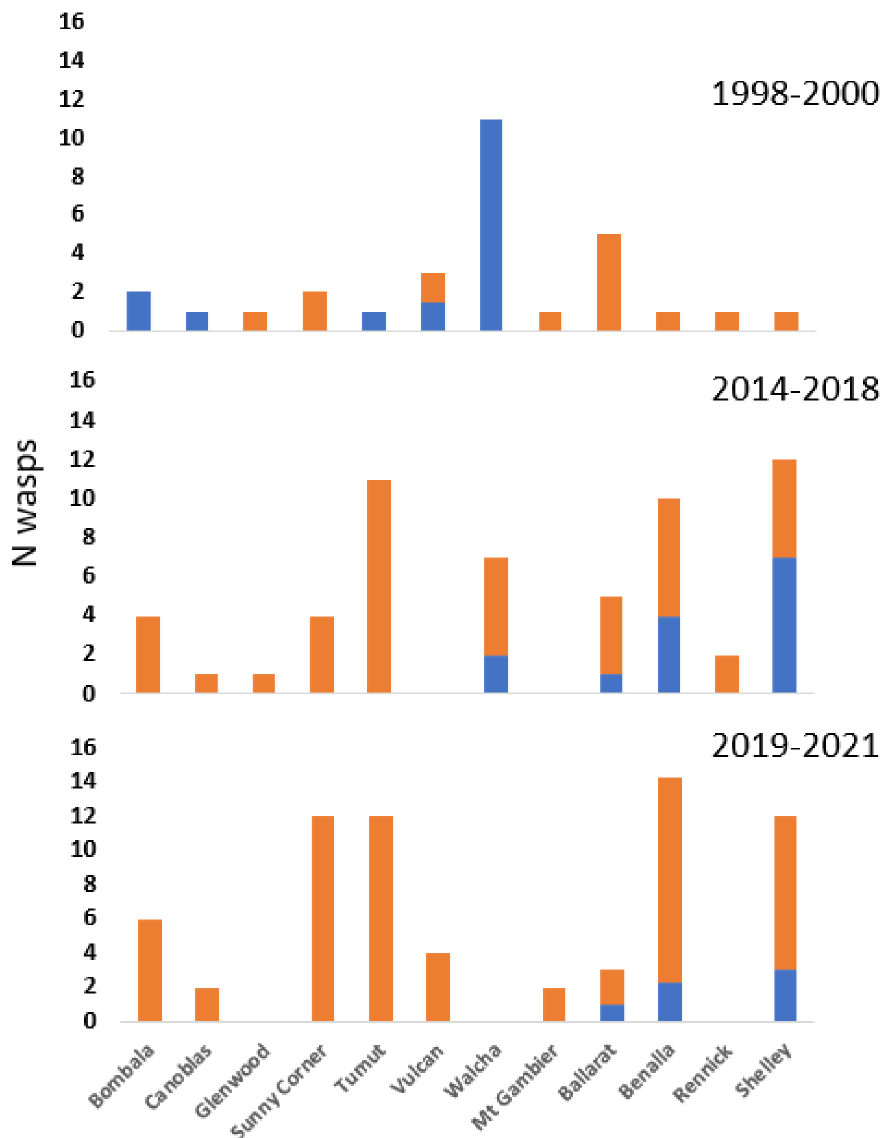

Figure 6: Results at the region level for genetic profiling of background nematodes in 1998-2000 (using RFLP), 2014-2018 (using mtCOI and SSR), and in 2019-2021 (using mtCOI) Nematodes were classified as Kamona (blue) or not Kamona (orange).

Potential drivers of nematode recovery rates

While nematodes in wasps from directly-inoculated trees are more likely to be Kamona (77%), nematodes from background populations are more likely to be Lineage D (83%), however the reasons behind the failure of Kamona to persist beyond its immediate inoculation generation are unknown. There was no evidence that the inoculation history at the block level influenced background nematode rates, with four blocks that had received Kamona 10-13 times since 2005, and three blocks that were inoculated <10 times all only having Lineage D in background populations. Similarly, there was no block-level relationship between the number of trees inoculated in 2018 and 2019 (P2017-18-19 and P2018-19-20) and the number of background Kamona recovered in 2020-21 (P2018-29-20 and P2019-20-21) (Spearman rank correlation, rho=0.22, P=0.49) (Figure 7).

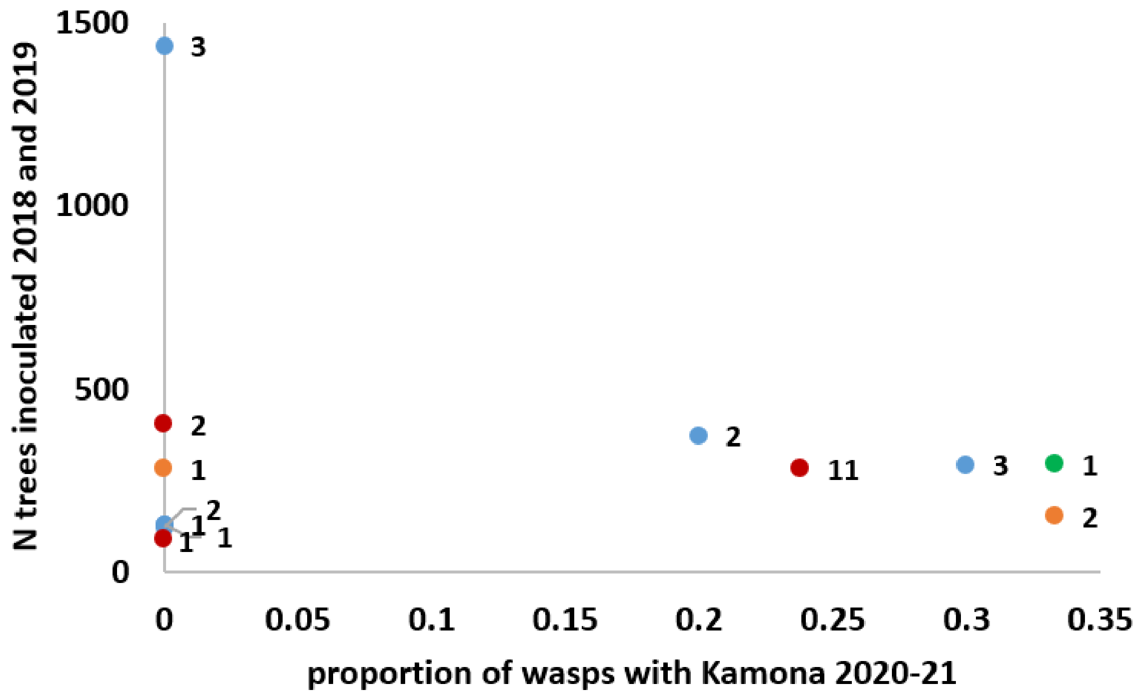

Figure 7: Number of trees inoculated with Kamona in P2017-18-19 and P2018-19-20 and the proportion of background wasps infected with Kamona in P2018-19-20 and P2019-20-21 from Ballarat (green), Benalla (blue), Myrtleford (red), and Shelley (orange). Number beside each datapoint represents the number of bins.

At the region level, there was also no relationship between the number of trees inoculated in 2017-18 and the number of wasps containing Kamona collected in background populations in 2020-21 (Spearman rank correlation, rho=-0.06, P=0.91) (Figure 8).

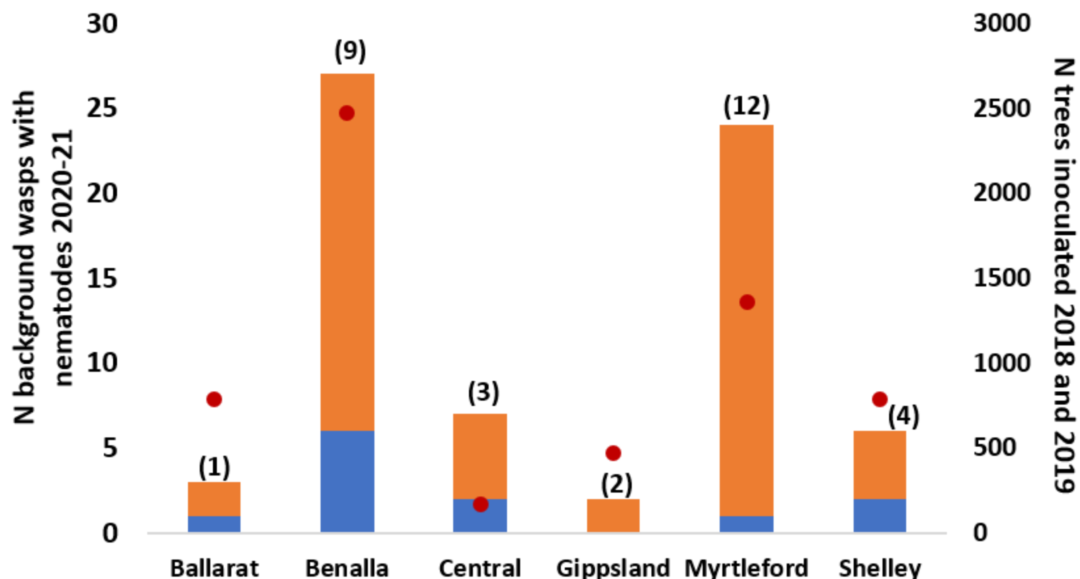

Figure 8: Number of background wasps parasitised with Kamona (blue) or Lineage D (orange) nematodes from different regions in Victoria, and number of trees inoculated with Kamona in the preceding seasons (red dot) (P2017-18-19 and P2018-19-20). The number above bars indicates the number of bins from which wasps emerged.

Inoculations themselves are ostensibly working, however, as Kamona recovery immediately after inoculation averaged 80% at these sites (for which inoculated bins were sampled) (Figure 9). Thus, it appears that these wasps are not successfully transmitting the nematodes they carry into the next generation to be detectable in background populations.

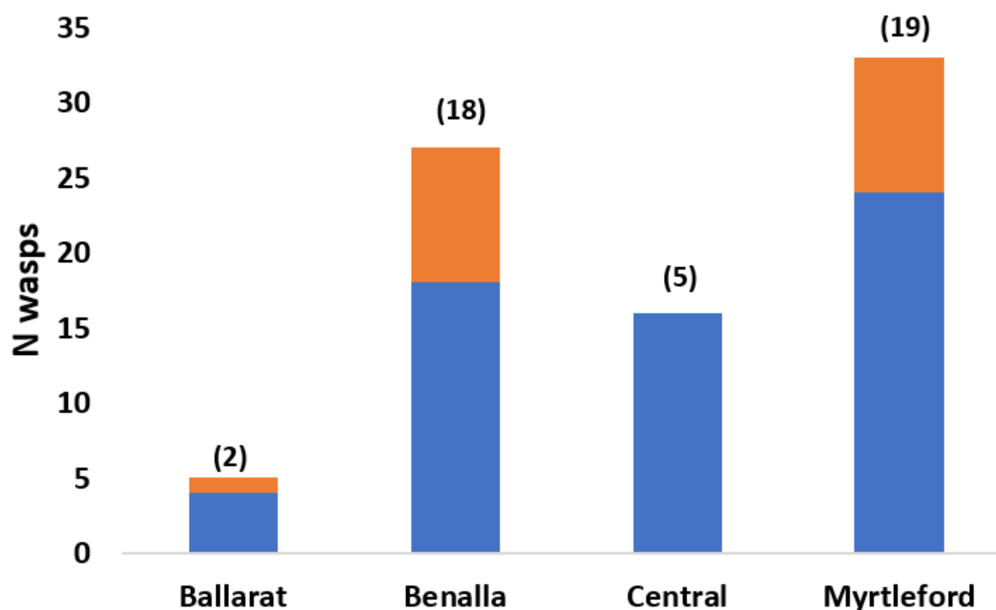

Figure 9: Number of wasps (P2018-19-20 and P2019-20-21) parasitised with Kamona (blue) or Lineage D (orange) nematodes immediately following inoculation (P2017-18-19 and P2018-19-20, respectively). The number above bars indicates the number of bins from which wasps emerged.

Tree status (NST/TT) did not affect parasitism rates (Mann-Whitney U-test, MW=689, P=0.32), number of Sirex per billet (MW=629, P=0.22), or Kamona recovery (MW=595, P=0.96). Inoculation did not affect parasitism rates of emerging Sirex (MW=588, P=0.76; inoculated: 66 ± 5%, uninoculated 55 ± 6%), but, unsurprisingly, inoculated trees yielded a significantly higher proportion of Kamona-infected adults (70 ± 6%) than uninoculated trees (18 ± 6%) (MW=341, P<0.001). A significantly lower number of adults emerged per inoculated billet (10.2 ± 1.3) than from uninoculated billets (14.5 ± 1.7) (MW=990, P=0.03). Adults emerging from bins from which >10 wasps of a single lineage emerged did not differ in their parasitism rates (t_{53}=1.6, P=0.84 - Figure 10).

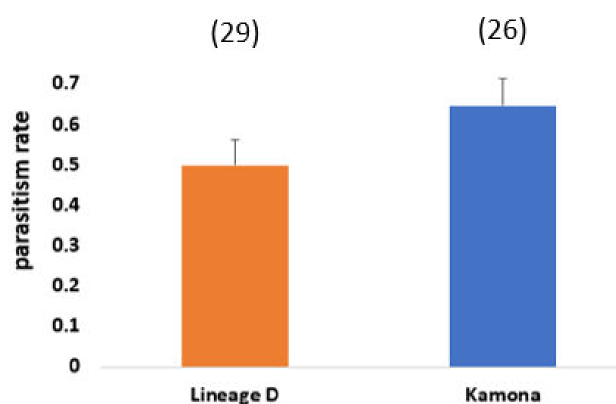

Figure 10: Average + SE parasitism rate (proportion of wasps with nematodes) of wasps emerging from bins from which a single genotype was recorded from at least 10 wasps of Lineage D (orange) or Lineage B (blue). The number above the bars is the number of drums used for the calculation.

Nematode reproduction rate

From the results from Eshetu et al. (2022) growth of the four selected *Amylostereum* strains varied significantly and impacts the success of *D. siricidicola*. It appeared that the best media to conduct further reproductive studies was MEA and using the *Amylostereum* strain (CMW49995, NSW, N72; Figure 11). Eight *D. siricidicola* cultures from Lineage D and five cultures from Lineage B were selected for the reproduction trials. Each trial had three replicates and the trial was conducted twice. Results (Figures 12,13; Table 5) demonstrate that the reproduction rate varies significantly (F_9=32,644;P=2,00E-16) between the different nematode strains. Looking more specifically at Lineage B, the Kamona strain was one of the isolates with the lowest reproduction rate, whereas isolates V484 and V473 had the highest reproduction rate, varying from 500 to 4000 mean number of nematodes. Looking at Lineage D the reproduction of certain isolate is higher compared to Lineage B. The S14 isolate had the lowest reproduction rate, whereas V457 had the highest rate, varying from 300 to 7500 mean number of nematodes. Trial 1 and trial 2 show the same trend. Unfortunately, trial 2 failed for isolate N99 and N104 due to contamination and issues regarding eggs not hatching.

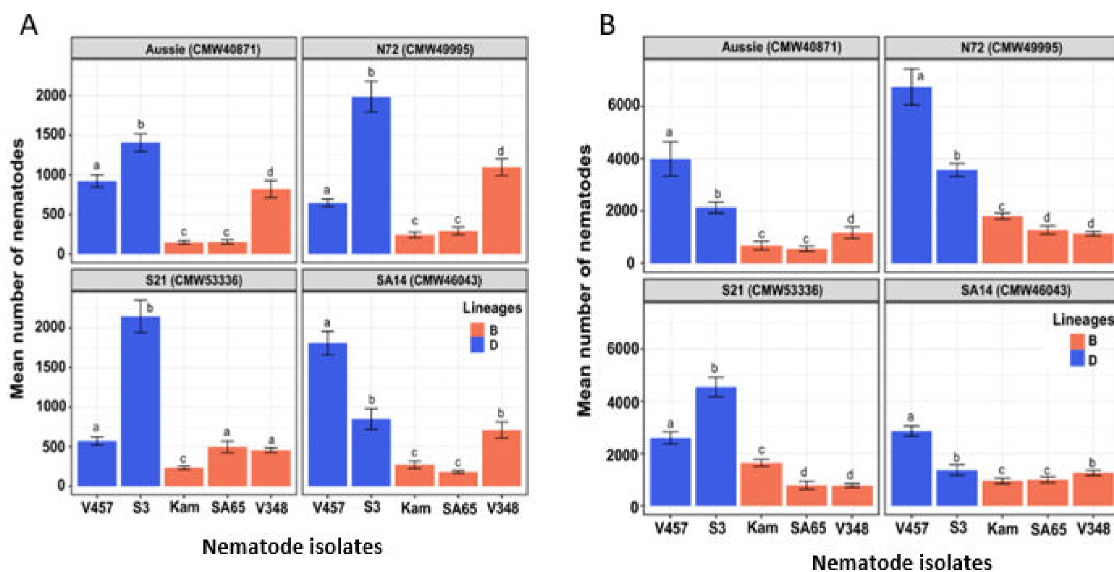

Figure 11: Variation in productivity of *D. siricidicola* on four different *A. areolatum* strains. *Deladenus* productivity in two experimental replica and four fungal strains; on (A) PDA and (B) MEA culture media. Kam, Kamona (Lineage B); S3 (Lineage D); SALY65 (2012) (Lineage B); V348 (Lineage B) and V457 (Lineage D). Error bars represent mean numbers of nematode (+SE) produced after 25 days of nematode egg inoculation on four different fungal strains.

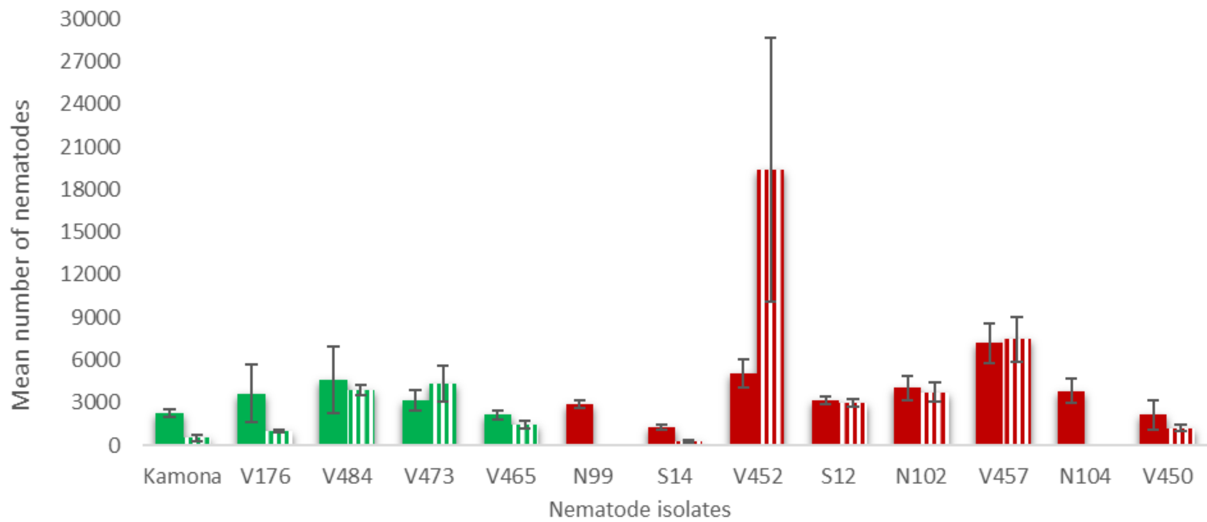

Figure 12: Mean ± SE number of *D. siricidicola* from approximately 500 eggs on MEA after 25 days on the Australian *A. areolatum* strain N72 (CMW49995). The green colour represents the Lineage B nematode strains and the red colour represents Lineage D. The full colour represents trial 1, the patterned samples represent trial 2.

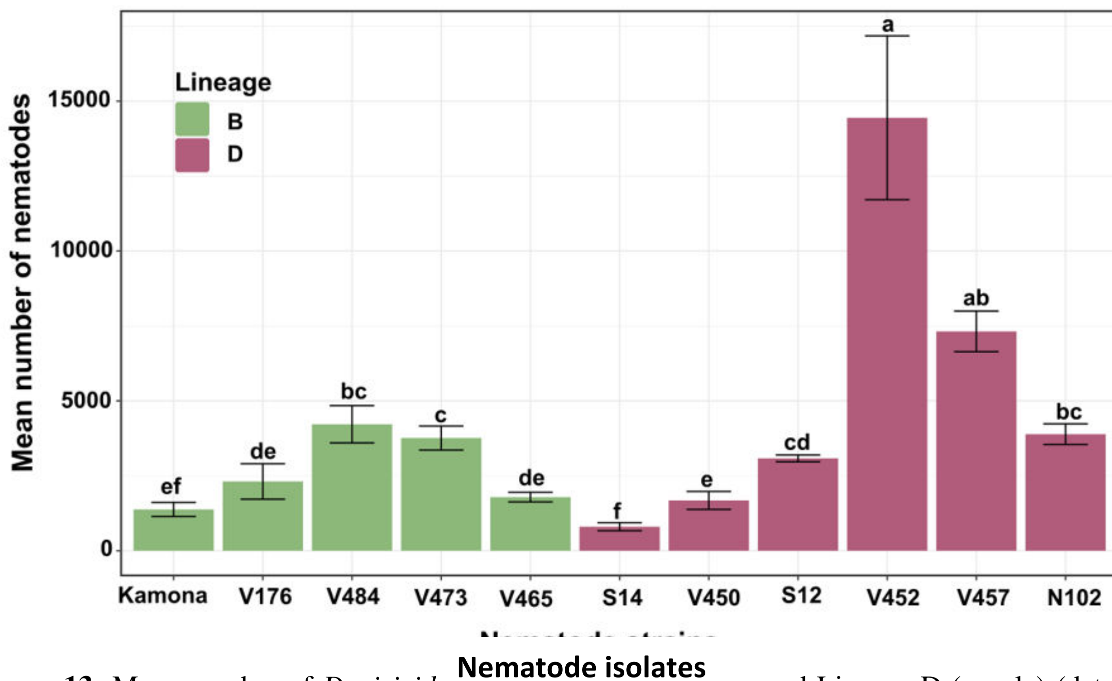

Figure 13: Mean number of *D. siricid* [illegible] and Lineage D (purple) (data from both trials represented separately in Fig 3 combined, with N99 and N102 discarded because there were no data from the second trial) from approximately 500 eggs grown on MEA for 25 days on the Australian *A. areolatum* strain N72 (CMW 49995). Different letters above bars represent significant difference between the nematode isolates.

Table 5. Statistical analysis of the combined data from both trials with lineage as main factor and without strains N99 and N102.

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Signif.
Lineage	1	7.96	7.958	19.025	2.19e-05	***
Isolates	9	122.89	13.655	32.644	2,00E-16	***
Plate	5	4.71	0.942	2.252	0.0512	.
Lineage:Plate	5	6.52	1.303	3.115	0.0101	*
Residuals	177	74.04	0.418			

Field inoculation trials

A total of 284 wasps (56F/228M) emerged from the caged field-collected billets and were dissected. For all the female wasps it was observed that the nematodes had parasitised the eggs for all the treatments. An unusually high level of parasitism - over 90 % - was observed for all the treatments, including the control, with no significant differences in parasitism rates between treatments (Figure 14). This indicated very high background parasitism, with results predominantly due to the nematodes already present at the study site before inoculation, i.e. background parasitism. This was confirmed by the genotyping results from the subset of parasitised wasps, as all but one of the parasitised wasp examined were infected with lineage B (Table 8). This includes wasps emerging from billets inoculated with Lineage D.

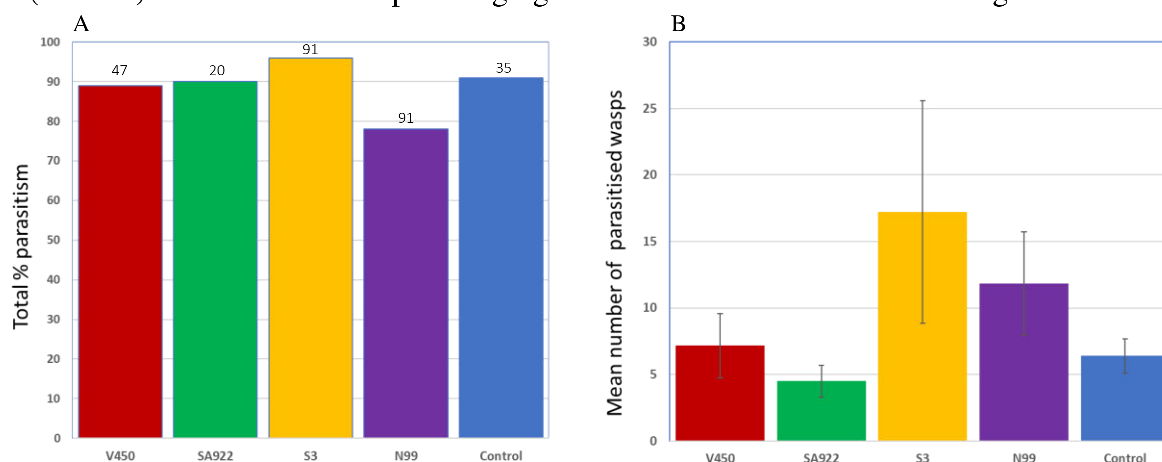

Figure 14: (A) Total % parasitism of all five treatments. The number above the bar represents the total number of wasps that emerged from the billets. (B) Mean ± SE number of parasitised wasps per treatment. Trees were inoculated with Lineage D (V450, S3, N99) or Lineage B (SA922), and the control was uninoculated.

Table 8: Number of nematode samples sequenced for the individual treatments and their haplotype allocation.

Source	# Samples	Results
V450	12	1 Lineage D
		11 Lineage B
SA922	2	2 Lineage B
S3	14	14 Lineage B
N99	5	5 Lineage B
Control	4	4 Lineage B

These results appear to parallel the field situation in Australia, with the established background population outperforming inoculated nematodes. Similar results were found in the US, where background nematodes far outperformed Kamona in parasitism rates of emerging wasps also (Bittner et al. 2019). All else being equal, this suggests that there may simply be a temporal advantage to establishment within a tree.

Sterilising ability

Of the 61 Sirex females dissected to examine egg sterilisation, 35 were parasitised with Kamona (all but one from inoculated trees), and 26 with Lineage D (all but two from uninoculated trees). Nematodes were present in eggs of wasps parasitised with both lineages, and there was no notable difference in egg sterilisation rates, apart from two wasps which did not have any parasitised eggs (Lineage B) (Figure 15).

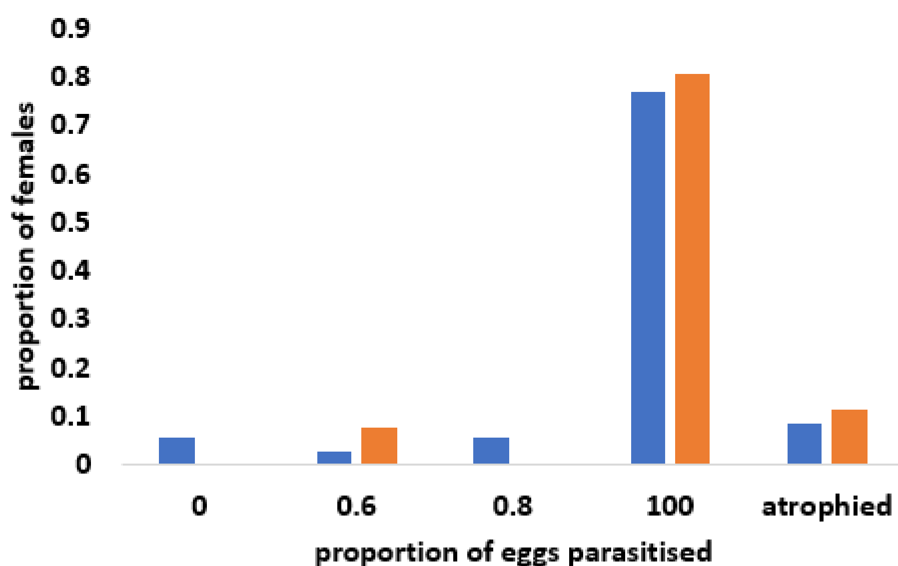

Figure 15: Frequency histogram showing the proportion of female Sirex with zero, 60%, 80% and 100% of sampled eggs (N=5/wasp) parasitised, and with ovaries completely atrophied, from those parasitised with either Kamona (blue; N=35) and Lineage D (orange; N=26).

Thirteen samples of Kamona were noted as having “many nematodes in ovaries, very few in body cavity” while no Lineage D had such an observation. Five Lineage D and three Kamona- parasitised females were noted as having “no nematodes in distal eggs” (a similar result found in Nahrung & Griffiths 2015). No other notable differences were observed between nematode lineages during dissection; both lineages had a 2-3 individuals with many parent nematodes noted, and with “many” and “few” nematodes recorded in ovaries. Importantly, we have confirmed the sterilising ability of Lineage D is similar to that of Kamona.

Nematode interaction trial

All eight *D. siricidicola* lineage crosses produced viable offspring. The total number of eggs for the F1 and F2 for both trials is shown in Figure 16. As the profile of both SSR markers was homozygous for the parental isolates, any crosses between lineage B and D would be seen as heterozygous markers in individual offspring. According to the Hardy – Weinberg calculations in the F1 generation both crosses with V457 had lower actual heterozygous numbers than the expected numbers (Table 9). The crosses K * S14 had the expected number

of heterozygous offspring. In the F2 generation all crosses had higher number of heterozygous loci than the expected numbers. From a previous study (Fitza et al. 2022) all crosses with Lineages A, B and C contained at least some heterozygous individuals, with proportions ranging from 3-56%. In this study, we used the lowest and the highest scoring nematode isolates of Lineage B from the reproduction trial to cross with four isolates of Lineage D. Interestingly all the crosses contained heterozygous individuals, with proportions ranging from 0-98% in the F_1 and 37-100% in the F_2 (Figure 17). In the F_2 generation the number of hybrids further increased. Lineage B and D seem to have a very high interbreeding capability, therefore no mating barriers. In Eshetu et al. (2022) hybrids between Lineages B and D, and C and D, were observed from the field. The current experiment under laboratory conditions, starting off with the same number of eggs from both Lineages, is obviously not a true reflection as to what is observed in nature. The results confirm that there are no mating barriers and that both lineages are compatible. It is a curious but consistent observation, however, that hybrids in the field is rare. That observation suggests that there is either an as yet undefined ecological or mating barrier amongst the lineages, or that hybrids are less 'fit' than their parents. Together with the excessive homozygosity in field populations, these results points towards a non-Mendelian and non-random mating system.

Table 9: Proportion of F1 and F2 nematodes from crosses that were heterozygous or homozygous for alleles of either parent. Under random mating, it is expected to have 25% homozygotes resembling each parent and 50% heterozygous offspring in the F1 cross given the equal starting population of equal number of homozygous parental isolates.

F1	Parent 1 (p)	Parent 2 (q)	#single F1 nematodes screened	Homozygous for alleles of parent 1	Homozygous for alleles of parent 2	Heterozygous
	K	457	50	47	0	3
	K	N102	66	0	3	63
	K	S14	67	1	0	66
	K	N99	71	31	1	39
	484	457	68	6	33	29
	484	N102	70	0	4	67
	484	S14	69	69	0	0
	484	N99	68	8	4	56

F2	Parent 1 (p)	Parent 2 (q)	#single F1 nematodes screened	Homozygous for alleles of parent 1	Homozygous for alleles of parent 2	Heterozygous
	K	457	71	3	0	68
	K	N102	67	3	2	62
	K	S14	69	2	1	66
	K	N99	66	25	0	41
	484	457	68	0	0	68
	484	N102	70	0	0	70

484	S14	67	43	0	24
484	N99	67	1	0	66

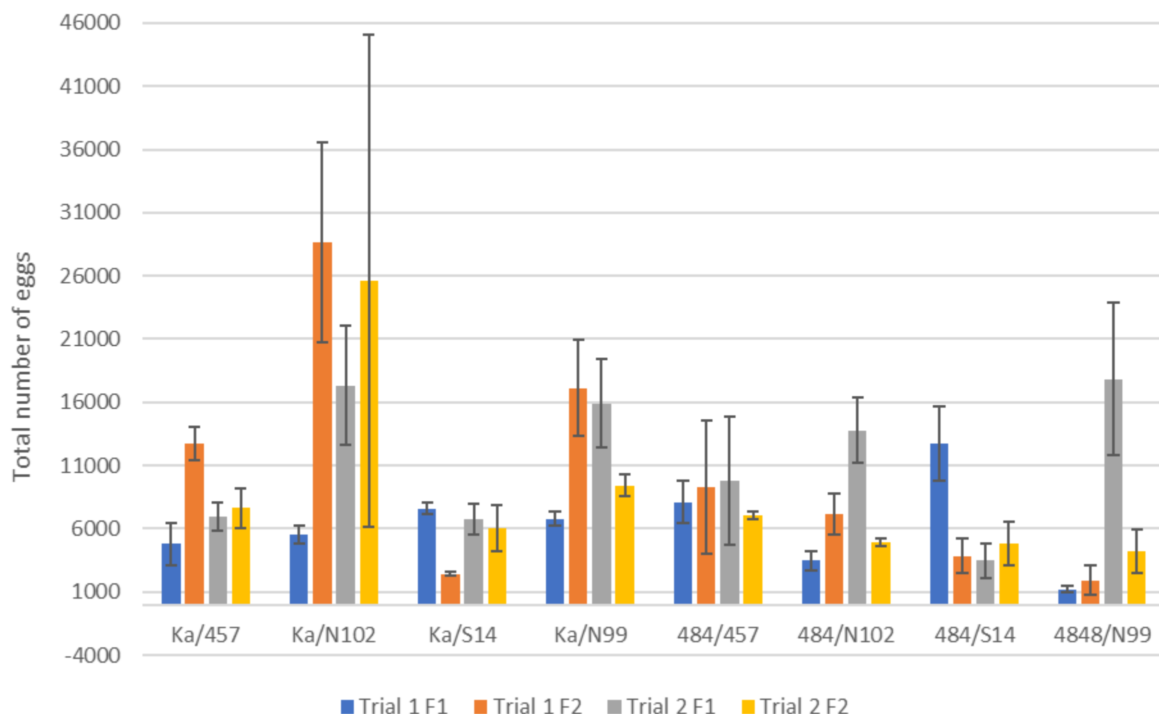

Figure 16: Total number of eggs from the different crosses grown on MEA after 20 days on the Australian *A. areolatum* strain N72 (CMW49995). The different colours represent the different generations F1 and F2 of both trials.

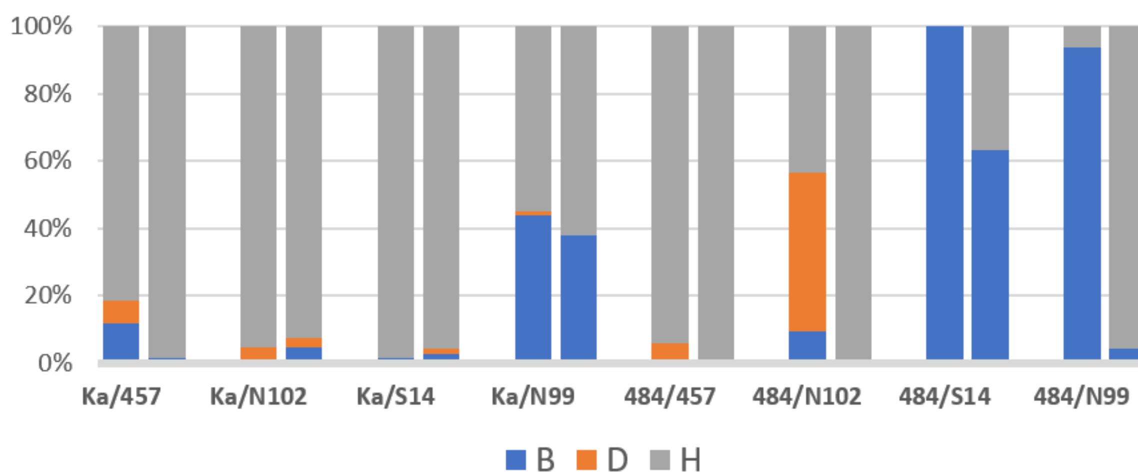

Figure 17: Percentage of *D. siricidicola* belonging to the two Lineages or hybrids. Approximately 500 eggs grown on MEA Petri dish and after 20 days on the Australian *A. areolatum* strain N72 (CMW) eggs were collected. This was done for F_1 and F_2. The blue colour represents the Lineage B nematode strains, the orange colour represents Lineage D and the grey colour represents hybrids (heterozygous). The left column represents F_1 and the right column represents F_2.

Inoculation trials and infection studies

At the end of October 2021 70 *Pinus radiata* logs (80 cm long) were obtained from a plantation (26°57'36,13"S 30°33'27,22"E) in Mpumalanga, South Africa. These were placed

into emergence cages at the FABI Biocontrol Centre. Over the two and a half months the logs were kept moist and female *S. noctilio* collected and brought to FABI. Twenty females emerged and brought to the laboratory to check for parasitism and for egg activation studies. About 60% were parasitized. The eggs were cleaned and spread out on a slide and pressure was exerted for one minute. The eggs were kept moist over the next few days. After 10-12 days signs for egg activation were determined (Figure 18). A success rate of less than 1% was achieved per wasp. These were placed onto nutrient diet media (Figure 19). However, there was no success in the eggs to develop into larvae. Further testing and optimization need to be done.

Initial results from a different area (Riversdale, Western Cape, South Africa) conducted in March/April 2021, indicated an egg activation success rate of between 20-90%. *Sirex noctilio* wasps from this area were generally larger in size than wasps collected from Mpumalanga and showed little to now parasitism. A portion of the activated eggs developed fully into first instar larvae. The first instar larvae were reared further for a short duration on an artificial diet. This egg activation technique has promise for future work to obtain *S. noctilio* larvae from female wasps via dissection. Credit for this work goes to Dr Gudrun Dittrich-Schröder and Ms Elmarie Cronje at FABI.

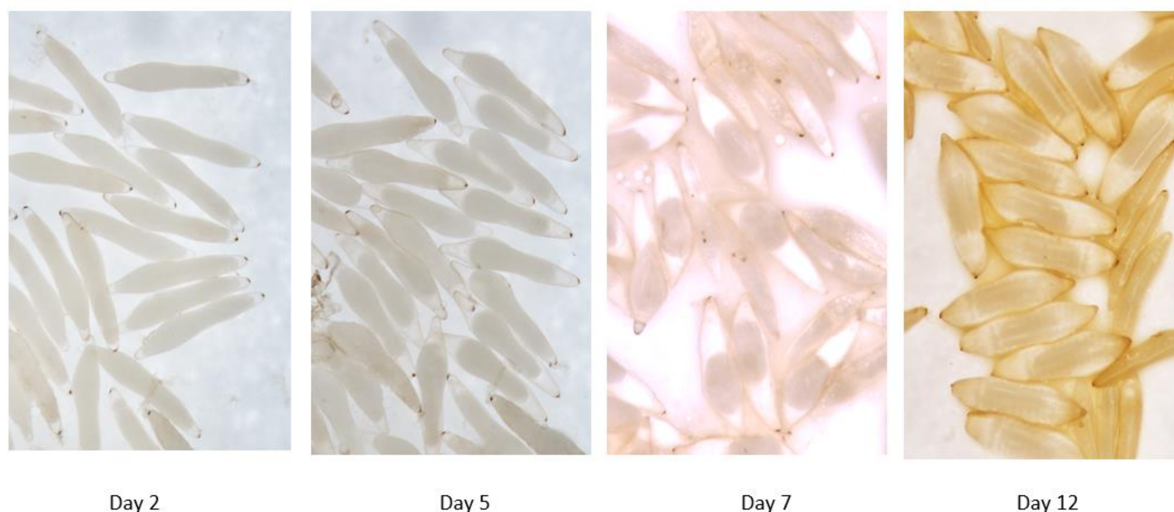

Figure 18: Pictures of activated, developing eggs over time period of twelve days taken by Gudrun Dittrich-Schröder at FABI.

Figure 19: Bottle of the Asian Long-Horn Beetle diet used to place activated eggs and allow them to develop into larvae.

Conclusions

This study confirms the results of Eshetu et al. (2020) who found a novel lineage (Lineage D) of *D. siricidicola* parasitising *S. noctilio* background (uninoculated) populations. Inoculated trees yielded 77% Kamona, suggesting that inoculation themselves are working but that Kamona is not persisting significantly beyond the emerging generation. In this context, Kamona could be considered an inundative biocontrol agent (impacting the generation against which it was released) while Lineage D acts as a classical biocontrol agent (released once (although its origin is unknown) and continues to exert control with no intervention).

There is significant variation in the reproductive rate of the various nematode isolates in culture. One of the consistent outcomes, however, is that the Kamona strain appears to be reproducing slower, at least in culture, compared to a number of other isolates from background populations.

The fastest reproducing strains appear to be from Lineage D. It should be remembered that this culture-based results do not necessarily reflect the situation in the field. If nothing else, it could increase laboratory production numbers, and it might be hoped that this trait will be maintained in the field. However, it should also be noted that faster-reproducing nematodes may consume more *Amylostereum*, potentially competing with Sirex larvae for this food source and leading to smaller adults that lay fewer eggs and have a smaller dispersal distance.
It would make sense to incorporate some of the Lineage D strains into production and inoculation efforts. What gains this would provide remains to be tested, both in production and inoculation. This should not be seen as a silver bullet, but rather a sensible use of the genetic diversity present in the environment. A system such as that used in South Africa where strains from highly parasitised billets are isolated each year and introduced into the nematode production system could be considered. Not only does it sample from the potentially adapted and fittest genotypes in the environment, but it avoids any potential long term cultural or storage effects.

Mating between Lineage B and D isolates have been demonstrated before, but the current experiments highlight that there appear to be no mating barrier in culture. For this reason, our interaction trials did not yield any insight into possible competition, but rather produced mixed cultures and a majority of individuals with recombined genetics. The barriers that maintain the apparent lineage separation in the field will have to be determined. This might relate to as yet undetermined ecological barriers, or artificial barriers introduced via distribution and inoculation protocols.

The high level of homozygosity observed in previous studies was confirmed in this study and suggests a non-random mating or genetic sorting mechanism. It is possible that a genetic system such as meiotic parthenogenesis, as observed in *Meloidogyne* spp., or other inbreeding mechanisms could be present, but this remains to be tested.

Recommendations

Trap tree plots should be re-evaluated for their value and the technique used to inoculate them. They may be seen as more valuable as monitoring plots, as opposed to primarily for the inoculation and spread of *D. siricidicola*. Inoculation technique of trap trees should be continued to be reviewed to improve the yield (numbers of parasitised females emerging).

This and the related study by Eshetu et al. demonstrates the value of ongoing sampling for storage (and potential culturing) from the pest and biological control populations, as is done elsewhere (e.g. in South Africa), and for occasionally studies to characterise the diversity and potential impact of *D. siricidicola*.

The NSCC (industry and technical experts) met on 18-19 October 2022 in Canberra, with the main business item discussion of options for ongoing Sirex management (Table 10). It was broadly agreed that current evidence confirms that inoculation with Kamona is working (ie parasitised females are being produced) but not in sufficient numbers to persist in background populations in the presence of Lineage D. The agreed short-term plan was to continue TTPs with Kamona for the next season, while sourcing Lineage D to substitute into the rearing program in future.

Table 10: Options for ongoing Sirex management program considered at NSCC meeting 19 October 2022.

Consider the options

Option	Supporting	Negating
Maintain status quo (TTPs, Kamona) – continue trying to improve/understand TTP yield	Sirex isn't outbreaking… Kamona parasitism rates as good as L-D (at bin level); TTPs provide good data	TTP yield is v low and variable. What else can we try!? Kamona isn't persisting anyway TTPs ~$1000 each
Continue TTPs, replace Kamona with Lineage D	Would faster repdve rate improve culturing?? And in-tree parasitism wrt larger dose? TTPs provide good data "Good news" story!	Yield may not be affected by nematode (strike rate, sex ratio, wasp para etc); can too many be bad (size?) Difficult to determine success
Discontinue TTPs, use NSTs with Kamona/Lineage D	TTP yield low, no evidence of contribution (unless direct)	Vic mean 12/billet for NST and TT
Discontinue routine TTPs. Emphasis on surveillance and response via inoculation on demand (TTPs/NSTs) Improve silviculture (thinning etc)	TTP yield low, $0.5Mpa Sirex isn't outbreaking	Ongoing availability of nematodes, viability of production No evidence that 'flooding' worked in SC Rarely attacks healthy/dominant trees
Use TTPs only for monitoring – not inoculating	Keeps track of populations and Ibalia parasitism rates	Emergence facility results may not reflect field results

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Appendix 1: COI protocol to distinguish nematode lineages

Protocol to distinguish *Deladenus siricidicola* strains using COI sequencing

Firehiwot B. Eshetu[a], Irene Barnes[a], Helen F. Nahrung[b], Katrin N.E. Fitza[a], Nicolas Meurisse[c], Bernard Slippers[a *]

[a]Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, 0002, South Africa

[b]Forest Industries Research Centre, University of the Sunshine Coast, Queensland, Australia

[c]Scion (New Zealand Forest Research Institute), 49 Sala Street, Rotorua 3046, New Zealand

DNA extraction

The NucleoSpin® Tissue (Macherey-Negel, Germany) high DNA recovery and purity kit was used for total genomic DNA extraction from ethanol-preserved nematodes and ethanol-preserved infected wasp eggs, as well as from Petridish cultures on *Amylostereum areolatum*, following the manufacturer's instructions. The concentration of the genomic DNA harvested was quantified with a Nano-Drop ND-1000 UV/Vis Spectrometer (NanoDrop Technologies, Wilmington, DE 19810 USA). A minimum of 30 ng/µl genomic DNA concentrations was preferred as final working concentration for downstream PCR amplification.

Mitochondrial (mtCOI) sequencing

A portion of the 5' end of the mitochondrial cytochrome *c* oxidase subunit one (mtCOI) gene is amplified using mtCOI-F and mtCOI-R primers specifically designed for *Deladenus* spp. (Morris et al., 2013). The PCR reaction master mix was prepared in a 25 µl total volume using the MyTaq™ DNA polymerase protocol consisting of 0.5 µl of 1.5-unit MyTaq™ DNA polymerase (Bioline Ltd. UK), 5 µl of 10x MyTaq™ PCR buffer, 1µl of 0.5 M of each primer, 2 µl of template DNA (30 ng/µl) and 15.5 µl of sterilized PCR grade SABAX water. PCR cycling was performed using the method described in Fitza et al. (2019). Gel electrophoresis was performed on 2% (w/v) agarose using 3 µl of PCR product mixed with 2 µl GelRed™ (Biotium, California) in a sodium-borate buffer system and visualized under ultraviolet light. PCR products purification, sequencing PCR, and precipitation were the same as those described by Fitza et al. (2019). Precipitated PCR products were sent for sequencing at the DNA Sequencing Facility, Faculty of Natural and Agricultural Science, University of Pretoria, South Africa).

Sequences are edited and manually checked in Bioedit version 7.2.5 (Hall 1999) and aligned using the MAFFT free online alignment program version 7 (Katoh et al., 2017).

Mitochondrial (mtCOI) analyses

Above protocol yields a 523 bp amplicon of the mtCOI gene region. Single nucleotide polymorphisms at nucleotide positions 23, 208, 314, and 385, distinguish lineages D, A, C and B, respectively, from one another (Table 1).

Table 1: Mitochondrial cytochrome *c* oxidase subunit 1 (mtCOI) sequence-based single nucleotide polymorphism between different lineage groups in *D. siricidicola* populations.

Lineage groups	Site 23[a]	Site 208[b]	Site 314[c]	Site 385[d]
Lineage A	C	T	C	C
Lineage B	C	C	C	T

Lineage C	C	C	T	C
Lineage D	T	C	C	C

[a] Site 23: Polymorphic site at position 23 and distinguishes lineage D from 3 the other lineages
[b] Site 208: Polymorphic site at position 208 and distinguishes lineage A from the 3 other lineages
[c] Site 314: Polymorphic site at position 314 and distinguishes lineage C from the 3 other lineages
[d] Site 385: Polymorphic site at position 385 and distinguishes lineage B from the 3 other lineages

Lineage B reference sequence (539 bps - Fitza et al. 2019) is:

```
  1 gttgtcctga tataaggttt cctcgtttaa ataacttaag tttttggcta ttgccaactg
 61 ctttagtttt ggtcttatct tcaggttttg ttgatatagg tgggggtact agttgaacgg
121 tttacccacc tttaagaact tttggtcatt tgggtgctag tattgattta gttattttta
181 gtttgcattg tgctgggatt agttctattt tgggtggtat taattttata tgcacagtaa
241 agaatttgcg tagtagatct ttatctttgg aacatataag tttatttgtt tgaagtgttt
301 ttgtaacagt ttttttgttg gttttgtctt taccagtact agctggtgct attactatgt
361 tattgacaga tcgtaatttt aatacttctt tttttgatcc tagttttggt ggtaaccctt
421 tgatttatca acacttgttt tgatttttg gtcatccgga ggtttatatt ttgattttgc
481 ctgcttttgg tattttaagt catagtgcct tgagacttac aggtaaaaaa gagattttt
```

Lineage A reference sequence (Fitza et al. 2019)

```
  1 gttgtcctga tataaggttt cctcgtttaa ataacttaag tttttggcta ttgccaactg
 61 ctttagtttt ggtcttatct tcaggttttg ttgatatagg tgggggtact agttgaacgg
121 tttacccacc tttaagaact tttggtcatt tgggtgctag tattgattta gttattttta
181 gtttgcattg tgctgggatt agttctattt tgggtggtat taattttata tgcacagtaa
241 agaatttgcg tagtagatct ttatctttgg aacatataag tttatttgtt tgaagtgttt
301 ttgtaacagt ttttttgttg gttttgtctt taccagtact agctggtgct attactatgt
361 tattgacaga tcgtaatttt aatacttctt tttttgatcc tagttttggt ggtaaccctt
421 tgatttatca acacttgttt tgatttttg gtcatccgga ggtttatatt ttgattttgc
481 ctgcttttgg tattttaagt catagtgcct tgagacttac aggtaaaaaa gagattttt
```

Lineage C reference sequence (Fitza et al. 2019)

```
  1 gttgtcctga tataaggttt cctcgtttaa ataacttaag tttttggcta ttgccaactg
 61 ctttagtttt ggtcttatct tcaggttttg ttgatatagg tgggggtact agttgaacgg
121 tttacccacc tttaagaact tttggtcatt tgggtgctag tattgattta gttattttta
181 gtttgcattg tgctgggatt agttctattt tgggtggtat taattttata tgcacagtaa
241 agaatttgcg tagtagatct ttatctttgg aacatataag tttatttgtt tgaagtgttt
301 ttgtaacagt ttttttgttg gttttgtctt taccagtatt agctggtgct attactatgt
361 tattgacaga tcgtaatttt aatacttctt tttttgatcc tagttttggc ggtaaccctt
421 tgatttatca acacttgttt tgatttttg gtcatccgga ggtttatatt ttgattttgc
481 ctgcttttgg tattttaagt catagtgcct tgagacttac aggtaaaaaa gagattttt
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More details

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