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Efficacy of Kamona strain *Deladenus siricidicola* nematodes for biological control of *Sirex noctilio* in North America and hybridisation with invasive conspecifics

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Abstract

Sirex noctilio is an invasive woodwasp that, along with its symbiotic fungus, has killed pine trees (*Pinus* spp.) in North America and in numerous countries in the Southern Hemisphere. We tested a biological control agent in North America that has successfully controlled *S. noctilio* in Oceania, South Africa, and South America. *Deladenus siricidicola* nematodes feed on the symbiotic white rot fungus *Amylostereum areolatum* and can switch to being parasitic on *S. noctilio*. When parasitic, the Kamona nematode strain can sterilise the eggs of *S. noctilio* females. However, in North America, a different strain of *D. siricidicola* (NA), presumably introduced along with the woodwasp, parasitises but does not sterilise *S. noctilio*. We tested the sterilising Kamona biological control strain of *D. siricidicola* against *S. noctilio* in North America. Interactions between the biological control strain and the NA strain could include competitive exclusion, co-infection within hosts or hybridisation. We reared *D. siricidicola* Kamona on an *A. areolatum* strain native to North America (IGS-BE) and another strain (IGS-BDF) used commercially to mass-produce the nematode in Australia. We inoculated Kamona reared on either strain of *A. areolatum* into logs infested with *S. noctilio* larvae and compared parasitism rates against control logs. Individual nematodes were isolated from *S. noctilio* hemocoels and from sterilised eggs and were genotyped with eight microsatellite loci. A high rate of parasitisation of *S. noctilio* by *D. siricidicola* NA was found for all treatments and we found evidence of both co-infection and hybridisation. Surprisingly, sterilisation rates were not related to the rates of parasitisation by *D. siricidicola* Kamona.

Keywords

hybridization, coinfection, biological control, *Deladenus siricidicola*, *Sirex noctilio*, *Amylostereum areolatum*, parasitic nematode

Introduction

Invasive species management often employs biological control agents, such as predators, parasitoids or disease organisms, to slow invasive population growth. Many factors should be taken into consideration when evaluating biological control agents, including potential interactions with closely-related organisms which become sympatric upon introduction. Competition amongst closely-related organisms could affect the long-term success of the agent (Messing et al. 2006, Guzmán et al. 2016, Cebolla et al. 2018), but unpredictable and potentially irreversible effects may also occur through hybridisation (Szűcs et al. 2011, Havill et al. 2012). Hybridisation between introduced and native biological control organisms could have effects ranging from inhibition, to no impact, to enhancement of control, but direct links between hybridisation and control efficacy have not been demonstrated. We confirmed intraspecific hybridisation between two strains of an insect-parasitic nematode and investigated the efficacy of infection and sterilisation of the invasive target insect.

The Eurasian woodwasp, *Sirex noctilio* and its symbiotic white rot fungus, *Amylostereum areolatum,* can kill pine trees (*Pinus* spp.). Both organisms have been introduced to North America (Hoebeke et al. 2005, Nielsen et al. 2009) and pine-growing regions in the Southern Hemisphere (reviewed in Hurley et al. 2007). The nematode *Deladenus siricidicola* has been used in the Southern Hemisphere as a biological control agent since the 1970s because it can kill eggs in female *S. noctilio* (Hurley et al. 2007). A commercialised strain of *D. siricidicola*, called Kamona, is used there, but a non-sterilising strain of this nematode species (hereafter called the "North American strain;" NA) was unintentionally introduced to North America, presumably along with invasive *S. noctilio* and is well-established (Yu et al. 2009, Williams et al. 2012, Kroll et al. 2013). Although not found inside *S. noctilio* eggs, the NA strain is transferred amongst trees along with viable eggs (Kroll et al. 2013).

Deladenus siricidicola can live for many generations as free-living mycophagous forms, feeding on *A. areolatum* within pines, but can be triggered to change to the infective form and enter *Sirex* larvae. Higher CO_2 and lower pH in close proximity to larvae are associated with conversion of *D. siricidicola* from mycophagous to infective forms (Bedding 2009). A strain of *D. siricidicola* used for biological control was originally isolated from *Sirex juvencus* in Sopron, Hungary, but after 15–20+ years in lab culture in the mycophagous phase, it lost the ability to switch to the infective form. Therefore, *D. siricidicola* was re-isolated in 1991 from the Kamona forest in Tasmania, where the Sopron strain had been released in 1970 (Hurley et al. 2007, Bedding and Iede 2005) and this strain is now referred to as Kamona. However, having been grown in culture for many generations, the Kamona strain has undergone repeated bottlenecks and is not genetically diverse (Mlonyeni et al. 2011). While morphologically indistinguishable, there are genetic differences between the Kamona strain and the NA strain in nuclear and mitochondrial DNA sequences (Yu et al. 2009, Morris et al. 2013) and they can be distinguished with microsatellite loci (Mlonyeni et al. 2011).

Native North American species of *Sirex*, *Deladenus* and *Amylostereum* interact with the invasive species in pine trees. During *Sirex* oviposition, adult females inoculate trees

with *Amylostereum* which subsequently surrounds larval *Sirex* galleries, assisting with larval nutrition (Thompson et al. 2014). *Deladenus* nematodes also feed on this fungus when in the mycophagous phase. Bedding and Akhurst (1978) suggest that *Deladenus* species are more specific to fungi than to insect hosts and feeding and reproduction of *Deladenus* species and strains differ based on *Amylostereum* species and strain (Morris et al. 2012, 2014, Caetano et al. 2016). Thus, the fungal species or strain accompanying a *Sirex* larva could impact whether a particular *Deladenus* genotype is abundant and near enough to larvae to transform to the parasitic phase. Previous studies have suggested that numerous strains of *A. areolatum* were introduced with *S. noctilio* to North America (Nielsen et al. 2009, Bergeron et al. 2011) and that there is a separate native genotype of *A. areolatum* (Nielsen et al. 2009, Olatinwo et al. 2013). While siricids are less tightly associated with fungal strains than previously thought (Nielsen et al. 2009; Hajek et al. 2013, 2018; Wooding et al. 2013), an exception appears to be *S. noctilio* in North America which primarily uses *A. areolatum* and only rarely *A. chailletii* (Wooding et al. 2013). *Amylostereum chailletii* is usually found associated with native siricid species, such as *Sirex nigricornis* (Bedding 1974, Morris et al. 2013).

The purpose of this study was to further evaluate the biological control potential of Kamona in North America, where treatments in Pennsylvania and New York were not efficacious against *S. noctilio* in the presence of the NA strain of *D. siricidicola* (Williams and Hajek 2017). We reared cultures of *D. siricidicola* Kamona on two *A. areolatum* fungal strains to investigate whether preconditioning, while the nematodes are mycophagous, would help the nematodes survive, reproduce and ultimately infect *S. noctilio* larvae. We followed procedures similar to those used in the Southern Hemisphere against *S. noctilio* (e.g. Carnegie and Bashford 2012), injecting Kamona into woodwasp-infested pine logs in the local environment where *D. siricidicola* NA and *A. areolatum* were already present. We evaluated the emerging woodwasp adults for nematode parasitism and sterilisation of eggs and identified a subsample of nematodes to strain using genetic markers. This is the first study in which single *Deladenus* nematodes have been isolated from *Sirex noctilio* woodwasps and their eggs for fine-scale genetic identification, including hybridisation.

Methods

Parental generation

In spring and early summer of 2013 and 2014, trees infested with *S. noctilio* were collected from field sites and cut into logs about 70 cm long. We sealed both cut ends with wax to retain moisture and placed them inside cardboard barrels (91 cm high x 61 cm diam.) with covers made of window screening. Barrels were kept in an unheated barn and were checked for emergence daily from late June through September. We collected adults of *S. noctilio* in 29 ml clear plastic cups and stored them at 4 ± 1 °C to extend their life span.

Sirex spp. are haplodiploid, so successful mating is required to obtain female offspring to test the ability of *D. siricidicola* to sterilise female hosts. Ten males per fe-

male were mated outdoors in cages (60 × 60 × 60 cm; BugDorm 2; Bioquip, Rancho Dominguez, CA) following methods described by Caetano and Hajek (2017). After mating, we kept females at 4 °C for at least 24 hours before allowing them to oviposit in trees prepared for nematode injection trials.

Tree preparation and oviposition

In 2013 and 2014, mature red pine trees, *Pinus resinosa*, were selected at Arnot Teaching and Research Forest, Cornell University (Tompkins County, New York, USA). To make these trees attractive to female woodwasps for oviposition, they were weakened by injection with the herbicide Banvel (49.4% diluted in water 1:1) in early July. We drilled holes into the trunks 50 cm above ground level and about 5 cm deep at a 45° angle, spaced 10 cm apart from each other around the circumference of the tree trunk (Zylstra et al. 2010), then injected 1 ml of the herbicide solution into each hole.

We enclosed a 1 m section of each treated tree with a cage made of window screening, with the bottom of the cage approximately 80 cm above the ground (see Figure 1). In July and August 2013, 4 weeks after the herbicide had been injected, we released *S. noctilio* females into the cages to oviposit. We placed two or three females at a time in each cage for three to four days and then added new females for another three to four days, for a total of 4 or 5 females per cage. Woodwasps released in the same cage at the same time had their pronota marked with different colours of paint to identify individu-

Figure 1. Oviposition cage design. Cages enclosed 1 m of a mature red pine bole and were approximately 50 cm in diameter.

als for later dissection. After exposure to woodwasps, standing experimental trees were left to overwinter in the forest and were cut in mid-April of 2014. We excised the portion of each tree that had been caged, waxed the cut ends and inoculated these bolts with nematodes by the end of April (see below). Each tree had a single cage, so the terms "tree" and "log" are interchangeable in this study. This protocol was repeated in 2014–2015.

Origin of nematode and fungal cultures

Cultures of *D. siricidicola* Kamona grown on *A. areolatum* BDF were imported in January each year from Ecogrow Environment (Westgate, NSW), the Australian commercial producer of this nematode for biological control of *S. noctilio*, following USDA APHIS permits. We reared colonies of this nematode strain in the Sarkaria Arthropod Research Laboratory, a quarantine facility under USDA APHIS permit at Cornell. Nematodes were grown in 100 mm diameter Petri dishes on ½ PDAh (Morris et al. 2012). Half of the imported nematodes were transferred to Petri dishes containing *A. areolatum* BE, a fungal strain found in North America (Nielsen et al. 2009), which was cultured from *S. nigricornis* collected in Warrensburg, NY (SAC132). We transferred nematodes of each strain on to new media every two weeks until we obtained at least 30 well-colonised plates for each fungal strain. During 2014–2015, only *D. siricidicola* Kamona on *A. areolatum* BE was used.

Nematode inoculation

Suspensions of nematodes were prepared by rinsing culture plates into 50 ml centrifuge tubes with autoclaved tap water. We counted all nematodes (both juvenile and adult) within five 20-µl drops under a dissecting scope at a magnification of 60× and adjusted suspension volumes to obtain 40 nematodes per drop, which resulted in an average of 2000 nematodes per ml. We added 0.5 g of polyacrylamide gel powder to each 50 ml tube of adjusted suspension and allowed the gel to hydrate (Williams and Hajek 2017).

On the same day, following methods of Bedding (2009), we used a punch hammer to punch holes (1 cm wide × 1 cm deep) into the logs that had been within oviposition cages the previous summer. We punched two rows of holes along the length of each log on opposite sides of the log; these holes were placed 20 cm apart in a row. Each of the holes was filled with 1 ml of the nematode suspension or with control gel (see Table 1 for numbers of trees/treatment in both years). After inoculation, we placed treated logs inside cardboard barrels with window screening covers; logs that were inoculated with a suspension of *D. siricidicola* Kamona were kept in the quarantine facility and control logs were kept in an unheated barn located about 1.6 km from the quarantine. During the following summer, we checked barrels daily to collect adult (offspring) woodwasps and these were stored at 4 °C until dissection.

Table 1. Experimental design. BDF = fungal strain of commercially-produced nematodes, BE = fungal strain found in North America.

Year	Treatment	Number of trees
2013–2014	*D. siricidicola* Kamona grown on *A. areolatum* BDF	4
	D. siricidicola Kamona grown on *A. areolatum* BE	4
	Control gel without nematodes	4
2014–2015	*D. siricidicola* Kamona grown on *A. areolatum* BE	6
	Control gel without nematodes	5

Woodwasp dissections

Dissections of *Sirex* mothers and offspring were performed under a dissecting microscope at a magnification of 60×. For male offspring, abdomens were removed and cut open. Two drops of deionised water were added to the abdominal contents and internal organs were removed and spread apart in the dissecting dish. If present, we collected nematodes with disposable sterile pipettes. For females, abdomens were removed, cut lengthwise on both sides and dorsal sclerites were removed, exposing internal organs. Three drops of deionised water were added to the abdomen. Taking care to avoid breaking the venom gland, we spread eggs in the water in a 5.5 cm diameter glass dissecting dish. At this point, eggs were counted. We preserved eggs and nematodes (if present) in 1.5 ml centrifuge tubes containing 95% ethanol at -20 °C. For verification of sterilisation status, eggs were later spread in a 35 mm diameter gridded Petri dish and placed on an inverted compound microscope to count sterilised and unsterilized eggs at 200×. It was found that this method would detect nematodes in eggs when the number of nematodes per egg was low.

For this study, we use the term "parasitised" to indicate that nematodes are present anywhere inside the woodwasp body and "sterilised" to only indicate the presence of nematodes inside of inviable woodwasp eggs. "Partial sterilisation" refers to less than 100% of eggs being sterilised within a woodwasp.

DNA extraction, PCR and data analysis

Initially, we took samples of multiple nematodes from each woodwasp hemocoel and extracted their DNA in groups using DNeasy kits (Qiagen, Germantown, MD). The mitochondrial COI DNA barcoding locus was amplified using these DNA preparations from multiple nematodes per woodwasp using methods similar to Morris et al. (2013), sequenced at the Cornell University Institute of Biotechnology (Ithaca, NY) and compared against sequences available in GenBank to identify nematode strains.

For analysis of single nematodes, we spread an aliquot from a suspension of live or preserved nematodes on to 1.5% agar in a 5 cm diameter Petri dish. While viewing through a dissecting scope, we picked up single nematodes using a tool consisting of a minuten pin mounted on the end of a glass rod. We placed each nematode into a 10–20 µl drop of 10× PCR buffer (Qiagen) diluted to 1× in 0.025% Tween on a clear plastic dish. To avoid cross-contamination, tools were cleaned with 10% bleach then rinsed with

water between picking up individual nematodes. After viewing through the microscope to confirm the presence of a single nematode per drop, each drop was transferred to a well of a PCR strip tube using a sterile pipette tip. For analysis of sterilised eggs, we selected an intact sterilised woodwasp egg, washed away any external nematodes and split the egg open on a clean dish of agar. Single nematodes were individually selected as above.

Reference samples were collected in the same way for both Kamona (selected from a pure colony sample) and NA (selected from three sites in New York and one site in Pennsylvania, USA).

Nematodes in PCR buffer were frozen at -80 °C for a minimum of 30 minutes or up to several days to begin the lysing process. Thawed nematodes were treated with 1 µl of Proteinase K (Qiagen) and lysed chemically overnight at 56 °C, followed by heat inactivation of the enzyme at 95 °C. These template DNA preparations were stored at 20 °C until use in PCR reactions.

Based upon Mlonyeni et al. (2011), we chose 8 microsatellite loci most likely to show variation in *D. siricidicola* NA and designed two multiplex panels of four loci each (Panel A: Ds1, Ds105, Ds323 and Ds388 and Panel B: Ds83, Ds201, Ds366 and Ds325). Forward primers were 5'-labelled with fluorescent dyes (FAM, VIC, NED or PET; Applied Biosystems, Foster City, CA, USA). Reverse primers (IDT, Coralville, Iowa) had a 5' GTTTCTT pigtail (Brownstein et al. 1996). The panels were amplified using the Type-It PCR kit (Qiagen) with the standard manufacturer-recommended reagent concentrations. For PCR, we used a temperature profile of 95 °C for 5 min followed by 32 cycles of annealing at 57 °C for 90 sec. All denaturing steps were 95 °C for 30 sec and cycle extensions were 72 °C for 30 sec, with a final extension at 60 °C for 30 min. Products were diluted with Hi-Di Formamide and GeneScan 600 LIZ Size Standard v. 2.0 (Applied Biosystems) and visualised with an ABI 3730×1 at the Cornell University Institute of Biotechnology. Allele calls were checked by two authors using both Geneious and GeneMarker software.

For microsatellite data, we selected only those nematodes with successful amplification at five or more of the 8 loci. We used Structure v. 2.3.4 (Pritchard et al. 2000) with K=2 groups using correlated allele frequency and an admixture model to distinguish the parental genotypes. NewHybrids v. 1.1 (Anderson and Thompson 2002) was used to distinguish parental and F1, F2 and backcross hybrid classes. Both Structure and NewHybrids were run with 20,000 burn-in iterations followed by 1 million sample iterations.

Results

Woodwasp emergence and dissections

The 12 trees from 2013–2014 produced a total of 86 *S. noctilio* (Table 2). Of these, 39 emerged from control logs (none parasitised), 46 from logs treated with *D. siricidicola* Kamona grown on *A. areolatum* BDF (13 parasitised males, 57% and 12 parasitised females, 52%) and only one from logs treated with *D. siricidicola* Kamona grown in *A. areolatum* BE (1 parasitised female, 100%).

The 11 trees, inoculated in 2014, produced a total of 220 woodwasps in 2015. The five control trees produced 213 *S. noctilio*, with 86 males (63%) and 45 females (58%) parasitised with nematodes. Only seven woodwasps emerged from Kamona-treated logs and this included 4 parasitised males (67%) and one parasitised female (100%) (Table 2).

The parental female (mother) woodwasps that were recovered from cages after oviposition were also dissected and many of these were found to be already carrying NA nematodes, which could have been transferred to the tree during oviposition. Thus a large number of offspring woodwasps from control trees were parasitised with NA. The mothers' parasitism status is shown for experimental woodwasps in Table 3.

Table 2. Emergence and parasitism levels. BDF = fungal strain of commercially-produced nematodes, BE = fungal strain found in North America.

Emergence year	Treatment	Total number of woodwasps	Number of males	Number (percent) of males parasitised with any strain of *D. siricidicola*	Number of females	Number (percent) of females parasitised with any strain of *D. siricidicola*	Total percent of eggs sterilised
2014	*D. siricidicola* Kamona grown on *A. areolatum* BDF	46	23	13 (57)	23	12 (52)	50
	D. siricidicola Kamona grown on *A. areolatum* BE	1	0	–	1	1 (100)	80
	Control	39	24	0	15	0	0
2015	*D. siricidicola* Kamona on *A. areolatum* BE	7	6	4 (67)	1	1 (100)	0
	Control	213	137	86 (63)	76	45 (59)	0

Table 3. Experimental offspring (woodwasps from treated trees) used in microsatellite analysis of nematodes. Nematodes from control trees were not included in the microsatellite analysis.

Host wasp ID	S5	S5 egg	S10	S10 egg	S11	S11 egg	S13	S16	S18	S36 egg	S1	S1	S35	S37	S38	S44	S2
Year emerged	2014	2014	2014	2014	2014	2014	2014	2014	2014	2014	2015	2014	2014	2014	2014	2014	2015
Number of nematodes sampled	30	23	27	16	28	22	19	16	26	23	42	30	23	21	24	18	30
Host wasp sex	F	–	F	–	F	–	F	F	F	F	F	M	M	M	M	M	M
Potential mothers of host wasp with NA?	Y	–	Y	–	Y	–	N	Y	Y	Y	Y	N	N	N	N	Y	Y
Fungus used to culture Kamona	BE	–	BDF	–	BDF	–	BDF	BDF	BDF	BDF	BE	BDF	BDF	BDF	BDF	BDF	BE
Number of eggs	25	–	72	–	61	–	87	27	21	60	22	–	–	–	–	–	–
% of host eggs sterilized	80	–	69	–	90	–	0	0	0	82	0	–	–	–	–	–	–
% of (Kamona + hybrid) nematodes	96	–	5	–	24	–	11	0	0	0	2	–	–	–	–	–	–

Nematode identification

The analysis of COI from grouped nematodes per woodwasp showed that 94% of parasitised experimental woodwasps carried the NA strain (29 out of 31 parasitised woodwasps), as well as 100% of the control woodwasps (131 parasitised woodwasps).

Microsatellite analyses were performed on a subsample of nematodes from a subsample of experimental woodwasps (not on those from control trees). It was not possible to isolate single nematodes from every woodwasp emerging from treated wood, either because preservation/extraction failed or the number of nematodes was not sufficient for analysis. A total of 418 single nematodes from 14 parasitised experimental woodwasps (8 females and 6 males) were genotyped at 5 or more loci (Table 3). Of these, 84 nematodes were isolated from inside of 8 woodwasp eggs. We also genotyped 44 pure, cultured Kamona and 155 nematodes from 25 woodwasps from 4 sites. Structure analysis revealed that the microsatellite genotypes of NA found in the region were distinctly different from the cultured Kamona and placed them in two separate genetic clusters (Figure 2). NewHybrids provided probabilities of assignment to hybrid class including F1, F2 and NA backcrosses for each individual nematode (Figure 3).

Within woodwasps, mixtures of parental strains and hybrids were found in 8 of 14 woodwasps (Figure 3). The NA strain predominated overall with the exception of one female woodwasp (S5) which had mostly Kamona. Over all 418 nematodes sampled from experimental woodwasps, 15.1% were pure Kamona, but these were concentrated in three woodwasps. The hybrid classes combined were all found at low frequencies (usually less than 10% per woodwasp and 2.1% overall). Within eggs, the composition of nematodes was generally similar to that inside the woodwasp hemocoel, although that information was not available for one of the eggs (S36) because nematodes from the hemocoel failed to amplify.

Egg sterilisation

Twelve parasitised females emerging from the Kamona/BDF treatment in 2014 had 50% of all eggs sterilised (Table 2); six of those females had sterilised eggs with a mean of 71% and range of 25–100% sterilisation per female, the other six females had unsterilised eggs. Within the microsatellite sample group (8 females over all treatment groups), egg sterilisation ranged from 0 to 90 percent. A high proportion of nematodes found inside of eggs were the NA strain, which has not been reported previously within eggs in North America (Figure 3). There was no relationship between the combined proportion of Kamona and hybrid genotypes and the proportion of eggs sterilised within the 8 female woodwasps sampled (Table 3). In some cases, most eggs were sterilised even when Kamona-related genotypes were few or undetected.

Discussion

The objective of this study was to test a biological control agent that is already successfully controlling *Sirex noctilio* in Oceania, South Africa and South America against this invasive woodwasp in North America. In the 1970s, many different strains of *D. siricidicola* were tested against *S. noctilio* in Australia and New Zealand, before the

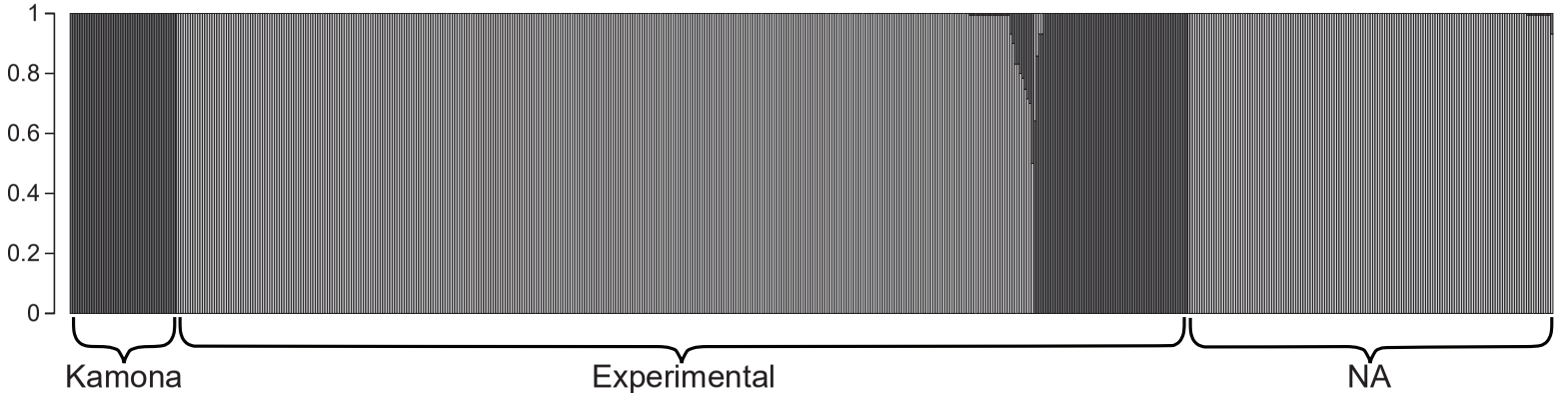

Figure 2. Structure results based on microsatellites, complete data set. Structure analysis produced two distinct genetic clusters for Kamona (dark) and NA (light). Vertical bars represent each individual's proportion of membership in the cluster(s); thin black vertical bars separate the individuals. Woodwasps from experimental logs were predominantly NA with few hybrids and Kamona.

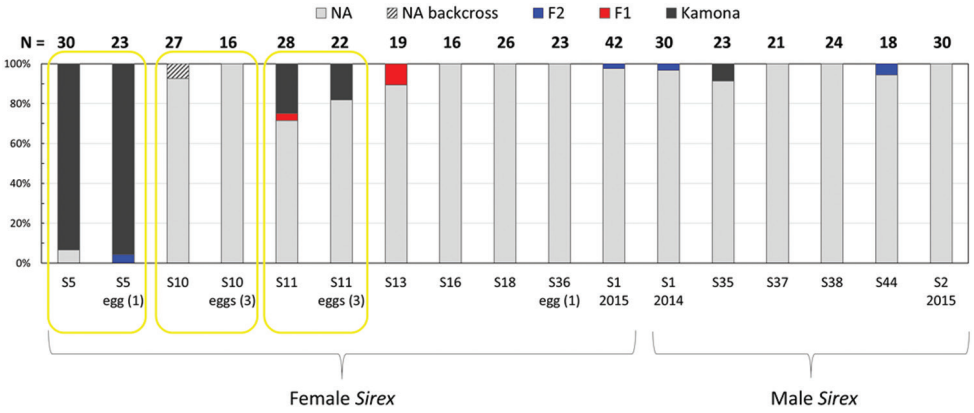

Figure 3. The percentage of nematodes belonging to parental and hybrid classes based on analysis of 8 microsatellite loci with NewHybrids. Number of nematodes sampled above bar. Nematodes were found within the woodwasp hemocoel, except those within eggs, as labelled below the bar (number of eggs sampled in parentheses). Yellow highlight shows samples from which both hemocoel and egg(s) were sampled from the same female.

Sopron/Kamona strain was chosen for biological control (reviewed in Bedding 2009). These investigations revealed great variability amongst strains in parasitism and sterilisation, which seemed to depend on interactions between the woodwasp and nematode strains (Bedding 1972, 2009). Timing of host ovarian development in relation to juvenile nematode release within the host is an important factor affecting sterilisation of woodwasp eggs (Bedding 1972). The *S. noctilio* found in North America are genetically different from those introduced to Oceania, for which the Kamona strain was selected (Boissin et al. 2012, Bittner et al. 2017). Unlike in the Southern Hemisphere, the use of the Kamona strain for control of *S. noctilio* in North America could be complicated by the presence of *D. siricidicola* NA or native species of *Deladenus* which are not known to sterilise *S. noctilio*.

Based on the high growth rates of Kamona on *Amylostereum areolatum* IGS BE in Morris et al. (2014), we hypothesised that rearing Kamona on BE would improve the survival and/or reproduction of Kamona in the experimental trees, which could already contain the native BE strain of *A. areolatum*. Unfortunately, our conclusions about this treatment effect are limited by sample size. The BE treatments produced far fewer woodwasps overall, yet the woodwasps that did emerge had a higher parasitism rate compared to the treatment reared on *A. areolatum* BDF (Table 2). However, these parasitism rates also included much more parasitism by the NA strain than the Kamona strain, which grew equally well on BE in lab culture (Caetano et al. 2016). More research is needed to understand the best rearing technique for Kamona in order to optimise inoculations, were this strain to be used for *S. noctilio* control in North America.

A long term study similar to this one was conducted using trees that were naturally infested with *S. noctilio* (Williams and Hajek 2017). These trees were cut down and injected with Kamona in autumn and left on the ground until spring, when the

injected portions were transferred to rearing barrels. Although the methods differed, both studies obtained low parasitism rates by Kamona.

Overall, the number of woodwasps emerging from Kamona-treated trees in this study was also low, especially in 2015. Some of the factors that may affect the success of woodwasp development and/or nematode parasitism include the use of herbicide (D.W. Williams, pers. comm.), the presence of competing blue stain fungi from bark beetles (Yousuf et al. 2014, Williams et al. 2012), the timing of injection and tree cutting (Williams et al. 2012), the strain of *A. areolatum* for nematode development (Bedding 2009) and the moisture content of wood (Hurley et al. 2008). Moisture content may have differed in the current experiment, because the quarantine storage conditions were warmer and drier than the unheated barn where controls were stored. However, in their injection trials, even when moisture levels were adequate, Hurley et al. (2008) also observed low rates of parasitism by Kamona which could not be fully explained.

Sequencing of COI on grouped nematodes was used to determine if any Kamona were successful at infecting North American *S. noctilio* and this method cannot detect hybridisation. When COI was sequenced for groups of nematodes per parasitised woodwasp, it most often indicated the NA strain, rarely the Kamona strain and sometimes showed ambiguities that suggested co-infection. Thus it provided a coarse-grained picture of the overall success of the Kamona strain and suggested that only about 6% of parasitised woodwasps in experimental trees had Kamona. Microsatellite genotyping of single nematodes from selected woodwasps, both inside and outside of eggs, provided a more detailed picture. This method provided clear evidence that both hybridisation and co-infection with different strains did occur during this study.

Near *Sirex* larvae, some nematodes become parasitic or "infective," but sexual reproduction amongst nematodes is only known to occur outside of the *Sirex* larva (both in mycophagous and infective forms, Bedding 1972), so different strains must arrive at and enter a larva independently. Multiple mated female infectives can enter each woodwasp larva (from 1 to over 100, Bedding 1972), thus co-infection of different nematode strains is not surprising. Infective females absorb nutrients directly from the host and release fertilised eggs or hatched juveniles when triggered by host pupation and, depending upon appropriate timing, the juveniles may enter host eggs (Bedding 1972). Thus, even at the egg level, co-infection of nematode strains can occur, as seen in a single egg from woodwasp S5 (Figure 3). As the cycle continues, the composition of nematodes within a female woodwasp (and her eggs) affects the next generation of woodwasps by direct transfer of nematodes during oviposition. However, even in the experimental trees where no maternal-generation woodwasps were intentionally parasitised with NA, woodwasps (S1.2014, S13, S35, S37 and S38, Figure 3) still contained predominantly NA nematodes. This shows the strong prevalence of the NA strain in areas that have already been exposed to *S. noctilio*, such as our study site, even as the trees appeared unaffected at the time of the study. Possibly treatment with herbicide made the trees attractive to *S. noctilio* in the environment or they had been used by *Sirex* for oviposition in a previous season; either way, nematodes could have migrated through the tree vascular tissue to woodwasp larvae in the experimental section.

During the mycophagous phase, nematodes within a tree produce many generations, "breeding wherever there is growing fungus" (Bedding 1972) and this is where the opportunity for hybridisation occurs. This is not the first demonstration of between-strain hybridisation in *Deladenus*. Using seven species of *Deladenus*, Akhurst (1975) compared inter- and intraspecific hybridisation, based on successful production of F1s and F2s of the mycophagous phase. Hybridisation between strains of the same *Deladenus* species was only tested using *Deladenus rudyi* strains from Turkey and Japan. Hatch of eggs from F1s was diminished compared with crosses within the same strain, but remained > 40%.

The possible outcomes of hybridisation range from hybrid instability or reproductive failure to hybrid vigour and selective advantage. Interspecific hybridisation of parasites can produce new combinations of genetic diversity that may result in increased fitness, infectivity, host range/diversity or geographic range (e.g. Boissier et al. 2016). Intraspecific hybridisation (amongst strains) may be likely to succeed due to fewer genetic barriers and similarity of host biology.

Conclusions

If the Kamona strain were to be used as a biological control agent in North America, its success could be limited by competition with the NA strain or may be either enhanced or reduced by hybridisation. However, the most unexpected result of this study was that, even when Kamona was very low or undetectable, the NA strain was able to enter the eggs, something that had never been recorded previously. In 6 out of 8 females, we detected less than 10% Kamona/hybrid genotypes yet two of these had high egg sterilisation levels (Table 3). To our knowledge, the NA strain has never been detected inside of *S. noctilio* eggs in North America prior to this study. Our data reveal that a relatively small presence of Kamona strain can result in either no sterilisation or fairly high sterilisation rates. Future work on the developmental and biochemical mechanisms of sterilisation could tell us whether there is potential for hybridisation between NA and Kamona to affect sterilisation of *S. noctilio* on a landscape scale.

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