Diversity, specificity and admixture in the *Sirex - Amylostereum - Deladenus* symbiosis

by

Katrin Nathalie Elsbeth Fitza

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Supervisor: Prof. Bernard Slippers

Co-supervisor: Dr. Jeff Garnas

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DECLARATION

I, Katrin Nathalie Elsbeth Fitza, declare that the thesis, which I hereby submit for the degree *Philosophiae Doctor* at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this institution or any other tertiary institution.

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Katrin Fitza

September 2019

PREFACE

The invasive woodwasp *Sirex noctilio* and its fungal wood-decaying symbiont *Amylostereum areolatum* has significant global economic impact on the pine-growing industry. The wasp originates from Eurasia and has spread to various other parts of the world. The application of biological control agents is an important means of managing the pest. The main biological control agent is the entomopathogenic nematode, *Deladenus siricidicola*. Previous studies have indicated extreme homozygosity within populations of this biological control agent in the Southern Hemisphere. This is a concern, because the broad geographic spread of the pest means that the biological control agents need to perform optimally under a broad variety of conditions. It is also unclear how this low genetic diversity impacts the efficacy of *D. siricidicola* against genetically diverse *Sirex* populations. At the start of this thesis it was not known what genetic diversity is present amongst available global populations of the nematode.

The **aim of this PhD** study was to understand diversity, specificity and admixture in the *Sirex-Amylostereum-Deladenus* complex. This was achieved by investigating the global genetic diversity and structure of *D. siricidicola*. Experimental admixture amongst identified lineages was explored as a method to increase diversity in *D. siricidicola* populations, including its potential impact on reproduction in the nematode in culture. Lastly, the diversity and specificity in the wasp-fungus association in the native region of Japan was examined, as this could impact the use of *Deladenus* spp. for biological control.

Chapter 1 comprises a literature review in which we reflect on the role of genetic diversity in invasion (including biological control as an assisted form of invasion), as well as factors that affect genetic diversity during this process. The uniform framework used in invasion biology is used to consider the mechanisms that affect genetic diversity during different phases of invasion, and how it relates to biological control. The importance of genetic diversity for the success of invasion is also discussed. We furthermore consider ways to mitigate processes that reduce genetic diversity, as well as methods that might help increase genetic diversity in biological control programs. Finally, we summarize what is known regarding diversity in the invasive and biological

control populations of *S. noctilio* and *D. siricidicola* and how this might be related to successful management of the pest.

No study has thus far characterised the genetic diversity of global populations of *D. siricidicola*, despite its potential importance for consideration in biological control programs. In **Chapter 2** of this thesis we collect populations of the nematode from across its distribution in the Southern Hemisphere, as well as from a potentially introduced range in North America and its native range in Europe (represented by collections from Spain). We conduct a population genetics study on these samples using mitochondrial and nuclear data.

A key question raised by previous studies that point out a lack of diversity in *D. siricidicola*, high diversity in *S. noctilio* populations, and specificity in interactions between the nematode and wasp-fungal complex, is how to increase diversity in biological control populations. In **Chapter 3** we investigate the potential of interbreeding between different lineages identified in Chapter 2. This is important both for considering the intentional mixture of different strains for testing in biological control programs, and for considering the consequences of accidental spread of the nematode between regions. Furthermore, we test the change in reproductive rate in admixed populations compared to parental nematode strains.

Recent studies have shown a lack of fidelity in symbionts associated with some introduced Siricid wasps, specifically in North America. Whether such infidelity also occurs in native Siricid populations is not known. Such information is important to consider the possible influence of using biological control agents such as *D. siricidicola*, which interacts with both the wasp and fungus, in different areas. In **Chapter 4** we examine the host specificity and diversity of *Amylostereum* associated with native Japanese Siricids.

Final conclusions, implications and future prospects are summarized and discussed at the end of this thesis in the **Concluding Remarks** section.

The following papers, posters and congress presentations were produced based on the results from this study:

Publications

Papers

Fitza, KNE., Tabatat, M., Kanzaki, K., Garnas, J., Slippers, B., 2016. Host specificity and diversity of *Amylostereum* associated with Japanese siricids. *Fungal Ecology* **24**, 76-81.

Kanzaki, N., Tanaka, S.E., Fitza, KNE., Kosaka, H., Slippers, B., Kimura, K., Tsuchiya, S., Tabata, M., 2016. *Deladenus nitobei* n. sp. (Tylenchomorpha: Allantonematidae) isolated from *Sirex nitobei* (Hymenoptera: Siricidae) from Aomori, Japan, a new member of the siricidicola superspecies. *Nematology* **18,** 1199-1217.

Lombardero, M., Ayres, M., Krivak-Tetley, F., Fitza, KNE., 2016. Population biology of the European woodwasp, *Sirex noctilio*, in Galicia, Spain. *Bulletin of Entomological Research* **106,** 569-580.

Fitza KNE, Garnas JR, Lombardero MJ, Ayres MP, Krivak-Tetley FE, Ahumada R, Hurley BP, Wingfield MJ, Slippers B. 2019. The global genetic diversity of *Deladenus siricidicola*, in native and non – native populations. *Biological Control* **132**, 57-65.

Posters

Slippers B., Hurley BP., Boissin E., Garnas JR., X. Mlonyeni OX., Fitza KNE., Postma A., Yek S., Wingfield MJ. 2014 Global patterns of diversity of the *Sirex-Amylostereum-Deladenus* symbionts and its implications for control. IUFRO 24th World Congress, Salt Lake City, USA Oct5-Oct11

Fitza KNE., Mlonyeni O.X., Hurley B.P., Yek S.H., Ahumada R., Ayres, M.P., Dodds K., Lombardero M.J., Brockerhoff E.G., Wingfield M.J., Slippers B. 2014. Diversity of the parasitic

nematode *Deladenus siricidicola* and its relevance as a biological control agent. IUFRO 24th World Congress, Salt Lake City, USA Oct5-Oct11

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Orals

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Mlonyeni O.X., Fitza KNE., Wingfield BD., Greeff J., Wingfield MJ., Slippers B. (2016) Global *Deladenus* diversity: genetics and host interaction. XXV International Congress of Entomology, Orlando, Florida, United States of America, 25-30 September 2016.

Fitza KNE, Garnas JR, Ahumada R, Ayres MP, Krivak-Tetley FE, Dodds K, Lombardero MJ, Brockerhoff EG, Wingfield MJ, Slippers B. (2017). Genetic diversity of *Deladenus siricidicola*, the biological control agent of the woodwasp *Sirex noctilio.* Combined Congress of the Entomological and Zoological Societies of Southern Africa, CSIR ICC, Pretoria, South Africa, 3-7 July 2017.

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All the chapters have been written up as a publishable unit and will be submitted for publication. Chapter 2 has been published in *Biological Control* and Chapter 4 has been published in *Fungal Ecology*.

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Chapter 1

Importance of genetic diversity in biological control agents of invasive insect pests, with a special focus on plantation forestry

Katrin N.E. Fitza, Jeff Garnas, Bernard Slippers

Abstract

Increasing trade and travel has facilitated an increased rate of spread of plant insect pest invasions globally, including in plantation forestry. Consequently, the development of biological control is required over shorter time scales and for deployment across different regions around the world. This wide scale application of biological control agents requires adaptability to different environmental conditions and variable pest populations. Understanding the genetic diversity of both pest and biological control populations becomes increasingly important in this context. In this review we consider the role of genetic diversity in biological control. We do so by considering what is known from invasion biology, where this question has also been addressed, and because there are fundamental overlaps between accidental invasion processes and biological control development and deployment. We particularly consider the relevance of considering genetic diversity in the *Sirex noctilio* (invasive pest) -*Deladenus siricidicola* (biological control) system as a general example, as well as for its relevance to the rest of the thesis.

1.1 Introduction

Biological control is critical for invasive pest management in plantations (Kenis et al., 2017). The development of biological control is, however, time-consuming, expensive and the outcome is uncertain. Given the increasing rate of global spread of plantation pests, the demand for biological control is also increasing (Garnas et al., 2012; Wingfield et al., 2015). Not only is the rate of development a challenge, but biological control needs to be applicable over an ever-increasing distribution and complexity of environments.

To ensure successful application of biological control agents across different regions and in different pest populations, it is important to consider adaptability of the biological control agent and to understand the genetic processes underlying adaptation and selection of natural enemies. The genetic basis of the traits associated with the efficiency of the biological control agent, as well as the genetic diversity within and among populations further need to be understood, warranting successful biological control agents (Le Hesran et al., 2019; Roderick et al., 2012; Roderick and Navajas, 2003).

Implementation of biological control often occurs through the collection of biological control agents from a limited area, resulting in the random selection of a subset of genetic variation, compared to the native population (genetic bottleneck). As a result, biological control agents might contain limited genetic variation on which selection can act. Biological control agent populations are also subject to genetic drift, inbreeding, selection and founder effects, especially during rearing and introduction, leading to further decrease in diversity (Hopper et al., 1993; Sol et al., 2012; Tayeh et al., 2015; Wright and Bennett, 2017). Decreases in genetic diversity can have negative implications for the adaptability of the biological control agent to changing environments, changing pest populations and for the general fitness and success of the agent (Booy et al., 2000; Malausa et al., 2017).

Increasingly inexpensive and powerful tools, including microsatellite and single nucleotide polymorphism (SNP) markers, are available for rapid screening of genetic diversity in populations of biological control agents (Guichoux et al., 2011; Mondini et al., 2009). The growing availability of genomes of pests and biological control

organisms is improving the discovery of these markers (Abdelkrim et al., 2009). For example, the genetic diversity of the biological control agent, *Aphidius ervi*, a parasitoid wasp, was studied using mitochondrial DNA sequencing and microsatellite markers to investigate the population origin and diversity, in native and introduced populations (Hufbauer et al., 2004). The authors could show that the introduced wasp population had fewer rare alleles due to the occurrence of a mild genetic bottleneck. Piombo et al. (2018) sequenced and characterized the genomes of two strains of the biological control yeast, *Metschnikowia fructicola*, used against postharvest diseases, enabling them to investigate their genetic diversity. The authors applied genome sequencing and assembly to utilize the genomic information for identifying numerous high impact SNPs.

The impact of genetic diversity on colonization success has been more studied in invasion biology than in biological control systems (Dlugosch and Parker, 2008; Hufbauer et al., 2013, 2017). Although an invasion is most often due to an accidental introduction process and biological control agents are intentionally introduced, both go through similar stages: transport, introduction, establishment and spread (Abram and Moffat, 2018; Blackburn et al., 2011; Ehler, 1998; Wingfield et al., 2017). Various evolutionary mechanisms impact genetic diversity during these stages, including mutations, admixture, gene flow, inbreeding, genetic drift and selection (Ellegren and Galtier, 2016; Hufbauer and Roderick, 2005; Li et al., 2018). Understanding the impact of these mechanisms at various stages of deploying biological control agents is important, albeit underexplored.

This review aims to combine knowledge from general invasion biology and biological control systems on the role of genetic diversity in invasion. We consider how genetic diversity can be influenced during each of the stages of an invasion process. Finally, we examined the relevance of the current understanding of the role of genetic diversity in invasion biology and biological control in the well-studied global invasion of *Sirex noctilio* (Hymenoptera: Siricidae; woodwasp) and *Amylostereum areolatum* (Russulales: Amylostereaceae; the mutualistic fungus associated with the wasp), as well as its biological control agent *Deladenus siricidicola* (Nematoda: Neotylenchidae).

1.2 Stages of invasion and processes that affect diversity in biological control programs

Blackburn et al. (2011) defined a unified framework to structure our understanding of the stages of invasion (Fig.1). This framework was predominately developed using literature from accidental introductions of animal and plant systems. While this framework is generally applicable to all invasions, specific details may vary among the different types of invasion. Zenni et al. (2017) specified the unified framework to tree invasion, including evolutionary mechanisms which play important roles in the transition of the invader from one stage to the next and could lead to the failure of the invasion. Wingfield et al. (2017) applied the unified framework to forest fungal pathogen invasions, discussing aspects such as symbiosis that could influence invasion success; e.g. insects that depend on symbionts for nutrient acquisition.

Biological control can be considered as a deliberate invasion, which explicitly selects the invader, i.e. the biological control agent (Ehler, 1998; Fagan et al., 2002; Marsico et al., 2010). The development and deployment of biological control adhere broadly to key stages identified by Blackburn et al. (2011), namely transport, introduction, establishment and spread (Heimpel and Mills, 2017). Some of these stages include unique processes such as quarantine and mass rearing that place specific constraints and selection pressures on the biological control populations (Freedman and Harms, 2017; Nordlund, 1998; Wang et al., 2014; Fig. 1). It is important to consider the evolutionary dynamics that play a role during these different stages, as these have an impact on the diversity of the biological control agent.

1.2.1 Transport: sampling and genetic bottlenecks

Biological control agents are usually collected from a limited region within the native environment of the pest or from known populations in other introduced regions. Alternatively, biological control agents can be sourced from known populations in other introduced regions. Consequently, the biological control agents typically represent a population with reduced genetic diversity.

Sampling regions are often, but not always, identified based on ideal climate matching of the target invader and biological control agent, as well as other characteristics such as rate of parasitism and growth rate, which are important for pest suppression (Kenis et al., 2017; Kruitwagen et al., 2018; Robertson et al., 2008; Sheppard et al., 2003). Knowledge of the genetic diversity of a biological control agent in the native area could guide the collection of individuals, but is often lacking. Most biological control projects are challenged by the lack of knowledge whether the sampled genetic diversity, represented among the collected individuals, will be sufficient to ensure persistence, spread or pest suppression (Bartlett, 1994; Kenis et al., 2017; Sheppard et al., 2003). It is tempting to theorize that more genetic diversity is better, as it should enhance adaptive potential, but this can currently not be confirmed. What might constitute optimal levels of variation or even minimum targets, or how these vary across organisms and systems, is virtually unknown.

1.2.2 Introduction: quarantine process and mass rearing

After identifying a potential biological control agent, the quarantine process is the next step in the intentional invasion. The quarantine process is a controlled secure environment preventing accidental release, where risk assessment (e.g. examining the host specificity) for the release permitting processes can be conducted. This process may take some time/several generations, increasing the chances of inbreeding and genetic drift, as the initial population size may be small (Bennett, 2018; Fauvergue et al., 2012; Unruh et al., 1983). The genetic makeup could also be impacted by adaptation to laboratory conditions impacting the selection of favourable traits for such conditions. Hence, care should be given to preserving genetic diversity in laboratory colonies (Bartlett, 1994; Bennett, 2018) including by starting with a large population, keeping large numbers during breeding and imposing outcrossing among sub-colonies (Bartlett, 1994; Lommen et al., 2017).

During mass rearing loss of variation may occur due to adaptation to artificial rearing conditions and subsequent prolonged rearing. At the beginning of mass rearing, factors such as food source (including alternative food sources, such as artificial diet) and ideal growth temperature need to be determined and optimized (Paynter et al.,

2016). Once this is achieved, laboratory conditions can be seen as stable environment with little heterogeneity, leading to directional selection causing loss of genetic variation (Berlocher and Friedman, 1981; Guzmán-Larralde et al., 2013).

In a recent study on *Amblyseius swirskii*, a predatory mite of whiteflies, it was shown that not only did the heterozygosity level of a commercially long-term mass reared population decline 2.5-fold compared to the wild population, but also that the commercial population was highly differentiated from the eight natural populations that it originated from (Paspati et al., 2019). Oher examples of traits shown to have deteriorated due to mass rearing is in entomopathogenic nematodes, where virulence, heat tolerance and fecundity were affected (Bilgrami et al., 2006). These effects were based on changes in the nematode, its symbiotic bacteria or on both partners (Bilgrami et al., 2006). To identify the effects of rearing on the performance of the biological control agent, quality control and exploring molecular traits linked to phenotypes associated with effective mass production, as well as effectivity against the pest are needed (Sørensen et al., 2012).

1.2.3 Establishment and spread: release

Once the safety of the biological control agent has been approved and it has been successfully reared, the agent is released into the field. By now the biological control agent has undergone various stages that could have reduced its genetic diversity, from bottlenecks to drift and selection. The genetic diversity of the agent likely represents a subset of the genetic diversity present in its native area. The geographical location, variability in temperature, availability of food source, as well as the presence of enemies (usually not known to occur in the native region of the invasive organism, biological control agent), are now key selection pressures exerted onto the control agent (Fauvergue et al., 2012). Consequences of these selection pressures could lead to asynchronization of the agent with pest populations. Inbreeding levels are also expected to be higher during this stage and hence increased homozygosity can be observed. If the number of biological control agents released is not high enough, mate finding difficulties could also cause Allee effects (Hopper and Roush, 1993).

It is well known that founding events cause the establishment of a fraction of the native genetic diversity occurring in the native region and occur during various stages of the invasion process (Barrett and Husband, 1990; Dlugosch and Parker, 2008; Nei et al., 1975; Szűcs et al., 2019). Potential consequences of the founder effect are short- and long-term impacts on fitness, as well as dispersal and reduced evolutionary potential. In the case of a biological control invasion, founder effects reoccur throughout the invasion process including during release and establishment (Fauvergue et al., 2012).

1.3 Why does genetic diversity matter for invasion and biological control agents?

1.3.1 Inbreeding

Inbred offspring are generally assumed to be less fit in comparison to outbred offspring (Charlesworth and Willis, 2009; Keller and Waller, 2002). Inbreeding is associated with excess homozygosity which can result in the phenotypic expression of rare recessive alleles. Some organisms have mechanisms to avoid inbreeding (e.g. assortive mating), though in small populations such strategies may be of limited utility. Inbreeding can also have positive effects by exposing recessive alleles to selection (e.g. when they become homozygous) resulting in higher probabilities of purging deleterious alleles from the population (Hedrick, 1994; Hedrick and Garcia-Dorado, 2016). However, alleles with negative effects may also become fixed due to drift (García-Dorado, 2003; Lynch et al., 1995).

1.3.2 Adaptability

Adaptability in the field of invasion has been the subject of an increasing number of studies. Adaptive evolution can have a cascading effect by positively impacting fitness traits such as reproductive rate or survival (Szűcs et al., 2019) which can be seen within two to three generations (Stewart et al., 2017; Szűcs et al., 2017b). Dlugosch et al. (2015) highlighted the role of adaptive genetic variation in the invasion process. Attributes of adaptive genetic variation such as founding populations, its genetic architecture, interaction with different environments and interaction to different host

populations, are seen as avenues that allow us to study and understand evolutionary changes during the invasion process. Ideally, intraspecific adaptive genetic variation can be utilized in biological control programs (Lommen et al., 2017).

1.3.3 Genotype x genotype interaction

The genotype of both the host (pest) and the biocontrol agent play an important role in the successful spread of the biological control agent. The specificity of the interactions between the host genotype and the biological control agent genotype are important, because only specific genotypes (host + agent) are compatible in some cases (Carius et al., 2001; Hudson et al., 2016; Schulenburg and Ewbank, 2004). For example, matching the host-pathogen genotypes was shown to be important in the gypsy moth-baculovirus system (Hudson et al., 2016). The gypsy moth varied in its mean susceptibility to different isolates of the baculovirus and disease susceptibility among the host families.

Complex patterns of pest invasion have often resulted in high diversity of pest populations, as well as the presence of unique admixed populations of pests in invaded areas (Garnas et al., 2016). This may reduce the effectiveness of the biological control agent if genotype x genotype specificity occurs and needs to be considered in control programs. A key focus for biological control development should be linking compatible genotypes of the host and the control agent (Lambrechts et al., 2006). This is challenging due to typical limited understanding of wild populations of the biological control agent and the host, time and complexity of sampling. A narrow genetic base, therefore, can be limiting to the success of genotype matching.

Resistance development in the pest against the biological control agent could become a threat in control programs. Genotype x genotype interactions not only influence the performance level of the biological control agent, but also the development of more resistant pest genotypes (Mills, 2017; Tomasetto et al., 2017, 2018). A recent example of resistance development in a pest against its control agent, is the Argentine stem weevil *Listronotus bonariensis,* which causes severe impact on ryegrass, and its biocontrol agent *Microctonus hyperodae,* that has been applied for 25 years (Tomasetto et al., 2017, 2018). Rapid evolution of resistance in the pest and the lack

of reciprocal evolution by the control agent has threatened the viability of the control agent. Tomasetto et al. (2017, 2018) argue that agricultural intensification and expansion have led to accelerated evolution in the pest. It is expected that this is also true for other agricultural systems.

1.3.4 Genotype x environment interaction

Genotype x environment interactions of biological control agents are important to increase their invasion success (Hufbauer and Roderick, 2005; McDonald, 1976). Climatic mismatch between the native range of the biological control agent and the area of application has led to failure of past biological control programs. For example, Stiling et al. (1993) reported that climate has contributed to 34.5% failure of biological control agent introductions globally. Cowie et al. (2016) ascribed the restricted survival and establishment of the biological control agent *Anthonomus santacruzi* (flowerbud-feeding weevil) after release in the Highveld region of South Africa, to regional low temperature and low humidity. Climate matching software as well as identifying sites most suitable for release may assist in improving genotype x environment matching by helping to identify sites most suitable for release (Senaratne et al., 2008; Sutherst and Maywald, 1985).

Evolutionary adaptation to variable environments can also play a role in biological control success. Climate matching can contribute to the initial successful adaption (Robertson et al., 2008), but might not be relevant across a diverse landscape, where the biological control agent is expected to act or spread (Reed et al., 2003). The flowerhead weevil, intended to be released against musk thistle in Australia, was collected from three regions (southern France, northern Italy, eastern France; Cullen and Sheppard, 2012). It was expected that the population from southern France would be the best to establish in Australia, due to ideal climate matching. However, the population from eastern France, with no close climatic match to Australia, was the best established throughout the invaded range of musk thistle. Cullen and Sheppard (2012) attributed the greater success of the eastern population to the fact the eastern France population had undergone evolutionary adaptation as the population had moved from France via Canada and New Zealand before being released in Australia.

1.4 Drivers of genetic diversity in biological control populations

1.4.1 Loss of genetic diversity

Various evolutionary processes (i.e. genetic drift, strong selection and inbreeding) lead to reduced genetic diversity in a population of biological control agents. An understanding of when these processes occur during the introduction and knowledge of these processes could aid in optimising pest management through biological control.

1.4.1.1 Genetic drift

Genetic drift is an evolutionary process whereby allele fixation occurs due to chance events such as random sampling. The magnitude of this evolutionary process is dependent on the population size, and in the long–term, drift reduces genetic variation within populations (Szűcs et al., 2019). Due to this reduction in genetic variation, differences between populations can become more pronounced. As the populations of the biological control agents are generally small, the influence of genetic drift is large and alleles can become fixed or lost. As an example, a study on the pinewood nematode, *Bursaphelenchus xylophilus*, in three different areas in Japan, has shown that the most secluded forest had the lowest genetic diversity in the nematode population. The authors suggest that the low genetic diversity may have occurred as a result of genetic drift, causing reduced genotypic diversity when compared with the originally-introduced nematode population (Zhou et al., 2007).

1.4.1.2 Selection

During the quarantine phase of the invasion process, selection for particular traits, such as high levels of parasitism and fast growth, can occur to benefit mass rearing. In most instances, biological control agents are reared on alternative hosts or even artificial diets, as pests are often difficult to maintain in a laboratory environment. As a consequence of the rearing procedure, genetic diversity might be negatively impacted

and often becomes reduced (e.g. the selection of altered host preference; Hopper et al., 1993). The biocontrol agents could struggle in the field, as important traits for the field may have been lost through this process (Stouthamer, 2017).

1.4.1.3 Inbreeding

Inbreeding causes extreme homozygosity, which can expose recessive deleterious alleles and ultimately lead to decreased fitness. As the population size of the biological control agent is generally small, mating between relatives becomes more likely. Research on the impact of inbreeding on establishment success comparing inbred and outbred populations of whitefly on natal to novel hosts, showed an 11-fold decrease of establishment of the inbred line on a novel host (Hufbauer et al., 2013). Similar observations were made investigating the consequences of inbreeding on growth rate, persistence and adaptive ability on biological control agents (Szűcs et al., 2017a).

Inbreeding and its impact on species with either complementary sex determination (CSD) or non-CSD is particularly important for the application of parasitoid wasps. In hymenopterans, haplodiploid sex determination is common, whereby the males are haploid and the females diploid. Therefore, females are heterozygous at the *csd* locus and males are either homozygous or hemizygous. The occurrence of inbreeding in those systems leads to an increase of diploid males, hence a loss of diversity at the *csd* locus. Eventually the population may go extinct, as the diploid males are sterile, or lead to sterile triploid female offspring (Stouthamer et al., 1992; Van Wilgenburg et al., 2006; Zayed and Packer, 2005). In non-CSD parasitoid wasps inbreeding influences sperm production as well as sperm functionality, causing the population to propagate less effectively, whereas inbreeding does not seem to affect life-history traits such as body site or life-span (Leung et al., 2019).

1.4.2 Increase in genetic diversity

Genetic variation present in different populations can be integrated into genetically depauperate populations, to minimize potential negative fitness consequences of low

genetic diversity (Hedrick and Garcia-Dorado, 2016). The process whereby genetic diversity is increased, is referred to as genetic rescue. Genetic rescue is a mechanism which restores genetic diversity and improves the fitness in small populations by introducing new alleles into the population (Whiteley et al., 2015). This tool is ideal for use in biological control when introducing a population.

1.4.2.1 Admixture

Admixture is the interbreeding of populations within a species that are usually geographically isolated and may generate novel allelic combinations in the offspring (Rius and Darling, 2014; Roman and Darling, 2007). The consequences and potential benefits of admixture are largely unknown, but in a few cases the process has been identified as a driver of invasion success (Zenni et al., 2017). Specifically biological control intraspecific admixture could contribute to the establishment success of the biological control agents (Li et al., 2018; Rius and Darling, 2014), as admixed offspring can have increased growth rate, fecundity and reduced development time compared to the parental strains (Szűcs et al., 2012b, 2019). Admixture also accelerates range expansion, due to more rapid adaptive evolution, which can be effective against the range expansions of the pest (Ochocki and Miller, 2017; Szűcs et al., 2017b; Weiss-Lehman et al., 2017). To improve the success of biological control agent, exploiting admixture may be necessary (Lommen et al., 2017; Szűcs et al., 2019).

The primary advantage of admixture is its positive contribution towards adaptive potential. Admixture can lead to increased genetic diversity within a lineage (Kolbe et al., 2004; Lavergne and Molofsky, 2007), resulting in favourable new phenotypes for the introduced environment (Facon et al., 2008). Subsequently, the fitness of admixed offspring can be increased (Benvenuto et al., 2012; Seko et al., 2012; Szűcs et al., 2012a). A laboratory cross of Swiss and Italian populations of *Longitarsus jacobaeae*, indicated hybrid advantage in the F2 offspring, which was evident in the increase of both lifespan and oviposition period, compared to parents (Szűcs et al., 2011, 2012a). Questionable is however the long-term value of admixture, as hybrid advantage typically declines in subsequent generations.

Admixture can also have negative fitness costs, such as reduced viability or fertility, observed through breakdown of admixed offspring (Rius and Darling, 2014). Such negative impacts are usually more associated with admixture between genetic incompatible populations or interspecific crosses (Johansen-Morris and Latta, 2006; Niehuis et al., 2008). Admixture in biological control agents is negatively associated with the loss of host specificity, however, proof is still needed (Louda et al., 2003; Simberloff and Stiling, 1996).

1.4.2.2 Transformation

Genetic diversity can be augmented through the incorporation and uptake of novel genetic material (Herrera-Estrella et al., 1990; Nigro et al., 1999; Ossanna and Mischke, 1990). Through the development of improved transformation techniques, transformation systems have become of interest in biological control programs (Herrera-Estrella et al., 1990). The system would ideally be applied to add beneficial traits to the biological control agent (Ossanna and Mischke, 1990). The most well-studied examples of gene transformation are of baculoviruses used in biocontrol. Genes, such as the insect toxin 1 from *Buthus eupeus*, have been transferred to *Autographa californica* to improve their effectiveness as biological control agent, as the gene expression causes a faster death of the host insect (Possee et al., 1997; Stewart et al., 1991). Similarly, the entomopathogenic nematode *Heterorhabditis bacteriophora*, was manipulated by inserting the *hsp70A* gene from *Caenorhabditis elegans* to improve its heat tolerance (Hashmi et al., 1998).

1.4.2.3 Mutation

Mutations occurring at a high enough frequency may generate useful diversity in invasive populations (Estoup et al., 2016). However, the rate at which mutations occur varies across species. It has been suggested that higher mutation rates could result in greater adaptation success in a new environment. As life history traits and other fitness associated traits are governed by a large number of loci, the chance that a mutation will occur and have an impact on such complex traits is increased (Houle et

al., 1996; Hufbauer and Roderick, 2005). A study, which examined genetic differences on asexually reproducing *Microctonus hyperodae,* concluded that mutation was the only evolutionary process responsible for differences within and between biotypes, resulting in adaptation of the eastern South American biotype to New Zealand conditions compared to the western South American biotype (Phillips et al., 2008).

Bartlett et al. (1994) argued that the occurrence of spontaneous mutations should not be ignored, as on average of 50 mutations per 100 individuals (assuming a diploid individual with 27 000 loci) can occur, which could be significant. Mutations may also be introduced into laboratory populations through mutagenic methods, such as irradiation or other mutagenic agents (Aastveit, 1966; Bartlett, 1966, 1994; Scossiroli and Scossiroli, 1959). Such approaches have been successful in inducing genetic variability. For example, UV-treated *Trichoderma harzianum* had higher protease production, resulting in enhanced biocontrol activity (Szekeres et al., 2004). Similarly, UV-treated *Alcaligenes xylosoxydans* had higher chitinase production, increasing its effectiveness against soil borne fungal pathogens (Vaidya et al., 2003).

There are, however, several disadvantages associated with the introduction of mutations that are likely to result in either the death or reduced fitness of an organism (Bartlett, 1994; Loewe and Hill, 2010). Mutations can be detrimental recessive or dominant lethal, leading to the death of the population, unless selection acts against such mutations.

1.5 The importance of studying genetic diversity in the *Sirex-Deladenus* complex

The woodwasp *Sirex noctilio* (Fig. 2) and its biological control agent *Deladenus siricidicola* is an economically important system to study. *Sirex noctilio* (Hymenoptera, Superfamily Siricoidea, Siricidae) was first discovered as an invasive species in New Zealand in 1900 (Miller and Clark, 1935). Since then, the wasp has invaded various other regions in the Southern Hemisphere (Beèche et al., 2012; Carnegie et al., 2005; Hurley et al., 2007; Iede et al., 1988; Klasmer and Botto, 2012; Rebuffo, 1990; Tribe, 1995), followed by northern America (Bergeron et al., 2008; Hoebeke et al., 2005) and lastly, China (Li et al., 2015). The nematode *D. siricidicola* (Nematoda,

Neotylenchidae) was discovered in New Zealand in 1962 in infected *S. noctilio* eggs (Kampfer et al., 1998; Zondag, 1962, 1969). Since then, a Sirex control program has been developed using this nematode species throughout the Southern Hemisphere.

Molecular characterization of the invasion history of *S. noctilio* has revealed higher levels of genetic diversity naturally in invaded areas than previously expected (Boissin et al., 2012). Molecular markers, applied to both the fungal symbiont of the wasp, *Amylostereum areolatum,* and *S. noctilio* allowed for the investigation of invasion routes (Boissin et al., 2012; Mlonyeni et al., 2018; Slippers et al., 2001). Two source populations were identified for the global invasion; one of European origin, and an unsampled population of unknown origin. Multiple invasions from these origin populations, as well as subsequent spread between non-native regions, were suggested by this analysis (Boissin et al., 2012). In the invaded areas, such as North America, Oceania, South Africa and South America, unusually high genetic diversity was identified.

The discovery of *Deladenus siricidicola* in New Zealand led to a large-scale screening of various nematode species and strains to identify a nematode with high levels of parasitism. The chosen strain was called the Sopron strain, originally collected from the Sopron town in Hungary. This strain was first used as a biological control agent in Australia, and parasitism rates of 90% promised efficient control of *S. noctilio*. However, continuous laboratory mass rearing of the Sopron strain on *A. areolatum* resulted in a loss of virulence (Bedding and Iede, 2005). A recollected strain, referred to as the Kamona strain, after the Kamona forest in Tasmania, replaced the Sopron strain as the biological control agent. This Kamona strain was used as a biocontrol agent throughout the Southern Hemisphere, i.e. South America and South Africa. To ensure that the virulence of the nematode was maintained the original and subsequently reared strains are stored in liquid nitrogen (Bedding, 1993).

Deladenus siricidicola is the biological control agent of choice against *S. noctilio* due to its unique bicyclic lifecycle (Bedding, 1967). During the mycetophagous phase the nematode feeds and reproduce on the wasp's fungal symbiont, *Amylostereum areolatum* (Bedding, 1972; Zondag, 1969). This phase is used to maintain the nematode in culture and for mass rearing. The parasitic phase is triggered by a rise in CO_2 and a decrease in pH levels. In this phase, the nematode enters the larvae and

later enters the developing *Sirex* eggs, rendering them infertile. In various regions of the Southern Hemisphere, the parasitism performance has, however, varied significantly (Hurley et al., 2007). Possible reasons include wood moisture content, loss of virulence and incompatibility of the nematode to *S. noctilio* and *A. areolatum* populations (Hurley et al., 2008).

A diversity study on *D. siricidicola* from the Southern Hemisphere revealed extreme homozygosity in contrast to the high genetic diversity of *S. noctilio* (Mlonyeni et al., 2011). The release of a single strain, the Kamona strain, followed by genetic bottlenecks, drift and inbreeding during the mass rearing process, are expected to have led to the low genetic diversity. The low genetic diversity may result in challenges associated with adaptation to variable host populations and environmental variables, thereby reducing the effectiveness of the biocontrol agent (Hurley et al., 2008, 2007).

There is currently no global comparative study on the diversity of *D. siricidicola* populations. However, this is needed to explain the variable levels of success of the nematode in different parts of the world (Hurley et al., 2007). It might also offer ways to augment diversity in populations with reduced diversity. In addition, the presence of a distinct non-sterilizing strain of *D. siricidicola* in the field in North America is concerning and its relationship to strains in other parts of the world is important to understand. It is also important to understand potential admixture between lineages, should the Kamona or other strains be released in North America (Williams et al., 2009; Yu et al., 2009).

1.6 Conclusion

Biological control is a preferred approach for controlling invasive pests. It is an environmentally friendly approach, whereby known natural enemies from the geographic origin of the pest are introduced to reduce the pest population in non-native regions. The success of these programs is, however, often low. Considering the rising numbers of invasive pests, improved predictability and strategic application of biological control agents are important for the future of control programs.

Knowledge gained from invasion biology can be applied to better understand and steer intentional invasion processes, such as biological control. A focus on evolutionary

mechanisms, which either negatively or positively impact the genetic diversity present in a population, could be particularly advantageous. It is important to consider the influence of evolutionary processes, such as genetic drift, selection and inbreeding during collection, rearing and deployment of biological control agents. Mechanisms such as transformation, admixture and mutation can also be exploited to counteract detrimental effects associated with reduced genetic variability.

This review highlights the importance of understanding the genetic diversity in biological control programmes and its potential role in adaptation of both the pest and biological control agent. The increasing knowledge of pest and biological control agent diversity opens many options to attempt improving the success of biological control programs.

A system where this combined knowledge could be applied is the *Sirex-Amylostereum-Deladenus* system. Information of the distribution history of the nematode indicated very low genotypic diversity in the Southern Hemisphere, while the wasp and fungus are diverse across the range where the nematode is used. Understanding the diversity and interaction between global populations of the nematode, complementing knowledge of the rest of the system, holds the promise of helping the success of the program.

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1.8 Figures

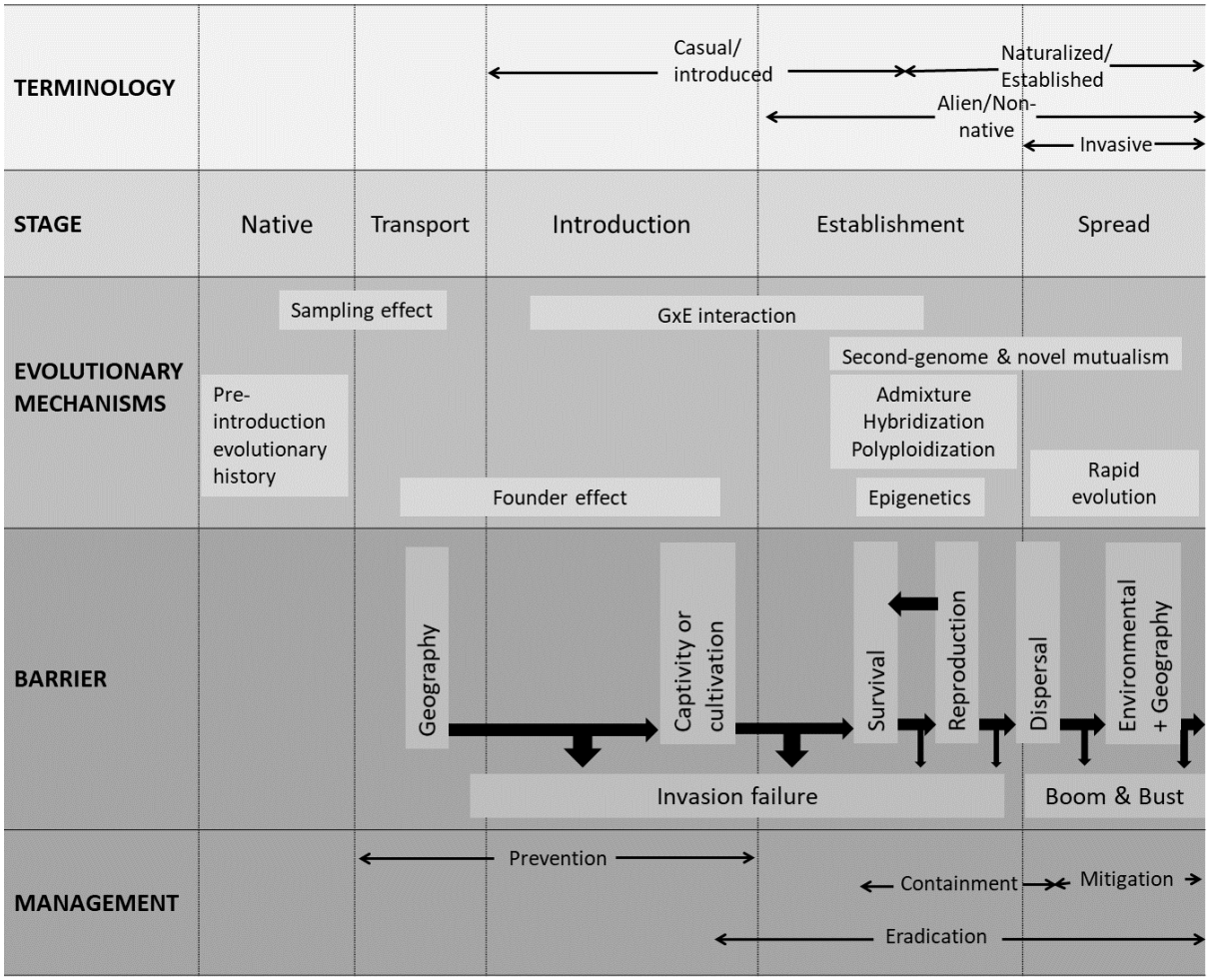

Figure 1. The expanded unified framework for biological invasion (Blackburn et al., 2011; Zenni et al., 2017). For biological control, a deliberate form of invasion, the introduction stage would be split into quarantine stage and the mass rearing stage. In terms of evolutionary mechanisms, genotype x genotype interaction needs to be added, which would be important across the quarantine, mass rearing, establishment and spread stages.

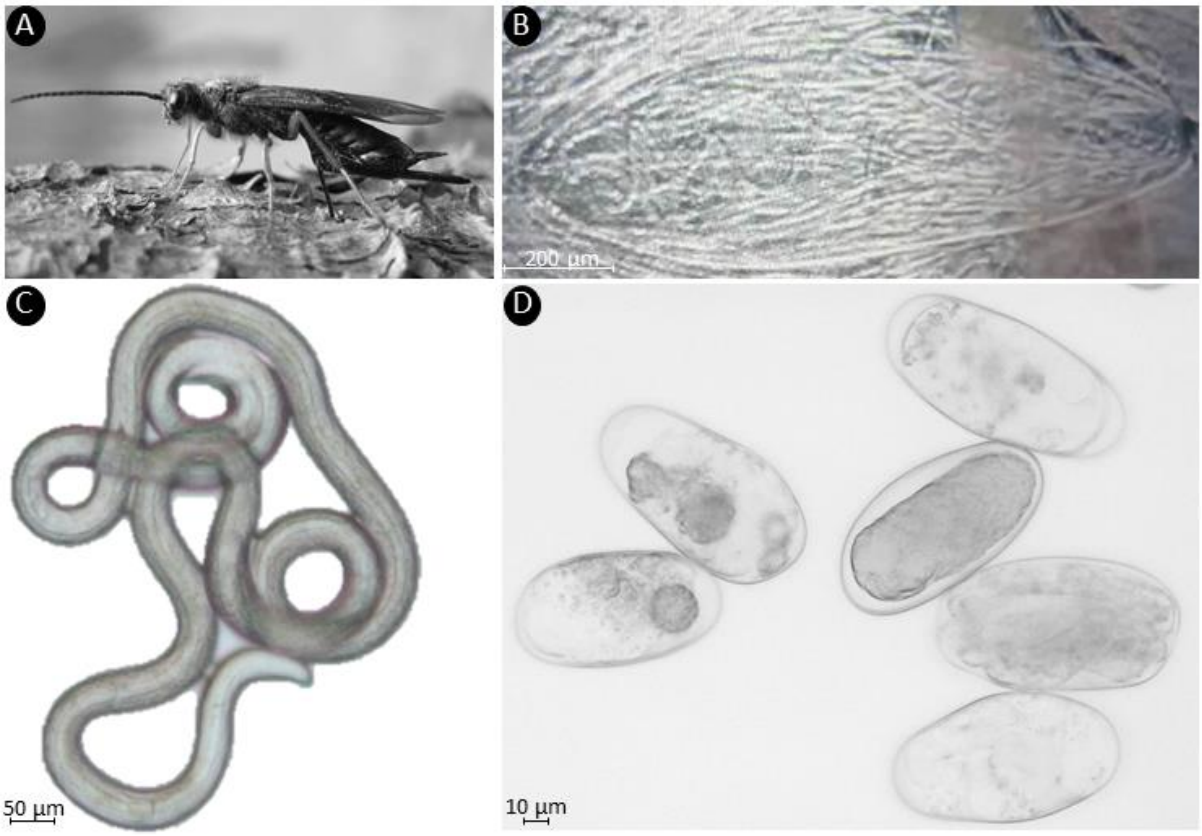

Figure 2. Pictures of the *Sirex noctilio – Deladenus siricidicola* system. **A.** Female *S. noctilio* **B.** Egg of *S. noctilio* invested with *D. siricidicola* **C.** Single *D. siricidicola* nematode **D.** Eggs of *D. siricidicola*.

Chapter 2

The global diversity of *Deladenus siricidicola* in native and non-native populations

Katrin N.E. Fitza[a], Jeff R. Garnas[b,c], Maria J. Lombardero[d], Matthew P. Ayres[e], Flora E. Krivak-Tetley[e], Rodrigo Ahumada[f], Brett P. Hurley[b], Michael J. Wingfield[a], Bernard Slippers[a]*

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

[b]Department of Zoology and Entomology, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria 0002, South Africa

[c]Department of Natural Resources and the Environment, University of New Hampshire, New Hampshire, USA

[d]Departamento de Producción Vegetal, Universidad de Santiago, 27002 Lugo, Spain

[e]Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, USA

[f]Bioforest S.A., Casilla 70-C, Concepción Chile

Abstract

The nematode *Deladenus siricidicola* is the primary biological control agent of *Sirex noctilio,* a globally invading woodwasp pest of *Pinus* species. Preliminary studies on the diversity of populations of *D. siricidicola* revealed very low diversity in the Southern Hemisphere where they have been introduced for the purpose of biological control. The potential to augment biocontrol efficacy by increasing genetic diversity in biocontrol programs motivated this study, which investigated the patterns of genetic diversity in *D. siricidicola* across eight countries, including the presumed native range (Spain), areas of accidental introduction (Canada and the USA) and countries *D. siricidicola* has been intentionally released (Argentina, Brazil, Chile, New Zealand and South Africa). Nematodes were screened using mitochondrial COI sequence data and twelve microsatellite markers. Analyses of these data identified three distinct lineages from North America (Lineage A), the Southern Hemisphere (Lineage B) and Spain (Lineage C). Strains from Chile were an exception as they appear to represent an admixture of lineages A and B. This suggests a common origin of populations throughout the Southern Hemisphere, with a second introduction from North America into Chile. The introduction into North America is distinct from that in the Southern Hemisphere and probably originated from Europe. It is evident that substantial genetic diversity exists in *D. siricidicola* globally, which could be exploited to augment the reduced diversity in some populations used in biocontrol programs.

Keywords: Biological control, *Deladenus siricidicola*, *Sirex noctilio*, invasive pest, population genetics

2.1 Introduction

Many biological control organisms are successful despite passing through a genetic bottleneck. There is also little concrete evidence that genetic diversity is necessary for biocontrol success (Holt and Hochberg, 1997; Stouthamer and Kazmer, 1994), although some examples exist (Hufbauer, 2002; Phillips *et al.*, 2008; Tomasetto *et al.*, 2017). However, conventional wisdom posits that genetic diversity generally has fitness advantages for populations (Roderick and Navajas, 2003), and there are many reasons to think that augmenting genetic diversity might be beneficial in biocontrol systems. Firstly, genetic diversity of biological control agents can impact establishment and efficiency (Forsman, 2014; Szűcs *et al.*, 2017), as well as the potential for co-evolution with hosts. Furthermore, genetic diversity can also positively affect adaptive potential to a range of climates (Booy *et al.*, 2000), or the reproductive fitness of the biological control agent (Joron and Brakefield, 2003; Reed and Frankham, 2003). Additionally, diversity reduces risks of negative effects from inbreeding. On the negative side, high genetic diversity in biological control agents could also increase the probability of non-target effects via host-switching, though empirical examples of this relationship are lacking (Roderick and Navajas, 2003).

The diversity of biological control agents can be especially important when considering the complex patterns of invasion of the pests against which they are deployed (Garnas *et al.*, 2016). Multiple introductions of invasive pests often result in increased genetic diversity and population substructure across the invasive range, which could complicate pest management efforts (Garnas *et al.*, 2016). Such is the case for the target pest of the current study; the woodwasp *Sirex noctilio* that is an invasive pest of *Pinus* on four continents. Boissin *et al*. (2012) analysed the global diversity of *S. noctilio* populations and considered potential routes of invasion. The results suggested that multiple introductions occurred in various parts of the world, leading to a high degree of admixture.

The main biological control agent used for the management of pest populations of *S. noctilio* in the Southern Hemisphere is the parasitic nematode *Deladenus siricidicola* (Hajek and Morris, 2014; Slippers *et al.*, 2015). *Deladenus siricidicola* was identified in New Zealand in 1962, putatively after an accidental introduction from Europe. Characterisation and screening of numerous strains resulted in the selection of a

virulent strain from Hungary (the Sopron strain) for deployment in biological control programs in Australia (Bedding, 1972; Bedding and Akhurst, 1974; Bedding and Iede, 2005). The strain was highly effective, often sterilizing more than 90% of females in inoculated logs (Bedding and Akhurst, 1974). Continuous mass rearing in culture on *Amylostereum areolatum*, the fungal symbiont of *S. noctilio*, is thought to have eventually led to the loss of virulence in this strain (Bedding and Iede, 2005; Eskiviski *et al.*, 2003; Eskiviski *et al.*, 2004). A strain was then obtained from sites where the Sopron strain was originally released, in the Kamona forest in Tasmania. The 'Kamona strain' has since been released in Australia, South America and South Africa in the 1990s (Hurley *et al.*, 2007). Apart from the Kamona strain, a strain referred to as "Encruziliado do Sul", isolated from infested wasps in Brazil, has been used in biological control programs in Brazil and Argentina (Eskiviski *et al.*, 2003).

The diversity of *D. siricidicola* used in biological control programs stands in contrast to the diversity of invasive *S. noctilio* against which it is deployed. Mlonyeni *et al.* (2011) have shown that the samples from the biological control programs were virtually all homozygous and identical across the Southern Hemisphere (Argentina, Brazil and South Africa). Low genetic diversity may have resulted from genetic bottlenecks as well as inbreeding and selection during mass rearing and the distribution process. This low diversity in the nematode populations, and the potential lack of adaptability to different environments and different *S. noctilio* populations, might be one of the reasons that contribute to the variable success rate in inoculation programs using the nematode across the Southern Hemisphere (Hurley *et al.*, 2008; Hurley *et al.*, 2007).

Deladenus siricidicola has been identified from invading populations of *S. noctilio* in Canada and the USA in the late 2000s (Kroll *et al.*, 2013; Leal *et al.*, 2012; Morris *et al.*, 2013; Ryan *et al.*, 2012; Yu *et al.*, 2009). It appears that the nematode arrived with *S. noctilio* into both countries (Yu *et al.*, 2009). Mitochondrial cytochrome oxidase subunit 1 (CO1) sequence data on *D. siricidicola* strains from North America showed that it is distinct from the Kamona strain (Leal *et al.*, 2012). PCR-RFLPs also distinguished between the North American strains and the Southern Hemisphere strains (Mlonyeni *et al.*, 2011; Morris *et al.*, 2013). The North American strain is often referred to in literature as 'non-sterilizing', because the nematodes do not enter the eggs of the majority of wasps that they infect (Williams and Mastro, 2010; Yu *et al.*,

2009). In contrast, the Kamona strain is nearly always found within eggs of *S. noctilio* wasps in the Southern Hemisphere (Bedding, 2009).

The impact of low genetic diversity in biocontrol populations of *D. siricidicola* on its efficacy in suppressing *S. noctilio* populations is not known. But it is plausible that the lack of additive variation might constrain local adaptation to variable climate and/or host tree or insect traits across its Southern Hemisphere range (Hufbauer and Roderick, 2005; Roderick and Navajas, 2003). Currently, very little is known regarding variation within or among populations of this nematode worldwide, despite its importance to global pine production (Slippers *et al.*, 2012; Slippers *et al.*, 2015). The aim of this study was to characterise the genetic diversity of *D. siricidicola* across its invaded range and in one part of its presumed native range, to infer patterns of both intentional and accidental spread. Additionally, this work contributes to efforts to catalogue and store living, dormant strains of *D. siricidicola*, of which very few existed previously.

2.2 Materials and methods

2.2.1 Nematode strains and DNA extraction

Fifty-seven nematode strains were collected from Argentina, Brazil, Canada, Chile, New Zealand, South Africa, Spain and United States of America (Table 1). They represent the presumed native range (Spain), areas of accidental introduction (Canada, the USA and New Zealand) and countries where *D. siricidicola* has been intentionally released to control *S. noctilio* (Argentina, Brazil, Chile and South Africa). Sample numbers collected from intentionally introduced regions were substantially higher due to easier access. In this study a strain represents a culture of nematodes isolated from a single wasp. All the strains used in this study are maintained at the Tree Protection Co-operative Program (TPCP) in the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

For DNA extraction, nematode cultures were reared in 500 ml Erlenmeyer flasks for one month. These flasks contained a mixture of sterilized wheat and rice and were inoculated with *A. areolatum* as a feeding substrate (Bedding and Akhurst, 1974).

Nematodes were harvested from flasks using the method described by Mlonyeni *et al*. (2011). Briefly, the flasks were washed with sterile nanopure water and sieved. To allow nematodes to settle, the water was left for 30 min. The sediment was later pipetted into 1.5 ml Eppendorf tubes which were centrifuged at 13 000 rpm for 3 min. The supernatant was removed and the pellet used for DNA extractions.

DNA was extracted from the nematodes using a phenol-chloroform extraction method (Sambrook and Russell, 2006). Through phase separation proteins were eluted and DNA precipitated using 0.1 volume of 2 M NaAc and 2 volumes of 100% EtOH overnight. Samples were then centrifuged for 30 min at 8000 rpm (Eppendorf Centrifuge 5417C), followed by washing of the pellet with 70% EtOH. After vacuum drying (Concentrator 5301), pellets were resuspended in 50 µl Sabax water (Adcock Ingram Ltd., Bryamston, RSA). Digestion of RNA was done by adding 5 µl of RNaseA and incubating at room temperature (22°C) for one hour. DNA concentrations of samples were determined using a ND-1000UV/Vis Spectrometer (NanodDrop Technologies, Wilmington, DE 19810 USA). Sample concentrations were adjusted to a final concentration of 100 ng/µl.

2.2.2 Confirmation of nematode identity

North American and Spanish collections are from areas where more than one *Deladenus* species co-occur. To confirm identity of the collected strains, the mitochondrial cytochrome oxidase subunit 1 (CO1) gene region was sequenced for each strain. Primers CO1F and CO1R were used for PCR amplification (Morris *et al.*, 2013). The PCR reaction mixture consisted of 5 µl 5x MyTaq™ buffer, 0.5 M of each primer, 1.5 units of MyTaq™ DNA Polymerase (Bioline Ltd. UK), 2 µl of the DNA template (approximately 200 ng) and sterile Sabax water to make up a final volume of 25 µl. The PCR cycling procedure included 4 min initial denaturation at 95°C followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 1 min, and a final extension step of 72°C for 45 min. From the PCR products, 2 µl were mixed with 1 µl GelRed™ (Biotium, California) followed by electrophoresis on 2% (w/v) agarose gels in a TAE buffer system and visualized under ultraviolet light. PCR amplicons were purified using 6% Sephadex G-50 (Sigma-Aldrich, Germany) according to the manufacturer's instructions, followed by

sequencing with the help of the Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California, USA). Sequencing products were run on the ABI Prism^{TM} 3500xl automated DNA sequencer (Applied Biosystems USA, Foster City, California). The sequences generated were submitted to GenBank (MF179729 – MF179784).

The available sequences of three described *Deladenus* spp. (*D. siricidicola*, *D. proximus* and *D. canii)* were obtained from GenBank and included in the dataset (Supplementary Table 1). Assembly of the sequencing reads was done in CLC Bio Main Workbench v6 (CLC Bio, www.clcbio.com). MAFFT v7 was used to align the entire dataset, including sequence data of described *Deladenus* spp. from GenBank, using the E-INS-I algorithm and additionally confirmed with ClustalW in Mega v6 (Tamura *et al.*, 2011). To identify the best fit nucleotide substitution model, jModelTest v2.3.1 (Darriba *et al.*, 2012; Guindon and Gascuel, 2003) was run. A maximum likelihood (ML) analysis was performed in PhyML v3.1 (Guindon and Gascuel, 2003). Branch support was calculated through 1000 bootstrap replicates. Additionally a maximum parsimony tree (MP) was constructed in PAUP v4.0b10 (Swofford, 2003) and tree length, consistency index (CI), rescaled consistency index (RC) and the retention index (RI) calculated. Furthermore to determine relatedness between populations in this study, a CO1 haplotype network based on the median-joining algorithm using NETWORK v4.6.1.3 (Bandelt *et al.*, 1999) was constructed based on the 539 bp amplicon.

2.2.3 Microsatellite identification and primer design

Twelve microsatellite markers were used in this study. Ten were developed by Mlonyeni *et al.* (2011). The draft genome of *D. siricidicola* was used to identify two additional microsatellite repeats applying the program Msatcommander v0.8.2 (Faircloth, 2008) and flanking primers were designed using Primer 3.0 software (Rozen and Skaletsky, 1999). Ds33, a trinucleotide repeat (TGA), was amplified using primers Ds33F (5’ CACCATCAACATCACCTCAT 3’) and Ds33R (5’ TGCTGTTGCGCATTATCATT 3’). The second locus, Ds38, also a trinucleotide repeat (ACA) was amplified using the primers Ds38F (5’ CGAGCGAAAACAACAACAAC 3’) and Ds38R (5’ AGCATCATCATCCTCAGCATT 3’). All labelled primers for fragment

analysis were sourced from Thermo Fisher Scientific (Life Technology, RSA). PCR reactions were set up as described for the CO1 gene region. The following PCR cycling parameters were applied: a 4 min. initial denaturation step at 95°C followed by 35 cycles of denaturation at 95°C at 30 sec., annealing at 58°C for 30 sec., extension at 72°C for 1 min. and a final extension of 72°C for 45 min. followed by a cooling step to 4°C for 10 min. The products were then visualized on 2% (w/v) agarose gels. Fluorescently labelled primers were arranged into two panels (Table 2) based on size and fluorescent dye colour, each consisting of either 7 or 5 primer pairs to conduct fragment analyses using GeneScan (Applied Biosystems, Foster City, California). PCR products were diluted to a ratio of 1:100 using Sabax water. The various amplicons from different primers were pooled according to the panel arrangement. 1 µl of this pooled mix was added to 10 µl of formamide and 0.2 µl GeneScan-500 Liz size standard (Applied Biosystems). These samples were run on the ABI PRISM^{TM} 3500xl DNA analyser to determine product size (DNA sequencing facility, University of Pretoria). The software GeneMapper® v4.1 (Life Technologies, Foster City, CA) was run on the GeneScan data to score allele fragment sizes.

The different alleles were confirmed by sequencing the PCR fragments from strains that were homozygous for the alleles (Inqaba Biotec South Africa). This was done to confirm the amplification of the right locus and the variation in allele sizes observed. PCR and sequencing conditions were applied as described above. The sequences were submitted to GenBank (MF179717 – MF179728).

2.2.4 Population structure analysis

Population structure amongst the *D. siricidicola* strains was inferred using Bayesian clustering principles in the program `STRUCTURE` v2.2 (Pritchard *et al.*, 2000). A correlated allele frequency model allowing for admixture was performed. Burn-in length was set to 100 000 followed by a run length of 700 000 simulations. Twenty iterations were performed for each value of *K* (from *K*=1 to *K*=10), where K is the number of distinct populations. The optimal value for *K* was determined using the Evanno's method (Evanno *et al.*, 2005) based on Δ*K* and visualized using both `STRUCTURE` Harvester (Earl, 2012), as well as CLUMPAK (Kopelman *et al.*, 2015).

For each run of *K* CLUMPAK identifies possible major and minor modes representing alternative clustering of samples. We also performed a principal coordinate analysis (PCoA) using GenAIEx v6.2 with the average genetic distance among population to further consider possible population structure (Peakall and Smouse, 2006).

2.2.5 Genetic diversity based on microsatellites

The presence of unique alleles, number of effective alleles, as well as the gene diversity (Nei, 1973) were investigated using GenAIEx v6.2 (Peakall and Smouse, 2006). Genotypic diversity (Ĝ) indices were calculated by applying Stoddard and Taylor's method (1988) to the full dataset, while analyses of genetic diversity were performed on the clone-corrected dataset by removing identical genotypes. We compared genetic diversity among populations using sample rarefaction at N=4 to account for differences in strain number/sampling intensity. Observed and expected genetic diversity (H), G'' and Jost's D (Jost, 2008) were computed both within and among populations, using GENODIVE to determine differentiation among populations.

2.2.6 Scenario testing

Population introduction history was inferred using DIYABC (Cornuet *et al.*, 2014; Cornuet *et al.*, 2008). Using historical data on the timing and directionality of *D. siricidicola* introductions and population clustering results from STRUCTURE, the number of hypothesized introduction scenarios tested was limited to 11. For these analyses Chile, North America, Southern Hemisphere and Spain were defined as separate populations. An unsampled population as the source for all defined populations was also considered. In other scenarios, the source was represented by one of the defined populations from which the remaining populations diverged.

Default prior parameter settings were applied using the DIYABC program. The set of microsatellite markers used were either di- or trinucleotide repeats with different mutation rates. As such, two different mutation models were used according to the group of repeats. For dinucleotide repeats, the mean mutation rate was set at $1E^{-5}$ – $1E^{-4}$ and the individual locus mutation rate at $1E^{-5}$ – $1E^{-4}$. For trinucleotide repeats, the mean mutation rate was set at $1E^{-5}$ – $1E^{-4}$ and the individual locus mutation rate at $1E^{-}$

^{5} - 1E^{-4} selected based on a study on the nematode *Pristionchus pacificus* (Molnar *et al.*, 2012). For the one sample summary statistics, the mean number of alleles, mean genetic diversity and mean size variance were selected. For two-sample summary statistics the F_{st}, classification index, shared allele distance and $(d)^2$ distance were chosen. To evaluate prior scenario combinations 1 000 000 simulated data sets were generated and used, with the help of principal component analysis (PCA). Posterior probabilities of the 11 scenarios were analysed using the closest 1% simulated data sets and selected summary statistics. Logistic regression analysis was applied to determine the confidence in scenario choice (Cornuet *et al.*, 2014).

2.3 Results

2.3.1 Confirmation of nematode identity

The alignment of the cytochrome oxidase subunit 1 (CO1) fragment consisted of 539 base pairs including 59 parsimony-unimformative, 70 parsimony-informative and 410 constant characters. The 1000 most parsimonious trees were identified (TL = 0.93, CI = 0.87, RI = 0.99, and RC = 0.86). The phylogenetic analysis assigned all 57 nematode strains used in this study to *D. siricidicola* (Fig. 1).

The strains were separated into three haplotypes based on three informative sites (Fig. 2). Haplotype and nucleotide diversity was low (Hd = 0.343, Pi = 0.002). Each haplotype could be linked to distinct geographic regions. The first lineage (A) included all the strains from North America and Chile. The second lineage (B) included all but one of the strains from the Southern Hemisphere and the last lineage (C) represented all the strains from Spain, representing the native region. The solitary exception to this trend was that some Chilean strains grouped with the North American haplotype, while others grouped with the Southern Hemisphere haplotype.

2.3.2 Microsatellite identification and primer design

In total 73 332 microsatellite regions were identified in the *D. siricidicola* genome. Of these microsatellites, the most abundant motif were the dinucleotides (31%) followed

closely by the mononucleotide (30%). The trinucleotides made up 24%, the tetranucleotides 12% and the pentanucleotides and hexanucleotides combined constituted 3%. A total of 12 microsatellite markers were applied (Table 3), including both dinucleotide and trinucleotide regions. Of the seven new microsatellite markers tested, two were polymorphic and were added to the existing ten microsatellite markers.

2.3.3 Population structure analysis

STRUCTURE analysis grouped the data in two or three clusters (Fig 3). According to Evanno's statistics (Evanno *et al.*, 2005) the most likely number of populations (*K)* was *K* = 2. However, taking the CO1 data and the historical background into consideration the most likely *K* was *K* = 3. The STRUCTURE analysis at *K* = 3 differed from *K* = 2 by splitting the Northern Hemisphere nematode strains into the North American lineage (Canada and United States) and the Spanish lineage. The Southern Hemisphere lineage consisted of strains from Argentina, Brazil, South Africa and New Zealand. The Chilean strains appeared to represent an admixed population of the North American and the Southern Hemisphere lineages. At *K* = 4 the presence of a fourth lineage of unknown geographic origin was identified that seemed to be part of the admixed population in Chile. The Principal Coordinate Analysis separated the data into the same three clusters as STRUCTURE, with PC1 and 2 explaining 64.4% and 18.6% of the variation in the dataset respectively (Fig. 4).

2.3.4 Microsatellite diversity

Thirteen of the 134 alleles identified were unique to a specific lineage (Table 3). The Spanish lineage had the highest number of unique alleles (n = 12), whereas the Southern Hemisphere lineage had no unique alleles (Table 4). Gene diversity of the Southern Hemisphere lineage was the lowest (H = 0.119), while the Chile lineage had the highest gene diversity (H = 0.500) (Table 4). The same pattern was reflected in the genotypic diversity, whereby the Southern Hemisphere lineage showed the lowest diversity ($e\hat{G}$ = 2.4) and Chile had the highest rarefied genotypic diversity ($e\hat{G}$ = 4, tied with Spain; Table 4).

Analysis of population differentiation using AMOVA showed that 86% of the molecular variation was due to variation among populations (Table 5). Pairwise comparison of the population diversity (Table 6) indicated the highest level of genetic differentiation between the North American and the Southern Hemisphere populations (G'' = 0.894). The majority of alleles were shared between the Southern Hemisphere and Chilean populations indicating a lower level of genetic differentiation (G'' = 0.584; Table 6).

The microsatellite data generated were used to construct a genotype network in which 38 genotypes were identified (Fig. 5). These genotypes grouped into three major clusters representing the North American population (Canada and United States of America), the Spanish population and the Southern Hemisphere population (Argentina, Brazil (Encruziliada do Sul), New Zealand and South Africa). The Chilean strains grouped in between the Southern Hemisphere and the North American clusters.

2.3.5 Scenario testing

The best support in terms of posterior probability [*P* = 0.773; 98%, (CI) 0.762-0.783], using Approximate Bayesian computation (DIYABC), strongly favoured a model in which 1) the Southern Hemisphere and the Spanish populations diverged first from an unsampled population, 2) where the Spanish population later gave rise to the North American population, and 3) where the Chilean population diversity arose via admixture between North American and Southern Hemisphere populations (Fig. 6).

2.4 Discussion

This is the first study to consider the genetic diversity of *D. siricidicola* at a global scale, including nematodes from the presumptive native range in Europe. Three lineages were identified that mapped to North America (Lineage A), the Southern Hemisphere (Lineage B, except for Chile which grouped with both Lineages A and B) and Spain (Lineage C). Interestingly, strains from Chile represented an admixed sample of both the Southern Hemisphere and North American lineages. The results re-affirm the lack of diversity in Southern Hemisphere populations of the nematode. They also provide

a framework for understanding of the histories of introduction and invasion, and suggest opportunities to strategically amend biological control programs with additional genetic diversity.

All the datasets emerging from this study showed a clear distinction between the strains from North America and those from other areas of the world. The North American lineage (Lineage A) included strains from the USA and Canada and all twelve loci investigated could discriminate these strains from those collected in Spain and the Southern Hemisphere. North American samples were also represented by only one CO1 sequence haplotype, which differed from that of the Southern Hemisphere and the Spanish populations. *Deladenus siricidicola* was discovered soon after the first reports of *S. noctilio* in New York in 2005 (Hoebeke *et al.*, 2005) when it was reported in 2006 from parasitized *S. noctilio* (Shields, 2009; Williams *et al.*, 2009; Yu *et al.*, 2009). Subsequent studies highlighted the distinction between North American strains and strains from the Southern Hemisphere (Leal *et al.*, 2012; Mlonyeni *et al.*, 2011; Morris *et al.*, 2013). Since these populations so clearly differ genetically from Southern Hemisphere populations, it is prudent to avoid generalizations from studies on nematodes in North America when considering populations of *D. siricidicola* elsewhere in the world, and *vice versa*. Biocontrol programs using *D. siricidicola* should ideally be studied in the context and environment where they are intended to be used and in relation to local fungal and Siricid populations as well as local climatic conditions.

The lack of diversity in Southern Hemisphere populations is not surprising since these biocontrol strains were isolated from a single source (Tasmania, Australia, where biocontrol strains collected from Hungarian wasps were originally released) and shared with countries and pine growers. However, the relatively high genetic diversity in the North American population was unexpected. Diversity in North America was comparable to that seen in the Spanish population, which represents part of the presumed native region. There have been no reports of intentional introductions of *D. siricidicola* strains in North America; the species is assumed to have arrived with the *S. noctilio* introduction into North America (Bittner *et al.*, 2017; Yu *et al.*, 2009). From this perspective, such high diversity is intriguing, especially given the likelihood of a bottleneck (Roderick and Navajas, 2003). Work done on the fungal symbiont *A. areolatum* (Bergeron *et al.*, 2011; Castrillo *et al.*, 2015; Nielsen *et al.*, 2009) and *S.*

noctilio (Boissin *et al.* 2012; Bittner *et al.* 2017) suggests multiple introductions of the wasp into North America. This suggests that there could have also been multiple introductions of *D. siricidicola* into North America. In the case of the wasp and the fungus these introductions appear to have come from at least two distinct geographic areas, whereas the North American strains of *D. siricidicola* appear to come from a single geographic origin. Such unexpectedly high levels of diversity, possibly due to multiple or large introductions, appears to be a pattern amongst many invasive pests (Garnas *et al*. 2016). A broader *D. siricidicola* population study across North America would allow for a better understanding of origin and number of introductions of the nematode in that region of the world.

The origin of the North American population of *D. siricidicola* cannot be conclusively inferred from the results of this study. However, DIYABC analyses suggests that the North American lineage is derived from the Spanish lineage (Lineage C) and is related but not identical to our Spanish samples. Previous studies on the introduction of *S. noctilio* and *A. areolatum* into North America support an introduction from Europe (Bergeron *et al.*, 2011; Bittner *et al.*, 2017; Boissin *et al.*, 2012; Nielsen *et al.*, 2009) though a second introduction from an unknown source, possibly South America, is likewise supported (Boissin et al. 2012).

The existence of a single lineage (B) of *D. siricidicola* in Brazil, Argentina, New Zealand and South Africa was expected given the extensively deployed biological control program across these regions from a single source population. The Kamona strain of the nematode has been repeatedly released in these countries since the early 2000's (Bedding and Iede, 2005; Hurley *et al.*, 2007; Slippers *et al.*, 2012). The effect of this reduced diversity present in the Southern Hemisphere should be considered in light of (1) the diversity of the *S. noctilio* populations across the Southern Hemisphere (Boissin *et al*. 2012), (2) variation in interactions between nematode strains and *S. noctilio* populations (Bedding and Iede, 2005), and (3) the diversity of tree species and environmental conditions across this range (Hurley *et al.*, 2007). Furthermore, the small sample sizes are unlikely to represent the full regional diversity and further sampling in these regions would add valuable information.

The Southern Hemisphere lineage (B) had the lowest genetic diversity of all the regions in this study and contained no private alleles. Low diversity (and high

homozygosity, also observed in this study and in Mlonyeni *et al*. 2011) suggests some degree of inbreeding. This is likely to have occurred given the serial sub-culturing and intensive rearing inherent to this and other augmentative biological control programs (Bedding and Iede, 2005; Slippers *et al.*, 2012).

Isolates from Chile represented an admixed population containing diversity from both the North American and Southern Hemisphere lineages. While this population had a low number of unique alleles, it had the highest level of gene diversity of all populations sampled, likely as a direct consequence of admixture. Historically, Chile obtained *D. siricidicola* strains from Brazil and New Zealand for use in their biological control program, with no known intentional introduction from North America (personal observation). Thus, it appears that there might have been an accidental introduction of *D. siricidicola* from North America into Chile. An alternative hypothesis is that other source populations of the Chilean population share diversity with North American populations. This finding aligns with the potential link in populations of *S. noctilio* in North America and South America (Boissin *et al*., 2012, Bittner *et al*., 2017).

The population of *D. siricidicola* from Spain was more diverse than other populations in this study. It also appears to be part of the population of origin of the North American populations of the nematode. Spain forms part of the presumed native range of *D. siricidicola,* which extends across Europe and also includes parts of Asia (Bedding and Akhurst, 1978). While the nematode is thought to have been introduced into North America (Bittner *et al.*, 2017; Yu *et al.*, 2009), the high diversity in that country could suggest a native origin, or at least a longer biogeographic history. Because our sampling in the native range of *S. noctilio* was limited, fine scale phylogeographic interpretations were not possible. Broader studies of *D. siricidicola* diversity across Eurasia would be needed to understand the origin of introduced populations. Furthermore, Lombardero *et al*. (2016) shows that the Spanish population of *D. siricidicola* sterilizes the local *S. noctilio* female wasps, whereas the North American strains of *D. siricidicola* do not sterilize the local *S. noctilio* (Williams and Mastro, 2010; Yu *et al.*, 2009). It would be interesting to consider in future studies to what extent this variation in the sterilization ability of the nematode is genetically determined and conserved versus being affected by environment or subject to rapid evolutionary change.

2.5 Conclusions

The use of molecular markers has made it possible to identify three distinct lineages amongst *D. siricidicola* global populations. Consequently, it has been possible to characterise and confirm patterns of spread around the world. The markers developed in this study and the curated strains that now reside in long-term storage provide tools and raw material for further exploration of the diversity of this important biological control agent of *S. noctilio*. Such studies hold the ultimate goal of developing increasingly effective and resilient biological control programs. The discovery of admixture in the Chile population of *D. siricidicola* confirms that inter-lineage cross-breeding is possible and occurs with some frequency in the wild. This has relevance both for efforts to increase diversity in biological control programs, for example by selecting or breeding high-efficiency strains for specific regions and pest populations. The effects of the genetic diversity *of D. siricidicola* on the effectiveness of biological control programs remains to be investigated, and can now be directed using the information provided in this study.

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2.8 Tables and Figures

Table 1. Collection locations of the *Deladenus siricidicola* strains used in this study.

Country	Location	No. samples
Argentina	Misiones	1
Brazil	Santa Catarina	1
Canada	Ontario	9
Chile	Los Lagos	2
	BíoBío	1
	BíoBío	1
New Zealand	North Island, Rotoru-Kaingaroa Forest	2
South Africa	Eastern Cape	2
	Western Cape	2
	Southern Cape	3
	KwaZulu Natal	6
	Mpumalanga	8
Spain	Galicia	12
United States of America	Pennsylvania	6
	Vermont	1

Table 2. Microsatellite primers used for the fragment analysis, their fluorescent labels and the panel arrangement. The two primers developed in this study are highlighted in grey.

SSR locus	SSR Motif	Forward primer 5'-3' Reverse primer 5'-3'	Panel No.	Fluorescent label	Tm (°C)
Ds1	GTA	CAATGTGCTGCGTCAATTTT ACCCAACGCGTAGTGATAGC	1	FAM	59
Ds33	TGA	CACCATCAACATCACCTCAT TGCTGTTGCGCATTATCATT	1	FAM	60
Ds38	ACA	CGAGCGAAAACAACAACAAC AGCATCATCATCCTCAGCATT	2	VIC	60
Ds54	CA	CAGCCACAACAATTCACACC GCACAAAAATCTCGCCTCAT	2	FAM	59
Ds105	AG	TGGTAGCAATCGATCGAAAA CGTGTCCACTTGTCCCTCTC	1	NED	59
Ds201	TG	TGCATAGCTGGCGATAAATG CGAGTCACGTACGCATTAGC	2	NED	59
Ds316	GA	TGCGGATATCTTCTCATTGTAA TCAAATGTTATGCGAAATTCTG	2	VIC	59
Ds323	TG	TTTACCTGTTGGCTGTTACCG TGGGGTAAAAGTGGATTGGA	1	VIC	60
Ds325	GT	ACGCTTATGTGTGCCACTTG GGGTCTCTTGATGATGTTTCG	2	PET	59
Ds366	GT	CGCTGCTGTACTGCTGTTTT CACACAAATGCACACATGGA	1	PET	59
Ds375	AC	GGCAGCTGAAATGATGACAA ATCATCATCAGCAATATCCTCA	1	VIC	60
Ds388	(GT)(TG)	AAGTCAGCTGAAAGGCGAAG TGTGTGCATGAAAACGGAAC	1	NED	60

Table 3. Alleles scored at each locus for each strain of *D. siricidicola.* The -1 implies that these alleles could not be scored.

Sample No.	Ds 105		Ds366		Ds1		Ds323		Ds375		Ds388		Ds33		Ds38		Ds54		Ds201		Ds325		Ds316	
SA(D3)	151	151	158	158	155	155	167	167	185	185	225	225	244	244	152	209	152	152	170	170	304	304	386	387
SA4	151	151	158	158	155	155	163	167	185	185	225	225	244	244	209	209	143	152	169	170	304	304	387	387
SA11	151	151	158	158	155	155	167	167	185	185	225	225	244	244	209	209	152	152	170	170	304	304	387	387
SA24	151	159	158	158	155	155	167	167	185	185	225	225	244	244	209	209	143	152	170	170	304	304	387	387
SA47	151	151	158	158	155	155	167	167	185	185	225	225	244	244	209	209	152	152	170	170	304	304	387	387
SA107	151	151	158	158	155	155	167	167	185	185	225	225	244	244	152	209	152	152	170	170	304	304	387	387
SA735	151	159	158	158	155	155	167	167	185	185	225	225	244	244	209	209	143	152	170	170	304	304	387	387
SA753	151	151	158	158	155	155	167	167	185	185	225	225	244	244	209	209	152	152	170	170	304	304	387	387
SA757	151	151	158	158	155	155	167	167	182	182	225	225	244	244	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
SA761	151	151	158	158	155	155	167	167	185	185	225	225	244	244	120	209	152	152	170	170	304	304	387	387
SA785	151	151	158	158	155	155	167	167	185	185	225	225	244	244	209	209	152	152	170	170	304	304	387	387
SA701	151	151	158	158	155	155	167	167	185	185	225	225	244	244	152	209	152	152	170	170	304	304	387	387
SA500	151	151	158	158	155	155	167	167	185	185	225	225	244	244	152	209	152	152	170	170	304	304	387	387
SA587	151	151	185	185	155	155	167	167	185	185	225	225	244	244	152	209	152	152	170	170	304	304	387	387
SA599	151	151	158	158	155	155	167	167	185	185	225	225	244	244	152	209	152	152	170	170	304	304	387	387
SA417	151	151	158	158	155	155	167	167	185	185	225	225	244	244	152	209	152	152	170	170	304	304	387	387
SA450	151	151	158	158	155	155	167	167	185	185	225	225	244	244	152	209	152	152	170	170	304	304	387	387
SA693	151	151	158	158	155	155	167	167	185	185	225	225	244	244	152	209	152	152	170	170	304	304	387	387
SA750	151	151	158	158	155	155	167	167	185	185	225	225	244	244	152	209	152	152	170	170	304	304	387	387
SA399	151	151	158	158	155	155	167	167	185	185	225	225	244	244	152	209	152	152	170	170	304	304	387	387
SA410	151	151	158	158	155	155	167	167	185	185	225	225	244	244	209	209	152	152	170	170	304	304	387	387
ARG	151	151	158	158	152	152	167	167	185	185	225	225	244	244	209	209	152	152	170	170	304	304	387	387
BRL	151	151	158	158	155	155	167	167	185	185	225	225	244	244	152	209	152	152	170	170	304	304	387	387
BF1	151	151	158	158	143	152	163	167	179	185	222	225	244	244	144	144	143	152	170	170	304	304	387	387
BF3	151	159	158	160	155	155	163	163	179	185	222	222	244	244	144	144	143	152	169	169	304	306	386	391
BF5	151	159	158	158	143	152	163	167	179	185	225	225	244	244	144	209	152	152	170	170	304	306	387	391
Chile	151	151	158	158	143	152	163	167	185	185	225	225	244	244	143	143	143	143	170	170	304	304	387	387

Spain73	171	171	160	160	152	152	163	163	179	182	222	222	265	265	120	135	152	152	169	169	308	308	386	386
Spain74	169	173	160	160	152	152	163	163	179	182	222	222	284	284	120	120	152	152	169	169	308	308	386	386
Spain110	169	171	160	160	155	180	163	163	179	179	222	222	265	265	120	152	152	152	169	169	308	308	386	386
Spain277	163	163	160	160	180	180	163	163	179	182	222	222	265	265	120	129	152	152	169	169	308	308	386	386
Spain300	161	163	160	160	180	183	163	163	179	179	222	222	265	265	129	129	152	152	169	169	308	308	386	386
spain264	169	169	160	160	180	180	163	163	182	182	222	222	265	265	120	120	152	152	169	169	308	308	386	386
Spain272	171	171	160	160	180	180	163	163	182	182	222	222	265	265	129	129	152	152	169	169	308	308	386	386
Spain263	169	171	160	163	180	180	163	163	182	182	222	222	265	265	120	129	152	152	169	169	306	308	386	386
spain297	169	171	160	160	180	180	163	163	182	182	222	222	250	265	120	120	152	152	169	169	308	308	386	386
spain267	169	171	160	160	180	180	163	163	182	182	222	222	265	281	120	129	152	152	169	169	-1	-1	386	386
Spain299	163	163	160	160	180	180	163	163	179	182	222	222	265	265	120	129	152	152	169	169	308	308	-1	-1
Spain313	163	163	160	160	180	183	163	163	179	182	222	222	265	265	120	120	152	152	169	169	308	308	386	386
USA1	159	159	160	160	168	168	163	163	179	179	222	222	299	299	141	141	143	143	169	169	306	306	391	391
USA4	159	159	160	160	168	180	163	163	179	179	222	222	299	299	135	141	143	143	169	169	306	306	391	391
USA6	159	159	160	160	143	143	163	163	179	179	222	222	293	293	135	141	143	152	169	169	306	306	391	391
USA38	159	159	162	162	168	180	164	164	180	180	222	222	293	293	135	143	143	143	169	169	306	306	391	391
USA49	159	159	160	160	180	180	163	163	179	179	222	222	299	299	135	135	143	143	169	169	306	306	391	391
USA44	159	159	160	160	168	180	163	163	179	179	222	222	299	299	135	141	137	143	169	169	306	306	391	391
USA51	159	159	160	160	168	180	163	163	179	179	222	222	293	299	141	141	143	143	169	169	306	306	391	391
Can323	151	159	160	160	155	168	163	163	179	185	222	222	244	299	135	141	143	152	169	169	306	306	391	391
Can470	159	159	-1	-1	180	180	163	163	-1	-1	222	222	-1	-1	135	141	143	143	169	169	306	306	391	391
Can468	159	159	160	160	180	180	163	163	179	179	222	222	299	299	135	141	143	143	169	169	306	306	391	391
can1085	159	159	160	160	168	180	163	163	179	179	222	222	299	299	135	141	143	143	169	169	306	306	391	391
Can392	159	159	160	160	143	143	163	163	179	179	222	222	299	299	135	141	143	143	169	169	306	306	391	391
Can280	159	159	160	160	143	143	163	163	179	179	222	222	299	299	135	141	143	143	169	169	306	306	391	391
Can278	159	159	160	160	180	180	163	163	179	179	222	222	299	299	135	141	143	143	169	169	306	306	391	391
Can268	159	159	160	160	155	180	163	163	179	179	222	222	-1	-1	135	141	143	143	169	169	306	306	391	391
Can224	159	159	160	160	155	180	163	163	179	185	222	222	299	299	135	141	143	152	169	169	306	306	391	391
NZL6	151	151	158	158	152	152	167	167	185	185	225	225	244	244	209	209	152	152	170	170	304	304	387	387
NZL9	151	151	158	158	152	152	167	167	185	185	225	225	244	244	209	209	152	152	170	170	304	304	387	387

Table 4. Summary statistics of the populations in *D. siricidicola* using the microsatellite markers.

Population	No. of strains	No. of effective alleles	No. of unique alleles	No. of genotypes	Gene diversity[a]		Genotypic diversity	
					H_o	H_e	$\hat{G}$[b]	e$\hat{G}$[c]
Southern Hemisphere	25	1.08	0	7	0.119	0.109	3.2	2.4
Chile	4	1.76	2	4	0.500	0.458	4.0	4.0
Spain	12	1.42	11	12	0.167	0.210	12.0	4.0
North America	16	1.30	6	12	0.188	0.183	9.1	3.7

[a] The gene diversity calculated using all microsatellite markers based on Nei (1973)

[b] The method of Stoddart and Taylor (1988) was applied to estimate genotypic diversity ($\hat{G}$)

[c] e$\hat{G}$ refers to the genotypic diversity obtained using rarefaction based on the lineage with the smallest number of samples, in this study being Chile with four individuals

Table 5. Molecular variance analysis (AMOVA) of microsatellite marker data from all *Deladenus siricidicola* strains and populations.

Source	df	Estimated variance	Proportion of variance (%)	Statistic	Value	Probability
Among populations	3	590.3	86%	φ_{PT}	0.864	0.001
Within populations	53	125.7	14%			
Total	56	716.0	100%			

Table 6. Pairwise genetic difference values amongst the four populations. The upper right corner shows the Jost's D values and the lower left values are G''.

	Southern Hemisphere	Chile	Spain	North America
Southern Hemisphere	-	0.188	0.898	0.965
Chile	0.584	-	0.727	0.560
Spain	0.882	0.648	-	0.498
North America	0.894	0.636	0.684	-

Figure 1. Phylogenetic analysis of *Deladenus* spp. using both Maximum Likelihood (ML) and Maximum Parsimony (MP) based on cytochrome c oxidase subunit 1 (CO1) sequence data. The tree is rooted to *Howardula aoronymphium*. All bootstrap values above 70% are indicated for ML (roman) and MP (italics) at the nodes.

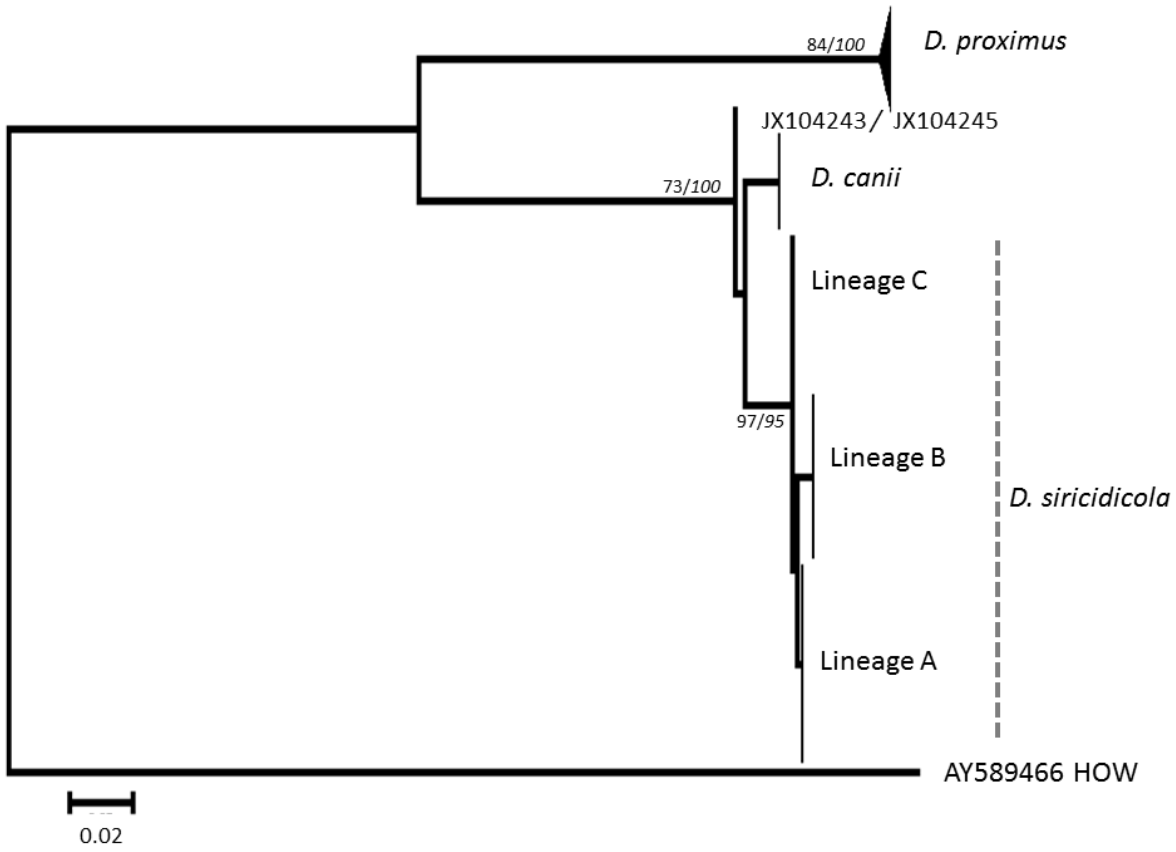


Figure 2. A haplotype network based on analysis of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene sequence data. The network was constructed using a median-joining algorithm. The circles represent the different haplotypes and the size of the circles is linked to the number of strains per haplotype. The colours represent the different geographic origins of the isolates.

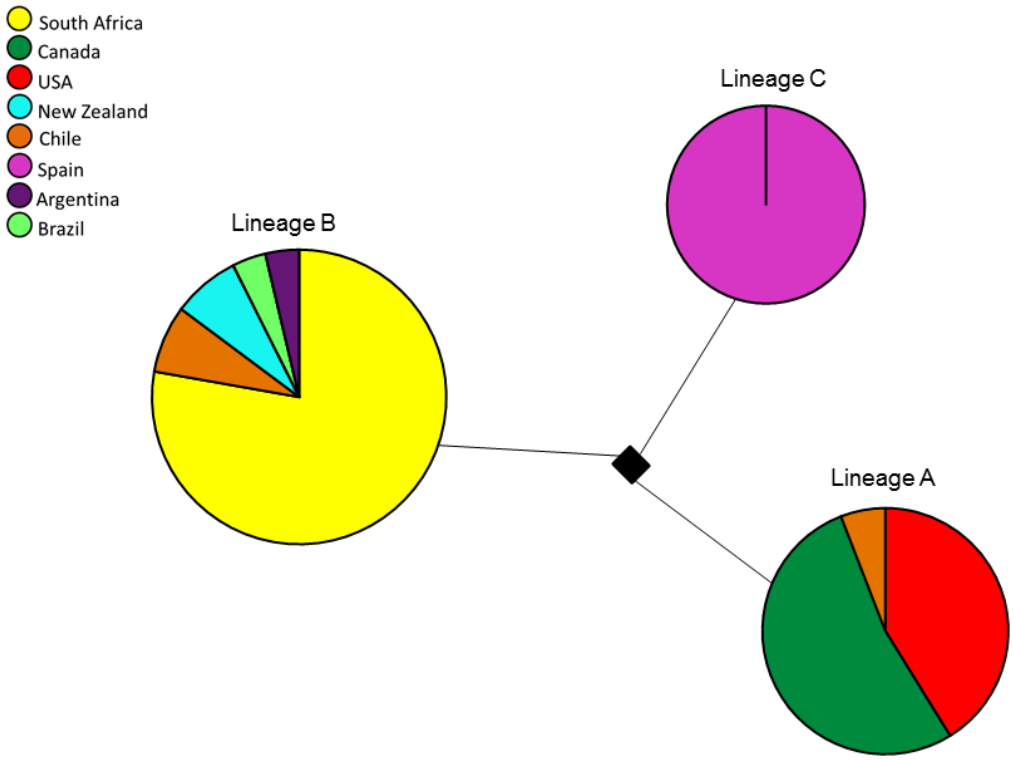


Figure 3. Bayesian clustering analysis (STRUCTURE) conducted using microsatellite marker data from all isolates (n = 57). Vertical columns represent individual strains and the different colours represent identified populations. The countries of origin of the isolates are indicated by the horizontal lines. (ARG = Argentina, BRA = Brazil, CHL = Chile, CAN = Canada, ESP = Spain, NZL = New Zealand, RSA = South Africa and USA = United States of America).

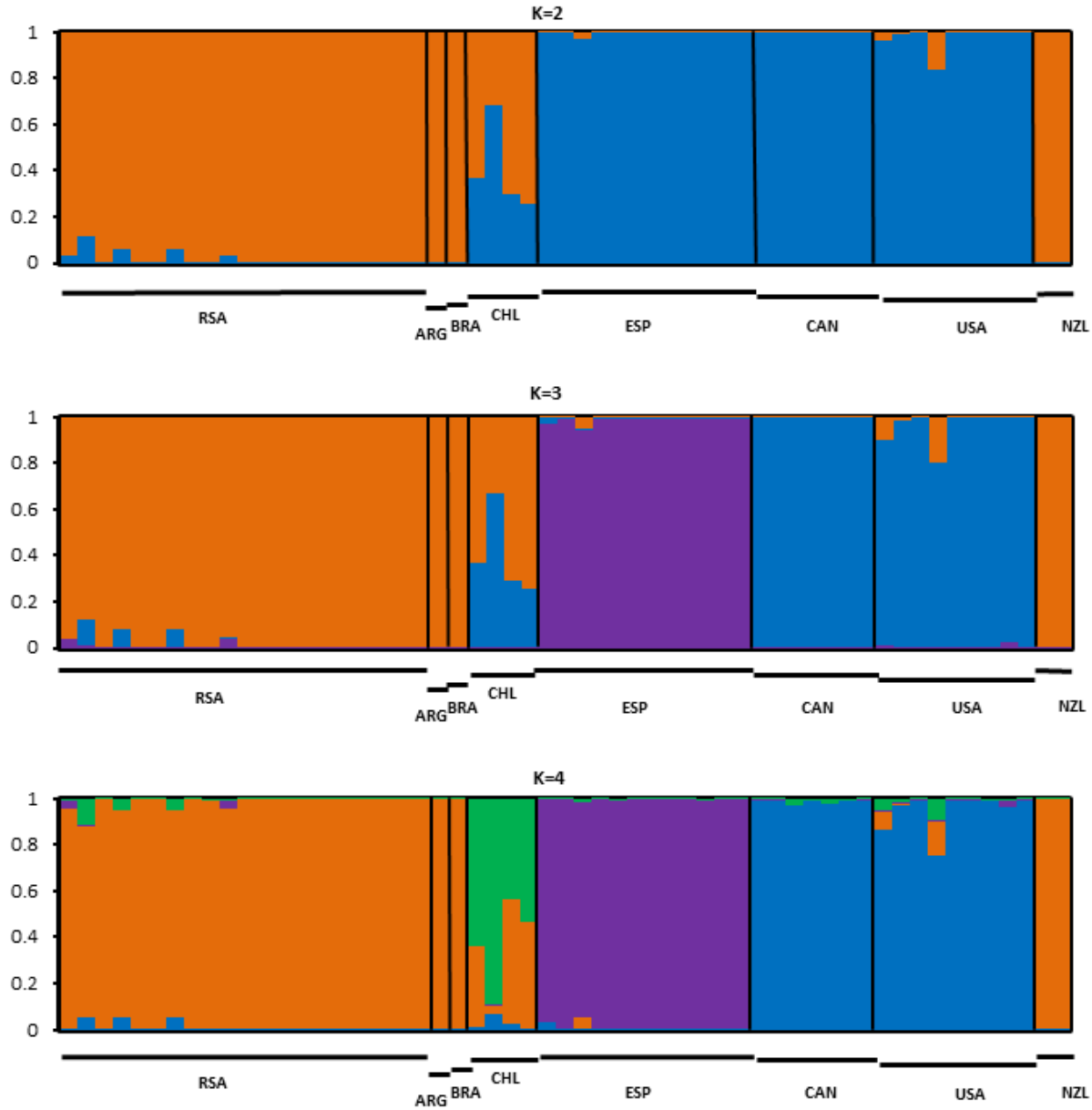


Figure 4. Principal Coordinates analysis plot of the microsatellite data set using Nei's genetic distance (Nei, 1973). The different shapes represent the four different lineages (ESP = Spain, CHL = Chile, NAM = North America and SH = Southern Hemisphere). The first two principal coordinates provided the best separation.

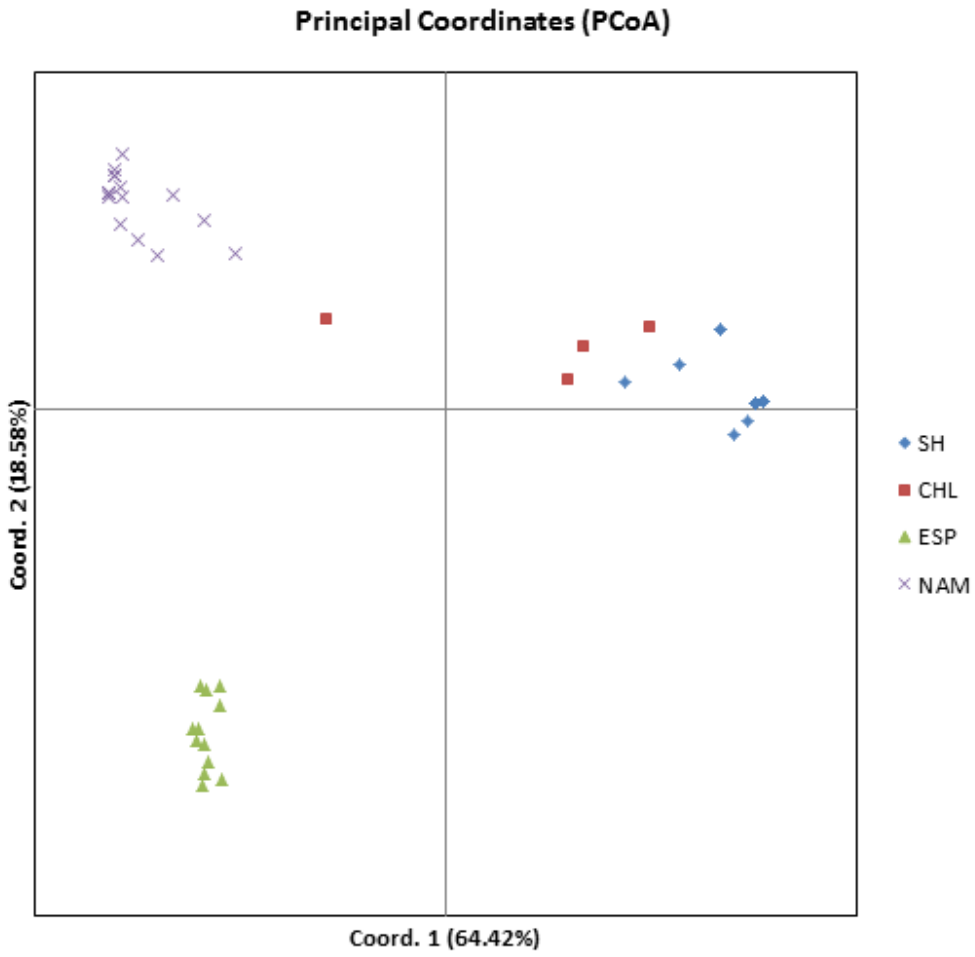


Figure 5. Genotype network drawn using microsatellite markers. The network is based on a median-joining algorithm. Coloured circles represent the different genotypes and the size of the circles is linked to number of strains with that genotype. The colours represent the different geographic origins of the isolates. The small black circles indicate undetected intermediate genotypes. Solid lines are used to group the genotypes into the three lineages, while the dashed line groups the Chilean strains.

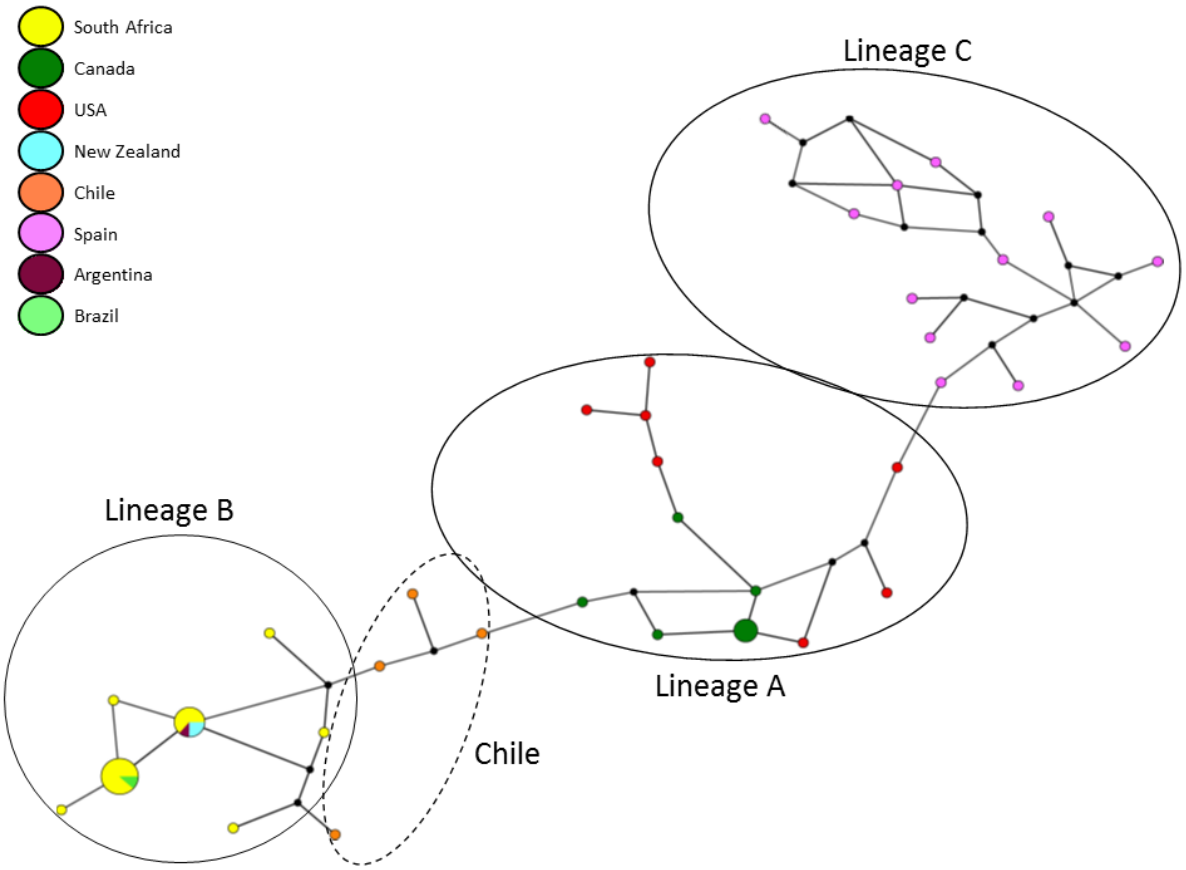
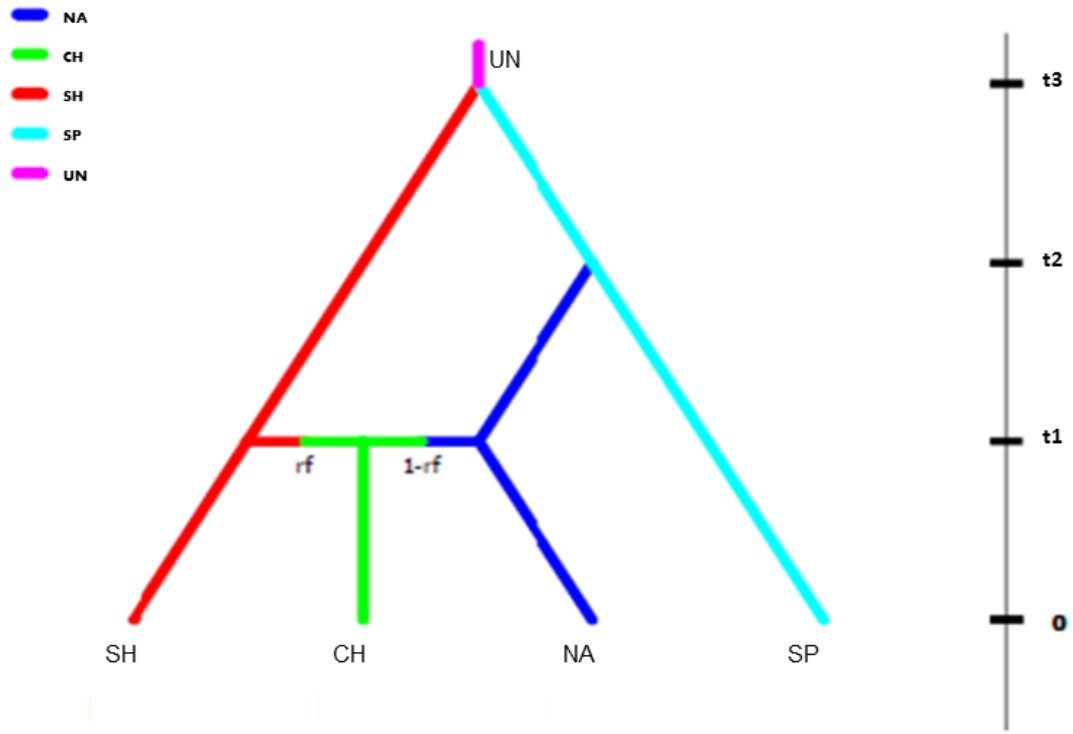


Figure 6. Representation of the most likely scenario (P=0.79) among the 11 scenarios tested in our DIYABC analysis. The figure represents the most likely scenario (SP =Spain, CH = Chile, NA = North America, SH = Southern Hemisphere and UN = Unsampled region). The vertical axis represents time but is not to scale.



Supplementary table 1. Nematode samples used for the mitochondrial cytochrome oxidase 1 subunit (CO1) phylogenetic analysis.

# sample	Species	Origin	Referance
JX104240	*Deladenus proximus*	USA, PA, Mt. Morris	Morris et al., 2013
JX104241	*Deladenus proximus*	USA, PA, Mt. Morris	Morris et al., 2013
JX104279	*Deladenus proximus*	USA, NY, Fabius	Morris et al., 2013
JX104242	*Deladenus proximus*	USA, PA, Garards Fort	Morris et al., 2013
JX104269	*Deladenus proximus*	USA, NY, Warrensburg	Morris et al., 2013
JX104278	*Deladenus proximus*	USA, NY, Fabius	Morris et al., 2013
JX104266	*Deladenus proximus*	USA, NY, Warrensburg	Morris et al., 2013
JX104277	*Deladenus proximus*	USA, NY, Fabius	Morris et al., 2013
JX104274	*Deladenus proximus*	USA, LA, Grants Parrish	Morris et al., 2013
JX104271	*Deladenus proximus*	USA, NY, Warrensburg	Morris et al., 2013
JX104270	*Deladenus proximus*	USA, NY, Warrensburg	Morris et al., 2013
JX104267	*Deladenus proximus*	USA, NY, Warrensburg	Morris et al., 2013
JX104273	*Deladenus proximus*	USA, LA, Grants Parrish	Morris et al., 2013
JX104272	*Deladenus proximus*	USA, LA, Grants Parrish	Morris et al., 2013
JX104243		USA, NY, Newcomb	Morris et al., 2013
JX104245		USA, NY, Newcomb	Morris et al., 2013
JX104253	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013
JX104237	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013
JX104248	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013
JX104238	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013
JX104250	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013
JX104244	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013
JX104252	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013
JX104236	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013
JX104254	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013
JX104239	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013

JX104249	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013
JX104246	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013
JX104232	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013
JX104247	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013
JX104251	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013
JX104235	*Deladenus canii*	USA, NY, Newcomb	Morris et al., 2013
JX104233	*Deladenus canii*	USA, NY, Oswego	Morris et al., 2013
MF179769	*Deladenus siricidicola*	Spain, Galicia	
MF179780	*Deladenus siricidicola*	Spain, Galicia	
MF179768	*Deladenus siricidicola*	Spain, Galicia	
MF179767	*Deladenus siricidicola*	Spain, Galicia	
MF179766	*Deladenus siricidicola*	Spain, Galicia	
MF179765	*Deladenus siricidicola*	Spain, Galicia	
MF179763	*Deladenus siricidicola*	Spain, Galicia	
MF179762	*Deladenus siricidicola*	Spain, Galicia	
MF179761	*Deladenus siricidicola*	Spain, Galicia	
MF179759	*Deladenus siricidicola*	Spain, Galicia	
MF179760	*Deladenus siricidicola*	Spain, Galicia	
MF179764	*Deladenus siricidicola*	Spain, Galicia	
MF179742	*Deladenus siricidicola*	South Africa, Southern Cape	
MF179778	*Deladenus siricidicola*	South Africa, KwaZulu Natal	
MF179735	*Deladenus siricidicola*	South Africa, Southern Cape	
MF179734	*Deladenus siricidicola*	South Africa, KwaZulu Natal	
MF179772	*Deladenus siricidicola*	South Africa, Eastern Cape	
MF179733	*Deladenus siricidicola*	South Africa, Mpumalanga	
MF179732	*Deladenus siricidicola*	Argentina	
MF179731	*Deladenus siricidicola*	New Zealand, North Island	
MF179729	*Deladenus siricidicola*	South Africa, Mpumalanga	
MF179730	*Deladenus siricidicola*	South Africa, Mpumalanga	
MF179744	*Deladenus siricidicola*	South Africa, Southern Cape	

JX104275	*Deladenus siricidicola*	USA, NY, Manlius	Morris et al., 2013
MF179747	*Deladenus siricidicola*	South Africa, Eastern Cape	
MF179782	*Deladenus siricidicola*	Brazil	
JX104259	*Deladenus siricidicola*	USA, PA, Tioga	Morris et al., 2013
MF179781	*Deladenus siricidicola*	Chile, Las Trancas	
MF179777	*Deladenus siricidicola*	South Africa, KwaZulu Natal	
MF179776	*Deladenus siricidicola*	South Africa, KwaZulu Natal	
MF179775	*Deladenus siricidicola*	South Africa, Southern Cape	
MF179774	*Deladenus siricidicola*	South Africa, Mpumalanga	
MF179773	*Deladenus siricidicola*	South Africa, Mpumalanga	
MF179746	*Deladenus siricidicola*	New Zealand, North Island	
MF179745	*Deladenus siricidicola*	South Africa, Western Cape	
MF179743	*Deladenus siricidicola*	South Africa, KwaZulu Natal	
MF179741	*Deladenus siricidicola*	South Africa, Mpumalanga	
MF179738	*Deladenus siricidicola*	South Africa, Mpumalanga	
MF179736	*Deladenus siricidicola*	South Africa, KwaZulu Natal	
MF179737	*Deladenus siricidicola*	South Africa, Mpumalanga	
JX104281	*Deladenus siricidicola*	USA, NY, Huron	Morris et al., 2013
MF179740	*Deladenus siricidicola*	Chile, Santa Ines	
MF179739	*Deladenus siricidicola*	Chile, Los Nogales	
MF179748	*Deladenus siricidicola*	Canada, Ontario	
MF179749	*Deladenus siricidicola*	USA, PA	
MF179750	*Deladenus siricidicola*	USA, VT	
MF179751	*Deladenus siricidicola*	USA, PA	
MF179752	*Deladenus siricidicola*	Canada, Ontario	
MF179784	*Deladenus siricidicola*	Canada, Ontario	
MF179783	*Deladenus siricidicola*	Canada, Ontario	
MF179779	*Deladenus siricidicola*	Canada, Ontario	
JX104234	*Deladenus siricidicola*	USA, NY, Oswego	Morris et al., 2013
JX104255	*Deladenus siricidicola*	USA, NY, Oswego	Morris et al., 2013

JX104256	*Deladenus siricidicola*	USA, NY, Oswego	Morris et al., 2013
JX104257	*Deladenus siricidicola*	USA, NY, Oswego	Morris et al., 2013
JX104258	*Deladenus siricidicola*	USA, NY, Oswego	Morris et al., 2013
JX104260	*Deladenus siricidicola*	USA, NY, Onondaga	Morris et al., 2013
JX104261	*Deladenus siricidicola*	USA, NY, Onondaga	Morris et al., 2013
JX104262	*Deladenus siricidicola*	USA, NY, Onondaga	Morris et al., 2013
JX104263	*Deladenus siricidicola*	USA, NY, Onondaga	Morris et al., 2013
JX104264	*Deladenus siricidicola*	USA, NY, Onondaga	Morris et al., 2013
JX104265	*Deladenus siricidicola*	USA, NY, Oswego	Morris et al., 2013
JX104276	*Deladenus siricidicola*	USA, NY, Manlius	Morris et al., 2013
JX104280	*Deladenus siricidicola*	USA, NY, Huron	Morris et al., 2013
JX104282	*Deladenus siricidicola*	USA, NY, Triangle	Morris et al., 2013
JX104283	*Deladenus siricidicola*	USA, NY, Huron	Morris et al., 2013
JX104284	*Deladenus siricidicola*	USA, NY, Oswego	
MF179771	*Deladenus siricidicola*	USA, PA	
MF179770	*Deladenus siricidicola*	USA, PA	
MF179758	*Deladenus siricidicola*	Canada, Ontario	
MF179757	*Deladenus siricidicola*	Canada, Ontario	
MF179756	*Deladenus siricidicola*	Canada, Ontario	
MF179755	*Deladenus siricidicola*	Canada, Ontario	
MF179753	*Deladenus siricidicola*	USA, PA	
MF179754	*Deladenus siricidicola*	USA, PA	
AY589466	*Howardula aoronymphium*	USA, NY, Rochester	Morris et al., 2013

Chapter 3

Experimental admixture among distinct lineages of *Deladenus siricidicola*, the biocontrol agent of *Sirex noctilio*

Katrin N.E. Fitza[a], Jeff Garnas[b,c], Bernard Slippers[a]

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

[b] Department of Zoology and Entomology, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria 0002, South Africa

[c] Department of Natural Resources and the Environment, University of New Hampshire, New Hampshire, USA

Abstract

Deladenus siricidicola is a principal biological control agent used to suppress populations of the globally invasive pine pest, the woodwasp *Sirex noctilio*. Previous studies have reported low genetic diversity in *D. siricidicola* populations in biological control programs in the Southern Hemisphere (Southern Hemisphere lineage) and identified two additional, distinct lineages in North America and Spain. In this study, we tested the ability of these three lineages to interbreed and produce viable offspring. We used microsatellite markers to confirm admixture in offspring. Secondly, we examined whether the resulting admixed offspring in one of these crosses varied with respect to its growth rate on different isolates of the *Amylostereum areolatum* fungus, compared to parental nematode strains. The experimental evidence shows that all the lineages were able to interbreed, with the genotypic profile of the offspring generally skewed towards one of the parents. The offspring from the one cross showed significant variation in growth rate on different isolates of *A. areolatum*, compared to the parental strains. The results of this study have relevance for the introduction of more genetic diversity into biological control programs, but also inform expectations of accidental introductions of *D. siricidicola* into new regions.

Keywords: Hymenoptera, genetic diversity, nematoda, invasive pest, population genetics

3.1 Introduction

Biological control is an important tool for the management of invasive pest insects (Heimpel and Mills, 2017). Given the increasingly global distribution of many pests of agriculture and forestry (Wingfield et al., 2008), biological control agents are frequently applied among regions or even continents as a management tool. Biological control populations can spread “naturally” via co-introduction with hosts, or be purposely introduced through sharing of laboratory reared or field collected strains (Garnas et al., 2012).

Classical biological control agents are often collected from a narrow geographic range, in some cases from a single site or region within their native distribution, which stands in contrast to the various conditions they will likely encounter in their adventive range (Roderick and Navajas, 2003). Additionally, best practices before releasing candidate agents dictate rearing for at least a few generations to ensure colony purity (e.g. absence of hyperparasitoids) and to perform appropriate host specificity and other impact testing (Bush, 2018; Van Driesche and Hoddle, 2009). Therefore, during collection and rearing, biological control agent populations very likely undergo population bottlenecks, drift and inbreeding, as well as selection to laboratory conditions (Yek and Slippers, 2014). As a result, biological control agent populations are often likely to have reduced additive genetic variation, which can (but decidedly does not always, Garcia-Rossi et al., 2003; Garnas et al., 2016; Hufbauer, 2001; Mills, 2017; Rasmussen et al., 2018; Salt and van den Bosch, 1967) result in reduced adaptive potential (Roderick and Navajas, 2003). A lack of adaptive potential of the biological control agent could in turn limit fitness in novel environments or response to variable host phenology of defences, among myriad other selective pressures (Fowler et al., 2015).

Numerous authors have highlighted genetic diversity in introduced natural enemy populations as a potential threat to biological control programs (Hufbauer, 2002; Phillips et al., 2008; Tomasetto et al., 2017, 2018). Until recently, there was scant evidence for evolution of resistance to macroparasites (which develop but do not multiply within infected hosts) in biocontrol systems (Garcia-Rossi et al., 2003; Hufbauer, 2001; Mills, 2017). However, it has been documented that populations of the Argentine ryegrass stem weevil (*Listronotus bonariensis*) have evolved resistance

to the South American braconid parasitoid, *Microctonus hyperodae*, which was used as a biocontrol agent (Tomasetto et al., 2017, 2018). The authors have speculated that reduced genetic diversity in biocontrol agent populations limit its responses to evolved resistance observed in the weevil pest, though definitive tests are lacking. In another example, the application of a static parasite population (endospore-forming bacterium, *Pasteuria penetrans*) over four generations showed a decreased attachment ability against its host, the root-knot nematode *Meloidogyne javanica*, as a results of host resistance development (Tzortzakakis et al., 1996).

Where a lack of additive genetic variation is found to limit adaptive potential in biocontrol populations, a number of potential mitigation strategies exist. Proposed strategies are collection across multiple biological control agent populations from a broader geographic range, multiple introductions, and maintenance of large or multiple populations in quarantine, though costs can be prohibitive (Yek and Slippers, 2014). Intentional genetic admixture (direct cross-breeding among multiple rearing lines) has also gained attention in the recent years as a method to introduce diversity in biological control programs (Myers and Cory, 2017; Rasmussen et al., 2018; Reed and Frankham, 2003; Reed et al., 2003; Rius and Darling, 2014). Genetic admixture results in gene flow between isolated lineages resulting in interlineage hybrid offspring (Benvenuto et al., 2012a; Rius and Darling, 2014). Interspecific hybrid individuals often show elevated fitness over parental sources at least in the short-term, via “hybrid advantage”, or heterosis (Crow, 1948; Lippman and Zamir, 2007). In principle, similar benefits could be conferred to admixed individuals of the same species, particularly where source populations represent evolutionarily distinct or isolated lineages.

The long-term positive (or negative) effects of admixture on adaptive potential via increased additive genetic diversity or through the creation of novel phenotypes are largely unknown (Abbott et al., 2013; Barton, 2001). A few examples are emerging, such as the improved establishment success (via enhanced reproduction, hunger and cold tolerance) of the biocontrol agent, *Cryptolaemus montrouzieri*, achieved through experimental admixture of two laboratory maintained populations (Li et al., 2018). In another biocontrol system, admixture between the invasive (North American) population of *Harmonia axyridis* and a local biocontrol strain used in Europe appears to have improved life-history traits (i.e., larval survival, fecundity and age at first hatching), possibly facilitating the insect’s subsequent invasion in Europe (Turgeon et

al., 2011). Positive effects of admixture, however, can either be short-lived (Benvenuto et al., 2012b; Dlugosch et al., 2015; Lynch, 1991) or negative, resulting in local maladaptation (Burke and Arnold, 2001; Edmands, 2007; Johansen‐Morris and Latta, 2006).

An important forestry biological control system is the entomoparasitic nematode, *Deladenus siricidicola* Bedding (Tylenchida: Neotylenchidae), deployed in annual augmentative release to control the European woodwasp (Hymenoptera: Siricidae), *Sirex noctilio* Fabricius in many parts of Southern Hemisphere (Hurley et al., 2008; Slippers et al., 2015). *Deladenus siricidicola* was first identified in New Zealand in 1962 apparently having been co-introduced with *S. noctilio* (Zondag, 1962, 1969). During the nematode's mycetophagous phase it feeds and reproduces asexually on the basidiomycetous mycangial fungus (*Amylostereum areolatum),* inoculated into trees by ovipositing wasps. Upon encountering wasp larvae, the juvenile nematodes develop into a parasitic form which eventually enters the ovaries of the female or testes of the male wasp, sterilizing the female (Bedding, 1972, 1974; Zondag, 1969). The mycetophagous form of *D. siricidicola* is ideal for mass rearing on fungal cultures, though source populations must periodically be re-collected from the field (after having passed through a wasp host) in order to maintain virulence (Slippers et al., 2012). Nematodes reared too long on fungal cultures alone experience strong selection for rapid growth in laboratory rearing conditions and can lose the ability to convert to the parasitic form upon encountering a wasp host (Bedding and Iede, 2005).

Deladenus siricidicola is thought to be native to Eurasia (Bedding and Akhurst, 1978; Spradbery and Kirk, 1978), but today is distributed globally, having been moved both intentionally for biological control and accidentally with *S. noctilio* introductions. In countries such as Australia, Chile, Brazil, Argentina and South Africa, a specific strain of *D. siricidicola*, known as the Kamona strain, forms the cornerstone of biological control program of *S. noctilio* (Hurley et al., 2007). The Kamona strain was obtained from Tasmania, the region where the Sopron strain from Hungary had previously been released (Bedding and Iede, 2005; Nahrung, 2017). Studies on biological control strains of the nematode have shown that the biological control agent has limited diversity (Mlonyeni et al*.,* 2011; Fitza et al., 2019). The restricted range of the nematodes original collection and subsequent recollection, as well as the occurrence

of bottlenecks and inbreeding, which are expected during mass rearing, could explain the limited genetic diversity of the biological control strain (Mlonyeni et al., 2011).

A recent study on the diversity of *D. siricidicola* identified three distinct lineages amongst 57 globally-collected nematode strains (Fitza et al., 2019). These lineages broadly conform to the accidental introduction of nematode populations in North America (lineage A), to biological control populations in the Southern Hemisphere (lineage B), and a population from the native range in Spain (lineage C). Additionally, Chilean samples appear to comprise of an admixed population between lineage A and an accidentally introduced lineage B (Fitza et al., 2019). Beéche et al. (2012) reported the introduction of two nematode strains, Encruziliada do Sul from Brazil and one strain from Tangoio, New Zealand, but to which lineage they belonged was not known at that stage. This latter discovery laid the foundation for the augmentation of diversity of *D. siricidicola* populations using inter-strain admixture since lineage interbreeding was possible and resulting progeny did not exhibit observable fitness disadvantage. The readily accessible admixed individuals in Chile suggest that fitness consequences of mixing were at least largely neutral, if not beneficial. The question remained, however, whether all these nematode strains from evolutionary distinct lineages would readily interbreed and if so, how such admixture would influence fitness.

Variable levels of parasitism of *D. siricidicola* around the Southern Hemisphere have raised the question about its adaptive capacity and/or phenotypic plasticity (Hurley et al., 2007; Slippers and Wingfield, 2012; Villacide and Corley, 2008). To be successful, *D. siricidicola* populations must perform well under various environmental conditions including climate (e.g., in the summer and winter rainfall regions of South Africa), tree species, fungal isolates, and wasp hosts. Boissin et al. (2012) showed that the invasive populations of *S. noctilio* are highly variable and genetically distinct from each other across their invasive range and that global movement is complex. Variation in *A. areolatum* is also likely to be important – recent studies have shown significant variation in the growth of nematodes on different isolates of the fungus (Mlonyeni et al., 2018b; Morris et al., 2012, 2014).

The aim of this study was to investigate the ability of the different lineages of *D. siricidicola,* identified by Fitza et al. (2019), to interbreed in culture. We considered whether such admixture would impact reproductive rate on different isolates of *A.*

areolatum, using two nematode strains shown to vary in this trait (Mlonyeni, 2018). We use previously designed microsatellite markers (Fitza et al., 2019; Mlonyeni et al., 2011) to type both parental and putatively admixed lineages.

3.2 Materials and methods

3.2.1 Nematode strains

Strains of *D. siricidicola* used in this study have been established from a single female *S. noctilio* for each strain from the regions studied. All strains are maintained at the Biocontrol Centre of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria (Table 1). Two strains from each of the three identified lineages in Fitza et al. (2019) (lineage A, B and C) and two Chilean strains from an admixed population were selected for this study. The strains have been maintained on *A. areolatum* (CMW40871) using malt extract agar (MEA) in a 22°C incubator.

3.2.2 Fungal isolates

Four fungal isolates were selected based on data from a previous study (Mlonyeni, 2018; Table 1). Two isolates were collected in South Africa (CMW46043, CMW47563), one from the USA (CMW40703) and one from Australia (CMW40871). The two South African isolates represent one genotype and the Australian and USA isolates each are different genotypes, based on 11 microsatellite markers (Mlonyeni et al., 2018b). The Australian isolate is generally used for rearing in the *Sirex* biological control program in South Africa. Isolates differ in mean growth rates on PDA at 23°C, where the South African isolate (CMW46043) has the slowest growth rate and the USA isolate the fastest growth rate (Mlonyeni, 2018). These isolates have been maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria.

3.2.3 Crosses between *D. siricidicola* lineages in culture

Six crosses were made between the three *D. siricidicola* lineages (lineage A, B, C; Fitza et al. 2019) and two Chilean admixed strains (Fig. S1, Table 3). Cultures of the individual nematode strains were grown on 90 mm diameter $^1/_2$ potato dextrose agar Petri dishes (PDA, Difco^{TM} Potato Dextrose Agar, LOT 2347578) (19.5 g/L potato dextrose extract, 17.5 g/L agar) on the CMW40781 fungal isolate. Petri dishes were left for 25 days, after which eggs from the F_0 generation were collected. To remove nematodes and eggs from agar plates, Petri dishes were flooded with 2 ml sterile water and decanted into a small excavated glass block. After gently swirling the water, the eggs were separated from the nematodes by sedimentation. After swirling, 10 µl of the sediment with eggs for each strain was pipetted with a Gilson pipette, and all eggs were counted using a counting slide with a 3 mm^2 grid under a dissecting microscope. Nematode concentration (individuals ml^{-1}) was estimated as the average of three counts of 10 ul of well-mixed nematode-water suspension and a volume containing approximately 500 eggs was collected. For each cross, approximately 500 eggs per strain from two parental strains were put together on one spot of the plate. Plugs (5 mm diameter) of *A. areolatum* CMW40871 were placed on the agar surface, approximately 70 mm from the nematode eggs. Cultures were left to develop for 20 days after which the eggs representing the F_1 generation were harvested and transferred onto a new Petri dish. An additional cross (2xUSA1/RSA450; Table 3) was carried out with approximately 1000 eggs from USA1 with approximately 500 eggs of RSA450 to investigate if doubling the number of eggs of USA1 would increase the presence of the parental genotype in the offspring.

3.2.4 Molecular characterization of interbreeding between lineages

For each cross, single nematodes were picked up using a micro-dissection needle from the F_1 generation for DNA extraction. The DNA extraction protocol used by Barstead et al. (1991) and Williams et al. (1992) was adapted for this study. A single nematode was placed in 15 µl of lysis buffer (5 µg proteinase K, 1 µl of 10x PCR buffer and 9 µl of SABAX). The sample was briefly spun down at 2000 rpm for 2 min using a centrifuge (Eppendorf Centrifuge 5417C) and then frozen at -80°C for 10 min. After

freezing, the sample was placed into the PCR machine for proteinase K activation at 65°C for 60 min and deactivation at 95°C for 15 min.

Fragment analysis from microsatellite markers was used to determine potential admixed offspring. The microsatellite genotype profile of each parental strain was known from previous work (Fitza et al., 2019) and all were known to be polymorphic across populations. As single nematode DNA was limited, only one PCR could be done for individual collected offspring. For each cross between 31-224 individual nematodes were screened, using two microsatellite markers for each cross and dividing the collected individual nematodes roughly equally between the two markers (Table 4; Figure S1A). that were homozygous for different alleles in each parental strain, such that any heterozygosity could be uniquely interpreted as admixture.

PCR reactions were conducted in a total volume of 21.5 µl made up with 5 µl 5x MyTaq™ buffer (Bioline USA, Taunton, Massachusetts), 5 µM of each primer, 2.5 units of MyTaq™ DNA Polymerase (Bioline USA, Taunton, Massachusetts) and 15 µl of the DNA template. PCR cycling conditions were a 4 min initial denaturation step at 95°C, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 1 min, and a final extension of 72°C for 45 min, followed by a cooling step of 4°C for 10 min. The products were then visualized on 2% (w/v) agarose gels using Gel Doc™ EZ Imager (Bio-Rad, USA). PCR products were diluted to a ratio 1:100 using SABAX water. One µl of this diluted DNA was added to 10 µl of HiDi™ Formamide and 0.2 µl GeneScan-500 Liz size standard (Applied Biosystems). These samples were run on the ABI PRISM™ 3500xl DNA Analyser (Applied Biosystems, USA) to determine product size (DNA sequencing facility, University of Pretoria). The software GeneMapper® v4.1 (Life Technologies, Foster City, CA) was applied on the GeneScan data to score allele fragment sizes.

3.2.5 Nematode productivity assay

To assess the consequence of admixture on one component of fitness, population growth assays were performed with one admixed *D. siricidicola* strain (USA6/CHL1) and its two parental strains (USA6, CHL1; Fig. 1). Of course, even the admixed strain contained the offspring from within-lineage mating, as genotyping individuals is

destructive and could not be performed. The USA6/CHL1 was chosen because the proportion of admixed offspring was the highest among all of our experimental crosses (56%), as expected under random mating.

Nematode growth rate assays were conducted on 19.5 g/L PDA in 90 mm diameter Petri dishes using the four isolates of *A. areolatum* (Table 1). A five mm diameter plug was transferred from the growing edge of an *A. areolatum* culture to the edge of a new Petri dish and left to grow for five days at 23°C. Eggs from the two parental strains, reared on the CMW40871 fungal isolate were collected as described above. Approximately 1000 eggs per parental strain were placed together on different plates with the four fungal isolates. After 20 days F_1 eggs were collected and approximately 300 eggs were placed on a new plate with the respective fungal isolate. For the parental control 300 eggs of each parental strain were placed separately on the four fungal isolates. A total of 20 Petri dishes (5 replicates for each of the four *A. areolatum* isolates) were incubated at 23°C in the dark for 25 days. Subsequently, the nematodes were harvested by washing with 2 ml sterile water and decanting into the glass excavator block. A second wash was done to ensure the collection of all nematodes. Nematodes were allowed to settle and water was removed. One ml sterile water was added to the sediment and mixed well, after which a 100 µl subsample was taken and all nematodes were counted using a counting slide with a 3 mm^2 grid under a dissection microscope. The whole trial was then repeated to ensure consistency of results. To confirm the success of interbreeding, 40 single nematodes were screened using a microsatellite marker that was homozygous but different between the parental strains (Figure S1B).

3.2.6 Statistical analysis

Analysis of Variance (ANOVA) comparing the interaction between the independent variables (fungal isolate and nematode strain) on the dependent variable (growth rate), was performed. The data were square-root transformed to conform to the assumption of constant variance and normality of the ANOVA test. Tukey's Honestly Significant Difference (HSD) post-hoc tests were performed to compare pairwise treatment

combinations. These analyses were run using the statistical programme JMP® Statistical Software (SAS, USA).

3.3 Results

3.3.1 Characterization of crosses of *D. siricidicola* lineages in culture

All seven *D. siricidicola* lineage crosses produced viable offspring, and all contained at least some heterozygous individuals (indicating admixture), with the proportion ranging from 3-56% (unshaded rows, Table 3). Heterozygotes were rarer than expected by chance (2.5%-28.8%) in five of these seven crosses. Both crosses using CHL1 (with USA6 and ESP313) produced an expected proportion of heterozygotes (55.7% and 43.3%, respectively). One of two subsequently repeated crosses USA6/CHL1 likewise yielded the expected proportion of heterozygotes (48.6%; Table 3, shaded rows), with the other still yielding more heterozygotes than any other cross (36.1%). Most crosses resulted in significant asymmetry in the representation of parental strain among the homozygous (non-admixed) nematodes. With the exception of USA6/CHL1 cross, all showed over-representation of one parent ranging from 42.1% to 96.4%, with the lowest percentage coming from the CHL1/ESP313 cross (which yielded the expected number of heterozygotes).

3.3.2 Nematode productivity assay

Looking first at pooled results across the two experimental iterations performed (Fig. 1a), a two-way ANOVA model including fungal isolate, nematode strain, and their interaction accounted for over 52% of variation in square root-transformed nematode abundance after 25 days of incubation ($F=10.8$; $df=11,107$, $P<0.0001$; $R^2=0.522$ [adjusted-$R^2= 0.476$]). Both main effects were highly significant (fungal isolate: $F=14.6$; $df=3,107$; $P<0.0001$; nematode strain: $F=29.9$; $df=2,107$; $P<0.0001$) and the nematode x fungal interaction was moderately significant ($F=2.2$; $df=6,107$; $P=0.0461$). The USA6/CHL1 admixed strain showed a significantly higher growth rate on all fungal isolates (Tukey's HSD post-hoc test on the nematode strain main effect, alpha=0.05). When all pairwise comparisons between treatment levels were considered (Fig. 1a)

the growth rate was only statistically significant on the CMW46043 (RSA) fungus, driving the interaction effect. This admixed strain had an average abundance that was 256% (±51% SD) and 259% (±87% SD) greater than its parental Chilean and US strain, respectively (maximum of 386% as abundance). Abundance did not differ significantly among the two parental strains overall or on specific fungal isolates.

When included as a blocking factor, experimental iteration resulted in a stronger model overall based on AIC ($F=8.8$; $df=23,95$; $P<.0001$; $R^2=0.68$ [adjusted-$R^2=0.603$]); Experimental iteration was significant as a main effect and in all its two and three-way interactions, with the exception of the two-way interaction with fungal isolates. Figure 2b shows all pairwise treatment comparisons based on Tukey's HSD. While current sample size and the number of comparisons under consideration made it difficult to discriminate among treatment levels, overall there was a high degree of qualitative agreement among trials. Nematode abundance was higher in Trial 1 relative to Trial 2 ($F=14.3$; $df=1,95$; $P=0.003$). In both iterations, the USA6/CHL1 cross performed best, particularly when grown on the CMW46043 (RSA) fungus, though the performance of this admixed strain was more strongly differentiated in Trial 1.

3.4 Discussion

In this study, *D. siricidicola* strains representing three distinct lineages (from the US, South Africa, and Spain) plus two Chilean strains, which appear to be admixed between North America and the Southern Hemisphere lineage, were shown to be capable of interbreeding. SSR genotyping of F_1 offspring of multiple individuals, however, revealed substantial asymmetries in the proportion of both parental strains and hybrid strains resulting from these crosses. The frequency of heterozygotes (admixed individuals) present in the F_1 generation varied from 3% to 56% in the different crosses. The absence of one parental (homozygous) genotype in sampled offspring in 4 of 9 crosses was surprising – in all but one cross (one of the three USA6/CHL1 crosses) there was a significant over-/under-representation of one of the parental strains in the resulting offspring. In two-thirds of the crosses (6 of 9), there were significantly fewer hybrid individuals than would be predicted under Hardy-Weinberg equilibrium. Variation in the cross-breeding results can be expected as

some genotypes may be more cross-compatible than others (Akhurst, 1975). Differences could also be attributed to the fungal isolates preference or reproductive rate differences between the different strains. Caetano et al., (2016), Morris et al., (2012) and Mlonyeni et al., (2018a) all indicated that the reproductive performance of the nematode strain was depended on the fungal isolate on which the nematode strain was grown.

All of the crosses where the proportion of hybrids conformed to random expectations included CHL1 as one of the parental strains. This is particularly interesting given that CHL1 is hypothesized to itself have undergone one or more admixture events (Fitza et al., 2019). This suggests the possibility that the barriers to admixture are lower in this strain, though whether this is likely to be a cause or a consequence of admixture in Chilean populations is unknown. Examples in literature of barriers to admixture can be grouped into pre- (e.g. gametic isolation, mechanical isolation, sexual isolation) and post-zygotic (inviability, sterility, breakdown) reproductive isolation (Coyne and Orr, 2004). For example, in the cereal rust mite *Abacarus hystrix* intraspecific hybrid inviability in crosses seems to be host species dependent. If crosses were done on the host where the males developed on, no progeny was found. Crosses conducted on hosts on which females developed, only male progeny was obtained. One possible mechanism could be incapability of the mite sperm from the one host to fertilize mite eggs from the other host (Skoracka, 2008). A postzygotic barrier was observed during interbreeding of strains of the nematode *Haemonchus contortus* from different geographic regions, as there were less than the mendelian expectation of L1 offspring, even though egg genotyping indicated no inter-strain hybrid genotype deficit (Sargison et al., 2019).

Reproductive fitness of the resulting strains varied both by nematode strain and the fungal isolate on which they were grown. Additionally, clear, though comparatively weaker, nematode x fungal isolate interaction effects were observed. Interestingly, in both trials, the hybrid USA6/CHL1 strain grew better than either parental strain, suggesting some form of hybrid advantage. This advantage was to some degree context dependent, as the differences in reproductive output were mostly on one fungal isolate (CMW46043, RSA). CMW 46043 is a fungal isolate obtained in 2013 and identified by a previous study to be the slowest growing isolate of the four fungal isolates tested (Mlonyeni, 2018). All nematodes and fungi for this experiment were

collected from wasps that were obtained from plantation or naturally-growing trees, so none have lost its ability to persist under field conditions. Still, why this strain of fungus was particularly good for nematode growth, and disproportionately good for the admixed strain, is not clear.

Development of a method to conduct crosses with various nematode strains, as well as the modification of a single nematode DNA extraction protocol, allowed for the screening of offspring using microsatellite markers to indicate admixture. These cross-breeding experiments showed clear interbreeding potential of all three lineages. Recent evidence has shown that lineages interbreeding in *D. siricidicola* also occurs in nature, as a Chilean population appear to represent admixture between nematodes of North American and Southern Hemisphere origins (Fitza et al. 2019). Akhurst (1975) likewise showed that intraspecific crosses of *D. siricidicola* produced viable eggs with a hatching success rate of 75%, however the genotypes of the parental *D. siricidicola* cultures used were not known. Akhurst (1975) further reported that egg deposition and hatching success varied substantially among interspecific crosses of *Deladenus* spp. These studies suggest a high likelihood of interbreeding between the different lineages.

Interbreeding capabilities of various nematode strains has implications for the introduction of the biological control nematode strain. Admixture has the potential advantage of adaptive enrichment, which can result in increased hybrid fitness due to selection of favourable phenotypes (Facon et al., 2008; Szűcs et al., 2012, 2019). However, studies of the long-term impact of admixture on population fitness are lacking, and where they exist, results are equivocal (Garnas 2016, 2018). In many cases, short-term hybrid advantage (heterosis) is a phenomenon that disappears with further generations and with back-crossing (Johansen‐Morris and Latta, 2006; Lippman and Zamir, 2007).

The opportunity to use controlled lineage crossing to improve aspects of biological control efficacy is rooted in following two fundamental ideas: first, that increasing additive genetic diversity is generally positive for populations (Hahn and Rieseberg, 2017; Lommen et al., 2017); and second, that desirable traits could be intentionally imported into otherwise well-adapted and established populations (Lirakis and Magalhães, 2019; Shi et al., 2018). On the first point, admixture clearly has the

potential to increase additive genetic diversity in otherwise low-diversity, often inbred strains, assuming that survivorship of hybrid offspring is equivalent to or greater than parental stains. Whether such diversity would result in novel desirable traits or trait combinations that could be selected for (either in the laboratory or in the field), is largely unknown. However, many native and invasive populations are highly tolerant to inbreeding and sustain low levels of diversity (Frankham, 2005; Roman and Darling, 2007; Simberloff, 2009). In fact, it has been challenging to demonstrate a strong positive correlation between additive genetic diversity and population fitness (Estoup et al., 2016; Johansen‐Morris and Latta, 2006; Rius and Darling, 2014).

The potential to intentionally import desirable traits into local populations via admixture is an intriguing possibility. This approach requires considerably more knowledge of strain fitness values and/or traits that are variable across populations, and also assumes some degree of local adaptation (otherwise strain replacement would be preferred). Additionally, desirable traits could be distributed among lineages such that admixture could combine such traits. In the *D. siricidicola* system, traits such as conversion efficiency (from the mycetophagous to the parasitic form) are important (Mlonyeni et al., 2018a), as well as more obvious traits such as population growth rate or tolerance to different environmental conditions. The ability to sterilize *S. noctilio* by entering eggs (once in the ovary) also varies in among *D. siricidicola* populations, with clear negative consequences for the control of wasp populations (Kroll et al., 2013; Yu et al., 2009).

The capability of the USA strain from our study to interbreed with strains from other parts of the world raises possibilities to breed for the sterilizing ability in the USA population. Alternatively, this trait could also be exported from USA populations given the unintentional movement among global populations of this nematode and the propensity to hybridize (Fitza et al., 2019). While non-sterilization of the wasp host is highly undesirable from a biocontrol perspective, this trait may enhance nematode fitness considerably, since female wasps still act as vectors and the lack of sterilization would mean more wasps in the tree serving as host for offspring. If this trait were to escape the USA population, it therefore has the potential to spread quickly.

Admixture among independently evolving lineages can also break up co-evolved allele combinations resulting in negative fitness consequences. In a biocontrol context, this

could manifest as a reduction in the infectivity rate of the Kamona strain or dilution of desirable characteristics (Hajek and Morris, 2014; Williams and Hajek, 2017; Williams et al., 2012). Interbreeding as a strategy to improve control efficacy, therefore, needs to be carefully considered for future development of biological control programs in the USA and elsewhere.

The USA6/CHL1 hybrid offspring showed a higher reproductive rate compared to the parental nematode strains, indicating the potential to increase fitness through admixture, at least in the short term. This likely represents hybrid vigour (heterosis), often attributed to elevated heterozygosity at loci were deleterious, often recessive alleles were present. The Chilean populations had higher genetic diversity compared to other Southern Hemisphere populations, perhaps as consequence of an earlier admixture event (Fitza et al., 2019), but did not perform better than the USA6, non-admixed parent.

3.5 Conclusion

Biological control programs rely on effective parasitism, successful establishment and spread, high fecundity and efficient mass rearing capabilities of the control agent. The current study demonstrated the interbreeding potential amongst various lineages of *D. siricidicola.* This provides a means to introduce more genetic diversity to the *Sirex* biological control programs, and potentially the opportunity to introduce and subsequently select for desirable traits or trait combinations. Increased genetic diversity could be especially valuable in the Southern Hemisphere, where previous studies have identified high homozygosity in the nematode population (in contrast to comparatively high diversity in the *S. noctilio* populations). However, high variability and systematic asymmetries in the success and fitness consequences of admixture demonstrate that careful study is warranted prior to its implementation as a biocontrol enhancement strategy.

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3.8 Tables and Figures

Table 1. Isolate codes**,** country of origin, date of collection and CMW accession numbers (fungus only) for *D. siricidicola* and *A. areolatum* used in this study. Each strain was established as a culture from a single, field-collected *S. noctilio* female.

Isolate code	Organism	Origin	Collection date	Accession #
RSA410	*D. siricidicola*	South Africa, Mpumalanga	2013	
RSA450	*D. siricidicola*	South Africa, Mpumalanga	2013	
CHL1	*D. siricidicola*	Chile, Valparaíso	2013	
CHL3	*D. siricidicola*	Chile, BioBio	2013	
USA1	*D. siricidicola*	USA, Pennsylvania	2013	
USA6	*D. siricidicola*	USA, Pennsylvania	2013	
ESP264	*D. siricidicola*	Spain, Galicia	2014	
ESP313	*D. siricidicola*	Spain, Galicia	2014	
SA2013	*A. areolatum*	South Africa, KwaZulu Natal	2013	CMW46043
SA2012	*A. areolatum*	South Africa, KwaZulu Natal	2012	CMW47563
USA	*A. areolatum*	USA, Pennsylvania	2014	CMW40703
AUS	*A. areolatum*	Australia	2003	CMW40871

Table 2. The allele scores for the F1 offspring for each cross. The hybrids are highlighted in grey. The parental allele sizes are given as a reference.

Cross		#nematode	Ds105		#nematode	Ds105		#nematode	Ds325		#nematode	Ds325	
Parent 1 (p)	**Parent 2 (q)**												
ESP264	**RSA410**	***ESP264***	169	169	***8***	151	169	***ESP264***	308	308	***24***	304	304
		RSA410	151	151	***9***	151	151	***RSA410***	304	304	***25***	304	304
		1	151	151	***10***	151	151	***17***	304	304	***26***	304	304
		2	151	151	***11***	151	151	***18***	304	308	***27***	304	304
		3	151	151	***12***	151	151	***19***	304	304	***28***	304	304
		4	151	169	***13***	151	151	***20***	304	304	***29***	304	304
		5	151	151	***14***	151	151	***21***	304	304	***30***	304	308
		6	151	151	***15***	151	151	***22***	304	304	***31***	304	304
		7	151	151	***16***	151	169	***23***	304	304			

Cross		#nematode	Ds151		#nematode	Ds151		#nematode	Ds325		#nematode	Ds325	
Parent 1 (p)	**Parent 2 (q)**												
USA1	**RSA450**	***USA1***	159	159	***59***	151	151	***USA1***	306	306	***166***	304	306
		RSA410	151	151	***60***	151	151	***RSA410***	304	304	***167***	304	304
		1	151	151	***61***	151	151	***108***	304	304	***168***	304	304
		2	151	151	***62***	151	151	***109***	304	304	***169***	304	304

3	151	151	***63***	151	151	***110***	304	304	***170***	304	304	
4	151	151	***64***	151	151	***111***	304	304	***171***	304	304	
5	151	151	***65***	151	151	***112***	304	304	***172***	304	304	
6	151	151	***66***	151	151	***113***	304	304	***173***	304	304	
7	151	151	***67***	151	151	***114***	304	306	***174***	304	304	
8	151	151	***68***	151	151	***115***	304	304	***175***	304	304	
9	151	151	***69***	151	151	***116***	304	304	***176***	304	304	
10	151	151	***70***	151	151	***117***	304	304	***177***	304	304	
11	151	151	***71***	151	151	***118***	304	304	***178***	304	304	
12	151	151	***72***	151	151	***119***	304	304	***179***	304	304	
13	151	151	***73***	151	151	***120***	304	304	***180***	304	304	
14	151	151	***74***	151	151	***121***	304	304	***181***	304	304	
15	151	151	***75***	151	151	***122***	304	304	***182***	304	304	
16	151	151	***76***	151	151	***123***	304	304	***183***	304	304	
17	151	151	***77***	151	151	***124***	304	304	***184***	304	304	
18	151	151	***78***	151	151	***125***	304	304	***185***	304	304	
19	151	151	***79***	151	151	***126***	304	304	***186***	304	304	
20	151	151	***80***	151	151	***127***	304	304	***187***	304	304	
21	151	151	***81***	151	151	***128***	304	304	***188***	304	304	
22	151	151	***82***	151	151	***129***	304	304	***189***	304	304	
23	151	151	***83***	151	151	***130***	304	304	***190***	304	304	
24	151	151	***84***	151	151	***131***	304	306	***191***	304	304	
25	151	151	***85***	151	151	***132***	304	304	***192***	304	304	

26	151	151	***86***	151	151	***133***	304	304	***193***	304	304
27	151	151	***87***	151	151	***134***	304	304	***194***	304	304
28	151	151	***88***	151	151	***135***	304	304	***195***	304	304
29	151	151	***89***	151	151	***136***	304	306	***196***	304	304
30	151	151	***90***	151	151	***137***	304	304	***197***	304	304
31	151	151	***91***	151	151	***138***	304	304	***198***	304	304
32	151	151	***92***	151	151	***139***	304	304	***199***	304	304
33	151	151	***93***	151	151	***140***	304	304	***200***	304	304
34	151	151	***94***	151	151	***141***	304	304	***201***	304	304
35	151	151	***95***	151	151	***142***	304	304	***202***	304	304
36	151	151	***96***	151	151	***143***	304	304	***203***	304	304
37	151	151	***97***	151	151	***144***	304	304	***204***	304	304
38	151	151	***98***	151	151	***145***	304	304	***205***	304	304
39	151	151	***99***	151	151	***146***	304	304	***206***	304	304
40	151	151	***100***	151	151	***147***	304	306	***207***	304	304
41	151	151	***101***	151	151	***148***	304	304	***208***	304	304
42	151	151	***102***	151	156	***149***	304	306	***209***	304	304
43	151	151	***103***	151	151	***150***	304	304	***210***	304	304
44	151	151	***104***	151	151	***151***	304	304	***211***	304	304
45	151	151	***105***	151	151	***152***	304	304	***212***	304	304
46	151	151	***106***	151	151	***153***	304	304	***213***	304	304
47	151	151	***107***	151	151	***154***	304	306	***214***	304	304
48	151	151				***155***	304	304	***215***	304	304

		49	151	151		***156***	304	304	***216***	304	304
		50	151	151		***157***	304	304	***217***	304	304
		51	151	151		***158***	304	304	***218***	304	304
		52	151	151		***159***	304	304	***219***	304	304
		53	151	151		***160***	304	304	***220***	304	304
		54	151	151		***161***	304	304	***221***	304	304
		55	151	151		***162***	304	304	***222***	304	304
		56	151	151		***163***	304	304	***223***	304	304
		57	151	151		***164***	304	304	***224***	304	304
		58	151	151		***165***	304	304			

Cross		**#nematode**	**Ds151**		**#nematode**	**Ds151**		**#nematode**	**Ds316**		**#nematode**	**Ds316**	
Parent 1 (p)	**Parent 2 (q)**												
		USA1	159	159	***20***	151	151	***USA1***	391	391	***60***	387	387
		RSA410	151	151	***21***	151	151	***RSA410***	387	387	***61***	387	387
		1	151	151	***22***	151	151	***41***	387	387	***62***	387	387
		2	151	151	***23***	151	151	***42***	387	387	***63***	387	387
USA1*	**RSA450**	***3***	151	151	***24***	151	151	***43***	391	391	***64***	387	387
		4	151	151	***25***	151	151	***44***	387	387	***65***	391	391
		5	151	151	***26***	151	151	***45***	387	387	***66***	387	387
		6	151	151	***27***	159	159	***46***	387	387	***67***	391	391
		7	151	159	***28***	151	151	***47***	387	387	***68***	387	391

		8	151	151	***29***	151	159	***48***	391	391	***69***	387	391		
		9	151	159	***30***	151	151	***49***	391	391	***70***	387	387		
		10	151	159	***31***	151	151	***50***	387	391	***71***	387	387		
		11	151	159	***32***	151	159	***51***	391	391	***72***	387	387		
		12	151	151	***33***	151	151	***52***	387	387	***73***	387	387		
		13	151	159	***34***	151	151	***53***	387	387					
		14	151	151	***35***	151	151	***54***	387	387					
		15	151	159	***36***	151	151	***55***	387	387					
		16	151	151	***37***	151	151	***56***	387	391					
		17	151	151	***38***	151	159	***57***	387	387					
		18	159	159	***39***	151	151	***58***	387	391					
		19	151	151	***40***			***59***	387	387					

Cross		**#nematode**	**Ds151**		**#nematode**	**Ds151**		**#nematode**	**Ds151**		**#nematode**	**Ds151**	
Parent 1 (p)	**Parent 2 (q)**												
USA6	**ESP264**	***ESP264***	169	169	***10***	169	169	***21***	169	169	***32***	169	169
		USA6	159	159	***11***	169	169	***22***	159	159	***33***	169	169
		1	169	169	***12***	169	169	***23***	169	169	***34***	169	169
		2	169	169	***13***	159	169	***24***	169	169	***35***	169	169
		3	169	169	***14***	169	169	***25***	169	169	***36***	169	169
		4	169	169	***15***	169	169	***26***	169	169	***37***	169	169
		5	169	169	***16***	169	169	***27***	169	169	***38***	169	169
		6	169	169	***17***	169	169	***28***	169	169	***39***	169	169

		7	169	169	***18***	169	169	***29***	169	169	***40***	169	169
		8	169	169	***19***	169	169	***30***	169	169			
		9	169	169	***20***	169	169	***31***	169	169			

Cross		#nematode	Ds105		#nematode	Ds1055		#nematode	Ds325		#nematode	Ds325	
Parent 1 (p)	**Parent 2 (q)**												
		CHL1	151	151	***20***	151	159	***CHL1***	304	304	***60***	304	306
		USA6	159	159	***21***	151	159	***USA6***	306	306	***61***	304	306
		1	151	159	***22***	151	159	***41***	304	306	***62***	304	306
		2	151	151	***23***	151	159	***42***	304	306	***63***	304	304
		3	151	151	***24***	151	159	***43***	304	306	***64***	304	304
		4	151	159	***25***	151	159	***44***	304	306	***65***	306	306
		5	151	159	***26***	151	151	***45***	306	306	***66***	304	304
		6	151	159	***27***	151	159	***46***	304	306	***67***	304	304
CHL1	**USA6**	***7***	151	159	***28***	151	159	***47***	306	306	***68***	304	306
		8	151	159	***29***	151	159	***48***	304	304	***69***	304	306
		9	151	159	***30***	151	159	***49***	306	306	***70***	304	306
		10	151	159	***31***	151	159	***50***	304	306	***71***	304	304
		11	151	159	***32***	151	159	***51***	304	306	***72***	306	306
		12	159	159	***33***	151	151	***52***	304	306	***73***	304	306
		13	151	159	***34***	151	151	***53***	304	306	***74***	304	304
		14	159	159	***35***	159	159	***54***	304	304	***75***	306	306
		15	151	151	***36***	151	159	***55***	304	306	***76***	304	304

		16	151	151	***37***	151	159	***56***	306	306	***77***	304	304
		17	159	159	***38***	151	159	***57***	304	304	***78***	304	306
		18	151	159	***39***	151	151	***58***	304	304	***79***	306	306
		19	151	159	***40***	159	159	***59***	304	304			

Cross		#nematode	Ds105		#nematode	Ds151		#nematode	Ds3255		#nematode	Ds325	
Parent 1 (p)	**Parent 2 (q)**												
		CHL1	151	151	***44***	151	163	***CHL1***	304	304	***132***	304	308
		ESP313	163	163	***45***	151	163	***ESP313***	308	308	***133***	304	308
		1	151	163	***46***	163	163	***89***	308	308	***134***	308	308
		2	151	163	***47***	151	163	***90***	304	308	***135***	304	308
		3	151	163	***48***	151	163	***91***	304	308	***136***	304	308
		4	163	163	***49***	163	163	***92***	304	308	***137***	304	304
		5	163	163	***50***	151	163	***93***	308	308	***138***	304	304
CHL1	**ESP313**	***6***	151	163	***51***	163	163	***94***	304	308	***139***	304	304
		7	163	163	***52***	163	163	***95***	308	308	***140***	304	308
		8	163	163	***53***	151	163	***96***	308	308	***141***	304	308
		9	163	163	***54***	163	163	***97***	308	308	***142***	304	304
		10	163	163	***55***	163	163	***98***	304	308	***143***	304	304
		11	151	163	***56***	151	151	***99***	308	308	***144***	304	304
		12	163	163	***57***	151	163	***100***	308	308	***145***	308	308
		13	163	163	***58***	163	163	***101***	304	304	***146***	304	304

14	163	163	***59***	163	163	***102***	304	308	***147***	304	304
15	151	163	***60***	151	163	***103***	308	308	***148***	304	308
16	151	163	***61***	151	163	***104***	308	308	***149***	304	308
17	151	163	***62***	151	163	***105***	304	308	***150***	304	308
18	163	163	***63***	163	163	***106***	304	308	***151***	304	308
19	151	163	***64***	151	151	***107***	308	308	***152***	304	304
20	163	163	***65***	151	163	***108***	308	308	***153***	304	304
21	163	163	***66***	151	163	***109***	308	308	***154***	304	308
22	151	163	***67***	163	163	***110***	304	304	***155***	304	308
23	151	163	***68***	163	163	***111***	308	308	***156***	308	308
24			***69***	151	163	***112***	304	308	***157***	304	308
25	151	163	***70***	163	163	***113***	304	304	***158***	304	308
26	151	163	***71***	151	163	***114***	308	308	***159***	308	308
27	151	163	***72***	163	163	***115***	308	308	***160***	304	308
28	151	163	***73***	163	163	***116***	304	308	***161***	304	304
29	151	163	***74***	163	163	***117***	308	308	***162***	304	304
30	163	163	***75***	151	163	***118***	308	308	***163***	304	308
31	163	163	***76***	151	151	***119***	308	308	***164***	304	304
32	163	163	***77***	151	163	***120***	304	308	***165***	304	308
33	163	163	***78***	163	163	***121***	304	308			
34	151	163	***79***	163	163	***122***	304	308			
35	151	163	***80***	163	163	***123***	304	308			
36	151	163	***81***	151	163	***124***	308	308			
37	163	163	***82***	163	163	***125***	308	308			

		38	151	151	***83***	163	163	***126***	308	308			
		39	151	151	***84***	163	163	***127***	304	308			
		40	151	163	***85***	163	163	***128***	304	308			
		41	151	163	***86***	163	163	129	304	308			
		42	151	163	***87***	163	163	130	304	308			
		43	151	163				131	308	308			

Cross													
Parent 1 (p)	**Parent 2 (q)**	**#nematode**	**Ds316**		**#nematode**	**Ds316**		**#nematode**	**Ds388**		**#nematode**	**Ds388**	
		CHL3	391	391	***20***	387	391	***CHL3***	221	221	***60***	225	225
		RSA410	387	387	***21***	387	391	***RSA410***	225	225	***61***	225	225
		1	387	391	***22***	387	391	***41***	221	225	***62***	225	225
		2	387	391	***23***	387	391	***42***	221	225	***63***	225	225
		3	387	391	***24***	387	391	***43***	225	225	***64***	225	225
		4	387	391	***25***	387	391	***44***	225	225	***65***	225	225
CHL3	**RSA410**	***5***	387	391	***26***	387	391	***45***	225	225	***66***	221	225
		6	387	391	***27***	387	391	***46***	225	225	***67***	225	225
		7	387	391	***28***	387	391	***47***	225	225	***68***	225	225
		8	387	391	***29***	387	391	***48***	225	225	***69***	225	225
		9	387	391	***30***	387	391	***49***	225	225	***70***	225	225
		10	387	391	***31***	387	391	***50***	225	225	***71***	225	225
		11	387	391	***32***	387	391	***51***	221	225	***72***	225	225

		12	387	391	***33***	387	391	***52***	225	225	***73***	225	225						
		13	387	391	***34***	387	391	***53***	225	225	***74***	221	225						
		14	387	391	***35***	387	391	***54***	225	225	***75***	225	225						
		15	387	391	***36***	387	391	***55***	221	225	***76***	225	225						
		16	387	391	***37***	387	391	***56***	225	225	***77***	225	225						
		17	387	391	***38***	387	391	***57***	221	225	***78***	225	225						
		18	387	391				***58***	225	225	***79***	225	225						
		19	387	391				***59***	221	225									

Cross (Trial1)

Parent 1 (p)	**Parent 2 (q)**	**#nematode**	**Ds105**		**#nematode**	**Ds105**		**#nematode**	**Ds105**		**#nematode**	**Ds105**	
CHL1	**USA6**	***CHL1***	151	151	***9***	159	159	***19***	151	159	***29***	159	159
		USA6	159	159	***10***	151	159	***20***	159	159	***30***	151	159
		1	151	151	***11***	151	151	***21***	159	159	***31***	151	159
		2	151	151	***12***	151	159	***22***	151	159	***32***	151	159
		3	151	159	***13***	151	159	***23***	159	159	***33***	151	151
		4	159	159	***14***	151	159	***24***	151	151	***34***	159	159
		5	159	159	***15***	159	159	***25***	151	151	***35***	151	151
		6	159	159	***16***	151	159	***26***	151	151	***36***	151	151
		7	151	159	***17***	159	159	***27***	159	159			
		8	159	159	***18***	151	151	***28***	151	159			

Cross (Trial2)															
Parent 1 (p)	**Parent 2 (q)**	**#nematode**	**Ds105**		**#nematode**	**Ds105**		**#nematode**	**Ds105**		**#nematode**	**Ds105**	
CHL1	**USA6**	***CHL1***	151	151	***9***	151	151	***19***	151	159	***29***	151	159
		USA6	159	159	***10***	151	159	***20***	151	151	***30***	151	159
		1	151	159	***11***	151	151	***21***	151	151	***31***	151	159
		2	151	159	***12***	159	159	***22***	151	159	***32***	151	151
		3	151	151	***13***	151	159	***23***	151	151	***33***	151	151
		4	159	159	***14***	151	159	***24***	151	159	***34***	151	159
		5	151	151	***15***	159	159	***25***	159	159	***35***	151	159
		6	151	151	***16***	159	159	***26***	151	151			
		7	151	151	***17***	151	151	***27***	151	159			
		8	151	159	***18***	151	159	***28***	151	159			

Table 3. Proportion of F_1 nematodes from hybrid crosses that were heterozygous (representing successful admixture) or homozygous for alleles of either parent. Expected proportions were calculate using the Hardy Weinberg equation (p2 + 2pq + q2 = 1) with lower and upper 95% confidence intervals depending on the number of nematodes screened. All parental nematodes were homozygous for all SSR alleles; the presence of two alleles at any of the two SSR loci screened were considered to be admixed. Numbers in red indicate statistically lower proportion of offspring in a given category (based on 95% Cis); blue indicated higher than expected proportion and black is undistinguishable from random. The numbers in the parentheses are lower and upper 95% CI values, respectively, and were derived using random resampling (10,000 iterations). Dashes represent the same expected frequency for parent 2 as in parent 1. Blue shading indicates two repeat crosses of between the CHL1 and USA6 nematode strains that was the most successfully admixed in the original crosses. Asterisk refers to the only asymmetric cross (USA1/RSA450) performed with 1000 and 500 eggs respectively (p = 0.67, q=0.33).

Parent 1 (p)	Parent 2 (q)	#single F_1 nematodes screened	Heterozygous (Admixed)	Homozygous for alleles of Parent 1	Homozygous for alleles of Parent 2	Expected Hz (2pq)	Expected homozygosity, Parent 1 (p^2)	Expected homozygosity, Parent 2 (q^2)
ESP264	**RSA410**	31	**16,1%**	**0.0%**	**83.9%**	50.0% (32.3%, 67.7%)	25.0% (9.7%, 41.9%)	-
USA1	**RSA450**	224	**3.6%**	**0.0%**	**96.4%**	50.0% (43.3%, 56.7%)	25.0% (19.6%, 30.4%)	-
USA1*	**RSA450**	72	**19.4%**	**11.1%**	**69.4%**	44.4% (33.3%, 55.6%)	44.4% (33.3%, 55.6%)	11.1% (4.2%, 18.1%)
USA6	**ESP264**	40	**2.5%**	**0.0%**	**97.5%**	50.0% (35.0%, 65.0%)	25.0% (12.5%, 37.5%)	-
CHL1	**USA6**	79	**55.7%**	**26.6%**	**16.5%**	50.0% (39.2%, 60.8%)	25.0% (16.5%, 34.2%)	-
CHL1	**ESP313**	164	**43.3%**	**14.6%**	**42.1%**	50.0% (42.4%, 57.6%)	25.0% (18.8%, 31.5%)	-
CHL3	**RSA410**	77	**20.8%**	**0.0%**	**79.2%**	50.0% (39.0%, 61.0%)	25.0% (15.6%, 35.1%)	-
CHL1	**USA6**	36	**36.1%**	**27.8%**	**36.1%**	50.0% (42.4%, 57.6%)	25.0% (18.8%, 31.5%)	-
CHL1	**USA6**	35	**48.6%**	**37.1%**	**14.3%**	50.0% (39.0%, 61.0%)	25.0% (15.6%, 35.1%)	-

Table 4. Microsatellite markers and their primer sequence used for the screening of the various *D. siricidicola* crosses.

Cross (Parent 1 / Parent 2)	Primer 1	Primer 2
USA1/RSA450	DS105	DS325
	F5' TGGTAGCAATCGATCGAAAA 3'	F5' ACGCTTATGTGTGCCACTTG 3'
	R5' CGTGTCCACTTGTCCCTCTC 3'	R5' GGGTCTCTTGATGATGTTTCG 3'
USA6/CHL1	DS105	DS325
RSA410/CHL3	DS316	DS388
	F5' TGCGGATATCTTCTCATTGTAA 3'	F5 'AAGTCAGCTGAAAGGCGAAG 3'
	R5' TCAAATGTTATGCGAAATTCTG 3'	R5' TGTGTGCATGAAAACGGAAC 3'
CHL1/ESP31	DS105	DS325
ESP264/RSA410	DS105	DS325
ESP264/USA6	DS105	DS325

Figure 1. Mean nematode abundance of parental and hybrid *D. siricidicola* nematodes in 100 µl (±SE) grown per Petri dish after 25 days on four strains of *A. areolatum*. One-way ANOVA Tukey HSD at $P<0.05$ was used to determine statistical differences of the nematode strains reproducing on different fungal isolates. Different letters represent significant difference between the nematode strains. The various nematode strains are represented through different shapes and the error bars show standard error. (A) Mean productivity of nematodes of both trials combined with the three nematode sources and the four fungal isolates (CMW46043 = RSA2013; CMW47563 = RSA2012; CMW40703 = USA; CMW40871 = AUS). (B) Mean productivity for the first and second trial.

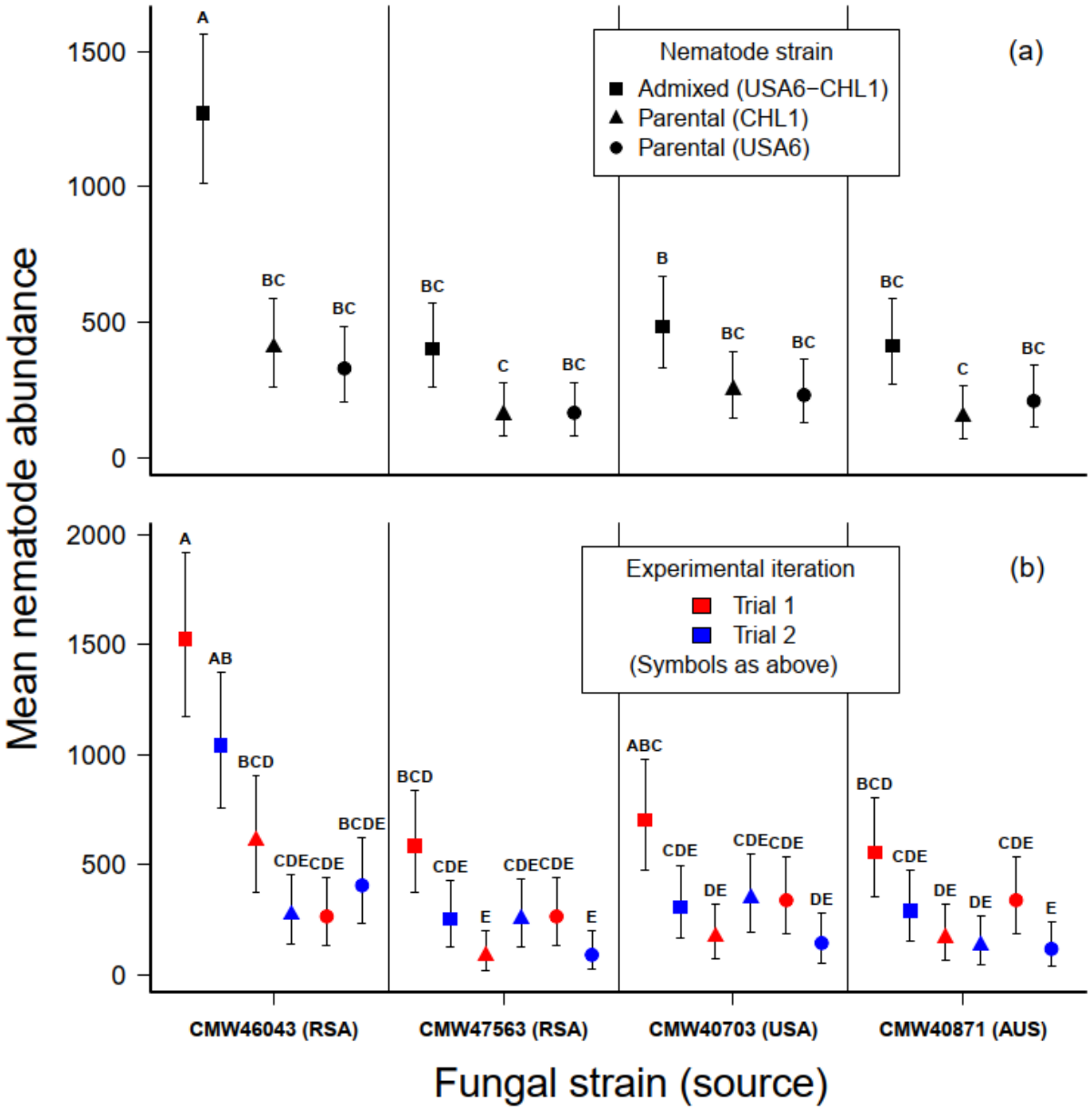
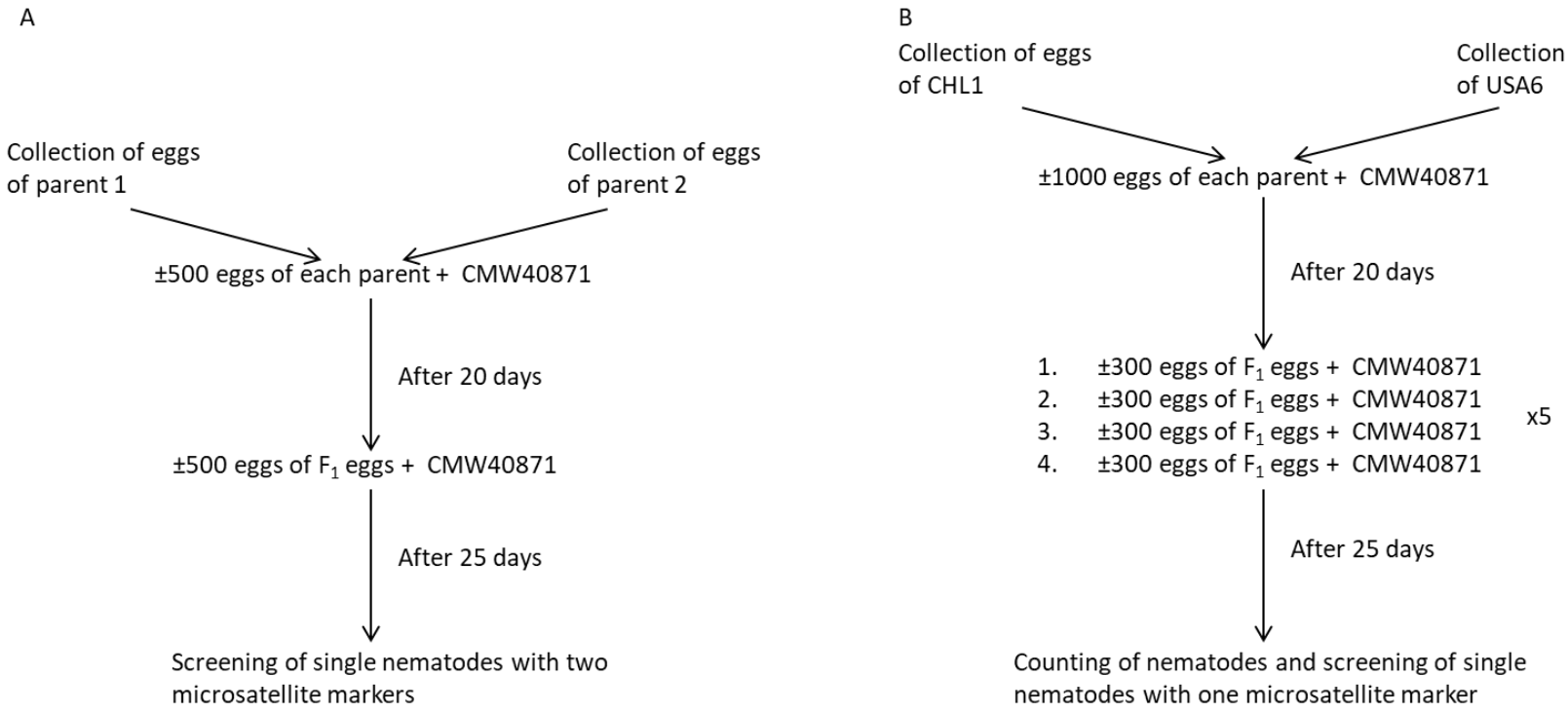

Nematode strain
Admixed (USA6−CHL1)
Parental (CHL1)
Parental (USA6)
(a)
Experimental iteration
Trial 1
Trial 2
(Symbols as above)
(b)
Mean nematode abundance
Fungal strain (source)
CMW46043 (RSA)
CMW47563 (RSA)
CMW40703 (USA)
CMW40871 (AUS)

Figure S1. Cross-breeding and nematode productivity assay experiments in *Deladenus siricidicola*. (A) Interbreeding and screening of F_1 offspring to determine heterozygous offspring. (B) Growth performance of F_1 offspring on four different *Amylostereum areolatum* isolates.



Chapter 4

Host specificity and diversity of *Amylostereum* associated with Japanese siricids

Katrin N. E. Fitza[a], Masanobu Tabata[b], Natsumi Kanzaki[c], Koki Kimura[d], Jeff Garnas[e], Bernard Slippers[a]

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

[b]Forestry & Forest Products Research Institute, Tohoku Research Center, 92-25 Nabeyashiki Shimo-Kuriyagawa, Morioka, Iwate 020-0123, Japan

[c]Forestry & Forest Products Research Institute, 1 Matsunosato, Tsukuba, Ibaraki 305-8687, Japan

[d]Forestry & Forest Products Research Institute, Aomori Prefectural Industrial Technology Research Center, 46-56 Kominato-Shinmichi, Hiranai, Higashi-Tsugaru, Aomori 039-3321, Japan

[e]Department of Zoology and Entomology, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa

Abstract

The mutualism between siricid woodwasps and *Amylostereum* fungal symbionts has long been considered to be species-specific. Recent studies from North America have challenged this assumption, where native siricids and the introduced *Sirex noctilio* are clearly swapping symbionts. Whether this pattern is a consequence of invasion or an underappreciated property of siricid biology is unknown. Here we show that the native Japanese siricid, *Sirex nitobei,* carries both *Amylostereum areolatum* and *Amylostereum chailletii*, rather than only *A. areolatum* as long assumed. Furthermore, all samples from a *Urocerus* sp. unexpectedly carried *A. chailletii* and not *Amylostereum laevigatum.* Vegetative compatibility group tests revealed extensive clonality, with one VCG present amongst three *A. areolatum* isolates and six VCGs present amongst 61 *A. chailletii* isolates. These results contribute to the understanding of insect-fungal fidelity in the siricid-*Amylostereum* association and, together with other studies, suggest that host tree influences *Amylostereum* species occurrence, perhaps more strongly than wasps.

Keywords: Insect-fungal symbiosis, host fidelity, clonal population, Siricidae, Basidiomycota

4.1 Introduction

Siricid woodwasps and *Amylostereum* fungi live in mutualism that is obligatory for the wasp but not for the fungus. The numerous studies of this symbiosis have been particularly stimulated by the significant damage that a particular Eurasian species pair, *Sirex noctilio* and *Amylostereum areolatum*, have caused in areas where it is invasive and attacks commercially-grown *Pinus* trees (Slippers and Wingfield, 2012). The invasion outside the native Eurasian range was first noted around 1900 in New Zealand, followed by spread over the next decade to all major pine-growing areas of the Southern Hemisphere. In 2005, *S. noctilio* was also reported from the USA, which has brought much research interest to the field in recent years.

The siricid-*Amylostereum* mutualism is maintained across wasp generations by transmission of asexually produced propagules of the fungus by the female wasp (Slippers *et al.*, 2003; 2015). Female siricid wasps have specialized internal organs called mycangia in which they carry asexual arthrospores of *Amylostereum* which they introduce into the tree host whilst laying their eggs. This mode of reproduction and spread of asexual spores results in the widespread distribution of fungal clonal lineages. While not strictly vertically-transmitted, the offspring acquire fungal inoculum from the larval environment which was inoculated at the time of oviposition by the mother. In most cases, therefore, the maternal strain is re-acquired, during development, though the possibility of acquiring other strains during tunnelling does exist.

Prior to the widespread availability of rapid molecular tools, fungal clones were often identified and delineated using compatible growth assays to establish Vegetative Compatibility Groups (VCGs). For example, Vasiliauskas *et al*. (1998) found a VCG of *A. areolatum* across more than 600 km and from isolates made 12 years apart in Scandinavia. They also found that clonality was more prevalent in *A. areolatum* (12 VCGs from 53 isolates) than in *Amylostereum chailletii* (47 VCGs from 57 isolates). This aligns with the fact that *A. chailletii* sexual fruit bodies appear to be more common than *A. areolatum*. Slippers *et al*. (2001) used VCGs to identify a clone that has been spread with invasive *S. noctilio* across the Southern Hemisphere. In another part of the invasive range in Canada, Wooding *et al.* (2013) identified 14 VCGs from 27 isolates of *A. areolatum*. Slippers *et al*. (2015) suggested that wasps could acquire

sexually produced genotypes of the fungus, which could help explain the higher VCG diversity observed in North America.

Since its first description in 1929 by Carthwright, the *S. noctilio-A. areolatum* symbiosis had been viewed as highly specific (Slippers *et al*., 2003). Strict co-speciation is not supported in the siricid-fungal association as the same fungus is often carried by multiple siricid species. However, it was well-entrenched in the literature that each siricid carries but a single *Amylostereum* fungus, suggesting the absence of contemporary symbiont swapping, perhaps enforced by the biology of the wasps. Recent studies from North America have called this into question, however, by showing that *A. areolatum* that presumably arrived with the invasive *S. noctilio* in 2004 is regularly carried by several native siricids. For example, *Sirex nigricornis* and *Sirex nitidus*, thought to exclusively carry *A. chailletii*, in fact regularly carry *A. areolatum*, that it putatively acquired from *S. noctilio* (Hajek *et al.*, 2013; Olatinwo *et al.*, 2013; Wooding *et al.*, 2013). Both Hajek *et al*. (2013) and Olatinwo *et al*. (2013) also found *A. areolatum* associated with *S. nigricornis* outside the range of invasive *S. noctilio,* raising the possibility that the association predates the known arrival of *S. noctilio*.

Native siricids worldwide are generally understudied, owing to their low abundance and habit of attacking only dead or highly stressed trees with little economic value. As a consequence of the global importance of *S. noctilio* – a species capable of killing healthy trees in the Southern Hemisphere – some of these native species and their fungal symbionts have received recent attention. In North America, there is a diverse native siricid fauna. However, the question of fidelity in the wasps-symbiont relationship is complicated by the invasion of *S. noctilio* since novel interactions among wasps, trees and fungi may yield different patterns that may differ in a native, co-evolved context.

In Japan, four genera of the family Siricidae, namely *Sirex*, *Urocerus*, *Xeris* and *Xoanon*, have been reported (Takeuchi, 1962; Tabata *et al.*, 2012). All species are believed to be native to the region. Two *Amylostereum* species have been confirmed to be present in Japan, namely *A. areolatum* and *Amylostereum laevigatum*. *A. laevigatum* has been isolated from *Urocerus antennatus* and *Urocerus japonicus* and confirmed using DNA sequence data (Tabata and Abe, 1997, 1999). *Sirex nitobei* and *Xanon matsumurae* were shown to carry *A. areolatum*, also supported by sequence

data (Tabata *et al*., 2000, 2012). *A. chailletii* has been described from *U. antennatus*, but only based on culture morphology (Sano *et al.*, 1995) and was likely misidentified (Tabata, *pers. obs.*). The identification of *Amylostereum* species based on culture morphology is complicated and has led to mistaken identities in the past (Thomsen and Harding, 2011).

The aim of this study was to identify the *Amylostereum* spp. associated with two common Japanese siricid species, namely *S. nitobei* and an unknown *Urocerus* species. We used sequence data of both the nuclear internal transcribed spacer (ITS) rDNA and mitochondrial small subunit (mtSSU) rDNA to identify fungal isolates obtained from these wasps. We also considered the diversity and clonality of the *Amylostereum* isolates using VCGs. Using 64 samples across two sites and three tree species, we examined the degree of specificity between *Amylostereum* fungi in these insects in the context of the native pine ecosystem with diverse siricid fauna where no known siricid invaders occur.

4.2 Materials and methods

4.2.1 Fungal sources and DNA extraction

Fifty five *Amylostereum* isolates were collected from mycangia of the *Urocerus* sp. specimens outside Komoro city in the Nagano prefecture in Japan. Nine *Amylostereum* isolates were collected from mycangia from *S. nitobei* outside Takko Town in the Aomori prefecture in Japan. Isolations were made using the techniques reported by Thomsen and Harding (2011). The nine *S. nitobei* isolates in this study were all associated with *Pinus densiflora*, while the 55 isolates from the *Urocerus* sp. were obtained from two tree hosts, *Abies homolepis* and *Larix leptolepis.* The *Urocerus* sp. in this study is a new species (unpubl. data) of woodwasp in Japan and will be described in a future study (Tabata, *pers. comm*.).

All isolates were maintained on potato dextrose agar (PDA; Merck (Pty) Ltd South Africa) and are deposited in the Culture Collection of the Tree Protection Cooperative Programme (CMW) (Table 1). The mycelia were collected and phenol-chloroform extraction performed. DNA was precipitated overnight using 0.1 volume of NaAc and

1 volume of 100% $EtOH_2$. After centrifugation (Eppendorf 5417C, Hamburg, Germany) and clean-up with 70% $EtOH_2$ the samples were vacuum dried (Concentrator 5301, Eppendorf, Hamburg, Germany) at 45°C for 5 min. The dried DNA was resuspended in 100µl sterile distilled water.

4.2.2 Primer amplification and DNA sequencing

To identify the *Amylostereum* isolates the mtSSU rDNA primer pair MS1 and MS2 (White *et al.*, 1990) were used to amplify a portion of the mitochondrial small subunit, as well as the ITS1 and ITS4 primers to amplify the internal transcribed spacer (ITS rDNA) region (White *et al.*, 1990). PCR volume for MS1 and MS2 was 25 µl, 5 µl of MyTaq™ Reaction Buffer (Bioline USA, Taunton, Massachusetts), 0.1 µM of both MS1 and MS2, 0.5 µl of MyTaq™ DNA polymerase (Bioline USA, Taunton, Massachusetts) and around 100 ng of the template DNA was added. PCR conditions were as follows: preincubation of 95°C 3 min, 35 cycles of 95°C for 45 s, 58°C for 30 s and 72°C for 1 min, and a final extension of 72°C for 10 min. For amplification of the ITS rDNA gene the PCR protocol and cycling conditions from Wooding *et al.* (2013) were followed. The PCR amplicons were sequenced using the ABI Prism™ 3500xL automated DNA sequencer (Applied Biosystems USA, Foster City, California). All sequence data was submitted to GenBank (ITS: **KU870238 -KU870275**; mtSSU: **KU870276 - KU870311**).

4.2.3 Phylogenetic analyses

Sequence data representing authentic isolates of described *Amylostereum* spp. were obtained from GenBank and alignment of the entire dataset constructed online by means of MAFFT v7 (Katoh and Standley, 2013). This was verified with ClustalW in MEGA v6 (Tamura *et al.*, 2011). While ITS and mtSSU data were present for all species, both could not always be found for the same isolates. The best fit model was selected for the resulting datasets using jModelTest v2.1.3 (Guindon and Gascuel, 2003; Darriba *et al.*, 2012) and used to construct the maximum likelihood tree (ML) using PhyML v3.1 (Guindon and Gascuel, 2003). Maximum parsimony (MP) phylogenetic analyses were also done using PAUP v4.0b10 (Swofford, 2003).

Measures such as tree length (Lombardero *et al.*), consistency index (CI), rescaled consistency index (RC), and the retention index (RI) were recorded. Statistical support for branching for both analyses were determined through 1000 bootstrap replicates.

4.2.4 Vegetative compatibility analysis

VCG analysis was used to investigate clonality among *A. areolatum* and *A. chailletii* isolates as described by Vasiliauskas *et al*. (1998) and Slippers *et al*. (2001). All the isolates from each of the two prefectures were crossed in all possible combinations on PDA. Hyphal plugs of 0.5 cm^2 were cut from the edge of actively growing cultures and placed around 2 cm apart on a 9 cm Petri dish with PDA, and incubated at 23°C. After 3 - 4 weeks compatibility was determined by scoring the presence or absence of a brown demarcation zone. The presence of the demarcation zone was considered as an incompatible reaction whereas the mergence of the two cultures showed compatibility between the cultures.

4.3 Results

4.3.1 DNA sequencing and phylogenetic analyses

Based on sequence data the ITS amplicon was 604 bp for *A. chailletii* and 607 bp for *A. areolatum*. For the mitochondrial small subunit region the amplicon was calculated to be 519 bp for *A. areolatum* and 516 bp for *A. chailletii.* Analyses on the two gene regions were performed separately. The dataset for ITS after alignment consisted of 439 characters (30 parsimony-uninformative, 24 parsimony-informative, 385 constant characters). One thousand most parsimonious trees were found (TL = 38, CI = 0.763, RI = 0.906, and RC = 0.692). The mtSSU dataset consisted of 493 characters after alignment (23 parsimony-uninformative, 28 parsimony-informative, 442 constant characters). Forty-two most parsimonious trees were found (TL = 40, CI = 0.900, RI = 0.970, and RC = 0.873).

Based on phylogenetic analyses, 61 of 64 isolates in our study were grouped strongly with known *A. chailletii* sequences (ML/MP; Fig. 1). The remaining three samples were

confirmed as *A. areolatum* (ML/MP; Fig. 1). The *A. chailletii* isolates were isolated from both species of woodwasps, *S. nitobei* and the *Urocerus* sp. *A. chailletii* was collected from both the Nagano and Aomori prefecture (Fig 2). The three *A. areolatum* isolates all originated from *S. nitobei* wasps attacking *P. densiflora* collected from the Aomori prefecture.

4.3.2 Vegetative compatibility analysis

Seven VCGs were identified from the 64 *Amylostereum* isolates (Table 1). Six out of the seven VCGs came from the *A. chailletii* isolates. There was geographic structure in the distribution of VCGs in *A. chailletii,* with VCG1 being found only in the Aomori prefecture and the remaining five in the Nagano prefecture (Fig. 2). The most frequent VCG in *A. chailletii* contained 36 isolates, all from the Nagano prefecture. One *A. chailletii* VCG (VCG5) was collected from both *L. leptolepis* and *A. homolepis*. In *A. areolatum,* one VCG was identified representing three isolates from the Aomori prefecture. The VCGs did not overlap between samples from *S. nitobei* and the *Urocerus* sp.

4.4 Discussion

The *S. nitobei* wasps sampled for this study from *P. densiflora* trees in the Aomori prefecture in Japan were associated with both *A. chailletii* and *A. areolatum*. This result confirms recent North America findings that the paradigm of obligate fidelity to a single fungus per wasp species should be discarded, even in a native context devoid of non-coevolved invaders. The native range of *S. nitobei* is eastern Asia. Terashita (1970) first identified *A. areolatum* as a symbiont of *S. nitobei* in Japan using morphological data. This was later confirmed by Tabata *et al*. (2000) using DNA sequence data that grouped three *Amylostereum* isolates from *S. nitobei* (Kochi prefecture) with European isolates of *A. areolatum*. These results illustrate a lack of host-symbiont fidelity in *S. nitobei* that is similar to that observed in *S. nigricornis* and other North American species in the past 2 yrs. It is not clear, however, how widespread or prevalent either of these species are over the wider distribution of *S. nitobei*.

Urocerus species are common throughout Japan. In this study the unknown *Urocerus* species yielded only isolates of *A. chailletii.* Kanamitsu (1978) and Sano *et al*. (1995) described *A. chailletii* from mycangia of *U. antennatus* from *Abies sachalinensis* and *Picea jezoensis* in Hokkaido and *Cryptomeria japonica* in the Mie prefecture using culture morphology. *Amylostereum* spp. are difficult to separate in culture, so it is hard to confirm this identification of *A. chailletii*. In subsequent studies from the Ibaraki, Kochi and Nagasaki prefectures, the fungal species associated with this woodwasp species was recognised as *A. laevigatum* (Tabata and Abe, 1997, 1999) using both morphological and DNA sequence data (Tabata *et al*. 2000). These sampling areas for the studies prior to 2000 are located in the central and southern part of Japan, stretching over an area of around 1000 km. One of the new sampling sites of this study now incorporates the northern part of Japan, whereas the second site adds to the existing data from the central area of Japan. The data from this study and that of Tabata *et al*. (2000) confirm that *Urocerus* species in Japan are associated with both *A. laevigatum* and *A. chailletii*.

VCG assays revealed a high degree of clonality in the 64 *Amylostereum* isolates studied. All three *A. areolatum* isolates were of the same VCG, while all 61 *A. chailletii* isolates belonged to 5 VCGs. Previous studies have revealed much higher levels of diversity of *A. chailletii* in native regions such as Europe (Vasiliauskas *et al*. 1998). In Europe *A. chailletii* frequently produces sexual structures and apparently spreads through basidiospores as well as in association with the woodwasp. In Japan the fruit bodies of neither *A. areolatum* nor *A. chailletii* have been recorded. Together with the clonality observed here, it seems most likely that these species are spread predominantly in association with woodwasps rather than via sexual spore production and wind, at least in the areas that we studied here. There was no VCG overlap in the *A. chailletii* isolates carried by *S. nitobei* and the *Urocerus* sp., but this could be driven by the fact that they were not sampled in the same site. Further isolates are required to understand clonal distribution and the degree to which clones are specific to wasp or tree species.

The collection data from this and other studies in Japan and Europe (Tabata and Abe, 1997; Vasiliauskas *et al.*, 1998; Tabata and Abe, 1999; Vasiliauskas, 1999; Vasiliauskas and Stenlid, 1999; Tabata *et al.*, 2000) appears to suggest that the tree host may be a non-trivial driver of the fungal and wasp species association. For

example, *A. chailletii* always appears to be associated with tree hosts in the Pinaceae, but outside the genus *Pinus* (i.e. *Picea* spp., *Larix* spp., *Abies* spp. and rarely *Pinus* spp.), irrespective of the wasp species. On the other hand, *A. areolatum* is most commonly found in association with *Pinus*. The Cupressaceae (*Cupressus* spp., *C. japonica* and *Chamaecyparis* spp.) appears to primarily support *A. laevigatum*, which may drive the association of this fungus with siricids of the genus *Urocerus* that attack trees within this family. While *Urocerus* carries *A. laevigatum* on Cupressaceae (Tabata and Abe, 1997, 1999), it carried *A. chailletii* on Pinaceae (Tabata *et al*., 2000 and results from this study). While interesting, the extent of the role of tree species in structuring siricid-*Amylostereum* interactions awaits further study.

Apart from the unexpected prominence of *A. chailletii* and the absence of *A. laevigatum*, the study reinforced the recent discovery that woodwasps do not always carry the same fungal species. How dynamic the dominance of a particular species as fungal symbiont is over time, is an intriguing question. The mechanism through which such switching happens has not been directly observed, but is likely through wasps ovipositing in trees that have already been attacked by other woodwasps or infected by *Amylostereum* by basidiospores (Wooding *et al*. 2013, Slippers *et al*. 2015). The frequency, mechanisms and conditions of symbiont switching, as well as its ecological consequences deserves closer study. The Japanese forest resource appears to offer ideal opportunities to do so. As pointed out by Wooding *et al*. (2013), this could be significant in terms of virulence of associated fungi, or changes in interactions with parasites such as *Deladenus* spp. or *Ibalia* spp. that are dependent on the fungus for food or signals to find its host.

4.5 Acknowledgement

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4.7 Tables and Figures

Table1. *Amylostereum* isolates used for phylogenetic analysis in this study.

Species	Isolate No.*	Other isolate No.	Host	Insect	Location	Vegetative compatibility groups	GenBank Accession Nos. (ITS)	GenBank Accession Nos. (mt-SSU-rDNA)
A. areolatum		B1350	*Pinus densiflora*	*S. nitobei*	Japan		AF218389	
A. areolatum		B1351	*Pinus densiflora*	*S. nitobei*	Japan			
A. areolatum	Sn1	CMW42090	*Pinus densiflora*	*S. nitobei*	Japan	VCG7	KU870238	KU870299
A. areolatum	Sn2	CMW42091	*Pinus densiflora*	*S. nitobei*	Japan	VCG7	KU870239	KU870307
A. areolatum		DAOM:239281	*Pinus sylvestris*		Canada			HM461086
A. areolatum		DAOM Francke Sirex 1			Germany			HM461088
A. areolatum		DAOM 239284	*Pinus sylvestris*		Canada			HM461087
A. areolatum		CBS:655.93	*Picea abies*		Denmark			HM461090
A. areolatum		AtII28			Austria			HM461089
A. chailletii		B1387		*U. gigas*	Germany		AF218392	
A. chailletii		B1355	*Abies balsamea*		Canada		AF218393	
A. chailletii		DOAM X-589			Canada			HM461091
A. chailletii		54-95	*Tusga* sp.		Canada			AF238458
A. chailletii		DOAM 21327	*Abies balsamea*		Canada			AF238459
A. chailletii		L234	*Picea abies*		Lithuania			AF238460
A. chailletii		SC62.8	*Picea sitchensis*		United Kingdom			AF238461
A. chailletii		CBS 483.83		*U. gigas*	United Kingdom			AF238457
A. chailletii	Sn7	CMW40400	*Pinus densiflora*	*S. nitobei*	Japan	VCG1	KU870241	
A. chailletii	Sn10	CMW40401	*Pinus densiflora*	*S. nitobei*	Japan	VCG1	KU870240	KU870305
A. chailletii	Sn14		*Pinus densiflora*	*S. nitobei*	Japan	VCG1		KU870306
A. chailletii	Usp1	CMW42092	*Larix densiflora*	*Urocerus sp.*	Japan	VCG2		KU870306
A. chailletii	Usp6		*Larix densiflora*	*Urocerus sp.*	Japan	VCG2	KU870268	KU870303
A. chailletii	Usp26	CMW40402	*Larix densiflora*	*Urocerus sp.*	Japan	VCG2	KU870245	KU870280
A. chailletii	Usp10	CMW40403	*Larix densiflora*	*Urocerus sp.*	Japan	VCG3	KU870249	KU870276

A. chailletii	Usp33	CMW42096	*Larix densiflora*	*Urocerus sp.*	Japan	VCG3		KU870281
A. chailletii	Usp34		*Larix densiflora*	*Urocerus sp.*	Japan	VCG3	KU870257	
A. chailletii	Usp22	CMW40404	*Larix densiflora*	*Urocerus sp.*	Japan	VCG4	KU870247	KU870279
A. chailletii	Usp108	CMW40406	*Abies homolepis*	*Urocerus sp.*	Japan	VCG5	KU870248	KU870277
A. chailletii	Usp109	CMW40407	*Abies homolepis*	*Urocerus sp.*	Japan	VCG5	KU870244	
A. chailletii	Usp13		*Larix densiflora*	*Urocerus sp.*	Japan	VCG6	KU870271	KU870311
A. chailletii	Usp15	CMW40409	*Larix densiflora*	*Urocerus sp.*	Japan	VCG6	KU870258	KU870278
A. laevigatum		B1361	*Cryptomeria japonica*	*U. japonicas*	Japan		AF218395	
A. laevigatum		B1362	*Cryptomeria japonica*	*U. japonicas*	Japan		AF218395	
A. laevigatum		B1371	*Juniperus nana*		France		AF218396	
A. laevigatum		B1372	*Juniperus nana*		France		AF218396	
A. laevigatum		CBS 626.84	*Juniperus nana*		France			HM461092
A. laevigatum		CBS 624.84	*Juniperus nana*		France			AF238462
A. laevigatum		Voucher F2	*Juniperus* sp.		Finland		JX049990	
A. ferreum		B1359	*Podocarpus lambertii*		Brazil		AF218390	
A. ferreum		B1360	*Podocarpus lambertii*		Brazil		AF218390	
A. ferreum		CBS 637.84	*Podocarpus lambertii*		Brazil		HM461082	HM461093
A. ferreum		CBA 633.84	*Podocarpus lambertii*		Brazil			AF238464
A. sp.		B1393			USA		AF218391	
E. tinctorium		B1122					AF218397	
E. tinctorium		DAOM16666						U27035

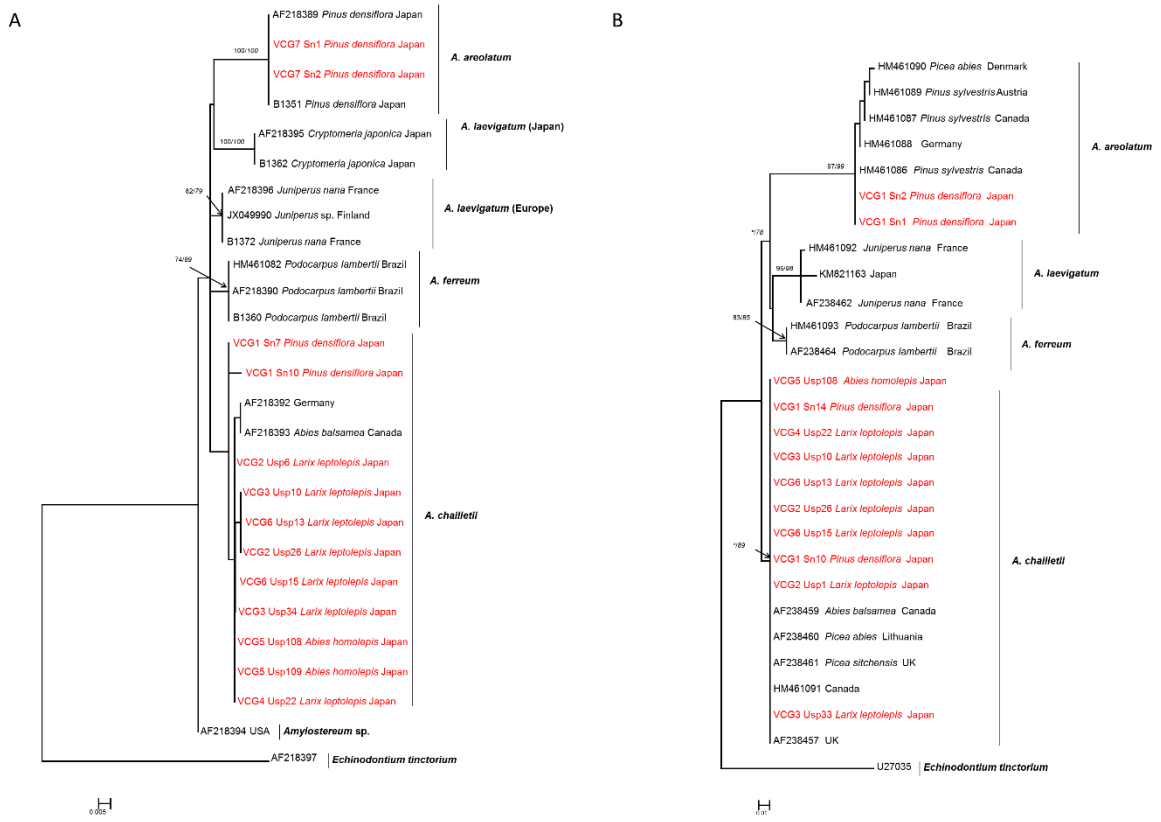

Figure 1. Identification of *Amylostereum*. Phylogenetic analysis using both Maximum Likelihood (ML) and Maximum Parsimony (MP). (A) A Maximum Likelihood phylogenetic tree of a subset of sequences of the internal transcribed spacer region (ITS-rDNA), based on 439 bp and (B) the mitochondrial small subunit (mtSSU rDNA), based on 493 bp. Both trees are rooted to *Echinodontium tinctorium.* Samples obtained from *Urocerus* sp. are abbreviated with Usp, whereas the samples from *Sirex nitobei* with Sn. Statistical support was determined by 1000 bootstrap replicates and all the bootstrap values above 70% are indicated for ML (roman) and Maximum Parsimony (italics) at the nodes. * indicates that the bootstrap values were below the threshold. Isolates in red are the isolates from this study.

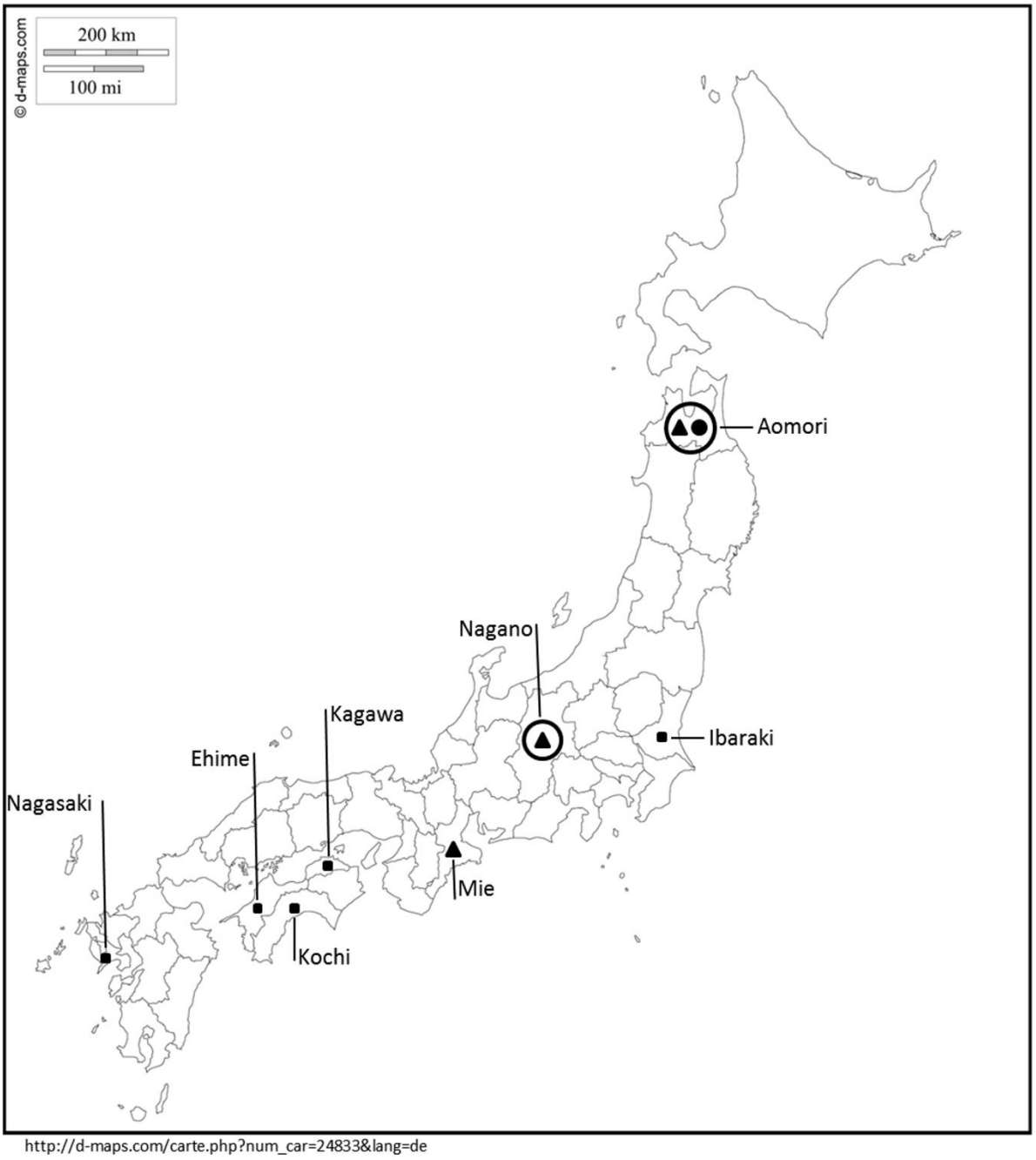

Figure 2. Map of Japan highlighting the sampling areas from the various studies. The various shapes represent the three different *Amylostereum* species present in Japan: the square (■) represents *A. laevigatum*, the triangle (▲) *A. chailletii* and the circle (●) *A. areolatum*. The two encircled locations identify the areas sampled for the current study. Names of the prefectures of sampling sites are indicated.

Concluding remarks

The nematode *Deladenus siricidicola* is the primary biological control agent applied for management of the globally invasive pine pest *Sirex noctilio*. Together with *S. noctilio's* symbiotic fungus, *Amylostereum areolatum*, the pest causes severe losses in the pine industry worldwide. The nematode was discovered in New Zealand in 1962 in the eggs of a female wasp, but is native to Eurasia, where *S. noctilio* also originates. A virulent strain was selected in Australia and subsequently used widely as part of the *S. noctilio* control program in Australia, South Africa and South America and considered for release in North America. Little information is available on the genetic diversity of this nematode across its range in native and non-native regions.

A previous study reported extreme homozygosity in some biological control populations of *D. siricidicola* the Southern Hemisphere. These results highlighted the need to better understand the global diversity given the potentially important role that genetic diversity can play in the ability to adapt to variable environments and hosts. In this thesis we undertook to study the population structure of *D. siricidicola* using samples from parts of the native range, as well as non-native populations from North America and across the Southern Hemisphere. The results revealed three lineages, namely lineage A from Canada and North America, lineage B from the Southern Hemisphere (used in biological control programs), and lineage C from Spain in the native region. Interestingly, there seemed to have been an admixed population of lineage A and B, discovered in Chile. The limited collections from the native region (Eurasia) and some invaded regions (e.g. Australia) would be valuable to address in future studies.

We studied the ability of the distinct lineages identified in this study to interbreed. Experiments were done through mixing strains in culture and tracking interbreeding through microsatellite marker analysis. The offspring were screened for heterozygosity at loci known to be homozygous in the parental strains. A reproductive rate study was performed with one of the nematode crosses on four *A. areolatum* isolates. An increase in the rate of reproduction in the hybrid offspring was observed on all fungal isolates. A significant higher reproductive rate was recorded for the parental and hybrid offspring on the slowest growing fungal isolate. This tool of interbreeding between the lineages can be used to augment diversity in biological control programs, but other crosses and the persistence and performance of the hybrids in the field need to still be determined.

The fidelity between the host and its symbiont is believed to be strict for the purpose of the stability of the symbiosis. In the *S. noctilio-A. areolatum* symbiosis, fungal spores are released into tree during oviposition. The larvae than acquire the fungus through the surrounding environment and furthermore the fungus degrades the wood into a digestible form for the wasp larvae. In this thesis the specificity of the mutualism between the Siricid wasp and the *Amylostereum* spp. in the native region Japan was investigated. It was found that native Japanese siricids could not only carry one specific *Amylostereum* sp. but also alternative species. In context of the biological control agent and the control program this may have consequences in terms of the interaction of the nematode with the wasp. Furthermore, the lack of fidelity may point to a stronger role of the host tree to determine the fungal species the wasp could carry.

During the period of my PhD studies I participated in two related projects that did not form part of the main PhD project:

Part of the global nematode collection originated from samples of *Sirex nitobei* obtained from Japan as representatives of native Asian region. These samples were not included in this study as sequencing of the mitochondrial cytochrome oxidase subunit 1 gene region revealed that they were not *D. siricidicola*. We collaborated with Japanese colleagues to describe a new species, *D. nitobei* (Appendix A). Furthermore, we collaborated with Spanish colleagues to characterize the diversity, host preference and natural enemy prevalence on *S. noctilio* in this native part of its range (Appendix B).

The work done in this thesis relied heavily on the support of collaborators for sampling and expertise, and demonstrates the importance of international collaboration for the study of invasive insect pests.

Appendix

Related articles published during the period of the PhD study

Supplementary paper A

BRILL

Nematology 00 (2016) 1-19

Nematology
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Deladenus nitobei n. sp. (Tylenchomorpha: Allantonematidae) isolated from *Sirex nitobei* (Hymenoptera: Siricidae) from Aomori, Japan, a new member of the *siricidicola* superspecies

Natsumi KANZAKI [1,*], Suguru E. TANAKA [1,2], Katrin FITZA [3], Hajime KOSAKA [4], Bernard SLIPPERS [3], Koki KIMURA [5], Satoshi TSUCHIYA [5] and Masanobu TABATA [6]

[1] *Forestry and Forest Products Research Institute (FFPRI), 1 Matsunosato, Tsukuba, Ibaraki 305-8687, Japan*
[2] *Laboratory of Forest Botany, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8685, Japan*
[3] *Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa*
[4] *Kyushu Research Center, FFPRI, 4-11-16 Kurokami, Chuo, Kumamoto, Kumamoto, 860-0862, Japan*
[5] *Forestry and Forest Products Research Institute, Aomori Prefectural Industrial Technology Research Center, 46-56 Kominato-Shinmichi, Hiranai, Higashi-Tsugaru, Aomori 039-3321, Japan*
[6] *Tohoku Research Center, FFPRI, 92-25 Nabeyashiki, Shimo-kuriyagawa, Morioka, Iwate 020-0123, Japan*

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Summary – *Deladenus nitobei* n. sp., a parasite of a woodwasp species, *Sirex nitobei*, is described based on its typological characters and molecular profiles of part of the small subunit D2-D3 expansion segments of the large subunit and internal transcribed spacer region of the ribosomal RNA gene, as well as part of the mitochondrial cytochrome oxidase subunit I (mtCOI) gene. Adult host woodwasps emerging from dead Japanese red pine logs, *Pinus densiflora*, collected at Aomori, Japan, were dissected and examined for nematode association. The new species was isolated from the body cavity and reproductive system of *S. nitobei* as large parasitic females and small parasitic juveniles. The nematodes were cultured successfully on 1.0% malt extract agar medium, inoculated with *Sirex*-associated fungus, *Amylostereum areolatum*. The mycophagous adult nematodes were characterised by the relative position of the excretory pore, located at 25 (19-28) and 25 (18-30) μm anterior to the hemizonid in the male and female, respectively, and a broad female tail with a rounded distal end. Typologically, the new species forms a cryptic species complex with *D. siricidicola* and *D. canii*. In addition, the new species and *D. siricidicola* share the same host wasp, tree and fungal species in Japan. However, the cryptic species can be separated from each other based on the described morphological and molecular sequence differences in the mtCOI gene.

Keywords – cryptic species, *Deladenus canii*, *Deladenus siricidicola*, molecular, morphology, morphometrics, new species, species complex, taxonomy.

The genus *Deladenus* Thorne, 1941 is a group of insect-parasitic/fungal-feeding nematodes, and currently contains 25 species, including two superspecies, *D. siricidicola* Bedding, 1968 and *D. wilsoni* Bedding, 1968, each containing four and two species, respectively (Chitambar, 1991; Shahina & Maqbool, 1992; Chizov & Sturhan, 1998; Siddiqi, 2000; Nasira *et al.*, 2013; Yu *et al.*, 2013, 2014; Tomar *et al.*, 2015). The nematodes have a characteristic life cycle with two different phases, *i.e.*, insect-parasitic and fungal-feeding phases. These characteristics make these nematodes ideal as biological control agents against woodwasps (Siricidae), and hence have attracted the interests of many researchers (*e.g.*, Bedding, 1967, 1972, 2009; Bedding & Iede, 2005).

The interesting biological characteristics of *Deladenus*, *i.e.*, the four different types of adult (male, mycophagous female, infective female and parasitic female) and the similarity of morphological characters amongst species, complicate the taxonomy of the genus (Blinova & Korenchenko, 1986; Fortuner & Raski, 1987; Chita-

* Corresponding author, e-mail: nkanzaki@affrc.go.jp

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mbar, 1991; Remillet & Laumond, 1991; Siddiqi, 2000). A growing body of molecular data for the genus assists in the distinction of species, although data are not available for many of the described species (*e.g.*, Yu *et al.*, 2009, 2011, 2013, 2014; Morris *et al.*, 2013; Tomar *et al.*, 2015).

In the present study, a species of *Deladenus*, according to the definition by Fortuner & Raski (1987) and Kosaka & Ogura (1995), was isolated from a Japanese native woodwasp, *Sirex nitobei* Matsumura. The species is considered to belong to the *siricidicola* superspecies, and the species is described, based on morphological characters and molecular markers, as *D. nitobei* n. sp.

Materials and methods

Nematode isolation

Five dead Japanese red pine trees, *Pinus densiflora* Sieb. & Zucc., were collected from Takko, Aomori, Japan (40°17′07.52″N; 141°05′59.33″E; 373 m a.s.l.), in May 2015. The trees were cut into *ca* 1 m-long logs on site, and brought back to the Forestry and Forest Products Research Institute, Tsukuba, Ibaraki, Japan. The logs were individually enclosed in a plastic mesh bag and placed in a wired mesh cage. The emergence of woodwasps was recorded. The emerged wasps were sexed and examined for their nematode association, *i.e.*, the wasps were either dissected in M9 buffer to isolate internal parasitic nematodes, or dissected on a sterile glass slide to establish nematode cultures. To establish nematode cultures, an infected ovary was transferred onto the edge of a 1.0% malt extract agar plate (MEA: malt extract 10 g; agar 20 g; distilled water 1000 ml), opposite to an earlier inoculated culture of the fungal symbiont, *Amylostereum areolatum* (Fr.: Fr.) Boidin, which was isolated from *S. nitobei* collected from Takko, Aomori, Japan (same locality as present study), in 2015. The plate was kept at 15-20°C overnight. The nematodes emerged from the ovary and migrated to the edge of the fungal colony from where they were transferred to new MEA together with mycelium of the original fungal culture. The transferred plates were observed occasionally and ones without contamination by non-*Amylostereum* fungi or bacteria were subcultured and kept as laboratory cultures of the nematode.

The parasitic females were collected from the dissected wasps in M9 buffer, transferred to small test tubes, heat-killed at 55°C for 1 min and fixed in TAF (7.0% formalin, 2.0% triethanolamine, 91% distilled water). After a week of fixation, nematodes were then processed into glycerin using a modified Seinhorst's method (Minagawa & Mizukubo, 1994) and mounted in glycerin (permanent slides) according to the method of Maeseneer & d'Herde (see Hooper, 1986).

Morphological observations

The infective female occurred in relatively old (3 weeks or older) cultures of *D. nitobei* n. sp.; thus, males, mycophagous females and infective females were collected from 2- to 3-week-old cultures. Some live material served for the observation of typological characters with an Eclipse 80i light microscope (Nikon) equipped with DIC optics using the methods of Kanzaki (2013). The rest of the nematodes were heat-killed at 60°C for 1 min, fixed in TAF, processed into glycerin and mounted as described above for the parasitic female material. The mounted type materials of all four forms of nematodes were used for photomicrographs, drawings and measurements. Photomicrographs were taken with a microscope digital camera system, DS-Ri1 (Nikon), and drawings were scanned to digitalise. The photomicrographs and drawings were edited with PhotoShop Elements 9 (Adobe) to construct the figures.

Molecular profiles and phylogenetic analysis

DNA samples were prepared as described by Kikuchi *et al.* (2009) and Tanaka *et al.* (2012). The near-full-length of the small subunit (SSU), internal transcribed spacer region (ITS) and the D1-D2-D3 expansion segments of large subunit (D1-D2-D3) of ribosomal RNA (*ca* 3.8 kb), and the partial code of the mitochondrial cytochrome oxidase subunit I (mtCOI: *ca* 0.7 kb) were sequenced as the molecular barcodes for the nematode species. The molecular sequences were determined by PCR direct sequencing following the methods of Kanzaki & Futai (2002) and Ye *et al.* (2007).

The molecular phylogenetic analyses were conducted based on SSU and mtCOI to determine the phylogenetic status of new species among entomoparasitic tylenchids and within *Deladenus*, respectively. The analyses were conducted separately as the number of sequences deposited in the GenBank database is different for these two genes and the model of evolution also differs between them. The D2-D3 and ITS regions are also used for phylogenetic analyses (*e.g.*, Morris *et al.*, 2013), but accumulate mutations slower than the mtCOI and thus contain very

little variation from the close relatives *D. siricidicola* and *D. canii* Bedding, 1974 (Fig. S1).

Sequence alignments were performed using the online version of MAFFT (Katoh *et al.*, 2002: http://mafft.cbrc.jp/alignment/server/). Maximum likelihood phylogenetic analyses were performed in an online version of PhyML (Guindon *et al.*, 2010: http://www.atgc-montpellier.fr/phyml/). The nucleotide substitution model was selected automatically in the online PhyML, and the tree topology was evaluated with 1000 bootstrap pseudo-replicates. The sequences used in comparison with *D. nitobei* n. sp. were determined based on the results from BLAST homology search (http://blast.ddbj.nig.ac.jp/blastn?lang=en) and Kanzaki *et al.* (2016) and Morris *et al.* (2013) for SSU and mtCOI, respectively.

Results

Deladenus nitobei [*] **n. sp.**
(Figs 1-8)

Measurements

See Table 1.

Description

Male

Medium-sized for genus, *i.e.*, 1375-1708 μm long. Body cylindrical, slender, smoothly ventrally arcuate when killed by heat. Cuticle thin, annulated, lateral field with typically 12 (11-13) incisures at mid-body, posteriorly connected to bursa. Deirid clearly observed in middle of lateral field *ca* three body diam. posterior to hemizonid. Head weakly offset from body, separated by a shallow constriction, lip region in lateral view squarish-round, *ca* twice as broad as high. Stylet with narrow lumen, comprising a short cone *ca* one-third of total stylet length and a shaft with a clear, small and rounded basal knob. Dorsal gland orifice clearly visible at *ca* one-third of stylet length posterior to stylet knobs. Subventral gland orifice unclear, located at two-thirds of pharynx length posterior to stylet base. Pharynx not muscular, forming cylindrical tube, without distinct median bulb, but slightly expanding around level of dorsal gland orifice. All three glands (dorsal gland and two subventral glands) located on dorsal side of body, *i.e.*, subventral glands extending dorsally, and thus three gland cell nuclei observed on dorsal side of body, although separation between each gland often unclear. Nerve ring and barely visible pharyngo-intestinal junction either overlapping each other, or junction sometimes slightly posterior to nerve ring. Excretory pore ventral, varying in position between almost immediately to *ca* one body diam. posterior to pharyngo-intestinal junction/nerve ring level. Hemizonid visible *ca* one max. body diam. posterior to excretory pore. Gonad single, on right of intestine, anteriorly outstretched, reaching to level of pharyngeal glands or more anterior. Spermatocytes arranged in double rows in anterior part of testis, in multiple rows in middle section, and as well-developed spermatocytes in a single row in posterior testis. Sperm formed in posterior region of testis, *i.e.*, immediately posterior to single-rowed spermatocytes, where two different types of sperm, tentatively termed as large amoeboid (*ca* 7-12 $\times$ 5-6 μm) and small rounded (*ca* 1.5 μm in diam.) sperm, were observed. *Vas deferens* visible, consisting of rounded cells, sometimes containing well developed sperm. Spicules typical of genus, *i.e.*, moderately wide and smoothly arcuate ventrally consisting of squared capitulum (manubrium) lacking clear condylus and rostrum, and smoothly curved horn-like blade with bluntly pointed distal end in lateral view. Spicules separate but with tips angled towards each other, appearing narrowly V-shaped in ventral view. Gubernaculum present, crescent-shaped with slightly dorsally recurved anterior end in lateral view. Bursa beginning around anterior end of spicules, well developed, peloderan with small blunt projection at tip in ventral view. Tail conical, bluntly pointed in lateral view.

Mycophagous female

Anterior part similar to mycophagous male. Medium-sized for genus, *i.e.*, 1708-2000 μm long. Body cylindrical, slender, smoothly ventrally arcuate, or sometimes C-shaped when killed by heat treatment. Gonad single, on right of intestine, anteriorly outstretched, reaching to level of pharyngeal glands or more anterior. Gonad organs arranged as ovary, oviduct, spermatheca, crustaformeria, uterus and vagina/vulva. Post-uterine branch absent. Oocytes arranged in double-triple rows in anterior half of ovary with well-developed oocytes arranged as single row in remainder. Oviduct narrow tube-like, with a cluster of rounded cells presumed to be oviduct-spermatheca junc-

[*] The species epithet is derived from the species epithet of the host woodwasp, *Sirex nitobei*. The common name of the wasp in Japanese is 'Nitobe-kibachi', and 'Nitobe woodwasp' in English. Thus, although the species epithet *nitobei* was originally dedicated for the wasp species to Inao Nitobe, the scientific and common names of host and parasite are consistent.

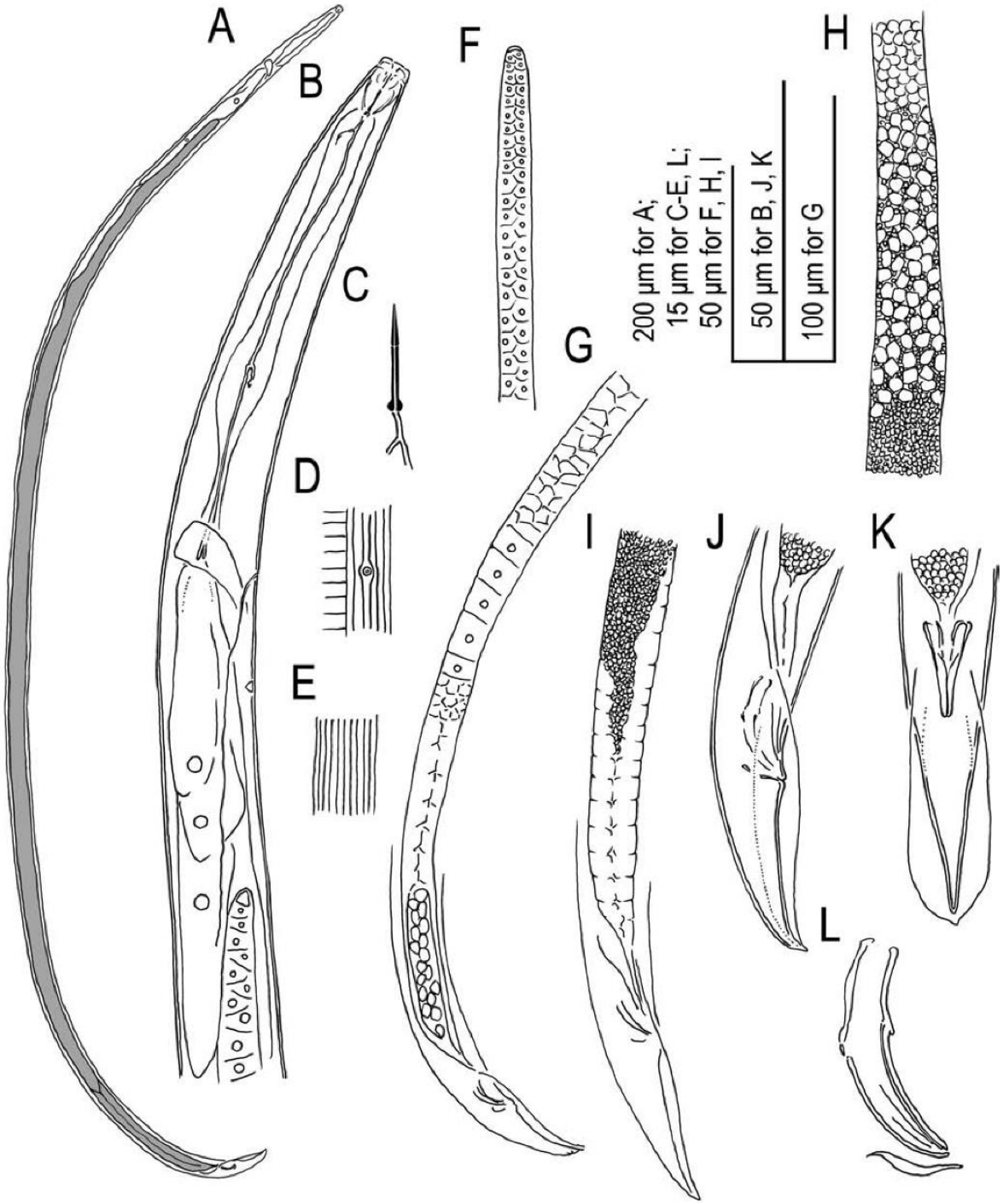

Fig. 1. Mycophagous male of *Deladenus nitobei* n. sp. A: Entire body, right lateral view; B: Anterior region, right lateral view; C: Stylet; D: Body surface showing deirid, lateral field and annulations; E: Lateral field at mid-body; F: Anterior end of testis; G: Posterior part of testis and *vas deferens* producing large amoeboid sperm in right lateral view; H: Middle-posterior part of testis producing small sperm; I: *Vas deferens* containing small sperm, right lateral view; J, K: Male tail, right lateral view (J) and ventral view (K); L: Spicule and gubernaculum, right lateral view.

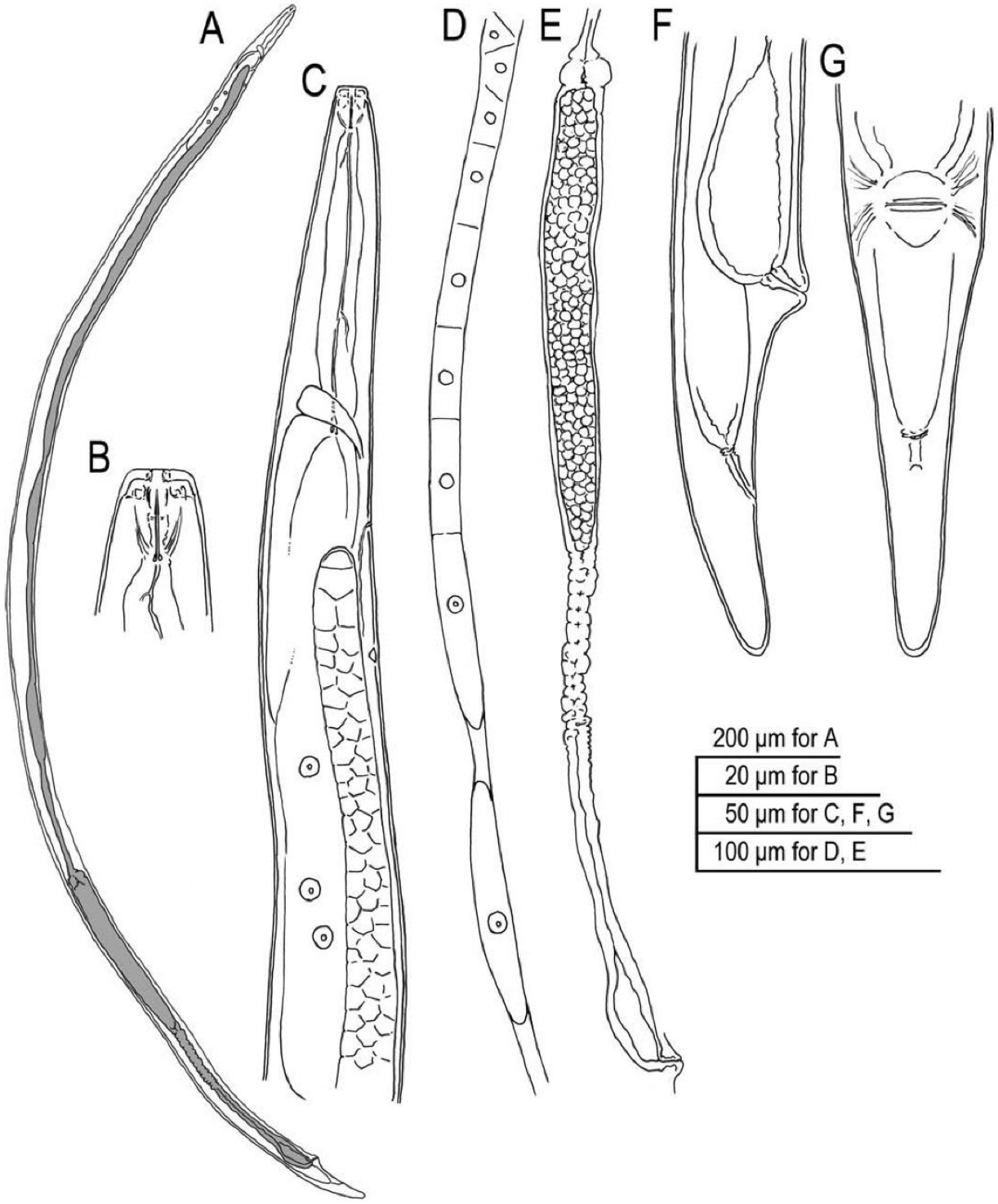

Fig. 2. Mycophagous female of *Deladenus nitobei* n. sp. A: Entire body, right lateral view; B: Lip region, right lateral view; C: Anterior part, right lateral view; D: Posterior part of ovary containing well developed oocytes; E: Posterior part of gonad (spermatheca to vagina/vulval region), right lateral view: F, G: Vulval and anal region, right lateral view (F) and ventral view (G).

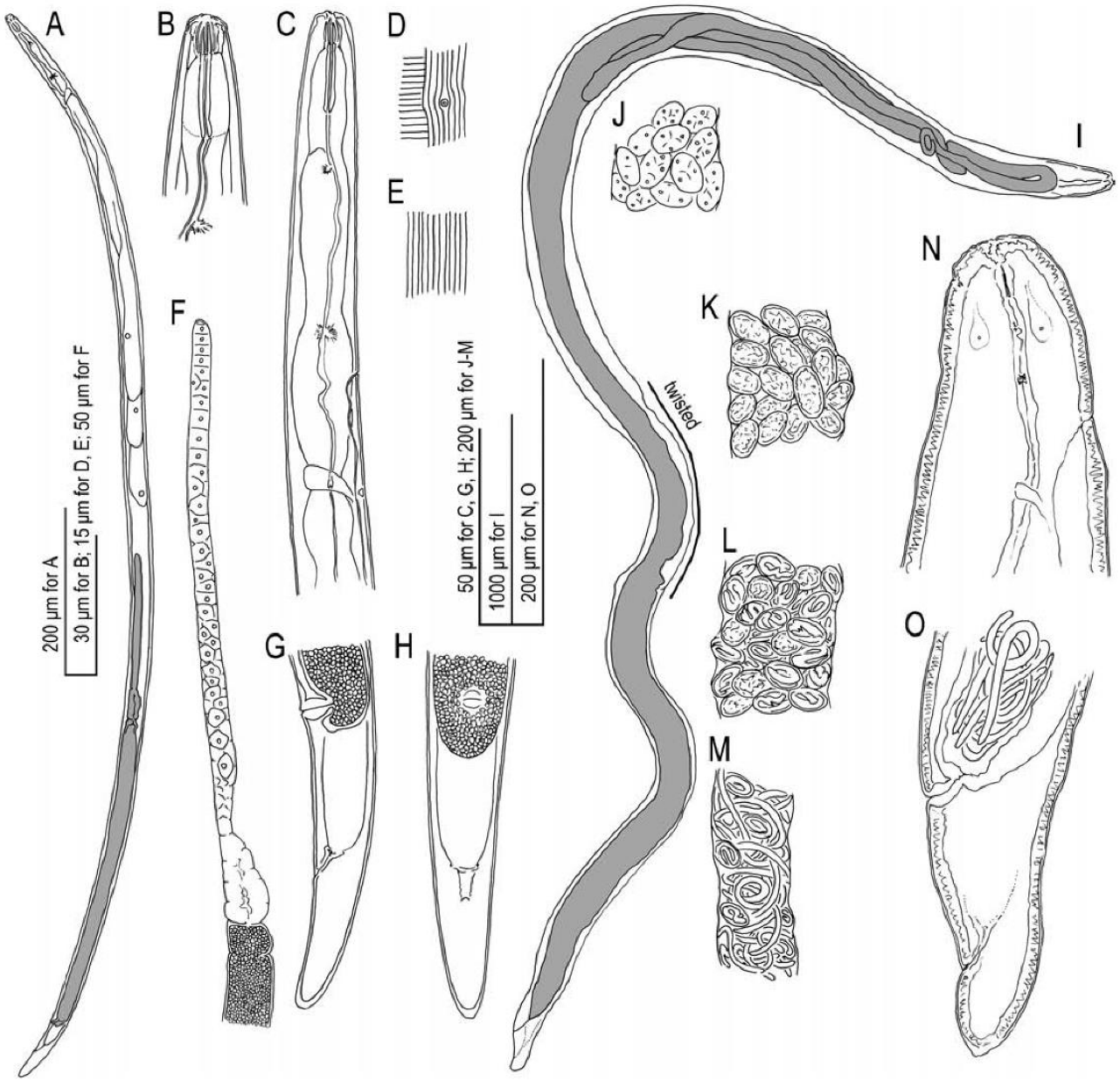

Fig. 3. Infective and parasitic females of *Deladenus nitobei* n. sp. A: Entire infective female, left lateral view; B: Lip-stylet region of infective female, left lateral view; C: Anterior part of infective female, right lateral view; D: Body surface showing deirid, lateral field and annulations; E: Lateral field at mid-body; F: Anterior part of gonad (ovary to anterior end of uterus); G, H: Vulval and anal region, left lateral view (G) and ventral view (H); I: Entire body of parasitic female (the specimen was twisted at mid-body, showing right lateral side of anterior part and left lateral side of posterior part); J-M: Embryo in uterus from anterior to posterior part, where the uterus contains single- and two-cell stages in anterior region (J), developing embryo in multiple-celled stage (K) or forming juveniles (L) in middle section, and hatched juveniles (M) in posterior region; N: Anterior end of parasitic female, right lateral view; O: Vulval and anal region of parasitic female, left lateral view.

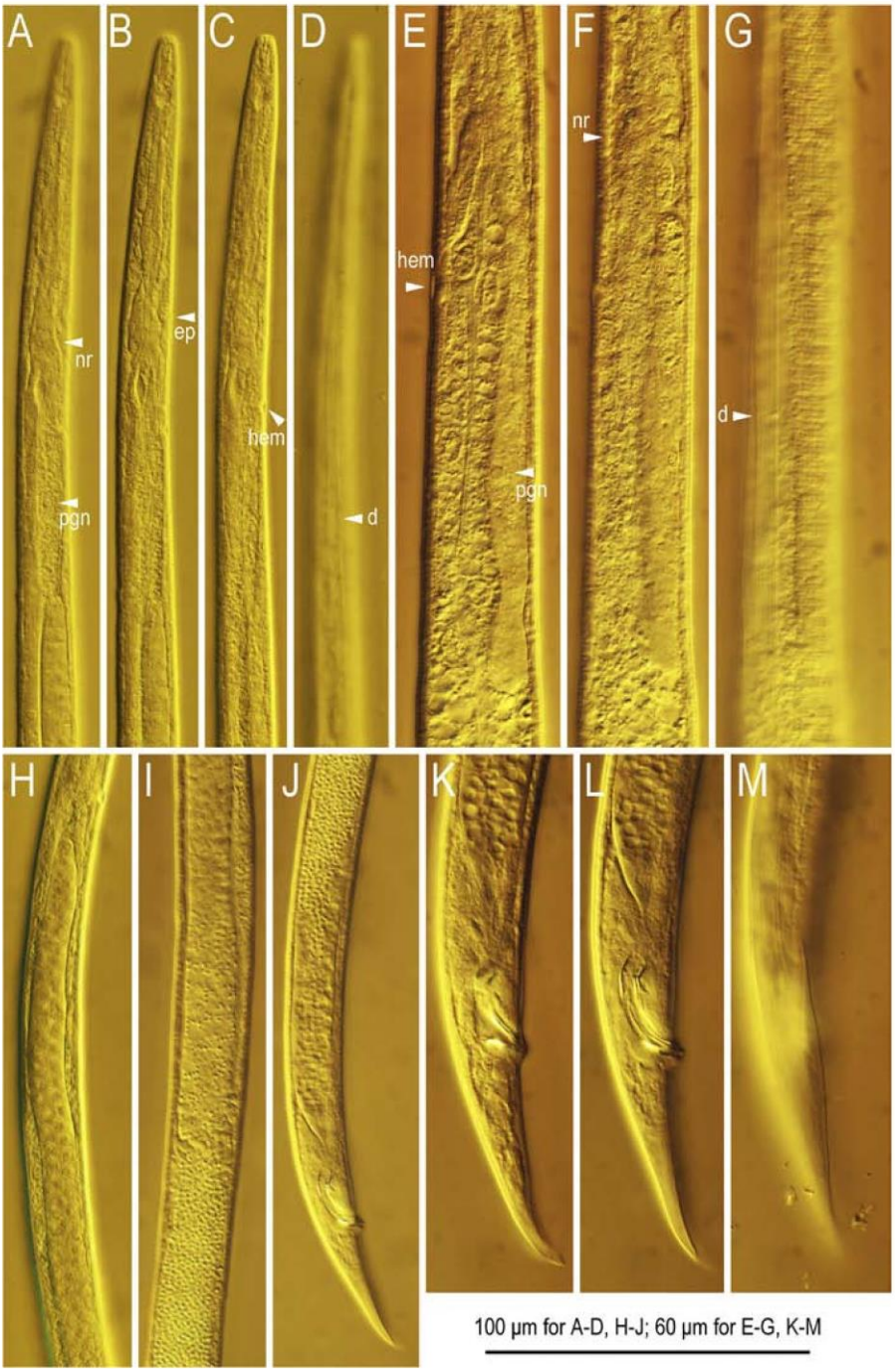

Fig. 4. Mycophagous male of *Deladenus nitobei* n. sp. A-D: Anterior region, right lateral view in different focal planes; E-G: Close-up of nerve ring-pharyngeal gland part, left lateral view in different focal planes; H: Anterior part of testis; I: Middle-posterior part of testis producing small sperm; J: *Vas deferens* containing small sperm, right lateral view; K-M: Male tail, right lateral view showing tail tip (K), spicule and gubernaculum (L) and bursa (M). Abbreviations: d = deirid; ep = excretory pore; hem = hemizonid; nr = nerve ring; pgn = pharyngeal gland cell nucleus. This figure is published in colour in the online edition of this journal, which can be accessed *via* http://booksandjournals.brillonline.com/content/journals/15685411.

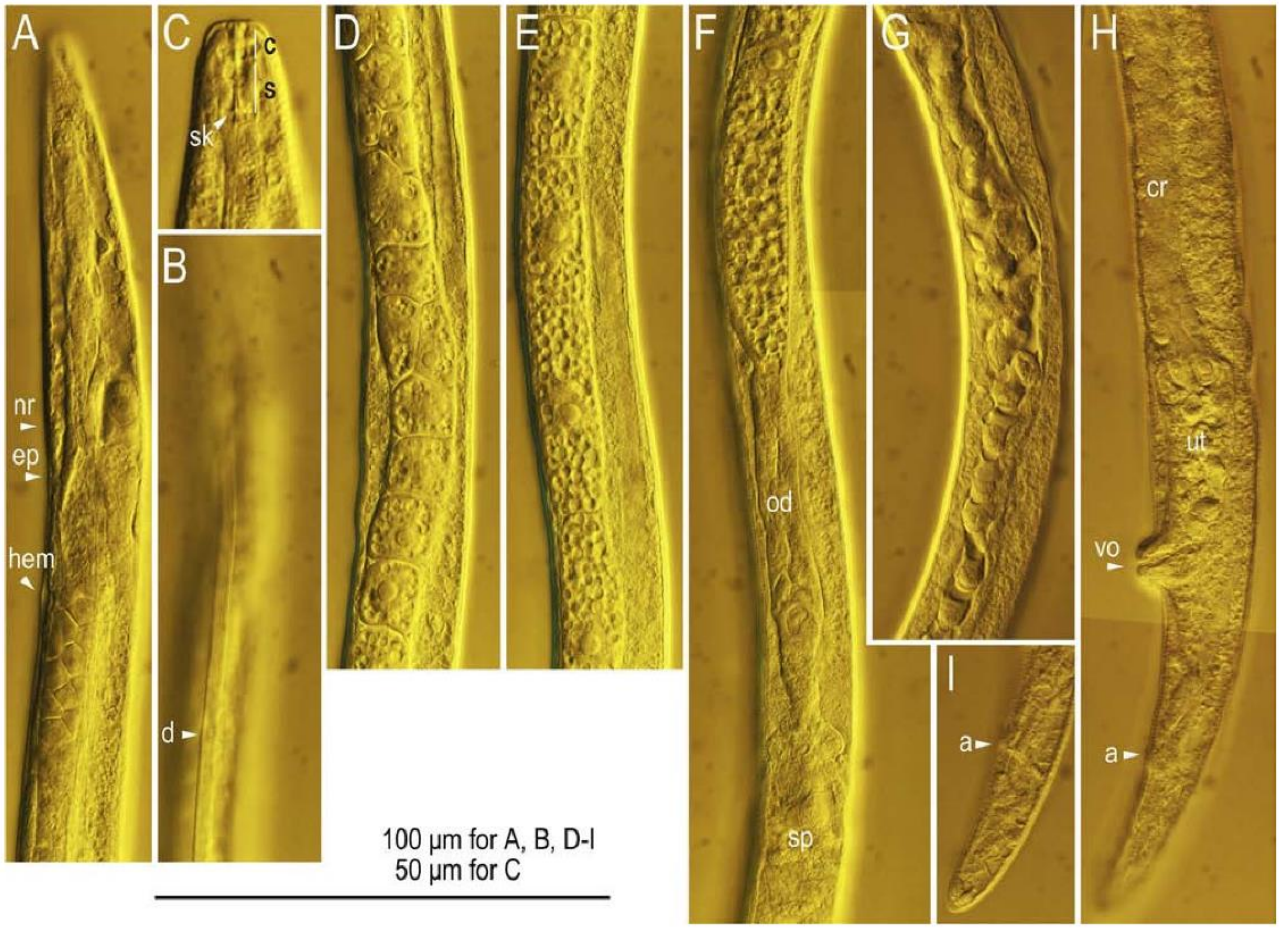

Fig. 5. Mycophagous female of *Deladenus nitobei* n. sp. A, B: Anterior region, left lateral view in different focal planes showing anterior end of ovary (A), lateral field and relative position of deirid (B); C: Lip region, right lateral view; D, E: Anterior (D) and middle (E) part of ovary containing developing oocytes; F: Posterior part of ovary, oviduct and anterior end of spermatheca; G: Spermatheca containing large amoeboid sperm; H: Posterior part of body, left lateral view; I: Tail, left lateral view. Abbreviations: a = anus; c = stylet conus; cr = crustaformeria; d = deirid; ep = excretory pore; hem = hemizonid; nr = nerve ring; od = oviduct; s = stylet shaft; sk = stylet knob; sp = spermatheca; ut = uterus; vo = vulval opening. This figure is published in colour in the online edition of this journal, which can be accessed *via* http://booksandjournals.brillonline.com/content/journals/15685411.

tion tissue. Spermatheca long tube, not branched (= part of gonadal tube), with thin wall, filled with large sperm. Uterus a thick-walled tube, sometimes containing an egg. Vagina a slightly inclined anteriorly, with muscular constriction at uterus-vulva junction. An X-shaped vulval muscle observed in ventral view. Vulva located in very posterior part of body, *i.e.*, V = 94.9-95.9, forming a horizontal slit. Vulval region clearly offset, in lateral view body narrowing immediately after vulval region. Rectum and anus present, functional. Rectum *ca* one anal body diam. in length, with muscular constriction at intestine-rectal junction. Anus a small dome-shaped slit at *ca* three-fifths vulva-tail tip length posterior to vulval opening. Tail short, broad with rounded terminus.

Infective female

Mid-sized for genus, *i.e.*, 1250-1583 μm long. Body cylindrical, slender, smoothly ventrally arcuate when killed by heat treatment. Cuticle thin, annulated, lateral field with more than ten incisures at mid-body. Deirid clearly observed laterally in middle of lateral field at *ca* 1.5 body diam. posterior to hemizonid. Lip region not clearly offset, *i.e.*, head dome-shaped. Because of robust stylet, stomatal opening surrounded by sclerotised cepha-

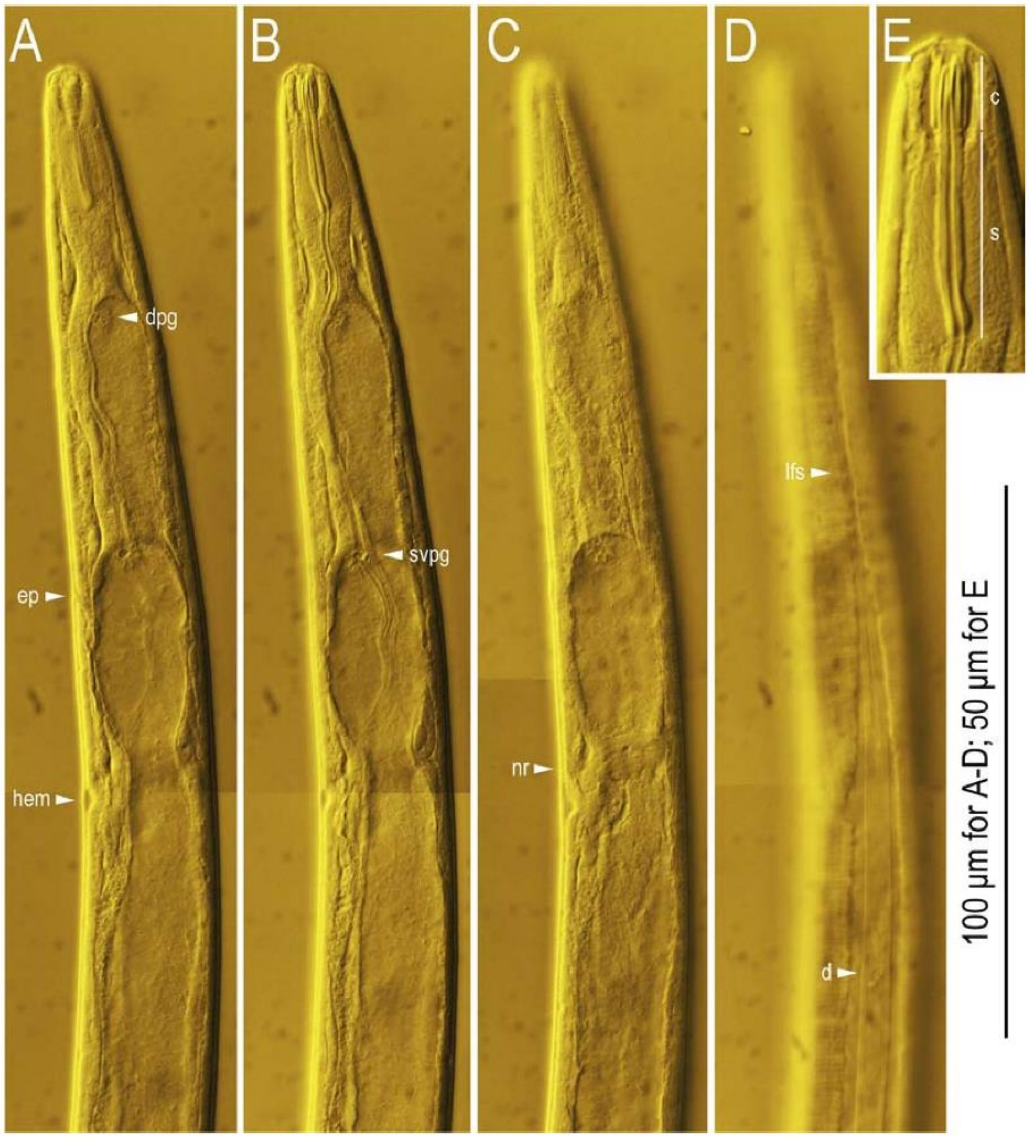

Fig. 6. Infective female of *Deladenus nitobei* n. sp. A-D: Anterior region, left lateral view in different focal planes; E: Lip region and stylet, left lateral view. Abbreviations: c = stylet conus; d = deirid; dpg = dorsal pharyngeal gland orifice; ep = excretory pore; hem = hemizonid; lfs = start of lateral field; nr = nerve ring; s = stylet shaft; svpg = subventral pharyngeal glands orifice. This figure is published in colour in the online edition of this journal, which can be accessed *via* http://booksandjournals.brillonline.com/content/journals/15685411.

lic framework occupying *ca* one-third of lip diam. Stylet with moderately wide lumen and thick stylet wall, comprising a short cone, *ca* 25% of total stylet length, dorsal side slightly longer than ventral side, shaft with slightly dorsally bent posterior end, a feature considered to be a basal knob. Dorsal gland orifice clearly visible at *ca* one stylet length posterior to stylet knobs. Subventral gland orifice clearly visible at two-thirds of pharynx length posterior to stylet base. Pharynx not muscular, forming broad tube, without distinct median bulb. All three glands (dorsal gland and two subventral glands) very well developed, located on dorsal side of body, *i.e.*, subventral glands extending dorsally, and thus all three gland cell nuclei observed on dorsal side of body. Excretory pore ventral,

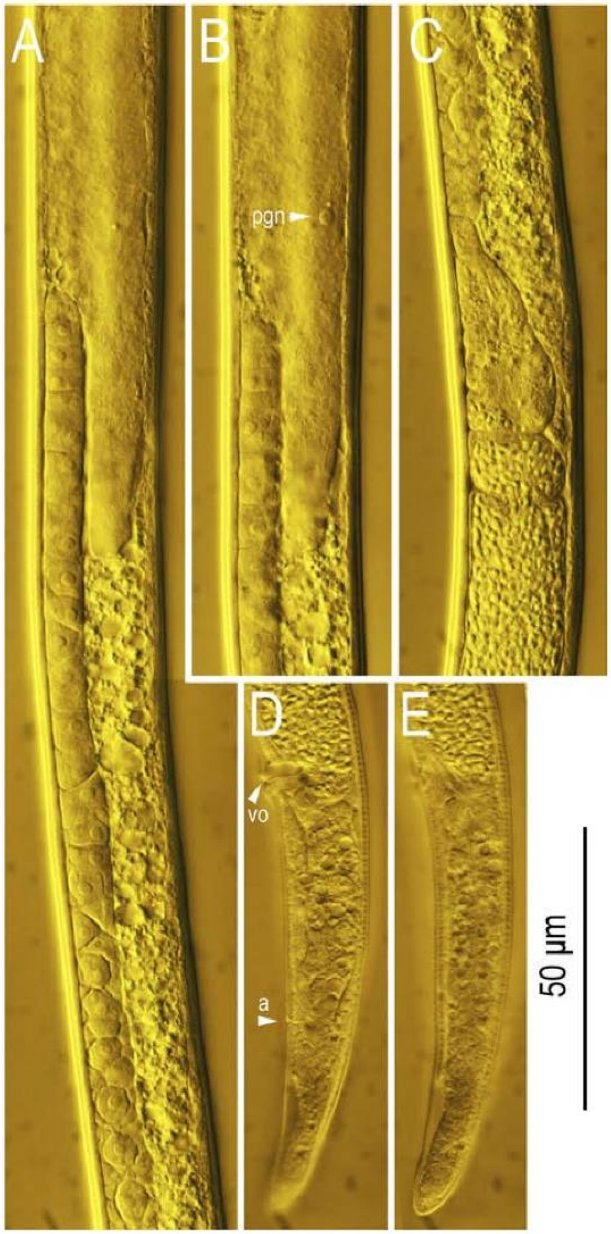

Fig. 7. Infective female of *Deladenus nitobei* n. sp. A, B: Anterior region of ovary in different focal planes showing relative position of ovary and pharyngeal gland cell nucleus; C: Mid-region of gonad (posterior end of ovary to anterior part of uterus); D, E: Vulva to anus region, left lateral view in different focal planes. Abbreviations: a = anus; pgn = subventral pharyngeal gland nucleus; vo = vulval opening. This figure is published in colour in the online edition of this journal, which can be accessed *via* http://booksandjournals.brillonline.com/content/journals/15685411.

0.5-1.0 body diam. posterior to subventral gland orifice. Nerve ring and vague pharyngo-intestinal junction either overlapping or junction sometimes slightly posterior to nerve ring. Hemizonid ventrally visible at *ca* 1.5 max. body diam. posterior to excretory pore or slightly posterior to nerve ring. Gonad single, on right of intestine, anteriorly outstretched, reaching to level of mid-body. Gonad organs arranged as ovary, ovary-uterus connective tissue assumed to be derived from oviduct, spermatheca and crustaformeria, uterus and vagina/vulva. Oocytes arranged in single to double row(s) in entire ovary. Connective tissue comprising various sizes of rounded cells, but its structure not clearly separated into organs. Remainder of gonad seemingly consisting of a uterus filled by small sperm, posterior end of which expanding posteriorly beyond vulva/vagina to form a post-uterine sac-like expansion. Vagina perpendicular to body axis or slightly inclined anteriorly, surrounded by a little sclerotised tissue. Vulva located very posteriorly, *i.e.*, V = 93.8-94.8, in form of a short horizontal slit. Rectum and anus present, not degenerate. Rectum slightly less than one anal body diam. in length, with muscular constriction at intestine-rectal junction. Anus a small dome-shaped slit located at *ca* 60% of vulva-tail tip distance posterior to vulval opening. Tail short, broad with rounded terminus.

Parasitic female

Mid-sized for genus, *i.e.*, 4574-8298 μm long. Body elongated-obese, cylindrical. Almost all parts of body occupied by gonad as in other parasitic females in insect-parasitic tylenchids. Cuticle with thin surface later having coarse and very shallow annulations, often very difficult to observe by light microscopy, and a soft, thick inner layer that likely facilitates nutrient uptake. Lateral field and deirid not observed, probably obscured or lost by expanding cuticle. Head region around stomatal opening overgrowing aperture, the latter therefore appearing to be sunken into head region, overgrown tissue seemingly separated into several sections, but not clearly observed. Stylet seemingly separated into conus and shaft, retracted into body, detailed structure and length of each section of stylet not observed as masked by overgrown tissue of head region. Two anterior glands observed at level of dorsal pharyngeal gland orifice. Dorsal pharyngeal gland orifice present at *ca* one stylet length posterior to stylet base. Subventral gland orifice visible *ca* three stylet lengths posterior to stylet knob. Simple tube-like pharynx observed, but unclear. Nerve ring not clearly observed in some individuals, located about 1-2 body diam. from anterior end. Excretory pore observed in some

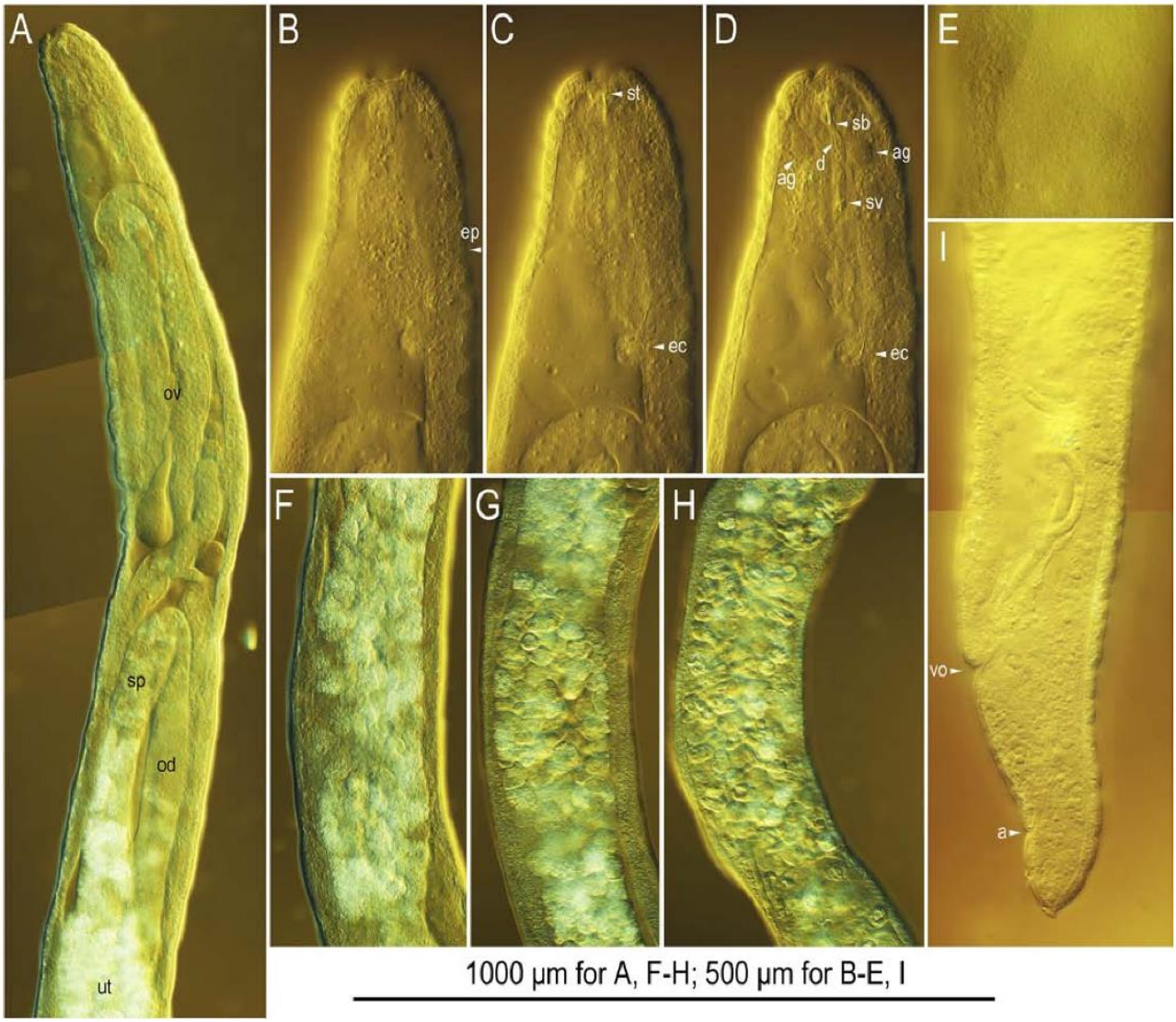

Fig. 8. Parasitic female of *Deladenus nitobei* n. sp. A: Anterior region, right lateral view; B-D: Head to pharynx region in different focal planes; E: Body surface showing weak and coarse annulation; F-H: Embryos deposited in anterior to posterior part of uterus; I: Vulval and anal region, left lateral view. Abbreviations: a = anus; ag = anterior glands; d = dorsal pharyngeal gland orifice; ec = excretory cell; ep = excretory pore; od = oviduct; ov = ovary; sb = stylet base; sp = spermatheca; st = stylet tip; sv = subventral pharyngeal glands orifice; ut = uterus; vo = vulval opening. This figure is published in colour in the online edition of this journal, which can be accessed *via* http://booksandjournals.brillonline.com/content/journals/15685411.

individuals, located just posterior to level of subventral gland orifice. Hemizonid not observed. Gonad very long, comprising ovary, oviduct, spermatheca, uterus and vagina/vulva. Ovary long, tangled in anterior part of body with multiple rows of oocytes. Oviduct a simple, broad tube. Spermatheca short, simple, connecting oviduct and uterus, seemingly not functional. Uterus occupying majority of gonad (= majority of nematode body), harbouring thousands of embryos from single-celled stage to hatched juveniles, *i.e.*, parasitic female is ovoviviparous. Vagina clearly inclined anteriorly, uterus-vulval junction not clearly observed. Vulva open, located very posteriorly, *i.e.*, V = 97.0-97.9. Rectum and anus present, seemingly non-functional, *i.e.*, intestine-rectal junction not clear and

Table 1. Morphometrics of *Deladenus nitobei* n. sp. All measurements are in μm and in the form: mean ± s.d. (range).

Character	Mycophagous female		Mycophagous male	Infective female	Parasitic female
	Holotype	Paratypes	Paratypes	Paratypes	Paratypes
n	–	9	10	10	5
L	1875	1842 ± 90 (1708-2000)	1538 ± 110 (1375-1708)	1433 ± 115 (1250-1583)	6660 ± 1407 (4574-8298)
a	57.8	60.4 ± 3.7 (54.6-65.2)	64.3 ± 4.7 (55.8-74.1)	52.2 ± 4.6 (42.6-56.9)	30.5 ± 4.3 (25.0-36.8)
b	26.3	26.0 ± 1.6 (23.7-28.5)	21.5 ± 1.6 (19.5-24.3)	15.0 ± 1.2 (13.5-17.5)	N/A[1]
c	54.4	50.8 ± 4.3 (42.7-56.9)	37.7 ± 3.4 (31.6-42.7)	40.5 ± 3.3 (34.8-45.0)	108.3 ± 15.0 (89.7-128.0)
c′	2.3	2.4 ± 0.2 (2.2-2.6)	3.0 ± 0.2 (2.7-3.3)	2.9 ± 0.4 (2.4-3.5)	1.2 ± 0.1 (1.1-1.3)
T or V	95.3	95.4 ± 0.3 (94.9-95.9)	84.3 ± 3.4 (80.6-90.5)	94.4 ± 0.3 (93.8-94.8)	97.4 ± 0.3 (97.0-97.9)
M	38.5	37.3 ± 2.4 (35.7-42.8)	36.9 ± 2.2 (33.3-38.5)	28.8 ± 2.4 (25.6-34.2)	30.1 ± 2.6 (27.6-34.5)
Max. body diam.	32	31 ± 3.0 (26-36)	24 ± 2.0 (21-27)	28 ± 3.8 (23-35)	217 ± 31 (183-266)
Stylet length	9.0	9.4 ± 0.3 (9.0-9.7)	9.0 ± 0.5 (8.3-9.7)	26 ± 0.8 (24-27)	25 ± 0.7 (24-26)
Stylet conus	3.4	3.5 ± 0.2 (3.4-4.1)	3.3 ± 0.3 (2.8-3.4)	7.4 ± 0.7 (6.9-9.0)	7.5 ± 0.7 (6.8-8.5)
Dorsal pharyngeal gland orifice from anterior	29	28 ± 2.0 (25-32)	27 ± 2.3 (23-30)	34 ± 2.0 (30-36)	61 ± 21 (43-94)
Excretory pore from anterior	80	77 ± 5.4 (65-85)	69 ± 3.4 (65-76)	76 ± 8.1 (65-89)	133 ± 26 (104-162)
Nerve ring from anterior	175	186 ± 8.7 (175-199)	198 ± 11 (186-219)	96 ± 4.9 (90-106)	196, 219[2]
Hemizonid from anterior	106	102 ± 4.2 (95-107)	94 ± 4.3 (85-99)	102 ± 8.0 (90-114)	N/A[3]
Subventral pharyngeal gland orifice from anterior	117	114 ± 6.4 (100-123)	121 ± 12 (100-136)	68 ± 2.9 (62-72)	109 ± 23 (89-147)
Excretory pore to hemizonid	25	25 ± 3.8 (18-30)	25 ± 2.8 (19-28)	27 ± 4.7 (22-36)	N/A[3]
Vulval body diam.	27	29 ± 2.3 (27-34)	–	20 ± 3.4 (17-26)	85 ± 24 (60-111)
Cloacal/anal body diam.	15.2	15.3 ± 0.6 (14.5-16.6)	13.7 ± 0.7 (12.4-14.5)	12.3 ± 0.5 (11.7-13.1)	53 ± 9.6 (43-66)
Tail	35	36 ± 1.8 (34-40)	41 ± 2.3 (35-43)	36 ± 4.6 (30-46)	62 ± 15 (47-83)
Spicule (chord)	–	–	47 ± 1.6 (44-49)	–	–
Spicule (curved median line)	–	–	40 ± 1.8 (38-44)	–	–
Gubernaculum (chord)	–	–	17.5 ± 1.7 (16.0-20.3)	–	–

[1] Pharyngo-intestinal junction not observed clearly.
[2] Nerve ring observed in only two individuals.
[3] Hemizonid not observed.

degenerate. Tail short, broad with rounded, short conical terminus.

Type host and locality

Parasitic females and juveniles of *D. nitobei* n. sp. were isolated from the ovary of *S. nitobei* which emerged from a log of dead Japanese red pine, *P. densiflora*, in July 2015. The logs were collected at Takko, Aomori, Japan (40°17′07.52″N; 141°05′59.33″E; 373 m a.s.l.), in May 2015. The male, mycophagous female and infective female materials were collected from cultured material derived from the parasitic juveniles isolated from *S. nitobei*.

Type material

Holotype mycophagous female, four paratype mycophagous females, five paratype males, five paratype infective females and five paratype parasitic females deposited in the USDA Nematode Collection, Beltsville, MD, USA, with USDANC collection numbers *Deladenus nitobei* T-696t (holotype mycophagous female), T-6798-6801p (paratype mycophagous females), T-6802-6806p (paratype males), T-6807-6811p (paratype infective females) and T-6812-6816p (paratype parasitic females); and five paratype mycophagous females, five paratype males and five paratype infective females deposited in the Forest Pathology Laboratory Collection, FFPRI, Tsukuba, Japan, with collection numbers PF01-05 (paratype mycophagous females), *Deladenus nitobei* PM01-05 (paratype males) and IF01-05 (paratype infective females). In addition to type materials, several more unmounted specimens of all four morphs (in dehydrated glycerin) are available from the Forest Pathology Laboratory Collection FFPRI (N. Kanzaki) upon request.

Diagnosis and relationships

In addition to its generic characters, the new species is characterised by the relative position of the excretory pore in the mycophagous forms, which is 25 (19-28) and 25 (18-30) μm anterior to the hemizonid in the male and female, respectively, and by the shape of the mycophagous female tail, which is broad with a rounded tip.

The species belonging to the genus *Deladenus* have four adult forms, but not all four forms have been described for all species, *i.e.*, in many cases, only the mycophagous male and female forms have been described. Based on the description of type material and/or cultured material, Chitambar (1991) evaluated 37 morphological characters for their taxonomic and diagnostic significance. Most of the previously suggested diagnostic characters were concluded to be unreliable, *i.e.*, most of the characters have too large variation within the species, which overlap among the species, or the typical characters were likely to be the results of artefacts (distortion or deformation), which occurred during fixation and/or treatment of material. The characters considered to be valuable were: *i*) the position of the excretory pore in relation to hemizonid (anterior to or posterior to hemizonid); *ii*) the distance between hemizonid and the excretory pore; and *iii*) the female tail tip morphology (although the female tails still vary within each species and overlap among several species). For these reasons the typological comparison was first conducted based on these three characters of the mycophagous males and females, after which we examined other characters in the most closely related species.

Based on the position of the excretory pore, *i.e.*, 18-30 (19-28 in male; 18-30 in female) μm anterior to the hemizonid, the newly described species overlaps with the following eight species, *D. apopkaetus* Chitambar, 1991, *D. canii*, *D. cocophilus* Nasira, Shahina & Firoza, 2013, *D. indicus* Singh, 1976, *D. ipini* Massey, 1974, *D. siricidicola* and *D. valveus* Yu, Popovic & Gu, 2014 (Bedding, 1968, 1974; Massey, 1974; Singh, 1976; Chitambar, 1991; Nasira *et al.*, 2013; Yu *et al.*, 2014).

Deladenus nitobei n. sp. is distinguished from *D. apopkaetus* by the relative position of the excretory pore (overlapping with or posterior to *vs* anterior to nerve pharyngo-intestinal junction/nerve ring), the number of lateral lines at mid-body (>10 *vs* 6-7) and female tail shape (posterior part consistently broad *vs* somewhat conical) (Chitambar, 1991); from *D. cocophilus* by the shape of the stylet knob in mycophagous adults (small and rounded *vs* wing-like) and in the infective female (dorsally bent and somewhat squared *vs* large ring-shaped), and female tail shape (posterior part consistently broad *vs* somewhat conical) (Nasira *et al.*, 2013); from *D. indicus* by the number of lateral lines (<14 *vs* 22) (Singh, 1976; Chitambar, 1991) (the original description of *D. indicus* (Singh, 1976) was not available for the present study and we used the information provided in Chitambar, 1991); from *D. ipini* by the absence *vs* presence of a post-uterine sac (Massey, 1974); and from *D. valveus* by the female tail shape (posterior part consistently broad *vs* conical with pointed tip) (Yu *et al.*, 2014). Based on the typological

characters, *D. nitobei* n. sp. overlaps with two other species belonging to the *D. siricidicola* superspecies, namely *D. siricidicola* and *D. canii* (Bedding, 1968, 1974). These three species share the relative position of the excretory pore to hemizonid, *i.e.*, 19-30, 21-60 and 21-70 μm anterior to hemizonid, and all three species have a broad female tail (Bedding, 1968, 1974). However, the new species can be distinguished from both close relatives by the molecular markers, in particular the mtCOI marker (see below).

Molecular profiles and phylogeny

Newly-determined sequences were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) with accession numbers KX365181 and KX365194 for SSU + ITS + D1-D2-D3 LSU and mtCOI of type strain, respectively, and KX365170-KX365180 and KX365182-KX365193 for ITS and mtCOI of 11 parasitic females picked up from different host individuals.

Molecular phylogenetic analysis based on SSU suggested that new species belongs to the *Deladenus* clade within the Tylenchomorpha entomoparasites with 100% bootstrap support, but the status within the genus was not clearly resolved (Fig. 9). On the other hand, the analysis based on mtCOI to estimate the species status within closely related species, *D. nitobei* n. sp. showed clear separation between *D. canii*, *D. siricidicola* and *D. proximus* (Fig. 10), *i.e.*, the intraspecific variations were less than 0.5% in each species, while the interspecific variation between new species and two close relatives was 3.8-4.0%, which is higher than that between *D. siricidicola* and *D. canii* (2.3%).

Discussion

Phylogeny and taxonomic status

The *D. siricidicola* superspecies currently contains five species, namely *D. siricidicola*, *D. canii*, *D. imperialis* Bedding, 1974, *D. rudyi* Bedding, 1974 and *D. nitobei* n. sp. Amongst these five species, *D. siricidicola*, *D. canii* and *D. nitobei* n. sp. are not clearly distinguished from each other by typological characters. Bedding (1974) suggested the distance between the excretory pore and hemizonid, as well as some morphometrics, as being diagnostic characters to separate *D. siricidicola* and *D. canii*, but the characters overlapped in the extensive analysis by Chitambar (1991). Thus, *D. siricidicola*, *D. canii* and *D. nitobei* n. sp. form a cryptic species complex. However, Akhurst (1975) demonstrated the species-level difference between *D. siricidicola* and *D. canii* with several hybridisation experiments, *i.e.*, using the biological species concept. In the present study, we did not have authentic material of *D. siricidicola* or *D. canii* and thus could not conduct a hybridisation study. However, based on the mtCOI-based phylogenetic analysis, the level of variation between our new species and two close relatives (3.8-4.3%) is larger than the intraspecific variation of other species (<0.5%) and the difference between *D. siricidicola* and *D. canii* (2.3%). We consider these molecular differences as indicative of the species status of *D. nitobei* n. sp.

Further detailed examination of biological characters (*e.g.*, condition for production of infective stage) and hybridisation tests will doubtless corroborate the species status of *D. nitobei* n. sp.

Distribution of *Deladenus* spp. in Japan

Worldwide surveys of *Deladenus* species have been conducted by previous authors. For example, Bedding & Akhurst (1978) summarised the biogeographical information of the genus based on the literature and their own collections. Other authors have also more recently reported on the distribution and intraspecific variation of *Deladenus* spp. (*e.g.*, Yu *et al.*, 2009, 2011; Morris *et al.*, 2013).

In Japan, the type locality of *D. nitobei* n. sp., four *Deladenus* species have previously been reported, namely *D. nevexii* Bedding, 1974, *D. rudyi*, *D. siricidicola* and *D. wilsoni* (Akhurst, 1975; Bedding & Akhurst, 1978). Although detailed locality information within Japan was not given, *D. siricidicola* appeared to be widely distributed in Japan and associated with *S. nitobei* and *Xeris spectrum spectrum* (L.) (Siricidae) and *Pinus* spp., *i.e.*, sharing the same host woodwasp and tree species as *D. nitobei* n. sp. (Bedding & Akhurst, 1978). However, it is possible that all or some of the previously reported Japanese '*D. siricidicola*' records could actually be conspecific with *D. nitobei* n. sp., although it is not possible to confirm this hypothesis. *Deladenus rudyi* has been identified from four woodwasp species: *S. juvencus* L., *Urocerus japonicus* Smith, *U. antennatus* Marlatt and *X. s. spectrum*, and some Pinaceae and Cupressaceae trees; *D. nevexii* is associated with *X. s. spectrum*; and *D. wilsoni* was reported from many different genera of Pinaceae trees and the parasitic wasps, *Rhyssa jozana* Matsumura and *R. per-*

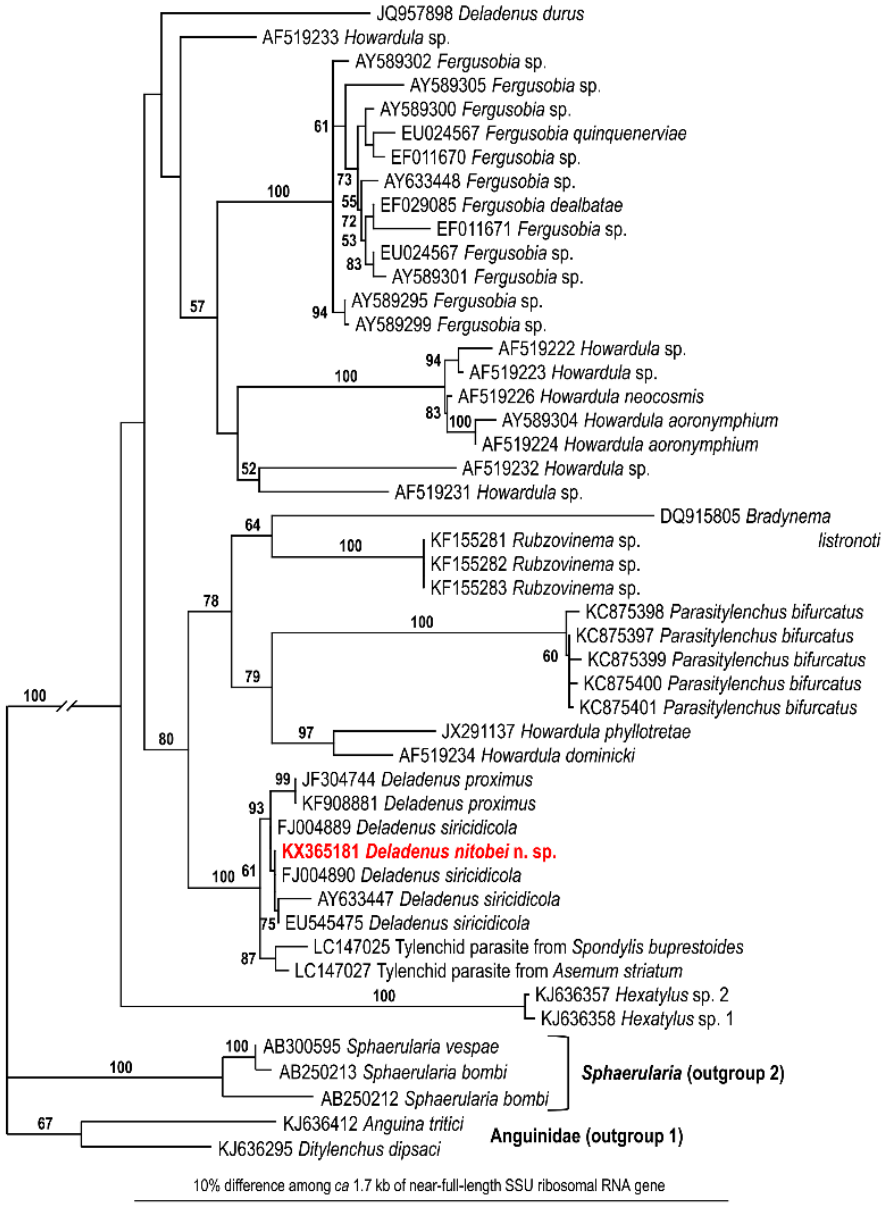

Fig. 9. Molecular phylogenetic relationship among several tylenchid insect-parasitic species. The Maximum Likelihood tree was inferred from near-full-length SSU. The substitution model selected was GTR + I + G (ln L = −7760.16, AIC = 15726.31) and parameters are: Pinvar = 0.451; Shape = 0.639. Bootstrap support exceeding 50% is shown for appropriate clades. This figure is published in colour in the online edition of this journal, which can be accessed *via* http://booksandjournals.brillonline.com/content/journals/15685411.

suasoria L., although the host woodwasp information in Japan has not been provided (Akhurst, 1975; Bedding & Akhurst, 1978). In addition to these above-mentioned species, Yushin *et al.* (2007) isolated a *Deladenus* sp. from *U. japonicus*. The latter species shares the host wasp species with *D. rudyi*, but it was distinguished by feeding preference, *i.e.*, *D. rudyi* has a clear preference for *A. chailletii*, while the *Deladenus* sp. was successfully cultured on *A. laevigatum* (Fr.) Boidin (Bedding & Akhurst, 1978; Yushin *et al.*, 2007).

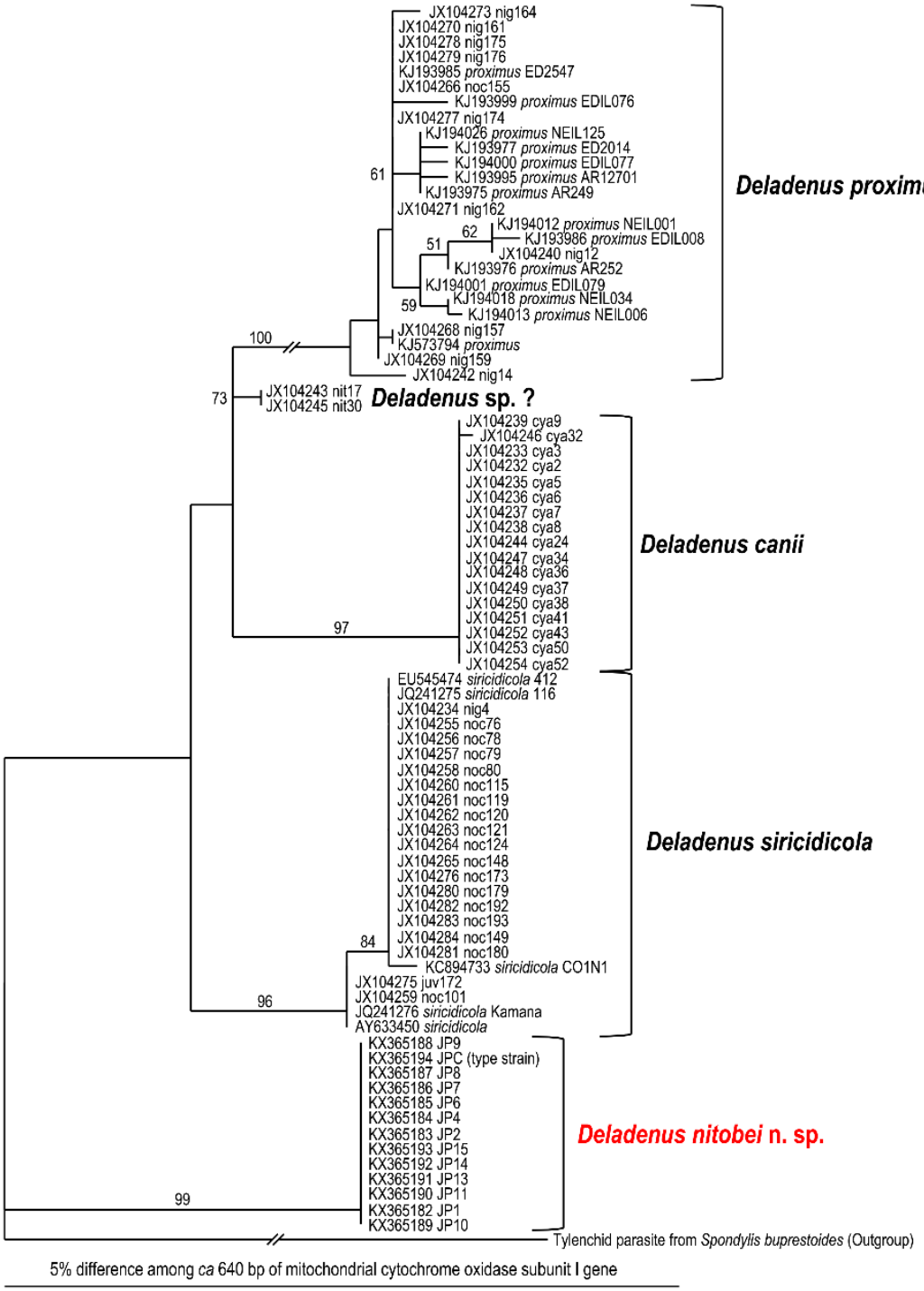

Fig. 10. Molecular phylogenetic relationship among several *Deladenus* species. The Maximum Likelihood tree was inferred from mitochondrial cytochrome oxidase subunit I sequences. The substitution model selected was GTR + G (ln L = −1853.06, AIC = 3998.13) and shape parameter is 0.175. Bootstrap support exceeding 50% is shown for appropriate clades. This figure is published in colour in the online edition of this journal, which can be accessed *via* http://booksandjournals.brillonline.com/content/journals/15685411.

In addition to the typological character (distance between the hemizonid and the excretory pore in mycophagous adults), *D. siricidicola* and the new species are distinguished from the other *Deladenus* species reported from Japan by their preferences in host wasp and food source. Bedding & Akhurst (1978) reported that *Deladenus* species were fungus-specific. For example, *D. rudyi* could feed and reproduce on *A. chailetii* and *D. siricidicola* was specific to *A. areolatum*, while only *D. wilsoni* could utilise both fungal species. A detailed field survey of these wasp-nematode-fungi associations is necessary to understand the bionomics of the new species and interspecific relationship, *e.g.*, competition, habitat segregation and/or host preference.

Acknowledgements

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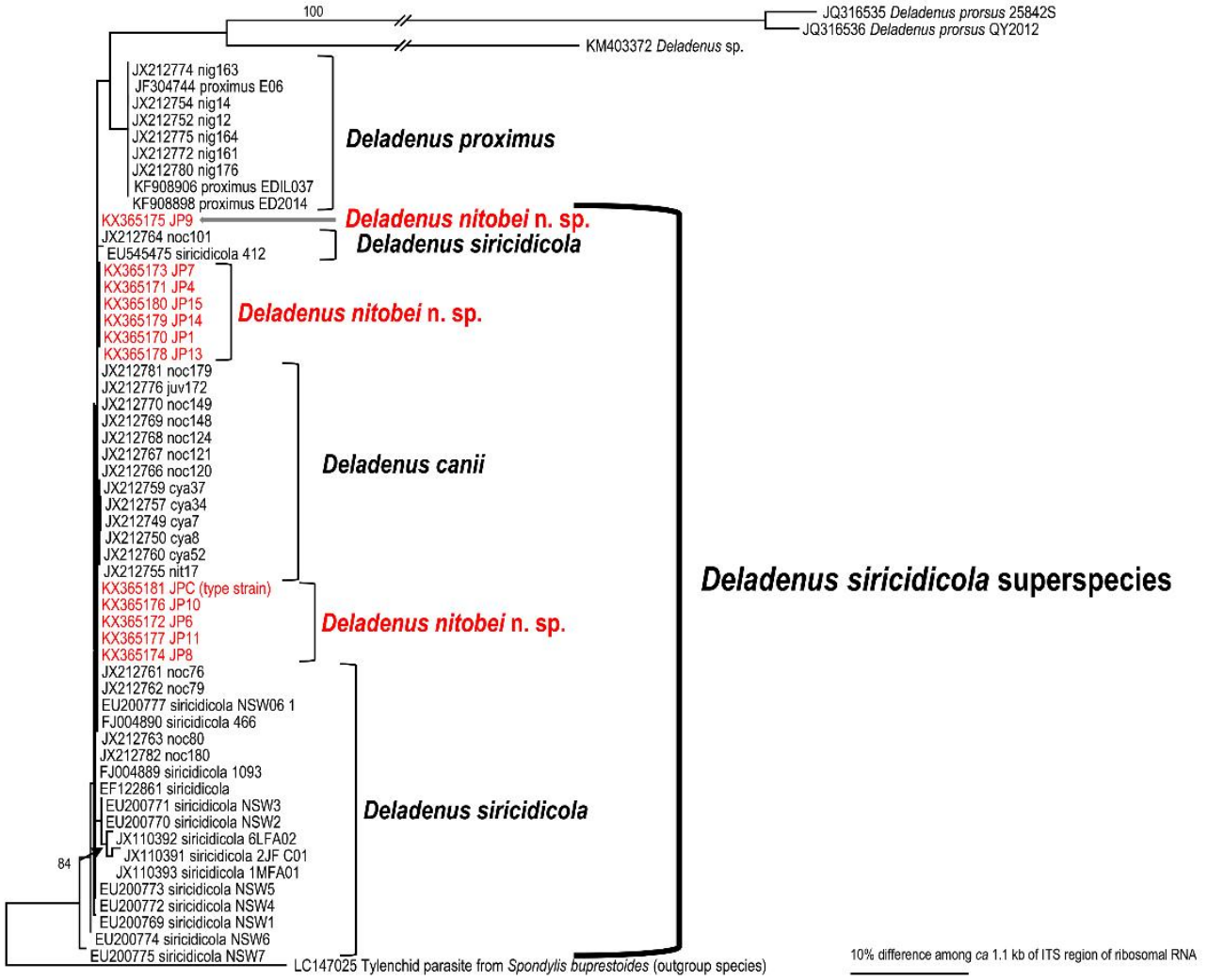

Fig. S1. Molecular phylogenetic relationship among several *Deladenus* species. The Maximum Likelihood tree was inferred from internal transcribed spacer region of ribosomal RNA. The substitution model was selected by JMODELTEST as HKY + G ($\ln L = -2898.38$, AIC = 6090.22) and shape parameter is 0.175. Bootstrap support exceeding 70% is shown for appropriate clades. This figure is published in colour in the online edition of this journal, which can be accessed *via* http://booksandjournals.brillonline.com/content/journals/15685411.

Supplementary paper B

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Population biology of the European woodwasp, *Sirex noctilio*, in Galicia, Spain

M.J. Lombardero[1]*, M.P. Ayres[2], F.E. Krivak-Tetley[2] and K.N.E. Fitza[3]

[1]Departamento de Producción Vegetal, Universidad de Santiago de Compostela, 27002 Lugo, Spain: [2]Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, USA: [3]Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0028, South Africa

Abstract

Sirex noctilio Fabricius (Hymenoptera, Siricidae) is rare and rarely studied where it is native in Eurasia, but is a widespread pest of pines in the Southern Hemisphere. Here we report on the abundance, basic biology, host use patterns and natural enemies of native *S. noctilio* in Galicia, Spain. Most trees attacked by *S. noctilio* failed to produce any adult progeny: >90% of emergences came from <20% of the attacked trees. The highest reproduction was in *Pinus pinaster*, followed by *Pinus sylvestris* and *Pinus radiata*. The proportions of *S. noctilio* requiring 1, 2 or 3 years for development were 0.72: 0.24: 0.04. Delayed development could be an adaptation to avoid parasitic nematodes, which sterilized 41.5% adults with one year generation time but only 19% of adults with 2 years generation time. Hymenoptera parasitoids accounted for 20% mortality. Sex ratios were male biased at 1: 2.9. Body size and fecundity were highly variable and lower than previously reported from the Southern Hemisphere. On attacked trees, there were 5–20 attacks per standard log (18 dm^2), with usually 1–3 drills per attack. Attack densities and drills per attack were higher in trees that subsequently died. The production of *S. noctilio* per log was positively related to total attacks, and negatively related to: (1) attack density, (2) incidence of blue stain from *Ophiostoma* fungi and (3) frequency of lesions in plant tissue around points of attack. A preliminary life table for S. *noctilio* in Galicia estimated effects on potential population growth rate from (in decreasing order of importance) host suitability, unequal sex ratio, parasitic nematodes and Hymenoptera parasitoids.

Keywords: abundance, *Amylostereum*, *Deladenus*, host suitability, invasive insect, invasive pest, *Pinus*, population ecology, sex ratio

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Introduction

Sirex noctilio Fabricius (Hymenoptera, Siricidae) is a woodwasp native to Eurasia (Taeger & Blank, 2008). It has been accidentally introduced into multiple locations in the Southern Hemisphere, where it has frequently become an important pest in plantations of pine species that originate from the Northern Hemisphere (Slippers *et al.*, 2012). Most recently, in 2004, the species was also discovered in the USA (Hoebeke *et al.*, 2005) and Canada (de Groot *et al.*, 2006). Despite a century of research and well-established ongoing management programmes, control of *S. noctilio* in the Southern Hemisphere remains unpredictable and spread continues to new areas (Slippers *et al.*, 2015). Understanding why a species is not a pest in one ecosystem can help to understand how it becomes a pest in other ecosystems (Ayres *et al.*, 2014).

S. noctilio larvae rely for their development on the alteration of wood by their specialized symbiotic fungus,

*Author for correspondence
Phone: 34 982 823 150
Fax: 34 982 285 926
E-mail: mariajosefa.lombardero@usc.es

Amylostereum areolatum (Chaillet ex Fries) Boidin, a basidiomycete (Talbot, 1977; Madden, 1988; Thompson *et al.*, 2014). This fungal species is inoculated into trees during oviposition, together with a phytotoxic venom containing noctilisin (Coutts, 1969*a*, *b*; Coutts & Dolezal, 1969; Bordeaux *et al.*, 2014). The combined action of the insect and its symbiont may cause up to 80% mortality in pine plantations of the invaded area (Talbot, 1977; Neumann *et al.*, 1987). Control strategies have included removal of infested trees and silvicultural practices that limit tree competition (Neumann *et al.*, 1987; Dodds *et al.*, 2007, 2014), but the most widely used strategy has been classic biological control (Hurley *et al.*, 2007). Several species of enemies have been introduced to control populations of *S. noctilio*. Hymenoptera parasitoids that have been introduced from the native range of *S. noctilio* include *Ibalia leucospoides leucospoides* (Hochenwarth) and *Rhyssa persuasoria persuasoria* (Linnaeus) and from North America *Megarhysa nortoni nortoni* (Cresson). These species have become established in most regions where they were introduced and are thought to play a role in reducing populations of *S. noctilio* (Hurley *et al.*, 2007; Collett & Elms, 2009; Cameron, 2012). However, the most widely employed control agent is the parasitic nematode *Deladenus* (*Beddingia*) *siricidicola* Bedding, which can produce parasitism of up to 90%, although with substantial variation among years and areas (Hurley *et al.*, 2007).

There has been great growth in knowledge of *S. noctilio* and its associated species in the Southern Hemisphere (e.g., Slippers *et al.*, 2012, 2015). However, there have been relatively few studies of *S. noctilio* in its native range and the species has been consistently characterized as rare and at most a secondary pest that colonizes suppressed trees or trees weakened from primary damage by other insects, fungi or abiotic effects. Ratzeburg (1844) reported woodwasps from a pine forest that was experiencing severe defoliation; he regarded them as secondary pests but suggested that they could occasionally kill trees if they reach high local abundance. Similarly, Hartig (1860) described woodwasps as far less important than bark beetles. Later descriptions from Europe have continued to describe siricids as mainly scavengers of trees dying for other reasons (Judeich & Nitsche, 1895; Chrystal, 1928; Benson, 1943; Wolf, 1969*a*). Spradbery & Kirk (1978) provided the most comprehensive study of *S. noctilio* in Europe. More recently, Tarasco & Notte (2003) reported some damage by *S. noctilio* in *Pinus pinea* in Italy, but Wermelinger *et al.* (2008) described them as restricted to greatly weakened trees.

There is similarly limited knowledge of siricids on the Iberian Peninsula. The most prominent works are by Hall (1968) and Spradbery & Kirk (1978). Previous literature referred to occasional records of Siricidae on the Iberian Peninsula, but often with questionable or erroneous identifications (Llorente & Gabuyo, 1984), as has also occurred in other areas of Europe (Chrystal, 1928). In Spain, *S. noctilio* and *S. juvencus* have often been confused (Ceballos, 1963). The main hosts of *S. juvencus* are various species of *Picea* (Wolf, 1969*a*; Spradbery & Kirk, 1978; Schiff *et al.*, 2012) and these tree species are not naturally distributed in Spain except as ornamentals in gardens and some small recent plantations in the Basque Country and Pyrenees (IFN4, 2013). According to Kirk (1974) and Spradbery & Kirk (1978), *S. noctilio* is the only species of *Sirex* occurring in the Mediterranean bioclimatic area. The Fauna Europaea Organization cites *S. juvencus* as absent in the Spanish mainland (Taeger & Blank, 2008).

Although it is frequently assumed that most invasive species exhibit enhanced performance in their introduced range, there are few tests (Parker *et al.*, 2013). In the case of *S. noctilio* these comparisons are currently impossible because the biology of native populations is too poorly known. Here we report studies of the abundance, basic biology, host use patterns and natural enemies of *S. noctilio* on the Iberian Peninsula, where it is native and rare. We address the question of why it is rare by summarizing the information in a preliminary life table that permits comparisons of effects on abundance from different sources. This information may help to understand what is different in the population dynamics of *S. noctilio* in the Southern Hemisphere, which may suggest strategies for control and management.

Material and methods

Study area

Our studies were conducted in Galicia (located in Northwestern Spain), which is a major region of commercial forestry. The most common pine species in the area are the native maritime pine (*Pinus pinaster* Aiton) which occupies 217,281 ha of pure stands, followed by the non-native *Pinus radiata* D. Don with 96,177 ha and finally the native *Pinus sylvestris* L. with 32,736 ha (IFN4, 2013). To locate study sites, we searched pine stands of all three species during spring of 2013 to locate stands supporting detectable populations of *S. noctilio* (as evidenced by emergence holes and resin drips from attacks by *S. noctilio*; Ayres *et al.*, 2014). We located populations of *S. noctilio* within four different stands with pine plantations in the province of Lugo. Two stands contained *P. pinaster*: Pena de Rodas (43.121°N, 7.679°W; tree age = 16 years, DBH ± SD of attacked trees = 12 ± 2 cm) and Cova da Serpe (43.097°N, 7.753°W; 23 years old, DBH = 12 ± 2 cm). One stand contained *P. radiata*: Begonte (43.201°N, 7.691°W; 19 years old, DBH = 10 ± 3 cm), and one contained *P. sylvestris*: Faro (42.631°N, 7.892°W; 23 years old, DBH = 11 ± 3 cm). In March of 2013, and again in March of 2014, we located individual trees within each stand that had been attacked by *S. noctilio* during the previous flight season (late summer or early autumn of 2012 or 2013). A total of 134 attacked trees were located based on external drippings of resin; we confirmed that the attacks were from *S. noctilio* based on characteristic drillings of the inner bark from female ovipositors. In July of 2013 and again in 2014, we noted whether or not each tree was still alive or had died since March, then felled the attacked trees, cut them into logs and transported them to emergence containers within a building in Lugo. The number of study trees in 2013 and 2014 (respectively) were 26 and 26 from Cova da Serpe, 23 and 0 from Pena de Rodas, 13 and 14 from Begonte, and 19 and 13 from Faro. The bole of each tree (from just above ground to the base of the live crown) was cut into 8–23 logs (depending on tree height) of 60–70 cm length. Each log was labelled individually to record its position in the bole of the tree. Before being placed in emergence cans, the logs were measured (length and diameter), waxed on both ends and examined to confirm the absence of emergence holes.

Genetic analyses of *S. noctilio*

A subset of the collected Galician wasps was sequenced to confirm species identity. This was done using the mitochondrial cytochrome *c* oxidase subunit I (COI) locus as described by Wooding *et al.* (2013). Sequences were deposited in

GenBank under accession numbers KT780457, KT780458, KT780459 and KT780460. Sequencing data were parsed by JModelTest v. 0.1.1 (Posada, 2008) to identify the best nucleotide substitution model, followed by the maximum likelihood analysis through PhyML 3.0.1 inputting the model parameters obtained (Guindon & Gascuel, 2003).

Flight period, natural enemies and body size

Cans were checked for emerging insects every two days after emergence started until late November when emergences ceased. We resumed checking for new emergences the following summer after it became evident that additional *S. noctilio* were emerging in the second flight season after oviposition. We collected all individual insects emerging from logs, including *S. noctilio*, its natural enemies and associates. For each adult *S. noctilio*, we measured body size with three metrics (length from head to cornus, tibia length, and pronotum width) and dissected the abdomens to search for parasitic nematodes in the gonadal tissue. We collected additional measurements on a subset of 46 females to count the total number of eggs produced and examine the eggs for infective nematodes.

Log dissections

In the spring of 2014, from the attacked trees that we felled in 2013, we examined and dissected a subset of 171 bolts from 11–13 trees per site. Most trees were represented by 2–3 logs, but for 1–3 trees per site we dissected all of the logs (9–13). Before dissection, we: (1) estimated the xylem moisture content (≈percentage) of each log as the average of 2 measurements per log using a Delmhorst RDM-3 moisture meter (Delmhorst Instrument Co. Towaco NJ); and (2) recorded the number of points on the surface of the log that had produced drippings of resin from drillings by *S. noctilio*. Then we removed the bark, counted the number of oviposition galleries from individual pairs of the bark beetle *Tomicus piniperda* (L.) (Coleoptera, Curculionidae), and counted and identified any other insects that had colonized the inner bark. Then, by examination of the full exterior surface of the xylem on each log, we marked and counted the individual attacks from *S. noctilio* (each from one female wasp at one location on the tree) and recorded the number of drillings (usually 1–3) associated with each attack. Each drilling was scored as filled with oleoresin or not. We also estimated the total proportion of surface area of each log with visible blue staining (characteristic of Ophiostomatoid fungi). Some of the drills from *S. noctilio* induced the formation of a visible lesion in the xylem tissue surrounding it. These lesions matched the description of Coutts & Dolezal (1966), who interpreted them as a barrier of polyphenols that represent a defensive reaction of the trees against the mutualistic fungus of *S. noctilio* (*A. areolatum*). We scored each drill for whether it had or had not induced a lesion. We recorded the number of exit holes from each log, each representing the emergence of an adult *S. noctilio* or an adult of one of its parasitoids, which had developed from feeding on a larva of *S. noctilio*. We also measured the diameter of exit holes.

After measurements were completed from the outer surface of logs, we cut the logs twice transversely (at ≈20% of the total length from each end) to expose two cross-sectional surfaces that could be examined for the characteristic white staining of wood ('white rot') from *A. areolatum* and the presence of blue stain from *Ophiostoma* (blue–grey colour from pigments produced by *O. minus* and *O. ips*). We took a digital photograph of both exposed surfaces on each log and analysed them with ImageJ software (Schneider *et al.*, 2012) to quantify the proportion of cross-sectional area occupied by both *A. areolatum* and *Ophiostoma* spp.

Statistical analyses

We used maximum likelihood estimation and log-likelihoods (VGAM package for R; Yee, 2015) to compare the fit of the frequency distribution of wasp emergence holes per tree to four competing binomial models: Poisson, zero-inflated Poisson, negative binomial and zero-inflated negative binomial. We evaluated patterns among sites and trees in the adult size of *S. noctilio* with a statistical model that included site, sex and individual trees within site, with trees as a random effect. We tested for differences among sites in sex ratio of *S. noctilio*, and the incidence of nematode parasites, with Pearson chi-square statistics from contingency analyses. We estimated the proportion of *S. noctilio* killed by wasp parasitoids as the slope of a regression (with intercept = zero) of number of emerging parasitoids per tree vs. the number of *S. noctilio* plus parasitoids emerging per tree (Cochran, 1977).

Except as noted, we analysed results from log dissections with a general linear model that included site, tree status (alive or dead) and tree (as a random effect, nested within site and tree status); logs were nested within tree to avoid pseudoreplication. To satisfy assumptions of normality, we applied square root transformations to log-specific measurements of attacks by *T. piniperda*, attacks by *S. noctilio*, total drills, attacks/dm^2, drills/dm^2, and total exit holes, and we applied arcsin transformations to proportions of log area (surface area and cross-sections) with blue stain, proportion of log cross-sections with white rot, and proportions of drills that were filled with resin or produced lesions. We compared the average number of drills per attack among sites with a linear regression ratio estimator (Cochran, 1977) using logs as sample units. To further evaluate hierarchical patterning in drills per attack, we also performed an analysis that explicitly considered each of 3,256 attacks distributed across 171 logs from 11 to 13 trees at each of four sites; the response variable, number of drills per attack, was evaluated with a generalized linear mixed model (Poisson link function) that included site, tree (as a random effect nested within site) and log within tree (as a random effect nested within site, tree status and tree) (Bates *et al.*, 2014). Except where indicated, other analyses were performed with JMP, version 11.0, SAS Institute, Cary, NC. Because we have unequal sample size we reported least-square means (lsmeans) throughout.

Results

Adult morphology and life history of *S. noctilio* in Galicia

Adult females from our study population had darkly tinted wings, black femora and pale tibiae, as has been described for other populations of *S. noctilio* from Southern Europe (Schiff *et al.*, 2012). Sequencing of the COI locus and subsequent maximum-likelihood analyses confirmed that the wasps obtained were *S. noctilio*.

Generation development time of *S. noctilio* in our study populations was usually 1 year (72%), but some individuals required 2 years (24%) or even 3 years (4%). In fall of 2013, 1

year after our study trees were attacked, we collected a total of 187 emerging adults (138 males and 49 females): median emergence dates (and 10th–90th percentiles) for males and females were 20 September (2 September–23 October) and 30 September (4 September–30 October), respectively. The next year, from 17 July to 7 September (logs held at room temperature), a second emergence occurred from the same logs (47 males and 15 females). During the winter of 2014–15, while dissecting the same logs for other measurements, we observed another ten *S. noctilio* larvae that were early to mid-instar (2–5 mm long) and destined to emerge no sooner than summer of 2015 (3 years after oviposition by parental females).

Adult body size was quite variable with females tending to be longer than males: overall means ± SE (10th–90th percentile) = 20.0 ± 0.5 (14–25) and 17.3 ± 0.3 (12–24) mm, respectively. Males emerging the 2nd year after attack (in 2014 from trees attacked in 2012) were larger than those that emerged from the same trees in the 1st year: mean ± SE = 14.3 ± 0.4 vs. 19.5 ± 0.8 mm for 1st- and 2nd-year emergence, respectively. However, female length did not differ between those emerging one vs. 2 years after oviposition: mean ± SE = 20.0 ± 0.7 vs. 20.5 ± 0.9 mm. There was no difference in body size of either sex between those emerging from trees attacked in 2012 vs. 2013. There were no apparent differences in body size among sites: $F_{3,137} < 1.1$, $P > 0.3$ for effects of site and site × sex (for adults emerging in 2013 from trees attacked in 2012, where all sites and both sexes were represented). There was modest variation among trees in male body size (23% of total variance; 95% confidence interval for variance = 0.3–11.2; $n = 172$ males from 21 trees) but not in female body size (8% of total variance; estimated variance = −2.8 to 5.5; $n = 64$ females from 14 trees). The dbh of trees was unrelated to adult body size ($P > 0.6$; $n = 14$ and 21 trees for females and males) (fig. 1, Supplemental materials).

The number of eggs per female was highly variable, ranging from 0 to 270 (mean ± SD = 88 ± 54), and was related as a power function to all three linear dimensions of body size: $Eggs = 0.010 \cdot BodyLength^{2.89}$ ($r^2 = 0.53$); $Eggs = 7.49 \cdot Pronotum^{2.01}$ ($r^2 = 0.33$); $Eggs = 1.83 \cdot Tibia^{2.37}$ ($r^2 = 0.39$) ($P < 0.0001$ and $n = 46$ females for all). Compared with females emerging 1 year after oviposition, females emerging 2 years after oviposition tended to have more eggs: 83 vs. 70 eggs given the same (average) body length of 22.1 mm ($t = 2.28$, df = 43, $P = 0.027$ for comparison of residuals; see fig. S1 in Supplementary Materials).

Overall sex ratio (females: males) of *S. noctilio* emerging from trees attacked in 2012 was 1: 2.9. Among the four plots, sex ratios ranged from 1: 2.3 (Begonte) to 1: 4.7 (Faro), but plots were not statistically distinguishable ($\chi^2 = 1.66$, df = 3, $P = 0.65$). The sex ratio did not differ between animals emerging 1 vs. 2 years after attack (1: 2.8 vs. 1: 3.1, respectively). The sex ratio of *S. noctilio* emerging from trees attacked in 2013 was similar to that of trees attacked in 2012 (1: 2.4).

Parasitism

We recorded two species of Hymenopteran parasitoids in Galicia: *I. l. leucospoides* (38 and 25 individuals from trees attacked in 2012 and 2013, respectively) and *R. persuasoria persuasoria* (26 from trees attacked in 2012). The first emergence of *I. l. leucospoides* (1 year after attack by *S. noctilio*) was slightly earlier than that of *S. noctilio*: median (10th–90th percentile) = 13 September 2013 (4 September–22 October) and 8 September 2014 (1 September–18 September). We also recorded emergence of some *I. l. leucospoides* 2 years after attack by *S. noctilio* (six individuals from trees attacked in 2012 emerging from 17 July to 7 September in 2014). Apparent parasitism of *S. noctilio* by *I. l. leucospoides* was 10 ± 3 and 6 ± 4% during emergence of 1 and 2 years, respectively, after attacks in autumn of 2012, and 30% during emergence of 1 year after attacks in autumn of 2013. Adults of *R. p. persuasoria* emerged about 1.5 years after attack by *S. noctilio* (during late April and early May of 2014 from trees attacked in fall of 2012); $n = 26$ individuals relative to 186 *S. noctilio* and 38 *I. l. leucospoides* emerging from the same material in the previous autumn.

We recorded frequent parasitism of *S. noctilio* by the nematode *Deladenus siricidicola*: 39% of 67 females and 36% of 181 males were parasitized, overall. The nematodes were sterilizing to females in that ≈90% of eggs within infected females contained 27–302 nematodes per egg. Animals parasitized by *D. siricidicola* were present at all four sites and in trees attacked in both 2012 and 2013. For animals emerging 1 year after attack, the frequency of parasitized adults was similar between years: 62 of 144 (43%) and 16 of 40 (40%) for trees attacked in 2012 and 2013, respectively. However, the frequency of parasitism by *D. siricidicola* was only 12 of 64 (19%) in adults emerging 2 years after attack. Parasitism was lower at Faro than at the other three sites: one of 14 adults at Faro vs. five of ten at Begonte, 36 of 89 at Cova da Serpe and 25 of 43 at Pena de Rodas ($\chi^2 = 11.80$, df = 3, $P = 0.008$). There was no difference in body size between parasitized and non-parasitized *S. noctilio:* $F_{1,177} < 1$, $P > 0.5$ for effects of nematodes and nematode × sex; restricted to *S. noctilio* emerging 1 year after attack).

Reproductive success by species and tree

Slightly over half of the trees attacked by *S. noctilio* in the fall of 2012 had died by the time we cut them in July of 2013: 8 of 13 *P. radiata* from Begonte; 8 of 19 of *P. sylvestris* from Faro, 14 of 26 *P. pinaster* from Cova da Serpe and 18 of 23 *P. pinaster* in Pena de Rodas. Of the 48 trees that died following attack by *S. noctilio* in the fall, 41 were colonized in the winter after attack by the bark beetle *T. piniperda*. The other seven trees did not show any other causes of death other than attack by *S. noctilio*. Among trees attacked in fall of 2013, 12 of 14 *P. radiata* (Begonte) and 12 of 13 *P. sylvestris* (Faro), had died by the time we cut them the following July, but mortality of *P. pinaster* (Cova da Serpe) was only ten of 26.

The production of adult progeny of *S. noctilio* was highly concentrated in a minority of the attacked trees (fig. 1). About 70% of the attacked trees produced 0 adult progeny. More than 90% of the progeny emerged from less than 20% of the attacked trees. The probability density function for production by tree was well described by a zero-inflated negative binomial (ZINB) with $\phi = 0.41$ (proportion of excess zeros), $\mu = 4.3$ (central tendency for production), and $k = 0.47$ (overdispersion parameter for production). Most emergences came from *P. pinaster*: 15 of the 23 trees from Pena de Rodas had emergences and 17 of 52 trees from Cova da Serpe. Only five of 27 trees from Begonte (*P. radiata*) produced *S. noctilio*, and only one of 32 trees from Faro (*P. sylvestris*). Only five of the 48 trees that were still alive in the summer after attack produced adults of *S. noctilio* or their parasitoids, and this only accounted for 9% of total emergence.

Dissection of logs

In dissecting 171 logs, we found 3,256 attacks with 6,115 drillings. The number of attacks per 18 dm^2 (the surface area

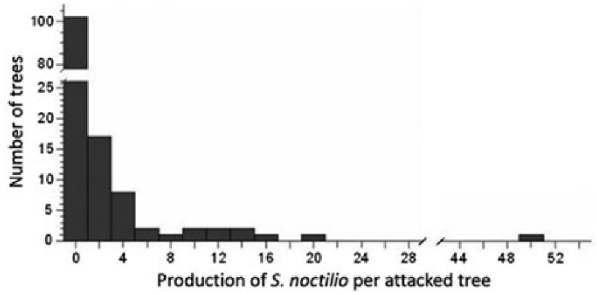

Fig. 1. Frequency distribution of the production of *S. noctilio* adults from attacked trees Data are for 139 trees representing four sites, three species and 2 years of attacks. Production per tree was standardized to the average volume of 50 dm^3 of logs per attacked tree.

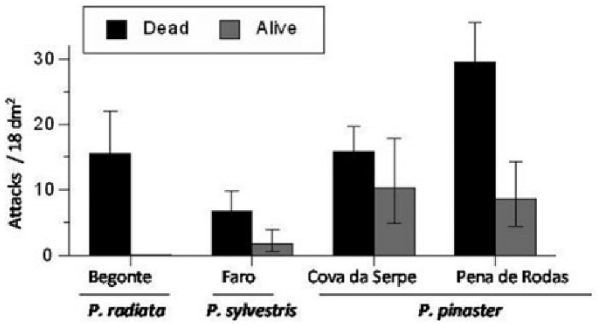

Fig. 2. Density of attacks by *S. noctilio* in pine trees of three species from four sites in Galicia, Spain. Trees at each site were separated into those that did and did not die by mid-summer following attacks the previous fall (dead vs. alive). Attack densities were standardized to the surface area of an average log. The figure represents means and standard error bars which were back-transformed (from $\sqrt{x}$).

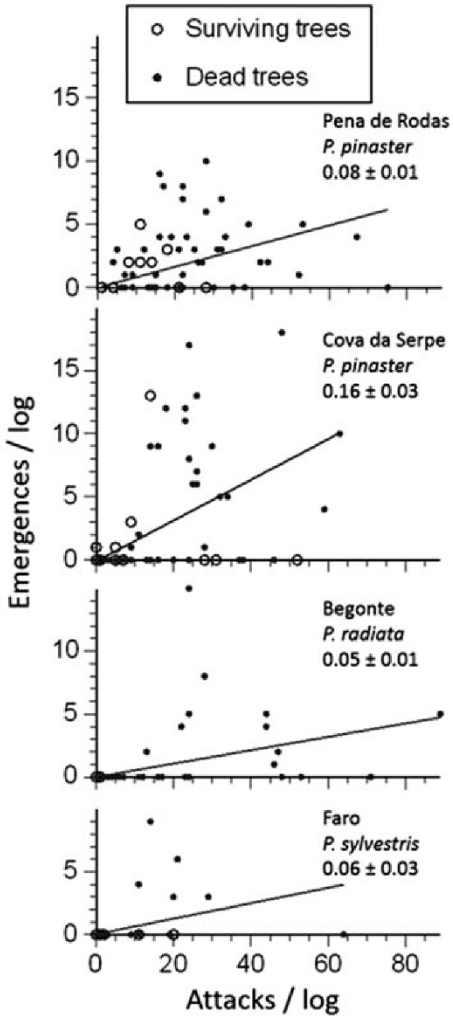

Fig. 3. Emergences relative to attacks for dissected logs from pines at four study sites in Galicia, Spain. Values indicate estimates of the population averages for emergences/attack ± SE at each study site (from slopes of regressions forced through the origin). Open symbols indicate trees that were still alive the summer after attack; others had died subsequent to attack.

of an average log) differed among study sites ($F_{3,40} = 4.47$; $P = 0.008$; fig. 2) being highest in the two sites with *P. pinaster* (Pena de Rodas and Cova da Serpe) and lowest at Faro (*P. sylvestris*) and Begonte (P. *radiata*). The density of attacks was much higher (average of about 2×) in trees that were dead the summer after attack than in trees that were still alive ($F_{3,40} = 16.06$; $P = 0.0003$). The average number of adult *S. noctilio* emerging per attack also varied among sites: 16 adults per 100 attacks at Cova da Serpe and 5–8 adults per 100 attacks at the other three sites (fig. 3). Logs that produced the most *S. noctilio* also tended to be most colonized by *T. piniperda* during January and February after the autumn when they were attacked: slope of *T. piniperda* attacks per log vs. *S. noctilio* attacks per log ± SE (both square root transformed) = 0.23 ± 0.07 ($P = 0.001$, $n = 168$ logs). We did not observe any tendency for attacks or emergences of *S. noctilio* to vary from the lower to upper bole of attacked trees.

The number of drillings per attack was 1, 2, 3 or occasionally more (to a maximum of 8): overall proportions for drills = 1: 2: 3: more = 0.43: 0.34: 0.18: 0.05. The average number of drillings per attack was highest in the two sites with *P. pinaster*, lowest at the site with *P. sylvestris*, and intermediate at the site with *P. radiata* (fig. 4). The average number of drills/attack was higher in trees that died by the following July compared with those that were still alive: least square means ± SE = 1.84 ± 0.07 vs. 1.53 ± 0.11 drills/attack ($F_{1,\ 52.9} = 5.26$, $P = 0.026$; no interaction between site and tree status). These patterns notwithstanding, most of the variance in drills/attack was among nearby attacks within the same logs: ≈87% of the total random variance was within individual logs as opposed to among logs within trees or among trees within sites (from a nested analysis of log-transformed data). Similarly, the generalized linear mixed model indicated only modest variance among trees within sites and no variance among logs within trees.

About 62% of the total drillings were filled with resin and 35% induced lesions. The proportion of drills filled with resin was unrelated to whether trees died or not but varied among sites ($F_{3,\ 33.2} = 3.12$, $P = 0.039$: lsmeans ± SE = 1.08 ± 0.08, 0.77 ± 0.08, 1.05 ± 0.13 and 0.81 ± 0.10 for Cova da Serpe, Pena de Rodas,

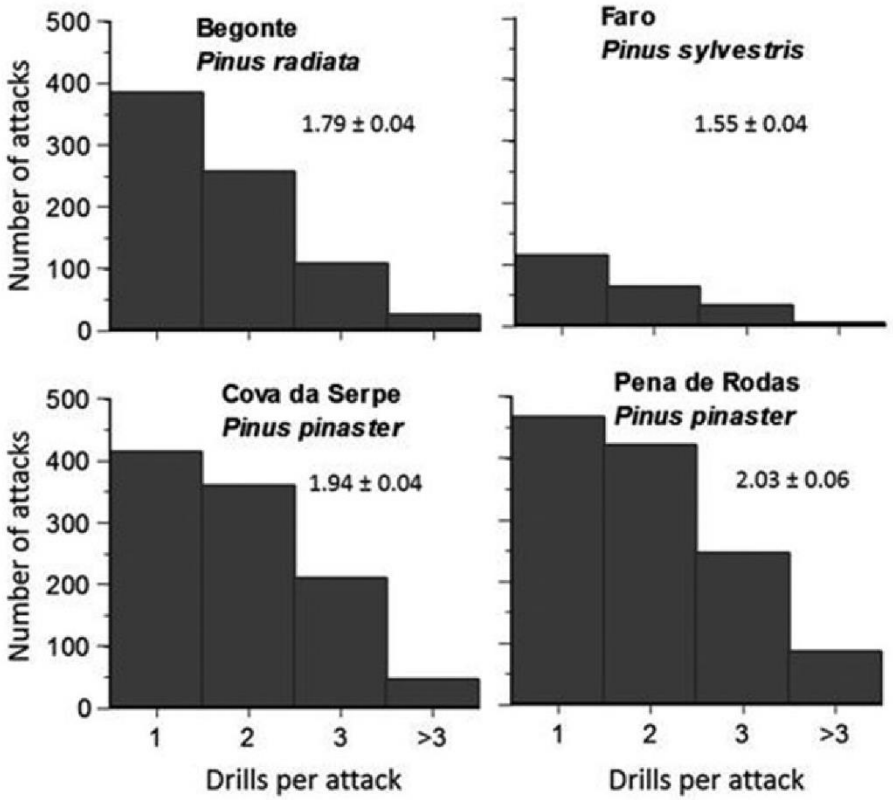

Fig. 4. Frequency distributions of drills per attack by *S. noctilio* females attacking pines at four sites in Galicia, Spain. Values indicate mean ± SE of drills/attack.

Begonte and Faro, respectively; units = arcsin-transformed proportions). In contrast, the proportion of drills that produced lesions was lower in trees that died ($F_{1,\ 39.6} = 5.50$, $P = 0.024$: lsmeans ± SE = 0.59 ± 0.06 vs. 0.86 ± 0.10, respectively) and did not vary among sites ($F_{3,\ 34.7} = 1.07$, $P = 0.27$).

Whiteness of the xylem from disappearance of lignin ('white rot'; apparently from *A. areolatum*) was only evident in a small part (average of 3% of cross-sectional area) of a minority of logs (55 of 171), and not until ≈6 months after *S. noctilio* emergence. There were no differences in the extent of white rot among sites ($F_{3,\ 75.4} = 0.90$, $P = 0.44$), but white rot was significantly higher in trees that had died by the summer after attack ($F_{1,\ 76.2} = 6.03$, $P = 0.016$: lsmeans ± SE = 0.091 ± 0.017 vs. 0.016 ± 0.025, respectively; units = arcsin-transformed proportions). The area of white rot was weakly but significantly related to attacks/dm^2 (Pearson's $r = 0.18$, $P = 0.014$, $n = 168$ logs). Production of *S. noctilio* per log was positively correlated with the extent of white rot, as expected, but the relationship was weak and there were many logs with no visible white rot that had relatively high production of *S. noctilio* (fig. 5).

Blue stain from *Ophiostoma* spp. was ubiquitous within trees attacked by *S. noctilio* (visually evident in all but 2 of 171 logs). There was little or no variation in blue stain among sites ($F_{3,\ 36.6} = 1.37$, $P = 0.26$). As with white rot, there was a relationship with whether trees were still alive in the summer after attack, but the pattern was opposite that of white rot: lsmeans ± SE = 0.85 ± 0.09 vs. 0.55 ± 0.07, for trees that were alive vs. dead, respectively (units = arcsin-transformed proportions; $F_{1,\ 36.6} = 7.13$,

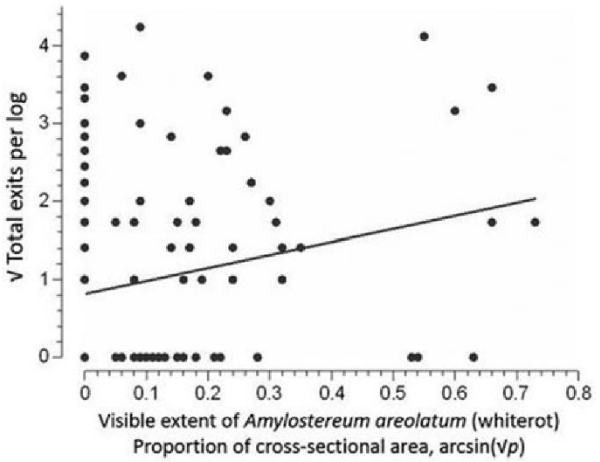

Fig. 5. Production per log of *S. noctilio* relative to visible extent of their mutualistic fungus, *A. areolatum*. Production tended to be higher in logs with more white rot, but there were also many logs with no visible white rot that still had relatively high production of *S. noctilio*. Values of 0.1, 0.4 and 0.7 on the *x*-axis correspond to 1, 15 and 41% of the cross-sectional area.

$P = 0.011$). There was further conspicuous variation in blue stain among trees within site × status (42% of total random variance; estimated 95% confidence interval for variance among trees = 0.02–0.15). High blue stain within logs was correlated

with lower extent of white rot ($r = -0.16$, $P = 0.027$) and lower productivity of S. *noctilio* ($r = -0.36$, $P < 0.0001$) (Pearson's correlation coefficients from $n = 169$ logs for both).

Determinants of *S. noctilio* emergence in *P. pinaster*

Our dissections included a total of 101 logs from *P. pinaster*, representing 26 trees attacked by *S. noctilio*. There was considerable structure to the correlation matrix of the various measurements (table S1, Supplemental materials). From these data, we identified a four-parameter model that explained 33% of the variance in emergences per log and in which the coefficients for all four parameters were significantly different from 0 (fig. 6). Emergence was positively related to total attacks per log (fig. 6a), but negatively related to the density of attacks (fig. 6d). Independent of total attacks and attack density, exits per log were negatively associated with the extent of blue stain and with the proportion of drills that induced lesions in the surrounding xylem tissue (fig. 6b, c). An alternative model in which total attacks and attacks/dm^2 were replaced by total drills and drills/dm^2 provided equivalent goodness of fit (delta AIC < 2; all four parameters also significant). There were no alternative models with equivalent or additional information from including any combination of the following other variables: extent of white rot, proportion of drills that were resin filled, attacks by *T. piniperda*, external resin drips from attacks by *S. noctilio* and moisture content of logs.

A preliminary life table of *S. noctilio* in Galicia

Data permitted a simple model of the demography of *S. noctilio* in Galicia (table 1). We estimated that an average female (with 88 eggs; data reported above) can generate up to 84 individual attacks on host trees (with drills per attack of 1–4 as in fig. 4, and eggs per drill as previously reported (Madden, 1974; Spradbery, 1977). Given the observed production of new adults per attack (fig. 3), this yields an average of about 7.9 potential adult progeny per parental female. The reduction from 88 eggs to 7.9 potential progeny was mostly attributable to larval mortality within the host tree and partly attributable to sterilization of 36% of eggs by the parasitic nematode, *D. siricidicola*. The reproductive potential of our study population was further depreciated by 20% incidence of Hymenoptera parasitoids. The male biased sex ratio (1: 2.8) further reduced λ by a factor of 0.48 relative to what it would be with a sex ratio of 1: 1 (table 1).

Discussion

Adult morphology and life history of *S. noctilio* in Galicia

Adult females of *S. noctilio* in our study area had dark femora, in contrast to the original species description and reports from most other areas (Schiff *et al.*, 2006). This likely contributed to some earlier taxonomic confusion (Ceballos, 1963; Llorente & Gabuyo, 1984). Our specimens match descriptions of Schiff *et al.* (2012) for *S. noctilio* in the Azores, Portugal, Spain, Italy and Turkey that are distinguishable from the more widespread pale legged form. Consistent with this, analyses of COI sequence data placed specimens from our study clearly within *S. noctilio*, but also within their own cluster separate from that of the pale legged *S. noctilio* in South Africa.

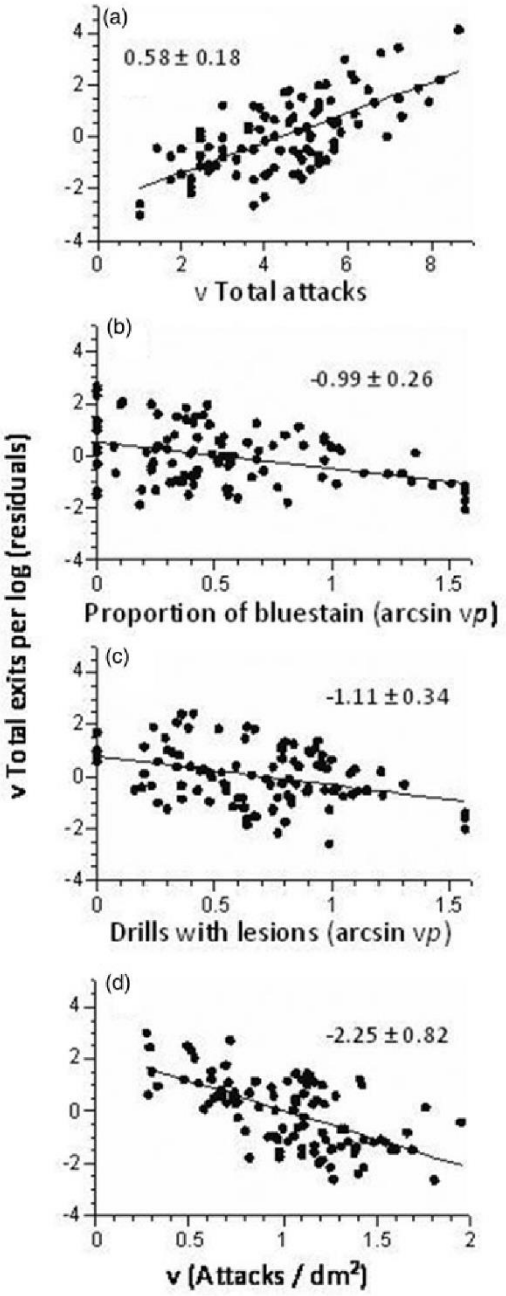

Fig. 6. Production of *S. noctilio* from 101 logs of *P. pinaster* relative to total attacks (a), proportion of blue stain (b), proportion of drills with lesions (c) and the density of attacks (d). Values within each frame indicate the coefficient ± SE for that parameter in a model that includes all four parameters (all significantly different from 0 at $P < 0.05$; intercept for the multiple linear regression = 2.32 ± 0.48; $r^2 = 0.33$). Exits per log in each panel are residuals after adjustment for effects of the other three parameters. The full model explained 33% of the variation in production of *S. noctilio* per log.

Generational development time of most *S. noctilio* in our study area was one year, but sometimes 2 years (24%) or even longer (≈4%). This is longer than reported by Spradbery & Kirk (1978) for Europe (98% univoltine), shorter

Table 1. A preliminary life table of *S. noctilio* in Galicia. An average female can generate up to about 84 attacks, with an average of about one egg per attack. With the consideration of impacts from parasitic nematodes, host suitability, parasitoids and skewed sex ratio, this suggests a maximum reproductive potential of λ = 1.65 (vs. 1.00 for replacement). The largest demographic impacts were from host suitability, followed by skewed sex ratio, parasitic nematodes and Hymenoptera parasitoids.

Demographic metric	Units	Data source	Nature of demographic impact	Size of demographic impact[1]
88	Eggs/female	This study: 88 = fecundity of the average-sized female		
84	Attacks/female	Attacks/female = eggs/female · attacks/egg. See footnote[2]		
0.094	Adult progeny/attack	This study: average of 0.094 adult progeny/attack (fig. 3); 79 of 217 *S. noctilio* sterilized by *D. siricidicoia* (36%)	Parasitic nematodes, Host suitability	0.26[3] 0.87[3]
0.80	Survival from parasitoids (proportion of *S. noctilio* brood not killed by Hymenoptera parasitoids)	This study: 20% parasitism = 38/. *leucospoides*, 26 *R. persuasoria* and 249 *S. noctilio* emerged from trees attacked in 2012	Hymenopter a parasitoids	0.20
0.26	Sex ratio (proportion of *S. noctilio* adults that are female)	This study: overall emergences of S. *noctilio* were 81 females and 226 males (M:F = 2.8:1)	Unequal sex ratio	0.48[4]
1.64	≈λ: adult females/adult female	Product of rows 2–5 in column 1		

[1]Proportional reduction in potential number of female progeny per female adult due to the corresponding demographic factor.
[2]Eggs/attack = Σ(*d*·*Pd*·*Ed*), where *d*, number of drills per attack (1–4); *Pd*, proportion of attacks with drills = 1–4; and *Ed*, expected eggs/drill with drills = 1–4. Eggs/attack = 1.05 = midpoint of 0.64 and 1.45 as calculated using *Pd* = 0.43, 0.34, 0.18 and 0.05 from fig. 4 (this study) and *Ed* as reported by either Madden (1974; 0.04, 0.34, 0.52 and 0.55) or Spradbery (1977; 0.25, 0.63, 1.33 and 1.00).
[3]Adult progeny/attack is lowered by: (1) parasitic nematodes that have sterilized the eggs introduced by attacking females; and (2) by mortality of immatures woodwasps within the host tree. With 84 attacks/female × 0.094 adult progeny/attack, there were 7.9 adult progeny/female; in the absence of 36% egg sterilization by nematodes, we can estimate that this would have been 10.7 adult progeny per attack. Thus, the estimated demographic impact from nematodes = 1–7.88/84 = 0.26, and the estimated impact from dying within the host tree = 1–10.7/84 = 0.87.
[4]Relative to sex ratio of 1:1.

than reported for Tasmania (50% semivoltine; Neumann & Minko, 1981) and similar to New Zealand (Morgan, 1968) and Argentina (Corley & Villacide, 2012). Emergence of *S. noctilio* in Galicia started in mid-August and lasted about 3 months similar to Australia (Nahrung *et al.*, 2015). In other populations emergence is concentrated in a few weeks (Ryan & Hurley, 2012). Voltinism and phenology can be expected to vary with climate (Neumann *et al.*, 1987; Corley *et al.*, 2007). The Galician climate is oceanic with a continental influence in the study plots (Martínez-Cortizas & Pérez-Alberti, 1999) with a mean annual temperature of ≈11°C and mean annual precipitation of ≈1,000 mm. Emergence times could be relatively protracted in Galicia because of cool rainy conditions in autumn when *S. noctilio* are emerging (Neumann *et al.*, 1987). Unlike Wolf (1969*a*) and Morgan (1968) we did not find longer development times in lower trunks or larger trees.

There has been speculation (e.g., Hanson, 1939) about whether multi-year life cycles in *Sirex* spp. involve developmental quiescence (≈ diapause) vs. slow growth vs. extended growth. The occurrence in our material of early instars two years after oviposition provides strong evidence of prolonged developmental quiescence at the egg stage or early larval stage; this pattern is similar to reports of prolonged diapause in Argentina (Corley *et al.*, 2007). However, we also found evidence of extended growth in that male adults emerging in the 2nd year were about 50% longer than those emerging after one year, and females emerging in the 2nd year, though not longer, had ≈18% more eggs for their body size than females that were univoltine.

Our adults were smaller than reported from some areas: female lengths of 8–38 mm in Galicia contrast with 21–72 mm in Tasmania (Madden, 1974) and 10–44 mm in South Africa (Hurley *et al.*, 2008), but similar to Victoria, Australia (Neumann & Minko, 1981). The diameters of exit holes from our material were also small compared with other reports for this species: 1–6 mm in Galicia vs. 3–7 mm in Zondag & Nuttall (1977) and Schiff *et al.* (2012). Concomitant with the smaller body size, fecundity of our population was lower than in the Southern Hemisphere: range for eggs/female = 0–270 vs. 30–450 (Madden, 1974), 50–500 (Zondag & Nuttall, 1977), 21–458 (Neumann & Minko, 1981) and 40–466 (Corley *et al.*, 2007). We found an average of 88 eggs/female vs. 220 reported by Neumann *et al.* (1987). Compared with Tasmanian material (Madden, 1974), Galician females had as many eggs as expected based on pronotum width, but only about half as many eggs on average because of their smaller size. In terms of table 1, the potential factor of population increase (λ) would be ≈3.3 instead of ≈1.6 if Galician females were as large as in Tasmania.

The overall sex ratio in Galicia was 1:2.9 (female: male), which is somewhat more male biased than reported by Spradbery & Kirk (1978) for Europe (1:1.8); but not nearly as biased as in some invasive populations (1:32 in Brazil: Iede *et al.*, 1998; 1:12 in South Africa: Hurley *et al.*, 2008). Even the relatively modest sex ratio bias in Galicia was enough to be the second strongest demographic factor of those we could parse (48% reduction in population growth potential compared to a 1: 1 sex ratio; table 1).

Reproduction success by tree in Galicia

Maybe the most remarkable result from our study was the low abundance of *S. noctilio* in Galicia. The species appeared to be restricted to suppressed trees (Hall, 1968; Ayres *et al.*, 2014), which are scarce in the actively managed forests that prevail in our study area. Most pine stands were devoid of resin drips from attacks or holes from emerging adults. Our four study plots contained the most conspicuous populations of *S. noctilio* that we could find, and still the abundances were very low: only 313 adult progeny from 134 attacked trees (compare for example to 2,558 progeny from 18 attacked trees in New York (Eager *et al.*, 2011) or 7,018 individual from 60 attacked trees in Canada (Ryan *et al.*, 2012)).

Even among the suppressed trees that were attacked by *S. noctilio*, it was only a fraction that produced progeny. We could not find comparable frequency distributions of emergence per tree from other locations. We hypothesize that outbreak populations have a lower fraction of attacked trees with 0 emergences than in Galicia ($\phi = 0.41$ from ZINB). In terms of the ZINB fit to our data, a modest reduction in ϕ or increase in μ would increase emergences per attack, which could influence abundance of *S. noctilio* because of the sensitivity of λ to progeny per attack (table 1). Understanding host suitability seems important for understanding variable abundance of *S. noctilio*.

Regression analyses of dissected logs suggested some apparent drivers of host suitability for *S. noctilio*. The number of attacks (and drillings) per tree was positively related to emergence per tree. This is at least partly because more attacks means more eggs (estimated average of about 1 egg per attack). So emergences would be positively related to attacks given similar survival of egg to adult. However, more attacks and more drills also means more inoculation of the phytotoxin (noctilisin) and the fungus (*A. areolatum*), which should increase the probability of tree death, and thereby increase the success of the fungus and larvae within trees (Coutts & Dolezal, 1969; Madden, 1977; Corley *et al.*, 2007). Indeed, trees that experienced higher attack densities tended to be those that died following attack and those that accounted for most production of *S. noctilio*. If there is positive feedback in the population dynamics from increasing attack success with increasing abundance (Corley *et al.*, 2007), it could explain the tendency for *S. noctilio* to switch quickly between low and high abundance. This is consistent with there being particularly low production of *S. noctilio* in our study sites where *S. noctilio* was least abundant (Faro and Begonte). However, our data do not demonstrate that higher attack densities cause higher tree mortality; the pattern could also indicate that *S. noctilio* attack at higher densities on trees that are already more likely to die. If outbreaks of *S. noctilio* are partly a result of positive feedback from larger populations having greater success in killing trees, then we could expect higher attack densities in outbreak populations than we observed in Galicia. Such data seem to be limited, but Coutts & Dolezal (1966) reported up to '500 tunnels per square foot of bark surface', which is $\approx$30-fold higher than our observations of no more than 1–2 attacks/dm^2.

The suitability of *P. pinaster* for *S. noctilio* was inversely related to the tendency for induced production of polyphenols (lesions) around points of drilling by attacking females. Overall, more than a third of drillings (2,141 of 6,041) induced visible lesions. Our results support the hypothesis that these lesions are a defensive response against *A. areolatum* (Coutts & Dolezal, 1966; Hillis & Inoue, 1968; Talbot, 1977; Madden, 1988; Thompson *et al.*, 2014). Notably, lesions were more frequent in trees that were still alive the summer after attacks.

The success of S. *noctilio* within logs was inversely related to the presence of blue stain fungi. This is consistent with the hypothesis of competition between blue stain fungi and *A. areolatum* (Wolf, 1969*a*; Ryan *et al.*, 2011; Hurley *et al.*, 2012; Yousuf *et al.*, 2014*a*, *b*), but we question whether this interpretation applies in our case. The main vector of blue stain to our pines is the bark beetle, *T. piniperda*, which flies several months after *S. noctilio*. Thus the introduction of blue stain should generally come well after *A. areolatum* has had a chance to establish itself in host trees.

After accounting for effects of other factors, an additional negative correlation emerged between production per log and attack density, which suggests larval competition. Competition for space within the xylem was not evident in our dissections, but there could be competition for limited patches of *A. areolatum*, especially in trees with low carbohydrate content (Titze, 1965). Another possibility is that concentrations of attacks within a region of pine bole could trigger more effective inducible defences in those regions.

Top-down effects

In addition to effects from host suitability and skewed sex ratios, there are strong effects on the abundance of *S. noctilio* in Galicia from natural enemies (table 1). The strongest top-down effects were from the parasitic nematode, *D. siricidicola*. This is consistent with the attention that has been afforded *D. siricidicola* as a biological control agent (Hurley *et al.*, 2007). Ours is the first work that we know since Wolf (1969*b*) reporting the frequency of *D. siricidicola* in a native population of *S. noctilio*. We found *D. siricidicola* to be ubiquitous (infecting 36–39% of adults at all study sites). This is consistent with earlier studies in Belgium (Wolf, 1969*b*). Parasitism by *D. siricidicola* in invaded areas, where it has been repeatedly introduced as a control agent, can be both higher and lower than we found in Galicia. In Australia infection can reach 100% of the population within inoculated trees (Bedding & Akhurst, 1974, but see Nahrung *et al.*, 2015); infection levels in Brazil are 70 to 80% (Iede *et al.*, 1998), and in South Africa infection ranges from 23 to 96% (Tribe & Cillié, 2004). However there have been less successful introductions in Brazil (Fenili *et al.*, 2000), Argentina (Eskiviski *et al.*, 2003) and South Africa (Hurley *et al.*, 2007). The abundance of *D. siricidicola* could be affected by environmental conditions – including any factors that influence growth of *A. areolatum*, on which the nematodes feed within trees before infecting larvae (Hurley *et al.*, 2008; Carnegie & Bashford, 2012; Nahrung *et al.*, 2015).

D. siricidicola in Galicia readily entered female eggs and caused nearly complete sterilization of infected females in our study; this differs from North American populations (Kroll *et al.*, 2013). Understanding the maintenance of sterilization in native, unmanaged, populations of *D. siricidicola*, such as in Galicia, could have value in managing the chronic loss of sterilization in populations of nematodes introduced for biological control (Bedding & Iede, 2005).

D. siricidicola offers a potential explanation for the prolonged life cycle displayed by about 35% of Galician *S. noctilio*. Females that emerged two years after attack tended to have about 35% higher fecundity, and also had only about half the risk of being sterilized by *D. siricidicola*. The reduced parasitism could be a result of higher nematode performance in moist

wood (Bedding & Akhurst, 1974; Hurley *et al.*, 2008), and wood moisture decreases with time following attack by *S. noctilio* and colonization by *A. areolatum* (Coutts & Dolezal, 1965).

S. noctilio in Galicia are also regularly parasitized by two species of Hymenoptera. These same species of parasitoids have been reported at comparable frequencies elsewhere in Europe (Wolf, 1969*b*; Spradbery & Kirk, 1978). The overall proportion of *S. noctilio* killed by parasitoids in Galicia (≈20%), was less than reported in several studies from the Southern Hemisphere (25–55% in New Zealand, Victoria, New South Wales and South America; Neumann *et al.*, 1987; Iede *et al.*, 2000; Carnegie *et al.*, 2005) and on the low end for reports from North America of ≈10–40% (Ryan *et al.*, 2012; Zylstra & Mastro, 2012; Kroll *et al.*, 2013). It is tempting based on table 1 and comparisons with other continents to infer that Hymenopteran parasitoids are less important than low host suitability and parasitic nematodes as explanations for low abundance of *S. noctilio* in Galicia in particular and Europe in general. However, we still have limited understanding how the factors identified in table 1 vary with the abundance of *S. noctilio*. Understanding the direction and strength of density-dependent feedbacks is an emerging challenge for understanding the global ecology and management of *S. noctilio*.

Supplementary material

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0007485316000043

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Summary

Thesis title: Diversity, specificity and admixture in the *Sirex - Amylostereum - Deladenus* symbiosis
Student: Katrin Nathalie Elsbeth Fitza
Supervisors: Prof. Bernard Slippers
Dr. Jeff Garnas

Department: Biochemistry, Genetics and Microbiology
Degree: PhD Genetics

Biological control is an important management tool to deal with the rapidly increasing number of invasive pests of plantation forests globally. It is important to consider the genetic diversity of both the pest and biological control populations to understand geographic population structure, patterns of invasion, genotype-genotype interactions and potential adaptability. This thesis examined patterns of genetic diversity and specificity of the *Sirex–Deladenus–Amylostereum* complex. Mitochondrial sequence data and nuclear microsatellite markers were used to characterize the diversity in a global collection of *D. siricidicola* from both native and non–native regions. The data revealed the presence of three distinct lineages, from North America (Lineage A; non-native), the Southern Hemisphere (Lineage B; non-native) and Spain (Lineage C; native). Interestingly, samples from Chile represented an admixed population of lineages A and B. The global study showed evidence of substantially genetic diversity present globally which could be used to augment the reduced genetic diversity in the Southern Hemisphere biological control populations. The three *D. siricidicola* lineages were shown to be able to interbreed in culture. The admixed offspring of one of the crosses showed a significant increase in its reproductive rate on the slowest growing fungal isolate, when compared to the parental strains. Experimental admixture suggests the possibility and advantage of introducing more genetic diversity in biological control programs. As the symbiotic fungus *A. areolatum* of the pest wasp *S. noctilio* plays a crucial role in the mass production and influences the performance of the biological control agent, the fidelity of the *Sirex –Amylostereum* association was studied in native Siricids in Japan and their fungal associates. It was shown that the association was not species specific. *Sirex nitobei* was associated not only with

Amylostereum areolatum, but also *Amylostereum chailletii*. *Urocerus* sp., previously associated with *A. laevigatum*, carried *A. chailletii*. Vegetative compatibility test revealed high clonality both among *A. areolatum* and *A. chailletii* in association with these wasps. Together with previous studies it seems that the host tree plays a more critical role in selection of *Amylostereum* species than the wasp. The thesis illustrates the importance of studying the genetic diversity of biological control agents and the potential of augmenting the genetic diversity in these populations as a mean to improve adaptability.