NOTE / NOTE

Distinguishing isolates of *Deladenus siricidicola*, a biological control agent of *Sirex noctilio*, from North America and the Southern Hemisphere using PCR–RFLP

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Abstract: The woodwasp *Sirex noctilio* Fabricius, along with its obligate symbiotic fungus *Amylostereum areolatum* (Chaillet ex Fr.) Boidin, is amongst the most damaging invasive species to many commercial pine plantations. The most effective biocontrol agent for management of this woodwasp has been the nematode *Deladenus siricidicola* Bedding. Before this agent can be used in North America, answering key questions about its interaction with native siricids and other strains of the nematode is essential, as would be the need to track its spread after release. The aim of this study was to develop tools to differentiate between the North American *D. siricidicola* isolates and the Southern Hemisphere Kamona strain of this species. We sequenced a region from ribosomal DNA and the cytochrome oxidase subunit 1 and developed a PCR–RFLP method based on a single nucleotide polymorphism flanking a microsatellite sequence. These markers will be useful for science-based operational biocontrol of *S. noctilio*.

Résumé : La guêpe perce-bois *Sirex noctilio* Fabricius ainsi que son symbiote fongique obligatoire *Amylostereum areolatum* (Chaillet ex Fr.) Boidin est parmi les espèces invasives qui causent le plus de dommages dans plusieurs plantations commerciales de pin. L'agent de lutte biologique le plus efficace pour la gestion de ce sirex est le nématode *Deladenus siricidicola* Bedding. Avant que cet agent puisse être utilisé en Amérique du Nord, il est essentiel d'élucider d'importantes questions au sujet de son interaction avec les siricidées indigènes et d'autres races de nématodes, de même qu'il est nécessaire de suivre sa dispersion après son lâcher. Le but de cette étude était de développer des outils pour distinguer les isolats nord-américains de *D. siricidicola* de la race Kamona de cette espèce originaire de l'hémisphère sud. Nous avons séquencé une région de l'ADN ribosomique ainsi que la sous-unité 1 de la cytochrome oxydase et élaboré une méthode PCR–RFLP basée sur un seul polymorphisme nucléotidique encadrant une séquence microsatellite. Ces marqueurs seront utiles pour la lutte biologique opérationnelle sur des bases scientifiques contre *S. noctilio*.

[Traduit par la Rédaction]

Introduction

Sirex noctilio Fabricius (Hymenoptera: Siricidae) is a woodwasp native to Eurasia and North Africa (Morgan 1968; Spradbery and Kirk 1978) that has caused extensive tree mortality in plantations of exotic pines (*Pinus* spp.) in the Southern Hemisphere (Hurley et al. 2007) where it has been introduced inadvertently in several countries, such as New Zealand (Rawlings 1948), Australia (Neumann et al. 1987), Uruguay (Maderni 1998), Argentina (Klasmer et al. 1998), Brazil (Iede et al. 1988), South Africa (Tribe 1995), and Chile (Beèche et al. 2012). The recent discoveries of *S. noctilio* populations in North America, more specifically in New York (Hoebeke et al. 2005) and Ontario (de Groot et al. 2006), have raised some concerns about the impact that this insect could have on this continent given the extent of the pine populations (Ciesla 2003; Hoebeke et al. 2005; Haugen 2006).

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Deladenus siricidicola Bedding is a parasitic nematode of *S. noctilio* that has two morphologically different forms: a fungal-feeding form and a parasitic form. The fungal-feeding form feeds on *Amylostereum areolatum* (Chaillet ex Fr.) Boidin, the symbiotic fungus injected by *S. noctilio* females into trees at the time of oviposition. The parasitic form infects *S. noctilio* larvae where it completes its development and produces juveniles that migrate into the reproductive organs of the woodwasp, sterilizing *S. noctilio* females (Bedding 1967, 1972; Bedding and Akhurst 1974). The Kamona strain of this nematode was isolated in the Kamona region, Tasmania, Australia (from a site where the initial release of this originally Hungarian strain was made), and has been used to inoculate infested pine plantations in Australia and Brazil with great success (Bedding and Iede 2005; Iede et al. 2012). In other areas of the Southern Hemisphere, such as in South Africa, however, this strain of *D. siricidicola* has not been as effective (Hurley et al. 2007). Hurley et al. (2008, 2012) ruled out the inoculation techniques and nematode virulence as the source of this loss in parasitism effectiveness and hypothesized that other factors, such as wood moisture and fungal competition, may have been the cause of this diverging result. Bedding (2009) also hypothesized that the low success in South Africa may have been due to warm, dry winters, which caused Sirex-infested trees to dry out before the nematode populations could proliferate throughout the tree.

The Kamona strain of *D. siricidicola* is the most widely used biological control agent against *S. noctilio* in the Southern Hemisphere. Yu et al. (2009) reported the presence of a different strain of *D. siricidicola* in Canada using morphology and sequence data of ribosomal DNA (rDNA) and cytochrome oxidase subunit 1 (CO1). Mlonyeni et al. (2011) confirmed this finding using microsatellite markers. The effect that this strain has or may have on North American populations of *S. noctilio* is not clear. Neither is it clear whether this strain from North America could be used as an alternative to the Kamona strain nor whether these strains would compete or could interbreed. Research is currently underway to determine whether the Kamona strain could be used as a biocontrol agent in North America (Williams et al. 2012).

The aim of our study was to develop a fast, effective, and highly reliable molecular method to discriminate between the Kamona strain and the populations from North America. This tool would be essential to distinguish the impact or effectiveness of the different strains in populations of *S. noctilio* from North America.

Materials and methods

Nematode sources and collection

A survey of 335 sites in southern Ontario, Canada, reported that 39 were positive for *S. noctilio*. Twelve of these sites were sampled to collect *S. noctilio*, from which 46 strains of *D. siricidicola* were isolated and cultured. In addition, we obtained 10 *S. noctilio* specimens from northern New York State, USA, which were positive for *D. siricidicola*. These were not cultured but were kept in 95% EtOH until needed. Nematodes were harvested from cultures grown on (2%) potato dextrose agar plates onto which *A. areolatum* had been inoculated (Bedding and Akhurst 1974).

DNA extraction

Twenty microlitre nematode samples were placed in a 1.5 mL microcentrifuge tube, frozen in liquid N_2, and ground using a Kontes pellet pestle (Kimble-Chase, Vineland, New Jersey). DNA was extracted using a Qiagen DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, California) according to the manufacturer's directions and eluted in 100 μL of 10 mmol/L Tris, pH8.

The 10 *S. noctilio* containing *D. siricidicola* were frozen and ground in a Retsch mixer mill type MM2 (Retsch, Germany) in a stainless steel capsule with two stainless steel ball bearings. DNA was extracted as above and its concentration was determined using a NanoDrop ND-1000 spectrophotometer prior to performing polymerase chain reaction (PCR) analysis.

PCR of the rDNA region and CO1

Primers for SSU and ITS amplification used are the forward primers SSU-F-2, SSU-F-4, SSU-F-22, and ITS1 (Ferris et al. 1993) and the reverse primers SSU-R-9, SSU-R-13, SSU-R-81, and ITS2 (Vrain et al. 1992). All SSU primer sequence information can be found in the Blaxter laboratory web site (http://www.nematodes.org/research/barcoding/sourhope/nemoprimers.html).

The 25 μL PCR reaction mix contained 5 μL of 5× PCR buffer, 1 unit of GoTaq DNA polymerase (Promega Corporation, Madison, Wisconsin), 1.6 mmol/L $MgCl_2$, 1.6 μg of BSA, 0.2 mmol/L dNTPs, 1 μmol/L forward and reverse primers, and 1 μL of DNA template. The amplification protocol was carried out as specified by Vrain et al. (1992) and carried out in a Biometra T-Gradient thermocycler. CO1 primers and PCR amplification for the CO1 gene was carried out as in Ye et al. (2007).

The PCR products were sent for sequencing to the CHUL (Université Laval, Québec, Québec). The sequence data obtained were analyzed using Sequencher 4.8 software (Genecodes Corporation, Ann Arbor, Michigan).

PCR – Restriction Fragment Length Polymorphism (RFLP)

Samples were amplified using microsatellite primers DS1F (CAATGTGCTGCGTCAATTTT) and DS1R (ACCCAACGCGTAGTGATAGC) (Mlonyeni et al. 2011). PCR cycles were carried out in a Biometra T-Gradient thermocycler according to the protocol used by Mlonyeni et al. (2011). Five microlitres of PCR product was then digested using 10 units of restriction enzyme *Hae*III (New England Biolabs) and incubated at 37 °C overnight. The digested PCR product was then run on a 3% agarose MS molecular screening gel (Roche, Canada) containing 0.25 μg/mL EtBr at 3 V/cm for 4 h, visualized under UV light, and photographed. The PCR product of a few isolates was sent for sequencing to the CHUL (Université Laval, Québec, Québec). The sequence data obtained were analyzed using Sequencher 4.8 software (Genecodes Corporation, Ann Arbor, Michigan).

Results and discussion

We sequenced 2700 bp of the rDNA region (partial 18S-ITS1 – 5.8SRNA – partial ITS2) and 686 bp of the CO1 from 46 *D. siricidicola* isolates from Ontario and 10 *D. siri-*

cidicola isolates from the United States and compared these sequences with those from the Kamona strain. Comparison of the rDNA region cannot differentiate between the North American isolates and the Kamona strain because a portion of the Canadian population and all of the US isolates are identical in sequences of this locus to the Kamona strain. Out of the 46 Ontario isolates, 14 isolates had the same sequence as the Kamona strain (type 1) (accession No. FJ004890), 20 isolates were type 2 (accession No. FJ004889), and 12 isolates contained types 1 and 2. All 10 isolates from the United States were of type 1. The changes between types 1 and 2 include three substitutions and one insertion. All 56 North American isolates have the same CO1 sequence (accession No. JQ241275) but differ at two positions with the Kamona strain (accession No. JQ241276) and with the abovementioned Australian isolate.

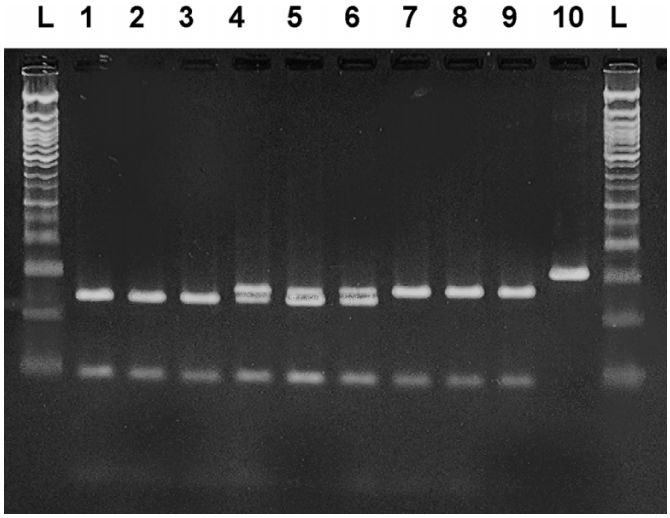
The observation that one cannot differentiate between the North American isolates and the Kamona strain by sequencing the rDNA region is not completely unexpected because this rDNA region generally results in significant interspecific variation in DNA sequences and, in principle, a comparatively small intraspecific variance (Hillis and Dixon 1991). Even though there are two base pair substitutions in the CO1 sequence between the North American isolates and the Kamona strain, this will require sequencing to identify the strains, which is time consuming and expensive. For this reason, it was important to develop a more practical, rapid, and effective molecular method to differentiate between these two populations.

Apart from sequence data of the rDNA and CO1 loci, we characterize the differences between the North America isolates and the Kamona strain using the microsatellite markers developed by Mlonyeni et al. (2011). The 12 loci exhibiting polymorphisms between the Southern Hemisphere sources (Argentina, Australia, Brazil, and South Africa) and the Canadian sources were screened for restriction sites. Locus Ds1 contained a single nucleotide polymorphism (A/G at position 48) in the microsatellite flanking region, which created a *Hae*III site in the North America isolates (Canada and the United States) that was absent in the Kamona strain and the other Southern Hemisphere sources. This locus was thus chosen to test as a diagnostic PCR-RFLP tool.

The PCR product of the Ds1 locus from the 56 North American isolates was 169–181 bp, whereas the PCR product of the Kamona strain was 157 bp. The difference in size is due to either 12 or 16 (GTA) simple sequence repeats (SSRs) in the North American samples compared with the Southern Hemisphere samples, including the Kamona strain, which have 10 (GTA) SSRs. This PCR-RFLP assay, therefore, did not merely distinguish the North American isolates from the Kamona strain but also differentiated three allele types amongst the North America isolates. For the Canadian isolates, the RFLP pattern for 24 isolates contained two fragments of 133 and 48 bp (type A, 16 GTA repeats), for four isolates it contained two fragments of 121 and 48 bp (type B, 12 GTA repeats), and for 18 isolates it contained three fragments of 133, 121, and 48 bp (type AB) (Fig. 1). All 10 isolates from the United States were type A.

It is thought that *D. siricidicola* was inadvertently introduced with *S. noctilio* into both Canada and the United States, since it was reported to be absent in North America

Fig. 1. Polymerase chain reaction – restriction fragment length polymorphism of *Deladenus siricidicola* isolates from southern Ontario, Canada and the Kamona strain. L, 50 bp ladder; lane 1, isolate 392; lane 2, isolate 451; lane 3, isolate 468; lane 4, isolate 116; lane 5, isolate 352; lane 6, isolate 1088; lane 7, isolate 172; lane 8, isolate 403; lane 9, isolate 1119; lane 10, Kamona strain.



(Bedding and Akhurst 1978) prior to the *S. noctilio* introductions into these two countries. Data on the genetic diversity of the nematode, such as those produced in this study, can thus reflect the origin(s) of the invasive woodwasp. This is potentially useful, as uncertainty remains about the origins of the introduction of *S. noctilio* into North America, other than the clear indications that there has most likely been more than one introduction from different origins (Nielsen et al. 2009; Bergeron et al. 2011). For such questions to be addressed, further collections across the native range of *D. siricidicola* in Eurasia are needed.

The existence of distinct strains in the Southern Hemisphere and North America presents opportunities for biological control. Mlonyeni et al. (2011) has raised concerns regarding the high homozygosity present in Southern Hemisphere populations of *D. siricidicola*, suggesting that this absence of diversity can have an impact on the ability of this nematode to adapt to different environments and host types in regions where it is being considered for use as a biological control. The existence of the levels of diversity revealed in this and other studies creates the opportunity to exchange cultures and increase diversity. The tools presented here can help study the integration and spread of such cross-introduced strains.

The PCR-RFLP method presented here is a faster and more cost-effective assay than any other assay currently available for strains of *D. siricidicola*. In addition, we have shown that even though the DNA extracts from the US samples contained a mixture of insect and nematode DNA, the nematodes could be identified without the need to subculture, since the primers are specific for the nematode. These tools will be invaluable in experiments in coming years aiming to address the need for and potential risks of introducing the Kamona strain as a biological control in North America (see Williams et al. 2012).

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