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

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Highlights

• The most comprehensive global study of the genetic diversity of *D. siricidicola*.

• Three lineages associated with North America, the Southern Hemisphere and Europe.

• An admixed North American and Southern Hemisphere population identified in Chile.

• An opportunity exists to augment the reduced diversity in some biocontrol programs.

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

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 1990's (Hurley *et al.*, 2007). Apart from the Kamona strain, a strain referred to as "Encruzilhado do Sol", 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 global genetic diversity of *D. siricidicola* and to infer patterns of movement and spread, both intentional and accidental. Additionally, this work contributes to efforts to catalog and store living, dormant strains of *D. siricidicola*, of which very few existed previously.

2. Material and methods

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 and the USA) and countries where *D. siricidicola* has been intentionally released to control *S. noctilio* (Argentina, Brazil, Chile, New Zealand 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.

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

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 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 of MyTaq^{TM} buffer, 0.5 M of each primer, 1.5 units of MyTaq^{TM} 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^{TM} (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™ 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. 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.

2.3 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™ 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).

Table 2. Microsatellite primers used for the fragment analysis, their fluorescent labels and the panel arrangement

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

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 ΔK 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).

2.5 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.

2.6 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).

3. Results

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).

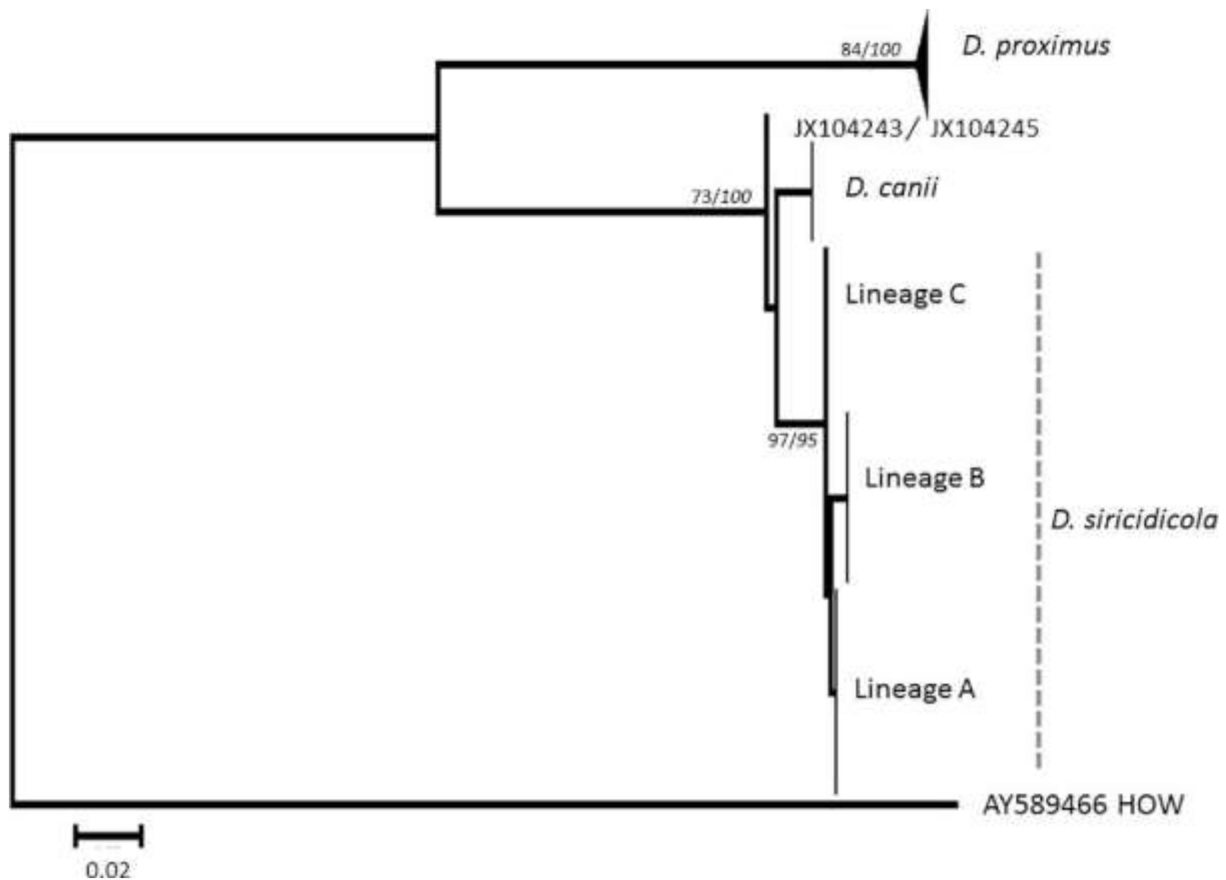

Figure 1. Phylogenetic analysis of *Deladenus* spp. using both Maximum likelihood (ML) and Maximum Parsimony (MP) based on cytochrome c oxidase subunit 1 (CO1) sequence data. The tree is rooted to *Howardula aoronymphium*. All bootstrap values above 70% are indicated for ML (roman) and MP (italics) at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

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.

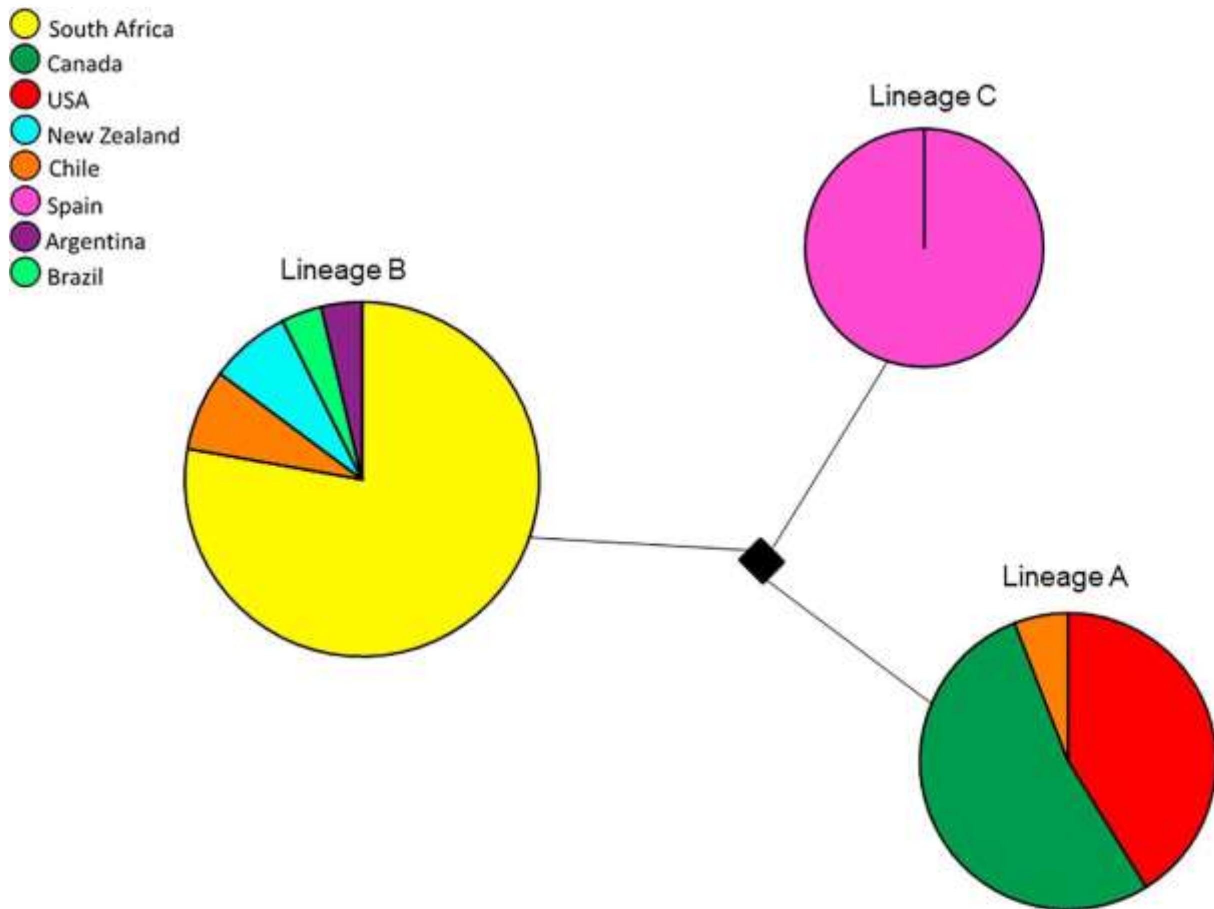

Figure 2. A haplotype network based on analysis of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene sequence data. The network was constructed using a median-joining algorithm. The circles represent the different haplotypes and the size of the circles is linked to the number of strains per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

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, 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.

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).

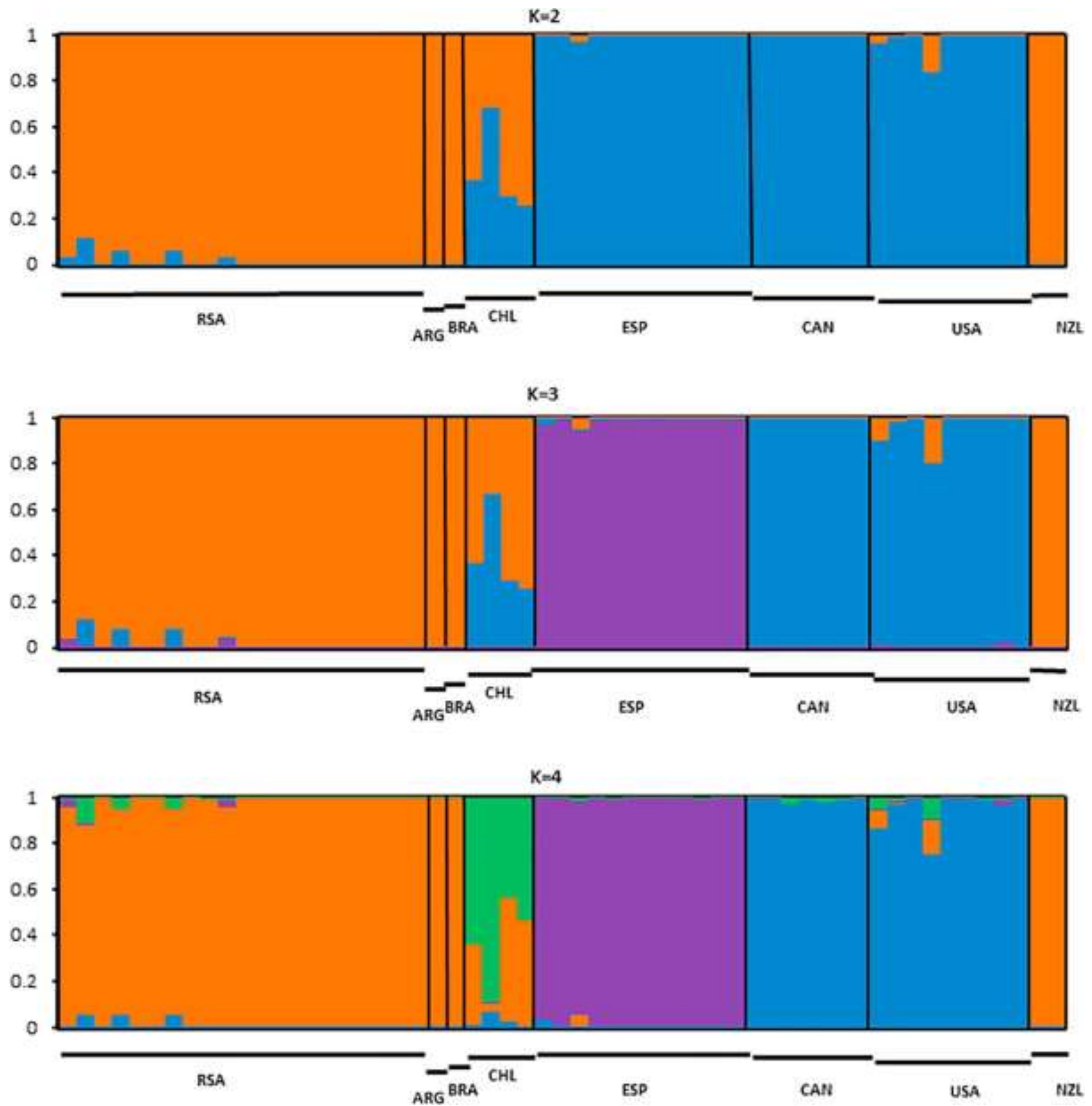

Figure 3. Bayesian clustering analysis (STRUCTURE) conducted using microsatellite marker data from all isolates (n = 57). Vertical columns represent individual strains and the different colours represent identified populations. The countries of origin of the isolates are indicated by the horizontal lines.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

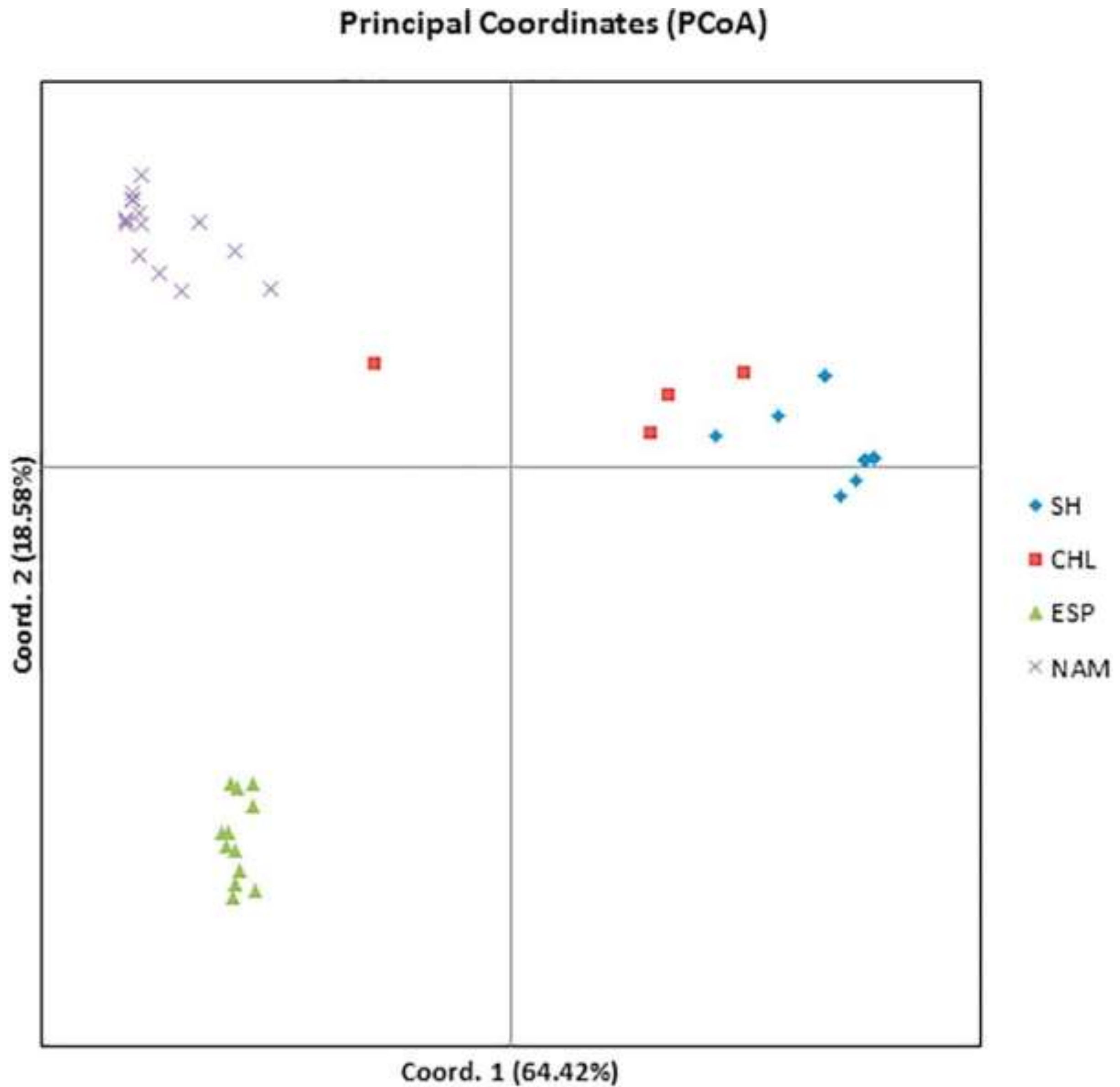

Figure 4. Principal coordinates analysis plot of the microsatellite data set using Nei's genetic distance (Nei, 1973). The different shapes represent the four different lineages (ESP = Spain, CHL = Chile, NAM = North America and SH = Southern Hemisphere). The first two principal coordinates provided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

3.4 Microsatellite diversity

Thirteen of the 134 alleles identified were unique to a specific lineage. The Spanish lineage had the highest number of unique alleles (n = 12), whereas the Southern Hemisphere lineage had no unique alleles (Table 3). 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 3). The same pattern was reflected in the genotypic diversity, whereby the Southern Hemisphere lineage showed the lowest diversity (eĜ = 2.4) and Chile had the highest rarefied genotypic diversity (eĜ = 4, tied with Spain; Table 3).

Table 3. 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	Ĝ[b]	eĜ[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 (Ĝ)

[c] eĜ 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

Analysis of population differentiation using AMOVA showed that 86% of the molecular variation was due to variation among populations (Table 4). Pairwise comparison of the population diversity (Table 5) 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 5).

Table 4. 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 5. 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	-

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, New Zealand and South Africa). The Chilean strains grouped in between the Southern Hemisphere and the North American clusters.

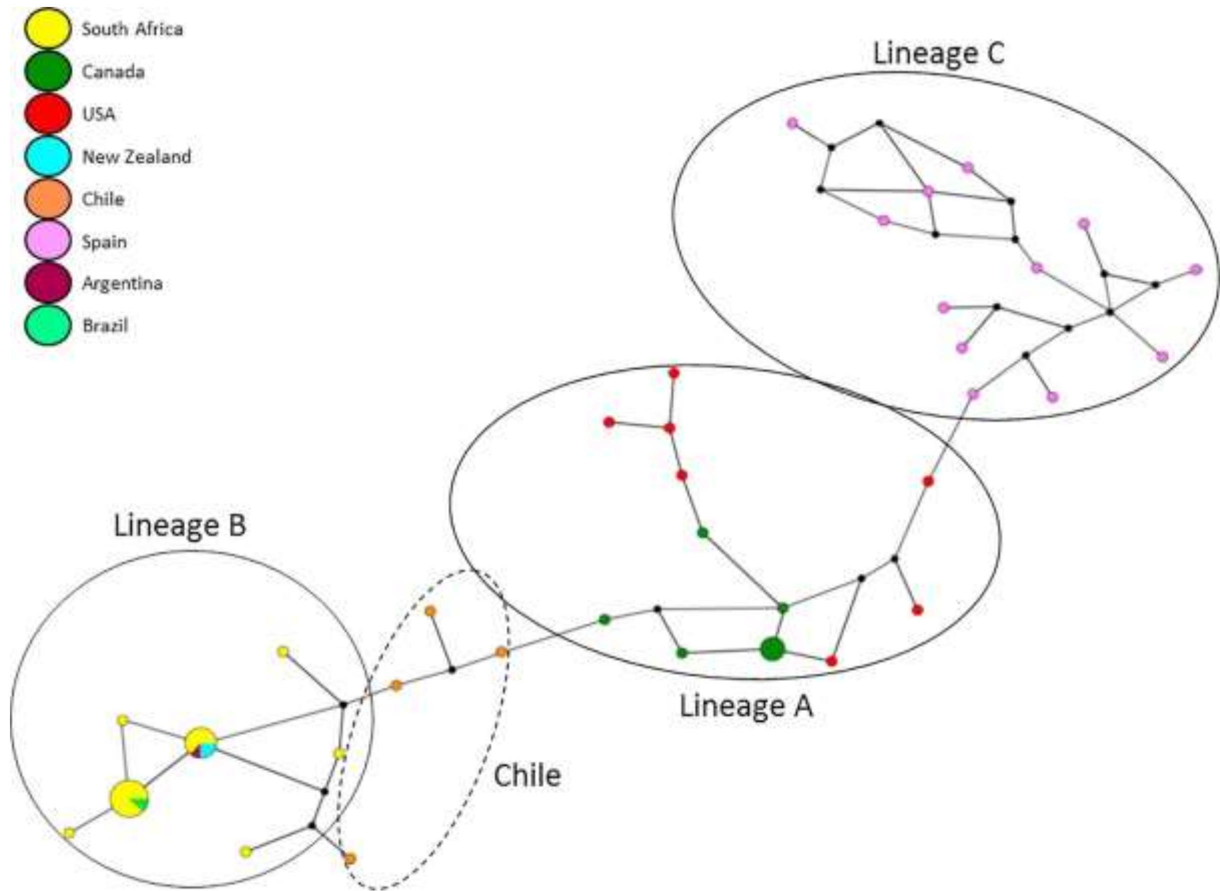

Figure 5. Genotype network drawn using microsatellite markers. The network is based on a median-joining algorithm. Coloured circles represent the different genotypes and the size of the circles is linked to number of strains with that genotype. The colours represent the different geographic origins of 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.

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).

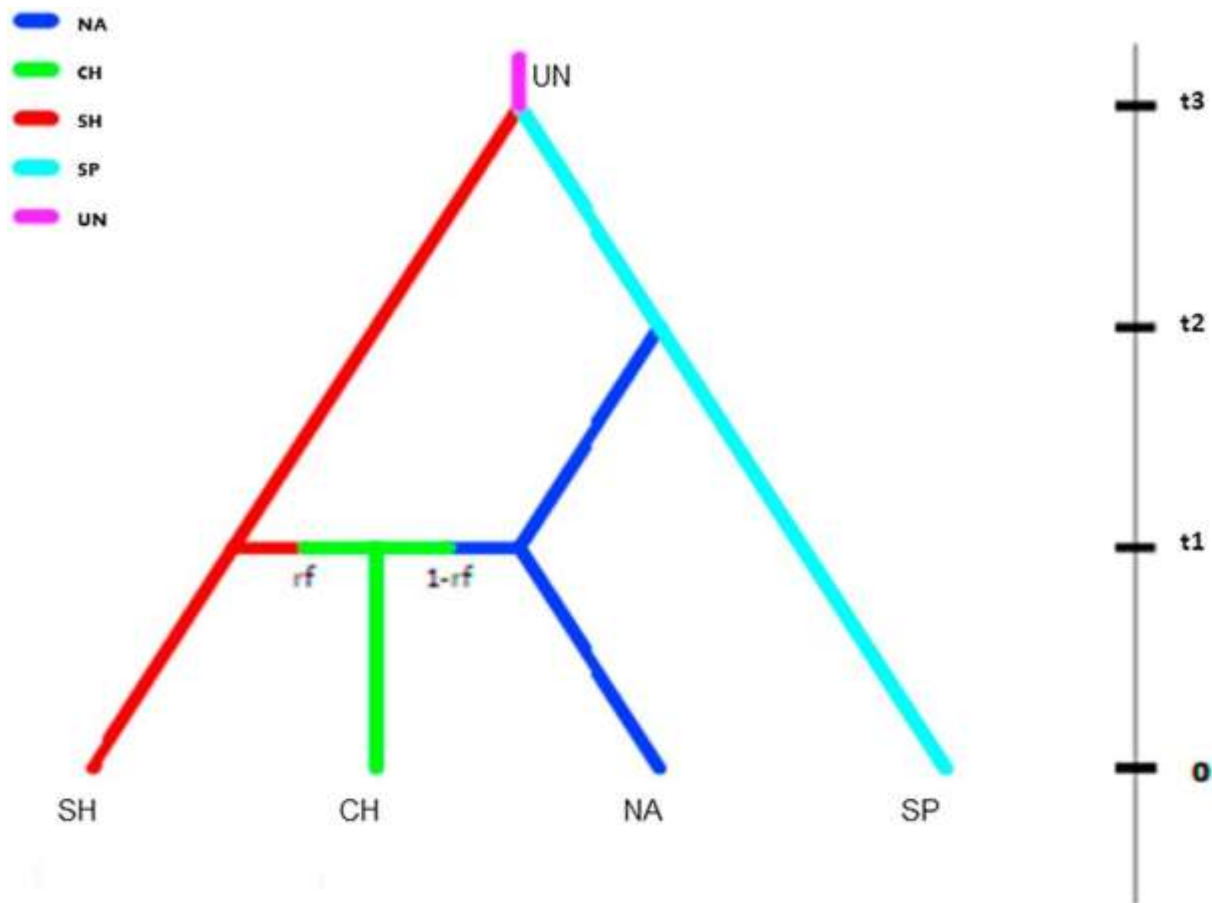

Figure 6. Representation of the most likely scenario (P=0.79) among the 11 scenarios tested in our DIYABC analysis. The figure represents the most likely scenario (SP =Spain, CH = Chile, NA = North America, SH = Southern Hemisphere and UN = Unsampled region). The vertical axis represents time but is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

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.

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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Supplementary material

Supplement 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	
MF173163	*Deladenus siricidicola*	Spain, Galicia	
MF176462	*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