Unexpected diversity in historical biological control programs: Population genetics of the nematode *Deladenus siricidicola* in Australia and New Zealand

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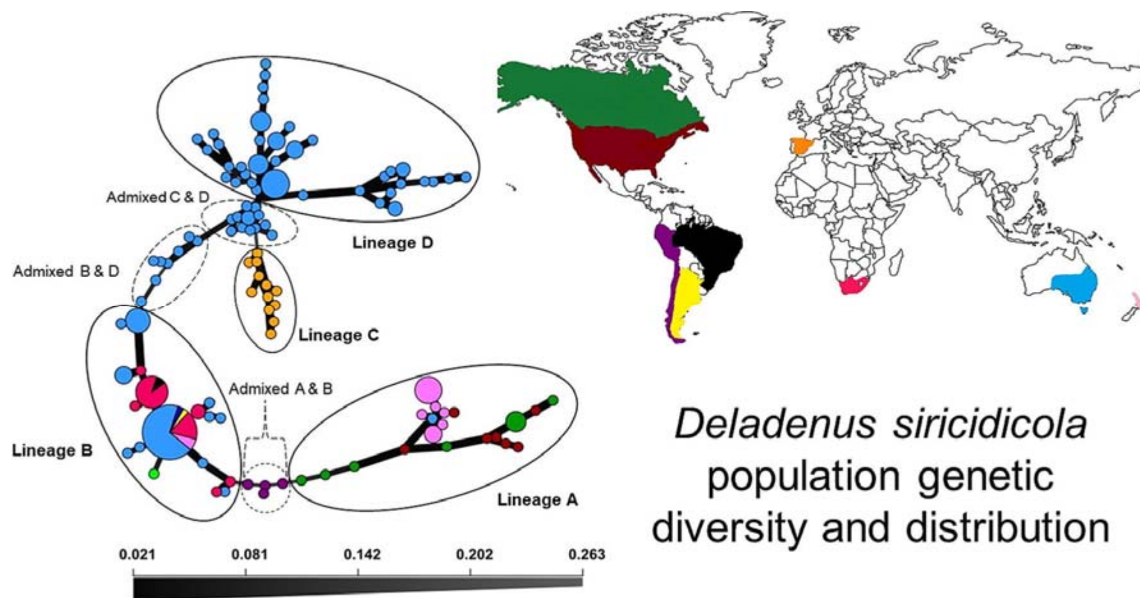
HIGHLIGHTS

- Australian and New Zealand *D. siricidicola* populations have unexpectedly high genetic diversity.
- A novel lineage of *D. siricidicola* is dominant in Australia.
- New Zealand and North American nematode populations share a common origin.
- Genetic patterns may reflect original introductions and selection over biocontrol releases.
- Newly found *D. siricidicola* diversity may improve biological control programs globally.

ABSTRACT

The nematode *Deladenus siricidicola* is used for the biocontrol of the invasive woodwasp, *Sirex noctilio,* that infests *Pinus* globally. In New Zealand, *D. siricidicola* was accidentally introduced along with *S. noctilio*, after which its potential for biological control was realized in the 1960s. Additional strains of the nematode were collected from across its Eurasian distribution for the development of a biological control program in Australia in the 1970s. A strain from Hungary (Sopron) was originally selected for large-scale releases and later replaced by a strain that was collected from previously released populations in Tasmania (Kamona). The Kamona strain is still used today in biological control programs in Australia, South Africa and South America. Despite the long history of biocontrol, the nematode's genetic diversity has not been studied in Australia and New Zealand. We assessed the population genetic diversity and structure of *D. siricidicola* in these countries using mitochondrial COI sequence data and 12 microsatellite markers. We also compared our data to that of a recent global diversity study. The results show a higher than expected diversity of the nematode in these Australasian countries, especially Australia. Three lineages previously defined from North America (A), Southern Hemisphere (B) and Spain (C) were identified in Australia (A, B, C) and New Zealand (A, B). A previously undescribed lineage (D), was identified in both Australia and New Zealand. Despite the evidence of admixture with other lineages, including the widely distributed biocontrol lineage (B; Kamona), lineage D remained distinguishable and dominant in these populations, even where Kamona has been released intensively for decades. Surprisingly, the results also revealed a shared history between populations in New Zealand and North America. These findings highlight the importance of understanding and monitoring genetic diversity in biological control programs and hold opportunities to improve both the selection and deployment of this nematode for the management of diverse populations of *S. noctilio*.

GRAPHICAL ABSTRACT



Keywords: *Sirex noctilio* woodwasp; Kamona strain; Sopron strain; Genetic diversity; Admixture; Biological control

1 Introduction

Sirex noctilio F. (Hymenoptera: Siricidae) and its symbiotic fungus *Amylostereum areolatum* (Chaillet ex Fr.) Boidin (Russulales: Amylostereaceae) is native to Eurasia and Northern Africa. This insect-fungus complex threatens commercial pine plantations in the Southern Hemisphere (Bedding and Iede, 2005; Nahrung et al., 2016; Slippers et al., 2012) while, in North America, both native and non-native *Pinus* spp. are at risk (Yemshanov et al., 2009; Dodds et al., 2010; Ayres et al., 2014). *Sirex noctilio* was accidentally introduced to New Zealand in the early 1900s (Bain et al., 2012; Slippers et al., 2015) and was reported in Australia in 1952 (Gilbert and Miller, 1952). In addition to native populations, *S. noctilio* from these two countries served as source populations for new introductions in the Southern Hemisphere (Boissin et al., 2012). *Sirex noctilio* was reported in several countries in South America in the 1980s (Argentina, Brazil, Chile and Uruguay) and in South Africa in 1994 (Hurley et al., 2007; Castillo et al., 2018; Wilcken et al., 2018). The most recent introductions were recorded in the USA, Canada and China, in 2004, 2005 and 2013,

respectively (Zylstra et al., 2010; Ayres et al., 2014; Li et al., 2015). In North America and East Asia, *S. noctilio* is not yet a serious pest to *Pinus* spp., partly because other siricids natural enemies in these regions help maintain *S. noctilio* populations at low levels.

Deladenus (=*Beddingia*) *siricidicola* (Tylenchida, Neotylenchidae), is a parasitic nematode of *S. noctilio* (Bedding and Akhurst, 1974; Bedding, 1993, 2009). Its life cycle comprises a free-living mycetophagous form and a parasitic form associated with structural dimorphism (Bedding, 1972; Bedding and Akhurst, 1974; Bedding and Iede, 2005). In the mycetophagous cycle, the nematodes graze and multiply on *A. areolatum*, a behaviour that is exploited for laboratory-based mass production of the nematode for management programs (Bedding, 1972). During the parasitic cycle, *D. siricidicola* infects woodwasp larvae, multiplies, and enters the developing eggs of emerging *S. noctilio* females (Bedding, 1972). The nematodes are spread to other trees when the woodwasp lays these infected eggs.

Deladenus siricidicola was first discovered in populations of *S. noctilio* in the North Island of New Zealand in 1962 (Zondag, 1962, 1969). In New Zealand, there are no native Siricidae, and a plausible scenario is that *D. siricidicola* has been introduced alongside *S. noctilio* in imported timber or wood products. This discovery stimulated its use as a biological control agent, and it was subsequently deployed on the South Island, leading to high levels of parasitism (Bedding, 1968; Zondag, 1969; Hurley et al., 2007; Bain et al., 2012). There has not been widespread deployment of the nematode since then in New Zealand, and a combination of silvicultural control and classical biological control through the nematode and parasitic wasps keep *S. noctilio* populations under control (Bain et al., 2012). It is not known, however, whether one or several strains of *D. siricidicola* were present in New Zealand at the time of the species discovery and first isolations for biological control, or whether additional accidental introductions continued over time.

In Australia, the Commonwealth Scientific and Industrial Research Organisation (CSIRO) began investigating *D. siricidicola* as a biological control agent in the 1960s (Spradbery and Kirk, 1978; Bedding and Iede, 2005; Bedding, 2009). An extensive collection and subsequent screening were done from areas where siricids are native (Bedding and Akhurst, 1974; Spradbery and Kirk, 1978; Bedding and Iede,

2005; Bedding, 2009). These studies confirmed *D. siricidicola* as the most suitable species for the biological control of *S. noctilio* and the first experimental releases of the Sopron strain (from Hungary) were made in Tasmania in 1970 (Bedding and Iede, 2005). The Sopron strain and three additional strains (from Corsica, Greece and New Zealand) were also released in the state of Victoria from 1973, with the Sopron strain (Sopron 198) ultimately selected for ongoing release in mainland Australia (Bedding and Akhurst, 1978; Spradbery and Kirk, 1978; Bedding, 2009; Collett and Elms, 2009). The Sopron strain, isolated from *S. juvencus*, was chosen because of its minimal impact on the size (and therefore flight range and egg laying ability) of parasitized adult female *S. noctilio* (Bedding, 2009).

In the late 1980s, the Sopron strain in mainland Australia appeared to have lost its ability to keep Sirex populations at a low level, with recorded parasitism levels of less than 30% (Haugen and Underdown, 1993). This reduction in virulence was attributed to an unintentional selection process during mass rearing favouring the mycetophagous form of the nematode at the expense of its parasitic form (Bedding and Iede, 2005; Collett and Elms, 2009). The more virulent form of the Sopron strain was, therefore, recollected from *S. noctilio* from the Kamona Forest in Tasmania, renamed as the Kamona strain and used for subsequent releases (Bedding, 1972; Bedding and Akhurst, 1978). The defective Sopron strain was unwittingly released in Brazil in 1989-90, followed by the introduction of Kamona in 1994 (Iede et al., 2012). Nematodes were subsequently distributed from Brazil to Argentina (1996-99), Chile (2006-09) and Uruguay (Martinez, 2020; Hajek et al., 2021). Kamona was also sent from Australia to South Africa (1995-96 and 2004-06), while nematodes from New Zealand were sent to Uruguay (1987), Argentina (1999, 2001) and Chile (2006-09) (Hajek et al., 2021). These shared resources and possible inbreeding in culture have led to genetically similar and remarkably homozygous populations of the nematode across the Southern Hemisphere (Mlonyeni et al., 2011). This lack of diversity holds potential risks for the long-term sustainability of the biocontrol program and limited opportunities for selection.

The success of the biological control program of *S. noctilio* using *D. siricidicola* has been variable among countries and over time, and there are factors other than loss of virulence that influence this variation (Hurley et al., 2008, 2012). Biological factors that are thought to influence *D. siricidicola* application success include variable

rates of reproduction on different fungal haplotypes of *A. areolatum* (Hurley et al., 2012; Morris et al., 2012; Caetano et al., 2016; Mlonyeni et al., 2018b), competition with co-occurring fungi (such as sapstain fungi; Hurley et al., 2012), variability in the ability to convert to an infective form (Mlonyeni et al., 2018a), variation in resistance between different *S. noctilio* populations (Bittner et al., 2019; Morris et al., 2020), and variation amongst different *Pinus* spp. (Nahrung et al., 2016; Williams and Hajek, 2017). Non-biological factors, such as local climatic conditions, wood moisture content, inoculation techniques and spatial arrangements of release can influence the consistency of *D. siricidicola* parasitism (Hurley et al., 2008; Corley et al., 2014, 2019; Yousuf et al., 2014).

The population genetics of *D. siricidicola* have been studied in both introduced and native areas using mitochondrial and microsatellite markers (Mlonyeni et al., 2011; Fitza et al., 2019; Morris et al., 2020). Mlonyeni et al. (2011) designed 10 microsatellite markers and characterized a limited number of nematode populations from Canada and Southern Hemisphere (Argentina, Australia, Brazil and South Africa). Fitza et al. (2019) added mtCOI sequence data, as well as two additional microsatellite markers to additional collections of *D. siricidicola* from Argentina, Brazil, Canada, Chile, New Zealand, Spain and the USA. That study confirmed the lack of diversity of the nematode in the Southern Hemisphere populations and identified three distinct lineages based on the sampled populations: lineage A, related to the accidental introduction of nematode populations in North American, lineage B, biological control populations in the Southern Hemisphere and lineage C, a population from the native European range (Fitza et al., 2019). The evidence of an admixed population in Chile together with evidence of potential reproduction between lineages Fitza et al. (2022), is an opportunity to introduce diversity into nematode populations in management programs.

In the light of the importance of understanding diversity in this globally practiced biological control program, we aimed to study the diversity of the *D. siricidicola* populations in the areas where it was identified and has been used for more than 50 years, in New Zealand and Australia, respectively. Our collections from across this region allowed us, for the first time, to consider whether the Sopron and Kamona strains are the only established population across pine plantations in Australia and how this diversity has been influenced by the long-term biological control program. We

also explored the historical connections between populations of *D. siricidicola* from New Zealand and Australia, and between these populations and those elsewhere in the world.

2 Materials and methods

2.1 Sampling

To cover a large geographic area, sampling of *D. siricidicola* was made from four states in Australia (New South Wales, South Australia, Victoria and Tasmania) and the North Island of New Zealand where the introduction of the nematode was accidental (Figs.1A & B). Each nematode strain represents an isolate recovered from an individually infected wasp either as a living culture or as ethanol-preserved wasp eggs or testes, and these are all from uninoculated trees (Supplementary Table S1). To compare changes over time, the commercially available Kamona strain of *D. siricidicola* (provided and produced by EcoGrow Australia) and the defective form of the Sopron strain (the first commercially used strain and provided by CSIRO and retrieved from liquid N storage) were included in this study. The mycetophagous form of the nematode cultures are maintained at the Forestry and Agricultural Biotechnology Institute (FABI), Biocontrol Centre, University of Pretoria, South Africa according to the protocol described in Bedding and Akhurst (1974).

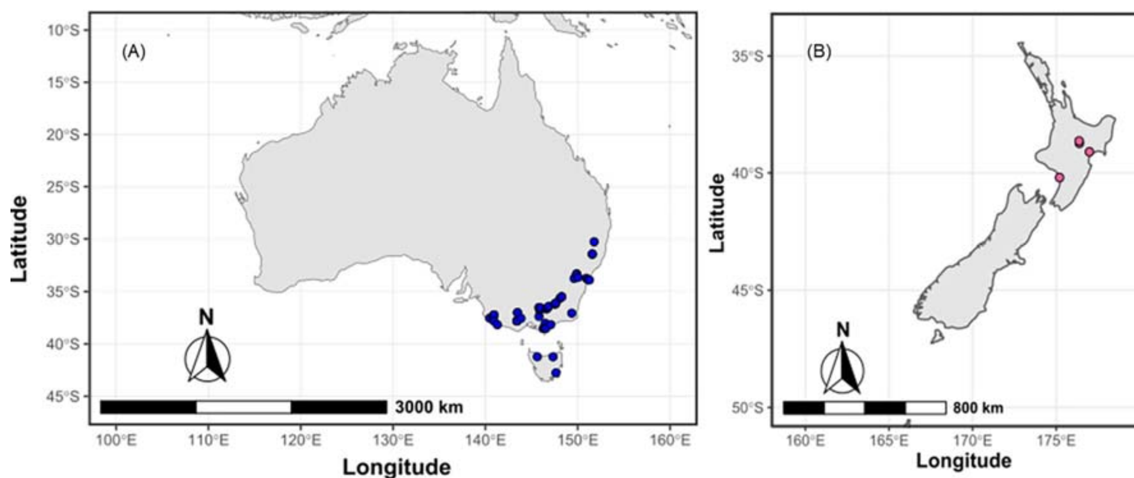


Fig. 1. *Deladenus siricidicola* collection sites. (A) Tasmania and three states (i.e., Victoria, New South Wales, and South Australia) from mainland Australia, and (B) North Island of New Zealand.

2.2 DNA extraction

Genomic DNA was either extracted from each nematode strain reared (bulked up) in 500 ml Erlenmeyer flasks containing wheat, rice and *A. areolatum*, or on Petri dish plates containing Malt Extract Agar (MEA, 20 g l^{-1} malt extract, 25 g l^{-1} purified agar) and *A. areolatum*, nematodes preserved in ethanol, or nematodes from ethanol-preserved infected wasp eggs and testes. For the nematode cultures reared in a flask and Petri dish, harvesting and preparation were performed according to Mlonyeni et al. (2011) and Fitza et al. (2019), and DNA was extracted using phenol-chloroform as described in Sambrook and Russell (2006). Genomic DNA precipitation, further cleaning and dilutions were performed as described by Fitza et al. (2019). The NucleoSpin® Tissue (Macherey-Negel, Germany) high DNA recovery and purity kit was used for DNA extracted from ethanol-preserved nematodes and ethanol-preserved infected wasp eggs following the manufacturer's instructions. The concentration of the genomic DNA harvested using both methods was quantified with a Nano-Drop ND-1000 UV/Vis Spectrometer (NanoDrop Technologies, Wilmington, DE 19810 USA). Genomic DNA concentrations were adjusted to a final working concentration of 15 ng/µl for downstream PCR amplification.

2.2.1 Mitochondrial (mtCOI) diversity analyses

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

Sequences were edited and manually checked in Bioedit version 7.2.5 (Hall 1999) and aligned using the MAFFT free online alignment program version 7 (Katoh et al., 2017). Segregation diversity (S), the average number of nucleotide differences (K), haplotype diversity (Hd) and nucleotide diversity indices (Pi), were calculated using DNA sequence polymorphism DnaSP version 6.11.1 (Rozas et al., 2017). To estimate the evolution of mtCOI sequences amongst the Australian and New Zealand populations in comparison to the previously identified lineages, nucleotide divergence was calculated in DnaSP version 6.11.1 (Rozas et al., 2017). The median-joining haplotype network was constructed using NETWORK version 5.0.0.3 to investigate the relationships among populations. In the analyses, data generated by Fitza et al. (2019) from strains of South America, New Zealand, North America, Spain and South Africa were included in this study.

2.2.2 Application of microsatellite markers

A total of 12 previously developed microsatellite markers (Mlonyeni et al., 2011; Fitza et al., 2019) were used in this study (Supplementary Table S2). The PCR reaction mixture and thermal cycler conditions were used as in Fitza et al. (2019). For fragment analyses, a 1:100 dilution of PCR amplicon with sterile SABAX water was made for all strains and pooled according to the panel arrangement as in Fitza et al. (2019). LIZ500 (GeneScan™ 500 LIZ™ dye Size Standard, Thermo Fisher Scientific, Waltham, MA, USA) was used as the standard marker. The GeneScan mix per lane was prepared by mixing 0.2 µl 500 LIZ™, 10 µl Hi-Di™ Formamide and 1 µl of pooled PCR mix. Denaturation was performed at 95°C for 3 min and the amplicons (1 µl per lane) were run on the ABI PRISM TM 3500xl DNA analyzer to determine product size (DNA sequencing facility, University of Pretoria). The GeneScan data was analysed using GeneMapper® v4.1 (Life Technologies, Foster City, CA) to score allele fragment sizes.

2.3 Microsatellite analysis

2.3.1 Genetic diversity analysis

Indices to measure genetic diversity were calculated using the data generated in this study for the strains from Australia and New Zealand and the dataset of Fitza et al. (2019). Indices calculated included: number of multilocus genotypes (MLG), number of expected multilocus genotypes based on rarefaction (eMLG) (Hurlbert,

1971; Heck Jr. et al., 1975), standard error (SE) based on eMLG rarefaction, MLG diversity (H) using Shannon-Wiener Index (Shannon, 1948), MLG diversity (G) using Stoddart and Taylor's index (Stoddart and Taylor, 1988), Evenness (E5) (Grünwald et al., 2003), Nei's unbiased gene diversity (Nei, 1978) and the index of association. These analyses were conducted in R applying the Poppr package (Kamvar et al., 2014). Estimates of heterozygosity, F-statistics (F_{st}, F_{is} and F_{it}), Nei's genetic distance, and Nei's unbiased genetic distance were estimated using GenAlEx version 6.505 (Peakall and Smouse, 2012). The levels of population differentiation were evaluated using analysis of molecular variance (AMOVA) using the R package Poppr (Kamvar et al., 2014). The allelic richness (AR) and private allelic richness were calculated in Hp-rare version1.1 Kalinowski (2005) for each population at every locus which uses rarefaction to account for differences in sample size.

2.3.2 Population structure and genetic relatedness analysis

Minimum spanning networks (MSN) were constructed using Nei's distance (Nei, 1978) to assess the genetic relatedness among the observed multilocus genotypes (MLGs) in this study with previously defined haplotypes in Fitza et al. (2019). Furthermore, to determine if there is any structure in the population, a model-based Bayesian clustering algorithm was implemented in the STRUCTURE program version 2.3.4 (Pritchard et al., 2000). The parameters were set with the assumption of admixture (ancestry model), whereby individuals may have mixed ancestry and/or individuals have inherited a proportion of their genome from each of the K populations. The K values were tested from 1 to 10, each with twenty independent runs, 700 000 Markov Chain Monte Carlo (MCMC) iterations, and a burn-in of 100 000. The optimal cluster for K, that best fit the data was calculated in STRUCTURE using the Evanno method (Evanno et al., 2005), and the clusters were assessed and visualized in STRUCTURE HARVESTER (Earl and vonHoldt, 2012) and CLUMPAK (http://clumpak.tau.ac.il/). Principal coordinate analysis (PCoA) was analysed with a 9999-permutation test using GenAlEx version 6.505 (Peakall and Smouse, 2012) to further investigate the population sub-division without assuming the Hardy-Weinberg equilibrium.

2.3.3 Scenario testing to infer routes of introduction of *D. siricidicola*

To infer the population introduction history of lineage D, the Approximate Bayesian Computation, DIY ABC analysis was performed (Cornuet et al., 2014). Combining previous knowledge of the introduction history of *D. siricidicola* (Fitza et al., 2019) with STRUCTURE outputs in this study, 31 scenarios were hypothesized (Supplementary Fig. S1). Previously identified lineages A (North American), B (the biocontrol population, Australia, South Africa, New Zealand and South America) and C (Spain), and the new lineage D (Australia) were defined as separate populations and an unsampled population was also considered as the possible source population. For DIY ABC analysis, the SSR primers were grouped as in Fitza et al. (2019) and had been calibrated at two different mutational rates. The two different sets of mutational rates used were based on the *Caenorhabditis elegans* and *Pristionchus pacificus* nematode mutational rates (Denver et al., 2009; Molnar et al., 2012) and as described in Fitza et al. (2019) with minor modifications. For dinucleotide repeats, the mean mutational rate and the individual locus mutation were set at $1E^5$- 1 E^3. For trinucleotide repeats, the mean mutational rate was set at $1E^6$– $1E^3$ and the individual locus mutation rate at $1E^7$ – $1E^3$. The sample summary statistics were set as follows i) for the one sample summary statistics variables: the mean number of alleles, mean genetic diversity and mean size variance were chosen and, ii) for the two-sample summary statistic variables: F_{st}, classification index, shared allelic distance and d^2 distance were chosen. To further evaluate the combination of hypothesized prior scenarios, the data set was simulated at 7 000 000 permutations. For the posterior analyses, the hypothesized 31 scenarios were categorized into two groups: i) scenario 1 to 16 and ii) scenario 17 to 31. Out of 31, a total of 7 scenarios were chosen for further posterior analyses from the two groups, and three strongly favoured scenarios were chosen for final test. The analyses of posterior probability were then further performed using the closest 1% of simulated data (Fitza et al., 2019). To again choose a strongly favoured scenario amongst the above three scenarios, model checking was further performed for each scenario the same as described in Fitza et al. (2019). Analyses to evaluate confidence in scenario choice, estimates of posterior distributions parameters and bias and precision on parameter estimation were performed using a default prior distribution.

3 Results

3.1 Samples

A total of 129 *D. siricidicola* strains were obtained from the North Island in New Zealand (n = 14), and four states in Australia (n = 34 from New South Wales; n = 12 from South Australia, n = 3 from Tasmania; n = 66 from Victoria) (Supplementary Table S1). Both the Sopron and Kamona strains (from EcoGrow Australia) that have been used historically in the biological control program in Australia were also included in the study (Supplementary Table S1).

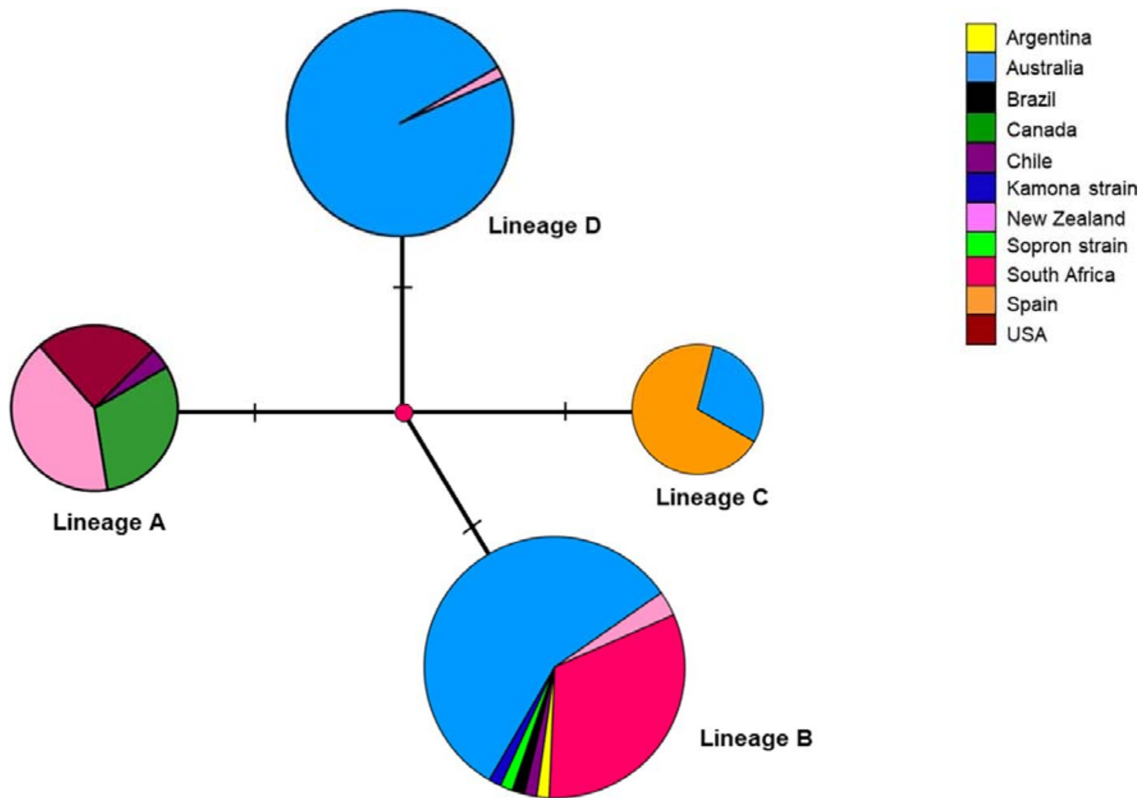


Fig. 2. Mitochondrial cytochrome *c* oxidase subunit 1 (mtCOI) sequence-based median-joining haplotype network of *D. siricidicola* populations. Each node represents strains that share a similar mtCOI genetic profile and colours represent geographic origin.

3.2 Mitochondrial (mtCOI) diversity analyses

A 523 bp amplicon of the mtCOI gene region was successfully sequenced for 119 of the *D. siricidicola* strains collected from Australia and New Zealand. mtCOI sequence revealed nucleotide base polymorphism at four different nucleotide positions 208, 385, 314 and 23, each distinguishing lineages A, B, C and D, respectively (Supplementary Table S3). All four identified lineages were present in Australasia (Fig. 2). The dominant haplotype in New Zealand was lineage A. Lineage B contained the Kamona and Sopron strains and was identified from other all Southern Hemisphere countries. Lineage C was present in Australia and not identified in New Zealand. Lineage (D) observed for the first time in this study primarily consisted of strains from Australia (58/106), and to a lesser extent (1/14) strains from New Zealand.

3.2.1 Estimation of genetic diversity using mtCOI sequence

Nucleotide differences (K) were low between all populations of *D. siricidicola* (Supplementary Table S4). The highest K values (K = 0.004) were seen mostly when comparing populations from Australia with New Zealand, North America and Spain, reflecting 0.4 % nucleotide differences of different haplotypes across these countries (Supplementary Table S4). There was 100 % nucleotide similarity between *D. siricidicola* from South Africa and the commercially used strains. In general, the Australian *D. siricidicola* population was found to be genetically similar to the two commercial strains, South Africa and South American *D. siricidicola* populations, whilst the New Zealand *D. siricidicola* population had high similarity with the North American population (K = 0.001).

Genetic diversity indices were highest for Australia and New Zealand (Table 1). The haplotype diversity in the Australian population (Hd = 0.529) was the second highest after South America (Hd = 0.600, including strains from Argentina, Chile and Brazil), followed by New Zealand (Hd = 0.362). For the average number of nucleotide differences and nucleotide diversity analysis, Australia had the highest values followed by South America and New Zealand. Sampling efforts across these regions were not equal, which might account for some of the variation in diversity indices between regions.

Table 1. Genetic diversity indices were simulated using cytochrome *c* oxidase subunit 1 (mtCOI) sequence data of *D. siricidicola* populations from Australia and New Zealand and compared to previous collections (Fitza et al., 2019).

Country	N[a]	S[b]	H[c]	Hd[d]	K[e]	Pi[f]
Australia	98	3	3	0.529	1.059	0.002
New Zealand	15	2	3	0.362	0.381	0.000
South Africa	20	0	1	0.000	0.000	0.000
South America	5	1	2	0.600	0.600	0.001
Spain	12	0	1	0.000	0.000	0.000
North America	16	0	1	0.000	0.000	0.000
Commercial	3	0	1	0.000	0.000	0.000
Total	169	3	4	0.701	1.108	0.002

[a]N: Sample size

[b]S: Number of polymorphic (segregating) sites

[c]H: Number of haplotypes

[d]Hd: Haplotype diversity

[e]K: Average number of nucleotide differences

[f]Pi: Nucleotide diversity

3.3 Microsatellite analysis

3.3.1 Estimates of allelic richness

In Australia, a population from Victoria had the greatest number of both observed (A_R = 44) and private (Pa = 9) alleles followed by New South Wales (A_R = 38 observed, Pa = 4 private) (Table 2). The allelic richness was further analyzed by comparing the Australian and New Zealand population with populations from South America, North America, Spain and South Africa (Table 2). The allelic richness in Australia was the highest with a 4.33 average number of observed alleles per loci (Supplementary Table S5), 70 multilocus genotype groups (MLGs) and expected multilocus genotypes (eMLGs) of 8.67, but is most likely skewed due to the larger sample size. The number of private alleles in the population from Australia (Pa = 25), for example, was greater than populations from native areas (Spain, (Pa = 10); Table 2). Allelic richness in the New Zealand population was moderate based on the

Table 2. Genetic diversity indices for *D. siricidicola* populations from Fitza et al. (2019) and collections for this study from Australia and New Zealand.

	Population	N[a]	A_R[b] (Pa[c])	MLG[d] (eMLG[e])	H[f]	G[g]	E5[h]	He[i]
All populations	Australia	117	54 (25)	70 (8.67 ± 1.10)	3.77	20.4	0.46	0.51
	New Zealand	16	26 (3)	7 (5.34 ± 0.87)	1.63	3.88	0.70	0.29
	South Africa	21	20 (0)	7 (4.62 ± 0.96)	1.50	3.32	0.67	0.07
	South America	6	25 (1)	6 (6.00 ± 0.00)	1.79	6.00	1.00	0.40
	Spain	12	22 (10)	12 (10.00 ± 0.00)	2.48	2.00	1.00	0.19
	North America	16	25 (7)	13 (8.49 ± 0.85)	2.43	9.14	0.79	0.16
	Total	188		111 (8.92 ± 1.02)	4.17	27.06	0.41	0.60
Australian sub-populations	New South Wales	34	38 (4)	27 (10.96 ± 0.89)[e]	3.20	21.41	0.87	0.36
	South Australia	12	27 (0)	8 (8.00 ± 0.00)[e]	1.98	6.55	0.89	0.37
	Victoria	66	44 (9)	41 (9.30 ± 1.40)[e]	3.19	10.84	0.42	0.52
	Tasmania	3	24 (0)	3	-	-	-	-
	Commercial	2	17 (1)	2	-	-	-	-

[a] N: Number of strains per collection site

[b] A_R: Allelic richness observed per collection site

[c] Pa: Number of private allelic richness observed per collection site

[d] MLG: Number of multi locus genotypes observed per collection sites

[e] eMLG: Number of expected multilocus genotypes at the smallest sample size (12) based on rarefaction ± standard error)

[f] H: Shannon-Wiener Index of MLG diversity

[g] G: Stoddart and Taylor's Index of MLG diversity

[h] E5: Evenness (distribution of genotype abundances)

[i] He: Nei's unbiased gene diversity

numbers of observed and private alleles (A_R = 26 and Pa = 3, respectively) (Table 2). Among the regions that use *D. siricidicola* as a biocontrol agent, the South African population had the lowest mean number of observed alleles per loci (n = 1.58) (Supplementary Table S5) and no private alleles, reflecting the establishment of a single MLG for the biocontrol program.

3.3.2 Genotypic diversity analyses

A total of 73 MLGs were observed in Australia and New Zealand (Table 2). In Australia, the highest number of MLG groups was observed in Victoria (41 MLG) followed by New South Wales (27 MLG), with the same patterns reflected in allelic richness. The MLG observed in South Australia and New Zealand was, MLG = 8 and MLG= 7, respectively (Table 2). However, based on the number of eMLG, New South Wales (10.96 eMLG), Victoria (9.3 eMLG), South Australia (8 eMLG) and New Zealand (5.94 eMLG) ranked first to fourth in their eMLG diversity. Comparing states within Australia and New Zealand, New South Wales was shown to potentially have a greater diversity at H (3.20) and G (21.41) followed by Victoria at H (3.19) and G (10.84). Comparing the diversity of the Australian population to New Zealand, H and G were greater for Australia (3.77 and 20.40 vs 1.63 and 3.88). The E5 estimates of genotype abundance distribution were 0.70 and 0.46 for Australia and New Zealand populations, respectively. eMLG at the smallest sample size based on rarefaction at eMLG = 8.67 vs eMLG = 10.00 for Australia and Spain respectively, and the distribution of genotype abundance (E5 = 0.46 vs E5 = 1.00) were both greater for the Spanish population.

3.3.3 Estimates of genetic differentiation

The overall F_{st} value observed in the global *D. siricidicola* population was > 0.5 (Table 3), supporting a high degree of genetic differentiation among populations and limited gene flow. This was supported with the AMOVA analyses ($p \leq 0.01$) (Table 3) that showed most of the variation observed was between populations (45.90 %). The pairwise F_{st} value between *D. siricidicola* populations from different regions showed genetic differentiation that ranged from F_{st} = 0.1 between New Zealand and North American populations to F_{st} = 0.81 between South Africa and the North American populations. The estimate of F_{st} between the Australian and the New Zealand populations showed 26.8 % of genetic differentiation of the total observed genetic diversity (F_{st} = 0.27). The Australian population was least differentiated from those

Table 3. Analysis of molecular variance (AMOVA) and genetic differentiation (F_{st}) estimate of *D. siricidicola* collections.

	Source of variation	Degree of Freedom	Sum of Square	Mean Square	Estimates of Variance	Percentage (%) of the total variance	P-value
All populations	Between population	5.00	958.77	191.76	4.23	45.90	0.01
	Between strains within population	181.00	1613.57	8.92	3.92	42.53	0.01
	Within population	187.00	199.56	1.07	1.07	11.57	0.01
	Total	373.00	2771.90	7.43	9.23	100.00	
	Total F_{is}				0.55 ± 0.07		
	Total F_{st}				0.58 ± 0.03		
Australian and New Zealand sub-populations	Between populations	3.00	314.91	104.97	1.76	26.78	0.01
	Between strains within population	124.00	1109.72	8.95	4.14	62.91	0.01
	Within population	128.00	86.80	0.68	0.68	10.31	0.01
	Total	255.00	1511.42	5.93	6.57	100.00	
	Total F_{is}				0.86 ± 0.04		
	Total F_{st}				0.29 ± 0.06		

F_{st} = (H_t - Mean He) / Ht. F_{st} = the genetic differentiation coefficient as estimated by mean F_{st} and the standard error, F_{is}= inbreeding coefficient as estimated by mean F_{is} and the standard error

from South America (Argentina, Brazil and Chile) (F_{st} = 0.19) (Supplementary Table S6).

The majority of the variation and the diversity that existed in the states in Australia and New Zealand were explained by the variation between strains within the population (62.91 %) (Table 3). The variation that existed both between New South Wales, Victoria, South Australia, Tasmania and New Zealand (26.78 %) explains the distribution of different populations (lineage A) in New Zealand (Table 3). This was further confirmed in a pairwise F_{st} analysis which resulted in minimal genetic differentiation amongst states. The pairwise F_{st} analysis between the commercial strain and New South Wales, South Australia, New Zealand resulted, in F_{st} = 0.45, F_{st} = 0.44, and F_{st} = 0.58, respectively, reflecting relatively limited gene flow (Supplementary Table S7).

Table 4. Hierarchical analysis of heterozygosity of *D. siricidicola* populations. Mean and standard error of observed heterozygosity (Ho) expected heterozygosity (He) and unbiased expected heterozygosity (uHe) as estimated over 12 SSR loci for each population.

Country	N	Ho[a]	He[b]	uHe[b]
Australia	117	0.066 ± 0.021	0.503± 0.0503	0.505 ± 0.0505
New Zealand	16	0.052 ± 0.047	0.278 ± 0.039	0.287 ± 0.040
South Africa	21	0.083 ± 0.049	0.067 ± 0.036	0.069 ± 0.037
South America	6	0.306 ± 0.050	0.367 ± 0.048	0.400 ± 0.052
Spain	12	0.153 ± 0.061	0.183 ± 0.074	0.191 ± 0.078
North America	16	0.147 ± 0.070	0.156 ± 0. 057	0.161 ± 0.059
Total	188	0.134 ± 0.023	0.259 ± 0.027	0.269 ± 0.028

[a] Ho: Observed heterozygosity ± standard Error

[b] He: Expected heterozygosity ± standard Error

[c] uHe: Unbiased expected heterozygosity

3.3.4 Estimates of heterozygosity

The overall observed heterozygosity (Ho = 0.134 ± 0.023) for *D. siricidicola* globally was much smaller than that of the expected heterozygosity (He = 0.259 ± 0.027) (Table 4). Despite the diversity that exists within Australia, the level of observed heterozygosity Ho (Ho = 0.066 ± SE= 0.021) was found to be nearly 100 times less than the He (He=0.503± SE= 0.050) and uHe (uHe = 0.505 ± SE = 0.0505). Likewise, in New Zealand, the observed heterozygosity was lower than expected Ho = 0.052 ± SE= 0.047, He = 0.278 ± SE = 0.039, uHe = 0.287 ± SE=0.040, for Ho, He and uHe, respectively. However, in the South American population heterozygosity was prominent and the differences observed between each of the heterozygosity values, Ho and He/uHe were relatively low (Table 4).

3.3.5 Inference of population structure and network analyses

Based on microsatellite data, the Australian population showed evidence of a shared history with almost all existing mtCOI lineages, but with the majority of strains forming a defined cluster akin to mtCOI lineage D (Fig. 3). Lineage D of *D. siricidicola* was unique and found in Australia using both mtCOI haplotype and SSR genotyping, whereas in New Zealand it was only found in the mtCOI haplotype network analysis. Lineages A, B, C and D all exist in Australia. The two lineages (B) and the new lineage (D) appeared to be the dominant lineages in Australia (Fig. 3). Evidence for admixture between existing SSR lineages was also evident in Australia (Fig. 3 & Supplementary Table S2). New Zealand had two SSR lineages and some strains were grouped with North American strains.

The population structure and strain distribution were revealed using PCoA and highlighted four dominant SSR lineages globally (Fig. 4). PCoA showed two principal coordinates describing 69.64 % of the total observed variation (Fig. 4). In Australia, more than 50 % of the strains assessed grouped with lineage D. However, the distribution of each lineage in Australia were not specific to specific regions.

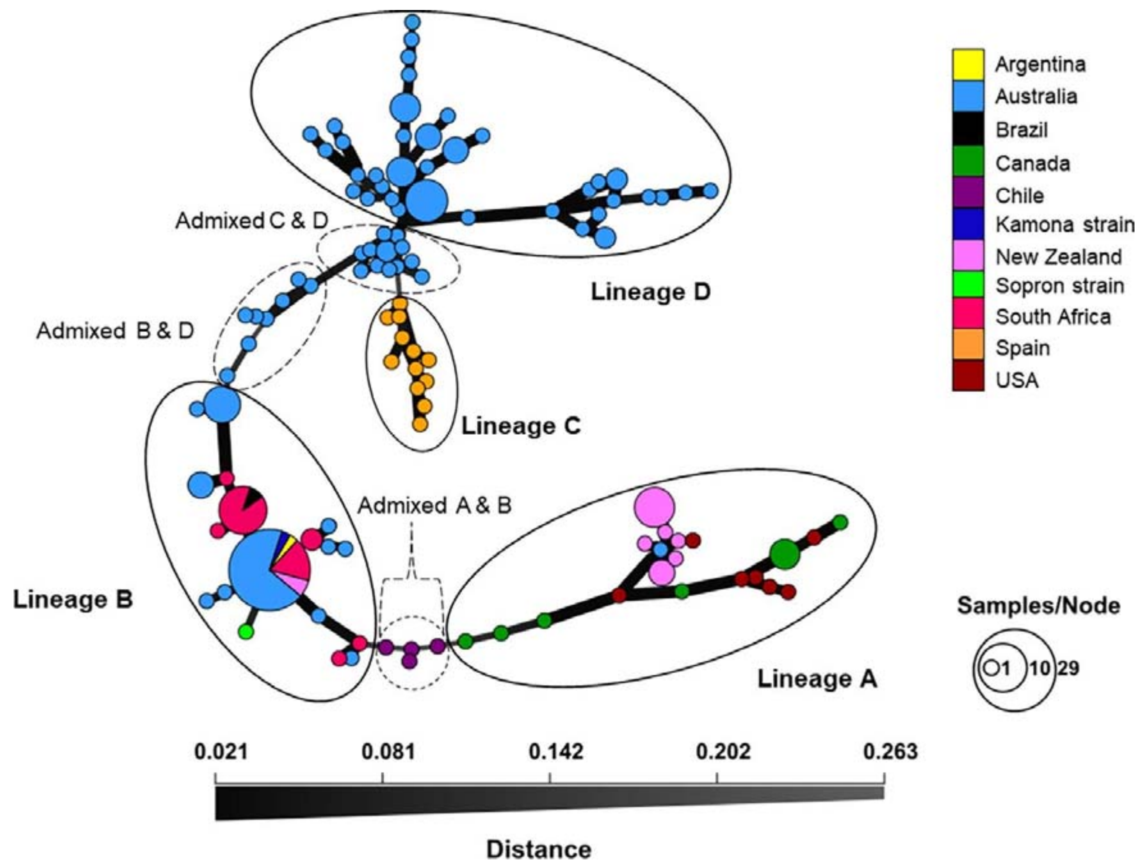


Fig. 3. Minimum spanning network constructed using Nei's distances showing four distinct clusters (lineages) separated by a large genetic distance. The sizes of the nodes are proportional to the number of strains representing the MLG and the thickness of the lines represent the Nei genetic distance between two nodes (thicker lines denote smaller genetic distance). The outer solid circle illustrates MLGs that share a lineage based on mtCOI. Dashed circles represent admixed strains based on STRUCTURE analyses and colours represent geographic origin.

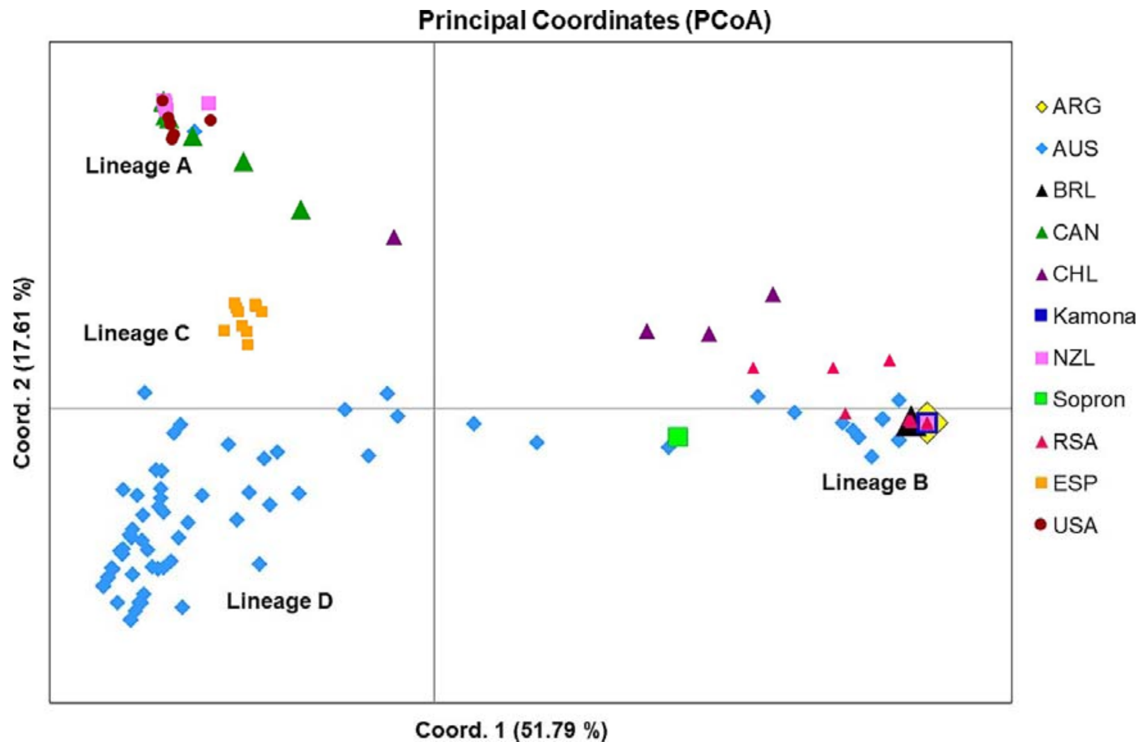


Fig. 4. Principal coordinate analysis plot (PCoA) of *D. siricidicola* populations, constructed using Nei's genetic distance (Nei 1978). The percentage at coordinates 1 and 2 indicates the extent of observed genetic variation in the simulated dataset. Colours represent the geographic origin of strains. ARG, Argentina; AUS, Australia; BRL, Brazil; CAN, Canada; CHL, Chile; Kamona, currently used commercial strain; NZL, New Zealand; Sopron, historical commercial strain (defective); RSA, South Africa; SA, South America; ESP, Spain; and USA, United States of America.

The population structure was further analyzed using STRUCTURE analyses and the results of NETWORK, PCoA and STRUCTURE, were highly congruent showing establishment of four distinct populations globally. Assessment of the ΔK statistics (Evanno et al., 2005) for the best K search was, however, incongruent with STRUCTURE and exhibited three populations (K = 3) globally (Supplementary Fig. S2A) and two (K = 2) within Australian and New Zealand sub-populations (Supplementary Fig. S2B). Given results observed in NETWORK and PCoA analyses, the most likely Ks were K = 4 and K = 3 globally and in Australian and New Zealand sub-populations, respectively. At K = 4, the four global lineages (A, B, C and D) were distinguished (Fig. 5A), while at K = 3 value the three lineages (A, B and D) were

identified from Australian and New Zealand sub-populations (Fig. 5B). There was evidence of admixture within the Australian population.

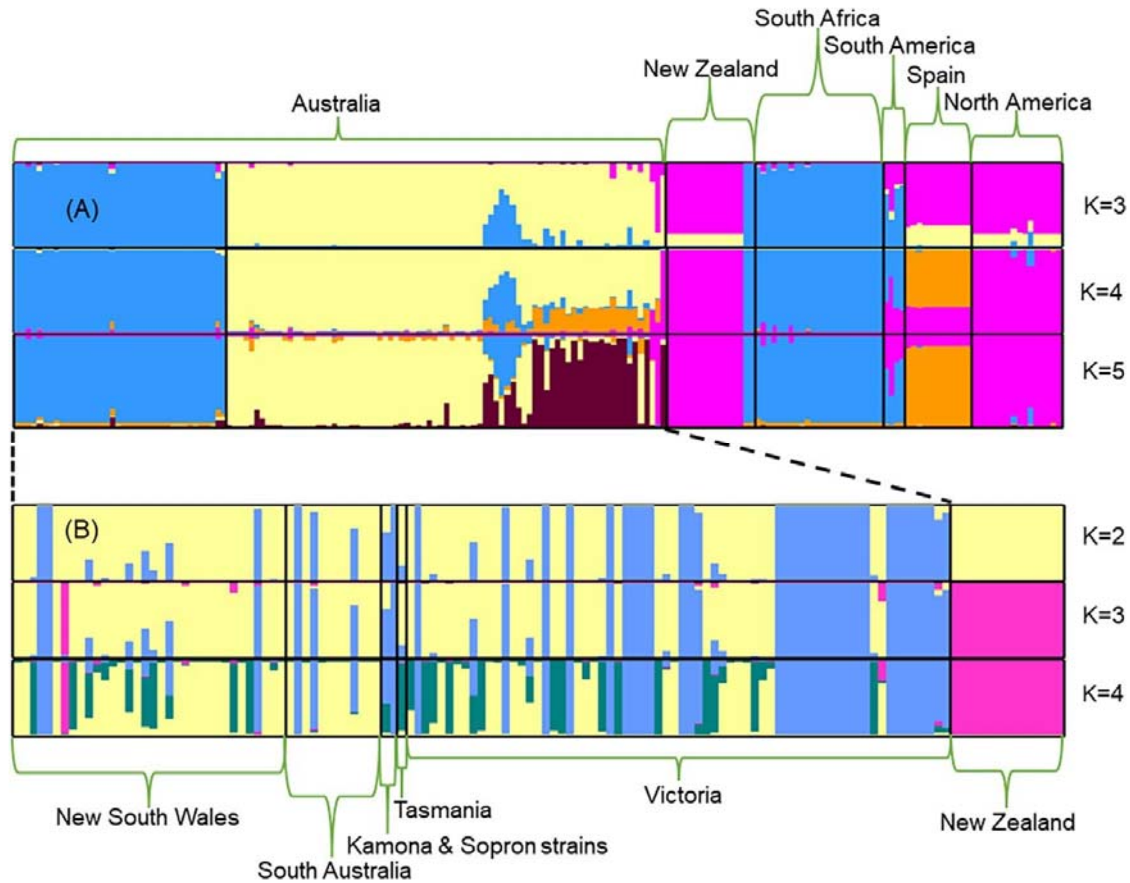


Fig. 5. Bayesian clustering of *D. siricidicola* populations using STRUCTURE. Clustering inferred (A) at Δk = 3, Δk = 4 and Δk = 5 using all data and (B) at Δk = 2, Δk = 3 and Δk = 4 using Australian and New Zealand *D. siricidicola* populations. Different clusters are divided into K colours and the vertical bar represents individual strain that lies within each cluster.

3.3.6 Evolutionary history and scenario testing between lineage groups

Of the 31 hypothesized scenarios tested in DIY ABC analyses (Supplementary Fig. S1), 12, 28 and 29 were the three most strongly favoured scenarios in explaining the simulated data set in posterior-based analyses (Fig. 6). In Scenario 12 (Fig. 6), lineages B and D diverged first from an unsampled population. Lineage D then gave rise to lineage A and C. In scenario 28 (Fig. 6), there is an unsampled population that

gives rise to two lineages: the oldest lineage of C and then a younger lineage B. Subsequently, lineage C gave rise to lineage D and lineage A. Each independent introduction in the analyses is indicated with sub-populations, B2 and C3 and the y-axis indicate a time point of divergences (i.e., t0, most recent populations; tn-db, a time when independent divergences occurred and to t4, an ancestral population). Scenario 29 is the same as 28 except that lineage A diverges off lineage C before lineage D (Fig. 6). The posterior probability of scenarios estimated through a direct approach, logistic regression and model fit evaluation showed the strongest support for scenario 28 (Supplementary Figs. S3 & S 4-6). The posterior probability analyses with a 95 % credibility interval, the so-called *type I error* for scenario 28 have also validated the scenario choice with 1000 simulations in the direct approach and logistic regression, p=0.418 and p=0.356 respectively (Supplementary Table S8). Analyses of confidence in the scenario choice also showed the strongest support for scenario 28. Our analysis inferred lineage D in Australia is likely sourced from Europe and established through an independent introduction from the biocontrol population of lineage B. As with mtCOI based network analysis the DIY ABC analysis supports at least three independent introductions to Australasia.

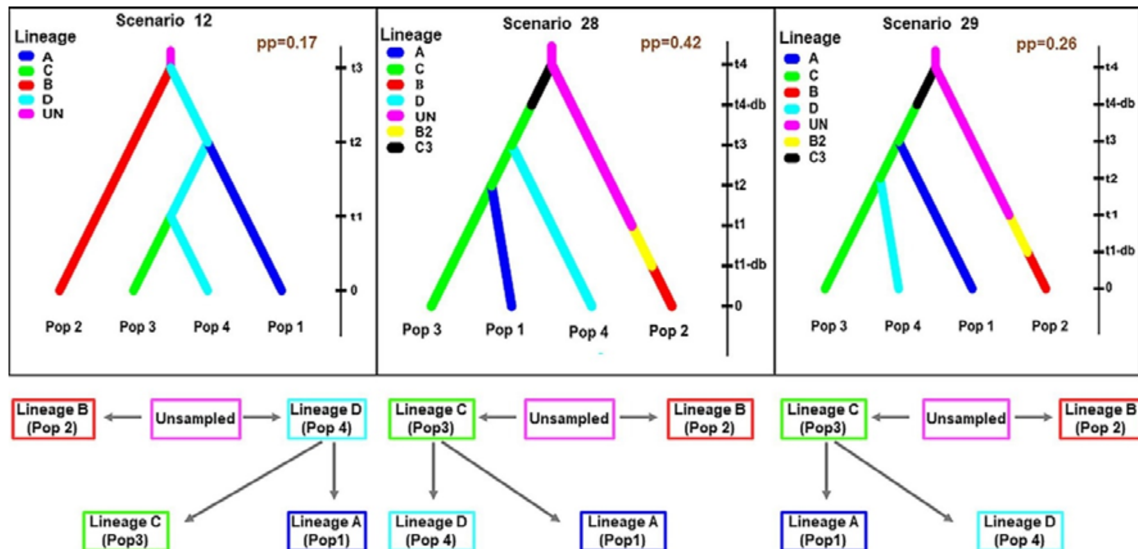


Fig. 6. Scenarios tested in DIY ABC analysis to infer *D. siricidicola* introduction routes into Australia and New Zealand. The three best scenarios out of 31 hypothesized scenarios are displayed. Arrows illustrate the possible routes of introduction hypothesized under each scenario. Pop1 (Lineage A: North America and New Zealand strains), Pop2 (Lineage B: South America, South Africa, commercial strains, and Kamona relative Australian strains), Pop3

(Lineage C: Spanish lineage) and Pop4 (Lineage D: Australian strains). B2 and C3 in scenarios 28 and 29 show independent introduction of population from their ancestral origin.

4 Discussion

This study confirmed the presence in Australia and New Zealand of a globally widespread lineage that includes the Kamona and Sopron strains used for biological control programmes in South Africa and South America (lineage B). It also confirmed the presence of another lineage in New Zealand that was only identified elsewhere from Chile and North America (A). More surprisingly, a lineage only known from Spain was also identified in Australia (C), while a previously unknown lineage was also found in Australia and New Zealand (D). There is evidence of limited admixture between lineages (B and D, and D and C) in Australia, but not obscuring the distinction between them. This, and the widespread abundance of lineage D, is curious, especially in Australia where the strains extensively and exclusively released over five decades were identified to lineage B. The results paint a unique picture of intentional and unintentional introductions of *D. siricidicola* around the world and open many questions relating to the adaptation of strains and opportunities to explore the diversity in these populations.

Both mtCOI sequence analysis and SSR data supported multiple unintentional introductions of *D. siricidicola* into New Zealand, given that no deliberate releases were made there other than the redistribution of the original strain from North Island to South Island in the 1970s (Hurley et al., 2007; Bain et al., 2012). The presence of at least three distinct lineages of *D. siricidicola* in New Zealand (A, B and D) provides a possible explanation for a curious result of Fitza et al. (2019) which identified admixture between lineage A and lineage B in Chile. *Deladenus siricidicola* populations in Chile were established from intentional introductions from New Zealand and Brazil (Hurley et al., 2007; Corley et al., 2014, 2019). This explained the presence of the widely disseminated lineage B across South America, but there was no clear connection between Chile and lineage A which at the time was only known from North America. Our results suggest a more plausible scenario where the presence of lineage A in Chile results from intentional introductions from New Zealand.

While the relation between New Zealand and South American populations can now be more clearly established with the release of nematodes from New Zealand to Uruguay, Argentina and Chile from the 1980s (Martinez, 2020; Hajek et al., 2021), there are still no reports of shared history between populations of *S. noctilio* or *D. siricidicola* in New Zealand and North America to fully explain the dissemination patterns of lineage A (Hurley et al., 2007; Boissin et al., 2012). Different scenarios can be proposed based on accidental introductions: directly from New Zealand to North America, indirectly from South America, or alternatively, both New Zealand and North American populations could originate from the same unknown Eurasian source. Another scenario relates to possible direct introduction(s) of infected parasitoid wasps from North America to New Zealand. During the 1960s and 1970s several species of parasitoid wasps were collected in the USA and introduced as biological control agents in New Zealand (Cameron, 2012). *Deladenus* spp. are known to be associated with rhyssine wasps (Bedding, 1968, 2009), including *D. siricidicola* parasitising *Rhyssa persuasoria* (Morris et al., 2020).

This study is the first to explore the population genetic structure on a large collection of *D. siricidicola* in Australia since it became widely used in biological control in the early 1970s. The discovery of significant genetic diversity, including four major lineages, was surprising. Four strains of nematodes were originally released in limited numbers in Victoria, Australia (from Corsica, Greece, Hungary and New Zealand), before Sopron 168 (originating from *S. juvencus* from Hungary) was selected for mass release because of its complete sterilization of *S. noctilio* females, high parasitism rates and minimal impact on the host *S. noctilio* adult size (Bedding, 2007, 2009). Sopron 168 was the only strain released in Tasmania, in the early 1970s, before the re-isolation of the Kamona strain from there (R. Bedding, pers. comm.). The early releases from collections in Europe might have persisted in Australia and might explain the presence of lineage (C) (collected from Spain).

The Sopron strain eventually lost its virulence, presumably due to prolonged culturing on the fungus in the laboratory (Bedding, 2009; Collett and Elms, 2009), and its subsequent replacement with the Kamona strain. This latter strain was expected to be similar to the Sopron strain genetically, as it was isolated from a population where the Sopron strain was released previously. Our study showed differences at three SSR loci (Ds01, Ds105 and Ds375) between these strains, and RAPD analysis in the early

2000s was also able to distinguish between them (Collett and Elms, 2009; Carnegie and Bashford, 2012). Thus, while these strains might have originated from a similar source (lineage B), they are clearly not identical.

Considering the long history of lineage B (Sopron and Kamona) in Australian biological control programs, in particular extensive releases of Kamona in south-eastern Australia designed to “flood out” the defective strain (Carnegie et al., 2005), and from prior work Fitza et al. (2019), we expected that the Australian populations would be dominated by lineage B. On the contrary, an additional three lineages co-occur, and lineage D was dominant (>50 % of strains collected from Australia). A similar result was found in the early 2000s with strains other than Kamona or Sopron dominating in Victoria (Collett and Elms, 2009; Carnegie and Bashford, 2012). The unexpected population diversity found in Australia could simply reflect the persistence of all or some of the four strains initially released from Hungary, Corsica, Greece and New Zealand (Bedding, 2009), or a combination of some of them with accidental introductions of *D. siricidicola* from different source populations, either through infected *S. noctilio* or infected parasitoid wasps. Whatever the origin, strains other than lineage B in Australia - in particular lineage D – must have successfully competed with the mass released strains (Sopron and Kamona) used in biological control programs to have persisted this long.

The genetic diversity of *D. siricidicola* populations in Australia was unique compared to other non-native populations studied thus far. This is not only due to the four lineages with potentially distinct origins, but also because there was evidence of admixture between these lineages creating new diversity not seen elsewhere. In contrast to the diversity revealed in this study in Australia, other Southern Hemisphere *D. siricidicola* populations were genetically similar, limited in diversity, and homozygous (Mlonyeni et al., 2011; Fitza et al., 2019). The discovered diversity provides a pool of strains from which to select for future trials for use in biological control programs, while admixture between them provides the opportunity to increase the potential diversity even further.

We used DIY ABC analysis to compare models of the ancestral history of lineages in Australia and New Zealand. The ABC analysis supports the results from NETWORK, STRUCTURE and PCoA analyses in three main aspects. Firstly, lineages

A and D, in Australia and New Zealand, shared a common ancestral origin from Europe. Secondly, lineages B and D in Australia were established independently from two different ancestral origins. Finally, lineages A and B in New Zealand were established through independent introductions, likely from two different source populations. These results confirm the conclusions from other analyses of multiple introductions of *D. siricidicola* over time into Australia and New Zealand, independent from the intentional introduction as part of a central biological control program in Australia.

Allelic richness and numbers of private alleles were unexpectedly high both in Australia and New Zealand. Despite the diversity and numbers of MLG groups, the level of heterozygosity was, however, still low. This is curious as outcrossing is the only known reproductive mode in *D. siricidicola* (Bedding, 1972), although questions have been raised about the possibility of parthenogenesis (Clark, 1994). Although admixture occurs between strains, the potential for selfing (parthenogenesis) needs to be further investigated (Thomas et al., 2012). The pattern of homozygosity could also be caused by inbreeding amongst related individuals, potentially in mass-rearing programs starting from small starter cultures, as hypothesized in Mlonyeni et al. (2011). The majority of strains isolated in our study, however, have not gone through a mass rearing program. There are, however, no obvious mechanisms that would promote prolonged inbreeding in the naturally occurring populations, and this could be investigated in the future. A possible alternative explanation is a genetic mechanism such as meiotic parthenogenesis that is known in other nematode species, such as *Meloidogyne hapla* (Liu et al., 2007). Information is not available on how *D. siricidicola* negates the possible negative effects of genetic bottleneck and/or inbreeding depression (Caballero and García-Dorado, 2013; Greenbaum et al., 2014).

5 Conclusion

Despite 50 years of the consistent release of specific strains of the nematode *D. siricidicola* (Sopron, followed by Kamona strain, both within lineage B) substantial additional and apparently unrelated genetic diversity persists in the *D. siricidicola* populations in Australia. This diversity is possibly linked to original releases of other strains and/or to unknown introductions, which must have a fitness advantage to not have been replaced by the mass-released strain. Interbreeding between the lineages

is demonstrated, leading to an admixed and unique population of *D. siricidicola* in Australia. Similarly, New Zealand appears to have had more than one accidental introduction of the nematode, in contrast to previous findings. One of these strains in New Zealand shares an origin of *D. siricidicola* with North America. The information emerging from this study will be invaluable to evaluate the efficacy and need for adaptation of current management strategies. The information also opens many new questions for future studies, such as the possibility of co-infestation of lineages that it is known to occur (Bittner et al., 2019), but not observed in this study, and the mechanisms that cause the excessively high levels of homozygosity. A better understanding of the potential fitness differences between the different strains and their progeny, including performance on different fungal isolates, could be valuable for selecting more efficient biological control agents for different environments and populations of *S. noctilio*.

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