A COMPARISON OF POPULATIONS OF *ARMILLARIA HINNULEA* IN NEW ZEALAND AND AUSTRALIA

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ABSTRACT

Armillaria hinnulea has been recorded from the northwest quadrant of the South Island of New Zealand, Tasmania and Australia. In New Zealand, *A. hinnulea* is rare, being restricted to decaying wood in South Island *Nothofagus* forests. A preliminary study of the relationship between the populations of *A. hinnulea* was performed using DNA sequence data from the internal transcribed spacer (ITS) region of the ribosomal DNA. Two major ITS clades were observed, each of which included representative sequences from Australian collections. Within one ITS clade, one Australian sequence was 100% identical with the New Zealand isolates while in the other ITS clade eight New Zealand isolates, representing five locations, were 100% identical but the Australian sequence differed by five base substitutions and was in a separate sub-clade. These results suggest at least two introductions of *A. hinnulea* have occurred, one relatively recently and one in the more distant past.

Keywords: *Armillaria hinnulea*, population genetics.

INTRODUCTION

Species of *Armillaria* (Fr.) Kummer are globally important agents of root disease (Shaw & Kile 1991). In New Zealand, three named species, *Armillaria hinnulea* Kile & Watl., *A. novae-zelandiae* (Stev.) Herink and *A. limonea* (G. Stev.) Bosewinkel, as well as a rare, un-named species, are present (Hood 1989). Globally, *A. hinnulea* is restricted to the South Island of New Zealand (Coetzee et al. 2001), Tasmania (Kile 1986) and south-eastern Australia (Kile & Watling 1983). In Australia, *A. hinnulea* is a weak primary pathogen, but important secondary pathogen, of wet sclerophyll eucalypt forests (Kile 1986). Based on the association with native forests of Australia, Kile & Watling (1983) suggested that *A. hinnulea* is indigenous there.

Phylogenetic analysis of *Armillaria* spp. from Australia and New Zealand with other global species, using the ITS region, implied that *A. hinnulea* is more closely related to Northern Hemisphere species than Southern Hemisphere species (Coetzee et al. 2001; Dunne et al. 2002). However, more recent phylogenetic analysis based on the elongation factor 1-alpha gene clustered *A. hinnulea* with other Southern Hemisphere species (Maphosa et al. 2006). *Armillaria hinnulea* is the only Southern Hemisphere species to form clamp connections in the subhymenial layer and is morphologically similar to European *A. bulbosa* Velen. (Kile & Watling 1983).

Because *A. hinnulea* has a limited distribution in New Zealand, and has been hypothesised to be indigenous to Australia, this fungus was selected as a model to study the dispersal of a basidiomycete from Australia to New Zealand. This paper reports the results of an investigation into the populations of *A. hinnulea* in New Zealand and Australia to further understand the relationship between these populations and to assess whether trans-Tasman dispersal of the fungus has occurred.

MATERIALS AND METHODS

Field collection

Armillaria hinnulea was collected from *Nothofagus* forests of the northern portion of the South Island (Table 1). Collection of basidiomes was guided by location records from the Landcare (PDD) and Forest Research (NZFRI-M) herbaria. Field collections were made under a Department of Conservation permit. Basidiomes were identified based on morphology.

Cultures

Haploid cultures of *A. hinnulea* were obtained by casting basidiospores onto 3% malt extract agar amended with 10 mg/ml benomyl (MEA-B). Using a dissecting microscope, single germinated basidiospores were transferred to 3% malt extract agar (MEA). Diploid cultures were obtained by placing small cubes of tissue from the cap and stipe on 3% MEA-B. All cultures were incubated at 20°C in the dark. All cultures were given unique culture numbers and were added to the NZFS culture collection at Scion. Australian isolates of *A. hinnulea* were imported into the Scion quarantine facility under MAF permit.

DNA extraction

Haploid cultures were plated onto 3% MEA that was overlaid with sterilised cellophane (Bio-Rad, Hercules, CA, USA, GelAir Cellophane support) and incubated at 20°C in the dark until the plate was covered by mycelium. Mycelium was scraped from the cellophane using a sterile scalpel blade and placed in a 2.0 ml Eppendorf tube and DNA was extracted using the Bio101 FastDNA kit and the FastPrep instrument (Q-Biogene, Carlsbad, CA, USA). Mycelium was homogenised with lysing matrix A and CLS-Y in the FastPrep instrument on setting 5 for 20 s, repeated twice. DNA was then extracted following the manufacturer's protocol.

PCR amplification and sequencing of the ITS

The ITS repeat (partial 18S rRNA, ITS-1, 5.8S rRNA, ITS-2 and partial 26S rRNA) was amplified using the universal PCR primer combination ITS1-F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). PCR conditions were 0.5 µM ITS1-F, 0.5 µM ITS4, 200 µM dNTP, 0.5 U *Taq* DNA polymerase (Roche Applied Science, Mannheim, Germany), 1x supplied Roche buffer with a final MgCl₂ concentration of 1.5 mM and 1-10 ng of template DNA. PCR conditions were 95°C for 6 min, 35 cycles of 95°C for 30 s, 60°C for 40 s and 72°C for 40 s, followed by 72°C for 5 min and a 4°C hold cycle. PCR products were purified for sequencing by incubating 5 µl PCR product with 10 U exonuclease I (Fermentas Life Sciences, Burlington, ON, Canada) and 1 U shrimp alkaline phosphatase (Fermentas Life Sciences, Burlington, ON, Canada) at 37°C for 15 min and 85°C for 15 min. The complete ITS PCR product sequence was determined under contract by Macrogen Inc. (Seoul, Korea) by sequencing the PCR product in both directions. Forward and reverse sequences were assembled using Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI, USA). Isolates that were sequenced in this study are presented in Table 1.

UPGMA analysis

A phylogeny was generated to compare isolates of *A. hinnulea* from New Zealand and Australia using UPGMA analysis (Unweighted Pair Group Method using Arithmetic averages) with 5,000 bootstrap replications (Sneath & Sokal 1973) based on evolutionary distances calculated using the maximum composite likelihood method. All analyses were performed using the MEGA 4.0 software package (Tamura et al. 2007). Additional ITS sequences from *A. hinnulea* were obtained from GenBank (Table 2). All ITS sequences were trimmed to remove the flanking 18S rRNA and 26S rRNA sequences so that they started at 5'-GAGARCTGTT and finished at TTTATTGAC-3' and then aligned using Clustal W within MEGA 4.0. The gap opening and gap extension penalties were adjusted manually to obtain the best visual alignment.

TABLE 1: Collection locations and ITS sequence lengths of *A. hinnulea* isolates used in this study.

NZFS#	Field number	GenBank accession	Collection location	Collection date	ITS size	ITS type
2137	46		Warbeck, Maruia Valley, NZ	8 May 04	760	Ila
2153	47		Warbeck, Maruia Valley, NZ	8 May 04	760	Ila
2164	48		Warbeck, Maruia Valley, NZ	8 May 04	760	Ila
2185	52		Lake Christabel Track, NZ	9 May 04	760	Ila
2207	57	EU734744	Station Creek, near Maruia, NZ	10 May 04	759	Ia
2227	58		Station Creek, near Maruia, NZ	10 May 04	759	Ia
2301	81		4-Mile Rd, Charleston, NZ	12 May 05	760	Ila
2308	82		4-Mile Rd, Charleston, NZ	12 May 05	760	Ila
2330	72		Lake Christabel Track, NZ	9 May 05	759	Ia
2332	73		Karamea, NZ	11 May 05	759	Ia
2358	79		Charleston, NZ	12 May 05	760	Ila
2376	86	EU734746	Lake Rotoroa, NZ	13 May 05	760	Ila
2384	87		Lake Rotoroa, NZ	13 May 05	759	Ia
2394	88	EU734745	Lake Rotoroa, NZ	13 May 05	760	Ib
2685	GR		Britannia Walk, north of Waimangaroa, NZ	8 May 06	759	Ia
2939*	Lot2(2)		Australia	Unknown	759	Ia
2940*	Lot2(3)		Australia	Unknown	760	Ib
2942*	119	EU734747	Australia	Unknown	760	Ilb

*Isolates were provided; haploid or diploid status and collection details unknown.

TABLE 2: *Armillaria hinnulea* sequences from GenBank that were used in this study.

GenBank #	Collection location	Reference	ITS size	ITS type
AF329905	South Island, NZ	Coetzee et al. 2001	759	Ia
AF329906	South Island, NZ	Coetzee et al. 2001	758	Ia
AF329907	South Island, NZ	Coetzee et al. 2001	759	Ia
AF329908	Australia	Coetzee et al. 2001	758	Ia
AF394918	NSW, Australia	Smith-White et al. 2002	760	Ib
AF454740	Tasmania, Australia	Dunne et al. 2002	759	Ilb

RESULTS

Field collection

A total of 104 haploid and 32 diploid isolates, representing 15 individuals of *A. hinnulea*, were collected from eight locations in the South Island (Table 1). The fungus was only ever observed and collected from decaying wood or stumps, never in association with recently killed or dying trees, suggesting a saprophytic rather than pathogenic role for this fungus in New Zealand. Although this species has previously been collected from the Westland region, near Haast (PDD61935), it was not found in this region during this study despite extensive searching.

ITS analysis

UPGMA analysis of ITS sequences of the *A. hinnulea* isolates included in the study indicated that there were two main ITS types (I and II), represented by two major clades in the cladogram, each with two subtypes (a and b), represented by subclades (Fig. 1, Table 1). The two ITS types had very similar base pair (bp) composition and sizes (Type I, 759 bp vs Type II, 760 bp), but there were 14 polymorphic sites that differentiated the ITS types. Each ITS type had a unique conserved site that could be used to distinguish it from the other ITS types; Types Ia (a deletion at position 27), Ib (A at position 528), and IIb (C at position 729), while Type IIa had four unique conserved sites (T at 103, T at 570, A at 595, T at 733). Polymorphic sites were shared by the four different ITS types as outlined in Table 3. Of the 24 individuals analysed, 11 were of Type Ia, 3 of Type Ib, 8 of Type IIa and 2 of Type IIb. A representative of each ITS type has been deposited in GenBank (Table 1). The DNA sequence composition of isolates within the Type Ia, Ib and IIa subclades were not 100% identical. For the Type Ia subclade, sequence AF329908 differed from the other Type Ia isolates by deletions at positions 94 and 403 and an insertion at position 590, while AF329906 differed by a deletion at position 592. The remainder of the Type Ia sequences were 100% identical. In subclade Type IIb, the two sequences differed from each other by one deletion and two base substitutions. Type IIb sequence AF45470 had a polymorphism at position 412 (Y=C or T); all Type I sequences had a C at this position and all Type II sequences had a T at this position. Within the Type IIa subclade, all sequences were 100% identical with the exception of isolate 2308, which had a polymorphism at position 172 (K=G or T). Every other sequence used in this study had a T at position 172. All of the Type Ib sequences were 100% identical.

To determine if there were multiple nuclear types present in one parent isolate, the ITS region of twelve haploid cultures and two diploid cultures, that were obtained from field collection number 57 (Table 1), were sequenced in the forward direction and compared. All ITS sequences were 100% identical.

TABLE 3: Number of polymorphisms shared (S) between different ITS Types and number of polymorphisms that differentiate (D) ITS types.

ITS Type	Type Ia	Type Ib	Type IIa	Type IIb
Type Ia	--	2 D	12 D	9 D
Type Ib	12 S	--	12 D	9 D
Type IIa	2 S	2 S	--	5 D
Type IIb	5 S	5 S	9 S	--

When the geographic source of the different ITS types was analysed, it was found that both Types Ia and Ib included isolates from Australia and New Zealand, but within Type II, Type IIa included only isolates from New Zealand, while Type IIb included only isolates from Australia. At Lake Rotoroa, ITS types Ia, Ib and IIa were present and at Lake Christabel, ITS types Ia and IIa were present, while a single ITS type was recorded at the remainder of the collection locations (Table 1).

FIGURE 1: The ITS sequence data were compared using the maximum composite likelihood method and then clustered using UPGMA analysis. An unrooted bootstrapped consensus tree (5000 replications) is presented. Numbers at nodes represent percentage of trees in which the isolates clustered together. The scale represents the number of base substitutions per site. Isolates 2939, 2940 and 2942 as well as sequences AF329908, AF394918 and AF454740 are Australian in origin while the remainder of isolates and sequences are from New Zealand. Isolate and sequence sources are listed in Tables 1 and 2.

DISCUSSION

In this study a comparison of the populations of *Armillaria hinnulea* within New Zealand and Australia was conducted using DNA sequence data from the ITS regions of the rDNA repeat. Population genetic studies typically use high resolution DNA markers such as AFLP (Vos et al. 1995), microsatellites or RAPD (Williams et al. 1990) to assess the variation within and between populations of the organism of interest. The ITS was utilised for this study because (1) the small number of individuals available for study, (2) DNA sequence information from additional isolates of *A. hinnulea* was publicly available from GenBank, (3) populations were being compared at a broad geographic scale, and (4) intraspecific variation in the ITS has been observed in Australian *Armillaria* spp. (Dunne et al. 2002). Unlike some Northern Hemisphere species, such as *Armillaria ostoyae*, that primarily spread clonally via root contacts and rhizomorphs (Dettman & van der Kamp 2001), *A. hinnulea* has been found to have high genotypic variation within a stand because it spreads primarily via basidiospores (Kile 1986). Basidiospores are produced as a result of sexual recombination, which ensures frequent recombination within *A. hinnulea*.

The phylogeny reported herein assumed that all ITS sequence data were derived from isolates of the same species, and thus represented different alleles from the ITS of *A. hinnulea*. The UPGMA analysis clearly resolved the intraspecific variation within the ITS region of *A. hinnulea* and suggested that at least two ITS types exist. *Armillaria hinnulea* was selected for this study because it has a restricted geographic distribution in New Zealand that may have arisen as a result of trans-Tasman dispersal from Australia. However, directionality of movement cannot be inferred from this study because of the small number of representative samples from Australia. The finding that six isolates from New Zealand were 100% identical with one isolate from Australia may represent a relatively recent introduction. A putative earlier introduction is signalled by the presence in New Zealand of eight isolates that had 100% identical ITS Type IIa sequences yet were different from the Australian isolate by five base substitutions. A mechanism by which this could occur is the introduction of an ITS type from Australia, followed by reproductive isolation, mutation and then expansion in New Zealand. Alternatively, an identical ITS type may be present in Australia but hasn't yet been detected because of the small sample size.

An earlier study by Kile & Watling (1988), using cultural methods, indicated that *A. hinnulea* isolates from Australia and New Zealand were incompatible. This was because the cultures used by the authors were from the un-named species of *Armillaria* that is macro-morphologically similar to *A. hinnulea* (I. Hood, pers. comm.). In this study, an Australian isolate was found to be 100% identical to six isolates of *A. hinnulea*, confirming that *A. hinnulea* is present in New Zealand.

A study of the New Zealand population of *A. hinnulea* using a high resolution DNA marker is required to understand gene flow between and within New Zealand populations of *A. hinnulea*. Gene flow between New Zealand populations is suggested because there were two geographic locations with multiple ITS types present, but variation between or within populations was not statistically analysed. The results also indicate that genetic movement of *A. hinnulea* between Australia and New Zealand has occurred.

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