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Molecular variation among isolates belonging to eight races of *Phytophthora clandestina*

X. L. Ma[A], *P. Kong*[B], *M. P. You*[C], *Hua Li*[A], *K. Sivasithamparam*[A] *and M. J. Barbetti*[A,C,D]

[A]School of Plant Biology, Faculty of Natural and Agricultural Sciences, The University of Western Australia, Crawley, WA 6009, Australia.

[B]Department of Plant Pathology and Weed Sciences, Virginia Polytechnic Institute and State University, Hampton Roads Agricultural Research and Extension Centre, 1444 Diamond Springs Road, Virginia Beach, VA 23455, USA.

[C]Department of Agriculture and Food Western Australia, Baron-Hay Court, South Perth, WA 6151, Australia.

[D]Corresponding author. Email: mbarbett@cyllene.uwa.edu.au

Abstract. *Phytophthora clandestina*, a serious pathogen of subterranean clover (*Trifolium subterraneum*), has only been recorded in Australia. A rapid method was used to characterise genetic variation among 61 isolates of *P. clandestina* belonging to eight pathogenic races based on DNA sequencing and single-strand conformation polymorphism (SSCP) analyses of the β-tubulin gene of the pathogen. The β-tubulin gene among those tested displayed a high degree of variability among isolates. Cluster analysis of the SSCP profiles grouped the 61 isolates into two main clusters (A and B). Cluster A with 3 subclusters *viz*; I (race 177), II (race 000) and III (races 001, 121, 101, 143 and 157) and B with race 173 alone. In addition, SSCP of β-tubulin also successfully differentiated between races 173 and 177, the two most prevalent and most virulent of the races studied. This is the first time that the β-tubulin gene has been used to study intra-species variation in this pathogen. In addition to showing relationship among the strains, it also provides a practical means for rapid monitoring of current and future differences in the distribution of *P. clandestina* strains, giving subterranean clover breeders and farmers a sound basis for the selection/breeding and deployment of appropriate cultivars to counter the predominant strain populations in specific localities.

Introduction

Rain-fed subterranean clover (*Trifolium subterraneum*) is the most important forage legume found across southern Australia (Morley 1961; Powell 1970; Gladstones and Collins 1983) where it has been sown over an estimated area of more than 22 million ha (Sandral *et al*. 1997), of which at least 6 million ha occur in south-west Western Australia (Gladstones 1975).

While several soilborne pathogens can cause root disease on subterranean clover across southern Australia, the most important pathogen associated with root disease is currently believed to be *Phytophthora clandestina* (Taylor and Greenhalgh 1987; Barbetti *et al*. 2007). You *et al*. (2006) demonstrated that the race distribution for *P. clandestina* in south-west Western Australia was strongly associated with the rainfall zones, with the majority and most diverse *P. clandestina* races occurring in the high rainfall zone with 700–1000 mm annual rainfall.

Phytophthora species can either be homothallic and/or heterothallic but this has not been clearly determined for *P. clandestina* (Taylor *et al*. 1985). Even though it has been previously stated that *P. clandestina* is homothallic (e.g. Purwantara *et al*. 2001), evidence to this effect was not provided. However, it is known that a low percentage (<5%) of outcrossing *in vitro* can occur in some other *Phytophthora* species known to be homothallic (Bhat and Schmitthenner 1993; Forster *et al*. 1994).

The main method of control of this disease is reliant upon utilising host resistance (Barbetti *et al*. 2007). Initially, four races of the pathogen were reported for southern Australia, but these were only based on the very restricted set of subterranean clover host differentials available at that time (Purwantara *et al*. 1996, 1998). However, it was not until the work of You *et al*. (2005*b*), who characterised a total of 10 pathogenic races among ~100 Western Australian isolates, that the first clear picture of the racial distribution of *P. clandestina* in south-west Western Australia became evident. This research allowed You *et al*. (2005*a*) to establish the availability of subterranean clover genotypes with resistance to *P. clandestina* race 001, to race 173, and even with combined resistance to both races 001 and 173. Subsequently, You *et al*. (2006) demonstrated that cv. Denmark was highly resistant to races 001, 101, 141, 151 and 143, and moderately resistant to race 121, whereas cv. Meteora was highly resistant to race 151, moderately to highly resistant to races 001 and 101 and moderately resistant to races 121, 141 and 143. Differences in the distribution of race populations (You *et al*. 2006) are critical for both subterranean clover breeders and for farmers, as this provides the only sound basis for the selection/breeding of cultivars with appropriate resistance against the predominant race populations of the pathogen in specific localities in the Mediterranean ecosystems of southern Australia.

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Molecular approaches have been applied for the study of the taxonomy and genetics of *Phytophthora* species. These include protein electrophoresis (Bielenin *et al.* 1988; Pane *et al.* 2000), isozymes (McHau and Coffey 1994; Mirabolfathy *et al.* 2000), DNA finger printing (Yamak *et al.* 2002; Camele *et al.* 2005; Irzykowska *et al.* 2005; Bhat *et al.* 2006), Single-strand conformation polymorphism (SSCP) analysis (Kong *et al.* 2003*a*, 2004) and direct DNA sequences (Ivors *et al.* 2004; Kroon *et al.* 2004; Kim *et al.* 2005). As a sensitive, inexpensive, and rapid method to detect single base substitutions (Orita *et al.* 1989; Sekiya and Hayashi 1993; Slabaugh *et al.* 1997), SSCP has a potential for differentiation of sequence variation within species. SSCP involves denaturation and electrophoresis of PCR products of a DNA fragment through a non-denaturing polyacrylamide gel. The polymorphisms produced by differential folding of single-stranded DNA due to subtle differences in sequence were detected by their mobility variations. A group of closely related sequences with substitutional sequence differences and/or indels, therefore, can be separated.

However, while internal transcribed spacer (ITS)-based sequencing and/or SSCP are widely used in studying inter-specific phylogeny of various pathogens, intra-specific variation has not been detected although subspecies can sometimes be differentiated on the basis of morphology (Forster *et al.* 2000; Crespo *et al.* 2002; Kong *et al.* 2003*b*, 2004; Ivors *et al.* 2004). In contrast, the β-tubulin gene has been reported to show high genetic divergence between very closely related species (Bilodeau *et al.* 2007). It has been used to detect fungicide resistance mutations within a species (Ma and Michailides 2005), as well as to study intra-species phylogenetic relationships in various pathogens, such as *Leptosphaeria maculans* (Voigt *et al.* 2005) and *Parmelia saxatilis* (Crespo *et al.* 2002).

The aim of the research reported in this paper was to identify key characteristics from β-tubulin and other genomic regions in order to develop a rapid and reliable molecular method to define the intra-specific variation among and between strains of *P. clandestina*.

Materials and methods

Pathogen isolates and DNA extraction

Sixty-one isolates from eight published races (You *et al.* 2005*b*) were used in this study. The isolates and their origin are listed in Table 1. All isolates were collected from south-west Western Australia.

A one-step boiling method (Kong *et al.* 2003*a*) and DNeasy Plant Mini Kit (Qiagen, Melbourne, Australia) was used to extract DNA from isolates. DNA extracted from cultured duplicates of individual isolates was used as control to detect potential contamination during culturing.

Primers design and PCR amplification

Primers ITS1 and ITS4 were used to amplify the entire ITS1, 5.8 ribosomal gene and ITS2 (White *et al.* 1990). Primers ITS6 and ITS7 (Cooke *et al.* 2000) were used to sequence the ITS1 region. Primers (TUB1: 5′-GAT CCC GTT CCC GCG TCT-3′; TUB2: 5′-CGC TTG AAC ATC TCC TGG AT-3′) for amplifying and sequencing the β-tubulin gene were designed based on the alignment of homologues of the gene in oomycetes.

Table 1. *Phytophthora clandestina* isolates used, their race details, and the location of their origin in south-west Western Australia

Isolate No.	Race No.[A]	Location[B]	Isolate No.	Race No.[A]	Location[B]
134	000	Bennelaking	103	173	Quininup
195	000	North Dandalup	217	173	Waroona
58	001	Augusta	218	173	Waroona
56	001	Capel	219	173	Waroona
C4954	001	Munglinup	223	173	Waroona
65	001	Nannup	224	173	Waroona
C4597	101	Munglinup	228	173	Waterloo
249	121	Kendenup	78	173	Wharton
234	143	Meelon	79	173	Wharton
202	157	Muradup	168	173	Yallingup
60	173	Augusta	169	177	Boyanup
174	173	Boyanup	171	177	Boyanup
57	173	Capel	172	177	Boyanup
153	173	Cardiff	173	177	Boyanup
154	173	Cardiff	243	177	Boyanup
155	173	Cardiff	148	177	Cardiff
40	173	Cornijup	150	177	Cardiff
41	173	Cornijup	151	177	Cardiff
42	173	Cornijup	43	177	Cornijup
73	173	Cornijup	46	177	Cornijup
203	173	Dardanup	47	177	Cornijup
84	173	Margaret River	86	177	Margaret River
187	173	Mayanup	89	177	Munglinup
189	173	Mayanup	201	177	Muradup
190	173	Mayanup	178	177	Warrening
105	173	Muchea	179	177	Warrening
107	173	Muchea	180	177	Warrening
91	173	Munglinup	181	177	Warrening
95	173	Munglinup	184	177	Warrening
197	173	North Dandalup	Williams	177	Williams
102	173	Quininup			

[A]Race as determined by differential host responses by You *et al.* (2005*b*).
[B]Nearest town in south-west Western Australia.

PCR amplification was performed in a total volume of 25 μL with 1× GoTaq Green Master Mix (Promega, Sydney, Australia), 0.4 μL of the DNA and 0.4 μM of a primer pair (0.2 μM each). Amplification was performed with an initial denaturing at 96°C for 2 min, followed by 30 cycles of 94°C for 30 s, 50–55°C for 45 s, and 72°C for 1 min.

SSCP

All 61 isolates were tested by SSCP. The procedures for the denaturation, electrophoresis and visualisation were followed as described by Kong *et al.* (2003*a*), except for using 1–4 μL of PCR products mixed with 6–9 μL (i.e. always a total 10 μL) of the denaturing buffer for each lane loading, and running the gel at 200 V for 3 h at 4°C using a Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad, Gladesville, Australia).

DNA sequencing and analysis

Eight isolates representative of six *P. clandestina* races were selected for DNA sequencing of the PCR product amplified by primers TUB1/2 (Table 2). PCR products amplified by primers ITS1/4 from three of the eight isolates were also sequenced. Sequencing of both DNA strands was carried out with reagents

supplied with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Melbourne, Australia) with AmpliTaq DNA Polymerase FS (Perkin Elmer, Waltham, MA, USA) using forward and reverse primers ITS1 and ITS4 for ITS region and TUB1 and TUB2 for the β-tubulin gene. Both DNA strands were sequenced on an ABI 373 sequencer (Applied Biosystem Industries). Sequences from the ITS region and the β-tubulin gene were trimmed of primer-binding sequences before alignment.

Data analysis

The SSCP markers were scored visually. The banding patterns of individual isolates were determined with the aid of an ssDNA ladder (Kong *et al*. 2003*a*). All visible DNA fragments of the same migration distance were registered as one genetic character. All fragments were translated into a binary matrix (0 for absence, 1 for presence of specific DNA band). Cluster analysis of the binary data was performed using the neighbour-joining method (Saitou and Nei 1987) with total distance implemented in PAUP 4.0b10 (Swofford 2002), and a dendrogram was constructed with a distance scale.

DNA sequences were aligned using ClustalW (Thompson *et al*. 1994) and were refined manually. BLAST was employed to compare the resulting sequences with those available in GenBank.

ANOVA were performed to analyse the association between genetic variations among the isolates and their geographic origins and rainfall zones using GENSTAT (9th edn, Lawes Agricultural Trust, Hertfordshire, UK).

ArcMap 9.2, 2007 (Environmental Systems Research Institute Inc., Redlands, CA, USA) was used to analyse the geographical location of the isolates and relate them with rainfall data.

Results

Evaluation of SSCP and sequences from β-tubulin gene and ITS of P. clandestina *isolates*

TUB1 and TUB2 primers successfully amplified the β-tubulin gene of all 61 isolates of *P. clandestina* that were assessed. In all PCR reactions, a single band of 316 bp in length was obtained. No length variation was observed among isolates. SSCP analysis of denatured PCR products resulted in distinct groups of bands, with one band consistent across all isolates. Different banding patterns were obtained among isolates within same race (Fig. 1). These patterns were reproducible across a series of gels and consistent between different PCR reactions. The duplicated culture material from each individual isolate produced the same SSCP pattern in each instance.

The β-tubulin gene of eight isolates representative of six *P. clandestina* races were further sequenced. Out of expected 316 bp of DNA sequence obtained, seven substitutions were observed among eight isolates across six races (GenBank accession numbers shown in Table 2). No two isolates had identical nucleotide sequences (Fig. 2). The SSCP pattern also showed a high degree of diversity across isolates (Fig. 1).

There were also six single base ambiguities (double peaks) observed in the β-tubulin gene sequence, of which four were in the third codon position and two were in the first codon position and, consequently, the translated amino acid sequence had only one amino acid difference. Out of the six ambiguities, three were observed in more than half of the isolates sequenced.

Amplification of DNA from the 61 isolates with ITS6/7 primers also produced single identical bands on agarose gel, and the SSCP patterns of the ITS1 were identical (Fig. 3).

The ITS regions of three isolates were further sequenced. The 834-bp nucleotides of all three sequences were identical

Table 2. GenBank DNA sequence accession numbers of *Phytophthora clandestina* isolates sequenced

Isolate No.	Race No.	GenBank accession number for internal transcribed spacer region	GenBank accession number for β-tubulin gene
195	000	EU257134	EU257139
C4954	001	EU257135	EU257142
65	001	ND[A]	EU257143
C4597	101	ND	EU257136
202	157	ND	EU257137
154	173	ND	EU257138
184	177	ND	EU257140
46	177	EU257132	EU257141

[A]ND = not determined.

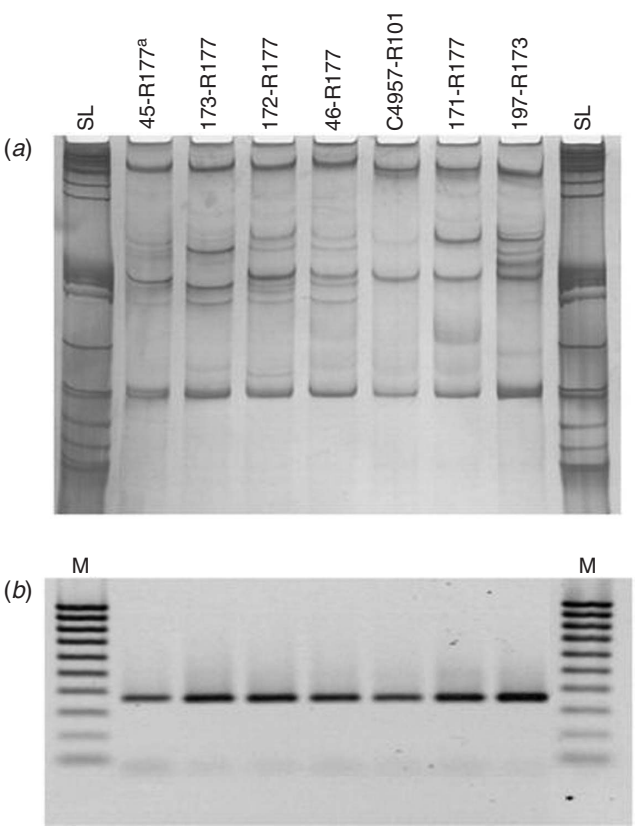

Fig. 1. (*a*) Single-strand conformation polymorphism (SSCP) banding patterns of the β-tubulin gene for the representative isolates of three races of *Phytophthora clandestina*. The isolate number and races are indicated on the top of lanes. SL is the ssDNA ladder. The band on the bottom is consistent across all isolates. (*b*) A single band detected from the agarose gel electrophoresis of PCR products used for SSCP analysis as evident in Fig. 3*a*. M is a 100-bp DNA ladder. [a]45 = isolate number and R177 = race 177.

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C4957       TGACGTCGCGTGGCTCRCAGCAGTACCGCGCCCTGACGGTACCCGAGCTGACCCAGCAGC
202         TGACGTCGCGTGGCTCRCAGCAGTACCGCGCCCTGACGGTACCCGAGCTGACCCAGCAGC
154         TGACGTCGCGTGGCTCRCAGCAGTACCGCGCCCTGACGGTACCCGAGCTGACCCAGCAGC
195         TGACGTCGCGTGGCTCGCAGCAGTACCGCGCCCTGACGGTACCCGAGCTGACCCAGCAGC
184         TGACGTCGCGTGGCTCRCAGCAGTACCGCGCCCTGACGGTACCCGAGCTGACCCAGCAGC
46          TGACGTCGCGTGGCTCGCAGCAGTACCGCGCCCTGACGGTACCCGAGCTGACCCAGCAGC
C4954       TGACGTCGCGTGGCTCRCAGCAGTACCGCGCCCTGACGGTACCCGAGCTGACCCAGCAGC
65          TGACGTCGCGTGGCTCRCAGCAGTACCGCGCCCTGACGGTACCCGAGCTGACCCAGCAGC
            *************** ******************************************

C4957       AGTTCGATGCCAAGAACATGATGTGCGCTGCTGACCCTCGTCACGGTCGYTATTTAACTG
202         AGTTCGATGCCAAGAACATGATGTGCGCTGCTGACCCTCGTCACGGTCGYTATTTAACTG
154         AGTTCGATGCCAAGAACATGATGTGCGCTGCTGACCCTCGTCACGGTCGYTATTTAACTG
195         AGTTCGATGCCAAGAACATGATGTGCGCTGCTGACCCTCGTCACGGTCGYTATTTAACTG
184         AGTTCGATGCCAAGAACATGATGTGCGCTGCTGACCCTCGTCACGGTCGYTATTTAACTG
46          AGTTCGATGCCAAGAACATGATGTGCGCTGCTGACCCTCGTCACGGTCGYTATTTAACTG
C4954       AGTTCGATGCCAAGAACATGATGTGCGCTGCTGACCCTCGTCACGGTCGTTATTTAACTG
65          AGTTCGATGCCAAGAACATGATGTGCGCTGCTGACCCTCGTCACGGTCGYTATTTAACTG
            ********************************************** **********

C4957       CCGCATGTATGTTCCGCGGACGTATGAGCACAAAGGAGGTKGATGAGCAGATGCTGAACG
202         CCGCATGTATGTTCCGCGGACGTATGAGCACAAAGGAGGTKGATGAGCAGATGCTGAACG
154         CCGCATGTATGTTCCGCGGACGTATGAGCACAAAGGAGGTKGATGAGCAGATGCTGAACG
195         CCGCATGTATGTTCCGCGGACGTATGAGCACAAAGGAGGTKGATGAGCAGATGCTGAACG
184         CCGCATGTATGTTCCGCGGACGTATGAGCACAAAGGAGGTKGATGAGCAGATGCTGAACG
46          CCGCATGTATGTTCCGCGGACGTATGAGCACAAAGGAGGTKGATGAGCAGATGCTGAACG
C4954       CCGCATGTATGTTCCGCGGACGTATGAGCACAAAGGAGGTGGATGAGCAGATGCTGAACG
65          CCGCATGTATGTTCCGCGGACGTATGAGCACAAAGGAGGTGGATGAGCAGATGCTGAACG
            ************************************** ******************

C4957       TGCAGAACAAGAACTCGTCGTACTTCGTCGAGTGGATCCCCAACAACATCAAGGCTAGCG
202         TGCAGAACAAGAACTCGTCGTACTTCGTCGAGTGGATCCCCAACAACATCAAGGCTAGCG
154         TGCAGAACAAGAACTCGTCGTACTTCGTCGAGTGGATCCCCAACAACATCAAGGCGAGCG
195         TGCAGAACAAGAACTCGTCGTACTTCGTCGAGTGGATCCCCAACAACATCAAGGCKAGCG
184         TGCAGAACAAGAACTCGTCGTACTTCGTCGAGTGGATCCCCAACAACATCAAGGCKAGCG
46          TGCAGAACAAGAACTCGTCGTACTTCGTCGAGTGGATCCCCAACAACATCAAGGCTAGCG
C4954       TGCAGAACAAGAACTCGTCGTACTTCRTCGAGTGGATCCCCAACAACATCAAGGCTAGCG
65          TGCAGAACAAGAACTCGTCGTACTTCGTCGAGTGGATCCCCAACAACATCAAGGCTAGCG
            *************************** ***************************** ****

C4957       TGTGTGATATCCCGCCCAAGGGCCTGAAGATGAGCACCACGTTCATCGGTAACTCTACAG
202         TGTGTGATATCCCGCCCAAGGGCCTGAAGATGAGCACCACGTTCATCGGTAACTCTACAG
154         TGTGTGATATCCCGCCCAAGGGCCTGAAGATGAGCACCACGTTCATCGGTAACTCTACAG
195         TGTGTGATATCCCGCCCAAGGGCCTGAAGATGAGCACCACGTTCATCGGTAACTCTACAG
184         TGTGTGATATCCCGCCCAAGGGCCTGAAGATGAGCACCACGTTCATCGGTAACTCTACAG
46          TGTGTGATATCCCGCCCAAGGGCCTGAAGATGAGCACCACGTTCATCGGTAACTCTACAG
C4954       TGTGTGATATCCCGCCCAAGGGCCTGAAGATGAGCACCACGTTCATCGGTAACTCTACAG
65          TGTGTGATATCCCGCCCAAGGGCCTGAAGATGAGCACCACGTTCATCGGTAACTCTACAG
            ************************************************************

C4957       CTATCCAGGAGATGTT
202         CTATCCAGGAGATGTT
154         CTATCCAGGAGATGTT
195         CTATCCAGGAGWGGTT
184         CTATCCAGGAGWGGTT
46          CTATCCAGGAGWGGTT
C4954       CTATCCAGGAGATGTT
65          CTATCCAGGAGATGTT
            **********  ***
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Fig. 2. Alignment of partial β-tubulin gene sequences of eight isolates from six races of *Phytophthora clandestina*. Bold letters represent the sites with ambiguities (double peaks) (S = G, C; *r* = G, A; Y = T, C; K = G, T; W = A, T).

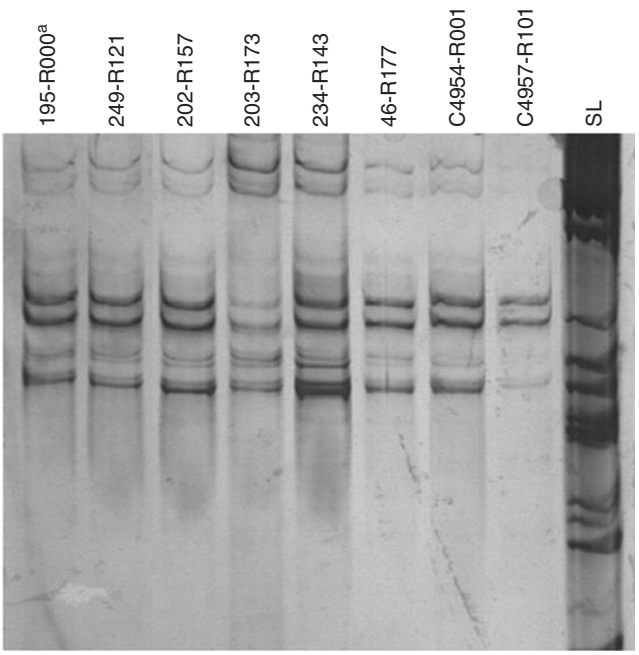

Fig. 3. Identical single-strand conformation polymorphism patterns of internal transcribed spacer-1 region for each representative isolate of eight races of *P. clandestina*. The isolate number and the races are indicated on the top of lanes and SL is the ssDNA ladder. [a]195 = isolate number and R000 = race 000.

(accession numbers shown in Table 2). Utilising GenBank BLAST, the degree of similarity between the ITS sequence we submitted and the ITS sequence of the *P. clandestina* isolates in GenBank was ≥98%.

Phylogenetic analysis of β-tubulin gene SSCP

Fifty-seven distinct SSCP bands were produced across the 61 *P. clandestina* isolates and of these 51 (89%) were polymorphic. The dendrogram deduced from the genetic distance matrix (total character difference) broadly divided the isolates into two main clusters, A and B. Cluster B contained all the *P. clandestina* isolates from race 173. Cluster A was further divided into three subclusters as follows: all isolates from race 177 formed subcluster I, all isolates from race 000 formed subcluster II, and isolates from races 001, 121, 101, 143 and 157 together formed subcluster III (Fig. 4).

Variations of β-tubulin gene among and within P. clandestina *races*

All the races of *P. clandestina* containing multiple isolates produced more than one SSCP banding pattern and no race-specific marker(s) were generated. The genetic distance among isolates ranged from 0 to 0.404 substitutions/site (between isolates 224 and 102, 224 and 243, and 102 and 243) with an overall mean genetic distance of 0.223 substitutions/site. Isolates from the same race were more closely related (mean genetic distance 0.186 substitutions/site) than those from different races (mean genetic distance 0.244 substitutions/site). The isolates within race 173 or race 177 were more variable (mean genetic distance 0.192 substitutions/site and 0.179 substitutions/site, respectively), compared with isolates within other races.

Analysis of genetic variations with geographic origin and rainfall zone

Despite the clustering of isolates, there was no overall partitioning of genetic diversity that corresponded to specific geographical locations. Neither was there any association observed between specific β-tubulin gene SSCP profile(s) and one or more particular rainfall zones from You *et al*. (2006). Of the 57 bands generated, none correlated with any particular geographic region. However, by analysis of the genetic variation against the rainfall zones (You *et al*. 2006), it is noteworthy that the level of genetic variation among *P. clandestina* isolates was significantly higher in those from the 600–800-mm rainfall zone with mean genetic distance of 0.246 than in those from the 400–600-mm or 800–1100-mm rainfall zones where the mean genetic distances were 0.211 and 0.214, respectively, with no significant difference between these latter two mean genetic distances (analyses not presented).

Discussion

This investigation using β-tubulin gene SSCP identified clear differences among strains of *P. clandestina*. These strains were allocated into clusters A and B and those from cluster A into three subclusters I, II and III. In particular, this technique was especially accurate in molecularly allocating strains into previously defined races 000, 173 and 177.

Genetic divergence and sequence of ITS and β-tubulin gene and evaluation of SSCP

The SSCP data in particular demonstrated that the β-tubulin gene displayed a high degree of variability between isolates of *P. clandestina* and can be used for studying the genetic variation within this species. Our results agree with the previous reports of Bilodeau *et al*. (2007) who concluded that divergence was higher for the β-tubulin gene than the ITS region in closely related species.

Phytophthora species can either be homothallic and/or heterothallic but this has not been clearly determined for *P. clandestina* (Taylor *et al*. 1985). The double peaks we observed in the β-tubulin gene sequence(s) may have been a consequence of the occurrence of one or more multi-isotypes of the β-tubulin gene, as multi-gene families with distinct tubulin coding regions exist within eukaryotic organisms (Sullivan 1988), or these double peaks could represent alleles of the same sequence. While fungi, oomycetes and unicellular organisms often have one or two isotypes of the β-tubulin gene, only a single copy of the β-tubulin is reported in *P. cinnamomi* (Weerakoon *et al*. 1998). However, there is no previous report on multi-β-tubulin gene isotypes in *P. clandestina* or in any other *Phytophthora* species. In contrast, two isotypes of the β-tubulin gene have been reported in various anamorphic states of fungi, such as *Aspergillus nidulans* (May *et al*. 1987), *Colletotrichum graminicola* (Panaccione and Hanau 1990) and *Trichoderma viride* (Goldman *et al*. 1993). Further studies need to be undertaken to confirm the presence of multi-isotypes of the β-tubulin gene and/or heterozygocity of *P. clandestina*.

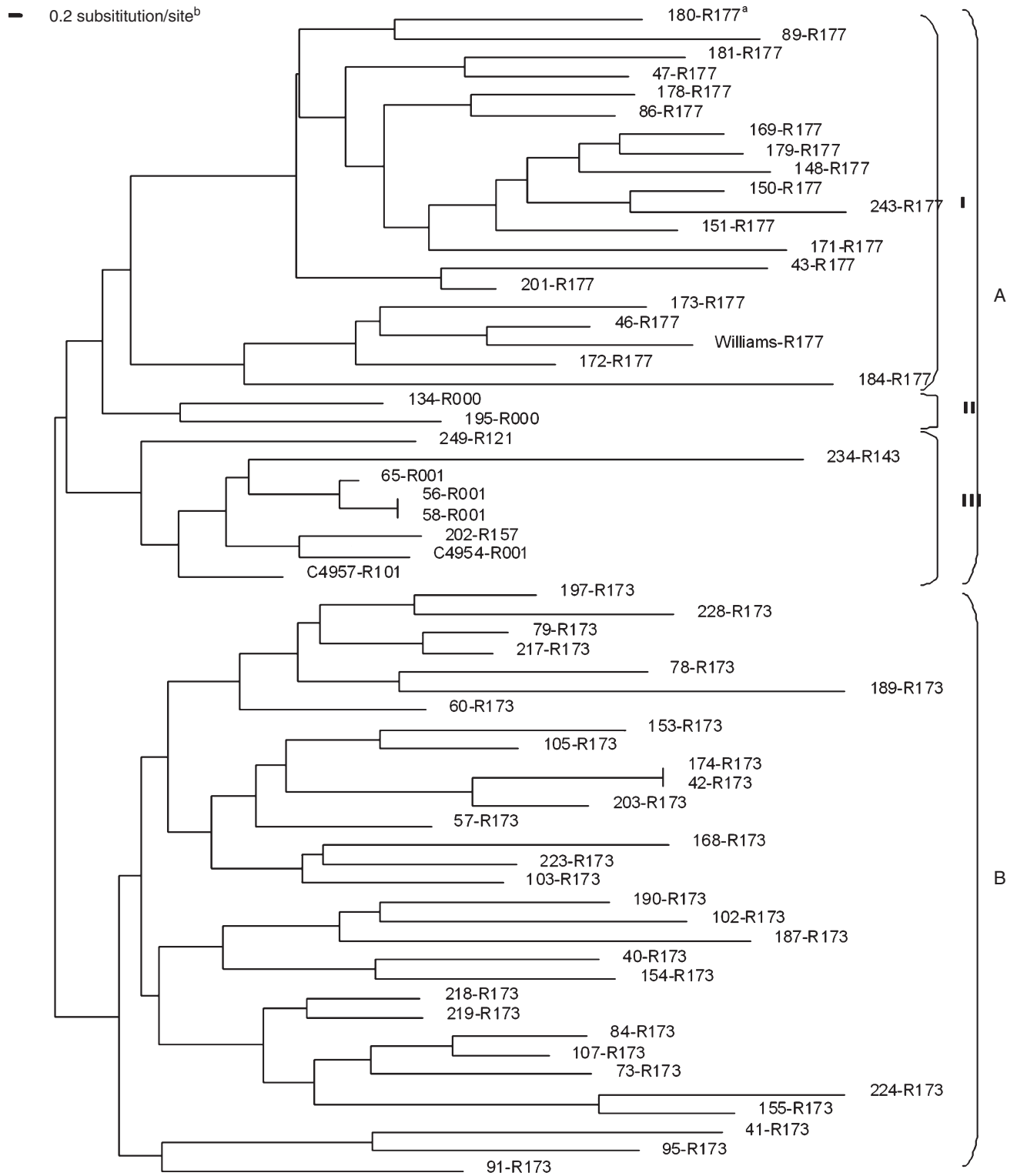

Fig. 4. Neighbour-joining tree based on single-strand conformation polymorphism data from the β-tubulin gene, showing clusters A and B, and the three subclusters within cluster A, for the 61 isolates. [a]180 = isolate number and R177 = race 177. [b]= scale bar for genetic distances as shown by length of the branches.

Genetic diversity of P. clandestina

Our research presented in this paper shows that the *P. clandestina* population in south-west Western Australia falls into two distinct clusters based on the SSCP pattern of the β-tubulin gene.

Races 177 (subcluster I of cluster A) and 173 (cluster B) are the most widely distributed and most common of all the races in south-west Western Australia, together constituting 80% of the 101 isolates characterised earlier by You *et al*. (2005*b*). More importantly, while all differentials used by You *et al*. (2005*b*) were resistant to race 000 and six of the seven host differentials were resistant to isolates belonging to less common races such as 001, only one host differential was resistant to race 173 and none of the seven host differentials were resistant to race 177. Using β-tubulin gene SSCP, we identified extra bands that allowed strains to be allocated into either race 177 or race 101. There is clearly a large level of variation, at least within some races, and this indicates the delineation of *P. clandestina* races by You *et al*. (2005*b*) was clearly conservative, allowing room for characterisation of strains within races by utilisation of molecular techniques such as SSCP that can not only detect and quantify genetic variation to a higher level, but do so relatively rapidly. It is likely that the conservative approach taken by You *et al*. (2005*b*) is the reason why some race(s) clustered with more than one branch in one subcluster (e.g. race 001 in subcluster III). Since most DNA substitutions were observed in the third codon position, the deduced amino acid variation was lower than that of the nucleotide. As a consequence, most of the nucleotide substitution detected in the selected region of the β-tubulin gene is unlikely to result in a change in protein function. For this reason, the genetic diversity may not always directly reflect the pathotype. In future, more detailed and more accurate genetic diversity corresponding to pathogenicity can be generated by examining the pathogenicity related genes of the nuclear genome.

The genetic variation we found was highest in the *P. clandestina* isolates from medium annual rainfall zone (600–800 mm) and lower in those from either the lower (400–600 mm) or the higher (>800 mm) annual rainfall zones. This could be a reflection of the fact that the pastures in >800-mm rainfall zone are mainly permanent with infrequent changes of cultivars, while the 400–600-mm rainfall zone is where there is less subterranean clover cultivars utilised due to lower annual rainfall and the inability of subterranean clover to cope with the more intensive cropping rotations utilised in that rainfall zone, as compared with the 600–800-mm rainfall zone. In contrast to rainfall zones, the genetic variation was not related to the geographic distribution pattern of the isolates of *P. clandestina*.

The high degree of genetic diversity we found suggests that *P. clandestina* continues to evolve. This is most likely in response to the fact that, in Australia, breeding/selection of subterranean clover cultivars with resistance to *P. clandestina* root rot has relied upon incorporation of what now appears to be major gene-based resistance (You *et al*. 2005*b*). With the widespread deployment of new sources of major gene-based resistance in new cultivars (You *et al*. 2005*b*; M. J. Barbetti, unpubl. data), further proliferation of new strains and/or races of *P. clandestina* seems inevitable. This happened previously with *P. sojae* on soybean in eastern Australia (Ryley *et al*. 1991; Ryley and Obst 1998) and in the USA where the introduction of soybean cultivars with major gene-based resistance resulted in the rapid appearance of additional new races (Walker and Schmitthenner 1984).

Our study has not only provided, for the first time, a means of further identifying differences in the distribution of strainal variation within *P. clandestina*, but will also allow easy monitoring of their distribution patterns in the future. Both are critical for clover breeders and for farmers, as they provide the only sound basis for the selection/breeding and deployment of appropriate cultivars to counter the predominant race populations of the pathogen in specific localities in the Mediterranean ecosystems of southern Australia.

The PCR-SSCP based method we used for rapidly characterising isolates of *P. clandestina* opens up, for the first time, the opportunity to monitor the evolution, distribution and spread of current and future strains of this pathogen. We believe this is the first time this technology has been used to successfully characterise intra-species variation in any *Phytophthora* species. This technique will not only be useful in *P. clandestina* but also in intra-specific characterisation of other *Phytophthora* species.

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References

Barbetti MJ, You MP, Li H, Ma X, Sivasithamparam K (2007) Management of root diseases of annual pasture legumes in Mediterranean ecosystems – a case study of subterranean clover root diseases in the south-west of Western Australia. *Phytopathologia Mediterranea* **46**, 239–258.

Bhat RG, Schmitthenner AF (1993) Genetic crosses between physiologic races of *Phytophthora sojae. Experimental Mycology* **17**, 122–129. doi: 10.1006/emyc.1993.1011

Bhat RG, Colowit PM, Tai TH, Aradhya MK, Browne GT (2006) Genetic and pathogenic variation in *Phytophthora cactorum* affecting fruit and nut crops in California. *Plant Disease* **90**, 161–169. doi: 10.1094/PD-90-0161

Bielenin A, Jeffers SN, Wilcox WF, Jones AL (1988) Separation by protein electrophoresis of six species of *Phytophthora* associated with deciduous fruit crops. *Phytopathology* **78**, 1402–1408. doi: 10.1094/Phyto-78-1402

Bilodeau GJ, Levesque CA, de Cock AWAM, Duchaine C, Briere S (2007) Molecular detection of *Phytophthora ramorum* by real-time polymerase chain reaction using TaqMan, SYBR Green, and molecular beacons. *Phytopathology* **97**, 632–642. doi: 10.1094/PHYTO-97-5-0632

Camele I, Marcone C, Cristinzio G (2005) Detection and identification of *Phytophthora* species in southern Italy by RFLP and sequence analysis of PCR-amplified nuclear ribosomal DNA. *European Journal of Plant Pathology* **113**, 1–14. doi: 10.1007/s10658-005-8915-1

Cooke DEL, Drenth A, Duncan JM, Wagels G, Brasier CM (2000) A molecular phylogeny of *Phytophthora* and related Oomycetes. *Fungal Genetics and Biology* **30**, 17–32. doi: 10.1006/fgbi.2000.1202

Crespo A, Molina MC, Blanco O, Schroeter B, Sancho LG, Hawksworth DL (2002) rDNA ITS and β-tubulin Gene sequence analyses reveal two monophyletic groups within the cosmopolitan lichen *Parmelia saxatilis. Mycological Research* **106**, 788–795. doi: 10.1017/S095375620020 610X

Forster H, Tyler BH, Coffey MD (1994) *Phytophthora sojae* races have arisen by clonal evolution and by rare outcrosses. *Molecular Plant-Microbe Interactions* **7**, 780–791.

Forster H, Cummings MP, Coffey MD (2000) Phylogenetic relationships of *Phytophthora* species based on ribosomal ITS I DNA sequence analysis with emphasis on Waterhouse groups V and VI. *Mycological Research* **104**, 1055–1061. doi: 10.1017/S0953756200003087

Gladstones JS (1975) Legumes and Australian agriculture. *Journal of the Australian Institute of Agricultural Science* **41**, 227–240.

Gladstones JS, Collins WJ (1983) Subterranean clover as a naturalized plant in Australia. *Journal of the Australian Institute of Agricultural Science* **49**, 191–202.

Goldman GH, Temmerman W, Jacobs D, Contreras R, Van Montagu M, Herrera-Estrella A (1993) A nucleotide substitution in one of the beta-tubulin genes of *Trichoderma viride* confers resistance to the antimitotic drug methyl benzimidazole-2-yl-carbamate. *Molecular & General Genetics* **240**, 73–80. doi: 10.1007/BF00276886

Irzykowska L, Irzykowski W, Jarosz A, Golebniak B (2005) Association of *Phytophthora citricola* with leather rot disease of strawberry. *Journal of Phytopathology* **153**, 680–685. doi: 10.1111/j.1439-0434.2005.01037.x

Ivors KL, Hayden KJ, Bonants PJM, Rizzo DM, Garbelotto M (2004) AFLP and phylogenetic analyses of North American and European populations of *Phytophthora ramorum. Mycological Research* **108**, 378–392. doi: 10.1017/S0953756204009827

Kim K-J, Eom S-H, Lee S-P, Jung H-S, Kamoun S, Lee YS (2005) A genetic marker associated with the A1 mating type locus in *Phytophthora infestans. Journal of Microbiology and Biotechnology* **15**, 502–509.

Kong P, Hong C, Richardson PA, Callegly ME (2003*a*) Single-strand-conformation polymorphism of ribosomal DNA for rapid species differentiation in genus *Phytophthora. Fungal Genetics and Biology* **39**, 238–249. doi: 10.1016/S1087-1845(03)00052-5

Kong P, Hong C, Richardson PA (2003*b*) Rapid detection of *Phytophthora cinnamomi* using PCR with primers derived from the *Lpv* putative storage protein genes. *Plant Pathology* **52**, 681–693. doi: 10.1111/j.1365-3059.2003.00935.x

Kong P, Hong CX, Tooley PW, Ivors K, Garbelotto M, Richardson PA (2004) Rapid identification of *Phytophthora ramorum* using PCR-SSCP analysis of ribosomal DNA ITS-1. *Letters in Applied Microbiology* **38**, 433–439. doi: 10.1111/j.1472-765X.2004.01510.x

Kroon LPNM, Bakker FT, van den Bosch GBM, Bonants PJM, Flier WG (2004) Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal Genetics and Biology* **41**, 766–782. doi: 10.1016/j.fgb.2004.03.007

Ma Z, Michailides TJ (2005) Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. *Crop Protection (Guildford, Surrey)* **24**, 853–863. doi: 10.1016/j.cropro.2005.01.011

May GS, Tsang MLS, Smith H, Fidel S, Morris NR (1987) *Aspergillus nidulans* beta tubulin genes are unusually divergent. *Gene* **55**, 231–243. doi: 10.1016/0378-1119(87)90283-6

McHau GRA, Coffey MD (1994) Isozyme diversity in *Phytophthora palmivora*: evidence for a southeast Asian centre of origin. *Mycological Research* **98**, 1035–1043. doi: 10.1016/S0953-7562(09)80430-9

Mirabolfathy M, Alizadeh A, Rahimian H (2000) Morphological, physiological and isozymic comparison of *Phytophthora megasperma* from pistachio and other hosts. *Iranian Journal of Plant Pathology* **36**, 15–20.

Morley FHW (1961) Subterranean clover. *Advances in Agronomy* **13**, 57–123. doi: 10.1016/S0065-2113(08)60957-8

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989) Detection of polymorphism of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 2766–2770. doi: 10.1073/pnas.86.8.2766

Panaccione DG, Hanau RM (1990) Characterization of two divergent beta tubulin genes from *Colletotrichum graminicola. Gene* **86**, 163–170. doi: 10.1016/0378-1119(90)90275-V

Pane A, Agosteo GE, Cacciola SO (2000) *Phytophthora* species causing crown and root rot of tomato in southern Italy. *Bulletin OEPP. EPPO Bulletin. European and Mediterranean Plant Protection Organisation* **30**, 251–255. doi: 10.1111/j.1365-2338.2000.tb00890.x

Powell SC (1970) Subterranean clover, our most important pasture legume. *Journal of Agriculture, Victoria* **68**, 274–277.

Purwantara A, Flett SP, Keane PJ (1996) Resistance of nineteen cultivars of subterranean clover to four races of *Phytophthora clandestina. Euphytica* **91**, 351–358. doi: 10.1007/BF00033097

Purwantara A, Flett SP, Keane PJ (1998) Variation in pathogenicity among isolates of *Phytophthora clandestina. Journal of Phytopathology* **146**, 587–591. doi: 10.1111/j.1439-0434.1998.tb04759.x

Purwantara A, Drenth A, Flett SP, Guppy W, Keane PG (2001) Diversity of *Phytophthora clandestina* isolated from subterranean clover in southern Australia: analysis of virulence and RAPD profiles. *European Journal of Plant Pathology* **107**, 305–311. doi: 10.1023/A:1011272419130

Ryley MJ, Obst NR (1998) Changes in the racial composition of *Phytophthora sojae* in Australia between 1979 and 1996. *Plant Disease* **82**, 1048–1054. doi: 10.1094/PDIS.1998.82.9.1048

Ryley MJ, Obst NR, Stovold GE (1991) A new race of *Phytophthora megasperma* f. sp. *glycinea* on soybean in Australia. *Australasian Plant Pathology* **20**, 97–100. doi: 10.1071/APP9910097

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406–425.

Sandral GA, Dear BS, Brennan J, Ewing MA (1997) The economic value of investment in annual plant improvement for cropping systems of Australia. Report to the Grains Research and Development Corporation, Canberra, Australia.

Sekiya TMY, Hayashi K (1993) Detection of mutant sequences by single-strand conformation polymorphism analysis. *Mutation Research* **288**, 79–83. doi: 10.1016/0027-5107(93)90209-X

Slabaugh MB, Huestis GM, Leonard J, Holloway JL, Rosato C (1997) Sequence-based genetic markers for genes and gene families: single-strand conformational polymorphisms for the fatty acid synthesis genes of *Cuphea*. *Theoretical and Applied Genetics* **94**, 400–408. doi: 10.1007/s001220050429

Sullivan KF (1988) Structure and utilization of tubulin isotypes. *Annual Review of Cell Biology* **4**, 687–716. doi: 10.1146/annurev.cb.04.110188.003351

Swofford K (2002) 'PAUP*: Phylogenetic Analysis Using Parsimony and other methods.' (Sinauer Associates: Sunderland, MA)

Taylor PA, Greenhalgh FC (1987) Significance, causes and control of root rots of subterranean clover. In 'Temperate pastures: their production, use and management'. (Eds JL Wheeler, CJ Pearson, GE Robards) pp. 249–251. (Australian Wool Corporation/Commonwealth Scientific and Industrial Research Organisation: Melbourne)

Taylor PA, Pascoe IG, Greenhalgh FC (1985) *Phytophthora clandestina* new-species in roots of subterranean clover. *Mycotaxon* **22**, 77–86.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680. doi: 10.1093/nar/22.22.4673

Voigt K, Cozijnsen AJ, Kroymann J, Pöggeler S, Howlett BJ (2005) Phylogenetic relationships between members of the crucifer pathogenic *Leptosphaeria maculans* species complex as shown by mating type (MAT1–2), actin, and β-tubulin sequences. *Molecular Phylogenetics and Evolution* **37**, 541–557. doi: 10.1016/j.ympev.2005.07.006

Walker AK, Schmitthenner AF (1984) Comparison of field and greenhouse evaluations for tolerance to *Phytophthora* rot in soybean. *Crop Science* **24**, 487–489.

Weerakoon ND, Roberts JK, Lehnen LP Jr, Wilkinson JM, Marshall JS, Hardham AR (1998) Isolation and characterization of the single beta-tubulin gene in *Phytophthora cinnamomi. Mycologia* **90**, 85–95. doi: 10.2307/3761016

White TJ, Bruns Y, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal RNA genes for phylogenetics. In 'PCR protocols: a guide to methods and applications'. (Eds M Innis, D Gelfand, J Sninsky, I White) pp. 315–322. (Academic Press: San Diego, CA)

Yamak F, Peever TL, Grove GG, Boal RJ (2002) Occurrence and identification of *Phytophthora* spp. pathogenic to pear fruit in irrigation water in the Wenatchee River Valley of Washington State. *Phytopathology* **92**, 1210–1217. doi: 10.1094/PHYTO.2002.92.11.1210

You MP, Barbetti MJ, Nichols PGH (2005*a*) New sources of resistance in *Trifolium subterraneum* L. to root rot caused by two races of *Phytophthora clandestina* Taylor, Pascoe and Greenhalgh. *Australian Journal of Agricultural Research* **56**, 271–277. doi: 10.1071/AR04293

You MP, Barbetti MJ, Sivasithamparam K (2005*b*) Characterization of *Phytophthora clandestina* races on *Trifolium subterraneum* in Western Australia. *European Journal of Plant Pathology* **113**, 267–274. doi: 10.1007/s10658-005-1226-8

You MP, Barbetti MJ, Sivasithamparam K (2006) Occurrence of *Phytophthora clandestina* races across rainfall zones in south-west Western Australia. *Australasian Plant Pathology* **35**, 85–87. doi: 10.1071/AP05090

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