DNA-BASED DETECTION OF WESTERN GALL RUST

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ABSTRACT

The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA of the western gall rust fungus (*Peridermium harknessii*) was amplified using the basidiomycete-specific PCR primers ITS1-F and ITS4-B. The PCR product was then sequenced and aligned with other pine stem rust ITS sequences and a conserved region within *P. harknessii* was targeted with the novel PCR primer Phar1. Our PCR protocol was able to differentiate *P. harknessii* from *Cronartium comandrae* and *C. coleosporioides* and detected *P. harknessii* within infected host tissue. However, *P. harknessii* was not distinguishable from *C. quercuum* f. sp. *fusiforme*. The method provides a rapid and sensitive detection protocol for *P. harknessii* and *C. quercuum* f.sp. *fusiforme* within infected host tissue.

Keywords: Western gall rust, *Peridermium (Endocronartium) harknessii*, DNA detection.

INTRODUCTION

Western gall rust (caused by *Peridermium harknessii* J.P. Moore syn. *Endocronartium harknessii* (J.P. Moore) Y. Hiratsuka), is an autoecious rust disease of hard pines (Hiratsuka 1969), including *Pinus radiata* D. Don (Parmeter & Newhook 1967). *Peridermium harknessii* is a close relative of the rust genus *Cronartium*, members of which cause disease on many species of *Pinus* throughout the world. It is hypothesised that *P. harknessii* is an autoecious derivative of its closest relative, *Cronartium quercuum* f. sp. *banksianae,* or a recent common ancestor (Vogler & Bruns 1998). Aeciospores produced on galls in spring and early summer directly infect young elongating pine shoots, causing formation of galls on branches or the main stem (van der Kamp 1989). These galls begin to sporulate within one-to-two years of infection (Old 1981). Colonisation of the main stem can lead to breakage at the infection point (Peterson 1960) or formation of characteristic hip cankers (Old 1981). In North America, western gall rust ranges from the Yukon to Baja California, and from the Pacific to the Atlantic Coasts (van der Kamp 1989). Western gall rust poses a potential threat to *P. radiata* in the Southern Hemisphere, where it is grown extensively in plantations (Parmeter & Newhook 1967; Old 1981; Old et al. 1986; van der Kamp 1989).

Rapid detection and implementation of an incursion response is necessary to prevent establishment of a disease (Hosking et al. 1999). Therefore, a method of detection that does not rely upon spore production would be advantageous because infected material could be identified and destroyed quickly, and prior to sporulation and potential spread of the fungus. Although *P. harknessii* has been grown in axenic culture, fungal growth from infected tissue is slow and the fungus does not always emerge from infected host material (Allen et al. 1988). Additionally, Lundquist et al. (1994) found that the fungus emerged only from galls that were collected immediately prior to aeciospore production. These limitations, and the advent of polymerase chain reaction (PCR) based molecular diagnostic techniques, have spurred the development of a species-specific DNA marker for western gall rust. A successful molecular detection technique must detect at the species, not the individual level, and it must not amplify host DNA.

The objective of this study was to develop a rapid PCR-based protocol that could detect *P. harknessii* mycelium within infected host tissue prior to sporulation. This rapid diagnostic test could be used by inspecting agencies in countries where gall rust is not known to occur, in the event of discovery of pine tissue with suspicious gall formation.

MATERIALS AND METHODS

Collection of fungal material

Western gall rust-infected lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.), ponderosa pine (*P. ponderosa* P. Laws. ex C. Laws) and shore pine (*P. contorta* Dougl. ex Loud. var. *contorta*) branches, as well as samples of *Cronartium coleosporioides* Arth. and *C. comandrae* Peck, were collected from locations throughout British Columbia in May and June 2002. The peridial layer was either intact or recently ruptured and aeciospores were present. Galls with evidence of hyperparasitism were not collected. In the laboratory, aeciospores were removed by rupturing the peridial layer, if required, and tapping the spores into sterilised weigh boats. Spores were purified from contaminating wood and debris by sieving through nylon mesh with a 1 mm pore size, followed by further sieving through 105 μm mesh (Small Parts Inc., Miami Lakes, FL). Purified aeciospores were stored at -80ºC until DNA was extracted.

Extraction of DNA

DNA extraction from aeciospores of *P. harknessii*, *C. coleosporioides* and *C. comandrae* followed the minipestle (Pellet Pestles® from Kontes-Kimble, Vineland, NJ)/diatomaceous earth method of Zambino (2002) without modification. Total DNA was isolated from galled *P. contorta* var. *latifolia* and *P. contorta* var. *contorta* branches, and fresh leaders of *P. contorta* var. *latifolia*, *P. contorta* var. *contorta* and *P. ponderosa* to provide samples that included host and pathogen DNA, and pure host DNA, respectively. A 2x CTAB extraction protocol based on the method of Vogler & Bruns (1998) was used with the following modification: initially, the outer bark was removed using a sterile scalpel, and the inner tissues were cubed and then frozen and ground to a fine powder in liquid nitrogen. DNA of *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Cumm.) Burds. et Snow and *P. radiata* was provided.

ITS PCR amplification and *P. harknessii* primer design

The universal basidiomycete PCR primers ITS1-F and ITS4-B were used to amplify the internal transcribed spacer regions and the 5.8s rRNA gene of the ribosomal DNA repeat from *P. harknessii*, *C. comandrae* and *C. coleosporioides* following the method of Gardes & Bruns (1993). The PCR products were purified using StrataPrep® PCR purification columns (Stratagene, La Jolla, CA), ligated into the pPCR-Script Amp (Stratagene, La Jolla, CA) vector, inserted into XL1-Blue MRF' kan electroporation competent cells (Stratagene, La Jolla, CA) and sequenced using M-13 forward and reverse primers on an ABI 3100 automated sequencer. The ITS sequences of three isolates of *P. harknessii*, each from a different host, and *C. comandrae* and *C. coleosporioides*, as well as ITS sequences from GenBank (Table 1), were aligned using Gene Runner (Hastings Software).

A region that was conserved within *P. harknessii*, yet was variable from other rust species (Table 2) was used to design the *P. harknessii* specific PCR primer Phar1. An Eppendorf MasterCycler gradient was utilised to empirically determine that the optimum annealing temperature of Phar1 was 55ºC. The amplification conditions were therefore 10 μM Phar1, 10 μM ITS4-B, 200 nm dNTP, 0.5 units *Taq* DNA polymerase (Roche New Zealand Ltd.), the supplied Roche buffer with a final $MgCl_2$ concentration of 1.5 mM, and 1 ng target DNA. Thermal cycling conditions were as follows: 94ºC for 2 min, followed by 30 cycles of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 1 min with a final extension at 72ºC for 7 min.

Sensitivity determination

The sensitivity of the method was determined by adding decreasing amounts of *P. harknessii* target DNA to PCR reactions that contained 10 ng of *P. radiata* DNA to determine the limit of detection. A total of 10 ng, 5 ng, 1 ng, 0.5 ng, 0.1 ng, 0.05 ng, 0.01

ng, 0.001 ng and 0.0001 ng of *P. harknessii* DNA was added to PCR reactions, prepared as described, resulting in ratios of 1:1 to 100,000:1 (host DNA:pathogen DNA).

TABLE 1: Rust ITS sequences downloaded from GenBank.

Rust species	GenBank accession number
Chrysomyxa arctostaphyli	L76488
Cronartium appalachianum	L76484
Cronartium arizonicum	L76504
Cronartium comptoniae	L76487
Cronartium conigenum	L76486
Cronartium flaccidum	X83911
Cronartium quercuum	L76495
Cronartium occidentale	L76507
Cronartium ribicola	L76499
Cronartium strobilinum	L76482
Peridermium bethelii	L76479
Pucciniastrum goeppertianum	L76509

TABLE 2: Sequence alignment of PCR primer Phar1 and rust DNAs.

Rust species	Sequence
Phar1 primer sequence	5' ATGTCACCTTGTGCTGTTATTTC 3'
Cronartium quercuum	5' ––––––––––A––G––––––––– 3'
Cronartium conigenum	5' ––––––A––––––T––––G––––3'
Cronartium arizonicum	5' ––––T–A––––––T––––G––––3'
Cronartium strobilinum	5' ––––T–A––––––T––––G––––3'
Cronartium appalachianum	5' –C––T–A––––––T––––G––––3'
Cronartium coleosporioides	5' –C––T–A––––––T––––G––––3'
Cronartium comandrae	5' –C––T–A––––––T––––G––––3'
Cronartium comptoniae	5' –C––T–A––––––T––––G––––3'
Cronartium flaccidum	5' –C––T–A––––––T––––G––––3'
Cronartium occidentale	5' –C––T–A––––––T––––G––––3'
Cronartium ribicola	5' –C––T–A––––––T––––G––––3'
Peridermium bethelii	5' –C––T–A––––––T––––G––––3'
Chrysomyxa arctostaphyli	5' –C–CAT–A–GTGAA–T––TAA–T3'
Pucciniastrum goeppertianum	5' –C–CAT–A–GAGCT–TA–TC–––3'

RESULTS

Amplification of DNA with ITS1-F and ITS4-B from *P. harknessii*, *C. comandrae*, *C. coleosporioides* and *C. quercuum* f. sp. *fusiforme*, as well as from *P. harknessii* and *C. coleosporioides*–infected *P. contorta* var. *latifolia*, resulted in a band of approximately 1000 base pairs (bp) in all cases (Fig. 1). The 1000 bp band was not amplified from uninfected *P. contorta* var. *latifolia* or *P. radiata* DNA (Fig. 1). When the primer Phar1 was used in conjunction with ITS4-B, a 700 bp band was amplified from *P. harknessii*, *P. harknessii*–infected *P. contorta* var. *latifolia* and *C. quercuum* f. sp. *fusiforme*. DNA from *Cronartium comandrae*, *C. coleosporioides, P. contorta* var. *latifolia* and *P. radiata* was not amplified by Phar1 and ITS4-B (Fig. 2). All isolates of *P. harknessii* that were collected in this study were amplified by Phar1 and ITS4-B, regardless of host origin.

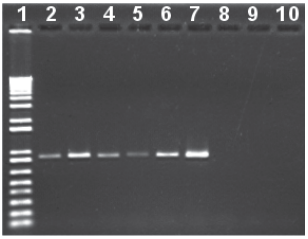

FIGURE 1: Amplification products generated by ITS1-F and ITS4-B. 1. 1 kb+ ladder, 2. *P. harknessii*, 3. *C. comandrae*, 4. *C. coleosporioides*, 5. *C. quercuum* f. sp. *fusiforme*, 6. *P. harknessii*–infected *P. contorta* var. *latifolia*, 7. *C. coleosporioides* infected *P. contorta* var. *latifolia*, 8. *P. contorta* var. *latifolia*, 9. *P. radiata*, 10. Water negative control.

Although gradient PCR indicated that *P. harknessii* DNA was amplified with annealing temperatures up to 63.5ºC and *C. quercuum* f.sp. *fusiforme* was only amplified at annealing temperatures up to 60.8ºC, the diagnostic band was too faint at 63.5ºC to be considered as a reliable marker. Therefore, the annealing temperature was lowered to 55ºC, which resulted in strong amplification of both rust species. When the sensitivity of detection was determined by adding decreasing amounts of pathogen DNA to PCR reactions that contained host DNA, it was found that the diagnostic band was amplified when the amount of pathogen DNA was between 10 ng and 0.05 ng.

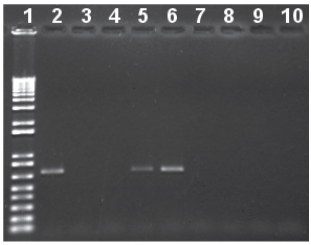

FIGURE 2: Amplification products generated by Phar1 and ITS4-B. 1. 1 kb+ ladder, 2. *P. harknessii*, 3. *C. comandrae*, 4. *C. coleosporioides*, 5. *C. quercuum* f. sp. *fusiforme*, 6. *P. harknessii*–infected *P. contorta* var. *latifolia*, 7. *C. coleosporioides*–infected *P. contorta* var. *latifolia*, 8. *P. contorta* var. *latifolia*, 9. *P. radiata*, 10. Water negative control.

DISCUSSION

Western gall rust, caused by *Peridermium harknessii*, is a serious threat to *P. radiata* plantations in New Zealand due to the amenable climate and the fact that no alternate host is required for completion of the rust life cycle (Parmeter & Newhook 1967; Old 1981; Old et al. 1986; van der Kamp 1989). Western gall rust is not present in New Zealand; therefore, a sensitive detection technique is needed to respond to an inadvertent introduction of the exotic pathogen. Our main objective was to develop a diagnostic protocol that could detect the presence of *P. harknessii* within infected host tissue.

The primers ITS1-F and ITS4-B have been utilised to amplify rust DNA in previous studies (Vogler & Bruns 1998) and the ITS provides a genomic region that is variable

enough to differentiate between species but is conserved within species (Zambino & Szabo 1993). The PCR primer Phar1, in conjunction with ITS4-B, was shown to differentiate *P. harknessii* from *C. comandrae* and *C. coleosporioides*. Unfortunately, DNA from other closely related *Cronartium* species was unavailable for empirical testing of the novel primer pairs. However, according to sequences lodged in GenBank the protocol will putatively differentiate *P. harknessii* from *C. appalachianum*, *C. comptoniae*, *C. flaccidum*, *C. occidentale*, *C. ribicola* and *P. bethelii* because alignment with sequences from the GenBank database indicated that these species are identical to *C. comandrae* and *C. coleosporioides* at the Phar1 recognition site. *Cronartium quercuum* f. sp. *banksianae* is the nearest sibling species to *P. harknessii* and is hypothesised to share with it a most-recent common ancestor (Vogler & Bruns 1998). As the primer recognition site varies by only two base pairs between *P. harknessii* and *C. quercuum*, it is not surprising that *C. quercuum* f.sp *fusiforme* DNA was also amplified by Phar1. The method developed in this study was able to detect the presence of as little as 50 pg *P. harknessii* DNA amongst 10 ng of host DNA. It is concluded that the PCR primers Phar1 and ITS4-B can be utilised to identify *P. harknessii* and *C. quercuum* f.sp. *fusiforme* within suspicious galls. Currently, this diagnostic test is being refined to allow *P. harknessii* and *C. quercuum* f.sp. *fusiforme* to be distinguished at the DNA level.

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