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Comparison of three varieties of *Leptographium wageneri* using Random Amplified Polymorphic DNA

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Leptographium wageneri (Kendrick) Wingfield is the cause of black stain root disease in western North America. The fungus has been separated into three host-specialised morphological variants, namely *L. wageneri* var. *wageneri* (Kendrick) Wingfield, var. *pseudotsugae* Harrington & Cobb and var. *ponderosum* (Harrington & Cobb) Harrington & Cobb. These varieties have been well-characterised on the basis of both morphological and molecular data. The aim of this study was to determine whether RAPD analysis might be used in distinguishing between the three varieties of *L. wageneri*. Screening was performed for thirty primers, and four primers that produced unique DNA fingerprints for each variety were selected. RAPD analysis separated the three varieties of *L. wageneri* into distinct groups. The RAPD analysis was reliable, reproducible and might be useful in separating the three varieties of *L. wageneri*, which are morphologically very similar.

Keywords: *Leptographium wageneri* var. *wageneri*, var. *pseudotsugae*, var. *ponderosum*, L. *wingfieldii*, Ophiostomataceae, PCR, RAPD.

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Introduction

Leptographium wageneri [Kingdom: Eumycota (Kendrick, 1992); Order: Ophiostomatales; Family: Ophiostomataceae (Benny & Kimbrough 1980)], the cause of black stain root disease, is one of the most important pathogens of conifers in western North America (Harrington & Cobb 1983, 1988). The disease results in a black staining of colonised sapwood of the roots and lower stems due to the presence of pigmented fungal hyphae in infected tissue. The causal agent of the disease was, for many years, known as *Verticicladiella wageneri* (Kendrick 1962) but was later transferred to *Leptographium* as *L. wageneri* (Wingfield 1985).

Leptographium wageneri only occurs in western North America and has been separated into three host-specialised morphological variants based on differences in conidiophore morphology, maximum growth temperatures and cultural appearances (Harrington & Cobb 1986, 1987). Vegetative compatibility (Zambino & Harrington 1990) and isozyme variation (Zambino & Harrington 1989) have also been used to distinguish between these varieties. The three varieties are thus *L. wageneri* var. *wageneri* which occurs on pinyon pine (*Pinus monophylla* and *P. jeffreyi*) (Harrington & Cobb 1986), var. *pseudotsugae* on Douglas-fir (*Pseudotsugae menziesii*) (Harrington & Cobb 1987) and var. *ponderosum* on hard pines (*P. ponderosa, P. edulis* and *P. contorta*) (Harrington & Cobb 1986).

Leptographium wageneri is one of the best characterised species in the ophiostomatoid group of fungi, which are significant tree pathogens in many parts of the world. Random Amplified Polymorphic DNA (RAPD) (Williams *et al.* 1990; Welsh & McClelland 1990) is a PCR based method, generating DNA fingerprints through amplification of DNA with single, short arbitrary nucleotide sequences. RAPD analysis has proven successful in comparisons between fungi at the species level (Lehmann *et al.* 1992; Yoon & Glawe 1993). It has also been used to compare varieties (Strongman & MacKay 1993), strains (Kwan *et al.* 1992; Ouellet & Seifert 1993) and races (Pipe *et al.* 1995). The aim of this study was to test the potential for characterising *Leptographium* species using RAPDs, but more specifically to compare the three well-defined and host specific varieties of *L. wageneri*.

Material and Methods

Three isolates of *L. wageneri* var. *ponderosum*, four isolates of *L. wageneri* var. *pseudotsugae*, two isolates of *L. wageneri* var. *wageneri*, and one isolate of *L. wingfieldii* Morelet were included in this study (Table 1). Cultures were grown on malt-extract agar plates (20 g/l) covered with sterile cellophane disks. The disks, covered with mycelia, were removed from the plates and lyophilised. DNA was extracted from the dried mycelium using the method described by Viljoen *et al.* (1993). The study included duplicate DNA preparations from different batch cultures of the same isolate to ensure the reproducibility of the results.

Amplification reactions were performed as described by Williams *et al.* (1990). Screenings were performed for 30 arbitrary sequence oligonucleotides, namely OPA1–20 and OPB1–10 (Operon Technologies, California, U.S.A.). The optimum $MgCl_2$ concentration was found to be 2,5 mM and this was used for all DNA amplifications. Amplification reactions were done using a Hybaid Omnigene Temperature Cycler (Hybaid, Middlesex, UK). An initial denaturation was done at 96°C for 5 minutes, followed by 35 cycles of 92°C denaturation for 15 seconds, 34°C annealing for 1 minute and thereafter the temperature was increased at a rate of 1°C/1,5 seconds to 72°C. The reaction was then allowed to proceed for 2 minutes. The reaction was completed with a 72°C chain elongation for 5 minutes.

The DNA amplification products were separated on 5% polyacrylamide gels and silver stained (Sambrook *et al.* 1989) to visualise the DNA. A binomial data matrix was constructed from the presence (1) or absence (0) of RAPD bands. Phylogenetic Analysis Using Parsimony (PAUP) (Swofford 1993) was used to generate a phylogram. The confidence intervals of the branch points was determined using bootstrap.

Results and Discussion

Six of the thirty primers screened produced single bands. These are not useful as DNA fingerprints, since no variation within the population could be observed. Twenty primers gave no amplification of the DNA from the three varieties of *L. wageneri*. Four primers produced banding patterns that were meaningful in distinguishing between the varieties and these were selected for RAPD analysis of the *L. wageneri* isolates. These four primers are OPA2 (5'TGCCGAGCTG3'), OPA4 (5'AATCGGGCTG3'),

Table 1 List of isolates, their origins and hosts

Organism		Source of Isolates[1]	Host	Origin
L. wageneri var.	*wageneri* (WAG1)	CMW402,CAS4,ATCC64194	*Pinus monophylla*	California, USA
	wageneri (WAG2)	CMW2331,CAS15,ATCC64195	*Pinus monophylla*	California, USA
L. wageneri var.	*pseudotsugae* (PSE1)	CMW127,BCH1,ATCC42953	*Tsuga heterophylla*	British Columbia, Canada
	pseudotsugae (PSE2)	CMW154,CAD55	*Pseudotsuga menziesii*	California, USA
	pseudotsugae (PSE3)	CMW266,IDD2	*Pseudotsuga menziesii*	Idaho, USA
	pseudotusgae (PSE4)	CMW390,NMD1	*Pseudotsuga menziesii*	New Mexico, USA
L. wageneri var.	*ponderosum* (PON1)	CMW307,BCL3	*Pinus contorta*	British Columbia, Canada
	ponderosum (PON2)	CMW279,CAP36	*Pinus ponderosa*	California, USA
	ponderosum (PON3)	CMW280,ORP1	*Pinus ponderosa*	Oregon, USA
L. wingfieldii (WING)		CMW2096	*Pinus sylvestris*	France

[1] ATCC - American Type Culture Collection, CMW - Culture Collection of M.J. Wingfield, Department of Microbiology and Biochemistry, UOFS, Bloemfontein. All other isolate numbers refer to isolates from the culture collection of Dr. T.C. Harrington, Department of Plant Pathology, Iowa State University, Iowa.

OPA9 (5'GGGTAACGCC3') and OPA11 (5'CAATCGCCGT3') Visual inspection of the banding patterns on the silver stained polyacrylamide gels was sufficient to distinguish the three varieties of *L. wageneri*. Different numbers of bands were scored depending on the isolate and the primer. For OPA2, 20 different bands were scored; for OPA4, 22 bands; OPA9, 14 bands; and for OPA11, 22 bands. Therefore, the binomial data matrix was produced using 78 characters.

The phylogram generated using PAUP is presented in Figure 1. The 1000 bootstrap repeats are also indicated. These results clearly show that the isolates within a variety of *L. wageneri* group closely together, with each of the three varieties *L. wageneri* var. *ponderosum*, var. *wageneri* and var. *pseudotsugae* grouping distinctly apart. *L. wingfieldii*, which was included as an outgroup in the analysis, grouped apart from all the *L. wageneri* isolates.

The analyses of the DNA fingerprints produced in the amplification with four primers OPA2, OPA4, OPA9 and OPA11 show that *L. wageneri* var. *ponderosum* isolates group closer to *L. wageneri* var. *wageneri* than to *L. wageneri* var. *pseudotsugae*.

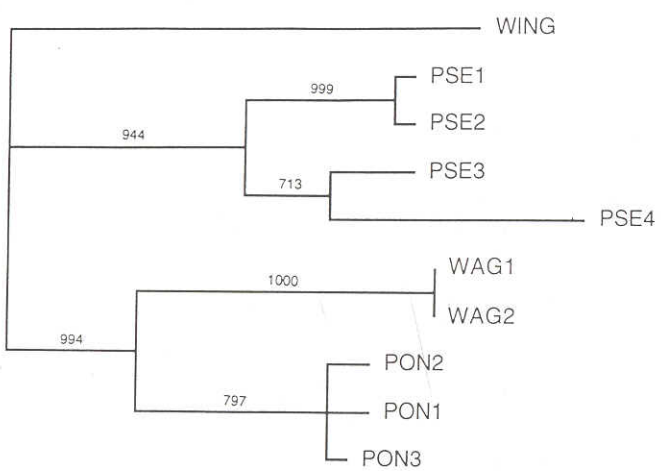

Figure 1 Phylogram produced from the binomial data matrix using PAUP analysis. Bootstrap values for 1 000 repeats are given. WING, *L. wingfieldii*; PSE, *L. wageneri* var. *pseudotsugae*; WAG, *L. wageneri* var. *wageneri* and PON, *Leptographium wageneri* var. *ponderosum*.

The same clustering pattern was found by Zambino & Harrington (1992) using isozyme analyses. The two clusters found within *L. wageneri* var. *pseudotsugae* further confirms the results of the isozyme analysis.

The results of the RAPD analyses conducted in this study show that the three varieties of *L. wageneri* group distinctly apart from each other and separate from *L. wingfieldii*, the outgroup species. The technique therefore appears to be useful in confidently distinguishing between the morphologically similar varieties of *L. wageneri*. RAPD analyses can prove to be a convenient diagnostic technique, given that the bands are reproducible between laboratories.

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