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Differentiation of sapstain fungi by restriction fragment length polymorphism patterns in nuclear small subunit ribosomal DNA

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Abstract

A useful DNA fingerprinting method for wood staining fungi is described. The polymerase chain reaction (PCR) was used to amplify nuclear small subunit ribosomal DNA (SSrDNA) from 55 fungal isolates of 13 sapstain species belonging to the *Aureobasidium, Ceratocystis, Leptographium*, and *Ophiostoma* genera. To find polymorphisms useful in differentiating the isolates, the amplified SSrDNAs were digested with 10 selected restriction enzymes. Genus-specific restriction fragment length polymorphism (RFLP) patterns were determined by *Rsa*I, *Sty*I, and *Taq*I for the four genera. This PCR-RFLP analysis of SSrDNA offers an easy and rapid tool for differentiation of the major sapstain organisms on stained wood.

Keywords: Sapstain fungus; Restriction fragment length polymorphism; Nuclear small subunit ribosomal DNA (18S SSrDNA)

1. Introduction

Wood discoloration caused by sapstain fungi is a long-standing world-wide problem for the wood industry. This cosmetic defect reduces the infected softwood's suitability for use in high value end products. The presence of sapstain fungi in exported wood products can lead to the rejection of a shipment by the importing country, resulting in financial losses for the exporting partner. To maintain and increase Canadian softwood exports it might be necessary to establish a quality control program confirming that wood products are free from unwanted microorganisms.

Sapstain fungi are early colonizers of freshly felled trees. They produce dark-blue or brown stain either superficially or deeply in the sapwood. Although most staining fungi are saprophytic, a few species are pathogenic to trees and crops. Recently, we conducted a detailed survey of sapstain fungi at sawmills across Canada and showed that species belonging to the genera *Aureobasidium, Ceratocystis, Leptographium* spp., and *Ophiostoma* spp. were frequently isolated from logs and lumber of five economically important softwood species [1].

Until recently, differentiation of sapstain fungi has

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been performed using morphological and physiological characteristics, including growth on cycloheximide media and pairing of unknown cultures with known tester species on sapwood media. This procedure required 8–12 weeks to be completed and the skills of an experienced mycologist. Among sapstain fungi, particularly with the genera *Ceratocystis, Leptographium* and *Ophiostoma*, species are difficult to differentiate due to the similarity of morphological traits, the insufficiency of biological species with compatible mating groups, and the lack of genetic and ecological information on many of these species [2]. The development of molecular techniques, like ribosomal DNA fingerprinting, offered a useful tool for detecting and identifying forest fungi [3]. However, these techniques have not yet been applied to the differentiation of sapstain fungi.

In this paper, we utilized polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) techniques to develop rapid and direct molecular tools for fingerprinting the four major Canadian sapstain genera, *Aureobasidium, Ceratocystis, Leptographium*, and *Ophiostoma* on wood.

2. Materials and methods

2.1. Fungal cultures and DNA preparation

Species belonging to the four genera *Aureobasidium, Ceratocystis, Leptographium* and *Ophiostoma* were frequently found and concomitantly occurred in stained wood in the five major softwood species that we surveyed across Canada [1]. Thus, these major genera were typed into 11 species of 55 isolates representing different geographic regions. Selected foreign strains were included as references. The details on the origin and sources of typed species used in this study are given in Table 1. The fungal cultures are maintained in the Culture Collection of the Wood Science Department at the University of British Columbia. The fungi were pre-grown on 2% malt extract agar (MEA) for 7–10 days at 20°C. Spore suspensions prepared from MEA cultures were spread onto sterile sheets of cellophane over-layered on MEA plates. After 2–3 days of incubation at 20°C, about 0.3 g of mycelium was harvested from the cellophane sheet by scraping the surface with a scalpel. The harvested mycelium was stored in a 2-ml sterile cryogenic vial (Sarstedt, Nümbrecht, Germany). Fungal genomic DNA was extracted directly from the mycelium in cryogenic vials using stainless steel drill bits and a hand drill [4], and the concentration was determined by GeneQuant II DNA/RNA Calculator (Amersham Pharmacia).

2.2. PCR amplification and restriction enzyme digestion analysis of PCR products

Two universal rDNA primers, NS1 and NS8 [5], were used for a PCR reaction to amplify the nuclear small rDNA (18S SSrDNA). The PCR amplifica-

Table 1
Fungal isolates used in this study

Fungal species	Isolate number	Origin	Host tree
Aureobasidium pullulans	AU72	B.C., Canada	Lodgepole pine
	AU73	B.C., Canada	White spruce
	AU123-436	Sask., Canada	Jack pine
	AU123-449	Sask., Canada	Jack pine
	AU156-127	B.C., Canada	Lodgepole pine
	Pr3-167	B.C., Canada	Lodgepole pine
	NOF1540	Alta., Canada	Poplar
	NOFC1542	Alta., Canada	Poplar
Ceratocystis coerulescens	AU123-22-12	Sask., Canada	Jack pine
	AU123-314	Sask., Canada	Jack pine
	AU123-437	Sask., Canada	Jack pine
	AU125-214	Sask., Canada	White spruce
	AU157-22-14	B.C., Canada	White spruce
	AU157-152	B.C., Canada	White spruce

Table 1 (*continued*)
Fungal isolates used in this study

Fungal species	Isolate number	Origin	Host tree
	AU157-223	B.C., Canada	White spruce
	Pr3-54	B.C., Canada	Lodgepole pine
	130/3p	UK	Corsican pine
Leptographium spp.	AU55-5	BC., Canada	Lodgepole pine
	AU71-15	B.C., Canada	Lodgepole pine
	AU123-239	Sask., Canada	Jack pine
	AU156-234	B.C., Canada	Lodgepole pine
	AU157-253	B.C., Canada	White spruce
L. procerum	199/3pb(L14)	Thetford Forest, UK	Corsican pine
L. wingfieldii	S/1/2 (LW1)	Bramskill, UK	Scots pine
Ophiostoma flexuosum	OS-4/1-A-1	Que., Canada	Unknown
	DS1-3B-2	Que., Canada	Unknown
O. floccosum	AU55-1	B.C., Canada	Lodgepole pine
	AU88-1-1	Sask., Canada	Jack pine
	AU156-211	B.C., Canada	Lodgepole pine
	AU197-3	Alta., Canada	Lodgepole pine
	387N	Forintek C.C.	Unknown
O. ips	124-43-13	Q.B., Canada	Unknown
O. minus	AU58-4	B.C., Canada	Lodgepole pine
AU123-43-13	Sask., Canada	Jack pine	
AU123-151	Sask., Canada	Jack pine	
AU198-4	Alta., Canada	Lodgepole pine	
0/909	Brandon, UK	Scots pine	
C/106	Nebraska, USA	Jack pine	
O. piceae	AU55-3	B.C., Canada	Lodgepole pine
	AU123-142	Sask., Canada	Jack pine
	AU153-5	B.C., Canada	Lodgepole pine
	AU157-241	B.C., Canada	White spruce
	AU187-1	Alta., Canada	Lodgepole pine
	H2009	UK	Scots pine
	H2181	UK	Sitka spruce
O. piliferum	AU55-2	B.C., Canada	Lodgepole pine
	AU80-3	Sask., Canada	Jack pine
	AU156-112	B.C., Canada	Lodgepole pine
	AU199-4	Alta., Canada	Lodgepole pine
O. setosum	AU55-6-1	B.C., Canada	Lodgepole pine
	AU160-21	B.C., Canada	Hemlock
	AU160-38	B.C., Canada	Hemlock
Species E	AU57-2	B.C., Canada	Lodgepole pine
	AU125-238	Sask., Canada	White spruce
	AU195-7	Alta, Canada	Lodgepole pine

Species E is an *O. piliferum*-related species and can be clearly divided into different individual taxa based on its unique mating pattern [1]. Forintek C.C.: Culture Collections of Forintek Corp. Canada. Alta.: Alberta; B.C.: British Columbia; Sask.: Saskatchewan; Que.: Quebec.

tions were performed in 0.5-ml PCR tubes, using a Hybaid Touch Down thermal cycler. PCR reaction mixtures (50 μl each) contained 100–200 ng of fungal genomic DNA, 40 pmol of each primer, 1×PCR buffer (10 mM Tris-Cl [pH 8.0], 1.5 mM $MgCl_2$, 50 mM KCl), 50 μM (each) of the four deoxynucleoside triphosphates (dNTPs), and 1 unit Thermostable DNA polymerase (Rose Scientific). Thermal cycling conditions were as follows: initial denaturation (94°C, 4 min), 30 cycles of denaturation (94°C, 50 s), annealing (52°C, 60 s), and primer extension (72°C, 60 s), followed by one final cycle of primer

extension (72°C, 10 min). Negative controls (no DNA template) were used in every experiment to check the presence of DNA contamination in reagents and reaction mixtures. After amplification, the PCR products (5 μl) were checked by electrophoresis on a 1.4% agarose gel. Amplified DNA (10–15 μl, approximately 0.5 μg) was digested, without further purification, using 3 units of various restriction enzymes (Pharmacia Biotech and Boehringer Mannheim), following the manufacturers' instructions. The products were separated on a 2.5% agarose gel in Tris-acetate-EDTA buffer with ethidium bromide and visualized under UV light. PCR amplifications and restriction digestion analyses were repeated at least two or three times.

3. Results and discussion

3.1. PCR amplification of rDNA regions

RFLP analysis of PCR-amplified rDNA products has been successfully used for distinguishing *Tylospora fibrillosa* ectomycorrhizas [6] and identifying the *Gaeumannomyces-Phialophora* complex [7]. The first step of this approach requires PCR amplification of target rDNA molecules. In previous work, we have shown that it is possible to differentiate two sapstain species, *Ophiostoma piceae* and *O. quercus*, by using species-specific primers designed based on the unique ribosomal internal transcribed spacer (ITS) DNA sequences [4]. However, the generalization of this approach to all the sapstain genus was limited by the inability of the universal ITS primers to amplify the ITS regions from some of the *Ophiostoma* and *Leptographium* species. Similar difficulty was also reported by Strydom et al. in the amplification of ITS sequence in *Leptographium* species [8].

Therefore, we targeted alternative rDNA regions for PCR amplification. Initially two common primer pairs, NS1-NS8 and NS7-NL4, known to specifically target fungal rDNA regions of about 1.4 kb and 1.8 kb, were selected. The expected sizes of PCR products amplified by these primers were likely suitable for RFLP analysis. NS1-NS8 targeted the entire SSrDNA region [5] while NS7-NL4 targeted the 5′ end of the SSrDNA, the 5.8S rDNA, both ITS1 and ITS2, and 5′ end of 26S large subunit rDNA regions [5,9]. The specificity of these PCR primers was evaluated by observing the presence or absence of expected sizes of PCR-amplified products. The NS1-NS8 primer pair produced PCR products from all the isolates typed (Table 1), but the NS7-NL4 primer pair produced little or no amplified DNA from some isolates. Thus, the NS1-NS8 primer pair was used in this PCR-RFLP study.

All the isolates typed produced the same amplification product of about 1.75 kb with the primer pair NS1-NS8. The amplified band corresponded to the expected size of the 18S SSrDNA region which is usually found in most fungal species. This indicated that all the sapstain species typed possessed no intervening sequences in the 3′ region of 18S SSrDNA, which could generate a larger PCR product size. Fig. 1 shows an example of the agarose gel electrophoresis pattern of the PCR products generated from some representative isolates from each species. One of these 1.75-kb PCR products amplified from *O. floccosum* was purified from an agarose gel, subcloned into pCR-TOPO vector (Invitrogen), and sequenced. Through nucleotide database searches, the 1.75-kb PCR product was identified as a SSrDNA gene based on its very high DNA sequence homology (95–99%) with the 18S SSrDNA of *Ophiostoma*

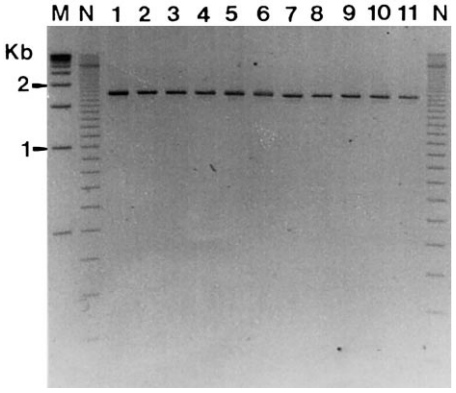

Fig. 1. PCR amplification of the 18S SSrDNA from mycelia of 13 sapstain species of the four fungal genera. The general rDNA primer pair, NS1-NS8 [8], was used. The PCR products (about 1.75 kb) were electrophoresed on a 1.4% agarose gel. Lane M: 500-bp DNA size marker (Bio/Can); lane N: 100-bp DNA size marker (Bio/Can); lane 1: *A. pullulans*; lane 2: *C. coerulescens*; lane 3: *O. minus*; lane 4: *Leptographium* spp.; lane 5: *O. floccosum*; lane 6: *O. flexuosum*; lane 7: *O. ips*; lane 8: *O. piceae*; lane 9: *O. piliferum*, lane 10: *O. setosum*; lane 11: species E.

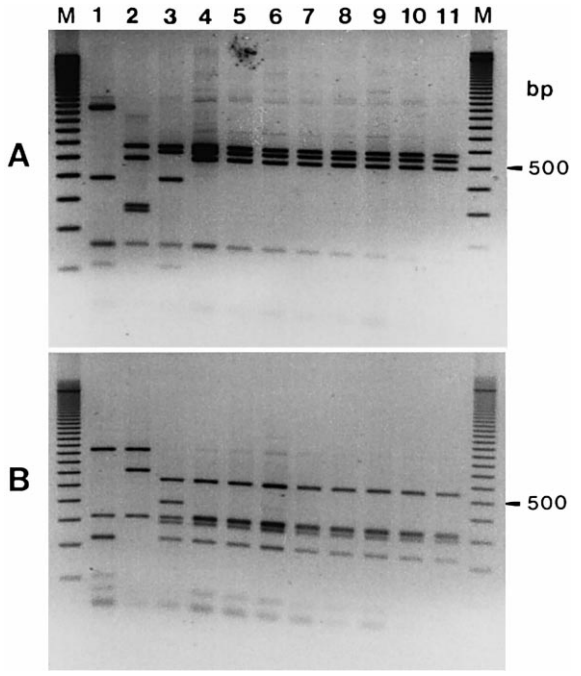

Fig. 2. Differentiation of the sapstain genera, *Aureobasidium, Ceratocystis, Leptographium*, and *Ophiostoma* by the RFLP patterns of the PCR-amplified 18S SSrDNA from mycelia of 13 sapstain species. PCR products were digested with *Rsa*I (A) and *Sty*I (B) and electrophoresed on a 2.5% agarose gel. Lanes M: 100-bp DNA size marker (Bio/Can); lane 1: *A. pullulans*; lane 2: *C. coerulescens*; lane 3: *Leptographium* spp.; lane 4: *O. flexuosum*; lane 5: *O. floccosum*; lane 6: *O. ips*; lane 7: *O. minus*; lane 8: *O. piceae*; lane 9: *O. piliferum*, lane 10: *O. setosum*; lane 11: species E.

species such as *O. piceae* (DDBJ accession number AB007663), *O. stenoceras* (GenBank M85054), and *O. ulmi* (GenBank M83261). The sequence of *O. floccosum* 18S SSrDNA was deposited in GenBank (accession number AF139810). The identity of the PCR-amplified products in Fig. 1 as SSrDNA was confirmed by Southern blot analysis using the [α-^{32}P]ATP-labeled *O. floccosum* SSrDNA as a probe (data not shown).

3.2. RFLP analysis of SSrDNA from cultures grown on artificial media

The information on the full length sequences of SSrDNA in sapstain and sapstain-related fungi is limited to *A. pullulans, O. ulmi, O. stenoceras*, and *Sporothrix schenckii* species. Thus, to select useful restriction enzymes allowing us to generate diagnostic RFLP patterns, potential restriction cutting sites existing in SSrDNA sequences of above four species and *O. floccosum* from this study were analyzed using PC-Gene software program (Intelligenetics). Ten restriction enzymes (*Bam*HI, *Bgl*II, *Eco*RI, *Hae*III, *Hin*dIII, *Rsa*I, *Sac*I, *Sty*I, *Taq*I and *Xba*I) were chosen.

The first tests of the selected enzymes for RFLP analysis were performed using SSrDNA amplified by PCR from genomic DNA samples of all the isolates in Table 1 grown on MEA. The enzymes *Bam*HI, *Bgl*II, *Eco*RI, and *Hin*dIII did not cut the 18S SSrDNA or only very small fragments were cut from the ends, resulting in no reliable RFLP patterns. The 18S SSrDNA was cut into two fragments

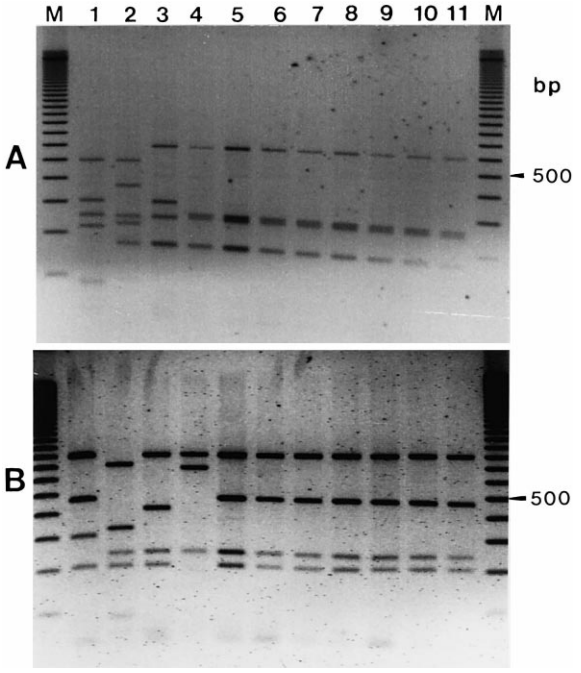

Fig. 3. Differentiation of *O. minus* by the RFLP patterns of the PCR-amplified 18S SSrDNAs from mycelia of 13 sapstain species of *Aureobasidium, Ceratocystis, Leptographium*, and *Ophiostoma* genera. PCR products were digested with *Hae*III (A) and *Taq*I (B) and electrophoresed on a 2.5% agarose gel. Lanes M: 100-bp DNA size marker (Bio/Can); lane 1: *A. pullulans*; lane 2: *C. coerulescens*; lane 3: *O. minus*; lane 4: *Leptographium* spp.; lane 5: *O. floccosum*; lane 6: *O. flexuosum*; lane 7: *O. ips*; lane 8: *O. piceae*; lane 9: *O. piliferum*, lane 10: *O. setosum*; lane 11: species E.

of approximately 650 and 1050 bp by *Sac*I, and of 150 and 1550 bp by *Xba*I. But all the isolates tested gave the same RFLP patterns (data not shown). By digestion with *Hae*III, *Rsa*I, *Sty*I, and *Taq*I, more than three fragments were generated in all the isolates in Table 1. The RFLP patterns by *Rsa*I and *Sty*I separated clearly all the species belonging to the four genera *Aureobasidium, Ceratocystis, Leptographium* and *Ophiostoma*. The representative results are given in Fig. 2. Within the *Ophiostoma* genus, only *O. minus* was differentiated from the other species using the RFLP patterns generated by *Taq*I and *Hae*III. Fig. 3 shows the representative results of *Taq*I and *Hae*III digestions. The rarity of fragment polymorphisms between the species tested was also apparent in the *Leptographium* genus. The *Taq*I RFLP patterns were the best choice for the differentiation of the four genera and *O. minus*.

There were no intra-species variations in the RFLP patterns by *Hae*III, *Rsa*I, *Sty*I, and *Taq*I. Consequently, we found no difference in the RFLP patterns either between domestic cultures from different provinces or between cultures from domestic and foreign origins.

3.3. RFLP analysis of SSrDNA from sapstain species present on wood

Based on the results in Section 3.2, we proceeded to test the use of PCR-RFLP analysis in differentiation of sapstain fungi growing on wood. For this, some isolates of *A. pullulans* (AU72, AU73), *O. piceae* (AU55-3, AU123-142), *C. coerulescens* (AU123-22-12, AU157-152), and *Leptographium* spp (AU55-5, AU71-15) were artificially inoculated either individually or as a mixture into lodgepole pine wood. After growth on the inoculated wood, fungal DNA samples for PCR were prepared directly from spores or mycelia present on the wood surface. This was achieved within 10 min, using a microwave heating method [4]. As shown in Fig. 1, SSrDNA genes were amplified by PCR from all these fungal DNA samples and subjected to restriction enzyme digestions with *Taq*I, *Rsa*I, and *Sty*I. The RFLP patterns generated by the three restriction enzymes were identical to those generated from the same species shown in Figs. 2 and 3 grown in artificial media.

Based on the *Taq*I RFLP patterns of SSrDNA, we were also rapidly able to detect and differentiate the four sapstain genera, *Aureobasidium, Ceratocystis, Leptographium*, and *Ophiostoma* from the randomly sampled fungal spores at a sawmill in Princeton, B.C. This showed that the PCR-RFLP method worked well and was reproducible with artificially and naturally infected wood.

The sensitivity of the PCR-RFLP method is an important consideration, as demonstrated by the fact that the minimal number of spores needed for rDNA amplification by PCR directly from an *Ophiostoma* species on wood is more than 100 spores [4]. We did not optimize this method as most synemata present on wood contained more than 100 spores. Although traditional methods might detect some sapstain fungi with less than 100 spores, there is no certainty that all wood endogenous fungi would grow on artificial media.

We conclude that PCR-RFLP of SSrDNA could be applied to fingerprinting of the four major Canadian sapstain genera, *Aureobasidium, Ceratocystis, Leptographium*, and *Ophiostoma* from laboratory cultures as well as, more significantly, on wood surface in the field. This latter approach was performed without isolating and growing the fungal cultures on media, and without harmful and tedious DNA preparation steps. For *A. pullulans* detection, the method described in this work was also faster and more convenient than the method previously published using a radio-labeled oligonucleotide probe [10]. To our knowledge this is the first application of PCR-RFLP to do a rapid and reliable detection of the major discoloring organisms on wood. We expect that this detection tool will be easily applicable in forest products industry to monitor the presence of the major sapstain fungal groups on softwood wood products.

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