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The β-tubulin gene is a useful target for PCR-based detection of an albino *Ophiostoma piliferum* used in biological control of sapstain

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Abstract

The potential of Cartapip™, an albino *Ophiostoma piliferum*, as a biocontrol agent against sapstain in logs has been tested in Germany. To detect the albino strain in field-tested wood, the usefulness of the β-tubulin gene as a target region for developing PCR-based assays was evaluated with 102 strains of *O. piliferum* and 31 strains of other wood-inhabiting species. A partial β-tubulin gene sequence of *O. piliferum* strains from different geographic origins was amplified by PCR and analyzed by restriction enzyme digestions and DNA sequencing. Variation in size and nucleotide sequences was found in intron regions indicating that intraspecific variation is present in the β-tubulin gene. Consequently, β-tubulin gene-derived PCR methods using PCR–RFLP patterns generated by *Hin*fI and *Spe*I and sequence-specific primers Cat1 and Cat2, were developed and their specificity for Cartapip™ was accessed with field-tested logs and lumber. The β-tubulin gene-based PCR methods were found to be valuable tools for rapid and reliable identification of Cartapip™ in field-tested logs and lumber in Germany. Specificity tests against other wood-inhabiting species and wild type *O. piliferum* strains from diverse nations showed that the Cat1 and Cat2 primers have potential to be used in other European countries, New Zealand, Alberta and British Columbia.

Introduction

Sapstain is a cosmetic damage in wood. In most wood-exporting countries, wood is stained at the harvesting sites, during storage and transportation by fungal species belonging to the Ophiostomatoid group. Fungicide-based sapstain control methods are either not feasible for all markets or problematic because of their broad spectrum of action. Government and public concerns on the environmental impact resulting from pesticide usage have prompted the search for alternative approaches to sapstain control. Thus, altered management practices as well as the utilization of natural products, induced resistance and the use of biological control have been encouraged as alternative control strategies.

Biological protection of wood from fungal stain using microorganisms has received intensive investigation since the mid1960s (Stilwell, 1966; Seifert et al., 1988; Kreber and Morrell, 1993; Behrendt and Blanchette, 1997; Yang, 1999). More recently, the use of albino strains of *Ophiostoma* species has been suggested as a potential biological control strategy for sapstain (Farrell et al., 1998). Effective albino agents to sapstain should be able to colonize the substrate quickly and resist any displacement by sapstain fungi. In addition, these albinos should not affect the wood properties or cause adverse effects to the environment. Cartapip™ (AgraSol Inc.), an albino form of the sapstain fungus *Ophiostoma piliferum* (Fr.) H. & P. Syd., was isolated from southern yellow pine (*Pinus taeda*) in South Carolina and Virginia in the USA (Farrell et al., 1993). This albino was initially used for pitch (i.e. wood extractives) reduction in the pulp and paper industry (Blanchette et al., 1992; Farrell et al., 1993). Recently, field trials with Cartapip™ in New Zealand

showed that it was effective in protecting cut red pine (*Pinus resinosa*) logs from bluestain (Behrendt et al., 1995a,b). Limited biocontrol trials with Cartapip™ have also been carried out in Germany and Alberta (Uzunovic et al., 1999a). However, no quick and reliable tools are available to detect the albino strain during field trials in these regions.

Nucleotide sequence variations can provide useful targets for the development of molecular tools to separate and detect fungi. The small and large subunits and internal transcribed spacer (ITS) regions of the nuclear rRNA gene have been analyzed for differentiating, detecting and establishing the phylogenetic profiles of the Ophiostomatoid fungi (Hausner et al., 1993; 2000; Spatafora and Blackwell, 1994; Okada et al., 1998; Kim et al., 1999a,b). All these analyses are based on interspecific variation in the rDNA sequences. In field trials, Cartapip™ needs to be differentiated from other fungal species and from wild type strains. Thus it is necessary to target the genes showing interspecific and intraspecific variations. In previous work, *O. piliferum* including Cartapip™ was differentiated from other species using PCR–RFLP analysis of the large subunit of the rRNA gene (Schroeder et al., 2001). We also found that in *O. piliferum* intraspecific variations occur in the ITS sequences rather than in the subunit sequences, and that the variations were related to the geographic origin of the *O. piliferum* strains. However, the rRNA gene was not suggested as a feasible target region for developing molecular probes for Cartapip™ because a large numbers of PCR amplification or DNA sequencing of the ITS regions failed (Schroeder et al., 2001).

The aim of the present work was to evaluate the potential of the β-tubulin gene for developing PCR-based molecular tools for the detection of Cartapip™. PCR-derived fragments of the β-tubulin gene were analyzed by restriction enzyme digestion and DNA sequencing, and β-tubulin gene-based PCR methods were developed to detect Cartapip™ in wood field trials in Germany.

Materials and methods

Fungal cultures and DNA preparation

Over 100 isolates of *O. piliferum* from Belgium, Canada, Germany, New Zealand, France, Sweden, the UK and the USA were studied, together with *O. floccosum* (5 isolates), *O. flexuosum* (2 isolates), *O. ips* (4 isolates), *O. minus* (4 isolates), *O. piceae* (4 isolates), *O. piceaperdum* (2 isolates), *O. setosum* (3 isolates), Species E (3 isolates, an *O. piliferum*-related species) (Uzunovic et al., 1999b), *Alternaria alternata*, *Hormonema dematioides* (2 isolates) and *Diplodia* sp. The species and isolates of Canadian origin were isolated from an extensive survey in Canadian softwood and maintained in the Culture Collection of the Wood Science Department at the University of British Columbia (Kim et al., 1999b; Uzunovic et al., 1999b). For DNA preparation, fungal isolates were inoculated on 2% malt extract agar (MEA, Oxoid) media overlaid with cellophane sheets and cultivated for 3 days at room temperature. DNA extraction was carried out by the methods described by Kim et al. (1999b).

Three methods were used to extract DNA from wood blocks or logs colonized by Cartapip™ and/or other fungi. About 3–5 cm^3 wood samples were ground in liquid nitrogen. In the first method, DNA was extracted from the ground materials using the DNeasy Plant Kit (Qiagen) according to the manufacturer's instructions. In the second method, DNA was extracted using a phenol/chloroform procedure (Sambrook et al., 1989). The ground materials (30–50 mg) were treated with 900 µl DNA-extraction buffer containing 1% (vol : vol) β-mercaptoethanol, incubated at 65 °C for an hour, and extracted with 0.5 volumes of Roti-Phenol/Chloroform (Roth). The extracts were treated with 3 M sodium-acetate solution (30 µl) and 0.5 volumes of isopropanol, incubated at −20 °C for 30 min and centrifuged at 13,000 rpm for 20 min. The resulting DNA-pellet was washed with 70% ethanol, air-dried and resolved in 50 µl TE-buffer. In the third method, DNA was extracted directly from the fungi present on wood surface. The wood block or log disks were incubated for 2 or 3 days in a humid chamber at 20 °C. Mycelia and/or spores developed on the surface of wood blocks were scraped with a sharp blade, and DNA was directly prepared from the scraped mycelia or spores using a microwave heating method (Kim et al., 1999b).

PCR amplification and RFLP

The primers T10 (5′-ACGATAGGTTCACCTCCA GAC-3′) and T222 (5′-GACCGGGGAAACGGAGA CAGG-3′) were used for the amplification of the partial β-tubulin gene (O'Donnell and Cigelnik, 1997). PCR amplification was carried out with a total volume of 25 µl in 0.6-ml reaction tubes and a Touchdown Thermocycler (Hybaid). The reaction

cocktail contained 1× reaction buffer (10 mM Tris–HCl (pH 8.0), 1.5 mM $MgCl_2$, 50 mM KCl), 80 μM of each deoxynucleotide, 20 pmol of each primer, 0.5 U Thermostable DNA polymerase (Rose Scientific) and 100 ng genomic DNA. The standard reaction conditions were as follows: initial denaturation at 94 °C for 4 min, 30 cycles of denaturation at 94 °C for 50 s, primer annealing at 58–60 °C for 50 s and DNA elongation at 72 °C for 50 s, and a final cycle of DNA elongation at 72 °C for 10 min. Aliquots of 5–10 μl of the PCR products were visualized by electrophoresis on a 1.4% agarose gel containing ethidium bromide. PCR reactions were repeated at least twice.

The amplified DNA fragments were digested with restriction enzymes without further purification. The PCR-products of the partial β-tubulin gene were cut with two different restriction enzymes, *Hin*fI and *Spe*I. Restriction enzymes were purchased from Pharmacia Biotech. Digestion reactions contained 15 μl of the PCR-products and 3–5 U of the enzymes. The concentration of reaction buffer and incubation temperature were controlled according to the enzyme suppliers' protocols. After incubation for 2–5 h, the digests were subjected to electrophoresis on 2.5% agarose gels (Eclipse Molecular Biologicals) in Tris–acetate–EDTA buffer with ethidium bromide (BioRad) at 60 V for 2 h. Restriction analysis was repeated at least twice. Banding patterns were visualized under UV light and documented using a digital camera and a Sigma Scan software (SPSS Scientific Inc.).

DNA sequencing and phylogenetic analysis

The PCR-amplified β-tubulin (1020 bp) gene products were gel-purified using Qiaquick Gel Extraction Kit (Qiagen), subcloned into pCR® 2.1-TOPO-vector using TOPO™ TA Cloning Kit (Invitrogen) and sequenced. Sequencing reactions were carried out using a PRIAM Ready Reaction DYEDEOXY Termination Cycle Sequencing Kit (Uzunovic et al., 2000). M13 forward and reverse primers were used as sequencing primers. The reactions were loaded onto an acrylamide gel and nucleotide sequences were analyzed on Applied Biosystem's ABI 373 DNA sequencer. All the nucleotide sequences were determined in both directions. The β-tubulin sequences of *O. piliferum* strains (AU122-1, Cartapip™, CBS129.32, 201/la, 2/97 and AU55-4) determined in this work have been deposited in the GenBank DNA sequence database under the accession numbers AF221626–AF221631. Nucleotide and amino acid sequences were aligned by the CLUSTAL W, version 1.8 (Thompson et al., 1994). Sequence relationships were analyzed from the alignment with the programs contained within PAUP*, version 4.0b6 (Swofford, 1998), and cladograms were constructed using PUZZLE program with 1000 puzzling steps.

Development of a set of Cartapip™-specific primers and PCR detection in wood

Cartapip™-specific primers, Cat1 and Cat2, were designed by targeting the β-tubulin gene. The partial β-tubulin sequences obtained from six *O. piliferum* strains from different geographic origin including Cartapip™ were aligned by using CLUSTAL W program (Figure 2). We chose possible target-regions with the same length (18 bases) and approximately the same GC-content for the forward and the reverse primers (see Figure 2).

For PCR detection in wood, fungal samples were collected from the wood blocks, lumber and logs that were artificially inoculated in British Columbia or from field trials in Germany. In Canada, lodgepole pine wood blocks ($0.5 \times 2 \times 5\ cm^3$) were sterilized by γ-radiation or autoclaving, inoculated with Cartapip™ alone or with a mixture of wild type *O. piliferum* strains and other species, and incubated at room temperature for 1–2 weeks in humid conditions. In logs,

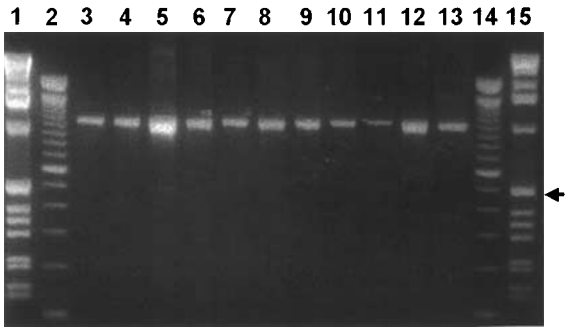

Figure 1. Representative gel electrophoresis of PCR products amplified with the primer pair T10 and T222 from geographically diverse *O. piliferum* strains. Lanes 1 and 15: 1-kb DNA marker (Life Technologies); Lanes 2 and 14, 100-bp ladder (Life Technologies); Lane 3: 2/97 (New Zealand); Lane 4: AU54-3 (British Columbia); Lane 5: AU191-5 (Alberta); Lane 6: AU102-4 (Saskatchewan); Lane 7: TAB 28 (USA); Lane 8: MNHN92.2860 (France); Lane 9: DSMZ4843 (Germany); Lane 10: CBS129.32 (assumed from Europe); Lane 11: 201/1a (UK); Lane 12: 192/1 (UK); Lane 13: Cartapip™ (USA). The arrow indicates 500-bp size.

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2/97          ACGATAGGTTCACCTCCAGACCGGTCAGTGCGTACGTATATCATCCCCAGCT---     52
AU55-4        ACGATAGGTTCACCTCCAGACCGGTCAGTGCGTACGTATATCATCCCCAGCT---     52
CBS129.32     ACGATAGGTTCACCTCCAGACCGGCCAGTGCGTACGTATATCATCCCCAGCC---     52
201/1a        ACGATAGGTTCACCTCCAGACCGGTCAGTGCGTACGTATATCATCCCCAGCC---     52
AU122-1       ACGATAGGTTCACCTCCAGACCGGTCAGTGCGTACGTATATCATCCCCAGCCAGC     55
CARTAPIP      ACGATAGGTTCACCTCCAGACCGGTCAGTGCGTACGTATATCATCCCCAGCCAGC     55
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                    T10

2/97          -CATCGCGAGACGACGGCGTTCTGCCAGATCTCGCTTGACTCTTTCCTCTTGCGA    106
AU55-4        -CATCGCGAGACGACGGCGTTCTGCCAGATCTCGCTTGACTCTTTACTCTTGCGA    106
CBS129.32     -CATGGCGAGACGACGGCGTTCTGCTAGATCTCGCTCGAGTCTTTACTCTTGCGA    106
201/1a        -CATGGCGAGACGACGGCGTTCTGCCAGATCTCGCTCGAGTCTTTACTCTTGCGA    106
AU122-1       TCTTGGCGAGACGACGGCGTTCAGCCAGATCTCGCTCGACTCTTTGCTCTTGCGA    110
CARTAPIP      TCTAGGCGAGACGACGGCGTTCAGCCAGATCTCGCTCGACTCTTTGCTCTTGCGA    110
               *   ***************** ** ********** ** ***** *********

                  Cat1

2/97          CGACGGTGCATTGTTGCTAACTCTGTCCCAGGGTAACCAAATCGGTGCCGCTTTC    161
AU55-4        CGACGGTTCATTGTTGCTAACTCTGTCCCAGGGTAACCAAATCGGTGCCGCTTTC    161
CBS129.32     CGACGGTTCATTGTTGCTAACACTGTCCCAGGGTAACCAAATCGGTGCCGCTTTC    161
201/1a        CGACGGTTCATTGTTGCTAACACTGTCCCAGGGTAACCAAATCGGTGCCGCTTTC    161
AU122-1       CGACGGTTCATTGTTGCTAACACTGTCCCAGGGTAACCAAATCGGTGCCGCTTTC    165
CARTAPIP      CGACGGTTCATTGTTGCTAACTCTGTCCCAGGGTAACCAAATCGGTGCCGCTTTC    165
              ******* ************* ***********************************

2/97          TGGTATGCTCAGCCCAACCCCTAGCCGCCTCCGACGGTCTTCGCCGCGGAATCAG    216
AU55-4        TGGTATGCTCAGCCCAACCCCTAGCCGCCTCCGACGGTCTTCGCCGCGGAATCAG    216
CBS129.32     TGGTATGCTCAGCCCAACCCCTAGCCACACCCGACGGTCTTCGCCGCGGAATCAG    216
201/1a        TGGTATGCTCTACCTAACCCCTAGCCACACCCGACGGTCTTTGCCGCGGAATCAG    216
AU122-1       TGGTATGCTCAGCCCAACCCCTAGCCACGTCCGACGGTCTTCGCCGCGAAACCAG    220
CARTAPIP      TGGTATGCTCAGCCCAACCCCTAGCCACCCCCGACGGTCTTCGCCGCGGAACCAG    220
              ********** ** *********** * ********** ****** ** ***

                                                          Cat2

2/97          CACGACGACCCGATCTCGATGCACGACTCTGTTCGACGACGACTAGGCTTGGTAC    271
AU55-4        CACGACGACCCGATCTCGATGCACGACTCTGTTCGACGACGACTAGGCTTGGTAC    271
CBS129.32     CACGACGACCCGATCTCGATGCACGACTCTGTTCGACGACGACTAGGCTTGGTAC    271
201/1a        CACGACGACCCGATCTCGGTGCACGACTCTGTTCGACGACGACTAGGCTTGGTAC    271
AU122-1       CACGACCGCCCGATCTCGATGCCCGACTCTGTTCGACGACTACTAGGCCTGGTAC    275
CARTAPIP      CACGACCGCCTGATCTCGATGCCCGACTCTGTTCGACGACGACTAGTCTTGGTAC    275
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2/97          TAACCTCATCTACAGGCAGCAGATTTCCGGCGGGCACGGCCTCGACAGCAATGGC    326
AU55-4        TAACCTCATCTACAGGCAGCAGATTTCCGGCGAGCACGGCCTCGACAGCAATGGC    326
CBS129.32     TAACCTCATCTACAGGCAGCAGATTTCCGGCGAGCACGGCCTCGACAGCAATGGC    326
201/1a        TAACCTCATCTACAGGCAGCAGATTTCCGGCGAGCACGGCCTCGACAGCAATGGC    326
AU122-1       TAACCTCATCTACAGGCAGCAGATTTCCGGCGAGCACGGCCTCGACAGCAATGGC    330
CARTAPIP      TAACCTCATCTACAGGCAGCAGATTTCCGGCGAGCACGGCCTCGACAGCAATGGC    330
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2/97          GTGTATGTATATCTGAATCTGATCCTCGACGTCCATGTCTGCATCGCTTTGGCAC    381
AU55-4        GTGTATGTATATCTGAATCTGATCCTCGACGTCCCTGTCTGCATCGCTTTGGCAC    381
CBS129.32     GTGTATGTATATC------TGATCCTCGACGTC------TGCATCGTTTT--CAC    367
201/1a        GTGTATGTATATC------TGATCCTCGACGTC------TGCATCGTTTT--CAC    367
AU122-1       GTGTATGTATATC------TATTCCTCGACGTC------TGCATCGCTTTGGCAC    373
CARTAPIP      GTGTATGTATATC------TATTCCTCGACGTC------TGCATCGCTTTGGCAC    373
              *************      *  ***********      ******* ***  ***

2/97          CCAGCTAACATCGCCCAGGTACAACGGTACCTCCGACCTCCAGCTGGAGCGCCTG    436
AU55-4        CCAGCTAACATCGCCCAGGTACAACGGTACCTCCGACCTCCAGCTGGAGCGCCTG    436
CBS129.32     CTAGCTAACATCGCCTAGGTACAACGGTACCTCCGACCTCCAGCTGGAGCGCCTG    422
201/1a        CTAGCTAACATCGCCTAGGTACAACGGTACCTCCGACCTCCAGCTGGAGCGCCTG    422
AU122-1       CCAGCTAACATCGCCCAGGTACAACGGTACCTCCGACCTCCAGCTGGAGCGCCTG    428
CARTAPIP      CCAGCTAACATCGCCCAGGTACAACGGTACCTCCGACCTCCAGCTGGAGCGCCTG    428
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holes (6 mm diameter × 5 mm depth) were made on the surface of lodgepole pine logs (20 cm diameter × 30 cm length) and inoculated with two wheat grains on which Cartapip™ was pre-grown. After 3 weeks of incubation at 20 °C, these infected logs were cross-sectioned into disks with 1 cm thickness. The presence of the fungi in each wood disk was examined by sampling from wood surfaces and culturing the sampled fungi on MEA plates. Samplings were randomly performed both right after sectioning and 2 days after incubation of the sectioned disks. In Germany, field trials with Cartapip™ were carried out using scots pine lumber and logs from a sawmill in Brandenburg in the summer of 2000. Four or six weeks after inoculation, wood samples were randomly taken from the Cartapip™-treated wood and used for fungal samplings. Parallel to the PCR detection with the Cat1/Cat2 primers, classical cultural methods were also used to detect Cartapip™. Template DNA preparation, PCR reaction and the analysis of the resulting PCR products were performed as described in previous sections. For PCR reaction, 58 °C was used as the annealing temperature.

Results and discussion

Test of universal β-tubulin gene primers against O. piliferum *strains and sequence analysis of the PCR products*

In order to determine the usefulness of the β-tubulin gene as a target, we first assessed the ability of the PCR primer pair T10–T222 to amplify the β-tubulin gene. This primer pair produced an approximately 1020-bp band from all the *O. piliferum* strains tested (Figure 1). To confirm the identity of the amplicons, we sequenced the PCR products from Cartapip™ and the five wild type *O. piliferum* strains (one from unknown origin and four from different geographic origins). The strains used were the same strains that were utilized in our ITS–rDNA sequence analysis (Schroeder et al., 2001). Through nucleotide database searches, we found that the amplicons have very high sequence homology (86–98%) with the sequence of known fungal β-tubulin genes. Our results confirmed that in *O. piliferum* the β-tubulin gene was easier to amplify by PCR than the ITS region.

The nucleotide sequence alignment of the β-tubulin amplicons from Cartapip™ and five representative *O. piliferum* strains showed that the partial β-tubulin gene sequences of the six *O. piliferum* strains were composed of four exons and four introns (Figure 2). A similar number of introns and exons are found in the T10–T222 primer-generated partial β-tubulin gene sequence of the rye glass choke pathogen, *Epichloe typhina* (Byrd et al., 1990). Comparison of the deduced β-tubulin protein sequences showed that the six *O. piliferum* strains shared between 99% to 100% sequence identity. Table 1 shows the deduced protein sequence identity of β-tubulin of *O. piliferum* and other fungi. Obviously, *O. piliferum* has higher sequence identity with the ascomycetes (91–97%) than with basidiomycetes (84–87%). Phylogenetic relationships among the ascomycetes in Table 1 revealed that

Table 1. Protein sequence identity of β-tubulin between *O. piliferum* and some fungi belonging to Ascomycota and Basidiomycota. Sequences were analyzed based on 240 deduced amino acid sequences from exons encoding β-tubulin, using PALIGN program in the PC/Gene sequence analysis software (IntelliGenetics)

Species	GenBank accession No.	Identity (%)
Ascomycota		
Aspergillus nidulans	AAA33328	94
Erysiphe pisi	AAA34230	95
Rhynchosporium secalis	CAA56936	97
Botryotinia fuckeliana	CAA93254	96
Erysiphe pisi	CAA57491	95
Blumeria graminis	CAA35709	96
Pestalotiopsis microspora	AF22514	97
Epichloe typhina	S14121	96
Colletotrichum gloeosporioides f.sp. *aeschynomene*	AAA62875	97
Penicillium digitatum	BAA11229	92
Gibberella fujikuroi	AAB18275	95
Trichoderma viride	CAA78765	91
Pneumocystis carinii	AAA33786	91
Basidiomycota		
Melampsora lini	AAG33239	87
Pleurotus sajor-caju	AAD21093	84
Schizophyllum commune	CAA44972	84
Coprinus cinereus	BAA19057	84

Figure 2. Nucleotide sequence alignment of the partial β-tubulin gene of Cartapip™ and five *O. piliferum* strains from different geographic origin. Intron sequences are in italic. The Cartapip-specific primer (Cat1: forward, Cat2: reverse) and universal β-tubulin gene primer (T10: forward) sites are underlined. Mismatches with the target sites of the Cat1 and Cat2 are marked in boldface. Perfectly conserved nucleotide positions and missing nucleotides in the alignment are shown with characters * and −, respectively.

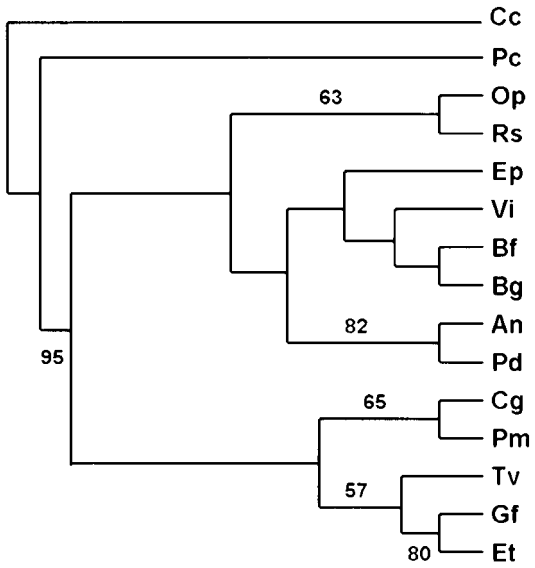

Figure 3. Quartet-puzzling tree based on 240 amino acid sequences of the β-tubulin from ascomycete and basidiomycete. The tree length is 96. 1000 puzzling steps were used. Puzzling support values (percentage) are presented at the node. Puzzling support values of less than 50% are not shown. The β-tubulin sequence of *Coprinus cinereus* (Cc) was used as outgroup. Number of quartet to examine is 1365. Number of parsimony-informative characters is 14. All characters have equal weight. Pc: *Pneumocystis carinii*; Op: *Ophiostoma piliferum*; Rs: *Rhynchosporium secalis*; Ep: *Erysiphe pisi*; Vi: *Erysiphe pisi*; Bf: *Botryotinia fuckeliana*; Bg: *Blumeria graminis*; An: *Aspergillus nidulans*: Pd: *Penicillium digitatum*; Cg: *Colletotrichum gloeosporioides* f.sp. *aeschynomene*; Pm: *Pestalotiopsis microspora*; TV: *Trichoderma viride*; Gf: *Gibberella fujikuroi*; Et: *Epichloe typhina*.

O. piliferum closely grouped with *Rhynchosporium secalis* (Figure 3).

Since no significant variations were found in the exon sequences of Cartapip™ and the five *O. piliferum* strains, the combined sequences of the exons and the introns were analyzed for intraspecifc relationships. The absence of a conserved mark signal in the nucleotide positions in the aligned sequences in Figure 2 indicates that intraspecific variation exists in the β-tubulin gene of *O. piliferum*. As expected, sequence variation was commonly higher in introns than exons. Additional intraspecific variation in the nucleotide sequences of the six *O. piliferum* strains was also observed in the length of intron. The phylogenetic tree based on the β-tubulin sequences also clearly shows that intraspecific variation occurs among the six strains (Figure 4). Cartapip™ was closely

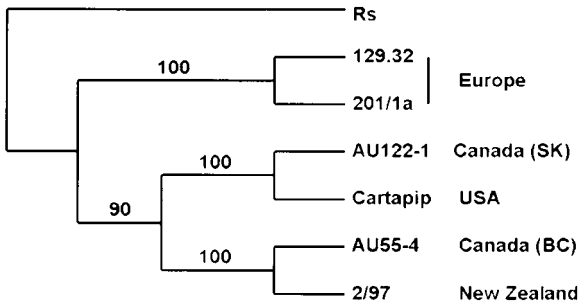

Figure 4. Intraspecific relationships among *O. piliferum* strains from diverse geographic origins. Quartet-puzzling tree was generated by Puzzle analyses using the nucleotide sequences of the β-tubulin gene. The tree length is 351. 1000 puzzling steps were used and puzzling support values (percentage) are presented at the node. Puzzling support values of less than 50% are not shown. The nucleotide sequence of the β-tubulin gene of *Rhynchosporium secalis* (Rs) was used as outgroup. Number of quartet to examine is 35. Number of parsimony-informative characters is 30. All characters have equal weight.

grouped with the Saskatchewan strain AU122-1, and this group was clearly separated from four other strains from Europe, New Zealand and western Canada. The European strain 201/1a was closely grouped with strain CBS129.32 (which has an unknown origin but is assumed to be from Europe), and this group was also separated from the sister grouped strains of New Zealand (2/97) and British Columbia (AU55-4). In the six strains, the relatedness shown in the β-tubulin gene was similar to that shown in the ITS–rDNA sequences (Shroeder et al., 2001). Overall, these results suggest that the β-tubulin gene could be used to develop a useful marker for differentiating Cartapip™ from the wild type *O. piliferum* strains in Germany.

Design and testing of the β–tubulin gene sequence-based Cartapip™-specific RFLP markers and PCR primers

PCR–RFLP analyses on the β-tubulin gene fragment derived by PCR using the T10–T222 primers were performed on all the strains used in this work. For these analyses, we identified with PC/Gene software *Hin*fI and *Spe*I as potential cutters for generating geographically variable patterns in the β-tubulin gene of the six strains sequenced. The RFLP patterns by *Hin*fI generated two main groups (Figure 5A). One group included the strains from Europe, Saskatchewan and USA, and the other group included the strains from British

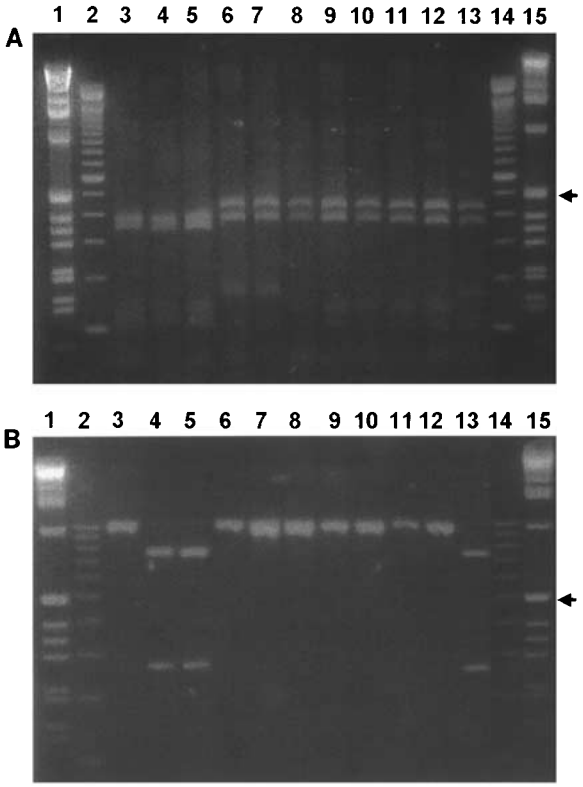

Figure 5. Representative restriction digestion patterns of the partial β-tubulin gene amplified with the primer pair T10 and T222 from geographically diverse *O. piliferum* strains. The arrow indicates 500-bp size. (A) RFLP with *Hin*fI; Lanes 1 and 15: 1-kb DNA marker (Life Technologies); Lanes 2 and 14, 100-bp ladder (Life Technologies); Lane 3: New Zealand; Lane 4: British Columbia; Lane 5: Alberta; Lane 6: Saskatchewan; Lane 7: USA; Lane 8: France; Lane 9: Germany; Lane 10: CBS129.32; Lanes 11 and 12: UK; Lane 13: Cartapip™ (B) RFLP with *Spe*I; Lanes 1 and 15: 1-kb DNA marker (Life Technologies); Lanes 2 and 14, 100-bp ladder (Life Technologies); Lane 3: 2/97 (New Zealand); Lane 4: AU102-4 (Saskatchewan); Lane 5: TAB 28 (USA); Lane 6: U54-3 (British Columbia); Lane 7: AU191-5 (Alberta); Lane 8: MNHN92.2860 (France); Lane 9: DSMZ4843 (Germany); Lane 10: CBS129.32; Lane 11: 201/1a (UK); Lane 12: 192/1 (UK); Lane 13: Cartapip™ (USA).

Columbia, Alberta and New Zealand. Cartapip™ was grouped with the strains from USA, Saskatchewan and Europe. This grouping suggests that the β-tubulin gene could be used for differentiating Cartapip™ from the wild type *O. piliferum* in New Zealand and in two Canadian provinces, Alberta and British Columbia. Digestion with *Spe*I permitted further differentiation of Cartapip™ from all the strains from Europe as

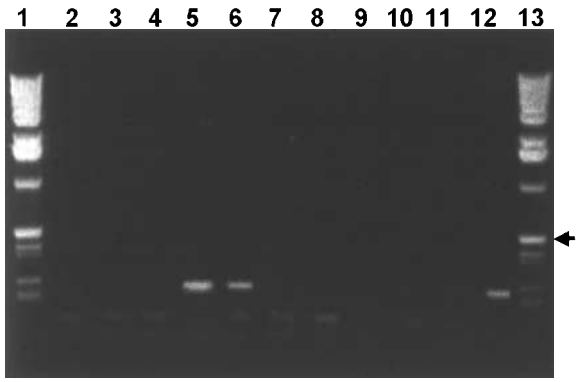

Figure 6. Representative gel electrophoresis of PCR products amplified with the primer pair Cat1 and Cat2 from geographically diverse *O. piliferum* strains. Lanes 1 and 13: 1-kb DNA marker (Life Technologies); Lane 2: New Zealand; Lane 3: British Columbia; Lane 4: Alberta; Lane 5: Saskatchewan; Lane 6: USA; Lane 7: France; Lane 8: Germany; Lane 9: CBS129.32; Lanes 10 and 11: UK; Lane 12: Cartapip™. The arrow indicates 500-bp size.

well as strains from New Zealand, British Columbia and Alberta, and most strains from Saskatchewan and USA (Figure 5B). The RFLP analyses of the β-tubulin gene with the two restriction enzymes suggested that the intraspecifc variation in *O. piliferum* is related to the geographic origin of the strains. Then, we assessed the specificity of the *Spe*I-generated RFLP pattern of the β-tubulin gene against other sapstainers such as *O. floccosum*, *O. minus*, *O. piceae*, *O. setosum*, *H. dematioides*, *Diplodia* sp., and *A. alternata*, which were frequently isolated from wood. Since no cutting patterns similar to those of Cartapip™ were observed with all the tested fungi (data not shown), it appears that the PCR–RFLP of the β-tubulin gene using *Hin*fI and *Spe*I could also be used as a molecular marker for Cartapip™ against other sapstain species.

To simplify the detection of Cartapip™ by PCR, we designed a Cartapip™-specific primer pair, Cat1–Cat2, based on the alignments of part of the six β-tubulin gene sequences (Figure 2). Then, we determined whether this primer pair could amplify an expected DNA fragment of 180 bp from the genomic DNAs of all the *O. piliferum* strains used. A PCR-product of 180 bp was produced with the strains from Quebec, Saskatchewan and USA including Cartapip™ but not with the strains from Europe, New Zealand, British Columbia and Alberta (Figure 6). When the Cat1–Cat2 primer pair was used in PCR reactions with the genomic DNAs of *O. floccosum*, *O. minus*, *O. piceae*,

O. ips, *O. piceaperdum*, *O. setosum*, *H. dematioides*, *Diplodia* sp., and *A. alternata*, no band of 180 bp was produced. This confirmed that the Cat1–Cat2 primer pair was specific for Cartapip™ and strains from the USA, Saskatchewan and Quebec. Consequently, the Cat1–Cat2 primer pair could be used to detect Cartapip™ in Germany, as well as in other European countries, New Zealand, Alberta and British Columbia.

Detection of Cartapip™ on wood by PCR and PCR–RFLP methods

To test the relevance of the developed molecular methods for detecting Cartapip™ present on wood, we first analyzed wood blocks and logs inoculated with Cartapip™ alone or with a mixture of fungi including an European *O. piliferum* strain and other sapstain species such as *O. setosum*, *O. piceae*, *Diplodia* sp., *Leptographium* sp. Using the microwave method (Kim et al., 1999b) and the Cat1–Cat2 primer pair, we were able to amplify the 180-bp band (Figure 6) directly from the fungal mycelia or spores present on the surface of the wood (lumber or log disks). As long as fungal mycelia were present on the wood surface, Cartapip™ detection by PCR was reproducible and feasible within 4–6 h, while 6–8 h were necessary to carry out the detection by PCR–RFLP of the β-tubulin gene with *Spe*I. These results showed that both the designed β-tubulin gene-derived primer pair and the *Spe*I-generated RFLP pattern of the β-tubulin gene could be used as molecular markers for the detection of Cartapip™ in mixed fungal communities on wood.

We extended our Cartapip™-detecting tests to samples collected from field trials with the biocontrol agent. Sixteen wood samples were obtained from lumber and logs treated with Cartapip™ in the summer of 2000 in Brandenburg, Germany. We were able to amplify the expected 180-bp band from the wood surface but not from internal wood samples (data not shown). Despite increasing the number of PCR cycles up to 45, the 180-bp band was still not produced from internal wood samples. Since we confirmed the quality of the template DNA through successful amplification of the 26S fungal rDNA using the rDNA primers NL1-LR5 (Vilgalys and Hester, 1990; O'Donnell, 1992), the failure to detect Cartapip™ from internal wood samples is likely due to low amount of the target β-tubulin DNA. The presence of insufficient level of target DNA in the template DNA could be explained by the fact that the rDNA is present in multi-copy genes while the β-tubulin is considered to be a single-copy gene with exceptions in a few fungal species (Thon and Royse, 1999).

However, when the wood samples (blocks or log disks) were incubated under humid conditions for 1–3 days, the Cartapip™ mycelia present inside the sample grew on the surface of the wood. The mycelia accumulated were enough to prepare sufficient amounts of template DNA for Cartapip™ detection by PCR using the Cat1–Cat2 primers. In field conditions, the intensity of fungal colonization in lumber and log varies greatly, and only rarely mycelia or spores are found in high amounts on wood surface. Since we are mainly concerned by the detection of the biocontrol agent alive, incubating the treated wood in a humid condition at room temperature might be the appropriate additional step to monitor Cartapip™ more easily using PCR techniques with Cat1–Cat2 primers and RFLP analysis.

In conclusion, we have demonstrated that the β-tubluin gene can be used as a target to develop molecular methods to detect Cartapip™. The β-tubulin gene-based PCR methods were successfully used in Germany and could also be used in other European countries, New Zealand, Alberta and British Columbia where field tests with Cartapip™ have been performed and/or are underway.

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