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Full Length Research Paper

Development of PCR method for fast detection of *Ophiostoma floccosum* in wood chips

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Ophiostoma floccosum **is one of blue stain fungi that cause cosmetic damage in softwood. To develop a molecular maker for the detection of** ***O. floccosum*****, we designed Oflo1 and Oflo2 primers based on the β-tubulin gene. PCR assay with eleven isolates of** ***O. floccosum*** **and nine other blue stain and discoloring fungal species (moulds) proved that the designed primers specifically amplified a 333 bp sized DNA band only from genomic DNA of** ***O. floccosum*** **isolates. Together with microwave heating extraction of spore DNA, we could detect** ***O. floccosum*** **within 2 h on artificially inoculated wood chip. We believed that the developed PCR method will be useful where detection of target species such as** ***O. floccosum*** **is required.**

Key words: *Ophiostoma floccosum*, Species-specific primer, β-tubulin gene.

INTRODUCTION

Ophiostoma is a genus of fungi within the Ascomycota. This genus contains numerous species some of which are associated with bark beetles. Several *Ophiostoma* species are known as plant pathogens (for example, *Ophiostoma ulmi*, *O. novo-ulmi*), while many can cause blue stain on living trees, logs and freshly cut or dried and rewetted wood (for example, *O. piceae*, *O. floccosum*, *O. piliferum*, *O setosum*). Blue stain, a cosmetic defect in wood that results in significant economical loss to forest products industries, is caused by growth of melanized hyphae or sporing structures inside or on the wood. The *Ophiostoma piceae* complex includes nine species based on morphology, culture characteristics, mating compatibility and sequence of internal transcribed spacer regions of the rDNA (de Beer et al., 2003). In the complex, *O. piceae*, *O. canum*, *O. floccosum* and *O. setosum* have been reported as coniferous group (Harrington et al., 2001), while *O. quercus*, *O. catonianum*, and the Dutch elm disease fungi: *O. ulmi*, *O. novo-ulmi*, and *O. himal-ulmi* as hardwood group. They are known to be a polyphyletic group of morphologically similar fungi. For this reason, delineation and identification of *Ophiostoma* species requires fine mycology skills and modern techniques.

Molecular approach has been used for the differentiation of *Ophiostoma* species by PCR and Restriction Fragment Length Polymorphism (RFLP) analysis of the 18S rRNA gene (Kim et al., 1999a), the species-specific detection of *O. piceae* and *O. quercus* by PCR using the internal transcribed spacer (ITS) DNA sequence-derived primers (Kim et al., 1999b), and the differentiation of *O. piliferum* from other blue stain *Ophiostoma* species by PCR and RFLP (Schroeder et al., 2001).

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Table 1. Blue stain fungal isolates and other fungal species used in this study.

Species	Isolate no. [a]	Host	Origin/source
Ophiostoma floccosum	DUCC0514	Japanese black pine	Korea
	DS1/3B-2	Unknown	Canada/AU
	KUC2032	Radiata pine	New Zealand
	KUC2319	Japanese red pine	Korea
	KUC2114	Nut pine	Korea
	KUC2420	Nut pine	Korea
	KUC2317	Japanese red pine	Korea
	KUC2759	Radiata pine	New Zealand
	KUC2417	Japanese red pine	Korea
	KUC2415	Japanese red pine	Korea
	KUC2419	Japanese red pine	Korea
O. piceae	H2009	Scotch pine	United Kingdom/JW
O. quercus	H1042	Oak	United Kingdom/JW
O. canum	CBS118668	Pine shoot beetle	Austria
O. ips	AU123-456	Jack pine	Canada
O. setosum	AU160-25	Unknown	Canada
O. minus	AU123-151	Jack pine	Canada
Leptographium terebrantis	CBS118620	Japanese black pine	United states
Penicillium sp.	DUCC408	Japanese black pine	Korea
Trichoderma sp.	DUCC409	Japanese red pine	Korea

[a]DUCC, Dankook University Culture Collection, Korea; CBS-KNAW, Centraalbureau voor Schimmelcultures - an Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW), The Netherlands; KUC, Korea University Culture Collection, Korea; AU, Dr. Adnan Uzunovic's personal collection, FP Innovations - Forintek Division, Canada. JW, Dr. Joan Weber, Centre for Forestry and Climate Change, Forest Commission, UK.

Currently, there has been no known molecular marker for *O. floccosum*, one of common blue stain species reported worldwide including Korea (Yun et al., 2009). In this study, we report a PCR-based molecular marker for differentiating *O. floccosum* from other blue stain fungi. The specificity of the developed molecular maker was demonstrated through specificity test on DNA samples and fungal samples on wood chips.

MATERIALS AND METHODS

Cultures of the *Ophiostoma* species and other fungi used for this study are shown in Table 1. The identification of all the obtained fungal isolates was verified before being used for the present study based on morphological characters and molecular data based on the nucleotides sequence of the β-tubulin gene. They were maintained in a deep freezer at -70°C until used. For genomic DNA extraction, all the fungi were grown on cellophane-layered 2% malt extract or potato dextrose agar for 5 days at 20 or 25°C. Mycelial harvest, DNA extraction using the drilling method, and PCR were performed as described by Kim et al. (1999b). For the design of species-specific primers, the β-tubulin gene was amplified by PCR with primers T10 (O'Donnell and Cigelnik, 1997) and BT12 (Schroeder et al., 2001) using Techne TC 3000 Thermal Cycler. Amplification of the β-tubulin gene was performed as follows: 95°C for 3 min, followed by 30 cycles consisting of 95°C for 30 s, 56°C for 30 sec and 72°C for 30 s, and one final cycle of extension at 72°C for 5 min. *Taq* DNA polymerase was purchased from Roche Diagnostics Korea Co. Ltd. (Seoul, Korea). The PCR amplicons were sequenced at Macrogen INC, Seoul, Korea. The determined nucleotide sequences was compared through Blast-N on GenBank database and aligned with those of other *Ophiostoma* species using Clustal X v1.8 program (Thompson et al., 1997) and used for primer development. The specificity of the developed PCR primers was tested by PCR against genomic DNAs from the target species and other fungal species which were known to occur on wood as sapstainers (*Ophiostoma* spp. and *Leptographium* sp.) or common mould species (*Penicillium* sp. and *Trichoderma* sp.).

For detection in wood samples, we used a method described in Kim et al. (1999b). *O. floccosum* spores were inoculated on a wood chip and incubated at 20°C

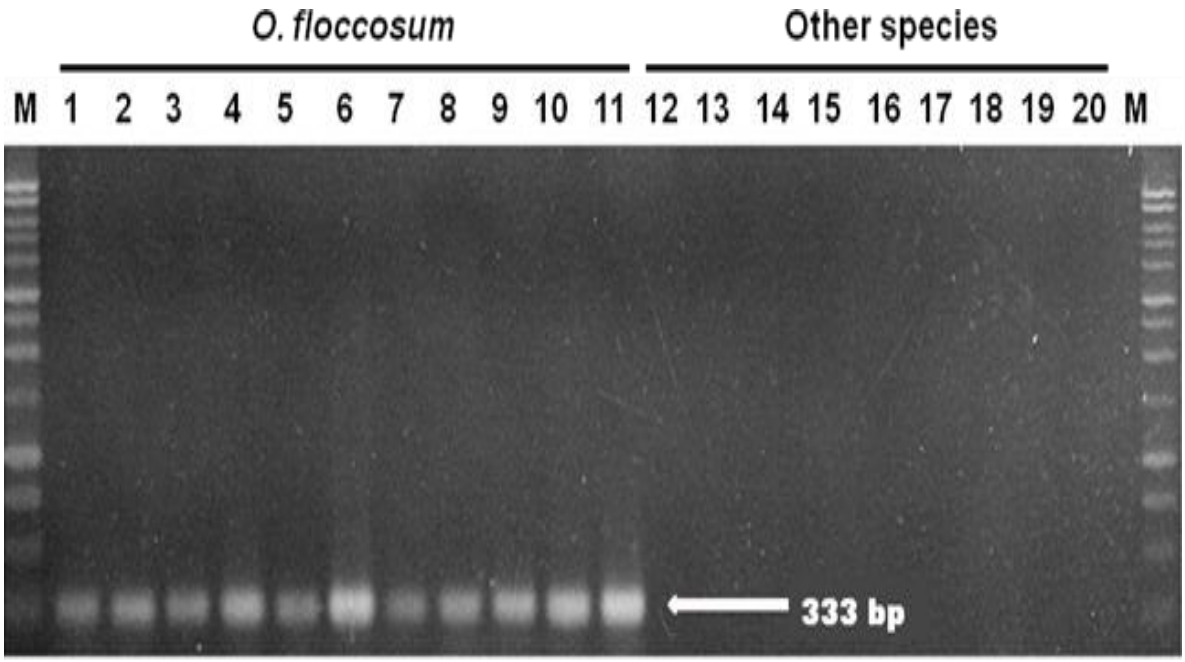

Figure 1. Gel electrophoresis of PCR products from *O. floccosum* with the primer pairs Oflo1-Oflo2. Lane M: 1 kb DNA ladder marker (Promega). Lanes 1-20; 1: DS1/3B-2, 2: OS-4/1-A-1, 3: KUC2072, 4: KUC2219D, 5: KUC2206, 6: KUC2420A, 7: KUC2207C, 8: KUC2225A, 9: KUC2217D, 10: KUC2215B, 11: KUC2719, 12: H2009, 13: H1042, 14: CBS118668, 15: AU123-456, 16: AU160-25, 17: AU123-151, 18: CBS118620, 19: DUCC408, 20: DUCC409.

for 2 weeks. After incubation, the spore masses at the tip of synnemata were randomly harvested and genomic DNA was extracted by a microwave heating method (Kim et al., 1999b). With the extracted genomic DNA, the specificity of the developed primers was evaluated.

RESULTS AND DISCUSSION

Nucleotide sequence sites which are specific for *O. floccosum* were searched and selected from the Clustal_X program-aligned nucleotide sequences of the β-tubulin gene obtained from this study and those from *Ophiostoma* species registered in GenBank DNA database. Based on the selected sites of nucleotide sequences, two primers were designed and named as Oflo1 (forward primer 5'-CCC CTC CTC CAA ATT TAA GAG A-3') and Oflo2 (reverse primer 5'-TAG TTT CGT ATA TCA AAA CGC GTG-3'), respectively. Oflo1 targeted 20 to 41 bp position and Oflo2 targeted 329 to 352 bp position in the β-tubulin gene sequence of *O. floccosum* 0514 (GenBank accession number JQ925340). These primers amplified only a 333 bp sized DNA band of the target gene from the genomic DNA of *O. floccosum* (Figure 1). Specificity of the Oflo1 to Ofl2 primers was tested by PCR against the prepared genomic DNAs from several *Ophiostoma* spp. and common mould species listed in Table 1. The primers did amplify the 333 bp-sized target DNA band only from eleven *O. floccosum* isolates and did not amplify other test species (*O. piceae*, *O. quercus*, *O. ips*, *O. canum*, *O. setosum*, *O. minus*), *L. terebrantis, Trichoderma* sp. and *Penicillium* sp. These results show that the Oflo1-Ofl2 primers are specific for *O. floccosum.* The developed Oflo1 to Ofl2 primers also successfully detected *O. floccosum* grown on wood chip (Figure 2). Since we used microwave heating for DNA extraction (Kim et al., 1999b), PCR detection of *O. floccosum* using the designed primers was feasible within 2 h, even with DNA obtained from a single synnema. The detection was possible with 10 pg of DNA *O. floccosum* 0514. The species belonging to the *O. piceae* complex has very similar morphology. Thus, morphology-based identification of *O. floccosum* is not easy and time-consuming process. Therefore, our PCR-based method will improve its diagnostics and save time.

Conclusion

Overall, PCR with designed markers in this study is rapid, specifiable and easy method for *O. floccosum* detection. The markers are expected to be applied where detection and identification of this species is needed.

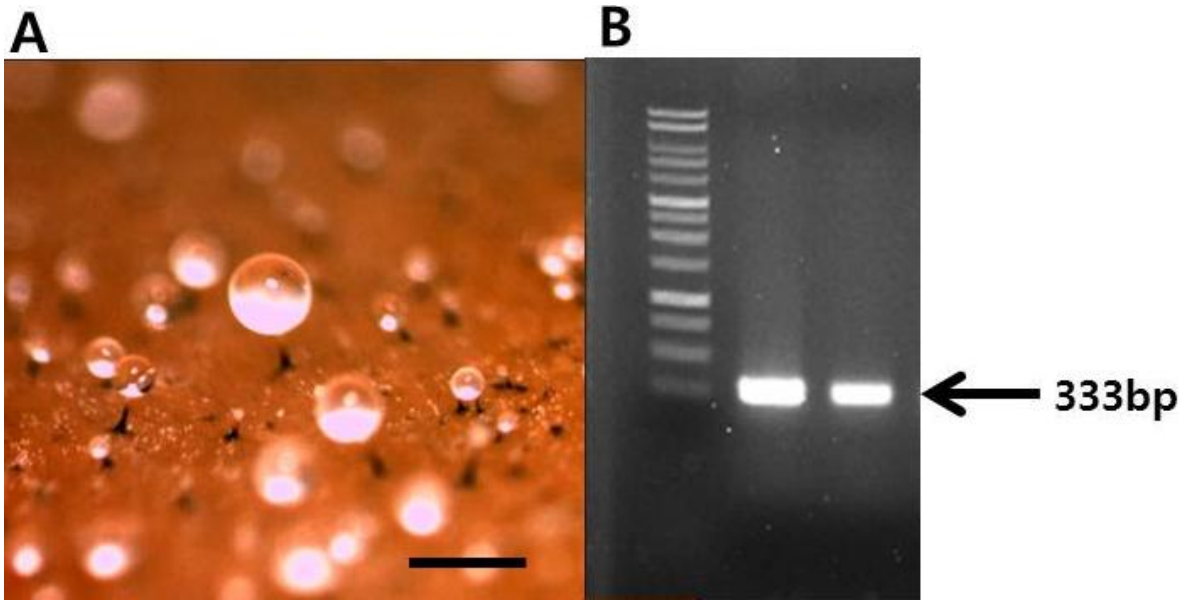

Figure 2. Evaluation of rapid detection of *O. floccosum* by PCR in artificially inoculated wood samples. A: Synnemata structure of *O. floccosum* formed on wood chip, B: Gel electrophoresis of PCR amplicons from conidial masses formed on the top of *O. floccosum* synnemata using the Oflo1-Oflo2 primers. Lane M: 1Kb DNA ladder marker (Promega), lane 1: genomic DNA of *O. floccosum*, lane 2: conidial masses of *O. floccosum* from artificially inoculated wood chip. Scale bar = 2mm.

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