J Wood Sci (2005) 51:83–88
DOI 10.1007/s10086-004-0617-3

NOTE

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Specific detection of a basidiomycete, *Phlebia brevispora* associated with butt rot of *Chamaecyparis obtusa*, by PCR-based analysis

Received: June 20, 2003 / Accepted: January 5, 2004

Abstract In order to monitor the basidiomycetous fungus *Phlebia brevispora* isolated from butt rot of *Chamaecyparis obtusa* (Japanese cypress) in 1997 in Nagasaki Prefecture, a sensitive polymerase chain reaction (PCR)-based assay was developed to specifically detect the fungus on-site. A species-specific primer for *P. brevispora* was derived from the internal transcribed spacer (ITS) region (containing 5.8S ribosomal DNA, ITS1 and ITS2) sequences of the fungus. The PCR assay was able to detect down to 1 fg DNA (per 1 μl PCR reaction mixture) and down to 0.2 mg mycelium of *P. brevispora* (per 1 g of decayed wood). The samples for on-site monitoring were collected in 2002 from the decayed tree stump in which *P. brevispora* had first been isolated. From the decayed tree tissue, *P. brevispora* could be detected by PCR assay even when its mycelium could not isolated from the tree tissue by culturing. This indicates that the PCR amplification using the specific primer developed here is a useful method for monitoring *P. brevispora* on-site.

Key words Japanese cypress (Hinoki) · On-site monitoring · PCR · White-rot basidiomycete

Introduction

Chamaecyparis obtusa (Sieb. & Zucc.) Endl., Japanese cypress, is one of the most economically and ecologically important tree species in Japan. Recently, butt rot damage of *C. obtusa* has increased in the Kyushu district.[1,2] Kubayashi et al.[3] recognized that there were several types of butt rot and several isolates with different cultural characteristics were obtained from the decay of *C. obtusa* in Nagasaki Prefecture. They also demonstrated that *Tinctoporellus epimiltinus* (Berk. & Broome) Ryvarden is one of the causal agents of butt rot. Kubayashi and Maekawa[4] classified the fungal isolates from the decayed tissues into several groups based on the macroscopic and microscopic characteristics of their cultural mycelia. They identified two of these groups as *T. epimiltinus* and *Phlebia chrysocreas* (Berk. & M.A. Curtis) Burds., and demonstrated that the latter is also a causal agent of butt rot of *C. obtusa*. Later, one of the remaining groups was identified as *Phlebia brevispora* Nakasone.[5] In 1997, two isolates of *P. brevispora* were obtained from decayed tissues of *C. obtusa* with butt rot in Nagasaki Prefecture. Since then, however, *P. brevispora* has not been reported from Japan, and the relationship of its pathogenicity to butt rot of *C. obtusa* and its ecological features are unknown.

Detection and monitoring of the target fungus, especially on-site, are important to check the infection of pathogenic fungi to the living tree or to study the fungal ecological features. An alternative approach using molecular techniques could offer several advantages by providing more specific and sensitive detection of the target fungi. To detect target fungi more specifically and sensitively, different methodologies have been used to identify DNA sequences that can function efficiently as probes or primers for target fungal species; these methodologies included random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR),[6,7] and the sequencing of DNA regions.[8–11] The development of PCR and taxon-specific primers[12,13] is making it increasingly feasible to detect fungal species in natural substrates. The technique has been used widely in medical and forensic work and allows very small amounts of DNA to be detected and identified.

Specific PCR primers have been developed to monitor target microorganisms, including the internal transcribed spacer (ITS) for *Biscogniauxia mediterranea* (De Not.)

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Table 1. Organisms and DNA Data Bank of Japan (DDBJ) accession number of the strains examined in this study

Fungi	Strain no.	DDBJ accession no.
Ceriporia lacerata[a]	MZ-340 (SFFPS)	AB091675
Ceriporia purpurea[a]	KKN-223-Sp (USDA)	–
Ceriporia spissa[a]	62024 (ATCC)	–
Ceriporia viridans	–	AF347109
Ceriporiopsis aneirina[a]	FP-100665-Sp (USDA)	–
Hericium erinaceus[a]	SFFPSC02 (SFFPS)	AB084622
Phanerochaete sordida[a]	YK-624 (SFFPS) 90872 (ATCC)	–
Phlebia brevispora[a]	HHB-7024-Sp (USDA)	AB084616
Phlebia brevispora[a]	TMIC33929 (TMI)	AB084614
Phlebia brevispora[a]	TMIC34596 (TMI)	AB084615
Phlebia chrysocreas[a]	TMIC31891 (TMI)	AB084617
Phlebia livida[a]	HHB-4609-Sp (USDA)	AB084618
Phlebia radiata[a]	HHB-5324-Sp (USDA)	AB084619
Phlebia subserialis[a]	HHB-9678-Sp (USDA)	AB084620
Phlebia tremellosa[a]	BMC-9152 (SFFPS)	–
Phlebia uda[a]	Kropp-1 (USDA)	AB084621
Trametes hirsuta[a]	YK-505 (SFFPS)	–
Xylobolus spectabilis[a]	7650 (IFO)	–

SFFPS, Laboratory of Systematic Forest and Forest Products Science; USDA, United States Department of Agriculture; ATCC, American Type Culture Collection; TMI, the Tottori Mycological Institute; IFO, Institute for Fermentation, Osaka
[a] Used as control to check primer specificity (see Experimental)

Kuntze in natural oak tissues[14] and for ectomycorrhizal fungi in *Eucalyptus* L'Her.[15] Schmidt and Moreth[16] developed species-specific primers based on the sequence of ITS2 for indoor rot fungi. The species-specific primers based on the sequence of ITS1 and ITS2 have been developed for a white-rot fungus, *Ceriporia lacerata* N. Maek., Suhara & R. Kondo, which was applied to bioremediation of polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in fly ash.[17] The ITS region is the most popular target region for phylogenetic analysis and taxonomic identification. This region is amenable to PCR because of the high copy number and the conserved flanking regions and discriminates well between taxa because of their variability.

The aim of the present study was to design PCR primers in the ITS region for the specific amplification of *P. brevispora* and to evaluate the primers for detection and identification of this fungus on-site.

Experimental

Fungal strains

Two strains of *Phlebia brevispora* (TMIC 33929 & 34596) described in a previous study[5] were used in this study. A third *P. brevispora* strain HHB-7024-Sp was obtained from the Center for Forest Mycology Research, Forest Products Laboratory, United States Department of Agriculture (USDA) Forest Service. The strain was isolated from *Pinus elliottii* Engelm. on 9 August 1972 at Long Pine Key, Everglades National Park, Florida, USA. Eleven fungal species were selected as closely related species (*Ceriporia lacerata, Ceriporia purpurea* (Fr.) Donk, *Ceriporia spissa* (Schwein. ex Fr.) Rajchenb., *Ceriporiopsis aneirina* (Sommerfeld) Domanski, *Phlebia chrysocreas*, *Phlebia livida* (Pers.) Bres., *Phlebia radiata* Fr., *Phlebia subserialis* (Bourdot & Galzin) Donk, *Phlebia tremellosa* (Schrad.) Nakasone & Burds., *Phlebia uda* (Fr.) Nakasone, and *Phanerochaete sordida* (P. Karst.) J. Erikss. & Ryvarden) and three fungal species (*Xylobolus spectabilis* (Klotzsch) Boidin, *Trametes hirsuta* (Wulfen) Pilát, and *Hericium erinaceus* (Bull.) Pers.) were selected as species distantly related to *P. brevispora* according to phylogenetic analysis.[5] These strains were obtained from the American Type Culture Collection (ATCC), USDA Forest Service, Institute for Fermentation, Osaka (IFO), the Tottori Mycological Institute (TMI), and the Laboratory of Systematic Forest and Forest Products Science, Kyushu University (SFFPS). Fungal strains used in this study are listed in Table 1.

Cultural condition

Fungal cultures were maintained at 4°C and subcultured at 30°C on a 1.5% (w/v) malt extract (Difco, Detroit, MI, USA) and 1.5% (w/v) agar (Wako, Osaka, Japan) medium (MEA). To prepare mycelium for DNA isolation, fungal mycelium was cultured in potato dextrose broth (PDB, Difco) medium at 30°C for 2 weeks, harvested, washed with distilled water, and freeze-dried.

Phlebia tremellosa BMC-9152 was used as competitive fungus for specific detection of *P. brevispora*. *Phlebia tremellosa* was inoculated onto sterilized wood chip media (WCM), which is composed of wood chips of *Castanopsis cuspidate* Schottky *var. sieboldii*, rice bran, and water [wood chips : rice bran = 8 : 2 (w/w; dry weight); moisture content

60%] in 3.5-l high-density polyethylene culture bags capped with Tyvek film (Dupont, Wilmington, DE, USA). Cultures were incubated at 22°C in the dark until the whole surface of the medium was covered with mycelium.

DNA extraction

DNA extraction from cultural mycelium was performed by a method described previously.[5]

DNA extraction from decayed wood and WCM culture was performed as follows. Five grams (fresh weight) or 2 g (dry weight: equal to 5 g of fresh weight) of sample was placed with liquid nitrogen in a mortar and ground with a pestle into a fine powder. The powder was transferred to another mortar and ground in 20 ml of CTAB2 buffer [2% (w/v) cetyltrimethylammonium bromide (CTAB, Wako); 1.4 M NaCl; 0.1 M Tris-HCl; 0.1% (v/v) β-mercaptoethanol; 20 mM ethylenediaminetetraacetic acid (EDTA, Wako); 2% (w/v) polyvinylpyrrolidone (PVP, Wako): pH 9.0]. The mixture was incubated at 65°C for 30 min with occasional inversion. The lysate was extracted once with the same volume of 0.1 M Tris-HCl (pH 8.0) saturated phenol/chloroform [1:1 (v/v)]. The mixture was centrifuged at 5000 *g* for 15 min, and the upper aqueous phase was extracted with the same volume of chloroform. The aqueous phase was recovered, and the same volume of 2-propanol was added to precipitate DNA. The precipitate pellet was collected by centrifugation at 10000 *g* for 15 min at 4°C, and was rinsed with 70% (v/v) ethanol. The pellet was dissolved with 1 ml of RNase A solution (50 μg/ml of RNase A) and incubated at 45°C for 1 h. The mixture was then repeatedly purified by chloroform extraction and 2-propanol precipitation. The DNA pellet was dissolved in MilliQ water (100 μl). The DNA mixture was further purified with the QIAquic PCR purification kit (Qiagen, Hilden, Germany). The purified DNA was then eluted with MilliQ water (100 μl). Finally, the purified DNA mixture was further diluted by 100 times with MilliQ water.

Primer design

To design the PCR primers to detect *P. brevispora,* ITS sequences in Table 1 with DNA Data Bank of Japan (DDBJ) accession number were aligned using Clustal X version 1.63b.[18] Approximately 20 mer of unique sequences on ITS1 and ITS2 for *P. brebispora* were synthesized as specific primers. The sequences of the respective primers were P.b.ITS-F; 5′-GTA GGG CTG ACC TTC GCA GGT C-3′ and P.b.ITS-R; 5′-AGC AAC ACT ATT ATA CTT CAC GG-3′.

PCR amplification

As a first step, to check the PCR specificity, the following experiment was carried out. PCR was performed in a 20-μl reaction mixture containing 1 ng of total genomic DNA from mycelia of each strain, 10-pmol of the primer set P.b.ITS-F/-R, 0.5 unit of Takara EX-Taq DNA polymerase (Takara Bio, Shiga, Japan), 2 mM of $MgCl_2$ solution, and 0.2 mM of each dNTP. Amplifications were performed in a thermal cycler (PC801; Astec, Fukuoka, Japan). PCR reactions for specific amplification of *P. brevispora* consisted of an initial denaturation at 94°C for 1 min, 10 cycles of preamplification, 22 cycles of amplification, and a final extension at 72°C for 5 min; each cycle of preamplification consisted of denaturation at 94°C for 30 sec, annealing at 65°C for 30 s, and extension at 72°C for 1 min. Each cycle of amplification was the same as those in preamplification except for annealing at 62°C for 30 s. A positive control for the ITS region using published universal primers, ITS1 and ITS4,[13] and a negative control without a DNA template were used in each amplification. Electrophoresed gel was photographed on a UV transilluminator, and the photograph was scanned using a scanner. The scanned picture was expanded and converted to black/white contrast using Photoshop 4.0 (Adobe).

To confirm the achievement of greater PCR specificity and PCR sensitivity, the following three experiments were carried out.

1. PCR sensitivity was tested by amplifying the dilution series of the genomic DNA of *P. brevispora* from 1 ng to 1 fg per 1 μl of reaction mixture. The template DNA concentration was estimated by gel electrophoresis using a 1-kb DNA ladder marker (New England Biolabs, Beverly, MA, USA).
2. The PCR was performed in a reaction mixture containing 1/10 volume of total DNA extracted from 5 g of WCM inhabited by *P. tremellosus* and a DNA concentration series from *P. brevispora* (ranging from 1000 pg/μl to 1 fg/μl at final concentration in PCR reaction mixture).
3. DNA was extracted from 5 g of WCM inhabited by *P. tremellosus*, which contained a weight series of freeze-dried mycelium of *P. brevispora* (0.2–10 mg mycelium per gram of WCM). WCM culture was regarded as environmental background containing various organic extract and abundant microorganisms.

On-site monitoring

To carry out specific detection of *P. brevispora* for on-site samples, decayed tissue samples were harvested from a tree stump from which *P. brevispora* had been isolated. Sampling was carried out on 14 October 2002. Four 10-g samples of decayed tissue were harvested from the outer bark of the tree stump at the four points of the compass (viz., north, south, east, and west), and a fifth sample was collected from the rotted heartwood. A section of the root of a neighboring tree was also harvested. Butt-rotted tissue was also harvested from the tree stump of *Chamaecparis obtusa* in the forest at Kunimi-cho, Nagasaki Prefecture. In this forest, no basidioma and no isolate of *P. brevispora* were obtained from butt-rotted trees. Isolation of basidiomycetous fungi from decayed tissue was carried out using MEA with antibiotics (10 ppm of benomyl, Sigma, St. Louis, USA; 50 μg/ml

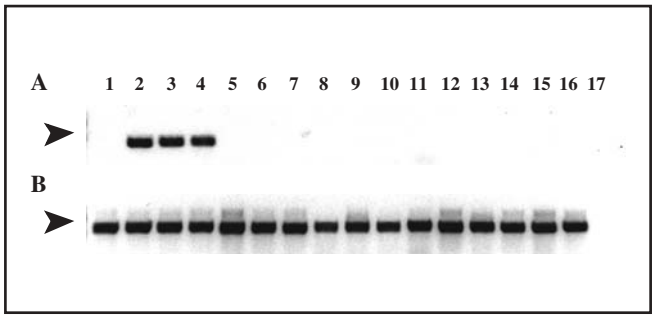

Fig. 1. Specificity of the designed primer. Polymerase chain reaction (PCR) was carried out using *Phlebia brevispora* specific primers (**A**) and ITS1/ITS4 (**B**). *Lane 1*, *Phlebia chrysocreas* TMIC31891; *lane 2*, *P. brevispora* TMIC33929; *lane 3*, *P. brevispora* TMIC34596; *lane 4*, *P. brevispora* HHB-7024-Sp; *lane 5*, *Phlebia subserialis* HHB-9678-Sp; *lane 6*, *Phlebia uda* Kropp-1; *lane 7*, *Phlebia livida* HHB-4609-Sp; *lane 8*, *Phlebia radiata* HHB-5324-Sp; *lane 9*, *Phanerochaete sordida* YK-624; *lane 10*, *Ceriporia lacerata* MZ-340; *lane 11*, *Ceriporia purpurea* KKN-223-Sp; *lane 12*, *Ceriporia spissa* ATCC 62024; *lane 13*, *Ceriporiopsis aneirina* Fp-100665-Sp; *lane 14*, *Trametes hirsuta* YK-505; *lane 15*, *Hericium erinaceus* SFFPSC02; *lane 16*, *Xylobolus spectabilis* IFO 7076; *lane 17*, no template. *Arrowheads* indicate amplified fragments

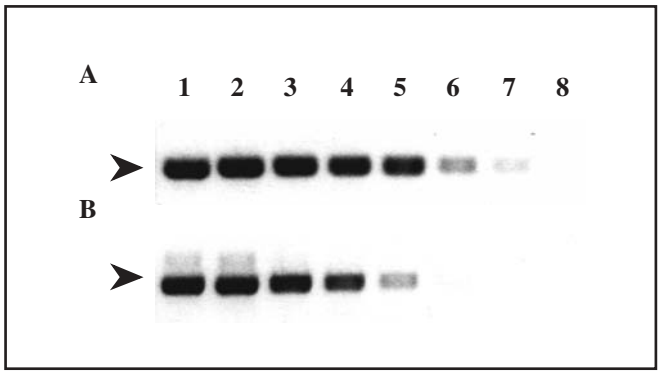

Fig. 2. PCR amplification in different amounts of template DNA. PCR was carried out using *Phlebia brevispora* specific primers (**A**) and ITS1/ITS4 (**B**). Concentrations of DNA from *P. brevispora* for *lanes 1–7*: 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg per microliter in reaction mixture, respectively. *Lane 8* contains no template DNA (negative control). *Arrowheads* indicate the amplified fragment

of ampicillin sodium, Wako; and 10 μg/ml of tetracycline hydrochloride, Wako). For DNA extraction, 5-g samples were freeze-dried and then milled with a wood chipper to 2–3 mm^3 in size. DNA extraction and species-specific PCR were carried out as described above except that the PCR reaction mixture contained 250 ng/μl of bovine serum albumin (BSA, Wako).

Results and discussion

Specificity of designed primer

In the test to check primer specificity, a single band (approximately 400 bp) was amplified for *Phlebia brevispora* by PCR using specific primers. No amplification was observed in any other fungal species used in this experiment (Fig. 1A). On the other hand, PCR products could be produced for all fungal strains examined in this experiment using the primer set of ITS1/4 (Fig. 1B). This indicates that the specific primer set developed here is useful for specifically detecting *P. brevispora*.

Sensitivity

The sensitivities of specific primers were tested by amplifying dilutions of the DNA template from 1000 pg/μg to a final concentration of 1 fg/μl. At the final concentration, PCR product was produced using the primer set of P.b.ITS-F/-R (Fig. 2A), whereas the ITS region was not amplified at the same concentration of the template DNA (Fig. 2B).

The sensitivity of PCR reactions was also tested in the presence of impurities (Figs. 3, 4). Total DNA extract from

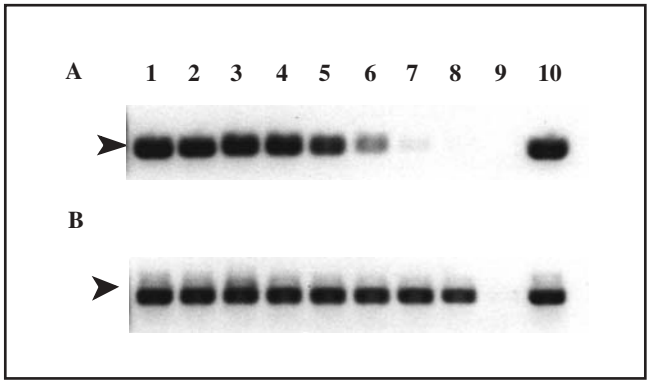

Fig. 3. PCR amplification in different amounts of template DNA from *Phlebia brevispora*. The PCR reaction mixture also contains total DNA isolated from wood chip media (WCM) inhabited by *Phlebia tremellosus*. PCR were carried out using *P. brevispora* specific primers (**A**) and ITS1/ITS4 (**B**). Concentrations of DNA from *P. brevispora* for *lanes 1–7*: 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg per microliter in reaction mixture, respectively. *Lane 8*, no template DNA from *P. brevispora*; *lane 9*, no template DNA (negative controls); *lane 10*, DNA from *P. brevispora* of 1 ng/μl at final concentration (positive controls). *Arrowheads* indicate the amplified fragment

WCM culture may contain aqueous wood extract and competitive DNA from other organisms. The sensitivity of these primer sets was maintained at equal levels (Fig. 3A). Notably, the P.b.ITS-F/-R primer set was able to amplify its target with an even smaller amount of mycelium (0.2 mg of dry weight per 1 g of WCM) (Fig. 4A). The PCR reactions developed here are sufficiently sensitive to amplify from 1 fg (per 1 μl reaction mixture) of target DNA, and are able to amplify the target sequences while coexistent with impurities. Despite some limitations such as the need to dilute the total DNA extract to obtain an appreciable amplicon, this method was shown to be sensitive. The sensitivity of specific primers was found to be greater than that found in previous reports.[10,14,17]

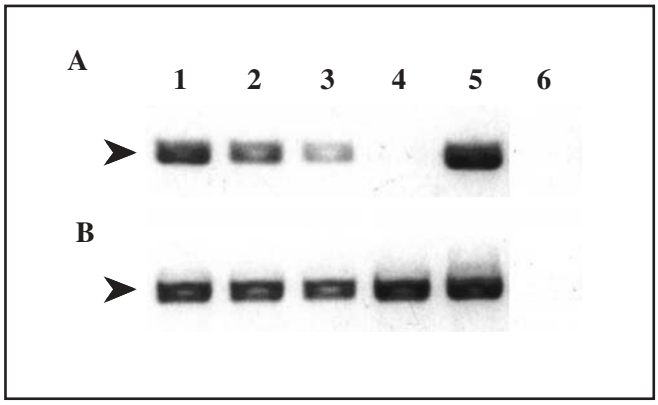

Fig. 4. PCR amplification of DNA isolated from WCM inhabited by *Phlebia tremellosa* and from different amounts of dried *Phlebia brevispora* mycelia. PCR was carried out using *P. brevispora* specific primers (**A**) and ITS1/ITS4 (**B**). The amounts of dried mycelium per gram of wood culture for *lanes 1–3*: 10, 2, and 0.2 mg, respectively. *Lane 4*, no mycelium of *P. brevispora*; *lane 5*, 100 pg/μl DNA from *P. brevispora*; *lane 6*, no template DNA (negative control). *Arrowheads* indicate the amplified fragment

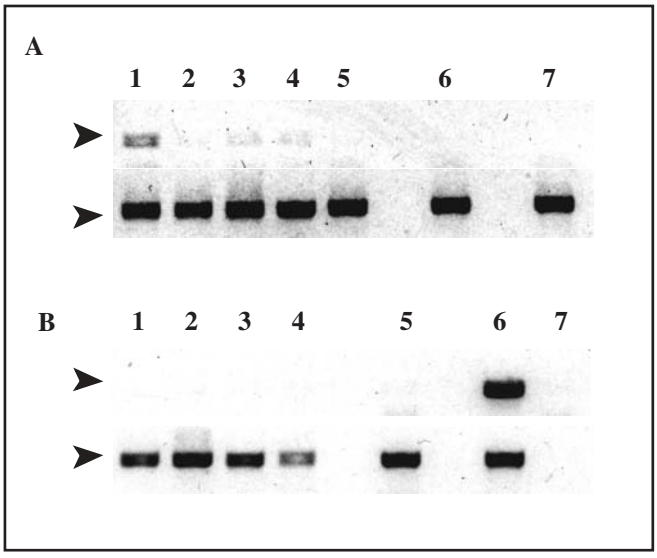

Fig. 5A,B. Species-specific PCR using samples from decayed tree tissue. **A** DNA was extracted from decayed tissues of tree stump or root tissue of living tree. *Lane 1*, sample of decayed stump from the southern side; *lane 2*, the western side; *lane 3*, the heartwood; *lane 4*, the northern side; *lane 5*, the eastern side; *lane 6*, sample from the root tissue of living tree; *lane 7*, sample from the decayed stump at Kunimicho. **B** DNA was extracted from fungal mycelial samples isolated from decayed tissues of the tree stump. *Lane 1*, sample from the southern side; *lane 2*, the western side; *lane 3*, the heartwood; *lane 4*, the eastern side; *lane 5*, from root tissue of the living tree; *lane 6*, 100 pg genomic DNA from *Phlebia brevispora* (positive control); *lane 7*, no template DNA (negative control) as template. PCR was carried out using primers indicated by the *upper arrowhead*; *P. brevispora* specific and *lower arrowhead*: ITS1/4 (control). *Arrowheads* indicate the amplified fragment

On-site monitoring

Two strains, TMIC33929 and 34596, obtained from the butt-rotted stump of Japanese cypress in Nagasaki Prefecture in 1997, were identified as *P. brevispora*.[5] After 5 years, no basidioma of this fungus was observed on or around this stump. Wood tissue samples harvested from all regions of the stump were considerably decayed and resembled soil or leaf mold in appearance. *Phlebia brevispora* isolate was not successfully obtained from all wood tissues on the MEA plates, and two or more unknown filamentous fungi grew from each wood tissue sample except for the wood tissue sample collected from the northern side. Furthermore, to detect *P. brevispora* from the cultural mycelia in the contaminated filamentous fungi, species-specific PCR was carried out for the DNA extracted from each mycelial mat of the filamentous fungi on the media. As shown in Fig. 5B, no amplification was observed in species-specific PCR for *P. brevispora*. On the other hand, results of PCR for DNA extracted directly from each wood tissue sample were different from those of the fungal isolates from the same wood tissue samples (Fig. 5). A *P. brevispora*-specific band was detected in samples from the southern and northern sides, and the heartwood of the stump (Fig. 5A, lanes 1, 3, and 4, upper arrowhead). Despite the fact that no isolates of the fungus could be obtained from the wood tissues, these results suggest that *P. brevispora* could be successfully monitored on-site with the specific primer.

Isolation of fungal strains from decayed tissue needs experience, accuracy, and due to competition among a wide variety of microorganisms inhabiting the rotted wood, good fortune during sample preparation; samples are often contaminated with other fungi (Ascomycetes, Deuteromycetes, Zygomycetes etc.) despite the use of agar media containing an inhibitor (e.g., benomyl and thiabendazole). In attempts to avoid contamination, reducing the size of tissue samples also reduces the possibility of finding the target organism inhabiting the tissue fragment. Consequently, a large number of tissue fragments must be examined.

The use of species-specific PCR amplification will overcome these problems and enable detection of the target organisms, despite the presence of other competitive organisms in the decayed tissue. In the decayed tree tissue samples, *P. brevispora* was detected by PCR assay even when its mycelia could not be isolated from the samples by culturing. In addition, the PCR method probably detects the target species much faster than the culturing method. The PCR approach, however, has the disadvantage that the quantification of fungal biomass is not easy. Although some PCR-based quantification methods have been previously described,[7,19] they are too laborious for routine analysis. Whatever PCR method based on DNA is used, it does not distinguish between dead and living fungal cells.

This molecular biology technique represents not only a replacement for the traditional one applied to identify pathogenic fungi, but can also contribute to diagnosis of infection by pathogenic fungi of a living tree or clarification of some specific aspects related to fungi life cycle or distribution. Although it has been difficult to investigate the distribution of a basidiomycete species without its basidiomata it will be possible to investigate the distribution in detail by using the PCR method. In addition, the PCR

method would be applicable to the ecological studies of many basidiomycetes.

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