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SHORT COMMUNICATION

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Identification of the basidiomycetous fungus isolated from butt rot of the Japanese cypress

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Abstract To identify a basidiomycetous fungus isolated from butt rot of *Chamaecyparis obtusa*, Japanese cypress, its cultural features were examined, and sequences of its nuclear ribosomal 18S and ITS1–5.8S–ITS2 regions were analyzed. In culture, this fungus is characterized by the occurrence of chlamydospores, blastoconidium-like cells, and clavate-to-spathulate hyphal ends at the tips of aerial hyphae, and production of a small basidioma on the mycelial mat after 3 months of incubation. The morphological features of the basidioma are identical to those of *Phlebia brevispora*. Furthermore, molecular data of the sequences of these strains and *P. brevispora* showed a high level of similarity. These results appear to justify determining the present fungus as *P. brevispora*. This is the first report of this species for Japan and outside of southeastern USA.

Key words *Chamaecyparis obtusa* · Corticiaceae · Cultural features · *Phlebia brevispora* · Ribosomal DNA sequence

Chamaecyparis obtusa (Sieb. & Zucc.) Endl., the 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 southwestern Japan (Kyushu District) (Kawabe et al. 1983; Kubayashi 1995). Kubayashi et al. (2001) recognized that there were several decay types of butt rot and several isolates with different cultural characteristics 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 (2001) classified the isolates from the decayed tissues into several groups based on the macro- 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 also is a causal agent of butt rot of *C. obtusa*. However, the other groups of the isolates remain unidentified. This study was carried out to identify one of the unidentified groups on the basis of cultural features and DNA sequence data.

Two isolates, TMIC33929 and TMIC34596, were obtained from decayed tissues of *C. obtusa* with butt rot on August 29, 1997, at Tomikawa, Isahaya-city, Nagasaki Prefecture. Fungal strains used in this study and their sources are given in Table 1, which also lists the DNA Data Bank of Japan (DDBJ)/GenBank/European Molecular Biology Laboratory (EMBL) accession number of the 18S Hibosomal DNA (18S rDNA) sequence used in this study. We obtained these strains from American Type Culture Collection (ATCC), Center for Forest Mycology Research, Forest Products Laboratory, USDA Forest Service (USDA), Institute for Fermentation Osaka (IFO), the Tottori Mycological Institute (TMI), and Laboratory of Systematic Forest and Forest Products Science, Kyushu University (SFFPS).

Cultural characteristics were examined using the methods described by Nakasone (1990). For DNA extraction, mycelia were grown on potato dextrose agar (PDA; Difco) or a 2% (w/v) malt extract (Difco) agar plate. Each mycelium was placed in a mortar with liquid nitrogen and ground with a pestle into a fine powder. The mycelium powder was transferred to another mortar and ground with CTAB2 buffer [2% (w/v) cetyltrimethylammonium bromide (CTAB; Wako, Osaka, Japan); 1.4 M NaCl; 0.1 M Tris-HCl; 0.1% (v/v) β-mercaptoethanol; 20 mM ethylenediaminetetraacetic acid (EDTA); 2% (w/v) polyvinylpyrrolidone (PVP; Wako): pH 9.0] with 50 μg/ml RNase A (TaKaRa, Shiga, Japan). The mixture was put into a 1.5-ml tube and incubated at 65°C for 30 min. The lysate was extracted with the same volume of chloroform twice. The same volume of

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

Fungus	Strain no.	DDBJ accession no.
Bjerkandera adusta		U59065
Ceriporia purpurea	KKN-223-Sp (USDA)	AB084586
Ceriporia reticulata	24995 (ATCC)	AB084587
Ceriporia spissa	62024 (ATCC)	AB084588
Ceriporia viridans	HHB-9594-Sp (USDA)	AB084592
Ceriporiopsis aneirina	FP-100665-Sp (USDA)	AB084589
Dacrymyces stillatus		L22258
Gloeophyllum trabeum	6430 (IFO)	AB084611
Hericium erinaceum	SFFPSC02 (SFFPS)	AB084622
Laxitextum bicolor		AF026605
Phanerochaete avellanea	HHB-9371-Sp (USDA)	AB084594
Phanerochaete brunnea	TMIC33977 (TMI)	AB084595
Phanerochaete chrysosporium		AB026593
Phanerochaete magnoliae	TMIC33868 (TMI)	AB084596
Phanerochaete sanguinea	FP-105385-Sp (USDA)	AB084597
Phanerochaete sordida	YK-624 (SFFPS)	AB084593
Phanerochaete stereoides	TMIC33983 (TMI)	AB084598
Phanerochaete velutina	RLG-11272-Sp (USDA)	AB084599
Phlebia brevispora	HHB-7024-Sp (USDA)	AB084600
Phlebia chrysocreas	TMIC31891 (TMI)	AB084601
Phlebia livida	HHB-4609-Sp (USDA)	AB084602
Phlebia radiata	HHB-5324-Sp (USDA)	AB084603
Phlebia subochracea	HHB-8494-Sp (USDA)	AB084604
Phlebia subserialis	HHB-9678-Sp (USDA)	AB084605
Phlebia uda	Kropp-1 (USDA)	AB084606
Pycnoporus coccineus	SFFPSC01 (SFFPS)	AB084612
Trametes hirsuta	YK-505 (SFFPS)	AB084607
Trametes versicolor	6482 (IFO)	AB084608
Xylobolus annosus (Stereum annosum)		AF026587
Xylobolus frustulatus	4932 (IFO)	AB084609
Xylobolus subpileatus	7076 (IFO)	AB084610

USDA, United States Department of Agriculture, Forest Products Laboratory; ATCC, American Type Culture Collection; TMI, the Tottori Mycological Institute; IFO, Institute for Fermentation Osaka; SFFPS, Laboratory of Systematic Forest and Forest Products Sciences, Kyushu University

isopropanol was added to the aqueous layer to precipitate DNA. Precipitated pellets were dissolved in sterilized MilliQ water at 4°C overnight. The mixture was further centrifuged at 10000*g* or higher for 15 min and the supernatant was used.

Polymerase chain reaction (PCR) amplification was performed for the 18S rDNA using the primers EukNS20F (5′-TGTAGTCATATGCTTGTCTCAA-3′) and EukNS1750R (5′-TCCTCTAAATGACCAAGTTTG-3′) and for the internal transcribed spacer region (ITS) using the primers ITS-1 (5′-TCCGTAGGTGAACCTG CGG-3′) and FO-514 (5′-**CAGGAAACAGCTATGAC CTAT**GCTTAAGTTCAGCGGGTAGTCC-3′) (Derms et al. 1988). Bold letters show the sequence that codes M13 (RV), the universal primer. Amplifications were performed using TaKaRa EX-Taq DNA polymerase (TaKaRa) in a thermal cycler (PC801; Astec, Fukuoka, Japan). PCR reactions consisted of an initial denaturation at 94°C for 1 min 30 s, 32 cycles of amplification, and a final extension at 72°C for 5 min. Each cycle of amplification consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min. A negative control using MilliQ water instead of DNA was set up for each experiment. PCR products were purified with the QIAquick PCR purification kit (Qiagen, Tokyo, Japan). Direct sequencing of PCR products was conducted for both strands using the ABI Prizm 377 Genetic Analyzer (P.E. Biosystems, Tokyo, Japan). The sequence reaction was conducted using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (P.E. Biosystems). Amplified 18S rDNA fragments were sequenced with the following primers: EukNS600R (5′-ATACGCTATTGGAGCTGGAA-3′); EukNS581F (5′-TCCCAGCTCCAATAGCGTAT-3′); EukNS1165R (5′-CCTGGTGGTGCCCTTCC-3′); and EukNS1149F (5′-GGAAGGGCACCACCAGG-3′), in addition to the above primers. The ITS sequence was sequenced with the same primer as already described. Sequences were edited with the Genetyx-MAC 9.0 program (Software Development, Tokyo, Japan). Sequence data of U59065 [*Bjerkandera adusta* (Fr.) P. Karst.], L22258 [*Dacrymyces stillatus* Nees: Fr.], AF026605 [*Laxitextum bicolor* (Pers.) Lentz], AF026593 [*Phanerochaete chrysosporium* Burds.], and AF026587 [*Xylobolus annosus* (Berk. & Broome) Boidin (=*Stereum annosum* Berk. & Broome)] were obtained from DDBJ/GenBank/EMBL. Alignment was conducted using Clustal X version 1.63b [parameter sets as follows: gap opening was 10.00, gap extension was 0.05, delay divergent sequences (%) was 40, DNA transition weight was 0.5, use negative matrix was off, and IUB DNA weight matrix] (Thompson et al. 1997) and SeqPup 0.6f

(Gilbert 1996). Phylogenetic relationships were inferred using the "Bootstrap N-J [neighbor-joining (NJ) method; Saitou and Nei 1987] tree" program in Clustal X. To evaluate the strength of support for the branches of the NJ trees, 100 replications of bootstrap (Felsenstein 1985) analysis were performed. The tree was displayed using TreeView PPC 1.6.6 (Page 2001).

To determine optimum growth temperature, TMIC33929 was grown on malt extract agar [MEA, 1.5% (w/v) malt extract agar, Difco; 2% (w/v) Bacto agar, Difco] plates at 12 different temperatures (4°, 10°, 15°, 18°, 21°, 24°, 27°, 30°, 33°, 36°, 39°, and 42°C). Six replicates were made for each temperature. Colony growth rate was calculated as radial growth of the mycelium for each 48 h.

The optimal temperature for the isolate was between 30° and 33°C. This fungus could grow between 10° and 39°C, but no visible growth was observed at 4°or at 42°C. Its growth on MEA was 25–28 mm in radius at 30°C for 48 h. The macroscopic and microscopic features of a colony on MEA after incubation for 2 weeks are as follows: mycelial mats white; around inocula and surrounding area appressed and subfelty, then becoming moderately thin, slightly raised, arachnoid to cotton-woolly toward margin; margin even, appressed to raised; odor absent; agar partially or entirely bleached; hyphal system monomitic; hyphae hyaline, 2–6 μm diameter, smooth, thin-walled, nodose-septate, sparsely branched, sometimes becoming clavate to spathulate at the tip of aerial hyphae (Fig. 1B), sometimes encrusted with crystalline or resinous materials (Fig. 1D); chlamydospores arising terminally or intercalary, subglobose to globose, 11–24 × 9–18 μm, smooth, thick-walled (up to 1 μm) (Figs. 1A; 2); blastoconidium-like cells arising terminally or laterally from undifferentiated hyphae, ovoid to pyriform, sometimes with a truncate base, 5–9 × 4.5–8 μm, smooth, thin- to slightly thick-walled (up to 0.5 μm) (Figs. 1C, 2).

The initial phylogenetic analyses used to search for the taxonomic positions of TMIC33929 and an additional strain, TMIC34596, examined the partial sequence of 18S rDNA (approximately 1700 bp). A phylogenetic analyses based on these sequences using Clustal X revealed that the two isolates were nested within the clade of *Phlebia* Fr.,

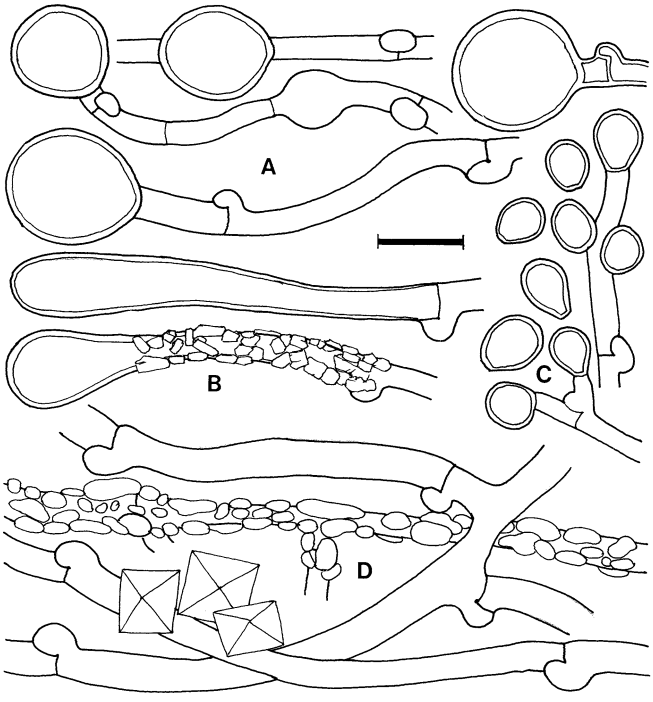
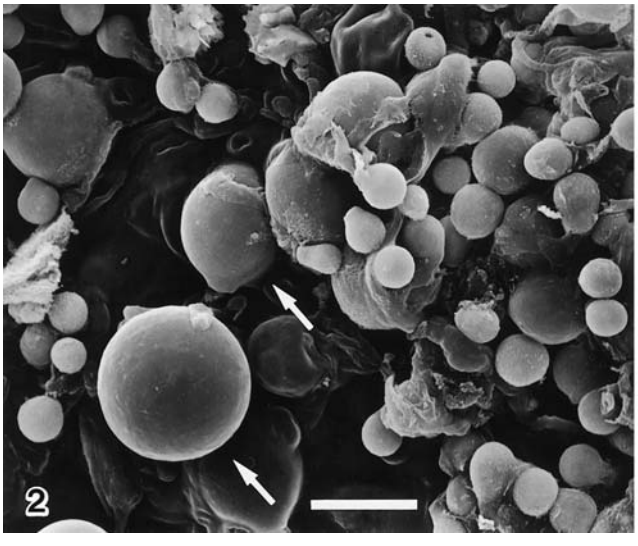


Fig. 1. Microscopic characteristics of TMIC33929 in a 2-week-old culture. **A** Chlamydospores. **B** Clavate to spathulate hyphal tip cells in the aerial mycelium. **C** Blastoconidium-like cells, arising terminally or laterally from undifferentiated hyphae. **D** Hyphae, sometimes encrusted with crystalline or resinous materials. *Bar* 10 μm



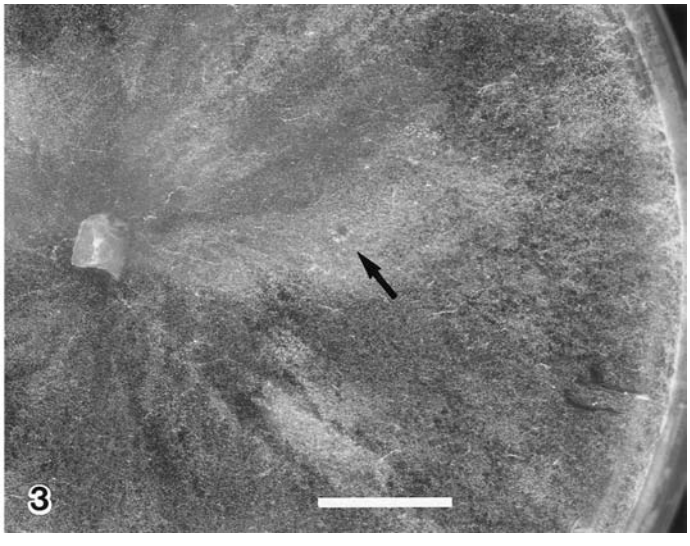


Fig. 2. Scanning electron microscopy (SEM) micrograph of TMIC33929 in a 2-week-old culture shows chlamydospores (*arrows*) and abundant blastoconidium-like cells. *Bar* 10 μm

Fig. 3. Basidioma (*arrow*) produced on malt extract agar (MEA) in a 3-month-old culture of TMIC33929. *Bar* 1 cm

Table 2. Comparison of internal transcribed spacer (ITS) sequences between *Phlebia brevispora* HHB-7024-Sp (AB084616) and other strains

Fungal strain (DDBJ accession no.)	Number of nucleotides used in this study (bp)	Identity with *P. brevispora* (%)
TMIC33929 (AB084614)	593	98.8
TMIC34596 (AB084615)	587	98.1
P. radiata (AB084619)	594	88.5
P. uda (AB084621)	592	85.0
P. chrysocreas (AB084617)	598	85.5
P. subserialis (AB084620)	597	81.9
P. livida (AB084618)	560	80.0
Hericium erinaceum (AB084622)	601	69.7

which is a member of the family Corticiaceae *sensu lato* (see Fig. 4). In this tree, the clade of *Phlebia* was supported by a high bootstrap value (83/100). Among *Phlebia* spp. examined in this study, the sequence of 18S rDNA from the two isolates were identical to those of a polysporous strain (HHB-7024-Sp) isolated from a basidioma of *Phlebia brevispora* Nakasone. According to Nakasone and Eslyn (1981), Nakasone (1990), and Wang and Zabel (1990), *P. brevispora* is characterized by the production of blastoconidium-like cells (described as chlamydospores by Wang and Zabel 1990), chlamydospores, and cystidium-like clavate cells in the aerial hyphae in culture. We confirmed that both TMIC33929 and TMIC34596 formed these structures, which resembled those seen in HHB-7024-Sp in shape and size. In addition, the other cultural features of the present isolates were also coincident with the previous descriptions. Further, the ITS sequences of TMIC33929 and TMIC34596 showed high levels of similarity, 98.8% and 98.1%, respectively, to that of HHB-7024-Sp, whereas its levels of similarity to the ITS sequences of the other *Phlebia* spp. and *Hericium erinaceum* (Fr.) Pers., which belongs to Hericiaceae Donk, were low, 88.5%–80.0% and 69.7%, respectively (Table 2). Analyses of the ITS sequence data indicated that the two Japanese isolates and *P. brevispora* (HHB-7024-Sp) are conspecific.

In culture, after 3 months of incubation, a small basidioma was formed on the mycelial mats of TMIC33929 (Fig. 3). The basidioma is characterized as follows: the hymenial surface "Buff," "Honey," "Hazel," "Isabelline" to "Olivaceous" (according to Rayner 1970), smooth; hyphal system monomitic; hyphae 2.5–8 μm diameter, smooth, thin- to thick-walled (up to 2 μm), nodose septate (Fig. 5D); leptocystidia obclavate to subfusiform, 52–75 × 4.5–8 μm, with a basal clamp, sometimes encrusted with resinous materials, projecting up to 40 μm beyond the hymenial surface (Fig. 5C); basidia clavate, 16–24 × 4–5 μm, with a basal clamp, producing four sterigmata (Fig. 5B); basidiospores ellipsoid to short cylindrically, 4–4.5 × 2–2.5 μm, smooth, thin-walled, nonamyloid (Fig. 5A). These macro- and microscopical features were identical to those of *P. brevispora* as described by Nakasone and Eslyn (1981).

Although we have collected no basidiomata under natural conditions, it appears justifiable to identify the Japanese isolates as *P. brevispora* on the basis of their cultural features and rDNA sequence data. *Phlebia brevispora* has previously been known only in the southeastern USA, viz. Florida, Illinois, Louisiana, Maryland, Mississippi, Oklahoma, South Carolina, and Virginia (Nakasone and Eslyn 1981; Nakasone 1990). Therefore, this is the first report of this species for Japan, and the first outside the southeastern USA.

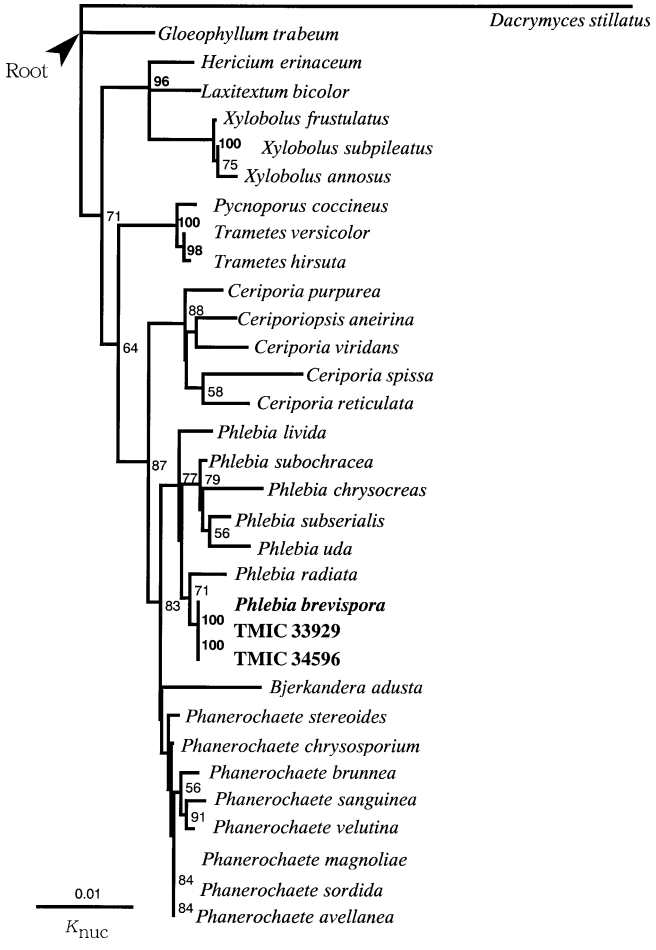


Fig. 4. Neighbor-joining tree derived from the18S rDNA sequences. *Arrowhead* indicates the root. The tree was rooted by the outgroup using sequence of a heterobasidiomycete, "*Dacrymyces stilatus*." Bootstrap values ≥50% are indicated

Phlebia brevispora has never been reported to cause decay of living trees. However, we suggest that this may be one of the causal agents of butt rot of *C. obtusa*. An inocu-

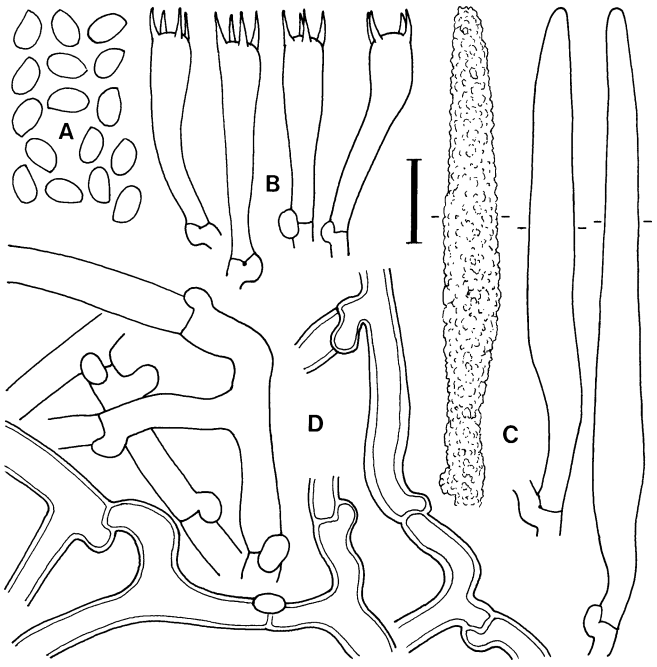


Fig. 5. Microscopic characteristics of a basidioma produced in a 3-month-old culture of TMIC33929. **A** Basidiospores. **B** Basidia. **C** Leptocystidia, encrusted with resinous materials. *Short lines* indicate the level of the hymenial surface. **D** Subicular hyphae. *Bar* 10 μm

lation experiment is required to confirm the pathogenicity of *P. brevispora* to *C. obtusa*.

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References

Derms E, Hendriks L, Van de Peer Y, Neefs J-M, Smits G, Vandenbempt I, De Wachter R (1988) Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res 16:r87–r173

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Gilbert D (1996) SeqPup version 0.6f. Biocomputing Office, Biology Department Indiana University, Bloomington

Kawabe Y, Hashimoto H, Dozono Y, Matsuo T, Miyazaki T (1983) Butt-rot of Japanese cypress in Nagasaki Prefecture (in Japanese). Trans Kyushu Br Jpn For Soc 36:231–232

Kubayashi T (1995) Butt-rot of Japanese cypress in Nagasaki Prefecture (in Japanese). For Pests 44:162–167

Kubayashi T, Maekawa N (2001) Identification and pathogenicity of the fungus isolated from butt rot of Japanese cypress, *Chamaecyparis obtusa*. J Gen Plant Pathol 67:262–267

Kubayashi T, Hattori T, Kawabe Y (2001) *Tinctoporellus epimiltinus*, a causal fungus of butt rot of Japanese cypress. For Pathol 31:43–52

Nakasone KK (1990) Cultural studies and identification of wood-inhabiting Corticiaceae and selected Hymenomycetes for North America. Mycol Mem 15:1–412

Nakasone KK, Eslyn WE (1981) A new species, *Phlebia brevispora*, a cause of internal decay in utility poles. Mycologia 73:803–810

Page RDM (2001) TreeView PPC Version 1.6.6. Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow

Rayner RW (1970) A mycological colour chart. Commonwealth Mycological Institute, Kew, Surrey, and British Mycological Society

Saitou M, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882

Wang CJK, Zabel RA (1990) Identification manual for fungi from utility poles in the eastern United States. American Type Culture Collection, Rockville, MD, pp 68–69