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**Sakae Horisawa · Yoh Sakuma · Katsuhiko Takata**
**Shuichi Doi**

# Detection of intra- and interspecific variation of the dry rot fungus *Serpula lacrymans* by PCR-RFLP and RAPD analysis

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**Abstract** We investigated a genotype-based assay to discriminate the dry rot fungi *Serpula lacrymans*. DNAs were extracted from 74 isolates from the northern half of Japan, and internal transcribed spacers (ITS) were amplified by polymerase chain reaction. Genotypes of isolates were checked by restriction fragment length polymorphism (RFLP) using two enzymes, *Taq* I and *Hha* I. Among the 74 isolates identified as *S. lacrymans* in terms of morphologic features, 5 isolates were shown to have been misidentified. Random amplified polymorphic DNA (RAPD) analysis was conducted in order to detect the intraspecific diversity of *S. lacrymans* isolated in Japan. Because no relation between geographical origin and genetic distances was observed, the intraspecific diversity of *S. lacrymans* is suggested to be small.

**Key words** Serpula lacrymans · PCR-RFLP · ITS · RAPD

## Introduction

The dry rot fungus *Serpula lacrymans* is very important as an indoor decay fungus. This fungus has been reported to cause a great amount of damage in Europe, Australia, and Japan.[1–3] One of the physiological features of *S. lacrymans* is that it can obtain water from a water source and grows in dried wood even if the wood is below the fiber saturation point.[3] In regard to the characteristics of the growth temperature of *S. lacrymans*, its optimum growth temperature is around 20°–22°C, while exposure at 40°C for 15 min kills it.[4,5] In Japan, this fungus is observed in the northern half of the main island of Honshu and in Hokkaido. It is not clear how *S. lacrymans* infects, although case studies have suggested that *S. lacrymans* usually lives in soil and invades buildings through wood chip or scraped wood left on the surface of the soil.[2,6] This hypothesis is supported by a report showing nitrogen consumption in the soil by *S. lacyrmans*.[7] Soil surveys and identification of fungi in the soil should clarify the infection route of the fungus.

Basidiomycetes have so far been identified by means of traditional techniques such as visual and microscopic observation. Cultural features also did in identification depending on the experience of the examiner. Identification by these methods is difficult and sometimes erroneous. Recently, molecular biology methods for classification and identification of basidiomycetes have been developed and used. For example, in *S. lacrymans*, methods such as sodium dodecyl sulfate polyacry amide gel electrophoresis (SDS-PAGE) and Western blotting according to protein information,[8,9] restriction fragment pattern of ribosomal DNA amplified by polymerase chain reaction (PCR),[10] and randomly amplified polymorphic DNA (RAPD) analysis due to total genome information[11,12] have been reported.

The full length of the internal transcribed spacer (ITS) region (ITS I, ITS II, and 5.8S) is between the conserved 18S and 28S subunit regions. Restriction fragment length polymorphism (RFLP) of ITS is an adaptable tool for identification between species or genera.[13–16] RAPD analysis is based on PCR using single primer arbitrary nucleotide sequences, with the amplified fragment presenting differences in the DNA between samples as polymorphisms. This technique is sometimes utilized for the detection of intraspecific variation.[12,17–20]

Using the molecular biology methods, fungi in the soil can be detected without obtaining isolates. We have kept and subcultured a collection of *S. lacrymans* isolated from a

S. Horisawa[1] (✉) · K. Takata · S. Doi
Institute of Wood Technology, Akita Prefectural University, Noshiro 016-0876, Japan

Y. Sakuma
Biological Resources Division, Japan International Research Center for Agricultural Sciences, Tsukuba 305-0851, Japan

*Present address:*
[1] Department of Environmental Systems Engineering, Faculty of Engineering, Kochi University of Technology, Kami-gun, Kochi 782-8502, Japan
Tel. +81-887-57-2519; Fax +81-887-57-2520
e-mail: horisawa.sakae@kochi-tech.ac.jp

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**Table 1.** Geographical origin of isolates tested

| Isolates code | Geographical origin | Isolates code | Geographical origin |
|---|---|---|---|
| 7701 | Asahikawa | 8204 | Asahikawa |
| 7702 | Asahikawa | 8205 | Asahikawa |
| 7802 | Asahikawa | 8206 | Takanosu |
| 7803 | Sapporo | 8207 | Asahikawa |
| 7805 | Sapporo | 8208 | Asahikawa |
| 7806 | Biei | 8209 | Sapporo |
| 7901 | Mikasa | 8401 | Asahikawa |
| 7902 | Asahikawa | 8403 | Kenbuchi |
| 7904 | Asahikawa | 8404 | Sapporo |
| 7905 | Asahikawa | 8501 | Muroran |
| 7906 | Kimobetsu | 8504 | Asahikawa |
| 7907 | Asahikawa | 8505 | Asahikawa |
| 8001 | Takikawa | 8506 | Asahikawa |
| 8002 | Hiroshima | 8601 | Asahikawa |
| 8003 | Toubetsu | 8602 | Asahikawa |
| 8005 | Kushiro | 8603 | Saku |
| 8006 | Ashibetsu | 8604 | Ina |
| 8007 | Asahikawa | 8605 | Asahikawa |
| 8008 | Asahikawa | 8606 | Sapporo |
| 8010 | Asahikawa | 8607 | Asahikawa |
| 8011 | Asahikawa | 8701 | Asahikawa |
| 8013 | Asahikawa | 8702 | Asahikawa |
| 8014 | Asahikawa | 8703 | Asahikawa |
| 8015 | Kushiro | 8704 | Toyama |
| 8016 | Nayoro | 8705 | Asahikawa |
| 8101 | Higasiasahikawa | 8706 | Asahikawa |
| 8102 | Asahikawa | 8708 | Asahikawa |
| 8103 | Asahikawa | 8709 | Toyama |
| 8106 | Asahikawa | 8710 | Asahikawa |
| 8107 | Nayoro | 8802 | Asahikawa |
| 8109 | Asahikawa | 8803 | Asahikawa |
| 8110 | Asahikawa | 8804 | Asahikawa |
| 8111 | Asahikawa | 8805 | Asahikawa |
| 8112 | Asahikawa | 9201 | Nakashari |
| 8113 | Kushiro | 9301 | Tsubetsu |
| 8201 | Asahikawa | 9302 | Asahikawa |
| 8203 | Asahikawa | 9401 | Akikawa |

**Table 2.** Type strains used as reference

| Species | Isolates | Geographical origin |
|---|---|---|
| *Serpula lacrymans* | IFO 08697 | Nara |
| *Serpula lacrymans* | IFO 30955 | Asahikawa |
| *Gloeophyllum sepiarium* | IFO 04944 | – |
| *Gloeophyllum sepiarium* | IFO 06267 | – |
| *Gloeophyllum trabeum* | IFO 06430 | – |
| *Gloeophyllum trabeum* | IFO 06509 | – |
| *Coniophora puteana* | IFO 06275 | – |
| *Coniophora puteana* | B004 | – |


**Fig. 1.** Sampling sites of isolates

damaged building in the northern half of Japan (the northern half of Honshu and Hokkaido) during the period 1977–1996. Doi[6] made an invaluable database of collection sites, physiological features, and decay capacities. The goal of the present study was to examine a reliability of the PCR-RFLP analysis against the 74 isolates from Japan, identified as *S. lacrymans* by means of traditional methods. Intraspecific variation in the isolates was also investigated using RAPD analysis.

## Materials and methods

### Isolates

Seventy-four isolates, obtained from the northern half of Japan and identified as *Serpula lacrymans*, were examined (Table 1, Fig. 1). *Serpula lacrymans* IFO08697 and IFO30955, *Gloeophyllum sepiarium* IFO04944 and IFO06267, *Gloeophyllum trabeum* IFO06430 and IFO06509, and *Coniophora puteana* IFO06275 were used as type strains (Table 2). *Coniophora puteana* B004, isolated in our laboratory, was also examined for reference. Each isolate was subcultured on slanted potato dextrose agar (PDA) medium and was kept at 4°C until examination.

### DNA extraction from culture

Mycelia of isolates cultured on PDA plates were collected. DNA was extracted from mycelia using a method employing benzyl chloride.[21] To each sample, about 0.1 g of mycelia was placed in a 1.5-ml microtube and 200 $\mu$l of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 9.0), 300 $\mu$l of benzyl chloride, 100 $\mu$l of 10% SDS, and 5 $\mu$l of mercaptoethanol were added. The tube was vortexed and incubated at 50°C for 30 min with shaking or repeated vortexing at 5 min intervals. After centrifugation at 20000 $g$ at 4°C for 15 min, the supernatant was collected, and DNA was precipitated with isopropanol using 3 M sodium acetate solution. The precipitate was rinsed with 70% ethanol and dried. The precipitate was then resuspended in 200 $\mu$l of TE buffer. RNase (10 mg/tube) was added to the suspension at 37°C to remove contaminating RNA. For purification, the DNA solution was processed using polyvinylpyrrolidone.[22,23]

### PCR and digestion by restriction enzymes

The full length of the ITS region was amplified using the primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′).[13] Three primer pairs, NS1 (5′-GTA GTC ATA TGC TTG TCT C-3′) and NS2 (5′-GGC TGC TGG CAC CAG ACT TGC-3′), NS3 (5′-GCA AGT CTG GTG CCA GCA GCC-3′) and NS4 (5′-CTT CCG TCA ATT CCT TTA AG-3′), and NS5 (5′-AAC TTA AAG GAA TTG ACG GAA G-3′) and NS8 (5′-TCC GCA GGT TCA CCT ACG GA-3′) were employed to amplify partial 18S rDNAs.[13] PCR mixture was prepared from the Expand High Fidelity PCR system (Roche-Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. The mixture contained 10 ng of template DNA, each primer at a concentration of 0.5 pM, each dNTP at a concentration of 200 nM, 1.5 U/100 $\mu$l of DNA polymerase, and Expand reaction buffer with 1.5 mM $MgCl_2$. PCR was performed with a GeneAmp 2400 thermal cycler (Applied Biosystems, CA, USA) with the following parameters: an initial denaturization of 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and a final extension of 7 min at 72°C. All PCR products were stored at 4°C until used. Aliquots of PCR products were examined by running on 0.8% agarose gel.

Amplified DNA of ITS was digested by restriction enzymes *Taq* I, *Hae* III, and *Hha* (Takara Bio, Shiga, Japan)[10] according to the manufacturer's instructions. Digested fragments were separated on a 1.0 mm thick vertical gel containing 8% polyacrylamide (acrylamide–bisacrylamide, 29:1) gel. Electrophoresis was performed in 0.5 × TBE (tris, boron, EDTA) buffer at 11 V/cm. Separated fragments were stained with ethidium bromide, and detected with an ultraviolet (UV) transilluminator.

### Cloning and sequencing

PCR products of the ITS region and the 18S rDNA were cloned into pCR2.1-TOPO using a TOPO TA Cloning Kit (Invitrogen, Leek, the Netherlands) according to the manufacturer's instructions. Nucleotide sequences were determined using a model 377 ABi automated sequencer (Applied Biosystems, CA, USA). Sequencing reactions were facilitated by an ABi BigDye Prism dideoxy sequencing dye terminator kit (Applied Biosystems) as recommended by the manufacturer. Nucleotide sequence data were compared against the DDBJ nucleotide sequence database using the BLAST program.

### RAPD analysis

Amplification reactions were performed in 0.2-ml PCR tubes using an Expand High Fidelity PCR system. Each reaction had a total volume of 10 $\mu$l containing 1 × PCR buffer with 1.5 mM $MgCl_2$, 200 $\mu$M each of dNTP (Takara), 0.2 $\mu$M primer, 20 ng of genomic DNA, and 0.4 U DNA polymerase. Thirty-nine random primers (Operon, CA, USA) were tested. PCR was performed in a GeneAmp 2400 thermal cycler with the following parameters: an initial denaturization of 3 min at 94°C, 45 cycles of 45 s at 94°C, 1 min at 37°C, 1.5 min at 72°C, and a final extension of 7 min at 72°C.[12] Amplification products were separated by electrophoresis on 2.0% agarose gels with 1 × TBE buffer at 7 V/cm, and were visualized by staining with ethidium bromide. Three replications were performed for each primer tested.

DNA fragments were scored based on photographs of the gels. Data were recorded as a binary matrix, and similarity was calculated with the formula of Nei and Li:[24]

$$F_{xy} = 2n_{xy} / (n_x + n_y)$$

where $F_{xy}$ is the proportion of the reproducible bands common to the patterns compared, $n_{xy}$ is the number shared by both isolates, and $n_x + n_y$ is the total number of reproducible bands in both strains. The distance ($d$) between two isolates is calculated with the formula $d = 1 - F_{xy}$. A dendrogram was generated using the unweighted pair groups method (UPGMA) employing the PHYLIP program package.[25]

## Results

### PCR-RFLP of ITS region

ITS fragments were amplified from all isolates and type cultures tested, and they had similar lengths of about 650 bp.

The restriction enzymes tested in this report were selected in accordance with an earlier report,[10] but no ITS fragment was digested by *Hae* III. Therefore, this report describes the results regarding *Taq* I and *Hha* I (Fig. 2). In regard to *Serpula lacrymans* type strains IFO08697 and IFO030955, the ITS fragments were digested into fragments with lengths of 310, 190, 65, 42, and 32 bp by *Taq* I, and 340 and 290 bp by *Hha* I. The digested fragment patterns of isolates agreed with those of the *S. lacrymans* type strain except for 8012, 8501, 8708, 8709, and 8802, which were distinct in regard to both restriction enzymes.

Isolates 8710 and 8802 were estimated to be *Coniophora puteana* by comparing them with the digested fragment patterns of IFO06275 and B004 of both enzymes. Isolates 8501, 8708, and 8709 did not show agreement with any type strain tested.

### Determination of species of isolates from sequences of ITS regions and 18S rDNA

The ITS sequence of *S. lacrymans* IFO03955 was already determined and registered in the DDBJ/EMBL/GenBank (Accession No. AJ419909). We also sequenced the ITS of IFO08697 and IFO30955 and confirmed high homologies of 99% and 100%, respectively, to the ITS sequence of *S. lacrymans* in the database (Table 3). Therefore, IFO08697 and IFO30955 should be eligible as type strains. The PCR-RFLP pattern of the ITS region of isolates 8501, 8708, and

**Table 3.** Sequence analysis of internal transeribed spacers (ITS) regions and 18S rDNA

| Isolates | Sequenced fragment | Closest species (Accession No.) | Identity |
|---|---|---|---|
| 8501 | ITS | *Antrodia vaillantii* (AJ249266) | 650/655 (99%) |
| 8708 | ITS | *Oligoporus placentus* (AJ416069) | 628/636 (98%) |
| 8709 | ITS | *Irpex lacteus* (AB079265) | 651/665 (97%) |
| IFO 30955 | ITS | *Serpula lacrymans* (AJ419909) | 610/610 (100%) |
| IFO 08697 | ITS | *Serpula lacrymans* (AJ419909) | 610/611 (>99%) |
| 8015 | 18S rDNA | *Serpula lacrymans* (AJ536023) | 1779/1780 (>99%) |
| 8704 | 18S rDNA | *Serpula lacrymans* (AJ536023) | 1778/1781 (>99%) |
| 8804 | 18S rDNA | *Serpula lacrymans* (AJ536023) | 1779/1780 (>99%) |
| IFO 30955 | 18S rDNA | *Serpula lacrymans* (AJ536023) | 1779/1781 (>99%) |

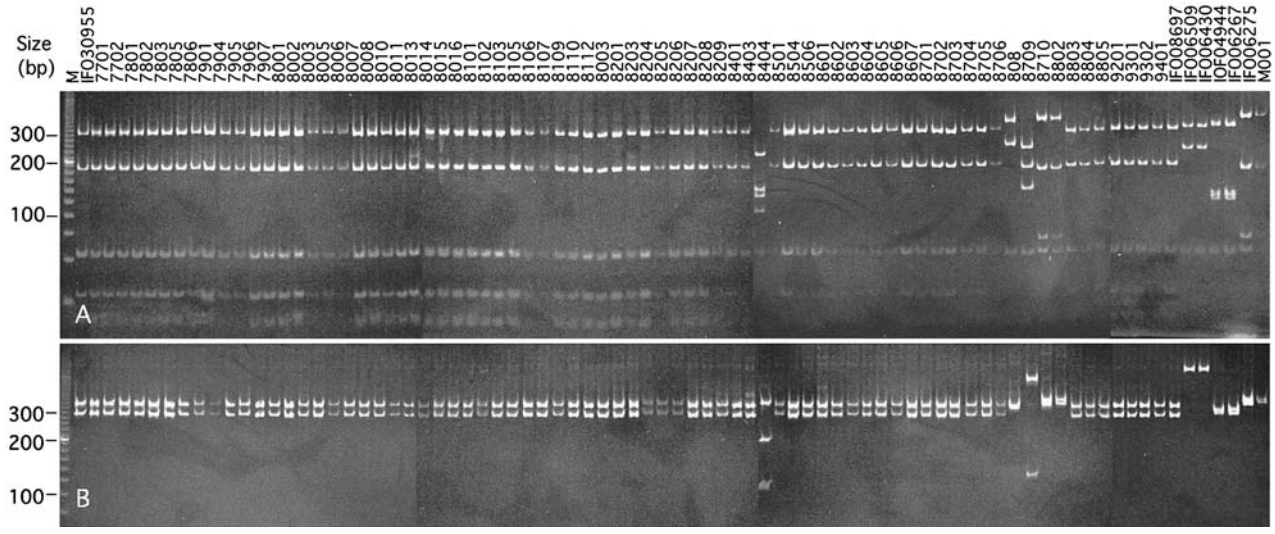


**Fig. 2.** Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) patterns obtained by digestion of internal transcribed spacers (ITS) fragments by *Taq* I (*A*) and *Hha* I (*B*). Electrophoresis on 8% polyacryamide gel in 1 × TBE (tris, boron, EDTA) buffer. The *left outer lane* is a DNA size marker (*M*). Isolate numbers are shown *above each lane*

8709 did not agree with any type strains used in this study. To infer these unidentified isolates, ITS regions were sequenced. The results of sequence analysis were compared with sequences in a DNA database. The ITS sequences of 8501, 8708, and 8709 showed high homology to *Antrodia vaillantii* (99%), *Oligoporus placentus* (98%), and *Irpex lacteus* (97%), respectively. Consequently, isolates 8501, 8708, and 8709 are most likely to be those species (Table 3).

To ascertain identity of *S. lacrymans*, the entire 18S rDNA sequences of isolates 8015, 8704, 8804, and IFO30955 were analyzed. Each sequence showed high homology to *S. lacrymans* strains, with more than 99% identities (Table 3). Therefore, those four isolates can be confirmed as *S. lacrymans*.

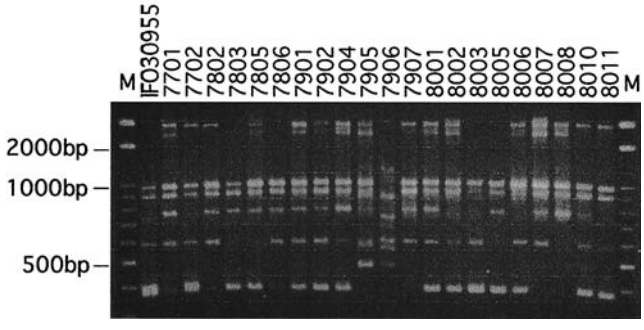


**Fig. 3.** An example of random amplified polymorphic DNA (RAPD) analysis (using primer OPH-03). Electrophoresis on 2% agarose gel in 1 × TBE buffer. Both *outer lanes* are DNA size marker (*M*). Isolate numbers are shown *above each lane*

## RAPD analysis

Of the 39 primers used, 5 primers (OPA-18, OPB-10, OPC-02, OPC-05, and OPH-03, Table 4) successfully yielded many polymorphic fragments. A typical example of a resulting image is shown in Fig. 3. In a total of 74 isolates and 4 type strains (IFO04944, IFO06430, IFO08697, IFO30955), 71 informative fragments were scored from the 5 primers. A dendrogram generated from genetic distances shows that isolates and type strains not demonstrated to be *S. lacrymans* in PCR-RFLP were at a distance from the cluster of *S. lacrymans*, except for 8708 (Fig. 4). Isolates 8015, 8704, 8804, and IFO30955, which were placed in different clusters

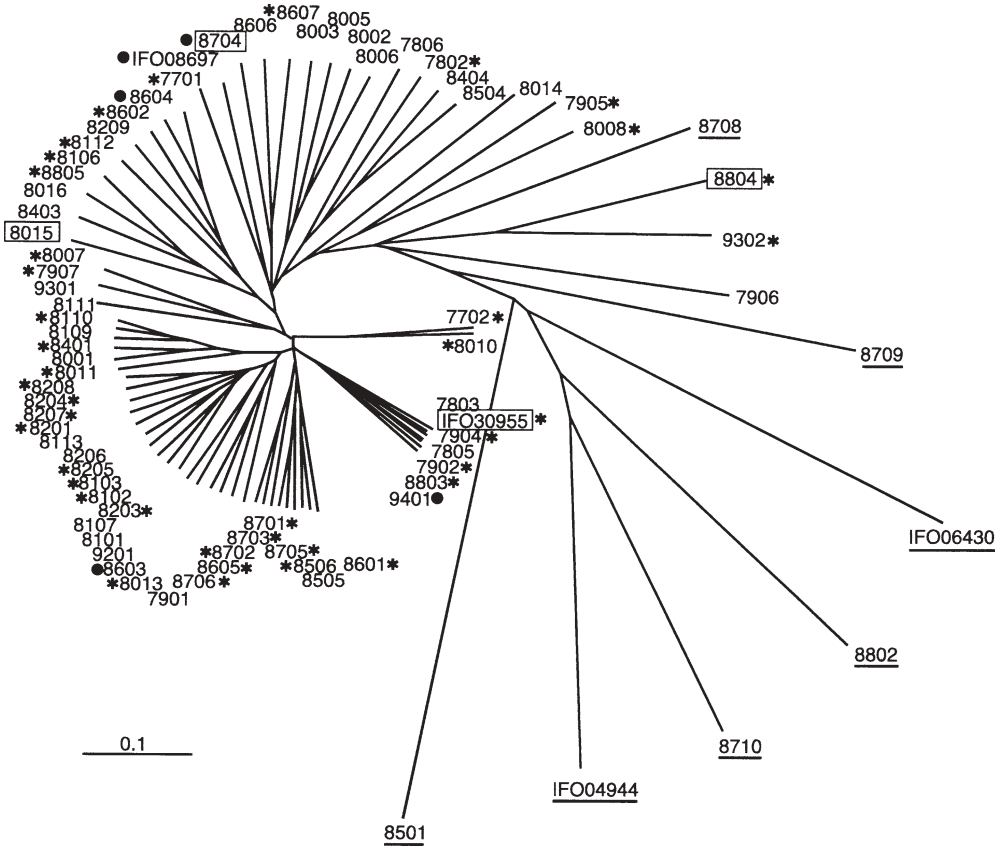


**Fig. 4.** Dendrogram based on the RAPD fragment patterns of isolates tested. The dendrogram was generated using the unweighted pair groups method (UPGMA). *Underlined isolates*, other species of *Serpula lacrymmans*, *asterisks*, isolates from Asahikawa, *circles*, isolates from Honshu. 18S rDNA of isolates in *boxes* were sequenced and showed high homology to *S. lacymans* in DDBJ database

**Table 4.** Sequence and number of informative fragments of random amplified polymorphic DNA (RAPD) primer

| Primer | Sequence | Number of informative fragments |
|---|---|---|
| OPA-18 | AGGTGACCGT | 10 |
| OPB-10 | CTGCTGGGAC | 18 |
| OPC-02 | GTGSGGCGTC | 17 |
| OPC-05 | GATGACCGCC | 17 |
| OPH-03 | AGACGTCCAC | 9 |

of *S. lacrymans,* were checked for sequences of 18S rDNA. Therefore, isolates examined by RAPD analysis were separated into *S. lacrymans* and others. The dendrogram revealed no relationship between the geographical origins of the isolates. For example, isolates from Asahikawa in Hokkaido or Honshu were not separately localized in the dendrogram.

## Discussion

Using PCR-RFLP analysis of the partial rDNA (ITS region), 74 isolates from the northern half of Japan were judged to be as *Serpula lacrymans* or related species. Double digestion using a pair of enzymes *Taq* I and *Hae* III as described in an earlier report[10] was not effective for this study; however, a result sufficient for discrimination was obtained only by *Taq* I supported by digestion by *Hha* I. Therefore, it is suggested that these two enzymes can provide information for the identification of *S. lacrymans*. In this study, some isolates were recognized as *Coniophora puteana* by comparing their RFLP patterns with type strain. It is possible to infer the species of unknown isolates by comparing their RFLP patterns with identified strains if the type strains are obtained.

Even if type strains for comparison were not prepared, it was possible to determine the species by searching the database in term of homology. The species can be identified with a high degree of certainty from rDNA sequencing; however, the cost of sequencing is high. To reduce the number of target fragments for sequencing, previous information such as PCR-RFLP patterns is cost effective.

Clustering generated from RAPD information separated a group of isolates inferred as *S. lacrymans* and related species by PCR-RFLP, except for one isolate. The placement of isolate 8708 in the dendrogram, which was diagnosed as *Oligoporus placentus* with the ITS sequence, is incorrect. This is thought to be due to an insufficient amount of informative fragments for the detection of interspecific diversity. However, more fragments were not obtained because the aim in this analysis was the detection of intraspecific diversity in *S. lacrymans*. From the result showing that geographical origin was not related to the clustering, it is suggested that *S. lacrymans* has very little intraspecific difference. The cause of this small degree of diversity could be considered to be due to similar environ-

mental conditions such as those found indoor, or due to the small geographic scale. Moreover, the intraspecific variation of *S. lacrymans* is reported to be less than that of *Serpula himantioides* .[12] The small degree of variation in *S. lacrymans* suggests the reliability of identification by PCR-RFLP. It also suggests that considerations degrading geographical condition are not necessary for pest control of *S. lacrymans*. From those, DNA-based assay will allow the detection of *S. lacrymans* before it spreads to or infects buildings.

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