Development of a Multiple Detection Technique for Fungi by DNA Microarray with the Simultaneous Use of Internal Transcribed Spacer Region of Ribosomal RNA Gene and β-Tubulin Gene Probes

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Received 27 May, 2013/Accepted 21 January, 2014

We offer the first description of the development of a multiple detection technique for fungi by DNA microarray with the simultaneous use of internal transcribed spacer region (ITS) of ribosomal RNA gene and β-tubulin gene probes. The assay uses 12 oligonucleotide probes and multiplex amplification to detect fungal species belonging to various sections of *Aspergillus*, the *Eurotium* genus, and the *Penicillium* genus. The specificity of each probe was tested using 231 reference fungal strains, including 79 target and 152 non-target strains in 102 species of 24 genera. We determined the optimum concentration of the primer pairs for multiplex PCR to be 0.5 μM for the β-tubulin gene and 0.125 μM for the ITS region. In the field trial using 76 specimens containing 323 fungi (up to five fungal strains were included in one specimen), the concordance rate between the DNA microarray and the DNA sequencing results was 97.4% at the species or genus levels.

Key words: DNA microarray / Multiplex detection / ITS region / β-tubulin gene / Fungi.

Fungi are widely distributed in the natural environment and fungal spores are easily dispersed into the air, with many causing serious biodeterioration and health problems. The main fungal species responsible for serious biodeterioration are included in the xerophilic fungi, which can grow in restricted water conditions, such as the *Eurotium* sp., *Aspergillus penicillioides*, and the non-xerophilic fungal species of *Penicillium* and other *Aspergillus*. People exposed to these airborne fungi (e.g. *Aspergillus fumigatus*), especially at high concentrations of spores, may suffer a variety of adverse health effects, including infectious diseases (Baedana, 1980). Furthermore, many fungal species that thrive in damp indoor environments are potent mycotoxin producers (e.g. *Aspergillus flavus*) and may play a role in reported adverse health effects (Jarvis and Miller, 2005).

The rapid detection of these fungal species existing in communal environments would aid in the prevention of fungal biodeterioration and public health problems. Conventional methods for the identification of fungi rely on microscopic or culture techniques that are time consuming and error-prone, especially when utilized for high-throughput analysis. Many molecular techniques have provided promising approaches that are complementary to the conventional methods. However, the immense diversity in fungi causes difficulties in true group-specific and species-specific detection via selective primer-based PCR amplification alone. In addition, processing many samples collected by an air sampling instrument or other methods, even via DNA sequence analysis, is not suitable because it is expensive and
requires the step of isolating a single strain from a culture containing several fungi, which is time consuming.

Recently, a two-step detection strategy utilizing probe hybridization techniques including DNA microarrays was developed to identify fungi (Neugebauer et al., 2009; Sato et al., 2010; Tambong et al., 2006; Wu et al., 2002). In this study, we adapted the DNA microarray technique to enable the analysis of samples of multiple numbers of fungi. Our aim was to develop an accurate and rapid assay system for the multiplex detection of the fungal species belonging to various sections of Aspergillus, the Eurotium genus, and the Penicillium genus. To this end, we utilized a multiplex PCR-based DNA microarray targeted to the ITS region and \( \beta \)-tubulin gene at the species or genus levels.

A total of 79 target strains representing 28 species (3 genera) from the genus of Eurotium (e.g. E. herbariorum), Aspergillus section Restricti (e.g. A. penicillioides), Fumigati (e.g. A. fumigatus), Flavi (A. flavus), Nidulantes (A. versicolor), Penicillium subgenus Penicillium (e.g. P. chrysogenum), and 152 non-target strains representing 74 species (20 genera) from the genus of Acremonium, Alternaria, Arthrinium, Byssolachyus, Chaetomium, Chrysosporium, Cladosporium, Colletotrichum, Diaporthe, Epicoccum, Fusarium, Monascus, Pythium, Scopulariopsis, Trichoderma, Trichonycton, Trichothecium, Verticillium, and an other Aspergillus section Nigri (e.g. A. niger), Terrei (e.g. A. terreus), Circumdati (e.g. A. ochraceus), Clavati (e.g. A. clavatus), Cremei (e.g. A. dimorphicus), and other Penicillium subgenus Aspergilloides (e.g. P. sclerotiorum) were used to evaluate the applicability of the probe with respect to specificity and sensitivity. The classification of Aspergillus referred to the paper of Peterson (2008), and that of Penicillium referred to the publication of Samson and Pitt (2001), respectively. These strains were obtained from the American Type Culture Collection (ATCC), Japan Collection of Microorganisms (JCM), and NITE Biological Resource Center (NBRC).

A total of 323 fungi used in field trials were collected from many facilities in Japan by air samplers (Biotest RCS Air Sampler). These fungi were grown on M40Y agar, containing a high concentration of sucrose (400 g/L) or potato dextrose agar (PDA) at 25°C for 1-2 weeks. Small fragments of the fungal cultures were punched out using a capillary glass tube with internal diameter of 1 mm. The genomic DNA was directly extracted from the punched-out fungi by using the QuickGene-810 system (Kurabo Industries, Osaka, Japan). Prior to DNA extraction, the samples were pretreated by disrupting the mycelia in a 2-mL screw-capped tube containing Zirconia beads (a mixture of 2 types of beads with different diameters; AMR Inc.; Gifu, Japan) by using the mini bead-beater BSP-3110BX (Wakenibtech Co., Kyoto, Japan) for 20 s at 2500 rpm.

A total of 104-probes were designed from the ITS region and \( \beta \)-tubulin gene sequences available in the Genbank database or our own sequencing data. Designed probes were synthesized and modified with 5′-NH\(_2\) to increase the binding to the silicon chip surface by Life Technologies Corporation (Tokyo, Japan). The DNA microarrays with diamond-like carbon coating (GENE SILICON\textsuperscript{TM}) were prepared at the Technical Research Laboratory, Toyo Kohan Co. (Yamaguchi, Japan). For multiplex PCR, the universal fungal primers Bt2a (5′-GGTAACAAATCGGTGCTGGTTC-3′) and Bt2b (5′-ACCCCTAGTGTAGTACCCGTCG-3′) described by Glass and Donaldson (1995) were used to amplify the \( \beta \)-tubulin gene, and the universal fungal primers ITS1-Fw (5′-TGGTCTATTTAGGAAAGTAAGAATC-3′) and ITS1-Rv (5′-CTCGGTTCCTCCATCGATGC-3′) were used to amplify the ITS regions. ITS primer sets were designed from ITS1-F (Gardes and Bruns, 1993) and ITS-2 (White et al., 1990) primer sequences with minor modifications, respectively.

Reaction mixtures (20 \( \mu \)L) contained about 100-400 pg of genomic DNA, 0.5 \( \mu \)M \( \beta \)-tubulin gene primer (each), ITS region primers (each, 0.5 \( \mu \)M, 0.25 \( \mu \)M, 0.125 \( \mu \)M, 0.0625\( \mu \)M, or 0.03125\( \mu \)M), 1 U of NovaTaq\textsuperscript{TM} Hot Start DNA polymerase (Merck, KGaA, Darmstadt, Germany), dNTP mixture (50 \( \mu \)M dATP, 50 \( \mu \)M dTTP, 50 \( \mu \)M dGTP, 40 \( \mu \)M dCTP; Toyobo Co., Osaka, Japan), 10 \( \mu \)M Cy-5 labeled-dCTP (GE Healthcare Limited, Buckinghamshire, United Kingdom), and reaction buffer (1 \( \times \) Ampdirect\textsuperscript{®} G/C, Amp Addition-4; Shimadzu Corporation, Kyoto, Japan). Reactions were performed in a PCR Thermal Cycler Dice\textsuperscript{®} Gradient (Takara Bio Inc., Shiga, Japan), with initial denaturation at 95°C for 10 min, followed by 40 cycles (30 s at 95°C, 30 s at 56°C, and 1 min at 72°C). The amplicons were confirmed by MCE-202 MultiNA (Shimazu) analysis. The amplicons for ITS sequencing were generated by PCR using the two primers: ITS1 (5′-TCCGTAAGTTAACCCTCGG-3′) and ITS4 (5′-TCCTCCGGCTATTGATATGC-3′) (Gardes and Bruns, 1993; White et al., 1990).

For hybridization analysis, amplicons (3\( \mu \)L) were added to a tube containing 1.5\( \mu \)L hybridization solution (3 \( \times \) SSC containing 0.3% SDS and 15 \( \times \) Denhardt solution). The mixture in the tube was then transferred to the DNA microarray and incubated at 45°C for 1 h. After hybridization, unbound amplicons were washed out twice with 2 \( \times \) SSC/0.2% SDS at room temperature for 5 min, and twice with 2 \( \times \) SSC at room temperature for 5 min. The fluorescent signal was detected with a fluorescent scanner, BIOSHOT\textsuperscript{TM} (Toyo Kohan). Image processing and calculation of the signal
intensities were performed with the "Shot Analyzer" program version 1.133 (Toyo Kohan).

First, extensive screening revealed that many designed probes cross-reacted with heterologous species or produced weak hybridization signals with homologous species (data not shown). As a result, twelve probes (ITS: 6, β-tubulin: 6) could be selected for the target species (Table 1). The specificity of the β-tubulin gene probes was found to be higher than that of the ITS probes by using single DNA as a template (Table 1). The graph in Figure 1 shows the fluorescence intensity of the DNA microarray analysis. Only probes of Ap-n2 and Apen-beta2r for detecting A. penicillioides belonging to Aspergillus Section Restricti were species-specific (these probes did not react with A. restrictus belonging to the same section), whereas the other probes were genus or group-specific. Af1-1and Afum-beta2 probes could detect A. fumigatus, Neosartorya fischeri, and N. hiratsukae belonging to Aspergillus section Fumigati. Af1a-1r and Afla-beta7 probes could detect A. flavus, A. oryzae, A. parasiticus, and A. sojae belonging to Aspergillus section Flavi. Av1-2 and Aver-beta1r probes could detect A. versicolor and A. sydowii belonging to Aspergillus section Nidulantes. Eur1-2 and Eur-beta2 probes could detect E. herbariorum, E. amstelodami, E. carnoyi, E. chevalieri, E. echinulatum, E. intermedium, E. niveoglaucum, E. repens, E. rubrum, E. tonophilum, and A. proliferans belonging to the Eurotium genus. Pae1-2 and Pall2-beta1r probes could detect P. chrysogenum, P. aurantiogriseum, P. commune, P. expansum, P. griseofulvum, P. hisricum, and P. roqueforti belonging to the Penicillium subgenus Penicillium. These probes did not react to closely-related species, for example, Afla1-1r and Afla-beta7 did not react to A. niger belonging to Aspergillus section Nigri, Pae1-2 and Pall2-beta1r did not react to P. sclerotiorum belonging to Penicillium subgenus Aspergilloides (data not shown).

In the evaluation of primer pair concentration in simultaneous amplification for target fungal strains, the concentration of β-tubulin primer was fixed at a final concentration of 0.5 μM. On the other hand, that of the ITS primer set was changed to 0.03125 μM from 0.5 μM as the final concentration, because its copy number of the ITS region in the genome was significantly greater than that of the β-tubulin gene (Einsele et al., 1997; May et al., 1985). The primer concentration was adjusted by analyzing the fluorescence intensity and confirming the simultaneous amplification of the ITS region and β-tubulin gene. The fluorescence intensity of the β-tubulin probes were mostly weak at the final concentration of 0.5μM each (Fig 1, lane 1) compared with the primer pair concentration (Fig 1, lane 2-5), especially of Afum-beta2 for A. fumigatus and Afla-beta7 for A. flavus. On the other hand, the fluorescence intensity of ITS probes decreased dependently on concentration, and especially Pae1-2 for P. chrysogenum was not detected. Best results were obtained with the final primer pair concentration of the ITS region between 0.25 μM to 0.0625 μM, and that of the β-tubulin gene at 0.5 μM. Furthermore, mixture samples, each containing 3-5 wild type fungi in one tube, were used for evaluation. The trend of the fluorescence intensity of the ITS or β-tubulin probes was similar to the result using the reference strains. In the electrophoretic profile obtained for each target region, an approximately 450-bp PCR fragment, predicted with the partial region of the β-tubulin gene, was observed at the primer pair concentration of

| Probea | Sequence (5’-3’) | Number of false-reaction strains per tested 231 strains |
|--------|------------------|--------------------------------------------------------|
| A1     | Ap-n2            | GAGACCTAACCATGACACTCATCATCATGACACGCACGC | 2 |
|        | Apen-beta2r      | CATCCTGAGCATGACACGCACGC | 1 |
| A2     | Af1-1            | GAAGCTGTTGCTGAAGTATGACATCAGATCGTCGTCG | 5 |
|        | B: Aflm-beta2    | AACATCTACGATCTGACCTGACCTG | 0 |
| A3     | Afla1-1r         | GCAACTAAGGTACAGTAACAAAC | 11 |
|        | Afla-beta7       | TGAACAAGCTTTTGAACACTCC | 0 |
| A4     | Av1-2            | ACTACTGAACCTTCATGACAGATGG | 2 |
|        | Aver-beta1r      | TTGTAGCTGAGTTTGAGGAGG | 0 |
| EU     | Eur1-2           | GCTGTAGGTGTTTAGTAACCAATT | 5 |
|        | Eur-beta2        | AGGGTCACACCCACAAATATG | 0 |
| PE     | Pae1-2           | AGTCCTGAGAAAATATAATTATTA | 0 |
|        | B: Pall2-beta1r  | TGTCATGTGACAACACGCACGC | 1 |

| Detection target group (main target species) | Probea | Sequence (5’-3’) | Number of false-reaction strains per tested 231 strains |
|---------------------------------------------|--------|------------------|--------------------------------------------------------|
| A1   Aspergillus Section Restricti (Aspergillus penicillioides) | Ap-n2  | GAGACCTAACCATGACACTCATCATCATGACACGCACGC | 2 |
|      | Apen-beta2r      | CATCCTGAGCATGACACGCACGC | 1 |
| A2   Aspergillus Section Fumigati (Aspergillus fumigatus) | Af1-1  | GAAGCTGTTGCTGAAGTATGACATCAGATCGTCGTCG | 5 |
|      | B: Aflm-beta2    | AACATCTACGATCTGACCTGACCTG | 0 |
| A3   Aspergillus Section Flavi (Aspergillus flavus) | Afla1-1r | GCAACTAAGGTACAGTAACAAAC | 11 |
|      | Afla-beta7       | TGAACAAGCTTTTGAACACTCC | 0 |
| A4   Aspergillus Section Nidulantes (Aspergillus versicolor) | Av1-2  | ACTACTGAACCTTCATGACAGATGG | 2 |
|      | Aver-beta1r      | TTGTAGCTGAGTTTGAGGAGG | 0 |
| EU   Aspergillus Section Aspergillus (Eurotium herbariorum) | Eur1-2 | GCTGTAGGTGTTTAGTAACCAATT | 5 |
|      | Eur-beta2        | AGGGTCACACCCACAAATATG | 0 |
| PE   Penicillium subgenus Penicillium (Penicillium chrysogenum) | Pae1-2 | AGTCCTGAGAAAATATAATTATTA | 0 |
|      | B: Pall2-beta1r  | TGTCATGTGACAACACGCACGC | 1 |

<sup>a</sup> I : ITS, B : β-tubulin
**FIG. 1.** Fluorescence intensity of ITS and β-tubulin probes in multiplex PCR. Lanes 1, 0.5 μM: 0.5 μM (combination of final primer concentration, β-tubulin: ITS); 2, 0.5 μM: 0.25 μM; 3, 0.5 μM: 0.125 μM; 4, 0.5 μM: 0.0625 μM, 5, 0.5 μM: 0.03125 μM. MIX WT, Mixture sample of wild-type fungi isolated from the indoor environment. Target species. Non-target species.

| Species (strain No.) | Fluorescence intensity |
|----------------------|------------------------|
|                      | ITS                    | β-tubulin               |
| A. penicillioides    | [Graph]                | [Graph]                |
| A. fumigatus         | [Graph]                | [Graph]                |
| A. flavus            | [Graph]                | [Graph]                |
| A. versicolor        | [Graph]                | [Graph]                |
| E. herbariorum       | [Graph]                | [Graph]                |
| P. chrysogenum       | [Graph]                | [Graph]                |
| A. flavus            | [Graph]                | [Graph]                |
| A. niger             | [Graph]                | [Graph]                |
| Cladosporium sp.     | [Graph]                | [Graph]                |
| MIX WT               | [Graph]                | [Graph]                |
| A. penicillioides    | [Graph]                | [Graph]                |
| Eurotium sp.         | [Graph]                | [Graph]                |
| Penicillium sp.      | [Graph]                | [Graph]                |
| Cladosporium sp.     | [Graph]                | [Graph]                |

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| Species (strain No.) | Fluorescence intensity |
|----------------------|------------------------|
|                      | ITS                    | β-tubulin               |
| A. penicillioides    | [Graph]                | [Graph]                |
| A. fumigatus         | [Graph]                | [Graph]                |
| A. flavus            | [Graph]                | [Graph]                |
| A. versicolor        | [Graph]                | [Graph]                |
| E. herbariorum       | [Graph]                | [Graph]                |
| P. chrysogenum       | [Graph]                | [Graph]                |
| A. flavus            | [Graph]                | [Graph]                |
| A. niger             | [Graph]                | [Graph]                |
| Cladosporium sp.     | [Graph]                | [Graph]                |
| MIX WT               | [Graph]                | [Graph]                |
| A. penicillioides    | [Graph]                | [Graph]                |
| Eurotium sp.         | [Graph]                | [Graph]                |
| Penicillium sp.      | [Graph]                | [Graph]                |
| Cladosporium sp.     | [Graph]                | [Graph]                |

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**Species (strain No.)**

- **A1**: A. penicillioides (JCM22971)
- **A2**: A. fumigatus (JCM10253)
- **A3**: A. flavus (JCM10252)
- **A4**: A. versicolor (NBRC30338)
- **EU**: E. herbariorum (JCM1575)
- **PE**: P. chrysogenum (JCM22555)
- **MIX WT**: Mixed sample of wild-type fungi isolated from the indoor environment.

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**Legend**

- **Ap-n2**: Ap-n2
- **Apen-beta2r**: Apen-beta2r
- **Af1-1**: Af1-1
- **Afla-beta7**: Afla-beta7
- **Av1-2**: Av1-2
- **Eur-beta2**: Eur-beta2
- **Pae1-2**: Pae1-2
- **Pall2-beta1r**: Pall2-beta1r

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**Notes**

- **Target species**
- **Non-target species**
0.03125 μM - 0.25 μM for ITS, and 0.5 μM for β-tubulin (data not shown). An approximately 280-bp PCR fragment predicted with the partial ITS region was observed at the primer pair concentration of 0.125 μM - 0.5 μM for ITS, and 0.5 μM for β-tubulin (data not shown).

Finally, we determined the optimum primer pair concentration for simultaneous amplification in one tube to be 0.5 μM for Bt2a/Bt2b (β-tubulin gene) and 0.125 μM for ITS1-Fw/ITS1-Rv (ITS region). Although the final concentration of the ITS primer (0.125 μM) utilized in this study was low compared with the concentration used in standard PCR conditions, this concentration could co-amplify the ITS region with the β-tubulin gene because of its high copy number in the fungal genome. To examine the sensitivity and specificity of the DNA microarray assay system, we collected airborne fungi on the M40Y or PDA medium, and genomic DNAs were batch-extracted from the cultured medium directly (up to five fungal strains were included in one tube). The hybridization signals were calculated using the signal-to-noise ratio [SNR; (Signal median - Background)/Background]. Evaluation of signals, positive or negative, are described below. If the SNR values of both of ITS region and β-tubulin probes were greater than 3.0, the assay was scored as positive for existence of target fungi. If the SNR values of both or either were less than 3.0, the assay was scored as negative. The performance of the DNA microarray was validated by comparison with the results obtained from the ITS region sequence analysis.

Figure 2 summarizes the hybridization reactions of the 76 specimens, that contained a total of 323 fungi. Among the 76 specimens, 74 (97.4%) were accurately found to be the target fungal species (positive reaction shown in black squares) and non-target species (shown in blank). The concordance rate between the DNA microarray results and DNA sequencing was 97.4% (74/76), whereas that between the results of morphological observation and DNA sequencing was 93.4% (71/76; data not shown).

If we used either the ITS probes or β-tubulin probes for detecting the target fungi, the concordance rate of the sequencing results and DNA microarray results was reduced from 97.4% to 78.9%. The ITS probes Av1-2 (for A. versicolor) for sample numbers 25 and 31; Afla1-1r (for A. flavus) for sample numbers 60, 62, and 67; Pae1-2 (for P. chrysogenum) for sample numbers 60 and 67 were cross-hybridized to some non-target species or non-specific amplicons (shown in white squares). Furthermore, some false reactions were also observed with the β-tubulin probes, although these probes showed high specificity when evaluated during the probe selection using a singleplex PCR assay. The probes Apen-beta2r (for A. penicillioides) for sample numbers 10 and 13 and Eur-beta2 (for Eurotium spp.) for sample numbers 12, 17, 43, 44, 54, 58, and 72 were cross-hybridized to some non-specific amplicons (shown in white squares).

These results demonstrate that a single probe alone is not sufficient for effective species or genus level identification by using the DNA microarray assay for samples which contained various fungal species. The advantage of using multiple probes is that false reactions due to cross-hybridization can be avoided to a larger degree, and a greater number of fungi can be analyzed in one test.

We also observed false-negative reactions (shown in white triangles) despite the presence of the target species. Comparatively, despite the ITS sequences of Eurotium sp. in sample number 74, and P. chrysogenum in sample number 76 perfectly matching the corresponding probes for the detection of Eurotium species (Eur1-2, Eur-beta2) and Penicillium subgenus Penicillium. (Pae1-1, Pall2-beta1r), they did not display clear positive results. We do not know why these 2 samples showed false-negative results despite inclusion of the target fungal species. The probe-binding site of the amplicons might have formed strong secondary structures, which do not open under the conditions of hybridization. Indeed, previous studies have utilized parameter analyses to reveal that false-negative results were mainly due to reduced accessibility of probe-binding sites caused by the secondary structures of the target molecules (Peplies et al., 2003; Wei et al., 2012). Another possible reason for the false-negative results is that the concentration of the target DNA was too low in the mixed DNA sample, and thus, non-target amplicons were preferentially amplified.

On the other hand, the false-positive judgment was not indicated in all probes. For example, we confirmed that P. sclerotiorum belonging to Penicillium subgenus Aspergilloides, A. restrictus belonging to Aspergillus section Restricti, and A. sclerotiorum belonging to Aspergillus section Circumdati as non-target fungal species were included in sample No. 36, No.40, and No.46 by the ITS region sequencing, but the false-positive reaction was not detected in probes for the target fungal species of Aspergillus, and Penicillium. The fungal species A. fumigatus and A. flavus were not collected in this field trial, but the probe of Afl-1 and Afum-beta2 for A. fumigatus, Afla1-1r and Afla-beta7 for A. flavus at least did not show false-positive reaction.

The common probes for fungi, namely the universal ITS probe Fs-4 (5’-CAACAACGGATCTTGGGTTTTC-3’), reacted with all samples, whereas the samples numbers 14, 20, 40, 42, 59, 61, and 66 did not react with the universal β-tubulin probe Fun-beta2 (5’
FIG. 2. Summary of hybridization patterns. Results are indicated by the following symbols: ■, Positive (SNR>3); □, Non-specific signal (SNR>3); △, False-negative (SNR<3). A1: A. penicillioides, A2: A. fumigatus, A3: A. flavus, A4: A. versicolor, EU: E. herbariorum, PE: P. chrysogenum, X: non-target species e.g. Cladosporium sp., Alternaria sp. CF: Fungi common. I: ITS probes, B: β-tubulin probes.
-GAGCCCGGTACCATGGACGC-3’). This might be because the amplicon of β-tubulin gene was not amplified or amplified at only a low level by the mismatch of sequences between the forward primer Bt2a and template DNA. For example, we confirmed that the Bt2a and its binding site sequence of Cladosporium sp. or Alternaria sp. were a 2-4 nucleotide mismatch (data not shown). In the case of various inspection scenes (e.g. food and environment samples), these probes, especially Fs-4 for detecting ITS seem to be helpful when judging whether the provided foreign body samples are fungi or not.

This study presents a multiplex PCR-based DNA microarray assay system for the multiplex detection of fungi with the simultaneous use of ITS region and β-tubulin gene probes. In this protocol, the genomic DNA was directly batch-extracted from cultured fungi without the step of isolation to a single strain. We confirmed at least that our assay is able to detect target fungal species from the batch extraction of DNA from up to five fungal strains mixed with other various non-target fungal species. This assay system is an accurate and rapid technique for the simultaneous high-throughput detection of target fungi in a mixed sample which contains many different fungal species. The primer set used in the multiplex PCR is fungal universal, and thus applicable to other fungal species. More detailed mycoflora analysis will be enabled by increasing the detection of target fungal species by further development of probes.

ACKNOWLEDGMENTS

We thank Dr. Kousuke Takatori, NPO Center for Fungal Consultation, for his technical help with the morphological identification, for providing the fungal species, and for his helpful comments. We also thank Dr. Tetsuaki Tsuchido, Department of Life Science and Biotechnology, Kansai University, Dr. Rika Kigawa, independent administrative institution, National Research Institute for Cultural Properties, Tokyo, Dr. Yuko Kumeda, Osaka Prefectural Institute of Public Health, Sadatoshi Miura, Japan Institute of Insect Damage to Cultural Properties, and Kazushi Kawagoe, Ikari Corporation, for their advice and help in the selection of the target fungi.

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