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Identification of Medically Important Molds by an Oligonucleotide Array†

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Infections caused by fungi have increased in recent years. Accurate and rapid identification of fungal pathogens is important for appropriate treatment with antifungal agents. On the basis of the internal transcribed spacer 1 (ITS 1) and ITS 2 sequences of the rRNA genes, an oligonucleotide array was developed to identify 64 species (32 genera) of clinically important filamentous (or dimorphic) fungi. These 64 species included fungi causing superficial, cutaneous, subcutaneous, and invasive infections. The method consisted of PCR amplification of the ITS regions using a pair of universal primers, followed by hybridization of the digoxigenin-labeled PCR products to a panel of species- or group-specific oligonucleotides immobilized on a nylon membrane. Of 397 fungal strains (290 target and 107 nontarget strains) tested, the sensitivity and specificity of the array was 98.3% (285/290) and 98.1% (105/107), respectively. Misidentified strains were usually those belonging to the same genus of the target species or having partial homology with oligonucleotide probes on the membrane. The whole procedure can be finished within 24 h starting from isolated colonies; reproductive structures, which are essential for the conventional identification methods, are not needed. In conclusion, the present array is a powerful tool for identification of clinically important filamentous fungi and may have the potential to be continually extended by adding further oligonucleotides to the array without significantly increasing the cost or complexity.

The identification of molds (filamentous fungi) can be challenging, and accuracy will depend on the organism and the experience of the clinical microbiologist (20). Conventional methods for fungal identification in the clinical laboratory are based on morphological and physiological tests. These methods often require several days or even weeks and may be inaccurate (30). In the last few decades, invasive mycoses have become a major cause of infectious morbidity and mortality in patients receiving immunosuppressive chemotherapy for cancer or organ transplantation and in immunodeficient patients, such as individuals with AIDS (2, 3, 9, 14, 33). Since invasive mycoses are often associated with a poor prognosis, the early, rapid, and accurate identification of the pathogenic fungi is important for timely and appropriate management.

In recent years, numerous DNA-based methods have been developed to diagnose mycotic infections and to identify pathogenic fungi (6). PCR methods are particularly promising because of their simplicity, specificity, and sensitivity. Genes of the 18S rRNA (27, 41, 47) and 28S rRNA (15, 28, 38) have been extensively used for molecular identification. Recently, Hall et al. (20) evaluated the commercial MicroSeq D2 large-subunit rRNA gene fungal sequencing kit (Applied Biosystems, Foster City, Calif.) and drew the conclusion that the kit offers the promise of being an accurate identification system. However, they noted that 33% of clinical isolates could not be identified due to a limited sequence database in this commercial system. In addition, the MicroSeq kit requires an automatic sequencer, which is not yet standard equipment for most clinical microbiology laboratories. A number of studies have described probes, restriction fragment length polymorphism, or other methods to identify unique ribosomal DNA sequences (26, 35, 44). Although these published methods are useful for the identification of fungal species, they can identify only one or a limited number of species. The ribosomal internal transcribed spacer (ITS) regions also have been widely used as targets to detect and identify human fungal pathogens (5, 7, 10, 21, 23, 24, 31–33, 43).

DNA array (or DNA chip) technology has been found to be a useful tool to identify a variety of bacteria, especially for those bacteria that are difficult to differentiate by conventional methods or when the identification procedures may take a long time (1, 17, 45). Wu et al. (47) recently developed an array of oligonucleotide probes to detect airborne fungi. The probes were designed on the basis of the 18S rRNA gene and were used to detect 31 species belonging to 15 genera. The aim of this study was to investigate the feasibility of using a panel of oligonucleotide probes designed on the basis of the ITS regions to identify 64 mold species (32 genera) of clinical importance.

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MATERIALS AND METHODS

Fungal strains. A total of 397 strains including 290 target (64 species) and 107 nontarget strains were used (see Table S1 in the supplemental material) (sup-

plemental Table S1 and supplemental Fig. S2 from this study are supplied as supplemental files). Of the 290 target strains, 233 were reference strains obtained from culture collection centers and 57 were clinical isolates. Of the 107 nontarget strains, 93 were reference strains and 14 were clinical isolates. Reference strains were obtained from the American Type Culture Collection (Manassas, Va.), Bioresources Collection and Research Center (BCRC, Hsichu, Taiwan, Republic of China), and CBS (Centraalbureau voor Schimmelcultures, The Netherlands). Clinical isolates were obtained from the Mycology Reference Centre, Division of Microbiology University of Leeds, Leeds, UK (strain designated with a prefix of LM), and from the Laboratory of Parasitology and Mycology of Angers University Hospital (Angers, France) (strain designated with a prefix of LMA). Morphological identification of clinical isolates to the species level was accomplished using established procedures including microscopic and macroscopic characteristics (11, 30). All fungal strains were subcultured on Sabouraud dextrose agar (Difco, Detroit, Mich.), incubated at 30°C until there was evidence of apparent hyphal growth, and then used for DNA extraction.

DNA extraction. Mycelium (approximately 0.5 × 0.5 cm) was scraped into a 2-ml screw cap tube (Azygen Sientific, Union City, Calif.) containing 300 mg of 0.5-mm-diameter zirconium/silica beads (Biospec Products, Bartlesville, Okla.) in 1 ml of sterilized water using an inoculation loop. The mycelial suspension was shaken for 5 min at a speed of 4,200 rpm in a mechanical cell disrupter (Mini-Beadbeater, Biospec Products). An aliquot (0.1 ml) of the disrupted cell suspension was transferred to a 1.5-ml centrifuge tube and centrifuged at 8,000 × *g* for 10 min in a microcentrifuge. Fungal DNA in the supernatant was extracted by a genomic DNA extraction kit (Viogene, Taipei, Taiwan).

ITS amplification and sequencing. The ITS sequences of some species that were not available in the database of GenBank were determined in this study (see Table S1 in the supplemental material). The fungus-specific, universal primers ITS 1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS 2 (5′-GCTGCGTT CTTCATCGATGC-3′) (46) were used to amplify a small conserved portion of the 18S ribosomal DNA, the adjacent ITS 1, and a small portion of the 5.8S ribosomal DNA. An additional pair of primers, ITS 3 (5′-GCA TCGATGAAG AACGCAGC-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) (46), were used to amplify a conserved portion of the 5.8S ribosomal DNA, the intervening ITS 2, and a small portion of the 28S ribosomal DNA. PCR was performed with 5 μl (1 to 5 ng) of template DNA in a total reaction volume of 50 μl consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.8 mM deoxyribonucleoside triphosphates (0.2 mM each), 0.7 μM primer (each), *Taq* DNA polymerase (1.25 U), and 50 μl of a mineral oil overlay. PCR was carried out with an OmniGen thermal cycler (Hybaid Limited, Middlesex, UK) under the following conditions: initial denaturation, 94°C, 3 min; 35 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 1 min); and final extension, 72°C, 5 min. A negative control was performed with each test run by replacing the template DNA with sterilized water in the PCR mixture. PCR products were purified with a PCR-M CleanUp kit (Viogene, Taipei, Taiwan) and were sequenced on a model 377 sequencing system (Applied Biosystems, Taipei, Taiwan).

To amplify the ITS regions for array hybridization, the primer pair ITS 1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGA TATG-3′) (46) was used to amplify a fragment encompassing ITS 1, the 5.8S rRNA gene, ITS 2, and partial regions of the 18S and 28S ribosomal DNA. The reverse primer ITS 4 was labeled with a digoxigenin molecule at its 5′ end and was synthesized by MDBio Inc. (Taipei, Taiwan). PCR conditions were the same as described in the previous section. DNA extracted from *Saccharomycodes ludwigii* BCRC 21378 was used as a positive control for each run of PCR and array hybridization.

Design of oligonucleotide probes. Species- or group-specific oligonucleotide probes (21- to 30-mers) used for identification of 64 fungal species are listed in Table 1. Probe design was based on sequence data from the ITS 1 or 2 regions; these sequences were either available in the GenBank database or determined in this study. Probe selection was facilitated by using visual sequence alignment employing the PrettyBox command of the Wisconsin Genetics Computer Group package (version 10.3; Accelrys Inc., San Diego, Calif.). Areas displaying sequence divergence among different species were analyzed for probe selection. A total of 58 probes, including one positive control (a probe designed on the basis of the ITS 2 region of *Saccharomycodes ludwigii* BCRC 21378), were used for fabrication of the oligonucleotide array (Table 1). The designed probes were checked for internal repeat, self-biding, secondary structure, and GC content by using Vector NTI software (Invitrogen Corporation, Carlsbad, Calf.). Seven additional bases of thymine were added to the 3′ end of each probe (4). The specificity of the prospective sequence was first analyzed with a fungal ITS database developed in our laboratory and with sequences available in the GenBank using BLAST.

Fabrication of oligonucleotide arrays. The arrays (1.1 by 0.9 cm) (Fig. 1) were made in batches of 20. The oligonucleotide probes were diluted 1:1 (final concentration 10 μM) with a tracking dye solution (30% [vol/vol] glycerol, 40% [vol/vol] dimethyl sulfoxide, 1 mM disodium EDTA, 0.15% [wt/vol] bromophenol blue, and 10 mM Tris-HCl, pH 7.5). The probe solutions were drawn into different wells of a round-bottom 96-well microtiter plate and spotted onto a positively charged nylon membrane (Roche, Mannheim, Germany) with an automatic arrayer (Wittech, Taipei, Taiwan) using a solid pin (500 μm in diameter). The array contained 80 dots, including 58 dots for identification of 64 medically relevant molds (32 genera), 1 for positive control (probe code PC), and 5 for negative controls (probe code NC; tracking dye only) (Fig. 1). In addition, 16 dots (probe code M) contained 5′-digoxigenin-labeled ITS 4 (final concentration 0.16 μM); these dots formed a cross on the array after hybridization and were used as markers to visually locate hybridized probes (see Fig. S2 in the supplemental material). Once all probes had been applied, the membrane was air-dried and exposed to shortwave UV (Stratalinker 1800; Stratagen, La Jolla, Calf.) for 30 s. Unbound oligonucleotides were removed by two washes (2 min each) at room temperature in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS). The arrays were stored at room temperature for further use.

Hybridization procedures. Except where otherwise indicated, the hybridization procedures were carried out at room temperature (approximately 27°C) with a shaking speed of 60 rpm. Most reagents, except buffers, were included in the DIG Nucleic Acid Detection kit (catalog no. 1175041; Roche). Each array was prehybridized at 50°C for 2 h with 1 ml of hybridization solution (5× SSC, 1% [wt/vol] blocking reagent, 0.1% *N*-laurylsarcosine, 0.02% SDS) in an individual well of a 24-well cell culture plate. The digoxigenin-labeled PCR product amplified from an isolate was heated in a boiling water bath for 5 min and immediately cooled on an ice bath. Ten microliters of denatured PCR products of the test organism and of the positive control (*Saccharomycodes ludwigii*) were diluted with 0.3 ml of hybridization solution and added to each well. Hybridization was conducted at 55°C for 90 min. After removing the nonhybridized PCR products, the array was washed four times (5 min each) in 1 ml of 0.25× SSC–0.1% SDS, followed by incubation for 1 h with 1 ml of blocking solution (1% [wt/vol] blocking reagent dissolved in maleic acid buffer [0.1 M maleic acid, 0.15 M NaCl, pH 7.5]). After removal of the blocking solution, 0.3 ml of alkaline phosphatase-conjugated sheep antidigoxigenin antibodies (diluted 1:2,500 in blocking solution) was added to each well and incubated for 1 h. The array was washed three times (each 15 min) in 1 ml of washing solution (0.3% [vol/vol] Tween 20 in maleic acid buffer), followed by washing in 1 ml of detection buffer (0.1 M Tris-HCl, 0.15 M NaCl, pH 9.5) for 5 min. Finally, 0.3 ml of alkaline phosphatase substrate (a stock solution of nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate diluted 1:50 in detection buffer) was added to each well and incubated at 37°C without shaking. Color development was visible between 30 min and 1 h after the start of the reaction.

Definition of sensitivity and specificity. A fungal strain was identified as one of the 64 target species when the probe (or one of several probes) designed for the species and the positive control probe (see Fig. S2 in the supplemental material) were hybridized. Sensitivity was defined as the number of target strains correctly identified (true positives) divided by total number of target strains tested (36). When a strain hybridized to its corresponding group-specific probe, the strain was considered to be correctly identified. Specificity was defined as the number of nontarget strains producing negative hybridization reactions (true negatives) divided by total number of nontarget strains tested (36). Occasionally, the PCR product of one strain hybridized to more than one species-specific probe on the array (i.e., cross-hybridization). Under these conditions, the strain was identified as the species corresponding to the probe that produced the most intense hybridization signal as judged visually. When a strain produced discrepant identification results between the conventional methods and the array, the ITS 1 and 2 regions of the isolate were amplified by PCR and sequenced for species clarification.

RESULTS

Probe development. In the beginning of this study, between one and seven probes (data not shown) were designed for identification of each species (or a group of species) and a total of 213 probes were synthesized to identify 64 fungal species listed in Table S1 in the supplemental material. Through extensive hybridization screening, many probes cross-reacted with heterologous species or produced no hybridization signals

TABLE 1. Oligonucleotide probes used in this study

Microorganism	Probe code[a]	Sequence (5′ to 3′)[b]	Length (bp)	T_m (°C)	Location[c]	GenBank accession no.
Absidia corymbifera	Abcor3	CTGGGCTTCTAGTTGATGGCATTTAGTTGC	30	57.9	146–175 (2)	AF117938
Acremonium falciforme	Acfal1	GGAAGCCCCCTGCGGGCACAA	21	49.7	39–59 (2)	AY830120[d]
Acremonium kiliense	Ackil2	TCTGATTTTATTGTGAATCTCTGAGGGGCG	30	55.2	111–140 (1)	AJ621775
Acremonium kiliense	Ackil3	TGATTTTATTGTGAATCTCTGAGGGGCGAA	30	53.8	113–142 (1)	AJ621775
Acremonium strictum	Acstr2	CAGCCTCCCCTGCGTAGTAGCA	22	55.3	422–443 (2)	AJ621771
Alternaria alternata	Alalt3	CGCACTCTCTATCAGCAAAGGTCTAGCATC	30	57.9	461–490 (2)	AY625056
Aspergillus clavatus	Ascla3	CGACACCAACCCAATYTTTCTAAGGT	26	52.1	518–543 (2)	AY373847
Aspergillus flavus	Asfla4	CGAACGCAAATCAATCTTTTTCCAGGT	27	51.6	512–538 (2)	AY373848
Aspergillus fumigatus	Asfum2	GCCAGCCGACACCCAACTTTATTTTTCTAA	30	55.2	213–242 (2)	AY230140
Aspergillus nidulans	Asnid2	GCGTCTCCAACCTTATTTTTCTCAGGT	27	53.1	483–509 (2)	AY373888
Aspergillus niger	Asnig2	ACGTTTTCCAACCATTCTTTCCAGGT	26	51.3	517–542 (2)	AY373852
Aspergillus terreus	Aster2	CCGACGCATTTWTTGTGCAACTTGTTT	27	51.6	493–520 (2)	AJ001333
Aspergillus versicolor	Asver4	ACGTCTCCAACCATTTTCTTCAGGT	25	50.9	486–510 (2)	AY830119[d]
Aureobasidium pullulans	Aupul2	ATTTCTAACAACGCTCTTTGGGTCGGTACG	30	56.5	454–483 (2)	AF121283
Aureobasidium pullulans	Aupul3	TCAAAGGAGAGGACTTCTGCCGACTGAAAC	30	57.9	456–485 (2)	AY139395
Aureobasidium pullulans	Aupul4	GGCGTAGTAGAATTTATTCGAACGTCTGTC	30	55.2	428–457 (2)	AY139395
Beauvera bassiana	Bebas5	GGGACCTCAAACTCTTGTATTCCAGCATC	29	56.4	166–194 (1)	AJ560691
Bipolaris spicifera	Bispi	TCTTTGGCCCGCCAAAGACTCGCCTTAAA	29	64.1	395–423 (2)	AY253918
Blastomyces dermatitidis	Blder2	CCGCTAGAACTTCTGGTGAACGATTGACAT	30	56.5	163–192 (1)	AF038358
Chaetomium cochlioides/ *C. globosum*/*C. funicola*	Chcgf1[e]	GGCCTCTCTGAGTCTTCTGTACTGAATAAG	30	56.5	157–186 (1)	AJ279450
Chaetomium funicola	Chfun2	CGTAGTAGCATATCTTTGTCTCGCTCAGG	29	56.4	436–464 (2)	AJ279450
Cladophialophora bantiana	Clban5	TCTTCTCCCTCATGTGGGAAACATTGCA	28	54.8	544–571 (2)	AB091211
Cladophialophora carrionii	Clcar2	AGGCCACGGTCCTCTCCTCTAA	22	53.4	524–545 (2)	AB109180
Cokeromyces recurvatus	Corec2	TTTAGACTTTGGGGCCGCCCAAATAATACT	30	55.2	157–186 (2)	AY830118[d]
Cunninghamella berthalletiae	Cuber3	CACTCTCGGCCTAAATATAAGGCTCGAC	28	56.3	590–617 (2)	AF254930
Cunninghamella spp.	Cun4	GAGATAAATTATTACTGGTCCTGGTGATTC	30	52.4	527–556 (2)	AF254931
Epidermophyton floccosum	Epflo2	TCCATAGGTGGTTCAGTCTGAGCGTT	26	54.4	279–304 (1)	213646
Exophiala dermatitidis	Exder1	ACTCTTGAATCAAATCGTGTCCAATGTCTG	30	53.8	157–186 (1)	AF050270
Exophiala werneckii (Hortaea werneckii)	Hower2	GTCCGTCTCTAAGCGTTGTGAATAGCGATC	30	57.9	387–416 (2)	AY128704
Exophiala jeanselmei	Exjea	CCAAACGTGTCTTGTCTGAGTAAACGTC	28	54.8	160–187 (1)	AY163556
Fonsecaea compacta/ *F. pedrosoi*	Focp[e]	CTCACGGGAACACTTTTTTTTTTAAGGT	28	50.4	167–194 (2)	AY830117[d]
Fusarium moniliforme/ *F. oxysporum*/ *F. pallidoroseum*	Fumop[e]	AGTAGTAAAACCCTCGTTACTGGTAATCGT	30	53.8	448–477 (2)	AY462580
Fusarium moniliform	Fumon	CGAGTCAAATCGCGTTCCCCAAATTG	26	54.4	395–420 (2)	AY533376
Fusarium solani	Fusol2	AGTAGCTAACACCTCGCGACTGGAGA	26	56.0	446–471 (2)	AF129105
Geotrichum candidum	Gecan	CTCTCTTGGAATTGCWTTGCTYTTCTAAA	29	51.5	291–319 (2)	AJ279451
Geotrichum capitatum	Gecap3	GTGCAACAAGCTGTGTTGAATCTTTC	26	49.7	369–388 (2)	AF455443
Malbranchea filamentosa	Mafil4	CGGCGCTGGTCAGAACCAAATCTTTTA	27	54.6	463–489 (2)	AY177301
Microsporum audouinii/ *M. canis*/*M. ferrugineum*	Macf1[e]	TGGCCTAACGCACCATGTATTATTCAGGT	29	55.0	706–734 (2)	AJ252333
Microsporum cookei	Micok4	GCCTTGACTGGACTCCTTTGTCCGTTAAAT	30	56.5	152–181 (2)	AY830116[d]
Microsporum gallinae	Migal3	GGCCTCGTTTCAATAATTGTCGTTAGAGAAT	31	54.0	536–566 (2)	AJ000620
Microsporum gypseum	Migyp3	CCGGTTTTCTGGCCTAGTTTTAGTTAGGGAT	31	56.6	582–612 (2)	AG168128
Microsporum nanum	Minan1	AGGAACGATCAAAACACGCGAACAC	25	52.6	115–139 (1)	AB049927
Paracoccidioides brasiliensis	Pabra2	TTCGGAGCTTTGACGTCTGAGACCTATCAT	30	56.5	158–187 (1)	AY631237
Paecilomyces javanicuss	Pajav	TAGTACTCCAACGCGCACCGGGAA	24	55.7	99–122 (2)	AY830115[d]
Penicillium marneffei	Pemar2	GTCACCACCATATTTACCACGG	22	50.6	468–487 (2)	AB049132
Phialophora richardsiae	Phric3	CCACTAAAACTCTTCTGTATCTCGCGTACC	30	56.5	111–140 (1)	AY179948
Phialophora verrucosa	Phver3	CCAGGACCCGGTCCTTCTCCTTTAAC	26	57.6	521–546 (2)	AF397135
Piedriaia hortai var. *hortai*	Pihor2	AAACGATCCGCCCAGCGAGAAT	22	51.6	106–127 (2)	AY839755[d]
Pseudallescheria boydii	Psboy3	GGTTGCCTTCTGCGTAGTAAGTCTCTTTTG	30	56.5	579–608 (2)	AY228118
Rhizopus oryzae	Riory4	GCAGGAATATTACGCTGGTCTCAGGATCTT	30	56.5	486–515 (2)	AB097271
Rhizomucor pusillus	Ripu4	ATCCGTTCAAGCTACCCGAACAATTTGTAT	30	53.8	169–198 (2)	AF117934
Saccharomycodes ludwigii	Salud[f]	CTTGAAATTGTTGCCTAGCAAAGAAGAA	28	55.4	522–549 (2)	AB056135
Scopulariopsis brevicaulis	Scbre3	TGCGTAGTAGATCCTACATCTCGCATCG	28	56.3	500–527 (2)	AY625065
Scytalidium dimidiatum/ *S. hyalinum*	Sydh[e]	AACTCCGGTCAGTGAACGTTGCC	23	53.7	161–183 (2)	AY213688
Scedosporium prolificans	Scpro4	GGCCAGCCGTCAAACCCTCTATTCTTAT	28	56.3	467–494 (2)	AY228117

Continued on facing page

TABLE 1—*Continued*

Microorganism	Probe code[a]	Sequence (5′ to 3′)[b]	Length (bp)	T_m (°C)	Location[c]	GenBank accession no.
Trichophyton rubrum/ *T. soudanense*/ *T. violaceum*	Trrsv3[e]	CCGCCCTGGCCCCAATCTTTATA	23	53.7	614–636 (2)	Z97993
Trichophyton verrucossum	Trver	GCCTTCCCCCAAATCTCTCTGAGATW	26	54.4	607–632 (1)	Z98003
Trichophyton mentagrophytes/ *T. tonsurans*/ *T. schoenleinii*	Trmmist[e]	GCCTCAAAATCTGTTTTATACTTATCAGGT	30	51.1	619–648 (2)	Z97999
Ulocladium consortiale	Ulcon2	CCAAGGTCAGCATCCACAAAGCCTT	25	54.2	477–501 (2)	AY278837

[a] Oligonucleotide probes are arranged on the array as indicated in Fig. 1.
[b] Seven additional bases of thymine were added to the 3′ end of each probe.
[c] The location of probe is shown by the nucleotide number of either ITS 1 or ITS 2; the number (1 or 2) in parenthesis indicates the ITS region from which the probe was designed.
[d] ITS sequences determined in this study and submitted to GenBank.
[e] A group-specific probe used to detect several closely related species.
[f] The probe on the array was used as a positive control of assay.

with homologous species. Finally, 58 probes were selected for fabrication of the array (Fig. 1). For most fungi, a single probe was designed for each species. However, some group-specific probes (Fig. 1, probe codes underlined; Table 1) were designed, i.e., two or more closely related species shared a probe that could not differentiate species within the group. This is due to the fact that some genetically related species have high similarities of both ITS 1 and 2 sequences. For example, the probe Chcgf1 was used to identify three species (*Chaeotomium cochlioides*, *C. globosum*, and *C. fumicola*). Other group-specific probes were Focp (*Fonsecaea compacta*/*F. pedrosoi*), Fumop (*Fusarium moniliforme*/*F. oxysporum*/*F. pallidoroseum*), Macf1 (*Microsporum audouinii*/*M. canis*/*M. ferrugineum*), Sydh (*Scytalidium dimidiatum*/*S. hyalinum*), Trmst1 (*Trichophyton mentagrophytes*/*T. schoenleinii*/*T. tonsurans*), and Trrsv3 (*Trichophyton rubrum*/*T. soudanense*/*T. violaceum*). The probe Cun4 was a genus-specific (*Cuminghamella*) probe.

Furthermore, strains of some species were found to have high intraspecies divergence of ITS sequences and multiple probes (Fig. 1, probe codes in bold face; Table 1) were used to identify a single species. For example, probes Ackil2 and Ackil3 were used to identify *Acremonium kiliense*, and probes Aupul2, Aupul3, and Aupul4 were designed for identification of *Aureobasidium pullulans*. The melting temperatures (T_m) of probes ranged from 49.7 to 57.9°C (Table 1).

Hybridization of reference strains to the oligonucleotide array. A total of 326 reference strains, including 233 target and 93 nontarget strains, were tested by hybridization to the array. Fig. S2 in the supplemental material shows the hybridization results of reference strains of different species. Of the 233 target strains, 228 (97.9%) were correctly identified to the species or group level, 2 (*Acremonium falciforme* CBS 101427 and *Acremonium strictum* BCRC 32290) were misidentified, and 3 (*Acremonium strictum* CBS 102295, *Bipolaris spicifera* CBS 418.67, and *Hortaea werneckii* ATCC 58301) were not identified (no hybridization signal) (see Table S1 in the supplemental material and Table 2). Strains that produced discrepant identification by the conventional methods and the array method were further analyzed in Table 2.

A. falciforme CBS 101427 hybridized to probes Acfal1 (designed for *A. falciforme*) and Fusol2 (designed for *Fusarium solani*) with almost equal hybridization signals, and no unambiguous species designation for the strain was obtained by the array method. *A. strictum* BCRC 32290 was misidentified as *A. kiliense* (Table 2). The ITS 1 and 2 regions of *A. strictum* BCRC 32290 were sequenced and compared with sequences in the

	1	2	3	4	5	6	7	8	9	10
A	Abcor3	Acfal1	**Ackil2**	**Ackil3**	Acstr2	M	Alalt3	**Aupul2**	**Aupul3**	**Aupul4**
B	Ascla4	Asfla4	Asfum2	Asnid2	Asnig2	M	Aster2	Asver4	Bebas5	Bispi
C	Blder2	Chcgf1	Chfun2	Clban5	Clcar2	M	NC	Corec2	Cun4	Cuber3
D	Epflo2	Exder1	Exjca	Focp	Fumon	M	Fumop	Fusol2	Gecan	Gecap3
E	M	M	M	M	M	NC	M	M	M	M
F	Hower2	Mafil4	Macf1	Migyp3	Micok4	M	Migal3	Minan1	Pabra2	Pihor2
G	Pajav	NC	NC	NC	Pemar2	M	Phric3	Phver3	Psboy3	PC
H	Ripu4	Riory4	Scbre3	Scpro4	Sydh	M	Trmst1	Trver	Trrsv3	Ulcon2

FIG. 1. Layout of oligonucleotide probes on the array (1.1 by 0.9 cm). The probe "PC" (G10) was designed on the basis of the ITS 2 region of *Saccharomycodes ludwigii* BCRC 21378 and used as a positive control. Probes coded "NC" (E6 and G2 to G4) were negative controls (tracking dye only). Probes coded "M" were ITS 4 labeled with digoxigenin at its 5′ end and were used as position markers. Group-specific probes are underlined, and multiple probes used to identify a single species are in bold face. The corresponding sequences of probes are listed in Table 1.

TABLE 2. List of fungal strains that produced discrepant identification by the conventional methods and array hybridization

Microorganism	Species identified by array hybridization	ITS sequence analysis result[a]	GenBank accession no. or strain no.[b]
Target species			
Acremonium falciforme CBS 101427	*Acremonium falciforme* or *Fusarium solani*	*Acremonium falciforme* (98%/99%)	CBS 475.67
		Fusarium solani (98%/99%)	AY569560
Acremonium strictum BCRC 32290	*Acremonium kiliense*	*Acremonium strictum* genogroup II (100%/95%)	AY138846
		Acremonium kiliense (100%/100%)	CBS 122.29
Acremonium strictum CBS 102295	Not identified	*Acremonium strictum* genogroup II (99%/100%)	AY138844
		Acremonium strictum (69%/70%)	BCRC 32239
Bipolaris spicifera CBS 418.67	Not identified	*Exserohilum rostratum*[c] (98%/99%)	AF163066
		Bipolaris spicifera (84%/75%)	CBS 274.52
Exophiala dermatitidis LM 8	*Exophiala jeanselmei*	*Exophiala jeanselmei* (99%/99%)	AY163552
		Exophiala dermatitidis (48%/56%)	ATCC 38714
Exophiala jeanselmei LM 68	Not identified	*Exophiala mesophila* (97%/98%)	AF542377
		Exophiala jeanselmei (60%/38%)	CBS 835.95
Horteae werneckii ATCC 58301	Not identified	*Exophiala* sp. strain 700-03 (98%/98%)	AY591350
		Horteae werneckii (37%/45%)	ATCC 36317
Trichophyton soudanense LMA 951336	*Trichophyton mentagrophytes*/*T. schoenleinii*/*T. tonsurans*	*Trichophyton tonsurans* (95%/100%)	AF170479
		Trichophyton soudanense (95%/74%)	ATCC 24583
Nontarget species			
Aspergillus oryzae BCRC 30102	*Aspergillus flavus*	*Aspergillus oryzae* (97%/95%)	AY373857
		Aspergillus flavus (97%/95%)	AF453893
Aspergillus sydowii BCRC 33347	*Aspergillus versicolor*	*Aspergillus sydowii* (99%/95%)	AY373868
		Aspergillus versicolor (97%/95%)	AY373882
Rhizomucor miehei BCRC 31627	*Rhizomucor pusillus*	*Rhizomucor miehei* (100%/100%)	AJ278360
		Rhizomucor pusillus (67%/70%)	BCRC 33122

[a] Values in parenthesis are the ITS 1/ITS 2 sequence similarities of the discrepant strain with the indicated fungal species.
[b] The ITS sequences of the discrepant strain were compared with the indicated GenBank accession number or the closest reference strain in our database.
[c] *Exserohilum rostratum* is a new name of *Bipolaris rostratum*.

GenBank and in our database. The results revealed that both ITS sequences of *A. strictum* BCRC 32290 had 100% similarity with those of the type strain (*A. kiliense* CBS 122.29) in our database. However, *A. strictum* BCRC 32290 had sequence similarities of 100% (ITS 1) and 95% (ITS 2), respectively, with *A. strictum* genogroup II (GenBank accession no. AY138844) (Table 2).

Of the three strains not identified (*Acremonium strictum* CBS 102295, *Bipolaris spicifera* CBS 418.67, and *Hortaea werneckii* ATCC 58301), *A. strictum* CBS 102295 had sequence similarities of 99% (ITS 1) and 100% (ITS 2), respectively, with *A. strictum* genogroup II (AY138844), but the similarities were only 69% (ITS 1) and 70% (ITS 2), respectively, with type strain *A. strictum* BCRC 32239 (Table 2). *B. spicifera* CBS 418.67 had sequence similarities of 98% (ITS 1) and 99% (ITS 2), respectively, with *Exserohilum rostratum* (a new name of *B. rostratum*) (AF163066). However, *B. spicifera* CBS 418.67 displayed sequence similarities of only 84% (ITS 1) and 75% (ITS 2), respectively, with *B. spicifera* CBS 274.52 in our database. Therefore, the designation of CBS 418.67 as *B. spicifera* may be questionable. *H. werneckii* ATCC 58301 had high ITS similarities (98 to 99%) with *Exophiala* sp. strain NYSDOH 700-03 (AY591350) by a BLAST search in GenBank (Table 2). However, *H. werneckii* ATCC 58301 had sequence similarities of only 37% (ITS 1) and 45% (ITS 2), respectively, with *H. werneckii* ATCC 36317 (type strain) in our database. For this reason, the designation of the strain ATCC 58301 as *H. werneckii* may be incorrect. Despite the above-described inconsistent results, a third strain of *A. strictum* (BCRC 32239) and a second strain of *B. spicifera* (CBS 274.52) were correctly identified (see Table S1 and Fig. S2, panels 4 and 19, in the supplemental material).

Of the 93 nontarget reference strains, two were misidentified by the array. *Aspergillus sydowii* BCRC 33347 and *Rhizomucor miehei* BCRC 31627 were misidentified as *A. versicolor* and *R. pusillus*, respectively (see Table S1 in the supplemental material and Table 2). A BLAST search revealed that the species designation of the two reference strains is correct. Misidentifications of the two strains were apparently caused by their partial ITS sequence homology with the above-indicated target species (Table 2). Although *R. miehei* BCRC 31627 had low ITS similarities (67 to 70%) with *R. pusillus*, sequence alignment indicated that the probe (Ripu4) designed for *R. pusillus* had an 85% similarity with a partial region of the ITS 2 of *R. miehei* BCRC 31627.

In addition to hybridization to their species-specific probes, each of the strains *Acremonium falciforme* CBS 475.67 (see Fig. 2, panel 2, in the supplemental material), *Alternaria alternata* BCRC 30501 (panel 5), *Aspergillus nidulans* ATCC 11267 (panel 12), *Aspergillus versicolor* BCRC 30225 (panel 15), *Scopulariopsis brevicaulis* ATCC 7903 (panel 55), and *Ulocladium consortiale* CBS 105.21 (panel 65) cross-hybridized to an extra heterologous probe on the array. However, the hybridization signals caused by the heterologous probes were much

weaker than those produced by their homologous probes, and according to the protocol, these species were correctly identified by the array. The hybridization results for three selected nontarget strains (*Absidia coerulea* BCRC 30897, *Penicillium lividum* BCRC 31673, and *Rhizopus azygosporus* BCRC 31158) are also shown (see Fig. S2, panels 68 to 70, in the supplemental material). For the three nontarget species, no probes were hybridized except the probe for positive control.

Hybridization of clinical isolates to the oligonucleotide array. A total of 71 clinical isolates, including 57 target and 14 nontarget strains, were tested. Of the 57 target strains, 54 were correctly identified (see Table S1 in the supplemental material). *Exophiala dermatitidis* LM 8 and *Trichophyton soudanense* LMA 951336 were identified as *E. jeanselmei* and *Trichophyton mentagrophytes*/*T. schoenleinii*/*T. tonsurans*, respectively, by the array method (Table 2). ITS sequence comparison revealed that the identifications of *E. dermatitidis* LM 8 and *T. soudanense* LMA 951336 might have been misidentifications of *E. jeanselmei* and *T. tonsurans*, respectively, because of their high ITS similarities with the later two species and low similarities with their respective reference strains (Table 2). *E. jeanselmei* LM 68 was not identified, and ITS sequence analysis demonstrated that its ITS had similarities of 97% (ITS 1) and 98% (ITS 2), respectively, with *E. mesophila* (GenBank accession no. AF542377), which was not a target strain of this study (Table 2). Therefore, the clinical isolates *E. dermatitidis* LM 8, *E. jeanselmei* LM 68, and *T. soudanense* LMA 951336 should represent misidentifications of *E. jeanselmei*, *E. mesophila*, and *T. tonsurans*, respectively, or their close relatives. On the basis of these results, 100% (57/57) of clinical isolates were correctly identified by the array method. All 14 nontarget clinical isolates produced no hybridization signal with all probes on the array (see Table S1 in the supplemental material).

Performance of the array for fungal identification. Of the 233 target reference strains, 228 (97.9%) were correctly identified, and of the 57 target clinical isolates, 57 (100%) were correctly identified (see Table S1 in the supplemental material). Irrespective of strain sources, an overall sensitivity of 98.3% (285/290) was obtained by the array method. Of the 93 nontarget reference strains, two (*A. sydowii* BCRC 33347 and *Rhizomucor miehei* BCRC 31627) were misidentified and a specificity of 97.9% (91/93) was obtained. For the 14 nontarget clinical isolates, a specificity of 100% (14/14) was obtained. In summary, a specificity of 98.1% (105/107) was obtained irrespective of the sources of nontarget strains.

Hybridization of multiple microorganisms to an array. An array could be used to simultaneously identify several different microorganisms if these microorganisms belonged to distant taxa. For example, the PCR products of *Absidia corymbifera* BCRC 33078 and *Aspergillus fumigatus* BCRC 32120 could concurrently hybridize to their respective probes on an array (see Fig. S2, panel 66, in the supplemental material). Another example was that *Aspergillus flavus* BCRC 30007 and *Penicillium marneffei* CBS 334.59 could be identified on a chip (see Fig. S2, panel 67, in the supplemental material).

Detection limit of the array. Serial 10-fold dilutions of DNAs extracted from two strains (*Aspergillus fumigatus* BCRC 32120 and *Rhizopus oryzae* BCRC 31108) were used to determine the detection limit. The present method was able to detect fungal genomic DNA at a level as low as 10 pg (data not shown).

DISCUSSION

In this study, an oligonucleotide array was developed to identify 64 species (32 genera) of clinically relevant molds. The 64 species covered pathogens causing superficial, cutaneous, subcutaneous, and invasive infections. Several species of dimorphic fungi (*Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Penicillium marneffei*) (see Table S1 in the supplemental material) were also included. The prominent feature of the present method is that it combines the various methods into a single standardized protocol encompassing DNA extraction, PCR amplification of the ITS regions, and hybridization of the PCR products to the array. Another important characteristic is that visualization of fungal reproductive structures, which is essential for classical identification, is not needed for the present method. Since the primer pairs (ITS 1 and 4) used in this study can also amplify the ITS regions of yeasts (5, 31, 46), it is expected that clinically relevant yeasts also could be identified by the same approach used in this study.

Traditionally, the identification of molds causing infections involves isolation of organisms and relies on the expertise of the clinical microbiologist. Identification of filamentous fungi is therefore technical demanding and time consuming, and inaccurate identification is not rare. Taylor et al. (42) have pointed out that there are distinctions between the "evolutionary species" (theoretical species) concept and the "operational species" concept that may include the "morphological species" concept, "biological species" concept, and "phylogenetic species" concept. It has been noted that in general a single morphological species can contain multiple biological or phylogenetic species (42). Besides, strains belong to the same species may display different morphological characteristics at different growth stages. In this study, it was found that even some reference strains (*Bipolaris spicifera* CBS 418.67 and *Hortaea werneckii* ATCC 58301) obtained from collection centers may be misidentified.

The good performance (sensitivity and specificity) of the present array might be due to the fact that the fungal ITS sequences have low intraspecies variation and high interspecies divergence (10, 19, 21, 24, 32). Until now, there has been no report defining the criteria for maximum intraspecies variation in the ITS regions. However, the criteria have been established for the D1/D2 region of large-subunit rRNA genes. Fell et al. (16) established a database of 230 species of basidiomycetous yeasts and suggested that strains differing by two or more nucleotides in the D1/D2 region represented different taxa. Kurtzman and Robnett (29) studied approximately 500 yeast species and proposed that strains showing greater than 1% substitutions in the approximately 600-nucleotide D1/D2 region were likely to be different species. However, the fungal ITS region is more variable than the D1/D2 region. We have sequenced both ITS 1 and 2 regions of many fungal species to establish the intraspecies variation of medically important molds (data not shown) and found that, in general, 2% divergence of both regions (ITS 1 and 2) seems to be a reasonable threshold to differentiate species. Some intraspecies variations

of ITS 1 (or ITS 2) may be >2%; however, it was rarely found that the divergence of both ITS regions is >2%.

Although a single probe was designed to identify each individual species under most conditions, some exceptions existed. For example, multiple probes were used to identify *Acremonium kiliense* (Ackil2 and Ackil3) and *Aureobasidium pullulans* (Aupul2, Aupul3, and Aupul4) (Table 1 and Fig. 1) because strains within the two species have high intraspecies divergence of ITS sequences. Fig. S2 (panel 16) in the supplemental material shows that *A. pullulans* BCRC 32064 hybridized to the probe Aupul2, while *A. pullulans* BCRC 31981 hybridized to other two probes (Aupul3 and Aupul4) (panel 17). In contrast, some species within a genus displayed high ITS homology and the design of species-specific probes was difficult. Therefore, several probes were designed to identify a "group" of closely related species. For example, the probe Chcgf1 (Table 1) was used to identify species of *Chaeotomium cochlioides*, *C. funicola*, and *C. globosum*. However, *C. funicola* had its own species-specific probe (Chfun2) (Table 1). Therefore, when both probe Chcgf1 and probe Chfun2 were hybridized, the microorganism was *C. funicola* (see Fig. S2, panel 22, in the supplemental material). If only the probe Chcgf1 was hybridized, the strain might belong to *C. cochlioides* or *C. globosum* (panels 21 and 23). Similarly, the probe Fumop was designed to identify *Fusarium moniliforme*, *F. oxysporum*, and *F. pallidoroseum*, but an additional probe (Fumon) was designed for *F. moniliforme* (Table 1). Therefore, *F. moniliforme* could be differentiated from *F. oxysporum* (panel 34) and *F. pallidoroseum* (panel 35) by hybridization to probes of both Fumop and Fumon (panel 33).

In this study, four strains (*Acremonium strictum* CBS 102295, *Bipolaris spicifera* CBS 418.67, *Exophiala jeanselmei* LM 68, and *Hortaea werneckii* ATCC 58301) were not identified (Table 2). The anamorphic characteristics of *A. strictum* CBS 102295 are as follows: conidiophores simple, occasionally branched, phialides slender (arising from submerged or slightly fasiculate aerial hyphae), conidia grouped in slimy heads (cylindrical or ellipsoidal, hyaline). These characteristics are typical of the species *A. strictum* (11). *A. strictum* CBS 102295 was not identified, and this might be due to genetic diversity among isolates of the species. Novicki et al. (39) found a greater-than-expected variation among strains previously identified as *A. strictum*. In sequence analyses of the ITS regions and the D1/D2 variable domain of the 28S ribosomal DNA, five clinical isolates phenotypically identified as *A. strictum* were found to have <99% similarities to the *A. strictum* type strain (CBS 346.70) at the ITS and 28S loci. But a sixth isolate phenotypically identified as an *Acremonium* sp. had >99% similarities to the type strain at both loci. They concluded that five out of the six clinical isolates belong to species other than *A. strictum* or that the *A. strictum* taxon is genetically diverse.

Bipolaris spicifera CBS 418.67 had sequence similarities of 98% (ITS 1) and 99% (ITS 2), respectively, with *Exserohilum rostratum* (a new name of *B. rostratum*) (GenBank accession no. AF163066). However, *B. spicifera* CBS 418.67 displayed sequence similarities of only 84% (ITS 1) and 75% (ITS 2), respectively, with *B. spicifera* CBS 274.52 in our database. Therefore, the designation of CBS 418.67 as *B. spicifera* may be questionable. The conidiophores of CBS 418.67 are erect, unbranched, septate, and regularly zig-zagged in the apical part. The conidia of CBS 418.67 are brown, cylindrical with rounded ends, and have three distosepta (conidia subdivided by inner wall layer only). The above-described characteristics of CBS 418.67 are typical of the species of *B. spicifera* (11) and may be why CBS 418.67 was given the name *B. spicifera*.

For *Exophiala jeanselmei* LM 68 (a not-identified clinical isolate), its ITS sequences had similarities of 97% (ITS 1) and 98% (ITS 2), respectively, with *E. mesophila* (GenBank accession no. AF542377), which was not a target strain of this study (Table 2). Members of genus *Exophiala* are often difficult to identify to the species level because of their variable morphological appearances (40). The microscopic morphologies of *E. jeanselmei* LM 68 and *E. mesophila* are similar; both produce annellides that are slender, tubular, sometimes branched, and characteristically tapered to a narrow, elongated tip. Their conidia are oval and gather in clusters at the end and sides of the conidiophore.

Hotaea werneckii ATCC 58301 had high ITS similarities (98 to 99%) with *Exophiala* sp. NYSDOH 700-03 (AY591350) by a BLAST search in GenBank (Table 2). At the early stage, ATCC 58301 produce pale or dark brown yeast-like cells. These cells (2 to 5 by 5 to 10 μm) are actually annellides and are round at one end while tapered and elongated with striations at the other end where conidia are formed. The one- or two-celled annelloconidia form and accumulate at annellidic points along the hyphae. The above-described microscopic characteristics of ATCC 58301 are typical of the species *H. werneckii* (11), and this might explain why ATCC 58301 was given the name *H. werneckii*.

Invasive aspergillosis is a leading cause of infection among patients undergoing treatment for hematological malignancies, solid organ transplantation, and hematopoietic stem cell transplantation (8, 10, 37). *Aspergillus fumigatus* remains the most frequent cause of invasive aspergillosis, followed by *A. flavus*, *A. terreus*, *A. niger*, *A. nidulans*, and *A. versicolor* (12, 22). These six important *Aspergillus* species were all included in this study, and probes designed for them showed satisfactory hybridization results (see Fig. S2, panels 10 to 15, in the supplemental material). The only exception was one strain (BCRC 302225) of *A. versicolor* that cross-hybridized with the probe Asnid2 designed for *A. nidulans* (see Fig. S2, panel 15, in the supplemental material). However, the probe Asver4, designed to identify *A. versicolor*, produced much a stronger hybridization signal than the probe Asnid2.

Recently, Husain et al. (22) prospectively studied 53 liver and heart transplant recipients and found that invasive infections due to filamentous fungi other than *Aspergillus* species were significantly more likely to be associated with disseminated and central nervous system infections than were those due to *Aspergillus* species. The associated mortality rate was 100% for zygomycosis, 80% for non-*Aspergillus* hyalohyphomycosis, and 54% for aspergillosis. Non-*Aspergillus* molds causing sever infections in organ transplant recipients include zygomycetes, *Fusarium* spp., *Pseudallescheria boydii* (*Scedosporium apiospermum*), *Scedosporium prolificans*, and dematiaceous molds (6). Probes for most of these important non-*Aspergillus* species were included in the current study (Table 1).

Some species of dermatophytes are phylogenetically and taxonomically very closely related (19, 25, 34). A high level of ITS homology was observed for several common dermatophyte

species. Our in-house ITS database demonstrates that the sequence similarities between *Trichophyton rubrum* ATCC 28188 and *T. violaceum* ATCC 28944 are 0.98 (ITS 1) and 0.95 (ITS 2), respectively, whereas the similarities are 1.0 (ITS 1) and 0.97 (ITS 2), respectively, between *T. rubrum* ATCC 28188 and *T. soudanense* ATCC 24583. Sequence similarities of 0.96 (ITS 1) and 0.99 (ITS 2) were observed between *T. mentagrophytes* CBS 361.62 and *T. tonsurans* ATCC 56186, and were 0.95 (ITS 1) and 0.94 (ITS 2) between *T. schoenleinii* ATCC 22775 and *T. tonsurans* ATCC 56186. For this reason, the group-specific probe Trrsv3 was designed to identify species of *T. rubrum*, *T. soudanense*, and *T. violaceum*, whereas Trmst1 was used for identification of *T. mentagrophytes*, *T. schoenleinii*, and *T. tonsurans*. Additional probes designed from other loci such as the D1/D2 or ribosomal intergenic regions (13) may help to generate species-specific probes of dermatophytes. However, separate PCR or multiplex PCR using different sets of primers will be needed to amplify these different regions (13).

Although the T_m values of some probes listed in Table 1 were lower than the array hybridization temperature (55°C), clear hybridization signals were still obtained for these probes (see Fig. S2 in the supplemental material). For example, probes Acfal1 (T_m = 49.7°C) and Gecap3 (T_m = 49.7°C), designed to identify *Acremonium falciforme* and *Geotrichum capitatum*, respectively, produced discernible signals (see Fig. S2, panels 2 and 38, in the supplemental material). This might be partially due to the use of relatively low stringency buffer (high ionic strength [5× SSC] and low detergent concentration [0.02% SDS]) for hybridization reaction. Volokhov et al. (45) used several probes having T_m values (40 to 44°C) lower than the hybridization temperature (45°C) for identification of *Listeria* spp. and still obtained good hybridization results. In contrast, probes Acstr2 (T_m = 55.3°C) and Blder2 (T_m = 56.5°C), used to identify *Acremonium strictum* and *Blastomyces dermatitidis*, respectively, produced relatively weak hybridization signals (see Fig. S2, panels 4 and 20, in the supplemental material). The secondary structure of probes may influence the hybridization efficiency (13).

In conclusion, species identification of clinically important molds by the present oligonucleotide array is reliable and can be used as an effective alternative to the conventional identification methods. The whole procedure can be finished within 24 h starting from isolated colonies, and visualization of fungal reproductive structures is not required. The array used in this study has the potential to be expanded by including more probes in order to identify more species.

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