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SHORT COMMUNICATION

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Primers based on specific ITS sequences of rDNAs for PCR detection of two fairy ring fungi of turfgrass, *Vascellum pratense* and *Lycoperdon pusillum*

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Abstract *Vascellum pratense* and *Lycoperdon pusillum* cause fairy ring disease on turfgrass in golf courses. For effective disease control, detection of the mycelia of the two fungi in the soil is important. Comparing the sequence data of the internal transcribed spacer (ITS) regions of these fungi with each other and with those from the database, we designed four pairs of polymerase chain reaction (PCR) primers for each fungus. The primers allowed amplification of the DNA of the objective fungi singly, but of no other DNA from field-collected mushroom-forming fungi or soil-borne turfgrass pathogenic fungi.

Key words Fairy rings · Internal transcribed spacer (ITS) · *Lycoperdon pusillum* · Polymerase chain reaction · *Vascellum pratense*

Fairy rings of established turf are caused by any of the 60 species of basidiomycetes, the mushroom-forming fungi (Dernoeden 1995). The fungi cause circles of mushrooms, rings or arcs of dark green grass due to luxuriant growth, or rings of dead grass (Couch 1995). In Japan, the main species of fairy ring fungi in golf courses are *Lycoperdon perlatum* Pers., *L. gemmatum* Batch, *Marasmius oreades* (Bolt.: Fr.) Fr., and *Lepista sordida* (Schum.: Fr.) Sing. (*Lepista subnuda* Hondo) (Tani 1991; Phytopathological Society of Japan 2000). Moreover, two other fungi, *Vascellum pratense* (Pers. Em. Quél.) Kreisel and *Lycoperdon pusillum* Batsch: Pers., were reported to cause death in *Agrostis* spp., which are commercially important as the greens of golf courses (Terashima and Fujiie 1999). The mycelia of each fungus have an ecologically different lifestyle in the soil, and both fungi form fruit-bodies just before causing turfgrass to die (Terashima and Fujiie 1999). To control the diseases more effectively in the early stages by either biological or chemical methods, identifying the species of the causative mycelia and detecting the existence of the mycelia before recognizing the fruit-bodies are important.

Molecular techniques offer sensitive means for detecting soil-borne fungi. The internal transcribed spacers (ITS) of nuclear rDNA (rRNA genes) are used especially to analyze molecular differences among fungi, because inter- and intraspecific variation is observed in the ITS of related fungal species. The ITS of rDNA is nested between conserved sequences of the 18S, 5.8S, and 28S subunits, and is present in multiple, tandemly arranged copies in fungal genomes. We aimed to design polymerase chain reaction (PCR) primers to detect the mycelia of the fairy ring fungi *V. pratense* and *L. pusillum* separately using the ITS regions.

The mycelial isolates and fruit-bodies of the two fairy ring fungi (*V. pratense* and *L. pusillum*), five other mushroom-forming fungi, and eight pathogenic fungi (Phytopathological Society of Japan 2000), four Basidiomycotina, two Ascomycotina, and two Deuteromycotina, were used for DNA extraction (Table 1). For the DNA sequences, the *V. pratense* isolate, Vp1, and *L. pusillum* isolate, Lp1, were used. The isolates of the mushroom-forming fungi were grown in modified Czapek–Dox liquid medium, which contains 15 g glucose, 15 g sucrose, 5 g polypeptone (Nihon, Osaka, Japan), 5 g yeast extract (Difco, Michigan, USA), 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, and 0.01 g $FeSO_4 \cdot 7H_2O$ in 1l distilled and deionized water (ddw). The pathogenic fungi were grown in PS liquid medium,

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Table 1. Mycelial isolates or fruit-bodies of mushroom-forming fungi and pathogenic fungi used for DNA extraction

Fungus	Disease name	Isolate code	DNA extraction source	Origin			
				Location in Japan	Year	Turfgrass	Source for mycelial isolation
Mushroom-forming fungus							
1 *Vascellum pratense*	Fairy rings	Vp1	Mycelia	Chiba	1996	(Unknown)	Fruit-body
2 *Vascellum pratense*	Fairy rings	Vp2	Mycelia	Chiba	1996	(Unknown)	Fruit-body
3 *Vascellum pratense*	Fairy rings	Vp3	Mycelia	Chiba	1996	(Unknown)	Fruit-body
4 *Vascellum pratense*	Fairy rings	Vp4	Mycelia	Chiba	1996	(Unknown)	Fruit-body
5 *Vascellum pratense*	Fairy rings	Vp6	Mycelia	Chiba	1998	*Agrostis* sp.	Fruit-body
6 *Vascellum pratense*	Fairy rings	Vp7	Mycelia	Chiba	1998	*Agrostis* sp.	Fruit-body
7 *Vascellum pratense*	Fairy rings	Vp8	Mycelia	Chiba	1998	*Agrostis* sp.	Fruit-body
8 *Vascellum pratense*	Fairy rings	Vp9	Mycelia	Chiba	1998	*Agrostis* sp.	Fruit-body
9 *Vascellum pratense*	Fairy rings	Vp10	Mycelia	Chiba	1998	*Zoysia matrella*	Fruit-body
10 *Vascellum pratense*	Fairy rings	Vp11	Mycelia	Chiba	1998	*Z. matrella*	Fruit-body
11 *Vascellum pratense*	Fairy rings	Vp12	Mycelia	Chiba	1998	*Z. matrella*	Fruit-body
12 *Vascellum pratense*	Fairy rings	Vp13	Mycelia	Chiba	1998	*Agrostis* sp.	Fruit-body
13 *Vascellum pratense*	Fairy rings	Vp14	Mycelia	Chiba	1998	*Z. matrella*	Fruit-body
14 *Vascellum pratense*	Fairy rings	Vp16	Mycelia	Chiba	1998	*Agrostis* sp.	Mycelial cord
15 *Vascellum pratense*	Fairy rings	Vp19	Mycelia	Chiba	2000	*Z. matrella*	Fruit-body
16 *Vascellum pratense*	Fairy rings	Vp22	Mycelia	Chiba	2001	*Poa pratensis*	Fruit-body
17 *Vascellum pratense*	Fairy rings		Fruit-body	Chiba	2000	*Z. matrella*	
18 *Vascellum pratense*	Fairy rings		Fruit-body	Chiba	2000	*Z. matrella*	
19 *Vascellum pratense*	Fairy rings		Fruit-body	Chiba	2000	*Z. matrella*	
20 *Vascellum pratense*	Fairy rings		Fruit-body	Chiba	1998	*Agrostis* sp.	
21 *Vascellum pratense*	Fairy rings		Fruit-body	Chiba	1998	*Z. matrella*	
22 *Vascellum pratense*	Fairy rings		Fruit-body	Chiba	1998	*Agrostis* sp.	
23 *Vascellum pratense*	Fairy rings		Fruit-body	Mie	2000	(Unknown)	
24 *Lycoperdon pusillum*	Fairy rings	Lp1	Mycelia	Chiba	1998	*Agrostis* sp.	Fruit-body
25 *Lycoperdon pusillum*	Fairy rings	Lp3	Mycelia	Chiba	1998	*Agrostis* sp.	Fruit-body
26 *Lycoperdon pusillum*	Fairy rings	Lp4	Mycelia	Chiba	1998	*Agrostis* sp.	Fruit-body
27 *Lycoperdon pusillum*	Fairy rings	Lp5	Mycelia	Chiba	1998	*Agrostis* sp.	Fruit-body
28 *Lycoperdon pusillum*	Fairy rings	Lp6	Mycelia	Chiba	1998	*Z. matrella*	Fruit-body
29 *Lycoperdon pusillum*	Fairy rings	Lp7	Mycelia	Chiba	1998	*Z. matrella*	Fruit-body
30 *Lycoperdon pusillum*	Fairy rings	Lp9	Mycelia	Chiba	1998	*Agrostis* sp.	Fruit-body
31 *Lycoperdon pusillum*	Fairy rings	Lp10B	Mycelia	Chiba	1998	*Agrostis* sp.	Fruit-body
32 *Lycoperdon pusillum*	Fairy rings	Lp10C	Mycelia	Chiba	1998	*Agrostis* sp.	Mycelial cord
33 *Lycoperdon pusillum*	Fairy rings	Lp10D	Mycelia	Chiba	1998	*Agrostis* sp.	Mycelial cord
34 *Lycoperdon pusillum*	Fairy rings	Lp11	Mycelia	Chiba	1999	*Agrostis* sp.	Fruit-body
35 *Lycoperdon pusillum*	Fairy rings	Lp13	Mycelia	Chiba	2001	*Z. matrella* and *Lolium perenne*	Fruit-body
36 *Lycoperdon pusillum*	Fairy rings	Lp15	Mycelia	Chiba	2001	*Z. matrella*	Fruit-body
37 *Lycoperdon pusillum*	Fairy rings	Lp16	Mycelia	Chiba	2001	*Z. matrella*	Fruit-body
38 *Lycoperdon pusillum*	Fairy rings	Lp17	Mycelia	Chiba	2001	*P. pratensis*	Fruit-body
39 *Lycoperdon pusillum*	Fairy rings	Lp18	Mycelia	Chiba	2001	*P. pratensis*	Fruit-body
40 *Lycoperdon pusillum*	Fairy rings	Lp19	Mycelia	Osaka	2001	(Unknown)	Fruit-body
41 *Lycoperdon pusillum*	Fairy rings	Lp20	Mycelia	Chiba	2001	*P. pratensis*	Fruit-body
42 *Lycoperdon pusillum*	Fairy rings	Lp21	Mycelia	Chiba	2001	*Z. matrella* and *Lolium perenne*	Fruit-body
43 *Lycoperdon pusillum*	Fairy rings		Fruit-body	Chiba	2000	*Z. matrella* and *L. perenne*	
44 *Lycoperdon* sp.		Ly1	Mycelia	Chiba	2000	*Z. matrella*	Fruit-body
45 *Lycoperdon* sp.		Ly2	Mycelia	Chiba	2000	*Z. matrella*	Fruit-body
46 *Lepista sordida*	Fairy rings	Ls1	Mycelia	Chiba	2000	*Z. matrella*	Fruit-body
47 *Conocybe lactea*		Cl5	Mycelia	Chiba	1999	*Z. matrella*	Fruit-body
48 *Conocybe lactea*		Cl10	Mycelia	Chiba	1999	*Z. matrella*	Fruit-body
49 *Conocybe lactea*		As1	Mycelia	Chiba	1999	*Z. matrella*	Fruit-body
50 *Agaricus campestris*		Ac5	Mycelia	Chiba	1999	*Z. matrella*	Fruit-body
Pathogenic fungus of turfgrass							
51 *Rhizoctonia solani* AG2-2 LP	Rhizoctonia rot	Large patch-①		Chiba	1992	*Z. matrella*	
52 *Rhizoctonia solani* AG2-2 LP		92-7-HR-2		Chiba	1992	*Z. japonica*	
53 *Rhizoctonia solani* AG2-2 IIIVB	Summer blight	93-8-BP-1		Chiba	1993	*Agrostis* sp.	
54 *Rhizoctonia solani* AG2-2 IIIVB	Summer blight	93-6-BR-1		Chiba	1993	*Agrostis* sp.	
55 *Sclerotinia homoeocarpa*	Dollar spot	T-91351		Chiba	1991	*Agrostis* sp.	
56 *Sclerotinia homoeocarpa*	Dollar spot	92-④-7		Chiba	1992	*Agrostis* sp.	
57 *Curvularia* sp.	Curvularia leaf blight	950801		Chiba	1995	*Agrostis* sp.	
58 *Curvularia* sp.	Curvularia leaf blight	950803		Chiba	1995	*Agrostis* sp.	
59 *Curvularia* sp.	Curvularia leaf blight	94-9-dog 19-3		Chiba	1994	*Agrostis* sp.	
60 *Curvularia* sp.	Curvularia leaf blight	CK-11-7		(Unknown)			
61 *Ceratobasidium* sp. (binucleate *Rhizoctonia* AG-D)	Rhizoctonia patch	93-8-WL-5		Chiba	1993	*Z. matrella*	
62 *Ceratobasidium* sp. (binucleate *Rhizoctonia*)	Winter patch	94-3-YP-2		Chiba	1994	*Agrogstis* sp.	
63 *Colletotricum* sp.	Anthracnose	K9968		Gifu	1999	*Agrostis* sp.	
64 *Gaeumannomyces graminis*	Take-all	94-1-Gaeu-1		Chiba	1994	*Agrostis* sp.	

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     ITS1-F
1'   CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATTGAATACTCTTGATGAGTTGTAGCTGGCTCTCCGGGGCATGTGCACGCTTG
1"   CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATTGAATATTCTTGATGGGTTGTAGCTGGCTCTTCGGGGCATGTGCACGCCTG
                                                                                                                HIM-F2
121' TCTTGATTTTATTCATCCACCTGTGCACCTTTTGTAGTCTTGGGGGTTGAAAGCAGTCGACTATCGGATGGC-TATGGCTTTTCCGGATGTGAGAAATGCTGAGTGCATAGACGCATACA
121" TCTTGACTTTATTCATCCACCTGTGCACCTTTTGTAGTCTTGGGGGTTGAGAGCAGTCAATTATCGGATGGCTTATAGCCTTTTCGGATGTGAGGAATGCTGAGTGC--GAAAGCATACA
            HIM-F1                                                                                       CHI-F2
240' GCTCTTCTCA-AATGACTTGCATACCCTT-----GAGTACTATGTATTCATATACCACGTCGTATGTTGTAGAATGTGATCAATGGGTCTATGTACCTATAATAATCATATACAACTTTC
239" GCTCTCCTCAGAATGACTTGTAAACCTCTCCCTCGAGTACTATGTATTCATATACCACATAGTATGTTGTAGAATGTGATCAATGGGCCTCTGTGCCTATAATAATCATATACAACTTTC
          CHI-F1
354' AGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCNGAAATGCGATAAGTAATGTGAATTGCANGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTA
359" AGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCNGAAATGCGATAAGTAATGTGAATTGCA-GAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTA
                                           HIM-R2 HIM-R3
474' TTCCGAGGAGCATGCCTGTTTGAGTGTCATTAAATTCTCAACCCCTCCAG---TTG--------TGATGGGGCTTGGATATGGGAGTTNTGCNGGGTNCCTTTATCAGTAAAGGTCAGCTC
478" TTCCGAGGAGCATGCCTGTTTGAGTGTCATTAAATTCTCAACTCCTCTAGCTTTTGCGAGCTATGATGGGGCTTGGATTTGGGAGTT-TGC---GGGCCTTTATTAAT-AAGGTCAGCTC
                                                       CHI-R1             CHI-R2
584' TCCTGAAATACATTAGCGGAACCGTTTGCAGTCCCGTCACTAGTGTGATAATTATCTACACTGTGATGACTGCTCTCTGACTAGTTCAGCTGCTAATCGTCCGTTACGGACAATGTTTAA
593" TCCTGAAATGCATTAGCGGAACCGTTTGCAGTCCCGTCACTAGTGTGATAATTATCTACACTGTGATGATTGCTCTCTGATCAGTTCAGCTGCTAATCGTCCACTATGGACAACACTTAA

704' TGAACTTCTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCA
713" TGAACTTCTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCA
                                                                ITS4-B
824' AATTTAAAATCTGGCAGTCTTTGGCTGTCCGAGTTGTAATCTAGAGAAGTGATGCCCGCGCTGGACCGTGTACAAGTCTCCTG
833" AATTTAAAATCTGGCGGTCTCTGGCCGTCCGAGTTGTAATCTAGAGAAGTGATGCCCGCGCTGGACCGTGTACAAGTCTCCTG
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Fig. 1. Nucleotide sequences of two internal transcribed spacer (ITS) regions including parts of small-subunit ribosomal RNA gene (SrDNA) and large-subunit ribosomal RNA gene (LrDNA), and 5.8S rDNA of *Vascellum pratense* (*upper row*, 906 bp) and *Lycoperdon pusillum* (*lower row*, 915 bp). *Asterisks* show that the nucleotides of the upper and lower rows are the same; *boxes* indicate the forward (ITS1-F) and reverse (ITS4-B) primers used. *Solid* and *broken underlines* indicate the designed forward (HIM-F1 and HIM-F2) and reverse (HIM-R2 and HIM-R3) primers for *V. pratense*, and forward (CHI-F1 and CHI-F2) and reverse (CHI-R1 and CHI-R2) primers for *L. pusillum*, respectively

containing extract from 100 g fresh potato and 20 g sucrose in 1 l ddw. The former were incubated at 25°–30°C for 7–14 days according to the optimum condition for each isolate, and the latter were incubated at 25°C for 3 days before harvesting. The fruit-bodies collected were freeze-dried.

The fresh mycelium was cut into pieces with a blade, and the freeze-dried fruit-body was granulated. About 100 mg of these pieces was placed in a 1.5-ml centrifuge tube, and the DNA was extracted using Isoplant (Nippon Gene, Toyama, Japan) according to the manufacturer's protocol. The ITS fragments of Vp1 and Lp1 were amplified with the primer pair, ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4-B (5′-CAGGAGACTTGTACACGGTCCAG-3′), which specifically enhanced basidiomycetes (Gardes and Bruns 1993). PCR was performed in a final aqueous volume of 25 μl containing 0.2 mM of each deoxynucleotide triphosphates (dNTP) (Ultrapure dNTP Set; Pharmacia, Uppsala, Sweden), 2.5 mM $MgCl_2$, 0.5 μM each primer, 1.25 U Taq DNA polymerase (Promega, Wisconsin, USA), buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0), and 0.1% (v/v) Triton X-100] supplied by the manufacturer, and 25 ng of the extracted template DNA. Reactions were carried out in a thermal cycler (Thermo processor TR-100; Taitec, Saitama, Japan) programmed as follows: 95°C for 5 min; 30 cycles of 95°C for 1 min, 53°C for 1 min and 72°C for 1 min; and 72°C for 10 min. To confirm the amplified fragments, a 3-μl portion of the products was analyzed by running in 2% agarose gels (Nusieve; BMA, Maine, USA), using 1 × TBE buffer. Gels were stained with ethidium bromide and photographed under UV light.

For cloning and sequencing, 50-μl portions of the PCR products from Vp1 and Lp1 were subjected to electrophoresis. The fragments were cut from the gel, collected using Suprec-01 (Takara, Osaka, Japan), and ligated into the pT7Blue T-Vector (Novagen, Wisconsin, USA) using DNA Ligation Kit Ver. 1.2 (Takara). Using *Escherichia coli*

JM109-competent cells (Takara), the transformed colonies were selected by the color selection method. The cloned products were sequenced using the T7 promoter primer #69348-1 and the U-19mer primer #69819-1 (Novagen), and BigDye terminator with ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Norwalk, CT, USA). The sequence data were aligned using the computer software Genetyx-Mac Ver. 5 (Software Development, Tokyo, Japan). The aligned sequence data were subjected to homology search among the database of the DNA Data Bank Japan (DDJB). With Genetyx-Mac, separate primers were designed to contain specific parts each for *V. pratense* and *L. pusillum*, comparing each to the other and to the highly homologous 13 fungi among those in the database. The designed primers for the two fungi were confirmed so as not to be specific for the ITS sequences of the other mushroom fungi.

The 906-bp and 915-bp nucleotide sequences of the ITS regions, including parts of small subunit rDNA and large subunit rDNA, and 5.8S rDNA from the *V. pratense* isolate, Vp1, and the *L. pusillum* isolate, Lp1 (DDBJ accession no. AB067725 and AB067724, respectively) are listed in Fig. 1. The specific primers for *V. pratense*, two forward (HIM-F1, 5′-TGACTTGCATACCCTTGAGT-3′, and HIM-F2, 5′-GCATAGACGCATACAGCTC-3′) and two reverse (HIM-R2, 5′-CATCACAACTGGAGGGGT-3′, and HIM-R3, 5′-TATCCAAGCCCCATCACAACTG-3′), and those for *L. pusillum*, two forward (CHI-F1, 5′-GAATGACTTGTAAACCTCTCCCTCG-3′, and CHI-F2, ATGTGAGGAATGCTGAGTGCGAAAG) and two reverse (CHI-R1, GCCCCATCATAGCTCGCAAAAG, and CHI-R2, AAGGCCCGCAAACTCCCAAATCCA), are also shown in Fig. 1.

The expected sizes of the fragments were amplified from all template DNAs extracted from the fungi listed in Table 1 with the universal primers ITS1 and ITS4 (White et al. 1990), and then the template DNAs were subjected to amplification with the specific primer sets designed for *V. pratense* and *L. pusillum*. Annealing temperatures were determined by shifting at every degree from 50° to 63°C. The other PCR and electrophoresis conditions were the same with those for the primers ITS-F and ITS4-B.

The four primer pairs, HIM-F1 and HIM-R2, HIM-F1 and HIM-R3, HIM-F2 and HIM-R2, and HIM-F2 and HIM-R3, amplified parts of the ITS of the *V. pratense* isolates at annealing temperatures ranging from 53° to 57°C. None of the other fungal isolates, including *L. pusillum*, the closely allied species of this fungus, or the two *Lycoperdon* spp. were amplified. Figure 2A shows the amplified fragments of *V. pratense* and the absence of other fungi with the primer pair HIM-F2 and HIM-R3 with the annealing temperature of 57°C. The four primer pairs, CHI-F1 and CHI-R1, CHI-F1 and CHI-R2, CHI-F2 and CHI-R1, and CHI-F2 and CHI-R2, amplified only parts of the ITS of the *L. pusillum* isolates at annealing temperatures ranging from 57° to 63°C. Figure 2B shows the amplified fragments of *L. pusillum* with primers CHI-F1 and CHI-R2 at the annealing temperature of 63°C.

With the pairs of primers designed for *V. pratense* and *L. pusillum*, each objective fungus was successfully detected.

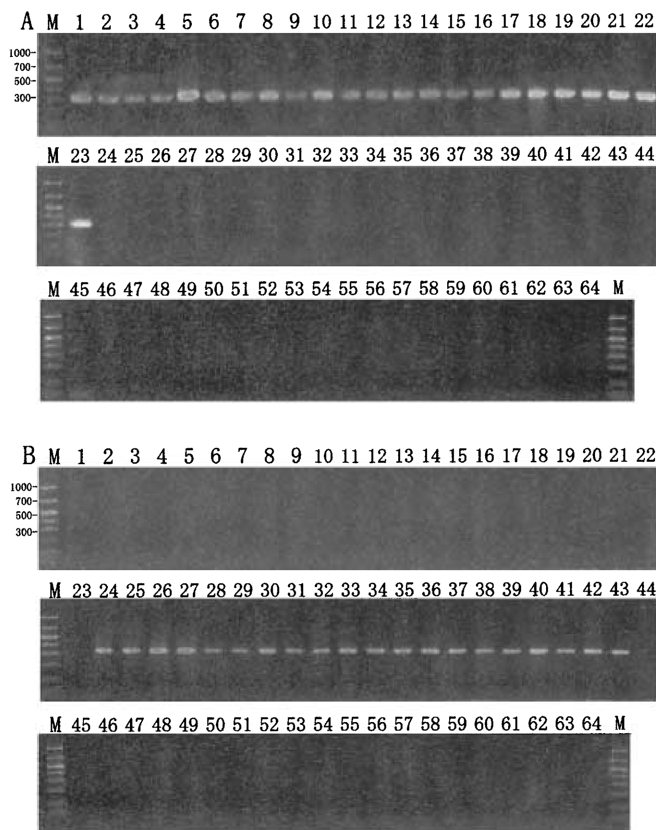

Fig. 2. DNA fragments amplified with HIM-F2 and HIM-R3 (**A**) and with CHI-F1 and CHI-R2 (**B**). *M*, 50- to 1000-bp marker (1000, 700, 500, 400, 300, 200, 100, 50); *sample numbers* are as listed in Table 1. Annealing temperatures for **A** and **B** were 57° and 63°C, respectively

The individual fungus that is capable of developing fairy rings has a different lifestyle. Although the habitats of the mycelia of *Marasmius oreades* (Shantz and Piemeisel 1917) and *Agaricus arvensis* (Edwards 1984) in soil have been described, those of other fairy ring fungi are still unknown. A characteristic sign of fairy rings is the presence of the fruit-bodies of the associated fungi (Couch 1995); the fruit-bodies are the only means for the identification of the specific fungi. The detection of the existence of the mycelia in the early stages of development is important for earlier diagnosis and effective disease control. This molecular technique might allow further fungal study in the soil and lead to effective disease control.

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