Mycoscience (2012) 53:396–401
DOI 10.1007/s10267-012-0182-3

SHORT COMMUNICATION

Rapid and simple preparation of mushroom DNA directly from colonies and fruiting bodies for PCR

Kosuke Izumitsu · Kanako Hatoh · Takuya Sumita · Yuki Kitade · Atsushi Morita · Abdul Gafur · Akira Ohta · Masataka Kawai · Takashi Yamanaka · Hitoshi Neda · Yuko Ota · Chihiro Tanaka

Received: 2 November 2011 / Accepted: 17 January 2012 / Published online: 7 February 2012

Abstract We have optimized a simple and rapid preparation procedure for mushroom DNA extraction from colonies on media or from fruiting bodies for PCR amplification. The protocol combines microwaving twice for 1 min, cooling for 10 min, and centrifuging for 5 min. By using this procedure, more than 100 samples of mushroom DNA can be prepared within 1 h. The DNA obtained can be used for (1) identifying mushroom species by PCR and subsequent sequencing, (2) amplifying low copy number genes (at least 2,000 bp), and (3) screening genetic transformants. This technique will contribute to the mycology of mushroom species.

Keywords Basidiomycetes · Colony PCR · Direct PCR · Fungi · ITS spacer region

Polymerase chain reaction (PCR) has become a common tool for field mycology. For example, internal transcribed spacer (ITS) regions may be amplified by PCR and subsequently sequenced for molecular phylogenetic analysis in various mushroom species (Vilgalys and Sun 1994; Oda et al. 1999; Wu et al. 2000; James et al. 2001). DNA sequences obtained by PCR are now used for population genetics and biodiversity research of mushroom species. The current DNA barcoding approach has also attracted attention (Meyer and Paulay 2005; Hajibabaei et al. 2007; Min and Hickey 2007; Chase and Fay 2009; Seifert 2009). DNA barcoding is a novel system designed to provide rapid and accurate species identification by using short, standardized gene regions (barcodes) as internal species tags (Hebert and Gregory 2005). Mushrooms constitute at least 14,000 and perhaps as many as 22,000 known species, most of which will be barcoded in the future (Lindequist et al. 2005). Thus, PCR and subsequent DNA sequencing are now the essential techniques of taxonomy, population genetics, molecular phylogenetics, and barcoding of mushroom species (Hajibabaei et al. 2007). For these studies, the assembly of a large number of tissue samples and the subsequent isolation and archiving of genomic DNA are generally required. Simple and high-throughput methods for isolation of genomic DNA from mushroom tissue samples for PCR are urgently required.

PCR is important for molecular mycology. Genetic transformation of several mushroom species has also become possible (Binninger et al. 1987; Rhee et al. 1996; Honda et al. 2000; Kuo et al. 2004). In experiments of genetic transformation (e.g., gene replacements in fungi), PCR-based screening of many transformants is generally required (Shiotani and Tsuge 1995; Nayak et al. 2006; Izumitsu et al. 2009). Thus, simple and rapid techniques for PCR that allow the convenient screening of many transformants of mushroom species are also needed.

Although PCR amplification can be performed directly on various bacterial cultures, for fungi prior isolation of

K. Izumitsu · K. Hatoh · T. Sumita · Y. Kitade · A. Morita · C. Tanaka (✉)
Laboratory of Environmental Mycoscience, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
e-mail: chihiro@kais.kyoto-u.ac.jp

A. Gafur
APRIL Fiber R&D, Pangkalan Kerinci 28300, Indonesia

A. Ohta
Shiga Forest Research Centre, Yasu, Shiga 520-2321, Japan

M. Kawai
Nara Forest Research Institute, Takatori, Nara 635-0133, Japan

T. Yamanaka · H. Neda · Y. Ota
Forestry and Forest Products Research Institute, Tsukuba, Ibaraki 305-8687, Japan

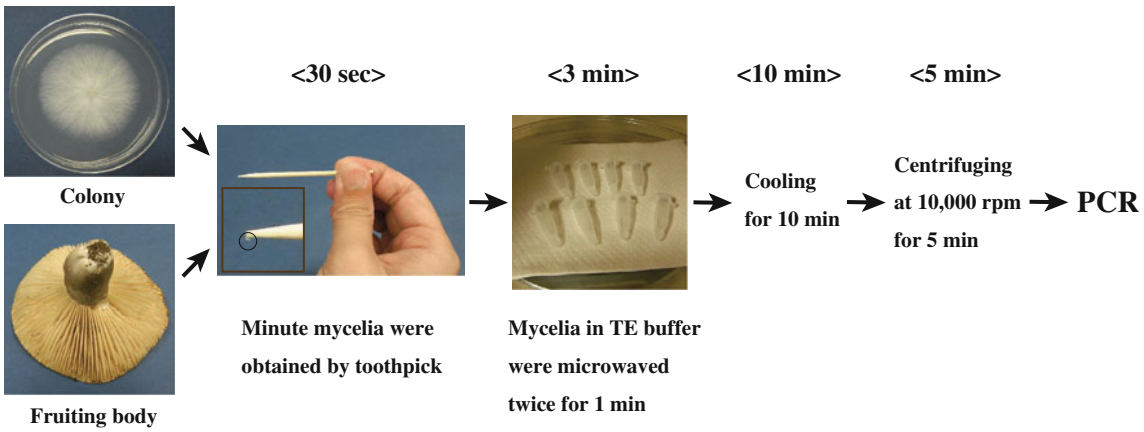

Fig. 1 Scheme of DNA preparation procedures of mushroom species for polymerase chain reaction (PCR). *Open circle* in inset shows minute mycelia being picked up

DNA is often preferred. Various currently available methods for extracting fungal genomic DNA (Raeder and Broda 1985; Leceilier and Silar 1994; Nakada et al. 1994) require grinding (with or without liquid nitrogen) and purification steps and, thus, are time consuming and not suitable for analyzing a large number of samples. Simple methods for preparation of genomic DNA for PCR are available only for some model fungal species, e.g., *Aspergillus nidulans* and *Magnaporthe grisea*, but not in mushroom species (Tendulkar et al. 2003; Suzuki et al. 2006).

Here, we have optimized a simple and rapid preparation procedure of mushroom DNA extraction from colonies on media or from fruiting bodies for PCR amplification (Fig. 1). This procedure includes the following steps. First, minute quantities of mycelium (0.1–1 μg) (Fig. 1) were obtained from a colony on medium or from a fruiting body (stipe) of a mushroom species using a sterilized toothpick. The mycelium was suspended in 100 μl TE buffer in a 1.5-ml tube. For extraction of DNA from colonies on a medium, we used relative young colonies [4–6 days after inoculation on potato dextrose agar (PDA) media] and obtained mycelia from the edge of the colonies, taking care not to include media. For extraction of DNA from fruiting bodies, we used the stipe of relatively fresh fruiting bodies. The tubes were microwaved (600 W) for 1 min. After being stored at room temperature for 30 s, the tubes were microwaved (600 W) again for another 1 min. The tubes were cooled at −20°C for at least 10 min. The tubes were then centrifuged at 10,000 rpm for 5 min. This step is omissible in some cases. The supernatants can be directly used for templates of PCR. Using this procedure, more than 100 samples of mushroom DNA can be prepared in 1 h. In this study, we used an ExTaq (Takara Bio, Shiga, Japan) PCR enzyme kit; similar results were also obtained using KodDash (Toyobo, Osaka, Japan).

Table 1 Polymerase chain reaction (PCR) primers used in this study

ITS4	5′-TCCTCCGCTTATTGATATGC-3′
ITS5	5′-GGAAGTAAAAGTCGTAACAAGG-3′
HPH-inchk-f1	5′-TGCTGCTCCATACAAGCCAACCACG-3′
HPH-inchk-r1	5′-TTCGACAGCGTCTCCGACCTGATGC-3′
LeExg2-f1	5′-TCGCAACGTCAAGGATTTCGGTGC-3′
LeExg2-r1	5′-TCTGATTTGTGTACCACTGTGCGC-3′
LeExg2-r2	5′-CCTGCAGGTATGGTGAGGGTCTGG-3′
HmHsp70-f1	5′-TGCGTGTCCGTCATGGAGGGCAAG-3′
HmHsp70-r1	5′-ACCACGATGGGTGTCACCATTCG-3′
HmHsp70-r2	5′-TCCTCCTTCTTCTCCTCAGACTCG-3′
FvMIP-f1	5′-CCTCCCTCATCGCATCTGCATCC-3′
FvMIP-r1	5′-TACTCGCTGCGCTACCGACTTCG-3′
FvMIP-r2	5′-TCATAGATCTCGCAGAGACGGTG-3′

With the currently described method we obtained genomic DNA from pure cultures of eight mushroom species (*Lentinula edodes*, *Grifola frondosa*, *Agaricus bisporus*, *Pleurotus eryngii*, *Mycena chlorophos*, *Flammulina velutipes*, *Hypsizygus marmoreus*, and *Pleurotus pulmonarius*) on PDA media. ITS regions were amplified by PCR using the primer pair of ITS4 and ITS5 (Table 1) (White et al. 1990). ITS regions were successfully amplified in all eight mushroom species (Fig. 2a). The amplicons were suitable for DNA sequencing. Analysis of the DNA sequences confirmed that the ITS regions of respective mushroom species were successfully amplified from colonies (not shown). The DNA was sequenced with the CEQ 2000 DNA Analysis System sequencer (Beckman Coulter) manually, using a Dye Terminator CEQ-DTCS Quick Start kit (Beckman Coulter).

In general, ITS spacer regions are present in high copy numbers. We also examined low copy number genes *Exg2* (GenBank accession number HQ906601) of *L. edodes*,

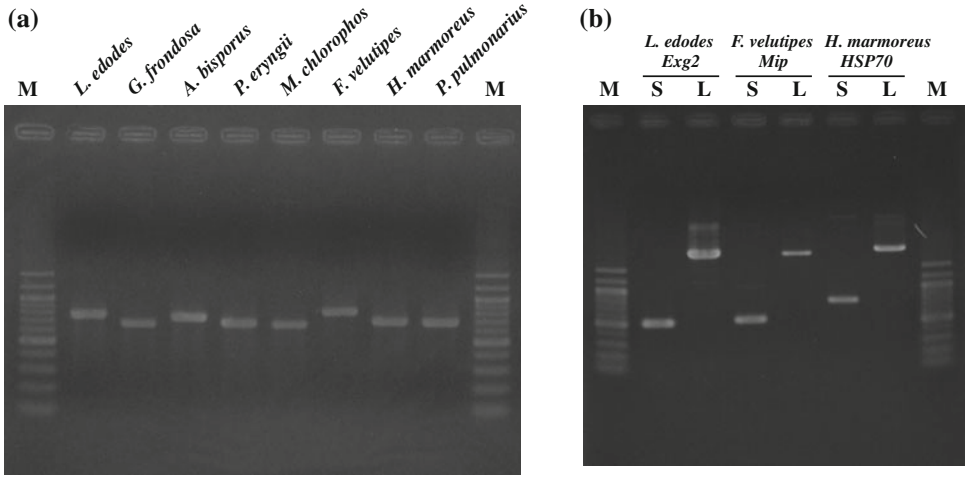

Fig. 2 PCR of genomic DNA prepared from colonies of mushroom species on potato dextrose agar (PDA) medium. **a** Internal transcribed spacer (ITS) regions were amplified by PCR. **b** Low copy number genes [*Exg2* of *Lentinula edodes*, *HSP70* of *Hypsizygus marmoreus*, and *Mip* of *Flammulina velutipes*] were amplified by PCR. *M* 100-bp ladder marker (Nacalai Tesque, Kyoto, Japan), *S* short-length amplification, *L* long-length amplification

HSP70 (GenBank accession number GQ246176) of *H. marmoreus*, and *Mip* (GenBank accession number HQ630589) of *F. velutipes*, for PCR (Fig. 2b). Two primer pairs were designed for respective genes: one primer set, f1 and r1, is for amplification of short-length regions (500–700 bp) of genes, and the other primer set, f1 and r2, is for amplification of long-length regions (about 2,000 bp) of genes (Table 1). All genomic regions examined were successfully amplified by PCR (Fig. 2b), indicating that our procedure of DNA preparation from colonies on media is also suitable for amplifying low copy number genes by PCR.

We also examined genomic DNA obtained from fruiting bodies. Fruiting bodies of six mushroom species (*L. edodes*, *G. frondosa*, *A. bisporus*, *P. eryngii*, *F. velutipes*, and *H. marmoreus*) were obtained commercially. The genomic DNA was prepared from both stipe and lamella of each fruiting body. ITS regions were successfully amplified from both stipe and lamella of all mushroom samples (Fig. 3a). Similarly, we also investigated low copy number genes for PCR. As with colonies on media, all genomic regions examined were successfully amplified from both stipe and lamella of fruiting bodies (Fig. 3b). These results indicated that our method of DNA preparation from fruiting bodies is also suitable for PCR analysis.

To further confirm the procedure, we explored 20 mushroom samples collected in the field (Table 2). Before genomic DNA preparation, the mushroom samples were not washed or decontaminated in any manner. Genomic DNA was extracted directly from stipes and lamellae of relatively fresh fruiting bodies. The remaining parts of the fruiting bodies were dried in a dehydrator (48°C, 24 h) and deposited as voucher specimens at the Natural History Museum and Institute, Chiba (CBM). ITS regions were amplified by PCR using the primer set of ITS4 and ITS5.

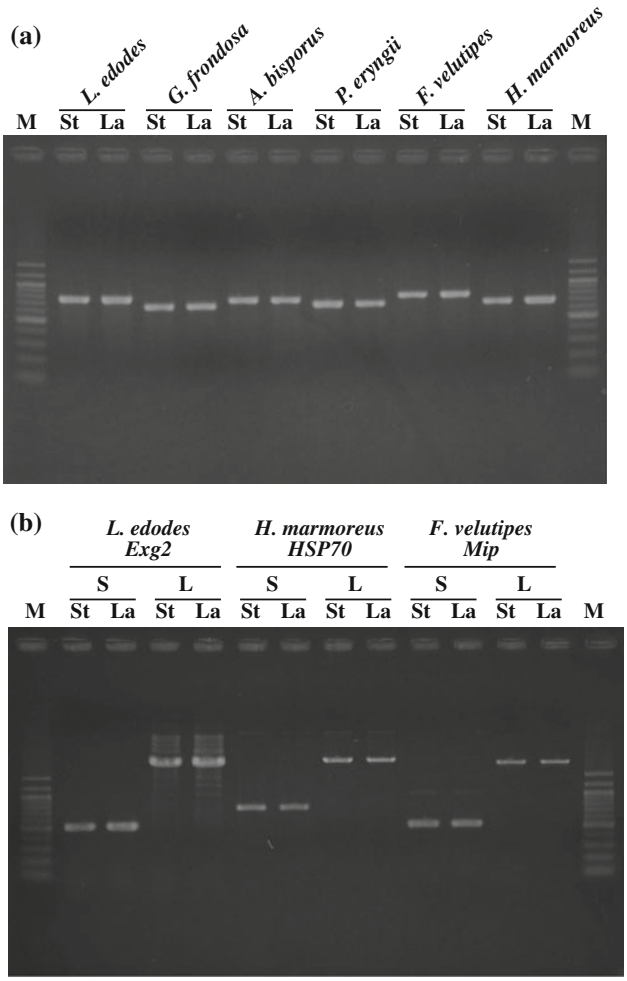

Fig. 3 PCR from genomic DNA prepared from fruiting bodies of mushroom species. **a** ITS regions were amplified by PCR. **b** Low copy number genes [*Exg2* of *L. edodes*, *HSP70* of *H. marmoreus*, and *Mip* of *F. velutipes*] were amplified by PCR. *M* 100-bp ladder marker, *St* sample from a stipe, *La* sample from a lamella, *S* short-length amplification, *L* long-length amplification

Figure 4 shows a part of the results of ITS amplifications. In this experiment, ITS regions were successfully amplified from stipes of 16 mushroom species, although several samples could not be amplified from lamellae. We also prepared genomic DNA in duplicate or triplicate from stipes and lamellae of these 16 mushroom samples. ITS

Table 2 Identification of mushroom samples by BlastN search

Sample no.	Voucher no.	Identification by observation	Best hit species by BlastN	*E* value
1	CBM-FB-38800	*Inocybe* sp.	*Inocybe sphaerospora*	5.0E−149
2	CBM-FB-38801	*Amanita fuliginea*	*Amanita fuliginea*	0
3	CBM-FB-38802	*Amanita* sp.	*Amanita oberwinklerana*	1.0E−60
4	CBM-FB-38803	*Amanita* aff. *flavipes*	*Amanita flavipes*	0
5	CBM-FB-38804	*Amanita farinose*	*Amanita farinose*	0
6	CBM-FB-38805	*Amanita* sp.	*Amanita imazekii*	5.0E−149
7	CBM-FB-38806	*Cantharellus cibarius*	*Cantharellus cibarius*	0
8	CBM-FB-38807	*Russula subnigricans*	*Russula subnigricans*	0
9	CBM-FB-38808	*Agrocybe cylindracea*	*Agrocybe chaxingu*	0
10	CBM-FB-38809	*Inocybe* sp.	Uncultured *Cortinariaceae*	0
11	CBM-FB-38921	*Amanita fritillaria*	*Amanita fritillaria*	0
12	CBM-FB-38922	*Russula* sp.	*Russula crustosa*	0
13	CBM-FB-38923	*Amanita* sp.	*Amanita* sp. 2	7.0E−73
14	CBM-FB-38924	*Boletus* sp.	*Boletus rubropunctus*	9.0E−101
15	CBM-FB-38925	*Russula mariae*	*Russula mariae*	0
16	CBM-FB-38926	*Hymenopellis* sp.	*Hymenopellis raphanipes*	0
17	CBM-FB-38927	*Amanita vaginata* var. *punctata*	*Amanita vaginata*	3.0E−122
18	CBM-FB-38928	*Amanita sychnopyramis* f. *subannulata*	*Amanita sychnopyramis* f. *subannulata*	0
19	CBM-FB-38929	*Russula* cf. *subnigricans*	*Russula subnigricans*	0
20	CBM-FB-38930	*Tricholoma bakamatsutake*	*Tricholoma bakamatsutake*	0

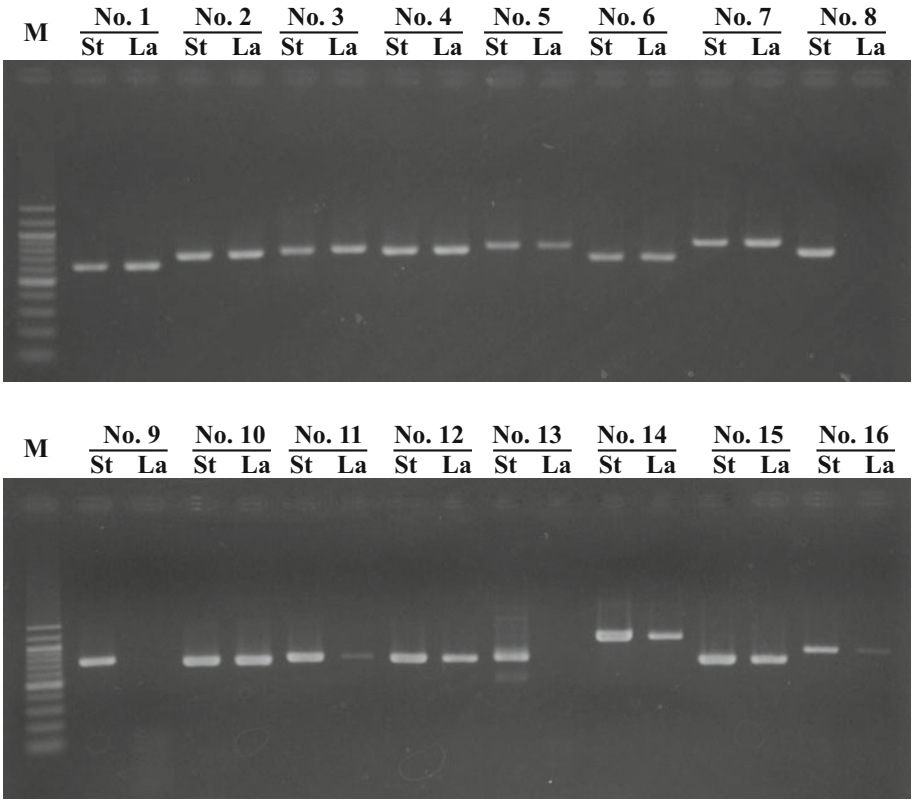

Fig. 4 PCR from genomic DNA prepared from stipes and lamellae of 16 wild mushroom fruiting bodies collected in Japan. ITS regions were amplified by PCR. *M* 100-bp ladder marker, *St* sample from a stipe, *La* sample from a lamella, *S* short-length amplification, *L* long-length amplification, *No.* q. v. sample no. in Table 2

regions were successfully amplified from stipes in 95.3% (41/43) and from lamellae in 73.5% (25/34), indicating that our preparation procedure from a stipe is suitable for PCR from mushroom samples collected in the field. The amplicons were suitable for sequencing. We sequenced all 20 samples and searched using BlastN search in GenBank (Table 2). The results of our tentative identification by observation and the results from BlastN search resembled each other, indicating that our procedure is also suitable for DNA barcoding analysis.

Genetic transformation of mushroom species has been possible for the past two decades (Binninger et al. 1987; Rhee et al. 1996; Honda et al. 2000; Kuo et al. 2004). Before this study, we had obtained genetic transformation mutants in two mushroom species, *H. marmoreus* and *F. velutipes*, using *Agrobacterium tumefaciens*-mediated transformation (our unpublished data). In these experiments, the hygromycin phosphotransferase (*HPH*) gene was used as a selectable genetic marker (Carroll et al. 1994; Walton et al. 2005). Using the procedure previously described, we prepared genomic DNA of the mutant strains from colonies on PDA media and amplified the *HPH* gene by PCR using primers HPH-inchk-f1 and HPH-inchk-r1 (Table 1). These regions were successfully amplified in all

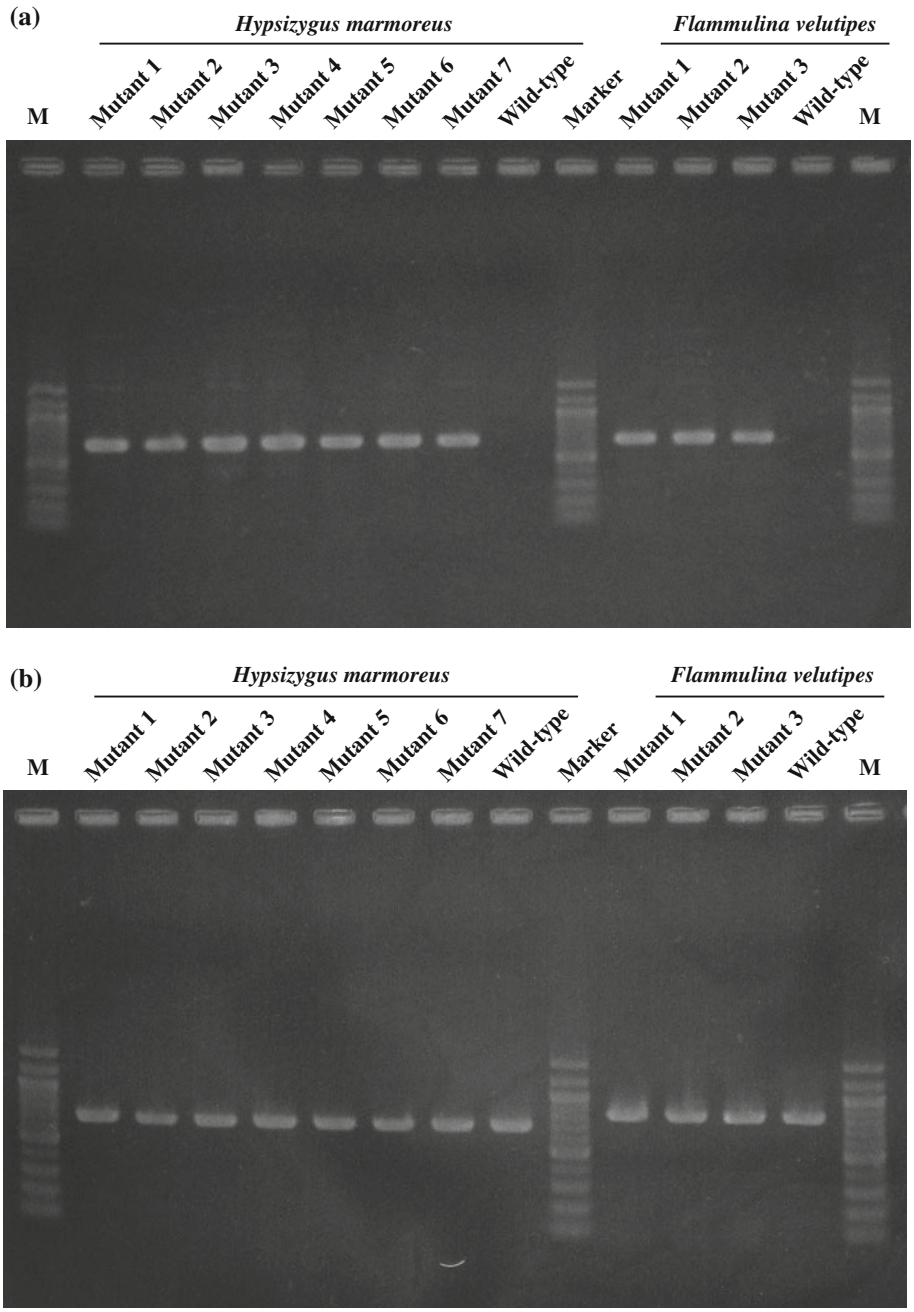

Fig. 5 PCR from genomic DNA prepared from colonies of gene transformants and wild-types of *H. marmoreus* and *F. velutipes*. **a** *Hph* genes were amplified by PCR. **b** ITS regions were amplified by PCR. *M* 100-bp ladder

seven mutants of *H. marmoreus* and three mutants of *F. velutipes*, whereas no amplicons were obtained in respective wild-type strains (Fig. 5a). ITS regions were successfully amplified in the respective wild-type strains (Fig. 5b). These results clearly indicated that our procedure of DNA extraction is suitable for screening genetic transformants in mushroom species.

In conclusion, our procedure for preparing mushroom DNA from colonies on media and fruiting bodies is suitable for (1) identifying mushroom species by PCR and sequencing, (2) amplifying low copy number genes (at least 2 kb), and (3) screening genetic transformants.

Acknowledgments A part of this work was financially supported by Research and Development Projects for Application in Promoting New Policies in Agriculture, Forestry, and Fisheries (No. 21080) and Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science (Nos. 22880017 and 22380084).

References

Binninger DM, Skrzynia C, Pukkila PJ, Casselton LA (1987) DNA-mediated transformation of the basidiomycete *Coprinus cinereus*. EMBO J 6:835–840

Carroll AM, Sweigard JA, Valent B (1994) Improved vectors for selecting resistance to hygromycin. Fungal Genet Newsl 41:22

Chase MW, Fay MF (2009) Barcoding of plants and fungi. Science 325:682–683

Hajibabaei M, Singer GAC, Hebert PDN, Hickey DA (2007) DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends Genet 23:167–172

Hebert PD, Gregory TR (2005) The promise of DNA barcoding for taxonomy. Syst Biol 54:852–859

Honda Y, Matsuyama T, Irie T, Watanabe T, Kuwahara M (2000) Carboxin resistance transformation of the homobasidiomycete fungus *Pleurotus ostreatus*. Curr Genet 37:209–212

Izumitsu K, Yoshimi A, Kubo D, Morita A, Saitoh Y, Tanaka C (2009) The MAPKK kinase ChSte11 regulates sexual/asexual development, melanization, pathogenicity, and adaptation to oxidative stress in *Cochliobolus heterostrophus*. Curr Genet 55: 439–448

James TY, Moncalvo JM, Li S, Vilgalys R (2001) Polymorphism at the ribosomal DNA spacers and its relation to breeding structure of the widespread mushroom *Schizophyllum commune*. Genetics 157:149–161

Kuo CY, Chou SY, Huang CT (2004) Cloning of glyceraldehyde-3-phosphate dehydrogenase gene and use of the gpd promoter for transformation in *Flammulina velutipes*. Appl Microbiol Biotechnol 65:593–599

Leceilier G, Silar P (1994) Rapid methods for nucleic acids extraction from Petri dish-grown mycelia. Curr Genet 25:122–123

Lindequist U, Niedermeyer THJ, Julich WD (2005) The pharmacological potential of mushrooms. Evidence-Based Compl Alt Med 2:285–299

Meyer CP, Paulay G (2005) DNA barcoding: error rates based on comprehensive sampling. PLoS Biol 3:e422

Min XJ, Hickey DA (2007) Assessing the effect of varying sequence length on DNA barcoding of fungi. Mol Ecol Notes 3:365–373

Nakada M, Tanaka C, Tsunewaki K, Tsuda M (1994) RFLP analysis for species separation in genera *Bipolaris* and *Curvularia*. Mycoscience 35:271–278

Nayak T, Szewczyk E, Oakley CE, Osmani A, Ukil L, Murray SL, Hynes MJ, Osmani SA, Oakley BR (2006) A versatile and efficient gene-targeting system for *Aspergillus nidulans*. Genetics 172:1557–1566

Oda T, Tanaka C, Tsuda M (1999) Molecular phylogeny of Japanese *Amanita* species based on nucleotide sequences of the internal transcribed spacer region of nuclear ribosomal DNA. Mycoscience 40:57–64

Raeder U, Broda P (1985) Rapid preparation of DNA from filamentous fungi. Lett Appl Microbiol 1:17–20

Rhee MD, Graca PMA, Huizing HJ, Mooibroek H (1996) Transformation of the cultivated mushroom, *Agaricus bisporus*, to hygromycin B resistance. Mol Gen Genet 250:252–258

Seifert KA (2009) Progress towards DNA barcoding of fungi. Mol Ecol Resour 9:83–89

Shiotani H, Tsuge T (1995) Efficient gene targeting in the filamentous fungus *Alternaria alternata*. Mol Gen Genet 248:142–150

Suzuki S, Taketani H, Kusumoto K, Kashiwagi Y (2006) High-throughput genotyping of filamentous fungus *Aspergillus oryzae* based on colony direct polymerase chain reaction. J Biosci Bioeng 102:572–574

Tendulkar SR, Gupta A, Chattoo BB (2003) A simple protocol for isolation of fungal DNA. Biotechnol Lett 25:1941–1944

Vilgalys R, Sun BL (1994) Ancient and recent patterns of geographic speciation in the oyster mushroom *Pleurotus* revealed by phylogenetic analysis of ribosomal DNA sequences. Proc Natl Acad Sci USA 91:4599–4603

Walton FJ, Idnurm I, Heitman J (2005) Novel gene functions required for melanization of the human pathogen *Cryptococcus neoformans*. Mol Microbiol 57:1381–1396

White TJ, Bruns TD, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego

Wu QX, Mueller GM, Lutzoni FM, Huang YQ, Guo SY (2000) Phylogenetic and biogeographic relationships of Eastern Asian and Eastern North American disjunct *Suillus* species (Fungi) as inferred from nuclear ribosomal RNA ITS sequences. Mol Phylogenet Evol 17:37–47