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# Fungal Diversity in Composting Process of Pig Manure and Mushroom Cultural Waste Based on Partial Sequence of Large Subunit rRNA

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**Fungal diversity during composting was investigated by culture-independent rDNA sequence analysis. Composting was carried out with pig manure and mushroom cultural waste using a field-scale composter (Hazaka system), and samples were collected at various stages. Based on partial sequence analysis of large subunit (LSU) ribosomal RNA (rRNA) and sequence identity values, a total of 12 different fungal species were found at six sampling sites; *Geotrichum* sp., *Debaryomyces hansenii*, *Monographella nivalis*, *Acremonium strictum*, *Acremonium alternatum*, *Cladosporium sphaerospermum*, *Myriangium durosai*, *Pleurotus eryngii*, *Malassezia globosa*, *Malassezia restricta*, *Rhodotorula glutinis*, and *Fusarium sporotrichioides*. *Geotrichum* sp. of the class Saccharomycetes was the most predominant fungal species throughout the composting process (185 out of a total of 236 identified clones, or 78.4%), followed by *Acremonium strictum* (7.6%), *Monographella nivalis* (5.1%), and *Pleurotus eryngii* (3.8%). The prevalence of *Geotrichum* sp. was the lowest (61.1%) at the beginning of composting, and then gradually increased to 92.5% after 10 days of composting.**

**Keywords:** Fungal diversity, composting, LSU (26S) rRNA, *Geotrichum* sp.

Composting is an aerobic process in which crude organic materials are degraded by a complex network of successive groups of microorganisms. It has been widely used in bioremediation of heavy metals and organic toxicants [4], biodegradation of green wastes [36], and as a potentially safe processing for disposal of genetically modified organisms [29]. The major effects of composts have been reported by many researchers, concerning reduction of disease in agricultural crops by suppressing various soil-borne plant pathogens [3, 20]. Fungi play an important role in composting by breaking down tough debris and enabling bacteria to continue the decomposition process. Fungi grow and spread vigorously by producing many cells and filamentous hyphae, and they can attack organic residues that are too dry, acidic, or low in nitrogen for bacterial decomposition [1]. Much of the research on the compost environment to date has been focused on measuring and modeling changes in physical and chemical parameters [6, 11–13, 28, 38] and on bacterial community dynamics [7, 14, 18, 22, 23, 26, 27, 31, 32]. Despite the importance of fungi in composting, the fungal population involved in the process has rarely been examined [11, 23].

Knowledge of the microbial diversity of the compost ecosystem is necessary for the most efficient application of the process, and the ability to predict the presence of microbial species in such an ecosystem can facilitate the isolation of useful new strains [1, 15, 17]. Various cultivation-based techniques have been employed to investigate microbial diversity. However, these methods are time-consuming, and they limit the investigation to the cultivable portion of the microbial flora that are present. It is well known that less than 1% of extant microbial species can be cultivated and characterized. Therefore, many recent studies of microbial diversity have been carried out using culture-independent methods that rely on rapid PCR-based techniques [11, 15, 17, 21, 33]. The use of these techniques is expected to expand our understanding of various ecosystems and to

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facilitate the isolation of genes responsible for the production of novel biocatalysts and bioactive molecules.

The Hazaka system is a field-scale composter equipped with a mechanical agitator and a forced air-supplying system. During the operation of the system, the temperature of the compost is elevated to 60–76°C [22]. Hazaka system composters have been used to treat various substrates and are presumed to be the best for composting pig manure and mushroom culture waste (MCW) [27]. The current work was designed to study the fungal species present at various stages of pig manure and MCW composting in a Hazaka system field-scale composter and to determine their phylogenetic relationships *via* partial sequence analysis of large subunit (LSU) ribosomal RNA (rRNA).

## Materials and Methods

### Composting and Sampling

Composting was carried out at the Yonghyeon Nonghyup Compost Factory (Yonghyeon-myeon, Sacheon, Korea) with a field-scale Hazaka system composter (40 m in length×1.5 m in width). The raw materials, consisting of pig manure and MCW (8:2, v/v), were mixed, stacked, and naturally pre-fermented for one month in the storage area (5 m in length×3 m in width×2 m in depth). The pre-fermented mixture was added to the composter and processed with mechanical agitation and forced aeration for 2 weeks. Samples were collected from 6 sites at a depth of 10–20 cm from the surface (Fig. 1). The duration of fermentation was 0 day for site S0, 1 day for S1, 3 days for S2, 7 days for S3, 10 days for S4, and 14 days for S5. The temperature of the fermenting compost at each site was directly measured at the collection depth. The pH was measured after mixing the sample with distilled water. The moisture content was determined after drying the sample at 105°C overnight.

### Total DNA Extraction and PCR

Samples were diluted in HTE buffer (50 mM Tris, 2 mM EDTA, pH 8.0), agitated for 4–5 h, and then allowed to settle for 12 h to remove solid particles. The supernatants were centrifuged at 14,000 ×*g* for 5 min at 4°C, and then the wet pellets (approximately 0.3 ml each) were subjected to total DNA extraction using the G-spin Genomic DNA Extraction Kit (Intron Biotechnology, Suwon, Korea). The total DNA samples thus obtained were purified by electrophoresis on a 0.7% agarose gel and extracted with a gel purification kit (Intron Biotechnology, Suwon, Korea). The purified DNA was used as a template for PCR with primers that are based on the eukaryotic LSU rRNA sequence and that amplify a region corresponding to positions 184–818 in *Saccharomyces cerevisiae* [37]. The forward primer is a 21 mer (NLF184, 5'-ACCCGCTGAAYTTAAGCATAT-3') and the reverse primer is a 25 mer (NLR818, 5'-CTCCTTGGTCGT GTTTCAAGACGG). The PCR mixture (50 µl) contained 1 µl of *Taq* polymerase (2.5 unit, Super-Therm DNA polymerase, JMR, Side Cup, Kent, U.K.), 3 µl each of the primers NLF184 and NLR818 (10 pmol), 5 µl of reaction buffer, 1.5 mM $MgCl_2$, 5 µl of 2 mM dNTP, 5 µl of template DNA, and 28 µl of sterile water. Thirty cycles (denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 90 sec) were followed by a final extension at 72°C for 10 min. The anticipated fungal rDNA PCR products, approximately 600 bp in length, were separated by agarose gel electrophoresis and purified with a PCR purification kit (Intron Biotechnology, Suwon, Korea).

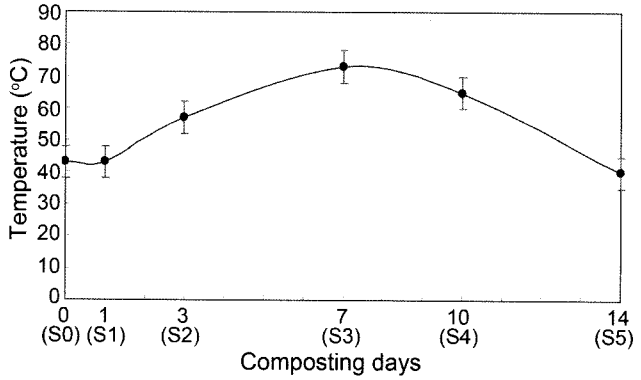


**Fig. 1.** Changes in temperature during composting of pig manure and MCW in a field-scale composter.
The duration of composting for the sites was 0 day for S0, 1 day for S1, 3 days for S2, 7 days for S3, 10 days for S4, and 14 days for S5.

### rDNA Library Construction, Sequencing, and Sequence Analysis

Amplified fungal rDNAs inserted into the pGEM-T easy vector (Promega, Madison, WI, U.S.A.) and transformed into *Escherichia coli* DH5α. Recombinant clones were randomly picked and recombinant plasmids were extracted using a plasmid purification kit (Intron Biotechnology, Suwon, Korea). The insert size in purified plasmids was confirmed *via* standard vector-targeted PCR and gel electrophoresis. Samples for nucleotide sequencing were prepared by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp., Norwalk, CN, U.S.A.), and analyzed on an automated DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Assembly of the nucleotide sequences was performed with the DNAMAN analysis system (Lynnon Biosoft, Quebec, Canada). All reference sequences were obtained from the National Center for Biotechnology Information (NCBI) and Ribosomal Database Project (RDP) databases (http://rdp8.cme.msu.edu). rDNA sequence identity searches were performed using the BLASTN as well as PSI-BLAST tools in the NCBI Web site [19]. Sequences were aligned using the multiple sequence alignment program, CLUSTAL W [35]. Phylogenetic analysis was performed by neighbor-joining methods [25]. Gaps and positions with ambiguities were excluded from the phylogenetic analysis. Bootstrap analysis was performed on data resampled 1,000 times using the DNAMAN analysis system.

### Nucleotide Sequence Accession Numbers and Nomenclature

Nucleotide sequences have been deposited in the GenBank database under the accession numbers DQ365320 to DQ365555. The prefixes of the clones are 0F for the library from the S0 site, 1F for S1 site, 2F for S2 site, 3F for S3 site, 4F for S4 site, and 5F for S5 site.

## Results

### Changes in Abiotic Factors During the Composting Process

The temperature of the compost at sites S0 and S1 was about 43°C. After one day of composting, the temperature

began to rise, reaching 73±5°C after 7 days of composting at S3, then cooling to 40±5°C at the end of fermentation at site S5 (Fig. 1). The pH of the compost increased from 8.9±0.04 at site S0 to 9.2±0.01 after 1 day of composting at site S1, remained constant until 10 days of composting at site S4, and then decreased to 9.1±0.01 at the end of fermentation at site S5 (data not shown). The moisture content of the compost gradually decreased from 52% at site S0 to 37% at site S5 (data not shown).

**Fungal Diversity and Phylogenetic Relationships**

A total of 236 clones, about 40 clones from each site (S0 to S5), were obtained from the compost collected at different stages of the composting process. The clones could be divided into 12 groups based on their rDNA sequences (Table 1). Among the identified fungi, 7 species were assigned to the phylum Ascomycota (*Geotrichum* sp. MTCC 3974 and *Debaryomyces hansenii* of the class Saccharomycetes, and *Monographella nivalis*, *Acremonium strictum* genogroup I, *Acremonium alternatum*, *Cladosporium sphaerospermum*, and *Myriangium durosai* of the class Pezizomycetes), 4 species to the phylum Basidiomycota (*Pleurotus eryngii* of the class Hymenomycetes, *Malassezia globosa* and *Malassezia restricta* of the class Ustilaginomycetes, and *Rhodotorula glutinis* of the class Urediniomycetes), and 1 species to the subdivision Deuteromycotina (*Fusarium sporotrichioides* of the class Hyphomycetes). Most of the rDNA sequences showed 98–100% identity with those of the species listed in the databases; however, the rDNA sequence of the group 7 clone showed only 89% similarity to that of *Myriangium durosai*.

Phylogenetic relationships among the identified composting fungi and other related fungi were analyzed by allocating those fungi having a reasonable degree of confidence to particular taxa and clarifying their taxonomic position (Fig. 2)

**Changes in Fungal Species Composition During the Composting Process**

Changes in fungal species composition during the composting of pig manure and MCW are shown in Table 2. Six or seven groups of fungi were present at the early to mid phases (S0 to S3) of composting; however, only 2 or 3 groups of fungi were found at the late to end stages of composting (S4 to S5). *Geotrichum* sp. of the class Saccharomycetes was the most predominant fungal species found throughout the composting process, accounting for 185 of the 236 sequenced clones (78.4%). The prevalence of *Geotrichum* was the lowest (61.1%) at the beginning of composting at site S0, and then gradually increased to 92.5% after 10 days of fermentation at site S4. *Acremonium strictum* of the class Pezizomycetes was the second most abundant species (7.6%) and was found at all phases of the composting process. *Monographella nivalis* of the class Pezizomycetes (5.1%) and *Pleurotus eryngii* of the class Hymenomycetes (3.8%) were the third and fourth most abundant species, respectively, and were found at the early to mid phases (S0 to S3) but not at the late or end phases (S4 to S5) of composting. Table 3 shows the changes observed in fungal class during the composting process.

## DISCUSSION

Physical characteristics, such as temperature, pH, nutrient availability, and moisture content, give a general indication of the level of decomposition reached during the composting process [34], and it is suggested that the temperature and available substrates are the key factors in the selection of species in microbial communities during composting [23, 31–33]. In the present study, the temperature of the compost began to rise after one day of fermentation, reached the highest temperature (73±5°C) after 7 days of fermentation, and then

**Table 1.** Fungal species identified during composting of pig manure and mushroom cultural waste by rDNA sequence analysis.

| Group | Phylum | Class | Species | Accession No. | Identity (%) |
|---|---|---|---|---|---|
| I | Ascomycota | Saccharomycetes | *Geotrichum* sp. MTCC3974 | AY225313 | 94–100 |
| II | | | *Debaryomyces hansenii* | AY497693 | 99–100 |
| III | | Pezizomycetes | *Monographella nivalis* | AF452030 | 95–99 |
| IV | | | *Acremonium strictum* genogroup I | AY138485 | 95–100 |
| V | | | *Acremonium alternatum* | U57349 | 98 |
| VI | | | *Cladosporium sphaerospermum* | AB100654 | 98 |
| VII | | | *Myriangium durosai* | AY016365 | 89 |
| VIII | Basidiomycota | Hymenomycetes | *Pleurotus eryngii* | AY450347 | 99–100 |
| IX | | Ustilaginomycetes | *Malassezia globosa* | AF064025 | 98 |
| X | | | *Malassezia restricta* | AY743607 | 99 |
| XI | | Uredinomycetes | *Rhodotorula glutinis* | U57349 | 99 |
| XII | Deuteromycotina | Hyphomycetes | *Fusarium sporotrichioides* | AY188917 | 99 |

[a]Accession number of the nearest relative was given.

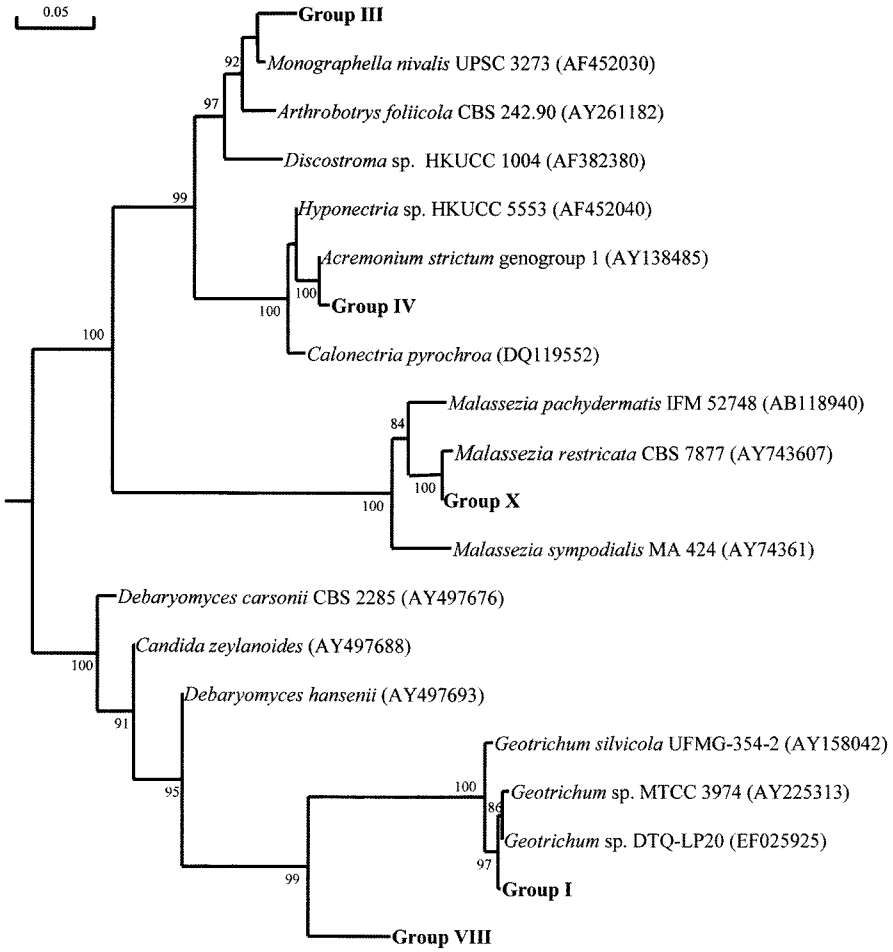


**Fig. 2.** Phylogenetic relationships between composting fungi and other fungi based on rDNA sequences.
Numbers above each node are confidence levels (%) generated from 1,000 bootstrap trees. The scale bar is in fixed nucleotide substitution per sequence position.

cooled down to 40±5°C at the end of fermentation (Fig. 1). This result indicates that the compost passed through several phases of fermentation, as has been previously reported [22]. The pH of the compost increased slightly only at the beginning of the composting, while the moisture content decreased continuously throughout the composting

**Table 2.** Changes in fungal species during composting of pig manure and mushroom cultural waste.

| Species | S0 | S1 | S2 | S3 | S4 | S5 | Total |
|---|---|---|---|---|---|---|---|
| *Geotrichum* sp. MTCC3974 | 22 (61.1%) | 28 (70.0%) | 31 (77.5%) | 31 (77.5%) | 37 (92.5%) | 36 (90.0%) | 185 (78.4%) |
| *Debaryomyces hansenii* | | 1 (2.5%) | 1 (2.5%) | | | | 2 (0.8%) |
| *Monographella nivalis* | 4 (11.1%) | 3 (7.5%) | 4 (10.0%) | 1 (2.5%) | | | 12 (5.1%) |
| *Acremonium strictum* genogroup I | 1 (2.7%) | 6 (15.0%) | 1 (2.5%) | 4 (10.0%) | 3 (7.5%) | 3 (7.5%) | 18 (7.6%) |
| *Acremonium alternatum* | | | 1 (2.5%) | 1 (2.5%) | | | 2 (0.8%) |
| *Cladosporium sphaerospermum* | 1 (2.7%) | | | | | | 1 (0.4%) |
| *Myriangium durosai* | | | | | | 1 (2.5%) | 1 (0.4%) |
| *Pleurotus eryngii* | 5 (13.9%) | 1 (2.5%) | 2 (5.0%) | 1 (2.5%) | | | 9 (3.8%) |
| *Malassezia globosa* | 2 (5.5%) | | | | | | 2 (0.8%) |
| *Malassezia restricta* | 1 (2.7%) | 1 (2.5%) | | | | | 2 (0.8%) |
| *Rhodotorula glutinis* | | | | 1 (2.5%) | | | 1 (0.4%) |
| *Fusarium sporotrichioides* | | | | 1 (2.5%) | | | 1 (0.4%) |
| | 36 (100%) | 40 (100%) | 40 (100%) | 40 (100%) | 40 (100%) | 40 (100%) | 236 (100%) |

The duration of composting for the sites was 0 day for S0, 1 day for S1, 3 days for S2, 7 days for S3, 10 days for S4, and 14 days for S5.

**Table 3.** Changes in fungal class during composting of pig manure and mushroom cultural waste.

| Class | S0 | S1 | S2 | S3 | S4 | S5 | Total |
|---|---|---|---|---|---|---|---|
| Saccharomycetes | 22 (61.1%) | 29 (72.5%) | 32 (80.0%) | 31 (77.5%) | 37 (92.5%) | 36 (90.0%) | 187 (79.2%) |
| Pezizomycetes | 6 (16.7%) | 9 (22.5%) | 6 (15.0%) | 6 (15.0%) | 3 (7.5%) | 4 (10.0%) | 34 (14.4%) |
| Hymenomycetes | 5 (13.9%) | 1 (2.5%) | 2 (5.0%) | 1 (2.5%) | | | 9 (3.8%) |
| Ustilaginomycetes | 3 (8.3%) | 1 (2.5%) | | | | | 4 (1.7%) |
| Uredinomycetes | | | | 1 (2.5%) | | | 1 (0.4%) |
| Hyphomycetes | | | | 1 (2.5%) | | | 1 (0.4%) |
| Total | 36 (100%) | 40 (100%) | 40 (100%) | 40 (100%) | 40 (100%) | 40 (100%) | 236 (100%) |

The duration of composting for the sites was 0 day for S0, 1 day for S1, 3 days for S2, 7 days for S3, 10 days for S4, and 14 days for S5.

process. The slight rise in pH is thought to be due to ammonification and mineralization of organic matter through the activities of microorganisms [39].

To date, only a few fungal species have been identified in samples from compost [23] and other environments [2, 5, 30] using a molecular approach, and it was concluded that several problems contributed to this result, including insufficient DNA extraction, non-optimal primer selection, incompleteness of gene databases, and low taxonomic resolution of DNA sequences. Previous attempts to study the phylogeny of fungi have relied on PCR amplification followed by restriction endonuclease analysis of the internal transcribed spacer (ITS) and non-transcribed spacer (NTS), the V8–V9 region of fungal 18S rRNA, and the large subunit rRNA, specifically region D1/D2 [8, 10, 16, 23]. In the present study, primers that were designed to amplify a wide range of eukaryotic nuclear LSU rRNA genes [37] were employed, and the amplified sequences were used to determine fungal diversity in the compost.

A total of 12 groups of fungi were identified from the samples retrieved at different stages of pig manure and MCW composting (Table 1). Several fungal species, including *Geotrichum* sp., *Geotrichum candidum*, *Backusella* sp., *Pichia* sp., *Saccharomyces* sp., *Candida boidinii*, *Candida krusei*, and *Candida tropicalis*, have previously been identified in various compost samples by 18S rRNA sequencing [9, 11, 23, 24]. Differences in the primers used for amplification, the raw materials, and the manner of composting may have caused the differences in the fungal species that were identified. In the present study, the number of fungal species decreased from 6 or 7 species at the early to mid phases to 2 or 3 species at the late to end phases of composting. *Geotrichum* sp. of the class Saccharomycetes was the predominant fungal species throughout the composting process, and its prevalence gradually increased from 61.1% at the beginning to 92.5% after 10 days of composting (Table 2). The genus *Geotrichum* is known to be involved in degradation and spoilage of cheese, paper, and sewage [7]. The role of *Geotrichum* in composting and its potential application to rapid and high-quality compost production needs to be investigated further.

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