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ORIGINAL PAPER

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Succession and phylogenetic profile of eukaryotic communities in the composting process of rice straw estimated by PCR-DGGE analysis

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Abstract The succession and phylogenetic profile of eukaryotic communities during the composting process of rice straw (RS) were studied by applying polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis followed by sequencing of 18S rDNA. Principal component analysis and cluster analysis of the DGGE band patterns of eukaryotic communities resulted in exactly the same grouping as found with phospholipid fatty acid (PLFA) analysis (Cahyani et al. in Soil Sci Plant Nutr 48:735, 2002) and by the DGGE pattern analysis of the bacterial communities (Cahyani et al. in Soil Sci Plant Nutr 49:619, 2003) for the same samples, namely the communities characterizing the pre-composting stage (initial RS materials), and thermophilic, middle, and curing stages of the compost. Different eukaryotic members characterized the respective stages as follows: fungi (Ascomycota) for the initial RS materials, protozoans (Apicomplexa) as well as the fungi (Ascomycota) of the initial RS materials for the thermophilic stage, fungi (Ascomycota and Basidiomycota), protozoans (Opalozoa, Ciliophora and Leptomyxida), nematodes and stramenopiles for the middle stage, and fungi (Ascomycota, Zygomycota and Oomycota), algae (Haptophyceae and Chrysophyceae), and nematodes for the curing stage, respectively. Temperature, moisture content, and substrates available seemed to play a key role in determining the composition of eukaryotic members present at the respective stages of the composting process of RS.

Keywords Denaturing gradient gel electrophoresis · Eukaryote · Phylogenetic profile · Rice straw compost · Succession

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Introduction

Composting is the microbial degradation of organic solid materials that involves aerobic decomposition and passing through a thermophilic stage (Finstein and Morris 1975). Decomposition of organic materials by composting achieves the following objectives: sanitation, mass and bulk reduction, and resource recovery (Finstein and Morris 1975; Miller and Donahue 1990). To evaluate the degree of composting and to improve the quality of the end product, the characterization of biota present throughout the composting process is important. Several studies have indicated that the presence of certain microorganisms exerts a distinctive effect on quality improvement of compost or acceleration of the composting process (Nakasaki and Uehara 1996; Requena et al. 1996; Kuo-Shu et al. 1998; Badr El-Din et al. 2000).

The world produces 540 million tonnes of rice annually from an area of about 148 million hectares, with 92% of the total production coming from Asia (Piggin 2001). As a crop residue, rice straw (RS) has been commonly subjected to composting in Asian countries such as Japan, Taiwan, Korea, China, the Philippines and Indonesia (Takahashi et al. 1978). The use of RS compost incorporated into rice fields for sustainable rice production is well known (Sakon et al. 1982; Inoko 1984; Shiota et al. 1984; Songmuang et al. 1997) and it is sometimes used as the growth media for mushrooms (Chiou et al. 1972; Takahashi et al. 1978; Lim 1981; Rambelli 1989). However, the biota responsible for the composting process of RS have not been elucidated fully.

The present study is the continuation of two previous studies revealing the biota responsible for the composting process of RS. Two cultivation-independent methods, phospholipid fatty acid (PLFA) analysis and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis followed by sequencing of eubacterial 16S rDNA were conducted in our previous studies (Cahyani et al. 2002, 2003).

PLFA analysis detected the succession of biota including bacteria and eukaryotic organisms throughout the composting process of RS. Biomarker PLFAs of fungi and Gram-negative bacteria predominated in the RS materials used. At the thermophilic stage (the first 2 weeks), biomarker PLFAs of Gram-positive bacteria and actinomycetes predominated. After the thermophilic stage, biomarker PLFAs of other Gram-positive bacteria became dominant. Finally at the curing stage, the proportion of biomarker PLFAs of Gram-negative bacteria and eukaryotes increased, indicating the co-contribution of bacteria and eukaryotic organisms to the decomposition process at this stage (Cahyani et al. 2002).

PCR-DGGE analysis followed by sequencing of 16S rDNA detected the succession of eubacterial communities which accorded with the profile of biomarker PLFAs of Gram-positive and negative bacteria and actinomycetes. Different bacterial members characterized respective stages of composting: α-proteobacteria in RS materials, thermophilic *Bacillus* spp. and actinomycetes at the thermophilic stage, and *Cytophaga* and clostridial members at the middle and curing stages, respectively. In addition, mesophilic *Bacillus* members were always present throughout the composting process (Cahyani et al. 2003).

For complete elucidation of the biota responsible for the composting process of RS, the present study aimed at revealing the succession and phylogenetic profile of eukaryotic organisms by applying molecular (18S rDNA) methods using PCR-DGGE analysis, followed by sequencing.

Many earlier studies have revealed the presence of eukaryotic organisms in the composting process, such as the proliferation of fungi by culture methods (Waksman et al. 1939; Fergus 1964; Chang and Hudson 1967; Kane and Mullins 1973; Strom 1985; Wiegant 1992; Straatsma et al. 1994; Klamer and Søchting 1998), of microfauna including protozoans by MPN methods and of nematodes by flotation in colloidal silica (Keeling et al. 1995) or by funnel methods (Akgül and Ökten 2000). In the composting of a mixture of *Miscanthus* straw and pig slurry (Klamer and Bååth 1998) and of various feed materials from municipal solid waste (Herrmann and Shann 1997), PLFA analysis showed that the biomarker PLFA of fungi (18:2ω6c) appeared at the mesophilic stage before peak heating and at the curing stage of composting. In addition, Herrmann and Shann (1997) also reported the appearance of the biomarker PLFA of protozoans (20:4ω6c) at the curing stage. Thus, there is still little information about the succession and phylogenetic profile of eukaryotic organisms in the composting process. So far, most molecular studies of composting focused mainly on bacterial communities (Beffa et al. 1996a,1996b; Blanc et al. 1999; Ishii et al. 2000; Pedro et al. 2001; Ueno et al. 2001; Haruta et al. 2002a,2002b).

Materials and methods

All the samples used in the present study were the same as those used in the two previous studies on revealing the biota responsible for the composting process of RS, either in the detection of the succession of biota by using PLFA analysis (Cahyani et al. 2002), or the succession of bacterial communities by using PCR-DGGE analysis followed by sequencing of 16S rDNA (Cahyani et al. 2003).

Experimental site

Composting of RS was conducted in a storehouse at Aichi-ken Anjo Research and Extension Center, central Japan (latitude 34°8′N, longitude 137°5′E).

Setup of the compost pile and sampling method

Composting was performed based on a conventional method used in Japan with RS and ammonium sulfate ($[NH_4]_2SO_4$) as materials (Cahyani et al. 2002, 2003). The size of the compost pile was approximately 210 cm long, 180 cm wide, and 80 cm high. At each sampling time, five sub-samples were collected randomly at a depth of about 30 cm, where the measured temperature was not different from that of the center. The samples were then stored at −20°C.

Dried RS (compost material) piled in a storehouse was sampled twice, 45 days before the onset of composting and on the day of composting. Samples were taken 12 times during the 145-day period of composting.

DNA extraction and purification

DNA was extracted with DNA extraction buffer [100 mM Tris-HCl (pH 8.0), 100 mM EDTA, 1.5 M NaCl, and 1% cetyltrimethylammonium bromide (CTAB)], followed by freeze (−196°C) and thaw (65°C) treatments, and lysozyme addition, and was purified with Sephadex G-200 as described previously (Cahyani et al. 2003). Five sub-samples were collected every sampling time during composting. All the procedures were performed in duplicate using a mixture of five sub-samples of RS compost for each sampling time.

PCR-DGGE analysis

The 18S rDNA was amplified with PCR using the eukaryotic primer set of forward primer with GC-rich clamp (*Saccharomyces cerevisiae* position: 1,427–1,453, 5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCTCTGTGATGCCCTTAGATGTTCTGGG-3′; the underlined sequence corresponded to the GC-rich clamp) and reverse primer (*S. cerevisiae* position: 1,616–1,637, 5′-GCGGTGTGTACAAAGGGCAGGG-3′; Van Hannen et al. 1998). PCR was performed in a total volume of 50 μl in 200-μl microtubes, which contained 0.25 μl each primer (100 pmol each), 5 μl 2.5 mM dNTP mixture, 5 μl 10× *Ex Taq* buffer (20 mM Mg^{2+}; TaKaRa, Tokyo, Japan), 0.25 μl 5 units/μl *Ex Taq* DNA polymerase (TaKaRa), 1 μl DNA template (about 15 ng) and 38.5 μl milli-Q water. Cycle conditions for the amplification were as follows: an initial denaturation at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 0.5 min, annealing at 52°C for 1 min, extension at 68°C for 1.5 min, then a final extension at 68°C for 10 min with TaKaRa PCR Thermal Cycler Model TP 240 (TaKaRa). The PCR product was analyzed on 2% (w/v) agarose gels as described previously (Cahyani et al. 2003). The DNA fragments of the PCR products were separated on a DGGE gel which was prepared according to Muyzer et al. (1998) with a denaturing gradient from 25% to 65%. The electrophoresis was performed in an electrophoresis cell D-code™ System at 60°C and 75 V for 16 h (Van Hannen et al. 1998). Visualization of DGGE bands was performed as described previously (Cahyani et al. 2003). We applied the duplicate of PCR products for DGGE at first, and found exactly the same patterns for each replication of sample from each sampling time. To show the succession of eukaryotic communities during the composting process of RS, we presented a single replicate of sample from each sampling time for the figure of DGGE patterns (Fig. 1).

Statistical analysis

To estimate the succession of eukaryotic communities during the composting process of RS, the data obtained from the DGGE patterns, based on band intensity and position, were analyzed by cluster analysis and principal component analysis. Principal component analysis was performed using EXCEL STATISTICS 1997 for Windows (SRI, Tokyo). A correlation matrix was used in the analysis. Cluster analysis was performed according to the Blackbox program (Aoki 1996). Ward method was used in the analysis.

Sequencing of the selected DGGE bands

The selected DGGE bands which had large Eigen values in PCA analysis or which were specific at each stage of composting were excised from the polyacrylamide gel and reamplified with the same primer. The re-amplified PCR products were confirmed by comparing their position with the original DGGE patterns. The PCR products that matched the position to the excised band in the original DGGE patterns were then sequenced with the Thermo

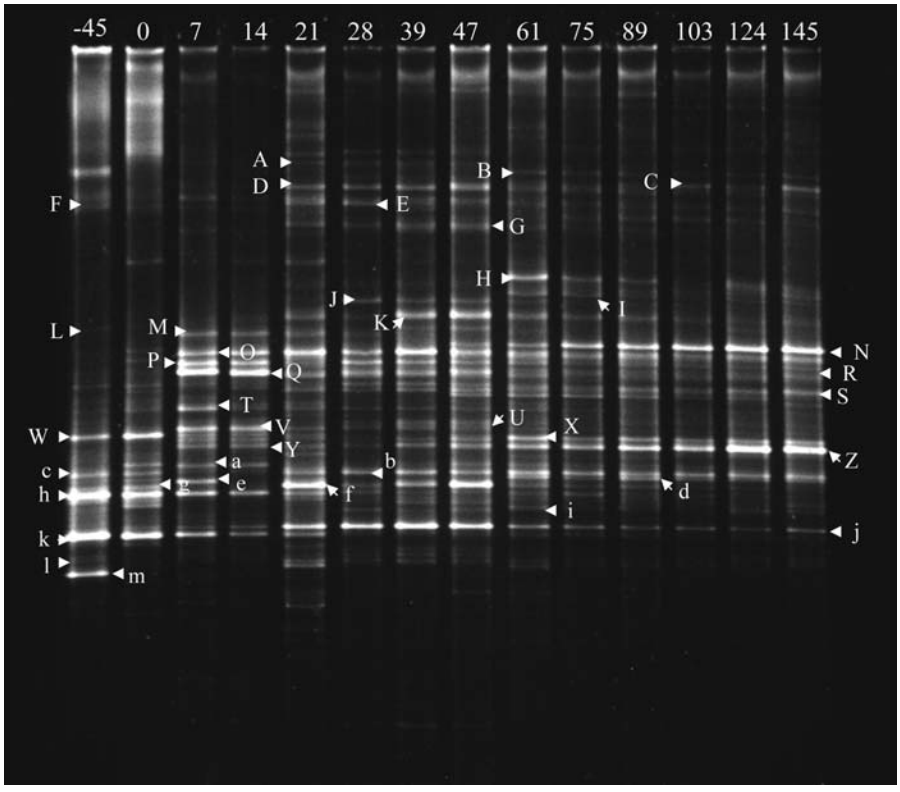

Fig. 1 DGGE patterns of the eukaryotic communities associated with the composting process of rice straw. *Numbers above the lane* denote the composting time (days)

Sequenase™II Dye Terminator Cycle sequencing kit (Amersham, Tokyo, Japan) according to the instructions, by using the set of two primers described above (forward primer without GC clamp and reverse primer) with the 373S DNA sequencer (ABI, Urayasu, Japan).

Phylogenetic analysis

Sequences of DGGE bands were compared to the 18S rDNA sequences obtained with the BLAST search from the database of the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/E-mail/homology.html).

Nucleotide sequence accession number

The 18S rDNA partial sequences obtained in this study are available in the DDBJ database under the accession numbers AB120134–AB120172.

Results

DGGE band patterns of eukaryotic communities during the composting process of RS

Four stages were recognized in the composting process of RS based on the temperature changes of the composting pile and the succession patterns of PLFA composition and eubacterial communities as described in our previous studies, namely pre-composting stage (the initial RS materials), thermophilic stage, middle stage, and curing stage (Cahyani et al. 2002, 2003).

DGGE band patterns shown in Fig. 1 illustrated the profile of eukaryotic communities in the composting process of RS. The number of DGGE bands ranged from 15 to 19 for the RS materials (45 days before composting and on day 0), from 19 to 23 during the thermophilic stage (day 7 to day 14), from 30 to 37 during the middle stage (day 21 to day 47), and from 27 to 38 during the curing stage (day 61 to day 145), respectively (Fig. 2).

Statistical analysis of the DGGE patterns of eukaryotic communities in the initial RS materials and RS under the composting process was performed by principal component analysis and cluster analysis. Principal component analysis showed that the total contribution percentages of the first and second principal components were 26.7% and 25.7%, respectively. The DGGE bands with positively and negatively large Eigen values in the first principal component were T and Q, and d, I, B, S, R, H, and N, respectively. On the other hand, the DGGE bands with positively and negatively large Eigen values in the second principal component were j, D, K, b, G, f, and J, and h, k, and F, respectively (Fig. 3). Closest relatives of the DGGE bands with positively and negatively large Eigen values in the first and second principal components are listed in Table 1.

Cluster analysis identified four groups for the DGGE patterns of the eukaryotic communities in the initial RS materials and RS under the composting process (Fig. 4). The DGGE patterns were first divided into three clusters: the first cluster was further divided into two sub-clusters of the initial RS materials and the RS compost at the thermophilic stage; the second cluster was the RS compost at the middle, and the third cluster the RS compost at the curing stages. Domains A–D shown in Fig. 3 corresponded to the respective clusters and sub-clusters obtained in the cluster analysis.

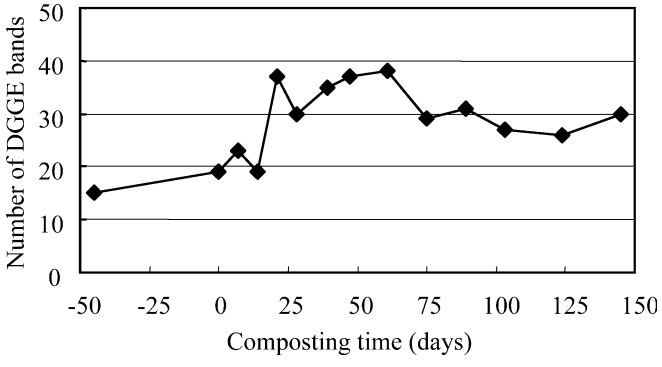

Fig. 2 Number of bands in the DGGE patterns of the eukaryotic communities associated with the composting process of rice straw

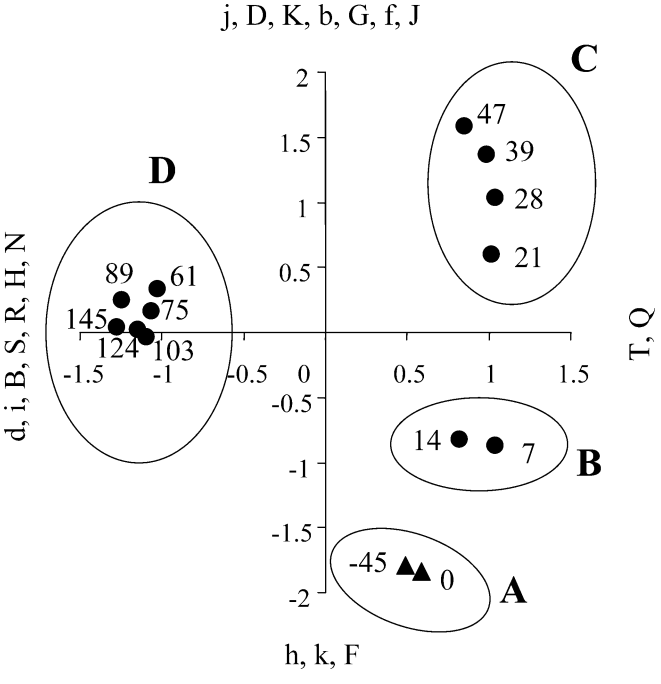

Fig. 3 Principal component analysis of the DGGE patterns of the eukaryotic communities associated with the composting process of rice straw. *A* Rice straw materials at the pre-composting stage, *B* rice straw compost at the thermophilic stage, *C* rice straw compost at the middle stage, *D* rice straw compost at the curing stage. *Numbers next to the symbol* denote the composting time (days)

Table 1 Closest relatives of the eukaryotes associated with the composting process of rice straw that were characterized by respective DGGE bands [*1st PC(+)* positively large Eigen value in the first principal component, *1st PC(−)* negatively large Eigen value in the first principal component, *2nd PC(+)* positively large Eigen value in the second principal component, *2nd PC(−)* negatively large Eigen value in the second principal component, *R* initial RS materials, *T* thermophilic stage of composting, *M* middle stage of composting, *C* curing stage of composting, *v* presence]

DGGE band	PCA analysis Eigen value	Seq bp	Stages of composting				Closest relatives			Similarity (%)	Alignment
			R	T	M	C	Microorganisms	Phylogenetic affiliations	Accession number		
L		160	v				*Trigonocryptus conus*	Platyhelminthes	AJ287584	94	52/55
							Antorchis pomacanthi	Platyhelminthes	AJ287476	94	52/55
W		164	v				*Raciborskiomyces longisetosum*	Ascomycota	AY016351	100	164/164
							Cladosporium herbarium	Ascomycota	AJ301721	100	164/164
c		164	v				*Euascomycetes* sp.	Ascomycota	Y18694	98	161/164
							Exophiala nigra	Ascomycota	X91896	98	161/164
g		166	v				*Ustilago* sp.	Basidiomycota	AJ244777	97	162/166
l		164	v				*Oryza sativa* 18S ssu rRNA	Spermatophyta	AF069218	98	161/163
m		164	v				*Aspergillus restrictus*	Ascomycota	AB008407	100	164/164
e		162		v			*Pichia membranifaciens*	Ascomycota	AB053242	100	162/162
							Candida ethanolica	Ascomycota	X96455	100	162/162
Y		165		v			*Echinamoeba thermarum*	Euamoebida	AJ489266	97	68/70
A		169			v		*Echinamoeba thermarum*	Euamoebida	AJ489266	98	167/169
E		231			v		*Osyris lanceolata*	Spermatophyta	L24409	94	48/51
B	1st PC(−) −0.205	162				v	*Chlamydomyxa labyrinthuloides*	Chrysophyceae	AJ130893	92	150/162
C		164				v	Eukaryote marine clone ME1-24	Eukaryote	AF363207	93	140/150
							Heteromita globosa	Cercozoa	U42447	91	144/157
H	1st PC(−) −0.190	164				v	*Lagenidium giganteum*	Oomycota	X54266	96	158/164
I		164				v	*Phytophthora megasperma*	Oomycota	X54265	98	161/164
N	1st PC(−) −0.189	162				v	*Rhabditoides inermiformis*	Nematoda	AF083017	91	148/162
R	1st PC(−) −0.205	164				v	*Backusella ctenidia*	Zygomycota	AF157122	100	164/164
S	1st PC(−) −0.205	163				v	Unidentified eukaryote	Eukaryote	AJ130850	95	154/161
							Uncultured fungus clone	Fungi	AF372717	95	153/161
d	1st PC(−) −0.205	162				v	*Colletotricum truncatum*	Ascomycota	AJ301945	98	159/162
							Hypocrea jecorina	Ascomycota	AF510497	98	159/162
i	1st PC(−) −0.205	162				v	*Cruciplaccolithus neohelis*	Haptophyceae	AJ246262	84	125/148
							Coccolithus pelagicus	Haptophyceae	AJ246261	84	125/148
F	2nd PC(−) −0.164	166	v	v			*Geopyxis carbonaria*	Ascomycota	AF104665	94	157/166
a		165	v	v			*Echinamoeba thermarum*	Euamoebida	AJ489266	97	68/70
T	1st PC(+) 0.172	162	v	v	v		*Colpodella* sp. ATCC50594	Apicomplexa	AY142075	87	142/162
M		163		v	v		*Gregarina chortiocetes*	Apicomplexa	L31841	92	151/164
Q	1st PC(+) 0.166	163		v	v		*Apusomonas proboscidea*	Opalozoa	L37037	92	151/163
U		164		v	v		*Dendroligotrichum dendroides*	Bryophyta	Y16519	91	149/162
							Atrichum undulatum	Bryophyta	X85093	91	149/162
V		165		v	v		*Echinamoeba thermarum*	Euamoebida	AJ489266	97	68/70
O		244		v	v	v	*Plasmodiophora brassicae*	Plasmodiophorida	Y12831	97	48/49
							Spongospora subterranea	Plasmodiophorida	AF310901	97	48/49
P		245		v	v	v	*Plasmodiophora brassicae*	Plasmodiophorida	Y12831	97	48/49
							Spongospora subterranea	Plasmodiophorida	AF310901	97	48/49
D	2nd PC(+) 0.192	164			v	v	Unidentified eukaryote	Eukaryote	AJ130861	98	161/164
							Platyophrya vorax	Ciliophora	AF060454	92	151/164

Table 1 (continued)

DGGE band	PCA analysis Eigen value	Seq bp	Stages of composting				Closest relatives			Similarity (%)	Alignment
			R	T	M	C	Microorganisms	Phylogenetic affiliations	Accession number		
G	2nd PC(+) 0.174	163			v	v	Uncultured marine stramenopile	Eukaryote, stramenopiles	AF290083	92	149/161
							Eukaryote marine clone ME1-17	Eukaryote	AF363186	92	150/163
J	2nd PC(+) 0.162	164			v	v	*Paraflabellula hoguae*	Leptomyxida	AF293899	89	112/125
K	2nd PC(+) 0.192	161			v	v	*Halicephalobus gingivalis*	Nematoda	AF202156	95	153/161
X		164			v	v	*Rhizophlyctis rosea*	Chytridiomycota	AF164252	98	161/164
Z		162			v	v	Uncultured rhizosphere ascomycete	Ascomycota	AJ506032	99	161/162
							Ascovaginospora stellipala	Ascomycota	U85087	98	160/162
b	2nd PC(+) 0.178	162			v	v	*Ascovaginospora stellipala*	Ascomycota	U85087	99	161/162
							Chaetomium elatum	Ascomycota	M83257	99	161/162
f	2nd PC(+) 0.174	164			v	v	*Calvatia gigantea*	Basidiomycota	AJ237864	100	164/164
							Lycoperdon umbrinum	Basidiomycota	AJ237860	100	164/164
							Bovistella radicata	Basidiomycota	AJ237856	100	164/164
h	2nd PC(−) −0.176	162	v	v	v	v	*Pestalotiopsis* sp.	Ascomycota	AF346561	97	158/162
							Amphisphaeria sp. MAFF235878	Ascomycota	AF346545	97	158/162
j	2nd PC(+) 0.200	162	v	v	v	v	*Ascovaginospora stellipala*	Ascomycota	U85087	98	159/162
							Chaetomium elatum	Ascomycota	M83257	98	159/162
k	2nd PC(−) −0.164	164	v	v	v	v	*Oryza sativa* 18S ssu rRNA	Spermatophyta	AF069218	98	161/163

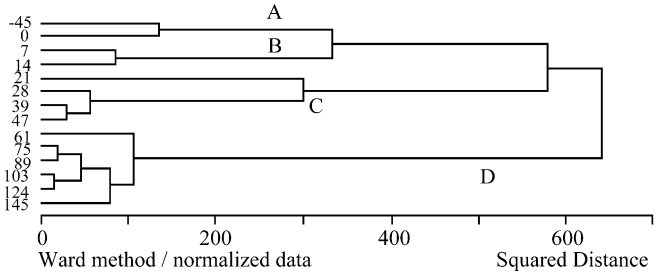

Fig. 4 Cluster analysis of the DGGE patterns of the eukaryotic communities associated with the composting process of rice straw. *A* Rice straw materials at the pre-composting stage, *B* rice straw compost at the thermophilic stage, *C* rice straw compost at the middle stage, *D* rice straw compost at the curing stage. *Numbers next to the cluster* denote the composting time (days)

Succession of eukaryotic communities

Based on the distribution of the score plots of the DGGE bands of eukaryotic communities in the initial RS materials and RS under the composting process (Fig. 3, Table 1), the succession of eukaryotic communities during the composting process of RS was summarized as follows.

RS materials used

The score plots of DGGE bands of the initial RS samples are located at the lower right-side in Fig. 3. Bands h, k, and F (negatively large Eigen values in the second principal component) characterized the eukaryotic communities in the initial RS materials. These characteristic bands corresponded to members of the Ascomycota (bands h and F) and *Oryza sativa* (band k).

Thermophilic stage

The score plots of DGGE bands in the RS compost at the thermophilic stage shifted upwards from the score plots of DGGE bands in the initial RS materials in the same fourth quadrant (Fig. 3). Besides the three sustained bands (h, k, and F) characterizing eukaryotic communities from initial RS materials to the thermophilic stage, band T (positively large Eigen value in the first principal component) was a characteristic band at the thermophilic stage. Band T corresponded to members of the protozoan Apicomplexa.

Middle stage

The score plots of DGGE bands in the RS compost at the middle stage are located at the upper right-side in Fig. 3. Band Q (positively large Eigen value in the first principal component) and bands j, D, K, G, f, and J (positively large Eigen values in the second principal component) characterized the eukaryotic communities at this stage. Three characteristic bands (Q, D, and J) corresponded to members of the Protozoa (Opalozoa, Ciliophora, and

Leptomyxida). Two characteristic bands (j and f) corresponded to fungal members (Ascomycota and Basidiomycota). Bands G and K corresponded to stramenopiles and nematodes, respectively.

Curing stage

The score plots of DGGE bands in the RS compost at the curing stage are located at the middle left-side in Fig. 3. All bands with negatively large Eigen values in the first principal component and band b (positively large Eigen value in the second principal component) characterized the eukaryotic communities in the RS compost at the curing stage. Five characteristic bands (b, d, S, R, and H) corresponded to fungi (Ascomycota, Zygomycota, and Oomycota). Two bands (i and B) corresponded to algae (Haptophyceae and Chrysophyceae). Band N corresponded to nematodes.

Eukaryotic members common at respective stages

There were 88 different DGGE bands in total (Fig. 1). Thirty-nine of these were successfully excised and sequenced, including the characteristic bands in the first and second principal components described above. All sequenced data of these bands which appeared commonly at respective stages are shown in Table 1.

Common bands in the RS materials and RS under the composting process

Bands h, j, and k were always present from the initial RS materials until the end of the composting process (Fig. 1). Changes in band intensities resulted in these three bands appearing as the characteristic bands in principal component analysis as described above. Bands h and k appeared with high intensity in the initial RS materials, while band j showed a strong intensity at the middle stage. Bands h and j corresponded to fungal members (Ascomycota), and band k corresponded to rice 18S rDNA (*O. sativa*).

Common bands in the RS under the composting process

Bands O and P appeared from the thermophilic stage to the end of composting. The sequences of these two bands showed longer DNA fragments (244/245 bp) without any clear affiliation, and only short sequences matched to the available DNA database. These two bands seemed to belong to the same unknown organisms.

Common bands in the RS materials

As shown in Table 1, among 12 sequenced bands in the RS materials, 6 were specific bands which were restricted in the RS materials. Sequence analysis indicated that eukaryotic communities in the RS were dominated by fungi (7 bands), followed by rice 18S rDNA (2 bands), and a protozoan (1 band), and 2 bands showed no clear affiliation to any known eukaryote.

Common bands at the thermophilic stage

Two out of 14 sequenced bands at the thermophilic stage appeared specifically at this stage only. The 14 bands were affiliated to fungi (4 bands), protozoans (3 bands), rice 18S rDNA (1 band), and a moss (1 band), and 5 bands showed no clear affiliation to any known eukaryote.

Common bands at the middle stage

Twenty bands at the middle stage were sequenced, two of which appeared at this stage only. Sequence analysis indicated these bands affiliating to fungi (6 bands), protozoans (6 bands), a moss (1 band), a nematode (1 band), a stramenopile (1 band), and rice 18S rDNA (1 band), and 4 bands showed no clear affiliation to any known eukaryote.

Common bands at the curing stage

Among 22 sequenced bands at the curing stage, 9 exclusively existed at this stage. The closest relatives of these bands were affiliated to fungi (11 bands), protozoans (3 bands), algae (2 bands), nematodes (2 bands), a stramenopile (1 band), and rice 18S rDNA (1 band), and 2 bands showed no clear affiliation to any known eukaryote.

Discussion

DGGE band patterns of eukaryotic communities during the composting process of RS

Principal component analysis and cluster analysis of the DGGE patterns of eukaryotic communities during the composting process of RS in Fig. 1 resulted in exactly the same grouping as those found by PLFA analysis (Cahyani et al. 2002) and DGGE pattern analysis of the eubacterial communities (Cahyani et al. 2003) using the same samples: communities associated with the pre-composting stage (initial RS materials), and thermophilic, middle, and curing stages of the compost (Fig. 5). These similar groupings irrespective of the target organisms indicated that bacterial and eukaryotic communities were affected in

a similar manner by environmental factors such as temperature, moisture content, pH, and substrates.

The number of DGGE bands was low in the initial RS materials and at the thermophilic stage, increased at the middle stage, then decreased a little and remained relatively constant at the curing stage (Fig. 2). Similar patterns of the number of DGGE bands were also observed in the succession of eubacterial communities in the same composting samples (Cahyani et al. 2003). Dry conditions (moisture content of RS 8%) in the initial RS materials and high temperature (42–66°C) at the thermophilic stage of composting might stimulate the growth of specific biota only. By contrast, the composting conditions at the middle and curing stages prepared habitats favorable to more diverse organisms.

Succession of eukaryotic communities responsible for the composting process of RS

The succession of eukaryotic communities during the composting process of RS was reflected in the anti-clockwise shifting of the score plots of the DGGE bands at four stages of composting (Fig. 3). Figure 3 indicated the following characteristic eukaryotes in the succession: fungi (Ascomycota) characterized the initial RS materials, the thermophilic stage was characterized by protozoans (Apicomplexa) as well as the fungi (Ascomycota) of the initial RS materials, the middle stage by fungi (Ascomycota and Basidiomycota), protozoans (Opalozoa, Ciliophora and Leptomyxida), nematodes and stramenopiles, and finally the curing stage by fungi (Ascomycota, Zygomycota and Oomycota), algae (Haptophyceae and Chrysophyceae), and nematodes. Since the present study neglected the mesophilic stage which occurs generally for a short period before the thermophilic stage, further study of this stage is necessary for understanding the total succession of eukaryotic communities in the composting process of RS. The phylogenetic profile of eukaryotic communities throughout the four stages observed in the present study was as follows.

RS materials used

The characteristic band k corresponded to *O. sativa*, the rice 18S rDNA. Band l which appeared only in the RS material taken 45 days before composting was also related to *O. sativa*. The appearance of these two bands in the first RS material sample and the gradual decrease in intensity of band k indicated the decomposition of RS materials during composting (Fig. 1).

Characteristic bands h and F corresponded to fungi, indicating that the dry conditions during the storage period of RS materials seemed to be favorable for fungal growth. The other bands which existed specifically in the RS materials belonged mainly to fungi (bands W, c, g, and m). Except for band g, which was affiliated to the Basidiomycota, all those bands were affiliated to the Ascomycota. Mycelial Ascomycota are common saprophytes that degrade cellulose and other structural polymers (Deacon 1997). The finding that the majority of DGGE bands in the RS materials were closely related to fungi agreed well with the results of PLFA analysis (Cahyani et al. 2002) in which the biomarker PLFA of fungi (18:2ω6c) predominated in the RS materials.

Chang and Hudson (1967) investigated fungi in wheat straw compost and also observed fungal colonization of wheat straw that was stored under dry conditions for varying periods, namely *Aspergillus repens*, *A. amstelodami*, *A. versicolor*, *A. candidus*, *A. nidulans*, and *Penicillium* spp. However only band m corresponded to the genus *Aspergillus* in the present study. The closest relative of band W, *Cladosporium herbarium*, was one of the commonest primary saprophytes that grew actively on wheat straw at the time of harvesting or after harvest (Chang and Hudson 1967).

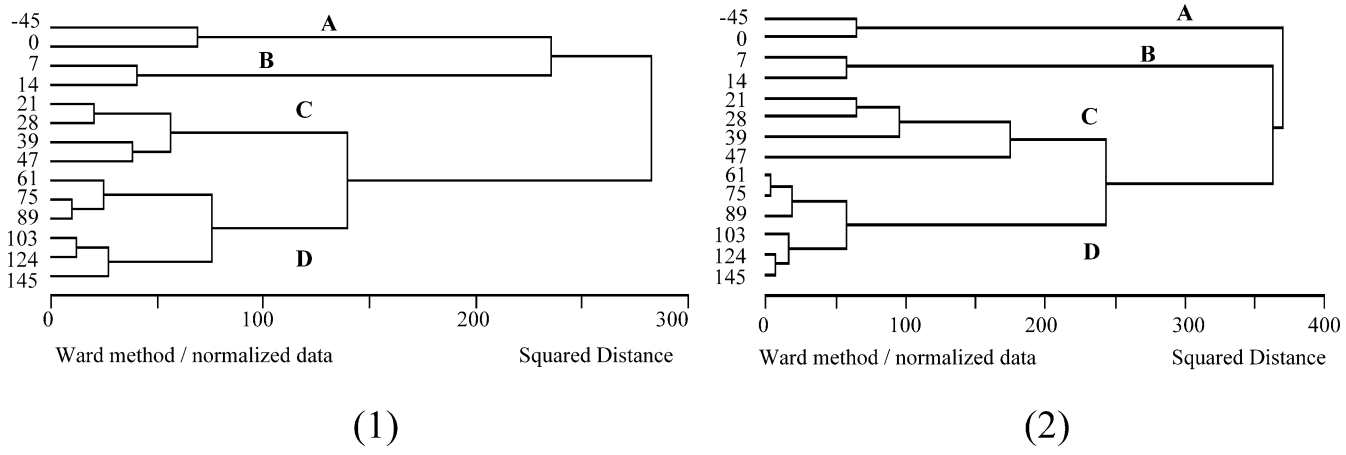

Fig. 5 Cluster analyses of PLFA composition in the rice straw under the composting process (*1*) and DGGE patterns of the bacterial communities associated with the composting process of rice straw (*2*). *A* Rice straw materials at the pre-composting stage, *B* rice straw compost at the thermophilic stage, *C* rice straw compost at the middle stage, *D* rice straw compost at the curing stage. *Numbers next to the clusters* denote the composting time (days)

Bands L and a showed no clear affiliation (Table 1) to any known eukaryote, and only short sequences were matched to those from the available DNA database, which might result from the scarcity of the sequence in the database related to these eukaryotes.

Thermophilic stage

Decrease in intensity of the characteristic bands h and F might reflect the suppressive effect of high temperature upon the growth of these fungi, as several bands which corresponded to fungal members in the RS materials (bands W, c, g, and m) disappeared at the thermophilic stage. This finding seemed to reveal the sanitation effect of a high temperature phase in the composting process. No thermophilic or thermotolerant fungus was reported to grow at 60°C in the composting of mushroom (Fergus 1964). Hassen et al. (2001) found that a high temperature (55–60°C) significantly decreased the populations of yeast and filamentous fungi from 4.5×10^6 to 2.6×10^3 cells g^{-1} waste dry weight in the composting of municipal solid waste. Akbar et al. (2001) also reported that no mesophilic fungus was obtained after 20 days of composting of municipal solid waste due to high temperature (60–68°C), though some types of yeast and molds were isolated at the initial stage of composting. According to the study by Chang and Hudson (1967) on the composting of wheat straw, mesophilic fungi were killed after the temperature had reached 40°C and above, and the thermophilic and thermotolerant species were also killed above 60°C.

Band e appeared specifically in the sample taken on day 7 when the temperature of the compost pile reached a peak (66°C) and corresponded to a yeast (Ascomycota). Peters et al. (2000) reported that only the genus *Candida* was detected during the thermophilic stage (60–80°C) of composting with an organic agricultural substrate by using PCR primers specific to fungi (NS7 and NS8).

According to Chang and Hudson (1967) and Klamer and Søchting (1998), the fungi developing during the composting process could be divided into two groups, one appearing before peak heating and the other appearing after that. As the mesophilic stage before peak heating was not examined in the present study, a comparison could be made at the stages after peak heating only. However, none of the thermophilic and thermotolerant fungi that had been frequently found in many earlier studies by culture methods were detected as the closest relatives in the present study, such as *Aspergillus fumigatus*, *Chaetomium thermophile*, *Mucor pusillus*, *Humicola lanuginose*, and *Thermomyces lanuginosus* in mushroom compost (Fergus 1964; Straatsma et al. 1994), municipal waste compost (Kane and Mullins 1973), a mixture of Miscanthus straw and pig manure compost (Klamer and Søchting 1998), wheat straw compost (Chang 1967; Chang and Hudson 1967), and horse manure compost (Waksman et al. 1939). This apparent discrepancy might indicate the differences between the two methodological approaches, the culture method and the molecular method, by using directly extracted DNA from environmental samples. Isolation and cultivation conditions seemed to allow the proliferation of some fungi with small biomass from a small number of spores of which the biomass was too small to be detected by the PCR-DGGE method.

The closest relatives of seven bands that were sequenced in the present study were protozoans, among which three appeared at the thermophilic stage, six at the middle stage, and three at the curing stage, respectively. Although bands V, Y, and a matched to the protozoan group for their short sequences, they were neglected in the following discussion. Among three protozoan bands at the thermophilic stage, bands M and Q appeared first at this stage. Although band T appeared from the initial RS materials, it showed strong intensity only on day 7 at the highest observed temperature. Thus they were regarded as thermophilic/thermotolerant. Keeling et al. (1995) also detected protozoans at the thermophilic stage of composting for mixed garden-waste.

Interesting was the appearance of several bands at the thermophilic stage which showed no clear affiliation to any known eukaryote, and only short sequences matched to them in the available DNA database (Table 1). Noteworthy was that band O had similar sequences to band P, and three other bands (V, Y and a) also showed similar sequences to each other. One possible reason is the scarcity of the sequence in the database related to these eukaryotes, but further investigation should be carried out to clarify this phenomenon.

Although the temperature of the compost pile on day 14 decreased to 42°C, no new band appeared. At this time any recovering or newly appeared members might not yet be at a level detectable by the PCR-DGGE method.

Middle stage

All fungal bands which appeared at the middle stage (f, j, X, Z, b, and h) were continuously present in the curing stage until the end of the composting process. This indicated that the conditions for growth such as habitats and substrates were similar for fungi during the middle and curing stages.

Besides fungal bands belonging to Ascomycota and Basidiomycota, a fungal band X belonging to *Rhizophlyctis rosea* (Chytridiomycota) was detected in this stage. This fungus has been reported to be an active cellulose decomposer in soil (Deacon 1997).

Several bands that appeared at the middle stage corresponded to protozoans (bands Q, D, J, A, T, and M), among which bands D, J, and A newly appeared at this stage. The role and activity of the protozoans characterized by either the three bands which newly appeared at this stage or the other three bands which showed a decrease in intensity at this stage is still unclear. Some activities of protozoans in soil are well known, such as stimulation of the nitrification process (Clarholm 1985; Griffiths 1986, 1989, 1994; Alphei et al. 1996), and possibilities for biocontrol of plant pathogens in the

interaction with soil microflora (Pussard et al. 1994), but their role and activity in the composting process of RS remains to be elucidated.

In addition, the closest relative of band K was a nematode *Halichepalobus gingivalis* in the order Rhabditida. This was the first stage when a nematode was detected. The presence of several species of nematodes in the compost used for cultivation of mushrooms was also reported by Akgül and Ökten (2000).

Curing stage

The curing stage was characterized by DGGE bands which corresponded to fungi, algae and nematodes. Although none of the characteristic bands belonged to protozoans, band C (which appeared specifically at this stage but was not listed as one of the characteristic bands in Table 1) corresponded to the protozoan Cercozoa. Bands D and J that appeared from the middle stage and corresponded to protozoans showed a decrease in intensity at the curing stage.

The fungal population at the curing stage was of a complex composition, and besides Ascomycota, Basidiomycota, and Chytridiomycota, two other groups appeared at this stage, namely Oomycota and Zygomycota. The closest relative of band H, *Lagenidium giganteum*, a member of the Oomycota, is known as a parasite of invertebrates (on nematode, mosquito larvae, etc.; Deacon 1997).

Two characteristic bands at this stage belonged to algae, band B belonging to Chrysophyceae and band i to Haptophyceae. Presence of algae at the curing stage was probably due to the low temperature of the compost pile, as some freshwater species of Chrysophytes showed maximum growth in cool water (Perry et al. 2002). Figure 1 shows that the two bands related to algae appeared clearly on day 61 when the temperature of the compost pile was 18°C, and the intensity of these two bands decreased gradually to the end of composting.

The characteristic band N corresponded to a nematode, *Rhabditoides ineformis*. This species was in the same order (Rhabditida) as the closest relative of band K. Thus, nematodes found in the composting process of RS by PCR-DGGE method were only members belonging to the order Rhabditida. In contrast, Akgül and Ökten (2000) isolated 13 species of nematodes from compost used for cultivation of mushrooms and reported that 4 species belonged to the order Tylenchida, 3 to Aphelenchida, 2 to Dorylaimida, and 5 to Rhabditida.

In conclusion, principal component analysis and cluster analysis of the DGGE band patterns of eukaryotic communities associated with the composting process of RS resulted in exactly the same grouping as found by the previous PLFA analysis (Cahyani et al. 2002) and DGGE pattern analysis of the bacterial communities (Cahyani et al. 2003) for the same samples, namely communities associated with the pre-composting stage (initial RS materials), and thermophilic, middle, and curing stages of the compost. Different eukaryotic members characterized the respective stages as follows: fungi (Ascomycota) for the initial RS materials, protozoans (Apicomplexa) as well as the fungi (Ascomycota) of the initial RS materials for the thermophilic stage, fungi (Ascomycota and Basidiomycota), protozoans (Opalozoa, Ciliophora and Leptomyxida), nematodes and stramenopiles for the middle stage, and fungi (Ascomycota, Zygomycota and Oomycota), algae (Haptophyceae and Chrysophyceae), and nematodes for the curing stage. Temperature, moisture content, and substrates available seemed to play a key role in determining the composition of eukaryotic members present at the respective stages of the composting process of RS.

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