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Succession of Eukaryotic Communities during Traditional Composting of Domestic Waste based on PCR-DGGE Analysis

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The succession of eukaryotic communities during traditional composting of domestic waste was analyzed through denaturing gradient gel electrophoresis (DGGE) combined with 18S rRNA gene fragment sequences. The 18S rRNA gene fragments were amplified through culture-independent and culture-dependent approaches. DGGE profiles showed that there were variations of microorganism community profiles during the initial of mesophilic, thermophilic, cooling and maturation phases. Further analysis based on 18S rRNA gene sequence showed that eukaryotic communities observed in the initial mesophilic phase were dominated by *Ascomycota* in addition of *Stramenopiles* and *Uncultured Eukaryotes*. The diversity of eukaryotes decreased at thermophilic phase, in addition dominant bands in mesophilic phase were decreased in the intensity. The number of DGGE bands increased at cooling and maturing phases where some selected bands characterized as *Ascomycota*, *Basidiomycota*, *Zygomycota*, *Gymnamoeba*, *Chlorophyta* and *Arthropods*.

Key Words: Domestic waste, Composting, 18S rRNA, DGGE analysis.

In recent years, composting process attracted many attentions as viable and environmentally sensible alternative for treating of organic waste[1]. Composting, the biological degradation of organic wastes to humus, saves natural resources. Composted organic wastes can be beneficial as fertilizer in agriculture and as soil enrichment. The use of compost is also known to exert positive effects on the physical properties of soil, such as increasing the soil porosity, enhancing microbial activities and the ability to suppress plant diseases, and therefore reducing the need for pesticides[2].

Composting is the biological conversion of organic waste into stable material under self-heating and aerobic condition. The self-heating is due to the heat liberation from microbial metabolic activity. The heating-up is determined by the degradability and energy content of the substrates, the availability of moisture and oxygen, and the mode of energy conservation[3]. Furthermore, these parameters are strongly connected. On the other hand, increasing temperature is consequence as heat production during composting and almost completely derived from biological activity as direct response to heat production[4]. Solid organic waste is degraded to soluble low molecules by passing through four thermal phases that are mesophilic, thermophilic, cooling and maturation steps[5]. The composting process is generally characterized by short mesophilic period and then fast transition to the thermophilic period. After the subsequent

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decreasing temperature, the curing period or compost stabilization stage is started[6].

In the initial mesophilic stage, the temperature is same as on the environment, and the pH is low. As the material started to be degraded, the numbers of mesophilic microbes, often lactic acid bacteria and yeasts, increase. The proliferation of acid-producing microbes could cause further decrease of the pH-level. As the temperature rises, thermophilic microbial flora take over the degradation, and normally this event coincides with the pH turning to alkaline[7]. The temperature starts to decrease as the resources for the thermophilic microbes become scarce, signifying the beginning of the cooling phase, which is followed by the maturation phase[8].

Understanding of microbial succession during composting process is critical to improve the process and the final product[1], and also to find new microbial source for biotechnological application[9,10,11]. A few reports have been studied on bacterial community dynamics during composting process[12,13,14,15,16], however very limited on the eukaryotic community dynamics[17,18,19]. Since eukaryotes are major degrader of compost biomass such as carbohydrates, lipids, proteins and lignin, research on the dynamic of eukaryotic communities during the composting process could indirectly lead to the increased of using compost on agricultural land.

Several method, based on culture dependent and independent techniques, have been used for investigating the microbial diversity during composting process[2,3,12,20,21]. It is well known that culture dependent shows limitation on coverage identification of microbes diversity, however, culture independent have been found only coveraging most abundant cells in the niche, while the numerically rare organisms are generally not detected[22]. The use of DGGE technique to separate rRNA gene fragment from cultivated isolates and direct extracted DNA could enlarge the microbial community analysis and reducing the gap between the result from culture dependent and independent[23,24,25]. In this report we would like to present the dynamics of microbial communities, especial the eukaryotes during the traditional composting process of domestic waste.

MATERIALS AND METHODS

Composting set-up and sampling

Domestic wastes, as compost raw material, were collected from the local temporary shelter at Kelurahan Cibangkong (Non Government Organization, NGO), Bandung with the distance at around 5 km from the Laboratory. The composting process was carried out by traditional process. The size of one compost pile was 80 x 120 cm and 80 cm heigh. Aeration was provided through open-ended air intake bamboo pipes inserted into the pile. Temperature was measured every 2 days and samples were taken from three points of compost pile: top, middle and bottom. The period of composting process is about 2 months. Samples were collected from each point at day 0, 1, 3, 11, 15, 28, and 56. Samples were immediately used for further analysis.

Physicochemical analysis

Extract compost were prepared by shaking, approximately 30 grams of the fresh sample in 270 ml of distilled water, and then filtrated. The pH was measured with pH meter in water extract supernatant. The moisture content was obtained by drying the sample at 70°C until reached constant weight.

Isolation and cultivation of microbes

Supernatant of extract composts were re-filtered through 0.22-µm-pore-size cellulose membrane filter (Sartorius, Germany). Microbes pellet in the membranes were resuspended in STE buffer (10 mM Tris-HCl pH 8.0, 0.1 M NaCl, 1 mM EDTA) and then stored at -20°C until used for DNA isolation. For the cultivation, extract composts were added into PDB (Potatoes Dextrose Broth) media (LabLemco) (1% v/v). The cultures were incubated at 37°C for compost samples taken at day 0 and 56; 50°C for samples taken at day 2; 60°C for samples taken at day 3 and 28; 70°C for sample taken at day 11 and 15. The cultures were harvested after 3-7 days.

Isolation of total DNA Amplification of 18S rRNA gene fragments

Total DNA from filtration samples were extracted using the Mobio Power soil DNA extraction kit (Mobio Inc., Carlsbad, California) according the manufacture instruction. Total DNA from cultivation samples were extracted using Zhou methods[26] with slight modifications. The pellet cells

were suspended in DNA extraction buffer [100 mM Tris-HCl (pH 8.0), 100 mM EDTA, 1.5 M NaCl, and 1% cetyltrimethylammonium bromide (CTAB)] containing proteinase K (10 mg/ml) and incubated at 37°C for 30 min. After vortexing, 20% SDS was added to the mixture and incubated at 70°C for 1 h, freezed at -20°C for 15 min and thawed at 70°C for 15 min three times. An equal volume of chloroform isoamylalcohol (24:1, v/v) was added to the mixture. The aqueous phase was recovered by centrifugation. The upper phase of the solution was transferred to new Eppendorf tube and the DNA was precipitated with 0.6 volume of isopropanol at room temperature for 1 h. The pellet of crude DNA were obtained by centrifugation at 16.000 g for 20 min at room temperature, washed twice with cold 70% ethanol, and dissolved in sterile deionized water. The DNA was stored at -20°C until further used.

The 18S rRNA gene fragments were amplified by PCR method using a set of eukaryotic primers as described by Diez[27]. One primer lies on conserved region among members of the eukarya (*Saccharomyces cerevisiae* positions 1423 to 1438) incorporated with a 40-base GC clamp: 5' CCGGGGGCGCGCCCCGGGCGGGGCGGGGGC ACGGGGGGCCCAGGTCTGTGATGCCC-3'. The other primer is universal primer (*Saccharomyces cerevisiae* positions 1641 to 1627): 5'-GACGGGCGGTGTGTAC-3'. These primer amplify a fragment at approximately 210 bp long. PCRs were performed by using *Taq* DNA polymerase according to the instructions provided by the manufacturer (Fermentas). A touchdown PCR program was implemented as follows: first denaturation was performed at 94°C for 5 min, followed by 30 cycles, where denaturations were performed at 94°C for 30 s, the annealing temperature was performed for 1 min, and elongation was performed at 72°C for 1 min. In the first 10 cycles, the annealing temperature was decreased by 1°C from 55°C to 45°C every cycle. The annealing temperature was 45°C in the last 20 cycles. The final extension was at 72°C for 10 min. The product was visualized on 1% agarose gel.

DGGE analysis and reamplification of DGGE bands

DGGE was performed using 40 µl of the PCR product loaded into a 8% polyacrilamide-bisacrilamide (37.5:1) denaturing gel with gradient from 30% to 70% in 1 x TAE buffer (100% of denaturant corresponded to 7M urea and 40% (v/v) deionized formamide). Electrophoresis was performed at 150 V for 6 h at 60°C by the DCode System (Bio-Rad, USA). After the electrophoresis, the gels were stained by Silver Staining methods[28]. The selected DGGE bands which are specific at each stage of composting were carefully excised and extracted with TE buffer (10 mM Tris-Cl, 1 mM EDTA) in boiling water for 5 min and then incubated overnight at 37°C. The extracted DNA was reamplified by using the same primer without addition of 40 bp GC clamp. PCRs were carried out with the following conditions: hot start and 5 min of denaturation at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C, and single addition extension step of 10 min at 72°C. All of PCR products were subjected to DNA sequencing, carried out by ABI PrismR 3100 Genetic Analyzer (Applied Biosystems), the Macrogen Sequencing Service (Korea).

Phylogenetic analysis

The sequencing results were compared to 18S rRNA gene sequences from GenBank database at NCBI (National Centre of Biotechnological Information) through web site http://www.ncbi.nlm.nih.gov using BLAST program for screening of sequence similarity. Sequences alignments were performed by ClustalW program. Aligning sequences were visualized using GenDoc program. Phylogenetic reconstruction was accomplished with the phylogeny inference package (PHYLIP version 3.62). Evolutionary distances were calculated by F84 method with the DNADIST program. Phylogenetic trees were constructed from distance matrices by the neighbor-joining method, which was implemented with the NEIGHBOR program. The node reproducibility for tree topology was estimated by bootstrap analysis, which included 1000 replicate data sets. The trees were visualized by Treev32 program.

RESULTS

Physicochemical properties of composting process and 18S-rRNA gene fragment

Measurement of physicochemical parameters such as temperature, pH, and moisture content were performed during the composting process. Four phases were recognized in the

process based on temperature changing as follows: mesophilic, thermophilic, cooling, and maturing phases. Few samples were collected during composting process; one from the early mesophilic phase and four from thermophilic phase. Two samples were collected from cooling and maturing phases respectively (Fig. 1). During composting process, maximum temperature reached at 72°C on day 15th, and then slowly decreased up to 40°C during the next 40 days. Variation of pH during the composting process was detected in the range of 6 – 9. Water content decreased from 68.3% to 33.9% at maturing phase (Table 1).

Table 1. Physicochemical properties of samples during composting process

Sampling point	Temperature at the middle of compost pile (°C)	pH	Moisture content (%)
1	35	5.9	68.3
2	52	6.7	70
3	59	7.4	55.5
4	72	8.3	40.9
5	65	8.5	42.4
6	50	9	41.8
7	39	7.6	33.9

The DNA from filtration and cultivation samples were extracted and appeared as single band on etidium bromide stained agarose gel (data not shown). Total chromosomal DNA from each sample was used as template to amplify 18S-rRNA gene fragment using primer pairs as described in the methods. The agarose gel electrophoresis showed that amplifications of the fragment were successfully amplified for both culture-dependent and independent samples resulting single band with the size at approximately 210 bp as expected (Fig. 2).

DGGE profile and Phylogenetic Analysis

In order to detect non-dominant eukaryotic member in compost habitat, culture-dependent technique in PDB media was done to accompany culture-independent approach. The DGGE profiles of the eukaryotic communities in both strategies showed variation of bands on each lane (Fig. 3). Most of intense bands were appeared at middle sides of the both gels. The different pattern was clearly observed between the gels at thermophilic phase, where some bands in the culture independent gel (lane S2-S5) disappeared or decreased in intensity, while some bands appeared with strong intensity in the culture-dependent gel (lane P2-P5). To asses eukaryotic community during composting process based on

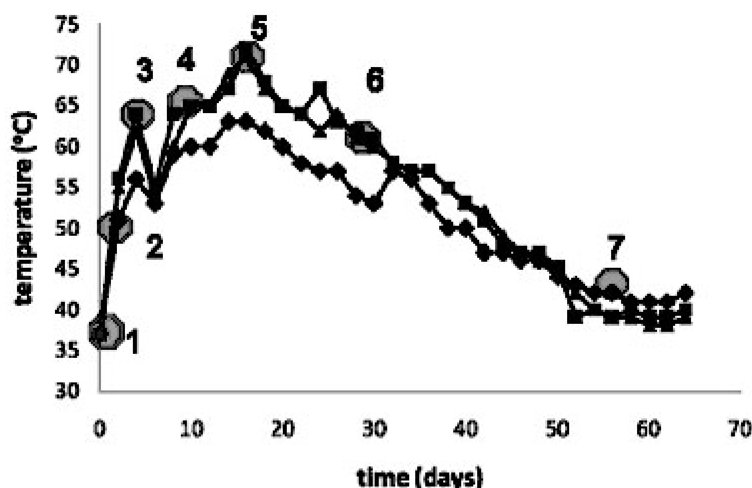

Fig. 1. Temperature changes during composting at three points of compost pile: (?) top, (- %-) middle and (♦) bottom. 1-7 show sampling time during temperature changing at mesophilic, thermophilic, cooling, and maturing phase.

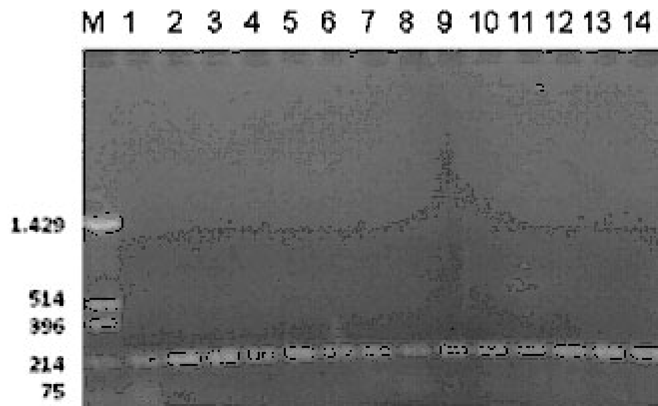

Fig. 2. Agarose gel electrophoresis of PCR product. M, pUC19/*Hinf*I DNA marker; (1-7) amplicons from filtration samples; (8-14) amplicons from cultivation samples.

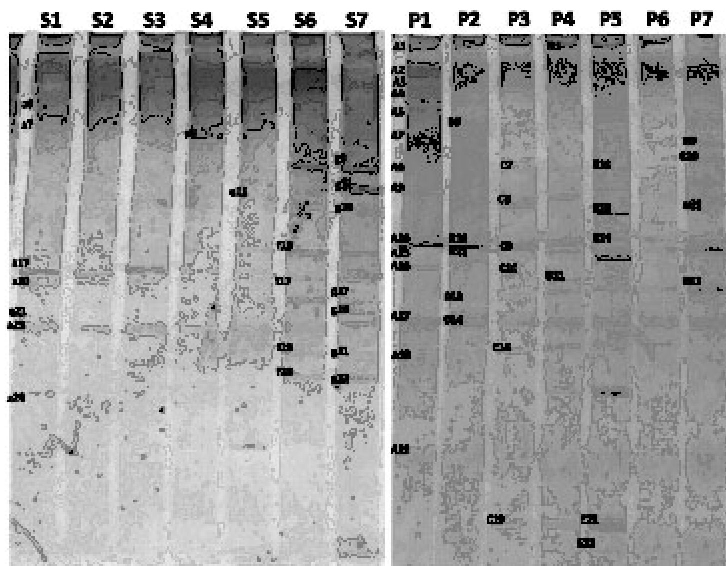

Fig. 3. DGGE band profiles of the compost sample. Left, culture-independent samples. S1-S7, compost sample from sampling point 1-7. Right, culture-dependent samples. P1-P7, compost culture from sampling point 1-7

the 18S rRNA gene sequence, 56 selected DGGE bands (20 bands from culture-independent and 36 bands from culture-dependent) were successfully re-amplified and sequenced. Most of the commonly bands belonged to fungi (*Ascomycota*, *Basidiomycota* and *Zygomycota*), *Uncultured Eukaryote* and *Gymnamoeba* (Table 2 and 3). Meanwhile, some of thin bands were characterized as *Stramenopiles*, *Chlorophyta*, *Apicomplexa*, *Arthropods* and *Chordata*. Eukaryotic diversity of culture-dependent was lower compared to that the culture-independent samples, where most of the bands characterized as *Ascomycota* dominantly. Surprisingly, three of the sequences were characterized as bacterial organisms (band B13, C14 and E21), and four other sequences showed no clear affiliation to any clear eukaryotes.

Based on the DGGE profile and the phylogenetic analysis of the selected bands, a shift of eukaryotic community was observed from the early mesophilic phase to thermophilic, cooling and maturing phases. Eukaryote members detected during the early mesophilic phase belonged to *Ascomycota* phylum dominantly, which were also

Table 2. Sequence similarities of excised DGGE bands from culture independent samples

DGGE band	Most closely related microorganisms	Taxonomic group	Accession number	Similarity (%)
a6	*Pichia membranifaciens*	Ascomycota	EF550365.1	87
a7	*Thraustochytrium sp.*	Stramenopiles	DQ834738.1	74
a17	*Pichia kudriavzevii* EM12	Ascomycota	JF274497.1	100
a20	*Saccharomyces sp.*	Ascomycota	GQ166761.1	100
a21	Uncultured eukaryote	Eukaryote	GU824516.1	86
a23	Uncultured Pichia m1	Ascomycota	HM151325.1	89
a24	*Candida sorboxylosa* NRRL Y-17669	Ascomycota	EF550391.1	95
d8	*Saturnispora hagleri* NRRL Y-27828	Ascomycota	EF550359.1	75
e11	*Kazachstania* sp. TATR11-1	Ascomycota	GQ181169.1	87
f13	Uncultured eukaryote 18S rRNA gene	Eukaryote	AB120148.1	96
f17	*Hartmannella vermiformis* isolate CT5.1	Gymnamoeba	DQ407588.1	79
f19	Hartmannella vermiformis isolate CT5.1	Gymnamoeba	DQ407588.1	84
f20	Uncultured Chlorophyta	Chlorophyta	HQ191425.1 1	82
g9	Uncultured eukaryote18S rRNA gene	Eukaryote	AB120148.1	76
g11	*Chaetosphaeria ciliata*	Ascomycota	GU180614.1	97
g13	*Ceratosphaeria lampadophora* strain CBS	Ascomycota	GU180618.1	91
g17	*Fusarium solani*	Ascomycota	AB473810.1	97
g19	*Thamnocephalis sphaerospora*	Zygomycota	AB016013.1	87
g21	*Culex quinquefasciatus*	Arthropoda	XM_001863698.1	92
g22	*Hartmannella vermiformis*	Gymnamoeba	DQ190271.1	80

found throughout the composting phase. Two other bands were detected as *Stramenophiles* and *Uncultured Eukaryote*. Following the increasing temperature at the thermophilic phase, dominant bands in mesophilic phase were decreased in the intensity. Some new bands were appeared in the bottom side of the gel. Some cultured *Basidiomycota* were also observed in this phase, indicated that these species present non-dominantly in thermophilic compost habitat but could be enriched in PDB media.

Following on decreasing temperature, the diversity of eukaryotes species increased significantly especially on the cooling and maturing phases. Species of *Zygomycota*, *Gymnamoeba*, *Arthropoda*, *Bacillariophyta* besides *Ascomycota* were detected in these phases. Phylogenetic analysis indicated that these organisms on cooling and maturing phases were found in different cluster from that of the organism on mesophilic and thermophilic phases (Fig. 4 and 5). DGGE profiles showed that bands affiliated to *Basidiomycota* were decreased in intensity or disappeared at these phases.

DISCUSSION

Rapid change in physicochemical conditions during composting of domestic waste was correlated with a succession of different communities of microorganisms. DGGE analysis of 18S rRNA gene fragments amplified from both culture-dependent and culture-independent approaches were used to observe population shift of eukaryotes. DGGE applications in combination with PCR and sequencing have been reported to detect the profile of prokaryotes in many samples of diverse habitats, such as compost, deep sea water, hot springs, and desert[24,29,30,31]. However, in contrast with prokaryotic microorganism, phylogenetic studies of eukaryotes based on the difference of 18S rRNA gene sequence are inhibited by the limitations of the available sequence in the GenBank. Many large relatedness groups of eukaryotes known from classical studies are not characterized phylogenetically yet[32]. In this study, three sequences from DGGE bands, A1, A14 and A19, are close to *Pichia kudriavzevii* but showing significant difference in the percent similarity of

Table 3. Sequence similarities of excised DGGE bands from culture-dependent samples

DGGE band	Most closely related microorganisms	Taxonomic group	Accession number	Similarity (%)
B5	*Gregarina polymorpha*	Apicomplexa	AF457129.1	84
C19	*Gregarina niphandrodes*	Apicomplexa	FJ459747.1	100
A1	*Pichia kudriavzevii*	Ascomycota	AB536782.1	98
A2	*Candida pseudolambica*	Ascomycota	AB536781.1	98
A3	*Issatchenkia hanoiensis*	Ascomycota	HQ659554.1	77
A4	*Kodamaea ohmeri*	Ascomycota	GU597327.1	75
A5	*Saccharomyces sp.*	Ascomycota	FJ644448.1	98
A7	*Thraustochytrium sp.*	Stramenophiles	DQ834738.1	74
A8	*Saccharomyces sp.*	Ascomycota	GQ166761.1	98
A9	*Saccharomyces sp.*	Ascomycota	GQ166761.1	100
A14	*Pichia kudriavzevii*	Ascomycota	AB536777.1	100
A15	*Candida pseudolambica*	Ascomycota	AB536781.1	100
A16	*Saccharomyces sp.*	Ascomycota	GQ166761.1	100
A17	*Saccharomyces sp.*	Ascomycota	GQ166761.1	99
A18	*Saccharomyces sp.*	Ascomycota	GQ166761.1	92
A19	*Pichia kudriavzevii*	Ascomycota	AB536782.1	100
B10	*Saccharomyces sp.*	Ascomycota	GQ166761.1	100
B11	*Melanotaenium endogenum*	Basidiomycota	DQ789980.1	81
B13	*Mycobacterium avium*	Actinobacteria	EF514855.1	76
B14	*Mycobacterium marinum*	Actinobacteria	CP000854.1	100
C7	*Petromyzon marinus*	Chordata	GQ215662.1	80
C8	*Uncultured eukaryote*	Eukaryote	FJ611225.1	93
C9	*Saccharomyces sp.*	Ascomycota	GQ166761.1	98
C10	*Saccharomycetales sp.*	Ascomycota	EF060843.1	69
C14	*Branchiostoma floridae*	Chordate	XM_002594305.1	89
D1	*Oophila amblystomatis*	Chlorophyta	HM590634.1	81
D11	*Secale cereal*	Angiosperms	JF489233.1	89
E10	*Tuber panniferum*	Ascomycota	AF054903.1	79
E13	*Lentinula lateritia*	Basidiomycota	AF026596.1	91
E14	*Saccharomyces sp.*	Ascomycota	GQ166761.1	99
E21	*Pseudomonas fluorescens*	Proteobacteria	AM181176.4	86
E22	*Yarrowia lipolitica*	Ascomycota	CR382132.1	95
G9	*Candida rugosa*	Ascomycota	GU144663.1	86
G10	*Candida sp.*	Ascomycota	DQ177818.1	87
G12	*Candida sp.*	Ascomycota	DQ177818.1	99
G17	*Candida rugosa*	Ascomycota	GU144663.1	98

the sequence (Table 3). The same results are also found from A5, A16, A17, and A18 band sequences. Based on the results of BLASTn, all the sequences showed close to *Saccharomyces sp* with the percent of similarity 96, 100, 99 and 92 respectively. Although these sequences are phylogenetically grouped in the same phylum, limited sequence data restrict our representation of their deeper branch in species level. ClustalX program used in this study could only align 46 out of 56 sequences to further construct its phylogenetic tree. The other sequences located at different position are too divergent to calculate their distance matrices by neighbor-joining program. This can be explained since many more sequences of 18S rRNA with unusual lengths, ranging from about 1.5 kb to 4.5 kb, have been documented in GenBank recently[33].

Similar DGGE patterns were observed from the cultivation and filtration samples at the initial mesophilic phase. At this phase, eukaryote diversity was low as characterized by the present of *Ascomycota* dominantly. Several studies with

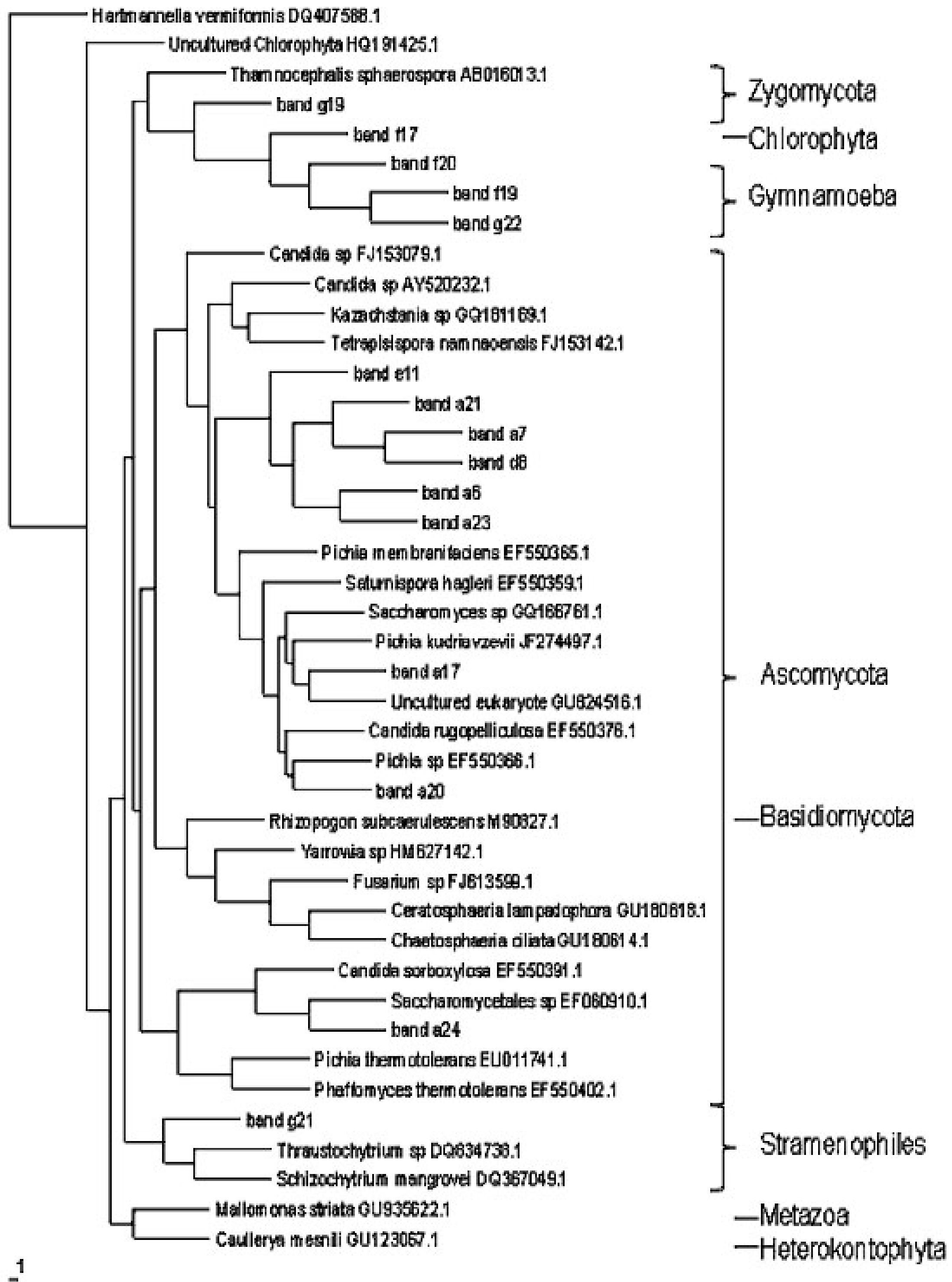

Fig. 4. Phylogenetic relationship of the 18S rRNA gene fragment sequences obtained from the filtration samples and related sequence, based on a distance analysis (neighbor-joining algorithm with F84 model.

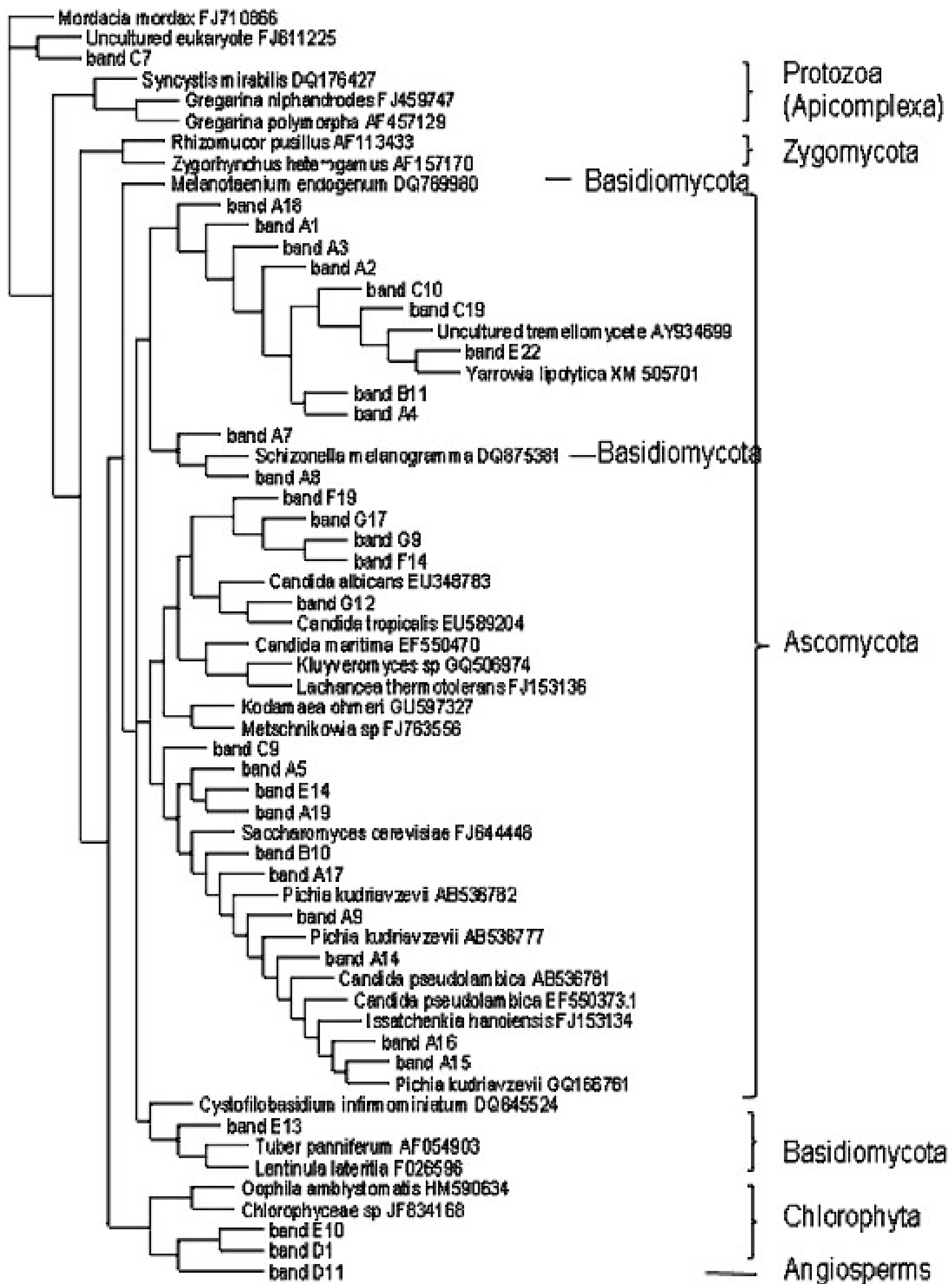

Fig. 5. Phylogenetic relationship of the 18S rRNA gene fragment sequences obtained from the cultivation samples and related sequence, based on a distance analysis (neighbor-joining algorithm with F84 model.

different type of composting process also found the dominance of this yeast[34,35]. The dominance of *Ascomycota* at mesophilic phase in various type of composting suggests that this fungi is initial fungal colonizer of compost. Some bands of the cultivation sample at the mesophilic phase disappeared or showed a decrease intensity when entering the thermophilic phase. These results were in accordance with other studies found that diversity of microorganisms at the thermophilic phase was generally decreased[17,18]. *Eukaryote* cultures in this phase were dominated by *Ascomycota* in addition of *Basidiomycota* found in small number. Some unique bands found in the bottom of DGGE gel suggest that several thermophilic microorganisms present at this phase.

Some DGGE bands at early thermophilic phase were identified close to yeast of biotechnological interest, e.g. *Yarrowia lipolitica* (Table 3), however the DNA sequence showed low similarity compared to the sequence from NCBI database. The present of this yeast was unsurprised since this organism was degrades complex hydrocarbons such as lipid[36]. As the temperature increase, lipid was rapidly degraded during the thermophilic phase of composting[37]. Such thermophilic fungi are commonly found in composts, and along with thermophilic bacteria, the organisms play vital role in the composting process[7]. However, the present of this yeast on cultivation culture at 70°C was surprising since yeast often found at around 60°C as maximum temperature for growth[38].

Entering the cooling and maturing phases, increasing diversity of microorganisms was indicated by the appearence of several new bands, while the bands at the bottom of the gel representing thermophilic microorganisms were disappear. Alignment of the sequences in this phase to NCBI database showed that most of the sequences belonged to *Ascomycota*, *Basidiomycota*, *Zygomycota*, *Gymnamoeba*, *Bacillariophyta*, *Arthropods* and *Uncultured Eukaryote*. The types of microorganisms in these phases were slightly different from that found dominant in the early mesophilic phase. Several studies in compost biodiversity are accordance with this result[18,19]. The variety was found in the composition of the dominant microorganisms. Many factors affected on biodiversity changing during composting process, such as raw materials and composting scale used, tropical climate condition and composting techniques. All of the factors presumably affect the pattern of succession of eukaryotes during composting process.

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