Microeukaryotic Community and Oxygen Response in Rice Field Soil Revealed Using a Combined rRNA-Gene and rRNA-Based Approach

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Irrigated rice field soil is subjected to frequent changes in oxygen status due to the water regime by agricultural management. In this study, the community response of microeukaryotes in rice field soil to the oxygen status was explored in a microcosm experiment under defined conditions. Water-saturated soil was incubated under a two-level factorial design of oxygen and organic enrichment with plant residue. The eukaryotic microbial community composition, which was either present or potentially active in the soils, was analyzed using denaturing gradient gel electrophoresis (DGGE) targeting the 18S rRNA gene or reverse-transcribed 18S rRNA. Oxygen availability was a primary factor shaping the microeukaryotic community in both DNA- and RNA-based analyses, revealing a shift within a week of incubation. Plant residue also affected the microeukaryotic community, which was more notable in the active community showing rRNA expression with time. Sequences of amplicons in DGGE bands indicated that protozoa (ciliates, flagellates, and amoebae) were the most prominent microeukaryotic community, which was more notable in the active community showing rRNA expression with time. The use of a modified primer for soil protozoa suggested the functional importance of Heterolobosea amoeba in rice field soil, particularly in anoxic soil with organic enrichment.

Key words: DGGE, microeukaryotes, rice field soil, protists, rRNA

Microbial eukaryotes play various roles in soil as decomposers, predators, and primary producers. However, understanding the diversity and functions of eukaryotes still represents a major challenge in soil microbiology. Recent studies on microeukaryotic communities in aquatic ecosystems using culture-independent molecular approaches have demonstrated the markedly high diversity covering most lineages of the Eukarya (9, 36). In contrast, the community structure of microbial eukaryotes in soil, except for fungi (1), have been much less thoroughly studied using molecular approaches. Available studies, however, have demonstrated the eukaryotes’ high diversity and geographical heterogeneity, which suggests that they are endemic (2, 10, 23).

The rice field is one of the best-studied model ecosystems of wetlands, which show distinct characteristics from other terrestrial environments in terms of biogeochemistry and microbial ecology (5, 19, 21). The ecological significance of microbial eukaryotes in rice field soil have been demonstrated, e.g., fungi as decomposers of plant residues (32, 33, 41) and protozoa for bacterial predators (26, 29, 30). However, the microeukaryotic community and its response to environmental factors in rice field soil have not been well studied (37).

One of the most important controls of microeukaryotes in the environment is the availability of O2 (11, 12). Molecular studies in aquatic environments have demonstrated unexpectedly diverse groups of microeukaryotes in the water layer and sediments that are permanently anoxic (e.g., 6, 18, 39, 40, 42), which indicated an adaptation of the microeukaryotic community to anoxia. Irrigated rice field soil experiences repeated changes in oxygen status due to the agricultural water management of the field, and the microbial eukaryotic community may adapt to this adverse oxygen status. For example, when rice field soil is drained, fungi dominate among the microbial eukaryotes inhabiting the rice straw, and when the rice field is flooded, protozoa are predominant (41). However, the oxygen response of the microeukaryotic community in soil has not been directly addressed; in the field, the change in oxygen status in soil is caused by the change in water potential (flooded vs. drained), which may also affect the diversity and activity of soil microorganisms.

In this study, we explored the oxygen response of the microeukaryotic community of a rice field soil in a microcosm incubation experiment. The community structure of the microbial eukaryotes was analyzed using denaturing gradient gel electrophoresis (DGGE) targeting the 18S rRNA gene and 18S rRNA to understand the oxygen response of the “seed” community and the potentially active community. The universal primer that has been commonly used for molecular analysis of the microeukaryotic community (7) was modified to detect a wide range of soil microeukaryotes, including heteroloboseans, one of the major clades of soil amoeba (38). Our results demonstrated that both present and active microeukaryotes in rice field soil exhibited a rapid response to different (oxic/anoxic) oxygen statuses at the community level with an additional response to organic enrichment with rice straw, which is a major plant residue supplied to the soil.

Materials and Methods

Soil

Yellow soil (Oxiaquic Dystrochrepts) was collected from the

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Oxygen Response of Soil Microeukaryotes

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DNA- and RNA-based DGGE of microeukaryotic community

The DNA-based DGGE banding patterns of the soil microeukaryotic community were distinct from the RNA-based DGGE banding patterns in all four different soil incubations (Supplementary data, Fig. S2). The DGGE profiles of amplified DNA extracted independently or together with RNA were nearly identical, which indicated that the DNA extraction procedures did not affect the results of DGGE. We also tested random hexamers as primers in the reverse transcription of 18S rRNA and found that the DGGE profiles were close to those obtained using the PCR reverse primer for reverse transcription (data not shown).

We amplified the 18S rRNA genes of microeukaryotes in the soil at the start of incubation and then weekly or biweekly for 10 weeks (a representative DGGE gel is shown in Fig. 2; see Supplemental Fig. S3 for DGGE profiles of all triplicate samples. DGGE bands detected in common in triplicate were regarded as representatives.). We detected 44 bands of different mobility. Each sample had 16–24 bands, 7 of which were commonly observed in all samples. The soils incubated under oxic conditions yielded some additional bands with time, and most of these additional bands were detected in amplified DNA from soils with and without added rice straw. The soil incubated under anoxic conditions yielded a relatively stable DGGE banding pattern. Cluster analysis of the DGGE profiles demonstrated distinct differences in the microeukaryotic community in soil incubated under oxic and anoxic conditions after one week, although the average similarity was high (approximately 80%) (Fig. 4A). Clustering also indicated that the DGGE profiles of amplified DNA from oxic soils shifted with the time of incubation, but rice straw in the soil had little influence. The DGGE profiles of the amplified DNA from anoxic soil also shifted temporally, but this shift was much weaker compared to the shifts in oxic soil, and rice straw had little effect on these profiles.

The RNA-based DGGE banding patterns were relatively simple compared to those of DNA-based DGGE (Fig. 3; see Supplemental Fig. S4 for DGGE profiles all of the triplicate samples). We detected 56 bands of different mobility. Each sample had 10–22 bands, and 5 bands were commonly observed in all of the samples throughout and before incubation (Supplementary Fig. S4). The effect of oxygen status and rice straw on the microeukaryotic community was more pronounced in RNA-based analysis than DNA-based analysis, which was clearly shown by less similarity between treatments in cluster analysis (Fig. 4B).

An ANOSIM2 test indicated that the effect of the treatment (oxic/anoxic; with/without rice straw) on both DNA- and RNA-based DGGE profiles was significant (P<0.001). In DNA-based analysis, pairwise R-values between soil with or without added rice straw were lower than those of other comparisons (Table 1). The effect of rice straw on DGGE
profiles evaluated by pairwise R-values was the most prominent in RNA-based analysis of anoxic soils.

**Phylogenetic affiliation of sequences of the amplicons in DGGE bands**

Amplicon sequences in the bands commonly observed in DNA-based analysis were affiliated with microalgae, amoebae, and flagellates (Table 2). Sequences of DGGE fragments
exclusively observed under oxic conditions were affiliated with amoebae, Rhodophyta, and Metazoa, including Ostracoda, Nematoda, and Gastricha. Among these eukaryotes, the bands that were prominent in the late period of incubation in oxic soils (bands 8B and 6A, Fig. 2) represented lobose amoebae and nematodes. Moreover, a few bands were transiently dominant in oxic soils with added rice straw in the early period of incubation (bands 2A and 2B), representing lobose amoebae. DNA sequences in prominent bands from oxic soils with added rice straw were closely affiliated with anaerobic/microaerophilic heteroloboseans (Metopus), and oomycetes. Sequences affiliated with testate amoebae and Polymyxa were obtained from the bands of anoxic soil samples with and without added rice straw.

### Discussion

In this study, we saturated rice field soil with water for both oxic and anoxic incubation to analytically study the response of microbial eukaryotes to oxygen while avoiding the effect of soil moisture, which would also affect the community composition and activity of soil microorganisms (27, 43). Analysis of Fe(II) validated that oxic and anoxic conditions were maintained in each incubation, although the oxygen level of water-saturated soil in the oxic incubation may have been lower than in unsaturated soil under upland conditions.

### Table 2. Similarities of sequences obtained from the excised bands of rRNA-gene-based DGGE to sequences in the NCBI database

<table>
<thead>
<tr>
<th>Band</th>
<th>Appearance</th>
<th>Seq. bp</th>
<th>Closest relative</th>
<th>Accession number</th>
<th>Similarity (%)</th>
<th>Phylogenetic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 A</td>
<td>Common</td>
<td>444</td>
<td><em>Flamella arnhemensis</em></td>
<td>EU186022</td>
<td>83</td>
<td>Amoebae, Lobosea</td>
</tr>
<tr>
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<td>Common</td>
<td>527</td>
<td><em>Scotiellopsis terrestris</em></td>
<td>AB012847</td>
<td>100</td>
<td>Green algae, Chlorophyta</td>
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<td>517</td>
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<td>80</td>
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<tr>
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<td>525</td>
<td><em>Arachnula sp. ATCC 50593</em></td>
<td>EU273440</td>
<td>86</td>
<td>Flagellates, Rhizaria</td>
</tr>
<tr>
<td>4 O</td>
<td>Common</td>
<td>531</td>
<td>Soil flagellate AND21</td>
<td>AY965866</td>
<td>97</td>
<td>Flagellates, Rhizaria</td>
</tr>
<tr>
<td>4 P</td>
<td>Common</td>
<td>539</td>
<td>Lobosea species Mb 5C</td>
<td>AB425950</td>
<td>97</td>
<td>Amoebae, Lobosea</td>
</tr>
<tr>
<td>10 J</td>
<td>Common</td>
<td>405</td>
<td>Desmodesmos communis</td>
<td>X73994</td>
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<td>Green algae, Chlorophyta</td>
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</tr>
<tr>
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<td>Common</td>
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<td>Stramenopile species MAST-12 KKTS_D3</td>
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<td>Stramenopiles</td>
</tr>
<tr>
<td>10 H</td>
<td>Common</td>
<td>537</td>
<td>Ceratiomyxella tahitensis H104-93-1-1</td>
<td>FJ544419</td>
<td>85</td>
<td>Amoebae, Lobosea</td>
</tr>
<tr>
<td>2 O</td>
<td>Oxic</td>
<td>523</td>
<td><em>Ilyocryptis japonica</em></td>
<td>AB076616</td>
<td>99</td>
<td>Ostracoda, Metazoa</td>
</tr>
<tr>
<td>2 F</td>
<td>Oxic</td>
<td>485</td>
<td><em>Cyanidioschyzon sp. Y16</em></td>
<td>HM161754</td>
<td>86</td>
<td>Rhodophyta</td>
</tr>
<tr>
<td>2 H</td>
<td>Oxic</td>
<td>487</td>
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<td>HM161754</td>
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<td>Rhodophyta</td>
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<tr>
<td>3 A</td>
<td>Oxic</td>
<td>537</td>
<td><em>Nolandella sp. ATCC CRA-27</em></td>
<td>EU273456</td>
<td>87</td>
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<tr>
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<td>500</td>
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</tr>
<tr>
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<td>Oxic</td>
<td>554</td>
<td><em>Tracheleughyla dentata</em></td>
<td>AJ418790</td>
<td>99</td>
<td>Testate amoeba, Rhizaria</td>
</tr>
<tr>
<td>3 E</td>
<td>Oxic</td>
<td>524</td>
<td>Chaetonotus sp.</td>
<td>AJ001735</td>
<td>95</td>
<td>Gastrotricha, Metazoa</td>
</tr>
<tr>
<td>4 G</td>
<td>Oxic</td>
<td>537</td>
<td><em>Ilyocryptis japonica</em></td>
<td>AB076616</td>
<td>99</td>
<td>Ostracoda, Metazoa</td>
</tr>
<tr>
<td>4 J</td>
<td>Oxic</td>
<td>537</td>
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<td>99</td>
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<tr>
<td>6 A</td>
<td>Oxic</td>
<td>525</td>
<td><em>Eumonhystera filiformis EumoFil2 829+830</em></td>
<td>AY593937</td>
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<td>596</td>
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<tr>
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<td>Oxic</td>
<td>536</td>
<td><em>Heterocryptis incongruens C3</em></td>
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<td>Ostracoda, Metazoa</td>
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<tr>
<td>2 A</td>
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<td>460</td>
<td><em>Flamella fluviatilis</em></td>
<td>EU186024</td>
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<td>8 K</td>
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<td>627</td>
<td><em>Naegleria pagei strain 4830/I</em></td>
<td>DQ768721</td>
<td>98</td>
<td>Amoebae, Heterolobosea</td>
</tr>
</tbody>
</table>

1 Common, commonly observed; Oxic, prominent in oxic soils; Anoxic, prominent in anoxic soils; Oxic RS (+), prominent in oxic soils with added rice straw

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In this study, we saturated rice field soil with water for both oxic and anoxic incubation to analytically study the response of microbial eukaryotes to oxygen while avoiding the effect of soil moisture, which would also affect the community composition and activity of soil microorganisms (27, 43). Analysis of Fe(II) validated that oxic and anoxic conditions were maintained in each incubation, although the oxygen level of water-saturated soil in the oxic incubation may have been lower than in unsaturated soil under upland conditions. Soils incubated under anoxic conditions with added rice straw rapidly accumulated Fe(II) and reached a plateau in 4 weeks, which suggested that organic enrichment with rice
Table 3. Similarities of sequences obtained from the amplicons in excised bands of rRNA-based DGGE gels to sequences in the NCBI database

<table>
<thead>
<tr>
<th>Band</th>
<th>Presence in treatment&lt;sup&gt;1&lt;/sup&gt;</th>
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<th>Closest relative</th>
<th>Accession number</th>
<th>Similarity (%)</th>
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<td>Oxic RS (-)</td>
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<td>0–10</td>
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<td>0–10</td>
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<sup>1</sup> Incubation time (weeks) when each band was detected.
straw stimulated anaerobic respiration, giving further reduced conditions after 4 weeks, where methanogenesis was the terminal process (20).

Both DNA- and RNA-based analyses clearly demonstrated that oxygen status shaped the community structure of microbial eukaryotes both in the present and active forms in the soil. DGGE bands of amplicons specific either to aerobic soil or anaerobic soil were identified. These results suggested that microbial eukaryotes with different physiologies in response to oxygen inhabited the rice field soil and the community adapted to the given oxygen status, which developed within a short period of time (<1 week). The rapid response of the bacterial community in rice field soil to the oxygen gradient after flooding has also been previously reported (34).

Although less influential than the oxygen status, the response of the microeukaryotic community to organic enrichment was also manifested using cluster analysis, particularly for RNA-based DGGE. Various functional groups of micro-eukaryotes, such as decomposers (fungi) and bacterial predators (protozoa), expressed the 18S rRNA gene in response to the addition of rice straw. This demonstrated the sensitivity of the RNA-based approach in studying the effect of different environmental factors on functionally important microbial eukaryotes in soil. It is generally assumed that RNA-based analysis targets the population of microorganisms that are actively growing in these environments. However, this is not always true (3); specific levels of 18S rRNA are maintained in the resting form of microbial eukaryotes, such as protozoan cysts (44) and fungal spores (15). Indeed, some major DGGE bands in RNA-based analysis that were included in all of the samples (e.g., band 3C and 6A in Fig. 3, related to ciliate and fungi Mortierella, respectively) were also detected in the control soil that had been stored at 4°C for more than one year, which indicated they were most likely in dormancy. In this study, we regarded DGGE bands in RNA-based analysis that appeared in samples during incubation as representing active forms of microbial eukaryotes.

Combined DNA- and RNA-based community analyses demonstrated a distinct difference between the seed bank and active community of microbial eukaryotes in rice field soil. For example, one prominent DGGE fragment exclusively observed in RNA-gene-based analysis (band 10J in Fig. 2) demonstrated a close relationship with planktonic green alga Desmodesmus (formerly Scenedesmus), which is commonly observed in rice fields (14). This suggested that rice field soil stores not only the seeds of soil microeukaryotes but also that of aquatic microeukaryotes that proliferate when the soil is flooded for rice cultivation.

Most amplicon sequences in DGGE bands, particularly those from RNA-based analysis, were affiliated with different types of protozoa (ciliates, flagellates, lobosea amoebae, heteroloboseans), while only a few of these sequences were affiliated with fungi. These results were in contrast with our previous results that fungi are actively involved in the decomposition of plant residue in the same rice field soil that was unsaturated with water (33, 41). Such differences suggest that water potential is another environmental factor that potentially shapes the active community of microeukaryotes in rice field soil and that protozoa are functionally more important in water-saturated rice field soil. This is plausible given that soil protozoa inhabit the water film of soil particles (16). The fungal community in rice field soil may also respond to water conditions; in this study, all fungal-related sequences from oxie soil with added rice straw were affiliated with flagellated fungi (Chytridiomycota), which are characterized by zoospores and are commonly observed in aquatic ecosystems as well as in soil (17). In contrast, the fungal community in aerated rice field soil is often dominated by other groups such as Ascomycota, Mucoromycotina, and Zoopagomycotina (32). Further studies are required to elucidate the effect of soil moisture conditions on the microeukaryotic community structure in rice field soil.

Ciliate-related sequences were not identified in amplicons in prominent bands of DNA-based DGGE, while some were identified in amplicons in prominent bands of RNA-based DGGE (e.g., band 6B and 8F in Fig. 4). In many soils, ciliates are less numerous than flagellates and amoebae (8); however, our results suggested that the activity of ciliates might be comparable to that of other protozoa in terms of rRNA expression. Because different sequences related to ciliates were identified in samples incubated for different periods and with different treatments, ciliates could potentially be used as bioindicators in rice field soil to show the impact of agricultural practices such as soil and water management, fertilization, and pest control on soil microorganisms (13).

Our RNA-based analysis revealed the involvement of anaerobic protozoa in response to the addition of rice straw. In particular, the detection of sequences related to Harpagon confirms the utility of the modified primer adapted to amplify Heterolobosea 18S rRNA, which was not amplified with the original primer (Euk 516r), and also suggests the stimulated activity of this group in anaerobic rice field soils with organic enrichment. Harpagon is an amoeboid-flagellate and a member of the Psalteriomonadidae, which consists of anaerobic/microaerophilic heterolobosea (35). The Harpagon species are often isolated from freshwater sediments and feed on bacterial prey. This species may play an important role as a grazer in anaerobic rice straw decomposition because the anaerobic microbial food chain must be much shorter than the aerobic chain, due to low growth efficiency (11). Importantly, no cysts have been reported for this family (35). Thus, it would be interesting to study their survival strategy in rice field soil under aerobic conditions.

Conclusion

The combined DNA/RNA-based approach revealed a distinct difference between the seed bank and the active portion of microbial eukaryotes in rice field soil. Both present and active communities of microeukaryotes demonstrated a rapid response to the oxygen status, resulting in a shift within one week. The use of a primer modified for soil protozoa suggested the ecological importance of heteroloboseans, particularly in the anoxic decomposition of plant materials. In this study, we adopted two contrasting conditions in oxygen status, while actual wetland soil consists of layers of different oxygen contents. Future studies will focus on the effect on the oxygen gradient on microbial eukaryotes in soil. The ecological functions of anaerobic protozoa in rice field soil should also be addressed further.
Oxygen Response of Soil Microeukaryotes

Acknowledgements
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References