Active community structure of microeukaryotes in a rice rhizosphere revealed by RNA-based PCR-DGGE

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Abstract

The rhizosphere is one of the hot spots in soil ecosystems for varieties of microorganisms. In this study, we explored the seasonal change of microeukaryotic community of rice rhizosphere focusing on the active members through a RNA-based molecular approach. Rice plants (*Oryza sativa* L.) were grown in a pot where the rhizosphere was compartmented from bulk soil with a nylon gauze. The *Eh* in the rhizosphere compartment indicated that the rhizosphere was under oxic conditions in the initial stage of plant growth and then suddenly became anoxic or suboxic. Denaturing gradient gel electrophoresis targeting 18S rRNA-transcribed cDNA demonstrated that the active community of microeukaryotes in the rice rhizosphere was different from that in the bulk soil. The rhizosphere community showed a temporal shift in accordance to the shift of the redox conditions having three stages: the oxic before maximum tillering stage, anoxic/suboxic stage before maximum tillering stage, and anoxic/suboxic stage in the panicle initiation stage and thereafter. Active members specific to the rhizosphere either oxic or anoxic/suboxic stage were found: Heterolobosea amoeba, ciliates, and Chytridiomycota fungi for the oxic stage and oomycetes, ciliates, and Ascomycota fungi in the anoxic/suboxic stage. The present results demonstrate that a specific group of microeukaryotes inhabit the rice rhizosphere even under anoxic/suboxic conditions and play various ecological roles as plant parasites, microbial grazers, and organic decomposers.

Keywords: microbial food web, paddy soil, protists, protozoa, rice

Introduction

The region of soil in the vicinity of plant roots, in which the chemistry and microbiology are influenced by root growth, respiration, and nutrient exchange, is first defined by Hiltner in 1904 as “rhizosphere”. In the rhizosphere, quantities and types of substrates are different from those in the non-rhizosphere soil, which leads to colonization by different populations of microorganisms (Lynch 1990).

Wetland rice field soil has distinct biogeochemical cycles and microbial communities from upland soils (Conrad and Frenzel 2002; Kirk 2004). In a flooded (anoxic) paddy field, rice roots supply oxygen to the rhizosphere (Goto and Tai 1956; Ando *et al.* 1983). Besides oxygen, rice roots supply organic matter into the rhizosphere, which support active microbial metabolisms (Kimura *et al.* 1979). The redox conditions in the rice rhizosphere of submerged soil is thus controlled by the balance between the oxidizing power of roots and oxygen consumption by inhabiting microorganisms; the rice rhizosphere is more oxic than bulk soil in the early plant growth stage (Trolldenier 1988), while it becomes more reduced than bulk soil in the late plant growth stage (Kimura *et al.* 1979). The microbial community inhabiting the rhizosphere of submerged rice plants is often characterized by a group of bacteria and methanogenic archaea that were distinctive from the community in bulk soil and other environments in a rice field ecosystem suggesting their close association with rice roots (Asakawa and Kimura 2008; Edwards *et al.* 2015; Breidenbach *et al.* 2016).

Microeukaryotes are another inhabitants of the rice rhizosphere. Asiloglu *et al.* (2015) recently studied the microeukaryotic community associated with rice roots through a DNA-based molecular approach; the community distinctive in the rhizosphere

63 included heterotrophic protists (flagellates), plasmodiophorid fungi (*Polymyxa*), and
64 oomycetes. Heterotrophic protists are the essential element of the microbial loop in soil
65 due to their grazing effects (Clarholm 1981). Model experiments using amoeba isolates
66 showed that protistan grazing shapes the rhizospheric bacterial community structure,
67 changes the root architecture, and increases the growth of rice (Bonkowski and Brandt
68 2002; Kreuzer *et al.* 2006; Herdler *et al.* 2008). Plasmodiophorid fungi and oomycetes
69 are well known as root parasites (Kanyuka *et al.* 2003; Latijnhouwers *et al.* 2003;
70 Furuya *et al.* 2007). Oomycetes may also saprophytically grow on rice roots-derived
71 organic materials (Murase *et al.* 2012).

72 DNA-based analysis enables us to study the community present in the samples,
73 but they may not be all active. It is generally assumed that RNA-based analysis targets
74 the actively growing microorganisms. Murase *et al.* (2014) showed that RNA-based
75 analysis is more sensitive in studying the microeukaryotic community involved in
76 decomposition of organic matter (rice straw) in a rice field soil under anoxic conditions.
77 Thus, a RNA-based approach may be more beneficial to study the effect of the
78 environmental change in the rice rhizosphere on the microeukaryotes in more detail. So
79 far, the microeukaryotic community in the rice rhizosphere was demonstrated by
80 DNA-based approaches (Ikenaga *et al.* 2004; Asiloglu *et al.* 2015) and still little is
81 known about the active community structure of the rice rhizosphere.

82 One of the main problems with the RNA-based molecular studies is that RNA is
83 more prone to degradation by nucleases than DNA. Minimizing the degradation of RNA
84 in the samples is therefore a prerequisite for the RNA-based analysis of microbial
85 community. Field experiments potentially give the information on the microbial
86 community *in situ*, but the collecting and processing root samples from the fields need

certain time, which has a potential risk to affect the results of RNA-based analysis of the active community in the rhizosphere due to degradation of RNA in the process. Model experiments can be an alternative way as long as they are carefully designed. A rhizosphere compartment is used to separate the rhizosphere from the bulk soil (Gilbert and Frenzel 1995). Eller *et al.* (2005) made a direct comparison of the compartment and field studies and showed that the compartment study can be extrapolated to the field environment. In this study, we explore the active microeukaryotic community structure of a rice rhizosphere by a same pot experiment with a rhizosphere compartment, which minimizes the sampling time. PCR-based molecular analysis combined with a locked nucleic acid oligonucleotide (LNA) clamping technique (Ikenaga and Sakai 2014) was conducted to avoid the amplification of rice plant-originated gene and its transcripts that may underestimate the microeukaryotic community inhabiting the rhizosphere. The results indicate that the rice plants have a discriminative impact on the both present and active communities of microeukaryotes in the rhizosphere soil.

Materials and Methods

Soil sample

Soil was taken from the plow layer (0-10 cm depth) of a rice field (site D2) at Anjo Research and Extension Center, Anjo, Aichi, Japan (20 m asl, 34°48'N, 137°30'E) on April 18, 2013. The field site and soil properties have been described elsewhere (Lu *et al.* 2002). Prior to use in the pots, the soil was sieved (< 2 mm) and fertilized with a basal fertilizers; $(NH_4)_2SO_4$, calcium super-phosphate and KCl at the rates of 0.1 g N kg^{-1} soil, 0.1 g P_2O_5 kg^{-1} soil, and 0.1 g K_2O kg^{-1} soil.

110

111 ***Rice cultivation in compartmented pots***

112 We cultivated rice plants in compartmented pots according to Eller and Frenzel
113 (2001). A perforated stainless steel cylinder (height, 12 cm; diameter, 4 cm; hole
114 diameter, 1 mm) covered with nylon-gauze (mesh size, 30 μm) for the inside was
115 placed in the center of a 1-liter plastic pot (height, 15cm; diameter, 12 cm). The
116 cylinder was filled with 150 g of the fertilized soil; the compartment was regarded as a
117 rhizosphere. The rest space in the pot was filled with 850 g of the fertilized soil, which
118 was regarded as a bulk soil. The soil was submerged with deionized water and two
119 32-day-old rice seedlings (*Oryza sativa* L., Nipponbare) were transplanted in the
120 rhizosphere compartment on June 11, 2014. In total 15 pots were set in the open air.
121 The soil was kept flooded and weeds and insects were manually removed. Ammonium
122 sulfate was top-dressed 40 days after transplanting (DAT) (on July 21) at a rate of 0.1 g
123 N kg^{-1} soil.

124 Three pots were used for monitoring the plant height, tiller number and soil
125 redox potential (*Eh*). The number of the tillers and height of the plants were recorded
126 weekly. Platinum electrodes were kept inserted in the rhizosphere compartment and
127 bulk soil at 5 cm depth and soil *Eh* was measured in triplicate twice a week.

128

129 ***Collection of rhizosphere and bulk soil samples***

130 Destructive sampling of the rhizosphere and bulk soil samples was conducted in
131 triplicate for four times: before the maximum tillering stage, July 7 and 16 (26 and 35
132 DAT); before the panicle initiation stage, August 12 (62 DAT); before the harvesting
133 stage, September 12 (93 DAT). After transferred to the laboratory, the surface water of

134 the pots was removed. Then, the rhizosphere compartment was taken out from the pot
135 and the rhizosphere soil was placed in a 300-mL sterilized beaker. Based on the results
136 of our previous study that the microeukaryotic communities of rhizosphere soil and the
137 roots shared the same members of microeukaryotes distinct from the bulk soil
138 (Asiloglu *et al.* 2015), rice roots were sampled together with the rhizosphere soil in the
139 compartment to minimize the sampling time. The rice roots were cut (<5 mm) with
140 sterilized scissors and mixed through with the rhizosphere soil. The bulk soil remained
141 in the pots was also sampled after mixing. Immediately after mixing, 0.5 g of
142 rhizosphere and bulk soil samples were put into a 2-mL tube with 0.7 g of zirconia
143 beads for nucleic acids extraction and shock frozen in liquid nitrogen. The soil samples
144 in tubes were stored at -80^{o}C until use.

145 Fragmented rice roots were collected from 20 g of rhizosphere soil samples by
146 screening in water using a 500-μm mesh sieve. The weight of the collected roots was
147 measured after drying at 60^{o}C for 24 h; the root biomass was expressed as dry weight
148 of roots per pot.

149

150 ***Nucleic acid extraction***

151 Total nucleic acid was extracted according to Lueders *et al.* (2004) with
152 modification (Murase *et al.* 2015). Briefly, the stored soil sample was treated with
153 RNA*later*®-ICE (Life Technologies, Carlsbad, CA, USA) prior to nucleic acid
154 extraction. Total nucleic acid was extracted by beads mill homogenization and
155 dissolved in 110 μL TE buffer.

156 After purification of the nucleic acid extracts on a filtration column (Zymo-Spin^{TM}
157 IV-HRC Column, Zymo Research, Irvine, CA, USA), 50 μL of each extract was

158 subsequently treated with DNaseI (Promega, Madison, WI, USA) to remove DNA. The
159 resultant RNA was precipitated with ethanol, dissolved in 30 μl TE buffer, and stored at
160 −80 °C. The remainder of the nucleic acid extract, not treated with DNase, was used for
161 DNA-based analysis.
162
163 ***(RT-)PCR-DGGE analyses of the microeukaryotic community***
164 DGGE of amplicons from 18S rRNA and its gene in extracted nucleic acids was
165 performed according to Murase *et al.* (2014) with modifications. For cDNA synthesis,
166 RNA samples (2 μL) diluted with RNase-free H_2O (9μL) were first heated at 70°C for
167 10 min and then cooled on ice for 10 min. Then the sample was mixed with the
168 reaction mixture for reverse transcription (RT) that contained 4 μL of 5 × reaction
169 buffer, 2 μL dNTP (10mM), 1 μL of RNase inhibitor, 1 μL of random primer (2.5
170 pmol/μL) and 1 μL of ReverTra Ace (Toyobo, Osaka, Japan) in a total volume of 20 μL.
171 The thermal protocol of reverse transcription was 10 min of annealing of primers
172 (30°C), followed by 20 min of reverse transcription (42^{o}C) and termination of the
173 reaction at 99 ^{o}C for 5 min.
174 The reverse transcripts and extracted DNA samples were used in the PCR
175 amplification as templates for RNA-based and DNA-based analysis. PCR amplification of
176 cDNA of 18S rRNA and its gene was performed as described in Murase *et al.* (2014) with
177 one exception; LNA complimentary to the partial sequence of 18S rRNA gene specific to
178 family *Poaceae*, which inhibits amplification of rice roots-originated 18S rRNA and its gene,
179 was added in the mixture at the concentration of 2 μM. The sequence was same as the
180 peptide nucleic acid (PNA) that was used in our previous study (Asiloglu *et al.* 2015):
181 (G(L)5(L)G(L)5(L)cccT(L)cccG(L)G(L)A(L)A(L)), where A(L), G(L), T(L), and 5(L) are

LNA of adenine, guanine, thymine, and 5-methylcytosine, respectively. The effectiveness of LNA was verified by negative amplification of 18S rRNA gene in nucleic acid extracted from rice plants by PCR (data not shown).

DGGE analysis of amplicons was performed as described before (Asiloglu *et al.* 2015). Principal component analysis (PCA) and cluster analysis of DGGE patterns was performed using the PRIMER-E software (Plymouth Marine Laboratory, Plymouth, United Kingdom). Binary data (presence or absence) of DGGE bands were used for the analysis and the Bray-Curtis similarity was calculated for cluster analysis. ANOSIM (analysis of similarity) test was performed to test the null hypothesis that there was no difference in DGGE profiles between samples and pairwise comparisons were made. Statistical difference in the number of DGGE fragments between samples was tested by using a one-way analysis of variance (SPSS for Windows, version 22.0). The relationship between DGGE banding patterns and environmental parameters related to the growth of rice plant (the number of the tillers, height of the plants, root biomass, and *Eh* of the rhizosphere) was determined for rhizosphere samples by canonical correspondence analysis (CCA) in CANOCO 5 (Ter Braak and Šmilauer 2002). The Monte Carlo permutation test (1000 random permutations) was performed to analyze significant effects of environmental variables on the observed DGGE banding patterns.

Sequencing amplicons in the DGGE bands

DGGE fragments were excised from the gels for RNA-based analysis. After two rounds of re-amplification and confirming the mobility of the amplicons on a DGGE gel, the amplicons were sequenced directly as previously described (Murase *et al.* 2012).

206 ***Nucleotide sequence accession numbers***

207 The sequences are available in the DDBJ/EMBL/GenBank nucleotide sequence
208 databases under the accession numbers LC160274-LC160289.

209

210 **Results**

211 ***Plant growth and soil redox potential***

212 The rice plants in the compartment grew healthy and reached the maximum
213 tillering stage at 51 DAT with the height of 73 cm (Fig. 1A). Root biomass linearly
214 increased from 0.55 g pot^{-1} at 26 DAT (the first sampling) to 1.38 g pot^{-1} at 93 DAT
215 (the fourth sampling) (Fig. 1B).

216 The redox potential of the bulk soil gradually decreased after submergence (Fig.
217 1C). The redox potential of the rhizosphere compartment increased in 16 DAT keeping
218 higher than that of the bulk soil until 27 DAT, and then, dropped suddenly to a lower
219 value than that of the bulk soil and to below 0 in 34 DAT. The redox potential of the
220 rhizosphere compartment and bulk soil became similar in 69 DAT and showed a
221 gradual decrease from -100 to -190 mV.

222

223 ***DGGE fingerprints of microeukaryotic community***

224 The RNA-based DGGE banding patterns of the microeukaryotic communities in
225 the rhizosphere and bulk soils are shown in Fig. 2. The total number of identified
226 DGGE bands with different mobility was 46 and it ranged from 7 to 22 for each
227 sample. The number of DGGE bands in the rhizosphere soil increased with time from
228 9-10 on July 7 to 16-22 on August 12 ($P< 0.05$, as determined by Tukey's honestly

229 significant difference test), while no significant difference was detected between bulk
230 soil samples.

231 PCA and cluster analysis of DGGE banding patterns both demonstrated that the
232 active eukaryotic community of the rhizosphere soil samples were distinct from that of
233 the bulk soil samples (Figs. 3 and S2). The rhizospheric community showed a seasonal
234 change. Most strikingly, the rhizosphere community showed the greatest change within
235 9 days in the early period of plant growth before the maximum tillering stage (between
236 July 7 and 16), when the *Eh* of the rhizosphere soil suddenly dropped changing from
237 oxic to anoxic conditions (Fig. 1C). The rhizospheric community showed a further
238 change in August (before a panicle initiation stage) with a less extent than in the early
239 stage of plant growth. The difference of active eukaryotic community between the
240 rhizosphere and bulk soil and the temporal shift of the rhizospheric community were
241 supported by ANOSIM test ($P< 0.001$ and the pairwise R values=1.000). On the other
242 hand, the seasonal shift of eukaryotic community in the bulk soil was less prominent
243 (Fig. 3).

244 CCA showed the seasonal change in the DGGE banding patterns of active
245 microeukaryotic community in the rhizosphere in accordance with the results of PCA
246 and cluster analysis (Fig. 4). Correlation between soil *Eh* and the active
247 microeukaryotic community was observed in the initial stage (July 7) and plant height
248 and root biomass were related with the active community in the late stage of plant
249 growth (August 12 and September 12). The tiller number was less related to the active
250 microeukaryotic community.

251 The difference of eukaryotic community between the rhizosphere and bulk soils
252 was also demonstrated by DNA-based analysis but the shift of rhizospheric community

253 in the early stage of plant growth was less prominent than RNA-based analysis (Figs.
254 S1 and S2).
255
256 ***Phylogeny of characteristic DGGE bands***
257 We obtained sequence information from 16 DGGE fragments, of which 8 could be
258 affiliated to heterotrophic protists (ciliates and amoeba in Amoebozoa, and
259 Heterolobosea), 4 to fungi (Ascomycota and Chytridiomytoca), 3 to oomycetes, and 1 to
260 algae (Table 1). PCA allowed us to classify the fragments based on the Eigenvectors.
261 The fragments with large negative Eigenvectors for PC1 were specific to the
262 rhizosphere community in the late growth stage of rice plants and their sequences were
263 affiliated to oomycetes (bands M and I), ciliates (bands B, D, and E), and Ascomycota
264 fungi (bands H and F). Fragments with large positive Eigenvectors of PC2 were specific
265 to the rhizosphere community in the early stage of rice growth, which included
266 Heterolobosea amoeba (band P), ciliates (band J), and Chytridiomycota fungi (band N).
267 The DGGE fragment commonly observed in the rhizosphere and bulk soil throughout
268 the period was affiliated to Heterolobosea amoeba (band O).
269
270 **Discussion**
271 ***Rhizosphere effect of rice on the microeukaryotic community***
272 In this study, the potentially active microeukaryotic communities in the rice
273 rhizosphere soil were explored by RNA-based DGGE as well as DNA-based analysis of the
274 present community. To our knowledge, this study was the first attempt to monitor the active

275 microeukaryotic community in the rhizosphere. Both approaches demonstrated that the
276 rhizospheric community is distinct from the bulk soil community, which had been also
277 reported in the field study targeting 18S rRNA genes (Asiloglu *et al.* 2015). RNA-based
278 analysis gave additional insight regarding the seasonal shift of microeukaryotic community
279 in the rhizosphere soil, in particular in the early growth period of rice plants. Thus,
280 RNA-based analysis was more responsive than DNA-based analysis to detect the
281 community shift of microeukaryotes in the rice rhizosphere. This was in agreement with our
282 previous study that the effect of organic enrichment on the microeukaryotic community in an
283 anoxic rice field soil was demonstrated by RNA-based analysis but not by DNA-based
284 analysis (Murase *et al.* 2014).

285 The change in the active microeukaryotic community of the rhizosphere in the early
286 growth stage of rice plants was coincided with the drop in redox potential of the rhizosphere
287 soil. The accelerated decline of the redox potential in the rice rhizosphere is consistent with
288 the previous study (Kimura *et al.*, 1979). It is most likely that the microeukaryotic
289 community responded to the change in oxygen tensions in the rhizosphere; oxygen is a key
290 factor that shapes the microeukaryotic community in a rice field soil (Murase *et al.* 2014). It
291 is not clear, however, if the rhizosphere was under anoxic or suboxic conditions even though
292 the *Eh* value in the rhizosphere compartment was negative. Recently, Takenouchi *et al.*
293 (2016) reported that phagotrophic soil protists can grow in a wide range of oxygen tension
294 from the atmospheric level down to below the typical Pasteur point (0.21 %) accompanied
295 with a community level shift.

296 A further shift of active microeukaryotic community in the rhizosphere was
297 demonstrated between the early and late growth stages of rice plants. The results of CCA

298 suggests that the active community in the late growth stage is related with plant growth
299 demonstrated by root biomass and plant height rather than soil *Eh*. Increased root biomass
300 may have increased the amount of root exudates and slough off cells available for microbes
301 in the rhizosphere of the late growth stage. Chemical components of the exudates may
302 change depending on the growth stages of plants (Rovira 1959; Fitter 1991), which may also
303 be a potential reason for the shift of microeukaryotic community.

304 Altogether the present results suggest that the environmental conditions in the rice
305 rhizosphere change in relation to the plant growth and the active microeukaryotic
306 community responsively show a shift.

307

308 ***Phylogeny of microeukaryotes in the rice rhizosphere***

309 Heterotrophic protists (amoeba and ciliates), fungi (Chytridiomycota and
310 Ascomycota) and oomycetes characterized the active rhizosphere community of
311 microeukaryotes, suggesting their various ecological roles as either parasites, bacterial
312 grazers, or organic decomposers.

313 Chytridiomycota are aquatic fungi but commonly observed in both soil and
314 aquatic ecosystems (Gleason *et al.* 2008). Many species of Chytridiomycota are known
315 to be saprobic growing on a variety of substrates. Indeed, our previous study
316 demonstrated that Chytridiomycota is active in the water-saturated but oxic] rice field
317 soil with rice straw applied (Murase *et al*. 2014). Chytridiomycota-related sequences
318 were also retrieved from the rice straw compost under decomposition in a rice field
319 (Hatamoto *et al.* 2008). These findings confirm the function of Chytridiomycota in

decomposition of dead organic materials in soil. Chytridiomycota are also known to be parasitic infecting various organisms including phytoplankton, zooplankton, fungi, invertebrate animals and plants (Gleason *et al.* 2008). In this study Chytridiomycota-related sequences were detected in the rhizosphere in early stage of plant growth. This may suggest another role of Chytridiomycota fungi as plant parasites in a rice field ecosystem as it is unlikely that the rice roots at this stage were already dead and under decomposition processes, though their saprobic growth on root exudates and slough off should be also taken into account. The result that Chytridiomycota-related sequences were not retrieved from the rice roots in the late growth stage having a low *Eh* implies that the role of Chytridiomycota in the decomposition of rice roots may be rather minor as they are thought to be mostly obligate aerobes (Gleason *et al.* 2008).

The DGGE bands related to Ascomycota fungi (bands H and F) were specific to the late stage of plant growth, which suggests these fungi may be active in the anoxic or suboxic rhizosphere. This is supported by that the sequences show a closed relationship to the sequence of a DGGE fragment originated from the anoxic layer of rice field soil (Murase *et al.*, 2006).

Oomycetes-related sequences were found in the rhizosphere at the late growth stage of rice plant. The result is consistent with our previous study through DNA-based molecular approach that oomycetes was specific to the rice rhizosphere with increased prominence at the harvesting stage (Asiloglu *et al.* 2015). The present result further indicated that oomycetes were active in the rice rhizosphere at the late stage of plant growth where oxygen tension was very low. Oomycetes are a well-known group of

343 plant parasites but saprobic oomycetes inhabit aquatic and moist soil habitats (Kamoun
344 2003). The role of oomycetes in the decomposition of organic matter is substantial in
345 anoxic soil (Murase *et al.* 2014).

346 Ciliates were involved in the active community of the rhizosphere in the late stage
347 of plant growth, which suggests that ciliates function as grazers in the rice rhizosphere
348 even under anoxic or suboxic conditions. Indeed, the sequence of band D was closely
349 related to *Paramecium aurelia*-complex, which is a member of the ciliate community in
350 alpha-mesosaprobic fresh water environments (Foissner and Berger 1996). Various
351 species of ciliates can be found in oxic and anoxic rice field soils (Schwarz and Frenzel
352 2003; Murase *et al.* 2014). The rice roots have specific rhizosphere effects on both
353 aerobic and anaerobic microorganisms (Kimura *et al.* 1979), which potentially makes
354 the rice rhizosphere a suitable habitat for various species of ciliates by promoting the
355 growth of prey microbes for ciliates. Ciliates are known to regulate microbial
356 community composition (Louiser and Bamforth 1988) and nutrient mineralization
357 (Griffiths 1986). Ciliates feed on prokaryotes, fungi, and other protists, suggesting that
358 not only protists-prokaryotes interactions but also protists-microeukaryotes interactions
359 may have important ecological roles in the rice rhizosphere.

360 Our approach with a rhizosphere compartment not only minimized the risk of
361 RNA degradation during sample collection but also achieved the emphasized
362 rhizosphere effect as shown in the soil redox potential. RNA-based molecular approach
363 successfully demonstrated that rice roots and the growth stage of rice plant are
364 important factors affecting the active microeukaryotic community. Seasonal changes in
365 the active community of microeukaryotes may indicate that fungi, oomycetes and
366 heterotrophic protists may have different roles in the different growth stages. Our

knowledge on functions of microeukaryotes in the rhizosphere was, however, still very limited as it has been mostly obtained by controlled laboratory experiments where model microeukaryotic species were used or by model experiments like the present study. Microeukaryotes are very diverse and each group, even each species, may play different roles, and interactions between them in the rhizosphere may give significant feedback on plant performance (Herdler *et al.* 2008). We suggest that future studies should consider the functions of actively present microeukaryotes in the rhizosphere and the effects of their interactions on plant growth. Field-based study combined with molecular approach like stable isotope probing (Qiu et al. 2008) would give a direct evidence of the rhizosphere effect on microeukaryotes *in situ*.

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489

Table 1. Similarities of sequences obtained from the excised DGGE fragments to sequences in the NCBI database

Band	Eigenvectors*		Seq. bp	Closest relatives		Phylotenetic group	Alignment	Identity (%)
	PC1	PC2		Microorganisms	Accession no. no.			
M	-0.263	0.117	530	*Lagenidium* sp. SLG-2014b	KJ716873	Stramenopiles; Oomycetes	523/530	99
B	-0.199	-0.005	520	*Trochiliopsis australis*	JQ918367	Ciliophora (ciliates)	484/519	93
D	-0.188	0.153	446	*Paramecium* cf. *aurelia*	LN869961	Ciliophora (ciliates)	440/446	99
I	-0.186	-0.045	528	*Lagenidium* sp. SLG-2014b	KJ716873	Stramenopiles; Oomycetes	521/530	98
H	-0.170	-0.082	527	*Boudiera acanthospora*	U53373	Fungi, Ascomycota	468/513	91
E	-0.160	-0.218	501	*Engelmanniella mobilis*	AF508757	Ciliophora (ciliates)	494/501	99
F	-0.040	-0.215	525	*Boudiera acanthospora*	U53373	Fungi, Ascomycota	501/511	98
G	-0.037	-0.108	530	*Lagenidium* sp. SLG-2014b	KJ716873	Stramenopiles; Oomycetes	522/530	98
A	-0.001	-0.165	531	*Ceratiomyxella tahitiensis*	FJ544419	Amoebozoa	484/533	91
O	0.000	0.000	617	*Naegleria lovaniensis*	U80062	Heterolobosea	595/617	96
P	0.022	0.224	614	*Naegleria australiensis*	AF338421	Heterolobosea	606/614	99
C	0.032	0.164	527	*Chytridium polysiphoniae*	AY032608	Fungi; Chytridiomycota	504/531	95
J	0.044	0.220	501	*Aspidisca fusca*	JX025168	Ciliophora (ciliates)	462/508	91
N	0.044	0.220	576	*Nowakowskiella hemisphaerospora*	EU828462	Fungi; Chytridiomycota	274/318	86
K	0.185	0.140	350	*Blepharisma undulans*	KP970233	Ciliophora (ciliates)	345/350	99
L	0.185	0.140	526	*Characiopodium* sp. Mary 9/21 T-3w	AY197622	Viridiplantae(Microalgae)	500/526	95

*Eigenvectors in PCA of the RNA-based DGGE banding pattern (Fig. 3)

490 **Figure legend**

491

492 Fig. 1. Temporal change in (A) plant height and tiller numbers, (B) root biomass and (C)
493 soil redox potential in a pot experiment. Bars indicate the standard error (n=3).

494

495 Fig 2. The DGGE banding pattern of active microeukaryotic community. Rhizo,
496 rhizosphere soil; Bulk, bulk soil. Alphabets by the bands indicate the name of the bands
497 from which sequences were retrieved.

498

499 Fig. 3. PCA of DGGE fingerprints of microeukaryotic community. Clusters with the
500 group average similarity of 50% and 70% were overlaid with the solid and dotted
501 circles.

502

503 Fig. 4. Canonical correspondence analysis (CCA) of DGGE banding patterns of the
504 active community of microeukaryotes in the rhizosphere compartment. Vectors indicate
505 the different weight and orientation of environmental variable. DGGE bands from
506 which the sequences were retrieved are overlaid in the ordination in triangles with the
507 name (Fig. 2). Pseudo-F statistic was obtained in CANOCO 5 by testing all constrained
508 axes using the Monte Carlo permutation procedure (n = 1000).

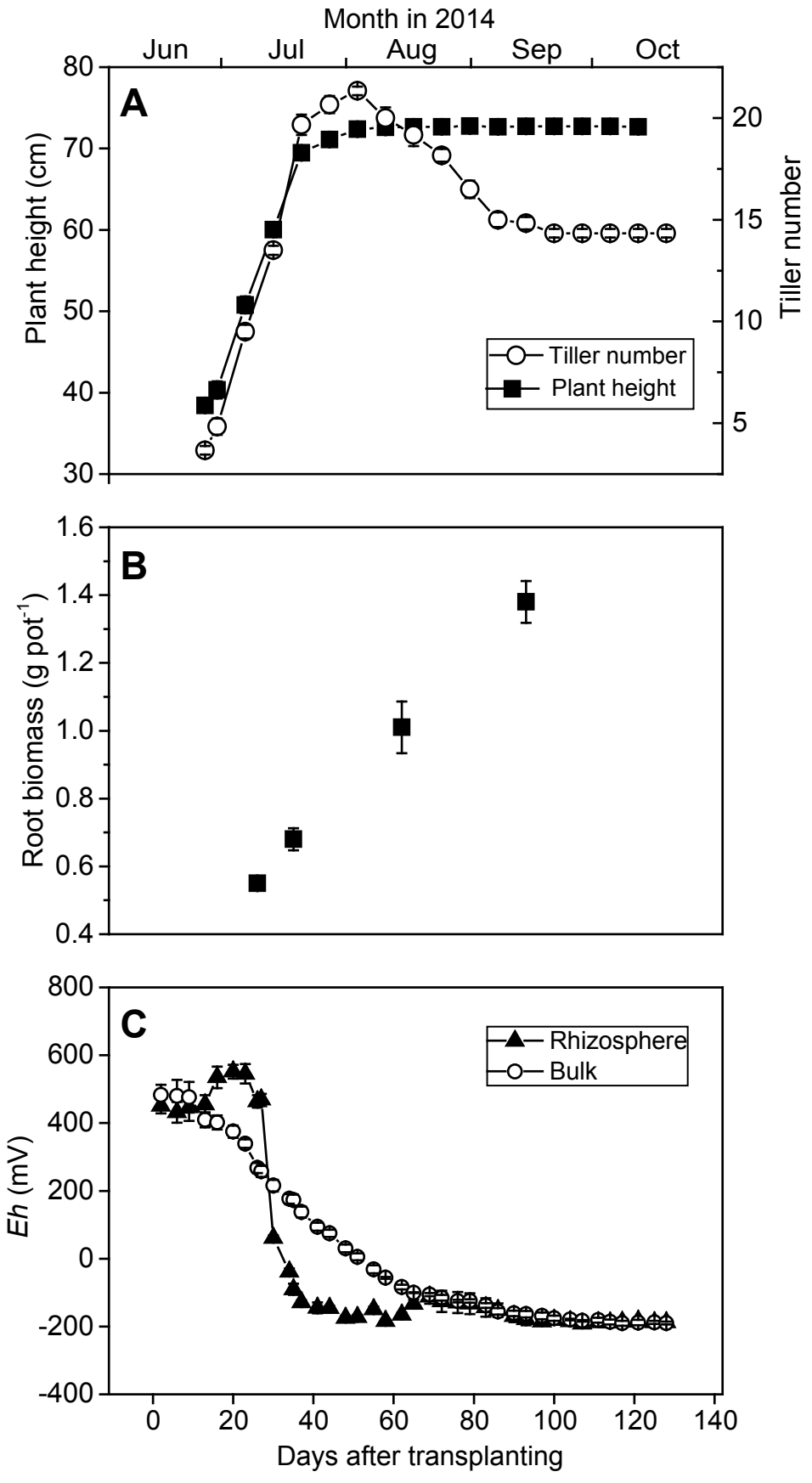

Fig. 1. Asiloglu & Murase

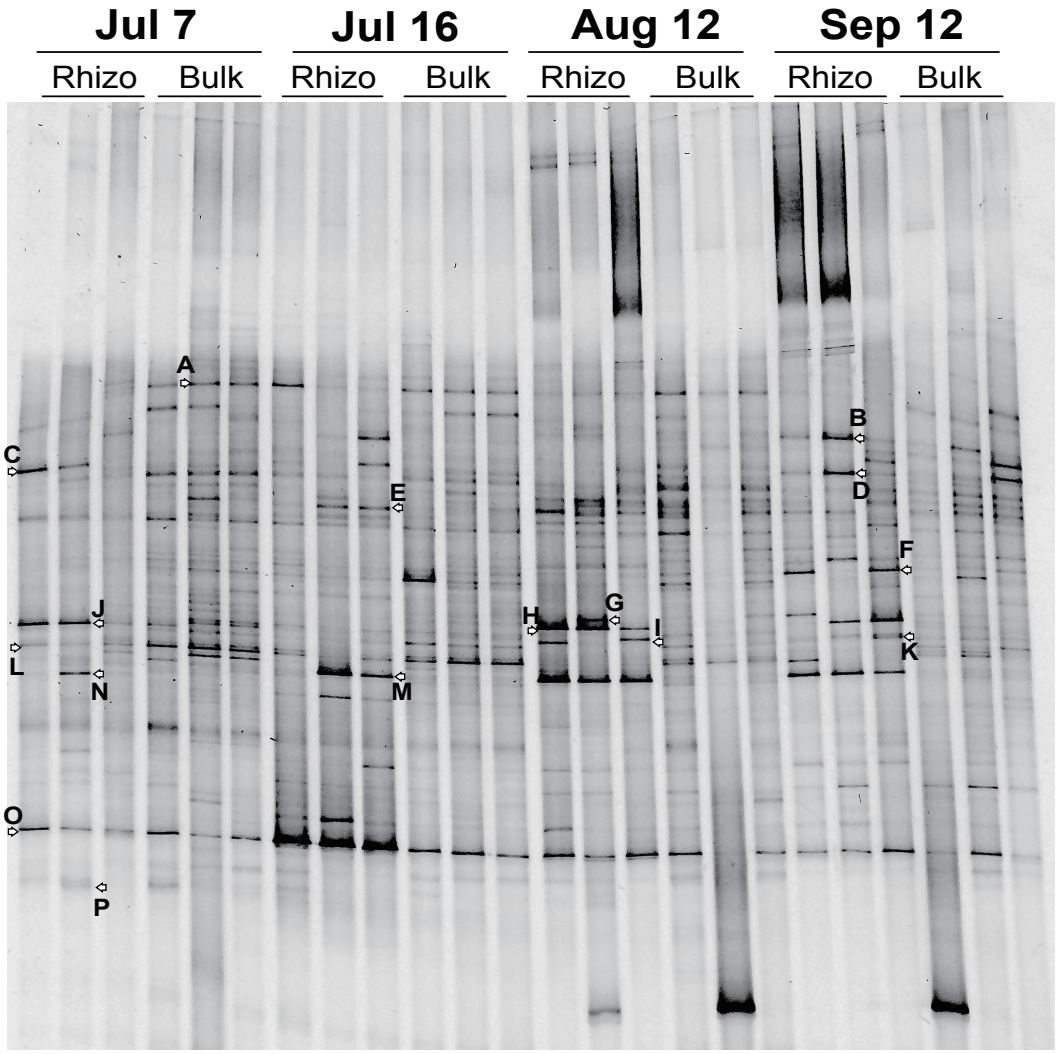

Fig. 2. Asiloglu and Murase

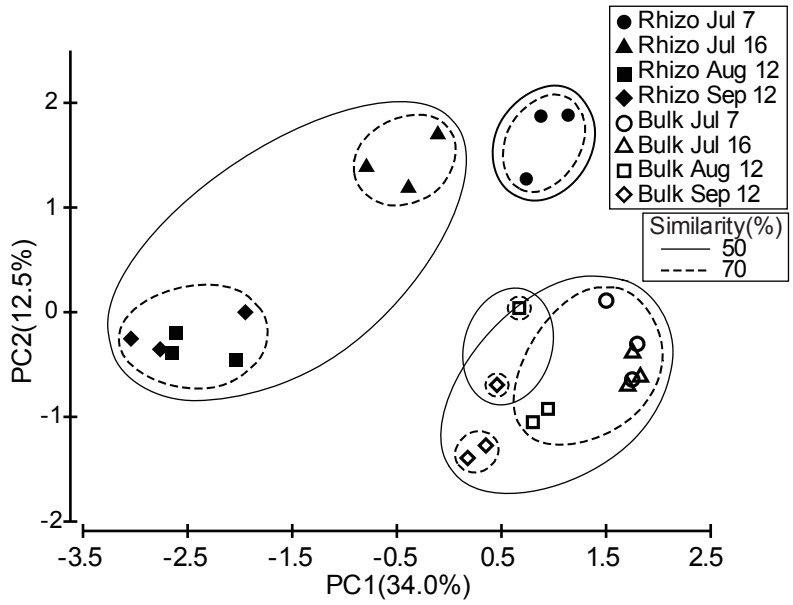

Fig. 3. Asiloglu and Murase

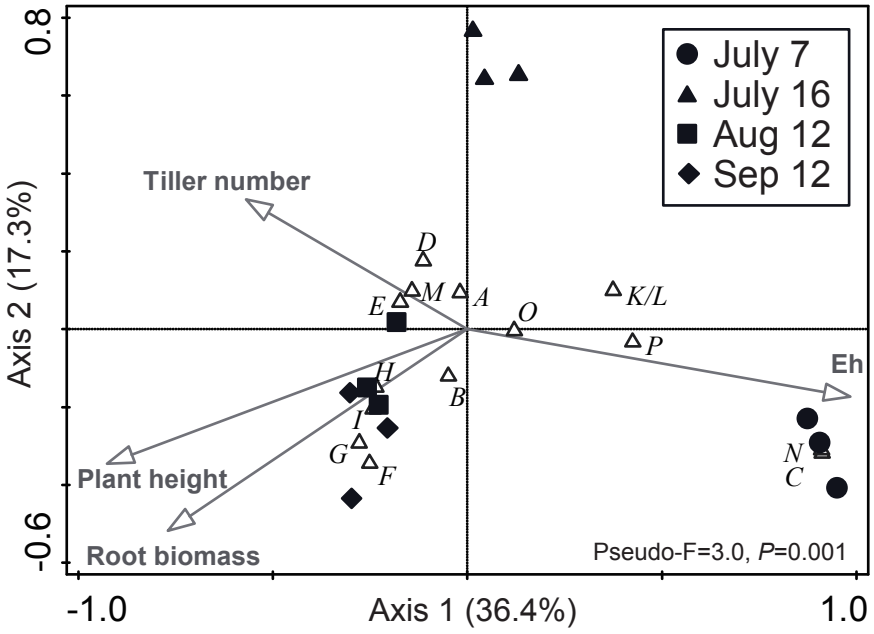

Fig. 4. Asiloglu and Murase