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**Title:** Community structures of arbuscular mycorrhizal fungi associated with pioneer grass species *Miscanthus sinensis* in acid sulfate soils: habitat segregation along pH gradients

**Short running title:**

AM fungal community in acid sulfate soil

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1     **Abstract**

2     Acid sulfate soil shows extremely low-pH, and revegetation of the soil is difficult due  
3     to high concentration of toxic elements such as aluminum and poor nutrient availability.  
4     Community compositions of arbuscular mycorrhizal (AM) fungi that associate with  
5     *Miscanthus sinensis*, pioneer grass species in acid sulfate soil, were investigated to  
6     clarify environmental factors that regulate the community structure. The rhizosphere  
7     soils of *M. sinensis* grown in acid sulfate soil were collected from three sites that  
8     distributed in subarctic, temperate and subtropical zones in addition to those of the  
9     plants grown in a sandy soil site in a subarctic zone. *M. sinensis* seedlings were grown  
10    on these soils in a greenhouse for 2 months, and large subunit ribosomal RNA gene of  
11    the fungi was amplified from DNA extracted from the roots. Based on the nucleotide  
12    sequences of the gene, 20 phlotypes across 6 genera were detected from the four sites  
13    in total. The similarity indices of AM fungal communities among the sites did not  
14    correlate with geographical distance. Ordination analysis (principal component  
15    analysis) on the communities suggested that the first principal component reflected  
16    edaphic factors, particularly soil pH. Plotting of soil pH data at which respective  
17    phlotypes occurred and subsequent statistical analysis revealed that the ranges of  
18    preferential pH were significantly different among the phlotypes. The distribution of  
19    AM fungal phlotypes along pH gradients was further recognized by plotting the first  
20    principal component scores of the phlotypes against their preferential pH. The

21 phylotypes that showed higher scores along the second principal component were  
22 detected from three or more sites and occurred in a wide range of pH. These  
23 observations suggested that the preference and range of substrate pH to which the  
24 fungi could adapt were different among the phylotypes and thus soil pH might be a  
25 likely driving force for structuring AM fungal communities in acid sulfate soils.

26

27 **Key words:** Acid sulfate soil, arbuscular mycorrhizal fungi, community structure,  
28 *Miscanthus sinensis*, pioneer plants.

29

## 30 INTRODUCTION

31

32 Acid sulfate soil is widely distributed in coastal and volcanic areas in the world and  
33 generated by chemical and microbial oxidation of sulfide-rich materials, mainly pyrite  
34 originated from marine sediment, estuarine depositions or volcanic depositions that are  
35 exposed by large-scale land development and reclamation (Prasittikhet and  
36 Gambrell 1989). The environmental impacts of acid sulfate soil have become major  
37 concern: drainage from the soil that shows extremely low-pH and high-levels of iron  
38 threatens terrestrial and marine ecosystems (Appleyard *et al.* 2004; Powell and  
39 Martens 2005). Thus, there is an urgent need for the development of restoration  
40 strategies for acid sulfate soil.

41 Pioneer plants that consist of primary vegetation in disturbed areas may have  
42 developed diverse strategies to adapt to severe environment during evolution. It has  
43 been suggested that phosphorus (P) deficiency is one of the primary factors that limit  
44 the growth of plants in acidic soil as well as high levels of phytotoxic elements  
45 (reviewed by Kochian *et al.* 2004). Associating with symbiotic microorganisms that  
46 improve P nutrition is likely to be one strategy of pioneer plants to confer acidic soil-  
47 stresses. Arbuscular mycorrhizal (AM) fungi associate with up to 80% of terrestrial  
48 plants and supply P to the host through extensive hyphal networks constructed in soil  
49 (Smith and Read 1997). It has been found that many of herbaceous pioneers in early  
50 primary succession after volcanic eruption formed AM association (Oba *et al.* 2004;  
51 Wu *et al.* 2007), and these observations suggest that AM fungi play significant roles in  
52 the establishment of pioneer vegetation. On the contrary, (Allen 1991) claims that non-  
53 mycotrophic or at least facultative mycotrophic plants dominate in the early stage of  
54 primary succession. Given the fact that P-deficiency is crucial for plant survival and  
55 growth in acidic soil, however, it is likely that P-acquisition via AM associations is an  
56 important strategy for pioneer plants that colonize acidic soil. Little is known, however,  
57 about the physiology and ecology of AM fungi in acidic soil so far.

58 Recovering vegetation by using native pioneer plants could be one direction to  
59 the restoration of acid sulfate soil, and it is expected that the application of AM fungi  
60 to the restoration program may contribute to the establishment of vegetation.

61 *Miscanthus sinensis* Andersson, major pioneer grass species in acid sulfate soil in  
62 Eastern Asia, has a high potential of photosynthesis as a C<sub>4</sub> plant and distributes the  
63 roots deeply in soil for the efficient uptake of water and nutrients (Ohtsuka *et al.* 1993).  
64 The plant releases citrate to protect the root tips from aluminum-toxicity under acidic  
65 conditions and thus is highly acid-tolerant (Kayama 2001). It has been known that *M.*  
66 *sinensis* grown in a semi-natural grassland harbors a variety of AM fungi (Saito *et al.*  
67 2004) and showed greater mycorrhiza-dependency than other grasses (Murakoshi *et*  
68 *al.* 1998).

69         The objectives of this study are to clarify AM fungal community  
70 compositions in acid sulfate soil and to explore driving forces structuring the  
71 community in such extreme environment. Two questions are addressed for these  
72 purposes: i) whether climate and/or geographical isolation are the critical factors to  
73 determine the community compositions and ii) how soil acidity affects the  
74 communities. To answer these questions, three fields in which acid sulfate soil had  
75 been found were chosen from subarctic, temperate and subtropical zones in Japan in  
76 addition to a non-acid sulfate soil site from a subarctic zone. We focused on AM fungi  
77 associated with *M. sinensis* that was commonly found in all of the fields, and the soil  
78 trap culture technique was employed for the analysis of the communities (Bever *et*  
79 *al.* 1996; Brundrett *et al.* 1999a). The community compositions were determined based  
80 on the nucleotide sequences of the D1/D2 domains of large subunit ribosomal RNA

81 gene (LSU rDNA) directly amplified from DNA extracted from *M. sinensis* roots.  
82 Recently, this method has been successfully applied to AM fungal community analyses  
83 in various ecosystems (e.g. Gollotte *et al.* 2004; Pivato *et al.* 2007; Turnau *et al.* 2001).

84

## 85 MATERIALS AND METHODS

86

### 87 Sampling sites

88 The sampling sites were located in Rankoshi, Hokkaido Isl. (30-50 m altitude,  
89 42°42'N, 140°21'E, subarctic zone), Hazu, Achi, Honshu Isl. (100-200 m altitude,  
90 34°47'N, 137°07'E, temperate zone) and Nago, Okinawa Isl. (26°35'N, 127°58'E,  
91 subtropical zone) and Atsuma, Hokkaido Isl. (30 m altitude, 42°43'N, 141°52'E,  
92 subarctic zone) in Japan (Fig. 1). The former three were the sites for acid sulfate soil,  
93 and the latter was a control site for non-acid sulfate soil. The climates and surrounding  
94 vegetation of these sites are summarized in Table 1.

95           Acid sulfate soil in Rankoshi was originated from pyroclastic sediment and  
96 exposed by quarrying. The site consists of a steep slope (30-40° angle, 50 m in height)  
97 with several terraces (20-30 × 3-4 m, W/D) and has not been disturbed severely  
98 since 1980'. The ground of the site was not fully covered with vegetation, but patchy  
99 distributions of *M. sinensis*, *Petasites japonicus* (Sieb. et Zucc.) Maxim var. *giganteus*  
100 (Fr. Schm) Kitam., *Polygonum sachalinense* Fr. Schm. and the young seedlings of *B.*

101 *platyphylla* var. *japonica* and *Salix sachalinensis* Fr. Schm. were observed. Acid  
102 sulfate soil in Hazu was originated from marine sediment and was exposed by  
103 quarrying. This site had been quarried for a long time so that the bottom ground of the  
104 field (ca 10 ha) is 20-100 m below the top of the surrounding hills and flat except for  
105 the two ponds (30-50 m in diam, 2-4 m in depth). Quarrying had been ceased in 1999,  
106 and the field has not been severely disturbed since then. *Alnus firma* Sieb. et Zucc. (0.1  
107 to 3 m height) was observed around the two ponds and along the border between the  
108 quarry and surrounding forest. The rest of the area was not fully covered with  
109 vegetation, but patchy distributions of herbaceous plants, *Artemisia princeps* L., *M.*  
110 *sinensis* and *Solidago altissima* L., and shrub, *Lespedeza cyrtobotrya* L. were observed.  
111 Nago site was located in Nago branch, Okinawa Prefectural Agricultural Research  
112 Center. Acid sulfate soils in the site was originated from marine sediment and exposed  
113 on the steep slopes created by the reclamation of sugarcane field. The slopes (50-60°  
114 angle, 4-5 m in height) surrounded the north (80 m) and west (50 m) sides of the  
115 sugarcane field. The ground of the slopes was not fully covered with vegetation, but  
116 scattered distributions of *M. sinensis*, *Sasa kurilensis*, *S. veitchii* and *Melastoma* spp.  
117 were observed. Atsuma site was located on a terrace along Abira River. After clear-  
118 cutting for the construction of a high-voltage power line tower in 1980', *M. sinensis*  
119 grassland (50 m × 30 m) had been established on sandy soil originated from volcanic  
120 pumice and ash, and a thick (7-8 cm) organic layer consisted of a gradient form litter to

121 humus had been developed on the top layer.

122

### 123 **Soil sampling and trap culture**

124 Two to 3 kg rhizosphere soil (top 5-10 cm in depth, 30-40 cm in diam) of *M. sinensis*  
125 was collected from randomly chosen 12 plants grown in Rankoshi (May 2005), Hazu  
126 (June 2005) and Nago (April 2006) sites. From the Atsuma site, rhizosphere soil  
127 samples were collected from 12 intersections of 10 m × 5 m interval grid lines drawn  
128 on the grassland after removing the litter layer (3-4 cm) in October 2006. Each soil  
129 sample was sieved with a 4.5 mm stainless mesh and stored at room temperature  
130 separately.

131           The seed of *M. sinensis* (Kaiseisha Co., Ltd., Otofuke, Hokkaido) was sown  
132 onto each of the soil samples in 9 cm plastic pots (350 mL in vol). Four pots were  
133 prepared for each soil sample: one was for DNA extraction, and the other three were  
134 for the assessment of AM colonization. After sowing, the soil surface was covered with  
135 a thin layer of autoclaved river sand to avoid soil cluster formation on the surface. The  
136 seedlings were grown only with tap water in a temperature/light-controlled (26°C/14 h  
137 day-length) greenhouse with steel flooring and thinned to 10 plants pot<sup>-1</sup> two weeks  
138 after sowing. The cultivation periods of Rankoshi and Hazu samples overlapped for a  
139 month, but they were cultured in different rooms in the facility to avoid cross  
140 contamination. The roots were harvested after 2 months from each pot separately,

141 washed with tap water and blotted on a paper towel. The samples for DNA extraction  
142 was frozen in liquid nitrogen immediately, freeze-dried for 2 days and stored at  $-30^{\circ}\text{C}$ ,  
143 whereas those for the assessment of AM colonization were cut into ca 1 cm segments  
144 and stored at  $-30^{\circ}\text{C}$ .

145 The levels of AM colonization was assessed after clearing with 10% KOH  
146 and staining with Trypan blue by the gridline intersection method (Giovannetti and  
147 Mosse 1980)

148

#### 149 **Soil chemical properties**

150 The soil samples were air-dried, crushed and passed through a 2 mm sieve. Soil pH  
151 ( $\text{H}_2\text{O}$ ) was measured at a 1:2.5 soil to water ratio (w/v) by an electrode after shaking  
152 for 1 h at 160 rpm. Soil pH ( $\text{H}_2\text{O}_2$ ) was measured after over night oxidation with 30%  
153  $\text{H}_2\text{O}_2$  at a 1:5 soil to  $\text{H}_2\text{O}_2$  ratio (w/v). Total carbon (C) and nitrogen (N) were analyzed  
154 by the vario MAX CNS (Elementar, Tokyo). Available phosphate (Truog-P) was  
155 measured by the vanado-molybdate method after extraction with 0.001 M  $\text{H}_2\text{SO}_4$  at  
156 ratio of 1:200 (w/v) (Truog 1930).

157

#### 158 **Molecular identification**

159 The dried root sample was first cut into small segments (less than 1cm) and mixed well  
160 in a plastic bag, and then 10 to 20 mg sample was transferred to a 2 mL tube with a O-

161 ring sealed cap (Yasui Kikai, Osaka) and ground by the Multi-Beads Shocker (Yasui  
162 Kikai, Osaka) with a metal cone at 2,500 rpm for  $2 \times 60$  s at room temperature. DNA  
163 was extracted from each sample by using the DNeasy Plant Mini Kit (Qiagen, Tokyo)  
164 according to the manufacturer's instructions and stored at  $-30^{\circ}\text{C}$ . A part of the large  
165 subunit ribosomal RNA gene (LSU rDNA) was amplified in a 25  $\mu\text{L}$  reaction mixture  
166 with the Expand High-Fidelity PLUS PCR System (Roche Diagnostics, Tokyo)  
167 containing 0.5  $\mu\text{M}$  primers and 1  $\mu\text{L}$  of the template DNA. The LSU rDNA-universal  
168 forward primer LR1 (van Tuinen *et al.* 1998) and fungal LSU rDNA-specific reverse  
169 primer FLR2 (Trouvelot *et al.* 1999) were used for the amplification of 700-760 bp of  
170 the fungal gene. The PTC-225 DNA Engine Tetrad thermal cycler (MJ Research,  
171 Tokyo) was employed, and the program was as follows: initial denaturation at  $94^{\circ}\text{C}$  for  
172 2 min, followed by 30 cycles of denaturation at  $94^{\circ}\text{C}$  for 15 s, annealing at  $50^{\circ}\text{C}$  for 40  
173 s and polymerization at  $72^{\circ}\text{C}$  for 80 s, and final elongation at  $72^{\circ}\text{C}$  for 10 min. In the  
174 case that fungal LSU rDNA could not be directly amplified from the DNA extract,  
175 nested PCR was employed. The first reaction was performed with the primer pair of  
176 LR1 and NDL22 (van Tuinen *et al.* 1998) with the same program. The second reaction  
177 was performed with the LR1 and FLR2 using 2.5  $\mu\text{L}$  of a 1:100 dilution of the first  
178 PCR product.

179           The PCR products were cloned into the pT7Blue T-vector (Novagen/Merch,  
180 Tokyo) according to the manufacturer's instructions. As the first step, 16 clones were

181 randomly chosen from each sample, and the nucleotide sequence were determined by  
182 the dideoxy-sequencing method using the BigDye Terminator v3.0 or v3.1 Cycle  
183 Sequencing Kit with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems,  
184 Tokyo). The DNA sequences were aligned with published data by the ClustalX  
185 program, and those showed high similarity to organisms outside Glomeromycota were  
186 excluded from the subsequent analysis. The neighbor-joining tree was displayed using  
187 the NJplot software. The confidence limits of each branch in the phylogeny were  
188 assessed by 1,000 bootstrap replications and expressed as percentage values. AM  
189 fungal phylotypes were defined based on sequence similarity to known species, tree  
190 topology and a bootstrap value more than 70%. Then, potential phylotype richness was  
191 estimated by the EstimateS 7.5 software for each sample based on rarefaction curves  
192 with 95% confidence intervals (CI) (Colwell *et al.* 2004). In this computation, each  
193 clone was regarded as one sample in which the phylotype of the clone was scored '1'  
194 and the other phylotypes were scored '0'. For the samples of which 95% CI of  
195 phylotype richness was more than 2, additional 16 clones were randomly chosen and  
196 sequenced.

197

### 198 **Statistical analysis**

199 Rarefaction curves and Chao's Sørensen incidence-based similarity indices were  
200 computed by the EstimateS 7.5. Principal component analysis (PCA) was performed

201 by the SPSS statistical software ver. 10.0 (SPSS Inc, Chicago). In these analyses, the  
202 presence or absence of each phylotype was scored as '1' or '0', respectively, in each  
203 root sample, and these data were pooled within the respective sites. The root samples  
204 from which the PCR product of AM fungi could not be obtained were excluded from  
205 the analyses. Prior to PCA, Hellinger transformation (Legendre and Gallagher 2001)  
206 was applied to the data set that contained many zeros and rare phlotypes. This  
207 transformation was expected to make the data set more suitable for community  
208 analysis by giving less weight to rare phlotypes (Legendre and Gallagher 2001;  
209 Ramette 2007). All pH values were transformed to real number before statistical  
210 treatments. In the analysis of the pH preference of AM fungal phlotypes, analysis of  
211 variance (ANOVA) with Tukey-Kramer test as a post hoc test was performed by the  
212 SPSS. In this analysis, the transformed pH values (real number) were to be  
213 retransformed to logarithmic values for normalization, and only the data sets that  
214 showed no significant difference in the variance were subjected to ANOVA. Simple  
215 correlation analysis was performed by the SPSS.

216

## 217 **RESULTS**

218

### 219 **Soil chemical properties**

220 Soil pH of *M. sinensis* rhizosphere soils from Rankoshi ranged from 2.7 to 5.4, and the

221 values in between 3.5 – 3.9 were observed most frequently (Fig. 2a). The pH values of  
222 the soils from Hazu ranged from 3.7 to 6.8 with the highest frequency in between 4.0-  
223 4.4. The pH values of the soils from Nago and Atsuma were higher than those from the  
224 others and ranged from 3.5 to 5.7 with the highest frequency in between 5.5 – 5.9 in  
225 Nago and from 5.4 to 6.1 with the highest frequency in between 5.5 – 5.9 in Atsuma.  
226 Since the pH values observed in Nago samples were higher than expected, pH (H<sub>2</sub>O<sub>2</sub>)  
227 of the soils that showed pH higher than 5.5 was measured. Oxidation of the soils by  
228 H<sub>2</sub>O<sub>2</sub> dramatically decreased the pH values to less than 2.5, implying that the soils in  
229 Nago were ‘potential acid sulfate soil’ (data not shown). The levels of available P were  
230 lower in Rankoshi and Atsuma soils, followed by those in Nago and Hazu soils (Fig.  
231 2b). Total N contents in the Hazu soils were lowest, followed by those in the Rankoshi  
232 and Nago soils, and Atsuma soils showed highest N content (Fig. 2c). Total C contents  
233 in both of Rankoshi and Hazu samples were within a range of 1.4 – 4.9 g kg<sup>-1</sup> and  
234 lower than the other samples (Fig. 2d). The C levels in Nago soils were highly variable  
235 among the samples and ranged from 5.4 to 57.7 g kg<sup>-1</sup> with the highest frequency in  
236 between 7.0 – 7.9 g kg<sup>-1</sup>. Total C contents in the Atsuma samples were considerably  
237 higher than those in the others and more than 10.0 g kg<sup>-1</sup> up to 26.0 g kg<sup>-1</sup>.

238 All of these parameters were combined irrespective of the sites and subjected  
239 to simple linear correlation analysis, and significant correlations were observed among  
240 pH, total N and C. The correlation coefficients (*r*) were: 0.682 between pH and total N,

241 0.699 between pH and total C and 0.975 between total N and C ( $P < 0.01$ ). None of the  
242 parameter showed significant correlation with Truog-P levels.

243

#### 244 **AM fungal phlotypes**

245 The levels of AM fungal colonization on *M. sinensis* seedlings in the trap culture were:

246 Rankoshi, 0 – 63.1%; Hazu, 16.8 – 53.5%; Nago, 22.9 – 55.7%; Atsuma, 1.9 – 15.6%.

247 Fungal LSU rDNA were successfully amplified from seven root samples by one-step

248 PCR and from one sample by nested PCR out of the 12 root samples of Rankoshi. The

249 PCR products were also obtained by one-step PCR from eight, nine and nine samples

250 of Hazu, Nago and Atsuma, respectively. The root samples from which PCR product

251 could not be obtained were those showed zero or lower levels of colonization in

252 general. After cloning of the PCR products, 16-32 clones from each sample were

253 sequenced, and in total, 134, 139, 128 and 107 clones from the Rankoshi, Hazu, Nago

254 and Atsuma samples, respectively, were found to be of AM fungal origin. Overall, 20

255 different phlotypes (including 3 subgroups) across 6 genera were detected based on

256 phylogenetic analysis. Fig. 3 shows a phylogenetic tree constructed with the

257 representative sequences from each root sample. The number of phlotype (phlotype

258 richness) detected from Rankoshi, Hazu, Nago and Atsuma sites were 6, 8, 11 and 12,

259 respectively. ARC1 and PAR1 were related to *Archaeospora gerdemannii* and

260 *Paraglomus occultum*, respectively, and found from all of the four sites. PAR1 was,

261 however, further separated into two subgroups, group-I and -II, with a bootstrap value  
262 of 100%. The group-I consisted of the clones detected from Rankoshi, Hazu and Nago,  
263 the acid sulfate soil sites, while the group-II consisted of those only from Atsuma, the  
264 sandy soil site. ARC1 was detected from 8 out of the 9 root samples grown on the  
265 Nago soils thus was one of the dominant types in the site. PAR1 group-II, an Atsuma-  
266 specific phylotype that were detected from 6 out of the 9 samples, was one of the  
267 dominants in the site. GLO5 showed no similarity to known species but related to  
268 uncultured *Glomus* sp. hr11. This type was also detected from all of the four sites.  
269 GLO1 was related to *Glomus intraradices* and detected from Rankoshi, Hazu and  
270 Atsuma sites. This phylotype was further separated into two subgroups, group-I and -II,  
271 with a high bootstrap value, and the group-II consisted of the clones only from  
272 Rankoshi, while the group-I consisted of those from Rankoshi, Hazu and Atsuma.  
273 GLO1 (group-I and -II) was the most abundant type in Rankoshi site. GLO3 showed  
274 high-sequence similarity to *Glomus* sp. HR1 and *Gl. manihotis* and was found from  
275 more than halves of the Hazu, Nago and Atsuma samples. ACA1 was related to  
276 *Acaulospora mellea*, and detected from the Hazu, Nago and Atsuma sites. But this  
277 phylotype was further separated into two subgroups, group-I and -II, with a bootstrap  
278 value of 100%. The group-I consisted of the clones from Nago and Atsuma, whereas  
279 the group-II consisted of the clones only from Hazu and was the most abundant type in  
280 the site. GLO6 showed no similarity to known species but related to uncultured

281 *Glomus* sp. rp2 and was detected from Rankoshi and Atsuma sites. GIG1 was related to  
282 *Gigaspora margarita* and found both from Hazu and Atsuma. GLO2 and ACA2 were  
283 related to *Gl. clarum* and *Ac. longula*, respectively, and found both from Nago and  
284 Atsuma sites. In Atsuma, ACA2 was found from 5 out of the 9 samples thus one of the  
285 dominants in the site. UNC2 showed sequence similarity to uncultured glomeromycete  
286 6.8 and detected only from Hazu. ACA3, SCT1, GIG2 and GLO7 showed similarity to  
287 uncultured *Acaulospora* sp. S175, *Scutellospora* sp. hr83, *Gi. gigantea* and *Gl.*  
288 *claroideum*, respectively, and were found only from Nago. UNC1 and GLO4 showed  
289 similarity to uncultured glomeromycete Cp193 and *Gl. mosseae*, respectively, and  
290 found only from Atsuma. The rarefaction curves for the evaluation of sampling  
291 efficiencies on the recovery of AM fungal phylotype from the four sites appeared to  
292 reach plateaus (data not shown).

293

#### 294 **Community analysis**

295 Similarity of AM fungal communities between every combinations of two out of the  
296 four sites was first assessed with respect to geographical distance between the sites  
297 (Fig. 4). The highest value of the similarity index was observed between Nago and  
298 Atsuma, the most distant sites, and the lowest value was observed between Rankoshi  
299 and Atsuma, the nearest sites, reflecting that no significant correlation was found  
300 between the indices and geographical distance.

301 To explore environmental factors that affect AM fungal community, PCA was  
302 employed (Fig. 5). The communities in Rankoshi and Hazu sites were closely located  
303 on the site plot and showed positive scores along the first principal component (PC1)  
304 axis that accounted 40.2% of the total variance (Fig. 5a). In contrast, the communities  
305 in Nago and Atsuma sites were closely located and showed negative scores with the  
306 PC1. All the community showed positive scores along the PC2 that accounted 27.1%  
307 of the total variance. The PC1 and PC2 score plot of the phylotypes obtained by the  
308 PCA was shown in Fig. 5b. GLO1 group-I and -II, PAR1 group-I, GLO5 and ACA1  
309 group-II showed relatively higher positive scores with the PC1, whereas ARC1, GLO3,  
310 ACA1 group-I, ACA2, PAR1 group-II and GLO2 showed relatively higher negative  
311 scores. Along the PC2 axis, ARC1, GLO3, GLO1 group-I, PAR1 group-I, GLO5,  
312 ACA1 group-I and ACA2 showed positive scores, and the rest of the phylotypes  
313 showed nearly zero or negative scores.

314 Based on the PCA, it was assumed that the PC1 might reflect soil chemical  
315 parameters particularly pH, total C and N, because these values of the soils from  
316 Rankoshi and Hazu were relatively lower compared with those from Nago and Atsuma.  
317 Since these parameters were correlated each other, soil pH was employed as a  
318 representative for subsequent analyses. The pH values of the soils in which the  
319 respective phylotypes occurred were combined irrespective of the sites and plotted (Fig.  
320 6). Only the phylotypes that occurred in four or more soil samples were included in

321 this analysis. Although the most of the phylotypes distributed in a broad range of pH, it  
322 was found that GLO1 group-I occurred preferentially in a more acidic range (average  
323 pH 3.8) than GLO3 (average pH 4.4) ( $P < 0.01$ ). It should be noted that the variances  
324 of the pH values of ACA2 and PAR1 group-II that occurred in the soils with average  
325 pH 5.4 and 5.6, respectively, were significantly smaller than those of the other  
326 phylotypes ( $P < 0.05$ ) (data not shown), and thus they were excluded from the analysis.  
327 The distribution of the phylotypes along pH gradients was more clearly recognized by  
328 plotting the PC1 scores of these phylotypes against the average pH values of the soils  
329 in which they occurred (Fig. 7). GLO1 group-I, GLO1 group-II, ACA1 group-II,  
330 GLO5 and PAR1 group-I that showed the positive scores occurred preferentially in  
331 between pH 3.8 – 4.2, whereas those with the negative scores were separated into two  
332 groups: one (ARC1, ACA1 group-I and GLO3) occurred in less acidic range (pH 4.3 –  
333 4.4) than those with the positive scores and the other (ACA2 and PAR1 group-II)  
334 occurred in pH more than 5. The phylotypes that were detected from three or more  
335 sites e.g. GLO1 group-I, PAR1 group-I, ARC1 and GLO3 showed higher PC2 scores  
336 (Fig. 8). It is noteworthy that these phylotypes are the ones that occurred in a wide  
337 range of pH (Fig. 6).

338

## 339 **DISCUSSION**

340

### 341 **Soil pH as a driving force for structuring AM fungal communities**

342 To our knowledge, the present study provides first information about AM fungal  
343 community in the pioneer vegetation of acid sulfate soil with respect to different  
344 climatic zones and soil pH gradients. It was found that the influences of climate and/or  
345 geographical isolation on AM fungal communities are minimum. Instead, it is most  
346 likely that soil chemical properties, probably soil pH, are the major driving force for  
347 structuring the community compositions in acid sulfate soil based on the observations  
348 that soil pH was correlated with the community compositions and AM fungal  
349 phylotypes, at least that of dominant phylotypes, distributed along pH gradients. It has  
350 been known that edaphic factors are of importance in regulating the species  
351 composition of AM fungal communities (Abbott and Robson 1991; Johnson *et*  
352 *al.* 1992). Although the influences of soil fertility e.g. soil C and N (Johnson 1993) and  
353 organic/conventional farming systems (Hijri *et al.* 2006; Oehl *et al.* 2005) on the  
354 communities were studied extensively, information on the influence of soil acidity  
355 (pH) is rather scarce (Abbott and Robson 1991). Although soil pH, total C and N were  
356 well correlated each other in this study, we consider that soil acidity is a likely primary  
357 factor that structures the communities in extremely acidic soil (or disturbed area) due  
358 to the following reasons. Firstly, the levels of C and N in soil may be a reflection of  
359 vegetation history in acidic soil i.e. the growth of pioneer plant is much slower and  
360 poorer in more acidic soil, resulted in less C and N accumulation in more acidic soil.

361 Secondly, it is unlikely that soil C status affects AM fungal colonization or community  
362 composition directly due to the nature of the fungi as an obligate biotroph. Thirdly, the  
363 effects of soil C and N on AM community compositions in arable soils shown by  
364 Johnson (1993), an only report that claimed that soil C and N affected the community  
365 so far, were not unequivocal, and the change in the community could not be attributed  
366 solely to soil C and N levels because available P levels were also different between the  
367 treatments. However, the possibility that C and N levels regulate, directly or indirectly,  
368 AM fungal community composition in acid sulfate soil could not be excluded by the  
369 results obtained in the present study. Therefore, the hypothesis that soil pH is the  
370 primary factor for structuring the community in acidic soils should be further examined  
371 experimentally.

372           The fact that all of the AM fungal communities of the four sites showed  
373 similar scores along the PC2 axis may imply that the community compositions are  
374 fundamentally similar to each other. It was initially expected that the effects of  
375 surrounding vegetation, which is largely defined by climatic factors, on AM fungal  
376 community composition could be substantial, because the top soils of the surrounding  
377 forests would be the most likely inoculum source of AM fungi for these disturbed areas.  
378 Particularly, it was surprising that the fungal community compositions were strikingly  
379 similar between Nago and Atsuma sites of which climate and plant species  
380 compositions in the surrounding vegetation were quite different. It is well known that

381 plant species composition is a major regulatory factor of AM fungal community  
382 composition (e.g. Johnson *et al.* 2004; Johnson *et al.* 1992; Pivato *et al.* 2007;  
383 Vandenkoornhuyse *et al.* 2002), and this may be due to the fact that every phases in the  
384 life history of AM fungi is dependent on the host plants (Bever *et al.* 1996; Eom *et al.*  
385 2000). Therefore, there was no doubt that the forests surrounding the four sites might  
386 harbor different AM fungi and that AM fungal communities of *M. sinensis* grown  
387 within the sites (disturbed areas) could reflect the difference. The most likely and  
388 simplest explanation for our results is that particular AM fungal genotypes with which  
389 *M. sinensis* associates preferentially may have been selected. The most of individual *M.*  
390 *sinensis* plants grown in the three acid sulfate sites were isolated each other due to poor  
391 vegetation coverage, and this might provide less opportunity to associate with other  
392 plant species for the fungi. If this is true, the surrounding forests may retain extremely  
393 high AM fungal diversity that enables *M. sinensis* to select the similar communities  
394 irrespective of the plant species composition of the forest. AM fungal diversity in the  
395 surrounding forests needs to be assessed to examine this assumption.

396         There is a possibility that the soil trap culture biased the AM fungal  
397 communities thus the species compositions were similar to each other. It has been  
398 known that different analytical methods e.g. field-collected samples- and trap culture-  
399 based analyses gave different results (Bever *et al.* 1996; Brundrett *et al.* 1999a;  
400 Brundrett *et al.* 1999b), and a diversity of approaches is suggested to be employed for

401 more complete descriptions of AM fungal community compositions (Bever *et al.* 1996).  
402 In soil trap culture, the main components of AM fungal propagules may be spores and  
403 mycorrhizal root fragments, suggesting that AM fungi that are capable of regenerating  
404 more rapidly i.e. more competitive fungi (Brundrett *et al.* 1999a) will be detected,  
405 compared with analysis on the field-collected root samples that are grown in the  
406 existing mycelial networks of the fungi (Fitter *et al.* 2000). On the other hand, seasonal  
407 variation in AM fungal communities in host plant roots was reported by the PCR-based  
408 method targeting fungal SSU rDNA (Heinemeyer *et al.* 2004), implying that plants  
409 with different growth phases harbor different AM fungi. In the present study, field  
410 samples were to be collected from the four experimental sites belonged to subarctic to  
411 subtropical zones, and difficulty in collecting field-grown root samples from the plants  
412 with the same growth phase in different climatic zones was considered. The soil trap  
413 culture, in contrast, was expected to minimize the sampling biases caused by difference  
414 in the host growth phase and thus employed in the present study. The facts that AM  
415 fungal phylotype richness detected in the present study was comparative to those in the  
416 pioneer vegetation of volcanic desert of Mt. Fuji (Wu *et al.* 2007) and more than  
417 double of the Lahar area of Mt. Pinatubo (Oba *et al.* 2004) may validate our approach.

418

#### 419 **Difference in pH preference of AM fungi**

420 The present study demonstrated that the ranges of substrate pH to which the fungi were

421 capable of adapting were different. The significantly smaller variances of the soil pH  
422 values of ACA2 and PAR1 group-II that occurred at pH more than 5 may imply that  
423 these phylotypes could adapt to only a narrow range of pH. In contrast, it was  
424 surprising that several phylotypes ARC1, GLO3, GLO1 group-I, GLO5 and PAR1  
425 group-I occurred in many of the geographically isolated sites that showed a large  
426 variation of soil pH. These fungi could be categorized as ‘generalists’, although slight  
427 difference in preferential pH among the generalists was observed. The  
428 physiological/genetic backgrounds of the generalists are of interest not only from the  
429 viewpoint of basic biology but also from that of applied science/technology for  
430 environmental restoration. It has been known that *Gl. intraradices* which is closely  
431 related to one of the generalist GLO1 in this study showed enormous intraspecific  
432 functional (genetic) diversity e.g. large variations in hyphal growth rates and spore  
433 productivity were found among the isolates from the single experimental field (Koch *et*  
434 *al.* 2004). It is considered that the generalists may have greater intraspecific  
435 functional/genetic diversity as observed in *Gl. intraradices*, thus they could adapt to a  
436 wide range of pH and dominate even under severe environment.

437

#### 438 **Conclusion**

439 In contrast to our results, Öpik *et al.* (2006) suggested that the region/location effect on  
440 AM fungal community might be significant through the meta-analysis of 26

441 publications reporting the community compositions in different regions of the world.  
442 They also pointed out, however, that there were too few studies on the fungal  
443 communities of the same plant species at different sites. Further surveying the AM  
444 fungal communities of primary vegetation in acid sulfate soil that occurs in diverse  
445 regions and climatic/vegetation zones may contribute to understanding the significance  
446 of the region/location effects on the community as well as that of edaphic factors.

447           Our study may also contribute to the future application of AM fungi to  
448 restoration of acid sulfate soil. The finding of the ‘generalists’ that can colonize the  
449 host plant over a range of pH is novel, and it is expected that these fungi may have a  
450 great potential as ‘universal fungi’ for environmental restoration if they can be isolated  
451 successfully.

452

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454

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458 seed.

459

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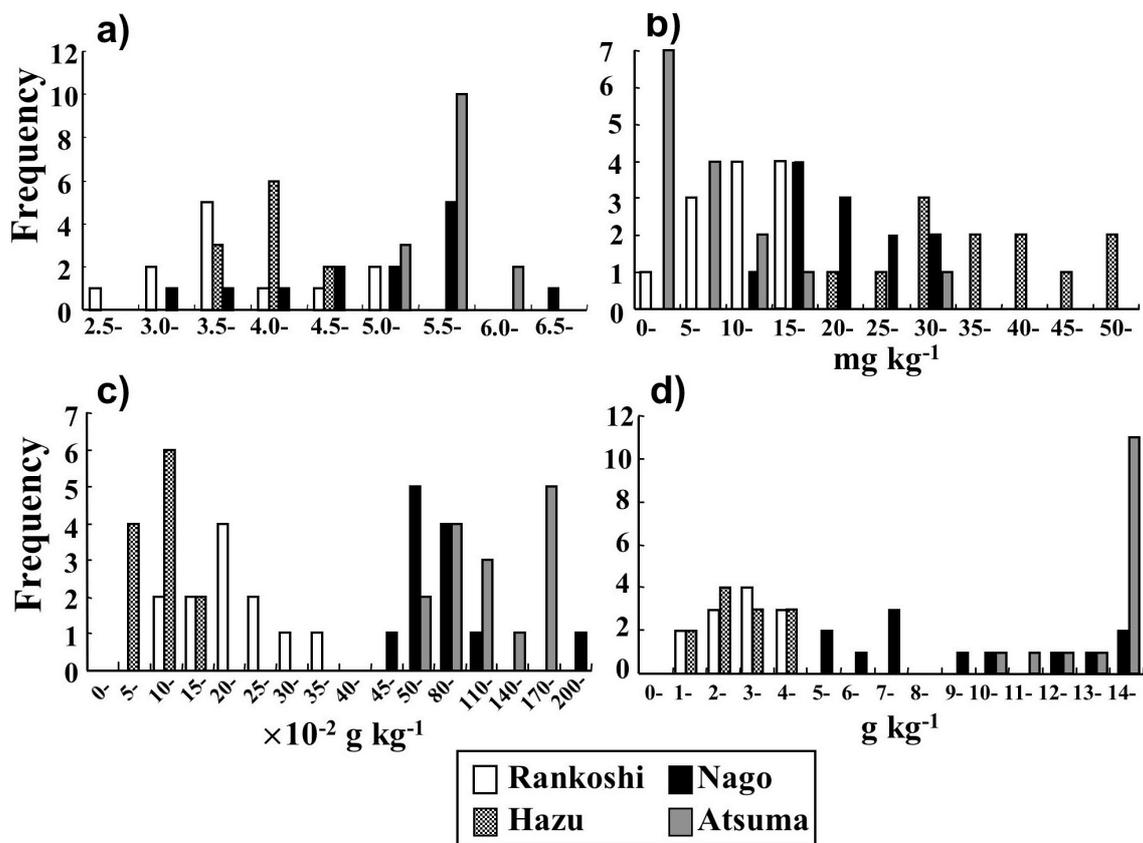


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569 **Figure 1** Location of the sampling sites of the rhizosphere soils of *Miscanthus sinensis*

570 grown on acid sulfate soil (Rankoshi, Hazu, Nago) and sandy soil (Atsuma) in Japan.

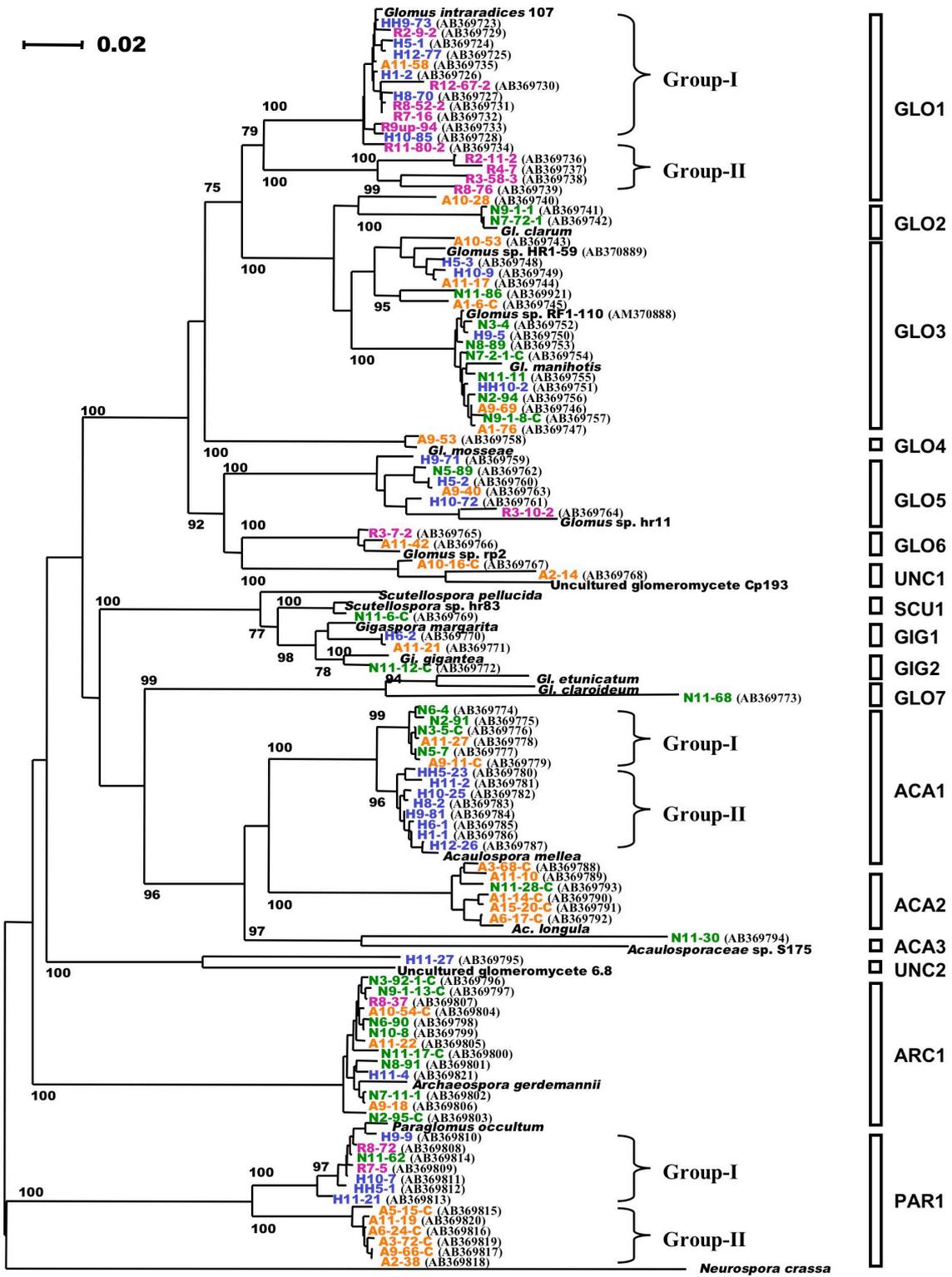


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573 **Figure 2** Frequency distributions of pH (a), Troug-P (b), total nitrogen (c) and total574 carbon (d) of the rhizosphere soil of *M. sinensis* collected from Rankoshi, Hazu, Nago

575 and Atsuma sites.

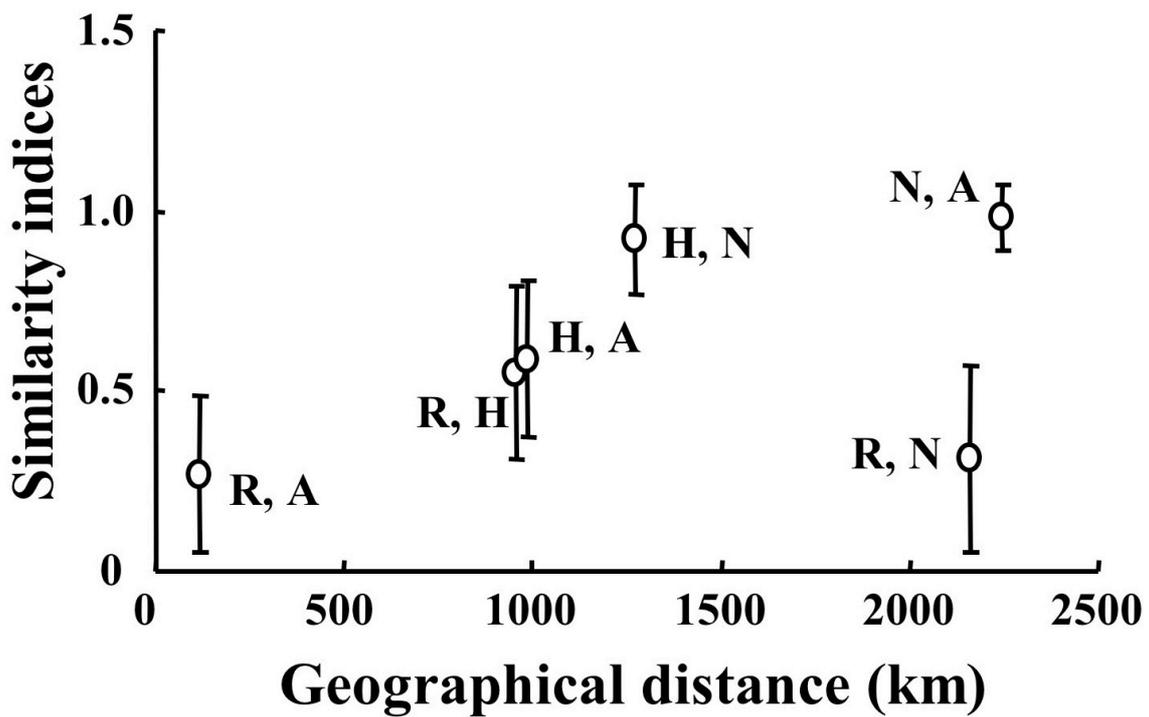


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579 **Figure 3** Phylogenetic analysis based on the sequences of 700-760 bp fragment of AM  
580 fungal LSU rDNA obtained by the *M. sinensis* trap culture using the rhizosphere soils  
581 collected from Rankoshi (purple), Hazu (blue), Nago (green) and Atsuma (orange).  
582 The neighbor-joining tree is drawn by the NJplot. The bootstrap values more than 70%  
583 are indicated. The representative sequences from each root sample are incorporated.  
584 The clone names are followed by the GenBank accession numbers of the sequences  
585 obtained in this study. The accession numbers of the reference sequences are: *Glomus*  
586 *intraradices* 107, AY639221; *Gl. clarum*, AJ510243; *Gl. manihotis*, AM158947;  
587 *Glomus* sp. hr11, AM040407; *Glomus* sp. rp2, AM040435; *Gl. caledonium*,  
588 AM040317; *Gl. mosseae*, DQ469129; *Gl. etunicatum*, AF145749; *Scutellospora*  
589 *pellucida*, AY639326; *Scutellospora* sp. hr83, AM040378; *Gigaspora margarita*,  
590 AF396783; *Gi. gigantea*, AY900504; *Acaulospora mellea*, AY900513;  
591 *Acaulosporaceae* sp. S175, AB206249; *A. longula*, AM040293; Uncultured  
592 glomeromycete 6.8, AY639357; Uncultured glomeromycete Cp193, AB206207;  
593 *Archaeospora gerdemanni*, AJ510234; *Palaglomus occultum*, AJ271713; *Neurospora*  
594 *crassa*, AF286411.



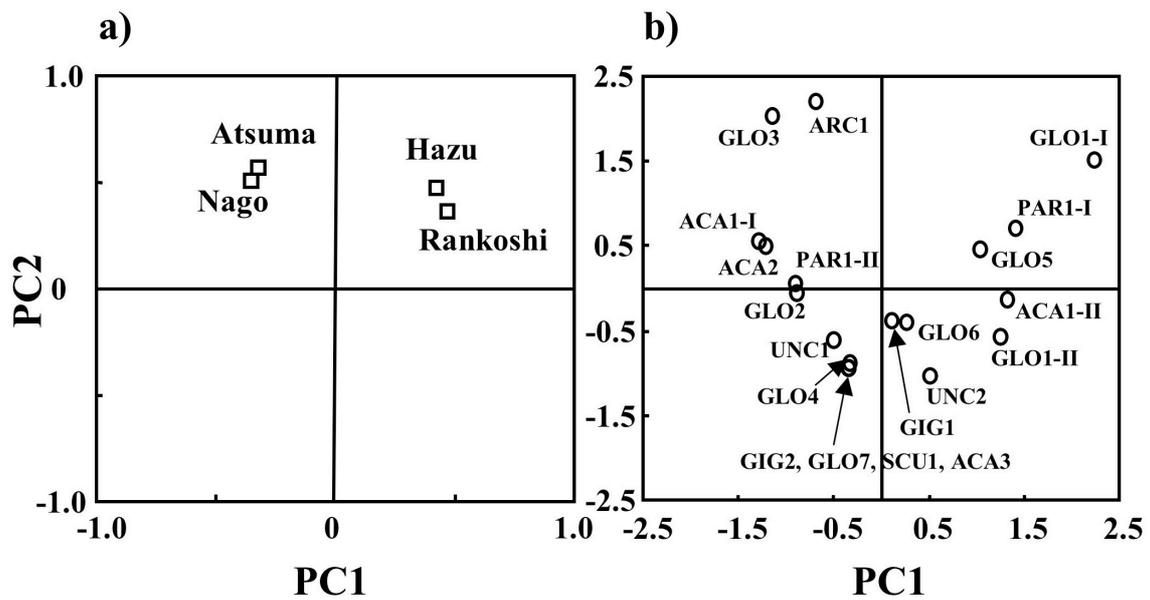
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597 **Figure 4** Scatter plot of Chao's Sørensen similarity indices of AM fungal communities

598 against geographical distance between two sampling sites. R, Rankoshi; H, Hazu; N,

599 Nago; A, Atsuma. No significant correlation between the two parameters was found ( $P$ 600  $< 0.05$ ). Vertical bars indicate SD.



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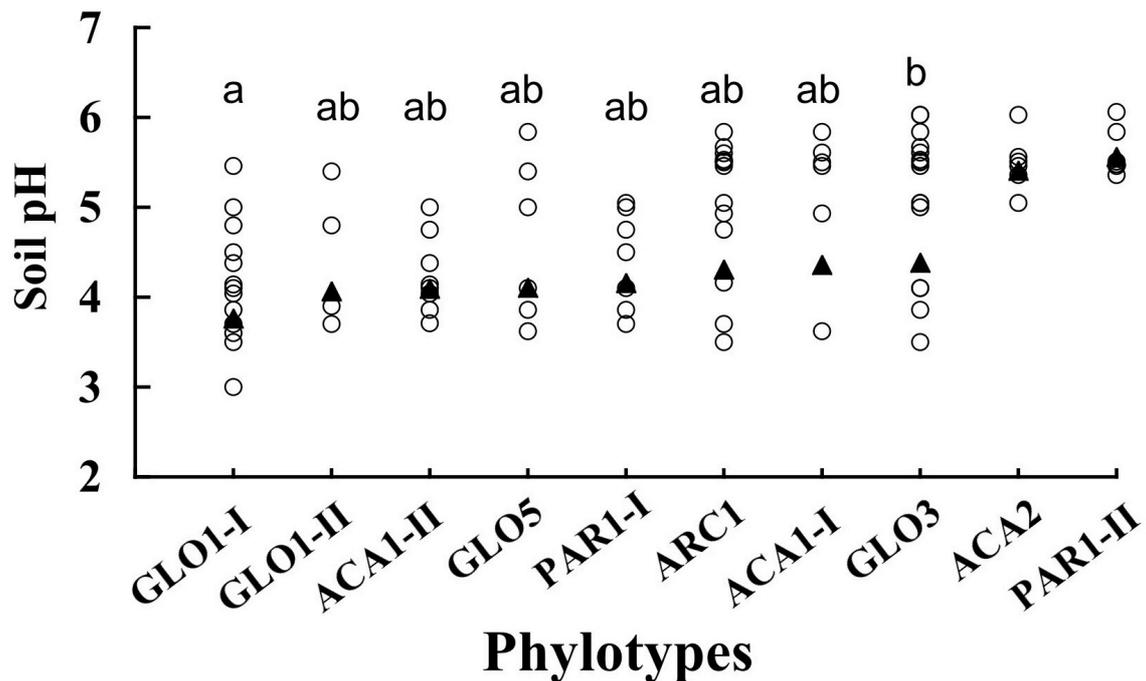
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603 **Figure 5** Principal component analysis on AM fungal communities in Rankoshi, Hazu,

604 Nago and Atsum sites. a) Component plot of experimental sites. The first (PC1) and

605 second (PC2) principal components explained 40.2% and 27.1% of the total variation,

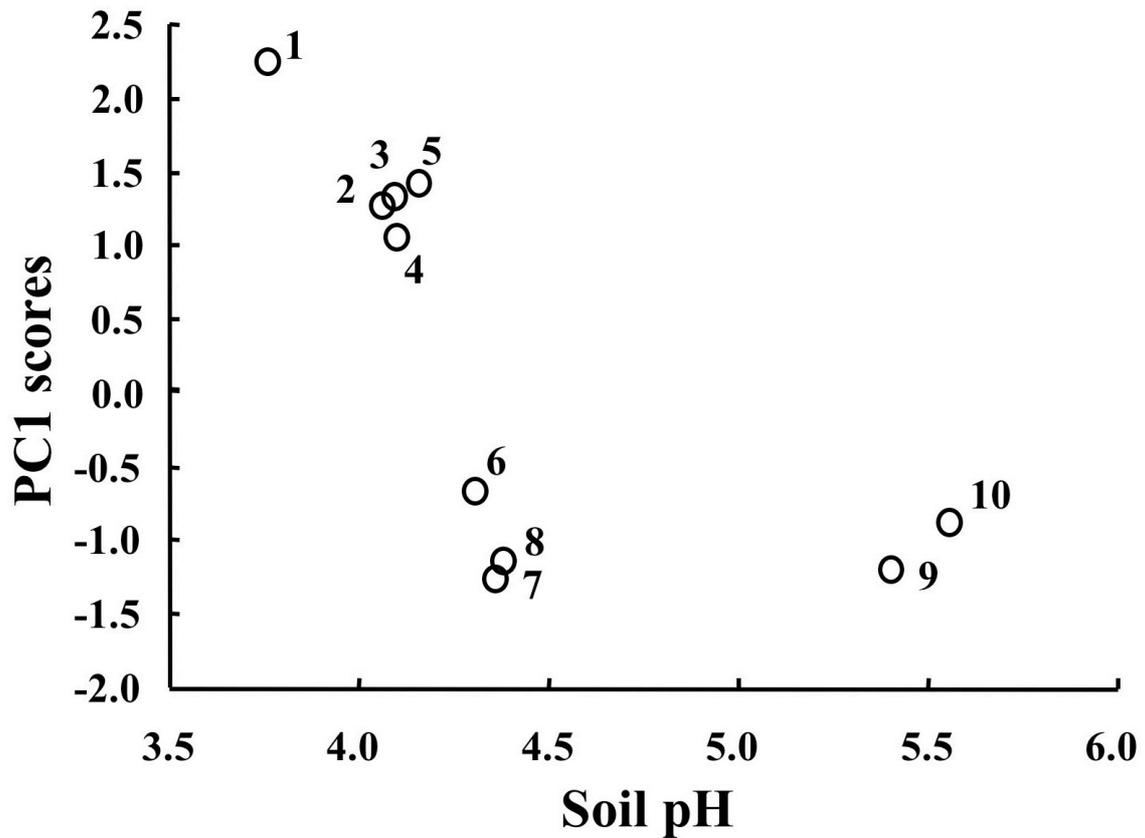
606 respectively. b) Scatter plot of the PC1 and PC2 scores of AM fungal phylotypes.



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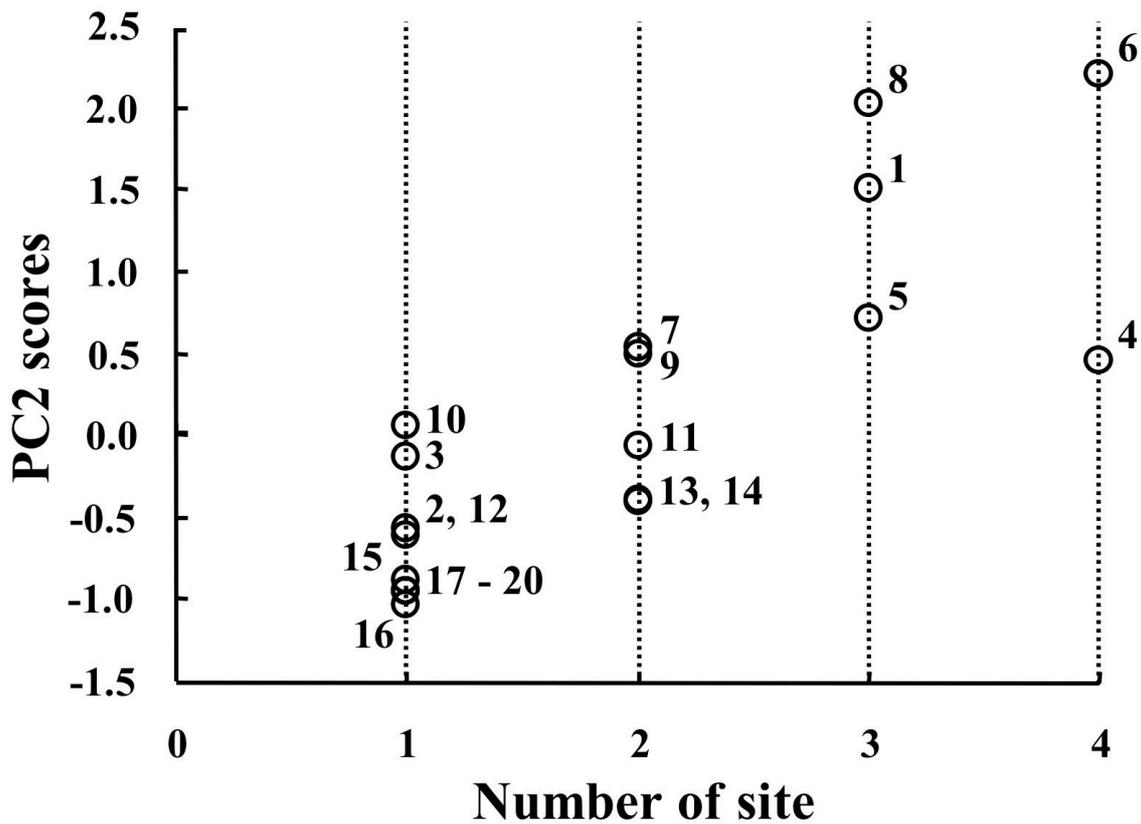
609 **Figure 6** Difference in the range of pH in which AM fungal phylotypes occurred. The  
 610 pH values of rhizosphere soils from which each AM fungal phylotypes were detected  
 611 were plotted (open circle). Only the phylotypes that occurred in four or more soil  
 612 samples (across the all sites) were included in this analysis. The test of equality of  
 613 variance showed the data sets of ACA2 and PAR1 group-II showed significantly  
 614 smaller variances than those of the other phylotypes, thus these two phylotypes were  
 615 excluded from subsequent ANOVA. Different letters indicate significant difference in  
 616 pH distribution (Tukey-Kramer test,  $P < 0.05$ ). The pH values were transformed to real  
 617 number for the calculation of mean value (closed triangle) but were to be  
 618 retransformed to logarithmic values for ANOVA for normalization.



619

620

621 **Figure 7** Scatter plot of the first principal component (PC1) scores of AM fungal  
 622 phylotypes against the average pH values of the soils in which they occurred. Only the  
 623 phylotypes detected from four or more soil samples (across the all sites) were included  
 624 in this analysis. The phylotypes are identified by numbers as follows: 1, GLO1 group-  
 625 I; 2, GLO1 group-II; 3, ACA1 group-II; 4, GLO5; 5, PAR1 group-I; 6, ARC1; 7, ACA1  
 626 group-I; 8, GLO3; 9, ACA2; 10, PAR1 group-II.



627

628

629 **Figure 8** Scatter plot of the second principal component (PC2) scores of the

630 phylotypes against the number of the sites from which they were detected. All of the

631 phylotypes were included in this analysis. The phylotypes are identified by numbers as

632 follows: 1, GLO1 group-I; 2, GLO1 group-II; 3, ACA1 group-II; 4, GLO5; 5, PAR1

633 group-I; 6, ARC1; 7, ACA1 group-I; 8, GLO3; 9, ACA2; 10, PAR1 group-II; 11,

634 GLO2; 12, UNC1; 13, GLO6; 14, GIG1; 15, GLO4; 16, UNC2; 17, GLO7; 18,

635 SCU1; 19, GIG2; 20, ACA3.

636 **Table 1** Climates and surrounding vegetation of the experimental sites.

Sites (Soils)	Locations / Climatic zones	Temperature / rainfall (Max. snow) <sup>a</sup>	Surrounding vegetation (Dominant species)
Rankoshi (Acid sulfate soil)	Hokkaido Isl. / Subarctic	7.4°C / 1,169 mm (150 cm)	Deciduous broad-leaved ( <i>Betula platyphylla</i> var. <i>japonica</i> and <i>Quercus</i> spp.) and conifer ( <i>Larix leptolepis</i> ) trees and <i>Picea jezoensis</i> plantation
Hazu (Acid sulfate soil)	Honshu Isl. / Temperate	16.0°C / 1,600 mm	Evergreen broad-leaved trees ( <i>Q. glauca</i> , <i>Q. myrsinaefolia</i> and <i>Castanopsis cuspidate</i> )
Nago (Acid sulfate soil)	Okinawa Isl. / Subtropical	22.5°C / 2,437 mm	<i>Casuarina equisetifolia</i> plantation and sugarcane field
Atsuma (Sandy soil)	Hokkaido Isl. / Subarctic	6.5°C / 1,017 mm (100 cm)	Deciduous broad-leaved trees ( <i>Quercus</i> spp., <i>B. platyphylla</i> var. <i>japonica</i> and <i>Acer</i> spp.)

637 <sup>a</sup>Annual mean temperature and rainfall. Maximum snow depth in February is indicated  
638 in parentheses in the case of the sites in subarctic zone.

639