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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Abstract

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

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

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

61 *Miscanthus sinensis* Andresson, major pioneer grass species in acid sulfate soil in
62 Eastern Asia, has a high potential of photosynthesis as a C_4 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

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

MATERIALS AND METHODS

Sampling sites

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

Acid sulfate soil in Rankoshi was originated from pyroclastic sediment and exposed by quarrying. The site consists of a steep slope (30-40˚ angle, 50 m in height) with several terraces (20-30 × 3-4 m, W/D) and has not been disturbed severely since 1980'. The ground of the site was not fully covered with vegetation, but patchy distributions of *M. sinensis, Petasites japonicus* (Sieb. et Zucc.) Maxim var. *giganteus* (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^{-1} 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˚C,
143 whereas those for the assessment of AM colonization were cut into ca 1 cm segments
144 and stored at –30˚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 (H_2O) 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 (H_2O_2) was measured after over night oxidation with 30%
153 H_2O_2 at a 1:5 soil to H_2O_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 H_2SO_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 × 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˚C. A part of the large
165 subunit ribosomal RNA gene (LSU rDNA) was amplified in a 25 μL reaction mixture
166 with the Expand High-Fidelity PLUS PCR System (Roche Diagnostics, Tokyo)
167 containing 0.5 μM primers and 1 μL 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˚C for
172 2 min, followed by 30 cycles of denaturation at 94˚C for 15 s, annealing at 50˚C for 40
173 s and polymerization at 72˚C for 80 s, and final elongation at 72˚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 μL 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

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

Statistical analysis

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

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

RESULTS

Soil chemical properties

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_2O_2)
227 of the soils that showed pH higher than 5.5 was measured. Oxidation of the soils by
228 H_2O_2 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^{-1} 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^{-1} with the highest frequency in
236 between 7.0 – 7.9 g kg^{-1}. Total C contents in the Atsuma samples were considerably
237 higher than those in the others and more than 10.0 g kg^{-1} up to 26.0 g kg^{-1}.

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 phylotypes**
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 phylotypes (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 phylotype (phylotype
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.

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

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

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

DISCUSSION

Soil pH as a driving force for structuring AM fungal communities

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

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

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

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

There is a possibility that the soil trap culture biased the AM fungal communities thus the species compositions were similar to each other. It has been known that different analytical methods e.g. field-collected samples- and trap culture-based analyses gave different results (Bever *et al.* 1996; Brundrett *et al.* 1999a; 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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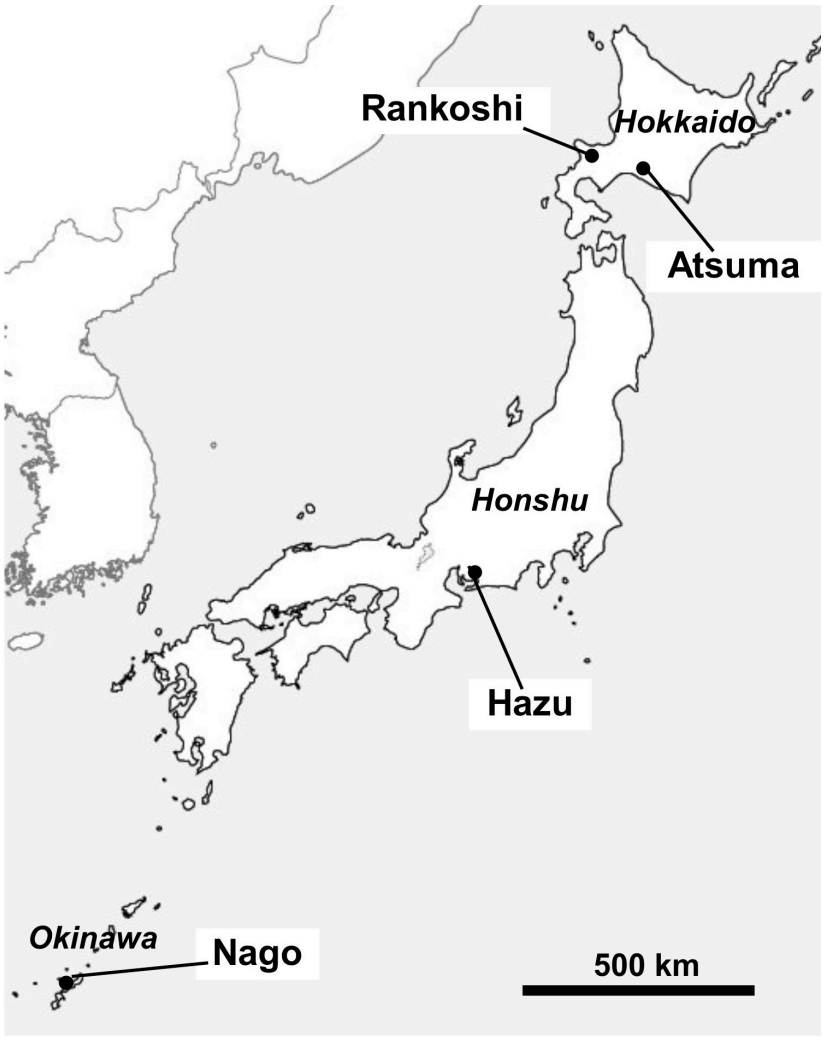
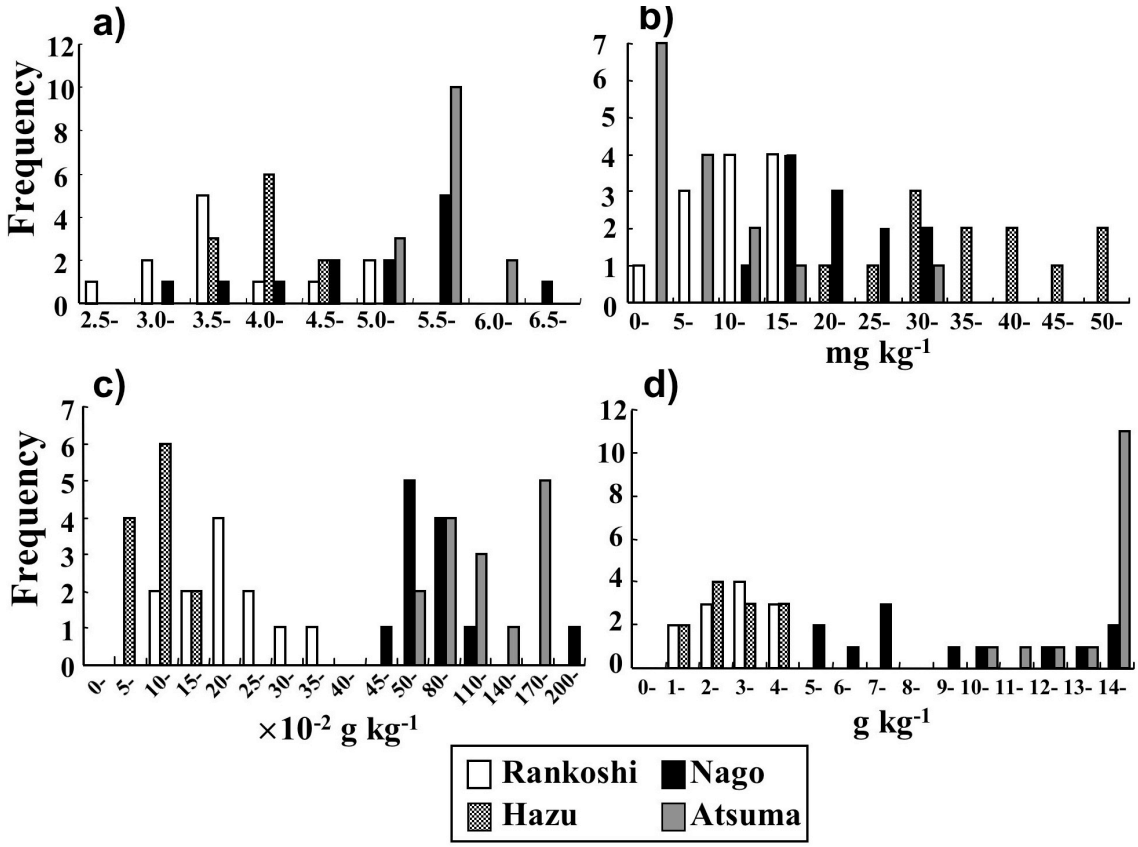


567

568

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.



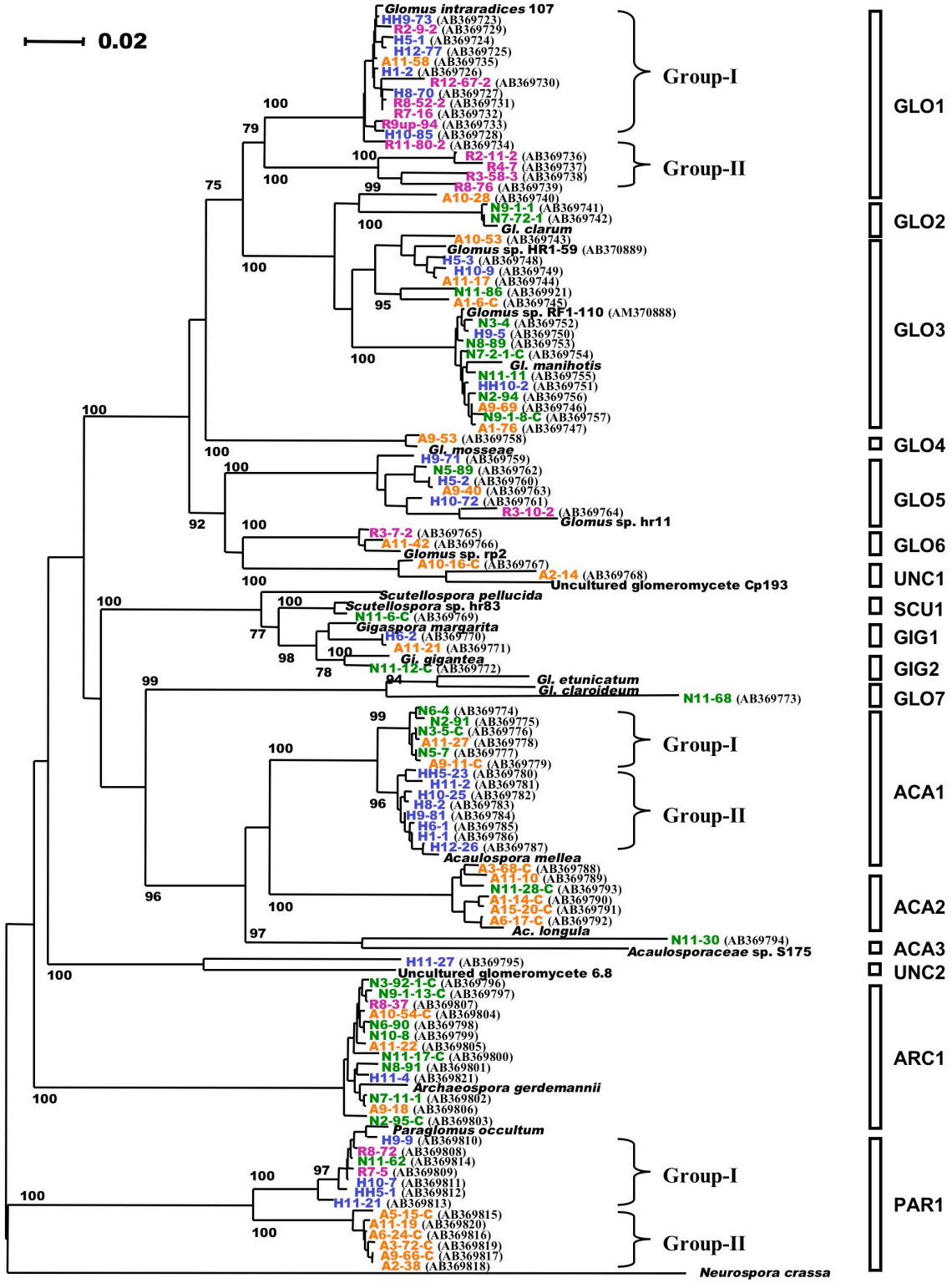
571

572

573 **Figure 2** Frequency distributions of pH (a), Troug-P (b), total nitrogen (c) and total

574 carbon (d) of the rhizosphere soil of *M. sinensis* collected from Rankoshi, Hazu, Nago

575 and Atsuma sites.

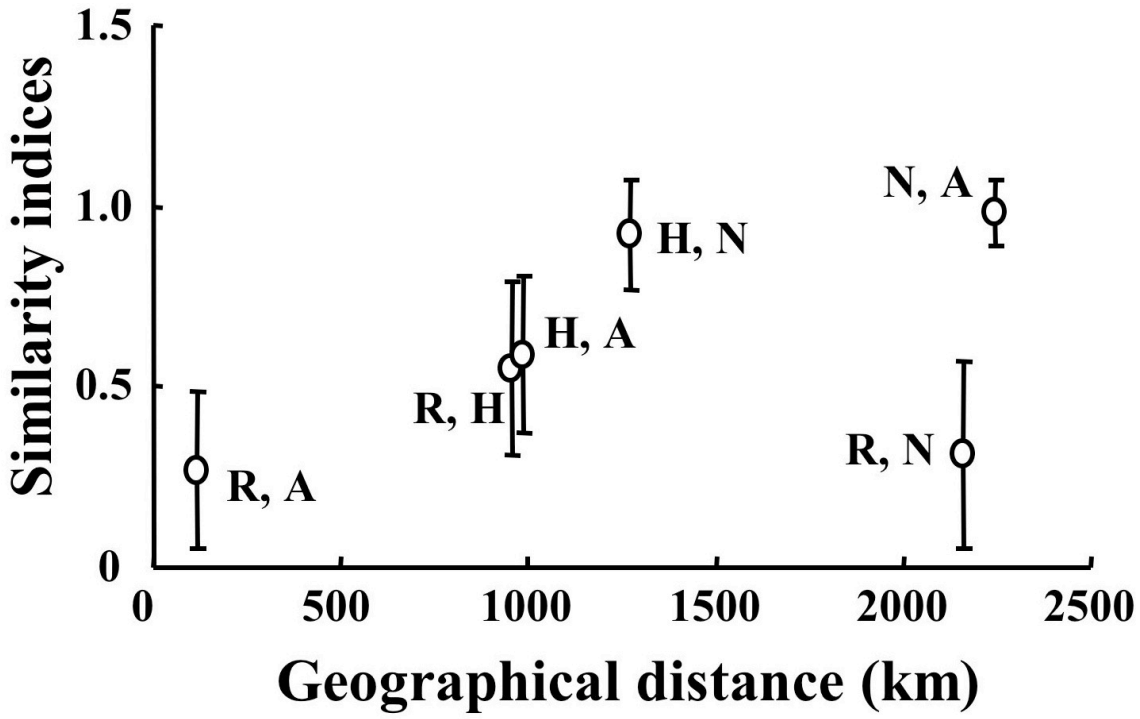

0.02
Glomus intraradices 107
HH9-73 (AB369723)
R2-9-2 (AB369729)
H5-1 (AB369724)
H12-77 (AB369725)
A11-58 (AB369735)
H1-2 (AB369726)
R12-67-2 (AB369730)
H8-70 (AB369727)
R8-52-2 (AB369731)
R7-16 (AB369732)
R9up-94 (AB369733)
H10-85 (AB369728)
R11-80-2 (AB369734)
R2-11-2 (AB369736)
R4-7 (AB369737)
R3-58-3 (AB369738)
R8-76 (AB369739)
A10-28 (AB369740)
N9-1-1 (AB369741)
N7-72-1 (AB369742)
Gl. clarum
A10-53 (AB369743)
Glomus sp. HR1-59 (AB370889)
H5-3 (AB369748)
H10-9 (AB369749)
A11-17 (AB369744)
N11-86 (AB369921)
A1-6-C (AB369745)
Glomus sp. RF1-110 (AM370888)
N3-4 (AB369752)
H9-5 (AB369750)
N8-89 (AB369753)
N7-2-1-C (AB369754)
Gl. manihotis
N11-11 (AB369755)
HH10-2 (AB369751)
N2-94 (AB369756)
A9-69 (AB369746)
N9-1-8-C (AB369757)
A1-76 (AB369747)
A9-53 (AB369758)
Gl. mosseae
H9-71 (AB369759)
N5-89 (AB369762)
H5-2 (AB369760)
A9-40 (AB369763)
H10-72 (AB369761)
R3-10-2 (AB369764)
Glomus sp. hr11
R3-7-2 (AB369765)
A11-42 (AB369766)
Glomus sp. rp2
A10-16-C (AB369767)
A2-14 (AB369768)
Uncultured glomeromycete Cp193
Scutellospora pellucida
Scutellospora sp. hr83
N11-6-C (AB369769)
Gigaspora margarita
H6-2 (AB369770)
A11-21 (AB369771)
Gi. gigantea
N11-12-C (AB369772)
Gl. etunicatum
Gl. claroideum
N11-68 (AB369773)
N6-4 (AB369774)
N2-91 (AB369775)
N3-5-C (AB369776)
A11-27 (AB369778)
N5-7 (AB369777)
A9-11-C (AB369779)
HH5-23 (AB369780)
H11-2 (AB369781)
H10-25 (AB369782)
H8-2 (AB369783)
H9-81 (AB369784)
H6-1 (AB369785)
H1-1 (AB369786)
H12-26 (AB369787)
Acaulospora mellea
A3-68-C (AB369788)
A11-10 (AB369789)
N11-28-C (AB369793)
A1-14-C (AB369790)
A15-20-C (AB369791)
A6-17-C (AB369792)
Ac. longula
N11-30 (AB369794)
Acaulosporaceae sp. S175
H11-27 (AB369795)
Uncultured glomeromycete 6.8
N3-92-1-C (AB369796)
N9-1-13-C (AB369797)
R8-37 (AB369807)
A10-54-C (AB369804)
N6-90 (AB369798)
N10-8 (AB369799)
A11-22 (AB369805)
N11-17-C (AB369800)
N8-91 (AB369801)
H11-4 (AB369821)
Archaeospora gerdemannii
N7-11-1 (AB369802)
A9-18 (AB369806)
N2-95-C (AB369803)
Paraglomus occultum
H9-9 (AB369810)
R8-72 (AB369808)
N11-62 (AB369814)
R7-5 (AB369809)
H10-7 (AB369811)
HH5-1 (AB369812)
H11-21 (AB369813)
A5-15-C (AB369815)
A11-19 (AB369820)
A6-24-C (AB369816)
A3-72-C (AB369819)
A9-66-C (AB369817)
A2-38 (AB369818)
Neurospora crassa
Group-I
Group-II
Group-I
Group-II
Group-I
Group-II
GLO1
GLO2
GLO3
GLO4
GLO5
GLO6
UNC1
SCU1
GIG1
GIG2
GLO7
ACA1
ACA2
ACA3
UNC2
ARC1
PAR1

576

577

578

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 (pupple), 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 gerdemannii*, AJ510234; *Palaglomus occultum,* AJ271713; *Neurospora*
594 *crassa,* AF286411.



595

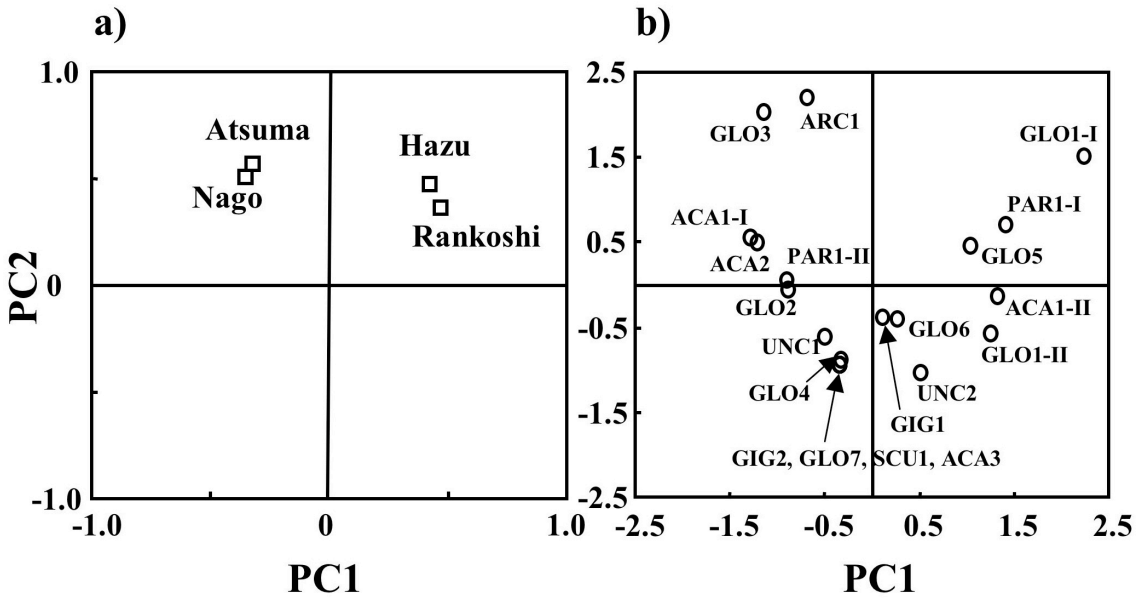
596

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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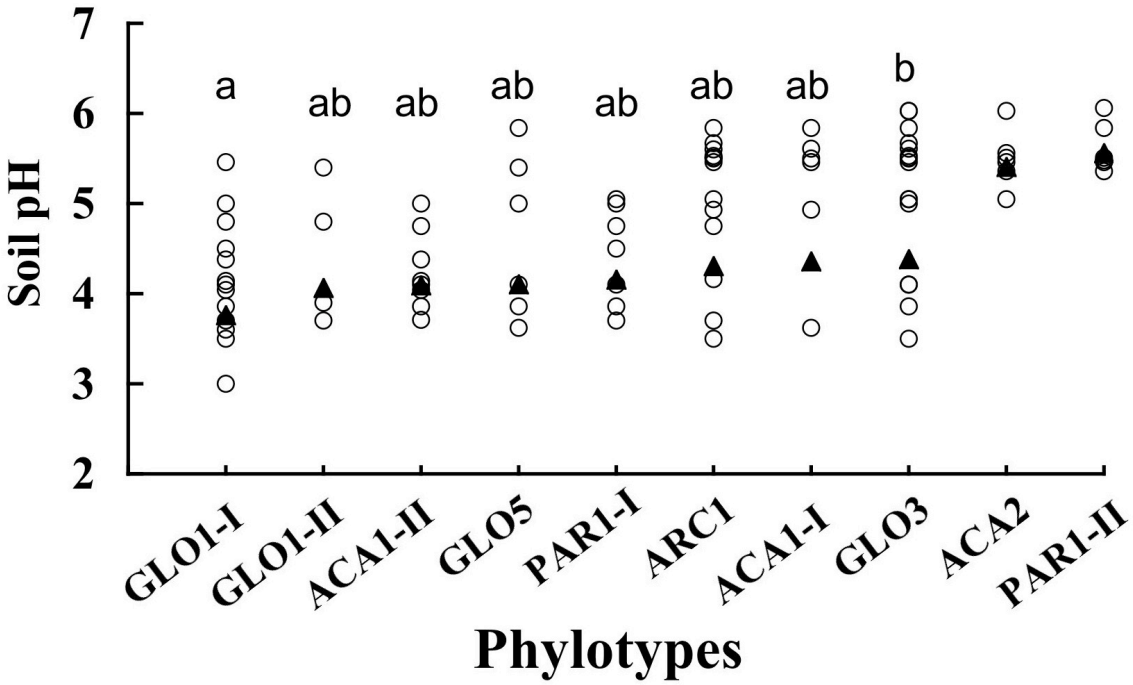
602

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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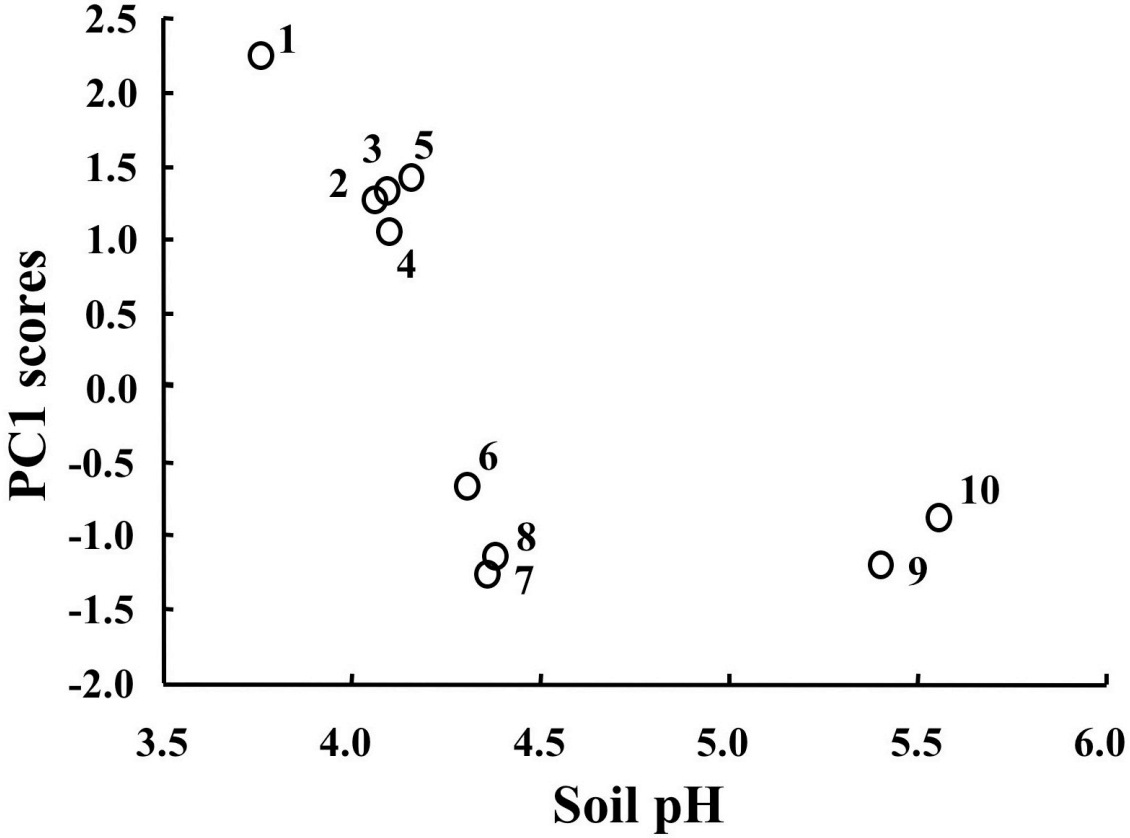
608

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.



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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.

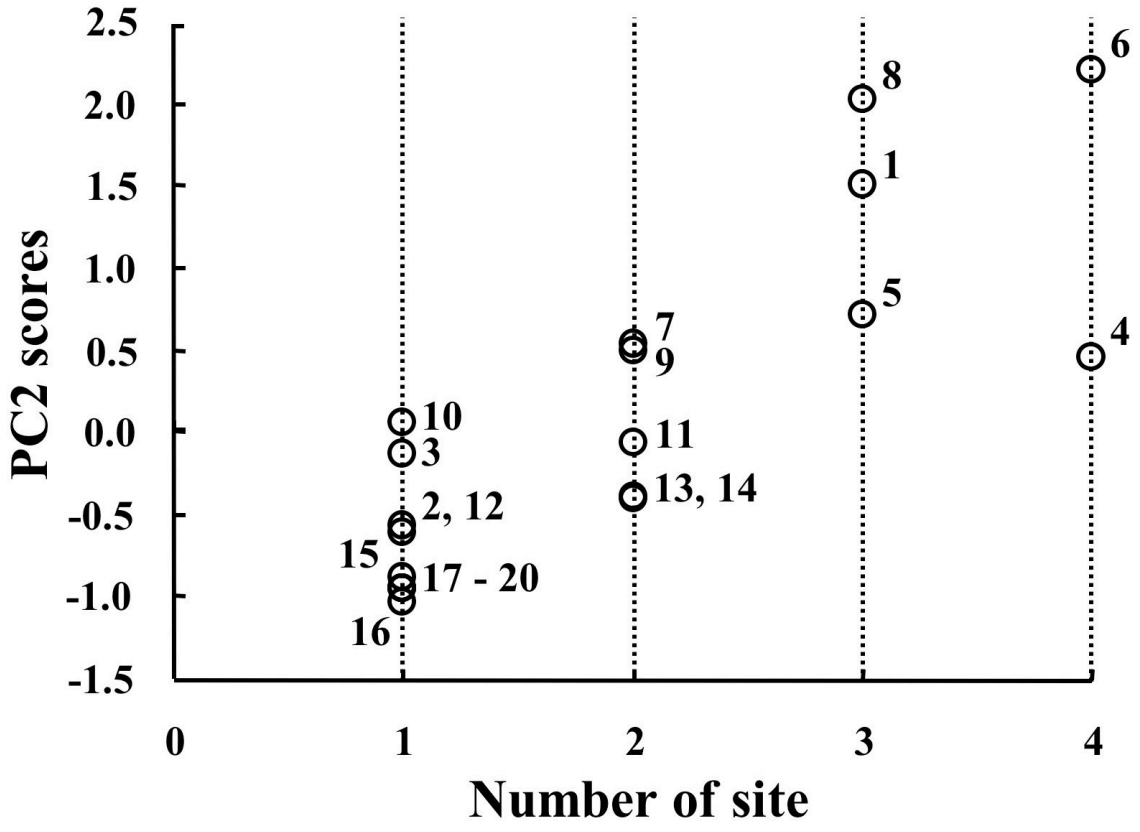

Figure 8 Scatter plot of the second principal component (PC2) scores of the phylotypes against the number of the sites from which they were detected. All of the phylotypes were included in this analysis. The phylotypes are identified by numbers as follows: 1, GLO1 group-I; 2, GLO1 group-II; 3, ACA1 group-II; 4, GLO5; 5, PAR1 group-I; 6, ARC1; 7, ACA1 group-I; 8, GLO3; 9, ACA2; 10, PAR1 group-II; 11, GLO2; 12, UNC1; 13, GLO6; 14, GIG1; 15, GLO4; 16, UNC2; 17, GLO7; 18, 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)[a]	Surrounding vegetation (Dominant species)
Rankoshi (Acid sulfate soil)	Hokkaido Isl. / Subarchtic	7.4˚C / 1,169 mm (150 cm)	Deciduous broad-leaved (*Betula platyphylla* var. *japonica* and *Quercus* spp.) and conifer (*Larix leptolesis*) trees and *Picea jezoensis* plantation
Hazu (Acid sulfate soil)	Honshu Isl. / Temperate	16.0˚C / 1,600 mm	Evergreen broad-leaved trees (*Q. glauca*, *Q. myrsinaefolia* and *Castanopsis cuspidate*)
Nago (Acid sulfate soil)	Okinawa Isl. / Subtropical	22.5˚C / 2,437 mm	*Casuarina equisetifolia* plantation and sugarcane field
Atsuma (Sandy soil)	Hokkaido Isl. / Subarchtic	6.5˚C / 1,017 mm (100 cm)	Deciduous broad-leaved trees (*Quercus* spp., *B. platyphylla* var. *japonica* and *Acer* spp.)

637 [a]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