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ORIGINAL PAPER

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Defoliation effects on the community structure of arbuscular mycorrhizal fungi based on 18S rDNA sequences

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Abstract The effects of defoliation on arbuscular mycorrhizal (AM) associations in the field were investigated in terms of the community structure of AM fungi colonizing roots of grassland plants; the carbohydrate balance of the host plants was also determined. We focused on two plant species dominating Japanese native grasslands: the grazing-intolerant species *Miscanthus sinensis* and the grazing-tolerant species *Zoysia japonica*. Community structures of AM fungi were determined from 18S rRNA gene sequences. The dominant fungal group in both plant species was the *Glomus* clade, which was classified into several phylogenetic groups based on genetic distances and topology. In *Miscanthus* roots, the *Glomus*-Ab, *Glomus*-Ac, and *Glomus*-Ad groups were detected almost equally. In *Zoysia* roots, the *Glomus*-Ab group was dominant. Defoliation effects on the community structure of AM fungi differed between the plant species. In *Miscanthus* roots, the percentage of root length colonized (%RLC) by the *Glomus*-Ac and *Glomus*-Ad groups was significantly reduced by defoliation treatment. On the other hand, AM fungal group composition in *Zoysia* roots was unaffected by defoliation except on the last sampling date. Decreased %RLC by *Glomus*-Ac and *Glomus*-Ad coincided with decreased non-structural carbohydrate (NSC) levels in host plants; also, significant positive correlations were found between the %RLC and some NSC levels. On the other hand, the %RLC by *Glomus*-Ab in both plant species was unaffected by the NSC level. These results suggest that AM fungal groups have different carbohydrate requirements from host plants.

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Introduction

In grassland ecosystems, plants frequently lose their photosynthetic parts as a result of mowing, locusts, or grazing. Under these conditions, mycorrhizal colonization is likely to be affected, as the photosynthate available for the fungi is reduced (Gehring and Whitham 1994, 2002). Some studies have shown that mycorrhizal colonization declines following grazing under field conditions (Bethlenfalvay and Dakessian 1984; Bethlenfalvay et al. 1985; Trent et al. 1988). Daft and El-Giahmi (1978) also observed decreased mycorrhizal colonization in defoliated plants, suggesting the importance of photosynthate supply for controlling development of AM fungi. However, several studies have indicated that mycorrhizal colonization increased with grazing or defoliation intensity (Wallace 1981; Bayne et al. 1984; Eom et al. 2001), while others show that mycorrhizal colonization was unaffected by defoliation (Reece and Bonham 1978; Borowicz 1993; Busso et al. 2001). To explain these inconsistent results, Gehring and Whitham (1994) proposed three hypotheses: (1) differential defoliation intensity, (2) differential plant defoliation tolerance, and (3) differences in the AM fungal species colonizing the plant roots.

To resolve whether differences in defoliation intensity and plant defoliation tolerance affect AM association, an experimental approach using grazing-tolerant and grazing-intolerant plant species with different defoliation frequencies was taken (Allsopp 1998). That study showed that mycorrhizal colonization on both types of plant species decreased with defoliation frequency, but that the pattern of decrease varied among the plant species. Factors determining variation among plant species are unknown, but they might be related to carbon reserves,

regrowth ability, or the carbohydrate balance of the plant species.

Another explanation for the differential response of AM associations to defoliation among plant species is based on differences among the AM fungal species that colonize the roots. AM fungi are known to consist of functionally distinct fungi although, to date, AM fungi have been assumed to be a single functional group (Dodd et al. 2000). The functional differences may possibly indicate the importance of clarifying which AM fungal species colonize plants. Defoliation certainly affects the species composition of ectomycorrhizal fungi in conifers (Saikkonen et al. 1999; Cullings et al. 2001). In addition, the species composition of AM fungi in grazing areas has been investigated by spore surveys, which indicated that AM fungal species diversity decreases with grazing (Bethlenfalvay and Dakessian 1984; Eom et al. 2001). It has been suggested, however, that species composition determined from spore surveys poorly represents the actual AM fungal community in plant roots (Clapp et al. 1995; Kowalchuk et al. 2002).

Molecular methods combined with the polymerase chain reaction (PCR) have been used in studies of the diversity and phylogeny of AM fungi, and may be useful tools for identifying AM fungi in roots (Sanders et al. 1996; Clapp et al. 2002). The 18S rRNA genes have been the main targets in this approach because information about these genes has been accumulated for a wide range of organisms, and because they are present in relatively high copy numbers. In several field experiments, these sequences have been useful in studies of AM fungal ecology and diversity (Clapp et al. 1995; Helgason et al. 1998, 1999, 2002; Daniell et al. 2001; Kowalchuk et al. 2002; Vandenkoornhuyse et al. 2002). Using molecular techniques, Clapp et al. (1995) first showed multiple colonization in the same root by different AM fungal genera. Moreover, several studies have investigated AM fungal species diversity and seasonal variation in arable lands, seminatural grasslands, and woodlands (Helgason et al. 1998, 1999, 2002; Daniell et al. 2001; Vandenkoornhuyse et al. 2002).

The present study investigated the effects of defoliation on AM associations in a field in terms of (1) the community structure of AM fungi colonizing the plant roots, as determined using molecular methods, and (2) carbohydrate balance in grazing-tolerant and grazing-intolerant plant species. We focus on the two major C_4 grasses in Japanese native grasslands: *Miscanthus sinensis* Anderss., a bunch-type tall grass that is grazing intolerant, and *Zoysia japonica* Steud., a turfgrass that is grazing tolerant. Ecological succession between *Miscanthus*- and *Zoysia*-dominated communities is well documented. When *Miscanthus*-type grassland is subjected to intensive cattle grazing or mowing, the community of the grassland changes to that of a *Zoysia*-type grassland (Numata 1969).

Materials and methods

Study area, experimental design, and sampling procedure

The experiment was conducted at the Experimental Farm of Tohoku University, Miyagi, Japan (38°44′–38°46′ N, 140°44′–140°45′ E). The area has a temperate climate with a mean annual temperature of 10.0°C, and 1,633 mm mean annual precipitation. The study was carried out from July to October 1998 on *Miscanthus*- and *Zoysia*-type grasslands separated by a distance of 4.3 km. The soil was a nonallophanic andisol (pH 5.5) with low available phosphorus concentrations of 13.0 μg g^{-1} and 3.3 μg g^{-1} (Truog method) in the *Miscanthus*-type and *Zoysia*-type grasslands, respectively.

Six 9 m^2 plots and six 1 m^2 plots were randomly located in the *Miscanthus*-type and *Zoysia*-type grasslands, respectively. In the *Zoysia*-type grassland, the four sides of the plots were cut to disconnect physiological integration through rhizomes. Within each grassland, all plots were within approximately 30 m of one another and were protected from grazing by livestock. Half of the plots were assigned as defoliation-treated plots; the remainder were control plots. Defoliation treatments to *Miscanthus* were carried out on 21 July and 14 August by removing all leaves with shears. Defoliation treatments to *Zoysia* were carried out on 28 July, 6 August, and 21 August by removing all plant material 0 cm above ground level with a turfgrass clipper. Because leaf regrowth of *Zoysia* is active, defoliation treatments were performed three times to retain the treatment effect.

Miscanthus plants from both control and defoliation-treated plots were removed with three 20-cm-diameter ×20-cm-deep soil cores on 16 July, 11 August, 7 September, and 13 October. *Zoysia* plants were removed with three 8-cm-diameter ×6-cm-deep soil cores on 22 July, 20 August, 17 September, and 19 October. Sampling was performed at a consistent time of day (10:00 to 11:00) in consideration of diurnal changes in non-structural carbohydrate (NSC) content. Plant parts were separated from soil and subdivided into above-ground portions (stems, leaves, and heads) and below-ground portions (roots and rhizome). Roots were divided into three subsamples (about 30 roots of 1 cm length) and used for DNA extraction, measurement of mycorrhizal colonization, and weight of dry matter. Plant parts, except for the two root subsamples, were heated at 105°C for 30 min to inactivate plant enzymes, dried at 70°C for 48 h, and then weighed.

The size of the experimental plots and sampling cores differed between *Miscanthus* and *Zoysia* because of their plant types: *Miscanthus* is a large bunch-type grass that reaches a height of about 3 m, and its density is low (about two stands/m^2), while *Zoysia* is a small rhizomatous plant (about 20 cm tall), and its shoot density is very high.

DNA extraction, PCR amplification, and cloning of PCR products

DNA was extracted from lateral roots of a root subsample using a boiling procedure (Di Bonito et al. 1995; Saito et al. 2000, 2001). DNA extracted from three cores per plot was pooled and stored at −20°C until subsequent PCR amplification.

The oligonucleotide primers used in PCR amplification were designed based on alignments of partial 18S rRNA gene sequences obtained from the DDBJ/EMBL/GenBank database. Two nested pairs of oligonucleotide primers (Fig. 1) were designed with the primer analysis software OLIGO 4 (National Biosciences). The outer primer pair in the first reaction is AMV4.5F (5′-AAT TGG AGG GCA AGT CTG G-3′) and AMV4.5R (5′-AGC AGG TTA AGG TCT CGT TCG T-3′). The inner primer pair in the second reaction is AMV4.5NF (5′-AAG CTC GTA GTT GAA TTT CG-3′) and AMV4.5NR (5′-CAC CCA TAG AAT CAA GAA AGA-3′). In a BLAST search, the primer pair AMV4.5F-AMV4.5R identically matched many Eukaryota sequences but mismatched 8 out of 130 AM fungal sequences in the database by one base indel or substitution. The primer pair AMV4.5NF-AMV4.5NR identically matched 200 out of 232 AM fungal sequences and the *Pipto-*

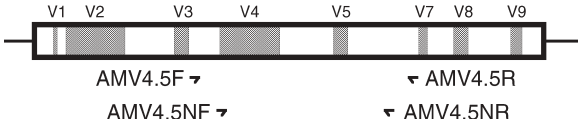

Fig. 1 Location of PCR primers on 18S rDNA. Primer pairs AMV4.5F-AMV4.5R and AMV4.5 NF-AMV4.5NR were used for the initial and second amplifications, respectively. Variable regions are highlighted in *gray*

cephalis corymbifera sequence (Zygomycota, accession no. AB016023) in the database. Of 232 AM fungal sequences (mostly Archaeosporaceae), 27 mismatched the primer pair by one base, and five sequences (mostly Paraglomaceae) mismatched it by two bases (for alignments of sequences with the primer sequence, see Electronic Supplementary Material).

The PCR mixtures (50 µl) contained 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.025 U μl^{-1} AmpliTaq Gold (Applied Biosystems), 0.5 µM of each primer, and 5 µl template DNA. The following thermal profile was used: 95°C for 10 min, then 94°C for 30 s, 60°C for 30 s, 72°C for 1 min for 20 or 40 cycles, and finally 72°C for 10 min. The first reaction was performed with the primer pair AMV4.5F and AMV4.5R for 20 cycles. Next, the second (nested) PCR was performed with the primer pair AMV4.5NF and AMV4.5NR for 40 cycles, with 5 µl diluted (1:50) amplification product from the first reaction as a template.

Amplified products were separated by electrophoresis on 2% NuSieve GTG agarose (BMA) gels and visualized with a UV transilluminator after staining with ethidium bromide. The products were purified from agarose gels with a QIAquick gel extraction kit (Qiagen, Hilden, Germany). Purified products were inserted into the pT7BlueT Vector (Novagen, Madison, Wis.) using Ligation-kit No. 2 (TaKaRa, Kyoto, Japan) and transformed into *Escherichia coli* bacteria.

First PCR-RFLP

As a first characterization of the cloned sequences, PCR products (AMV4.5NF-AMV4.5NR) of about five clones per root subsample were digested with a series of restriction endonucleases: *Hin*fI (Promega), *Cfr*13I, *Mbo*I (TaKaRa), *Mva*I, *Taq*I, and *Vsp*I (Fermentas). Digestions were performed using 5 µl PCR product in a total volume of 7.5 µl, following the manufacturers' protocols. Electrophoresis was performed using 2% NuSieve 3:1 agarose (BMA) gels and TBE buffer. Clones were characterized by different RFLP types.

Sequencing and phylogenetic analysis

Up to three clones for each RFLP type were sequenced with a DYEnamic direct cycle sequencing kit (Amersham), with a Li-Cor 4000 automated DNA sequencer (Li-Cor) and with the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems), using an ABI PRISM 310 genetic analyzer (Applied Biosystems). Procedures were performed according to the manufacturers' instructions. All sequences were submitted to DDBJ (accession nos. AB076266–AB076346).

The DNA sequences were aligned with published data using Clustal X (Thompson et al. 1997) with some manual adjustment. Phylogenetic reconstruction was performed using PAUP* 4.0b8 (Swofford 2001). Analyses were performed using neighbor-joining algorithms (Saitou and Nei 1987) with the Kimura two-parameter model (Kimura 1980). Bootstrap resampling was performed using 1,000 replicates.

Second PCR-RFLP

Sequences determined in this study were classified into several groups and subgroups based on phylogenetic topology and pairwise distances. A combination of *Hsp*92II (Promega), *Hpy*CH4IV (New England Biolabs), and *Mbo*I (TaKaRa) was suitable for distinguishing sequences into phylogenetic subgroups (for details, see Electronic Supplementary Material). An additional restriction endonuclease, either *Hin*fI or *Taq*I (Fermentas), was required for some cloned sequences to be separated into phylogenetic subgroups. The PCR products (AMV4.5NF-AMV4.5NR) of about 20 clones per root subsample were digested. Digestion procedures followed that of the first PCR-RFLP described above. Electrophoresis was performed using 4% NuSieve 3:1 agarose (BMA) gels and TBE buffer. When a clone showed an unexpected RFLP pattern, the sequence of the clone was determined and classified into a subgroup. After cloned sequence characterization, we calculated the relative occurrence frequency of sequences representing RFLP types of each phylogenetic group in a root subsample.

Mycorrhizal colonization

Fresh lateral roots were cleared with 10% KOH and stained with Trypan Blue [a phenol-free modification of the method of Phillips and Hayman (1970)]. The percentage of root length colonized (%RLC) by all AM fungal materials was determined using a magnified intersection method at 150× (McGonigle et al. 1990). The %RLC by AM fungi belonging to a phylogenetic group was estimated by multiplying the %RLC of all AM fungal materials by the relative occurrence frequency of the phylogenetic group. The diversity of the AM fungal phylogenetic subgroups in the plant roots was calculated using the Shannon-Weiner diversity index (Pielou 1974), $H' = -\sum Pi \ln P_i$, where P_i is the relative occurrence frequency of a phylogenetic subgroup i.

Non-structural carbohydrate

Dried plant materials were ground; soluble sugar was extracted from 100 mg ground material with shaking in 80% ethanol at 40°C for 17.5 h (Akiyama 1999). The extracted solution was evaporated at 60°C, and then 1 ml distilled water was added along with 1 ml 5% $ZnSO_4$ and 1 ml 4.8% $Ba(OH)_2$. After centrifugation, the supernatant was analyzed for reducing sugar using arsenomolybdate (Somogyi 1952) and for total sugar using anthrone sulfuric acid (Trevelyan and Harrison 1952). Non-reducing sugar concentration was calculated by subtracting reducing sugar concentration from total sugar concentration. Starch was dissolved by heating the residual materials from ethanol extraction at 100°C for 10 min. Dissolved starch was extracted by incubating at 20°C for 15 min with 4.6 *N* perchloric acid three times (Pucher et al. 1948). After centrifugation, the supernatant was hydrolyzed at 100°C for 120 min and analyzed using anthrone sulfuric acid as a standard of glucose. Starch concentration was calculated by multiplying the hydrolyzed starch concentration by 0.9 to take hydrolysis into account. Total NSC concentration was calculated as the sum of reducing sugar, non-reducing sugar, and starch concentrations.

Statistical analysis

Measurements from three cores were averaged. The %RLC by all AM fungal materials was arcsine-transformed so as not to violate normality and homoscedasticity assumptions. Repeated-measures analysis of variance (repeated-measures ANOVA) of the split-plot design were conducted separately for each plant for %RLC and NSC content (reducing sugar, non-reducing sugar, and starch) to determine effects of defoliation treatment sampling date, and their interaction, using the GLM procedure of SAS (SAS Institute 1989). Data for July, when defoliation treatment was not carried out, were not included in the ANOVA. Significance was tested using *F*

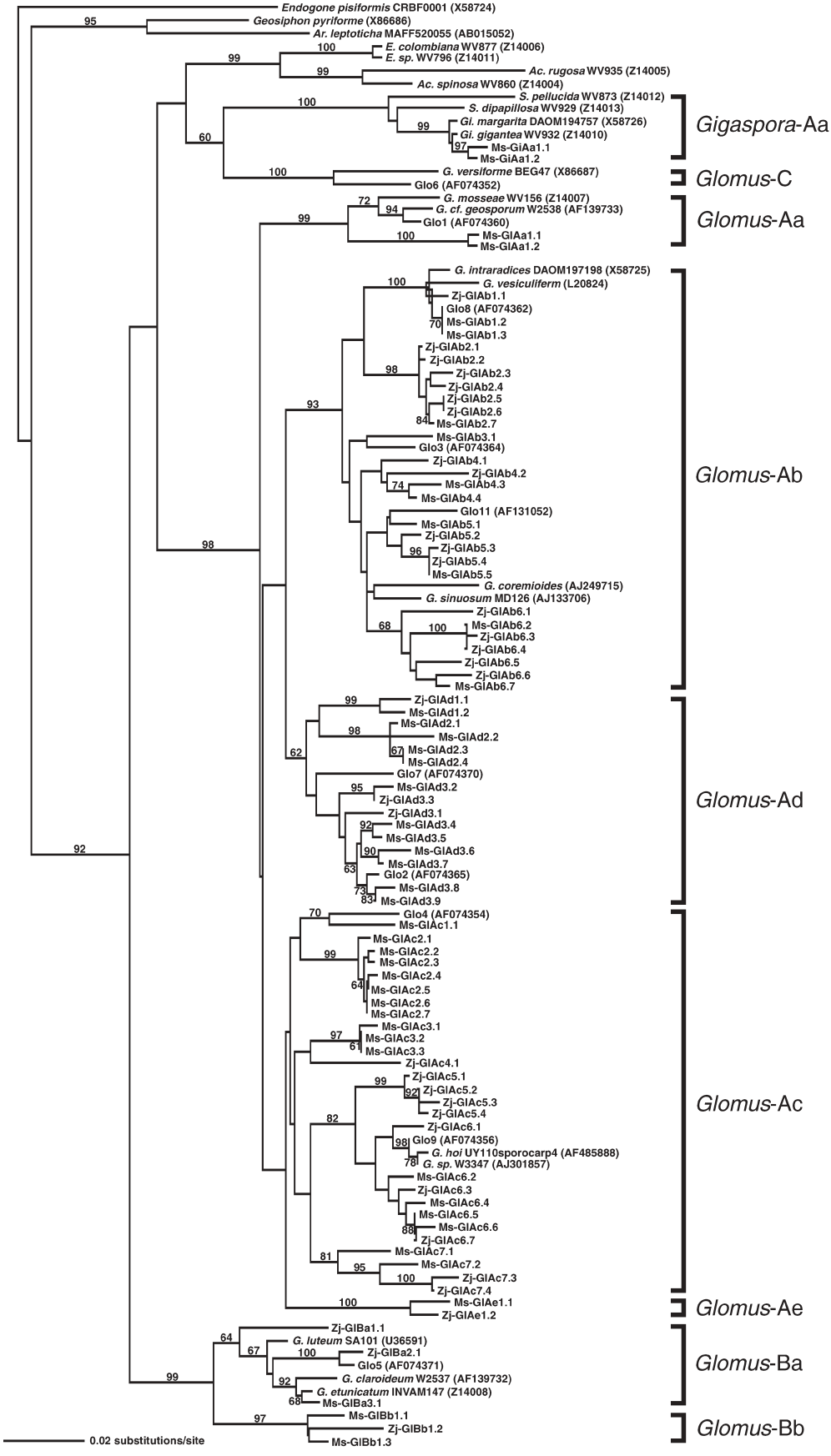

Fig. 2 Neighbor-joining tree based on partial 18S rDNA sequences obtained from *Miscanthus sinensis* and *Zoysia japonica* roots using the primer pair AMV4.5NF-AMV4.5NR, aligned with 31 other fungal sequences from the database (accession numbers in parentheses); 447 positions certain to be in alignment were used for tree construction. The tree is rooted with *Endogone pisiformis*. Bootstrap values (from 1,000 replicates) greater than 60% are shown above or below the branches. Phylogenetic groups and subgroups are identified based on topology and pairwise distance (within group, <0.06; within subgroup, <0.03). Individual sequences determined in this study are identified by host plant (*Ms Miscanthus*, *Zj Zoysia*), group (*GiAa Gigaspora*-Aa, *GlAa–GlAe Glomus*-Aa–*Glomus*-Ae, *GlBa–GlBb Glomus*-Ba–*Glomus*-Bb), subgroup number, and sequence identifier. Phylogenetic group assignments are given on the right side of the tree

adjusted with the Greenhouse-Geisser (G-G) method (von Ende 1993). To test the effect of defoliation treatment on diversity and estimated %RLC by a phylogenetic group, the Mann-Whitney test was carried out within each sampling date. Spearman rank correlation coefficients were calculated to determine the relationship between estimated %RLC by a phylogenetic group and the NSC content. In this correlation analysis, all data from July to October were used separately for each host plant.

Results

Phylogenetic analysis of AM fungi in roots

Nested PCR amplification products (about 650 bp) were successfully obtained from fungal DNA from both *Miscanthus* and *Zoysia* roots. After cloning of amplification products, about five clones per root subsample were analyzed by PCR-RFLP (*Cfr*13I, *Hin*fI, *Mbo*I, *Mva*I, *Taq*I and *Vsp*I) as a first characterization of the cloned fragments. From 247 cloned fragments, 57 RFLP types were detected. Up to three cloned fragments of each RFLP type were sequenced and aligned with published sequences obtained from the database. From phylogenetic analysis, 64 out of 67 sequences determined were clustered into the Glomeromycota clade, indicating that the primer pairs used were highly specific to the 18S rDNA of Glomeromycota. One of the sequences not clustered into the Glomeromycota clade showed high similarity (99%) to some plant sequences, and two other sequences showed the greatest similarity to some Basidiomycota sequences (99% and 98% similarity), based on the BLAST sequence similarity search. Figure 2 shows the neighbor-joining tree of the AM fungal sequences, which included several sequences determined after the second PCR-RFLP typing (see below). In this phylogenetic tree, most of the sequences belonged to the *Glomus* clade. Only two sequences were in the family Gigasporaceae, and no sequences were detected in the family Acaulosporaceae. In the *Glomus* clade, several phylogenetic groups and subgroups were recognized (Fig. 2). In this study, definitions of groups were based on tree topology and pairwise distance: pairwise distances within a phylogenetic group and within a subgroup were <0.06 and <0.03, respectively.

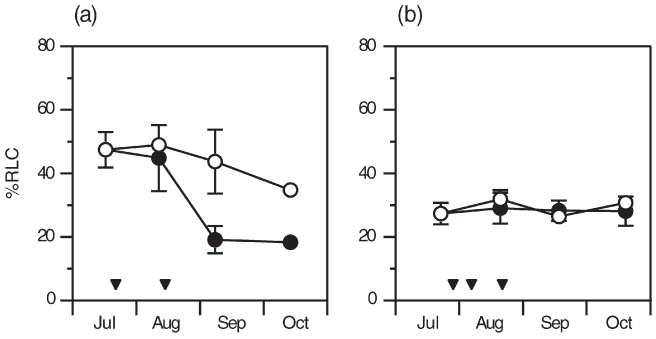

Fig. 3 Colonization of **a** *M. sinensis* and **b** *Z. japonica* roots, measured as percentage root length colonized (%RLC) by all arbuscular mycorrhizal (AM) fungal materials. Values are means ±1SE (n=3). *Open circles* Control, *closed circles* defoliation treatment, *inverted triangles* dates of defoliation

Dynamics of phylogenetic group composition

To investigate phylogenetic group composition, previously cloned fragments were analyzed by a second PCR-RFLP with combinations of three or four restriction endonucleases that distinguished the sequences in each phylogenetic subgroup; 449 and 443 sequences derived from *Miscanthus* and *Zoysia* roots were analyzed, respectively. From *Miscanthus* roots, sequences representing RFLP types of the *Glomus*-Ab, *Glomus*-Ac, and *Glomus*-Ad groups (see Fig. 2) were frequently detected, and their relative occurrence frequencies were nearly equal (Table 1). Relative occurrence frequencies of other groups in *Miscanthus* roots were less than 5%. The phylogenetic subgroups that showed high relative occurrence frequency were *Glomus*-Ab1, -Ab6, -Ac1, -Ad2, and -Ad3 (12–16%). From *Zoysia* roots, sequences of the *Glomus*-Ab group were dominantly detected, and sequences of the *Glomus*-Ac group were sometimes detected (Table 1). Sequences of other groups were barely detected in *Zoysia* roots. The phylogenetic subgroups showing high relative occurrence frequency were *Glomus*-Ab2 (43%) and -Ab6 (30%).

The effects of defoliation on AM were examined by comparing the %RLCs by all AM fungal materials and estimated %RLCs by phylogenetic groups in the control and defoliation-treated plots. The %RLC by all AM fungal materials from *Miscanthus* was significantly reduced with defoliation treatment (treatment: $F_{1,4}$= 11.66, P<0.05; Fig. 3a). On the other hand, the %RLC in *Zoysia* was not influenced by defoliation treatment

Table 1 Phylogenetic group composition (%) of arbuscular mycorrhizal (AM) fungi in *Miscanthus sinensis* and *Zoysia japonica* roots, measured as relative occurrence frequency of sequences representing RFLP types of each phylogenetic group. Values are means ±SD of all sampling data

	Gigaspora-A	*Glomus*-A					*Glomus*-B	
	a	a	b	c	d	e	a	b
Miscanthus	1.1±3.0	0.7±3.4	32.4±31.9	27.0±23.0	30.5±29.0	5.0±6.8	1.6±3.8	1.8±5.4
Zoysia	1.5±7.2	1.0±4.0	78.6±25.8	14.3±16.7	3.5±7.1	0.2±1.2	1.0±3.7	0.0±0.0

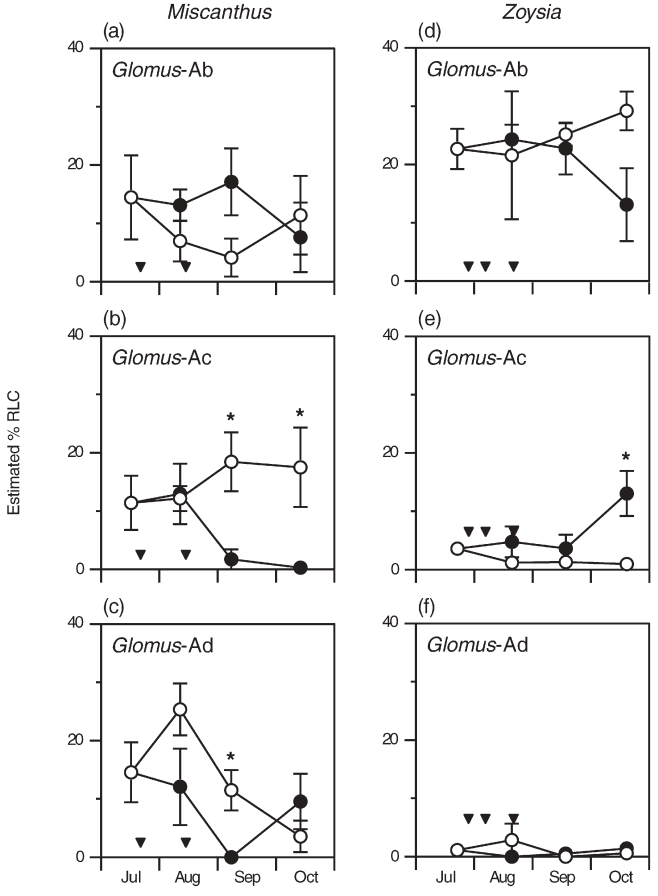

Fig. 4 Estimated %RLC by AM fungi belonging to *Glomus*-Ab (**a**, **d**), *Glomus*-Ac (**b**, **e**), and *Glomus*-Ad (**c**, **f**) in *M. sinensis* (**a**–**c**) and *Z. japonica* (**d**–**f**). Estimated %RLC was calculated by multiplying the %RLC of all AM fungal materials by the relative occurrence frequency of sequences representing RFLP types of a phylogenetic group. Values are means ±1SE (n=3). *Open circles* Control, *closed circles* defoliation treatment, *asterisks* significant difference between treatments within a sampling date at P<0.05 (Mann-Whitney test), *inverted triangles* defoliation treatment dates

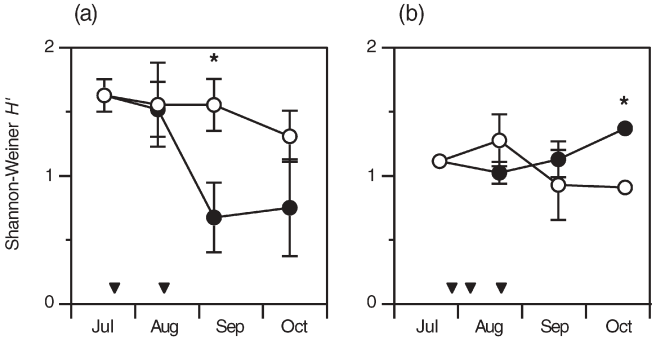

Fig. 5 Diversity of phylogenetic subgroups in **a** *M. sinensis* and **b** *Z. japonica* roots, measured as Shannon-Weiner diversity index (H'). Diversity indices are based on relative occurrence frequency of sequences representing RFLP types of each subgroup. Values are means ±1SE (n=3). *Open circles* control, *closed circles* defoliation treatment, *asterisk* significant difference between treatments within a sampling date at P<0.05 (Mann-Whitney test), *inverted triangles* defoliation treatment dates

(treatment: $F_{1,4}$=0.17, P=0.70; Fig. 3b). Next, estimated %RLCs by the major phylogenetic groups, *Glomus*-Ab, -Ac, and -Ad, were analyzed (Fig. 4). The estimated %RLC by the *Glomus*-Ac group of *Miscanthus* was significantly lower in defoliation-treated plots than it was in control plots in September and October (Fig. 4b). Also, the estimated %RLC by the *Glomus*-Ad group was significantly lower in defoliation-treated plots relative to controls in September (Fig. 4c). Conversely, the estimated %RLC by the *Glomus*-Ab group of *Miscanthus* was slightly greater in defoliation-treated plots than in control plots, but there were no significant differences between treatments (Fig. 4a). The estimated %RLCs by the *Glomus*-Ac and -Ad groups of *Zoysia* were very low, but the value of *Glomus*-Ac in defoliation-treated plots was higher than that in control plots in October (Fig. 4e, f). On the other hand, the estimated %RLC by the *Glomus*-Ab group of *Zoysia* was high, and values were similar between control and defoliation-treated plots for the experimental period (Fig. 4d).

The diversity of phylogenetic subgroups in AM fungi in the control and defoliation-treated plots was compared (Fig. 5). Subgroup diversity in *Miscanthus* roots decreased with defoliation treatment, and the diversity index in September was significantly lower than that of the control (Fig. 5a). On the other hand, subgroup diversity in *Zoysia* roots was found to be similar in the control and defoliation-treated plots during the experimental period, with the exception of October, when the diversity index was significantly higher in defoliation-treated plots than in control plots (Fig. 5b), due to increasing occurrence frequency of *Glomus*-Ac (Ac1, Ac4, Ac5, and Ac6).

Relationship between estimated %RLC and NSC in plants

The contents of reducing sugar, non-reducing sugar, and starch in *Miscanthus* plants were 14.6, 23.3, and 13.4 mg g^{-1} dry matter (28.5, 45.4, and 26.1% of the total NSC) during the experimental period, respectively. *Zoysia* had small amounts of reducing sugar (6.1 mg g^{-1}, 8.3%) and large amounts of starch (32.5 mg g^{-1}, 44.2%). The non-reducing sugar content in *Zoysia* plants was 35.0 mg g^{-1} (47.6%). These data indicate that *Zoysia* had larger amounts, or higher ratios, of storage carbohydrates than did *Miscanthus*. Conversely, *Miscanthus* had relatively high levels of reducing sugar.

Defoliation effects on the NSC content of host plants were investigated for above-ground and below-ground parts. Non-reducing sugar, starch, and total NSC content in each *M. sinensis* plant part were significantly lower in defoliation-treated plots than they were in control plots (P<0.05; Fig. 6a–c). Differences in reducing sugar in *M. sinensis* between treatments in the whole plant (treatment: $F_{1,4}$=5.19, P=0.085) and in the above-ground parts (treatment: $F_{1,4}$=4.06, P=0.114) were marginally signifi-

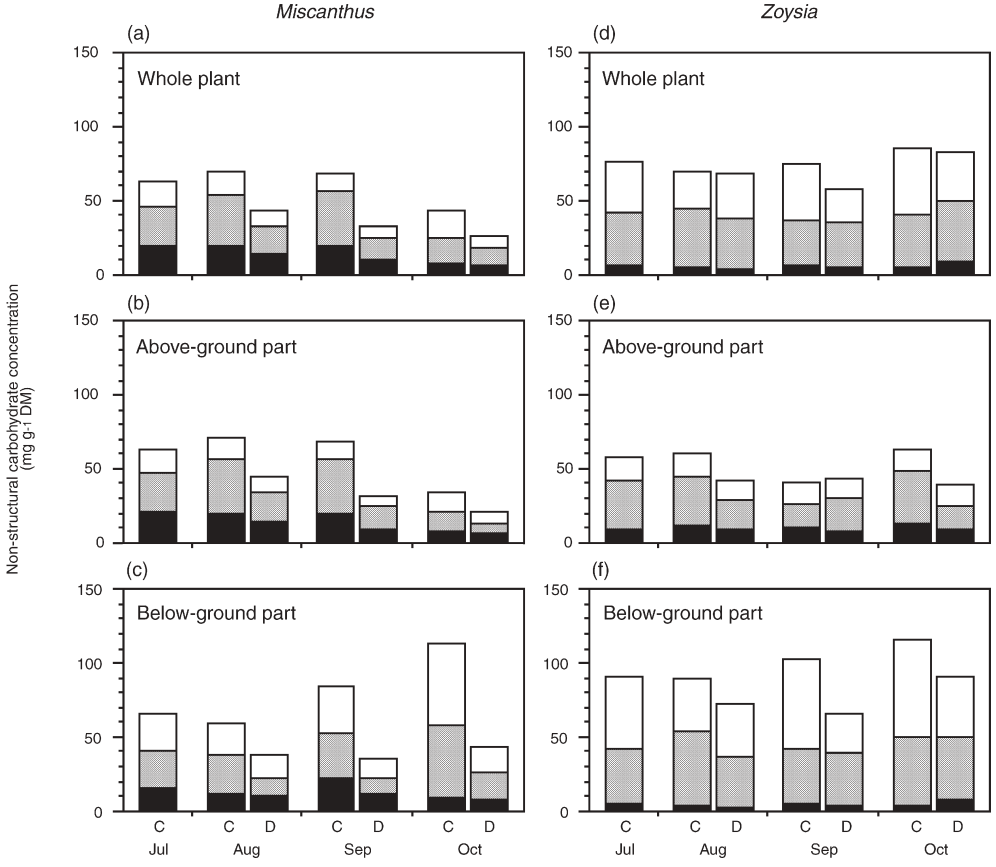

Fig. 6 Contents of non-structural carbohydrates (NSC) in whole plants (**a**, **d**), and above-ground (**b**, **e**) and below-ground (**c**, **f**) parts of *M. sinensis* (**a**–**c**) and *Z. japonica* (**d**–**f**). Total NSC comprises reducing sugar (*black column*), non-reducing sugar (*gray column*), and starch (*white column*). *C* Control, *D* defoliation treatment

Table 2 Relationships between estimated percentage root length colonized (%RLC) by AM fungi belonging to *Glomus*-Ab, *Glomus*-Ac, and *Glomus*-Ad and contents of non-structural carbohydrates (NSC) in *M. sinensis* and *Z. japonica* roots. Total NSC comprises reducing sugar (*R*), non-reducing sugar (*N*), and starch (*S*). Values are Spearman rank correlation coefficients

Miscanthus	Whole plant (*n*=24)				Above-ground (*n*=23)				Below-ground (*n*=24)			
	R	N	S	Total NSC	R	N	S	Total NSC	R	N	S	Total NSC
Glomus-Ab	–0.103	–0.211	0.046	–0.138	–0.115	–0.141	0.021	–0.199	–0.233	–0.213	–0.221	–0.225
Glomus-Ac	0.429**	0.639***	0.415**	0.577***	0.348	0.521**	0.229	0.493**	0.225	0.353*	0.405**	0.473**
Glomus-Ad	0.253	0.334	0.218	0.359*	0.293	0.348	0.488**	0.424**	0.307	0.135	0.055	0.090
Zoysia	Whole plant (*n*=22)				Above-ground (*n*=24)				Below-ground (*n*=22)			
	R	N	S	Total NSC	R	N	S	Total NSC	R	N	S	Total NSC
Glomus-Ab	–0.121	–0.123	–0.133	–0.090	–0.250	–0.387*	–0.050	–0.456**	0.258	0.059	–0.093	0.059
Glomus-Ac	0.407*	0.138	–0.196	0.009	0.360*	0.366*	0.381*	0.479**	0.325	–0.037	–0.200	–0.124
Glomus-Ad	–0.125	–0.021	0.083	0.029	0.048	0.013	–0.075	–0.045	–0.367*	0.035	0.008	0.061

*P<0.1, **P<0.05, ***P<0.01

cant. In contrast to *Miscanthus*, no NSCs of *Zoysia* were influenced by defoliation treatment (Fig. 6d–f). Only in total NSC level, in below-ground parts, was there significant difference between treatments (treatment: $F_{1,2}$=25.57, P<0.05); this was due mainly to the starch level in below-ground parts, which was marginally lower in defoliation-treated plots than in controls (treatment: $F_{1,2}$=13.47, P=0.07).

Relationships between estimated %RLCs of fungal phylogenetic groups and NSC concentration levels in plants were analyzed (Table 2). The %RLC by the *Glomus*-Ac group of *Miscanthus* positively correlated with NSC levels for most items. Also, the %RLC by the *Glomus*-Ad group showed a significant positive correlation with starch and total NSC levels in above-ground parts of *Miscanthus*. On the other hand, no significant correlation was shown between the *Glomus*-Ab group and NSC levels. For *Zoysia*, obvious correlations were few because *Zoysia* had little variation in NSCs and %RLC. However, as with *Miscanthus*, the %RLC by the *Glomus*-

Ac group of *Zoysia* was positively correlated with NSC levels in above-ground parts, and the %RLC by the *Glomus*-Ab group was either unrelated or negatively correlated with NSC levels.

Discussion

Loss of plant photosynthetic parts by mowing or grazing affects mycorrhizal associations. However, reported defoliation effects on mycorrhizal colonization have been inconsistent (Gehring and Whitham 1994). Allsopp (1998) suggested that mycorrhizal associations of grasses with different grazing tolerances responded differently to defoliation. Our results also indicate that defoliation effects on mycorrhizal colonization differ between the grazing-intolerant *Miscanthus* and the grazing-tolerant *Zoysia*. Furthermore, responses to defoliation of the community structure of AM fungi colonizing roots also differed between *Miscanthus* and *Zoysia*. The %RLC by all AM fungi of the grazing-intolerant *Miscanthus* was significantly decreased by defoliation treatment. This decrease was due largely to the sensitive response of the *Glomus*-Ac and -Ad groups to defoliation, resulting in decreased diversity of the AM fungal subgroup in *Miscanthus* roots. On the other hand, the %RLC of the grazing-tolerant *Zoysia* plant did not differ between treatments despite severe defoliation. Also, AM fungal group composition in *Zoysia* roots was unaffected by defoliation, except in October. *Zoysia* largely allocates dry matter to below-ground parts, which consequently contain high levels of starch. After severe defoliation, *Zoysia* utilizes stored carbohydrates for leaf regrowth and quickly begins to photosynthesize. This ability to regrow rapidly and retain NSC levels could result in maintenance of %RLC and AM fungal group composition.

Miscanthus showed little regrowth after defoliation. In such conditions, where newly photosynthesized carbohydrate was lacking, NSC levels in *Miscanthus* decreased, indicating that reserve carbohydrates such as starch and non-reducing sugar were used for maintenance respiration and for supporting reducing sugar levels. This reduction in NSC levels in defoliated *Miscanthus* decreases the amount of NSC for mycorrhizal roots. In mycorrhizal roots, it has been suggested that mycorrhizal fungi take up and use glucose, which is a product of sucrose hydrolyzed in the apoplast by invertase (Hampp et al. 1995; Shachar-Hill et al. 1995; Solaiman and Saito 1997). Although we could not measure carbohydrate influx into AM fungi and instead measured the concentration of carbohydrates in the plants, a reduction of influx in AM fungi was expected to occur due to evidence of a decreasing total NSC pool level in the plants. This might have led to the decrease in fungal colonization in the *Glomus*-Ac and the *Glomus*-Ad groups, probably because the fungi failed to obtain enough NSC to survive, and it was hypothesized that the *Glomus*-Ac and -Ad groups consisted of fungi requiring large amounts of NSC from the host plant. On the other hand, the *Glomus*-Ab group in *Miscanthus* and *Zoysia* roots was independent of NSC levels, and occasionally negatively correlated with NSC levels, and it was hypothesized that the *Glomus*-Ab group might contain fungi that were unaffected by the amount of NSC available from the host plant. Similarly, Saikkonen et al. (1999) also suggested the presence of ectomycorrhizal fungi exhibiting high or low costs for the host plant. Our hypothesis may explain differences in AM fungal composition in *Miscanthus* and *Zoysia*. That is, *Miscanthus* has a larger pool size of reducing sugar than does *Zoysia*, and this might lead to coexisting fungi (*Glomus*-Ab, -Ac, and -Ad) that exhibit different energy requirements. On the other hand, *Zoysia* has a limited reducing sugar pool and is probably colonized by fungi (*Glomus*-Ab) that are unaffected by carbohydrate levels. To ascertain whether the host plant energy requirement differs among AM fungal groups, physiological studies using mycorrhizal plants inoculated with these fungal groups are needed.

Glomus is a diverse genus (Simon et al. 1993; Simon 1996; Schüßler et al. 2001). Schüßler et al. (2001) suggested that at least three distinct *Glomus* clades (GlGrA, GlGrB, and GlGrC) exist. Furthermore, GlGrA is composed of three subclades: GlGrAa, GlGrAb, and GlGrAc (Schwarzott et al. 2001). Our sequence data also showed distinct *Glomus* clades, *Glomus*-A, -B and -C, which correspond to GlGrA, GlGrB, and GlGrC, respectively. *Glomus*-A was composed of at least five groups: *Glomus*-Aa, -Ab, -Ac, -Ad, and -Ae. *Glomus*-Aa, -Ab, and -Ac included some known species from voucher specimens and corresponded almost to GlGrAa, GlGrAb, and GlGrAc, respectively. These groups could consist of multiple fungal species. The *Glomus*-Aa group is closely related to *G. mosseae*, *G. geosporum*, or to the Glo1 group. Daniell et al. (2001) showed that the Glo1 group dominantly colonize arable crop roots. The authors suggested that this fungal group could be dominant even under conditions of severe physical soil disturbance such as plowing. In seminatural woodlands, fungi corresponding to the *Glomus*-Ab, -Ac, and -Ad groups were frequently found, along with Acaulosporaceae and Gigasporaceae (Helgason et al. 1998, 1999). *Glomus*-Ab, -Ac, and -Ad groups also dominantly colonized roots of *Miscanthus* or *Zoysia* in seminatural grasslands. These fungal groups might prefer nutrient-poor and less disturbed environments such as seminatural woodlands and grasslands. The *Glomus*-Ac and -Ad groups included few known species from voucher specimens, although these groups included some sequences from AM roots. Clapp et al. (1995) showed that *Acaulospora*, *Glomus*, and *Scutellospora* colonized bluebell (*Hyacinthoides non-scripta*) roots in seminatural woodlands, but spores of *Glomus* species were found infrequently in rhizosphere soil. It is possible that the *Glomus*-Ac and -Ad groups produce spores only infrequently, or that sequence data of the *Glomus*-Ac and -Ad groups have only just begun to be collected. It is necessary to survey spore compositions in the rhizosphere and to obtain DNA sequences from the spores to fully understand the phylogenetic data from mycorrhizal roots.

Several AM fungi-specific primers have been designed to obtain AM fungal 18S rRNA genes. One such primer is VANS1, which was designed based on three AM fungal sequences (Simon et al. 1992). The primer has been used for evolutionary studies of AM fungi (Simon et al. 1993; Simon 1996) and in several field studies (Clapp et al. 1995, 1999; Chelius and Triplett 1999; Saito et al. 2001). However, when accumulating information on AM fungal 18S rRNA genes, it should be recognized that the VANS1 site is not conserved within AM fungi (Sawaki et al. 1998; Clapp et al. 1999; Redecker et al. 2000; Schüßler et al. 2001). The general fungal primer AM1 was designed to amplify fungal DNA and exclude plant DNA sequences (Helgason et al. 1998). This primer, in conjunction with the universal eukaryotic primer NS31 (Simon et al. 1992), has been shown to amplify AM fungal DNA sequences from field-collected roots (Helgason et al. 1998, 1999; Daniell et al. 2001), although it has became apparent that the AM1 primer excludes a certain number of fungal types from Archaeosporaceae and Paraglomaceae (Redecker et al. 2000). In the present study, the AMV4.5NF-AMV4.5NR primer pair was designed as an AM-specific primer pair for nested PCR. However, the former and latter primers have one and two positions that differ in Archaeosporaceae and Paraglomaceae, respectively. Therefore, although almost all sequences amplified with this primer pair were clustered into the Glomeromycota clade, information may be limited concerning the AM fungal group excluding Archaeosporaceae and Paraglomaceae. Recently, some specific primers or primer sites for Archaeosporaceae and Paraglomaceae have been designed (Redecker 2000; Schüßler et al. 2001). These primers will be useful for field research.

Sequences obtained from mycorrhizal roots were classified by twice-performed PCR-RFLP analysis after cloning nested PCR products. The objective of the first PCR-RFLP analysis was to detect as many sequence types as possible using six restriction endonucleases. The second PCR-RFLP, after sequencing analysis, classified many sequences into several phylogenetic groups using three or four restriction endonucleases. This method enabled detailed and semiquantitative analysis of the AM fungal community in the roots. However, it is necessary to pay attention to the quantitative reliability of the method. This method depends on the assumption that the frequency of occurrence of sequences representing RFLP types in a phylogenetic group reflects the amount of corresponding fungal DNA in the root. It is well known that the quantitative ratio between sequence types changes as the number of cycles in PCR increases, even though the ordinal relationship between them does not. For estimating AM fungal composition in roots more quantitatively, further modifications of this method are required. One likely method is utilization of specific primers designed to detect major fungal group sequences that are characterized by the first PCR-RFLP and for sequencing analysis. It would be possible to quantify the fungal composition using specific primers for many root fragments or to carry out in-situ PCR for mycorrhizal roots.

The present study showed *Glomus* to be a principal group colonizing *Miscanthus* and *Zoysia* roots. The effects of defoliation on the community structure of AM fungi differed between these two plant species. Differential requirements of AM fungal groups for carbohydrate from host plants and/or differential plant grazing tolerance are thought to contribute to the different responses. *Miscanthus*- and *Zoysia*-type grasslands are major native grasslands in Japan and Eastern Asia. It is well known that a community of a *Miscanthus*-type grassland changes to that of a *Zoysia*-type grassland when the *Miscanthus*-type grassland is subjected to intensive mowing or cattle grazing (Numata 1969). When the *Miscanthus*-type grassland subjected to such disturbances is succeeded by the *Zoysia*-type grassland, the community structure of AM fungi will change with this succession; this implies that a close relationship exists between the AM fungal community and vegetative succession.

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