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Arbuscular mycorrhizal fungi on adjacent semi-natural grasslands with different vegetation in Japan

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Arbuscular mycorrhizal (AM) fungi on Japanese semi-natural grasslands were investigated at three adjacent sites with different vegetation. The predominant grasses at the three sites were 1) *Pleioblastus chino*, 2) *Miscanthus sinensis* and *Arundinella hirta* (*M. sinensis/A. hirta*), and 3) *Zoysia japonica*, respectively. The degree of colonization was higher in *M. sinensis/A. hirta* than in *P. chino* and *Z. japonica*. AM fungi were recovered by spore extraction and by pot cultures started from soil inoculum or from transplanting of field plants. Total spore number obtained by the spore extraction method was highest in the rhizosphere of *M. sinensis/A. hirta* and lowest in that of *P. chino*. A *Glomus* sp. resembling *G. geosporum* predominated in association with *M. sinensis/A. hirta* and *P. chino*. From *Z. japonica*, three species, *Acaulospora gerdemannii*, *Glomus leptotichum*, and a species resembling *G. clarum*, were isolated by pot culture from soil and two species, *A. longula* and *Scutellospora cerradensis*, by pot culture from transplanting of *Z. japonica*. From *M. sinensis/A. hirta*, one species, *A. longula*, was found by pot culture from soil. From *P. chino*, no AM fungus was detected by either method. Single-spore culture confirmed that *G. leptotichum* and *A. gerdemannii* are conspecific.

Key Words——AM fungus; dimorphism; semi-natural grassland; single spore culture; vegetation.

Arbuscular mycorrhiza is a widespread symbiosis that plays a significant role in phosphorus acquisition of many terrestrial plants, especially in soils with low levels of available phosphorus (Hetrick, 1984). In grassland the input of phosphorus fertilizer is much less than that in arable lands. Tillage, common in arable lands, reduces the function of arbuscular mycorrhizal (AM) symbiosis through the breakdown of their hyphal network in the soil (Jasper et al., 1989), whereas grassland soils are normally not tilled, and such disruption does not normally occur. The function of the AM symbiosis in phosphorus cycling may consequently be much more significant in grassland ecosystems than in more intensively managed agricultural systems. Furthermore, AM fungi may contribute to the plant community structure of grassland through their hyphal network connecting different plant species (Grime et al.,1987).

In Japan, semi-natural grasslands are distributed in hilly areas. They can be classified into several types based upon the dominant grass species, such as *Miscanthus sinensis* Anderss., *Zoysia japonica* Steud., and *Pleioblastus chino* Makino (Numata, 1961). These semi-natural grasslands developed from deciduous broad-leaved forest by traditional grazing and burning farming methods. Their soils are derived mainly from volcanic ash and are characterized by high phosphate-fixing capacity (Inoue, 1986). Such semi-natural grassland ecosystems have recently been documented from the viewpoint of their sustainability and biological diversity (Numata, 1993), though not for mycorrhizal fungi. Although AM symbioses in various grassland ecosystems have been extensively studied worldwide (e.g., Walker et al., 1982; Miller, 1987; Bentivenga and Hetrick, 1992; Sanders and Fitter, 1992), little has been reported on indigenous AM fungi in the semi-natural grasslands in Japan. In the present paper, therefore, the indigenous AM fungi were investigated in three adjacent different grassland sites by three different methods.

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Materials and Methods

Sampling sites The experimental site was located in the Mt. Fujinitayama experimental farm of the National Grassland Research Institute, Tochigi, Japan (36°55′N, 139°58′E). Three sampling sites with different vegetation were chosen. The first site (site Pc) retained its original vegetation of *P. chino* dominating the forest floor of the deciduous broad-leaved trees, *Quercus acutissima* Carruth., *Q. serrata* Murr., and *Castanea crenata* Sieb. & Zucc. The other two sites were adjacent to the first and had developed from the original vegetation by either cattle grazing for about 40 yr or annual burning for about 20 yr. The dominant grasses in one site (site MA) were *M. sinensis* and *Arundinella hirta* C. Tanaka, and that in the other (site Zj) was *Z. japonica*. The flora of these grasslands was reported in detail elsewhere

(Shiyomi, 1993). The soils in the experimental farm were derived from volcanic ash of Mt. Nasu and classified as Entic Haplumbrepts. The surface soils were loam with pH (H_2O) of 4.8–5.0 and available phosphate (Troug method) of 20–50 mgP/kg dried soil.

Root colonization by AM fungi Roots of the predominant grasses at three sites, (1) *P. chino*, (2) *M. sinensis* and *A. hirta* (*M. sinensis/A. hirta*), and (3) *Z. japonica*, were collected in June and September 1994. Since *M. sinensis* and *A. hirta* were present in the same density at the sampling site and have similar morphological and eco-physiological characteristics, we grouped these plant species together. The roots were dug out from three to five locations at each site, carefully washed free from soil particles and other plant roots, blotted dry and stained to reveal mycorrhizas with trypan blue (Phillips and Hayman, 1970). Root colonization of AM fungi was examined on fifty 1 cm pieces of roots under a compound microscope. Mycorrhizal colonization was estimated by colonization density and relative abundance of mycorrhizal structures, including vesicles, arbuscules, and others (Trouvelot et al., 1986).

Retrieval of AM fungi The AM fungi were recovered from the soils by three different methods: (1) spore extraction; (2) soil trap culture, namely, pot culture started from soil inoculum; (3) plant trap culture, namely, pot culture started from transplanting of field plants. Rhizosphere soils of *P. chino*, *M. sinensis/A. hirta*, and *Z. japonica* at each site were collected with their root systems.

In the first method, the samples were sieved through a 2 mm mesh, and 10 g portions were used for the spore extraction. The soils were wet-sieved by using 106 μm mesh as a final sieve (Daniels and Skipper, 1982). The spores were then concentrated by sucrose centrifugation (Nishio, 1987), collected under a dissecting microscope, and further classified into morphological types. Attempts were made to produce pot cultures from the dominant spore types with sorghum (*Sorghum bicolor* Moench) and white clover (*Trifolium repens* L.) as host plants grown in autoclaved soil and sand mixture (1 : 1 v/v) under growth cabinet conditions.

In the soil trap culture method, 100 g of the sieved soil was layered at about 5 cm depth in disinfected substrate in a plastic pot (1 L) with 0.72 g/L of KNO_3 and 0.29 g/L of calcium superphosphate. The fertilizer was added because the available phosphate in the potting medium was too low to support initial growth of the host plants. Mixed seedlings of sorghum, white clover, orchard grass (*Dactylis glomerata* L.), and alfalfa (*Medicago sativa* L.) were transplanted into the pots, which were placed in a glasshouse. Sporulation of the AM fungi and growth of host plants were evaluated after 80 d. The spores formed in the pot culture were recovered by the wet-sieving described above.

In the plant trap culture method, the three types of dominant grasses were dug up, and their roots washed thoroughly to remove adhering spores and soil particles. Each plant was transplanted to a pot as described in the soil trap culture method and grown in the glasshouse. After 5 mo, the sporulation was evaluated.

Spore morphology was studied for identification according to the methods of Walker (1983) and Morton (1988). Size, color, and shape were examined for fresh spores in water under a dissecting or a compound microscope. Broken and unbroken spores were mounted with poly-vinyl-lactoglycerol (PVLG) and examined under compound microscope equipped with Nomarski optics. The reaction of the wall components to Melzer's regent was also examined.

Single-spore isolation The fungi recovered by the soil trap culture method were further purified by single-spore isolation. Seeds of *Z. japonica*, *M. sinensis*, and *S. bicolor* were surface-sterilized for 3 min in saturated $Ca(OCl)_2$ solution and germinated on moist filter paper. Seven-d-old seedlings were transplanted singly into pots (200 ml, disposable polyvinyl chloride) containing autoclaved soil-sand mixture. When transplanting, a single spore of an AM fungus isolated from the pot culture was put on a branching point of the root and covered with the soil under a dissecting microscope. Each inoculated pot was enclosed in a transparent plastic bag (Sun Bag, Sigma) to prevent cross-contamination (Walker and Vestberg, 1994) and incubated in a growth chamber at 25°C with light of 52–78 μmol $m^{-2} s^{-1}$. Sterile water was supplied once a wk. Root colonization and spore formation were examined after 90 and 180 d.

Results

Colonization by AM fungi in the grass roots The degree of colonization was higher in the roots of *M. sinensis/A. hirta* at site MA than in the roots of the other plants in both June and September (Table 1). In June, arbuscules were only observed in *M. sinensis/A. hirta* roots, while they were observed in all plants in September. Coiled hyphae were commonly observed in the roots of all plants in both June and September.

Table 1. Colonization and hyphal anatomical features of arbuscular mycorrhizal fungi colonizing grasses in the semi-natural grasslands.

Plant species (Site)	*P. chino* (Pc)		*M. sinensis/A. hirta* (MA)		*Z. japonica* (Zj)	
Sampling date	June 23	September 2	June 23	September 2	June 23	September 2
Colonization (%)	16	9	44	61	21	26
Morphology[a)]	C	A, V, C	A, V, C	A, V, C	C	A, V, C

a) A; arbuscule, V; vesicle, C; coil.

Table 2. Frequency of AM fungal spores in the rhizosphere of three grasses in the semi-natural grasslands.

Morphological spore type[a]	Number of spores (per 10 g fresh soil)[b]		
	The soil collected beneath		
	P. chino at site Pc	*M. sinensis* at site MA	*Z. japonica* at site Zj
1	6.3a	21.7a	25a
2	0.7a	1a	2.3a
3	4.3a	101.7b	36.7c
4	2a	1a	2a
5	0a	0a	0.7a
6	0a	0.7a	0a
7	0a	0.3a	0a
8	0a	0.3a	0.3a
9	5.3a	0b	0.7b
10	0a	0.3a	0.7a
11	1a	1.7a	0a
12	0a	0a	0.3a
13	0a	0a	2.7a
Total No.	19.7a	128.7b	61.3c

a) Morphological type: 1, pale yellow *Scutellospora* sp., 150–250 μm; 2, apricot globose spores, 250–300 μm; 3, brown *Glomus* sp., 110–160 μm; 4, white hyaline globose spores, 150–200 μm; 5, white hyaline ellipsoid spores, 150–200 μm; 6, white hyaline *Scutellospora* sp., 500 μm; 7, dark red *Sclerocystis* sp., 100–150 μm; 8, large white globose spores, 500 μm; 9, white hyaline ellipsoid *Scutellospora* sp., 250–300 μm; 10, large brown *Scutellospora* sp., 500 μm; 11, deep yellow globose spores, 100 μm; 12, brown clavate spore, 100 μm; 13, red globose spores, 50 μm. (Spore size are approximate.)

b) Mean of 3 replicates. Means followed by the same letter do not differ significantly (l.s.d., $P<0.05$).

Spores of AM fungi in the rhizosphere of the grasses

The spores were classified into thirteen different morphological types and the numbers of each spore type in the experimental plots were recorded (Table 2). Total spore numbers were the highest in *M. sinensis/A. hirta* at site MA and the lowest in *P. chino* at site Pc. The composition of morphological spore types in *M. sinensis/A. hirta* was characterized by the predominance of spore type 3. About 80% of spores were type 3 in *M. sinensis/A. hirta*, while about 60% in *Z. japonica* at site Zj. The spore type 3 resembled *Glomus geosporum* Nicolson & Gerdemann (Fig. 1). However, no new spores were produced in the pot culture attempts from those spores, so that their identity could not be confirmed. Spore type 9, white ellipsoid *Scutellospora* sp., was found only with *P. chino*, but we could neither collect enough spores for identification nor culture it.

Table 3. Plant growth in the soil trap culture method.[a]

Trapping plant[c]	Plant dry weight (mg/pot)[b]		
	Inoculum source from		
	site Pc	site MA	site Zj
S. bicolor	400a	380a	1475b
D. glomerata	11a	23a	21a
T. repens	11a	18a	29b
M. sativa	20a	19a	45b
Spore numbers[d]	1.5a	50b	174b

a) Pot culture started from soil inoculum.

b) Mean of 4 replications. Means followed by the same letter do not differ significantly (l.s.d., $P<0.05$).

c) One plant of *S. bicolor* and *D. glomerata* and four plants of *T. repens* and *M. sativa* were grown together in a pot.

d) Numbers per 10 g fresh soil.

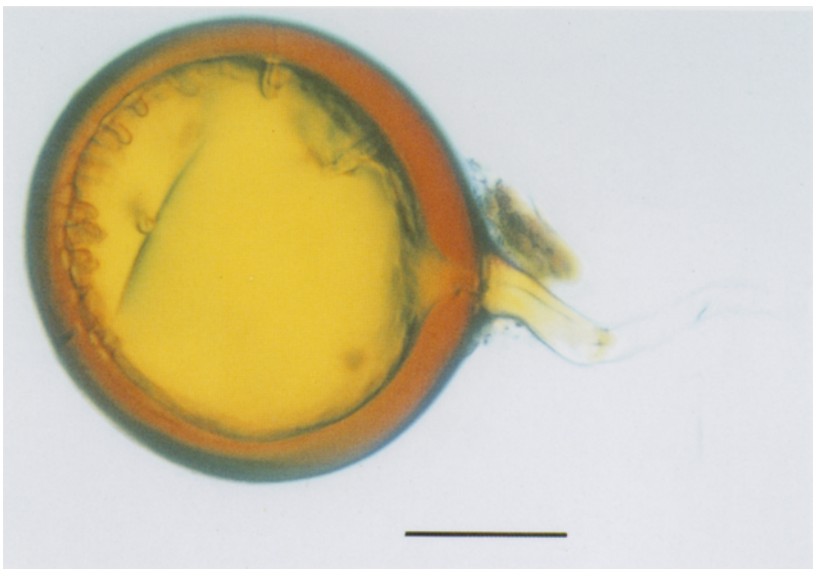

Fig. 1. Spore type No. 3 extracted from the rhizosphere soil of *Miscanthus sinensis* and *Arundinellara hirta* in PVLG. Bar = 50 μm.

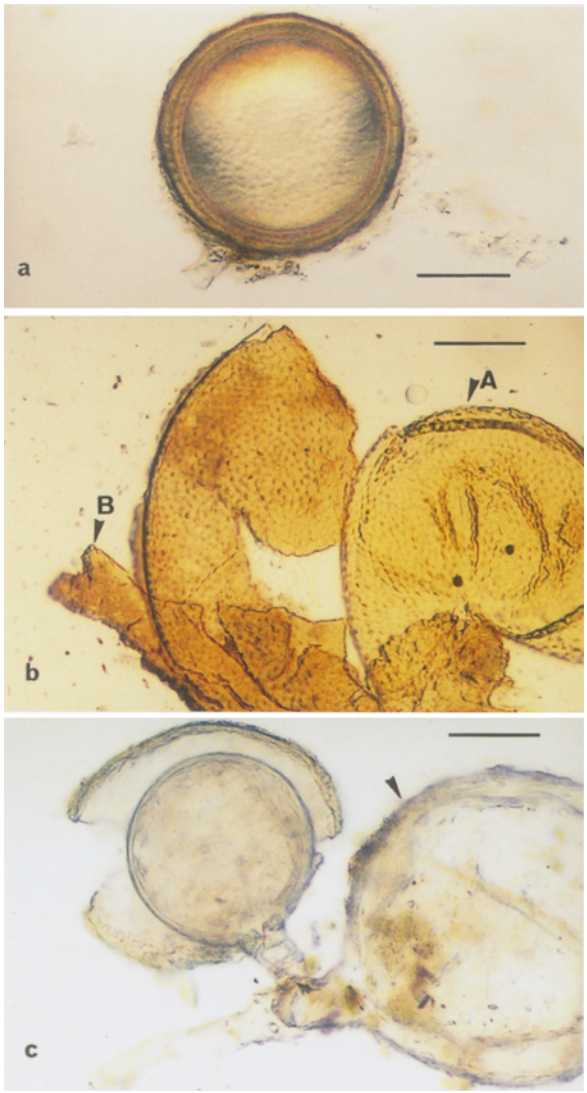

Fig. 2. Spores of *Acaulospora gerdemannii* from soil trap culture inoculated with the soil of *Zoysia japonica*.
a. Intact spore in water. Bar=80 μm. Spore diam, (102–)226(–262) μm. b. Broken spore in Melzer's reagent. The outer wall (arrow head A) was stained rust and the inner wall showed the ornamentation with concave depressions (arrow head B). Bar=80 μm. c. Spore with sporiferous saccule (arrow head) in PVLG. Bar=80 μm.

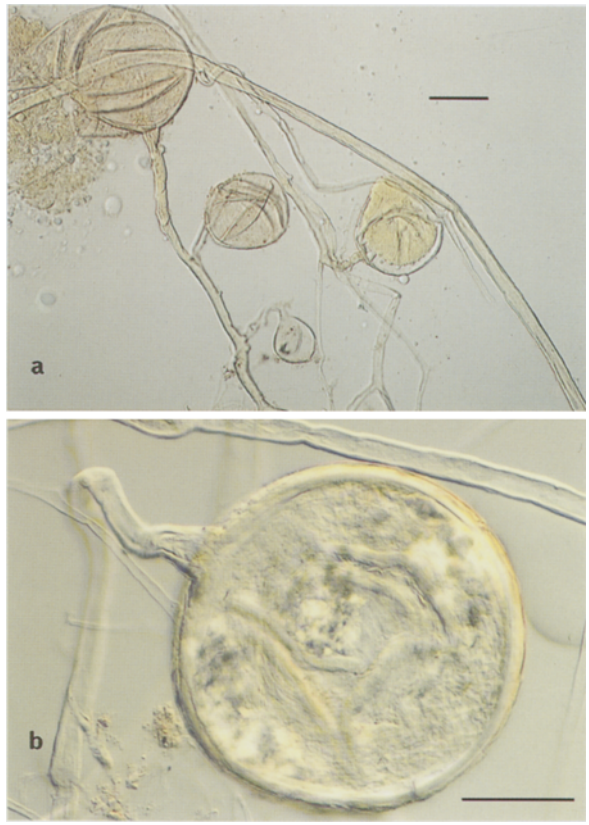

Fig. 3. Spores of *Glomus leptotichum* from soil trap culture inoculated with the soil of *Zoysia japonica*.
a, b. Spores in PVLG. Bar=50 μm. Spore size was variable, (120–)174(–283)×(120–)191(–242) μm. Composite spore wall thickness, (2–)11(–20) μm. The spore surface became sienna to rust with Melzer's reagent.

Trapping cultures of AM fungi Table 3 shows the growth of trapping host plants and the spore numbers produced in pots by the soil trap culture method. The soil inoculum from site Zj increased the growth of the trap plant and produced different spores from other sites. Spore types found by the soil trap and plant trap culture methods are summarized in Table 4. From site Zj, three species were isolated by the soil trap culture method and two species by the plant trap culture. These were

Table 4. Arbuscular mycorrhizal fungi isolated from the semi-natural grasslands by two different trap culture methods.

Sampling site (Dominant grass species)	Soil trap culture[a)]	Plant trap culture[b)]
Pc (*P. chino*)	Not trapped	Not trapped
MA (*M. sinensis/A. hirta*)	*Acaulospora longula*	Not trapped
Zj (*Z. japonica*)	*Glomus leptotichum* *Acaulospora gerdenannii* *Glomus* sp. like *G. clarum*	*A. longula* *Scutellospora cerradensis*

a) Pot culture started from soil inoculum.
b) Pot culture started from transplanting of field plants.

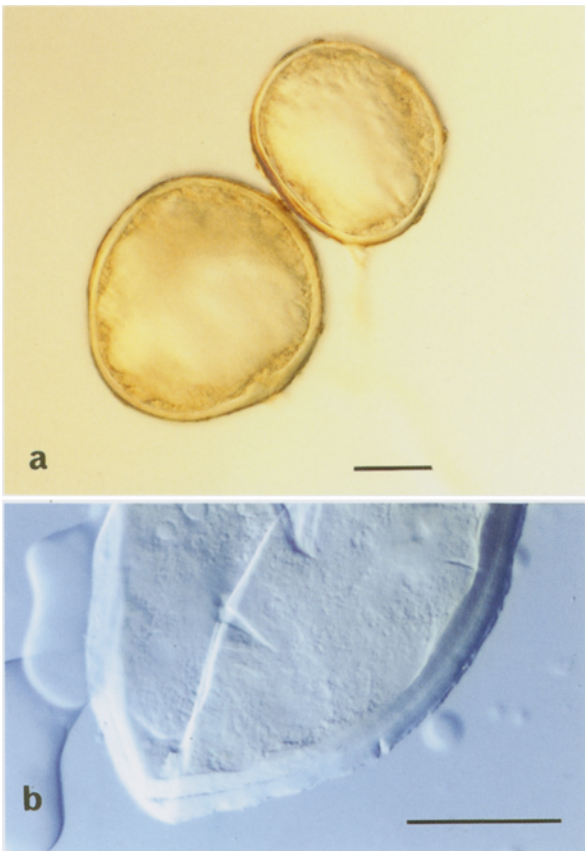

Fig. 4. Spores of *Glomus* sp. like *G. clarum* from soil trap culture inoculated with the soil of *Zoysia japonica*.
a. Spores in PVLG. Bar=50 μm. b. Broken spore showed bi-layered wall structure. Nomarski optics. Bar=50 μm. Spore diam, (119–)150(–179) μm. Spore wall was composed of two inseparable walls.

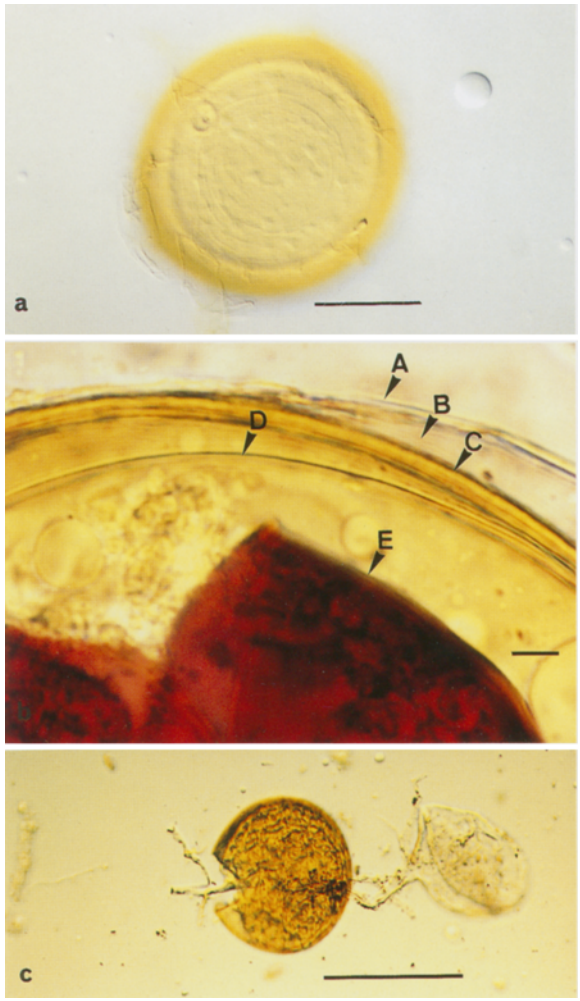

Fig. 5. Spores of *Acaulospora longula* from soil trap culture inoculated with the soil of *Miscanthus sinensis* and *Arundinellara hirta*.
a. Spore in PVLG. Bar=50 μm. Spore diam, (110–)118(–140) μm. b. Broken spore stained with Melzer's reagent. Arrow heads indicate five walls (A–E). Bar=30 μm. c. Spore with collapsed sporiferous saccule. Bar=40 μm.

Acaulospora gerdemannii Schenck & Nicolson (Morton et al., 1996) (Fig. 2), *Glomus leptotichum* Schenck & Smith (Morton et al., 1996) (Fig. 3), a species similar to *Glomus clarum* Nicolson & Schenck (Fig. 4), *A. longula* Spain & Schenck (Schenck et al., 1984) (Fig. 5), and *Scutellospora cerradensis* Spain & Miranda (Spain and Miranda, 1996) (Fig. 6). From site MA, only one species, *A. longula*, was recovered by the soil trap culture but no AM fungus was found in the plant traps. From *P. chino*, no fungus was recovered by either method.

Single-spore culture Four species, *A. gerdemannii*, *A. longula*, *G. leptotichum*, and the *Glomus* sp. isolated

Table 5. Frequency of sporulation from single spores with different potential host plants.

AM fungal species	Frequency of sporulation with different potential hosts[a)]		
	Z. japonica	*M. sinensis*	*S. bicolor*
A. gerdemannii	0/19	0/18	2/25
A. longula	0/19	0/20	0/20
G. leptotichum	2/9	0/9	1/16
G. clarum-like sp.	3/20	5/17	4/19

a) Number of pots showing sporulation/number of pots tested.

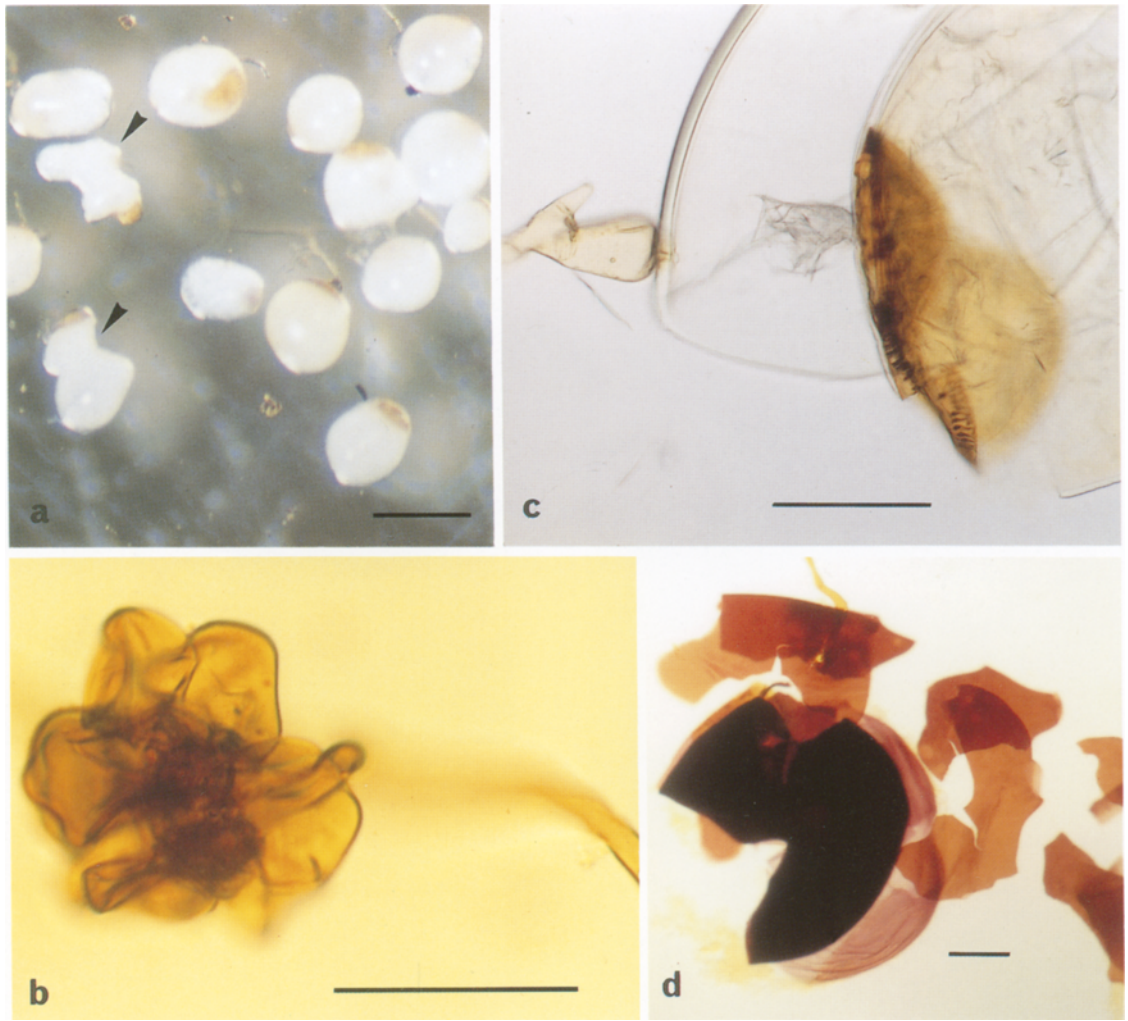

Fig. 6. Spores of *Scutellospora cerradensis* from plant trap culture of *Zoysia japonica*.
a. Intact spores in water. Arrow heads indicate irregular shaped spores. Bar=300 μm. Spore size, (178–)292(–500)× (198–)297(–395) μm. b. Knobby auxiliary cell. Bar=40 μm. c. Broken spore with yellow germination shield and subtending hypha. Bar=100 μm. d. Broken spore stained with Melzer's reagent. Bar=100 μm.

from site Zj were examined for single-spore culture with three host plants, *Z. japonica*, *M. sinensis*, and *S. bicolor* (Table 5). The *Glomus* sp. resembling *G. clarum* colonized all three host plants. *Glomus leptotichum* colonized *Z. japonica* and sorghum. *Acaulospora gerdemannii* colonized only sorghum. The cultures started from single spore of *A. gerdemannii* produced the spores identical to those of *G. leptotichum*, proving that these two 'species' were conspecific. Spores of *A. gerdemannii* were also formed in the cultures started from single spore of *G. leptotichum*. Single-spore cultures from *A. longula* were unsuccessful with any host.

The cultures obtained, except for *S. cerradensis*, have been deposited in the culture collection of the Laboratory of Soil Microbiology, the National Grassland Research Institute.

Discussion

AM fungi in semi-natural grasslands The results of this study on the AM fungi in three adjacent, but different, semi-natural grasslands show that *M. sinensis* and *A. hirta* were the most heavily colonized hosts and had higher associated spore numbers in the rhizosphere than the other two grasses. This may be a manifestation of greater mycorrhizal dependency of *M. sinensis* and *A. hirta* than the other grasses, although we did not examine mycorrhizal dependency (Hetrick et al., 1988). Lower spore numbers and poor colonization in *P. chino* may indicate its low mycorrhizal dependency, though seasonal or other temporal effects (Walker et al., 1982) have not been taken into account. The spore extraction method from field soil did not reveal any differences in the species composition among experimental sites, whereas isolation by soil and plant trap cultures showed some such differ-

ences.

The grassland of *Z. japonica* developed under grazing pressure from farm animals to which *Z. japonica* is adapted (Numata, 1961). Generally, grazing of the top part of the plant reduces photosynthesis and results in the reduction of assimilate allocation into roots, which supports the growth of AM fungi. Although grazing can decrease mycorrhizal colonization of plants (Bethlenfalvay and Dakessian, 1984; Bethlenfalvay et al., 1985), heavy grazing pressure on *Z. japonica* did not decrease the degree of colonization with AM fungi (Yamane et al., unpublished). Perhaps the AM fungi colonizing *Z. japonica* have adapted to such environmental pressure. If so, the ecology and function of AM fungi isolated from *Z. japonica* may be of value to be studied as models for the interrelationship among grazing, host plant physiology, and AM fungi.

Identification of the AM fungi The *Glomus* sp. isolated from site Zj was different from *G. clarum* (Nicolson and Schenck, 1979; Ueda et al., 1992) in the following features. The walls of the fungus did not react with Melzer's reagent at least under the present culture conditions, although the presence of the outer sloughing layer was sometimes observed. Its spores differed from those of *G. clarum*. Furthermore, *G. clarum* forms spores within the root, but this feature was not observed in the present species.

The spores identified as *A. longula* can also be identified, from the original species descriptions, as *A. mellea* Spain & Schenck, *A. morrowiae* Spain & Schenck (Schenck et al., 1984) and *A. rugosa* Morton (Morton, 1986). From their protologues, these species are remarkably similar. Their spore dimensions are slightly different, but overlapping; *A. longula*, (55–)75–95(–100) μm in spore diam; *A. mellea*, (72–)95–105(–120) μm; *A. morrowiae*, (63–)79–92(–120) μm; *A. rugosa*, (49–)92(–118) μm. The differences in spore color seem to be not distinct. There were some differences in spore wall structure. However, in view of the ontogenic process of spore wall development (Franke and Morton, 1994), it is likely that these species show little difference in wall structure and may be synonymous (Morton, unpublished; Walker, unpublished). Our *A. longula* was within the range described for these species. It is necessary to re-describe those species including the present isolate. *Scutellospora cerradensis* was previously found in soybean fields in northern Japan by Saito and Vargas (1991), who tentatively identified it as *S. gilmorei* (Trappe & Gerdemann) Walker & Sanders. It has been collected in several sites in Japan and may be one of the more common AM fungi there (Saito, unpublished). *Glomus clarum* was previously isolated in Japan (Ueda et al., 1992). The *Glomus* sp. isolated in this study resembled *G. clarum* except for its lack of reaction to Melzer's reagent and its failure to sporulate in roots. It is likely that it is a biotype rather than a different species. However, the range of species in glomalean fungi has not been well defined, partly because only a small number of conspecific isolates have been examined.

Glomus leptotichum, *A. gerdemannii* and the *Glomus* sp. were successfully isolated from single-spore in pot culture. The single-spore culture confirmed that *G. leptotichum* and *A. gerdemannii* are conspecific (Morton et al., 1996). Our results increase the known geographic distribution of this fungus. Morton and Benny (1990) placed the genera *Glomus* and *Acaulospora* in different families. It is not reasonable for synanamorphs of one species to belong to different families, and there is an urgent need for a molecular-based study is to clarify the phylogenetic position of this species.

Isolation methods of AM fungi In the present study three different methods for isolation of AM fungi were used. Extracted spores have been often used for production of AM fungal cultures, but this method does not always succeed in producing cultures of the predominant spores in soil. In fact, culturing of the dominant spore type 3 (Fig. 1) failed. Dark spores, such as those of *G. geosporum*, may not decay rapidly, and their presence as dead spores, as found in this study, does not necessarily indicate that they are present as living organisms. They may be merely relict representatives of symbioses that existed before ecosystem changes were induced by the actions of mankind. The soil trapping culture method has been widely used for isolation, and it successfully gave several isolates in the present study. Both it and the plant trap culture method can be used for isolation of fungi living within roots but not producing propagules in soil (Miller et al., 1985). Plant traps were not successful in the present study, perhaps because the growth conditions of the transplanted grasses failed to simulate the field conditions. The unnatural conditions of trapping methods may result in cultures predominated by a group of microorganisms which play only a minor role in the complete ecosystem. So the trapping cultures only reflect a part of the flora of AM fungi in the samples. Any method of isolation of AM fungi may reveal only a part of the AM fungi in soils. Combined use of different isolation methods may give much valuable information about indigenous AM fungi in soil, but there are still difficulties in culturing AM fungi. For example, coiled hyphae were observed in the roots of all three plants in both June and September. Coiled hyphae were also found in the roots of *P. chino* (Yamabe, 1986). However, few coiled hyphae were observed in the trap cultures in the present study. Fungi may change their colonization patterns with different hosts or conditions, but if their mycorrhizal anatomy is consistent, then unknown AM fungi forming coiled hyphae were not isolated by any of the trapping methods used in this investigation.

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