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Sequence Analyses of PCR Amplified Partial SSU of Ribosomal DNA for Identifying Arbuscular Mycorrhizal Fungi in Plant Roots

Moon-Sung Tae[1], Ahn-Heum Eom[2] and Sang Sun Lee[1]*

[1]*Department of Biology, Korea National University of Education, Chungbuk 363-791, Republic of Korea*
[2]*Institute of Natural Science, Korea National University of Education, Chungbuk 363-791, Republic of Korea*
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The genomic DNAs were extracted from roots of *Glycine max* and *Sorghum bicolor*, and compared with those from spores of two arbuscular mycorrhizal (AM) fungi, *Glomus mosseae* and *Scutellospora heterogama*. The partial small subunit (SSU) of ribosomal RNA genes were synthesized and amplified by polymerase chain reaction with the fungal specific primers, AM1 and NS31. By the recent molecular techniques, the presence of another AM fungal DNA were confirmed in the roots of two plants, and three sequences of rDNA fragments amplified were identified to be close to those of *G. caledonium*, *G. fasiculatum* and *G. proliferum*. The two AM fungi, both, were found to colonize at the cortical layers of plant roots collected in the fields, together.

KEYWORDS: **Arbuscular mycorrhizal fungi, *Glycine max*, PCR-RFLP, *Sorghum bicolor***

Arbuscular mycorrhizal (AM) fungi have mutualistic associations with most vascular plant species and ubiquitous terrestrial ecosystem (Smith and Read, 1996). It has been documented that AM improve inorganic nutrient uptake, in particular phosphorus, plant growth and development, and tolerance to soil pathogens (Gianianazzi and Schuepp, 1994; Smith and Read, 1996). In despite of these advantages, the studies on AM have been limited because AM fungi are obligate symbionts which can only be cultured under the presence of their host.

Several studies have shown that individual species of AM fungi differ in their ability to promote plant growth (van der Heijden *et al.*, 1998) and also differ in their growth response to plant species (Bever *et al.*, 1996; Eom *et al.*, 2000). Thus, identification of AM fungi in plant roots is important in agricultural and ecological studies. Taxonomy and identification of AM fungi have been relied on microscopic observation of spores collected from soil (Hall, 1984; Hall and Fish, 1979; Schenck and Perez, 1990; Trappe, 1982). However, there is much evidence that the spore counts or microscopic observations of roots do not reflect composition or abundance of AM fungi (Clapp *et al.*, 1995). Also, it is not possible to predict active fungal symbionts of an individual plant from an AM fungal spore community around the roots because of complex root system in belowground. It has not been possible to identify intraradical mycorrhizal fungal hyphae to species.

In recent years, molecular techniques have been used to study phylogenetic relationships and genetic variations of AM fungi. Several attempts have made to use ITS region of rDNA as a tool for the identification of AM fungi (Redecker *et al.*, 1997) and phylogenetic studies of DNA extractions and sequencing from AM fungal spores (Redecker *et al.*, 2000). However, it was not possible to apply for AM fungal communities in plant roots since these studies were based on spores. Only a few studies have been aimed at the direct identification of mixed populations of AM fungi in active colonization within root system in the field. Several PCR based detection methods have been developed using specific primers (Helgason *et al.*, 1999; Simon *et al.*, 1992). The purpose of this study was to detect AM fungi in active colonized roots of *Glycine max* and *Sorghum bicolor* using PCR-RFLP technique and to investigate interactions between mycorrhizal fungal community and crop plant species.

Materials and Methods

The roots of *Glycine max* and *Sorghum bicolor* were collected at the cultivation fields in Chungbuk, Korea in October, 2000. Half of root samples from each species were stained with 0.05% trypan blue (Koske and Gemma, 1989) and colonization of AM fungi were observed. Spores of AM fungi, *Glomus mosseae* and *Scutellospora heterogama*, were extracted from soil using wet-sieving and sucrose density gradient centrifuge methods (Daniels and Skipper, 1982). A single AM fungal spore was washed with distilled water three times and spore wall was crushed to extract DNA. DNA from roots of *G. max* and *S. bicolor* were extracted using CTAB (Ausubel *et al.*, 1999), and the concentration of DNA was estimated by absorbance at 260 nm.

DNA from roots of two plant species, and spores of two AM fungal species were amplified using AccuPower

*Corresponding author <E-mail: sslee@cc.knue.ac.kr>

PCR Premix (Bioneer Co., 1 U *Taq* DNA polymerase, 250 μM dNTP, pH 9.0 10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$) with general fungal primer AM1(GTT TCC CGT AAG GCG CCG AA, 10 pmol) designed to exclude plant DNA (Helgason *et al.*, 1999) and universal eukaryotic primer NS31(TTG GAG GGC AAG TCT GGT GCC, 10 pmol; Simon *et al.*, 1992). The PCR was performed for 30 cycles (10 cycles at 95°C for 1min, 58°C for 1 min, 72°C for 2 min, 19 cycles at 95°C for 30 sec, 58°C for 1 min, 72°C for 3 min and 1 cycle at 95°C for 30 sec, 58°C for 1min and 72°C for 10 min). The PCR products were separated by electrophoresis on 1% agarose gel and stained with EtBr. The bands for use as insert DNA were cut from the gel and purified with gel purification Kit (Bioneer, Korea). The purified DNA were inserted to pGEM®-T Easy Vector and transformed to *Escherichia coli* strain JM109. Putative positive transformants were selected from the various transformed *E. coli* cells mentioned above. The recombinant plasmid DNA were extracted from the clone and amplified using primers AM1 and NS31. The PCR products were digested with restriction enzymes, *Hinf*I and *Alu*I according to manufacturers manual (Bioneer, Korea). Restriction fragments were separated on 3% agarose gel and the lengths were read using 1D main (Bioneer, Korea). One clone of each RFLP type was sequenced on automatic sequencer (ABIPRISM™, USA) using the sequencing primers SP6 and T7. DNA sequence analyses were performed with the BLAST software available through the National Center for Biotechnology Information (NCBI). Clustal X (ver 1.81) was used for alignment of DNA sequence and neighbour-joining phylogeny using *Geosiphon pyriforme* and *Aspergillus niger* as an outgroup.

Results and Discussion

In the past decades, many progresses have been made in agricultural and ecological studies. Host plants benefit from AM fungi in increased growth through phosphorus uptake, and also mycorrhizal associations is related to environmental aspects. However, in practice, AM fungal colonization in roots was able to observe under microscopes due to the morphological and histological differences from other pathogenic fungi. However, it was not possible to differentiate AM fungi in colonized roots at the below level of genus. Spore communities in soil do not reflect AM fungal populations in active symbionts. Recent progresses of molecular techniques allow being able to resolve these problems. This study investigated AM fungi in roots of two host plants, *G. max* and *S. bicolor*, in Korea, using fungal specific primers AM1 and PCR-RFLP analysis.

Partial SSU of rDNA fragment of AM fungi from roots and spores were amplified using AM1 and NS31 primers and the length of PCR products was approximately 550 bp (Fig. 1). The clones from *S. bicolor* were separated into two groups of restriction patterns, while clones from *G. max* had only one pattern from restriction analysis using *Hinf*I and *Alu*I (Fig. 1 and Table 1). Two sequences of AM fungi from roots of *S. bicolor* were similar to *G. caledonium* (NCBI accession number: Y17635) and *G. fasciculatum* (NCBI accession number: Y17640), and sequences from *G. max* were similar to *G. proliferum* (NCBI accession number: AF213462; Fig. 3 and Table 2). The size difference between total length of fragments (Table 1) and sequences (Fig. 2) may be the result of variations on agarose gels and difficulties of accurate read-

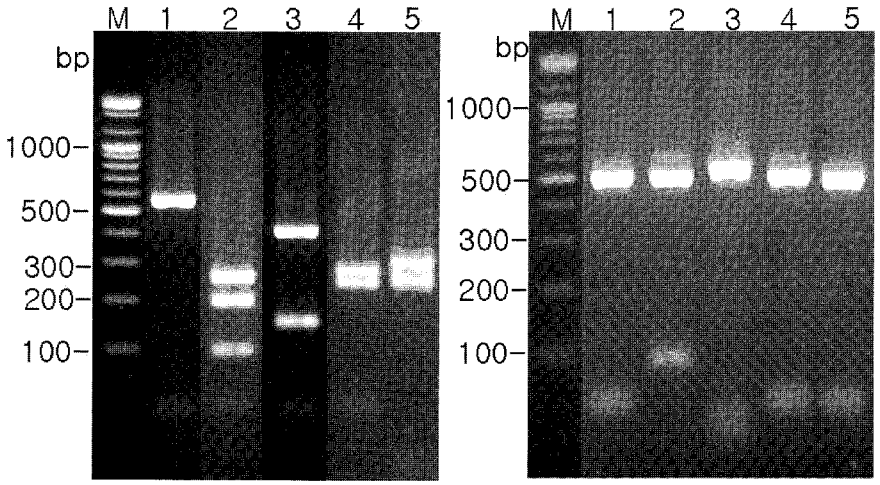

Fig. 1. The DNA fragments digested by the restriction enzymes, *Hinf*I (left) and *Alu*I (right in the figure) from the partial 18S rDNA bands were synthesized with the specific primers, AM1 and NS31, and loaded on 3% agarose gels; Lane M indicate molecular size marker (100 bp ladder), the DNA products of the lane 1 and 2 originated from the roots of *Sorghum bicolor*, lane 3 from the roots of *Glycine max* and those of lane 4 and 5 from the single spore of *Glomus mosseae* and *Scutellospora heterogama*, respectively.

Table 1. Length of restriction fragments of partial SSU rDNA bands of arbuscular mycorrhizal fungi synthesized with the specific primers from DNAs of plant roots[a] and fungal spores[b]

AM fungi (host plant)	Fragment lengths (bp) cleaved by the specific restriction enzymes	
	*Hinf*I	*Alu*I
1 (*Sorghum bicolor*)[a]	530, 20	500, 50
2 (*Sorghum bicolor*)[a]	280, 200, 100, 20	500, 100
3 (*Glycine max*)[a]	410, 150, 20	550, 30
Glomus mosseae[b]	280, 250, 20	500, 50
Scutellospora heterogama[b]	300, 250	500, 50

[a]The soil mixed with the roots of *Sorghum bicolor* were collected for this work. Different numbers indicate different colony from roots of each host plant. The plant roots collected from the soil were grounded to extract DNA.
[b]The single spores collected were grounded to extract DNA.

ings of fragment band on the gel worked here.

Changes in fungal spore populations in agricultural soils have been observed following different cropping histories (Johnson *et al.*, 1991) and some management practices, e.g. the use of fungicides or soil disinfection, can have negative effects on the symbiotic fungal population. It could be a reason for relatively low number of species found in roots of both crop species. Helgason *et al.* (1998) found significantly lower diversity of AM fungal communities in roots from arable fields than those from forest sites. Also, they found that the most dominant species was *G. mosseae* in arable study sites and not found in woodland. This study also found only species in genus *Glomus* species from roots. AM fungal spores collected from soils in the same site as this study (unpublished data) showed that seven *Glomus* species in *S. bicolor* and nine in *G. max*. This result suggested that these species had more

Table 2. 18S rDNA sequences of Glomales and other fungi obtained by database (NCBI) and this study[a]

Species	Marked	NCBI codes
Species of *Glomus*	Glo1	AF074358
	Glo2	AF131045
	Glo3	AF131049
	Glo4	AJ309460
	Glo8	AJ309462
	Glo9	AF074356
	Glo10	AJ309410
	Glo11	AF131053
	Glo1A	AJ309414
	Glo1B	AJ309438
	GLA006[b], GLA011[b]	Fern plants
	GLA016[b], GLA014[b], GLA032[b]	in our laboratory
	1(*S. bicolor*)[c], 2(*S. bicolor*)[c], 3(*G. max*)[c], 4(*G. mosseae*)[c], 5(*Sc. heterogama*)[c]	In this work[d]
Glomus fasciculatum		AF231760
Acaulospora sp.	Aco1	AF131036
	Aco2	AF074346
	Aco3	AF074349
	Aco4	AF074351
	Acau1	AJ309439
	TH30	AF074372
Acaulospora koskei		AF231762
Acaulospora laevis		AF074347
Scutellospora sp.	Scut1	AF131022
Scutellospora dipurpurenscens		AF131027
Scutellospora heterogama		AJ306434
Archaeospora leptoticha		AJ301861
Geosiphon pyriforme		X86686
Mucor indicus		AF113429
Rhizoctonia solani		AB000046
Tricholoma matsutake		AF385751
Hymenoscyphus ericae		AF069440
Aspergillus niger		AF109327

[a]The DNA sequences (AFO-series) were collected from NCBI (http://www.ncbi.nlm.nih.gov/).
[b]Unpublished data.
[c]Analyzed in this works (sslee@cc.knue.ac.kr or tms698@hanmail.net).
[d]Not published but see the thesis written by Lee, Jong Ki, in Korea National University of Korea (2002).

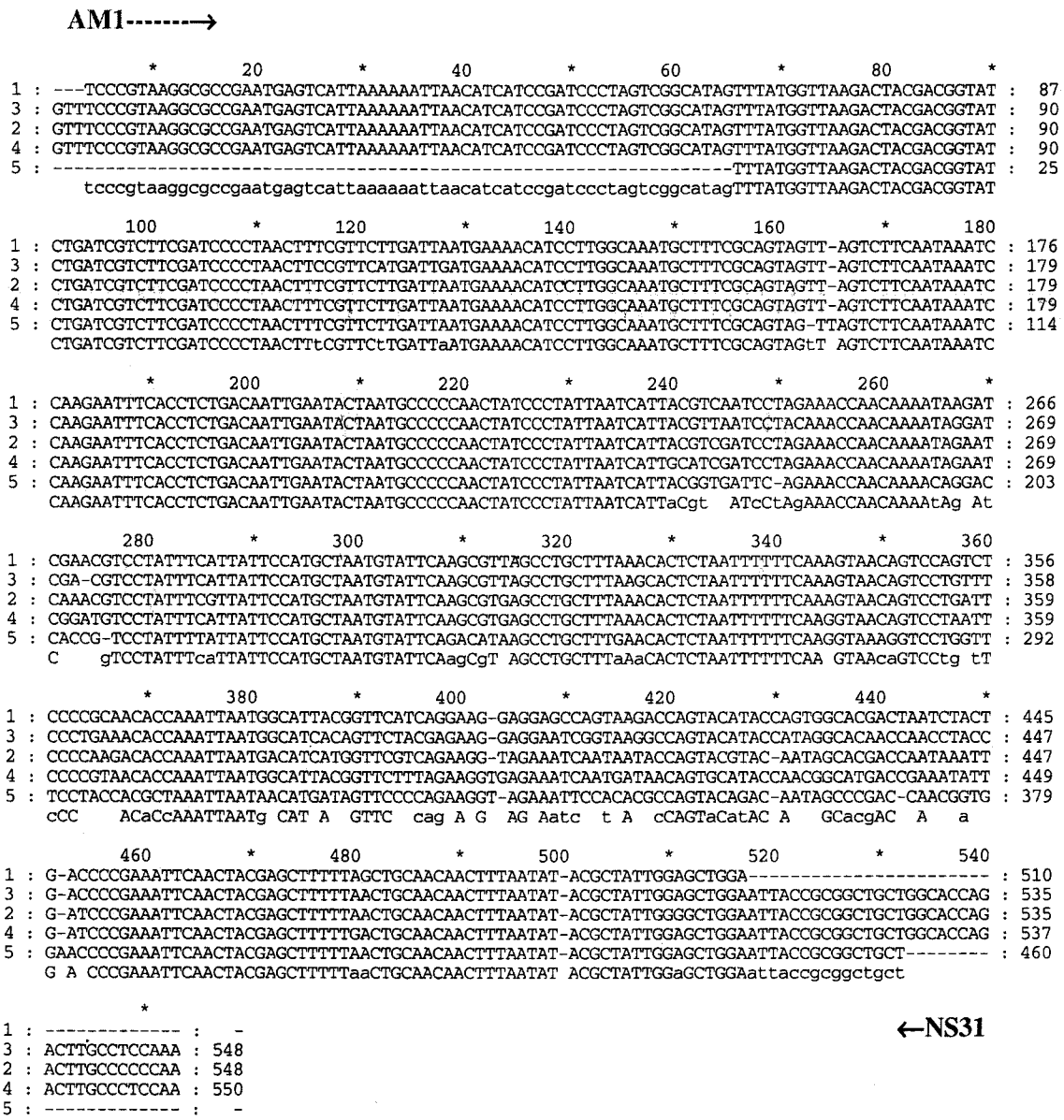

Fig. 2. The alignment data of partial 18s rDNA region (5'→3'). Sequences were aligned using CLUSTRAL X. The sequences were amplified from roots of *Sorghum bicolor* (1 and 2 marked), and *Glycine max* (3 marked), and from the spores of *Glomus mosseae* (4 marked) and *Scutellospora heterogama* (5 marked).

ability of sporulation and colonization in arable sites than natural field sites (Helgason, 1999). The lower number of species in roots than in spores in soils may be the result of difference in activity and association with other plants.

The sequences from spores were identical with sequences of *G. mosseae* (Accession numbers: U96141) and *S. heterogama* (Accession numbers:AJ306434) from NCBI (Table 2). This result confirmed that the procedures of this experiment were correct. Although these AM fungal species did not found in roots of two plant species in this study, more data from spores will help identification by comparisons of RFLP patterns of DNA from roots and those from fungal spores. As previously published specific primers, including AM1 primer used in this study for AM fungi have shown several mismatches, it was a particular interest to establish new primers which match to all the AM fungal species and exclude plant and other fungal DNA. Also, restriction enzymes for creating unique RFLP pattern is required for simple and sensitive identification of AM fungi within roots.

This study was able to identify AM fungi colonized in roots of host plant native in Korea. These have not been reported and were not consistent with previously conducted morphological studies. Also, it was observed that two different AM fungal species were colonized together in the roots of *S. bicolor*, and it was difficult to differentiate *Glomus* species in Glomales. The rDNA fragment analysis provided greater resolution to symbiotic relation-

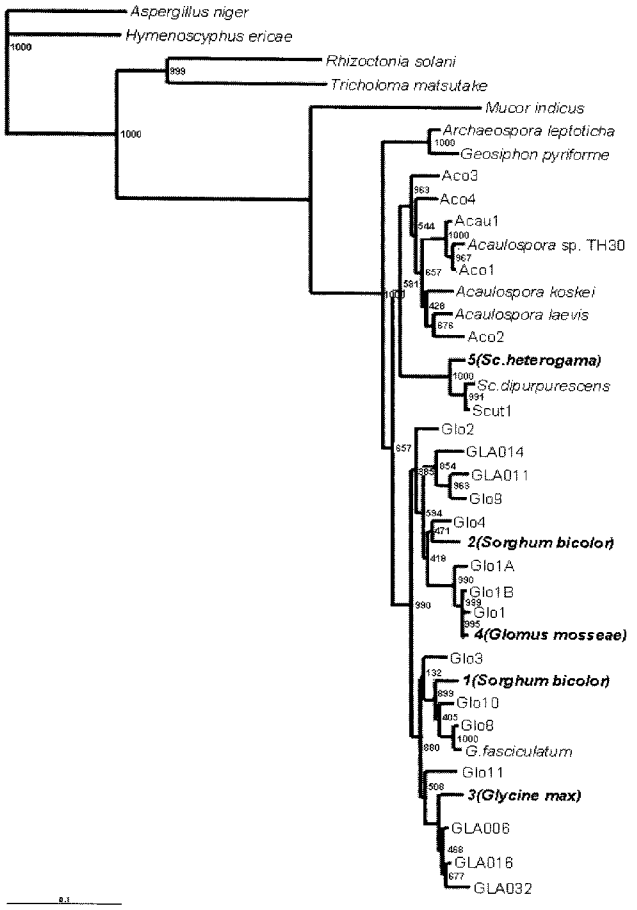

Fig. 3. Phylogenetic relationships among Glomales and other fungi inferred from 550 bp aligned sites of 18s rDNA sequence. The letter of bold italic indicates sequences analyzed in this study. Species names and simple markers or their related information in parenthesis indicate host plants and marked in Table 2.

ships between plant and AM fungi.

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References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. 1999. *Short protocols in molecular biology.* John Wiley and Sons, Inc, New York.

Bever, J. D., Morton, J. B., Antonovics, J. and Schultz, P. A. 1996. Host-dependent sporulation and species diversity of arbuscular mycorrhizal fungi in a mown grassland. *Journal of Ecology* **84**: 71-82.

Clapp, J. P., Young, J. P. W., Merryweather, J. W. and Fitter, A. H. 1995. Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. *New Phytologist* **130**: 259-265.

Daniels, B. A. and Skipper, H. A. 1982. Methods for the recovery and quantitative estimation of propagules from soil. Pp 29-35. *In*: Methods and principles of mycorrhizal research. N. C. Schenck (Ed.). American Phytopathological Society. St. Paul, Minn.

Eom, A. H., Hartnett, D. C. and Wilson, G. W. T. 2000. Host plant species effects on arbuscular mycorrhizal fungal communities in tallgrass prairie. *Oecologia* **122**: 435-444.

Gianinazzi, S. and Schuepp, H. 1994. *Impact of Arbuscular mycorrhizas on Sustainable Agriculture and Natural Ecosystems.* Birkhauser Verlag, Basel.

Hall, I. R. 1984. Taxonomy of VA mycorrhizal fungi. Pp 57-94. *In*: VA-Mycorrhizae. C. L. Powell & D. J. Bagyoargi (Eds.). Baca Raton. CRC.

_____ and Fish, B. J. 1979. A key to the Endogonaceae. *Trans. Br. Mycol. Soc.* **73**: 261-270.

Helgason, T., Fitter, A. H. and Young, J. P. W. 1999. Molecular diversity of arbuscular mycorrhizal fungi colonizing *Hyacinthoides non-scripta* (bluebell) in a seminatural woodland. *Molecular Ecology* **8**: 659-666.

Johnson, N. C., Pfleger, F. L., Crookston, R. K. and Simmons, S. R. 1991. Vesicular-arbuscular mycorrhizas respond to corn and soybean cropping history. *New Phytologist* **117**: 657-664.

Koske, R. and Gemma, J. N. 1989. A modified procedure for staining roots to detect VA mycorrhizas. *Mycological Research* **92**: 486-505.

Lee, J. K. 2002. Studies on the mycorrhizae found in the roots of Korean ferns and its relatives. Korea National University of Education, Chungbuk.

Redecker, D., Morton, J. B. and Bruns, T. D. 2000. Molecular phylogeny of the arbuscular mycorrhizal fungi *Glomus sinuosum* and *Sclerocystis coremioides*. *Mycologia* **92**: 282-285.

_____, Thierfelder, H., Walker, C. and Werner, D. 1997. Restriction analysis of PCR-amplified internal transcribed spacers of ribosomal DNA as a tool for species identification in different genera of the order Glomales. *Applied and Environmental Microbiology* **63**: 1756-1761.

Schenck, N. C. and Perez, Y. 1990 Manual for the Identification of VA Mycorrhizal Fungi. Synergistic Publications. Gainesville, Florida.

Simon, L., Lalonde, M. and Bruns, T. D. 1992. Specific amplification of 18S fungal ribosomal genes from vesicular- arbuscular endomycorrhizal fungi colonizing roots. *Appl. Environ. Microbiol.* **58**: 291-295.

Smith, S. E. and Read, D. J. 1996. *Mycorrhizal symbiosis.* Academic Press. London.

Trappe, J. M. 1982. Synoptic keys to the genera and species of Zygomycetous mycorrhizal fungi. *Phytopathology* **72**: 1102-1108.

Van der Heijden, M. G. A., Klironomos, J. N., Ursic, M., Moutoglis, P., Streitwolf, E. R., Boller, T., Wiemken, A. and Sanders, I. R. 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature* **396**: 69-72.