Genetic diversity and host plant preferences revealed by simple sequence repeat and mitochondrial markers in a population of the arbuscular mycorrhizal fungus *Glomus intraradices*

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Received: *24 October 2007*
Accepted: *21 December 2007*

Summary

• Arbuscular mycorrhizal fungi (AMF) are important symbionts of plants that improve plant nutrient acquisition and promote plant diversity. Although within-species genetic differences among AMF have been shown to differentially affect plant growth, very little is actually known about the degree of genetic diversity in AMF populations. This is largely because of difficulties in isolation and cultivation of the fungi in a clean system allowing reliable genotyping to be performed.

• A population of the arbuscular mycorrhizal fungus *Glomus intraradices* growing in an *in vitro* cultivation system was studied using newly developed simple sequence repeat (SSR), nuclear gene intron and mitochondrial ribosomal gene intron markers.

• The markers revealed a strong differentiation at the nuclear and mitochondrial level among isolates. Genotypes were nonrandomly distributed among four plots showing genetic subdivisions in the field. Meanwhile, identical genotypes were found in geographically distant locations. AMF genotypes showed significant preferences to different host plant species (*Glycine max*, *Helianthus annuus* and *Allium porrum*) used before the fungal *in vitro* culture establishment.

• Host plants in a field could provide a heterogeneous environment favouring certain genotypes. Such preferences may partly explain within-population patterns of genetic diversity.

Key words: arbuscular mycorrhizal fungi, genetic diversity, *Glomus intraradices*, host plant preference, mitochondrial markers, simple sequence repeats.

New Phytologist (2008) **178**: 672–687

doi: 10.1111/j.1469-8137.2008.02381.x

Introduction

Arbuscular mycorrhizal fungi (AMF) belong to an ancient fungal phylum, the Glomeromycota. They form obligate symbioses with the majority of land plants (Smith & Read, 1997; Schüssler *et al.*, 2001). The early colonization of land by plants was probably associated with the presence of AMF (Redecker *et al.*, 2000). Nutrient exchange and protection from pathogens are thought to be key elements in the symbiosis (Newsham *et al.*, 1995; Smith & Read, 1997). AMF community composition can determine plant biodiversity and productivity (Van der Heijden *et al.*, 1998). The host range of

AMF is thought to be very wide, as most AMF associate with a large number of plant species (Smith & Read, 1997). Morphological characterizations of AMF spores have distinguished < 200 species, although molecular studies suggest that a significantly larger number of species exist (Redecker, 2002). Difficulties in identification and cultivation, coupled with difficulties in obtaining uncontaminated DNA from AMF field samples (Redecker *et al.*, 1999; Corradi *et al.*, 2004), have greatly hindered studies of their ecology and genetic diversity in natural ecosystems.

Although there are many studies focusing on AMF species diversity, only a few studies have used molecular techniques to look at genetic diversity within AMF populations. However, knowledge of such diversity is relevant as (Koch *et al.*, 2006) demonstrated a genetic basis for differential effects on plant growth among isolates of the AMF *Glomus intraradices* originating from a single field. In glasshouse experiments, the biomass of the host plants *Brachypodium pinnatum* and *Prunella vulgaris* varied by up to 33% (Koch *et al.*, 2006). This suggests that, depending on the effect on the plant growth, a given AMF individual in a population could be favoured by certain plant species. Phosphate uptake by the host plant has also been shown to be significantly affected by different isolates of the same AMF species (Munkvold *et al.*, 2004), although within-population variation was not considered. Finally, as field studies identifying AMF associated with particular plant species have only focused on resolving AMF species identities and not intraspecific variations (Helgason *et al.*, 1998; Del Val *et al.*, 1999), a reliable identification of genotypes within and among field populations would allow the study of associations of particular AMF genotypes and plant species.

Several different approaches using molecular markers and different sampling designs have been used to study genetic diversity and structure in AMF populations. Stukenbrock & Rosendahl (2005a) used a hierarchical design to study multilocus genotypes of three *Glomus* species. Significant genetic structure was found at a small scale, among plots separated by a few metres, whereas among neighbouring field sites, with differing agricultural treatments, no differentiation was detected. Vandenkoornhuyse *et al.* (2001) used inter simple sequence repeat (ISSR) fingerprints and ribosomal gene polymorphisms to study differentiation among AMF from different sewage treatments in a field. A high degree of diversity was found for two *Glomus* species and the observed diversity was structured among field plots of different treatments. Using *in vitro* propagated *G. intraradices* from a field population, Koch *et al.* (2004) found high genetic diversity and differentiation among field plots. In the same population, Corradi *et al.* (2007) found polymorphism in copy numbers of ribosomal genes.

The ideal approach to studying genetic diversity in AMF populations would be to have a marker-based system that can be applied to DNA originating from single spores from field sites. One critical limitation of this approach is the quantity of available DNA for genotyping. While Stukenbrock & Rosendahl (2005b) developed multiplex PCR to reliably amplify three gene introns, the number of loci that can be genotyped is limited. Also, so far, use of multiplex PCR has been unsuccessful in genotyping some AMF spores. Furthermore, the development of reliable markers in intergenic regions remains a major challenge, because of the lack of conserved flanking sequences among related species. Conserved ribosomal gene sequences have been successfully used in resolving deep phylogenetic relationships in AMF (Schüssler *et al.*, 2001) but would be insensitive to within-population variation. Some rapidly evolving regions of ribosomal DNA sequences (e.g. internal transcribed spacer (ITS)) exhibit within-spore variability (Sanders *et al.*, 1995), and these regions are therefore unsuitable as isolate-specific markers. Amplified fragment length polymorphism (AFLP) was successfully used to genotype *G. intraradices* isolates from one field (Koch *et al.*, 2004). This method allows a large number of loci to be analysed. Nevertheless, a larger quantity of DNA is required for reproducible fingerprints than that contained in an AMF spore and observed loci are difficult to check for homoplasy. Furthermore, genetic markers without sequence-specific primers (such as AFLP or ISSR) can potentially be biased by the amplification of undetected contaminating microorganisms, if material from natural soils or glasshouse experiments is used (Hijri *et al.*, 2002).

To overcome the limitations of DNA quantity per individual and the problems of contaminating microorganisms, a reliable strategy is to obtain single-spore isolates of the fungi from a field site and then put them into an *in vitro* culture system. Such a culture system has been described (St.-Arnaud *et al.*, 1996) and if initiated with a single spore allows the clonal growth of a large amount of fungal material. Although this approach is extremely labour intensive and time consuming it allows the extraction of large amounts of contaminant-free AMF DNA from each isolate. Additionally, it permits multiple DNA extractions to be made from the same individual, allowing experiments to be properly replicated to control for artifacts. True replication is almost never applied to AMF population or community studies because the amount of DNA per spore allows no replication (but see e.g. Vandenkoornhuyse *et al.*, 2001).

Microsatellites or simple sequence repeats (SSR) are widely used for estimation of population substructures and relatedness among individuals (Queller *et al.*, 1993; Griffiths *et al.*, 1996; Jarne & Lagoda, 1996). SSR are composed of tandemly repeated sequence motifs from one to six nucleotides in length (Tautz & Renz, 1984). Polymorphism at these loci mostly arises through slipped-strand mispairing and subsequent errors during DNA replication (Tautz & Renz, 1984). A large number of SSR loci were found in fungal genomes (Karaoglu *et al.*, 2005). In *Neurospora*, mutational patterns were studied in detail using cross-species comparisons (Dettman & Taylor, 2004). To our knowledge, only primers consisting of repeat

motifs have been used to genotype AMF (Longato & Bonfante, 1997; Vandenkoornhuyse *et al*., 2001; Douhan & Rizzo, 2003) and suitable regions for primer design in flanking repeats have not been reported.

Genetic diversity of fungal mitochondria has rarely been studied in natural populations. Raab *et al*. (2005) reported the first sequences of a mitochondrial ribosomal gene in AMF. Knowledge of AMF-specific mitochondrial sequences is critical for the specific amplification of potentially polymorphic sequences in populations, as some species were shown to harbour endosymbiotic bacteria (Bianciotto *et al*., 1996). To our knowledge, no study has reported mitochondrial diversity in AMF populations.

Given the potential ecological importance of genetic diversity in single populations of AMF, the distribution of genetic diversity across different populations is relevant to the understanding of AMF–host plant interactions. A field population of *G. intraradices* in Tänikon, Switzerland, was chosen to establish what is, to our knowledge, the largest collection of *in vitro* cultures of one AMF population. The field site in Tänikon was divided into plots to which different agricultural treatments were applied. During the process of isolation of AMF spores from the field, different host plants were used for an initial round of cultivation. Establishment of *in vitro* cultures was rarely attempted for AMF, because of the time-consuming procedure required to isolate single spores for propagation under sterile laboratory conditions. Nevertheless, only *in vitro* cultures provide the necessary quantities of contamination-free DNA for the development of new markers in noncoding regions of the genome or the application of AFLP with appropriate levels of replication. Ten *in vitro* isolates were initially genotyped using AFLP, revealing strong genetic differentiation among three main genotypes, shown by an average of nearly 50% polymorphic loci among pairs of isolates (Koch *et al*., 2004). Phenotypic traits measured in 16 isolates revealed large variation in hyphal and spore production (Koch *et al*., 2004). Genetically different isolates from this field were shown to differentially affect plant growth (Koch *et al*., 2006), making it possible to test whether host plant preferences exist among genetically different isolates of *G. intraradices*. *Glomus intraradices* has a haploid genome (Hijri & Sanders, 2004), facilitating the development of SSR markers.

The aims of the present study were: to use nuclear SSR and mitochondrial markers to study a large collection of *in vitro* isolates from a single population; to test for associations of isolate genotypes with host plant species used for the establishment of *in vitro* cultures; and to compare identified genotypes with newly established *in vitro* cultures from two distant populations. In addition, *G. intraradices* is the first AMF species for which a whole-genome sequencing project has been initiated (Lammers *et al*., 2004). The project uses DNA from an isolate of Canadian origin (DAOM181602), allowing us to compare genetic differences within a population with the isolate that is currently being sequenced.

Materials and Methods

Field sites and establishment of single-spore isolates

A total of 47 *Glomus intraradices* (Schenk & Smith) single-spore isolates were established from three populations located in Switzerland (Table 1), including 41 from a field near Tänikon, three from a field near Eschikon and three from a field near Changins. Additionally, the isolate DAOM181602 from a field site at Pont Rouge, Quebec, Canada was included in the study. Geographical distances between sampling sites were 17 km between Eschikon and Tänikon, 220 km between Eschikon and Changins, and 236 km between Tänikon and Changins.

Forty-one isolates originated from a long-term experiment on the effect of tillage located at Hausweid, Tänikon (Jansa *et al*., 2002). The field site comprised seven sampling plots (6 m × 19 m) over a total area of 90 m × 110 m and was under a 4-yr crop rotation for 12 yr consisting of rapeseed (*Brassica napus* L.), winter wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), and winter wheat. All field plots were treated identically. Three field plots were subjected to tillage and one field plot to chiselling, and three field plots were left untreated (Jansa *et al*., 2002). For details on spatial arrangement of plots, see Koch *et al*. (2004). Thirty soil cores were sampled randomly from each plot, pooled and mixed to obtain a representative sample (Jansa *et al*., 2002). Trap cultures with different host plant species were established in replicates from each plot by mixing soil from each plot 1 : 4 (volume:volume) with autoclaved quartz sand and filling individual 300-ml pots with this mixture. Trap host species included soybean (*Glycine max* L.), sunflower (*Helianthus annuus* L.), leek (*Allium porrum* L.) and ribwort plantain (*Plantago lanceolata* L.) (Jansa *et al*., 2002; Koch *et al*., 2004). Plants were grown under standardized conditions in the glasshouse for 5 months. Single spores of *G. intraradices* were isolated from the trap cultures by wet-sieving and decanting and were then used to inoculate approx. 10-d-old seedlings of *P. lanceolata*. For a summary of numbers of isolates recovered from each the seven field plots and four trap host species, see the Supplementary Material.

Three isolates originated from a long-term fertilization experiment in Changins (Ryser & Vullioud, 2003; Mathimaran *et al*., 2005). Soil cores were taken from two plots (15 m × 8 m each) with different phosphate treatments to establish trap cultures with a mixture of maize (*Z. mays*), bushy rattlepod (*Crotalaria grahamiana* Wight & Am.), *A. porrum* and *H. annuus* (Mathimaran *et al*., 2005). Single spores of *G. intraradices* were used to inoculate sterile seedlings of either *Z. mays* or *A. porrum*. Three isolates originated from the model agricultural grasslands of the Swiss Free Air CO_2 Enrichment (FACE) experiment that was established to assess CO_2-induced changes in the plant community near Eschikon (Zanetti *et al*., 1996; Gamper *et al*., 2005). From one field

Table 1 Arbuscular mycorrhizal fungi (AMF) isolate identity and geographical origin, experimental treatment and trap culture plant species

Isolate	Population	Plot	Experimental treatment	Plant species for trap culture	Genotype
A1	Tänikon	A	No tillage	Sunflower (*Helianthus annuus*)	IV
A2	Tänikon	A	No tillage	Soybean (*Glycine max*)	XVIII
A3	Tänikon	A	No tillage	Soybean (*G. max*)	XVII
A4	Tänikon	A	No tillage	Soybean (*G. max*)	XVII
A5	Tänikon	A	No tillage	Sunflower (*H. annuus*)	VII
A6	Tänikon	A	No tillage	Sunflower (*H. annuus*)	XI
A7	Tänikon	A	No tillage	Soybean (*G. max*)	XVIII
B1	Tänikon	B	No tillage	Leek (*Allium porrum*)	IX
B2	Tänikon	B	No tillage	Leek (*A. porrum*)	I
B3	Tänikon	B	No tillage	Sunflower (*H. annuus*)	II
B4	Tänikon	B	No tillage	Sunflower (*H. annuus*)	III
B5	Tänikon	B	No tillage	Sunflower (*H. annuus*)	II
B6	Tänikon	B	No tillage	Sunflower (*H. annuus*)	V
B7	Tänikon	B	No tillage	Sunflower (*H. annuus*)	I
B8	Tänikon	B	No tillage	Sunflower (*H. annuus*)	I
B9	Tänikon	B	No tillage	Sunflower (*H. annuus*)	I
B10	Tänikon	B	No tillage	Leek (*A. porrum*)	I
B11	Tänikon	B	No tillage	Leek (*A. porrum*)	I
B12	Tänikon	B	No tillage	Leek (*A. porrum*)	XIII
B13	Tänikon	B	No tillage	Leek (*A. porrum*)	I
B14	Tänikon	B	No tillage	Soybean (*G. max*)	VII
B15	Tänikon	B	No tillage	Soybean (*G. max*)	VII
B16	Tänikon	B	No tillage	Soybean (*G. max*)	VII
B17	Tänikon	B	No tillage	Soybean (*G. max*)	I
C1	Tänikon	C	Tillage	Leek (*A. porrum*)	XV
C2	Tänikon	C	Tillage	Leek (*A. porrum*)	XV
C3	Tänikon	C	Tillage	Leek (*A. porrum*)	XVII
C4	Tänikon	C	Tillage	Leek (*A. porrum*)	XVII
C5	Tänikon	C	Tillage	Leek (*A. porrum*)	XVI
C7	Tänikon	C	Tillage	Leek (*A. porrum*)	XVII
D1	Tänikon	D	Tillage	Sunflower (*H. annuus*)	VIII
D2	Tänikon	D	Tillage	Sunflower (*H. annuus*)	VI
D3	Tänikon	D	Tillage	Sunflower (*H. annuus*)	VI
D4	Tänikon	D	Tillage	Leek (*A. porrum*)	II
D5	Tänikon	D	Tillage	Sunflower (*H. annuus*)	VI
D6	Tänikon	D	Tillage	Sunflower (*H. annuus*)	VI
E1	Tänikon	E	No tillage	Ribwort plantain (*Plantago lanceolata*)	X
F1	Tänikon	F	Chisel	Leek (*A. porrum*)	VII
G1	Tänikon	G	Tillage	Leek (*A. porrum*)	XIV
250	Tänikon	unknown	Unknown	Unknown	VI
251	Tänikon	unknown	Unknown	Unknown	VI
FACE#128	Eschikon		60 Pa pCO_2, 56 g/m^2/yr	Hawkbit (*Leontodon autumnalis*) and *Dipsacus fullonum*	V
FACE#484	Eschikon		60 Pa pCO_2, 56 g/m^2/yr	*Zea mays* and *P. lanceolata*	VII
FACE#494	Eschikon		60 Pa pCO_2, 56 g/m^2/yr	*Z. mays* and *P. lanceolata*	VII
S1	Changins		No phosphate addition	*Z. mays*, *Crotalaria grahamiana*, *A. porrum* and *H. annuus*	VIII
S6	Changins		2.6 g/m^2/yr exceeding crop removal	*Z. mays*, *C. grahamiana*, *A. porrum* and *H. annuus*	VIII
S7	Changins		No phosphate addition	*Z. mays*, *C. grahamiana*, *A. porrum* and *H. annuus*	VIII
DAOM181602	Pont Rouge, Québec, Canada		Unknown	Unknown	XII

Agricultural treatments included tillage and experimental treatments included ambient or elevated pCO_2, as well as phosphorus treatment. The multilocus genotype of each isolate of *Glomus intraradices* used in this study is shown (constructed from the data for all 13 loci included in this study; see Table 3).

plot, trap cultures were established using either a combination of autumn hawkbit (*Leontodon autumnalis* L.) and teasel (*Dipsacus fullonum* L.) or *Z. mays* and *P. lanceolata*. *Glomus intraradices* spores from these trap cultures were then used to inoculate sterile seedlings of *A. porrum*. In addition, *G. intraradices* isolate DAOM181602 was included in this study. DAOM181602 was isolated from a field site at Pont Rouge, Quebec, Canada (Biosystematics Research Centre, Ottawa, Canada). DAOM181602 is widely used as a commercial AMF inoculum for agricultural applications (Premier Tech Inc., Rivière-du-Loup, Canada). Furthermore, a *Glomus diaphanum* (Morton & Walker) isolate (FACE#107) from the Swiss FACE experiment near Eschikon (Gamper *et al.*, 2005) was used to test whether the newly developed markers amplify DNA of a closely related AMF species.

In vitro cultures and DNA extraction

Either single spores of each isolate (in the case of the Tänikon and Changins isolates) or 1-cm pieces of colonized leek roots (in the case of the Eschikon isolates) were surface-sterilized and transferred to plates containing M culture medium with root tumour-inducing plasmid T-DNA-transformed carrot roots (Bécard & Fortin, 1988; Koch *et al.*, 2004). Isolates were clonally subcultured every 15 wk by transferring pieces of medium containing hyphae and spores to fresh plates. Several generations of subculturing were necessary to grow sufficient fungal material to inoculate five to 10 two-compartment plates of each isolate. Two-compartment plates allowed the fungus to proliferate on one half of the plate in the absence of roots (St.-Arnaud *et al.*, 1996; Koch *et al.*, 2004). Freshly isolated hyphae and spores of each isolate were separately dried overnight at 48°C and ground into a fine powder using a Retsch MM300 mixer mill (Retsch GmbH, Haan, Germany). The DNA was extracted using a modified version of the Cenis method for fungal DNA extraction (Cenis, 1992). Ground mycelia were resuspended in 500 μl of extraction buffer (200 mM Tris, pH 8.5, 250 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% sodium dodecyl sulphate (SDS)) and incubated at 60°C for 45 min. To each sample, 250 μl of sodium acetate (pH 5.2) was added and then the samples were stored at −20°C for 20 min. The tubes were centrifuged at 13 400 ***g*** (10 min). Afterwards, the supernatants were transferred to new tubes and diluted with 750 μl of a 24 : 1 solution of chloroform and isoamyllic alcohol. After an additional centrifugation at 13 400 ***g*** (10 min), the top phase was collected and transferred to a new tube. DNA was precipitated by adding 650 μl of isopropanol, with storage at −20°C for 20 min and centrifugation (13 400 ***g***, 15 min). The supernatant was discarded and the pellet was washed with 100 μl of EtOH 70%. After the final centrifugation (13 400 ***g*** for 10 min), the ethanol was removed and the dried pellets were resuspended in TE (0.1 mM EDTA).

Species identification based on internal transcribed spacer sequences

Internal transcribed spacer (ITS) sequences have frequently been used in the identification of closely related AMF species because of their high degree of polymorphism. For our study, primers in the conserved flanking regions (18S and 25S) were used to amplify and sequence the ITS in the most genetically differentiated isolates found in the field population of Tänikon (determined according to Koch *et al.*, 2004). Sequences with accession numbers AJ557006–9 had already been obtained (Koch *et al.*, 2004) from isolates of the Tänikon population. Sequences AY842570 and AY842571 were obtained from the isolate of Canadian origin, DAOM181602, by J. Jansa *et al.* (unpublished; sequences available on GenBank). In order to determine the species identity of our isolates, additional sequences were retrieved from GenBank for *G. intraradices*, the most closely related species *G. diaphanum*, and *Glomus fasciculatum* (Thaxter *sensu* Gerd.). *Glomus claroideum* (Schenk & Smith) sequences were used as an outgroup to root the phylogenetic tree. All available sequences matching the ITS region were retrieved from GenBank for these species, with the exception of sequences published by Renker *et al.* (2005). These sequences were excluded as phylogenetic species identification of *G. intraradices* was performed with a more distantly related species (*Glomus* sp.) as an outgroup and two highly divergent clades of *G. intraradices* were identified by the authors. All retrieved sequences were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw). Phylogenetic relationships were inferred with PAUP 4.b10 (Swofford, 2002) using the neighbour-joining algorithm (uncorrected *p* distance) with heuristic search, and clade support was assessed by performing 1000 bootstrap replicates. Maximum parsimony analysis was performed using a heuristic search (swap through tree-bisection–reconnection, 10 rounds of random sequencing addition). Maximum parsimony clade support was based on 1000 bootstrap replicates, performed identically to the neighbour-joining analysis.

SSR locus recovery and analysis

Glomus intraradices spores were purchased from Premier Tech. Genomic DNA was isolated as described in Magrini *et al.* (2004) and used to construct a partial genomic Lambda Zap Express Library (Stratagene, Inc., La Jolla, CA, USA). Fragments from a complete digestion of genomic DNA with *Bgl*II were ligated with *Bam*H1-digested and CIAP-treated Lambda Zap vector, packaged and used to infect *Escherichia coli* VCS257 cells. The unamplified library had a cell titre of 3×10^8 pfu/ml. Mass conversion of pBK-CMV phagemids to plasmids was accomplished in *E. coli* XLOLR according to the Zap Express manual (Stratagene, Inc.). Plasmid DNA was isolated (Plasmid Mini Prep Kit; Qiagen, Inc., Hombrechtikon, Switzerland) from 400 randomly selected

Table 2 Nuclear and mitochondrial markers developed for this study

Locus	Accession	PCR cycles	Forward primer (5′–3′)	Reverse primer (5′–3′)
SSR				
Bg32-T7	CG431930	30	CAA CCA TTG GCT TCA GAA ACA G	TTG ATT GTT TCA GCA ACA AGT TTG
Bg42-T7	CG431913	28	ACT CGC TAA AAT TTG CCG ATC T	CGT TTA TCC GTT ATC CGG AAC AT
Bg62-T3	CG431880	32	CGT CAG TAA ACT TGA TGT GAT AAA AAT GA	GCC ACT TTG GAC ACA TAG AAC TAG C
Bg196-T7	CG431972	26	TTA TTA CTT GGA ATG GAC ACG CA	GGC TAA TAT CCA ATA CGA ATC TGT ACC T
Bg235-T7	CG432041	28	CAT TTA CAT CAC TTT TGC TTA TAT GTT CAC	TGA GAT CAT GTG GCT TCA GAT AAG TA
Bg273-T7	CG432137	26	CAG GCA ATT CGG GTA GAT ACA ATT	TCC GTT GGC ATA ACT AAG CAA AT
Bg276-T7	CG432062	26	ATG CAA GAT TTT AAA GGC AAA GCT	CTT GCT TCA AAT CTT TAT CCG GTA G
Bg303-T3	CG432175	32	TTT ACC ATC GGC ACT ATC TGG A	TTG ATG AGA CTA ACA ATT ATC TTG AAG TTT AA
Bg348-T7	CG432294	30	ACG TCC AAT CAT ATT TTG CGG	TGG AGC TTT ATG GAC TGA AGA TGT TAC
Bg355-T7	CG432269	26	TTC CTC TTT AAC AAA AAT CAT CTA TCC TT	AAA TTT TTT GAC ATG TTA TCG ATC ACT T
Nuclear gene intron				
nr int	BE603853	28	TCA ACG TCC ATT TTT GTT TCT ATT TAT GAT	TCC TTC ATT TAA ACT TAG ATC TTT TTT TGT AA
Mitochondrial LSU gene introns				
mtLSU int 1	AJ973189-93	26	AGA AAC GAC GTT CTG CTG CTC ATT AA	TCT TCG GAG TCC CTT GCA AC
mtLSU int 2	AJ973189-93	24	TGC TAT TCA ATC CTA ATT ACA TTA GTG GG	CGC CCT CAC CAG GCA ATA T

See Supplementary Materials for alignments of alleles for each locus, a description of the repeat polymorphism found and electropherograms. LSU, ribosomal large subunit; SSR, simple sequence repeats.

XLOLR clones. Insert sizes were estimated by agarose gel electrophoresis after *Xho*I and *Pst*I digestion. Bidirectional sequencing was performed on 331 clones with inserts larger than 2 kb using Li-Cor 4200 with 700- and 800-nm dye-labelled sequencing primers (T3 and T7, respectively; Li-Cor, Inc., Lincoln, NE, USA). Sequence results were deposited in dbGSS at National Center for Biotechnology Information (NCBI) with accession numbers CG431601–CG432299 (average length of 820 bp). Potential SSR markers were identified using the program TANDEM REPEATS FINDER (Benson, 1999). Clones Bg32-T7, Bg42-T7, Bg62-T3, Bg196-T7, Bg235-T7, Bg273-T7, Bg276-T7, Bg303-T3, Bg348-T7 and Bg355-T7 were chosen based on their high repeat motif scores given by TANDEM REPEATS FINDER. On the basis of these sequences, primers were designed that were expected to amplify this region. Primers developed for this study, the number of cycles used in PCR, and NCBI accession numbers are shown in Table 2. Clone sequences lying within the designed primers were compared to the nonredundant protein data base of GenBank using BlastX (Altschul *et al*., 1990) to identify potential homologous coding regions in other fungi. None of the repeat regions contained significant alignments.

Intron length variation in a nuclear gene and the mitochondrial large subunit

Using a published expressed sequence tag (EST) sequence (accession number BE603853) from 10-d-old germinating spores of *G. intradices* (Lammers *et al*., 2001), primers were designed to amplify the sequence in genomic DNA (F2 5′–3′ TAG GAC CAG CTT CAC CAC CTT C; R2 5′–3′ GTA ACC AAA ACA AAA GGG CGT T). Sequencing revealed the presence of an intron, not found in the EST. In order to detect intron length variation among isolates, new primers were designed that only amplify the intron sequence (Table 2). Raab *et al*. (2005) described the highly conserved ribosomal large subunit (LSU) gene sequence of mitochondria in the *Glomus* genus. Primer pairs amplifying each of the two identified introns were also designed (Table 2).

All PCR mixes contained 10 ng of genomic DNA, 1 μl of 10 × PCR buffer including 15 mM $MgCl_2$ (Qbiogene, Inc., Morgan Irvine, CA, USA), 0.19 mM dNTPs, 0.25 μM of each of the two primers and 0.15 U Taq DNA polymerase (Qbiogene, Inc.) in a total volume of 10 μl. Thermal cycling conditions included 3 min at 94°C, followed by cycles of 30 s at 94°C, 30 s at 53°C and 45 s at 72°C and a final elongation of 7 min at 72°C. The number of PCR cycles was optimized for each locus in order to obtain similar amounts of PCR product after amplification (see Table 2). Forward primers were either hexachlorofluorescein phosphoramidite (HEX) or fluorescein (FAM) fluorescence-labelled to allow capillary fragment length visualization on a ABI-3100 Genetic Analyzer™ (Applied Biosystems, Inc., Foster City, CA, USA). A mixture of 1 μl of PCR product, 0.3 μl of ROX-500 size standard and 13.8 μl of Hi-Di formamide was prepared for loading. Data from the ABI-3100 Genetic Analyzer™ were manually scored using GENEMAPPER 4.0 (Applied Biosystems, Inc.).

For each different allele found at each of the 13 loci, one representative isolate was chosen to sequence the locus to ensure homology and reproducibility of the fragment length analysis. For this, PCR products were purified using the MinElute PCR purification kit (Qiagen, Inc.). Purified and quantified PCR products were directly cycle sequenced with

BigDye Terminator v1.1 (Applied Biosystems, Inc.) following the supplier's instructions. Cycle sequence products were purified by ethanol precipitation. An ABI Prism™ 3100 Genetic Analyzer was used for automated sequencing. Sequence profiles were visually checked using VECTOR NTI 7.1 software (Invitrogen, Inc., Carlsbad, CA, USA).

Construction of minimum spanning networks

The complete data set containing allele lengths for all isolates at all 13 loci was used to construct a minimum spanning network using ARLEQUIN 3.11 (Excoffier *et al.*, 2005). With this method genotypes are treated as nodes rather than terminal branches as in a phylogenetic tree (Excoffier & Smouse, 1994). Distances in the minimum spanning network represent numbers of loci that are polymorphic between genotypes within any given pair of genotypes. A second network was constructed only using data from the two mitochondrial loci.

Relationship between number of sampled isolates and genotypic diversity

To estimate to what degree the sampling effort in the Tänikon population represented the total genotypic diversity in that population, a resampling simulation was performed. Two isolates at a time were randomly chosen and compared to see whether they were of different genotypes. This procedure was repeated, successively choosing three to 41 isolates (with replacement). The mean number of different genotypes observed and the standard deviation were calculated by performing a bootstrap over 10 000 repetitions of the procedure. Values of means were plotted against the number of randomly sampled isolates. To estimate the efficiency of sampling in the Tänikon population, a second resampling simulation was performed. Out of the seven field plots, one was randomly chosen and the number of different genotypes scored. Successively, two to seven field plots were randomly chosen (without replacement) and the total number of different genotypes was scored. The mean number of different genotypes for a given number of plots and the standard deviation were calculated by performing a bootstrap over 10 000 repetitions. All calculations were performed using R statistical software (R_Development_Core_Team, 2006). Total genotypic diversity was estimated using the nonparametric Chao-1 estimator for species richness (Chao, 1984). A 95% confidence interval for the total diversity estimate was calculated using a jackknife procedure (1000 randomizations). The software ESTIMATES version 8.0.0 was used for all calculations (Colwell, 2006).

Genetic differentiation within the field population

The degree of genetic structure among plots within the Tänikon field population was evaluated by estimating the proportion of the total genetic variation attributable to among-plot vs within-plot variation. The significance of the fixation index $F_{ST(plot\text{-}population)}$ was tested using 10 000 permutations of samples among plots. In three plots (E, F and G), only one isolate was obtained, and therefore no within-plot variation could be calculated and these plots were excluded from the analysis.

Four different trap host species were inoculated with soil cores from the field. To test whether the identity of trap host species had an effect on which genotypes were found among *G. intraradices* isolates, the degree of genetic differentiation among isolates from different trap host species was estimated. To account for a potential bias caused by the genetic differentiation among field plots, the effect of trap host species was tested within each field plot. Therefore, the fixation index $F_{ST(trap\ host\text{–}plot)}$ was calculated. The significance of the trap host species effect on genetic structure was tested using 10 000 permutations of samples among different trap host species nested within plots. *Plantago lanceolata* was excluded from the analysis as a host species as only a single isolate was recovered from these trap cultures. Furthermore, plots E, F and G were excluded as only single isolates were recovered (see above). All calculations were performed using HIERFSTAT (Goudet, 2005), a population genetics package for computation of hierarchical *F* statistics for R statistical software.

Correlation of pairwise genetic distances

Koch *et al.* (2004) assessed genetic diversity among 10 isolates of the Tänikon population and DAOM181602 using AFLP. Nine of those 10 isolates from Tänikon and DAOM181602 were also included in the present study. Therefore, genetic distances assessed using AFLP data of Koch *et al.* (2004) among all pairs of the nine isolates and DAOM181602 could be compared with genetic distances based on the newly developed SSR and intron markers. Genetic distances assessed by AFLP were calculated using PAUP 4.b10 (Swofford, 2002), representing percentages of shared AFLP bands (Euclidean distance). Genetic distances based on SSR and intron markers for the same pairs of isolates (representing a subsample of the total number of isolates from Tänikon) were calculated using the number of loci showing different alleles. The correlation between genetic distances based on AFLP and SSR and intron markers among all pairs of isolates was determined with a Mantel test performed using R statistical software (R_Development_Core_Team, 2006).

Results

Amplification of nuclear and mitochondrial markers

Ten SSR markers, one nuclear gene intron marker and two mitochondrial intron markers were successfully amplified from DNA extracted from 48 *in vitro* cultures of *G. intraradices* isolates. The number of different alleles at each locus varied

Table 3 Genotypes found among 48 isolates of *Glomus intraradices* of four different geographical origins

Genotype	*n*	Bg32	Bg42	Bg62	Bg196	Bg235	Bg273	Bg276	Bg303	Bg348	Bg355	nr int	mt genotype	mtLSU int1	mtLSU int2
I	8	261	271	303	301	324	249	307	142	199	331	272	iii	308	285
II	3	262	271	303	312	324	249	307	142	199	331	272	iii	308	285
III	1	261	271	303	312	324	249	278	142	199	331	272	iii	308	285
IV	1	261	271	303	301	324	249	307	142	199	331	270	iii	308	285
V	2	261	271	303	301	324	250	307	142	199	331	270	iii	308	285
VI	6	261	271	303	301	324	250	278	142	199	331	270	iii	308	285
VII	7	262	271	303	301	324	250	278	142	199	331	270	iii	308	285
VIII	4	262	271	303	301	324	250	307	142	199	331	270	iii	308	285
IX	1	261	271	303	301	324	250	307	142	206	331	272	iii	308	285
X	1	261	271	303	301	324	245	307	142	199	331	270	iii	308	285
XI	1	263	271	303	301	324	245	312	142	199	331	270	iii	308	285
XII	1	262	271	293	312	324	249	278	142	199	331	276	iii	308	285
XIII	1	262	271	303	312	324	250	278	142	199	331	270	iii	308	285
XIV	1	261	271	303	312	324	250	307	142	199	331	270	iii	308	285
XV	2	243	271	305	298	324	248	287	142	197	331	265	ii	305	285
XVI	1	243	271	305	298	324	247	287	142	197	331	265	ii	305	285
XVII	5	272	256	293	294	310	245	279	139	206	313	270	i	305	273
XVIII	2	263	248	298	281	303	245	298	139	194	319	273	i	305	273

Allele lengths (bp) are shown for each simple sequence repeat (SSR) locus (Bg32–Bg355), a nuclear intron (nr int) and two mitochondrial large subunit (mtLSU) introns. Genotypes I–XVIII (except XII) were found in the population from Tänikon. Genotype XII was only found in isolate DAOM181602, originating from Québec, Canada. Mitochondrial genotypes were constructed based on the two mtLSU introns.

from two to six (Table 3). Sequencing of loci in DNA from representative isolates showed that all alleles of each locus share highly similar or identical flanking regions, ensuring that the fragment length amplification is locus specific. Among the SSR markers, Bg276 was found to be the most polymorphic locus. Differences in length among alleles at SSR loci ranged from 3 to 31 bp. The intron identified in a nuclear gene exhibited five different alleles (range 265–273 bp) in the 48 isolates. The mitochondrial ribosomal large subunit gene introns each showed two alleles (Table 3). Several of the potential SSR loci were found to contain multiple repeat motifs, which were responsible for length polymorphism among alleles, as well as indel mutations occurring outside the repeat motif. This meant that polymorphism at these loci was not always observed in the repeat sequence that the software TANDEM REPEATS FINDER had identified. For a complete description of polymorphism among alleles, sequence alignments and electropherograms see the Supplementary Material. None of the markers could be amplified from the *G. diaphanum* (isolate FACE#107) DNA.

Genotypic diversity in populations

Eighteen distinct multilocus genotypes were found among all isolates in the study (Table 3). The same 18 genotypes could be identified if multilocus genotypes were constructed using only the 10 SSR markers and the nuclear gene intron. The Canadian isolate DAOM181602 showed a nuclear genotype (XII) that was not found in any of the three Swiss locations (Table 3). Three distinct mitochondrial genotypes were found based on the two mitochondrial LSU introns. All three isolates from Changins and all three isolates from Eschikon contained mitochondrial genotypes that were also found in the Tänikon population (genotypes V, VII and VIII). The most frequent mitochondrial genotype (iii), shared among all three Swiss locations, was also found in isolate DAOM181602. Meanwhile, the two rarer mitochondrial genotypes were exclusively found in the Tänikon population (Table 3). Sequences of the ITS region from five of the most differentiated genotypes (II, VI, XV, XVII and XVIII) and DAOM181602 (XII) confirmed that the isolates belong to the highly supported clade of *G. intraradices* (Fig. 1). The newly sequenced ITS region from isolate A2 was deposited in GenBank (accession number EU221582).

Based on all nuclear and mitochondrial loci, a minimum spanning network among genotypes of all locations was constructed (Fig. 2a). Distances in the network are based on the number of polymorphic loci between genotypes. The analysis revealed a large cluster of genotypes with one to seven loci polymorphic between genotypes within pairs of genotypes (I–XIII). Genotypes XV–XVIII were each clearly distinct from the large cluster of genotypes (Fig. 2a). The two mitochondrial loci distinguished the large cluster of genotypes from the two genotypes XV and XVI, as well as XVII and XVIII. Overall, in pairs of isolates the average number of polymorphic loci was 5.61 ± 2.74 ($\pm$ SD). A minimum spanning network based on the mitochondrial markers alone revealed three genotypes (Fig. 2b).

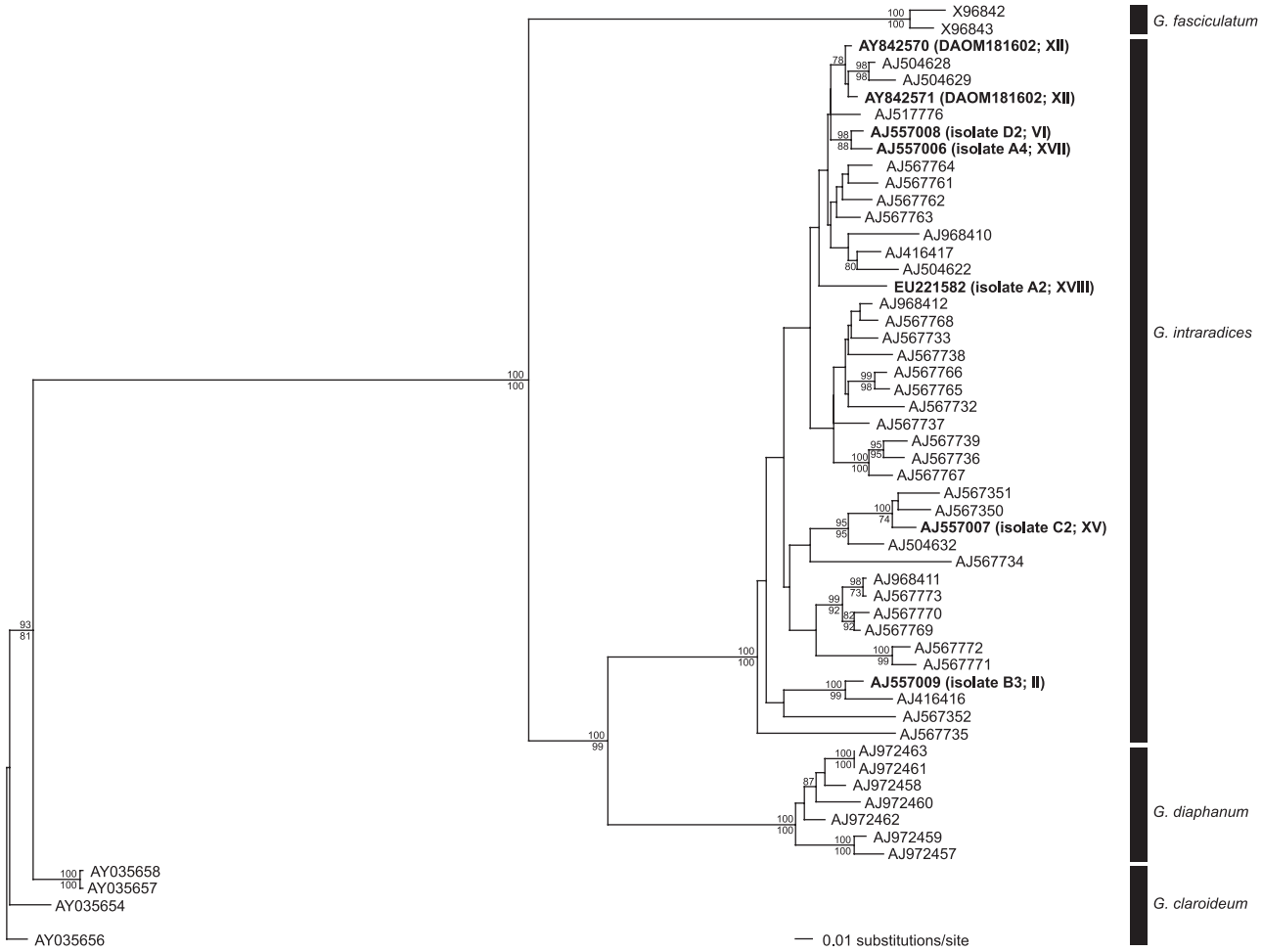

Fig. 1 Consensus of neighbour-joining (uncorrected *p* distance) and maximum parsimony analyses are shown for internal transcribed spacer (ITS) sequences of *Glomus intraradices*, *Glomus diaphanum*, *Glomus fasciculatum* and *Glomus claroideum* (defined as the outgroup). Numbers on branches indicate clade support in percentage of 1000 bootstrap replicates (only clade support > 75% is shown). Upper numbers correspond to the neighbour-joining analysis, and lower numbers correspond to the maximum parsimony analysis. Sequences are identified by their GenBank accession number. Sequences in bold were recovered from isolates used in this study. Isolate identities and simple sequence repeat (SSR) multilocus genotypes are indicated in parentheses.

Estimated total diversity in a field population

Our sampling in the Tänikon population recovered 17 different genotypes from 41 isolates. The most common genotype was found in eight isolates and the next most common in six isolates. A total of 10 genotypes were singletons. A resampling procedure showed that a substantial number of genotypes were covered by our sampling. Nevertheless, more genotypes are likely to exist (Fig. 3a). Using the Chao estimator statistics, the total diversity in the sampled field was estimated at 18 genotypes (9.92–59.9; jackknife 95% confidence interval). We also estimated sampling efficiency based on the field plot design. Resampling of field plots showed that sampling additional plots could greatly increase the number of genotypes, as the number of genotypes increases almost linearly with the number of field plots (Fig. 3b).

Spatial genetic structure within a field population

Isolates in the Tänikon population were sampled in seven different field plots, and in four of these more than one isolate was recovered (see the Supplementary Material), allowing the estimation of genetic differentiation indices. Genetic diversity was significantly structured among these four plots with a fixation index of $F_{ST(plot\text{-}population)} = 0.208$ ($P < 0.0001$; 10 000 permutations). Genetic structure in the field was further indicated as genotypes I, VI, XV and XVIII were only recovered from single plots (Fig. 4). This means that a given genotype is more likely to be found in certain plots than others.

Association of genotypes with host plant species

Four different host plant species (*G. max*, *H. annuus*, *A. porrum* and *P. lanceolata*) had been used to establish the isolates in the

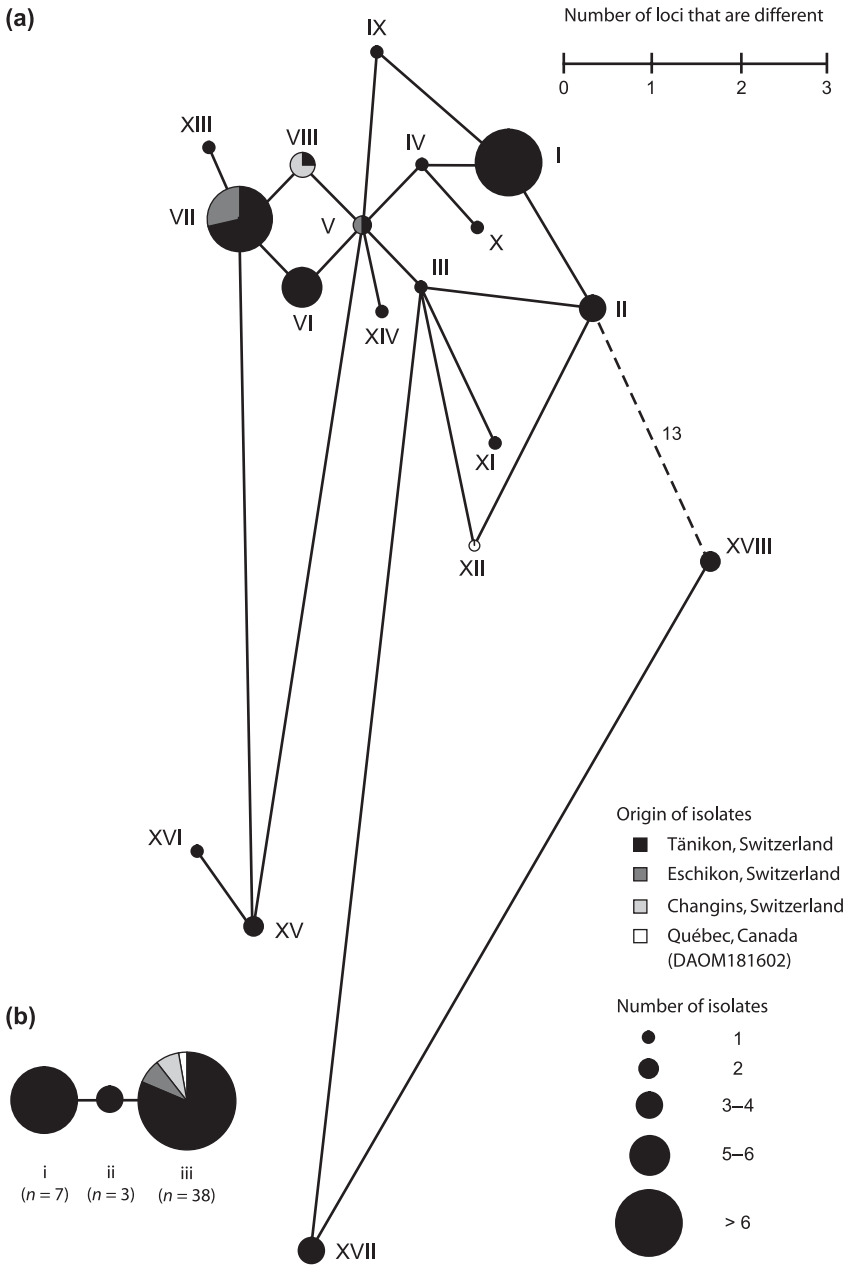

Fig. 2 (a) Minimum spanning network from 48 *Glomus intraradices* isolates based on 10 simple sequence repeats (SSR), one nuclear intron and two mitochondrial large subunit (LSU) gene introns from three Swiss locations and the Canadian isolate DAOM181602. Distances represent numbers of loci that are different between genotypes within any pair of genotypes (I–XVIII). Dashed lines represent distances between genotypes that could not be drawn to scale (numbers indicate numbers of loci that are different). Not all large distances were drawn in order to simplify the diagram. (b) Minimum spanning network based on the two mitochondrial LSU gene introns. Distances represent numbers of loci that are different between genotypes within any pair of mitochondrial genotypes (i–iii).

glasshouse before they were transferred to the *in vitro* cultures with transformed carrot roots. The experimental design in the Tänikon population and the isolation procedure allowed a test of whether host trap species identity affected which AMF genotypes were isolated. In four field plots, isolates were recovered using three different trap host species (see the Supplementary Material). Genetic differentiation among isolates originating from different trap host species was estimated within each plot by nesting trap host species identity within field plots. This hierarchical analysis corrects for the bias introduced by the genetic differentiation among field plots. We found a significant structuring of the isolate genotypes among trap host species ($F_{ST(\text{trap host–plot})} = 0.388$; $P = 0.0007$; 10 000 permutations). For a given trap host species, certain genotypes are, therefore, more likely to be recovered.

Discussion

We used newly developed SSR and mitochondrial markers to study genetic diversity in a field population of *G. intraradices*. To our knowledge, these are the first such markers to be established for AMF. In combination, the 13 loci allowed us to identify 17 distinct genotypes within one field population.

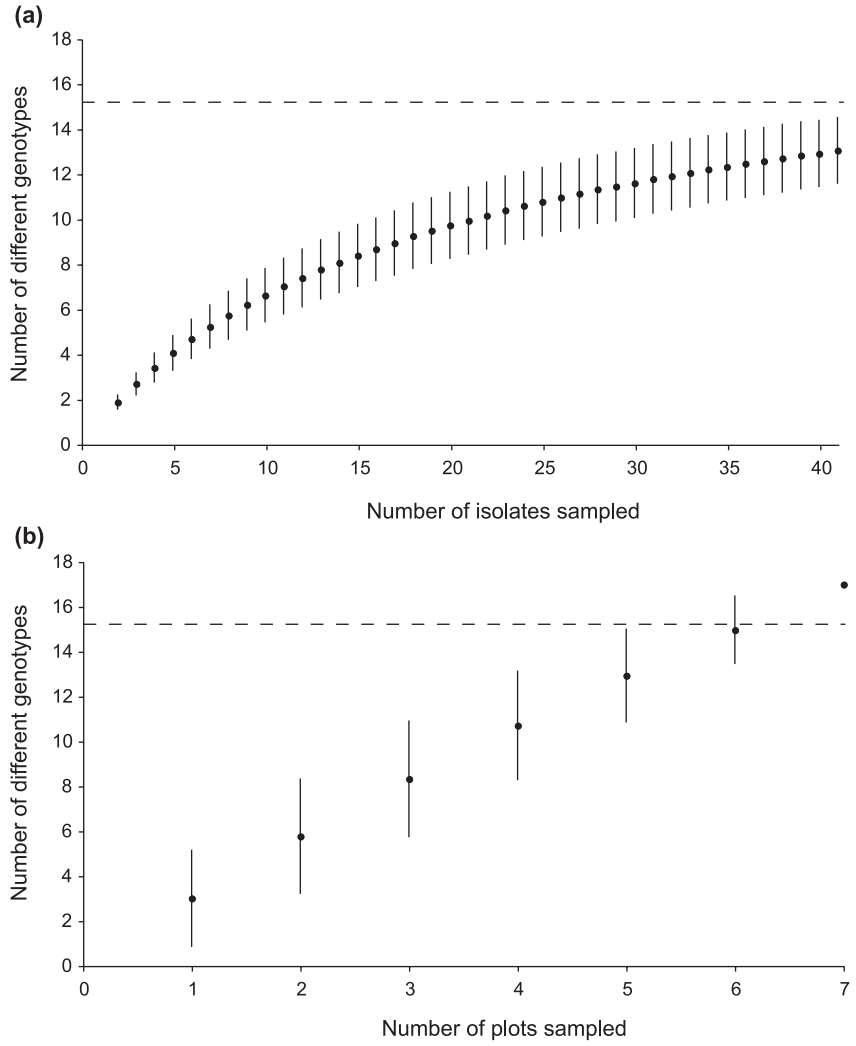

Fig. 3 (a) Relationship between the number of isolates sampled and the number of different genotypes found. Data points indicate the mean number of different genotypes found for different numbers of sampled isolates (with replacement) among all isolates from the Tänikon population. Error bars represent ± 1 SD based on a resampling procedure (10 000 permutations). (b) Sampling efficiency based on the field plot design in the Tänikon population. Data points indicate the mean number of different genotypes found in randomly sampled sets of field plots (without replacement). Error bars represent ± 1 SD based on a resampling procedure (10 000 permutations). The dashed lines represent 90% of the total genotypic diversity found in the field population.

A comparison with six isolates from two different locations in Switzerland revealed that very similar genotypes can be found in multiple locations. A comparison of ITS sequences of our isolates with sequences of closely related species confirmed that all isolates belong to the genetically diverse species of *G. intraradices*. *In vitro* cultivation allowed the extraction of pure fungal DNA in sufficient quantities to control locus specificity and reproducibility of the markers. The identification of multiple repeat motifs and additional indel mutations and single nucleotide polymorphisms within some loci suggests that allele length differences alone do not accurately reflect relatedness (see Supplementary Material). Nevertheless, the combination of the new markers permits a fine-scale genotyping of *G. intraradices* isolates. To our knowledge, this study shows for the first time the presence of polymorphism in mitochondria of a glomeromycotan species. Based on two introns of the mitochondrial ribosomal large subunit gene, we have shown that the cytoplasm of AMF is not genetically homogenous within a field population. These markers provide a basis on which to study potential associations of mitochondrial genotypes with phenotypic traits of the fungus or environmental factors.

Koch *et al.* (2004) used a subsample of nine *in vitro* isolates from the same field population and DAOM181602 to compare genetic diversity using AFLP. Four highly significant monophyletic groups of isolates were identified. This compares with seven distinct genotypes that can be identified among the same isolates based on the newly developed SSR and intron markers. There was a strong and significant correlation between genetic distances estimated by the two genotyping methods (Fig. 5). Interestingly, AFLP indicated genetic differences for pairs of isolates for which no differentiation was detected by SSR and intron markers. However, where large differences were observed between isolates using SSR markers, genetic differences were not detectable with AFLP. This is probably because at large genetic distances AFLP appear to saturate and no longer increase proportionally compared with genetic distances based on SSR and intron markers.

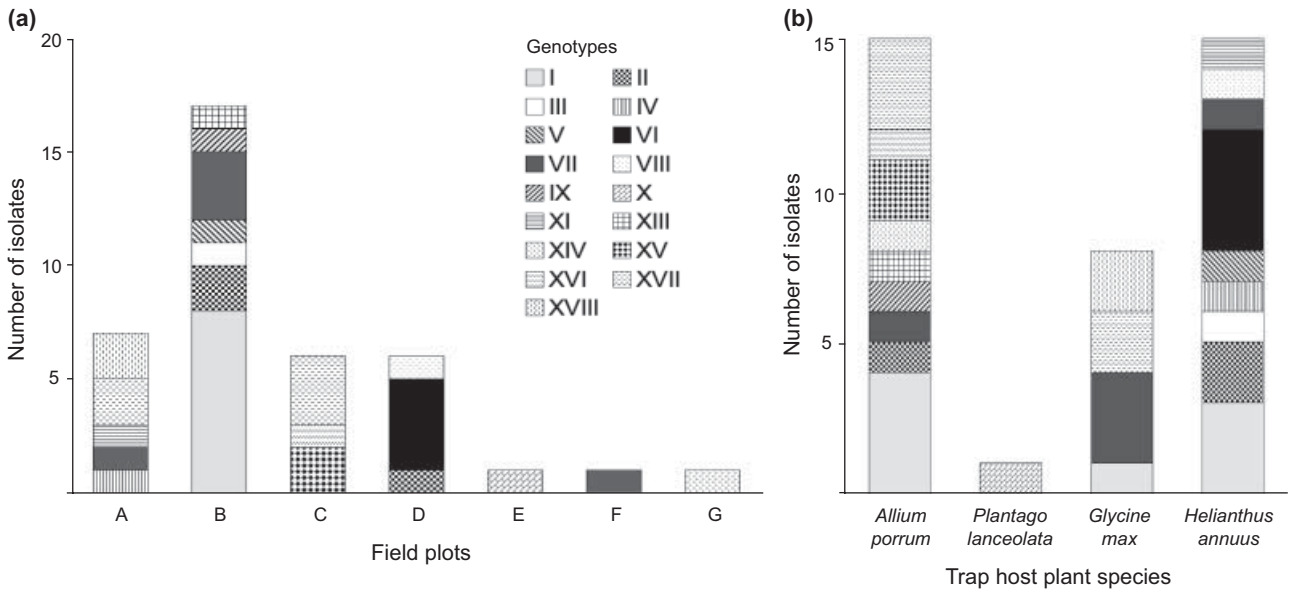

Fig. 4 (a) Number of isolates and different genotypes found in seven field plots (labelled A–G) of the Tänikon field site. Two isolates are not shown because their plot of origin is not known. Different shading indicates different genotypes. The isolates recovered from field plots E–G were not included in the statistical test for differentiation among plots. (b) Number of isolates and genotypes originating from trap cultures containing one of four different trap host species: *Allium porrum*, *Plantago lanceolata*, *Glycine max* and *Helianthus annuus*. Different shading represents different genotypes as in (a). The isolate recovered from *P. lanceolata* is shown in the graph although it was not included in the statistical test for a trap host species effect.

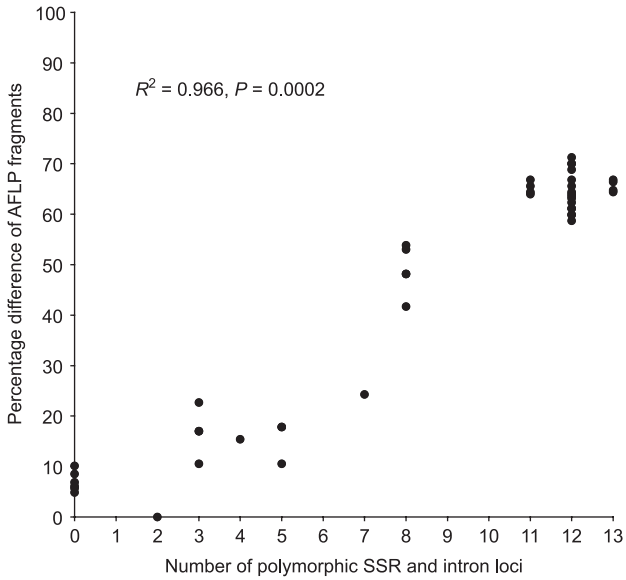

Fig. 5 Correlation of genetic distances among nine *Glomus intraradices* isolates from the Tänikon field population and DAOM181602 based on the percentage of different amplified fragment length polymorphism (AFLP) fragments (Koch *et al*., 2004) and genetic distances based on the 10 newly developed simple sequence repeats (SSR), one nuclear intron and two mitochondrial loci (number of loci that are polymorphic in pairs of isolates). Mantel test: R^2 = 0.966, P = 0.0002, 10 000 permutations.

Taken together, these results show that AFLP and SSR markers resolve fine-scale differences among isolates within populations of AMF, probably covering large sections of the genome. Nevertheless, SSR markers are advantageous because of their much lower requirements in terms of DNA quantity and the sequence specificity of the primers. Furthermore, no amplification was obtained for these loci in the most closely related species, *G. diaphanum*, suggesting that these markers are species-specific. Species specificity of markers is particularly important if soil samples are to be analysed that potentially contain a large diversity of AMF species.

Sequence-specific markers may potentially be used to address some of the remaining questions in AMF genetics concerning heterokaryosis and ploidy. In our study, only single alleles were amplified from each locus, suggesting a haploid genome and genetic uniformity among nuclei within isolates for those markers. Nevertheless, our results do not exclude the possibility that more than one allele might occur at low frequency in an isolate at an undetectable level in some nuclei. Conversely, our markers might not exhibit sufficient polymorphism to discriminate genetically diverse nuclei or different copies of a polyploid genome. Neither of these points can be directly addressed with this data set. Hybridization experiments to determine the presence and absence and numbers of markers per nucleus and the study of loci under higher mutation rates would allow firmer conclusions to be drawn regarding these issues.

The genetic diversity estimated using the seven SSR and nuclear intron polymorphisms is significantly lower than that reported by Vandenkoornhuyse *et al.* (2001) for field populations of two distantly related *Glomus* species. Meanwhile, Stukenbrock & Rosendahl (2005a) reported a slightly lower number of genotypes for three different *Glomus* species. One possible reason for this discrepancy is that different species may vary considerably in their levels of genetic diversity, but another is that markers without sequence-specific primers (such as ISSR or AFLP) were applied to single spores from field samples. Identifying contaminant sequences in spores can be difficult (Hijri *et al.*, 2002). The newly developed SSR markers are likely to be in noncoding regions, as BLAST results do not indicate significant matches in their flanking regions. Markers in noncoding regions are more likely to be selectively neutral than those in intron regions as these might be linked to genes under selection. Nevertheless, the neutrality of any noncoding region in the genome would depend on the occurrence of recombination.

Within the Tänikon field population the genetic diversity was structured among plots, meaning that genotypes were not found at random among the plots. The estimation of sampling efficiency indicates that additional field plots would probably increase the number of genotypes recovered. However, our analysis of genetic structure in the field is potentially biased by the finding that trap host plants affect the genetic diversity of recovered isolates. A further confounding factor in estimating total diversity in a field may stem from the fact that all population genetic studies need to isolate spores for genotyping. Considering the strong differences in hyphal and spore production among isolates of *G. intraradices* (Koch *et al.*, 2004), a potential bias may arise from the fact that poorly sporulating isolates may be underrepresented.

The comparison of isolates from the Tänikon population with isolates from two geographically distant locations and the isolate DAOM181602 showed that the same or highly similar nuclear and mitochondrial genotypes can be found over a distance of hundreds of kilometres, and different continents in the case of isolate DAOM181602. Considering the high degree of diversity found within the Tänikon population, such a finding is surprising. The isolate DAOM181602, identified by Koch *et al.* (2004) as belonging to one of the monophyletic groups of the Tänikon field population, can be distinguished from all other isolates based on SSR markers, but not based on mitochondrial markers. Nevertheless, the distance in the minimum spanning network between DAOM181602 (genotype XII) and the most closely related genotypes found in the Tänikon population (II and III; Fig. 2a) does not exceed the average distance between genotypes in the network. Therefore, our sampling across different locations suggests that a significant amount of genetic diversity is already found within one field population and differentiation among field populations, if any, is rather weak. A study of intercontinental diversity of AMF found very similar sequence types in temperate and tropical habitats, also suggesting that AMF may show high local diversity and weak geographic structure (Husband *et al.*, 2002).

Plant species have previously been shown to be associated with distinct AMF species assemblages (Vandenkoornhuyse *et al.*, 2003; Scheublin *et al.*, 2004), with some studies experimentally demonstrating a direct effect of the plant species on the AMF community composition (Johnson *et al.*, 1992; Johnson *et al.*, 2004). Effects on host plant growth have been shown for a subset of AMF genotypes in the Tänikon population (Koch *et al.*, 2006), indicating that there could be fitness benefits to evolving specificity with a given AMF genotype. Our study showed that particular AMF genotypes were significantly more frequently isolated depending on which trap host plant species was used for cultivation. Given the interaction between plant species and AMF genotypes, it is possible that past crop cultivation in the study field site may have had an impact on the abundance of particular genotypes. A more extensive sampling of AMF spores and a broader range of host species would be needed to allow generalizations to be made from our results. As single spores were isolated and transferred from mature trap cultures to establish single-spore cultures, the likelihood of recovering a particular isolate genotype from trap cultures is probably linked to the spore production of that genotype at the time of collection or to the preference of genotypes for a given host. Differential spore production among genotypes could result either from greater plant resources being available to a given isolate or from a competition effect caused by other AMF isolates or species present in the same trap culture. On the basis of our study, it cannot be concluded by which mechanism particular genotypes were favoured for spore production. To our knowledge, this study is the first to report preferences of host plants for particular genotypes within an AMF species, a factor that could influence the co-evolution of specific interactions between AMF genotypes and plants.

Explaining patterns of genetic diversity within AMF species remains a major challenge because of the difficulties in sampling and genotyping these fungi. To infer genetic diversity patterns at the inter-populational or global scale, underlying processes responsible for the genetic diversity at the local level need to be known. A first hypothesis for the distribution of genetic diversity is an isolation-by-distance model, postulating that dispersal of AMF genotypes is restricted in space over generations. A large proportion of offspring spores and hyphal networks would remain within close proximity of the parental mycelium, an assumption likely to be true based on current knowledge of AMF life cycles. If random survival of genotypes (i.e. genetic drift) acts over generations, spatial genetic heterogeneity may arise and be maintained through limited dispersal. Genetic diversity would, therefore, be associated with the geographic scale at which sampling is performed. More distant locations or populations would show greater genetic divergence. This hypothesis is weakened by the findings of

this study and those of Koch *et al.* (2004) because multiple isolates with very similar genotypes were sampled in geographically distant populations. Furthermore, genetic differences within the local population strongly exceeded differences among genetically distant populations. Nevertheless, a large-scale sampling of isolates of different populations in different locations is needed to confirm these findings.

A second hypothesis to explain patterns of genetic diversity concerns adaptation of AMF genotypes to host plants within populations. Taking together the finding of differential phosphate uptake of plants inoculated with different *Glomus* isolates (Munkvold *et al.*, 2004), the finding of different host plant growth depending on AMF genotypes (Koch *et al.*, 2006), and the results of our study indicating nonrandom association of isolate genotypes and host plants, there appears to be strong evidence for adaptation of AMF genotypes to host plants within populations. Therefore, the diversity of host plants in a particular field would represent a heterogeneous environment for AMF genotypes, based on preferential associations of AMF genotypes and host plant species. Such a process could lead to high genetic diversity within populations. Meanwhile, different AMF populations may not necessarily be genetically differentiated as the presence of similar host plants may have selected for similar AMF genotypes. The study by Koch *et al.* (2006) and the present study were based on a population sampled from an agricultural field subjected to crop rotation over 20 yr. These conditions may have favoured particular AMF genotypes.

A third hypothesis concerns hyphal fusions, or anastomoses, which were proposed as a potential factor shaping the evolution of genetic diversity in field populations (Sanders, 2002; Giovannetti *et al.*, 2004; Bever & Wang, 2005; Stukenbrock & Rosendahl, 2005a). It was speculated that frequent hyphal fusions among different isolates and eventual genetic exchange may erode genetic differentiation in field populations. While hyphal fusions within populations may play a role in shaping the evolution of genetic diversity, no hyphal fusions were reported among isolates of different geographical origins (Giovannetti *et al.*, 2003). Data from Stukenbrock & Rosendahl (2005a) showed strong linkage disequilibrium between alleles indicating a clonal evolution of genotypes in the three studied *Glomus* species. Data from Koch *et al.* (2004) and the present study show both strongly differentiated genotypes of *G. intraradices* and also genetically close genotypes. Anastomoses might, therefore, have played a role in shaping the genetic diversity of the field population, but the mechanism is clearly not strong enough to homogenize the different genotypes.

It would be possible to adapt SSR markers for amplification of DNA from single spores or root fragments, instead of DNA from *in vitro* cultures, using nested PCR primers based on flanking sequences of the repeat motifs. This should make it possible to carry out large-scale field studies across populations in order to study global distributions of genetic diversity and host plant associations. This would allow the testing of hypotheses about the evolution of genetic diversity within AMF species. Agricultural applications depending on the development of effective fungal inoculum should consider multiple sampling in field populations to obtain genetically diverse AMF as potential inocula and consider potential associations with particular plant species.

Acknowledgements

This work was supported by a grant from the Swiss National Science Foundation (grant number 3100AO-105790/1). We thank Jan Jansa for providing the Tänikon isolates and for help in establishing *in vitro* cultures. Swarnamala Ratyanaka and John Spalding are gratefully acknowledged for prepublication access to the Genome Survey Sequence data and for assistance with the TANDEM REPEATS FINDER program. We thank Rui Rodrigues Martins Candeias for help with culturing isolates and for extracting DNA, Beat Ruffner for help with sequencing, Jérôme Goudet for advice on data analysis and Tanja Renée Scheublin, Martine Ehinger, Alexander M. Koch and three anonymous reviewers for helpful comments on earlier versions of the manuscript.

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Supplementary Material

The following supplementary material is available for this article online:

Table S1 Summary of number of isolates from each field plot and trap host species.

Appendix S1 Alignment and description of sequence polymorphism at each locus.

Appendix S2 Electropherograms of alleles.

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