Genetic diversity of the arbuscular mycorrhizal fungus *Glomus intraradices* as determined by mitochondrial large subunit rRNA gene sequences is considerably higher than previously expected

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Received: *22 April 2008*
Accepted: *5 June 2008*

Summary

• *Glomus intraradices* is a widespread arbuscular mycorrhizal fungus (AMF), which has been found in an extremely broad range of habitats, indicating a high tolerance for environmental factors and a generalist life history strategy. Despite this ecological versatility, not much is known about the genetic diversity of this fungal species across different habitats or over large geographic scales.

• A nested polymerase chain reaction (PCR) approach for the mitochondrial rRNA large subunit gene (mtLSU), distinguished different haplotypes among cultivated isolates of *G. intraradices* and within mycorrhizal root samples from the field.

• From analysis of 16 isolates of this species originating from five continents, 12 mitochondrial haplotypes were distinguished. Five additional mtLSU haplotypes were detected in field-collected mycorrhizal roots. Some introns in the mtLSU region appear to be stable over years of cultivation and are ancestral to the *G. intraradices* clade.

• Genetic diversity within *G. intraradices* is substantially higher than previously thought, although some mtLSU haplotypes are widespread. A restriction fragment length polymorphism approach also was developed to distinguish mtLSU haplotypes without sequencing. Using this molecular tool, intraspecific genetic variation of an AMF species can be studied directly in field plants.

Key words: arbuscular mycorrhiza, Glomeromycota, *Glomus intraradices*, intraspecific diversity, mitochondrial haplotypes, molecular markers.

New Phytologist (2008) **180**: 452–465

doi: 10.1111/j.1469-8137.2008.02574.x

Introduction

Arbuscular mycorrhizal fungi (AMF) are associated with the broad majority of plant species and play an important role in mineral nutrient uptake. In exchange for photosynthates provided by the plant symbionts, the fungal partners improve the plants' access to phosphate, nitrogen and other mineral nutrients. The diversity of AMF correlates with diversity of plant communities suggesting that AMF influence competitive interactions among plants (Streitwolf-Engel *et al.*, 1997; van der Heijden *et al.*, 1998).

Molecular methods have been developed that allow identification of AMF within roots without the necessity of spore formation. In all studies to date addressing genetic diversity of AMF in roots in the field, only regions of nuclear-encoded ribosomal RNA genes have been used. Specific polymerase chain reaction (PCR) primers amplify diagnostic regions of these genes from colonized roots (Redecker, 2006). The resulting PCR products are characterized by various methods, including restriction fragment length polymorphism (RFLP) and DNA sequencing to identify the fungi. The applications of molecular identification methods in field settings have

yielded novel insights into the ecology of these fungi (Öpik *et al.*, 2006).

When using nuclear rRNA genes for phylogeny and identification of glomeromycotan fungi, the high variation among gene copies, present even within single spores of these organisms (Sanders *et al.*, 1995; Lloyd MacGilp *et al.*, 1996; Lanfranco *et al.*, 1999) impairs not only the identification of closely related morphospecies, but also differentiation of isolates within a morphospecies. Variation is more acute in the internal transcribed spacers (ITS) than in the more conserved regions of rRNA genes. For example, ITS sequences within a single spore isolate of *Glomus intraradices* were as divergent as sequences from other isolates (Jansa *et al.*, 2002b).

Intraspore rRNA gene variation could occur among rRNA gene copies in the genome of a single nucleus, as reported from other organisms (Buckler *et al.*, 1997) or among nuclei inhabiting the same cell. The genetics of multiple nuclei in the glomeromycotan mycelia is conflicting, with some evidence suggesting nuclear populations are heterokaryotic (Kuhn *et al.*, 2001; Hijri & Sanders, 2005) and other data indicating they are homokaryotic (Pawlowska & Taylor, 2004). A heterokaryotic genetic system implies absence of a fixed nuclear genotype for a fungal isolate, with populations of nuclei changing within a species. Rosendahl (2008) summarized recent progress in the budding field of AMF population biology.

Koch *et al.* (2004) characterized isolates of *G. intraradices* cultivated in root organ cultures using amplified fragment length polymorphism (AFLP), showing a high degree of genetic and phenotypic diversity among those isolates. With the exception of the Canadian isolate DAOM197198, all isolates originated from one field site in Tänikon, Switzerland (Jansa *et al.*, 2002b).

Croll *et al.* (2008) used a larger set of root organ cultures of *G. intraradices* isolates from the same field site to elucidate local genetic diversity. They used 10 simple sequence repeat (SSR) loci as molecular markers as well as introns of the mitochondrial large subunit rRNA gene (mtLSU; Raab *et al.*, 2005) and introns of a nuclear gene. Genetic diversity among fungal isolates was high, but isolates from other locations in Switzerland and Canada were not substantially different. These results complemented those of Koch *et al.* (2004) and indicated that much of the global genetic diversity of *G. intraradices* could be represented just within this field site. Multilocus genotypes also have been identified from individual field-collected spores of *G. mosseae* using markers from single copy nuclear genes GmFOX2, GmTOR2, and GmGIN1 (Stukenbrock & Rosendahl, 2005).

Mathimaran *et al.* (2008) used a set of 18 SSR markers to analyse genetic variation among eight isolates of *G. intraradices*. Only two isolates from this set appeared to be identical clones. Neither the SSR or AFLP markers listed earlier, nor the 'Single Nucleotide Polymorphisms' of Stukenbrock & Rosendahl (2005) have been applied to mycorrhizal roots from the field so far.

Mitochondrial DNA has a long history as a molecular marker (especially for the Metazoa), which precedes the era in which PCR facilitated the access to its sequences from a broad range of organisms (Bruns *et al.*, 1989). A region of the mtLSU was used so successfully for routine molecular identification of ectomycorrhizal fungal species from colonized roots that a large dataset is available for comparative study (Bruns *et al.*, 1998).

Use of mitochondrial genes avoids possible complications from heterogeneous sequences encountered with nuclear genes. Raab *et al.* (2005) provided the first sequences from the mitochondrial genome of the Glomeromycota and documented the absence of any substantial variation in an mtLSU region within isolates of *G. intraradices* and *G. proliferum*. However, sequences were polymorphic among isolates of this species. Most notable was the presence/absence of introns and sequence variation within introns. These results suggest that mtLSU sequences provide useful information that distinguishes closely related *Glomus* species as well as intraspecific variation. A practical aspect of this approach is using mtLSU data to determine haplotypes of fungal symbionts directly amplified from mycorrhizal roots, an essential criterion for population studies of nonculturable organisms. Specific primers have been designed which directly amplify mtLSU sequences from mycorrhizal roots (Raab *et al.*, 2005). Molecular analyses of field-collected mycorrhizal roots reveal high diversity and putative taxa that do not sporulate (Helgason *et al.*, 2002). However, sequence types corresponding to a few well-known morphospecies were also detected in a broad range of habitats. *Glomus intraradices* has been the most common species detected in a range of studies and it is one of the most extensively studied species in Glomeromycota. This species was found in mycorrhizal roots in habitats as different as high-input and low-input agricultural field sites (Hijri *et al.*, 2006) and species rich grasslands (Sýkorová *et al.*, 2007a) in Switzerland, phosphate-polluted sites (Renker *et al.*, 2005) and mountain meadows (Börstler *et al.*, 2006) in Germany, and geothermal soils in Yellowstone National Park, USA (Appoloni *et al.*, in press). All of this ITS sequence variation clustered phylogenetically within a clade of sequences originating from a single spore of *G. intraradices* (Jansa *et al.*, 2002b). Using more conserved rRNA gene regions *G. intraradices* was detected in grasslands of Estonia (Öpik *et al.*, 2003) and even in tropical trees in Panama (Husband *et al.*, 2002). This fungal species has been classified as a generalist because it is abundant across disturbed as well as more mature habitats (Sýkorová *et al.*, 2007a). It is widespread geographically and tolerates a wide range of habitats (Öpik *et al.*, 2006). It is also compatible with all culturing systems currently in use, from glasshouse pots to root-organ cultures (Jansa *et al.*, 2002a), and thus is one of the most common fungal components in commercial inocula (Corkidi *et al.*, 2004). Not surprisingly, therefore, it was chosen as the model AMF species for genome sequencing (Martin *et al.*, 2004). Given the importance and ubiquity of this species, a detailed understanding of population structure is essential.

Defining boundary conditions for *G. intraradices* has been problematic because variation in morphological features intergrades within and between isolates. Spore wall organization and structure is conserved and diagnostic, but number and color of layers are variable so that spore populations can vary considerably in size and color. Also, isolates can vary greatly in frequency and degree of aggregation in roots and/or soil.

This study addressed the following questions: Are mtLSU sequences polymorphic among isolates from different geographic locations? Do intron sequences provide stable markers that distinguish fungal haplotypes? An applied outcome of this work was an easy-to-use genotyping system based on mtLSU markers to study intraspecific genetic variation in the field.

Materials and Methods

Root organ cultures of *G. intraradices*

Isolate CC-4, originating from a fallow field in Clarence Creek, Ontario, Canada, was purchased from the Glomeromycota *in vitro* Collection (GINCO)/Belgium (ID codes MUCL43204, DAOM229456; for details see http://emma.agro.ucl.ac.be/ginco-bel/index.php). Isolate DAOM197198 originated from Pont Rouge, Québec, Canada, tree plantation/*Fraxinus americana* and was obtained independently from G. Bécard (University of Toulouse, France) in 1995 and from N. Requena (University of Karlsruhe, Germany) in 2005. This isolate also is known under the ID codes MUCL43194 and DAOM181602. Isolates JJ141, JJ145, and JJ183 originated from Hausweid, Tänikon, Switzerland (long-term field tillage experiment including crop rotation), and were obtained from J. Jansa (see Jansa *et al.*, 2002b). All isolates were propagated in root organ cultures (ROCs) on transformed carrot roots as previously described by Bécard & Fortin (1988). For DNA extraction, spores were dissolved in 10 mM sodium acetate–citrate buffer (pH 6.0) and washed in sterile water according to (Doner & Bécard, 1991). Croll *et al.* (2008) and Koch *et al.* (2004) used the isolate codes B7, C5, C2 and C3 for JJ291 (Raab *et al.*, 2005), JJ141, JJ145 and JJ183, respectively.

Inocula and pot cultures of *G. intraradices*

Isolates of *G. intraradices* from the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) were obtained as pot culture substrate-inoculum (see http://invam.caf.wvu.edu/index.html): AU212B (Australia), CA502 (California/USA), CR316A (Costa Rica), FL208A (Florida/USA), JA202 (Kitami Agricultural Station/Hokkaido/Japan from *Phaseolus vulgaris* crop), KE114 (Kenya), NB102C (Namibia; from a native bush in the Namib desert), SW205 (Switzerland; same strain as JJ141 contributed to INVAM, for details see ROCs above) and VA110 (Virginia/USA; soil from suburban home garden near Washington, DC). Isolate DD-4 (accession number DQ487216, nuclear-encoded small subunit rRNA gene) originated from a Dutch dry dune grassland (Provinciale Waterleidingduinen/Netherlands; 52°36′N, 4°38′E) and was obtained from M. G. A. van der Heijden (Vrije Universiteit Amsterdam) as pot culture substrate-inoculum. All pot culture substrate-inocula were stored at 4°C until DNA extraction. For isolate DD-4, a new pot culture was set up and cultured under the greenhouse conditions described in Tchabi *et al.* (2008). Substrate consisted of sterilized Terragreen (American aluminium oxide, Oil Dry US special, Type III R, < 0.125 mm; Lobbe Umwelttechnik, Iserlohn, Germany) and Loess from a local site mixed 9:1 (w : w). Approximately 1 g of contributed inoculum was layered under seeds of *Plantago lanceolata* and *Hieracium pilosella*. After 8 months of cultivation, a 10–15 ml sample from the original pot culture substrate-inocula and from the new culture of DD-4 was wet-sieved using a top sieve with 1 mm openings and a bottom sieve with 38 μm openings. The content of the bottom fraction was collected in 20 ml water, applied to a 70% (w : v) sucrose solution and centrifuged at 820 *g* for 2 min (Esch *et al.*, 1994). All organic matter suspended in the supernatant was decanted, repeatedly rinsed in a 38 μm sieve, transferred to 1.5 ml tubes, and stored on ice until DNA extraction. Single spores were collected separately, washed thoroughly in distilled water and placed in 0.2 ml tubes.

Field-collected root samples

The DNA extracts of mycorrhizal roots collected from the 'Ramosch'-meadow in the Engadin region of Switzerland were provided by Z. Sýkorová (Sýkorová *et al.*, 2007b). Samples S6 (*Trifolium* sp.) and S10 (*Trifolium* sp.) correspond to the samples 11-2v and 11-3a, respectively, of Sýkorová *et al.* (2007b) in the Supporting Information, Table S1. DNA extracts from samples of two *Plantago lanceolata* mycorrhizal root systems originating from the Gyöngyösoroszi mine spoil in the Matra mountains of Hungary (47°50′44″N, 19°53′05″E) were provided by I. Parádi (Eötvös Loránd University, Budapest, Hungary). These two samples were designated da2 and da4.

DNA extraction

The DNA of 1–15 spores was extracted according to Redecker *et al.* (1997): spores were crushed in 2 μl 0.25 N NaOH, heated to 95°C, and incubated for 2 min. One microliter of 0.5 M TrisHCl (pH 8.0) and 2 μl 0.25 N HCl were added to this crude extract, which was heated again for 2 min and then used directly as PCR template. DNA was extracted from: spore populations (> 15); 50–80 mg (wet weight) of the organic fraction extracted from inocula/pot culture substrates and 50–80 mg (wet weight) of plant roots using the DNeasy plant mini kit (Qiagen, Hilden, Germany). Liquid nitrogen was used to grind frozen root samples. Depending on success of amplification, extracts were further diluted 1:10 and 1:100 in

Table 1 The sequences of RNL primers used to amplify regions of the mitochondrial rRNA large subunit gene (mtLSU) gene

RNL primer	sequence	RNL primer	sequence
1*	AGACCCGAARCCWRGTGATCT	31	TTMGTGCCGCCACTTATTAG
2*	GGRAASAGCCCAGAAYA	33	CTGCCCTATAGAAGAGTTAC
2c	TCGTGATAAGGCGATTCGTC	35	TAACCCCTCAACGACCACAC
3*	TGCATMATGGGTCAGCGAGT	36	CACCTGTTCTGGGCTGTTGC
5*	GAGCTTCCTTTGCCATCCTA	36b	CACCAGTTCTGGGCTGTCGC
7*	CTTCTGCTTTCGGCGAAGAG	37	TAGCTGGGCTAAGAATGCTG
7b	CAGCTATGTCCACCGGCTCA	38	AGCTTGGACTAACCCACTAATG
9*	CAGTAAAGCTGCATAGGGTCT	39	CGGACCTATTGCCCAATACT
10*	AGAAGAAAGAGCTGGCTGTG	40	CCTAGAAGTGCTGGCGTGTT
10h	CCTAATAATCCTCCTACAAG	41	GGCTCTTTCCYGAACCTTAC
11*	AAGGCAACACGCCAGCACTT	42	TAGTACACTGCTAAGCTAGA
11b	AGGGCAACACGCCAGCACTT	43	CCCCTAACAGTTAATAACTC
12*	GATAGCGTAACAGCTCAGTG	44	CACTGCTAAGCTAGATTACT
12b	GATAGGGTAACAGCTCAGTG	45	ATACTCACCCTACCCTTACC
13*	TGGTCGATGGACGACGGATA	46	CCGACACAGGTCTGCAAGTA
13d	AGATCGMCGTAGTTCCTTCT	51	GATTCTGAAAGACGGAATAG
14*	AGGATAGGCCTGGAAACCAAGC	52	GGTTTTGCGTACGTAAGAGT
15*	CTGAGCTGTTACGCTATC	53	CATGGGTGTTGCTCTCAAATC
16*	ACCTGGAGATAGCTGGTCTT	54	AGCAACACCCATGTGCAAGT
17*	CCATAGAGTTGGCTCTAACA	55	ACTCTTACGTACGCAAAACC
17h	GCACGGAATTGAACCRTAGAG	57	CCTGTTAGGCGTACCTATGCC
24	GAGCATACTAAGGCGTAGAG	58	TAACCCATAATGCTCATCTA
25	ATCAGTGGGGAGAGGACAGT	59	GGAGATCATTGGGGTTATCC
26	CTTGTGCAAGTAGGCCTTCT	60	TAAGTAGGTGGACATAACTG
27	CCAACTATGCAACCGTAGG	61	GCCTAACCCTTCCCTATAGA
28a	CCATGGCCAAGTGCTATTTA	62	CCAGTGCCGTACCGTCTAGT
29	TAATAAGACTGAACGGGTGT	63	CATTATATGCTCCGGCGTAG
30	TAGCATCGGGCAGGTATCAG	64	AAGCACGGAATTGAACCATA

Shaded primers were used for the nested polymerase chain reactions; the others were used for sequencing.
*Primers designed by Raab *et al*. (2005).

TE (Tris-ethylenediaminetetraacetic acid) buffer and used again as PCR template (Table S1).

PCR amplification of mtLSU

Isolates CC4, DAOM197198 and JJ183 were amplified by nested PCR as described in Raab *et al*. (2005) with slight changes. For the first reaction the primer pair consisted of RNL-3 and RNL-9. Cycling parameters were 3 min at 95°C, 34 cycles of 1 min at 95°C, 1 min at 51°C, 4 min at 72°C, and finally 10 min at 72°C (parameter type 1). For the second reaction the primer pair was RNL-1 and RNL-5. Cycling parameters were 3 min at 95°C, 34 repeats of 1 min at 95°C, 1 min at 56°C, 4 min at 72°C and a final elongation of 5 min at 72°C (parameter type 2). The *Taq* polymerase from GE Healthcare (Otelfingen, Switzerland) included 2 mM $MgCl_2$, 0.5 μM of each primer and 0.25 mM of each desoxynucleotide in the master mix.

Based on these results and data from Raab *et al*. (2005), new primers with improved specificity were developed and applied in a nested PCR approach that was used for most other samples. Forward and reverse primers RNL-28a and RNL-5, respectively, were used in the first reaction and RNL-29 and RNL-30, respectively, in the second reaction (for primer sequences, see Table 1). *Taq* polymerase (master mix see above) or Phusion High-Fidelity DNA Polymerase from FINNZYMES (BioConcept, Allschwil, Switzerland) including 1× Phusion HF Buffer, 0.5 μM of each primer and 0.2 mM of each desoxynucleotide in the master mix, were used. Parameter type 1 (Table S1) was applied for both reactions when using *Taq* polymerase. Cycling parameters were changed when using Phusion polymerase: 30 s at 98°C, 33 cycles of 10 s at 98°C, 30 s at 55°C and 2 min at 72°C, followed by 10 min at 72°C (parameter type 3). Parameter type 4 (30 s at 98°C, 34 cycles of 10 s at 98°C, 30 s at 60°C, 2 min at 72°C, and 5 min at 72°C/Phusion polymerase) and parameter type 6 (see next paragraph) were used for exceptions (details summarized in Table S1). DNA was extracted at least twice from each isolate. If DNA was extracted from organic matter, results were confirmed by PCR products from spores in all isolates except JA202, NB102C and SW205.

PCR amplification of nuclear-encoded ITS rDNA

A nested PCR was performed according to Redecker (2000). The universal eukaryote primer pair NS5/ITS4 was used for

the first PCR reaction (White *et al.*, 1990). The *Glomus* group A-specific primer pair GLOM1310/ITS4i was used in the second PCR reaction. The *Taq* polymerase master mix contained following concentrations: 2 mM $MgCl_2$, 0.5 μM of each primer and 0.125 mM of each desoxynucleotide. Parameter type 5 cycling conditions for the first reaction were as follows: 3 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 51°C, 2 min at 72°C, and a final extension phase of 10 min at 72°C. The second nested step was performed under the same conditions, but with an annealing temperature of 61°C (parameter type 6). Depending on success of PCR amplification, 1 μl PCR product of the first reaction was used undiluted or at dilutions of 1:10 or 1:100 (in TE buffer) as template for the second reaction.

Cloning, sequencing and sequence analyses

The PCR products were purified using the High Pure Kit from Hoffmann LaRoche (Basel, Switzerland) and cloned into the pGEM-T vector (Promega/Catalys, Wallisellen, Switzerland) following the manufacturer's protocol. Before cloning, blunt-ended PCR products based on Phusion polymerase were incubated at 72°C for 13 min using *Taq* polymerase, 2 mM $MgCl_2$ and 0.125 mM dATP for adding 3′-adenines. Clones of mtLSU rDNA were amplified using the respective PCR primers of the second nested step or the vector primers M13fwd (GTA AAA CGA CGG CCA GTG) and M13rev (GGA AAC AGC TAT GAC CAT G). Products were purified and sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit (ABI, Foster City, CA, USA) and an ABI 310 capillary sequencer. Sequencing primers for isolates differing from the sequencing set for JJ291 (Raab *et al.*, 2005) are provided in the Supporting Information, Table 2. Complete sequences of the isolates JJ141 and JJ145 were composed by sequenced clones and directly sequenced PCR products (Tables S1 and S2). Clones of nuclear encoded ITS rDNA were sequenced in both directions using the primers of the second nested step or alternatively the universal forward primer ITS1F (CTT GGT CAT TTA GAG GAA GTA A) instead of GLOM1310. Sequences of mtLSU rDNA were aligned and corrected in BIOEDIT (Hall, 1999), sequences of the ITS rDNA were edited in SEQUENCE NAVIGATOR (version 1.0.1). Alignments were performed in BIOEDIT (Hall, 1999) and in PAUP* 4.0b10 (Swofford, 2001). DNA sequences were submitted to the European Molecular Biology Laboratory (EMBL) database under the accession numbers AM950203 to AM950227, and AM980833 to AM980863.

Isolates available as soil inoculum were extracted, amplified and analysed by RFLP or sequencing at least twice (Table S1).

Phylogenetic analyses

Phylogenetic trees were inferred using distance, parsimony or maximum likelihood criteria as implemented in PAUP*. Neighbor joining or heuristic search algorithms were applied for the respective criteria. Maximum likelihood models and parameters were estimated using MODELTEST 3.5 (Posada, 2004). In addition, Bayesian analyses were performed using MRBAYES 3.1.1 for Macintosh (Ronquist & Huelsenbeck, 2003).

Insertions and deletions in the introns were coded by appending binary characters (1 for deletion, 2 for insertion) to the sequence matrix. Each deletion of more than three bases was coded, resulting in 21 binary characters added to the whole dataset. Regions of exons and introns that could not be aligned unambiguously were excluded from the analyses. Phylogenetic networks were obtained using SPLITSTREE 4.8 (Huson & Bryant, 2006). The Neighbor Net option using uncorrected distances and equal angles was chosen.

RFLP analyses

Based on virtual restriction patterns of the sequence data of the mtLSU rDNA, a RFLP system was established in order to distinguish different sequence types. For RFLP analyses, 20 U *Dra*III, 2.5 U *Bsa*JI from New England Biolabs (BioConcept, Allschwil, Switzerland) and 2.5 U *Hin*dIII from MBI Fermentas (LabForce, Nunningen, Switzerland) were used per sample, respectively. For each reaction 8 μl of the final nested PCR products were digested overnight at 37°C (*Bsa*JI samples at 60°C) in a total volume of 15 μl. For visualization, 1.5% agarose gels (1% SeaKem-0.5% NuSieve; Cambrex Bio Science, Rockland, ME) were loaded with the total volume of the digestion products and run at 100 V for 1 h. Fragment lengths were determined using QUANTITY ONE (version 4.1.0). The RFLP patterns were compared with the virtual patterns using a modified spreadsheet developed by Dickie *et al.* (2003).

Results

Diversity of ITS sequences and mtLSU haplotypes in *G. intraradices* isolates

Sixteen isolates of *G. intraradices* originating from geographic locations on five continents were analysed. These isolates included two strains (JJ291 and BEG75) previously studied by Raab *et al.* (2005). In ITS-based phylogenies (Fig. 1), most isolates grouped into a clade that included sequences originating from a single spore of isolate JJ291. This group is designated as '*G. intraradices* main clade' because members have been used to genetically define the species in field studies (Hijri *et al.*, 2006; Sýkorová *et al.*, 2007a). Isolate FL208A from Florida (USA), showed a closer relationship to *G. proliferum*, clustering with sequences from the same isolate previously obtained by another group (P. Sudarshana *et al.*, unpublished), and with sequences from isolates VA110 and KS906 from Virginia and Kansas (USA), respectively. Isolate VA110 also was analysed in the present study, but the resulting sequences did not cluster with FL208A. Instead, it showed a close relationship

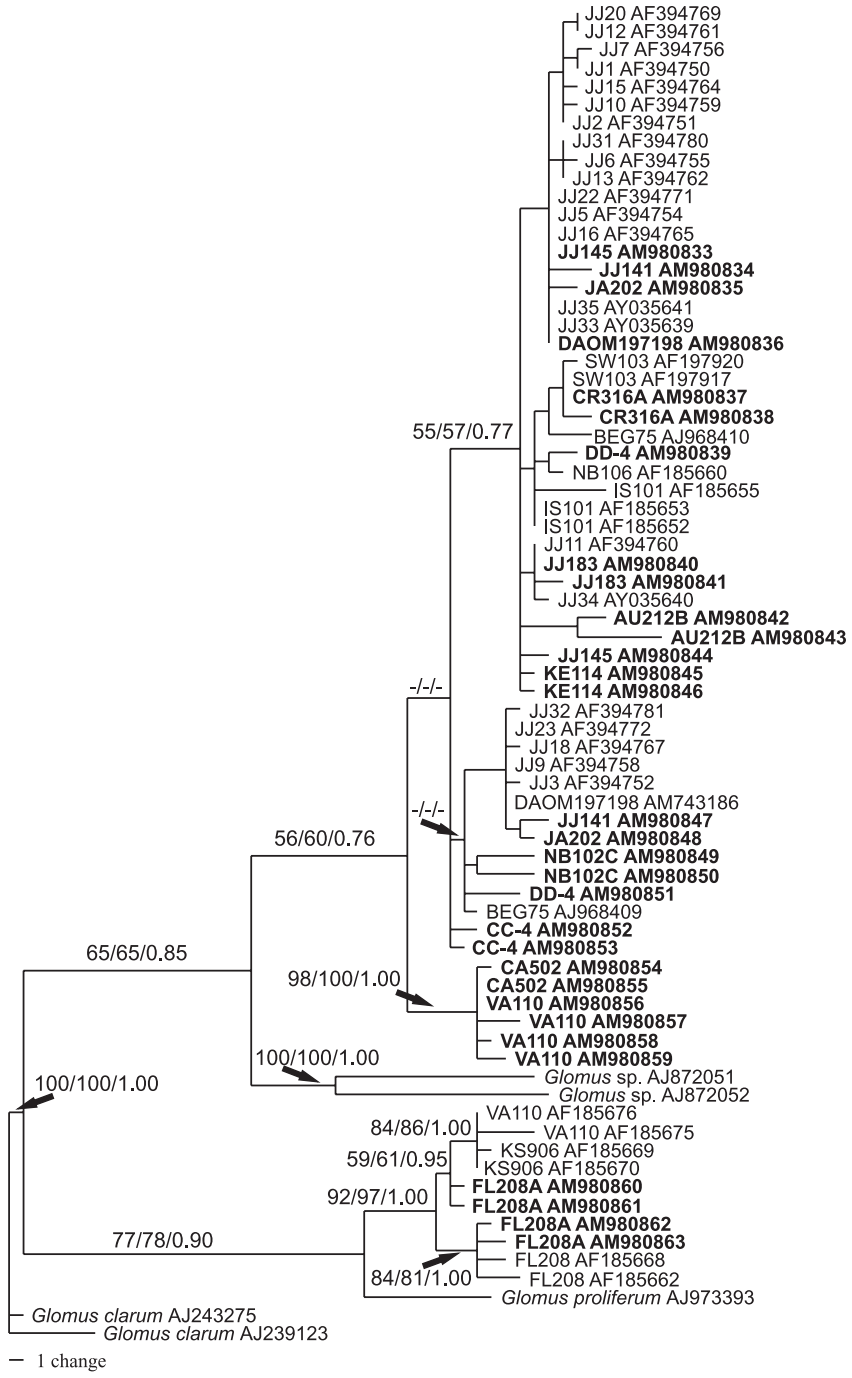

Fig. 1 Phylogenetic tree of *Glomus intraradices* isolates based on 5.8S rDNA and ITS2 sequences, with *Glomus clarum* as outgroup. The sequences JJ1 to JJ32 originated from a single-spore culture of *G. intraradices* JJ291 (Jansa *et al*., 2002b). The phylogenetic tree was generated following alignment of 300 characters and a heuristic search under the maximum parsimony criterion. Values at the nodes indicate: parsimony bootstrap values from 1000 replicates, neighbor-joining bootstrap values from 1000 replicates and Bayesian posterior probabilities. For clarity of the figure, only the values above 50% for the first five nodes from the root are provided. Sequences in bold type were unique to this study.

with CA502 from California (USA). The cluster containing VA110 and CA502 showed a tendency to group outside the *G. intraradices* main clade in distance and parsimony analyses, but this was not supported by bootstrap values or in Bayesian analyses. Sequences AJ872051 and AJ872052 constitute a clade of environmental sequences (Hijri *et al*., 2006) which is a clearly separated sister group to the *G. intraradices* main clade. Generally, bootstrap and posterior probability values were relatively low, which may be caused by a very strict alignment, which left only 300 bp for analyses. Nevertheless, the topology of the tree was highly consistent between analyses. Omitting the VA110/CA502 sequences raised the bootstrap value of the *G. intraradices* main clade, indicating that unresolved sister clades may deteriorate the support for the main clade. To confirm that ITS and mtLSU sequence data originate from the same fungal genotype, sequences were amplified from the same spore for several isolates (Table S1).

Table 2 Mitochondrial rRNA large subunit gene (mtLSU) rDNA sequence structure of *Glomus intraradices* isolates and root colonizing *G. intraradices* (shaded) within the priming sites of RNL-29/RNL-30

	Clones		Introns			Exon region	
Isolate/plant sample (Origin)	Accession number	Haplotype	Pos. 1 (-type, bp)	Pos. 2 (-type, bp)	Pos. 3 (-type, bp)	Complete/parts between intron positions (bp)	Fragment length (bp)
DAOM-197198 (Canada)	AJ841804	I	1-1, 1057	2-1, 401	No	1073/363, 672, 38	2531
	AJ841808**		1-1, 1058	2-1, 401	No	1063/363, 670, 30	2522
	AM950203		1-1, 1057	2-1, 401	No	1071/363, 670, 38	2529
JJ291* (Switzerland)	AJ973189		1-1, 1056	2-1, 401	No	1071/363, 670, 38	2528
	AJ973190		1-1, 1056	2-1, 401	No	1071/363, 670, 38	2528
	AJ973192		1-1, 1057	2-1, 401	No	1071/363, 670, 38	2529
CC-4 (Canada)	AM950204	II	No	2-2, 415	3-1, 809	1071/363, 670, 38	2295
	AM950205		No	2-2, 415	3-1, 808	1071/363, 670, 38	2294
	AM950206		No	2-2, 415	3-1, 809	1071/363, 670, 38	2295
CR316A (Costa Rica)	AM950207		No	2-2, 415	3-1, 809	1071/363, 670, 38	2295
BEG75* (Switzerland)	AJ938171	III	No	2-2, 414	No	1071/363, 670, 38	1485
	AJ938173		No	2-2, 414	No	1071/363, 670, 38	1485
	AM040984		No	2-2, 414	No	1071/363, 670, 38	1485
JJ141 (Switzerland)	AM950208	IV	1-2, 1107	2-1, 401	3-2, 1356	1071/363, 670, 38	3935
JJ145 (Switzerland)	AM950209		1-2, 1107	2-1, 401	3-2, 1356	1070/363, 669, 38	3934
JJ183 (Switzerland)	AM950210	V	1-3, 1094	2-3, 389	No	1071/363, 670, 38	2554
	AM950211		1-3, 1094	2-3, 389	No	1071/363, 670, 38	2554
	AM950212		1-3, 1094	2-3, 389	No	1071/363, 670, 38	2554
DD-4 (Netherlands)	AM950213	VI	1-4, 425	2-4, 303	3-3, 944	1067/363, 666, 38	2739
AU212B (Australia)	AM950214	VII	No	2-5, 430	3-4, 850	1071/363, 670, 38	2351
JA202 (Japan)	AM950215	VIII	No	2-1, 401	No	1071/363, 670, 38	1472
KE114 (Kenya)	AM950216	IX	No	No	No	1070/363, 670, 37	1070
NB102C (Namibia)	AM950217	X	1-1, 1057	2-6, 339	3-1, 809	1071/363, 670, 38	3276
CA502 (California)	AM950218	XI	1-5, 489	2-7, 233	3-5, 667	1087/377, 672, 38	2476
VA110 (Virginia)	AM950219		1-5, 489	2-7, 233	3-5, 667	1088/377, 673, 38	2477
FL208A (Florida)	AM950220	XII	1-6, 662	No	No	1124/358, 728, 38	1786
Plantago lanceolata da4 (Hungary)	AM950221	XIII	1-7, 444	2-4, 303	3-6, 739	1067/363, 666, 38	2553
Trifolium sp. S6 (Switzerland)	AM950222	XIV	1-7, 444	2-4, 303	No	1067/363, 666, 38	1814
	AM950223		1-7, 444	2-4, 303	No	1067/363, 666, 38	1814
Trifolium sp. S10 (Switzerland)	AM950224		1-7, 445	2-4, 303	No	1067/363, 666, 38	1815
	AM950225		1-7, 444	2-4, 303	No	1067/363, 666, 38	1814
Plantago lanceolata da2 (Hungary)	AM950226	XV	No	2-4, 303	3-3, 944	1068/363, 667, 38	2315
	AM950227	XVI	1-3, 1094	No	3-7, 938	1067/363, 666, 38	3099
*Festuca pratensis** (Switzerland)	AJ841288	XVII	1-8, 1108	No	No	1069/366, 665, 38	2177
	AJ841289		1-8, 1108	No	No	1068/366, 664, 38	2176

Haplotypes and intron types were distinguished by sequence differences. Introns containing putative open reading frames for LAGLIDADG are shaded.

*Raab *et al*. (2005); **sequence incomplete at 3′-end.

Among the 16 cultivated isolates, 12 mitochondrial haplotypes were distinguished (Table 2). An additional five haplotypes were identified in five root samples. The exon–intron structure of the gene region among the isolates is graphically depicted in Fig. 2. The length of the analysed region of the mtLSU varied between 1070 bp and 3935 bp among isolates because of the presence/absence of introns at three locations, and considerable length variation within introns. One isolate, KE114 from Kenya, did not contain any of the three introns. Isolate DAOM197198 from Canada, which is used in the genome sequencing project, grouped with isolate JJ291 from Switzerland in haplotype I. Two additional haplotypes were found in isolates JJ183, JJ141 and JJ145, which originated from the same field site as JJ291 in Switzerland. In addition to DAOM197198/JJ291, three pairs of isolates showed the same haplotypes, respectively (JJ141/JJ145, VA110/CA502, CC4/CR316A).

Sequence polymorphism of the introns correlated with length polymorphism. In other words, variation in intron lengths mirrored differences in intron sequences (Fig. 2, Table 2).

In order to further confirm sequence homogeneity of the mtLSU rDNA within the same isolate (Raab *et al*., 2005), at least three cloned PCR products obtained from two different

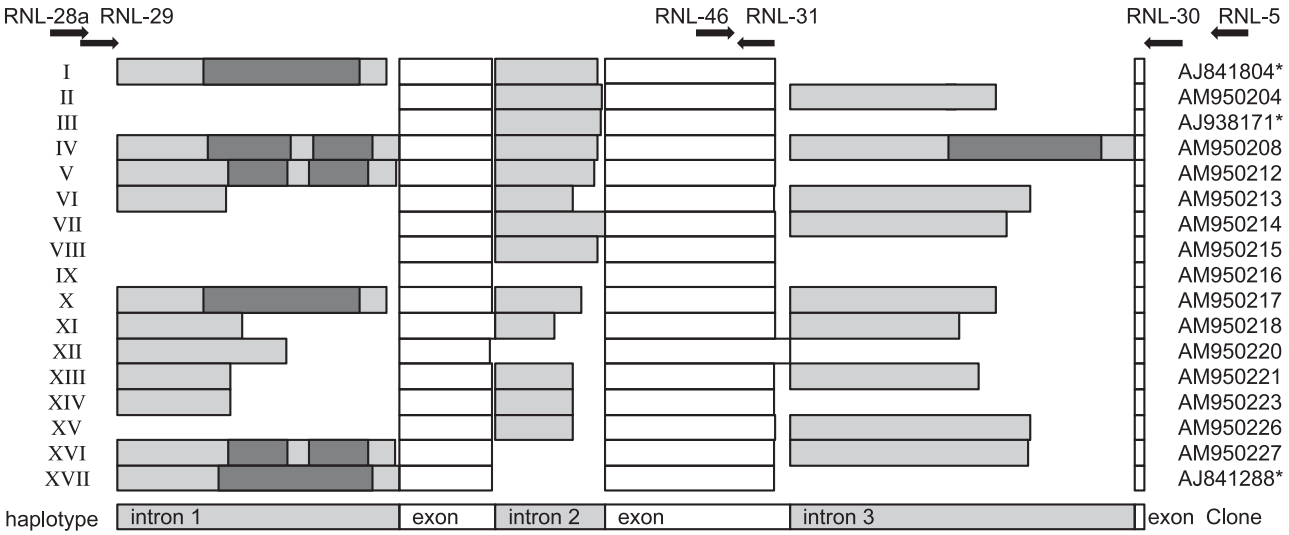

Fig. 2 Organization of the mitochondrial rRNA large subunit gene (mtLSU) gene region containing three exons and two to three introns for *Glomus intraradices* haplotypes I–XVII in 5′–3′ orientation. Introns are shaded in light grey and putative LAGLIDADG open reading frames (ORFs) in dark grey. Arrows show location and orientation of primers. *, Clones from Raab *et al*. (2005). Approximately to scale.

ROC plates were sequenced for each of the isolates CC-4, JJ183 and DAOM197198. The clones (Table S1) differed from the consensus sequence on average by 0.35% (CC-4) 0.24% (JJ183) and 0.28% (DAOM197198), which is within the range of the misincorporation error of *Taq* polymerase (Cline *et al*., 1996).

MtLSU exon and intron phylogeny

The MtLSU exon sequences clearly separated the FL208A isolate from all other *G. intraradices* isolates in the phylogenetic tree (Fig. 3), a pattern that was in agreement with ITS phylogeny. Isolate FL208A grouped closer to *G. proliferum*. Isolates CA502/VA110 and two sequences obtained from colonized roots of *Festuca pratensis* in a calcareous grassland (Raab *et al*., 2005) also grouped in clades with high bootstrap support. The exon and intron sequences of CA502 and VA110 did not differ by more substitutions than expected from *Taq* polymerase error.

All other *G. intraradices* isolates were quite similar in their mtLSU exon sequences (Fig. 3). A subclade containing mostly environmental sequences from grasslands received some bootstrap support in the exon tree (Fig. 3) and was also distinct in intron length (Fig. 2) and sequence (Table 2). Position 2 introns all were 303 bp in length and differed by only a few point mutations. All position 1 introns in this subclade were 425–444 bp long and lacked an open reading frame (ORF) for a homing endonuclease, which was detected in other isolates (see below).

Overall, sequences of the position 2 intron showed considerable similarity. This intron was present in 14 of 16 isolates and 6 of 9 environmental sequences. Phylogenetic analysis of the position 2 intron (Fig. 4) confirmed trends obtained from exon sequences. NeighborNet networks were used to provide more detailed visualization of any potential conflicts between intron phylogenies that might be caused by reticulate evolution. Four major groups of isolates were distinguished: the group of predominantly environmental sequences discussed above; a clade comprising all Tänikon isolates, DAOM197198 and JA202; a clade comprising BEG75, CC4 and CR316; and CA502/VA110. Some isolates did not fall into any of these groups, such as NB102C and AU212B. Isolate NB102C was not positioned on a distinct branch in the NeighborNet network in Fig. 4, possibly because the sequence region distinguishing the BEG75 and Tänikon isolate group was missing in this isolate.

A position 1 intron occurred in 10 of 16 isolates and 8 of 9 environmental sequences. A number of sequences containing this intron contained ORFs for homing endonucleases of the LAGLIDADG 2 type (Dalgaard *et al*., 1997). Furthermore, a LAGLIDADG type 1 ORF was found in isolates JJ141 and 145 in a position 3 intron. The position 2 intron did not contain any putative ORFs. Interestingly, some ORFs consisted of two regions separated by a putative noncoding sequence (Fig. 2). Phylogenetic analysis of position 1 intron sequences (Fig. 5) indicated that they were homologous. Some isolate groups described above were verified and the group comprising the Tänikon isolates and DAOM197198 was differentiated further into subgroups. Isolate NB102C associated closely with DAOM197198/JJ291, clarifying its ambiguous grouping in Fig. 4.

The position 3 intron was present in only 56% of the isolates. Although highly polymorphic in the central region, sequences of this intron were homologous in regions adjacent to the exons. In JJ141/JJ145, this intron contained an ORF for a type 1 homing endonuclease. Conflicts among exon and intron

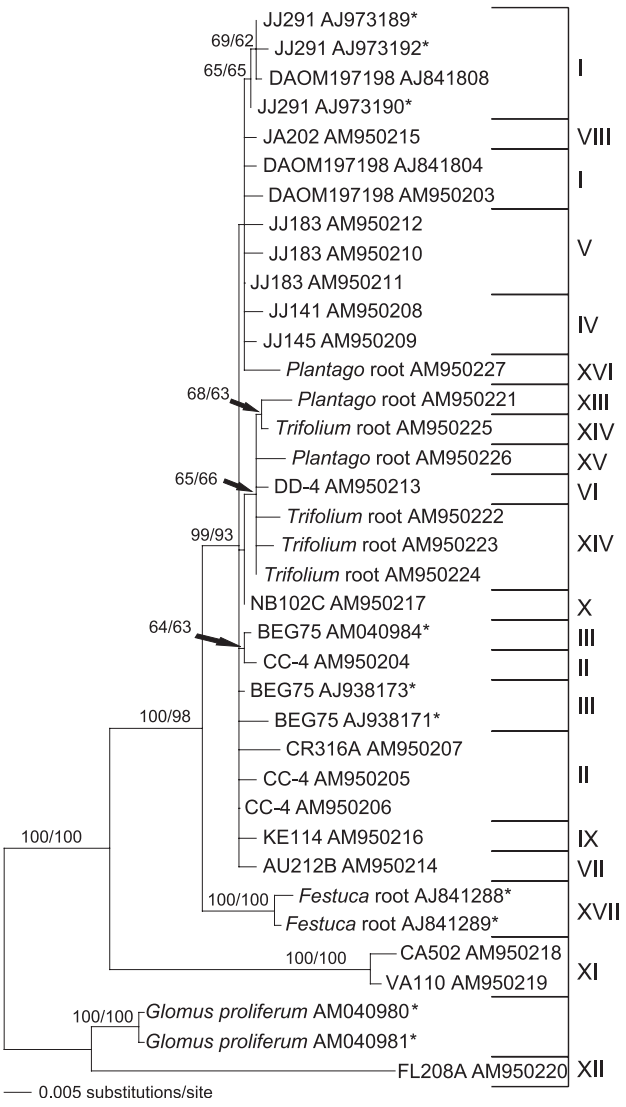

Fig. 3 Phylogeny of *Glomus intraradices* isolates and *Glomus proliferum* based on mitochondrial rRNA large subunit gene (mtLSU) exon sequences. The tree was rooted by midpoint rooting. Roman numerals indicate mtLSU haplotypes. The phylogenetic tree was obtained from 943 characters using a heuristic search under the maximum likelihood criterion. Values on the nodes indicate: neighbor-joining bootstrap values from 1000 replicates and maximum parsimony bootstrap values from 1000 replicates. Sequences from cultured isolates of *G. intraradices* are labeled with isolate codes and accession numbers. For sequences from roots, host species and accession number are indicated. *, Sequences from Raab *et al*. (2005).

phylogenies were not detected, ruling out frequent transfer of these noncoding regions that could impair their use as intraspecific molecular markers.

Intron stability in the mtLSU of *G. intraradices*

Comparisons of multiple culture lineages of the same isolates did not reveal any changes in the intron length or sequence of any isolate. For example, two lineages of isolate DAOM197198 were identical in both exon and intron sequences, even though one was obtained directly from G. Bécard (Toulouse, France) and has been propagated in the Botanical Institute in Basel since 1995 and the other was obtained from N. Requena (Karlsruhe, Germany) in 2005. The same result was obtained for two lineages of JJ291 cultivated independently over a 2-yr period. JJ141 and SW205 are two culture lineages originating from the same isolate and showed identical haplotypes. SW205 was pot cultured repeatedly at INVAM, whereas JJ141 was obtained as root organ culture. Identical haplotypes in populations from geographically distant locations (Switzerland/Canada, Virginia/California, Costa Rica/Canada) provided additional evidence for stability of markers.

Detection of haplotypes in field-collected roots

The mtLSU region could be amplified from field-collected roots using the improved primer combinations RNL-28a/RNL-5 and RNL-29/RNL-30. All amplified sequences clustered in the *G. intraradices* clade (Fig. 3). This result clearly demonstrated high primer specificity for the target clade. Interestingly, grassland isolate DD-4 was the only cultivated genotype clustering in the exon clade, which contained most environmental sequences from grassland communities. Among root samples, only one from *Plantago lanceolata* (sample code da2) yielded sequences of two different haplotypes (XV and XVI, Table 2).

RFLP analyses

From sequence data of the DNA region between primers RNL-29 and RNL-30, a combination of restriction enzyme sites were identified that unambiguously separated mtLSU haplotypes without sequencing (Table S3). A single conserved target site for *Dra*III was present in each mtLSU rDNA sequence. The restriction site was located in the exon region adjacent to the 5′-insertion site of position 2 intron. Restriction sites for *Bsa*JI were more abundantly distributed, but situated mainly nearer the 5′ ends of sequences. Restriction sites for *Hin*dIII are more abundant near the 3′ end of the DNA region.

Use of *Bsa*JI alone distinguished all haplotypes, whereas *Dra*III or *Hin*dIII clearly identified 59% and 71%, respectively, of these haplotypes. To avoid ambiguous identification as a result of similar restriction patterns, using all three enzymes provided the most accurate assessment of haplotypes.

This RFLP approach was applied successfully to all isolates from INVAM, isolate DD-4 and all of the other sample types in this study (see Table S1, selected examples in Fig. 6). RFLP patterns were diagnostic for all of the haplotypes present in this study.

Discussion

We showed in this study that intraspecific groups within *G. intraradices* were resolvable by variation and organization

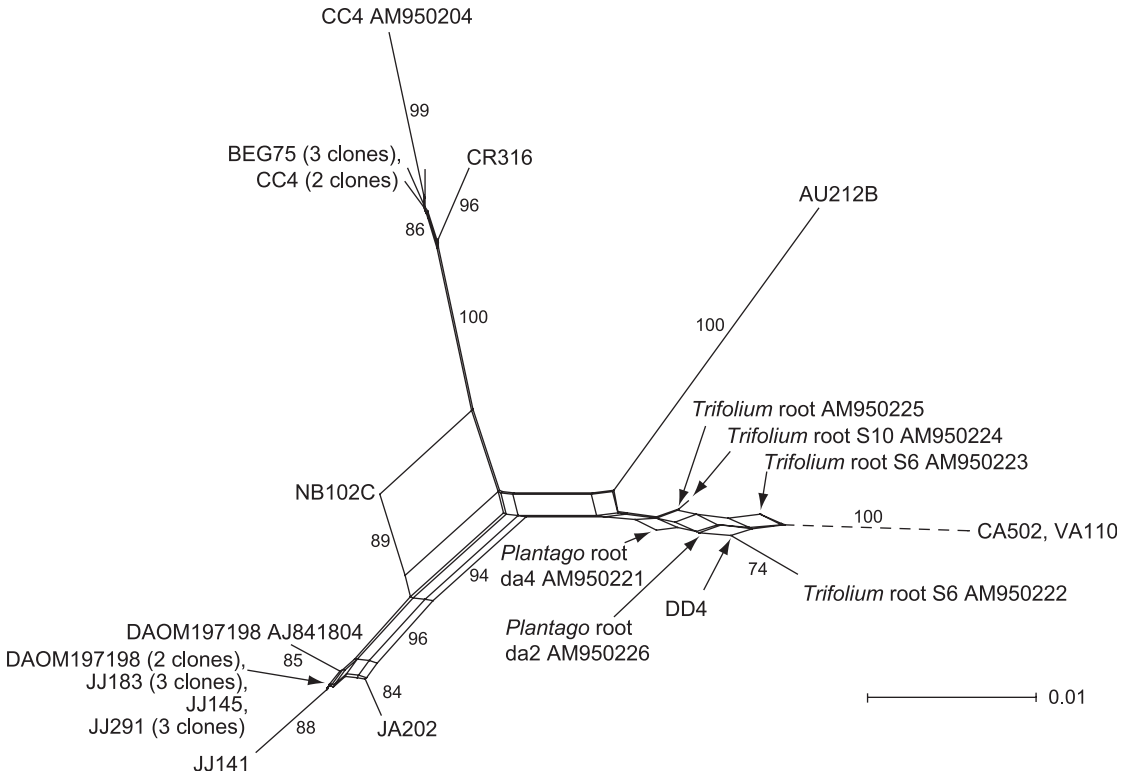

Fig. 4 NeighborNet network obtained from sequences of the position 2 intron. The dashed line indicates a branch that was reduced in length by a factor of 10 to improve readability of the figure. Numbers on the branches are bootstrap values from 1000 replications.

in a region of the mtLSU gene. Both exons and introns of this region provided stable molecular markers to identify haplotypes of this species from spores, colonized roots from field sites and even root fragments and mycelium mixed with organic matter obtained by sucrose extraction from pot cultures.

The AFLP markers were used initially to genetically differentiate intraspecific groups in *G. intraradices* (Koch *et al.*, 2004), but they had limitations in that markers were nonspecific and could be analysed only using pure DNA from the target organism. DNA from microorganisms present associated with pot-cultured or field-collected AMF spores could confound AFLP results, so root organ cultures (Bécard & Fortin, 1988) provided the means to obtain contamination-free DNA. *Glomus intraradices* was one of a limited range of AMF species compatible with this culture environment.

Recently, microsatellite simple sequence repeats (SSR) were developed for *G. intraradices* (Croll *et al.*, 2008; Mathimaran *et al.*, 2008). Five of the *G. intraradices* isolates resolved as five mtLSU haplotypes in this study were also resolved as distinct genotypes by SSRs (Mathimaran *et al.*, 2008). The *G. intraradices* isolates from the Tänikon field site used in the present study allow the comparison with the multilocus genotypes of Croll *et al.* (2008), because some isolates were used in both studies. Based on the introns in positions 1 and 2, Croll *et al.* (2008) distinguished the same three mitochondrial haplotypes (JJ291, JJ145/141 and JJ183). In addition, JJ145 and JJ141 could be distinguished by a single base pair length difference of one SSR locus. JJ291 and DAOM197198 were also differentiated by SSR data. These authors, like Raab *et al.* (2005), were not aware of the position 3 intron present in JJ141 and JJ145, which offers additional resolving power (e.g. among BEG75 and CC4).

It is not yet clear if microsatellite SSR variation (Croll *et al.*, 2008) will elucidate the whole range of intraspecific diversity characterized by the mtLSU locus, because primers specific to *G. intraradices* genotypes were designed exclusively from isolates in root organ cultures, most of them originating from the Tänikon field site. Generally, multilocus population analyses from environmental samples face the problem that genotypes from different loci cannot be linked to each other if more than one target organism occurs in a sample. Mathimaran *et al.* (2008) showed that SSR markers could be applied to measuring fungal diversity in colonized roots, but not yet under field conditions. Specificity must be tested exhaustively to exclude amplified products from plants and other associated microorganisms. Despite these potential problems, comparisons of both genotyping systems in more detail in future studies will offer new and intriguing insights.

Based on a greater breadth of isolate sampling, mtLSU data clearly show that intraspecific diversity within *G. intraradices* is considerably higher than previously reported. Croll *et al.* (2008) reported that genotypic diversity in isolates from the

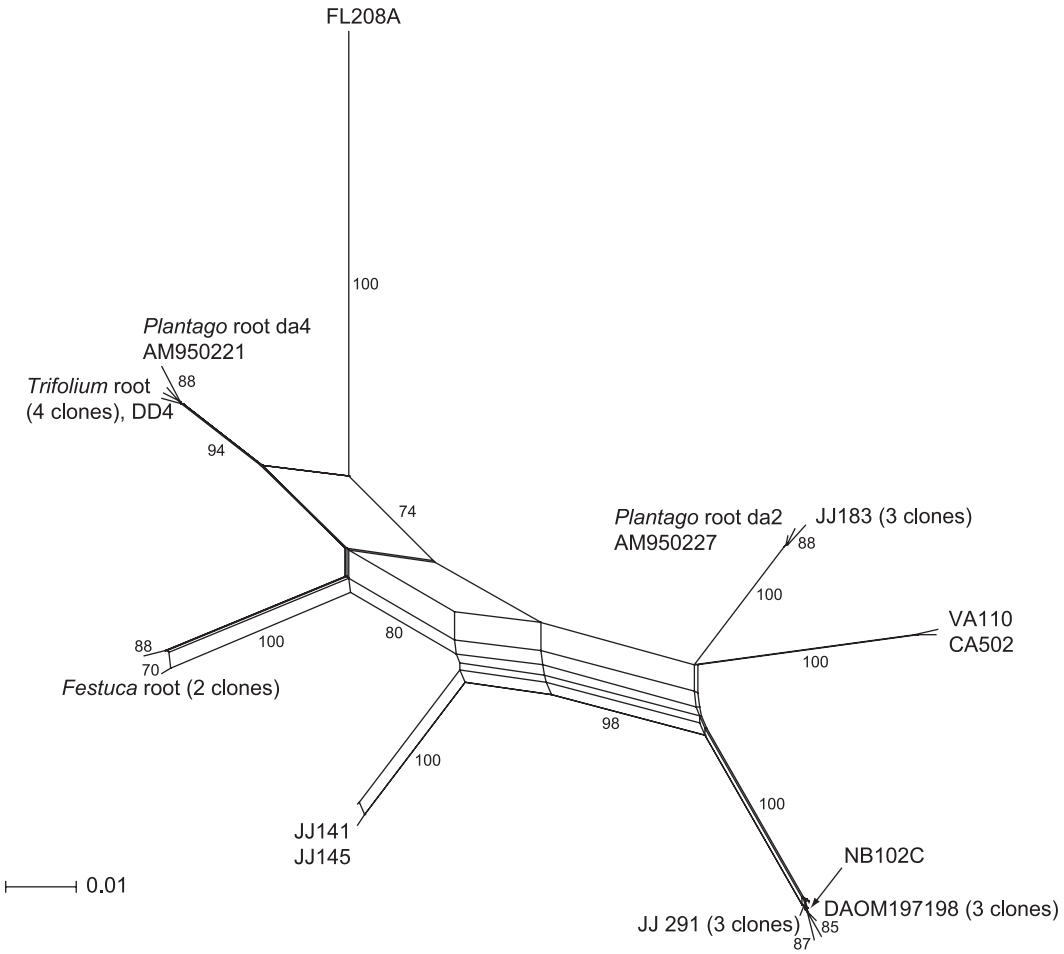

Fig. 5 NeighborNet network obtained from sequences of the position 1 intron. Numbers on the branches are bootstrap values from 1000 replications.

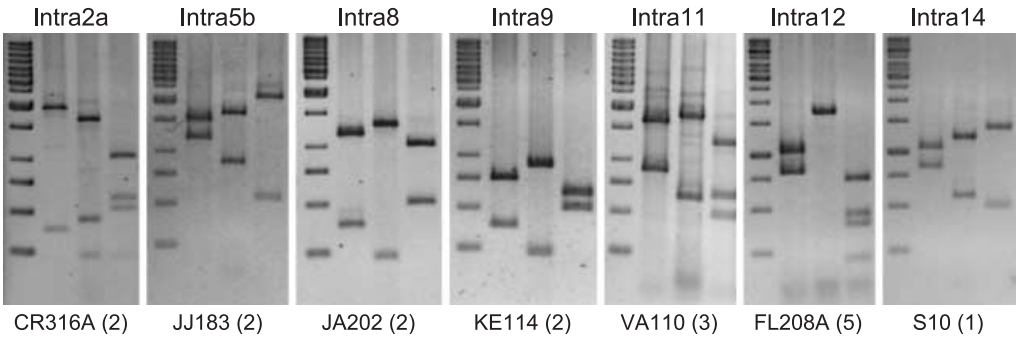

Fig. 6 Banding patterns of selected *Glomus intraradices* restriction fragment length polymorphism (RFLP) types. Respective isolate/root sample and polymerase chain reaction (PCR) product ID (in brackets) are shown below the pictures (for details see the Supporting Information, Table S1). The PCR products of the mitochondrial rRNA large subunit gene (mtLSU) rDNA generally were amplified using the primer pair RNL-29/30 in the second PCR step. RNL-1/5 was used for isolate JJ183, the only example of a cloned PCR product. DNA was digested with restriction enzymes *Dra*III, *Bsa*JI, *Hin*dIII and loaded in the same order onto gels. DNA ladder in left-most lane of each gel (bp): 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10 000.

Tänikon site was higher than that characterized in other isolates from Switzerland and one isolate from Canada (DAOM197198). Based on these comparisons, they concluded that intraspecific variation in *G. intraradices* is more diverse locally than globally, a hypothesis also put forward by Koch *et al.* (2004).

The finding that almost every *G. intraradices* isolate we sampled from a broad geographical range constitutes a different mtLSU haplotype certainly is surprising. Among 16 cultured fungal isolates, 75% comprised distinct haplotypes. Moreover, unique haplotypes were identified in most of the root samples

analysed. Conversely, evidence also suggests that some haplotypes are distributed over a very broad geographical distribution, the most striking of them being haplotype I. Considerable effort, including sampling of multiple isolates from each of a number of field sites around the world, will be needed to obtain a comprehensive overview of local versus pandemically distributed haplotypes in *G. intraradices*.

In contrast to SSR or AFLP fingerprinting methods, mtLSU sequences can be analysed phylogenetically, providing insights into the evolutionary relationships of the isolates and allowing to confirm the origin of the sequences from the target taxon.

Phylogenetic resolution within the *G. intraradices* clade was higher with mtLSU exon than ITS sequence data. The clade comprising sequences JJ1–JJ32 in the ITS trees has been used as a molecular grouping criterion for *G. intraradices* in field studies (Sýkorová *et al.*, 2007a). Moreover, this clade also contains the isolate currently being sequenced to represent the genome of *G. intraradices* (Martin *et al.*, 2004). MtLSU exon data clearly distinguish well-separated lineages within this group, among them the CA502/VA110 lineage, which is present in the ITS tree but its separation from the 'main clade' is not supported by bootstrap analysis.

A second clade of *G. intraradices* isolates can be distinguished in the ITS-5.8S phylogeny constructed in this study. It comprises isolates FL208, VA110 and KS906 grouping with *G. proliferum*. Some of these sequences have been present in the public database for years (e.g. AF185662, AF185668, AF185669, AF185670, AF185675 and AF185676 in Fig. 1). We confirmed that isolate FL208 groups in this clade, but even after repeated sequencing of multiple clones, ITS sequences from VA110 spores we obtained did not group with FL208A. Instead, its ITS and mtLSU sequences consistently grouped with CA502. The reason for this remains unclear. In any case, the mtLSU exon phylogeny confirms the genetic distance of FL208A from the other *G. intraradices* isolates. Based on unequivocal evidence from the ITS phylogeny, where a true outgroup could be used, the root of the tree is located between FL208/*G. proliferum* and the remaining taxa, which is consistent with the mtLSU exon phylogeny rooted by midpoint rooting (Fig. 3).

With its taxonomic resolution superior to ITS, the mtLSU exon region will be a useful molecular marker to contribute to a taxonomic consolidation of *G. intraradices.* Data from this and other gene regions will expand discovery of other isolates and clarify their interrelationships and distribution.

Fungal lineages generally are thought to lose introns more quickly rather than to gain them (Goddard & Burt, 1999). The complete absence of mtLSU introns in the KE114 isolate of *G. intraradices*, and in *G. proliferum* may therefore represent a derived condition. The hypothesis that the introns were inserted as independent events in all other isolates is not parsimonious. Introns in similar positions (1 and 2) have also been found in the more distantly related isolates of *G. mosseae* (O. Thiéry, unpublished). Possibly, these introns evolved in an ancestor common to both lineages. As in the study by Raab *et al.* (2005), ORFs coding for putative homing endonucleases were detected in some of the introns. Most of them were present in position 1 introns but an endonuclease ORF was also found in the position 3 intron. Homing endonucleases catalyse the spread of the intron-containing allele that encodes them to other intron-less alleles (Dalgaard *et al.*, 1997).

Evidence has been accumulating that not all symbiotically active AMF necessarily sporulate in field settings (Hempel *et al.*, 2007). The frequently used 'trap culturing' approach to propagate AMF from field samples in order to obtain spores for morphological analyses may bias the range of species-level taxa detected (Sýkorová *et al.*, 2007a). A similar bias may be expected among isolates of a species within a population. Thus, it is highly desirable to develop specific molecular tools as culture-independent techniques to analyse intraspecific genetic diversity of glomeromycotan fungi directly within mycorrhizal roots. The possibility of amplifying and then characterizing glomeromycotan fungi that do not show evidence of sporulation in a field setting (Rosendahl & Stukenbrock, 2004) and therefore are not culturable is a significant asset of mtLSU primers. MtLSU markers targeting higher-level phylogenetic taxa can be developed to easily obtain sequence data from other species. By contrast, design of primers to characterize SSR loci in isolates of other species will require considerably more genomic information.

As the RFLP method to detect haplotype variation within *G. intraradices* was tested using a worldwide sampling of isolates, it can be expected to be applicable to the whole range of diversity found in this species. No laborious cloning steps are necessary to analyse PCR products, and direct sequencing is possible unless several haplotypes are present in the same root. In some cases sequencing may still be useful to obtain additional information about the haplotypes present or to confirm the RFLP results. Such an approach will facilitate sampling over a broader geographic range and more diverse habitats. Biogeographic patterns can be elucidated and the role of human intervention can be examined in such studies, and the hypothesis that *G. intraradices* is a true generalist can be tested. Moreover, it will be interesting to determine whether some of the mtLSU haplotypes or haplotype groups correspond to ecotypes and how these correlate with degree of culturability.

Acknowledgements

This work was funded by a grant by the Swiss National Science Foundation to the senior author, which is gratefully acknowledged. The authors would like to acknowledge Thomas Boller and Andres Wiemken at the Botanical Institute for continuing support, Jan Jansa and Marcel van der Heijden, Natalia Requena and Guillaume Bécard for providing fungal isolates, Kurt Ineichen for cultivating *G. intraradices*, Zuzana Sýkorová and Istvan Paradi for root DNA extracts, and Zuzana Sýkorová,

Pascal Bittel, Tobias Mentzel and the technical staff at Hebelstrasse for helpful discussions and making things work.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Polymerase chain reaction (PCR) conditions for isolates of *Glomus intraradices* and mycorrhizal plant roots

Table S2 Sequencing primer sets for *Glomus intraradices*

Table S3 Restriction fragments lengths of mtLSU rDNA sequences for sequenced clones of *G. intraradices* isolates and field samples

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