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ORIGINAL PAPER

Long-term tracing of *Rhizophagus irregularis* isolate BEG140 inoculated on *Phalaris arundinacea* in a coal mine spoil bank, using mitochondrial large subunit rDNA markers

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Abstract During the last decade, the application of arbuscular mycorrhizal fungi (AMF) as bioenhancers has increased significantly. However, until now, it has been difficult to verify the inoculation success in terms of fungal symbiont establishment in roots of inoculated plants because specific fungal strains could not be detected within colonized roots. Using mitochondrial large subunit ribosomal DNA, we show that *Rhizophagus irregularis* (formerly known as *Glomus intraradices*) isolate BEG140 consists of two different haplotypes. We developed nested PCR assays to specifically trace each of the two haplotypes in the roots of *Phalaris arundinacea* from a field experiment in a spoil bank of a former coal mine, where BEG140 was used as inoculant. We revealed that despite the relatively high diversity of native *R. irregularis* strains, *R. irregularis* BEG140 survived and proliferated successfully in the field experiment and was found significantly more often in the inoculated than control plots. This work is the first one to show tracing of an inoculated AMF isolate in the roots of target plants and to verify its survival and propagation in the field. These results will have implications for basic research on the ecology of AMF at the intraspecific level as well as for commercial users of mycorrhizal inoculation.

Keywords *Rhizophagus irregularis* · mtLSU · Inoculation · Isolate tracing · Arbuscular mycorrhiza · Spoil banks · *Glomus intraradices*

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Introduction

Arbuscular mycorrhiza is formed by obligately symbiotic soil fungi from the phylum Glomeromycota (Schüßler et al. 2001) and represents the most frequent and ubiquitous type of mycorrhizas, colonizing the roots of the majority of terrestrial plant species. In this symbiosis, the spectrum of host plants ranges from liverworts and pteridophytes to gymnosperms and angiosperms. Root colonization by arbuscular mycorrhizal fungi (AMF) can considerably affect the growth and health of host plants that benefit from improved nutrient uptake, higher resistance to

drought, heavy metals, or pathogens (Smith and Read 2008).

Some AMF isolates are used for commercial applications (Gianinazzi and Vosátka 2004). Promising areas for these large-scale inoculations are reclamations of anthropogenically disturbed ecosystems, horticulture, and sustainable plant production. The purpose is to increase crop yield, accelerate the process of soil revitalization and plant establishment, increase the soil inoculum potential, and reduce the industrial fertilizers input and cultivation costs.

An example of anthropogenically disturbed ecosystems are spoil banks in the north-western part of the Czech Republic which arose as a consequence of extensive brown coal mining. The surface of the spoil banks is mostly formed by Miocene clays with unfavorable physical properties, in particular high compactness and low water permeability. AMF inhabit almost all undisturbed ecosystems; however, mechanical disturbance of soil connected with mining activities has been shown to cause a decrease in AMF spore abundance as well as AMF species diversity and natural inoculation potential (Waaland and Allen 1987). Thus, inoculation with AMF might improve the establishment, growth, and survival of plants on these stands by reducing various kinds of stress (Püschel et al. 2008; Rydlová et al. 2008).

Neither different AMF species nor different isolates of the same species can be distinguished microscopically based on fungal structures formed in roots. It has thus been impossible so far to detect and trace inoculated AMF isolates in roots under field conditions, as the AMF inoculants are generally applied to soils already containing a resident AMF community. Therefore, there is a strong need for molecular tools able to distinguish different AMF species and isolates, to detect them in the field, and to verify inoculation success. Moreover, ecological implications of AMF inoculation are still largely unresolved, despite recent efforts in this field (Antunes et al. 2009; Mummey et al. 2009). Any risk of releasing AMF species or isolates that could become invasive should also be neglected (Rosendahl et al. 2009; Schwartz et al. 2006).

During the past 15 years, molecular methods have been developed for the determination of AMF from plant roots, soil, or spores at the family and species level (e.g., Gollotte et al. 2004; Helgason et al. 1999; Krüger et al. 2009; Redecker 2000) and their use is nowadays well established. Recently, considerable effort has been put into the development of AMF isolate-specific molecular markers and population studies (Rosendahl 2008), and also because it has been frequently shown that different isolates of the same AMF species might exhibit different functional effects (Koch et al. 2006).

One of the most widespread AMF species has been referred to as *Glomus intraradices*. However, a recent study by Stockinger et al. (2009) has highlighted the need to redefine this species based on molecular phylogeny and morphology because its type strain from Florida (FL208) appeared to be distinct from this ubiquitous fungal clade. These authors revealed that this species fell into the same phylogenetic clade as *Glomus irregulare*, a species described recently by Blaszkowski et al. (2008), as also shown by Sokolski et al. (2010) using three protein-coding genes. In the recently published revised classification of the Glomeromycota by Schüßler and Walker (2010), *G. irregulare* became *Rhizophagus irregularis* and *G. intraradices* became *R. intraradices.* We adopt this new nomenclature throughout this publication.

Among different markers (Croll et al. 2008b; Koch et al. 2004; Raab et al. 2005) tested for *R. irregularis*, only the mtLSU could be used to characterize and distinguish different haplotypes of this fungus in the field (Börstler et al. 2008, 2010). Raab et al. (2005) showed that the mtLSU is homogeneous within the same isolate, but that it can distinguish different isolates. Using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach and sequencing, Börstler et al. (2008, 2010) revealed that the diversity of mtLSU haplotypes of *R. irregularis* is very high both in cultured isolates collected world-wide as well as in field populations. Moreover, a specific detection assay for one particularly frequent *R. irregularis* haplotype was already reliably applied on field roots (Börstler et al. 2010). Isolate-specific detection of other AMF species (e.g., *Funneliformis mosseae*, formerly known as *Glomus mosseae*) in field roots is currently not feasible due to a lack of polymorphism and scarcity of data in the mtLSU (Thiéry et al. 2010) or markers established so far only for spores (putative single copy protein-coding genes *GmFOX2*, *GmTOR2*, and *GmGIN1*; Rosendahl et al. 2009; Stukenbrock and Rosendahl 2005).

As the use of mtLSU as a molecular marker opens new possibilities for tracing the typically non-indigenous AMF strains used as bioenhancers in agriculture, we developed and applied strain-specific molecular assays for *R. irregularis* inoculated in a field experiment on a coal mine spoil bank—a disturbed ecosystem with low AMF infection potential. The high biomass biofuel crop *Phalaris arundinacea* (Gryndler et al. 2008; Wrobel et al. 2009) was studied, and fertilization was complemented with inoculation of AMF. Both AMF colonization of roots and AMF infection potential of soil were recorded. Particular aims of this work were (1) to characterize mtLSU of the AMF isolate *R. irregularis* BEG140, which was inoculated into the field experiment; (2) to establish a routine and reliable method for specific long-term tracing of this AMF isolate in the roots of *P. arundinacea* grown in the field experiment; and (3) to evaluate the persistence and proliferation of the inoculated strain in the field over 3 years.

Materials and methods

The field experiment: design and sampling

The field experiment was established in May 2006 on the coal mine spoil bank Merkur near Chomutov, North Bohemia, Czech Republic (50°25′55″ N, 13°17′25″ E). The spoil bank originated from 2000 to 2005. The aims of this experiment were to test the possibility of reclamation of this spoil bank using selected fast-growing high biomass crops and to evaluate the influence of organic matter as well as microbial inoculation on the growth of these crops.

First, the upper soil horizon already colonized by plants was removed mechanically to a minimum depth of 10 cm in order to level the site and to remove the majority of AMF potentially present on this site. The experimental plots (size 7.5×4 m) were arranged in a rectangle grid and separated by footpaths of a width of approximately 50 cm. The plots were components of a larger factorial experiment of more complex randomized design (Gryndler et al., in preparation). The studied plots (1) were inoculated with a mixture of three AMF species isolated from temperate anthropogenic sites in the Czech Republic (*R. irregularis* BEG140—formerly known as *G. intraradices* BEG140; *F. mosseae* BEG95—formerly known as *G. mosseae* BEG95; *Claroideoglomus claroideum* BEG96—formerly known as *Glomus claroideum* BEG96) using expanded clay as a carrier (15 l/plot) or (2) remained uninoculated as a control. The inoculum of the selected isolates was produced by the company Symbiom, Lanškroun, Czech Republic. Two levels of organic fertilizer (mixture 1:1 of industrial compost and lignocellulose substrate) at a quantity equivalent to 40 or 400 t/ha were applied on the plots as well. For chemical characteristics of the organic fertilizer components as well as spoil bank clay, see Gryndler et al. (2008). Altogether, 12 plots were studied (i.e., inoculated/uninoculated combined with two levels of organic fertilization in triplicate). *P. arundinacea* var. "Palaton S", as a biofuel crop was sown on the field (20 kg seeds/ha).

During 3 years, nine soil cores of ca. 10 cm diameter and ca. 15 cm depth containing roots of *P. arundinacea* were collected from each plot in each sampling time (spring, summer, and autumn, respectively). Roots from three sampling points from each plot were pooled, washed, cut into pieces, and mixed and 100 mg of them were stored at −20°C before the DNA extraction, which yielded three true replicates from each treatment at each time point. The total number of root samples used for preparing DNA extracts was thus 108. They were bulk root samples, supposedly representative for the experimental plots.

Three root subsamples of the pooled remainder were stained using 0.05% trypan blue in lactoglycerol (Koske and Gemma 1989). Root mycorrhizal colonization was quantified using the modified segment method (Giovannetti and Mosse 1980) under a compound microscope at ×100 magnification and was expressed as a mean percentage of the three subsamples. Mycorrhizal infection potential (MIP) of each plot was assessed after each sampling by cultivating maize in two mixed soil samples (obtained by pooling the soil cores from each plot mentioned above) in 500 ml pots in the greenhouse for 5 weeks. The maize mycorrhizal colonization was evaluated in the same way as the field roots and MIP was expressed as a mean percentage of the two soil samples. The influence of inoculation, fertilization, and sampling time on colonization and MIP was evaluated using ANOVA with repeated measures using the statistical software S-Plus 6.2 (Insightful Corp., USA). The data for colonization levels (in percent) were arcsine-transformed prior to the analyses in order to fulfill the assumptions of ANOVA.

Molecular characterization of the inoculated strain *R. irregularis* BEG140

R. irregularis BEG140 was isolated in 1998 from a pyrite waste deposit with high concentrations of Mn, Fe, and other heavy metals near Chvaletice/Czech Republic (Rydlová et al. 2004) and was subcultured as a multispore culture. It was deposited in the International Bank for the Glomeromycota (BEG) and maintained at the Institute of Botany, Academy of Sciences of the Czech Republic (IB/ASCR). Its cultures were established in the greenhouse in open pots with *Zea mays*, *Trifolium pratense*, or *Plantago lanceolata* as host plants in sterilized substrate from the collection site (7 pots) as well as in inert substrate (sterilized zeolite/sand 1:1 or 1:2, in 18 pots). The cultures were occasionally checked under the stereomicroscope for their purity and were subcultured using a mixture of substrate, roots, and spores as inoculum. This isolate is also being continuously produced as inoculum by the Symbiom company.

At the time of this study, only a subsample (ca. 35 g, stored at −20°C) of the original inoculum used in the field experiment in May 2006 was available. As we considered this amount not sufficient to characterize the inoculum properly, we decided to also screen all accessible cultures of *R. irregularis* BEG140. Spores from the original inoculum sample and the 25 greenhouse cultures of *R. irregularis* BEG140 deposited at IB/ASCR, as well as at the Symbiom company (8 samples provided in February 2009), were wet-sieved (Gerdemann and Nicolson 1963). From each pot, crude DNA extractions were performed from 2 to 11 samples (1–3 spores per sample; Redecker et al. 1997). DNA was also extracted from the roots found in the inoculum sample (ca. 50 mg) using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and from the roots of *Z. mays* from a selected pot culture using the Ultra Clean Soil DNA kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions.

In order to check the success of the DNA extraction and to prove the identity of the isolates as members of the Glomeraceae (*Glomus* group A, Schwarzott et al. 2001), 1 μl of each DNA extract was used as a template for a nested PCR procedure with Glomeraceae-specific primers (NS5/ITS4 in the first reaction and GLOM1310/ITS4i in the second reaction) targeting ITS and SSU nrDNA as described by Redecker (2000). DNA extracts yielding PCR products were then used as a template for amplification of the mtLSU using the general nested PCR approach for haplotypes of *R. irregularis* and its close relatives (Börstler et al. 2008): the primers RNL5/RNL28a were used in the first PCR, the primers of the second PCR were RNL29/RNL30. For this approach, the optimized PCR conditions described by Börstler et al. (2010) were used. PCR products were digested using the enzymes *Dra*III, *Bsa*JI, and *Hin*dIII as described in detail by Börstler et al. (2008); the restriction fragments were separated on agarose gels.

As two different RFLP patterns (subsequently called RFLP types Intra A and Intra B; Fig. 1) were repeatedly found in the cultures, the corresponding mtLSU PCR product of each RFLP type was cloned and sequenced completely in both directions in order to determine the respective sequence type (haplotype). Thus, the PCR products were purified, cloned, re-amplified, and sequenced as described in Börstler et al. (2008) and Fehrer et al. (2009). For sequencing of haplotype A, the forward primers M13fwd, RNL-29, 16, 12, 24, and 25 and the reverse primers M13rev, RNL-30, 26, 27, 7, 7b, and 36 were chosen. Sequencing forward primers for haplotype B were M13fwd, RNL-11, 13, 16, 35, 37, 24, and 25 and sequencing reverse primers were M13rev, RNL-26, 27, 7, 38, 36, 70 (5′-AGCTCGGAATTGAACCATAG-3′), and 71 (5′-TCCTCCTACGAGGATTTCAC-3′). For previously published primer sequences, see Raab et al. (2005) and Börstler et al. (2008).

After editing the sequences in Sequence Navigator (version 1.0.1) or Sequence Scanner (version 1.0), they were aligned in BioEdit (Hall 1999) along with all mtLSU haplotypes already characterized by Börstler et al. (2008, 2010). Exon parts of the mtLSU sequences were analyzed phylogenetically using distance, parsimony, and maximum likelihood criteria as implemented in PAUP* 4.0b10 (Swofford 2003). Neighbor-joining or heuristic search algorithms were applied, respectively. Maximum likelihood models and parameters were estimated using Modeltest 3.5 (Posada and Crandall 1998).

Primer design, PCR conditions, and optimization of the specific nested PCR assays for BEG140

The mtLSU sequences of BEG140 were screened for unique primer annealing sites differing from most or all other mtLSU sequences known so far. The suitability of selected sequence motifs as targets of potential primers was analyzed using the Primer Designer software (version 3.0; Scientific & Educational Software, Cary, NC, USA). For haplotype A, the primer pair RNL-16 (forward; Raab et al. 2005)/RNL-89 (reverse, 5′-CTATGCAACCGTAGGTAAGC-3′) was designed for the first PCR of the nested PCR assay. These priming sites are present in several mtLSU haplotypes (Fig. 2a); however, the first PCR should exclude all haplotypes lacking the intron at position 3, as the primer site of RNL-89 is located in this intron (Supplementary Fig. S1). The primer pair RNL-2c (forward; Börstler et al. 2008)/RNL-145 (reverse, 5′-ATAGGTACGCCTAAGAGGGT-3′) was designed for the second PCR. For haplotype B, the primers RNL-85 (forward, 5′-CAGCCAATTATGGTGTTAGT-3′) and RNL-38 (reverse; Börstler et al. 2008) were designed for the first PCR, and RNL-87 (forward, 5′-AAGCAGGAAGAAGGAAGACT-

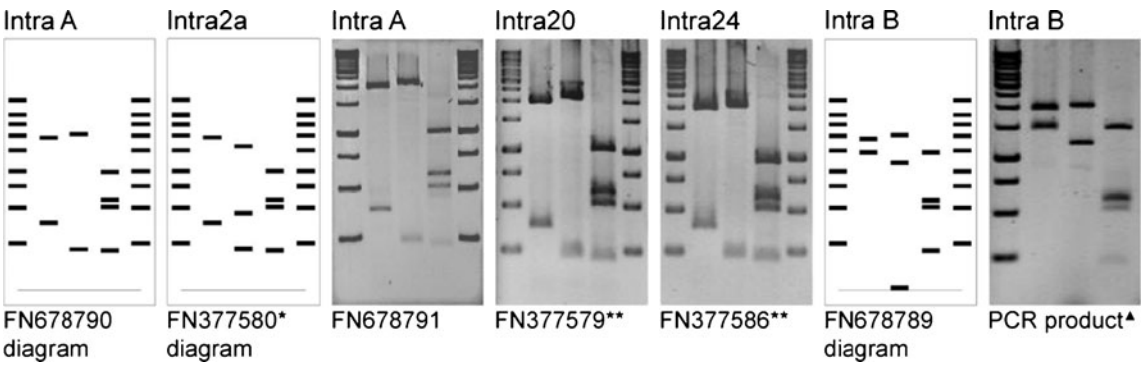


Fig. 1 RFLP banding patterns of haplotypes A and B (Intra A and Intra B) of the mtLSU in comparison with the respective patterns of Intra2a, Intra20, and Intra24. PCR products were amplified using the primer pair RNL-29/RNL-30 and digested using the restriction enzymes *Dra*III, *Bsa*JI, and *Hin*dIII and loaded in the same order per sample onto the gels. DNA ladder in the left-most lane of each gel (bp): 250, 500, 750, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 5,000, 6,000, 8,000, and 10,000. The size standard in the left-most lane of the diagrams produced in silico (bp): 250, 500, 750, 1,000, 1,500, 2,000, 2,500, 3,000, and 4,000. Accession numbers of the respective clones are shown below the patterns. *Triangle*, original digested PCR product from which the clone FN678789 was derived. *Single asterisk*, from Börstler et al. (2008); *double asterisk*, from Börstler et al. (2010)

3′) and RNL-142 (reverse, 5′-GTTGCCTCTGTCCTTA TAGC-3′) for the second PCR (Supplementary Fig. S1, Fig. 2b). Both primers of the second PCR would most likely also detect the most similar haplotype XVII besides haplotype B. No DNA template was available to assess this in vitro. However, haplotypes B and XVII differ by four base changes in the second PCR product and can be distinguished unequivocally by sequencing.

Cycling parameters were optimized by gradient PCRs using DNA extracts from spores as well as colonized roots of the corresponding haplotype. The following reactions of a nested PCR assay were set up: 1.25 units of *Taq* DNA polymerase (Fermentas, St. Leon-Rot, Germany) was used with 2 mM $MgCl_2$, 2.5 μl of 10× *Taq* buffer (with KCl/ without $MgCl_2$), 0.5 μM of each primer, and 0.25 μM of each deoxynucleotide (0.125 μM in the second PCR, respectively) in a volume of 25 μl per reaction. Cycling parameters for the first PCR were set on 3 min at 95°C, 32 cycles of 30 s/95°C, 30 s/61°C and 2 min 30 s/72°C, followed by 10 min at 72°C. The conditions for the second PCR were optimized as follows: 3 min at 94°C, 34 cycles of 30 s/94°C, 30 s/64.5°C or 64°C (haplotypes A/B) and 1.5 min/72°C, followed by 10 min at 72°C. All PCRs were conducted using the Mastercycler ep gradient S (Eppendorf, Hamburg, Germany). The expected product lengths for haplotypes A/B were 1,440/1,441 bp in the first PCR and 403/484 bp in the second PCR.

In an in silico approach (Amplify, Bill Engels, version 3, University of Wisconsin 2005), one representative of all mtLSU haplotypes completely sequenced so far was additionally tested for potential amplification success. These tests have the advantage that even large numbers of haplotypes can be included of which no DNA template is available, which is often the case for haplotypes found in colonized roots from the field. In subsequent in vitro PCRs, selected templates were used which had given positive or questionable results in the in silico analysis. Template quality and identity of all samples used in these PCRs were verified using the general PCR approach for mtLSU haplotypes of *R. irregularis* and its close relatives (see above). In silico amplifications were in our experience a valid approximation of in vitro PCRs: in no case we have obtained PCR products in a reaction when no amplification was predicted in silico. Supplementary Table S1 shows the results for the primer pairs designed for haplotypes A and B, respectively. Only an additional haplotype II can be amplified with the primers for haplotype A, but can be distinguished by a 42-bp insertion in "intron position 2". Supplementary Fig. S3 shows that a product from haplotype II amplified with these primers. Therefore, a combination of the nested PCR assay, PCR product length information, and sequencing analyses guarantees the identification of haplotype A, in case haplotype A and II should occur in the same field site. However, this was probably not the case in the field experiment analyzed here, as we did not find the haplotype II RFLP pattern in any of our field samples.

Tracing the introduced *R. irregularis* BEG140 in *P. arundinacea* roots

Root samples (100 mg fresh weight) of *P. arundinacea* from 12 selected plots from 9 harvests were ground in liquid nitrogen, and DNA was extracted using the Ultra Clean Soil DNA kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA extracts were screened using nested PCRs with the specific primers and conditions for the two haplotypes of *R. irregularis* described above. PCR products were checked on 1% agarose gels, and all positive reactions were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced with the primers RNL2c or RNL142. The edited sequences were aligned in BioEdit (Hall 1999) with haplotype A or B sequence, respectively.

The presence/absence of each *R. irregularis* BEG140 haplotype in the roots was scored in each sample. The differences of its frequency of occurrence in each plot with respect to inoculation, fertilization, and sampling time were evaluated using generalized linear models with binomial family and logit link and analyzed by analysis of deviance. For tests of treatments, error degrees of freedom reflected the actual number of replicates (plots), not the number of samples (plots×harvests). Statistical analyses were performed in S-Plus version 6.2 (Insightful Corp., USA).

DNA extracts from *P. arundinacea* roots positive with the haplotype-specific primers were additionally screened using the general PCR-RFLP approach for mtLSU haplotypes of *R. irregularis* and its close relatives (see above). The RFLP profiles were checked for bands corresponding to the BEG140 RFLP types Intra A and B known from the corresponding pot cultures or field-applied inoculum sample. One selected PCR product was purified and cloned as described above for the inoculum sample, and a representative clone with the RFLP type Intra A of BEG140 was sequenced for confirmation using the sequencing primers of haplotype A listed above.

Complementary nrLSU-based assays for general abundance of AMF and all three inoculated AMF species

Isolate-specific detection of other AMF species than *R. irregularis* in field roots is currently not feasible; thus, we applied the species-specific approach to monitor the presence/absence of species that were present in the inoculum in the field experiment. Samples from the first four samplings were screened using a nested PCR approach targeting the variable D2 domain of the nuclear ribosomal

large subunit gene (nrLSU) in order to follow the development of Glomeromycota in general and each species supposed to be present in the original inoculum in the *P. arundinacea* roots. The first PCR step was performed using the primer pair LR1 and FLR2 (van Tuinen et al. 1998). Four second step PCRs were performed from each product of the first step diluted 1:100 using an AMF general primer pair (250f: 5′-AGTTGTTTGGGATTG CAGCT-3′/FLR4; Gollotte et al. 2004) and primers specific for the *C. claroideum* clade (GC410f: 5′-ATTGAAGTCA GTCGTGCTGG-3′/GC650r: 5′-ACCAAAGAGAAGCCA GGTG-3′), for *F. mosseae* (GM440f: 5′-TCAACCTTTTGA GCTCGGTC-3′/GM620r: 5′-TATCCGTTGCAAT CCTCGG-3′), and *R. irregularis* (GI410f: 5′-GATTGAAG CCAGTCGTACCT-3′/GI710r: 5′-CGTTCTAACCTATT GACCATC-3′). Primers for *R. irregularis* and *C. claroideum* have been already applied in a greenhouse study (Janoušková et al. 2009). For primer development, localization, and specificity, see Supplementary Notes S1. Cycling parameters were 4 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 58°C/59°C/60°C (first/AMF general/taxon-specific PCRs) and 1.5 min at 72°C, followed by 10 min at 72°C.

Results

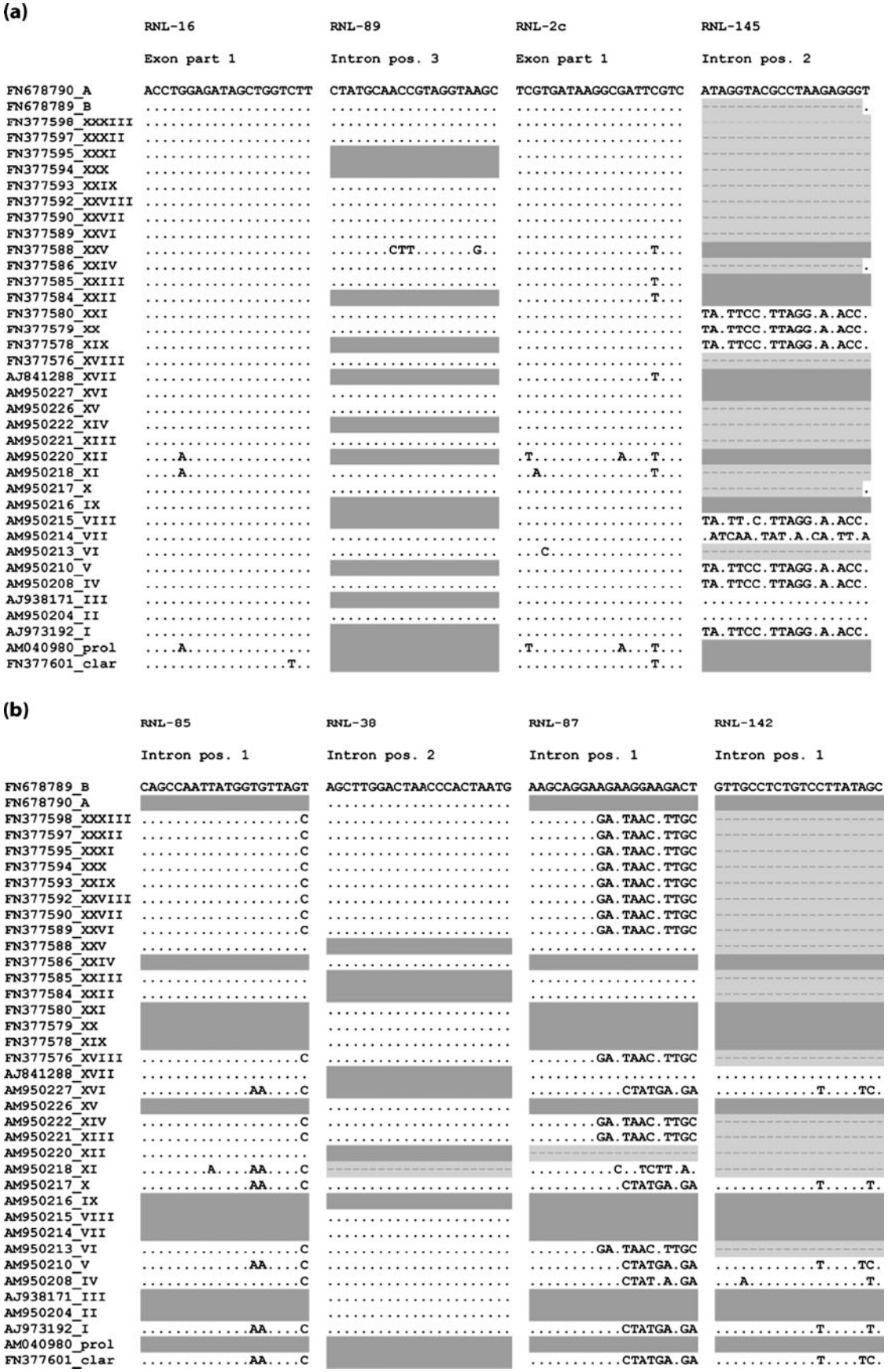
Molecular characterization of the inoculated strain *R. irregularis* BEG140

DNA was extracted from 129 spore samples and 2 root samples originating from 31 different cultures of this fungus including the inoculum applied to the field in 2006. No spores were found in three cultures. MtLSU PCR products were obtained from 17 cultures, and 3 different RFLP types were recovered. One of them corresponded to haplotype I according to Börstler et al. (2008), which has a wide distribution in different field locations (e.g., Börstler et al. 2010; Lee and Young 2009). It was found in two spores from a single open pot culture established at the IB/ASCR (where the BEG140 pot cultures are placed in the vicinity of several other *R. irregularis* isolates) and was therefore considered as a greenhouse contamination. The two remaining RFLP types (Intra A and B; Fig. 1) were found repeatedly in 9 and 10 cultures, respectively. In two cultures from Symbiom, both types were detected; however, mostly only a single RFLP type was found in a particular culture. In spores as well as in the roots of the inoculum sample from 2006, only Intra A was detected.

Cloning and sequencing of PCR products of the general approach obtained from roots of the inoculum (haplotype A) and a pot culture from the IB/ASCR (haplotype B) yielded sequences 2,253 and 3,288 bp in length, respectively. The sequences were submitted to public databases under accession numbers FN678790 (haplotype A) and FN678789 (haplotype B). Neither of these mtLSU haplotypes had been detected in previous studies analyzing this gene region or parts of it (Börstler et al. 2008, 2010; Croll et al. 2008b; Lee and Young 2009; Raab et al. 2005). Their sequence structure is shown in comparison to all other currently known mtLSU haplotypes of *R. irregularis* in Supplementary Fig. S1: in haplotype A, the intron "position 2" lacks a sequence region of 42 bp, which is present in the otherwise identical introns at this position of haplotypes II and III as defined by Börstler et al. (2008). An intron "position 1" is missing in haplotype A and the intron "position 3" is identical to those found in haplotypes II, X, XXIII, XXIV, and haplotype B. The latter contains an intron "position 2", which is identical to those of haplotypes X and XXIV, but also revealed a unique intron at position 1. This intron is most similar to intron "position 1" found in haplotype XVII, but lacks a sequence region of 39 bp and differs furthermore by four base pair changes. Phylogenetic analyses of the mtLSU exon sequences (Supplementary Fig. S2) showed that haplotypes A and B both belong to the main cluster of isolates of the extremely widespread fungal clade that has been referred to as phylotype GLOM A-1 based on nuclear rDNA sequence data (Börstler et al. 2008, 2010), which largely corresponds to *R. irregularis*, and that they can be distinguished from a subclade containing mostly sequences from grasslands ("grassland clade" in Supplementary Fig. S2).

Based on the sequence results, precise restriction fragment sizes of both haplotypes were calculated in silico: haplotype A is characterized by 373, 1,920 (*Dra*III), 223, 2,070 (*Bsa*JI) and 218, 508, 585, and 982 bp (*Hin*dIII) fragments. Haplotype B is characterized by 1,442, 1,886 (*Dra*III), 106, 1,186, 2,036 (*Bsa*JI) and 218, 508, 570, 585, and 1,447 bp (*Hin*dIII) fragments. Despite the overall sequence similarity between haplotypes A and II, the corresponding RFLP patterns (Intra A and Intra2a) are easy

Fig. 2 Annealing sites of the nested PCR primers developed for mtLSU haplotypes A and B in comparison to sequences of all other available mtLSU haplotypes. **a** Haplotype A primers RNL-16/RNL-89 (first PCR) and RNL-2c/RNL-145 (second PCR); **b** haplotype B primers RNL-85/RNL-38 (first PCR) and RNL-87/RNL-142 (second PCR). The alignments are shown in 5′–3′ orientation of the primers. Accession numbers are provided for representatives of the mtLSU haplotypes A, B, I–XI, XIII–XXXIII (*R. irregularis* and its close relatives, see Supplementary Fig. 2), and haplotype XII (*R. intraradices*) as defined by Börstler et al. (2008, 2010), as well as *R. proliferus* (*prol*; Raab et al. 2005) and *R. clarus* (*clar*; Thiéry et al. 2010). Character states identical to those of haplotype A (**a**) or B (**b**) are shown as *dots*. Sequences which do not contain the respective intron at the primer annealing site are shown in *dark gray*. Gaps at the primer locations are shown as *shaded hyphens*


(a)
RNL-16
Exon part 1
RNL-89
Intron pos. 3
RNL-2c
Exon part 1
RNL-145
Intron pos. 2
FN678790_A ACCTGGAGATAGCTGGTCTT CTATGCAACCGTAGGTAAGC TCGTGATAAGGCGATTCGTC ATAGGTACGCCTAAGAGGGT
FN678789_B
FN377598_XXXIII
FN377597_XXXII
FN377595_XXXI
FN377594_XXX
FN377593_XXIX
FN377592_XXVIII
FN377590_XXVII
FN377589_XXVI
FN377588_XXV .....CTT.....G.. ....T...
FN377586_XXIV
FN377585_XXIII ....T...
FN377584_XXII ....T...
FN377580_XXI TA.TTCC.TTAGG.A.ACC.
FN377579_XX TA.TTCC.TTAGG.A.ACC.
FN377578_XIX TA.TTCC.TTAGG.A.ACC.
FN377576_XVIII
AJ841288_XVII ....T...
AM950227_XVI
AM950226_XV
AM950222_XIV
AM950221_XIII
AM950220_XII ....A..... .T.....A...T...
AM950218_XI ....A..... ..A.....T...
AM950217_X
AM950216_IX
AM950215_VIII TA.TT.C.TTAGG.A.ACC.
AM950214_VII .ATCAA.TAT.A.CA.TT.A
AM950213_VI ...C.....
AM950210_V TA.TTCC.TTAGG.A.ACC.
AM950208_IV TA.TTCC.TTAGG.A.ACC.
AJ938171_III
AM950204_II
AJ973192_I TA.TTCC.TTAGG.A.ACC.
AM040980_prol ....A..... .T.....A...T...
FN377601_clar ....T.. ....T...
(b)
RNL-85
Intron pos. 1
RNL-38
Intron pos. 2
RNL-87
Intron pos. 1
RNL-142
Intron pos. 1
FN678789_B CAGCCAATTATGGTGTTAGT AGCTTGGACTAACCCACTAATG AAGCAGGAAGAAGGAAGACT GTTGCCTCTGTCCTTATAGC
FN678790_A
FN377598_XXXIII ....C .....GA.TAAC.TTGC
FN377597_XXXII ....C .....GA.TAAC.TTGC
FN377595_XXXI ....C .....GA.TAAC.TTGC
FN377594_XXX ....C .....GA.TAAC.TTGC
FN377593_XXIX ....C .....GA.TAAC.TTGC
FN377592_XXVIII ....C .....GA.TAAC.TTGC
FN377590_XXVII ....C .....GA.TAAC.TTGC
FN377589_XXVI ....C .....GA.TAAC.TTGC
FN377588_XXV
FN377586_XXIV
FN377585_XXIII
FN377584_XXII
FN377580_XXI
FN377579_XX
FN377578_XIX
FN377576_XVIII ....C .....GA.TAAC.TTGC
AJ841288_XVII
AM950227_XVI .....AA....C ....CTATGA.GA .....T....TC.
AM950226_XV
AM950222_XIV ....C .....GA.TAAC.TTGC
AM950221_XIII ....C .....GA.TAAC.TTGC
AM950220_XII
AM950218_XI .....A....AA....C .....C..TCTT.A.
AM950217_X .....AA....C ....CTATGA.GA .....T.....T.
AM950216_IX
AM950215_VIII
AM950214_VII
AM950213_VI ....C .....GA.TAAC.TTGC
AM950210_V .....AA....C ....CTATGA.GA .....T....TC.
AM950208_IV ....C ..........CTAT.A.GA ...A.....T.
AJ938171_III
AM950204_II
AJ973192_I .....AA....C ....CTATGA.GA .....T.....T.
AM040980_prol
FN377601_clar .....AA....C ....CTATGA.GA .....T....TC.

to distinguish as Intra 2a has an additional restriction site for *Bsa*JI (Fig. 1). The Intra A pattern is similar to the RFLP patterns Intra20 and Intra24 of haplotypes XX and XXIV (Börstler et al. 2010) and might be difficult to distinguish if the quality of the gel is low (Fig. 1). In contrast, Intra B is so far unique and easy to distinguish from all other RFLP patterns.

We conclude that due to the multispore origin of BEG140, it is in fact a mixture of two different haplotypes A and B. However, in the sample of the inoculum applied to the field in 2006, only haplotype A could be detected. As this sample was very small and haplotype B was found in several cultures of *R. irregularis* BEG140, we concluded that this haplotype likely was present in low quantity in the original inoculum used and decided to develop a specific assay to ensure the tracing of both haplotypes.

Influence of experimental treatments on the mycorrhizal fungal colonization of *P. arundinacea* roots and the MIP of the soil

Mycorrhizal root colonization reached 18–83% of the root length in the first year in the 12 studied plots (Fig. 3). However, the variance was relatively high in the first months after sowing, and due to the lack of available root material (the plants were still rather small), only one to two replicate samples per plot were collected. From 2007, the plants covered the experimental plots, enabling the harvest of enough roots, and the root colonization was >80% in most samples. ANOVA with repeated measures revealed that the sampling time (Df=1; F=57.7; p<0.001) and fertilization dose (Df=1; F=7.1; p<0.05) had a significant influence on the level of root colonization. Inoculation showed only a trend to higher root colonization (Df=1; F=5.6; p=0.05), and interactions among the factors were not significant.

In July 2006, the roots from the control plots showed a tendency towards a lower (37±16%, mean±SD; p=0.06) colonization than those from the inoculated plots (65±18%, mean±SD). This difference was not found in the samples from September 2006 and was recovered again in May and September 2008. In September 2006, the colonization level dropped to approx. 45±18%, probably due to fast growth of the roots (the AMF colonization process might have been slower than the root growth). Afterwards, the colonization reached 85±8% in May 2007 and remained on this level until the end of the experiment with an exception in November 2007, when it dropped to 75±14%, probably due to intensive rainfalls and excessive soil moisture (Fig. 3). In May and September 2008 and May 2009, there was a significant positive impact of fertilization on the mycorrhizal colonization.

The MIP of the soil followed largely a similar kinetics as the colonization levels of the roots (data not shown). Sampling time (Df=1; F=52.3; p<0.001), inoculation (Df=1; F=9.3; p<0.05), and fertilization dose (Df=1; F=9.7; p<0.05) all had a significant influence on root colonization. The effects of fertilization dose and mycorrhizal inoculation on plant yield parameters, including experimental treatments not considered in the present study, will be published elsewhere (Gryndler et al., in preparation).

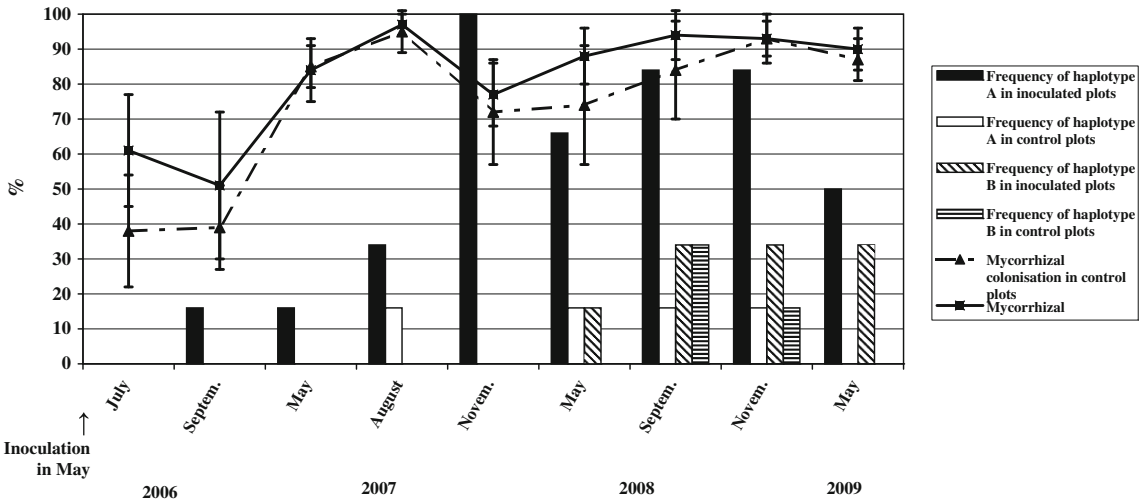


Fig. 3 Time course of mycorrhizal colonization and frequency of occurrence of haplotypes A and B in the roots of *P. arundinacea* during 3 years of field experiment (2006–2009). The frequency of occurrence of each haplotype is shown as the percentage of plots tested positive with each specific nested PCR assay for the corresponding haplotype within the six plots of the respective inoculation treatment investigated at each harvest. Mycorrhizal colonization of *P. arundinacea* roots is depicted as the percentage of mycorrhizal colonization±standard deviations (n=6 for each inoculation treatment at each harvest). The data were pooled over both fertilization treatments because those did not have significant effects on the occurrence of haplotype A

Tracing of the introduced *R. irregularis* BEG140 in the roots of *P. arundinacea*

The screening of the 108 *P. arundinacea* field root extracts with the haplotype A-specific assay yielded 31 positive nested PCR amplicons. After sequencing of all 31 amplicons with primer RNL2c, all sequences matched exactly the diagnostic sequence region of haplotype A originating from the inoculum sample (data not shown). Thus, the primers did not amplify other so far known haplotypes present in the field, such as haplotype II (Börstler et al. 2008). During the experiment, haplotype A was detected for the first time in a single sample in September 2006, then in another single sample in May 2007. From November 2007, its frequency increased to ca. 50% of all 12 plots before decreasing at the end of the experiment (Fig. 3). Haplotype A PCR products were detected 27× in inoculated plots, but only 4× in control plots. Their distribution among the two fertilization treatments was relatively balanced: haplotype A was found 13× in plots fertilized with an equivalent of 40 t of the organic fertilizer/ha and 18× in plots fertilized with a quantity equal to 400 t/ha. The analysis of deviance revealed that the frequency of haplotype A differed significantly between the inoculated and non-inoculated plots (*Df* error=11; F=24.43; $p<0.001$); in contrast, there was no difference between the two fertilization levels (*Df* error=11; F=1.37; p=0.28). Temporal variation in the frequency of haplotype A was also significant (*Df*=104; F=13.55; $p<0.001$).

All except one of the 31 DNA extracts, which tested positive with the specific assay for haplotype A, also yielded a positive PCR product with the general PCR-RFLP approach. Their RFLP profiles were compared visually with banding patterns of different haplotypes of *R. irregularis* and its close relatives described in Börstler et al. (2008, 2010), and the presence of the Intra A banding pattern was scored. In 11 of the 30 samples, only this banding pattern was observed. In two samples, the Intra A bands were prevalent and clearly distinguishable in a mixed pattern also comprising bands of other haplotypes. In other 11 samples, the Intra A pattern was weak in mixed profiles dominated by other haplotypes. In six samples, Intra A bands were not discernable, and instead, patterns of other known haplotypes were observed.

Interestingly, samples producing clear and dominant Intra A bandings originated predominantly from the first 2 years of the field experiment (2006/2007). In samples of the years 2008/2009, mixed RFLP patterns, often containing *R. irregularis* RFLP types Intra1, Intra3a, Intra6, Intra8, Intra13, and Intra14 (RFLP types defined by Börstler et al. (2008, 2010)), were predominant (data not shown). Altogether, clear or weak Intra A banding pattern was observed in approximately 77% of samples yielding a PCR product with the specific PCR assay. For one selected sample, yielding a strong banding pattern of haplotype A, the general nested PCR approach was repeated and the cloned PCR product was sequenced with the sequencing primer set for haplotype A. The sequence (accession number FN678791) matched exactly the sequence obtained from the inoculum from 2006 on which haplotype A was defined.

The PCR assay for haplotype B yielded 10 amplicons, all originating from samplings in 2008 and 2009. Seven of them were found in the inoculated treatment and three in the control treatment. We did not test the frequency of haplotype B statistically, as its occurrence was too low. All sequences matched exactly the diagnostic sequence region of this haplotype originating from the pot culture of *R. irregularis* BEG140. Additional analysis of all 10 samples with the general PCR-RFLP approach revealed mixed RFLP patterns, that is, the samples contained more than one haplotype. In six samples, very weak bands were observed, possibly belonging to haplotype B, but difficult to interpret. Therefore, we conclude that whenever haplotype B was present, it did not occur as the dominant haplotype.

Detection of the three inoculants with taxon-specific PCRs targeting nrLSU

For the first four samplings, nested PCRs targeting the nrLSU revealed members of Glomeromycota in 13 samples from inoculated and 5 samples from control plots, 9 each per fertilization treatment. *R. irregularis*-typical PCR amplicons were detected in 12 samples predominantly in inoculated plots (9), first in September 2006. All samples except one tested positive with both the *R. irregularis* BEG140-specific mtLSU and *R. irregularis*-specific nrLSU assays. Furthermore, *R. irregularis* was detected in seven additional samples with nrLSU indicating that other *R. irregularis* variants were present along with BEG140. The two remaining species from the inoculum (*F. mosseae* and *C. claroideum*) were found less frequently: *F. mosseae* only four times (twice in inoculated and twice in control plots) and *C. claroideum* in a single control plot in the fourth sampling from August 2007. The inoculum sample was also tested for nrLSU, and only *R. irregularis* and *C. claroideum* were detected.

Discussion

Inoculation of AMF is increasingly used as a biofertilization tool for agriculture and horticulture (Gianinazzi and Vosátka 2004). However, it has been difficult to prove the effectiveness of this treatment in terms of inoculum

establishment, i.e., the competitivity of the inoculated strains in relation to AMF already present at the respective site. Molecular methods have been available for some time to detect species level taxa of AMF, but these could not be used to verify inoculation success if local strains of the inoculated species were already present, which is usually the case as species most frequently used for inoculation (*R. irregularis*, *F. mosseae*) are ubiquitous (e.g., Sýkorová et al. 2007). Nevertheless, Farmer et al. (2007) could show that the frequency of the occurrence of inoculated species was increased by inoculation. The advent of more sensitive molecular markers (Börstler et al. 2008; Croll et al. 2008a; Mathimaran et al. 2008) now allows the assessment of the intraspecific variation and to distinguish and trace genotypes and isolates.

At our experimental locality, the upper soil horizon had been removed to minimize the abundance of indigenous AMF before the setup of the experimental plots. In spite of this, the MIP analysis performed on the substrate sampled at the locality prior to inoculation revealed a relatively high natural inoculation potential. Our inoculated AMF had thus to compete with indigenous strains. In principle, the haplotypes of *R. irregularis* BEG140 might have been naturally present at the field site before inoculation as the isolate originated from a locality approximately 200 km from the experimental site. However, the analysis of *P. arundinacea* roots from the uninoculated control plots indicates that the inoculated *R. irregularis* isolate was not native to this site as the haplotype A, characteristic for BEG140, was detected significantly more often in inoculated than control plots. Furthermore, from the vicinity of the experimental site, within several tenths of meters, *R. irregularis*, *F. caledonium*, and *C. claroideum* were isolated. This *R. irregularis* isolate was characterized using the mtLSU markers and was distinct from both haplotypes of BEG140.

The temporal occurrence and first appearance of haplotype A in roots suggest a relatively slow establishment (possibly involving dormancy of its propagules), and its occasional occurrence in control plots indicates the possibility of dispersal of the haplotype across the experimental field into uninoculated areas. This could have happened by hyphal growth and/or propagule carryover by harvesting machines for *P. arundinacea*. The slightly declining frequency and the decreasing dominance of the Intra A pattern relative to other haplotypes towards the end of the sampling period might even provide hints that the isolate was about to be outcompeted in later stages by the spreading of additional *R. irregularis* haplotypes from neighboring spoil bank terrain, which was covered by pioneer vegetation already by the time of the setup of the field experiment. This interpretation would be in agreement with the fact that haplotype A in the phylogenetic analysis does not fall within a clade of haplotypes preferentially found in grasslands which might constitute an ecotype adapted to the conditions in less disturbed settings (Börstler et al. 2010). However, additional samplings from later years are required to address this question. A different sampling scheme with several sampling points within each plot would be necessary to address spatial fungal spreading, which was not in the focus of the present study.

The lower frequency of haplotype B in comparison to haplotype A (10 versus 31 samples), which was also almost equally found in inoculated and uninoculated samples, indicates that it was not efficiently inoculated. This could be due to its low abundance in the inoculum, to a lack of competitivity in the field, or even a combination of both. The sparse colonization found by haplotype B might even be explained by an origin other than the inoculum—it might already have been present at the site or might have migrated into it from the surroundings.

We demonstrate that mtLSU is a useful molecular marker for specific tracing of different haplotypes of *R. irregularis* and its close relatives: using root organ cultures, the within-strain homogeneity of mtLSU rDNA for *R. irregularis* has been proven (Raab et al. 2005). This marker has already been used successfully for the characterization of *R. irregularis* strains in pure cultures (Börstler et al. 2008; Raab et al. 2005) as well as for diversity studies in the field (Börstler et al. 2010). Our results show that the specific assay, targeting specific haplotypes, is more sensitive than the general PCR-RFLP approach, as in approximately 30% of the samples tested positive for BEG140 using the specific assay, no RFLP profile corresponding to any of the two BEG140 haplotypes was detected. This is to be expected as BEG140 banding patterns may be concealed by other, possibly more dominant haplotypes in mixed restriction profiles.

We conclude that haplotype A of the inoculated strain *R. irregularis* BEG140 established well in the roots of the target plants on the experimental field site and proliferated there for 3 years even though several native haplotypes of the same species co-existed and established in the roots as well. Considering recent findings on the exchange of nuclear gene markers among isolates of *R. irregularis* (Croll et al. 2009), it will be interesting to see whether nuclear molecular markers (Croll et al. 2008a; Mathimaran et al. 2008) will provide comparable patterns to mitochondrial ones once they are adapted to the tracing of isolates in the field.

This study demonstrates for the first time the feasibility to trace an inoculated *R. irregularis* isolate in the roots of target plants and to verify its establishment and survival in the field. A well-developed and verified technique for the long-term tracing of inoculated AMF strains has the potential to contribute considerably to a breakthrough of the commercial application of mycorrhizal technology. The

users of AMF inoculation will thus be able to prove the persistence of introduced AMF strains in roots of perennial crops even years after inoculation. At the same time, our findings are of general interest for mycorrhizal ecology as we demonstrate for the first time that an introduced isolate of AMF is able to compete and persist in the field among indigenous AMF.

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