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Research

Nonself vegetative fusion and genetic exchange in the arbuscular mycorrhizal fungus *Glomus intraradices*

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Summary

• Arbuscular mycorrhizal fungi (AMF) form symbioses with the majority of plants and form extensive underground hyphal networks simultaneously connecting the roots of different plant species. No empirical evidence exists for either anastomosis between genetically different AMF or genetic exchange.

• Five isolates of one population of *Glomus intraradices* were used to study anastomosis between hyphae of germinating spores. We show that genetically distinct AMF, from the same field, anastomose, resulting in viable cytoplasmic connections through which genetic exchange could potentially occur.

• Pairs of genetically different isolates were then co-cultured in an *in vitro* system. Freshly produced spores were individually germinated to establish new cultures. Using several molecular tools, we show that genetic exchange occurred between genetically different AMF. Specific genetic markers from each parent were transmitted to the progeny. The progeny were viable, forming symbioses with plant roots. The phenotypes of some of the progeny were significantly different from either parent.

• Our results indicate that considerable promiscuity could occur in these fungi because nine out of 10 combinations of different isolates anastomosed. The ability to perform genetic crosses between AMF experimentally lays a foundation for understanding the genetics and evolutionary biology of these important plant symbionts.

Introduction

The arbuscular mycorrhizal fungi (AMF), belonging to the Glomeromycota, are a monophyletic group representing one of the main fungal phyla (James *et al.*, 2006). This phylum must have diverged from other fungi at least 400 million yr ago, although some studies indicate it could be much earlier (Heckman *et al.*, 2001; Corradi *et al.*, 2004). Despite its basal position in fungal evolution, diversification is extremely low. The AMF form a symbiosis with the majority of plants, having important consequences for plant nutrition and plant diversity (Smith & Read, 1997; Van der Heijden *et al.*, 1998). AMF live inside plant roots but also produce extraradical hyphae that extend from the roots into the surrounding soil. The fungi form extensive underground hyphal networks, simultaneously connecting the roots of different plant species (Giovannetti *et al.*, 2004). The hyphae are coenocytic and were shown to be heterokaryotic, possessing genetically different nuclei coexisting in a common cytoplasm (Kuhn *et al.*, 2001). However, the evidence for heterokaryosis is controversial in AMF (Pawlowska & Taylor, 2004; Hijri & Sanders, 2005; Rosendahl, 2008). Hyphae form below-ground networks, connecting many plants, even of different species, genera and families (Giovannetti *et al.*, 2004). Knowledge of the genetics and mating systems, if any, is completely lacking. One reason for this is that AMF have been presumed to be asexual for at least 400 million yr (Remy *et al.*, 1994; Judson & Normark, 1996). AMF populations were shown to be highly diverse and

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the individuals were assumed to form clonal networks in the field that are separate from each other (Koch *et al.*, 2004; Stukenbrock & Rosendahl, 2005). However, anastomosis has been observed between hyphae of the same isolate (Giovannetti *et al.*, 1999), and hyphae of the same AMF isolate growing from different plants were shown to anastomose to create larger networks (Giovannetti *et al.*, 2004). Hyphal fusions among genetically identical hyphae are a ubiquitous phenomenon in filamentous fungi and are thought to be crucial in improving within-network homeostasis and intra-hyphal communication (Glass *et al.*, 2004). However, nonself recognition mechanisms have been shown to prevent most vegetative fusions among genetically different fungi (Glass *et al.*, 2000). If anastomosis occurs between genetically different AMF, this could allow the formation of extensive genetically diverse networks and also possibly allow exchange of nuclei. Genetically different nuclei could fuse and recombine in a common cytoplasm (Pontecorvo, 1956). Even low degrees of parasexuality (i.e. fusion of nuclei and recombination in the absence of meiosis) could dramatically alter the genetic structure of populations and, therefore, their evolutionary trajectory (Hoekstra, 1994). In order to understand the genetics of these important plant symbionts it is necessary to identify whether exchange of nuclei occurs between genetically different individuals of AMF, leading to viable offspring.

The aims of this study were to test whether hyphae of genetically different isolates in a population form anastomoses; whether genetic material is exchanged through potential hyphal fusions resulting in genetically heterogeneous mycelia; and whether any observed genetic exchange could influence the phenotype of the fungus.

Materials and Methods

Isolation and culturing of co-occurring arbuscular mycorrhizal fungi (AMF)

Single spores of *Glomus intraradices* from one agricultural field site in Tänikon, Switzerland, were used to establish axenic cultures (St-Arnaud *et al.*, 1996; Koch *et al.*, 2004). Sampling was performed on four different plots each separated by 30–85 m. Isolates A4, B3 and D1 originate from different plots, while isolates C2 and C3 originate from the same plot. All isolates used in this work were previously shown to be genetically distinct and differ in their phenotypic traits (Koch *et al.*, 2004; Croll *et al.*, 2008b). The chosen isolates cover the most divergent genotypes found in the field population. For each isolate, the internal transcribed spacer region was sequenced and compared with deposited sequences of *G. intraradices* to confirm species identity (Croll *et al.*, 2008b). Before all experiments, the fungi were cultivated over 3 yr in identical environmental conditions with Ri T-DNA-transformed carrot root on standard M growth medium (Koch *et al.*, 2004, 2006).

Expt 1: observation of hyphal anastomosis and measurement of anastomosis frequency

The experiment on anastomosis between AMF isolates of *G. intraradices* was carried out twice using two different culture conditions; water and M medium (Bécard & Fortin, 1988). In both systems, spores were placed on to 30 × 40 mm sterile cellophane membranes (Hoefer, San Francisco, CA, USA) that were then put into 9-cm-diameter sterile Petri dishes. In the water culture system, the membranes were placed in the bottom of Petri dishes and moistened with sterile distilled water (SDW) by means of a sterile wet cotton roll. In the M medium culture system, the membranes were placed on to M medium lacking sucrose and vitamins.

Anastomosis frequency In order to estimate anastomosis frequency among and within the isolates of *G. intraradices*, a cluster of six spores of one isolate was placed on a cellophane membrane next to a cluster of six spores of the same or a different isolate (Fig. 1a). The spore clusters were placed 3 mm apart on parallel lines. This arrangement of spores was repeated five times per membrane. There were three replicate membranes per pairing of isolates. All possible combinations of pairings were made with the isolates A4, B3, C2, C3 and D1, giving a total of 15 combinations (10 between different isolates and five within-isolate pairings). This whole procedure was repeated at least five times for each pairing and with both water and M medium (Table 1 and Supporting Information, Table S1).

Petri dishes were then sealed with parafilm and incubated at 25°C in the dark. After 35 d incubation, the germinated hyphae were observed for contact. To do this, hyphae that touched each other were observed under a dissecting microscope. Hyphae were traced back to the original spore to ensure that the contact was between two hyphae originating from two different spores. Viability of the hyphae at the contact point was then assessed by staining for the presence of succinate dehydrogenase (SDH) activity (Giovannetti *et al.*, 2004). Deposition of formazan salts in hyphae allowed the visualization of viable mycelia and of protoplasmic continuity between fusing hyphae. Membranes bearing contacts that stained positively with SDH were mounted on microscope slides and stained with 0.05% Trypan blue in lactic acid and observed under a Polyvar microscope (Reichert-Jung, Vienna, Austria). Hyphal contacts were scored at magnifications of ×125–500 and verified at ×1250 for the status of the contact. A contact between two hyphae was scored as noninteracting when hyphae touched, but no morphological changes were observed and no fusion occurred. Fusion was scored as perfect when anastomosis occurred, streaming of the protoplasm could subsequently be observed between the two hyphae, and staining with SDH showed metabolic activity at the hyphal bridge. Two other intermediate states were observed and scored. Pre-fusion incompatibility was scored where morphological changes

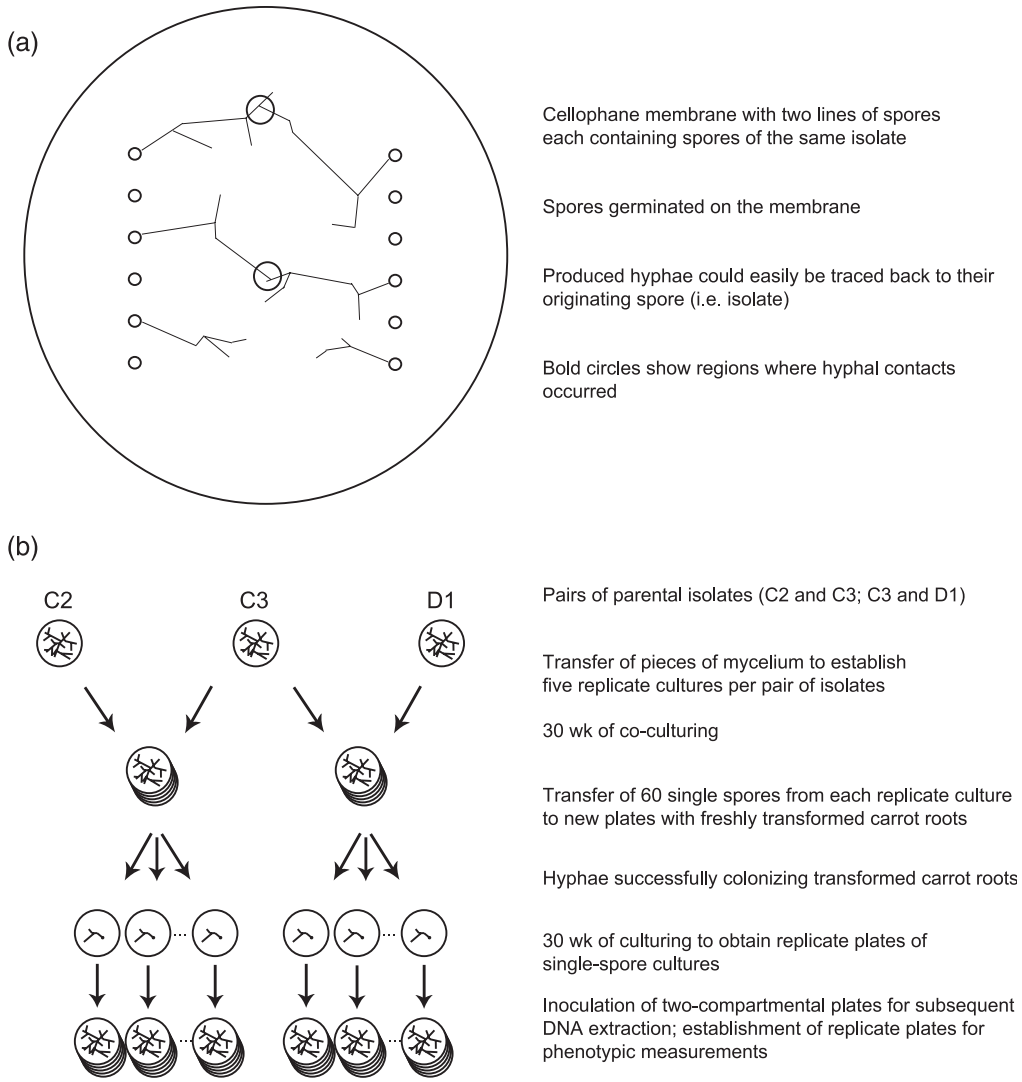

Fig. 1 Conceptual drawings of the experimental design. (a) Observation of hyphal contacts between different isolates showing the ease of tracing the two fused hyphae back to the original parental spores. (b) Culturing procedure to obtain single-spore progeny from plates containing pairs of parental isolates. For further details see the Materials and Methods section.

occurred between the two hyphae, indicating the first stages of anastomosis but then the protoplasm of one hypha retracted. Post-fusion incompatibility was scored when, following anastomosis, the protoplasm of one hypha withdrew and a septum formed between the two hyphae.

To observe the process of incompatibility in more detail in hyphal contacts, the presence of wall thickenings and retraction septa was assessed on hyphae on membranes stained with DAPI, which were mounted in a 0.01% (w/v) solution of Calcofluor White (Sigma-Aldrich s.r.l., Milano, Italy). These were observed under epifluorescence with the filter combination U1. Images were captured through a three-CCD colour video camera connected to a computer, by using the software Pinnacle 8.

Time-lapse microscopy of hyphal anastomosis formation Continuous observations of *in vivo* hyphal growth and anastomosis were performed in the water system culture conditions. Plates were incubated in the dark at 25°C and observed daily under an inverted microscope equipped with a video camera and connected to the computer (DMIRB, Leica, Milano, Italy). Movies showing anastomosis formation, protoplasmic streaming through hyphal connections and incompatibility responses were recorded using the Pinnacle software. Anastomosing hyphae were monitored, and pre- and post-fusion events and elapsed times recorded.

Correlation of perfect fusion frequency and genetic distance between isolates Amplified fragment length polymorphism (AFLP) data from Koch *et al.* (2004) was used to calculate genetic distance among the five isolates from the population. Euclidean distances were calculated using PAUP 4.b10 (Swofford, 2002), representing percentages of shared AFLP

Table 1 Frequency of anastomosis (fusion) between pairs of arbuscular mycorrhizal fungi

	C[a]	PF[b]	PFI[c]	PrFI[d]	NI[e]
Within isolate					
A4–A4	156	75 (48.1%)	0 (0%)	0 (0%)	81 (51.9%)
B3–B3	158	78 (49.4%)	0 (0%)	0 (0%)	80 (50.6%)
C2–C2	202	103 (51.0%)	0 (0%)	0 (0%)	99 (49.0%)
C3–C3	101	48 (47.5%)	0 (0%)	0 (0%)	53 (52.5%)
D1–D1	144	66 (45.8%)	0 (0%)	0 (0%)	78 (54.2%)
Between isolates					
A4–B3	106	2 (1.9%)	9 (8.5%)	9 (8.5%)	86 (81.1%)
A4–C2	108	5 (4.6%)	15 (13.9%)	2 (1.9%)	86 (79.6%)
A4–C3	97	10 (10.3%)	11 (11.3%)	4 (4.1%)	72 (74.2%)
A4–D1	82	0 (0%)	11 (13.4%)	6 (7.3%)	65 (79.3%)
C2–B3	106	2 (1.9%)	13 (12.3%)	7 (6.6%)	84 (79.2%)
C2–C3	95	5 (5.3%)	18 (18.9%)	0 (0%)	72 (75.8%)
C2–D1	108	2 (1.9%)	22 (20.4%)	9 (8.3%)	75 (69.4%)
C3–B3	103	1 (1.0%)	11 (10.7%)	6 (5.8%)	85 (82.5%)
C3–D1	98	1 (1.0%)	15 (15.3%)	4 (4.1%)	78 (79.6%)
D1–B3	91	4 (4.4%)	8 (8.8%)	3 (3.3%)	76 (83.5%)

[a]Number of hyphal contacts (C) observed either between hyphae of the same isolate or between hyphae of genetically different isolates growing in water.
[b]Contacts resulting in perfect fusion (PF).
[c]Contacts resulting in prefusion incompatibility (PrFI).
[d]Contacts resulting in post-fusion incompatibility (PFI).
[e]Contacts showing no interaction (NI).

bands. The correlation between genetic distances and percentages of perfect fusions among all pairs of isolates was calculated using the Mantel test implemented in FSTAT 2.9.3 (Goudet, 2001).

Expt 2: detection of genetic exchange among isolates

Two pairs of isolates (C3 and C2, originating from the same field plot; C3 and D1, originating from different field plots) were co-cultured together on Petri dishes containing M medium in order to obtain progeny that could potentially be the result of genetic exchange between isolates. Five replicate cultures of each pairing were established by transferring blocks of *c.* 4 cm^2 of media containing hyphae and spores of each isolate (Fig. 1b). Ten days after the initial transfer, new hyphae growing out of each of the transferred blocks were observed. After 30 wk of co-culturing (two growth periods of 15 wk each), 60 single spores were isolated from each culture and individually transferred to new plates containing M medium (one spore per plate), with a nonmycorrhizal Ri T-DNA-transformed carrot root. Spores isolated from cultures of isolate pairings germinated at rates of 68–82% and 63–72% for the pairings C2–C3 and C3–D1, respectively. Ten to 18 single spores of each replicate culture of the pairings of isolates C2 and C3 successfully colonized the transformed carrot roots. Eight to 14 spores from each of the pairings of isolates C3 and D1 successfully colonized the transformed carrots. Single-spore cultures were further cultivated for 60 wk through clonal subculturing (four growth periods of 15 wk) by transferring *c.* 4 cm^2 of media containing mycelium and roots. One single-spore culture from each replicate culture of the two pairings was chosen randomly (if hyphal growth and spore production were sufficient for subsequent culturing). For each single-spore culture, six to 10 two-compartment plates were inoculated (St.-Arnaud *et al.*, 1996). The two-compartment plates allow the proliferation of the fungus in one compartment that is root-free, while remaining connected to the roots in the other compartment (St.-Arnaud *et al.*, 1996). After 15 wk, the root-free fungal compartments of all plates were removed and pooled per single-spore line for extraction of hyphae and spores (Koch *et al.*, 2004). Five single-spore lines each of different replicate co-cultures of C2 and C3 were successfully cultured for sufficient DNA extraction, while four single-spore lines of different replicate co-cultures of C3 and D1 were successfully cultured.

Establishment of single-spore lines from parental isolate C3
Single spores were isolated from an *in vitro* culture of isolate C3 in the same way that single spores were isolated from cultures in Expt 1. Genetic analysis of single spore lines originating from a single isolate allows one to control for consistency in genotypes among spores. Furthermore, this allowed us to test whether variation in the genetic fingerprint among spores originating from a single spore culture could be as strong as variation seen among spores originating from pairings of isolates. A total of four single-spore lines from isolate C3 were successfully cultivated for DNA extraction.

With these cultures, AFLP could be performed on single-spore lines that could potentially be the result of genetic exchange. In addition, we already knew that these lines were viable as they had colonized the plant roots and produced new extraradical hyphae and spores. In parallel, the isolates C2, C3 and D1 used to establish the pairings were also cultivated on two-compartment plates to grow material for DNA extraction.

Genotyping of the single spore lines and parental isolates

DNA extraction Freshly isolated hyphae and spores were used for extraction of DNA from each single-spore line (originating from pairings of isolates and from isolate C3) and the parental isolates. All fungal material was separately dried overnight at 48°C and ground into a fine powder using the Retsch MM300 machine from Qiagen Inc. (Hombrechtikon, Switzerland). The DNA was extracted using a modified version of the Cenis method for fungal DNA extraction (Cenis, 1992) with an additional step of 1 : 1 dilution with a solution of 24 : 1 of chloroform isoamyl alcohol before the final precipitation, to remove remaining impurities.

Amplified fragment length polymorphism AFLP was used to score total genetic variation using 200 ng of DNA for each of two replicate reactions. All DNA quantifications were performed using Picogreen reagents (Invitrogen, Inc., Carlsbad, CA, USA). The AFLP protocol by Koch *et al.* (2004) was used, except for the following. Digestion was performed in 25 μl reaction volumes containing 15 U *Eco*RI and 15 U *Mse*I restriction enzymes, 0.025 μg BSA and *Eco*RI reaction buffer (all from New England Biolabs Inc., Ipswich, UK). Incubation was at 37°C for 3 h. Ligation was performed overnight at 10°C with 1 U of T4 ligase and T4 ligase reaction buffer (both from New England Biolabs) in a total volume of 30 μl. Ligated reaction products were diluted 1 : 1 with TE buffer. The preamplification reaction contained 2.5 μl of the diluted ligation product, 2.5 μl 10× PCR buffer plus Mg (Qbiogene, Inc., Morgan Irvine, CA, USA), 0.08 mM dNTPs, 0.324 μM E-0 primer, 0.36 μM M-0 primer and 2.5 U Qbiogene Taq DNA polymerase in a total volume of 25 μl. The reaction product was diluted 1 : 10 with ddH_2O. Selective amplification was in a 10 μl mix containing 2.5 μl of the diluted preamplification product, 1 μl 10× PCR buffer plus Mg (Qbiogene), 0.1 mM dNTPs, 0.3 μM EcoRI fluorescence-labelled selective primer, 0.1125 μM MseI selective primer, 0.5 U Qbiogene Taq DNA polymerase. Five combinations of selective primers were used for the amplification of fragments: *Eco*RI-TC (FAM)/*Mse*I-TT, *Eco*RI-GTG (FAM)/*Mse*I-TTG, *Eco*RI-AAG (FAM)/*Mse*I-CCT, *Eco*RI-TT (HEX)/*Mse*I-AT and *Eco*RI-TT (HEX)/*Mse*I-TT.

Selective PCR products labelled with different fluorescent dyes (HEX or FAM) were multiplexed in pairs before being run on am ABI-3100 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA, USA). From each of the two PCR products, 1 μl was used and added to 12.8 μl of Hi-Di formamide and 0.2 μl of ROX-500 size standard. AFLP fragments between 75 and 500 bp were manually scored using GeneMapper 3.7™ software (AppliedBiosystems).

Scoring procedure for AFLP loci For all loci, band presence was scored if bands of both replicate runs exceeded 50 relative fluorescence units (RFU) out of approx. 10 000 RFU detected in capillary electrophoresis. The setting of this threshold was necessary because band scoring can be unreliable at very low abundance. If bands of either of the two replicates of a sample were below 50 RFU, the locus was scored as an unknown state for that sample. Therefore, only unequivocal loci were considered to score genetic variation among single-spore lines and their respective parental isolate. A complete overview of fingerprints of parental isolates and single-spore cultures of pairings of isolates can be found in Tables S2–S4.

Control for scoring accuracy and potential variation within parental lines The four single-spore lines originating from isolate C3 were scored in the same way as for the other lines originating from pairings of isolates, by applying the same threshold of 50 RFU to all loci. For each locus of parental isolate C3, we checked whether all four single-spore lines showed band presence. In this way, it was possible to ensure that our method of scoring genetic variation by AFLP was reliable and did not produce artefacts by applying the scoring threshold of 50 RFU (potentially excluding bands showing marginally smaller peak heights than 50 RFU) and that single spores from parental lines did not give significantly different fingerprints from the parental genotype. Comparing all four single-spore lines from C3, a total of 489 loci were scored, of which 443 (91%) showed clear presence of a peak (both replicates of each of the four single-spore lines showed a peak above 50 RFU; see earlier).

In Table S3, a complete overview of AFLP fingerprints of parental isolates and single-spore progeny cultures of pairings of isolates is shown as in Table S2, but all loci showing inconsistent scorings among single spore cultures of C3 were excluded (see earlier). This most conservative assessment of fingerprints shows that evidence for genetic exchange as presented in Table 2 and Table S2 is not significantly affected by the scoring method.

Sequence-based markers We used six previously described nuclear loci to genotype parental isolates and single-spore progeny (Croll *et al.*, 2008b). These loci were sequenced and shown to exhibit length polymorphism among the parental isolates by Croll *et al.* (2008b). Loci Bg26, Bg62, Bg196, Bg273, Bg348 and Bg355 were amplified, and fluroescence-labelled fragments were visualized on a ABI-3100 Genetic Analyzer according to Croll *et al.* (2008b). Alleles of parental isolates and progeny were scored manually using GeneMapper 3.7 software.

Table 2 Detecting genetic exchange between pairs of arbuscular mycorrhizal fungi

	Number of parental isolate specific AFLP loci[a]		
	C2	C3	D1
Parental isolates			
Isolate C2	223	0	
Isolate C3	0	127	
Single-spore cultures[b]	Presence of parental isolate-specific loci in offspring[c]		
S1[d]	76	83	
S2	104	69	
S3[d]	73	83	
S4	136	45	
S5[d]	75	85	
Parental isolates	Number of parental isolate-specific AFLP loci[d]		
Isolate C3		176	0
Isolate D1		0	259
Single-spore cultures[e]	Presence of parental isolate-specific loci in offspring[c]		
Sa[d]		94	73
Sb[d]		98	72
Sc[d]		99	72
Sd[d]		84	67

[a]A number of loci observed in AFLP fingerprints were specific to one or other parental isolates.
[b]S1–S5 denote single-spore cultures established from spores produced in co-culture of parental isolates C2 and C3.
[c]Cultures initiated with single spores that were produced after co-culturing two parental isolates were fingerprinted with AFLP. The number of parental isolate-specific loci from both parents was observed in these cultures.
[d]The genotypes of the three single-spore cultures S1, S3 and S5 from pairings of parental isolates C2 and C3 are similar in that they not only possess similar numbers of parental-specific loci but also inherited the same parental-specific loci. Meanwhile, Sa–Sd also inherited the same parental specific loci from their parental isolates C3 and D1.
[e]Sa–Sd denote single spore cultures established from spores produced in co-culture of parental isolates C3 and D1.

Additionally, PCR reactions were set up for each of the six loci containing a 50 : 50 mixture of DNA from the parental isolates C2 and C3. Electropherograms revealed the presence of both alleles, each being specific to either of the two parental isolates (data not shown). This confirmed that the amplifications of alleles are sensitive to amplify two parental specific alleles in one reaction.

Alleles in progeny were cloned with the TOPO TA Cloning Kit (Invitrogen) following the manufacturer's instructions. Sequencing was performed with the BigDye 3.1 Terminator cycle sequencing kit (Applied Biosystems, Inc.) using M13 primers and run on an ABI Prism 3100 Genetic Analyzer.

Copy number polymorphisms in ribosomal RNA genes Isolates of the *G. intraradices* population were recently shown to harbour different copy numbers for three ribosomal RNA genes (Corradi *et al.*, 2007). In that study, isolate C2 was shown to harbour significantly more copies of the 5.8S, 18S and 25S ribosomal RNA genes than isolate C3. Meanwhile, the single-copy gene *Rad15* was used to control for homogeneity in copy number estimates among different DNA samples. Following the established protocol (Corradi *et al.*, 2007), the relative copy numbers for the ribosomal gene 5.8S were measured in parental isolates C2 and C3, as well as in the single-spore line S2. The single-copy gene *Rad15* was used as a control in both parental isolates and S2 (Corradi *et al.*, 2007). All primer sites were shown to be conserved among isolates of *G. intraradices* (Corradi *et al.*, 2007). Two replicate amplifications were performed for each sample and averaged for plotting.

Measurement of phenotypic traits on parental and single-spore cultures Parental isolates C2, C3 and D1, as well as the four single-spore cultures obtained from each of the two pairings of parental isolates, were measured for hyphal and spore density as described in Koch *et al.* (2004). Progeny S2 was not included in the measurements of phenotypic traits, as there were insufficient numbers of spores and hyphae on culture plates at the moment of the setup of replicate cultures. Nevertheless, at the subsequent round of clonal subculturing, hyphal and spore densities of progeny S2 did not appear to be lower than in other progeny. Consistent differences in phenotypic traits among parental isolates were observed over several years of cultivation in this axenic system (Koch *et al.*,

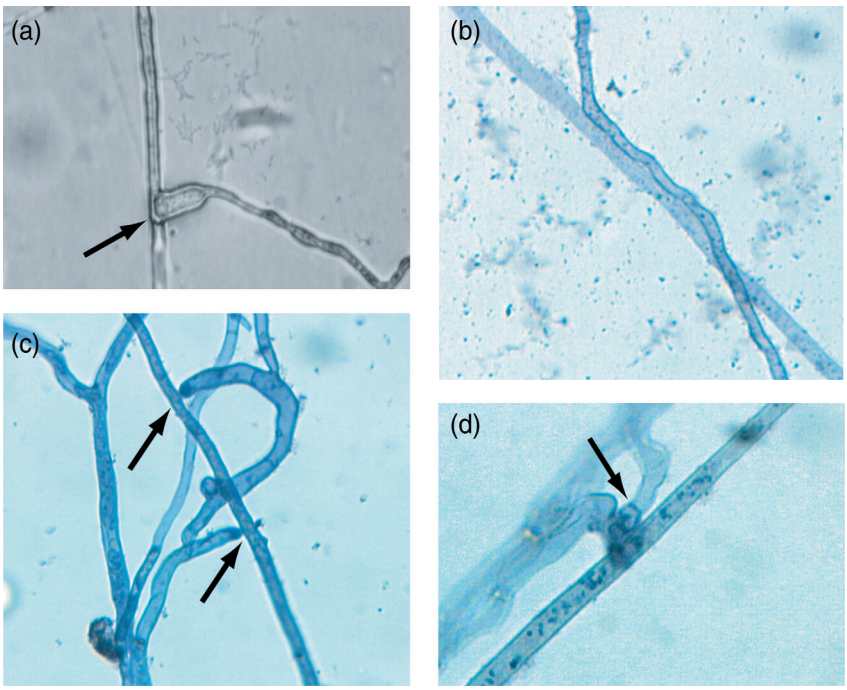

Fig. 2 Contacts between hyphae of genetically different isolates. (a) Perfect fusion (arrow), protoplasmic streaming occurs within the fusion bridge (also see Supporting Information, Videos S1 and S2). (b) No interaction; no morphological change is observed in hyphae even though there is contact between hyphae. (c) Pre-fusion incompatibility; protoplasm withdrawal occurs in the approaching hypha. Arrows show two contact points. (d) Incompatible fusion; protoplasm withdrawal and septum formation occur (arrow) in one of the fused hyphae after anastomosis. Staining for succinate dehydrogenase (a) and with Trypan blue (a–d).

2004, 2006). To reduce potential maternal effects resulting from the establishment of single-spore cultures from pairings of parental isolates, single-spore cultures were grown for 60 wk on culture medium. During this period, *c.* 4 cm^2 pieces of media containing hyphae and spores were transferred to fresh plates every 15 wk. Sixteen replicate plates were established for each parental isolate and each of the single-spore cultures from pairings of isolates for phenotypic measurements. After a further growth period of 15 wk, all plates were measured for hyphal and spore density as described in Koch *et al.* (2004). A total of 176 plates were included in the statistical analysis. The data were analysed separately for parental isolates C2, C3 and progeny S1, S3–S5 and parental isolates C3, D1 and progeny Sa–Sd by a one-way analysis of variance (ANOVA). Pairwise comparisons among parental isolates and progeny were performed by a Tukey–Kramer test ($\alpha = 0.05$). A Dunnett's test was also performed using the parental isolates as control samples ($\alpha = 0.025$). The results were qualitatively identical.

Results

Anastomoses among isolates of a population (Expt 1)

We tested the occurrence of anastomosis among all possible combinations of pairs of five *G. intraradices* isolates, analysing between 82 and 202 hyphal contacts per pair of isolates (Table 1). When hyphae from spores of the same isolate grew together, between 46 and 51% of the contacts resulted in perfect fusion (Table 1). All other contacts, where hyphae touched but no morphological changes were observed and no fusion occurred, were scored as noninteracting. Pairings between genetically different isolates resulted in much lower numbers of anastomoses, but in all pairings, except for the pairing of isolate A4 with D1, some perfect fusions were observed (Table 1, Fig. 2a). At the hyphal bridge of a perfect fusion, rapid cytoplasmic streaming occurred, either in one direction (Video S1) or in both directions (Video S2). Although the majority of contacts resulted in no interaction (Fig. 2b), a large number of hyphal contacts produced intermediate interactions that were not observed in pairings between hyphae of the same isolate (Table 1). These were scored as pre-fusion incompatibility, where morphological changes occur within two hyphae, indicating the first stages of hyphal recognition but then the protoplasm of one hypha retracts (Table 1, Fig. 2c). Post-fusion incompatibility also occurred, where, following anastomosis, the protoplasm of one hypha withdraws and a septum forms between the two hyphae (Table 1, Fig. 2d). The experiments of hyphal fusion were repeated in water and gellan gum-based culture medium and yielded very similar results (Table S1).

The percentage of perfect fusions between genetically different isolates was negatively correlated with the genetic distance between the isolates in the water system ($R^2 = 0.41$; $P < 0.05$), but not in the M medium system ($R^2 = 0.03$; $P = 0.7$).

Molecular evidence for genetic exchange (Expt 2)

AFLP markers Perfect fusions (and possibly fusions that result in post-fusion incompatibility) offer the possibility for exchange of nuclei between genetically different AMF. In

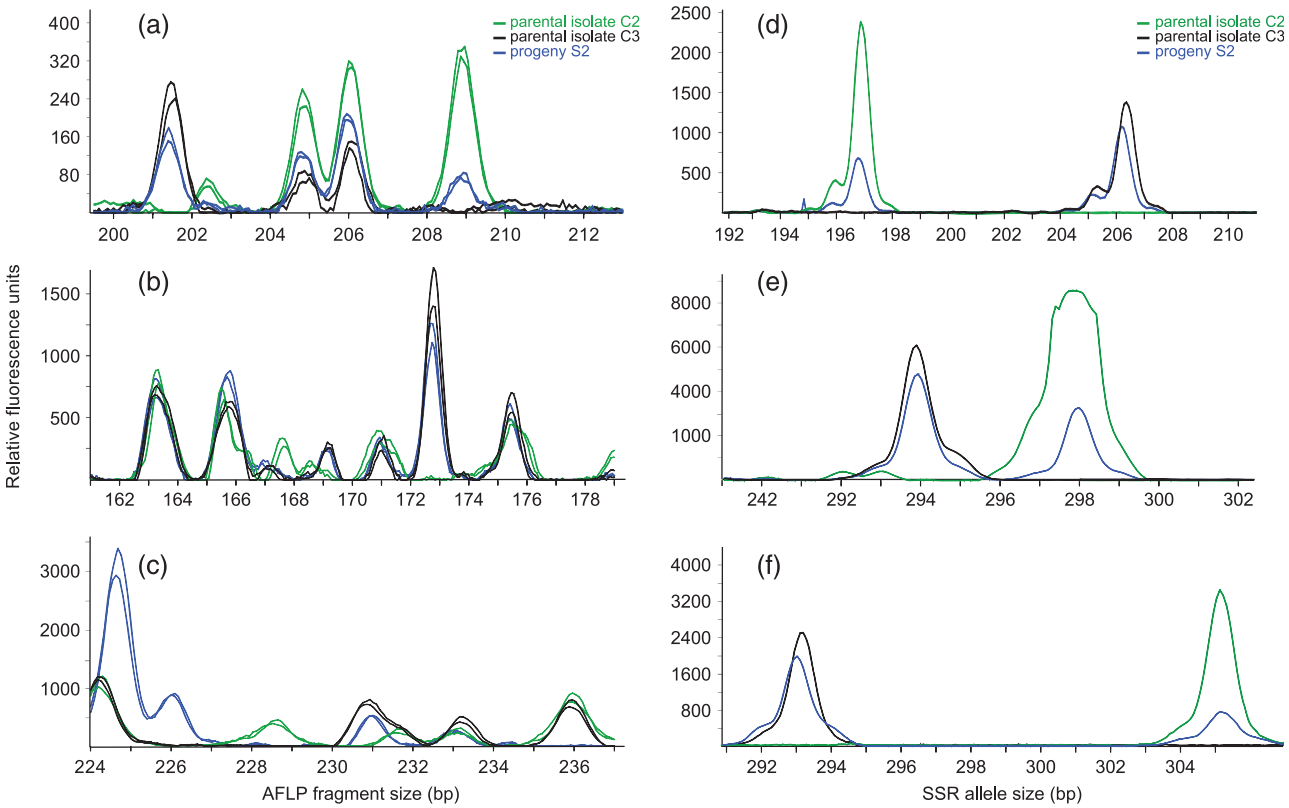

Fig. 3 Detecting genetic exchange using genetic markers. (a) Part of an electropherogram of an AFLP fingerprint showing five loci, two of which are specific to parental isolate C2 (202 bp, 209 bp; shown in green), one of which is specific to parental isolate C3 (201 bp; shown in black) and two that are not parental-specific (205 bp, 206 bp). The fingerprint of the single-spore culture S2 (progeny of co-culture of parental isolates C2 and C3; shown in blue) shows the presence of two parental-specific loci, one from each parental isolate. Lines of the same colour represent replicate fingerprints of the same sample. (b, c) Two additional examples of electropherograms of AFLP fingerprints showing biparental inheritance of parental-specific loci. For numbers of all loci observed, see Table 2 and Supporting Information, Tables S2 and S3. (d–f) Electropherograms of amplified alleles from the polymorphic loci Bg348, Bg196 and Bg62, respectively. Parental isolates C2 and C3 exhibit alleles of different sizes (shown in green and black, respectively). For all three loci, the fingerprint of DNA from an arbuscular mycorrhizal fungal (AMF) culture initiated from single spore S2 (shown in blue) shows the presence of both alleles. Alleles were scored as present in all genetic analyses when the relative fluorescence units for a peak were > 50.

order to test for genetic exchange between genetically different AMF, we analysed the AFLP banding patterns of DNA from the progeny of the co-cultured parental AMF. We looked for the presence and absence of markers that were shown to be specific for each of the parental isolates (Fig. 3a–c, Table 2). All five progeny from the pairing of parental isolates C2 with C3 possessed many markers that were specific to each of the two parental isolates (Table 2). Three of the five progeny (S1, S3 and S5) had almost identical genotypes (Table 2), even though they originated from crossings performed on independent plates. All four progeny from the pairing of parental isolates C3 with D1 showed biparental inheritance, as each progeny possessed several markers that were specific to each of the two parental isolates (Table 2). Additionally, all four progeny appeared to harbour almost identical genotypes even though they also originated from crossings performed on independent plates. AFLP analysis showed that a fraction of the loci detected in progeny was not found in either of the two respective parental isolates (Table S2). Similarly, a fraction of AFLP loci common to the two respective parental isolates was not found in the progeny (Table S2).

In the control experiment, four single spores from parental isolate C3 were cultivated and genotyped in the same way as progeny from the crossing experiment. In total, 9% of all loci were found to be variable among single-spore progeny from the parental isolate C3. This corresponds to approximately only 10 AFLP fragments. This number is therefore much lower than the differences seen between progeny of the co-cultures and the parental isolates (> 100 fragments). This shows that the genetic differences among progeny from the co-cultures must be the result of genetic exchange, as the variation could not be explained by genetic drift occurring during spore formation, in the absence of genetic exchange. Table S3 shows data where these variable loci were excluded to obtain a conservative estimate of polymorphism among parental isolates and single-spore progeny from the crossing experiment. Both

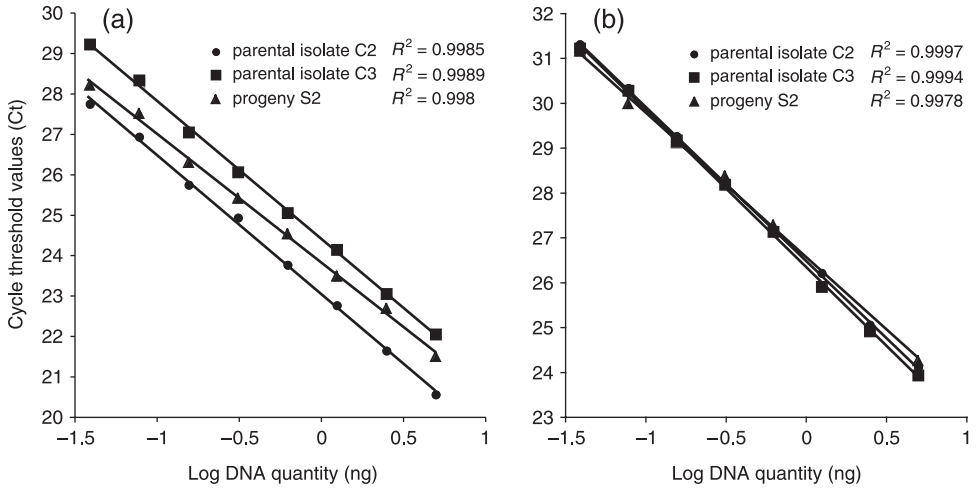

Fig. 4 Real-time quantitative PCR results showing linear regressions of cycle threshold values and log DNA quantity. Each line represents the average of two replicate amplifications. (a) Amplification of the 5.8S ribosomal gene shows copy number polymorphism between parental isolates C2 and C3, while single-spore culture S2 shows an intermediate copy number. (b) Amplification of Rad15, a single-copy gene in parental isolates C2 and C3 and single-spore culture S2.

scoring methods show that each progeny possesses several markers that were specific to either of the two parental isolates and that the scoring method does not bias the results.

Sequence-based markers We also used six different sequence-based markers that distinguish nuclear genomes of parental isolates C2, C3 and D1 by length differences among the specific alleles (Croll *et al.*, 2008b) to test whether progeny inherit DNA from both parents. One progeny (S2) from the pairing of parental isolates C2 and C3 showed clear evidence for biparental inheritance (Fig. 3d–f). For each of these six loci, parental-specific alleles from both parents co-occurred in the progeny S2. The progeny S1, S3, S5 and Sa–Sd showed the presence of only alleles from parental isolate C3. The progeny S4 showed only alleles from parental isolate C2. Alleles in the progeny were sequenced and this confirmed their identity with parental isolates.

Copy number polymorphism The parental isolates were shown to differ in ribosomal gene copy number (Corradi *et al.*, 2007). We therefore measured copy number of the 5.8S gene of progeny S2 that showed evidence for genetic exchange based on the six sequence-based markers. The copy number estimate was compared with that of the parental isolates C2 and C3. A single-copy gene (*Rad15*) was used as a control amplification for all samples (Corradi *et al.*, 2007). Copy number of progeny S2 was intermediate between that of the parental isolates C2 and C3 (Fig. 4). Relative copy numbers of three single-spore progeny from pairings of parental isolates C2 and C3 (S1, S3, S5) indicated similar copy numbers to parental isolate C3, and one single-spore progeny (S4) indicated a similar copy number to parental isolate C2 (data not shown).

Phenotypic traits of parental isolates and single-spore progeny

The parental isolates showed large and heritable differences in hyphal and spore densities similar to that previously seen by Koch *et al.* (2004, 2006) (Fig. 5). Hyphal and spore densities among progeny varied significantly among offspring, which would not be expected if they were genetically similar to the parental isolates (Fig. 5). The factor isolate/progeny was highly significant ($P < 0.0001$) for both ANOVA. Progeny S1–S4 showed a range of hyphal and spore densities exceeding the difference between the parental isolates C2 and C3. Hyphal densities of two progeny (S3 and S5) from the pairing of isolates C2 and C3 were significantly higher than in both parental isolates (Fig. 5). All other progeny of both pairings exhibited either intermediate hyphal and spore densities compared with the parental isolates or did not differ significantly from one of the parental isolates.

Discussion

The results of the three different molecular analyses on the progeny of the pairings between parental isolates show that genetic exchange had indeed occurred. It is notable that, using AFLP markers, we found evidence of genetic exchange in all nine progeny from two pairings, even though anastomosis frequency in these pairings was low. Moreover, one progeny out of nine gave strong evidence of exchange using six independent genetic markers. Two lines of evidence suggest that, following anastomosis and exchange of nuclei, replication of DNA originating from the two different individuals is sustained, rather than the genetic material of one isolate outcompeting all of that from the other isolate. First, the genetic

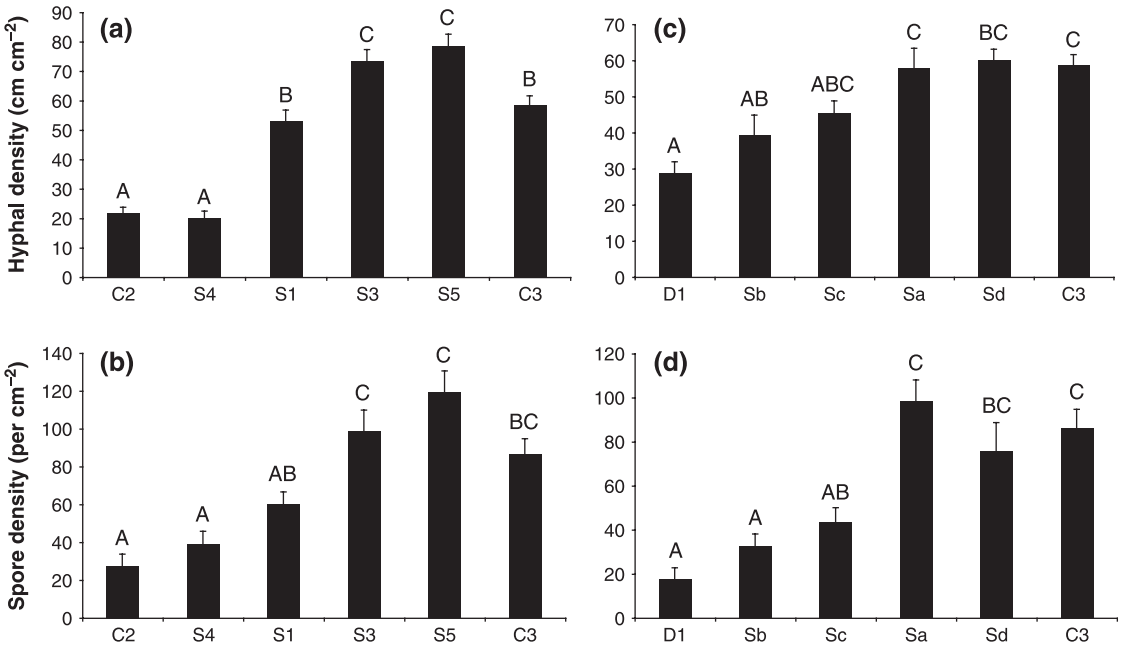

Fig. 5 Phenotypic trait characterization of progeny cultures. (a, b) Mean hyphal and spore densities of parental isolates C2, C3 and single-spore progeny cultures S1, S3, S4 and S5 from pairings of the parental isolates. (c, d) Mean hyphal and spore densities of parental isolates C3 and D1 and single-spore progeny cultures Sa–Sd. Error bars represent +1SE. Different letters indicate significant differences based on a Tukey–Kramer test ($\alpha = 0.05$).

analyses were performed on a large amount of spores and hyphae that have regrown clonally from an individual spore after having successfully formed a symbiosis with a plant root. Second, the progeny were also maintained in symbiosis over a period of 18 months before genetic analyses.

Anastomoses among isolates in a population

Anastomoses play a critical role in many filamentous fungi, including improving network interconnectedness within a growing mycelium and during the encounter between genetically different individuals during sexual reproduction (Glass *et al.*, 2004). Hyphal fusions within mycelia of AMF were observed in isolates of *G. mosseae*, *G. caledonium* and *G. intraradices* (Giovannetti *et al.*, 1999, 2001, 2004), but vegetative incompatibility mechanisms, known from Ascomycota and Basidiomycota, were hypothesized to prevent hyphal fusions among genetically different isolates (Pawlowska & Taylor, 2005). Indeed, vegetative incompatibility was found to occur between isolates of *G. mosseae* from different geographic locations (Giovannetti *et al.*, 2004). Our results show that genetically well-characterized isolates of one population form functional anastomoses. Such fusions have the potential to allow exchange of nuclear material among growing mycelia. The negative correlation of genetic distance and perfect fusion frequency identified in the water system suggests that vegetative incompatibility may be a gradual process in AMF, that is, that genetically similar isolates are more likely to fuse than genetically distant isolates. The relative ease of tracing hyphae back to the original spore, not requiring flurorescence-labelling, make the experimental system ideal to study larger numbers of different isolates, originating from different locations. Furthermore, the ease of tracing the fused hyphae back to the parents avoids the need to develop molecular labelling of nuclei to show such fusions in fungi that form a dense mat of mycelium. However, potential anastomoses among mature mycelia should be studied if reliable tracking of different isolates of AMF becomes feasible. Anastomosis frequencies among secondary mycelia could be different from those for germinating mycelia and be under the influence of various environmental factors.

Inheritance of genetic markers from parental isolates

AFLP analysis of progeny The AFLP fingerprints of single-spore cultures established from spores isolated from pairings of parental isolates showed evidence of biparental inheritance and exchange of genetic material. AFLP gives a genome-wide scan of polymorphic loci among individuals, thus providing a large number of reproducible loci for genotyping. All nine tested cultures showed evidence of genetic exchange using AFLP fingerprinting. None of the single-spore progeny showed exactly one of the parental genotypes. This suggests that hyphal fusions and eventual exchange of genetic material occur relatively frequently in *in vitro* cultures.

A fundamental concern with the application of arbitrary genetic markers, such as AFLP, to genotyping single-spore

progeny is that processes other than genetic exchange may influence the genotype of the progeny. In particular, in the case of a heterokaryotic mycelium where genetically different nuclei coexist (Kuhn *et al.*, 2001; Hijri & Sanders, 2005), spores inheriting nuclei through a random process (i.e. genetic drift) could be genetically different, even though they originate from the same mycelium (Sanders, 2002). However, the heterokaryosis hypothesis has been disputed in AMF (Pawlowska & Taylor, 2004) and no data on genetic differences among nuclei are available for *G. intraradices*. In our control experiment, we were able to distinguish between potential segregation of nuclei within isolates and genetic exchange. Relative to the genetic difference among the different parental isolates, only a small amount of genetic variation was found among progeny spores from one isolate. Our results corroborate the occurrence of genetic exchange among parental isolates because the AFLP data cannot be explained solely by segregation of genetically different nuclei that coexist within an isolate. Furthermore, this control experiment also shows that potential artefacts from AFLP genotyping, such as random shifts in frequency of markers or detection thresholds, are negligible.

Genetic entities in AMF comprise nuclei in a continuous cytoplasm, but also mitochondria and potentially endosymbiotic bacteria. Even though endosymbiotic bacteria were not observed in *in vitro* cultures of *G. intraradices* (M. Hijri & I. R. Sanders, unpublished), AFLP does not provide information about the location in the genome where particular loci occur. Thus evidence for genetic exchange based on AFLP may be partly the result of exchange of mitochondria and/or endosymbiotic bacteria. However, the nuclear DNA content is approx. 15 Mb (Hijri & Sanders, 2004). The *G. intraradices* genome must therefore be significantly larger than the mitochondrial genome. Thus, the large proportion of parental specific loci that were inherited by offspring spores suggests that the genetic exchange is likely to be mostly based on nuclear AFLP markers.

Not all specific AFLP loci of each of the two parents were found in the progeny, suggesting either shifts in frequency or complete disappearance of these particular genetic markers (see Tables S2–S4 for full AFLP data). If a given locus in a DNA sample becomes rare, AFLP fingerprints may indicate that locus as missing, although it may be present but below the detectable limit of AFLP. Similarly, AFLP fingerprints may indicate an appearance of a putatively new locus in the case where a locus at low frequency simply becomes more frequent. These two scenarios might account for disappearance or appearance of loci in fingerprints of progeny. In this way, the AFLP data could reflect changes in frequencies in loci rather than novel loci, or complete disappearance (see columns 'Loci common to parental isolates missing in progeny' and 'Loci found in progeny not observed in parental isolates' in Tables S2 and S3).

Several single-spore progeny showing evidence of biparental inheritance were very similar based on their AFLP genotype (S1, S3, S5 and Sa–Sd), as they share a large proportion of loci (Table S4). It is notable that S1, S3 and S5, as well as Sa–Sd were isolated from independent replicate plates of pairings of parental isolates. Single-spore progeny showing evidence of genetic exchange and having very similar AFLP genotypes suggest that nonrandom processes could play a role during genetic exchange between two parental isolates. These processes could include compatibility mechanisms selecting for specific combinations of parental genomes. In other fungi, genomic conflict was suggested to occur after fusion of genetically different individuals (Roca *et al.*, 2003, 2004). Whether mixing of parental genomes in progeny provides an opportunity for conflict in AMF remains to be investigated.

In order to overcome the limiting amounts of DNA from single spores and to ensure high reproducibility, cultures originating from germinated spores were clonally subcultured. Even though we were able to control for potential segregation among single-spore lines during the subculturing, the ideal experimental design would be based on genotyping hyphae and spores directly produced by a fused mycelium of different isolates.

Detecting genetic exchange through sequence-based markers

The sequence-based markers used in this study were previously developed to show genetic differences in a much larger sample of the same *G. intraradices* population (Croll *et al.*, 2008b). The large number of SNPs and indels makes these sequence-based markers suitable for a reliable identification of isolates (Croll *et al.*, 2008a). The lengths of short repeat motifs or indels at each locus were identified by capillary electrophoresis (see Supporting Information in Croll *et al.*, 2008b) and the electropherograms exhibit typical stutter peaks of repeat locus amplifications (Griffiths, 1996; Fig. 2). The sequencing of the alleles in the progeny further confirmed the results of our analysis. The results obtained with the sequence-based markers showed biparental inheritance in one single-spore line (S2) from the pairing of isolates C2 and C3 but not in others. This shows that, in at least one single-spore line, nuclei of genetically different parents mixed and formed viable offspring. This is not contradictory to the results from AFLP showing biparental inheritance in all nine lines, as it could mean either that not all nuclei from both parental isolates were inherited simultaneously or that the alleles from one parental isolate were present at very low frequency, below detection by amplification. Relative heights of inherited alleles in offspring S2 suggest that parental nuclei were not inherited in equal proportions. Alleles specific to parental isolate C3 appear to be more abundant than alleles specific to parental isolate C2. This suggests that parental nuclei potentially mix in different proportions to form single-spore progeny.

There was a general congruence between AFLP fingerprints and sequence-based marker genotypes across all single-spore progeny, to the extent that all progeny of the parental

isolates C3 and D1 showed very similar AFLP fingerprints and, in all cases, the specific sequences of parental isolate C3. Among single-spore progeny of C2 and C3, S4 showed an AFLP fingerprint closest to parental isolate C2 and inherited the specific allele from the same parent. Furthermore, single-spore progeny S1, S3 and S5 all showed AFLP fingerprints most similar to parental isolate C3, in accordance with their sequence-based marker genotype. The general correlation between AFLP fingerprints and sequence-based marker genotypes was also shown to occur within a population of *G. intraradices* isolates (Croll *et al.*, 2008b).

Using ribosomal gene copy number, single-spore progeny S2 showed an intermediate copy number, suggesting that a mixing of genomes of the parental isolates can produce intermediate copy numbers for ribosomal genes. The intermediate copy number shown by progeny S2 supports the genetic analyses based on the other markers in our study.

Phenotypic traits of parental isolates and single-spore progeny

Hyphal densities and spore production were shown to be strongly heritable traits over many generations of clonal subculturing (Koch *et al.*, 2006). Indeed, hyphal densities and spore production of parental isolates C2, C3 and D1 showed very similar phenotypes compared with measurements of the same isolates in two previous studies in the same *in vitro* culture system (Koch *et al.*, 2004, 2006). Single-spore progeny of pairings of parental isolates show a range of distinct hyphal and spore densities compared with their respective parental isolates. Progeny S4 shows very similar phenotypic traits to parental isolate C2, corroborating the genetic analysis showing that S4 inherited most of the specific AFLP loci of parental isolate C2. Meanwhile, progeny S3 and S5 showed significantly higher hyphal densities than both parental isolates. The finding of phenotypic traits in progeny that exceed both parental isolates suggests that the inheritance of these traits is not strictly additive, that is, progeny did not inherit an intermediate phenotype of parental isolates. Also, S1, S3 and S5 showed significantly different hyphal and spore densities, despite their genotypes being very similar. This suggests that either minor genetic differences (as detected by AFLP) can result in clearly distinct phenotypic traits or epigenetic mechanisms play a role in determining these traits in the progeny. Similarly, progeny Sa–Sd show significant differences in hyphal and spore densities among each other. Analysis of genetic markers associated with particular phenotypes may help to elucidate the genetic basis of these phenotypic traits. Progeny showing distinct phenotypes from the respective parental isolates could have important consequences for the symbiosis with host plants, as genetic differences among isolates were already shown to influence plant growth and phosphate uptake (Munkvold *et al.*, 2004; Koch *et al.*, 2006).

Fate of genetically different nuclei in a common mycelium

Genetically different nuclei might undergo recombination in a common mycelium following potential nuclear fusion. The genotyping methods used in this study would not allow the detection of whether the mixing of genetic material in single spores resulted in recombination among genetically different nuclei in the mycelium. Novel loci found by AFLP could be explained either by frequency shifts or by recombination events. The presence of both parent-specific alleles at several loci in single-spore culture S2 could reflect the coexistence of genetically different nuclei without nuclear fusion. However, recombination may have occurred among nuclei, but all daughter nuclei resulting from such events could have remained in the common cytoplasm. As a result, alleles specific to both parental may be found in single-spore progeny. Parent-specific labelling of nuclei would be required to track eventual recombination events. Furthermore, segregation among single spores produced by a progeny could shed light on the fate of nuclear genotypes following exchange through hyphal fusions.

Genetic exchange and evolution of genetic diversity

Our results suggest that AMF are promiscuous in the sense that an individual will form hyphal connections with several other members of the population and this creates the possibility for genetic exchange. In the absence of a sexual reproductive cycle, such promiscuity could allow many genetically different individuals of a population to connect, thereby forming a large, genetically diverse hyphal network. Exchange of nuclei among isolates provides the opportunity for fusion of genetically different nuclei and recombination. Genetic exchange could also act as an important mechanism in maintaining the multigenomic state (Bever & Wang, 2005; Hijri & Sanders, 2005).

Knowledge of the compatibility of isolates and their potential to recombine is critical in the understanding of their effects on plants, as genetic differences among isolates were shown to influence costs and benefits to the host (Koch *et al.*, 2006). Genetic diversity within AMF networks may maintain the unusually low host specificity seen in these symbionts (Sanders, 2003). Because we were able to create viable genetic crosses between genetically and phenotypically different AMF, our study lays a foundation for experimentally investigating the genetics, control of compatibility/incompatibility and the genetic architecture of phenotypic traits in this important fungal phylum. Finally, the evolution and diversification of species in the phylum Glomeromycota should no longer be assumed to be the result of the long-term absence of genetic exchange.

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Supporting Information

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

Table S1 Frequency of anastomosis (fusion) between pairs of arbuscular mycorrhizal fungi

Table S2 Summary of AFLP loci detecting genetic exchange between pairs of arbuscular mycorrhizal fungi

Table S3 Summary of AFLP loci detecting genetic exchange between pairs of arbuscular mycorrhizal fungi using a more conservative analysis in which all loci showing inconsistent scoring among single spore cultures of isolate C3 were removed

Table S4 Summary of AFLP loci detecting exchange between pairs of parental isolates using AFLP (S1–S5 originating from pairings of isolates C2 and C3; Sa–Sd originating from pairings of isolates C3 and D1)

Video S1 The video shows two hyphae of genetically different *Glomus intraradices* isolates that have fused.

Video S2 The video shows two living hyphae (stained with fast green) of two genetically different *Glomus intraradices* isolates that have fused.

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