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ORIGINAL PAPER

Impact of multispores in vitro subcultivation of *Glomus* sp. MUCL 43194 (DAOM 197198) on vegetative compatibility and genetic diversity detected by AFLP

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Abstract Vegetative compatibility and amplified fragment length polymorphism (AFLP) genotyping of in vitro multispores clonal lineages, issued from the same ancestor culture of the arbuscular mycorrhizal fungal strain MUCL 43194 and subcultured several generations in different locations, was assessed. Vegetative compatibility was studied by confronting the germ tubes of two spores from the same or different clonal lineages and stained with nitrotetrazolium blue–Trypan blue and diamidinophenylindole to detect hyphal fusions and nuclei, respectively. Further AFLP analysis of single spores was performed to assess the genetic profile and Dice similarity between clonal lineages. Germ tubes of spores distant by as many as 69 generations were capable of fusing. The anastomosis frequencies averaged 69% between spores from the same clonal lineage, 57% between spores from different clonal lineages, and 0% between spores belonging to different strains. The AFLP patterns showed similarities averaging 92% within clonal lineages and 86% between clonal lineages. Each spore presented unique genotype and some of them shared more markers with spores from different lineages than within the same lineage. We showed that MUCL 43194 maintained self-recognition for long periods of subcultures in vitro and that spores involved in compatibility tests had different genotypes. Our findings suggest that MUCL 43194 maintains genetic diversity by means of anastomoses.

Keywords Arbuscular mycorrhizal fungi (AMF) · In vitro culture · Vegetative compatibility · Anastomosis · Genetic drift · AFLP

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Introduction

Arbuscular mycorrhiza is one of the most ancient and widespread symbiosis in the plant kingdom. It is a mutualistic association between the roots of plants and the mycelium of arbuscular mycorrhizal fungi (AMF) belonging to the Glomeromycota (Schüßler et al. 2001). Through exchange of carbon, from plant to fungus, and nutrients (e.g., phosphorus), from fungus to plant, these organisms play a key role in plant production, biodiversity, and in the functioning of ecosystems (van der Heijden et al. 1998).

These obligate biotrophs develop a profuse extraradical mycelium within the soil capable of interconnecting plants by spreading from mycorrhizal roots and colonizing new plants with which they come into contact (Voets et al. 2008). Anastomosis may also contribute to this underground web by interconnecting different AMF networks.

This phenomenon was decisively demonstrated within the same isolate of *Glomus mosseae* (Giovannetti et al. 2004) and *Glomus intraradices* (Voets et al. 2006), but never reported between different isolates of the same species. Germinating spores were also considered for anastomosis studies. Anastomosis was observed between spores of the same isolate (Giovannetti et al. 1999, 2003) within the *Glomeraceae* and recently between five isolates of one population of *G. intraradices* sampled from the same agricultural field (Croll et al. 2009). Although anastomosis occurrence between the isolates was minimal in this study, its presence suggested that genetic exchange may occur and that in the absence of a sexual reproductive cycle, genetically different individuals in a population may reconnect to form a genetically diverse network of hyphae (Croll et al. 2009). The low values of nonself-vegetative fusion (between 0% and 10%) also emphasized that anastomosis formation is regulated by highly specific recognition mechanisms.

Although anastomosis between different isolates of a population of *G. intraradices* sampled from the same agricultural field was recently demonstrated (Croll et al. 2009), neither their filiation to a potential common ancestor nor the distance (i.e., number of generations) separating the isolates from this potential ancestor and among themselves were identified. Therefore, nothing is known on vegetative compatibility between different generations of the same AMF strain. It is obvious that in field conditions, several generations of the same strain may coexist, exchange genetic information, and possibly undergo a genetic drift across successive generations (Sanders 2002).

In the last two decades, the root organ culture (ROC) system has become a major tool to investigate the physiology of the AMF and plant/AMF association (Fortin et al. 2002). It was recently used to investigate the anastomosis process within the *Glomeraceae* and *Gigasporaceae* (de la Providencia et al. 2005, 2007) and between different AMF extraradical mycelium networks belonging to the same isolate (Voets et al. 2006). By using the ROC system, many generations can be produced starting with multi- or single spores and monitored under controlled conditions. Although the ROC conditions allow for the long-term cultivation of AMF (Fortin et al. 2002), the subcultivation process, for most species belonging to the *Glomeraceae*, is often intense (e.g., every 3 to 4 months for *G. intraradices*), and the inoculum used to start new generations represents only a subsample of the mother culture since hundreds to thousands of spores are often relegated from possible association, development, and genome perpetuation (Cranenbrouck et al. 2005). Therefore, it cannot be excluded that subcultivation may result in a genetic drift and loss/modification of some attributes. For instance, Plenchette et al. (1996) observed a decrease in the capacity of AMF produced over successive generations in vitro to colonize plants. This trend may most likely increase between descendants of a single mother culture grown under diverse conditions (e.g., host root species, composition of growth medium, temperature of incubation, frequency of subcultivation).

At present, several *Glomeraceae* strains have been maintained in vitro in different laboratories for many years (Glomeromycota in vitro Collection (GINCO), personal communication) and circulated worldwide. However, it has never been established whether successive clonal lineages of the same strain are still capable of self-recognition. The arbuscular mycorrhizal strain MUCL 43194 (DAOM 197198) originating from Canada was isolated from a white ash tree plantation and established in vitro for the first time in 1992 and continuously cultured on ROC in the Biosystematics Research Centre (lodged by the AAC Eastern Cereal and Oilseed Research Centre, Ottawa, ON, Canada; Chabot et al. 1992). One culture of this strain was distributed to several laboratories and has been maintained under varying growth conditions. MUCL 43194 has in the past been determined as *G. intraradices* (Chabot et al. 1992), but recent work has shown that it belongs to a different clade in the Glomus GlGrAb grouping of Schwarzott et al. (2001) and is now known to be phylogenetically in the same clade as *Glomus irregulare* (Stockinger et al. 2009). The relationships among fungi in this group are not yet clear, and consequently, we continue to use the MUCL designation rather than to use a Linnaean binomial that may turn out to be in need of revision in the future.

The objective of this study was to investigate the genetic relationships between in vitro clonal lineages of MUCL 43194 sharing a common ROC ancestor and maintained under different conditions in five different laboratories in Europe and Canada. The experiments aimed (a) to detect vegetative compatibility between the different clonal lineages and (b) to explore intraspecific molecular diversity by comparing amplified fragment length polymorphisms (AFLP).

Materials and methods

AMF material

The AMF used was MUCL 43194 (DAOM 197198) formerly identified as *G. intraradices* and presently reclassified in a clade that contains the recently described species *G. irregulare* Błaszk., Wubet, Renker, and Buscot (Stockinger et al. 2009). Different clonal lineages of this strain were established over time in five different laboratories (Fig. 1; see Online Resource Table S1 for extended affiliations).

MUCL 43194 strain originating from Canada (referred to as CAN) was established in vitro, using five spores (i.e.,

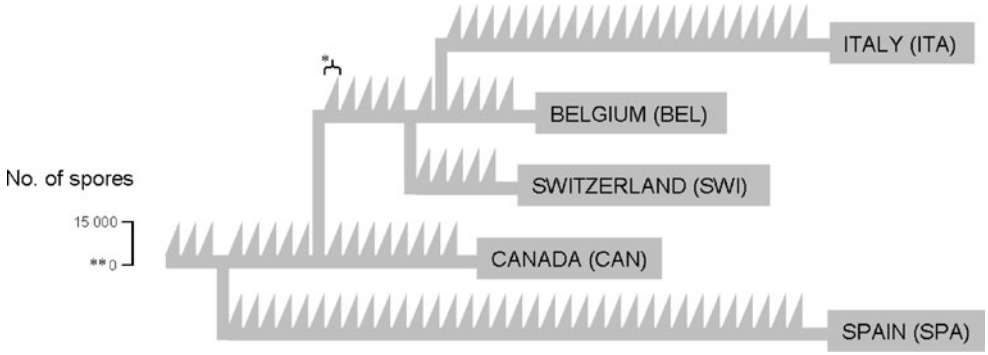

Fig. 1 Diagram showing the development of the MUCL 43194 (DAOM 197198) clonal lineages used in this study. *Single asterisk* one generation (subculture). *Double asterisk*s logarithmic scaling bar representing the total spores involved in the culturing process; the number of spores produced (per plate) in every generation is averaged to 15,000 (according to the data provided for every lineage), from which a 100-spore inoculum was taken to start the following generation. In terms of nuclei, spore numbers should be multiplied between 1.5 to 5.0×10^3 times (Giovannetti et al. 1999; Viera and Glenn 1990)

multiple spores) as a starter inoculum (Chabot et al. 1992). The initial subculture of the strain was supplied to Spain in 1995 (referred to as SPA) and the second one to GINCO in 2001 (referred to as BEL). The strain was further distributed from GINCO to Switzerland (referred to as SWI) and to Italy in 2005 (referred to as ITA). In each location, the strain was maintained in vitro over several generations. Details of the cultural conditions in the five locations are summarized in Table 1.

In 2007 and 2008, in vitro cultures were obtained from these locations. Spores were extracted with forceps, and an average of 100 spores were associated with transformed carrot (*Daucus carota* L.) roots in a bicompartmented Petri plate (St-Arnaud et al. 1996) on the modified Strullu–Romand (MSR) medium (Declerck et al. 1998) solidified with Phytagel (3 g l^{-1}; Sigma-Aldrich, USA) as described by Cranenbrouck et al. (2005). The Petri plates were incubated in an inverted position in the dark at 27°C. Another strain belonging to the same clade as *G. irregulare*, MUCL 41833, was received from GINCO and used as a control. This strain was isolated in the Canary Islands and established in vitro in 1999 starting from a single vesicle (GINCO, personal communication).

Vegetative compatibility tests

After 4 months of culture, spores from each location were extracted from the MSR medium with 0.1 M citrate buffer as described by Bécard and Fortin (1988) and rinsed with sterile distilled water.

Four-square centimeter sterilized (Giovannetti et al. 1999) cellophane membranes (Z377600-1PAK, Sigma, USA) were laid on Petri plates (90 mm diameter) on the surface of MSR medium (20 ml) without sucrose and solidified with Phytagel (3 g l^{-1}). Two spores belonging either to the same or different clonal lineages were paired on each membrane approximately 0.5 cm apart with their germ tubes oriented toward each other. The following combinations were tested, i.e., BEL × BEL, CAN × CAN, ITA × ITA, SPA × SPA, SWI × SWI, BEL × CAN, BEL × ITA, BEL × SPA, BEL × SWI, CAN × ITA, CAN × SPA, CAN × SWI, ITA × SPA, ITA × SWI, and SPA × SWI. Two additional combinations were used as controls, CAN × MUCL 41833 and MUCL 41833 × MUCL 41833. Thirty-six replicates were prepared for each combination. The Petri plates were sealed and incubated in the dark at 27°C in normal position.

After 21 days of incubation, the number of contacts and fusions between the spore germ tubes in the different pairings were scored. Then the frequency of anastomosis was evaluated by determining the probability of fusion between the two clonal lineages involved in each pairing (see "Statistical analysis of anastomosis frequency" section). Hyphal fusion and protoplasm continuity between fusing hyphae were confirmed, and the deposition of formazan salts within the active cytoplasm was observed, as soon as the membranes supporting the pairings were stained with nitrotetrazolium blue chloride (NBT) and Trypan blue. The membranes were removed from the MSR medium and incubated at 27°C overnight, in the dark, on a filter paper saturated with a 1.2-mM NBT solution (Smith and Dickson 1997). The samples were then counterstained at 50°C overnight, in the dark, on filter paper saturated with Trypan blue 1% (Kormanik and McGraw 1982) to observe the hyphal cell wall. The hyphal contacts and fusions were registered and confirmed under a bright-field microscope (Olympus BH2-RFCA, Japan) at ×40 to ×400 magnifications. To observe nuclei in hyphal bridges, 14- to 21-day incubated membranes supporting mycelium with active cytoplasmic flow were mounted on microscope slides in diamidinophenylindole (DAPI; Sigma, Germany) at 5 μg ml^{-1} in 50% glycerol (Giovannetti et al. 1999) and were immediately observed at ×400 and ×1,000 magnifications under an epifluorescence microscope (Olympus BH2-RFCA, Japan) by using the UG-1 filter

Table 1 Summary of the in vitro culture methods and growth conditions of MUCL 43194 and 41833 used to study vegetative compatibility

Strain	Subcultivation			Growth conditions		
43194	Host	Frequency (months)	Cultivation method	Medium	pH	Temperature (°C)
Canada	Transformed carrot roots DC1	~5–7	Colonized root apex transplanting[a]	MSR MM	5.5	25–27
Spain	Nontransformed tomato roots cv. vendor Transformed carrot roots DC2	~4	Multispores association with new host	MM	5.5	24
Belgium	Transformed carrot roots DC1	~7	Multispores association with new host	MSR	5.5	27
Switzerland	Transformed carrot roots DC1 and DC2	~5	Multispores association with new host	MSR MM	5.5	26
Italy	Transformed carrot roots DC1 Transformed chicory roots	~1	Colonized root apex transplanting	MSR	5.5	26
MUCL 41833	Transformed carrot roots DC1	~6	Multispores association with new host	MSR	5.5	27

DC1 Ri-TDNA transformed carrot (*D. carota* L.) root clone no. 1 (Declerck et al. 1998), *DC2* Ri-TDNA transformed carrot (*D. carota* L.) root clone no. 2 (Bécard and Fortin 1988), *MSR* modified Strullu–Romand medium (Declerck et al. 1998), *MM* minimal medium (Bécard and Fortin 1988)

[a] Method consisting in the transfer of healthy mycorrhizal root apexes with extraradical hyphae and multiple spores onto fresh medium (Cranenbrouck et al. 2005)

(BP 330–385 nm, DM 400 nm). All images were recorded by digital camera (Leica DFC320, Germany) with the software DFC V6.3.0 (Leica, UK, 2004).

Statistical analysis of anastomosis frequency

The anastomosis frequency data were analyzed by means of a logistic regression using, for each contact, the presence/absence of fusion as a response variable and the strains pairings as a categorical independent variable. This analysis models the probability of fusion following contact in each of the 15 pairings. The strain MUCL 41833 was excluded from this test because it was considered that it has no probability of fusion with MUCL 43194 as shown in the "Results" section. The effect of the categorical variable was assessed with a likelihood ratio test, taking the equality of the 15 fusion probabilities as a null hypothesis. A contrast was computed to compare the probability of fusion of the same lineage pairings to that of different lineage pairings (Wald chi-squared test). The analysis was performed with the SAS/STAT Software 9.1.3 (SAS Institute, Inc., USA).

Single-spore whole-genomic DNA amplification for molecular analysis

Single-spore genomic DNA amplification was performed using the GenomiPhi V2 DNA Amplification Kit (GE Healthcare, UK). Five single spores of every clonal lineage for MUCL 43194 and five for MUCL 41833 were extracted from a ROC culture and crushed in 1 µl of sterile water using a sterile needle and directly subjected to DNA amplification, according to the manufacturer's instructions (GE Healthcare, UK). To confirm amplification, 2 µl of the product was visualized on a 1% agarose gel stained with Gelred™ (Biotium, Hayward, CA, USA). Before storage at −20°C, the amplification product was precipitated with ethanol and EDTA/sodium acetate solution and suspended in water (30 µl) for quantification using the NanoDrop™ spectrophotometer (Thermo Scientific, USA). To check the absence of contamination, each single-spore genomic DNA amplicon was controlled by sequencing the 18S region between the primers GeoA2-NS2.

Characterization of the rDNA SSU

The amplified genomic DNA of each single spore from MUCL 43194 was controlled by amplification and sequencing of a 500-bp fragment (using the primers Geo A2, NS2) of the rDNA SSU, followed by blastn search (Altschul et al. 1997) in the GenBank database. One spore from each clonal lineage of MUCL 43194 and MUCL 41833 was randomly selected to obtain their full 18S sequences and put them into a phylogenetic tree. Details on polymerase chain reaction (PCR) and sequence analysis are given with Figure S1 (Online Resources).

AFLP analysis

The AFLP analysis was conducted as described by Voyron et al. (2009) except the restriction–ligation step for each

spore that was performed on 150 ng of the amplified genomic DNA. Preselective PCR was carried out in 20 µl instead of 25 µl (final volume) with *Eco*RI + 0 and *Mse*I + 0 used as preselective primers, and selective PCR was executed with the couples (a) *Eco*RI + AC–*Mse*I + C and (b) *Eco*RI + AC–*Mse*I + T as selective primers (*Eco*RI primers labeled with D4 WellRED dye, Sigma-Proligo, Beckman Coulter license). To detect the DNA resulting fragments, 2 µl of the selective PCR product was run on the CEQ™ 2000xl DNA Analysis System (Beckman Coulter, USA) under the conditions detailed also by Voyron et al. (2009). Finally, one random sample of each AMF strain was submitted to three independent rounds of restriction–ligation–amplification to estimate the variability generated by the AFLP procedure. Additionally, three samples of culture medium from AMF plates, in each case submitted and not submitted to whole-genome amplification (WGA), were run under the same AFLP conditions as negative controls.

AFLP data analysis

Raw electropherograms were analyzed with the Fragment analysis software (CEQ8000, Beckman Coulter) and corrected manually. Only fragments between 60 and 280 bp with relative fluorescent units (RFU) above 500 were scored. Fragments present in negative controls were excluded from the analysis. The matching fragments were analyzed in a binary matrix with the Dice (Bonin et al. 2007) similarity coefficient and the derived dendrogram generated with the unweighted pair group method of arithmetic averages (UPGMA) using the XLSTAT software v.2008.5.01 (Addinsoft 1995–2008).

Reproducibility of profiles

In order to assess whether the WGA and AFLP procedures generated reproducible profiles (i.e., avoid significant variability of DNA fragments), both procedures were tested in a two-side assay: (a) five replicates of three DNA aliquots, i.e., 1, 10, and 30 µg of *Arabidopsis thaliana* DNA, were submitted to a GenomiPhi kit-mediated WGA and then (b) were run in two independent rounds of AFLP for one couple of selective primers: *Eco*RI + AGA and *Mse*I + GGT (Gadkar and Rillig 2005). Both WGA and AFLP procedures were carried out with the same methodology as previously described for AMF single-spore samples. Two sets of AFLP profiles of five replicates per aliquot were obtained. The electropherograms were analyzed with the Fragment analysis software (CEQ8000, Beckman Coulter) and corrected manually. The fragments between 60 and 520 bp with RFU above 500 were scored.

The *A. thaliana* profiles and the AMF profiles reserved for AFLP error estimation were analyzed in respective binary matrixes under simple match (*m*; Bonin et al. 2007) similarities. Dissimilarities (i.e., the variation between two profiles), were calculated $d = (1 - m) \times 100$, where d=0% corresponds to identical profiles and d=100% to completely different profiles. The average dissimilarities between the five profiles from the different WGA-amplified samples in each DNA dilution allowed us to estimate the variability introduced by the WGA technique, while dissimilarities between profiles in the same sample and dilution from the independent AFLP runs allowed us to estimate the AFLP introduced variations.

Results

Vegetative compatibility tests

For both strains (i.e., MUCL 43194 and MUCL 41833) and all lineages, the spores placed on the cellophane membrane started to germinate after 3 days of incubation. The overall rate of germination, measured 21 days after incubation, varied among the clonal lineages from low for CAN and SWI (50% and 57%, respectively) to medium (78%) for MUCL 41833 and high (100%) for BEL, ITA, and SPA. Only the membranes where the two spores germinated and hyphae came into contact were considered for the vegetative compatibility tests (VCT). The first contacts between hyphae from germinating spores were observed 7–10 days after germination. Hyphal fusions, i.e., cell wall and protoplasmic continuity, between contacting hyphae from the same or different clonal lineages of MUCL 43194 spores were detected in all the pairings. However, the estimated frequency of anastomosis varied between the clonal lineages from 41% (CAN × BEL) to 78% (BEL × BEL; Fig. 2) and reached 89% for MUCL 41833×41833. Shared genetic material (detected by DAPI fluorescence microscopy) through hyphal bridges was also observed in the different pairings tested (Fig. 3b). The highest average (69%) of hyphal fusions between different MUCL 43194 spores was observed when pairings were formed between spores of the same clonal lineage (e.g., BEL × BEL; Fig. 4) with values ranging between 58% (SPA × SPA) and 78% (BEL × BEL). In contrast, the frequency of anastomosis between combinations of spores from different clonal lineages (e.g., BEL × CAN) averaged 57%. Both averages (same clonal lineage group, i.e., five pairings grouped and different clonal lineage group, i.e., ten pairings grouped) were statistically different (p=0.006). When the 15 MUCL 43194 pairings were compared individually, six were found to be statistically different (p values shown in Fig. 4): BEL × BEL (78%), CAN × ITA (44%), CAN × BEL (41%), CAN × SWI (48%), BEL × SPA (77%), and SPA × SWI (48%). Finally, no anastomosis was observed between the

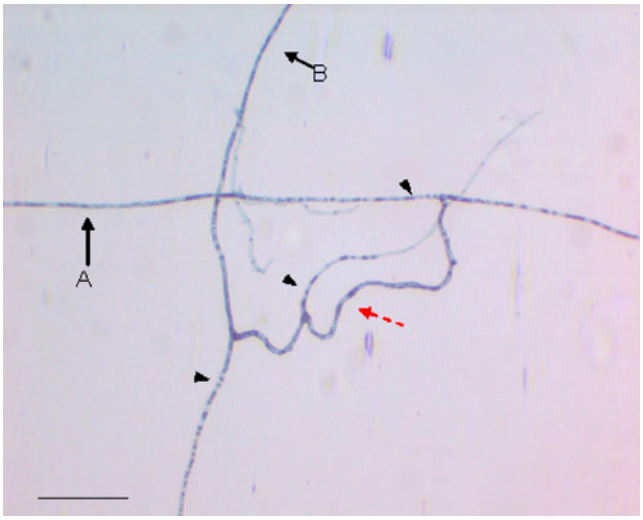

Fig. 2 Bright field micrograph of a hyphal fusion between hyphae from two different spores of MUCL 43194 (SPA) stained with NBT and Trypan blue. *A*, *B* Hyphae arising from spores A and B, respectively. The cytoplasmic continuity in the hyphal bridge (*red dotted arrow*) can be observed with the formazan salts deposition (*arrowheads*). *Bar*=20 µm

spores from two different strains belonging to the same clade as MUCL 43194 AFTOL (i.e., MUCL 43194 and MUCL 41833). No evidence of incompatible reactions (i.e., vacuolization, septa formation, and cell wall thickening or growth arrestment) was observed in any of the analyzed pairings either before or after hyphal fusion.

SSU-based phylogenetic analysis

The 500-bp sequences of the rDNA SSU recovered from the spores of the MUCL 43194 clonal lineages showed identities of 99% with MUCL 43194 AFTOL after the blastn search (data not shown) indicating that the WGA products were not contaminated. The rDNA SSU complete sequence of the five clonal lineages from MUCL 43194 and MUCL 41833 (FN600534-39) was used to construct a tree showing the phylogenetic relationship (Online Resource Figure S2). The similarity within the MUCL 43194 isolates averaged 99.81% and 99.62% between MUCL 43194 and MUCL 41833. Both strains are situated within the MUCL 43194 AJ852526 and MUCL 43194 AFTOL AY635831 clade supported by an 82% bootstrap.

AFLP analysis

The selective PCR with both couples of primers (*Eco*RI + AC–*Mse*I + C and *Eco*RI + AC–*Mse*I + T) produced electropherograms with approximately 140 fragments (peaks) each, among the range of 50 to 350 bp and between 20,000 to 90,000 RFU. The most reliable fragments ranging between 60 and 280 bp (i.e., a total of 253) were scored for the AFLP analysis.

Among the 29 samples analyzed, no identical profiles in both couples of primers were found, indicating polymorphisms between strains and between clonal lineages within the same strain. Among the 253 fragments obtained, 13 were shared by every sample in both strains, eight were exclusive to MUCL 43194, and six were exclusive to MUCL 41833.

The whole set of AFLP markers was considered to estimate the Dice similarity index between samples. The similarities obtained fall into three categories: (a) between spores within the same filiation, where the highest similarities averaged 92%, (b) between clonal lineages within the same strain, with similarities averaging 86%, and (c) between strains of the same species, with an average similarity of 65%. By comparison, similarities between species of the same genus in a separate study (MUCL 43194 and 41833 and *Glomus clarum* 46238) averaged 51%.

A dendrogram based on the Dice similarities between samples is shown in Fig. 5. The samples from MUCL 43194 did not form clusters according to their origins. No correlations were observed between the number of generations separating the spores of each MUCL 43194 filiation and the anastomosis frequency (r=0.09) or the Dice genetic similarity (r=0.45).

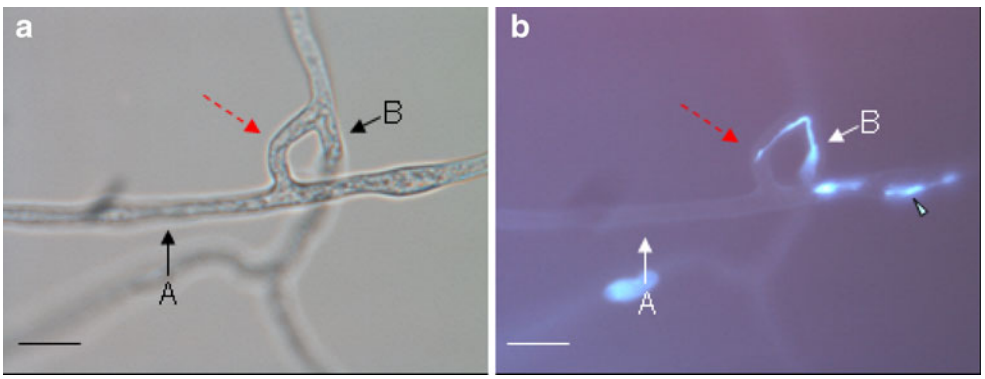

Fig. 3 Hyphal fusion between hyphae from two different spores (CAN × SWI) of MUCL 43194. **a** Bright field micrograph and **b** fluorescence micrograph with DAPI staining. *A*, *B* hyphae arising from spores A and B, respectively. Observe the fluorescent nuclei (*arrowheads*) and a shared nucleus within the hyphal bridge (*red dotted arrows*). *Bars*=10 µm

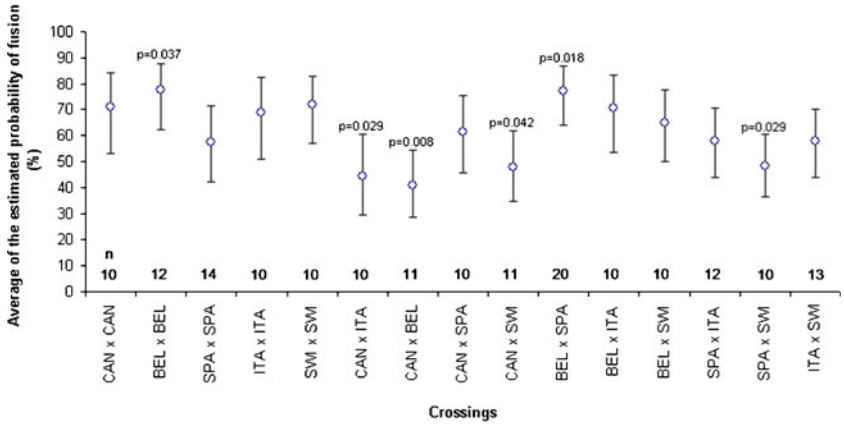

Fig. 4 Average of the estimated probability of fusion (anastomosis frequency) of hyphal contacts between all the MUCL 43194 combinations tested. *Error bars* represent the confidence intervals (α=5%). Averages with values of *P* are significantly different from the value expected. *n* number of pairs analyzed per pairing

Reproducibility of profiles

The ability of the WGA–AFLP to produce reliable profiles was tested with *A. thaliana* DNA (technique control) and AFLP for MUCL 43194 DNA (experiment control). The profiles generated by the primer couple tested (E*co*RI + AGA–M*se*I + GGT) had 33 DNA fragments that were compared in a similarity matrix. When the profiles from the different *A. thaliana* DNA aliquots (1, 10, and 30 µg; WGA replicates) were compared, they averaged dissimilarities (variations) of 2.2% and 1.1% in the first and second rounds of AFLP, respectively (3.65% overall). When the same replicates were compared between the two independent rounds of AFLP, dissimilarities averaged 2% representing a total variation of 4.1% and 3.0%, respectively (WGA + AFLP; 3.55% overall).

Since the aim of the analysis was to characterize single-spore DNA fingerprints, no estimation of WGA-introduced variability could be estimated; however, the AFLP variation was estimated by carrying out three rounds of independent restriction–ligation–amplification. When both couples of selective primers were analyzed, MUCL 43194 presented variations of 3.6% (with 14 variable fragments out of 253) while MUCL 41833 variations averaged 2.4% (with 12 variable fragments out of 253).

Discussion

Vegetative compatibility tests have been used for decades to identify and classify filamentous fungi with the so-called compatibility groups (Carling 1996; Barros et al. 2007), but rarely considered in terms of AMF population structure. Pioneer work by Giovannetti et al. (1999) paved the way to investigate vegetative compatibility between germinating spores belonging to different species (i.e., *G. mosseae*, *G. caledonium*, *G. intraradices*, *Gigaspora rosea*, and *Scutellospora castanea*) and isolates within a species of

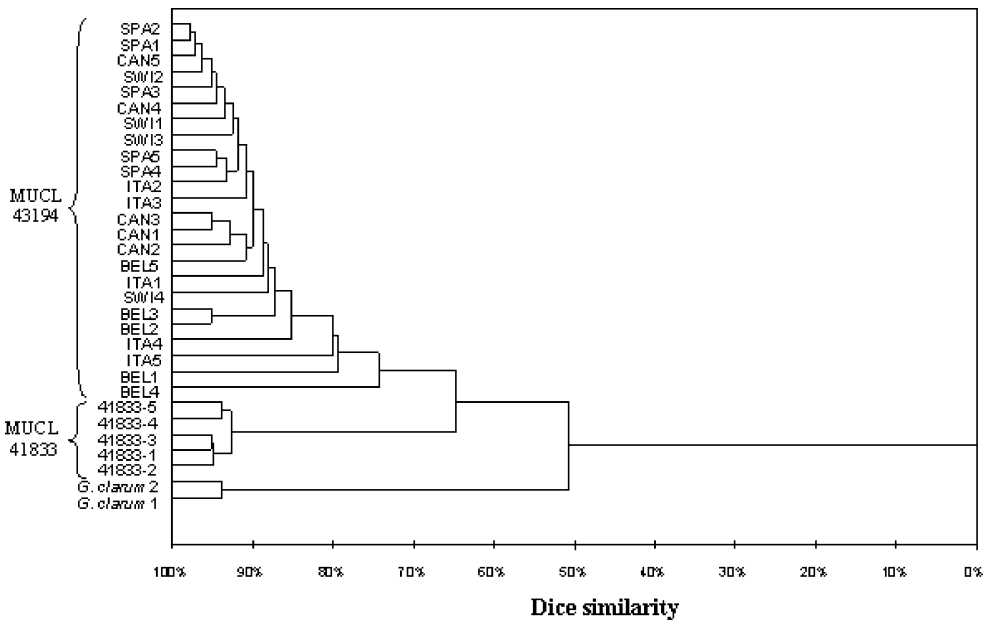

Fig. 5 Dendrogram generated with UPGMA cluster analysis of the Dice similarity index based on single-spore AFLP profiles of the 29 samples of MUCL 43194 and 41833 and two additional samples of *G. clarum* (MUCL 46238). Labels in the MUCL 43194 brace correspond to the origin of the spore: *BEL* Belgium, *CAN* Canada, *ITA* Italy, *SPA* Spain, and *SWI* Switzerland

G. mosseae (Giovannetti et al. 2003) and *G. intraradices* (Croll et al. 2009). However, no study considered the vegetative compatibility between descendants of a single mother culture. In our study, we demonstrated for the first time that several clonal lineages of a single strain, maintained in vitro since 1992 through subcultivation in different locations (i.e., under different growth conditions), were able to anastomose. However, the frequency of anastomosis was significantly higher within clonal lineages than between clonal lineages, suggesting susceptibility to genetic drift.

Vegetative compatibility has been reported among different *Glomeraceae* groups in presymbiotic and symbiotic mycelia originating from spores belonging to the same isolate (Giovannetti et al. 1999) and recently from spores belonging to different isolates of one population of *G. intraradices* sampled from the same agricultural field (Croll et al. 2009). Conversely, vegetative compatibility has never been observed between different species (Giovannetti et al. 1999) or in isolates from the same species originating from highly distant (i.e., avoiding any risk of a recent identical ancestor) ecosystems (Giovannetti et al. 2003). This observation was corroborated in our study. No anastomosis was detected between two strains (i.e., MUCL 41833 and 43194) belonging to the same clade sampled in the Canary Islands (GINCO, personal communication) and in Québec, respectively. Though numerous contacts between hyphae were established, neither hyphal fusion nor vegetative incompatibility reaction, preceding or subsequent to hyphal contact, was observed, as reported in the study by Giovannetti et al. (2003). The absence of incompatibility reactions in our experiment presupposed that no recognition/rejection signal between the two tested strains was released or perceived. So far incompatibility reactions in AMF have only been reported for *G. mosseae* (Giovannetti et al. 1999, 2003) and *G. intraradices* (Croll et al. 2009); hence, further compatibility tests involving a large spectrum of strains are required to corroborate this finding.

In our study, VCT showed that several clonal lineages of the same strain (MUCL 43194) were capable of self-anastomosing as well as anastomosing with each other. Self-anastomosis frequency ranged from 58% to 78% in MUCL 43194 and 89% in MUCL 41833. These data are similar to those previously reported by Croll et al. (2009) for five isolates of *G. intraradices*.

The ability to anastomose of different clonal lineages originating from the same mother culture and separated from each other by nine (BEL × SWI) up to 69 generations (ITA × SPA; Fig. 1) supports the hypothesis that vegetative compatibility is maintained over long periods. Nevertheless, the differences found in probability of fusion for some crossings (CAN × ITA, CAN × BEL, CAN × SWI, and SPA × SWI) and the differences observed for the pairings within the same clonal lineage group (69%) compared to the pairings between different clonal lineage group (57%) suggested a genetic drift presumably because of a modification in the frequencies of the genes responsible for either compatibility (in the case of increased probability of fusion) or incompatibility (in the case of a decreased probability of fusion). The differences in anastomosis frequencies further support the hypothesis that anastomosis represents a fundamental strategy for the fungus to survive and perpetuate. It also shows that the ROC with multiple spores is a satisfactory system to maintain AMF over several generations (at least 70), without loss of vegetative compatibility (but not exempt from decrease along generations). Additionally, our study points out that VCT combined with the ROC represent nowadays, the most powerful tool to track AMF under strictly controlled and versatile conditions such as different host roots or different culture media.

Based on the rDNA SSU, MUCL 43194 and MUCL 41833 clustered with MUCL 43194 AFTOL (Figure S2) and could not be separated. However, these strains were unable to anastomose, suggesting that they are sufficiently distant to be considered different. At present, powerful molecular tools such as DNA microsatellite markers that can resolve differences between strains are available for AMF (Mathimaran et al. 2008). It is obvious that combining specific markers with VCT could help decipher the species concept in AMF and provide clues to the functional implications of the genetic differences between strains (van der Heijden and Scheublin 2007).

To assess genotypic differences between clonal lineages, single spores of each clonal lineage were scrutinized for fragment length polymorphic DNA after a full genome amplification with the strand displacement technique. This technique proved suitable to perform a reliable analysis of molecular markers of single spores such as AFLP or microsatellites (Gadkar and Rillig 2005; Mathimaran et al. 2008). Our results supported the robustness of this technique since the WGA error for *A. thaliana* averaged 1.65% and AFLP error 2% (3.65% total). The variability obtained in the AFLP for AMF was slightly higher, 3.6% and 2.4% for MUCL 43194 and MUCL 41833, respectively. However, the error rates obtained in our experiments are reasonably acceptable since they do not exceed 5% (Meudt and Clarke 2007). The single-spore genetic similarities observed within and between strains (MUCL 43194 and 41833) and between species (MUCL 43194 and 41833 vs. *G. clarum*) were consistent with the ranges reported by Rosendahl and Taylor (1997) for other species of *Glomus*. However, this comparison should be considered with caution because our methodology to produce the polymorphic markers differed, e.g., genomic DNA amount, primers for preselective and selective PCR among others. When the AFLP profiles of each spore were compared by UPGMA of Dice similarity, we observed that clusters were

formed at species and strain level, while no apparent clusters of the spores analyzed were formed by clonal lineage of MUCL 43194. This showed that some spores from different clonal lineages shared more markers than others belonging to the same filiation. These findings (altogether with the VCT) suggest that the ROC system used allows for the perpetuation of the genetic variants (of different nuclei) during tens of generations although, as mentioned before, the changes in probability of fusion might suggest the fluctuation of variant frequencies caused by drift. A determinant in genetic drift is the effective population size (N_e). When N_e is small, genetic drift (besides inbreeding and accumulation of deleterious mutations) leads to the lose of genetic variability (Palstra and Ruzzante 2008). For AMF, N_e could be considered in terms of nuclei numbers in propagules rather than in terms of the number of propagules (Fig. 1). The population of nuclei within a single spore, in *Glomus* species, has been estimated at between 1,000 and 5,000 (Viera and Glenn 1990; Giovannetti et al. 1999). During germination, a large fraction of nuclei migrates to the germinating hyphae (Bécard and Pfeffer 1993). Thus, in a ROC of *Glomus*, using multiple spores as inoculum, in which anastomosis occurs at considerable rates (Giovannetti et al. 1999, 2001, 2003; de la Providencia et al. 2005; Voets et al. 2006) and where the exchange of nuclei exists (Giovannetti et al. 1999, 2001, 2003), the probability of gene fixation should be limited (Bever and Wang 2005). If a random distribution of nuclei occurs during spore formation, the different nuclei would be prone to drift (Sanders 2002). However, once inoculated as multiple spores, the spores germinate and the germ tubes (and later the extraradical mycelium) actively fuse, pooling their nuclei again, thus increasing the nuclear N_e, and thereby inheriting the different variants for generations (Bever et al. 2008) while preserving the vegetative compatibility traits. Another possible explanation to the fluctuations in the anastomosis frequency could be a selection pressure acting over the capacity of the clonal lineages to anastomose. For instance, the environmental conditions (e.g., host root, temperature, growth medium), or a selection applied during subculturing, may preserve some anastomosis phenotypes over others. However, since the inocula selected for subculturing are not chosen based on their capacity to anastomose, this would be a side effect selection from a pressure applied to clonal lineages, for instance, selecting the fittest colonies (e.g., the most spore or mycelium productive) for in vitro culture.

Surprisingly, no identical AFLP profiles were obtained in the strains, whatever their origin or clonal lineage. These results support the finding that not only different nuclei inhabit the *Glomus* mycelium and spores but that not every variant of nuclei is present in every spore. Some reports support the heterokaryotic nature of AMF (Bever and Morton 1999; Kuhn et al. 2001; Bever and Wang 2005). Nevertheless, nothing is known about the abundance of different nuclei that inhabit the spores nor to what extent they are represented, either during germination or during sporogenesis. Bever and Morton (1999) proposed a model of inheritance in spore shape for *Scutellospora pellucida* based on different nuclei inhabiting the spores and anastomosis. They suggested that segregation of nuclei through hyphae maintains genetic variation between progeny spores. Nevertheless, the molecular and physiological processes responsible for the spatial distribution and segregation of nuclei in AMF mycelium and spores remain poorly known. Similarly, ploidy and DNA recombination in AMF are still a matter of debate (Rosendahl 2008).

In this study, we demonstrated that descendant spores of a single mother culture propagated in vitro with multispores have the potential to maintain their capacity to anastomose each other over long periods (at least for 70 generations) even when cultured in different conditions. Nonetheless, we also found evidence of genetic drift through the fluctuations of anastomosis frequencies between clonal lineages. We showed further evidence that different genotypes (nuclei) coexist within MUCL 43194 and that vegetative compatibility is achieved between germ tubes of spores harboring nonidentical genotypes. These results suggest that anastomosis is a fundamental process for the fungus to exchange genetic information and to pool genetic variability. It also substantiates the adequacy of the ROC to maintain AMF over several generations without the loss of vegetative compatibility, which makes it a suitable system to trace the offspring of a single individual.

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