Divergence of *Funneliformis mosseae* populations over twenty years of laboratory cultivation, as revealed by vegetative incompatibility and molecular analysis

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Abstract

Arbuscular mycorrhizal fungi (AMF) are widespread, important plant symbionts. They absorb and translocate mineral nutrients from the soil to host plants through an extensive extraradical mycelium, consisting of indefinitely large networks of non-septate, multinucleated hyphae which may be interconnected by hyphal fusions (anastomoses). This work investigated whether different lineages of the same isolate may lose the ability to establish successful anastomoses, becoming vegetatively incompatible, when grown separately. The occurrence of hyphal incompatibility among five lineages of *Funneliformis mosseae*, originated from the same ancestor isolate and grown in vivo for more than 20 years in different European locations, was assessed by systematic detection of anastomosis frequency and cytological studies. Anastomosis frequencies ranged from 60 to 80% within the same lineage and from 17 to 44% among different lineages. The consistent detection of protoplasm continuity and nuclei in perfect fusions showed active protoplasm flow both within and between

lineages. In pairings between different lineages, post-fusion incompatible reactions occurred in 6-48% of hyphal contacts and pre-fusion incompatibility in 2-17%. Molecular fingerprinting profiles showed genetic divergence among lineages, with overall Jaccard similarity indices ranging from 0.85 to 0.95. Here, phenotypic divergence among the five *F. mosseae* lineages was demonstrated by the reduction of their ability to form anastomosis and the detection of high levels of vegetative incompatibility. Our data suggest that potential genetic divergence may occur in AMF over only 20 years and represent the basis for detailed studies on the relationship between genes regulating anastomosis formation and hyphal compatibility in AMF.

Keywords

Arbuscular mycorrhizal fungi; Anastomosis; Hyphal compatibility; Post-fusion incompatibility; Pre-fusion incompatibility; Genetic diversity.

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi (AMF) are important plant symbionts which are considered fundamental biofertilizers and bioenhancers, increasing plant nutrition and tolerance to biotic and abiotic stresses (Smith and Read 2008; Rouphael et al. 2015). Moreover, they enhance plant biosynthesis of secondary metabolites with health-promoting activities (Sbrana et al. 2014) and affect gene expression of the relevant key enzymes (Battini et al. 2016). AMF absorb and translocate mineral nutrients from the soil to the host plants by means of an extensive extraradical mycelium (ERM) which develops around colonized roots in the surrounding soil (Smith and Read 2008). Such belowground mycelium consists of a complex and indefinitely large network of non-septate, multinucleated hyphae that may be interconnected by hyphal fusions (anastomoses), and which can link the roots of different plants in a community (Giovannetti et al. 2004).

Anastomosis, a common cellular process in filamentous fungi, has been largely studied in Basidiomycetes and Ascomycetes, given its essential role in sexual fusion and in the establishment and maintenance of interconnectedness, homeostasis and intra-hyphal communication in the fungal colony (Gregory 1984; Glass et al. 2000, 2004; Hutchison and Glass 2012; Rayner 1991, 1996).

The occurrence and frequency of anastomoses in AMF, as well as the cytological events involved, have been widely investigated since 1999 (Giovannetti et al. 1999), and their importance for nuclear intermingling, genetic exchange and interplant nutrient flow has been demonstrated by different authors (see Giovannetti et al. 2015). AMF mycelia carry thousands of nuclei at all times (Bécard and Pfeffer 1993; Cooke *et al*. 1987; Pawlowska and Taylor 2004), and their genetic organization has been debated for years (Boon et al. 2015; Croll et al. 2008; Hijri and Sanders 2005; Kuhn et al. 2001; Pawlowska and Taylor 2004; Lin et al. 2014; Tisserant et al. 2013). Recent data, however, have shown that AMF mycelium contains nuclei that are either highly similar or of two dominant genotypes, depending on the isolate (Riley and Corradi 2013; Ropars et al. 2016).

In AMF, successful anastomoses, characterized by complete fusion of hyphal walls, protoplasm continuity and occurrence of nuclei in the middle of hyphal bridges, have been detected between: i) hyphae of the same germling and of different germlings of the same/different isolates (de Novais et al. 2013; Cárdenas-Flores et al. 2010, 2011; Croll et al. 2009; de la Providencia et al. 2013; Giovannetti et al. 1999; Purin and Morton 2011, 2013), ii) asymbiotic and symbiotic mycelium of the same isolate (Sbrana et al. 2011), and iii) extraradical hyphae of the same isolate developing from colonized roots of plants belonging to different species, genera and families (Giovannetti et al. 2004). Such a process contributes greatly to hyphal interconnectedness and integration in these obligate biotrophs, as the percentage of perfect anastomoses may exceed 90% in some isolates belonging to the genus *Glomus sensu lato* (de Novais et al. 2013) and the bidirectional flow of cellular particles and organelles in fusion bridges may proceed at a maximum speed of 1.8 μm s^{-1} (Giovannetti et al. 2015).

78 By contrast, hyphal contacts which do not lead to anastomoses may result in either no
79 interactions or incompatible hyphal interactions. Pre-fusion incompatible responses, consisting of
80 protoplasm retraction and septa formation before anastomosis, have been reported to occur between
81 germlings of geographically different isolates of the same AMF species, highlighting the existence of
82 early self/non-self discriminating signals (Croll et al. 2009; de la Providencia et al. 2013; Giovannetti
83 et al. 2003; Purin and Morton 2013). On the other hand, incompatibility reactions occurring at post-
84 fusion stages, consisting of protoplasm withdrawal and septa formation in fused hyphae, occurred in
85 hyphal interactions between asymbiotic and symbiotic mycelia and between genetically different
86 germlings belonging to the same species, showing that such events are finely regulated by specific
87 recognition mechanisms (Croll et al. 2009; de la Providencia et al. 2013; Sbrana et al. 2011).
88 The consequences of vegetative incompatibility have not been adequately investigated,
89 despite the important role they may play in population divergence of AMF isolates in natural and
90 agricultural ecosystems and in AMF isolates maintained in culture collections worldwide or utilized
91 as inoculants in agriculture. Indeed, within-population patterns of genetic differentiation have been
92 detected in native AMF isolates of *Funneliformis mosseae* and *Funneliformis caledonium* from
93 agricultural soils in Denmark and of *Glomus intraradices* (now identified as *Rhizophagus irregularis*
94 or *Rhizoglomus irregulare*) in Switzerland (Croll et al. 2008; Koch et al. 2006; Rosendahl 2008;
95 Stukenbrock and Rosendahl 2005). Other authors found evidence that segregation may occur in one
96 population of *R. irregulare,* leading to segregated lines with different phenotypic traits (Angelard et
97 al. 2010; Angelard and Sanders, 2011). Unfortunately, the quoted studies did not investigate whether
98 the differentiated/segregated lineages were still capable of self-recognition, leading to the production
99 of perfect anastomoses.
100 To the best of our knowledge, only one work reported the maintenance of hyphal recognition
101 ability among different lineages of a single AMF isolate, in an in vitro experiment carried out on
102 descendant clonal root organ cultures (ROCs) of the same *R. irregulare* ancestor, maintained under
103 different laboratory conditions for about a decade (Cárdenas-Flores et al. 2010).

104 In this study, we tested five lineages of *F. mosseae* originating from the Rothamsted isolate
105 previously denominated Yellow Vacuolate (Mosse and Bowen 1968), later ascribed to the species
106 *Glomus mosseae* (now *F. mosseae*), that had been separately grown for more than 20 years in five
107 European laboratories, where different selective pressures were operating (number and identity of
108 host plant species, chemical and physical characteristics of the soil, temperature and light conditions
109 in the greenhouses). The aims of the study were: (i) to evaluate, by culture assays, phenotypic
110 differences, such as germination and growth ability, of the five different lineages; (ii) to detect the
111 occurrence of vegetative incompatibility in hyphae belonging to the same and to the five different
112 lineages, by systematic detection of anastomosis occurrence and frequency and by cytological studies;
113 and (iii) to assess the genetic distances of the five different lineages, by DNA fingerprinting methods.
114
115 **MATERIALS AND METHODS**
116 **Fungal material**
117 Five different culture lines of the AM fungal species *F. mosseae* (T.H. Nicolson & Gerd.) C. Walker
118 & A. Schüßler, established from the Rothamsted isolate originally described as Yellow Vacuolate
119 (Mosse and Bowen 1968, hereafter *F. mosseae* YV) were used. The lineages were grown in different
120 European laboratories for at least 20 years (Table 1). Spores were extracted from pot culture soil by
121 wet sieving and decanting, flushed into petri dishes and collected with a capillary pipette under a
122 dissecting microscope (Wild, Leica, Milano, Italy). Intact, healthy spores were sonicated (60s) in a
123 B-1210 cleaner (Branson Ultrasonics, Soest, The Netherlands), washed three times in sterile distilled
124 water (SDW) and incubated for germination on a cellulose ester membrane (Millipore HAWP04700,
125 0.45 μm diameter pores) placed on moist sterile quartz grit. To confirm that all lineages were
126 originating from *F. mosseae* isolate YV, the occurrence of the distinctive 214bp fragment produced
127 by restriction of the ITS region with the enzyme *Taq*I was assessed on a batch of 10 spores for each
128 lineage by DNA extraction (Online resource 1), ITS amplification with the primers ITS1 and ITS4
129 and analysis of the restriction products (Giovannetti et al. 2003). With the aim of removing a possible

130 "maternal effect" induced by different culture conditions, such as soil composition and host plant,
131 germinated spores from each lineage were used to inoculate *Allium porrum* L. seedlings (leek) and to
132 establish pot-cultures using a 1:1 mixture of sterile calcinated clay (Terragreen) and sandy soil. Pots
133 were maintained in Sun-transparent bags (Sigma Aldrich s.r.l., Milan, Italy) in a greenhouse for four
134 months. Then the newly produced spores were collected and used for the subsequent tests.
135
136 **Phenotypic traits of the five lineages**
137 The different *F. mosseae* lineages were tested for their germination and growth ability. Sporocarps
138 were extracted from pot culture soil by wet sieving and decanting, collected with forceps under the
139 dissecting microscope, rinsed five times in SDW and transferred to Millipore membranes placed on
140 moist sterile quartz grit in 9-cm diameter petri dishes. After 14 days of incubation in the dark at 25°C,
141 mycelium growing on the membranes was stained with trypan blue (0.05% in lactic acid) and
142 observed under the dissecting microscope. Germination percentage and hyphal length of each
143 germling were evaluated by the grid line intersect method (Giovannetti and Mosse 1980) on at least
144 50 germinated sporocarps for each lineage.
145
146 **Table 1.** Culture lineages used in this work, descending from the same ancestor isolate of
147 *Funneliformis mosseae* (Yellow Vacuolated) obtained by all donors from Rothamsted Research
148 Station (UK)

Lineage	Isolate code	Repository	Acquisition date	Culture substrate	Donor
PI	IMA 1	Pisa, Italy	1979	Sandy soil	M. Giovannetti
TO	BEG 12 TO	Turin, Italy	1980	Sandy soil	A. Schubert
DJ	INRA LPA5	Dijon, France	1976	Field soil-sand	V. Gianinazzi-Pearson
GR	BEG 119	Granada, Spain	1973	Sand-vermiculite-soil	C. Azcon-Aguilar

BA	BEG 116	Barcelona, Spain	1981	Sandy soil	V. Estaun

Vegetative compatibility assays and cytological studies of hyphal compatibility/incompatibility

To minimize the effects due to differential germination and hyphal growth among the five lineages, newly germinated sporocarps, belonging either to the same or to different lineages and showing comparable hyphal lengths, were selected for compatibility test pairings. Paired sporocarps were placed on a 47 mm Millipore membrane, approximately 1 cm apart, and at least 30 replicate membranes were prepared for each pairing. The five self pairings and all possible (10) pairing combinations among different lineages were tested. Membranes were placed on moistened membranes of the same type laid on sterile quartz grit in 14-cm-diameter petri dishes, which were sealed with Parafilm and incubated at 25°C in the dark. After 20 days of incubation, occurrence of anastomoses was assessed on germlings by staining for the localization of succinate dehydrogenase (SDH) activity (Smith and Gianinazzi-Pearson 1990). Deposition of formazan salts in hyphae allowed the visualization of viable mycelia and of protoplasmic continuity between fusing hyphae. Membranes bearing two germinated sporocarps whose hyphae came into contact were mounted on microscope slides with 0.05% trypan blue in lactic acid and observed under a Reichert-Jung (Vienna, Austria) Polyvar microscope. To verify the maintenance of viability of perfect fusions some membrane pairings were incubated for longer periods, up to 70 days, before staining as previously described. All hyphal contacts were scored at magnifications of x125 to 500 and the frequency of perfect anastomoses was calculated by determining the proportion of hyphal contacts that led to hyphal fusions. Each hyphal contact was verified at a magnification of x1,250 and assigned to a hyphal interaction class, *i.e.* non-interacting hyphae, pre-fusion incompatibility, post-fusion incompatibility and perfect fusion (Sbrana et al. 2011). Briefly, contacts were scored as non-interacting when hyphae crossed with no cytological reactions, while they were scored as

incompatible when one or both hyphae underwent protoplasm vacuolization and septa formation, separating live from dead hyphal compartments. When incompatible reactions occurred before fusion, in hyphae showing tip swelling and/or homing (preanastomosis attraction between hyphae), indicative of hyphal recognition, contacts were assigned to pre-fusion incompatibility, while contacts showing protoplasm withdrawal isolating hyphal compartments of hyphae after anastomosis were assigned to post-fusion incompatibility.

To visualize nuclear mingling in perfect fusions or hyphal incompatible responses, germlings paired on membranes were stained: (i) for hyphal viability and protoplasmic continuity, by the localization of SDH activity or with Cell tracker (Molecular Probes, USA); (ii) for the visualization of nuclei, using diamidinophenylindole (DAPI), 5 μg/ml in a 1:1 (vol/vol) water-glycerol solution, or Syto-13, 1 μM in distilled water; and (iii) for the localization of hyphal cross-septa and wall thickening, in a 0.01% (wt/vol) solution of Calcofluor White (Sigma Aldrich, Milan, Italy). All stains were observed in visible light or under epifluorescence with the Polyvar microscope using the filter combination U1 (BP 330-380, LP 418, DS 420) or B1 (BP 330-380, LP 418, DS 420).

DNA extraction and PCR optimization

Since optimal random amplification of polymorphic DNA (RAPD) and inter-simple-sequence repeat (ISSR) amplification depend on several factors, including DNA template, reagent concentrations and temperature profile, several PCR mixes and thermocycler parameters (DNA template, $MgCl_2$, primers and dNTPs concentrations, annealing temperature and cycle number) were optimized before molecular analyses (Online resource 1). For molecular analyses of the different lineages, 100 intact, healthy spores per lineage were selected, surface-sterilized and subjected to DNA extraction (Online resource 1).

RAPD and ISSR-PCR assays

198 RAPD-PCR amplification of DNA from the five *F. mosseae* lines was performed using random 10-
199 mer primers (OP-A, MWG Biotech AG, Germany, Online resource 1). The reaction was carried out
200 in an optimized mix with a final volume of 25 µl, containing 1x reaction buffer, 2 mM $MgCl_2$, 200
201 µM dNTPs, 100 ng primer, 5 ng DNA and 0.125 U *Taq* DNA polymerase (TaKaRa *Taq*™ Bio Inc.).
202 Reactions were performed in a thermocycler Mastercycler personal 5332 (Eppendorf). The optimized
203 thermocycling program consisted of 1 min at 94°C, 40 cycles of denaturation at 94 °C for 30 s,
204 annealing at 34 or 35°C (depending on the oligomer) for 1 min, extension at 72°C for 1 min and a
205 final extension step of 10 min at 72°C. Amplification products were electrophoresed in 1.5% NuSieve
206 3:1 agarose gel in 1x TBE buffer with 0.05% ethidium bromide.
207 The same DNA extracts were used as templates for RAPD-PCR reactions carried out with
208 oligonucleotide primers 19-26 bp in length (Online resource 1) (Sebastiani et al. 2001). The
209 amplification reactions were performed in a total volume of 10 µl. Each reaction consisted of 5 ng of
210 template DNA, 1x reaction buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 250 ng of primer, 0.2 U of *Taq*
211 DNA polymerase (Roche Diagnostics) and 0.001% of gelatin (Roche). Reactions were carried out in
212 the thermal cycler Mastercycler personal 5332 (Eppendorf) with the following thermal programme:
213 denaturation at 94°C for 90 s, 45 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 1 min
214 and extension at 75°C for 2 min, followed by two final extension steps, at 75°C for 10 min and at
215 60°C for 10 min. All the amplification volume was loaded onto a 2.5% agarose gel (1% NuSieve 3:1
216 plus 1.5% Agarose-1000, Invitrogen, UK) with 1x TBE containing 0.05% ethidium bromide.
217 Random microsatellite and flanking regions were amplified with ISSR primers (Online
218 resource 1). The amplification reactions were performed in a 25 µl mixture volume containing 1x
219 reaction buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 200 ng of primer, 5 ng DNA and 0.125 U of *Taq* DNA
220 polymerase (TaKaRa *Taq*™ Bio Inc.). The thermocycler Mastercycler personal 5332 was
221 programmed as follows: 10 s at 95°C; 5 cycles of 30 s at 94°C, 1 min at 50°C, and 1 min at 72°C; 35
222 cycles of 30 s at 94°C, 1 min at 48°C, and 1 min at 72°C; a final extension of 10 min at 72°C.

223 Amplification products were loaded onto a 1.5% NuSieve 3:1 agarose gel in 1x TBE buffer with 0.05%
224 ethidium bromide.
225 For each primer used and each lineage, three replicate amplifications and electrophoreses were
226 carried out and analyzed.
227
228 **Statistical analyses**
229 Frequency data of perfect fusions, post-fusion and pre-fusion interactions obtained for each replicate
230 pairing were arcsine transformed, checked for fulfillment of ANOVA assumptions (by Shapiro-Wilk
231 and Levene's tests) and then submitted to two-way and/or one-way ANOVA followed by Tukey's
232 HSD multiple range test (homogeneous variances) or Welch's test (not-homogeneous variances) to
233 assess: (i) significant differences between self and non-self hyphal interactions, independently of the
234 lineage's combination; (ii) differences among lineages in perfect fusions, comparing the different self
235 pairings; (iii) differences among lineages in perfect fusions, comparing the different non-self pairings;
236 (iv) differences among lineages in perfect fusions, comparing self and non-self pairing combinations
237 involving each lineage; and (v) differences among lineages in incompatible post-fusion and pre-
238 fusion interactions, comparing non-self pairings involving each lineage. For perfect fusions data, the
239 following relative perfect fusions ratio was also calculated for each replicate pairing: $F_{ij}/((F_{ii}+F_{jj})/2)$,
240 where F_{ij} is the inter-lineage frequency while F_{ii} and F_{jj} are average self-anastomosis frequencies of
241 parents' lineages. Such ratios and arcsine-transformed average frequencies obtained from
242 compatibility tests were used to compute Manhattan distance values, which are particularly useful for
243 the study of closely related populations (Nei 1987) and a Bray-Curtis similarity matrix.
244 At the end of the PCR optimization procedure, presence (1) or absence (0) of specific
245 DNA amplification products, considering intense and reproducible bands, was scored for each
246 replicate and primer selected, and Jaccard similarity indices among replicate profiles were calculated.
247 Data obtained from optimized RAPD and ISSR profiles for each lineage replicate and primer selected
248 were assembled to be used to compute the pairwise genetic distances among the lineages. Manhattan

and Bray-Curtis indices were used to compute genetic distance/similarity and to assay by Mantel tests the relationship between the molecular data matrix and matrices based on hyphal compatibility data. Analyses were performed using IBM SPSS software version 23 and diversity and similarity indices and Mantel tests (9999 permutations) were computed using PAST version 3.

RESULTS

Phenotypic traits of the five lineages

Sporocarps of the five *F. mosseae* culture lines showed distinct germination percentages and mycelial lengths (Table 1). The lineage BA exhibited the lowest mean germination percentage, which was significantly different ($P < 0.01$) from those recorded for the other culture lines. No significant differences were detected among lineages PI, TO, DJ and GR, for which germination rates ranged between 35 and 50%. One-way ANOVA and Tukey's HSD carried out on hyphal length data showed that values detected for lines TO and BA were significantly lower than those observed for the lineages PI and GR ($F_{4,20}=14.11$, $P<0.001$). Only germinated sporocarps showing a similar number of germinating spores (average 6±2 per sporocarp) and comparable hyphal lengths were selected for compatibility assays.

Vegetative compatibility assays

Self pairings. After 20 days' incubation, hyphae originating from germlings of the same *F. mosseae* lineage showed perfect hyphal fusions, characterized by the establishment of protoplasm continuity, which was visualized by formazan salt depositions in hyphal bridges (SDH activity), and by shared nuclear material (visualized by DAPI fluorescence microscopy). High percentages of fusions were detected in all *F. mosseae* self pairings (Online resource 2) and one-way ANOVA showed significant differences (Welch test=3.61, $P = 0.015$) between self-anastomosis rates of lineage PI (60.4±2.6), compared with DJ (79.6±3.8) (Tukey's HSD, $P=0.014$), whereas rates of lineages TO, GR and BA (64.3±4.7, 66.6±3.1 and 69.0±4.7, respectively) did not differ from the others. In self pairings, all

hyphal contacts which did not lead to anastomoses showed no interactions, and the rate of non-interacting contacts ranged from 20.3±3.8% (lineage DJ) to 39.6±2.6% (lineage PI).

Table 2. Germination percentages and mycelial length from sporocarps produced by five different *Funneliformis mosseae* lineages originating from the same ancestor isolate.

Lineage	Germination (%)		Hyphal length (mm)
PI	46.2	(40.4-52.1)[a]	245.5 ± 6.5[b] a
TO	43.4	(37.6-49.3)	180.2 ± 7.4 c
DJ	39.9	(34.2-45.8)	205.6 ± 8.0 bc
GR	41.4	(36.5-46.5)	220.4 ± 9.5 ab
BA	17.1	(13.6-20.7)	176.9 ± 6.3 c

[a] In parenthesis, 95% confidence limits of means are reported (P=0.01)

[b] standard error of means are reported. Means followed by the same letter do not differ significantly at $P \leqslant 0.05$ by Tukey's HSD test.

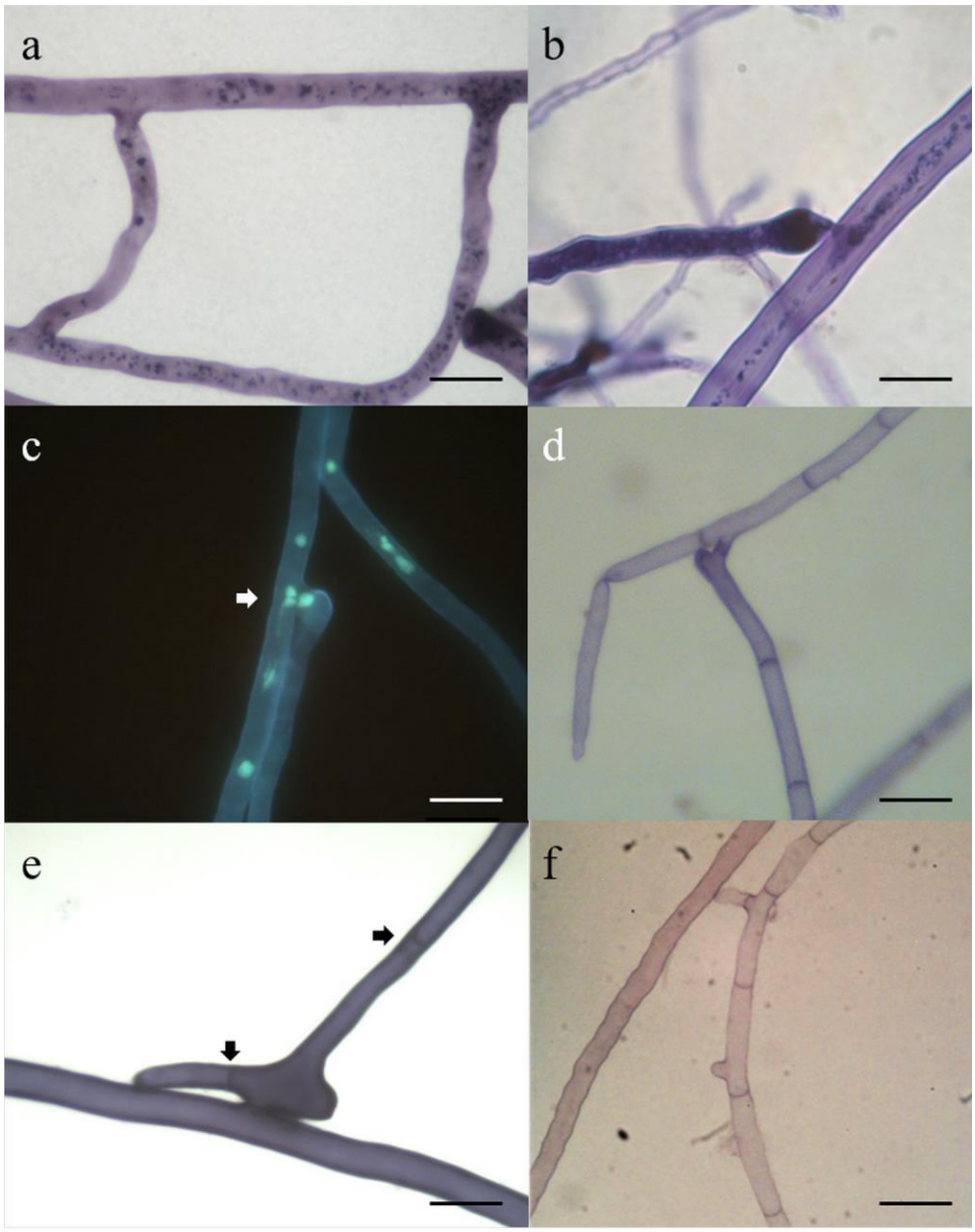

Fig. 1 Micrographs showing hyphal interactions occurring in both self- and non-self pairings among germlings belonging to different *Funneliformis mosseae* lineages, after succinate dehydrogenase (SDH) localization and trypan blue staining (a, b, d, e, f) or after 2,4-diamidinophenylindole (DAPI) staining (c). a) perfect fusion occurring between germlings of the PI lineage (scale bar = 10 µm); b-c) perfect fusions occurring between hyphae originating from spores of BA and GR lineages: of note, the protoplasm continuity between fused hyphae demonstrated by SDH and nuclear mingling (arrow) (b, scale bar = 10 µm; c, scale bar = 8 µm; d) cross-walls (septa) indicating incompatibility, occurring after fusion between hyphae belonging to lineages TO and GR (scale bar = 12 µm); e-f) incompatibility response, with protoplasm withdrawal and septa formation (arrows), occurring before hyphal fusion in pairings PI-DJ (e, scale bar = 10 µm) and TO-BA (f, scale bar = 12 µm)

297 Long-term maintenance (40-70 days of incubation) of perfect fusions viability was confirmed
298 by assessing anastomosis frequencies on SDH-stained self-pairings, which were not significantly
299 different from those detected at 20 days' harvests.
300 **Non-self pairings.** Perfect fusions also were detected among all the different *F. mosseae* lineages:
301 such fusions showed nuclear mingling through hyphal bridges by DAPI fluorescence microscopy
302 (Fig. 1a-c), and their viability and protoplasm continuity were maintained in long-term incubated
303 pairings. Percentages of perfect hyphal fusions ranged from 17±4.2%, in TO-GR pairings, to 44±7.2%,
304 in PI-DJ pairings (Fig 2). One-way ANOVA showed that, independently of lineages' combination, a
305 significantly lower number of perfect hyphal fusions was obtained from non-self pairings compared
306 with self-pairings (Welch's test$_{1,247}$=125.10, P<0.001), while it failed to detect significant differences
307 among the different non-self pairings. Similarly, one-way ANOVA carried out with relative perfect
308 fusion ratios did not show significant differences among all the non-self combinations ($F_{9,148}$=1.44,
309 P=0.175).
310 One-way ANOVA followed by Tukey's HSD also was used to detect significant differences
311 among self/non-self pairings for each lineage: all non-self pairings involving the DJ lineage showed
312 a significantly lower number of perfect hyphal fusions, compared with DJ self pairings ($F_{4,86}$=4.56,
313 P=0.002), while non-self pairings involving the other *F. mosseae* lineages differed from those of the
314 relevant self-pairings only in some combinations (Online resource 2). TO-GR and TO-BA fusion
315 frequencies were significantly lower than those found in TO self pairings ($F_{4,84}$=10.58, P<0.01), PI-
316 GR perfect fusion values were significantly lower than PI self anastomosis rates (Welch's
317 test$_{4,31}$=14.29, P<0.001), GR-TO and GR-PI fusion rates were significantly lower than those of GR-
318 GR (Welch's test$_{4,44}$=22.33, P<0.001), and BA-TO and BA-DJ anastomosis frequencies were lower
319 than those of BA-BA (Welch's test$_{4,15}$=13.12, P<0.001).
320 Post-fusion incompatibility responses were detected in hyphal interactions between germlings
321 of the different *F. mosseae* lineages, and were characterized by protoplasm retraction and multiple
322 septa formation leading to cellular death, hindering protoplasmic continuity between hyphae of the

different lineages (Fig. 1d). Frequencies of post-fusion incompatible contacts ranged from 6.0±1.6 to 47.8±6.7% in DJ-BA and in TO-GR pairings, respectively (Fig. 2). One-way ANOVA followed by Tukey's HSD, used to detect significant differences among frequencies obtained from all non-self combinations, showed that incompatible fusion rates of TO-DJ pairings were significantly lower than those of TO-GR ($F_{3,64}$=6.176, P=0.001) (Fig. 2). Interestingly, when analysing groups of pairings involving each lineage, incompatibility values found in DJ-BA and DJ-TO were significantly lower than those of DJ-PI pairings (Welch's test$_{3,38}$=11.59, P<0.001) and among pairings involving TO, and post-fusion incompatibility rates found in TO-DJ were significantly lower than those of TO-GR ($F_{3,64}$=6.18, P=0.001) (Online resource 2).

Pre-fusion incompatibility responses were detected in non-self hyphal interactions between germlings of all *F. mosseae* lineages tested. The main feature of such a cellular event was represented by the formation of hyphal swellings by the contacting hypha on the surface of the contacted one, followed by wall thickenings, protoplasm retraction and septa formation, hindering anastomosis formation (Fig. 1e). When pre-fusion incompatibility occurred in tip-to-side contacts, a recognition response often was observed, consisting of the differentiation of lateral tips by the side of a contacted (recipient) hypha, in correspondence to the approaching tip of the contacting one (Fig. 1f). Pre-fusion incompatible interactions showed frequencies ranging from 1.6±1.0% to 17.3±5.0% in TO-BA and DJ-GR pairings, respectively, and one-way ANOVA followed by Tukey's HSD, used to detect significant differences among data obtained from all non-self pairings, showed significant differences among them (Fig. 2). In addition, frequencies of pre-fusion incompatible contacts detected in TO-BA and DJ-BA pairings were significantly higher than those of the other pairings involving TO ($F_{3,64}$=4.56, P=0.006) and DJ (Welch's test$_{5,41}$=5.99, P=0.001) lineages, respectively (Online resource 2). On the contrary, no significant differences were found among pairings involving the other *F. mosseae* lineages (Online resource 2).

During non-self interactions, hyphae appeared to intersect without any reaction in 17.9±5 (PI-DJ) to 47.9±5.5% of contacts (BA-DJ). No significant differences in the rates of non-interacting

contacts were detected among non-self pairings involving TO, DJ, GR and BA, compared with their relevant self-pairings, while the pairing PI-DJ showed a significantly lower no-interaction rate than PI-PI ($F_{4,79}$=3.32, P=0.014).

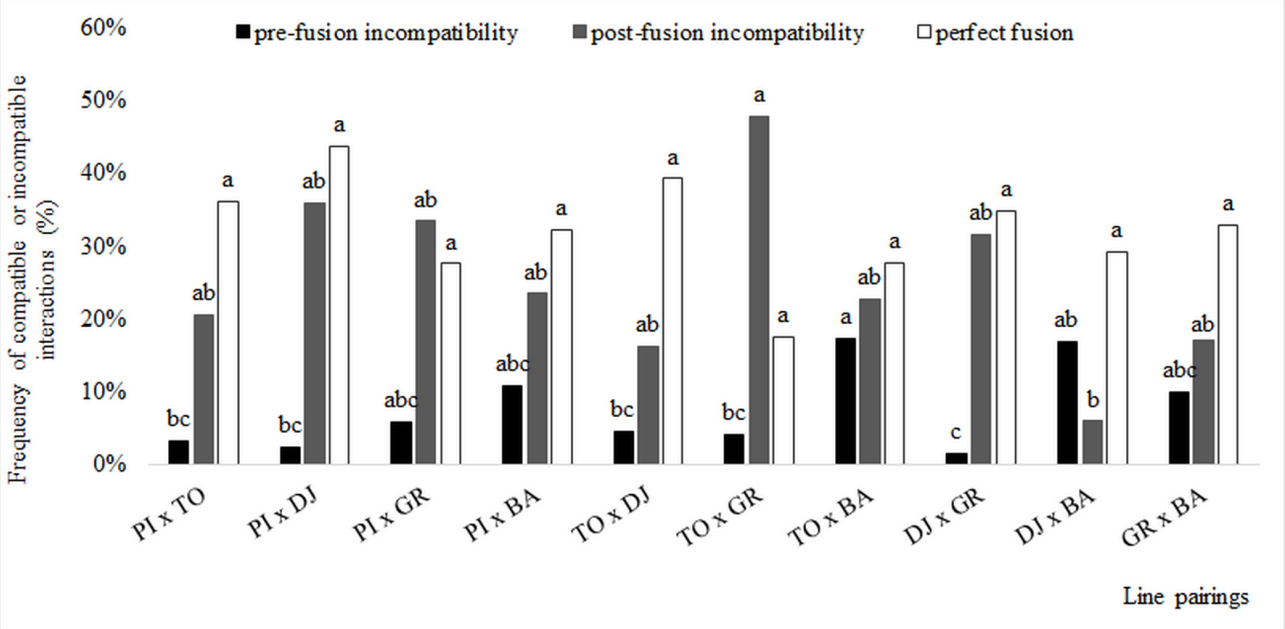

Fig. 2 Mean frequencies of contacts showing perfect anastomoses, pre-fusion or post-fusion incompatible hyphal interactions detected in pairings among germlings belonging to different *Funneliformis mosseae* lineages originating from the same ancestor isolate. Bars with the same letter do not differ significantly (Tukey's HSD, P=0.04)

Fingerprinting analyses

The YV-distinctive 214bp fragment produced by restriction of ITS region with the enzyme TaqI was detected in all the lineages analyzed. Optimization procedures allowed the selection of PCR conditions ensuring the most reproducible electrophoretic profiles, as assessed by correlation analyses. Correlation coefficients among replicate profiles were very high (Online resource 1), ranging from 0.988 to 1 for the entire set of data. The optimized profiles generated by three PCR replications for each lineage and oligonucleotide selected (8 RAPD 10-mer, 6 RAPD 20-mer, 7 ISSR) were combined and analyzed. The analysis of 196 amplified fragments, with an average of 8.2±0.3 (mean±standard

error of the mean) bands per primer, showed a high proportion of uniformly shared bands among *F. mosseae* lineages, with low numbers of polymorphic fragments. RAPD carried out with 10-mer oligonucleotides showed the occurrence of 3.3±0.7% (TO, GR) to 14±0% (PI) polymorphic fragments, whereas RAPD with 19-26-mer oligonucleotides produced 4.8±0.1% (BA) to 11.6±0% (DJ and GR) polymorphic fragments (Fig. 3). More homogeneous profiles were obtained by ISSR-PCR, with a maximum of 6±0% polymorphic bands, observed in the DJ lineage (Fig. 3).

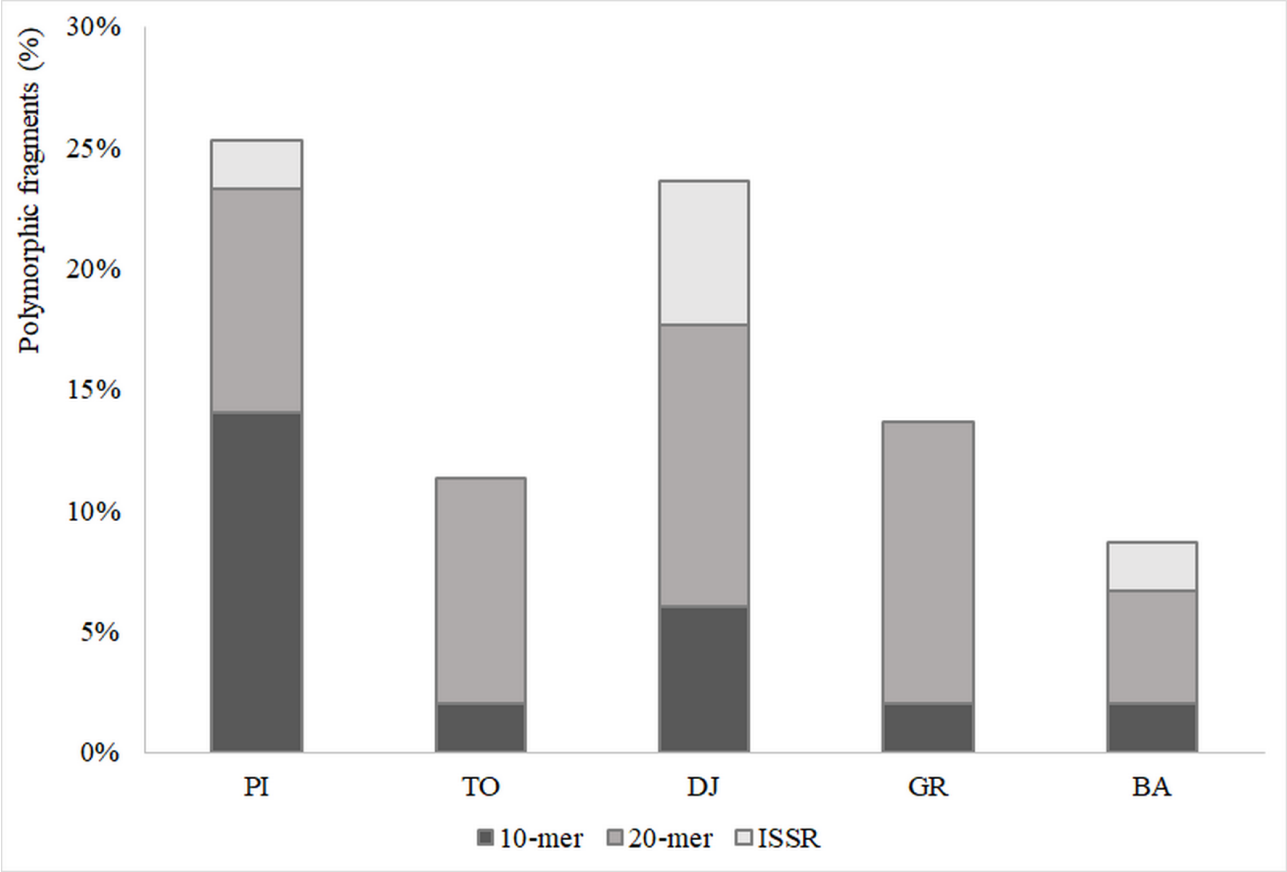

Fig. 3 Percentages of polymorphic fragments detected in the different *Funneliformis mosseae* lineages by using 10-mer or 20-mer RAPD and ISSR primers

Diversity among *F. mosseae* lineages

The Bray-Curtis indices matrix calculated on the basis of perfect fusion rates obtained from compatibility tests carried out on the different *F. mosseae* lineages showed similarities ranging from 0.57 (PI-TO) to 0.84 (BA-TO), with an average value of 0.80, while the matrix calculated on the basis of the relative perfect fusion ratio showed the maximum similarity in the pairing PI-DJ and the minimum for the pairing BA-DJ (Fig. 4a). Rates of unsuccessful fusions, *i.e.* followed by hyphal death, produced a similarity matrix with values ranging from 0, for all pairings involving the lineage BA, to 0.72 (TO-PI) (Fig. 4b). The distance matrix computed using pre-fusion incompatibility rates showed similar values to the post-fusion one, with the exception of the pairing TO-DJ, showing similarity indices of 0.83 and 0.61, respectively (Fig. 4c). A Mantel test carried out to assess correlation among the diversity matrices obtained from data of relative perfect fusion ratio, pre-fusion incompatibility and post-fusion incompatibility showed an R value of 0.90 (P=0.008), indicating highly significant correlation.

The Bray-Curtis similarity matrix calculated on the basis of *F. mosseae* lineages fingerprinting profiles showed values ranging from 0.92 to 0.98, with maximum similarity between PI and DJ and maximum divergence between TO and GR (Fig. 4d).

No correlation was detected between diversity (Manhattan) or similarity (Bray-Curtis) matrices calculated from compatibility assay results and fingerprinting analyses, because Mantel tests were never significant (P ranging from 0.69 to 0.92).

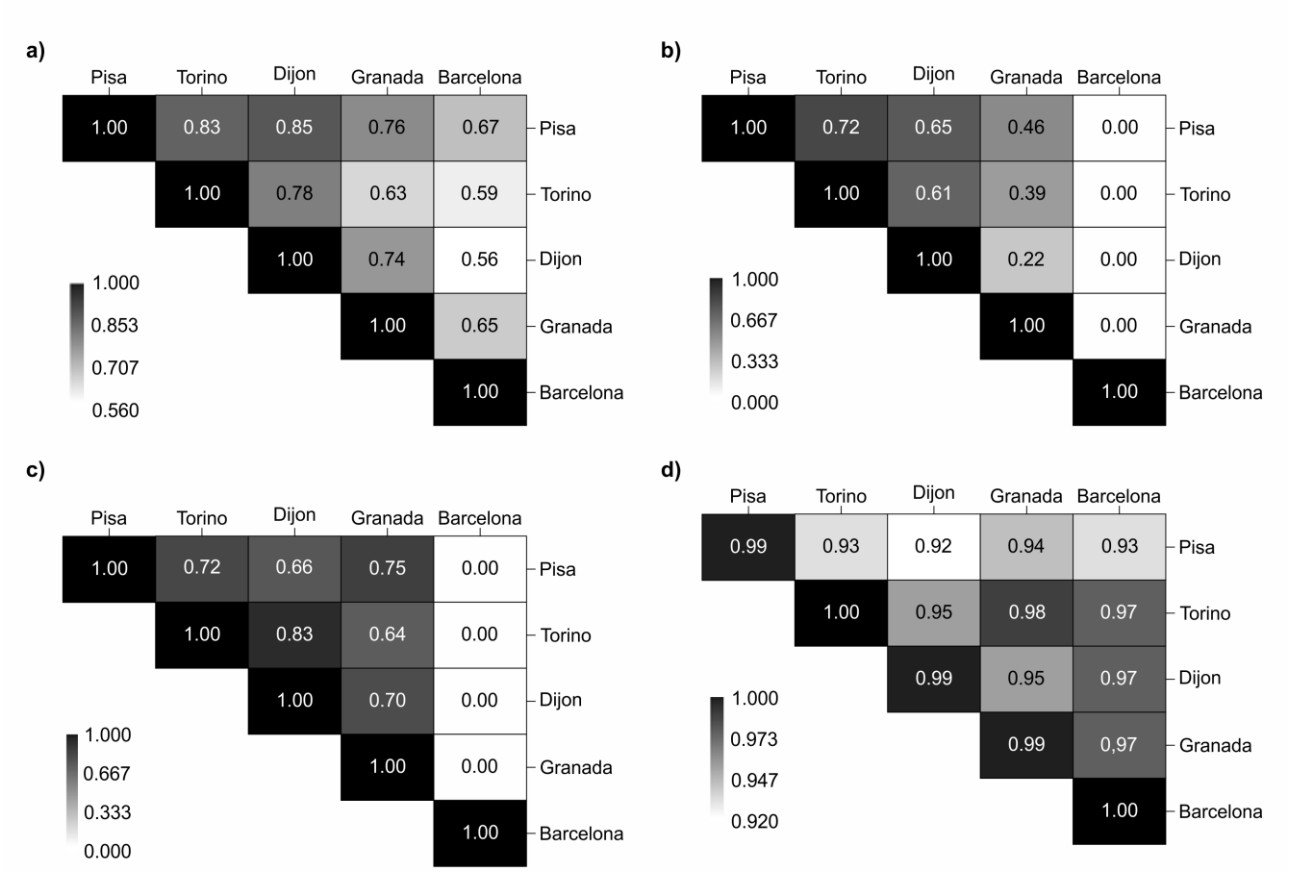

Fig. 4 Bray-Curtis similarity matrices computed on the basis of either phenotypic or genotypic traits of the different *Funneliformis mosseae* lineages. a) matrix based on the relative ratio between the perfect fusions frequency occurring in non-self pairings and the mean self-pairings frequencies of the relevant parent lineages; b) matrix based on the frequency of contacts showing post-fusion incompatibility; c) matrix based on the frequency of contacts showing pre-fusion incompatibility; d) matrix based on cumulative results of RAPD and ISSR analyses

Discussion

The data obtained in this work revealed divergence in *F. mosseae* lineages which originated from the same isolate and were propagated for 20 years in different locations, in vivo. The pairings between different lineages showed high percentages of incompatible hyphal fusions and a reduction in the

421 ability to form perfect fusions (compatible anastomoses). Moreover, we detected pre-fusion
422 incompatible interactions, absent in self pairings, which, by reducing nuclear intermingling and
423 protoplasm flow between lineages, may represent, together with post-fusion incompatibility, the
424 outcome of the phenotypic and molecular divergence among the different *F. mo*sseae lineages.

425 Here, we assessed hyphal compatibility of *F. mosseae* cultures descending from the same
426 Rothamsted isolate, originally named "Yellow Vacuolated" by Barbara Mosse, which were cultured
427 in vivo for more than 20 years in different European laboratories. In AMF, the ability of hyphae
428 belonging to the same isolate to fuse, producing interconnected networks both in asymbiotic and in
429 symbiotic stages, has been confirmed in different experimental systems (Giovannetti et al. 1999, 2003,
430 2004; Croll et al. 2009; Cárdenas-Flores et al. 2010, 2011; de Novais et al. 2013, 2017; Pepe at al.
431 2017; Purin and Morton 2011, 2013; de la Providencia et al. 2013). Self-anastomosis frequencies of
432 *F. mosseae* lineages analysed here occurred in 60-80% of contacts, consistent with fusion rates
433 previously obtained for other *F. mosseae* isolates, ranging from 40 to 85% of hyphal contacts
434 (Giovannetti et al. 1999, 2003). Among isolates of other Glomeraceae species, a wider variability in
435 self-fusion frequencies was observed, as low perfect anastomosis rates were reported for isolates of
436 *Rhizoglomus clarus* and *Funneliformis coronatus* (6.3 and 4.1% of contacts, respectively) whereas
437 high interconnectedness was detected in *R. irregulare* MUCL41833 and *Glomus formosanum*
438 germlings (89 and 91.4% of contacts, respectively) (de Novais et al. 2013; Cardenas-Flores et al.
439 2010; Pepe et al. 2016; Purin and Morton 2013).

440 In this work, germlings of the different lineages showed anastomosis frequencies significantly
441 lower than those belonging to the same lineage, an event possibly representing the first detectable
442 sign of lineages' segregation and genetic drift. Such findings are consistent with those reported in a
443 previous study, where germlings of *R. irregulare* originating from different clonal lineages of a single
444 strain, maintained in vitro through subcultivation in different locations, showed the ability to form
445 hyphal anastomoses, although their fusion frequencies were significantly higher within clonal
446 lineages than between lineages (Cárdenas-Flores et al. 2010). Genetic drift occurred in one population

447 of *R. irregulare*, leading to segregated lines with different phenotypic traits (Angelard et al. 2010,
448 Angelard and Sanders 2011) and in *Claroideoglomus etunicatum*, where individual spores contained
449 a subset of the total allelic variation from the parent isolate (Boon et al. 2013), although, no data are
450 available on the ability of such germlings to anastomose. Germlings of *R. irregulare* isolates
451 originating from the same agricultural field were able to establish perfect fusions when paired in vitro,
452 although their anastomosis frequencies did not exceed 10% of contacts (Croll et al. 2009). On the
453 contrary, no anastomoses were detected in pairings of *F. mosseae* or *R. irregulare* isolates originating
454 from different geographic locations (Cárdenas-Flores et al. 2010; Giovannetti et al. 2003). The
455 divergence that we detected here in the five lineages originated over a 20 years period, which although
456 rapid, is consistent with that observed in other AMF and fungal taxa, among in vitro subcultures.
457 Indeed, genetic and phenotypic changes and variations in fungal characters, such as pathogenicity,
458 growth rate and symbiotic ability were reported in pathogenic, arbuscular and ectomycorrhizal fungi
459 over similar or even shorter time intervals (Angelard et al. 2014; Marx 1981; Richter et al. 2010).
460 When the different *F. mosseae* lineages were paired, a particular cellular event occurred: after
461 hyphal fusion and protoplasmic mingling a high number of anastomoses produced many consecutive
462 cross-walls leading to the cellular death of fused hyphae. Such an event was previously detected in
463 other fungal taxa when pairing isolates belonging to the same anastomosis group and was named
464 vegetative (or heterokayon) incompatibility (VI) (Hutchinson and Glass 2012; Silar 2012). In
465 Glomeraceae, post-fusion incompatibility was first detected and described in non-self pairings of *R.*
466 *irregulare* in an in vitro experimental system, where the anastomoses, though viable only for a short
467 time period, allowed cytoplasmic flow and genetic exchange between genetically different lineages
468 (Croll et al. 2009). In other works, post-fusion incompatibility was not detected either in non-self
469 spore pairings of *R. clarus* and *R. irregulare* isolates or in *R. irregulare* lineages originated from a
470 single mother culture (Cárdenas-Flores et al. 2010; de la Providencia et al. 2013; Purin and Morton
471 2013). In model fungal species, the mechanisms involved in self/non-self discrimination and in the
472 process of cell death induced by heterokaryon incompatibility have been widely investigated, but so

473 far the molecular evolution of the relevant genes and alleles and the specific roles of their products
474 have not been disclosed completely in non-model species (Aanen et al. 2010; Glass and Dementhon
475 2006; Paoletti et al. 2007; Paoletti 2016). In AMF genomes, no *het* or *vic* loci, which are the main
476 components of fungal VI systems (Paoletti 2016), were detected. In *R. irregulare mat*-like loci
477 (Corradi and Brachmann 2017), which are rarely involved in fungal VI, and putative pheromone-
478 sensing genes (Halary et al. 2013) have been reported, though it is still not known whether they may
479 have a role in the occurrence of fusions or VI. In the future, genome-wide association studies may
480 disclose genetic traits involved in regulating AMF hyphal compatibility/incompatibility, while
481 transcript analyses may reveal the biochemical changes associated with cellular processes occurring
482 during anastomosis formation and the establishment of protoplasmic flow or VI, in both asymbiotic
483 and symbiotic interacting hyphae.

484 Pre-fusion incompatibility, that is protoplasm retraction and septa production before
485 anastomosis formation, represented a further cellular event hindering protoplasm intermingling
486 among the different lineages, thus increasing the chance of their possible divergence. The contacts
487 leading to pre-fusion incompatibility ranged from 1 to 17% among all the lineages tested, a frequency
488 much lower than that previously detected between germlings belonging to geographically different *F.*
489 *mosseae* isolates of 32-51% (Giovannetti et al. 2003), indicating an overall lower diversity of our
490 lineages, all originating from the same mother culture. Interestingly, recognition responses previously
491 described in geographically different *F. mosseae* isolates (Giovannetti et al. 2003), represented by the
492 differentiation of lateral tips in the contacted hyphae in correspondence of the approaching ones, were
493 often detected here during contacts between different lineages' hyphae.

494 The frequencies of non-interacting hyphal contacts were not statistically different among self-
495 and non self interactions in the majority of lineages, although a significantly lower rate was found in
496 PI-DJ pairings compared with PI-PI ones. In non-self pairings, the frequencies of hyphal encounters
497 showing no interaction (29-49%, depending on lineage combinations) were consistent with those
498 reported for in vitro assays on different lineages of a single *R. irregulare* isolate, which reached a

maximum of 59% (Cárdenas-Flores et al. 2010) but lower than those previously described in assays among geographically different isolates of *F. mosseae* (49-68%, Giovannetti et al. 2003) and isolates of *R. irregulare* originating from the same field (65-86%, Croll et al. 2009), showing that, even though they were maintained in different cultural conditions for a long time, our lineages, sharing a common origin, were still able to retain high hyphal recognition ability.

The coexistence of hyphal compatibility and incompatibility within the same non-self crossing combination has been reported rarely (e.g. in *Rosellinia necatrix*, Uwamori et al. 2015) and never observed in model filamentous fungi, such as *Neurospora crassa* or *Podospora anserina* (Glass and Dementhon 2006; Glass et al. 2000; Saupe 2000). In asymbiotic non-self pairings of AMF, variable behaviors were described: only pre-fusion incompatible hyphal interactions were detected in pairings among geographically different isolates of *F. mosseae* (Giovannetti et al. 2003), while pre- and post- fusion incompatibility and perfect fusions co-occurred in non-self pairings of *R. irregulare* isolates (Croll et al. 2009; de la Providencia et al. 2013). On the other hand, only perfect fusions were described in non-self pairings among *R. clarus* isolates and *R. irregulare* lineages originating from the same isolate (Cardenas-Flores et al. 2010; Purin and Morton 2013).

Here, we assessed genetic diversity using RAPD and ISSR-PCR analyses. Such methods, utilized to detect fungal genetic variations because of their highly informative profiles (Khan et al. 2017; Molinier et al. 2016), may show some pitfalls, which we avoided by optimizing PCR parameters affecting reproducibility, obtaining consistent and informative profiles for each lineage's DNA. As demonstrated by Jaccard similarity, this procedure allowed us to obtain highly similar results from replicate amplifications with which to carry out genetic diversity analyses. When studied by RAPD and ISSR, the five lineages showed a high proportion of uniformly shared bands, with 10 to 25% polymorphic fragments among them, suggesting a low but detectable genetic divergence. The genetic distances matrix obtained by cumulative analysis of RAPD and ISSR profiles suggested that the PI lineage diverged more than the other lineages did, possibly due to specific environmental conditions. It is interesting to compare the similarity obtained from fingerprinting analyses of our five

525 *F. mosseae* lineages with those calculated from the analyses of six geographically different *F. mosseae*
526 isolates, incapable of hyphal fusion, including the PI lineage (isolate IMA1, Avio et al. 2009): Jaccard
527 similarity of the five lineages ranged from 0.85 to 0.95, while that of the geographically-different *F.*
528 *mosseae* isolates ranged from 0.06 to 0.35.

529 Fingerprinting analyses of members of the same vegetative compatibility group (VCG) of
530 different pathogenic fungal species originating from the same geographic area have shown
531 inconsistent banding patterns by RAPD and AFLP: overall, more similar profiles were produced by
532 fungal isolates belonging to the same VCG, versus those obtained from isolates of other VCGs
533 (Amaradasa et al. 2015; Cilliers et al. 2000; Okabe and Matsumoto 2000). Accordingly, if we consider
534 the *F. mosseae* lineages that we analyzed as belonging to a single VCG because all pairings showed
535 hyphal fusions, it is worth comparing their genetic divergence with anastomosis ability. Results
536 obtained from Mantel tests show that the phenotypic divergence observed is not mirrored by the
537 genetic divergence detectable with the fingerprinting methods used, because no significant correlation
538 was found between distance matrices computed on hyphal interactions and on molecular data.
539 Likewise, no apparent relation previously was detected among genetic similarity and anastomosing
540 aptitude when asymbiotic mycelia originated from different *R. clarus* isolates were paired in vitro,
541 but fusions occurred between hyphae of spores collected close to each other in the same habitat (Purin
542 and Morton 2013). Moreover, AFLP analyses of *R. irregulare* subcultures showed that even if average
543 genetic similarity within the same lineage was higher than between clonal lineages, some spores from
544 different clonal lineages shared more AFLP markers than those of the same one (Cárdenas-Flores et
545 al. 2010). On the contrary, in asymbiotic mycelium of different *R. irregulare* isolates from the same
546 field, cultured in vitro, a significant negative correlation between genetic distance, assessed by AFLP,
547 and perfect fusion frequencies was reported, suggesting a higher anastomosing ability between
548 genetically similar isolates (Croll et al. 2009). Such variability in results suggests that molecular
549 markers used to genotype AMF isolates tested so far are not strictly representative of the unknown
550 genetic factors regulating anastomosis formation in AMF, and specific regions encoding for

551 transcripts involved in hyphal recognition and fusion are needed to link this particular phenotypic
552 trait with genetic diversity. Indeed, studies on the genetic basis of AMF phenotypic/functional features
553 are particularly challenging, as the wide genetic diversity detected in AMF may be driven by genetic
554 recombination (den Bakker et al. 2010), by the occurrence of heterokaryosis in MAT-like high-
555 mobility group proteins (Riley et al. 2014) and by putative activity of meiosis-related genes (Halary
556 et al. 2011).

557 In conclusion, divergence among *F. mosseae* lineages, originated from the same isolate and
558 propagated for 20 years in different conditions, was here demonstrated by the reduction of their ability
559 to form anastomosis and the detection of high levels of VI, both pre-fusion and post-fusion,
560 suggesting that the phenomenon of genetic drift, possibly due to the diverse distribution of nuclei
561 during spore formation, may also involve genes that regulate anastomosis formation.

562

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570

571 **Conflict of interest**

572 The authors declare that they have no conflict of interest.

573

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