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Research

Significant genetic and phenotypic changes arising from clonal growth of a single spore of an arbuscular mycorrhizal fungus over multiple generations

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Summary

- Arbuscular mycorrhizal fungi (AMF) are highly successful plant symbionts. They reproduce clonally producing multinucleate spores. It has been suggested that some AMF harbor genetically different nuclei. However, recent advances in sequencing the *Glomus irregulare* genome have indicated very low within-fungus polymorphism.
- We tested the null hypothesis that, with no genetic differences among nuclei, no significant genetic or phenotypic variation would occur among clonal single spore lines generated from one initial AMF spore. Furthermore, no additional variation would be expected in the following generations of single spore lines.
- Genetic diversity contained in one initial spore repeatedly gave rise to genetically different variants of the fungus with novel phenotypes. The genetic changes represented quantitative changes in allele frequencies, most probably as a result of changes in the frequency of genetic variation partitioned on different nuclei.
- The genetic and phenotypic variation is remarkable, given that it arose repeatedly from one clonal individual. Our results highlight the dynamic nature of AMF genetics. Even though within-fungus genetic variation is low, some is probably partitioned among nuclei and potentially causes changes in the phenotype. Our results are important for understanding AMF genetics, as well as for researchers and biotechnologists hoping to use AMF genetic diversity for the improvement of AMF inoculum.

Introduction

Arbuscular mycorrhizal fungi (AMF) are very successful symbionts of plants (Cavagnaro *et al.*, 2005). They have persisted for over 400 million yr and may have played a crucial role in the colonization of land by plants (Redecker *et al.*, 2000). AMF are abundant in most terrestrial ecosystems and are obligate biotrophs forming symbioses with the roots of the majority of plant species. They improve plant nutrient acquisition and plant growth, and promote floristic diversity (Grime *et al.*, 1987; Newsham *et al.*, 1995; Harrison, 1997; Smith & Read, 2008). Populations of AMF have been shown to be highly variable, both phenotypically and genetically (Bever, 1999; Pringle *et al.*, 2000; Koch *et al.*, 2004; Pawlowska & Taylor, 2004). The study of AMF genetics is relevant for understanding plant growth because differences in symbiotic efficiency have been shown among different isolates of the same AMF species (Munkvold *et al.*, 2004; Gamper *et al.*, 2005; Koch *et al.*, 2006).

Unlike most other organisms, genetic variation in AMF stems not only from differences among individuals, but also from genetic heterogeneity found within individuals. Genetic variation at ribosomal genes within isolates has been documented in several AMF species (Sanders *et al.*, 1995; Clapp *et al.*, 1999, 2001). Experimental evidence has shown that some of the genetic variation within the AMF *Scutellospora castanea* and *Glomus etunicatum* is attributable to genetic differences among nuclei (nucleotypes) within a common cytoplasm (Kuhn *et al.*, 2001; Hijri & Sanders, 2005).

Recent experiments on the AMF *Glomus irregulare* have shown that hyphal fusion between two genetically different AMF can lead to genetic exchange, creating genetic hybrids or crossed lines (Croll *et al.*, 2009). Single spore lines (SSLs) derived from crossed or hybrid lines have been shown to be genetically and phenotypically different from each other and from the parental lines (Angelard *et al.*, 2010). The observed genetic differences among the SSLs could be categorized into two classes. First, amplified fragment length polymorphism (AFLP) identified predominantly losses of genetic markers in the SSLs (52–82% of the genetic differences), but also gains (13–43% of changes). Second, an analysis at the Bg112 locus showed quantitative changes in the frequency of four alleles among the SSLs. Neither losses nor gains of alleles were observed. The changes in allele frequency among the AMF lines

could only be explained if at least some of the alleles were located on different nuclei (Angelard *et al.*, 2010). The rationale for this was that some alleles increased in frequency, whereas others decreased, and this could not happen if the alleles were co-located together on the same nuclei. The changes in allele frequency among the AMF lines have been defined as partial segregation (Sanders & Croll, 2010). In a study on a different AMF species, no loss of alleles during spore formation was found (Pawlowska & Taylor, 2004). However, it is unknown whether allele frequencies were affected. In the study by Angelard *et al.* (2010), both genetic exchange and segregation in AMF gave rise to genetically novel AMF lines which exhibited a range of phenotypes that were significantly different from their parents. The new lines were distinct in their phenotypes and, most importantly, in their effect on plant growth and plant gene expression (Croll *et al.*, 2009; Angelard *et al.*, 2010; Angelard & Sanders, 2011; Colard *et al.*, 2011).This highlights the importance of studying genetic variation in AMF and the dynamics of AMF genetics in order to understand plant growth.

Despite their importance for plant growth, little is known about genetic processes in AMF (Sanders & Croll, 2010). Although evidence for the segregation of nucleotypes was inferred by Angelard *et al.* (2010), segregation occurred in crossed or hybrid AMF lines where different nuclei may have been mixed within a fungus by the hybridization process (Angelard *et al.*, 2010). Quantitative genetics experiments are required on defined AMF isolates to understand how much genetic and phenotypic variation occurs within a single AMF spore, and how it is partitioned and inherited from one generation to the next. This is particularly topical as recent efforts to sequence the genome of *G. irregulare* (isolate DAOM 197198) have revealed very little polymorphism in the assembled parts of the genome, indicating only one haplotype, although significantly large and currently unassembled parts of the genome contain many repeated elements and retrotransposons (F. Martin, INRA, Nancy; pers. comm.).

AMF grow and reproduce clonally. Therefore, under the null hypothesis that there are no genetic differences among nuclei (i.e. a single haplotype), no significant genetic or phenotypic variation would be expected among clonal SSLs generated from an AMF culture that started from one AMF spore. Furthermore, no further significant variation in genotypes or phenotypes would be expected in the second and third generations of SSLs. To test this, we established single spore AMF lines (SSLs) repeatedly over successive generations. All SSLs originated from an AMF culture that had been initiated from a single AMF spore and maintained in stable culture conditions for several years. Thus, we were able to test the null hypothesis by measuring any genetic or phenotypic variation arising among the offspring of a single spore and over successive generations. To observe quantitative genetic changes, we measured changes in the frequency of alleles at the previously studied Bg112 locus. The design of our experiment also allowed us to investigate whether any genetic or phenotypic changes seen at a given generation were caused by changes that had already occurred at the previous generation or whether they represented newly arising genetic or phenotypic changes. Our results demonstrate that the AMF lines changed significantly both genetically and phenotypically over several successive generations. Such differences in quantitative phenotypic traits and quantitative genetic changes originating from a single spore and occurring repeatedly over several generations are remarkable for a clonally growing organism.

Materials and Methods

Experimental set-up: AMF isolate and establishment and propagation of SSLs

Single spore cultures of *Glomus irregulare* Blask., Wubet, Renker & Buscot from an agricultural field site in Tänikon, Switzerland were used to establish axenic cultures (Anken *et al.*, 2004; Koch *et al.*, 2004). These fungal isolates were previously ascribed to the species *Glomus intraradices* (Stockinger *et al.*, 2009). *Glomus irregulare* has also recently been renamed as *Rhizophagus irregularis* (Blask., Wubet, Renker & Buscot) C. Walker & A. Schuessler (Krüger *et al.*, 2011). The single spore cultures were maintained clonally in an axenic culture system on standard M growth medium with a clone of transformed *Daucus carota* L. roots (Bécard & Fortin, 1988). The fungi were propagated for > 3 yr under standardized axenic conditions, minimizing maternal and environmental effects. One single spore isolate, referred to as C3 (G0: parental generation), was chosen to start the experiment and to establish SSLs (Koch *et al.*, 2004).

Three successive generations (G1, G2 and G3) of SSLs were established using the same procedure as described in Angelard *et al.* (2010) (Fig. 1). (For full details of the numbers of SSLs established, see Supporting Information Notes S1). To reduce maternal effects, and to obtain sufficient fungal material, each line was subcultured twice onto replicate plates before starting a new generation of SSLs. One round of subculturing involved the transfer of a piece of medium (*c.* 2 cm^2), containing colonized roots, spores and hyphae, onto a new Petri dish containing sterile M medium. The fungus was then left to grow clonally for 15 wk before the next round of subculturing.

At each new single spore generation, two-compartment plates were also inoculated (8–12 replicates per line) by taking fungal material from replicate plates of each of the clonally grown SSLs. This followed the procedure described in Koch *et al.* (2004). This allowed us to extract fungal DNA from each SSL, which was free of carrot DNA.

For reasons of feasibility, for all three generations (G1, G2 and G3), only a subset of all newly established lines was randomly chosen from the successfully germinating, root-colonizing and sporulating SSLs from the previous generation (see Notes S1). The number of lines was chosen to keep the design as balanced as possible.

Molecular analyses on SSLs

Molecular analyses were performed on all successive generations (from G0 to G3; Fig. 1). Hyphae and spores of each SSL were removed from the root-free compartment and DNA was extracted as in Koch *et al.* (2004). DNA extractions of all the lines of isolate C3 for the G0, G1 (five lines) and G2 (12 lines) generations were replicated by splitting the root-free compartments into two. In the

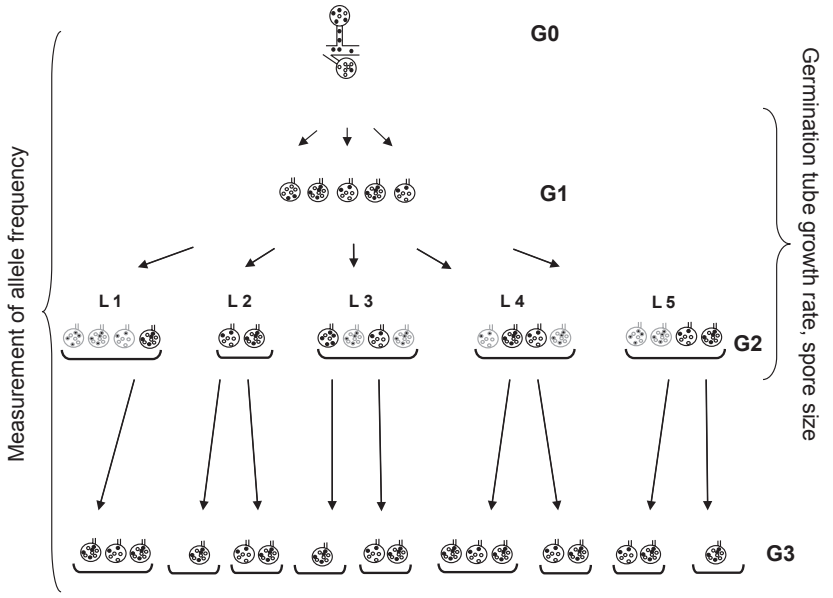

Fig. 1 Design of the experiment. Successive single spore lines of *Glomus irregulare* (isolate C3) were set up for three generations. The figure shows which phenotypic and molecular measurements were made over the generations. Spores shown in light gray at G2 were lines that were not propagated further in G3. Only those lines shown in black at G2 were propagated in G3.

G3 lines (17 lines), DNA extraction was replicated for seven lines. All fungal material from each generation and each isolate was separately processed as in Croll *et al.* (2009).

We used the established semi-quantitative sequence-based marker Bg112 to investigate quantitative genetic changes occurring among SSLs at each generation. The marker is very suitable as it reproducibly detects differences in allele frequencies among crossed and segregated lines (Angelard *et al.*, 2010). Although this method does not allow us to make conclusions on the absolute number of alleles, it is a good method for investigating relative changes in allele frequency among SSLs in this organism. Isolate C3 harbors four alleles at the Bg112 locus. The reliability of the technique was first assessed by Angelard *et al.* (2010), as they used two independent DNA extractions of each AMF line and observed no significant differences in allele frequency among replicates. We also conducted an analysis that confirmed this reproducibility, as we found that only a small percentage of the variance was explained by residual errors (potential methodological errors) compared with variance explained by the segregated lines (see Notes S2 and Table S2).

Four alleles of the Bg112 locus (allele 1, 174 bp; allele 2, 178 bp; allele 3, 208 bp; allele 4, 214 bp), which all co-occur in the isolate C3, were amplified following a previously published method (Croll *et al.*, 2009; Angelard *et al.*, 2010). For 21 of the 34 AMF lines, we performed two independent DNA extractions. For each independent DNA extraction, we replicated the PCR twice, thus providing four PCR data points per sample. Ten PCR replicates failed to amplify, so that 10 samples lacked replicate PCRs for each DNA extraction. In the 13 lines in which only one DNA extraction could be made, we replicated the PCR four times to give four values per allele. This meant that, for subsequent data analyses, there were data from 11 AMF lines with both replicate DNA extractions and replicate PCRs, and a further 10 AMF lines where there were only replicate PCRs but not replications of the DNA extractions. An exact amount of DNA (5 ng) was used in every PCR. PCR amplifications of all samples were carried out simultaneously and the PCR products were all run on the same capillary sequencer.

For each allele of the locus Bg112, we calculated the relative fluorescence measured as the height of a peak of fluorescence on an electropherogram generated after capillary electrophoresis on an automatic capillary sequencer, as in Angelard *et al.* (2010). This allowed us to obtain an estimate of the frequency of each allele relative to the others, but not the absolute numbers of each allele (see Supporting Information of Angelard *et al.* (2010) for more details on this method).

Measurement of phenotypic traits

Phenotypic measurements were carried out on the spores from generations G1 and G2. Germination tube length was measured as the increase in length of the germination tube, 6 d after single spores were randomly chosen and transferred onto new M medium. The length was measured on the germination tube of each single spore using a graduated eyepiece with a 16× magnification on a binocular dissecting microscope. Spore diameter was measured using a graduated eyepiece with a 40× magnification. The values were then converted to the germination tube growth rate (see Notes S3). All measurements were carried out blind to the sample line identity. Germination length and spore diameter were measured on the progeny of the G1 and G2 generations, whilst establishing the SSL lines of the G2 and G3 generations. As both traits are correlated ($R^2 = 0.485$, $P < 0.0001$) and the results are qualitatively similar, we only present the results for the germination tube growth rate. Results on spore diameter are presented in Supporting Information Fig. S1.

Statistical analysis of variation in the relative allele frequencies

We performed pairwise correlations between the fluorescence values for each allele to measure the independence of alleles relative to each other. The relative fluorescence values of each line are shown in Supporting Information Table S1. If some of the allele

frequencies are significantly correlated, it is possible to reduce these values to a smaller number of variables for further statistical analysis.

Significant correlations were found between the frequencies of some alleles, meaning that not every allele was independent from all the others. Thus, we performed a principal component analysis on the relative fluorescence values for the alleles to reduce the number of variables to a smaller number for further data analysis. We reduced the dataset from four variables (four alleles) to two principal components (PCs), whilst retaining most of the information (see Notes S4 and Table S2). The first two PCs explained 97.3% of the variance. The first PC represented 52.6% of the variance and was highly correlated with the frequency of alleles 1 and 2 ($R = 0.7609$ and 0.7869) and negatively correlated with the frequency of alleles 3 and 4 ($R = -0.5619$ and -0.7691). The second PC represented 44.7% of the variance and was highly correlated with the frequency of alleles 2 and 3 ($R = 0.5846$ and 0.8145) and negatively correlated with the frequency of alleles 1 and 4 ($R = -0.63$ and -0.6203). All of these correlation coefficients were highly significant.

Statistical analysis of the molecular data at G1

For all statistical analyses, both replicate DNA extractions and replicate PCRs on the same DNA extraction were considered as true replicates. This was because differences among DNA extractions introduced a negligible amount of variance (see Notes S2 and Table S2).

To test whether SSLs at G1 differed from each other in their allele frequencies at the Bg112 locus, we performed a univariate one-way ANOVA on the two variables obtained from the PC analysis.

Statistical analysis of the molecular data at G2

To test whether SSLs at G2 differed from each other in their allele frequencies at the Bg112 locus, and whether we could still detect the effect of a previous genetic change (at G1), we performed a nested ANOVA on the two variables obtained from the PC analysis, with two random factors ('G2 nested within G1' and 'G1').

Statistical analysis of the molecular data at G3

To test whether SSLs at G3 differed in their allele frequencies at the Bg112 locus, and whether we could still detect the effects of genetic change that occurred at G1 and G2, we performed Mantel tests. We could not perform a nested ANOVA because, through the course of the experiment, the design became too imbalanced after the production of three SSL generations. We computed three matrices: two matrices of the pairwise genetic distance between lines after three generations of SSLs for each of the two PCs; and one matrix of the pairwise genetic relatedness (see Notes S4), which comprises the distance between any two SSLs. Mantel tests were then used to test whether any of the matrices were significantly correlated.

However, in order to test whether the lines after three generations of segregation were genetically different, we performed five *t*-tests and one one-way ANOVAs separately, where we had multiple G3 lines from one parent, as this is not investigated by the Mantel test.

Statistical analysis of phenotypic variation at G1

To test whether the phenotypes of SSLs at G1 differed, we performed a univariate one-way ANOVA on the germination tube growth rate and spore diameter of 128 individually chosen single spores from five G1 lines (Fig. 1). Four spores did not germinate and were removed from the analysis, leaving a total of 124 spores. The model used was a one-way random model with the main factor G1 lines (random). To meet the requirements of the statistical tests, the following transformation of the fungal growth traits was used: $(\text{weighted germination length})^{0.5}$ (Sokal & Rohlf, 2000).

Statistical analysis of phenotypic variation at G2

To test whether the phenotypes of SSLs at G2 differed, and whether this was a result of differences already occurring among G1 lines, we performed a nested ANOVA on the two variables germination tube growth rate and spore diameter, with two random factors ('G2 nested within G1' and 'G1'). We took 758 individually chosen spores from 18 G2 lines. Twenty-two spores did not germinate and were removed from the analysis, leaving a total of 736 spores. Data on both spore diameter and hyphal tube growth rate were square root transformed (Sokal & Rohlf, 2000).

All statistical analyses were performed with the programs JMP 5.0 (SAS Institute Inc., Cary, North Carolina, USA) and R version 2.13 (R Development Core Team, www.R-project.org).

Results

Overall molecular variation

We found strong and significant negative correlations between some pairs of alleles. The frequencies of alleles 1 and 3 and alleles 2 and 4 were strongly correlated (Table 1). Because this meant that some of the alleles were not independent from each other, it was possible to reduce the four variables of allele frequency to two variables (PCs 1 and 2) that captured most of the variation in allele frequency at the Bg112 locus.

Changes in allele frequency at G1

The allele frequencies of five SSLs at G1 differed significantly from each other (Fig. 2). This was true for both PCs (first PC: $F_{4,15} = 5.55$, $P = 0.006$; second PC: $F_{4,15} = 323.05$, $P < 0.0001$; Table S3). For the first PC, lines L2 and L3 were significantly different from line L1 (Fig. 2). All five lines exhibited significantly different allele frequencies for the second PC (Fig. 2).

Changes in allele frequency at G2

Allele frequencies of SSLs at G2 also differed from each other (Fig. 2). Allele frequencies among the G2 SSLs (nested within G1 lines) differed significantly for the second PC ($F_{7,22} = 111.5814$, $P < 0.0001$; Table S3). Many differences among the lines were observed (Fig. 2). Differences also occurred between SSLs originating from the same parental G1 line. For example, allele

Table 1 Pairwise correlation coefficients between the relative frequency of four alleles (variables) of the Bg112 locus in single spore lines of *Glomus irregulare* isolate C3

	Allele 1	Allele 2	Allele 3
Allele 1			
Allele 2	0.199 ($P = 0.03$)		
Allele 3	−0.918 ($P < 0.0001$)	0.005 ($P = 0.95$)	
Allele 4	−0.218 ($P = 0.02$)	−0.937 ($P = 0.0001$)	−0.095 ($P = 0.29$)

$n = 122$. P values are shown in parentheses.

frequencies differed between line L2-1 and L2-2, even though they both originated from the same SSL (L2) in the previous generation (Fig. 2). In addition, allele frequencies differed among several lines at G2 that originated from different G1 parents. We did not detect any statistically significant differences in allele frequency among G2 lines nested within G1 lines for the first PC ($F_{7,22} = 1.112$, $P > 0.05$; Table S3).

Changes in allele frequency at G3

We tested whether the G3 lines increased in genetic distance from each other and whether the pedigree of the lines explained an increase in genetic differentiation among the lines. We expected that two G3 lines should be genetically more similar if they arose from the same parent at G2 than if they only shared a common G1 grandparent. We tested this by correlating the distance in the pedigree (i.e. relatedness or number of generations to the last common line parental line) with the genetic distance measured at the Bg112 locus. We found significant correlations between the matrices of relatedness and genetic distance (Fig. 3). The Mantel test between the class of relatedness and the average pairwise distance between lines was significant for both PCs. The coefficient was positive between the first PC and the relatedness class ($R = -0.124$, $P = 0.026$) and negative between the second PC and the relatedness class ($R = 0.181$, $P = 0.027$). This means that lines at G3 which shared the same G2 parent were more similar to each other than to other lines for which they only shared a G1 grandparent. The pattern was the reverse for the second PC. The opposite sign of the coefficient of the two correlations was expected as PCs are orthogonal. Figure S2 shows the proportion of each allele in each line at G3, as well as the relationship of each G3 line to the G1 and G2 lines. The G3 dataset was too unbalanced to perform an ANOVA on the whole dataset. However, we performed *t*-tests or ANOVAs on either two or three G3 lines that shared the same parent. Most G3 lines showed significant differences in allele frequency for both PCs (Table S4). Only two tests were not significant for the first PC.

Differences in phenotypes at G1 and G2

We measured germination tube growth rates and spore diameter among the different lines at G1 and G2 of the experiment. The germination tube growth rate was significantly different among the

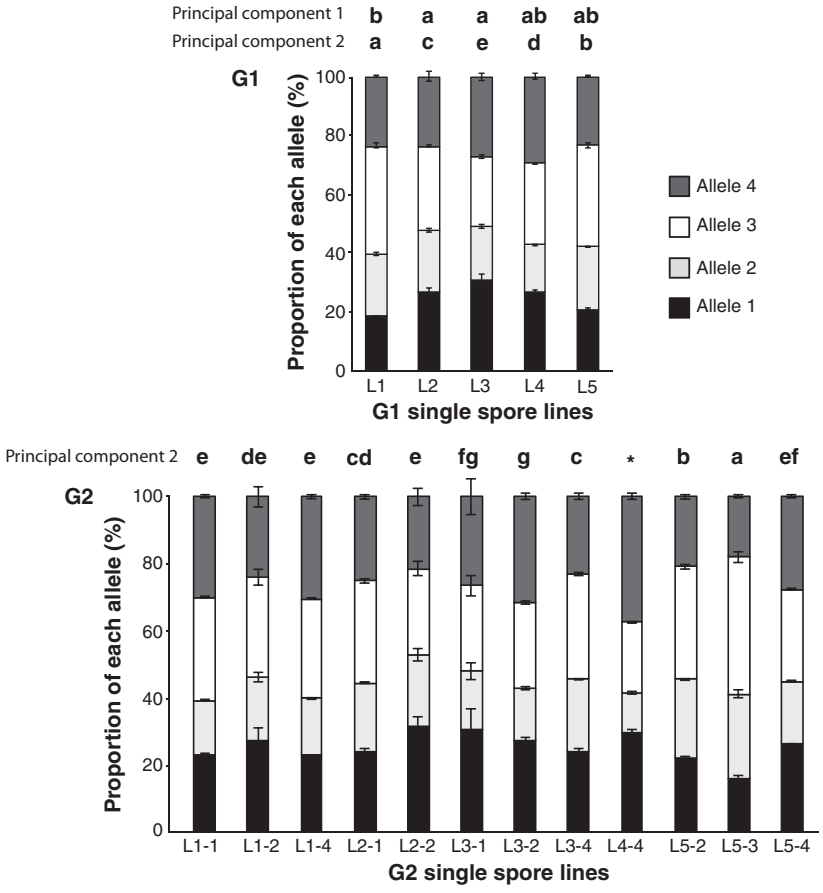

Fig. 2 Relative allele frequencies of the Bg112 locus in *Glomus irregulare* over two successive generations of single spore lines (SSLs). G1 is the first single spore generation; G2 is the second single spore generation. Different shading within bars represents the four different alleles. Different letters above the bars at G1 represent significant differences at $P \leq 0.05$ according to a Tukey–Kramer honestly significant difference (HSD) test performed on the principal components 1 and 2 (see Table S3 for details of ANOVA). Different letters above the bars at G2 represent significant differences at $P \leq 0.05$ according to a Tukey–Kramer HSD test performed on the principal component 2 (Table S3). No means comparisons were made for the principal component 1, as this variable showed no significant differences at G2 (Table S3). *, SSL not analysed statistically as there was only one line at G2 originating from the G1 L4 parental line.

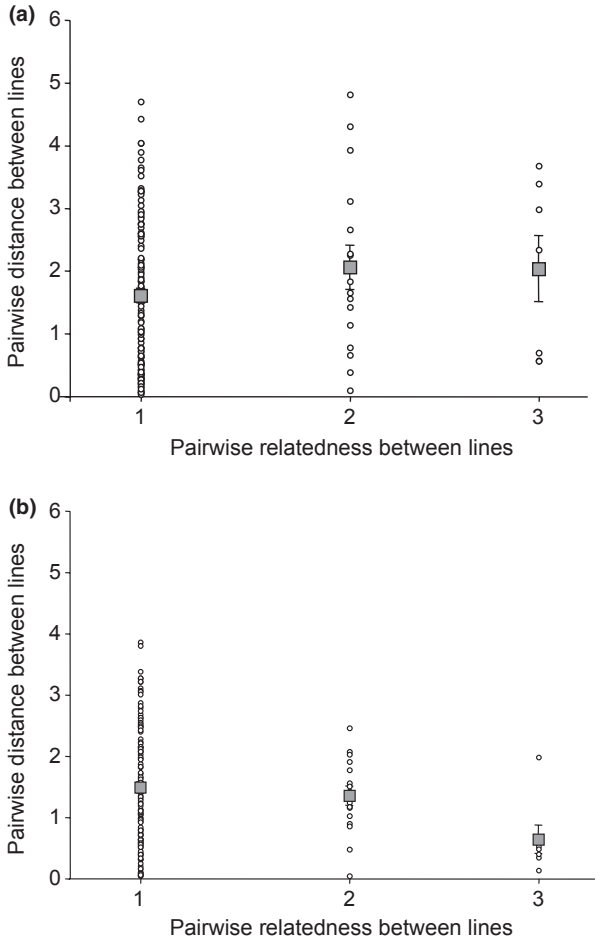

Fig. 3 Relationship between the matrices of pairwise distance and pairwise relatedness of *Glomus irregulare* single spore lines after three generations (G3) using the data on allele frequencies at the Bg112 locus. (a) First principal component (PC) (open circles, pairwise distance between single spore lines; closed squares, mean difference of the relatedness class). (b) Second PC (open circles, pairwise distance between single spore lines; closed squares, mean difference of the relatedness class). Error bars represent ± 1SE.

SSLs at G1 ($F_{4,119}$ = 8.536, $P \leq$ 0.0001; Fig. 4a; Table S5a). The lines L5 and L1 exhibited the fastest growth rates after 6 d, whereas lines L2 and L3 exhibited the slowest growth rates (Fig. 4a). Additional differences among SSLs arose at G2. G2 lines originating from the same G1 parental line differed significantly in their germination tube growth rates ($F_{13,698}$ = 8.455, $P \leq$ 0.0001; Fig. 4b; Table S5b). For example, line L1-1 exhibited significantly faster germination tube growth rates than line L1-4, even though both lines originated from the same G1 parental line (Fig. 4b).

Statistically and qualitatively similar results were obtained for the spore diameter (see Fig. S1 and Table S5a,b).

Discussion

We observed considerable genetic and phenotypic differences in *G. irregulare* SSLs over several generations. Given that AMF are clonal, and that all the material analyzed in this experiment originated from one single AMF spore, the rapidly occurring genetic and phenotypic differences are quite remarkable. We are not aware of any other clonal organisms in which the generation of such variation has been reported. An explanation of how such genetic and phenotypic differences can arise successively among clonal progeny from a single spore is clearly warranted and is important for researchers working on the mycorrhizal symbiosis.

Explanation of genetic differences observed over successive clonal generations

The molecular analyses of successive SSLs showed that genetic differences occurred among the lines at each new single spore generation. As expected, the differences were not qualitative (i.e. presence or absence of alleles), but quantitative. Relative allele frequencies differed among SSLs. Our results are also in agreement with those of Pawlowska & Taylor (2004) who did not observe a loss of sequence variants after one generation of SSLs in the AMF *G. etunicatum*. Allele frequencies and phenotypes were not measured in that study. As in Angelard *et al.* (2010), the most parsimonious explanation for the changes in allele frequency among lines is that some of the alleles are located on different nuclei. If each nucleus had contained all four alleles in equal amounts, no significant changes in relative frequency of the different alleles should have been observed. Thus, our results do not support the null hypothesis.

The genetic variation measured could be an underestimate as the molecular analyses are restricted to fungal lines that successfully formed single spore cultures. Approximately 70% of single spore cultures failed to establish at each new generation, representing an unquantifiable proportion of additional genetic variation that could have been missed.

Our results are intriguing given the current knowledge regarding the genome of *G. irregulare*. Recent data on the genome sequence of *G. irregulare* (isolate DAOM 197198) indicate only a small amount of polymorphism in the assembled part of the genome. The assembled sequence data suggest one haplotype, although considerable parts of the genome containing many repeat regions and retrotransposons remain unassembled (F. Martin, INRA, Nancy; pers. comm.). Several factors may explain why these seemingly opposing results may be complementary. First, the Bg112 locus was discovered using a strategy to detect potentially polymorphic loci specifically occurring in or near repeat regions, using publicly available sequences of the DAOM 197198 genome, the same strategy as described previously (Croll *et al.*, 2008). Thus, Bg112 may represent a within-isolate multi-allele locus that is not in the assembled part of the *G. irregulare* genome. This would highlight the importance of filling the gaps in the genome that are difficult to assemble as they may contain important polymorphism. Second, even in well-established reference genomes that are considered to be complete, very similar copies of genes that show slight sequence differences are often missing or fail to assemble, especially if they occur in duplicated regions (Eichler, 2001). However, variation in such complex regions of the genome is considered to be a primary source of evolutionary innovation and can have major effects on the phenotype (Lynch & Katju, 2004; Dennis *et al.*, 2012). Resolving such small polymorphic differences in or among genomes requires

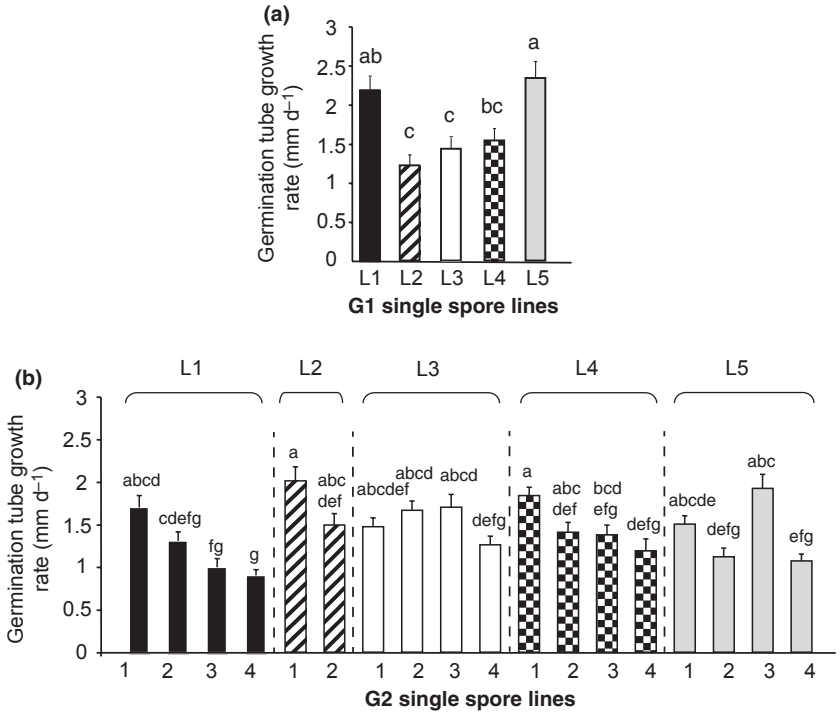

Fig. 4 Phenotypic measurements of the *Glomus irregulare* single spore lines (SSLs) over two successive generations. (a) Mean germination tube growth rate of single spore lines at G1. (b) Mean germination tube growth rate of single spore lines at G2. Error bars represent + 1SE. The five SSLs at G1 are shown by different shading within the bars. Shading within bars at G2 shows which SSLs are derived from a given G1 parent. Letters above the bars show differences according to a Tukey–Kramer honestly significant difference (HSD) test where $P \leq 0.05$.

ultrahigh sequence coverage coupled with novel strategies that can specifically target polymorphic regions (Dennis *et al.*, 2012). Our data suggest that such an approach is warranted for the study of genetic variation in AMF genomes and its functional significance.

Quantity of genetic changes observed over successive generations

Our results showed that the locus Bg112 is well suited to differentiate among lines. The locus provided semi-quantitative measurements of allele frequencies during spore formation. We found a random association between the frequencies of certain pairs of alleles (alleles 2 and 3, and alleles 3 and 4). However, there was a strong correlation between the frequencies of alleles 1 and 3, as well as alleles 2 and 4. From these data, we can hypothesize a minimum number of independently segregating nuclei at the Bg112 locus, and we would expect at least two nucleotypes. The highly correlated pairs of alleles could represent duplicate copies of the locus on the same nucleus. Alternatively, the highly correlated alleles could exist because of the dependence of one nucleotype on another.

The *G. irregulare* isolate used had previously undergone two successive single spore culturing events before we started this experiment. Thus, the number of single spore culturing events leading to the G3 generation is five instead of three. The genetic variation within *G. irregulare* may be considerable, because we detected genetic differences arising among sibling lines even after these five single spore generations. Alternatively, the strength of the bottleneck on nucleotype diversity imposed by spore formation may not be sufficient to deplete genetic diversity over a few single spore generations.

One particularly interesting feature of the dataset on allele frequencies at the three generations concerns the amount of variation in allele frequency observed at each generation. There were clear differences in allele frequency among the lines at G1. At G2, there were also clear differences between SSLs. The statistical analysis of allele frequencies showed that significant differences in allele frequency had occurred at G2 among lines that shared the same G1 parent. This means that genetic differences occurred at G1 and that additional genetic differences occurred again at G2.

At G3, we found that the most recently diverged lines (e.g. sharing the same parental line) were more similar to each other than by randomly choosing any other line. These results show that the genetic bottleneck imposed by spore formation is detectable over multiple generations of SSLs. Taken together, our molecular results indicate a very dynamic genetic system in AMF.

Explanation of the phenotypic differences observed over successive clonal generations

We also detected considerable phenotypic changes among lines undergoing at least two single spore generations. The phenotypic changes appeared to be random, as line L2 had the slowest germination tube growth rates and, in the next generation, one of its offspring, L2-2, had the fastest germination tube growth rates. After two successive single spore culturing events, the phenotypes of the SSLs showed a similar pattern of divergence to the genetic data.

The large differences in phenotypes generated among SSLs that share the same parent are also unusually high for a clonal organism. It is possible that some of the variation in phenotypes among AMF lines could be attributable to differences in the developmental stages at which the spores were chosen for germination experiments. However, the large sample size, with strong statistical support, makes such an effect unlikely. Furthermore, as the cultures were all of the same age, differences in the rate of development

would also represent a difference in phenotype among the lines. Although we have observed both genetic and phenotypic changes arising simultaneously among the lines, it is not possible to say from our results whether genetic changes among the lines are responsible for the observed phenotypic differences. The variation observed in phenotypes could be a result of genetic or epigenetic differences among the AMF lines, or a combination of both.

Genetic and phenotypic stability in *G. irregulare*

In our previous studies of AMF genotypes and phenotypes, the same fungus was subcultured onto replicate plates by transferring many spores and hyphae, rather than just one spore. Replicate subcultured material prepared in this way was very stable, showing very little phenotypic or genetic variation or variation in effects on plant growth among replicates of the same culture (Koch *et al.*, 2004, 2006). To measure the phenotypes in this study, each SSL was also replicated onto multiple Petri dishes by transferring a large amount of hyphae and spores of each SSL onto new medium. Indeed, although we demonstrate clear phenotypic and genetic differences among SSLs, the variation in allele frequencies and phenotypes was very small among subcultured replicates of each SSL. This suggests that, following genetic and phenotypic changes occurring at spore development, there is a possibility to stabilize the phenotypes of the genetically novel SSLs. This has important implications for researchers of mycorrhiza to ensure reproducibility in experiments, as well as commercial implications. The production of 'designer' mycorrhiza by raising genetically novel AMF lines has been proposed as a tool to develop inoculum with the desired symbiotic properties (Sanders, 2010). The creation of SSLs from only one isolate of *G. irregulare* is a way of rapidly producing a range of AMF lines with novel genetic composition and phenotype. Given that the genetic composition of AMF has been shown to alter the fungal phenotype and the fungal effects on plant growth, it is potentially useful that, from one single spore, several new genetic and phenotypic variants can be produced.

Conclusions

We conclude that surprising amounts of genetic and phenotypic variation occur successively from a single AMF spore through clonal growth. This is an unexpected amount of variation to be derived from a clonal organism, and for which we see no other parallels in nature. Despite the latest findings on the *Glomus* genome, the differences arising at each generation are difficult to explain without considering the heterokaryosis hypothesis. This highlights the need to fill the unknown gaps in the *Glomus* genome, as well as to obtain a better understanding of gene variation in unassembled complex parts of the genome. Our results have important consequences as they show that, depending on the cultivation method, the genetic make-up of the fungus can be altered significantly. Future research should be aimed at obtaining an understanding of how to maintain the genetic makeup of novel AMF lines and investigating the exciting prospect of how the segregation of genetic diversity can be used effectively to improve the symbiotic properties of AMF.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Phenotypic measurements of the single spore lines over two successive generations.

Fig. S2 Relative allele frequencies of the Bg112 locus over three generations of single spore lines.

Table S1 Mean ± 1SE of the relative fluorescence used to compare the relative frequency of the different alleles shown in Fig. 2

Table S2 Percentage of the variance explained by the different factors of random one-way univariate ANOVA (using REML) on the two principal components encompassing the relative allele frequencies of the four alleles of isolate C3 at the locus Bg112

Table S3 Results of ANOVAs on the two principal components of the allele frequencies

Table S4 Results of one-way ANOVAs on the two principal components of the allele frequencies at the G3 generation, performed separately on each set of single spore lines (SSLs) that share the same G2 parent

Table S5 Results of ANOVA on the phenotypic traits

Notes S1 Establishment of three generations of single spore lines (SSLs).

Notes S2 Testing the reproducibility of allele frequency measurements at the Bg112 locus.

Notes S3 Phenotypic measurements and analysis.

Notes S4 Molecular analysis: principal component analysis, correlations between pairs of alleles and Mantel tests.

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