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Correspondence and requests for materials should be addressed to R.A. (e-mail: raphael.arlettaz@nat.unibe.ch).

Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi

Gerrit Kuhn*†, Mohamed Hijri*† & Ian R. Sanders*

* *Institute of Ecology, University of Lausanne, Biology Building, 1015 Lausanne, Switzerland*
† *These authors contributed equally to this work*

Ancient asexuals directly contradict the evolutionary theories that explain why organisms should evolve a sexual life history[1,2]. The mutualistic, arbuscular mycorrhizal fungi are thought to have been asexual for approximately 400 million years[3,4]. In the absence of sex, highly divergent descendants of formerly allelic nucleotide sequences are thought to evolve in a genome[2]. In mycorrhizal fungi, where individual offspring receive hundreds of nuclei from the parent, it has been hypothesized that a population of genetically different nuclei should evolve within one individual[5,6]. Here we use DNA–DNA fluorescent *in situ* hybridization to show that genetically different nuclei co-exist in individual arbuscular mycorrhizal fungi. We also show that the population genetics techniques[4] used in other organisms are unsuitable for detecting recombination because the assumptions and underlying processes do not fit the fungal genomic structure shown here. Instead we used a phylogenetic approach to show that the within-individual genetic variation that occurs in arbuscular mycorrhizal fungi probably evolved through accumulation of mutations in an essentially clonal genome, with some infrequent recombination events. We conclude that mycorrhizal fungi have evolved to be multi-genomic.

Arbuscular mycorrhizal fungi (Class Zygomycetes; Order Glomales) are extremely successful fungi that form mutualistic symbioses with the roots of approximately 60% of all plant species[7]. They improve plant nutrition and promote plant diversity[8]. These fungi have been assumed to be asexual[7]. This is supported by measurements of the degree of linkage disequilibrium, which indicated that genetic variation among the spores of arbuscular mycorrhizal fungi deviates significantly from that expected from a recombinant population[4]. Genetic diversity in the ribosomal DNA occurs inside individual spores[9–12], even though it is thought that several copies of rDNA are kept the same by concerted evolution[13]. It has been hypothesized that by accumulation of mutations, in the absence of recombination, individual arbuscular mycorrhizal fungi have evolved to comprise genetically divergent nuclei, or that one individual contains several genomes[5]. Here we refer to an arbuscular mycorrhizal fungal spore as an individual.

We tested the hypothesis that individuals contain genetically different nuclei by performing specific fluorescent DNA–DNA *in situ* hybridization (FISH) on nuclei from spores of the arbuscular mycorrhizal fungus *Scutellospora castanea* (BEG 1). We used hybridization probes that specifically recognize two divergent sequences of the ITS2 region, known as T2 and T4, that were previously shown to co-occur within individual spores of this fungus[14]. Probes were only used for variant ITS2 sequences that had previously been shown to

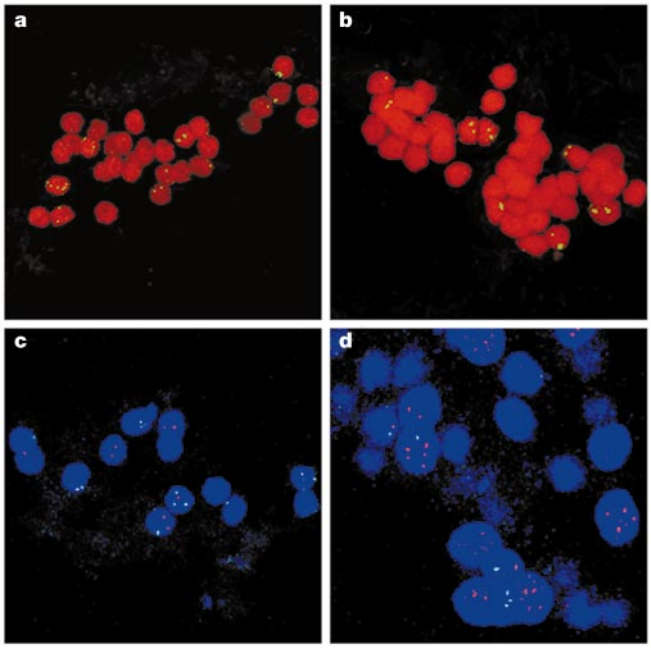

Figure 1 Nuclei of *Scutellospora castanea* taken with scanning laser confocal microscopy after single-target and double-target DNA–DNA FISH. **a**, Hybridization signals (green) of the probe T2-DIG to nuclei (red). **b**, Hybridization signals (green) of the probe T4-DIG to nuclei (red). **c**, Hybridization signals of the probes T2-DIG (light blue) and T4-biotin (red) to nuclei (purple). **d**, Hybridization signals of the probes T2-biotin (red) and T4-DIG (light blue) to nuclei (purple). The colours of images **c** and **d** have been adjusted to give better contrast between the colour of the two probes and the nuclei.

Table 1 DNA–DNA FISH on *S. castanea* nuclei

Treatments	Number of nuclei observed	% nuclei hybridizing with T2	% nuclei hybridizing with T4	% nuclei hybridizing with T2 and T4	% nuclei unlabelled
Single FISH with T2 (digoxigenin)	1,662	40.26 (3.04)	—	—	59.74 (3.04)
Single FISH with T4 (digoxigenin)	3,405	—	17.03 (2.77)	—	82.97 (2.77)
Double FISH with T2 (biotin) and T4 (digoxigenin)	1,120	40.16 (2.87)	6.52 (1.04)	8.20 (2.30)	45.12 (3.46)
Double FISH with T2 (digoxigenin) and T4 (biotin)	1,364	41.02 (3.38)	9.64 (2.48)	9.63 (2.39)	39.71 (4.16)

Numbers in parentheses represent ± 1 s.e.m. Five spores were crushed on each slide and the number of sides observed were 34, 43, 28 and 23 for the treatments single-target FISH (T2-DIG), single-target FISH (T4-DIG), double-target FISH (T2-biotin and T4-DIG) and double-target FISH (T2-DIG and T4-biotin), respectively. Proportions of nuclei that were labelled with T2 or T4 did not differ from the presented results in experiments with a smaller sample size, where one spore was placed on each side. Student's *t*-tests showed significant differences between the percentage of nuclei labelled with T2 and T4 in single-target FISH ($t_{1.75} = 30.31, P \leq 0.0001$) and between T2 and T4 in both double-target FISH experiments ($t_{1.44} = 121.76, P \leq 0.001$ and $t_{1.54} = 56.05, P \leq 0.0001$). The *t*-tests revealed no significant differences in the percentage of nuclei hybridizing to either T2 or to T4 as a result of different labelling and also showed no significant differences in hybridization percentage with a given probe in single-target FISH experiments compared to double-target FISH experiments.

be of glomalean origin[15]. Single-target FISH showed that significantly more nuclei in *S. castanea* spores contained sequence T2 (40%) than T4 (17%) (Fig. 1a, b; Table 1). Double-target FISH using T2 and T4 probes showed that the divergent sequences T2 and T4 were indeed segregated in different frequencies among the nuclei (Fig. 1c, d; Table 1). Approximately 40% of nuclei contained only the T2 sequence and between 6 and 9% of nuclei contained only the T4 sequence. T2 and T4 co-occurred in between 8 and 9% of nuclei (Fig. 1c, d; Table 1). These results support the hypothesis that arbuscular mycorrhizal fungal spores contain a population of genetically different nuclei.

We need to know whether this genetic variation has been brought about by lack of recombination. Because several genomes exist within individuals, recombination could potentially occur among nuclei within individuals and this has not previously been considered. Using a theoretical approach we assessed whether the prediction from previous population genetic studies that mycorrhizal fungi are clonal (based on fingerprinting and calculations of index of association[4]) is valid. We constructed artificial data sets representing presence or absence of alleles at 15 loci for a population of recombining nuclei within 30 spores. We then calculated the total number of nuclei containing an allele at each of the loci, for each spore. However, a fingerprinting technique performed on whole spores cannot take into account within-individual polymorphism (Fig. 2a). Therefore, whether the presence or absence of an allele at a given locus would be observed for each spore would depend on the number of nuclei in which that allele is present and the sensitivity of the fingerprinting technique (Fig. 2b). We therefore calculated the presence of alleles at each locus for each spore, simulating different levels of sensitivity of the fingerprinting technique. We then calculated the index of association[16] for the population spores. All observed values of the index of association deviated significantly from zero (Fig. 3), allowing us to reject the null hypothesis that the population of hypothetical arbuscular mycorrhizal fungal spores were a recombining population. We concluded that any potential recombination was hidden within individuals and was not detectable using this technique.

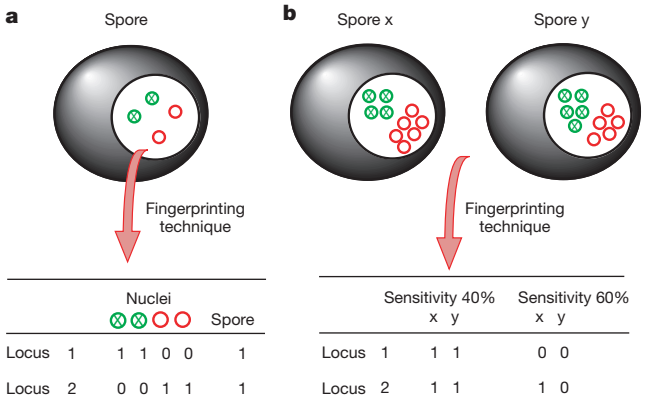

Figure 2 Within- and among-spore polymorphism. **a**, A spore of a hypothetical arbuscular mycorrhizal fungus contains a population of two genetically different nuclei (crossed and open circles). Within-individual polymorphism caused by differences among nuclei is masked in a fingerprinting method on the whole spore because the presence of both alleles is detected. **b**, Two hypothetical spores of an arbuscular mycorrhizal fungus, x and y, contain a population of two genetically different nuclei that are present in different frequencies. At a sensitivity of fingerprinting of 40%, where at least four nuclei carrying a given allele are required to show its presence in the spore, spores x and y appear to be genetically identical. At sensitivity 60%, where six nuclei are required to show the presence of an allele, spores x and y appear to be genetically different. Thus, spores can appear to be polymorphic or identical, depending on the fingerprinting sensitivity. Binary matrices show presence of alleles (1) or absence (0) during detection using a fingerprinting method at two different sensitivities.

Thus, the genomic structure of arbuscular mycorrhizal fungi gives rise to difficulties in detecting recombination with techniques that are based on linkage disequilibrium. To solve this problem, we used a phylogenetic technique known as character incompatibility analysis[17] to detect whether genetically divergent nuclei in these fungi are likely to have arisen by the accumulation of mutations in clonal nuclear lineages or by recombination events. We looked at sequence variation in ITS regions (including the 5.8S gene) within isolates of the arbuscular mycorrhizal fungi *Glomus geosporum*, *Glomus mosseae* and *Gigaspora margarita* and in the 28S gene in *G. geosporum*, *Glomus coronatum*, *Glomus constrictum* and *G. mosseae*[18] (Table 2). Calculation of the Le Quesne probability[17] showed that for a large proportion of the variable characters the incompatibility count differed significantly from that which would be expected if it had arisen from recombination. A small proportion of the variation in rDNA sequences could be explained by recombination events, although this was higher in *Gi. margarita* than for the other fungi. Using a jacknife procedure we showed that only a small proportion of the sequences contributed nearly all the variation that is explained as being the result of recombination events (Table 2). This was also true for the variation seen in *Gi. margarita*. The results show, therefore, that most of the variant sequences are the result of accumulation of mutations in a clonal genome.

Until now, reports of within-individual sequence variation in arbuscular mycorrhizal fungi have been restricted to rDNA sequences. Our results demonstrate genetic differences only among nuclei for one region of rDNA. Therefore, we also provide evidence to support genetic differences among fungal nuclei, clonality and Muller's ratchet. We analysed the sequence of part of a gene encoding for a binding protein (*BiP* gene) that has high amino-acid sequence similarity (86% and 78%) to that of *Aspergillus niger* and *Saccharomyces cerevisiae*, respectively[19]. This gene is highly conserved in eukaryotes and is a single-copy gene in other fungi. We sequenced 15 variant sequences of this gene from genomic DNA of one isolate of *Glomus intraradices*. Along a length

Table 2 Incompatibility analysis on the variation in rDNA sequences

Species	Number of variable sequences	Number of clonal characters (% of variant characters)	Number of recombinant characters (% of variant characters)	MIC 50‡	MIC 10‡
Analysis performed on variable ITS sequences (comprising ITS1, 5.8S gene and ITS2)					
*G. geosporum**	26	50 (83%)	10 (17%)	3%	7%
G. geosporum†	26	17 (85%)	3 (15%)	—	—
*G. mosseae**	22	102 (68%)	47 (32%)	13%	54%
G. mosseae†	22	47 (70%)	20 (30%)	—	—
*Gi. margarita**	18	53 (52%)	48 (48%)	11%	33%
Gi. margarita†	18	10 (31%)	22 (69%)	—	—
Analysis performed on variable sequence of 28S gene					
*G. geosporum**	15	122 (92%)	10 (8%)	13%	26%
*G. coronatum**	19	80 (86%)	13 (14%)	15%	21%
*G. constrictum**	12	21 (51%)	20 (49%)	8%	8%
*G. mosseae**	17	57 (90%)	5 (8%)	5%	10%

*The analysis was performed with insertions or deletions in the data set representing a fifth character.
†The analysis was performed on sequences that varied only due to substitution.
‡MIC 50 and MIC 10 are defined as the percentage of sequence variants that need to be removed to reduce the matrix incompatibility to less than 50% and less than 10% of the total matrix incompatibility in the data set. This is performed by sequentially eliminating the sequences that contribute the most to the total matrix incompatibility and then recalculating the total incompatibility contained in the remaining data set.

of 680 base pairs (bp), these variants ranged in similarity at the nucleotide level from 92% to 99% from one of the sequences that was randomly chosen as the comparison sequence (see the Supplementary Information). If this gene is also single-copy in arbuscular mycorrhizal fungi then the sequence variants that we observed must be segregated among nuclei. Incompatibility analysis indicated that no variation in these sequences was likely to be due to recombination. Furthermore, both synonymous and non-synonymous substitutions occurred in the sequences; that is, not all of the substitutions are selectively neutral. The mean number of substitutions compared to the comparison sequence was 26.6 (range 3 to 48) with a mean ratio of 1.02 (s.e.m. ± 0.10) synonymous to one non-synonymous substitution. We would expect a higher rate of synonymous substitutions to non-synonymous substitutions unless Muller's ratchet is in operation, because selection should act to conserve the sequence at non-synonymous sites. These data are consistent with evidence for Muller's ratchet in genomes of endosymbiotic prokaryotes[20].

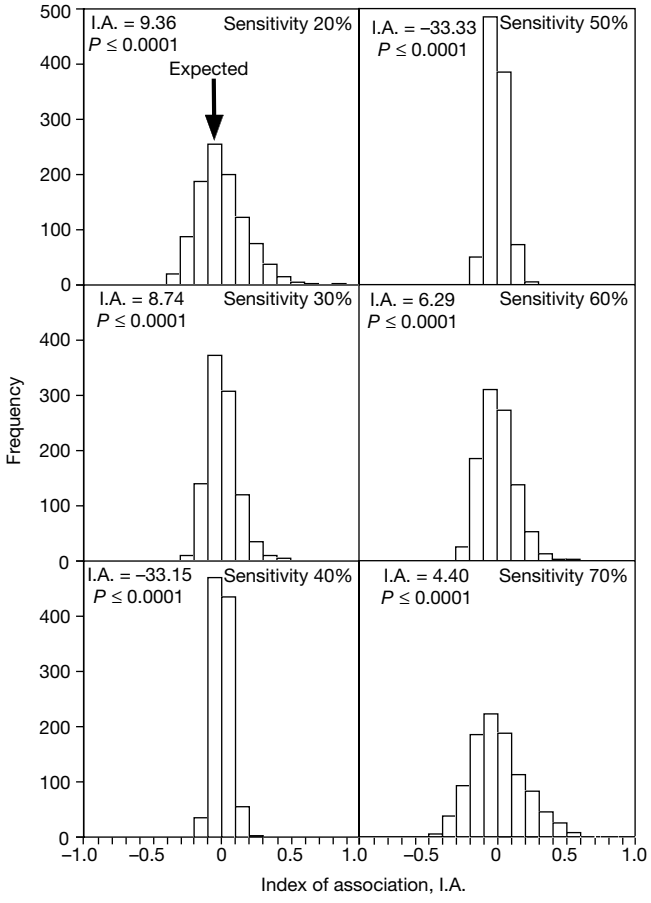

Figure 3 Frequency distributions for the index of association for a population of 30 hypothetical spores of an arbuscular mycorrhizal fungus. Each spore contains a population of recombining nuclei, calculated at 20% to 70% sensitivities of the fingerprinting. Data calculated with 80% sensitivity did not give a normal frequency distribution and is not shown. Each graph also shows the observed I.A. for the data set: in each case this differed significantly from the expected I.A.

From our results, we predict that genetic variation is generated by accumulation of mutations in a predominantly clonal genome, leading to the creation of a population of genetically different nuclei. The evidence for (infrequent) recombination events is unsurprising in an organism that contains genetically different nuclei that co-exist coenocytically. However, our analyses indicate that these recombination events are rare and do not purge the majority of mutations in the genomes. Many other fungi have stages of heterokaryosis, where more than one nuclear genotype coexists, although genetic bottlenecks occur in the life cycle of each individual at each generation, limiting the number of nuclei that are transferred to the next generation to one per individual. No such stage in the life history of arbuscular mycorrhizal fungi is known and new spores receive many nuclei from the mother hyphae. Spores may also receive genetic material by fusion of hyphae[21,22], although the studies suggest that cross-incompatibility is most frequent. We suggest, therefore, that such a mechanism would not reduce the potential problems associated with Muller's ratchet as the nuclei received would also be accumulating mutations. Most theories of evolutionary and population genetics assume that one individual contains a single genome and so new models for evolution of multi-genomic organisms need to be developed. Our results may stimulate research on mycorrhizal symbiosis at the molecular and physiological levels, because several variants of the same gene exist in an individual and could lend to variation in expression. Our results provide essential information that is needed before any studies on genomics or proteomics of arbuscular mycorrhizal fungi are undertaken. □

Methods

Slot-blot and DNA–DNA FISH

Hybridization probes were constructed for two different sequences of the ITS2 region, subsequently referred to as T2 and T4, that were known to be variable in *S. castanea* (GenBank accession numbers SCAJ2872 and SCAJ2874)[14,15]. Probes were produced from cloned DNA that was amplified with the forward primer (5′-CACCTGCTTGAGGGT-

CAGT-3′) and the reverse primer ITS4 (ref. 23). The size of the probes were 274 bp and 258 bp for T2 and T4, respectively. Probes were labelled by polymerase chain reaction (PCR) with DIG-11-dUTP and Biotin-16-dUTP (Roche) and purified with QiaQuick PCR purification kit (Qiagen).

Spores of *S. castanea*, *G. geosporum* (BEG 18) and *Glomus* sp. (BEG 19) were collected from pot cultures and immediately fixed (in 4% formaldehyde, 100 mM Tris/HCl, pH 8, 100 mM NaCl, 2 mM $MgCl_2$ and 0.05% Triton X100, for 2 h at room temperature). Five spores or single spores were crushed on Frost Plus slides (Polylabo) and then dried overnight. For single-target FISH, 25 μl of the hybridization solution was loaded per slide. For double-target FISH, a mixture of digoxigenin and biotin probes in a 1:1 ratio was added to the hybridization solution. Hybridization was carried out at 37 °C overnight. The post-hybridization washes were made twice with 50% formamide in double-strength SSC (42 °C, 10 min), then twice with double-strength SSC, (37 °C, 5 min) and then rinsed again twice with half-strength SSC with 0.1% SDS (60 °C, 15 min). The post-hybridization washes were identical to those used for the slot-blot procedure. For full details regarding slot-blot conditions and slide preparation for FISH and all controls see the Supplementary Information.

For signal detection in single-target FISH, slides were incubated with anti-DIG-fluorescein conjugate antibody (Roche) and counterstained with propidium iodide. In double-target FISH experiments, signals were detected with a mixture of anti-DIG-fluorescein conjugate antibody (Roche) and Streptavidin Texas-Red conjugate. The slides were counterstained with TOTO-3 (molecular Probes). Slides were examined using a scanning confocal microscope (further details are given on signal detection and microscopy in the Supplementary Information).

Testing for detection of recombination

Thirty identical data sets were constructed, representing 30 spores of arbuscular mycorrhizal fungi, each containing a population of 50 genetically different nuclei that were variable at 15 loci. The nuclei within each spore were then recombined by the random rearrangement of alleles among nuclei, assuming that loci were independent of each other. The frequency of genetically different nuclei occurring in each spore was then altered by randomly selecting 10 nuclear genotypes that were then replicated a random number of times in the spore, with a maximum limit of 10 replicates of a given nuclear genotype per spore. The number of nuclei showing the presence of an allele at each locus was summed for each spore. The presence or absence of an allele at each locus was then calculated for each whole spore using different sensitivities of the fingerprinting method to give binary data sets. Sensitivity was defined by the percentage of nuclei containing a given allele that was required to give a positive signal with a fingerprinting technique. The sensitivities ranged from 100% (where the presence of an allele in 1 nucleus in a spore was sufficient to give a positive signal with a fingerprinting technique) through to 0% (where even if every nucleus contained the allele the technique would not be sensitive enough to detect presence of the allele). Polymorphism among the 30 spores at the 15 loci was calculated for data sets at 20%, 30%, 40%, 50%, 60%, 70% and 80% sensitivities. Sensitivity was calculated on a percentage basis rather than actual numbers of nuclei to account for differences in the numbers of nuclei among spores. Each of these seven data sets were then separately used to calculate the index of association[16].

Incompatibility analysis on rDNA and *BiP* gene sequences

Incompatibility analysis was performed on sequences of ITS and 28S rDNA from different arbuscular mycorrhizal fungi species. ITS sequences of *G. geosporum* (BEG 18) were obtained by extracting genomic DNA from spores (Qiagen DNeasy Plant Mini Kit) from a culture that originated from a single spore and amplified by PCR[9]. The 640-bp product containing ITS1, the 5.8S gene and ITS2 was purified (Qiagen QiaQuick purification kit), ligated into a pGEM-T vector and transformed into *Escherichia coli* JM109 (Promega). Clones were selected at random and both strands were sequenced using a dye-terminator cycle sequencing kit. Sequences were carefully edited by hand. ITS sequences from *G. geosporum* (BEG 18) and from *G. mosseae*[24] and *G. margarita*[24] were aligned. Sequences of the 28S gene for the species *G. geosporum* (BEG 11)[18], *G. coronatum* (BEG 49)[18], *G. constrictum* (BEG 130)[18] and *G. mosseae* (BEG 25)[18] were also aligned and used for the analysis. All isolates originated from single spores except *G. coronatum* and *G. constrictum*. Further details on the compatibility analysis on rDNA and the gene encoding a binding protein are given in the Supplementary Information.

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Correspondence and requests for materials should be addressed to I.R.S. (e-mail: Ian.Sanders@ie-bsg.unil.ch).

Transmission potential of smallpox in contemporary populations

Raymond Gani & Steve Leach

Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, UK

Despite eradication[1], smallpox still presents a risk to public health whilst laboratory stocks of virus remain[2,3]. One factor crucial to any assessment of this risk is R_0, the average number of secondary cases infected by each primary case. However, recently applied estimates have varied too widely (R_0 from 1.5 to >20) to be of practical use, and often appear to disregard contingent factors such as socio-economic conditions and herd immunity[4–8]. Here we use epidemic modelling[9] to show a more consistent derivation of R_0. In isolated pre-twentieth century populations[10–12] with negligible herd immunity, the numbers of cases initially rose exponentially, with an R_0 between 3.5 and 6. Before outbreak controls were applied, smallpox also demonstrated similar levels of transmission in 30 sporadic outbreaks in twentieth century Europe[1], taking into account pre-existing vaccination levels[13,14] (about 50%) and the role of hospitals in doubling early transmission. Should smallpox recur, such estimates of transmission potential (R_0