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Genetic processes in arbuscular mycorrhizal fungi

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Abstract

Arbuscular mycorrhizal (AM) fungi (Glomeromycota) colonize roots of the majority of land plants and facilitate their mineral nutrient uptake. Consequently, AM fungi play an important role in terrestrial ecosystems and are becoming a component of sustainable land management practices. The absence of sexual reproductive structures in modern Glomeromycota combined with their long evolutionary history suggest that these fungi may represent an ancient asexual lineage of great potential interest to evolutionary biology. However, many aspects of basic AM fungal biology, including genome structure, within-individual genetic variation, and reproductive mode are poorly understood. These knowledge gaps hinder research on the mechanisms of AM fungal interactions with individual plants and plant communities, and utilization of AM fungi in agricultural practices. I present here the current state of research on the reproduction in AM fungi and indicate what new findings can be expected in the future.

Keywords: Arbuscular mycorrhizal fungi; Genetic makeup; Glomeromycota; Homokaryosis; Population structure; Reproductive mode; Vegetative incompatibility

1. Introduction

Arbuscular mycorrhizal (AM) fungi (phylum Glomeromycota) colonize roots of the majority of land plants, facilitate plant mineral nutrient uptake [1], and suppress plant diseases [2]. They account for up to 50% of the total soil microbial biomass [3] and contribute to the creation and maintenance of soil aggregate structure [4]. Because of these characteristics, AM fungi are perceived as one of the most important components of a paradigm shift from conventional to sustainable land management practices [5]. Yet despite the obvious significance of AM fungi in ecosystem functioning, we lack the basic understanding of the genetic processes involved in their reproduction. The main reason for this deficiency is AM fungal obligate biotrophy and recalcitrance to axenic cultivation [6]. As a result, virtually no genetic tools are available to study the mechanisms involved in the recognition of compatible hosts, the root penetration process or the nutrient exchange with the host. Furthermore, the dynamics and regulation of the population-level interactions with plant communities are unknown.

Glomeromycota are one of the oldest terrestrial organisms. Their fossil record dates back to the Ordovician (460 million years ago) [7], and the molecular clock-based inferences place the origin of Glomeromycota between 1200 and 1400 mya [8]. Given that fossil evidence for sexual reproduction in ancestral AM fungi is lacking and that modern AM fungi seem asexual, it has been suggested that they may represent one of the oldest groups of clonally reproducing eukaryotic organisms on Earth [9]. If the extant AM fungi are indeed clonal and have been clonal throughout their evolutionary history, their existence

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would be a challenge to the evolutionary dogma that clonality leads to extinction due to the inability to offload deleterious mutations from a population [10].

The reproductive mode of AM fungi is directly related to organization of genetic variation in these organisms and both questions are actively studied and debated. Asexual spores formed by AM fungi contain hundreds of nuclei and harbor uncommonly polymorphic sequences of rRNA coding genes, which confounds genetic makeup of these propagules. In other organisms, tandemly repeated copies of rRNA genes present in each nucleus are subject to a sequence homogenization process known as concerted evolution [11]. Unequal crossing-over and gene conversion in somatic nuclei (mitotic recombination) and during meiosis are the mechanisms responsible for concerted evolution of rRNA genes within and among individuals of a recombining species. The rare instances of the within-individual rDNA polymorphism include rRNA pseudogenes existing alongside functional rRNA gene copies [12], or parental rRNA types carried by hybrid genomes [13]. Interestingly, putative asexual species seem to have a propensity for within-individual rDNA variation [14,15]. Because of the multinucleate structure of AM fungal spores, the source of the intrasporal rDNA polymorphism is unclear. The entire intrasporal rRNA variation could be either contained in every single nucleus (homokaryosis), or distributed among different nuclei (heterokaryosis).

In this review, I will summarize the current state of research on the genome and genetic processes involved in the reproduction of AM fungi and point out the important unresolved questions.

2. Sexual and parasexual genetic recombination

Except for one instance [16], sexual reproductive structures have not been reported in AM fungi. However, in many other fungi that lack morphological evidence of sexual exchanges, genetic recombination has been detected in nature through the analysis of their population structure using methods of population genetics and phylogenetics [17]. It is unclear whether the signatures of recombination observed in natural populations of seemingly asexual fungi are consequences of cryptic but conventional sexual exchanges of syngamy and meiosis, or whether they are products of the illegitimate hyphal fusions of a parasexual cycle.

Parasexual recombination has been studied in many asco- and basidiomycetous fungi under laboratory conditions [18], and it has been postulated to occur in nature in AM fungi [19]. In the laboratory, parasexual recombination in asco- and basidiomycetes is a product of fusions of hyphae or protoplasts that are forced between genetically distinct mutant individuals by cocultivation under selective conditions requiring complementation of mutant phenotypes to restore their ability to grow. Such fusions result in a temporary coexistence of genetically different nuclei within a common cytoplasm (heterokaryosis) and eventually lead to karyogamy. Once diploid heterozygous nuclei are formed, they proliferate mitotically, which enables recombination between nonsister chromatids of homologous chromosomes. Sister chromatid non-disjunctions, also occurring during nuclear divisions, lead initially to aneuploidy, and eventually to haploidization yielding recombinant segregants. Under laboratory conditions, chemicals that disrupt the mitotic spindle are often used to speed up the process of haploidization.

Although the rate of mitotic crossovers per nucleus during the parasexual process is up to three-fold lower than that of meiotic crossovers, the creation and coexistence of heterokaryons, heterozygotes, and various euploid and aneuploid segregants may be an effective way to reassort and maintain genetic variation [20]. Pontecorvo [20], a pioneer of parasexual genetics, proposed that in wild populations of asexual fungi, genetic and evolutionary advantages of parasexuality might be comparable to those derived by sexual fungi from syngamy and meiosis. Yet, half a century of pursuit for evidence to support this hypothesis yielded surprisingly few examples of naturally occurring parasexuality with the majority of cases representing somatic interspecific hybridization events [21]. The reason may be that most fungi, like many other organisms, possess mechanisms that prevent conspecific fusions among genetically differentiated individuals outside the brief periods when these individuals are engaged in sexual interactions [22,23].

3. Vegetative incompatibility

Although fusions between genetically differentiated individuals are a hallmark of sexual reproduction, genetically distinct mycelia in a non-reproductive mode do not anastomose readily because of vegetative (somatic) incompatibility barriers. The existence of these barriers has been explained as an adaptation that hinders transmission of infectious cytoplasmic elements [24], or eliminates parasitic nuclei [25]. In filamentous ascomycetes, haploid vegetative mycelia fuse successfully only if they carry identical alleles at each of several loci defining an allelic heterokaryon incompatibility system and, in some species, compatible alleles at different loci constituting a non-allelic heterokaryon incompatibility system [23]. Otherwise, heterokaryotic cells arising during the initial interaction between two vegetatively incompatible individuals are isolated from the remaining mycelium by an occlusion of septal pores, their content is rapidly degraded, and the continuity between the interacting mycelia is disrupted. In basidiomycetes, contacts be-

tween primary homokaryotic mycelia are governed by sexual compatibility mechanisms that require different alleles at mating-type loci to form a dikaryon [22]. Encounters between secondary dikaryotic mycelia are controlled by somatic incompatibility systems responsible for the antagonistic interactions if the participants are genetically dissimilar.

Glomeromycota do not appear to differ from other fungi in their ability for self versus non-self recognition [26,27]. Hyphal fusions occur readily within individual mycelia or mycelia of individuals from the same isolate [28–30]. Yet, they are prevented between genetically different individuals [26]. For example, hyphae of *Glomus mosseae* isolates from different geographic locations home towards each other, which indicates their ability to recognize individuals of the same species. However, retraction of cytoplasm from interacting hyphal tips and formation of cross-walls before or during hyphal contact precludes formation of a heterokaryotic mycelium [26].

4. Are AM fungi clonal or recombining?

Thus far, three studies have been conducted with the use of population genetic and phylogenetic methods to detect recombination or clonality in AM fungi but they yielded rather inconclusive results [19,31,32]. Taylor and Rosendahl [31] analyzed AFLP (amplified fragment length polymorphism) marker variation in soil-borne populations of individual spores of *Glomus caledonium* and *G. mosseae*. For each population, they calculated: (1) the index of association, i.e. the variance of similarities for all pair-wise combinations of multilocus genotypes and (2) the length of a parsimony tree constructed by treating AFLP loci as phylogenetic characters and alleles as character states. A comparison of the observed parameters with those expected in a freely recombining population indicated that the populations of both species were clonal. Although this is not an unexpected outcome, it should be considered with caution. The AFLP technique can amplify DNA from other organisms that are associated with soil-borne glomalean spores in addition to the markers specific to AM fungi thus confounding the results. AM fungal spores are known to harbor endosymbiotic bacteria [33], as well as chytridiomycetous and ascomycetous associates [34]. In fact, the latter were shown [35,36] to be responsible for data misinterpretation in analyses of genetic markers derived from AM fungi in other studies.

A multi-locus fingerprinting of ISSR (inter simple sequence repeat) markers was used by Vandenkoornhuyse et al. [19] to analyze population structure in *Glomus claroideum* and *Glomus* DAOM225952. For each of the two species, the authors sampled three populations representing three different environments; each sample contained 100 soil-borne spores. In both taxa, the observed values of the index of association indicated recombination in two of the three analyzed populations. The authors speculated that either sexual or parasexual genetic exchanges were responsible for this outcome. However, spore-associated microorganisms or within-individual exchanges among the multiple copies of rRNA coding genes could be other likely sources of the observed signatures of recombination. The latter possibility deserves serious consideration because recombination among rDNA repeats in AM fungi has been inferred in several studies [27,37,38], and its occurrence would not be inconsistent with an exclusively clonal reproductive mode.

Recently, Stuckenbrock and Rosendahl [32] revisited the problem of a reproductive mode in populations of *G. caledonium* and *G. mosseae*. Using a combination of multiplex and nested PCR, they retrieved from individual spores fragments of the LSU (large subunit) rRNA gene and of two protein-coding genes (*FOX2*, *TOR2*) that contain highly variable introns. The spores were genotyped by SSCP (single strand conformation polymorphism) electrophoresis and the identity of SSCP alleles was verified by sequencing. This approach eliminated the uncertainty about marker homology that could plague AFLP and ISSR approaches described earlier, where DNA fragment sizes rather than sequences were used as indicators of marker homology. All tandemly repeated rRNA gene copies in a spore were represented by a single SSCP pattern, which simplified the analysis by eliminating any potential signature of mitotic recombination. Calculation of the index of association for the recovered genotypes revealed a significant association among the SSCP alleles in both populations, which is a strong indication of a clonal population structure. However, it could also signify a genetic linkage among the investigated loci in an otherwise recombining population. Increasing the number of loci analyzed should resolve this dilemma in the future.

5. Are the Glomeromycota a non-Mendelian genetic system?

Population genetics tools used to examine the reproductive mode of Glomeromycota rely on the assumption that AM fungal individuals contain genetically homogeneous populations of nuclei, i.e. the entire within-individual genetic variation is contained in every nucleus. This assumption is well supported by several lines of evidence, including: (1) PCR amplifications of several polymorphic rDNA sequences from individually micro-dissected nuclei of *Glomus etunicatum* and *Glomus intraradices* [27], (2) patterns of within- and among-isolate variation of rDNA and a highly variable genetic marker PLS1 (*POL1*-like sequence) in *G. etunicatum*

[27], and (3) the absence of within-individual genetic variation in single copy markers (*GmFOX2*, *GmTOR2*, *GmGIN1*) that are variable among the isolates of *G. mosseae* [39].

Contrary to the data supporting genetic homogeneity of AM fungal individuals, a novel non-Mendelian genetic system has been postulated to exist in Glomeromycota [37,40]. Rather than relying on meiotic reassortment of genetic traits brought together during sexual syngamy, AM fungi are hypothesized to reassort entire haploid nuclei and, as a consequence, maintain diverse nuclear populations in their mycelia and spores. Conceptually, this process resembles early events of the parasexual cycle, where genetically dissimilar vegetative individuals fuse and combine their nuclei in a common mycelium. However, instead of giving rise to recombinant nuclei, the interacting nuclei are expected to remain genetically unaltered except for occasional mutations. Like parasexuality, the nuclear reassortment process requires fusions among genetically differentiated individuals to create and maintain their heterokaryotic structure [41,42]. Yet, it is unlikely that such fusions occur in nature because vegetative mycelia of Glomeromycota appear to be under a strong selection to avoid them [26] as described earlier. Furthermore, current support for the operation of the nuclear reassortment process relies on inferences of a static condition of nuclear diversity in individual spores of AM fungi and involves data that may also be explained in other ways without invoking heterokaryosis. For example, polymorphism of RAPD (random amplified polymorphic DNA) fingerprints among soil-borne spores in single-spore isolates of *Gigaspora margarita* [43], which was interpreted as evidence of heterokaryosis, could well be caused by non-specific amplification of DNA from spore-associated microorganisms. Such contaminations were documented [35,36] in other experiments where internuclear marker polymorphism was claimed to reflect heterokaryosis [44–46].

Heterokaryosis was also proposed as an explanation for heritability of different shapes and sizes of spores in several isolates of a morphological species *Scutellospora pellucida* [41]. Ratios of nuclei carrying different genetic determinants of spore shape or size were postulated to determine spore phenotype, e.g. "spores with predominance of nuclei with genes for round spores would be expected to produce a majority of round spores and a few narrow spores..." [41]. This speculation was not accompanied by evidence that spores of intermediate phenotypes can be regenerated by fusions of hyphae derived from spores with opposite sizes or shapes. Furthermore, evidence was lacking that the morphologically divergent isolates in fact represented the same phylogenetic lineage. AM fungal taxonomy is largely based on morphological characters, and it is unclear whether and to what extent the morphologically defined species correspond to phylogenetically distinct lineages.

Other evidence for heterokaryosis came from the heterogeneity in hybridization patterns of rDNA probes among nuclei in FISH (fluorescent in situ hybridization) experiments with spores of *G. intraradices*, *G. mosseae*, *Gigaspora rosea* [47] and *Scutellospora castanea* [37]. However, as any nucleic acid denaturation-based technique, FISH experiments have inherent limitations caused by the effect of target DNA conformation on its accessibility to the probes and consequently on the probe signal intensity [48]. Extensive empirical studies on in situ hybridization of fluorescent rRNA probes in bacteria demonstrated that controlling for target accessibility is intrinsically difficult, and controls that involve probe hybridization to denatured targets in blot hybridization experiments are poor predictors of probe behavior during in situ hybridization [49]. Consequently, until better controls are available, the FISH results indicating heterokaryosis in AM fungi should be considered with caution.

More recently, an inference of a low number of copies per genome of a genetic marker known as PLS1 [27] was offered as evidence of heterokaryosis in *G. etunicatum* [40]. The intrasporal polymorphism of PLS1 has been documented in several single-spore isolates of *G. etunicatum* with each spore containing 13 variants of the marker [27]. However, the genome structure of *G. etunicatum* and the physical organization of PLS1 variants are not known and open to speculation. Consequently, evidence that different PLS1 variants are single-copy sequences distributed among different nuclei would support heterokaryosis. The inference of PLS1 variant copy number per nucleus was based on a genome size estimate in *G. etunicatum* by flow cytometry and a PLS1 template copy number reconstruction by quantitative PCR [40]. Surprisingly, the size of *G. etunicatum* genome estimated in this study was significantly smaller than earlier genome size estimates in other species of *Glomus* obtained using a variety of methods [50,51]. This discrepancy will have to be resolved before conclusive estimates of gene copy number can be made. In addition, the conclusion that only one or few copies of PLS1 are present in each nucleus [40] is in disagreement with genetic and phylogenetic analyses of PLS1 variation within and among geographically isolated populations of *G. etunicatum* [27]. Within each isolate, all analyzed spores contained a set of identical PLS1 variants, which would not be expected under heterokaryosis because of the random loss of nuclei due to genetic drift. Furthermore, there was no difference in the number of PLS1 variants among the isolates; each of them harbored a series of 13 PLS1 variants (Fig. 1). Such retention of the same number of variants in spores from geographically isolated populations would be unlikely in a heterokaryotic organism where each variant is ex-

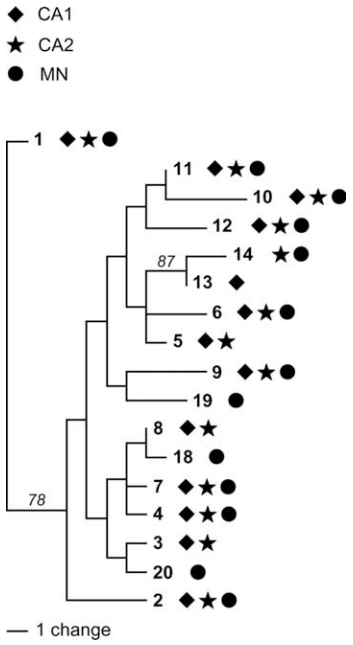

Fig. 1. A single most parsimonious phylogram of the PLS1 variants detected in *G. etunicatum* isolates OT-3-5-4 (CA1), OT-13-4-2, OT-21-1-2, OT-73-3-4 (CA2) from California, and in PE-14-8-557 and PE-14-8-558 isolates from Minnesota (MN). Numbers above branches represent bootstrap support. This figure has been modified from Pawlowska and Taylor [27].

pected to reside in a different nucleus. Molecular divergence patterns among some of the variants from different isolates attest to the fact that these populations were not recent migrants dispersed by human activities but have evolved in geographic isolation.

6. Genome size and structure in AM fungi

Conflicting genome size estimates are one of the sources of uncertainty that hinder the progress of AM fungal genetics. Data obtained by Bianciotto and Bonfante [50] and by Hosny et al. [51] indicated that AM fungal genomes are uncommonly large in comparison to other fungi. Based on these estimates, Pawlowska and Taylor [27] speculated that AM fungal genomes may have a duplicated structure and that genome polyploidization may be a way of avoiding mutational meltdown if AM fungi are in fact clonal. A rising number of monomorphic gene sequences recovered from individuals of *G. etunicatum* (Pawlowska, unpublished data), *G. caledonium*, *G. geosporum*, and *G. mosseae* [39] indicate that the extent of genome duplications in AM fungi may be more limited and only involve specific genomic regions. Recent genome size estimates by Hijri and Sanders [40,52] suggest that AM fungal genomes may be much smaller and more similar in size to other fungal genomes than previously believed. While all the existing estimates may be correct, more experiments are needed to resolve and explain the discrepancies and to elucidate the extent of genome duplications in AM fungi.

7. Somatic mosaicism in Glomeromycota

Although the hypothesis of a persistent multigenomic structure in AM fungi is not well supported, a transient genetic mosaicism resulting from somatic mutations in some of the nuclei would not be unexpected in overall genetically homogeneous individual mycelia. Evolutionary consequences of somatic mutations have been studied extensively in multicellular organisms that engage in both clonal and sexual reproduction [53]. Mathematical models indicate that somatic mutations may be an important source of evolutionary innovation [54,55], and in exclusively clonal organisms, with selection acting at the within-individual level, may facilitate reduction of a population mutation load [56].

Models considering accumulation of deleterious somatic mutations and interplay of selection forces acting at the within- and among-individual level in multicellular clonal organisms generated interesting predictions about the evolution of clonal reproductive structures, including the number of cells that they contain and the extent of genetic similarity among these cells [56,57]. These predictions may be extendable to the number and origin of the nuclei in AM fungal spores. When there is no within-individual selection, i.e. cells with deleterious mutations divide at the same rate as other cells and selection acts only on individuals, unicellular propagules are expected to be favored as the most effective way to reduce the mutation load [57]. Multicellular reproductive structures derived mitotically from one primordial cell would be a close equivalent of unicellular propagules. Interestingly, multicellular propagules containing cells sampled randomly from a body of a parent are predicted to be severely disadvantageous when selection acts only on individuals. In contrast, when a moderate to strong selection exists at the within-individual level, multicellular propagules representing a random sample of parental cells would be better suited to eliminate deleterious mutations than the unicellular ones [56]. Understanding the fate of nuclei during the AM fungal life cycle and their origin during spore formation combined with a better understanding of AM fungal reproduction will enable the testing of these predictions.

8. Transposable elements in AM fungi

Genomes of bdelloid rotifers, a 35–40 million year old clonal lineage [58], seem to lack most of the known retrotransposable elements [59]. This observation gave rise to the hypothesis that sexual reproduction evolved as a mechanism to purge transposable elements from

genomes, and thereby to avoid their deleterious effects [60]. In a broad sense, this hypothesis bridges a gap between the Muller's ratchet hypothesis (recombination as a mechanism that purges deleterious mutations from populations) [61], and the Red Queen hypothesis (recombination facilitates adaptation to rapidly coevolving parasites, in this case genomic parasites) [62]. Interestingly, transposable elements are often sexually transmitted. Transition to asexual reproduction should therefore eliminate the acquisition of new elements although it would not be expected to eliminate transposition altogether. Continued activity of existing transposable elements after the loss of sexual purging mechanisms may be one of the causes behind the rapid demise of asexual lineages unless such elements are inactivated by the accumulation of mutations or by a host suppressive genetic background [63]. On the other hand, in asexual fungi, transposition-related ectopic recombination was postulated to generate genetic variation and facilitate adaptation to environmental changes [64]. With a putative asexual status, Glomeromycota are ripe for the investigation of the role of transposable elements in their genetics and evolution.

9. Conclusion

AM fungi are involved in one of the oldest and most widespread mutualistic symbiotic associations on the planet. Understanding genetic processes that govern their reproduction is critical for: (1) elucidation of the mechanisms responsible for AM fungal interactions with plant individuals and communities, (2) verification of a putative ancient asexual status of Glomeromycota, and (3) development of commercial products containing mycorrhizal fungi. Studies undertaken so far have not been entirely successful in explaining transmission genetics in Glomeromycota. AM fungi are a difficult group of organisms to study because of their obligate biotrophy and their recalcitrance to axenic cultivation [6]. However, several tools for genetic enquiry have been created or are in the process of being tailored for AM fungi, including the mutiplex [39] and global PCR amplification strategies for individual spores [65] (Pawlowska and Taylor, unpublished), or a genetic transformation system (Natalia Requena, personal information). Furthermore, completion of the ongoing project to sequence the genome of a representative AM fungal species, *G. intraradices*, will vastly expand the resources available to the mycorrhizal research community [66]. Rigorous data acquisition to verify the conflicting findings is necessary in several research areas, including: (1) the genome size and structure with an emphasis on rRNA gene array organization, (2) the individual genetic makeup and behavior of nuclei during a life cycle, and (3) the reproductive mode in nature. Other urgent research problems include (4) the understanding of vegetative incompatibility barriers and mechanisms, and (5) a reconciliation of the morphological species recognition system with the phylogenetic species recognition approach.

Discovery of the AM fungal reproductive mode in nature will generate another set of questions that would depend on whether AM fungi turn out to be clonal or recombining. In the case of clonality, the research priorities would involve a verification of the putative ancient asexual status and understanding the mechanisms responsible for the long-term evolutionary survival of Glomeromycota. If, instead, signatures of recombination were confirmed in nature, unearthing the nature of the recombination processes would be in order.

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