Trends in Plant Science

Review

Nuclear Dynamics in the Arbuscular Mycorrhizal Fungi

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Arbuscular mycorrhizal fungi (AMF) are plant root symbionts that continuously carry thousands of nuclei in their spores and hyphae. This unique cellular biology raises fundamental questions regarding their nuclear dynamics. This review aims to address these by synthesizing current knowledge of nuclear content and behavior in these ubiquitous soil fungi. Overall, we find that that nuclear counts, as well as the nuclei shape and organization, vary drastically both within and among species in this group. By comparing these features with those of other fungi, we highlight unique aspects of the AMF nuclear biology that require further attention. The potential implications of the observed nuclear variability for the biology and evolution of these widespread plant symbionts are discussed.

Highlights

Arbuscular mycorrhizal fungi (AMF) can carry thousands of nuclei in their cells at all times.

The number, shape, and frequency of these nuclei vary substantially among and within species.

Some AMF strains, referred to as AMF dikaryons, carry two distinct nuclear genotypes within their cells.

The frequency of two coexisting genotypes may vary across AMF dikaryons, raising questions about its significance for mycorrhizal symbiosis.

A Fungus That Carries Thousands of Nuclei in One Cell

Arbuscular **mycorrhizal** (see Glossary) fungi (AMF), subphylum Glomeromycotina of the phylum Mucoromycota [1,2], are widespread root symbionts of land plants [3]. The obligate symbiosis between the fungus and its host is established via **arbuscules**, which are the main site for the bidirectional exchange of nutrients between the two mycorrhizal partners. AMF increase the uptake of phosphorous and nitrogen [3] for their plant hosts and in return receive carbon sources (sugars, lipids) [4]. Arbuscules seem to be an evolutionary pinnacle since similar structures were observed in fossil rhizome of early land plants from the Lower Devonian [5,6]. Molecular clock estimates place the origin of the Glomeromycota between the Ediacaran or Cryogenian (600–720 Ma) [7] and the Tonian period of the Neoproterozoic (~980 Ma) [8].

Compared with other fungi, AMF cells are highly unusual, as each of their spore and hyphae continuously carry hundreds to thousands of nuclei. Because AMF produce **coenocytic hyphae**, virtually millions of nuclei can coexist in one large cytoplasm at any given time. Furthermore, recent data has shown that, at least in model AMF from the species *Rhizophagus irregularis*, the nuclear organization is either homokaryotic (nuclei with one genotype present in the cytoplasm) or dikaryotic, where the mycelium contains thousands of nuclei originating from two parental strains [9–11].

The present review aims to provide a synthetic view of the multinucleate state of AMF. It will highlight the high nuclear variability that exists within and among species and discuss its potential effect on plant hosts. A comprehensive view of nuclear dynamics in AMF is important to better understand their biology and genetic organization. Some of the questions that will be addressed in this review include: how many nuclei are typically found in the perpetually multinucleated AMF and are there any stages where only one or two nuclei are found? Is there evidence of intraspecific variability in nuclear size, shape, and **ploidy** within the AMF mycelium and spores and where do nuclei localize within hyphae and spores? Finally, how can this variability affect the plant hosts?

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Inter- and Intraspecific Variability in Nuclear Counts and Forms

Do AMF species carry stable nuclear populations and how do their nuclear counts compare with those of other multinucleate fungi? Generally, AMF have a higher content of nuclear DNA

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compared with other fungi, ranging from 0.13 (*Cetraspora pellucida*) to 3.4 picograms (pg) (*Diversispora versiformis*) of DNA per nucleus (Table 1) [12]. Nuclear size (diameter) and DNA content may show high variation both between and within species. Notably, flow cytometry data showed that nuclei can vary up to eightfold in DNA content between members of the family Gigasporaceae [13]. However, some species analyzed in [13] were later reclassified (to *Racocetra*), reducing the intrafamily DNA content variation to twofold. Nuclear size can also vary between life cycle stages [intraradical stage, extraradical mycelium (ERM), and spores] within a species [14], with larger nuclei being carried by spores while smaller relatives prevail in the extra- and intraradical mycelium. What triggers the variation in nuclear size within a single organism is unknown, but it could be linked to functional activity or is simply an adjustment to the available space. Indeed, nuclei can expand in size when surrounded by large cytoplasmic volume without changing their nuclear content, as seen in other organisms [15,16]. It is unknown, however, whether nuclear expansion or reduction requires energy, or what exact purpose it may serve (e.g., relation to expression or ploidy).

One common misconception for non-mycologists is that AMF are the only fungal group that carries multinucleate spores. In reality, other members of the phylum Mucoromycota, as well as members of Zoopagomycota [1], produce spores that are also multinucleate. For example, 10–16 nuclei per spore are found in *Zoophthora aquatica* (syn. *Erynia aquatica*) [17] and a mean number of four nuclei are carried in the sporangia of *Rhizopus* sp. What sets AMF apart from any other group is the number of nuclei carried by their cells, which is one to two orders of magnitude higher than any fungal relative, with spores carrying between 130 and 35 000 individual nuclei, depending on the species and physiological state (Figure 1A,B). The number of nuclei per spore varies among species (Table 2), but remarkably also between sister spores of the same individual [18,19]. While the number of nuclei increases proportionally with spore size, similarly sized sister spores can still vary in the number of nuclei they contain [19]. The final number of nuclei in each sister spore is not related to spore viability or spore **germination** ability [19]. The shape of nuclei can also vary between and within spores, with some nuclei showing a globose shape while others have irregular shapes [20].

In summary, when it comes to nuclear counts, the numbers found in AMF are unmatched by any other fungal relative and there is, to date, no evidence that stages with one or two nuclei exist in these organisms. Furthermore, the number of nuclei per spores also vary dramatically both within and between species. Altogether, these nuclear features are one of the reasons AMF are still considered highly unusual eukaryotic organisms.

Nuclear Migration, Morphology, Mitosis, and Localization

The high and wide inter- and intraspecific heterogeneity in nuclear count we described beg a fundamental question: is nuclear migration and division tightly controlled in AMF, or do these processes follow stochastic mechanisms? Similarly, what type of morphological structures are found in AMF and do all these carry a high number of nuclei?

During AMF spore germination, multiple nuclei (but not all) migrate into newly formed **germ tubes**. This allows for multiple regermination events to occur in case of failure to encounter a host [21,22] or physical disturbance of the tube [23]. In such cases, AMF can also retract the cytoplasm and nuclei back into the spore, contributing to nuclear preservation, leaving a hollow hypha behind that is separated from the viable hyphae by retention **septa** (Figure 1C). Based on the observations, it is possible that nuclear division (mitosis) might occur prior to germination [24], but there is no experimental evidence of nuclear division at that stage. Nuclear division occurs postgermination in the absence of a plant host [25,26] but is not required for the germination

Glossary

Appressorium: specialized fungal cell that allows hyphae to penetrate the plant tissue.
Arbuscules: highly branched haustoria-like structures formed in cortical root cells. Arbuscules are the centers of the bio-directional nutrient exchange between the AMF and the host.
Ascomycetes: group of higher fungi that have septate hyphae and spores borne in microscopic cells called asci.
Autophagy: function which allows for degradation of cellular components.
Basidiomycetes: group of higher fungi that have septate hyphae and spores borne on a basidium.
Coenocytic hyphae: also known as nonseptate hyphae, tube-looking structures, created by cell walls containing large amount of chitin. They contain multiple nuclei organelles and cytoplasm. Coenocytic hyphae are considered as large multinucleate cells.
Filamentous fungi: fungi that grow as a mass of branching filamentous structures called hyphae.
Germ tube: the germinating hyphae originating from a spore or a spore subtending hyphae.
Germination: the process in which new growth in the form of hyphae is produced from a fungal spore.
Karyogamy: the fusion of two, normally haploid, compatible nuclei.
Karyorrhexis: destructive fragmentation of the nucleus, usually during cell death.
Mating-type loci: genetic regions that govern sexual compatibility in fungi.
Meiosis: type of cell division in sexually reproducing organisms that results in four cells carrying one copy of each chromosome.
Mycorrhiza: the symbiotic association between a mycorrhizal fungus and the root system of a compatible plant host.
Nucleophagy: autophagy targeting nuclei.
Nucleotype: group of nuclei sharing the same genetic information.
Parasynchronous mitosis: nuclei undergoing mitosis featured by a graded spatial variation (mitotic wave).
Ploidy: the number of chromosome sets, present in a single nucleus.
Septa: (singular, septum); internal walls separating fungal hyphae into hyphal cells (or compartments).
Sexual compatibility: following hyphal fusion, compatible haploid nuclei can

stage since spores exposed to rapamycin (mitotic inhibitor) can still germinate [25]. Regardless, mitosis that occurs during spore germination can significantly increase the number of nuclei (e.g., in spores of *Gigaspora margarita* nuclear counts can increase from 2000 to 26 000 following postgermination mitosis) [18].

Following symbiosis establishment, AMF can produce multiple multinucleate spores, which are formed at the tips of the hypha or intercalary. During spore formation, multiple nuclei flow freely into the spore as it grows in size. Remarkably, nuclei can also move out of the spore during spore formation, yet despite this bidirectional movement the spore nuclear content always increases over time [27]. The nuclear population in developing spores further increases via mitosis [19]. Thus, the mechanism leading to the accumulation of nuclei in the AMF spore combines features seen in the **ascomycete** *Aspergillus oryzae* (i.e., many nuclei flow into the spore) and that of *Rhizopus* sp., where a single nucleus undergoes mitosis leading to multinucleate spores [28].

Nuclear morphology varies substantially in AMF, ranging from globose to ellipsoid, fusiform, or reniform based on observations of the ERM [20,27,29], as well as the intraradical mycelium

fuse and generate novel genetic combinations. Sexual compatibility involves recognition of compatible mates (govern by mating type loci in fungi), karyogamy and ploidy changes, and meiosis.
Somatic compatibility: successful hyphal fusion (also called anastomosis) between two genetically distinct strains.
Strigolactones: group of plant hormones that stimulate spore germination and hyphal elongation and branching of some AMF.
Zygospore: sexual spores of zygomycetes following fusion of haploid cells.

Table 1. Variation in Nuclear Size[a,b] (Diameter) and DNA Content between and within AMF Species

Species	Size (μm)	Nuclear content (pg)	Genome size (Mb)	Method	Refs
Acaulospora longula	n.d.	0.34	n.d.	Flow cytometry after DAPI staining relative to *Gigaspora margarita*	[13]
Acaulospora laevis	n.d.	0.54	n.d.	Flow cytometry after DAPI staining relative to *G. margarita*	[13]
Acaulospora scrobiculata	n.d.	0.33	n.d.	Flow cytometry after DAPI staining relative to *G. margarita*	[13]
Cetraspora pellucida	n.d.	0.13	n.d.	Flow cytometry after DAPI staining relative to *G. margarita*	[13]
Dentiscutata heterogama	n.d.	0.22	n.d.	Flow cytometry after DAPI staining relative to *G. margarita*	[13]
Diversispora versiformis	2.36	0.26	n.d.	Cytofluorometry using chicken red blood cells as an internal standard	[14]
D. versiformis	2[a]	n.d.	n.d.	Flow cytometry after DAPI staining	[42]
Funneliformis caledonium	n.d.	0.38	n.d.	Flow cytometry after DAPI staining relative to *G. margarita*	[13]
G. margarita	4.23	0.76	740	Cytofluorometry using chicken red blood cells as an internal standard	[14,76]
Gigaspora rosea	4.8[a]	0.65	598	Flow cytometry after DAPI staining relative to *G. margarita*	[13,38,76]
G. rosea	n.d.	0.65	n.d.	Flow cytometry after DAPI staining relative to *G. margarita*	[13]
Racocetra castanea	n.d.	0.88	n.d.	Flow cytometry after DAPI staining relative to *G. margarita*	[13]
R. castanea	n.d.	1	n.d.	Flow cytometry	[77]
Racocetra gregaria	n.d.	1.08	n.d.	Flow cytometry after DAPI staining relative to *G. margarita*	[13]
Racocetra persica	n.d.	1.7	n.d.	Fluorescent DNA stain Hoechst 33258	[78]
Rhizophagus clarus	n.d.		116.4	Illumina and PacBio sequencing	[79]
Rhizophagus irregularis	3.3[a]	0.16	154.8 ± 6.2	Cytofluorometry using *Arabidopsis thaliana* as an internal standard	[80]
R. irregularis (DAOM197198)	1.8[a]	n.d.	153	Sequenced using Sanger, 454, Illumina, and PacBio platforms	[81]
R. irregularis (Strain A4)	n.d.	n.d.	138.3	Flow cytometry	[9,10]
R. irregularis (Strain A5)	n.d.	n.d.	131.5	Flow cytometry	[9,10]
R. irregularis (Strain C2)	n.d.	n.d.	122.8	Flow cytometry	[9,10]
R. irregularis (Strain SI1)	n.d.	n.d.	211.5	Flow cytometry	[9,10]
R. irregularis (Strain A1)	n.d.	n.d.	125.8	Flow cytometry	[9,10]

[a]Abbreviation: n.d., no data or unknown.
[b]When size of nuclei was not reported, it was calculated using the software FIJI (ImageJ version 1.52e) based on the scale of the images in each publication when possible.

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and arbuscules [14]. This variation may represent fungal mitotic division phases as seen in other fungi [30–32], or may indicate distinct nuclear functions or even **karyogamy**; an event that is still elusive in AMF. For example, elongated nuclei are consistently anchored to the plasma membrane and may thus potentially create functionally separated areas in the coenocytic hyphae [29]. Such a situation is reminiscent to what happens in the ascomycete *Ashbya gossypii*, where the regulated internuclear distance creates hyphal territories governed by a single nucleus and this nonrandom distance plays an important role in regulating nuclear division [33]. Direct evidence of karyogamy could be revealed using fluorescence *in situ* hybridization procedures based on nucleus-specific probes.

To our knowledge, elongated nuclear shapes commonly seen in AMF are rare in **filamentous fungi** and, when encountered, such deformities are related to space limitations or nuclear degradation. For example, appressorial nuclei of the pathogenic ascomycete *Magnaporthe oryzae* constrict in order to migrate through the space-limited penetration pegs [34]. The nuclei in AMF spores are usually localized at the periphery of the innermost spore wall, possibly due to space limitation created by the abundant lipid droplets (Figure 2A). AMF spores contain nuclei that are usually round but can also have irregular shapes. It is unknown whether the irregular shapes observed are linked to a functional response or to spore cytoplasmic degradation (Figure 2B).

AMF nuclei can travel in pulses, bidirectionally or against the cytoplasmic flow [22,35,36]. How nuclear movement is controlled in AMF is not known, but presumably fungal cytoskeletal features such as the motor proteins dynein and kinesin, actin filaments, and microtubules likely play a role [32,37]. Generally, higher numbers of nuclei are concentrated in the AMF hyphal tips, where the mitotic activity takes place to sustain the hyphal expansion [38]. Mitotic activity of AMF increases with the establishment of the symbiosis in order to sustain the hyphal growth and tip expansion and, indeed, plant-derived signals (such as **strigolactones**) have the potential to directly stimulate fungal mitotic activity [38].

Mitotic division of nuclei in multinucleate filamentous fungi, other than AMF, can be **parasynchronous** (sequential), synchronous, or asynchronous [39] and division patterns can vary greatly even within a common cytoplasm (i.e., it can become synchronous in some parts of the mycelium while remaining asynchronous in others; see [39] and references within). Still, information on nuclear division in AMF is very limited and mostly originates from observations rather than experimental studies of this phenomenon. Based on available data, AMF nuclei seem to divide asynchronously during the asymbiotic and symbiotic stages, indicating the presence of self-regulated nuclei (i.e., nuclei are able to divide independently of neighboring relatives) [27,29]. In the future, it will be particularly important to examine whether asynchronous divisions are taking

Figure 1. Multinucleate Spores and Hyphae of Arbuscular Mycorrhizal Fungi (AMF). Nuclei were stained with SYTO 13 green fluorescent nucleic acid dye and observed with a confocal microscope (ZEISS LSM 800). Images consist of multiple pictures (approximately 300 z-stacks with 0.35 μm intervals) merged to a single 2D image. Z-stacks were taken with an oil immersion 60× lens and the final merged images were color coded along the z-axis to allow for depth recognition. Color coding uses a color pallet (appears in image A) to transform the green color produced by the fluorescent dye to a color gradient along the z-axis, with blue colored nuclei being closer to the observer and red colored nuclei being the furthest. (A) Multinucleate spores of a homokaryotic, and (B) a dikaryotic strain of the AMF *Rhizophagus irregularis*. It is unknown whether dikaryotic strains have higher nuclear counts than homokaryotic strains. (C) Multinucleate coenocytic hyphae and spores of a homokaryotic strain of *R. irregularis*. It is possible that the observed coupled nuclei could represent different mitotic stages. While AMF are considered aseptate fungi, they do form multiple septa like structures called 'retention septa' (orange arrow). The retention septa are not normally distributed across the hyphal network and are not compartmenting the hyphae to individual cells similar to basidiomycetes. Retention septa are accompanied by cytoplasmic retraction, leaving a hollow, nonviable hyphae behind (orange arrow is also pointing to a hollow hypha while the white arrow points to a viable hypha filled with cytoplasm, nuclei, and organelles). The AMF material for this analysis originated from *in vitro* transformed root cultures with *Daucus carota* as a host.

Table 2. Number of Nuclei[a] Recorded in Spores from Different AMF Species

Species	Number of nuclei	Spore mean diameter (μm)	Method	Refs
Dentiscutata erythropus	3850	292 × 310 (irregular shape)[b]	Counts of nuclei stained with acetoorcein	[45]
D. erythropus	17 000[c]	280	Based on the model by [82]	[78]
Diversispora versiformis	15 000[c]	150	Based on the model by [82]	[78]
D. versiformis	1000	80	Flow cytometry	[42]
Funneliformis caledonium	9000[c]	200	Based on the model by [82]	[78]
F. caledonium	1000	224[b]	Stained spores with DAPI (estimation)	[82]
Gigaspora decipiens	35 000[c]	400	Based on the model by [82]	[78]
Gigaspora gigantea	2600	300[b]	Counts of nuclei stained with acetoorcein	[45]
G. gigantea	27 000[c]	350	Based on the model by [82]	[78]
Gigaspora margarita	2000	250	Image analysis	[18]
G. margarita	20 000[c]	357[b]	Estimation model based on spore size	[82]
Glomus aggregatum	230	90	Confocal microscopy and fluorescence dye	[19]
Glomus cerebriforme	130	80	Confocal microscopy and fluorescence dye	[19]
Oehlia diaphana	320	90	Confocal microscopy and fluorescence dye	[19]
Racocetra castanea	725	320[b]	(via personal communication)	[83]
R. castanea	650	298[b]	Flow cytometry	[13]
Racocetra persica	22 707[c]	320[b]	Based on the model by [82]	[78]
Rhizophagus irregularis	220	90	Confocal microscopy and fluorescence dye	[19]

[a]For the data from [19] we used the average number of nuclei of mature sized spores. For all studies when a range was reported we calculated the average number of nuclei.
[b]If spore diameter was not reported in the studies, it was completed with information from the book 'Glomeromycota' by Blaszkowski *et al*. [90].
[c]Reported values are suggested to be an overestimation of the actual spore nuclear content [18].

place between the coexisting **nucleotypes** present in AMF dikaryons (see section Nuclear Genome Size, Ploidy, and Genome Organization). In particular, an asynchronous division could skew the ratios of the two coexisting genotypes across in AMF dikaryons and potentially affect fungal and host fitness and their evolutionary fate [40,41].

In addition to the intra/extraradical hyphae and spores, AMF also develop tree-shaped structures called arbuscules [3] in root cells. These structures represent the site of nutrient exchange between the fungus and the host and nuclei have been observed in the large trunks of arbuscules but not in the finest branches [42]. BrdU-labeled nuclei in arbuscule trunks were found to be active at the early stage of arbuscule development and able to undergo mitotic division, but these shortly undergo **karyorrhexis** (fragmentation), a mechanism that was previously correlated with the ephemeral and collapsing nature of arbuscules [43]. Extraradical hyphae can create branched absorbing structures (BAS) [35], which also contain numerous nuclei but, to our

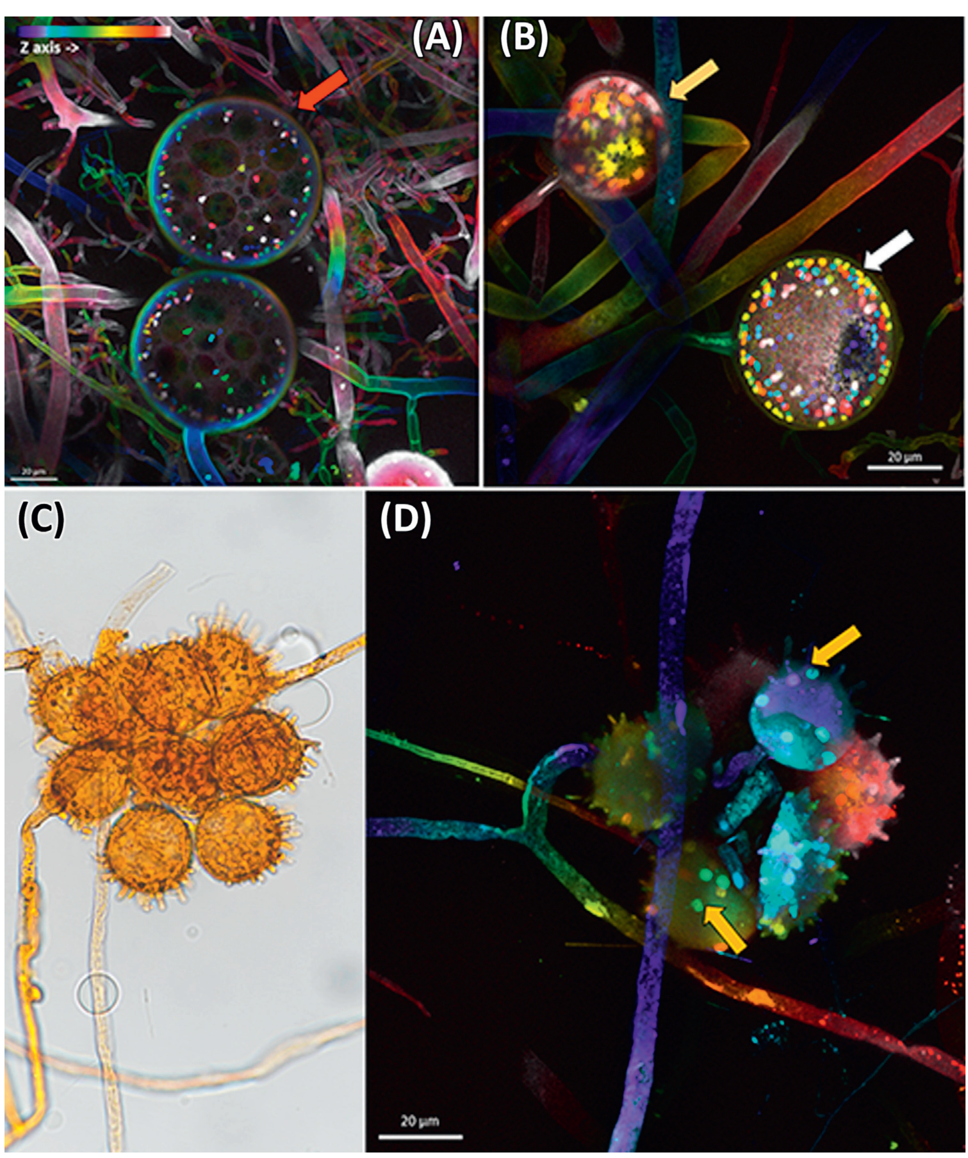

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Figure 2. Localization of Nuclei in Spores and Auxiliary Cells. (A) Localization of nuclei peripheral to the innermost spore wall (red arrow). Lipid droplets are also visible within the spores, along with abundant hyphae in the background. (B) Irregular shaped nuclei within a spore (yellow arrow) compared with regular round nuclei found commonly in arbuscular mycorrhizal fungi (AMF) spores (white arrow). (C) Auxiliary cells as seen with an optical microscope. The function of auxiliary cells is not known to this day and it is possible that they are remnants of a former functional structure. (D) Confocal microscopy of the same auxiliary cells observed in (C) with nuclei stained with SYTO 13 green fluorescent nucleic acid dye (orange arrows). Images consist of multiple pictures (approximately 300 z-stacks with 0.35 μm intervals) merged to a single 2D image. Z-stacks were taken with an oil immersion 60× lens and the final merged images were color coded along the z-axis to allow for depth recognition. Color coding uses a color pallet (appears in Figure 1A) to transform the green color produced by the fluorescent dye to a color gradient along the z-axis, with white colored nuclei being closer to the observer and violet colored nuclei being the furthest. The AMF material for this analysis originated from *in vitro* transformed root cultures with *Daucus carota* as a host.

knowledge, there is no detailed information on their longevity or activity. In addition to BAS, some AMF species can produce terminal swellings called vesicles in the intraradical growth stage. While vesicles frequently are mentioned as storage units, they also contain multiple nuclei [3] and are

able to act as propagules that can produce infectious hyphae [44]. In *Scutellospora* and *Gigaspora* AMF species, vesicles are absent, but these species possess distinct extraradical structures called auxiliary cells (Figure 2C) that, remarkably, contain a handful of nuclei (Figure 2D) [45]. The low number of nuclei found in these cells contrasts with the rest of the mycelium, raising the intriguing possibility that auxiliary cells have a unique function such as **meiosis**, perhaps similar to **zygospores** [46].

When it comes to migration and division, AMF show few similarities with distant filamentous fungi, but unique characteristics also stand out. In particular, there is evidence that individual nuclei represent genetically independent units, dividing asynchronously along the mycelium. Similarly, each germinating spore acquires different numbers of nuclei, an indication that nuclear migration is not tightly controlled, if not for specific structures such auxiliary cells where the nuclear counts are surprisingly always small.

Nucleophagy and Nuclear Degradation in AMF?

Considering that millions of nuclei float within the same cytoplasm in AMF, it is safe to assume that many would degrade over time. However, if, how and when this phenomenon occurs in AMF is not understood. When facing starvation, the ascomycete *A. oryzae* can enable mechanisms that degrade its own nuclei and redistribute nutrients to ensure colony survival [47]. This phenomenon of targeted nuclear degradation is called '**nucleophagy**', which is the selective **autophagy** of nuclei. It has been hypothesized that nuclear DNA degradation can provide a stable flux of phosphorus and nitrogen [48], which are usually the main nutrients that limit fungal growth [49]. Nucleophagy has also been observed in the yeast *Saccharomyces cerevisiae* [50] and the fungus *M. oryzae* [51], a plant pathogen, where it plays an important role in plant infection during the **appressorium** formation. It has also been observed in the ascomycete *Fusarium oxysporum* during vegetative hyphal fusion, where the invading nucleus remains and the resident nucleus gets degraded, possibly as a result of heterokaryon incompatibility [52]. Besides the role in nutrient recycling, nucleophagy also holds an important role in damaged DNA regulation [53]. Any of these processes can theoretically occur in AMF.

While nucleophagy has not been directly studied in AMF, there is reported evidence of nuclear degradation [27,29,36] in *R. irregularis* [36] and *Gigaspora rosea* [29]. It was hypothesized that nucleophagy happens as a way to preserve resources for future regermination events when the germ tubes fail to encounter a host [29]. In AMF, nuclear degradation is also accompanied by cytoplasmic retraction and degradation of neighboring organelles, as well as isolation of the hyphal compartment with septa formation [21,22]. This means that nuclear degradation is likely to be a programmed cell death mechanism in AMF [54], as opposed to being the result of targeted nucleophagy. Still, degraded nuclei and diffused DNA strands and mitotic spindles have also been observed in viable parts of symbiotic, aseptate hyphae [27]. This degradation likely represented selective nucleophagy because it was neither induced by UV light during the observation [55], nor was it accompanied by overall cytoplasmic degradation.

Considering the significant phosphorus and nitrogen quantities stored in the thousands of nuclei coexisting in AMF cells, nucleophagy could be a critical yet severely overlooked process in these organisms. For example, nucleophagy might play a role in the early establishment of the symbiosis, specifically by providing an early boost in phosphorus availability during the formation of arbuscules and prior to the extensive spread of ERM, where nutrient exchange is important for successful interaction. Nucleophagy could also be important during the association with the host because of the essential role of phosphorus in the stability of the symbiosis [3]. Lastly, this process could also contribute to the genetic stability of AMF via targeted nuclear degradation

of damaged/mutated DNA and may play an active role in maintaining stable nuclear frequencies in AMF dikaryons.

Nuclear Genome Size, Ploidy, and Genome Organization

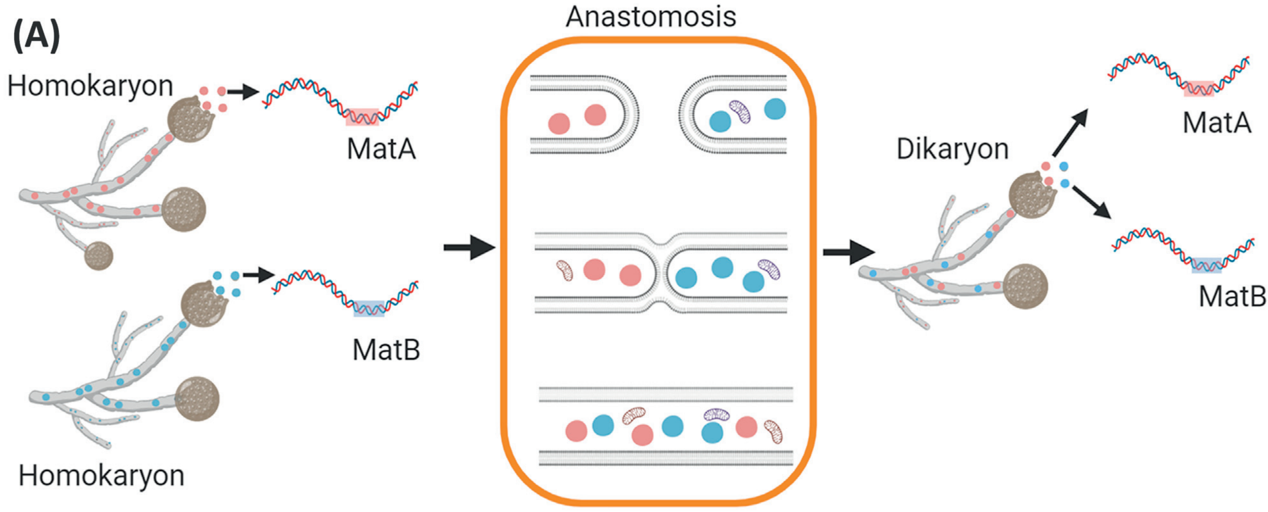
AMF nuclear genomes can vary significantly in size, from approximately 140 megabases in species within genus *Rhizophagus* (formerly known as *Glomus* spp.) to several hundreds of megabases in the family Gigasporaceae (Table 1). Although the size of nuclei can vary within the mycelium, all studies performed to date found that AMF carry haploid nuclei and no evidence of diploidy or aneuploidy has been published to date for this group.

Recent genome and single-nucleus analyses also revealed that model AMF carry either nuclei with one shared genotype (homokaryons) or two distinct genotypes (dikaryons). In AMF dikaryons, each nucleotype also carries divergent genomic regions with similarities to those that govern sexual identity in other fungi (i.e., a **mating-type locus)** [9,10] (Figure 3A). In **basidiomycetes** and ascomycetes, the emergence of dikaryosis is driven by **somatic** and **sexual compatibility** of homokaryotic strains following hyphal fusion (Figure 3A), suggesting that AMF may undergo sexual reproduction. Still, the dikaryotic state can be maintained indefinitely without the necessity to proceed through sexual reproduction (i.e., karyogamy and meiosis). Thus, it is possible that in the AMF dikaryotic stage the genetically distinct nuclei never undergo meiosis and remain instead in a dikaryotic stage indefinitely or until stochastic processes lead to homokaryosis, as seen in the ascomycete *Neurospora crassa* [56]. Note that the presence of the dikaryotic stage can also allow fungi to generate diversity somatically [41,57] and similar mechanisms may also occur in the AMF dikaryotic stage [58].

Nuclear Ratios in AMF Dikaryons: An Effect on the Plant Partner?

All AMF dikaryons analyzed to date harbor allele frequencies that hover around 50%. Still, variation in the allele spectrum exists among dikaryons, with some strains harboring lower frequency alleles than others (Figure 3B). This variation in allelic frequencies may indicate that the ratio of each coexisting nucleotype fluctuates among strains and, to some extent, among their spores and across their mycelium within each strain. To date, information about nucleotype frequency in the AMF dikaryotic mycelium has been limited to a single analysis based on the PCR amplification of a very small noncoding locus (*bg112*) in spores of one *R. irregularis* strain [59]. This study suggested that the two coexisting genotypes can be unbalanced in AMF dikaryons, with each of two *bg112* alleles ranging between 40 and 60%. It must be noted that these frequencies are close to a balanced 50% nuclear ratio, so this apparent nuclear unbalance can also be explained by known variability across PCR reactions.

Some coexisting alleles belonging to each nucleotype can be transcribed simultaneously in AMF dikaryons [60]. If the existence of unbalanced nuclear ratios is confirmed in all AMF dikaryons and validated with other tools, it would be important to understand if such ratios vary across developmental stages (temporally or spatially), between or within species, or even as a response to the environment. The identification of a link between balanced and/or unbalanced nuclear ratios in AMF dikaryons and environmental cues (e.g., host, soil conditions) could have far-reaching consequences for understanding the mycorrhizal symbiosis. In theory, two nucleotypes could be equally represented across the mycelium (1:1 ratio) or be unbalanced with one nucleotype being more abundant than the other overall (or spatially). Both scenarios offer opportunities for AMF dikaryons and their hosts and this certainly warrants a deeper understanding of how each coexisting nucleotype coexists within the mycelium and contributes to the fungal and plant phenotype.



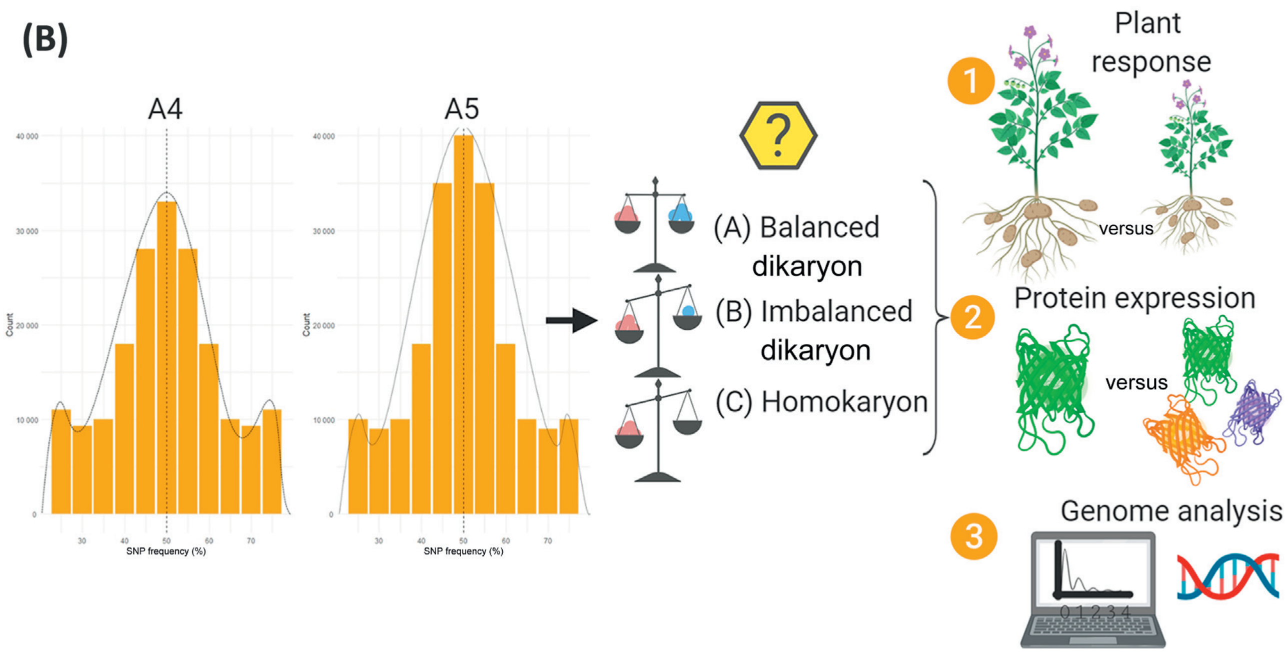


Figure 3. Homokaryotic and Dikaryotic Strains of Arbuscular Mycorrhizal Fungi (AMF). (A) Anastomosis and cytoplasmic exchange between two homokaryotic strains of *Rhizophagus irregularis*. Like the majority of fungi, closely related AMF strains can physically interact and proceed to hyphal fusion (anastomosis). During anastomosis, cytoplasm is exchanged (plasmogamy), including nuclei and mitochondria [84]. The establishment of a successful anastomosis between compatible strains can happen rather rapidly (in 35 min), as observed with time lapse light microscopy, and the cytoplasmic exchange takes place at a rate of 1.8–2.6 $\mu m\ s^{-1}$ [85]. Compatibility has been observed between the same strains and even between genetically distinct strains [70,84,86–89]. Successful anastomosis leads to plasmogamy and exchanges of nuclei. It is unknown whether somatic compatibility in AMF is controlled by genetic distance or/and mating type related genes. MatA and MatB refer to nucleotypes with unique mating type loci. (B) SNP frequency of dikaryotic *R. irregularis* strains DAOM 664343 (A4) and DAOM 664344 (A5), based on data produced by [5,68], indicating that nuclear ratios between the two nucleotypes could vary. Variation in nuclear ratios could possibly affect host response, protein expression, and genome analysis.

Interestingly, each scenario also comes with intrinsic complexities. For example, when nuclear genotypes are equally represented in the mycelium (1:1 ratio) (Figure 3B), it may be assumed *a priori* that their contribution to the transcriptome/host phenotype would

be identical. Yet, this is not what happens in conventional fungal dikaryons (two nuclei/cell) like the basidiomycete *Agaricus bisporus*. Specifically, in this species, gene expression of each nuclear type is actually unbalanced, even when their nuclear ratios are identical, and there is evidence that each coexisting nucleotype controls distinct cellular functions [61]. It is plausible that similar mechanisms are also present in AMF dikaryons, whereby each nucleus could coordinate a different cellular pathway. In cases where one nucleotype is more abundant, then the effect on the transcriptional output would be obvious: one nucleus would dominate the proteome generated by the AMF dikaryon, thereby driving fungal growth and possibly directing the establishment of the mycorrhizal symbiosis.

If we assume that coexisting nucleotypes are relatively divergent and carry distinct genes and effectors in AMF dikaryons, then interstrain variability in nuclear ratios could also play a driving role in the establishment of the mycorrhizal symbiosis and plant growth. In some cases, one ‘nucleotype A’ may be genetically adapted to interact with ‘plant species X’, shifting nuclear ratios in its favor upon establishment of symbiosis with that particular species (unbalanced; Figure 3A). In others, both nucleotypes carry complementary effectors (balanced; Figure 3B).

Interaction between Nuclei in AMF Dikaryons: Cooperation versus Dominance?

The long-term coexistence of two divergent nucleotypes in AMF dikaryons indicates that cooperation may be at play in these organisms. Still, is there evidence that genotype could constantly dominate the other?

In conventional dikaryotic fungi, genetically unique nuclei interact in multiple ways. For example, they compete against each other, leading to the elimination of one nucleotype within the timespan of a single generation [62,63]. Environment can also play a role in nuclear selection, resulting in fluctuating nuclear ratios [64–66]. Similar examples of nuclear dominance have yet to be observed in AMF dikaryons (i.e., all strains analyzed to date have been carrying two genotypes for more than two decades) [67].

As seen in other dikaryotic fungi, cooperation could occur by exchanging genetic information through somatic recombination [41,68], a process that can maximize the fitness of fungal dikaryons [40]. Cooperation between the two nucleotypes can also become apparent through the stabilization of nuclear ratios, as seen, for example, in the dikaryotic basidiomycete *A. bisporus* (Basidiomycota). In this fungus, nuclei appear paired in cells (karyollele pairs) and equally distributed in each (i.e., two nuclei per cell, at a 1:1 ratio), but each nucleotype is functionally complementary, expressing genes at different levels depending on the fungus life-cycle [61]. Lastly, nuclear cooperation can also lead to variation in transcription as a result of physical proximity of the nuclei, as seen in dikaryotic strains of *Schizophyllum commune* where the relative positioning of nuclei can alter gene expression [69].

Whether the two distinct genotypes cooperate in AMF dikaryons is unknown at this time. To tackle his question, we must first understand how each contribute (equally or not) to the AMF transcriptome and phenotype and whether each targets distinct cellular functions, as seen in *A. bisporus*. In parallel, mathematical models could be built to understand whether the origin and the coexistence (and the potential frequency variability) of the two distinct genotypes in AMF dikaryons is better explained by random processes, as opposed to cooperation or dominance.

Concluding Remarks and Future Perspectives

As expected for a perpetually multinucleate organism, nuclear dynamics are complex in AMF and are thus unsurprisingly not well understood. Nuclei exhibit variability at many levels, from shape and size to localization within the mycelium. Variation is always larger between species, but extensive variability among conspecific strains is also found. Common nuclear features among all AMF species analyzed to date include the presence of bidirectional nuclear movement [35,36] and the coexistence of different nuclear types within some strains [9,10]. Future analyses should investigate if some nuclei carry universal fungal features, such as nuclear coupling (as seen in conventional dikaryons) or whether one of the two coexisting nucleotypes (and mating types) dominate over the other in the spores and mycelium of AMF dikaryons. More generally, understanding if/how genetically distinct nuclei cooperate with each other will be important to understand why AMF dikaryons originate and why this unique genetic condition has been maintained for decades in some strains (see Outstanding Questions).

Nuclear features unique to AMF include the presence of a high nuclear spore content, as well as anchored and elongated nuclear shapes. It will now be important to understand if all nuclei share the same ploidy regardless of their shape and whether various shapes are linked with a specific karyotype (e.g., diploidy, aneuploidy).

Another understudied basic aspect of the AMF nuclear biology is the cellular mechanism that allows for continuous nuclear movements across the mycelium. Understanding how nuclei are passed between AMF strains is also essential to fully comprehend how these plant symbionts recognize one another and create nuclear diversity in nature. Within this context, it was proposed that interstrain genetic distance may act as a barrier against plasmogamy in AMF [70,71]. In the future, it will be interesting to see if plasmogamy and the production of stable dikaryotic strains is actually driven by compatibility around the putative mating-type loci, as opposed to genetic distances between isolates [9,10]. How nuclei coordinate movements or cooperate in AMF dikaryons is also unclear, so investigating their relative proportions in and transcriptional output will improve the understanding of the biological role of each coexisting nucleotype in mycorrhizal symbiosis. To this end, the use of high-resolution quantifying methods such as digital droplet PCR may be the best approach to measure with exactitude the number and frequency of each nuclear genotype within AMF dikaryons and thus detect their relative changes in abundance across varying experimental conditions.

Lastly, how do homokaryons and dikaryons compare in term of growth and symbiotic potential? In fungi, no general trend in either vegetative or functional differences is found between homokaryotic and dikaryotic strains. Rather, such differences seem species-specific across the fungal kingdom. For example, homokaryotic strains can grow faster than dikaryotic relatives in the basidiomycetes *Hericium coralloides* [72] and *Peniophora* sp. [73], but basidiomycete dikaryotic strains outgrow homokaryons in *Gloeophyllum trabeum* [74] and *Phellinus weirii* [75]. To date, no studies have compared similar patterns between AMF homokaryons and dikaryons. This is surprising, given the relevance of AMF for terrestrial ecosystems and for the agro-tech sector. As such, it will be essential for future studies to determine if dikaryosis provides a growth/functional or fitness benefit in AMF compared with homokaryotic relatives.

Outstanding Questions

Do AMF nuclei share the same ploidy regardless of their shape, or does nuclear shape correlate with a presence of aneuploidy?

Which cellular mechanisms control the movement of thousands of nuclei across the AMF mycelium?

What is the function of auxiliary cells in some AMF species and why do these harbor so few nuclei compared with the rest of the mycelium?

What is the relative transcriptional output of each nucleotype in AMF dikaryons,

How common are AMF dikaryons in nature and across the AMF phylogenetic tree?

Does the frequency of nuclear genotypes vary among AMF dikaryotic strains and, if so, how does this variation affect the plant hosts?

What controls somatic compatibility in these widespread plant symbionts?

How frequently do coexisting nuclei undergo karyogamy and can these events result in somatic recombination, as opposed to meiotic rearrangements, in AMF dikaryons?

Acknowledgments

Our research is kindly funded by the Discovery program of the Natural Sciences and Engineering Research Council (RGPIN-2020-05643), a Discovery Accelerator Supplements Program (RGPAS-2020-00033). N.C. is a University of Ottawa Research Chair and V.K. is supported by the MITACS Industrial PDF program (IT16902). We would also like to thank Keith Hubbard and Denise Chabot for providing excellent training and technical assistance with the confocal imaging.

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