1 Exploring the Genome of Glomeromycotan Fungi

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CONTENTS

I. Introduction

All fungi forming a mutualistic symbiosis with plant roots called arbuscular mycorrhiza were formerly grouped together in one order, the Glomales, placed in the Zygomycota (Morton 1993). Based on molecular analyses suggesting that arbuscular mycorrhizal fungi should be separated from other fungal taxa, they were transferred a decade ago to the **Glomeromycota**, a new phylum created specifically for them (Schüssler et al. 2001). Whilst members of this monophyletic group originated from the same common ancestor as the Ascomycota and Basidiomycota, they have **no obvious affinity to other major extant phylogenetic groups in the kingdom Fungi** (James et al. 2006) and they probably diverged from the other fungal lineages several hundred million years before plants colonized terrestrial habitats 400–500 million years ago (mya) (Heckman et al. 2001). Glomeromycotan fungi are complex but extremely successful organisms. They establish a compatible interaction with plants by either avoiding or suppressing plant defence reactions whilst redirecting host metabolic flow to their benefit without being detrimental to their host. The mechanisms by which this biotrophy is achieved are largely unknown but the Glomeromycota have accompanied land plants through evolution and survived across periods of important environmental change to become ecologically and agriculturally important symbionts which improve the overall fitness of very different plant taxa in terrestrial ecosystems world-wide (Smith and Read 2008). Substantial evidence has accumulated about how a rational use of the microsymbiont properties should significantly contribute to decreasing fertilizer and pesticide use in agriculture (Gianinazzi et al. 2010).

Although the Glomeromycota show considerable diversity between or within morphologically recognizable species (Rosendahl 2008), they share some singular biological traits

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which limit experimental approaches that can be exploited to characterize their complexity. One particularity is their **reproduction through large asexual spores**, each of which is a single cell harbouring several hundreds or thousands of nuclei. The main mechanism for filling a spore with so many nuclei appears to be a massive influx of nuclei from subtending mycelium into the developing spore (Jany and Pawlowska 2010). **No sexual stage is known** for these fungi but, although they are assumed to only reproduce asexually, recent transcriptome analyses indicate that they do possess genetic information essential for sexual reproduction and meiosis (Tisserant et al. 2012). Absence of a sexual cycle raises questions about how Glomeromycota deal with deleterious mutations usually eliminated through meiosis and how they have adapted to new hosts or habitats during evolution. High polyploidy, with multiple gene copies in the same genome, has been speculated as a possible mechanism to buffer against mutational events (Pawlowska and Taylor 2004), whilst **nuclear exchange through hyphal anastomosis between different individuals of a same species** (Casana and Bonfante 1988; Giovannetti et al. 2001) provides the possibility for genetic flux and recombination events (Croll et al. 2009; Angelard and Sanders 2011). However, basic information concerning ploidy, karyosis, number of chromosomes or whether meiosis does occur in the Glomeromycota is still lacking, and evidence for genetic exchange, recombination or segregation is very limited.

Another particularity is that **all glomeromycotan fungi are obligate symbionts** which have so far proved to be incalcitrant to pure culture in the absence of a host root on which they depend as a carbon source. This introduces inherent **limitations in the application of standard techniques like genetic transformation or mutant generation/characterization**, and it greatly hinders advances in the knowledge about gene function in these organisms. As previously pointed out (Gianinazzi-Pearson et al. 2001), there is converging evidence that the Glomeromycota are an unusual group of fungi, and information about their genome structure, complexity and function is essential to understanding the processes regulating their symbiotic attributes, their reproductive biology and their apparent stability during coevolution in symbiosis with many different plant taxa. Despite the fact that the biology of glomeromycotan fungi makes them extremely difficult to manipulate experimentally, the advent of powerful molecular techniques has considerably furthered research during the past decade. The present chapter updates on the state of the art on Glomeromycota genomics within the past decade (Gianinazzi-Pearson et al. 2001), as well as on progress made through targeted and high-throughput sequencing programmes into an understanding of their basic biology.

II. Glomeromycota Genome Organization

A. Nuclear Genome Characteristics

Values for the DNA contents of glomeromycotan fungi vary depending on the analytical method and the genome of reference used (see Gianinazzi-Pearson et al. 2001). The **haploid genome size** was previously estimated to range from 128 to 1,065 Mb, depending on the fungal species studied, which further underlines the **high diversity within this phylum** (Hosny et al. 1998a). Important differences also exist within the same genus: the genome size of *Glomus* species was estimated by similar methods to be, for example, 177 Mb for *G. geosporum* and 375 Mb for *G. caledonium* (Hosny et al. 1998a) and 14.0–16.5 Mb for *G. intraradices* DAOM 197198 (syn. *G. irregulare*; Hijri and Sanders 2004). The apparently small genome of the latter (see below), together with its cultivability in vitro on root organ cultures, has made it an appropriate candidate for first sequencing attempts of a glomeromycotan genome (Lammers et al. 2004). To date, several genome sequencing programmes, based mainly on extensive whole-shotgun (WGS) sequencing, have generated altogether 345 Mbp that have been assembled in 163,968 contigs in a total of 52.5 Mb (Martin et al. 2008b). This corresponds to about fourfold the initially expected genome of *G. intraradices* DAOM 197198 and an estimated genome space of >150 Mb. Recent data provided by Sedzielewska et al. (2011) have confirmed that the genome of

this fungus is in fact 10 times larger (155 Mb) than estimated by Hijri and Sanders (2004) and is within the range for the Glomeromycota. Correct genome assembly is hindered by the fact that critical genetic information, such as a genetic map, is not available for the Glomeromycota and that **multiple gene copies in the same genome** (Pawlowska and Taylor 2004) would lead to different assemblies with the same set of scaffold data. **High polymorphism**, which appears to be characteristic of glomeromycotan fungi (see Sanders and Croll 2010) and which has also been indicated from genome sequencing (Martin et al. 2008b), raises an additional question of functional genes versus pseudogenes, which are difficult to separate for most software packages used for whole genome assemblies. Recent information about functional genes is discussed below in Sect. IV.

The **very low GC content** (~30 %) observed from genome sequencing of *G. intraradices* DAOM 197198 is in agreement with the values of 30–35 % obtained by other methods for a range of Glomeromycota, and which are relatively low as compared to most other fungal taxa (Hosny et al. 1998b). The latter authors proposed that a mutational pressure from GC to AT may exist in the Glomeromycota and that, since these fungi proliferate under light-deprived conditions in soil and roots, their environment has not exerted any significant counter selection against AT-rich sequences so that GC contents have become low. The **relatively high proportion of methylated cytosine residues**, which are frequent in repeated sequences, is another particularity of the glomeromycotan genome (Hosny et al. 1998b). A number of repetitive non-coding DNA sequences have been characterized in several species in the Glomeromycota (Gollotte et al. 2006), and genome sequencing of *G. intraradices* DAOM 197198 has revealed that this fungus is also rich in small repeats. Interestingly, variable tandem repeats are considered to affect the rate of evolution of coding and regulatory sequences in other organisms (Gemayel et al. 2010).

B. Nuclear Ribosomal Genes

The **number of nuclear ribosomal gene copies** has been estimated to be 71–88 in representative glomeromycotan genomes (Gollotte et al. 2006), which is considerably less than in fungi with smaller genomes like yeast or *Cochliobolus heterostrophus* (see Gianinazzi-Pearson et al. 2001). The polymorphic characteristics of ribosomal genes have made them a choice target for phylogenetic and taxonomic studies over a wide range of eukaryotic organisms. The **three coding regions of ribosomal genes**, the small sub-unit (SSU) or 18S, the 5.8S unit and the large sub-unit (LSU) or 25S, are **separated by two internal transcribed spacers** (ITS1 and ITS2; Mitchell et al. 1995). The two non-coding regions (ITS), being under less functional pressure, are more variable and mutate more frequently (Sanders et al. 1995) than the three conserved coding regions. A comprehensive description of nuclear ribosomal genes in the Glomeromycota and their exploitation in phylogeny prior to 2001 is given by Gianinazzi-Pearson et al. (2001). **SSU, LSU and ITS regions are used for molecular phylogeny and taxonomy** of fungi in this phylum. The highly conserved SSU was the first region to be selected for phylogenetic analysis of the Glomeromycota, mainly because of the large number of eukaryotic sequences present in public databases (Simon et al. 1992). It enabled the initial dating back of this taxon to an ancestral *Glomus*-like fungus 353–462 mya (Simon et al. 1993). SSU sequences remain the most numerous available for glomeromycotan fungi and they are mainly at the origin of more recent taxonomic reorganizations (Schüssler et al. 2001; Schüssler and Walker 2010).

However, the SSU region does not allow species-level resolution in the Glomeromycota. Also, the first primer allowing identification of Glomeromycota in host roots, the SSU-based VANS1, is not well conserved across the phylum (Clapp et al. 1999). Consequently, other ribosomal regions, such as the ITS/5.8S region in combination with the SSU or the 5′ end of the LSU, have been exploited to generate more taxon-specific primers for monitoring fungal communities in roots (for example, see van Tuinen et al. 1998; Redecker 2000; Pivato et al. 2007). Comparison of the level of polymorphism between the different ribosomal regions of *G. mosseae* BEG12, *G. mosseae* BEG69, *G. coronatum* BEG22 and *G. intraradices*

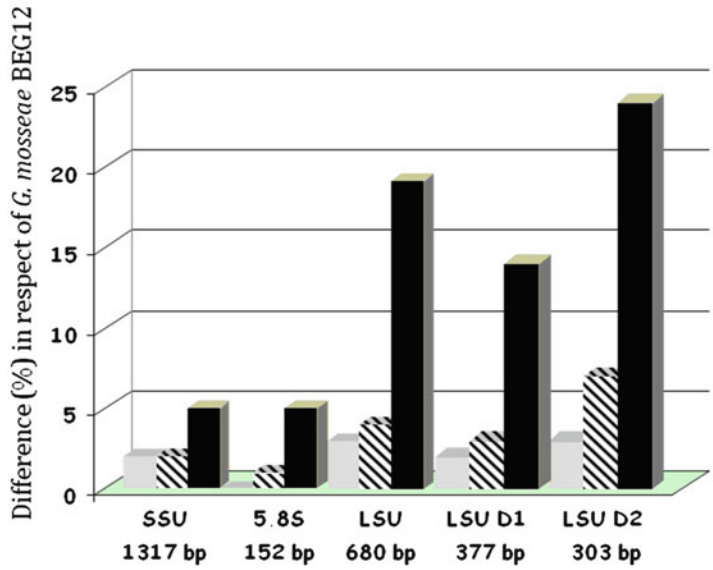

Fig. 1.1. Difference (expressed as a percentage) between sequences of *Glomus mosseae* (BEG12), *G. mosseae* BEG69 (*pale grey*), *G. coronatum* BEG22 (*hatched*) and *G. intraradices* AFTOL ID 48 (*black*) for the SSU, 5.8S, the 5′ end of the LSU (including the variable D1 and D2 domains) and the variable D1 and D2 domains alone

AFTOL ID48, clearly illustrates the **potentiality of the 5′ end of the LSU for diversity studies** (Fig. 1.1). Sequence differences between *G. mosseae* BEG12 and *G. coronatum* BEG22 or *G. intraradices* AFTOL ID 48 are greatest for the 5′ end of the LSU region and in particular for the variable D2 domain. In spite of this, a general caution applies to the use of nuclear ribosomal sequences for diversity studies in that they have a potentially high inter and intra-sporal heterogeneity which implies that several nuclear ribosomal variants may occur in the same isolate (Sanders et al. 1995; Sanders and Croll 2010).

C. The Mitochondrial Genome

The first **mitochondrially encoded genes** from the phylum Glomeromycota were identified from an amplified and sequenced region of the mitochondrial gene coding for the LSU of ribosomal RNA (Raab et al. 2005). In contrast to their nuclear-encoded counterparts, these genes do **not show variation within single spores and fungal isolates**. This is of particular importance as it has been proposed that the **nuclei in the coenocytic glomeromycotan mycelium are genetically heterogeneous**, resulting in a population of allelic variants with no fixed overall genotype (Kuhn et al. 2001). The first complete mitochondrial genome of a glomeromycotan fungus was sequenced from *G. intraradices* strain 494 by whole genome amplification and subsequent pyrosequencing (Lee and Young 2009). It has a size of 70.6 Kbp and a GC content of 37.2%, which is higher than the GC content of the nuclear genome. The genetic code used in the protein-coding genes is the standard code except that UGA is used for tryptophan, which is typical for many fungal mitochondrial genomes. The mitochondrial genome of *G. intraradices* contains a similar set of genes to that of other fungi: three subunits of ATP synthase (*atp6*, *atp8*, *atp9*), three of cytochrome c oxidase (*cox1*, *cox2*, *cox3*), seven of NADH dehydrogenase, and apocytochrome b (cob). Moreover, it contains the standard set of 26 tRNAs and ribosomal small subunit (*rns*) and large subunit (*rnl*) genes. Notably, all genes are encoded on the same strand. However, the *rps3* gene, which is found in the mitochondrial genome of other fungi, was apparently transferred to the nuclear

genome in *G. intraradices*, and there is evidence for the presence of nuclear copies of other mitochondrial sequences (Lee and Young 2009). **The *G. intraradices* mitochondrial genome bears little resemblance to that of other fungi**, but this comes as little surprise since gene order in fungal mitochondrial genomes is not conserved among distantly related taxa.

A large part of the *G. intraradices* mitochondrial genome consists of introns and other non-coding sequences; only 24.6 % are coding sequences. A total of 26 introns were identified in strain 494, most of them belonging to type 1. As in other fungi, the mitochondrial genome of this glomeromycotan fungus is rich in **homing endonucleases**, enzymes that are often coded in introns and thought to function **as mobile elements** in the insertion of introns containing an endonuclease open reading frame (ORF) into intron-free alleles (Dalgaard et al. 1997). Homing endonucleases of the LAGLIDADG family were first reported in introns of the *rnl* gene (Raab et al. 2005). The complete mitochondrial genome sequence shows that four of these ORFs are within introns and one is attached to the *nad3* gene, whilst two GIY-YIG type homing endonuclease ORFs are in intergenic spacers. As mobile genetic elements, homing endonucleases are presumably transferred laterally. Comparison of different isolates and species of *Glomus* has provided evidence for the existence in glomeromycotan fungi of **horizontal transfer of *rnl* introns and the "homing cycle"** (Chevalier and Stoddard 2001), involving insertion, degeneration and loss (Thiéry et al. 2010). Due to this activity, introns containing homing endonuclease ORFs seem to show higher degrees of polymorphism. This polymorphism has been used to distinguish between isolates of *G. intraradices* in field studies (Börstler et al. 2010) and to track an inoculated isolate over several years in a field site (Sýkorov et al. 2011).

The involvement of fungal mitochondria in spore germination and early signal exchange with host root colonization (Besserer et al. 2006) highlights the need to study the coding sequences of mitochondrial genomes. On the other hand, non-coding regions are of direct importance as molecular markers of these fungi in ecological studies and in biotechnological applications. In addition, **mitochondrial markers to study inheritance of these organelles could provide important baseline data to help to elucidate the genetics** of the Glomeromycota and possible previously unrecognized sexual processes.

III. Nuclear Genome Evolution in the Glomeromycota

The **Glomeromycota are traditionally thought to be ancient asexuals**. This assumption is based on the fact that no morphological structures conclusively indicative of sexual reproduction have been observed in the whole phylum; a study reporting zygosporangia in *Gigaspora* (Tommerup and Sivasithamparam 1990) has never been confirmed. The lack of basic knowledge about their genetics leaves the evolutionary biology of the Glomeromycota open to a lot of speculation. The inability to culture these fungi separately from their host plants and to obtain stable transformants has contributed to this situation.

Considering the well-known **benefits of sexual reproduction** in avoiding the accumulation of deleterious mutations in the genome, which should inevitably lead to evolutionary meltdown processes such as Muller's ratchet (Muller 1932), it seems hard to understand how these fungi could persist as important actors of terrestrial ecology for more than 400 million years. Morphological stasis resulting in a relatively low number of described morphospecies and striking similarities of extant Glomeromycota with 400 million year old fossils have been cited as other lines of evidence for the lack of diversification caused by long term clonal propagation in conjunction with the asexual lifestyle (Sanders and Croll 2010). Ancient asexuals are often considered as "scandalous" exceptions of the rule as they challenge current theories of sex (Judson and Normark 1996). The bdelloid rotifers, a lineage of invertebrates solely consisting of parthenogenetically reproducing females for at least 40 million years, are often cited as a striking

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