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The genome of *Geosiphon pyriformis* reveals ancestral traits linked to the emergence of the arbuscular mycorrhizal symbiosis

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Summary

Arbuscular mycorrhizal fungi (AMF) (subphylum Glomeromycotina) [1] are amongst the most prominent symbionts and form the Arbuscular Mycorrhizal symbiosis (AMS) with over 70% of known land plants [2] [3]. AMS allows plants to efficiently acquire poorly soluble soil nutrients [4] and AMF to receive photosynthetically fixed carbohydrates. This plant-fungus symbiosis dates back more than 400 million years [5], and is thought to be one of the key innovations that allowed the colonization of lands by plants [6]. Genomic and genetic analyses in diverse plant species started to reveal the molecular mechanisms that allowed the evolution of this symbiosis on the host side, but how and when AMS abilities emerged in AMF remains elusive. Comparative phylogenomics could be used to understand the evolution of AMS [7,8]. However, the availability of genome data covering basal AMF phylogenetic nodes (Archaeosporales, Paraglomerales) is presently based on fragmentary protein coding datasets [9]. *Geosiphon pyriformis* (Archaeosporales) is the only fungus known to produce endosymbiosis with nitrogen-fixing cyanobacteria (*Nostoc punctiforme*) presumably representing the ancestral AMF state [10–12]. Unlike other AMF, it forms long fungal cells (‘bladders’) that enclose cyanobacteria. Once in the bladder, the cyanobacteria are photosynthetically active and fix nitrogen, receiving inorganic nutrients and water from the fungus. Arguably, *G. pyriformis* represents an ideal candidate to investigate the origin of AMS and the emergence of a unique endosymbiosis. Here, we aimed to advance knowledge in these questions by sequencing the genome of *G. pyriformis*, using a re-discovered isolate.

Keywords: *Geosiphon pyriformis*, homokaryon, bladders, binning, metagenome, symbiosis, cyanobacteria, *Nostoc Punctiforme*

Results

General genome characteristics of Geosiphon pyriformis

The only known culture of *G. pyriformis* was lost over a decade ago (A. Schüßler, pers. comm). In an attempt to rediscover *G. pyriformis*, we searched and identified symbiotic bladders of the *G. pyriformis*-*N. punctiforme* symbiosis (Figure 1) at the only known stable habitat of this species in the Spessart Mountains near the village of Bieber in Germany [13].

Figure 1: a. Image of *G. pyriformis* bladders in soil from its natural habitat in the Spessart mountains. **b.** Schematic representation of *G. pyriformis* bladders containing *Nostoc* cells (based on M. Kluge 2002). *Nostoc* cells are shown in dark green, and heterocysts (differentiated cell that carries out nitrogen fixation) are shown in light green. Bladders contain *G. pyriformis* nuclei (orange), and several vacuoles (white). Aseptate hyphae reach out and extend from the bladders.

Upon cultivation, we extracted DNA and RNA from active bladders. Total DNA was subjected to 5Kb-mate-pair and 125 bp paired-end Illumina sequencing, producing respectively 47 and 81 million 125 bp paired ends and 5kb mate-pairs reads. *G. pyriformis* reads were identified using a read binning approach recently implemented to assemble the genome of *Diversispora epigaea* [14], and upon identification these were assembled into a 129 MB assembly and 795 scaffolds with an average read coverage of 118X. In parallel, total RNA was subjected to 150 bp paired-end Illumina sequencing. The resulting RNA-seq reads were mapped onto the *G. pyriformis* genome assembly using STAR [15] and used for genome annotation after implementing RepeatMasker [16]. This procedure identified of 24195 genes in *G. pyriformis*, resulting in a BUSCO gene repertoire completeness of 96.2% (3.1% complete duplicated). The gene counts, estimated genome size, and genome statistics are all similar to those of model AMF

species [17,18] and are indicative of high genome completeness (Table 1, see figure of “K-mer distribution of filtered illumina genomic reads of *G. pyriformis*” in Data S1 and see table of “Genome completeness using Busco” in Data S1). SignalP showed that, among all the genes identified in *G. pyriformis*, 365 represent putative secreted proteins (see table of “Secretome proteins from genomes” in Data S1) and 27% of these are candidate effectors (see table of “Effector prediction on *G. Pyriformis* genome “in Data S1). We also identified 19 putative secreted CAZymes in *G. pyriformis*; in line with numbers found in other AMF species (Table S1 and Table S2).

Figure 2: Bubble plot containing all transposable elements in the genomes of glomeromycotina and mucoromycotina genomes used in this study. The figure shows the expansion of Gypsy elements in *G. pyriformis*.

1
2 AMF genomes carry a substantial fraction of transposable elements (TE) [18–21], and we found
3 that *G. pyriformis* has undergone similar TE expansions. The expansion of Gypsy transposable
4 elements in *G. pyriformis* is evident in comparison to all other AMF genomes (Figure 2). With
5 regards to TE, we find no evidence that *G. pyriformis* (see figure of “Heatmap showing evidence
6 for a “one-speed genome” in *Geosiphon Pyriformis*” in Data S1) carries a two-speed genome
7 [22]. Two-speed genomes are characterized by the presence of TE-poor and gene dense regions
8 that a clearly separate from others that contain rapidly evolving genomic regions that usually
9 carry less genes, abundant TE, and other repeat elements [22,23].

10 Using genome data and single nucleus data it was recently shown that AMF carry two genome
11 organizations – i.e., homokaryotic (co-existing nuclei carry one parental haploid genotype) or
12 dikaryotic (two parental genotypes co-exist in the mycelium) [21,24–26]. Mapping reads onto
13 the *G. pyriformis* genome revealed reduced levels of polymorphism (0.5 SNP/Kb) and allelic
14 frequencies suggesting that this species is carries low a low nuclear diversity and likely
15 homokaryotic (see figure of “Genome wide allele frequency of *G. Pyriformis* shows it is a
16 homokaryon” in Data S1).

17 ***Placement of G. pyriformis based on phylogenomics***

18 The *G. pyriformis* genome annotation was used to identify the phylogenetic placement of this
19 species using amino-acid sequences. In this case, we used a set of 434 conserved fungal single
20 copy genes (data available at DOI: 10.5281/zenodo.1413687) to construct a phylogenetic tree of
21 the fungal kingdom. Phylogenomics supports the monophyly of Glomeromycotina and its close
22 relationship with Mortierellomycotina within the phylum Mucoromycota [1] (Figure 3). Within
23 Glomeromycotina, *G. pyriformis* groups within a monophyletic clade with *Ambispora leptoticha*
24 and *Paraglomus occultum*, which diverged around 287 MYA (Figure S1). This clade is distinct
25 from more diverged nodes that contain sequenced representatives from Glomerales and
26 Diversisporales [27]. The current placement of *G. pyriformis* as a sister lineage to *A. leptoticha*
27 and *P. occultum* has full statistical support, and is favored by 73% of the gene sequences we
28 used. Alternative topologies that place, for example, *G. pyriformis* at a basal node to all AMF
29 (Alt-T1; Figure S2, see table of “Results of alternative tree topology test for phylogenetic tree “ in
30 Data S1) or as being associated with Glomerales or Diversisporales (Alt-T2 and Alt-T3; Figure

S2, see table of “Results of alternative tree topology test for phylogenetic tree “ in Data S1) were all rejected significantly using statistical tests implemented in IQ-TREE, including the KH, SH, ELW, and AU tests (see table of “Results of alternative tree topology test for phylogenetic tree “ in Data S1).

Figure 3: Phylogenetic tree representing the evolutionary relationships of fungi and placement of *G. pyriformis* in Glomeromycotina clade. The tree was resolved using maximum likelihood phylogenetic reconstruction with IQ-TREE on a concatenated alignment of 434 protein coding genes. Numbers indicate nodes with less than 100% bootstrap support. Branches are coloured according to their phylum.

Phylogenetic tests for all alternative topological placements of *G. pyriformis* were rejected. The asterisk denotes the location where inferred gene losses and gains occurred in the MRCA of Glomeromycotina.

The genome of Geosiphon pyriformis uncovers shared gene features in the Glomeromycotina

Phylogenomics revealed that *G. pyriformis* is a member of a clade that diverged early from the lineage encompassing the already sampled Diversisporales and Glomerales. As such, the *G. pyriformis* genome fills the gap in the genomic coverage of major AMF phylogenetic clades. With this data in hand, we first searched for genetic features that arose in the most recent

1 common ancestors (MRCA) of all Glomeromycotina by comparing orthogroups from five
2 available AMF genomes and four other members of the Mucoromycota as outgroups using
3 OrthoFinder [28]. This analysis identified 661 gains and 344 losses that occurred before the
4 divergence of the Glomeromycotina (Table S3 and see table of “Orthogroups lost in the MRCA
5 of Glomeromycotina” in Data S1).

6 Among the 344 orthogroups classified as lost in the MRCA of the Glomeromycotina, we note the
7 missing key enzymes involved in essential metabolic functions, such as sugar and thiamine
8 metabolisms, or in the biosynthesis of fatty acids. These genes are also referred to as “Missing
9 Glomeromycotina Core Genes” (MGCGs; see table of “Orthogroups lost in the MRCA of
10 Glomeromycotina” in Data S1, see table of “Missing MRCA genes across glomeromycotina core
11 genes” in Data S1), and our analysis reveals that these have also been lost by *G. pyriformis*.
12 Other key losses that affect all sequenced Glomeromycotina include enzymes that actively
13 degrade plant cell wall (see table of “Presence and absence of plant and fungal cell wall related
14 CAZymes” in Data S1) . Among the 661 orthogroups gained in the MRCA, most encode for
15 proteins involved in signaling pathways (e.g., protein kinases), protein–protein interactions (e.g.,
16 the tetratricopeptide repeat, Sell1, the homodimerization BTB (Broad- Complex, Tramtrack and
17 Bric a brac), and WD- 40 domain- containing proteins) and High Mobility Box (HMG) (see
18 table of “Functions of genes which are gained in orthogroups” in Data S1).

19 Comparative genomics also showed that *G. pyriformis* carries the same signatures of sexual
20 reproduction found in AMF relatives. These include a complete set of meiosis-specific genes
21 (see table of “Identification of Meiosis specific genes” in Data S1) [29,30], and a highly
22 conserved genomic locus with architecture and sequence similarity to the mating-type (MAT)
23 locus of basidiomycetes [21,31,32] (see figure of “Transcriptional directions of the putative
24 AMF mating-type locus” in Data S1).

27 ***Regulation of orthogroups gained in the MRCA of Glomeromycotina***

28 We investigated available gene expression data from the model AMF *Rhizophagus irregularis*,
29 and found that 7 and 39 orthogroups (with a total of 8 and 272 genes; see table of “Upregulation

1 of ortholog genes from Rhiir2 genome “in Data S1 and see table of “Downregulation of
2 Orthogroups genes from Rhiir2” in Data S1) gained in the MRCA of *Glomeromycotina* are
3 respectively upregulated and downregulated across all four experimental conditions in the model
4 AMF *Rhizophagus irregularis*.

5 These conditions include symbiotic associations between *R. irregularis* and distinct plant hosts
6 (*Medicago truncatula*, *Brachypodium distachyon*) [33–35], and others based on laser-capture
7 microdissection arbuscule-specific gene expression [33,34,36]. We investigated the putative
8 function of these differentially regulated genes by identifying protein motifs along their coding
9 sequences, and found that these are involved in a myriad putative cellular function, though these
10 mostly include protein tyrosine kinases, cytochrome p450, as well as FAD binding (see table of
11 “Upregulation of ortholog genes from Rhiir2 genome” and “Downregulation of Orthogroups
12 genes from Rhiir2” in Data S1). Lastly, one differentially regulated OG (OG0001728) shows
13 evidence of originating from horizontal gene transfer from bacteria – i.e., this orthogroup is
14 shared between bacteria and fungi (Figure S3).

15 ***Distinct genomic features of Geosiphon pyriformis***

16 Besides the evolution of AMS, the acquisition of the *G. pyriformis* genome offers an opportunity
17 to identify innovations linked to the emergence of the only known cyanobacteria – fungus
18 endosymbiosis. To identify such innovations, hierarchical clustering and abundance of Pfam
19 domains was performed using available genomes in the Glomeromycotina and representatives of
20 Mucoromycotina and Mortierellomycotina (Figure S4). This analysis revealed a significant
21 overrepresentation of 16 protein domains in *G. pyriformis* compared to relatives in the
22 Mucoromycota – e.g., Lipase_3, RNase_H, Retrotrans gag domains, dUTPase, Spuma_A9PTase,
23 Myb_DNA-bind_6 (see table of “Pfam domain counts from genomes of mucoromycota used in
24 this study” and “p values calculated by fisher's test” in Data S1).

25 We also sought evidence of horizontal gene transfers (HGT) between partners of the unique
26 *Geosiphon-Nostoc* endosymbiosis, and found 18 genes with potential bacterial within in the *G.*
27 *pyriformis* genome (see table of “HGT containing genes with pfam domains” in Data S1).
28 Among putative HGT, two are protein encoding genes with significant sequence conservation
29 with *Nostoc* and Gamma proteobacteria homologues (see figure of “Phylogenetic tree showing
30 evidence of Horizontal gene transfer of Selenium binding protein” and “Phylogenetic tree

showing evidence of horizontal gene transfer in Molybdenum cofactor carrier” in Data S1). All putative HGT are located within contigs with average coverage and surrounded by genes of AMF origin, suggesting these do not represent contaminants.

Discussion

MRCA of all extant Glomeromycotina carried hallmarks of mutualism and obligate biotrophy

Genome data from a representative of the basal node of the AMF phylogeny filled an important gap in understanding the origin of AMS. Specifically, it allowed us to conclude that the MRCA of all extant Glomeromycotina carried hallmarks of mutualism and obligate biotrophy – i.e., a lack of genes for fatty acids and thiamine biosynthesis and nutrition, and a reduced number of genes that actively degrade plant cells. As such, the mechanisms involved in AMS appeared prior to the emergence of Glomeromycotina and represent a synapomorphy of this sub-phylum. *G. pyriformis* has also conserved genomic signatures of sexual reproduction, as well as an apparent low nuclear polymorphism. Both traits are thus conserved across Glomeromycotina and are in stark contrast with the notion that these organisms represent an ancient asexual lineage.

The retention of a conserved Glomeromycotina gene set in *G. pyriformis*, including a sub-set of these involved in plant cell wall degradation, indicates that this species might be possible to form mycorrhizae, although this has never been observed. As such, this retention could reflect an intrinsic capacity for *G. pyriformis* to undergo classic (but rare) mycorrhizal associations with plants under the right conditions. Although speculative at this point, this hypothesis is supported by the identification of rare *Geosiphon*-like sequences in environmental samples [37–39].

Novel symbiotic abilities and horizontal gene transfers in G. pyriformis

As a result of losses in fatty acid biosynthesis genes, AMF are entirely dependent on the host plant they associate with to obtain lipids [40–43]. Within this context, our findings suggest that, during the switch from regular AMS to a fungal- cyanobacteria symbiosis, *G. pyriformis* has evolved novel strategies to obtain lipids from its new host through the expansion of specific gene motifs. Specifically, the *G. pyriformis* genome carries a striking over-representation of Lipase 3 protein domains that hydrolyze ester linkages of fatty acids. As *Nostoc* spp is known to produce a wide variety of extracellular lipids in high amounts [40–43], it is possible that these abundant lipids are released in the environment (like many other cellular compounds released by

1 cyanobacteria [44–47]) and are then broken down by lipases to be used as an energy resource by
2 *G. pyriformis*.

3 As we find evidence of bacteria-like genes in the *G. pyriformis* genome, our work also suggests
4 that the co-existence of multiple endosymbionts and *G. pyriformis* nuclei within restricted
5 bladders offers some opportunities for horizontal gene exchange. Although none of the putative
6 bacterial genes we identified in *G. pyriformis* are functionally related, there is evidence that one
7 is differentially regulated during AMF symbiosis, supporting the notion that bacteria-like genes
8 can play a major role in fungal evolution [14,48].

9 ***Identification of a putative AMF core-symbiotic toolkit***

10 The *G. pyriformis* genome also enabled the identification of a putative core AMF symbiotic
11 toolkit conserved in all the sampled Glomeromycotina. This set of genes is differentially
12 regulated in model AMF during symbiotic interactions with different plant hosts, including
13 dicots, monocots and non-vascular plants, and thus provides a basis for future research on
14 symbiosis-related mechanisms in these plant symbionts. The identification of a core set of gene
15 gains specifically regulated during mycorrhizal symbiosis, and their conservation across the
16 Glomeromycotina phylogeny, also provides support for the early emergence of symbiosis-
17 specific gene functions in AMF over 400 million years ago, contemporaneously with the
18 evolution of the first land plants [49,50]. Lastly as genetic transformation is currently unfeasible
19 in Glomeromycotina, only assumptions can be proposed for the function of these putative core
20 genes. However, as some encode for chitin synthases, one attractive hypothesis could be that
21 some evolved for the production of short-chain chitoooligosaccharides or lipo-
22 chitololigosaccharides that are known symbiotic signals triggering the activation of the symbiotic
23 program on the host plant [2,51,52].

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Author Contributions

MMC, MK, CK, YW, JS, PM, and NC planned and designed the research, wrote the Manuscript and helped with data analysis. MMC carried out genome annotation and bioinformatics analysis. YW performed phylogenetic and molecular dating analysis. ECHC and GY produced repeat analysis with MMC. JK performed ortholog analysis. CR performed the transcriptome analysis. MVL helped improving the quality of images. MK and CK produced biological materials. NC supervised all process.

Declaration of Interests

The authors declare no competing interests.

Star Methods

Resource availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Nicolas Corradi (ncorradi@uottawa.ca).

Materials availability

This study did not generate new unique reagents.

Experimental Model and Subject Details

AM Fungi *G. pyriformis* were used for this study. Genome assembly is submitted in NCBI accession number of AAOMT000000000.

Data and Code Availability

Genome assembly is available in NCBI with accession number of JAAOMT000000000. Genome sequencing reads are submitted in SRA with accession number of SRR11466073, Bioproject

PRJNA610605, Biosample SAMN14307302. RNA-seq reads are available in SRA with accession of SRR12018969, SRR12018968, SRR12018970. All scripts used to analyze are archived in <https://github.com/madhubioinfo/Geosiphon>.

METHOD DETAILS

Cultivation of *G. pyriformis* samples from natural habitat

G. pyriformis was sampled during autumn in the only known stable habitat near the village Bieber in the Spessart (Germany). Active bladders of the *Geosiphon-Nostoc* endosymbiosis were found in slightly acidic soil (pH 5). The bladders occurred close to the hornwort *Anthoceros* spp. and the liverwort *Blasia pusilla* L., as these plants harbor the cyanobacteria needed to trigger the *Geosiphon-Nostoc* endosymbiosis. After sampling spores and bladders were transferred to the institute in Průhonice and cultured in beakers [53,54], which contain a small pot with a sterile mixture of sand and soil (from the original habitat). The cultures were grown in a climate chamber at 18°C with 14 h light and 10 h night. The substrate is kept wet by a filter paper, which reaches from the substrate into a water reservoir in the beaker. To be maintained over time, cyanobacteria be frequently added to the cultures. For our cultures, *Nostoc punctiformis* was obtained from the Culture Collection of Algae (SAG) at the University of Göttingen (Germany) as strain SAG69.79 [53,54].

Genome and transcriptome sequencing and assembly

High quality DNA was extracted from active bladders of *G. pyriformis* and *Nostoc punctiforme* using the NucleoSpinII Plant kit (Machery-Nagel) and purified with the genomic DNA clean-up kit (Machery-Nagel) using the manufactures recommendations. Total DNA was sent to Fasteris (Switzerland) for library Illumina library preparation and sequencing using on 150 paired end and 5kb mate pairs inserts (illumina Nextera mate pair kit). Sequencing was performed using the Illumina Hiseq 4000 platform. Total RNA was extracted using the RNeasy-Mini Kit (Qiagen) as per instructions of the manufacturer for library RNA-seq Illumina library preparation with sequencing of 150 cycles and paired ends.

Poor quality and adapter sequences were trimmed using Trimmomatic [55] with parameters the following parameters of ILLUMINACLIP:2:30:10 SLIDINGWINDOW:5:20 LEADING:5

TRAILING:5 MINLEN:50. The resulting 1 GB of non-redundant metagenome reads were assembled using metaSPAdes V3.12.0 [56]. Assembled contigs were binned on the basis of tetra nucleotide signature using CONCOCT [57], following part of the procedure used to assembly the genome of *Diversispora epigaea* [14]. Binned clusters were annotated using BLAST v 2.6.0+ [58,59] and clusters containing bacterial hits were removed. Using this approach, 21 bona-fide AMF clusters were retained, and used as reference to filter original paired-end and mate pair reads with BLAT v. 36x1 [60]. The reads which had mapped to the filtered contigs and matched by BLAT were then extracted to build cleaned sequence libraries that were assembled with MaSuRCA 3.3.0 [61]. Additional round of nr BLAST searches on MaSuRCA assembled contigs were performed to further remove contaminating bacteria. K-mer (k = 21) based methods were used on filtered reads to estimate genome size of *G. pyriiformis* using jellyfish 1.1.12 [62] and plotted in GenomeScope 2.0 [63] (see figure of “K-mer distribution of filtered illumina genomic reads of *G. pyriiformis*” in Data S1).

Genome annotation

Protein coding genes were predicted using Funannotate V1.7.4 (<https://funannotate.readthedocs.io/>) [DOI:10.5281/zenodo.3679386], which automates gene prediction. Assembled transcripts using Trinity [64] and Rnaseq reads mapped bam file were used as transcript evidence for gene call.

Transposable elements were predicted using TransposonPSI [65]. Repeat sequences were first identified using RepeatModeler [66] with multiple numbers of iterations. The iteration with the most number of repeats were then used for soft-masking the genome with REPEATMASKER (open 4.0.646) [16]. Output files generated from above procedures were used to identify repeat along the assembly. The completeness of genome assembly was assessed with BUSCO version 2.0 [67] with default parameters using the fungal gene dataset [fungi_odb9] (see table of “Genome completeness using Busco” in Data S1).

Putative gene functions were identified using Diamond BLASTX [59]. Pfam domain analysis were performed using Pfamscan [68,69] (Figure S4) (see table “Pfam domain counts from genomes of mucoromycota used in this study” and “pvalues calculated by fisher’s test” in Data S1) and Carbohydrate-active enzymes (CAZYme) were identified using the dbCAN CAZy database [70] (Table S2) (see table of “Presence and absence of plant and fungal cell wall

related CAZymes” in Data S1. Putative CAZymes were further verified through comparisons of data from Morin et al 2019 [17] (Table S1). Secretory proteins were identified using previously published pipelines [71,72] (see table of “Secretome proteins from genomes” in Data S1), and effectors were identified using EffectorP 2.0 [73] (see table of “effector prediction on *G. Pyriiformis* genome” in Data S1). The putative MAT loci of *Paraglomus* sp., and *R. irregularis* were identified by BLAST search procedures (see figure “Transcriptional directions of the putative AMF mating-type locus” and table “Identification of Meiosis specific genes” in Data S1). The tests for a two-evolutionary rates analysis was performed in part by measuring intergenic distance among genes in genome using a R script [22] (see figure of “Heatmap showing evidence for a “one speed genome” in *Geosiphon Pyriiformis*” in Data S1). For this study, published genomes of additional Glomeromycotina, Mortierellomycotina, and Mucoromycotina were downloaded from JGI portal MycoCosm database [74,75] [DOI: 10.1093/nar/gkt1183].

SNP calling and allele frequency analysis

Filtered Mate Pair and Paired End reads were mapped onto the assembled *G. pyriiformis* genome using the BWA-MEM v 0.7.17 algorithm [76] and sorted into a BAM file using samtools (v 1.9) [76]. Variants were called using FREEBAYES v1.2.0 [77] and filtered using vcftools [78]. Filtering cutoffs and procedures were as described by in Ropars et al. 2016 and Morin et al. 2019 [17,21] (see figure “Genome wide allele frequency of *G. Pyriiformis* shows it is a homokaryon” . Quality filtered variants and SNPs which passed filtering were used for constructing allele frequency plot using a custom R script (code available in GitHub repository).

Phylogenetic analysis and molecular dating

The phylogenomic analyses employed a set of 434 generally conserved and single-copy proteins in fungi (data available at DOI: 10.5281/zenodo.1413687), which were developed through efforts of the 1000 fungal genomes project and provided in the Joint Genome Institute MycoCosm site [1,75,79]. Profile-Hidden-Markov-Models of these markers were searched in the *Geosiphon* predicted protein sequences using HMMER3 (v3.1b2) [68] and recovered 393 homologs (out of the 434) in total. The 434 markers in 45 included fungal genomes were further collapsed into 57 partitions using a greedy search embedded in PartitionFinder v.2.1.1 for consistent phylogenetic signals [80]. Phylogenetic trees were produced using the PHYling pipeline (data available DOI:

1 10.5281/zenodo.1257002) and with maximum likelihood method implemented in IQ-TREE
2 (v.1.7-beta9) [81]. Concordance factors across the tree were calculated using the package
3 implemented in IQ-TREE.

4 The divergence time of *Geosiphon* sp. from the clade of "*Ambispora leptoticha* and *Paraglomus*
5 *occultum*" was estimated using the R8S v1.81 [82] with the phylogenetic tree reconstructed from
6 the earlier step. We employed five calibration constraints to calibrate the tree, including the
7 crown groups of Fungi (1100 MYA) [83], Dikarya (772 MYA) [83], Chytridiomycota (>573
8 MYA) [84–86], the MRCA of Chytridiomycota and terrestrial fungi (>750 MYA) [84–86], and
9 Glomeromycotina (>460 MYA) [11]. The divergence time of each clade was inferred using the
10 Langley-Fitch method with Powell algorithm [87–89].

11 **Alternative topology test and dating analyses**

12 To test the likelihood of other possible phylogenetic placements of *G. pyriformis*, we first
13 reconstructed the associated phylogenetic trees using constraint tree topology as illustrated in
14 Figure S2 via “-g” option of the IQTREE package (iqtree-1.7-beta9) [81] (see table “Results of
15 alternative tree topology test for phylogenetic tree” in Data S1). We then compared our best tree
16 (shown as Figure 3) with alternative topologies to compute the log-likelihoods of the trees using
17 Kishino-Hasegawa test, Shimodaira-Hasegawa test, expected likelihood weight, and
18 approximately unbiased test via “-zb” and “-au” parameters in IQTREE [90–94]. All tests were
19 performed with 10,000 resampling estimated log-likelihood (RELL) method for reliable results.
20 The best-fit substitution models for the genome-scale data matrix were estimated using
21 ModelFinder implemented in IQTREE package [95].

22 ***Detection of putative horizontal gene transfers***

23 To identify genes in *Geosiphon* that have potential origin in cyanobacteria, we compared the
24 *Geosiphon* sp. genome to the available fungal and cyanobacterial genomes. To highlight
25 potential HGT genes, we used a Python script (available in github repository) [96] to filter out
26 genome component in *G. pyriformis* with higher similarity score to cyanobacteria than any fungi,
27 excluding the *G. pyriformis* itself (see figure of “ Phylogenetic tree showing evidence of
28 horizontal gene transfer in selenium binding protein” and “Phylogenetic tree showing evidence
29 of horizontal gene transfer in Molybdenum cofactor carrier” in Data S1) (see table of “HGT
30 containing genes with pfam domains” in Data S1) .

Gene orthology and evolution of symbiotic specific genes

Orthogroups resulting from the OrthoFinder run were parsed using a custom Python script. To be retained, an orthogroup had to fill the following conditions: any sequence from the non-AMS fungi, at least one sequence of *Geosiphon pyriformis*, one sequence of either *Rhizophagus irregularis* or *Rhizophagus cerebriforme* and at least one sequence of *Gigaspora rosea* or *Diversispora epigaea* (see table of “Functions of genes which are gained in orthogroups” in Data S1). Reciprocally, orthogroups that could correspond to gene losses in the AMS fungi were extracted by retaining orthogroups with no sequences of AMS fungi and at least one sequence of each non-AMF fungi (Table S3) and (see table of “Orthogroups lost in the MRCA of Glomeromycotina” and “Missing MRCA genes across glomeromycotina core genes” in Data S1).

Orthogroups showing evidence of regulation in symbiotic conditions in *R. irregularis* were subjected to Maximum Likelihood (ML) analysis [92,97] to check for the absence of non-AMS species. First, proteins contained in orthogroups were searched against the nine proteomes of 9 distinct species using the BLASTp+ v2.9.0 [98] with default parameters and an e-value threshold fixed at 1e-05 (threshold was set to 1e-03 when no non-AMS species sequences were identified). Then, proteins were aligned using MUSCLE v3.8.31 [99] with default parameters and resulting alignment trimmed to remove positions with more than 80% of gaps using trimAl v1.4rev22 [100]. Prior to ML reconstruction, best fitting evolution model was tested using ModelFinder [95] and then ML analysis was performed using IQ-TREE v1.6.1 [81] with 10,000 replicates of SH-aLRT. Trees were visualized and annotated with the iTOL platform v5.5 [101,102] (Figure S3).

After first round of phylogeny, orthogroups showing an AMF specific pattern were blasted against the full MycoCosm database (1565 proteomes, last accessed: 03/01/2020) to confirm the AMF-specific pattern and a phylogenetic analysis was performed following the procedure described above.

Differential expression analysis and combination of expression data to orthogroups

Expression data of *Rhizophagus irregularis* in four conditions were used to select orthogroups containing gene significantly deregulated in symbiosis for further analysis. Paired-end reads were trimmed and fragments mapped onto Rhiir2_1 genome assembly of *R. irregularis*

(https://genome.jgi.doe.gov/Rhiir2_1/). Stringent settings of mapping were used (similarity and length read mapping criteria at 98% and 95%, respectively). Genes differentially expressed (DEG) in planta compared to extraradical mycelium were identified after EdgeR [103] normalization with a false discovery rate (FDR) correction using CLC Genomic Workbench (Qiagen). We retained genes showing an expression > 2- or < -2-fold times in planta compared to extraradical hyphae (FDR ≤ 0.05). Sets of 2683, 2518 and 2410 DEG were found in *M. truncatula*, *B. distachyon* and *L. cruciata* respectively (see table “Upregulation of ortholog genes from Rhiir2 genome” and “Downregulation of ortholog genes from Rhiir2 genome” in Data S1). Detailed information on the data are available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) portal (accession no GSE67926). The analysis performed on RNA-seq data from arbuscocytes in *M. truncatula* [36] presented 6359 DEG.

Quantification and Statistical analysis

Statistical analysis and graphs were generated using R studio version 4.0.1 (2020-06-06)

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Table 1: Summary statistics for genome assembly of sequenced *G. pyriformis* and other species from Glomeromycotina and selected Mucoromycota used in this study

Genomes	Assembly size	No of scaffolds	Scaffold N50	Largest scaffold (Kb)	Total Gap%	Repeat %	Busco completeness %	GC %
<i>Geosiphon Pyriformis</i>	129	795	703	2733.91	0.023	64.35	96.2	29.25
<i>Gigaspora rosea</i> V1.0	597.95	7526	734	1204.75	7.92	63.44	97.9	28.81
<i>Rhizophagus ceribriforme</i> DAOM227022 V1.0	136.89	2592	266	709.02	17.60	24.77	98.3	26.55

<i>Rhizophagus irregularis</i> DAOM 197198V2.0	136.80	1123	129	1375.86	5.06	26.38	98	27.53
<i>Diversispora versiformis</i> strain IT104	147	731	434	2010.39	0.061	43.6	98.2	25.1
<i>Rhizopus microsporus</i> ATCC11559 VI	25.97	131	8	2782.17	2.41	4.68	98.6	37.48
<i>Mucor Circinelloides</i> CBS 277.49 V2.0	36.59	26	4	6050.25	0.00	20.38	97.2	42.17
<i>Phycomyces Blakesleeanus</i> NRRL1555 V2.0	53.94	80	11	4452.46	1.06	9.74	96.9	35.78
<i>Mortierella Elongata</i> AG-77	49.86	473	31	1526.29	0.30	4.63	99.7	48.05

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