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The mitochondrial genome of the arbuscular mycorrhizal fungus Gigaspora margarita reveals two unsuspected trans-splicing events of group I introns

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Summary

- Arbuscular mycorrhizal fungi (AMF) are ubiquitous organisms that benefit ecosystems through the establishment of an association with the roots of most plants: the mycorrhizal symbiosis. Despite their ecological importance, however, these fungi have been poorly studied at the genome level.
- In this study, total DNA from the AMF Gigaspora margarita was subjected to a combination of 454 and Illumina sequencing, and the resulting reads were used to assemble its mitochondrial genome de novo. This genome was annotated and compared with those of other relatives to better comprehend the evolution of the AMF lineage.
- The mitochondrial genome of G. margarita is unique in many ways, exhibiting a large size (97 kbp) and elevated GC content (45%). This genome also harbors molecular events that were previously unknown to occur in fungal mitochondrial genomes, including trans-splicing of group I introns from two different genes coding for the first subunit of the cytochrome oxidase and for the small subunit of the rRNA.
- This study reports the second published genome from an AMF organelle, resulting in relevant DNA sequence information from this poorly studied fungal group, and providing new insights into the frequency, origin and evolution of trans-spliced group I introns found across the mitochondrial genomes of distantly related organisms.

Introduction

Arbuscular mycorrhizal fungi (AMF) are ecologically relevant organisms that are involved in one of the most widespread terrestrial symbioses with the roots of most land plants: the mycorrhizal symbiosis. In this symbiosis, the plant partners provide AMF with the sugars necessary for their survival, and the fungal partner increases the acquisition and uptake of several nutrients (e.g. phosphates and nitrates) by the plant from the soil. The presence of AMF in the soil therefore benefits many plants, and has been repeatedly correlated with an increase in the overall biodiversity of terrestrial ecosystems worldwide (Harrison, 1999; Sanders, 2002, 2003; Rosendahl, 2008; Bonfante & Genre, 2010; Corradi & Charest, 2011). In addition to their tremendous ecological importance, AMF have also been studied for their potentially atypical evolutionary history and genetic system. Indeed, AMF spores and hyphae are coenocytic and multinucleated throughout their entire life cycle, a feature unknown from other fungal relatives, and the real genetic structure of these co-existing nuclei (heterokaryons vs homokaryons) is currently a matter of extensive debate (Kuhn et al., 2001; Pawlowska & Taylor, 2004; Hijri & Sanders, 2005; Stukenbrock & Rosendahl, 2005; Rosendahl, 2008). AMF are also thought to have propagated in the absence of sexual
reproduction for at least 500 million years (My) (Redecker et al., 2000; Humphreys et al., 2010), a controversial view that has been challenged recently by reports of recombination in some of their populations (Vandenkoornhuyse et al., 2001; Croll & Sanders, 2009; den Bakker et al., 2010; Sanders & Croll, 2010), and by the presence of meiosis-related genes in many AMF species (Halary et al., 2011).

To date, publicly available sequence data from AMF include two mitochondrial genomes from species in the genus Rhizophagus (Lee & Young, 2009; Krüger et al., 2012) and a number of expressed sequence tag (EST) sequences (Tisserant et al., 2012). These data have been most instrumental in analyzing the position of the different fungal phyla (Liu et al., 2006, 2009; Lee & Young, 2009), and have revealed some of the genetic and molecular peculiarities of this ecologically critical lineage (Tisserant et al., 2012). In this study, we expanded the current sampling of available AMF sequence data by acquiring the complete mitochondrial genome sequence from another representative of this group: Gigaspora margarita. This species represents an additional model in AMF research, but belongs to a lineage (Diversisporales) for which very little nuclear and organellar sequence information is currently available. This evolutionary lineage is also very distinct from that of better studied species in the genus Rhizophagus (Glomerales) (Krüger et al., 2012), so that inspection of DNA sequences from its members may reveal new insights into their phylogenetic relationships.

Mitochondrial genomes often harbor group I and/or group II introns, self-splicing catalytic ribozymes that must be precisely excised from their surroundings (e.g. mRNA, rRNA or tRNA) for interrupted genes to regain their function. Both introns splice via a series of transesterification reactions (Johansen et al., 2006; Lambowitz & Zimmerly, 2011). Group I intron splicing starts with the binding of an exogenous guanosine to the catalytic core of the intron, whereas, in group II introns, the splicing mechanism starts with a nucleophilic attack on the 5′ splice site of the intron, either by an endogenous bulged adenosine or an exogenous hydroxyl residue. Trans- and cis-splicing of introns can occur (Glanz & Kuck, 2009). In trans-splicing, polynucleotide chains that are separated at the DNA level are joined together at the RNA level. The trans-splicing mechanism has long been thought to be exclusive to group II introns, but the first cases of trans-spliced group I introns have been reported recently, almost concomitantly, in three distantly related organisms, that is, a placozoan animal, a green plant and a green alga (Burger et al., 2009; Grewe et al., 2009; Pombert & Keeling, 2010).

Given the occurrence of trans-spliced group I introns in widely dispersed lineages, it is surprising that such events have yet to be found in the mitochondrial genomes of fungi (reviewed in Bullerwell & Lang, 2005; Lang et al., 2007). A probable reason for this may be the presence of sampling biases across the fungal phylogeny, so that trans-spliced introns may actually be present in some of the least-studied fungal lineages. Interestingly, AMF (phylum Glomeromycota) represent one of the fungal lineages whose mitochondrial genomes have been largely unexplored so far (Raab et al., 2005; Borstler et al., 2008; Lee & Young, 2009). The acquisition of the complete mitochondrial genome from G. margarita may therefore reveal the presence of a number of molecular features that were previously unsuspected to occur in fungal mitochondrial genomes.
Materials and Methods

**Gigaspora margarita** spore cultivation and harvesting

Spores of *Gigaspora margarita* Becker and Hall (isolate BEG34; deposited at the European Bank of Glomeromycota) were used for all experiments. These spores contain endobacteria, according to Lumini *et al.* (2007), and were produced in pot cultures in a climatic chamber, by inoculation on clover (*Trifolium repens* L.) pots, or *in-vitro* propagated in Petri dishes, on minimal medium, by inoculation of T-DNA-transformed chicory (*Cichorium intybus*) roots with a multipsporal inoculum of sterile spores (*Lumini et al.*, 2007). Spores were manually collected, rinsed three times with sterile water and surface sterilized with 3% (w/v) chloramine T and 0.03% (w/v) streptomycin, twice for 10 min, with one round of 10 s of sonication and, finally, rinsed three times for 10 min with sterile water, and stored at 4°C.

Nucleic acid extraction and reverse transcription-polymerase chain reaction (RT-PCR) procedures

Genomic DNA was extracted from c. 500 *G. margarita* spores, resulting in a total of 10 μg of total DNA. Briefly, spores were pelleted by centrifugation, resuspended in 300 μl of lysis solution (Epicentre Biotechnologies) containing proteinase K, mixed thoroughly using a vortex and crushed using a plastic pestle. The resulting mixture was immediately incubated at 65°C for 15 min, vortexed for 30 s every 5 min, and then cooled and incubated to 37°C for 30 min at the same temperature on addition of 2 μl of 5 μg μl⁻¹ RNase A. Following RNase treatment, the sample was placed on ice for 5 min, 150 μl of MPC Protein Precipitation Reagent (Epicentre Biotechnologies) were added and the solution was vortexed vigorously for 10 s. Protein debris was pelleted at 4°C for 10 min at a speed of ≥ 10 000 g and the supernatant was transferred to a new tube. DNA was then precipitated using isopropanol, rinsed twice using 70% ethanol and the DNA was finally suspended in TE buffer.

Total RNA was isolated using the QIAGen Plant RNA extraction kit following the manufacturer’s procedures. In this case, a total of 100 spores was pelleted by centrifugation, crushed using a plastic mortar and treated with a mixture of β-mercaptoethanol and a series of specific buffers supplied by the manufacturer. On extraction, total RNA was treated with RNase-Free DNase I (Epicentre Biotechnologies, Madison, WI, USA) to remove any traces of DNA contamination. In this case, the mixture was gently shaken at 37°C for 1 h in the presence of DNase I and specified buffer.

DNase-treated RNA was subjected to RT-PCR using the iScript kit (Bio-Rad Laboratories) following the manufacturer’s recommendations in order to produce cDNA. The cDNA was used for PCR with specific primers, and the resulting amplicons were subjected to Sanger DNA sequencing. This procedure was instrumental to detect and confirm the presence of *trans*-splicing in the *cox1* and *rns* genes of *G. margarita*, and to determine the exact location of several introns within the *rnl* gene. Specifically, a combination of primers located on exons 4 and 5 of the gene *cox1*, and on exons 1 and 2 of the *rns* gene, were used with DNase-treated cDNA as a template. PCRs produced amplicons whose sequences reconstructed a fully functional RNA molecule for both genes. The primers used to identify the *trans*-splicing events were as follows: *cox2F*, 5′-TCCTAGCTTGTTGATACATATGCTT-3′; *cox5R*, 5′-CGTGAAGTGATGAGGGAACGCTG-3′; *rnsF*, 5′-TCACACATGCGAGTCCATGA-3′; *rnsR*, 5′-TAAGGTGGACTAGGAGGATTTCTTA-3′;
**Genome sequencing and de novo assembly**

A total of 5 μg of *G. margarita* DNA was used to produce one Titanium DNA pyrosequencing library from Roche 454 Life Sciences (Branford, CT, USA) located at the Génome Québec Innovation Centre (McGill University, QC, Canada). A quarter of a Titanium 454 pyrosequencing run was performed, and 23% of the resulting reads were assembled using gsAssembler 2.6 (formerly known as Newbler) into 2828 contigs larger than 500 bp. The largest contigs were found to belong to the bacterial symbiont, *Candidatus Glomeribacter gigasporarum*, whose genome is publicly available (Ghignone *et al.*, 2012). Among all the contigs obtained, however, a total of six had clear homology with known mitochondrial sequences from other fungi.

In parallel, 500 ng of DNA from *G. margarita* was subjected to deep sequencing using the Illumina technology, with 100-bp paired-end reads (with an average insert of 337 bp), and a quarter of a channel on the GAIIx instrument located at Fasteris S.A. (Geneva, Switzerland). This procedure was used to increase the coverage for many regions of the mitochondrial genome, as well as to confirm the source and validate the assembly of the contigs assembled using 454 pyrosequencing data (i.e. additional quality check based on spatial information from the paired-end reads). The Illumina and 454 reads were reassembled together using the hybrid assembler MIRA 4 (Chevreux *et al.*, 2004), in combination with the mitochondrial contigs previously identified with the gsAssembler. The contigs resulting from this hybrid assembly were converted to, edited and re-assembled with CONSED 19.0 (Gordon *et al.*, 1998, 2001; Gordon, 2003), resulting in the assembly of three contigs of obvious mitochondrial origin (i.e. no significant homology with the endosymbiotic bacterial genome or with nuclear homologs from other species) with similar coverage. None of the 454 reads assembling onto the genome data of *Candidatus Glomeribacter gigasporarum* align with the mitochondrial genome of *G. margarita*.

**Genome completion, annotation and analysis**

PCR with a number of primer sets, bacterial cloning and Sanger sequencing were used in combination with CONSED to link the three contigs of mitochondrial origin into a single, circular molecule with an average coverage of 20× (Supporting information Fig. S1a). The mitochondrial genome of *G. margarita* was annotated manually using Artemis 13.2.0 (Carver *et al.*, 2008) in combination with BLAST (Altschul *et al.*, 1997, 2005) procedures against the ‘nonredundant’ repository at the National Center for Biotechnology Information (NCBI). tRNAs were detected using RNAweasel 5.1.2 and tRNAscan-SE 1.21 (Lowe & Eddy, 1997; Lang *et al.*, 2007). Group I and group II introns were manually detected, and repeats were searched with Tandem Repeats Finder (Benson, 1999). The mitochondrial genome of *G. margarita* is deposited in GenBank under the accession number JQ041882.

**Phylogenetic analyses**

In the present study, a phylogeny based on 14 mitochondrial genes from representatives of all extant fungal phyla was reconstructed. The mitochondrial genes include seven NADH dehydrogenase subunit units (*nad1*-6 and *nad4L*), three cytochrome oxidase subunits (*cox1*, 2 and 3), three ATP synthase subunits (*atp1*, 6, 8, 9/c) and the apocytochrome b (*cob*). Amino acid sequences of these genes were also obtained from representatives of the ascomycetes, basidiomycetes, chytridiomycetes, blastocladiomycetes, zygomycetes and the AMF *Rhizophagus irregularis* and *G. margarita*.

The amino acid sequences of the different genes were aligned using MUSCLE 3.6 (Edgar, 2004), and concatenated using bioinformatics tools implemented in Geneious 5.5. Best-fit models of
evolution were chosen using ProtTest 3 (Darriba et al., 2011). Phylogenies were reconstructed using the maximum likelihood algorithm implemented in PhyML 3.0 (Guindon et al., 2010) with 1000 bootstraps and the LG + I + Γ model of evolution. Bayesian analyses were performed using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001), the model WAG + I + Γ, with 1 000 000 generations and a ‘burn in’ of 100 000. In parallel, the CAT + Γ4 model of amino acid substitution implemented in PhyloBayes 3 (Lartillot et al., 2009) was also used. In this case, two concurrent chains were run in parallel and were terminated using the PhyloBayes automatic stopping rule (maxdiff < 0.1).

**Results**

High-throughput DNA sequencing using both the Illumina and 454 pyrosequencing platforms was performed using total DNA extracted from *G. margarita*, resulting in the acquisition of 233 365 400 bp (in 4 667 308 reads) and 95 500 945 bp (in 266 716 reads) of raw sequence data, respectively. Subsequent hybrid Illumina and 454 assemblies resulted in three contigs with clear homology with known mitochondrial genomes, which were linked by PCR and confirmed by Sanger sequencing. These systematic procedures resulted in the acquisition of a single, circular molecule, the mitochondrial map of *G. margarita*, which we annotated using available programs (Lowe & Eddy, 1997; Lang et al., 2007; Carver et al., 2008). The *G. margarita* mitochondrial genome (mtDNA) maps as a single circular molecule of 96 998 bp (Fig. 1) featuring strong sequence homology with that of other fungal relatives. The overall GC content (45%) of the *G. margarita* mtDNA is higher than that of many fungi, however, and this increase affects both its coding and noncoding regions equally (e.g. intergenic regions are 45.15% GC vs 43.55% for gene features; tRNA is 49.52% GC and rRNA is 47.12% GC; Table 1). The genes identified in the *G. margarita* mtDNA (Table 1) are scattered among the two DNA strands. We took advantage of this genome annotation to test recent hypotheses regarding the placement of AMF within the fungal phylogeny, and phylogenetic analyses resulted in very high statistical support for most branches (Fig. 2).
Figure 1. The mitochondrial genome map of *Gigaspora margarita* isolate BEG34. Filled boxes represent genes located across the genome. Genes located outside the map are transcribed clockwise, and genes located inside the map are transcribed counterclockwise. Introns are shown as white (group I) or gray (group II). tRNA genes are shown according to their one-letter amino acid code, followed by their anticodon. Open reading frames (ORFs) smaller than 100 amino acids are not shown. The regions of the genomes that are joined by trans-splicing are shown as red and green inner circles for *cox1* and *rns* exons, respectively.
Table 1. Genome features of *Gigaspora margarita* and other fungal mtDNAs

<table>
<thead>
<tr>
<th></th>
<th><em>Gigaspora margarita</em></th>
<th><em>Rhizophagus irregularis</em></th>
<th><em>Saccharomyces cerevisiae</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>Allomyces macrogynus</em></th>
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<tr>
<td>Size (bp)</td>
<td>96 998</td>
<td>70 606</td>
<td>85 779</td>
<td>57 473</td>
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<tr>
<td>GC content (%)</td>
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<td>36.79</td>
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<tr>
<td>Total</td>
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<td>37.18</td>
<td>17.11</td>
<td>39.5</td>
</tr>
<tr>
<td>Genes&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>tRNAs</td>
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<td>25</td>
</tr>
<tr>
<td>rRNAs</td>
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<td>2</td>
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<td>2</td>
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<tr>
<td>Group II</td>
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<td>4</td>
<td>2</td>
</tr>
<tr>
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<td>0.221</td>
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<tr>
<td>Coding %</td>
<td>15.5</td>
<td>24.3</td>
<td>28.5</td>
<td>38.5</td>
</tr>
</tbody>
</table>

1. <sup>a</sup>Corresponds to strain FY1674.
2. <sup>b</sup>Includes protein-encoding genes with known functions, structural RNAs, as well as free-standing and intronic open reading frames (ORFs).
3. <sup>c</sup>Cis/trans-spliced.
4. <sup>*</sup>*Rhizophagus irregularis* harbors two ORFs (GIY-YIG endonucleases) that are not present in *G. margarita*.
Figure 2. Fungal phylogeny inferred from the concatenation of 14 mitochondrial genes. The major fungal phyla are surrounded by rounded rectangles. The tree is based on an unambiguous alignment of 3668 amino acid positions, and rooted using Chytridiomycota as outgroup. Black circles highlight branches with posterior probabilities of unity for Bayesian analyses performed with MrBayes (WAG + I + Γ model of evolution) and PhyloBayes (CAT + Γ4 model of evolution). Black circles also indicate a bootstrap support greater than 98% for maximum likelihood analyses performed using PhyML (LG model of evolution), unless a numerical value is present above the black circle. In these cases (two in total), the numerical values represent the bootstrap support for the respective nodes. Scale bar corresponds to 0.2 amino acid substitutions per site. *Fusarium and Gibberella represent different anamorphs.

The coding capacity of the *G. margarita* mitochondrial genome is very similar to that of its closely related species *Rhizophagus irregularis*, with which it shares a complement of 14 protein-encoding genes with known functions, 25 tRNAs and two rRNAs. The *G. margarita* mtDNA features four intronic open reading frames (ORFs) located within the *cox1* and *nad5* genes, and two putative free-standing endonucleases (*orf129* and *orf132*) displaying degenerated single LAGLIDADG motifs (*Fig. 1*). A second LAGLIDADG motif found downstream of the *orf129* reading frame and separated by a stop codon suggests that this ORF is a pseudo-gene, on its way out of the genome. The *G. margarita* mitochondrial genome also harbors one group II intron within the *rnl* (large subunit of the rRNA) gene. Very little gene order conservation is observed between the two species, reflecting the rapid shuffling of genomic material typical of fungal mitochondrial genomes. Genes are encoded on the different strands in *G. margarita*, but not in *G. intraradices*, and most of the increased size in the *G. marginata* mtDNA relative can be accounted for by the presence of much larger intergenic regions. No evidence for the presence of extensive interspersed repeated elements (*Benson, 1999*) or heteroplasmy was found. In particular, all reads assembled on our reference map appeared to be monomorphic, and no relevant polymorphism could be identified.
The *G. margarita* mtDNA features a total of 22 introns (21 group I and one group II; Table 1) inserted within five genes. Of these, two are group I introns that are surprisingly spliced *in trans* (Fig. 3). These *trans*-spliced group I introns are inserted within the cytochrome oxidase I (*cox1*) and the small subunit of the rRNA (*rns*) genes, respectively. In both cases, exons from these genes are found scattered across the genome on opposite strands. These unusual features were confirmed both *in vitro* by specific PCR products and sequencing, and *in silico*, as these regions are covered by many reads from 454, Illumina and Sanger sequencing technologies (Fig. S1a,b). The first four *cox1* exons (of eight in total) are located 28 kb apart from the rest of the coding sequence on opposite strands, whereas the two *rns* exons are also on opposite strands, but separated by 24 kb (Fig. 3a). RT-PCRs using specific primers, followed by sequencing of the resulting products, confirmed that exons of both genes are linked at the RNA level *in trans* (Fig. 3b). Sequence inspection revealed the presence of potentially *trans*-spliced group I introns, which appear to be broken in the L8 loop in between the P8 pairings (Fig. 3c). Investigation of *cox1* intron insertion sites revealed that the *G. margarita* *trans*-spliced intron is inserted at the same location as the first of two *trans*-spliced group I introns recently found in *Trichoplax adhaerens* (a primitive metazoan) (Fig. 4). The *G. margarita* and *T. adhaerens* introns share only traces of sequence similarity within their conserved core. To our knowledge, no other *trans*-spliced group I intron has been reported in *rns*.

**Figure 3.** *Trans*-splicing of group I introns in *cox1* and *rns*. (a) Structure of the *cox1* (left) and *rns* (right) exons in the mitochondrial genome of *Gigaspora margarita*. Roman numbers indicate the order and location of the different exons (eight exons in total for *cox1*, left; two exons in total for *rns*, right). Arrows indicate the location and direction of the primers used in reverse transcription-
polymerase chain reaction (RT-PCR) procedures. (b) RT-PCR procedures demonstrating the presence of trans-splicing in the cox1 and rns genes. Specific primers produced a band of the expected size and sequence only when used on cDNA. gDNA was used as a control. An extremely faint band appearing using gDNA and rns-specific primers represents an unspecific product, which we confirmed by DNA sequencing. (c) Left, cox1 intron; right, rns intron. The GI structures were modeled according to the conventions described in Burke & RajBhandary (1982). The exon/intron splice junctions are denoted by arrows. Canonical Watson–Crick base pairings are indicated by dashes. Guanine–uracil pairings are indicated by dots. Numbers inside variable loops and above straight lines indicate their length. The putative junctions between the segments of the trans-spliced introns are denoted by asterisks (located in L8).

![Diagram](image)

**Figure 4.** Intron insertion sites in cox1 from selected organisms of various evolutionary lineages. Location of introns in the mitochondrial cox1 genes of Gigaspora margarita (fungus; this article), Trichoplax adhaerens (placozoan animal; Burger et al., 2009), Helicosporidium sp. (nonphotosynthetic green alga; Pombert & Keeling, 2010) and Isoetes engelmannii (vascular plant; Grewe et al., 2009). The cox1 coding regions are shown as dark gray bars; cis-splicing and trans-splicing group I introns are depicted by white triangles and Y-shaped symbols, respectively; group II introns are shown by gray triangles. Light gray shading highlights introns located at homologous sites among different organisms. nt, nucleotides. The cox1 gene in Selaginella moellendorffii (Hecht et al., 2011) also contains a trans-splicing intron at the same position as in the sister lycophyte, Isoetes engelmannii.

**Discussion**

**Structure and content of the G. margarita mitochondrial genome**

The mitochondrial genome of G. margarita represents the third AMF organelle genome ever sequenced, and the first from a representative of the Diversiporales. Its coding capacity is similar to that found in other fungi, and is almost identical to that of the AMF G. intraradices, the only exceptions being a few unknown, and possibly spurious, ORFs. In particular, the proteome encoded by the genome includes 14 proteins with known functions, involved in a restricted number of processes that are typically found in fungal mitochondria (i.e. electron transport, oxidative phosphorylation) (Bullerwell & Lang, 2005).
Surprisingly, however, this apparent conservation in the gene repertoire is not mirrored at other levels. For instance, at 97 kbp, the mitochondrial genome of *G. margarita* is at the high end of the size spectrum for the kingdom (*Bullerwell & Lang, 2005*). It is also intriguing that the *G. margarita* mitochondrial genome is very GC rich compared with those of most fungi. This variation in GC content is reflected in both coding and noncoding regions, and so this characteristic does not appear to be related to translational constraints. The underlying mechanism giving rise to this nucleotide bias is difficult to assess, but recent analyses of mitochondrial genomes from unicellular organisms characterized by a high GC content have led to suggestions that, rather than being under selective (i.e. adaptive) pressure, GC bias may be a result of neutral processes, such as biased gene conversion or ‘GC drive’ (loss of certain repair functions) (*Barth & Berendonk, 2011; Smith et al., 2011*).

**Phylogenetic relationships of extant fungal phyla based on mitochondrial protein-encoding genes**

The phylogenetic placement of AMF within the fungal tree of life has long been a matter of debate. These plant symbionts were recognized for some time as a potential sister lineage to the higher fungal phyla (i.e. Ascomycota and Basidiomycota; *Schwarzott et al., 2001; James et al., 2006*), but recent analyses of concatenated nuclear and mitochondrial sequences have suggested that AMF may represent one of many monophyletic phyla (Glomeromycota; *Hibbett et al., 2007*) composing the base of the fungal tree of life (*Corradi & Sanders, 2006; Liu et al., 2006, 2009; Lee & Young, 2009*).

In this study, the amino acid sequences of fungal mitochondrial protein-encoding genes were used to determine the relationships of AMF mitochondrial genomes (i.e. *G. margarita* and *G. intraradices*) with those of other members of the kingdom. Our phylogenetic analyses resulted in a robust fungal phylogeny (i.e. posterior probabilities of 1.0 and bootstrap support > 98% for most branches) that appears to be consistent with the most recent reconstructions of the fungal tree based on mitochondrial and nuclear sequence data (*Liu et al., 2006, 2009; Lee & Young, 2009*). Specifically, our phylogeny placed the AMF as a potential sister lineage to a basal fungal lineage, the Mortierellales, with high statistical support in most cases. Indeed, Bayesian reconstructions using different models resulted in a posterior probability of 1.0 for this particular node, whereas maximum likelihood analyses resulted in a statistical support higher than that reported by others using very similar datasets (*Lee & Young, 2009*).

Unfortunately, this strong phylogenetic signal could not be linked to the presence of shared genomic signatures (i.e. unique genes shared between the groups or conserved gene order) between the AMF and the Mortierellales, which is most probably a result of the high level of gene shuffling that characterizes fungal mitochondrial genomes. Certainly, similar analyses could strongly benefit from the use of concatenated mitochondrial and nuclear datasets, as a number of discrepancies have been reported between phylogenies reconstructed using organellar and nuclear genes. Together with the addition of many more species representative of the diversity of Glomeromycota and Mucoromycota, such analyses will be critical to resolve the origin and evolution of the most basal lineages of the fungal tree of life.

*Gigaspora margarita* shares a trans-spliced group I intron at a cognate site with a placozoan animal

The phenomenon of trans-splicing in organelles has long been thought to be restricted to the group II introns found in plant/algal mitochondria and chloroplasts (*Glanz & Kuck, 2009*), but we now know that similar trans-splicing events can also involve group I introns within the *cox1* genes of a
metazoan animal (*Trichoplax*), a vascular lycophytic plant (*Isoetes engelmannii*) and a
nonphotosynthetic alga (*Helicosporidium*) (*Burger et al., 2009; Grewe et al., 2009; Pombert &
Keeling, 2010*). In this study, we newly demonstrate that group I trans-splicing also occurs in
fungal organisms, and that it can also affect the maturation of the SSU rRNA; therefore, the
identification of these previously unsuspected events offers a unique opportunity to better
comprehend the origin and evolution of these rare mechanisms.

The *G. margarita* *cox1* trans-spliced intron (intron 4) is located at the same position as a *cis*-spliced
intron 8 homolog in the related fungus *G. intraradices* (*Lee & Young, 2009*). Introns of both
species share sequence similarity in their core region (e.g. can be folded into a similar group I
structure, but the sequence similarity is relatively low), but, in *G. intraradices*, the intron is
characterized by the presence of a complete LAGLIDADG ORF, suggesting that it has been
acquired recently. Homologous genomic locations have been invaded by *cis*-spliced group I introns
in a number of diverse fungi (e.g. *Schizosaccharomyces pombe, Gibberella zeae* (*Lang, 1984*), but
are intronless in the more closely related species *Rhizopus* and *Mortierella*. Together, these features
indicate that this specific genomic location is very prone to intronic invasions, and that the group I
intron at this location is likely to have originated recently in the AMF, undergoing subsequent
disruption and trans-splicing in the lineage leading to *G. margarita*.

Perhaps even more interesting is the fact that the *G. margarita* trans-spliced intron is located at a
position homologous to one of two trans-spliced introns identified in the *cox1* gene of *Trichoplax*, a
primitive animal (*Burger et al., 2009*). The *cis*-spliced *cox1* introns found at the same position in
other species share significant sequence similarity with their trans-counterparts. The structural
similarity between *G. margarita* and *T. adhaerens* also extends to the number of introns, as both
species share a total of four introns: that is, the same number seen between the two closely related
fungi *Gigaspora* and *Rhizophagus* (although only three are at cognate sites in all three organisms).
Together, these features are consistent with the view that, after invasion of a genome, copies of the
group I mobile element spread to additional compatible sites.

The trans-splicing event common to the fungus *G. margarita* and the placozoan *T. adhaerens* has
yet to be identified at the same location in other species, but it is worth noting that a similar event
has been observed in close vicinity. Indeed, in *Helicosporidium* sp., the *cox1* gene is trans-spliced
at a site that is 11 nucleotides upstream of that found in *G. margarita* (*Pombert & Keeling, 2010*).
This position represents a widely recognized ‘hot spot’ for group I introns in various fungi, and is
found in *T. adhaerens* and plants (*Cho et al., 1998; Burger et al., 2009*). The two closely located
*cox1* positions that harbor trans-spliced introns map to a highly conserved region of the
transmembrane domain VI of the Cox1 protein, and a functionally important histidine codon is
located between them (*Shapleigh et al., 1992*).

Although it may seem unlikely for trans-spliced introns located at identical positions to have
independent histories (i.e. in the *cox1* of a fungus and an animal), this site is a known homing site
for group I introns, and there is also precedence for the independent conversion of introns from a
cis- to a trans-form. The latter is exemplified by *nad1* intron 4 (group II type) in flowering plants,
where the break, in some cases, is upstream of the *matR* intronic ORF and, in other cases,
downstream of it (*Qiu & Palmer, 2004*). Thus, some group I/II introns may be more susceptible and
tolerant to a discontinuous gene structure than others, and this seems to be particularly true for those
located in the *cox1* gene. Overall, the notorious invasive nature of group I introns appears to be
compatible with an independent and recent acquisition of trans-spliced forms at cognate sites in
*G. margarita* and *T. adhaerens*. 
Trans-splicing of group I intron in the small subunit of the rRNA: a recent event?

Given the hundreds of group I introns that have been identified to date in bacterial and organellar genes, it is surprising that, so far, trans-splicing of group I introns has been found only in the coxl gene; to our knowledge, the Gigaspora rns gene represents the first example of trans-splicing of a structural RNA gene in organelles.

The G. margarita rns gene harbors all conserved core features found in homologs from other species, but appears to be atypical in other ways. First, the gene is separated into two exons at a very unusual position, the variable region 7, a region of the SSU rRNA structure that is not a known homing site for group I introns. Indeed, very few introns of any type have been found in this region of the SSU rRNA, and only one intron of 950 (790 of which were group I type) was identified nearby, and it was a spliceosomal intron in a nuclear SSU rRNA gene (Jackson et al., 2002). Accordingly, even in known cases of fragmented mitochondrial rns genes, none has been found to be broken within variable region 7 (cf. Chlamydomonas species V1, V2, V3, V6, Scenedesmus V3, Tetrahymena and Paramecium V2, Euglena V8; Waller & Jackson, 2009; Barbrook et al., 2010; Spencer & Gray, 2011). Second, the location of the rns trans-spliced intron is also unique because introns are typically found in the functionally important regions of the rRNA (Jackson et al., 2002). The model that is currently favored for the generation of scrambled rRNA molecules is by DNA recombination across repeats located within the variable V regions. In these cases, it has been suggested that the resulting separate RNA molecules can form base pairing within the conserved regions and still function in the ribosome. In G. margarita, however, this mechanism does not occur, as this species has used a more classical trans-splicing mechanism to link disrupted rns exons. So how did this novel trans-splicing event arise in the mitochondrial genome of G. margarita?

Although G. intraradices has a cis-splicing counterpart of the G. margarita coxl trans-intron, its SSU rRNA gene is intronless. This indicates that the trans-splicing event affecting the rns gene is unlikely to have derived from their common ancestor, and that it has probably appeared recently in the lineage leading to Gigaspora. Whatever the mechanism involved in the origin of this novel trans-splicing event, the location of the 3′ portion of the rns rRNA gene in G. margarita, which is close to, but on the opposite strand, relative to the rnl gene, indicates that it must have acquired a new promoter on rearrangement in order to be functional (rather than hitch-hiking along in a pre-existing transcriptional unit). Interestingly, the 3′ segment of the trans-splicing coxl gene is located beside the rns segment, and therefore it is plausible that the same promoter could have fuelled its expression following a recent disruption of the rns gene in Gigaspora.

Concluding remarks

The present study further highlights the importance of a broad taxonomic sampling in order to understand the frequency and nature of molecular mechanisms that have long appeared to be very rare, or restricted to a few lineages. Accordingly, the complete mitochondrial genome of G. margarita demonstrates that events of group I intron trans-splicing may be more common than previously thought. This genome sequence also represents valuable data from a group of organisms for which DNA sequences have been notoriously lacking. This new wealth of AMF sequence data may be used to develop a battery of molecular markers for studies of AMF biodiversity, or to further understand the function of this essential organelle in these ecologically critical organisms, especially during the establishment of the mycorrhizal symbiosis.
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