The mitochondrial genome of the ethanol-metabolizing, wine cellar mold
***Zasmidium cellare* is the smallest for a filamentous ascomycete**

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ABSTRACT

Fungi in the class *Dothideomycetes* often live in extreme environments or have unusual physiology. One of these, the wine cellar mold *Zasmidium cellare*, produces thick curtains of mycelial growth in cellars with high humidity, and its ability to metabolize volatile organic compounds including alcohols, esters and formaldehyde is thought to improve air quality. It grows slowly but appears to outcompete ordinarily faster-growing species under anaerobic conditions. Whether these abilities have affected its mitochondrial genome is not known. To fill this gap, its mitochondrial genome was assembled as part of a whole-genome shotgun-sequencing project. The circular-mapping mitochondrial genome of *Z. cellare*, at only 23,743 bp, is the smallest yet reported for a filamentous fungus. It contains the complete set of 14 protein-coding genes seen typically in other filamentous fungi, along with genes for large and small ribosomal RNA subunits, 25 predicted tRNA genes capable of decoding all 20 amino acids, and a single open reading frame potentially coding for a protein of unknown function. The *Z. cellare* mitochondrial genome had genes encoded on both strands with a single change of direction, different from most other fungi but consistent with the *Dothideomycetes*. The high synteny among mitochondrial genomes of fungi in the *Eurotiomycetes* broke down almost completely in the *Dothideomycetes*. Only a low level of microsynteny was observed among protein-coding and tRNA genes in comparison with *Mycosphaerella graminicola* (synonym *Zymoseptoria tritici*), the only other fungus in the order *Capnodiales* with a sequenced mitochondrial genome, involving the three gene pairs *atp8-atp9*, *nad2-nad3*, and *nad4L-nad5*. However, even this low level of microsynteny did not extend to other fungi in the

Dothideomycetes and *Eurotiomycetes*. Phylogenetic analysis of concatenated protein-coding genes confirmed the relationship between *Z. cellare* and *M. graminicola* in the *Capnodiales*, although conclusions were limited due to low sampling density. Other than its small size, the only unusual feature of the *Z. cellare* mitochondrial genome was two copies of a 110-bp sequence that were duplicated, inverted and separated by approximately 1 kb. This inverted-repeat sequence confused the assembly program but appears to have no functional significance. The small size of the *Z. cellare* mitochondrial genome was due to slightly smaller genes, lack of introns and non-essential genes, reduced intergenic spaces and very few ORFs relative to other fungi rather than a loss of essential genes. Whether this reduction facilitates its unusual biology remains unknown.

Keywords: filamentous, fungus, mitochondrial genome, smallest, synteny, *Zasmidium cellare*

Introduction

Mitochondrial genomes have been greatly reduced since their origin from a presumed α -prokaryotic ancestor (Gray 2012). Their remaining gene contents vary widely among eukaryotes, with from three to 94 genes (Lang et al. 1999), including those for coupled electron transport and oxidative phosphorylation, transcription and translation, RNA processing, protein import and maturation (Lang et al. 1999; Gray 2012). Gene contents of fungal mitochondrial genomes are much more uniform, typically containing 24-43 genes including 14 for oxidative phosphorylation, the large and small subunit ribosomal RNA genes (*rnl* and *rns*), and usually 20

or more genes for tRNAs. Among those for oxidative phosphorylation, there usually are seven for NADH:ubiquinone oxidoreductases (Complex I: *nad 1, 2, 3, 4, 4L, 5* and *6*), one for ubiquinol:cytochrome c oxidoreductase (Complex III: *cob*), three for cytochrome c:O₂ oxidoreductases (Complex IV: *cox1, 2* and *3*) and three for ATP synthase subunits (Complex V: *atp6, 8* and *9*). Genes of Complex II, found in plants and animals, are missing from the mitochondrial genomes of fungi. Other genes that are present in some fungal mitochondrial genomes include those for ribosomal protein subunits (*rps3* or *rps5*) and RNA processing (*rnpB*). In addition to these genes that were inherited vertically from the original bacterial donor, some fungi contain genes for DNA and RNA polymerases, reverse transcriptases, maturases and homing endonucleases that most likely are transferred horizontally and vary considerably among species.

Within the *Ascomycota*, mitochondrial gene order can vary widely, particularly in the *Saccharomycetes* (Aguileta et al. 2014), but can be much more uniform in other groups. For example, mitochondrial genomes within the *Sordariomycetes* and *Eurotiomycetes* tend to be highly syntenic, containing the same sets of 14 protein-coding genes, large and small ribosomal RNA subunit genes and clusters of tRNA genes in approximately the same order within each group (Kouvelis et al. 2004; Sun et al. 2011). In almost all ascomycetes, mitochondrial genes are encoded on a single strand so are transcribed in the same direction.

Ascomycetes that differ from the usual mitochondrial gene content often have unusual biology. For example, yeasts that produce energy through anaerobic fermentation rather than oxidative respiration often contain reduced sets of mitochondrial genes. Most yeasts, regardless of whether they divide by budding (*Saccharomyces cerevisiae*) or fission (*Schizosaccharomyces pombe*), have lost the *nad* genes (Complex I) that are present in the mitochondrial genomes of

almost all other fungi (Sekito et al. 1995; Koszul et al. 2003) and this loss can be stimulated by exposure to ethanol (Ibeas & Jimenez 1997). Loss of mitochondrial genes can be phylogenetically informative because it is a one-way process; once a gene has been lost from a mitochondrial genome it is not gained back (Adams & Palmer 2003) so the loss will be transferred vertically to future generations.

A filamentous fungus with unusual biology is *Zasmidium cellare*. This species, known as the wine cellar mold or, in Austria, as the “schwartze katze” (“black cat”) (Mandl et al. 2010), forms thick mats of mycelia in wine cellars when the humidity is high (Guéguen 1906; Tribe et al. 2006; Mandl et al. 2010) and ethanol vapor is present (Schanderl 1936). This fungus is able to metabolize and grow on vapors of alcohols (including ethanol), esters, formaldehyde and numerous other volatile organic compounds (Schanderl 1936; 1958) and, due to this ability, its presence is thought to improve the air quality within wine cellars (Schanderl 1936; Tribe et al. 2006). For this reason some vintners in Europe encourage its growth by spraying cellar walls with leftover wine (Tribe et al. 2006). Cellar walls in these wineries often are festooned with curtains of mycelial growth, which can be marketed as a positive selling point (Tribe et al. 2006). Despite this, spores of *Z. cellare* are not produced prolifically so are not detected in air samples (Haas et al. 2010; Mandl et al. 2010) and this species appears to have no negative effects on wine quality (Schanderl 1936; Mandl et al. 2010).

Other than its ability to metabolize volatile organic compounds (Schanderl 1958), very little is known about the biology or physiology of *Z. cellare*. Its growth in culture is slow and it often is overgrown by *Penicillium* and/or *Aspergillus* (Mandl et al. 2010), the two dominant members of the mycoflora in air samples from most wine cellars (Haas et al. 2010), but it was not overgrown by these competitors under anaerobic conditions (Mandl et al. 2010) where it may

have an advantage. Whether these abilities to metabolize ethanol vapor and other organic molecules and to outcompete ordinarily faster-growing fungi under anaerobic conditions are reflected in changes to its mitochondrial gene content or organization is not known.

The taxonomy and evolutionary relationships of *Z. cellare* have been cryptic until recently, as reviewed thoroughly by Tribe *et al.* (2006). The wine cellar mold was originally named *Racodium cellare* by Persoon in 1794 (Persoon 1794) with the generic name meaning “rag and tatter” (Tribe *et al.* 2006). In 1936 it was transferred to *Cladosporium* by Schanderl (1936) and was known as *C. cellare* for much of the last century until being moved to *Zasmidium* by Hawksworth & Riedl (1977). Analysis of sequences from the internal transcribed spacer (ITS) region of the ribosomal RNA genes showed that *Z. cellare* is most closely related to fungi in the class *Dothideomycetes*, particularly *Ramichloridium cerophilum* (Tribe *et al.* 2006), a black yeast isolated from dried leaves of bamboo plants (*Sasa* sp.) in Japan (de Hoog 1977). A recent, thorough analysis of *Ramichloridium* and related genera confirmed this relationship and showed that *Z. cellare* belongs in the family *Mycosphaerellaceae* of the order *Capnodiales* of the class *Dothideomycetes* (Arzanlou *et al.* 2007).

The *Dothideomycetes* is the largest and most ecologically diverse group of fungi, including saprobes and pathogens of plants and (more rarely) animals, lichens, endophytes and mycorrhizal associates, in both terrestrial and aquatic habitats (Goodwin 2014). Many of the saprobes are extremophiles that grow under conditions of exceptional heat or cold and the pathogens are known from virtually every major family of plants (Goodwin 2014). Substrates colonized range from rocks and statues on Mediterranean islands (Gorbushina *et al.* 1993) to mangrove roots that are alternately exposed to saltwater immersion and tropical sun (Alias & Jones 2000), to exposed faces of rocks under the harsh solar radiation, extreme cold and desiccation of Antarctica

(Selbmann et al. 2005).

Despite this high number of species and interesting ecological and physiological diversity, so far only two mitochondrial genomes have been published for members of the *Dothideomycetes*, those for the wheat pathogens *Phaeosphaeria* (synonyms *Parastagonospora* and *Stagonospora*) *nodorum* (Hane et al. 2007) and *Mycosphaerella graminicola* (synonym *Zymoseptoria tritici*) (Torriani et al. 2008). Both of these species have mitochondrial genomes of moderate size, ranging from 44 to 50 kb for *M. graminicola* and *P. nodorum*, respectively (Hane et al. 2007; Torriani et al. 2008). Unlike all other ascomycetes, genes on these two mitochondrial genomes were transcribed from both strands; each genome had a single change with approximately 40% of the genes oriented in the opposite direction, although the genes on each strand were not consistent between the species. Unlike most other fungal mitochondrial genomes, that of *P. nodorum* was missing the *atp8* and *atp9* genes (Hane et al. 2007), but it did contain a gene for ribosomal protein *rps5*, which was absent from that of *M. graminicola* (Torriani et al. 2008) and some other fungi. From this very limited sampling it appears that the mitochondrial genomes of species of *Dothideomycetes* have some significant differences from those in most other fungi. Whether these differences are common in the *Dothideomycetes* or are specific to the two sampled species is not known.

Due to its unusual biology and taxonomic placement in the *Dothideomycetes*, it seems likely that the mitochondrial genome of *Z. cellare* might be different from those in other fungi. The goal of this research was to analyze the mitochondrial genome of *Z. cellare* to test the hypothesis that its possible ability to grow anaerobically might have resulted in a reduced mitochondrial genome, specifically that the *nad* genes might have been lost as they have been in many yeasts. Secondary goals were to test whether a single change of direction is common in

Dothideomycetes mitochondrial genomes and whether a phylogeny of mitochondrial genes supports the placement of *Z. cellare* in the order *Capnodiales*.

Materials and methods

Mitochondrial genome sequencing and annotation

The mitochondrial genome of *Z. cellare* was sequenced through the Community Sequencing Program of the U.S. Department of Energy's Joint Genome Institute (<http://jgi.doe.gov/collaborate-with-jgi/community-science-program/>) and was assembled from sequence reads generated for the whole-genome shotgun sequencing project (<http://genome.jgi.doe.gov/Zasce1/Zasce1.home.html>). The *Zasmidium cellare* ATCC 36951 genome was sequenced using the Illumina platform. Two Illumina libraries (fragment, with 270-bp insert; and 3.5-kb-long mate-pair) were sequenced in 2×150- and 2×100-bp formats, respectively. Genome data were filtered for artifacts, processed for contamination and subsequently assembled with AllPathsLG release version R41043 (Gnerre *et al.* 2011). The mitochondrial genome was assembled separately from the nuclear genome and resulted in a single, 41.5×-read coverage of a 22,976-bp contig, which was then further improved into a circular 23,743-bp assembly.

Initial annotation of the mitochondrial genome was with MFannot (<http://megasun.bch.umontreal.ca/cgi-bin/mfannot/mfannotInterface.pl>) based on Genetic Code 4, and a custom Perl script was used to extract the annotations into a table. Predictions made by

MFannot were then verified and modified manually. Confirmation and further analysis of tRNA gene predictions was done with tRNAscan-SE 1.21 (Schattner *et al.* 2005) with Source set to Mito/Chloroplast and Genetic Code for tRNA Isotype Prediction set to Mold & Protozoan Mito.

A custom Perl script and Sequin (<http://www.ncbi.nlm.nih.gov/projects/Sequin/index.html>) were used to produce files in the sqn and gbk formats for further analysis and submission to GenBank. For visual representation of the resulting mitochondrial genome, information in the annotation table was rearranged manually and then used as input into CGView (Stothard & Wishart 2005). The resulting XML file was edited and then used to make a circular diagram of gene features and orientations with CGView. Final modifications to the figures were made with Adobe Illustrator.

Identification of repeated sequences was done with Tandem Repeats Finder (Benson 1999) and by manual *blastn* (Altschul *et al.* 1990) searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with *bl2seq* using each half of the sequence as a query separately against the complete sequence.

Testing for circularity of the Z. cellare mitochondrial genome

A culture of *Z. cellare* isolate ATCC 36951 was grown in yeast sucrose broth (10 g of yeast extract, 10 g of sucrose per L of water) for 10 days at room temperature with shaking. Mycelia were harvested and DNA was extracted using the Qiagen DNEasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). Four pairs of primers for polymerase chain reaction (PCR) analysis based on the initial *Z. cellare* mitochondrial genome assembly were designed using the PrimerQuest tool on the Integrated DNA Technologies (IDT, Coralville, Iowa, USA) web site (<http://www.idtdna.com/primerquest/home/index>) and subsequently ordered from IDT. Four

primers (Zcellmt-end1R: ACACTAGGACTTGAACCTAGAATAAC; Zcellmt-end2R: GTTGTAAGGGTATTAAGTTAGGTG; Zcellmt-end1F: GATCACTAGGCTTCGGGTCTAA; and Zcellmt-end2F: GGTTTCCCTAGATTCAAACAGATTT; all written 5' to 3') were designed proximal to inverted repeats to amplify over each end and both inverted repeats of the initial mitochondrial assembly to test for circularity of the sequence. Two sets of primers were designed in different regions of the interior of the mitochondrial sequence as controls. These were Zcellmt-int1F (5'-GCTATTATTGCCTTTATTGAAGTAGGTG-3') and Zcellmt-int1R (CTGAATAAGTTAACGGAAGGAAAGC), which amplified a portion of the *nad5* gene, and Zcellmt-int2F (5'-AGGGATGACCTTGAAATATGCT-3') and Zcellmt-int2R (TCGTTTATTTGCCACACGTTTA), which amplified the end of the *atp8* gene, all of *atp6*, and the beginning of the *cox3* gene. PCR was performed using all possible combinations of primers at the ends of the initial assembly (end1R/1F, end1R/2F, end2R/1F, end2R/2F) and using the int1R/1F and int2R/2F primer pairs. Each PCR was set up as follows: 12.5 µL of MyTaqRed (Bioline, Taunton, MA, USA), 7.5 µL of water, 2 µL of R primer (4 µM), 2 µL of F primer (4 µM), and 1 µL of template DNA (12.5 ng/µL) for a total of 25 µL per reaction. Thermal cycling was performed in an MJ Research (Waltham, MA, USA) PTC-100 thermal cycler for 2 min at 95 °C followed by 40 cycles of 1 min at 94 °C, 1 min at 51 °C and 2 min at 72 °C followed by 7 min at 72 °C and a 4 °C hold.

Amplification products from the end1F/1R and end2F/2R primer combinations were chosen for cloning and sequencing. Bands were excised from a 1% agarose gel containing 1× SYBR Safe DNA gel stain (Invitrogen Life Technologies, Grand Island, NY, USA) and purified using the Qiagen (Qiagen Inc., Valencia, CA, USA) QIAquick gel extraction kit. Insert DNA was

ligated into pCR2.1-TOPO vector and transformed into TOP10 chemically competent *E. coli* cells using the Invitrogen TOPO TA cloning kit (Invitrogen Life Technologies, Grand Island, NY, USA). After growth on color-selective medium, positive colonies were picked and grown in LB+carbenicillin broth (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, plus 50 mg of carbenicillin per L of water) and grown overnight at 37 °C on an orbital shaker at 250 rpm. Plasmid DNA was extracted from the resulting cultures using the QIAprep Spin Miniprep kit (Qiagen Inc., Valencia, CA, USA). The purified plasmid DNA was then digested with restriction enzyme *EcoRI* for 2 hours at 37 °C and the fragments were separated on a 1% agarose gel containing 1× SYBR Safe DNA gel stain (Life Technologies, Grand Island, NY, USA) to confirm the sizes of the inserts and vector. Three clones from each selected primer combination were sent to the DNA Sequencing Low Throughput Laboratory at the Purdue Genomics Core Facility (Purdue University, West Lafayette, IN, USA) for sequencing with universal M13 forward and reverse primers. Sequences were trimmed of vector and aligned using MacDNAsis (Hitachi Software, San Francisco, CA, USA).

After new sequence was obtained that joined and extended the two ends of the original assembly, new primers were designed in this region (ZcellmtnewF: TAGCCGACCTGACTCGTAATA; and ZcellmtnewR: GCACTCAGACTTGAAGTGAAGAA). PCR reactions were performed pairing these new primers with the original end primers in all possible combinations to test whether the mitochondrial genome was circular or linear.

Secondary structure prediction

Secondary structures of DNA and possible RNAs produced from a 110-bp inverted repeat

sequence were predicted with the RNAfold program (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>) of the Vienna RNA websuite (Gruber et al. 2008). The program was run with default values to predict the best secondary structure of possible RNA produced from each strand of the 110-bp repeat based on minimum fold energy.

Phylogenetic analysis

Published mitochondrial genome sequences of the two *Dothideomycetes* available, *M. graminicola* and *P. nodorum*, plus those of four species in the *Eurotiomycetes* (*Aspergillus fumigatus*, *A. nidulans*, *Penicillium digitatum* and *P. solitum*), and three *Sordariomycetes* (*Metarhizium anisopliae*, *Podospora anserina*, and *Verticillium dahliae*) were downloaded from GenBank (accession numbers NC_010222, NC_009746, NC_017016, NC_017896, NC_015080, NC_016187, NC_008068, NC_001329 and NC_008248, respectively) for comparison with *Z. cellare*. DNA sequences of the 14 protein-coding genes *nad1-6* and *4L*, *cob*, *cox1-3* and *atp6*, 8-9 were extracted from the GenBank files, aligned separately with MAFFT (<http://www.ebi.ac.uk/Tools/msa/mafft/>), concatenated manually and then a phylogenetic tree was constructed using the RAxML program with default values through the CIPRES gateway (<http://www.phylo.org/index.php/>). Alignments were verified and modified manually when necessary before phylogenetic analysis.

Synteny comparisons

To analyze the syntenic relationships among the mitochondrial genomes, the same species for the

phylogenetic analysis except without the *Sordariomycetes* were downloaded from GenBank and rearranged manually so that they all began at the 5' end of the *rns* gene. All genomes were then aligned, compared and visualized using Easyfig, a Python application that accepts multiple annotated sequences in standard formats and shows areas of alignment based on blast comparisons (Sullivan et al. 2011).

Testing for anaerobic growth of Z. cellare

Anaerobic growth was assayed by observing radial growth in nitrogen-purged serum vials containing potato-dextrose agar (PDA). Two ml of sterile melted PDA was added to sterile 75 × 20 mm flat-bottomed serum vials and solidified as horizontal slants under sterile conditions. Two cubes of actively growing *Zasmidium cellare* (ATCC 36951) cultures (approximately 3 mm per side) were excised from a PDA plate and placed on each slant surface. The vials were capped with sterile stoppers and sealed by crimping. Four replicate vials containing two cubes per vial were prepared. Two 18-gage needles were inserted through the stoppers and the vials were purged with nitrogen for 10 min. The needles were plugged and after 3 hours the nitrogen-purging process was repeated. The needles were then removed and the vials incubated at room temperature in the dark. Aerobic control cultures consisted of identical vials that were not purged with nitrogen. Growth originating from the cubes was observed *in situ* over a period of 11 days using a Leica MZ16 stereo microscope.

Results

Assembly and circularity of the Z. cellare mitochondrial genome

Assembly of the whole-genome shotgun reads of *Z. cellare* resulted in a mitochondrial genome of 22,976 bp that was identified as being linear. Initial annotation of this sequence revealed that it contained all of the genes present in the mitochondrial genome of its relative, *Mycosphaerella graminicola*, except for six tRNA genes that were mostly present in a single cluster in other species (data not shown). This assembly also contained a 110-bp identical repeat that was inverted near each end of the sequence, similar to inverted repeats seen in the telomeres of fungi with known linear mitochondrial genomes (Dinouël et al. 1993).

Based on this information, a second assembly was performed with different parameters and greater sequence coverage to test the linear-genome hypothesis and to see whether the mitochondrial sequence could be improved. This process resulted in a mitochondrial assembly of only 22,654 bp, the shortest for a filamentous ascomycete, which began and ended with the 110-bp inverted repeats. This second assembly appeared to confirm that the *Z. cellare* mitochondrial genome was linear, although the repeats could provide a means of circularization during replication.

To further test whether the *Z. cellare* mitochondrial genome is linear, PCR amplification products were cloned, sequenced and compared to the original two assemblies. Amplification of the two internal control regions gave products of the expected sizes (data not shown). However, the four primer combinations designed to amplify over the ends of the supposedly linear genome yielded products that were much larger than expected, which was not consistent with a linear genome. Sequencing of these products revealed that they were identical to the ends of the

original assembly but extended it by an additional 767 bases. Annotation of this additional sequence showed that it contained the six tRNA genes that are present in the mitochondrial genomes of related fungi but were missing from the original assembly. Amplifications with the original primers plus an additional pair within the newly generated sequence all gave products of the expected size. Therefore, the mitochondrial genome of *Z. cellare* appears to be circular mapping rather than the linear genome identified in the original assembly.

Annotation of the Z. cellare mitochondrial genome

The final circular mitochondrial genome of *Z. cellare* (GenBank accession number XYZ) was 23,743 bp long, the smallest for a filamentous fungus (Table 1). The only fungi with smaller mitochondrial genomes are yeasts, yeast-like or related to yeasts. *Ashbya gossypii* has a slightly smaller mitochondrial genome and grows filamentously but is in the order *Saccharomycetales* so is a yeast phylogenetically. True filamentous ascomycetes (*Pezizomycotina*) with the next-smallest mitochondrial genomes were in the class *Eurotiomycetes* (Table 1), but those were several hundred bases larger than the compact mitochondrial genome of *Z. cellare*.

Coding regions of the *Z. cellare* mitochondrial genome contained the usual complement of genes seen typically in filamentous fungi: the protein-coding genes *atp6*, *atp8-9*, *cob*, *cox1-3*, *nad1-6* and *nad4L*; ribosomal RNA genes *rnl* and *rns*; and 25 tRNA genes with at least one anticodon for each of the 20 amino acids. There was one ORF of unknown function coding for a potential protein of 238 amino acids. None of the genes appeared to contain any introns. Consistent with most other fungi and translation table 4, all of the protein-coding genes including the ORF began with ATG and ended with the ochre stop codon TAA except for *nad2*, which

started with TTG instead of ATG, and *nad6*, which had alternative start (TTG) and stop (TAG, amber) codons. There was a one-base overlap between the *nad4L* and *nad5* genes, i.e., the last base of the *nad4L* gene was the first base of *nad5*, and the *nad2* and *nad3* genes were consecutive with no separation between the end of *nad2* and the beginning of *nad3*. Anticodons for all 20 amino acids were represented at least once among the 25 tRNA genes. There were two tRNAs each with different anticodons for the amino acids arginine, leucine and serine, and three tRNAs for the single methionine anticodon. All but two of the 25 tRNAs had seven bases in their anticodon loop; the exceptions were phenylalanine and threonine, which each had a nine-base anticodon loop. As with mitochondrial genomes of other *Dothideomycetes*, genes were coded on both strands with a single change of direction.

Other than its very small size, the only unusual feature of the *Z. cellare* mitochondrial genome was a 110-bp inverted repeat that occurred near the location where the direction of transcription changed. Overall G + C content of the *Z. cellare* mitochondrial genome was 27.8%, which was lower than those seen in other *Dothideomycetes* but higher than for fungi in the *Eurotiomycetes* (Table 2).

Compared to related fungi, gene content of the *Z. cellare* mitochondrial genome was pared down slightly, with fewer ORFS, 2-3 fewer tRNA genes and no introns (Table 2). All of the extra genes (*rnpB*, *rps5*, RNA polymerases) that were identified in one or more related genomes were absent from that of *Z. cellare*. Lengths of the protein-coding genes in general were quite uniform across species (Table 3). Sizes of three genes, *atp8* (147 bp), *atp9* (225 bp) and *cox3* (810 bp), were identical among the *Dothideomycetes* and *Eurotiomycetes* compared (Table 3). The overall length of protein-coding genes in the mitochondrial genome of *Z. cellare* was slightly less than those for the other fungi, but only about 100 bp less than that of *Penicillium*

solitum, the species with the next-most-smallest total length of protein-coding genes. The *Z. cellare* mitochondrial genome also had a smaller length of tRNA and ribosomal RNA genes, but the difference in each was only a few hundred bases compared to the species with the next-most-smallest mitochondrial genome (Table 3). The main size difference in actual or potential coding regions was in the ORFs; the 717 bases of the single ORF in the mitochondrial genome of *Z. cellare* was less than half that of ORFs in *P. digitatum*, the species with the next-smallest total size of ORFS (Table 3), but was significantly less than the more than 7 kb of ORFs in the mitochondrial genomes of the other two *Dothideomycetes*. The mitochondrial genome of *Z. cellare* also had reduced sizes of intergenic spaces; the percent of the genome coding for genes or ORFs ranged from 61 to 82 for other *Dothideomycetes* and *Eurotiomycetes* compared to 85 for the mitochondrial genome of *Z. cellare* (Table 3).

Secondary structure of a 110-bp inverted repeat

Two copies of a 110-bp sequence were identified that were inverted and separated by 1089 bp. One copy was located between tRNA genes with anticodons for serine and alanine (Fig. 1). The second copy began 24 bases prior to the start and overlapped the first 86 bases of the *rnl* gene. The RNAfold analyses of the inverted repeats revealed significant secondary structures of predicted RNA produced by either strand (Fig. 2). Both strands form structures containing multiple stem loops (hairpins), with a double loop occurring in one structure (Fig. 2A). For the copy located between and transcribed from the same strand as the two tRNA genes (Fig. 2A), the centroid structure was identical to the minimal fold energy structure, indicating that the prediction is reliable (Gruber et al. 2008). However, for the copy that spanned the beginning of

the *rnl* gene (Fig. 2B) the centroid structure was very different, indicating that the quality of the prediction was poor.

Blastn analyses (Altschul et al. 1990) of this repeated sequence against the GenBank non-redundant database and against the mitochondrial genome sequence of *M. graminicola* identified alignment only to part of the sequence in two unrelated species. The best alignment was with 67 bases of the repeat to the mitochondrial genome of *M. graminicola*, at the beginning of the *rnl* gene (84% identical, e-value of $1e^{-15}$). The other hits were to 82 bases in mitochondrial genome sequences of the yeast *Saccharomyces cerevisiae*, in non-coding DNA between tRNA genes (76% identical, $6e^{-4}$). In each case only one copy was present so this duplication appears to be unique to the mitochondrial genome of *Z. cellare*.

Phylogenetic analyses of Z. cellare mitochondrial genes

The phylogenetic analysis showed a clear separation between the *Eurotiomycetes* and *Dothideomycetes* with different species in the two genera of *Eurotiomycetes* clustering together as expected (Fig. 3). This analysis also showed that *Z. cellare* clustered with *M. graminicola* in the *Capnodiales*, separate from *P. nodorum* in the *Pleosporales* (Fig. 3). Although the sampling depth of mitochondrial genomes was too low for firm conclusions, the mitochondrial phylogeny appears to confirm previous results based on analyses of nuclear genes.

Synteny comparisons of mitochondrial genomes

As expected, the mitochondrial genomes of species in the *Eurotiomycetes* were highly syntenic,

containing the same genes in the same order and orientation, and all coded on the same strand (Fig. 4). Exceptions were few and very minor, usually involving only movement of tRNA genes from one genome to another. There was some synteny between several genes in the mitochondrial genome of *P. nodorum* and fungi in the *Eurotiomycetes*, but this broke down almost completely in the *Capnodiales* where inversions and other more extensive rearrangements were very common (Fig. 4). Mitochondrial genomes in the *Dothideomycetes* were completely mixed up, in contrast to the high degree of synteny seen among those in the *Eurotiomycetes*.

Comparisons between the mitochondrial genomes of Z. cellare and M. graminicola

Comparisons between the two mitochondrial genomes that are available for species in the *Capnodiales* revealed a few similarities but many differences. The coding strand for each species had a single change of direction with approximately 40% of the genome transcribed in one direction and the remaining 60% from the opposite strand (Fig. 5). However, the genes on each strand were almost completely different. For example, the major genes on the “short” strand for *M. graminicola* were (in order) *rnl*, *cox3*, *nad1* and *cox1*, while those on the comparable strand for *Z. cellare* were *rns*, *cox2*, *nad1* and *nad4L-nad5*; only the *nad1* gene was in common between the two short strands.

Gene orders in general between the two genomes were almost completely different. Microsynteny extended to only a few small groups of genes: *atp8-atp9*; *nad2-nad3*; and *nad4L-nad5* (with the last base of the *nad4L* gene serving as the first base of the *nad5* gene) for protein-coding genes; plus two groups of four and three groups of two tRNA genes. The single ORF in the mitochondrial genome of *Z. cellare* was completely different from anything in the *M.*

graminicola genome, while all ten of those in *M. graminicola* were unique to that genome.

In addition to the ORFs of unknown function, the mitochondrial genome of *M. graminicola* contained genes for RNA polymerases and *rnpB* as well as two additional tRNA genes that were absent from the compact mitochondrial genome of *Z. cellare*.

Anaerobic growth of Z. cellare

The control cultures proliferated and grew during the 11 days of the experiment. However, hyphae under the nitrogen (anaerobic) treatment elongated, but then became very thin; by the end of 11 days growth and elongation seemed to have stopped. It was not clear whether the initial elongation was due to anaerobic growth, use of existing (stored) resources, or aerobic growth using residual or leaked oxygen in the culture system. Under the culture conditions tested *Z. cellare* did not show clearly discernable anaerobic growth. However, these experiments were rudimentary and did not provide a definitive answer.

Discussion

The mitochondrial genome of *Z. cellare*, at only 23,743 bp, is the smallest yet reported for a filamentous fungus. All fungi with smaller mitochondrial genomes are either yeasts, yeast-like or related to yeasts; so far no fungus in the *Pezizomycotina* has a published mitochondrial genome smaller than that of *Z. cellare*. Unlike many yeasts, where a small mitochondrial genome was due to loss of the *nad* genes (Sekito et al. 1995; Koszul et al. 2003), those genes were all present in the mitochondrial genome of *Z. cellare*. Therefore, there was no evidence for

convergent evolution for loss of the *nad* genes even though *Z. cellare* also appears able to grow anaerobically (Mandl et al. 2010). Instead, paring down of the *Z. cellare* mitochondrial genome was more general and included a complete lack of introns, slightly smaller gene sizes, loss of all protein-coding genes that are extraneous for the primary purpose of aerobic respiration, shorter intergenic spacers and a reduced amount of the genome containing ORFs of unknown function compared to all other fungi.

Like mitochondrial genomes of other fungi in the *Dothideomycetes* but unlike those for most other classes, there was a single change in the direction of transcription for genes on the mitochondrial genome of *Z. cellare*, leaving two contiguous blocks with about 40% of the coding region on one strand and the remaining 60% transcribed in the opposite direction. Interestingly, the particular genes on each strand among species were completely different, even between *Z. cellare* and *M. graminicola*, which are much more closely related compared to *P. nodorum* in the *Pleosporales*. The high degree of synteny seen among the mitochondrial genomes of fungi in the *Eurotiomycetes* broke down almost completely in the *Dothideomycetes*. In the *Capnodiales*, only very small areas of microsynteny remained, including the three gene pairs *atp8-atp9*, *nad2-nad3* and *nad4L-nad5*.

However, even this limited microsynteny was not conserved across the other groups. Genes *atp8* and *atp9* occurred separately in the *Eurotiomycetes* mitochondrial genomes, and were missing completely from the mitochondrial genome of *P. nodorum*. The *nad2-nad3* gene pair was conserved across all three *Dothideomycetes*, where they always occurred consecutively with no intergenic space, but this did not extend to the four *Eurotiomycetes* in which these genes were always separated. The only gene pair that was conserved across the *Dothideomycetes* and *Eurotiomycetes* was *nad4L-nad5*, but with three slightly different arrangements. In the two fungi

in the *Capnodiales* and the two species of *Aspergillus* in the *Eurotiomycetes*, these genes had the same structure including overlap between the last base of *nad4L* and the first base of *nad5*. In the two *Penicillium* species these genes were consecutive but without the one-base overlap, and in *Phaeosphaeria nodorum* they were separated by non-coding DNA plus an ORF of unknown function. Therefore, overall there was not a single pair of genes that was completely syntenic between the mitochondrial genomes of the four *Eurotiomycetes* and three *Dothideomycetes* compared. This most likely indicates that there is no functional significance of gene order in fungal mitochondrial genomes.

The phylogenetic analysis of all 14 protein-coding genes confirmed a much closer relationship between *Z. cellare* and *M. graminicola* and is consistent with both species being in the order *Capnodiales*. However, the sampling depth of species in the *Dothideomycetes* is still too limited for good resolution of phylogenetic relationships based on sequences from mitochondrial genomes alone. These analyses will become much more valuable as additional mitochondrial genomes are sequenced.

Other than its small size, the only really unusual feature of the *Z. cellare* mitochondrial genome was the 110-bp identical, inverted repeat that was separated by just over 1 kb of sequence containing several tRNA genes. This repeat originally fooled the assembly program into identifying the mitochondrial genome as linear and the error was only sorted out by designing PCR primers to amplify over the ends of the predicted assembly. Based on this experience, future reports of linear mitochondrial assemblies should be tested carefully to confirm whether they are linear or circular.

The origin and function of the 110-bp repeats are not known. The repeat sequence is capable of forming secondary structures including hairpins that are superficially similar to the telomeres

seen in organisms with known linear mitochondrial genomes (Dinouël et al. 1993; Forget et al. 2002; Nosek et al. 1998). However, the 110-bp sequence is much shorter than the 700 bp and up in other fungi (Dinouël et al. 1993; Forget et al. 2002; Nosek et al. 1995) and it does not contain the subrepeats that usually are present in mitochondrial telomeres (Dinouël et al. 1993; Nosek et al. 1995; Nosek et al. 1998). Furthermore, the circularity of the *Z. cellare* mitochondrial genome makes it highly unlikely that this sequence functions as a telomere. Forty percent of the sequence duplicates the first 44 bases of the *rnl* gene, which includes the part of this sequence that also was present in other fungi based on *blastn* analyses. One copy of the repeat occurred near the location where the direction of transcription changed. The lack of synteny among genes on the mitochondrial genomes of fungi in the *Dothideomycetes* implies that these genomes must be subject to frequent rearrangements. Therefore, the most likely explanation is that the 110-bp identical, inverted repeat arose as an artifact of mitochondrial rearrangements and probably has no functional significance.

The slight variations from the usual start and stop codons in the *Z. cellare* mitochondrial genes were mostly unique, indicating some plasticity for translation signals of mitochondrial genes. For example, all 14 protein-coding genes in the mitochondrial genome of *M. graminicola* began with the usual ATG initiation codon (Torriani et al. 2008) including the *nad2* and *nad6* genes which both started with a TTG codon in *Z. cellare*. Only one gene, *nad6*, ended with a codon (TAG) other than TAA in the mitochondrial genome of *Z. cellare*, compared to three genes, including *nad6*, that ended with TAG in *M. graminicola* (Torriani et al. 2008). Interestingly, the two tRNAs (for phenylalanine and threonine) with nine rather than seven bases in their anticodon loops also were the only two with nine among the *M. graminicola* tRNAs (Torriani et al. 2008), and this extended to *P. nodorum* but not the four *Eurotiomycetes*, where

the only tRNA with nine bases in the anticodon loop was for threonine. The only exception was *Aspergillus nidulans*, which had a second tRNA for arginine with a nine-base anticodon loop. Therefore, a tRNA for phenylalanine with nine bases in its anticodon loop seems to be specific for the *Dothideomycetes*.

The connection between the unusual biology of *Z. cellare* and its small mitochondrial genome is not currently known. Its ability to grow anaerobically could not be confirmed and appears to be limited at best, which may explain why the gene content of the *Z. cellare* mitochondrial genome is typical for a filamentous fungus. Its organization is similar to other *Dothideomycetes*, with two sections of contiguous genes coded on opposite strands and a scrambled gene order relative to other species. The mechanism of mitochondrial genome rearrangements in the *Dothideomycetes* is not known but appears to have left an inverted repeat in the *Z. cellare* mitochondrial genome as an artifact. Sequencing of mitochondrial genomes from many additional species of *Dothideomycetes* is needed for a more complete picture of mitochondrial genome evolution of fungi in this class.

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Table 1 – The position of *Zasmidium cellare* (in bold) among the 25 smallest fungal mitochondrial genomes in the GenBank**Fungi mitochondrial genomes database****(<http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=4751&opt=organelle>) and their taxonomic classifications.**

Species	GenBank accession	Genome length (bp)	Proteins	tRNAs	Class	Order	Configuration	Growth form	Reference
<i>Hanseniaspora uvarum</i>	NC_007780	18844	7	23	Saccharomycetes	Saccharomycetales	Linear	Yeast	Pramatef taki et al. 2006
<i>Schizosaccharomyces pombe</i>	NC_001326	19431	10	25	Schizosaccharomycetes	Schizosaccharomycetales	Circular	Yeast (fission)	Schäfer et al. 2005
<i>Candida glabrata</i>	NC_004691	20063	8	23	Saccharomycetes	Saccharomycetales	Circular	Yeast	Koszul et al. 2003
<i>Vanderwaltozyma polyspora</i> ^a	NC_009638	21684	8 ^b	26 ^c	Saccharomycetes	Saccharomycetales	Circular	Yeast	— ^d
<i>Candida orthopsilosis</i>	NC_006972	22528	14	24	Saccharomycetes	Saccharomycetales	Circular	Yeast	Kosa et al 2006
<i>Pneumocystis carinii</i>	NC_013660	22893	15	20	Pneumocystidomycetes	Pneumocystidales	Linear	Yeast-like	Sesterhenn et al. 2010
<i>Candida zemplinina</i> ^f	NC_005972	23114	8	25	Saccharomycetes	Saccharomycetales	Circular	Yeast	Pramatef taki et al. 2008
<i>Wickerhamomyces pijperi</i> ^g	NC_022166	23268	7 ^b	25 ^c	Saccharomycetes	Saccharomycetales	Circular	Yeast	—
<i>Ashbya gossypii</i> ^h FDAG1	NC_016756	23497	7 ^b	23 ^c	Saccharomycetes	Saccharomycetales	Circular	Filamentous but related to yeasts	—
<i>Ashbya gossypii</i> ^h ATCC 10895	NC_005789	23564	7 ^b	23 ^c	Saccharomycetes	Saccharomycetales	Circular	Filamentous but related to yeasts	—
<i>Kluyveromyces thermotolerans</i> ⁱ	NC_006626	23584	8	24	Saccharomycetes	Saccharomycetales	Circular	Yeast	Talla et al. 2005
<i>Zasmidium cellare</i>	TBD	23743	14	25^c	Dothideomycetes	Capnodiales	Circular	Filamentous	This report
<i>Arthroderma otae</i> ^j	NC_012832	23943	15	25	Eurotiomycetes	Onygenales	Circular	Filamentous	Wu et al. 2009

<i>Arthroderma obtusum</i> ^k	NC_012830	24105	15	25	Eurotiomycetes	Onygenales	Circular	Filamentous	Wu et al. 2009
<i>Candida metapsilosis</i>	NC_006971	24152	14	24	Saccharomycetes	Saccharomycetales	Linear ^l	Yeast	Kosa et al 2006
<i>Trichophyton mentagrophytes</i>	NC_012826	24297	15	25	Eurotiomycetes	Onygenales	Circular	Filamentous	Wu et al. 2009
<i>Lecanicillium muscarium</i>	NC_004514	24499	14	25	Sordariomycetes	Hypocreales	Circular	Filamentous	Kouvelis et al 2004
<i>Pneumocystis murina</i>	NC_020332	24608	14	28	Pneumocystidomycetes	Pneumocystidales	Linear	Yeast-like	Ma et al. 2013
<i>Metarhizium anisopliae</i>	NC_008068	24673	15	24	Sordariomycetes	Hypocreales	Circular	Filamentous	Ghikas et al 2006
<i>Cryptococcus neoformans</i> var. <i>grubii</i>	NC_004336	24874	15 ^b	21 ^c	Tremellomycetes	Tremellales	Circular	Filamentous	—
<i>Clavispora lusitaniae</i> ^m	NC_022161	24896	13 ^b	25 ^c	Saccharomycetes	Saccharomycetales	Circular	Yeast	—
<i>Cryptococcus neoformans</i> var. <i>grubii</i> H99	NC_018792	24919	15 ^b	22 ^c	Tremellomycetes	Tremellales	Circular	Filamentous	—
<i>Metacordyceps chlamydosporia</i> ⁿ	NC_022835	25615	14 ^b	25 ^c	Sordariomycetes	Hypocreales	Circular	Filamentous	—
<i>Saccharomyces castellii</i> ^o	NC_003920	25753	8	23	Saccharomycetes	Saccharomycetales	Circular	Yeast	Petersen et al 2002
<i>Candida sake</i>	NC_022168	26205	13 ^b	25 ^c	Saccharomycetes	Saccharomycetales	Circular	Yeast	—
<i>Candida theae</i>	NC_022169	26234	14 ^b	24 ^c	Saccharomycetes	Saccharomycetales	Linear	Yeast	—

^a Synonym: *Kluyveromyces polysporus*

^b Annotated with Mfanot

^c Determined with tRNAscan-SE

^d Not yet published or publication could not be found

^e Linear mitochondrial genomes occur in some isolates of this species

^f Synonym: *Starmerella bacillaris*

^g Synonym: *Pichia pipperi*

^h Synonym: *Eremothecium gossypii*

ⁱ Synonym: *Lachancea thermotolerans*

^j Anamorph: *Microsporum canis*

^k Anamorph: *Microsporum nanum*

^l Circular mitochondrial genomes also occur in this species

^m Anamorph: ⁿ Synonym: *Pochonia chlamydosporia*

^o Synonym: *Naumovozyma castellii*

Table 2 – Sizes and mitochondrial genome contents among all species in the class *Dothideomycetes* (orders *Capnodiales* and *Pleosporales*) with published information and four representatives of the *Eurotiomycetes*.

Species ^b	Order	Size (bp)	G + C content (%)	Number of genes ^a				
				Protein coding ^c	ORFs ^d	tRNA ^e	Missing genes	Introns
<i>Zasmidium cellare</i>	<i>Capnodiales</i>	23743	27.8	14	1	25	<i>rnpB</i> , <i>rps3</i>	None
<i>Mycosphaerella graminicola</i>	<i>Capnodiales</i>	43964	31.9	15	16	28	<i>rps3</i>	None
<i>Phaeosphaeria nodorum</i>	<i>Pleosporales</i>	49761	29.4	12 ^f	10	27	<i>atp8</i> , <i>atp9</i> , <i>rnpB</i> , <i>rps3</i>	5
<i>Aspergillus nidulans</i>	<i>Eurotiales</i>	33227	24.9	15	5	27	<i>rps3</i>	4
<i>Aspergillus fumigatus</i>	<i>Eurotiales</i>	30696	25.5	16	6	27	—	1
<i>Penicillium digitatum</i>	<i>Eurotiales</i>	28978	25.3	16	3	27	—	None
<i>Penicillium solitum</i>	<i>Eurotiales</i>	28601	25.5	15	2	27	<i>rps3</i>	None

^a All species contained genes for the large and small ribosomal RNA subunits (*rnl*, *rns*) so those are not included in these counts. Gene contents were verified with MFAnnot (<http://megasun.bch.umontreal.ca/cgi-bin/mfannot/mfannotInterface.pl>) and tRNAscan-SE (<http://lowelab.ucsc.edu/tRNAscan-SE/>) to make sure the annotation methods were consistent.

^b *Zasmidium cellare*, *Mycosphaerella graminicola* (synonym *Zymoseptoria tritici*), and *Phaeosphaeria* (synonyms *Stagonospora* and *Parastagonospora*) *nodorum* are in the class *Dothideomycetes*, while *Aspergillus fumigatus*, *A. nidulans*, *Penicillium digitatum* and *P. solitum* are in the *Eurotiomycetes*.

^c These include the 14 genes involved in oxidative respiration plus *rnpB* and *rps3*.

^d Open reading frames coding for potential proteins with no similarity to other genes in the Genbank non-redundant database. Some had similarity to RNA polymerase genes but those were considered as ORFs for this comparison.

^e Each species contained tRNA genes with anticodons sufficient to translate all 20 amino acids.

^f The *cox1* and *cox2* genes of *P. nodorum* are transcribed together with no stop between them so code for a polyprotein in this species.

Table 3 – Comparison of coding region sizes among three fungi in the class *Dothideomycetes* and four representatives of the *Eurotiomycetes* with sequenced mitochondrial genomes.

Gene	<i>Zasmidium cellare</i>	<i>Mycosphaerella graminicola</i>	<i>Phaeosphaeria nodorum</i>	<i>Aspergillus fumigatus</i>	<i>Aspergillus nidulans</i>	<i>Penicillium digitatum</i>	<i>Penicillium solitum</i>
<i>atp6</i>	774	774	774	771	771	774	774
<i>atp8</i>	147	147	—	147	147	147	147
<i>atp9</i>	225	225	—	225	225	225	225
<i>cob</i>	1164	1173	1200	1158	1164	1158	1158
<i>cox1</i>	1596	2010	1617 ^a	1716	1527	1707	1722
<i>cox2</i>	753	750	753 ^a	762	762	762	762
<i>cox3</i>	810	810	810	810	810	810	810
<i>nad1</i>	1062	1158	1116	1062	1059	1056	1056
<i>nad2</i>	1692	1743	1704	1689	1665	1689	1689
<i>nad3</i>	375	369	447	408	411	408	357
<i>nad4</i>	1482	1494	2313	1467	1467	1467	1467
<i>nad4L</i>	270	270	285	270	270	270	270
<i>nad5</i>	1974	2703	1977	1983	1980	1980	1980
<i>nad6</i>	645	678	576	651	687	651	651
<i>rnpb</i>	—	178	—	172	188	185	194
<i>rps5</i>							
<i>rnl</i>	3079	3615	3489	4858 ^b	4790 ^b	5370 ^b	5346 ^b
<i>rns</i>	1529	1608	1646	1437	1443	1457	1463
Total tRNA	1864	2003	1931	1929	2063	2001	2002
Total ORFs	717	10785	7359	2223	1659	1518	2163
Total respiratory	12969	14304	16090	13122	14043	13104	13068
Total RNA	4608	2401	5135	6467	6421	7012	7003
Total coding	20158	32493	30515	23741	24186	23635	22234
Percent coding	85	74	61	77	73	82	78

^a The *cox1* and *cox2* genes of *P. nodorum* have a 2-base pair overlap, i.e., the last two bases of *cox1* double as the first two bases of *cox2* in that species.

^b These sequences may contain uncalled introns.

Fig 1 – The circular mitochondrial genome diagram of *Zasmidium cellare*, produced using CGView. The diagram shows all genes, tRNAs, rRNAs and other open reading frames. The genome contains the 14 protein-coding genes cytochrome b (*cob*), cytochrome oxidase subunits 1-3 (*cyt1-cyt3*), atp synthase subunits 6, 8 and 9 (*atp6*, *atp8-9*), and nad dehydrogenase subunits 1-6 and 4L (*nad1-6*, *nad4L*), the large and small ribosomal RNA subunit genes (*rnl* and *rns*), and a single open reading frame (ORF) coding for a protein of unknown function, in addition to 26 tRNA genes *Z. cellare*, indicated by the amino acid encoded and the specific anticodon in parentheses. Approximately half of the genes are on each DNA strand.

Fig 2 – RNA secondary structure prediction of a 110-bp inverted repeat that occurs within the *Zasmidium cellare* mitochondrial genome assembly and is similar to telomeres seen in fungi with linear mitochondrial genomes. A and B show the minimum free energy prediction of the same sequence from opposite strands. Colors indicate the base-pair probabilities from 0 to 1.

Fig 3 – Maximum-likelihood phylogenetic analysis of concatenated DNA sequences from 14 mitochondrial protein-coding genes. Bootstrap support (percent of 100 replications) is indicated by each node. The tree agrees with previous phylogenies based on nuclear gene sequences. Species *Metarhizium anisopliae*, *Podospora anserina* and *Verticillium dahliae* are in the class *Sordariomycetes*, the two species of *Penicillium* and the two of *Aspergillus* are in the *Eurotiomycetes*, while *Stagonospora* (synonym *Parastagonospora*) *nodorum*, *Zasmidium cellare* (in bold), and *Mycosphaerella graminicola* (synonym *Zymoseptoria tritici*) are in the *Dothideomycetes*, the first in the order *Pleosporales*, the latter two in the *Capnodiales*.

Fig 4 – Synteny relationships of complete mitochondrial genomes based on *blastn* similarities. All genomes begin with the *rns* gene. A high degree of synteny exists among mitochondrial genomes of fungi in the *Eurotiomycetes* but this breaks down almost completely in the *Dothideomycetes*. Orders are indicated to the left. Positions of called genes are indicated by thick green arrows.

Fig 5 – Comparison of the circular mitochondrial genomes of *Zasmidium cellare* and *Mycosphaerella graminicola* (synonym *Zymoseptoria tritici*), drawn to scale. Both genomes have a single change in the direction of transcription. Seven groups of two and two groups of four genes that are in the same order and orientation on the two genomes are indicated by ovals A-C for protein-coding genes and by parallelograms D-I for tRNA genes. Although these two fungi in the *Capnodiales* have nine groups of 2-4 conserved genes, their orders and organization vary considerably.

Figure 1

Figure 2

A

B

Figure 3

Figure 4

Figure 5

Graphical Abstract