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Palindromic Genes in the Linear Mitochondrial Genome of the Nonphotosynthetic Green Alga *Polytomella magna*

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Abstract

Organelle DNA is no stranger to palindromic repeats. But never has a mitochondrial or plastid genome been described in which every coding region is part of a distinct palindromic unit. While sequencing the mitochondrial DNA of the nonphotosynthetic green alga *Polytomella magna*, we uncovered precisely this type of genic arrangement. The *P. magna* mitochondrial genome is linear and made up entirely of palindromes, each containing 1–7 unique coding regions. Consequently, every gene in the genome is duplicated and in an inverted orientation relative to its partner. And when these palindromic genes are folded into putative stem-loops, their predicted translational start sites are often positioned in the apex of the loop. Gel electrophoresis results support the linear, 28-kb monomeric conformation of the *P. magna* mitochondrial genome. Analyses of other *Polytomella* taxa suggest that palindromic mitochondrial genes were present in the ancestor of the *Polytomella* lineage and lost or retained to various degrees in extant species. The possible origins and consequences of this bizarre genomic architecture are discussed.

Key words: *Chlamydomonas*, inverted repeat, mitochondrial DNA, palindrome, telomere.

Introduction

The words mitochondrial genome often evoke the image of a simple, circular molecule with a few dozen neatly arranged genes (Boore 1999), but most mitochondrial DNAs (mtDNAs) are far more complex than this. Since their origin from a bacterial endosymbiont about two billion years ago, mtDNAs have adopted almost every shape, size, and organization imaginable (Burger et al. 2003). From the highly reduced mtDNA jigsaw puzzles of various protists (Nash et al. 2008; Vlcek et al. 2011) to the massive multichromosomal mtDNAs of certain flowers (Sloan et al. 2012), mitochondrial genomes are anything but ordinary. They have influenced our understanding of genomic architectural diversity across the eukaryotic tree of life and helped forge leading theories of genome evolution (Lynch et al. 2006; Gray et al. 2010).

Here, underscoring just how bizarre mtDNAs can be, we present a linear mitochondrial chromosome with palindromic genes. This unusual genome comes from the green algal genus *Polytomella*. Closely related to popular model organisms, such as *Chlamydomonas reinhardtii* and *Dunaliella salina* (Smith et al. 2010), *Polytomella* is a group of poorly studied nonphotosynthetic unicells, which bear four flagella and are found in habitats rich in dissolved organic matter, such as freshwater pools of rotting vegetation (Pringsheim 1955).

Previous work on *Polytomella parva*, *P. piriformis*, and *P. capuana* revealed atypical mtDNA features, including linear or linear fragmented conformations, closed-loop telomeres, scrambled and discontinuous rRNA-coding genes, and, in one case, an extreme nucleotide composition (Fan and Lee 2002; Fan et al. 2003; Smith and Lee 2008; Smith et al. 2010). In this study, genomic data from *P. magna* SAG 63-9—a heretofore genetically unexplored lineage, isolated from the sap of an elm tree in Cambridge, England (Pringsheim 1955)—provide a new take on gene and

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repeat arrangement in mitochondrial genomes, and genomes as a whole.

One Linear Chromosome, 10 Palindromic Repeats

Polytomella magna, like previously described *Polytomella* taxa, has a linear mitochondrial genome with inverted-repeat telomeres and ten unique genes, including fragmented and scrambled rRNA-coding regions (fig. 1*A*). The *P. magna* mtDNA is about twice the size (~28 kb) of those from other *Polytomella* lineages, which is the product of ten large and distinct duplications, each of which is in a tandem and inverted (i.e., palindromic) orientation (fig. 1*A*). These palindromes are AT-rich (avg. = 65%), ~240–3,100 nt long (avg. = 2,165 nt), and punctuated by short (2–102 nt) stretches of nonrepeated sequence (supplementary table S1, Supplementary Material online). The inverted repeats that form each palindromic element are identical to one another and separated by 2–13 nt of AT-rich (75–100%), but nonconserved, sequence (supplementary table S1, Supplementary Material online). When ignoring the telomeres, palindromes cover >98% of the *P. magna* mtDNA. Six of the palindromes contain a single coding region, and four harbor 2–7 coding segments. Consequently, all 10 genes in the *P. magna* mitochondrial genome are duplicated and in an inverted arrangement relative to their partners (fig. 1*A*). The duplicates of the tRNA- and rRNA-coding regions are identical and complete, suggesting that both copies are functional. For the seven duplicated protein-coding regions, however, only one of the pairs contains a stop and a start codon (supplementary table S2, Supplementary Material online), indicating that for every encoded protein there is a functional and nonfunctional version of the gene (fig. 1*A* and *B*). When the palindromic protein-coding genes are folded into putative stem-loop structures, the start codon is sometimes found in the apex of the loop with the stop codon located in the intervening sequence between the different palindromes (fig. 1*B*). Despite their palindromic nature, the genes in the *P. magna* mitochondrial genome have a similar organization to those of other *Polytomella* mtDNAs (fig. 1*A* and *B*).

To the best of our knowledge, *P. magna* is the first eukaryote shown to have an organelle genome consisting almost entirely of palindromes. Of the three other available *Polytomella* mtDNA sequences, those of *P. parva* and *P. piriformis* are devoid of palindromes, but that of *P. capuana* contains vestiges of palindromic genes: the 5′-ends of four protein- and five rRNA-coding regions form short (<30 nt) inverted repeats with the adjacent intergenic sequences (fig. 1*C*) (Smith and Lee 2008). The short palindromic elements in the *P. capuana* mtDNA have a similar orientation to those of *P. magna* (fig. 1*C*), and some can be folded into hairpin structures with the start codons of protein-coding genes positioned at the top of the loops (Smith and Lee 2008). Phylogenetic analyses (discussed later) support the view that palindromic genes arose early in *Polytomella* evolution and were subsequently maintained or lost to varying degrees in extant lineages.

Complex mtDNA Hybridization Pattern in Pulsed-Field Gels

Gel electrophoresis analyses support the linear conformation and ~28-kb size of the *P. magna* mitochondrial genome, but also hint at underlying structural complexity (fig. 2*A* and *B*; supplementary figure S1, Supplementary Material online). Pulsed-field gel electrophoresis (PFGE) of *P. magna* DNA followed by Southern blot hybridization with a *P. magna* mtDNA-derived probe (*cob*) consistently revealed an ~28-kb band, which co-migrated with linear markers (fig. 2*A* and *B*). This band was observed from in-gel digestion of *P. magna* cells (fig. 2*A*) as well as from samples of total cellular DNA and DNA isolated from a mitochondria-enriched fraction (fig. 2*B*). In addition, PFGE of the latter two samples resulted in a series of slow-migrating bands that hybridized with the mtDNA probe and co-migrated with linear markers of 165 to >300 kb (fig. 2*B*). These bands were not detected in the in-gel-lysis experiment, which, unlike the total DNA and mtDNA analyses, showed a strong mtDNA hybridization signal in the loading well, implying that a significant amount of mtDNA did not enter the gel (fig. 2*A*).

Although often depicted as genome-sized molecules, mtDNAs can have complicated and dynamic architectures (Bendich 1993, 2004). For example, the organelle genomes of various land plants and fungi are known to exist as complex, multigenomic linear-branched structures, which, through recombination, can generate unit-sized chromosomes (Oldenburg and Bendich 2004; Gerhold et al. 2010). These branched structures are frequently overlooked because they can get trapped in or close to the well during gel electrophoresis (Oldenburg and Bendich 2004).

The slow-migrating mtDNA bands in the *P. magna* PFGE experiments using mitochondrial and total cellular DNA could correspond to partially disrupted massive linear-branched mtDNAs, which remain more intact and therefore well-bound when derived from embedded cells. However, assemblies of these types of mtDNAs, because of their concatenated organization, typically give circular maps (Bendich 2004), not linear ones, like that derived for the *P. magna* mtDNA (fig. 1*A*). Moreover, cleavage of linear-branched organelle DNA with a single-cutter restriction enzyme normally produces a genome-sized fragment as well as various degrees of smearing (Bendich 2004). Digestion of *P. magna* mtDNA with a single-cutter (and two-cutter) restriction endonuclease, followed by PFGE and Southern blot analyses, gave sub-genome-sized bands, which migrated in accordance with the mitochondrial restriction map (supplementary fig. S1, Supplementary Material online); no genome-sized or high-molecular-weight bands or smears were observed.

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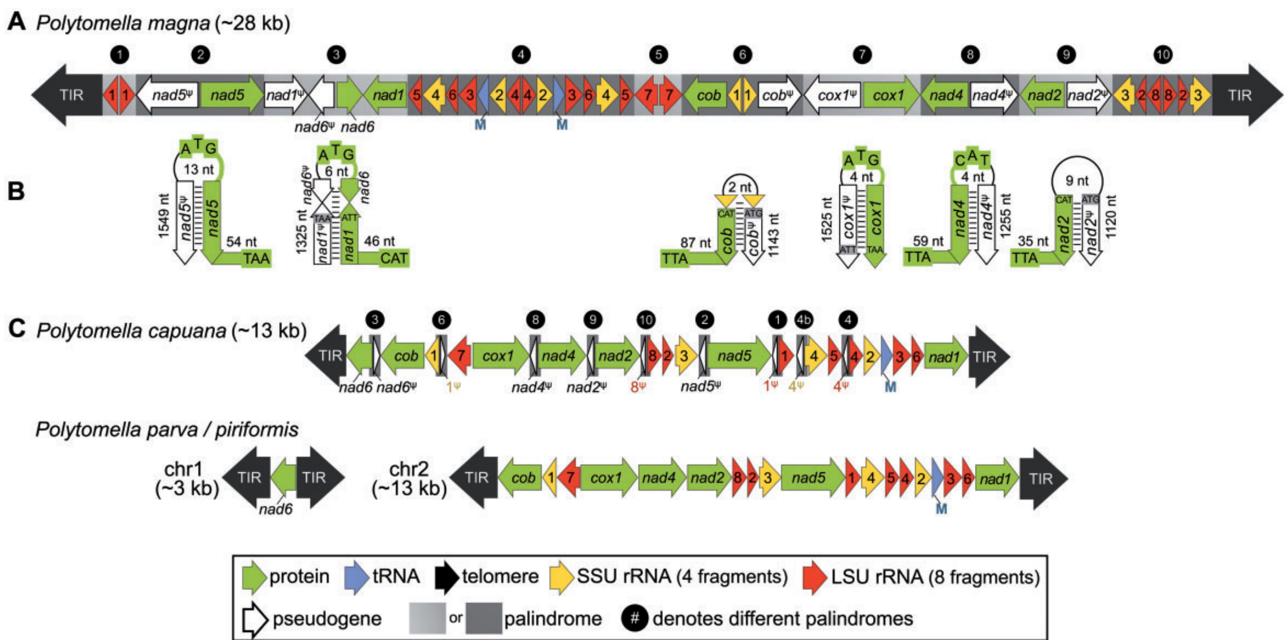

Fig. 1.—*Polytomella* mitochondrial DNA architecture. (*A*) The *P. magna* mitochondrial genome is an ~28-kb linear molecule with inverted-repeat telomeres (TIRs) and ten unique genes, including the small- and large-subunit rRNA genes, which are fragmented and scrambled into four and eight loci, respectively. The *P. magna* mtDNA contains ten palindromic repeats (boxed in dark or light gray and labeled with black circles). There is a functional (green) and nonfunctional copy (white and labeled with Ψ) of each protein-coding gene. (*B*) When the palindromic protein-coding regions are folded into putative stem-loop structures (stem and loop sizes as well as start/stop codons are shown), the start codon is often positioned in the apex of the loop and/or the stop codon is found in the intervening sequence between the different palindromes. (*C*) *P. capuana* has a 13-kb linear monomeric mtDNA with short palindromic repeats that include portions of coding DNA (boxed in gray and numbered with black circles based on their organization relative to *P. magna*). *Polytomella parva* and *P. piriformis* each have a linear bipartite mtDNA with chromosomes sizes of ~13 and ~3 kb and no palindromic coding regions.

Alternatively, the complex pattern of slower migrating bands could be a consequence of secondary structures. The palindromes within the *P. magna* mtDNA have the potential to form giant (>1.5 kb) cruciforms, which could slow DNA migration within gels (fig. 2*C*), as has been documented in a variety of other systems (Lilley and Clegg 1993; Oussatcheva et al. 1999; Sinden 1994; Stellwagen and Stellwagen 2009). The Gibbs free energy values, which can predict DNA duplex stability, of the folded single-stranded cruciforms are similar to the corresponding unfolded, double-stranded structures (supplementary table S3, Supplementary Material online), indicating that under the appropriate conditions stem-loops may form easily and stably with the *P. magna* mtDNA, but further experiments are required to test this hypothesis. If cruciform structures were impeding mtDNA PFGE migration, they could have formed during DNA sample preparation (Courey and Wang 1983), and may not reflect the in vivo genome architecture.

A New *Polytomella* Lineage

Maximum-likelihood phylogenetic analyses, using 18S rDNA and mitochondrial proteins, demonstrate that *P. magna* SAG 63-9 forms a distinct lineage within the *Polytomella* genus, sister to a clade containing all other known *Polytomella* species (fig. 3 and supplementary fig. S2, Supplementary Material online). This tree topology, when placed alongside available data on mtDNA architecture (fig. 3), supports the hypothesis that the ancestral *Polytomella* mitochondrial genome had palindromic loci, which were ultimately preserved in the *P. magna* lineage, but partially and completely lost in the *P. capuana* and *P. parva/piriformis* lineages, respectively. The origins of other *Polytomella* mtDNA features, including a linear bipartite structure, are highlighted in figure 3.

In his original description of *P. magna*, Pringsheim (1955) noted that it was larger than other identified *Polytomella* species and the only one with a discernable eyespot. Our experiences of culturing *P. magna* SAG 63-9 were consistent with Pringsheim's observations. We found that SAG 63-9, when grown on our standard *Polytomella* medium (Sheeler et al. 1968), was conspicuously larger than strains from the three other known *Polytomella* lineages (Smith et al. 2010), had a visible eyespot, and was pinkish in color (supplementary fig. S3, Supplementary Material online)—presumably because of the carotenoids in the eyespot (Kreimer 2009). The Culture Collection of Algae at the University of Göttingen (SAG) maintains, as of July 2013, one other strain labeled *P. magna*: SAG 63-4. Previous studies on the mtDNA sequence and

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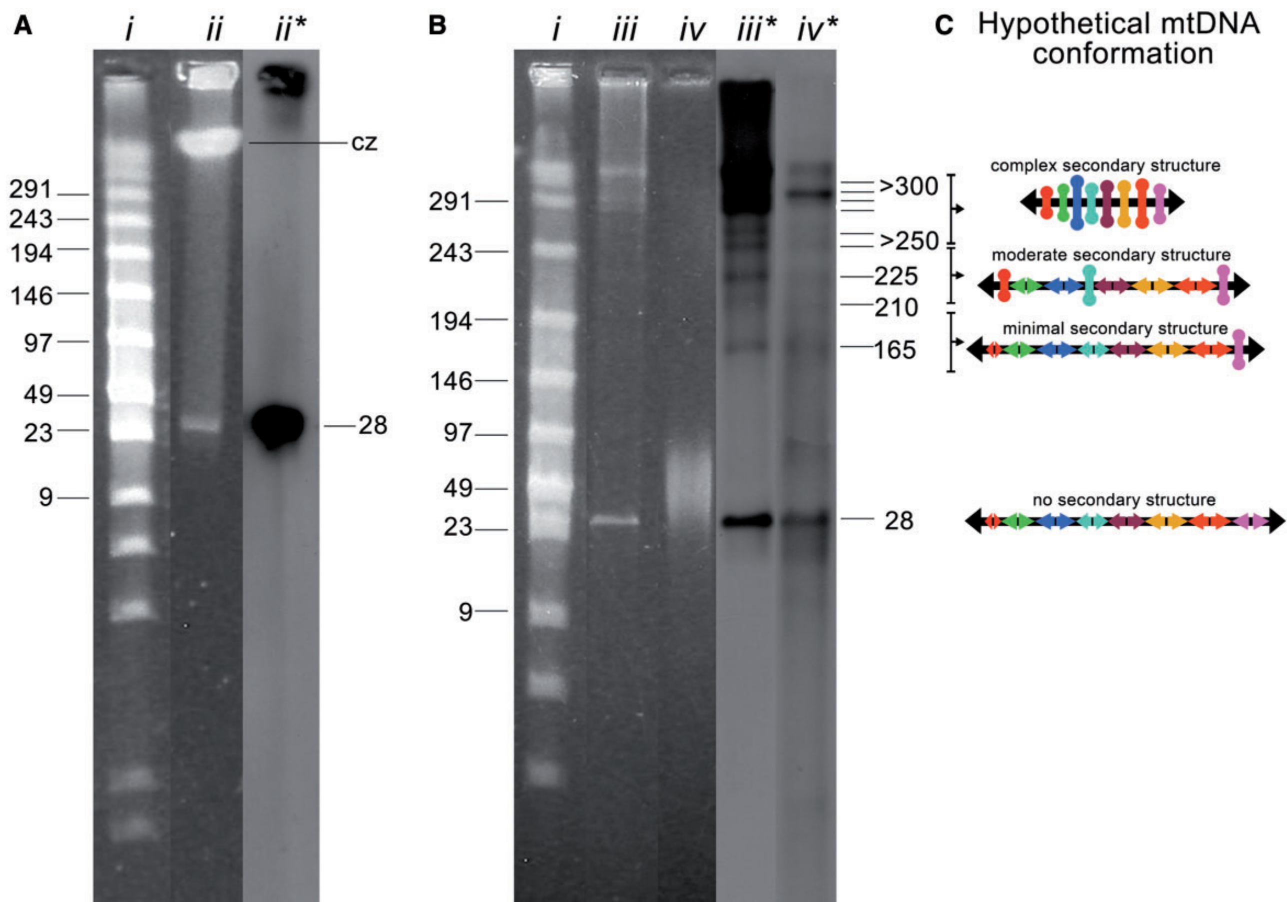

Fig. 2.—PFGE and Southern blot analysis of *P. magna* DNA. (*A*) PFGE and Southern blot of DNA resulting from the digestion of agarose-embedded *P. magna* cells (gel: 1% pulsed-field certified agarose; chiller temperature: 14 °C; running buffer: 0.5× TBE; run time: 10 h 30 min; initial switch time: 0.1 s; final switch time: 26 s; gradient: 6 V/cm; angle: 120°). (*B*) PFGE and Southern blot of purified mitochondrial and total cellular *P. magna* DNA (gel: 1% pulsed field certified agarose; chiller temperature: 14 °C; running buffer: 0.5× TBE; run time: 12 h; initial switch time: 15 s; final switch time: 15 s; gradient: 6 V/cm; angle: 120°). Lanes are as follows: (*i*) New England Biolabs low range PFG marker; (*ii*) agarose-embedded cells; (*iii*) purified mtDNA; (*iv*) total cellular DNA. Asterisk denotes Southern blot of given lane using an mtDNA-derived probe (*cob*). (*C*) Hypothetical mtDNA configuration. Note—cz = compression zone. Sizes of bands are labeled in kilobases.

structure of SAG 63-4 showed that it belongs to the *P. parva* lineage (Mallet and Lee 2006; Smith and Lee 2011). Consistent with this view, we found that SAG 63-4 had no discernable eyespot, was smaller than SAG 63-9 and indistinguishable in size from *P. parva* (SAG 63-3), and gave an off-white-colored pellet, with no visible carotenoids, similar to a pellet of *P. parva* (supplementary fig. S3, Supplementary Material online).

Origin of Palindromic Genes

How did *P. magna* acquire such a peculiar mtDNA organization? Palindromes can be found, to varying degrees, in most genomes, but rarely do they blanket entire chromosomes, as observed for *P. magna* (fig. 1*A*). Several elegant hypotheses have been put forth for the emergence of large palindromic DNA elements; for example, one model begins with strand annealing at a short DNA inverted repeat after a double-stranded break, whereas another involves cruciform extrusion and resolution (Tanaka and Yao 2009). Inspection of the *P. magna* mtDNA offers no obvious solution to the origin of its palindromes. However, the fact that all coding regions are duplicated suggests that the 10 palindromic units may have arisen by inter- and intra-recombination events following an mtDNA genome duplication, similar to the that proposed for the formation of the mtDNA replication-intermediate, head-to-head linear dimer in *Paramecium aurelia* (Pritchard and Cummings 1981). Alternatively, the genome-wide formation of palindromic sequences in the *P. magna* mtDNA may have been the result of repair activity to overcome DNA-replication-disrupting cruciform structures; these could have emerged following a heat shock from previously benign short inverted repeat sequences of canonical double-stranded DNA structure

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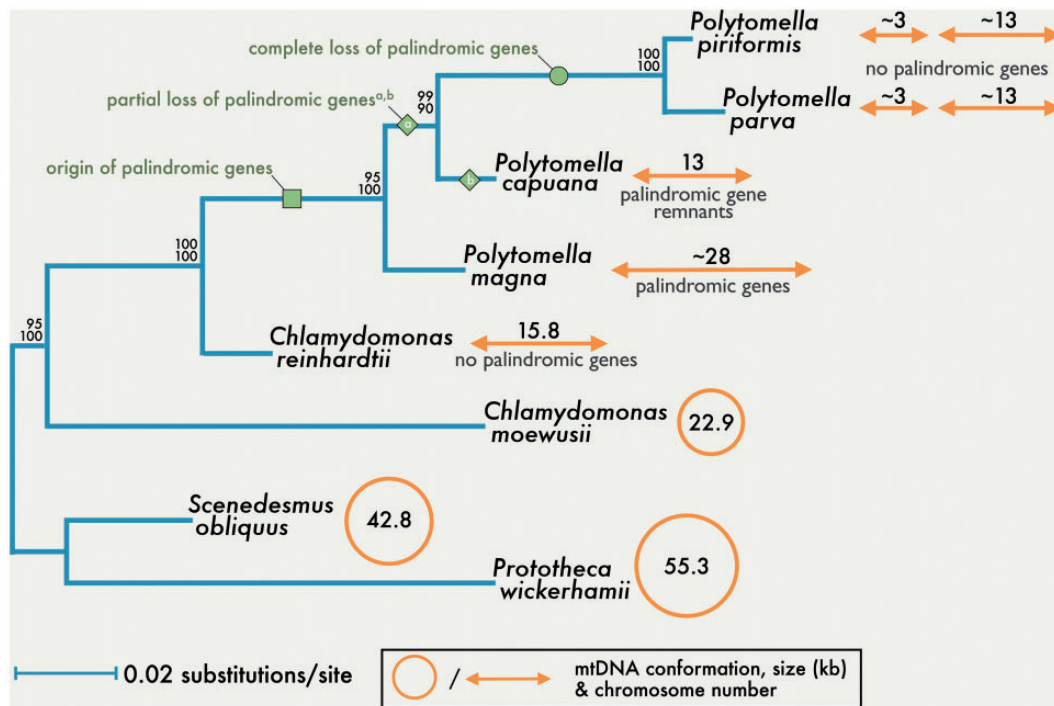

Fig. 3.—Maximum-likelihood phylogeny inferred from 18S rDNA sequences. Phylogenetic tree of chlamydomonadalean green algae, with the chlorophycean *Scenedesmus obliquus* and the nonphotosynthetic trebouxiophyte *Prototheca wickerhamii* used as outgroup. The same tree topology was obtained with a maximum-likelihood phylogeny inferred from the concatenated amino acid sequences of seven mtDNA-encoded proteins (supplementary fig. S2, Supplementary Material online). Bootstrap support values are indicated at each node: top ones and lower ones correspond to the 18S rDNA and mitochondrial protein trees, respectively. Hypothetical position of the origin, partial loss, and complete loss of palindromic genes are marked on the tree with a square, diamond, and circle, respectively. Note—Partial loss of palindromic genes could have occurred in the branch leading to *P. capuana*, *P. piriformis*, and *P. parva* (a) or alternatively it could have occurred in the lineage leading to *P. capuana* (b). Scale bar represents the estimated number of nucleotide substitutions per site.

(SantaLucia and Hicks 2004) that arose in some noncoding regions of the *P. magna* mtDNA. Homologous recombination might be quite high within the *P. magna* mtDNA: the inverted repeats that make up the palindromic elements are 100% identical (supplementary table S1, Supplementary Material online), which is likely the result of recurrent gene conversion between the palindromic units. High levels of gene conversion could also explain why the palindromes persist within the genome (Maréchal and Brisson 2010).

Palindromic organelle DNA repeats have been uncovered in other members of the Chlamydomonadales (the order to which *P. magna* belongs), including *C. reinhardtii*, *D. salina*, and *Volvox carteri* (Maul et al. 2002; Smith and Lee 2009; Smith et al. 2010), and are thought to have shaped their mtDNA evolution (Nedelcu and Lee 1998) and play a role in mitochondrial gene expression (Gray and Boer 1988). The fact that some of the palindromes within the *P. magna* mtDNA can be folded into hairpins, with the start codons of protein-coding regions located in the apex of the loop (fig. 1*B*), is suggestive of a role in gene expression. Unraveling the transcriptional architecture of *P. magna*, however, will not be a trivial task. Chlamydomonadalean transcriptome sequencing projects typically yield almost complete coverage of the mtDNA, including intergenic regions and telomeres, making it difficult to predict transcriptional units and processing sites (e.g., see GenBank accessions ERX177535–ERX177582).

Genetic palindromes are a hot topic. They are associated with a diversity of molecular processes, from DNA replication to major chromosomal rearrangements (Tanaka et al. 2002; Paek et al. 2009; Lavrov et al. 2012), are implicated in various human diseases (Tanaka et al. 2006; Voineagu et al. 2008), and have been the focus of international scientific meetings (Smith 2008). Overall, the *P. magna* mtDNA provides a fresh view of these important repetitive elements.

Methods and Materials

Polytomella magna SAG 63-9, made axenic following the method of Mallet and Lee (2006), was grown in darkness at 22 °C in the *Polytomella* medium of Sheeler et al. (1968) and harvested in the late logarithmic growth phase ($OD_{750\ nm} \approx 0.35$). A mitochondria-enriched fraction of

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P. magna was generated using Procedure B of Ryan et al. (1978), without the step employing CsCl gradient centrifugation. DNA was extracted from a cellular and a mitochondria-enriched fraction using the CTAB method (Reineke et al. 1998). DNA for PFGE plugs were prepared as previously described (Tanifuji et al. 2006). DNA was transferred to nylon membranes (Amersham Bioscience, NJ, USA) and nonradioactive Southern blot hybridizations were performed with a digoxigenin (DIG)-labeled, polymerase chain reaction-amplified *P. magna* mtDNA probe. Hybridization signals were detected using the standard protocol of the DIG detection kit (Roche Diagnostics, IN, USA) and Fuji Super RX medical X-ray film.

Library preparation and paired-end Illumina HiSeq 2000 sequencing (100 nt reads; ~500 nt inserts) were performed by BGI Americas (MA, USA), using total DNA, isolated with the DNeasy Plant Mini Kit (Qiagen, MD, USA). The *P. magna* sequence data (~5 Gb) were assembled de novo with Ray v1.2.1 (Boisvert et al. 2010), using a *k*-mer of 21, and separately with PASHA (Liu et al. 2011), using a *k*-mer of 31. The resulting Ray- and PASHA-generated contigs were independently scanned for mitochondrial sequences using BLAST and the *P. capuana* and *P. parva* mtDNAs as search queries. Contigs matching to mtDNA were identified in each data set. These contigs were extended using the paired-end data and the Map to reference program in Geneious v6.0.6 (Biomatters Ltd., Auckland, New Zealand), giving (in both cases) a complete, linear-mapping mitochondrial genome with telomeres (GenBank accession KC733827). Maximum likelihood phylogenetic analyses were performed with the PhyML 3.0 web server (Guindon et al. 2010), and the robustness of individual nodes on the tree were assessed using 100 bootstrap replicates.

Supplementary Material

Supplementary figures S1–S3 and tables S1–S3 are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

Acknowledgments

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