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

David Roy Smith¹,†, Jimeng Hua²,³, John M. Archibald², and Robert W. Lee³,*

¹Department of Biology, University of Western Ontario, London, Ontario, Canada
²Department of Biochemistry and Molecular Biology, Canadian Institute for Advanced Research, Integrated Microbial Biodiversity Program, Dalhousie University, Halifax, Nova Scotia, Canada
³Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada

*Corresponding author: E-mail: robert.lee@dal.ca.
†These authors contributed equally to this work.

Accepted: August 8, 2013

Data deposition: Sequence data from this article have been deposited in GenBank under the accession KC733827.

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. piniformis*, 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
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. 1A). 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. 1A). 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 noncon- stant by 2–13 nt of AT-rich (75–100%), but noncon-

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. 2A 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. 2A and B). This band was observed from in-gel digestion of *P. magna* cells (fig. 2A) as well as from samples of total cellular DNA and DNA isolated from a mitochondria-enriched fraction (fig. 2B). 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. 2B). 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. 2A).

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. 1A). 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.
Alternatively, the complex pattern of slower migrating bands could be a consequence of secondary structures. The palindromes within the \textit{P. magna} mtDNA have the potential to form giant (>1.5 kb) cruciforms, which could slow DNA migration within gels (fig. 2C), 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 \textit{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 \textit{P. magna} SAG 63-9 forms a distinct lineage within the Polytomella genus, sister to a clade containing all other known \textit{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 \textit{Polytomella} mitochondrial genome had palindromic loci, which were ultimately preserved in the \textit{P. magna} lineage, but partially and completely lost in the \textit{P. capuana} and \textit{P. parva}/\textit{piriformis} lineages, respectively. The origins of other \textit{Polytomella} mtDNA features, including a linear bipartite structure, are highlighted in figure 3.

In his original description of \textit{P. magna}, Pringsheim (1955) noted that it was larger than other identified \textit{Polytomella} species and the only one with a discernable eyespot. Our experiences of culturing \textit{P. magna} SAG 63-9 were consistent with Pringsheim’s observations. We found that SAG 63-9, when grown on our standard \textit{Polytomella} medium (Sheeler et al. 1968), was conspicuously larger than strains from the three other known \textit{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 \textit{P. magna}: SAG 63-4. Previous studies on the mtDNA sequence and
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. 1A). 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.

![Image of PFGE and Southern blot analysis of *P. magna* DNA](image-url)
(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 (Mare´chal 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. 1B), is suggestive of a role in gene expression. Unraveling the transcriptional architecture of P. magna, however, will not be a trivial task. Chlamydomonadales 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 ≈ 0.35). A mitochondria-enriched fraction of
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
The authors thank Goro Tanifuji and Naoko Onodera for technical assistance with PFGE and Southern blot protocols and sequence assembly. This work was supported by Discovery Grants to R.W.L. and J.M.A. from the Natural Sciences and Engineering Research Council (NSERC) of Canada. J.M.A. is a Fellow of the Canadian Institute for Advanced Research, Integrated Microbial Biodiversity Program, and is a New Investigator Award holder from the Canadian Institutes of Health Research.

Literature Cited
Bendich AJ. 1993. Reaching for the ring: the study of mitochondrial genome structure. Curr Genet. 24:79–290.

Bendich AJ. 2004. Circular chloroplast chromosomes: the grand illusion. Plant Cell 16:1661–1666.

Boisvert S, Laviolle F, Corbeil J. 2010. Ray: simultaneous assembly of reads from a mix of high-throughput sequencing technologies. J Comput Biol. 17:1519–1533.

Boore JL. 1999. Animal mitochondrial genomes. Nucleic Acids Res. 27:1767–1780.

Burger G, Gray MW, Lang BF. 2003. Mitochondrial genomes: anything goes. Trends Genet. 19:709–716.

Courey AJ, Wang JC. 1983. Cruciform formation in a negatively supercoiled DNA may be kinetically forbidden under physiological conditions. Cell 33:817–829.

Fan J, Lee RW. 2002. Mitochondrial genome of the colorless green alga Polytomella parva: two linear DNA molecules with homologous inverted repeat termini. Mol Biol Evol. 19:999–1007.

Fan J, Schnare MN, Lee RW. 2003. Characterization of fragmented mitochondrial ribosomal RNAs of the colorless green alga Polytomella parva. Nucleic Acids Res. 31:769–778.

Gerhold JM, Aun A, Sedman T, Joers P, Sedman J. 2010. Strand invasion structures in the inverted repeat of Candida albicans mitochondrial DNA reveal a role for homologous recombination in replication. Mol Cell. 39:851–861.

Gray MW, Boer PH. 1988. Organization and expression of algal (Chlamydomonas reinhardtii) mitochondrial DNA. Philos Trans R Soc Lond B Biol Sci. 319:135–147.

Gray MW, Lukes J, Archibald JM, Keeling PJ, Doolittle WF. 2010. Irremediable complexity? Science 330:920–921.

Guindon S, et al. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol. 59:307–321.

Kreimer G. 2009. The green algal eyespot apparatus: a primordial visual system and more? Curr Genet. 55:19–43.

Lawrov DV, Maikova OQ, Pett W, Belkov SI. 2012. Small inverted repeats drive mitochondrial genome evolution in Lake Baikal sponges. Gene 505:91–99.

Lilley DMJ, Clegg RM. 1993. The structure of the four-way junction in DNA. Annu Rev Biophys Biomol Struct. 22:299–328.

Liu Y, Schmidt B, Maskell DL. 2011. Parallelized short read assembly of large genomes using de Bruijn graphs. BMC Bioinformatics 12:354.

Lynch M, Koskella B, Schaack S. 2006. Mutation pressure and the evolution of mitochondrial genomes. Trends Genet. 19:709–716.

Lavrov DV, Polytomella parva

Mauther J, Sedman T, Jóers P, Sedman J. 2010. Strand invasion structures in the inverted repeat of Candida albicans mitochondrial DNA reveal a role for homologous recombination in replication. Mol Cell. 39:851–861.

Maul JE, et al. 2002. The Chlamydomonas reinhardtii plastid chromosome: islands of genes in a sea of repeats. Plant Cell 14:2659–2679.

Nash EA, Nisbet RER, Barbrook AC, Howe CJ. 2008. Dinoflagellates: a mitochondrial genome all at sea. Trends Genet. 24:328–335.

Nedelcu AM, Lee RW. 1998. Short repetitive sequences in green algal mitochondrial genomes: potential roles in mitochondrial genome evolution. Mol Biol Evol. 15:690–701.

Oldenburg DJ, Bendich AJ. 2004. Most chloroplast DNA of maize seedlings is circular. Curr Genet. 45:505:91–99.

Paek AL, et al. 2009. Fusion of nearby inverted repeats by a replication-based mechanism leads to formation of dicentric and acentric chromosomes that cause genome instability in budding yeast. Gene Dev. 23:2861–2875.

Pringsheim EG. 1955. The genus Polytomella. J Protozool. 2:137–145.
Pritchard AE, Cummings DJ. 1981. Replication of linear mitochondrial DNA from Paramecium: sequence and structure of the initiation-end cross-link. Proc Natl Acad Sci U S A. 78:7341–7345.

Reineke A, Karlovsky P, Zebitz CPW. 1998. Preparation and purification of DNA from insects for AFLP analysis. Insect Mol Biol. 7:95–99.

Ryan R, Grant D, Chiang KS, Swift H. 1978. Isolation and characterization of mitochondrial DNA from Chlamydomonas reinhardtii. Proc Natl Acad Sci U S A. 75:3268–3272.

SantaLucia J, Hicks D. 2004. The thermodynamics of DNA structural motifs. Annu Rev Biophys Biomol Struct. 33:415–440.

Sheeler P, Cantor M, Moore J. 1968. Growth characteristics of Polytomella agilis in batch culture. Life Sci. 7:289–293.

Sinden RR. 1994. DNA structure and function. San Diego (CA): Academic Press.

Sloan DB, et al. 2012. Rapid evolution of enormous, multichromosomal genomes in flowering plant mitochondria with exceptionally high mutation rates. PLoS Biol. 10:e1001241.

Smith GR. 2008. Meeting DNA palindromes head-to-head. Gene Dev. 22:2612–2620.

Smith DR, Hua J, Lee RW. 2010. Evolution of linear mitochondrial DNA in three known lineages of Polytomella. Curr Genet. 56:427–438.

Smith DR, Lee RW. 2008. Mitochondrial genome of the colorless green alga Polytomella capuana: a linear molecule with an unprecedented GC content. Mol Biol Evol. 25:487–496.

Smith DR, Lee RW. 2009. The mitochondrial and plastid genomes of Volvox carteri: bloated molecules rich in repetitive DNA. BMC Genomics 10:132.

Smith DR, Lee RW. 2011. Nucleotide diversity of the colorless green alga Polytomella parva (Chlorophyceae, Chlorophyta): high for the mitochondrial telomeres, surprisingly low everywhere else. J Eukaryot Microbiol. 58:471–473.

Smith DR, et al. 2010. The Dunaliella salina organelle genomes: large sequences, inflated with intronic and intergenic DNA. BMC Plant Biol. 10:83.

Stellwagen NC, Stellwagen E. 2009. Effect of the matrix on DNA electrophoretic mobility. J Chromatogr A. 1216:1917–1929.

Tanaka H, Bergstrom DA, Yao MC, Tapscott SJ. 2006. Large DNA palindromes as a common form of structural chromosome aberrations in human cancers. Hum Cell. 19:17–23.

Tanaka H, Tapscott SJ, Trask BJ, Yao MC. 2002. Short inverted repeats initiate gene amplification through the formation of a large DNA palindrome in mammalian cells. Proc Natl Acad Sci U S A. 99:8772–8777.

Tanaka H, Yao MC. 2009. Palindromic gene amplification—an evolutionarily conserved role for DNA inverted repeats in the genome. Nat Rev Cancer. 9:216–224.

Tanifuji G, Erata M, Ishida K, Onodera N, Hara Y. 2006. Diversity of secondary endosymbiont-derived actin-coding genes in cryptomonads and their evolutionary implications. J Plant Res. 119:205–215.

Vlcek C, Marande W, Teijeiro S, Lukes J, Burger G. 2011. Systematically fragmented genes in a multipartite mitochondrial genome. Nucleic Acids Res. 39:979–988.

Voineagu I, Narayanan V, Lobachev KS, Mirkin SM. 2008. Replication stalling at unstable inverted repeats: interplay between DNA hairpins and fork stabilizing proteins. Proc Natl Acad Sci U S A. 105:9936–9941.

Associate editor: Gertraud Burger